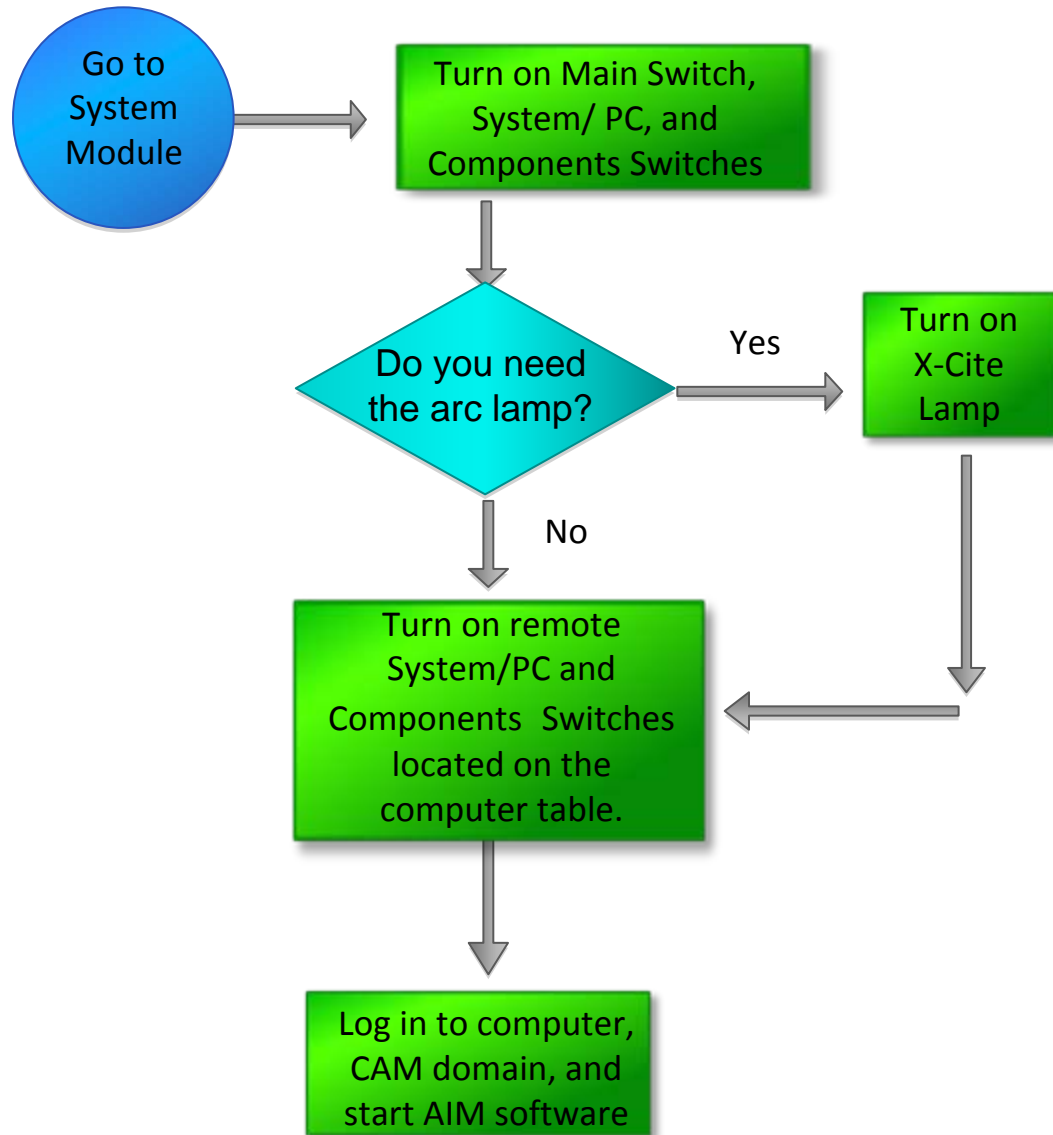


The left side of the slide features a series of vertical bars in blue, purple, yellow, and orange, with a green line running through them. To the right of these bars are four circles of different colors: a large purple one, a medium blue one, a small orange one, and a small yellow one.

# Zeiss LSM 510 Confocor III Training Notes

Center for Cell Analysis & Modeling

## Confocor 3 Start Up

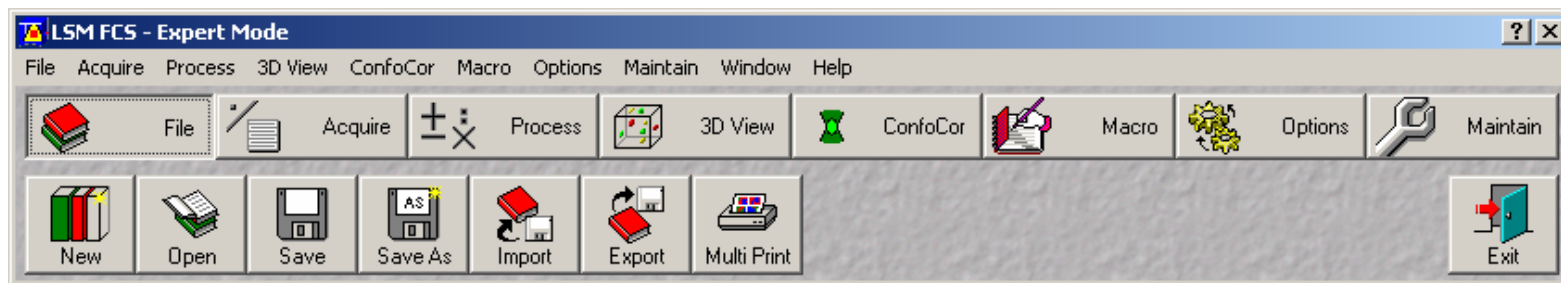


# Confocor III Training Notes

- When prompted to login, press Ctrl + Alt + Del, be sure to log into the CAM domain.
- Start the Zeiss Aim Software
- In the LSM Switchboard, select Scan New Images and Start Expert Mode. **DO NOT** select Use Existing Images.



# Confocor III Training Notes



## Creating/Accessing your database

- Select File, then New to create a new database or select Open for an existing database.
- Store all your data on the z partition (`//cfs02.cam.uchc.edu/home/CAM/your_ccam_account`) that has been set up for your lab. DO NOT store data on the desktop or in My Documents.
- Please back up your data on your own devices.

Note, there is a utility for Exporting your files to other file formats, however always be sure to save the raw data.



