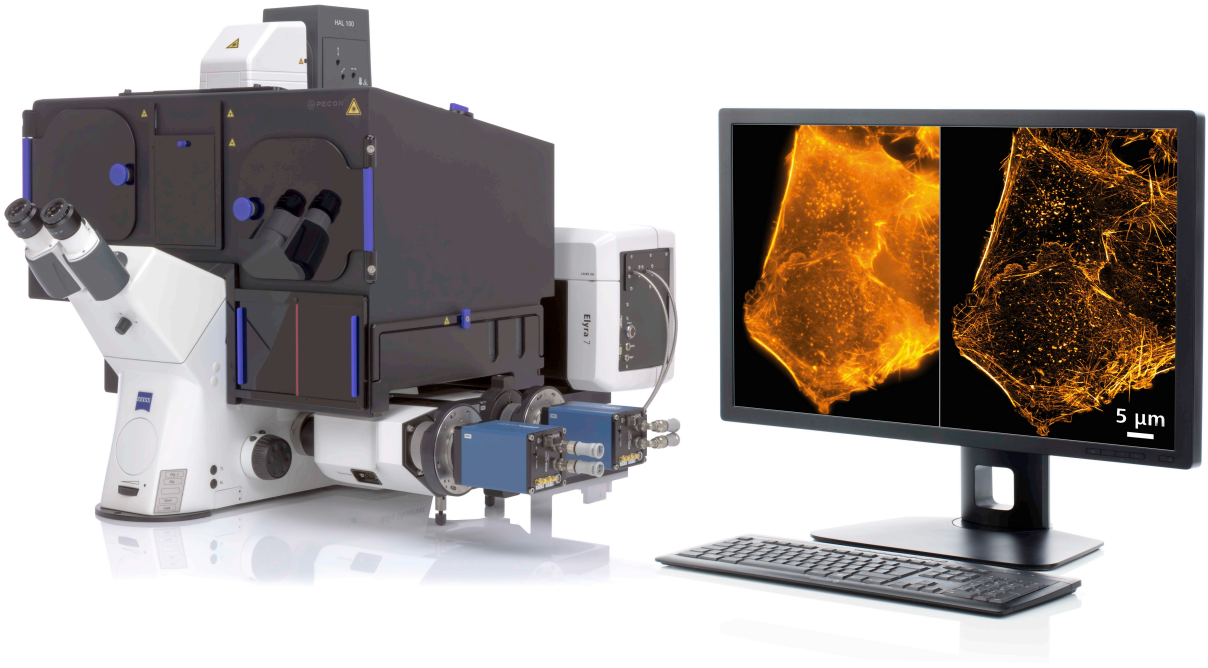
White Paper



ZEISS Elyra 7

Sample Preparation for Superresolution Microscopy – a Quick Guide

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ZEISS Elyra 7
Sample Preparation for Superresolution Microscopy – a Quick Guide

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General Guidelines for Superresolution Structured Illumination Microscopy (SR-SIM)

Sample support

In general, we recommend using high-precision coverslips (always no. 1.5 thickness) to mount the samples. High-precision cover glasses feature an exceptionally accurate thickness of 170 ± 5 µm (= 0,170 ± 0,005 mm). Also mind thickness issues when ordering glass-bottom petri dishes or multi-well plates.

For cover glasses see:

- ZEISS High precision Cover Glasses 18x18 mm; ZEISS order Number: 474030-9010-000
- Marienfeld Superior: www.marienfeld-superior.com
  - 18x18 mm (Cat.No. 0107032).
  - 22x22 mm (Cat.No. 0107052).
  - round with 18 mm diameter (Cat.No. 0117580).
- CellPath Ltd UK High performance coverslips no 1.5H 18x18 mm (Cat.No SAN-5018-03A)

For glass-bottom petri dishes and multi-well plates see:

- Willco (http://www.willcowells.com/)
- Lab-tek (offered through Thermo)
- MatTek (http://glass-bottom-dishes.com)

Please make sure that the coverslip is centered on the glass slide in order to fit into the sample holder.
See below pictures of the ZEISS level adjustable piezo stage insert.

