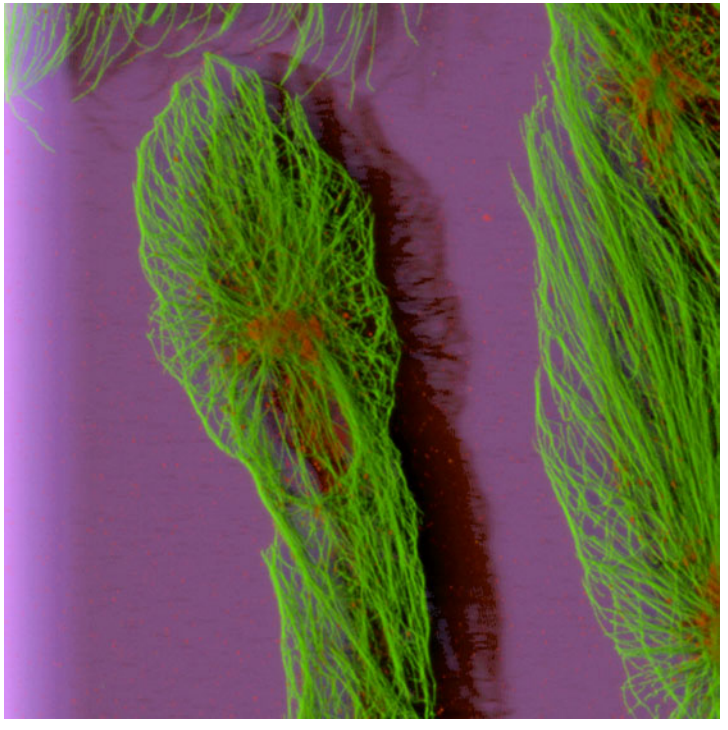
Confocal microscopy

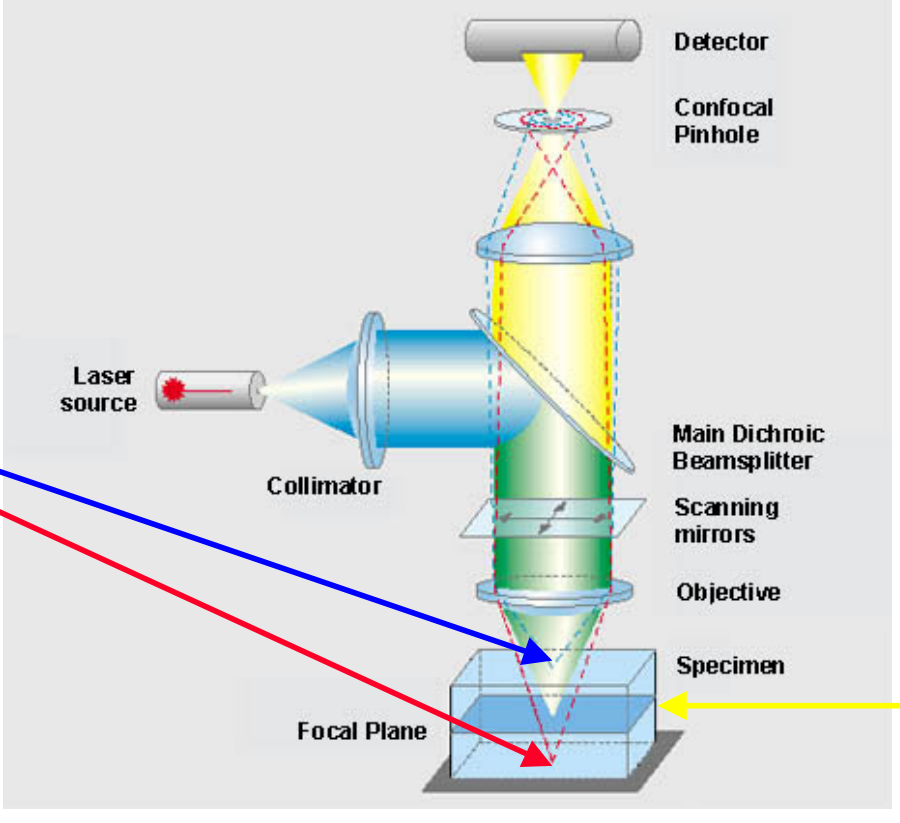
Zeiss LSM 510 and Zeiss LSM 510 META

Visualisation of biological structures in 3D



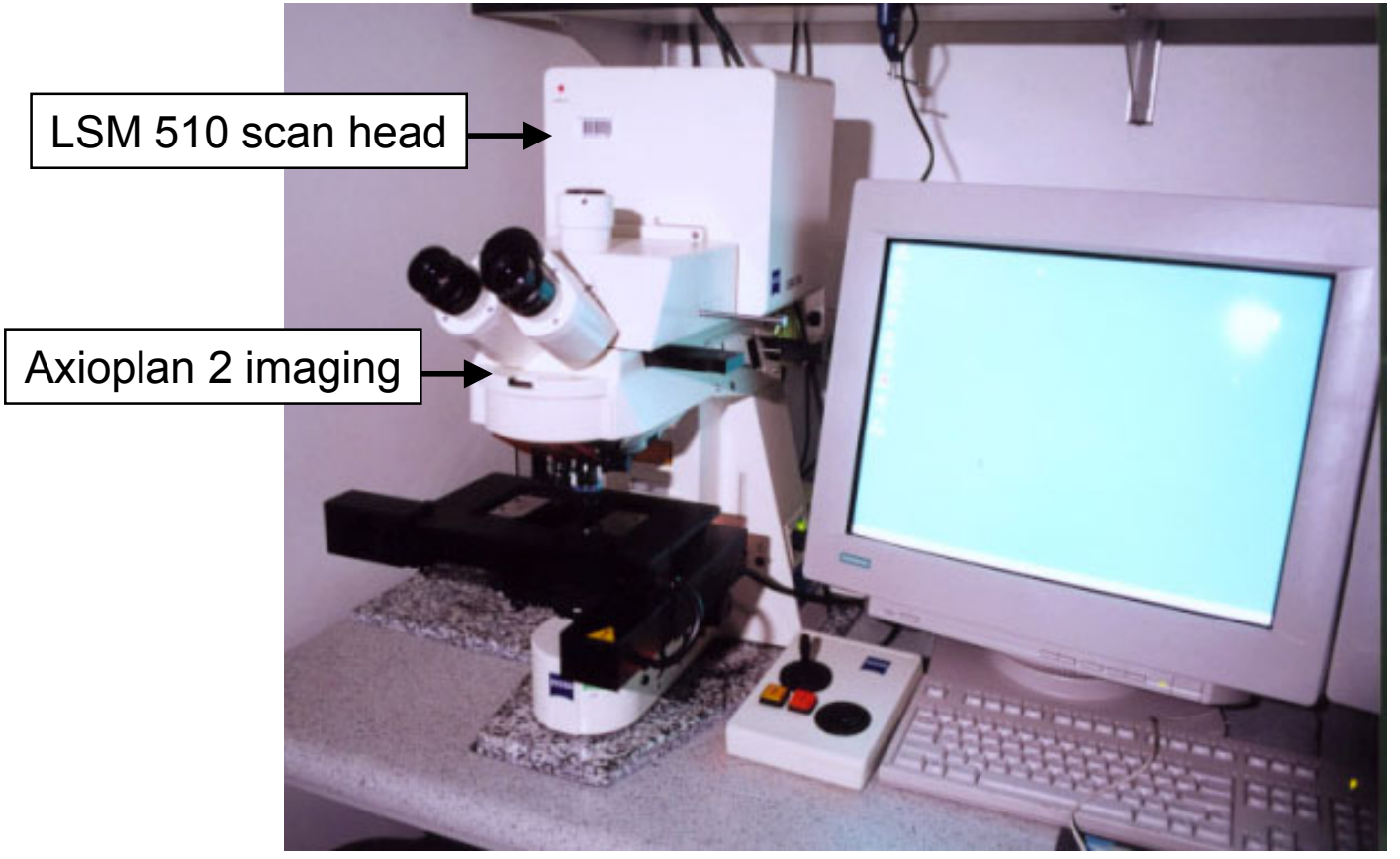
Confocal Principle

Signals from above and below the plane of focus fall outside the pinhole and are blocked

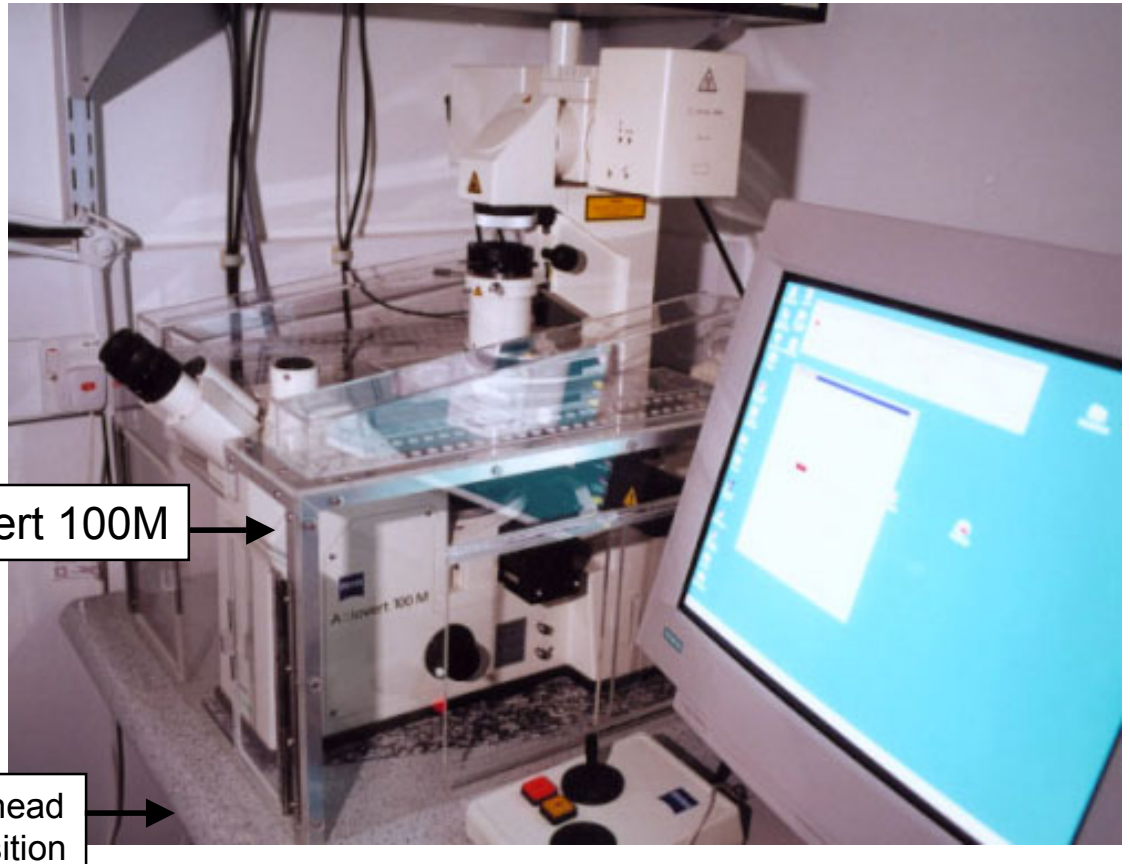


Only signals from plane of focus pass the pinhole and are detected - producing a “optical section”

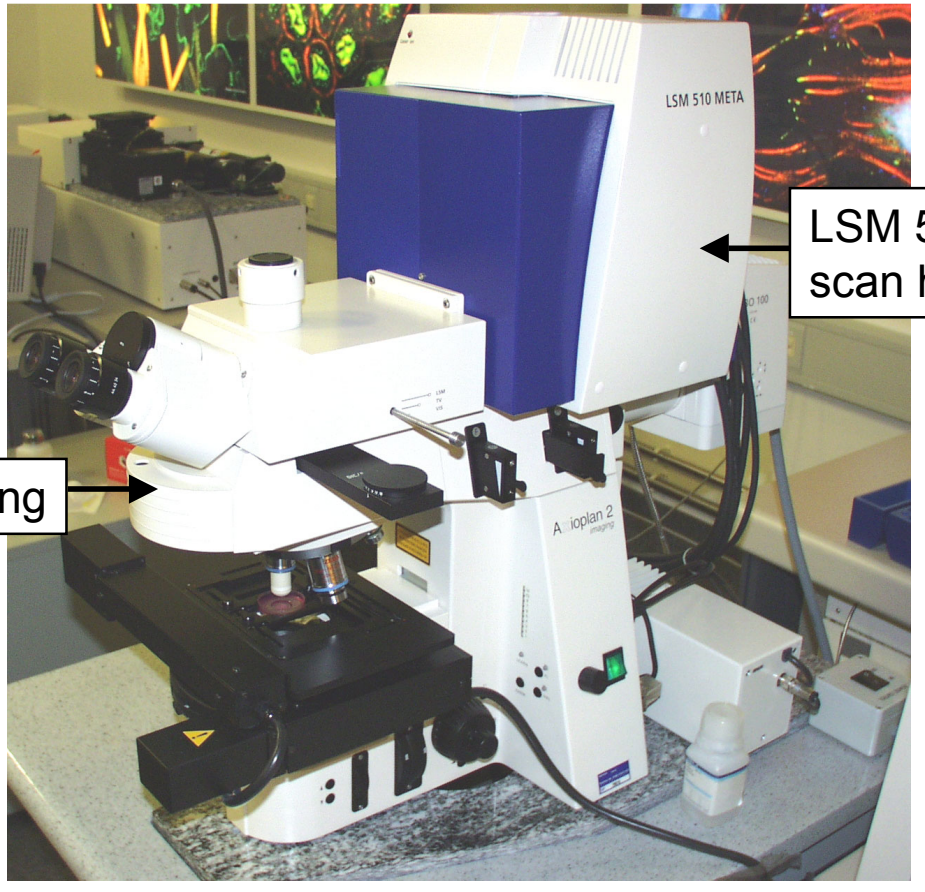
Upright Zeiss LSM 510 confocal microscope



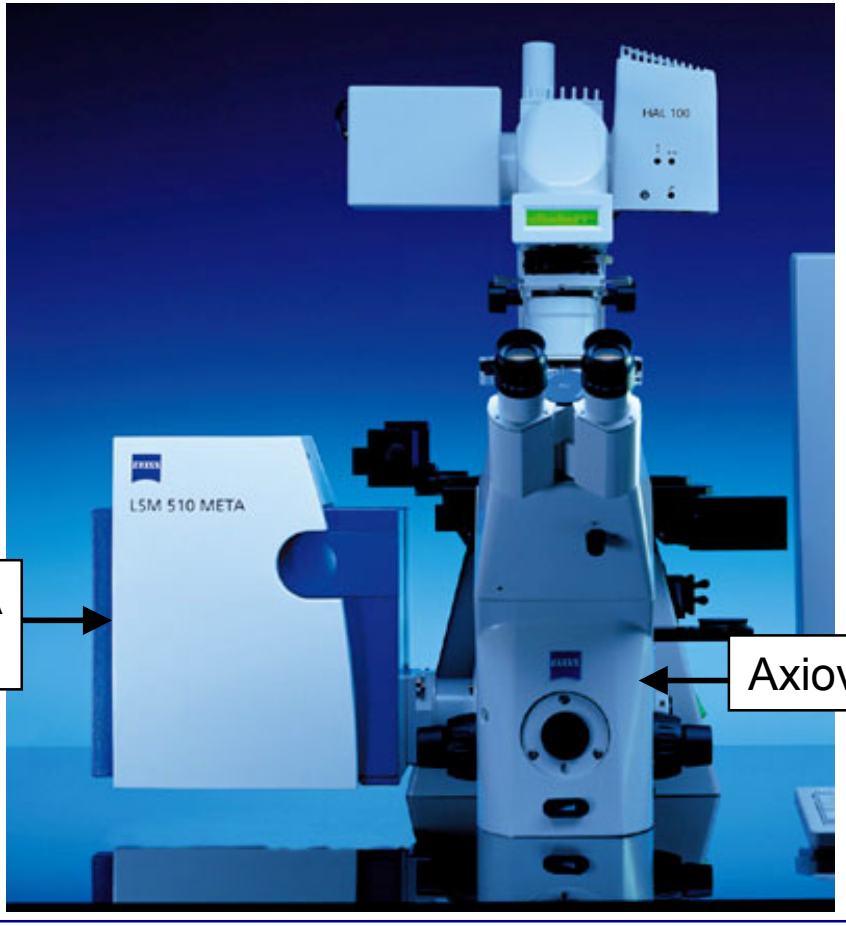
Inverted Zeiss LSM 510 confocal microscope



