March 2019
ZEN 3.0 SR (black edition)

ELYRA 7
Operating Manual

Knowledge of this manual is required for the operation of the instrument. Would you therefore please make yourself familiar with the contents of this manual and pay special attention to hints concerning the safe operation of the instrument.

The specifications are subject to change; the manual is not covered by an update service.

How to make best use of the ELYRA 7 operating instructions:

A few symbols in these operating instructions will help you to recognize the nature and purpose of information immediately:

The WARNING symbol warns against hazards for the user that might arise when operating the laser.

This WARNING symbol warns against hazards from dangerously high voltages.

The CAUTION symbol warns against faults and hazards that might arise during operation and which might cause damage to the unit.

The NOTE symbol will help you to optimally solve your work problem. It represents a practical tip which will help you to find out which settings and methods are capable of improving or accelerating a procedure.

The HOT SURFACE symbol warns against hazards for the user that might arise when touching the lamp housing during operation.

The MAINS PLUG symbol remembers service personal to pull the mains plug before opening the device housing.

Depending on the request, these operating instructions will supply you with various possibilities:

- If you want to know where to find certain general areas of information, refer to the following outline of sections to get a general overview.

- You will find a detailed table of contents at the start of every chapter. There you will see at a glance what topics are covered in detail.

Always remember: **The time you invest in getting acquainted with the product will pay for itself many times over in your application task.**

Contents

1 System Operation

In this section you will find the most important steps and procedures of the menu structure. The step-by-step description how to get an image will be shown by typical application examples including the WINDOWS graphic user environment.

2 Macros and Visual Basic

This section contains a description of the use of additional functions, e.g. maintenance, macros.

3 Additional Software Tools

This section contains a description of the use of additional tools for setting the microscope.

4 Annex

The annex contains the Application-specific Configurations, special notes and information for using the LSM microscope.

CHAPTER 1   SYSTEM OPERATION

CONTENTS

1 Purpose

This section describes the operation of the Laser Scanning Microscope with the ZEN 3.0 SR software.

When starting up and operating the microscope system, mind the operating instruction manuals for the Axio Observer7 microscope stand and microscope components:

- Axio Observer 3/5/7, Operating Manual (431004-7244-001)
- Definite Focus, Operating Manual (424533-7244-001)

1.1 Software

The ZEN software is used to

- control the microscope, the scanning module, the laser module, and the image acquisition process
- display, edit and analyze the images

It is a special user interface (desktop) based on the network-capable graphic 64-bit Microsoft® WINDOWS 7/10 operating system.

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The installation of the software for the Laser Scanning Microscope and the basic settings of the equipment components are carried out by ZEISS service staff. This job includes the creation of a customized software configuration in line with the specific hardware components of the customer's microscope system.

1.2 Convention for the Text in this Manual

All the originally used terms of the software interface, e.g.

- names of windows
- tool groups
- panels
- input boxes
- list / selection boxes
- check boxes
- menu items
- names of buttons and sliders
- keyboard keys

are displayed in **bold letters** to allow easier identification.

1.3   Backup

System backup

Prior to the delivery of each system, a backup of the entire system computer is created in the factory. This backup image belongs to the system and may be re-installed in order to restore the original state of the system computer. Please contact your local service representative if the backup image needs to be installed.

User files backup

The following user-generated files should be included in a backup procedure controlled and carried out on a regular basis by the user (keep directory structure):

- Carl Zeiss Image files: *.czi
- LSM Image files: *.lsm
- Exported images: *.* (*.Tiff, *.LSM-Tiff, *.BMP, …)
- Palette files: ZEN\Palette*.lut
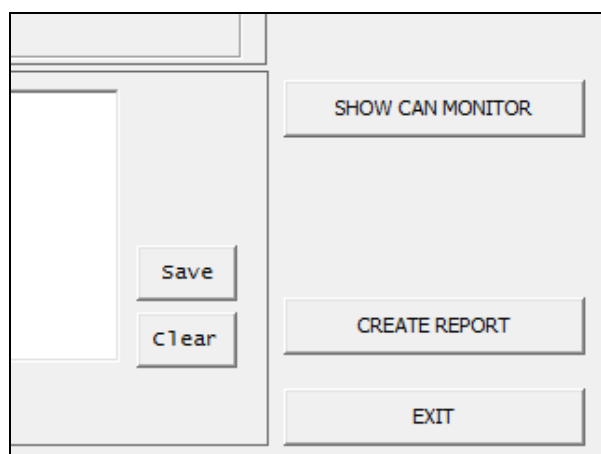
Fig. 1   Cut-out of the CanCheck window

The backup of further hardware calibration settings including the database and licence file can easily be created with the CanCheck tool (C:\ZEN\CanCheck).

- Click the **CREATE REPORT** button in the **CanCheck** window (Fig. 1).

A system report will be created and saved to the desktop on your computer as a zip file named report.zip. This report contains all the information needed for a system status report and to create a save copy in case the system has to be reset.

1.4   Software Operation

The ZEN software can be operated using the mouse, the PC keyboard, or both. The operation of the mouse and the keyboard is identical to that of the Microsoft® WINDOWS 7/10 operating system and is therefore not described in this manual. If required, see the Microsoft manual or online help for relevant information.

2 Hardware Aspects

2.1 Principles of Superresolution Microscopy (ELYRA)

A point object imaged by an objective lens will always be a blurred spot, the so called point spread function (PSF), due to the diffraction of light.

Therefore two points cannot come closer than a certain limit in order to still be resolved. This minimal distance in object space corresponds to a maximal cut-off frequency in the frequency space that can be transmitted through the objective lens.

The transmittable frequencies represent the so-called optical transfer function (OTF), which is the Fourier transform of the PSF. As a rule of thumb the resolution of a far field light microscope in the lateral direction is approximately half the wavelength, whereas it is 3 fold worse in the axial direction.

Hence, for a light microscope lateral and axial resolutions are approximately 200 and 600 nm, respectively. In order to enhance resolution, the PSF has to be narrowed, which is synonymous to the OTF to be expanded.

This exactly is accomplished by structured illumination microscopy (SIM) and photo activated localization microscopy (SMLM).

2.1.1 Principle of Structured Illumination Microscopy (SIM)
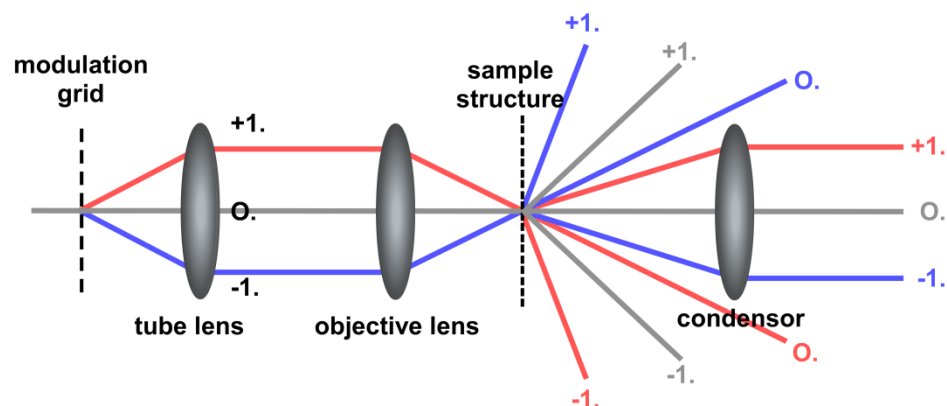
Fig. 2 **Principle of structured illumination – diffraction orders from the modulation grid (line grid pattern) and the sample structures are indicated**

In SIM a sinusoidal pattern, e. g. a line grid pattern (Stripe SIM), or a dot pattern (Lattice SIM) with a defined periodicity, is positioned in the excitation path in a plane that is conjugated to the image plane (Fig. 2).

Hence the pattern is projected onto the image and the excitation intensity is modulated along the pattern. This results in a modulation of the fluorescence as well.

The highest modulation contrast is obtained in the focal plane. The contrast gets weaker with distance to the focal plane so that depth discrimination in the axial direction is possible. The grid constant is ideally the cut off frequency of the system.

The interferences of the diffraction orders generated through the grid are used for the lateral and axial structuring of the light.
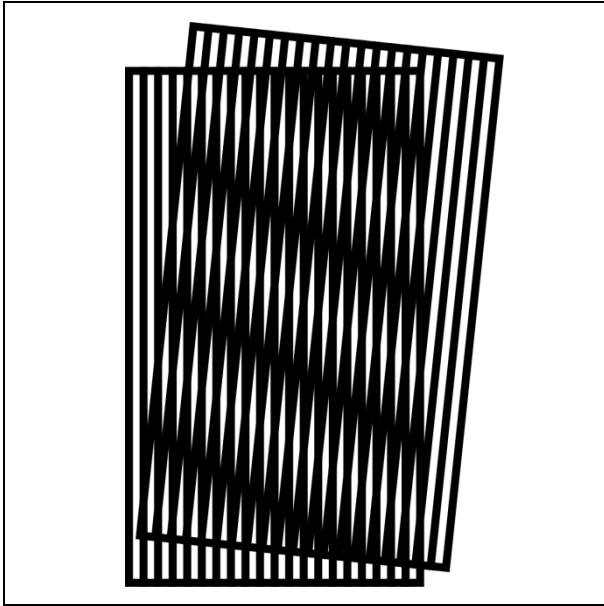
Fig. 3   **Moiré pattern generated by overlaying two line grids – the lower periodicity of the generated pattern is visible as thicker lines**

The interference of the structured light with object structures requires that at least part of the light is coherent and leads to so called Moirè fringes whose pattern has a longer periodicity and hence a lower space frequency compared to the object (Fig. 3).

Each sample or object structure can be regarded as a superimposition of many grids. Due to the structured illumination the object frequencies will be shifted to lower frequencies due to the Moiré effect. Therefore high frequencies that normally cannot be collected by the system can be transmitted due to their shift into lower frequencies.

Due to the diffraction nature of light the modulation grid frequency cannot have a periodicity that is smaller than half the wavelength, so resolution enhancement can be a maximum of two fold in each lateral and axial direction.

Due to the frequency shift the image frequencies are composed of non-shifted and shifted object frequencies and hence their contribution has to be determined.

A coherent image generated with a sinusoidal line grid pattern has generally 3 different orders created by interference: the 0th order from the 0th order non-diffracted beam, the 1st order created by interferenes of the $\pm 1^{st}$ order diffraction beams with the 0th order non diffracted beam (frequencies shifted by half the modulation frequency) and the 2nd order created by the interference between the $+1^{st}$ order with the -1st order diffracted beam (frequencies shifted by the modulation frequency). (Fig. 2).

It is the 2nd order that contains the high frequency information for resolution enhancement in X,Y (Fig. 4). The 1st order contains information for sectioning and better z-resolution.
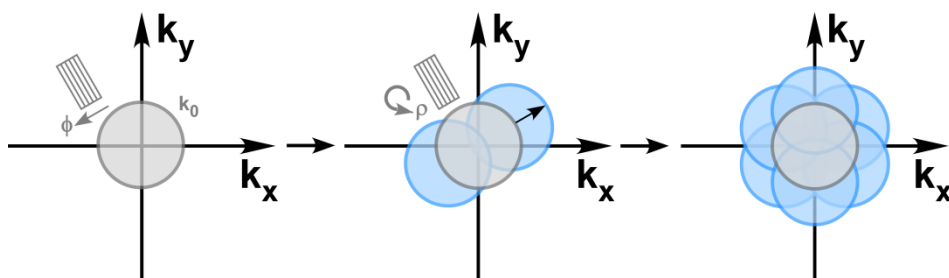
Fig. 4   **Expansion of the transmitted frequencies – the cut-off frequency is indicated by k_0, phase shifts by Φ and rotations by ρ; only the shift by the modulation frequency is shown as this contains the high resolution information**

To shift the frequencies to their correct location, a linear equation with 5 unknowns (2 x n-1, n=number of different orders, which is 3 in the case of a line grid) has to be solved. This can be pixel wise accomplished by recording 5 images with a phase shifted modulation pattern. Since resolution is obtained only in the orientation of the grid pattern, the grid has to be rotated to obtain a nearly uniform resolution in all directions. In generally one uses 3 rotations are sufficient. Hence, the reconstruction of a

SIM image requires a minimum of 3 x 5 = 15 images for a line grid pattern. Finally a back transformation from the frequency to the real space generates the high resolution image.

In Lattice SIM we have a 7 orders. Hence 13 (2 x 7 + 1) phase shifted images are minimally required. In ELYRA 7 15 phase images are used to have more information for reconstruction of the SIM image. Since the Lattice SIM pattern is symmetrical, no rotaion is necessary.

2.1.2 Principle of Photo Activated Localization Microscopy (SMLM)

In SMLM the sample is illuminated in a regime that statistically activates only one fluorescent molecule per PSF.

Hence, the centre of mass of the PSF can be determined, which can be done more precisely than the PSF itself. The accuracy of determination depends solely on the number of photons collected.

The resolution enhancement is by a factor of $1/\sqrt{N}$ with N representing the number of photons (Fig. 5).

To image molecules one at a time in a PSF requires certain properties of the fluorescence dye.

It has to exist in a bright and dark state, which can be converted between each other.

Most of organic dyes show such a behavior in certain reducing environments.

And there are also three classes of fluorescent proteins (FPs) with the required switching characteristics.

Fig. 5   The accuracy of the determination of the center of mass depend on photon number N

Photoactivatable FPs can be irreversibly switched from a dark to a bright state by violet light.

The bright state is than bleached by the imaging laser light. Photoconvertable FPs can be irreversible switched from one spectral state to another by violet light.

The latter one is than bleached by the imaging laser.

Photoswitchable FPs are reversibly switched from a dark state to bright state by violet light and will turn back to the ark state by illumination with the imaging laser light.

It is the correct balance between the activation light (mostly violet light) and the imaging light (mostly strong powers needed) to that determines how many molecules are in the on-state and how many molecules are in the off-state.

Fig. 6   **Principle of SMLM – Whereas in normal widefield images the PSF of closely juxtaposed point sources can overlap heavily (upper panel) their sequential illumination and plotting of their center of mass will result in their clear separation (lower panel)**

Since only a few molecules are active at a time the procedure of activating to convert molecules to the on-state followed by deactivation to convert them to the off-stateh has to be repeated many times in order to detect all labeled molecules (Fig. 6).

So the aim in SMLM is to activate a few molecules, calculate their center of mass, plot them in a new coordinate system and deactivate them quickly (preferentially after 1 to 3 frames) and repeat the process until statistically all molecules have been activated.

So a SMLM experiment easily needs 10 000 frames and more.

The resulting image is a plot of all molecules localizations with a certain precision depending on the photon numbers obtained from the molecule.

2.2   Optical Diagram of ELYRA (Schematic)
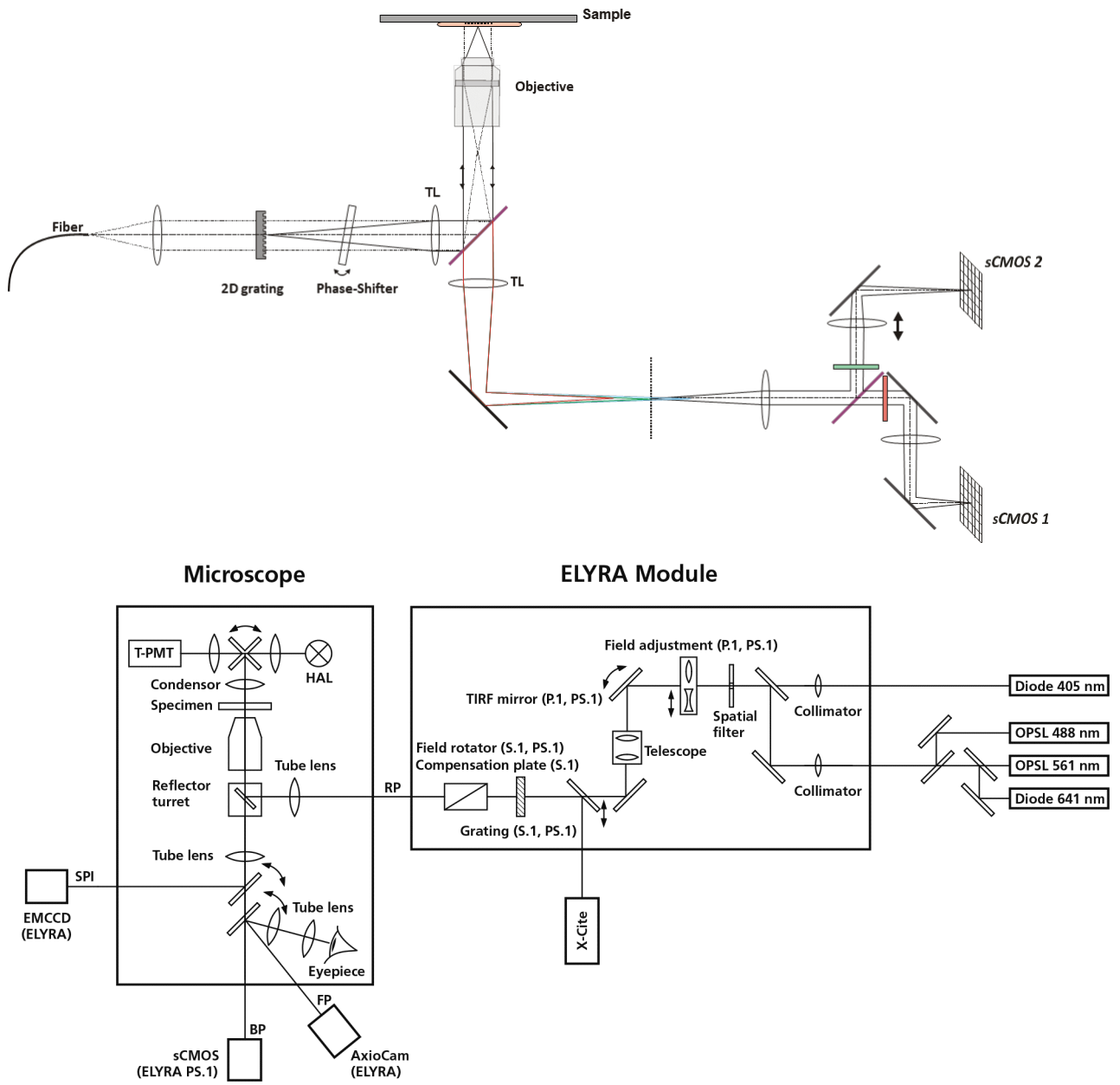
Fig. 7   Optical path, schematic (ELYRA 7)

The diagram is a schematic representation of the ELYRA system (Fig. 7).

Laser light is focused into the back focal plane of the objective to obtain widefield illumination in the specimen. For TIRF mode dependent on the angle of the TIRF mirror either Epi-, HILO- or TIRF-illumination is achieved.

For SIM mode only Epi-illumination is available as the TIRF mirror remains in a fixed position. Light emitted from the specimen is directed back via a reflector cube that separates the emission from the excitation light. The fluorescence signals are directed to individual EMCCD cameras.

Attachment sites for illumination modules and cameras

The ELYRA 7 module is always attached at the rear port (RP) of the Axio Observer 7 SR. An LSM module is always mounted to the left side port (SPl) of the stand. The Duolink will always be mounted, if present, on the right side port (SPr).

For ELYRA 7 type L systems an Andor iXon 897 Ultra EMCCD camera is mounted on the right side port (SPr).

For ELYRA type LS systems, the Andor iXon U897 Ultra EMCD camera is mounted to the rtght side port (SPr), whereas the pco.edge CHL sCMOS camera will be mounted to the base port (BP).

For ELYRA type S and eLS systems the pco.edge CHL sCMOS camera will be mounted to the right side port (SPr).

2.3   Performance and Features of ELYRA

Optical and mechanical aspects

The ELYRA 7 family members have only one illumination / detection unit, the ELYRA 7 module, attached to the rear port of the microscope.

The ELYRA module is designed for performing single molecule localization microscopy (SMLM) and/or Lattice structured illumination microscopy (Lattice SIM) SMLM. The ELYRA 7 family consists of ELYRA type L (SMLM only), ELYRA type S (SIM only) and ELYRA types LS and eLS (SMLM and SIM combination).

The highly integrated system design allows combining two super resolution illumination modes, SMLM and SR-SIM in one module. The ELYRA illumination module can be fitted to an inverted Axio Observer 7 SR microscope at the rear port.

The spectral range available extends from the V to the VIS region.

For the VIS (visible-light), the user can select from up to 3 lasers with wavelengths of 641, 561 and 488 nm. For the V (violet light) a 405 nm laser is available. Coupling of the laser light is through polarization-preserving single-mode optical fibers. One variable beam collimator provides adaptation of the respective laser wavelength to the objective used.

Acousto-optical tunable filters (AOTF) adjust the necessary brightness for all 4 laser lines within microseconds. The 405 laser is in addition equipped with neutral density filters that allow tuning the laser power further down.

The implemented cameras, the pco.edge CLHS sCMOS camera for Lattice SIM & SMLM and the Andor iXon 897 Ultra for SMLM, are optimized for the different technologies.

The high-NA Plan Apochromat 63x (NA 1.4), α-Plan Apochromat 63x (NA 1.46) and C-Apochromat 63x (NA 1.2) objectives are especially suited for performing Lattice SIM experiments. The α-Plan Apochromat 100x (NA 1.46) and α-Plan Apochromat 100x (NA 1.57) HI objectives with their back reflection blocker have been specifically designed for SMLM applications minimizing scatter light in TIRF mode.

For ELYRA types L, LS and eLS the TIRF mirror is moveable so the optimal TIRF angle for TIRF-illumination can be set. In ELYRA type S systems, this mirror is in a fixed position for Epi-illumination. ELYRA types S, LS and eLS systems have 5 grids with different grid constants to adapt laser wavelength, objective and penetration depth in Lattice SIM illumination. Galvo mirros are used to move the grids laterally.

The field of view (FOV) in SIM is ~ 75 x 75 μm (63 x objective, 1.6x Optovar, 1280 x 1280 pixel with a pitch of 6.45 μm) using the pco.edge CLHS sCMOS camera. The FOV in SMLM using the Andor iXon 897 Ultra with 512 x 512 pixel (16 μm x 16 μm pixel pitch) is ~ 50 x 50 μm and can be further reduced providing higher power densities.

2.4 Microscope Equipment of ELYRA Systems

The ELYRA 7 systems are equipped with the inverted Axio Observer 7 SR microscope with a modified rear port for the attachment of ELYRA illumination modules.

Only the differences from the delivered operating manual "Axio Observer" will be explained here.

(1) Stand

– The motorized objective nosepiece 5× H DIC is firmly fixed to the stand, where no operating elements can be found for the nosepiece. Operation will be performed via ZEN software control. The "Restriction of the nosepiece height to protect the objectives during motorized objective change" is inactivated. The nosepiece will be moved down automatically before each motorized objective change.

– The reflector mount is motorized and provided with the Axio Observer 7 SR reflector turret. The reflector turret has six positions: One transmitting light position (position 1), which is empty, one or more further positions (position 2-6) for push & click fluorescence filter sets or analyzer/polarizer cubes (reflector module) to be used for optical observation. For ELYRA four of those positions (positions 3-6) are adjusted and used in Lattice SIM and SMLM illumination regimes. For SMLM it is also possible to use a push & click filter set. If you want to use more conventional fluorescence filter sets, you need to employ a further reflector turret. When changing the reflector turret position you must make sure that the turret will click into position, since otherwise the image area will be cut. The reflector turrets are equipped with auto component recognition (ACR), so the actual filter sets will be updated and displayed in the ZEN software.

– The stand has a motorized focusing drive (fine & coarse). Sensitivity of the focusing drive is adjusted to the delivered objectives by the manufacturer but can be modified using the controls on the TFT display of the stand. The stand features an integrated power supply for the internal motors and stand electronics. External power supply units are used for the mercury vapor short arc lamp or the fiber coupled lamp.

– The analyzer slider for conventional DIC methods will be operated from the right side and is located just below the nosepiece.

– When the rod is pushed in, the analyzer is located in the beam path. In the laser widefield modes (Lattice SIM, SIM Apotome, SMLM, TIRF), the analyzer must not be located in the beam path, and the analyzer rod must be pulled out.

– When using SMLM, SIM Apotome and Lattice SIM, do not use a DIC slider.

(2) Specimen stages and fine focus drives

– XY-Piezo Scanning stage for motorized positioning in X and Y.

– Z-Piezo stage insert for motorized positioning in Z. The z-Piezo stage insert is obligatory for SR-SIM illumination. It is highly recommended for SMLM illumination when employing the holding focus. It is not compatible with Temp inserts and CO_2-incubators.

(3)   Transmitted-light illumination

- The illuminator support contains a security circuit which activates a shutter preventing laser light from reaching the stand when the support is moved to the back. A complementary shutter built in the stand prevents laser light from reaching the eyepieces during the scanning mode.

- The illuminator support is optionally equipped with a rotary polarizer. The Axio Observer description contains the adjustment for the DIC mode during conventional observation.

- For scanning in the transmitted-light DIC mode, the polarizer in the transmitted light support works like an analyzer and must be adjusted in such a manner that direct laser light will be blocked.

- The conventional analyzer slider in the stand must not be located in the beam path because the laser light is already polarized.

- The focusing screen for conventional transmitted-light is located in a support in front of the halogen lamp housing.

- Further information on the halogen lamp and the condensers is provided in the Axio Observer operating manual.

(4)   Reflected light fluorescence

For ELYRA mounted to the rear port of the stand the slots for sliders (reflector, iris stop, Apotome, Laser port adaptor) are not available. A fiber coupled lamp has to be used for epi illumination.

(5)   Imaging optics

The Optovar optics are motorized. Optovars in the stand are 1.0 x (for arc lamp widefield illumination and ocular observation) and 1.6 x (for SIM and laser widefield). Optovar sliders cannot be used.

The analyzer for the conventional DIC mode will be operated from the right side and is located just below the nosepiece.

Use of sliders with auxiliary objects (473704/14-0000-000) is not possible.

2.5   Computer Hardware and Software

ELYRA 7 is controlled via a standard high-end Pentium Intel Xeon PC. Linking to the electronic control system is made via Gigabit Ethernet interface. The PC comes with the WINDOWS operating system.

The instrument is fully motorized, permitting fast change-over between methods as well as automatic operation. Parameters once set or complex examination sequences once established can be saved and reproduced; therefore, complete application programs can be loaded and performed by pushbutton control.

The software of the ELYRA 7 runs on the ZEN (black edition) platform.

Conversion of the light signals into a digital image is effected by means of four 12-bit A/D converters; each of it can generate 4096 brightness levels.

The software provides an enormously wide range of image processing functions, including all standard 2D / 3D (stereo, projection) functions identical to sophisticated 3D reconstruction capabilities (surface and alpha rendering)and digital processing of voxels.

3   Startup and Shutdown of the System

3.1   Startup of the System

ELYRA 7 systems are equipped with three Multipoint connectors that are plugged via different sockets of the Electronic Control Unit.

The systems are equipped with a **Main** power switch on the Electronic control Unit (ECU). There is a further remote switch labeled **Components** that is connected to the ECU.

The **Main** switch has to be set to **ON** to be able to switch the system on and off. Components can be switched on and off seperately by using the **Components** switch. The **Main** switch can be used to switch off the complete system with one switch only.

The electronics to run the computer and the microscope are switched on with the **Main** switch. The laser module has its own key switch that has to be in the active position.

In order to be able to have access to the software, you have to switch the system on and start the computer with the following **Booting** procedure.

- Switch on the system via the **Main** switch of the ELYRA 7 system.

- Switch on all three Multipoint connectors if they are in the Off position.

- Switch on any device that is in the Off position of its own power supply switch.

- Switch on computer.

- Start ZEN software by double clicking the ZEN icon.

ZEN icon

Start ZEN software for operating the ELYRA and the microscope.

System Maintenance Tool icon

With a calibration objective and correct system configuration, the maintenance tool allows convenient self adjustment of the systems. The optical beam path, relative pinhole position and scanner adjustment can be set and checked automatically on the systems.

☞   Please not that the System Maintance Tool (SMT) is in case of ELYRA systems only available for service.

ZEN Configuration Tool icon

The **Configuration Tool** permits a new or updated database to be assigned to the ZEN software program. This function should preferably be performed by authorized service personnel. See CHAPTER 3: ADDITIONAL SOFTWARE TOOLS.
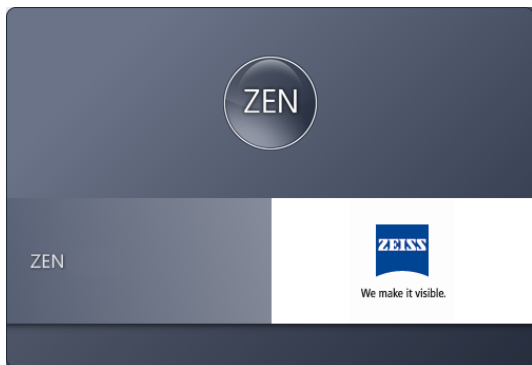
Fig. 8   **Starting the ZEN software –
                   The Loading "ZEN" window**
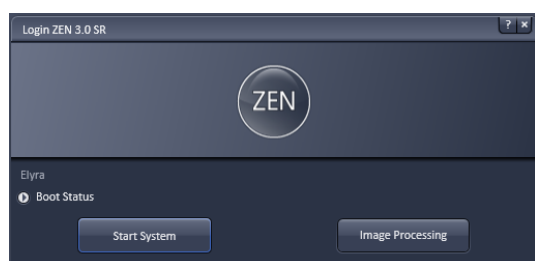
Fig. 9   **Starting the ZEN software –
                   The Login "ZEN" dialog**
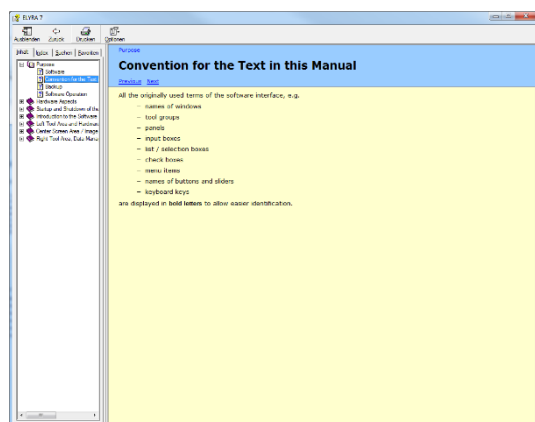
Fig. 10   **The Help menu dialog**

3.1.1   **Starting ZEN**

- To start the software double click the **ZEN** icon on your desktop!

  – During loading the program the **ZEN Access** window will appear (Fig. 8). It displays the ZEN symbol in the upper part, the designation of the current ZEN software version (lower left) and the Zeiss icon (lower right).

  – Once the program is loaded the **Login ZEN** dialog will appear (Fig. 9) in front of the ZEN interface. The number after ZEN designates the current version.

- By pressing the **? (question mark)** symbol, the context sensitive **Help menu** will appear (Fig. 10). Close the menu with the Windows commands if you do not want to search the help database by pressing the **x (cross)** symbol.

- Pressing the **x (cross)** symbol, the **Login ZEN** dialog will be closed and the ZEN software will not be started.

The **Login ZEN** dialog allows you to boot the Software in different modes. If the **Boot Status** is closed, the options are **Start System** and **Image Processing**.

A third option, **Offline/Demo**, becomes available (Fig. 11), once the **Boot Status** field is expanded by pressing the **expansion** symbol (triangle).

You can collapse the field by pressing the expansion symbol again.

Select:

– **Start System** for system control, data / image acquisition / recording and analysis. The whole microscope system will be initialized. The progress of the boot process will be displayed by the blue time tab. During booting the status of the system will be displayed in the **Boot Status** window and the single components will be listed which have been or are currently being checked during the boot process (Fig. 11).

– **Offline/Demo** for data analysis and simulation. **Offline/Demo** reads the active hardware database and simulates the respective hardware. This button is only available after opening the **Boot Status** display by clicking the little arrow left of the text "Boot Status" This option should be chosen when working at an offline station and if you want to test functionalities of the system. The progress of the boot process will be displayed by the blue time tab. The single components will be listed, which are checked during the boot process. Note that the software will not control the hardware in this mode.

– **Image Processing** for data analysis only. This mode ignores the hardware and activates only data handling and image processing functionality to analyze stored images. Note that only those software tools will be displayed that are needed for processing and analysis of the data. No hardware control tools will be shown.

– **Cancel** to abort the booting process and to leave the dialog. Note that the **Cancel** button will appear in the place of the **Image processing** button only if **Start System** or **Offline/Demo** was selected (Fig. 12).

– You can leave the software by choosing the **File** menu from the main tool area and selecting **Exit**.

– You can select a database before booting, if you wish to change the current one. To change the database open the **Hardware configuration database** expansion field (Fig. 13). The expansion field displays the currently loaded database and its location on the hard disc.
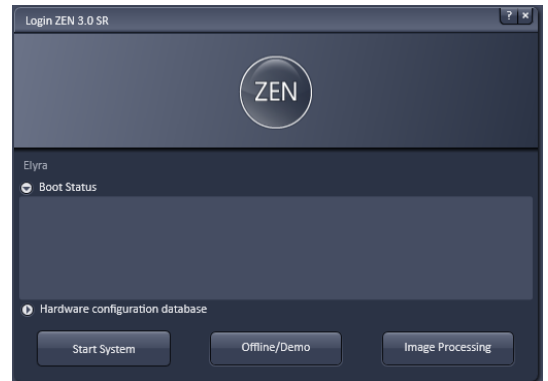
Fig. 11   **Starting the ZEN software – The Login "ZEN" dialog with Boot Status field expanded**
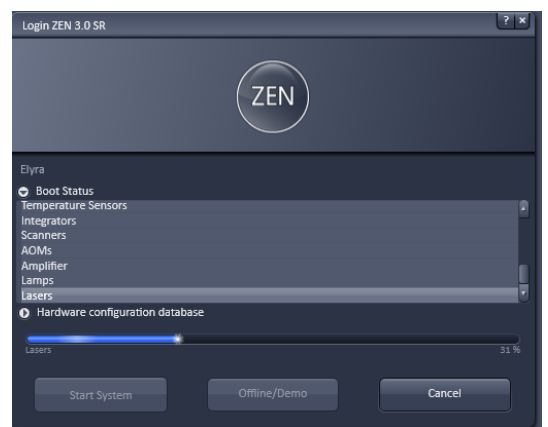
Fig. 12   **Starting the ZEN software – The Login "ZEN" dialog with Boot Status field expanded and during the process of booting**
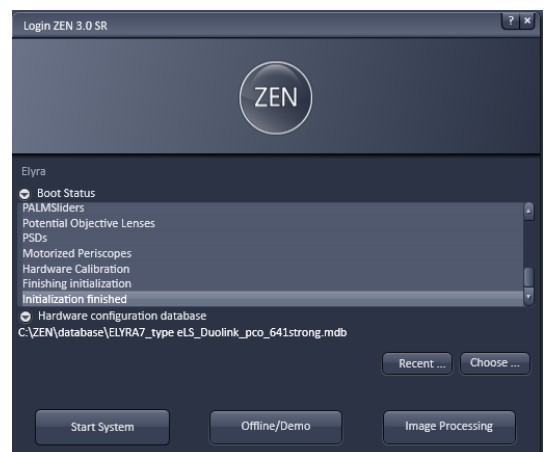
Fig. 13   **Hardware configuration database - The Login "ZEN" dialog allows you to boot the Software in different modes. If the Boot Status is closed, the options are Start System and Image Processing.**

– If **Recent...** is pressed the last couple of databases that were in use will be displayed (Fig. 14). Click on the one you want to select. By pressing the **Choose...** button Windows Explorer will open. Search for the folder file the database is stored and and open it. Select it for use. Select a database suited for your purpose from the Explorer window.

– You can also change the database after having booted the system. To this end choose from the **File** menu of the main tool area **Login...** and the **Login ZEN** dialog will appear. Select the database by pressing **Choose...** or **Recent...** and the system restarts.
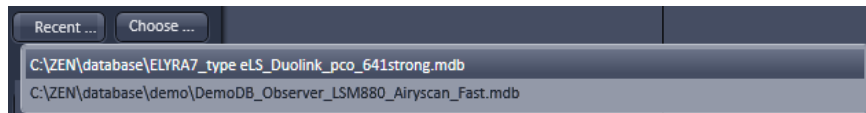
Fig. 14   **Recent databases**

3.2   Shutdown Procedure

⚠ Never shut down the computer by its main switch while the ZEN software is still active, or else you will lose the currently set operating parameters and the images just scanned.

☞ In the **Maintain** tab / **System Options** dialog window, activate **Laser off** on **Exit** in the **Shutdown** tab (section **Options tool group –** System Options).

3.2.1   Exiting ZEN Software
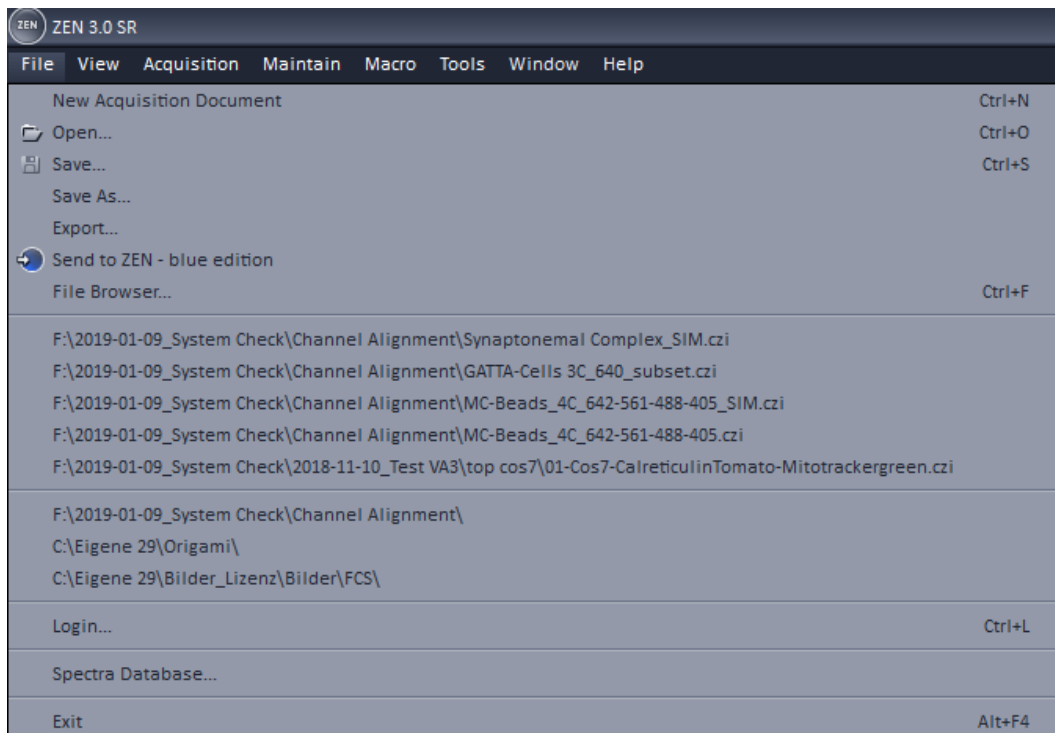
Fig. 15   Exit in the File menu

The software can be shut down from **File** menu of the Main toolbar by selecting **Exit** (Fig. 15) or by pressing the **Window Close** button (symbolized by a cross) of the ZEN window.

If in **Maintain** tab in the **System Options** dialog window the box **Laser off on exit** in the **Hardware** tab is ticked, the lasers will be automatically switched off when exiting the ZEN program.
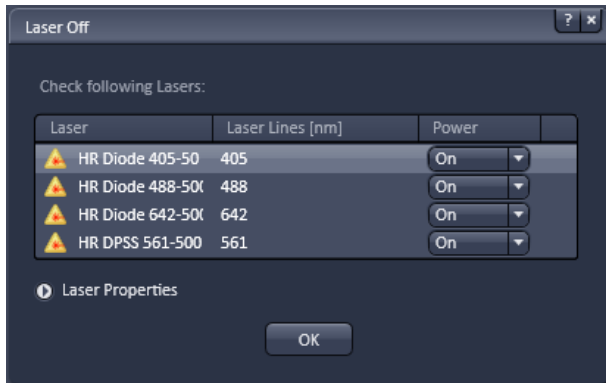
Fig. 16   Laser Off dialog box

If the box is not ticked, the **Laser Off** dialog box will appear that allows one to check on the listed lasers (Fig. 16).

By the **Power** drop down menu, if available, the laser can be left **On**, put in **Standby** or turned **Off**. Those lasers, for which no **Power** drop down menu is available, are switched off automatically when not in use. By expanding the Laser properties field you can view the current status of the selected laser. Press **Ok** after having made the desired changes to exit the ZEN software.

3.2.2   Switching System Power Off

After having exited the ZEN software, you may turn off the whole system with the following **Shutdown** procedure.

- Make sure you have saved all your image data.

- Switch of all the lasers which might still be running.

- Close the ZEN software window. This terminates the ZEN software.

- Shut down WINDOWS.

- About 20 seconds after WINDOWS is shut down your computer turns off.

For ELYRA systems you can now switch off the complete system by switching the **Main** switch to the off position.

Alternatively

- Switch off any device which have its own On/Off switch

- Switch off multipoint connectors

- Switch off lasers by the laser key switch

- Switch off system by the Main switch of the ECU

4   Introduction to the Software Application Layout

4.1   Overview on the Screen Layout
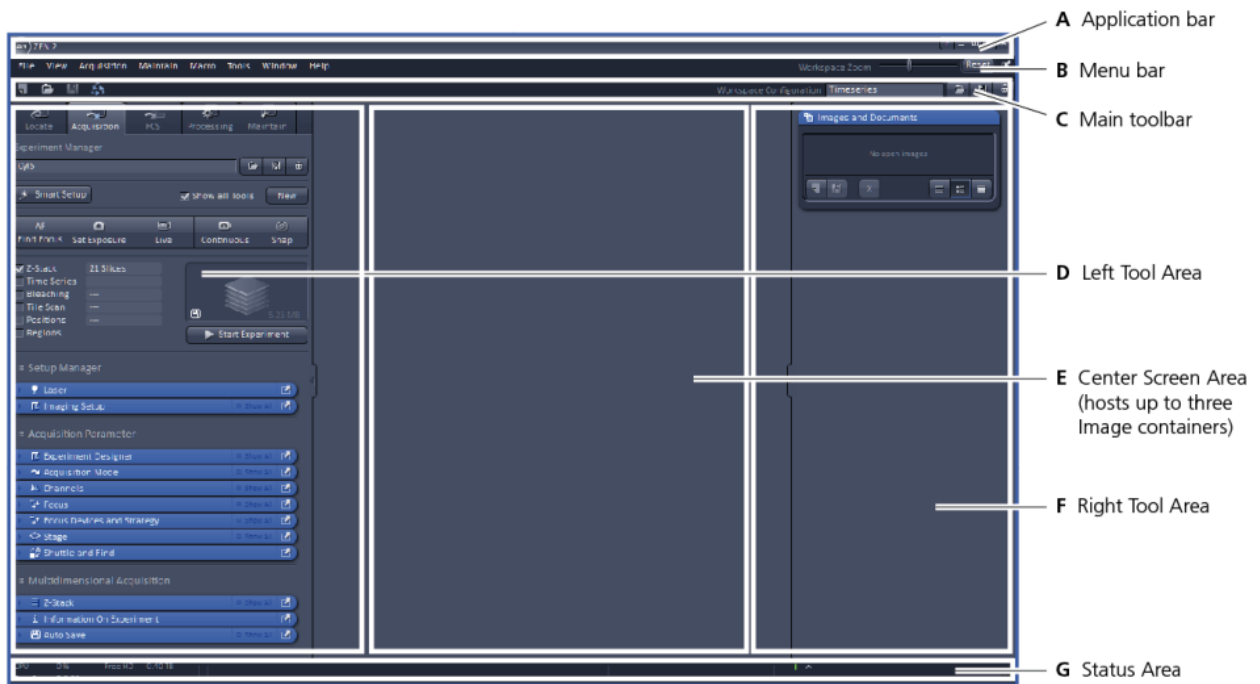
Fig. 17   ZEN Main Application window after Startup with empty image container

1 Main tool tabs	**4** Mutidimensional tool selection panel	**7** Tool	**10** Image tabs	**13** View controls
2 Configurations	**5** Starting multidimensional experiments	**8** Status bar	**11** Displayed image	
3 Action buttons	**6** Tool group	**9** View tabs	**12** File handling area	
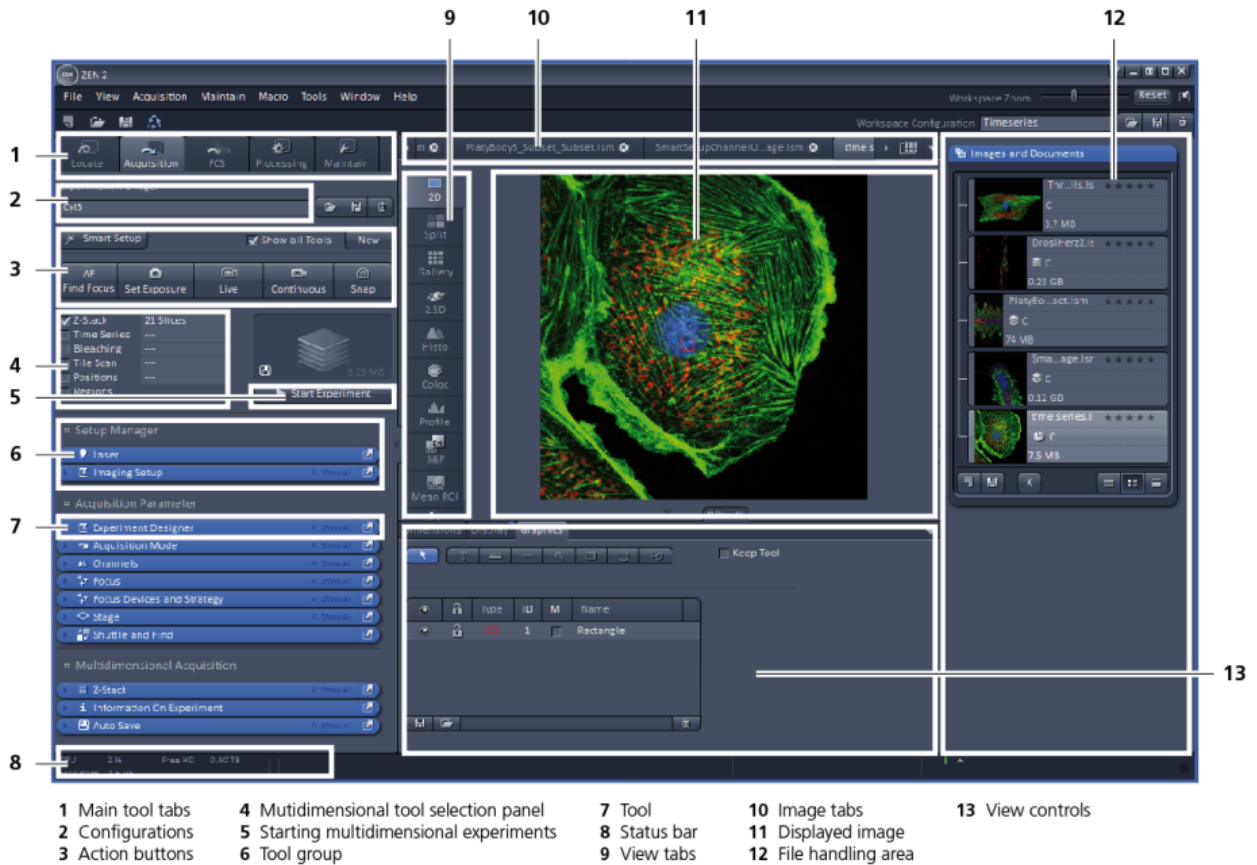
Fig. 18   ZEN Main Application window after Startup with several images loaded

4.2 Introduction to ZEN

ZEN - Efficient Navigation - is the new software for the LSM/ELYRA Family from ZEISS. With the launch of this software in 2007 ZEISS sets new standards in application-friendly software for Laser Scanning Microscopy.

The ZEN Interface is clearly structured and follows the typical workflow of the experiments performed with confocal microscopy systems:

On the **Left Tool Area** (Fig. 17/**D**) the user finds the tools for image acquisition, image processing and system maintenance, easily accessible via the **Main** tabs (Fig. 18/**1**). All functions needed to control the microscope can be found on the **Locate** tab, to acquire images use the **Acquisition** tools (Fig. 18/**3** and **4**). Arranged from top to bottom they follow the logic of the experimental workflow. The area for viewing and interacting with images is centered in the middle of the **Main Application** window: the **Center Screen Area**. Each displayed image can be displayed and/or analyzed with many view options available through "view tabs" which can be found on the left side of the image. According to the chosen view tab, the required view controls appear in View control blocks below each image. File management and data handling tools are found in the **Right Tool Area** (see Fig. 17 and Fig. 18).

Color and brightness of the interface have been carefully adjusted to the typical light conditions of the imaging laboratory, guaranteeing optimal display contrast and minimal stray light for high-sensitivity detection experiments. The **ZEN** software is optimized for a 30" TFT monitor but can also be used with dual-20" TFT setups.

A focus in the development of **ZEN** was to fulfill the needs of both basic users and microscopy specialists. Both types of users will appreciate the set of intuitive tools designed to make the use of a confocal microscope from ZEISS easy and fast:
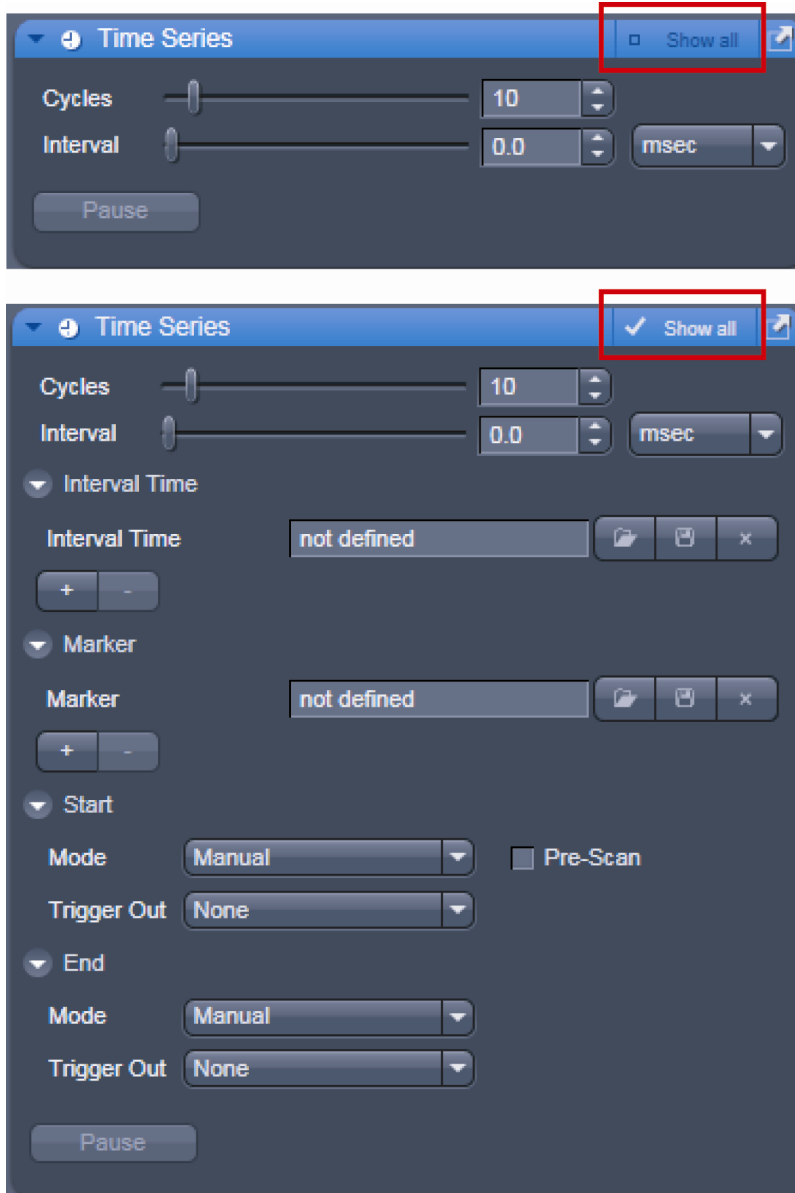
Fig. 19   Show all mode

The **Show all** concept ensures that tool panels are never more complex than needed. In the basic mode of the tools **Show all** is deactivated and the tools show only the most relevant functions, covering approximately 80% of the application. For each tool, the user can activate **Show all** to display and use additional functionality (Fig. 19).
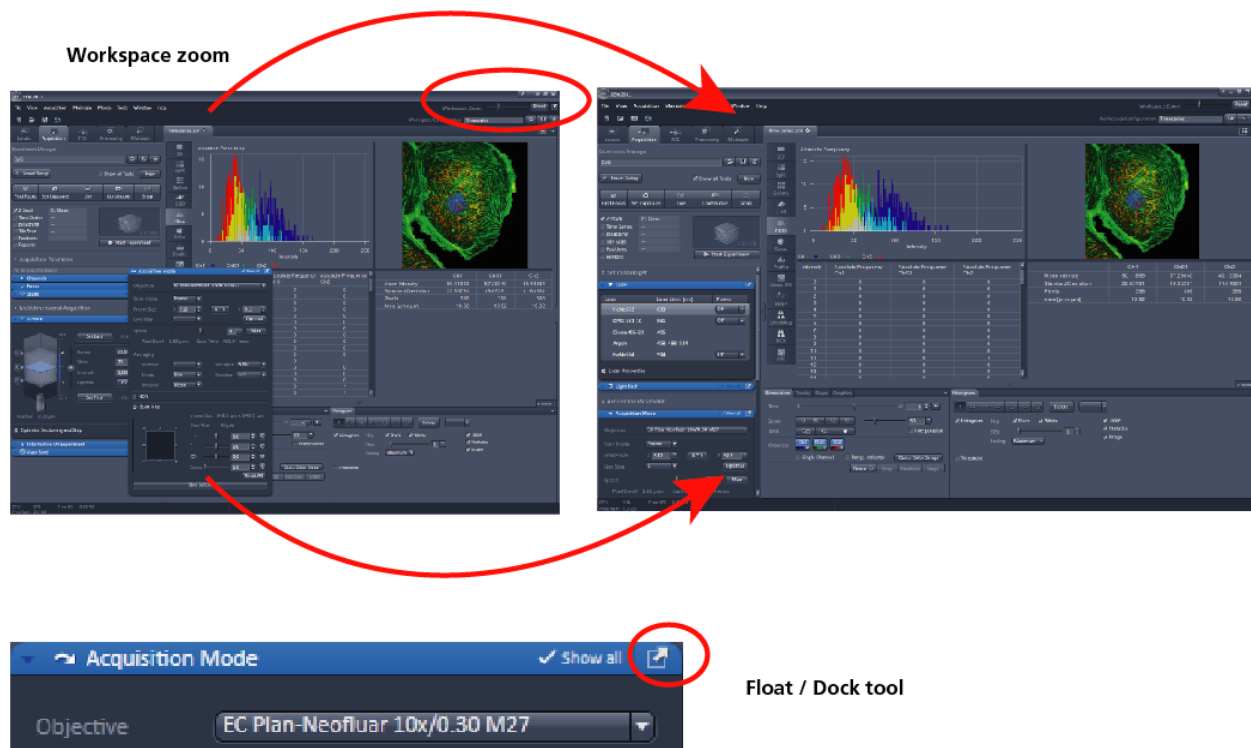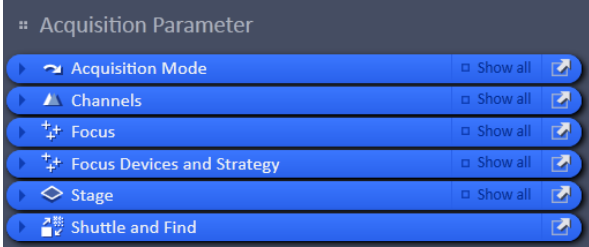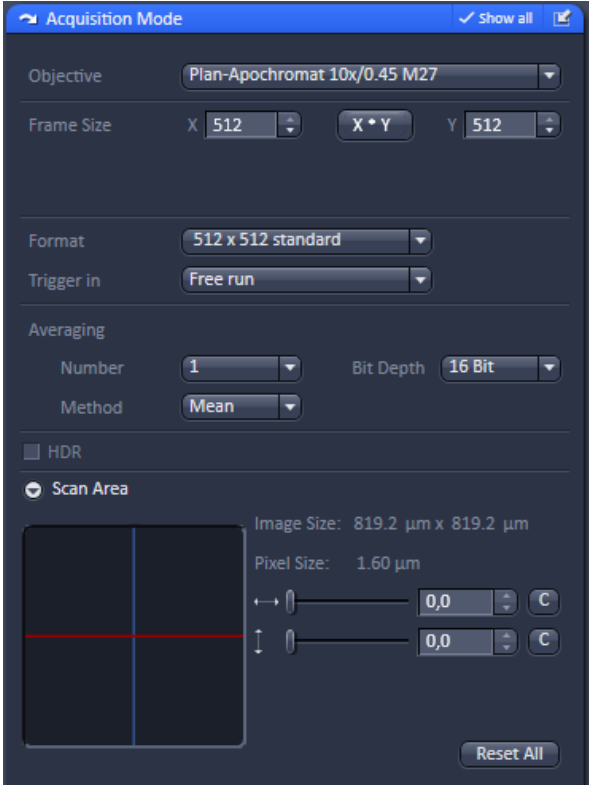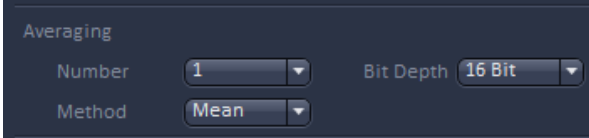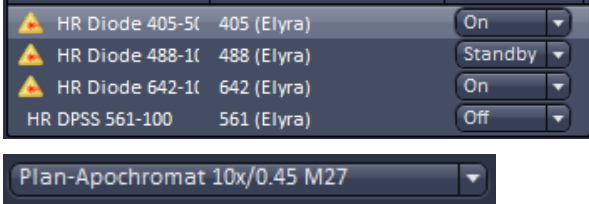
Fig. 20   **ZEN window Layout configuration**
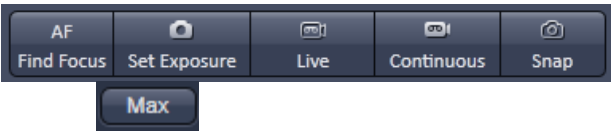
More features of **ZEN** include:

- The user can add more columns to the **Left Tool Area** or detach individual tools to position them anywhere on the monitor. To add a column, drag a **tool group** by the title bar (e.g. "Acquisition Parameter") to the right and a new tool column automatically opens. To detach a tool, click on the little icon on the very right end of the blue tool header bar or drag it by this blue header bar to where you want it (Fig. 20).

- Another feature is the scalable ZEN interface. This **Workspace Zoom** allows adjustment of the **ZEN** window size and fonts to the situational needs or your personal preferences (Fig. 20).

- Setting up conventional confocal software for a specific experiment can take a long time and is often tedious to repeat. With ZEN these adjustments have to be done only once – and may be restored with just two clicks of the mouse. For each type of experiment one can now set-up and save the suitable **Workspace Layout**. These configurations can also be shared between users.

- For most controls, buttons and sliders, a **tool tip** is available. When the mouse pointer is kept over the button, a small pop up-window will display which function is covered by this tool/button.

4.3   Function Elements

Function element	Description / Explanation
	Tool group
	Tool (closed) or tool window (opened)
	Panel (e.g.: **Averaging** panel) – Field with a subset of tools of a tool window
	List box or selection box – Selection of one of the displayed options via a mouse click. The selected option is highlighted. – Open the box by clicking on the arrow button.
	Input box with arrows ("spin box") – Input of text or numeric values via the keyboard or using the arrows.

Exposure Time [ms] ——— 100,00	Slider with input ("spin") box and arrows – Setting of numbers in the relevant input box by moving the slider or clicking on the arrow buttons or clicking on the slider and moving via the arrow keys of the keyboard. Press the **Shift** or **Ctrl** key while clicking on the arrow button to change the numeric values in coarse or fine steps.
AF Find Focus / Set Exposure / Live / Continuous / Snap / Max	Button – Selection / performance of a function via mouse click.
Dimensions Display Player Graphics	Tab – Selection of functions of a tab via mouse click (e.g.: **Dimensions** or **Display** tab).
	Load – **Save** – **Delete** – Buttons to load, save or delete a configuration, overlay set, track setting etc.
	New – **Save** – **Close** – Buttons to open a new acquisition document, save an image document or close it.

4.4   Application Bar

The application bar includes the following control elements of the ZEN software application window:

?	Starts the online help
–	Minimizes the application window
⊡	Maximizes over 2 screens
⧉	Switches between maximized-window mode and compressed-window mode
✕	Closes the window

4.5   Menu Bar

The menu bar contains the menu items and the controls for the **Workspace Zoom**, including the **Reset** button for the **Workspace Zoom** and the button to re-dock all free floating tools to the **Left Tool Area**.
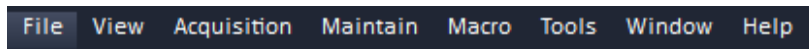
Fig. 21   **Menu bar, left side**

The menu functions are shown in the following menu overview (see next page).

Workspace Zoom

Moving the slider to left or right, changes the display size of the Left/Right Tool Area and the Center Screen Area.

Clicking the **Reset** button, sets the standard zoom factor for all areas.

Fig. 22   **Menu bar, right side**

Clicking on moves all undocked tools back to the **Left Tool Area** (see also section **Tool Groups and Tools**).

Menu overview

File

New Acquisition Document
Opens a new and empty image document.

Open...
Opens an existing document in the active image container.

Save...
Saves the active document.

Save As...
Saves the selected image with entering new formats.

Export...
Exports the currently active document using varius image formats.

Send to ZEN - blue edition
Sends the currently active document to ZEN blue edition.

File browser...
Opens a document containing a ZEN File Browser.

Open containing folder
Opens the folder, where the image file is saved.

Login...
Opens the Login ZEN dialog to change the software operation mode.

Spectra Database...
Opens the Spectra Database.

Exit
Closes the ZEN software.

View

Text View
Displays the image tabs with text only.

Small Thumbnail View
Displays the image tabs with small thumbnails.

Large Thumbnail View
Displays the image tabs with large thumbnails.

1 Container
Sets ZEN to work only with one image container.

2 Containers
Sets ZEN to work with two image containers.

3 Containers
Sets ZEN to work with three image containers.

Automatically layout container
The size of the containers is automatically adjusted depending on the screen space available.

Shared view Controls
Shares the view controls below the images between containers.

Separate view Controls
Each container is displayed with a separate view control panel.

"Show all" (global)
Sets all windows, tools, and control panels to "Show all", all controls are always visible in all tools.

Acquisition

New Acquisition Document
Opens a new and empty image document.

Set Exposure
Starts the Find procedure. This balances Gain settings.

Find Focus
Starts the auto focus. A Line / Z Scan is performed and the focus position is determined upon the contrast values.

Scan Overview Image ...
Scans an overview image

Live
Starts the fast scanning procedure, scans with lower resolution and higher speed.

Countinuous
Scans an image continuously with the currently active scanning parameters.

Snap
Scans one single image.

Start Experiment
Starts a multidimensional experiment (Z, T, ...).

Stop
Stops the scanning immediately.

Maintain

Camera
Opens the Camera Color Adjustment tool.

Remote Access Settings
For controlling LSM functions from iPad.

Hardware Administrator
For service only.

Test grid
For service only.

Macro

Macro
Opens the Macro Control window for creating, loading or handling macros.

Visual Macro Editor
Opens the Visual Macro Editor.

Tools

Paste Image
Pastes an image from clipboard for saving.

Window

Close
Closes the active window.

Close All
Closes all windows, a seperate save dialog will be displayed if images have not been saved.

Help

Help
Opens the ZEN help.

About
Opens a panel with information about the ZEN release.

☞　For detailed information about the image acquisition functions see section **Acquisition Tab**.

☞　The functionalities of the **Macro** menu are described in CHAPTER 2: MACROS AND VISUAL BASIC.

4.5.1　File

- In the menu bar, click on **File**.
    - This displays the menu shown in Fig. 23.
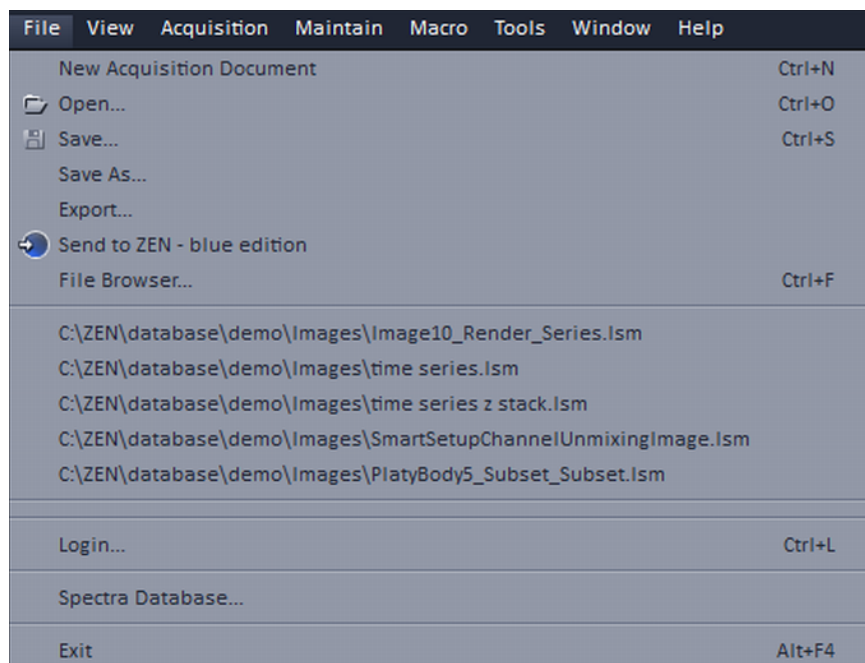
Fig. 23　　File menu

The items in this list reflect the standard windows files menus with the following exceptions:

- **File Browser** will open a ZEN File Browser in the currently active container (see section **ZEN File Browser**).

- In the second group of entries in the list, the five last accessed files are listed. Selecting a list entry will open the respective file.

- In the third group of entries, the four last accessed folders are listed. Selecting a list entry will open the ZEN File Browser and display the image files which are present in the folder.

- **Login** displays the **Login screen** and permits to change the operation mode of ZEN, e.g. from **Online** (with hardware started) to **Offline** (Image Processing or Demo mode), without quitting ZEN.

- **Spectra database** is starting the dialog used to handle previously recorded reference spectra.

☞　See also section Right Tool Area, Data Management and Storage for image file handling.
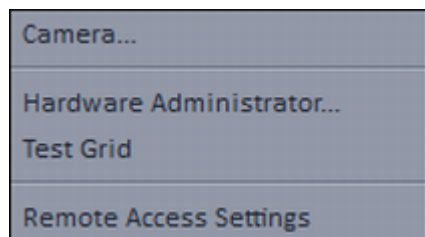
Fig. 24   Maintain list

4.5.2   Maintain

The **Maintain** menu from the **Menu** bar contains additional modules to check and guarantee the interference-free operation of all the software and hardware components of the systems. In the **Menu** bar, click **Maintain**. This opens the Maintain list (Fig. 24).

Some of the tools are only accessible by Service personnel (Hardware Administrator and Test Grid).

☞ See also section Maintain Tab for further information.
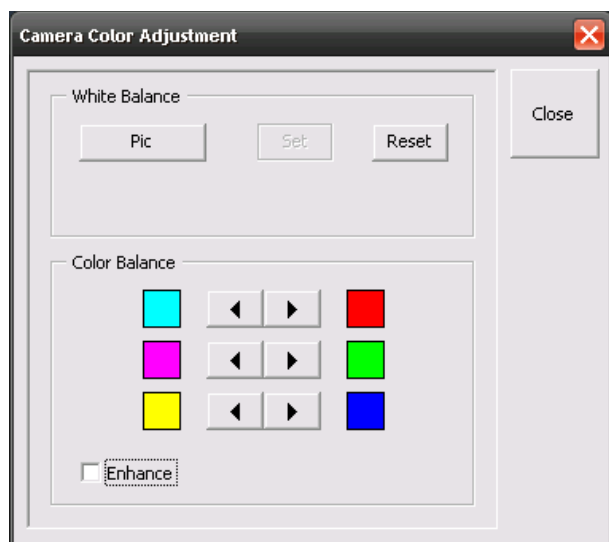
Fig. 25   Camera Color Adjustment window

4.5.2.1   Camera

This function allows the user to adjust the white balance and color balance of a connected camera.

- Click on **Camera** in the **Maintain** list.
  - The **Camera Color Adjustment** window appears on the screen (see Fig. 25).

Clicking the **Pic** button allows to set the white balance using the mouse cursor in the camera image. The **Set** button will become active. Press set to store new settings, press **Reset** to return to the old settings.

Use the arrow buttons to adjust the color balance of the camera.

4.5.2.2   Hardware Administrator

The **Hardware Administrator** function is for servicing purposes and may only be used by authorized service personnel. Its access is therefore password-protected.

4.5.2.3   Test Grid

The **TestGrid** function is for servicing purposes only and may only be performed by authorized personnel. Its access is therefore password-protected.

4.5.2.4   Remote Access Settings

The **Remote Access** tool (Fig. 26) allows you to control the ELYRA functions remotely from your iPad provided it is registered within the same (local) network as the ELYRA system.

Enable the Remote Access and define a user name and password for the access which then must be used to login form the iPad. Download the "ZEISS Remote" App from the App-Store and install it on your iPad.

A short tutorial comes with it to show you the first steps. Make sure that you connect to the right IP address of the system, namely the one that refers to the internet connected part.

Check with your local IT coordinator, IP addresses might even vary.
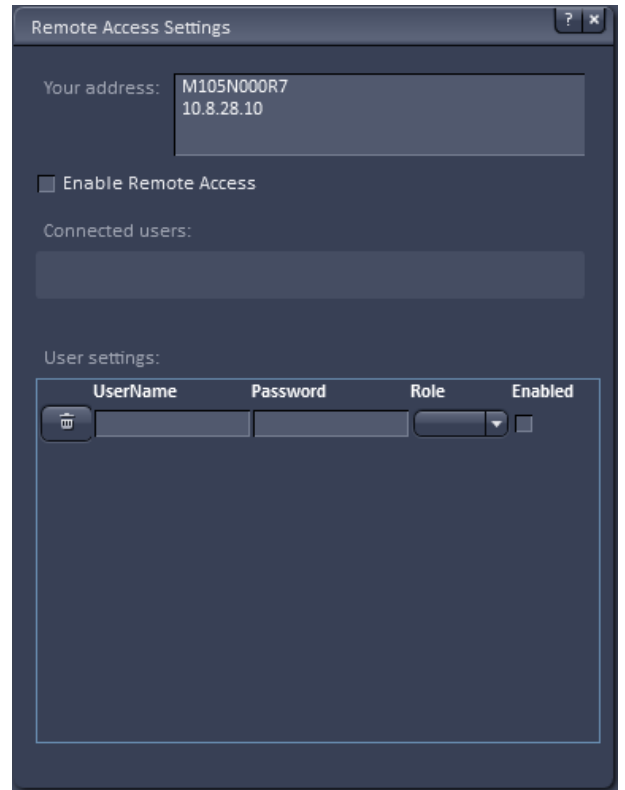
Fig. 26   Remote Access Settings window

4.5.3   Help – About

The **About** window can be accessed via the Help item in the menu bar clicking **About** opens the following dialog:
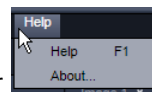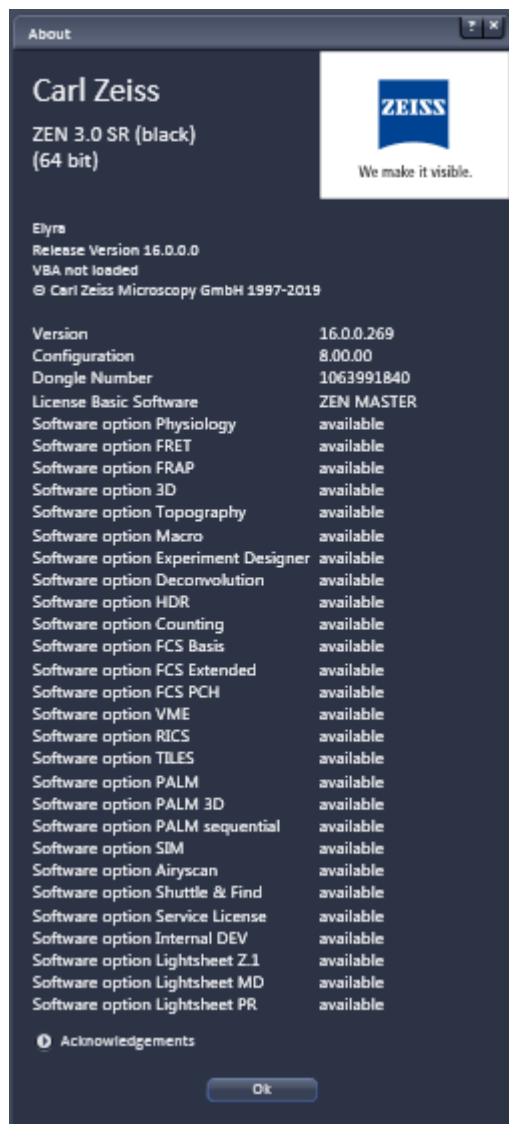
Fig. 27   **About window**

The panel hosts important information about the software version number, the license number and the available software modules.

4.6   Main Toolbar
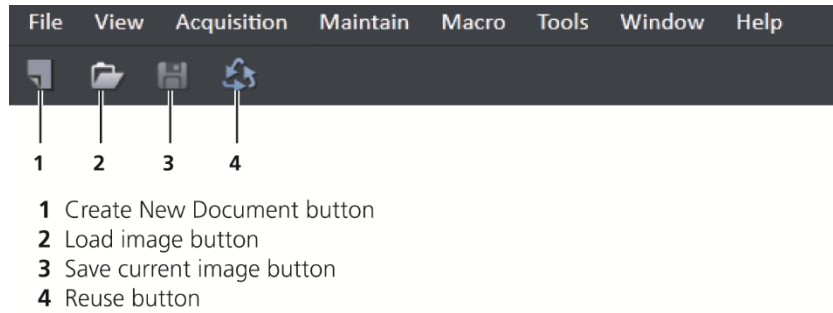
1 Create New Document button
2 Load image button
3 Save current image button
4 Reuse button

Fig. 28   Main toolbar, left side
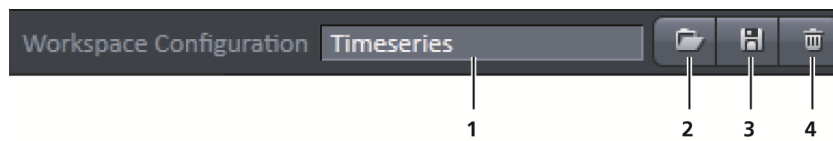
1 Workspace Configuration display box
2 Load Workspace Configuration button
3 Save Workspace Configuration button
4 Delete Workspace Configuration button

Fig. 29   Main toolbar, right side

Workspace Configuration

This function allows loading, saving or deleting a workspace configuration. These workspace configurations are saved settings that allow to restore a pre-defined layout of the whole ZEN application window, including status, size and position of tools and windows, workspace zoom, number of tool columns.

- To save a workspace configuration, click on the button, the following dialog will appear:
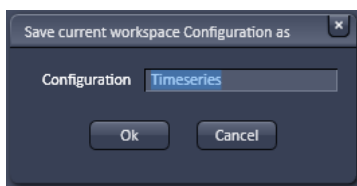
The name of the workspace configuration can be entered. Clicking **OK** saves the configuration.

- To load a workspace configuration, click on the [icon] button. The following list box will appear:
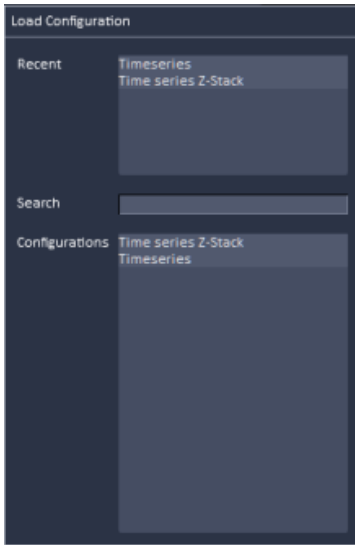
Selecting on of the list entries will load the respective configuration.

- To delete a workspace configuration, click on the [icon] button. The configuration to be deleted can be selected from a list.
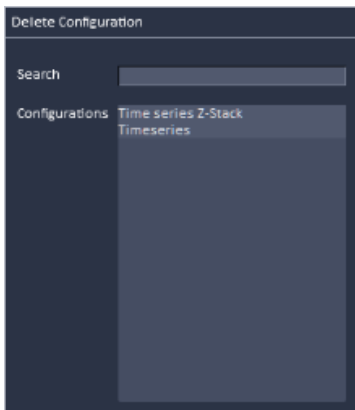
4.7   Status Bar

Fig. 30   Status bar

1 Progress bar
2 System information
3 Position and pixel intensity
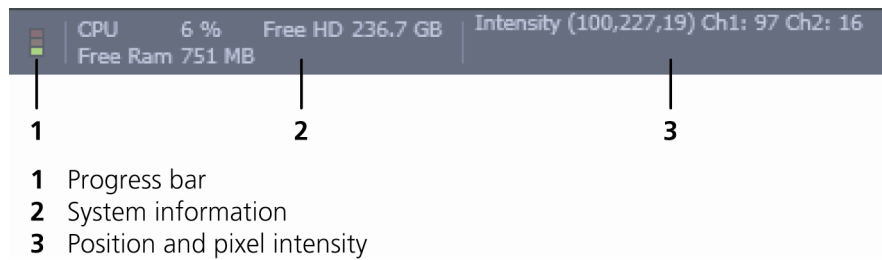
Progress bar

Shows an overview of all running processes. If only one process is running the details of this one are shown. If more than one process is running then the single process information will be shown in the gallery.

Fig. 31   Progress bar

System information

Shows information of the currently system tasks (state). The possible states are:

– CPU usage

– free hard disc capacity

– free RAM capacity

Position and pixel intensity

Shows the intensity values of the existing channels for the current X, Y and Z position of the mouse cursor in the image.
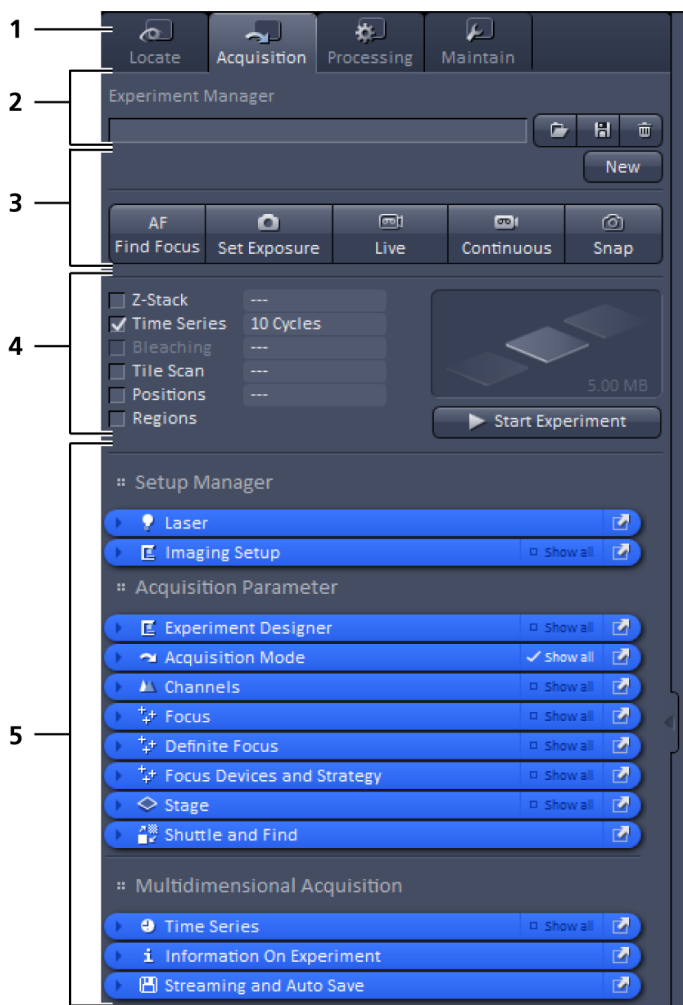
4.8 Left Tool Area

Main Tool tabs

Switches between the **Locate**, **Acquisition**, **Processing** and **Maintain** main tools to operate the included tool

Action buttons

Action buttons are only available for the **Acquisition Main** tool to control the image acquisition process.

Tool groups

Groups special tools, e.g. **Setup Manager** or **Acquisition Parameter**.

Setup Manager

Those tools are necessary if the system should be operated in full manual mode. The use of the automated functions of ZEN like smart setup includes the functions of those tools.

Acquisition Parameter

Tools continuously needed for all acquisition procedures.

1 Main tool tabs
2 Experiment Manager
3 Action buttons
4 Selection panel for multidimensional acquisition
5 Tool groups

Fig. 32   **Left tool area**

Fig. 33   **Expand slider with arrow button pointing left to collapse and right to expand**

Multidimensional Acquisition

Tools for all multidimensional imaging

The tools of this tool group will only appear if selected in the selection panel below the start buttons. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after hitting the **Start Experiment** button. The experiment type is represented above the button by a graphic.
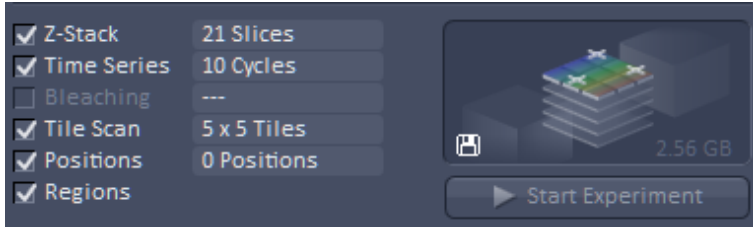
Tools / tools window

Used for setting the microscope and software functions.

Experiment Manager
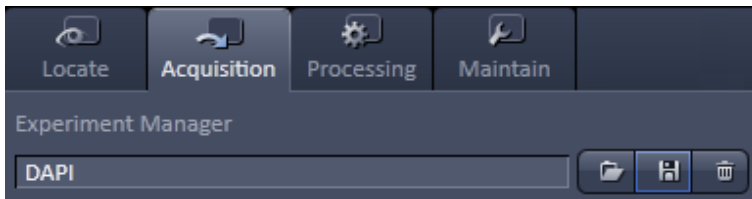
This function allows selecting an Experiment Manager imaging configuration. The configuration can be chosen in a list box. Clicking on a configuration loads the selection.
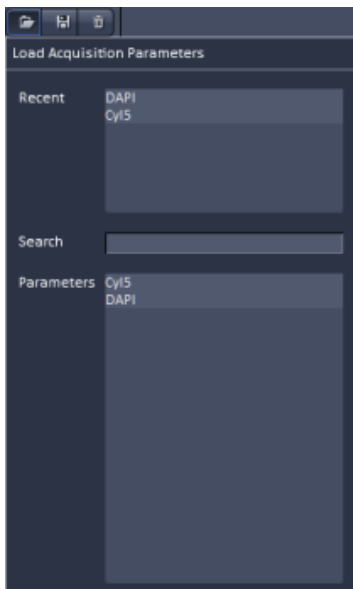
4.8.1 Tool Groups and Tools

Multiple Columns Layout of tool groups

This function allows moving one or several **tool groups** in a second or third column of the **Left Tool Area**. This is done by dragging and dropping the **tool groups** header. The second column opens automatically if a group is moved and closes if all groups are removed from it.

Undock function of tool windows

This function allows undocking and moving one or several tool windows from its home position in the Left Tool Area and further drags it to any position on the Monitor. This can be done by clicking the 🔲 button. The tools can also be undocked by simply dragging the header bar of the respective tool to the desired position. To place a tool back to its home position, click the 🔲 button again, drag the tool back to the **Left Tool Area** or click on the grey place holder at the home position of the tool. The last option is particularly useful if the screen gets too crowded and one can't find the tools any more. To float all tools back with one click use the 🔲 button in the top right corner of the menu bar.

To position the tool window freely on the desktop, use the drag and drop function.

Open a tool window

Clicking on the tool header opens or closes the tool pull down window. The state is indicated by a changing icon (🔽, ▶️) left hand in the tool window header.

If a tool is undocked from the **Left Tool Area** (float function) it will be opened automatically.

Show all function of the tool window

Clicking on the [□ Show all] button shows or hides additional functions of the tool.
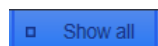
[□ Show all]   Only the basic functions are shown; this functionality is adequate for most applications.
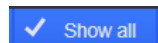
[✓ Show all]   All the functions are activated. The full functionality of the tool is available.

Activation of tools with respect to a multidimensional image acquisition functions of the tool window

The tools for multidimensional image acquisition can be activated and selected by ticking the respective tick boxes in the selection panel:
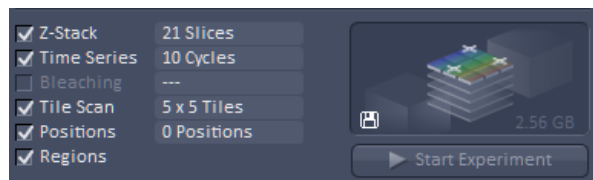
Clicking on the ☑ check box of the appropriate tool selects, activates and displays the tool for multidimensional image acquisition. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after hitting the **Start Experiment** button. The experiment type is represented above the button by a graphic.

4.8.2 Context Menu of the Left Tool Area

There are 2 different context menus in the Left Tool Area with different functionalities available.

New Acquisition Document opens an empty acquisition document in the active image container.

Find Focus starts the Auto focus. A Line – Z Scan is performed and the focus position is determined upon contrast values.

Set Exposure starts the **Find** procedure. This balances Gain settings.

Scan Overview Image … opens the **Scan Overview Image** dialog to set the parameters for an overview scan. Clicking the **Scan** button starts the procedure.

Live starts the fast scanning procedure – scans with lower resolution and higher speed.

Continuous scans an image continuously with the currently active scanning parameters.

Snap scans one single image.

Start Experiment starts a multidimensional experiment
(Z, T, ….).

Stop stops the scanning immediately

Fig. 34 **Context menu of multidimensional acquisition area**

Auto Close Mode means that the "oldest" open tool window will be closed automatically if opening a new one leads to a full tool column.

Right mouse click in the Left Tool Area opens a context menu to activate or deactivate the **Auto Close Mode**. A de-activated **Auto Close Mode** will lead to a scrollbar on the right hand side of the Left Tool Area, once a tool column is filled with open tool.

Move tool group to next column shifts an entire tool group to the adjacent column.

Fig. 35 **Context menu of the tool groups area**

4.9 Center Screen Area

The Center Screen Area is used for displaying scanned images or to show images in the available image views. Using the context menu of the Center Screen Area by clicking the background of a container, the view of this area can be varied individually.

The Center Screen Area can be split in a 1, 2 or 3 container view. So, it is possible to show several images in parallel.

Images can be moved from one container to another using the drag-and-drop function.

4.9.1 Overview of the Center Screen Area
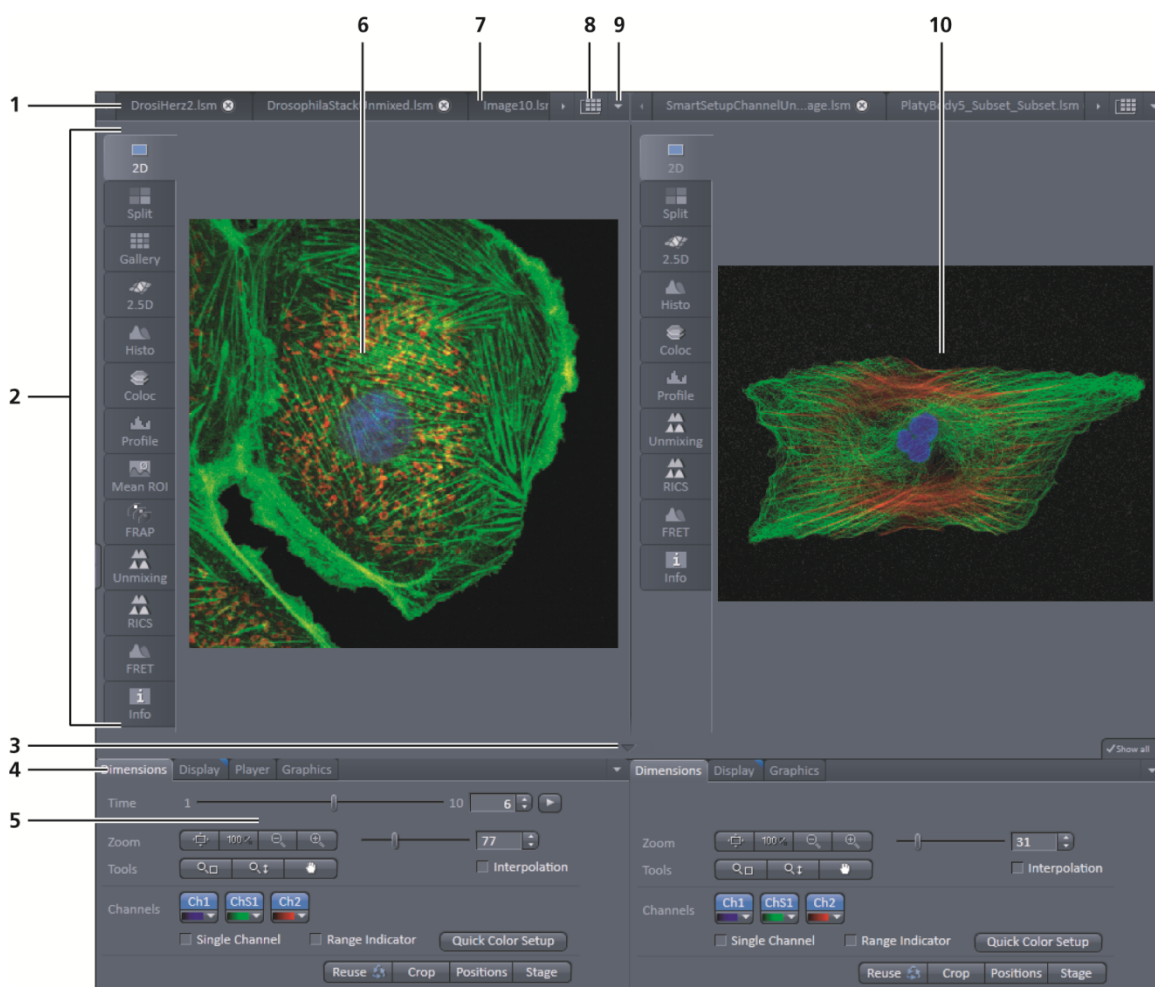
1 Image tabs with scroll arrow buttons left and right of container 1
2 Image view tabs of container 1
3 Arrow down or arrow up button to hide or show the image control area
4 Image view controls
5 Image control area
6 Image container 1 (with opened image window)
7 Background area of container 1
8 Expose mode button (overview)
9 Arrow down button for fast image selection
10 Image container 2

Fig. 36   Center screen area

Image tabs

For each opened image one image tab is shown in the header of the actual container. Three modes can be selected using the context menu of the **Center Screen Area** (see also section **Context Menu of the Center Screen Area**).
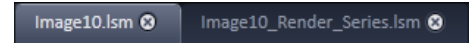
Fig. 37   Image tabs

Image view tabs

Used to select one of the various image views (see also sections **2D View** to **Information View**).
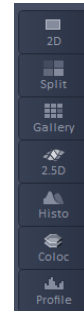
Arrow down or arrow up button to hide or show the image control area

Clicking on the arrow down button hides the image control area and increases the image view field of all containers in vertical direction for a larger image display. Clicking the arrow up button shows the image area in all containers once again.

Fig. 38   Image view tabs

Image view controls

Activates one or more of the various image display and analysis functions (see also section **2D View**).

Image control area

Shows the tools of the selected image display or analysis functions.
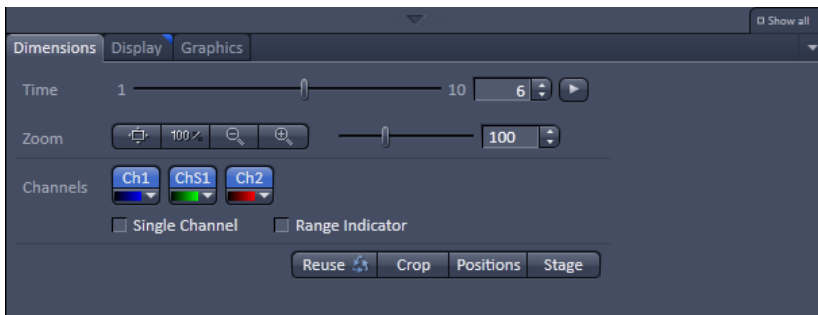
Fig. 39   Image control area

Expose Mode

Changes the one image display to the Expose display. Clicking on one of the image headers changes back to the one image display.

The main purpose of this function is to quickly find an image that might be only one of many open images which are all represented by image tabs on the top. Especially if the image tabs are configured to display only the image name.
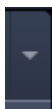
Arrow down button

Clicking on this button shows a list with all opened images for fast image selection.

☞ Using the drag and drop function, one or several images can be moved into another opened container. If closing a container, the included images will be moved automatically to another open container.
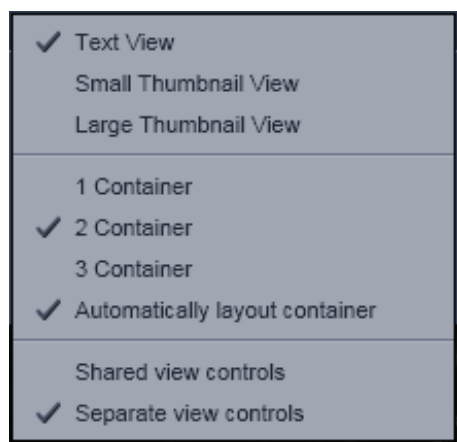
Fig. 40   Context menu

4.9.2   Context Menu of the Center Screen Area

Clicking the right mouse button on the background of the container opens the context menu of the Center Screen Area.

The following functions are available:

Display of the image tabs in the container header

Three options are available:

– Text View

– Small Thumbnail View

– Large Thumbnail View

Number of opened containers

Up to three containers can be opened in the Center Screen Area.

Shared or separate view controls

The display of the Center Screen Area can be changed between shared view controls mode and separate view controls mode.

Separate view controls shows a set of view control tabs for each container. In shared view controls mode, only one set of view control tabs is displayed, spanning the whole bottom area under all 2 or 3 containers. They always apply to the active image/container.

4.10 Right Tool Area

The Right Tool Area is used for displaying and handling the opened images, e.g. save or close. The view of this area can be varied individually, e.g. expand or hide the area, change between the view modes.

Expand slider

Dragging this slider to the left side expands the Right Tool Area by decreasing the center tool area.

Dragging the slider to the right side decreases the Right Tool Area up to the default size.

Save button

Saves the selected scanned or changed image. The **Save as** dialog appears.

New image document button

Opens an empty image document in the active container.

Close button

Closes the selected image. The **Close image** dialog appears to close the image with or without saving or to cancel the closing.

Textual View button

Shows images only with the textual characters.
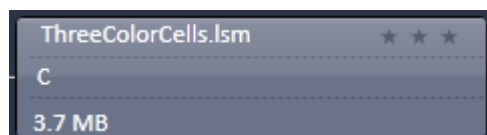
Thumbnail View button

Shows images with the textual characters and an image thumbnail preview.
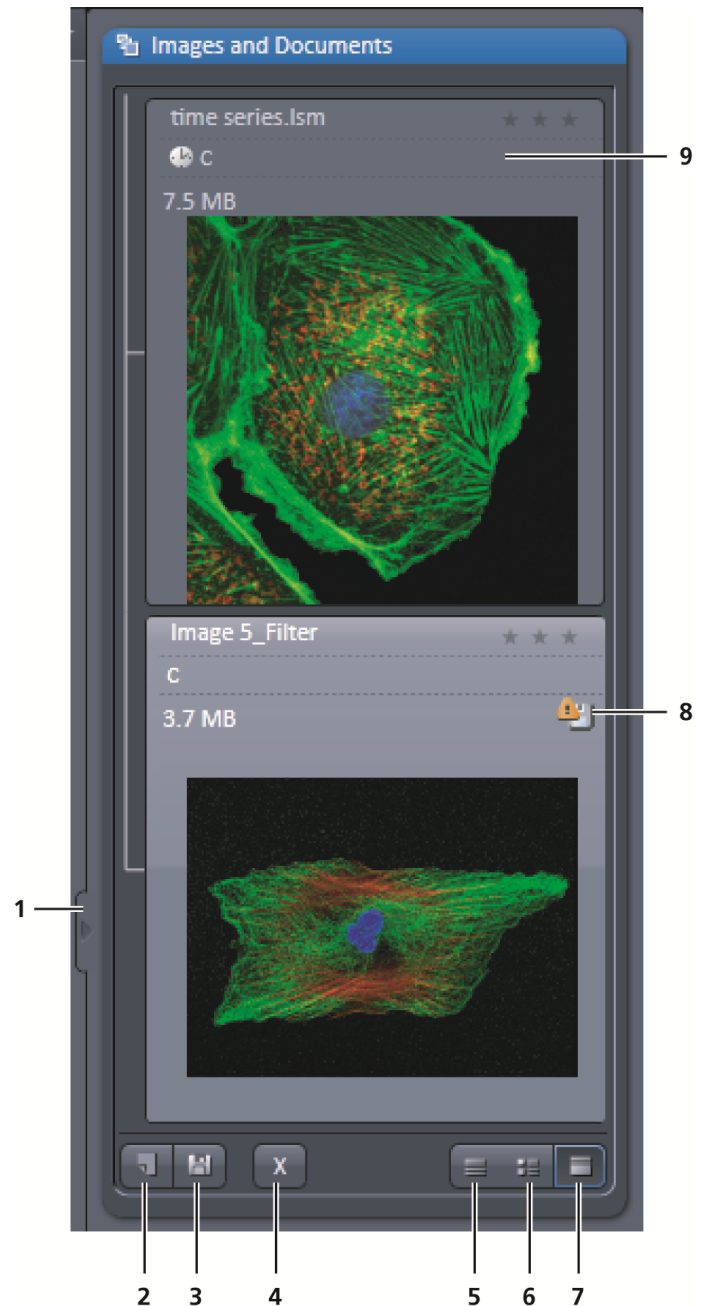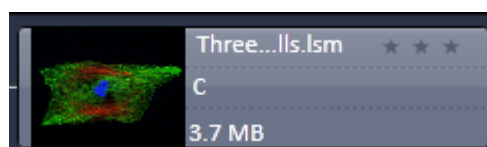
1 Expand slider
2 New image document button
3 Save button
4 Close button
5 Textual view button
6 Thumbnail view button
7 Big view button
8 Save status button
9 Image information field

Fig. 41   Right tool area (big view selected)

Big View button

Shows images with the textual characters and a big image preview.
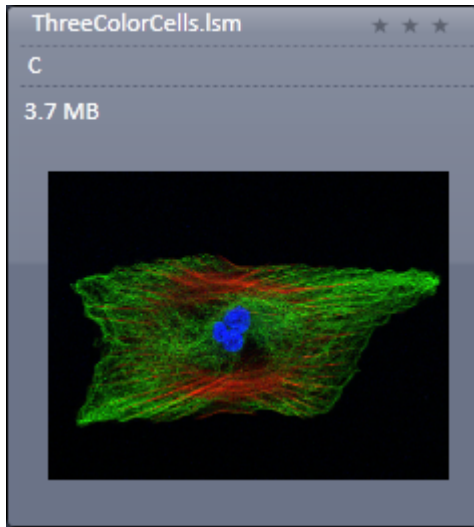
Save Status icon

This icon appears in the image that is not saved yet.

Image information

Displays the name, type and file size of the image.
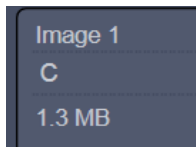
5   Left Tool Area and Hardware Control Tools

The Left Tool Area contains all tools for system operation, image acquisition, image processing and maintenance.

The major functions are organized in the four Main tool tabs (Fig. 42)

Locate

Visual and camera inspection in transmitted and epifluorescence light

Acquisition

Imaging with ELYRA

Processing

Processing superresolution and LSM images

Maintain

Setting Imaging and Firmware parameters
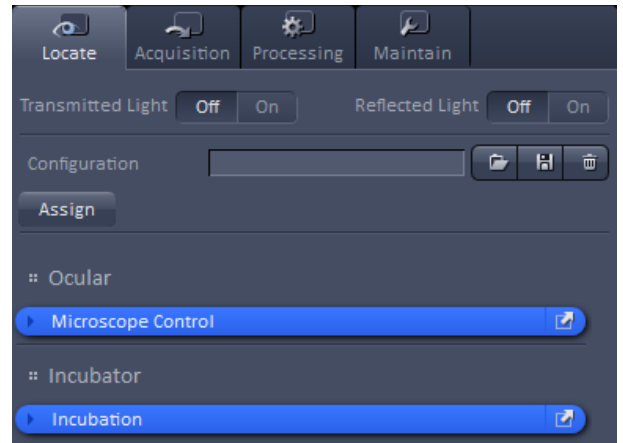
Fig. 42   Locate tab

By pressing the arrow left symbol of the **Expand slider** on the right border, the **left tool area** will collapse to the left side. The symbol changes to an arrow right symbol. By pressing the latter the **left tool area** will expand.
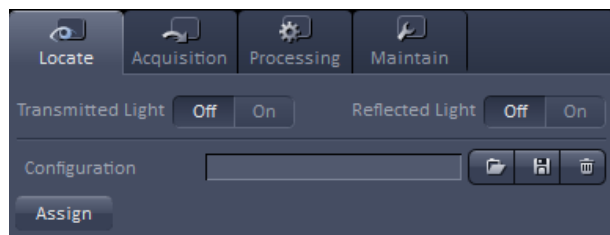
Fig. 43   Locate tab– Configuration panel
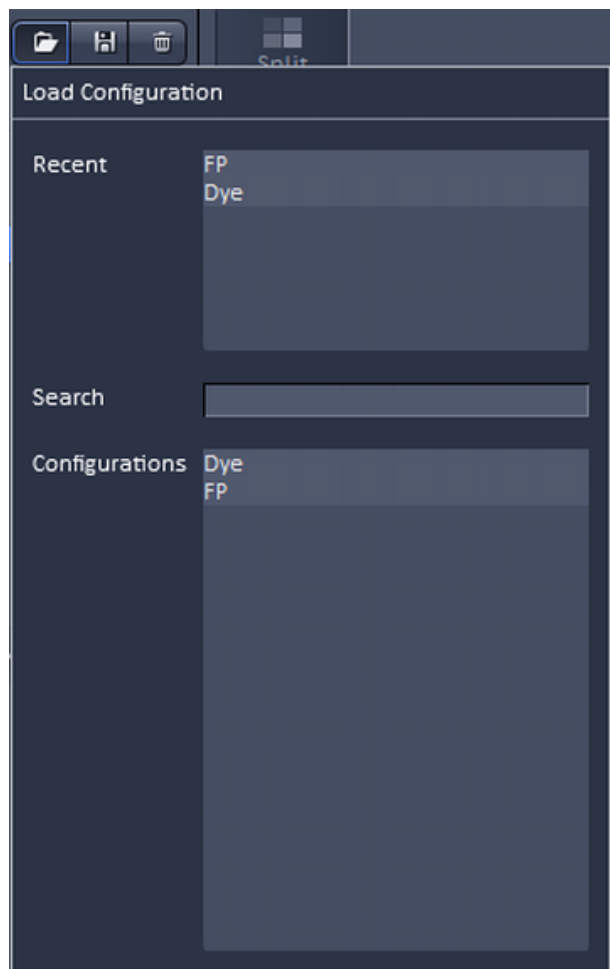
Fig. 44   Load Configuration panel

5.1   Locate Tab

Locate contains all functions of the microscope for visual inspection of the sample through the eyepieces (Fig. 43).

You can open the shutters for **Transmitted Light** (HAL lamp) and **Reflected Light** (X-Cite lamp) by pressing the respective **On** buttons in the Light control field.

Pressing the **Off** buttons will close the shutters, so no light can reach the eyepieces.

You can load a predefined ocular configuration from the **Configuration** selection box by pressing the **Load** button (   ).

All saved ocular configurations will be listed in the **Load Configuration** panel (Fig. 44).

☞   Please note that the selected objective lens and tube lens as well as the position of the 3D-SMLM slider will not be stored along a configuration.

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used ocular configurations or from the **Configurations** section, where all saved ocular configurations are displayed.

To search for a microscope configuration, type in its name or part of its name in the **Search** field and only those microscope configurations will be listed that contain the typed in string of letters.

The loaded configuration will be displayed in the **Configuration** display box.

Fig. 45   Delete configuration panel

Fig. 46   Delete Configuration pop up menu

Fig. 47   Save current configuration as panel

Fig. 48   Assign Pop up menu

To delete a configuration press the **Delete** button (🗑). This opens the **Delete Configuration** panel (Fig. 45).

Select the ocular configuration from the **Configurations** list by highlighting it or search for it by typing its name in the **Search** field. The **Delete Configuration** pop up menu will appear (Fig. 46). Press **Yes** to delete the configuration, **No** to keep the configuration or **Cancel** to close the dialog.

To save a microscope configuration press the **Save** button (💾). This opens the **Save current ocular configuration as** panel (Fig. 47). Type in an appropriate name in the **Configuration** field and press **Ok** to save the microscope configuration in the data base or press **Cancel** to abort the process. The configuration that is saved is the one as defined in the **Microscope Control** panel.

If you press the **Assign** button, the **Assign** pop up menu will appear (Fig. 48). You can select the button to which a configuration should be assigned in the **Button** selection drop down menu – up to 8 buttons are available. The configuration that should be assigned to a button can be selected from the **Configuration** selection drop down menu. By pressing **Assign** the configuration will be assigned to the button that will be displayed right to the **Assign** button. The buttons will carry the name of the selected configurations and appear from left to right and top to down according to their number. The ocular configuration assigned to that button will be automatically loaded by pressing that button. If you want to delete a predefined button, select the button (and its configuration), and press **Delete**.

The **Microscope Control** and **Incubation** tools are available in the **Locate** tab (Fig. 43). By pressing the expansion symbols in the bars the **Microscope Control** and **Incubation** tools become available. The **Microscope Control** tool permits configuring beam paths, which can be saved and reloaded in the **Configuration** field. Motorized functions controlled from the **Microscope Control** tool include objective, reflector cube and illumination mode (transmitted or reflected light) selection. The **Incubation** tool allows setting the temperature, CO_2 level, O_2 level and humidity.

☞ These microscope functions can also be operated directly on the microscope stand via assigned buttons or through the touch screen, if available. In that case, any changes are recorded by the software and displayed in the relevant windows / panels.

☞ For left side port access, the reflector has to be in a **None** position.

If the reflected light lamp is equipped with a software controlled shutter, the **Shutter On/Off** button in the **Fluorescence** field should be in the **On** position in order to open the shutter. In **Shutter Off** mode, the shutter is closed and will block the light.
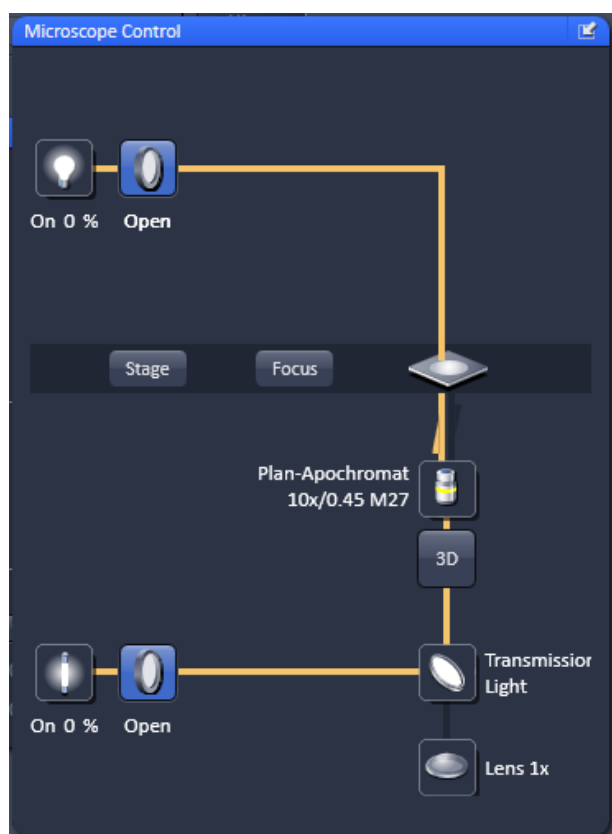
Fig. 49   Microscope Control tool
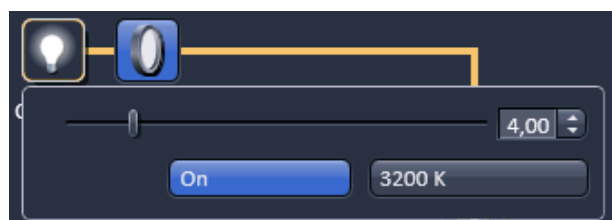
Fig. 50   Microscope Control – HAL lamp control

5.1.1   Microscope Control Tool for Axio Observer

To have access to the microscope functions to define a configuration, you have to expand the **Microscope Control** tool by pressing the expansion symbol in the **Microscope Control** tool tab (Fig. 49).

The **Microscope Control** panel contains the following functions:

HAL On/Off button for transmitted light: If the button is pressed, the control panel appears (Fig. 50).

If you press the **On** button it will be highlighted and the Halogen lamp will be switched on (symbolized by the halo in the lamp symbol).

In parallel the shutter for transmitted light automatically opens (**On** position in the **Light control** field and **Open** position in the **Ocular** panel). The light intensity of the HAL lamp can be varied via the input box or slider.

The light path will be highlighted by color in the diagram. Press the **On** button again to shut down the light. The button will go to the **Off** position and the shutter closes automatically.

The color temperature for photo documentation can be switched on via the **3200 K** button.

If the **3D** button (only available for systems equipped with the 3D-SMLM module) is pressed, the 3D-PALM slider will instantly move in its work position in the DIC slot of the objective. The button changes to indicate that the slider is in its active position. Press the button again to retract the slider to its load position.

HAL transmitted light Open/Close shutter button:

The shutter for transmitted light from the HAL lamp is switched on and off by repeated pressing (Fig. 51).

Note that it is equivalent to the Transmitted Light **On/Off** button from the **Light control** field. Whenever one of these buttons is pressed, the other buttons are updated. The shutter opens automatically, when the **HAL On/Off** button is switched to the **On** position, and closes automatically if the **HAL On/Off** button is switched to the **Off** position. However, it can be independently controlled, i.e. you can switch the shutter to the **Close** position with the halogen lamp still being switched on or vice versa you can open the shutter with the HAL lamp being switched off.

Condenser button:

The numerical aperture of the condenser is set via input box or slider (Fig. 52). The turret position of the filter can be selected from the **Filter** drop down menu (only for motorized condensers).

Stage button:

If the **Stage** button is pressed, the **Stage control** panel will appear (Fig. 53).

By pressing the **Stop** button you can interrupt any stage movement. The **Move to Zero** button will move the stage to the current zero positions of the x and y coordinates. The compass rose will move the stage in the direction of the arrowheads. Fast movement is obtained with the outer rose, slow movement with the inner rose.

The **X-Position (µm)** and **Y-Position (µm)** display boxes show the actual x and y coordinates. You can edit the numbers by typing or using the arrowheads. If numbers were altered, the first click on the compass rose will bring the stage to the altered position. The **Step Size (µm)** display box shows the currently used step size for stage movement. The value can be edited by typing or using the arrowheads. The **Set zero** button sets the x and y coordinates to 0.
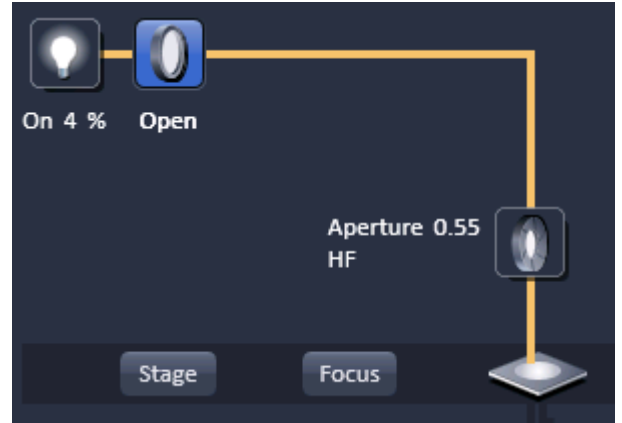
Fig. 51   **Microscope Control – HAL lamp switched on, shutter in the Open position**
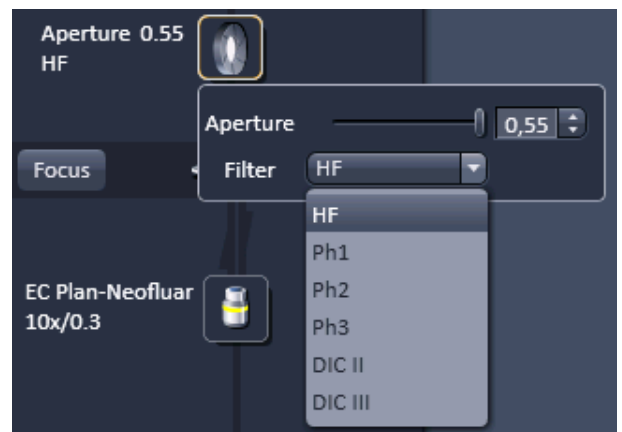
Fig. 52   **Microscope Control tool – Condensor control**

Fig. 53   **Microscope Control tool – Stage control**
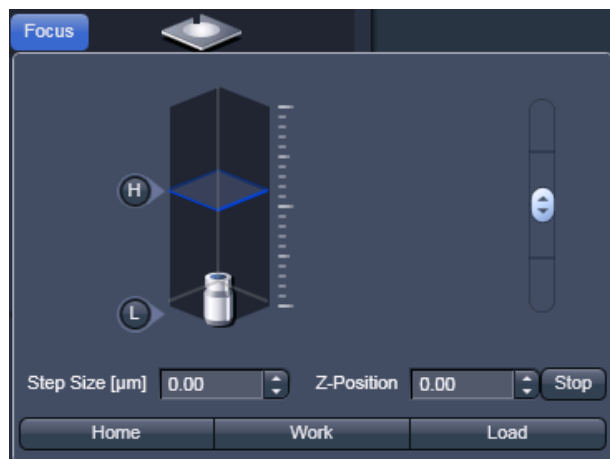
Fig. 54   Microscope Control tool – Focus control

Fig. 55   Microscope Control tool – Objective control

Fig. 56   Microscope Control tool – reflector turret control

Focus button:

If the focus button is pressed, the **Focus** control panel will appear (Fig. 54). The **Z-Position** display box shows the current z-position. The number can be edited by typing or using the arrowheads. The nosepiece or stage will automatically update the new position. By pressing the **Stop** button, you can stop the current movement. The **Step Size (µm)** display box shows the currently used step size for stage movement. The value can be edited by typing or using the arrowheads. The **Home** button sets the z-position to the predefined zero value. Pressing **Load** will lower the nose piece (z-position) for objective change. **Work** brings the z-position back to the actual used position if the focus was zeroed or in **Load** position. Home and Load positions can also be accessed from the z-position slider by pressing the **H** or **L** buttons. The z-position can be altered by grapping and sliding the **plane** or by using the arrow slider. The **plane** shows the actual z-position.

Objective button:

The objective can be selected via a graphical pop-up menu (Fig. 55). Please note, that for SIM the Plan-Apochromat 63x / 1.4 oil and for TIRF the alpha Plan-Apochromat 100x / 1.46 oil are the objectives of choice.

Reflector button:

The reflector cube can be selected from the reflector turret via a graphical pop-up menu (Fig. 56).

Tube Lens button:

The tube lens can be selected via a graphical pop-up menu (Fig. 57). **Lens 1x** is used for ocular observation and widefield imaging using an arc lamp, **Lens LSM** is used for laser scanning imaging and **Lens 1.6x** is used for widefield imaging using a laser source as well as for superresolution imaging. The tube lens for each application will be automatically chosen, but the selection can be overruled manually.

Fig. 57   Microscope Control tool – Tube lens control

Excite On/Off button for epifluorescence / reflected light:

Clicking the button shows the control panel (Fig. 58). Click **On** to open the shutter of the fiber coupled lamp (Fig. 59). In parallel the shutter for reflected light automatically opens. The light intensity of the lamp can be varied via the input box or slider (only if the lamp is controlled via SW) . The light path will be highlighted in the diagram. Press the **On** button again to shut down the light. The button will go to the **Off** position and the shutter closes automatically.
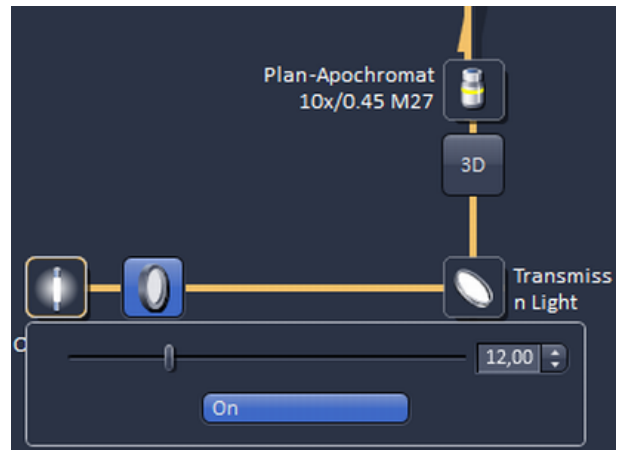
Fig. 58   Microscope Control tool – Excite control

Excite Reflected Light Open/Close Shutter button:

The shutter for reflected epi-fluorescent light from the lamp is switched on and off by repeated pressing (Fig. 59). Note that it is equivalent to the reflected light **On/Off** button from the **Light control** field. Whenever one of the buttons is pressed, the other buttons are updated The shutter opens automatically, when the **On/Off** button is switched to the **On** position, and closes automatically if the **On/Off** button is switched to the **Off** position. However, it can be independently controlled, i.e. you can switch the shutter to the **Close** position with the lamp still being switched on or vice versa you can open the shutter with the lamp being switched off. The light path will be highlighted.

Fig. 59   Microscope Control tool – Excite control

Visual inspection via binoculars

- In the main toolbar, activate the **Locate** tab.

- Choose the **Microscope Control** tool.

For transmitted-light observation (Bright-field)

- Switch the HAL lamp on by the **HAL On/Off** button and adjust the light intensity.

- Activate the condenser function with the **Condenser** button if applicable.

- Press the **Objective** button to swing the required objective into the working position. We recommend using the Plan-Apochromat 63x/1.4 oil for LATTICE SIM and 3D-SMLM and the α-Plan-Apochromat 100x/1.46 oil for SMLM due to their optimized optics.

- Select the desired tube lens by clicking the **Tube lens** button. Normally, the correct **Lens 1x** will be chosen automatically when the **Locate** tab of the main tool bar is pressed.

For reflected-light observation (Epi-fluorescence)

- Turn on the power supply switch of the epi illumination light source.

- Switch the refelected light **On/Off** button to the **On** position. As needed switch the **Reflected Light Open/Close Shutter** into the **Close/Open** position.

- Select the desired filter cube in the reflector turret by clicking the **Reflector** button. The filter will be automatically moved into the beam path to enable observation in epi-fluorescence.

- Select the desired tube lens by clicking the **Tube lens** button. Normally, the correct **Lens 1x** will be chosen automatically when the **Ocular** tab is pressed.

- Swing the required objective into the working position by selecting the objective with the **Objective** button. We recommend using the Plan-Apochromat 63x/1.4 oil for LATTICE SIM and 3D-SMLM and the α-Plan-Apochromat 100x/1.46 oil for SMLM due to their optimized optics.

(1) Conventional setting of the microscope Axio Observer

The Recording of microscope settings works as described for the microscope Axio Imager.

For the conventional setting of the Axio Observer, proceed as follows:

- Click the **Locate** tab in the **Main** toolbar.

- Place specimen on microscope stage.

  – The cover slip must be facing down.

- In the **Objective** list box, select the required objective.

- Use the focusing drive to focus the required specimen plane.

- Select specimen detail by moving the stage in X and Y via the XY stage fine motion control.

(2) Microscope settings on Axio Observer for transmitted-light observation

- Click on the **Transmitted light** button. Click the **On** button in the **Transmitted Light** panel and set the transmitted light intensity via the slider or click on **3200 K**.

Fig. 60   Axio Observer controls in the Microscope Control tool

- Click on the **Condenser** button and set the aperture via the slider in the **Condenser** panel. Set the filter in the **Filter** selection box.

- Click on the **Objective** button and select the objective by clicking on it.

- Click on the **Reflector** button and select **None**.

- Make sure there is no illumination from the reflected-light.

(3) Microscope settings on Axio Observer for reflected-light observation (Epi-fluorescence)

- Turn on the HBO 100 power supply switch.

- Click on the **Reflected Light** button and set the shutter to **Open**.

- Click on the **Reflector** button and select the desired filter set by clicking on it.

- The filter is automatically moved into the beam path to enable observation in epi-fluorescence.

- Click on the **Objective** button and select the objective.

- Make sure that the illumination from the transmitted-light is switched off.

5.1.2   Incubation Tool

To have access to incubator functions to define the atmosphere and temperature, you have to expand the **Incubation** tool by pressing the arrow in the **Incubation** tool (Fig. 61)

You will have access dependent on the attached equipment to

– Temperature (°C) control

– Atmosphere (%) control

– Y-module (°C) control

Fig. 61 **Incubation tool – Expanded fields for atmosphere and temperature control**

To have access to the respective controls, you have to expand the fields by pressing the triangles (Fig. 61).

The **Temperature (°C)** panel allows you to control up to 4 independent heating channels that are linked to certain devices (e.g. incubator XL, heating insert P, objective heater etc.) that are assigned to different channels. Enter the desired nominal temperature via the input boxes by typing in the numbers or by using the arrows to set the parameters. Check the corresponding box to activate the desired **Channel**. The numbers on the right will display the actual temperature. If a sensor is available, its temperature will also be displayed.

The **Atmosphere (%)** panel allows defining the O_2 and CO_2 concentration via the input boxes either type in the numbers or use the arrows to set the parameters. In order to monitor O_2, CO_2 and humidity levels, check the corresponding boxes. Actual values will be displayed on the right. If a humidifier is available it can be activated by checking the **Humidity** box. The actual humidity reading is displayed on the right,

The Y-Module (°C) panel allows setting the temperature for two independent thermostats (circulators), that can be selected via the **Selected** drop down menu. Each **thermostat** has two output channels, **01** and **02**. Type into the input boxes or use the arrows to define the appropriate temperature for each module. Activate the modules by checking the corresponding boxes. The actual values will be displayed to the right of each module.

☞   Note that using the Z-Piezo stage insert, which is required for both LATTICE SIM and SMLM, all incubation options are available.

5.2   Acquisition Tab

- **Acquisition** contains all functions of the microscope that are needed to set up and control widefield imaging by bright field or fluorescence, designated in this manual as widefield (**WF**), laser widefield imaging by epi-fluorescence and total internal reflection fluorescence (**Laser WF**) and widefield imaging with structured illumination microscopy (**SIM**).

- It depends on the hardware configuration, which of these functions are available. All widefield techniques use cameras.

- For excitation all imaging modes use lasers with the exception of **WF** that employs the epi illumination light source. If **Acquisition** is selected, the **Acquisition** tab tool becomes available (Fig. 62).

The **Acquisition** tab is divided into the

- **Experiment Manager** field for loading predefined acquisition parameters, action buttons and definition of the multidimensionality of the experiment and

- **Tool Group** field with the **tool tabs** for setting the parameters for imaging.
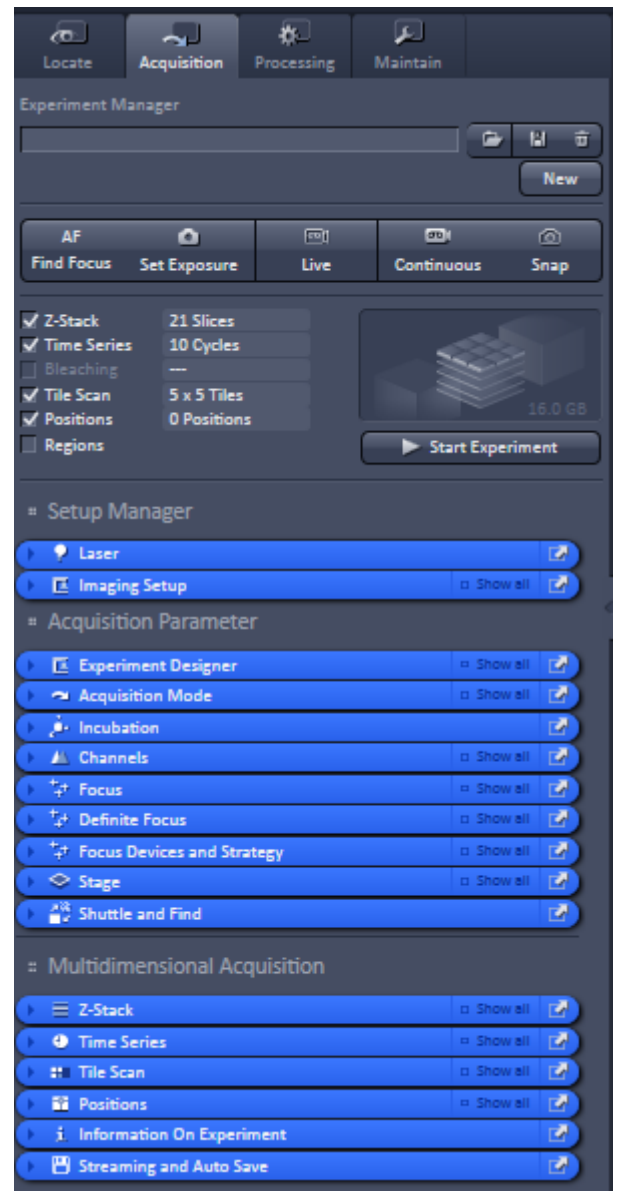
Fig. 62   **Appearance of Acquisition Tab tools for ELYRA**
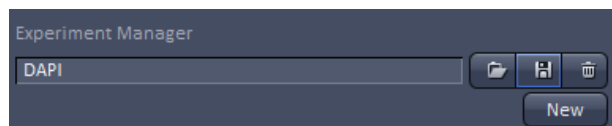
Fig. 63   **Experiment Manager field with loaded acquisition parameters**

Fig. 64   **Load Acquisition Parameters panel**

Fig. 65   **Delete Acquisition Parameters panel**

5.2.1 Configuration Functions

You can load predefined acquisition parameters from the **Experiment Manager** field (Fig. 63) by pressing the **Load** button ( ).

All saved acquisition parameters will be listed in the **Load Acquisition Parameters** panel (Fig. 64).

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used acquisition parameters or from the **Parameters** section, where all saved acquisition parameters are displayed.

To search for an acquisition parameter, type in its name or part of its name in the **Search** field and only those acquisition parameters will be listed that contain the typed in string of letters.

To delete parameters press the **Delete** button ( ). This opens the **Delete Acquisition Parameters** panel (Fig. 65).
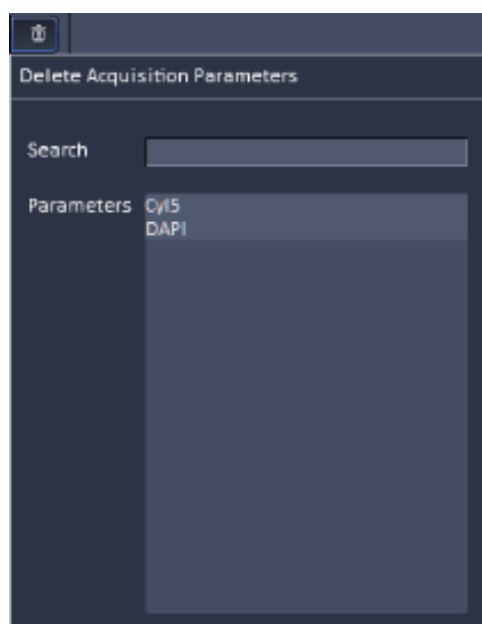
Select the acquisition configuration from the **Parameters** list by highlighting it or search for it by typing its name in the **Search** field. An **Info** menu pops up (Fig. 66). Press **Yes** to delete the acquisition parameters, **No** to keep the parameters. Close the menu by pressing the **cross** to abort the operation.

Fig. 66   Info panel

To save acquisition parameters press the **Save** button ( ). This opens the **Save Active Acquisition Parameters as** panel (Fig. 67). Type in an appropriate name in the **Name** field and press **Ok** to save the acquisition parameters in the data base or press **Cancel** to abort the process.

The following Acquisition Parameters are stored as a setting:

Fig. 67   Save Active Acquisition Parameters as panel

  – Time series: Cycles and Interval

  – Z-Stack: Range, Slices, Interval, Use of Piezo

  – Bleaching: Start and Repeat bleach settings, Iterations, Stop at specific intensity, Different scan speed, Trigger settings, laser wavelength and power

  – Tile scan: Number of tiles, Overlap, Bi-directional settings, Online stitching settings (Settings for Bounding Grid and Convex Hull are not saved as acquisition parameters)

  – All settings of the Acquisition Tool and Channels Tool.

  – All other parameters are ignored as acquisition parameters.

If the **New** button is pressed, a new **Image window** is opened in the **Image Container** into which the new image will be displayed. The next acquired image is displayed in this image frame, when it is the selected one. This function can be used to avoid that an old unsaved image (acquired by **Live**, **Continuous** or **Snap**) will be overwritten by a new image.
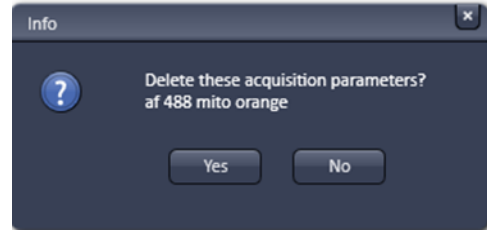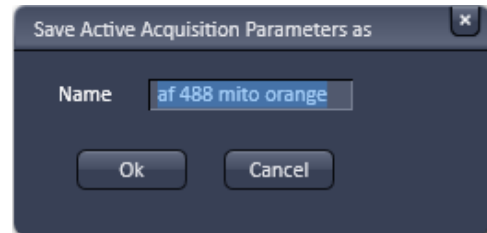
5.2.2   Action Buttons

Action buttons provide control for how the image is acquired (Fig. 68).
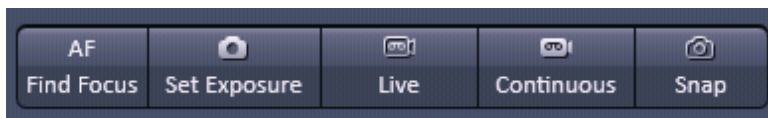
Fig. 68   Action buttons

They have the following functions, regardless of the imaging mode:

AF Find Focus triggers a frame z-Scan. The system selects the z-position associated with the frame of highest intensity or contrast. **AF Find Focus** can be disrupted by pressing the **Stop** button, to which the **Snap** button changes upon activation (Fig. 69).
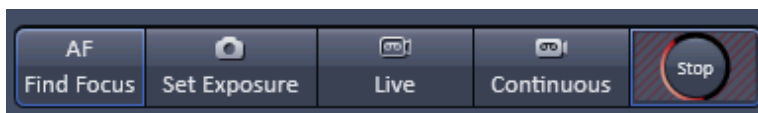
Fig. 69   Action buttons – AF Find Focus button active

Set Exposure optimizes the gain and offset settings (PMT only, for cameras only the exposure time will be adjusted) of all active channels and tracks dependent on the given laser power. **Set Exposure** can be disrupted by pressing the **Stop** button, to which the **Set Exposure** button changes upon activation (Fig. 70).
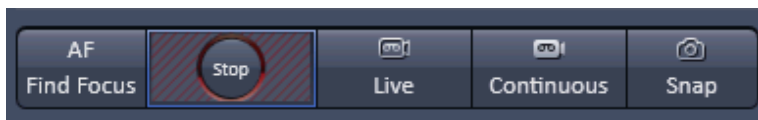
Fig. 70   Action buttons – Set exposure button active

Live uses the maximum speed for a given zoom setting for all active channels. It will set the resolution to 1280 x 1280 for the pco.edge CLHS sCMOS camera and 512 x 512 for the Andor iXon 897 Ultrja EMCCD camera. To disrupt **Live** acquisition, the **Stop** button has to be pressed into which the **Live** button changes after its activation (Fig. 71).

Fig. 71   Action buttons – Live button active

Continuous produce images from all active channels and tracks, thereby ignoring the settings for multidimensional acquisition, but taking into account the selected imaging mode. The last scanned frame will stay in the image window. To disrupt **Continuous** acquisition, the **Stop** button has to be pressed into which the **Continuous** button changes after its activation (Fig. 72).
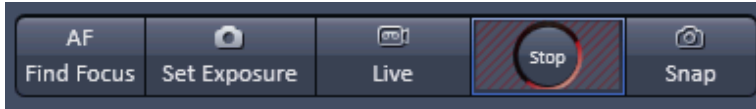
Fig. 72   **Action buttons – Continuous button active**

Snap produces one single image using all active channels and tracks thereby ignoring the parameters activated in the multidimensional acquisition tool group, but taking into account the imaging mode. **Snap** can be disrupted by pressing the **Stop** button into which the **Snap** button changes upon activation (Fig. 73).
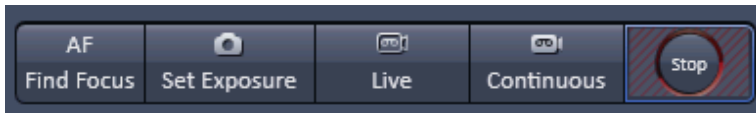
Fig. 73   **Action buttons – Snap button active**

☞   All actions buttons (with the exception of **AF Find Focus**) turn into **Stop** buttons once pressed, which can be used to stop the current process or experiment. In the case of the **AF Find Focus** button, the **Snap** button turns into **Stop**.

5.2.3 Multidimensional Acquisition Settings

In the **Multidimensional acquisition** field (Fig. 74), the multidimensionality can be defined by checking the corresponding boxes.
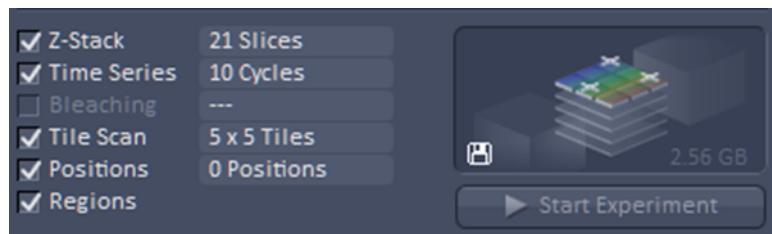
Fig. 74 **Multidimensional input field – Z-Stack (with 10 slices), Time series (with 10 images) and Regions active**

The following dimensions are available:

Z-Stack – Acquisition of an image series in z

Time series – Acquisition of an image series in time

Bleaching – Acquisition of a time series including definition of a region to be bleached.

Tile Scan – Acquisition of an image series with neighboring field of views (FOV).

Positions – Acquisition of an image series at different positions.

Regions – Imaging or manipulation of defined regions of interest (ROI)

The settings for multidimensional acquisition are accomplished within the tool groups **Acquisition Parameter** for **Regions** and **Multidimensional Acquisition** for all other functions. If available the settings are displayed beside the function, e.g. how many images are taken for the time series (Fig. 74). Corresponding tool bars within the tool groups only become available if the box of a multidimensional function is checked; otherwise the tool bar is not displayed (Fig. 75 and Fig. 76). The **graphical display** field above the **Start Experiment** button shows a representation of the experiment type with its dimensions is show. It also lists the estimated size of the final multidimensional image.
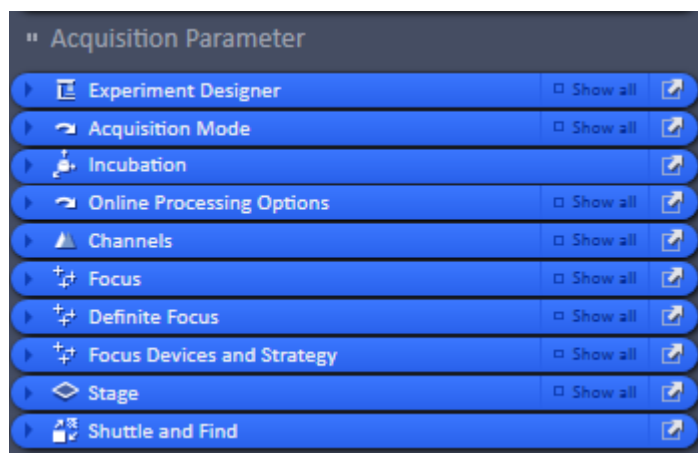
Fig. 75 **Acquisition parameter Tool group**
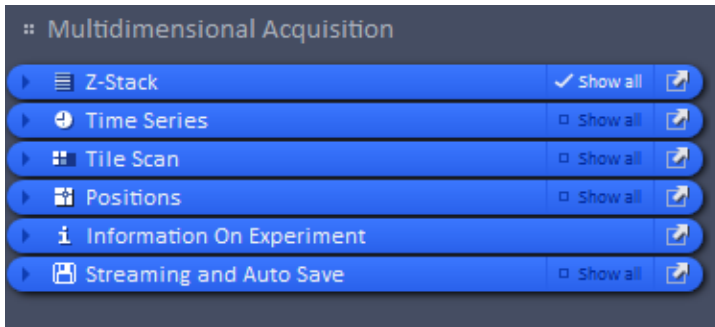
Fig. 76   **Multidimensional Acquisition Tool group**

☞   Note that not all functions are available for all imaging modes. Only those functions that are available can be activated; non-available functions are greyed out and inactive.

☞   Note that some functions are mutually exclusive. In this case checking one function will inactivate all other functions that are incompatible with the former.

☞   Note that some functions need other functions for a useful experiment. In this case checking that function will activate the other needed functions as well. The latter can be, however, manually deselected, if required.

Pressing the **Start Experiment** button will trigger the image acquisition, taking into account all activated functions. The progress of the experiment in regard to which image the system currently acquires will be shown updated to the right of the functions if available (Fig. 77).
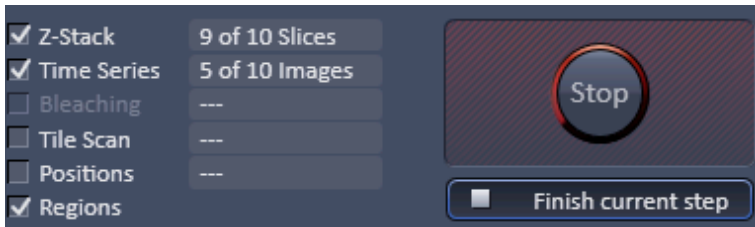
Fig. 77   **Multidimensional input field – display of the current status 9 from 10 slices taken from the 5th of the 10 images**

Upon pressing the **Start Experiment** button it turns into a **Finish current step** button, whereas the **graphical display** field turns into a **Stop** button. Pressing the **Stop** button immediately finishes the acquisition. Pressing the **Finish current step** button will interrupt the multidimensional acquisition after the currently performed step; e. g. when performing a Z-Stack over time it will finish the current Z-Stack and then stop the experiment.

☞   If **Start Experiment** is pressed and the camera is not yet cooled to its working temperature, an **Info** message pops up, telling that the temperature of the camera is not stabilized. This information tells a customer that the noise of the camera is not optimal (Fig. 78).

Fig. 78   **Info menu for camera temperature**
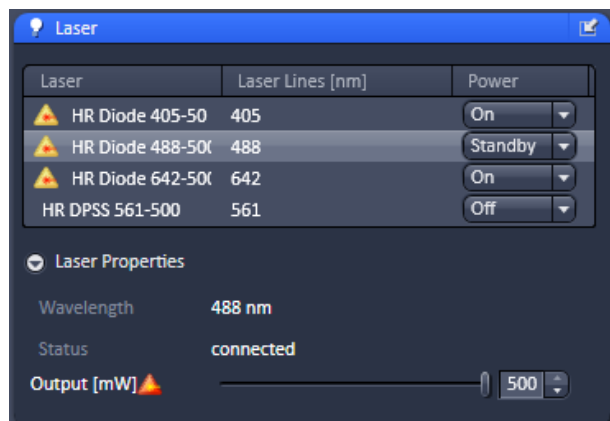
Fig. 79   Laser tool
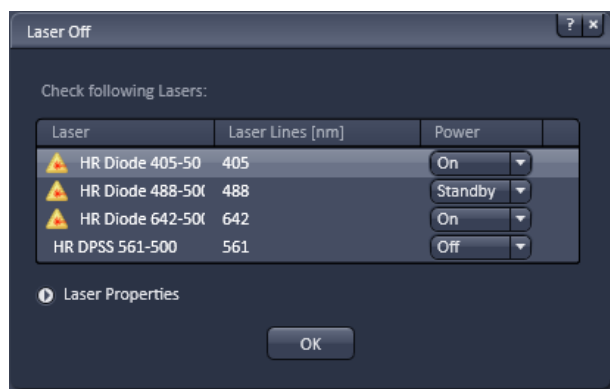
Fig. 80   Laser Off panel

5.2.4   Setup Manager – Laser

Open (and close) the **Laser** tool (Fig. 79) by clicking the **Laser** tab in the **Setup Manager** tool group.

Lasers are listed with their **Laser name**, their **Laser lines (nm)** and their **Power** state. Select the power state from the power state switch box. It shows the state of the laser: **On**, **Off** or **Standby**. **Standby** mode is available for all lasers. To activate a laser select **On** or **Standby** to switch the laser on for immediate use or for warm up, respectively. A laser that is in **Standby** will automatically go to **On** state, when in use and go back to **Standby**, when not in use.

The **Laser Properties** panel displays the current wavelength, status, and, if applicable, the **Output (mW)** power. It is recommended to set this to maximum to avoid laser fluctuation.

When exiting the Software, the **Laser Off** panel will be displayed - unless the checkbox **Lasers off on exit** in **Maintain / System Options / Hardware** is not ticked - showing the current power state of the lasers (Fig. 80). The user has the possibility to manually switch off still active lasers before the software closes. In case the status **On** is not changed, lasers are set into standby mode if applicable. Lasers without power status are switched off.

☞   After switching on the lasers in the laser control window and their status is displayed as ready (On) the system can be used for imaging. However, to minimize drift it is recommended to let the system warm up for 2 to 4 hours.

☞   Please bear in mind that a cooling phase of at least 5 minutes is required between switching off the laser via the software and switching off the entire system via the power

Laser life time

The absolute maximum power varies with the laser and declines over time as the laser is in use. A given tube current will over time produce less and less laser output power. Maximal power should only be set for short periods of time. Once the laser is no longer able to produce 100% output power with the maximal tube current available, the percentage of power and the tube current will also show up in red. With a tool tip you will be reminded, that the laser might reach the end of its lifetime. It can still be operated but its lifetime will decline even faster as it will be operated at high tube current to achieve the 100 or near 100% of power.

5.2.5   Setup Manager – Imaging Setup

The **Imaging Setup** tool (Fig. 81) allows new / existing beam path configurations and track definitions to be created / edited and saved for the different imaging modes.

- Click the **Imaging Setup** tool to open or close the tool window.

Different imaging modes can be selected by activating the respective tab.

The following imaging modes are available:

WF – Widefield imaging using the arc lamp for widefield illumination and a camera for detection

Apotome – SIM Apotome mode using laser for wideield illumination and a camera for detection

Lattice SIM – Structured Illumination Microscopy mode using a laser for widefield illumination and a camera for detection

Laser WF – Laser widefield imaging including epi-fluorescence (Epi), Highly Inclined and Laminated Optical sheet (HILO) and Total Internal Reflection Fluorescence (TIRF) modes using a laser for widefield illumination and a camera for detection

The imaging mode is selected by pressing the appropriate **Imaging mode** button. Below the **Imaging mode** button field the **Acquisition mode** field allows the selection of the acquisition mode. For all widefield imaging modes (WF, Apotome, Lattice SIM, Laser WF) only **Channel** mode is available.

☞ Note that the contents of tool groups and tool tabs will be specific for a selected imaging mode and hence change appearance, when imaging modes are switched. Thus it will depend on the selected imaging mode of the **Imaging Setup** tool, which functions will be available for other tools.

☞ The **WF**, Apotome, Lattice **SIM** and **Laser WF** light path tools have several information and control elements in common, whereas others are specific.
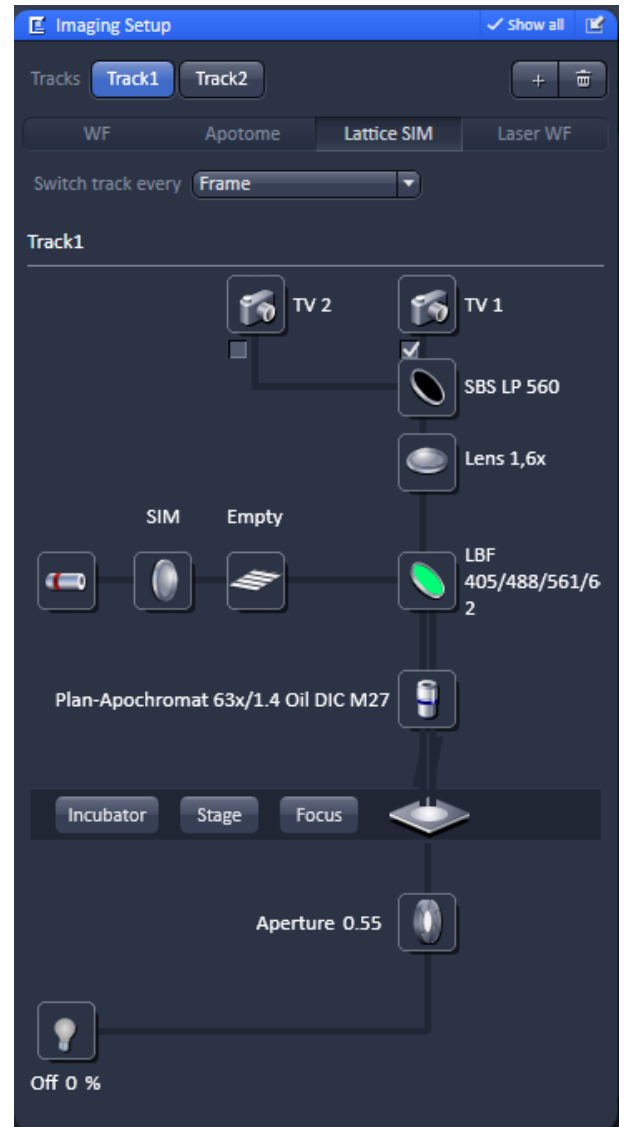
Fig. 81   Imaging Setup tool
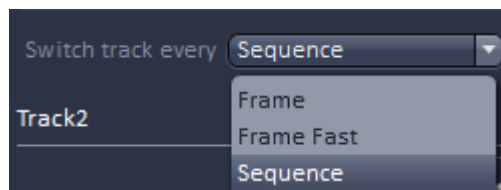
Fig. 82   **"Switch track every" pull down menu of the "Imaging Setup" tab with "Show all" active**
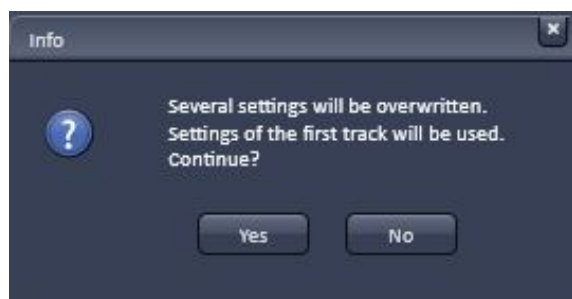
Fig. 83   **Info box of "Sequential" switch**
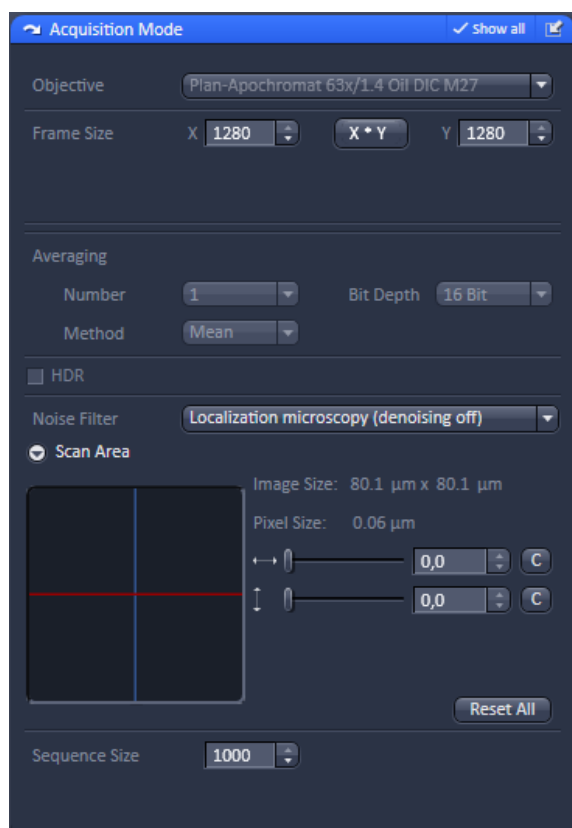
Fig. 84   **"Sequence size" input box of the "Acquisition" tab**

5.2.5.1   Sequential Laser Switching

Whenever at least two tracks are active, **Sequential** laser switching becomes available from the **Switch track every** drop down menu of the **Imaging Setup** tab (Fig. 82).

Select **Sequence** to enable this mode.

☞ This option is available in the **Switch track every** drop down menu only in the **Laser WF** imaging mode. It will be sustained when selecting **Time Series** from the experiment manager field. However, it will not be available whenever the **Z-Stack** check-box is active.

Since sequential laser activation is based on triggering, no mechanical switching of other devices, like filters, is possible. All mechanical settings for the first track will be applied to the second track as well and an **Info** message "**Several settings…**" will appear (Fig. 83).

Press **Yes** to continue with all tracks being updated to the configuration of track 1.

Press **No** if you do not want to continue. You can change after selection of any track these settings within the selected track. In this case the last changes will be applied to all other tracks.

You can set the number of frames per switch cycle in the **Acquisition Mode** tab in the **Sequence Size** input box (Fig. 84).

Type in a number or use the arrows for input. The input box will only be available, if **Sequence** was selected in the **Switch track every** drop down menu.

Please note that the number of cycles in **Time Series** must be an integer multiple of the **Sequence Size**. If changing the number of **Sequence Size** in a way that the cycles in **Time Series** is not any more an integer multiple of **Sequence Size**, the software will automatically update the number of cycles of **Time Series** to the next integer multiple. E.g. if the **Sequence Size** was 100 and the cycle number set to 10000 and now the **Sequence Size** is changed to 90, the next integer multiple of 10080 will be selected (Fig. 85).

After having defined your track configuration you can set the acquisition parameters in the **Channel** tab (Fig. 5). You can select for each track independent the laser lines used and their power settings. When setting **Exposure Time**, **EMCCD Gain**, **TIRF Angle** or **Collimator Position(s)** (the latter only visible in **Show all** mode) for one track, the other track will be automatically updated to the chosen settings. These parameters can therefore not be set individually for each track.

Note, that the **EPI** and **TIRF** button usage (pressed or depressed) will not be updated, but the values will. E. g. if in every track in **TIRF Angle** the button **EPI** was pressed and now in one track the **TIRF** button is pressed, only in this track the button usage is indicated. In all other tracks **EPI** stays selected, however, the last change will overrule all previous settings and each track will have TIRF illumination.

In the **Online Processing Options** tab you have the possibility to activate Online processing, however, **Automatic Laser Control** and **Holding Focus** will not be available for sequential laser switching and their functions are greyed out.

Once, the settings are made and the time series defined, press the **Start Experiment** button. One channel will be open for each track (Fig. 86) and sequentially filled with the images, whereby channels are switched every number of frames as defined in **Sequential Size**.