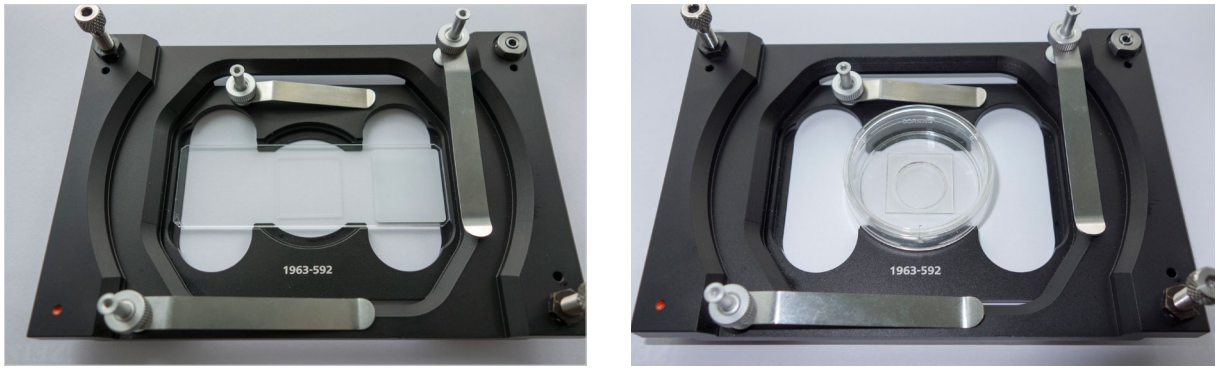


Figure 1 *ZEISS level adjustable piezo stage insert*

Fix your cells according to your standard protocol. For SR-SIM imaging, a thoroughly clean glass surface plays a crucial role. Therefore it is beneficial to seal or attach the coverslip in a way that facilitates cleaning with ethanol, without moving the coverslip.

Fluorescent Labels for SR-SIM

All common types of organic dyes usually conjugated to antibodies or fluorescent proteins are suitable to be used for SR-SIM. Make sure to have a highly specific labeling with low background to obtain a good signal to noise ratio.
For multicolor samples the fluorophores should be selected for minimal spectral overlap to avoid crosstalk. Available filter sets for Elyra 7 are are optimized for the available laser lines: 405, 488, 561 and 642 nm.

Note
Cytosolic or other non-specific fluorescent protein expression (e.g. GFP) will result in staining of extended areas. Since well-defined structures are missing to interfere with the grid pattern, modulation contrast will be low and the final image will lack high resolution information.

Embedding

Ideally, the sample should be embedded in a medium that matches the refractive index (RI) of the immersion oil (n=1,518). The following media perform well, despite having a lower refractive index.

- Fluoromout-G (SouthernBiotech) with RI of 1,40.
- 86% Glycerol with 2.5g/l DABCO (1,4-deazabicyclo[2.2.2] octane, Sigma Cat. D2522) in Tris-HCl. 1M pH 8.0. For further reading: http://www.nanoimaging.de/ → homepages → Sample requirements
- Non-hardening VECTASHIELD Mounting Medium with RI of 1,44.
- ProLong Gold (Life Technologies). Be aware this embedding medium needs 2 day to harden (in order to reach a constant RI). In addition your sample may shrink during this process. Refractive index (RI) of the cured product: 1,46.
- SlowFade Gold (Life Technologies) with RI of 1,42.
- 2,2'-thiodiethanol (TDE) – aqueous. RI can be varied ranging from being that of water (1.33) to that of immersion oil (1.52) by appropriately diluting with water.

Note

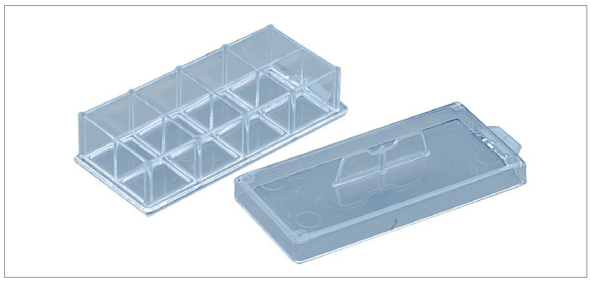
In order to have stable imaging conditions, especially concerning the RI of the mounting medium we would urge you to prepare the slides at least one week before use. Please note, that during curing the RI will rise.