Upright Zeiss LSM 510 META confocal microscope



Inverted Zeiss LSM 510 META confocal microscope



LSM 510 META scan head

Axiovert 200M

Contents

- Starting the Zeiss LSM 510 microscope, software and laser Selecting an objective and focusing the microscope
- Selecting an objective and focusing the microscope
- Configuring the laser scanning and detection for confocal image acquisition
- Acquiring a Z- and Time - Series
- Data storage

Descriptions also include the LSM 510 META

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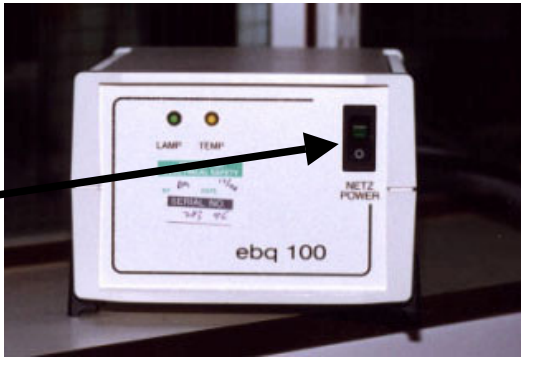
Start the Zeiss LSM 510 Confocal Microscope

1) First switch on the mercury lamp



2) Turn on the remote control switch

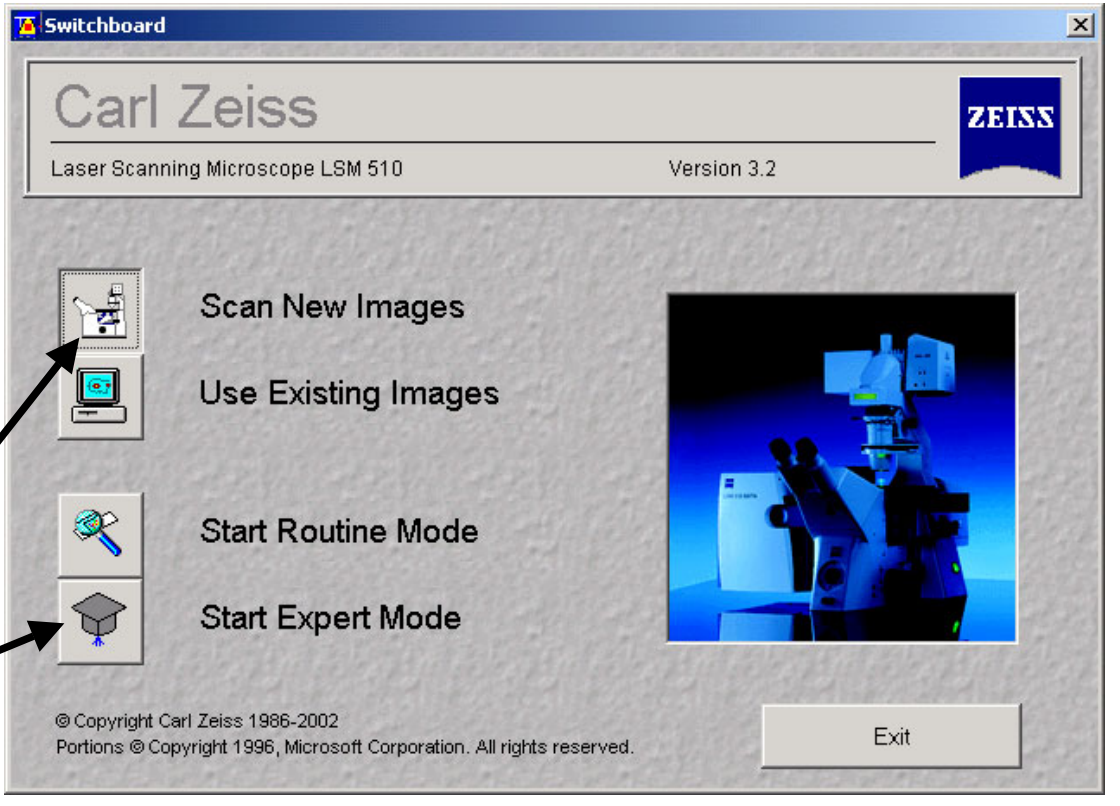
3) Wait for the computer to boot up and Login by simultaneously pressing the Ctrl, Alt and Delete keys

Starting the LSM 510 software

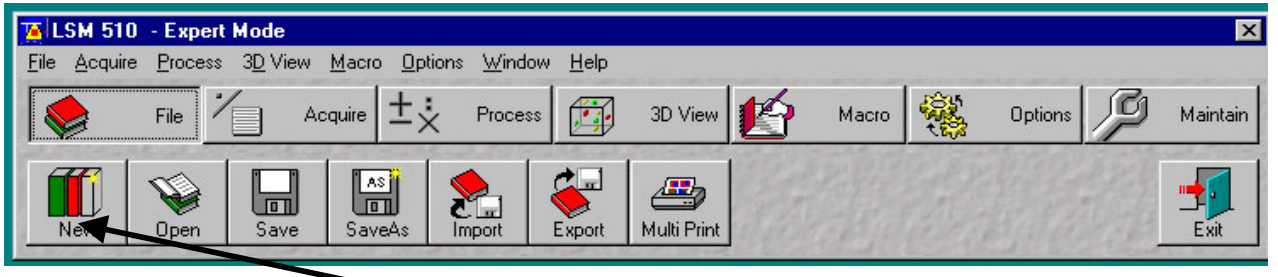
1) Double click the LSM 510 icon

2) Select “Scan New Images”

3) Select “Start Expert Mode”



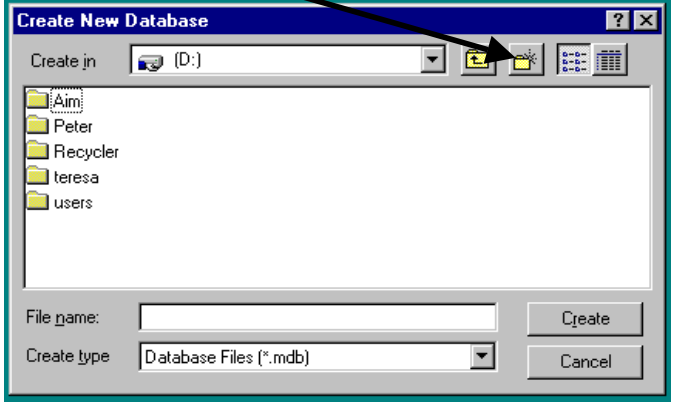
Creating a database for acquired images



1) In the main menu *File* select *New* database

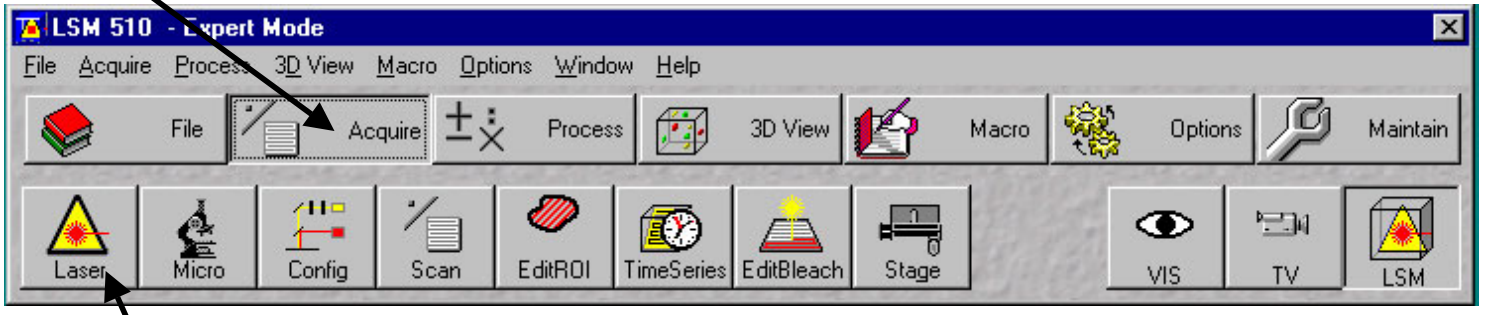
2) Select drive C or D: from pull down menu

3) Create a new directory if needed



Turning on the lasers

1) Select Acquire

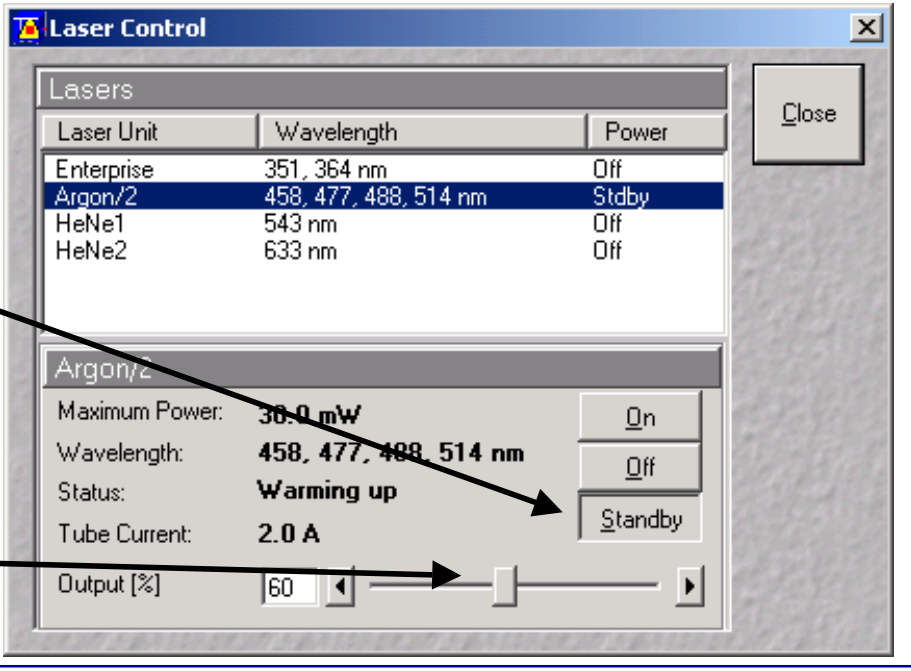


2) Select *Laser*

3) Switch required laser/s to *Standby*

4) When status is *Ready*, click *On*

5) Set Output [%] so that the tube current is between 5.5 and 6.5 A

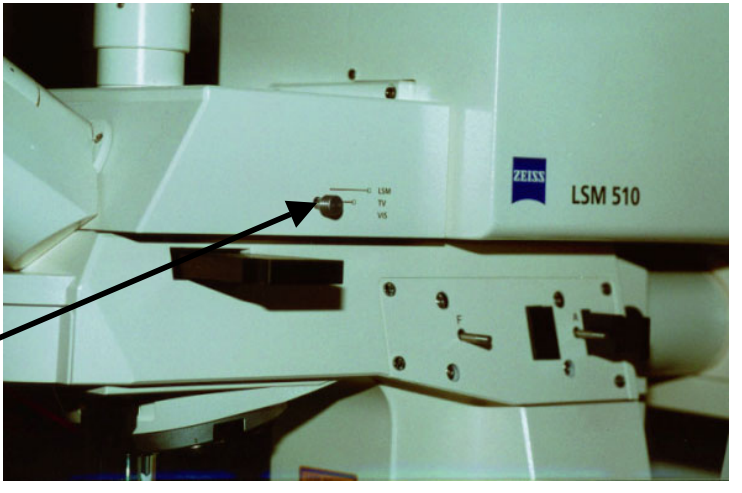


Change between direct observation and laser scanning

Upright Microscopes: Axioplan 2 imaging and Axioskop 2 FS

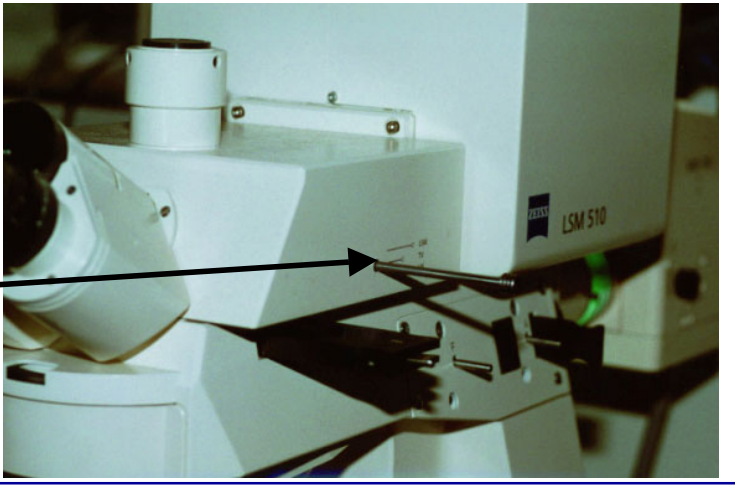
For direct observation of transmitted light and fluorescence:

Set slider to “VIS” (push it in)



For laser scanning image acquisition:

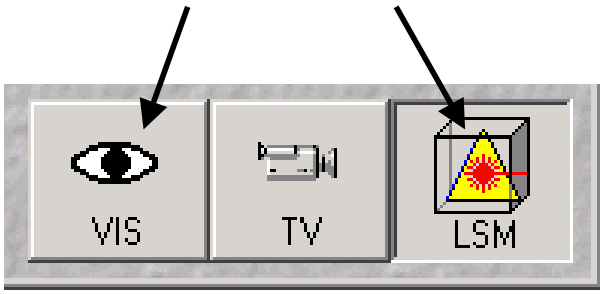
Set slider to “LSM” (pull slider out)



Change between direct observation and laser scanning

Inverted Microscope: Axiovert 200 M

Toggle between Vis and LSM button in main menu, automatic switching between direct observation and laser scanning (no slider)