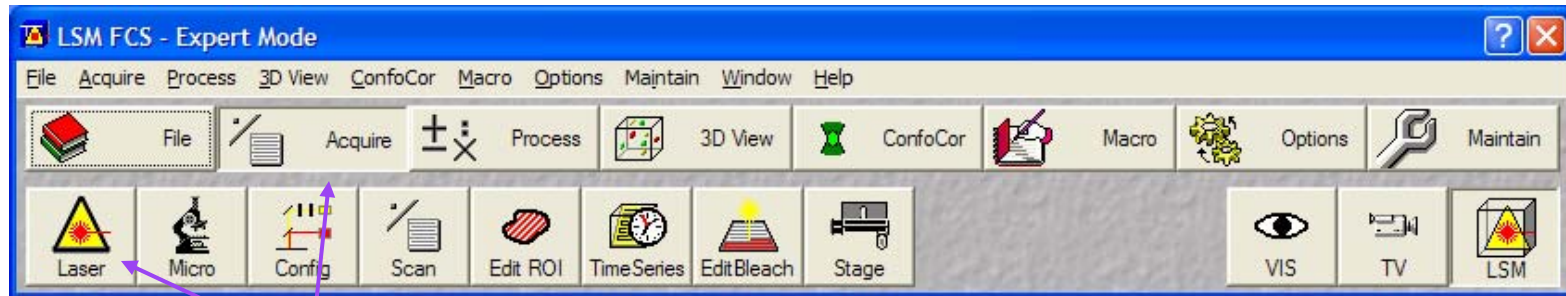
# Confocor III Training Notes

## Database and LSM Files

- When you create a new database, the software will automatically create a new folder with the database name. In that folder, you will find the database file and your .lsm image files. These files are tiff files with Zeiss acquisition information stored in the header of the file.
- If you want to delete files from your database, do so using the Zeiss software rather than manually deleting the files directly from the windows folder. In the past, deleting the files manually has created problems with the database.
- You can open the files via the database with the free Zeiss LSM Image Browser, available from their web site, <http://www.zeiss.com/micro>, or with an ImageJ plugin, the Zeiss LSM Reader, <http://rsb.info.nih.gov/ij/index.html>. Alternatively, the files themselves can be opened directly with Photoshop however you must use the “Open As” function and specify that it is a tiff file.
- Be aware that you can have two files with the same name in the database. The software will not warn you of file overwrite.



# Confocor III Training Notes



## Lasers

Go to the Acquire tab and select Laser to open the Laser Control dialog. Here you get access to the three lasers on the Confocor III:

- Argon (30mW) 458, 477, 488, and 514 nm
- DPSS (Diode Pumped Solid-State) (1.0 mW) 561 nm
- HeNe (Helium-Neon) (5.0 mW) 633 nm

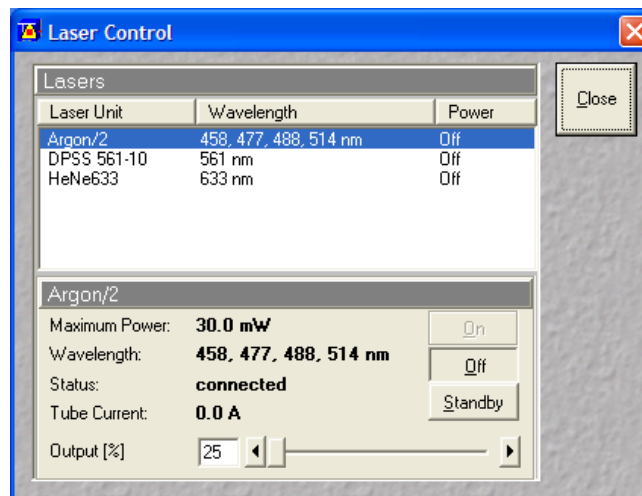
Generally it is suggested the lasers need about a 30 minute warm up period but you can start using them straight away for non-quantitative imaging purposes.



# Confocor III Training Notes

Select the laser(s) that would provide the correct wavelength(s) for exciting your specimen.

- Argon – This laser is fan cooled and has a specific start up and shut down procedure. Select Standby, when Status indicates Ready, click On. Set the output to ~ 50%, that should correspond to a tube Current of ~6.1 -6.5 amps. The Argon laser offers the following lines: 458, 477, 488 and 514 nm.
- DPSS 561 and HeNe 633 – The DPSS and helium-neon lasers are much weaker lasers and do not require fan cooling. Simply select the laser line required and press On to start the laser.
- Press Close, in the Laser Control dialog, once you have turned on the required laser(s).

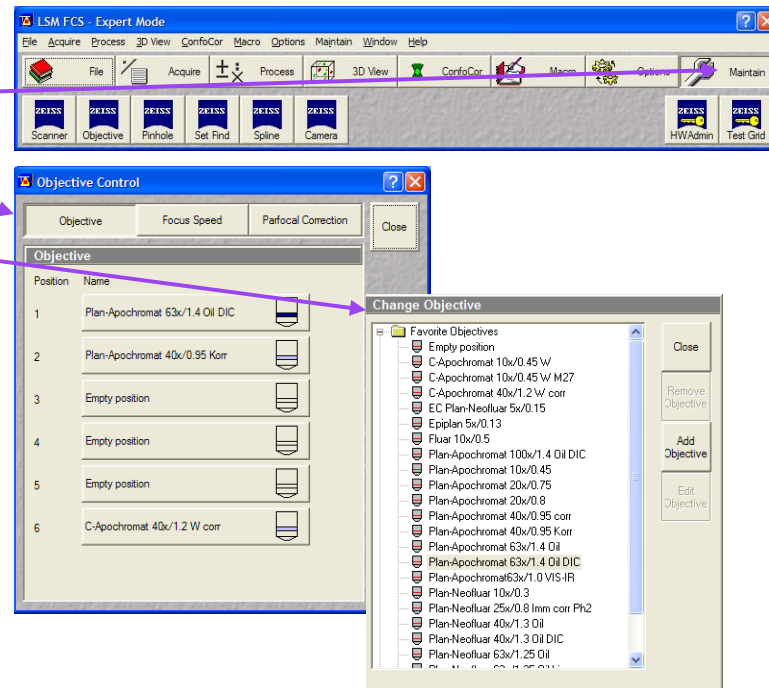


# Confocor III Training Notes

## Selecting Objectives

It is easiest to define which objective(s) you will be using in the software, and then use the Microscope Control dialog to select the correct turret position.

- Press Maintain>Objective to open the Objective Control dialog. Click once to select a turret position, 1-6.
- In the Change Objective dialog, find the objective you are using and double click your selection. Repeat this procedure for any other objectives you may have.
- Close both dialogs once you have made your selections and return to the Acquire tab.