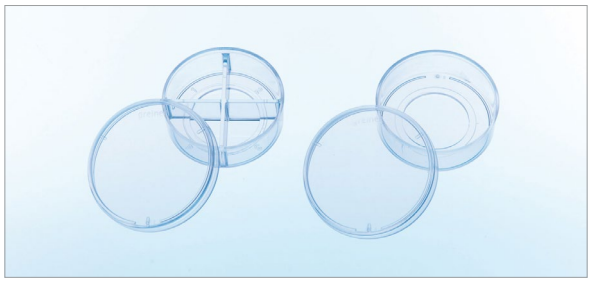
General Guidelines for Photoactivated Localization Microscopy (PALM)

Sample support

Samples for PALM and dSTORM are ideally prepared on Nunc Lab-Tek chambers or glass bottom dishes with cover glass thickness No 1.5.

- Nunc Lab-Tek II Chambered Coverglass no 1.5 (order no.: 155409)
- Greiner Bio-One (order no.: 672860 CELLview)
- Nunc Lab-Tek chambers (order no.: P35G-0.17-10-C or P35G-0.17-14-C)





All these formats will fit into the adjustable piezo stage insert.

Please be aware, in order to obtain ideal TIRF illumination you need a sufficient mismatch in refractive indices. In addition you may want to change imaging buffer concentration therefore we recommend no embedding after fixation of PALM and dSTORM samples.

Figure 2 *ZEISS level adjustable piezo stage insert*

Note

Nunc Lab-Tek chambers are well suited to transport samples between labs. They fit perfectly into a 50 ml Falcon tube, that can be filled with PBS and sealed with parafilm.

Fluorescent Proteins for PALM

The advantage of photoswitchable fluorescent proteins (PS-FPs) lies in their outstanding specificity and their small size, which is around 2 nm. The latter feature potentially allows for high labeling densities. Among photo-switchable proteins photoconvertable ones are the easiest to use as they - and the structure they stain – can be visualized at a different spectral range before conversion. Also one can easily check transfection efficiencies and expression levels. E.g. tdEOS or mEOS can be checked in the green spectral range, while the PALM experiment will be carried out detecting photo-switched EOS molecules in a more red shifted spectral band. Therefore tdEOS or its monomeric variant mEOS have been in extensive use as they also yield reasonable photon numbers.