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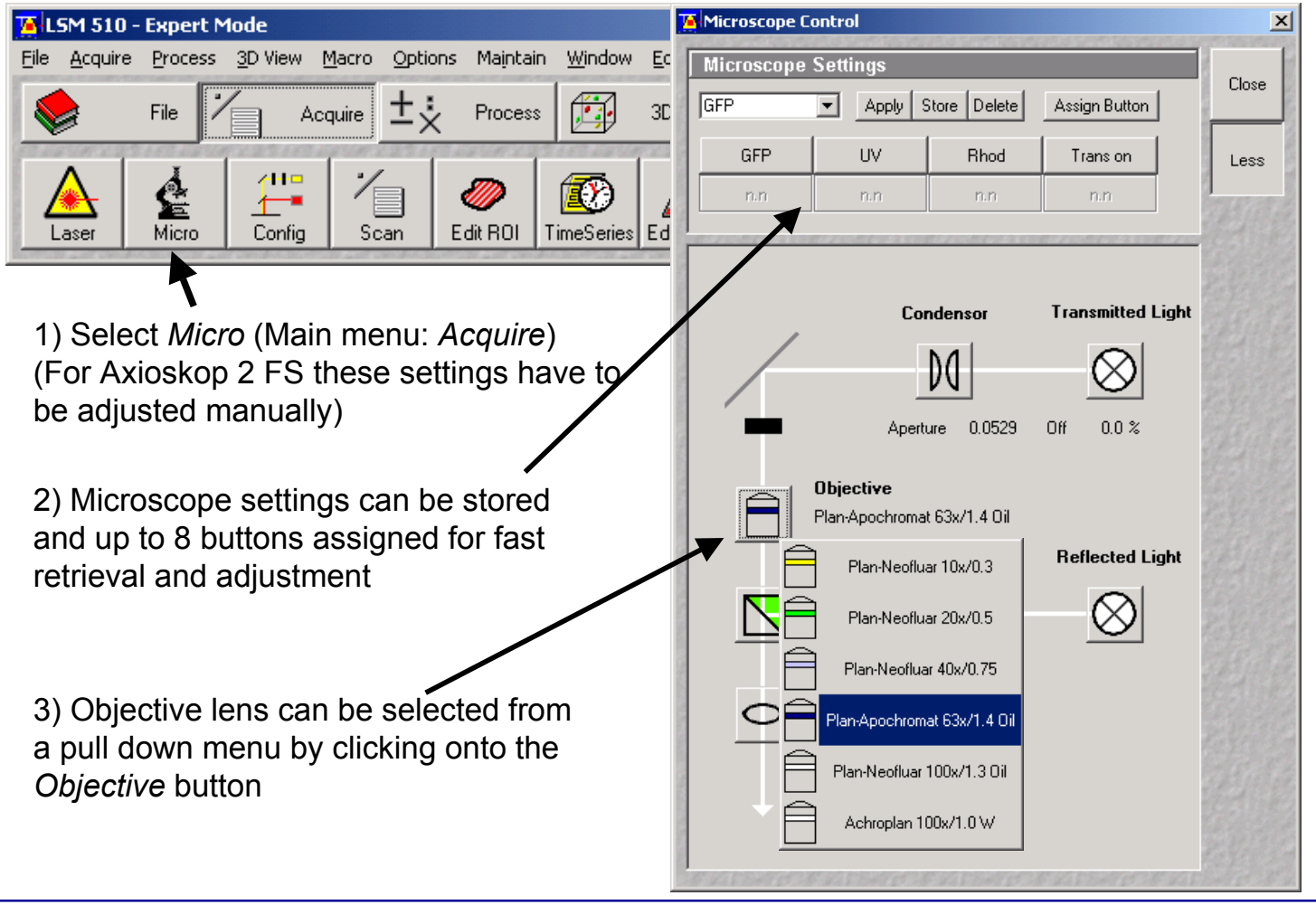
Selecting an objective and focusing the microscope

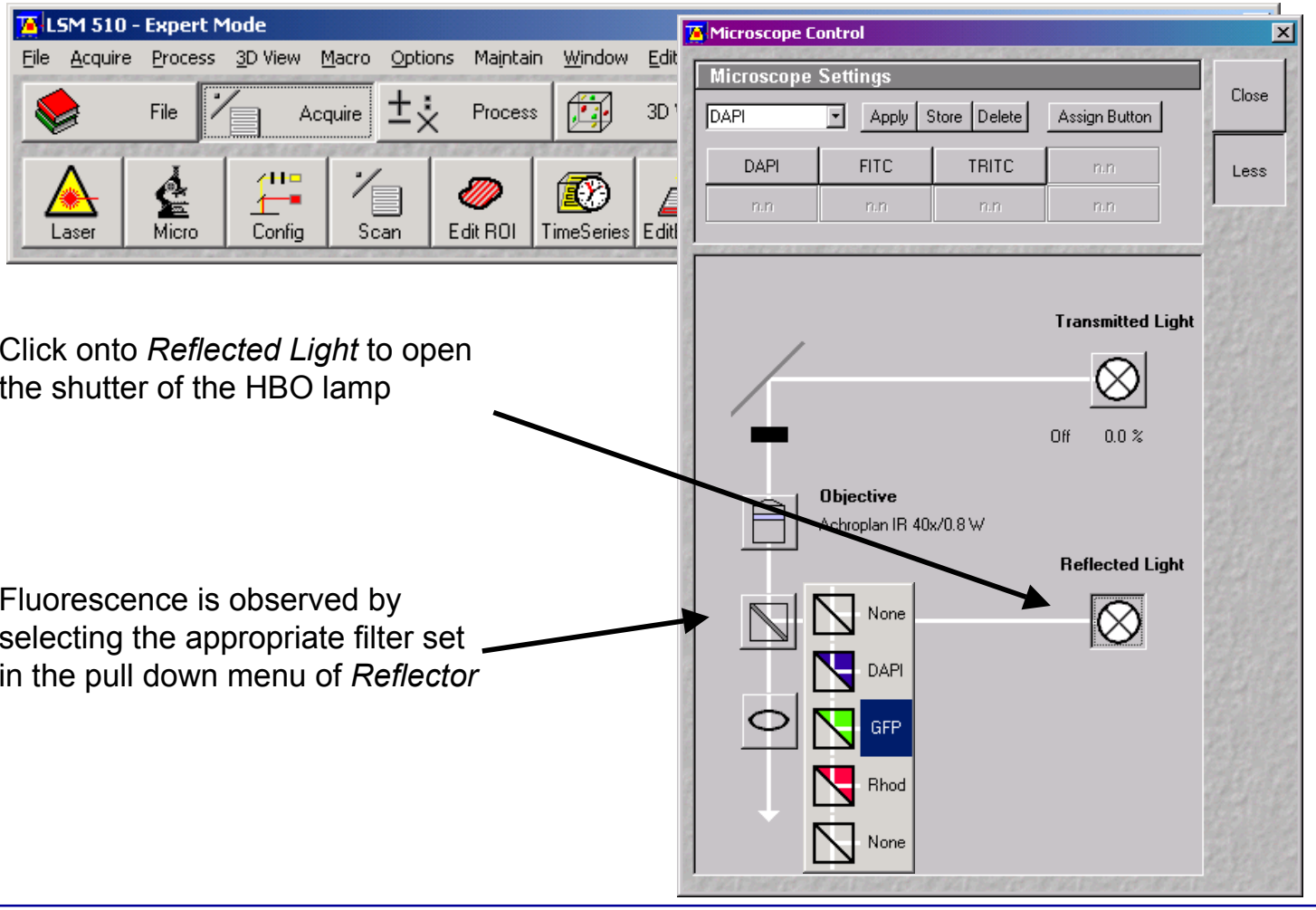


1) Select *Micro* (Main menu: *Acquire*) (For Axioskop 2 FS these settings have to be adjusted manually)

2) Microscope settings can be stored and up to 8 buttons assigned for fast retrieval and adjustment

3) Objective lens can be selected from a pull down menu by clicking onto the *Objective* button

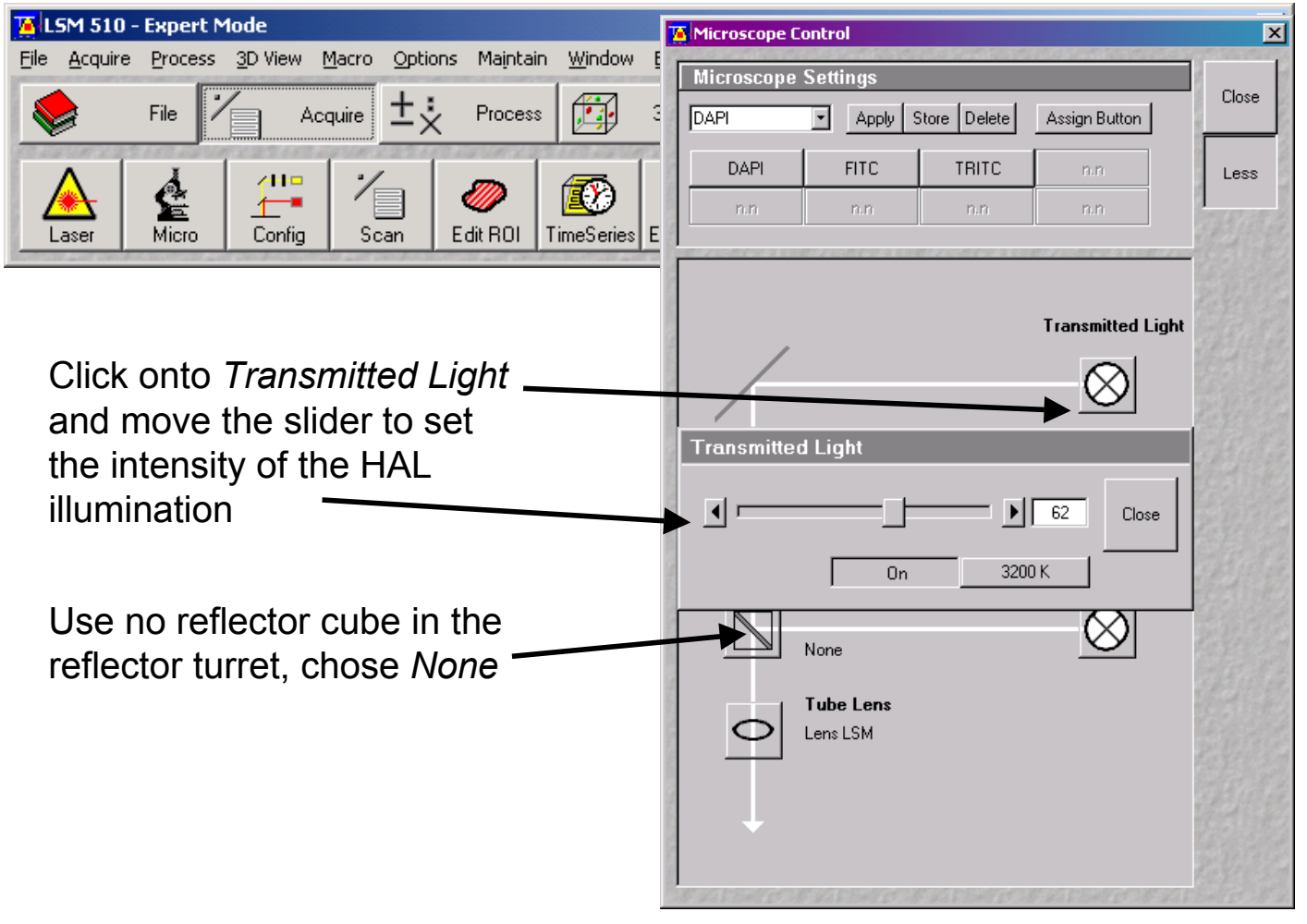
Focusing the microscope in fluorescence mode



Click onto *Reflected Light* to open the shutter of the HBO lamp

Fluorescence is observed by selecting the appropriate filter set in the pull down menu of *Reflector*

Focusing the microscope in transmitted mode



Click onto *Transmitted Light* and move the slider to set the intensity of the HAL illumination

Use no reflector cube in the reflector turret, chose *None*

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Choosing the configuration

SINGLE TRACK

Use for **single**, double and triple labelling

Simultaneous scanning only

ADVANTAGES

Faster image acquisition

DISADVANTAGES

Cross talk between channels

MULTI TRACK

Use for double or triple labelling

Sequential scanning, line by line or frame by frame

ADVANTAGES

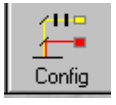
When one track is active, only one detector and one laser is switched on. This dramatically reduces crosstalk.

DISADVANTAGES

Slower image acquisition

Configuration of the filters and storage of the track configurations

SINGLE TRACK - lasers scan simultaneously

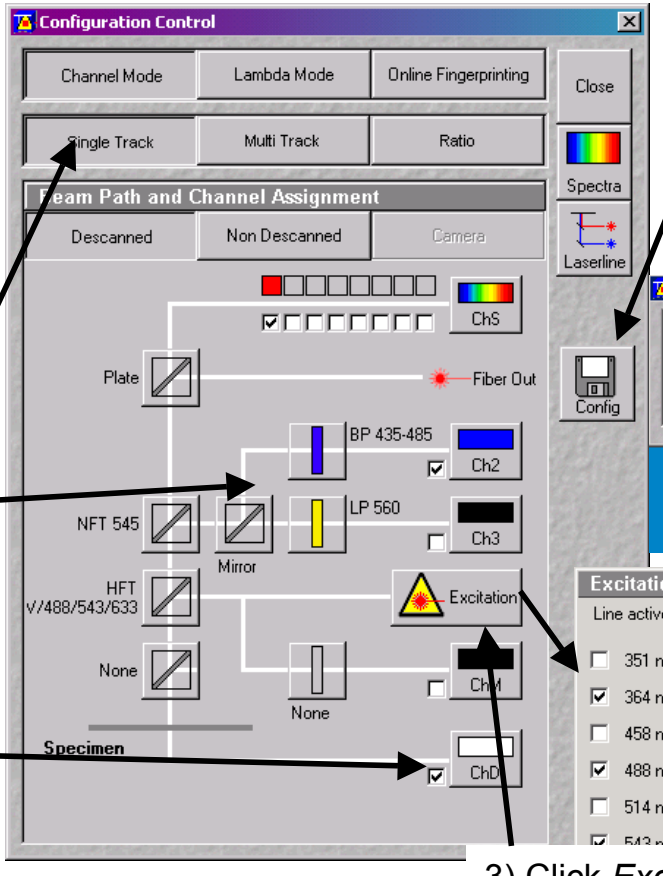


1) Select *Config* in the *Acquire* menu

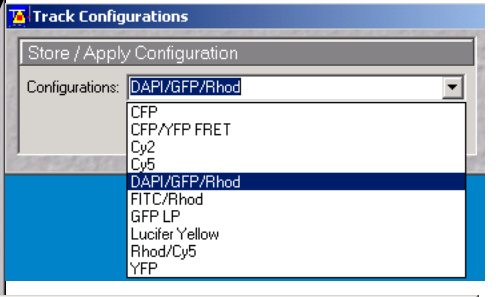
2) Select *Single Track*

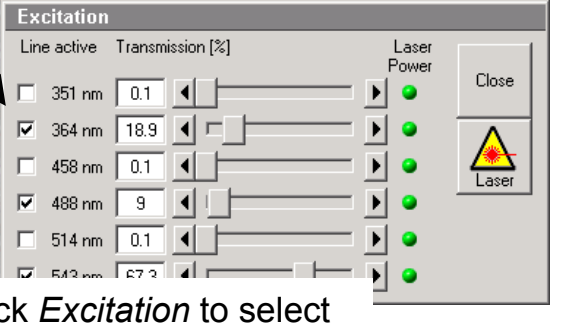
4a) Select the appropriate filters and activate the Channels

4c) Transmitted light image can also be generated. Transmission channel is usually set to white colour.