Frames for each channel are stitched together and treated as a continuous run, therefore absolute time information will be lost for each channel. The image will be treated as a two channel SMLM image and can be processed accordingly (Fig. 87).
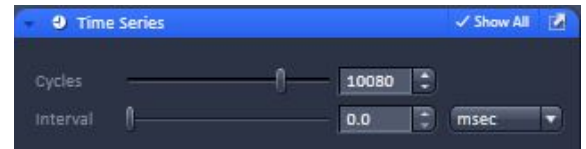
Fig. 85   Cycles of "Time Series" set to 10 080 after "Sequence size" has been changed from 100 to 90

Fig. 86   Track acquisition settings in the "Channels" tab
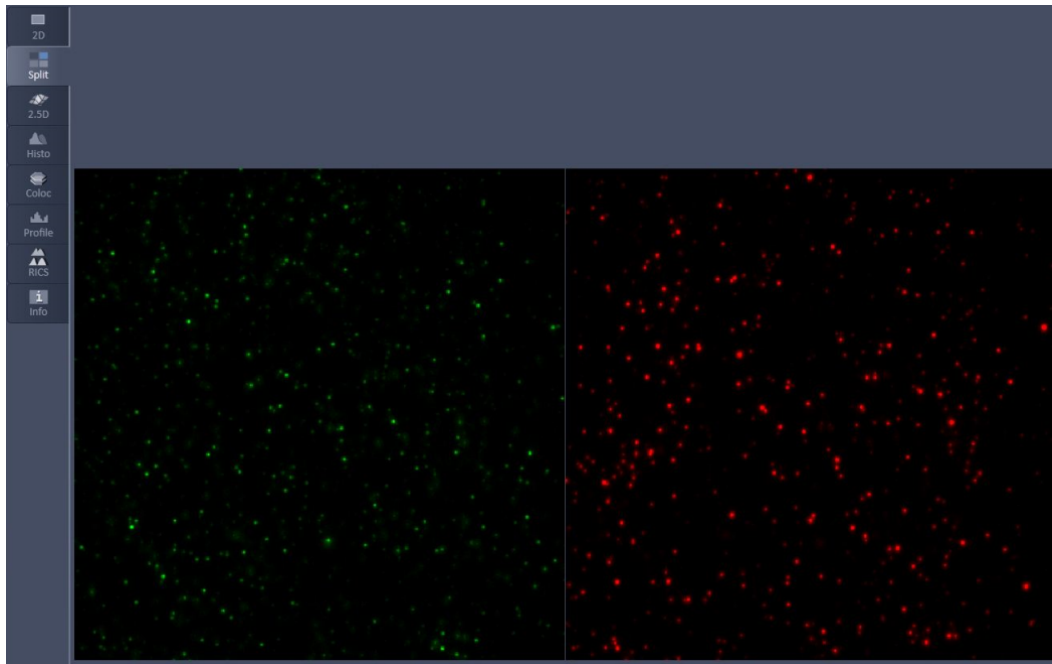
Fig. 87   **Image container in "Split" view for two sequential tracks**

☞   If **Online Processing** was active, SMLM and SWF images will be available for both channels. All Processing and Display options will be available for the SMLM image including Drift correction and Grouping. It should be noted that a continuous drift will be expressed as "jumps" at the transition points between the consecutive stitched blocks of frames, which are not accommodated by **model-based** drift correction. Therefore, **fiducial** based drift correction is the preferential method in this case.

5.2.5.2   Beam Path Configuration

In the **Beam path configuration** field the beam path can be selected (Fig. 88, Fig. 89 and Fig. 90). It depends on the ELYRA 7 type, the presence of Duolink and Imaging mode selected, what components are accessible. Some components are common to all imaging modes, whereas others will be specific.
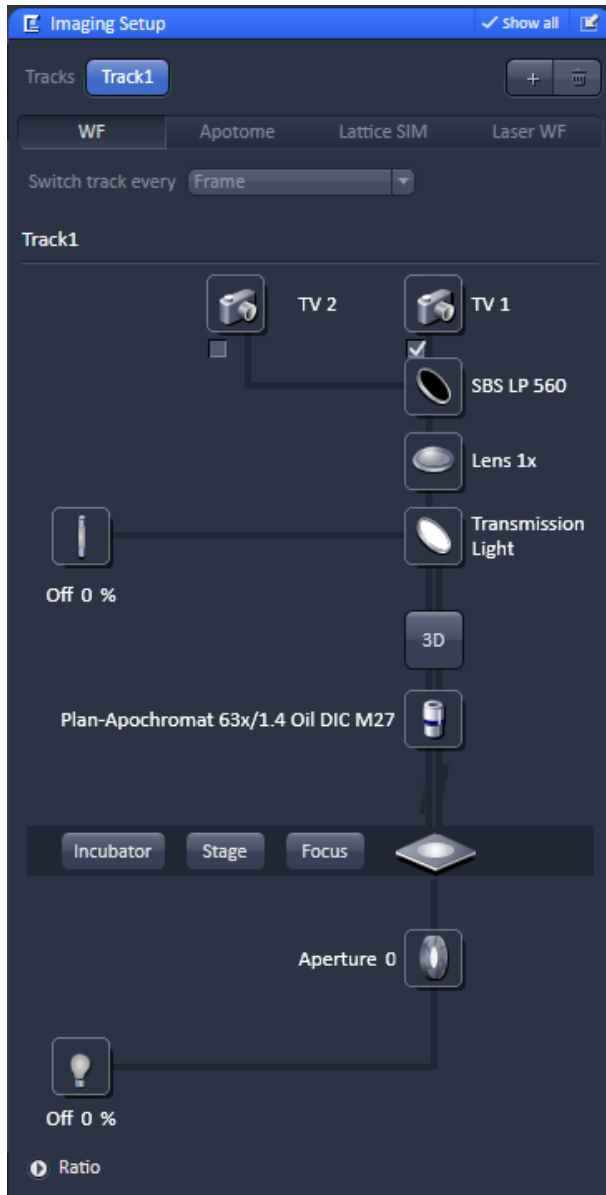
Fig. 88   Beam path configuration field – information and control elements for the WF imaging mode of ELYRA 7 type eLS, S and L with Duolink
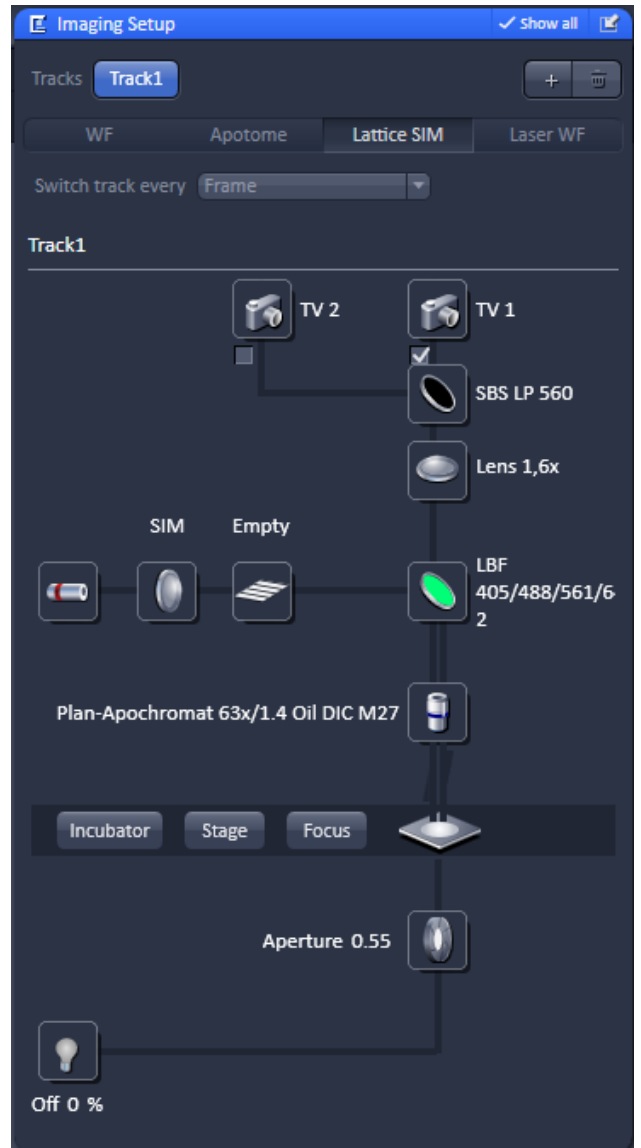
Fig. 89   Beam path configuration field – information and control elements for the Apotome & SIM imaging modes of ELYRA 7 types eLS, S and L with Duolink
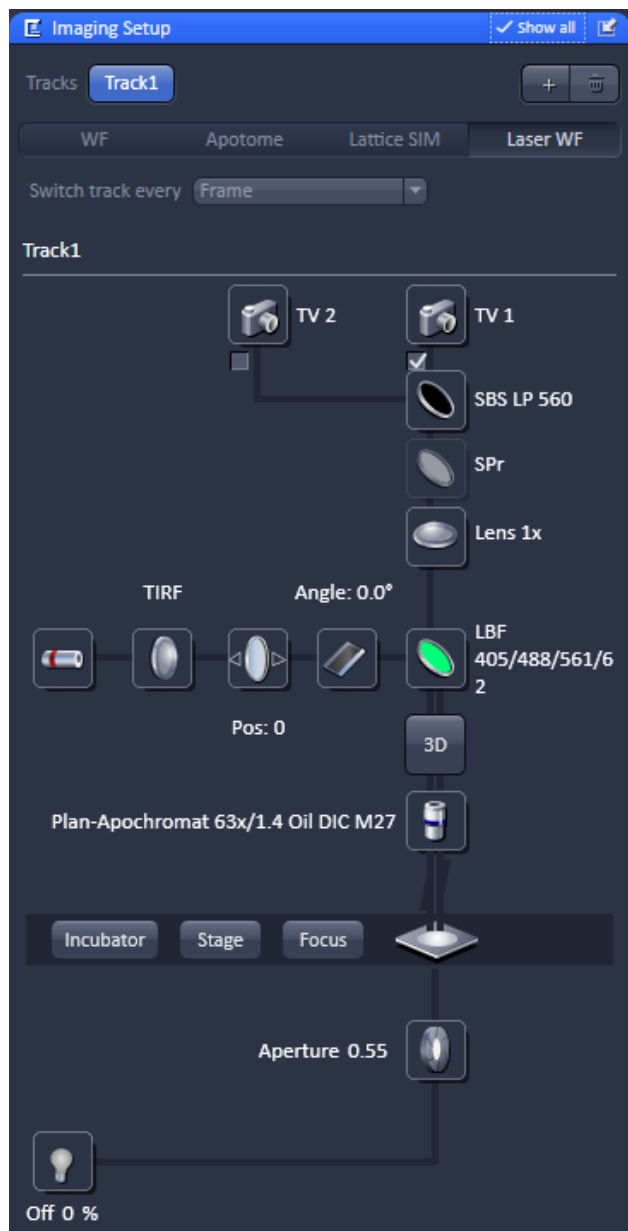
Fig. 90   **Beam path configuration field –**
               information and control elements for
               the Laser WF imaging mode of
               ELYRA type eLS with Duolink

☞ The **WF** and **Laser WF** light path tools have different appearances for different systems. The camera selection tabs are only available for ELYRA type LS systems. ELYRA types L, S and eLS systems without Duolink have only one camera (**TV1**) channel displayed without a checkbox. Those with Dualink have two camera (**TV1** and **TV2**) channels with a checkbox each displayed. For LS systems if the checkbox is deactivated, the TV icon will be greyed out and the checkbox will disappear. In this case, when triggering an image acquisition task the system will give a warning that "No active tractive track has been selected" and would not perform an image acquisition. To activate the camera again, you have to switch between different imaging modes. E.g. if you have inactivated the camera in the **Laser WF** imaging mode, you have to switch to **WF** imaging mode and than back to **Laser WF** imaging mode to have the camera active once more with a displayed ticked check box.

☞ The TIRF **Angle:#.#** adjustment button is only available for ELYRA P.1 and PS.1 systems; it is not available for ELYRA S.1 systems.

☞ The **3D-PALM** button is only available in WF and laser WF modes, if the system is equipped with a 3D-PALM module.

☞ Mixed modes are not supported. If you have one track defined in e .g. Laser WF mode and you select Lattice SIM in another track, the previous track will be inactivated.

5.2.5.3   Camera Selection

Dependent on the hardware configuration, one or two sCMOS (ELYRA 7 types S & eLS with and without Duolink, respectively), one or two EMCCD cameras (ELYRA type L with and without Duolink, respectively) or one EMCCD camera & one sCMOS camera (ELYRA type LS) (designated as **TV1** and **TV2**) are available (Fig. 91).

ELYRA type L systems have one Andor iXon 897 Ultra camera at the right sideport (SPr) (**TV1**) or two of these cameras on the right side port via Duolink (**TV1** and **TV2)**. TV1 is the camera to the front, TV to the rear.

ELYRA type S and eLS systems have one sCMOS pco.edge CLHS camera (**TV1**) at the right sideport (SPr) or two of these cameras on the right side port via Duolink (**TV1** and **TV2).** TV1 is the camera to the front, TV2 to the rear.

ELYRA type LS systems have an Andor iXon 897 Ultra at the right sideport (SPr) (**TV1: EM CCD Andor SMLM**) and an sCMOS pco.edge CLHS at the baseport (BP) (**TV2: sCMOS pco SIM**).

For ELYRA 7 type LS (1 EMCCD & 1 sCMOS) the camera can be activated by pressing the respective **TV1/2: EM CCD Andor SMLM / sCMOS pco SIM** button, which becomes highlighted, when selected. Always one camera will be active. Whenever one camera is active, the other will be inactive as they cannot be operated simultaneously. The system activates for each mode the best camera automatically; for **Laser WF** and **WF** imaging modes this will be the Andor iXon 897 and for **SIM** imaging mode the sCMOS pco.edge. However, which camera is used or not can be edited later in the **WF** and **Laser WF** imaging modes by the user.

When two cameras are available, the active camera is indicated by the highlighted **TV1/2: EM CCD Andor SMLM / sCMOS pco SIM**. Whenever one camera is active, the other will be inactive as they cannot be operated simultaneously. The system activates for each mode the best camera automatically; for **Laser WF** and **WF** imaging modes this will be the Andor iXon 897 and for **SIM** imaging mode the sCMOS pco.edge. However, which camera is used or not can be edited later in the **WF** and **Laser WF** imaging modes by the user.

Fig. 91   **Camera selection area – From top to bottom:**
(1) All imaging modes for ELYRA 7 types L, S & eLS with 1 camera
(2) All imaging modes for ELYRA types L, S, & eLS with 2 cameras (Duolink)
(3) Laser WF & SMLM imaging modes for ELYRA 7 type LS
(4) Apotome SIM & Lattice SIM imaging modes for ELYRA type LS

For ELYRA 7 types L (1 or 2 EMCCDs), S (1 or 2 sCMOS) and eLS (1 or 2 sCMOS), if one camera is present, it will be automatically active for a track. In case of two cameras check boxes are available that when ticked will activate the respective camera.
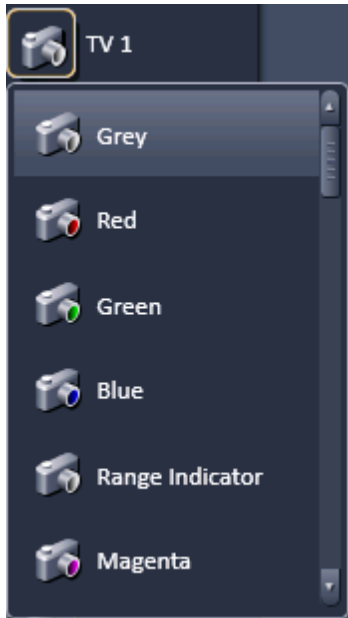
Fig. 92   **Selection of channel color of selected TV**

A color can be selected for the **TV** channel from a look up table that becomes available when the **TV** icon is pressed (Fig. 92). More than the displayed colors can be obtained by using the scroll bar in the pop up menu.

The position of the side port turret is indicated to the light path as follows:

– SP: light is directed 100% to the left side port (SPl) – only available for LSM

– SPr: light is directed 100% to the right side port (SPr) – all cameras for ELYRA 7 types L, S, and eLS. EMCCD camera for ELYRA 7 type LS

– BP: light is directed 100% to the base port (BP): sCMOS camera for an ELYRA type LS system.

5.2.5.4   Tube Lens Selection

The correct tube lens is automatically chosen for the corresponding imaging mode, but can be altered sub sequentially. The tube lens can be selected via a graphical pop-up menu of the **Tube lens** button (Fig. 93). For **WF** imaging mode, **Lens 1x** is appropriate, for **Laser WF, SIM Apotome** and **SIM** imaging modes **Lens 1.6x** should be used.
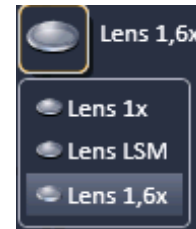
Fig. 93   Tube lens selection

5.2.5.5   Filter Cube Selection

The filter cube can be selected from the reflector turret via a graphical pop-up menu when the **Filter cube** button is pressed (Fig. 94).

There are six positions for the filter cube. 2 positions (positions 1 and 2) are for push and click filters. **Transmitted Light** (LSM/TL filter cube) is needed for LSM and Transmitted Light imaging and must be assigned to position 1 as this position will be automatically selected if Transmitted lightillumination is selected. The other position (position 2) can be equipped with a cube that is suitable for widefield excitation and observation through the eyepieces.

Fig. 94   Filter cube selection

Positions 3-6 hold calibrated filter cubes, which are used for widefield laser illumination in Lattice **SIM, SIM Apotome** and **Laser WF** imaging modes. They are permanently mounted in the turret and hence to use other filters need exchange of the whole filter turret.
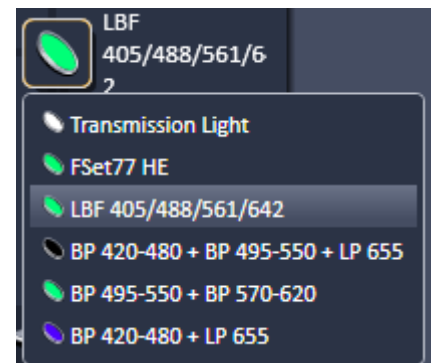
5.2.5.6   Objective Selection

The objective can be selected via the graphical pop-up menu when pressing the **Objective** button (Fig. 95).

Those objective, which are suited for a certain imaging mode are highlighted, but all objectives can be selected.

☞ Please note that for SIM the Plan-Apochromat 63x / 1.4 oil or the α-Plan Apochromat 63x / 1.4 oil and for TIRF the α-Plan Apochromat 63x / 1.4 oil (for sCMOS) and the α–Plan-Apochromat 100x / 1.46 oil (for EM-CCD) are the objectives of choice.

☞ For Stage and Focus control please refer to section Acquisition Parameter – Stage and section Acquisition Parameter – Focus.
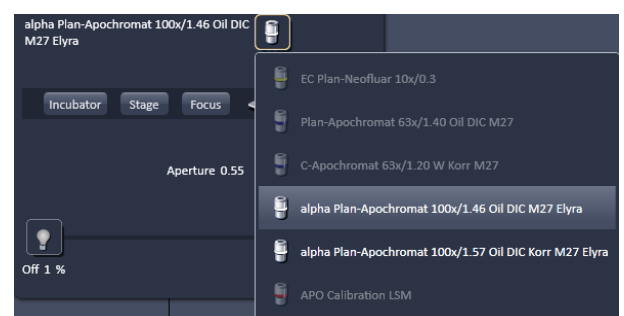
Fig. 95   Objective selection – for WF, SIM and Laser WF imaging mode
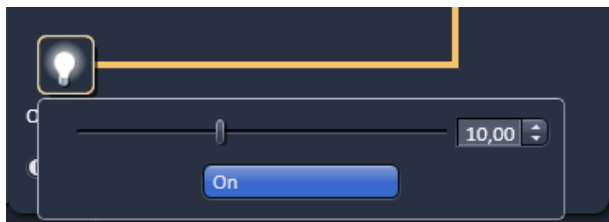
Fig. 96   HAL control

5.2.5.7   HAL Control

When the **HAL On/Off** button (available for **WF**, **SIM Apotome** and **Lattice SIM** and **Laser WF** imaging modes) for transmitted light of the halogen lamp is pressed, the control panel for the light settings is displayed (Fig. 96).

If you press the **On** button, the light intensity of the **HAL** lamp can be varied via the input box or slider. The light path will be highlighted by color in the diagram. Press the **On** button again to shut down the light.
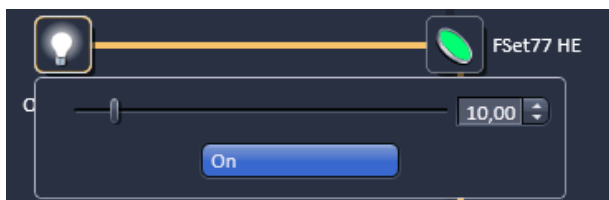
Fig. 97   X-Cite or HXP control panel

5.2.5.8   Epi Illumination Control

When the **Reflected Light On/Off** button (only available for **WF** imaging mode) for reflected light of the lamp is pressed, the control panel for the light settings is displayed (for software controlled lamps only) (Fig. 97).

If you press the **On** button, the light intensity of the lamp can be varied via the input box or slider. The light path will be highlighted by color in the diagram. Press the **On** button again to shut down the light.
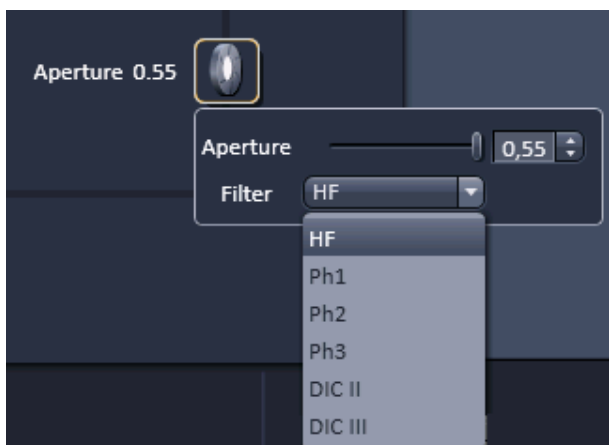
Fig. 98   Aperture control panel

5.2.5.9   Aperture Control

When the **Aperture** button (available for **WF**, SIM Apotome, Lattice **SIM** and **Laser WF** imaging modes) is pressed, the aperture size of the motorized condenser can be defined by typing or by using the slider or arrowheads (Fig. 98).

The **Filter** pull down menu allows to select the filter (available only in WF imaging mode) suitable for the objective in use.

5.2.5.10   Power Density Control

Press the **Power density** button (available only for **SIM Apotome**, **Lattice SIM** and **Laser WF** imaging modes) to open the selection panel for the requested telescope optics, which define the area the light is concentrated to (Fig. 99).

The higher the laser power density the less will be the field of view (FOV).

You have the option between **SIM** for SIM applications (least power density, default for **SIM** imaging modes) as well as **TIRF** (default for **Laser WF imaging mode**), **TIRF_HP** (high power, ~4 x higher density compared to **TIRF**) and **TIRF_uHP** (ultrahigh power, ~8 x higher density compared to **TIRF**). The selected mode will be displayed above the icon.

☞ Note that the optics for **TIRF_uHP** is not aligned and hence the FOV can be offset from a center position and the evenness of the field might be less in this case.

Fig. 99   Power density control – Laser WF imaging mode display

5.2.5.11   Grating Selection

Press the **Grating** (**Grid**) button (only available for **SIM** imaging mode) to open the **Grating selection** panel (Fig. 100).

You can choose between an **Empty** position and 5 grids **G1**-**G5** with increasing spacing, which is indicated in µm. The grating spacing depends on the laser line and objective used. The system will automatically load the grating for optimal resolution enhancement, which can be overruled by the user by selecting a different grid (only available in **Show all** mode).

☞ Note that those gratings that potentially can be used for resolution enhancement are highlighted.

Fig. 100   Grating selection control – SIM imaging mode
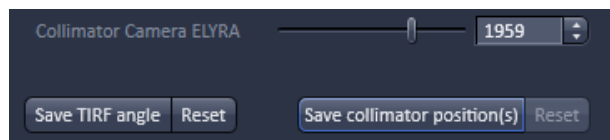
Fig. 101   **Collimator settings panel**
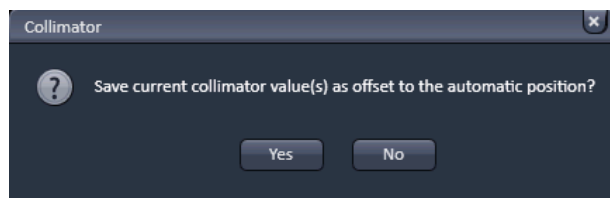
Fig. 102   **Save Collimator window**
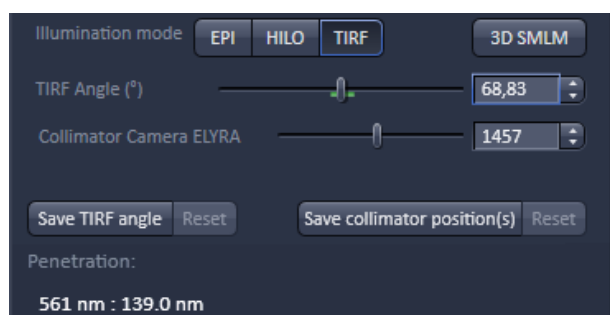
Fig. 103   **TIRF angle settings panel**
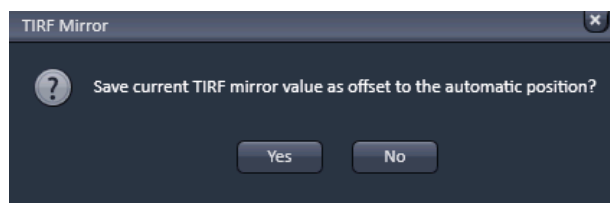
Fig. 104   **Save TIRF Mirror panel**

5.2.5.12   Collimator Setting

The Collimator setting is specific for the **Laser WF** imaging mode.

- Press the **Collimator** button to open the **Collimator settings** panel (Fig. 101).

- Adjust the Collimator position using the **Collimator Camera ELYRA** slider or by typing into the input box or using the arrows. The value in the input box and below the Collimator button display the current motor step position (**Pos.**).

- By pressing the **Save collimator position(s)** button, the current position is saved. You will be prompted in the **Collimator** window to confirm (Fig. 102). Press **Yes** to save the new position, **No** to abort the saving process. By pressing the **Reset** button (only active once a Collimator position was saved), the stored collimator position will be recalled.

5.2.5.13   TIRF Angle Setting

☞ Please be aware that for systems without 3D-PALM slider, you have for the **TIRF angle** settings panel **EPI** and **TIRF** mode available. In systems with 3D-PALM slider you have **EPI**, **HILO** and **TIRF** mode as options.

TIRF angle setting is specific for the **Laser WF** imaging mode. Press the **TIRF angle** button to open the **TIRF angle settings** panel (Fig. 103). You can switch between **EPI, HILO** and **TIRF** mode by pressing the respective button. In **TIRF** mode, a slider becomes available, with which one can set the TIRF angle.

☞ The HILO angle is arbitrarily offset from the TIRF angle by 10 mechanical steps of the TIRF mirror.

You can also input a value in the input box or use the arrows to change the value. The value displayed in the input box and above the **TIRF angle** button display the **Angle** in degrees (^{0}). The green outline defines the region with $\pm 1^0$ from the stored centered position.

By pressing the **Save TIRF angle** button you will be prompted to confirm saving the current position in the **TIRF Mirror** window (Fig. 104). Press **Yes** to save the new position, **No** to abort the saving process.

By pressing the **Reset** button (only active once a Collimator position was saved), the stored collimator position will be recalled. The theoretical calculated **Penetration** depth for each laser line is given in nm in the **Penetration** field.

☞ You can exced the boundaries of the slider only when going to one of its end and pressing **Save TIRF angle**, as than the current poisition will be the center position. Continue until you have reached the desired angle.

The TIRF angle can be adjusted using the **Specimen kit TIRF** (order number: 433800-0000-000).

New calibration might be necessary, if the TIRF angle was completely de-adjusted, if the TIRF objective is changed or after new installation of the software.

Proceed according to the protocol below:

Fig. 105   "TIRF angle (°)" adjustment slider with "EPI" (A-E) and "TIRF" (F) mode activated.

1.) Put a drop of TIRF beads from the bottle onto the glass section of a glass bottom dish.

2.) Dilute the TIRF beads with ca. 500 µl tap water; do not use distilled water, as the beads would settle only very slowly. Allow beads for a few seconds to settle. If beads settle too fast and only few remain in solution, you might dilute tap water with distilled water until you have a good ratio between settled and floating beads.

3.) Mount the glass bottom dish onto the microscope using an appropriate TIRF objective (e.g. objective alpha Plan-Apochromat 100x/1,46 Oil DIC M27; order number 420792-9800-720) and appropriate oil immersion.

4.) Select **EPI** mode (**TIRF angle** slider to the very left) in the **Channels** menu (Fig. 105/**A**) and focus on the settled beads using the 488 nm laser line (Fig. 106/**A**). Besides the settled in-focus beads you also should see blurry floating out-of-focus beads.

Fig. 106   TIRF beads imaged in "EPI" (A), "HILO" (B), "TIRF" (C), "hyperTIRF" (D) and "out of TIRF" (F) positions of the "TIRF angle (°)" adjustment slider

5.) Start moving the **TIRF angle** slider to the right. The image appearance will change as follows (Fig. 105/**B**-**E**):

a. **EPI** mode: on coverslip settled beads in focus plus floating blurry beads in solution with prominent haze from out-of-focus regions (Fig. 106/**A**)

b. Settled beads become brighter as HILO mode is approached and out-of-focus haze decreases.

c. **HILO** mode: settled beads and out-of-focus beads become brightest (Fig. 106/**B**). Haze is reduced further.

d. Brightness of settled and floating beads stay approximately the same, but out-of-focus haze becomes fainter as one approaches TIRF.

e. **TIRF** mode: Signal-to-noise ratio improves tremendously as out-of-focus haze disappears. Settled beads are clearly visible, whereas floating beads are not detected any longer (Fig. 106/**C**).

f. **HyperTIRF** mode. Signal of settled beads is fainting (Fig. 106/**D**).

g. **Out of TIRF** mode: Settled beads are no longer visible (Fig. 106/**E**).

6.) Go back to slider position, where beads are the brightest without noticeable out-of-focus haze. Recall TIRF angle position for the next step.

7.) Go to **TIRF** mode (Fig. 105/**F**) and enter the TIRF position determined in step 6 in the input box. Press **Enter** to move slider to the position.

8.) By using the arrows of the **TRIF angle (°)** input box fine-tune the optimal TIRF angle position by fine stepping back and forth.

9.) Press the **Save TIRF angle** button to store the setting. Whenever the same laser line is chosen, the TIRF angle will be set to the stored position.

10.) You might repeat with other laser lines, albeit the beads of the kit have an excitation maximum around 488 nm. Increase laser powers accordingly to visualize beads.

Please note, that the optimal TIRF angle is sample dependent. Therefore, it is recommended to fine-tune the TIRF angle for each sample. If the cover glass is tilted or uneven, TIRF will also depend on the actual position of the sample.
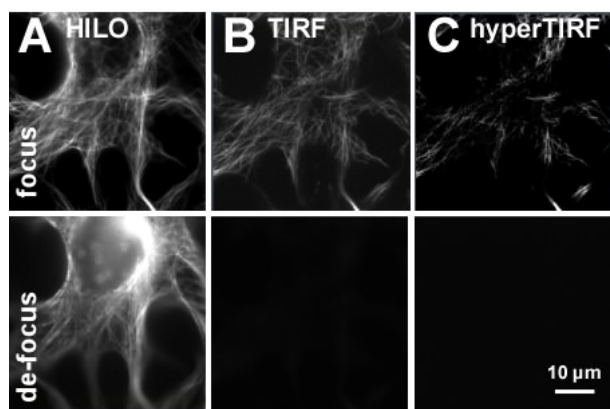
Fig. 107 **3T3 cells stained for tubulin imaged in "HILO" (A), "TIRF" (B) and "hyperTIRF" (C) mode.**

In order to fine-tune the TIRF angle, proceed as follows:

1.) Mount sample on microscope and focus in **EPI** mode.

2.) Switch to TIRF mode and move **TIRF angle (°)** slider back and forth to find the best TIRF setting. In **EPI** or **HILO** conditions, there is out-of-focus light visible and by defocusing up other structures, if stained, will appear (Fig. 107/**A**). In **TIRF** condition out-of-focus haze is not visible and signal-to-noise is maximal. By defocusing the signal is lost (Fig. 107/**B**). In **hyperTIRF** condition, the signal will be fainter and signal-to-noise will decay (Fig. 107/**C**).

3.) Set the TIRF angle to the optimal position and press the **Save TIRF angle** button if you want to recall the position later.

5.2.5.14   3D-PALM Slider

In order to place the 3D-PALM slider in the back aperture of the objective for the experiment, press the **3D** button (Fig. 108).

Press again to deactivate.

If activated or deactivated, the slider will not move immediately, but only if a Scan is triggered.

If you want to move the slider directly without having to perform a Scan, You have to resort to the **3D** button of **Locate**.

Fig. 108   3D-PALM slider activated
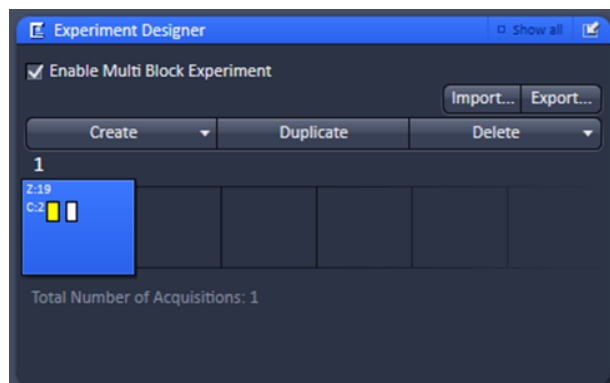
Fig. 109   Experiment Designer tool

5.2.6   Acquisition Parameter – Experiment Designer

Experiment Designer combines different multi-dimensional acquisition parameters with each parameter setting assigned to one imaging block in one total experiment described as Multi Block Experiment. Click the **Experiment Designer** tab to open or close the tool window (Fig. 109).

There is _always_ one blue Block (Active Acquisition Block) present with the active acquisition parameters assigned to it.

Changing acquisition parameters _always_ effects acquisition parameters of the active acquisition block even if the Experiment Designer tool is not open and/or not enabled.

When **Enable Multi Block Experiment** is checked, all functions within the tool become accessible.

The active acquisition parameters include all settings of the following tools:

- Imaging Setup
- Acquisition Mode
- Channels
- Focus Devices and Strategy
- Z-Stack
- Time Series
- Tile Scan
- Positions
- Regions
- Bleaching

The blue block is the active acquisition block. A Block can be activated (blue) and selected (enlarged) with a right mouse click or choosing **Activate** from the context menu of the left mouse click. Hardware and software is then changed to match the acquisition parameters of this activated (blue) block.

A left mouse click only selects a block (enlarged) but does not activate it (still grey). Hard- and software settings are not changed, they still represent the settings of the blue block. Wait and Delay blocks can be selected with a right or left mouse click. With a left mouse click a context menu opens which allows to **Disable/Enable** a block.

Disabled blocks are crossed out and will not be executed within the experiment. If only one acquisition block is present it cannot be disabled. Disabled blocks have to be enabled first before they can be activated.

New Blocks are added using the **Create** drop down list:

- – A **Acquisition Block** is used for image acquisition which is performed according to the acquisition parameter settings

- – A **Delay Block** sets a time delay between two blocks (not effective if defined as the very last block of a Multi block experiment). The delay time is set using the Delay slider which shows up when selecting a Delay Block. The time unit can be selected from the drop down list ranging from milliseconds (ms) to days (d).

- – A **Wait Block** pauses the acquisition until the user actively confirms the message box. The message of this box is typed into the editing field which shows up when a Wait Block is selected.

Use **Duplicate** to create a duplicate of the selected block. The duplicated block will be added as the last block. It will be active and selected.

Each Block icon shows as smaller icons or in writing its content like the delay time, the message or the acquisition dimensions (T for time, Z for Z-Stack, C for Channels, P for Positions and M for Tile scan).

The number of enabled acquisition blocks of the total Multi Block experiment is indicated underneath the row of block icons.

Loops and Repetitions are set (in **Show all** mode) in the **Loops and Repetition** panel. Use the arrow to open the panel (Fig. 110). The number in the **Loops** column states how often a sequence of Blocks defined by the number of the Block in **Start** and **End** is run. One loop/repetition is set as default and reflects the minimum of running the experiment once. Set the Loops value to 2 to perform the defined sequence twice. Repeat one Block by choosing the same number for Start and End. A yellow triangle shows up if a sequence cannot be performed for example if the end block is before the start block.

Fig. 110   Loops and Repetitions panel of the Experiment Designer tool

Whenever a block being part of a sequence is reached, the defined number of loops is performed. Thus outer loops also contribute to the number of total runs of an inner loop. Add a new row of loop settings by clicking the + button. Highlighted rows are removed by clicking the waste bin button. Rows of settings need to be checked in the **Enabled** column to be executed.

Use the **Export...** button to store a complete Multi Block experiment or selected Blocks under a **Name** of choice typed into the editing field. Within the **Export Experiment Blocks** dialogue you can **Select All** or **Deselect All** blocks using the corresponding buttons. Single blocks are selected/deselected with a mouse click. **Export** stores the selected block(s) under the chosen name.

Use the **Import...** button to load a Multi Block experiment or selected Blocks of an Experiment. Within the **Import Experiment Blocks** dialogue chose a name from the **Experiments** list with mouse click. **Select All** or **Deselect All** blocks using the corresponding buttons. Single blocks are selected/deselected with a mouse click. Delete removes the highlighted name/experiment from the list. **Import** adds the selected block(s) to the already existing block(s) of the Experiment Designer.

Pay attention to the following guidelines for the Experiment Designer

- Each Block generates a separate image file. Loops going back to a Block will add the newly acquired images to the already existing file of this Block. Hence, only one image per existing block irregardles of the set loops and repetitions will be generated. The time stamps of the final image represent the acquisition time of each image with the first image set to time point zero.

- Auto Save settings apply to the complete Multi Block experiment. Auto Save however produces a separate image for each Block and for each repetition of a Block. It serves as a back up. Images should therefore always be saved once the experiment is completed to save the data as one image file per Block.

- Image files are automatically named consecutively keeping the number for the image constant for a given Multi Block Experiment. The Block number always starts with 1 and the number is consecutive within the experiment.

- Use the Macro "ModifySeriesZEN.lvb" to combine auto-saved images into consecutive time series images if needed. See Chapter 2: MACROS AND VISUAL BASIC for further explanations.

- Set desired stage position(s) for each new block separately using the Positions Tool. For multi-position experiments in combination with Z-Stack acquisition the Z coordinate from the Position list is referring to the center position of the Z-Stack.

- Bleaching experiments are restricted to one position per Block. If more than one position is defined for a Block, Bleaching is disabled.

- Positions and Regions are not saved with the experiment, only with the image. Positions cannot be reused from the resulting image but have to be stored as separate position list(s). See also section 5.2.21.1 on using position lists.

- Autofocus Strategies use the center position of a Z-Stack as the reference position. For a given Z-Stack go to the center position and if necessary, click Center within the Center tab of the Z-Stack tool once to confirm the reference.

- Tile scan settings (number of tiles and % of overlap) are saved with the experiment. Settings within Bounding Grid and Convex Hull are not saved.

- All imaging modes defined in the Imaging Setup Tool (i.e. WF, SIM) can be assigned to different Blocks and used in mixed mode experiments.

5.2.7 Acquisition Parameter – Acquisition Mode

In the **Acquisition Mode** tool window, parameters for the acquisition can be set (Fig. 111,).

The user interface is adapted depending on the chosen Imaging mode in the **Imaging Setup** tool.

Controls include

– Objective selection

– Frame Size selection

– Format selection

– Averaging and color depth selection

– HDR High Dynamic Range (not available)

– Scan Area selection (not available)
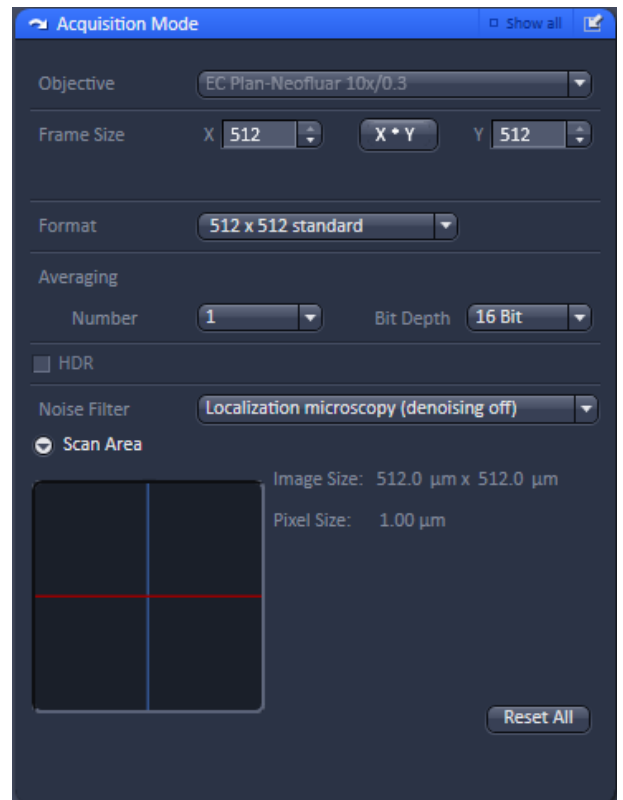
Fig. 111 Acquisition Mode tool window – appearance for WF imaging mode

5.2.7.1 Objective Selection

You can select an objective from the **Objective** drop down menu (Fig. 112). Objectives suited for a certain imaging mode will be highlighted, but every objective can be selected.

In case the system is equipped with an objective that has a motorized correction ring, an additional slider appears beneath the **Objective** selection (**Corr (%)**) once this objective is selected.

The slider is used to set the correction ring to the best position for minimal spherical aberration (Fig. 113).

Ideally use a line z scan (time series with maximum number of time points) to look at the reflection of

Fig. 112 Objective drop down menu – appearance for SIM imaging mode
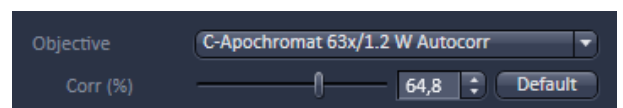
Fig. 113 Slider to set the correction ring

the coverslip. Set the slider to a position such that the image shows the reflection of the line to be as thin as possible. This is the optimal correction for minimum spherical aberration. **Default** sets the correction back to its middle position.

☞ Note that the default value set by the **Default** button is not always the middle of the correction collar, but the point where the optical design works at its theoretical best performance.

**Fig. 114   Format drop down menu –
appearance for Andor iXon 897
SMLM camera in WF and Laser WF
imaging modes (top) and for
sCMOS pco.edge SIM in SIM mode
(bottom)**
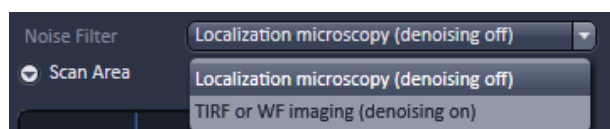
**Fig. 115   Noise Filter drop down menu –
appearance for ELYRA 7**

5.2.7.2   Format and Trigger Selection

Binning of the camera pixels can be set in the **Format** drop down menu with the Andor iXon cameras, but not for the sCMOS pco.edge camera (Fig. 114). In the **SIM** imaging mode only the standard (**1004 x 1002 standard;** numbers indicate pixels) is available. In the **WF** and **Laser WF** imaging modes you have additionally to the standard (**1004 x 1002 standard**) the options of 2x binning (**500 x 500 binned 2x2**) and 4x binning (**248 x 248 binned 4x4**). Selection of **Format** will update the possible **Frame Sizes**.

☞ When selecting the Andor iXon SMLM camera in the **WF** and **Laser WF** you have the options to select standard binning (**512 x 512 standard**), 2 x binning (**256 x 256 binned 2x2**) or 4 x binning (**128 x 128 4x4**).

☞ All cameras are run in exclusively in triggered mode, fre run is not available.

When selecting the sCMOS camera for Localization microscopy in laser WF mode the denoising should be switched off by selecting **Localization microscopy (denoising off)** in the **Noise Filter** drop down menu (Fig. 115). The SMLM software will correct the indicidual pixwel noise of the sCMOS camera. For TIRF and WF applications the entry **TIRF or WF Imaging (denoising on)** should be chosen.

5.2.7.3 Averaging and Color Depth Selection

You can select the number of image frames you want to average from the **Averaging Number** drop down menu (Fig. 116).

You can select a 2^n x averaging (n=0 – 4).

The way of averaging can be selected in the **Averaging Method** drop down menu (available only in **Show all** mode), where you can either select **Mean** for the mean average or **Sum** for adding the images up (Fig. 117).

The color bit depth can be selected from the **Bit Depth** drop down menu (Fig. 118).

Select **8 Bit**, **12 Bit** or **16 Bit** Data Depth, i.e. 256, 4096 or 65536 gray values.
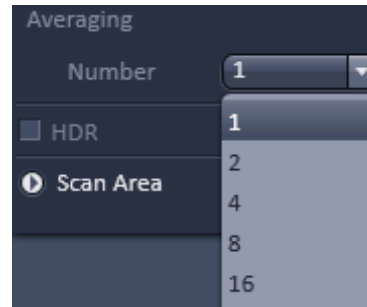
Fig. 116   **Averaging Number down menu**
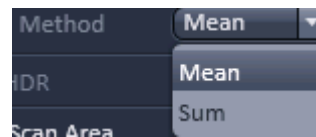
Fig. 117   **Averaging Method down menu**
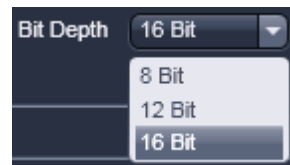
Fig. 118   **Bit Depth drop down menu**

5.2.8 Acquisition Parameter – Incubation

To have access to incubator functions you have to expand the **Incubation** tool by pressing the arrow in the **Incubation** tool (Fig. 119).

Dependent on the incubation equipment you have access to

- Temperature (°C) control
- Atmosphere (%) control
- Y-module (°C) control

by expanding the respective fields via pressing the triangles.

Respective input boxes allow to set the **Temperature (°C)** the **Atmosphere (%)** and the **Humidity** box. Actual readings if available will be displayed in the last column.

For more details please refer to section **Incubation Tool**.

Fig. 119   **Incubation tool – Expanded fields for atmosphere and temperature control**
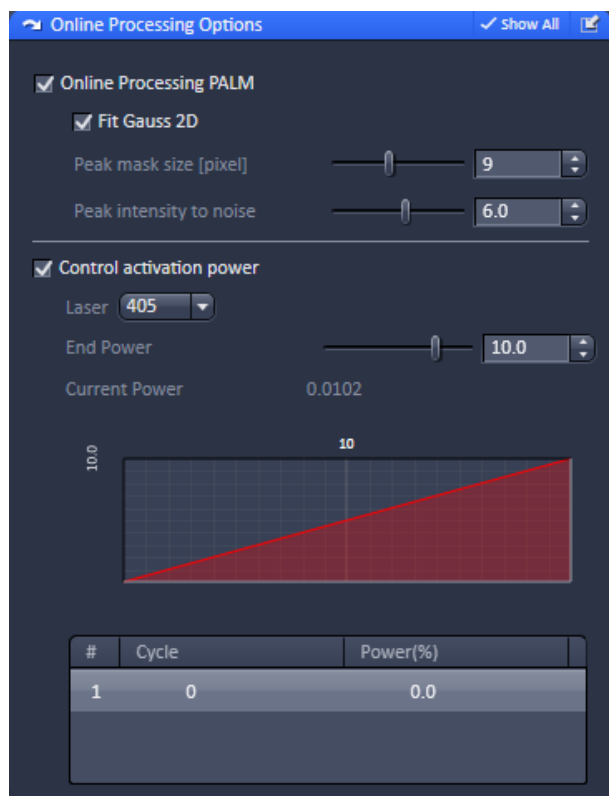
Fig. 120   Online Processing Options
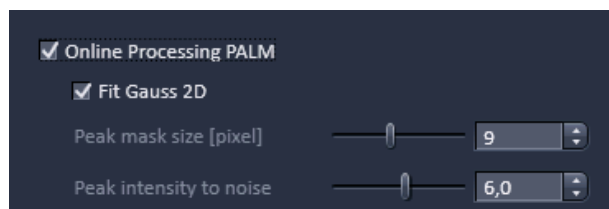
Fig. 121   Online Processing SMLM

5.2.9   Acquisition Parameter – Online Processing Options

The **Online Processing Options** tool is only available in the **Laser WF imaging** mode and allows to online process SMLM data, control the laser activation power and activate the holding focus, all processes that will take place parallel to data acquisition (Fig. 120).

5.2.9.1   Online Processing SMLM

If the **Online Processing SMLM** checkbox is ticked, the localization of molecules is computed and a SMLM image generated on the fly (for 3D-SMLM you will obtain a localization map only in 2D). You can decide, which kind of fit you want to use by checking the **Fit Gauss 2D** box to use a full 2D Gauss fit routine, or a **Mask** fit, with a predefined fixed Gaussian mask, if the box remains unchecked (Fig. 121).

With the **Peak mask size [pixel]** slider you can adjust the size of the mask that is used to cut an identified event out. As a default, the mask has a size of 9 x 9 pixels. You can alternatively type in numbers in the input boxes or use the arrowheads to define the size.

With the **Peak intensity to noise** slider, the noise threshold is set. An event will be considered as appropriate, if the standard deviation of its intensity against the rest image is smaller than the set value. The higher the number, the more discriminating the system will look for events. The default value is 6.

☞   When using **Online Processing SMLM**, a SMLM tab in the image will become available that contains the SMLM and the sum widefield (SWF) image.

5.2.9.2 Control Activation Power

Tick the **Control activation power** check box to have access to change the activation power during the experiment (Fig. 122).

The check box is only available if the **Online Processing SMLM** check box is ticked. The laser line to be controlled can be selected from the **Laser** pull down menu.

Only those lasers that are activated in the **Channel** or **Imaging Setup** tool will be displayed.

Use the **End power** slider to define the laser power reached at the end of the experiment.

It will depend on the experimental time and the initial laser power (set in the **Imaging Setup** or **Channel** tool tab), how steep the activation power change will be.

The graphical display (available only in the **Show all** mode) will depict the shape of the ramp and displays the end laser power in %.

If the initial laser power is higher than the end power, the slope of the ramp will be negative. Vice versa, if the initial laser power is lower than the end power, the slope will be positive.

Fig. 122   Control activation power field

☞ If the initial or end laser power are changed during the experiment the ramp will be recalculated. The system calculates the current point based on the assumption, that the changed values would have been the start values and takes the new calculated ramp starting from the point of change. All changes will be recorded in terms of at which **Cycle** the change was made and what the laser power was at the time of the change in the **Record** display field.

The **Current Power** displays the actual laser power in %.

5.2.10   Acquisition Parameter – Channels

In the **Channels** tool window the following options are available:

- Switching, activating, deactivating and naming/saving of tracks
- Defining laser and laser power (**SIM Apotome**, **Lattice SIM**, **Laser WF**)
- Defining the Field of View (FOV) (**SIM Apotome**, **Lattice SIM**, **Laser WF**)
- Defining Detector settings
- Defining Pinhole settings
- Defining Display options

Expand the **Channels** tool window to have access to all options (Fig. 123, Fig. 124 and Fig. 125).
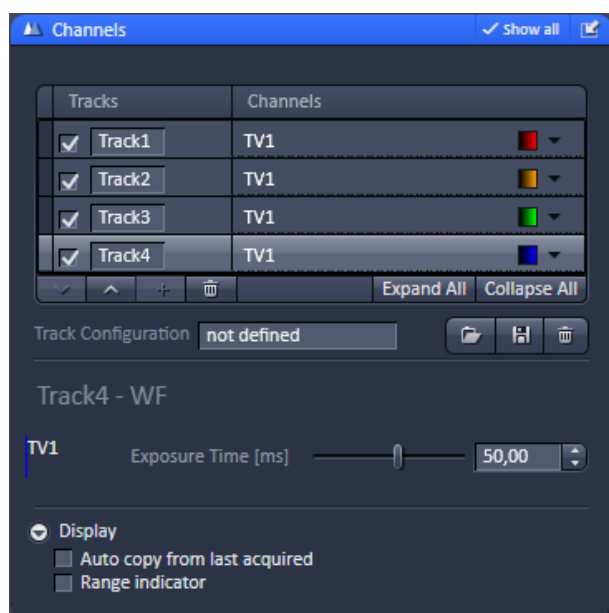
Fig. 123   Channels tool – Appearance for the WF imaging mode of ELYRA types S & eLS systems

☞ Note that mixed mode tracks are not supported, that is all tracks must be of the same mode. If a mode is changesd in onme of the tracks, allother tracks will be inactivated and have to be brought to the same mode in order to be active again. The last modified track will define the active mode.
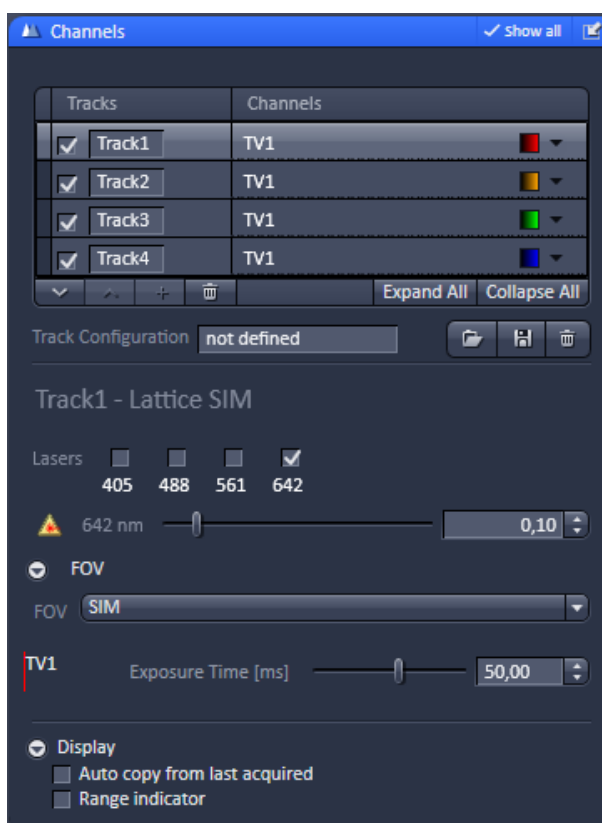
Fig. 124   Channels tool – Appearance for the SIM Apotome and Lattice SIM imaging modes of ELYRA types S & eLS systems
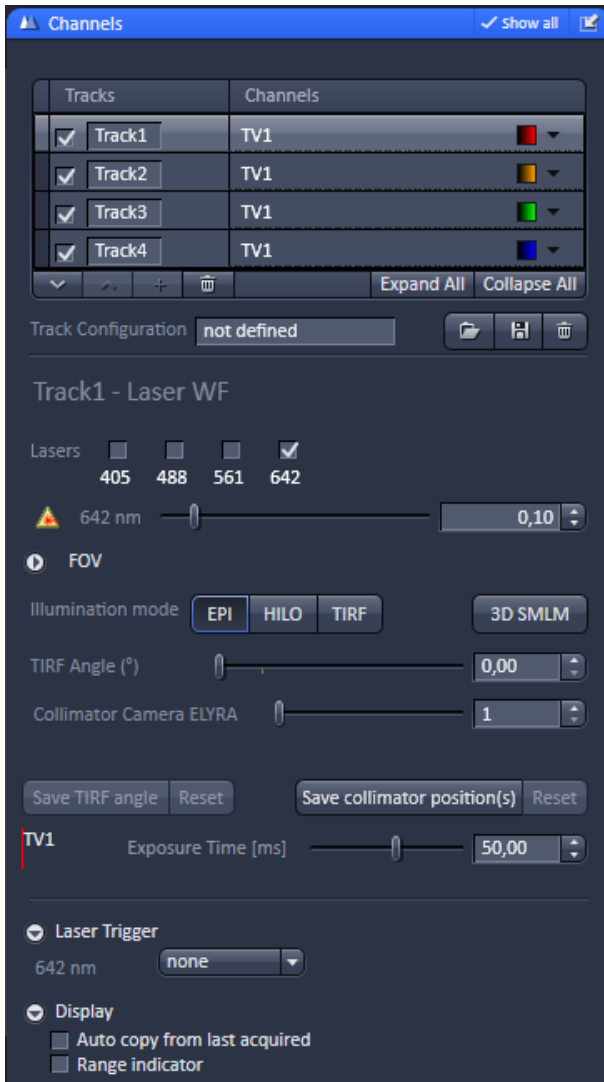
Fig. 125   Channels tool – Appearance for the Laser WF imaging mode of ELYRA types S & eLS systems
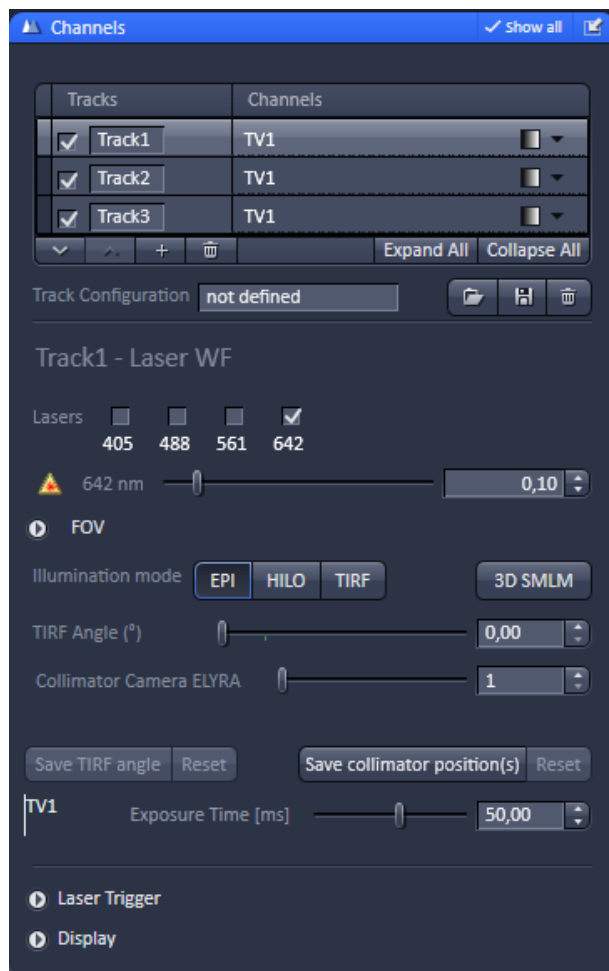
Fig. 126   Channels tool – Appearance for the Laser WF imaging mode of ELYRA tape eLS sytsem
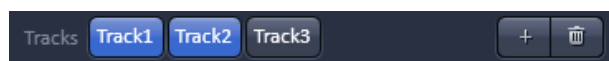
Fig. 127   Imaging Setup tool Tracks display– track selection of track 1 and 2 updated from the Channel tool

5.2.10.1   Defining Channels and Tracks

The **Channels** tool lists all tracks in the **Track Selection display** area that have been defined in the **Imaging Setup** tool.

The **Tracks** name and the used **Channels** for that track are indicated. Each track that is selected by mouse click will be highlighted and be displayed in the **Track Configuration display** area, which allows for individual settings of each track (Fig. 126).

Tracks are activated/deactivated for imaging with the check box.

Changes in the selection of the tracks in the **Channels** tool will automatically update the selection in the **Imaging Setup** tool and vice versa.

E.g. if Track 1 and Track 2 are selected in the **Channels** tool, they will be selected in the **Imaging Setup** tool as well (Fig. 127) and vice versa.

By clicking **Expand All**, all tracks will be selected and their settings displayed.

Collapse All de-selects all tracks and hides their settings.

You can either display one track or all tracks. There is no possibility to display several out of all tracks.

The color of a track can be set or changed selecting a LUT from the **Look Up Table (LUT)** drop down menu (Fig. 128).

The scroll bar allows access to all predefined colors.

The color is updated for the channels in the Imaging Setup tool of the corresponging track.

By pressing the **upwards pointing open arrow**, the highlighted track will move one position up in the displayed track array (Fig. 129).

Likewise, by pressing the **downwards pointing open arrow**, it will move one position down. The arrows are only active, if an upward or download movement is possible.

The sequence is updated in the Imaging Setup tool accordingly.

You can add up to 4 tracks by pressing the **+** button. You can delete tracks by highlighting them and pressing the **dust bin** button. The number of tracks is updated in the Imaging Setup tool accordingly.

You can load a predefined track configuration as a profile at the position of the highlighted track from the **Track Configuration** field (only available in the **Show all** mode) by pressing the **Load** button (symbolized by the **folder** icon) (Fig. 130).

☞ Please note that all tracks of a multitrack set up must be of the same imaging mode. If in a track one imaging mode will be changed, all other tracks will be deactivated untl they are set to the same imaging mode.
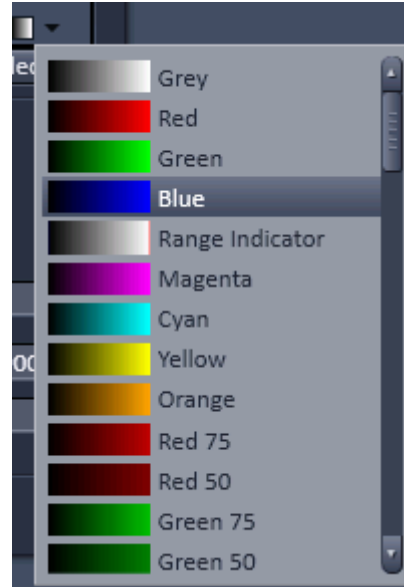
Fig. 128   **Track LUT drop down menu**

Fig. 129   **Track selection display – Track 2 moved to the top, no further upwards movement possible**

Fig. 130   **Track configuration**

All saved track configurations will be listed in the **Load Track Configuration** panel (Fig. 131).

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used track configurations or from the **Configurations** section, where all saved track configurations are displayed.

To search for a track configuration, type in its name or part of its name in the **Search** field and only those track configurations will be listed that contain the typed in string of letters.

To delete a track configuration press the **Delete** button (symbolized by the **dust bin** icon). This opens the **Track Configuration** panel (Fig. 132).

Select the configuration from the **Configurations** list by highlighting it or search for it by typing its name in the **Search** field.

Fig. 131   **Load Track Configuration panel**
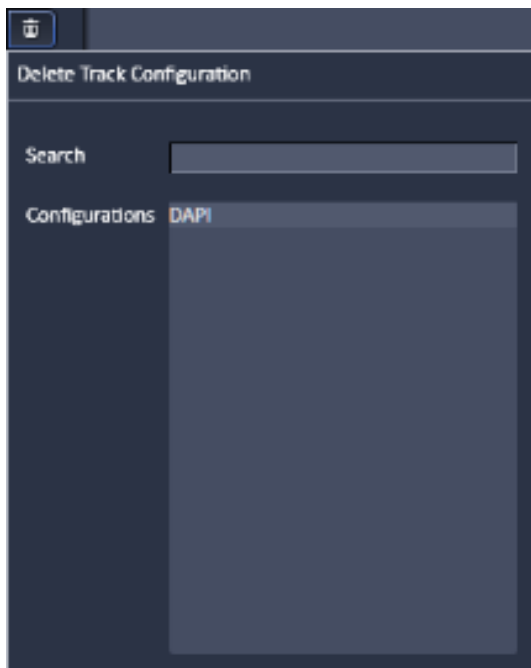
Fig. 132   **Delete Track Configuration panel**

The **Info** window pops up (Fig. 133).

Press **Yes** to delete the configuration, **No** to keep the configuration.

To save a configuration press the **Save** button (symbolized by the **disc** icon).

Fig. 133   **Delete information window**

This opens the **Save current Track configuration as** panel (Fig. 134).

Type in an appropriate name in the **Configuration** field and press **Ok** to save the configuration in the data base or press **Cancel** to abort the process.
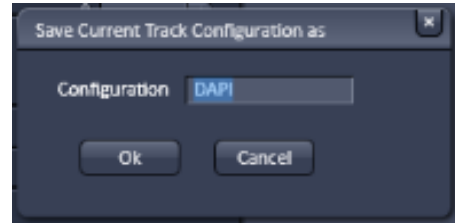
Fig. 134   **Save Current Track configuration as window**

The name of the track can only be changed when saving the track (Fig. 135).

If a highlighted track is saved with a certain name or a predefined track with a name is loaded into a highlighted track, the new name will appear in the **Tracks** designation box automatically.

Track designation in the **Imaging Setup** tool will automatically be updated (Fig. 136).
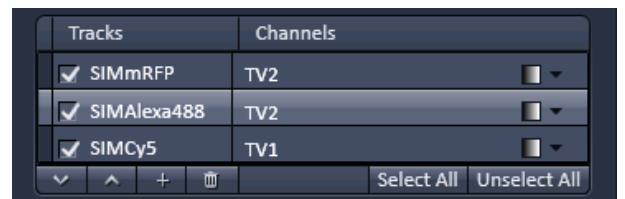
Fig. 135   **Track selection display – Tracks renamed**

The total set of parameters for one or multiple tracks can be stored as an acquisition configuration using the save option in the **Experiment Manager** field of the **Acquisition** panel.

Using multiple tracks is the preferred method to clearly separate weakly overlapping emission signals.

Fig. 136   **Imaging Setup tool Tracks display– Tracks renamed updated from the Channel tool**
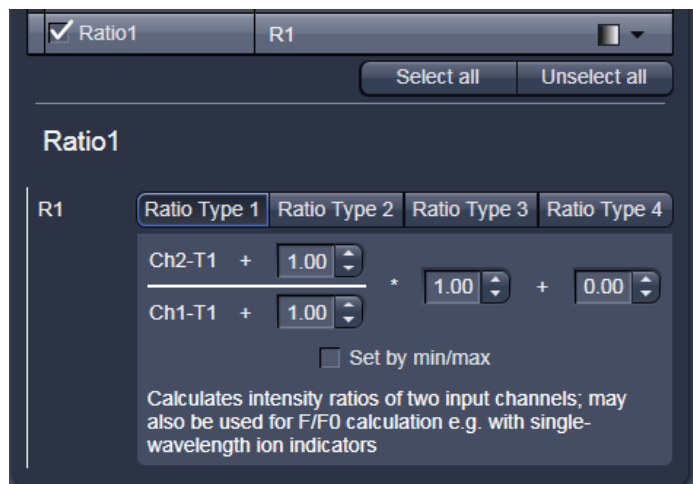
Fig. 137   **Channel Settings panel of a Ratio Channel**

5.2.10.2   Ratio Channels

The parameters for ratio metric imaging can be set when a ratio channel is selected.

- Click on the button of a ratio channel (e.g. **R1**).

- Clicking on the required tabs enables you to choose from four formulas (**Type 1** to **4**) for ratio calculation. The relevant decimal values can be entered in the input boxes. The entered values remain unchanged even after switchover to another formula and can be reactivated any time.

The formula type activated last is always used for ratio formation during the scan procedure. If the input box does not contain any value at all or no suitable value, the useful value last used will be activated.

The ratio channels are displayed as additional images.

Select the required formula and enter the relevant values.

Letters can be entered into the formula fields which will be valued as 1; it is also possible to make no entry, which will also be valued as 1, but will not be displayed.

Set by min/max allows the definition of the display scaling according to the expected minimal and maximal values.

- Laser lines and laser power are displayed according to each track. It is possible to vary laser intensities (in the same way as in the **Imaging Setup** tool).

☞   All parameters can be varied while imaging.

5.2.10.3 Track Control

Each laser can be selected for a track by checking the corresponding box (see Fig. 138, Fig. 139, Fig. 140).
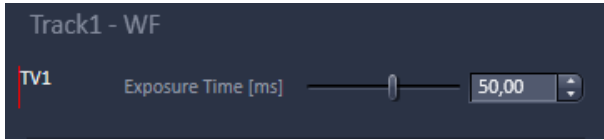
Fig. 138   Channel tool – track control; appearance for the WF imaging mode of ELYRA 7 Types S & eLS
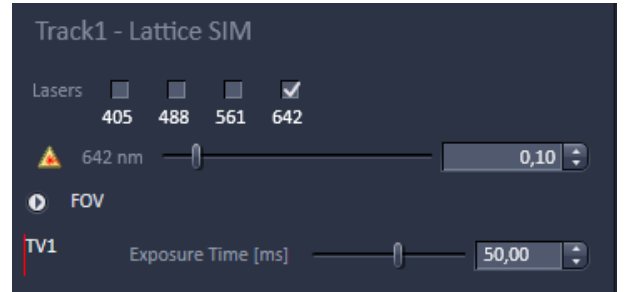
Fig. 139   Channel tool – track control; appearance for the Lattice SIM imaging mode of ELYRA 7 types S & eLS

For each checked laser the AOTF slider becomes available that allows to set the laser power via the slider or the input box.

☞ Note that for the **WF** imaging mode no laser line is available but the epi illumination is used.
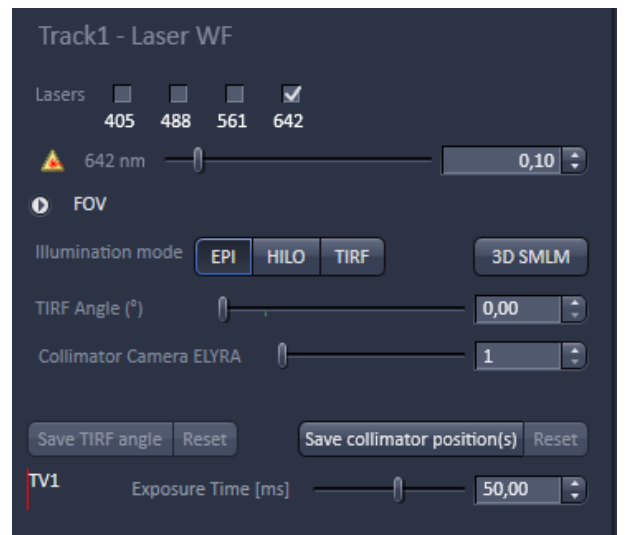
Fig. 140   Channel tool – track control; appearance for the Laser WF imaging mode of ELYRA 7 Types S & eLS
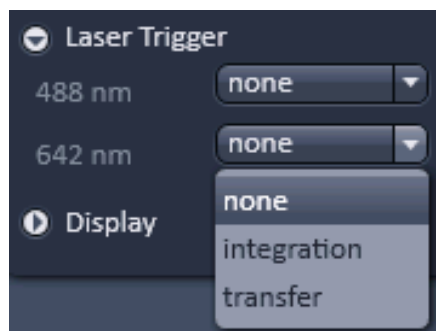
Fig. 141   Laser trigger expansion panel with FOV drop down menu

If in the **Laser WF** imaging mode at least one laser is selected, the **Laser Trigger** expansion field becomes available (see Fig. 141).

When expanding the box, all active lasers will be displayed with their **Trigger** drop down menus. You can select between

– **none**: no trigger

– **integration**: the laser is on only during the integration of the camera

– **transfer**: the laser is only on during the frame transfer of he camera

☞ Note that the laser trigger only works in the **Laser WF** imaging mode for the **Andor iXon SMLM** and the **CMOS pco.edge SIM** cameras.

For the **SIM Apotome**, **Lattice SIM** and **SMLM** imaging modes the Field of View (**FOV**) expansion field is available (see Fig. 142). By opening the field you can select the field of view via the **FOV** drop down menu (see Fig. 142). You have the following options

– **SIM** for the SIM imaging mode

– **TIRF** for the laser WF imaging mode

– **HP** (high power field) for the laser WF imaging mode

– **uHP** (ultra high power field) for the laser WF imaging mode
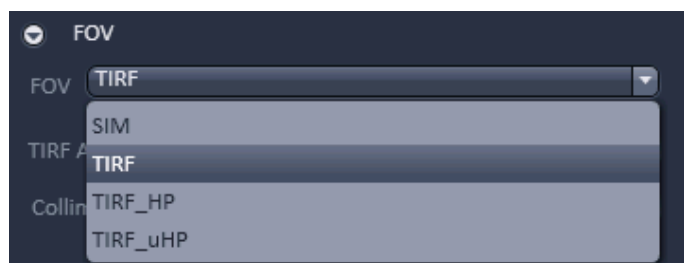
Fig. 142   FOV expansion panel with FOV drop down menu

In the **Laser WF** under **Illumination mode** you can select the illumination scheme you want to use. By pressing either of the buttons **EPI** (Epifluorescence), **HILO** (Highly Inclined Laminar Optical Sheet) or **TIRF** (Total Internal Reflection Fluorescence). The active button is highlighted (see Fig. 142). You can also press the **3D-PALM** button to activate the 3D-PALM slider to move into the back aperture of the objective once an active Scan is started.

The button is highlighted, if active. Press the button again to deactivate and to put the 3D-PALM slider in its rest position. When active online processing will be disabled.

By pressing the **Save TIRF angle** button the current set TIRF angle will be saved that will be recalled when pressing the **Reset** button on it right. By pressing the **Save Collimator position(s)** button the current set collimator position will be saved that will be recalled when pressing the **Reset** button on its right.

The **Channel** display field shows all selected channels with their name. Each channel can be controlled separately. In the **WF**, **SIM** and **Laser WF** an EMCCD camera serves as the detector for each channel. You can set the exposure time by and the EMCCD gain via the **Exposure Time [ms]** and **EMCCD Gain** sliders, respectively or their input boxes.

The **Camera Temperature** display field shows the current status of the camera's temperature.

5.2.10.4   Display Options

You can set display options by opening the **Display** expansion field (see Fig. 143).

Fig. 143   Display expansion field

If the **Auto copy from last acquired** box is checked, the settings from the last acquired image will be taken.

By checking the **Range indicator** box the image will be displayed with the range indicator look up table.

5.2.11   Acquisition Parameter – Focus

If the **Focus** tool tab is expanded the **Focus control** tool becomes available (Fig. 144).

The **Z-Position** display box shows the current z-position.

The number can be edited by typing or using the arrowheads. The nosepiece or stage will automatically update the new position.

By pressing the **Stop** button, you can stop the current movement. The **Step Size (µm)** display box shows the currently used step size for focus movement. The value can be edited by typing or using the arrowheads.

The **Home** button sets the z-position to the predefined zero value.

Pressing **Load** will lower the nose piece (z-position) for objective change.

Work brings the z-position back to the actual used position if the focus was zeroed or in **Load** position.

Fig. 144   Focus tool

Home and Load positions can also be accessed from the z-position display by pressing the **H** or **L** buttons.

The z-position can be altered by grapping and sliding the **plane** or by using the arrow slider. The **plane** shows the actual z-position.

Pressing **Manually** in the **Zero position** field will set the current z-position to 0.

If a **Z-Piezo stage** insert is in use and active then pressing **Leveling** in the **Piezo field** will center the Piezo stage position in regard to the microscope focus drive.

The **Piezo Position (µm)** input box allows the position of the Piezo to be altered.

The **Step Size (µm)** display box shows the currently used step size for focus movement of the **Z-Piezo stage**.
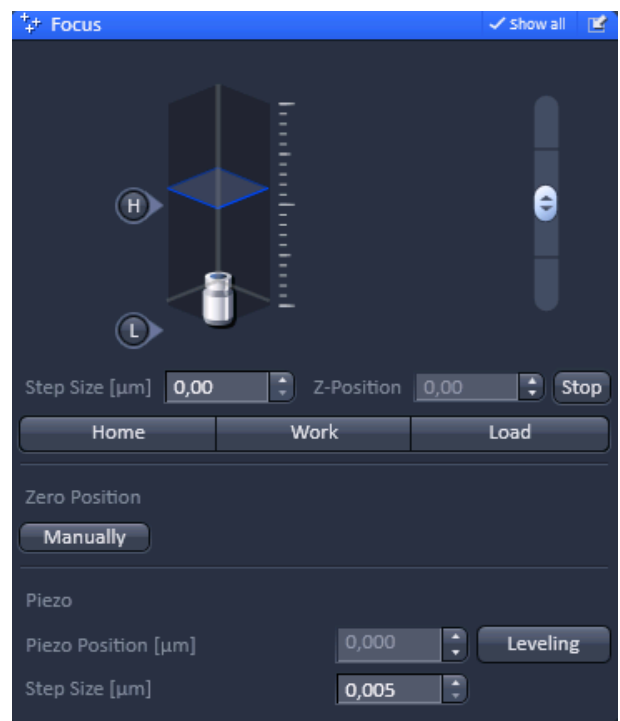
Use of an optional high resolution focusing device (Piezo objective focus or Z-Piezo stage insert)

The **Piezo Position** is indicated as a numerical value. This value and with it the focus position can be changed. A change of this value updates the value of the microscope focus accordingly.

The **Step Size**, by which the Piezo focus moves when clicking on the arrows, can be set between 0.005 µm and 10 µm.

As soon as the focus position of the microscope is changed (via hand wheel or software), the piezo objective focus is automatically levelled.

A click on the **Leveling** button moves the Piezo objective focus or Z-Piezo stage insert into the center position of its travel range and the focus position of the microscope is reset to compensate for the leveling. Therefore, the same Z-level remains visible (the current position is not set to zero).

The motor focus of the stand is operated in the same way via the relevant buttons. Moving into the **Work** or **Load** position is always performed via the motor focus and not via the Piezo objective focus.

☞ Please see printed manual CHAPTER 4: ANNEX for further information on the Piezo objective focus and Z-Piezo stage insert.

5.2.12 Acquisition Parameter – Definite Focus

The **Definite Focus** tool is available in the **Acquisition Parameter** tool group if a Definite Focus (DF) device is physically attached to the system (Fig. 145).

- Click on the left arrow to expand the **Definite Focus** tool area (Fig. 146). Click again to collapse the area.

- Activate **Show all** mode.

There are three areas in the **Definite Focus** control panel (Fig. 146):

- **Info** display (**Objective:**, **Status:** lines)

- **Action** buttons (**Find Surface**, **Store Focus**, **Periodic**, **Recall Focus**)

- **Z-Piezo** control (**Use Z Piezo** check box)

☞ The **Definite Focus** tool allows control of the Definite Focus during continuous scan and for experiments were the **Autofocus modus** of the Focus Devices and Strategy is **Off**.

☞ The **Definite Focus** tool is only available if a Definite Focus device of the 2nd generation (DF2) is attached to the system. The tool is not available for a Definite Focus device of the 1st generation (DF1).

In case the Definite Focus is switched off or has experienced some hard- or firmware problems the **Status:** line will display a **- Device not initialized -** message in red letters (Fig. 147) indicating that the device is not available for operation.

Fig. 145   Acquisition Parameter tool group with Definite Focus tool
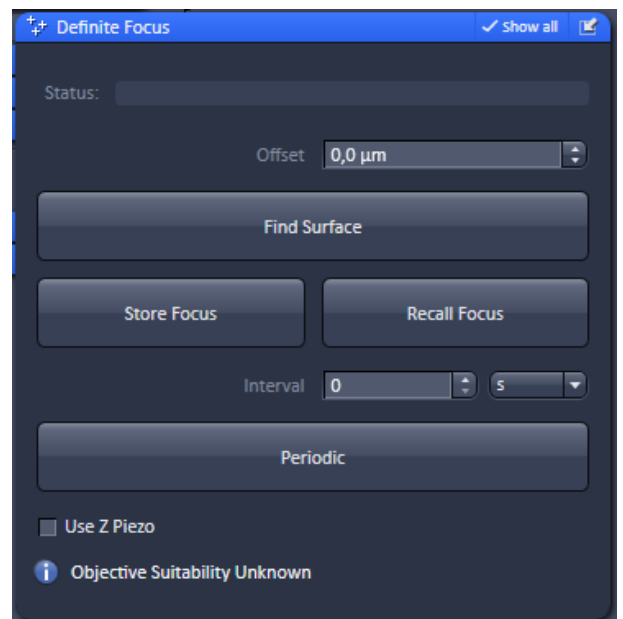
Fig. 146   Definite Focus tool: Expanded area with "Show all" active.
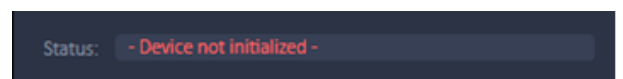
Fig. 147   Definite Focus tool with Definite

(1)   Action buttons

Action buttons are used to execute reflex finding and focus stabilization by the Definite Focus device.

☞   The last used Action button will be indicated by a blue rim.

☞   Action buttons can all be executed during a continuous scan.
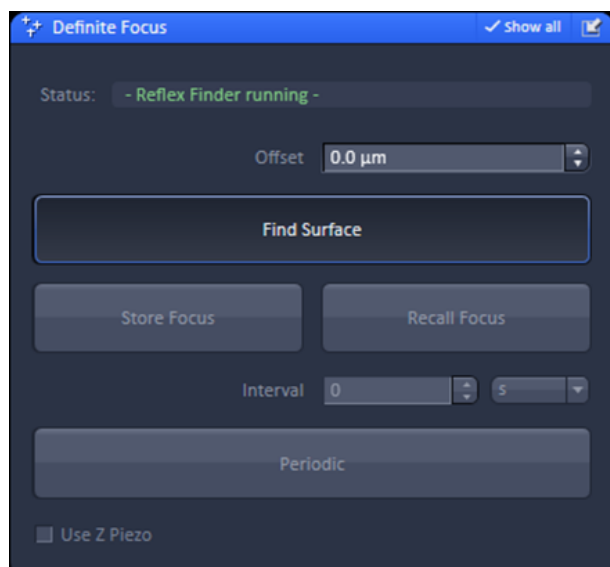
Fig. 148   **Definite Focus tool: Find Surface pressed and reflex finding running.**
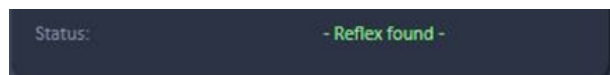
Fig. 149   **Definite Focus tool: Find Surface pressed and reflex found.**

(2)   Find Surface

When pressing the **Find Surface** button, the microscope stand's drive will perform a full range Z-Scan and the Definite Focus will look for the strongest reflection signal (Fig. 148). During operation, the **Find Surface** button stays actively pressed and the **Status:** line will display a blinking (fading and appearing) **- Reflex Finder running -** message in green letters. The Definite Focus will be adjusted to the highest reflection signal.

☞   Please note that even if the **Use Z Piezo** check box is activated, **Find Surface** will always use the microscope stand's Z-drive.

☞   Pressing the **Find Surface** button will inactivate the **Store Focus** and the **Recall Focus** buttons (greyed out buttons). Even if the **Periodic** button stays active, pressing the button will be ignored during the reflex finding run. After the run all buttons become active again.

Once a reflex is found and the Z-Scan stalled the **Status:** line will report **- Reflex found -** in green letters (Fig. 149).

If the Z-Scan during reflex finding should be aborted, the Find Surface button has to be pressed again during the operation. This will result in a **Status:** line **- Error -** message in red letters.

☞   The display on the Definite Focus will also show an error message in this case. The Definite Focus, however, can be used in subsequent operations without the need of a reboot. If the **Status:** line displays **- Error -** the reason for this error is reported in the error log-file. An error number is also displayed on the Definite Focus device and can be looked up in the Definite Focus Manual.

If the refractive index mismatch between the immersion medium and glass is low, a reflection might be too weak to be detected.

If the projected pattern is detectable but too weak, or the signal peak is not completely captured within the range of the Definite Focus the **Status:** line will display **- Error -** in red letters alike the situation, when the **Find Surface** procedure is interrupted (Fig. 150).

If however the grid cannot be unambiguously identified within the signal, the Status: line will display **- Reflex not found -** in grey letters (Fig. 151). In either way, the Definite Focus will resume normal operation the next time it will be used.

☞ If a reflection is not found, the system will re-scan the whole Z-range several times before reporting **- Error -** or **- Reflection not found -** upon which the operation will end. If you want to disrupt the process, you have to press the pressed **Find Surface** button during operation. In this case **- Error -** will immediately be reported on the **Status:** line and the run will end.

When a reflection is found the Definite Focus will have its focus at the reflection position. This does not necessarily mean that the sample is in focus. If you want to have the system focus on the sample after reflex finding, you for once in your session have to determine the offset between Definite Focus reflex and the focus position of the sample.

To this end perform **Find Surface** until **- Reflex found -** is displayed in the **Status:** line and then manually set the **Z-Position** to zero by pressing the **Manually** button in the **Zero Position** field of the **Focus** tool display window (Fig. 152).

Fig. 150   Definite Focus tool: Find Surface pressed and reflex not found because of insufficient signal strength.

Fig. 151   Definite Focus tool: Find Surface pressed and reflex not found due to insufficient grid recognition.

Fig. 152   Focus tool: Zero focus
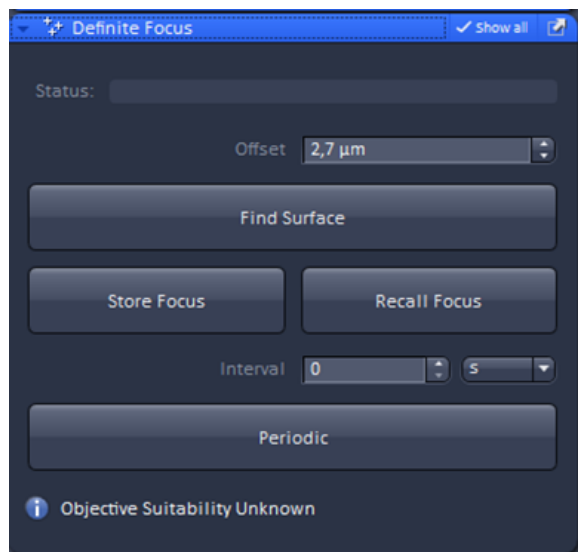
**Fig. 153   Definite Focus tool: Offset
entered in the Offset input box.**

**Fig. 154   Focal planes before (left) and
after (right) offset correction**
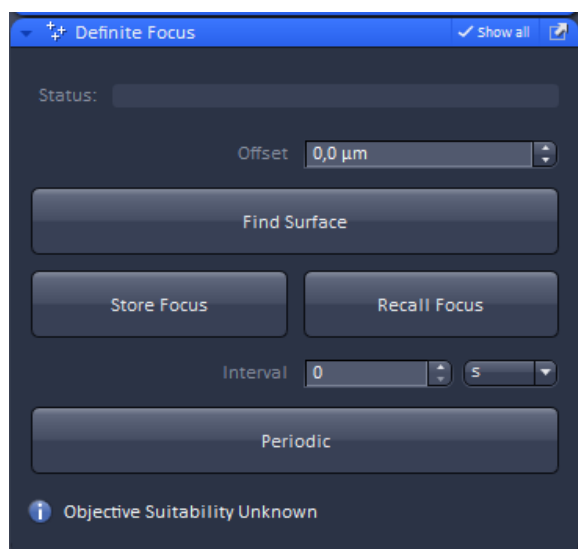
**Fig. 155   Definite Focus tool: Store Focus
executed.**

Slightly focus up and down using the stand's Z-drive until your sample is in focus.

Read the actual Z-position (offset between definite Focus and sample focus) in the **Z-Position** input box of the **Focus** tool display window and enter the number in the **Offset** input box of the **Definite Focus** tool window (Fig. 153).

The next time **Find Surface** is executed the offset will be added to the 'reflex' position (Fig. 154).

(3)   Store Focus

When pressing the **Store Focus** button (Fig. 155) the system will store the current sample Z-position as the position the Definite Focus will stabilize on when pressing **Periodic** or **Recall Focus**.

☞   There will be no feedback in the software which the current focus position is that was selected by pressing the **Store Focus** button. Nor will be the **Store Focus** button be highlighted. When the focus is changed and the new position should be used for the Definite Focus, the **Store Focus** button has to be pressed again, otherwise the old position will be used.

(4)   Periodic

When pressing the **Periodic** button, the system will periodically stabilize at the pre-set focal position. If **0** is entered in the **Interval** input window, stabilization will be continuous.

A blinking green message **- Stabilizing -** indicates that the Definite focus is permanently stabilizing (Fig. 156).

During operation in continuous mode the **Periodic** button is highlighted as pressed.

To stop periodic stabilization the highlighted **Periodic** button has to be pressed once again.

For time interval stabilization, a suitable interval time has to be entered in the **Interval** input box along with choosing a suitable time unit from the **units** drop down menu (Fig. 157). In this case the message **- Stabilizing -** will be permanently displayed during operation regardless if the Definite Focus is currently stabilizing or in waiting position. From the **units** drop down menu you can choose between seconds **s**, minutes **min** hours **h** and days **d** (Fig. 158). During operation in interval mode the **Periodic** button is highlighted as pressed.

To stop periodic stabilization the highlighted **Periodic** button has to be pressed once again.

If the Definite Focus receives no or a too weak signal during periodic stabilization the **Status:** line will display **- Error -** in red letters.

☞   A **Periodic** stabilization will be interrupted by pressing the **Find Surface** or **Recall Focus** buttons, but will be resumed after the reflex finding or stabilization procedures have been finished.

☞   When changing the focus during **Periodic** stabilization the new focus position will be the new stabilization position when the stand's Z-Drive is regulated by the Definite focus (**Use Z Piezo** check box inactive). In contrast, if the Definite Focus controls the Z-Piezo stage (**Use Z Piezo** check box active), the original focus position will be kept as the position to stabilize on.

☞   **Periodic** stabilization triggered from the **Definite Focus** tool will run asynchronously to image acquisition. In contrast, stabilization triggered from the **Focus Device and Strategies** tool will work synchronously.
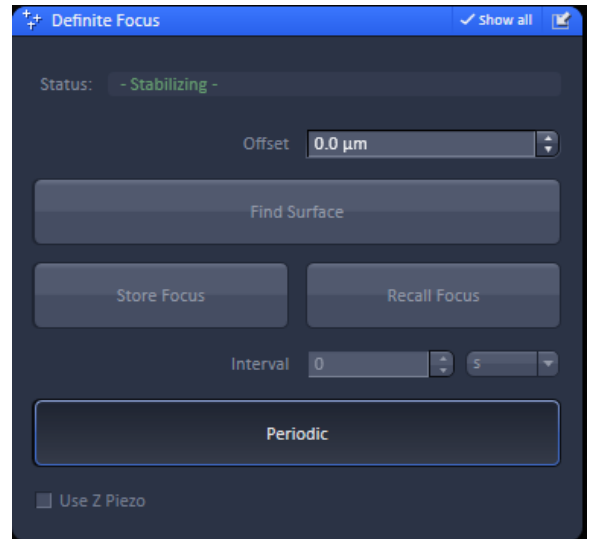
Fig. 156   Definite Focus tool: Periodic continuous stabilization executed.
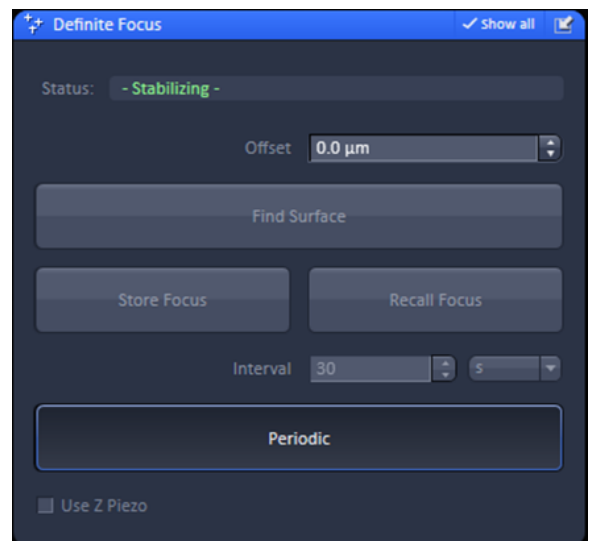
Fig. 157   Definite Focus tool: Periodic interval stabilization executed.
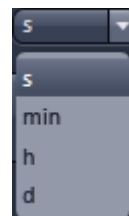
Fig. 158   Definite Focus tool: Units drop down menu for interval periodic stabilization.
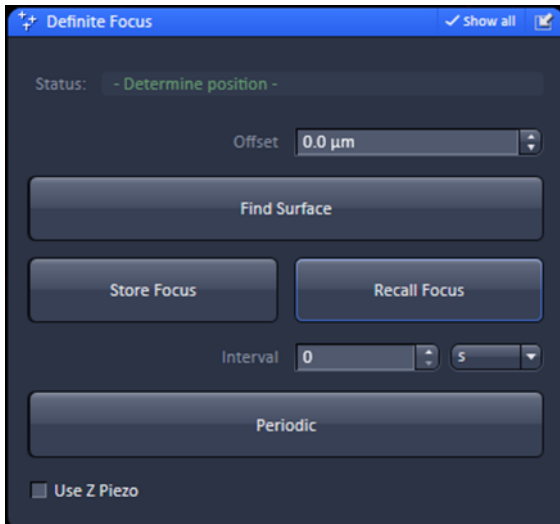
Fig. 159   Definite Focus tool: With "Recall Focus" a one-time stabilization is executed.

Fig. 160   Definite Focus tool: With "Recall Focus" being successful.

Fig. 161   Definite Focus tool: With "Recall Focus" being not successful.

(5)   Recall Focus

When pressing the **Recall Focus** button, the system will for one time stabilize at the pre-set focal position (Fig. 159). During stabilization the **Status**: line will display in green a blinking message **- Determine position -**, if the process takes more than 1 sec. Otherwise such a message is not displayed.

If the Definite Focus was able to stabilize, the **Status:** line will display the message **- Position stabilized -** in green letters (Fig. 160).

If the Definite Focus receives no or a too weak signal during stabilization the **Status:** line will display **- Position not stabilized -** in red letters (Fig. 161).

☞   Pressing **Recall Focus** will interrupt a **Periodic** stabilization, which will be resumed after the **Recall Focus** process is finished.

☞   If the focal plane is changed above a certain amount, the **- Position stabilized -** message will disappear. If **Recall Focus** is pressed again, the system will adjust and stabilize on the original focal position and the message reappears if the procedure was successful. If the focus change was too big, **Recall Focus** is likely to fail and in this case the message **- Error -** in red letters will be displayed.

(6) Z-Piezo control

If the **Use Z Piezo** check box is inactive the Definite Focus will execute stabilization via the microscope stand's Z-Drive. If the box is checked, the Definite Focus will control the Z-Piezo stage with the exception of **Find Surface** that always will use the stand's Z-drive (Fig. 162).

☞   Note that the **Use Z Piezo** check box is only available for systems with a Z-Piezo stage attached.

☞   The Z-Piezo stage is more precise and faster than the stand's Z-Drive and should be the preferred option when available.

The stand's Z-drive and the Z-Piezo stage can be controlled by separate hand wheels. If the Z-Piezo is moved via its hand wheel its range will not be centered around the focal position any longer. To center the range around the focal position you have to press the **Levelling** button in the **Focus** tool's **Piezo** area (Fig. 163). The **Piezo Position [µm]** input box will then be reset to **0**.

☞   Using the stand's Z-drive hand wheel will only affect the **Z-Position** input box displayed values and leaves the **Piezo Position [µm]** input box values unaffected. In contrast, using the Z-Piezo stage hand wheel will alter both display values.

The Z-Piezo and the stand's Z-drive affect the Definite Focus during **Periodic** stabilization in the following different ways:

–   Definite Focus controls the Z-Drive (**Use Z Piezo** inactive): Turning on the **Z-Drive's hand wheel** will alter the focus position the Definite Focus stabilizes on to the set new focus, which consequently will regulate to the new value.

–   Definite Focus controls the Z-Drive (**Use Z Piezo** inactive): Turning on the **Z-Piezo's hand wheel** will not alter the focus position the Definite Focus stabilizes on, which consequently will regulate to the old value.

Fig. 162   **Definite Focus tool: Z-Piezo in use in conjunction with Definite Focus.**
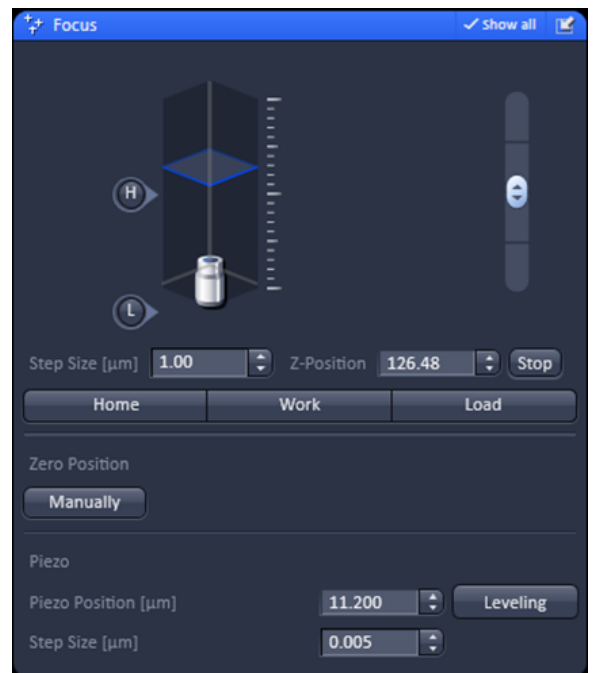
Fig. 163   **Focus tool: Focus and Piezo control panel.**

–   Definite Focus controls the Z-Piezo (**Use Z Piezo** active): Turning on the **Z-Drives's hand wheel** will not alter the focus position the Definite Focus stabilizes on, which consequently will regulate to the old value.

–   Definite Focus controls the Z-Piezo (**Use Z Piezo** active): Turning on the **Z-Piezo's hand wheel** will not alter the focus position the Definite Focus stabilizes on, which consequently will regulate to the old value.

In other words, whenever the Z-Piezo is involved during **Periodic** stabilization, either as the control element for the Definite Focus or as the device to alter the focus, the Definite Focus will regulate back to the pre-set focal position. So during **Periodic** stabilization the focus cannot be manually altered in these cases.
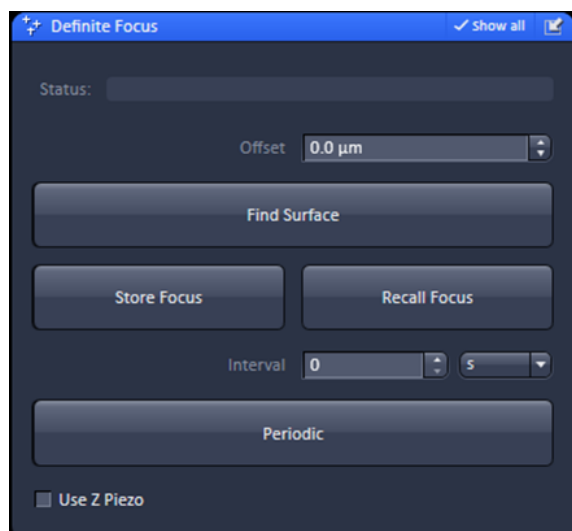
Fig. 164   Definite Focus tool: "Info" panel with "Objective:" info "suitable" and empty "Status: " line.
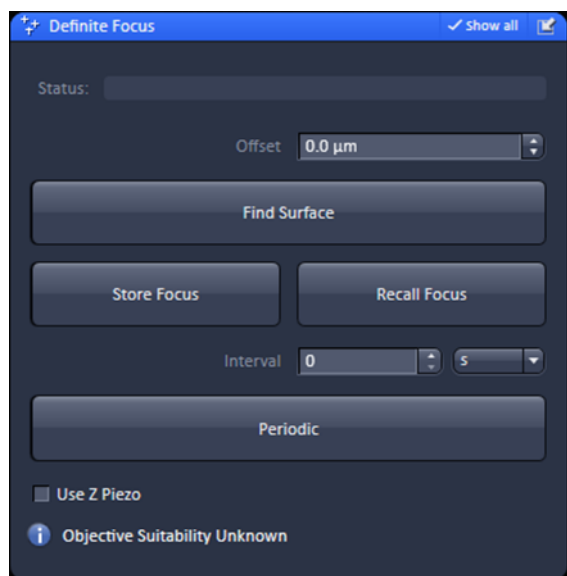
Fig. 165   Definite Focus tool: "Info" panel with "Objective Suitability unknown".

(7)   Info display

The info display area gives information on (Fig. 164):

- **i = information:** Suitability of the selected objective for Definite Focus

- **Status:** Current state the Definite Focus is in. The status line will be empty prior to use of the Definite Focus.

Info on objective

If the objective is suitable for Definite Focus no message is displayed in the **information** area, which stays collapsed (Fig. 164).

If the objective is unsuitable for Definite Focus or if there is no information available on its suitability, the information **Objective Suitability Unkown** appears (Fig. 165).

☞   In order for a correct display, the objective has to be correctly listed in the ZEN Software (in **Maintain / Objectives / Detection Objective**) as well as for the microscope stand (**Home / Settings/ Objectives** via the TFT).

☞   Please note, that the ZEN Black Software and the microscope stand do not update each other, so a new objective has to be entered in both listings. In case there is a mismatch between both databases a warning message will appear when booting the system (Fig. 166). Click **Ok** to continue and make necessary changes as required.
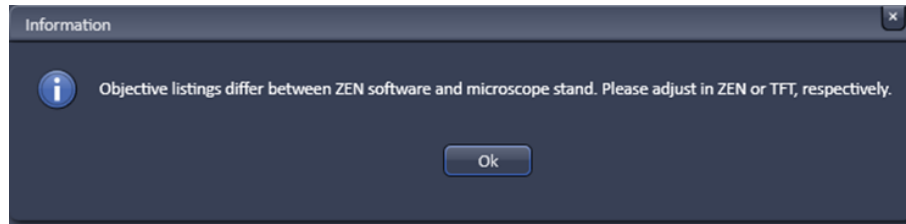
Fig. 166   Warning information at ZEN Software startup if the system detects differences in the ZEN and Firmware listed objectives

Info on Status

The **Status:** line indicates the current status of the Definite focus. It comprises the following messages:

– **Device not initialized** (in red): Definite Focus is switched off or experienced hard- or firmware problems during initialization.

– **Error** (in red): occurs when the Definite Focus has no sufficient signal or when there is an error on the hard- or firmware (Fig. 167).

– **Reflex Finder running** (in blinking green): whenever the **Find Surface** button was pressed and the system is actively looking for the glass reflex.

– **Reflex found** (in green): whenever after pressing the **Find Surface** button a glass reflex was found.

– **Reflex not found** (in grey): whenever after pressing the **Find Surface** button the glass reflex was not found (due to missing signal).

Fig. 167   Definite Focus tool: "Status: " line with "- Error -" message.

– **Stabilizing** (in blinking green): whenever the **Periodic** button was pressed for continuous stabilization

– **Stabilizing** (in green): whenever the **Periodic** button was pressed for interval stabilization.

– **Position stabilized** (in green): whenever the **Recall Focus** button was pressed and the Definite Focus found a sufficient strong signal to stabilize on.

– **Position not stabilized (in grey)**: whenever the **Recall Focus** button was pressed and the Definite Focus did not find a sufficient strong signal to stabilize on.

– **Determine position** (in blinking green): whenever the **Recall Focus** button was pressed and the Definite Focus screens for a sufficient strong signal to stabilize on.

☞   The **- Error -** message will be listed in the Error log display. The Definite Focus device shows in its display also **Error** followed by a number that can be looked up in the Definite Focus manual.
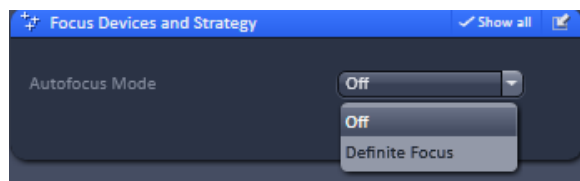
Fig. 168   Focus Devices and Strategy tool: "Autofocus Mode" drop down menu.
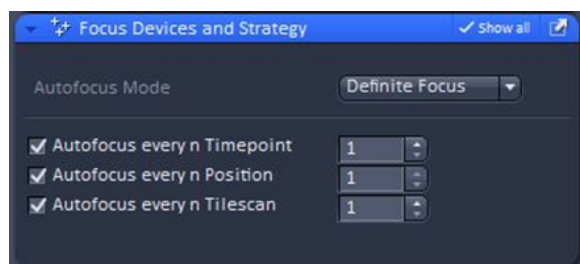
Fig. 169   Focus Devices and Strategy tool: Definite Focus selected

(8)   **The Definite Focus tool during synchronous stabilization**

Whenever the **Definite Focus** is selected from the **Autofocus Mode** drop down menu of the **Focus Device and Strategy** tool (Fig. 168) as the focus strategy, this setting will overrule the settings made in the **Definite Focus** tool whenever a scan is triggered by **Start Experiment**.

When Definite Focus is selected from the **Autofocus Mode** drop down menu, the **Autofocus every n Timepoint / Position / Tilescan** check boxes with their input boxes become available, dependent on the chosen dimensionality of the experiment.

To run in synchronous mode please check the boxes accordingly and enter an appropriate number in the input box defining before which events a stabilization should occur (Fig. 169).

☞   Stabilization before each time point (enter **1** in **Autofocus every n Timepoint**) is the most frequent stabilization scheme possible.

☞   No continuous stabilization can be triggered from within the **Focus Device and Strategy** tool.

☞   Stabilization from the **Focus Devices and Strategy** tool runs synchronously to the scan. In contrast, stabilization from the **Definite Focus** tool will be asynchronous.

☞   The state of the **Use Z Piezo** check box is applied for the stabilization via the **Focus Devices and Strategy**. That means if the box is checked, the Z-Piezo will be used.
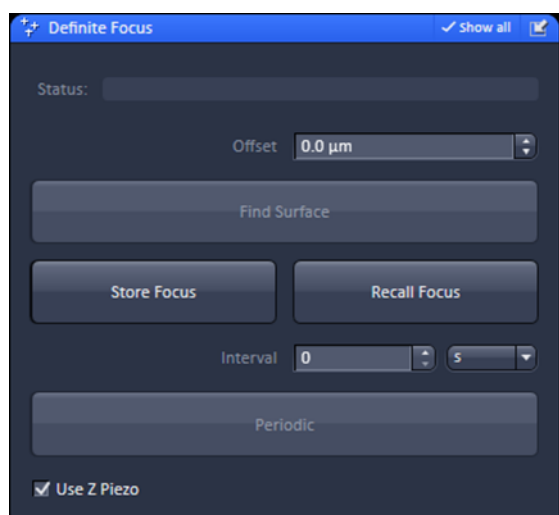
Fig. 170   Definite Focus tool: "Continuous scan" active

With **Definite Focus** selected in **Focus Devices and Strategy** tool the **Find Surface** and **Periodic** buttons will be deactivated during a scan in the **Definite Focus** tool (Fig. 170), no matter if the scan was triggered by **Continuous** or **Start Experiment**.

If **Periodic** is active and the **Continuous** scan button pressed, the stabilization, even if the **Periodic** button becomes inaccessible, will run with the respective settings in the background.

When the **Stop** scan button is pressed, the once active **Periodic** button becomes active again.

Stabilization will end only if the active button is pressed again. In contrast, if **Start Experiment** is pressed the **Periodic** button becomes inaccessible as well, but the stabilization regime is executed as defined under **Focus Devices and Strategy**.

After the end of the Experiment **Periodic** stabilization remains switched off.

An active **Find Surface** becomes deactivated by pressing both, **Continuous** or **Start Experiment** and remains inactive after the scan finished.

☞   Only if the **Start Experiment** button has been pressed synchronous stabilization will be effective.

5.2.13 Focus Devices and Strategies for Widefield Imaging Modes

You can opt for (Fig. 171):

– **Off**, no holding focus

– **Definite Focus**, hardware holding focus
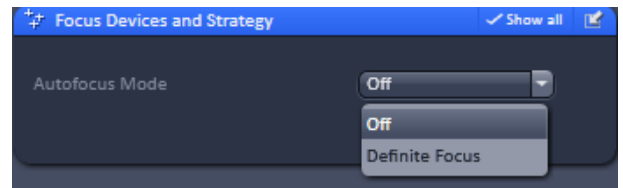
Fig. 171   **Focus strategies for Laser WF imaging mode**

If **Definite Focus** is selected, the **Autofocus every n Timepoint** check box becomes available.

Check the box to operate the Definite Focus in synchronous mode (Fig. 172).

Define the cycles in number of frames in which the Definite Focus will be activated in the **Autofocus every n Timepoint** input box.
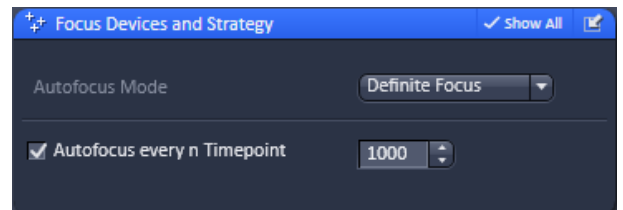
Fig. 172   **Definite Focus menu**

☞   We strongly recommend to use the Definite Focus in asynchronous and continuous mode operated by the TFT display for stabilization in SMLM experiments to minimize drift as best as possible. Note, in synchronous mode started from ZEN Software, the actual z-position at the time the Definite Focus will start is kept as the reference point. Hence, changing the focus will result in a compensation by the Definite Focus the next time it is active. In contrast, in asynchronous mode started from the TFT display, changing the focus by the stands focus drive will set this focus as the new reference point.
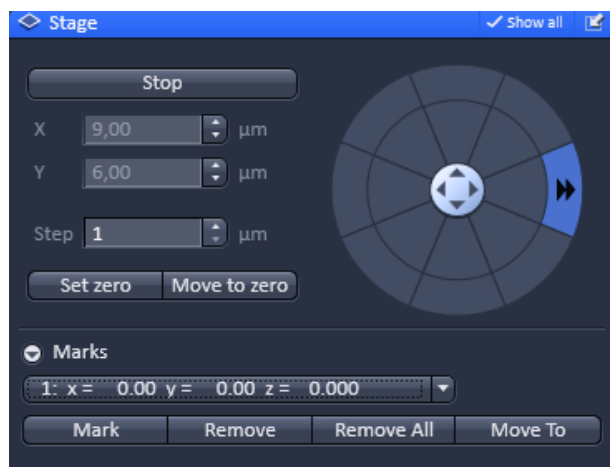
Fig. 173   **Stage control tool**
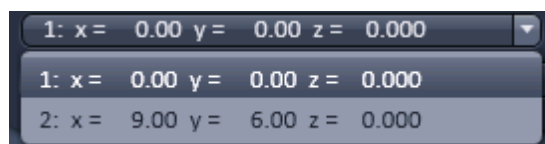
Fig. 174   **Marks position list drop down menu**

5.2.14   Acquisition Parameter – Stage

The following software description applies to systems which are equipped with a motorized scanning stage.

If the **Stage** tool window is expanded the **Stage control** tool becomes available (Fig. 173).

By pressing the **Stop** button you can interrupt any stage movement.

The **Move to Zero** button will move the stage to the current zero positions of the x and y coordinates.

The compass rose will move the stage in the direction of the arrowheads, which appear, when the mouse pointer is being in the field.

Fast movement is obtained with the outer rose, slow movement with the inner rose. The **X-Position (µm)** and **Y-Position (µm)** display boxes show the actual x and y coordinates.

You can edit the numbers by typing or using the arrowheads. If numbers were altered, the first click on the compass rose will bring the stage to the altered position.

The **Step Size (µm)** display box show the currently used step size for stage movement. The value can be edited by typing or using the arrowheads. The **Zero Position Set** button sets the x and y coordinates to 0.

If the **Mark** button is pressed, the current center position of the image will be stored. It can be recalled by selecting the position from the **Marks position list** drop down menu (Fig. 174).

Pressing the **Move to button** will bring the stage back to the position of the loaded position. By pressing **Remove**, the actual displayed position will be deleted. Pressing **Remove All** will delete all stored positions.

☞   If only one selected position is deleted, the position with the next number in sequence moves up one number.

☞   If a new **Zero** Position is **set** the coordinates of the marked positions are updated in relation to the new Zero Position.

The focus is moved to the marked position selected form the drop down menu by clicking **Move To**. Three different values for the **Speed Settings** are available. They can be assigned interdependently to **Speed 1-3**.

☞   The ELYRA systems are equipped with a Piezo stage, which is mounted on top of the microscope stage. If Koehler illumination was adjusted, the condensor can reach so deep that it can collide with the Piezo stage frame when the full travel range of the motorized microscope stage is used. Care should therefore be exerted in this case by restricting the travel range. If Koehler illumination is not required or if a 96 well plate is used it is recommended to move the condenser to the up position to avoid potential collisions.

5.2.15   Acquisition Parameter – Shuttle and Find

Shuttle and Find allows calibration and imaging of sample positions in laser wide field (laser WF) and SIM mode for reproducible repositioning after transfer to scanning electron microscopes (SEM).

For **Shuttle and Find** different types of sample holders are pre-designed. In addition the user can define other correlative sample holders.

The correlative sample holders have three fiducial markers enabling a three point calibration.

The calibration can be done in transmitted light or reflected light.

☞   A motorized microscope stage is mandatory.

☞   The workflow is unidirectional from SR (laser WF, SIM) to SEM only.

Different types of sample holders are available (Fig. 175), e.g.:

– Life Science cover glass 22x22

– Life Science for TEM Grids

– MAT Universal A

– etc.

☞   The Life Science cover glass 22x22 or the sample holder for TEM Grids should not be used together with Axio Imager and Axio Examiner equipped with a level adjustable sample holder. During the calibration procedure the objective or neighbouring objectives will collide with the level adjustable holder.
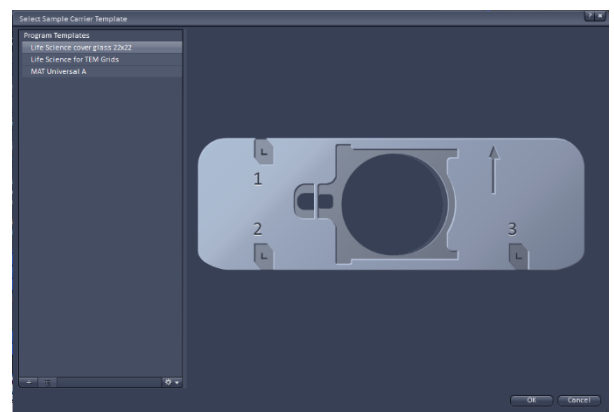
Fig. 175   Select Sample Carrier Template dialog

Creating of user-defined correlative holders

In the calibration dialog window new correlative holder files can be defined (Fig. 176).

• Click on the **+** button in the **Select Template** dialog.

• Type in a name for the new holder / sample carrier. An image of the new holder can be loaded.

In the holder file the distances (in millimeters) between the first and the second marker and between the second and third marker have to be inserted.

Fig. 176   New Sample Template dialog

The distances can be determined using the **Positions** button in the **Dimensions** control view and the functionality for **Marks** in the **Stage** tool in the Left Tool Area.

• Activate the **Positions** button and click on the first correlative marker.

• Determine the coordinates by clicking on **Mark** in the **Stage** tool.

- Go on with this procedure for marker two and three. Use coordinates for calculating the distances between the markers.

Mounting of cover glasses or TEM grids

- Place the specimen (sample carrier) in the suitable sample holder and fix it:

  Life Science Cover Glass 22x22:

  – Remove the clamping frame (Fig. 177/**1**) using tweezers.

  – Insert the cover glass (Fig. 177/**2**) in the sample holder (Fig. 177/**3**).

  – Slide in the clamping frame into the sample holder until the clamps are clicking into place (Fig. 177/**4**).

  Life Science for TEM Grids:

  – Lift the spring (Fig. 177/**5**) of the appropriate position and turn it side wards.

  – Insert the TEM grid (Fig. 177/**6**) into the provided holding spot of the holder (Fig. 177/**7**) and fix it with the spring.

Fig. 177   Mounting of cover glasses or TEM grids

Inserting a sample holder (various mounting frames are possible)

- Insert the sample holder into the mounting frame (Fig. 178/**3**) of the microscope stage in the following way:

    – for inverted stands, see Fig. 178/**1**

    – for upright stands, see Fig. 178/**2**
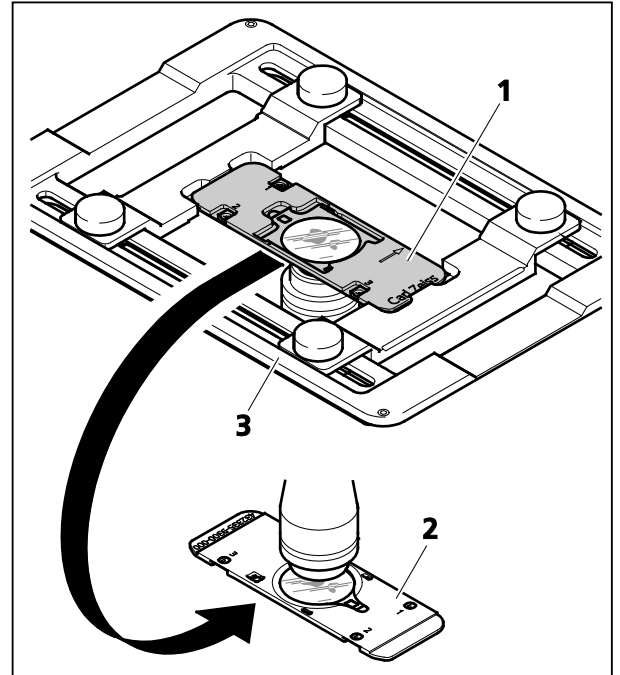
Fig. 178   Inserting a sample holder
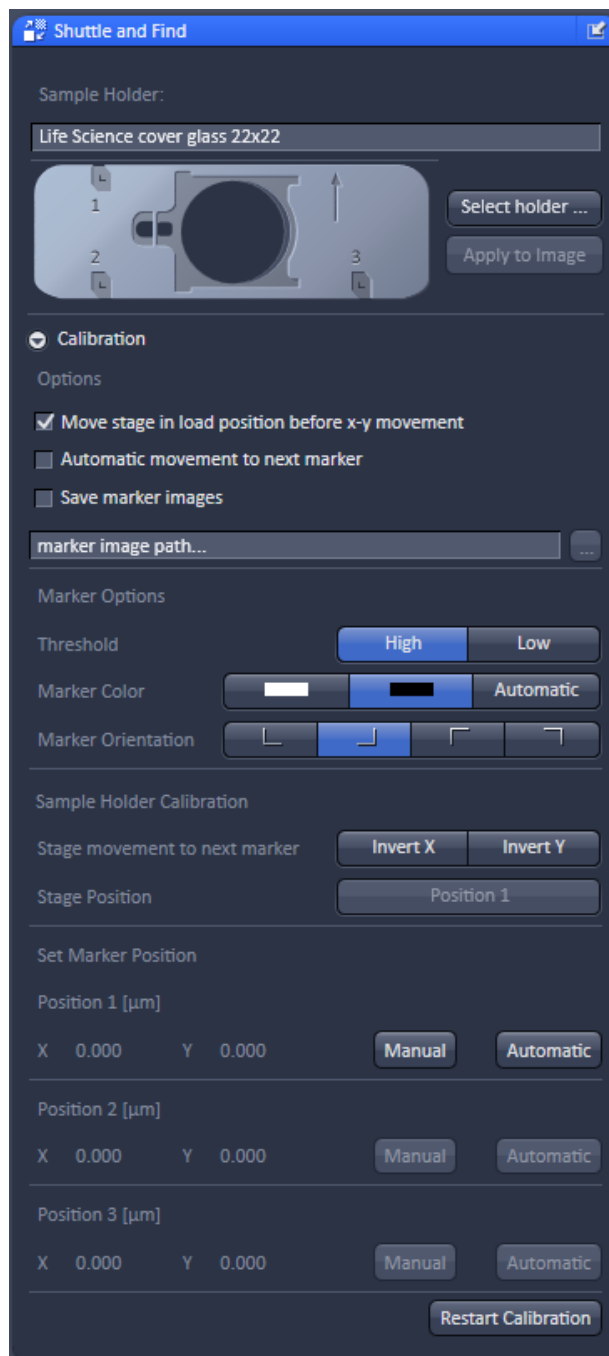
Fig. 179   Shuttle and Find tool

Calibration

The calibration markers consist of one small and a large L-shape marker. The bigger marker is used for coarse orientation, whereas the smaller marker is used for the calibration.

- Set the microscope for eyepiece observation.

- Use a dry objective.

- Move the microscope stage to the marker position **1** of the specimen holder.

- Focus on the small marker.

- Select the **Acquire** tab and open the **Shuttle and Find** tool in the **Acquisition Parameter** tool group (Fig. 179).

- Select the appropriate holder type from the list by clicking the **Select holder …** button, e.g.: **Life Science cover glass 22x22**.

The currently selected holder type is then shown in the **Sample Holder:** panel.

Apply to Image applies the current calibration to an acquired image. Do not remove the sample of the holder between image acquisition and calibration. Use this button only, when you forgot to calibrate the holder before you acquired the image.

- Choose general options for the calibration in the **Marker Options** field:

Move stage in load position before x-y movement moves the stage to load position before moving to the next correlative calibration marker. In case of using an Axio Observer, the objective revolver moves to load position.

Automatic movement to next marker moves the stage automatically to the next marker during the calibration procedure if the position of the correlative marker has been set manually or confirmed (automatic marker detection). If this checkbox is not activated, the stage has to be moved manually to the next marker.

Save marker images saves the marker images during the calibration. The images can be used to check the calibration afterwards. Click on the **Select Folder (...)** button to select a storage folder.

- Configure the beam path manually or select an appropriate configuration from the list (**Load Acquisition Parameters**).

- Click on the **Continuous** button to start the continuous scanning.

- Choose the options for marker recognition within the **Marker Options** field.

Threshold: High – Low: A low threshold for marker detection is used when the dimensions of the correlative L markers cannot be recognized precisely, e.g. when the sample holder is slightly filthy.

Marker Color: White – Black – Automatic: Select the color of the markers (as it appears in the image window) or leave it to automatic.

White: The marker is displayed white on a dark background.

Black: The marker is displayed dark on light background.

Automatic: The marker color is set automatically.

Marker Orientation: Select the correct orientation of the L-markers on your sample holder from the **Marker Orientation** field. Click on the corresponding button to set the same orientation like you see it in the live image.

Sample Holder Calibration field: The marker recognition during the calibration can be done either automatically or manually. During the calibration the stage can moved automatically to the next marker, when the **Automatic movement to next marker** checkbox is activated. If the stage is moving in the wrong direction, invert the direction by clicking on **X Invert** or **Y Invert** in the **Sample Holder Calibration** field.

If the first marker position was already set once and x/y coordinates are given, the stage can simply be moved to marker position one by clicking on the **Position 1** button.

a) Automatic marker recognition

The software proposes the position of the marker visible in the live image.

- Click on the **Continuous** button to start continuous scanning.

- Move the stage to the first marker manually or click **Position 1** in the software in case the first marker position was already set once and x/y coordinates are given.

- Click **Automatic** in the **Position 1** field (Fig. 180).

The software will propose the position of marker 1 in a separate window (**Marker location found** dialog (Fig. 181)).

- Click on **Accept** in the **Marker location found** dialog to confirm the proposed marker position. Click **Reject** if proposed position is not correct.

☞ Contamination of the markers or weak contrast settings may interfere with the marker recognition.

☞ If the proposed position is not correct, either optimize the contrast settings or use the manual marker recognition.

- If the **Automatic Movement to next Position** is activated, the stage will move automatically to the next marker (Position 2) after clicking **Accept**. Otherwise, the marker 2 has to be found manually.
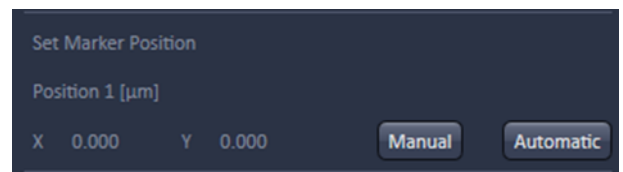
Fig. 180   Position 1 field
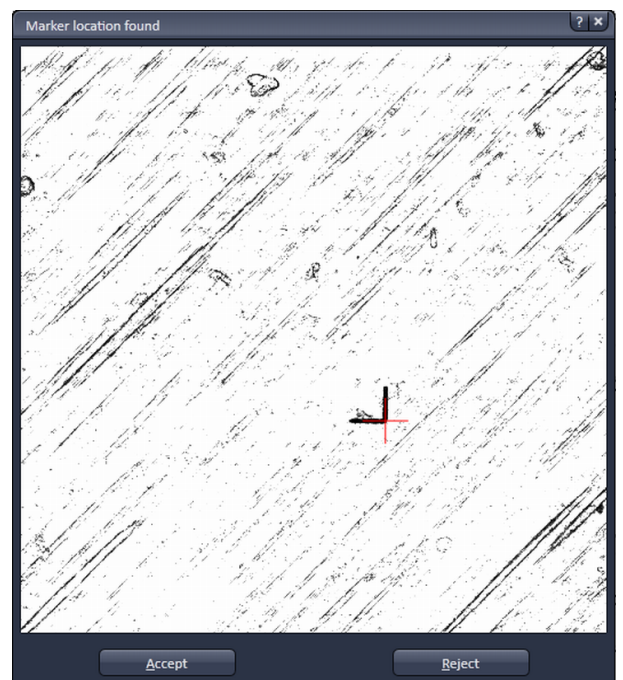
Fig. 181   Marker location found dialog

Fig. 182 Sample Holder Calibration field, Stage movement to next number

- If the stage is moving in the wrong direction, invert the direction by clicking on **X Invert** or **Y Invert** in the **Sample Holder Calibration** field (Fig. 182).

- When marker 2 is in position refocus and click on **Find Marker** in the Position 2 field.

- The system will propose the position for marker 2 in a **Marker location found** dialog. Confirm the proposal if the position is correct.

The stage moves automatically to marker 3.

- Click on **Find Marker** in the Position 3 field and confirm the position if correct.

The calibration is finished. The calibration can be started by clicking on **Restart Calibration** in the **Shuttle and Find** tool window.
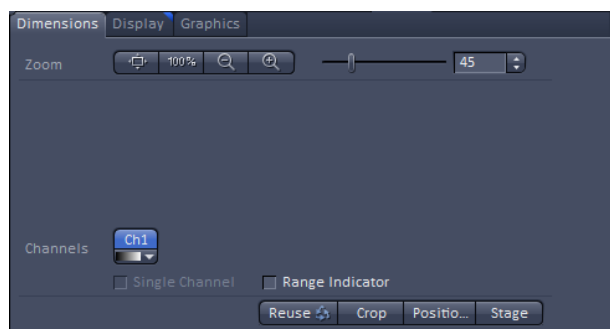
Fig. 183 Positions button in the Dimensions control tab of the 2D view

b) Manual marker recognition

If the marker on the sample holder is not recognized or user-defined markers are used, the marker positions can be set manually.

- Activate the Acquisition Parameter **Positions** within the Acquisition Parameter list.

- Click on the **Continuous** button to start continuous scanning.

- Move the stage to the first marker.

- Click on **Snap** to acquire a single image.

- Activate the **Positions** button in the **Dimensions** control tab (Fig. 183).

- Click either on the calibration marker or a user-defined marker to set a crosshair in the image and thus define the position of the marker.

- Confirm the position by clicking on **Manual** in the **Set Marker Positon** section within the **Shuttle and Find** tool.

- If the **Automatic movement to next marker** checkbox is not activated, move the stage to the second marker and got on as described above.

- Repeat the same procedure for the third marker.

Image acquisition and saving

- Acquire images of your sample as you are used to.

- For saving (Fig. 184) the images together with coordinates and defined regions of interest or points of interest use the .czi file format.

- Enter the name of the image and click on **Save**.

☞ Regions of interest (ROI) defined within the images will not be saved in the image format.

Mounting of the correlative sample holder into the SEM

- To use the specimen in a scanning electron microscope, remove the sample holder (Fig. 185/**2**) from the mounting frame of the microscope stage and insert it in the special SEM adapter (Fig. 185/**1**).
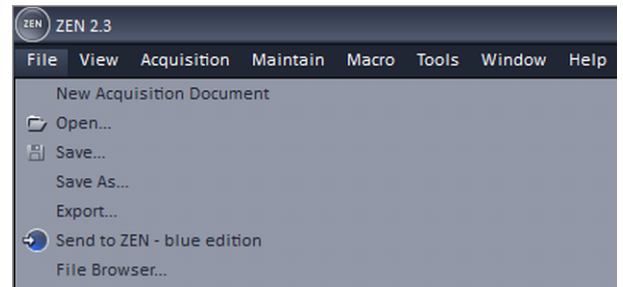
Fig. 184   File menu, Save As function
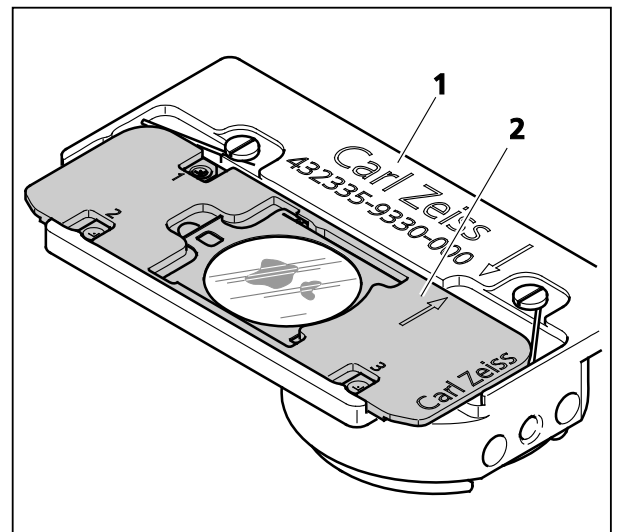
Fig. 185   Inserting the sample holder in the SEM adapter
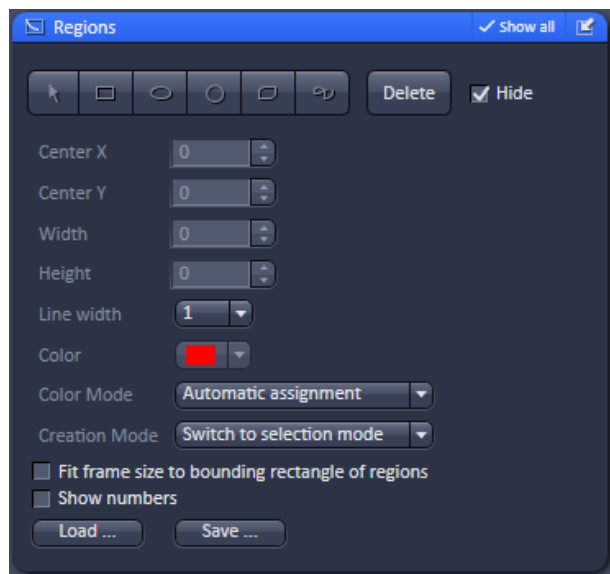
Fig. 186   **Regions tool**

5.2.16   Acquisition Parameter – Regions

The **Regions** tool tab is only available, if the Regions checkbox is ticked in the Multidimensional acquisition field. If the tool bar is expanded, the **Regions** tool becomes available (Fig. 186).

The **Regions** tool allows the user to define Regions of Interest (ROI) which are used for display for **WF**, **SIM Apotome**, **Lattice SIM** and **Laser WF imaging** modes.

In widefield techniques (**WF**, **SIM Apotome**, **Lattice SIM**, **Laser WF**), the whole sample is always illuminated, even if only the region is displayed.

The definition of the regions is done using an actual image of the sample and drawing the regions into this image (Fig. 187, Fig. 188). This makes it extremely easy to set the regions in the right position and dimension.
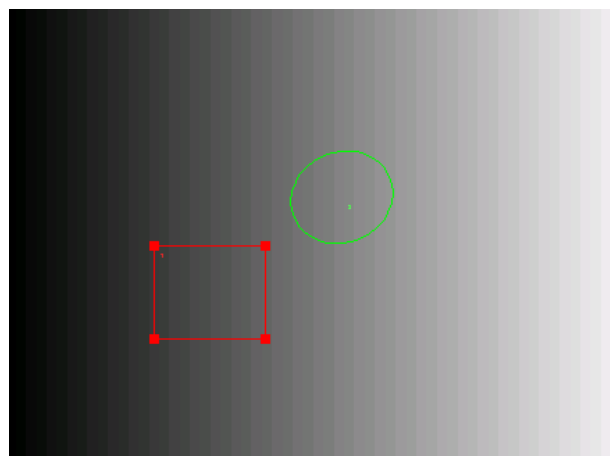
Fig. 187   **Image with two defined regions, the square one is selected**
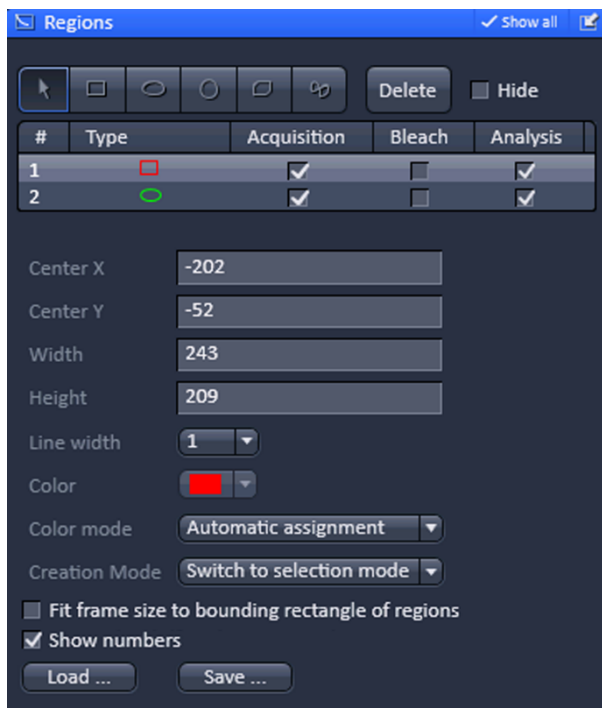
Fig. 188   **Regions tool**

The following tools are available in the **Tool selection** field:

Selects an existing region in the image. The outline of the region is marked with small rectangles to show it is active. The format and the position of the region can be changed clicking on the rectangles or the region outline and dragging it with the cursor. In addition the numerical values describing the position and size of the region are updated.

Rectangle: Drawing of a rectangle in the **Image Display** window; click and keep mouse button pressed, drag the rectangle in any direction, let go off the mouse button to end the procedure.

Ellipse: Drawing of an ellipse in the **Image Display** window; first click sets the center point, displayed line permits determination of the extension, second click sets the first dimension, then the second dimension and the rotation direction can be determined, third click sets the second dimension and direction and ends the procedure.

Circle: Drawing of a circle in the **Image Display** window; click and keep the mouse button pressed to set the center point, drag the diameter, let go off mouse button again to end the procedure.

Polyline: Drawing of a polyline figure in the **Image Display** window; first click sets the starting point, each further click adds a line, double-click on the starting point closes the figure and ends the procedure.

Closed **Bezier**: Drawing of a bezier figure in the **Image Display** window; first click sets the starting point, each additional click adds a line, double-click on the starting point closes the figure and ends the procedure.

By pressing the **Delete** button, the highlighted region will be removed. By ticking the **Hide** checkbox, the region will be hidden in the image.

All drawn regions will be listed in the **Region list** field. They are identified by an assigned number and its form.

The task (**Acquisition**, **Analysis**) for which a region will be used can be defined by ticking the respective boxes.
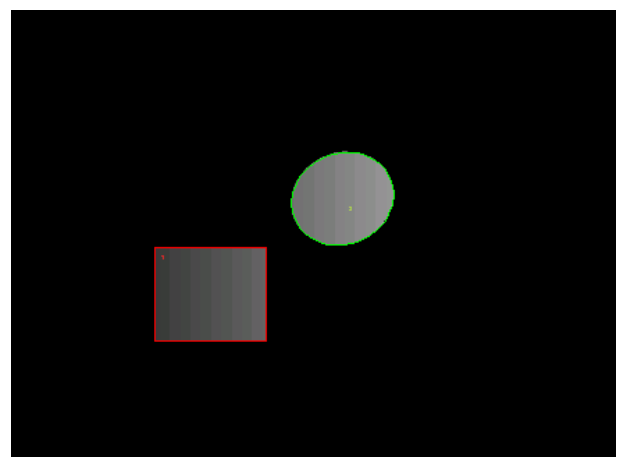
Fig. 189   Image with two defined regions used for imaging
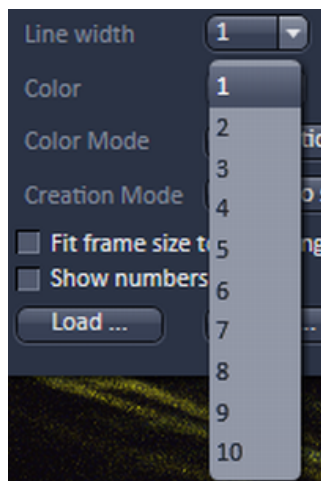
Fig. 190   **Line width drop down menu**

Fig. 191   **Color drop down menu**
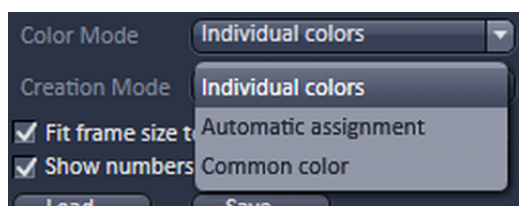
Fig. 192   **Color mode drop down menu**

When highlighting a ROI in the list, the numerical parameters for **Center X**, **Center Y**, **Width** and **Height** in pixels will be shown in **Region feature** field. In addition the ROI will be set to active in the image.

☞ Note, when a SMLM (**HR**) image is displayed numbers will refer to the SMLM image and not the **SWF** image, which normally do have different pixel resolutions.

It is possible to change the **Line width** from the Line width drop down menu (Fig. 190).

The **Color** of the outline of a ROI can be changed in the **Color** drop down menu (Fig. 191). The scroll bar gives access to all available colors.

Color selection is only possible if either **Individual colors** or **Common Color** is chosen in the drop down list of the **Color mode**.

The **Color mode** drop down menu allows defining how colors will be assigned to the outline of a region (Fig. 192). **Individual colors** allow the assignment of different colors to different regions.

If **Automatic Assignment** is chosen in the **Color mode** a color will be automatically assigned to each region as it comes.

The choice of Color is inactive in this mode. **Common Color** assigns the same color to all regions.

The **Creation Mode** drop down menu offers three different settings for defining ROIs (Fig. 192). **Switch to selection mode** activates the arrow tool once the region is drawn in the image. **Multiple elements** allow drawing multiple regions of the same type in a sequence. **One element only** restricts the number of ROIs to be drawn in the image to one. Any new region deletes the previous one.

The function **Fit frame size to bounding rectangle of regions** will be ignored.

For widefield modes (**WF**, **SIM Apotome**, **Lattice SIM**, **TIRF**) the Bleach function is not available and will be ignored.

The numbers of the different regions will be shown in the image if the check box next to **Show numbers** is checked.

Regions can be saved by pressing the **Save…** button using **Windows Explorer**.

This process stores all regions currently displayed in the Regions tool in one file. Saved images can be loaded later on by pressing the **Load…** button using the **Windows Explorer**.

5.2.17   Multidimensional Acquisition – Z-Stack

The **Z-Stack** tool tab is only available, if the **Z-Stack** checkbox is ticked in the **Multidimensional acquisition** field. If the tool bar is expanded, the **Z-Stack** tool becomes available (Fig. 193, Fig. 194, Fig. 195. When **Start Experiment** is pressed the system will perform a Z-Stack.
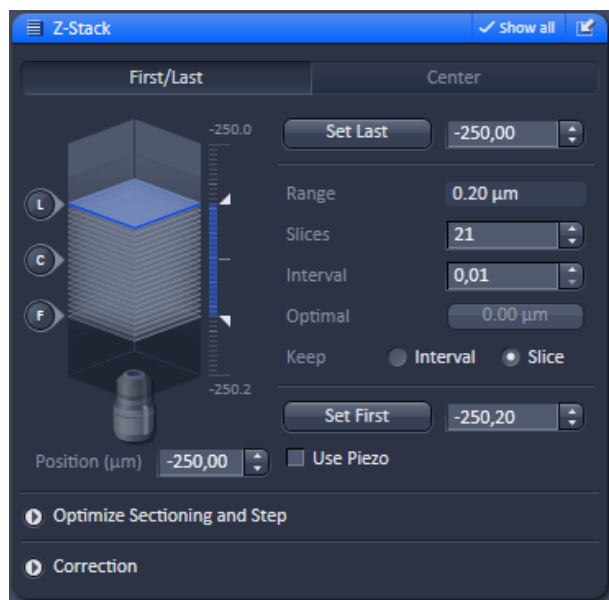
Fig. 193   **Z-Stack tool First/Last mode with Z-Stack Navigation displayed for the WF image mode**
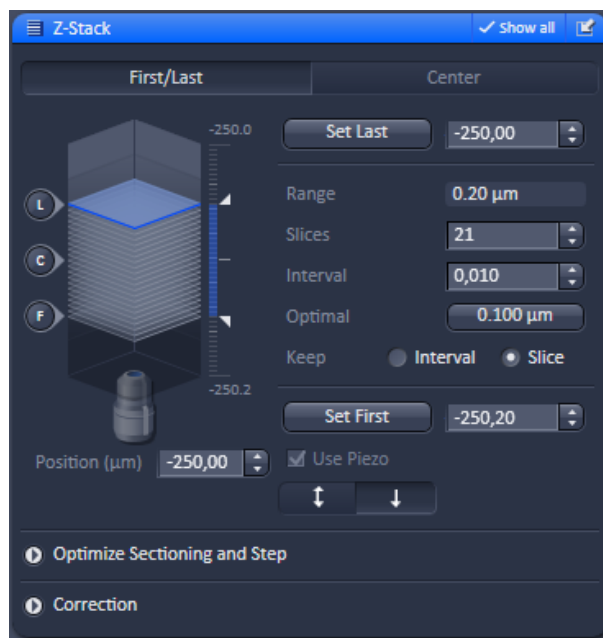
Fig. 194   **Z-Stack tool First/Last mode with Z-Stack Navigation displayed for the Lattice SIM image mode**
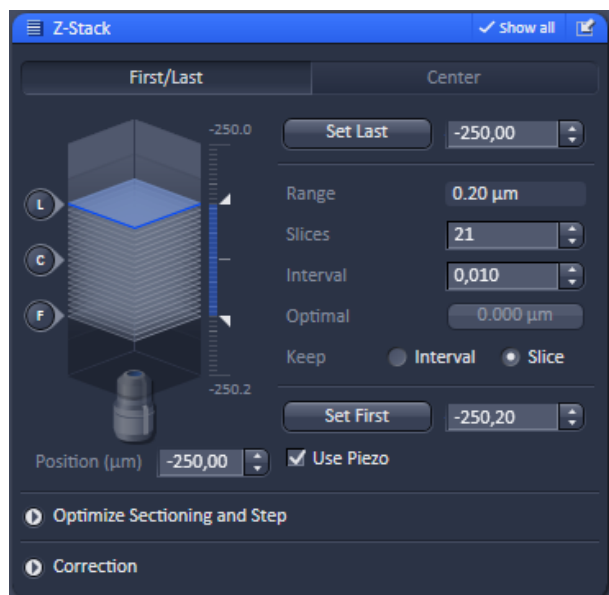
Fig. 195   **Z-Stack tool First/Last mode with Z-Stack Navigation displayed for the Laser WF image mode**

With the Z-Stack function a series of XY-images in different focus positions can be acquired resulting in a Z-Stack. In this way a 3 dimensional data set from the specimen is obtained.

The way in which the Z-Stack is acquired can be selected by pressing either the **First/Last** or **Center** mode buttons. The modes differ in the way the first and last images of the stack are selected. The **Center** mode is only available in the **Show all** mode.

In case a **Z-Piezo** stage insert focusing device is attached to the system, the Piezo device can be chosen for the acquisition of the stack.

5.2.17.1   Performing a Z-Stack Using First/Last Mode

Press the **First/Last mode** button to open the **First/Last definition** panel.

Press the **Continuous** action button and focus onto the specimen.

While scanning change the focus using the **Acquisition Parameter - Focus** tool, the **Z-Stack Navigation** (Fig. 196) or use the focus drive of the microscope.

When you reach the position within the specimen, where you would like to start the stack, hit **Set First** and this position (in µm referring to the 0 position of the Z drive) will be taken as the first position in the stack.

Move the focus into the other direction until the end position within the stack is reached, then hit **Set Last**. These focus positions are then indicated next to **Set First** and **Set Last** buttons. The **Range** display box shows the total height of the stack in micrometers.

Fig. 196   Z-Stack Navigation appearance for the SIM Apotome and Lattice SIM image modes

The **Slices** input box displays the number of slices of the stack. This number can be edited by typing or using the arrow keys. Changing the value for **Slices** automatically adjusts the **Interval** in a way that the total **Range** for the stack is maintained.

The **Interval** input box displays the z-distance (interval) between slices in **µm**. This number can be edited by typing or using the arrow keys. Changing the value for the **Interval** automatically adjusts the **Number of Slices** while keeping the total **Range** for the stack within the possibilities of fitting the calculated number of slices into the Range. The total Range may therefore vary to higher and lower numbers around the **initial value of the Range.**

Pressing the **(Opt)/(Leap)** button (only available in **SIM Apotome and Lattice SIM** imaging mode, you can select between Optimal (Nyquist) and Leap (3 fold broader spacing)). **Optimal** sets **Interval** to match the Nyquist criteria (2 fold over sampling) and accordingly adjusts the **Slices** to keep the **Range** within the limits defined by **Set First** and **Set Last**. The optimal interval is displayed in the button in **µm**. Leap sets the spacing 3 fold broader than Nyquist.

Within the **Keep** display field (only available in **Show all** mode) you have the option to switch between **Keep Interval** and **Keep Slice**. If **Keep Interval** is checked, the number of slices is adjusted to a new **Range** defined pressing **Set First** or **Set Last**. Likewise, when **Keep Slice** is checked, the value for the **Interval** between the slices is adjusted to a new Range defined by pressing **Set First** or **Set Last**.

The **Position (µm)** input box indicates the current position of the focus, no matter if the focus drive or a **Z-Piezo** stage insert was used for positioning. The focus is changed in small steps when clicking the arrow keys.

If the **Use Piezo** check box is ticked (only available, if a **Z-Piezo** stage insert is attached to the system), the **Z-Piezo** stage insert will be used to perform all Z movements triggered from the software. If the focus drive was used for **Set First** / **Set Last**, the system will transform this numbers for a matched **Z-Piezo** movement. In the **SIM** imaging mode, the **Use Piezo** check box is ticked by default and greyed out, so there is no choice to deselect the box and use the microscope focus drive for **SIM** imaging.

The rationale behind this is that **SIM** acquisition needs the accuracy of a **Z-Piezo** stage insert for taking Z-Stacks.

The **Fast Z Line** checkbox is only available in **Line Scan Mode** to be selected in the **Acquisition Mode** tool). It is not available with the **Z-Piezo**. If the check box is ticked and **Continuous** action button pressed a fast XZ scan for overviews is performed using a continuous movement of the microscope focus. The stack size is retained and the interval is adapted depending on the scan speed. The resulting center of the Line-Z stack can deviate from the center set in frame imaging mode by some microns.

The **Z-Stack Navigation** field displays a graphical representation of the Z-Stack. The **white frames** represent the number and localization of the slices. The **blue frame** indicates the current focus position, also during image acquisition. By dragging the **blue frame** with the cursor, the current focus position changes accordingly. The **ruler** immediate on the right indicates the dimensions (range) of the Z-Stack. One can also change the focus position by using the slider tool right to the **ruler**. Click on the circle symbol with the double arrow and move it up- or downwards to change the focus. This tool might also be used for defining the first and last position for the Z-Stack.

The **F**, **C** and **L** buttons indicate the positions of the first slice, center slice and last slice of the stack, respectively. If you press a button, a scan will be triggered at the respective focus position

☞ It is not relevant that the first and last position in the software match the absolute first and last position. The microscope will always move against gravity when acquiring the stack ignoring the assignment first and last.

☞ The precision of the Z-Stack position can be optimized for very critical applications by taking care that the approach to the first and the last slice when marking the positions will be made from the same direction as the Z-Stack is finally acquired. As the Z-Stack acquisition is always performed against gravity it is recommended to move the objective (for inverted stand Axio Observer and upright stand Axio Examiner) from a lower position to the first slice and then further up to the last slice. During this process be sure to move the nosepiece in this one direction only and that you do not refocus.

☞ To obtain best image quality with Z-Stacks using the nosepiece and which are critical regarding the focus impulse, a **Waitstate** can be activated. To this end go to the **Maintain** tab. In the **Option** field expand the **System Options** tab and select the **Hardware** tab. Tick the **Waitstate** check box. Such a wait state is only beneficial at certain acquisition speeds and not needed by default.

5.2.17.2   Performing a Z-Stack Using Center Mode

Press the **Center** mode button to open the **Center definition** panel (Fig. 197). This mode allows setting the Range of the Z-Stack in relation to the current **Focus Position** which is indicated as **Offset**.

The values for number of **Slices** and **Interval** are taken over from any settings done in **First/Last** mode. The **Offset** defines the **Center** position of the stack in relation to the **Focus Position**.

The **Slices** input box displays the number of slices of the stack. This number can be edited by typing or using the arrow keys. Changing the value for **Slices** leaves the **Interval** untouched, but the total **Range** is adjusted.

The **Interval** input box displays the z-distance (interval) between slices in **μm**. This number can be edited by typing or using the arrow keys. Changing the value for the **Interval** automatically adjusts the number of **Slices** while adjusting the total **Range**. The **Slices** number will remain unchanged.

Fig. 197   Z-Stack tool Center mode with Z-Stack Navigation

Pressing the **(Opt)/(Leap)** button (only available in **the SIM Apotome and Lattice SIM** imaging modes you can select between Optimal (Nyquist) and Leap (3 fold broader spacing). Optimal sets **Interval** to match the Nyquist criteria (2 fold over sampling) and accordingly adjusts the **Slices** to keep the **Range** within the limits defined by **Set First** and **Set Last**. The optimal interval is displayed in the button in **μm**. Leap sets the spacing 3 fold broader than Nyquist.

Within the **Keep** display field (only available in **Show all** mode) you have the option to switch between **Keep Interval** and **Keep Slice**. If **Keep Interval** is checked, the number of slices is adjusted to a new **Range** defined pressing **Set First** or **Set Last**. Likewise, when **Keep Slice** is checked, the value for the **Interval** between the slices is adjusted to a new Range defined by pressing **Set First** or **Set Last**.

The **Position (μm)** input box indicates the current position of the focus, no matter if the focus drive or a **Z-Piezo** stage insert was used for positioning. The arrow keys allow to change the focus in small steps. If the **Use Piezo** check box is ticked (only available, if a **Z-Piezo** stage is attached to the system), the **Z-Piezo** stage insert will be used to perform all Z movements triggered from the software. If the focus drive was used for **Set First / Set Last**, the system will transform this numbers for a matched **Z-Piezo** movement. In the **SIM** imaging mode, the **Use Piezo** check box is ticked by default and greyed out, so there is no choice to deselect the box and use the microscope focus drive for **SIM** imaging. The rationale behind this is that **SIM** acquisition needs the accuracy of a **Z-Piezo** stage insert for taking Z-Stacks.
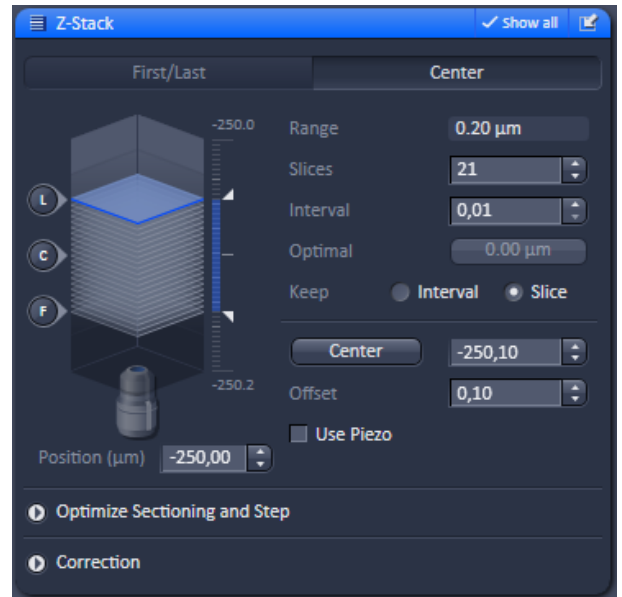
Pressing the **Center** button will define the current focus position (as defined in the **Position (μm)** input box) as the center position of the stack. After pressing **Center** the whole range will move by the difference between of the previous center position and the new one. The number in the **Center** input box will then equal the number in the **Position (μm)** input box.

The **Offset input** box allows to define an offset between the actual focus position as displayed in the **Position (μm)** input box and the current center position as displayed in the **Center** input box. One can edit the number by typing or using the arrow keys. When defining a specific **Offset** the **Center** position will be recalculated as the actual position plus the offset. Accordingly the range of the Z-Stack is shifted to a different position in Z. Pressing the **C** button of the **Z Navigation** representation moves the actual **Focus Position** to the **Center** position and acquires an image at that position. A Z-Stack is then acquired at the new position when starting the experiment. Hitting **Center** moves the **Center** position back to the actual **Focus Position** setting the **Offset** again to zero.