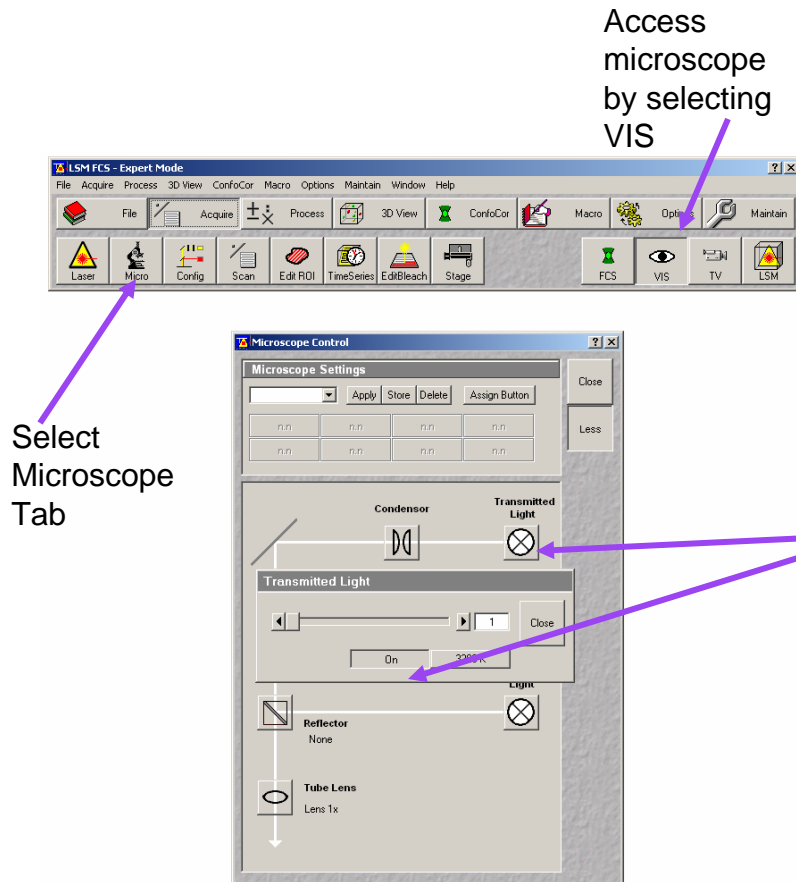
# Confocor III Training Notes

## Microscope Control

### Transmitted Light

While on the Acquire tab, select VIS for directing the light to the microscope, and then select Micro to access the Microscope Control dialog. From this dialog you can select your objective, and view your sample via transmitted light or fluorescent light.

- Press the Objective icon, and select your objective from the pull down menu.
- Make sure the Reflector cube is in the None position so the transmitted light does not have to pass through a colored filter.
- Press the Transmitted Light icon to access the Transmitted Light dialog. Press On and move the slider to adjust the intensity of the bulb. **BE CAREFUL WITH THE INTENSITY SLIDER.** You really shouldn't need to go above 1%.
- Deselect On to turn the bulb off and press Close to close the Transmitted Light box.



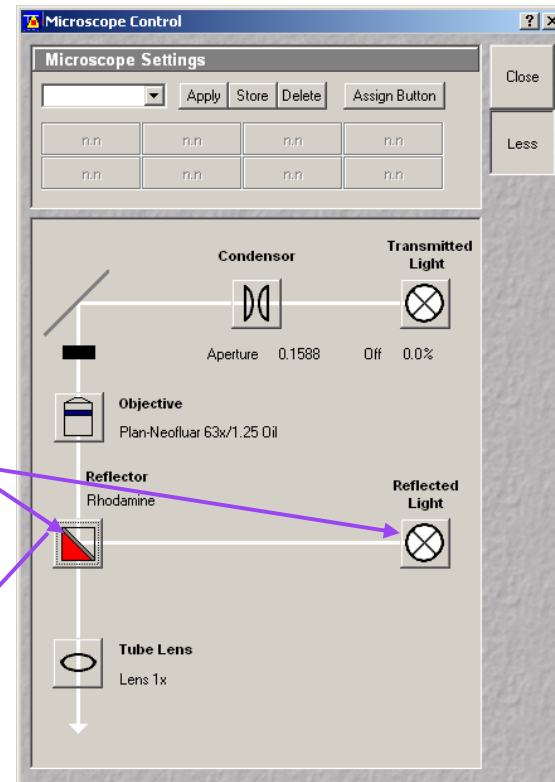
# Confocor III Training Notes

## Microscope Control (cont.)

### Fluorescent Light Control

You should still be in the VIS position on the Acquire tab, and have the Microscope Control dialog open.

- Press Reflector to select the appropriate fluorescent filter.
- Select Reflected Light icon to open the shutter, this will let fluorescent light reach the objectives for finding your sample. Be sure to center your sample in the middle of the field. The scan area is very small in comparison to the field of view from the microscope.
- When you are finished viewing your sample; deselect the Reflected Light icon to close the shutter.

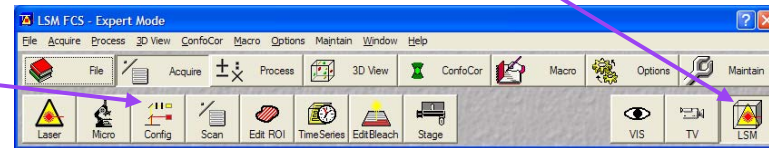


# Confocor III Training Notes

## Using the Confocal

- With the Acquire tab selected, select the LSM tab to change to confocal mode.
- Press Config to open the Configuration Control dialog. Here you will choose between Multi Track and Single Track configurations and make your laser, emission filter and detector selections.
- All configurations can be stored for future use.

Laser Scanning Mode



Single Track	Multi Track
Collects one probe or multiple probes simultaneously.	Collects multiple probes sequentially; by line or by frame.
Does not prevent bleed through between channels.	Avoids bleed through between channels
Better for sample because of reduced exposure to laser.	Exposes sample to increased laser light due to multiple scans.
Faster acquisition	Slower acquisition



# Confocor III Training Notes

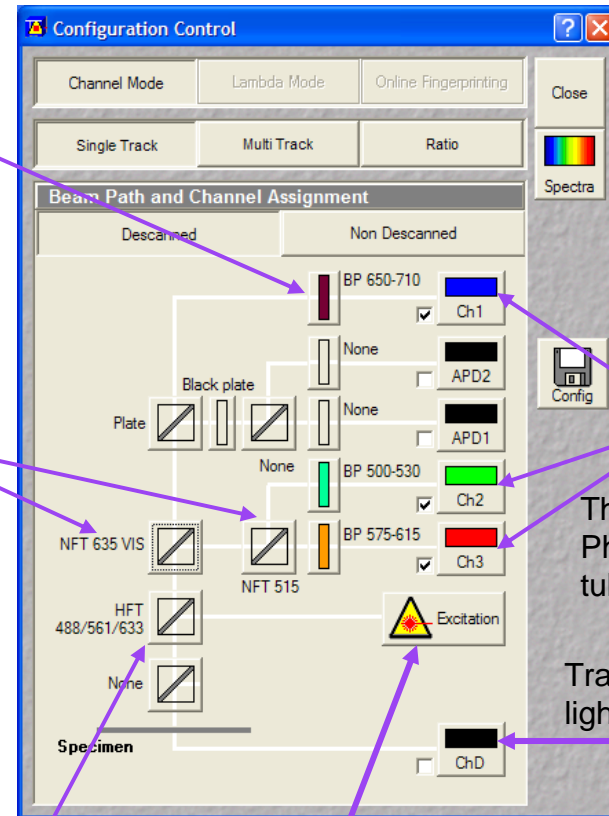
## Single Track

- Select Channel Mode and Single Track. Follow the excitation/emission pathway in the image to define your configuration.
- Press Excitation to select laser line and % transmission.
- Press and select the Excitation dichroic; this selection depends on the laser lines being used to excite the sample.
- Select 1<sup>0</sup> and 2<sup>0</sup> dichroics; selection is based upon wavelengths you want to capture. Dichroics reflect short wavelengths and pass long wavelengths.
- Activate detectors; Ch1, Ch2 and/or Ch3 and select pseudocolor for display. ChD is for transmitted light which is not confocal.