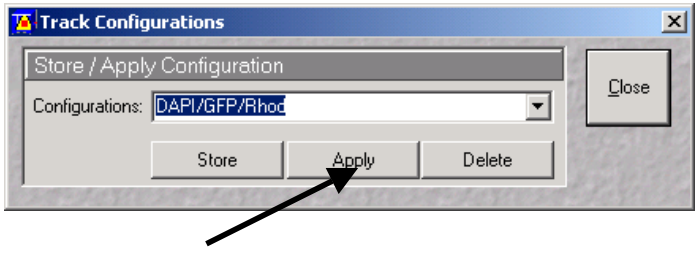
4b) The *Config* button opens the pull down menu to load/store Track configurations





3) Click *Excitation* to select the laser and attenuation

Applying a stored configuration and checking the settings



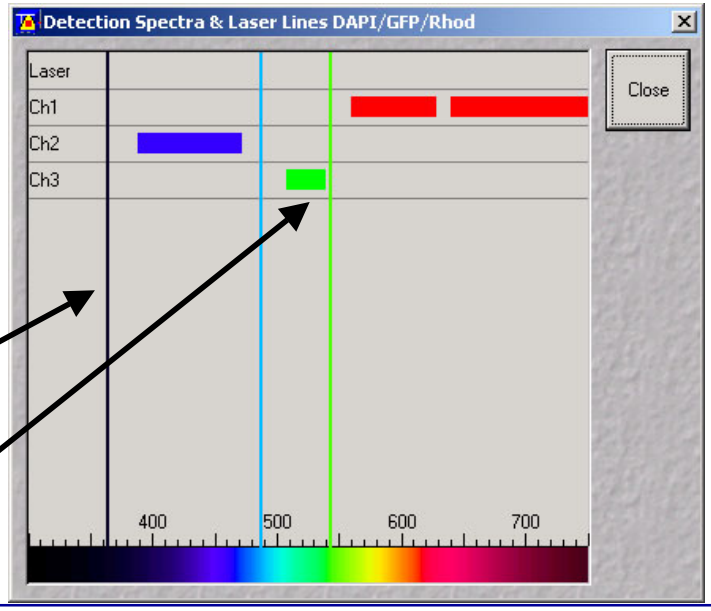
5) Chose a configuration in the *Track Configuration* menu. Select *Apply*

If you select *Store* by mistake, software will ask you, if you want to overwrite the configuration. **ANSWER *NO*!**

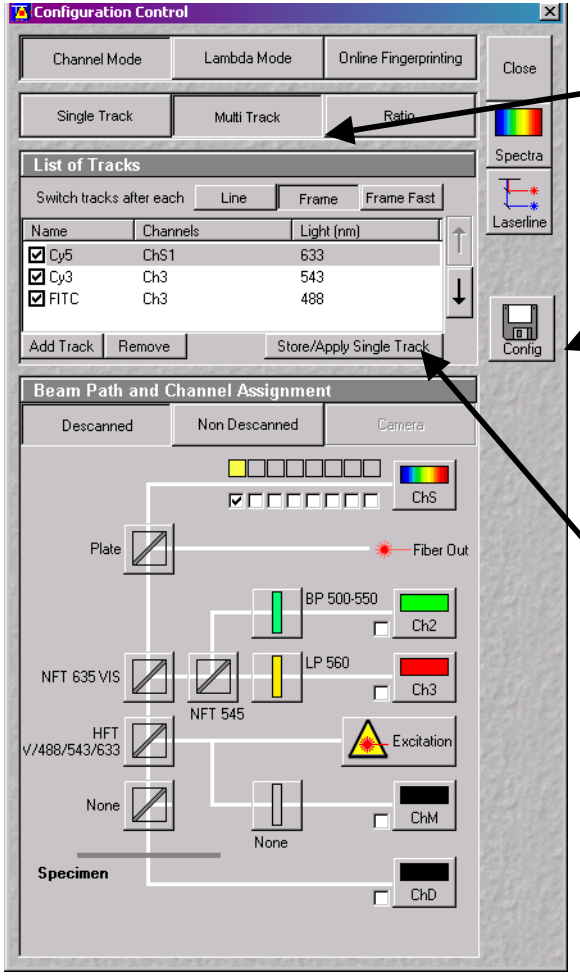
Each new login loads a predefined set of correct configurations.

6) To check for correct settings, click the *Spectra* button

The *Spectra* button opens a window to display the activated laser lines for excitation (colored vertical lines) and channels (colored horizontal bars)



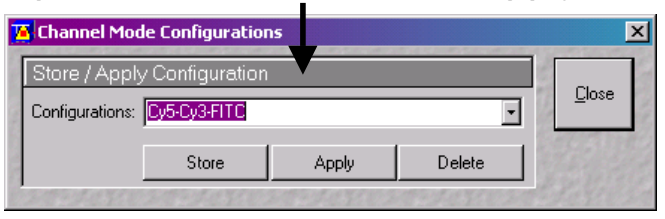
Multi Track Configuration



1) Select *Multi Track* for sequential scanning

2) Select *Config*

3) Select a stored track from the pull down menu, click on *Apply*

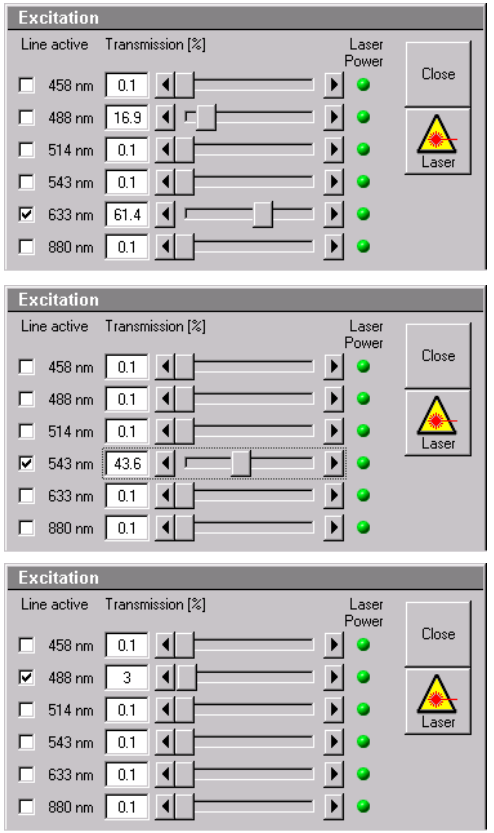


This button stores only the highlighted single track or applies a single track.

Cy5-Cy3-FITC Multi Track

Three laser lines and channels activated sequentially

Excitation

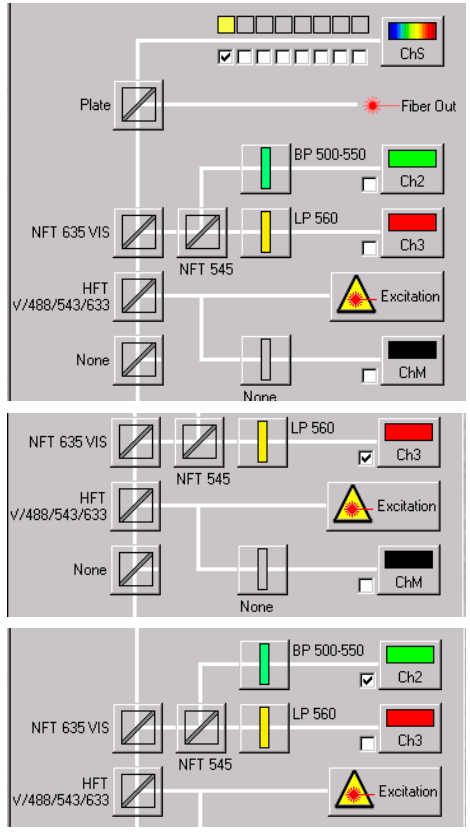


633 nm, using the META detector in Channel mode

543 nm

488 nm

Detection



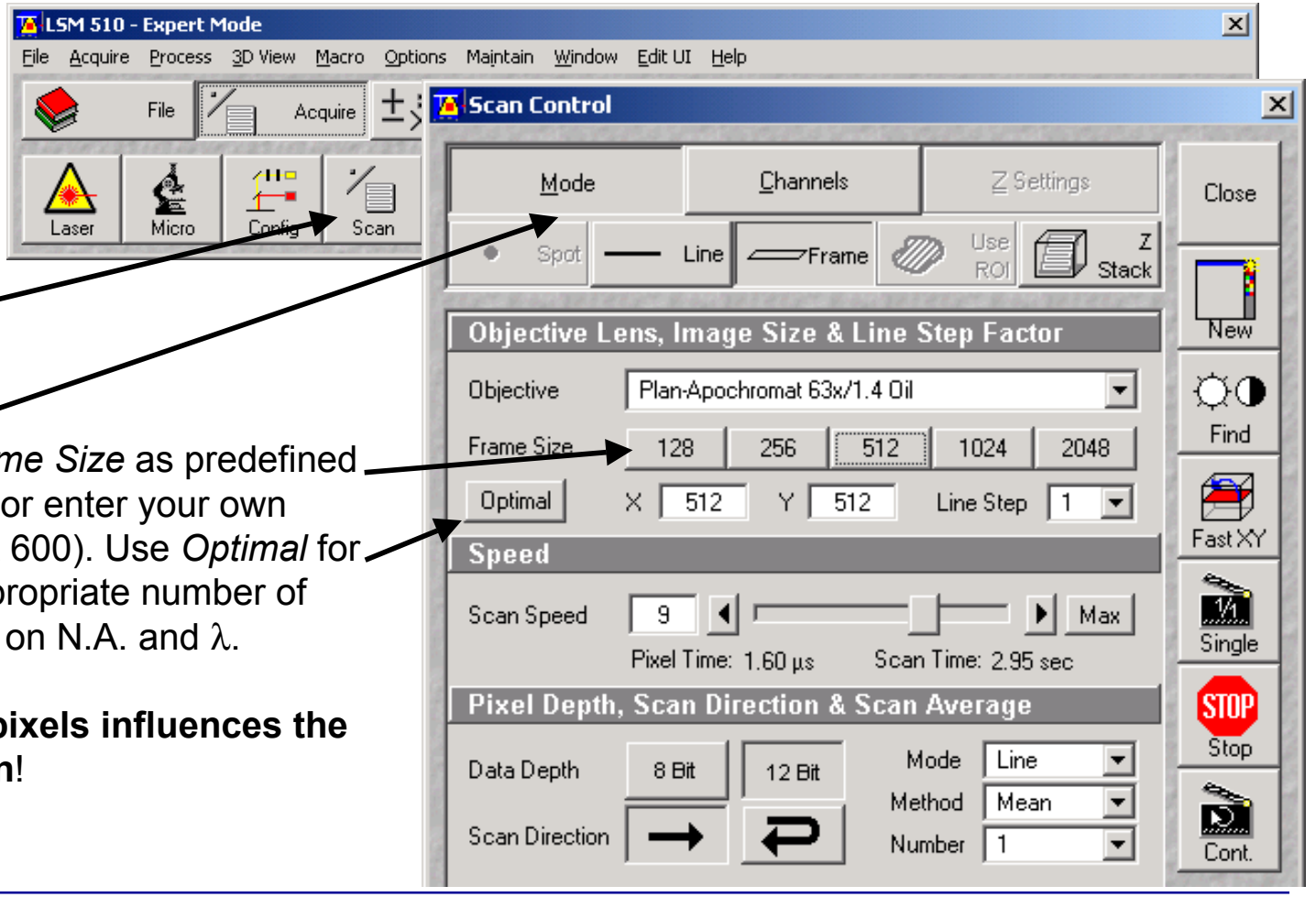
Setting the parameters for scanning

1) Select *Scan*

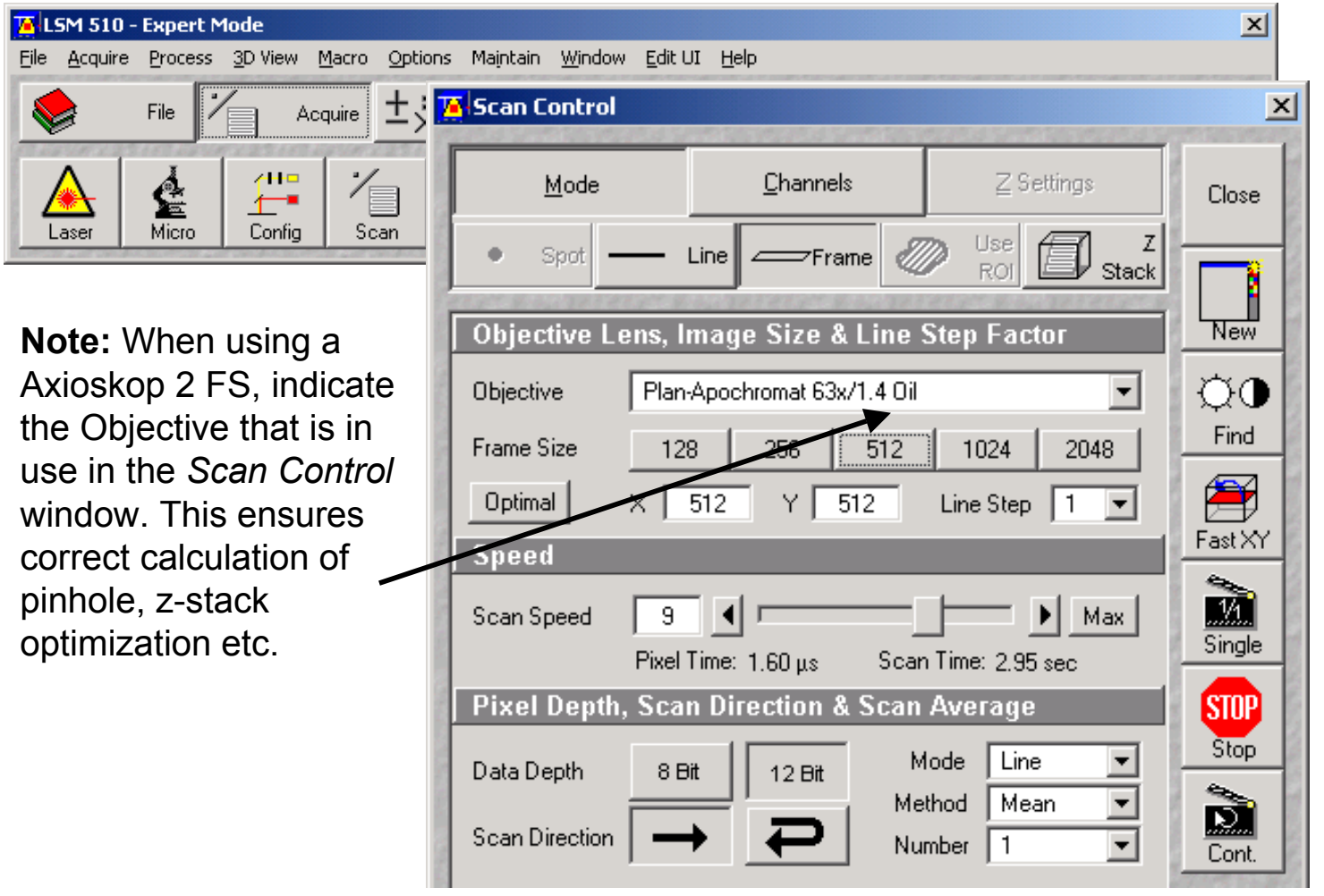
2) Select *Mode*

3) Select the *Frame Size* as predefined number of pixels or enter your own values (e.g 300 x 600). Use *Optimal* for calculation of appropriate number of pixels depending on N.A. and λ.

The number of pixels influences the image resolution!