Use the **F**, **C** and **L** buttons to get images from the first slice, center slice and last slice of the stack. The **Focus Position** updates accordingly. The **Offset** value then might be set to half of the Interval depending on the interval value and the number of slices, as with a lower number of slices or a large interval the real center position of the stack might be just between two slices.

Pressing the **Range Select** button will start a XZ-scan within the **Range** of the Z-Stack. This helps setting the **Range** of the Z-Stack without continuously scanning complete frames. The position of the first and last slice is indicated with a red line, the **Focus position**, which initially corresponds to the **Center** position, is indicated as a green line. Changing the **Offset** value moves the red lines in parallel to the new offset position, the green line, while the **Focus Position** is kept constant. Pressing the **C** button sets the **Focus Position** to the indicated **Center** position and the **Offset** back to zero. In parallel an image at the newly defined **Focus Position** is acquired and displayed. Alternatively, pressing **Center** sets the indicated **Center Position** back to the actual **Focus Position** moving the red lines back to the original position and setting the **Offset** back to Zero. The original values for Number of **Slices**, **Interval** and **Range** are always kept constant. The red lines can be moved independently from each other allowing an optimal adjustment of the Z-Stack range to the specimen. **Center** sets the green line to the center position between the red lines and adjusts the Focus Position accordingly. The original values for Number of **Slices** and **Range** are adjusted. The **Interval** is kept constant.

5.2.17.3   Performing a Z-Stack Using a Z-Piezo

A Z-Piezo allows Z-Stacks to be acquired considerably quicker compared to the focus drive of the microscope stand. The speed advantage depends on the total travel range in Z and the speed of the acquisition of the individual image of a Z-Stack. The available travel range of a Z-Piezo device is much smaller compared to the Z drive of the microscope. A Piezo device allows performing smaller steps in Z with a higher absolute precision for the individual steps. A Piezo device is recommended for 3D measurements of objects and for 3D superresolution imaging.

Fig. 198   Use Z-Piezo for Z-Stack acquisition

Tick the **Use Piezo** checkbox (only available if a Z-Piezo device is attached) to activate the usage of the **Z-Piezo** device in the **First/Last** or **Center** modes (Fig. 198). In the **SIM Apotome** & **Lattice SIM** imaging modes this is the obligatory mode and hence the checkbox is permanently ticked.

Clicking on the **Leveling** button in the **Focus** tool moves the Z-Piezo stage to its center position in respect to the current focus position of the microscope focus drive. This function is used to set defined initial conditions. The total Range of the Z-Stack is limited depending on the type of Piezo. Hence form the center position the Z-Piezo stage can move half the total range each up and down.

Define the first and last slice using **First/Last** mode by pressing the **Set First** and **Set Last** buttons at the respective focus positions. Adjusting the value for Number of **Slices** or **Interval** is independent form each other and affects the Range of the stack. The Focus Position is not changed.

When changes are made to **Interval** or Number of **Slices** in **First/Last** mode, the values are taken over for the **Piezo** mode provided they are within the Z-Piezo stage focus work range. This also works in the opposite direction.

In order to acquire an extended 3D SMLM data set, set the number of slices and the z-range using **First/Last** or **Center** mode. Choose **Laser WF** imaging mode in the **Imaging Setup** tab and choose from the **Trigger in** drop down menu of the **Acquisition** tab the entry **internal**. In this way the camera exposure is triggered to the slice.

☞   Please note that the software allows only a Z-Stack (inner loop) within a time series (outer loop), but no time series of a Z-Stack. This means that for each time point of the 3D SMLM experiment, only one image per slice is taken. It is not possible to take many images per slice before advancing to the next slice. As this procedure will be time consuming, it is advised to keep the number of slices to a minimum.
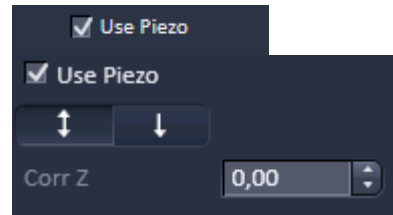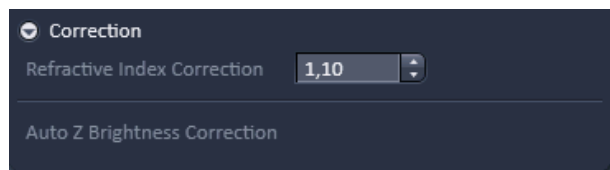
Fig. 199 Correction field – appearance for the WF, SIM and Laser WF imaging modes

5.2.17.4 Correction

Expand the **Correction** field by pressing the triangle (Fig. 199). The **Refractive Index Correction input box** allows you to consider the different refractive indices between the immersion medium of the objective (n') and the embedding medium of the specimen (n), which can be set between 0.5 and 3 by typing or using the arrow keys. The number should correspond to the ratio r=n/n'.

Fig. 200 Tile Scan tool – Centered grid active

Fig. 201 Tile Scan tool – Overlap defined

5.2.18 Multidimensional Acquisition – Tile Scan

When the **Tile Scan** tool tab is expanded the **Tile Scan** tool becomes available (Fig. 200).

This function permits a frame to be created as an overview image of the specimen. The size of the final tiled image depends on the settings for the image format set in the **Acquisition Mode** tool and the number of tiles.

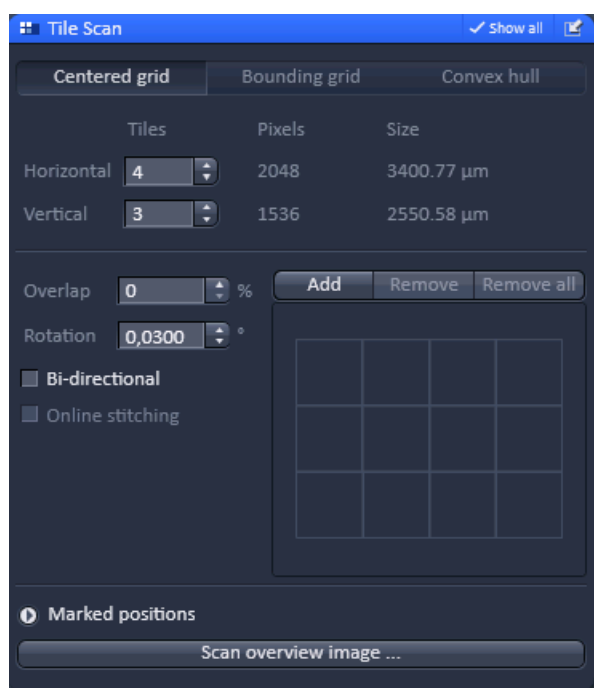The application of the **Tile Scan** function requires an objective with a minimum magnification factor of 2.5x.

The number of **Tiles** can be defined in the Tiles **Horizontal** and **Vertical** Input boxes. A maximum of 100 tiles can be set. The size of the final Tile Scan is displayed in **Pixels** and as a **Size** in μm. According to image format the number of **Pixels** changes. According to the objective used for imaging the **Size** of the Tile Scan changes.

The Tile Scan grid is displayed in the **grid display** area. It shows the number and location of the single tiles and also indicates any overlap.

The Tile scan is created around the absolute starting position of the stage. In case the stage does not move exactly horizontal to the scan field a **Rotation** value can be set in the **Rotation** input box to correct for this (available only in **Show all** mode). This can either be calibrated beforehand using a grid slide or any other specimen which displays clearly visible structures in horizontal direction.

Acquire smaller tile scans to see the effect of a specific value for **Rotation** until you find the right one. This value should be taken for the **Rotation**. Alternatively, use the **Tile Scan Rotation Macro** which provides an automatic **Rotation** calibration function. Take over this value to the main software before acquiring the tile scan.

When **Bi-directional** is active (available only in **Show all** mode) the image acquisition is performed also on the horizontal backwards movement of the stage.

Fig. 202   **Tile Scan tool – Marked positions**

If the system is equipped with the software option TILES, the **Overlap** input box is available (Fig. 201). Define the overlap of the tiles as the **%** of the tile size. The extent of overlap will also be displayed in the **grid display** area. As soon as the Overlap is > 0% the **Online stitching** checkbox becomes available. Online stitching cannot be activated if the HDR imaging is selected in the **Acquisition Mode** tool.

Fig. 203 Tile Scan tool – Bounding grid selected; two positions defined
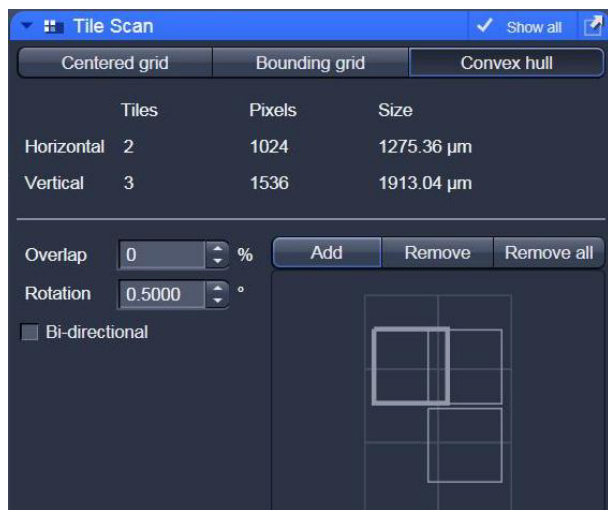
Fig. 204 Tile Scan tool – Convex hull selected
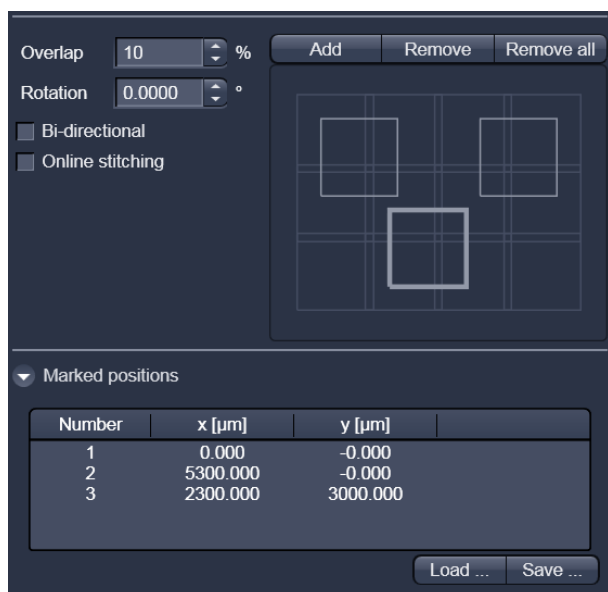
Fig. 205 Tile Scan: Marked positions

Tick the checkbox to activate online stitching. After ticking the checkbox the **Threshold** input box becomes available. Enter the desired threshold in **%**.

By pressing **Add** you can mark stage positions, which will be listed in the **Marked position** field in terms of Number as well as **x [μm]** and **y [μm]** coordinates (Fig. 202). By Pressing **Remove** the highlighted position will be removed from the list. Pressing **Remove all** will delete all positions. You can save a position list by Pressing the **Save...** button in the **Mark position** field via the Windows Explorer. You can load saved position list by pressing the **Load...** button via selection in Windows Explorer.

If **Centered grid** is selected, the current position will be the center of the Tile Scan.

If **Bounding grid** is selected positions that should be part of the tile scan have to be marked using **Add**. To this end click on the edge of the square to select the position, which will then be displayed with a thicker line width, and then press **Add**. Using these positions a bounding grid is created, which finally defines the dimensions of the tile scan (Fig. 203).

If **Convex hull** is selected positions that should be part of the tile scan have to be marked using **Add**. To this end click on the edge of the square to select the position, which will then be displayed with a thicker line width, and then press **Add**. Using these positions a convex hull is created, which finally defines the dimensions of the tile scan (Fig. 204).

Marked positions are listed up (Fig. 205). This list can be saved and loaded.

5.2.19   Scan Overview Image for Tiling

Pressing the **Scan overview Image…** button opens the **Scan overview Image** interactive window (Fig. 206).

Here you can define the number of **Tiles** by using the **Horizontal** and **Vertical** Input boxes (the extension of the scanned area will be displayed in **Pixel** and **Size (µm)** units), the objective from the **Objective** drop down menu and a potential zoom factor via the **Zoom** slider or Zoom input box with which to scan an overview image.

For widefield modes, only the zoomed in part of the camera chip will be displayed.

Changing **Objective** and **Zoom** will change the size, but leaves **Pixels** unchanged.

This overview image can then be used to navigate in the sample (i.e. define positions for imaging). **Scan** starts the tile scan.

Cancel closes the window without further action.

Fig. 206   **Scan Overview Image window**
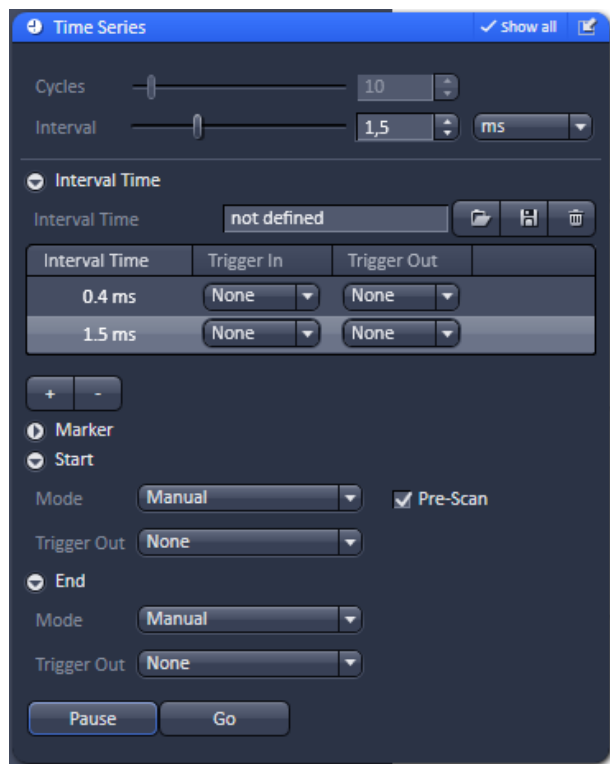
Fig. 207   Time Series tool

5.2.20   Multidimensional Acquisition – Time Series

When the **Time Series** tool tab is expanded the **Time Series** tool becomes available (Fig. 207). In the **Time Series** tool the parameters for the sequential acquisition of image frames as time series are defined. The options within the **Time Series** tool are limited and explained below.

To set up a simple time series acquisition it is enough to define the number of **Cycles**, which corresponds to the number of frames (or lines), imaged. This number can be defined with the **Cycles** slider or the **Cycles** input box. A maximum value of 100,000 can be set. The amount of data that can be handled by the system is limited. If the maximum amount is exceeded a warning will appear. In this case, it is necessary to either change the destination for data storage and/or reduce the amount of cycles. The limit also depends on the imaging setup and the acquisition mode. Note that there is an upper limit of handling data files, but this is beyond the value of 100 Gigabits.

Interval defines a potential time interval, set in milliseconds (ms), seconds (sec) or minutes (min) by the **Interval** drop down menu, to separate the beginning of two cycles. A time shorter than the scan time for a frame (or line) is not effective. The interval time can be changed by using the **Interval** slider or **Interval** input box, also while scanning.

There are different advanced tools (only available in **Show all** mode) including

– Interval Time
– Marker
– Start
– End

5.2.20.1   Interval Time

Expand the Interval field by clicking the triangle (Fig. 208). An **Interval Time** is a potential time interval between the end of one cycle and the beginning of the next cycle. The time and time unit for the delay time is set using the slider or input box of **Interval Time**. A cycle interval time is not effective if a cycle delay time is defined, since the interval time is always included in the delay. A total of six different delay times can be defined by adding further lines clicking on the **+** button.
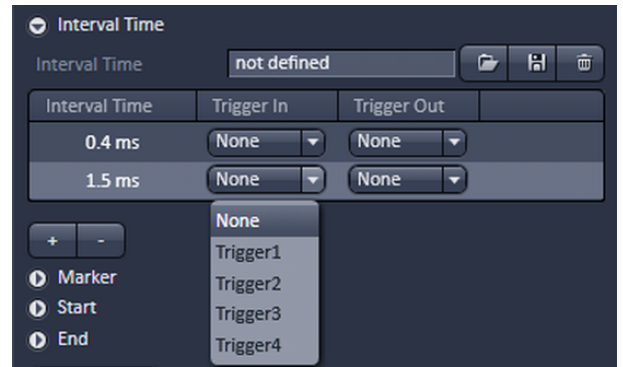

Fig. 208   Time Series tool – Interval Time

Clicking the **–** button deletes the currently highlighted delay time. Each delay time can be controlled using the **Trigger In** function.

(For a detailed description on the trigger use and interface see printed manual CHAPTER 4: ANNEX).

The trigger can be selected from the **Trigger In** pull down menu. Up to 4 triggers are available. If no trigger is set the highlighted delay time will be effective for the time series acquisition. Additionally it is possible to send a trigger signal out using the **Trigger Out** pull down menu at the start of each delay time. Up to 4 Triggers are available.

☞   Please note that Interval Time does not work with external triggering! So what is described in the next section is possible to select and to execute, but will not work.
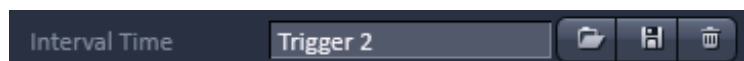
Fig. 209   Interval Time

You can load a predefined timer configuration from the **Interval Time** field by pressing the **Load** button (symbolized by the **folder** icon) (Fig. 209). All saved timer will be listed in the **Load configuration…** panel (Fig. 210). Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used timer configurations or from the **Configurations** section, where all saved timer configurations are displayed. To search for a timer configuration, type in its name or part of its name in the **Search** field and only those timer configurations will be listed that contain the typed in string of letters.

To delete a timer configuration press the **Delete** button (symbolized by the **dust bin** icon).

This opens the **Delete configuration…** window (Fig. 211).

Fig. 210   Load configuration… panel

Fig. 211   Delete configuration… panel
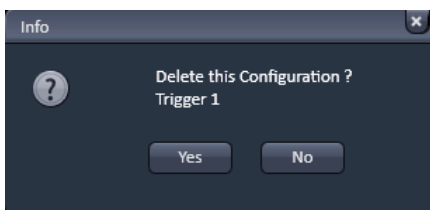
Fig. 212   Delete information window
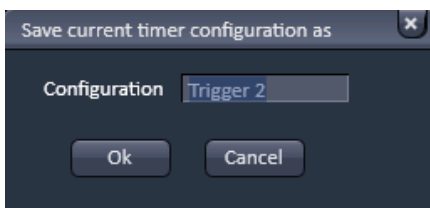
Fig. 213   Save current timer configuration as window

Select the timer configuration from the **Configurations** list by highlighting it or search for it by typing its name in the **Search** field. The **Info** window pops up (Fig. 212).

Press **Yes** to delete the configuration, **No** to keep the configuration.

To save a configuration press the **Save** button (symbolized by the **disc** icon). This opens the **Save current timer configuration as** panel (Fig. 213).

Type in an appropriate name in the **Configuration** field and press **Ok** to save the configuration in the data base or press **Cancel** to abort the process.

The delay time can be changed while scanning.

5.2.20.2   Marker

Expand the **Marker** field by clicking the triangle (Fig. 214). The **Marker** functionality provides the possibility to indicate the start or end of an external process in the data of the image series by connecting this external process to the selected **Trigger In** for that Marker. Up to 4 different triggers can be selected from the Trigger In pull down menu. You can edit a description of that marker in the **Description** box of the Marker, which will be visible in the image data using the Mean ROI functionality or the image gallery display mode. A **Trigger Out** signal can be set with each defined marker. Up to 4 different triggers can be selected from the pull down menu. By clicking **Set** a marker is set manually at any time during scanning. A maximum of seven different markers can be defined using the **+** button. The **–** button deletes the currently highlighted marker.

You can load a predefined timer configuration from the **Marker** field by pressing the **Load** button (symbolized by the **folder** icon) (Fig. 215).

Fig. 214   Time Series tool – Marker

Fig. 215   Marker

All saved marker configurations will be listed in the **Load configuration...** panel (Fig. 216).

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used marker configurations or from the **Configurations** section, where all saved marker configurations are displayed.

To search for a marker configuration, type in its name or part of its name in the **Search** field and only those marker configurations will be listed that contain the typed in string of letters.

To delete a marker configuration press the **Delete** button (symbolized by the **dust bin** icon). This opens the **Delete configuration...** window (Fig. 217).
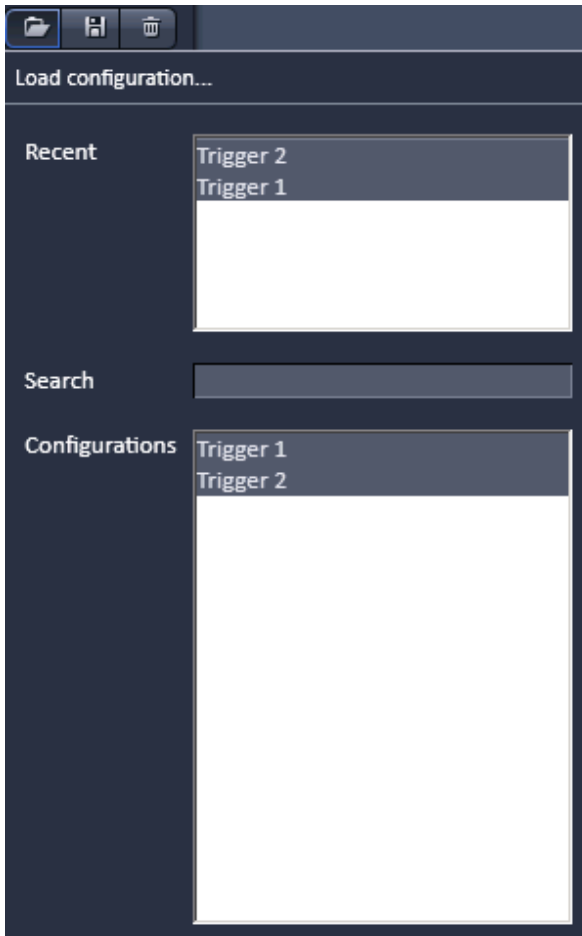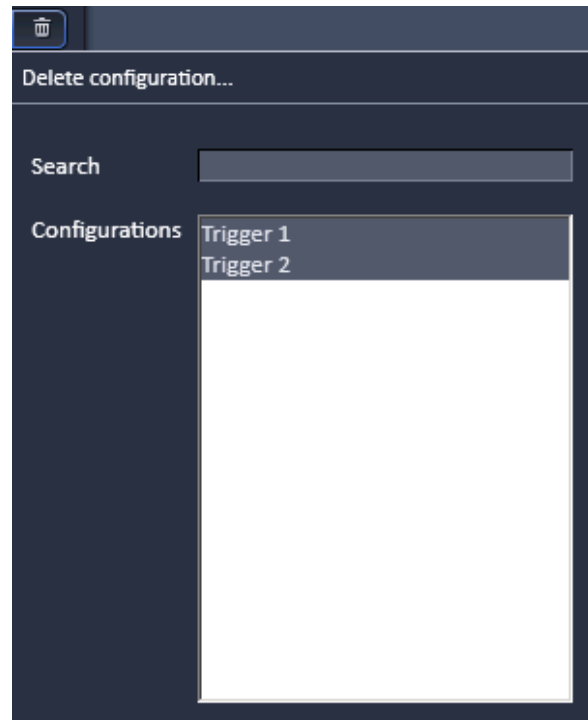
Select the marker configuration from the **Configurations** list by highlighting it or search for it by typing its name in the **Search** field.

Fig. 216   **Load configuration… panel**
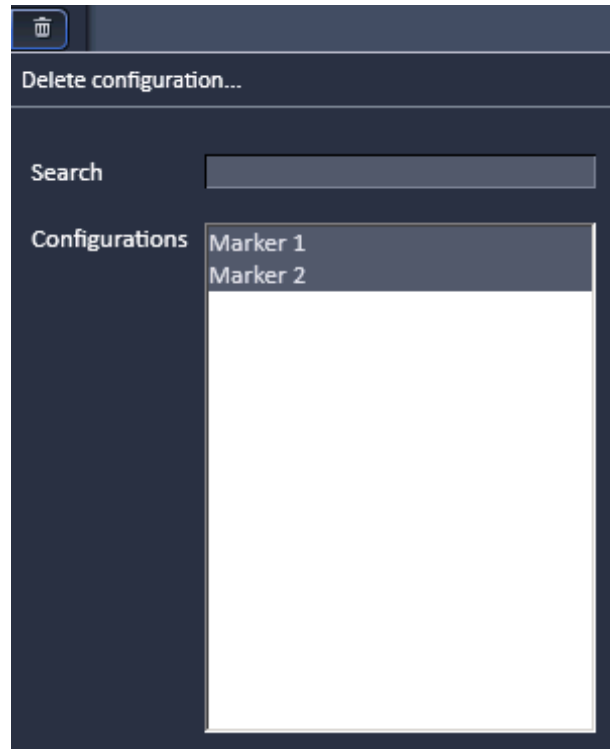
Fig. 217   **Delete configuration… panel**
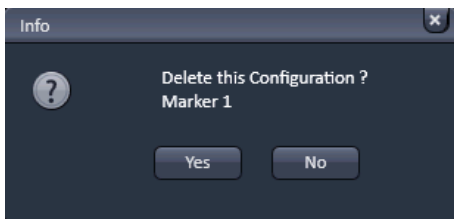
Fig. 218   **Delete information window**
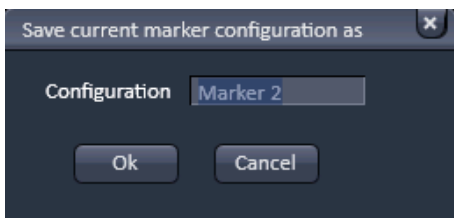
Fig. 219   **Save current marker configuration as window**

The **Info** window pops up (Fig. 218).

Press **Yes** to delete the configuration, **No** to keep the configuration.

To save a marker configuration press the **Save** button (symbolized by the **disc** icon).

This opens the **Save current marker configuration as** panel (Fig. 219).

Type in an appropriate name in the **Configuration** field and press **Ok** to save the configuration in the data base or press **Cancel** to abort the process.

When the image series is stored, all the markers, including the time indication and the comments, are stored along with the image contents.

The markers visible in the image series have different colors with the following meaning:

- **red**:   manually set marker with time indication and comments

- **blue**:   automatically set marker with change of delay

- **green**:   automatically set marker at the beginning and at the end of a bleaching procedure

5.2.20.3   Start

Expand the **Start** field by clicking the triangle (Fig. 220).

In the **Mode** drop down menu (Fig. 221) you can select the way the acquisition is triggered. You have the choice between Manual, Trigger and Time.

If **Manual** is selected the acquisition of a time series is started using the **Start Experiment** action button.
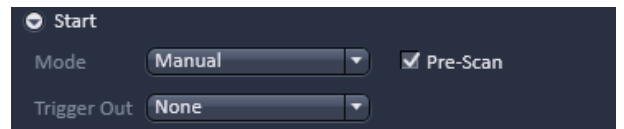
Fig. 220   Start field
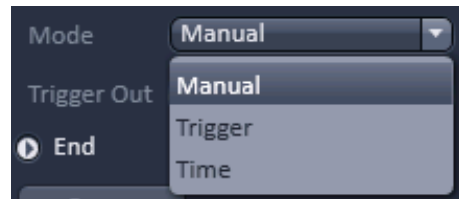
Fig. 221   Start Mode drop down menu
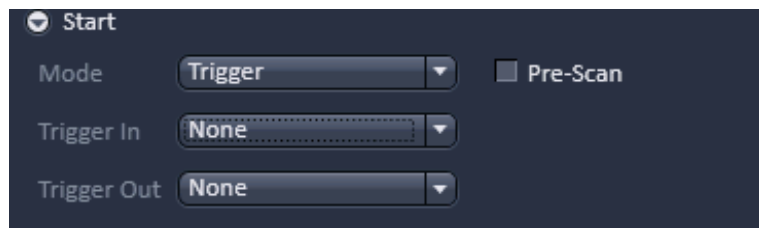
Fig. 222   Start Mode drop down menu– Trigger selected
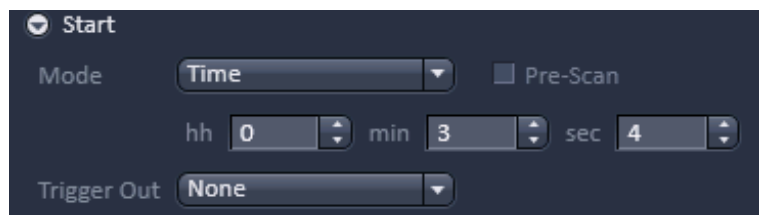
Fig. 223   Start Mode drop down menu – Time selected
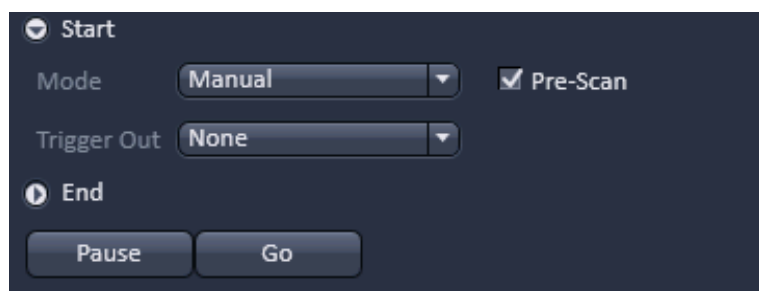
Fig. 224   Start – time series in process

Fig. 225   Start – time series interrupted

If **Trigger** is selected the **Trigger In** drop down menu becomes available (Fig. 222) from which up to 4 triggers can be selected. After pressing **Start Experiment** button the system will display the message **Waiting for Trigger** and starts acquisition once it received the trigger in signal.

If **Time** is selected the **hh**, **min** and **sec** input boxes become available (Fig. 223), in which the hours, minutes and seconds can be edited. After pressing **Start Experiment** button the system will display the message **Waiting for Start Time** and starts acquisition after the defined computer time has been elapsed.

If the **Pre-Scan** checkbox is ticked (Fig. 224) a continuous scan is performed when clicking **Start Experiment** button, but no image series is acquired until you hit the **Go** button, which becomes visible next to the **Pause** button in the lower part of the control window. Once the **Go** button was pressed it will disappear.

When Pressing the **Pause** button, which becomes active after **Start Experiment** was pressed, the time series is interrupted and the current scan is finished. The labeling of the button changes to **Resume** (Fig. 225) and if the **Resume** button is pressed the time series is immediately continued.

It is possible to send a **Trigger Out** signal with the start of the times series. The **Trigger Out** drop down menu allows defining up to 4 trigger out signals.

5.2.20.4 End

Expand the **End** field by clicking the triangle (Fig. 226).

Fig. 226   End field

In the **Mode** drop down menu Fig. 227) you can select the way the acquisition is triggered. You have the choice between Manual, Trigger and Time.

If **Manual** is selected the acquisition of a time series ends using the **Stop button** or after the defined number of cycles.

Fig. 227   End Mode drop down menu

If **Trigger** is selected the **Trigger In** drop down menu becomes available (Fig. 228) from which up to 4 triggers can be selected. The system will interrupt the acquisition once the trigger in signal is received.

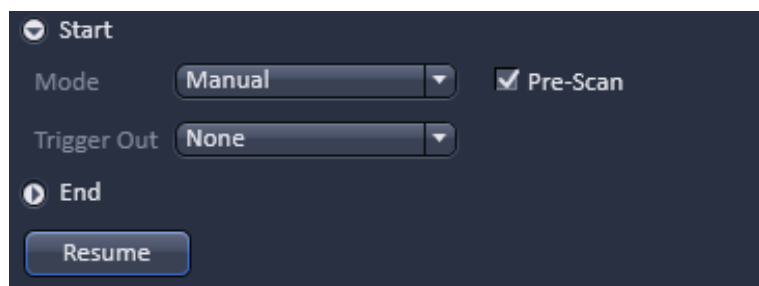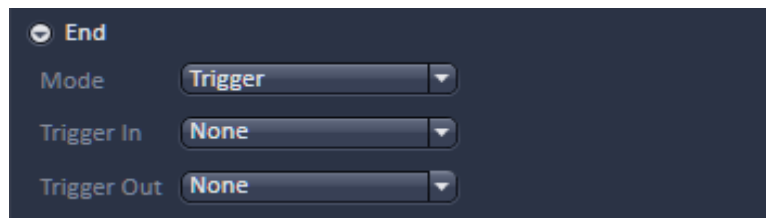**Fig. 228   End Mode drop down menu – Trigger selected**

If **Time** is selected the **hh**, **min** and **sec** input boxes become available (Fig. 229), in which the hours, minutes and seconds can be edited. The system interrupts the acquisition after the defined computer time has been elapsed.
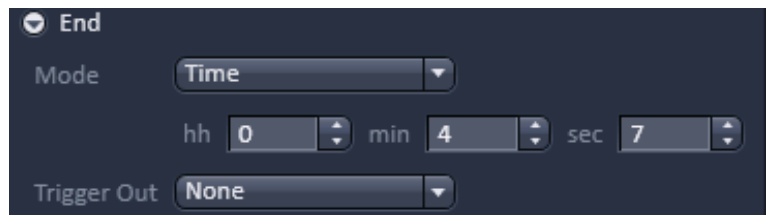
Fig. 229   End Mode drop down menu – Time selected

It is possible to send a **Trigger Out** signal with the end of the times series. The **Trigger Out** drop down menu allows defining up to 4 trigger out signals.

☞ If the entered number of cycles is processed without a set trigger impulse been given to end the procedure, the Time Series is finished.
If a trigger signal to end the scan arrives before the cycles have been processed, the Time Series will only be interrupted. **Waiting for Trigger** will be displayed in the status line. The Time Series can now be continued via a new trigger signal or ended via **Stop**.

☞ A Time Series is finished when the end **Time** is reached.
If the entered number of **Cycles** has been processed, the Time Series is finished before the end Time. If the number of cycles has not yet been processed until the end Time, the Time Series is also finished. The end time for the Time Series can be changed online. Bleach times will be added to the total time for image acquisition.

☞ If a time series is interrupted before its programmed end, the programmed number of images will be taken over in the database. However, only those images are stored which were created before interruption of the time series. This is due to the fact that the original image parameters are to be taken over via the **Reuse** function.

☞ It is possible to stop and restart a time series via TriggerIN and have the data concatenated in one file by alternating between the Start and End function. For example starting the time series with

TriggerIN 1 and stopping it via TriggerIN 2. Alternating between the two trigger inputs in essence pauses the time series for a desired time.
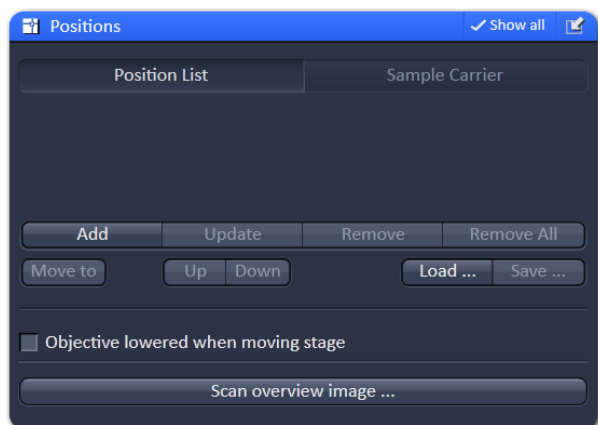
Fig. 230   **Positions tool**

5.2.21   Multidimensional Acquisition – Positions

When the **Positions** tool tab is expanded the **Positions** tool becomes available (Fig. 230).

This function comprises two main applications.

First, it is used to take images of a specimen at freely defined positions (**Positions List** mode).

Second, it is used to image specimens, typically cells, which are grown in sample carriers like plates with a set number of wells (**Sample Carrier** mode).

5.2.21.1   Position List

Press the **Position List** button to obtain the **Position List** control panel (Fig. 231).

Clicking the **Add** button adds the current position and its coordinates to the list of positions. Displayed are the number of the positions, their **x (µm)**, **y (µm)** and **z (µm)** coordinates and also their **offset** values (µm). See the description of **Setting individual offset values for positions in Position List mode** (below) and also the tool description of **Focus Devices and Strategy** for details on offset values (section **Focus Devices and Strategies for Widefield Imaging Modes**).

Highlighted positions can be removed by pressing the **Remove** button. Alternatively the whole list can be deleted by pressing the **Remove All** button.

Fig. 231   **Position List control panel**

It is also possible to move the stage to the highlighted position by clicking the button **Move to**. The order of positions in the list can be modified by pressing the buttons **Up** or **Down**.

You can save and load pre-saved positions by pressing the **Save…** and **Load…** buttons, respectively, which will open Windows Explorer.

The list of positions is consistent with the positions defined in the **Stage** tool.

☞   Note: When the list of positions is deleted in the **Position list**, it is also deleted in the **Stage** tool. This is not the case the other way round.

☞   It is recommended to save the position list as relative positions. The format "Positons list files (relative)" should be selected in the Windows Explorer. This format allows to reuse the positions for other experiments later on. Just make sure that the reference position for the list (for example the position 0) matches with the actual zero position in your sample. Start with positioning the sample at the desired zero position and set the stage to zero before loading the position list.

5.2.21.2 Sample Carrier

Press the **Sample Carrier** button to obtain the **Sample Carrier** control panel (Fig. 232).

The last used sample carrier will be displayed in the **Sample Carrier** display area. If no sample carrier is defined, the area remains empty.

You can define the sample carrier by clicking on **Properties** of the **Carrier definition** field.

By using the **Columns** and **Rows Number** input boxes, you can define the numbers of columns and rows of the carrier. In the Columns and Rows Distance (mm) input boxes, you can define the distance between the single wells of the carrier in horizontal and vertical direction, respectively (Fig. 233).

You can load a predefined carrier configuration from the **Carrier definition** field by pressing the

Load button (symbolized by the ⬚ icon) (Fig. 234).
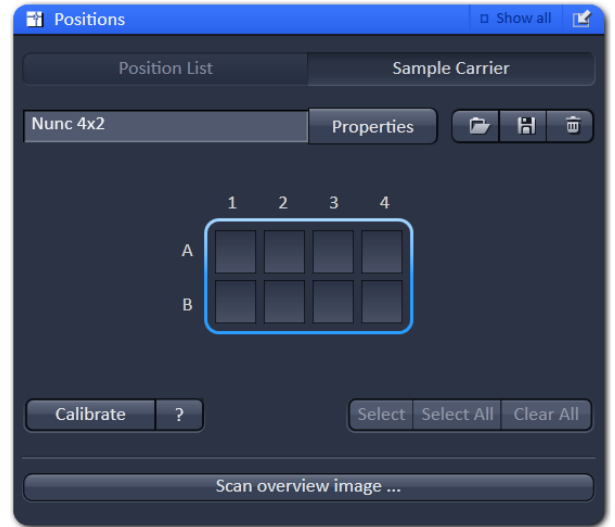
Fig. 232   **Sample Carrier control panel**

Fig. 233   **Properties Pop up window**

Fig. 234   **Carrier definition field**

All saved marker configurations will be listed in the **Load configuration…** panel (Fig. 235).

Select the appropriate one by highlighting it in the **Recent** section, which lists the recently used marker configurations or from the **Configurations** section, where all saved carrier configurations are displayed. To search for a carrier configuration, type in its name or part of its name in the **Search** field and only those carrier configurations will be listed that contain the typed in string of letters. The name of the currently loaded carrier configuration will be shown in the display box.

To delete a carrier configuration press the **Delete** button (symbolized by the **dust bin** icon). This opens the **Delete configuration…** window (Fig. 236).

Fig. 235   **Load configuration… panel**
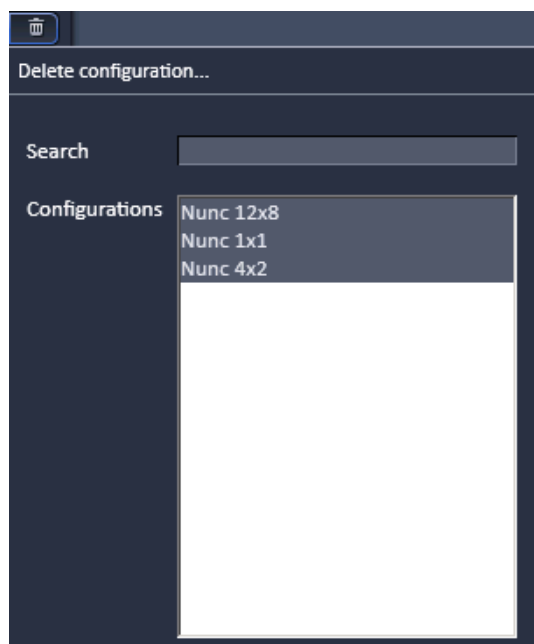
Fig. 236   **Delete configuration… panel**

Select the carrier configuration from the **Configurations** list by highlighting it or search for it by typing its name in the **Search** field. The **Info** window pops up Fig. 237).

Press **Yes** to delete the configuration, **No** to keep the configuration. To save a carrier configuration press the **Save** button (symbolized by the **disc** icon).

Fig. 237   **Delete information window**

This opens the **Save current carrier configuration as** panel (Fig. 238). Type in an appropriate name in the **Configuration** field and press **Ok** to save the configuration in the data base or press **Cancel** to abort the process.
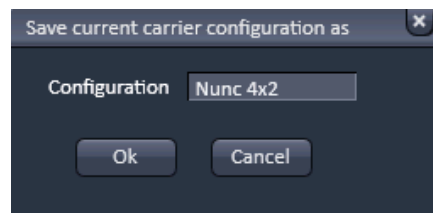
Fig. 238   **Save current carrier configuration as window**

To assign the actual position of the stage with a position in the sample carrier, move to the center of a well in the actual plate under the microscope then click **Calibrate** followed by clicking the field in the sample carrier representing this well of the plate. This assignment will be kept until changed repeating this procedure and that well will turn blue.

Pressing the **?** button (Fig. 239) will display a help text how to calibrate the position of the sample carrier.

If you click on any other than the blue highlighted well, the stage will move to this well based on the distances defined in the carrier properties. This will be than the blue highlighted one.

Fig. 239   **Calibration of sample carrier**

By pressing **Select** you can highlight those wells of the sample carrier that should be sequentially used by just clicking on them (Fig. 240). All selected wells will turn blue. By clicking on a selected blue highlighted well, it will be deselected. By pressing **Select all**, all wells will be selected for imaging. By pressing **Clear all**, all wells will be deselected. As long as Select stays pressed, the wells will be sequentially approached starting at the let upper position to the right lower one (A1, A2…;B1, B2,…).

Fig. 240   **Selection of wells in a sample carrier**

5.2.21.3 Lowering Objective when Moving the Stage

Some acquisition experiments require the objective lens to be lowered (or raised depending on the microscope) before moving the scanning stage to the next position (e.g. required if certain plate formats are used).

With the tool Positions, this is possible when activating ☑ Objective lowered when moving stage check box.

This feature is available to **Position List** and **Sample Carrier** if **Show all** is active.
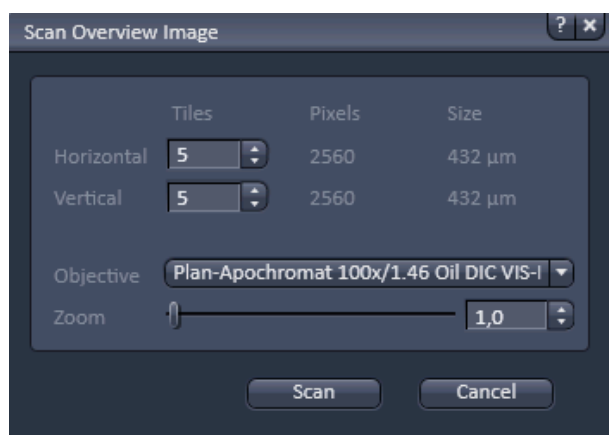
Fig. 241 Scan panel

5.2.21.4 Scan Overview Image

Pressing the **Scan overview Image…** button opens the **Scan overview Image** interactive window (Fig. 241). Here you can define the number of **Tiles** by using the **Horizontal** and **Vertical** Input boxes (the extension of the scanned area will be displayed in **Pixel** and **Size (µm)** units), the objective from the **Objective** drop down menu and a potential zoom factor via the **Zoom** slider or Zoom input box with which to scan an overview image. For widefield modes, only the zoomed in part of the camera chip will be displayed. Changing **Objective** and **Zoom** will change the size, but leaves **Pixels** unchanged. This overview image can then be used to navigate in the sample (i.e. define positions for imaging). **Scan** starts the tile scan. **Cancel** closes the window without further action.

5.2.22 Multidimensional Acquisition – Information on Experiment

The **Information on Experiment** tool (Fig. 242) displays the information of the current experiments in terms of

– Channels / Bit Depth

– Dimension

– Required Disk Space

– Duration for 1 frame
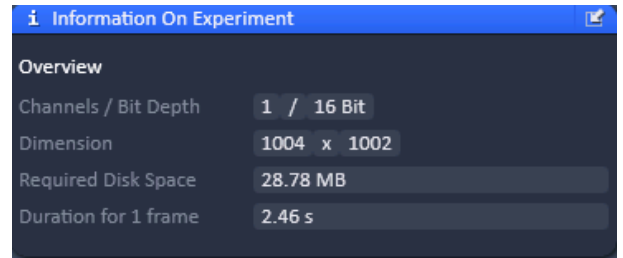
Fig. 242   **Information on Experiment tool**

Any changes in the settings will be automatically updated in regard to the displayed parameters.

5.2.23 Multidimensional Acquisition – Streaming and Auto Save

If the **Streaming and Auto Save** tool tab is expanded the **Streaming** and the **Auto Save** tools become available (Fig. 243).
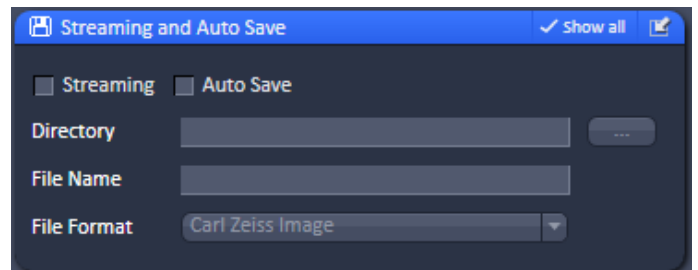
Fig. 243   **Streaming and Auto Save tool**
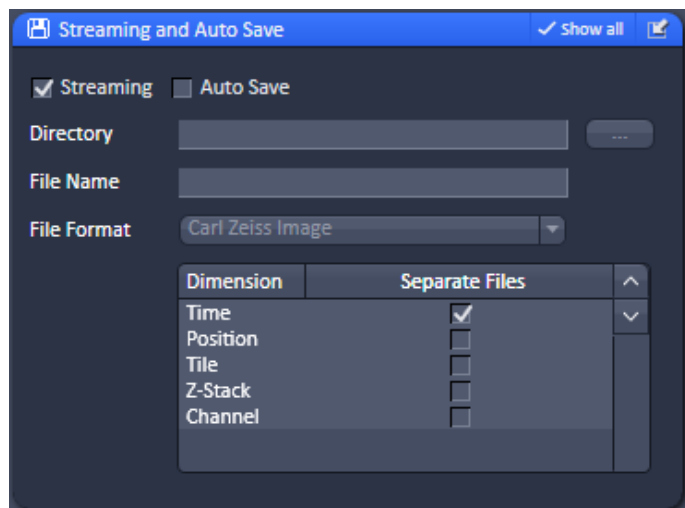
Fig. 244   **Streaming tool**

5.2.23.1   Streaming tool

If the **Streaming** checkbox is activated the **Streaming** tool is available (Fig. 244).

Acquisition settings

When **Streaming** is activated in the **Streaming** tool, pressing **Start Experiment** will open a Windows Explorer window (Fig. 245).

Here the file name and location can be defined as to where the experiment will be saved during the acquisition.
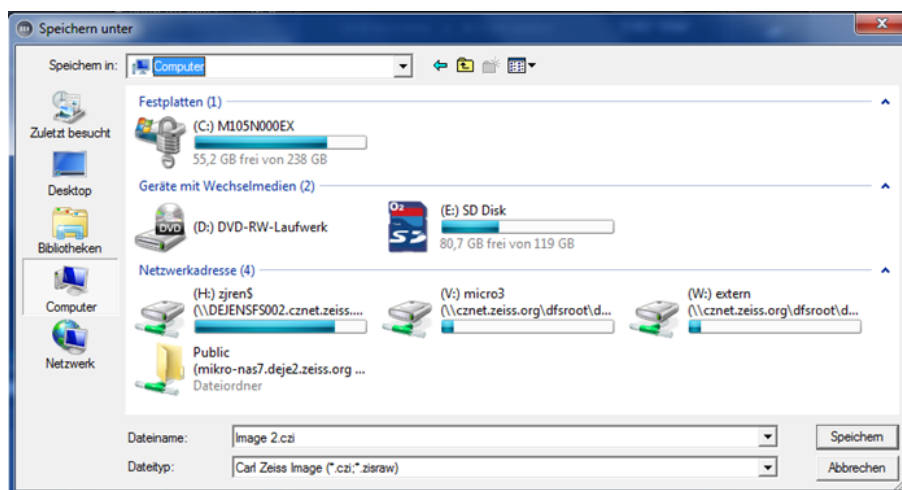
Fig. 245   **Windows Explorer window**

Online (external) Processing

It is possible to save dimensions of the experiment as individual files.

To do so, the dimensions can be chosen with the according check box in the **Streaming** tool window (see Fig. 244) or the **Set Streaming for processing during experiment** check box in the **Online Processing Options** tool window (see Fig. 246, for processing on a separate computer).

It is important that only the time checkbox is activated for external processing, which is the default setting.
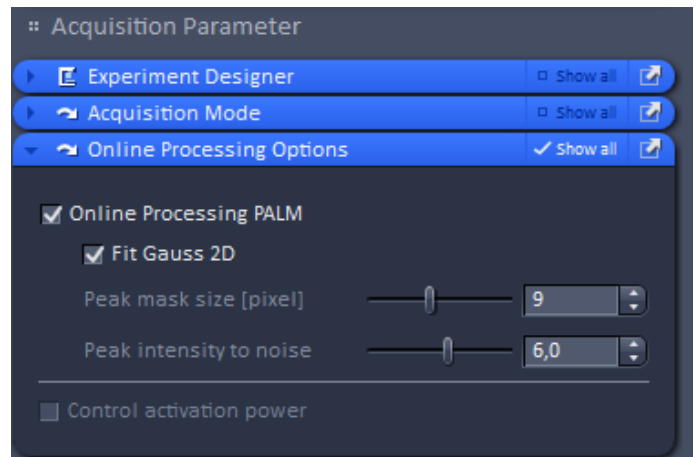
Fig. 246   Online Processing Options tool

Streaming

Open the **Streaming and Auto Save** tool window to activate this option by checking the box labelled **Streaming**. This will activate the function that data is saved and automatically written to the hard drive during the acquisition process, so when image acquisition is disrupted, the data up to this point will be available.

If you have an external processing PC you wish to stream onto, make sure that the directory is mapped.

When you turn on the PC for system control after the external PC is completely booted, the directory is mapped automatically.

Activated Streaming will enhance the speed by which .czi datasets are opened. Before an Experiment is started, a Windows Explorer window will open. Here the destination of the streaming and a file name can be chosen. The image series will then be saved with this file name extended by the exact date and time of the image acquisition.

The data can be separated into separate files, defined by chosen dimensions. More than one dimension can be selected. As a default for later external processing, time is activated and should remain this way. It will generate the file structure for later processing.

As an alternative, **Auto Save** is available as a simple way to secure files e.g. at an overnight acquisition without any special processing requirements.

☞   Note that **Auto Save** might slow down performance and does not allow external processing.

Comments on Data Management

To achieve the specified performance of the system, the Streaming option in the Streaming tool window has to be active. Please ensure the following for the drive you will be streaming your data onto:

- If you have no storage and analysis PC, use the D: or S: drive from your PC for system control. Using the C: drive will lead to an unstable system.

- If you have a storage and analysis PC: Ensure that you are using the 10 GbE network cable or fiber to connect the PC for system control to the storage and analysis PC. Ensure that the drive of the storage and analysis PC is mapped to the PC for system control. This will happen automatically when the storage and analysis PC is booted before the PC for system control is turned on.

☞ While streaming data (experiments) onto the storage and analysis PC, do not try to remove or load any other data from or to the storage and analysis PC. It will slow down the data stream from the PC for system control and will influence the acquisition speed of the experiment.

☞ Do not use the network of your institute or laboratory to stream data to the storage and analysis PC. Most likely, data transfer will be too slow and will jeopardize your experiment and will lead to an unstable system.

☞ Do not use any USB devices (external hard drives) to stream onto; data transfer will be too slow.

☞ Do not exchange single Raid elements of your storage and analysis PC. The system is designed to function as one unit. To remove already saved data from your storage and analysis PC (not during data acquisition), you can use USB devices (external hard drives) with USB connections; internal network connections, and the 10 GbE network connection.
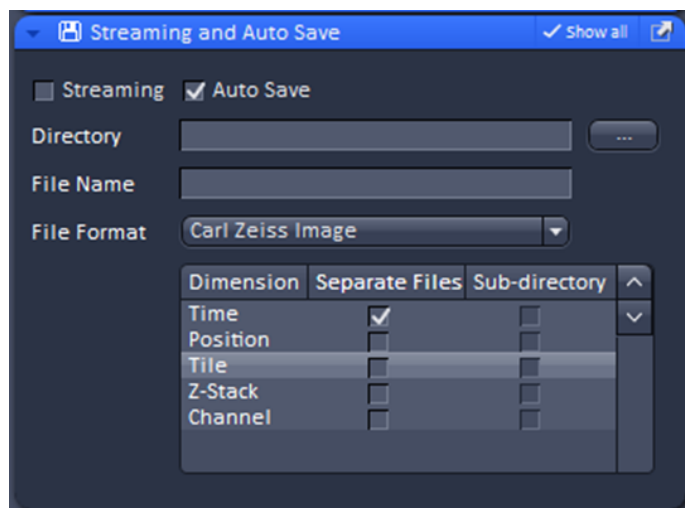
Fig. 247   Auto Save tool

5.2.23.2   Auto Save tool

If the **Auto Save** checkbox is activated the **Auto Save** tool is available (Fig. 247).

Images that are acquired by clicking the **Start Experiment** can be automatically written to the hard disc, so that when image acquisition is disrupted, the data up to the disruption point will be available.

If data should be automatically saved, the **Auto Save** check box has to be ticked. A directory, to which the file will be saved, has to be specified in the **Directory** field. You can specify the directory by clicking the **...** button in the Windows Explorer.

The selected directory will be displayed in the **Directory** display field. The name of the file has to be typed into the **File Name** display box. The image series will then be saved with this file name (or name of the sub-directory) extended by the exact date and time of the image acquisition.

The file format can be selected from the **File Format** drop down menu (Fig. 248). The scroll bar can be used to have access to all listed file formats.

In the **Dimension selection** field you can define how the image series is saved. For each dimension you can define by ticking the respective **Separate Files** checkbox to save this dimension in separate files. The file names of the single files will have extensions of a letter indicating the dimension (t for time series, z for Z-Stack, p for position etc.) preceding a number that indicates the frame number of the series. If none is checked, the whole image series will be saved in one file. If the **Sub-directory** checkbox is ticked in addition, all separate images of the specified dimension will be saved in a newly created sub-directory having the same name as the file. This option is not available for the Carl Zeiss Image (CZI) file format. If the Separate Files checkbox is not ticked, ticking the respective Sub-directory checkbox is of no consequence.

Fig. 248   **File format drop down menu**

The activation of Auto Save is indicated as a disc icon in the graphical scheme of the experiment next to the main tool group.

☞   Autosave does not save those image data which are acquired and processed with HDR function. Only the non processed data are stored.

5.3 Processing Tab

5.3.1 General Structure of the Processing Tab

Click on the **Processing** tab to have access to the processing functions. The list of available Processing tool will be displayed (Fig. 249). The scrollbar must be used to have access to all functions.
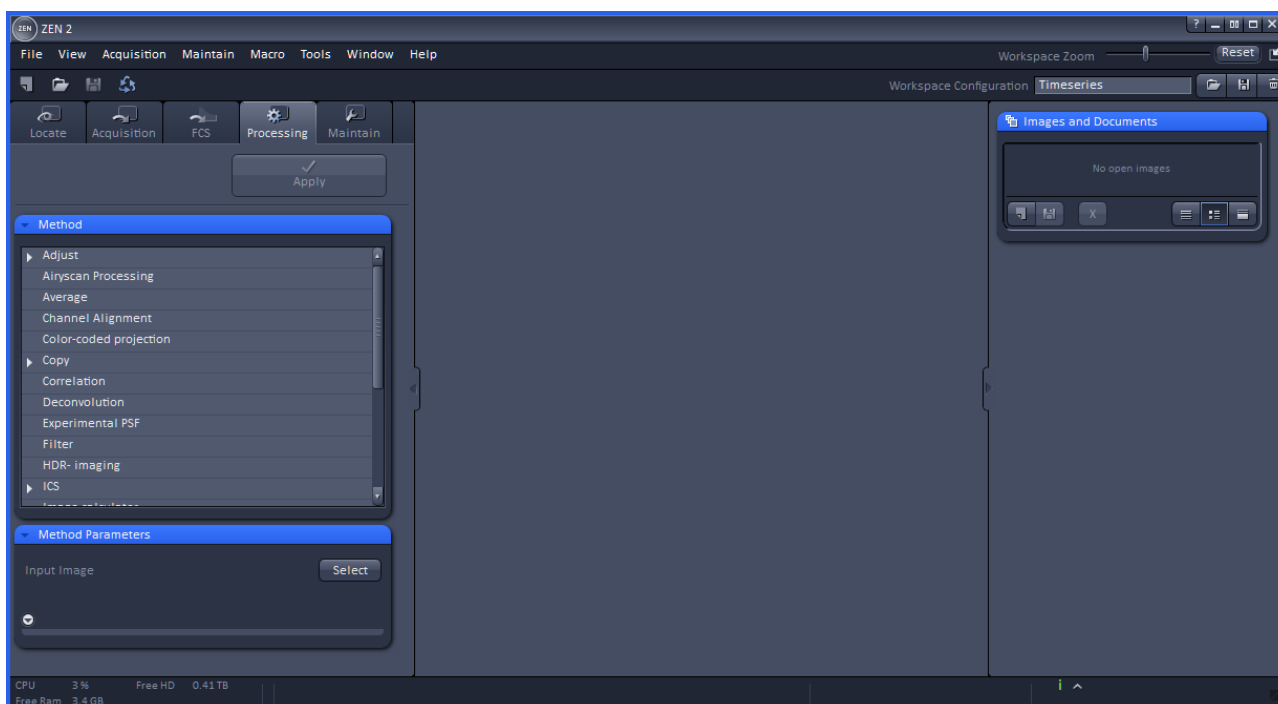
Fig. 249   **Processing tool**

The **Processing** tool provides a number of methods for image processing and analysis using mathematical operators and algorithms, which include:

– Adjust	– Airyscan Processing
– Average	– Channel Alignment
– Color-coded projection	– Copy
– Correlation	– Deconvolution
– Detrending	– Filter
– Experimental PSF	– ICS
– HDR-imaging	– Ion Concentration
– Image calculator	– Linear unmixing
– Lightsheet Processing	– Modify Series
– Maximum intensity projection	– Particle Tracking
– Localization Microscopy	– SIM
– Localization Microscopy Tools	– SIM Tools
– Stitch	

☞ All processing tools can be used with stored and reloaded images or with newly acquired images (that need not be stored before processing).

☞ Please note that for SMLM images, although stored in a .czi format, only the SMLM and Channel Alignment Processing tools are available, which can recognize tables. If you want to apply other Processing tools, the SMLM image based on the table has to be converted to a true image. Such a function is available as a sub-function in the SMLM Processing tool. Calculated SIM images, on the other hand, have the same format as normal widefield images.

☞ Note that all .lsm and .czi images can be loaded into a function, but in order for that function to make sense, the .lsm or .czi image format must have the right dimensions.
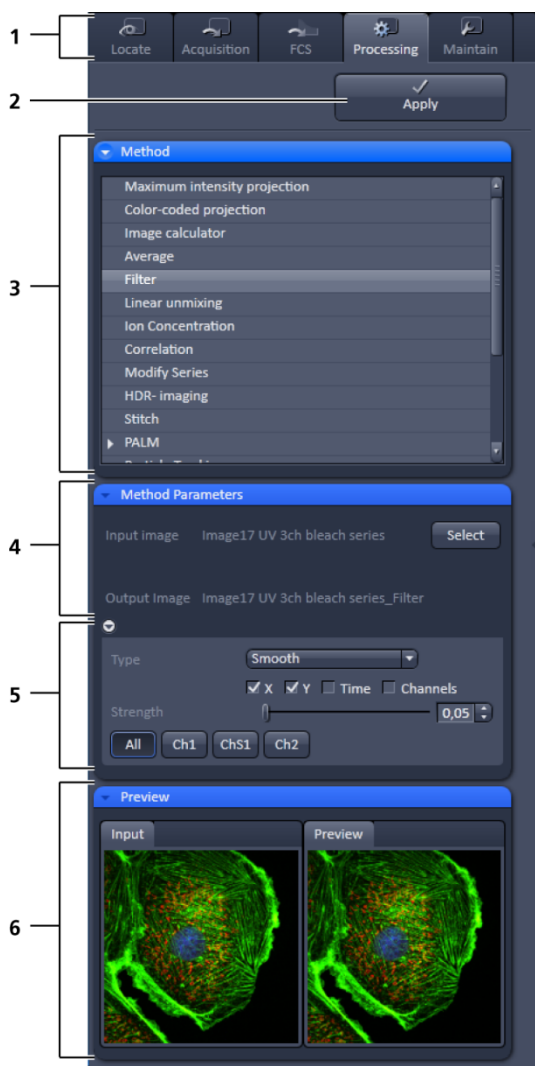
1   Main tool tabs
2   Apply button
3   Method panel with the available processing methods
4   Method Parameters panel with the input image and
    output image selection field
5   Method Parameters panel with with the
    method-specific settings
6   Preview panel with input and preview thumbnails

Fig. 250   Processing tool structure

The **Processing** tool tab (Fig. 250) is structured into different fields.

The top field or header (just beneath the **Main tool tab** area) contains the **Apply** button. The button is only active when an image is selected. If the **Apply** button is pressed, the selected or active processing tool operations will be applied to that image.

The next field contains the list of **Processing** tools. Select the desired tool by clicking on it. The active tool will be highlighted.

☞   Next is the image selection field. Highlight the image in the image container that you want to select. Load the **Input image** by pressing the **Select** button. **Select** will load the active image in the container. Alternatively, first press **Select** and then click on the image in the image container. With some image processing tools, two images can be loaded. Then select the first image to be loaded in the image container and press the **Select** button for **Input image**. Then select the second image to be loaded in the image container and press the Select button for **Input image 2**.

☞   Note that once an image is loaded by pressing **Select**, it cannot be unloaded but only be replaced by another one. This holds also true if the Processing tool is changed; the previous loaded image will stay selected. If another image is to be loaded, just highlight that image in the image container and press **Select** again. The newly selected image will replace the old one.

☞   If two input images are selected (**Input image** and **Input image 2**), they have to match each other in their dimensions. If they do not match, a warning is given. Acknowledge the warning to continue with the processing. The message will also be displayed if you load a new image pair that does not match in dimensions to the old pair. Since you can only load one image at a time, this will lead to a mismatch between the old **Input Image 2** and the new **Input Image**. Acknowledge the warning to continue loading the second new image.

☞   It is the responsibility of the user to check if an operation is suited for an image.

Once an image is selected by pressing the **Select** button, the **Output Image** name field with the name of the processed image will appear. The name can be later changed when the processed image is saved.

The next field is the method specific **Settings** panel that can be expanded / collapsed by pressing the triangle repeatedly. The triangle only appears when a processing tool is highlighted. It displays all the operational options for a specific processing function, if applicable, for the loaded image. Note that some

Processing functions do not contain additional options and the field remains empty. Only those functions will be displayed that match the dimensions of the image parameters and that can be applied to the loaded image.

Once an **Input image** is selected, the **Preview** panel becomes available at the bottom field. It shows the **Input image** (or **Input Image** and **Input Image 2**, which can be selected by pressing the respective thumbnail) on the left and the **Preview** image on the right. In addition, a new View tab – the **Preview** tab – is opened for the image in the **Center Screen Area** (Fig. 251).
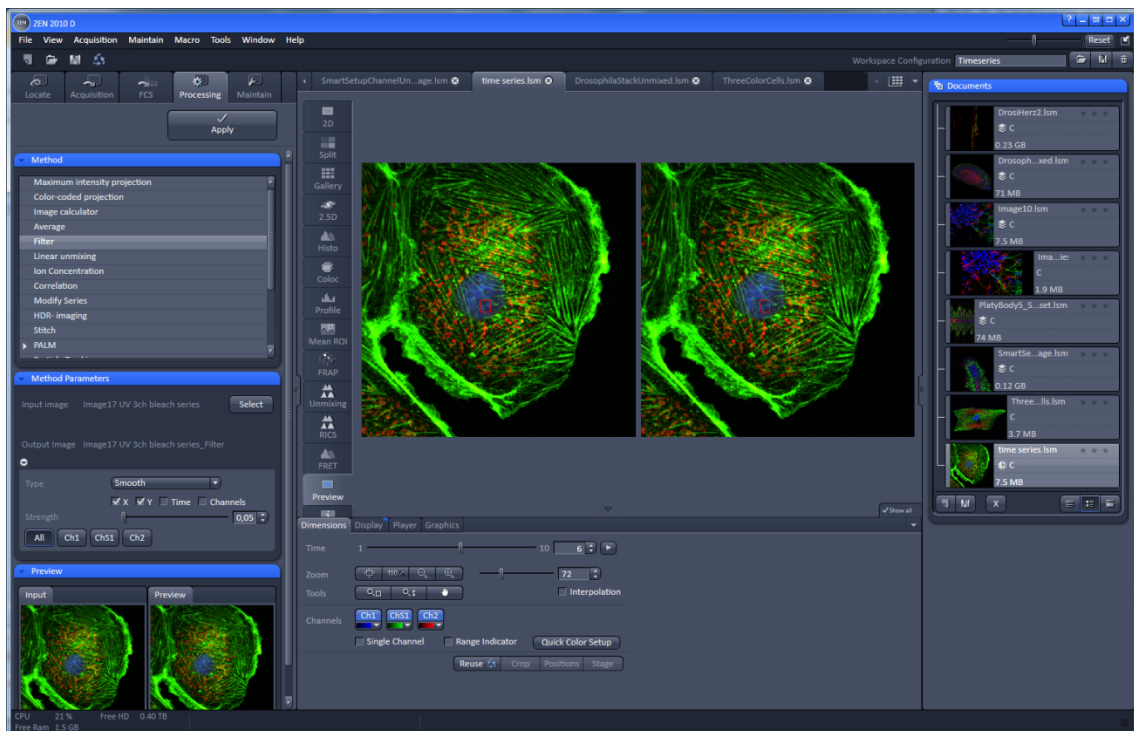
Fig. 251 **Preview view tab in the Left Tool Area**

The **Preview** tab shows the preview in more detail. The **Preview** thumbnail is always displayed in the **Processing** main tool tab, even when the view in the **Image Display** is changed. If in doubt which Image is selected as processing input, always refer to the displayed thumbnails. Both Previews – in the thumbnail as well as in the automatically generated Preview View tab in the Center Screen Area – are immediately updated.

5.3.2   Processing – Airyscan Processing

Licensing		Instrument		
standard	✓ optional	✓ LSM	ELYRA	Lightsheet Z.1
Tool accepts Z-Stacks and time series and combinations of both. See full description of tool for details.				
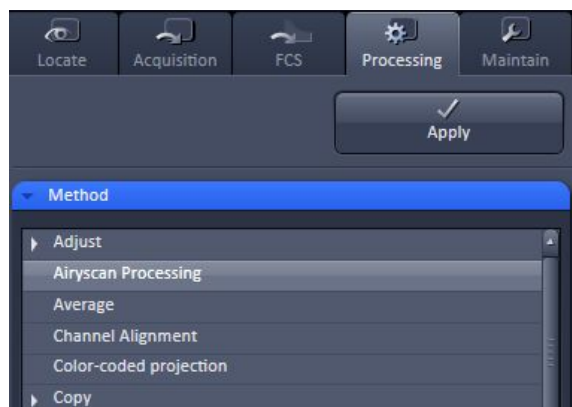
Fig. 252   Processing tab with Method selection list
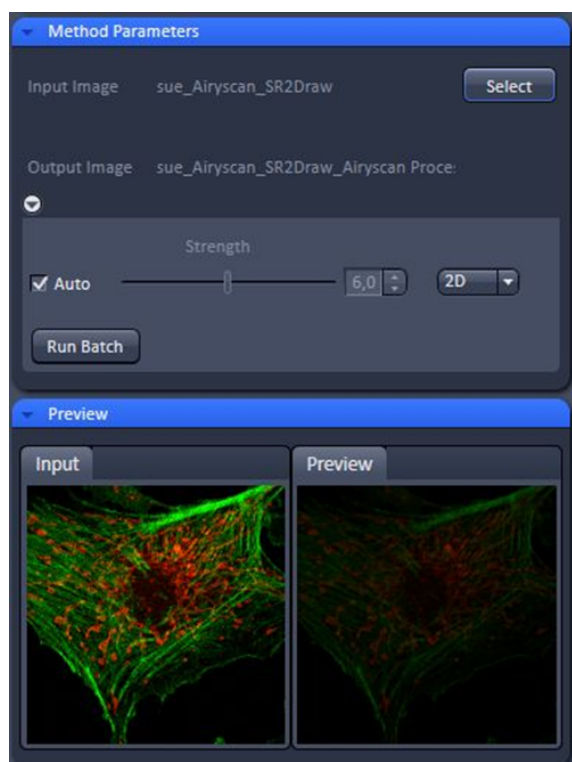
Fig. 253   Airyscan Processing tool: Method Parameters, Auto

Airyscan Processing provides the 2D and 3D processing function for image data acquired with Airyscan in superresolution (SR), resolution versus sensitivity mode (R-S) and virtual pinhole (VP) mode.

The highlighted image or image series in the central View container is selected upon clicking the **Select** button.

For images acquired in SR mode, the processing strength is per default preset with the **Auto** function activated.

For changing the parameter, uncheck the **Auto** function and use the slider or editing box to set a new value (Fig. 253).

2D processing is available for 2D and 3D data sets (also over time). 3D processing is available for 3D data sets (Z-Stacks also over time) only.

- To process Airyscan data, choose the **Airyscan Processing** function in the method list (Fig. 252).

Auto

- Klick on the open image you want to process and press **Select** (Fig. 253) in the method parameters dialogue. Make sure the right image is visible in the input window.

- For auto processing, activate the **Auto** checkbox. In the preview window you can check again whether the right image type is selected.

☞ Note that this preview will not show a full SR processing result.

- Press the **Apply** button at the top of the dialog.

Processing will start and might take a few minutes.

A new image will open with the result of the auto processing.

All images in a time series or Z-Stack will be processed.

Manual

- For manual processing, deactivate the **Auto** checkbox (Fig. 254).

- In the preview window you can check again whether the right image type is selected.

☞ Note that this preview will not show a full SR processing result.

- Use the strength slider to get a stronger or weaker result as with the auto processing. You might use the same values as used in the 2D preview processing in the **Airyscan** tab.

- Press the **Apply** button at the top of the dialog.

Processing will start and might take a few minutes.

A new image will open with the result of the manual processing (Fig. 254). All images in a time series or Z-Stack will be processed.

☞ Note that with 3D data you have the choice of a 2D or a 3D processing by selecting the method from the dropdown list (Fig. 255). A 3D processing will take longer but will result in a higher resolution increase since also the axial Airy data will be used for the processing.

☞ Use the same approach for R-S mode. However the achievable resolution will not match the resolution of the SR mode as the sampling in XY is not sufficient for superresolution. The images will still show higher resolution and a considerable increase in signal to noise ratio.

VP data processing

In case a VP mode image was selected, the manual processing slider will function for virtual pinhole parameter adjustment.

This allows to adjust the confocal sectioning after acquisition by binning the respective airy rings together.

- Press the **Apply** button at the top of the dialog.

Processing will start and might take a few seconds.

A new image will open with the result of the selected sectioning. All images in a time series or Z-Stack will be processed.
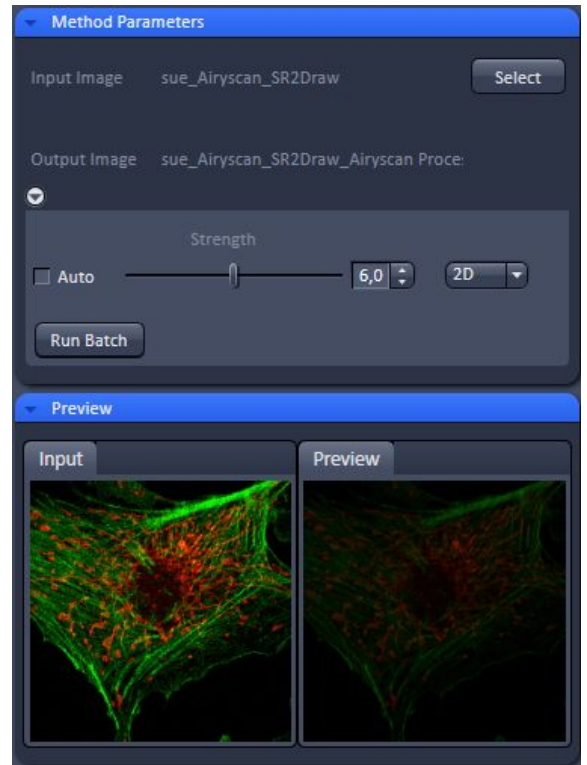
Fig. 254   Airyscan Processing tool: Method Parameters, Manual
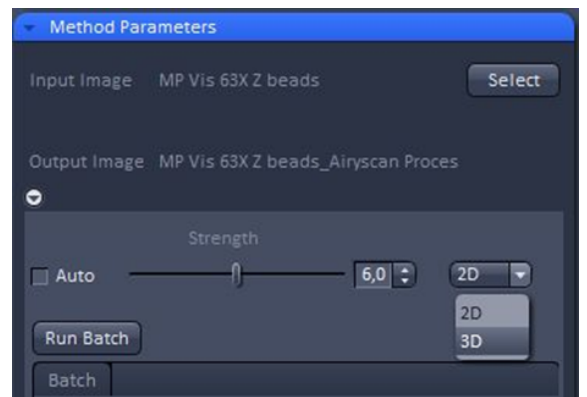
Fig. 255   Airyscan Processing tool: Method Parameters, 2D, 3D
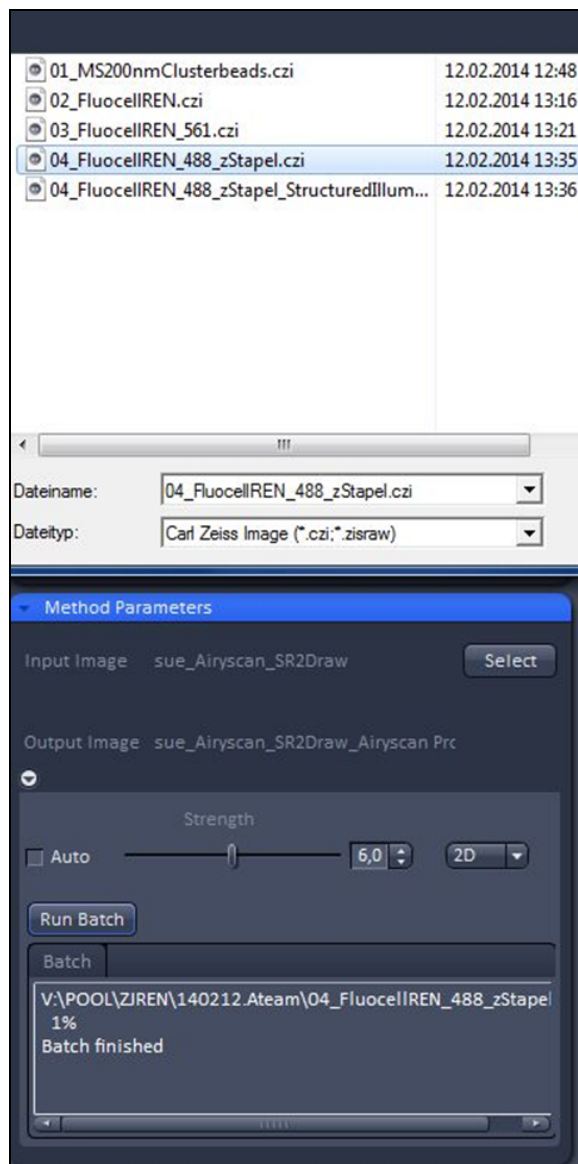
Fig. 256   Airyscan Processing tool:
Method Parameters, Run Batch

Batch

- For batch processing press the **Run Batch** button (Fig. 256) and select the respective directory and files from the file selection window which opens at the top.

- After confirming your selection the batch processing will start and might take a few minutes to hours depending on the number and type of files selected.

All files will be processed with the same processing strength and with the same mode: 2D or 3D. Do not mix the modes for processing as this will stop the processing (if 3D is chosen for 2D sets) or only do a 2D processing (if 2D is chosen for 3D data sets).

Processing can be stopped clicking **Stop Batch**.

The status of processing (% of progress/batch finished) is displayed in the Batch panel which appears in the Method tool once a batch process has been started.

Airyscan Fast processing

Fast Airyscan images are processed the same way as Airyscan images.

- Select the **Airyscan Processing** function from the list at **Processing / Methods**.

- Select the active image to be processed.
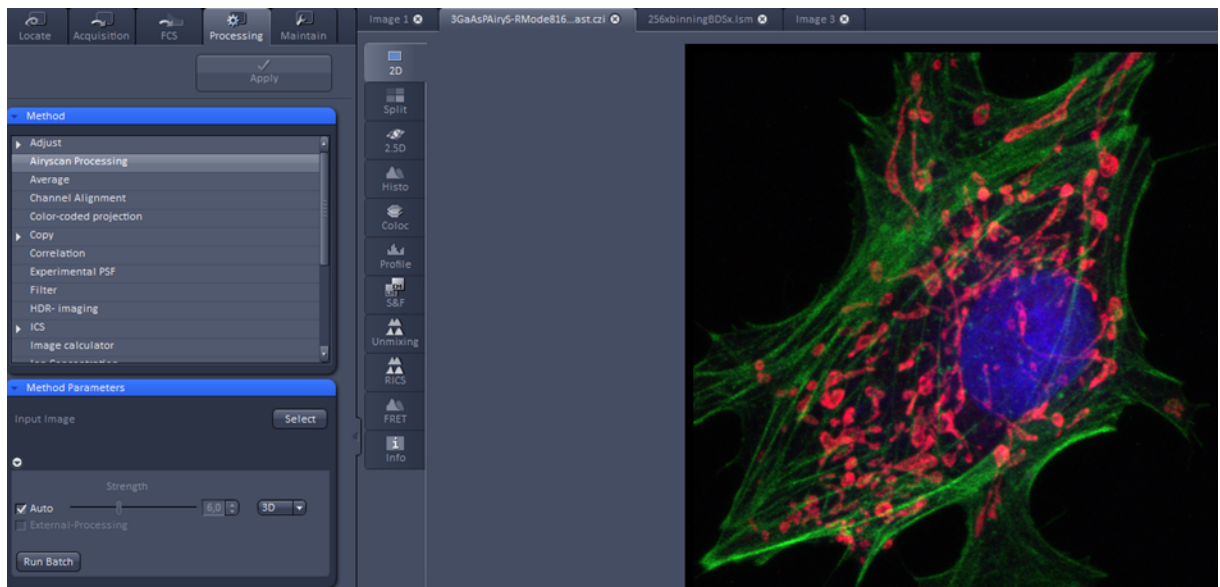
Fig. 257   **Airyscan Processing tool: Fast acquisition**

- Select auto or manual processing by using the **Auto** checkbox, and 2D or 3D mode depending on the dimensions of your data.

It is possible to use external processing on an external workstation or storage computer.

This allows to process streamed data while acquisition is still running on the system computer.

- Check **External-Processing** to activate this functionality.

Streaming itself is managed in the **Streaming and Auto Save** menu at **Multidimensional Acquisition** (see section 5.2.23).

☞ Note that batch processing (**Run Batch**) requires to select files with the same Airyscan type, e.g. only **Fast** or only **SR** Airyscan. It is also required to select an image of a matching type as the active image to get the right automatic processing method.
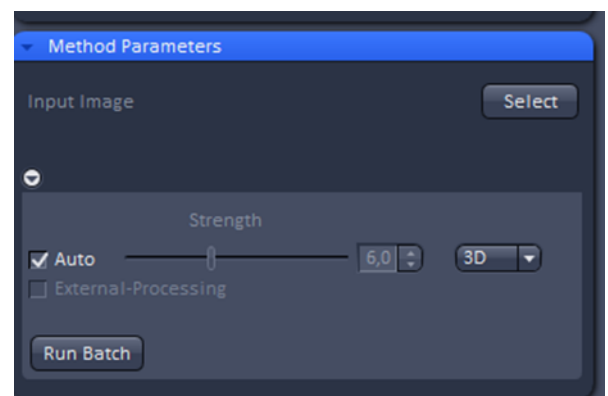
Fig. 258   **Airyscan Processing tool: Method Parameters, Fast acquisition**

5.3.3 Processing – Maximum Intensity Projection

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1

Tool accepts Z-Stacks and time series (incl. combinations with several other dimensions). See full description of tool for details.

The **Maximum Intensity Projection** function generates a maximum intensity projection image along the z-, time- or channel dimension of a multidimensional image data set (Fig. 259).

- To open the **Maximum Intensity Projection** Processing tool, select it from the list in the **Processing** main tab.

- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels of the function.

- Select the **Input Image** with the **Select** button and choose the dimension along which you want to generate a maximum intensity projection from the **Coordinate** pull down menu (z, time, channel) in the Settings panel.

- Clicking the **Apply** button creates the maximum intensity projection as a new image document using the name shown in the Output image display for the Select field.

The maximum intensity projection creates an output image whose pixels contain the maximum value over all images in the stack at the particular pixel location.

Fig. 259 Processing – Maximum Intensity Projection

5.3.4   Processing – Color-coded projection

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1
Tool accepts Z-stacks and time series (incl. combinations with several other dimensions). See full description of tool for details.				

The **Color-coded projection** function generates a maximum intensity projection image along the z-, or time- dimension of a multidimensional data set (Fig. 260). Instead of using the colors assigned to the channels, it displays the position in z or in time with a color gradient.

- To open the **Color-coded projection** Processing tool, select it from the list in the **Processing** main tab.

- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels of the function.

- Select the color gradient by choosing an appropiate look up table (LUT) from the **LUT** drop down menu.
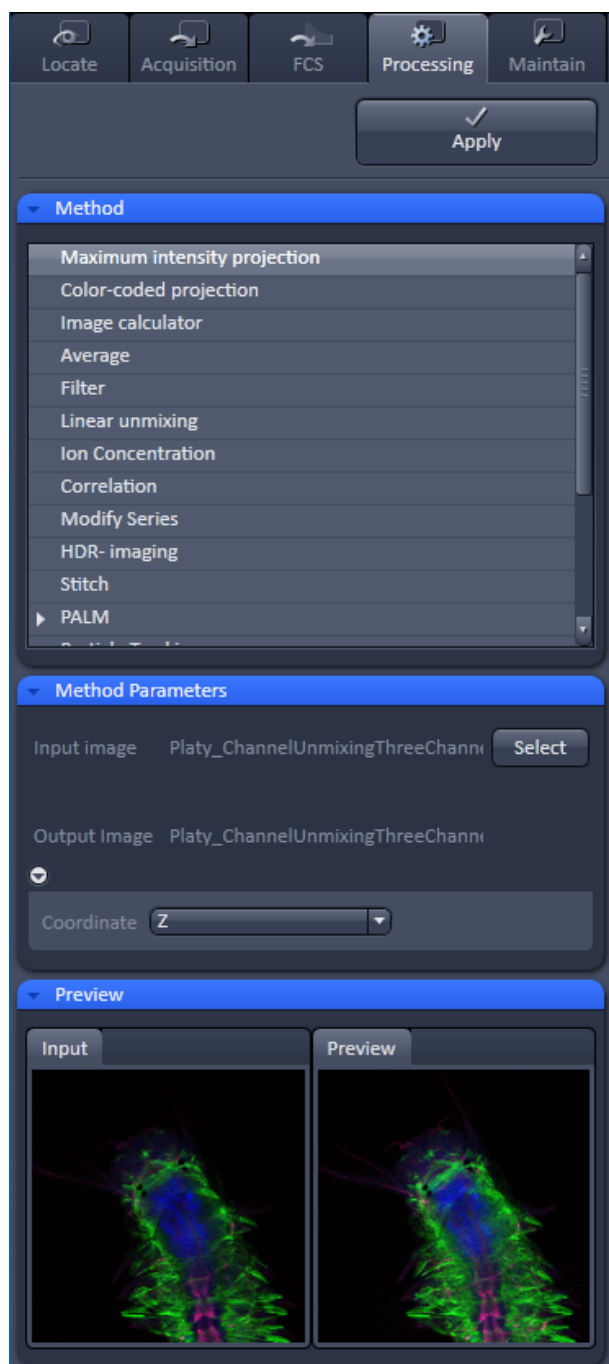
- You can select from the **dimension** drop down menu any dimension of the image. The color gardient will apply for the selcted dimension. These include **Z** (for a z-stack) or **T** (for a time series).

- Below the color gradients the corresponding depth or time is indicated in grey numbers and letters.

- Define the range (**ROI**) of the Z-Stack or time series to be used by using the sliders or the input boxes with arrows.

- Clicking the **Apply** button creates the color-coded projection as a new image document using the name shown in the Output image display for the Select field.
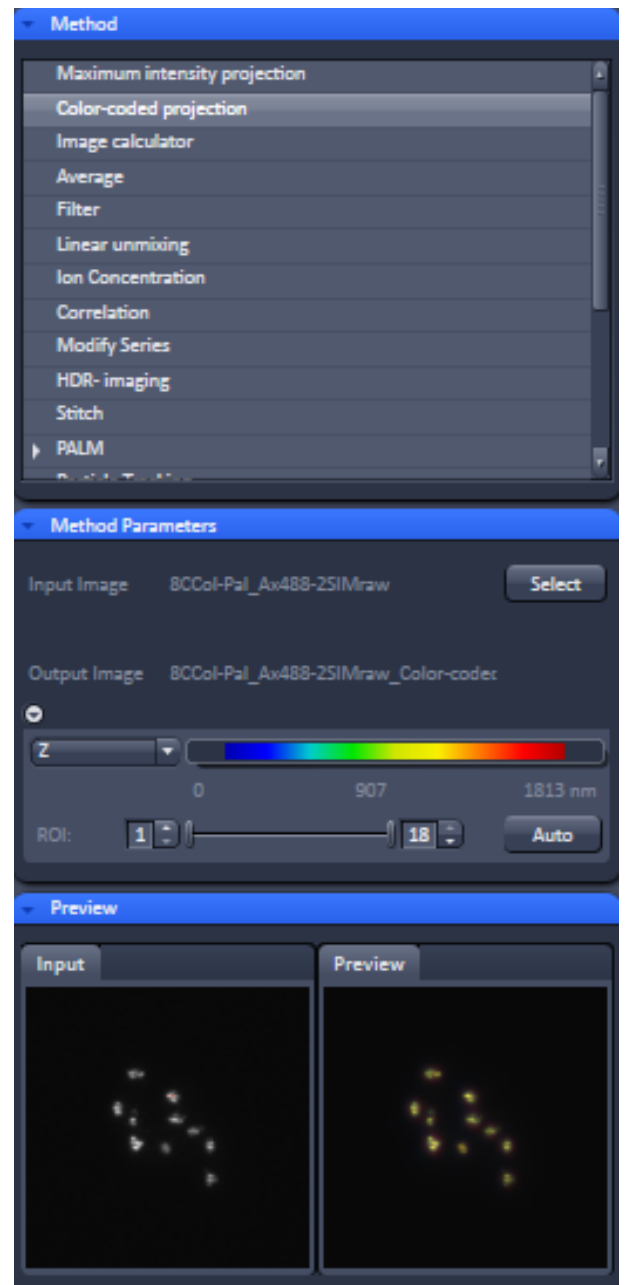

Fig. 260   Processing – Color-coded projection

5.3.5 Processing – Image Calculator

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1
Tool accepts all types of image data recorded with the listed instruments. See full description of tool for details.				
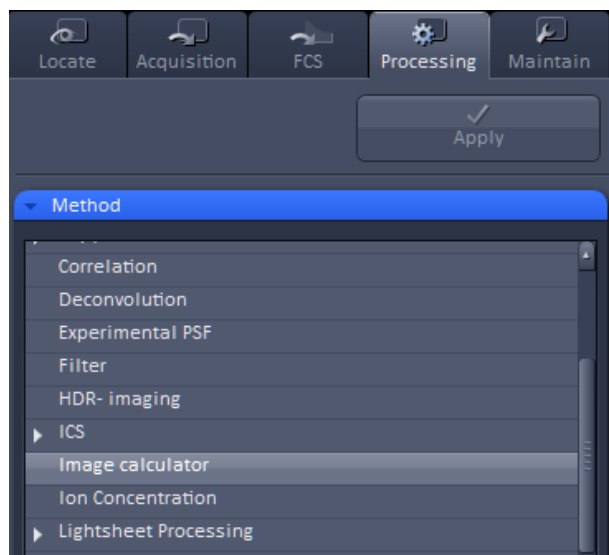
Fig. 261   Processing – Image Calculator

The **Image calculator** tool provides a calculator-style interface to apply arithmetic operators to the selected image.

- To open the **Image calculator** tool, click on the **Image calculator** in the list of the **Method** panel in the **Processing** main tool tab.

- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels (Fig. 261) for the function.

The two **Select** buttons in the **Selection** panel select the active image in the Image Display as **Input Image** and **Input Image 2**. The Output Image is automatically fed into a new image document. A specific input channel from a multi-channel image or series can be selected using the pull down menus next to the Select buttons.

The number in the **First image** display box, which can be edited, reduces the **Input 2 Image** series to the selected first frames of a time series. This can be used to normalize a time series to the values of its first image(s).

After loading 2 input images (source images S1 and S2) specifying the input channels, the calculator keyboard in the **Settings** panel allows the definition of an operator or a combination of operators which is represented by a formula in the formula display box. The operators will be inserted into the formula at the cursor position. The formula can also be typed in directly. By pressing **Input 1** or **Input 2** source image 1 or 2 will be loaded, respectively at the position of the cursor.

For an overview of the available more advanced operators, please refer to the formula panel that is shown in a pop-up window after clicking the **Operators…** button (Fig. 262).

By highlighting the selected operator, a description of the function of this operator is displayed in the lower part of the operators list (Fig. 262).

All image calculations in this tool work pixel by pixel and start with the upper left pixel regardless of the image size for the two images or image series.

☞ When choosing images of different data depth, the check box next to **Intensity range 0…1** should be marked. This normalizes the image intensity for all images to values between 0 and 1.

All actions performed in the Image Calculator have immediate effect on the previews. After pressing the Apply button on the top of the Processing **main tab**, a new image document with the resulting image is created.

A good example application for the Image Calculator is offsetting/normalizing two images or an image series with a reference image to enhance contrast for small changes in intensity:

- Select a time series as Input 1 and the first frames of these series (or another offset image) as Input 2.

- Then type/edit an operator as shown in Fig. 263 that enhances the relative changes in the image with respect to the relatively bright signal level, for example:
  S1-S2/2 = source 1 – ½ * source 2.

Fig. 262   Operators window

Fig. 263   Image calculation window

5.3.6   Processing – Average

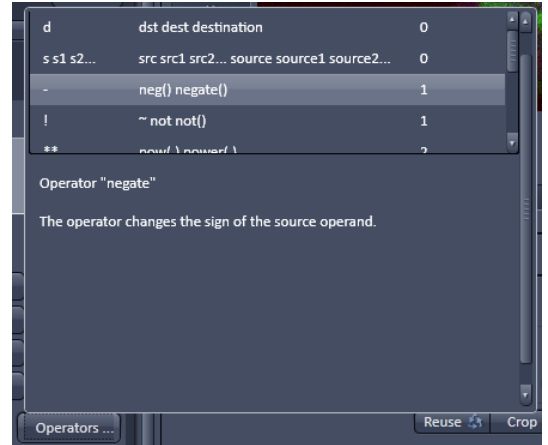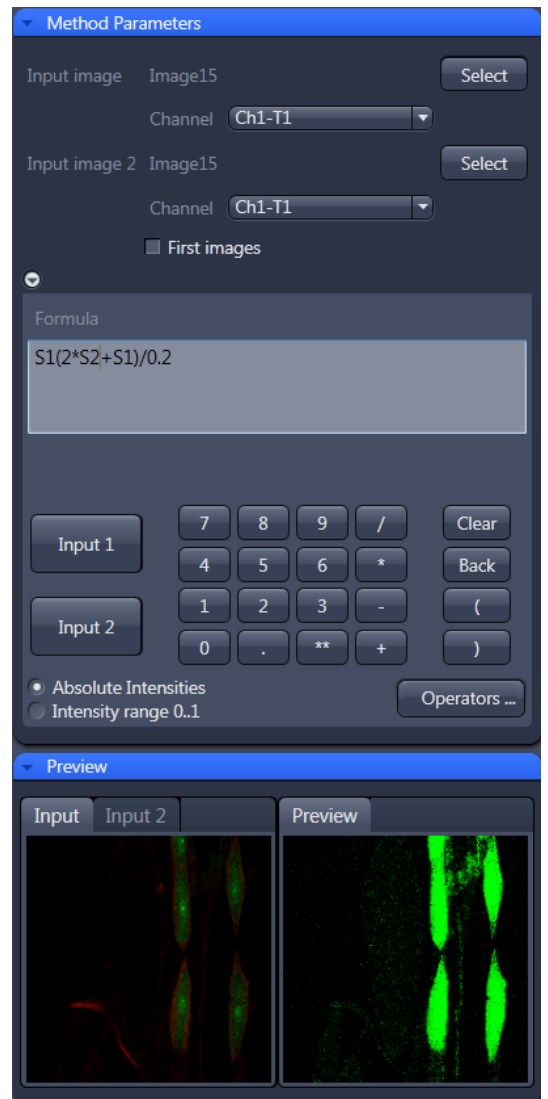| Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1
Tool accepts all types of image data recorded with the listed instruments. See full description of tool for details.				

The **Average** processing function will calculate an image that can be averaged in pixel and time.

- Activate **Average** in the **Processing** tool.

The **Average** menu will appear. The **Average** processing function will calculate an average image.

- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels (Fig. 264) for the function.

- To select an image, click on the image in the container and press the **Select** button. This will be your **Input** image.

- Select how many pixels you want to bin in x-direction, y-direction, and time points by setting the respective values in the **X Average Pixels**, **Y Average Pixels**, **Z Average Pixels** and **Time Average Pixels** input boxes, which can be opened by clicking on the arrows.

☞ Note that **Z-Average Pixels** and **Time Average Pixels** are only available for Z-Stacks and time series. You can either enter a value, or use the sliders, or arrows of the input boxes.

☞ If a moving average for **Time** should be performed, the respective **Moving Average** box must be checked. In this case, consecutive averages comprised of the specified number of frames in **Average Pixels** will be computed.
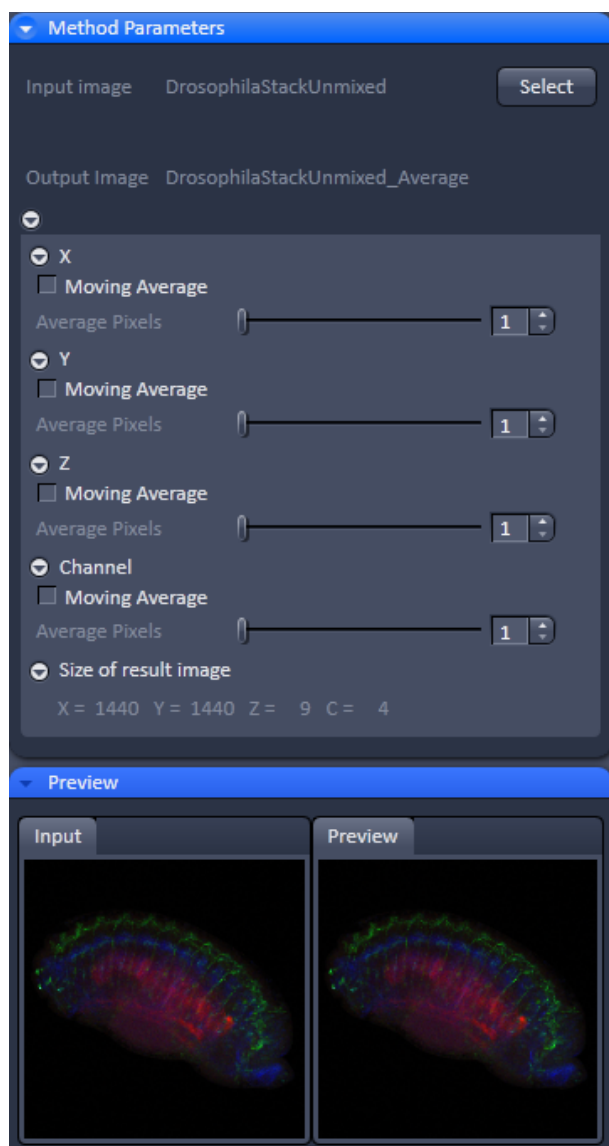
Fig. 264   Processing – Average

An average output image will be calculated according to the settings and the result presented in the **Preview** window.

The size of the resulting image in **X**, **Y**, **Z**, and **T** can be viewed in the **Size of result image** display.

If the **Apply** button is pressed, the averaged image will be opened as a new image in the container.

5.3.7   Processing – Filter

Licensing		Instrument		
✔ standard	optional	✔ LSM	✔ ELYRA	✔ Lightsheet Z.1

Tool accepts all types of image data recorded with the listed instruments. It is generally advisable to perform certain processing steps (e.g. linear unmixing or SIM processing) before applying filters. See full description of tool for details.

The filter function permits the processing of images with a Median, Smooth, Sharpen, or Bandpass filter.

- To open the **Filter** tool, select it from the list under the **Processing main tab** (Fig. 265).

- Select the Input Image with the **Select** button and choose the filter of your choice from the Type pull down (**Median**, **Smooth**, **Sharpen**, **Band**, and **Gradient**).

- Select the channels you want to process with the **All**, **Ch-T1** or **Ch-T2** buttons.

- Once a filter has been selected from the type drop down menu, filter specific settings are available.

Median Filter

With the median filter, the gray value of each center pixel is replaced with the median value of the neighboring pixels. The viewed neighbor pixels are defined by a square of a certain size called the kernel. The modified pixel now is the center pixel of this pixel matrix.

The median value is defined as the middle value (not average) of all the gray values sorted in ascending order within a matrix.

Image noise will be reduced by the application of the median filter. The cut-off of regions will slightly blur. Local maxima will be flattened. The dynamic range will be reduced considerably.
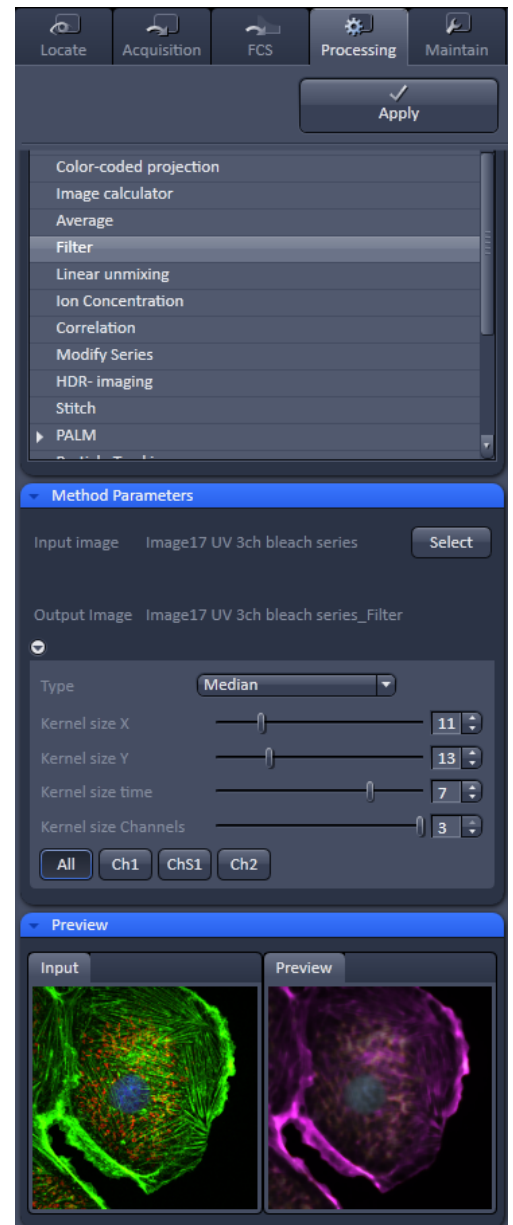

Fig. 265   Processing – Filter
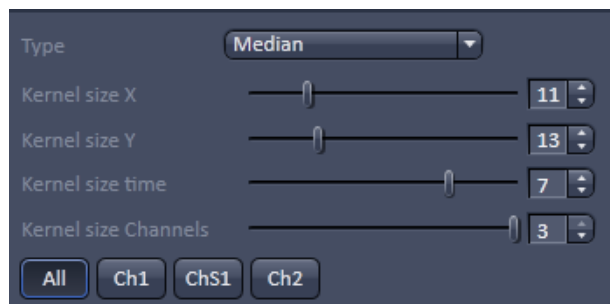
Fig. 266   **Median filter**

The effect of the filter increases with the matrix size. This matrix / kernel size can be set for each dimension with the three sliders **Kernel size X**, **Kernel size Y**, and **Kernel size Z** in the **Filter Type** panel or by setting the size in the spin-box on the right hand side of each slider (Fig. 266). A larger kernel, however, also increases the processing time. Other settings of this filter cannot be modified.
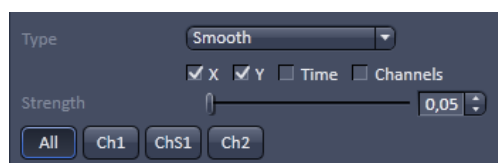
Smooth Filter

The smooth filter is a low pass filter with Gaussian characteristics. The gray value of each center pixel is replaced with the weighted average value of the surrounding neighbor pixels. The "neighborhood" is limited by the cut-off in pixels, set by the **Strength** slider. It has an analogous effect as the kernel size (Fig. 267).

Fig. 267   **Smooth filter**

If a dimension should be processed by this tool the respective **X**, **Y**, **Z**, and **Channels** check boxes must be ticked. The modified pixel now is the center pixel of the filter matrix.

Image noise will be reduced by the application of the low pass filter. Sharp edges of regions will blur. Local maxima will be flattened. The dynamic range will be reduced considerably.
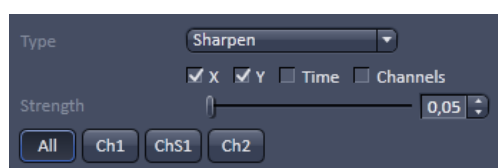
Sharpen Filter

With the sharpen filter, the original image is filtered with a low pass filter first. The result of this filtering is then subtracted from the original image. This will improve image sharpness.

Fig. 268   **Sharpen filter**

The "neighborhood" is limited by the cut-off in pixels, set by the **Strength** slider. It has an analogous effect as the kernel size (Fig. 268). If a dimension should be processed by this tool, the respective **X**, **Y**, **Z**, and **Channels** check boxes must be ticked.

Band Filter

With the band filter (Fig. 269), the original image is filtered with a "band blocker" filter. It filters out a certain frequency band with the position set by **Center**, width set by **Band Width** and a steepness of the edges set by **Strength**. This is particularly useful to remove periodic perturbances in the image.

If a dimension should be processed by this tool, the respective **X**, **Y**, **Z**, and **Channels** check boxes must be ticked.
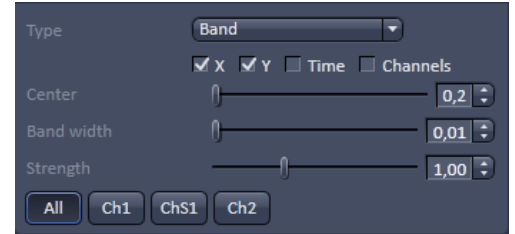
Fig. 269   **Band filter**

Gradient Filter

The Gradient Filter visualizes the direction and rate of brightness changes from one pixel to the other. It illustrates if brightness changes smoothly or abruptly in the image (Fig. 270).

The gradient filter can be used for all dimensions present in one dataset. You can select it if you wish to perform it for all channels with the **All** button or individual channels with the according (e.g. Cam1 or Ch1) buttons.

The dimensions can be chosen by checking the according check-boxes.
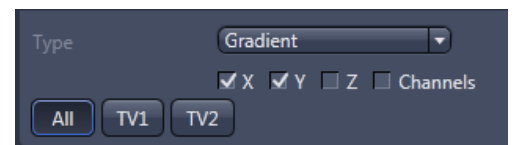
Fig. 270   **Gradient Filter**

5.3.8   Processing – Linear Unmixing

Licensing		Instrument		
✓ standard	optional	✓ LSM	ELYRA	Lightsheet Z.1

Tool accepts lambda stacks and multi-channel image data (single images and multi-dimensional image data). *Linear Unmixing* was developed for processing LSM data. Successful processing of multi-channel images obtained with other systems is potentially possible as well (provided this input meets the requirements for unmixing). See full description of tool for details.
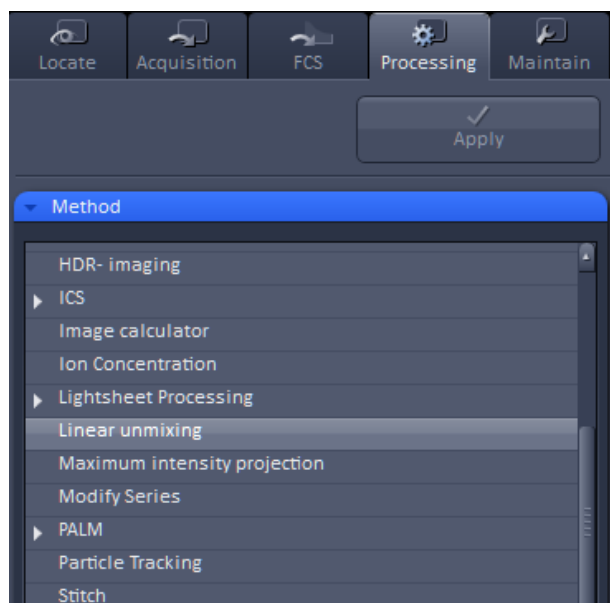
Fig. 271   Processing – Linear unmixing

The Linear Unmixing processing tool permits extracting the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from strongly overlapping multi-fluorescence data acquired in multi-channel images and so-called "Lambda stacks" (only available in LSM imaging mode).

In brief, with the knowledge of the spectral characteristic of individual components within a multi-component sample, even heavily overlapping individual spectral characteristics can be mathematically extracted from an experimental multi-channel data. This method is a strictly pixel-by-pixel image analysis procedure.

Experimentally, fluorescence spectra of mono-labeled samples are acquired and stored in the Spectra Database as an external reference. Then a multi-channel image or Lambda stack (available only in **LSM imaging** mode) from the multi-labeled sample is acquired.

Finally, the individual components are mathematically extracted using the information from the reference spectra. Up to ten different reference signals can be fed into the least-square-fit based algorithm to produce a 10-channel multi-fluorescence stack without any partial overlap between the channels.

If no mono-labeled samples are available, the references can be obtained by the following methods:

a)   Interactively by user-selection of regions in the image where only one fluorescence dye is present (only available in the **Unmixing View** tab).

b)   Automatically by software analyses of what the individual spectral signatures are. This processing function is called "Multi-channel Unmixing" or "Automatic Component Extraction" (ACE). Note: in some cases, spectrally acquired images are not appropriate for ACE or "Multi-channel Unmixing" and linear unmixing can lead to wrong results.

- To open the **Linear unmixing** tool, click on **Linear unmixing** in the **Processing** tool list under the **Processing** tab (Fig. 271).

- Pressing the **Select** button loads the active image from the **Image Display** and displays it as Input Image. Only Lambda stacks (only available in **LSM imaging** modes) or multi-channel images can be loaded.

- In the **Linear unmixing** panel (Fig. 272), the number of spectrally distinguishable fluorescent components within the imaged sample can be selected from the **Components** selection box. The number of extractable components cannot be higher than the number of acquired channels. The ZEN software is limited to a maximum of 10 components.
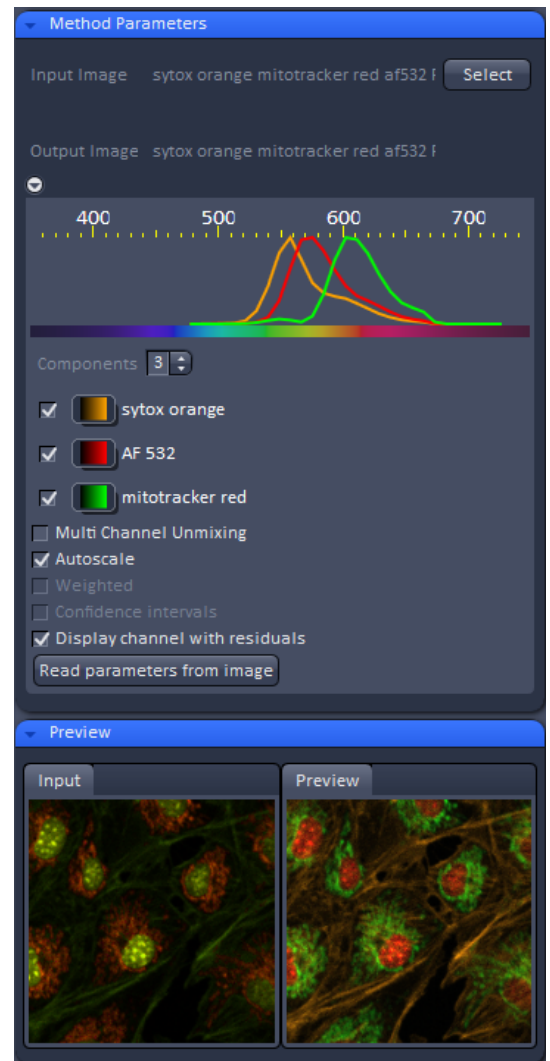

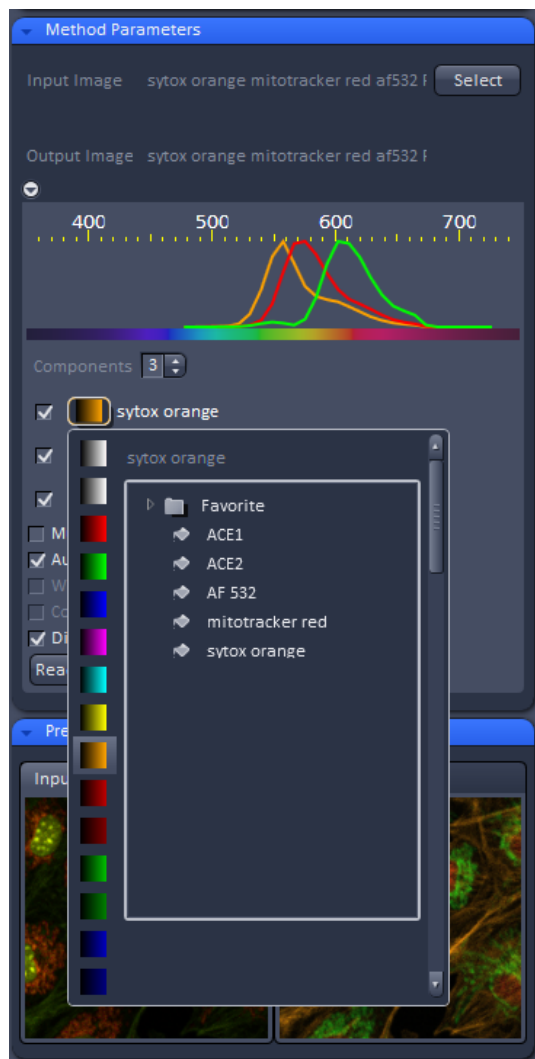**Fig. 272   Linear unmixing panel**

Fig. 273   Spectra and LUT assignment

- Select the sample-specific fluorescence dye reference spectra from the Spectra Database (Fig. 273).

- Assign appropriate colors to the fluorescence channels by clicking on the color flag and choose from the available lookup tables (LUTs)

- If no predefined reference spectra exist:

  – Prepare reference samples and create spectra via the **Save to Spectra DB** button in the Unmixing View (see section **Processing – Linear Unmixing**).

  or

  – Use the "Multi-channel Unmixing" functionality described below. (Note: in some cases, spectrally acquired images are not appropriate for "Multi-channel Unmixing" and linear unmixing can lead to wrong results – see discussion below.)

- After definition of the required reference spectra click **Apply**.

A new image with the resulting (unmixed) channels will be created (Fig. 274 and Fig. 275).

The following additional settings and functions are available for linear unmixing:

Auto scale balances the intensity of the unmixed channels to equal levels.

Display Channel with Residuals generates an additional channel in which the intensity values represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra. In essence, the residual value is the biggest remaining "residual" from the least square fit routine. The residuals are a general measure for how good the fit of the algorithm has performed.

The higher the intensity in this additional channel, the worse is the fit of the spectra to the dataset. This shows that either the acquired data is not representing the sample (e.g., when information is lost by pixel saturation) or wrong/insufficient reference spectra have been chosen. In this case, new image acquisition with no overexposed/ saturated pixels is strongly recommended. In the case of wrong reference spectra, choosing/acquisition of different/new reference spectra can be necessary to improve the results.

Weighted unmixing:

When this option is checked, spectral channels with high noise contribute less to the unmixing result. This option includes a statistical analysis of the signal-related (Poisson-) noise and weighs the respective contribution for the fitting with the combination of reference spectra to the experimental data. Note: this option involves a more sophisticated unmixing algorithm and therefore takes longer than the basic unmixing analysis. Weighted unmixing generates improved unmixing results when acquisition channels are not so well balanced but still have a good signal-to-noise ratio.
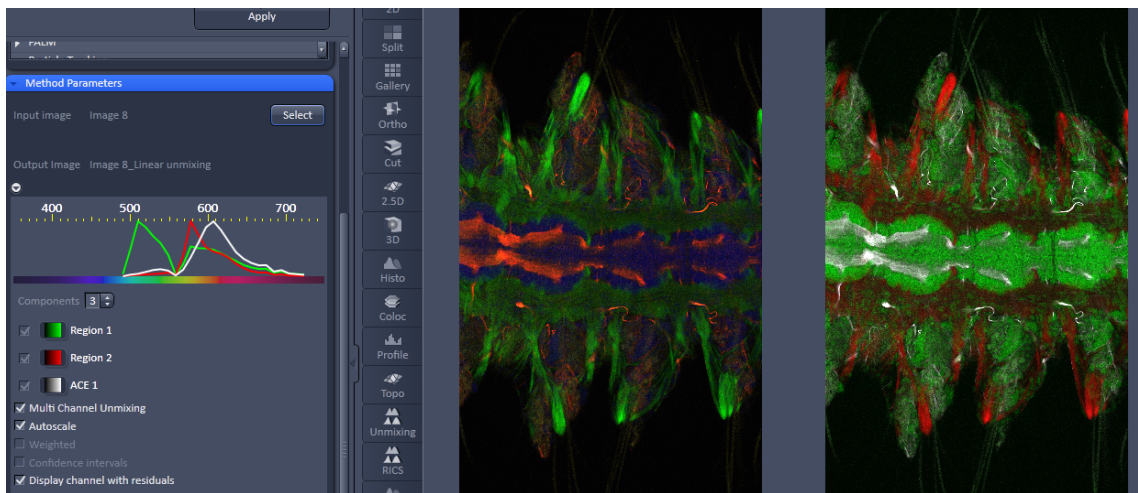
Fig. 274   Image Display window before unmixing in Preview
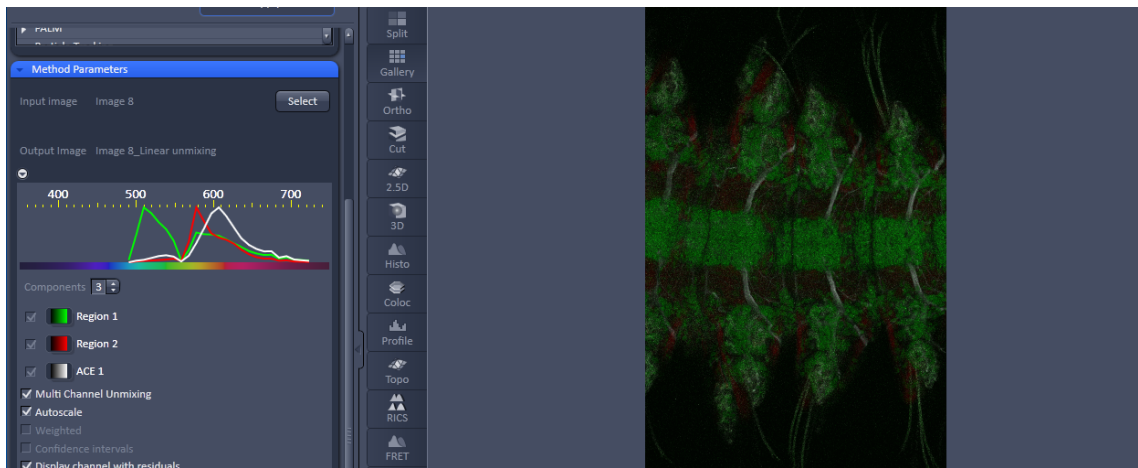
Fig. 275   Image Display window after unmixing

Display channels with statistical confidence:

This option displays an additional channel per unmixed component which shows the relative statistical error in each unmixed component channel. This statistical uncertainty of the pixel intensity in the unmixed channel is calculated based on the (Poisson-) noise from the acquired (input-) channels, the bandwidth and –position and the quality of the reference spectra. The display can be interpreted best when an appropriate false color LUT is chosen. The error is displayed as percentage of unmixed channel pixel intensity. For example: A "confidence value", i.e., pixel intensity of 50 represents a 50% statistical uncertainty of the corresponding intensity in the unmixed channel. The higher the statistical error for the acquired pixel intensity (the shot noise), the higher is the intensity value of this pixel.

Multichannel Unmixing:

When this option is chosen. the unmixing algorithm is applied to a multi-channel image (up to 10 channels) without the use of reference spectra.

The calculation of residuals and the subtraction of background based on a background spectrum are not available. For heavily overlapping signals this method will yield poor results.

☞ Avoid saturation of fluorescence signal in the data set to be unmixed. Saturation will generate a high signal in the residual channel. To get the best unmixing results, define an extra background channel, if possible.

☞ Reference Spectra used for Linear unmixing of the Lambda stacks processed in the Unmixing View are stored with the resulting image and can be re-loaded using the **Processing → Linear unmixing** tool with the Read parameters from image button. This facilitates processing of a series of images or Lambda stacks all with the same parameters / reference spectra.

5.3.9   Processing – Ion Concentration

Licensing		Instrument		
standard	✔ optional	✔ LSM	ELYRA	Lightsheet Z.1

Tool accepts single-channel or multi-channel, multi-dimensional data (typically time series data) of samples labeled with fluorescent ion indicators. *Ion Concentration* was developed for analyzing LSM data. Successful analysis of image data obtained with other systems is potentially possible as well (provided the input meets the requirements for intensity quantification). See full description of tool for details.

This Processing function permits the calibration of ion concentrations in physiological experiments.

To open the **Ion Concentration** tools click **Ion concentration** (Fig. 276).

The two Select buttons select the active image in the **Image Display** as **Input Image** and **Input Image 2** (for background subtraction). The name of the automatically generated Output Image is shown under the name of the Input Images.

Fig. 276   Processing – Ion concentration

In the **Preview** panel at the bottom of the Ion concentration tool window, thumbnails of the input images as well as a preview of the output image are displayed (Fig. 277).

After opening the **Method Parameters** panel full window by clicking the little arrow, the full tool window opens and the following functions are available (Fig. 278).
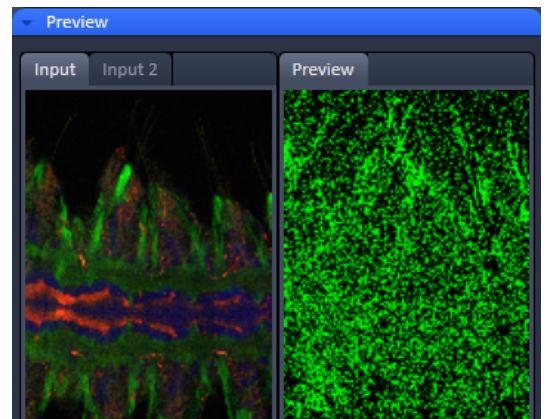
Fig. 277   Ion concentration panel

⚪ Calibration graph	Opens the display for the resulting calibration curve.
Load ... Save ... buttons	To load & save calibration curves from / to files.
✔ Background (Input 2) tick box	To select Input Image 2 as background image.
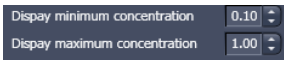
Spin boxes to set the Min and Max in the Output Image

Image scaling

Pull down menus for
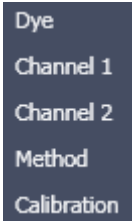
To set three different calibration options, according to the dyes (single wavelength, vs ratiometric), the calibration method and if the calibration is carried out in vitro/situ. Ch 1 and 2 select the channels from Input Image 1.
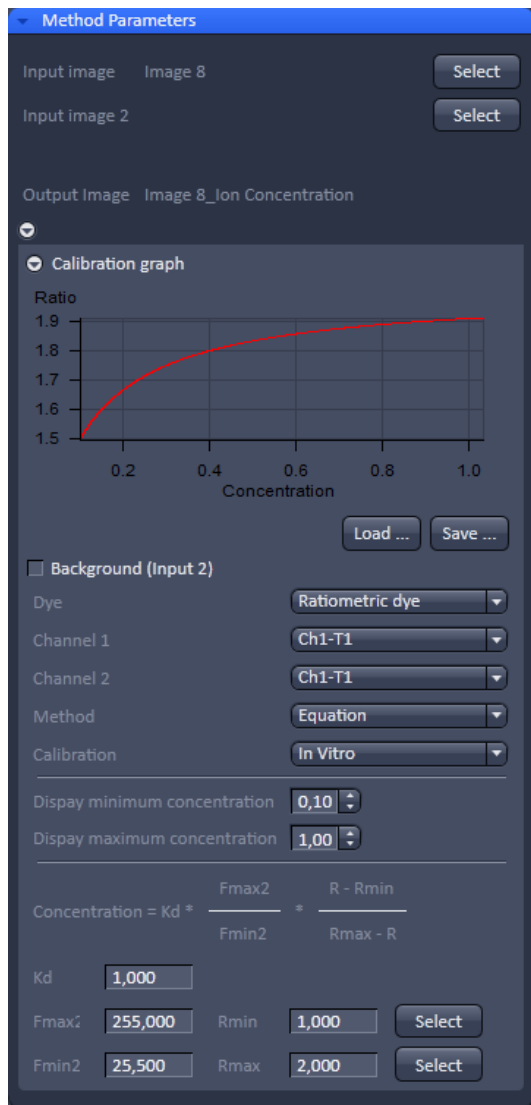
From these pull downs, the following combinations of parameters can be chosen:

Dye: single wavelength vs. ratiometric

Available methods for single wavelength: Titration or equation (both, **in vitro** and **in situ calibration**).

Available methods for **ratiometric** dyes: **Ratio**, **titration**, **equation**, and **unmixing**.

$$Concentration = Kd * \frac{Fmax2}{Fmin2} * \frac{R - Rmin}{Rmax - R}$$

Fig. 278 **Fully opened Ion Concentration tool window - Ratiometric dye in equation mode**

5.3.9.1 Single Wavelength Dyes – Offline Calibration

- Subtract background/ autofluorescence image from raw images to obtain better raw data to start with (Fig. 279).

- Perform equation or titration calibration (compare F with a calibration curve → titration calibration or put F values in calibration formula).
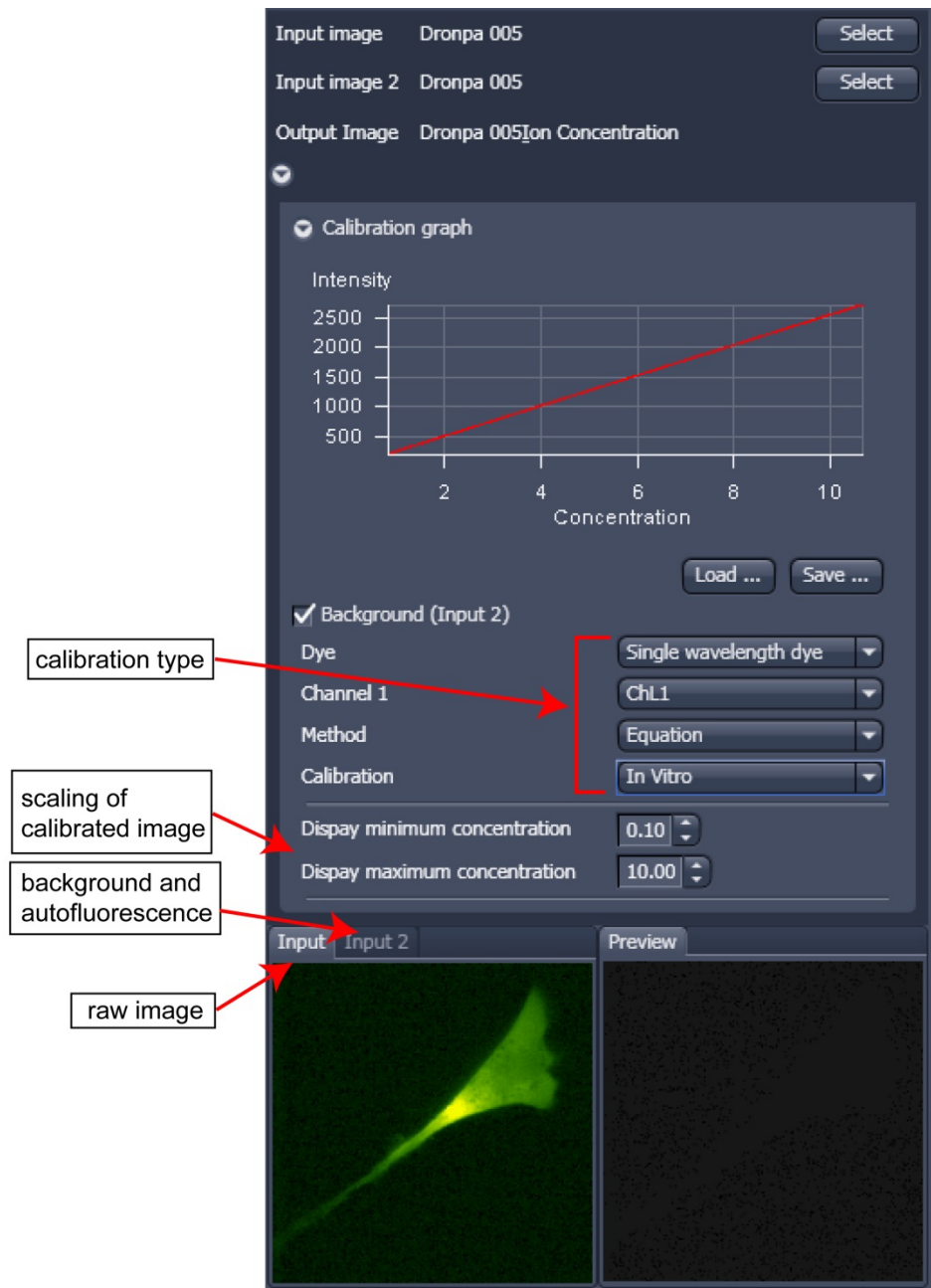
Fig. 279   Ion concentration panel

5.3.9.2   Ratiometric Dyes

- Fura-2, Indo-, SNARF, Cameleon, Ratiometric Pericam, Phluorin.

- Display fluorescence ratio R over time

- Display fluorescence ratio R corrected for background/autofluorescence over time

- Calculate absolute ion concentrations (pixel by pixel) via titration calibration (known ion concentrations applied to the cells – in situ – or in solutions – in vitro or equation calibration where possible [Fura-2, Indo-, SNARF]

- Calculation of R eliminates artifacts and uncertainties caused by

  – inhomogeneous dye distribution

  – photobleaching

  – may be applied with moving cells.

Ratiometric Dyes - Online Ratio (Fig. 280)

R(t1) = F1(t1) / F2(t1), R(t2) = F1(t2) / F2(t2) .
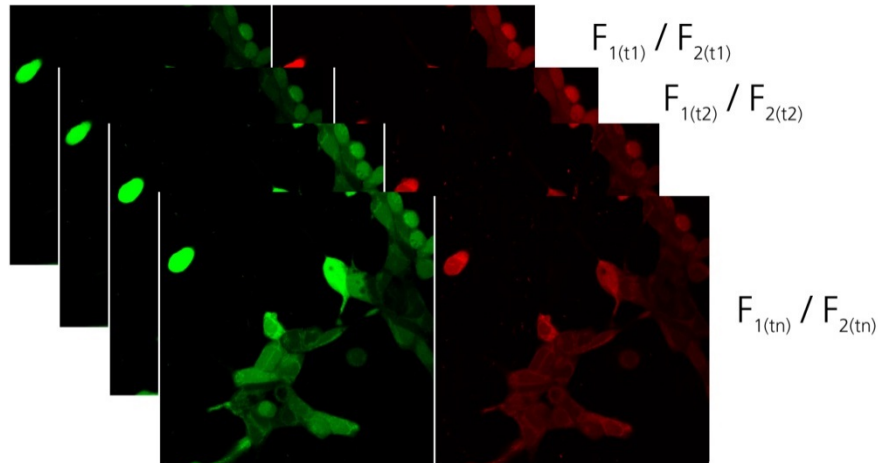
Fig. 280   **Ratiometric Dyes – online ratio**

Ratiometric Dyes - Calibration (Fig. 281)

- Subtract background/autofluorescence images from raw images to obtain

  R_{korr} [=F_1-$F_{1Background}$)/(F_2-$F_{2Background}$)]

  when calibration reference is not obtained with the experimental sample (in situ).

- Calculate ratio R.

- Perform equation- or titration calibration (compare R with a calibration curve → titration calibration or put R values in calibration formula).

Fig. 281   Ion concentration panel

Fig. 282 Ion concentration - Ratiometric dye, Equation Calibration

Ratiometric Dyes - Equation Calibration (Grynkiewicz) (Fig. 282)

Fura-2, Indo-1,

K_D (dissociation constant) taken from literature

R_{min}: derived from ion-free state of the dye (e.g. 0 Ca^{2+})

R_{max}: derived from ion-bound state of the dye (e.g. saturated with Ca^{2+})

F_{min2} and F_{max2} are the minimum and maximum fluorescence intensities at wavelength 2

R_{min}, R_{max}, F_{min2} and F_{max2} may be determined in the cells under investigation (in situ) or in solutions (in vitro)

Calibration parameters may be saved and reloaded (*.cal)

Options for Calibration Image Selection (Equation- or Titration Calibration)

- Click into image window.
- Select source channel(s).
- Optional background subtraction
- Optional calculation of parameters from overlay region(s)

5.3.10   Processing – Correlation

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1
Tool accepts potentially all types of image files. See full description of tool for details.				

- Activate **Correlation** in the **Processing** tool. The **Correlation** menu will appear (Fig. 283).

The **Correlation** processing function will display the spatial (only available in the **LSM imaging** mode) or temporal correlation of an image or image stack.

- To select an image, highlight the image in the image container and press the **Select** button. This will be your **Input** image.

As an output image the correlation image is computed and the result presented in the **Preview** window.

- You can select which kind of correlation you want to perform by activating the **X**, **Y**, **Z**, and **Time** check boxes to perform spatial correlations in x, y, and z-direction or a temporal correlation.

☞ Note **Z** and **Time** check boxes are only available for Z-Stacks or time series stacks.

- If the **Apply** button is pressed, the correlation image will be opened as a new image in the container.
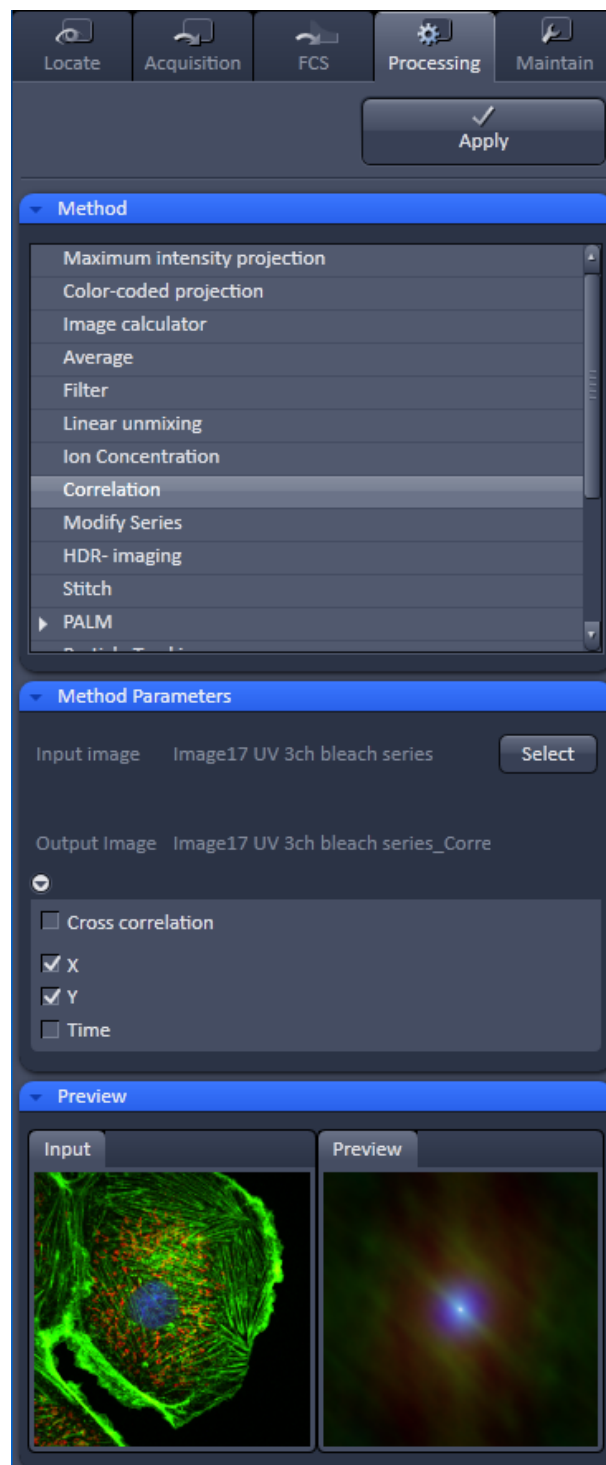
Fig. 283   Processing – Correlation

5.3.11 Processing – Modify Series

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1
Tool accepts single image files (for concatenation) and multi-dimensional image series (typically time series and Z-stacks). See full description of tool for details.				

This function allows modifying dimensions of images or image series (Fig. 284).

Depending on the input image (for example **Z-Stack** or **Time Series**) the available modifications vary, which are available in the Modify mode drop down menu (Fig. 285 and Fig. 286).

It allows modifying the acquisition date, rotating or mirroring the image(s), converting or reversing dimensions, renaming channels or making time series from Z-Stacks, tiles, or images from various positions. Furthermore it allows to concatenate time series or Z-stacks when two input files are selected.

☞ Do not apply the Modify Series function to raw image data from ELYRA or Airyscan – it might not be possible to process them any more. Do also not use the functions on SMLM images that have been processed already – some image dimensions will be lost which will render this images unusable.

Fig. 285 Modify mode drop down menu – Z-Stack options
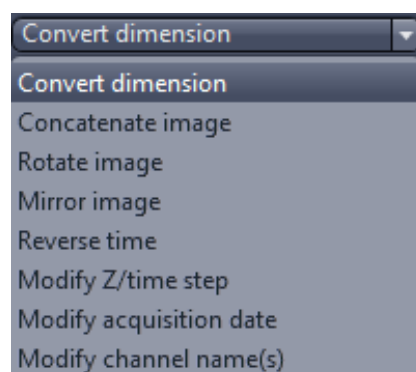
Fig. 286 Modify mode drop down menu – Time series options

Fig. 284 Processing – Modify series

5.3.12   Processing – HDR-imaging

Licensing		Instrument		
standard	✔ optional	✔ LSM	ELYRA	Lightsheet Z.1

Tool accepts image data recorded with the optional feature "HDR" of the acquisition tool *Acquisition Mode*. In addition, input acquired either without using the feature "HDR" or on instruments other than LSM systems is potentially suitable for this processing tool as well (provided the input meets the requirements for HDR-imaging). See full description of tool for details.

Please see also the description of the HDR (High Dynamic Range) function (part of the Acquisition Mode tool) for details on HDR image recording with LSM systems.

HDR creates an image with a depth of 32-bit and therefore covers a far broader dynamic range than conventional images (8 to 16 bit).
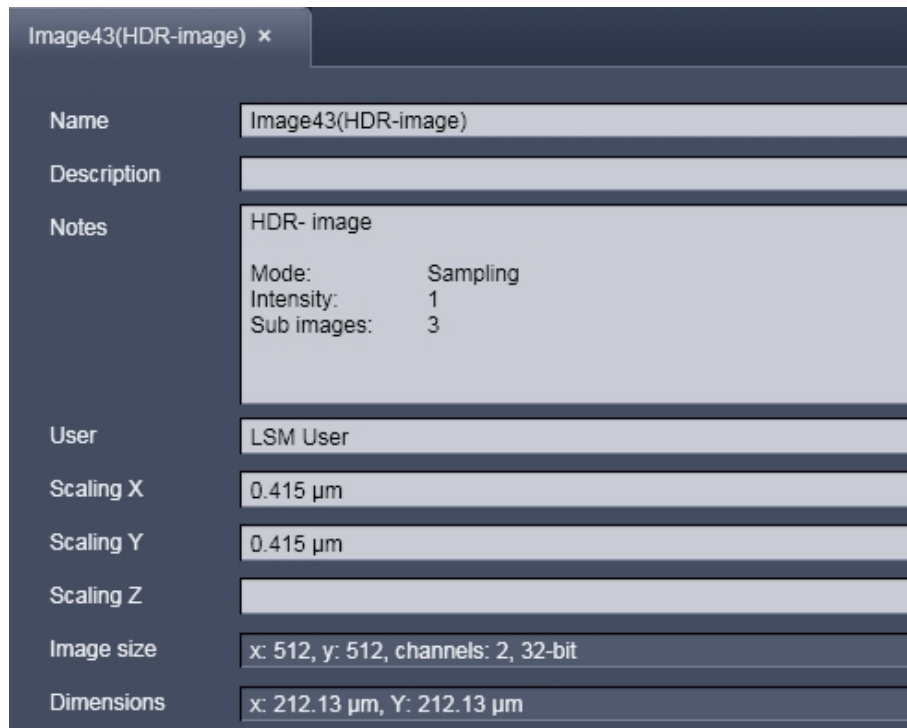
Fig. 287   Image info of a HDR image

As a result of the HDR process, the grey scale is independent from the initial bit depth, but covers a range from 0-1.

Also, the grey stepping is much finer, with much better visibility of weak structures (Fig. 288).

The HDR method employed is also shown in the image info (Fig. 287).
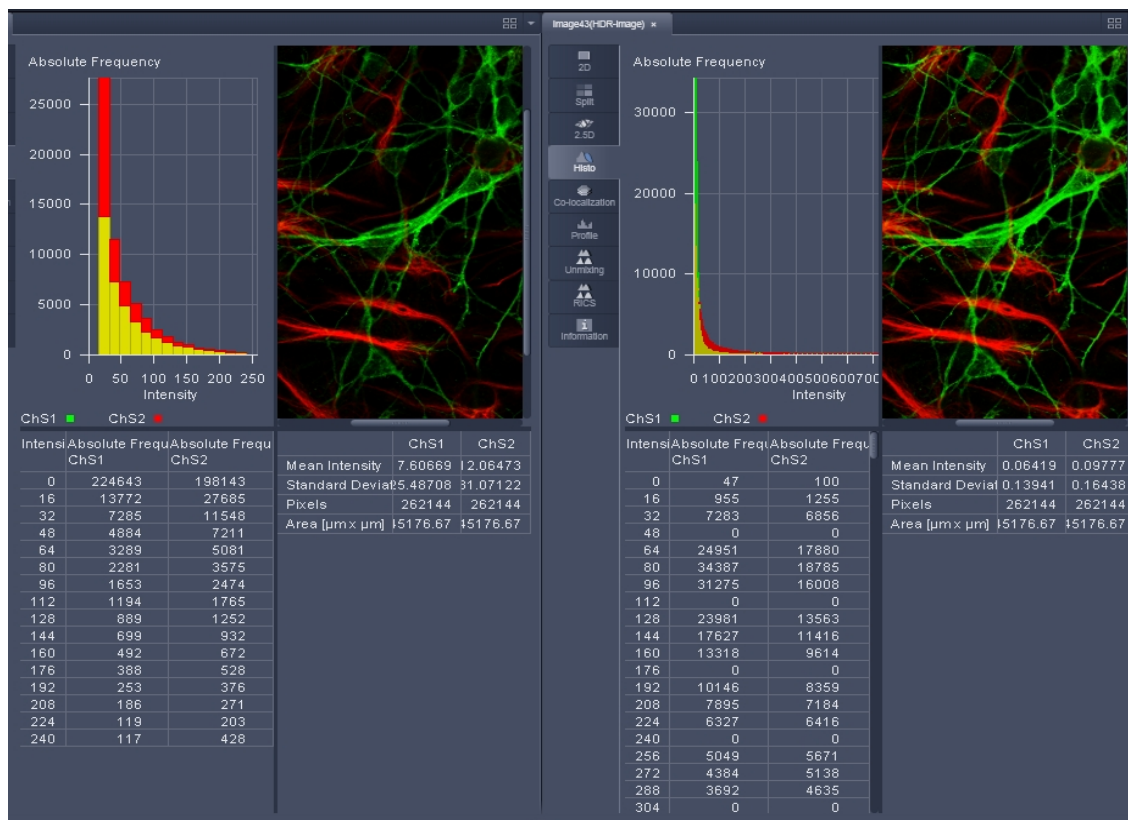
Fig. 288   HDR image display (left side without, right with HDR)

The **Sampling** mode is a meant to improve the image display as it equalizes the differences between the very high and the very low intensities in the image. However, it is not suitable for quantitative analysis as the level steps become non-linear.

The image re-calculation works already with a single image (**Frame** = 1) and is done in the following way taking **Intensity** 3.

Image x 1; cut off of grey levels above the dynamic range; Result divided by 1

Image x 2; cut off of grey levels above the dynamic range; Result divided by 2

Image x 3; cut off of grey levels above the dynamic range; Result divided by 3

The three resulting images are summed up and again divided by 3. This procedure is adapted accordingly if a higher **Intensity** number is set. If two images are **acquired** (**Frame** = 2) and Intensity is still at 3, the second image is rated lower and is multiplied and divided in three steps with the value 4,5 and 6; The then 6 resulting images are summed up and divided by 6.

The image acquisition parameters are not changed for the acquisition of the two (or more) **Frames**.

After acquisition, the grey dynamics of the image are automatically linearized (see description above) to match the range of the output display. This step visibly intensifies weak signals (Fig. 289).
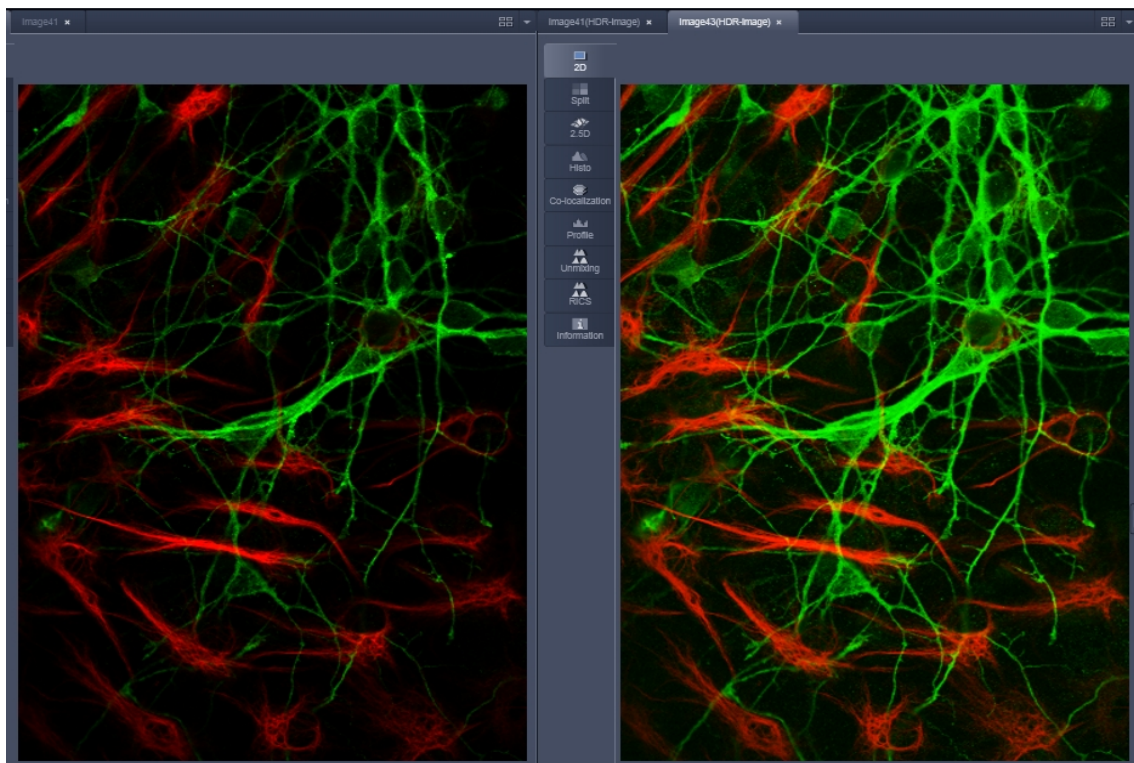
Fig. 289   HDR image display using Sampling mode (left side without, right with HDR)

Unlike the **Sampling** method, the **Illumination** method of HDR still provides a linear grey stepping which allows quantitative measurements. For the acquisition step with increased laser power, those regions of the image are excluded from exposures when no reasonable data can be obtained with any higher laser power.
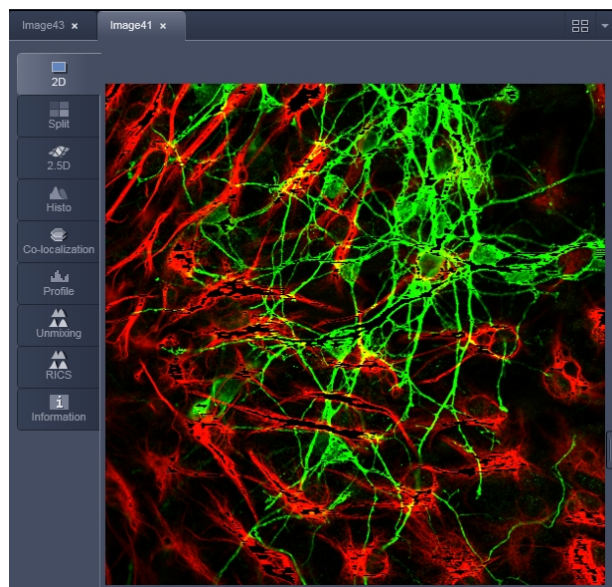
Fig. 290   HDR image display after using Illumination mode

During data recording with the **Illumination** method, masks are applied to protect the sample from laser irradiation. The respective ROIs are displayed in an additional image (Fig. 290).

The calculation of the resulting image follows the same procedures as for the sampling mode. However, the regions, which are excluded from a second and/or third illumination step are divided by 2 instead of 3.

After acquisition, the grey dynamics of the resulting image are not automatically compressed to match the range of the output display.

Use the **Gamma** and **Contrast** sliders of the image display (Fig. 291) to adjust the image.

For manual adjustment, a result can be achieved which fits the individual requirements, without affecting the actual raw data stored with the image.
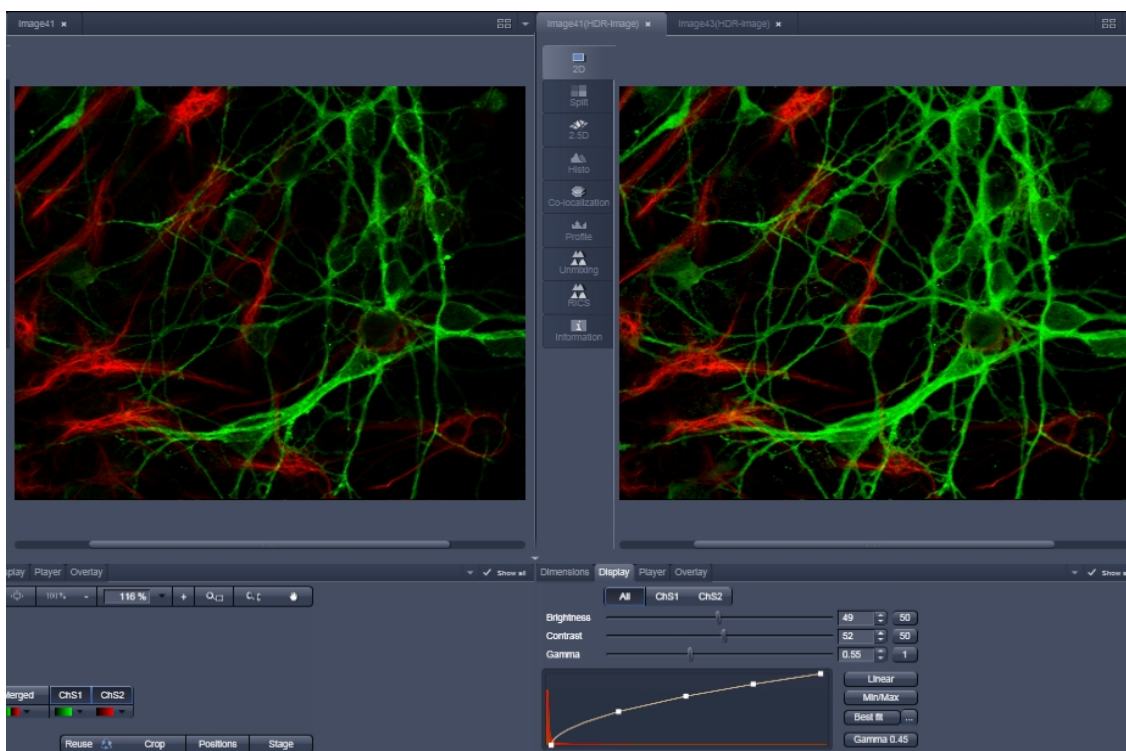
Fig. 291   Image adjustment of the HDR image (left side without, right with HDR)

5.3.13   Processing – Stitch

Licensing		Instrument		
standard	✔ optional	✔ LSM	✔ ELYRA	Lightsheet Z.1
Tool accepts image data recorded data recorded with the acquisition tool *Tile Scan* (incl. combinations with other multi-dimensional acquisition tools). Images recorded in SIM mode on ELYRA (SIM data) must be processed with the tool *Structured Illumination* before stitching. For optimal results, image data should have some degree of lateral overlap. See full description of tool for details.				

With this function tiled images acquired with Versions of ZEN 2012 and upwards and the tiling function can be stitched together in 2D and 3D.

Select a tiled image (or stack) with the **Select** button (Fig. 292).

The **Correlation Threshold** is defining the accuracy with which the algorithm detects similarities in the adjacent image planes.

If a 3D tile scan image has been acquired (Tile Scan combined with Z-Stack) then additional functions for the stitching procedure are available.

Fig. 292   Processing – Stitch

Ignore Z Correction will in some cases reveal better results for the 3D stitch. Depending on the image data the last step of the stitch algorithm, which makes a correction of the Z-Stacks in Z looking at the overall image, can lead to unwanted shifts of the stacks in Z. This can be avoided when this option is checked.