Emission Filter

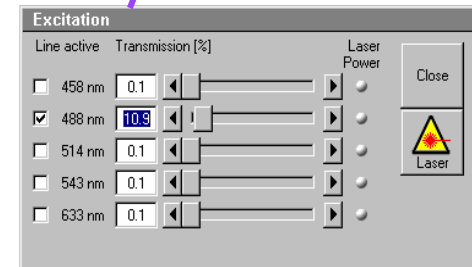
1<sup>0</sup> and 2<sup>0</sup> dichroics

Excitation Dichroic



Three Photomultiplier tubes

Transmitted light detector



# Confocor III Training Notes

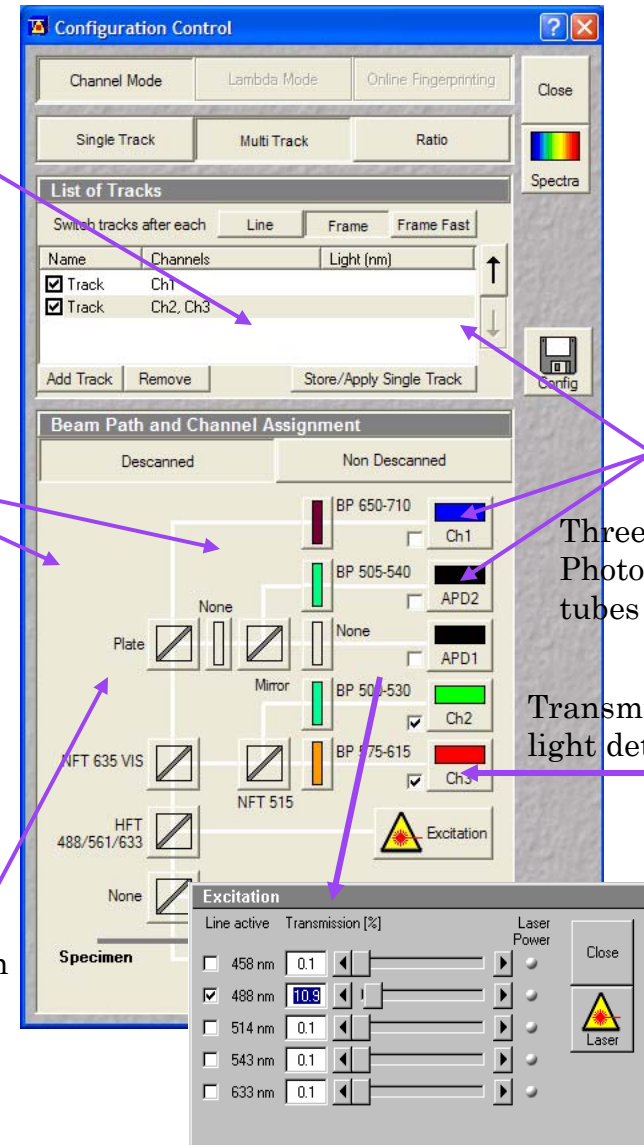
## Multi Track

- Select Channel Mode and Multi Track. Follow the excitation/emission pathway in the image to define your configuration.
- Press Add Track for each fluorescent probe or combination of probes.
- Activate the track by selecting the check box.
- Press Excitation to select laser line and % transmission.
- Press and select the Excitation dichroic; this selection depends on the laser lines being used to excite the sample.
- Select 1<sup>0</sup> and 2<sup>0</sup> dichroics; selection is based upon wavelengths you want to capture. Dichroics reflect short wavelengths and pass long wavelengths.
- Activate detectors; Ch1, Ch2 and/or Ch3 and select pseudocolor for display. ChD is for transmitted light which is not confocal.
- The individual tracks can be saved separately or the entire configuration can be saved for later use.

Emission Filter

1<sup>0</sup> and 2<sup>0</sup> dichroics

Excitation Dichroic



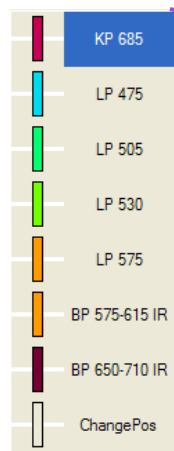
Three Photomultiplier tubes

Transmitted light detector

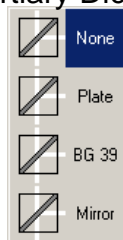
# Confocor III Training Notes

## Emission and Dichroic Filter Selections

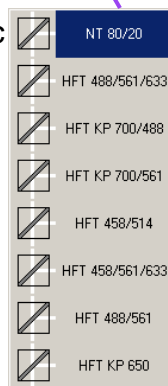
Channel 1



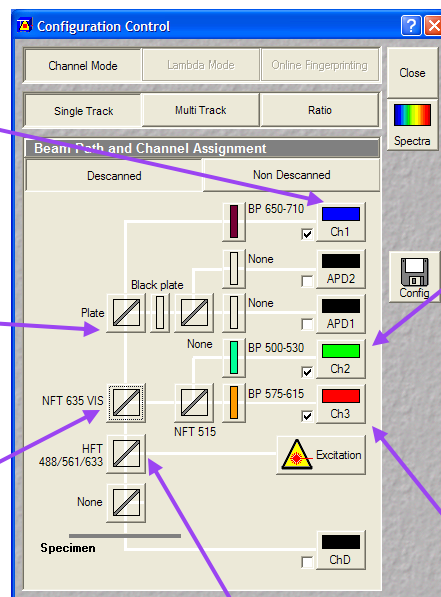
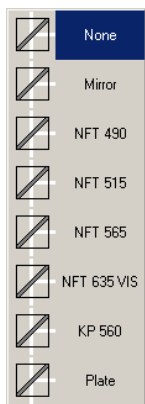
Tertiary Dichroic



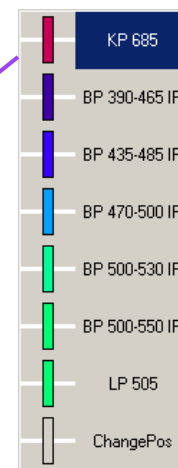
Primary Dichroic



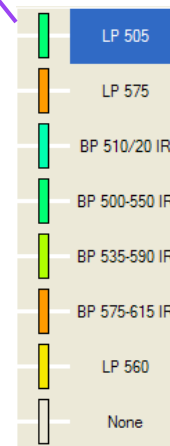
Secondary Dichroic



Channel 2



Channel 3



# Confocor III Training Notes

## Photomultiplier Tubes (PMTs) and Avalanche Photodiodes (APDs)

- PMTs
  - extremely sensitive detectors of ultraviolet, visible and near-infrared light.
  - Detectors can multiply the current produced by the light by as much as 100 million times.
  - Characteristics: high gain, low noise, high response, large area of collection.
  - Limited sensitivity in red and near infrared regions.
  