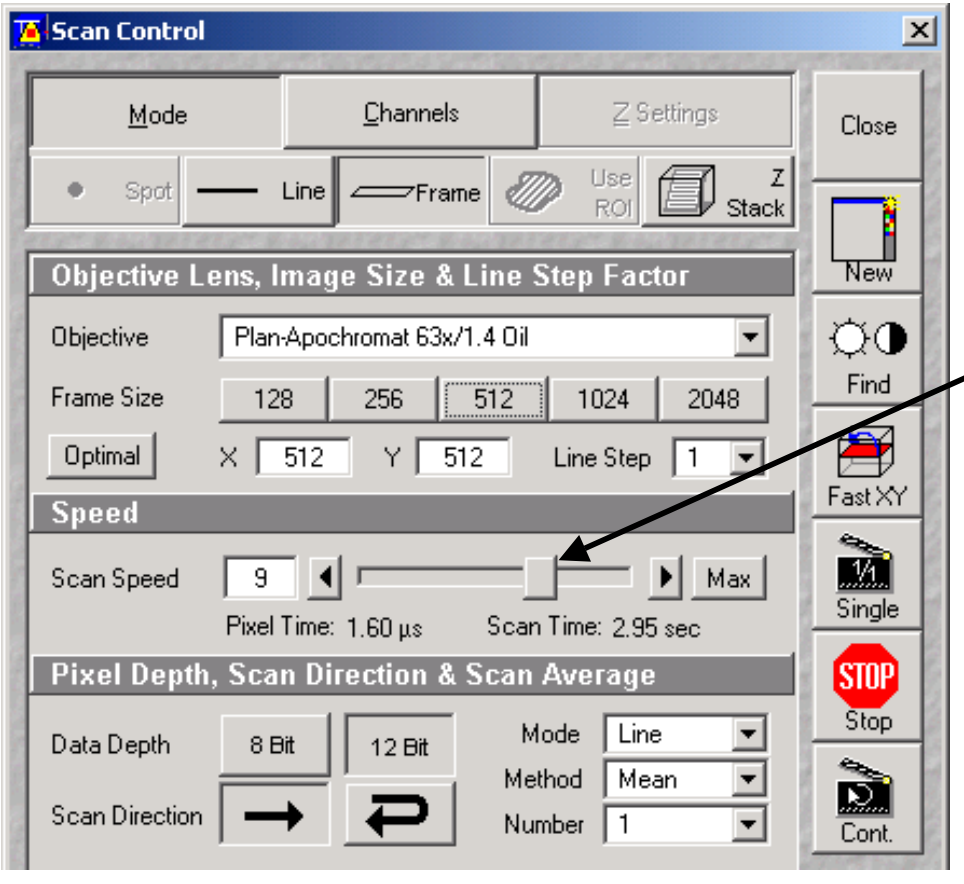


Setting the parameters for scanning



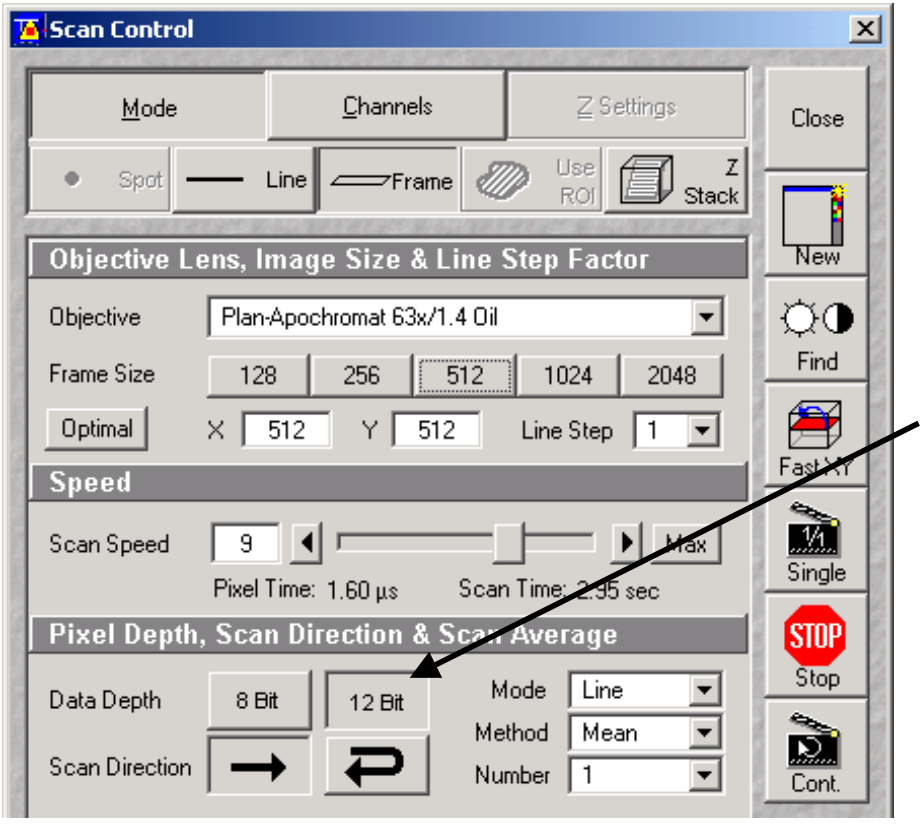
Note: When using a Axioskop 2 FS, indicate the Objective that is in use in the *Scan Control* window. This ensures correct calculation of pinhole, z-stack optimization etc.

Adjusting the scan speed



Adjust the scan speed - a higher speed with averaging results in the best signal to noise ratio. Scan speed 8 usually produces good results. Use 6 or 7 for superior images.

Choosing the Dynamic Range (8/12 Bit per pixel)

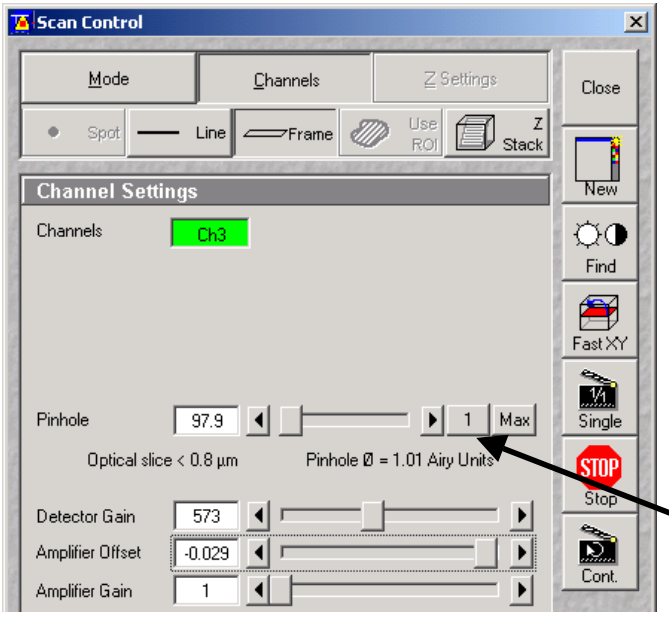


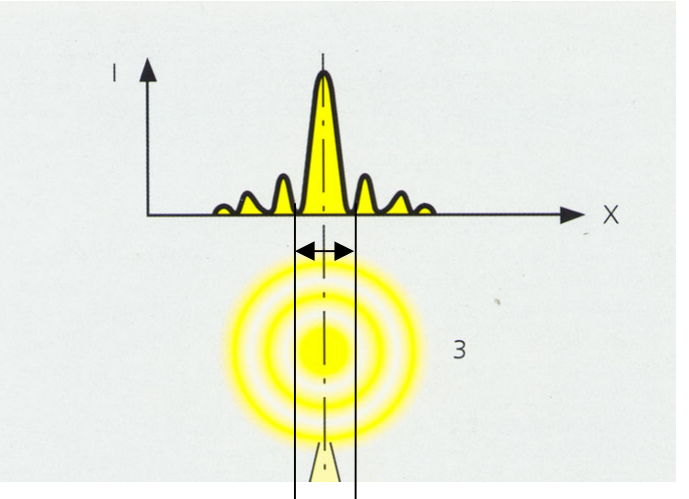
Select the dynamic range - 8 bit will give 256 gray levels, 12 Bit will give 4096 levels. Photoshop 5 will import 12 and 16 Bit images.

Publication quality images should be acquired using 12 Bit.

12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.

Channel Settings - Adjusting the Pinhole





Pinhole size
= 1 Airy unit

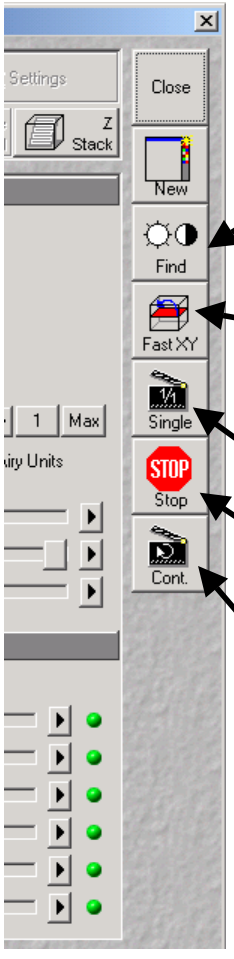
Set pinhole size to 1 Airy unit for best compromise between depth discrimination and efficiency.

Pinhole adjustment changes the “Optical slice”.

When collecting multi channel images, adjust the pinholes so that each channel has the same “Optical Slice”.

This is important for colocalization studies.

Image Acquisition



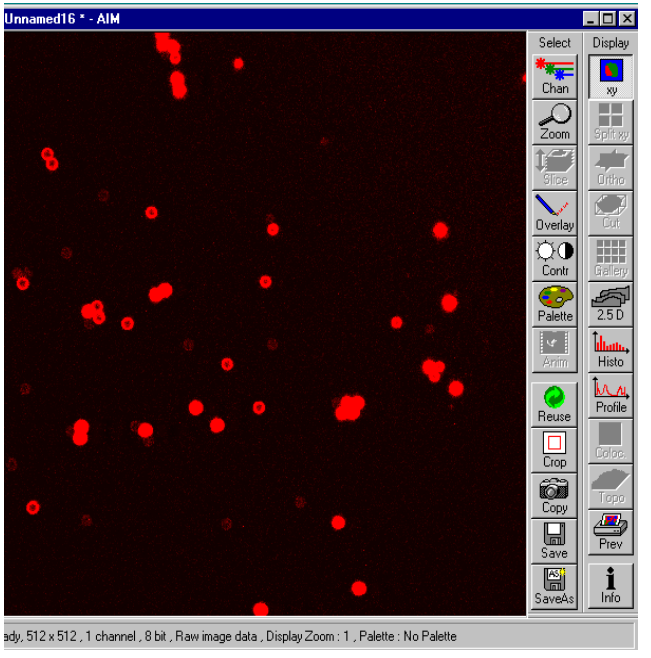
1) *Find* opens new image window and automatically pre-adjusts detector sensitivity

2) Select *Fast XY* for continuous fast scanning - useful for finding and changing the focus

3) *Single* records a single image

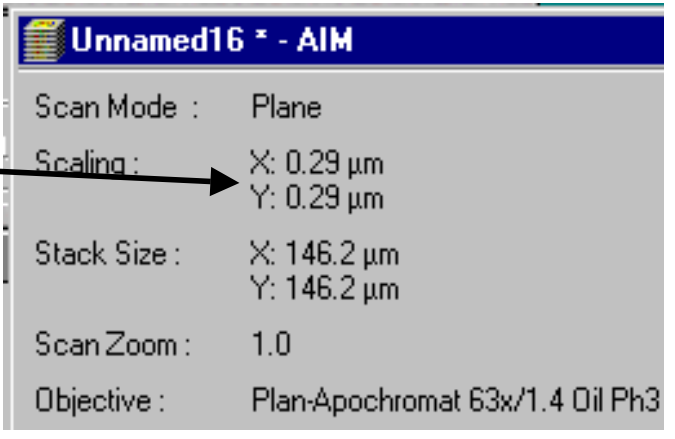
4) *Stop* blanks the laser beam and stops the scanning mirrors

5) Select *Cont.* for continuous scanning with selected scan speed



Minimal Pixel Size determined by Nyquist Sampling

Magnification	NA	Pixel size
5	0.15	1.03 µm
10	0.3	0.51 µm
20	0.5	0.31 µm
40	1.3 (oil)	0.12 µm
63	1.4 (oil)	0.11 µm
100	1.4 (oil)	0.11 µm



Values are for scan zoom = 1.0

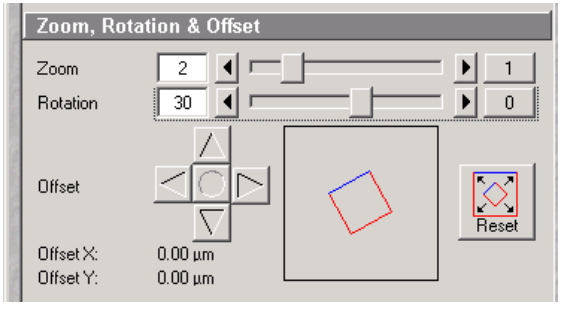
Adjusting the field size (“XY”) to 56 µm with the 63× lens, would produce a pixel size of 0.1 µm

Brightness of image = Magnification^2/NA^2

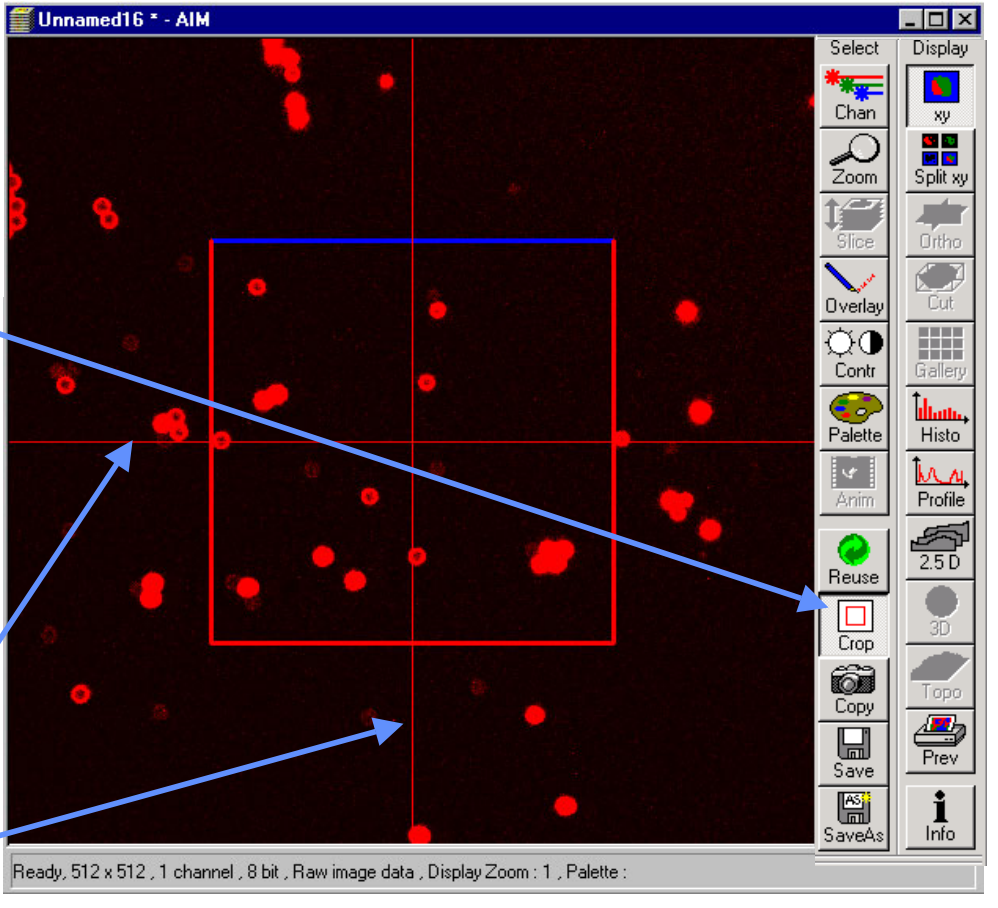
Field size can be adjusted by changing the objective magnification, or by optical zooming. Changing from 63 × to 100 × will reduce the field size, but will also reduce the amount of light available.

Optical Zooming

The level of zoom can be changed either by using the *Zoom, Rotation & Offset* control in *Mode* menu of the *Scan* Control, or by selecting *Crop* in the image menu.