5.3.14 Processing – Localization Microscopy Tools

Licensing		Instrument		
standard ✔ optional		LSM	✔ ELYRA	Lightsheet Z.1
Tool exclusively accepts SMLM data. See full description of tool set for details.				
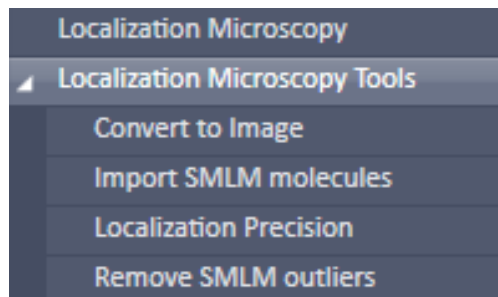
Fig. 293   **Processing Localization Microscopy**

The **Processing Localization Miscroscopy** tools (Fig. 293) is applied to data acquired in the **SMLM imaging** mode. It subdivides in 4 subcategories.

- **Convert to Image**: Converts the table (vector map) of a SMLM image to a bona fide image.

- **Import SMLM molecules**: Import function for molecule tables

- **Localization Precision**: Attaches to an Experimental PSF a localization precision look up table based on simulations.

- **Remove SMLM outliers**: Filter to select for molecules surrounded by a threshold number of other molecules

5.3.14.1   Localization Microscopy Tools – Convert to Image

The **Convert to Image** function allows converting the vector map of centroids to be transformed to a bona fide image.

Select the **Convert to Image** tab to select the **Convert to Image** processing tool (Fig. 294).

Highlight the SMLM image in the image container you want to convert and press **Select** (Fig. 295). Press **Apply** to convert the HR channel to a true image.

The new file has the extension "name_SMLM-ConverttoImage" as a default.

It contains the **SR** channel and **SWF** channel as grey value pixel images for 2D-SMLM. For 3D-SMLM it will contain the Z-Stack SR image file; no SWF image is available for 3D-SMLM.
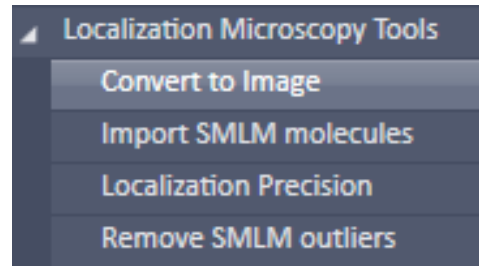
Fig. 294   Processing – Convert to Image tool selected
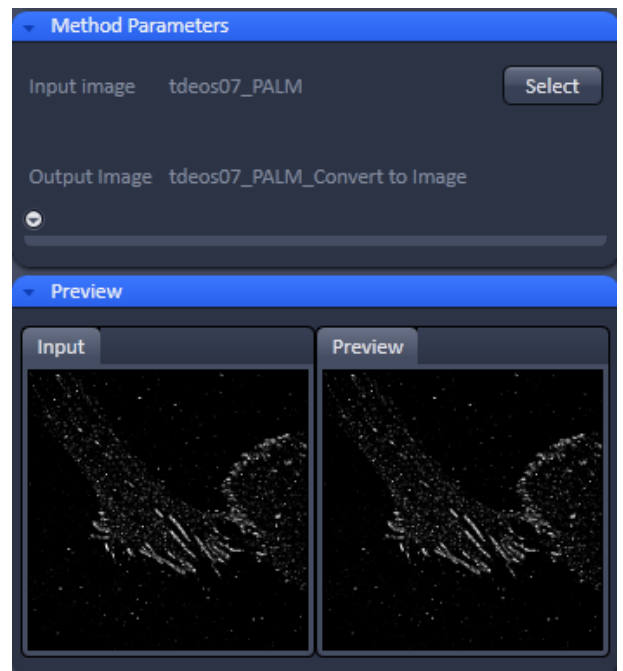
Fig. 295   Processing – Convert to image
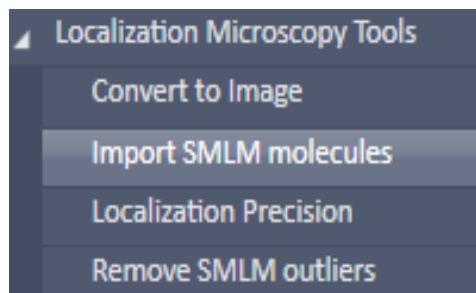
Fig. 296 Processing – Import SMLM molecules tool selected

Fig. 297 Processing – Import SMLM molecules

5.3.14.2 Localization Microscopy Tools – Import SMLM Molecules

The **Import SMLM molecules** function allows importing SMLM molecule tables saved as text files (extension .txt) and reconstruct a SMLM image from the table.

Select the **Import SMLM molecules** tab to select the **Import SMLM molecules** processing tool (Fig. 296).

Press **Select** (Fig. 297) to open **Windows Explorer** and select the molecule table you want to import. Press **Open** to load the table and then press **Apply** to create the SMLM and sum wide fied images (Fig. 298).

☞ There are no tools available if the **Methods Parameters** tool area is expanded.
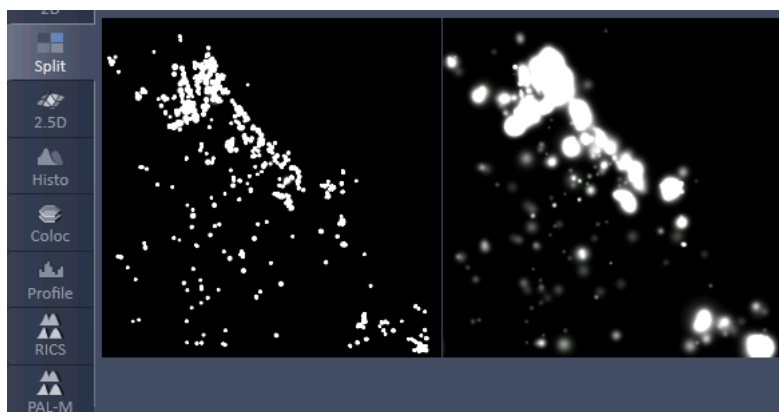
Fig. 298 SMLM image (left) and sum widefield image (right) in the Split mode after importing a molecule table

5.3.14.3 Localization Microscopy Tools – Localization Precision

Select **Localization Precision** to attach a Localization Precision look up table (LUT) to an Experimental PSF (Fig. 299).

Highlight the Image with the Experimental PSF (should be derived from the PSFs obtained by a 4 µm stack of 100 nm beads) and press **Select** (Fig. 300).

Press apply to obtain a file of the Experimental PSF with LUT attached. The file bears the extension "LocalizationPrecision".

☞ Please note that the localization precision will be estimated using a Monte-Carlo Simulation of the experimental PSF including noise. The generated lookup table will then contain the localization precision depending on the three parameters photon number, background variance and Z position.

☞ The Experimental PSF with attache LUT should be used when fitting 3D SMLM data. If only an Experimental PSF without LUT is used, a warning message pops up (Fig. 301). Press **No** to cancel processing or **Yes** if you want to continue. In this case, only a rough estimate of the localiszation precision will be given.
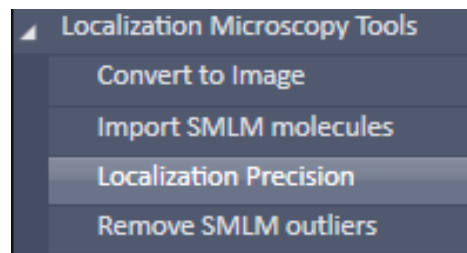
Fig. 299   Processing – Localization Precision tool selected
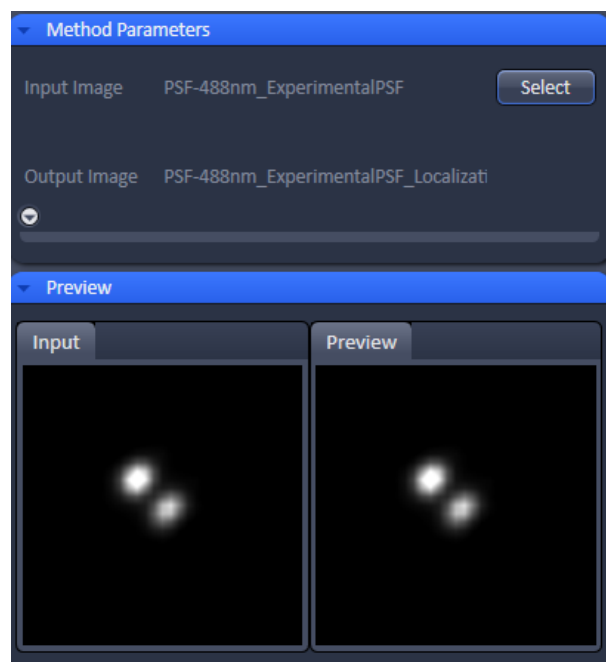
Fig. 300   Processing – SMLM - Localization Precision with double-lobed Experimental PSF loaded
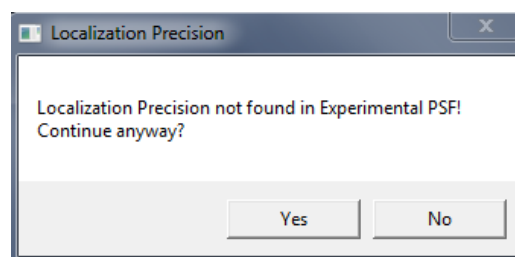
Fig. 301   Localization precision warning
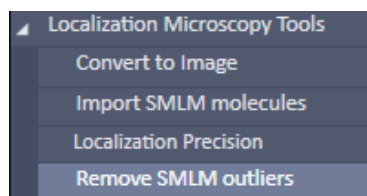
Fig. 302 Processing – Remove SMLM outliers tool selected
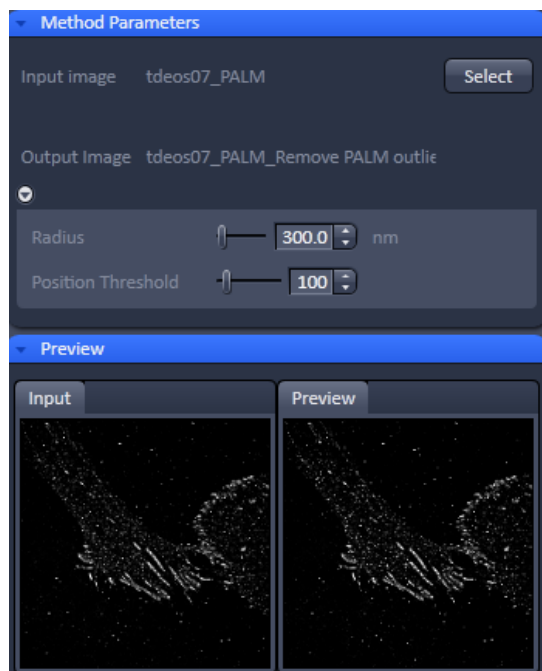
Fig. 303 Processing – SMLM Remove SMLM outliers
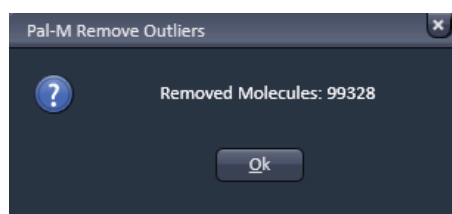
Fig. 304 Processing – SMLM Remove Outliers message box
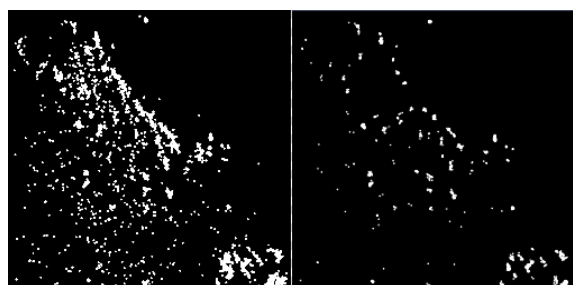
Fig. 305 SMLM images before (left) and after (left) removal of outliers; Radius set to 3000 and Position Threshold to 100. Only those peaks / molecules in denser structures remain.

5.3.14.4 Localization Microscopy Tools – Remove SMLM Outliers

The **Remove SMLM outliers** function allows removing all molecules that within a defined radius will have less than the specified numbers of neighboring molecules.

Select the **Remove SMLM outliers** tab to select the **Remove SMLM outliers** processing tool (Fig. 302). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to the filter functions (Fig. 303).

You can set two parameters by using the sliders or the spin input boxes:

Radius [nm]. The radius defines the distance from a localized position of a peak (molecule) in which all peaks falling within that distance are counted.

Position threshold. If the number of counted neighboring peaks (molecules) falls short of the set value, the respective peak (molecule) will be removed from the list and the SMLM image.

☞ This tool will emphasize densely labeled structures and can help remove floating molecules and ghost signals.

☞ This tool works only for SMLM images.

After setting the thresholds, press **Apply** to create the new image. A message will be displayed telling how many molecules have been removed from the input SMLM image (Fig. 304).

Press **Ok** to have access to the newly computed image (Fig. 305).

☞ Please note that for 3D-SMLM removing outliers applies only in 2D for a maximum intensity projection of the Z-Stack. Removing outliers does not apply for the z direction.

5.3.15 Processing – Localization Microscopy

The **Localization Microscopy** function allows setting filters for peak finding and choosing a fit model for the localization calculations.

Select the **Localization Microscopy** tab to select the **Localization Microscopy** Processing tool (Fig. 306). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to the **Peak Finder** and **Localizer** (Fig. 307).

Pressing the **Settings Default** button will reset all filter and parameter settings to the default values.

Pressing the **Settings Reuse** button will recall the parameter settings of the actively selected image.

The **Channel** drop down menu allows you in a multi-channel experiment to apply different settings to different channels, which are all listed (Fig. 308). If you select **All**, all channels will be fitted the same way. If you select a channel, and change settings, these setting will apply only to this channel. Note, even if only one channel is selected, every channel will be fitted according to the last settings.

The **Settings** drop down menu (Fig. 309) will allow you to define how peak events will be fitted. You have three options:

– **Discard overlapping molecules** (single emitter): only single emitters will be taken into account and fitted, whereas all multi-emitter events will be discarded.

– **Ignore overlap** (ignore emitter): all single- and multi-emitter events will be taken into account and fitted as single emitter events.
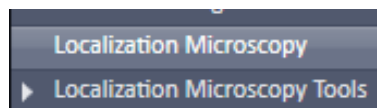
Fig. 306   Processing – Localization Microscopy
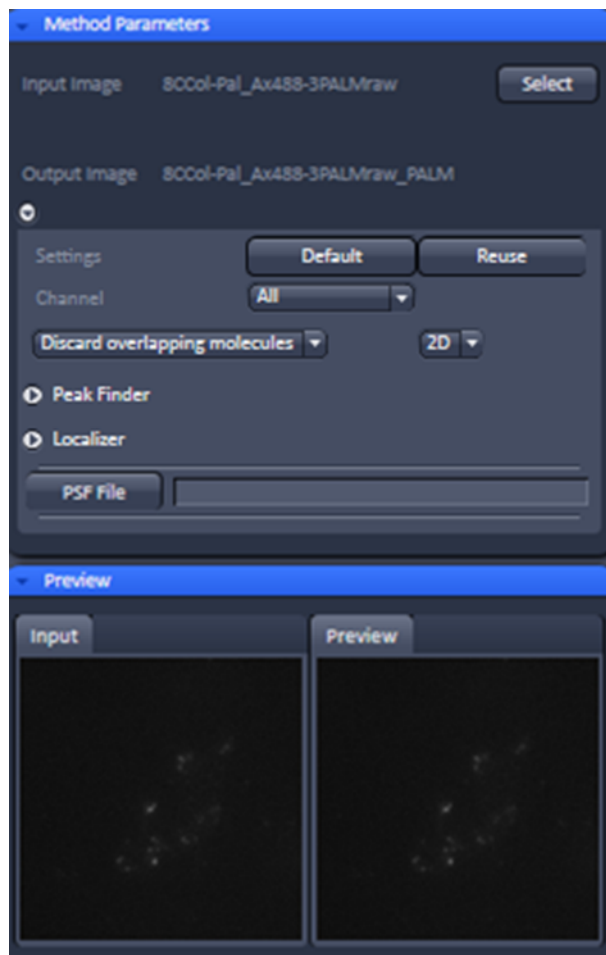
Fig. 307   Processing – SMLM – SMLM
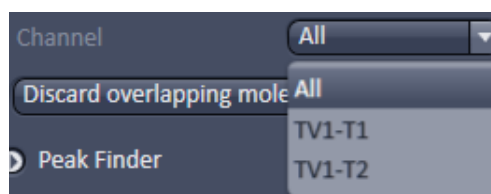
Fig. 308   Channel drop down menu of a dual channel data set
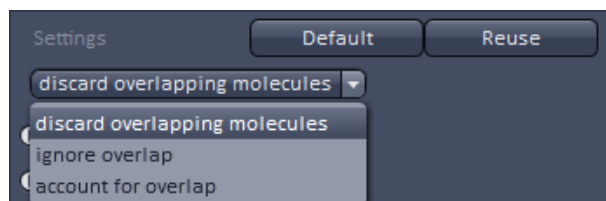
Fig. 309   Settings drop dpwn menu

Fig. 310   **Maximum cluster size field**

Fig. 311   **Dimension drop down menu**

Fig. 312   **Peak Finder Expansion field**

Fig. 313   **Double lobed PSF created by a double phase-ramp in the pupil plane**

– **Account for overlap** (multi-emitter): multi-emitter events will be fitted taking into account the overlap. If selected, the **Maximum cluster size** slider and input box become available (Fig. 310). Here you can select the maximum number of molecules in the cluster that are used for computation. So a number of 10 means, for example, that clusters with 10 or less peaks will be taken, and those exceeding 10 will be discarded. We recommend to limit custer size to 10 as otherwise computation can become time consuming.

White circles indicate that the particular emitters are used (if channel color is grey; otherwise the channel color is used), red circles that the emitters are discarded for the fitting process. For account of overlap the color stays white or in the channel color regardless if a cluster may be discarded due to the **Maximum cluster size** setting.

The **Dimension** drop down menu will display the dimensionality (2D or 3D) of the selected SMLM image, which is automatically recognized if a .czi format is loaded (Fig. 311). It is possible to overrule the automatically chosen value.

In the **Channel** drop down menu you can select the channel for which the Processing should be conducted for a multi-channel image.

Expand the **Peak Finder** field to have access to the peak selection filters (Fig. 312). Two filters are available:

– **Peak mask size**

– **Peak intensity to noise**

Use the sliders or input spin boxes to set the corresponding values.

☞ We recommend to start with the default values for the peak mask size (9 for 2D; 19 for 3D data) and then optimize on behalf of the results. Note that in 3D-SMLM using PRLIM (phase ramp localization imaging microscopy) with a double phase ramp, the PSF is split into two lobes (Fig. 313) that have a larger expansion than the classical PSF.
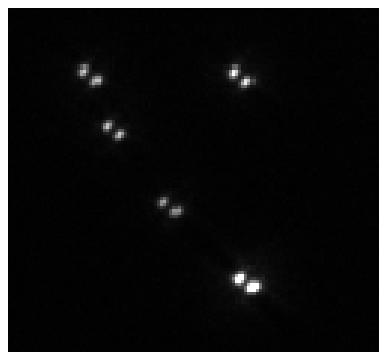
The **Peak mask size** filter defines the radius in **pixel** units for a detected peak intensity that will be used to cut out the peak and put the peak into a new coordinate system. The size will also be used when fitting a 2D Gauss function to the intensity distribution of the peak.

If the image was selected by pressing the **Select** button, the **Preview** window of the Image in the image container will show all identified peaks circled (Fig. 314). The radius of the circles will correspond to the set value of the **Peak mask size** (Fig. 315).
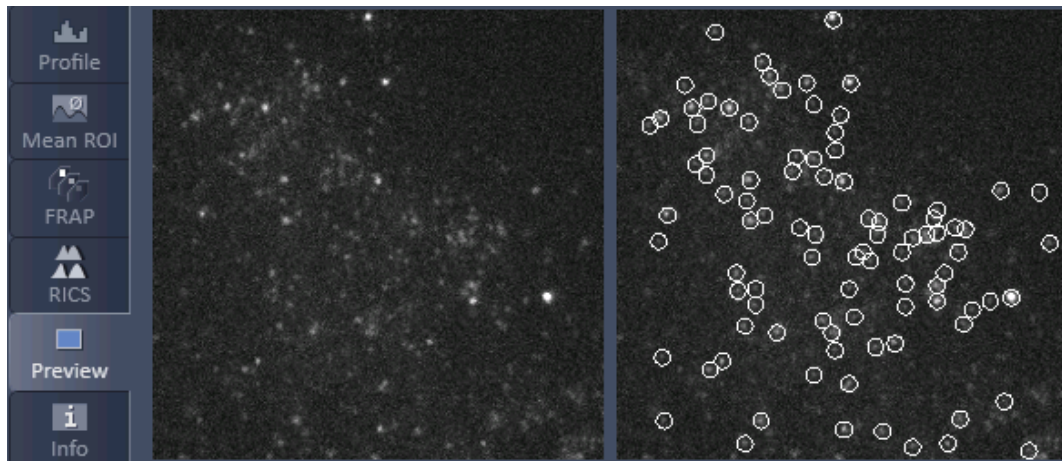
Fig. 314   Preview window of the image container for SMLM processing
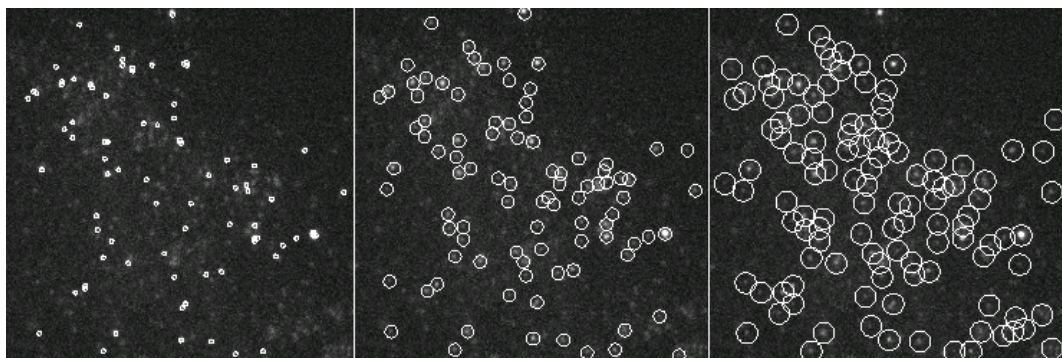
Fig. 315   Preview window of the image container for SMLM processing with the Peak mask size set to 3 (left), 9 (middle) and 15 (right) pixels

☞   The bigger the radius the more prone are closely juxtaposed peaks to overlap. The smaller the circles the more likely it is that one peak with a broad intensity distribution will be taken as two or multiple peaks. The preview can be taken to decide what the best radius will be. 9 and 19 pixels will work in > 95% of the cases for 2D and 3D SMLM experiments, respectively.

☞   If a circle around a peak lying at the borders of the image does not fit completely within the image, the peak is discarded.

Fig. 316 Preview window of the image container for SMLM processing with the discard molecules (A), ignore overlap (B) and account for overlap (C) selected

If the **discard overlapping molecules** was selected from the **Settings** drop down menu, all identified peaks with overlapping circles will be indicated in red and discarded (Fig. 316). If **ignore overlap** or **account for overlap** was selected, overlapping peaks will not be indicated and all peaks will be circled in white.

– If **discard overlapping molecules** is selected, all overlapping peaks, indicated by red circles will be discarded. This will result in the highest accuracy, which is defined as the deviation of the fitted to the real peak position and localization precision (the standard deviation from the fit) (Fig. 317, **A**). This method is therefore the method of choice for single emitters and little overlap.

– If **ignore overlap** is selected, all single and overlapping peaks will be taken into account. However, fit accuracy will be decreased when multi-emitter events are fitted as the distribution of one emitter will influence the distribution of an overlapping one without this fact taken into account. Hence accuracy might suffer for multi-emitter events whereas localization precision will be sustained (Fig. 317, **B**). This method is most suitable for slightly overlapping molecules.

– If **account for overlap** is selected all single and multi-emitter events will be taken into account. For multi-emitters the overlap of neighboring molecules is taken into account so losses in accuracy are minimized. There will be a slight decrease in the localization precision (Fig. 317, **C**). This method should be used, when molecules heavily overlap.
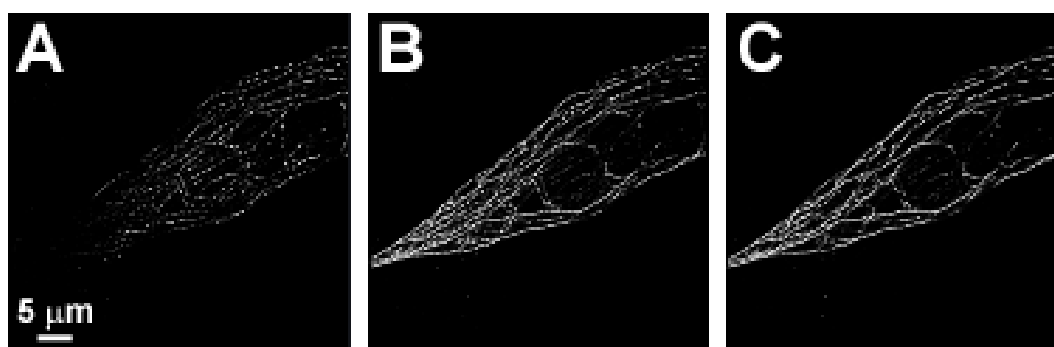
Fig. 317 Analyzed data set of Fig. 316 using discard overlapping molecules (A), ignore overlap (B) and account for overlap (C).

The **Peak intensity to noise** filter defines the threshold of the signal strength of an identified peak over background, at which the peak is regarded to be genuine. The filter works as follows:

- The highest intensity peak with the intensity I is identified in the frame
- The mean intensity M and the standard deviation SD is calculated from the frame
- The Peak intensity to noise (PI) is set.
- If $I - M > SD \times PI$ than the peak is regarded to be genuine
- The peak is cut out of the frame and the next highest intensity peak is selected.
- The procedure is repeated until $I - M < SD \times PI$. Than the peak finding process will end for that frame.

All identified peaks above the threshold will be displayed per frame in the Preview window (Fig. 318).

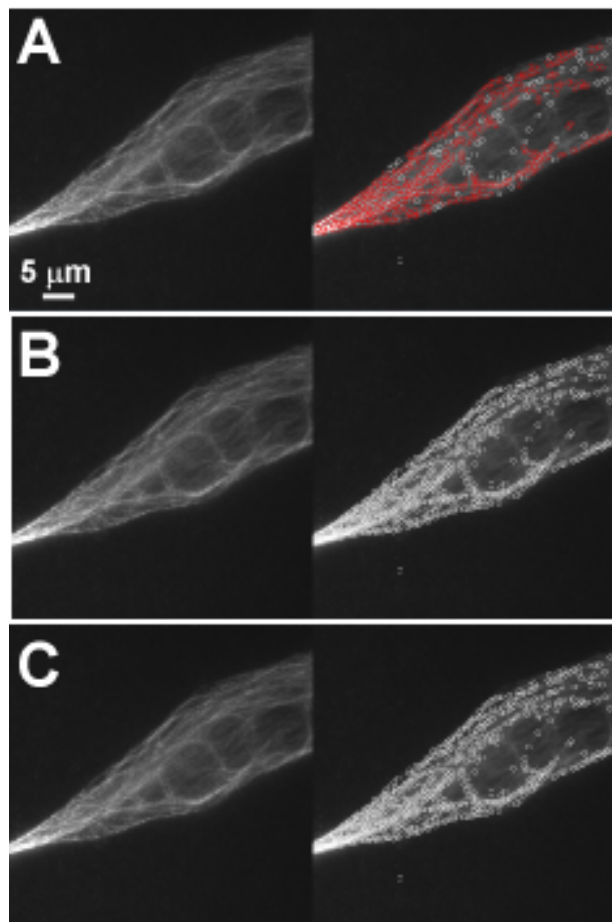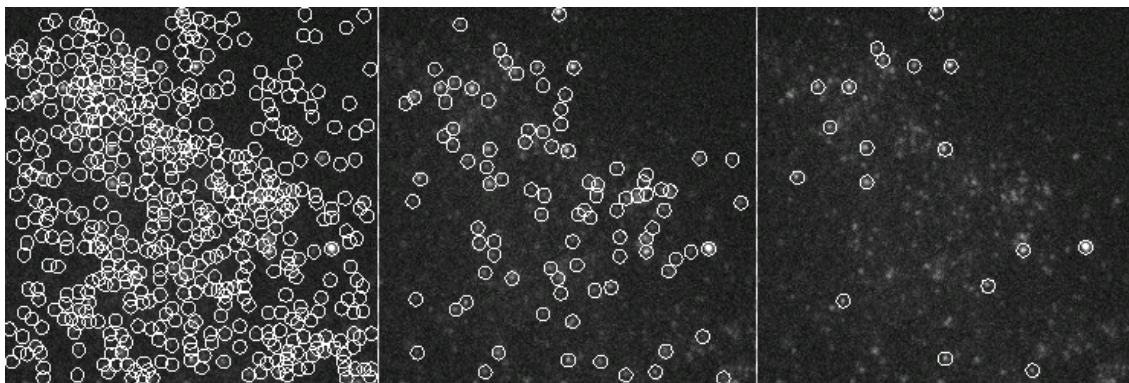**Fig. 318**   **Preview window of the image container for SMLM processing with the Peak intensity to noise set to 3 (left), 6 (middle) and 10 (right)**

☞ The higher the **Peak intensity to noise** factor, the more stringent is the peak selection. Too low numbers will result in noise being falsely identified as peaks; too high numbers will miss some genuine peaks. The preview can be taken to decide what the best value will be for the particular image series. The default value of 6 will work in > 95% of the cases.
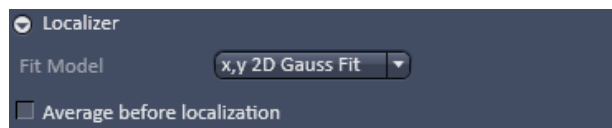
Fig. 319 Localizer Expansion field with x,y 2D Gauss Fit selected
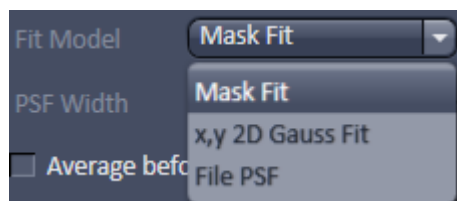
Fig. 320 Fit Model drop down menu
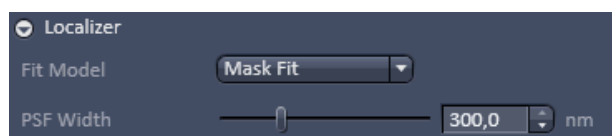
Fig. 321 Localizer Expansion field with Mask Fit selected
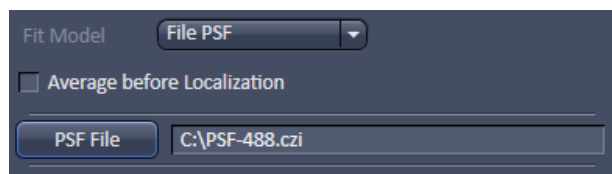
Fig. 322 File PSF menu

Expand the **Localizer** field to have access to the fit model used to localize peaks (Fig. 319). Three fit models are available that can be selected by the **Fit Model** drop down menu (Fig. 320) if 2D is selected from the Dimension drop down menu:

- **x,y 2D Gauss Fit**
- **Mask fit**
- **File PSF**

If **3D** is selected from the **Dimension** drop down menu, only **File PSF** is available. You should load an Experimenatl PSF with linked look up table for localization precision in order to obtain localization precisions in z in addition to x and y.

If **x,y 2D Gauss Fit** is selected, the intensity distributions of the identified peaks are fitted to a full 2D Gaussian function. The fit in each direction takes into account the influence of the other direction (x,y 2D Gauss).

If **Mask Fit** is selected, a Gaussian mask with a defined PSF width is used to match as best as possible the intensity distribution of a peak in x and y (Fig. 321). The PSF width can be set via the PSF Width slider or input spin box. Default value is the theoretical calculated PSF based on the objective lens and laser line used.

☞ Note that with the **Mask Fit** only one predefined PSF width will be used. Computation of the Fit will be faster for the **Mask Fit**, but less precise.

If **File PSF** is selected, you can select a PSF that will be used (Fig. 322). To this end press the **PSF file** button. This will open Windows Explorer, where you can select an approriate file, the name of which will be displayed in the **PSF file** display box.

☞ The fit model in 2D defines the PSF width, which will be used to fit to the experimental results. It does not correspond to the size of the peak that is defined by the Peak Mask Size and that in the end will define the overlapping degree of neighboring peaks. In 3D the PSF with the attached look up table is a simulation where the PSF was modulated.

☞ The settings of the **Localizer** will not influence the peak finding process and hence the preview image.

☞ In the Processing **SMLM** function, peak finding and localization are always linked. If you want to just view localized peaks (molecules), you might select in the **SMLM-Render** tab of the SMLM image in the **Display** drop down menu **Centroids**.

If the **Average before Localization** check box remains inactive, every peak (event) will be localized independently and is regarded as being derived from one molecule. Later on, peaks can be grouped belonging to one molecule in the image container in the **SMLM-Grouping** tab of the image (**localization before grouping**). If the check box is ticked, however, the system will group peaks (events) based on the filter settings that become available (Fig. 323) before localizing the grouped peaks (**grouping before localization**).

Fig. 323   Localizer Expansion field with Average before localization selected

☞   When **localization before grouping** is performed, the single peaks are first localized and then the localizations averaged. When **grouping before localization** is performed, than the peaks are first averaged and then localized.

There will be two filter settings when one performs grouping before localization:

– Filters for **Drift Correction** based on fiducial markers (like gold particles).

– Filters for **Grouping** the peaks (events).

Fig. 324   Drift correction panel

Expand the **Drift Correction** field to have access to the **Drift Correction** tool (Fig. 324). If you want to perform a drift correction the **Drift Correction** check box must be ticked.

☞   Note that for lateral drift correction the system needs to identify at least one fiducial marker based on the **Drift Correction** filter settings. Fiducial markers could be gold particles deposited on the glass surface of the dish. If the system cannot find a fiducial, no drift correction will be performed and in the **SMLM-Drift** tab of the image no fiducials will be listed in the **Fiducial** table.

You can set two parameters to be associated with a fiducial by using the sliders or the spin input boxes:

– **Min on time [%].** This defines the minimal number of frames out of the total frames acquired in percentage the molecule has to be on in order to be regarded as a fiducial. E. g. 75% means that the molecule has to be on at least ¾ of all the frames.

– **Capture radius [pixel].** This defines the radius around a fiducial in which no other fiducial shoud be localized in order to be taken as a fiducial. E. g. if 2 pixels was set and within two pixels another fiducial is present, than both fiducials will be discarded.

☞   Note that the settings of the **Drift Correction** will not influence the **Preview** image.

☞   Note that using **Drift Correction** in the Processing tool is irreversible. Therefore, in the created image all drift related functions in the **SMLM-Drift** tab will be inaccessible and greyed out.

Fig. 325   Grouping panel

Expand the **Grouping** field to have access to the **Grouping** tool (Fig. 325).

You can set two filters to be taken into consideration when peaks (events) are grouped:

– **Off Gap.** If peaks within the defined **Capture Radius** will be interrupted by more than the set value of consecutive frames, they will not be regarded belonging to the same molecules.

– **Capture radius [pixel].** If peaks in consecutive frames are not localized within the specified radius they are not regarded as belonging to the same molecule.

☞ Note that the settings of the **Grouping** will not influence the **Preview** image.

☞ Note that using **Grouping** in the processing tool is irreversible. Therefore, in the created **SMLM-Grouping** tab all functions will be greyed out and not accessible.

If all parameters and filters are set press the **Apply** button. This will create the SMLM image in the image container and the **SMLM** view tab becomes available that allows further processing and analysis of the image. The container will display SMLM image (**HR** channel) as well as the sum widefield image (**SWF** channel – not available for 3D-SMLM) (Fig. 326).

☞ Note that in contrast to the TIRF image, which is the raw image of the point spread functions (PSFs), the sum widefield image is the background corrected image of the PSFs and the SMLM image is a vector map of centroids.
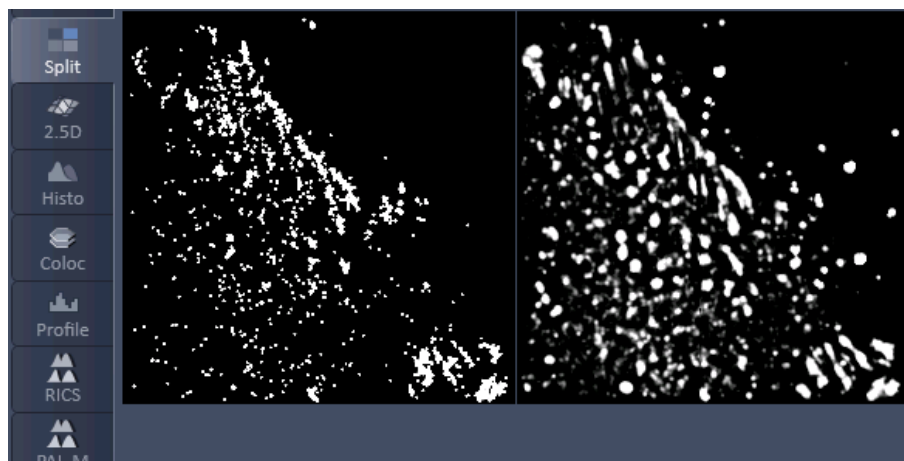
Fig. 326   SMLM image (left) and sum widefield image (right) after SMLM Processing

5.3.16   Processing – Particle Tracking

Licensing		Instrument		
standard	✓ optional	LSM	✓ ELYRA	Lightsheet Z.1
Tool was exclusively developed for tracking molecules or particles having a dot-like appearance in the image data.				

The Processing **Particle Tracking** tool allows the identification of single molecules and follows their movement. Trajectories will be displayed.

In the image you have than the possibility in the **Particle Tracking** tab to calculate the mean square displacement and diffusion coefficients of the single molecules.

Highlight the **Particle Tracking** field to have access to the **Particle Tracking** tool (Fig. 327).

Fig. 327   Processing – Particle Tracking tool selected

Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to the **Particle Tracking** tools (Fig. 328).

You can set different parameters to define the molecules and the calculation of the trajectories:

– **Peak intensity to noise.** Sets the signal over noise threshold according to I-M > SS x PI (I = intensity of particle; M = mean intensity of image frame; SD = standard deviation of mage frame; PI = peak intensity to noise).

– **Cut off.** Sets a filter to suppress molecules that deviate from the ideal Gauss shape, for example it there is a background structure.

– **Feature mask size [pixel].** Determines the mask used to cut out the particle.

– **Link range [frames].** Sets the lower limit in frames over which the particle has to be detected.

– **Displacement [pixel].** Sets the upper limit of distance the particle is lowed to have from one frame to the next.

– **Trajectory Length [pixel].** Sets the lower limit in the trajectory length a particle must have over the observed time period in order to be considered a particle.
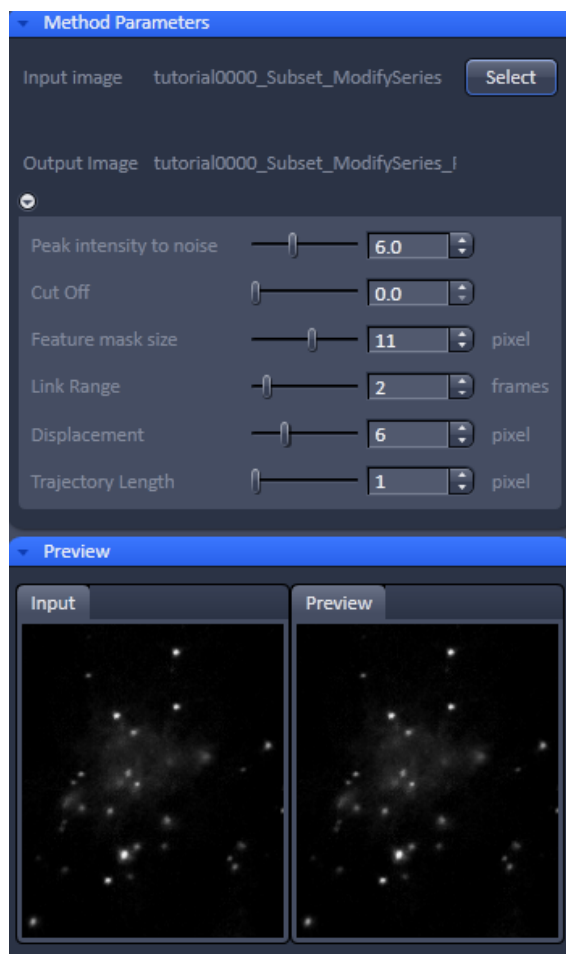
Fig. 328   Processing – Particle Tracking tool field
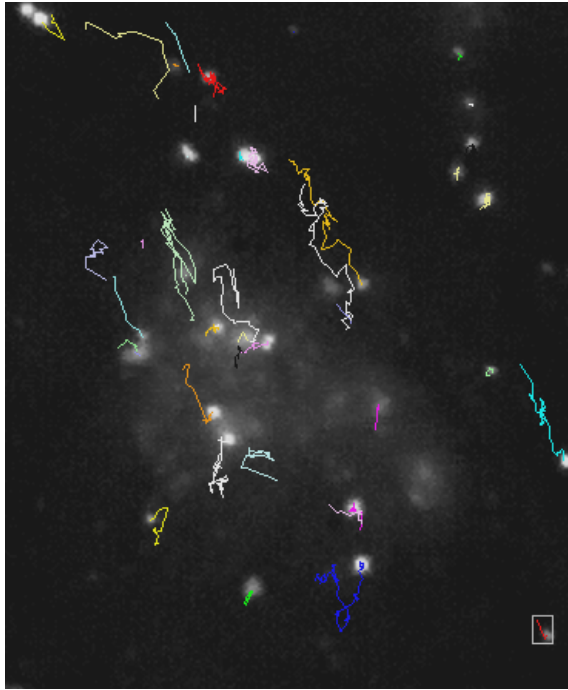
Fig. 329   **Image with trajectories of single molecules. The selected trajectory is boxed**

Once the settings are complete press **Apply** to obtain the trajectories of the single molecules (Fig. 329).

☞ Please note, that Particle Tracking only works for 2D data sets.

5.3.17 Processing – Experimental PSF

Licensing		Instrument		
✔ standard optional		✔ LSM	✔ ELYRA	✔ Lightsheet Z.1
Tool accepts Z-Stacks. See full description of tool for details.				
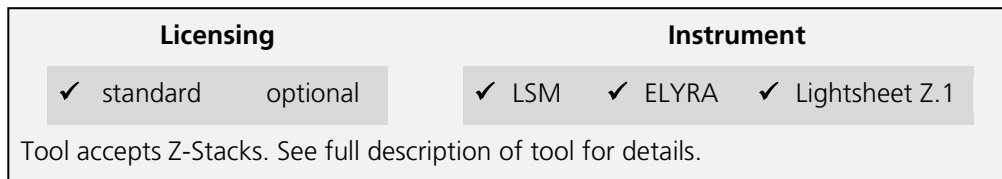
This function allows determining the point-spread function (PSF) of sub-resolution beads and using this experimentally determined PSF as input for Structured Illumination processing.

Select the **Experimental PSF** tab to select the **Experimental PSF** Processing tool (Fig. 330). Activate the image (a 3D stack of sub resolution beads) you want to process in the image container and press the **Select** button for **Input Image** to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 331).

☞ You can load a 3D stack of a widefield image or a non-processed raw data SIM image. LSM image formats are not accepted to determine the **Experimental PSF**.

You have the possibility to automaticaly compute the Experimental PSF by choosing **Automatic** from **Mode** drop down menu (Fig. 332).

If you want to process manually and set quality parameters, select **Adjust Parameters** (Fig. 333).

If you want to edit selected beads you can do so when selecting **Adjust ROIs**. Here, all selected beads (white circles) will be displayed in the input image as well. You can select a circle by clicking on it and delete it by pressing the Delete button of the keyboard. Alternatively, you can go from the Preview view tab to the 2D view tab were all selected beads are circled and listed and delete circles from there. The beads of deleted circles will then not be taken into account for the Experimental PSF calculation. On the other hand you can circle a bead in the 2D view not selected before and add it in this way for claculation of the Experimental PSF. Circles should be matched in radius to the preselected circles.

If you want to define suitable beads by yourself, you should select **Manual ROIs**. Here, no beads are selected which then can be done in the 2D view tab by choosing the circle graphic element for an overlay.

Fig. 330   Processing – Experimental PSF tool
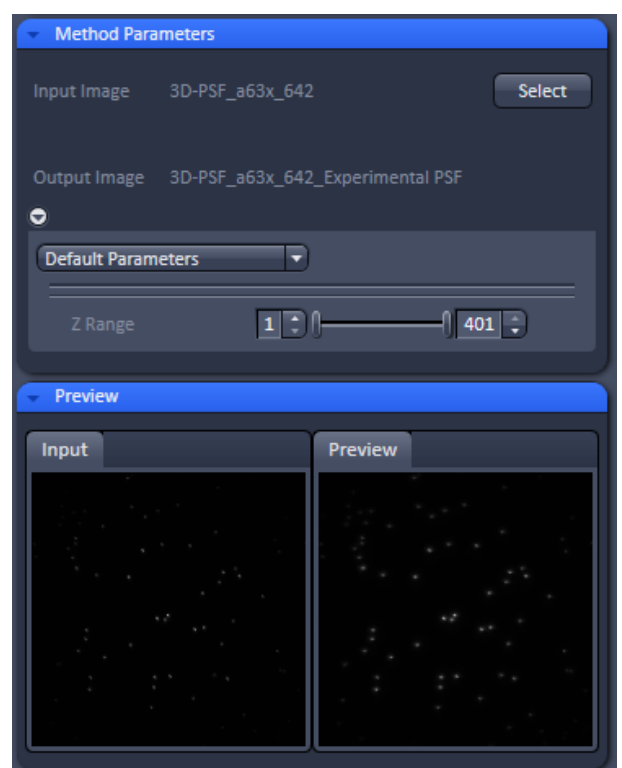
Fig. 331   Experimental PSF – Default Parameters mode
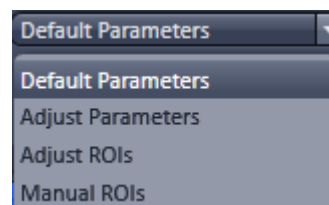
Fig. 332   Mode drop down menu

Being in the **Adjust Parameter** mode pressing the **Default** button will load Standard values. Data will only be reused, if the result image is selected. Than pressing the **Reuse** button will load the values of the selected image. The options **Symmetrical** and **Damp Edge** are not included in the Reuse function.

When the **Symmetrical** check box is ticked, a radial symmetrization of the PSF will be performed. That means that a perfectly round PSF is calculated, even out of an oval PSF.

When the **Damp edge** check box is ticked, the edge of the PSF will be smoothed (with a smooth transition to zero at the edge).
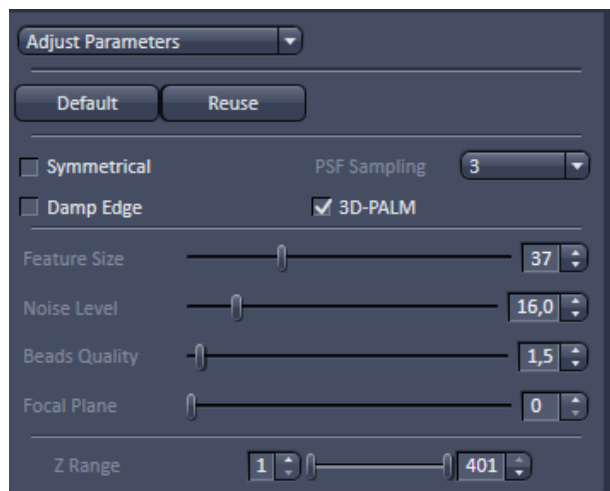
Fig. 333   Experimental PSF – appearance for Adjust Parameters, Adjust ROIs and Manual ROIs
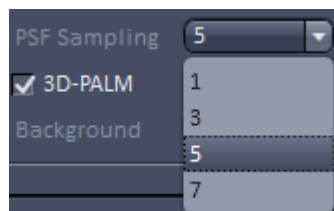
Fig. 334   PSF sampling drop down menu
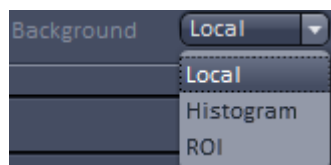
Fig. 335   Background drop down menu

The **PSF sampling** pull down menu allows you to interpolate the result to different degrees. Since the resulting PSF is an average of many PSFs corresponding to the localized beads and every bead is localized with the sub-pixel accuracy, one can obtain after averaging a PSF with higher resolution.

You can average the PSF by **1** (pixel size of the resulting PSF is the same or **1** times as in the original image), **3** (pixel size of the resulting PSF is **3** times smaller as in the original image), **5** (pixel size of the resulting PSF is **5** times smaller as in the original image) or **7** (pixel size of the resulting PSF is **7** times smaller as in the original image) times (Fig. 334).

The **3D SMLM** box will be automatically checked, if the image data was acquired with the 3D-PALM slider inserted. In this case the double lobe structure of each bead will be aligned to the right lobe.

In the **Background** drop down menu you can decide the way background is determined (Fig. 335).

You have three options:

- **Local**: Background is computed from the periphery area of the mask defined in **Peak Mask Size**.
- **Histogramm**: Background is calculkated from the histogram as at 10% cut off value.
- **ROI**: Backgrund is compited from ROIs in the image (**drawn** with the Graphics tool). Note, this option is only available if ROIs have been defined in the image.

- The **Z Range** slider can be used to set the range taken into account for the computation of the Experimental PSF.

There are different filters that can be set by corresponding sliders or spin input boxes to select sub-resolution beads.

Feature size sets the mask size that is used to cut out the bead and to create the **Experimental PSF**. The extensions are displayed in the **Preview** tab of the image container as circles (Fig. 336).

White circles indicate that the particular PSFs are used (if channel color is grey; otherwise the channel color is used), red circles that the PSF are discarded for the calculation of the Experimental PSF.
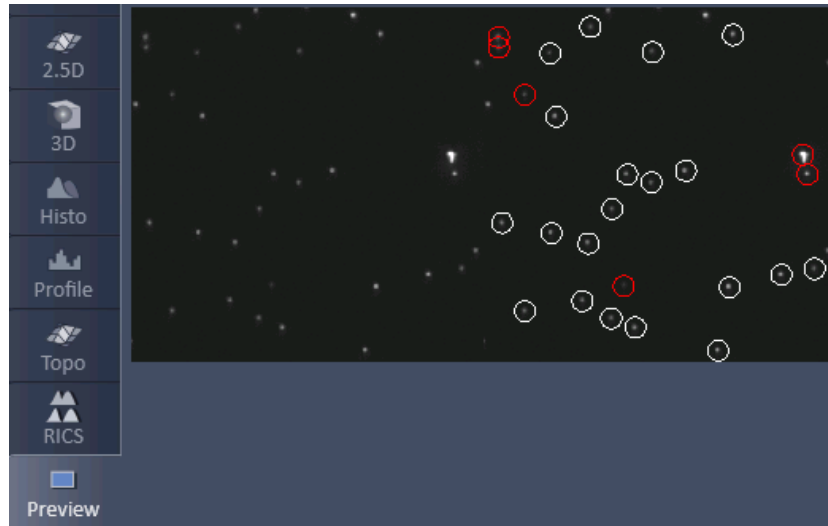
Fig. 336   Selection of beads in Preview tab

Noise Level sets the threshold of the intensity of the sub-resolution beads over background according to I – M > SD x NL (I = intensity of bead; M = mean intensity of image frame; SD = standard deviation of image frame; NL = noise level). Lowest number means that everything (even noise) is taken into account; highest number means only the brightest beads will be used.

Beads quality evaluates two characteristics, the intensity (first moment) and the size (second moment). These two moments are calculated for every localized bead. Then the beads are sorted according to these characteristics, resulting in two sorted lists of beads. The beads that are closer to the medians in both orderings are assumed to have higher quality. The **Beads quality** defines how far the beads characteristics (intensity and size) may be away from the median value. The smaller the threshold, the closer beads should be to the median. Beads quality equals to 0 is likely to result in a single bead right in the middle. In this way also the bead shape (aspect ratio) is assessed. Lower input number means that only perfectly shaped round beads will be taken into account. Highest input numbers will take into account all beads, even ellipsoid shaped ones.

Focal Plane determines which plane is the focal plane. The default is **-1** (automatic). Here the system determines the focal plane automatically. The user can also select a frame, which is presumably the focal plane, by input the number of the frame within the stack.

All sub-resolution beads that will be taken into account will be circled by a white line. Those that do overlap with their circles or are considered unsuitable (beads beyond the quality thresholds) are outlined in red and will not be taken into account (Fig. 336).

☞   Please note that you should use a 4 μm stack of 100 nm beads, if you want to use the Experimental PSF for 3D SMLM.

Once settings have been completed the **Preview** window displays beads that are not taken into account as red circles and those that are used in white circles (if channel color is grey) or in their channel color (if chanel color is other than grey) (Fig. 337). Press apply to compute the Experimental PSF. The created file will have the extension "… .ExperimentalPSF. …".
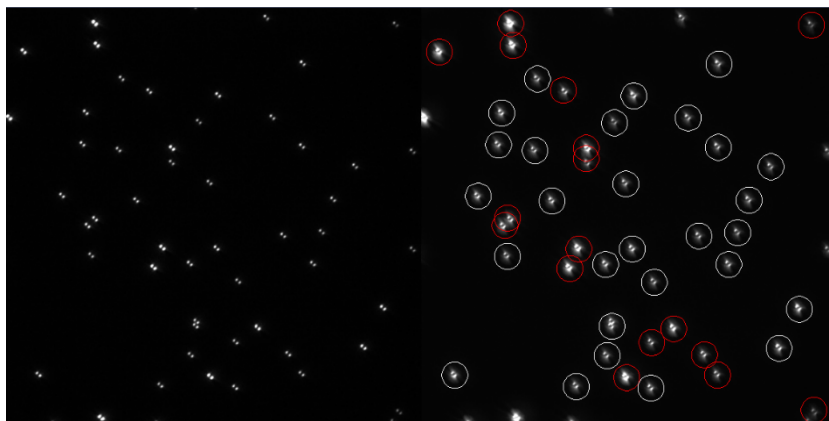
Fig. 337   **Preview window for bead selection using 3D SMLM**

☞   Best results for an Experimental PSF for 3D data will be obtained, if beads are seeded sparsely, for example 10 - 20 beads evenly distributed in a 512 x 512 image.
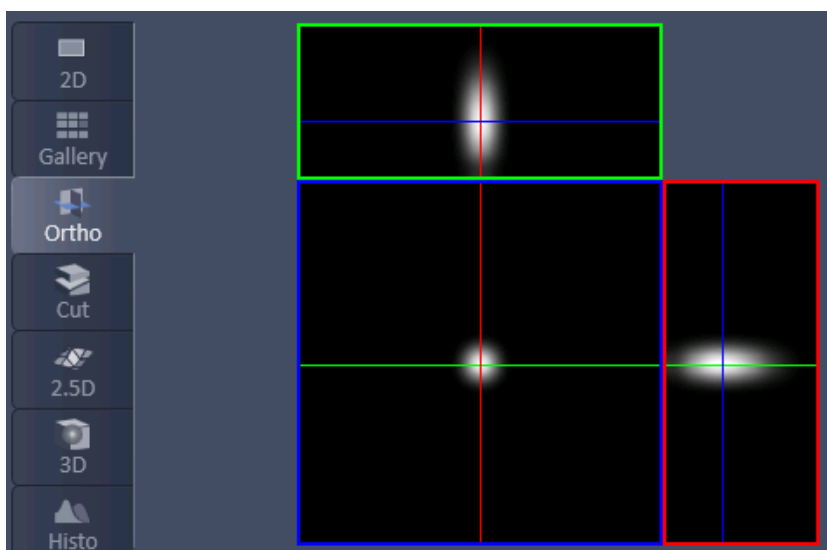
Fig. 338   **Experimental PSF in Ortho view**

If **Apply** is pressed an average PSF from all the beads taken into account will be computed (Fig. 338). For each channel a separate PSF will be displayed. The **Experimental PSF** can be saved (format .czi) and used for the **Structured Illumination - Structured Illumination** Processing tool in place of the **Theoretical PSF**. The size and pixel size of the **Experimental PSF** are recalculated and the PSF is interpolated to match the processed image.

5.3.18   Processing – SIM Tools

Licensing		Instrument		
standard ✔ optional		LSM ✔ ELYRA Lightsheet Z.1		

Tool accepts data recorded in SIM mode on ELYRA. In data sets containing other types of image data as well ("mixed mode" files), this tool selectively processes the SIM data. "Experimental PSF" (requires Z-stacks of point-like objects) and "Fourier Transform" accepts input other than SIM data as well. See full description of tool set for details.

The **Processing SIM tools** works exclusively for data acquired in the **SIM Apotome** and **Stripe** & **Lattice SIM imaging** modes with the exception of **Stripe SIM** tool that only works for Stripe SIM. It subdivides in 4 subcategories (Fig. 348):

Fig. 339   Processing – SIM tools

- **Fourier transform**: Calculates the spatial frequencies and displays them in an image. No phase information will be available.

- **Modulation contrast**: Determines the modulation contrast from an image with beads to serve as a diagnostic tool.

- **Stripe SIM**: calculates the high resolution image from the set of structured images acquoired with linear grid pattern.

- **TIRF filtered**: Will apply the TIRF sectioning to the SIM image if the TIRF was added as the last frame.

Fig. 340   **SIM – Fourier Transform tool selected**
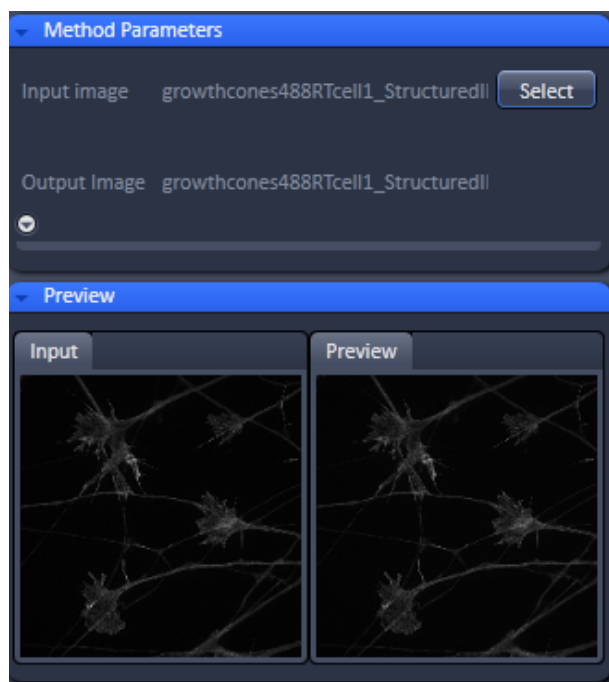
Fig. 341   **Structured Illumination – Fourier
Transform tool**

5.3.18.1   SIM – Fourier Transform

The **Fourier Transform** tool computes and displays the Fourier transform of the image (frequency space).

☞   Note that the Fourier image will contain the information on the spatial frequencies, but does not contain phase information.

Select the **Fourier Transform** tab to select the **Fourier Transform** Processing tool (Fig. 340). Activate the image you want to analyze in the image container and press the **Select** button for **Input image** to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 341).

If the **Log FT** check box is inactive, the result is the absolute value of the Fourier transform [sqrt (Real x Real + Imag x Imag) with Real and Imag being the real and imaginary part of the Fourier transform]. If **Log FT** is ticked, the result is the log-transform of the shifted absolute values [log (1+ Real x Real + Imag x Imag)]. Log-transformation squeezes the dynamic rage of the Fourier-transformed image, so that weaker intensities may become better visible.

Press **Apply** to compute the Fourier transforms of all image channels (Fig. 342).

☞   The Fourier images can be used to assess the quality of the SIM calculations and retrieve the resolution enhancement.
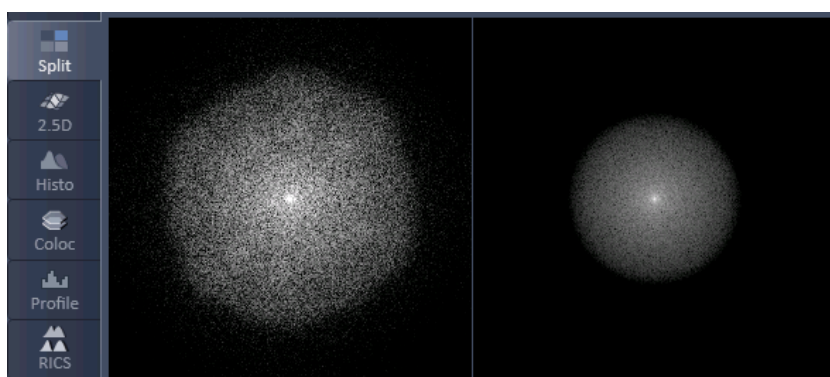
Fig. 342   **Fourier transform images of SR (left) and WF (right) image
in the Split view**

5.3.18.2   SIM – Modulation contrast

Modulation Contrast is a diagnostic tool to check on the modulation contrast of the system. Modulation Contrast is meaningful only for a Z-Stack of raw SIM data acquired with preferentially sub-resolution beads.

Fig. 343   SIM Tools – Modulation Contrast

Select the **Modulation Contrast** tab to (Fig. 344).

Pressing the **Default** button will save the present parameter values as default. By pressing **Reuse**, the currently saved parameter values will be loaded.

With the **Channel selection** drop down menu you can choose **All** channels or the channel you want to process if the image has more than 1 channel (Fig. 345). The Channel selection drop down menu is only available for multi-channel images, otherwise it is not displayed.

There are different filters that can be set by corresponding sliders or spin input boxes to select the (sub-resolution) beads.

Feature size: **Feature size** sets the mask size that is used to cut out the bead. The extensions are displayed in the **Preview** tab of the image container as circles (Fig. 346).

Fig. 344   SIM tools – Modulation contrast

Fig. 345   Channel selection drop don menu
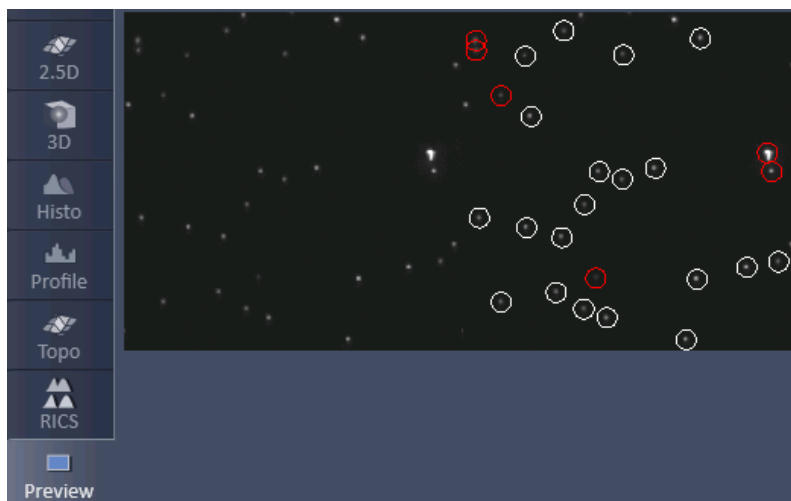
Fig. 346   **Selection of beads in Preview tab**

Noise Level: **Noise Level** sets the threshold of the intensity of the sub-resolution beads over background according to I – M > SD x NL (I = intensity of bead; M = man intensity of image frame; SD 0 standard deviation of image frame; NL = noise level). Lowest number means that everything (even noise) is taken, highest number means only the brightest beads will be taken.

Beads quality: **Beads quality** evaluates two characteristics, the intensity (zero moment) and the size (second moment). These two moments are calculated for every localized bead. They basically will assess the shape (aspect ratio of the beads). Then the beads are sorted according to these characteristics, resulting in two sorted lists of beads. The beads that are closer to the medians in both orderings are assumed to have higher quality. The Beads quality defines how far the beads characteristics (intensity and size) may be away from the median value. The smaller the threshold, the closer beads should be to the median. Beads quality equals to 0 is likely to result in a single bead right in the middle. Lower input number means that only perfectly shaped round beads will be taken into account. Highest input numbers will take into account all beads, even ellipsoid shaped ones.

All sub-resolution beads that will be taken will be circled by a white line. Those that do overlap with their circles or that are considered unsuitable (beads beyond the quality threshold) are outlined in red and will not be taken into account (Fig. 346).

If **Apply** is pressed "Line-scan" plots (and the corresponding table) will be displayed in the image container with which the modulation contrast can be assessed (Fig. 347).

Fig. 347   **Modulation contrast line plot and table**

The result is showing the dependence of the **Intensity** of the 1st and 2nd orders on z (**Distance [μm]**). The channel code **TV#-TX#-RX#-O#** is as follows:

- TV#-T# = channel name
- R# = number of rotation
- O# = number of order

The 1st orders should show periodicity (due to the Talbot effect), whereas the 2nd and higher orders should be more or less constant over the whole z-range. As an output there will also be two files in the **C:\\Temp** folder called **ModulationContrast.txt** and **ModulationPeriod.txt**. **ModulationPeriod.txt** contains the grating period for different rotations and z-planes as estimated from the modulation curves. This estimate is independent of the main SIM processing and can be used for cross-check. **ModulationContrast.txt** contains information for every localized bead: position, intensities and phase in the first and second orders. This file can be used for further possible quantitative analysis of the modulation.
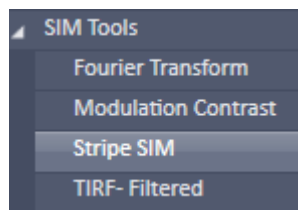
Fig. 348   **Processing – Stripe SIM tool selected**

Fig. 349   **Structured Illumination – Structured Illumination**

5.3.18.3   SIM – Stripe SIM

The **Stripe SIM** function allows automatic or manual processing of a set of structured images obtained by phase shift and rotation of a linear grid.

Select the **Stripe SIM** tab to select the **Structured Illumination** Processing tool (Fig. 348). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 349).

☞ You have to use the subdirectory **Structured Illumination** tab when selecting the image to have aces to the **Structured Illumination** tools. You will have no tools available is the directory **Structured Illumination** tab is selected.

If a region of interest (**ROI**) is defined in the image, then this **ROI** will be exclusively taken for image processing and the rest of the image will not be processed.

You can instead of processing only one selected image also load more images to be processed sequentially. To this end press the **Run Batch** button (Fig. 349). This will open **Windows Explorer** from which you can select all images you want to process. All selected files will be displayed in the **Batch display** window, which will appear when the files are opened by Windows Explorer. The **Batch display** window also shows the current state of batch processing.

The **Run Batch** button will turn into a **Stop Batch** button (Fig. 350) once pressed. If the button is pressed, batch processing will be stopped, no matter if **Apply** was pressed or not and the button turns back to **Run Batch**.

☞ In batch processing all images can only be processed with one parameter setting. If images, however, have been processed already once and are linked to processing parameters by pressing the **Attach** button, those settings will be taken and the general setting overruled. If you want to overrule attached settings and fit all Batch files with the set and displayed parameters, you have to check the **Use current for Batch** box.

If a Z-Stack was acquired you have the option to tell the system how the frames of the stack should be processed from the **Stack processing mode** drop down box (Fig. 351).

You have the option between **First-Last** and **Single**.

When **First-Last** is selected the double-slider or the left and right spin input boxes can be used to define the first and last frame of the stack that should be processed (Fig. 352).

When **Single** is selected, the slider or spin input box can be used to define one frame in the stack that should be processed (Fig. 353).

With the **Mode** drop down menu you have the option to select, how the single frames of a stack are processed (Fig. 354). You have the option between **2D**, **3D** and **3D large**.

☞ Note that **3D** and **3D large** are only available, when the image to be processed contains a stack of >1 and **First-Last** was selected as the **Stack processing** mode. Otherwise, the Mode drop down menu shows only **2D** and is greyed.

When **2D** is selected, every frame is reconstructed with the information of that frame only and the structuring in the thirds dimension is not used.
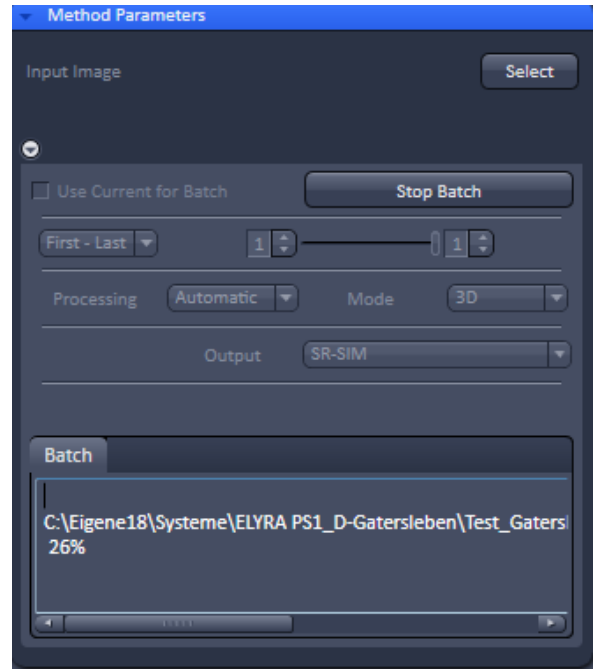
Fig. 350   Structured Illumination – Batch processing
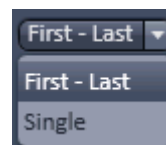
Fig. 351   Stack processing mode drop down box

Fig. 352   Setting of the first and last frame of a stack to be processed

Fig. 353   Selection of one frame from a stack be processed
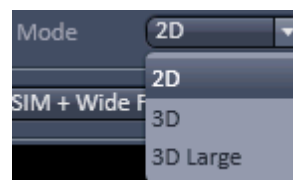
Fig. 354   Mode drop down box

If **3D** is selected, the information of the neighboring frames in regard to structuring information and out of focus contribution will be used in addition to the information of the frame itself. This will result in better x, y and z resolution and better contrast by removing the out of focus light to a greater extent at the cost of more computational power requirement.

Fig. 355   **Warning for memory shortage**
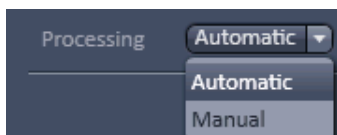
Fig. 356   **Processing drop down box**
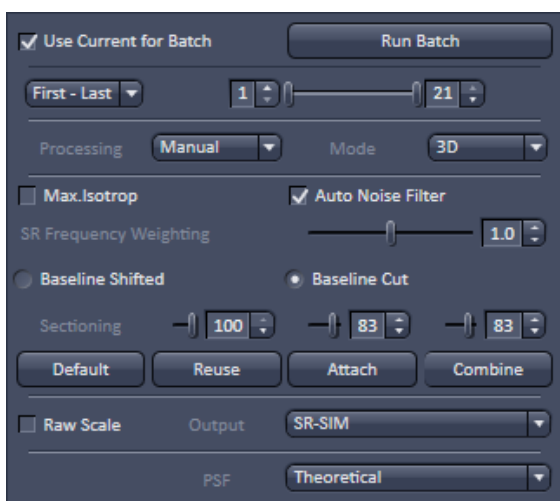
Fig. 357   **The Manual selection field**
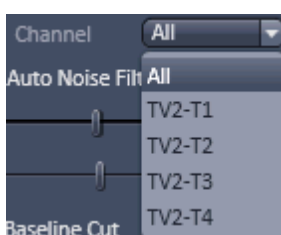
Fig. 358   **Channel drop down box**

If the stack is large, available memory might be limited. In this case a **Warning** is issued, recommending the usage of the **3D large** mode (Fig. 355). In this case, the stack is subdivided into individual smaller stacks, which are individually processed. The result images of the individual stacks are than automatically combined into one output image.

If one selects a region of interest (**ROI**) in the image, than that **ROI** will be used for image processing. This also can help in reducing memory problems when processing huge stacks.

In the **Processing** drop down menu one can select between **Automatic** and **Manual** processing (Fig. 356). If **Automatic** is selected, pre-set filter and parameter values will be used.

If **Manual** is selected these filters and parameters become accessible to be changed and the **Manual selection** field becomes accessible (Fig. 357).

☞   The **Automatic** mode is recommended for images with high modulation contrast. In case the modulation contrast is low one might benefit from changing the pre-set values.

In the **Channel** drop down menu (Fig. 358) **All** or single channels can be selected from a multi-channel image that should be processed. The **Channel** drop down menu is not displayed if the loaded image has only one channel.

Due to the limited number of rotations more frequencies and hence higher resolution information can be obtained exactly at the rotation angles. The directions between the rotational angles contain lesser frequencies. All available frequency information will be used if the **Max.Isotrop** checkbox is not ticked. This gives rise to the flower like appearance of the Fourier transform, which extends wider at the rotational angles.

If the **Max.Isotrop** checkbox is ticked, the software will compute the high resolution image with the same resolution in every direction that is it does not use the highest frequencies in the rotational directions and the Fourier transform will be circle like (Fig. 359).

If the **Auto Noise Filter** checkbox is not ticked, the **Noise Filter** slider is available, that can be used to set the noise filter for the so called Wiener de-convolution performed. The negative number indicates the power to 10 for the filter. The smaller the values (towards -11), higher frequencies are allowed leading to noisier and crispier images. If the **Auto Noise Filter** is ticked, the **Noise Filter** slider is not displayed.

The **SR Frequency weighing** filter (also known as HD goal filter) will affect more prominently the higher frequencies. You can set the value by using the slider or the spin input box. The filter is a kind of cone that multiplies the Fourier transform of the resulting image. Weighting changes the slopes of the cone from the convex (towards 0) to concave (towards 2) (Fig. 360). 0 is no filtering, 1 is a straight line, <1 is convex, >1 concave.

You can toggle between **Baseline Shifted** and **Baseline Cut**. In **Baseline Shifted** negative values that can arise as an artifact by the **Noise** or Wiener **filter** will be kept and an offset is added to the whole image, so that the minimal (negative) intensity becomes zero intensity (baseline shifted). In **Baseline Cut** negative values will be clipped by setting them to zero. No offset is applied.

The **Sectioning** (or Strength) **filter** removes the 0-frequencies for the 0th order with a pre-defined variance. It also removes the 0-frequencies from the non-shifted 1st (+1, -1) and 2nd (+2, -2) orders. Therefore bumps and deeps might be occasionally detected in the Fourier transformed image at the positions defined to the rotation angle and grating frequency. The higher the value, the more stringent the filtering will be and more of the lower frequencies will be removed (Fig. 361). Three filter sliders and spin input boxes for the **0** order, the **1st** orders and the **2nd** orders are available (from left to right) for input.

Fig. 359 Fourier transform with Max.Isotrop disabled (strong solid line) and enabled (blue stippled line). The inner circle (light solid line) is the Fourier transform of the non-structured image
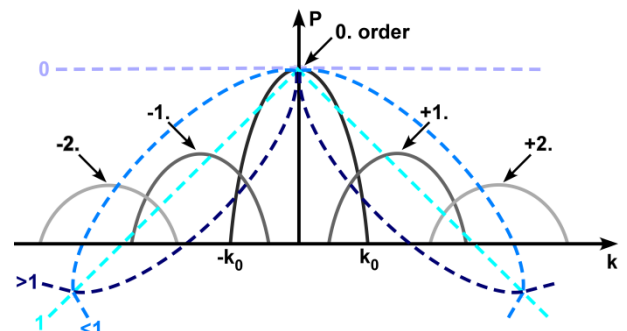
Fig. 360 Sketch of the influence of SR Frequency weighting filter (blue stippled lines) on the spatial frequencies

Fig. 361 Influence of sectioning filter (blue stippled lines) on the spatial frequencies. The open arrow indicates increasing strength, the double arrow the variance, which is pre-defined

All parameters will influence the Optical Transfer Function (OTF) used for reconstruction. The shape of the OTF in x, y is displayed in the **OTF display** window below the **Manual selection** field.

The **Preview** tab shows the **Input** image and a **Preview** of the output image.

By pressing **Default** the current parameter values will be stored as default values. By pressing **Reuse** the last stored default values will be loaded.
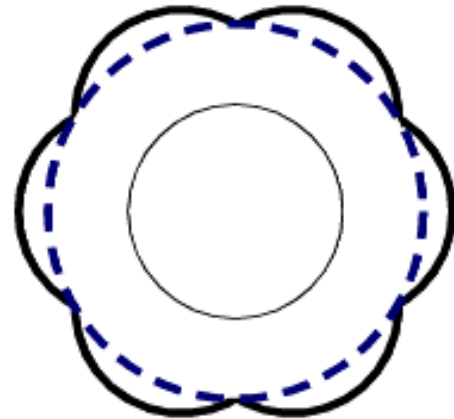
Pressing **Attach** allows parameter values to be attached to an image. In normal case if any of the SIM parameters will be changed, they will be applied accordingly for the processing, but may not be saved into the image. Then if the image is saved, the values of the parameters applied for the image will be lost. If you attach the corresponding tag to the image by pressing the **Attach** button parameter values used to process the image will be attached to the image, even if it is not explicitly saved again. They will then be available for further processing, in particular for Batch processing.
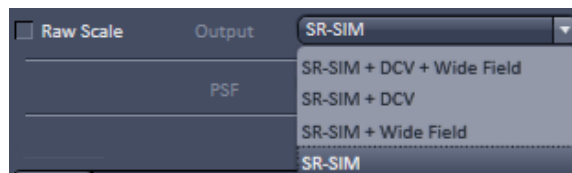
Fig. 362   **Output drop down menu**

Fig. 363   **PSF drop down menu**

Fig. 364   **PSF selection menu**

Fig. 365   **SIM image (left) and widefield image (right) in the Split mode after SIM processing**

In the **Output** drop down menu you can set, what kind of channels you want to display (Fig. 362). You have the option to display

– **SR-SIM + DCV + Widefield**

– **SR-SIM + DCV**

– **SR-SIM + Wide Field**

– **SR-SIM.**

Highlight the option you want to display. Channel designations are as follows: **SR** for SR-SIM, **WF** for Widefield and **D** for Deconvolution (DCV).

If the **Raw Scale** box is unchecked, output images will be normalized to the maximum dynamic range. Please note that in this case quantitative information is lost, however, images are displayed with best brightness and contrast. If you want to keep the original scaling for quantitative studies, you have to check the **Raw scale** box. In this case the orifginal dynamic range is kept.

You can select between theoretically computed or experimentally determined PSF by choosing **Theoretical** or **Experimental** in the **PSF** drop down menu (Fig. 363). In case **Experimenta**l is selected, the **PSF File** button becomes available (Fig. 364). Pressing the button will open Windows Explorer, which allows you to select any pre-saved Experimental PSF. The file name of the loaded file is displayed in the **PSF File** display window.

☞ Note, if the number of the Experimental PSF channels is less than the number of the WF or SIM channels, the theoretical PSF will be used for the missing channels and a warning is generated. The program also checks the match of the excitation wavelengths between the PSF and WF / SIM channels. In case of mismatching, it generates a warning, but the experimental PSF will still be used.

By pressing **Apply** the high resolution image will be computed with the current parameter values (Fig. 365).

If a value is changed you have the possibility to press **Combine**. Then a new calculation is only done for the changed parameter and combined with the previous calculations on the unchanged parameters, which will save dramatically computational time. If **Apply** is pressed, calculations will be done on all parameters regardless of being changed or unchanged.

5.3.18.4 SIM – TIRF-Filtered

This function works for SIM images (stack) in combination with a TIRF image. The lowest frame (first frame) of the stack, preferentially one taken close to the coverslip, will be taken and combined with the TIRF. The SIM image contributes the x,y enhancement, the TIRF image the z-resolution of the TIRF.

Select the **TIRF-Filtered** tab to select the **TIRF-Filtered** Processing tool (Fig. 366). Activate the SIM image you want to process in the image container and press the **Select** button for **Input image** to load the SIM image. Activate the TIRF image you want to use as the reference in the image container and press the **Select** button for **Input image 2**. Then expand the **Methods Parameters** tool window to have access to setting parameters (Fig. 367).

You can toggle between Input image 1 and 2 in the **Preview** tab window, which also displays a preview of the output image.

The **Image input** description field lists all the channels in the **SIM** column, the used lasers of a channel in the **Laser** column and the employed camera coded by a number in the **Camera** column.

The **Image input 2** description field lists all the channels in the **TIRF** column and the used laser of a channel in the **Laser** column.

☞ It is important to load the SIM image as **Input image** and the TIRF image as **Input image 2** as the software expects it that way. If in **Input image 2** an image stack is selected, the system gives an error message as it expects a single plane (Fig. 368).
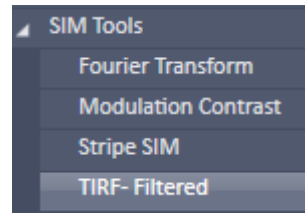
Fig. 366   **SIM – TIRF-Filtered tool selected**
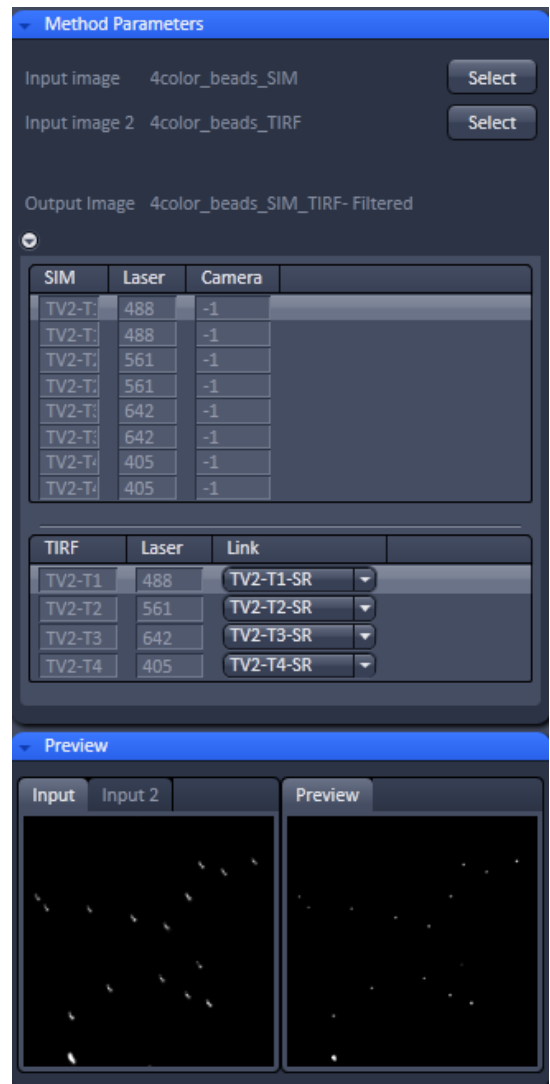
Fig. 367   **Structured Illumination – TIRF-Filtered tool**
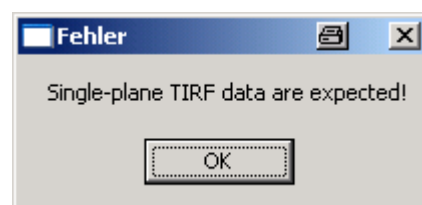
Fig. 368   **Error message for falsely loaded image dimensions in Input image 2**

The **Link** drop down menu available for each channel displays all channels of the SIM **Input Image** (Fig. 369).

You can now link the TIRF channel to one of the SIM channels by highlighting the latter. Than the respective TIRF channel will be used to compute its z-resolution into the linked SIM channel.

When **Apply** is pressed, the new SIM image with the improved z-resolution in the lowest frame will be computed.

Fig. 369   **Link drop down menu**

5.3.19   Processing – SIM

Licensing		Instrument		
standard ✔ optional		LSM	✔ ELYRA	Lightsheet Z.1
Tool accepts data recorded in SIM mode on ELYRA. In data sets containing other types of image data as well ("mixed mode" files), this tool selectively processes the SIM data. "Experimental PSF" (requires Z-stacks of point-like objects) and "Fourier Transform" accepts input other than SIM data as well. See full description of tool set for details.				

The **Processing SIM tool** works exclusively for data acquired in the **SIM Apotome** as well as **Stripe** & **Lattice SIM imaging** modes.

Fig. 370   Processing – SIM

The **SIM** function allows automatic or manual processing of a set of structured images (lattice SIM or Sripe SIM obtained by phase shift (Lattice SIM) or by phase shift and rotation of the grid (Stripe SIM). **SIM** processing tool can use SIM Apotome as well as Lattice SIM and Stripe SIM data.

Select the the **SIM** tab to select the **SIM** Processing tool (Fig. 370). Activate the image you want to process in the image container and press the **Select** button to load the image. Then expand the **Method Parameters** tool window to have access to setting parameters (Fig. 371).
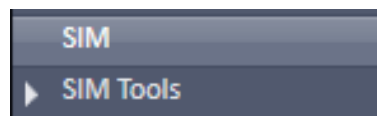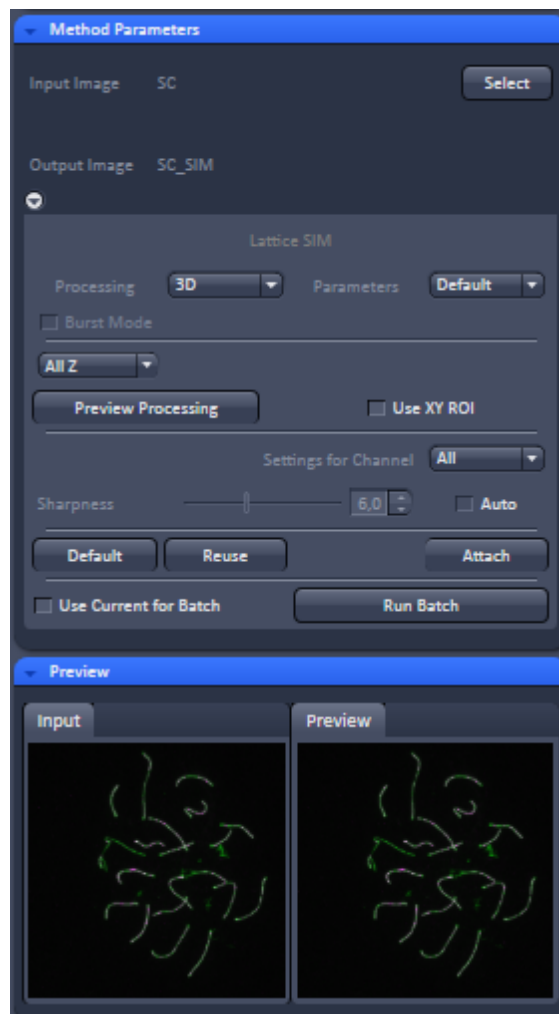
Fig. 371   Processing – SIM: Methods Parameter
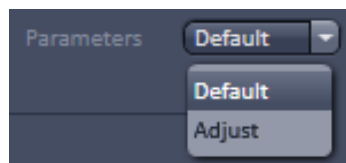
Fig. 372   Parameters drop
down menu

You have two options for Processing: Either **Default** or **Adjust**, which can be chosen from **Parameters** drop down menu (Fig. 372). **Default** uses standard settings, which will work in most of the cases; **Adjust** on the other hand gives access to additional parameters to be set.

The **Preview** tab shows the **Input** image and a **Preview** of the output image.

In **SIM** processing the mode of image is displayed in the image mode display area, e. g. **WF** (for wiedfield), **SIM Apotome**, **Stripe SIM** and **Lattice SIM**). It is also indicated, if the image was taken with **Leap** mode (**SIM Apotome Leap**, **Lattice SIM Leap**).

By pressing **Apply** the high resolution image will be computed with the current settings.
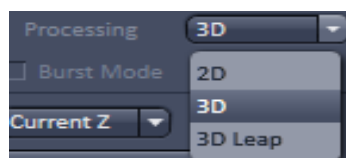
Fig. 373   Processing drop
down menu

5.3.19.1   Processing with Default setting

The tool indicates in the **Processing** drop down menu if the data set was taken in **2D** or **3D** or **3D Leap** (Fig. 373). For a 2D data set both 3D option are greyed out; for 3D datasets the **3D** or **3D leap** can be manually changed to **2D**. What is selected will determine how the data are processed.

When **2D** is selected, every frame is reconstructed with the information of that frame only and the structuring in the thirds dimension is not used.

If **3D** is selected, the information of the neighboring frames in regard to structuring information and out of focus contribution will be used in addition to the information of the frame itself. This will result in better x, y and z resolution and better contrast by removing the out of focus light to a greater extent at the cost of more computational power requirement.

If **3D leap** is selected, the system will reconstruct 3 planes instead of 1 between the original planes.

☞ Note that **3D** and **3D leap** are are only available, when the image to be processed contains a stack of >1 frames and **All Z** or **First-Last** was selected as the **Stack processing** mode. Otherwise, the **Processing** drop down menu shows only **2D** and other options are greyed.
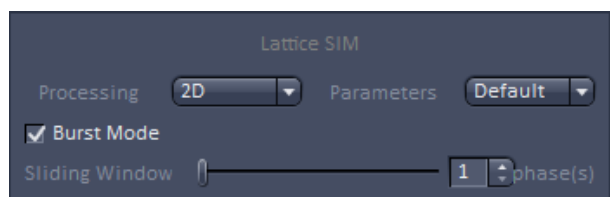
Fig. 374   Burst Mode active

☞ If the image was a 2D time series acquired with **Lattice SIM**, the **Burst Mode** check box becomes available (Fig. 374). If checked, the SIM calculation is not any more done by blocks of 15 phase images (i. e. 1-15, 16-30, …; **Block mode**), but by 15 phase images in a gliding or rolling manner with the number of phase image steps as defined in the **Sliding Windows…phase(s)** slider (**Burst mode**).

☞ For example, if the slider was set to 1 reconstruction will be performed on phase images 1-15, 2-16,…. Dependent on the slider settings, two events can be resolved to a better extend, however time resolution is restricted by the 15 images that have to be taken for one reconstructed image.

☞ If a region of interest (**ROI**) is defined in the image, and the **XY ROI** box is checked, then this **ROI** will be exclusively taken for image processing and the rest of the image will not be processed.

☞   Processing a ROI might result in a different noise filter as compared to processing the whole image dependent on the image content.

If a Z-Stack was acquired you have the option to tell the system, which planes of the stack should be processed from the **Stack processing mode** drop down box (Fig. 375).

You have the option between **All Z**, **First-Last** or **Current Z**.

If **All Z** is selected, the whole stack will be processed.

Fig. 375   Stack processing mode drop down

When **First-Last** is selected the double-slider or the left and right spin input boxes can be used to define the first and last frame of the stack that should be processed (Fig. 376).

If **Current Z** in **SIM** is selected, the current frame selected in the **2D view** will be processed.

Fig. 376   Setting of the first and last frame of a stack (plane) to be processed

In **SIM** processing tool you have the Option for fast Preview Processing by pressing the **Preview Processing** button (Fig. 377). The systems selects a ROI in XYZ by means of highest contrast in Z and highest intensity in XY and calculates the fitting parameters if in the **Adjust** window in the **Advanced Filters** field the **Parameters** drop down menu was set to **Fast**.

Fig. 377   Preview processing button

Note, if **Best** is selected the whole image will be taken and computation will be accordingly slower. If a ROI is defined and the Use ROI XY checkbox ticked the system will display the SIM image for this ROI only. Moving the ROI will update the SIM image on the fly. This might help to fast evaluate the fitted parameters and make adjustments for the processing of the total image. As previewed parameters will be taken for computation of the whole image, processing is much faster than processing an image without Preview Processing.

If more channels have been acquired the **Settings for Channel** drop down menu allows you to set parameters channel specific (Fig. 378). You have the option to select **All** or single channels from the multi-channel image that should be processed. If All is selected, the current settings will be applied to all channels. If one channel is selected, the settings defined for each channel will be applied to each of them. If a The **Settings for Channel** drop down menu is not greyed if the loaded image has only one channel.

Fig. 378   Channel drop down menu

If the **Sharpness Auto** box in **SIM** is not ticked, the **Sharpness** filter slider is available, that can be used to set the noise filter for the so called Wiener de-convolution (Fig. 379). The number indicates the negative number to the power to 10 for the filter.

The smaller the values (towards -11), the higher the frequencies that are allowed leading to noisier and crispier images.
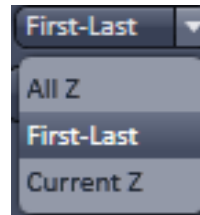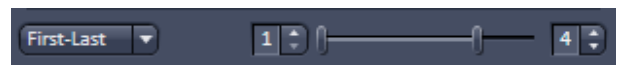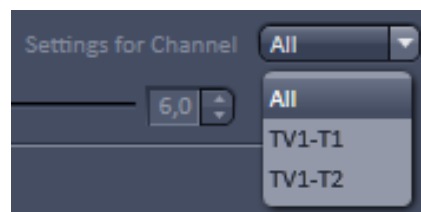

Fig. 379   Sharpness Filter with Auto box not ticked (upper panel) and ticked (lower panel)

If the **Sharpness Auto** box is ticked, the **Sharpness Filter** drop down menu with three pre-selections (Weak, Standard & Strong) for automatic noise filter estimation becomes available. Strong filtering allows for higher frequencies, leading to higher resolved, but in general noisier images.

☞ It depends on the sample structure and the signal-to-noise of the raw data image, which pre-selection gives the best result, which represents a compromise between resolution and background noise.

☞ In order to see, what filter was estimated when using auto noise filter settings, untick the **Sharpness Auto** box checkbox. The number displayed will be the noise filter from the previous Auto filter estimation.

Fig. 380   **Default, Reuse and Attach buttons**

By pressing **Default** the current parameter values will be stored as default values (Fig. 380). By pressing **Reuse** the last stored default values will be loaded.

Pressing **Attach** allows parameter values to be attached to an image. In normal case if any of the SIM parameters will be changed, they will be applied accordingly for the processing, but may not be saved into the image. Then if the image is saved, the values of the parameters applied for the image will be lost.

If you attach the corresponding tag to the image by pressing the **Attach** button parameter values used to process the image will be attached to the image, even if it is not explicitly saved again. They will then be available for further processing, in particular for Batch processing.

Fig. 381   **Run Batch button**

Fig. 382   **SIM – Batch processing**

☞ You can instead of processing only one selected image also load more images to be processed sequentially. To this end press the **Run Batch** button (Fig. 381). This will open **Windows Explorer** from which you can select all images you want to process.

☞ Once loaded the software immediately starts Batch processing and shows the current file (with directory) in the **Batch display** window, which is currently processed (Fig. 382). The **Batch display** window also shows the current state of batch processing in %. Once the button is pressed and the files loaded the system immediately begins to process the loaded files regardless if the **Apply** button is pressed or not.

The **Run Batch** button will turn into a **Stop Batch** button once pressed. If the button is pressed, batch processing will be stopped, no matter if **Apply** was pressed or not and the button turns back to **Run Batch**.

☞ In batch processing all images can only be processed with one parameter setting. If images, however, have been processed already once and are linked to processing parameters by pressing the **Attach** button, those settings will be taken and the general setting overruled. If you want to overrule attached settings and fit all Batch files with the set and displayed parameters, you have to check the **Use current for Batch** box.

☞ The **Default** mode is recommended for images with high modulation contrast. If **Default** is selected, pre-set filter and parameter values will be used. In case the modulation contrast is low one might benefit from changing the pre-set values and work in **Adjust** mode. If **Adjust** is selected all and new filters become accessible to be changed.

5.3.19.2 Processing with Adjust settings

If **Adjust** is selected the Adjust panel becomes available offering more options to process the data (Fig. 383).

Press the triangle of **Advanced Filters** to open to the **Advanced Filters** field (Fig. 384).

You can choose from the **Parameters** drop down menu how the parameters should be fitted (Fig. 385):

- **No Fit**: Theoretical parameters will be taken and fitting is skipped. This can be done channel specific and might help avoiding black planes due to poor raw data, where the system cannot converge on parameter values

- **Fast Fit**: Parameters are fitted by using a ROI of the image that is selected according to the highest intensity in XY and highest contrast in z.

- **Best Fit**: Parameters are fitted by using the whole image.

☞ In the **Advanced Filters** field a **Detrend** check box is available that becomes active for time series data only. If checked the algorithm compensates for bleaching or a decrease in fluorescence over time.

☞ Alternatively you can detrend time series images before or after processing in the **Detrending** processing tool. This tool allows in addition to set thresholds above which detrending should be performed.

With the **Sectioning** slider you can set the strength to remove the 0-frequencies for the 0th order with a pre-defined variance. The higher the value, the more stringent the filtering will be and more of the lower frequencies will be removed.

Press the triangle of **Advanced Settings** to open to the **Advanced Settings** field (Fig. 386).
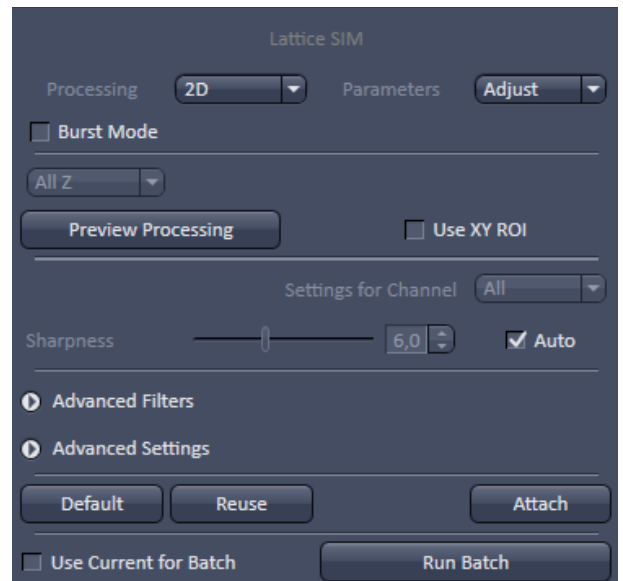
Fig. 383   The Adjust field with Advanced Filters and Advanced Settings collapsed

Fig. 384   Advanced Filters field
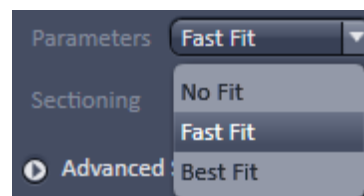
Fig. 385   Parameters drop down menu
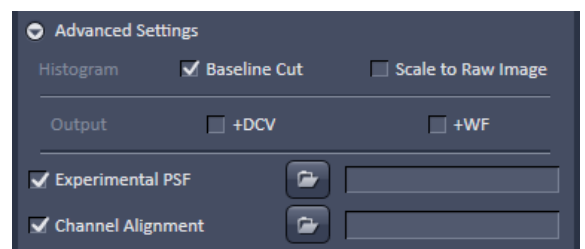
Fig. 386   Advanced Settings field expanded

In Advanced settings you can check or uncheck the **Histogram Baseline Cut** box to select or deselect baseline cut with baseline shift being used if the box is not ticked. In **Baseline Shift** negative values that can arise as an artifact by the **Noise** or Wiener **filter** (**Sharpness** filter) will be kept and an offset is added to the whole image, so that the minimal (negative) intensity becomes zero intensity. In **Baseline Cut** negative values will be clipped by setting them to zero. No offset is applied.

Fig. 387   Output +DCV and +WF checkboxes
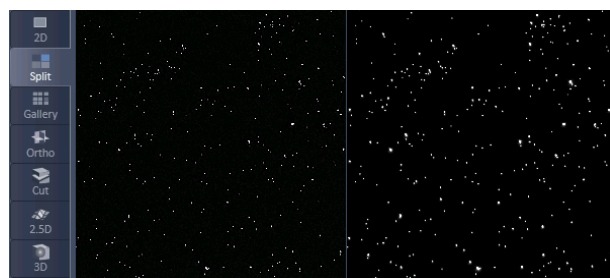
Fig. 388   SIM image (left) and widefield image (right) in the Split mode after SIM processing with checked +WF box
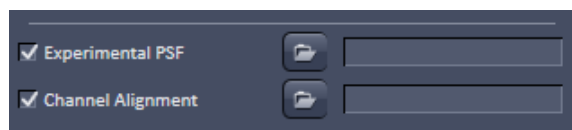
Fig. 389   Selection boxes for Experimental PSF and Channel Alignment

Output…+DCF and **Output…+WF** check boxes are available (Fig. 387) that if ticked will compute in addition to the SIM image the selected deconvolved (DCV) and widefield (WF) images. For example, if +WF was ticked the resulting computed image consists of a **SIM** and **WF** channel (Fig. 388).

If the the **Histogram Scale to Raw Image** is unchecked, output images will be normalized to the maximum dynamic range. Please note that in this case quantitative information is lost, however, images are displayed with best brightness and contrast. If you want to keep the original scaling for quantitative studies, you have to check the **Histogram Scale to Raw Image** box. In this case the original dynamic range is kept.

You can select between theoretically computed or experimentally determined PSF by ticking or unticking the **Experimental PSF** check box (Fig. 389). If not checked, the **Theoretical PSF** is taken. If checked, the Windows explorer **Open** icon becomes available. Pressing the button will open Windows Explorer, which allows you to select any pre-saved Experimental PSF. The file name of the loaded file is displayed in the **PSF File** display window along with the director path.

☞   Note, if the number of the Experimental PSF channels is less than the number of the WF or SIM channels, the theoretical PSF will be used for the missing channels and a warning is generated. The program also checks the match of the excitation wavelengths between the PSF and WF / SIM channels. In case of mismatching, it generates a warning, but the experimental PSF will still be used.

☞   In **SIM** processing tool, you have the possibility to also perform a channel alignment after processing. To this end check the **Channel Alignment** box (Fig. 389) and select a pre-defined template via Windows Explorer. The file name of the loaded file is displayed in the **Channel Alignment File** display window along with the directory path.

5.3.20   Processing – Channel Alignment

Licensing		Instrument		
✔ standard	optional	✔ LSM	✔ ELYRA	✔ Lightsheet Z.1
Tool accepts multi-color image data (including multi-dimensional data sets). See full description of tool set for details.				

The Processing **Channel Alignment** tool allows for correction of lateral drift and chromatic aberration between different channels of an image or between images of the same type.

Highlight the **Channel Alignment** field to have access to the alignment tools (Fig. 390).
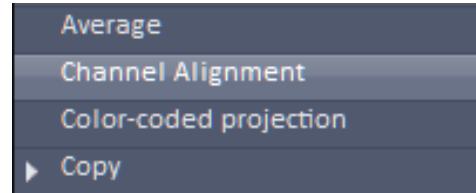
Fig. 390   **Processing – Channel Alignment tool selected**

Activate the first image you want to process in the image container and press the **Select** button for **Input image** to load the image.

If you want to load a second input, highlight it in the image container and press the **Select** button for **Input Image 2**.

You can toggle between the two input images in the **Preview** tab display field. Then expand the **Method Parameters** tool window to have access to the **Channel Alignment** tools (Fig. 391).

☞ Channel alignment works between identical types of images. Mixed mode images, like camera images and LSM images cannot be aligned towards each other. Use either LSM images or camera images with identical frame format respectively, as input to this processing tool.
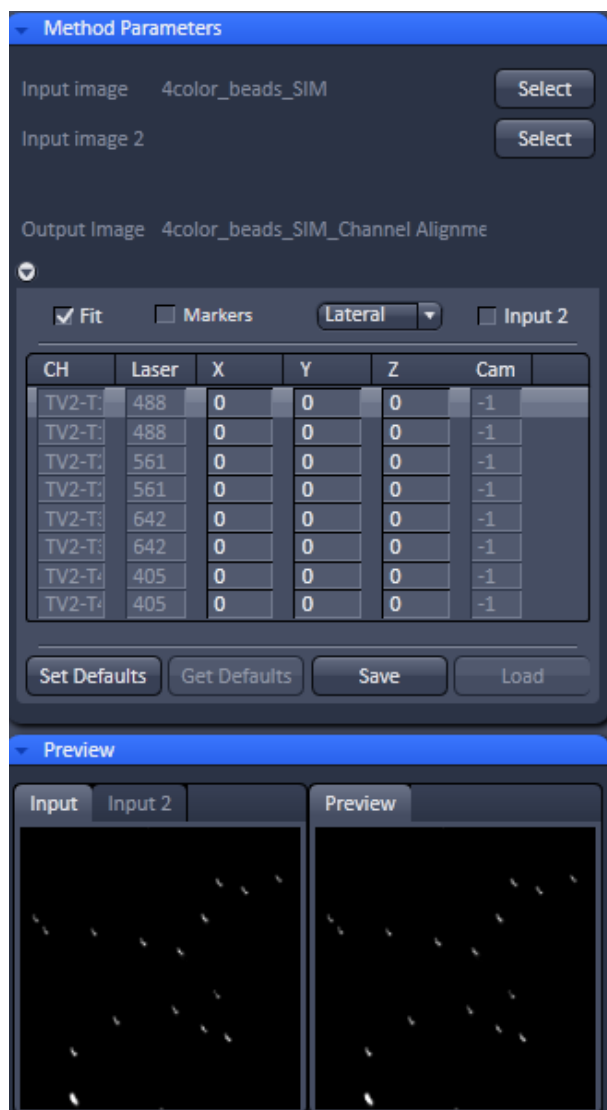
Fig. 391   Processing Channel Alignment tool field with one input image (with SIM and paired WF channels) selected

If the **Fit** check box is ticked and the **Input 2** checkbox inactive, than the software will align all channels of **Input image** having the same format to the first listed channel of the same format.

☞ Care should be taken about what channels will be aligned and in which order they appear. For SIM images the super resolution (**SR**) and the widefield (**WF**) channels are listed alternatively (pair wise). Since all channels are of the same format each channel (**SR** and **WF**) would be aligned to the first **SR** channel, since **SR** channels are normally listed before their corresponding **WF** channel. If this should be avoided, subsets of images containing only **SR** or **WF** channels have to be created in the Processing Tool **Copy Subset** and independently aligned. The situation is different for SMLM images. The high resolution (**HR**) and sum widefield (**SWF**) channels are also listed alternatively, with the **HR** channels preceding their corresponding **SWF** channels, however, the format of the **HR** channels is different to the one of the **SWF** channels. In this case the software aligns all **HR** channels to the first listed **HR** channel, which will be the first displayed channel, and all **SWF** channels independently to the first listed **SWF** channel, which is second in the list.

With the **Fit** check box ticked, the **coefficient table** lists as the first column the Channel name **Ch** and as the last two columns the used **Laser** line and camera channel **Cam**. In between are listed the corresponding correction coefficients. You have the choice from the **Alignment mode** drop down menu between **Lateral**, **Affine** and **Parabolic** alignment.

Markers do expect at least one ROI (Graphics function tab) in the image which will be used as the source for calculating the alignment.

Lateral (Fig. 392) is used to correct for linear lateral and axial drifts, that is in all directions. Linear alignments are performed in the x, y and z direction, when the **Applied** button is pressed. The respective offsets (coefficients) are displayed in the coefficient table in the **X**, **Y** and **Z** columns, respectively (Fig. 393).
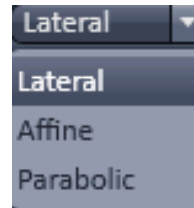
For **SIM**, **WF** and **SWF** images, the software will use significant structures for performing the alignment. You can set a ROI and then the structure within the ROI is used. For **HR** (SMLM) images at least one fiducial is expected. Without a fiducial, no alignment will be performed in this case.

Affine is used to correct for linear lateral (**X**, **Y**) and axial (**Z**) drifts, rotational offsets in the image plane in x and y (**RX**, **RY**) as well as a 1st order stretching in the image plane in x and y (**SX**, **SY**). The respective offsets (coefficients) are displayed in the **coefficient table** in the corresponding columns (Fig. 394). The scrollbar can be used to have access to all columns. For **SIM**, **WF** and **SWF** images, the software will use significant structures for performing the alignment. You can set a ROI and then the structure within the ROI is used. For **HR** (SMLM) images at least three fiducials are expected. Without fiducials, no alignment will be performed in this case.

Parabolic is used to correct for linear lateral (**X**, **Y**) and axial (**Z**) drifts as well as chromatic aberrations, where a 2nd order stretching in the image plane in x (**X1**, **X2**) and y (**Y1**, **Y2**) is performed. The respective offsets (coefficients) are displayed in the **coefficient table** in the corresponding columns (Fig. 395). The scrollbar can be used to have access to all columns. **Parabolic** only works with **HR** (SMLM) images and is displayed only in the **Alignment mode** drop down menu, when a **HR** (SMLM) image is selected as the **Input image**.

☞ The calculated coefficients in the **coefficient table** can be edited by hand. The correction will be exactly performed when pressing **Apply** according to the coefficients in the **coefficient table**.

☞ If the **Save** button is pressed, the **coefficient table** (with the coefficients) will be saved in a .bin file. These .bin file can be reloaded by pressing the **Load** button via the **Windows Explorer**. The **Load** button becomes only active when the **Fit** check box is not active.

Fig. 392   **Alignment mode drop down menu for selected SMLM image**

Fig. 393   **Correction table in the Lateral alignment mode**

Fig. 394   **Coefficient table in the Affine alignment mode**

Fig. 395   **Coefficient table in the Parabolic alignment mode**
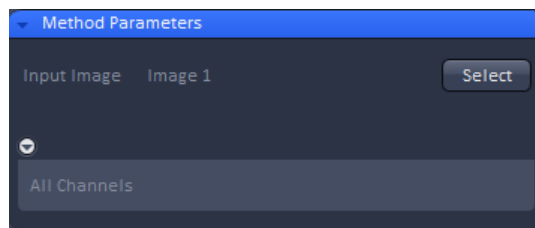
Fig. 396   Methods Parameter of the Copy Duplicat Processing tool
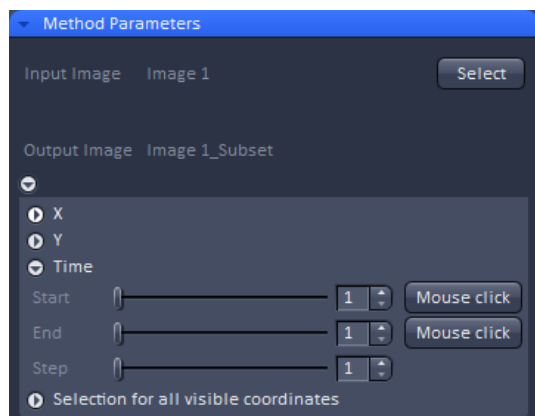
Fig. 397   Methods Parameter of the Copy Subset Processing tool
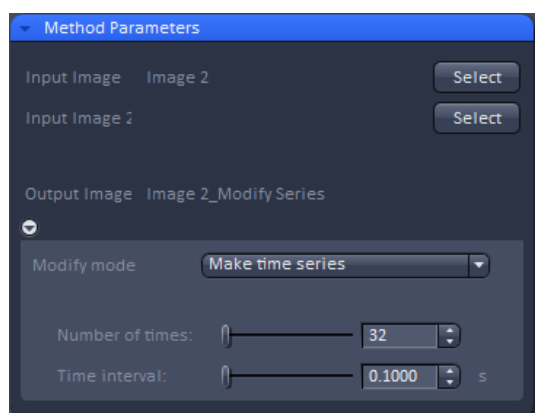
Fig. 398   Methods Parameter with Make time series selected in Modify mode

Using the channel alignment tool for drift corection

The following procedure can be used to remove lateral drift in an inage:

- Duplicate the time series. Select the time series in the processing tool **Copy** under the submenu **Duplication** (Fig. 396). Press **Apply** to obtain a copy from your time series.

- Generate reference image. Using the Processing tool **Copy** select the duplicate time series in the submenu **Subset**. Using **Time** select your reference image, for example the first image or the center image, by choosing for the **Start** and **End** value the same number (Fig. 397). Press **Apply** to extract that image from the time series.

- Generate reference time series. In the Proceeing tool **Modify** choose under **Modify mode** the entry **Make time series**. Select the exctracted image. Enter under **Number of times** and **Time intervals** the numbers from your original time series (Fig. 398) and press **Apply**. The result will be a time series with the same image at each time point.

- Apply drift correction. In the **Channel alignment** Processing function select the reference time series as **input 1** and the original time series as **input 2**. Check the **Fit** box and the **Input 2** box and leave **Marker** box unchecked (Fig. 399). Select **lateral** from the **correction mode selection** drop down menu. Press **Apply**. The resulting image will have a doubling in channels. The first half represents the original data set, the second half the drift corrected data set (Fig. 400). The number of channels depend on the number of channels of the original time series.

- Extract drift corrected channels. In the Processing tool **Copy** choose **Subset**. Select image with original and drift corrected channels. Select under **Channels** the second half of channels. Press **Apply** to obtain the final drift corrected image (without the original non-corrected channels).
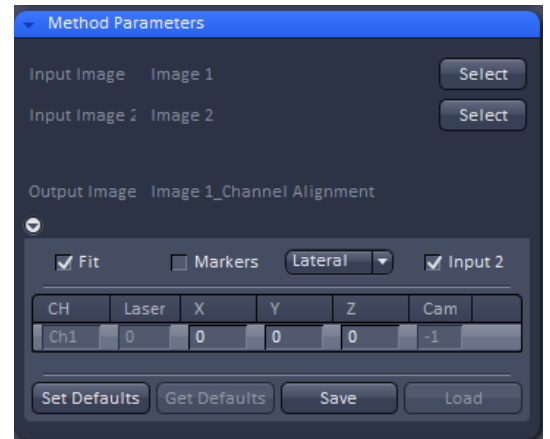
Fig. 399   **Methods Parameter of the Channel Alignment Processing tool**
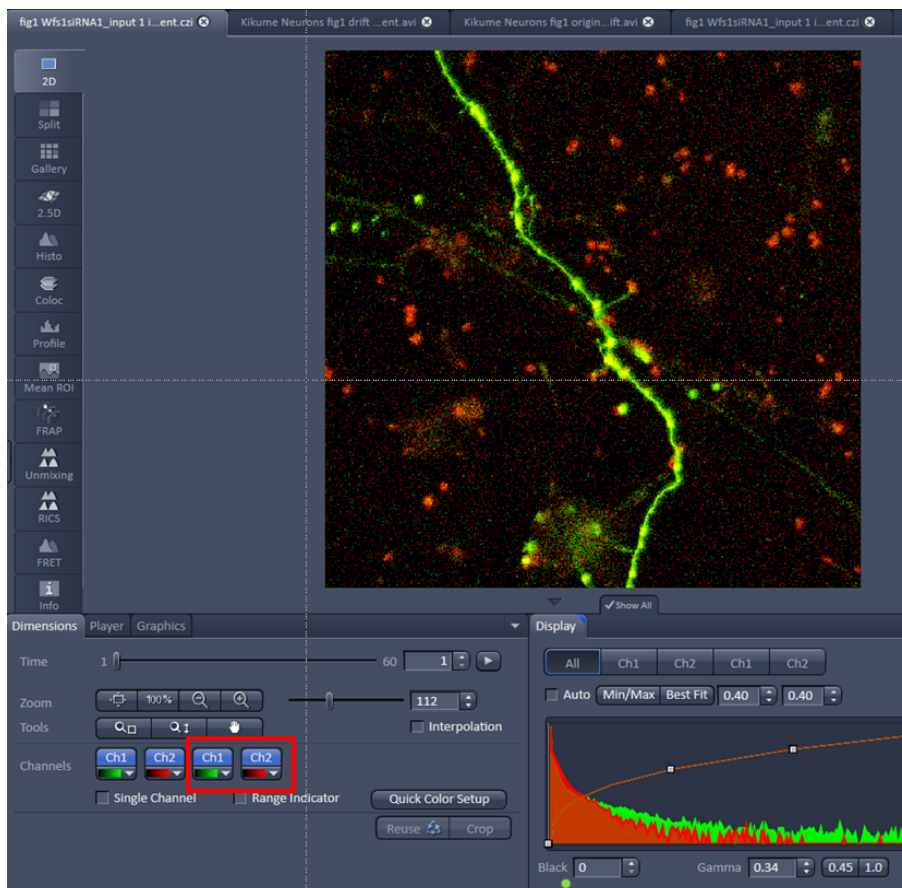
Fig. 400   **The boxed channels represent the drift corrected ones.**
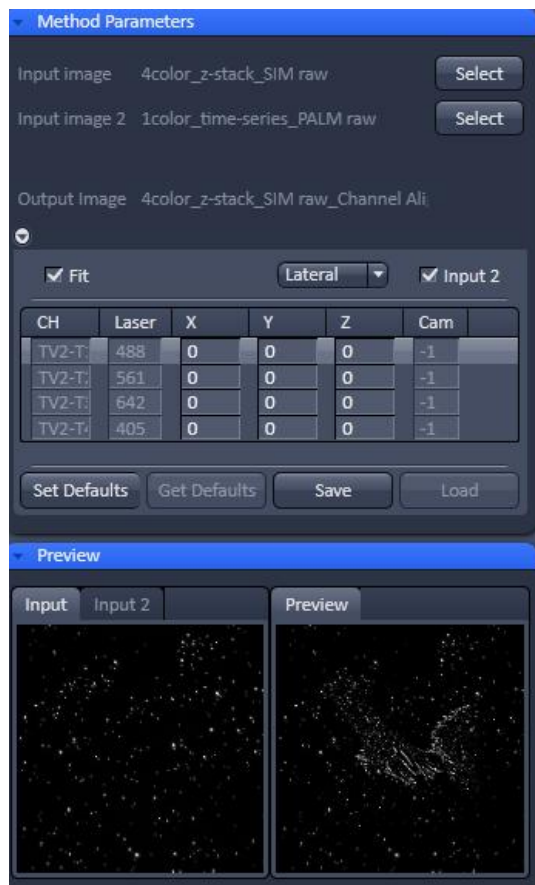
Fig. 401   **Two images mismatched in their dimensions (Z-Stack SIM raw and time-series SMLM) images loaded**

Fig. 402   **Coefficient table and assignment table**

☞ When the **Save Default** button is pressed, the present coefficients will be stored and can be recalled by pressing the **Restore default** button, which becomes active once the **Save default** button was pressed once.

If the **Input 2** check box remains inactive, the image selected for **Input image 2** is not considered for alignment. If the **Input 2** check box is ticked, than the channels of **Input image 2** are aligned to the corresponding channels of the **Input image** that is to those channels at corresponding positions in the list.

It is the responsibility of the user to align only images of the same dimensions and channels of the same format. If images will have different dimensions, for example a raw SIM Z-Stack and a raw SMLM time the system will give no warning message telling the user that the images do not match (Fig. 401). It will load both images and aligns the channels according to their position as long as there are matching channels.

☞ If no **Input image** was selected and only an **Input image 2**, no alignment will be performed.

☞ Loaded images cannot be unloaded but replaced by other images in selecting the respective image in the image container and then pressing the **Select** button for **Input image** or **Input image 2**.

If the **Fit check** box is not ticked a second table, the **assignment table** will be displayed below the **coefficient table** (Fig. 402). In this case the selected image will not be fitted directly, but a loaded alignment will be performed on the image. Columns in the **coefficient** and **assignment** tables can be expanded by dragging the borders of the columns.

The **coefficient table** in this case has to be loaded from a .bin file by pressing the **Load** button or is still available from a previous alignment or obtained by pressing the **Restore Default** button, if values have been previously restored by pressing the **Save default** button. In any case, the listed coefficients (no matter what **Alignment mode** is associated) will be applied to the selected image. If the **Input 2** check box is inactive, this will be the image selected for **Input image**. If the **Input 2** check box will be ticked, it will be the image selected for **Input image 2**.
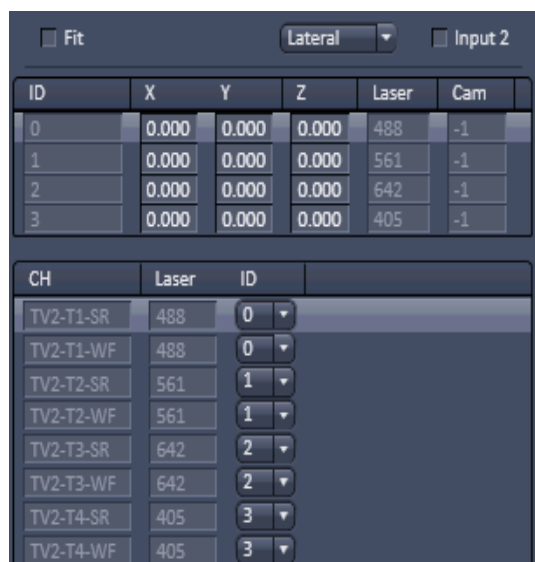
In either case, the other loaded image does not play a role. In the **coefficient table** the channel (**Ch**) assignment column is replaced by an identity (**Id**) column that numbers the channels in their ascending order starting with 0.

The assignment table represents the selected image, either **Input image** (**Input 2** check box inactive) or **Input image 2** (**Input 2** check box ticked).

Note that the dimensionality must not be the same as in the **coefficient** table. The **assignment table** lists the channels (**Ch**) and the laser lines used (**Laser**). With the identity (**ID**) drop down menu (Fig. 403), an ID from the **coefficient table** can be assigned individually to a channel. That channel will then be corrected exactly with the coefficients of that **ID**.

Fig. 403   **ID drop down menu**

If no alignment should be performed **do not align** can be chosen.

☞ The **channel alignment** tool is generically programmed to have high flexibility. Care should therefore be exerted in the control which channels are how aligned as there are no warnings, if unrelated channels are aligned to each other.

If you draw Regions of Interests (ROIs) in the image, they will be taken for alignemnt. For parabolic fit, you should have at least 3 well spaced regions. If you have fiducial markers, you can use them by setting the ROIs around them. Please be aware that in the z-direction the algorithm scans the whole z-Stack and takes the brightest area in th z-direction. If your beads are not the brightest structure, than the alignment will fail.

5.3.21   Processing – Lightsheet Processing

Licensing	Instrument
standard ✔ optional	LSM ELYRA ✔ Lightsheet Z.1

See full description of tool set for details.
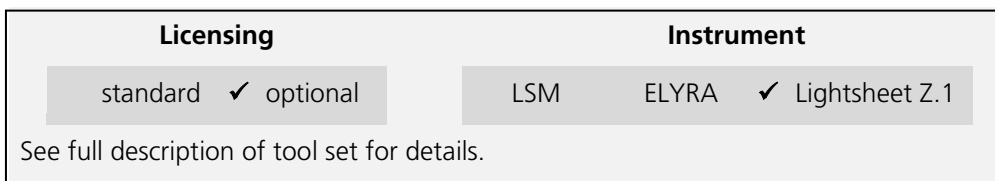
The **Lightsheet Processing** module allows one to process images that were acquired with the Lightsheet Z.1 using **Dual Side** illumination and/or with the **Multiview tool** tab.

Files need to be saved prior to any Lightsheet Processing task. If this is not the case the software will ask to save the data before proceeding.

☞   All images and registration files resulting from Lightsheet Processing can be found in a Result folder that is created in the folder of the original file, that was used for processing.
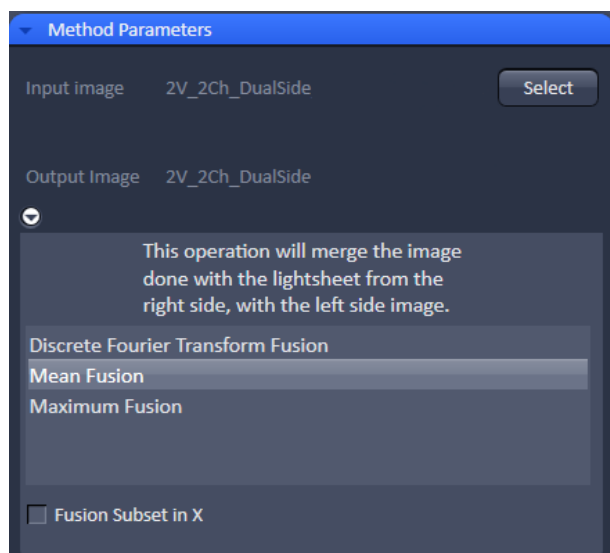
Fig. 404   Processing – Lightsheet Processing. Dual Side Fusion

5.3.21.1   Dual Side Fusion

This processing task can be used for the following datasets acquired with **Dual Side** illumination:

 – Image (Snap function)
 – Time Series
 – Z-Stacks
 – Combinations of Time Series and Z-Stacks
 – Multiview experiments as well in combination with Time Series

When a dataset has been acquired using **Dual Side** illumination, the dataset will have the illumination dimension saved in its metadata and the **Dimension slider** for Illumination is visible in the **Image Control area**. Both illumination sides are handled as single frames within the image. **Dual Side Fusion** provides three main options for merging both images from the different illumination sides into a single frame.

Choose the dataset you wish to perform the task by mark either option (Fig. 404).

Discrete Fourier Transform Fusion:

The fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003).

Fine structures and details are more visible after the fusion which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

After pressing Apply, a new .czi file will be generated. The original file name will be extended with "_Dual Side Fusion". For this resulting dataset, the illumination dimension is no longer available.

Mean Fusion:

The average intensity for each pixel is calculated using the values from both illumination sides.

Maximum Fusion:

The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X** can be activated (Fig. 405). With these controls it is possible to chose which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** within the image container (Fig. 406). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or spin box the area of the left illumination side is defined. This is marked at the slider itself with a red line, the spin box shows the pixel value of the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 406).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each view individually.
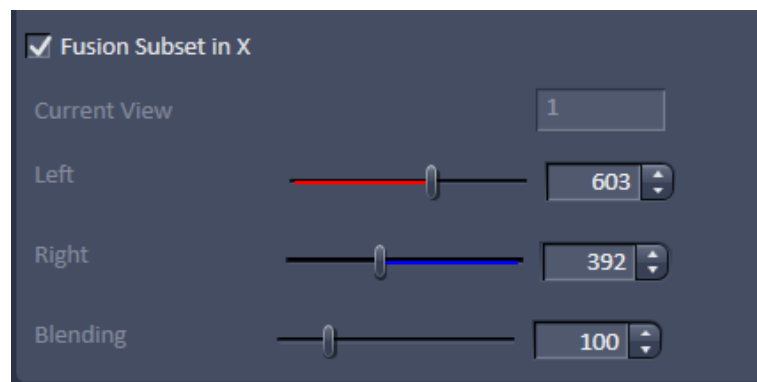
Fig. 405   Dual Side Fusion – Fusion Subset in X
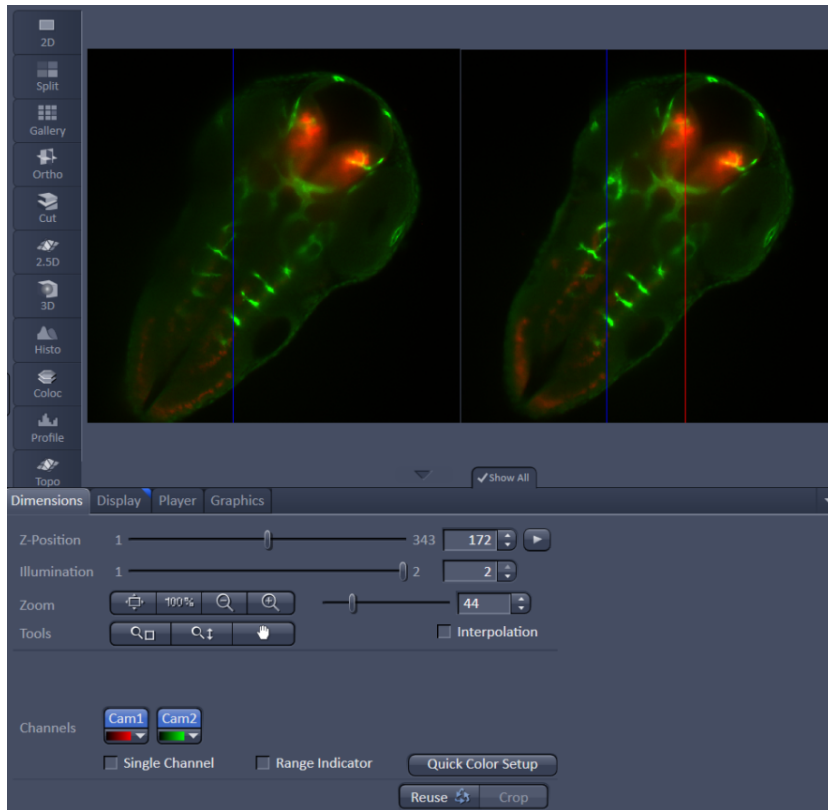
Fig. 406   Dual Side Fusion – Fusion Subset in X, Preview and Image control area

After pressing **Apply**, a new .czi file will be generated. The original file name will be extended with "_Dual Side Fusion". For this resulting dataset, the illumination dimension is no longer available.

5.3.21.2   Multiview Processing

5.3.21.2.1   Deconvolution

For the multiview datasets acquired with the Lightsheet Z.1, **Deconvolution** is an available option (Fig. 407).

The following datasets are available for this processing option:

a) Multiview experiments before registration and fusion (single or dual side illumination)

In this case, deconvolution is performed for each frame from all Z-Stacks and all Views. The process uses a point spread function (PSF) valid for single view images.

b) Multiview experiments after registration and fusion.

The deconvolution is performed on the fused dataset, hence only one Z-Stack needs to be processed. It uses a different point spread function (PSF) than what is used for the deconvolution before registration and fusion of a multiview experiment dataset.

The software automatically recognizes if the image has been registered and fused or not and adjusts the interfaces accordingly.

Fig. 407   **Processing – Lightsheet Processing. Multiview Processing, Deconvolution**

Two tabs are available for detailed configuring (**Deconvolutions** tab and **PSF settings** tab):

On the **Deconvolution** tab, select the desired algorithm and define the precise settings for it.

Use the drop-down list next to Algorithm to choose from the available deconvolution algorithms:

Nearest Neighbour: The Nearest neighbor method uses the simplest and fastest algorithm (Castleman, K.R., Digital image Processing, Prentice-Hall, 1079). Its function is based on subtraction of the out-focus information in each plane of a stack, taking the neighboring sections above and below the corrected Z-plane into account. This method is applied sequentially to each plane of the entire Z-Stack. It allows you to enhance contrast quickly, even if image stacks have not been put together optimally.

Regularized Inverse: The inverse filter is a genuine 3D method and generally achieves better results than the Nearest neighbor algorithm. It essentially involves dividing the Fourier transformation ("FT") of the volume by the FT or the PSF, which can be performed very quickly. In the real space, this corresponds to a deconvolution. In addition, a statistical method ("General Cross Validation – GCV") is applied which determines the noise component of the image and automatically sets the restoration strength to the optimum level in line with this. This process is also known as regularization. Z-Stacks must have been acquired at the correct (Nyquist or smaller) distance. The additional acquisition of z-planes above and below the structure of interest is recommended.
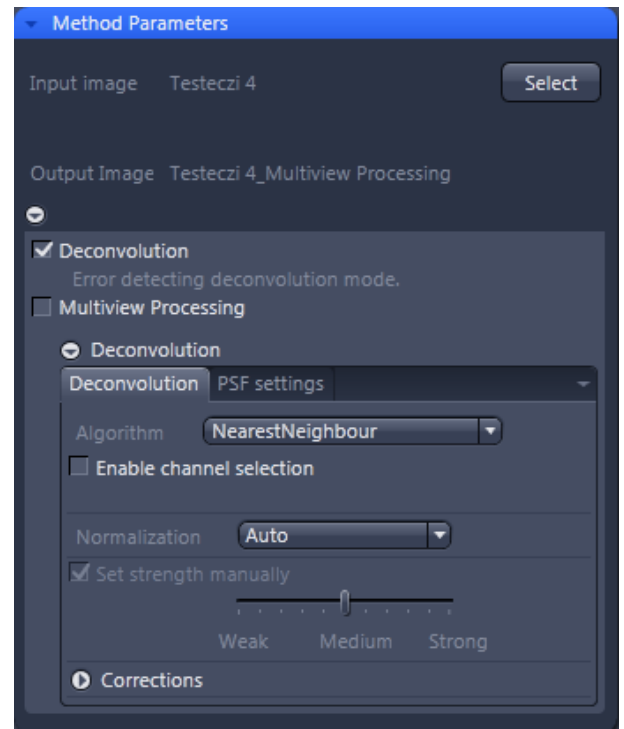
Fast Iterative: The "Fast Iterative" method is an iterative restoration method that uses only one iteration per convolution step (see Meinel, E.S.: Origins of linear and nonlinear recursive restoration algorithms. J. Opt. Soc. Am, 3 (&), 1986, 787-799). No regularization is used in this case. Due to the fast processing and convergence after just a few iterations, this method is suitable in particular for the processing of larger time lapse images. The results of the method can quickly lead to good results and remove most of the out-of-focus light. They do not create quantitative brightness conditions in the image. If under-sampled images are present, artifact formation may also result.

Constrained Iterative: The best image quality is achieved using the iterative maximum likelihood algorithm (see Schaefer et al.: "Generalized approach for accelerated maximum likelihood based image restoration applied to three-dimensional fluorescence microscopy", J. of Microscopy, Vo. 204, Pt2, November 2001, pp. 99ff.): This algorithm is able to calculate light from various focal planes back to its place of origin. Consequently, with this method it is possible to derive the 3D structure from fluorescence images with the correct brightness distribution and to visualize optical sections. It is also possible for missing information to be partially restored from neighboring voxels. The spatial resolution can be increased without artifacts up to a theoretical limit (one voxel). It is essential for Z-Stacks to have been acquired in accordance with Nyquist. Acquiring sufficient planes above and below the structure of interest is also imperative for achieving good results. As this is a complex mathematical method, the calculation can take longer, depending on the image size.
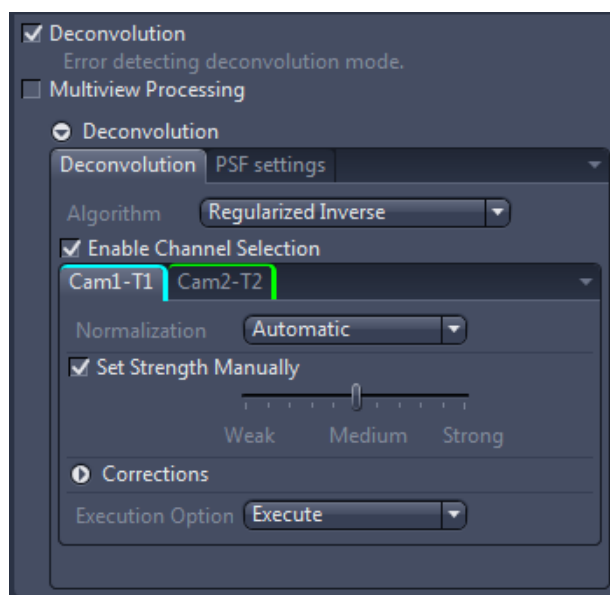
Fig. 408   Processing – Lightsheet Processing. Multiview Processing, Deconvolution, Enable channel selection

If the checkbox **Enable channel selection** is activated, the settings are applied on a channel-specific basis. This allows you to set parameters in a targeted way for each channel. You will see a separate colored tab for each of the channels (Fig. 408).

In the **Normalization** drop-down list, you can specify how the data from the resulting image are handled:

Clip: For non-registered and non-fused multiview images, this should be used as the default setting. It sets negative values to 0 (black). If the values exceed the maximum possible gray value of 65636 when the calculation is performed, they are limited to 65636 (pixel is 100% white).

Automatic: Normalizes the output image automatically. Not to be used for non-registered and non-fused multiview images.

In this case, the lowest value is 0 and the highest value is the maximum possible gray value in the image (gray value of 65636). The maximum available gray value range is always utilized fully in the resulting image.

Adjust Strength checkbox:

If you have selected the Nearest Neighbor algorithm or Fast Iterative, the set strength manually checkbox is always activated.

With the other Algorithm options, you can activate or deactivate this option using the check box next to **Set strength manually**. When activated, you can enter the degree of restoration using the slider.

To achieve strong contrast enhancement, move the slider towards Strong.

To achieve less contrast enhancement, move the slider towards Weak.

If the setting is too strong, image noise may be intensified and other artifacts, such as "ringing", may appear.

If **Set strength manually** is deactivated, it determines the restoration strength for optimum image quality automatically. The restoration strength is inversely proportional to the strength of the so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).

To expand the options for **Corrections**, use the black arrow-head button. Here you can find three additional corrections which can be activated or deactivated using the adjacent check boxes.

Lamp Flicker checkbox: Analyzes the total brightness of each z-plane. In the event of non-constant deviations in the total brightness between neighboring planes, a compensation factor is taken into account. Activate this function if you have acquired your images using an old fluorescent lamp that exhibits strong fluctuations in brightness.

Decay: Corrects changes in brightness (e.g. bleaching) of the sample during acquisition of the Z-Stack. This function should only be activated for widefield images. Use it if your sample undergoes strong bleaching during acquisition.

Background Correction checkbox: Analyzes the background component in the image and removes it before the DCV calculation. This can prevent background noise being intensified during DCV.

On the **PSF settings** tab (Fig. 409), you can see and change all key parameters for generating a theoretically calculated Point Spread Function ("PSF").

☞ If an image is used that is not registered and not fused, a point spread function (PSF) is used that is specific for single view images (single view PSF) for the Lightsheet system. If an image is loaded that has already been registered and fused, a specific PSF is used for this dataset. This multiview PSF is calculated using the information on how many views and which rotations were used to acquire this particular dataset, taken from the image metadata.

Ordinarily, images that have been acquired using ZEN (stored as *.czi) automatically contain all system parameters and image information, meaning that you do not have to configure any settings on this page. Most parameters are therefore grayed-out in the display. It is possible, however, that as a result of an incorrect microscope configuration, values may not be present or may be incorrect. You can change these here. The correction of spherical aberration can also be set here.
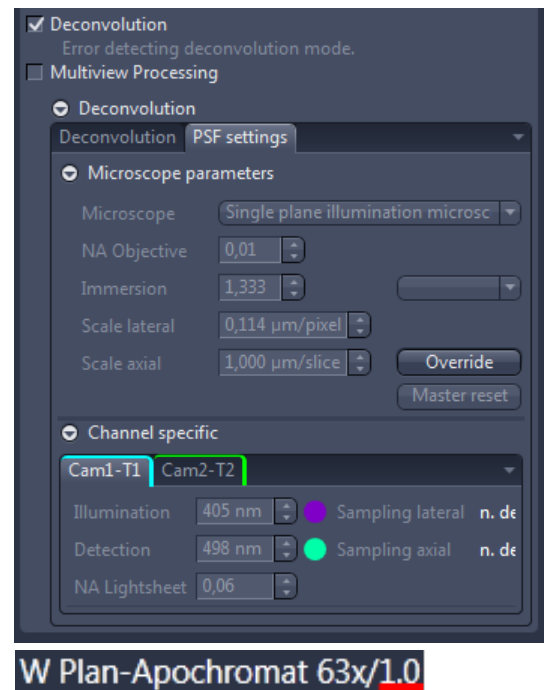
Fig. 409 Processing – Lightsheet Processing. Multiview Processing, Deconvolution, PSF settings

To edit any of the input boxes, press the **Override** button. To get back to the settings provided by the software, press the **Master Reset** button.

In the **Microscope Parameters** section are the most important system parameters for PSF generation that are not channel-specific displayed.

You can select the system used for data acquisition in the **Microscope dropdown** list. There are three options: Single plane illumination microscope, conventional microscope (also known as a widefield microscope), and confocal microscope for which the additional pinhole diameter parameter applies.

Dimension:

You can view and change the numerical aperture for the detection, which results as a combination of the detection optic and the used zoom setting, in the **NA Obj.** input field. So, it is not the numerical aperture of the detection optic alone which is indicated on the lens itself or displayed in the software.

The **Immersion input** field displays the refractive index of the immersion medium. Please note that this can never be smaller than the numerical aperture of the objective. You can make a selection from typical immersion media in the dropdown list next to the input field.

The **Scale lateral** input field displays the geometric scaling in the X/Y direction.

The **Scale axial** input field displays the geometric scaling in the Z direction.

When scaling in this window is changed it will override the settings chosen in the **Multiview Processing** window (Fig. 410).

In the **Channel specific** section, you will find all settings that are channel-specific. This means that they may be configured differently for each channel.

The **Illumination** input box holds the excitation wavelength for the channel [in nm]. The maximum is assumed. The color field corresponds to the wavelength (as far as possible).

The **Detection** input box represents the maximum of the emission wavelength for this channel dye. The color field corresponds to the wavelength (as far as possible).

The **NA Lightsh.** input box displays the numerical aperture (NA), which is resulting from a combination of the illumination optics and the zoom settings used during image acquisition.

5.3.21.2.2   Registration Options

The **Registration Options** check-box next to **Multiview Processing** opens the interface to define the settings for the Registration **Parameters**, the **Input settings**, and the **Fusion options** for the data (Fig. 410). Each interface is accessed by pressing the ⊙ button next to it.

The following datasets can be processed with this task:

– Multiview datasets, single side illumination

– Multiview datasets, dual side illumination, Dual Side without Fusion

– Multiview datasets, dual side illumination, Dual Side with Fusion

– Multiview datasets, with Deconvolution

– Multiview datasets, without Deconvolution

The first step is to select the **Registration Options** using the drop-down menu. The choice will influence the **Parameters** interface.

The **Registration** will generate the transformation matrices so that all views are matched to overlay. As a result, a dataset is provided in which all Z-Stacks of all views should already largely overlay. The reference at which registrations starts is always View 1 of the dataset. This image can be used as a control. If one or more views do not have the same alignment, registration was not successful and the parameters must be adjusted.
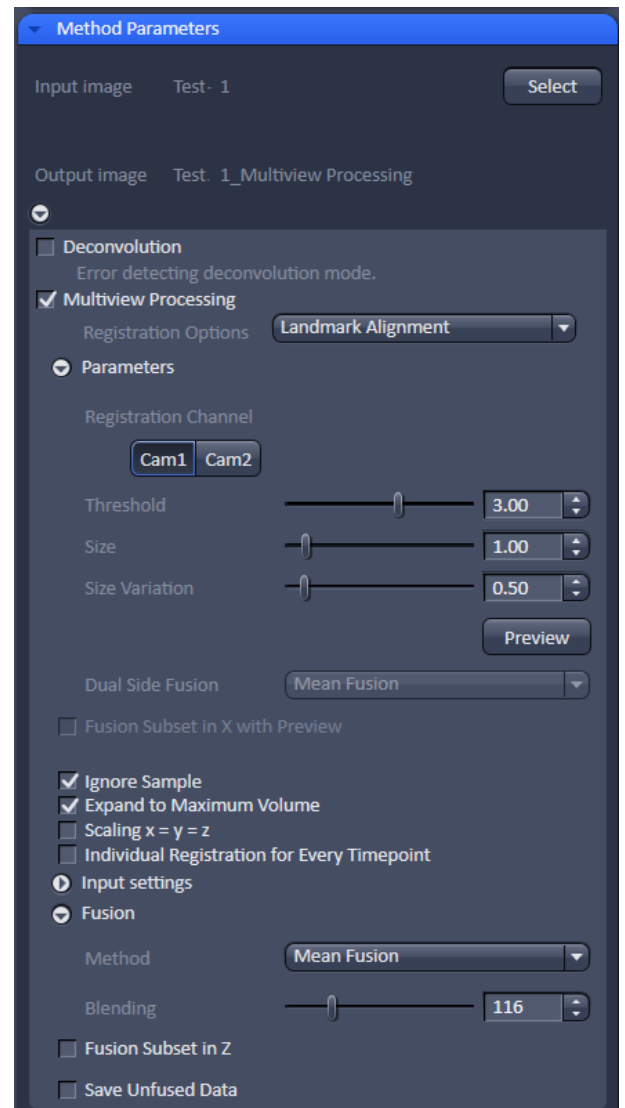
Fig. 410   **Processing – Lightsheet Processing. Multiview Processing**

When **Apply** is pressed and the registration process has finished, the transformation matrices are saved as an .xml file (Filename.czi.xml). Only the transformation matrix is saved if **Apply** is pressed and **None** is selected under **Fusion** (see below). This .xml file can be used for the **Use Registration From File** option.

The **Registration** must always be done before the **Fusion** of the data.

Registration Options are:

– Landmark Alignment

– Intensity Based Alignment

– Use Registration From File

– Interactive Registration
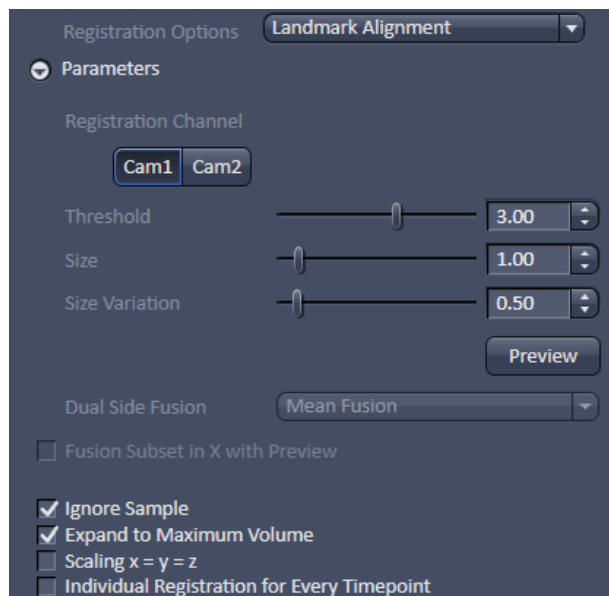
Fig. 411 Processing – Lightsheet Processing. Multiview Processing, Landmark Alignment Parameters

5.3.21.2.2.1 Landmark Alignment

For the **Landmark Alignment** (Fig. 411), the sample must have been embedded with appropriate fluorescent beads (fiducials) which can be recognized by the software. The beads can be present in the same channel as the image information. Another option is to image the beads alone in a second channel.

Select the Channel in which the beads are present by clicking the according **Registration Channel** button. One can select more than one channel for the registration process. For each chosen channel and individual registration will be performed; it is therefore advisable to use one channel for the final registration. To test which channel will lead to the best results, all channels can be chosen for later comparison of the results.

To define which structures within the images are the beads to be used for the Landmark Alignment, use the sliders and input boxes with arrows of

Threshold: brightness of beads,

Size: size of the beads, and

Size Variation: how much do the beads differ in size.

All values with these parameters are arbitrary units.

To check if the settings recognize the beads of the image, press the **Preview** button. This will circle all elements recognized as beads in the chosen and displayed image frame. Use the sliders in the **Dimensions View** control to change to a different view, z-slice, and time point and press again the **Preview** button. Control by using several different frames to see if the beads are recognized correctly before proceeding. Adjust the settings for **Threshold**, **Size**, and **Size Variation** until this is accomplished.

If your data was acquired with dual side illumination which has not yet been fused, the method to fuse both illumination sides is available in the **Dual Side Fusion** drop-down menu (**Mean Fusion**, **DFT Fusion**, **Maximum Fusion**).

Mean Fusion: The average intensity for each pixel is calculated using the values of both illumination sides.

DFT Fusion (Discrete Fourier Transform Fusion): The Fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003). Fine structures and details are more visible after the fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

Maximum Fusion: The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X with Preview** can be activated (Fig. 412). With these controls it is possible to define which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** in the image container (Fig. 413). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X with Preview** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or input box the area of the left illumination side is chosen. This is marked at the slider itself with a red line, the input box shows the pixel value along the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 413).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each view individually.
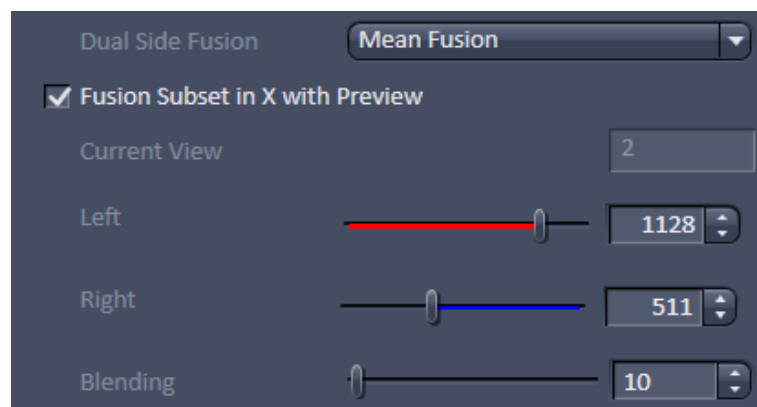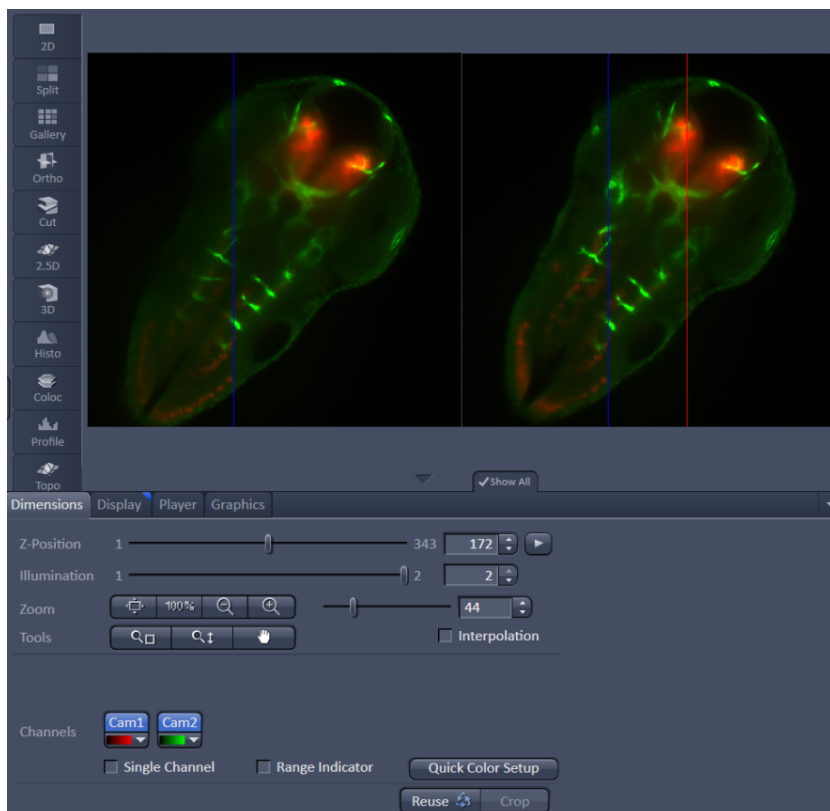

Fig. 412   Dual Side Fusion – Fusion Subset in X

Fig. 413   Dual Side Fusion – Fusion Subset in X, Preview and Image control area

Ignore Sample: This option activates an additional function to find structures within the sample that were incorrectly labeled as beads (fiducials). These structures are not taken into account during the Landmark Alignment process; but they are not removed from the **Preview**.

Expand To Maximum Volume: This checkbox is activated as default. In a multiview experiment not all views might include all the information. When **Expand To Maximum Volume** is activated, the volume of the resulting dataset after registration and fusion is determined and additional black pixel (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

Scaling x=y=z: The dimensions are downsized to the smallest to have the same size. If the scaling in **Deconvolution, PSF settings** (Fig. 409) is changed, the latter will override the scaling settings here.

Individual Registration For Every Time point: If the multiview experiment has been performed over time, this option becomes available.

– If **activated**, each time point generates a transformation matrix and each time point is registered individually. As a result, the time necessary for registration is multiplied by the number of time points and can rise substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the Individual Registration For Every Time point option becomes necessary.

– If **not activated**, one transformation matrix is generated for all the following time points based on the first time point. As a result, processing for registration has only to be done once which reduces processing time.

5.3.21.2.2.2   *Intensity Based Alignment*

The **Intensity Based Alignment** option does not need any fluorescent beads (fiducials) present in the sample since registration is done based on structural information within the image content. It is therefore advisable to select the **Registration Channel** which contains clearly defined fluorescent structures within the image. One can chose more than one channel for registration. For each chosen channel and individual registration will be performed; it is therefore advisable to use one channel for the final registration. To test which channel will lead to the best results, all channels can be chosen for later comparison of the results.

Registration can be done as a two-step process with a preceding **Pre-Registration** and afterwards the **Registration** (Fig. 414).

The **Pre-Registration** performs the process in a 2D maximum intensity projection of the Z-Stacks; while the **Registration** algorithm uses the complete 3D information of the Z-Stacks.

Fig. 414   **Processing – Lightsheet Processing. Multiview Processing, Intensity Based Alignment, Parameters**

☞ It is advisable to perform first a **Pre-Registration** to move similar structures already into close proximity, which might facilitate the **Registration**.

You can select the following **Pre-Registration Method**s from the drop down menu:

None: No Pre-Registration is performed. Registration is then only done with the settings chosen in the Registration Method menu.

Hardware Rotation: The Views are rotated solely based on the information from the rotation positioning of the sample holder which are saved in the metadata.

Trans Hardware Rotation: The Views are rotated solely based on the information from the rotation positioning of the sample holder which are saved in the metadata. Additionally the x, y, and z axis are moved to match the Views.

Correlation Hardware Rotation: The Views are rotated solely based on the information from the rotation positioning of the sample holder which are saved in the metadata. Additionally the x, y, and z axis are moved to match the Views. Cross-correlation is used to determine the translation in x, y, and z.