- APDs
  - Semiconductor device used as an alternative to PMTs.
  - Can measure light at lower levels; used for single photon counting.
  - Improved sensitivity in red and near infrared regions, 650 -1000nm.



# Confocor III Training Notes

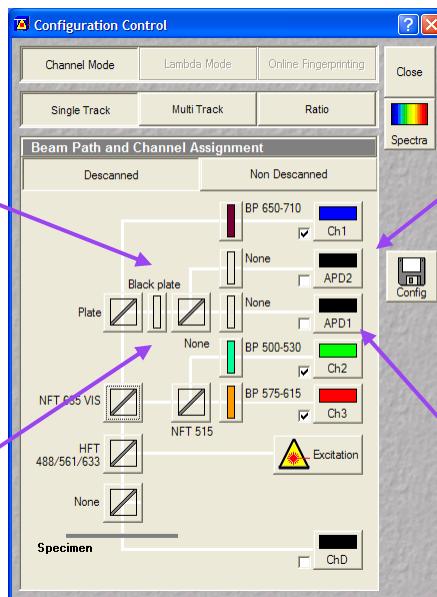
## Emission Filters

### Dichroics to APDs

	None
	NFT 500
	None
	NFT 545
	NFT 565
	None
	NFT 600
	NFT 635 VIS
	None
	NT 50/50
	Plate
	Mirror

Protects APDs  
when not in use

	Black plate
	None
	Black plate
	Black plate
	Black plate
	Black plate
	Black plate
	Black plate
	Black plate



	None
	None
	None
	BP 530-610 IR
	BP 560-610 IR
	LP 530
	BP 580-610 IR
	LP 580
	BP 615-680 IR
	BP 655-710 IR
	LP 655
	None
	None
	None
	Black plate

	None
	None
	BP 470-495 IR
	LP 470
	None
	None
	BP 505-540 IR
	BP 505-610 IR
	LP 505
	BP 530-610 IR
	LP 530
	LP 580
	None
	None
	Black plate

APD 2

APD 1





# Confocor III Training Notes

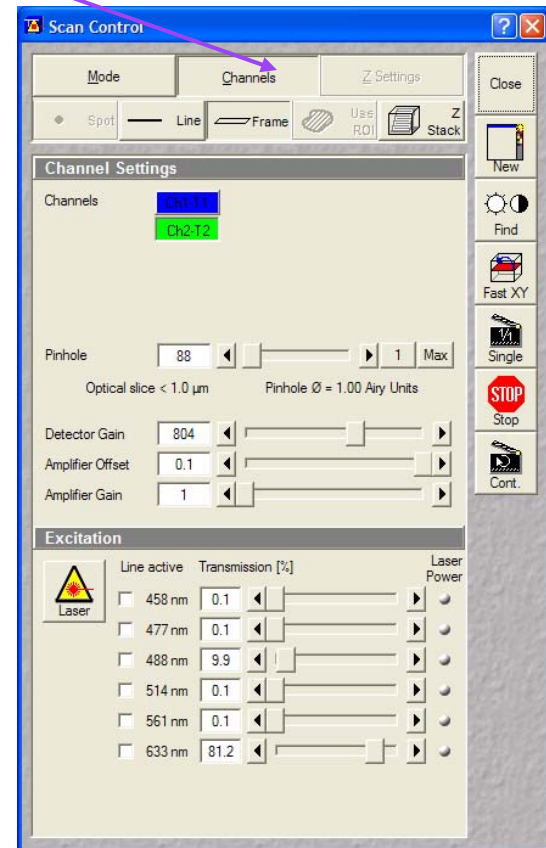
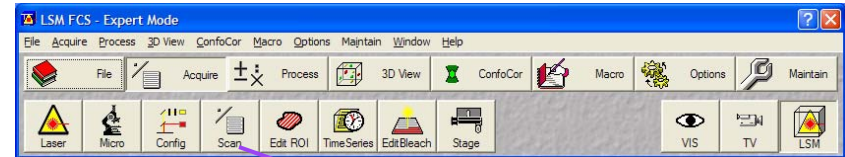
## Scan Control

### Channels Tab

- Under the Acquire tab, select the Scan button to get to the Scan Control Dialogue, press the Channels tab.
- Set the pinhole for each channel. Start at 1 airy unit for the best theoretical resolution. If you have multiple channels, start with the longest wavelength and set the airy unit to 1. Make sure the other channels have the same Optical Slice, adjust the pinhole if necessary.
- Adjust the Detector Gain, maximum sensitivity, and the Amplifier Offset, minimum intensity, for your image settings.



1 Airy Unit



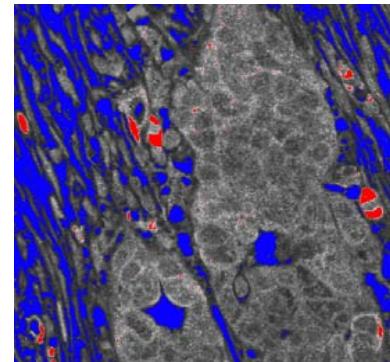
# Confocor III Training Notes

## Scan Control

### Channels Tab (cont.)

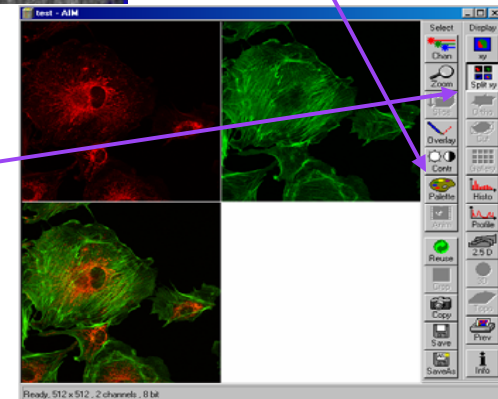
- Select the Palette tab, on the image display window, choose Range Indicator, then close the dialog. Depending on the settings, your image will display **Blue** pixels for minimum intensity and **Red** pixels for maximum intensity. ( 0-255 for 8 bit images and 0-4095 for 12 bit images.)
- Adjust the Detector Gain and Amplifier Offset to remove **red** and **blue** pixels. The minimum pixel intensity should be ~ 10-20 gray levels above 0 and the maximum pixel intensity should be ~10-20 gray levels below 255.
- If you have multiple channels, use Split x,y to view the channels separately as well as the over-lay image.
- In the MultiTrack configuration, you can deselect a channel in the Configuration Control to scan them one at a time when adjusting the settings. Once you have the settings for each channel, activate all the channels and perform a single scan generating the over-lay image.

Use the range indicator to aid with image settings.