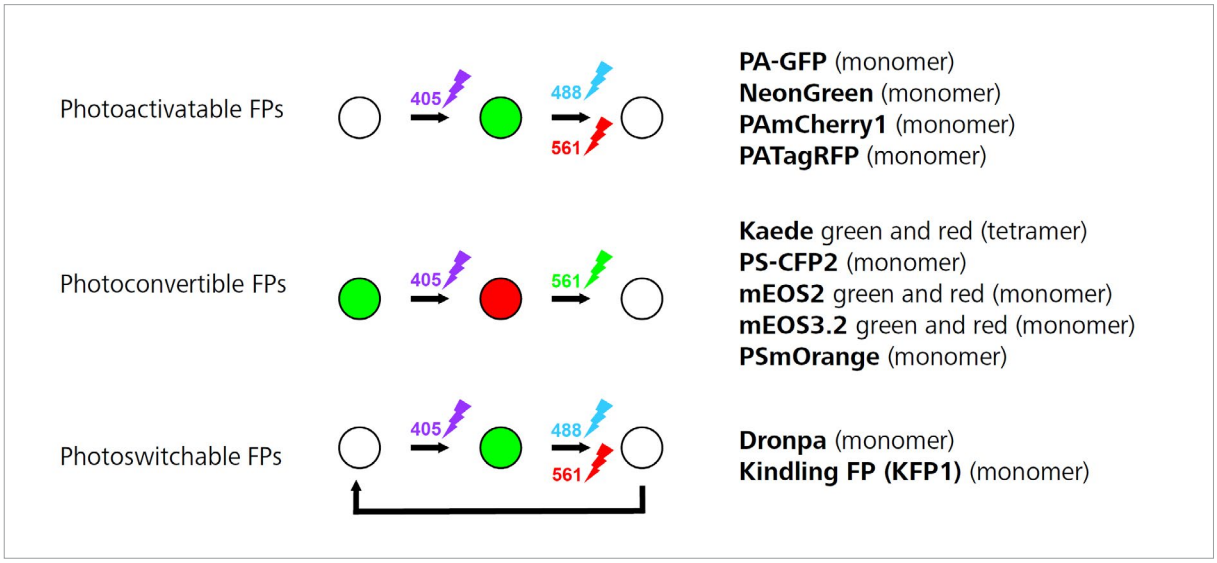


Figure 3 *Examples of fluorescent proteins that can be used for PALM imaging*

Most of these proteins are available through e.g. Addgene, Cambridge, MA 02139, USA

Recommended FP-Pairs for dual-color PALM

Pair 1: mEOS2 + Dronpa
Pair 2: NeonGreen + PA-mCherry1
Pair 3: Padron + Dronpa

If two differently stained molecules in the same sample are subjected to PALM, it is advisable to first image the higher wavelength dye as this does not cross-talk into the shorter wavelength channel. Hence, cross-excitation of the longer wavelength dye by the shorter wavelength and cross-emission of the shorter wavelength dye into the longer wavelength channel are minimized. Under experimental conditions many molecules of the shorter wavelength dye are equally activated with the 405 nm laser line, that is used for conversion / PALM imaging of the longer wavelength dye. Therefore reversible switchable fluorophores are the preferred choice for the shorter wavelength as they can be recovered and are not irretrievably lost.

Recommended Dyes for dSTORM

dSTORM uses organic dyes, which can be switched by employing reducing agents in the buffer. Dyes with a high photon yield per switching event, low on-off duty cycles, high survival fraction and a large number of switching cycles are preferential. Many Xanthene, Coumarin and Cyanine derivatives fulfill these criteria. Companies often trade these under special group names like Bodipy, Alexa Fluor (both from Invitrogen), Atto (from Sigma Aldrich), DyLight fluor (from Thermo Scientific) and FluoProbes (from Interchim). In the literature, Alexa 647 has been mostly used as it has proved to be a dye that matches all imaging criteria very well and can be switched easily between the dark and bright state.

The influence of the selected fluorophore has been nicely shown by G.T. Dempsey et al. (Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging, Nature Methods 2011). Please see below an extract of the data from this publication.