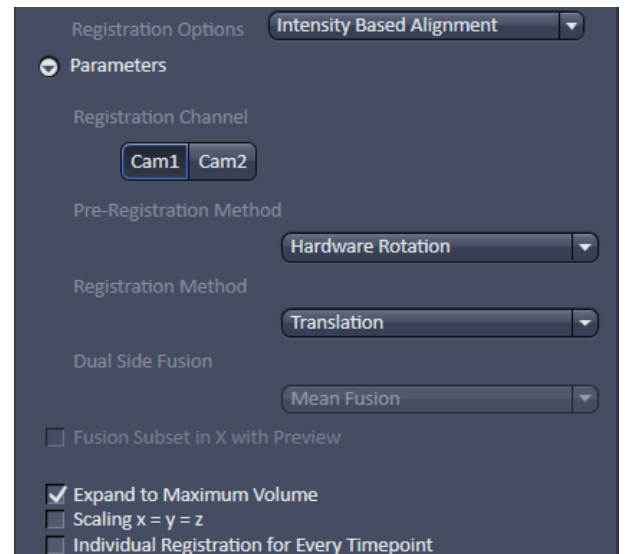To select the **Registration Method**, use the drop-down menu.

Use Pre Register Only: No Registration process is performed; only the result of the Pre-Registration is used.

Translation: The registration will correct along the x, y, and z axis only.

Trans Rotation: The registration will correct along the x, y, and z axis and also the rotation along x and y.

If your data was acquired with dual side illumination which has not yet been fused, the method to fuse both illumination sides is available in the **Dual Side Fusion** drop-down menu (**Mean Fusion**, **DFT Fusion**, **Maximum Fusion**).

Mean Fusion: The average intensity for each pixel is calculated using the values from both illumination sides.

DFT Fusion (Discrete Fourier Transform Fusion): The Fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003). Fine structures and details are more visible after fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

Maximum Fusion: The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X with Preview** can be activated (Fig. 415). With these controls it is possible to define which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** in the image container (Fig. 416). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X with Preview** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or input box the area of the left illumination side is chosen. This is marked at the slider itself with a red line, the input box shows the pixel value along the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 416).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each view individually.
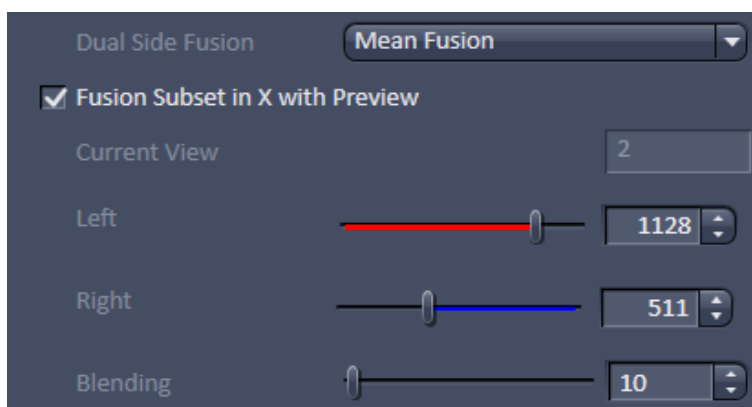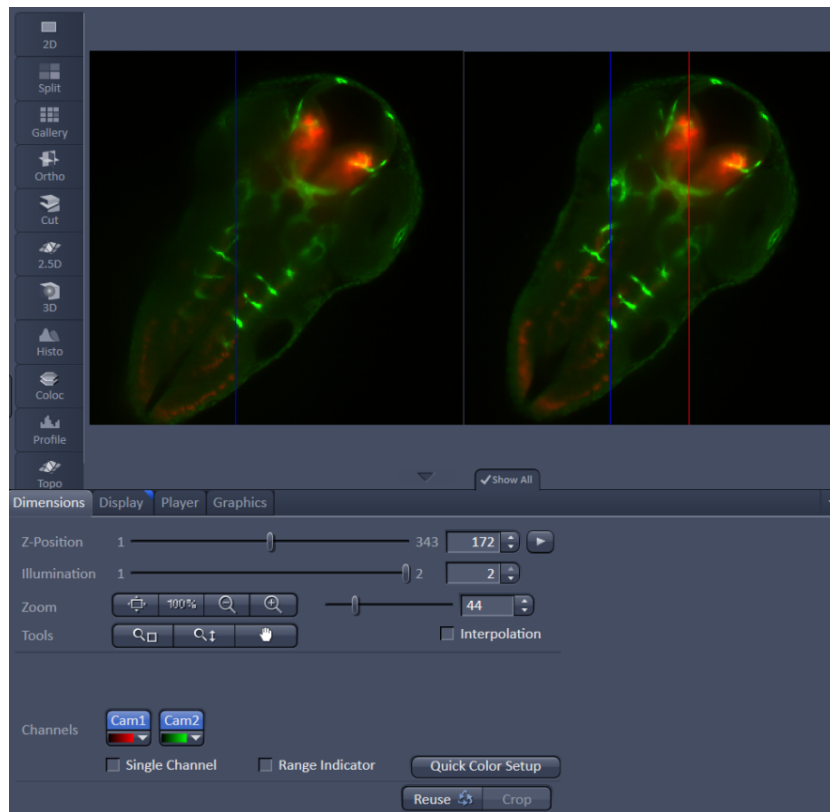

Fig. 415   Dual Side Fusion – Fusion Subset in X

Fig. 416   Dual Side Fusion – Fusion Subset in X, Preview and Image control area

Expand To Maximum Volume: This checkbox is activated as default. In a multiview experiment not all views might include all the information. When **Expand To Maximum Volume** is activated, the volume of the resulting dataset after registration and fusion is determined and additional black pixel (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

Scaling x=y=z: The dimensions are downsized to the smallest to have the same size.

Individual Registration For Every Time point: If the multiview experiment has been performed over time, this option becomes available.

– If **activated**, each time point generates a transformation matrix and each time point is registered individually. As a result, the time necessary for registration is multiplied by the number of time points and can rise substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the Individual Registration For Every Time point option becomes necessary.

– If **not activated**, one transformation matrix is generated for all the following time points based upon the first time point. As a result, processing for registration has only to be done once which reduces processing time.

5.3.21.2.2.3 Use Registration From File

When the Registration for the dataset has been determined already and the transformation matrix has been saved as an .xml file (Filename.czi.xml), this option can be chosen. Click on the button next to **Registration File** to open a Windows Explorer window in which you can find and select the file.

With the drop down menus, the **Interpolation** mode and **Dual Side Fusion** processing can be chosen (see as well **Landmark Alignment or Intensity Based Alignment** section).

Expand To Maximum Volume: This checkbox is activated as the default. In a multiview experiment, the Z-Stacks are often of different sizes. When **Expand To Maximum Volume** is activated, it will use the maximum slice number (from one View) present in the dataset for registration. The additional z-slices needed in other views will be filled with black frames. This will ensure that no data is lost from views with larger z-dimensions. If this checkbox is not activated, the number of z-slices from the first View is taken as the default and larger Z-Stacks will be cut; there is a risk of data loss.

Scaling x=y=z: The dimensions are downsized to the smallest to have the same size.
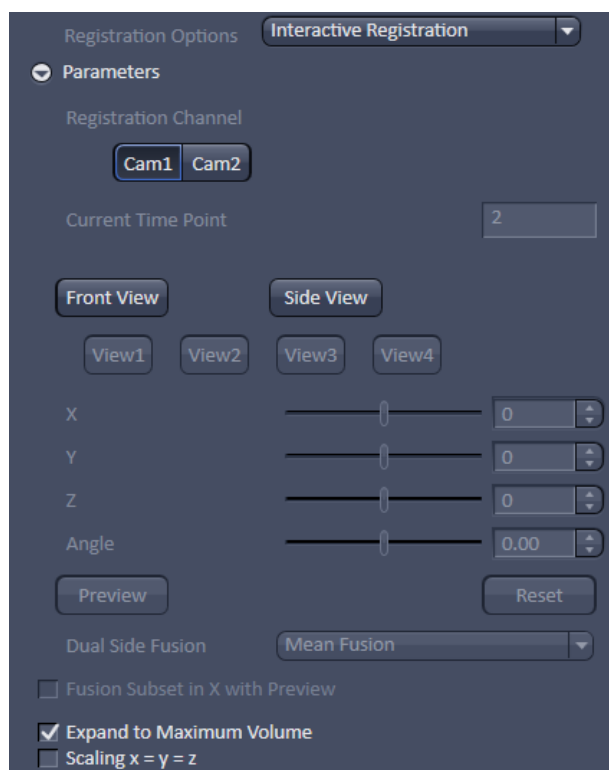
Fig. 417 **Registration Options: Interactive Registration**
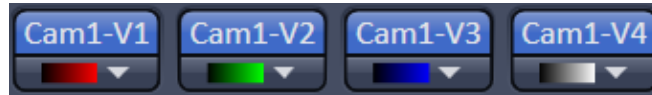
5.3.21.2.2.4 Interactive Registration

Interactive Registration allows to register the recoreded views of an experiment manually, when the other alignment options fail for that dataset (Fig. 417). The views of one channel at one time point of the experiment are the basis for the procedure. The z-stacks of the individual views are rotated based on the rotation information of the files meta data and transformed into channels resulting in one maximum intensity projection image. Within this newly generated image the views, now behaving as channels, can be moved in x, y, z and rotated to overlay each other. When all views are layed on top of each other, the **Apply** button will use the adjusted parameters to register, and when required fuse, the views for all time points of the experiment. An .xml file is created which can be used for the **Use registration from file** option. This .xml file can be found along with the processed data in the Result folder.

The first step is to chose the **Registration Channel** that should be used to manually overlay all views of the experiment by pressing the according button, e.g. **Cam1**. Only one channel can be used for this procedure, all other channels will not be diplayed. If the experiment consists of a time series, one time point is selected with the **Time** slider and spin box of the **image controal area**. This chosen time point is indicated in the **Current Time Point** box (Fig. 417).
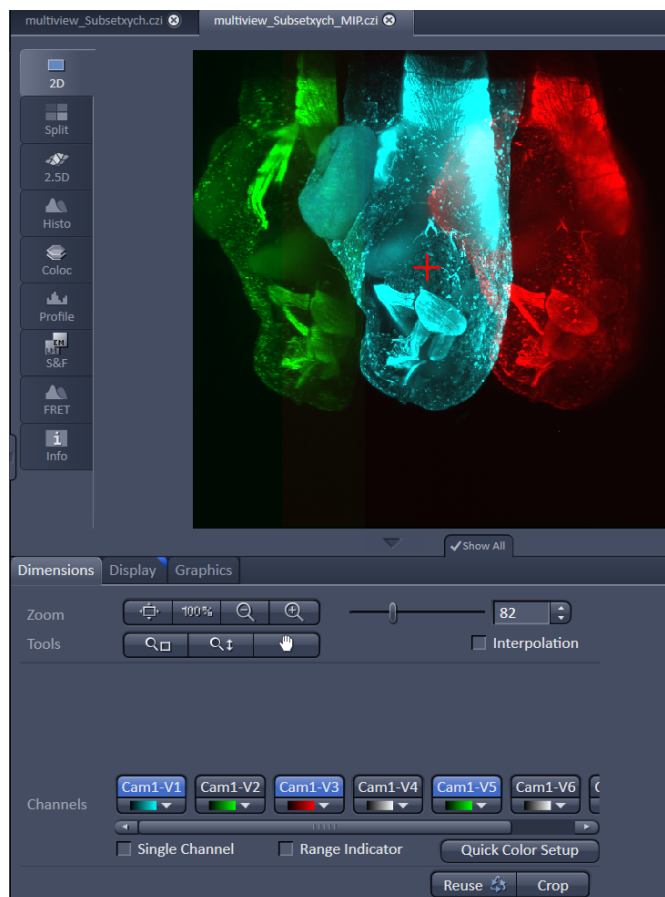
To generate the maximum intensity projection image either the **Front View** or the **Side View** button can be pressed. In the resulting maximum intensity projection image, each view is handeled as a channel (e.g. Cam1-V1, Cam1-V2) of the image, a color can be assigned to each channel and it can be turned on or off in the **image control area** (Fig. 418).

☞ Be aware, the calculation of the maximum intensity projection of the **Front View** or **Side View** will take some processing time, depending on the size of the used dataset.

Fig. 418   **Interactive Registration – Views into Channel conversion of the maximum intensity projection image**

It is of no consequence with which of the maximum intensity projections the process is started. In case of using the **Front View** button, the maximum intensity projection is calculated along the z-axis and the x,y axis are displayed in the resulting image (Fig. 419). The **Side View** button results in a maximum intensity projection along the x-axis, displaying the z- (horizontal) and y- (vertikal) axis. The maximum intensity projection image is opend in a new **image window**, named Filename_MIP.czi. The following steps are done on the basis of the maximum intensity projection, hence in this **image window**.

Fig. 419   **Interactive Registration – Maximum intensity projection (Front View) image with red cross overlay for view rotation (views Cam1-V1, Cam1-V3 and Cam1-V5 are visible in the image)**

View 1 is the master view and cannot be moved during the process, the button **View 1** is therefore inactive and grayed out. Any of the other views can be activated by pressing the according button (e.g. **View2**, Fig. 417). The activated view is automatically displayed in the image (the associated channel is turend on). To move the view relatively to View 1 the sliders and spin boxes can be used; **X** for the x-axis, **Y** for the y-axis, **Z** for the z-axis and **Angle** for a roation. The axis that is not present in the maximum intensity projection is grayed out and inactive (**Z** for the **Front View** and **X** for the **Side View**).

Addtitionally to the rotation **Angle**, the position of the rotation point can be defined. This is done by positioning the **red Cross overlay** within the maximum intensity projection image window to the desired position, the view should be rotated around (Fig. 419).

The **Reset** button moves all views back to the position they were at, as the maximum intensity projection was generated.

Each view should be activated once with its corresponding button and positioned, resulting in a matching overlay to **View 1**. Afterwards the maximum intensity projection is switched to either **Front View** or **Side View**; displayed in the same image window.

Again all views must be moved using the according sliders **Y**, **Z** and **Angle** to produce a perfect overlay to View 1.

Switching from **Front View** to **Side View** should be repeated at least twice, to adust the positioning of the views.

To controll if the manual alignment is satisfactory, the **Preview** button produces a new image of the channel and the time point, which is registered with the provided settings. The **Preview** image will contain fused views, when **Mean Fusion** has been selected at the **Fusion Method** drop down list.

If the result is satisfactory, one can proceed with the remaining settings for **Multiview Processing**.

If the data was acquired with dual side illumination, which has not yet been fused, the method to fuse both illumination sides is available in the **Dual Side Fusion** drop-down menu (**Mean Fusion**, **DFT Fusion**, **Maximum Fusion**).

Mean Fusion: The average intensity for each pixel is calculated using the values of both illumination sides.

DFT Fusion (Discrete Fourier Transform Fusion): The Fusion is performed based upon the published method ("Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003). Fine structures and details are more visible after the fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.

Maximum Fusion: The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image.

For **Mean Fusion** and **Maximum Fusion** the checkbox **Fusion Subset in X with Preview** can be activated (Fig. 420). With these controls it is possible to define which area of the left and the right illumination image should be used for fusion.

When this function is chosen, the image is displayed in the **Preview Tab** in the image container (Fig. 421). In this preview mode the left hand image shows the original dataset and the right hand image the preview of processing. Using the **dimension sliders** within the **image control area** the **Z-Position**, **Illumination** side and **View** can be chosen.

In **Fusion Subset in X with Preview** the currently chosen View is displayed in the **Current View** box.

With the **Left** slider or input box the area of the left illumination side is chosen. This is marked at the slider itself with a red line, the input box shows the pixel value along the y axis. Furthermore the cut off is shown in the preview image with a red line for easy orientation. It is best to set the **illumination slider** of the **image control area** to 1, showing the left side illumination of the original data.

Repeat the process with the **Right** slider and input box; here the marking lines are in blue and the **illumination slider** in the **image control area** should be placed to 2, showing the right side illumination of the original data.

The **Preview** in the image container will show the position of both sliders **Left** and **Right** as red and blue lines respectively (Fig. 421).

To avoid a visible change in intensity along the two cut off lines, for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image and the **Blending** slider should be used to define the number of pixels used within this overlap to crossfade both illuminations sides.

The position of the sliders **Left** and **Right** as well as the **Blending** value must be defined for each View individually.
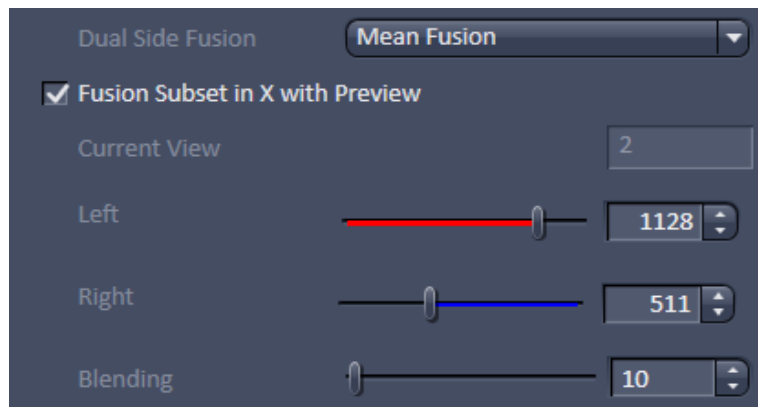
Fig. 420   Dual Side Fusion – Fusion Subset in X
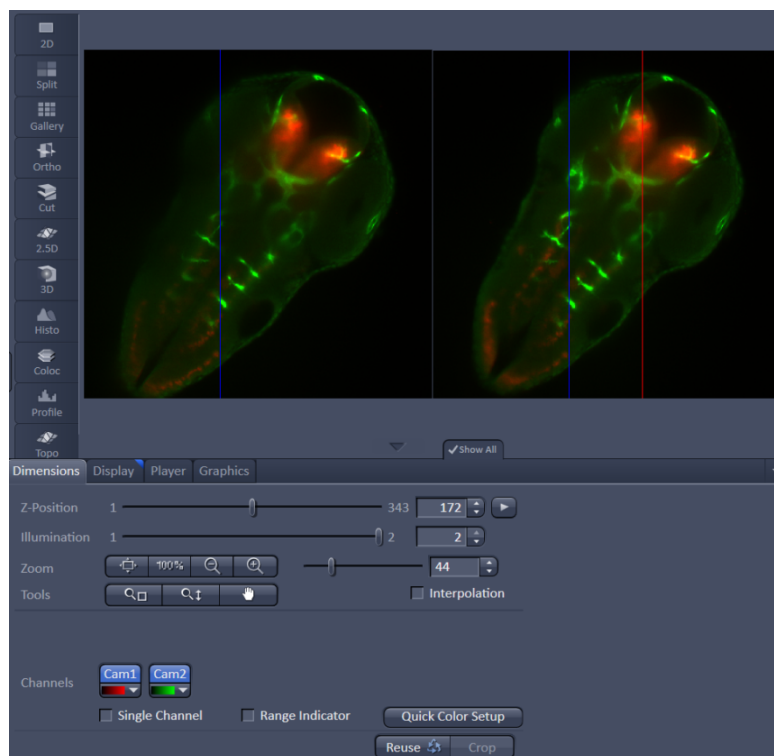
Fig. 421   Dual Side Fusion – Fusion Subset in X, Preview and Image control area

Ignore Sample: This option activates an additional function to find structures within the sample that were incorrectly labeled as beads (fiducials). These structures are not taken into account during the Landmark Alignment process; but they are not removed from the **Preview**.

Expand To Maximum Volume: This checkbox is activated as default. In a multiview experiment not all views might include all the information. When **Expand To Maximum Volume** is activated, the volume of the resulting dataset after registration and fusion is determined and additional black pixel (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

Scaling x=y=z: The dimensions are downsized to the smallest to have the same size. If the scaling in **Deconvolution, PSF settings** (Fig. 409) is changed, the latter will override the scaling settings here.

Individual Registration For Every Time point: If the multiview experiment has been performed over time, this option becomes available.

- If **activated**, each time point generates a transformation matrix and each time point is registered individually. As a result, the time necessary for registration is multiplied by the number of time points and can rise substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the **Individual Registration For Every Time point** option becomes necessary.

- If **not activated**, one transformation matrix is generated for all the following time points based on the first time point. As a result, processing for registration has only to be done once which reduces processing time.

Fig. 422   Processing – Lightsheet Processing. Multiview Processing, Input Settings

5.3.21.2.3   Input settings

In this sub-menu, a subset of a present time series can be chosen for the Registration and Fusion processing (Fig. 422).

Use the two sliders or input boxes with arrows on each side to truncate the **Time** points. If not every time point in this period should be taken, the **Steps** can be defined with the input-box with arrows.

If a subset of a time series is defined this way, the resulting images will be numbered according to their new sequence, which might not be the same as in the input file.

5.3.21.2.4 Fusion

After Registration of the Views, the **Fusion** process can be performed (Fig. 423). This will lead to one Z-Stack or Z-Stack over time.

You can choose the following methods using the drop-down menu:

None: no Fusion is performed after Registration and Dual Side Fusion.

Mean Fusion: The pixel in the fused image is determined by averaging the intensity level of the pixels for the involved views.
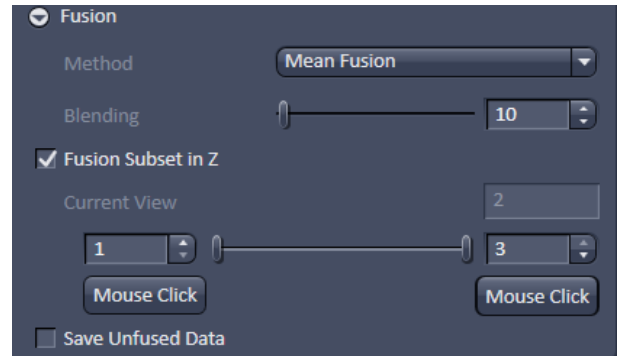

Fig. 423   **Processing – Lightsheet Processing. Multiview Processing, Fusion**

Mean Fusion + Multiview DCV: This option is only available if the dataset has not undergone deconvolution before. The pixel in the fused image is determined by averaging the intensity level of the pixels of the involved views. After fusion, a deconvolution is performed. The **Deconvolution** interface becomes available when chosen (see above).

When all Z-stacks (hence all views) are fused into one Z-stack, the intensity values at the Z-stack borders can lead to visible edges. To prevent this, the borders of all Z-stacks can be faded during the Fusion process. With the **Blending** slider and input box with arrows, the amount of blending (smoothing) can be controlled. The value for **Blending** should be as low as possible, with typical values from 20 to 80; but this needs to be tested for every dataset.

It is possible to define which portion of the Z-stacks of the contributing views of the experiment shall be used for the fusion process. To do so the checkbox **Fusion Subset in Z** must be activated. The view can be chosen with the according dimension slider of the **image control area**, and is displayed as **Current View**. For each of the views the z range can be chosen with the spin boxes or the sliders from each side. Alternatively a click on the **Mouse Click** button and afterwards onto the image (e.g. in the **Gallery View**) can be used to select the images to define the Z-range.

Whiles the whole Z-stacks are still used for registration (e.g. fiducials for Landmark based registration remain accessible), only the image information of the selected Z-range will be used for **Fusion**.

Portions of the Z-stacks, highly scattered and/or blurry content, can be discarded from the resulting fused image, to overall enhance the image quality. It must be considered for later deconvolution, that the calculated point spread function (PSF) of a multiview experiment, is based on the assumption that all views equally contribute to all areas of the image. This is not the case when using **Subset Z** and might result in artefacts of the deconvolution result.

If you want to keep the registered image as an interim result, even if the registration and fusion are done in one routine, activate the **Save Unfused Data** checkbox.

5.3.21.3   Online Multiview Processing

Processing of a multiview experiment dataset can be done online during data acquisition while the data is streamed onto a storage and analysis PC. ZEN software must be started on the storage and analysis PC and the master file (*.czi) of the experiment can be opened.
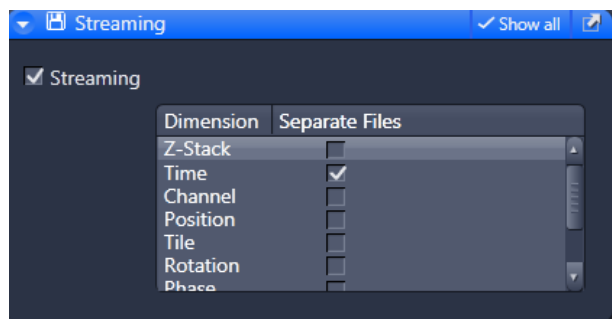

Fig. 424   Streaming tool window

It is possible to save dimensions of the experiment as individual files. To do so, the dimensions can be chosen with the according check box in the **Streaming** tool window (Fig. 424) of the **Maintain tab** (see as well section **Maintain Tab**).

☞ It is important that only the **Time** checkbox is activated for **Online Multiview Processing**, which is the default setting. If other dimensions are separated (e.g. channels) they are not included in the processing procedure and will be missing in the resulting file.

When time series are acquired with the Lightsheet Z.1, the first time point is a master file with the following appearance: Name.czi (e.g. FishEmbryogenesis.czi). For each time point, a separate file is additionally formed in the same folder with the nomenclature: Name(time point-1).czi (e.g. FishEmbryogensis(221).czi). This master file must be selected in the **Method Parameters** window. There, the settings for processing can be chosen as described in the section **Multiview Processing**.

When pressing the **Apply** button, processing will start for the already acquired images of the time series.

The registration option: **Interactive registration** is not available for **Online Multiview Processing** and is missing from the according drop down menu. To use this registration during the acquisition of the time series, please proceed as following. Open one time point of the experiment and choose the **Multiview Processing** option and press the **Select** button. Follow the description in section **Multiview Processing** to perform an interactive registration. When pressing the **Apply** button, an .xml file is created in the **Result** folder, which holds all registration information. The **Result** folder is created within the folder of the original dataset.

Proceed by opening the master file (first time point) of the ongoing experiment and choose **Online Multiview Processing**. Press the **Select** button and choose **Registration from file** from the drop down menu. Follow the instructions in section **Multiview Processing**.

5.3.22   Processing – Copy

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1
Tool accepts all types of image data as input. See full description of tool set for details.				

The Copy function provides means to copy or duplicate images.

Convert lamda to channels

Convert lamda to channels converts images acquired in Lamda Mode to multichannel images where each detector element represents a separate channel in the new image (Fig. 425). All view and processing options available for multichannel images apply to the new image.

Copy Channel

Copy Channel produces a copy of the selected image.

The first **Select** button in the selection panel selects the active image in the **Image Display** as Input Image. The **Channel** selection pull down menu specifies which channel to copy.

The second **Select** button is used to choose a destination image if the copied channel is to be added to an existing image document. In the second **Channel** selection pull down menu the destination channel is specified – or a new image document can be chosen as destination.

Pressing **Apply** starts the copy process.

☞ For Z-Stacks or Time Series, the entire series for the selected channel is copied.

Duplication

Duplication creates a new image document which is a duplicate of the selected input image.
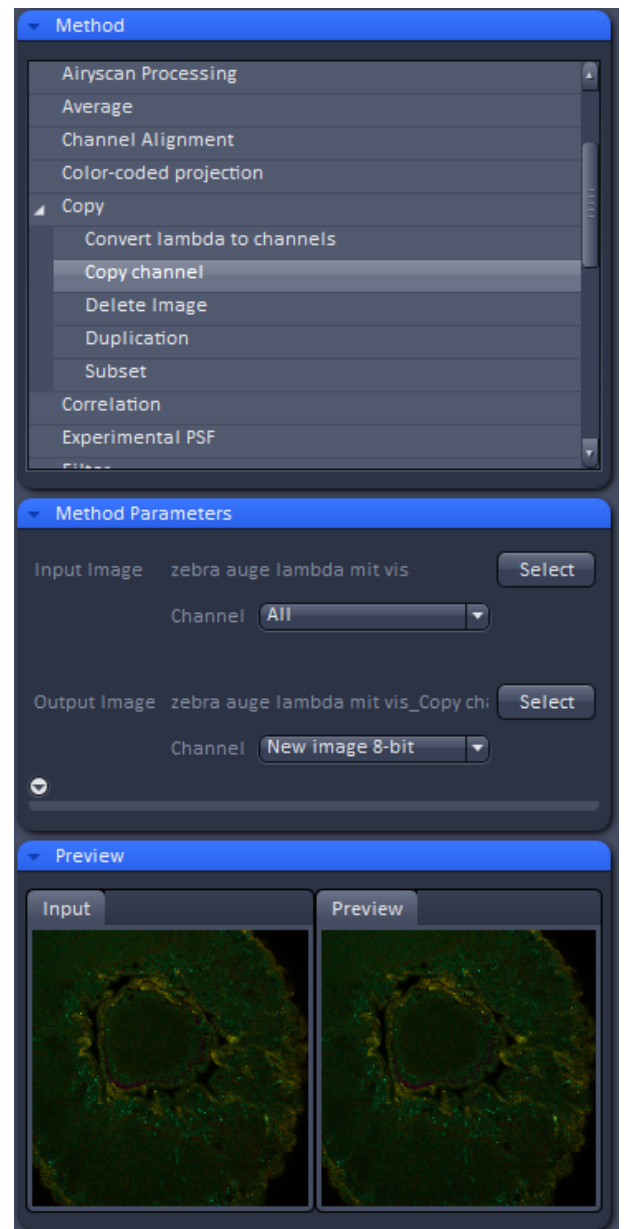
Fig. 425   Processing – Copy
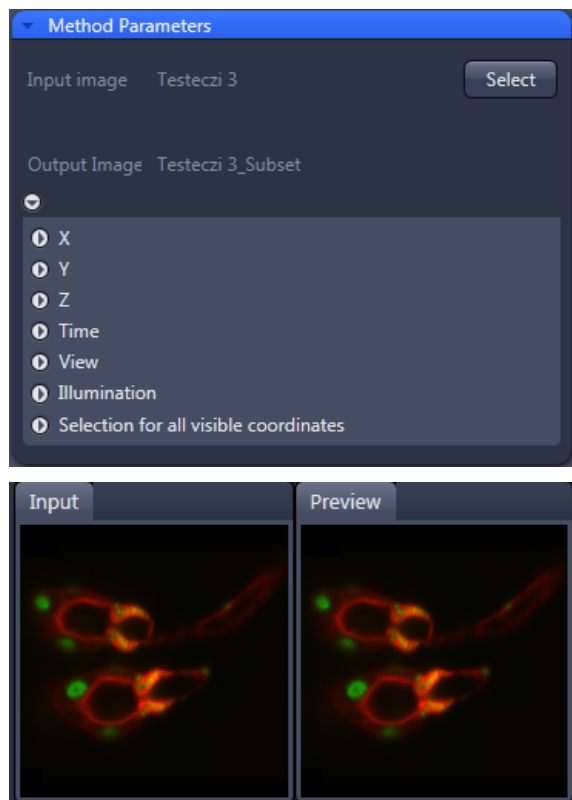
Fig. 426 Subset tool with loaded Multiview (Lightsheet Z.1) image
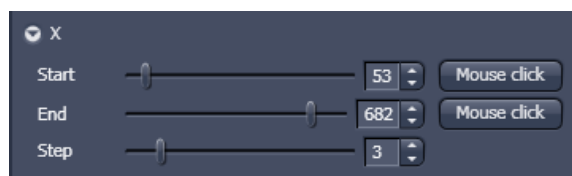
Fig. 427 Subset panel for x-coordinate
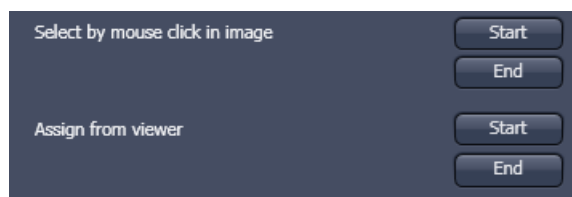
Fig. 428 Subset panel for "visible"

Delete Image

Single images or series of images can be deleted from the selected image series (input image) acquired as time series or Z-Stacks or combinations of those.

The **Select** button selects the active image in the **Image Display** as the **Input Image** to be processed.

In the tool-specific settings panel, the parameters for the interpolation procedure are set.

Opening for example the x-subset panel (Fig. 426) brings up three sliders to set the **Start** and **End** coordinates of the new data set.

Pressing the **Mouse click** button selects the respective coordinate by clicking on the original image in the **Preview View** in the image display area (Center Screen Area). The **Step** coordinate determines how many data points in the respective dimension are dropped out. For example: setting **Step** to 3 takes 1 and drops 2 pixels along the respective dimension, reducing the data to 1/3. The scaling is calculated to remain correct in the resulting image.

Subset

The **Subset** function is a set of tools to truncate a multidimensional data set in all available coordinates to the desired size (Fig. 427).

These subset panels are available for all coordinates of the selected image (x, y, z, t, view, illumination). The panel **Selection for all visible coordinates** is shown in Fig. 428. It always applies only to those coordinates open within the subset-panel (above).

The Select by mouse click in image option allows one to click in the Image Display and the respective coordinates are set (if displayed in the image: x, y in the 2D view, z, view and illumination in the gallery view, etc.).

The **assign from viewer** selects the coordinate from the slider in the **Dimensions View Options** control block.

5.3.23   Processing – ICS

Licensing		Instrument		
standard	✔ optional	✔ LSM	ELYRA	Lightsheet Z.1

Tool accepts exclusively input acquired with LSM 710 and LSM 780. See full description of the tool set and section on *RICS (Raster Scanning Image Correlation Spectroscopy)* for more details on requirements to be met.

- Activate **ICS** in the Processing tool by clicking on the arrow.

The **ICS** menu provides the following sub-processing functions: **Detrend**, **ICS correlation, Map filter, NandB Analysis and Remove structures** (Fig. 429).

Fig. 429   Processing – ICS

5.3.23.1   Detrend

- The Detrend function is a high pass recursive filter.

- Click **Detrend** in the **ICS** tool. The **Method Parameters** menu will show the input options for this function.

- Select the desired image in the image container, then click **Select** to define this image as the **Input** image.
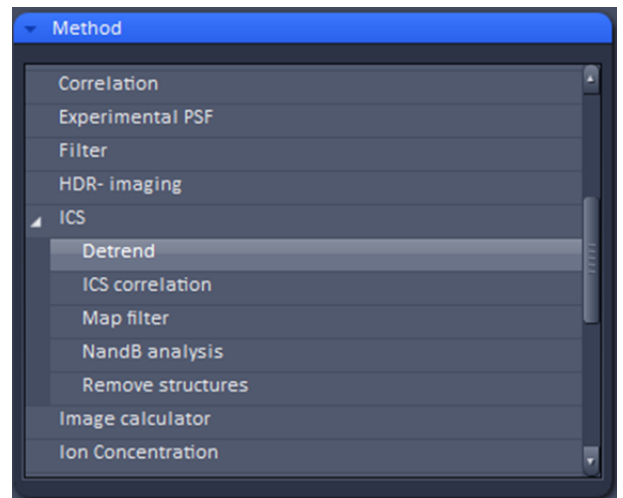
The resuling output image using the time constant parameter is shown in the **Preview** tool and the Preview View tab in the image container.

Input images are time series images.

The **Time constant** used to detrend the image is the number of images it takes for the recursive filter to decay to 1/e. Use a smaller number for detrending faster moving larger objects like vesicles and a higher number for detrending bleaching effect or slower cell movements.
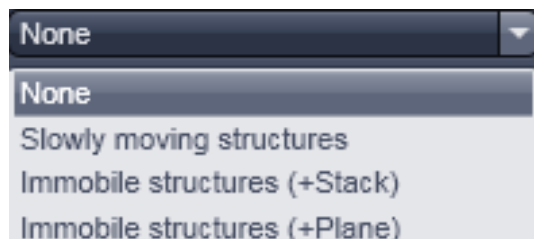
Fig. 430   Remove structure drop down menu

5.3.23.2   Remove Structure

- Activate **Remove structures** in the **ICS** tool. The **Remove structures** menu will appear.

- To select an image, highlight the image in the image container and press the **Select** button. This will be your **Input** image.

As an output image an image with the selected average subtracted is computed and the result presented in the **Preview** window.

- If the **Apply** button is pressed, the averaged subtracted image stack will be opened as a new image in the container.

You have four options that can be selected via the **Remove Structures** drop down menu (Fig. 430):

None, **Slowly moving structure**, **Immobile structures (+Stack)** and **Immobile structures (+Plane)**.

If **None** is selected, no average will be subtracted and the original image is kept. If **Slowly moving structure** is activated the **Average frames** input box becomes available. Type in the required number (minimum is 2) or use the slider or arrows for input. The number defines the number of frames that will be averaged and subtracted in a gliding way from the original stack in the following way:

- From a stack of n frames take successive m moving average frames with an overlap of m-1 and average these. The outcome is a new stack with n-m+1 gliding or moving average frames. Calculate from each of the average frames the overall average of all pixels, which is a scalar.

- Subtract the i-th of the gliding average frame from the (i-1)+m/2 original frame and add the i-th scalar. In this way, the first frame to be used from the original stack is m/2 and the last m/2+(n-m+1). This creates a new stack of n-m-1 frames that is the output image.

If **Immobile structures (+Stack)** is selected, the average frame of the stack will be computed and subtracted from each original frame. This creates a new average subtracted stack of the same size. An average value of all pixels from all frames is computed, which is a scalar, and added to each pixel of the single frames of the average subtracted stack to avoid negative numbers. The resulting stack is the output image.

If **Immobile structures (+Plane)** is selected, the average frame of the stack will be computed and subtracted from each original frame. This creates a new average subtracted stack of the same size. Average values of all pixels from each of the original frames is computed, which is a scalar, and added to each pixel of each corresponding single frame of the average subtracted stack to avoid negative numbers. The resulting stack is the output image.

5.3.23.3 ICS Correlation

- Activate **ICS correlation** in the **ICS** tool. The **ICS correlation** menu will appear (Fig. 431).

- Highlight the image in the image container and press the **Select** button. This will be your **Input** image.

- This function is only available in the LSM imaging mode

A correlation image will be computed based on the settings and displayed in the **Preview** window. As an output image an image with the selected average subtracted is computed and the result presented in the **Preview** window.

- The correlation to be computed can be selected from the **Correlation** drop down menu (Fig. 432). For one channel recordings, only **Spatial auto-correlation (S+A)** is available, for a two channel recording in addition **Spatial cross-correlation (S+C)**.

The way of average subtraction can be selected from the **Remove structures** drop down menu (Fig. 433).

- You can select between **None** (no average subtraction), **Slowly moving structures** (subtraction of moving average, the frame size of which can be selected in the **Average frames** input box), **Immobile structures (+Stack)** and **Immobile structures (+Plane)** (subtraction of total average and adding average scalar of all pixels from one frame to each corresponding frame).
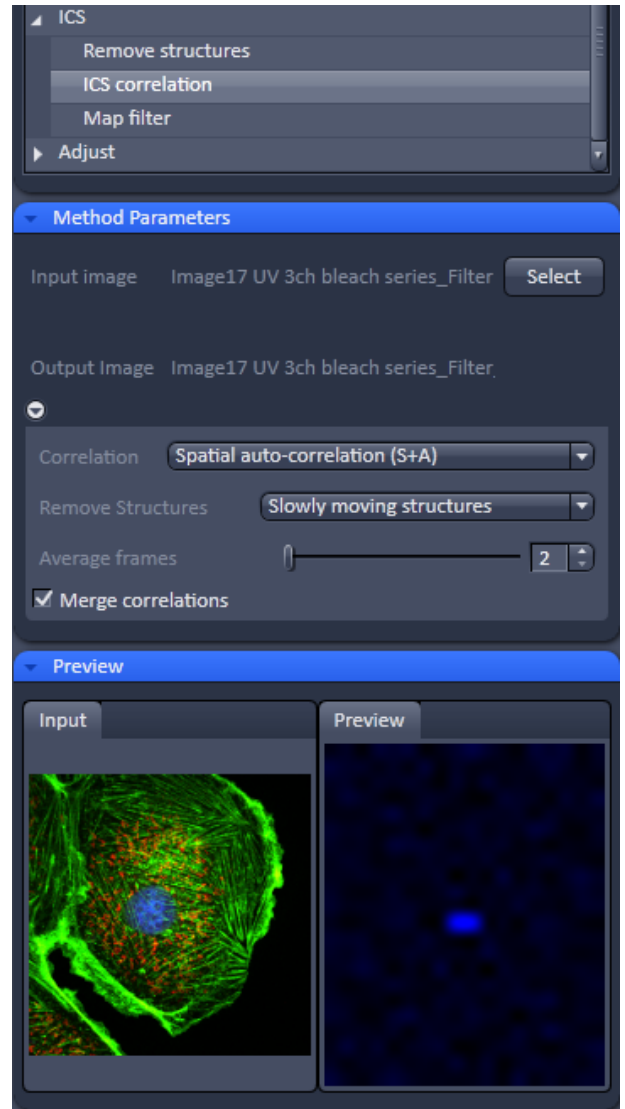
Fig. 431 Processing – ICS correlation
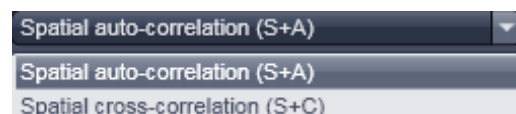
Fig. 432 Correlation drop down menu
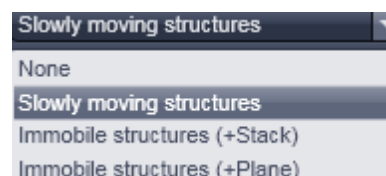
Fig. 433 Remove structures drop down menu
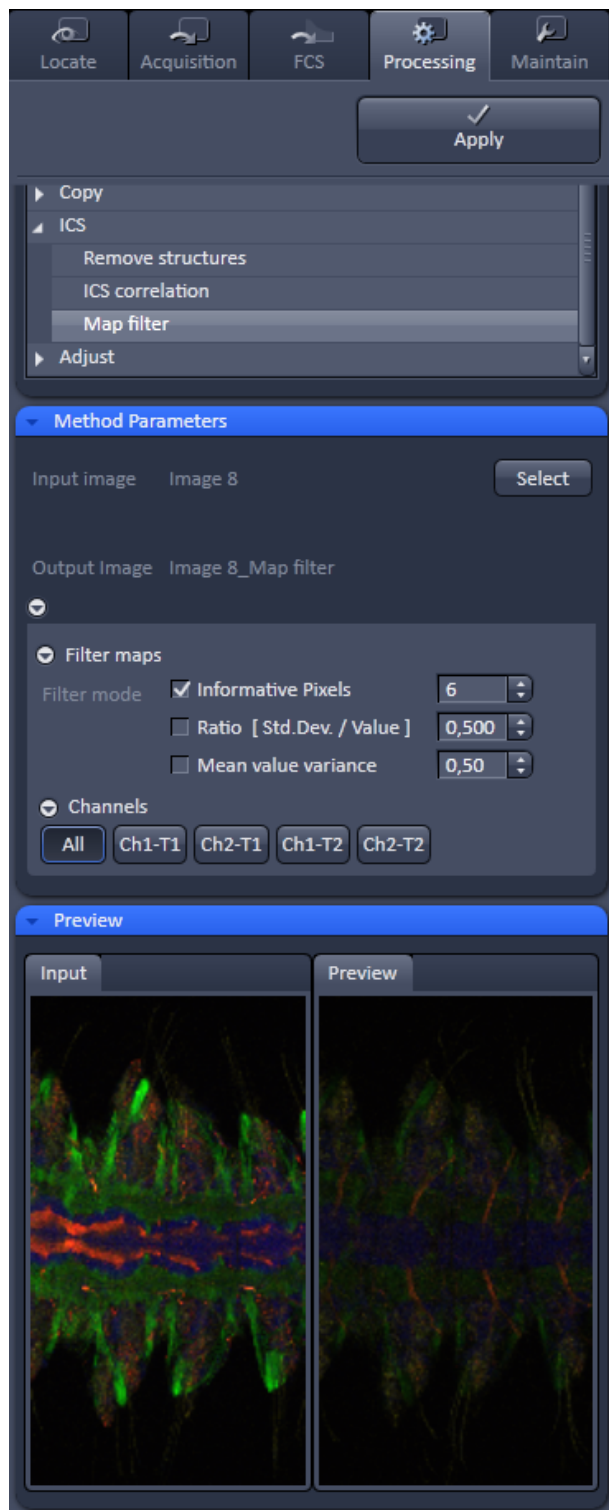
Fig. 434   Processing – ICS Map filter

5.3.23.4   Map Filter

- Activate **Map filter** in the **ICS** tool. The **Map filter** menu will appear (Fig. 434).

- To select an image highlight the image in the Image container and press the **Select** button. This will be your **Input** image map.

As an output image map an image map with the selected filters applied is computed and the result filtered image maps will be opened as a new image maps in the container.

The way of filters to be applied can be selected in the **Filter maps** menu, which can be opened by the arrow, by checking the **Ratio [Std.Dev./value]** and the **Mean value variance** check boxes.

☞ Note that both filters can be applied together by checking both boxes.

- Thresholds for the filters can be entered in the corresponding input boxes by typing or by using the arrows. Pixels with values exceeding the threshold will be displayed in black and will be discarded for the scaling of the map.

- You can define if all maps should be filtered by pressing **All** button in the available filter display area.

- If you want to apply filtering only to a specific map (**Number of particles**, its standard deviation **Std.Dev.**; **Diffusion coeff. [µm x µm /s]**, its standard deviation **Std.Dev.** and X^2) the **Filter all maps** box must not be ticked and the corresponding map button pressed, which is indicated by highlighting in blue.

☞ Note that filtering is never applied to the X^2 map, even if it was selected.

5.3.23.5   NandB Analysis

- The function **NandB** (Number&Brightness) **Analysis** calculates the image content in two ways:

  – It counts the photons per second per molecule referred to as the **Brightness**.

  – It counts the number of molecules referred to as **Number**.

- Click **NandB Analysis** in the **ICS** tool. The **Method Parameters** tool shows the input options for this function (Fig. 435).

- Select the desired image in the image container, then click **Select** to define this image as the **Input** image.

- The tool works with time series images which should be acquired along the following guidelines:

  – It is necessary to oversample the images in XY at least by a factor of 3 but better by up to a factor of 10. A pixel size of about 30 nm is beneficial as the method needs to be able to detect the fluctuation of the molecules within the confocal volume.

  – The imaging speed needs to be adapted to the speed of the molecules movement. For most experiments a speed of 6 or 7 (around 1 frame per second) has proven to be a fairly good setting. The scanner needs to be fast enough to "walk along" with the molecule: Too slow, and the molecule "escapes", too fast, and the molecule "stays behind".
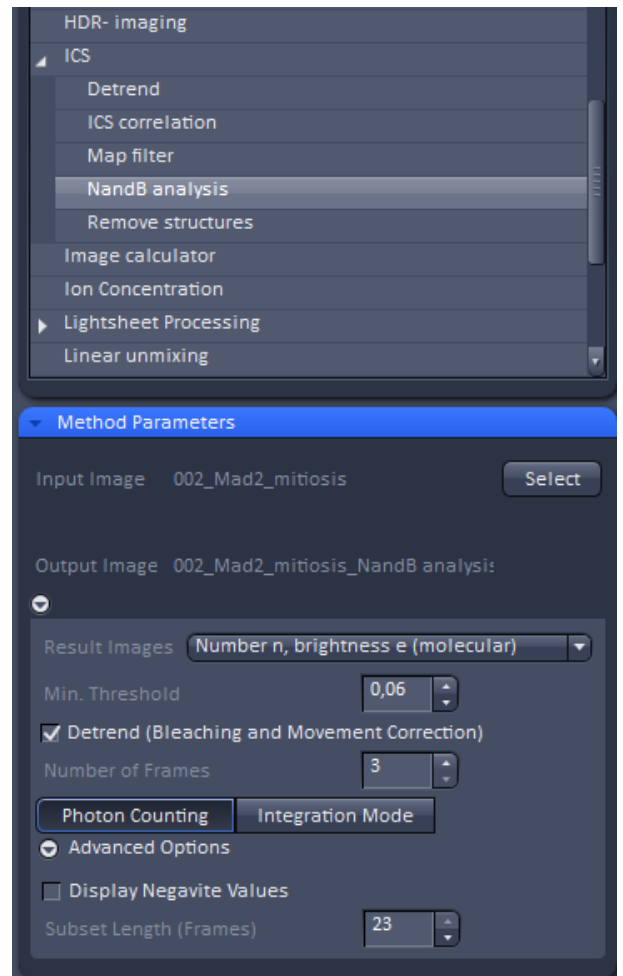
Fig. 435   **NandB Method Parameters tool**

For example:

EGFP (about 20nM) in buffer ph7.4: D=90 µm²/s -> Set the **Pixel dwell time to 25-32 µs/pixel and the Pixel size to 80-50nm**

- Within the **Method Parameters** tool the following choices can be made:

  – Result Images **Number N, brightness B (measured);** This method calculates the apparent number of molecules N and apparent brightness of molecules B according to the following formula:

$$B = \frac{\sigma^2 - \sigma_0^2}{(k) - offset} = \frac{S^2 \varepsilon^2 n + S^2 \varepsilon n}{S \varepsilon n} = S(\varepsilon + 1)$$

$$N = \frac{((k) - offset)^2}{\sigma^2 - \sigma_0^2} = \frac{\varepsilon n}{\varepsilon + 1}$$

- Result Images: **Number n, brightness ε (molecular);** This method calculates the number of molecules n and their brightness e according to the following formula:

$$n = \frac{((k) - offset)^2}{\sigma^2 - \sigma_0^2 - S((k) - offset)} \qquad n = \frac{((k)-offset)^2}{\sigma^2-\sigma_0^2-S((k)-offset)}$$

$$\varepsilon = \frac{\sigma^2 - \sigma_0^2 - S((k) - offset)}{((k) - offset)S} \qquad \varepsilon = \frac{\sigma^2-\sigma_0^2-S((k)-offset)}{((k)-offset)S}$$

Legend for the fomulas:

N and n	are apparent and corrected number of particles, respectively
B and ε	are apparent and corrected brightness, respectively
$\langle k \rangle$	is the mean signal expressed in digital levels (DLs)/pixel
σ^2	is the signal variance
σ_0^2	is the readout noise
offset	is the dark signal (DLs/pixel)
S	is the photon conversion factor (DLs/photon)

Min. Threshold sets a threshold for the intensity values to be taken into account for the calculation. This is useful if the background in the image is interfering with the calculation. If the pixel value is below [peak image value x Min. Threshold], the pixel is not processed. Intensity, number and brightness are set to 0.

Detrend (Bleaching and Movement Correction) is a high pass recursive filter that corrects for bleaching artefacts and the (much slower) movements for example of cells. When activated, the "trend" (moving average – average over the whole time series) will be subtracted from the input time series prior to N&B analysis. **Number of Frames** is the number of images it takes for the recursive filter to decay to 1/e. Use a smaller number for detrending faster moving objects like vesicles and a higher number for detrending bleaching effects or slower cell movements.

Photon Counting: If the input image has been acquired in Photon Counting mode, this button is automatically set active.

Integration Mode: If the input image has been acquired in Integration mode, this button is automatically set active. For images acquired in integration mode the following parameters are displayed and can be adapted:

Offset: Is set to 0 per default and does typically not need to be changed. The value 0 is taken for photomultiplying detectors. It is the value take for the calculation (see formulas above).

Readout Noise: Is set to 1 per default. This is a parameter typical for PMT type detector. Other detectors like cameras do have another value. It equals σ_0^2 within the formulas above.

Photon Factor: Is the photon conversion factor taken from the meta data of the image (if available) and describes the digital levels per photon (S in the formulas above).

Advanced Options

Display Negative Values shifts the offset such that also negative values are displayed as a greylevel above zero.

Subset Length (Frames)allows to define subsets of time series and have a value processed for each subset. This might be helpful for the analysis of time series with a high number of frames.

- The resulting output image using these settings is shown in the **Preview** tool and the Preview View tab in the image container.

- Click **Apply** to process the image series. For each input channel, the analysis produces three output channels: The original intensity image, I, the Number image, N, and the Brightness image, B or, if selected, the Number image n and the brightness image e.

- Use the **Coloc** View Tab to display the result as a scatter plot where either the Number or Brightness image is plotted against the intensity image.

- The table shows the mean number or brightness value along with the mean intensity value of the total image or a selected area. This allows for example to distinguish between regions where molecules are present as monomers or dimers.

See also the following literature for further information on N&B analysis:

Mapping the Number of Molecules and Brightness in the Laser Scanning Microscope, Michelle A. Digman et al, Biophysical Journal Volume 94 March 2008 2320–2332

Determination of Particle Number and Brightness Using a Laser Scanning Confocal Microscope Operating in the Analog Mode, ROOSHIN B. DALAL et al, MICROSCOPY RESEARCH AND TECHNIQUE 71:69–81 (2008)

5.3.24   Processing – Adjust

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1*****
Tool accepts all types of image data as input. See full description of tool set for details and potential restrictions. ***** Except Auto Adjust Shading Correction				
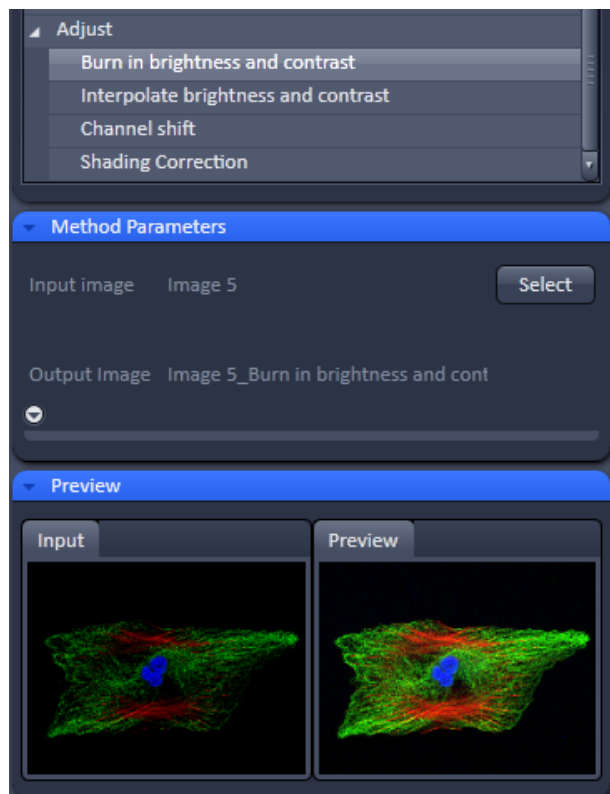
Fig. 436   **Processing – Adjust with Burn in brightness and contrast**

This tool allows brightness and contrast adjustments to your image as well as shifting two images against each other (Fig. 436).

5.3.24.1   Burn in Brightness and Contrast

The **Burn in brightness and contrast** function creates a new image document in which the current brightness and contrast settings from the Display View Options control block (see section **Center Screen Area / Image Containers - Display and Image Analysis**) are permanently written into the image file (Fig. 436).

The **Select** button selects the active image in the **Image Display** as the Input Image to be processed.

- After adjusting the brightness and contrast in the Display View Options control block, click Apply to create the new image document with the burnt in contrast and brightness settings.

5.3.24.2 Interpolate Brightness and Contrast

This function permits a continuous contrast and brightness adjustment in a Z-Stack or Z-Stacks over time by interpolation between the starting and end values of the respective image series. This permits a post-acquisition compensation of signal loss in, for example, thick tissue imaging where excitation and detection efficiency decrease significantly. Interpolation can be defined for the entire image or only for individual channels. In addition, a set of coordinates for which intensities are kept fixed during the interpolation calculation can be defined (Fig. 437).

The **Select** button selects the active image in the **Image Display** as the Input Image to be processed.

In the **Interpolation** panel (Fig. 438), the parameters for the interpolation procedure are set.

- In the **Coordinate** pull down, select the dimension for the interpolation. Depending on the image data type, x, y, z, and time are the possible options.

- Use the **List size** spin box to set the number of fixed data points. The table in the **Interpolation** panel will be extended to the selected number of rows.

- Highlight a row of your choice by clicking on it. Then use the **Brightness**, **Contrast** and Z (or t) slider to set the values for this fixed data point. The numbers in the table will be updated according to the slider position. Then highlight the next row, set the parameters and keep setting the fixed data points for all rows.

- Use the available **All**, **Ch1-T1** and **Ch2-T2** channel buttons to select the channel for interpolation or click on the **All** button if the entire image is to be interpolated.

- Having set the parameters, click on the **Apply** button. Interpolation will be performed in a new **Image Display** window.

- The newly created image (series) can be stored using the **Save As** function.

☞ If you de-activate the Extrapolate check box, only the slices lying between the first and last fixed data point (table rows) will be taken into account for interpolation. Otherwise, brightness and contrast will also be changed for the other slices.
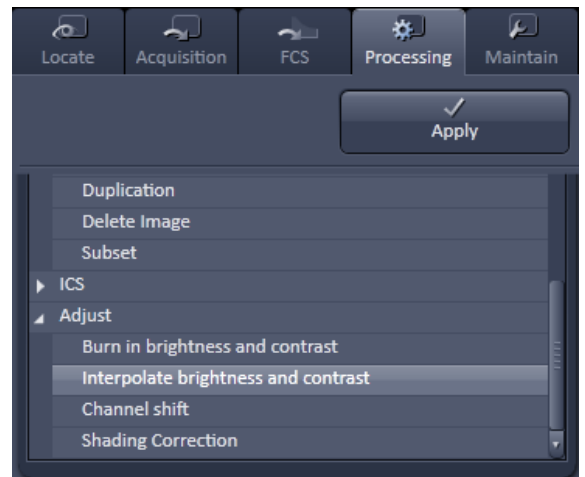
Fig. 437   Processing – Adjust with Interpolate brightness and contrast
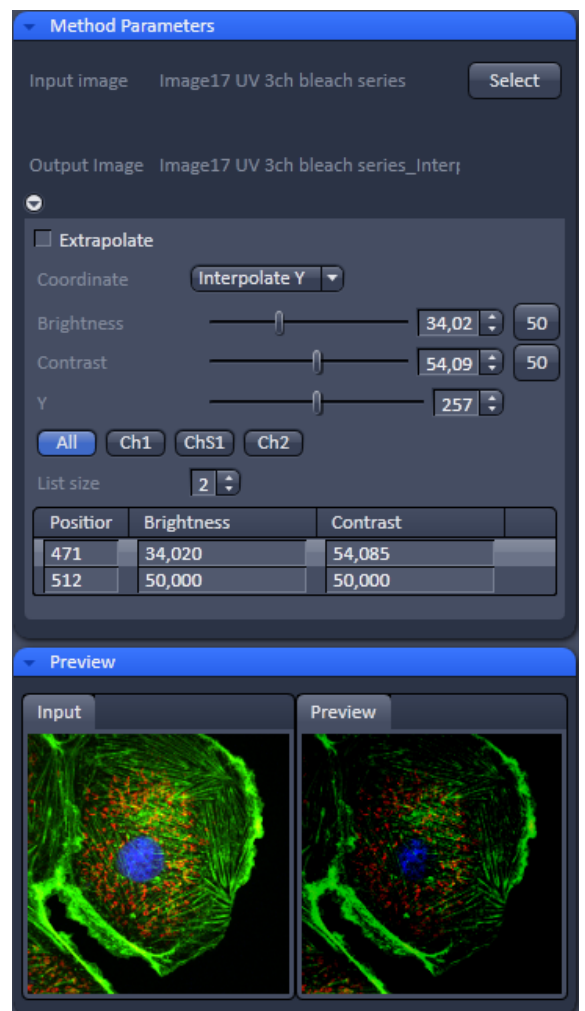
Fig. 438   Processing – Adjust with Interpolate brightness and contrast tool with loaded Z-Stack
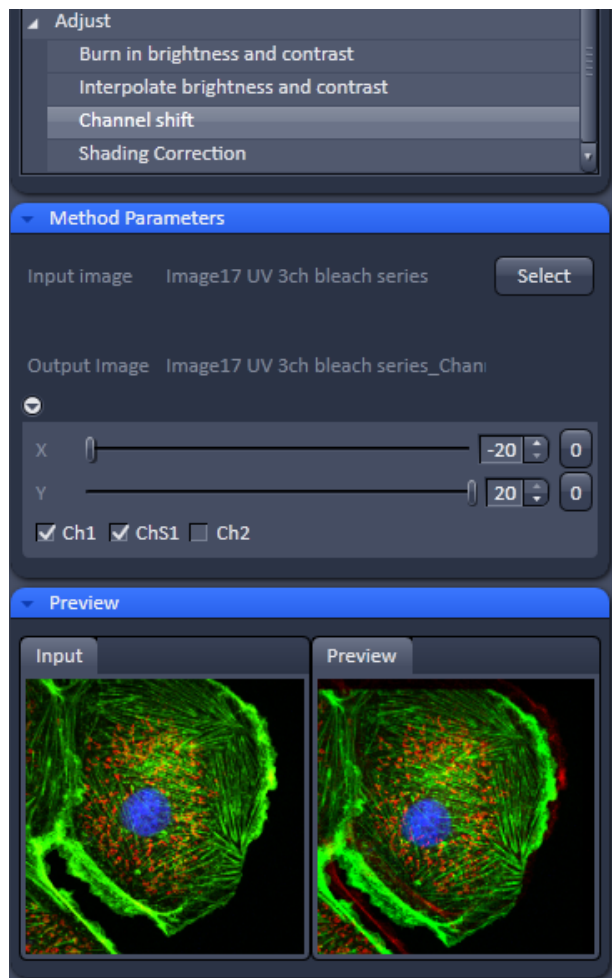
Fig. 439   Processing – Adjust with Channel shift

5.3.24.3   Channel Shift

Channel shift is used to produce a congruent image with relation to the pixels of the various channels (Fig. 439).

This pixel correction function is particularly important in UV applications.

☞ This is a manipulation of your data that has to be considered very carefully when co-localization analysis is part of your experiment.

The **Select** button selects the active image in the **Image Display** as the Input Image.

The image has to be a multi-channel image.

- Select the channels you want to shift by ticking the Ch1-T1 or Ch2-T2 check box:

- Use the X and Y sliders or spin boxes to define the shift in pixels to select the pixel shift in the horizontal and vertical direction. Clicking the Zero next to the spin-boxes resets the shift to the original position.

A preview is automatically generated at the bottom of the **Channel Shift** tool in the **Processing** tab of the Left Tool Area At the same time, a large preview is shown in the **Preview** view tab in the Image display in the Center Screen Area.

- Click on the **Apply** button to generate a new image with the set pixel shift.

5.3.24.4 Shading Correction

Shading Correction is a tool to manipulate images which are unevenly illuminated over the field of view. This works with single images, time series, Z-Stacks, or tiled images. Click **Shading correction** to open the interface for using that function (Fig. 440).

Select the image (or image series, tiled image, or Z-Stack) for which the shading correction should be applied by clicking **Select** next to **Input image**. When clicking **Apply** the system calculates a reference image from the input image(s) which is used to correct the input image(s). The corrected image is displayed in the center screen area.

In addition, the calculated **reference image** can be stored and used for further shading corrections of other images. Therefore the check box in the lower part of the tool box must be checked. Specify the folder where the image should be stored in the text box next to **Path**. **Browse** opens the windows folders from which you can select. A suggestion for the name of the image is made based on the original name of the image. Change this if needed.

This reference image can then be loaded and selected as a reference image for the shading correction of subsequent images, which are then processed much quicker.

The reference image must be taken from an image that was acquired with identical settings for objective, zoom, image format, and orientation.
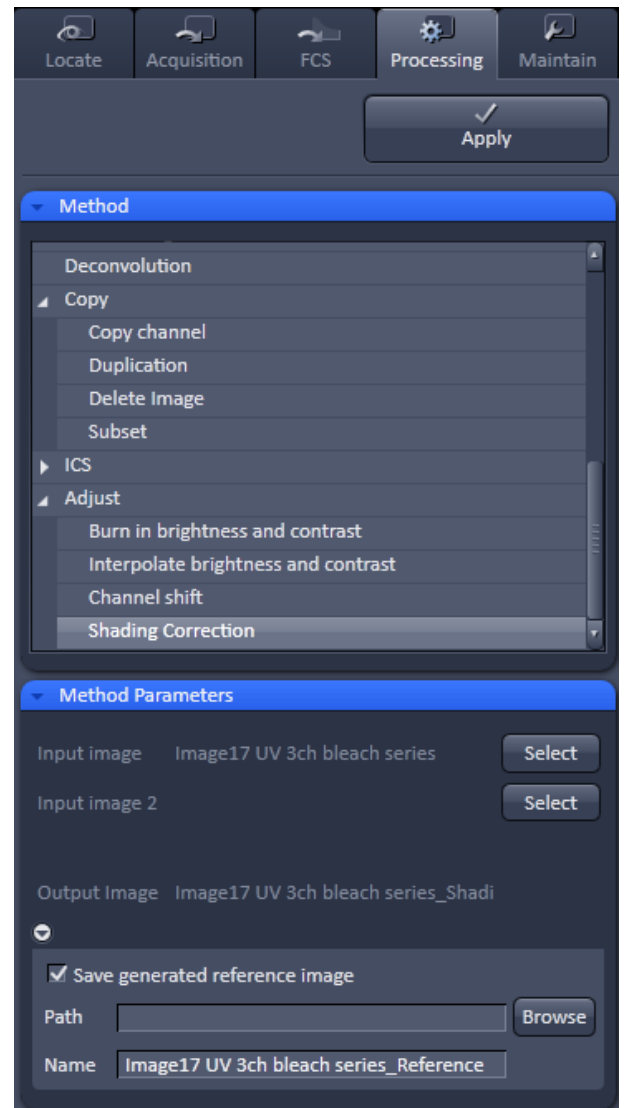
Fig. 440   Processing – Adjust with Shading correction

5.3.25   Processing – Detrending

Licensing		Instrument		
✓ standard	optional	✓ LSM	✓ ELYRA	✓ Lightsheet Z.1*****

Tool accepts all types of image data as input. See full description of tool set for details and potential restrictions.
* Except Auto Adjust Shading Correction

Fig. 441   **Processing – Detrending**

Fig. 442   **Detrending – Method Parameters**

The **Detrending Processing** function (Fig. 441) can be used to compensate for fluorescence loss during time lapse experiments that might be induced by bleaching.

Select the image you want to detrend by pressing the Select button. Note, if the image is not a time series, the **Select** button stays inactive.

Choose how you want to detrend the data. If the **Refer to Previous** checkbox is ticked, the software detrends the data of a frame in reference to the previous frame (Fig. 442). If the checkbox is not ticked, the software detrends the data in reference to the last image compared to the first image by linear interpolation.

If the **Use XY ROI** check box isticked and a ROI selected, detrendingwill be derived from the ROI but applied to the whole image.

You vcan input a threshold in the **Threshold** input box defining the intensity above which detrending occurs. This allows to avoid if signal is well abovebackground noise to raise background as well if the threshold is set above the background value.

5.4   Maintain Tab

Press the **Maintain** tab to have access to the **Maintain** tool group area (Fig. 443). The maintain tool group allow to adjust hardware settings.
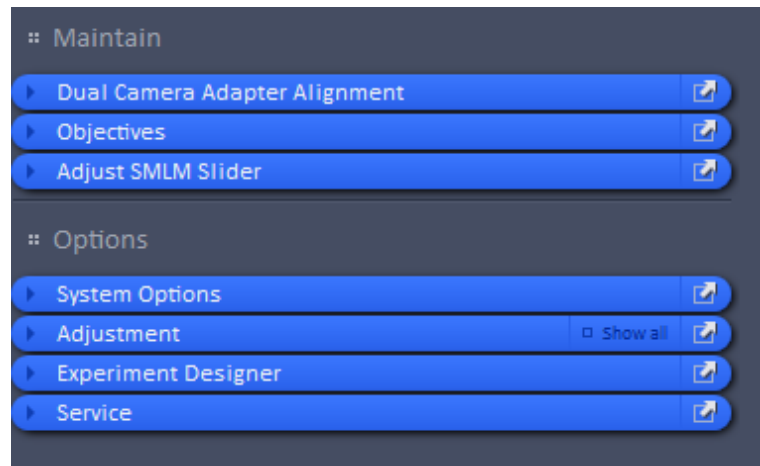
Fig. 443   Maintain tab – tool group area

The Maintain area is divided into the **Maintain** and **Options** field.

The tool tabs in the **Maintain** tool group allow the calibration and adjustment of hardware settings. The following options are available:

- **Dual Camera Adapter Alignment**
- Define **Objectives**
- **Adjust SMLM Slider** (only available for systems with 3D SMLM module)

The tool tabs in the **Options** tool group allow customize hardware and software settings. The following options are avialable:

- **System Options**:
  Options for **Camera**, **ELYRA**, **Load configuration**, **Reuse**, **Hardware**, **Image display** and **Streaming**
- **Adjustment**
- **Experiment Designer**:
  Automatic Document switch (enable/disable)
- **Service**:   Only for service use
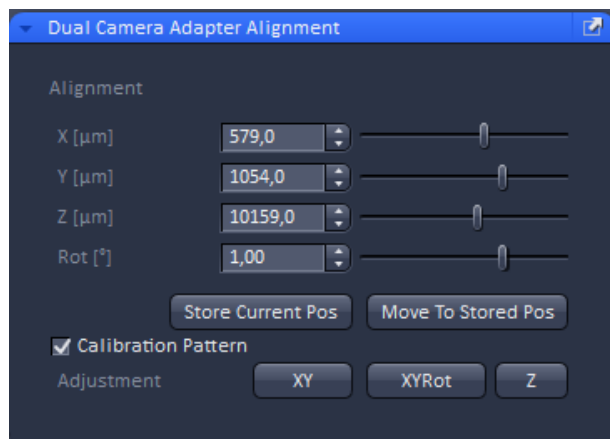
Fig. 444   **Maintain tool group – Dual Camera Alignment**
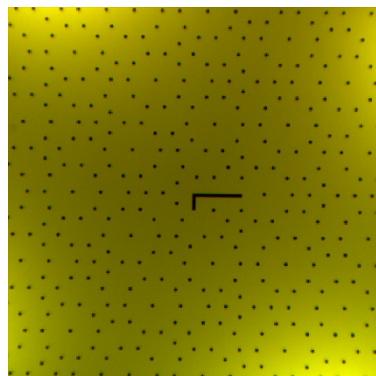
Fig. 445   **Penrose pattern illuminated by an LED and captured with an EMCCD camera. The hook serves for unambiguous alignment as the Penrose pattern is periodic.**

5.4.1   Maintain Toul group – Dual Camera Alignment

The **Dual Camera Alignment** tool (Fig. 444) allows to align two cameras of a Duolink to each other as the rear camera is motorized.

The current positions is displayed in **Alignment** for **X [μm]**. **Y [μm]**, **Z[μm]** as well as **Rot [°]**. The positions can be manually changed by the respective sliders or via typing numbers in the input boxes.

Pressing **Store Corrent Pos** will store the current positions. **Move To Stored Pos** will bring the positions to the stored ones.

In case auf automatic alignment a **Calibration Pattern** in the Duolink canbe used if the Calibration Pattern checkbox is ticked (Fig. 445). Inthis case no Continuous Scan or laser light iis needed, as an internal LED lamp will be used for illumination the pattern.

Press **Adustment XY**, **Adjustment XYRot** or **Adjustment Z** to trigger an automatic alignment in X and X, X, Y and Rotation or Z, respectively. If the alignment was successful (Fig. 446), press the **Store Current Pos** button to save the positions.

☞   You can also use a multi-color bead sample and laser light tomanually align the cameras. By Continuous scanning in a 2D view bring the beads in the 2 channels in aligbnment in X, Y, Z and Rot.

☞   The alignment is only usable for the beam path used doing the alignment. You can only store one setting. You cannot store multiple settings.
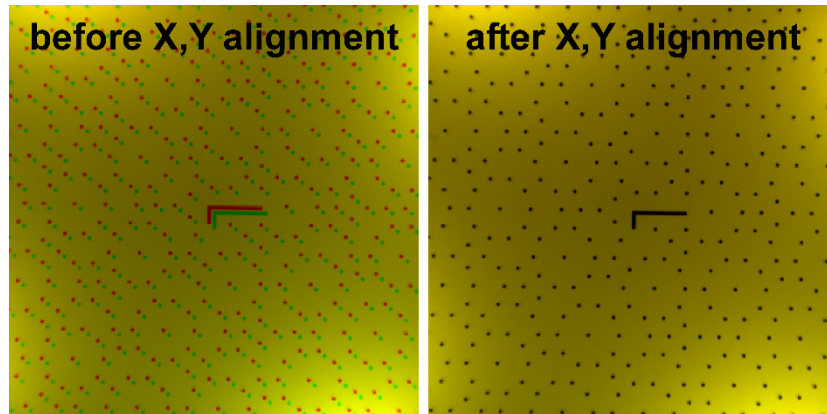
Fig. 446   **XY camera offsets before (left) and after (right)
alignment. The offset between camera 2 (green dots)
and camera 1 (red dots) visible before XY alignment
is minimized after the alignment.**

5.4.2   Maintain tool group – Objectives

Expand the **Objectives** tool to have access to objective definition and selection (Fig. 447).

With this function one can assign an objective to its respective position in the nosepiece, e.g. when an objective was added newly or replaced by another one without having to leave the software. Once the objective is activated its focus speed and parfocality can be set.

5.4.2.1   Detection Objective

Press the **Detection Objective** tab to have access to the Objective definition window (Fig. 447).

Press an Objective icon to have access to the **Change Objective** expansion window for the respective objective (Fig. 448) with the following functions

– Add Objective

– Show/Edit Objective

– Remove Objective

– Select objective.
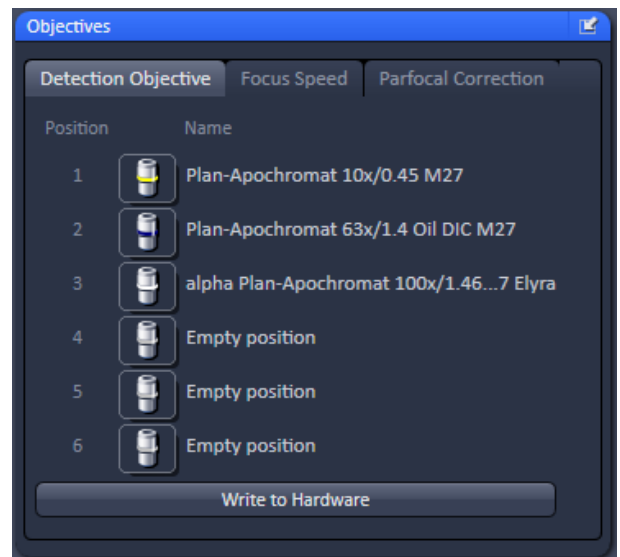
Fig. 447   **Maintain tool group – Objectives tool**
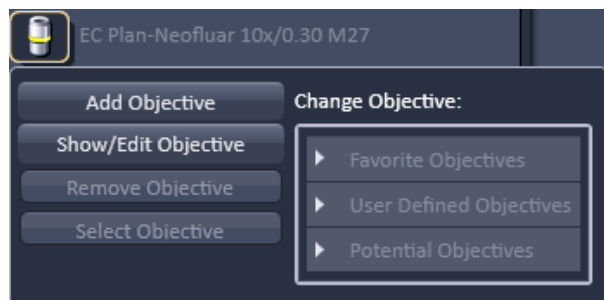
Fig. 448   Objective selection
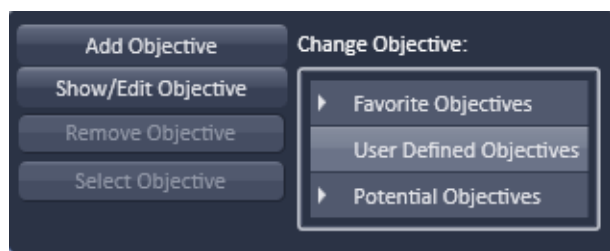
**Fig. 449   Objective selection – User Defined
                 Objectives with empty directory**
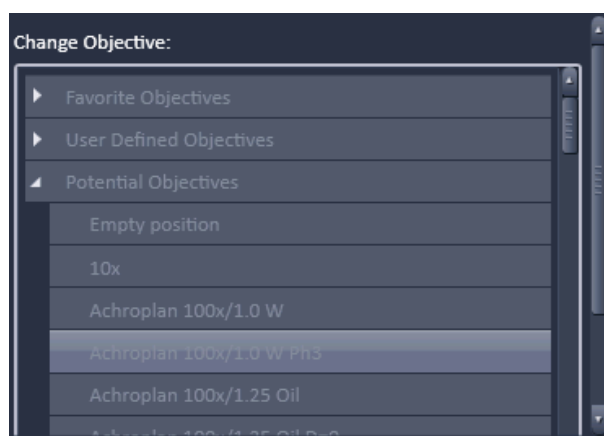
**Fig. 450   Objective selection – Potential
                 Objectives directory**

5.4.2.1.1   Select Objective

Select the **Objective** tab from the **Objective** tool, which will show the **Name** of all objectives with an assigned nosepiece **Position**. If **Automatic Component Recognition** (**ACR**) is available the correct objective Name and Position will be updated automatically. Otherwise you have to proceed manually as follows after screwing the objective into then nosepiece:

Click on the **Objective icon** button of the respective nosepiece position, which is indicated on the nosepiece. This will open the Objective selection menu (Fig. 449).

In the Change Objective area open one of the available directories of Favorite Objectives (showing all the objectives that have been previously selected), User Defined Objectives (showing all the objectives that have been previously defined by an user) (Fig. 448) or Potential Objectives (showing all objectives listed in the used ZEN database). Expanding the field opens the respective objective list directory (Fig. 450). An expansion triangle is only available, when objectives are available in the respective directory (Fig. 448). The scroll bars allow having access to all entries in the list.

- Select the correct objective by highlighting it. Press the **Select Objective** button. Note that the button becomes only active, if an objective was selected. The Name of the objective will appear at the corresponding position.

- Click anywhere outside the expansion window to close it.

5.4.2.1.2   Add Objective

This function allows adding objectives to the **User Defined Objective** database.

- Click on the Add Objective buttons. The Create new Objective expansion window will appear (Fig. 451). The scrollbar allows access to the whole field.

- Enter the parameters of the new objective in the appropriate display boxes or drop down menus. The text of those boxes that are inactive for editing, are greyed.

- Press the **Apply Add** button to store the newly defined objective in the **User Defined Objective** directory.

Fig. 451   Adding a new Objective – Editing field

If the **Non Zeiss** check box is ticked, objectives from other manufacturers can also be included in the database.

If the name of the objective already exists, a warning is displayed telling the user to rename (Fig. 452).
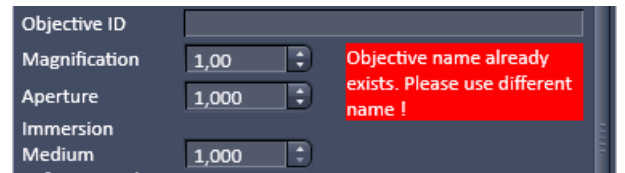
Fig. 452   Objective Name warning message
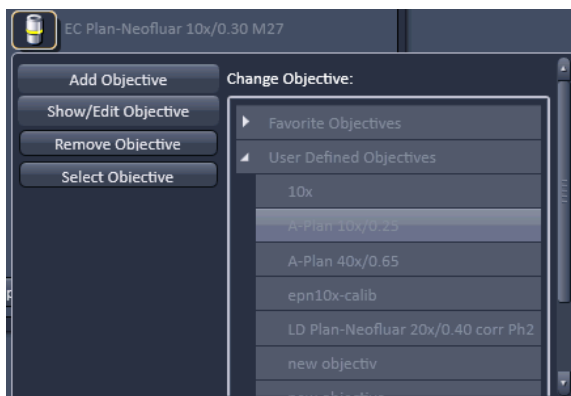
Fig. 453   Remove objective
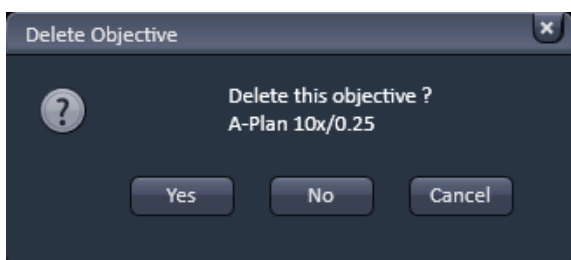
Fig. 454   Delete objective window
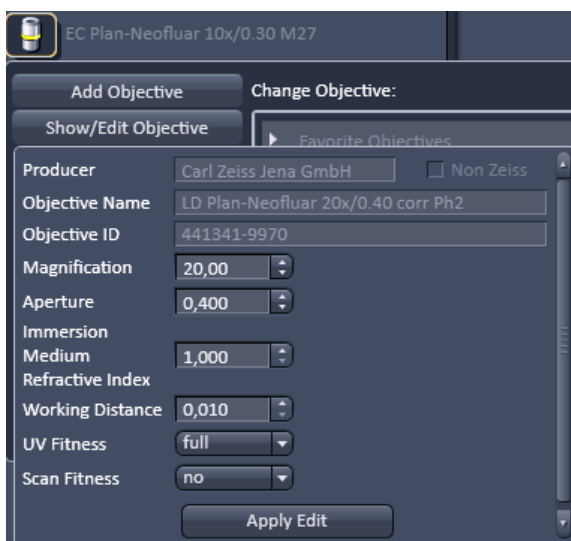
Fig. 455   Show/Edit objective

5.4.2.1.3   Remove Objective

This function allows removing objectives from the User **Defined Objective** database (Fig. 453).

Expand the **User Defined Objective** directory and highlight the objective you want to delete.

Press the **Remove Objective** button in order to delete the selected objective. Note that the Remove Objective button only becomes available, if an objective from the user Defined Objectives directory was chosen. You will be prompted to confirm to delete the objective in the **Delete Objective** pop up window (Fig. 454). Press **Yes** to delete the objective from, **No** to keep it in the database or **Cancel** to abort the process.

5.4.2.1.4   Show/Edit Objective

This function allows viewing the parameters of an objective from all objective directories. Editing the parameters is only possible for the **User Defined Objective** database (Fig. 455).

Expand the **Favorite Objective**, **User Defined Objective** or **Potential Objective** directory and highlight the objective you want to view. This will open the View tab.

In case of an objective listed in **User Defined Objective** directory, parameters can be edited within the display boxes (those parameters not to be edited will be greyed) or the drop down menus. Press the **Apply Edit** button (only available for user defined Objectives) to make the changes take effect and store the new annotations.

5.4.2.2 Focus Speed

Press the **Focus Speed** tab to have access to the **Focus Speed** definition window (Fig. 456).

The **Focus Speed** can be changed for every single listed objective. Use the speed slider beneath the objective's name or the input boxes to set the speed. By pressing the **Write** button the new settings will apply for computer and manual control.
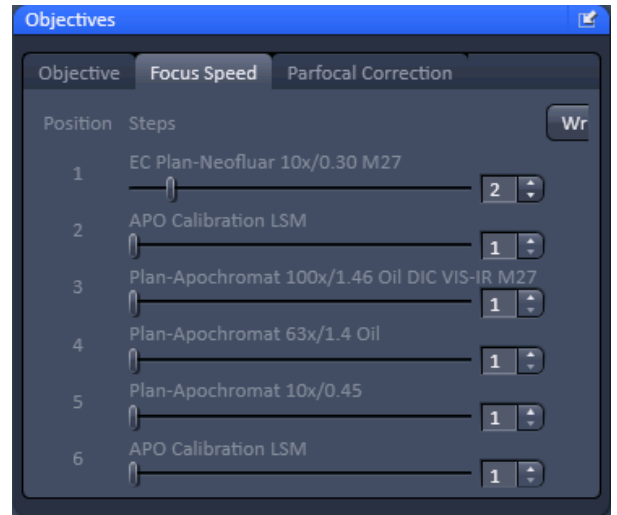
Fig. 456   **Focus Speed tab**

5.4.2.3 Parfocal Correction

Press the **Parfocal Correction** tab to have access to the **Parfocal Correction** definition window (Fig. 457).

If the **Parfocal correction active** check box is ticked in the **Activation** field the parfocal correction is active (Fig. 458).

⚠️ **CAUTION**
If Parfocal Correction is in use, a motorized stage can damage your objective! A respective warning is displayed in the **Activation** field.
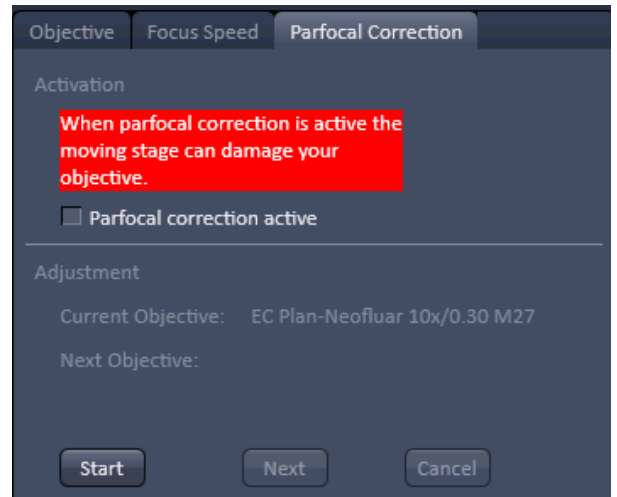
Fig. 457   **Parfocal Correction tab**
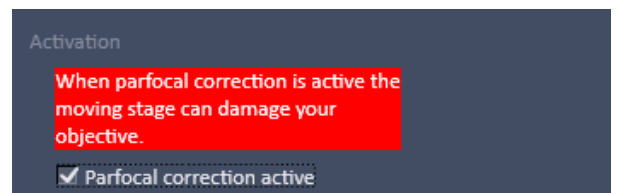
Fig. 458   **Parfocal Correction tab – Activation field**

For adjustment of the parfocal correction proceed as follows:
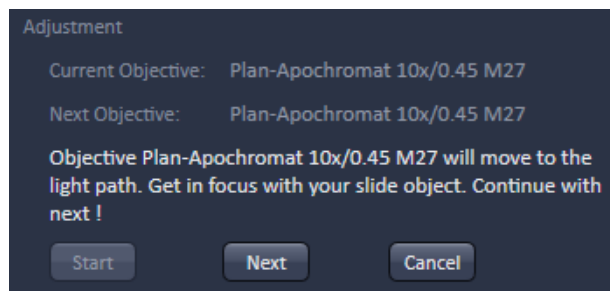
Fig. 459 Parfocal Correction tab – Adjustment field

Position a thin sample (cell or grid) in the sample holder of the stage and focus with the first objective in the nosepiece.

Press the **Start** button in the **Adjustment** field for adjusting the individual objectives (Fig. 459). The first objective in the list will be automatically selected and the **Start** button turns inactive. The **Current Objective** and **Next Objective** display areas list the respective objective.

A message is displayed asking the user to continue by pressing **Next**.

Fig. 460 Parfocal Correction tab – Adjustment finished

The **Next** button only becomes available when the Start button is pressed. If **Next** is pressed the next objective in the nosepiece is selected and the parfocal adjustment performed.

Press the **Cancel** button if you want to interrupt the Parfocal correction procedure. The **Cancel** button only becomes available when the **Start** button is pressed.

Press **Next** again until all objectives have been selected. You can cancel the process any time by pressing the **Cancel** button.

After the last objective has been corrected an info message informs the user that the Parfocal correction is finished (Fig. 460). The **Next** and **Cancel** buttons become inactive and the **Start** button active again.

Press **Start** to repeat the Parfocal correction.

5.4.3 Maintain tool group – Adjust PALM Slider

Open the **Adjust PALM Slider** tab to position the 3D-PALM slider at the correct position (Fig. 461).
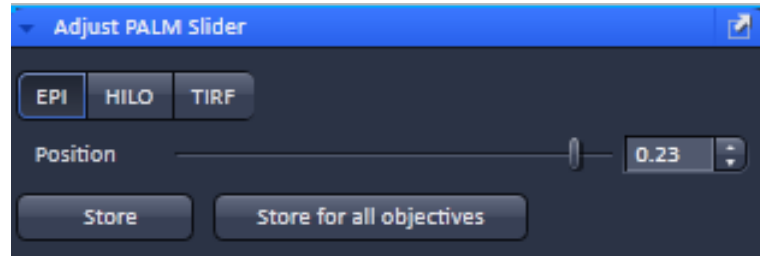
Fig. 461   Adjustment window for the 3D-PALM slider

You can select the illumination scheme by pressing the corresponding **EPI** (Epifluorescence), **HILO** (high inclined and laminated optical sheet) or **TIRF** (Total Internal Reflection Fluorescence) button. The selected button is highlighted.

With the **Position** slider or the **Position** input box, you can set the position of the 3D-PALM slider.

You can save the position for the objective in use by pressing the **Store** button, or for all objectives by pressing the **Store for all objectives button**. The saved positions will be used, whenever the respective objective is in use.

There are three ways to determine the optimal position of the slider (3D slider in active position):

– **HILO** illumination using 100 nm bead sample. In this case, focus on the beads (the position where the gap between the two lobes points in a 45° direction) and increase laserpower or camera gain to slightly oversaturate judging with Range Indicator. Position the slider so that both lobes become the same intensity (Fig. 462).
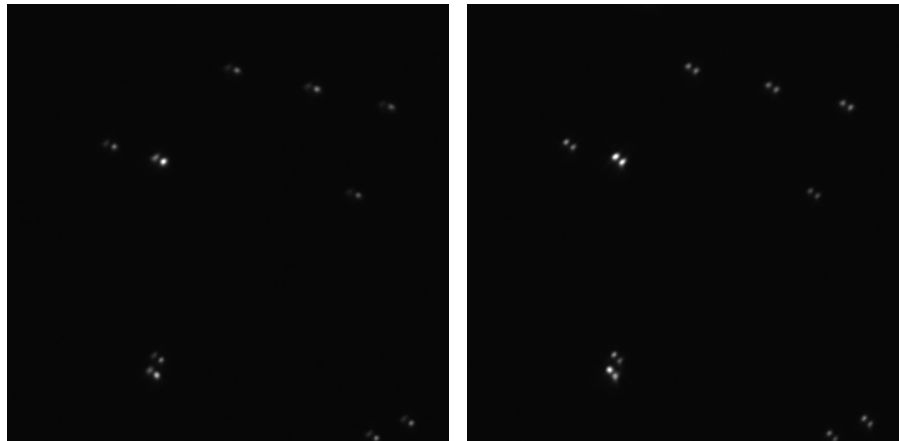
Fig. 462   Adjusting the 3D-PALM slider in HILO using equal illumination intensities of beads with non-optimal (left panel) and correct (right panel) positions.

– **EPI** illumination using 100 nm bead sample. In this case, focus on the beads and move slider until beads get obscured in the diagonal from left upper to right lower corner of the image. This will indicate, that the slider obscures the field of view and will be in the middle (Fig. 463).
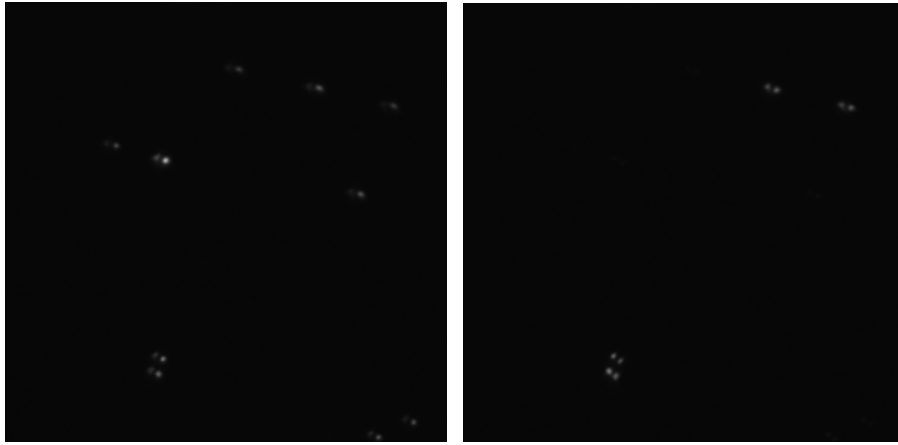
Fig. 463   Adjusting the 3D-PALM slider in Epi using obscuring of fluorescence in the diagonal of the image with non-optimal (left panel) and correct (right panel) positions.

– **EPI** illumination using 100 nm beads sample and using Bertrand lens to look into the back aperture og the objective. Use Tube lens 1x . Move the slider until it divides the puplil circle intio two equal halves (Fig. 464).
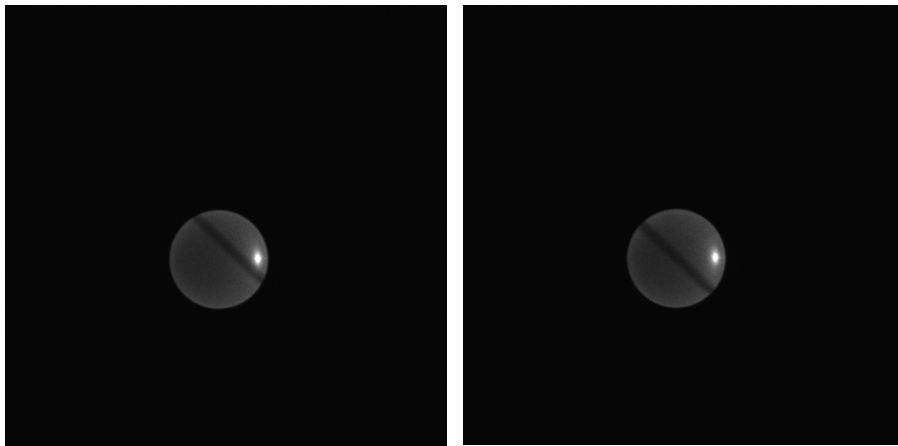
Fig. 464   Adjusting the 3D-PALM slider in Epi using imaging of the back focal plane with non-optimal (left panel) and correct (right panel) positions.

5.4.4   Options tool group – System Options

The **System Options** tool allows setting hardware parameters. Expand the tool to have access to the **System Options** setting window (Fig. 465).

The left and right pointing triangles give access to all functions by sliding the tabs to the left and right directions.

The downwards pointing triangle will list all the **Setting** functions in a drop down menu (Fig. 466). Selecting a function will automatically activate the respective tab.

The following user-accessible program **Settings** of the ZEN software are available

- **Camera**
- **ELYRA**
- **Load configuration**
- **Reuse**
- **Hardware**
- **Image display**
- **Streaming**

Click on the respective tab or select the function rom the Settings drop down menu to open the respective panel.
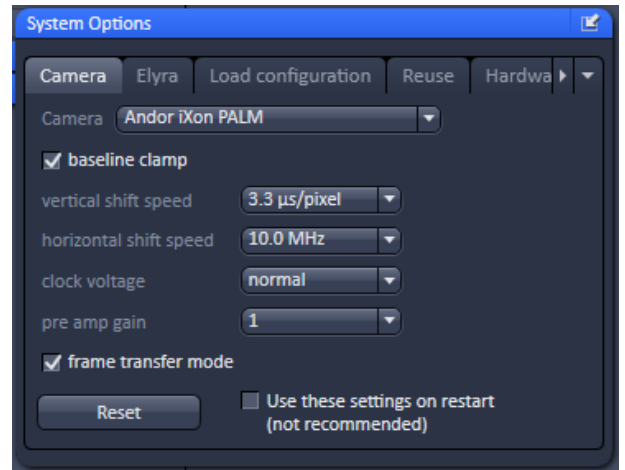
Fig. 465   System Options tool
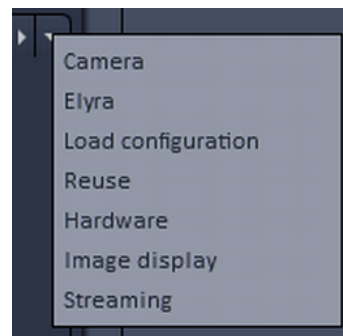
Fig. 466   Settings drop down menu
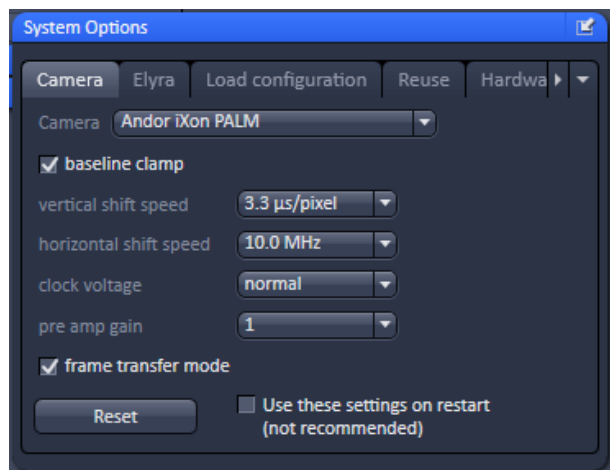
Fig. 467   System Options - Camera panel

Fig. 468   Camera selection drop down menu
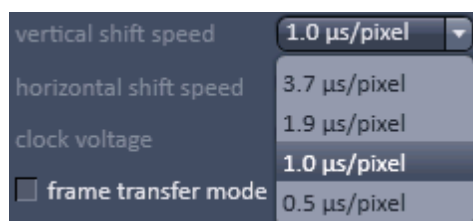
Fig. 469   Vertical shift speed drop down menu
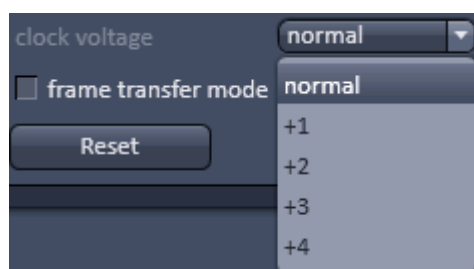
Fig. 470   Check voltage drop down menu

5.4.4.1   System Options – Camera

Press the **Camera** tab to open the **Camera** settings panel (Fig. 467).

Select the camera from the **Camera** selection drop down menu (Fig. 468). Dependent on the configuration the following cameras are available:

– AxioCam MRc 3.0 on the front port for IR transmitted light

– Andor iXon SIM (Andor iXon 885) on side or base port for SIM

– Andor iXon SMLM (Andor iXon 897) on side port for SMLM

Activate the baseline clamp by ticking the **Baseline clamp** checking box (Fig. 467). This will subtract the background in the display and add a certain offset to avoid negative pixels.

Select the vertical shift speed (not available for the AxioCam Mrc 3.0 camera) from the **vertical shift speed** drop down menu (Fig. 469). This will affect the speed the camera chip is read out vertically.

Select the horizontal shift speed (not available for the AxioCam Mrc 3.0 camera) from the **horizontal shift speed** drop down menu. This will affect the speed the camera chip is read out vertically.

Select the clock voltage (not available for the AxioCam Mrc 3.0 camera) from the **clock voltage** drop down menu (Fig. 470). This will affect the clock of the camera.

Activate the frame transfer by ticking the **frame transfer mode** checking box. This will synchronize the integration time with the transfer rate. If the integration time is smaller than the transfer rate (30 ms), it is recommended to inactivate frame transfer mode and use the free run mode.

By pressing the **Reset** button all parameters for the selected camera will be reset to the default values.

☞ For SIM and SMLM imaging the default settings of the camera are optimized and should not be changed. Changing camera parameters in general should be performed with great care.

☞ EMCCD cameras are sensitive to high light exposures. If an EMCCD camera is oversaturated for extended periods with gain settings above zero, the camera chip will age faster or can even be damaged. Therefore it is good practice to start always with gain zero and than adapt the gain to ones need. Also it is good advice to set the gain to zero when in SMLM experiments the bulk of molecules have to be switched off using high laser powers before they start to blink.

Activate the **Use these settings on restart (not recommended)** check box to start the system with the set parameters.

5.4.4.2   System Options – Elyra

Press the **Elyra** tab to open the **Elyra** panel (Fig. 471).

Tick the **Online pre-processing SR-SIM** check box to activate pre-processing of SIM data already during image acquisition. This will save time later when processing SIM data.

Tick the **Automatic collimator adjustment** check box to activate automatic adjustment of the collimator position in dependence of the z-focus position.

Fig. 471   System Options – Elyra panel

Check the **Use piezo for holding focus** box to use the **Z-Piezo** stage insert for the holding focus. If not ticked, the microscope focus drive will be used.

☞ It is strongly recommended to use the **Z-Piezo** stage insert for the holding focus as it is preciser and faster.

☞ Check the **Control holding focus position (only with fiducials)** box, if you want to use correlation amplitude and PSF width to find the focus of a fiducial using the holding focus. If unchecked, only the correlation amplitude will be used.

☞ It is recommended to use the **Control holding focus position (only with fiducials)** for the holding focus with a fiducial as it is preciser and faster. If blinking molecules are used it is better to deactivate the function.

Fig. 472   **System Options – Load configuration panel**

5.4.4.3   System Options – Load Configurations

Press the **Load configurations** tab to open the **Load configurations** panel (Fig. 472).

Load configuration lists parameters that, when their respective check box is ticked, will be taken into consideration when an image configuration is saved and loaded. The following parameters are available:

- Objective
- Frame size
- Speed
- Data depth
- Scan direction (not applicable)
- Average
- Pinhole diameter (not applicable)
- Detector gain and offset
- Zoom
- Rotation and offset (not applicable)
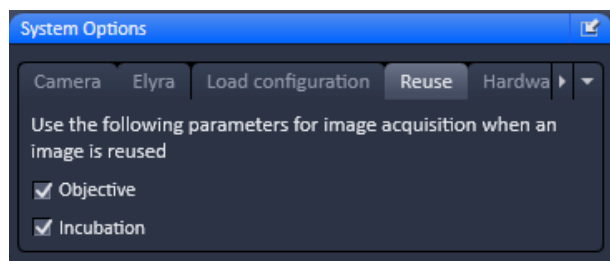
Fig. 473   **System Options – Reuse panel**

5.4.4.4   System Options – Reuse

Press the **Reuse** tab to open the **Reuse** panel (Fig. 473).

Reuse lists parameters that, when their respective check box is ticked, will be taken into consideration when the **Reuse** button for a loaded image is pressed. The following parameters are available:

- Objective
- Incubation

5.4.4.5 System Options – Hardware

Press the **Hardware** tab to open the **Hardware** panel (Fig. 474).

Hardware lists parameters that, when their respective check box is ticked, will set several hardware defaults.

By activation of the **Lasers off on exit** check box the lasers are automatically switched off when the ZEN software is closed. The lasers are allowed to cool for five minutes before they are switched off.

Fig. 474   System Options – Hardware panel

The Z-Stack **Waitstate** function delays the image acquisition during time series or Z-Stacks in case vibrations of the stage occur and are registered at the beginning of individual images. The default is off. The delay is set to 350 ms. This can occur for specific image acquisition settings and is dependent on the speed, the stage and the objective in use.

5.4.4.6 System Options – Image Display

Press the **Image display** tab to open the **Image display** panel (Fig. 475).

If the **Apply brightness and contrast changes to diagrams and tables** is ticked any changes in brightness and contrast of the image will also affect the image data shown in diagrams or tables. If not ticked any changes in the **Image Display** will not affect the original image data. The default setting is off.

The **Temporary files directory** can be chosen manually by pressing the **...** button via Windows Explorer. For large datasets it is advisable to use a drive **other** than the C-Drive of your computer.

Fig. 475   System Options – Image display panel

When ticking the **Interpolate** checkbox Interpolates the signal between pixels and thus smoothens the image. This interpolation is not saved with the original image data and can be active also during acquisition.

Ticking the **Invert x** check box will invert the x-coordinate in the display. The original data set is not changed.

Ticking the **Invert y** check box will invert the y-coordinate in the display. The original data set is not changed.

Ticking the **Invert z** check box will invert the z-coordinate in the display. The original data set is not changed.

Invert time (line scan only) - not available.

Ticking the **Exchange x and y** check box will exchange x for y coordinates and vice versa. The original data set is not changed. The **Fast** image acquisition works differently than conventional confocal point scanning. One difference is the 90° rotated scan direction. In order to match **Fast** images with other acquisition mode and the driving behavior of scanning stages, the **Exchange x and y** checkbox always needs to be checked.

Ignore file sets provides the possibility to open all files, including the master file, as a separate file. Master files are generated when images are saved in individual files along time point, stage position, tile, Z stack position and/or channel. This is defined within the Autosave Tool. Per default the option is not active and such subfiles are opened as one file if the master file (the first file set of all) is opened. Only the subsequent files can be opened individually unless this function is active.

Note: When a file set is opened via the masterfile and modified (i.e. cut region, subset etc.) and then saved again, the new data set will be saved only in one file and not in file subsets like the original data.

Airyscan Processing Baseline Shift - not available.
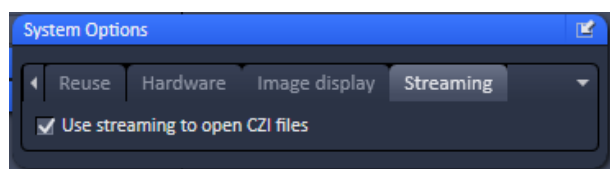
Fig. 476   System Options – Streaming panel

5.4.4.7   System Options – Streaming

Press the **Streaming** tab to open the **Streaming** panel (Fig. 476).

To increase the performance of the computers when opening and saving files, the **Use Streaming to open CZI files** checkbox needs to be checked.

This allows the system to access files faster from the streaming location.

Streaming in general is managed in the **Streaming and Auto Save** tool (see section 5.2.23).
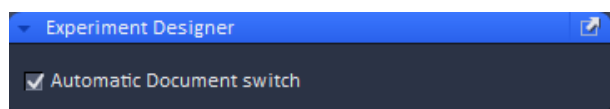
Fig. 477   Maintain, Experiment Designer

5.4.5   Options tool group – Experiment Designer

During the acquisition of multiblock experiments the currently acquired images of a block are displayed in the image container.

This leads to a constant switching between the images during the acquisition. If this is not wanted, it can be deactivated by clearing the **Automatic Document switch** check box in this tool window (Fig. 477).
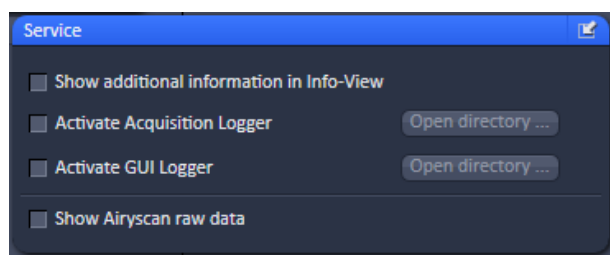
Fig. 478   Options, Service

5.4.6   Options tool group – Service

Only for service use.

6 Center Screen Area / Image Containers - Display and Image Analysis

6.1 Structure and Functional Concept of the Center Screen Area and the Image Display Container

6.1.1 General Structure

In this section, the Center Screen Area of the **ZEN Main Application** window is described. The **Center Screen Area** can be set up to hold 1, 2 or 3 **Image Containers**. Fig. 479 shows the layout with one or two Image Containers.
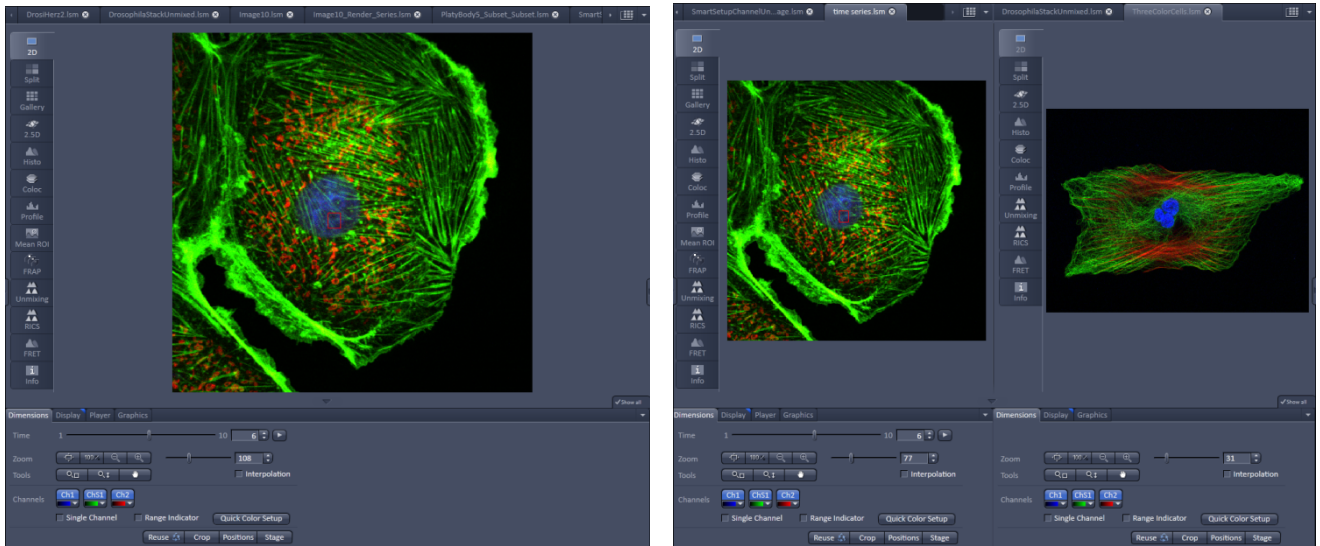
Fig. 479   **The Center Screen Area of the ZEN Main Application window –**
                  Left: 1 Image Container, Right: 2 Image Containers
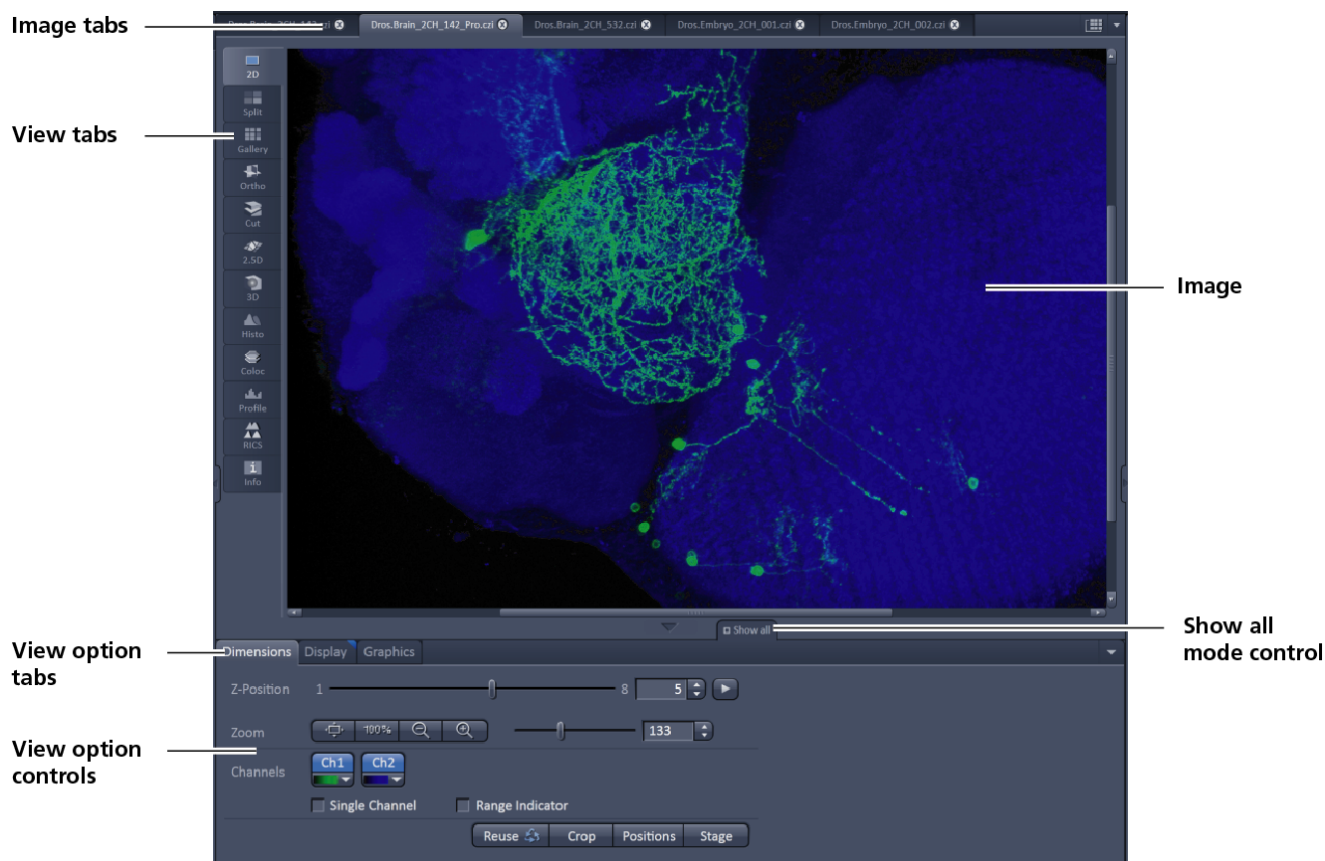
Fig. 480   Image Display window – image tabs show options -

View tabs:

The View tabs make all viewing options and image analysis functions directly available from the main view. Switching from one View tab to another, changes the view type for the currently active image, keeping the image in the foreground. This avoids several display windows for different analysis tools and keeps all information always right at hand.

☞   After switching between several open images, upon returning to a previously activated Image tab, the image document "remembers" which view type was activated before and displays the same parameters upon return to this image.

Image tabs:

The Image tabs show every opened window so they are easy to find and access even when the **Images and Documents** panel in the **Right Tool Area** is hidden/minimized. This way of organizing open images avoids the problem of many opened image display windows lying on top of each other. The **Image Container** concept allows large numbers of images open at the same time and having them all easily accessible while keeping the workspace organized and tidy. **Image** tabs can show the image file name only (Fig. 481/**a**), the name and a small image thumbnail (Fig. 481/**b**) or the file name and a larger image thumbnail (Fig. 481/**c**). This can be configured in the Container Context menu (see Fig. 483).
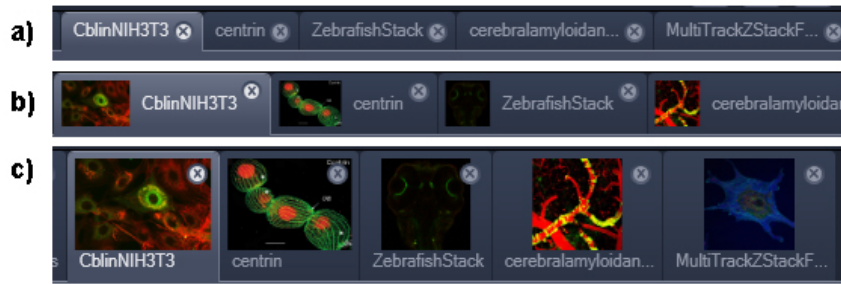
Fig. 481   **Image Display window; –image tabs show options**

View Option control tabs:

These tabs allow individual activation / deactivation of the available **View Option** control blocks by clicking on the tabs. Available but hidden View Option Control tabs are grey. Active tabs are displayed in front of the others:

The **View Option** control tabs:

The **View Option** control tabs are placed in the area under the image display. Each block hosts functionally related tools for image analysis, display modification and data manipulation. There are two groups of **View Option** control tabs: A general one which contains the blocks **Dimensions**, **Display**, **Player** and **Graphics**. The second group consists of **View** tab–specific tool tabs hosting tools that are functionally linked to only one view type. The View tab specific control tabs are marked with a blue triangle on their upper right corner. The control tabs of the general group and their respective tools are described in the section for **2D View** (the first and default **View** tab).

Tab mechanics:

All tabs of the two groups can be dragged out of their groups and placed freely in the **View Options Area**. Where ever it is possible to drop a grabbed tab – the underlaying area is displayed in blue:
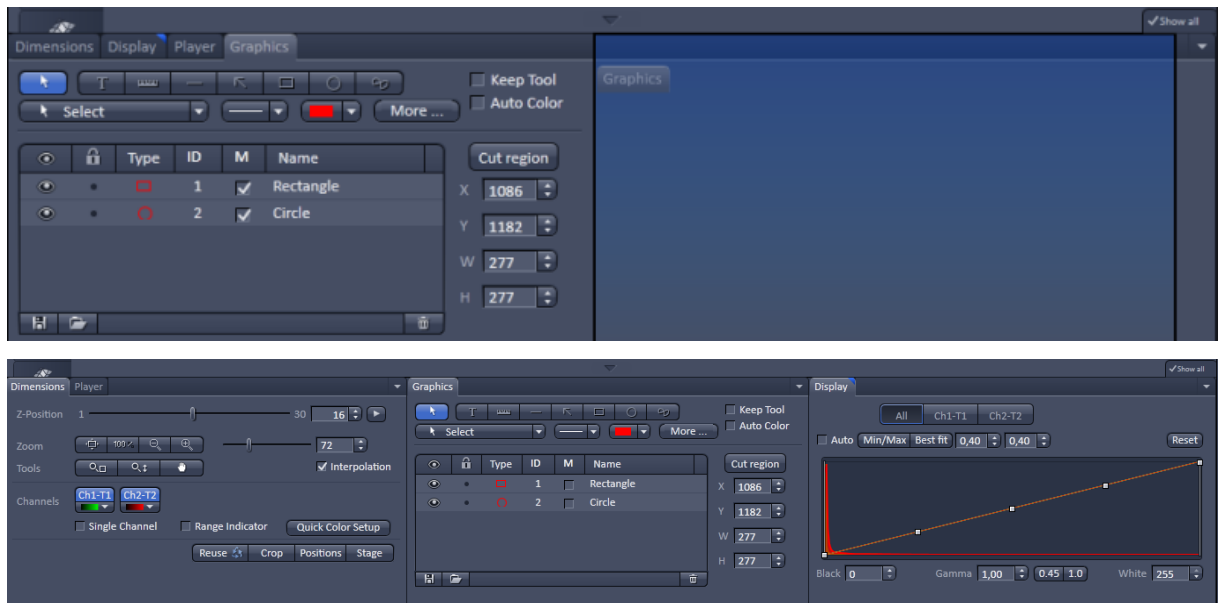
Fig. 482   **General View Options control blocks**

In order to reset the tab layout – use the entry **Reset Tab Layout** in the context menu or click on the

triangle [triangle icon] to access the View Options Area menu.
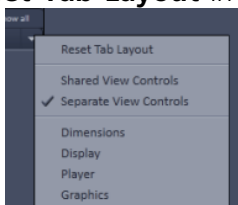
Show all mode of the View Options Area:

Activating the **Show all** mode [Show all button] of the View Options Area has two effects:

a) In every view options control tab all the available tools are shown; de-activating the **Show all** mode hides less often used tools.

b) All available view options control tabs are shown; de-activating the **Show all** mode hide less often used tabs.

Any changes done with these tools have immediate effect on the image display but not on the saved data. To permanently store your results you have to save the data as described in section **Processing – Copy**.

Fig. 483   Control area of the top of the image container

Fig. 484   Open Image pull down menu (left) and context menu of the Center Screen Area (right)

Image Handling and Container Layout Configuration:

The control area (Fig. 483) on the top right corner of the Image Display is for scrolling (click on the left or right triangle button) and listing the Image tabs which cannot be displayed if too many images are opened (left click on the down triangle).

This is just an alternative way of handling the open images (Fig. 484, left).

The main tool for this purpose is the **Images and Documents** panel (see section **Right Tool Area, Data Management and Storage**).

A right click on the image tab row opens the context menu (Fig. 484, right). for the layout of the Center Screen Area.

The Image Display:

The Image Display contains and displays the image data or –depending on the active view type – a combination of image data, overlays, graphs and tables. The content is automatically maximized to the available image or display size.

Expose Mode:

Clicking the **Expose Mode** button [icon] in the top right corner of the Image Display opens a field of thumbnails in the image display with one large thumbnail for each open image. Whenever you lost track of your open images, open the Expose Mode and click once in the image you are looking for and it will be immediately brought to the foreground of the Image Display (Fig. 485).
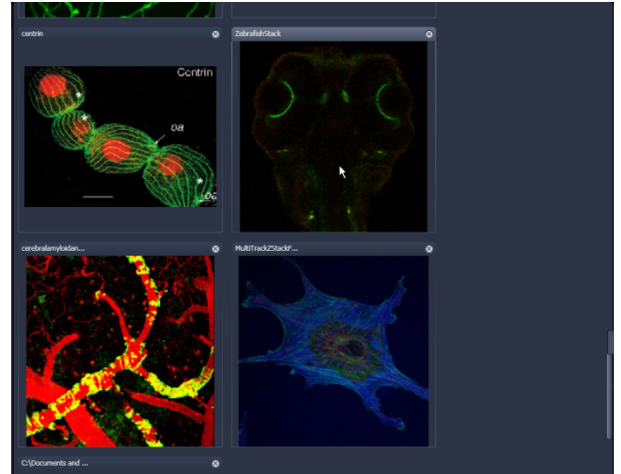
Fig. 485　　　　Expose Mode Display

6.1.2　　Container Configuration with the Container Context Menu

Clicking on the container background with the right-mouse button opens a context menu for:

– setting the view for the Image tabs (see above),

– choosing to split the Center Screen Area in 1, 2 or 3 containers and setting the automatic container layout

– choosing between separate or shared view controls between the containers.

The same menu is available in the menu bar in the **View** part of the menu.

Splitting the Center Screen Area in several containers has the advantage that side-by-side comparison of image data becomes very easy.

Fig. 486　　　　Container Context menu

The disadvantage is that the individual container necessarily becomes smaller. Up to 3 containers can be chosen. The "Automatically layout container" option is switched on by default and fixes the container width in layouts with multiple containers. In two-monitor setups it also fixes the right edge of the first container to the monitor edge.

If **Separate view Controls** are chosen, each container has its own set of View Options control blocks. If a **Shared view Controls** is chosen, the set of View Options control tabs spans the whole area under the containers. The tools and functions always apply to the currently active container / image.

6.2   2D View

This function allows to

– display a single image in frame mode,

– display multiple channel images in superimposed mode.

The following **View Option** control blocks apply to this View Type:

Dimensions, **Display**, **Player** and **Graphics**.

In the 2D view, only the general **View Option** control blocks are displayed. These view option blocks are also available in those other view options in which the functions are applicable.

The general view option control blocks are only described in this section.
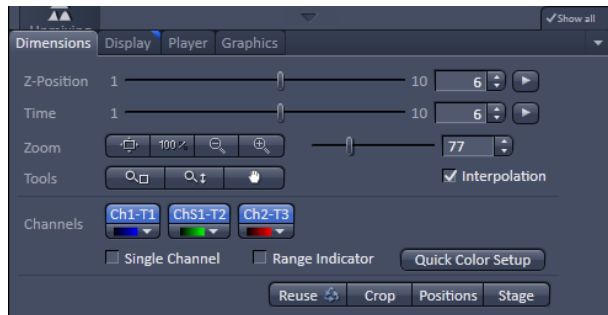
Fig. 487   View Options control block - Dimensions
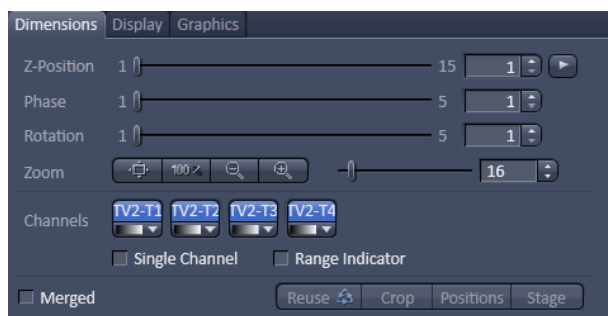
Fig. 488   View Options control block – Dimensions for SIM images

6.2.1   Dimensions

The **Dimensions View Options** control block holds the tools to modify the image display with respect to the (multiple) dimensions of the image data set.

The sections (slices) in the dimensions can be scrolled with sliders (**Z-Position** and **Time** in Fig. 487) and also directly addressed with setting numbers in the spin-boxes next to the sliders.

For **SIM** raw images you will have in addition available sliders for the Phases and Rotations of the grid (Fig. 488).
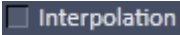
The small [▶] button on the right hand side of the spin-boxes (**Z-Position** and **Time**) starts and stops an animation in the respective dimension. This basic animation cannot be customized. For all other player-functionality refer to the **Player View Block**.

(1)   Dimensions – Zoom

In the **Dimensions View Options** control block, there are the **Zoom** and **Tools** function lines related to changing the zoom factor of a displayed image.

Any changes on these elements affect the displayed image immediately. The zoom function can be performed online.

The functions of the **Zoom** and **Tools** lines are:

Zoom Normal: The image is fitted automatically to size of the **Image Display** window which is always the originally displayed size.

Zoom 100%: displays one pixel of the image also as one pixel on the screen.

Zoom - reduces the image.

Zoom + enlarges the image.

Spin-box and slider: type in your magnification value in percent directly. The zoom factor can be set alternately by moving the slider. The spin-box displays the current zoom factor in %. 100% corresponds to the original size.

Zoom selection via mouse: draw the area to zoom in directly on the image.

Zoom-Mouse: allows you to enlarge / reduce the zoom factor of an image using the left / right mouse button, provided that the cursor is inside the image.

Navigation hand: allows you to scroll the position of a zoomed image inside of the image window.

Interpolation check box: activates / deactivates the zoom interpolation function.

(2) Dimensions – Channels

The **Channels** functions are designed to switch on/off channels or the display of the merged image as well as to assign color look-up tables (LUTs) to the individual channels:

Fig. 489   Image Display - Dimensions - Channel color lock-up-table

Clicking on the Ch1-T1 ChS1-T2 Ch2-T3 buttons switches the respective channels on/off in the display.

Clicking on the buttons or white arrow opens the channel color lock-up-table to select the color, LUT or no color for this channel (see Fig. 489).

The **Single Channel** check box activates / deactivates single mode operation.

The **Range Indicator** check box activates / deactivates the **Range Indicator** function.

Clicking on the **Quick Color Setup** button opens a selection menu. Here you can change the image view between **BGR**, **GRB**, **RGB**, **Grey** and **Range** indicator mode quickly.

Any changes on these functions affect the displayed image immediately.
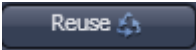
If the display area of the channels is not enough to display all channels a slider becomes available below the listed channels.

Fig. 490   Image Display - Dimensions

That allows you to scroll through all channels (Fig. 490).

(3) Dimensions – Reuse

Clicking the [Reuse] button transfers **ALL** acquisition parameters (exception: objective and collimator; see below) from the stored image data to the **Microscope Hardware Settings / Control tools** and applies those parameters directly to the system.

The acquisition parameters of an image are displayed in the Information View (section **Information View**).

In the **Maintain** tab (section **Options tool group – System Options – Reuse** tab), it can be set whether the objective and the collimator settings are also "re-used" and applied to the system. **Reuse** of the microscope objective only works in microscopes with motorized objective revolvers.
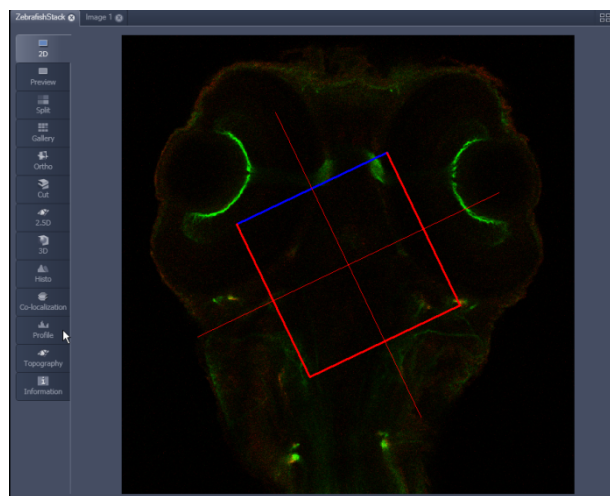
Fig. 491   **Image Display - Dimensions - Crop**

(4) Dimensions – Crop

This function allows defining the size and orientation of a rectangular scan area on the image displayed in the **Image Display** window interactively.

The defined area is synchronized with the **Zoom, Offset and Rotation** parameters in the Scan Area panel (partially **Show** all mode) in the Acquisition Mode tool (section **Acquisition Parameter – Acquisition Mode**).

Click on [Crop] will display the **Crop Rectangle** in the **Image Display** (Fig. 491). Any changes done with the **Crop Rectangle** are setting the scan parameters immediately. On the next execution of a scan (**Set Exposure**, **Live**, **Continuous**, **Snap**) these new scan parameters will be applied. To reset the crop function and use default values set **Zoom=1**, **Offset=0** and **Rotation=0** in the **Scan Control** window in the Scan Area panel in the **Acquisition Mode** tool (or click "reset all" in the same panel). When the [Crop] button is de-selected, the scan parameters are reset as well.

The **Crop Rectangle** is controlled via the following functional elements:

Offset   Click into the crop rectangle, keep the left mouse button pressed and drag the crop rectangle to the required position. Release the mouse button.

Zoom   Click on a corner of the crop rectangle, keep the left mouse button pressed and set the required size. Release the mouse button.

Rotation   Click on one end of the crosslines, keep the left mouse button pressed and set the required rotation angle. Release the mouse button. The first line scanned is highlighted in blue.

Side ratio   Click on any of the intersection points between crossline and crop rectangle, keep the left mouse button pressed and change the side ratio as required. Release the mouse button.

(5)   Dimensions – Positions

When clicking Positions a crosshair will be displayed at the cursor position in the image. With a mouse click the position of the crosshair in the image will be added to the Positions list in the Stage tool and the Positions tool.

(6)   Dimensions – Stage

Stage will show the cursor as a rectangle with a crosshair in the centre. With a mouse click the stage (and therefore the sample itself) will be repositioned. The image position with the cross hair will become the centre position of the stage and therefore the centre position of a next image

6.2.2   Display

Fig. 492   View Options control block - Display

In the **Display** View Option control block, the intensity minimum can set to the Black value and the intensity maximum to the White value of the image using the Min/Max button.

Clicking on the Best fit button sets the intensity minimum to Black and percentile value and the intensity maximum to White and percentile value.

Activating the ✓ Auto check box sets the intensity minimum and maximum values automatically.

With the 0,40 spin-boxes you can set the lower and upper percentile values.

The **Black**, the **Gamma** and the **White** values of the displayed image can be adjusted (Fig. 492) individually.

With the All Ch1-T1 ChS1-T2 Ch2-T3 buttons, the effect of the settings can be restricted to an individual channel or be applied to all channels. By default, the settings apply to all channels simultaneously.

The parameters can be changed using the spin-boxes – or directly by typing in numbers in the number-field. With the Reset button, the original settings are easily re-set.

The settings in this View control block apply only to the Image Display and are NOT changed in the original data set. To write the changed settings to the image data set, use the **Burn in Brightness and Contrast** function in the **Processing** tab (section **Processing Tab**).

Fig. 493　　　View Options control block - Player

6.2.3　　　Player

This function allows to

– animate frames of a Z-Stack or a time series

– specify animation parameters such as range and animation speed

When the image displayed in the **Image Display** window is neither a Z-Stack nor a time series this View Option control block is empty.

The **Player View Option** control block is controlled by the following elements:

 The current position slider is displayed in the View Option control block **Dimensions** and can also me moved manually or by entering the slice number in the input box. The slider can be accessed only, when the player animation is off.

 Start and **End** slider: The setting of the **Start** sliders limits the number of slices to be used for the animation. Slices before **Start** and after **End** are not taken into animated. These sliders can be changed during automatic animation.

 Starts the forward motion of the automatic animation. After the last slice has been passed, restart is made at the first slice.

 Starts backward motion of the automatic animation. After the first slice has been passed, restart is made at the last slice.

 Starts the combined forward / backward motion of the automatic animation, i.e. when the last slice has been reached, the backward motion is activated, and the forward motion is activated again on reaching the first slice.

 Stops the automatic animation.

 Move to the first slice.

 Move to the last slice.

 Speed can be set through a slider or spin-box (selection box).

 Multidimensional animations can be selected by including individual dimensions by checking tick boxes.

6.2.4 Graphics

This function allows to

– select from a set of drawing functions such as rectangles and arrows,

– add a scale bar to the image, as well as text annotations,

– use a set of interactive measurement functions for length, angle, area and size,

– add a text box that displays the coordinate of a hidden dimension (e.g., the z-position in a Z-Stack or the timestamp in a time series).
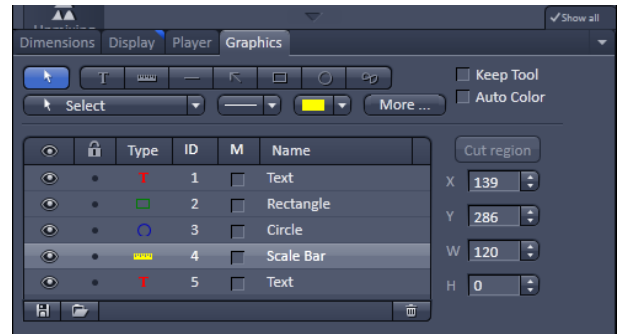
Fig. 494   View Options control block - Graphics

Functional Description

The graphics function uses a plane separate from the image plane (the graphics plane) and therefore does not change the content of the image(s).

The **Graphics** view option control block is available in all View Types except **2.5D**, **Lambda Coded** and **Preview**.

Any changes done with this function are effective immediately.

The overlay graphics can be stored in the images or separately in a file from where they can be re-loaded.

Available functions in the Graphics View Control Block
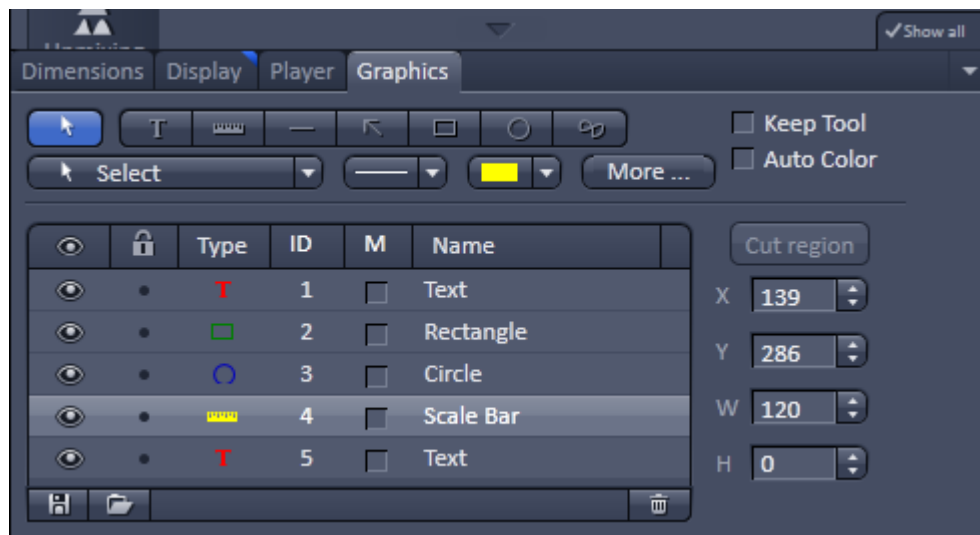
Fig. 495   Graphics view control block

The following list describes the most common functions in the **Graphics** view control block. More can be found in the [Select] pull down menu.

Select tool: Activation of the mouse cursor for selection, resizing or movement of an graphic element in the Image Display.
Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.
Movement: Click on the line and hold down the mouse button, move the entire element, release the mouse button.

Text (Annotation) tool: Creation of a text box in the Image Display.
After clicking on T, the Text window will be displayed, and text can be entered via the keyboard. The Font ... button enables you to select the font style and size in the Font window. The entered text will be displayed in the left upper corner of the Image Display window after clicking on OK and can be moved to the required position using the mouse. The Text window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.
Insert opens up a further window which allows you to annotate coordinates, time and Z-position with either automatic or user definable units and precision. This annotation is updated during image acquisition and can be exported with the image. The annotation can be stamped into already existing images.

Scale bar: Creation of a horizontal or vertical scale bar with default increments in the Image Display. Click and hold the mouse button for the starting point, drag horizontal or vertical scale, release the mouse button to end the procedure.

Line tool: Creation of a straight line in the Image Display.
Click and hold down the mouse button, draw a line in any required direction, release the mouse button to end the procedure.

Line with arrow button: Creation of a line with arrow in the Image Display window.
Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.

Rectangle tool: Creation of a rectangle in the Image Display.
Click and hold down the mouse button, draw a rectangle in any required direction, release the mouse button to end the procedure.

Circle tool: Creation of a circle in the Image Display.
Click and hold down the mouse button, draw a circle in any required direction, release the mouse button to end the procedure.

Closed Bezier tool: Creation of a closed bezier figure in the Image Display.
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure and closes the figure.
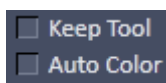
Keep Tool check box: keeps the selected tool for creation of further objects, if activated.

Auto Color check box: sets the color of the object automatically, if activated.

Line thickness pull down: sets the line thickness of the selected element.

Color selection pull down: In this pull down, different colors can be assigned to the graphic elements with a click of the mouse. A selected color is automatically assigned to the currently selected graphic element and to all elements created afterwards.

Cut Region tool: The region of a Z-Stack or 4D-image surrounded by an graphic element is extracted and displayed separately in a new Image Display. This function is only active if the active graphic element defines a closed contour or volume.
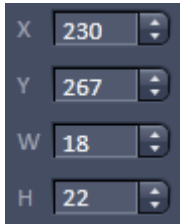
Coordinate and dimension display: shows the XY coordinates of the object center and the width and height of the graphic element.

Function elements of the graphics list box:

Activate/deactivate the display of the graphic element in the image.

Locks / unlocks the graphic element in the image

Shows the type of the graphic elements as an icon.

Shows the number of the graphic elements.

Activate/deactivate the display of the parameters of the graphic elements, e.g. A = area, for a closed object; L = Length of the line (or circumference of a closed object); Angle for a line/arrow or angle.

Shows the name of the graphic elements.

More ...: **The More** options (Fig. 496) allow the selection of the following text parameters in the appropriate list box:

– Text size

– Font name

– Font style

– Font character set

Clicking on the **S** or **L** buttons toggles between small or large font size.
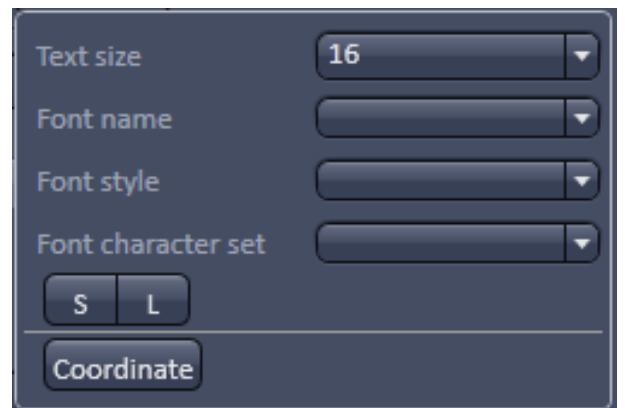
Fig. 496   **View Options Graphics - More**
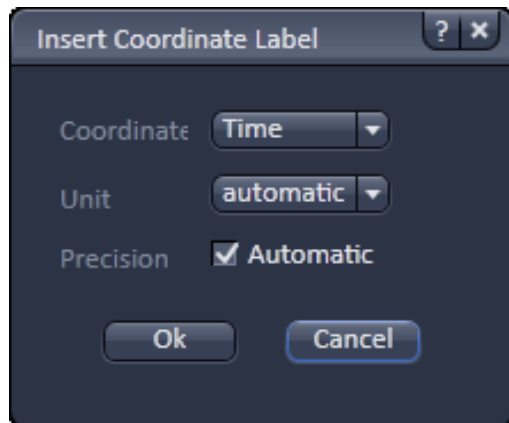
Fig. 497   View Options Grapics - Coordinate

To insert a **Coordinate Label** in the image display, click the [Coordinate] button in the **More …** dialog. The coordinate settings dialog (Fig. 497) offers the available settings and upon clicking **OK**, the selected coordinate is displayed as a text field in the image display. This text field is always immediately updated when the slice/section/time point is changed in the display by changing settings in the **Display View Option** control block or, for example, starting the **Player** animation. This feature is particularly useful to display time or z-position in an exported animation movie for presentations (see Fig. 495).

To load / save graphics from / to a file use the **Load** [ ] / **Save** [ ] buttons in this View Options control block. To delete a graphic element from the image select this element and click on the **Delete** [ ] button.

Naming and location in the file system can be set individually.

6.2.5   Preview View

The Preview View Type is an enlarged copy of the thumbnail Previews described in the section **Processing Tab**.

6.3   Split View

This function allows to

–   display the individual channels of a multi channel image as well as the superimposed image.

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** view options control blocks apply with the following additional features:

–   The **Dimensions** View Options control block shows the **Merged** tick box to activate / deactivate the display of the channel overlay and a **Zoom All** button is added synchronize zooming to the Dimensions view control block.

–   Graphic overlay elements are always displayed in all channel displays.
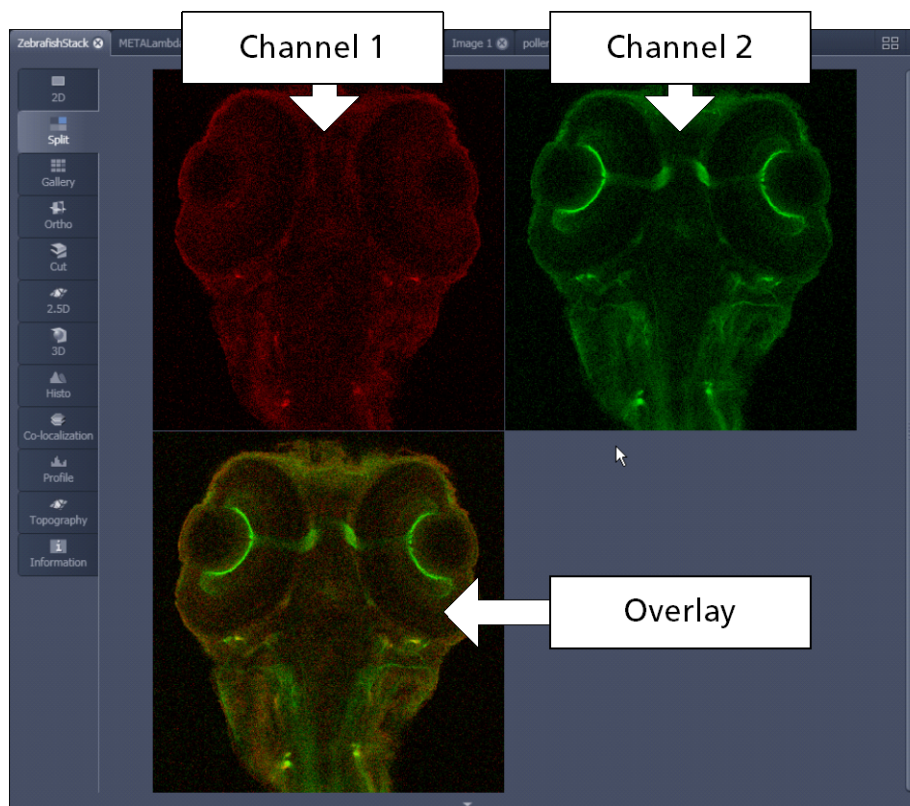
Fig. 498   Image Display, Split view type

☞   This function is useful to optimize the individual channels in a multi channel image acquisition together with the **Range Indicator** palette.

Fig. 499   Image Display - Dimensions – Channels with mixed modes

In the ELYRA systems channels of different imaging modes can be combined. For example, if SIM (SR channel) or SMLM (HR channel) images are computed they can be displayed in combination with widefield (WF channel) or sum widefield (SWF channel) images (Fig. 499).
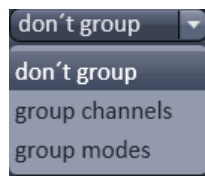
Fig. 500   **Mixed mode grouping drop down menu**

Channels if not rearranged by the user, are by default displayed by their color that is the different modes <u>are listed together.</u>

If the Software recognizes such **mixed mode** channels, the **mixed mode grouping** drop down menu becomes available (Fig. 500).

You have three options to group the **mixed mode** channels. If you choose **don't group**, all channels will be displayed split (Fig. 501).
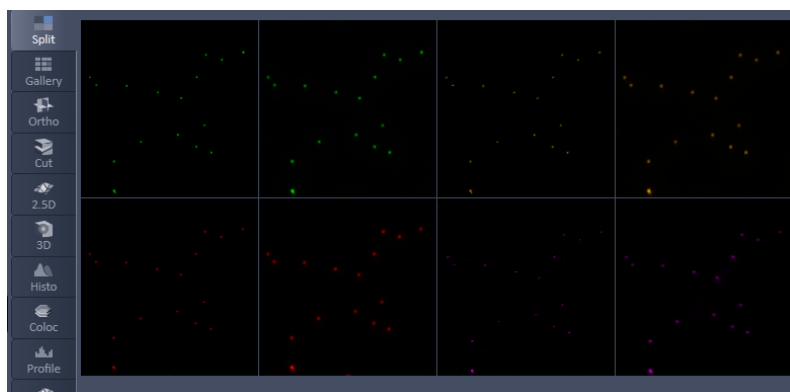
Fig. 501   **Image Display, Split view with mixed modes – don't group: 4 color channels with the 2 modes displayed together**

If **group channels** is selected all channels belonging to the same color will be merged in one image (Fig. 502).
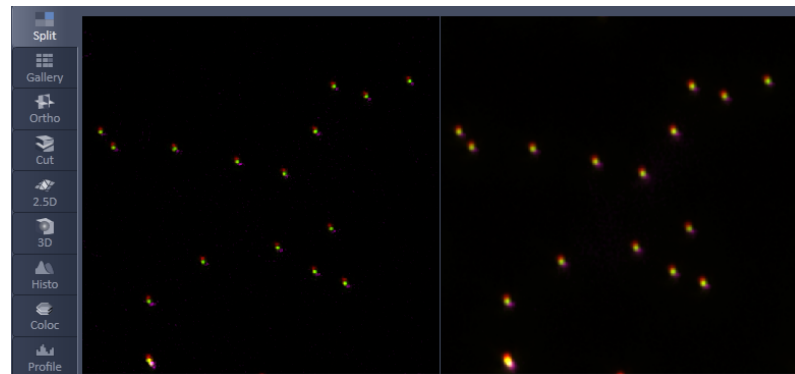
Fig. 502   Image Display, Split view with mixed modes – group channels: 4 color channels with 2 modes merged

If **group modes** is selected all channels belonging to one mode will be merged in one image (Fig. 503).
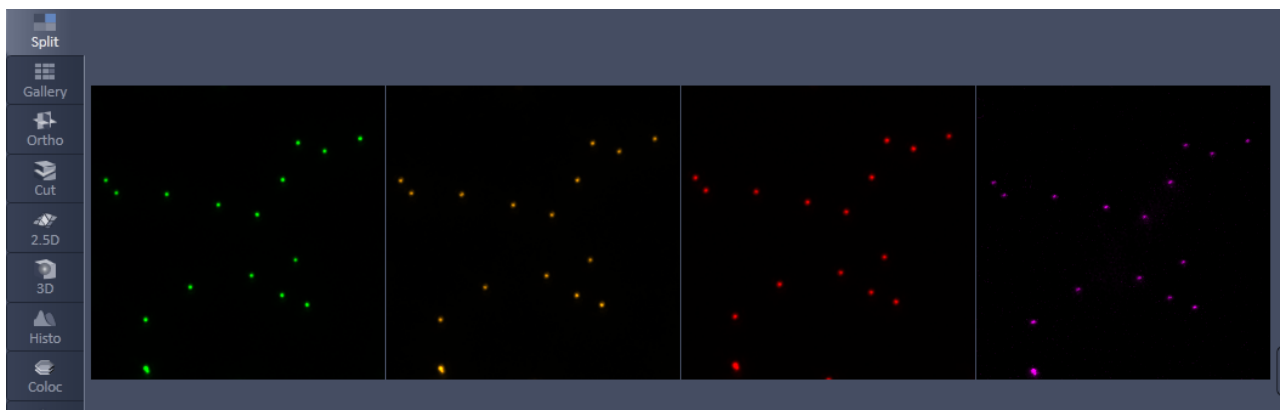
Fig. 503   Image Display, Split view with mixed modes – group modes: 2 mixed mode channels with 4 colors merged

6.4 Gallery View

This function allows to:

– display images (Z-Stack, time series, lamda scans and combinations of those) side by side in a tiled fashion,

– add data relevant to the displayed images (Z-Stack slice distance, time of acquisition or wavelength),

– extract a subset of images from the original stack and store the result as a new image (controls for this function are in the **Processing** tab function **Copy → Subset** (see section **Processing – Copy**).

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** View Options control blocks apply.

The additional view-specific **Gallery** View Option control block includes a tick box to switch on and off the display of the slice coordinates in the Gallery display. If this text is activated, transparent mode and color can be selected in this control block too (see Fig. 504).

In Gallery mode you will also see small indicators for the following actions that (might) have taken place during the acquisition:

– markers for changing intervals in time series

– markers for events set during a time series

– markers for bleach events during a time series

– markers for detector gain down regulation in case of detector overexposure

– a marker in case the detector has been shut down to protect against damage due to too much light
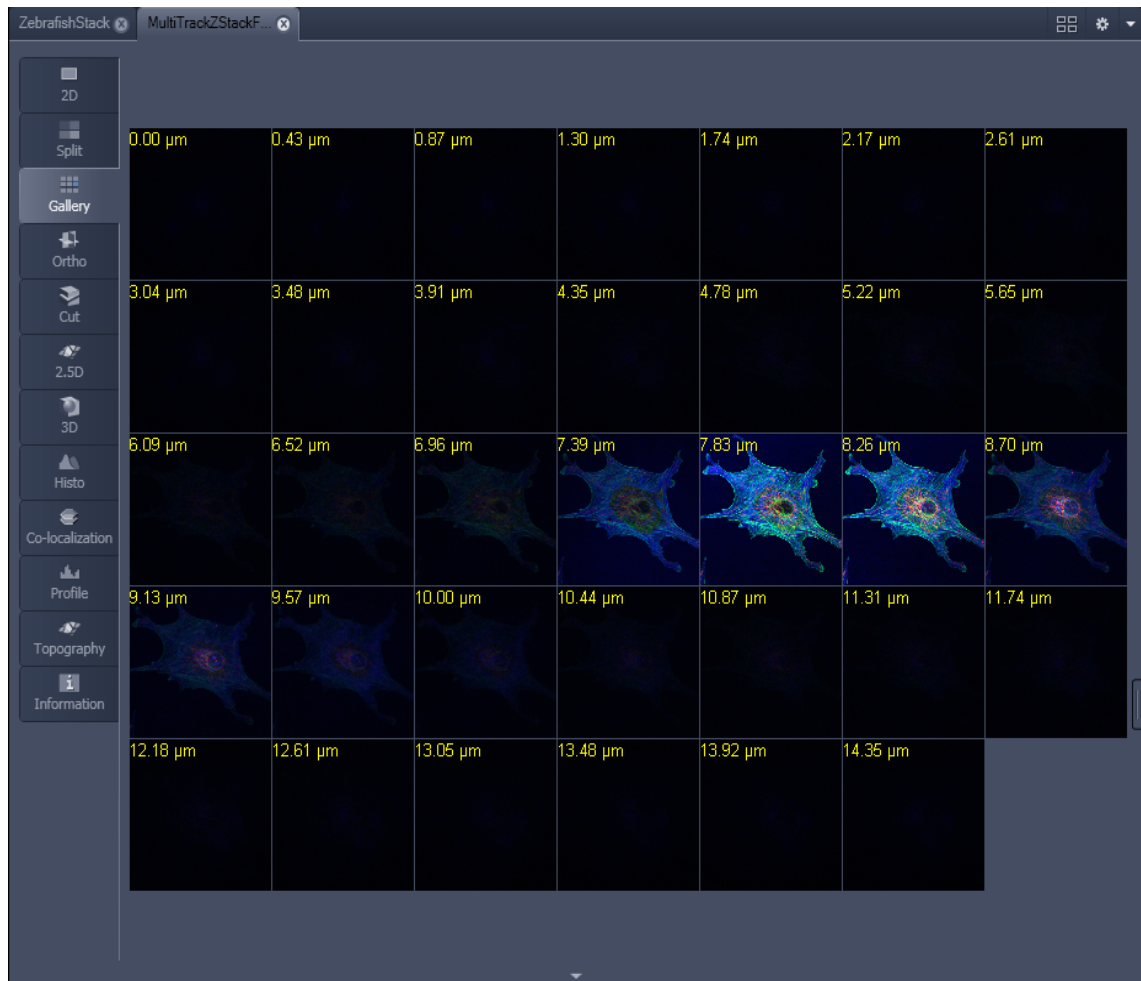
Fig. 504   Image Display window - Gallery display
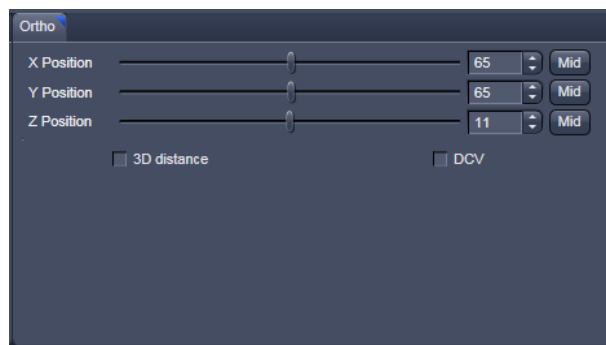
Fig. 505   View Options control block - Ortho

6.5   Ortho View

This function allows to

- display a Z-Stack of images in an orthogonal view

- measure distances in three dimensions

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** view options control blocks apply.