Palette>Range Indicator

Split x,y



# Confocor III Training Notes

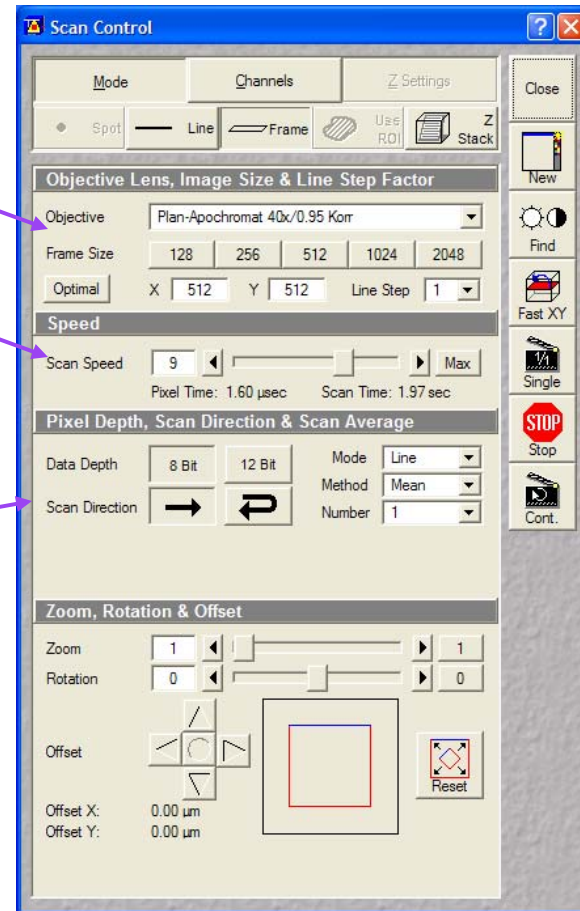
## Scan Control

### Mode Tab

- Objective – change lens from this window or from the microscope dialog (recommended)
- Frame Size – size of image, defaults to 512x512, ranges from 128 to 2048 pixels in x and y.
- Line Step – sampling rate of each frame. Recommended to leave at 1 for best image quality.
- Scan Speed – slower scan allows for longer pixel dwell time which will result in better image quality but result in increased chance of photobleaching.
- Data Depth – select between 8 bit (256 gray levels) and 12 bit (4095 gray levels) Use 12 bit for quantitative, data analysis.
- Scan Direction – use single direction for fixed cells, bidirectional for faster, live cell acquisition.
- Scan Average – improves signal to noise ratio
  - Mode – Line or Frame
  - Method – Mean or sum
  - Number – of lines or frames to average by.

Decreased scan speed and line averaging result in increased laser exposure.

Frame averaging reduced photobleaching but does not produce as clean an image as the other methods.

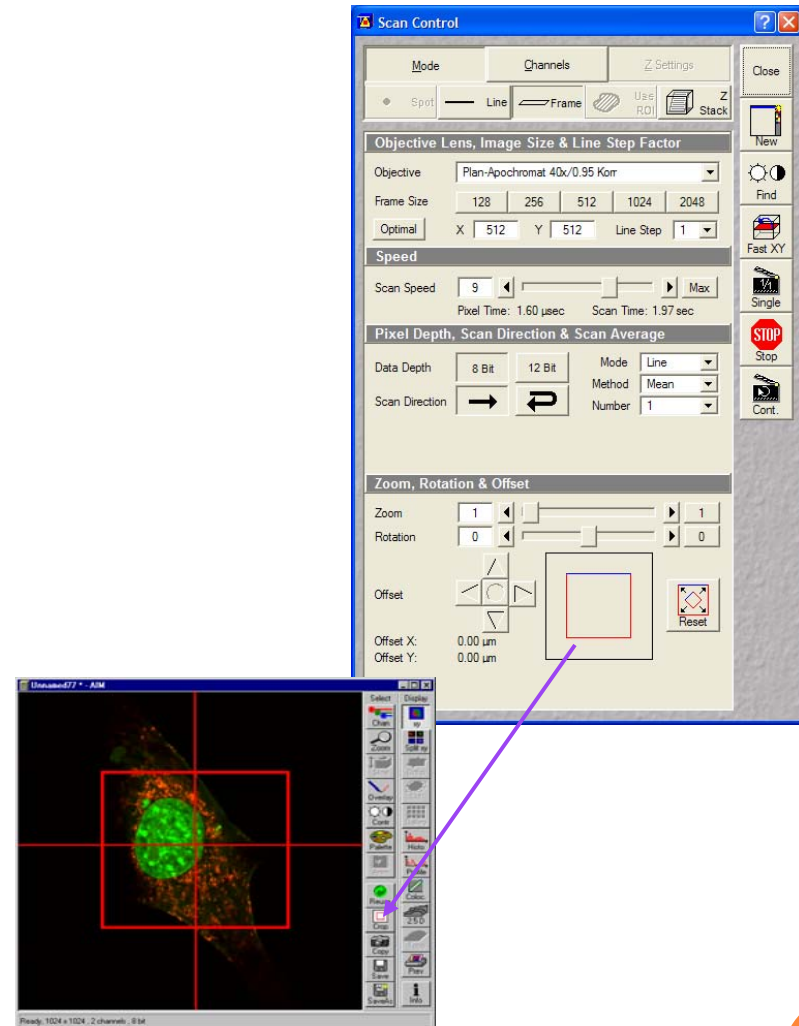


# Confocor III Training Notes

## Scan Control

### Mode Tab(cont.)

- Zoom
  - Use the Crop tool, on the image display window, to implement the zoom function, and/or rotation function.
  - Rest image to zoom 1 when you are finished. This will center the laser to optimal conditions.
  - The final magnification is zoom factor x magnification of the objective.



# Confocor III Training Notes

## Nyquist Sampling

- In order to preserve the spatial resolution in an image, the sampling rate should be twice that of the maximum frequency.
- Adjust the optical zoom in order to achieve the optimal x,y pixel size.

Objective	Numerical Aperture	Immersion	Coverslip Thickness (mm)	Working Distance (mm)	Z Resolution (μm) (1 Airy Unit)	*Optimal Z-Step (μm)	**XY Resolution (μm)	*Optimal XY pixel size	***Optimal Zoom
40x C-Apochromat	1.2	Water	.14-.18	.22	.7	.35	.25	.13	3.5
40x Plan-Apochromat	.95	Air	.31 - .21	.16	.8	.41	.31	.16	2.9
63x Plan-Apochromat	1.4	Oil	.17	.18	.6	.29	.21	.11	2.5
100x Plan-Apochromat	1.4	Oil	.17	.09	.6	.29	.21	.11	1.6
10x Plan-Neofluar	.3	Air	.17	5.6	9.0	4.48	1.0	.5	3.6
20x Plan-Neofluar	.5	Air	.17	2.0	3.2	1.59	.60	.3	3.0
63x Plan-Neofluar	1.25	Oil	.17	.10	.7	.37	.24	.12	2.3
10x Fluor	.5	Air	.17	2.0	3.2	1.59	.60	.3	5.9
40x Fluor	1.3	Oil	.17	.14	.7	.34	.23	.12	3.7