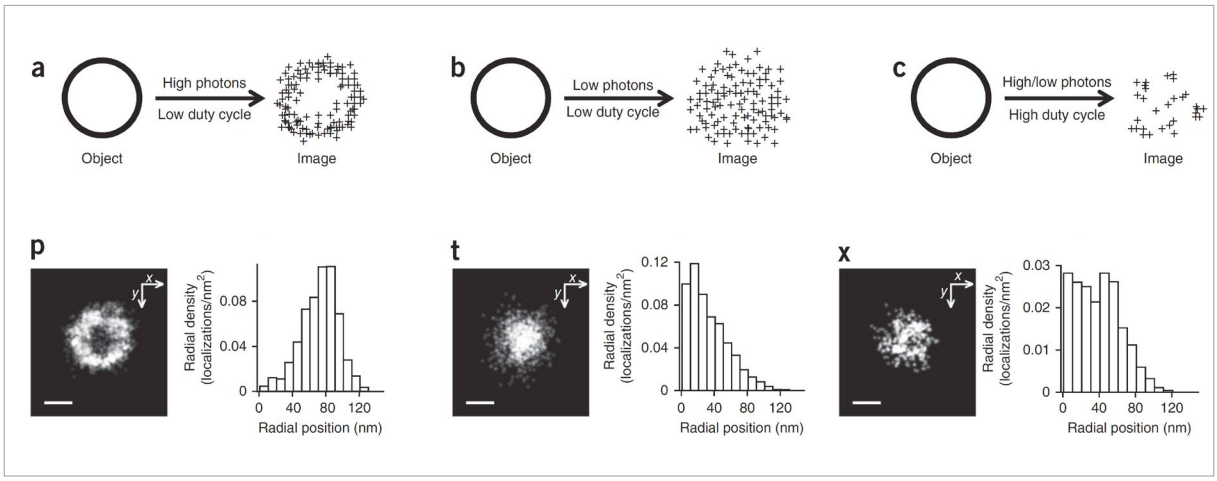


Figure 4 *(a-c): Effect of number of photons per on-switching event and the on-off duty cycle on STORM image quality for an example structure (a ring-like object). (p, t and x): Images of CCPs (clathrin-coated pits) using Alexa Fluor 647 (p), Atto 655 (t) and Cy5.5 (x). Shown are composite x–y cross-sections for ten CCPs aligned to their respective centers of mass along with the radial density distributions of localizations derived from the composite x–y cross-sections. Scale bars: 100 nm.*

For a multicolor experiment any combination between Atto 488, Cy3B/ Alexa 561 and Alexa 647/ DyLight 654 will work. Specifically the combination of Atto 488 with Alexa 647 has proven to be useful (see G.T. Dempsey et al.: Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging, Nature Methods 2011) Recommendations for double labeling: Atto 488 + Atto 565 or Alexa 647
A nice option could also be: tdEOS/mEOS + Alexa647

Antibodies for dSTORM

If we consider the size of antibodies it is preferable to do direct antibody labeling without a primary and secondary AB. Smaller antibodies such as nanobodies (cameloid like antibodies from camels, llamas and sharks) with a size in the range of 2 nm may be preferred.

Post-fixation Following Antibody Labeling

A post-fixation step can be valuable. Herein, you fix cells a second time after staining in order to improve the stability of the label. This can prevent the label from detaching and floating the imaging medium.

Imaging Buffer dSTORM

(please see: G.T. Dempsey, Nature Meth 2011 and S. van Linde, Nature Meth 2011)It is recommended to freshly prepare the imaging buffer every day. The oxygen scavenger system will only last for a few hours and is mainly needed for Cyanine dyes like Alexa 647 and Cy5 (Rhodamines and Oxazines do not require it.)

Imaging Buffer

100µl PBS 10x (Phosphate buffered saline ; e.g. from Sigma: D1408)
100µl MEA (Cysteamine Hydrochloride) stock concentration 1M (e.g. from Sigma M6500-25G) (toxic!!)

Optional:
Oxygen scavenger system (for cyanine dyes)
500µl Glucose 20% (e.g. in solution from Sigma (49163-100ML))
25µl Glucose Oxidase (e.g. 24 mg/ml GluOx from Aspergillus niger; Sigma G0543-50KU)
5µl Catalase (e.g.: 12.6 mg/ml Catalase from Bovine liver; Sigma C3155-50 MG)

Add H2O to final volume of 1ml

Very important: adjust pH to 7.5-8.5 with 5 M NaOH or 4.5 M KOH
(To test pH you can use pH paper in the indicated range)

Note

1 M MEA (Sigma M6500-25G): 0,113g in 1ml
Store MEA (solid) at 4°C. Prepare fresh as 1 M working stock solution in water. This stock can be kept at 4°C and used within 1-2 weeks of preparation. Alternatively you can freeze small aliquots at -20°C and keep them for several months.

The MEA concentration depends highly on the fluorophore and the experimental condition. Therefore the best concentration has to be tested by trial and error for each sample (between 10mM and 100mM).

If you use Cysteamine (not the Hydrochloride) you have to use 37% HCl to adjust the pH to 7.5-8.5

As a general advice:

- If blinking rates are too high: increase MEA concentration and/or increase pH
- If blinking rates are too low: decrease MEA concentration and/or decrease pH

Fiducial Markers for Localization Microscopy

Fiducial Markers (FM) are used to:
1) correct for small drifts (tens of nanometer range) during the course of an experiment
2) to align channels in multicolor experiments
3) to correct for chromatic aberrations in multicolor experiments
4) serve as a reference for the software autofocus

For **PALM imaging** we recommend fluorescent beads (e.g. Tetraspek beads from Invitrogen) or gold nanoparticles, dependant on the laser power, as fiducial markers. Choose the beads according to the fluorophores used in your experiment.