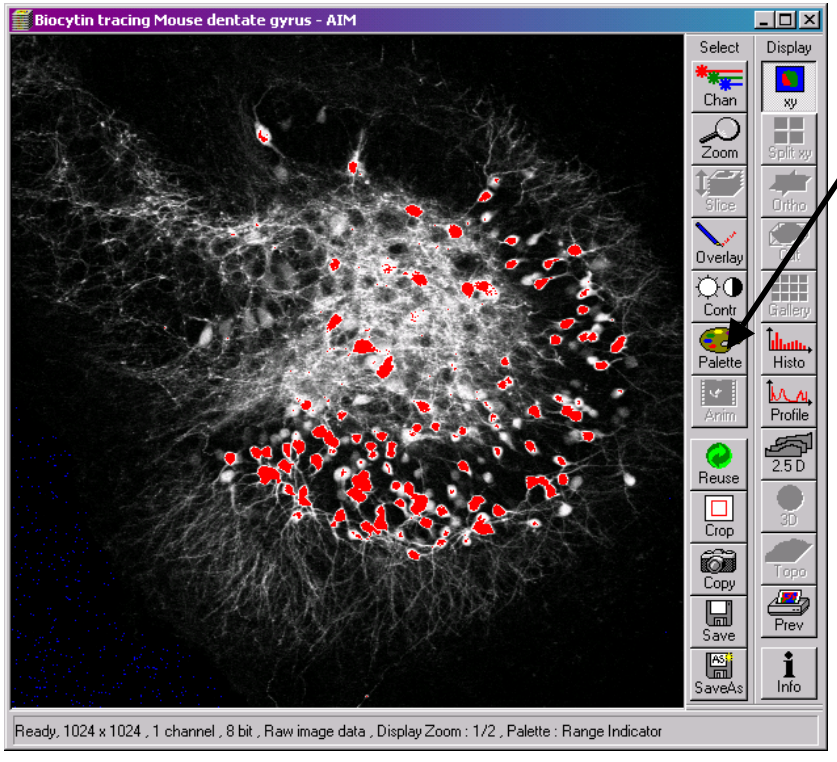
The image can also be rotated by selecting and dragging the bars

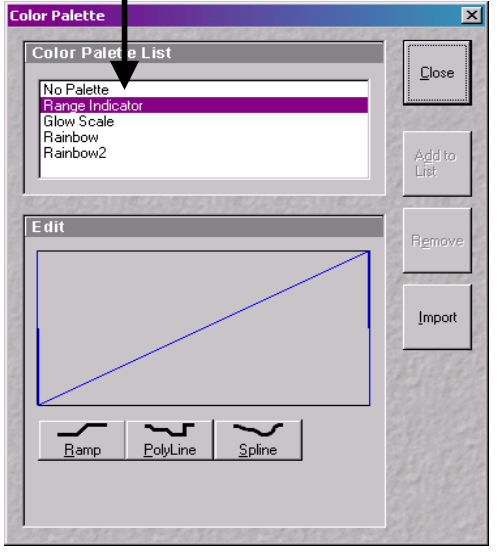


Selecting gain and offset – Choosing a lookup table

1) Select *Palette*

2) Select Range Indicator





Red = Saturation (maximum)

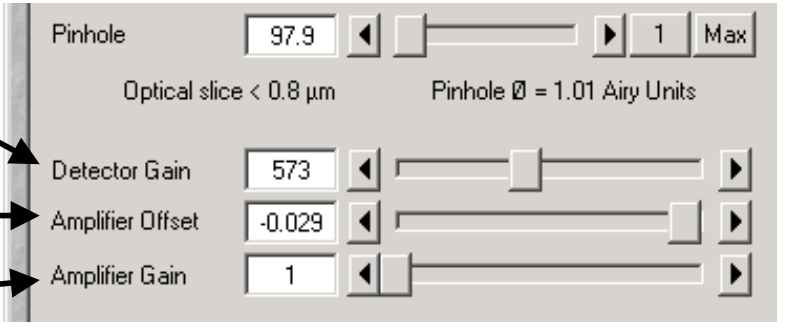
Blue = Zero (minimum)

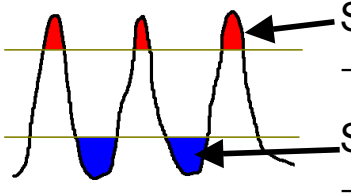
Scan Control – Setting Gain and Offset

Detector gain determines the sensitivity of the detector by setting the maximum limit

Amplifier Offset determines the minimum intensity limit

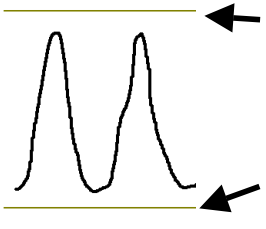
Amplifier Gain determines signal amplification





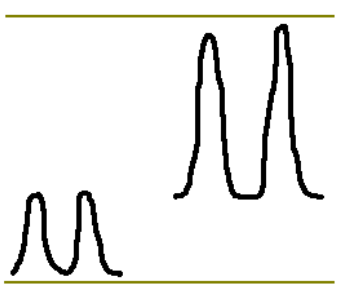
Saturation at the maximum
→ reduce *Detector Gain*

Saturation at the minimum
→ increase *Amplifier Offset*



Gain set correctly

Offset set correctly

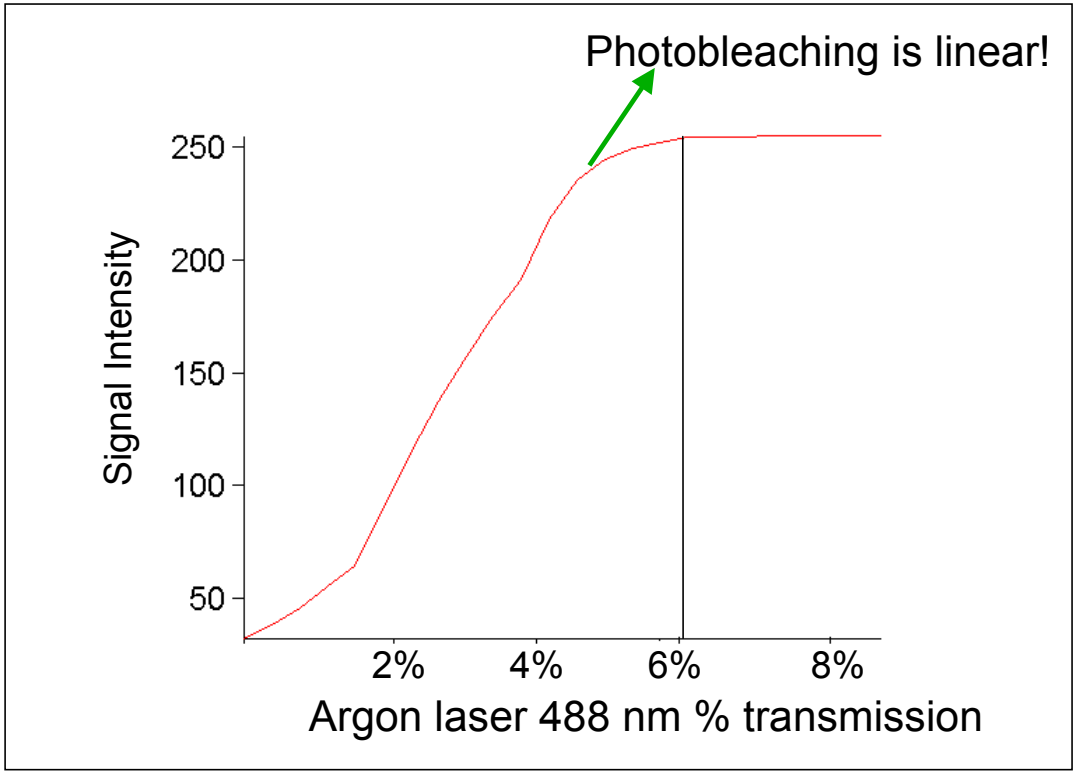


Gain

Offset

Amplifier Gain increases the whole signal, and the *Amplifier Offset* will need to be decreased.

Saturation of Signal Intensity with Laser Power



- Fluorophore saturates at 6% laser transmission
- Photobleaching is linear

Laser transmission should not be set higher than the saturation level.

Adjusting the Laser Intensity

1) Set *Pinhole* to 1 Airy unit

2) Set *Detector Gain* high

3) When the image is saturated, reduce AOTF transmission in the *Excitation* panel to reduce the intensity of the laser light at the specimen

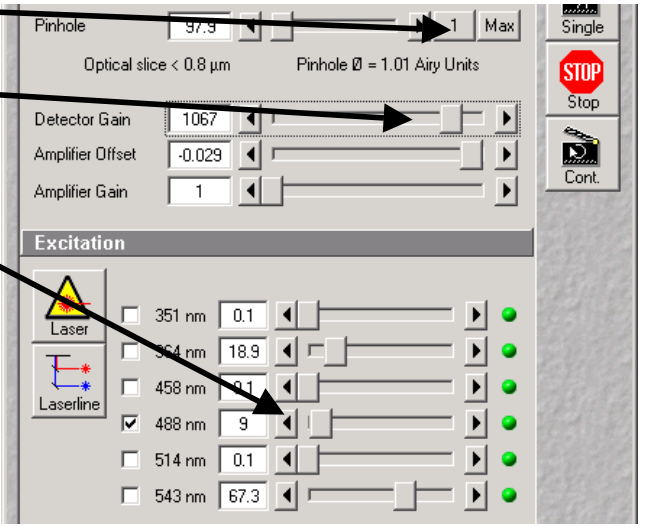
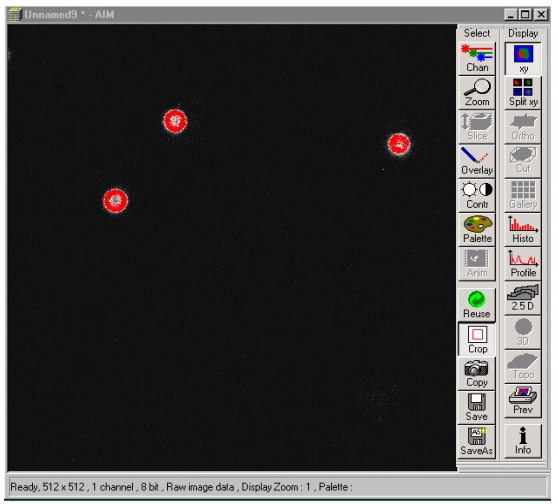
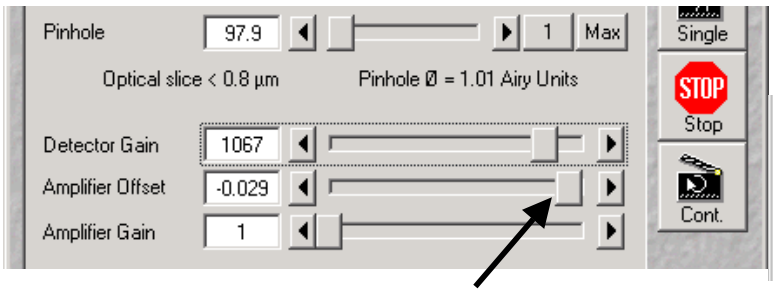


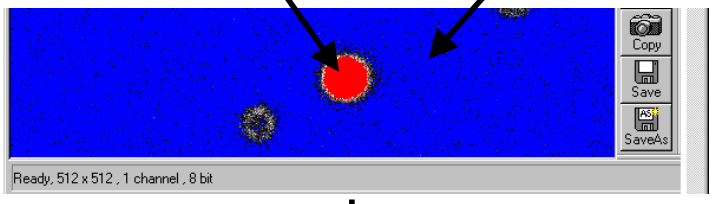
image with saturated pixels



Adjusting Gain and Offset



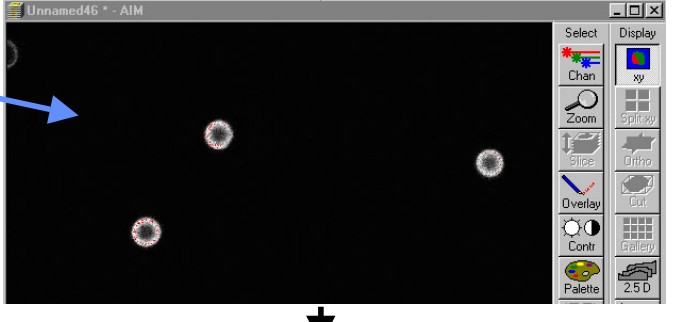
Both *Detector Gain* and *Amplifier Offset* saturated

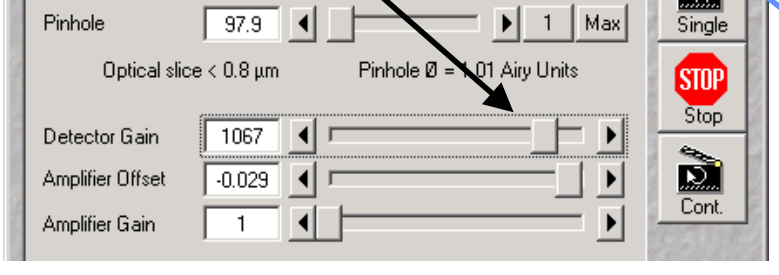


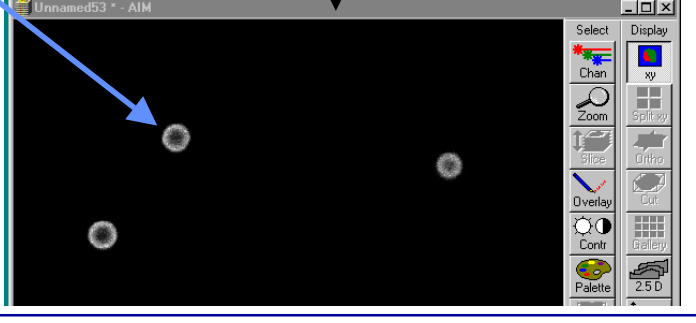
gain and offset **not** correct

1) Increase the *Amplifier Offset* until all blue pixels disappear, and then make it slightly positive.

2) Reduce the *Detector Gain* until the red pixels only just disappear.







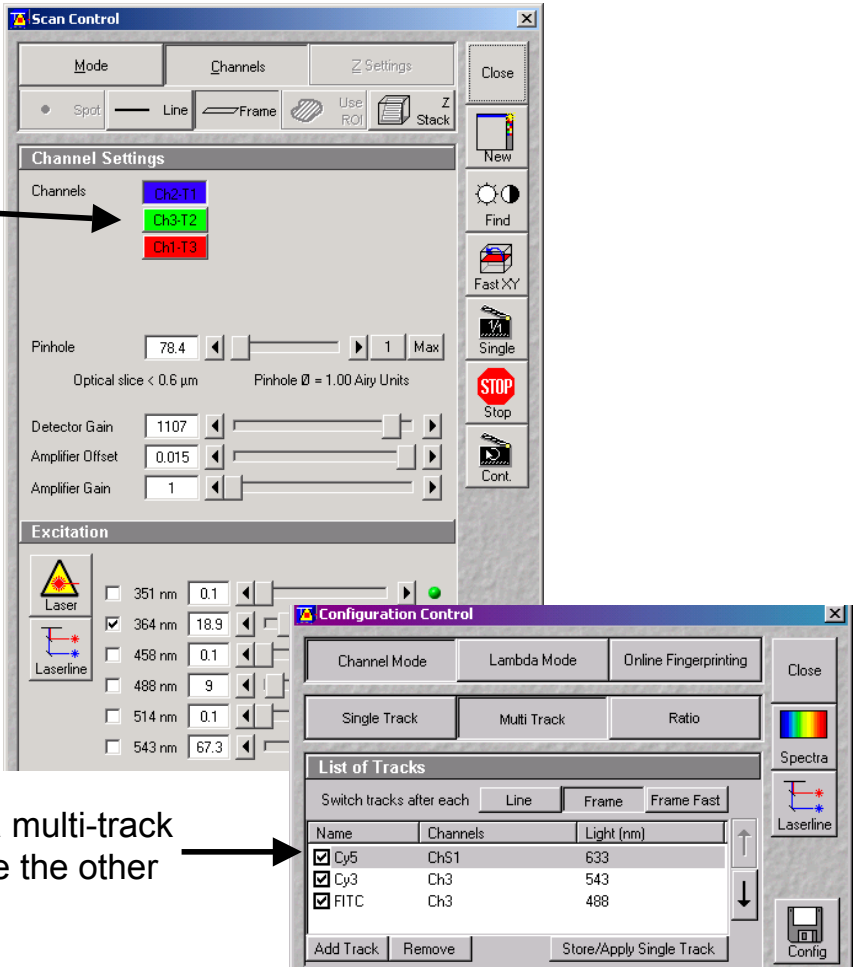
gain and offset correct

no blue
no red

Adjusting the Laser, Gain and Offset using a Multi Track Configuration

Each channel is selected independently by clicking on the colour button indicating the channel i.e. *Ch2-T1* (Channel 2, Track 1). The laser power and all other parameters are optimised as described in the previous slides for each selected channel.