In addition to the 4 general View Option control blocks, the view-specific **Ortho View Option** control block is available (Fig. 505).

- In the **Ortho** View, section sliders appear in the **Ortho View Option** control block together with orthogonal projections in the image (Fig. 506).
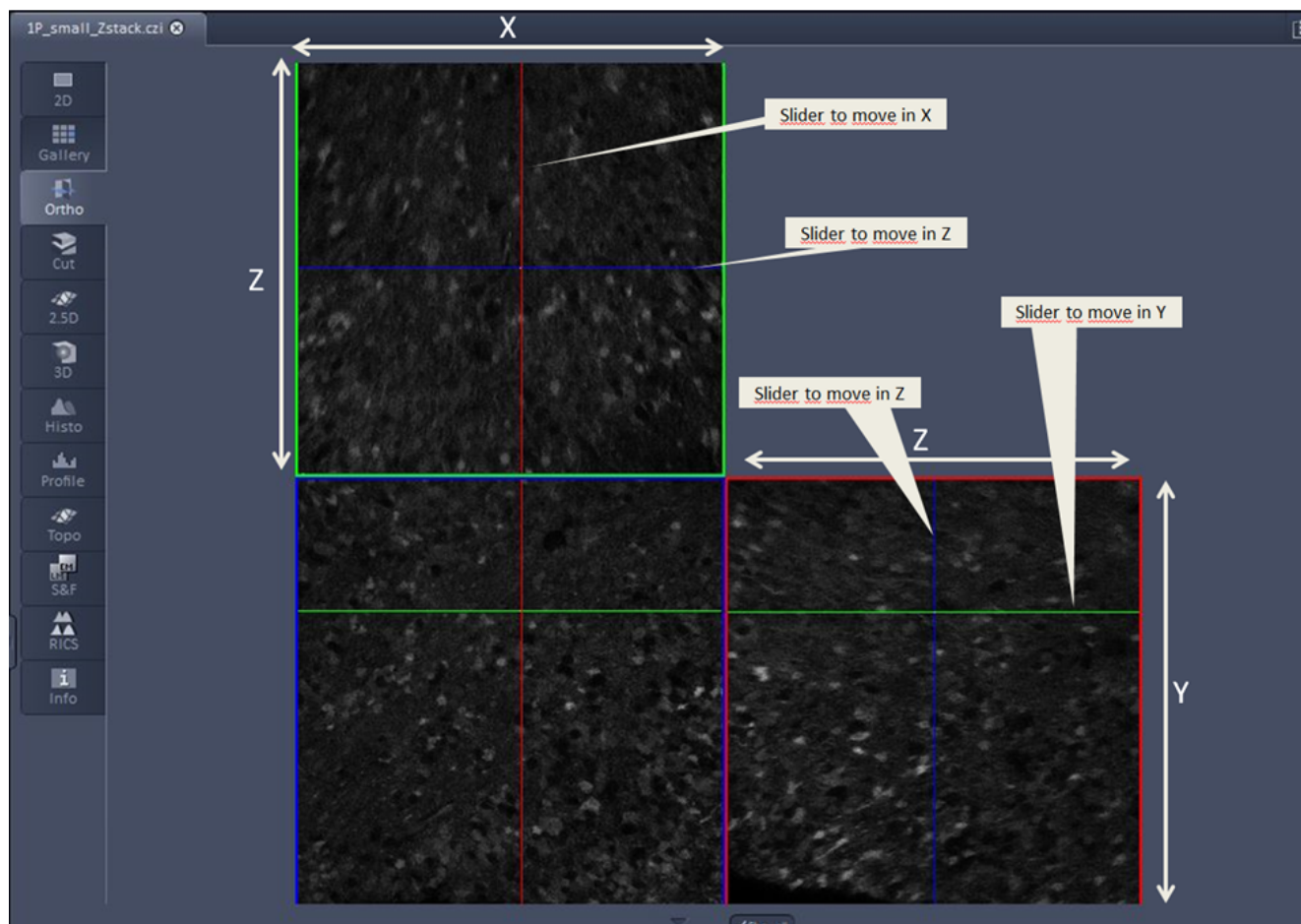
Fig. 506   Image Display - Ortho View

6.5.1   Ortho - Select Function

- By changing the parameters X, Y and Z in the **Ortho** View Option control block, the section plane can be positioned at any XYZ coordinate of the Z-Stack.

The position of section planes can be changed in various ways:

- By moving the sliders on the **Ortho** View Option control block.

  - X and Y settings may range from 1 up to the maximum number of pixels scanned (in the example shown: 512).

  - Z settings may range from 1 to a maximum of n, with n standing for the number of slices produced in the stack.

- By directly entering the relevant number value in the X-, Y- or Z-input box and pressing the **Tabulator** or **Enter** key.

- If you move the cursor into the **Image Display** window, it changes into a crossline symbol $\oplus$. By positioning this symbol with the mouse you can move the XZ and YZ section planes to any point of intersection with the XY plane. A click with the left mouse button places the intersection to the desired position.

- If you move the crossline symbol $\oplus$ onto the intersection of the red and green section planes, it changes into the: symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **simultaneously**.

- If you move the crossline symbol $\oplus$ onto the green section plane, it changes into the symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.

- You can reposition the (red) YZ plane in the same way using the symbol.

The result of an orthogonal section is visible at the image margin.

  - Section of the XZ plane (green line) through the stack: above the XY image.

  - Section of the YZ plane (red line) through the stack: right of the XY image.

  - Section of the XY plane (blue, slice plane of the stack): center image.

☞   If you zoom in one plane, the other planes will be zoomed with the same factor.

6.5.2   Ortho - Distance Function

- Activating the [✓ 3D distance] tick box permits length measurements in 3D space.

- Click on the [Mark] button to set the first XYZ-point for the measurement of the spatial distance.

- Set the second XYZ-point for measurement by moving the X-, Y-, Z-sliders or by moving the green, red and blue lines in the image.

  – The projections of the spatial distance are shown in the image planes by yellow lines (Fig. 507). The actual spatial distance is calculated and shown in µm next to the [Mark] button.
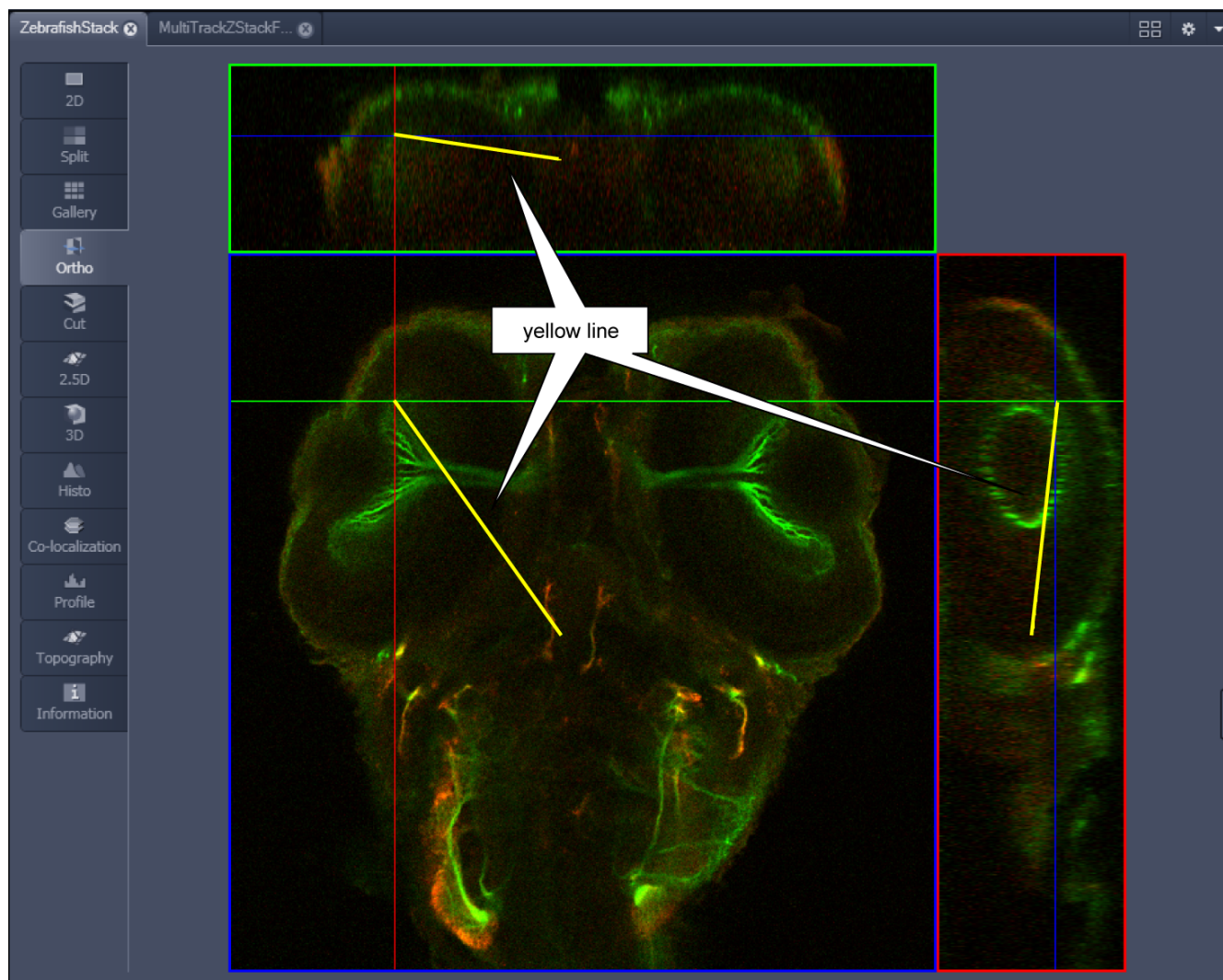
Fig. 507   **Image Display – Ortho View - 3D Distance Measurement display**

6.6   Cut View

This function allows to display a user defined section plane (= cut plane) of a Z-Stack. It automatically improves the image of the section plane by trilinear interpolation.

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** View Options control blocks apply.

Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

- By varying the parameters **X**, **Y**, **Z**, **Pitch** and **Yaw**, you can position a section plane of any orientation within the stack volume.

- Clicking the [Mid] [Mid] [Mid] [0] [0] buttons restores the original position.
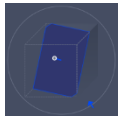
In order to place the Cut View Plane interactively use the interactive graphic in the cut view control tab.

Fig. 508   **Cut View – control block**

6.7   2.5 D View

This function allows to

- display the two-dimensional intensity distribution of an image in an pseudo 3D mode,
- show the intensity values in profile, grid or filled mode,
- show separate distribution for each channel in a multi-channel image.

☞   The **2.5 D** button can also be used online during scanning.

The settings of the **Dimensions** View Options control blocks apply. The settings of **Display**, **Player** and **Graphics** View Options control blocks do not apply.

Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

The image display in the **2.5 D View Type** is the same as in the **3D** View Type. For a description of the controls in the display window see section **3D Rendering Settings in VisArt**.

In brief, the viewing plane of the **Image Display** window can be rotated, tilted either directly with the mouse or by the scroll bars on the right-hand side and the bottom of the **Image Display** window.

a)

2.5 D Profile View

b)

2.5 D Grid View

c)

2.5 D Filled View

d)

2.5 D Surface View
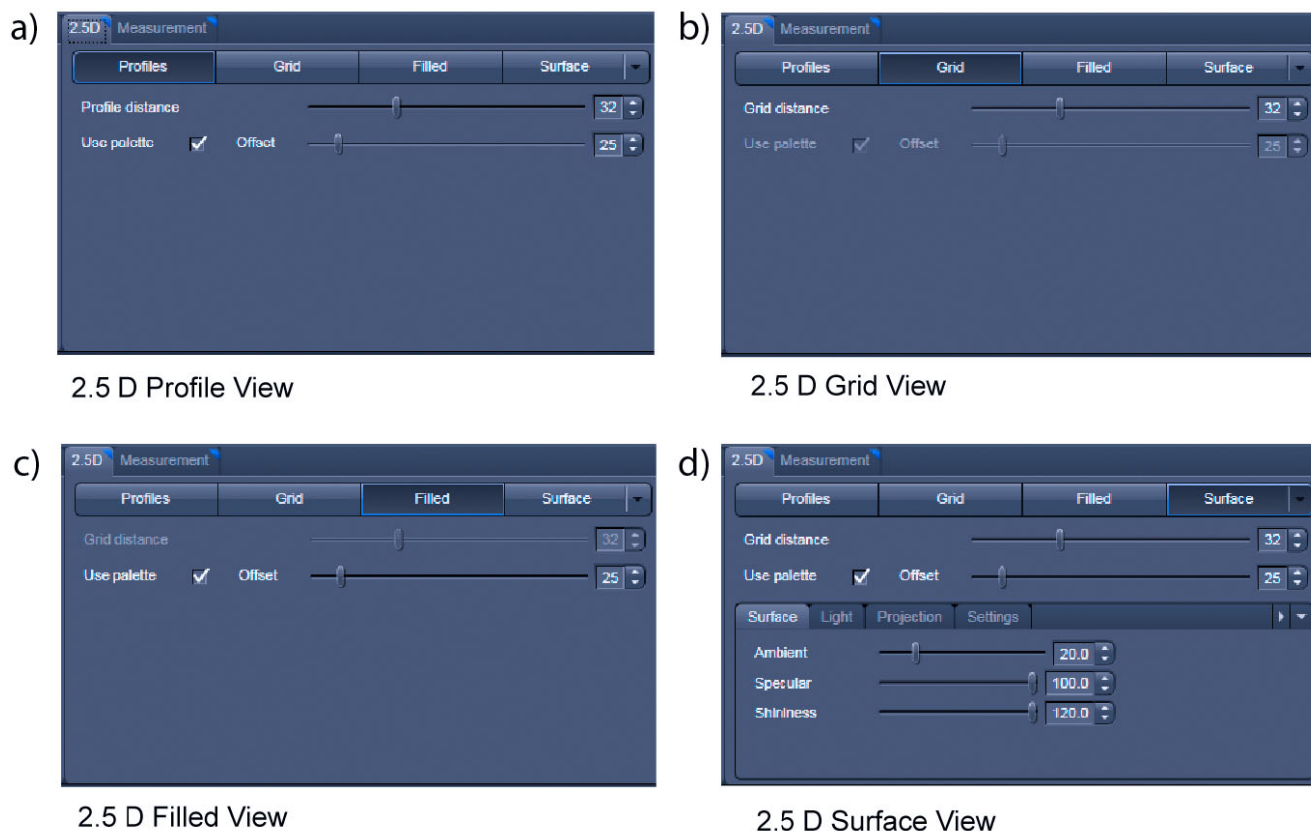
Fig. 509   2.5 D View Option control tabs

2.5 D Profile View   2.5 D Grid View

2.5 D Filled View   2.5 D Surface View
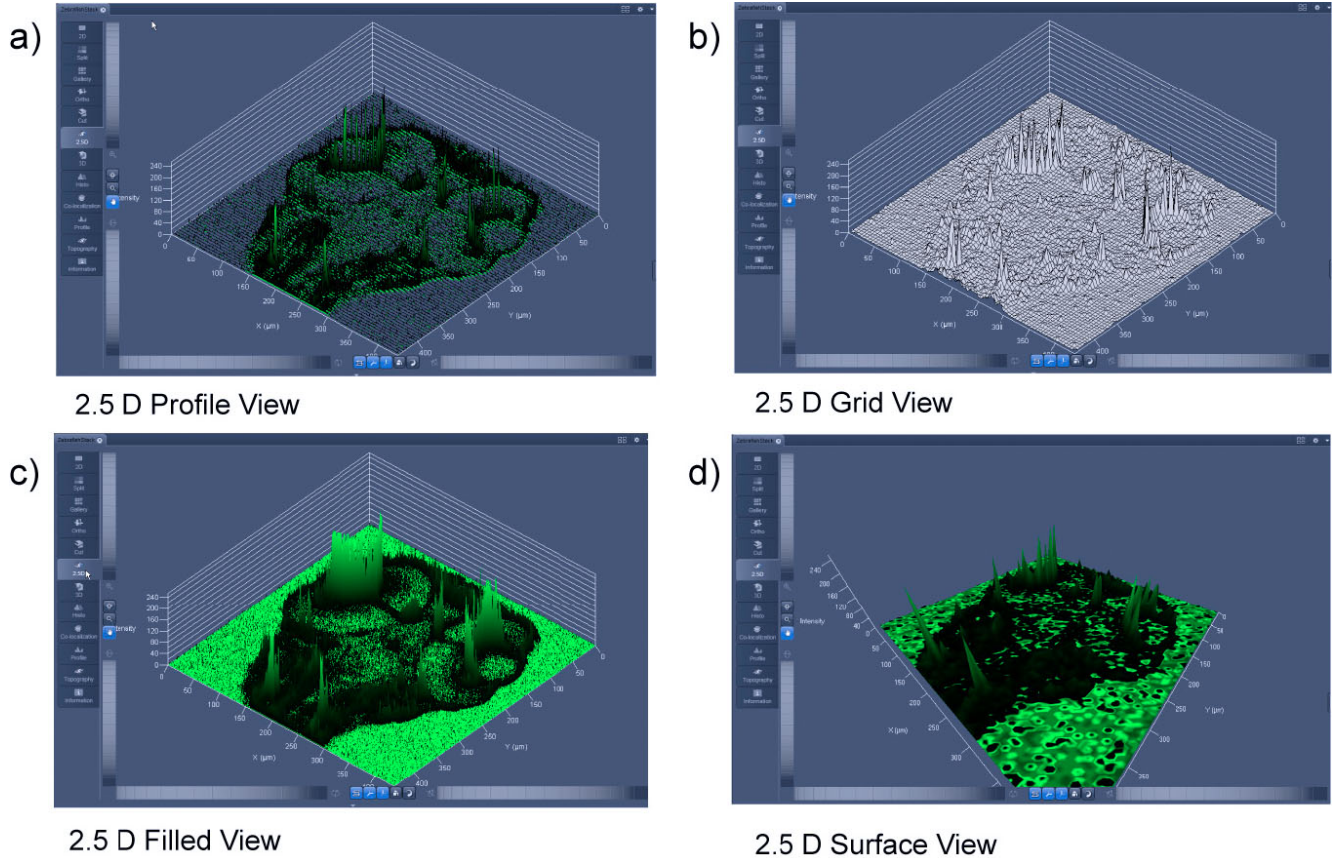
Fig. 510   **2.5 D Image Display**

The 2.5 D view control tab contains the following function elements:

Profile button   Profile display (vertical polygon display). Setting of the **Profile Distance** between 1 and 20 using the slider.

Grid button   Grid display (horizontal grid display). Setting of the **Grid Distance** between 1 and 20 using the slider.

Filled button   Color diagram (filled 3D diagram). Selection between the **Mono**, **Rainbow** and **Six Step** color palettes.

6.8   3D View

Use this View tab to display image Z-Stacks in three dimensions. Display and rendering of Z-Stacks with up to eight color channels are supported and the data can be a time lapse series (5D Image Stack). Advanced functions are linked to a license for the ZEN Module 3D VisArt.

The LUT for the channels set in the View option tab **Dimensions** and interpolation settings as well as the parameters set in the View option tab **Display** are applied. All other parameters from the **Player** and **Graphics View Option** tabs are not applied.

A click on **3D** will display the **3D** View Controls. Any changes done within these controls are effective immediately.

☞   The **3D** View can also be used online during scanning.
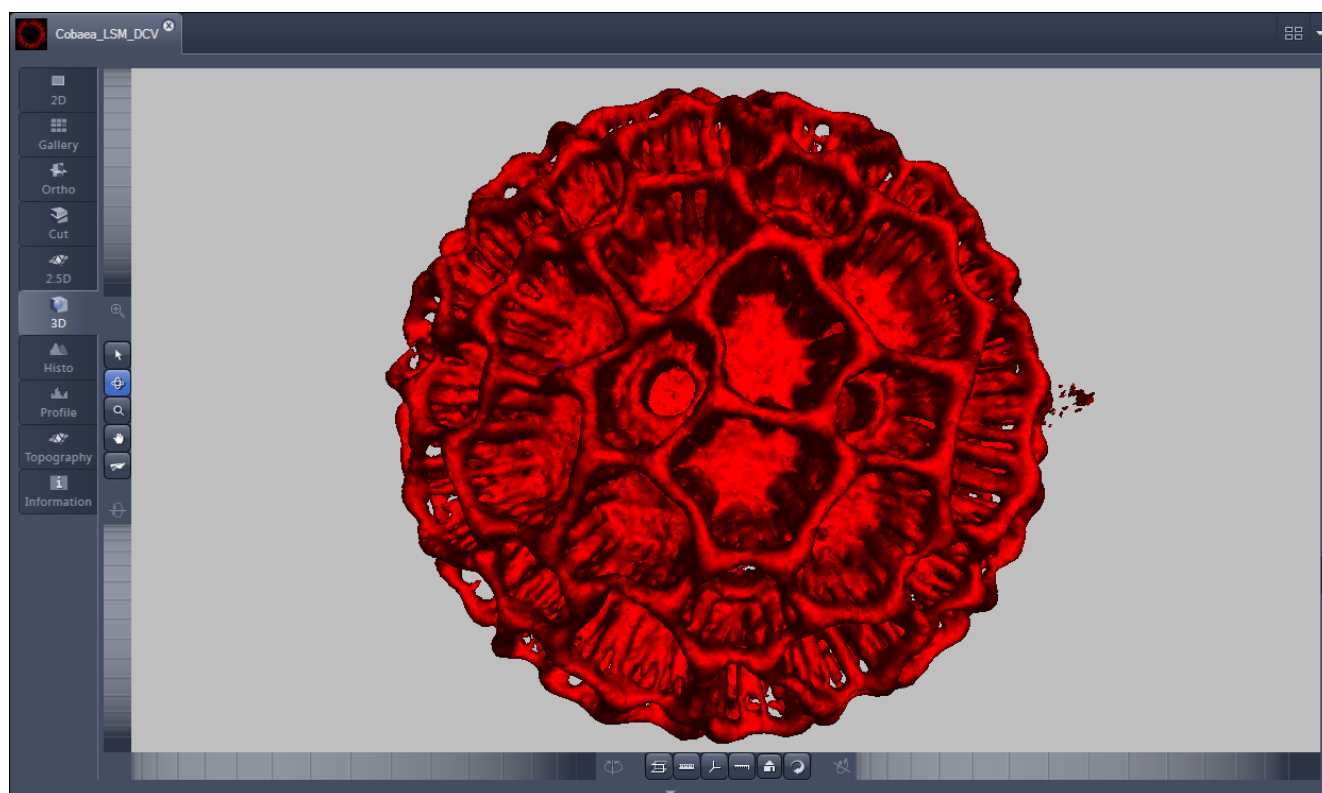
Fig. 511   Image Display window - 3D display

The **3D** View Controls contain the following functional elements:
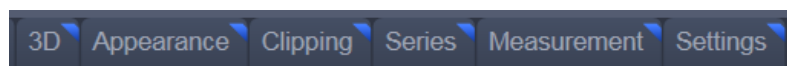
They are displayed when the tab is pressed in the order shown above.
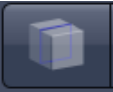
3D:
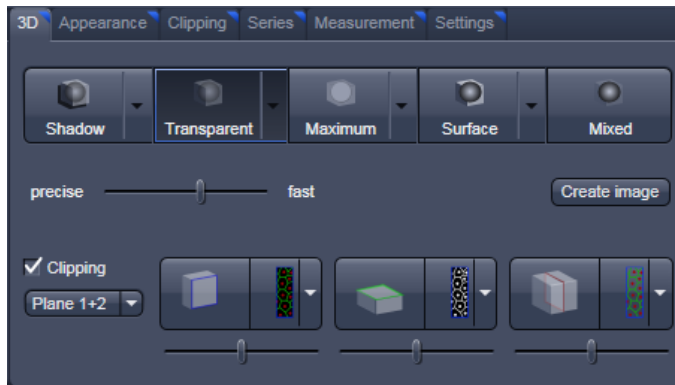
On the **3D** View control panel the different render modes are accessible:
Shadow, **Transparent**, **Maximum**, **Surface** and **Mixed**. The rendering quality can be adjusted by using
the ![precise ... fast slider] slider. The **Create image** button ![Create image] opens a new image
window and produces a 2D image of the currently used render mode. It uses the screen resolution unless
it is otherwise defined in the **Settings** tab (see there).

Clipping ![Clipping checkbox] activates the Clipping Plane functionality. 3 different Clipping Planes are displayed
and can be operated individually via 3 Buttons:
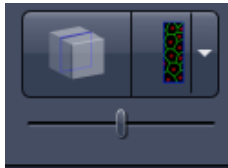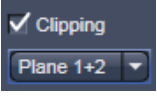
The Cut button toggles between different clipping behaviors of the respective planes. ![cube icon]

On the Clipping Planes different surfaces can be chosen from a pull down list: ![pulldown icon]

The Position of the clipping planes can be adjusted by the **Position Slider**.
Further functionality is available on the **Clipping Plane View control tab** → see there.

A wedge between 2 Planes can be clipped using the pull down ![Plane 1+2 pulldown] underneath the activation
check box for the clipping functionality.

Appearance:
On this view control panel the settings for the respective render mode are available.

Clipping:
3D VisArt has the option to cut off certain parts of the 3D information by using so called "clipping
planes". All the necessary tools to operate the clipping planes are found on this control panel.

Series:
3D Data can be visualized online by simply grabbing and turning the data stack with the mouse. In addition to this interactive way of interacting with the data 3D VisArt is able to perform render series where the dataset is turned around one of the axis, along a freely defined position list or a freely definable start and end point. The resulting series can be saved as movie files for presentations. A preview of the render series is available in the **position list** mode.

Measurements:
Measurements of distances, angles and polylines can be measured using the **Measure** panel in 3D VisArt.

Settings:
The **Settings** panel contains global settings for the 3D VisArt.
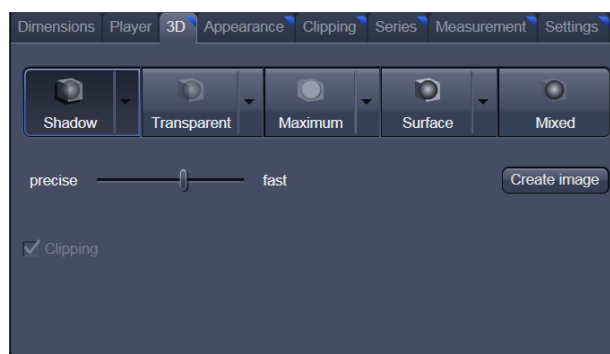
Fig. 512   3D panel - Shadow button
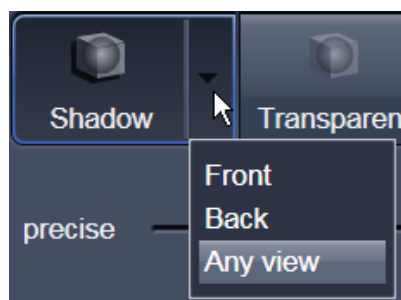
Fig. 513   Shadow pull down menu

6.8.1   Shadow Projection

In the Shadow render mode the structures in the image are illuminated by a virtual light source.

The volume is viewed as if looking through the eyepieces of the microscope and the light source projects a shadow onto a background plane.

This gives the data a reference in space, making visualization much easier. The rendering effect itself consists of a combination of light reflection and opacity (degree of transparency).

The display in this mode is calculated by the main processor (CPU) of the computer (Voxel mode). The different modes can be accessed by clicking on the pull down symbol at the right hand side of the **Shadow** button (Fig. 513).

- **Front** button: Shadow rendering front view
- **Back** button: Shadow rendering back view
- **Any View** button: Shadow rendering with user defined view

With a click on **Front**, the 3D reconstructed image is displayed in a shadow projection where it is illuminated at a 45° angle from the front left.

A click on the **Back** button creates the same projection with illumination from back left.

Detailed settings for shadow projection can be accessed via the **Appearance** tab.
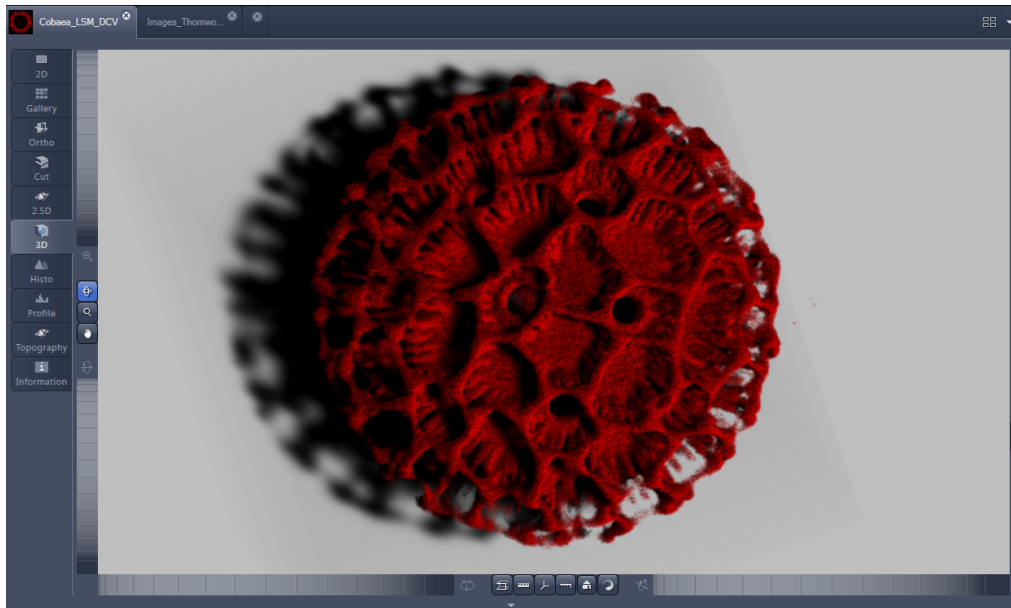
Fig. 514   Image Display window, 3D display, Shadow projection, Front view

The zoom wheel to the left of the **Image Display** window allows continuous zooming of the 3D reconstructed image.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.
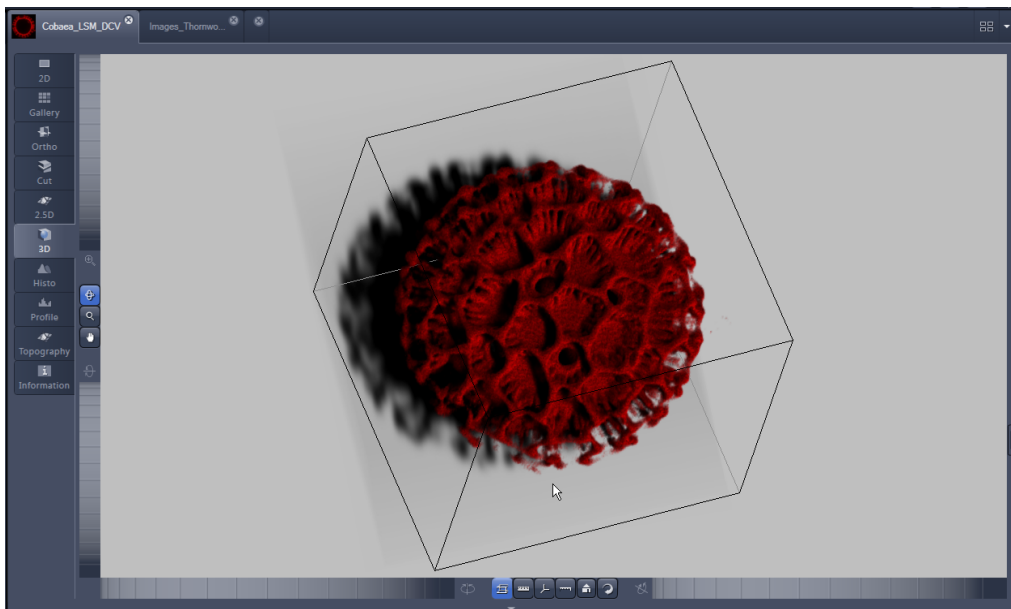
Fig. 515   Image Display window, 3D display, Shadow projection, Any View

The following additional buttons are available in the **Any View** shadow projection mode:

- After activation of the **Frame** button (below the image), a bounding box is drawn around the 3D reconstructed image.

☞ Depending on the used mode and hardware configuration, it can take several seconds until the 3D reconstruction is refreshed on the monitor after reorientation.

- A click on the **Coordinate System** button displays a colored coordinate system in the **Image Display** window, where the X axis is displayed in red color, the Y axis in blue and the Z axis in green.

- A click on the **Scale** button display an X-, Y- and Z-scale in the **Image Display** window.

- A click on the **Home** button resets the display parameters to the default values.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.

- A click on the **Animation** button activates the animation mode. The object can be pushed by dragging in the **Image Display** window and rotates continuously. Any new push with pressed left mouse button changes the rotation direction and speed of the animation.

6.8.2   Transparency Render Mode

Principle:

In the **Transparent** mode a three-dimensional image with a transparent effect is calculated. At least two two-dimensional texture stacks (computed from two different angles) are used depending on the volume orientation. In contrast to the Shadow mode the scene here is illuminated by diffuse white light from the rear. By changing the available parameters one can mix different channels and reveal relationships between information in those channels. This view is particularly useful for visualizing the three-dimensional relationships between structures within the volume. This mode can be displayed both in CPU based Voxel mode (**Basic**) and also (with the help of a suitable graphics card) with accelerated calculations in so-called OpenGL mode (**Advanced**).
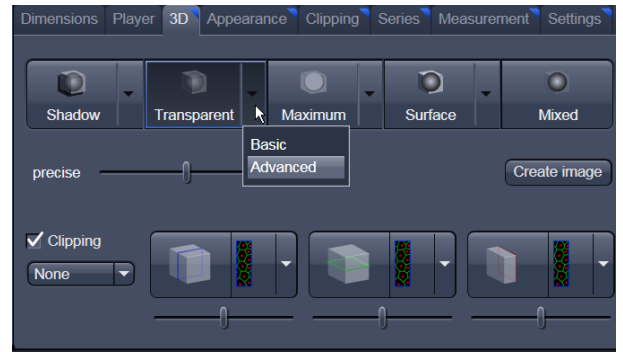
Fig. 516   3D View Control Panel, Transparent

Basic	Transparency rendering (voxel based) (CPU)
Advanced	Transparency rendering (voxel based) with textures (GPU)
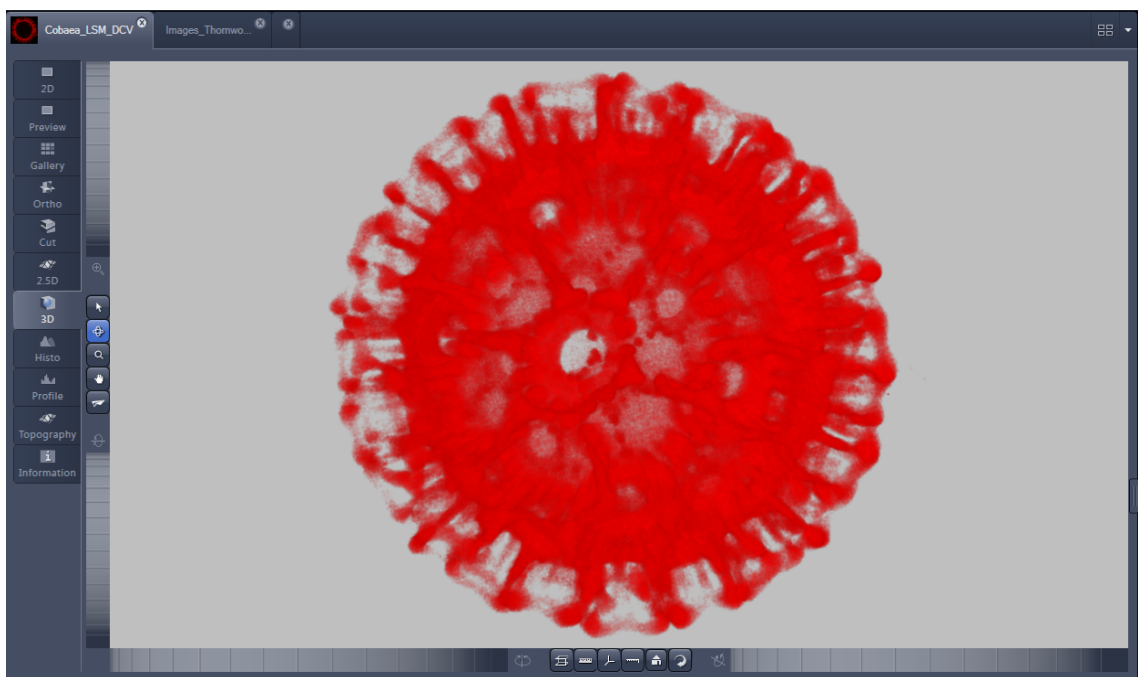
Fig. 517   Image Display window, 3D display, Transparency projection, Advanced
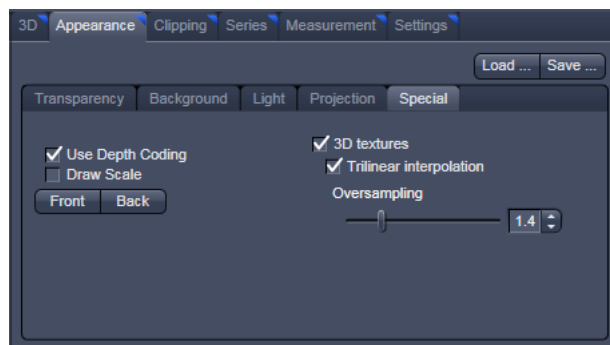
Fig. 518   3D toolbar – Transparent – Depth Coding

By means of the **Depth Coding** function (Fig. 518), the Z-information contained in a sequence can be colored with the colors of the rainbow, in which case "blue" stands for higher values and "red" stands for lower values.

The **3D Textures** option can be used for faster and better rendering of transparency renderings.

☞ Note that this function is only available in Transparency and Maximum Intensity Render Modes.

6.8.3   Maximum Mode
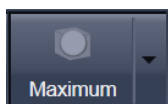
Clicking on the ⬜ button switches to **Maximum projection** mode, which calculates a three-dimensional view of the data by only showing pixels of the highest intensity along the projection axis. This view is particularly useful for displaying three-dimensional images in two dimensions, e.g. in publications, and guarantees a maximum possible degree of transparency. The display in this mode can be calculated by the main processor (CPU) of the computer (Maximum basic) or the graphics card (Maximum advanced) in OpenGL mode.

☞ Note that this is a rendering. If desired 1:1 Maximum Intensity Projections can also be done in the **Processing** tab in **Maximum Intensity Projections** (see section **Processing – Maximum Intensity Projection**). With these Images exact Intensity Measurements can be performed.

Maximum Basic   Maximum Intensity CPU based

Maximum Advanced   Maximum Intensity GPU based
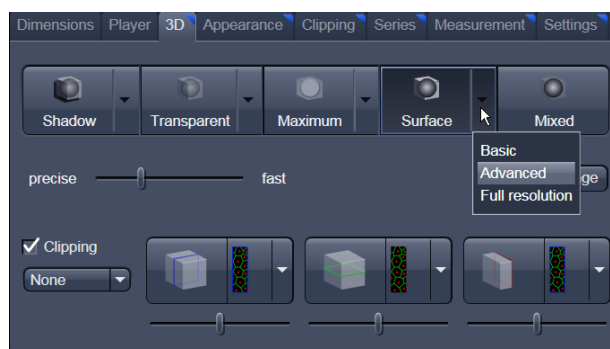
Fig. 519   3D toolbar – Surface

6.8.4   Surface Render Mode

In the **Surface** mode (Fig. 519) grey values in the image are computed as solid surfaces (also known as Iso surfaces) instead of soft transparent shades.

This display mode is useful to focus the attention on certain structures (e.g. plant cell walls, nucleoli, intracellular vesicles) while potentially hiding additional structures deeper within.
In this mode you also can select between Voxel (CPU) [basic] and OpenGL (graphics card) [advanced] display.

The [full resolution] projection is based on a high precision calculation method for 3D information on the basis of triangles with maximum resolution.

Basic button	Surface rendering (voxel based)
Advanced button	Surface rendering (triangle based)
Full Resolution button	High accuracy surface rendering (triangle based)

6.8.5   Mixed Render Mode

In **Mixed** mode both, transparency and surface mode, can be mixed in one volume display.

Using this mode one can render small structures within cells such as vesicles or speckles (e.g. FISH signals) as surfaces in one channel of a multichannel image while rendering the surrounding cytoplasm from another channel in transparency mode.

Fig. 520   3D toolbar – Mixed

This way one can also visualize more complex relationships convincingly.

The render mode (**Transparent**, **Surface**, **Surf.+Transp.**) can be chosen per channel.

☞ The channels can also be switched off individually by de-activating the channels in the **Dimensions** view control panel.
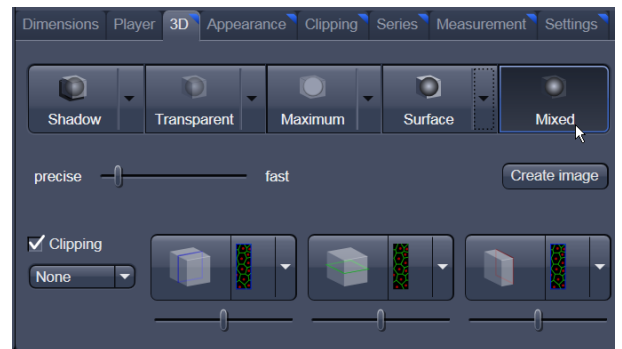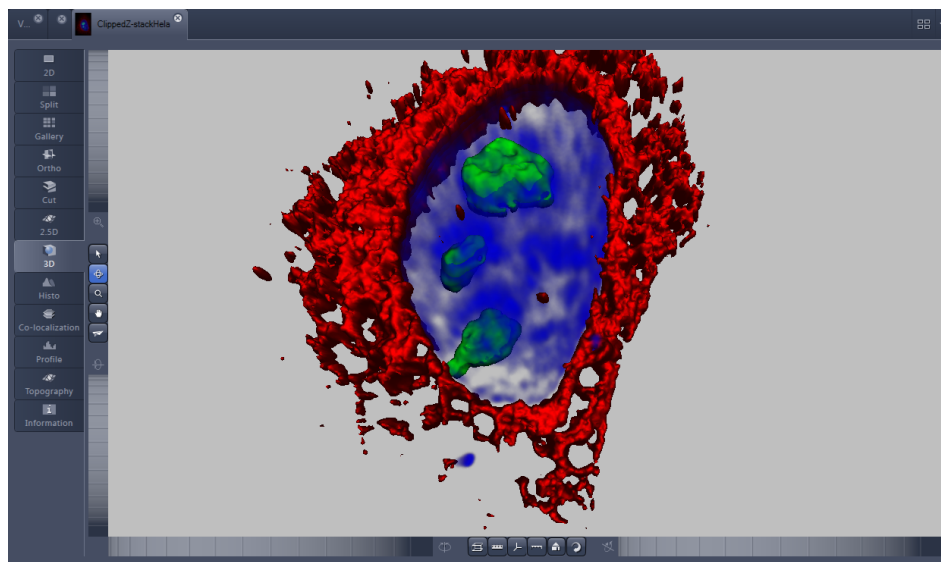
Fig. 521   Image Display window, 3D display, Mixed projection

6.8.6   Clipping Planes
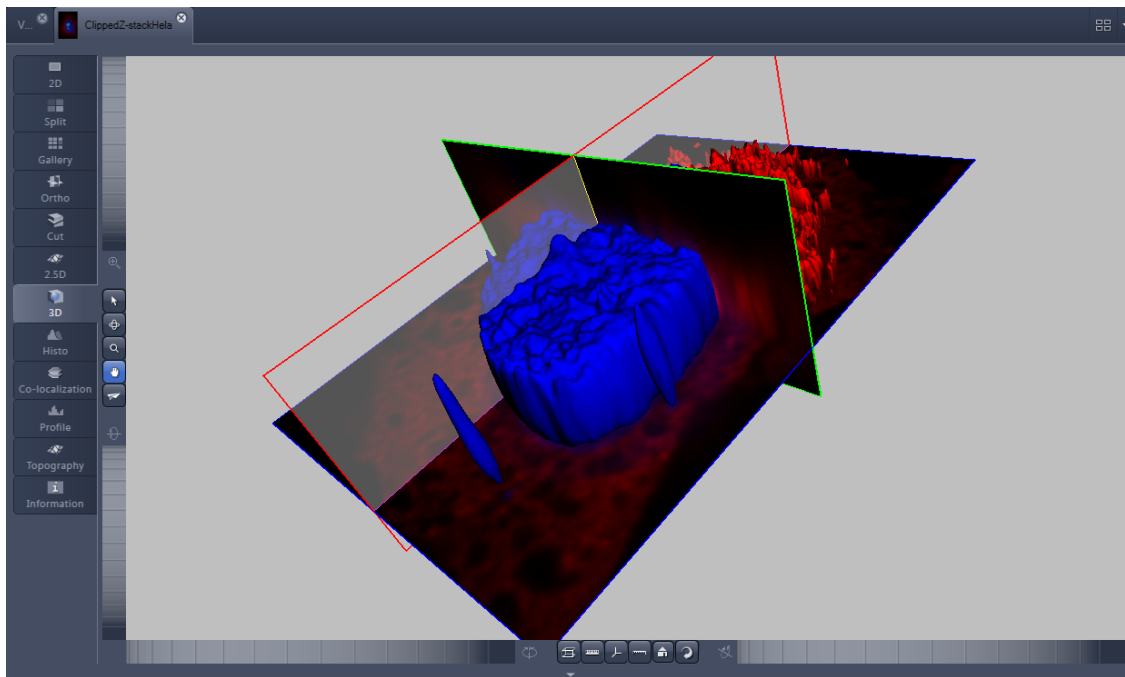
Fig. 522   Image Display window, 3D display, Clipping planes

3D VisArt can display up to three so called **Clipping planes**. These planes can be used to cut the volume open in order to visualize structures which are within a given volume. Clipping planes make most sense when used in the **Surface** and **Mixed** mode but can also be used with limited functionality with the rendering modes **Maximum** and **Transparency** as well as in the Split mode (see below)

The clipping planes can be accessed via the **3D** tab or the **Clipping** tab. On the 3D tab only the most commonly used functionality is available. The full functionality can be accessed on the **Clipping** tab.

☞   Note that the **Clipping** tab is only available with the **Show all** mode active.
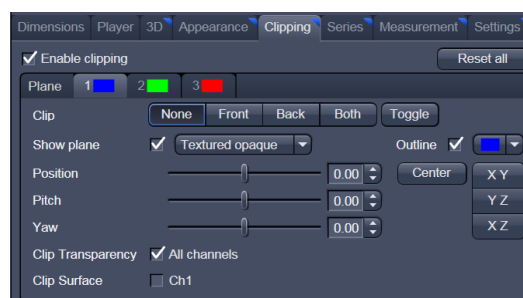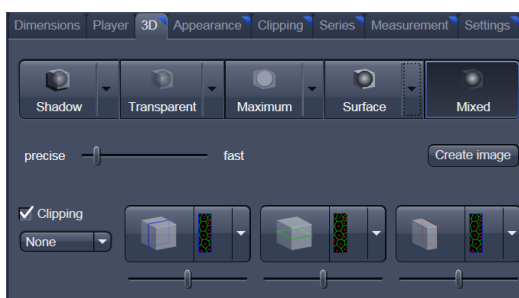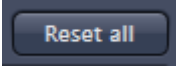
In **Surface** mode clipping planes can be used in a channel specific manner. This means, that in a multichannel image one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique is also applicable in **Mixed** mode.

A further possibility is, to use clipping planes in order to display only the grey values of the volume which touch the clipping plane.

All these functions can be used for creating a render series in order to show them and to make spectacular movies for presentations.

There are three clipping planes, which are labeled for ease of use with colored outlines. These outlines do NOT represent the channels. The standard orientations of the planes are **blue**: XY plane, **green**: YZ plane and **red**: XZ plane. This is linked to the default orientation of the volume axis.

Activating the **Clipping** panel displays the clipping planes and displays those with default settings if the image was not opened in the 3D module before. [Reset all] is setting back the appearance of the planes to the following settings:

- Each plane is placed into the center of the volume and oriented in orthogonal fashion.

- The planes have an opaque surface and colored outlines.

- The clipping functionality of the planes is not activated in this state.

Controlling the clipping functionality of the 3 planes

The clipping planes are operated by three buttons on the **3D** tab which are color coded in the same way the respective planes are color coded. The buttons are located below the **Render Mode Buttons** and can be activated by ticking the **clipping** tick box.

☞ Note that full functionality of the clipping planes can be found in the **Clipping View Control** tab!
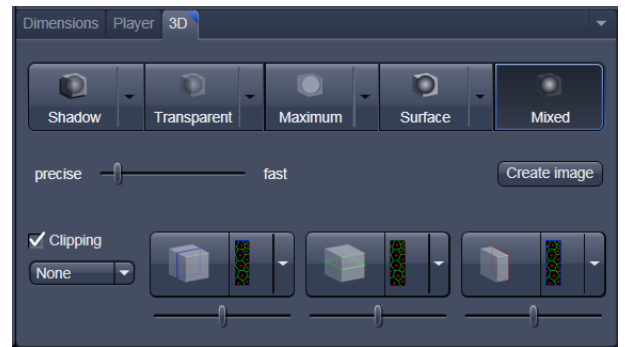
Fig. 523   Toggle buttons

By toggling these
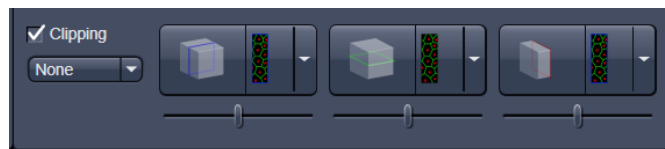
buttons, the clipping behavior of the planes can be set in the following manner:

- **None:** hides the plane completely

- **Front:** clips away the data on the front side of the plane

- **Back:** clips away the data on the back side of the plane

- **Both:** clips away the data on both sides of the plane

- **Show Plane** only displays the plane – no clipping

These adjustments can be made individually for each plane. The clipping planes can be chosen by selecting the respective button.
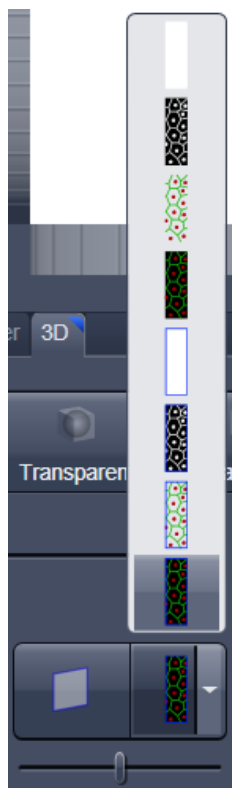
Fig. 524   Clipping planes

Appearance of the clipping planes

By default the clipping planes have an opaque surface and a colored outline. This can be changed by using the dropdown in the **3D** View Control tab. In the **Clipping** View Control tab, this function can be found in a pull down.

☞   This function is applicable to all 3 clipping planes separately.

Colored:
white - opaque surface of the plane

Binary:
displays the binary data on the clipping planes

Textured:
Data which are touched by the plane are displayed on the clipping plane as in the render mode **Transparent** but as a two-dimensional projection. This does include the settings (threshold, ramp etc) applying for **Transparent** render mode. Black pixels are transparent.

Textured fine:
Same as above but with 4x times the resolution

Tansparent:
Data which are touched by the plane are displayed on the clipping plane as in the render mode **Transparent** but as a two-dimensional projection. This does not include the settings applying for **Transparent** render mode. Black pixels are transparent.

Textured opaque:
as with **Transparent** (including the settings) but in this case black pixels are non-transparent, rendered volume data in the background do not shine through.

Interactive positioning of the clipping planes

The clipping planes can be moved interactively with the mouse by activating the ![button] button. If hovering over a plane outline of the plane turns into a different color, the cursor turns into a crosshair indicating that the plane can now be moved. Clicking and dragging then moves the plane. The clipping behavior (e.g. "front") is maintained during the movement.

☞   These movements can also be "recorded" using the render series function.

Clipping Planes Settings

Activate or deactivate the clipping functionality globally by the check box **Enable clipping**.
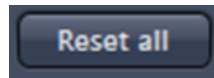
 displays the three clipping planes in the default mode (opaque surface, colored outlines, clipping planes active, no clipping).

With the **Plane** tabs the planes can be addressed individually and their settings can be adjusted independent from each other.

Outline displays or hides the colored outlines. The actual color can be chosen from a color chooser for each plane individually.

Show Plane hides or displays the plane.

☞ A plane can cut away information whilst being "invisible".

☞ For distance or angle measurements on a clipping plane the plane has to be displayed and a color has to be chosen for the clipping plane.

The appearance of the plane can be selected from a drop-down menu (see Fig. 524).

Clip names a row of toggle buttons switching between different clipping modes as named on the buttons .

Using the **Pitch**, **Yaw**, and **Position** sliders each plane can be freely orientated in three-dimensional space.
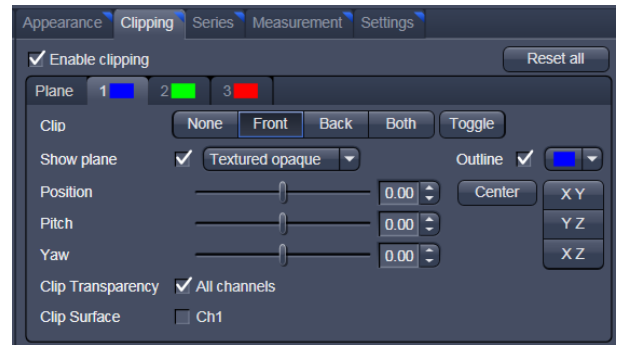
Fig. 525   Clipping planes settings
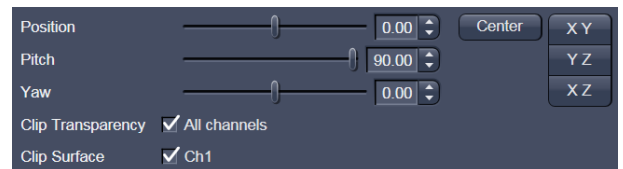
Fig. 526   Sliders Pitch, Yaw and Position

6.8.7 Flying Mode

The **Flying** mode is an interactive way to explore three-dimensional data. By pressing the mouse wheel you can fly into and through a dataset. Moving the mouse left / right and up / down controls the flying direction. Pressing the mouse wheel and the right mouse button at the same time allows for flying backwards.

☞ Once the **Flying** mode is activated, the rotation of the data switches modes: Whereas normally the data turns around an imaginative point in the center of the dataset, in the fly mode the data turns (if moved interactively with the mouse once one stops flying) around an imaginative point "behind" the operator. Pressing the [button] button switches back into the normal mode.

If a wheel-mouse is not available, pressing the [button] button turns the left mouse button into the **Flying** button.

☞ The **Flying** mode is only available in graphics card accelerated render modes.

6.8.8 3D Rendering Settings in VisArt

Depending on the render mode selected the following adjustments can be applied to the datasets:

All settings can be saved and re-applied to other datasets by using the [Load ... Save ...] buttons.
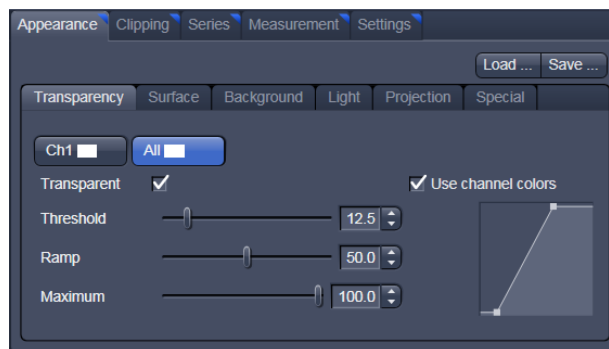
Fig. 527 3D Rendering window (e.g. Surface Advanced rendering mode)

– **Threshold** specifies a lower threshold as a percentage of the gray values displayed. This decides which data enter the rendered image. If one fluorescence channel contains a homogeneous fluorescence signal with high signal strength, one would set the threshold higher in order to "protect" signals from weaker channels from being quenched.

– **Ambient** describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 100).

– **Specular** sets a directional brightness (values on a scale between 0 and 100). Influences the differences between dark and bright structures, which are strongest at a value of 0.

– **Shininess** changes the surface gloss (on a scale between 0 and 100).

– **Ramp** controls the degree of the transition from completely transparent to completely opaque.

– **Maximum** influences the degree of opacity.

The settings are combined into a histogram [image]. The x axis corresponds to the gray values and the y axis to the degree of opacity. You can also use the mouse to interact with the histogram curve.

– **Roughness** (for Shadow mode only): You can also influence the roughness used to display the surface of the image structures. This changes the plasticity of the display (relative scale between 0 and 1). This setting quickly can produce artifacts if pixels reach saturation. The degree of roughness should be reduced accordingly.

– **Brightness** can be controlled via input field or slider bar.

– **Distance** determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").

– **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range: 0° to 90°).

– **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range: -180° to +180°).

– **Channels:** The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color and number).
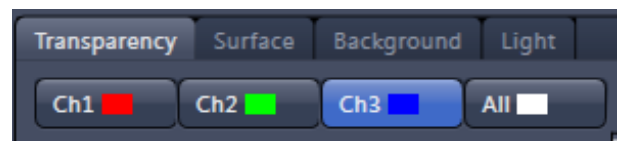
Fig. 528   **Channels**

– **Distance** (for Shadow mode only) sets the distance between the 3D object and the virtual background on a scale from 0.5 to 3.0.

– **Color**: The color of the background can be freely chosen from a table which appears when clicking the color chooser button .

– **View angle / Scale Z:** In hardware accelerated modes, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.
The Z-Scale of the dataset can be altered interactively by using the slider. The slider ranges from a factor of 0.1 to a factor of 5.
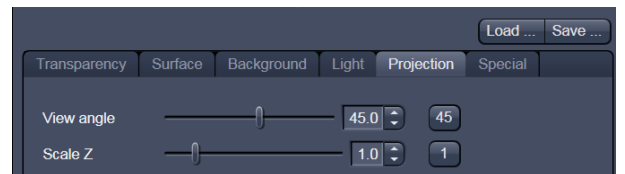
Fig. 529   **Viewing angle**

– **Background:** The background color can be altered by using the pull down menu. For some 3D Objects it can be beneficial to display a **Structured background** for better orientation within the dataset.
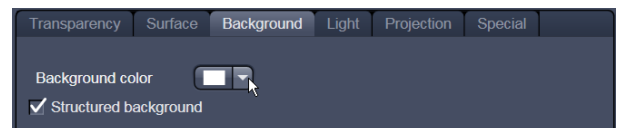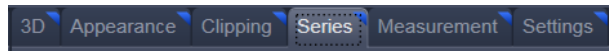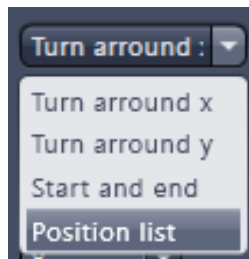

Fig. 530   **Background**

Fig. 531   **Series tab**

Fig. 532   **Render Series window**
(e.g. Turn around X mode)

Fig. 533   **Render Series modes**

6.8.9   Series

The **Series** tab (Fig. 531) displays the **Render Series** control panel. This panel allows settings for the axis to be used for rotation of the 3D reconstructed images. This feature is applicable to all render modes available in 3D VisArt.

There are four different modes for producing render series (see Fig. 533).

- Depending on the activated mode, directly set the parameters for animation in the **Render Series** window and the position of the 3D image in the **Image Display** window (zoom, rotation axes, rendering parameters).

- Click on **Apply** to start the animation

The animation is performed in a separate **Image Display** window, which permits the animation to be saved afterwards.

(1)   Turn around X and Turn around Y mode

In this mode, the image is turned around the X-axis or the Y-axis exclusively.

The values for **Number of Views**, **Difference Angle** and **First Angle** can be selected accordingly).
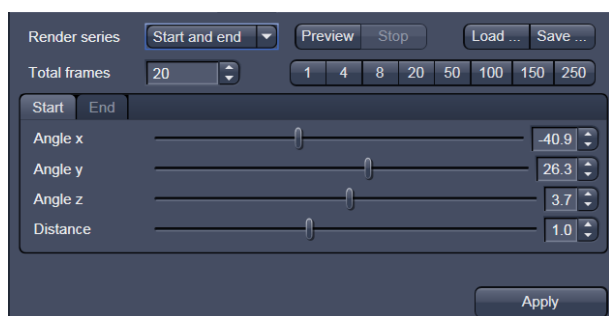
Fig. 534   **Start panel in the Render View control block**

(2)   Start and End mode

In the **Start and End** mode, the image is reconstructed between a start position and an end position.

The rotation angles for X, Y and Z and the distance (zoom) can be determined using the sliders.

The value for **Total frames** can be varied. The frames are distributed equally between the start and end points (Fig. 534).

(3)   Position List mode

In the **Position List** mode, the image is reconstructed between any required number of interim positions to be determined individually. Just rotate the reconstructed image with the mouse or do any other adjustment and add this as a "position" in the position list. The module will create a movie using those positions as a guideline. It will interpolate all the parameters ticked in the list.
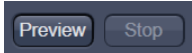
The rotation angles for X, Y and Z and the zoom can be determined directly in the image.

Every required interim position is included in the list of the **Render Series** window with a click on the **Add** button.

Fig. 535   **Render Series window - Position List mode**

Remove permits the contents of the list to be deleted. **Insert** inserts a position above the highlighted list entry.

The value for **Total frames** can be varied. A value of 20 produces a render series with 20 frames in total.

Interpolate lists variables that are interpolated during the Render Series.

Pressing the [Preview] [Stop] button creates a short and low resolution (no interpolation!) preview of the rendering using the positions currently in the list. This serves as a help to quickly review the rendering without actually starting the process (which might be time consuming in cases of long position lists.

Click on the **Apply** button calculates a spline along all the defined positions from the list and starts an animation along this spline track in space.

6.8.10 Interactive Measurements

3D VisArt features three tools for interactive measurements in Euclidian space. Measurements can be performed in all graphic-card accelerated modes (**Transparency**, **Surface**, **Maximum** and **Mixed**). The measured tools are shown as shapes in the rendered volume and can be changed there interactively. Measurement values can be exported as a table to the clipboard for downstream processing in other programs.

First a suitable tool has to be chosen. In order for measurements to work, the mouse cursor must "recognize" a structure. In the **Surface** mode this is a uniquely identifiable surface structure, for the transparent modes the tools look for the highest local gray level maximum.

As a sign, that the mouse cursor has "found" a structure, the cursor shape changes from ⊘ into ⊕.

The found measurement point is drawn as a blue circle 🔵.
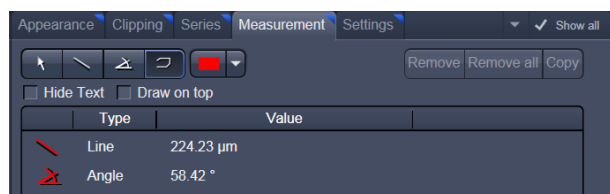
Fig. 536   3D Measurement View control block

Interactive 3D-Measurement

The measurement dialog contains a toolbar with the available measurement tools.

The **Line** tool measures the length along a line in µm. First click on a starting point and move the mouse to the desired end point while keeping the left mouse key depressed. The measurement is concluded upon releasing the left mouse key.

The **Angle** measurement tool defines an angle between two connected line segments. First define the starting point by clicking, and then draw the first line segment until you click on the first endpoint. Now draw the second line segment and conclude the measurement by clicking onto the second end point. This measures the angle in degree.

The **Curve** tool measures the total length (µm) along a variable multi-segmented polygon line. Please click from point to point and finish the measurement by clicking with the right mouse key.

Measurement values are compiled in a list. In order to delete a measurement, select it in the list and press the **Remove** button. One can also delete all measurements by clicking on **Remove all**.

The measurements can be copied into the clipboard with the **Copy** button.

Measurements are only transiently drawn into the volume and are not stored automatically with the image.

6.8.11   Settings

Flying mode

Ticking the tick box **Flying** mode activates the mode, unticking deactivates it.

To show or hide an overview image tick the **Show overlay image** box.

The fly-speed can be adjusted in a range from 1 to 100.
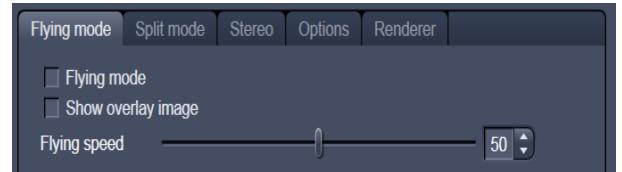
Fig. 537   **3D Renderer Settings – Flying mode**

Split Mode

Ticking this tick box activates a channel wise 3D split view of the data. This is only available in surface render mode only. An overview image with all channels combined can be shown optionally by ticking **Draw combined image**.
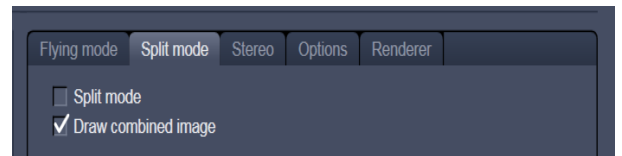
Fig. 538   **3D Renderer Settings – Split mode**

Stereo

Two Stereo views can be chosen from this sub-menu:

Anaglyph is a view in which the data can be examined in 3D using red/green glasses. The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

Fig. 539   **3D Renderer Settings – Stereo**

The stereoscopic effect can only be seen with the aid of red/green 3D goggles. The red lens is to be used for the right eye and the green lens for the left eye.

Camera offset: Defines the distance of both cameras from each other (units in meters).

Parallax shift: Determines the degree of shift which is necessary to correlate both camera views. The slider should be adjusted to maximize the stereo effect. Please note, that this setting will be different for each observer.
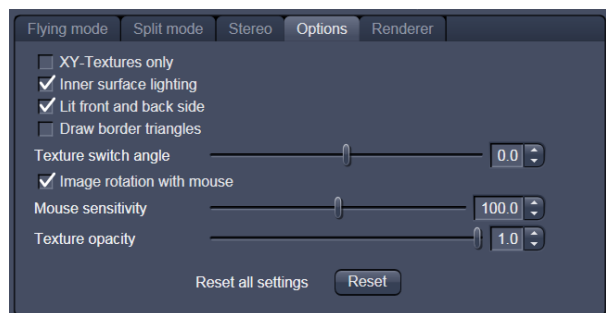
Fig. 540   3D Renderer Settings – Options

6.8.12   Options

XY Textures only: Instead of working with three texture stacks per dataset in transparent render mode, the system will only use one texture stack. This will result in faster renderings. Looking at the dataset from aside although will make the individual z-planes visible.

Inner surface lighting: If checked, illuminates the inside of a surface rendered volume. If unchecked, the inside is dark. If one views the inside of a volume by flying or zooming into it, the inside illumination is helpful. This option is only available for the **Surface** mode.

Lit front and back side: With activated **Inner surface lighting**, this check box colors the inside lighting with a bluish hue. This makes distinguishing inside from outside easier. This option is only available for the **Surface** mode.

Draw border triangles: When displaying the data touching the six sides ("borders") of the Z-Stack in 3D, the triangles used to do this are all located in one plane and orientation. This can cause artifacts, therefore the display of those border triangles can be switched off.

Texture switch angle: This parameter influences the volume angle, upon which the next set of 2D-Textures will be loaded into the graphics card (value range 1 to 100 degree). Depending on the object orientation, the number of z-slices and the chosen render settings this loading process can be visible and may be disturbing especially when rendering a series for movie export. Changing this angle can postpone or even avoid this switch during a series rendering. This option is only available for **Transparency** and **Maximum** modes.

Image Rotation with mouse: Activates a method to rotate the dataset interactively with the mouse. In addition to rotating it freely, when clicking and dragging at the sides of the image window the dragging rotates the data around one axis only. Just like the wheels on the 3D window frame.

Mouse Sensitivity: Influences the mouse sensitivity (value range: 1 to 200). Small values make the mouse actions precise but slow, large values less precise but faster.

Texture Opacity: With this slider the α-values of the dataset can be adjusted. The rendering is getting more or less transparent using this setting.

Reset: Sets all render settings back to the factory standard settings.
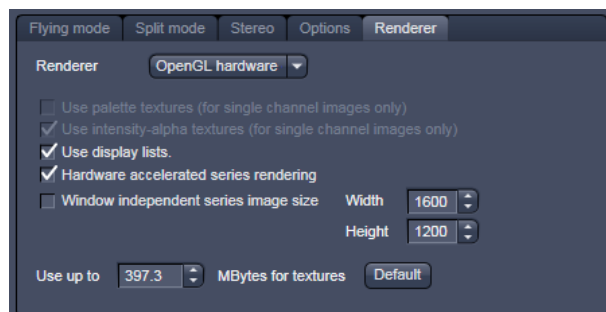
Fig. 541   3D OpenGL Renderer settings

Renderer Options:

Renderer: OpenGL Software is rendering the 3D data with the CPU; OpenGL Hardware uses the Graphics card to do this. Especially when having trouble with non Zeiss-certified graphics cards it is sometimes useful to switch to a pure CPU based rendering.

Palette textures: This feature is an optimization feature not supported by modern graphics cards any more. It used to optimize display of single channel images.

Intensity Alpha textures: This feature optimizes the usage of the graphic ram with single channel images.

Display Lists activates a batch processing of the operations the graphics card is doing. It results in faster rendering.

Hardware accelerated series rendering: Un-ticking this feature uses the Computer Processor for render series. In case of trouble with non-certified graphics cards render series can be performed with the CPU without risk. The use of memory of the graphics card can be adjusted in a dialog box.

Window independent series image size: Using this feature creates render series (see section **Series**) with the output size indicated in the two input boxes. The maximal resolution is 4096 x 4096 pixels. This feature is available in all Render Modes (CPU and GPU accelerated). This applies also to the "**Create Image**" button on the **3D View Options** tab.

Allocate Graphics Card RAM: The amount of Graphics card RAM can be freely defined in the last Item on the **3D | Settings** view control panel. **Default** sets this value to ~ 80% of the available RAM of the currently used graphics card.

6.8.13   3D View – Basic

Without a license for ZEN Module 3D VisArt the following features are available:

– Shadow view - Front view,

– Transparency render mode - Basic, rotate around x or y render series,

– Maximum brightness projection – Basic Mode only, only rotate around x or y, or start to end movies,

– Surface - Basic Render Mode, rotate around x or y movies,

– Stereo Anaglyph Mode.

6.9   Histogram View

The **Histogram View** (**Histo View**) allows to

- display a histogram (distribution of pixel intensities) of an image or Region of Interest,

- show the histogram values in table form,

- copy table to clipboard or save as text file,

- measure area and mean gray value and standard distribution in an area.

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** view options control blocks apply. The additional view-specific **Histo View Option** control block is shown in Fig. 542.

Any changes done with this View Option control block are effective immediately.
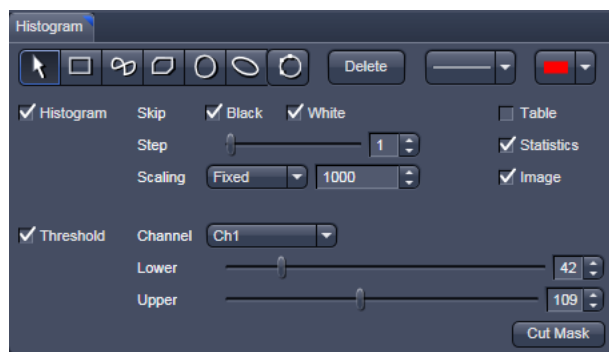
Fig. 542   View Option control block - Histo View
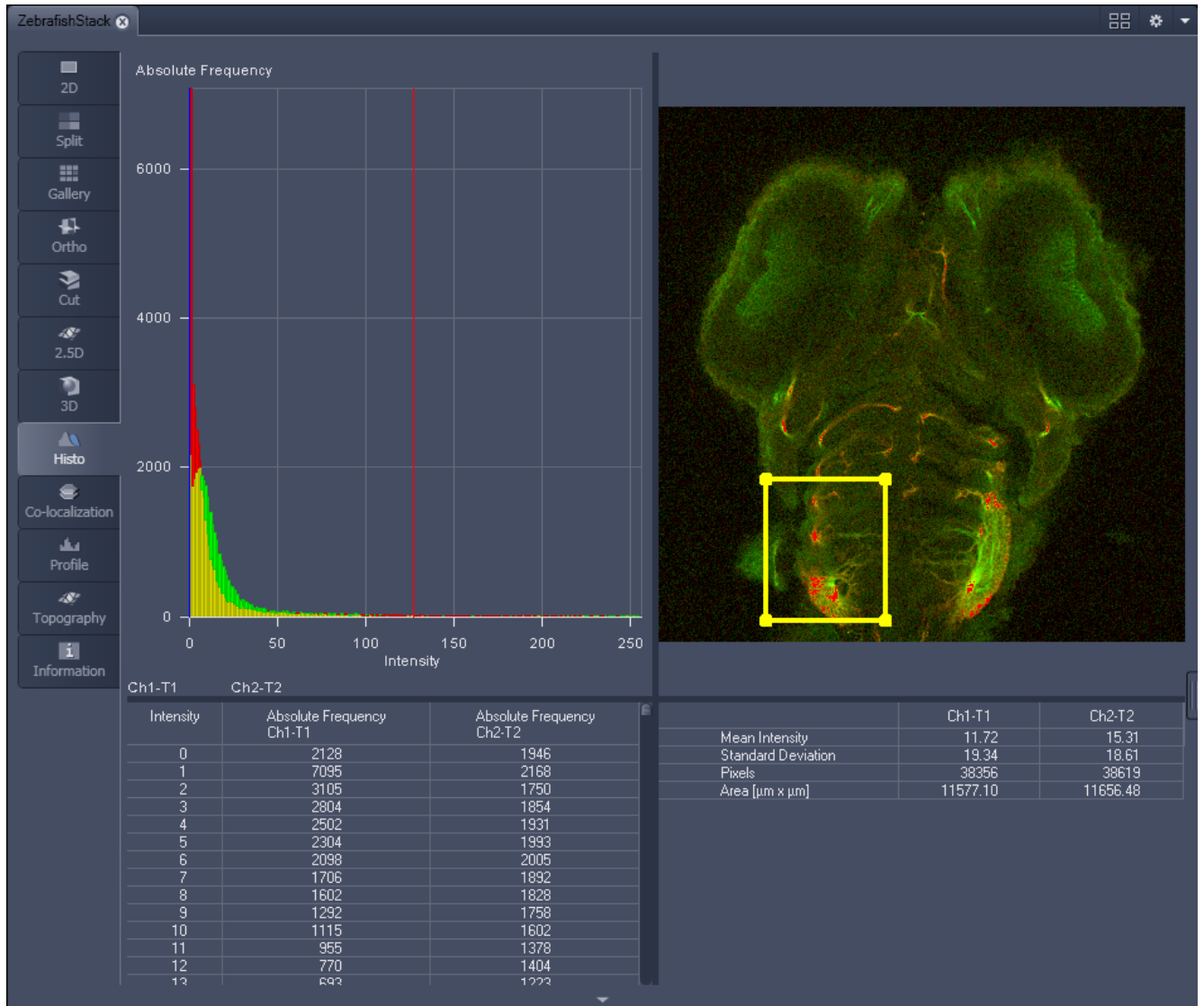
Fig. 543   **Image Display, Histogram view**

☞ The **Histo** button can also be used online during scanning.

Histogram functions:

Skip Black and **Skip White** tick boxes: Ignore black pixels (gray value 0) and Ignore white pixels (gray value 255 or 4096) in the histogram.

Step slider and spin box: Sets the number of intensity steps ("trays") of the histogram display. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps (for 8 bit images). Reduction is made by averaging.

Scaling pull down menu: Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed
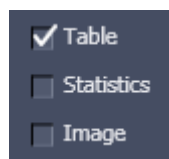
Show Table tick box: A table of the intensity values is shown in the **Image Display** window.
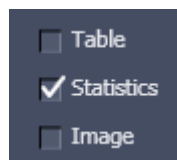
Show Statistics tick box: Displays statistical parameters (Mean Intensity, Standard Deviation, Number of Pixels and Size of Area) in an additional table. Area measurements of very small areas (<10 pixels) give only approximate values.
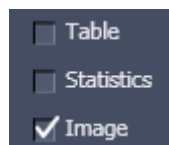
Show Image tick box: Shows the image in the **Image Display** window with the histogram graph.
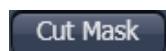
The **Cut Mask** tool creates a new image document which sets every pixel outside ROIs to Zero. Within the ROIs, only the pixels with values between lower and upper threshold are transferred to the new image.

☞ Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

Histogram functions and Overlays:

The functionality of the **Graphics View Option** control block fully applies to the **Histo View** Type. If a ROI is marked/selected in the Histo View Image display, only this ROI will be taken into account for the histogram display, the table and the statistics. If there are several ROIs, but none is marked/selected, the histogram display, the table and the statistics analyze all the pixels included in all ROIs.

Fig. 544   Histo View Option control block - Threshold

Histogram Threshold:

When the **Threshold** tick box is selected, the controls to set a lower and upper threshold for the histogram calculation are displayed (Fig. 544). The threshold can be set for each channel (channel selection pull down menu).

6.10   Colocalization View

The **Colocalization** function permits interactive analysis of two channels of an image by computing a scatter diagram (co-localization).

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** view options control blocks apply. The additional view-specific **Colocalization View Option** control block is shown in Fig. 545.

Any changes done with this View Option control block are effective immediately.

Fig. 545   Colocalization View Option control block

The Image Display in the Colocalization View shows 3 panels: the scatter diagram, the data table and the pseudo-colored image display (see Fig. 546 and Fig. 547).

☞   Colocalization is defined by the presence of two or more different molecules at the same location in a specimen. However, in the context of digital imaging, the term colocalization refers to colors emitted by fluorescent molecules detected by the same pixel in the image. It is important to be aware of the fact that colocalization cannot be analyzed for fluorophores with similar emission spectra. Accurate colocalization analysis is only possible if the fluorescence emission spectra are well separated between fluorophores and the correct filter sets (or spectral detection bands) are used for data acquisition. If spectral bleed-through artifacts are present because of spectral overlap between the fluorophore emission spectra, or due to the use of incorrect filter sets, colocalization measurements will be meaningless. To avoid this, the fluorophores must be carefully chosen and matched to the excitation laser lines to obtain the maximum excitation efficiency while still maintaining a useful degree of separation between emission wavelengths. The choice of fluorophores is crucial for colocalization analysis.

Fig. 546   Image Display, Colocalization view
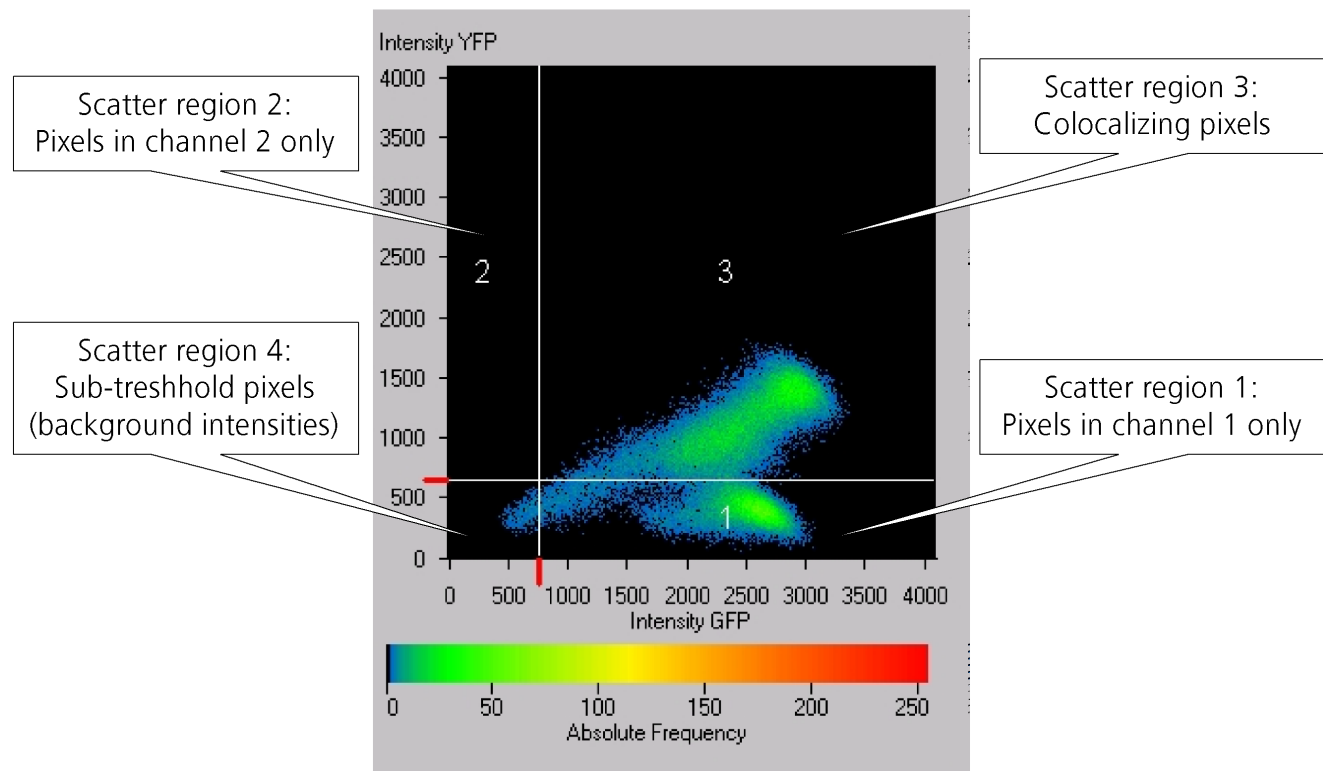
Fig. 547   Scatter diagram and threshold with crosshair

6.10.1 How a Scatter Diagram is Generated

All pixels having the same positions in both images are considered a pair. Of every pair of pixels (P1, P2) from the two source images, the intensity level of pixel P1 is interpreted as X coordinate, and that of pixel P2 as Y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred.

☞ Differences between the images cause irregular spots in the scatter diagram.

Identical images produce a clean diagonal line running from bottom left to top right, because only pixel pairs (0,0), (1,1), (2,2) with the same intensity can occur. Differences between the images cause an irregular distribution in the scatter diagram.

☞ Scatter diagram, image display and data table are interactively linked and immediately updated when settings are changed.

Available Controls:
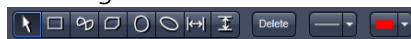
Drawing tools	With the drawing tools the colocalization analysis can be restricted to a region of the image. Tools in the Colocalization View Option control block and in the Graphics View Option control block work the same and can be combined.
	The Cross hair, Table and Image selection tick boxes: when selected, the respective element is displayed in the Image Display area. If off, the element is hidden.
	Scaling pull down menu: Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed
	Set the color LUTs for each quadrant in the image display to improve visibility of colocalized pixels.
	The Load and Save buttons can be used to save colocalization settings (like color settings for the quadrants and crosshair-positions) to a *.col file and re-load it when analyzing a different data set. This allows using the same parameter for the colocalization analysis of different images.
	The Cut Mask tool creates a new image document which sets every pixel outside Quadrant 3 (not colocalized) to Zero. ROIs don't play a role for this Cut Mask function (as opposed to the Cut Mask in Histo View).
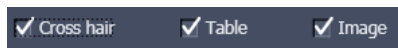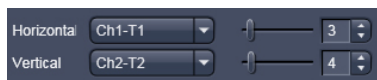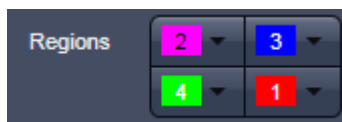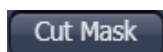
☞ Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

6.10.2   Quantitative Colocalization Parameters Shown in the Data Table

- **No. of pixels** in image ROI or scatter region
- **Area / relative area** of image ROI or scatter region
- **Mean intensities / SD** within image ROI or scatter region
- **Colocalization coefficients**
- **Weighted colocalization coefficients**
- **Overlap coefficient** after Manders
- **Correlation coefficients** (R and R^2)

6.10.3   Colocalization Coefficients

$$c_1 = \frac{pixels_{Ch1,coloc}}{pixels_{Ch1,total}} \qquad\qquad c_2 = \frac{pixels_{Ch2,coloc}}{pixels_{Ch2,total}}$$

- Relative number of colocalizing pixels in channel 1 or 2, respectively, as compared to the total number of pixels above threshold.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- All pixels above background count irrespective of their intensity.

Weighted colocalization coefficients

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_{i,total}} \qquad\qquad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_{i,total}}$$

- Sum of intensities of colocalizing pixels in channel 1 or 2, respectively, as compared to the overall sum of pixel intensities above threshold and in this channel.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- Bright pixels contribute more than faint pixels

Correlation coefficient, Pearson´s correlation coefficient

$$R_p = \frac{\sum_i (Ch1_i - Ch1_{aver}) * (Ch2_i - Ch2_{aver})}{\sqrt{\sum_i (Ch1_i - Ch1_{aver})^2 * \sum_i (Ch2_i - Ch2_{aver})^2}}$$

- Provides information on the intensity distribution within the colocalizing region
- Value range -1 to +1
  -1,+1: all pixels are found on straight line in the scatter diagram
  0:      pixels in scattergram distribute in a cloud with no preferential direction

6.10.4   Overlap Coefficient, Overlap Coefficient after Manders

(Manders, Verbeek and Aten, J. Microscopy 169:375-382, 1993)

$$r = \frac{\sum_i Ch1_i * Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 * \sum_i (Ch2_i)^2}}$$

- Another parameter used to quantify colocalization in image pairs
- Insensitive to differences in signal intensities between the two channels, photo-bleaching or amplifier settings

Value range 0 – 1 (0: no colocalization, 1:all pixels colocalize)

6.11   Profile View

This function allows to

- display the intensity distribution of an image along a straight or curved line,
- show the intensity values in table form and copy table to clipboard or save as text file,
- show separate profiles for each channel in a multi-channel image.

Fig. 548   View Option control block - Profile View

The settings of the **Dimensions, Display**, **Player** and **Graphics** view options control blocks apply. The additional view-specific **Profile View Option** control block is shown in Fig. 548.

Any changes done with this View Option control block are effective immediately.

The content of the overlay plane is temporarily hidden while the toolbar is displayed.

☞ The **Profile** View can also be used online during scanning.

- Draw a Profile line in the image display to define a profile. The intensity curves are shown in a separate graph (see Fig. 549).

- In the **Profile** View Option control block you can select the width and type of the profile line. The color setting is taken from the **Graphics** View Option control block.

- You can place two markers on the profile line to measure differences in intensities and distances in the XY plane.

- Tick the **Profile in Image Display** block to see an intensity graph superimposed on the image (see Fig. 550).

Fig. 549   Image Display - Profile View, Line Profile with markers
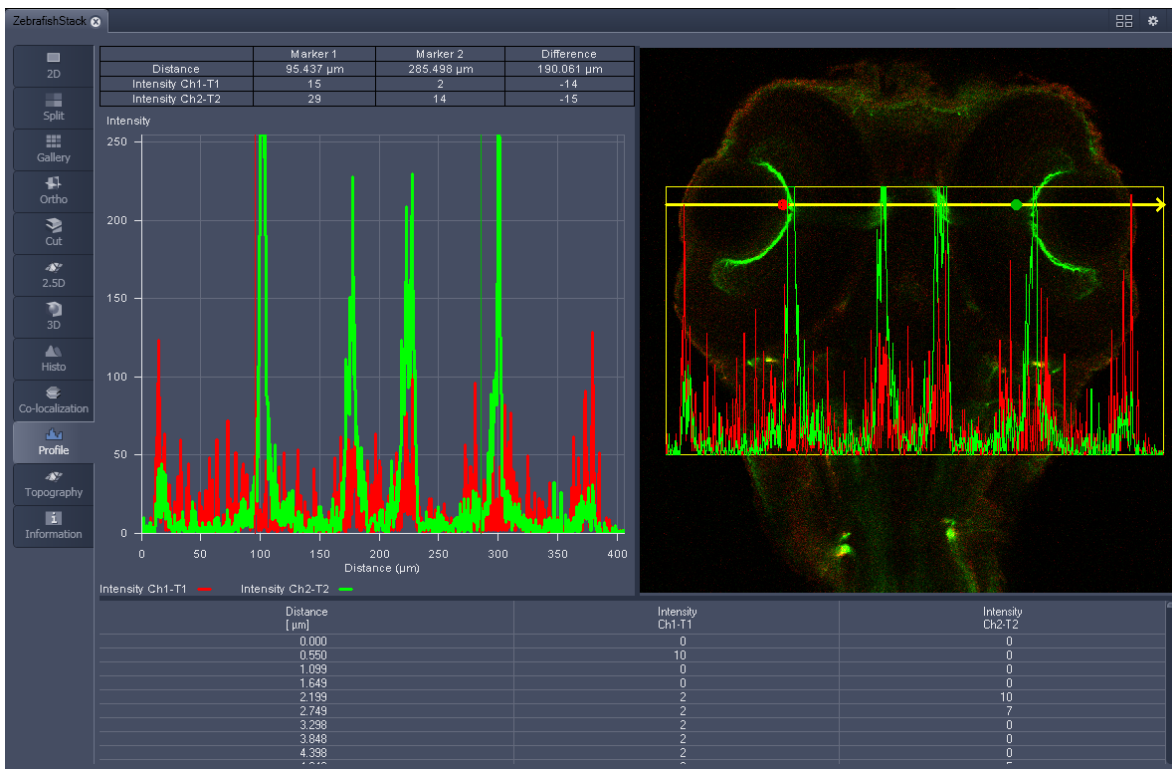
Fig. 550   Image Display - Profile View, Profile displayed in Image

Control block tools

The **Profile** toolbar contains the following tools:

Select button: Activates the mouse button for selection, resizing or movement of the profile line in the **Image Display** window.

<u>Resize:</u> Click on handle and hold down the mouse button, move the handle, release mouse button.

<u>Movement:</u> Click on line and hold down the mouse button, move the entire line, release mouse button.

Line with arrow button: (open arrow): Activates the straight profile drawing mode.

Click into the image and hold the mouse button, drag a line in any direction and release the mouse button to end the procedure.

Open polyline arrow button: Activates the open polyline drawing mode.

The first click into the image sets the starting point, each additional click adds a further line, right mouse click ends the procedure.

Line button: This button allows you to determine the line thickness of the profile line. It has no influence on the way the intensity profile is generated.

Show Table button: The profile diagram is displayed as a table at the bottom of the **Image Display** window.

☞   Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

6.12 Shuttle and Find View

Within the **S&F** control tab of the Shuttle and Find view (Fig. 551) regions of interests as well as points of interest can be defined. The regions and points can be relocated in the life image after moving the stage.

☞ Defined regions of interest and points of interest can only be relocated within one imaging session. In case the software was restarted, the positions of the image and of the defined regions and points are no longer valid.
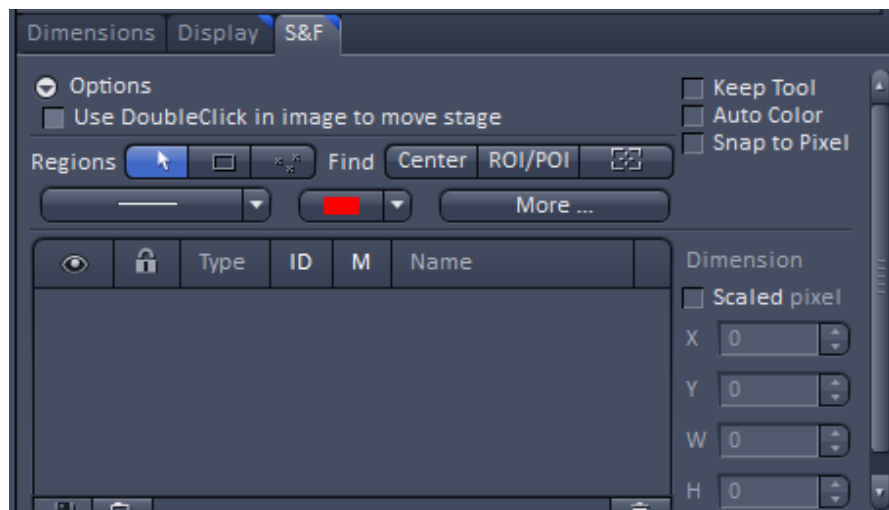
Fig. 551   Shuttle and Find control tab

Options provides settings for the general behaviour of the stage.
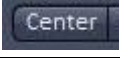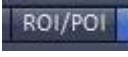
- **Use DoubleClick in image to move stage:** Moves the stage to the position you have double clicked on.

General settings for the graphical elements are set using the following features.

- **Keep Tool:** Keeps the current tool active. That's helpful if you want to draw in more than one ROI/POI.

- **Auto Color:** Uses a new color for each new element which is drawn in.

- **Snap to Pixel**: Draws in graphical elements using the pixel grid.

Within a snapped image multiple regions of interests (ROI) and points of interest (POI) can be defined using graphical elements. These ROIs or POIs can be relocated at the scanning electron microscope.

Regions and Find functions
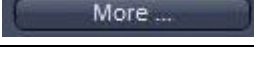
Button	Description
Select	By clicking on this button you activate the selection mode (button is marked blue) to select the ROIs or POIs in the image area (if drawn in). If you are currently in another mode, you can switch back to the selection mode using this button.
Draw Region of Interest	By clicking on this button you can draw in a rectangular ROI (Region of Interest).
Draw Point of Interest	By clicking on this button you can draw in a POI (Point of Interest) / marker point.
Center	Moves the stage to the center of the loaded image.
ROI/POI	Moves the stage to the selected ROI / POI.
Show stage position	Shows the current stage position as a rectangle in the image.

Dimension panel

Coordinates and dimensions of the selected graphical element are listed. If the Scaled pixel checkbox is activated, the unit is µm, otherwise Pixel.

- Parameter X: Shows the horizontal position (x coordinate) of the center of the graphical element.
- Parameter Y: Shows the vertical position (y coordinate) of the center of the graphical element.
- Parameter W: Shows the width of the graphical element.
- Parameter H: Shows the height of the graphical element.

Editing graphical elements

Button	Description
Line width	Dropdown list for choosing the line width.
Line Color	Dropdown list for choosing the color of the line.
More …	Opens a panel for setting annotations into the image.

Graphical elements list

List Columns	Description
Eye symbol	Shows or hides the ROI / POI in the image.
Lock symbol	Locks a ROI / POI to prevent changes.
Type	Displays the icon for the tool type (ROI/POI).
ID	Displays the ID for the graphic element. To do this, activate the checkbox at the corresponding list entry.
M	Displays measurement data for a graphic element. To do this, activate the checkbox at the corresponding list entry.
Name	Displays the name of the graphic element. To change the name, double-click in the Name field. Then enter the text of your choice.

Correlating a live image and a snapped image

The snapped image and the regions of interest drawn in the image can be relocated after the stage has been moved.

– Open a second container in the view tab

– Click on the **Continuous** button to start continuous scanning.

– Load the previously acquired image and click either on **Center** in the Find tool bar to center the live image or click on the ROI/POI within the Find tool bar to center the activated ROI or POI in the live image.

☞ Defined regions of interest and points of interest can only be relocated within one imaging session. In case the software was restarted, the positions of the image and the defined regions and points are no longer valid.

6.13 Mean of ROI: Additional View Type for Time Series

The **Mean of ROI** View Type allows to

– display the intensity - time diagram (mean intensity in user defined ROIs over time),

– use frame - time series and frame - Z-Stack - time series as input,

– show the intensity values in table form and copy table to clipboard or save as text file.

The **Mean of ROI** function permits interactive analysis and monitoring intensity over time.

The settings of the **Dimensions, Display**, **Player** and **Graphics** View Options control blocks apply. The additional view-specific **Mean of ROI View Option** control block is shown in Fig. 552.

Any changes done with this View Option control block are effective immediately.

The Image Display in the Mean of ROI View shows 3 panels: the intensity-over-time diagram, the data table with the intensity values for each ROI over time and the image display with ROIs in overlay (see Fig. 553 and Fig. 554).

Fig. 552   **Mean of ROI View Option control block**

To access the **Mean of ROI** View Type, load or acquire a time series data set and click on the **Mean ROI** View tab.

The mean intensity of one or several regions of interest is displayed over time. Time flags from bleaching experiments of trigger events are marked with little symbols on the time-axis of the graph (see Fig. 553).

The **Mean of ROI** analysis always refers to the currently displayed z-section of a Z-Stack (see **Dimension** View Option control block for which section it is).

Fig. 553   **Image Display window, Mean ROI display for time series in single plane**
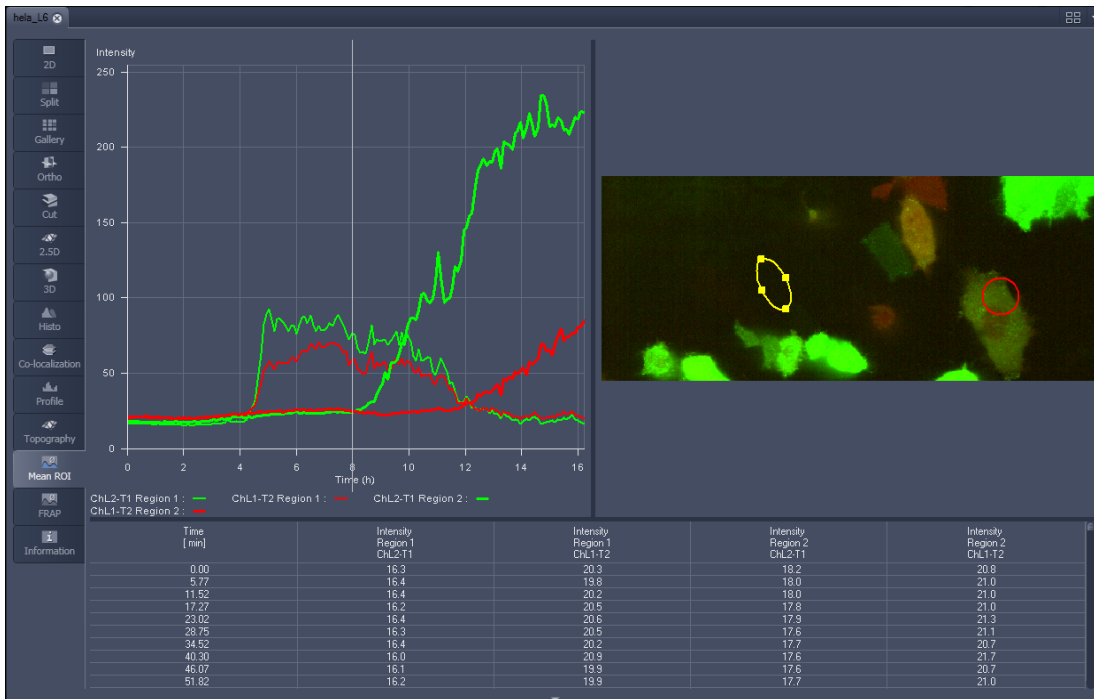
Fig. 554   **Image Display window, Mean ROI display for time series of Z-Stack**

The **Mean of ROI** view options control block contains the following function elements:

#	Type	X	Y	Width	Height	Lock	
☑ 1		147	65	51	37		
☑ 2		239	176	40	40		
☑ 3		376	121	84	113		

ROIs can be managed in the Graphics View Option control block.

Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.

Bezier button: Activates the Bezier figure drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

Circle button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.
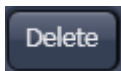
Recycle bin button: All the ROIs to the image are deleted.

Rectangle and 3D Rectangle button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.

Ellipse and **3D Elipse** button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

Polyline and **3D Polyline** button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.
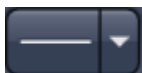
Line button: This button allows you to determine the line thickness of the ROI outline.
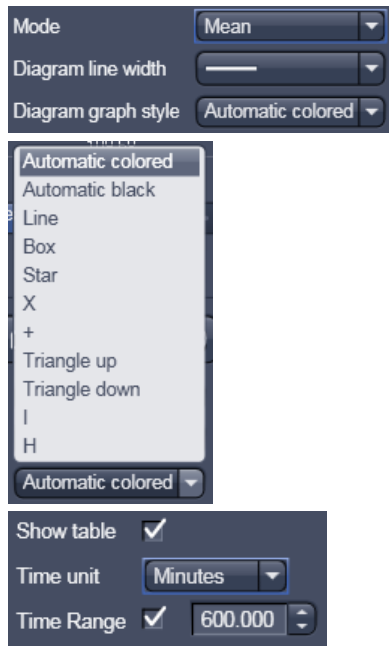
Buttons for diagram display options:

Mode pull down: choose between area and mean mode

Area: Display of the area of the ROI in the intensity time diagram, depending on the set threshold values. Area measurements of very small areas (< 10 pixels) give only approximate values.

Mean: Display of the mean values of the relevant ROI in the intensity time diagram.

Diagram line width pull down: sets the line with of the curves in the diagram.

Diagram graph style pull down**:** choose from a set of graph styles

Show Table tick box: The table of intensity values is displayed on the bottom of the Image Display window.

Time unit pull down: choose between seconds, minutes, hours or Automatic

Time Range check box and spin box: reduce the displayed time range to the selected value

In Area Mode: Ch1 / Ch3 / Ch4 pull down: Selection of the channel to be used.

In Area Mode: Threshold low and high slider: The intensity values below /above threshold are not displayed for the **Area** function.

6.14 Kinetic / FRAP View: Additional View Type for Time Series

The **FRAP** function permits interactive analysis of bleaching experiments, including

- Correction of FRAP data for bleaching and background,
- Fitting of FRAP data to a mono exponential or double exponential model for intensity.

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** View Options control blocks apply. The additional view-specific **FRAP View Option** control block is shown in Fig. 555.

Any changes done with this View Option control block are effective immediately.

The Image Display in the **FRAP** View shows 4 panels: the intensity-over-time diagram with the fitted curve, the table with the fit parameters, the intensity value data table and the image display (see Fig. 556, Fig. 558 and Fig. 559).

To access the Kinetic / FRAP analysis view type, load or acquire a time series data set and click on the **FRAP** View tab.
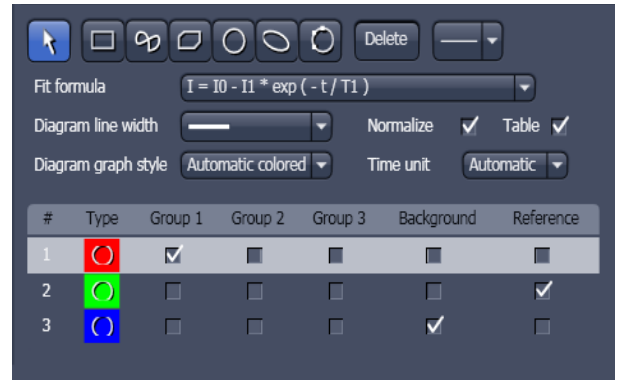
Fig. 555   **FRAP View Option control block**
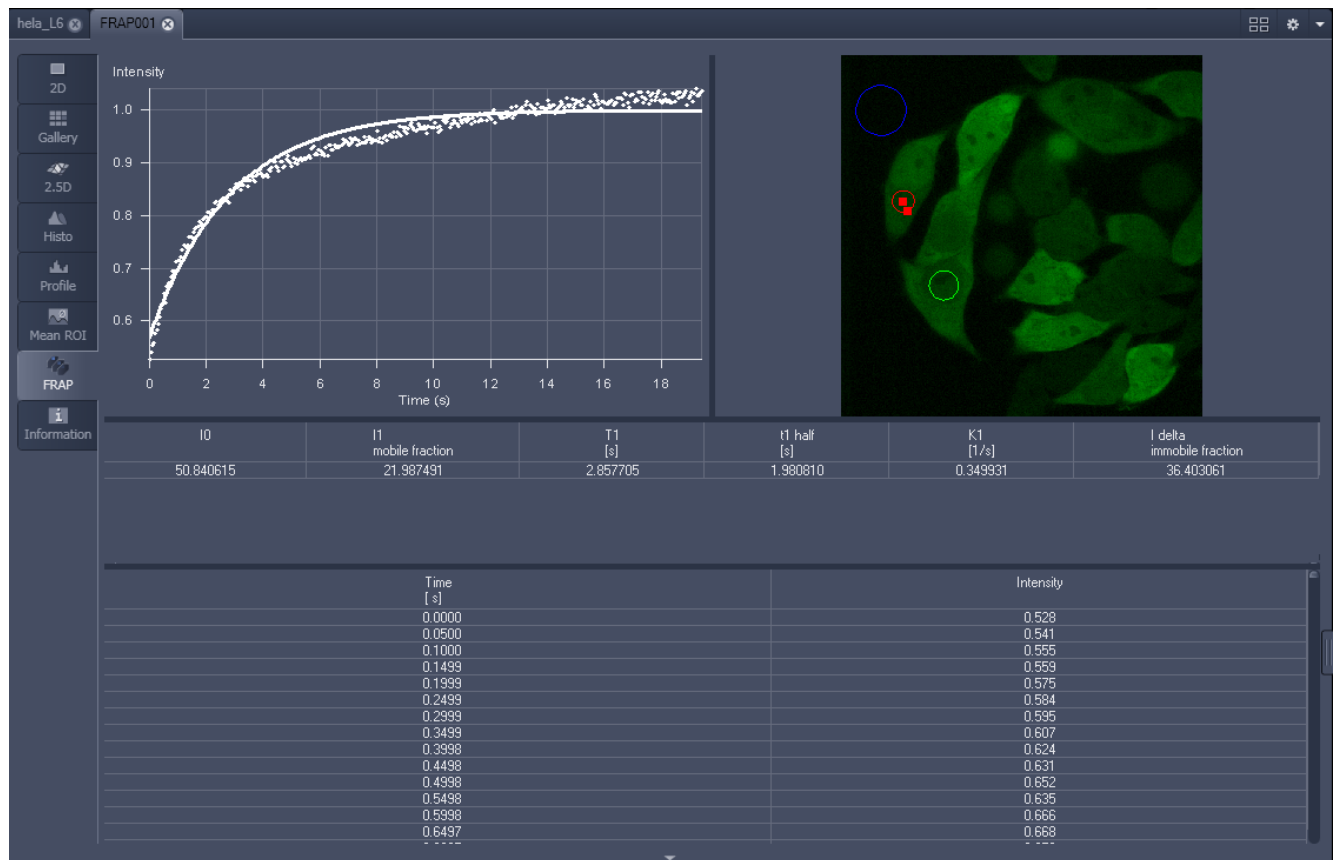
Fig. 556   **Image display of a time series in the FRAP View tab**

Available tools in the FRAP View:

Channels	Select single channels or all channels for analysis in the general **Dimensions** view options control box.
Kinetic Model / Fit formula	Pull down menu to select the mathematical model (mono or double exponential model) for fitting the data.
Analyis Areas / Regions of Interest (ROIs)	The **FRAP** View Options control block includes drawing tools that work identically to the graphics view options control block. ROIs from both control blocks can be combined.
Background Region	Check box in the list of ROIs: Mark the region of interest which represents the mean background intensity to be used for data correction.
Reference Region	Check box in the list of ROIs: Choose and mark the ROI which represents the fluorescence intensity of a reference cell that has not been bleached. The mean intensity within that region is used to correct the data at each time point for any bleaching artifact that occurred during the imaging process.
Combine Regions Group 1, 2, 3	Check boxes in the list of ROIs which allow to choose more than one ROI for analysis and to group them according to the experimental set up.

Example: FRAP Performed in a Nucleus Expressing GFP Labeled Proteins

Display of the image series in the **Mean ROI** display mode: The drawing tools are used to define the ROI to be analyzed (ROI 1), the background ROI (ROI 3), and the reference ROI (ROI 2) (see Fig. 557). The reference ROI must be a neighboring cell which has been imaged with the same laser intensity over time identical to the cell, which has been bleached to induce FRAP. Make sure the whole cell or cell compartment of interest is imaged and therefore illuminated.

☞ Use the **time** slider in the **Dimensions** view options control block of the general view options group to display the first image after the bleach event. This makes it easy to choose the ROI for analysis. The analysis region should be slightly smaller than the region that has been bleached. The latter is listed in the Mean of ROIs list in the graphics view options control block.

- Define your ROIs for analysis in the **Mean of ROI** View tab.
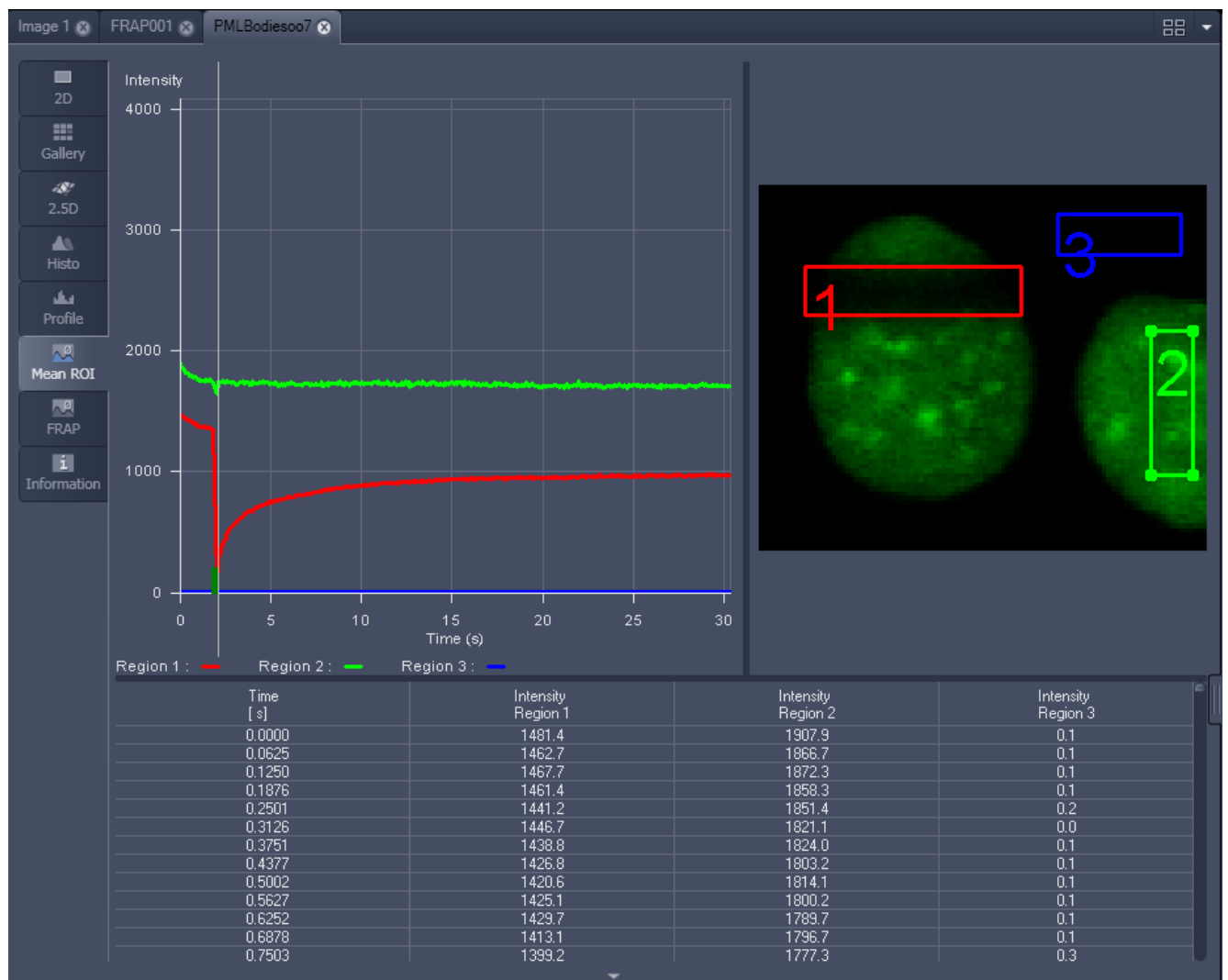
Fig. 557   **FRAP data displaying the first bleached image of an images series in the Mean of ROI View Type**

- Switch to the **FRAP** View tab.

- Mark the check boxes for background and reference region.

- The intensity values of the ROI to be analyzed will be corrected for background intensity and changes in intensity calculated for the reference region. The correction is done for each time point taking the actual intensity difference in the reference region into account.

  The remaining ROI or ROIs are used for analysis when checked active for either group 1, 2 or 3. ROIs assigned to the same group are analyzed added for analysis.

- Chose the **Kinetic Model** in the pull down list.

  - The result of the fit is displayed in the table. The result can be copied to the clipboard (Copy **Results**) or saved as a text file (**Save Results**) (right-mouse click). The following values are calculated and shown:

  - The final signal intensity in the analyzed ROIs following recovery **I0** (of the fitted curve).

  - The amplitude of the fitted curve (which equals the mobile fraction) **I1 mobile fraction**.

  - The fitted parameter **T1** (seconds).

  - The rate constant for the exchange of molecules between the bleached region and the surrounding area **K** (per second).

  - The part of the immobile fraction of the protein **I delta immobile fraction**.

Checking the **Table** tick box opens a further table in the image display area. It shows all intensity values for each channel and ROI analysis group over time. These values are corrected for background intensity and intensity loss of the reference region.

The values can be saved as a text file (**Save Table**) or copied into Excel via right-mouse click (clipboard) (**Copy Table**).

The data can be normalized optionally when marking the check box **Normalize** in the FRAP View Options control block.

☞ The calculation of the parameters is based on the same ROIs unless other ROIs or moved ROIs are selected again. The Kinetic Display is always available once the analysis has been performed.

☞ The analysis is not stored with the image.

☞ Note that this modeling is a very basic approach to your experiment. It offers a first hint on the possible presence of only one or, in case of a bad fit, more than one mobile fractions of the labeled protein within the cell or cell compartment examined. For a more advanced analysis refer to the scientific literature.

☞ The half time of the recovery can be calculated using the following formula: $-t_{half} = (\ln 0.5)*T1$
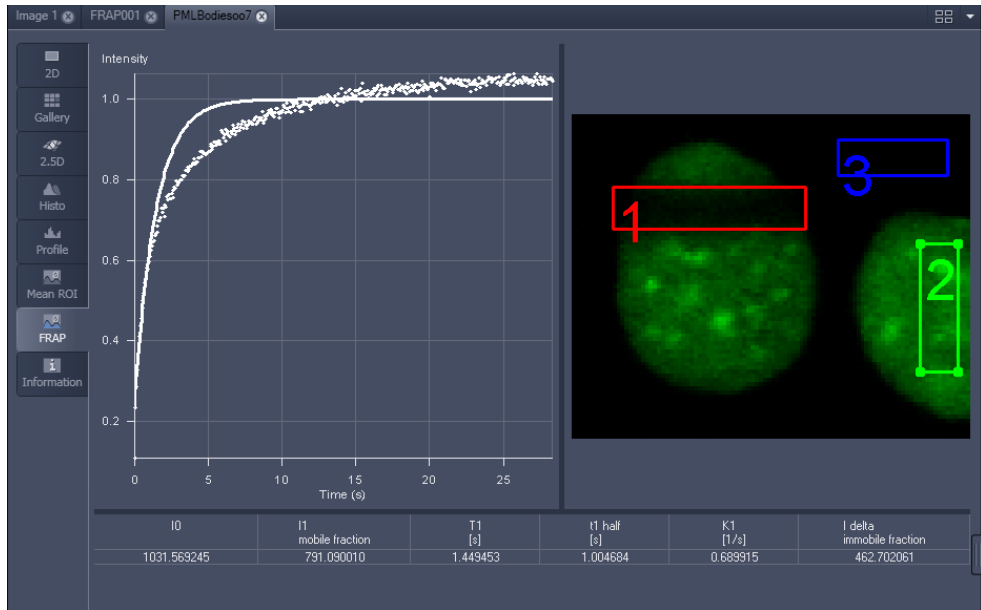
Fig. 558   Image window displaying the analysis of FRAP data using a mono exponential fit
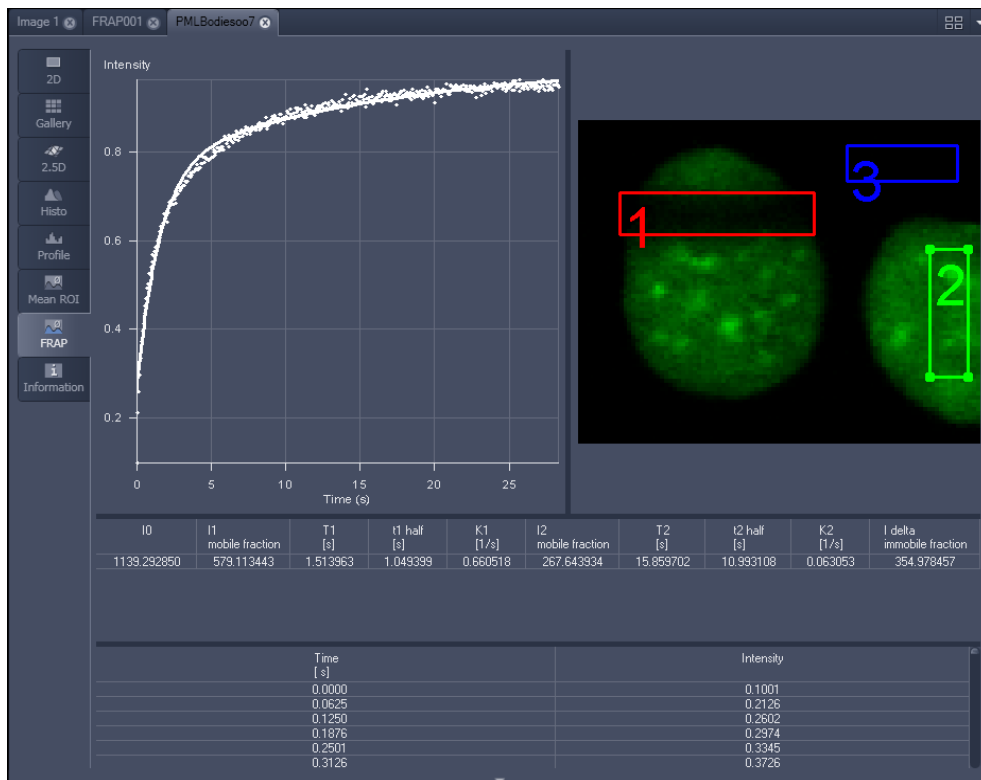
Fig. 559   Image window displaying the analysis of FRAP data using a double exponential fit

If the analysis is done using the double exponential fit the fitted curve displays the mean of the fitted values for the two different mobile fractions. The table shows the following additional parameters:

- The amplitude of the two curves, displayed as one (which corresponds to each part of the mobile fractions **I1** and **I2**).

- The fitted parameters **T1** and **T2** (seconds) for each mobile fraction.

- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1** and **K2** (per second) for each mobile fraction.

☞ The raw data of the experiment can be exported for further analysis using the Mean ROI display mode and, within this dialog, the table display of the results.

☞ Note that this modeling is also a very basic approach to your experiment. It offers a first hint on the possible presence of two mobile fractions of the labeled protein within the cell or cell compartment examined.

Please refer to relevant scientific literature or the website of the EAMNET (http://www.embl.de/eamnet) for further information on how to set up and perform FRAP experiments. A schematic curve marking the data points that are calculated performing the Kinetic Analysis is shown in Fig. 560. Please note, that the naming of the data points is not consistent with the information on the website.

A more general introduction to FRAP experiments can be found in the "Methods" brochure "Photomanipulation with Laser Scanning Microscopes" by Carl Zeiss Microscopy GmbH (order no. 45-0060 e/09.06) and the references cited therein.
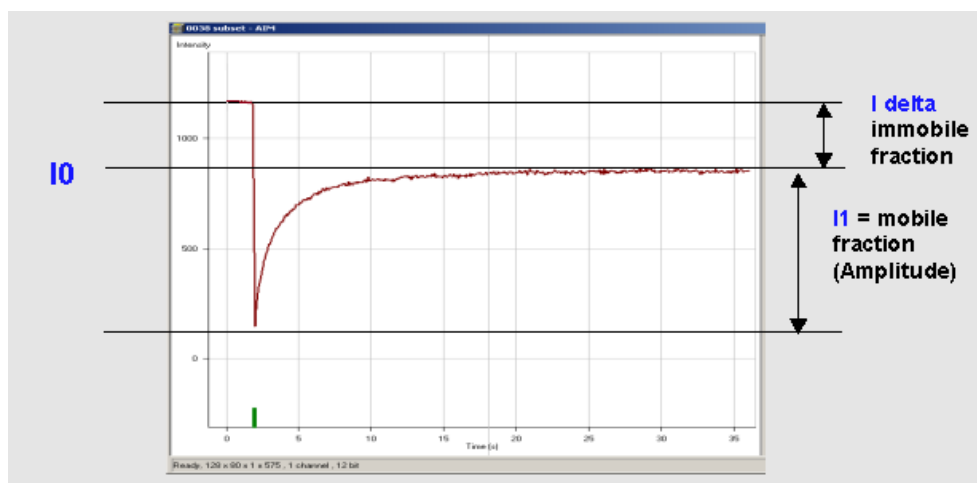
Fig. 560   **Schematic FRAP curve with marks at the relevant data points. I0, I1 and I delta which are calculated performing the Kinetic Analysis**

6.15 Lambda Coded: Additional View Types for Lambda Mode

The **Lambda Coded** View displays a Lambda Stack in a wavelength-coded color view. A color palette is automatically assigned to the individual images which are then displayed in a merge-type display. As a result, each pixel is represented similarly to a view through the oculars without emission filter (see Fig. 562).
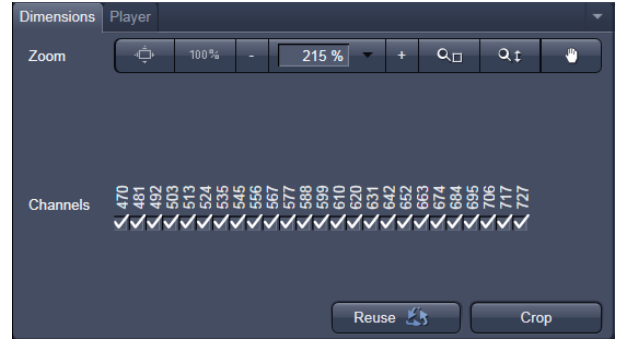
Fig. 561   Lambda Coded - specific Dimensions View Option control block

The settings of the **Graphics** View Options control block apply as in 2D view. In the **Display** View Options control block, the channel-specific settings of brightness, contrast and gamma don't apply since there are no channels. The **Dimensions** View Options control block is reduced since the wavelength is not a dimension any more when it is color-coded. The modified view-specific **Dimensions View Option** control block is shown in Fig. 561.

Any changes done with this View Option control block are effective immediately.
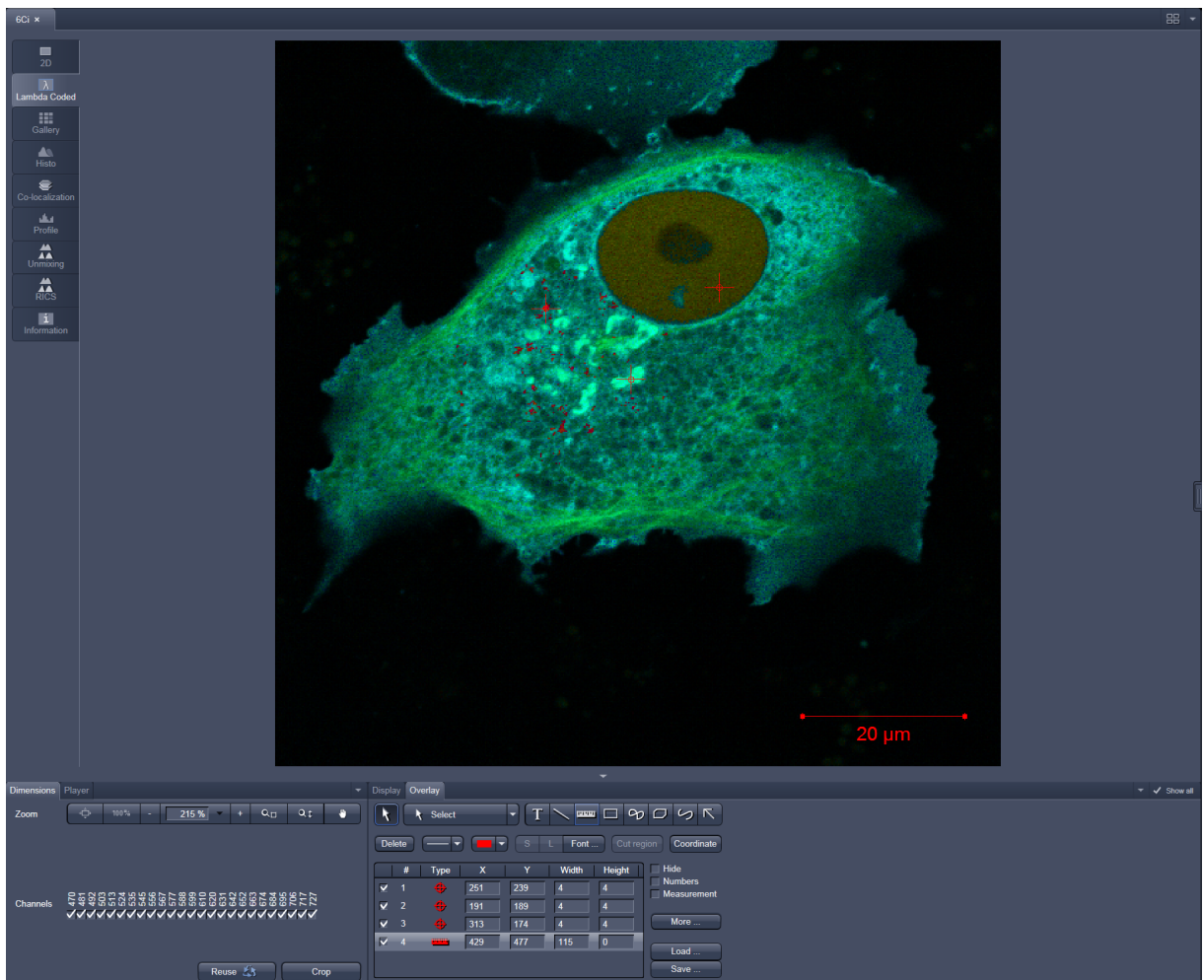
Fig. 562   Dimensions view option control block for Lambda Coded View
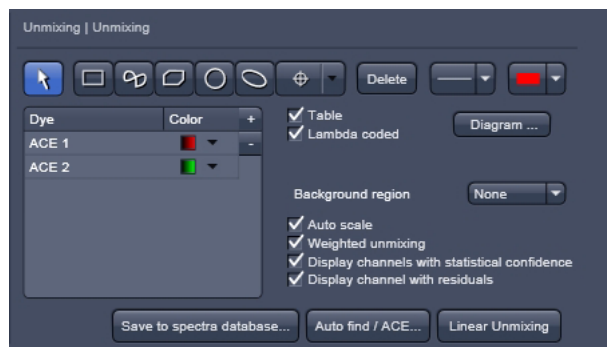
Fig. 563   Unmixing View Option control block
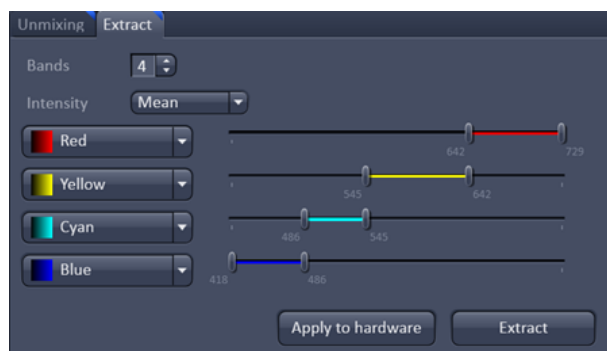
Fig. 564   Unmixing - Extract - View Option control block

6.16   Unmixing View

In the **Unmixing View**, Lambda or channel data sets can be analyzed and spectrally unmixed (for more information see as well section **Processing – Linear Unmixing**). In particular it allows to:

- display the spectra corresponding to user-defined ROIs (mean ROI intensity over Lambda),

- use all types of multi-channel images as input,

- show the intensity values in table form and copy table to clipboard or save as text file,

- generate unmixed multi-channel images.

The available tools and the functionality of the four general View Option control blocks **Dimensions**, **Display**, **and Graphics** is the same as in the **Lambda Coded** View (see the previous section on this view type). The two additional view-specific **Unmixing View Option** and **Unmixing Extract View Option** control blocks are shown in Fig. 563 and Fig. 564.

Any changes done with this View Option control blocks are effective immediately.

To access the Unmixing analysis view type, load or acquire a Lambda stack data set or a multichannel image and click on the **Unmixing** tab.

☞ Detector calibration is a service task. This calibration allows that reference spectra can be acquired at different detector gain settings than the multi-labeled image acquired for unmixing!

Tools in the Unmixing View Options Control Block

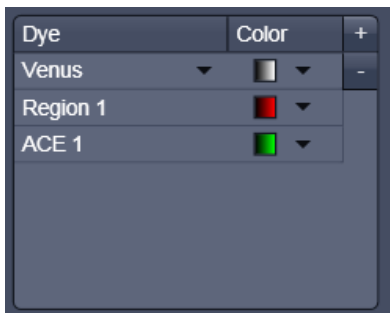– Tools for display customization and drawing:
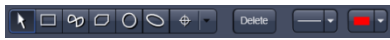
Drawing tools work the same as in the Graphics View Option control block
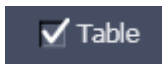
Show Table button: The table of intensity values over Lambda is displayed in the Image Display window.

☞ **Tables can always be saved or copied** to the clipboard by right-mouse clicking on the table display!

– Tools for unmixing:

Overview of spectral data selected, that is displayed and can be used for unmixing. Spectra saved in the spectra database can be loaded (here: Venus) by pressing the plus button in the upper right corner which creates a new row. In this row the left black arrowhead opens the spectra database. Manually defined ROIs (Region) and pixel selected by Automatic Component Extraction (ACE) can be added.

Select ROI for background subtraction

Automatically balances the intensity of unmixed channels to equal levels.
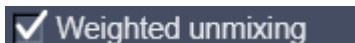
A statistical analysis of the signal-related (Poisson-) noise is performed and weighs the respective contribution to the fitting of the combination of reference spectra to the experimental data (see also section **Processing – Linear Unmixing**).

An additional channel per unmixed component is displayed which shows the relative standard deviation (RSD) in each unmixed component channel (see also section **Processing – Linear Unmixing**).

A separate channel in the unmixed image that displays the difference between fit and original data in grey values for the channel of the Lambda stack that shows the greatest deviation (see also section **Processing – Linear Unmixing**).
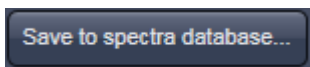
The values of the intensity Lambda diagram are saved to the spectra database in the ZEN\DyeDatabase directory. Make sure to have displayed only data of single dyes in the intensity Lambda diagram when executing this function. (The database interface is shown in Fig. 565).

Auto find / ACE...

Auto Find / ACE ("Automatic Component Extraction")
Automatically searches the image for regions with pure signals (Fluorescence originating from one single dye).

Linear Unmixing

Performs the unmixing with the selected spectra. Note: Those channels of the Lambda stack which are de-selected in the dimensions control tab are not included in the unmixing calculation.
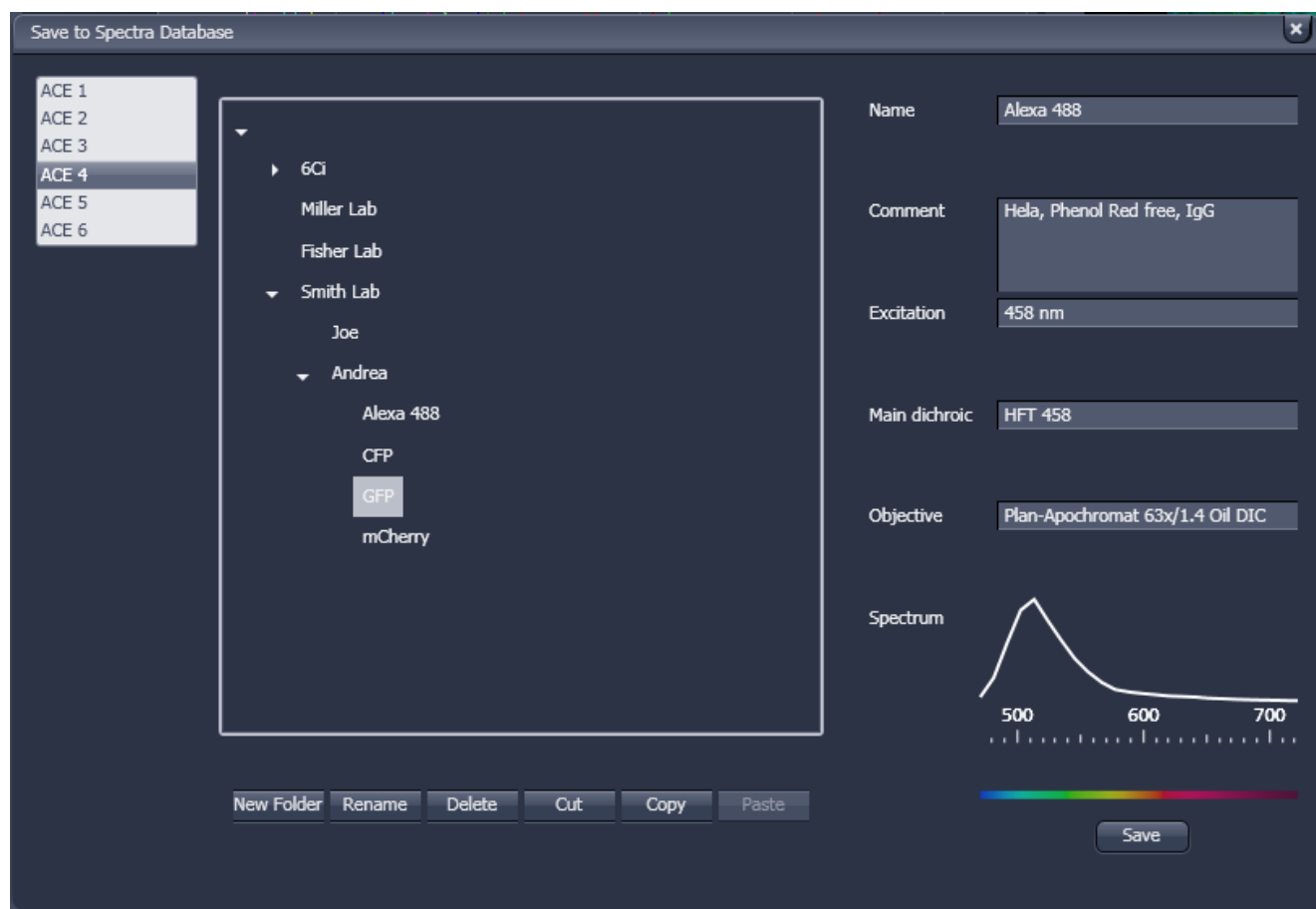

Fig. 565   Save to Spectra Database: database interface with subfolder structure

6.16.1   Automatic Component Extraction

The use of this function permits the automatic search for the individual reference spectra in a Lambda stack.

- Load or acquire a Lambda stack data set.

- Click on the **Unmixing View** tab.

- Click on the Auto find / ACE... button in the Unmixing View Option control tab (Fig. 567).

- Select the number of components in the sample and click **Ok** (Fig. 566). If a subset of existing spectra has been already defined by manual ROIs or by spectra from the spectra database, the number of remaining components is selected for ACE.

- Review the results in graph.

- When obtained spectra appear reasonable, unmix the data set by clicking the Linear Unmixing button (Fig. 567).

  – A new and spectrally unmixed multichannel image document is created (as many channels as previously defined components).
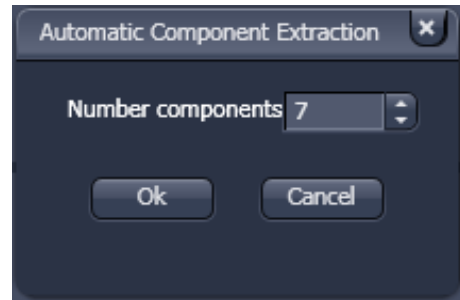
Fig. 566   ACE, number of components selection window
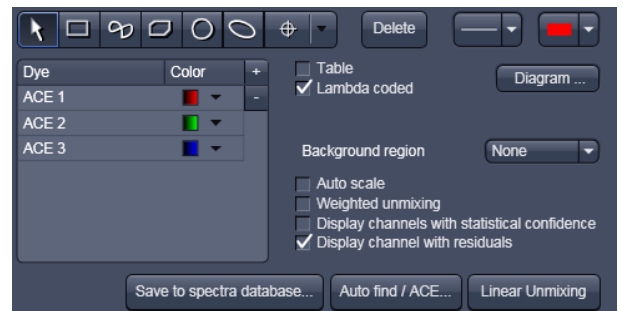
Fig. 567   Automatic Component Extraction function

Benefit and limitations of the ACE method:

Little to moderate spatial overlap of emission signals is required to obtain good results.

Because it relies on reference spectra extracted from the multi-channel / Lambda stack image itself, ACE could unmix on wrong assumptions and produce poor data.

However, it provides a solution for Emission Fingerprinting in those cases where reference spectra are not accessible via single-labeled controls.

If the resulting data is carefully checked for plausibility and quantification of the results is avoided carried out with the appropriate caution, this often is a helpful tool when working with spectrally overlapping dyes.

6.17 FRET View

In the **FRET** View type (Fig. 568), FRET data sets can be analyzed:

- Data gained performing Acceptor Photobleaching
- Data gained performing Sensitized Emission

The data sets are acquired setting up the imaging procedure manually.

To access the **FRET** View, load or acquire either a multichannel data set (minimum 3 channels) or a time series with bleach event and click on the **FRET** tab.

Depending on the type of data the Image Display in the **FRET** View shows 3 panels after initial analysis: the FRET image, the original data and the table with the analyzed data according to the method chosen.

☞ **Tables can always be saved or copied** to the clipboard by right-mouse clicking on the table display!
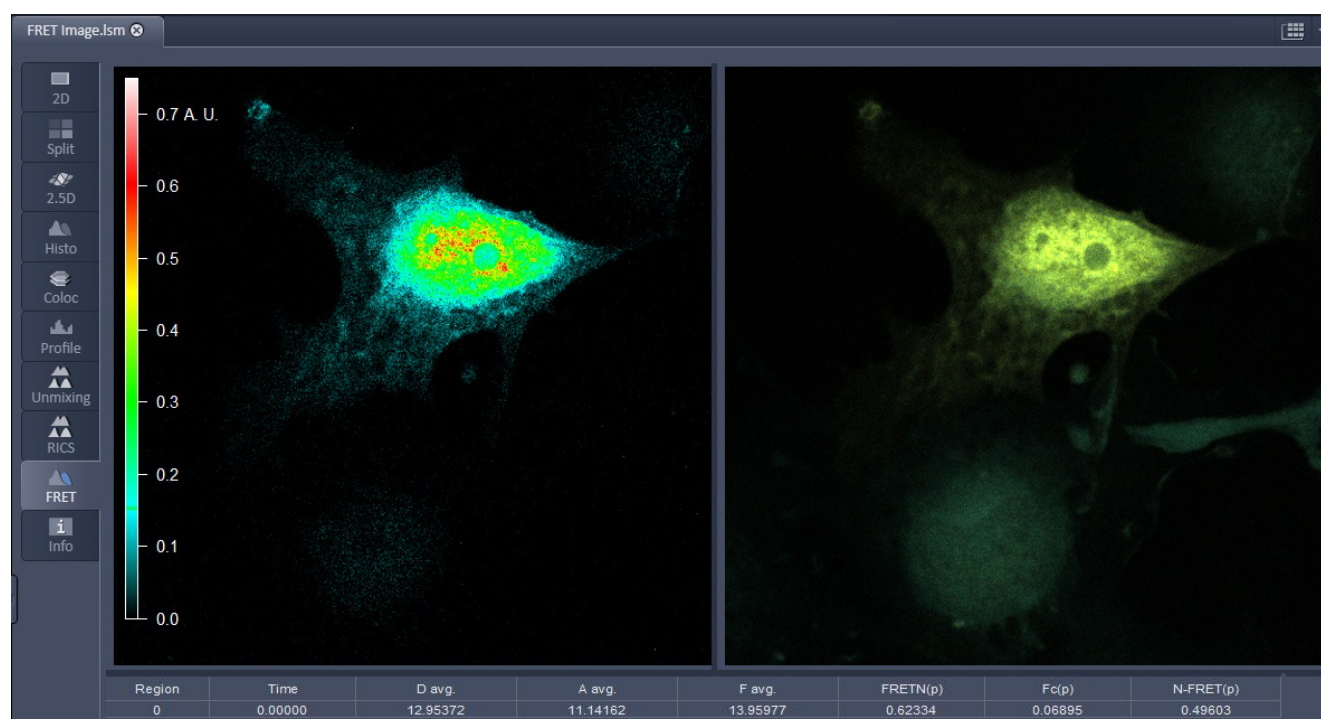
Fig. 568   FRET Display of Image after Sensitized Emission analysis

After a Sensitized Emission analysis the table shows the following parameters (Fig. 568):

Region: The identification numbers of regions assigned for analysis. Region zero refers to the whole image.

Time: When a time series is analyzed the time point is shown here.

D avg.: Average intensity of the region in the donor channel. These values are influenced by the chosen settings in the Settings Tab. They may therefore vary from values of the same regions in the Histo tab.

A avg.: Average intensity of the region in the acceptor channel. These values are influenced by the chosen settings in the Settings Tab. They may therefore vary from values of the same regions in the Histo tab.

F avg.: Average intensity of the region in the FRET channel. These values are influenced by the chosen settings in the Settings Tab. They may therefore vary from values of the same regions in the Histo tab.

FRETN (p): Result of analysis using Gordon method. The FRETN value is calculated for each pixel, afterwards the average for the chosen region is determined.

Fc (p): Result of analysis using Youvan method. The Fc value is calculated for each pixel, afterwards the average for the chosen region is determined.

N-FRET (p): Result of analysis using Xia method. The N-FRET value is calculated for each pixel, afterwards the average for the chosen region is determined.

Region	FRET(p) Eff.%	FRET Eff.%	D Pre	D Post	A Pre	A Post	Delta D	Delta A
0	-5.43673	5.94974	11.88683	12.63880	31.84916	29.66517	0.75198	-2.18671
1	16.39979	24.93387	43.65694	58.15797	93.28593	37.87503	14.50104	-55.41148
3	20.14109	26.91700	51.36111	70.27778	112.33334	43.75000	18.91667	-68.58334

Fig. 569   **Table after Acceptor Photobleaching analysis**

After an Acceptor Photobleaching analysis the table displays the following parameters (Fig. 569):

Region: The identification number of regions assigned for analysis. Region zero refers to the whole image.

FRET(p) Eff.%: FRET efficiency for each pixel is calculated and averaged for all pixels of the region.

FRET Eff.%: The averaged intensities of the region are used to calculate an averaged FRET efficiency for this region. Delta D / D Post * 100.

D Pre: Average donor intensity of the region in the pre-bleach image.

D Post: Average donor intensity of the region in the post-bleach image.

A Pre: Average acceptor intensity of the region in the pre-bleach image.

A Post: Average acceptor intensity of the region in the post-bleach image.

Delta D: Change in donor intensity of the region before and after the bleach event.
Delta D = D Post-D Pre.

Delta A: Change in acceptor intensity of the region before and after the bleach event.
Delta A = A Post- A Pre.

Independent of the FRET method, the FRET View Options Control Block offers four tabs:
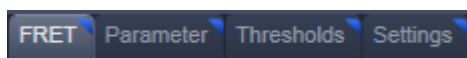
The available functions within each tab can vary according to the image loaded.

It is assumed that a time series with bleaching represents an experiment with Acceptor Photobleaching and a three channel image represents an experiment performing Sensitized Emission.
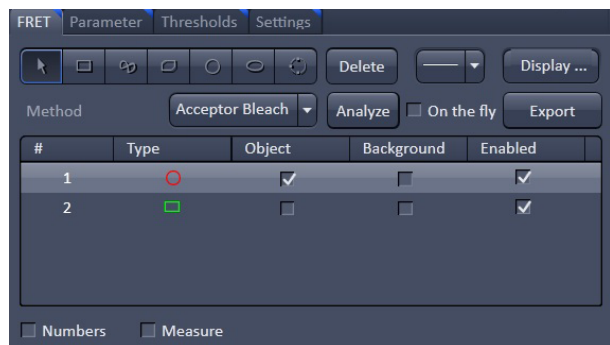
Fig. 570   FRET View Options Control Block, FRET tab

6.17.1   Tools in the FRET View Options Control Block for Acceptor Photobleaching

FRET tab (Fig. 570):

As **Method** for FRET Analysis, **Acceptor Photobleach** is automatically selected in the drop down list.

Within this tab the overlay regions present in the image (defined as bleach, background or control region of the bleach experiment) can be activated (The drawing tools correspond to the drawing tools described in section **Graphics**). Additional regions can be defined for analysis as well.

The checkboxes **Numbers** and **Measure** refer to the overlay regions and annotate the number of the region and its area to the overlay in the image.

Export allows the resulting FRET image to be saved as a separate image file.

The regions defined can be set as **Object** (Region of which FRET should be calculated) or **Background** (from which the threshold for the image analysis is deduced from and automatically used for analysis and displayed in the **Threshold tab**).

Regions and the assigned status as Object or Background can be selected or deselected for the individual analysis using the corresponding check box under **Enabled**.

To start analyzing the **Analyze** button must be clicked once. If all further changes shall be effective immediately the **On the fly** checkbox can be checked.
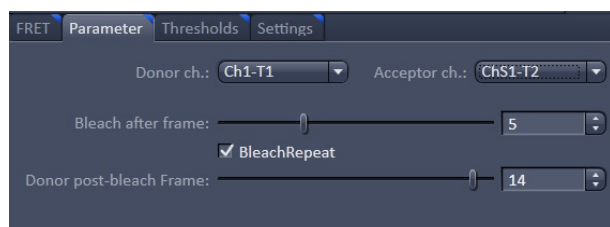
Fig. 571   FRET View Options Control Block, Parameter tab

Parameter tab (Fig. 571):

Various parameters of the image series can be set in this tab.

The image of the Donor and the Acceptor are assigned to the appropriate channel using the drop down menu next to **Donor ch.:** and **Acceptor ch.:**

The frame of the time series after the bleaching event (**Bleach after frame**) is set using the slider next to it or by editing the field next to the slider. If only one bleach event was performed, this defines the frame before and after the bleaching respectively for analysis.

In case of a bleach experiment with repeated bleaching time points the checkbox **Bleach Repeat** must be checked. This results in activation of the **Donor post-bleach Frame** slider and field. Here the frame can be chosen to provide the data of the donor intensity after bleaching (D post) for analysis.

Thresholds tab (Fig. 572):

The threshold for the image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor** or **All**, where **All** moves both sliders to the same value.

Alternatively, the threshold can be set by defining and enabling a background region in the FRET tab. The values for the thresholds are either displayed as grey value levels (**Raw data**) or **Normalized** to the value 1.
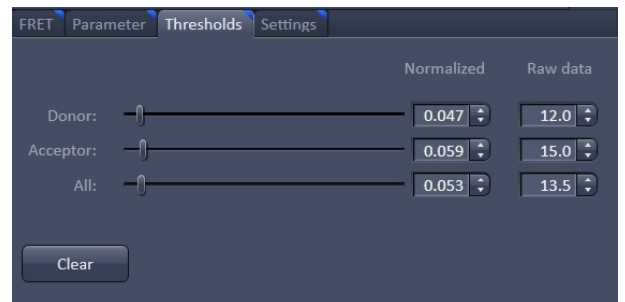
Fig. 572   **FRET View Options Control Block, Thresholds tab**

Settings tab (Fig. 573):

This tab provides access to the parameters used for the image analysis.

The **General** tab applies to both methods, Acceptor Photobleaching and Sensitized Emission.

If checked the listed options will be taken into account for the analysis of FRET.

Subtract background using thresholds: The previously defined grey values of the thresholds (FRET tab, Thresholds tab) are subtracted from each pixel prior to analysis. If not checked, pixel below the defined thresholds will not be displayed in the FRET Image and are not part of the analysis (value and area).

Fig. 573   **FRET View Options Control Block, Settings tab, General**

Exclude saturated (max intensity) pixels: Pixels that are saturated in at least one channel will not be considered for analysis.

Exclude zero intensity pixels: Pixels with zero intensity in any channel are not part of analysis. This checkbox is always checked.

Include thresholded pixels: Pixel with the same grey value as the defined thresholds will be part of analysis when checked. This is usually not activated.

Generate palette: The palette of the FRET image is displayed color coded when checked.

Show palette: This checkbox allows to choose if the palette of the FRET values is displayed within the FRET image or not.

The **Acceptor Photobleach** tab (Fig. 574) sets additional parameters to analyze the Acceptor Photobleaching experiment such as the choice for **Truncation** (as Remove Max or No Truncation) and the option not to display negative values in the image (**Don't show negative values in the FRET image**).
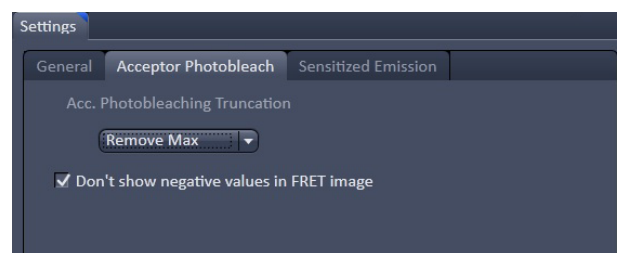
Fig. 574   **FRET View Options Control Block, Settings tab, Acceptor Photobleach**
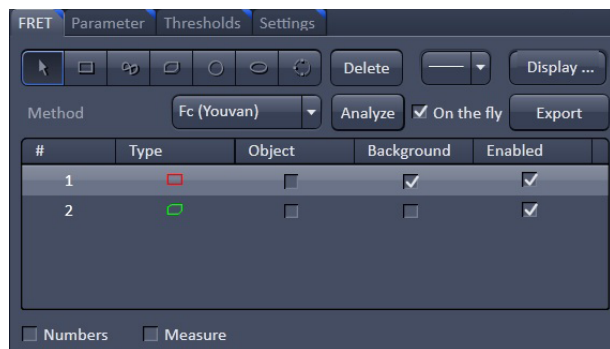
Fig. 575   **FRET View Options Control Block, FRET tab**

6.17.2   Tools in the FRET View Options Control Block for Sensitized Emission

FRET tab (Fig. 575):

Within this tab overlay regions can be defined. (The drawing tools correspond to the drawing tools described in section **Graphics**).

The checkboxes **Numbers** and **Measure** refer to the overlay regions and annotate the number of the region and its area to the overlay in the image.

Export allows the resulting FRET image to be saved as a separate image file.

The regions defined can be set as **Object** (Region where FRET should be calculated or the reference coefficient values are determined from) or **Background** (from which the threshold for the image analysis is deduced and automatically used for analysis and displayed in the **Threshold tab**).

Regions and the assigned status as Object or Background can be selected or deselected for the individual analysis using the corresponding check box under **Enabled**.

To start analyzing the **Analyze** button must be clicked once. If all further changes shall be effective immediately the **On the fly** checkbox can be checked.

Three different methods for FRET analysis are available in the drop down list:

- **Fc (Youvan)**,
- **FRETN (Gordon)** and
- **N-FRET (Xia)**.

Fc or **Youvan** method:

Youvan et al., Biotechnology et alia 3, 1 (1997)

Fc = Ff-Df(Fd/Dd)-Af(Fa/Aa)

Displays the Fc image with intensities converted from the FRET index calculated for each pixel using the Youvan method. This method assumes that the signal recorded in the FRET channel is the sum of real FRET signal overlaid by donor crosstalk and acceptor signal induced by direct (donor) excitation. There is no correction for donor and acceptor concentration levels and as a result the FRET values tend to be higher for areas with higher intensities.

FRETN or **Gordon** method:

Gordon et al., Biophys J 74, 2702 (1998)

FRETN = FRET1/ Dfd * Afa

Displays the FRET image with intensities converted from the FRET index calculated for each pixel using the Gordon method. This method calculates a corrected FRET value and divides by concentration values for donor and acceptor. This method attempts to compensate for variances in fluorochrome concentrations but overdoes it. As a result cells with higher molecular concentrations report lower FRET values.