For **dSTORM imaging** we recommend nanoparticles (gold colloids) of which the photoluminescence persists through the entire measurement and which should be immobilized on the coverslip.

Ideally one has about 1-3 fiducial markers in the field of view. You can either:

I) fix fiducial markers on coverslip before seeding of cells:
- sonicate FM solution in an ultrasound bath for at least 5 min
- add FM (100nm, BBI 1:1000 diluted) to dH2O solution
- vortex FM solution
- coat cover slips with Poly-L-lysine (PLL) for adhesion of FM
- add FM Mix solution onto PLL coated cover slips
- check density (1-3 FM in field of view)
- prepare sample on the slide

II) add fiducial markers to sample before experiment
- sonicate FM solution
- add 2-5µl FM solution into 2ml dH2O and vortex several minutes
- apply onto fixed cells
- wait ~1h for FM to settle

Note
Only dissolve FM in water (no salt solution), otherwise they will aggregate and not stick to the coverslip.

Fiducial Marker Order Info
- BB International [www.british-biocell.co.uk] & [http://www.bbigold.com]
  Gold Colloid 80 nm (Cat. No. GM.GC 80) and 100 nm (Cat. No. GM.GC 100)
- Microspheres-Nanospheres (http://www.microspheres-nanospheres.com/)
  40 nm (Cat. No. 790122-010) and 80 nm (Cat. No. 790120-010) nanospheres Au particles
- Nanopartz [www.nanopartz.com]
  Nanorods 550 (Cat. No. 30-25-550) and 600 (Cat. No. 30-25-600)
- Tetraspek Beads [www.invitrogen.com]

Fiducial coverslips
- Hestzig LLC (http://www.hestzig.com)
- Fiducial coverslips 500 ± 50 nm spectral range at two densities (Cat. No. 550-30AuF & 550-100AuF);
- Fiducial coverslips 600 ± 100 nm spectral range at two densities (Cat. No. 600-30AuF & 600-100AuF)

Literature References

Reviews on Superresolution Microscopy

1. G. Patterson et al.: Superresolution imaging using single-molecule localization, Annu Rev Phys Chem 2010
2. 2. B. Huang et al. Breaking the diffraction barrier: superresolution imaging of cells, Cell 2010
3. L. Schermelleh et al.: A guide to superresolution fluorescence microscopy, JCB 2010
4. D. Toomre and J Bewersdorf: A new wave of cellular imaging, Annu. Rev. Cell Dev. Biol. 2010

Superresolution Structured Illumination Microscopy (SR-SIM)

1. Schermelleh et al.: Subdiffraction imaging of the nuclear periphery with 3D structured illumination microscopy, Science 2008
2. Gustafsson et al.: Three dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination, Biophys. J. 2008

Fluorescent Proteins for PALM

1. G. Kremers et al.: Fluorescent proteins at a glance. J Cell Science 2011
2. G. Patterson: Highlights of the optical highlighter fluorescent protein, J of Microcopy 2011
3. Shroff et al: Current Protocols in Biology 2010

Sample Preparation and Fluorophore Properties for dSTORM

1. G.T. Dempsey et al.: Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging Nature Methods 2011 (Performance of fluorophores under different experimental conditions and imaging buffer solutions.)
2. S. van de Linde et al.: Direct stochastic optical reconstruction microscopy with standard fluorescent probes. Nature Methods 2011. (Detailed overview of fluorophores, experimental conditions and protocols for dSTORM)

Further Reading

Prof. Rainer Heintzmann (http://www.nanoimaging.de)

Prof. Markus Sauer Lab (http://www.super-resolution.biozentrum.uni-wuerzburg.de/)

ZEISS Online Campus edited by Mike Davidson (http://www.zeiss.com/campus)

This collection of protocols and information on superresolution sample preparation is just a general guideline. While all attempts are made to provide accurate, current and reliable information we cannot guarantee that the protocols will work or that the information contained here will be error-free.

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