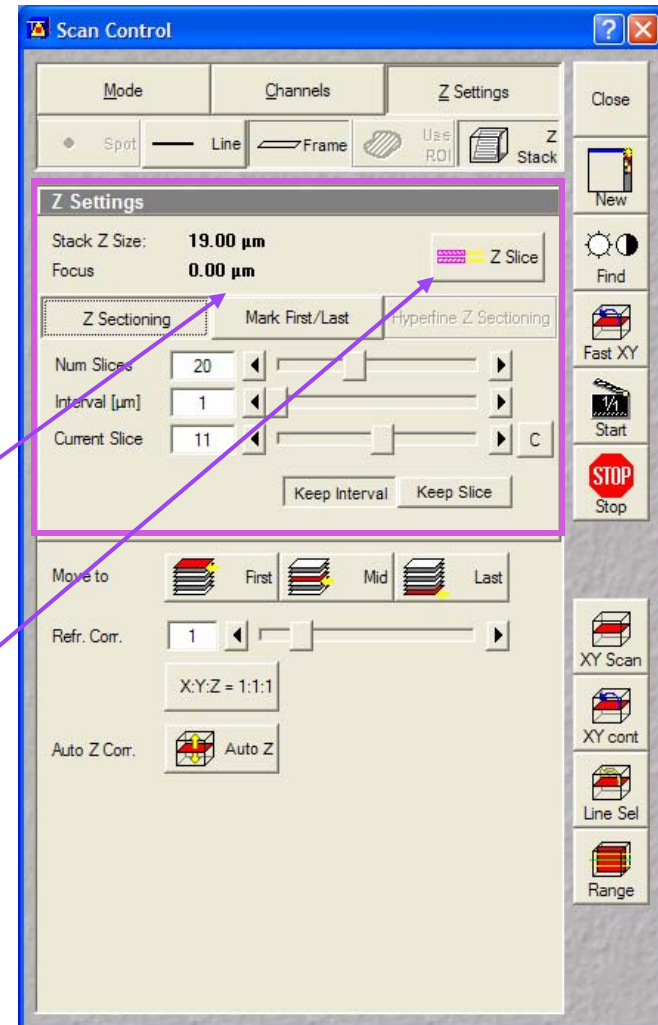


# Confocor III Training Notes

## Scan Control

### Z-Settings – Z-Stack

- Stack Z size – total size of stack
- Focus – current focus position
- Z-sectioning – You can define your stack by the number of slices, the step interval and the current slice position you are at. Collect the stack by either Keeping the Interval (number of slices will be modified) or Keeping the Slice (the interval will be modified).
- Mark First/Last – focus to the bottom of your sample and select Mark First, focus to the top of your sample, Mark Last.
- Interval – spacing at which you will acquire the slices. This should be based on the optimal Z-slice, which is equal to one half the z-resolution. Press the Z Slice button for this to be calculated automatically.



Note scan buttons are different under Z settings.

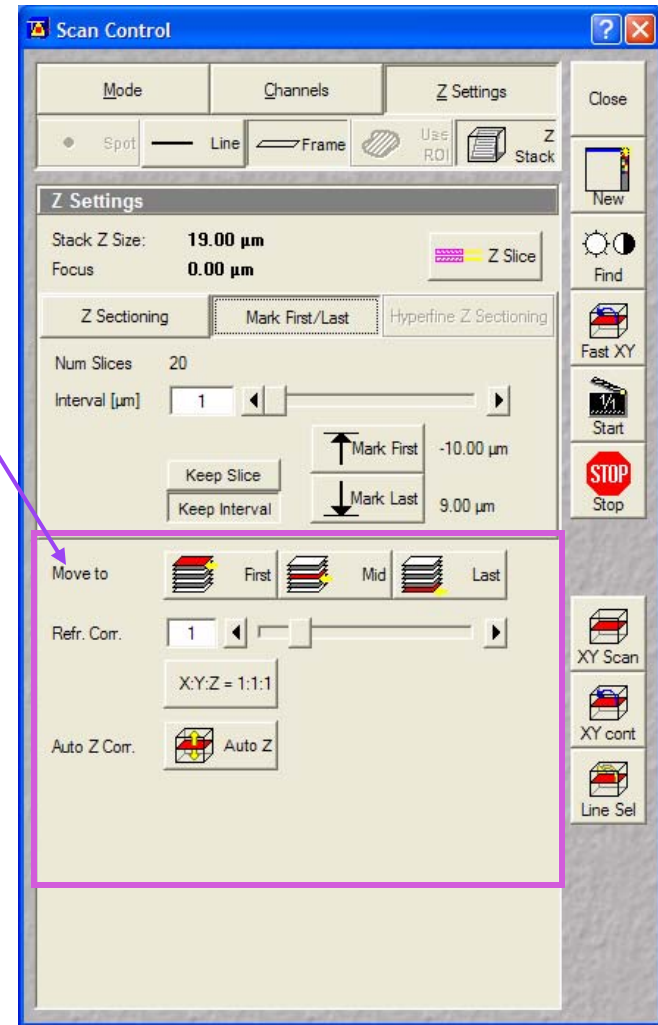


# Confocor III Training Notes

## Scan Control

### Z-Stack - Z-Settings (cont.)

- Move to First, Mid, Last – automatically move to position once the first and last positions have been set.
- Refr. Corr. – corrects for mismatch between immersion oil and mounting medium.
- Auto Z Corr. – compensates for signal loss in the volume by increasing laser power and detector gain.
- Move to First, Mid, Last – automatically move to position once the first and last positions have been set.
- Refr. Corr. – corrects for mismatch between immersion oil and mounting medium.
- Auto Z Corr. – compensates for signal loss in the volume by increasing laser power and detector gain.



Note scan buttons are different under Z settings.

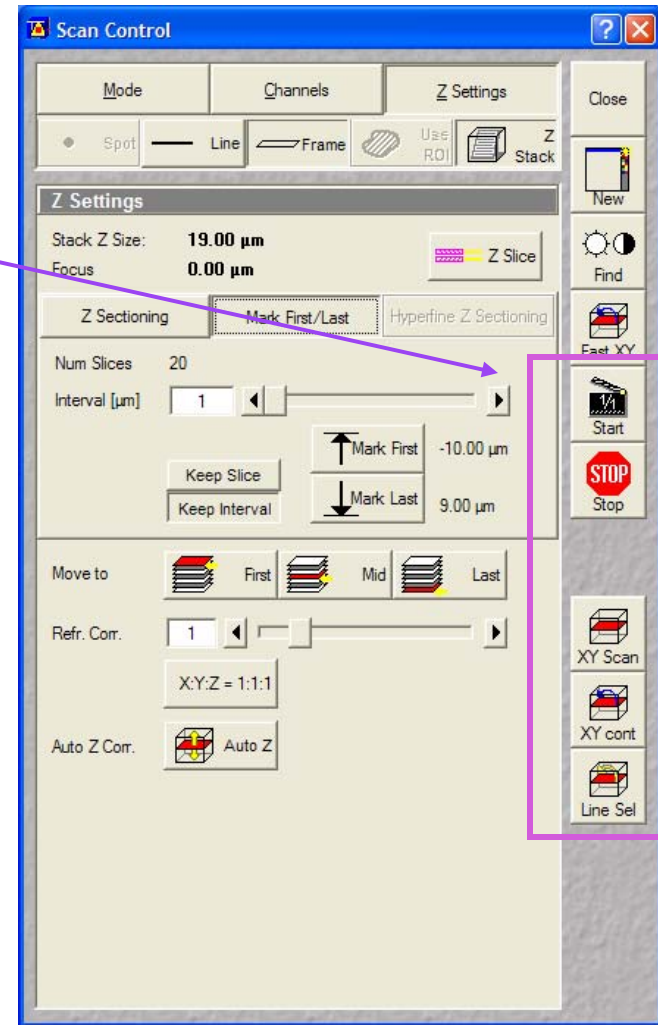
# Confocor III Training Notes

## Scan Control

### Z-Stack - Z-Settings (cont.)

Note the scan buttons change under the Z Settings tab

- Start – starts z-stack
- Stop – stops scans
- XY Scan – single scan
- XY cont – continuous scan
- Line Sel – line scan



Note scan buttons are different under Z settings.