For accurate colocalisation, adjust each *Pinhole* so that each channel has the same *Optical Slice*



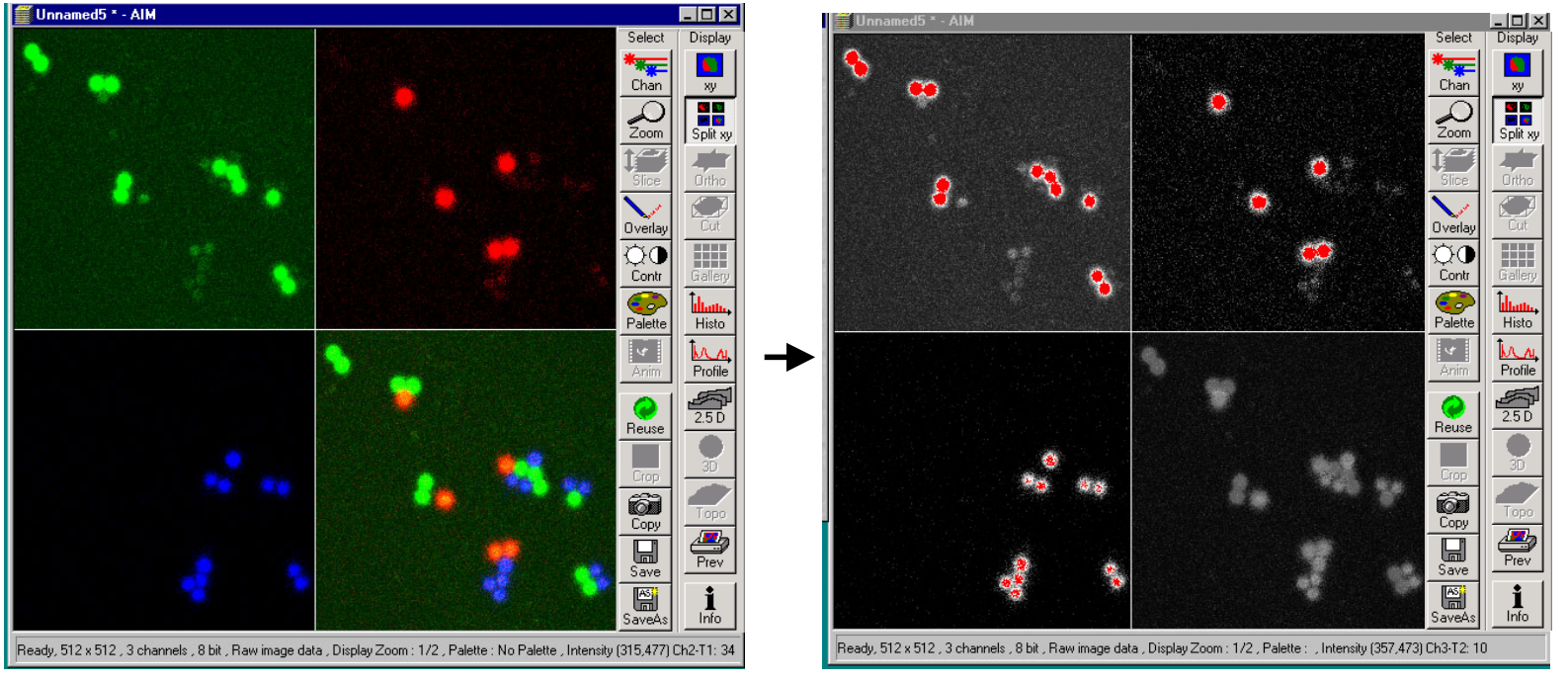
To adjust laser, gain or offset for a single track in a multi-track configuration it is possible to temporarily deactivate the other tracks in the *Configuration control*

Setting up Gain and Offset - Multi Track

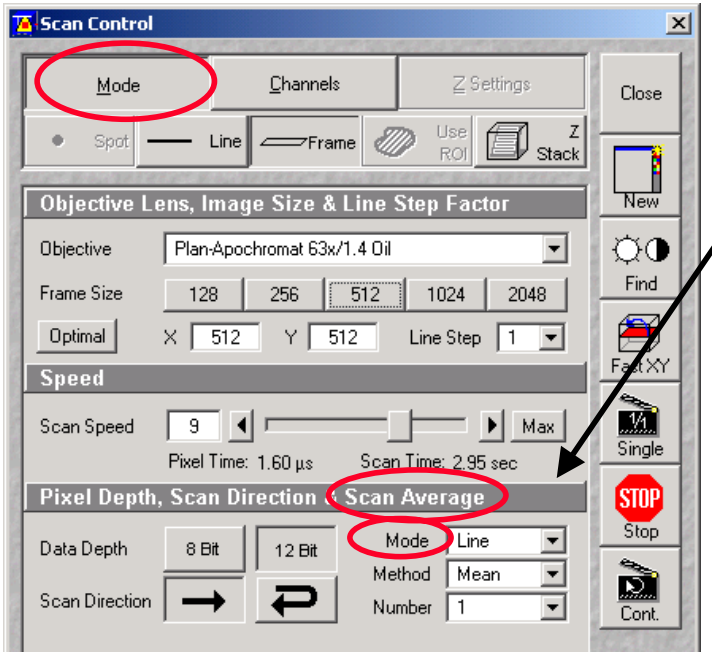
1) Select *Split XY* in the Image window

2) In *Palette*, select *Range indicator*

3) Select each channel separately under *Channels* in the *Scan control* window and adjust the Laser intensity, *Detector Gain*, and *Amplifier Offset* as described previously.



Line Averaging

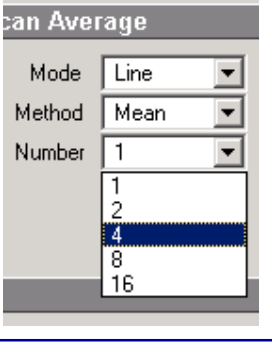


Averaging improves the image by increasing the signal : noise ratio

Averaging can be achieved line by line, or frame by frame

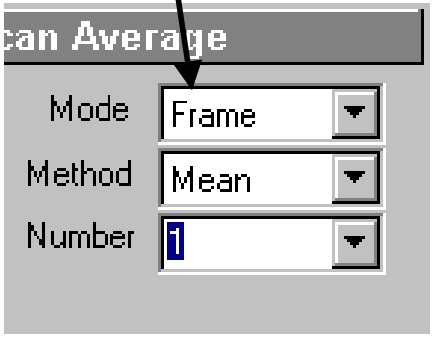
1) Select *Line* or *Frame* under *Mode* in *Scan Average* within the *Mode* panel of the *Scan Control* window

2) Select *Number* for averaging. The more the better for the signal to noise ratio (max 16) in this case, each line will be scanned 4 times. But: Averaging increases the exposure time of the sample!!

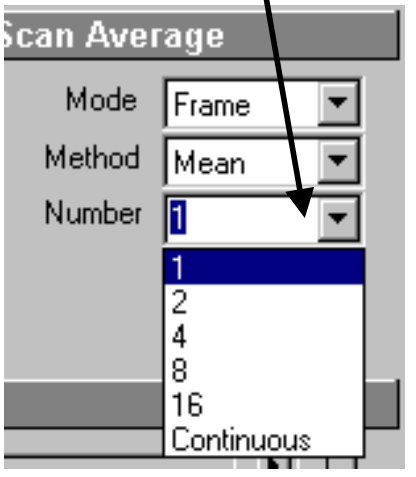


Frame Averaging

1) Select *Frame*

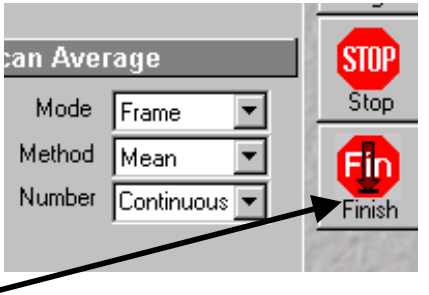


2) Select the *Number* for averaging - The more the better for signal to noise ratio (max 16). Continuous averaging is possible in this mode.



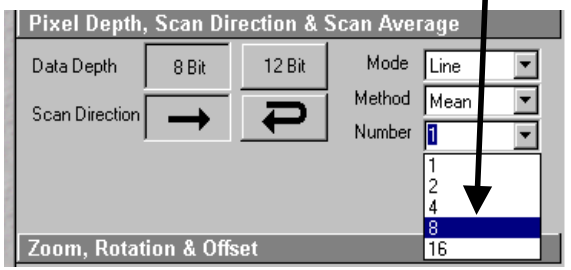
Frame averaging helps reduce photobleaching, but does not give quite such a smooth image. There is also a longer delay between each track when using “Multi Track”.

Continuous averaging has a *Finish* button which allows the scan currently in progress to be completed before stopping

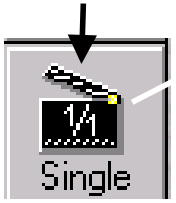


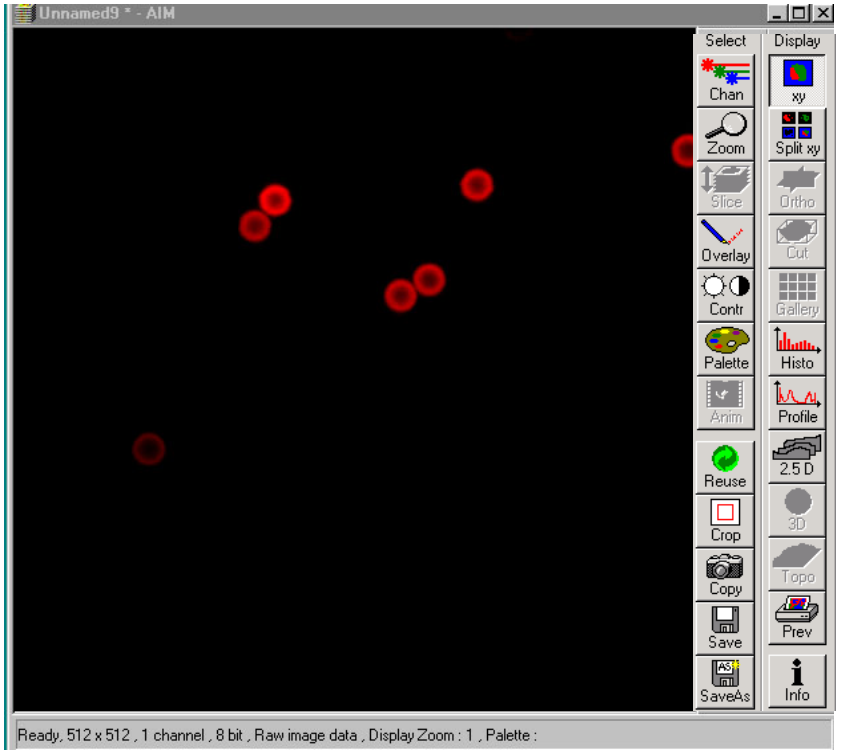
Collecting an Averaged Image

1) Under *Scan Average* select the *Number* for the average.

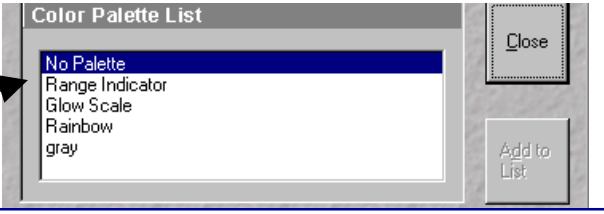


In the *Channels* panel of the *Scan Control* window select *Single*. An averaged image will be collected.





Range indicator set to *No Palette*

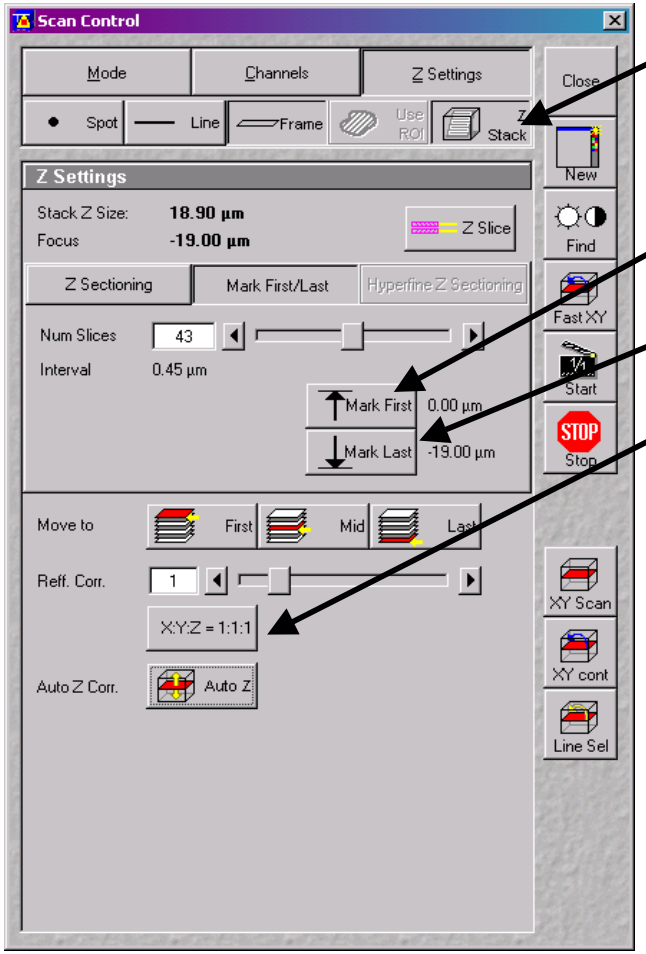


Contents

- Starting the Zeiss LSM 510 microscope, software and laser Selecting an objective and focusing the microscope
- Selecting an objective and focusing the microscope
- Configuring the laser scanning and detection for confocal image acquisition
- Acquiring a Z- and Time - Series
- Data storage

Descriptions also include the LSM 510 META

Scanning a Z-Series using *Mark First/Last*



1) Select *Z Stack*

2) Start scanning using *Fast XY* or *XY cont*

3) Keep your eye on the image and move the focus to the beginning of the Z-Series, then select *Mark First*

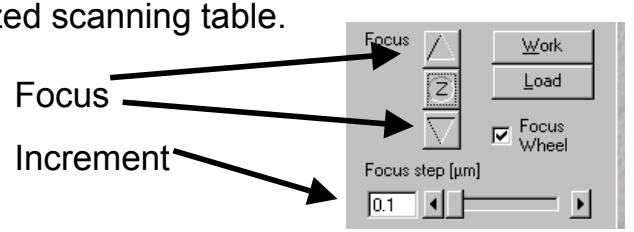
4) Move the focus back in the opposite direction to the end of the Z-Series, then select *Mark Last*

5) *X:Y:Z = 1:1:1* sets the Z-interval so that the voxel has identical dimensions in X, Y, and Z.