N-FRET or **Xia** method:

Xia and Liu, *Biophys J* **81**, 2395 (2001)

NFRET = FRET 1/ $\sqrt{(Dfd*Afa)}$

Displays an N-FRET image with intensities converted from the FRET index calculated for each pixel using the Xia method. This method is similar to the Gordon method with the difference that for concentration compensation the square root of donor and acceptor concentration is used. The resulting image is properly corrected for variances in the fluorochrome concentration.

Parameter tab (Fig. 576):

Various Parameters of the image can be set in this tab.

Prior to analysis the channels of the image need to be assigned appropriately using the drop down menu next to **Donor ch.:**, **Acceptor ch.:** and **FRET ch.:**.

To determine the **Donor coefficient** values by using a sample with only signal of the donor fluorophore, one or more regions in the image are defined and enabled as objects in the FRET tab. Of this region the Donor coefficient values are taken by clicking the **Analyze Donor** button.
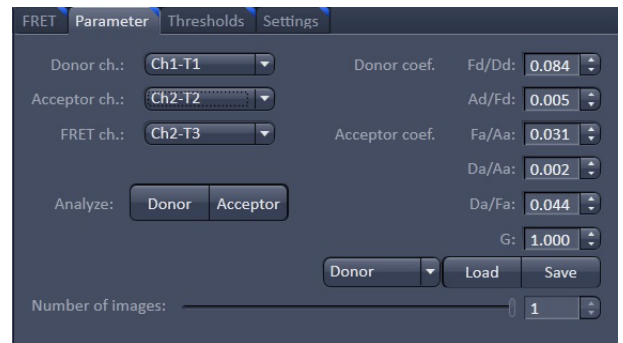
Fig. 576   **FRET View Options Control Block, Parameter tab, Last Analysis**

The **Donor coefficient** include the Donor signal detected in the FRET channel/Donor signal detected in the Donor channel (**Fd/Dd**) and the Donor Signal detected in the Acceptor channel/Donor signal detected in the FRET channel (**Ad/Fd**). These values are saved for future use as a .fret file by choosing **Donor** in the drop-down list and pressing afterwards the **Save** button.

To obtain the **Acceptor coefficient** values an image of a sample with only acceptor fluorescence is taken and one or more regions are defined as objects and enabled in the FRET tab and the **Analyze Acceptor** button is pressed.

The values for the **Acceptor coefficient** include the Acceptor signal detected in the FRET channel / Acceptor signal detected in the Acceptor channel (**Fa/Aa**), the Acceptor signal detected in the Donor channel / Acceptor signal detected in the Acceptor channel (**Da/Aa**), the Acceptor signal detected in the Donor channel/Acceptor signal detected in the FRET channel (**Da/Fa**). These values are saved for future use as a .fret file by choosing **Acceptor** in the drop-down list and pressing afterwards the **Save** button.

The Gordon factor **G** depends on the fluorphores used and is part of the Gordon and Xia analysis. Please refer to the original publications for further details (Gordon et al., Biophys J 74, 2702 (1998), Xia and Liu, *Biophys J* **81**, 2395 (2001)).

The total **Number of images** is set using the slider or by editing the field next to the slider, to choose how many images of a time series shall be taken into account. Their values will appear in the table below the image.

To load the saved Acceptor and Donor coefficient values for FRET analysis **Donor** or **Acceptor** are chosen in the drop-down list respectively. Afterwards the **Load** button is pressed and the previously saved .fret file chosen.
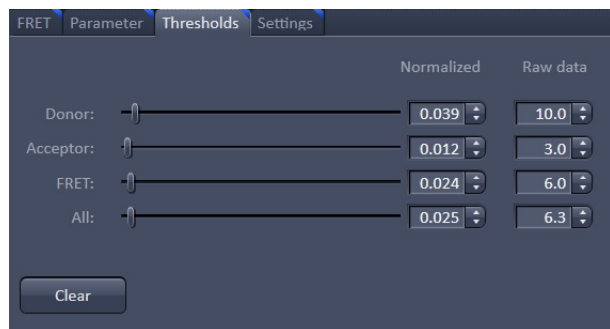
Fig. 577   **FRET View Options Control Block, Thresholds tab**

Thresholds tab (Fig. 577):

The threshold for the image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor**, **FRET** or **All**, where **All** moves all sliders to the same value. Alternatively, the threshold can be set by defining and enabling a background region in the FRET tab. The values for the thresholds are either displayed as grey value levels (**Raw data**) or **Normalized** to the value 1.
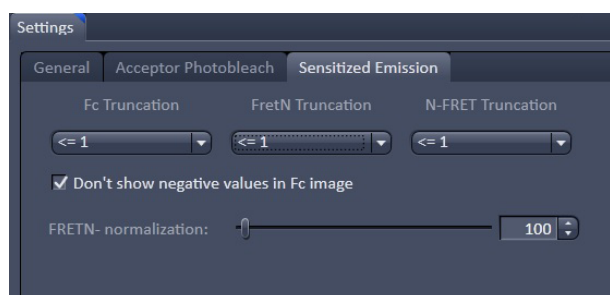

Fig. 578   **FRET View Options Control Block, Settings tab, Sensitized Emission**

Settings tab (Fig. 573 and Fig. 578):

This tab provides access to the definition of the parameters used for the image analysis.

The **General** (Fig. 573) tab applies to both methods, Acceptor Photobleaching and Sensitized Emission.

If checked the listed options will be taken into account for the analysis of FRET.

The **Sensitized Emission** (Fig. 578) tab sets additional Truncation parameters for each method to analyze the Sensitized Emission experiment such as the choice for **No truncation**, **Remove maximum** or the truncation of values above 1 (<=1), 2 (<=2) or 3 (<=3).

For the Fc analysis method, the option **Don't show negative values in the Fc image** can be selected using the check box. With the **FRET-N normalization** slider and text box a multiplication factor for the calculated FRET-N values can be set.

6.18   SMLM View

The **SMLM view** tab becomes available if **Online Processing** was active or for a SMLM processed image (Fig. 579).

The image displayed will be either a split between the raw data and the merged SMLM (**SR** channel) and sum widefield (**SWF** channel) images for an **Online Processed** image (Fig. 580), if the respective channels have been activated in the **SMLM-Render** and **Dimensions** view control tabs, respectively, or only the merged SMLM and sum widefield images for **Offline Processed** images (Fig. 579).

☞   For 3D SMLM images, only the processed HR image is available.

☞   A high resolution (**SR**) and sum widefield (**SWF**) image is only available for 2D SMLM images. There is only a HR image available for 3D SMLM.

Fig. 579   **View Tab – SMLM; overlayed SR and SWF channels for offlined processed images**

There are different **image view control** tabs associated with the **SMLM container view** tab, which are listed in the order one would normally proceed to process a SMLM image (Fig. 581).
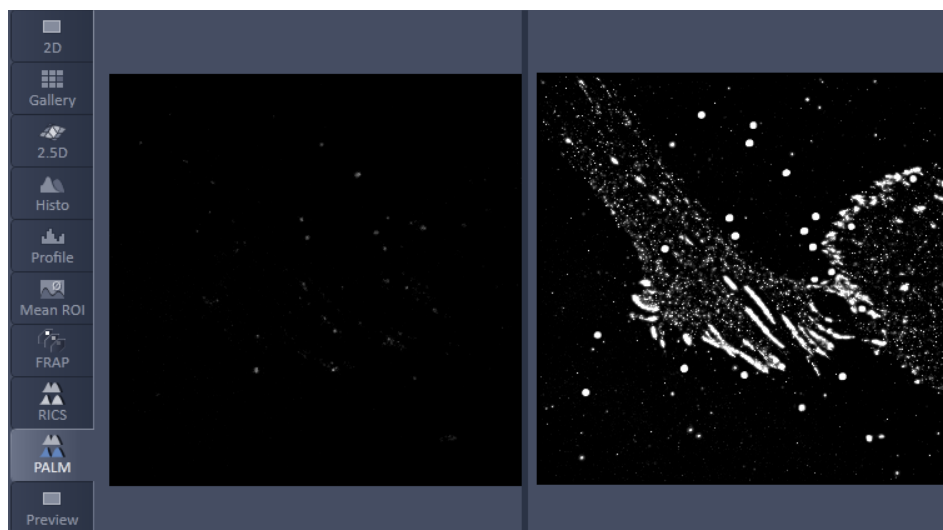
Fig. 580   **View Tab – SMLM; raw image (left) and overlayed HR and SWF channels for online processed images**
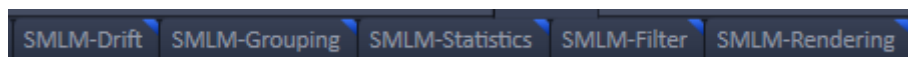
Fig. 581   **Specific view control tabs for SMLM**

SMLM-Drift: Setting parameters for drift correction using fiducial markers or models and performing drift correction. Viewing fiducials and corrected drift.

SMLM-Group: Setting parameters for grouping and performing grouping of identified peaks to one object.

SMLM-Statistics: Viewing Molecule table, statistic plots (histograms & scatterograms) and peak tracker (only available for **Online Processing**).

SMLM-Filter: Setting and executing filters including all parameters of the molecule table.

SMLM-Render: Setting pixel resolution, display and rendering of data.

Fig. 582   General view control tabs for SMLM

There are also **general control view** tabs available (Fig. 582), which functions have been described in section **2D View** including **Dimensions**, **Display** and **Graphics**, but that have special functions in relation to the **SMLM** view tab.

6.18.1   Dimensions View Control Tab

Time, **Zoom**, **Tools** and **Channels** as well as **Reuse**, **Crop**, **Positions** and **Stage** are general functions that have been described in section **2D View**.

If the data set like in 3D has a z dimension, the dimension will be displayed as Z-Position and with the Z-Position slider you can scroll through the data set (Fig. 583).
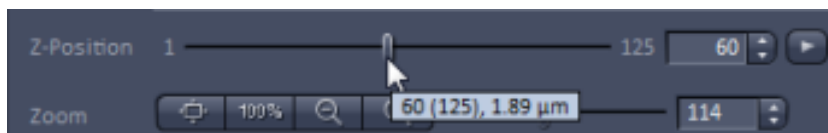
Fig. 583   Z-Position slider of the Dimensions tab with curser on the slider tool

☞   Please note that the spinbox display shows the number of the current slice. You can view the absolute Z-position in µm if you position the cursor on top of the slider button (Fig. 583). The display box is in the form "current slice # (total slice number), position # in µm". The position number in **SMLM-Filter** is given in nm. Hence the info box the slice # of the Z-Position slider of Dimensions can be linked to the position # of the **Position Z** slider of **SMLM-Filter** (Fig. 584).

Fig. 584   Position Z slider of the SMLM-Filter tab with minimum value set to a position corresponding to slice 60 of Fig. 583

☞   Care has to be exerted when relating relative slice numbers to absolute z positions. If z positions are restricted for absolute positions using the **Positioin Z** tool of **SMLM-Filter**, than the start slice is reset to 1 for the Z-Position and hence the slice number corresponds to the absolute position plus offset from a previous filtering. We recommend therefore that before resetting z-postions the **Position Z** filter tool is de-activated or using the **Position Z** histogram to find a useful z-range.

In addition there is the **Modify** field available for **Online Processed** data (Fig. 585).
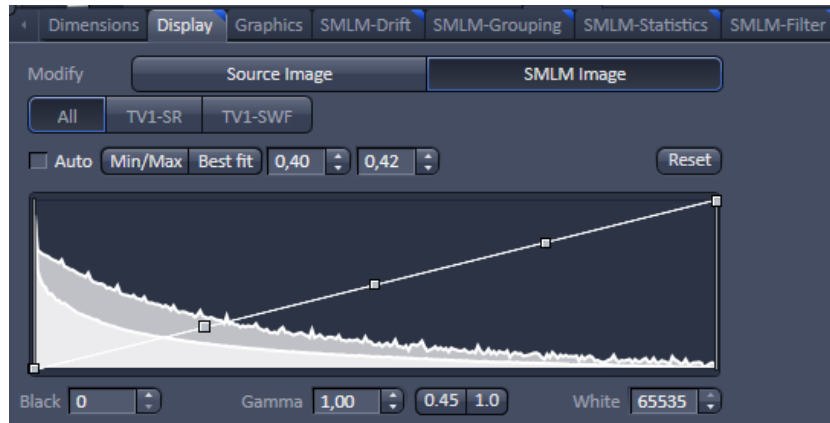
Fig. 585   Display view control tab of the SMLM view tab

☞ Note that for **Offline Processed** data the **Source Image** is not available and accordingly no **Modify** field displayed.

☞ Note that in 3D SMLM no online display is possible, so there is no distinction between Source and SMLM image.

In the **Modify** field you can press **Source Image** or **SMLM image**. If **Source Image** is pressed, the **TV** channels (time series) become available in the **Channels** field (Fig. 586, Fig. 587).
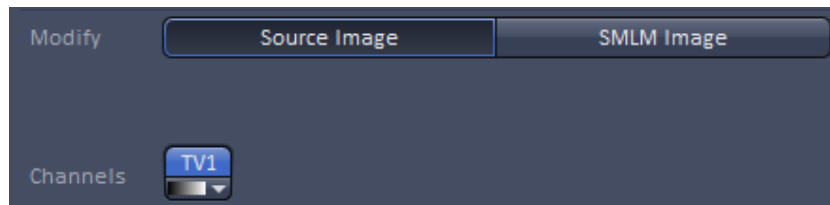
Fig. 586   Modify and Channels fields in the Dimension view control tab - Source Image selected (1 color channel)

Fig. 587   Channels fields in the Dimension view control tab - 2 color channels of Source image created by the Processing Tool Copy (Note that TV channels copied together by the Processing Copy tool are not numbered)

It will be the active **TV** channels that are also displayed in the other **View tabs**. In addition, all settings in the **control view tabs** will be applied to the active **TV** channels. If **SMLM Image** is pressed, the **HR** and **SWF** channels (SMLM and sum widefield images) become available in the **Channels** field (Fig. 588, Fig. 589).
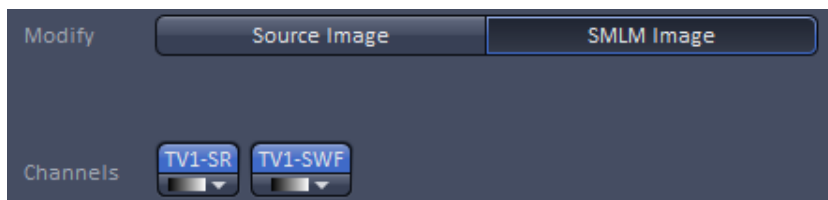
Fig. 588   **Modify and Channels fields in the Dimension view control tab - SMLM Image (1 color channel) selected**

Fig. 589   **Channels fields in the Dimension view control tab - 2 color channels of SMLM Image created with the Processing Tool Channel Alignment**

These can be selected individually or together in the **Channels** field. It will be the **HR** and **SWF** channels that are displayed in the other **View tabs**, if selected. In addition, all settings in the **control view tabs** will be applied to the **HR** and **SWF** channels, if selected.

☞ Two or more color channels of the **Source** image can only be created by copy two time series acquired with different colors in one file using the Processing tool **Copy**. Two or more channels of the SMLM image (mixed mode with **HR** and **SWF** channels) can either be obtained by processing a 2 color channel **Source** image in the Processing tool **SMLM** or by using the Processing Tool **Channel Alignment** with two SMLM images being **Input image** and **Input image 2**. Without a dual camera solution there is no way to acquire a 2 color channel image with **Online Processing**.

☞ Note that in the **SMLM** view tab you can only view either the **HR** or the **SWF** channels or the sum of them, dependent on which channels have been activated in the **Dimensions** view control tab of the **SMLM** view container tab and if **Merged** is active or not. If you want to view channels simultaneously and separated, or grouped in a specific mode, you have to go to the **Dimensions** view control tab of the **Split** view container tab (Fig. 590).
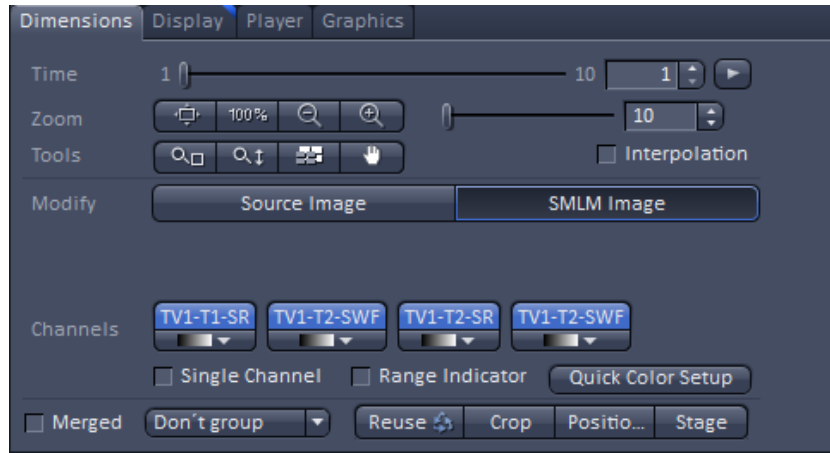
Fig. 590 **Dimension view control tab of the Split view tab**

☞ The **Group** drop down menu allows you to assign the way the mixed modes channels can be grouped (Fig. 591).
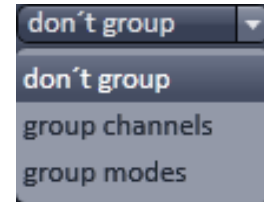
Fig. 591 **Group drop down menu**
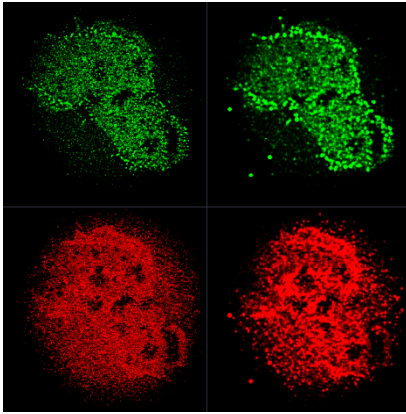
Fig. 592   **Split view tab - don't group selected**

Fig. 593   **Split view tab - group channels selected**
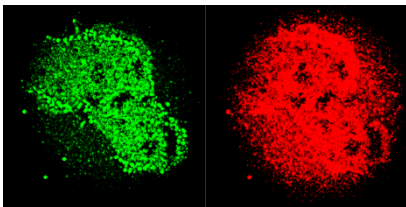
**Fig. 594**   **Split view tab - group modes selected**

☞ There are three options:

- **don't group**: each selected channel will be displayed individually (HR1, HR2, etc. single; SWF1, SWF2, etc. single) (Fig. 592).

- **group channels**: channels are grouped according to their modes (HR1, HR2, etc. together; SWF1, SWF2, etc. together) (Fig. 593).

- **group modes**: channels are grouped according to their color channel (HR1, SWF1 together; HR2, SWF2 together; etc.) (Fig. 594)

6.18.2   Display View Control Tab

The Display setting controls general functions are described in section **2D View**. In addition there is the **Modify** field available for **Online Processed** data (Fig. 595).
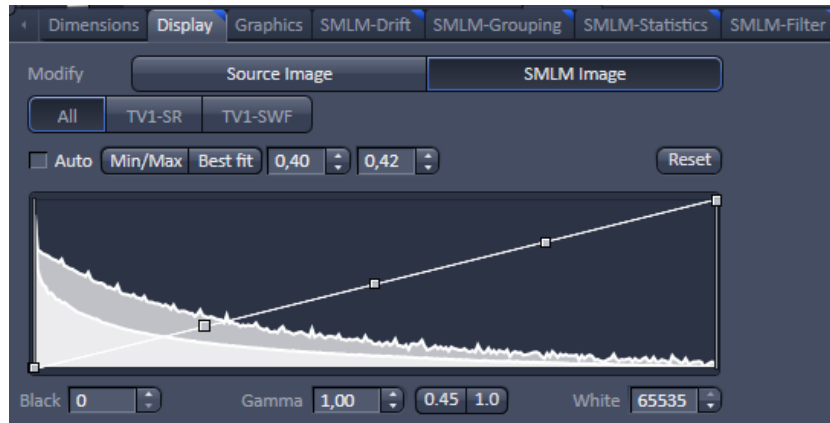
Fig. 595   **Display view control tab of the SMLM view tab**

☞ Note that for **Offline Processed** data the **Source Image** is not available and accordingly no **Modify** field displayed.

☞ Note that for 3D SMLM data the **Source Image** is not available and accordingly no **Modify** field displayed.

In the **Modify** field you can press **Source Image** or **SMLM image**. If **Source Image** is pressed, the **TV** channels (time series) become available in the **Channels** field (Fig. 596, Fig. 597).
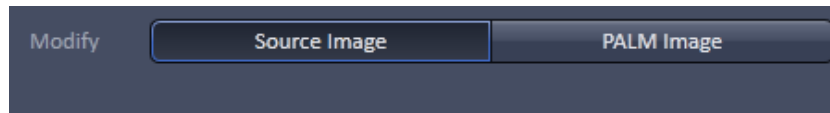
Fig. 596   **Modify and Channels fields in the Display view control tab - Source Image selected; only one TV channel available, hence no channel buttons displayed**
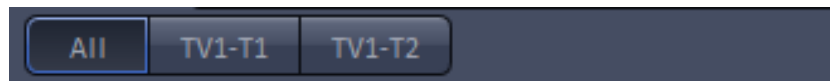
Fig. 597   **Channels fields in the Display view control tab - 2 color channels of Source image created by the Processing Tool Copy (Note that TV channels copied together by the Processing Copy tool are not numbered)**

If only one TV channel is available, no channel buttons are displayed for selection. Otherwise **All** and the channels name (**TV**, **TV** etc.) buttons will be displayed for selection. It will be the selected channels to which the display settings will be applied.

If **SMLM Image** is pressed, the **HR** and **SWF** channels (SMLM and sum widefield images) become available in the **Channels** field (Fig. 598, Fig. 599). These can be selected individually or together by pressing **All** in the **Channels** field. It will be the selected channels to which the display settings will be applied.

Fig. 598   Modify and Channels fields in the Display view control tab - SMLM Image selected; SR and SWF channels, that is more than one channel, available, hence All and channel buttons available
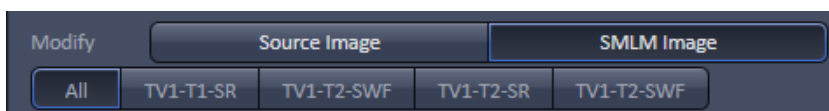
Fig. 599   Channels fields in the Display view control tab - 2 color channels of SMLM Image created with the Processing Tool Channel Alignment

6.18.3   Graphics View Control Tab

The selection and function of regions of interest (ROI) have been described in section **2D View** (Fig. 600).

All ROIs selected in **Graphics** will be used for reconstructing a SMLM image, if in the **SMLM-Filter** view control tab the **Use Region(s)** box is checked.
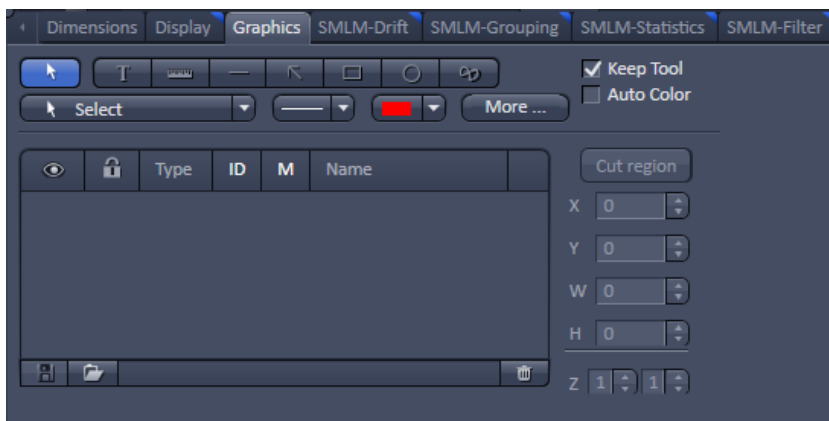
Fig. 600   Graphics view control tab of the SMLM view tab

6.18.4   SMLM-Drift View Control Tab

SMLM-Drift is used to perform a drift correction on the image. You can select the type of correction you want to perform by the **Correction Type** drop down menu, which is only available in the **Show all** mode (Fig. 601).

Fig. 601   SMLM-Drift view control tab of the SMLM view tab – Correction type drop down menu

You can select between

– **Fiducials**: drift correction will be performed on fiucial markers in the image (Fig. 602)

– **Model-based**: drift correction will be performed on sample structures (image self-alignments) (Fig. 603)
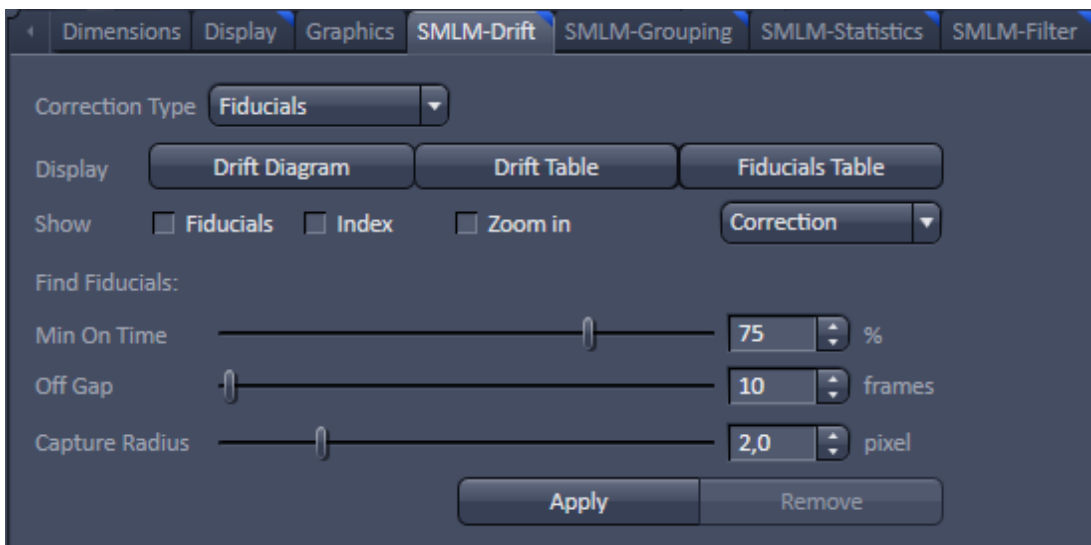
Fig. 602   SMLM-Drift view control tab of the SMLM view tab in Show all mode – Fiducials selected as Correction type
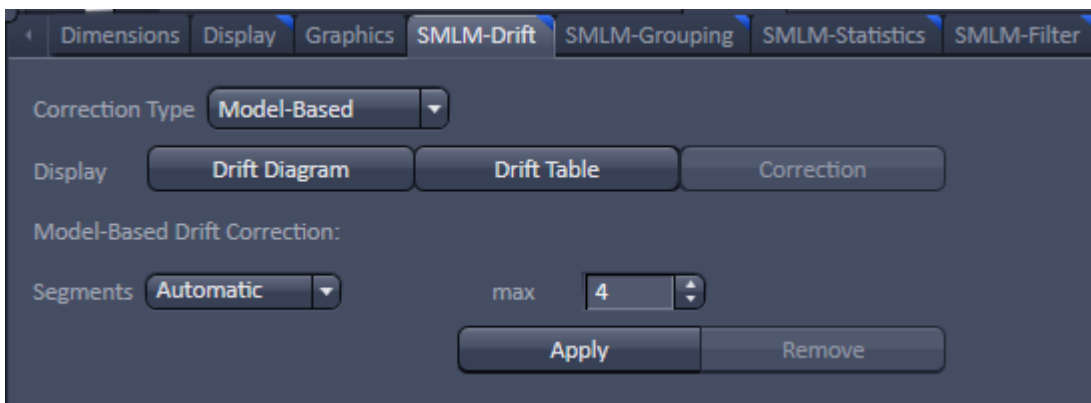
Fig. 603   SMLM-Drift view control tab of the SMLM view tab in Show all mode – Model-based selected as Correction type

If **Show all** mode is deselected, the display field shows the last selected correction type with certain options (Fig. 604 and Fig. 605).
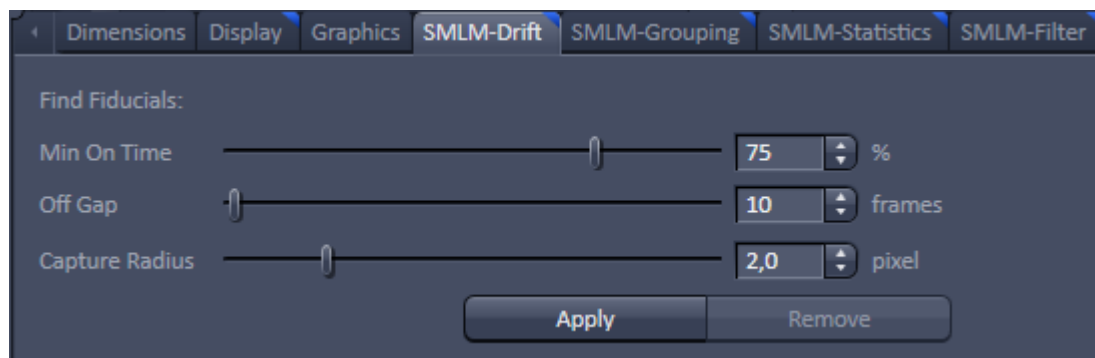
Fig. 604   **SMLM-Drift view control tab of the SMLM view tab with Show all mode not active – Fiducials selected as Correction type**
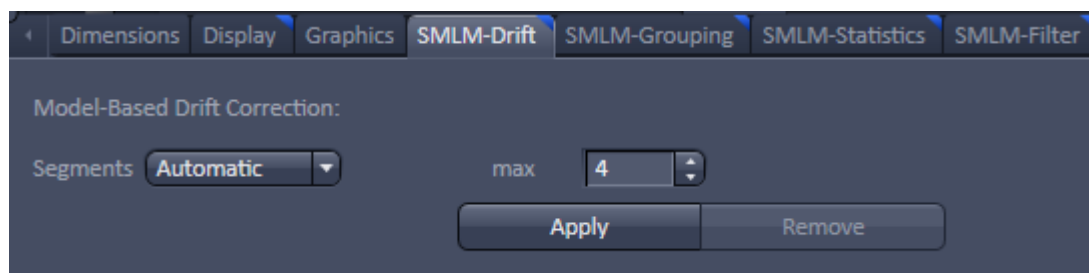
Fig. 605   **SMLM-Drift view control tab of the SMLM view tab with Show all mode not active – Model-based selected as Correction type**

☞   Please note that **Fiducials** and **Model-based** drift corrections are mutually exclusive. So if you switch from **Model-Based** with a drift correction performed to **Fiducials** and vice versa, the **Apply** button will be inactive and cannot be pressed. You first have to undo the drift correction by pressing the **Remove** button for a type of drift correction before the other type becomes available.

6.18.4.1   Fiducial Based Drift Correction

SMLM-Drift Fiducials selection is used to perform a drift correction based on fiducials like gold particles (Fig. 606).

What is to be considered to be a fiducial can be defined in the **Find Fiducials:** section, where the following parameters can be set:

- **Min On Time (%)**: Sets the minimum on time in percentage of the whole time series an object has to be detected in order to be regarded as a bona fide fiducial. 75% is the default value and means that the object has to be found in at least 75% of the recorded frames.

- **Off Gap (frames)**: Sets the maximum number of frames an object can be off consecutively in order to be still regarded as a fiducial.

- **Capture radius (pixel)**: Sets the maximum radius around an object, where another object is tolerated in order that the object is regarded as a fiducial.
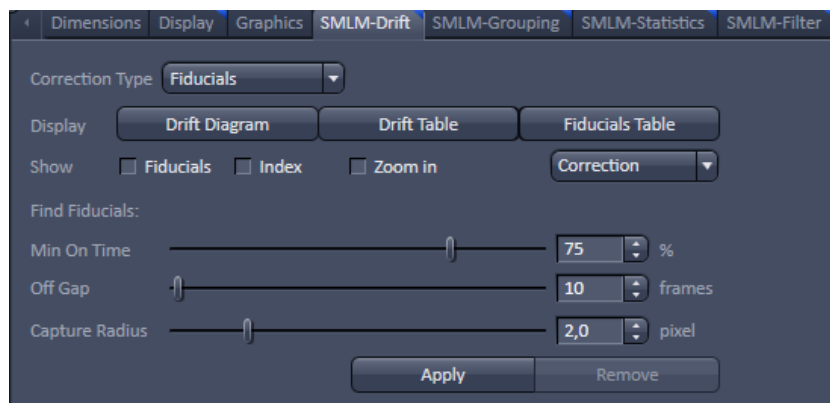
Fig. 606   SMLM-Drift view control tab of the SMLM view tab

When **Apply** is pressed, the software identifies on behalf of these criteria potential fiducials and uses them automatically to correct for their drift. The correction vector calculated from the average of all selected fiducials will then be applied to the SMLM image (**SR** channels).

Once **Apply** is pressed, the **Remove All** button becomes available, when fiducials have been identified and a drift correction has been performed (Fig. 607). Hence an inactive **Remove All** button is indicative of a SMLM image without drift correction. By pressing **Remove All** the drift correction will be undone and the button becomes inactive again.

☞ Drift correction is not applied to the sum widefield image (**SWF** channel), so there might be an offset between the **SR** and **SWF** channels after drift corection.

The **Display**, **Show** and **Drift Diagram** fields are only available in the **Show all** mode (Fig. 607).
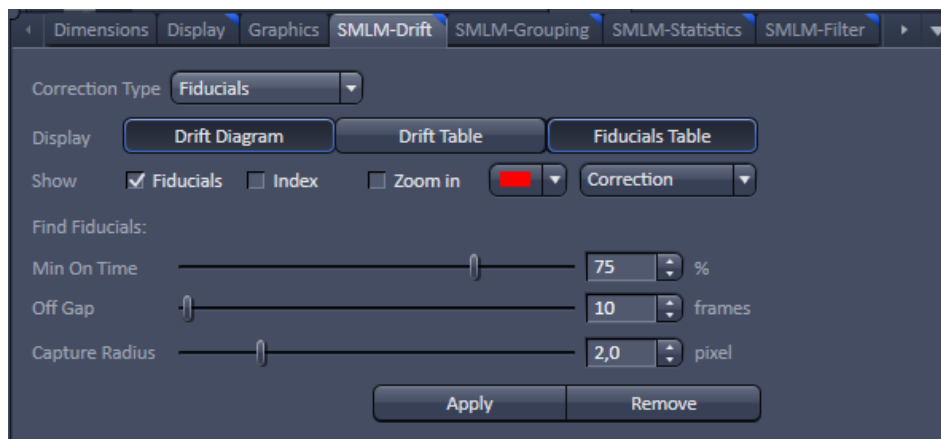
Fig. 607 SMLM-Drift view control tab – Drift correction performed with Fiducials Table and Drift table displayed and Fiducials selected

By pressing the **Fiducials Table** in the **Display** field the button will be highlighted and the fiducials table becomes visible beneath the image that lists all the identified fiducials of the image (Fig. 608).

Fig. 608 SMLM view – Fiducial table (left) and Drift table (right) displayed

By pressing the button again, the table will disappear. The table has different columns:

- **Use**: If the box is checked, the corresponding fiducial is selected and will be used for drift calculation. If the box is not checked, the fiducial is deselected and will not be used for drift correction.

- **Index**: Identifies the fiducial by a number.

- **x [μm]**: Position in x from the left upper corner of the image in μm.

- **y [μm]**: Position in y from the left upper corner of the image in μm.

☞   By pressing **Clear all** all fiducials will be deselected. If **Clear all** is pressed or one of the fiducials is deselected (**Use** box not checked) the **Select all** button becomes available. If **Select all** is pressed, all fiducials will be selected and the button becomes inactive. The button also becomes inactive, if all **Use** boxes are checked manually.

All identified fiducials (and those manually selected by **+**) are displayed as an overlay in the **HR** channel, when the **Fiducials** box in the **Show** field is checked (Fig. 609). The overlay identifies the fiducial by plotting its localizations by crosses and a circle around the localizations. The fiducial highlighted in the **Fiducials Table** has a circle with a thicker line.

☞   Not all positions of a fiducial are displayed in the overlay, just every 1000th position is plotted, to avoid crowding of the crosses in the image.

The color of the overlay can be selected by the **Color drop down** menu of the **Show** field. For more color images **color drop down** menus for each channel will be available. The original identified localized peaks corresponding to a fiducial will be deleted from the HR image (Fig. 610). The SWF channel will be uninfluenced.
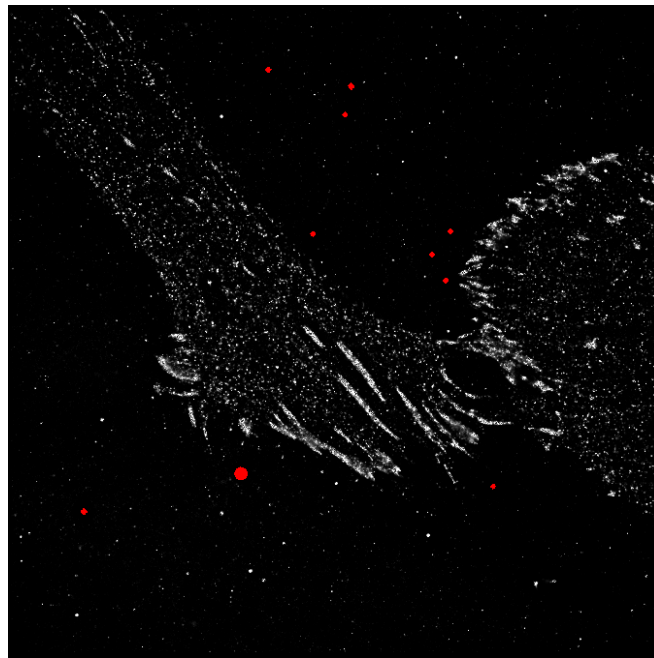
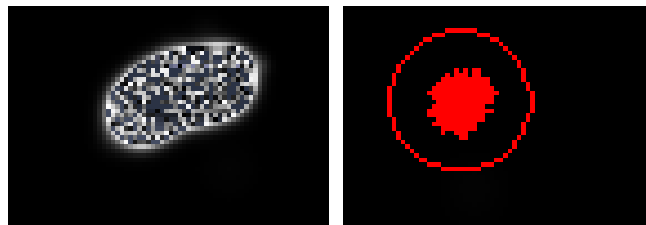**Fig. 609**   SMLM view - Fiducials displayed in the HR channel

Fig. 610   SMLM view - Zoomed fiducial before (left) and after (right) drift correction

You can zoom into a fiducial by checking the **Zoom** box in the **Show** field. The software will zoom into the fiducial that is currently highlighted in the Fiducials Table (Fig. 611).

When checking the **Index** box in the **Show** field, the number (index) of the fiducial will be displayed near the circle.

To toggle between the original drift and corrected drift of a fiducial, press **Clear All** to view original drift, then followed by **Select All** to view corrected drift wit the **Zoom** box checked (Fig. 612).

☞ Since drift correction is averaged even for one fiducial, drift correction will result in a circular cloud of positions rather than one position even if only one fiducial is used.

One can also manually select fiducials by pressing the **+** button. The buton then becomes active and when pointing with the mouse into the SMLM image, a crosshair becomes available as the mouse pointer (Fig. 611).

Move the crosshair over the position of the assumed fiducial and click into the image with the left mouse button. The fiducial at the selected position will be added to the table. To inactivate the **+** button, press it again.

☞ You do not need to have the fiducial exactly in the middle or fully circled. The software looks for the brightest peak around the crosshair.

Fig. 611 **SMLM view - Highlighted zoomed fiducial and crosshair (upper right) to manually select fiducials**
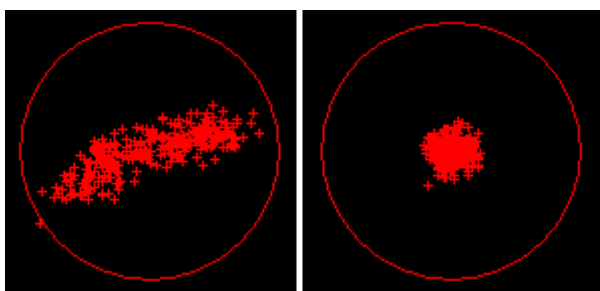
Fig. 612 **SMLM view - Zoomed fiducial with no fiducials selected (left) in the Fiducial table to display original drift and with fiducials selected (right) to view fiducial positions after drift correction**

All **-user defined-** fiducials are indicated in a yellow color and are indicated as such (Fig. 608). To remove a fiducial, highlight it in the **Fiducials Table** and press the **-** button.
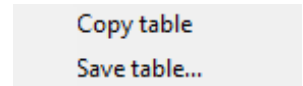
By pressing the **Drift Table** in the **Show** field the button will be highlighted and the drift table becomes visible that lists the average drifts of all selected fiducials (Fig. 608). By pressing the button again the drift table will disappear. The drift table has three columns:

– **Time**: Frame number in the time series

- **Drift x [nm]**: Drift in the x direction in nm.

- **Drift y [nm]**: Drift in the x direction in nm.

☞ If one wants to have the drift table for a specific fiducial, it should be the only one that is selected.

By pressing the right mouse button into the **Fiducials Table** or the **Drift Table**, a context menu becomes available (Fig. 613).
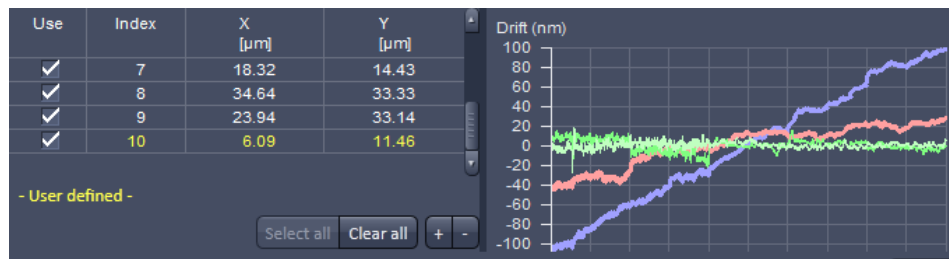
You have two options:

Copy table: data are saved to clipboard or

Save table...: data are saved as text (.txt) file.

By pressing the **Drift Diagram** in the Show field the button will be highlighted and the drift diagram becomes available (Fig. 614). By pressing the button again the drift diagram disappears.

Fig. 613   Fiducials Table and Drift Table context menu

Fig. 614   **SMLM view - Fiducials Table and Drift Diagram displayed**

☞ The displays of the **Drift Table** and **Drift Diagram** are mutually exclusive. Hence if the drift table was displayed and the **Drift Diagram** button is pressed, the drift diagram will replace the drift table and the **Drift Table** button will not be highlighted any more. Vice versa, if the drift diagram was active and the **Drift Table** button is pressed, the drift diagram will be replaced by the drift table and the **Drift Diagram** button will not be active any longer.

The drift diagram displays the drift in the x and y direction as separate graphs. You can select via the **Drift Diagram** drop down menu, how the drift and which drift is displayed (Fig. 614).

You have the following options (Fig. 615):

- **All**: Displays the average drift of all used fiducials and the drifts of all listed fiducials (with the exception of manually added fiducials) of the **Fiducial Table**.

- **Selected**: Displays the average drift of all used fiducials and the drift of the highlighted fiducial in the **Fiducial Table**.

- **Average**: Displays the average drift of all used fiduicials of the **Fiducial table**.

- **Used:** Displays the average drift of all used fiducials and the drifts of fiducials used (**Use** box checked) in the **Fiducial Table**.

- **Correction**: Displays the average drift of all used fiducials and the drift after correction of the highlighted fiducial of the **Fiducial Table**.
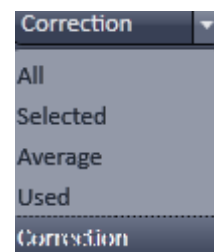
**Fig. 615**   Drift Diagram drop down menu
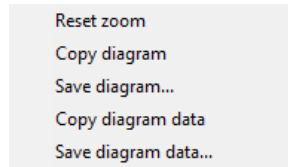
Fig. 616 **Diagram context menu**

By clicking with the right mouse button into the diagram, the Drift Diagram context menu becomes available (Fig. 616).

You can choose between the following options:

- **Reset zoom**: Will reset the zoom if the diagram was zoomed.
- **Copy diagram**: Copies the diagram into the clipboard.
- **Save diagram**: Saves diagram as a tagged image file (.tif), CompuServe GIF (.gif) or JPEG file interchange format (.jpg) file.
- **Copy diagram data**: Copies the diagram data (table) into the clipboard.
- Save **diagram data...**: Saves the diagram data (table) as a text (.txt) file.
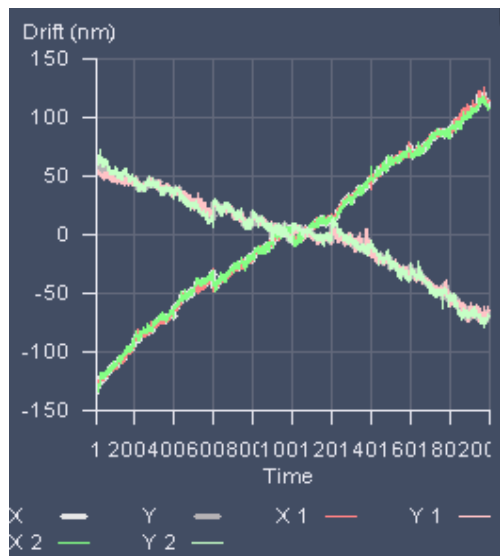
Fig. 619 **Drift Diagram – "All" selected**
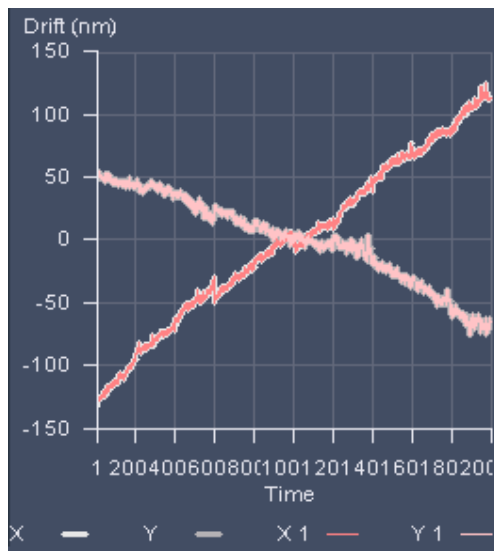
Fig. 617 **Drift Diagram – "Used" selected**
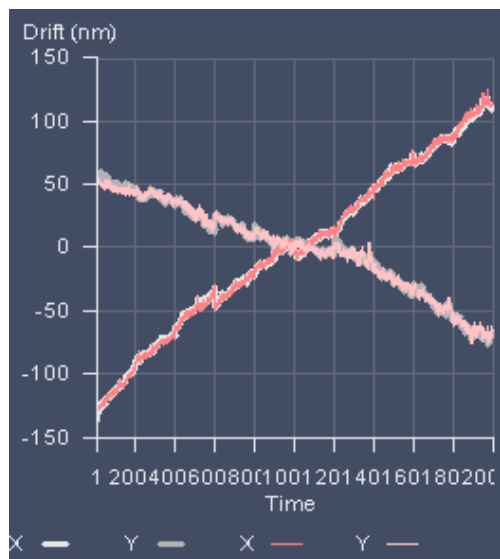
Fig. 620 **Drift Diagram – "Selected" selected**
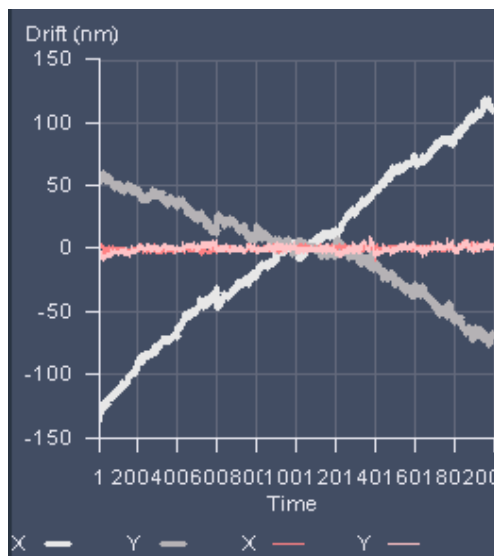
Fig. 618 **Drift Diagram – "Corrected" selected**
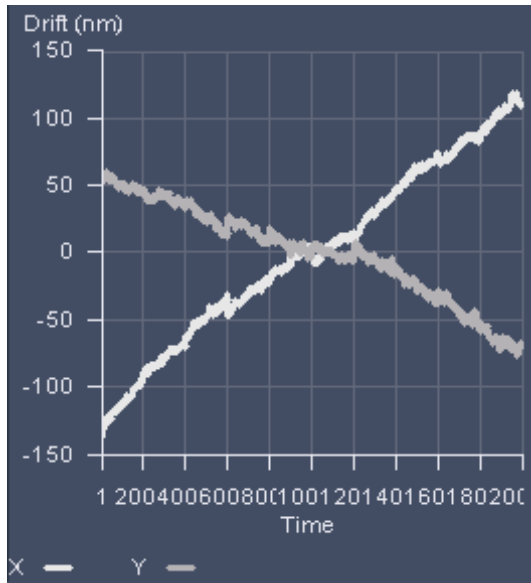
Fig. 621   Drift Diagram – "Average" selected
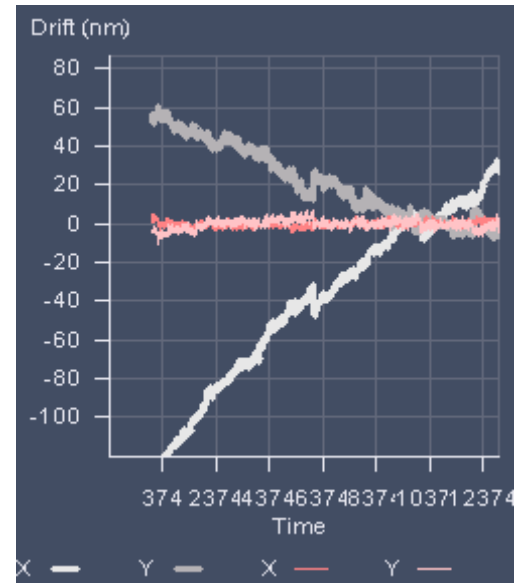
Fig. 622   Drift Diagram – "Corrected" selected and zoomed

With the left mouse button pressed you can by drawing a rectangle zoom into the defined region of the diagram (Fig. 622).

6.18.4.2   Model-based Drift Correction

When **Apply** is pressed, the software identifies on behalf of a model linear drift on sample structures and uses them to automatically correct for this drift. The correction vector calculated from the average of all structures will then be applied to the SMLM image (**HR** channels).

Once **Apply** is pressed, the **Remove** button becomes available, when a **Model-Based** drift correction has been performed (Fig. 623). Hence an inactive **Remove** button is indicative of a SMLM image without Model-Based drift correction.

By pressing **Remove** the drift correction will be undone and the button becomes inactive again. Also once **Apply** was pressed and a **Model-Based** drift correction was performed, the **Correction** button in the display field, only available in the **Show all** mode, becomes automatically active, which indicates that the image displayed is one where the drift correction has been applied.

By pressing the active **Correction** button it becomes inactive and the image will be displayed without the drift correction applied. The drift correction is hereby not undone in the molecule table, only in the displayed image. Hence, by repeatedly pressing the **Correction** button, you can switch the image display repeatedly between drift corrected and not corrected.
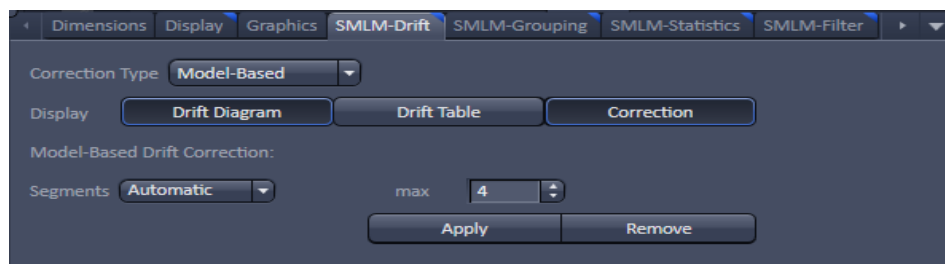

Fig. 623   **SMLM-Drift view control tab for Model-based Drift correction after a drift correction has been performed**

☞   Drift correction is not applied to the sum widefield image (**SWF** channel), so there might be an offset between the **SR** and **SWF** channels after drift correction.
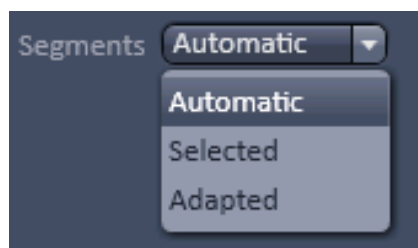

Fig. 624   **Segments drop down menu**

The **Model-Based** drift correction performs the drift correction in different segments, which can be set in the **Model-Based Drift Correction**: field in the **Segments** drop down menu and its input box (Fig. 624). The number of frames of the **SMLM** time series within each segment is determined by the total number of images divided by the number of segments rounded up or down to obtain integer numbers. Hence the last segment might contain less, equal or more frames than the preceding segments. The drift correction will be performed for each segment separately and than the drift between the segments corrected.

You can select between:

- **Automatic** (Fig. 625): The algorithm determines the optimal number of segments, but will not exceed the maximum (**max**) number as set in the **max** input box. Up to 50 segments can be selected.

- **Selected** (Fig. 626): The algorithm takes exactly the number of segments set in the input box. Up to 50 segments can be selected.

- **Adapted** (Fig. 627): This mode can (but must not) be used when the SMLM time series was interrupted by automatic z-focus drift control. Then the algorithm will take the frames between z-focus drift controls as segments. The input box is inactive and cannot be edited. It displays the number of segments of the SMLM series that will be used dependent on the number of z-drift controls; the number of segments equals number of z-drift controls plus 1. If no z-drift correction was performed, the number "1" is displayed, which than will be used as the number of segments.
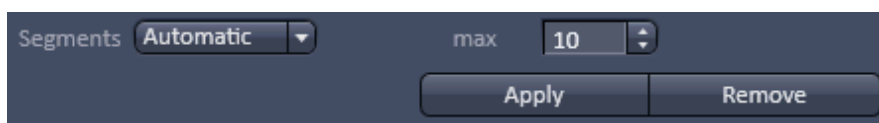
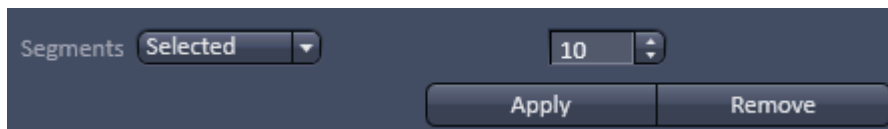**Fig. 625**   **Segment drop down menu with Automatic selected**

Fig. 626   **Segment drop down menu with Segments selected**

Fig. 627   **Segment drop down menu with adapted selected**

The **Display** field with the **Drift Diagram**, **Drift table** and **Correction** is only available in the **Show all** mode (Fig. 628).
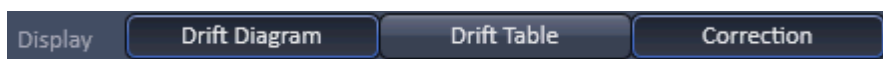

Fig. 628   **Display field**

☞ The number of optimal segments depends on the drift. If it is constant throughout the experiment, lower numbers of segments will give more robust fits. In contrast, if drift is changing for example direction during the experiment, more segments will be beneficial. If your drift curves are straight lines, you may reduce the number of segments. However, if kinks are visible, it can be beneficial to increase their numbers. If the number of kinks increases with increasing segments, you should stay with more segments, otherwise return to the number of segments from which the number of kinks does not increase any more.

By pressing the **Drift Diagram** button in the **Display** field the button will be highlighted and the drift diagram becomes available (Fig. 629). By pressing the button again the drift diagram disappears.

The drift diagram displays the drift in the x and y direction as separate graphs versus the frame number (**Time**).
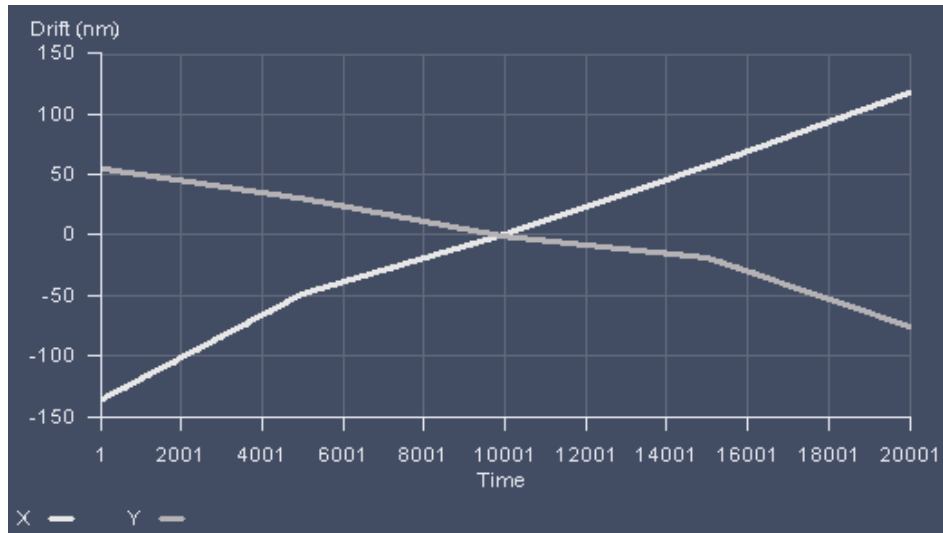
Fig. 629   **SMLM view – Drift diagram displayed for 2D SMLM data set**

For 3D SMLM data the drift in all directions x, y and z is displayed (Fig. 630).
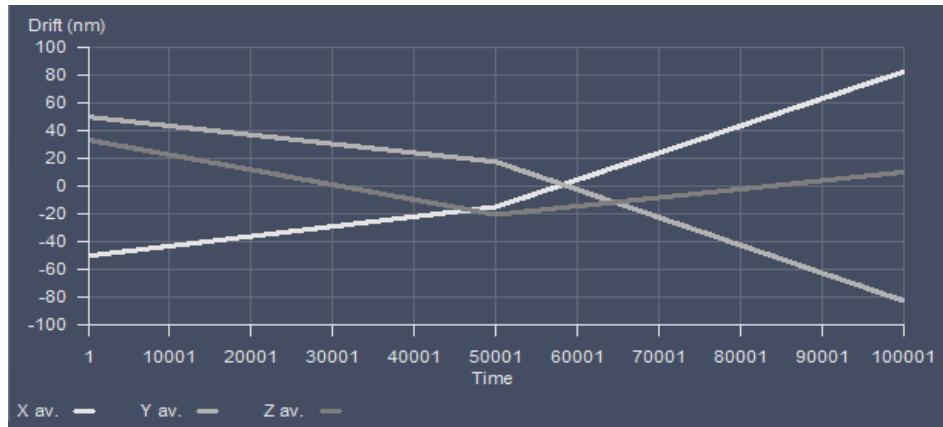
**Fig. 630**   **SMLM view – Drift diagram displayed for 3D SMLM data set**

By clicking with the right mouse button into the diagram, the Drift Diagram context menu becomes available (Fig. 631).

You can choose between the following options:

- **Reset zoom**: Will reset the zoom if the diagram was zoomed.

- **Copy diagram**: Copies the diagram into the clipboard.

- **Save diagram**: Saves diagram as a tagged image file (.tif), CompuServe GIF (.gif) or JPEG file interchange format (.jpg) file.

- **Copy diagram data**: Copies the diagram data (table) into the clipboard.

- Save **diagram data...**: Saves the diagram data (table) as a text (.txt) file.
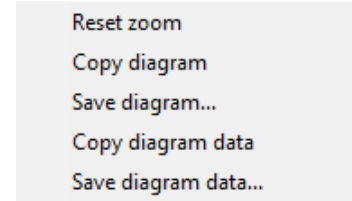
Fig. 631   **Diagram context menu**
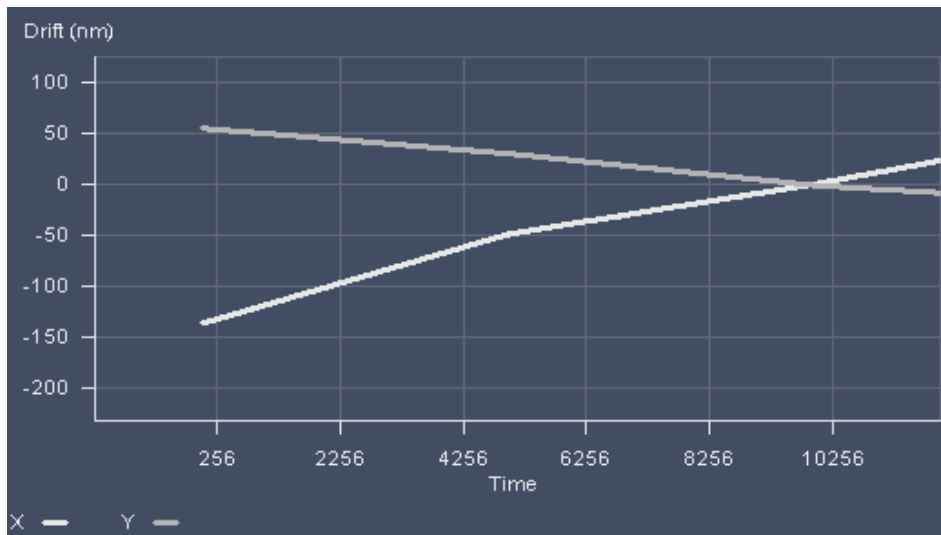
Fig. 632   **SMLM view – diagram displayed**

With the left mouse button pressed you can by drawing a rectangle zoom into the defined region of the diagram (Fig. 632). This can be reset to the original size by selecting reset Zoom from the context menu.
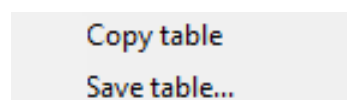
By pressing the **Drift table** button in the **Display** field the button will be highlighted and the drift table becomes visible beneath the image that lists the drift correction in nm in x (**Drift X [nm]**) and y (**Drift Y [nm]**) direction versus the frame number (**Time**) (Fig. 633).

Time	Drift X [nm]	Drift Y [nm]	Drift Z [nm]
0	-50.81	49.26	32.69
1	-50.81	49.25	32.69
2	-50.81	49.25	32.69
3	-50.81	49.25	32.69
4	-50.81	49.25	32.69
5	-50.81	49.25	32.69
6	-50.81	49.25	32.69
7	-50.81	49.25	32.68
8	-50.81	49.25	32.68
9	-50.81	49.25	32.68
10	-50.81	49.25	32.68
11	-50.81	49.25	32.68
12	-50.81	49.25	32.68
13	-50.81	49.25	32.68
14	-50.80	49.25	32.68

Fig. 633   **SMLM view – Drift table displayed for 3D SMLM data set**

By pressing the button again, the table will disappear. The table has different columns:

– **Time**: Identifies the frame by a number.

– **Drift X [nm]**: Drift correction in the x direction in nm.

– **Drift Y [nm]**: Drift correction in the y direction in nm.

– **Drift Z [nm]**: Drift correction in z direction in nm (for 3D SMLM data set only).

Copy table
Save table...

Fig. 634   **Fiducials Table and Drift Table context menu**

By pressing the right mouse button into the **Fiducials Table** or the **Drift Table**, a context menu becomes available (Fig. 634).

You have two options:

– **Copy table**: data are saved to clipboard or

– **Save table...**: data are saved as text (.txt) file.

☞ The displays of the **Drift Table** and **Drift Diagram** are mutually exclusive. Hence if the drift table was displayed and the **Drift Diagram** button is pressed, the drift diagram will replace the drift table and the **Drift Table** button will not be highlighted any more. Vice versa, if the drift diagram was active and the **Drift Table** button is pressed, the drift diagram will be replaced by the drift table and the **Drift Diagram** button will not be active any longer.
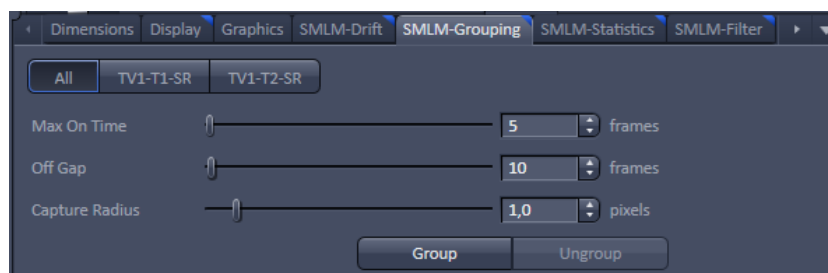
6.18.5   SMLM-Grouping View Control Tab

Fig. 635   SMLM-Grouping view control tab of the SMLM view tab

SMLM-Grouping is used to assign and group identified peaks to one object (molecule) (Fig. 635). What peaks should be considered to be derived from one molecule can be defined with the following parameters:

- **Max On Time**: Maximum number of frames that peaks within the defined capture radius are allowed to be detected in order to be considered to belong to one molecule. If peaks at the same location are found in more consecutive frames than all peaks are discarded.

- **Off gap**: Maximum number of frames peaks within the defined capture radius can be missing in order that the peaks are regarded to belong to the same molecule. If the defined number is exceeded, than the peaks will be sorted to two molecules and not grouped.

- **Capture radius:** Maximum radius in pixels (as defined by the set rendered localization precision in x,y and z) within which peaks of consecutive frames must lie in order to be regarded to belong to the same molecule.

If the **Group** button is pressed peaks are grouped to one molecule according to the set parameters. Since peaks are summed, the grouped image will have fewer objects (Fig. 636).

Since localization precision is encoded by the brightness of the color, often grouped images have brighter areas than ungrouped images.

Once grouped the **Ungroup** button becomes available. Pressing the **Ungroup** button will accordingly ungroup grouped peaks and the button will turn inactive again.

An active **Ungroup** button indicates a grouped SMLM image, an inactive one a non-grouped SMLM image.

Fig. 636   Grouped (left) and ungrouped (right) SMLM image

In a multi-channel experiment the channel selection buttons are available. Press **All** to group all channels with the same setting or press a channel button to set parameters specifically for that channel. Note, all channels will be grouped according to their actual settings.

☞   If a SMLM drift correction is performed in the **SMLM-Drift** view control tab, a grouped image will be automatically ungrouped and the **Ungroup** button will turn inactive. To re-group the **Group** button has to be pressed.

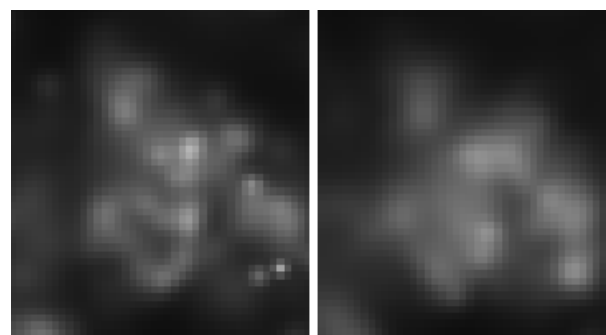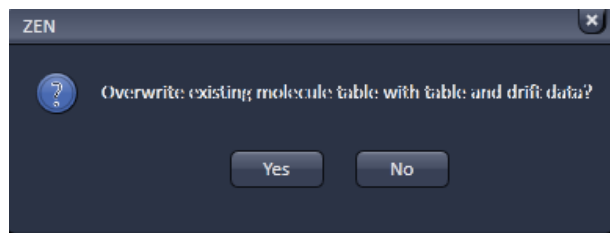☞   The table in the **SMLM-Statistics** view control tab is automatically updated.

Fig. 637   **ZEN pop-up menu when saving grouped data**

When saving the image after grouping the user is prompted by a **ZEN** pop-up menu to overwrite the existing molecule table with table and drift data (Fig. 637).

Press **Yes** to overwrite. In this case grouping and drift correction cannot be reversed or altered, if the saved image is loaded later. The controls in the **SMLM-Drift** and **SMLM-Grouping** view control tabs will be inactive.

If **No** is pressed, the molecule table and drift data are kept and grouping and drift correction are amenable to be changed.

The choice **Yes** is therefore only recommended, if no changes in drift correction and grouping are planned.

6.18.6   SMLM-Statistics View Control Tab
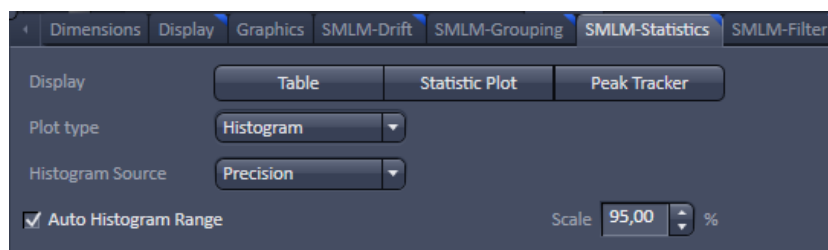
Fig. 638   **SMLM-Statistics view control tab of the SMLM view tab**

Fig. 639   **Display field with Table and Statistic Plot button active**

In **SMLM-Statistics** parameters associated with each peak or object can be viewed (Fig. 638).

If in the **Display** field the **Table** button is pressed, it becomes highlighted (Fig. 639) and the molecule table, which lists each peak (for ungrouped data, Fig. 640) or object (for grouped data, Fig. 641) will be displayed.
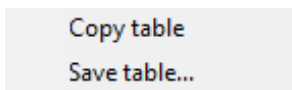
Index	First Frame	Number Frames	Frames Missing	Position X [nm]	Position Y [nm]	Position Z [nm]	Precision [nm]	Precision Z [nm]	Number Photons	Background variance	Chi square	Channel	Z Slice
451814	23158	1	0	18643.8	22659.8	2187.1	55.8	89.6	1247	47.13	0.72	1	1
451815	23158	1	0	14756.7	18598.7	2350.7	30.3	6.2	2998	60.60	1.53	1	1
451816	23158	1	0	23036.0	25638.1	2386.1	46.5	7.8	1793	25.17	0.64	1	1
451817	23159	1	0	22318.3	29404.1	1759.1	46.3	101.1	932	36.11	0.65	1	1
451818	23159	1	0	16687.3	20359.4	629.2	45.6	37.2	2482	107.77	1.41	1	1
451819	23159	1	0	22618.1	30853.4	2344.6	68.5	18.2	1148	36.11	0.65	1	1
451820	23159	1	0	15364.0	18596.7	2326.2	36.3	19.3	2384	107.77	1.41	1	1
451821	23159	1	0	29785.6	5498.7	1724.2	32.3	72.6	1309	37.79	0.59	1	1
451822	23159	1	0	22107.4	27689.8	1394.9	45.0	98.0	955	36.11	0.65	1	1
451823	23159	1	0	4371.8	1491.1	2107.4	49.8	87.6	1082	26.28	0.63	1	1
451824	23159	1	0	12865.9	29997.6	1733.5	44.5	98.9	861	21.35	0.69	1	1
451825	23159	1	0	30162.3	2949.3	1557.9	53.4	125.2	737	27.25	0.62	1	1
451826	23159	1	0	19435.3	26659.5	1512.1	93.2	203.7	443	32.91	0.63	1	1
451827	23159	1	0	15574.8	20462.7	771.6	48.6	42.9	2128	107.77	1.41	1	1
451828	23159	1	0	18534.8	22470.4	1811.9	45.1	94.8	1102	107.77	1.41	1	1

Fig. 640   **Molecule Table - appearance of ungrouped data**

Index	First Frame	Number Frames	Frames Missing	Position X [nm]	Position Y [nm]	Position Z [nm]	Precision [nm]	Precision Z [nm]	Number Photons	Background variance	Chi square	Channel	Z Slice
451814	17438	3	3	30352.4	1937.7	1535.5	22.3	64.8	2718	32.83	0.78	1	1
451815	17438	3	0	3048.0	9127.9	1436.4	28.1	73.7	2319	21.37	0.64	1	1
451816	17439	2	0	16857.4	28878.2	1251.2	24.2	55.7	2335	31.98	0.66	1	1
451817	17440	2	2	14982.3	19420.8	2495.0	27.4	19.9	5650	128.92	1.64	1	1
451818	17440	5	0	1827.5	3865.7	1604.2	23.3	46.8	5140	36.56	1.14	1	1
451819	17440	3	1	2231.0	27889.6	1762.3	24.5	47.3	3058	16.70	0.79	1	1
451820	17441	2	2	15887.0	19963.0	2367.0	34.6	43.6	4322	82.88	1.57	1	1
451821	17441	5	0	1377.8	28184.0	2146.7	12.9	44.7	9810	21.82	0.88	1	1
451822	17441	2	0	16060.2	25773.6	1329.7	25.6	70.0	2082	29.88	0.59	1	1
451823	17441	3	0	22275.9	19788.8	1311.2	20.3	46.7	4192	34.71	0.82	1	1
451824	17442	2	0	15706.5	19816.5	2354.4	32.8	40.7	4011	133.10	1.22	1	1
451825	17442	9	13	20064.9	25461.3	1379.1	19.9	64.6	8021	45.58	1.48	1	1
451826	17442	2	1	5897.5	1026.5	1972.4	36.6	103.7	1620	25.33	0.66	1	1
451827	17443	2	0	14367.4	27277.6	1964.0	25.3	72.2	2994	31.06	0.72	1	1
451828	17443	2	0	2773.2	22257.9	1871.6	18.1	51.3	2418	20.04	0.50	1	1

Fig. 641   **Molecule Table - appearance of grouped data**

By pressing the highlighted **Table** button, the molecule table will disappear and the button will not be highlighted any more. A vertical scroll bar allows access to each line of the table. A horizontal scroll bar becomes available, if the space for the table becomes too small to display all columns.

The molecule table has the following columns:

- **Index**: Assigns a number to each peak / object.

- **First Frame**: Number of frame in which the peak/object appears for the first time.

- **Number Frames**: Number of frames, the peak / object was consecutively detected. For ungrouped data this will always be 1.

- **Frames Missing**: Number of frames a peak / object was missing. For ungrouped data this will always be 0.

- **Position X [µm]**: Position in µm of a peak / object in the X direction with the origin at the upper left corner of the image.

- **Position Y [µm]**: Position in µm of a peak / object in the Y direction with the origin at the upper left corner of the image.

- **Position Z [µm]**: Position in µm of a peak / object in the Z direction with the origin at the lowest z plane (for 3D SMLM data sets only)

- **Precision [nm]**: Calculated localization precision of a peak / object in nm in x, y.

- **Precision z [nm]**: Calculated localization precision of a peak / object in nm in z.

- **Number Photons**: Total number of photons of a peak / object.

- **Background variance**: Variance of the number of background photons.

- **Chi square**: X^2 value of the Gauss fit for the localization precision.

- **PSF width [nm]**: Width of the point spread function (PSF) calculated from the Gauss fit in x,y (displayed for 2D data sets only).

- **Channel**: Designation of the channel by a number.

- **Z Slice**: Indicates the slice of a multi-stack experiment (3D SMLM data sets only).

Fig. 642   Table context menu

Fig. 643   Auto Plot Type drop down menu

By pressing the right mouse button into the molecule **Table**, a context menu becomes available (Fig. 642).

You have two options:

- **Copy table**: data are saved to clipboard or

- **Save table...**: data are saved as text (.txt) file.

If in the **Display** field the **Statistic Plot** button is pressed, it becomes highlighted (Fig. 639) and a plot will be shown. The type of the plot can be selected in the **Plot Type** drop down menu (Fig. 643).

You have the choice between

- **Histogram**: Plot of parameter values versus their frequencies.

- **Scatter Plot**: Plot of parameter values against other parameter values.

If **Histogram** is selected, the histogram specific functions will be displayed (Fig. 644).
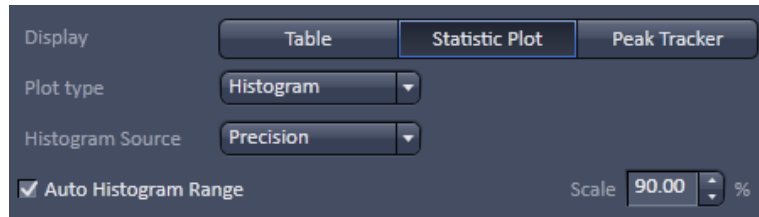
Fig. 644   Histogram view - Precision selected, Auto Histogram Range active and Scale set to 90%

With the **Histogram Source** drop down menu, you can select the parameter that should be plotted in the histogram (Fig. 645).
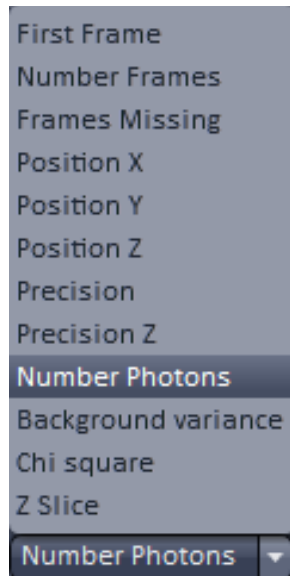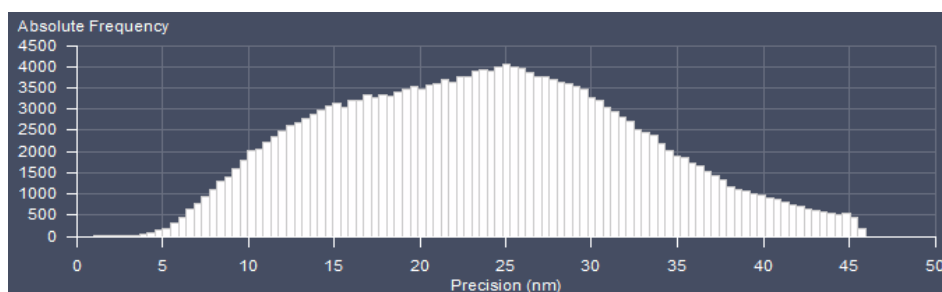

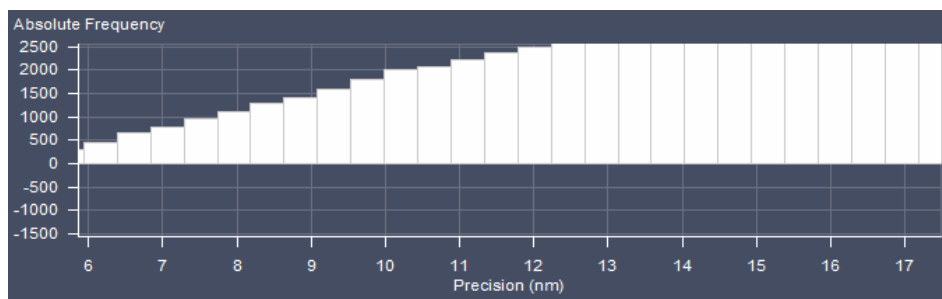**Fig. 645   Histogram Source drop down menu**

All parameters listed in the molecule **Table** are available. The selected parameter will be plotted versus its frequency (Fig. 646).
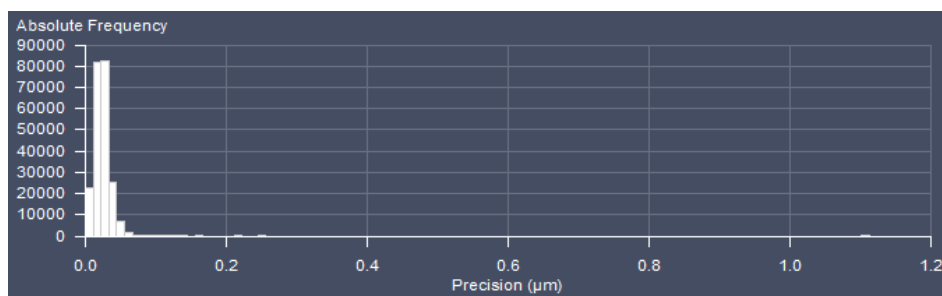
Fig. 646   **Statistic Plot - histogram of localization precision; no Auto Histogram Range**

Fig. 647   **Statistic Plot - histogram of localization precision; Auto Histogram range set to 90%**

Fig. 648   **Statistic Plot - zoomed histogram of localization precision; Auto Histogram range set to 90%**

By activating the **Auto Histogram Range** checkbox and input of a **Scale [%]** number (only available in the **Show all** mode) the Quantil level will be set for the data. There, a histogram will be rescaled (Fig. 647) in a way that only those x-values are displayed that accommodate the input% of the data starting from 0. Hence outliers with high values can be removed from the display.

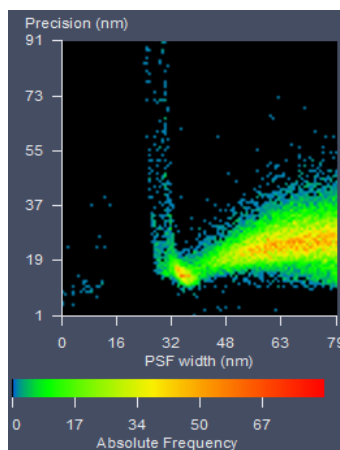You can zoom in an area by spanning a region with the pressed left mouse button (Fig. 648).

By clicking with the right mouse button into the diagram, the **Histogram** context menu becomes available (Fig. 649).

You can choose between the following options:

- **Reset zoom**: Will reset the zoom if the diagram was zoomed.

- **Copy diagram**: Copies the diagram into the clipboard.

- **Save diagram**: Saves diagram as a tagged image file (.tif), CompuServe GIF (.gif) or JPEG file interchange format (.jpg) file.

- **Copy diagram data**: Copies the diagram data (table) into the clipboard.

- Save **diagram data...**: Saves the diagram data (table) as a text (.txt) file.
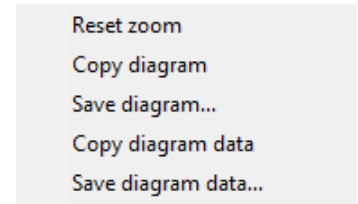
Fig. 649   Histogram context menu
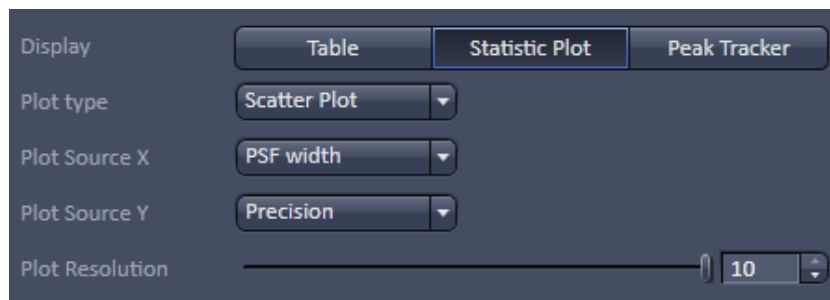
Fig. 650   Histogram view - PSF width and Precision selected, Plot resolution set to 10

If **Scatter Plot** is selected, the scatter plot specific functions will be displayed (Fig. 650).

With the **Plot Source X** and **Plot Source Y** drop down menus (Fig. 651) you can select the parameters that will be plotted against each other (Fig. 652).
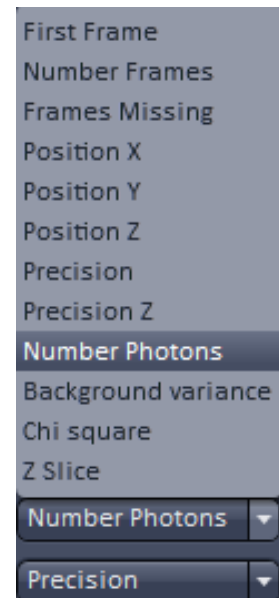
Fig. 651   Plot X and Y drop down menu
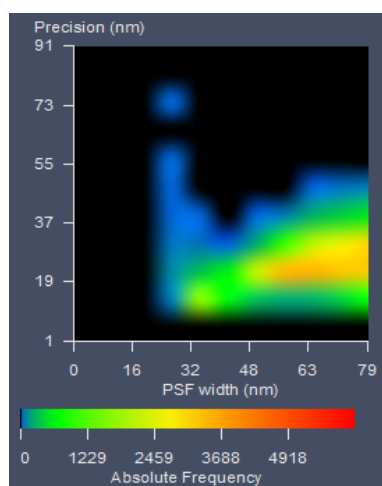
Fig. 652   Statistic Plot – scatter plot of PSF width versus localization precision; pixelresolution set to 1

The **Absolute Frequency** is color coded and represented by a scale bar beneath the diagram. You can also plot one parameter against itself. In this case, you will obtain a diagonal line. All parameters listed in the molecule **Table** are available.

Using the **Pixel Resolution** slider or its input spin box, the pixel resolution can be adjusted from 1 to 10, with 10 being the highest resolution (Fig. 653).

You can zoom in an area by spanning a region with the pressed left mouse button (Fig. 654).

By clicking with the right mouse button into the diagram, the **Histogram** context menu becomes available (Fig. 655).

Fig. 653   **Statistic Plot – scatter plot of PSF width versus localization precision; pixel resolution set to 10**

Fig. 655   **Scatter Plot context menu**
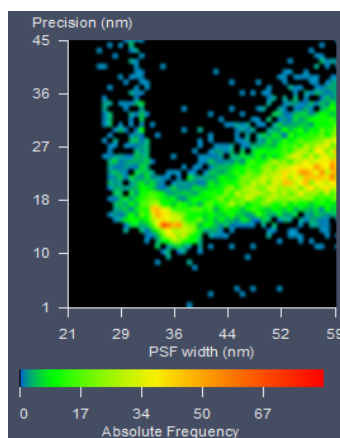
Fig. 654   **Statistic Plot – zoomed scatter plot of PSF width versus localization precision; pixel resolution set to 10**

You can choose between the following options:

- **Reset zoom**: Will reset the zoom if the diagram was zoomed.

- **Copy diagram**: Copies the diagram into the clipboard.

- **Save diagram**: Saves diagram as a tagged image file (.tif), CompuServe GIF (.gif) or JPEG file interchange format (.jpg) file.

- **Copy diagram data**: Copies the diagram data (table) into the clipboard.

- **Save diagram data...**: Saves the diagram data (table) as a text (.txt) file.

If **Peak Tracker** is selected, the peak tracking window will be displayed (Fig. 656).

Parameter	TV 1
Position X (µm)	14.65
Position Y (µm)	15.55
Number Photons	0.00
Precision [nm]	0.00
Chi square	0.00
PSF width	88.95
Background variance	0.00
molecules in current frame	0
molecules in all frames	0

Fig. 656   Peak tracking window

If peak tracking is active, the mouse cursor changes to a crosshair within the **Source Image** that allows one to select a spot by clicking with the left mouse button (Fig. 657), for example a fiducial, which will be displayed in a 11 x 11 pixel window. The selected spot is indicated by a cross. During **Online Processing** this would allow to constantly monitor the fiducial for axial and lateral drift.

The table below the peak tracking image has two or more columns. The first column is the **Parameter** list, the next columns are the values of each parameter for each channel (TV-1, TV-2 etc.). During **Online Processing** these values are frame wise updated.

Fig. 657   Source image (zoomed) with crosshair (right) and cross indicating selected spot (left)

The following parameter values are displayed:

- **Position X [µm]**: Position in µm of the selected spot in the X direction with the origin at the upper left corner of the image.

- **Position Y [µm]**: Position in µm of the selected spot in the Y direction with the origin at the upper left corner of the image.

- **Number Photons**: Total number of photons of a peak / object.

- **Precision [nm]**: Calculated localization precision of a peak / object in nm.

- **Chi square**: X^2 value of the Gauss fit for the localization precision.

- **PSF width [nm]**: Width of the point spread function (PSF) calculated from the Gauss fit.

- **Background variance**: Variance of the number of background photons.

Below the table are also listed the following two values:

- **molecules in current frame**: peak counts per frame
- **molecules in all frames**: accumulated peak counts to the present frame

These two values are, however, updated only every 1000th frame.

☞ The displays of the **Table** and **Peak Tracker** are mutually exclusive. Hence if the molecule table was displayed and the **Peak Tracker** button is pressed, the peak tracker will replace the molecule table and the **Table** button will not be highlighted any more. Vice versa, if the peak tracking was active and the **Table** button is pressed, the peak tracker will be replaced by the molecule table and the **Peak Tracker** button will not be active any longer.

6.18.7   SMLM-Filter View Control Tab

In the **SMLM-Filter** view control tab, parameters can be filtered in regard to their values (Fig. 658).
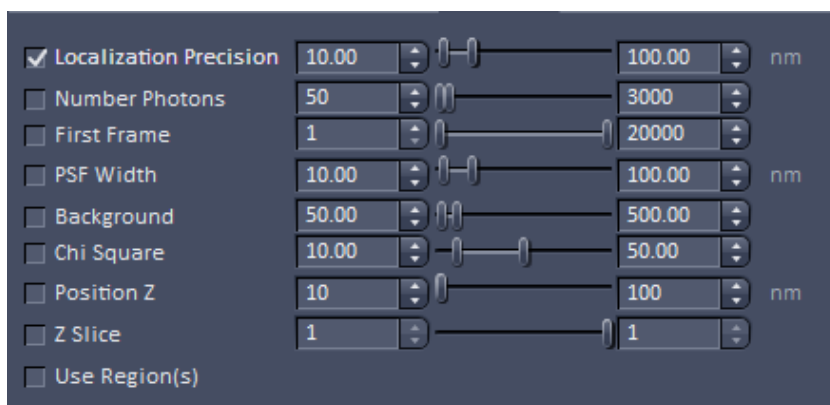
Fig. 658   SMLM-Filter view control tab of the SMLM view tab (displayed for 2D SMLM data)

It depends on the dimensionality (2D or 3D SMLM data sets), which parameters get accessible (Fig. 659).
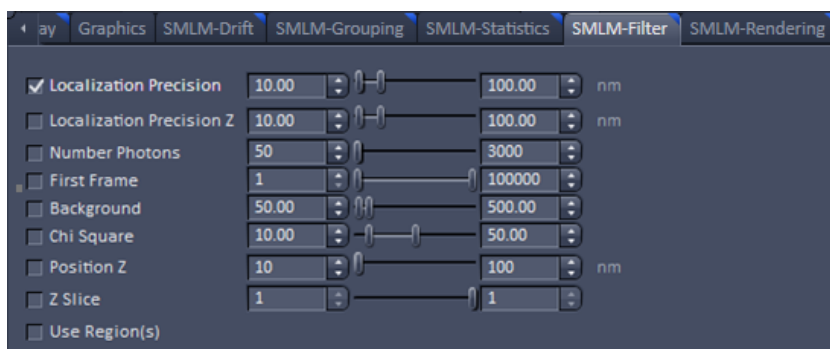
Fig. 659   SMLM-Filter view control tab of the SMLM view tab (displayed for 3D SMLM data)

Minimum (min) and maximum (max) values for each parameter can be entered via the corresponding left-hand and right-hand sliders or the respective input spin boxes. The following parameters are available for filtering:

- **Localization Precision [nm]**: Calculated localization precision of a peak / object in nm (in X, Y). min=1, max = 500. Note that a max.imum value of 1000 is hard coded in the software, so no molecules having a localization precision with > 1000 nm wil be displayed, even if the filter is inactive.

- **Localization Precision Z [nm]**: Calculated localization precision of peak / object in Z. min = 1, max = 500 (available only for 3D data sets)

- **Number Photons**: Total number of photons of a peak / object. min = 1, max = 10000

- **First Frame**: Number of frame in which the peak/object appears for the first time. min = 1, max = number of frames of the time series

- **PSF width [nm]**: Width of the point spread function (PSF) calculated from the Gauss fit (only available for 2D SMLM data sets). min = 1, max = 500

- **Background**: Variance of the number of background photons. min = 1, max = 5000; only available in **Show all** mode.

- **Chi square**: X^2 value of the Gauss fit for the localization precision. min = 0, max = 100; only available in **Show all** mode.

- **Position Z [nm]**: Position in nm of a peak / object in the Z direction with the origin at the lowest z plane (for 3D SMLM data sets only); only available in **Show all** mode. min = 0, max = defined number of slices from the SMLM-Render menu.

- **Z slice**: Indicates the slice of a multi-stack experiment (3D SMLM data sets only); only available in **Show all** mode. Min = 1, max = the defined number of slices.

- **Region**: Region(s) of interest (ROIs) drawn in the **Graphics** view control tab and defining the min. and max. x-positions as well as min. and max. y-positions in μm. Note that all active ROIs will be taken into account if the **Region** box is checked. **Region** is only available in **Show all** mode. If ROIs are selected, only that part of the image will be displayed and all calculations will be restricted to that part (Fig. 660).
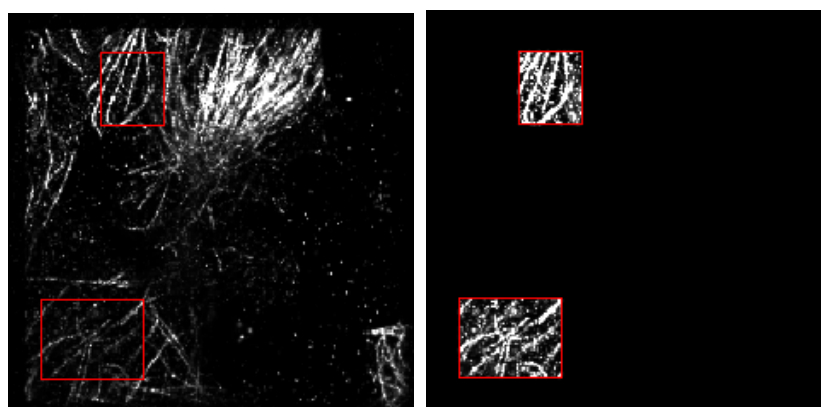
Fig. 660   **Image display with two ROIs selected and Use Region(s) box not checked (left panel) and checked (right panel)**

In order that a filter is applied to the data set, the corresponding box has to be checked. Checking the box leads immediately to a re-calculation of the data set and all tables, plots and SMLM images (**HR** channels) will be updated automatically (Fig. 661).
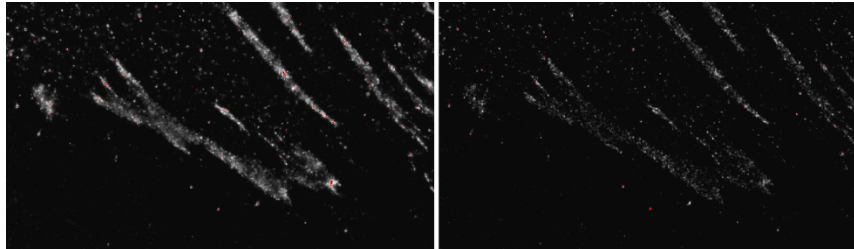

Fig. 661   **HR channel with no localization precision filter active (left) and with filter set to min = 10 nm and max = 20 nm (right)**

☞   All **SMLM-Filter** settings will be applied to images acquired during **Online Processing**.

☞   All **SMLM-Filter** settings will be independently applied for each slice of the **Z Slice**.

☞   Please be aware that the last filter settings during one imaging session will be kept for the next image (acquired or loaded from daved data). If these do not fit, an empty image could be the case. When experiencing images with too few or no localizations You are advised to check the filter settings first.

6.18.8   SMLM-Render View Control Tab

6.18.8.1   Rendering Data Sets

In the **SMLM-Rendering** view control tab, the display of the data can be set (Fig. 662).
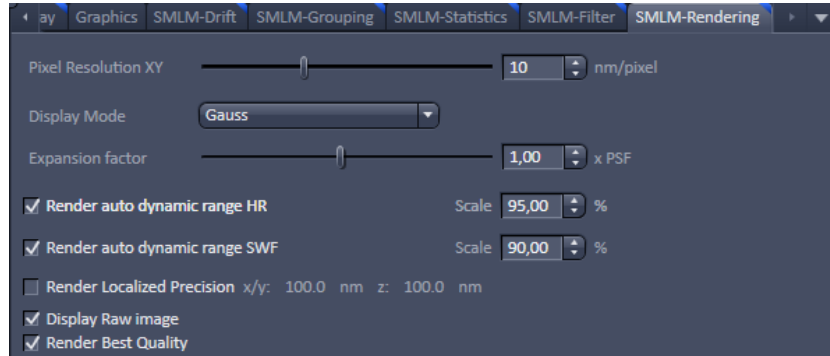
Fig. 662   SMLM-Rendering view control tab of the SMLM view tab
(displayed for 2D SMLM data)

Different settings are available for 2D SMLM (Fig. 662) or 3D SMLM (Fig. 663) data sets.
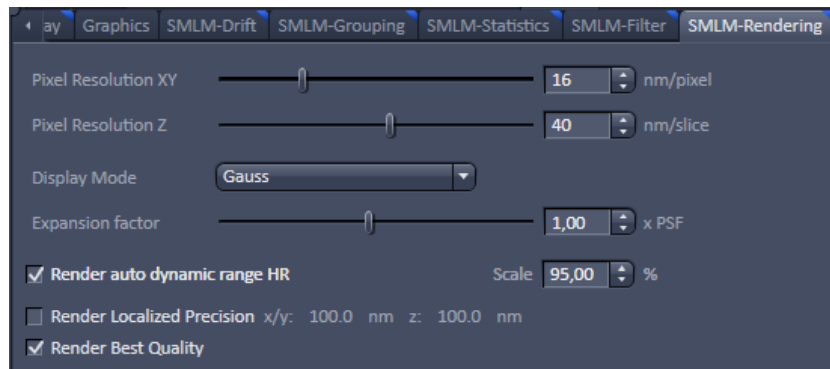
Fig. 663   SMLM-Rendering view control tab of the SMLM view tab
(displayed for 3D SMLM data)

With the **Pixel Resolution XY [nm/pixel]** slider (only available in **Show all** mode for all **Display Modes**) or its input spin box the size of the pixel in nm of the **HR** image can be set.

With the **Pixel Resolution Z [nm/pixel]** slider (only available in **Show all** mode for all **Display Mode**s and for **3D SMLM** data sets) or its input spin box the size of the voxel in z in nm of the **HR** image can be set.

☞ Note that according to Nyquist, the pixel or voxel size must be at least half of the displayed localization precision (and resolution) in order to obtain the displayed localization precision.
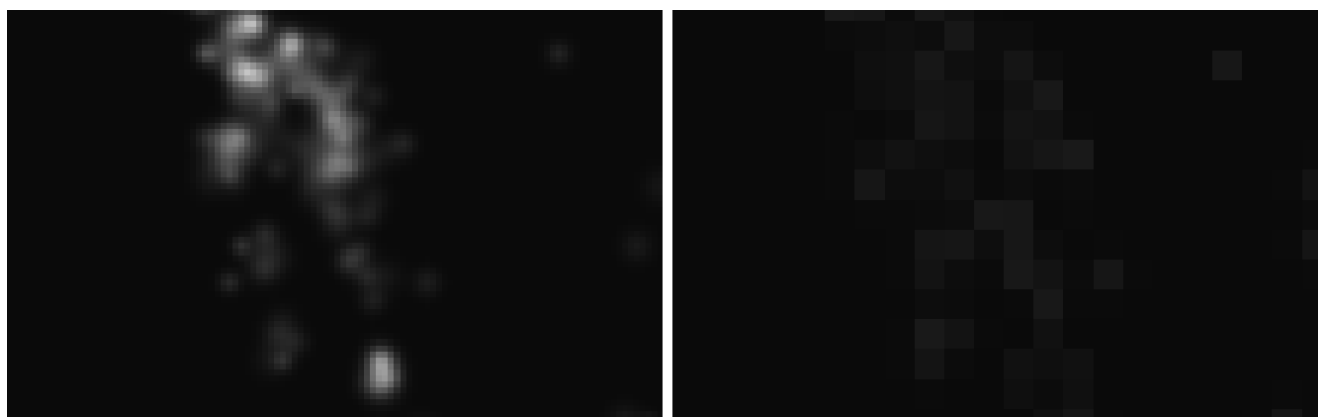
Fig. 664   HR channel with pixel resolution = 10 nm (left) and with pixel resolution = 50 nm (right)

☞ Localization precision (LP) is not identical to resolution (R), which also depends on the molecular density (MD). In order to obtain a resolution of 10 nm, the localization precision has to be at least 10 nm as well, but the molecules must be spaced not more than 10/2 nm = 5 nm apart from each other. In that sense the localization precision sets the maximum resolution possible. Hence if MD ≤ 1/2 x LP, than R=LP and if MD ≥ 1/2 x LP, than R = 2 x MD. These criteria apply to one, two and three dimensions.

If the **Display Raw Image** box (only available in **Show all** mode for all **Display Modes**) is checked, the **Source Image** will be displayed along the **HR** (SMLM) and/or **SWF** images (Fig. 665). If not checked, the raw image field is not displayed.
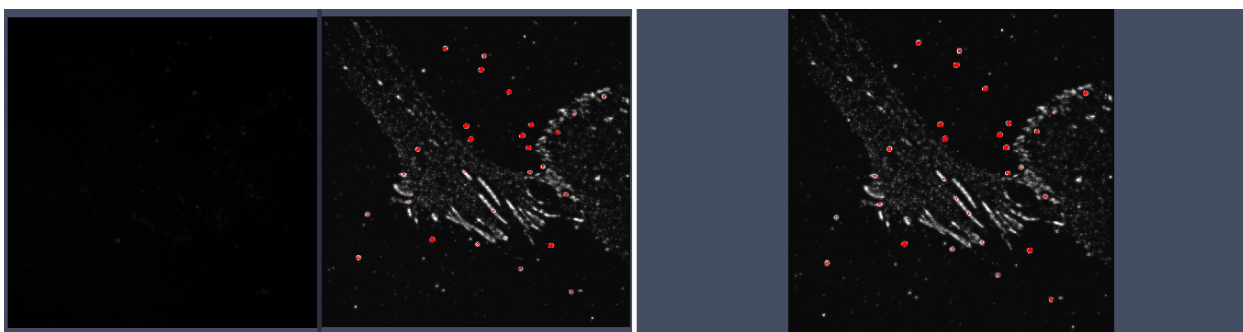
Fig. 665   **HR Image display with Show Raw Image check box active (left) and inactive (right) The raw or source image field is to the left, the HR and SWF image field to the right in case the raw image is displayed**

With the **Display Mode** drop down menu, you can select in which mode the **HR** channel should be displayed (Fig. 667).

You have 6 options (Fig. 666):

– **Gauss**: Localized peaks / objects are displayed with the localization error and rendered to a Gauss function. The higher the precision, the smaller the width of and the brighter the peak / object. Note that areas with higher molecule densities are also brighter.

– **Centroid**: Localized peaks / objects will be displayed by a dot at the localization point.

– **Gauss + Centroid**: Overlay of Gauss rendered peaks / objects and centroids.

– **Cross**: Localized peaks / objects will be displayed by a cross at the localization point.

– **Gauss + Cross**: Overlay of Gauss rendered peaks / objects and crosses.

– **Molecule Density**: Number of peaks / objects per area (in μm).

☞ Centroids are better visualized at lower zooms, whereas crosses are better observed at higher zooms.
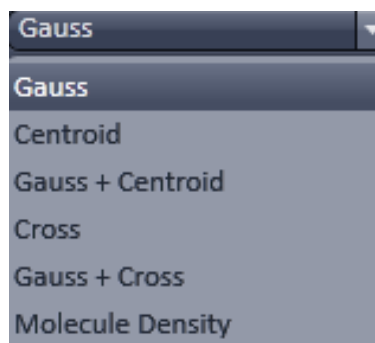
Fig. 666   **Display Mode drop down menu**
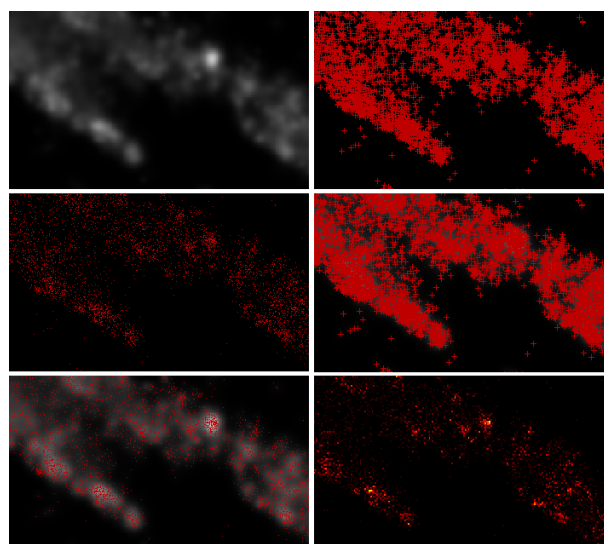
Fig. 667   **HR channel display modes - from top to bottom and left to right: Gauss, Centroids, Gauss + Centroids, Crosses, Gauss + Crosses, Molecule Density**

Associated with the Molecular Density map is a Scale bar that shows the color code of **Molecule Density in Molecules / µm²** according to the **HR** channel assigned look up table (LUT) (Fig. 668).

☞ Please note that molecular density applies for a plane only. Hence, for a 3D stack, a map for each slice is available. There is no density mapping in z-direction.

The available functionalities will alter with the **Display Mode** chosen (Fig. 669, Fig. 670, Fig. 671 and Fig. 673). Only the **Pixel Resolution [nm/pixel]** slider and the **Display Raw Image** are available for each **Display Mode**.

For all **Display Modes** displaying a centroid or cross (**Centroid**, **Gauss + Centroid**, **Cross**, **Gauss + Cross**) the **Display Mode Color** drop down menu is available (Fig. 670, Fig. 671), from which the color of the overlay (centroid or cross) can be selected (Fig. 672).

Fig. 672   Display Mode Color drop down menu

For all **Display Modes** displaying a Gauss rendered peak / object (**Gauss**, **Gauss + Centroid**, **Gauss + Cross**) the **Expansion factor [x PSF]** slider is available (Fig. 669, Fig. 671). The number set with the slider or by using the input spin boxes will be multiplied with the PSF (localization precision error), so the rendered peaks / objects become smaller for numbers < 1 and bigger for numbers > 1.
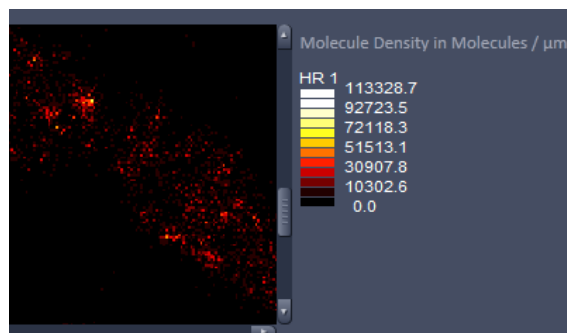
Fig. 668   Display Mode for Molecular density with part of the map (left) and scale bar (right)
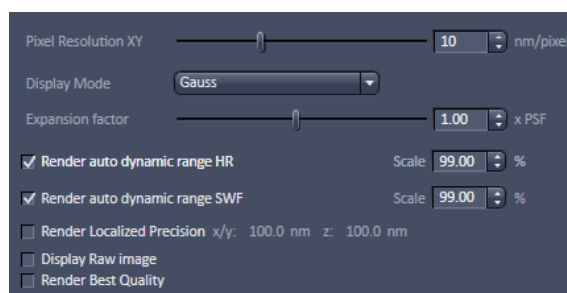
Fig. 669   Appearance of the SMLM-Render view control tab with Display Mode Gauss selected (for 2D SMLM data set)
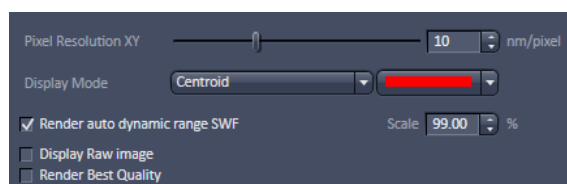
Fig. 670   Appearance of the SMLM-Render view control tab with Display Mode Centroid (or Cross) selected (for 2D SMLM data set)
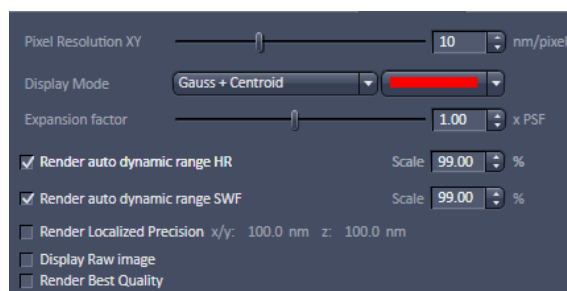
Fig. 671   Appearance of the SMLM-Render view control tab with Display Mode Gauss+Centroid (or Gauss + Cross) selected (for 2D SMLM data set)

☞ The **Expansion factor** slider can be used to correct data obtained with different fitting routines. For example, a full 2D Gauss fit applied to one lateral axis taken into account the other lateral axis will result in a factor of root 2 larger localization precisions than a Gauss fit that is independently performed on both lateral directions. By just multiplying a full 2D Gauss fit rendered data set by 1/$\sqrt{2}$ ≈ 0.71 one will obtain the localization precision one would have obtained by using a simple 2D Gauss (Fig. 674).
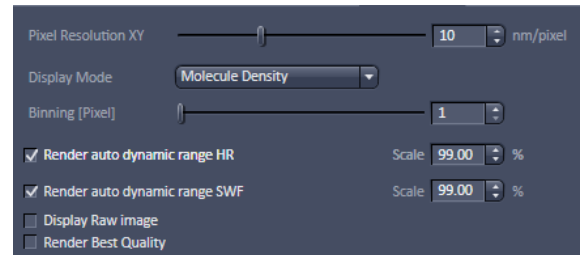
Fig. 673   **Appearance of the SMLM-Render view control tab with Display Mode Molecule Density selected (for 2D SMLM data set)**
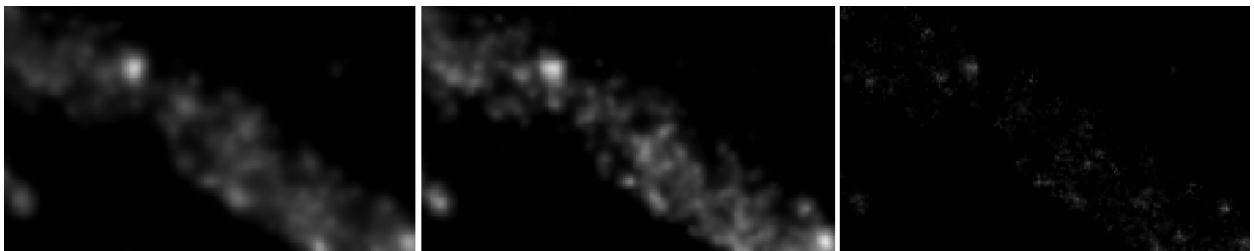
Fig. 674   **HR channel images with Expansion factor slider set 1 (left), 0.7 (middle) and 0.3 (right)**

☞ Using the **Expansion factor** slider just adapts the display but does not alter the table. A warning is given if the slider value drops below 0.71 as the data will be rendered more accurately than common fitting routines would allow (Fig. 675).

☞ The **Expansion factor** slider adapts the display in x, y and z.

☞ The Expansion factor and Render Localization Precision are mutually exclusive. If the Render Localization Precision box is checked, the Expansion factor is no longer available.

Fig. 675   **Expansion factor slider set 0.3 – red display of all values below 0.71**

For all **Display Modes** displaying a rendered peak / object and for density maps (**Gauss**, **Gauss + Centroid**, **Gauss + Cross**, **Molecule Density**) the **Render autodynamic range HR [%]** and **Render autodynamic range SWF [%]** sliders are available (only visible in **Show all** mode), which allow to set the display range for the HR and SWF channels individually.

The value indicates the Quantil, up to which percentage of data (stating with the lower values) will be displayed. The input number therefore sets the threshold for the maximum range, so the image will be fully scaled from 0% to input number%. Higher values out of the input% range would be displayed as saturated pixels (Fig. 676).

Note that the **Render autodynamic range HR [%]** is also vailable for molecule density maps (**Molecule Density**). You can input a range value by using the slider or the input spin boxes. In order that the range is applied to the **HR** or **SWF** channel, the corresponding slider box has to be checked.

☞ **Render automatic range** will be helpful, if the image contains very bright spots like fiducials. The range would be accordingly scaled from 0 to 100% and dimmer areas would not be visible. If the slider is set for example to 90% than the image would fully scale between 0% to 90% putting the higher values into saturation pixels. In this way dimmer regions will become brighter.
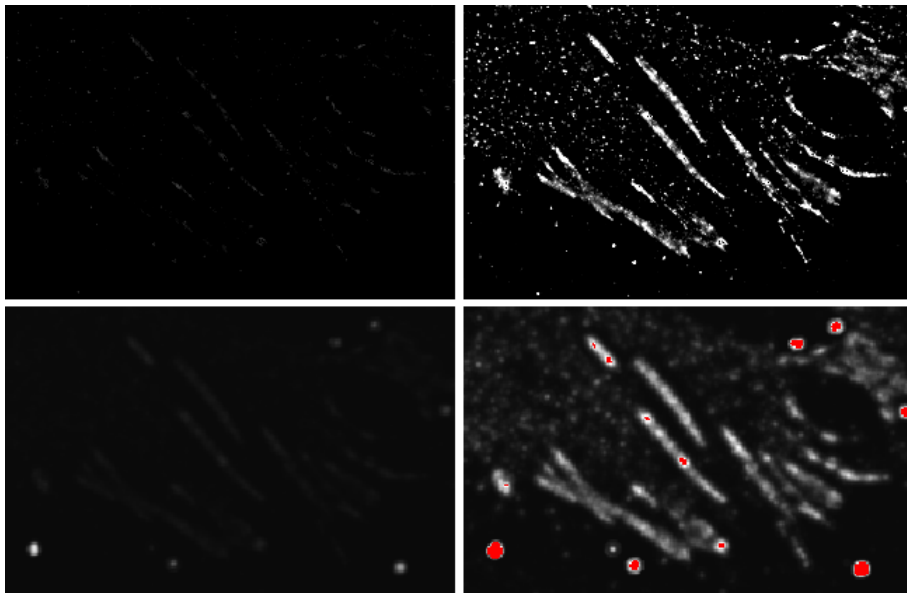
Fig. 676   **HR channels (upper panel) and SWF channels (lower panel) without (left images) and with (right images) autodynamic range applied (99.99% for HR; 99.00% for SWF).**

☞ Note that in case of the HR and SWF images the dimmer parts get brighter if autodynamic range is applied. In case of the SWF image the brightest spots will get into saturation.

For all **Display Modes** displaying a density map (**Molecule Density**) the **Binning [Pixel]** slider is available that allows to bin pixels in the molecule density map. You can set the bin value with the slider or using the input spin boxes. The map will be immediately rendered to the new bin settings (Fig. 677).
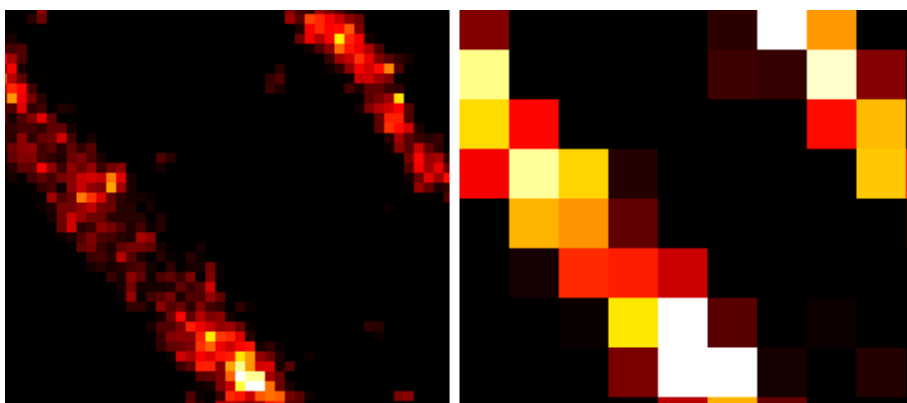
Fig. 677   **Molecule density map with Binning [Pixel] set to 1 (left) or 5 (right)**

☞ All **SMLM-Render** settings will be applied to images acquired during **Online Processing**.

If the **Render Localized Precision** box is checked (Fig. 678), all events will be rendered laterally and axially to the size indicated in the **x/y**: … nm and **z: … nm** display fields, respectively. The numbers in the **x/y: … nm** and **z: … nm** display fields correspond to the upper limits set in the **Localization Precision** and **Localization Precision Z** filters, repectively (Fig. 679), regardless if the corresponding filter box is checked for filtering or not.
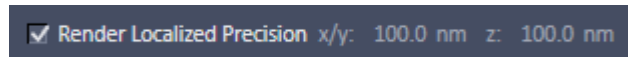
Fig. 678   **Render Localized Precision check box**

Fig. 679   **Localization Precision and Localization Precision Z filters with both upper limits set to 100**

☞ Note that the number displayed in **z: … nm** is irrelevant for 2D data sets.

☞ Note that the rendering according to the set values is arbitrary and does not correspond to true precision values as approximated by the fit. This function is therfore only a non-quantitative display option. For example, if 3D SMLM data have been processed with an **Experimental PSF** without attached Localization Precision Look Up Table (LUT), events will be displayed as dots, which can be vizualized larger using the **Render Localization Precision** tool.

☞ Note that if **Render Localized Precision** box is checked, the displayed **x/y:** and **z:** values will also be taken as pixel sizes for **Grouping**.

If the **Render Best Quality** box is checked (Fig. 680), data are renderd sub-pixel precise. That is, if the peak of the event is localized off center in a pixel, the peak of the Gaus used to render the event will be right on the localized position. If the **Render Best Quality** box is not checked, data are rendered pixel precise.That is, if the peak of the event is localized off center in a pixel, the peak of the Gaus used to render the event will nevertheless be in the center position of the pixel the vent was localized in.
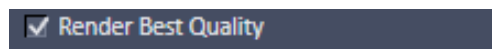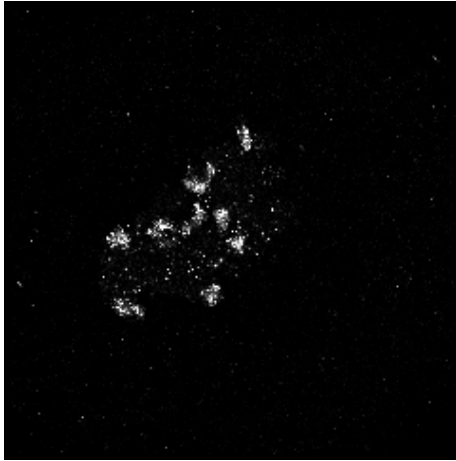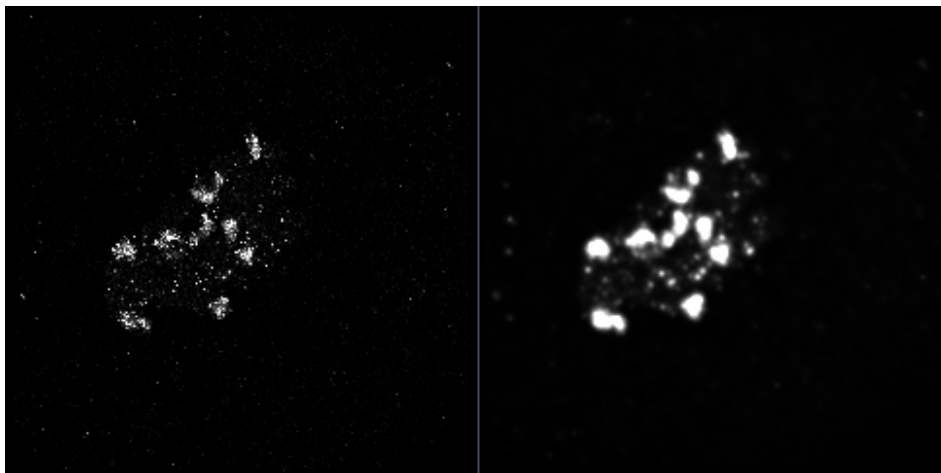
Fig. 680   **Render Best Quality check box**

6.18.8.2 Visualizing Data Sets

2D SMLM experiments can be displayed an visualized in the **2D** view tab (Fig. 681).

In the **Split** view tab both the HR and the SMW image (if both selected) can be viewd sied by side as two channels (Fig. 682).

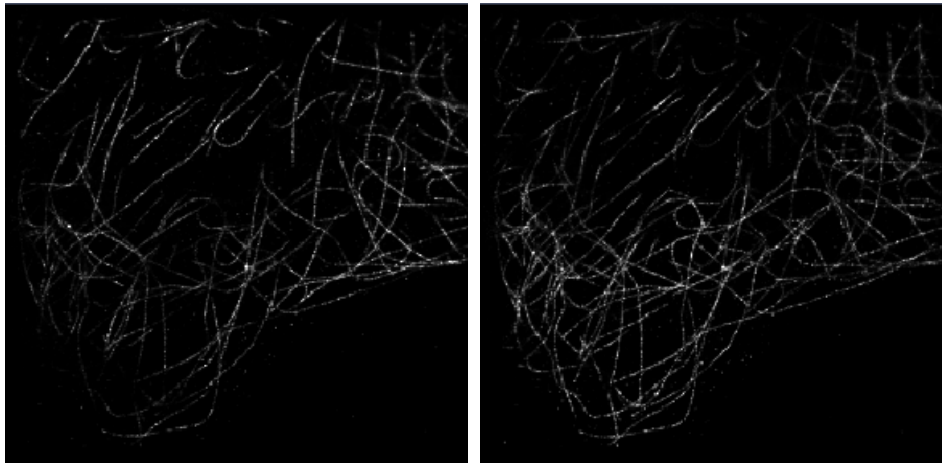**Fig. 681** **2D SMLM data set viewed in the 2D view tab with only HR channel active**

Fig. 682 **2D SMLM data set viewed in the Split view tab with both HR and SMW image active**

For 3D SMLM data you have several options to explore the axial dimension:

– 2D view with z position slider

– Color-coded map

– Ortho View

– 3D view with or without color cosing

In the **2D** view tab you can scroll the z slices with the Dimension **Z-Position** slider (Fig. 683).

Fig. 683   3D SMLM data set viewed in the 2D view tab at two different z positions

The z position can also be dsiplayed as a color map, were color hue codes for z position using the **Processing** function **Color-coded projection** (Fig. 684).
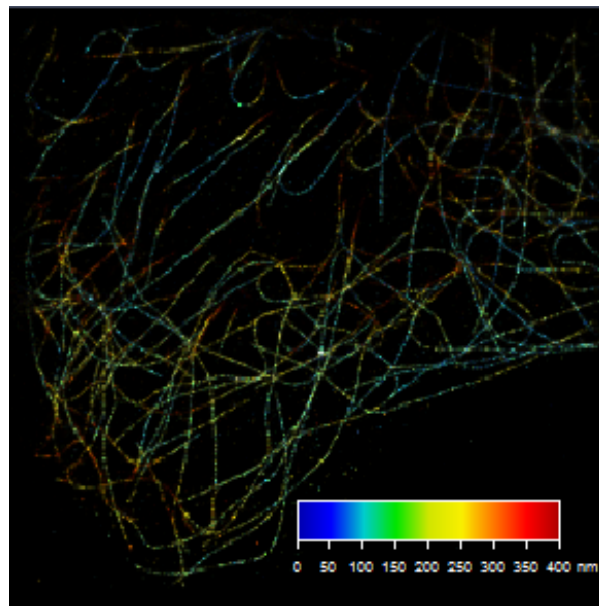
Fig. 684   3D SMLM data set viewed in Color-Coded Projection

To this end, select a suitable look up table. The resulting image is dispalyed as a three color RGB image (Fig. 685).

We recommend to restrict z positions to a minimum to obatin the best dynamic range of the color code. The color-code is displayed in a color-code bar.

Activate the color-code bar to alter its size (by drawing on the corners), format (by drawing on the corners and changing the extension) and position (by drag and drop). Font appearance and size can be changed under **More...** of the **Graphics** tool.

In the **Ortho** view tab XY, XZ and YZ views are avilable (Fig. 686).

☞ As XZ and XY have little extension for full fram images, **Cut** a region in the **Graphics** tool might help. The zoom function operates simulatneously on all images (XY, XZ and YZ).

☞ Ortho view allows you to take distance measurements.
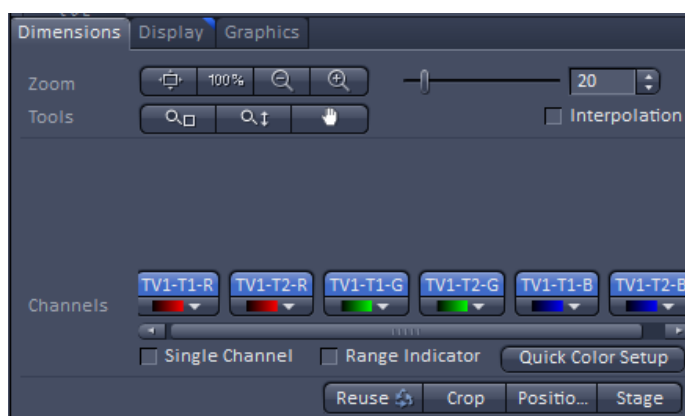
Fig. 685   Dimensions tab of a color-coded map
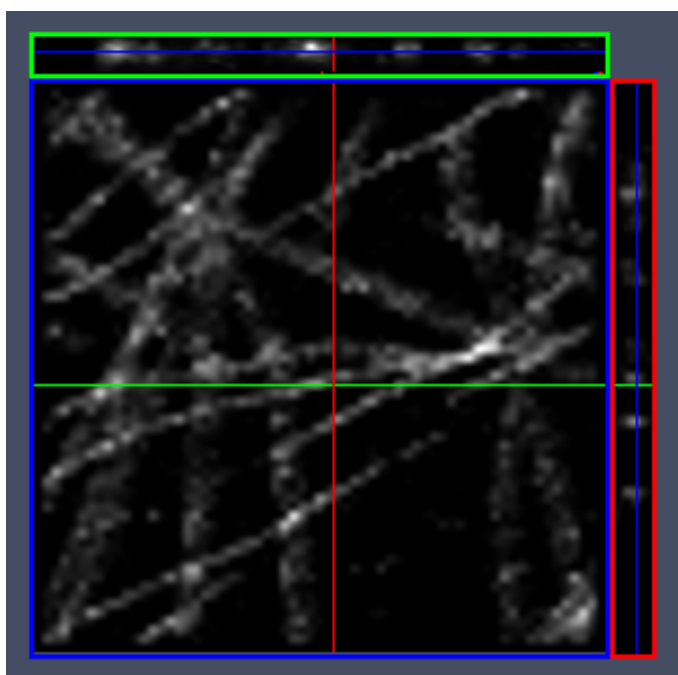
Fig. 686   3D SMLM data set viewed in Ortho view tab (64 x 64 pixels)

In the **3D** view tab the data set can be displayed in three direction with or without color coding (Fig. 687).
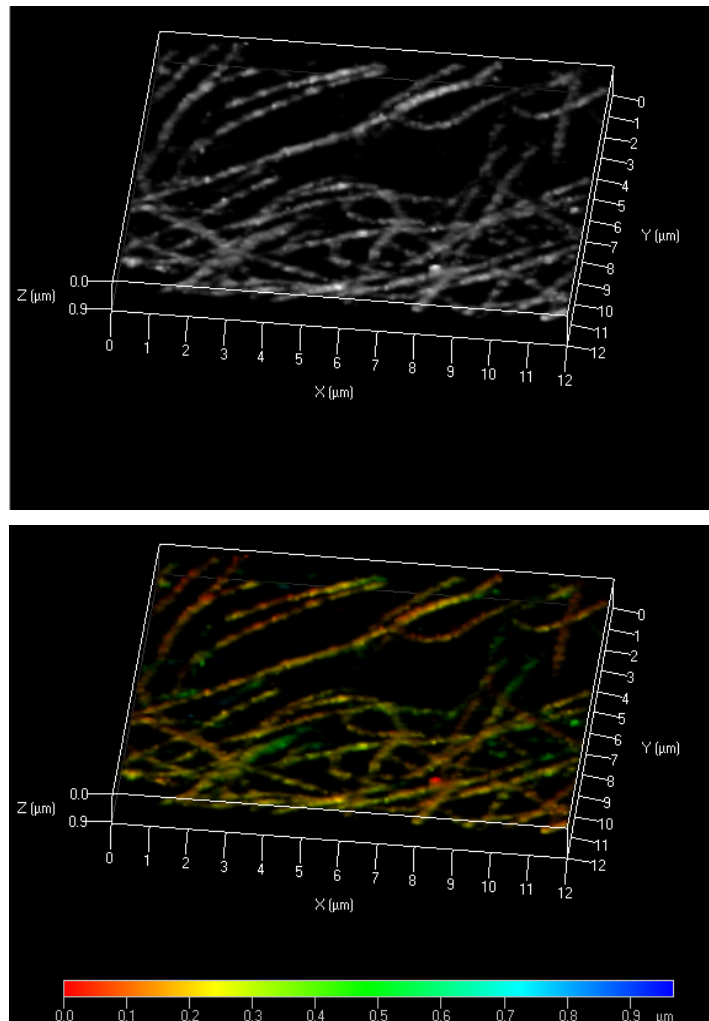
Fig. 687 **3D SMLM data set viewed in 3D view tab (128 x 128 pixels) without (upper panel) or with (lower panel) color code**

☞ As z extension can be small for full frame images, **Cut** a region in the **Graphics** tool might help.

☞ **3D** view allows you to take distance measurements.

☞ As 2D and 3D SMLM images are vector maps, which are not recognized in many Processing functions and bigger in their size than normal images, it might be beneficial to convert the vector maps to normal images with the SMLM **Convert to Image** processing function after Rendering. For example, the 3D display may be faster and facilitated after conversion.
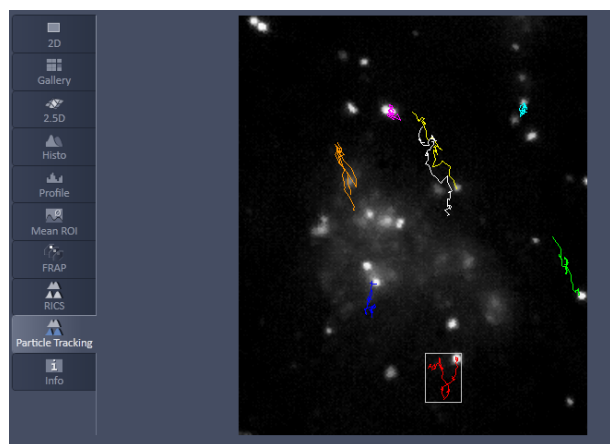
Fig. 688   View Tab – Particle Tracking

Fig. 689   General view control tabs for SMLM

6.19   Particle Tracking View

The **Particle Tracking** view tab is available for images that have been processed with the Particle Tracking Processing tool (Fig. 688).

The displayed image shows the tracks of identified particles in different colors as overlays to the first image of the time series.

Besides the general control view tabs **Dimensions**, **Display** and **Graphics** available which functions have been described in section **2D View**, there is a special Particle Tracking view tab available (Fig. 690).

6.19.1   Particle Tracking Control View Tab

With the **Trajectory Length** slider the minimal trajectory length in frames is defined, at which particles will be displayed (Fig. 689). A trajectory length of 64, for example, would mean, that only those particles will be displayed, that have been observed at least for 64 consecutive frames.
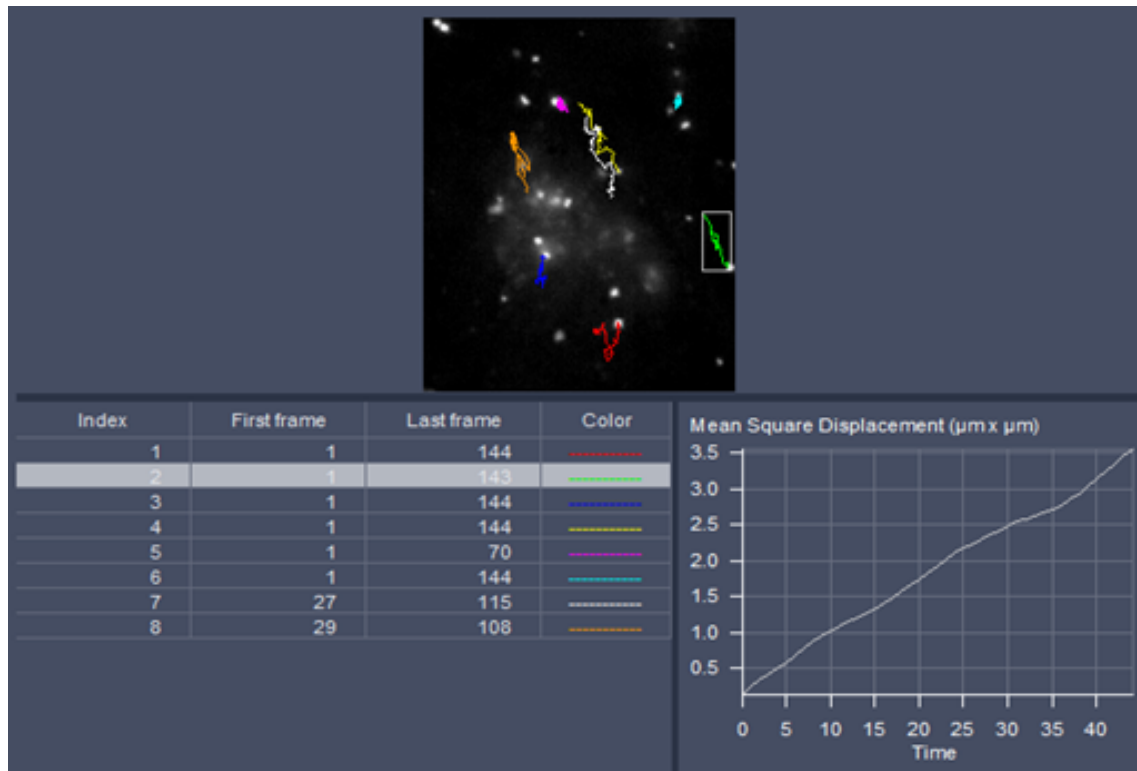
Fig. 690   View Tab – Particle Tracking; trajectories of moving particles displayed

In the **Show** field (only available in **Show all** mode) the particle **Table** and mean square displacement (MSD) **Diagram** can be displayed by pressing the corresponding **Table** and **Diagram** buttons. Once pressed, the buttons become highlighted and the table and diagram are displayed in the image container (Fig. 690). Pressing the buttons once more they become deactivated and the table and diagram are not displayed any longer.

The **Particle Table** lists all particles identified with the set minimal **Trajectory length**, of which the track is displayed in the image. It has for columns:

- **Index**: Identifies the Particle with a number

- **First frame**: The frame the particle was identified the first time.

- **Last frame**: The frame the particle was identified the last time.

- **Color**: Identifies the color of the trajectory of that particle in the image.

The track of the particle highlighted in the **Particle Table** will be framed by a rectangular in the image. Also its mean square displacement will be displayed in the MSD Diagram.

The MSD Diagram displays the Mean Square Displacement (µm x µm) of the particle that is selected and highlighted in the **Particle Table**.

6.20   Polarization Imaging

Linear (plane) polarized light, which is light whose wave goes only one direction, exciting a fluorescent molecule with a preferred dipole orientation results in polarized emitted light. It provides a contrast-enhancing method that is especially useful in the study of molecules that are fixed in their orientation or are greatly restricted in their rotational diffusion. Anisotropy is directly related to polarization and is defined as the ratio of the polarized light component intensity to the total light intensity.

In polarization microscopy using laser WF systems the sample is irradiated with vertical polarized light (in respect to the optical table) from a laser source. The emitted fluorescence is passed sequentially through emission polarizers (analyzers) that are housed in the **Notch filter cascade** and that transmit either the vertical (I_{VV}) or horizontal (I_{VH}) polarized emitted light onto the Quasar detector (L format fluorescence polarization). Since the vertical component of the emission light is parallel polarized to the vertical polarized excitation light, it is often also referred to as the parallel component (I_p, $I_{\parallel}$). Likewise, the horizontal polarized emission light is also designated as the perpendicular ("senkrecht" in German) component (I_s, $I_{\perp}$). In the Software the "p" and "s" designations are used.

Polarisation **P** and anisotropy **r** are defined as:

$$P = \frac{I_p - I_s}{I_p + I_s} \text{ and } r = \frac{I_p - I_s}{I_p + 2 \cdot I_s} \text{ with } 0 \leq r \leq 1.$$

Hence they can be interconverted to each other in the following way:

$$P = \frac{3 \cdot r}{2 + r} \text{ and } r = \frac{2 \cdot P}{3 + P}.$$

In a completely polarized sample ($I_s = 0$) the anisotropy r=1. In a completely non-polarized sample ($I_s = I_p$) anisotropy r=0.

The formulas for polarization **P** and anisotropy **r** as given above are strictly true only, if the optical transmission for both emission polarizers are identical. Any differences must be corrected by introducing a correction factor **G** that is multiplied with I_p. Hence the anisotropy **r** in such a case would be calculated according to:

$$r = \frac{I_p - G \cdot I_s}{I_p + 2 \cdot G \cdot I_s}$$

G can be measured using horizontally polarized excitation light and is defined as

$$G = \frac{I_{HV}}{I_{HH}}.$$

However, since in laser WF systems the polarization of the excitation light can not be changed easily from vertical to horizontal, G has to be determined with an isotropic fluorescent dye solution as the ratio between the mean intensities I_p and I_s, e.g. obtained from the histogram view at the image container.

☞   Please note that the G factor is not the mean intensity of the ratio (**R**) channel, where every pixel is computed seperately. It has to be calculated from the ratio of the mean intrensities of the I_p and I_s images.

As the formulas implies:

Anisotropy **r** is the preferential display as anisotropy of single species will be simply additive. Note that the ZEN Software provides for a formula to display the anisotropy directly in ratio imaging. Images could also be computed in the **Processing** tool`s **Calculator**.

For Laser WF polarization imaging, a dual camera setup with a polarizing beam splitter is required.
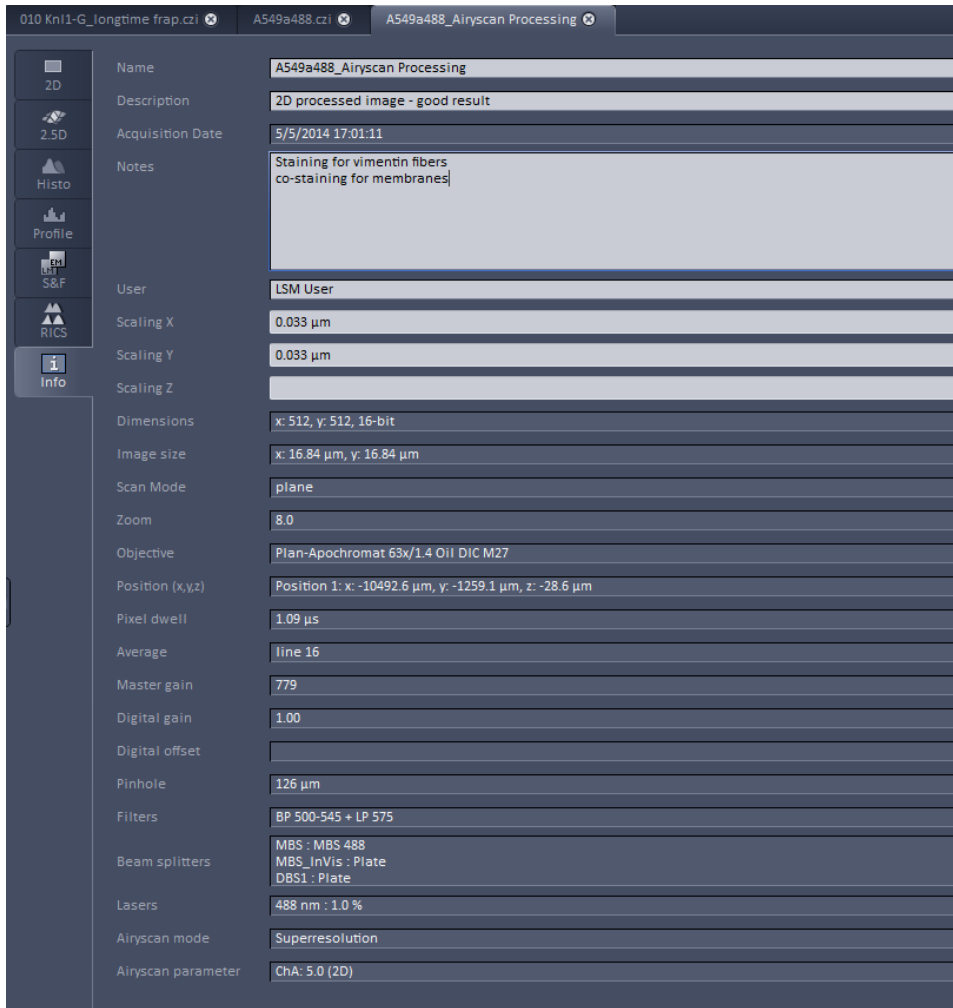
6.21   Information View

The **Information View** is a view type that shows a summary information sheet of all relevant image acquisition parameters. Parts of it can be edited – like, e.g., the **Notes** field.

The Information View displays:

- File name of the data set

- A brief description of the data set

- A notes field in which the user can note down essential information on the experiment

- The user name of the person who acquired the image

- The pixel scaling in X, Y, Z

- The image dimensions (pixels in x-y-z, channels, bit depth)

- Calibrated image size x-y-z in μm

- The scan mode (e.g. plane, time series)

- Tile scan information

- The zoom factor

- The objective lens

- The position of the individual images in values relative to the set zero position in the software (the Z position is indicating the center position of the Z-Stack)

- The position of the individual images in relative values

- The pixel dwell time of the scan

- The average mode used

- The Gain (Master) settings for all channels

- The Digital Gain settings for all channels

- The Digital Offset settings for all channels

- The pinhole sizes

- The filters and beam splitters in the beam path

- The used lasers with the settings of the respective AOTF

Fig. 691   Information View of a processed superresolution image

7 Right Tool Area, Data Management and Storage

7.1 General

Document Type

Images are stored as files (*.czi format) in folders just like in any other Windows application.

All information (metadata) will be saved with the .czi file. When exporting .czi files as .lsm files this metadata cannot be completely exported. When exporting .czi files as .lsm files, each z-stack will be saved as an individual .lsm file.

We provide further information about the .czi file format on request. Please contact your ZEISS representative.

In ZEN there are three ways to access your data:

- The File menu

- The ZEN File Browser

- Open Images are accessible in the Right Tool Area

ZEN File Browser

The File Browser allows access to image data of variable image formats and permits displaying them in three views:

- Gallery View,

- Table View and

- Form View.

Images can be loaded, copied and deleted within the ZEN File Browser.

The **Reuse** (see section **Dimensions**) function is available in the ZEN Image Browser: Hardware parameters can be loaded without opening the files by clicking the **Reuse** button.

Images and Documents

In the Right Tool Area the **Images and Documents** section is displaying the images which are loaded in the system memory. Double clicking brings the image to the front. Files can be saved and deleted/closed on this section.

Export

With the **Export** dialog in the **File** menu, the acquired data can be directly exported to various file types.

☞ In the **Save As** dialog in the **File** menu these formats can also be chosen as file type.

7.2   ZEN File Browser

The ZEN File Browser is a versatile tool to access your image data.

• In the menu bar, click on **File** and select the **File Browser …** item to open the **ZEN File Browser**.

It displays the hard drives and connected network drives available on the computer. Clicking on a folder opens it and lists the image files therein.

The ZEN File Browser can be operated in three views:

– Gallery View,

– Table View and

– Form View.
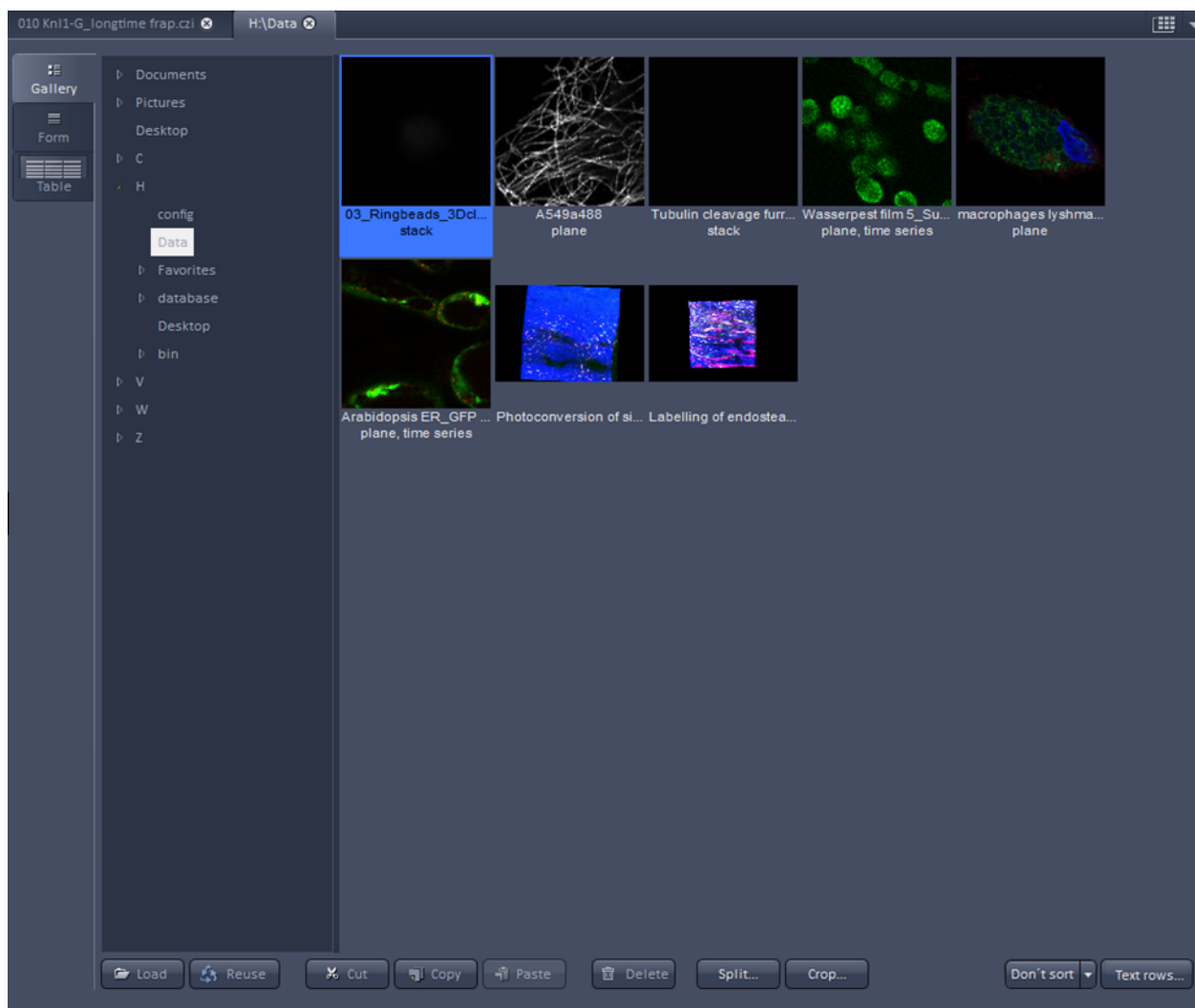
Fig. 692   ZEN File Browser

With the use of the button bar underneath the files can be loaded, the settings cen be re-used, images can be cut, copied, pasted and deleted.

Pressing the **Reuse** button loads the hardware settings of the .czi image highlighted (blue) in the browser window. You do not have to open the image to apply the **Reuse** function.

☞   Eventual info messages (Fig. 693) fading in the right hand corner inform about missing hardware if applicable. The message box disappears after ~ 10 seconds.
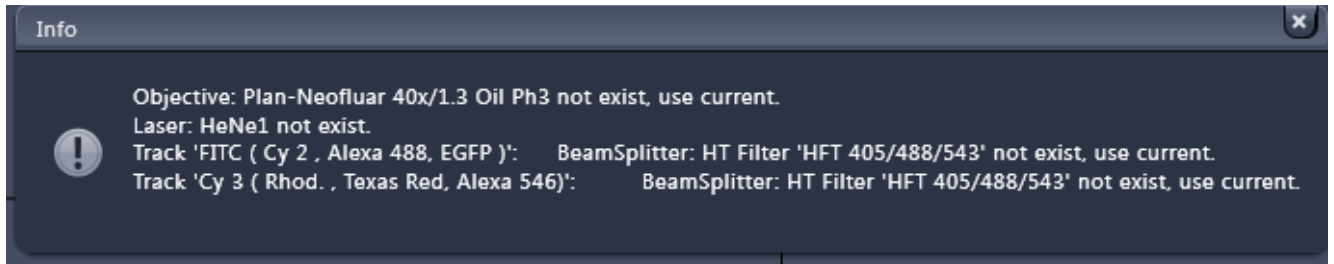
Fig. 693   **Info message**

The images can be sorted in three different ways, by name, the date the image was created (acquisition date/time) or the date, the images were accessed (Fig. 694).

When in Gallery View mode, images can be split according to their dimensions and stored as separate files. Click **Split…** to open the dialog.

Chose the **Size** of the dimension you want to split up the image.

The check box in the **Separate Files** column activates/deactives the split process for the individual dimensions (Fig. 695).

Click **Select file name and save…** to save the split up image dimensions as separate .czi or .lsm files.

Crop… allows you to save parts of acquired images as new files.

According to the available dimensions it is possible to chose a subset of this dimension with a selectable step size.

For example its possible to save every 2nd image of the first 100 images of a time series of 500 images.
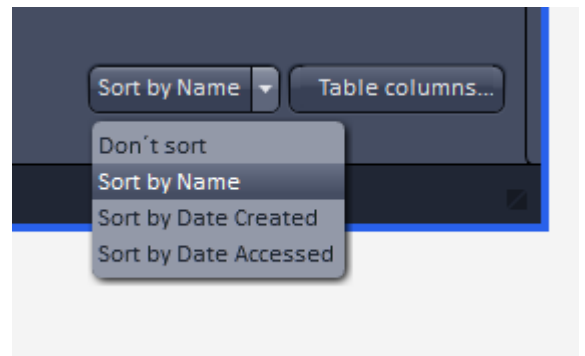
Fig. 694   **Sorting drop down menu in the File Browser**
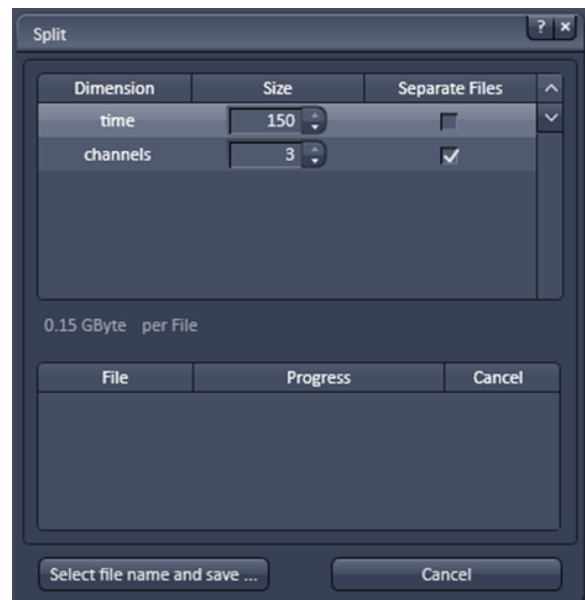
Fig. 695   **Split window of the Split function in the File Browser**
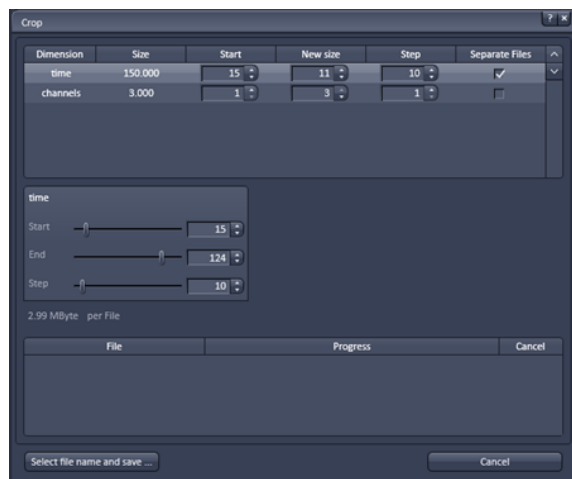
**Fig. 696   Crop window of the Split function in
the File Browser**

Within the dialog (Fig. 696) use the editing boxes or the sliders to set the desired values.

The slider box toggles with the highlighted dimension.

Click **Select file name and save...** to save the cropped images as separate .czi or .lsm files.