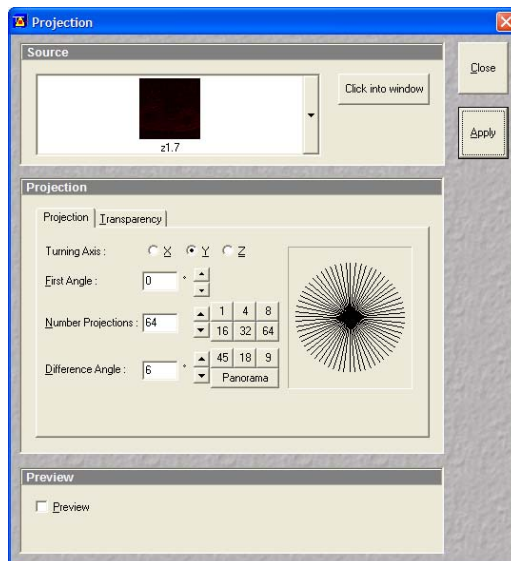
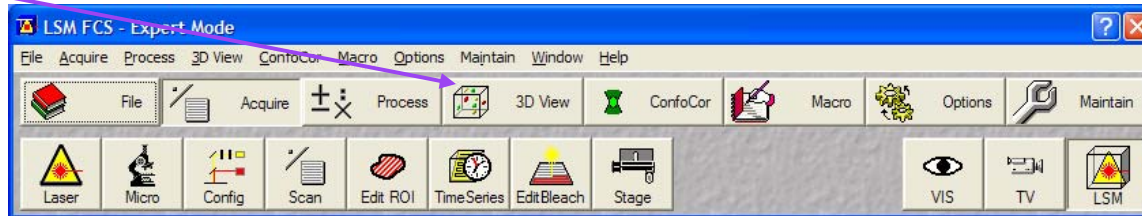




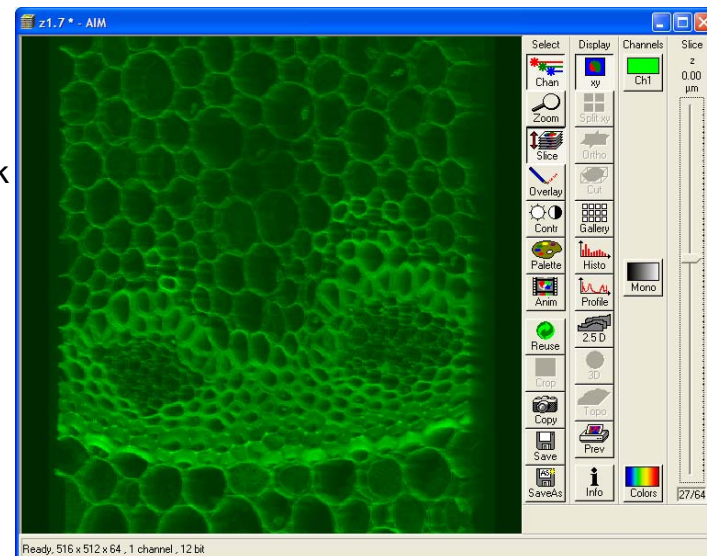
# Confocor III Training Notes

## 3D View

Under the 3D View tab, there are primitive functions that allow you to view your 3D data set.



Compile 3D stack  
for display



# Confocor III Training Notes

## Shutting down the Lasers

### Shutdown Procedure (Last User)

#### Argon Laser

- Lower power, select Standby, select Off.
- Wait until cooling cycle ends, ~ 3 minutes, Status will be reported as Connected.

#### HeNe 1 and HeNe 2

- Select Off for each laser

Shutdown Procedure (Between users; verify next user is coming. If in doubt, shut it down.)

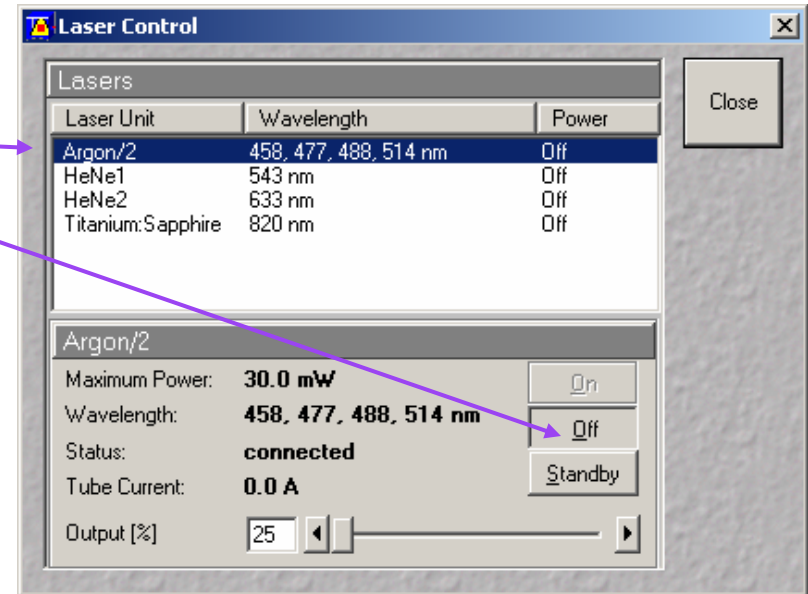
#### Argon Laser

- Lower power, select Standby.

#### HeNe 1 and HeNe 2

- Select Off for each laser

**Log off from computer, DO NOT shut the system off when the lasers are still on as you will damage the lasers.**



## Confocor 3 Shut Down

Check the CC3 sign up prior to shutting down the system.

Is someone signed up after you?

Yes

Did you verify they are coming?

Yes

Leave the system in a "stand by" mode and log off.

No

No

### SYSTEM SHUT DOWN:

- Turn off lasers, exit AIM software
- Shut down computer
- Turn off X-Cite bulb
- Turn off remote System/PC and Components Switches.

Go to  
System  
Module

Turn off System/ PC,  
Components Switches,  
and Main Switch.