7.2.1 Gallery View of the ZEN File Browser

In the **Gallery View** of the browser the images are displayed as thumbnails. One click selects a single image, press **Shift** and click to select multiple images. If the **CTRL** key is pressed, all clicked images are selected.
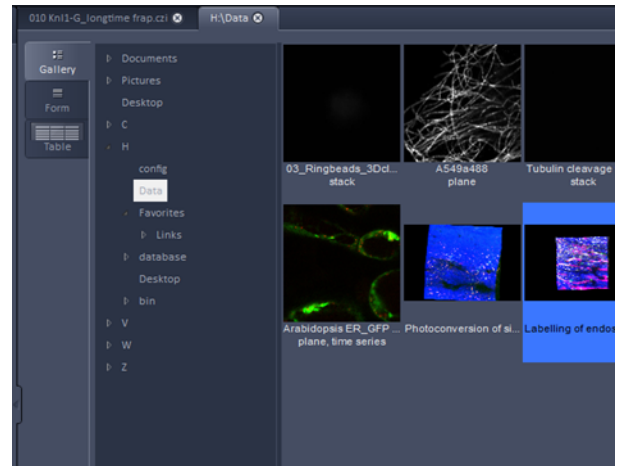
Fig. 697   Gallery view

Opening Images:

- To open an image, double click on it.
- For opening multiple images at a time, select the desired images and press **Load**.

The information stored in the .czi file format can be displayed underneath the thumbnail. By pressing the [Text rows...] button a choice of information is available (see Fig. 698).

The example below (Fig. 699) shows the use of the display of:

- file name,
- acquisition date,
- file size and
- scan mode.
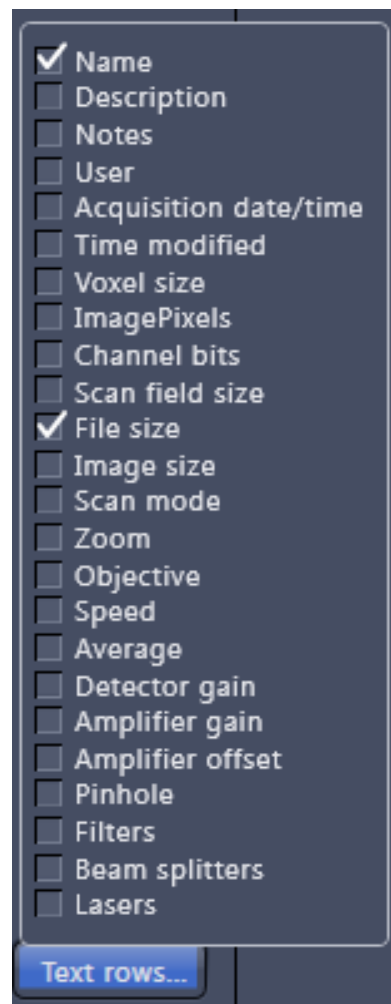
Fig. 698   Image information selection
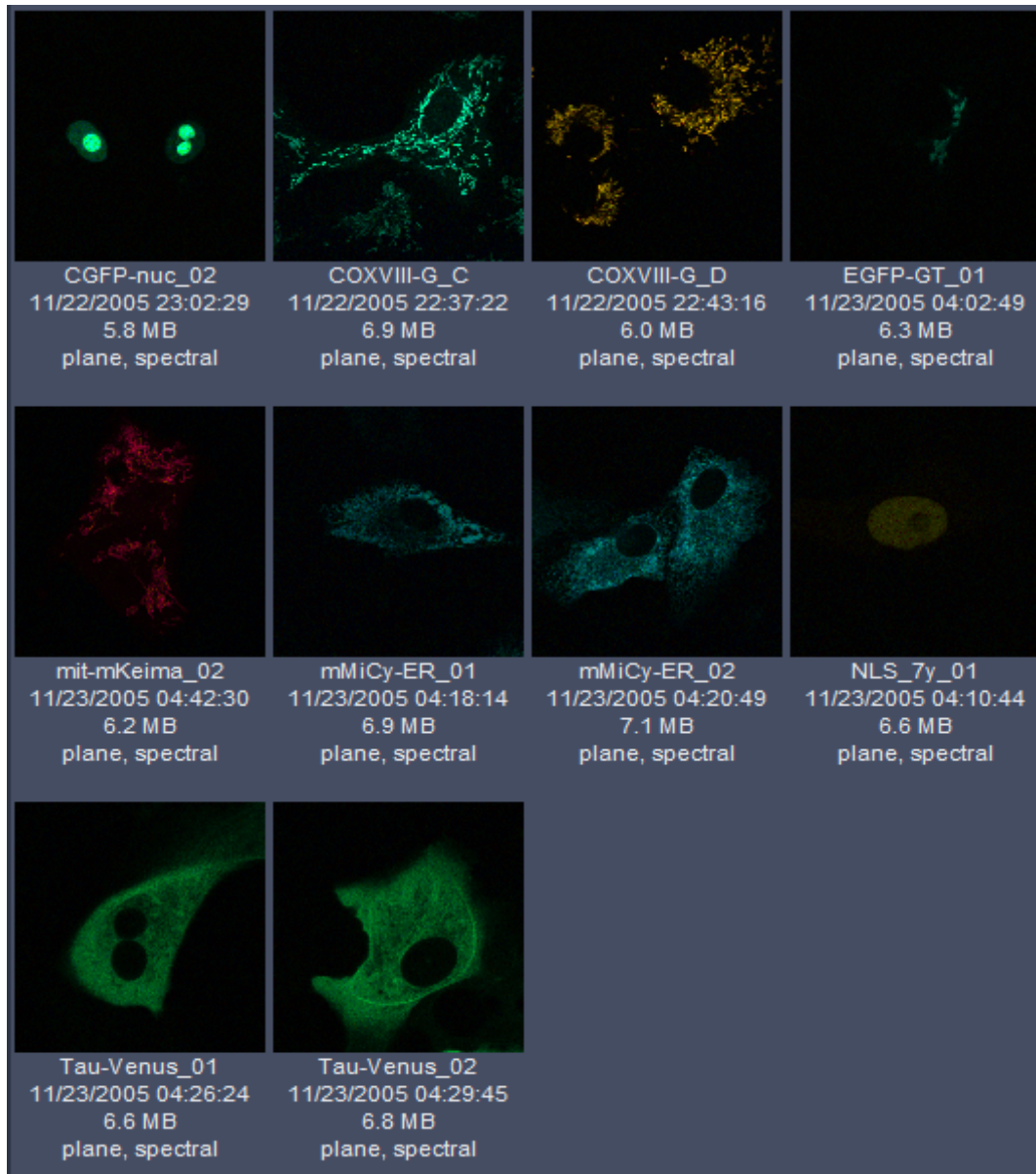
Fig. 699   Images with information

7.2.2 Form View of the ZEN File Browser

All images in a given folder can be accessed by using the slider below the Info-Panel or using the stepper on the right side of the slider. The total number of images in a folder is indicated on the right side of the slider .
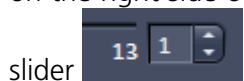
In the **Form View** of the browser all information available (or entered) in the **Info-tab** of the image is displayed.

☞ Note that the upper four fields (**Name**, **Descriptions**, **Notes** and **User**) are currently not saved. To edit these fields please load the image and edit the form in the info-view of the image. The information in those fields are – however – accessible by copy and paste.
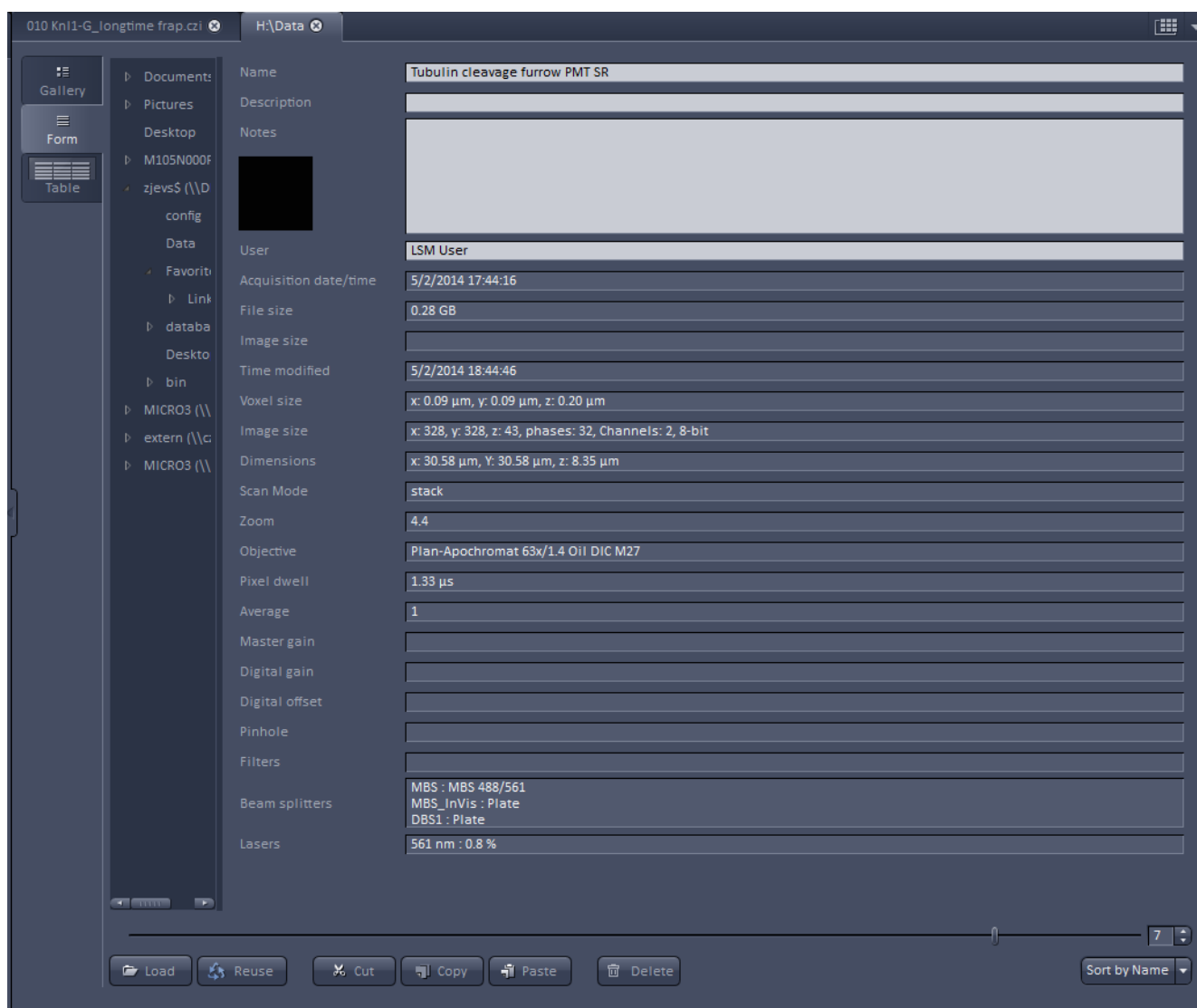
Fig. 700   Form view

7.2.3 Table View of the ZEN File Browser

The **Table View** is especially useful in case a lot of files are found in one folder.

Fig. 701 Table view

The columns displayed in the table are selectable from the **Table columns** button. The options shown in Fig. 702 are available.

The width of the columns is editable and the order of the columns can be freely shifted by dragging the header of the columns.
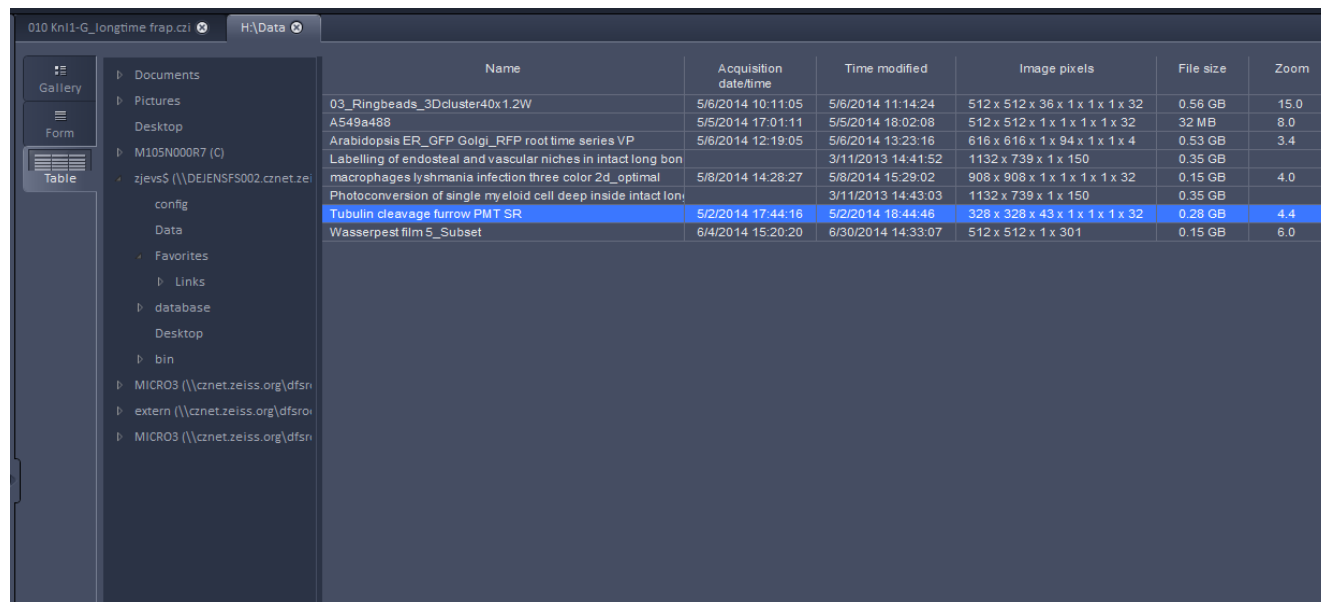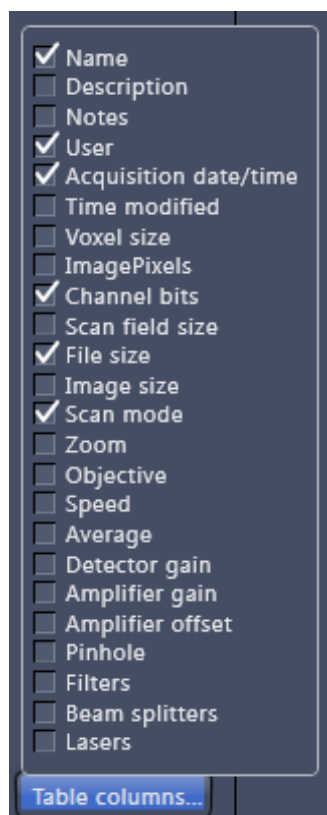
Fig. 702 Table view options

7.3   Images and Documents Panel

The **Images and Documents** panel (Fig. 703) is located in the Right Tool Area of ZEN. It displays all images and data which are open in ZEN. Its purpose is multi-fold:

– All opened images can be brought to the front by double clicking on them which makes the navigation through lots of images easy and fast.

– Basic file handling functions are implemented for fast operation with the acquired images. The images can be saved and closed using the following buttons:

– A new acquisition document can be opened using the button.

– The button saves the selected image.

– The button closes the selected image.

☞ On already saved images the button is closing the image, not deleting it.
Multiple images can be selected by holding down the **CTRL** key on the keyboard and clicking on the list entries.

– The whole section can be hidden by clicking on the black triangle at the left side of the panel.
Once hidden, a click on the right boarder of ZEN lets the **Images and Documents** panel slide back into the application.

– All files can be displayed in three ways by using the buttons underneath the section.

–   Textual View

–   Thumbnail View

–   Big View

The default setting is **Thumbnails view** as indicated on the screengrab.
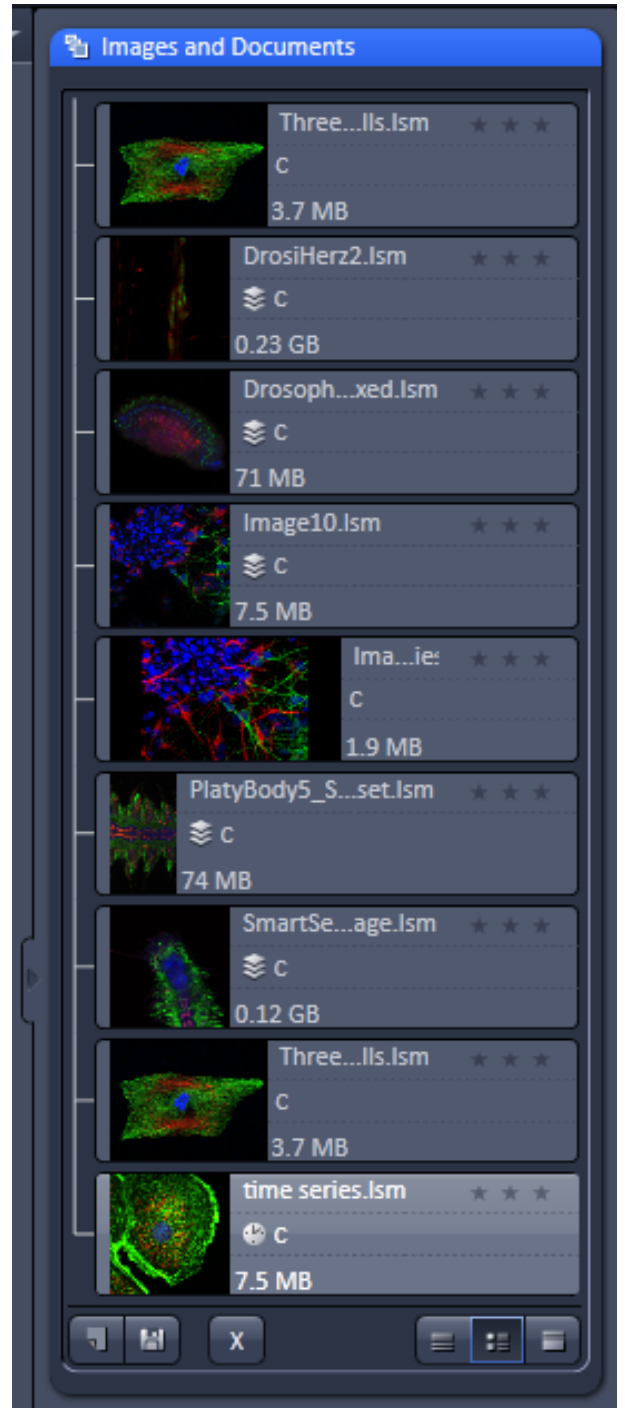
Fig. 703   Images and Documents panel
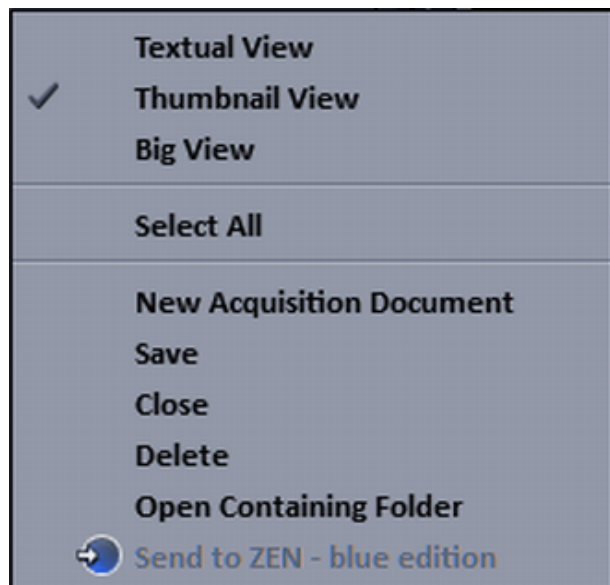
Fig. 704   **Context menu of the Images and Documents section**

The context menu of the **Images and Documents** section allows the following functions:

- – Selecting one of the view options **Textual View**, **Thumbnail View** or **Big View**
- – Selecting all opened image files by clicking on **Select All**
- – Opening a **New Acquisition Document**
- – Saving or closing the selected image files by clicking on **Save** or **Close**
- – Clicking **Delete** to delete the selected image file
- – Clicking **Open Containing Folder** opens the folder, where the selected image file ist saved.
- – Clicking **Send to ZEN – blue edition** sends the currently active document to ZEN blue edition.

☞ Not all image versions can be opened in ZEN (blue edition). Data acquired with ELYRA might not be displayed in the right way by ZEN (blue edition).

Regardless of which display type is chosen, the individual images contain the following information:

- – File name
- – Data type indicated by icons: like e.g. **Stack**, **Time**, (**Bleach**), **Lambda** or **C** for multichannel images. These icons are the same as underneath the start button when starting multidimensional experiments (section **Action Buttons**).

The file size is indicated in MB or GB.
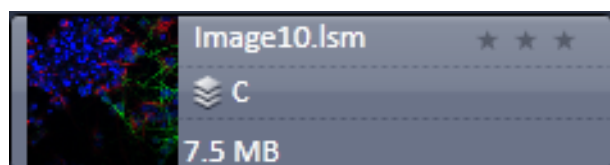
Fig. 705   **2 Ch. Z-Stack image**

- – Example of a 2 Ch. Z-Stack displayed with a small thumbnail in the **Images and Documents** section (Fig. 705).
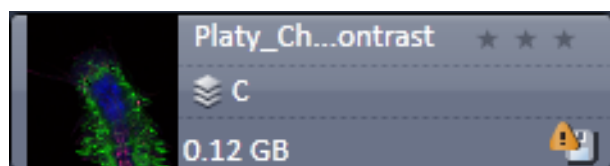
Fig. 706   **Image with warning symbol**

- – In case the image is not saved or has been processed, a warning symbol is diplayed on the right side (Fig. 706).

– A progress bar (Fig. 707) is shown for each image facilitating the overview if e.g. many processing function are running at the same time.
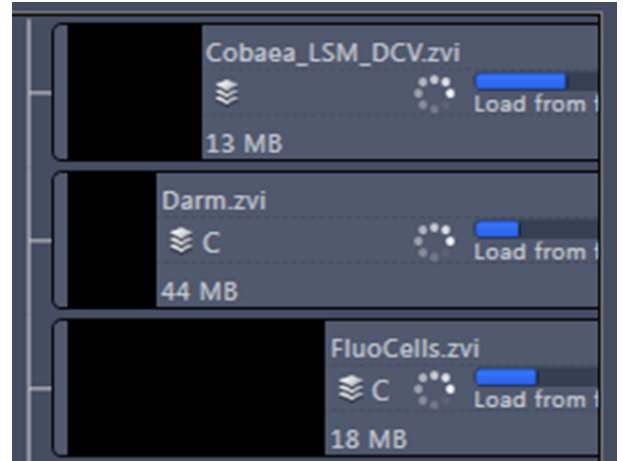
Fig. 707   **Image progress bar**

– In case long filenames are used the whole section can be dragged to the left and thereby enlarged (Fig. 708).

Fig. 708   **Enlarging the image view**

– The stars can be used to rate the pictures. They are activated by clicking on them.
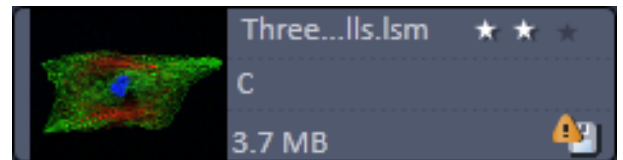
Fig. 709   **Image rating star function**

7.4   Opening of Files via the "Open" Command in the File Menu

- Click on the **File** menu of the menu bar and select **Open** or click on the [icon] icon of the main toolbar.
    - The standard windows open dialog appears.

- Select the desired file and click **Open**.

7.5   Save

The **Save** function allows to store an image together with the acquisition parameters (and processing information) to be stored in a .czi file.

Proceed as follows to save an acquired or an edited / processed image:

- Click on the **Save** or **Save As** button in the **File** menu of the menu bar or click on the [icon] icon of the main toolbar.
    - The **Save Image and Parameter As** window appears on the screen.

Save:
Stores a newly created or changed image. Newly created images must be given a name

Save As:
Stores a previously stored and called up image under a different name. If images are called up and stored again, the original data and time display will be retained.

- Clicking on either of these Menu Items opens the **Save As** window to create and open an image database.

- Click on the **OK** button in the **Save Image** window.

7.6 Export of Images

The **Export** function allows the export of acquired images and images loaded.

- Select the image to be exported.

- Click on the **Export** menu item in the **File** menu of the menu bar.

    - This opens the **Export** window (Fig. 710).
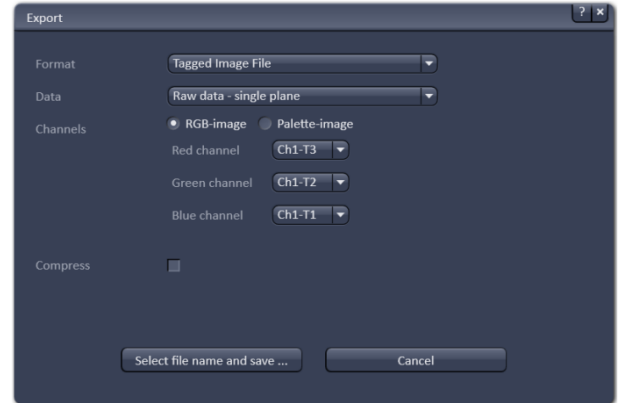
Fig. 710 Export window

- Under **Format**, select the format to which the image is to be exported to (Fig. 711).
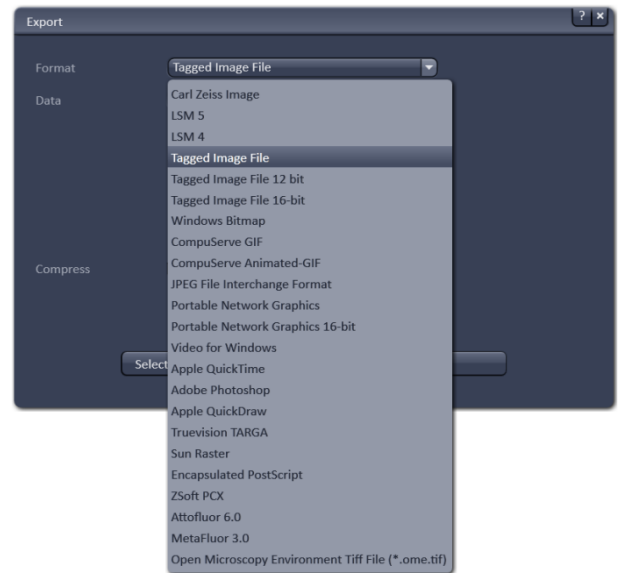
Fig. 711 Format selection

- Select the data type which the image is to be exported under **Data** (Fig. 712).

- Chose a compression level. For some file formats lossless compression or various other compression levels are available. The degree of losses for the image quality is listed according to the type of compression.

- Click on the **Select file name and save …** button. The standard Windows File saving dialog appears. Click **Save** to complete the export process.

☞ When stacks or time series are exported, each frame is stored as an individual image.
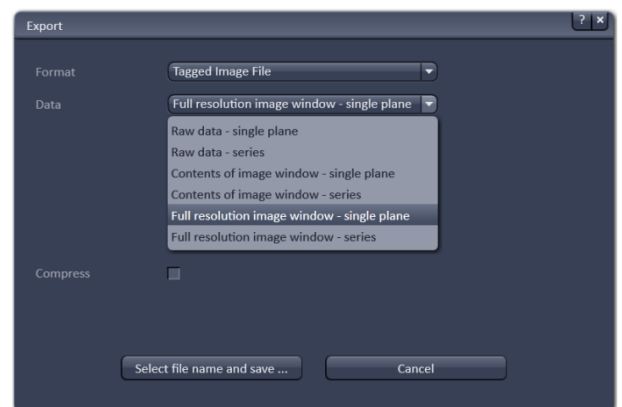
Fig. 712 Data type selection

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CHAPTER 2   MACROS AND VISUAL BASIC

CONTENTS

1 VBA Version

Both versions of VBA (Visual Basic for Applications) – version 6.4 (32 bit) and version 7.1 (64 bit) – are available.

Version 6.4 is registered by default to ensure compatibility to existing macros.

By registering version 7.1 the 64 bit processing will be supported. Other DLLs will be registered in this case. Old macros have to be converted to the new format.

The registered VBA version will be shown in the **Help/About** dialog.

2 Macros

The macro function permits the recording, running and editing of command sequences and their allocation to buttons in the **Macro** menu. Macros can be set up in two different ways: The Visual Macro Editor or alternatively they can be programmed in Visual Basic directly.

- In the **Menu** bar (see Fig. 1), click on **Macro**.
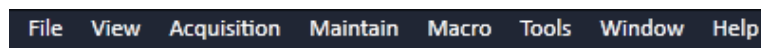
Fig. 1   **Menu bar**
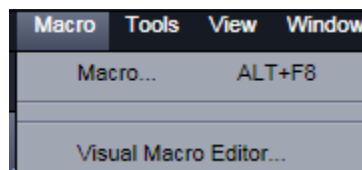
Fig. 2   **Macro menu**

– This displays the **Macro** menu (see Fig. 2).

- The Macro list is bipartite: **Macro** – editing and recording of macros; assign macros to list

- **Visual Macro Edito**r – edit automatic workflows with pre-defined modules

Selecting **Macro** opens a dialogue for editing and recording Macros based on VBA programming in the **Macro Control** window (see Fig. 3).

Using the **Visual Macro Editor** button (optional) allows to create automated work flows using the arrangement of symbols which depict the single steps within a work flow.

2.1 Macro Language

"Visual Basic for Applications", called VBA in the following, is used as the Macro language. This language is well known and widely used as Macro language in the "Microsoft Word for Windows" and "Microsoft Excel for Windows" products. Experience with "Microsoft Visual Basic" would also be beneficial for macro-programming ZEN.

An Integrated Development Environment (IDE), the "Microsoft Visual Basic Editor", is available for editing and debugging of macros. The IDE includes an "online help" with a detailed description of the VBA language.

Macros are stored in project files. One project file can include several macros.

☞ The VBA functionality described in this paragraph refers to VBA as implemented in the ZEISS LSM software 4.2. With the introduction of ZEN, the range of functions is and will be extended, while VBA can still be used with the functions described below. The extension of functionality applies mostly to display and access of image data. If you need assistance or further information please contact your local ZEISS LSM specialist.

2.2 Macro Control

To open the Macro Control, choose **Macro** from the **Macro** List in the main **Menu Bar**.

This opens the **Macro Control** window.

2.2.1 Edit Macro Function

This function allows you to manage project data. Macros can be recorded, stored, performed, edited and, if required, deleted.

Press the **Edit Macro** tab to switch to the **Macro** and **Recording** panels (see Fig. 3).
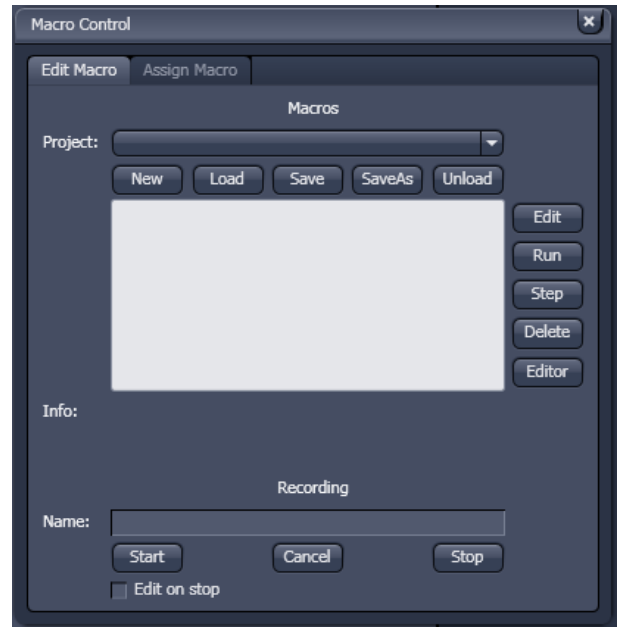
Fig. 3   **Macro Control Window**

(1) Macro panel

New button:	Creates a new project.
Load button:	Opens an existing project.
Save button:	Stores the project on the hard disk.
Save As button:	Stores an existing project under a new name.
Unload button:	Removes the selected macro from the **Macros** list.
Edit button:	Allows macros to be edited and debugged. The editor (**Microsoft Visual Basic**) is automatically located at the beginning of the relevant macro.
Run button:	Runs a macro.
Step button:	Opens the editor for line-by-line editing / debugging.
Delete button:	Deletes the selected macro.
Editor button:	Opens the editor. Displays the processed area of the macro edited last.

Fig. 4   Macro panel with new Macro assigned
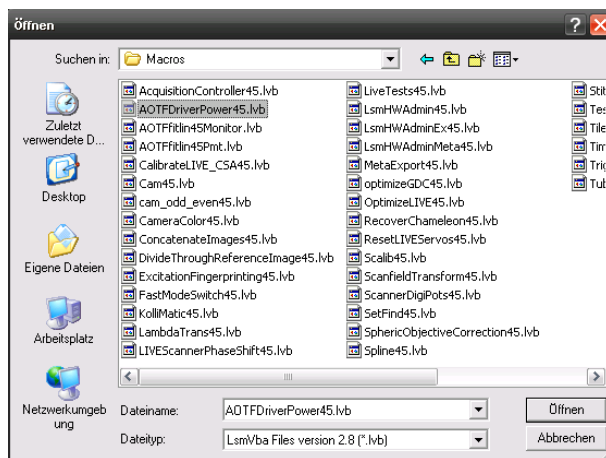
Fig. 5   Open window

Fig. 6   Macro panel with existing Macro assigned

Macros are stored and managed in project files (*.lvb). Before you can record or edit a macro, you have to create a project as follows:

• Press the **New** button to create a project file (see Fig. 4).

  – A new project is created and displayed in the **Project** selection box. The project name is automatically default (LSM followed by a number), but can be edited afterwards.

To activate an existing project, proceed as follows:

• Press the **Load** button.

  – The **Open** window will be opened (see Fig. 5).

• Select the relevant project file (data extension: ***.lvb**) from the **Macros** list box. Click on the **Open** button.

  – The project file will be opened and the macros contained in it are displayed in the **Project** selection box of the **Macro Control** window. You can use the pull down menu to load any listed Macro.

Recorded macros are stored in main memory first. Before the macros can be assigned to the list in the **Macro** submenu, the project must be stored on the hard disk.

• Press the **Save** button under the project name in the **Macro Control** window and determine the file name in the **Project** selection box, if required (see Fig. 6).

(2)   Recording panel

Before recording a command sequence, you can enter the name for the macro to be created in the **Name** input box of the **Recording** panel.

Start button: Starts recording.

Cancel button: Cancels the recording procedure.

Stop button: Stops recording.

☑ **Edit On Stop**:   On stopping the recording procedure, the macro editor is automatically opened at the relevant position.

Proceed as follows to record a macro:

- Enter a name for the macro to be created under **Name** in the **Recording** panel (see Fig. 7).

- Click on the **Start** button to start recording the macro.
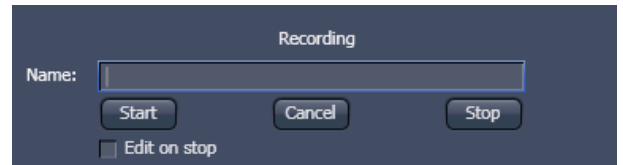
Fig. 7   Recording section

- Then perform the operations to be stored, e.g.:
  - Click on the LSM **Auto Exposure** action button. A find scan will be performed.
  - Click on the LSM **New** action button. A new **Image Display** window will be opened.
  - Click on the LSM **Snap** action button. A scan will be performed.
- Then click on the **Stop** button to end the recording (**Cancel** enables you to cancel recording).
  - If recording was successful, the entered **Name** will then also be available in the **Macros** list box of the **Macro** panel. The new macro is automatically assigned to the current project. It is possible to assign as many macros as required to a project.
- Click on the **Save** button to store the new macro.

Proceed as follows to run a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.

- Click on the **Run** button to start performing the macro.

☞   Provided that a macro is linked to a list in the **Macro** section of the **Menu Bar**, you only need to click on its name to perform it.

Proceed as follows to delete a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.

- Click on the **Delete** button. The macro will be removed from the list.

Proceed as follows to edit a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.

- Click on the **Edit** button. The Microsoft Visual Basic editing window will be opened.

- Make the required changes.

2.2.2 Assign Macro to the Macro list

This function permits stored macros to be linked to the assigned Macros list in **Macro** of the **Menu Bar**.

- Press the **Assign Macro** tab to switch to the **Assign Macro** panel (see Fig. 8).
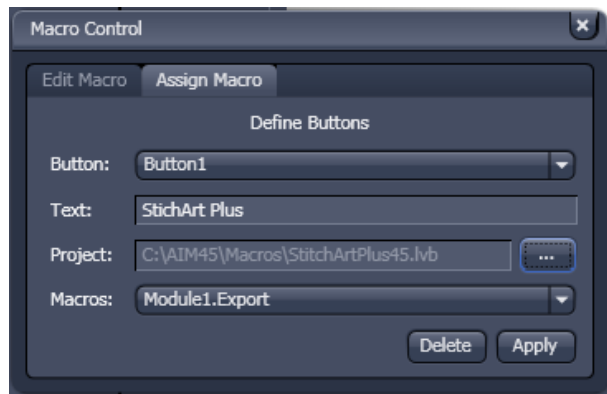
Fig. 8   Macro Control window – Assign Macro

Define Buttons panel

Proceed as follows to link a macro to the **Macro** list:

- Select the button number corresponding to the position in the list from the **Button** selection box.

- Enter the button labelling in the **Text** editing box.

- Select the name of the project file from the **Project** box using the "**...**" button.

- Select the macro name from the **Macros** box.

- Press the **Apply** button to assign the relevant macro to the specified list position in the **Macro** toolbar.

Delete a macro from the Macro list

Proceed as follows to delete a link from the **Macro** list position:

- Select the button number from the **Button** selection box.

- Press the **Delete** button to delete the list entry.

2.2.3 Editing and Debugging of Macros

The **Edit** in the **Edit Macro** panel button activates IDE (Integrated Development Environment), which allows macros to be edited and debugged. Under the **Help - Macrosoft Visual Basics Help** menu item, IDE contains detailed "online" help on its operation and on the VBA macro language. Therefore, only a few hints are provided in the following:

You should activate the required toolbars. We would recommend you to activate the **Debug** toolbar via the **View - Toolbars - Debug** menu item.

The following buttons in the toolbar can help you when debugging macros:

▶	Starts running the command lines.
■	Stops running the command lines.
‖	Interrupts processing of the command lines (pause).
🖐	Sets a breakpoint in the line with the text cursor.
⏏	Processes a command line and steps into subprocedures.
⏏	Processes a command line and steps over subprocedures.
⏏	Exits the subprocedure (step out).
👓	Displays the value of the marked expression (Watch). If nothing is marked, the value of the variable above the text cursor is displayed.
🔲	Activates the Watch window in which values of variables and expressions can be displayed. For this, text is marked in the code window and dragged into the Watch window. Variables can be modified in the Watch window.

In the left-hand edge of the code window you will find an arrow beside the current command line. A new current command line can be determined by moving the arrow via the mouse. This makes it possible to skip command lines or to process command lines several times.

2.3   Overview of Available Macros

Documentation files (*.rtf, *.doc) of advanced macros are located in the macro directory. Additional Macros for Service are listed in a separate folder.

Name	Description
CameraColor60.lvb (also Button in Maintain)	Color balance of Axiocam HRc
ConcatenateImages70.lvb	Enables the combination of all kinds of images irregardless of the size and data depth to be presented in a time series. Imported images can also be included. Type of concatenation (fitted to the smallest frame or ROI, fixed size or fitted to the largest frame) can be defined by the user.
FileExportZEN	For exporting files into different image formats in batch processing mode. A detailed description is provided in the macro folder as separate pdf file.
GDDReset.lvb	Enables or disables the use of the factory set GDD values. Resets the GDD settings to factory values if other values have been stored over time.
LsmHWAdmin60.lvb (also Button in Maintain)	Direct service hardware access (password protected)
ModifySeriesZEN.lvb	Modifies Z-Stacks and Time Stacks like Rotation of the stacks, being mirrored, Conversion of time stacks into Z-Stacks and vice versa; A more detailed description is available within the macro folder as separate pdf file.
Spline60.lvb	Aligns the actual scanner movement to the graphical line drawn in the image to mark the spline scan.
TileScanRotation60.lvb	A potential tilt of the stage leads to incorrect aligment of tiles. This can be calibrated using a grid sample (or the calibration objective) and the calibration function of the macro. The value is taken over by ZEN for the Tile Scan tool.
TestGrid60.lvb	Projects a test Grid as graphics overlay into the open image document (for Service only).
SphericObjectiveCorrection60.lvb	Calibrates the spheric component of objective error by automatic acquisition of a Z-Stack on a flat surface and a spherical fit of the topographic data.
TestGrid60.lvb	Projects a testgrid into the image window (password protected).
TileScanRotation60.lvb	Defines rotation angle of scanners during tile scan.

☞   During installation, default macros will be installed in **ZEN\Macros**. Self generated Macros can be saved in any folder.
In case of a new installation, old macros will be stored in **ZEN\Macros\BackupMacros** or **ZEN\Backup**, to avoid problems with identical names of existing and new macros.

2.4 Sample Macros

The ZEN software package includes a set of basic macros free of charge. Many of these can be used as samples for new macro development.

In the following paragraphs you find a short description of some representative and common macros. Please contact your local LSM specialist for more detailed and extensive information.

☞ Please check the **C:\ZEN\Macros** folder on your system for separate documentation in the more extensive macros in .pdf format.

2.5 ExcitationFingerprinting

With tunable multiphoton lasers (except OPO devices) it is possible to acquire Excitation Spectra and subsequently use these spectra to perform linear unmixing.

This process is called Excitation Fingerprinting.

To perform this task the software of the system provides the Macro "ExcitationFingerprinting60.lvb" (available in the folder: C:\ZEN\Macros).

Calibration of the AOM:

A typical Ti:Sa laser does not supply an even power level over the wavelength range. To use such a laser for Excitation fingerprinting it is recommended to calibrate for the average power at the sample plane using a power meter.

The resolution of the power meter should be below 1 mW.

For the calibration one channel must be active otherwise the scan will not start and no laser is available. When ZEN is started one channel is always active per default. If the system was in use, check in the **Imaging Setup** tool if a channel is active.

For the calibration it is not relevant which channel is active.

Activate the laser to be calibrated in the **Channels** tool.

Next, set up the power meter in specimen plane using the combination of objective and main dichroic that will be used for Excitation Fingerprinting. Both, objective and main dichroic, influence the average power at the sample as well and need to be taken into consideration for this step.

Set up the Acquisition mode for Frame scanning with maximum speed and bidirectional scanning at the highest zoom.

This mimics a spot scan and will result in a steady beam coming out of the objective. The read out of the power meter will be much more accurate.

It is not needed to measure absolute powers but relative powers influenced by the AOM settings.

Load and run the **Macro** from the **Macro Control** window.
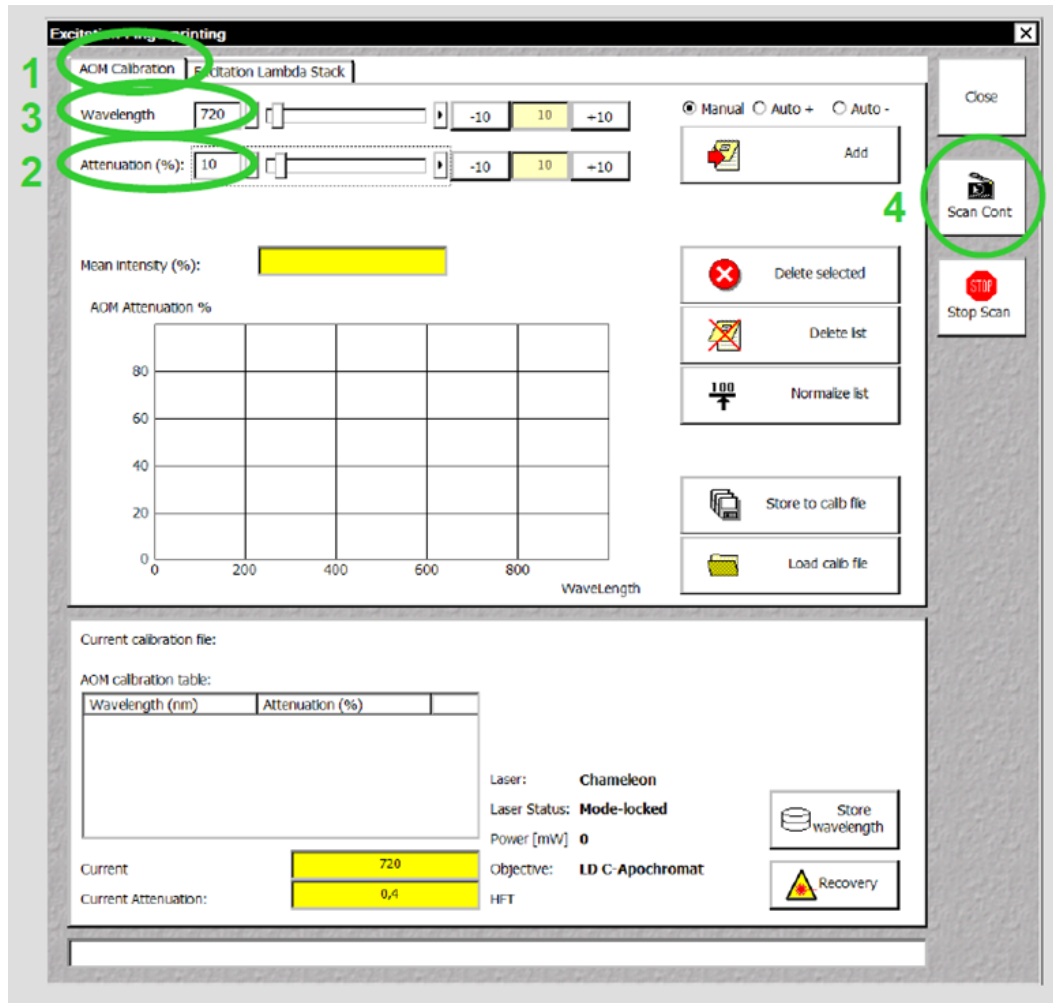
Fig. 9 **Macro: Excitation Fingerprinting**

1) Chose the **AOM Calibration** tab.

2) Set AOM Transmission to a value around 10.

3) Set the laser to the lowest wavelength possible (ideally this has already been done in the main software).

4) Start a Continuous scan.

5) Read out the value of the power meter. This value needs to be kept constant during the subsequent steps.

6) Activate the **Auto+** option (**Auto-** is chosen when starting with the highest wavelength available).

7) By clicking **Add and + 10 Steps** the AOM value will be added to the calibration list and the laser is tuned 10 nm further.

8) Now adjust the AOM transmission such that the power read out of the power meter shows the same value as with the previous wavelength.

9) Repeat step 2 and 3 until the end of the wavelength range is reached, then stop scanning.

To reuse the calibration for later use normalize the values before storing them as an .aom file in a folder of your choice

The normalized AOM values will provide the right AOM settings starting with the chosen setting at a given wavelength and calculating the relative values for all other wavelengths. Thus it is avoided to reach maximum or minimum AOM settings before the end of the wavelength range.

☞   The step size for setting the AOM and tuning the laser can be edited.
      Choosing **Manual** requires performing the tuning of the laser and the setting of the AOM by using the slider or typing in a value.
      Single highlighted values in the calibration list or the whole list can be deleted.
      Mean intensity (%) refers to the overall image intensity value; the value is displayed as % of the maximum intensity value at chosen data depth (8, 12 or 16 bit).

The lower part of the window shows the values of the currently used calibration file, the laser status, the objective and main dichroic.

The **Store wavelength** button will update the laser wavelength in the main SW.

Acquisition of an Excitation Lambda Stack:

1) Focus the specimen and set the AOM and detector gain to obtain a good image at the chosen wavelength. Switch to Excitation Lambda Stack.

2) Load the calibration file required for the used main dichroic and objective.

3) Set the wavelength range and step size at which the images should be acquired. (This is independent of the range and step size of the calibration curve.)

4) Start the image acquisition.

The Excitation lambda stack is displayed the same way as an emission lambda stack.

When checking the **Show Text** option the wavelength used for excitation of each image is displayed.

The Excitation Fingerprinting or the unmixing of the Excitation lambda stack can be performed in different ways.

It is possible to use already existing Excitation Spectra from previous experiments.

One can also use ACE (Automatic Component Extraction) to define the spectra from the newly acquired stack and use those for unmixing.

The spectra can also be defined manually by using the drawing tools.

The method to work best and is convenient to use depends on the labelling of the specimen.

If the different fluorescent dyes show no or only moderate spatial overlap in the specimen, ACE works well.

If the fluorescent dyes are strongly co-localized, it is recommended to use predefined Excitation Spectra of the individual fluorescent dyes.

2.6   ConcatenateImages

The mcacro allows concatenating various types of images into one image document showing the final image as a time series image. It is useful to combine images from Multi Block experiments into one image document showing the time series of one Block only. In this case the time stamps of the image are consecutive relating to the image acquisition time of the first image of the Block.
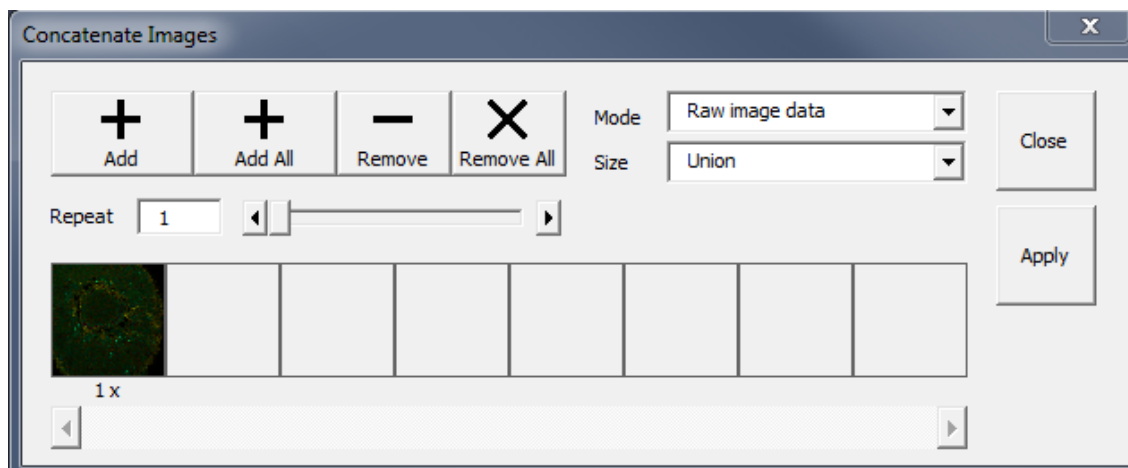
Fig. 10   Concatenate Images Macro

Use Raw image date **Mode** and **Size** Union for concatenating the images as raw data in a subsequent manner. **Add** opens up a small window showing all open image documents.

Chose the ones to be concatenated and click **Ok** to confirm the selection. **Apply** generates the final image document. Individual (highlighted) images or all can be removed clicking the corresponding buttons.

3   Object browser

To search the structure of an API object, use the object browser from the VBA editor. To explore an object from your VBA code, position the curser in the object and press "F2".

For the Lsm5Vba API, many classes and methods offer a short help-string explaining their meaning. The object browser can not only be used to explore the ZEN APIs, but also for all other VBA functionality.
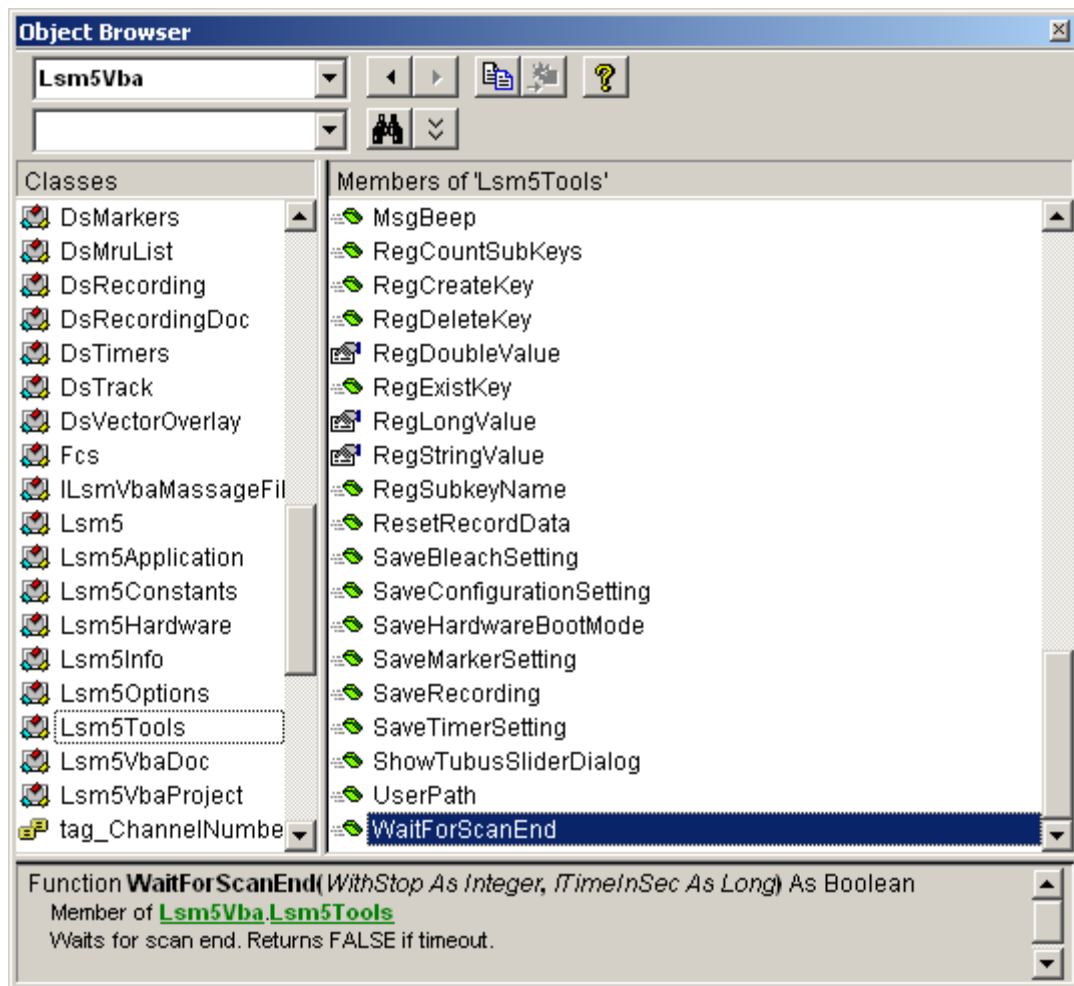
Fig. 11   Object Browser window

4   COM interface

The full range of ZEN VBA commands is also available via COM, by connecting a client program to a running ZEN system. There are two COM-automation objects available, for the Lsm5Vba interface and for the VBA-GUI interface. The COM object Lsm5Vba is called "Lsm5Vba.Application", and the one for the VBA-GUI interface is called "Zeiss.Micro.AIM.ApplicationInterface".

For example to connect a MATLAB session to ZEN, start ZEN first, start MATLAB, and then type

    >> ZENVBA = actxGetRunningServer('Lsm5Vba.Application')

    >> ZEN = actxGetRunningServer('Zeiss.Micro.AIM.ApplicationInterface.ApplicationInterface')

To start a scan, two commands are now available

    >> ZENVBA.Lsm5.StartScan

    >> ZEN.GUI.Acquisition.Snap.Execute

5   Glossary

- VBA: Visual Basic for Applications is an integrated programming environment that comes embedded into ZEN. It allows to automate routine operations by recording macros, and to write application programs that can use most features of the ZEN software.

- Macro: A program written in the VBA environment is usually called a macro.

- GUI: Graphical User Interface

6 Programming with the VBA-GUI interface

6.1 Introduction

The ZEN VBA-GUI interface allows you to program ZEN with the same comfort and ease as using the mouse to control the GUI. All capabilities of the ZEN system are accessible via an intuitive syntax.

The main idea behind the ZEN VBA-GUI interface is to make ZEN commands available in the same way as they are in the GUI. The image shows how this principle works: To set the acquisition frame size, one has to

1. Select the **Acquisition** tab
2. Go to the **Acquisition** mode box
3. Set **Frame Size X** and **Y**

In the VBA-GUI interface, this translates to "Acquisition.AcquisitionMode.FrameSizeX" and "FrameSizeY".
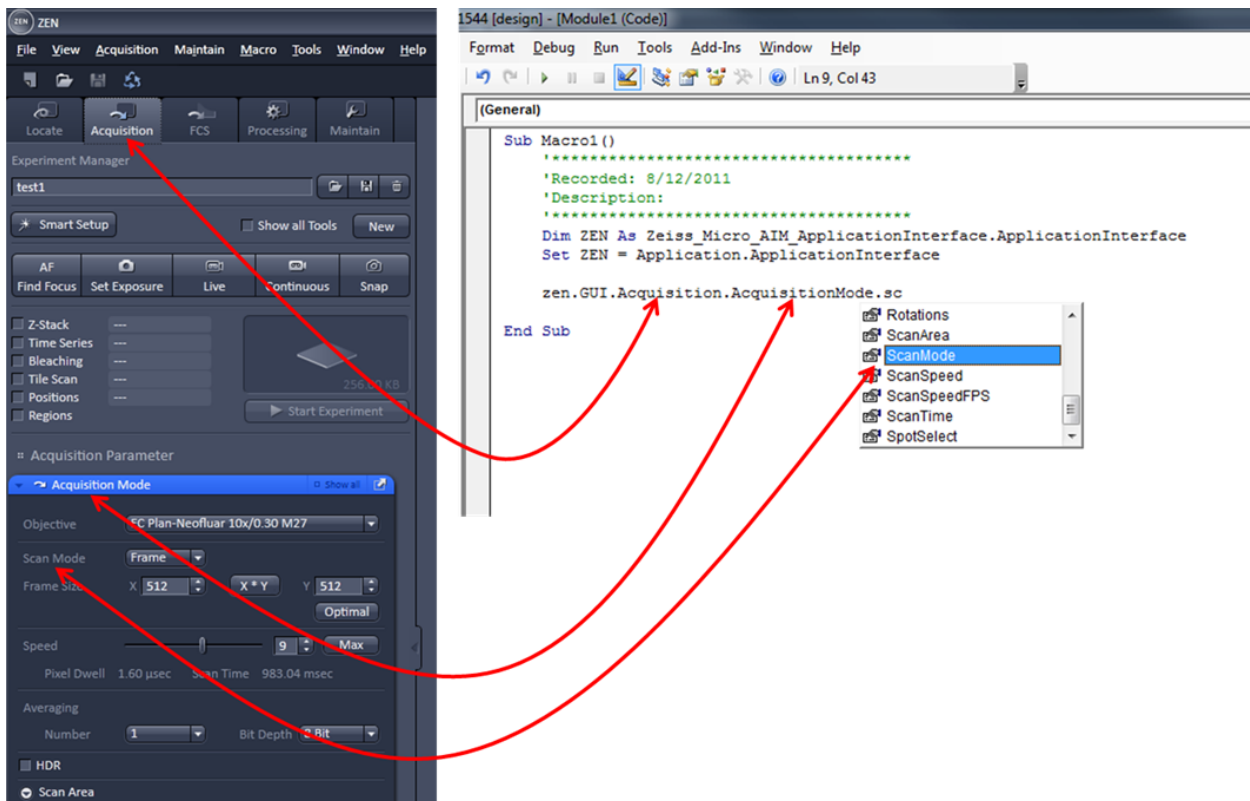
Fig. 12   **ZEN VBA-GUI Interface**

The easiest way to access the VBA-GUI interface is to start with a recorded macro. This contains a "ZEN" object, which has a "GUI" property. "ZEN.GUI" is the entry point to the VBA-GUI interface.

6.2 Structure of a VBA-GUI command

There are three different kinds of actions that can be performed in the ZEN GUI: Setting a value (either a number or a string, e.g. the frame size or configuration name), selecting from a list of options (e.g. the scan mode), and executing a command (e.g. Snap). To reflect this, different kinds of commands are available in the VBA-GUI interface.

All commands have a (Boolean, read-only) "IsEnabled" property that can be used to check whether the command is supported by the current system configuration. Also, commands have a (Boolean, read/write) "AsyncMode" property, that determines whether this command is executed asynchronously (i.e. program flow continues without waiting for the command to finish) or synchronously. The default is synchronous execution.

A command corresponding to a value in the GUI offers a "Value" property, which can be used to read or write the corresponding value. For numeric values, the command also offers (read-only) "Minimum" and "Maximum" properties.

A command corresponding to a range of options (i.e. a combo bvgbox or drop-down box) offers a selection using the "ByIndex" or the "ByName" properties. A list of available options (as name strings) can be queried from the "Items" property, and these name strings can be used in the "ByName" property.

A command corresponding to a button (e.g. Snap) offers two methods: "CanExecute" can be used to check whether the command can currently be executed, and "Execute" causes the specified action to happen.
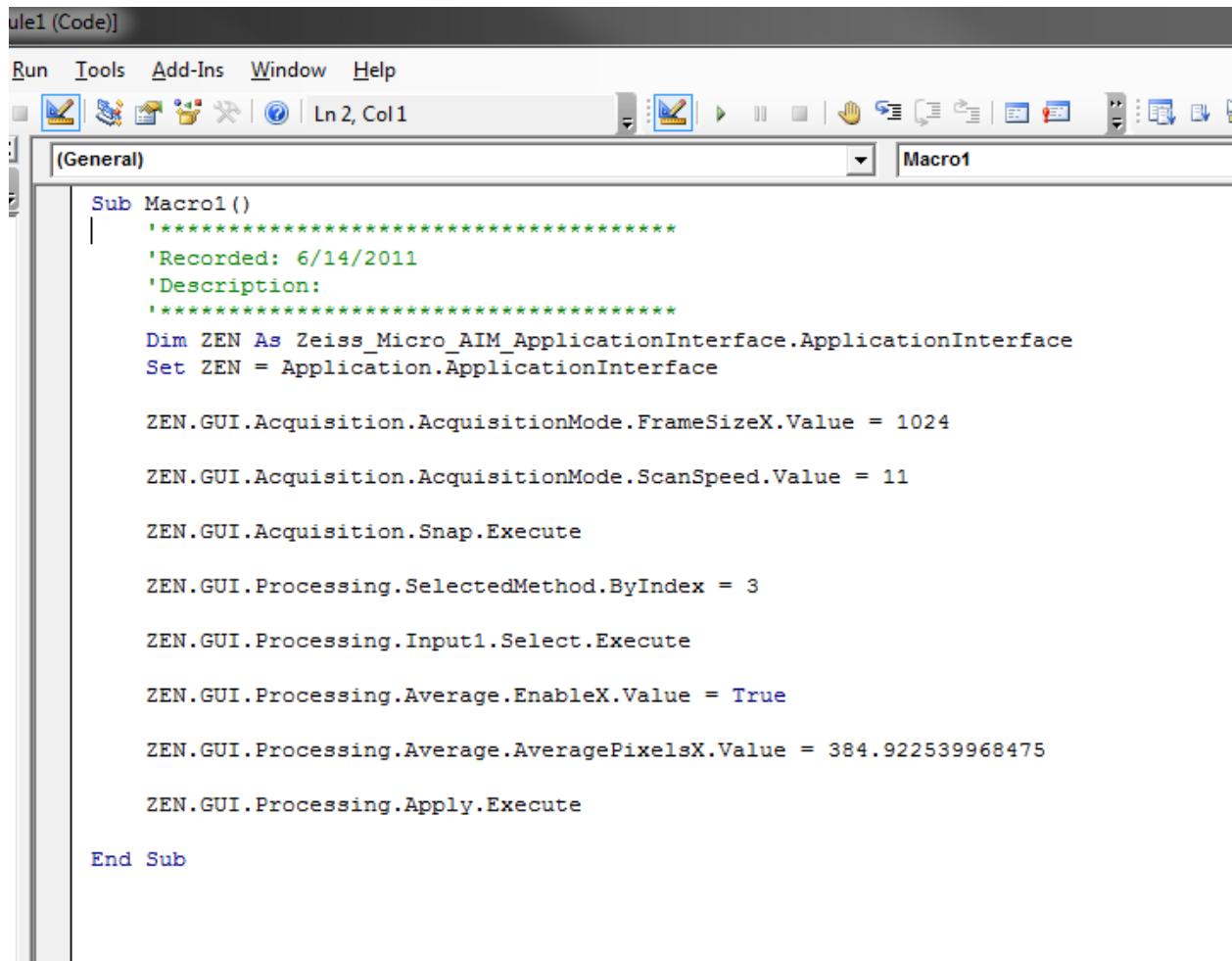
6.3 Recording a macro

When recording a macro, each click in the GUI is translated into a macro command in the editor. The image below shows an example in which the following steps are recorded:

- Set the frame size in X to 1024

- Set the scan speed to 11 (values are rounded)

- Take a Snap

- Select the processing function "Average"

- Select the currently active image as Input1

- Enable averaging in X-direction

- Set the averaging window in X-direction to a value of 385

- Start the processing function

The recorded macro can then be edited and executed just like a hand-written VBA program.

Fig. 13 **ZEN VBA-GUI Interface - Editor**

6.4 Selecting and managing image documents

Image document handling in the VBA-GUI interface happens via the "GUI.Document" command. From the list of open documents, images can be selected by index and by name, they can be closed and their acquisition parameters can be re-used. Also, a DsRecordingDoc pointer to the image object can be obtained directly from here.

6.5 Technical note

The ZEN VBA-GUI interface is a new component for the 2011 release of ZEN (black edition only). It is not backwards compatible with earlier releases of ZEN, which means that macros using this interface cannot run on earlier ZEN systems. However, macros using the conventional ZEN VBA commands can still be written and run on ZEN 2011. Thus, as long as no command in your macro begins with "ZEN.GUI", it is compatible to earlier releases.

Additionally, the VBA commands for ZEN black edition (including the VBA-GUI interface) are not compatible to ZEN blue edition.

7 Example Macros

This library of example macros is designed as a starting point for your own programming ventures. They are deliberately kept simple for quick understanding and hand-on. We recommend that you create copies the macros and then edit them to see how ZEN reacts to your commands.

You can find the macros in ZEN\Macros\ExamplesMacros.

7.1 Access to images

This macro demonstrates how to access the pixel values of an existing image.

[C:\ZEN\Macros\ExamplesMacros\access_to_images.lvb]

7.2 Backup recording settings

This macro demonstrates how to backup the acquisition settings and then later change the settings back to the original ones.

[C:\ZEN\Macros\ExampleMacros\backup_recording.lvb]

7.3 Event handling

Events can be used to react to actions by the user or by the hardware. Typically, events in ZEN are either

- Mouse events
- Scan state events
- Hardware events

This macro shows how to react to mouse events in a VBA macro.

[C:\ZEN\Macros\ExampleMacros\CaptureMouseEvents.lvb]

7.4 Export, Save and Load

This example demonstrates how to export an image (as TIFF), save it (in the CZI format), and load it (from CZI and TIFF). It can be easily adopted to other file formats.

[C:\ZEN\Macros\ExampleMacros\Export_Save_and_Load.lvb]

7.5 VBA-GUI API

We have not created example macros for the VBA-GUI API, because these can easily be generated by the macro recorder functionality. Activate macro recording and the execute the actions that you want to automate. Then edit the script to suit your needs.

Note: If you are afraid of working with an online-system, you can also record and edit macros in the ZEN Demo mode.

CHAPTER 3   ADDITIONAL SOFTWARE TOOLS

CONTENTS

Page

1   System Maintenance Tool

With a calibration objective and correct system configuration, the System Maintenance Tool allows convenient self-adjustment of LSM.

The optical beampath, relative pinhole position and scanner adjustment can be set and checked automatically with this tool. In systems that combine different imaging devices (e.g. ELYRA), the System Maintenance Tool calibrates only the LSM scan head.

☞   The System Maintainance Tool is for Service only. If a usewr tries to open the tool, the system warns that "For ELYRA 7 the SMT is or Servie use only" (see Fig.x)
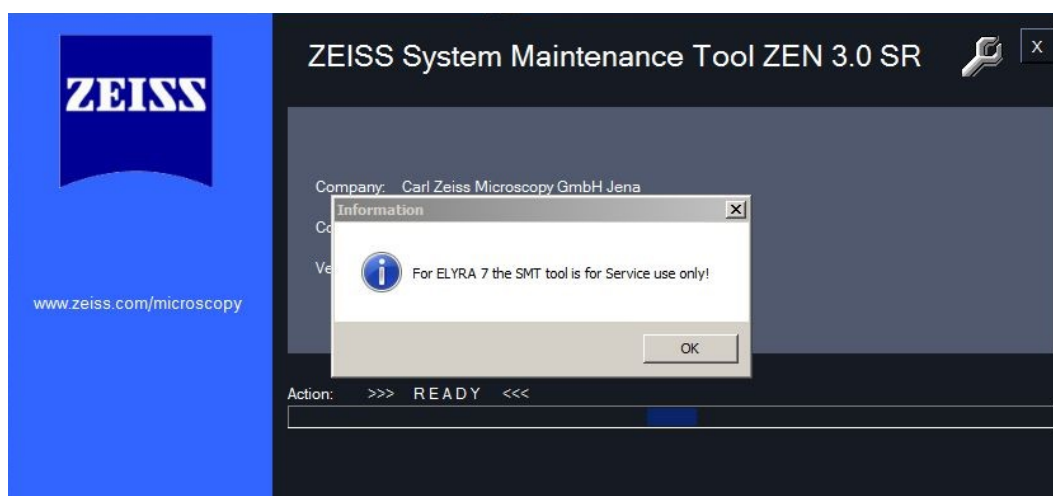
Fig. 1   System Maintainance Tool warning

The following sections therefore descrivbe selected tool for Service.

1.1   Calibration Objective

Observe the following notes:

− The calibration objective is not suitable for multiphoton excitation; there is a risk of bubble formation in the substrate.

− Using the HAL, the IR protection filter must be used, and the intensity of the HAL should only briefly be set to more than 70 %.

− With strong heat there is a risk of deformation of the substrate in the calibration objective.

− The lateral screws need to be tightened carefully without applying too much force.

− The calibration objective needs to be entered in the system database as "APO Calibration LSM" with SAP no. 420639-9000-700. It needs to be entered at ZEN \ Maintain \ Objectives and at the TFT display.

− Calibration objectives with other samples than shown in Fig. 2 shall not be used. The cap with the calibration sample can be obtained separately under SAP-No. 420639-9900-700.
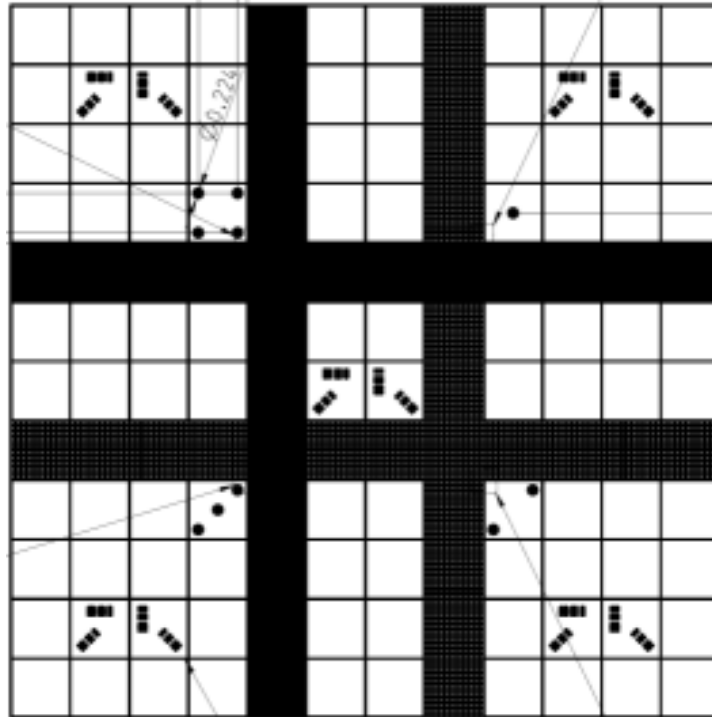
Fig. 2   Calibration objective

1.2   Starting the Maintenance Tool

Before starting the Maintenance Tool, the system hardware must be fully activated.

To do this, open ZEN and check the following settings: Correct entry of all existing lenses; the calibration objective needs to be entered as **APO Calibration LSM**.

- Correct entry of all existing lenses; the calibration objective needs to be entered as **APO Calibration LSM**.

- For LSM systems with a 405 nm laser, an approximate collimator position should be saved for the calibration objective.

- The focus plane needs to be approximately correct

- All light sources (Hal, LED's, lasers) need to be activated

- If all these settings are correct, close ZEN and then start the System Maintenance Tool by clicking the desktop icon (Fig. 3).

Fig. 3   Desktop icon of the Maintenance Tool

During the system initialization phase, the System Maintenance Tool will display the boot status (Fig. 4).

Fig. 4   Boot status of the Maintenance Tool

- After start, choose **Log in… Customer** (type-in any name) → not available.
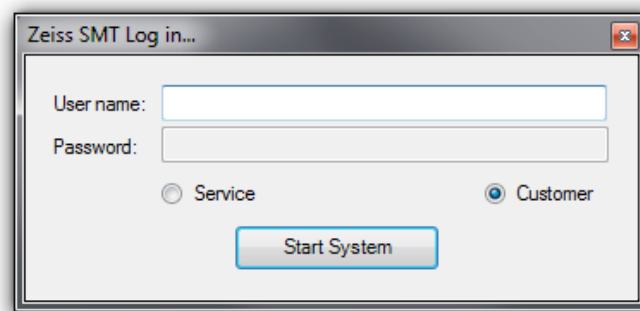
Fig. 5   Log in window

The Maintenance Tool can only be used, if the calibration objective is entered as **APO Calibration LSM**. If this is not the case, a message will remind the user to select the calibration objective.
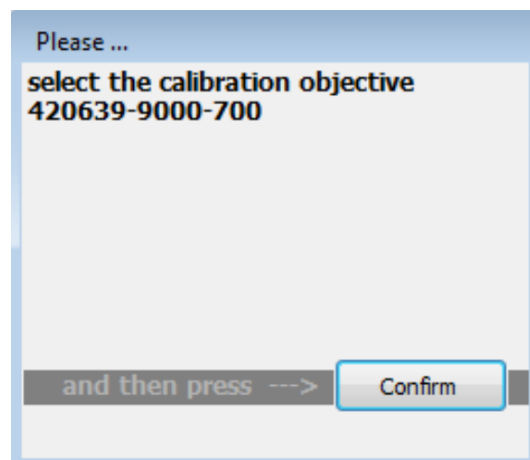
Fig. 6   Selection of the calibration objective

1.3   General Operation of the Maintenance Tool

The desired task can be chosen from the list and started with the start button.

Tasks available for LSM

- Sharpness Test
- Scan Field Test
- Scanner Calibration
- Pinhole to MBS Calibration

In case a task requires any manual adjustments, the Maintenance Tool guides users to all adjustment steps.

After the completion of a task, the result is displayed with a traffic light type green/yellow/red indicator.

- If green is indicated, the task has been fully accepted.

- If yellow is indicated, repeat the task and try to optimize the settings (e.g. check focus, KÖHLER illumination, halogen lamps, lasers (no standby) etc.

- If red is indicated, repeat the test one more time; if it still fails, contact your local LSM service engineer.
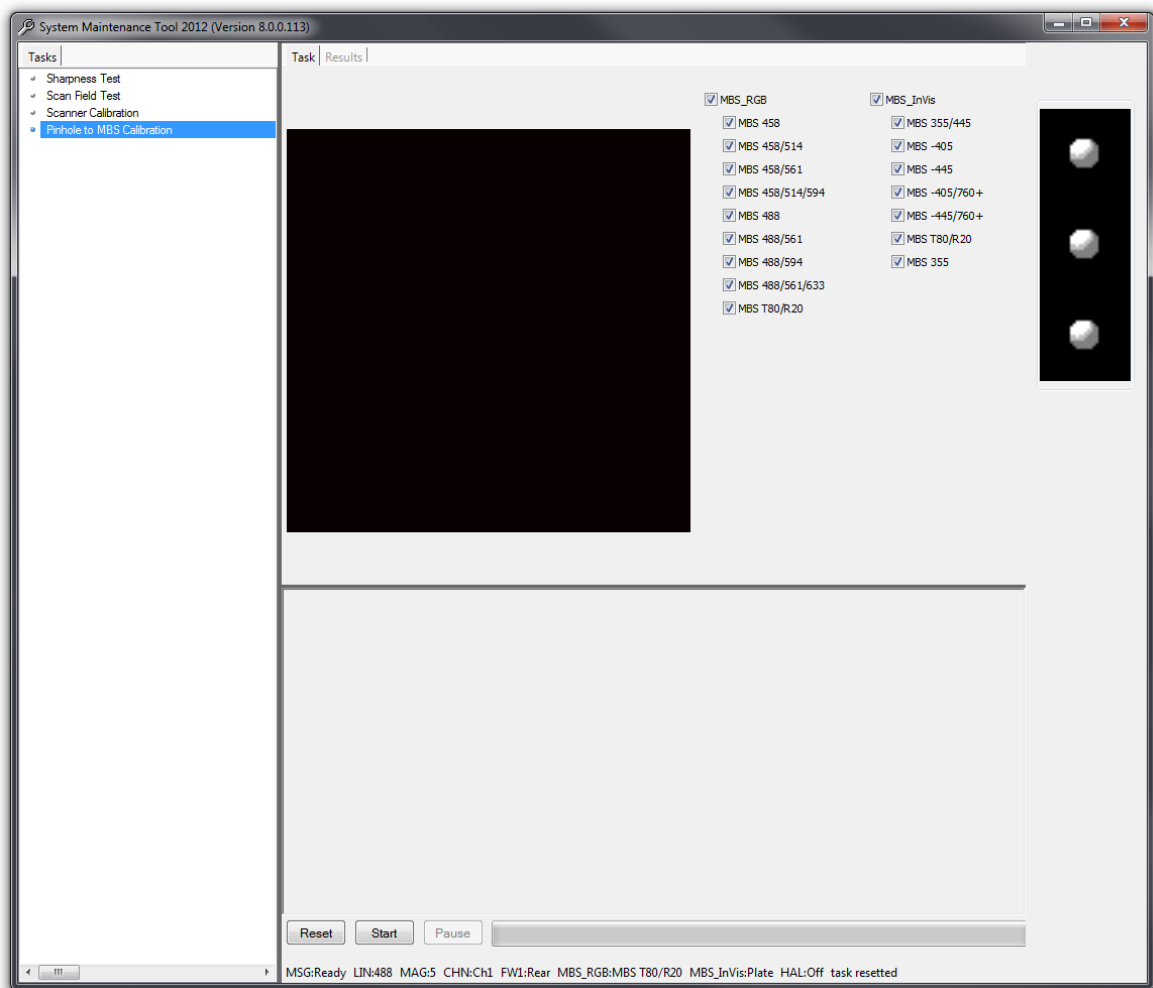
Fig. 7   **Maintenance Tool main window**
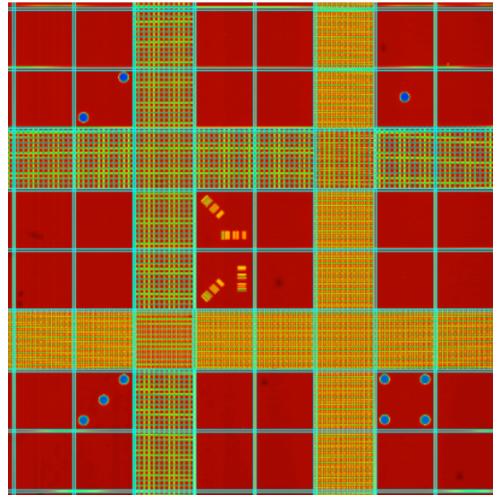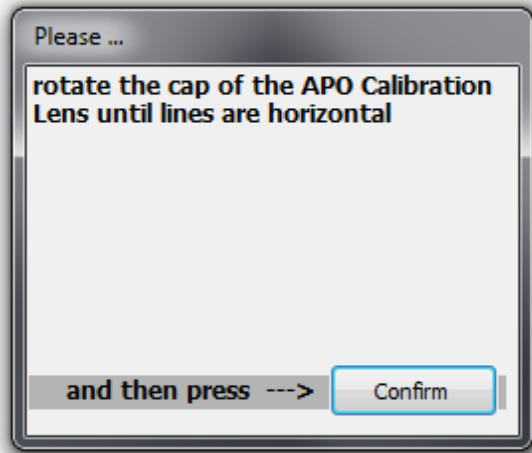
Fig. 8   **The Maintenance Tool guides users through required adjustment steps**

2   ZEN Configuration Tool

☞   The configuration tool provides access to the database of a system. Be aware that wrong entries can lead to a malfunction of the system. Always keep a backup of the original database. The database of the system is stored in C:/ZEN/database. Always make a copy of the database before you start to configure it.

Do not change any main settings or your system will not work anymore.

The following procedure describes how to define a new filter for external detectors like NDD or BiG.2. Once defined within the configuration tool (Fig. 9), these filters will also be accessible via ZEN.

- Open the tool; it will refer to the last database used which should be the systems database.
- Make sure you have a copy of this database.
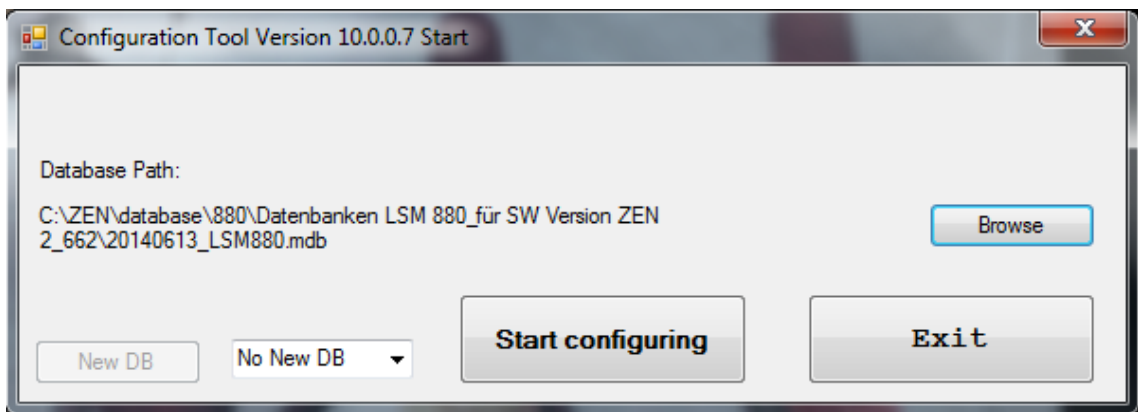
Fig. 9   Configuration Tool for LSM

- In case this is not the right database click **Browse** and choose the database to be configured.
- Click **Start configuring**.

- Within the appearing window (Fig. 10) choose the tabs **Scan Head**, **LSM 880**, **Filter**.
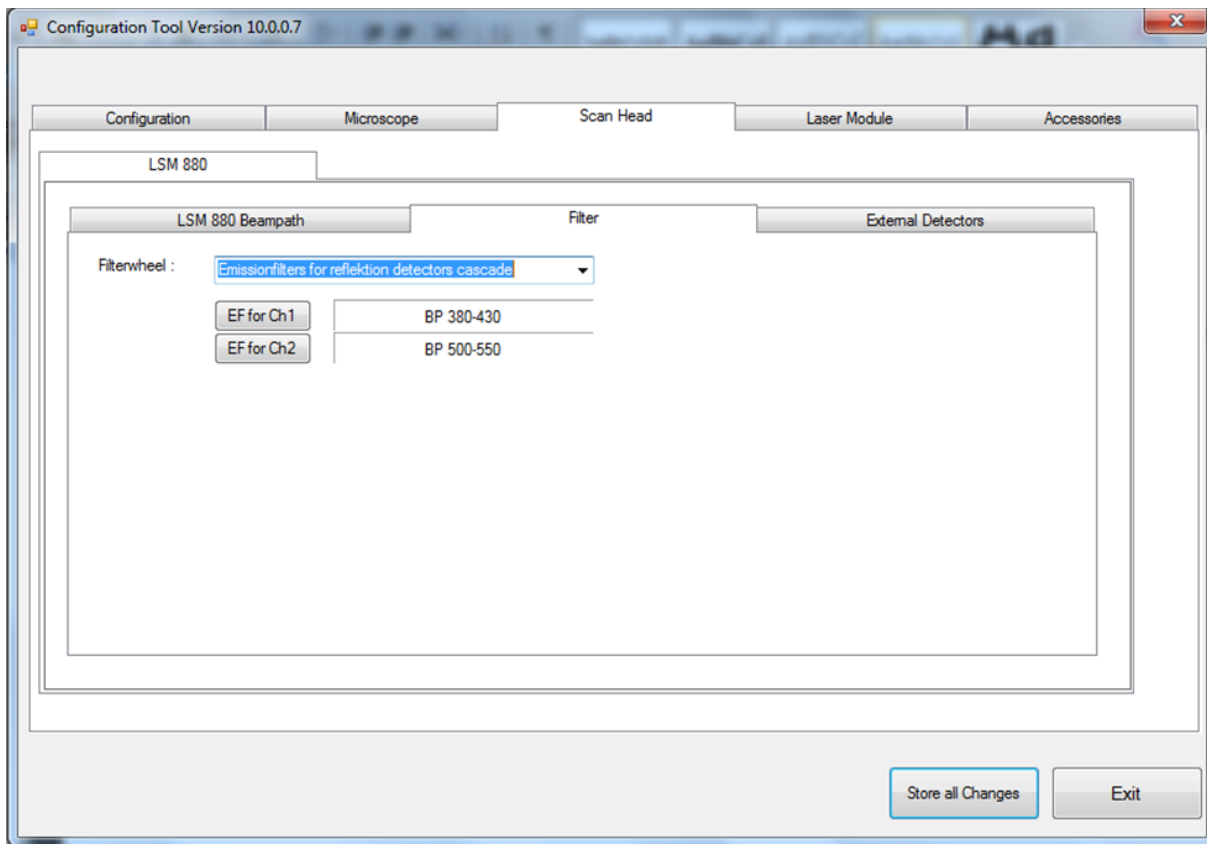
Fig. 10   Configuration Tool with seleceted tabs for access to filter settings

- Do not change any existing filters or filters from filterwheels inside the scan head. This leads to malfunctions of the system!

- From the drop down list next to **Filterwheel:** choose the device you want to define a new filter for.

This is either "Emissionfilters for DC-Out detectors" (BiG.2 on DCout port), "Emissionfilters for reflection detectors cascade" (any detector used on NDD port for reflection) or "Emissionfilters for transmission detectors cascade" (any detector used on NDD port for transmission) (Fig. 11).
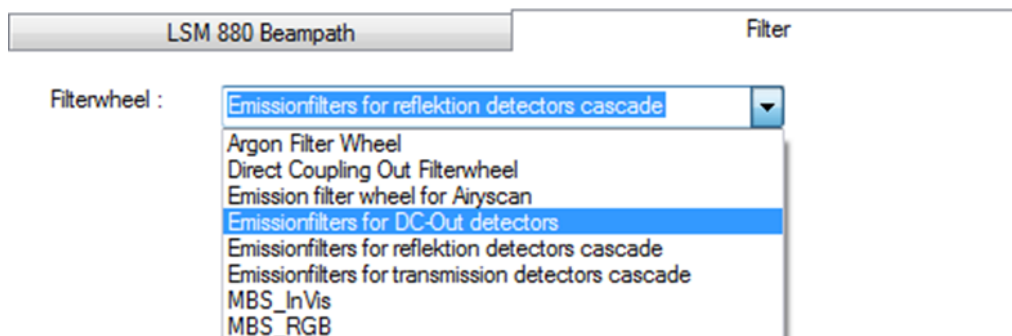
Fig. 11   Options of external detectors for which a new filter might be defined
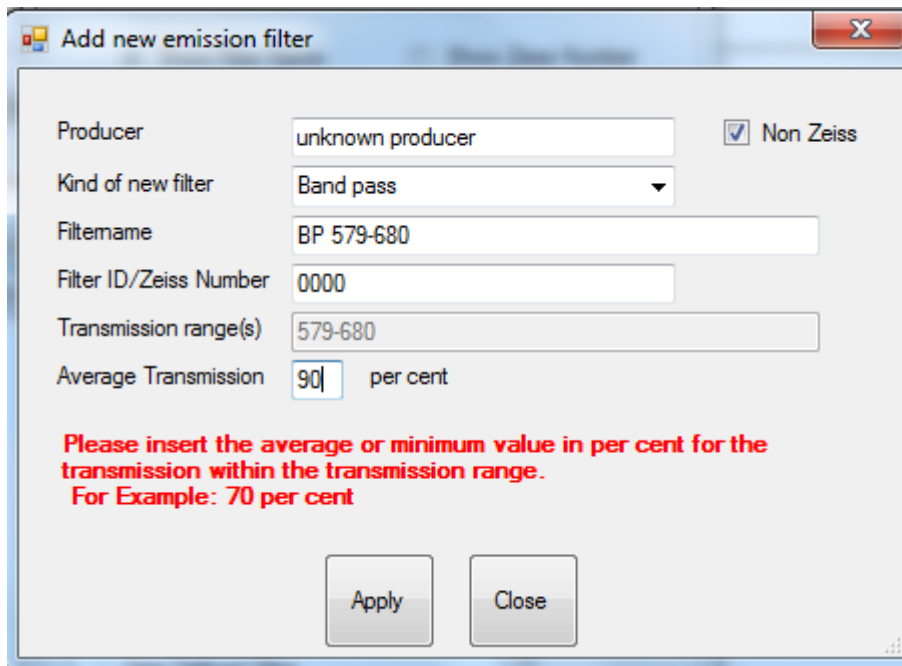
After selecting the "Filterwheel", the current filters are indicated each with a button next to it referring to the detection channel it is effective for (Fig. 10).

- Clicking this button opens the **Change Filter** window with a list of favourite, potential and user defined filters (Fig. 12).
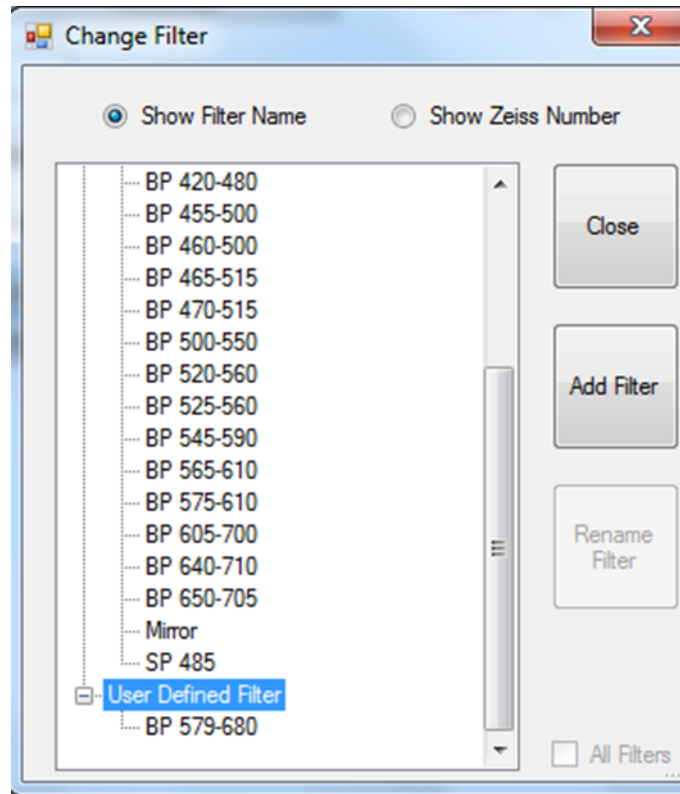
Fig. 12   List of Favourite, Potential and User Defined Filters

- To define a new filter, highlight the list point "User Defined Filter".
- The **Add** button becomes active and upon clicking **Add** the **Add new emission Filter** window opens (Fig. 13).

Fig. 13   Add new emission filter window

- Indicate the new filter as **Non Zeiss** and fill in the form to provide all necessary information to the system so it can use the information for image acquisition and processing.

The form on how to fill in the information is displayed as example in the lower part of the window when editing a special entry.

- Confirm the entry with **Apply** and then close the **Change Filter** window.

- Store all changes to the database before exiting the Configuration tool.

CHAPTER 4 ANNEX

CONTENTS

1   Software and Hardware Options

This section describes optional software and hardware configurations. Depending on your configuration, the content of dialogue and function may differ.

1.1   Software

The following software packages for ZEN 3.0 SR are available:

   – Software "ZEN Module PALM"
   – Software "ZEN Module PALM 3D"
   – Software "ZEN Module PALM Sequential"
   – Software "ZEN Module VBA"
   – Software "ZEN Module Shuttle & Find"
   – Software "ZEN Module Experiment Designer"
   – Software "ZEN Module 3D VisArt"
   – Software "ZEN Module SIM"
   – Software "ZEN Module Tiles & Positions"
   – Software "ZEN Module FRET"

If your configuration does not include the "ZEN Module FRET", the following functions are not available:

   – **FRET** view type

If your configuration does not include the "ZEN Module Shuttle & Find", the following functions are not available:

   – **Shuttle & Find** tool

If your configuration does not include the "ZEN Module VBA", the following functions are not available:

   – **New**, **Save** and **Save as** buttons in the **Macro Control** window
   – **Edit**, **Step**, **Delete**, **Editor** buttons in the **Macro Control** window

If your configuration does not include the "ZEN Module Experiment Designer", the following function is not available:

– **Experiment Designer** tool

If your configuration does not include the "ZEN Module 3D VisArt", the following functions are only available in a basic, stripped down version.

– **3D** view type in the **Image Display** document

If your configuration does not include the "ZEN Module 3D Deconvolution, DCV", the following function is not available in ZEN 2012)

– **DCV** button in **Processing**

If your configuration does not include the "ZEN Module Tiles & Positions", the following function is not available:

– **Stitch** function in **Processing**

1.2   Hardware

Depending on whether the following hardware components are available or not, the content of the screens may differ:

- Piezo objective focusing device / Z-Piezo stage
- X-Y Piezo scanning stage (different types available)
- Axio Observer.Z1
- Depending on the configuration the scan head equipment may differ in filters, beam splitters and the number of photomultiplier
- Transmitted-light PMT
- Non Descanned Detectors (NDD)

If your configuration does not include the Piezo objective focusing device or the Z-Piezo stage, the following functions are not available:

- **Use Piezo** in the **Z Stack** tool in the left tool area

If your configuration does not include an X-Y Piezo scanning stage, the following functions are not available:

- **Stage** tool, **Positions** tool and **Tile Scan** tool in the left tool area

Depending on the used microscope stand: Axio Imager.Z2, Axio Imager.M2, Axio Examiner or Axio Observer.Z1, the following dialogue and available functions may differ:

- Context and accessibility of the **Locate** tab.

2   Courses on "How to Operate the System in an Optimized Way"

ZEISS is offering training courses on how to operate the system in an optimized way.

- – Courses are held in our application center in Jena, Germany.
- – Courses are held in English or German language, respectively.

Check out:

www.zeiss.de/lsm

for the latest dates and ask your Zeiss representative for a quotation on courses.

3   Piezo Focusing Device

A Z-Piezo allows Z-stacks to be acquired considerably quicker compared to the focus drive of the microscope stand.

The speed advantage depends on the total travel range in Z and the speed of the acquisition of the individual image of a Z-stack.

The available travel range of a Z-Piezo device is much smaller compared to the Z drive of the microscope.

A Piezo device allows performing smaller steps in Z with a higher absolute precision for the individual steps.

A Piezo device is recommended for 3D measurements of objects and for 3D superresolution imaging (Airyscan).

Piezo Objective Focusing Device

For Axio Imager.Z2, Axio Imager.M2 and Axio Observer.Z1

Range: ≥ 200 µm

Minimum step size: 15 nm

Speed:

		Piezo objective focusing device
Slices	**Step size [µm]**	**xz-lines / s**
20	0.5	460

Objectives:

– M27

Installation:

• Screw in your microscope objective into Piezo Objective Focusing Device (see Fig. 1/**1**).

• Screw the thread-ring into your microscope (see Fig. 1/**2**).

• Easy clamp the Piezo Objective Focusing Device on the thread-ring (see Fig. 1/**3**).

1

2

3
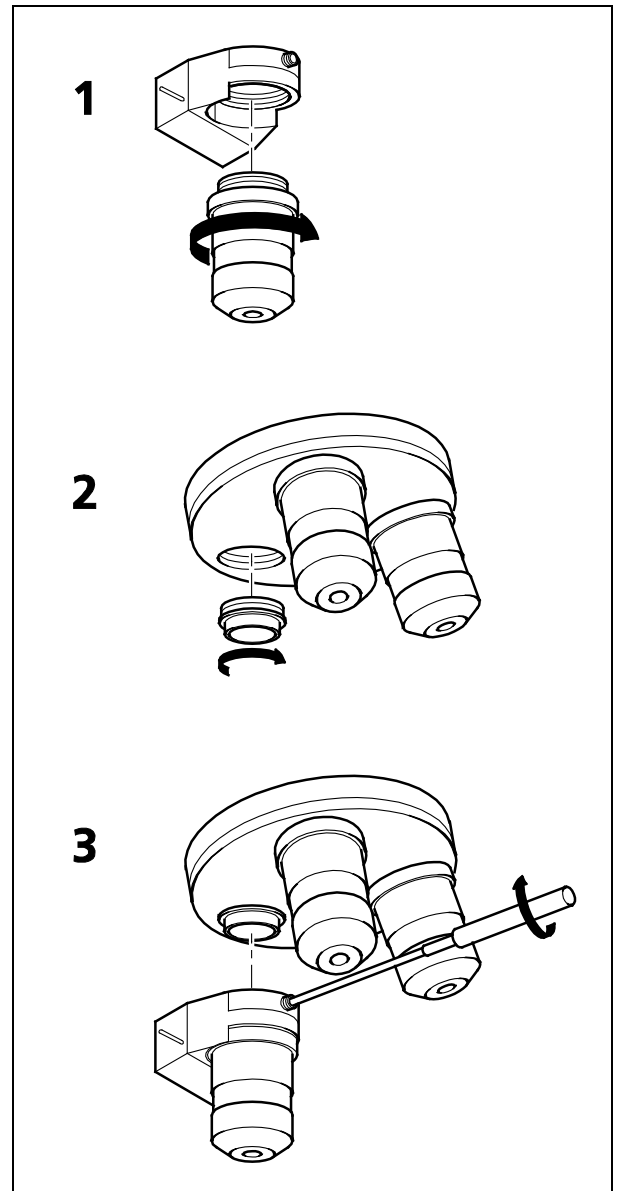
Fig. 1   **Installation of the Piezo Objective Focusing Device**

4   Specification of Trigger-Interface

The LSM provide two ways to control external devices or be controlled via external devices.

Synchronization signals:

This interface is provided directly by the electronics of the system and cannot be influenced via the user inter face. These signals are always present according to their specification. By connecting to the user interface connector these signals can be used to control external devices or synchronize external devices, like for example pulse generators, with the LSM. The detailed specification of these signals is described below.

The signal itself is identical to the signal of the pixelclock but is only set for one pixel.

Trigger signals:

The second interface is accessible via the software user interface in the tools **Time Series** and **Bleaching** and can be used to accept or provide signals for the following actions:

- Change interval of time series acquisition

- Set a marker within the time series acquisition

- Start Time Series

- End Time Series (and resume Time series when starting again with TriggerIN)

- Start Bleach event

Each signal itself is identical in length to the signal of the pixelclock but is only set for the time of one pixel.

Hardware Interface:

- User Port Adapter on the back of the Real Time Electronics in the System Electronic Rack

- Connector 2x **15-point** high density D-Type plug, 1x coax with outer shield
  (Triax Lemo S. A. Serie 00, EPL.00.650NLN)

Number of accessible interface signals:

- 4 x signal IN (asynchronous; able to generate an interrupt)

- 8 x signal OUT (synchronous)
  The label synchronous refers to the data stream PCIe2LVDS.
  The label asynchronous refers to the data stream PCIe2LVDS.   .

- 1x pixel clock

Connectors:

Pixelclock = Coax: Triax Lemo S. A. Serie 00, EPL.00.650NLN

Pin	Signal name	Description
Signal	PCLK_Out	PixelClock Output
Signal shield	GND	Digital ground
Outer shield	Shield	Chassis ground

The pixel clock is exactly symmetrical to the pixel. For example if the pixel rate is 10 kHz the high-level and the low level both have a time of 50 microseconds. The width of the TTL pulse therefore is 1/ (2 x pixel rate).

15-point high density D-Type plug: Plug "A"

Pin	Signal name	Description	Software interface
1	SyncOUT0 synch output	Synchronous output	Trigger 1 out
2	SyncOUT1	Synchronous output	Trigger 2 out
3	SyncOUT2	Synchronous output	Trigger 3 out
4	SyncOUT3	Synchronous output	Trigger 4 out
5	GND	Digital ground	-
6	AsyncOUT0 asynch output	Asynchronous output	-
7	AsyncOUT1	Asynchronous output	-
8	AsyncOUT2	Asynchronous output	-
9	+5 V	Va	-
10	GND	Digital ground	-
11	AsyncIN0 input	input	Trigger 1 in
12	AsyncIN1	input	Trigger 2 in
13	AsyncIN2	input	Trigger 3 in
14	AsyncIN3	input	Trigger 4 in
15	Connector detector*	Input / Connector detector	-

15-point high density D-Type plug: Plug "B"

Pin	Signal name	Description	Hardware output only
1	SyncOUT4 ** (stack)	Synchronous output	Sync. signal: Stack out
2	SyncOUT5 out Grabbing	Synchronous output	Sync. signal: Grabbing
3	SyncOUT outline **	Synchronous output	Sync. signal: Line out
4	SyncOUT outframe **	Synchronous output	Sync. signal: Frame out
5	GND	Digital ground	-
6	SyncOUT6	Synchronous output	-
7	SyncOUT7	Synchronous output	-
8	AsyncOUT6	Asynchronous output	-
9	+5 V	Asynchronous output	-
10	GND	Va	-
11	AsyncIN4 input	Digital ground	-
12	AsyncIN5	Asynchronous input	-
13	AsyncIN6	Asynchronous input	-
14	AsyncIN7	Asynchronous input	-
15	Connector detector*	Input / Connector detector	-

*) If the pin is connected to ground, the software recognizes that at least one connector is connected to the UserPort2.
**) The position of the two switches on the UserPortAdapter inside the RT-PC must switch thrown towards enclosure = default position.

Timing of the synchronization signals Stack out, Line out and Frame out

These signals are directly generated by the hardware and cannot be influenced by the software. They are also not seen by the user software and cannot be used to generate any markers within the image acquisition. They are highly precise tools to control external hardware. The signals are precise to the pixel and depending on the chosen pixel time the time between the signal and the start of the image acquisition varies.

The Line, Frame and Stack OUT signal is each set 16 pixels before the first image pixel of each line.

The signal itself is identical to the signal of the pixelclock but is only set for one pixel.

The Grabbing signal OUT is set to high-level with the start of data grabbing and set to low-level with the stop of data grabbing for each line.

☞   The Stack OUT signal is provided at the beginning of each Z-Stack if the Z-Stack is acquired with a Z-Piezo device (Z-Piezo stage frame or objective Piezo device)

Type/Voltage Range:

- TTL signal level 3.3 V, CMOS low power consumption
- 5.0 V tolerant input/output for interfacing with 5 V logic

Load:

Output: < 50 mA (internal serial 68 ohm resistor)

Input: 4.7 kOhm input impedance (internal 4.7 kOhm pullup to 3.3 V)

Trigger pulse description:

Signal output:

- Low level < 0.4 V, high level > 2.4 V (I = 10 mA)
- Slew rate 10 ns/V
- Reaction time trigger OUT 8 msec typical

Signal input:

- Low level <0.5 … 0.8 V, high level >2.0 … 5.5 V
- Falling edge force interrupt
- Pulse width to detect signal > 50 ns
- Reaction time trigger IN 16 msec typical

Caution:

- Never apply more than 5 V or negative voltages to avoid any damage.
- In and outputs are not galvanically decoupled.
- Therefore proper measures for galvanic decoupling of external devices have to be taken (opto-coupler etc.).

User interface box for synchronization and trigger signals (optional)

The interface box provides connections to the trigger IN and OUT functions accessible in the software user interface, the synchronization signals and the pixel clock. External devices can be connected to the LSM via standard BNC cables that are plugged into the user interface box. The box itself is delivered with the necessary cables to be connected to the user interface on the realtime electronics.
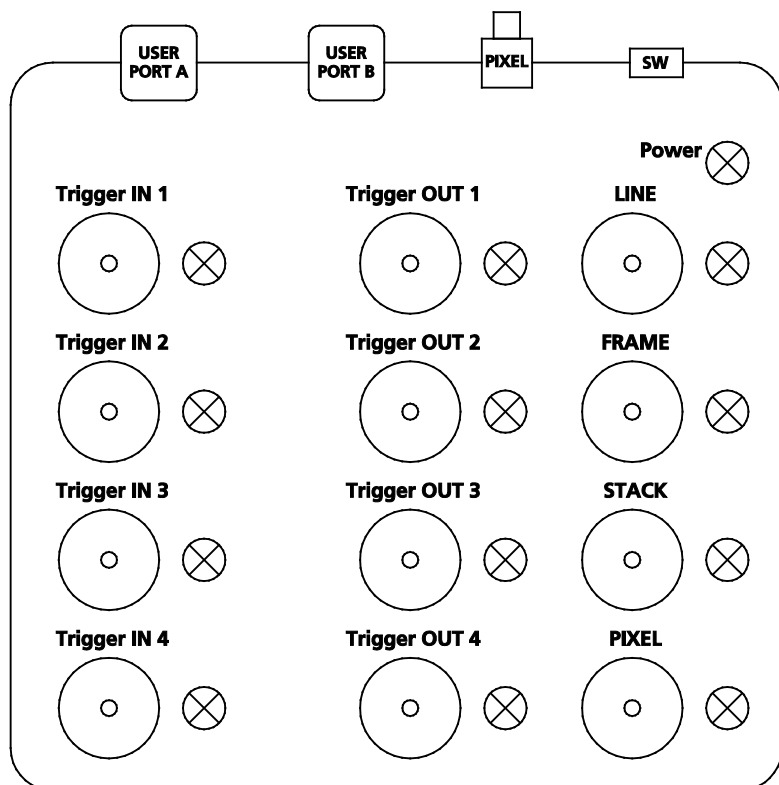
Fig. 2   **Panel of the User Interface box**

Trigger pulse description:

The pulses are identical to the pulses described for the user interface on the realtime electronics.

The LED lights signal the functionality of the individual plugs. The LED lights can be switched on or off with the switch **SW**. A LED blinks if a signal changes with a frequency of more than 2 Hz.

For a positive signal shorter than 500 ms, the LED is on for 500 ms. For a negative signal shorter than 500 ms, the signal is switched off for 500 ms.

The LED follows the level of the signals if these are longer than 500 ms. The LEDs react on pulses which are longer than 30 ns and have a frequency of less than 30 MHz.

5   Level-adjustable Sample Holder with Insert for Piezo Stage

⚠ Caution! The adjustment screws and other part of adjustable sample holders may collide with parts of the microscope, e.g. the condenser unit. When moving a motorized stage, avoid any collisions. Do not install inserts while the stage is moving. Be careful when switching the condenser front lens.

5.1   Level-adjustable Piezo Stage Insert

For the Piezo stage a level-adjustable stage insert is available. It consists of two parts (Fig. 3):

– Base plate (lower part) (Fig. 3, **bp**)
– Sample holder (upper part) (Fig. 3, **sh**)
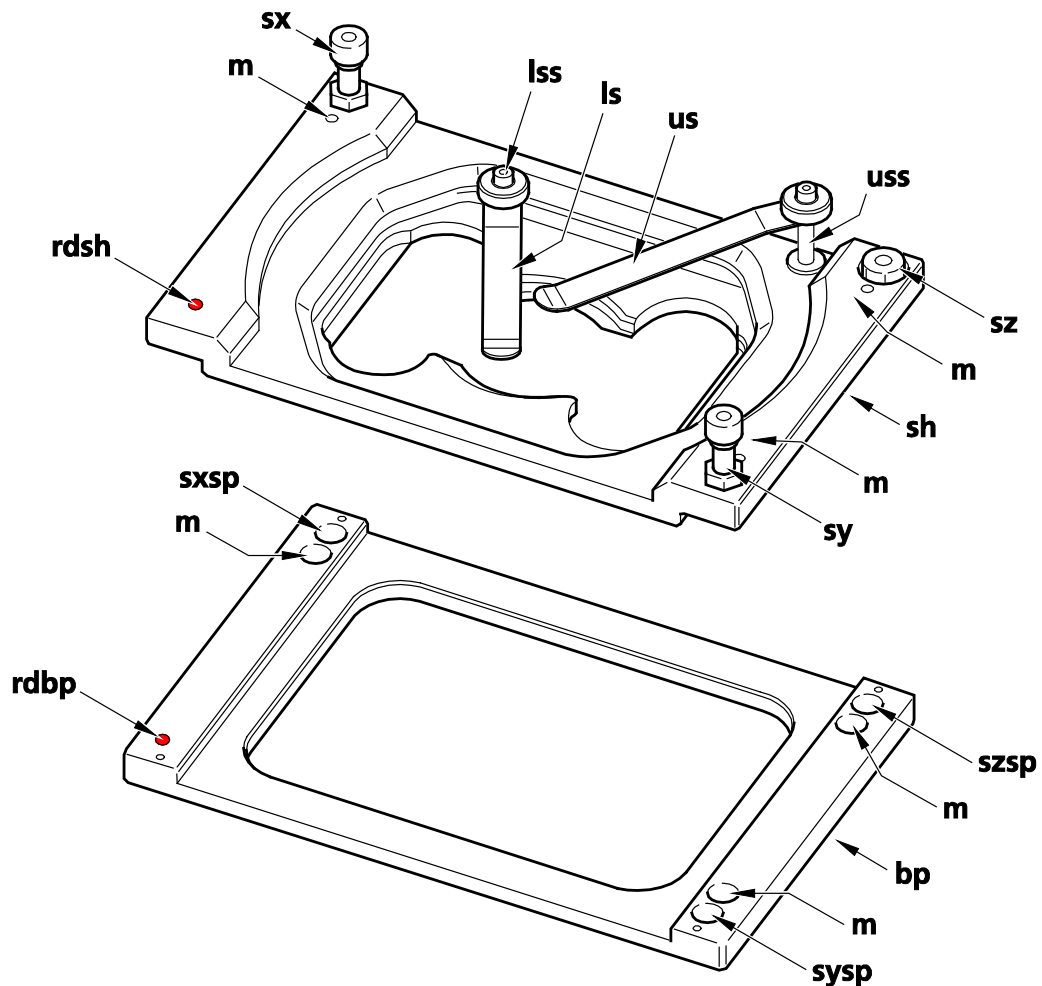
Fig. 3   **Level-adjustable Piezo stage insert – Sample holder and base plate separated**

The base plate is inserted into the Piezo stage. The sample holder is than placed on top of the base plate (Fig. 4). Both parts and both are held together by magnets (Fig. 3, **m**).

The sample holder is three-point mounted onto base plate via three screws (Fig. 3, **sx**, **sy** & **sz**) of the sample holder resting in their corresponding supporting points (Fig. 3, **sxsp**, **sysp** & **szsp**) (Fig. 4).

The front-left side of both, the base plate and the sample holder is indicated by a red dot (Fig. 3, **rdsh** & **rdbp**).

It should be ensured that the stage insert is mounted into the Piezo stage in the correct orientation.

When changing samples it is sufficient to take out only the sample holder, which is most conveniently done by pulling on the shafts of the upper and lower spring clamps (Fig. 3, **uss** & **lss**).

The strength of the magnets is weak enough that the sample holder detaches easily from the base plate when pulled. When inserted back the sample holder will attach to the base plate via the magnets (Fig. 3, **m**).

☞   If Koehler illumination was adjusted, the condensor can reach so deep that it can collide with the screws and upper spring shaft (Fig. 3, **sx**, **sy** & **uss**) when the full travel range of the motorized microscope stage is used. Care should therefore be exerted in this case by restricting the travel range. If Koehler illumination is not required it is recommended to move the condenser to the up position to avoid potential collisions.
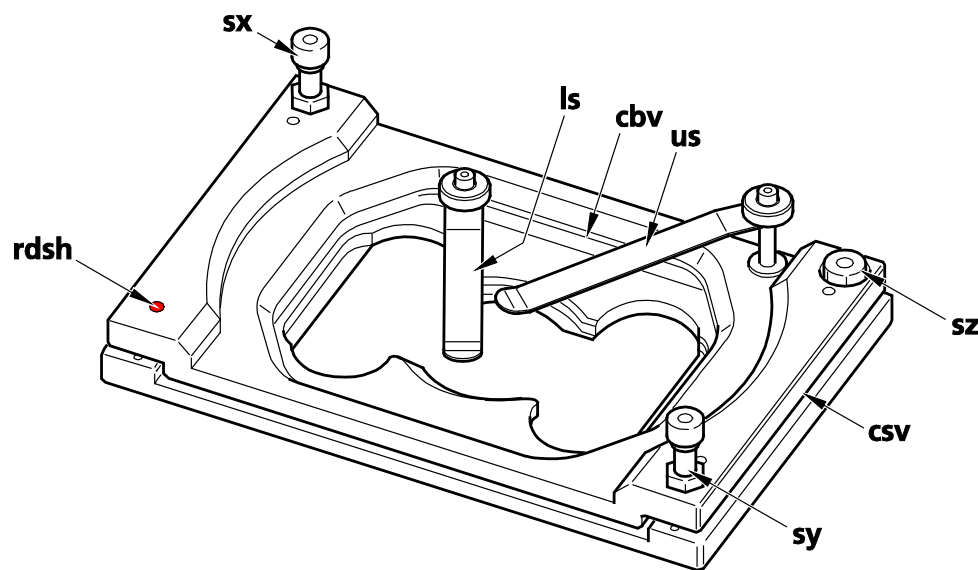
Fig. 4   Level-adjustable Piezo stage insert – Sample holder and base plate assembled
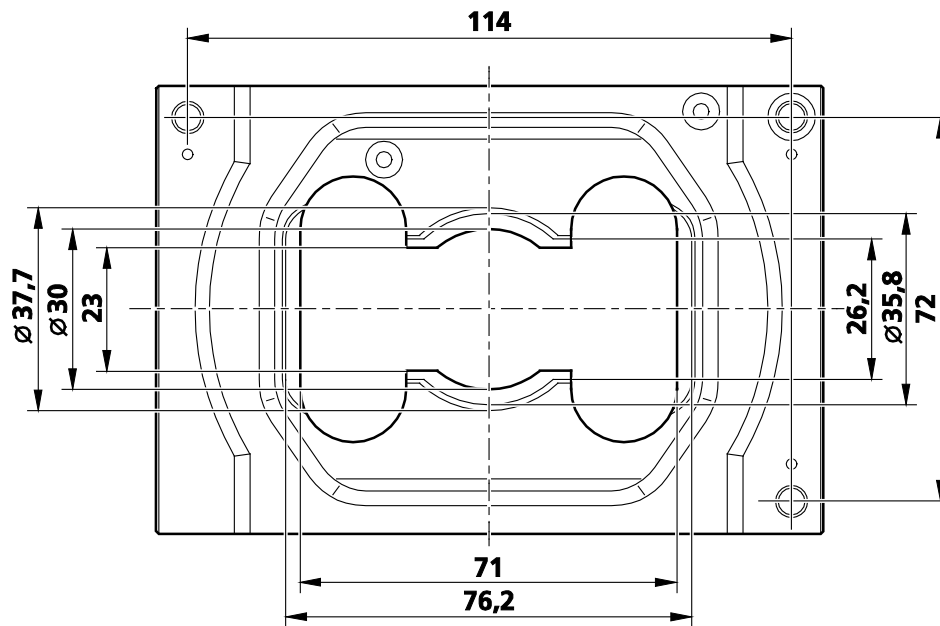
Fig. 5   Level-adjustable Piezo stage insert – Dimensions of the sample holder

The sample holder is designed to mount 36 mm Petri dishes, standard slides (76 mm x 26 mm) and Labtek™ chambers (Fig. 5). Slides can be clamped with the lower spring (Fig. 4, **ls**); dishes and Labtek™ chambers with the upper spring (Fig. 4, **us**). To secure a sample loosen the spring by pulling up, position the sample and clamp it by pressing the spring down until a resistance is experienced. To losen the sample, simply lift the spring.

☞   It is recommended not to place the lower clamp in a position directly over the objective lens to avoid back reflection of the excitation light. Rather clamp the sample at position offset from the lens position. Also one should restrain from using the lower spring for clamping dishes or Labtek™ chambers, as the induced strong strain on the spring can result in its damage. If not in use the springs can easily be stored by bringing them in a parallel position to the sample holder's longer edge. Secure in this position by pressing the spring down.

The three-point mounting allows for convenient leveling of the stage. Before leveling a sample one should make sure that the stage insert is correctly pre-adjusted. The cleft width measured at the corner of screw **sz** should be around 2 mm in size and is pre-set as such by the factory (Fig. 4).

If it happens to be substantially off, i.e. more than 1mm, one can adjust the distance with screw **sz** back to 2 mm. To pre-adjust the stage insert the cleft width viewed from the right (Fig. 4, **csv**) and back sides (Fig. 4, **cbv**) should be constant over their whole length; in other words the edges of the base plate and sample holder should be parallel. If this is not the case screws **sy** and **sx** (Fig. 4) can be used to adjust the edges to be parallel.

☞   When the cleft widths are constant and 2 mm in extension, there is enough range to adjust the leveling of the sample (about 1° tilt possible in both directions). If the cleft space is substantially larger or smaller and / or the edges are inclined to each other the range might be not sufficient for proper leveling.

In the following two procedures are given to adjust the level of the stage insert. Procedure 1 uses epi-fluorescence light, procedure 2 reflected light.

☞ For epi-fluorescence light either the X-Cite lamp or laser light can be used. Using the X-Cite lamp, if available, is recommended, since the incubator can be open and adjustment with the adjustment screws can be done under observation. In case of laser light the incubator has to be closed and adjustment cannot be done simultaneously with observation. Instead, having done an adjustment, the incubator has to be closed to monitor the outcome and the adjustment has to be done iteratively.

☞ The level-adjustable stage insert can become helpful when working with water objectives as their PSFs are sensitive to tilt in the sample.

5.2 Stage Leveling Using X-Cite or Laser in Fluorescence
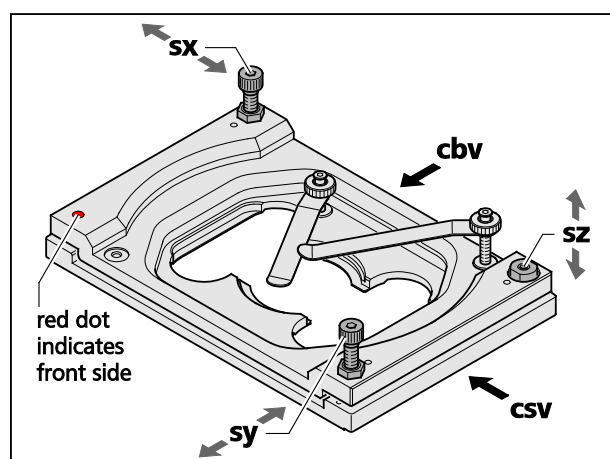
Fig. 6   Adjustable sample holder – schematic view
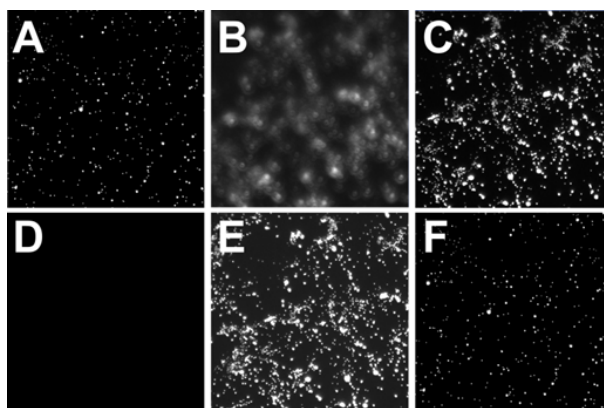
Fig. 7   Focus control in epi-fluorescence
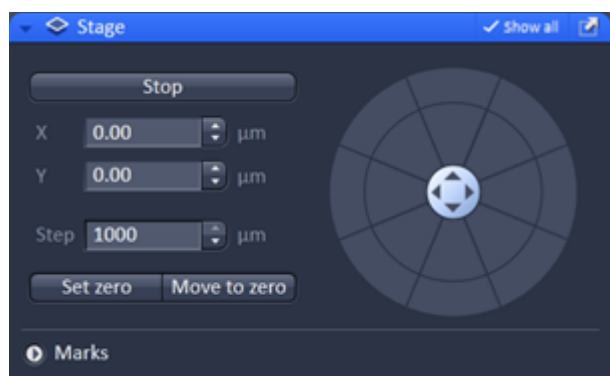
Fig. 8   Stage tool – x and y position set to zero

1. Take a fluorescent sample like embedded beads and mount centrally over the objective. (Note: you can also use your own fluorescent sample). Use WF illumination with the X-Cite or laser light and focus manually onto the sample using the stand's focus drive (Fig. 7/**A**).

2. Set stage to zero by pressing the **Set Zero** button in the **Stage** tool (Fig. 8). Set focus to zero by pressing the **Manual** button in the **Focus** tool (Fig. 9).

3. Adjust leveling in X in the following way:

    a) Enter in the **Stage** tool in the **Step [μm]** input box "1000" (Fig. 10). Press the upper or lower pointing arrow of the **X** input box, whatever is required to step 1000 μm to the right X direction.

    b) If the sample is out of focus (Fig. 7/**B**) refocus manually by using the stand's focus drive (Fig. 7/**C**); otherwise no further leveling is necessary. Record the relative change in the focus position by reading the value in the **Z-Position** input box of the **Focus** tool. If the focus is off more than 10 μm it is recommended to do a pre-adjustment. To this end adjust the edges of the clefts viewed from the right side (Fig. 6/**csv**) and back side (Fig. 6/**cbv**) to be parallel using screws **sy** and **sx**, respectively (Fig. 6). The cleft's width should be approximately 2 mm as preset by the factory, but can be adjusted with screw **sz** if needed.

    c) Multiply that value with **55** and manually set the focus onto this value using the stand's focus drive (Fig. 7/**D**). (Note: The factor **55** is only accurate, if the sample was positioned in the center.)

    d) Use the back left screw (Fig. 6/**sx**) of the sample holder to readjust the focus (Fig. 7/**E**). Turn clockwise if the focus position was positive, counter clockwise if it was negative. (Note that one turn corresponds to approximately 125 μm).

    e) Press the lower pointing arrow of the **X** input box of the **Stage** tool to return to the initial position. If the image is in focus (Fig. 7/**F**) X leveling is complete; otherwise repeat steps 3a) - e) iteratively. If applicable, the multiplication factor of 3c) can be adjusted.
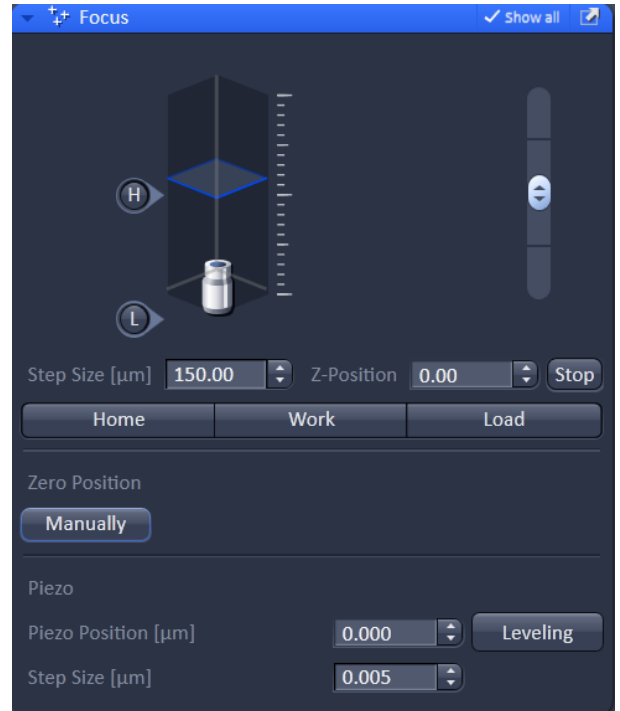
Fig. 9   **Focus tool – z-position set to zero**
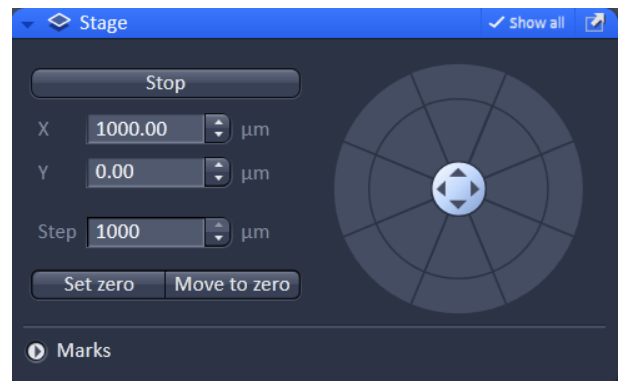
Fig. 10   **Stage tool – step size X set to 1000**

4. Adjust leveling in Y in the following way:

    a) Enter in the **Stage** tool in the **Step [μm]** input box "1000". Press the upper or lower pointing arrow of the **Y** input box, whatever is required to step 1000 μm upwards in the Y direction (Fig. 7/**A**).

    b) If the sample is out of focus (Fig. 7/**B**) refocus manually by using the stand's focus drive (Fig. 7/**C**); otherwise no further leveling is necessary. Record the relative change in the focus position by reading the value in the **Z-Position** input box of the Focus tool.

    c) Multiply that value with **40** and manually set the focus onto this value using the stand's focus drive (Fig. 7/**D**). (Note: The factor **40** is only accurate, if the sample was positioned in the center.)

d) Use the front right screw (Fig. 6/**sy**) of the sample holder to readjust the focus (Fig. 7/**E**). Turn clockwise if the focus position was positive, counter clockwise if it was negative. (Note that one turn corresponds to approximately 125 μm).

e) Press the lower pointing arrow of the **Y** input box of the **Stage** tool to return to the initial position. If the image is in focus (Fig. 7/**F**) Y leveling is complete; otherwise repeat steps 4a) - e) iteratively. If applicable, the multiplication factor of 4c) can be adjusted.

5.3   Stage Leveling Using X-Cite in Reflection
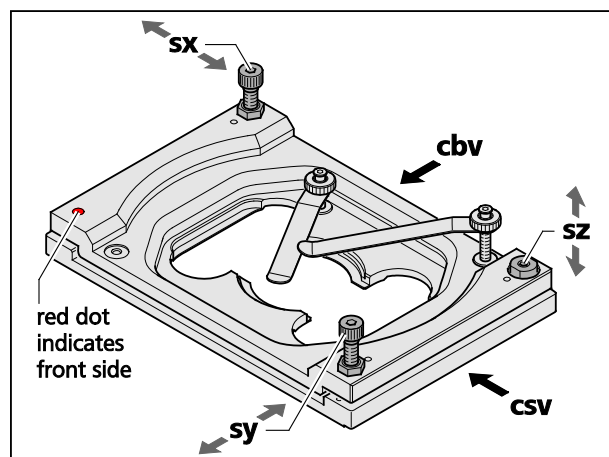
Fig. 11   **Adjustable sample holder – schematic view**
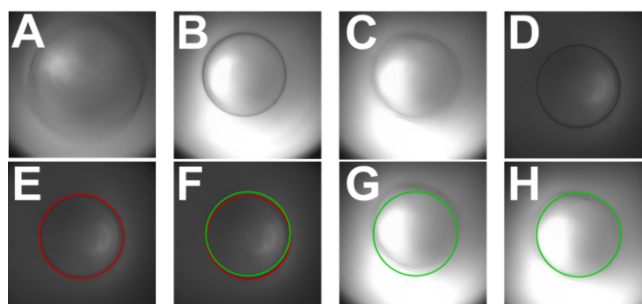
Fig. 12   **Focus control in reflection**

1. Assemble a fluorescent sample you want to level with sample bottom downwards into the sample holder. The sample site should be clean and free of any immersion oil. Position the sample such that the objective lens is in the middle of the sample. Use WF illumination with the X-Cite at 12 % and filter for excitation with the 561 nm line (optimal would be a small band pass to avoid too much light). Swing nosepiece to empty position (make sure no cover is in the empty position) and use preferentially Andor SIM camera, albeit the Andor PALM camera would also work. Take Optovar 1.0 and set EM gain to zero.

2. Draw fiber plug of X-Cite completely out from the X-Cite casing. Start acquisition. The image will be dark since fiber was pulled out. Put fiber back and slowly move in until you will see a coarsely adjusted image to have sufficiently reduced light levels you can work with (Fig. 12/**A**). (Note: with the fiber all in, the lamp is simply to bright leading to oversaturation of the camera). If the illumination is far from being centric, the sample is already strongly tilted and it is recommended to do a pre-adjustment. To this end adjust the edges of the clefts viewed from the right side (Fig. 11/**csv**) and back side (Fig. 11/**cbv**) to be parallel using screws **sy** and **sx**, respectively (Fig. 11). The cleft's width should be approximately 2 mm as preset by the factory, but can be adjusted with screw **sz** if needed.

3. Now pull (or push) fiber on the ELYRA site until the dark ring, which is already faintly visible in its contours, appears focused (Fig. 12/**B**). Dependent on the sample the focus could look worse, especially when the reflecting planes are wavy or you get reflection from different planes (Fig. 12/**C**).

4. Move in 10x objective and focus the already visible ring with the z-drive of the stand. The ring appears now well focused as long as the sample is not completely unsuitable (Fig. 12/**D**)

5. Mark the Ring with a ROI from the **Graphics** tool (3 point-circles will work best) (Fig. 12/**E** and Fig. 13).

6. Mirror the circle on the center of the image. (Note: If the circle is already centered in the middle that step is not required; deviations <20 pixel SIM camera or 10 pixel Palm camera are no problem and can be tolerated). To mirror the coordinates of the ROI **X, Y** (visible in the **Graphics** tab), calculate the new values as follows: X' = Xmax-X & Y' = Ymax-Y with Xmax and Ymax representing the number of pixels of the camera in X and Y-direction. E.g. if the coordinates X and Y would have been 502 and 512, respectively, the new coordinates X' and Y' would be calculated using the SIM camera to 1004-502=502 and 1002-512=490.

Fig. 13   Graphics tool – three point circle at position X=502 and Y=512

7. Draw a new circle with the same diameter in the original image with the new coordinates (Fig. 12/**F**). The old circle is not necessary anymore and can be switched off or deleted (Fig. 12/**G**).

8. Turn nosepiece back to free position. Than level stage by turning the screws of the sample holder (Fig. 11/**sx** & **sy**) until the more or less focused circle of the fiber overlaps with the ROI-circle (Fig. 12/**H**).

5.4   Stage leveling using X-cite in reflection and two glas plates
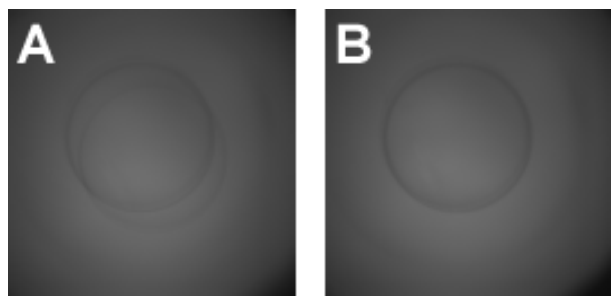
Fig. 14   **Focus control in reflection using two glass plates before and after stage leveling**

1. Swing nosepiece to empty position (make sure no cover is in the empty position). Place a glass plate evenly on the opening. Do not use coverslips as they tend to bend. Use a glass piece the thickness of a slide. You can also use a slide, though its big size makes placing it more difficult. Note that systems with Automated Component Recognition (ACR) will have a slightly elevated pin at the rim of the opening. Avoid placing the glass plate on top of it as it will cause the glass surface to be tilted.

2. Assemble a fluorescent sample you want to level with sample bottom downwards into the sample holder. The sample site should be clean and free of any immersion oil. Position the sample such that the opening is fairly in the middle of the sample. Use WF illumination with the X-Cite at 12% and filter for excitation with the 561 nm line (optimal would be a small band pass to avoid too much light). Use preferentially sCMOS pco.edge camera, albeit the Andor PALM camera would also work. Take Optovar 1.0 and set EM gain to zero.

3. Draw fiber plug of X-Cite completely out from the X-Cite casing. Start acquisition. The image will be dark since fiber was pulled out. Put fiber back and slowly move in until you will see a coarsely adjusted image to have sufficiently reduced light levels you can work with. (Note: with the fiber all in, the lamp is simply to bright leading to oversaturation of the camera). You might see already two unfocused rings; but there is also a chance that you cannot see the fiber outline at al. Pull the fiber on the ELYRA site until the two rings become clearly visible and are in focus (Fig. 8, **A**). If the illumination is far from being centric, the sample is already strongly tilted and it is recommended to do a pre-adjustment. To this end adjust the edges of the clefts viewed from the right side (Fig. 4, **csv**) and back side (Fig. 4, **cbv**) to be parallel using screws **sy** and **sx**, respectively (Fig. 4). The cleft's width should be approximately 2 mm as preset by the factory, but can be adjusted with screw sz if needed.

4. Level stage by turning the screws of the sample holder (Fig. 3, **sx** & **sy**) until the more or less focused circles overlap (Fig. 8, **B**).

6   Liquid Cooling System of ELYRA

The temperature of the scanners within the LSM scan head is controlled with a liquid cooling system. The liquid cooling system is also used to dissipate the heat generated by the electronics and the additional Peltier cooling of the detectors (GaAsP PMT detectors as well one of the side multi-alkali PMTs).

The liquid used for the device can be ordered via ZEISS service.

The system is equipped with an optic and acoustic warning if it does not function as needed. The following error code, which is also indicated on the unit itself, applies:

Green LED flashing or constant: System is working

Red LED flashing once: Cooling liquid is too warm. Check the temperature in the room. It should be at 22 °C plus/minus 3 °C. If it is more than 30 °C the cooling system cannot work.

Red LED flashing twice: Cooling liquid level is too low. Add liquid to the system.

Red LED flashing three times: Cooling fan is failing. Call service for maintenance.

Red LED flashing four times: No cooling liquid is in the system. Fill the system with liquid.

A corresponding acoustic signal is provided along with the optic signal.

It is recommended to exchange the liquid every two years. Refilling frequency is dependent on room conditions and adding liquid might be necessary more often.

In case the temperature within the scan head rises above 50 °C at any measuring point, the system will show and warning and shut itself down. Check the liquid cooling system before restarting the system.

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7   Autocorr Objectives - Installation

☞   When retrofitting autocorr objectives in most cases the firmware of the microscope has to be updated. Check with your local ZEISS support, whether this also applies to your system.

- Indicate the objective with its correct number (written on the objective) in the software of the microscope through the TFT interface.

- Shut down the microscope/system and connect the additional CAN distributor box to any free connector on the microscope.

- Screw the objective into the position of the objective turret that was set in the TFT interface. You might have to remove neighboring objectives to do so. It is not possible to use two adjacent positions for autocorr objectives; they have to alternate with standard objectives.

- Check whether turning the objective turret does not lead to a collision of the objective with the microscopes body. If so, loosen the small screws at the objective thread and slightly turn the objective such that it fits without colliding. Don't forget to retighten the screws.

- Connect the objective to the can distributor box using any free connector.

- Restart the microscope / system.

- Open the **ZEN Configuration Tool** and perform the following steps to indicate the autocorr objectives to the software:
  - Chose main tab: **Microscope**
  - Chose sub-tab: **Objectives**
  - Activate the checkbox: **motorized correction ring**
  - Click onto the button for the objective position (i.e. **Pos3**) which is identical to the one which now holds the autorcorr objective.
  - In the **Objectives** dialog chose the corresponding autocorr objective and select it for this position
  - Confirm the changes to the database by clicking "Store all changes".
  - Exit the **ZEN Configuration Tool**.

- Start ZEN.

- Within the **Acquisition** tool window the slider for controlling the correction ring is now visible when the autocorr objective is selected. See also the corresponding description in the SW chapter of this manual (*Chapter 1 System Operation*).

- The last position of the slider is stored when the system is shut down. When restarting the system again, the last positon of the slider is then set again.

- See also the corresponding description in the SW chapter of this manual (*Chapter 1 System Operation¸ Objective Selection*) for further instructions.

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8   ZEISS Microscopes Networked

8.1   About the Philosophy of our ZEISS Microscope System Computers

In 2016, ZEISS offers a number of capable workstation computers in all popular price classes which can be used in standalone, or in a networked configuration.

The common philosophy is to offer selected components with maximum data safety and good performance. The systems can run 24/7. Optional components allow to expand the working memory, fast OS file swap/transfer, storage and networking. Also, since 2016, we offer Cuda compatible graphics boards.

The system installation and OS is located on a redundant RAID on most of our computers. In case the speed of the RAID is not optimal for OS file swap/transfer, a fast PCIe or M.2 SSD is included to speed up processing performance.

With the introduction of the Z240 basis workstation, all computers allow to install a fast 10 GbE network connection for streaming and data exchange.

Storage computers are available as dedicated storage PCs (36TB RAID, up to 3x 10 GbE plus 1x 1GbE), or as upgraded Z840 workstations (max. 12TB HDD, 1x 10 GbE and 2x 1 GbE).

Nvidia Quadro M4000 graphics boards (8GB VRAM) with Cuda compatibility for fast deconvolution processing are available as an upgrade for older FirePro graphics boards.

8.2   Microscope System Computers and Software

Supermicro Storage

HP Z840 Workstation

HP Z Displays 24"/27"/32"

HP Z240 Workstation

8.3 Compatibility and Feature List of Components

System	HP Z240	HP Z840 single CPU	HP Z840 dual CPU	Supermicro Storage
CPU	Core i5 (4-core)	Xeon (4-core)	Xeon (8-core total)	Xeon (12-core total)
RAM max (with 16GB modules)	64GB (16GB default)	128GB (16 GB default)	256GB (64GB default)	256GB (64GB default)
HDDs by default	4TB no RAID	2TB RAID 1	4TB RAID 10	36TB RAID 5
HDD options	2x 4TB HDDs (need 5,25" mounts)	2-4x 4TB HDDs (need 5,25" mounts)	2x 4TB HDDs (need 5,25" mounts)	none
Swap	128GB M.2 SSD	256GB PCIe SSD	on RAID 10; optional 512GB PCIe SSD	256GB SSD
Graphics	Quadro K620 2GB	Quadro K620 2GB; option Quadro M4000	Quadro K2200 4GB; option Quadro M4000	Quadro K2200 4GB; option Quadro M4000
Network	1x 1GbE onboard; option 1x 10GbE card	2x 1GbE onboard; option 1x 10GbE card	2x 1GbE onboard; option 1x 10GbE card	2x 10GbE and 1x 1GbE onboard; option 1x 10GbE card
Remarks			ELYRA: either PCIe SSD or 10GbE card	

8.4 Networking of ZEISS Microscopy Computers

ZEISS microscope computers can be networked. The main purpose of such networking is storage of image data, faster processing, workspace economy and service.

Networking is not intended for experimental download of drivers, patches, OS system upgrades or untested third party software. This will very likely harm the system installation.

Networking also requires a certain work discipline, especially when handling file transfer and internet access. Any files, which might contain harmful code should not be transferred to a system computer. It is not recommended to connect the system computers to the internet without protection.

It is recommended to operate the ZEISS microscope computers in a kind of shielded LAN or e.g. via VPN, and to have only one or two (e.g. storage) computers connected to the internet.

These computer can handle download of service packs or remote access for the actual system computers.

Some third party software has been approved to run together with our ZEN software. Arivis is one of our approved partners. Ask your local ZEISS imaging specialist for the current software partners of ZEISS.
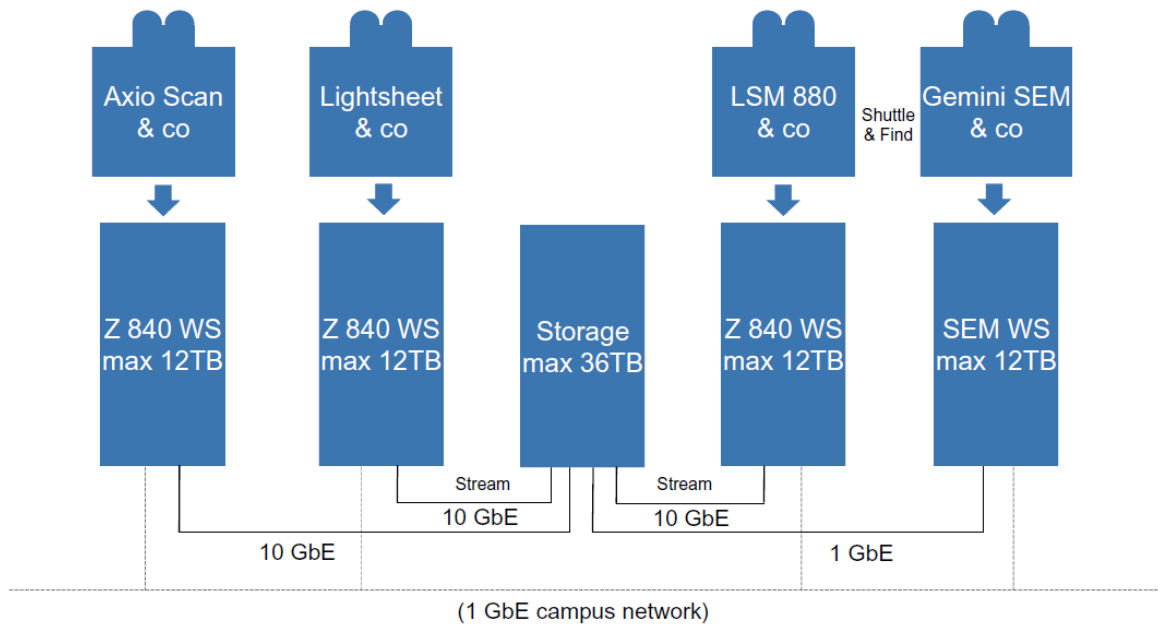
8.5   Solution 1: Peer to Peer
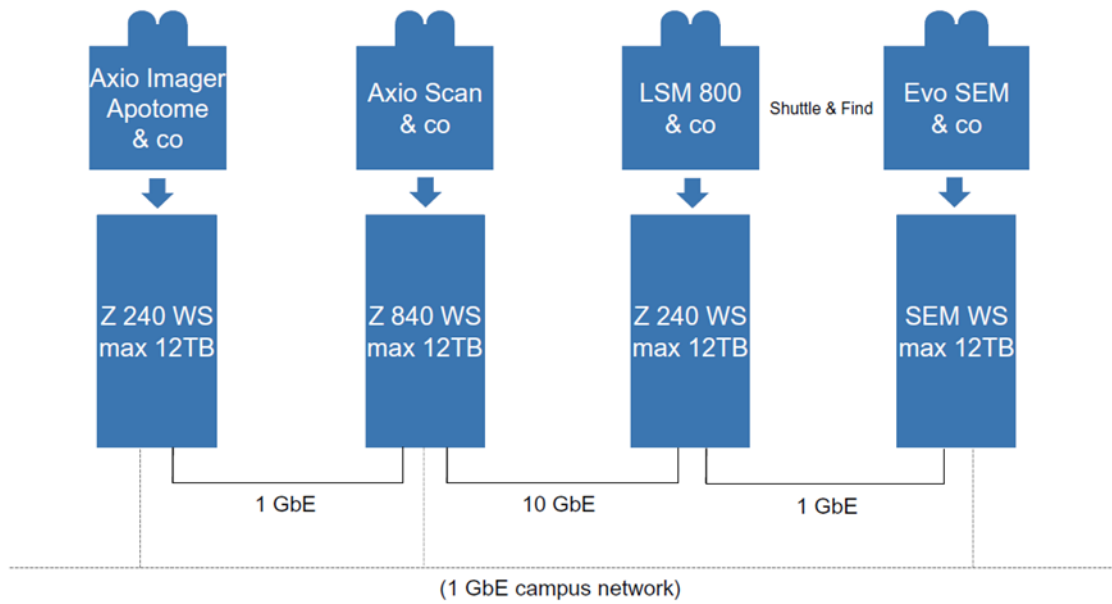
In this peer to peer configuration, a number of Imaging microscopes with workstations are networked via LAN connections. Optional 10 GbE adapters allow fast file transfer for preferred systems which share data of common experiments. Onboard 1 GbE adapters allow connection to a campus network. Optional 4TB HDDs allow to increase the shared storage space of the computers.
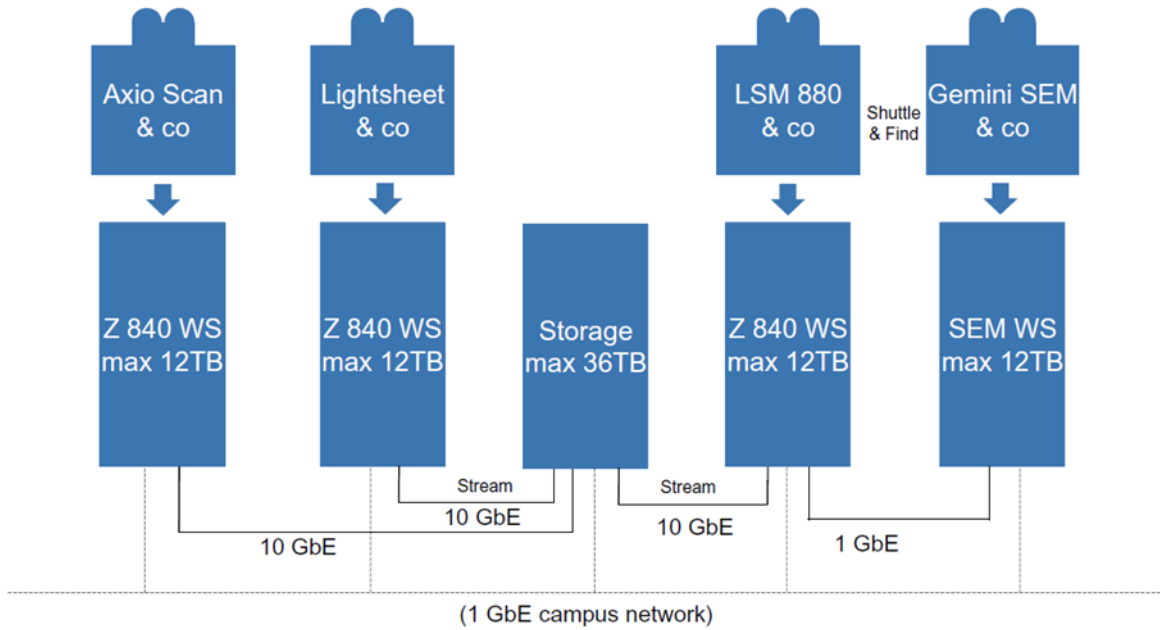
(*& co = "and comparable")

8.6   Solution 2a: Storage Shielded

In this storage PC configuration, Imaging systems with workstations are networked via 10 GbE LAN connections. Optional 10 GbE adapters allow fast file transfer for most of the systems. Onboard adapters allow connection to a campus network. The storage itself is shielded from the campus network. Optional HDDs allow to increase the storage space of the computers in addition to the storage PC.
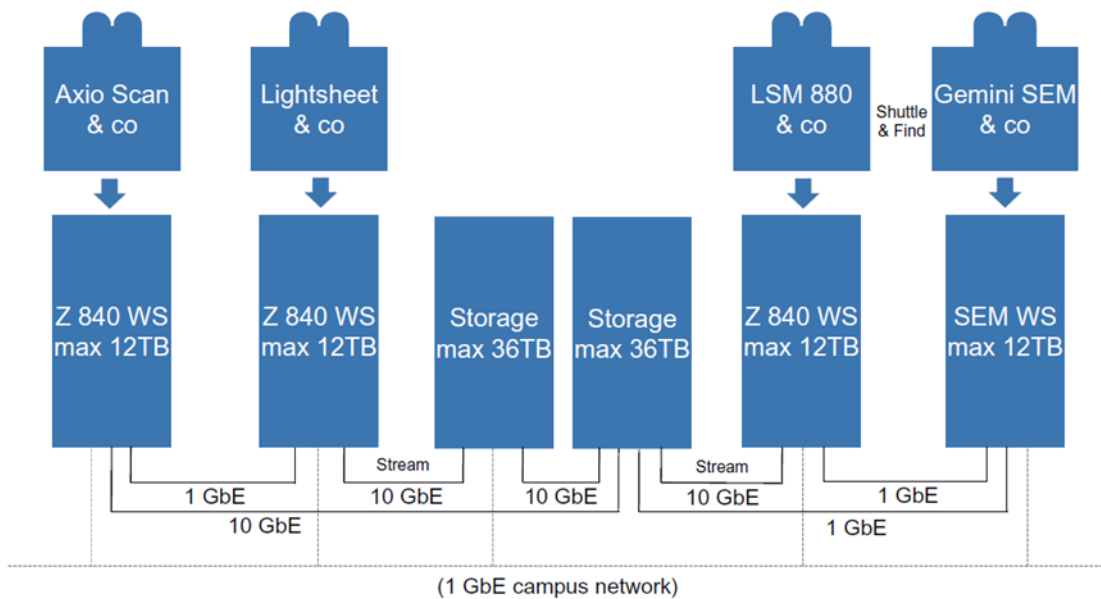
(*& co = "and comparable")

8.7   Solution 2b: Storage Open

In this alternative storage PC configuration, Imaging systems with workstations are networked via 10 GbE LAN connections. Optional 10 GbE adapters allow fast file transfer for most of the systems. Onboard adapters allow connection to a campus network. The storage itself is also open to the campus network. Optional HDDs allow to increase the storage space of the computers in addition to the storage PC.

(*& co = "and comparable")

8.8   Solution 3: Dual Storage

In this big dual storage PC configuration, Imaging systems with workstations are networked via 10 GbE LAN connections. Optional 10 GbE adapters allow fast file transfer for the systems. Onboard adapters allow additional connection to a campus network. The storages are partially open to the campus network. Optional HDDs allow to increase the storage space of the system computers.

(*& co = "and comparable")

8.9 ZEISS Microscopes Networked Solution 4: USB & WiFi
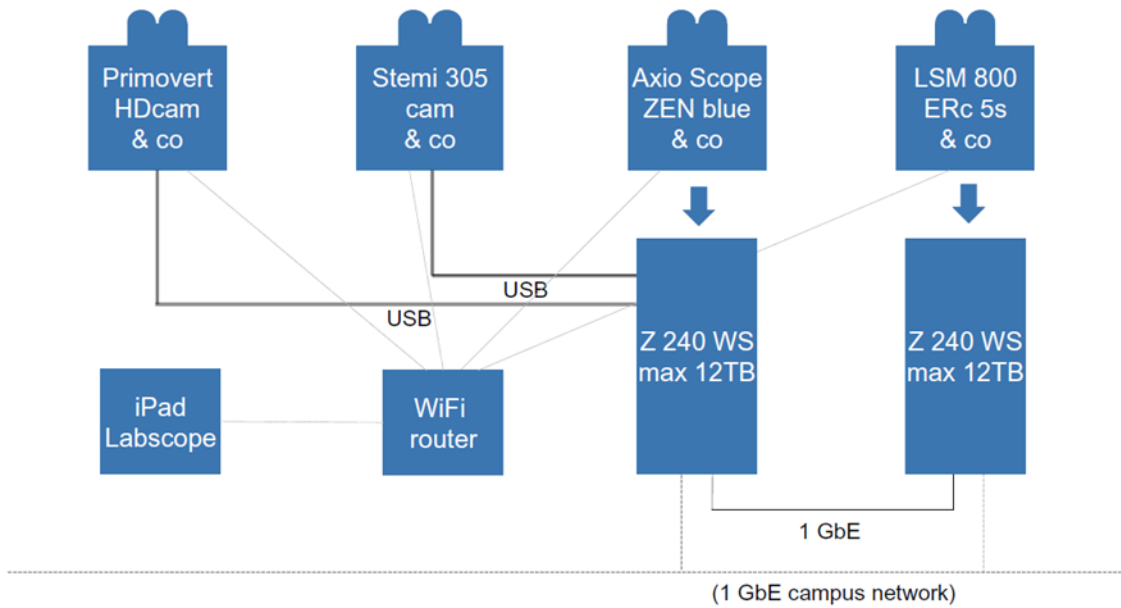
In this heterogeneous peer to peer configuration, a number of Imaging microscopes with and without workstation PCs are networked via WiFi, USB and LAN connections. Images can be viewed via iPads, or analysed on one of the workstations. Data share is possible via LAN or campus network. Optional 4TB HDDs allow to increase the shared storage space of the computers.

(*& co = "and comparable")