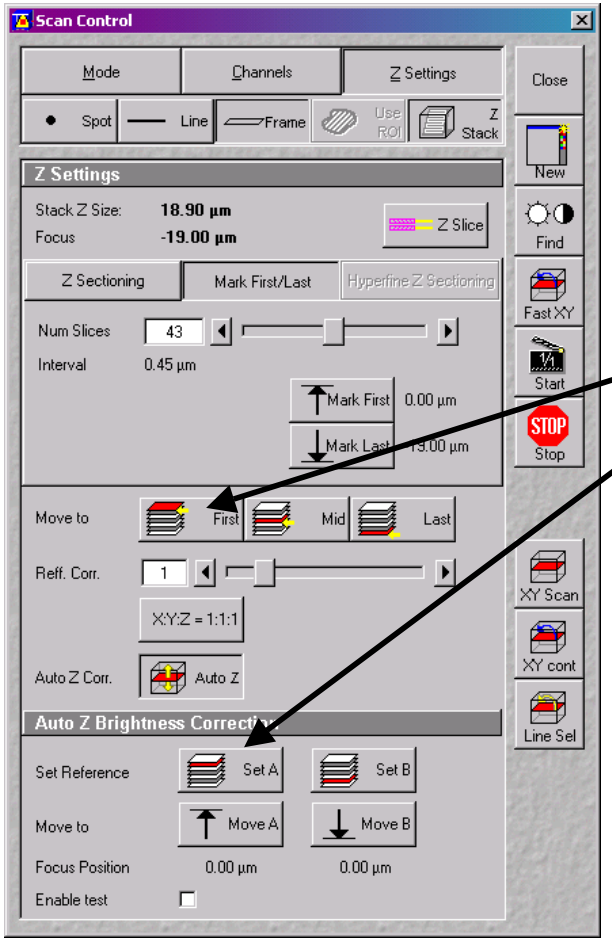
6) *Start* will initiate the acquisition of the Z-Stack. The acquisition can be stopped at any time.

NOTE

Focusing can be achieved manually (preferred), or using *Stage* in the LSM menu if there is a motorized scanning table.



Using Auto Z Brightness Correction



Auto Z provides an automatic gradual adjustment of *Detector Gain*, *Amplifier Offset, Amplifier Gain*, and Laser intensity setting between the first and last optical slice of a Z Stack.

1) After defining the Z position of the first and last optical slice activate *Auto Z.*

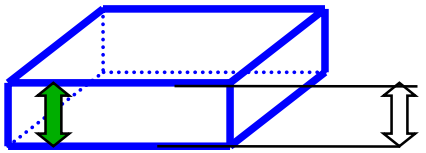
2) *Move to* the *First* Slice and adjust the parameter for the image acquisition in the *Channels* panel for each used channel as described in the previous slides. Then click on *Set A* to store the values.

3) Repeat the procedure after moving to the *Last* Slice. Click on *Set B* to store the parameters for the last slice.

Note: Positions A and B do not have to be the first and last slice of a stack and can also be defined simply by focussing to the appropriate positions, adjusting the parameters and pressing *Set A* or *Set B*.

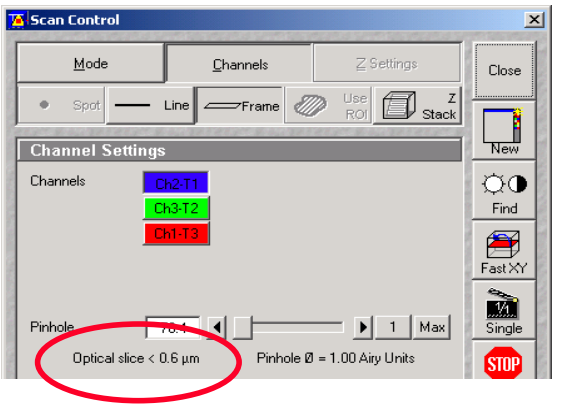
4) The parameters for image acquisition will be gradually and linearly adjusted between the first and last slice of the Z Stack. Thus signal intensity and image quality is comparable throughout the Z Stack.

Confocal Z Sectioning
Number of Sections for correct sampling

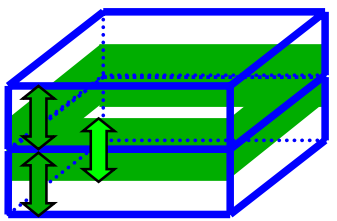


Optical thickness *d* depends on:

- Wavelength λ
- Objective lens, *N.A.*
- Refractive index *n*
- Pinhole diameter *P*



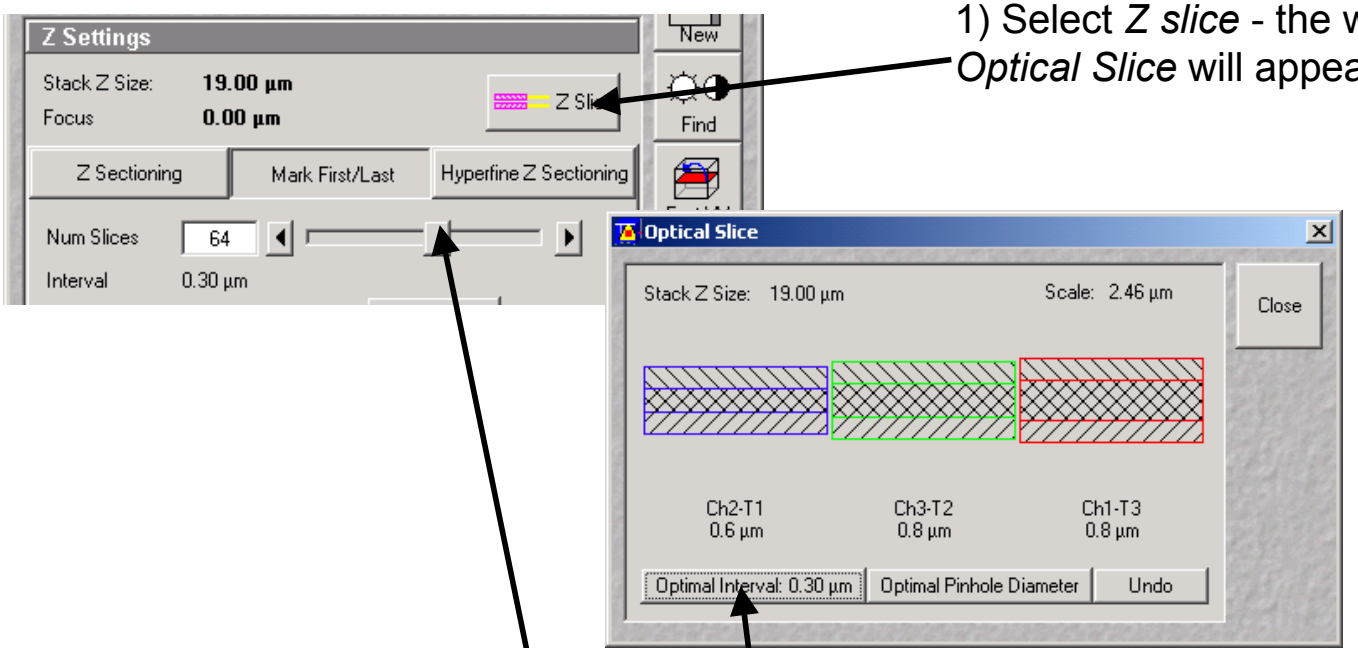
The optical slice thickness is displayed in the *Scan Control*



For Z-sectioning it is optimal to have:
no missing information @ minimal number of sections
Slices overlap by the half of their thickness

„Nyquist-“ or Sampling- Theorem

Z Stack – Number of Slices and Increment



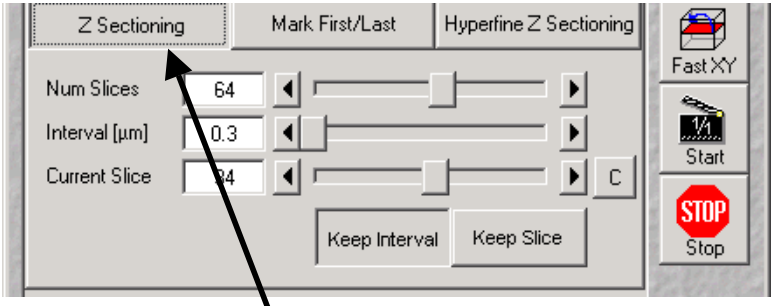
1) Select *Z slice* - the window *Optical Slice* will appear

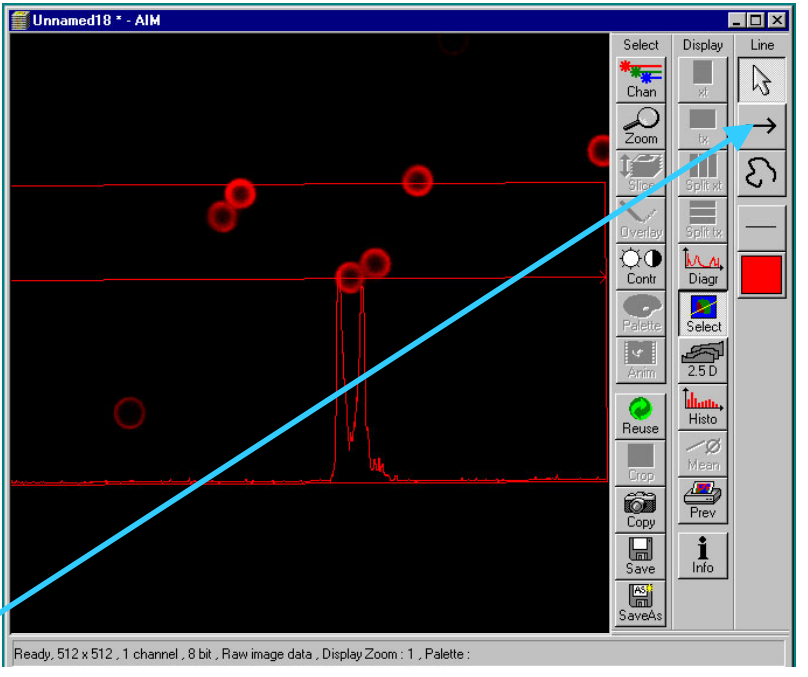
2) Select *Optimal interval* the computer will calculate the optimum number of sections

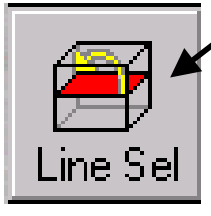
For more or less sections - adjust *Num Slices*

3) Select *Start* to acquire the complete stack

Z - Series using *Z Sectioning*







1) Select *Z Stack*

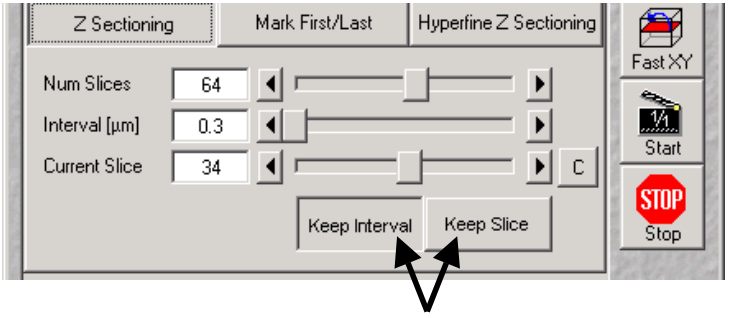
2) Select *Z Sectioning*

3) Select *Line Sel*

4) Select the large arrow button and position the XZ cut line

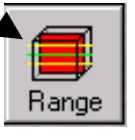
XZ cutline will be displayed as diagram within the XY image

Z Sectioning – Setting Range

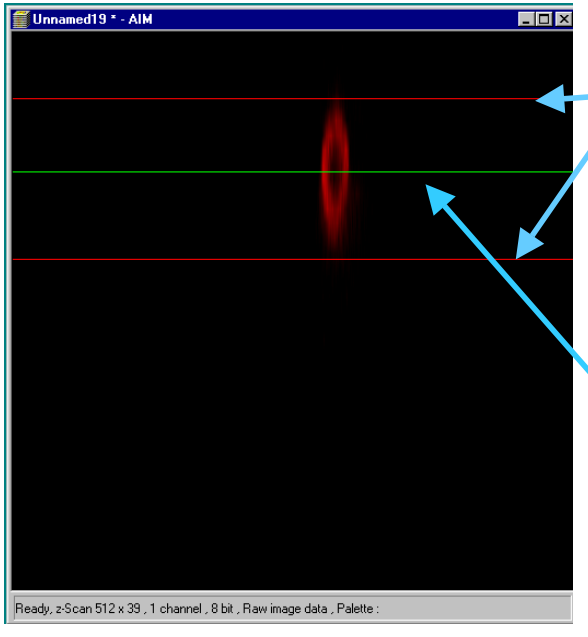


1) Decide whether to *Keep Interval* (number of slices will change) or *Keep Slice* (Interval between slices will be adjusted)

2) Select *Range* and position bars to decide where the Z - Series begins and ends



3) Select *Start* for image acquisition

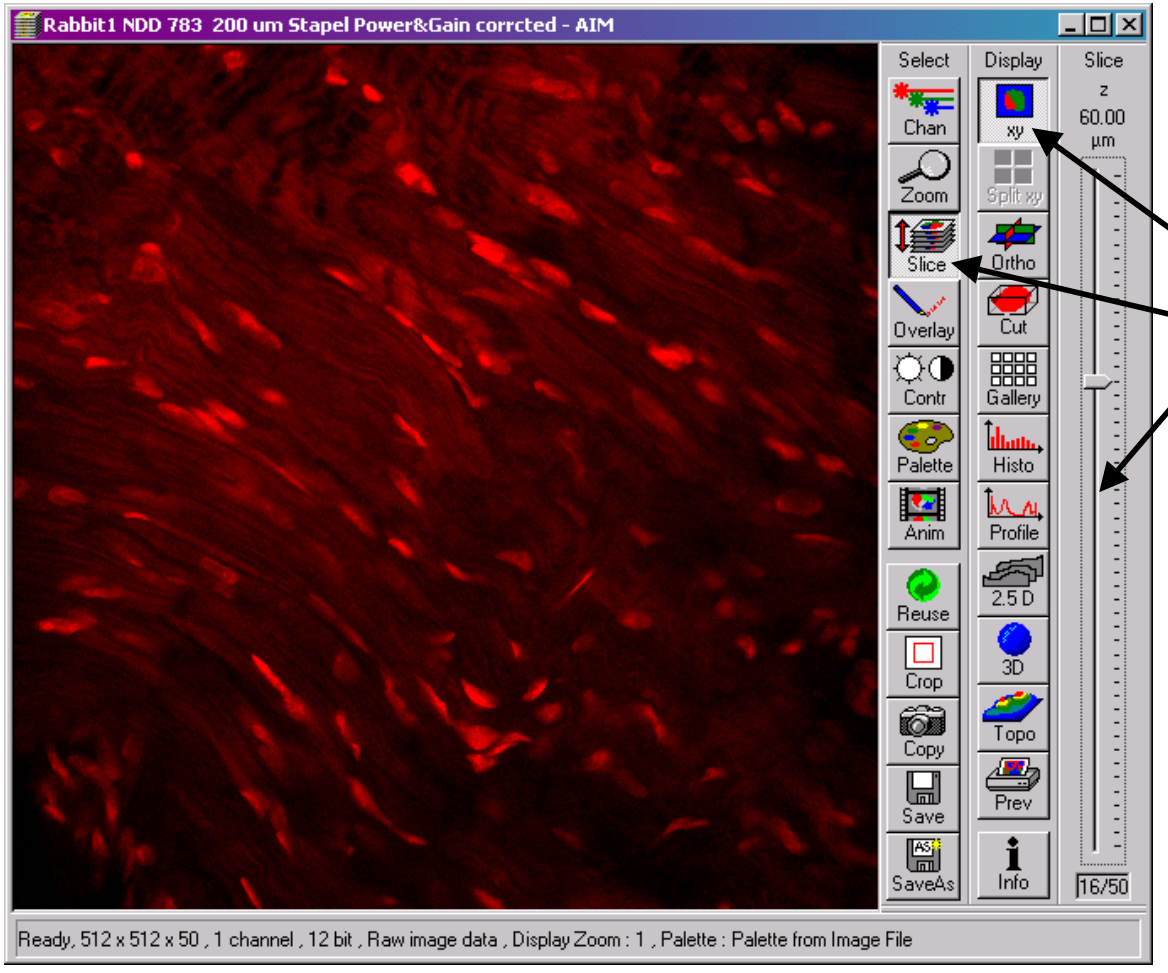


Pull red lines to set limits for Z-Series

Drag green line to change focus position

Pressing *Range* produces an XZ image of selected Z-range, plus 50% above and below the selected stack.

Viewing a Z - Series



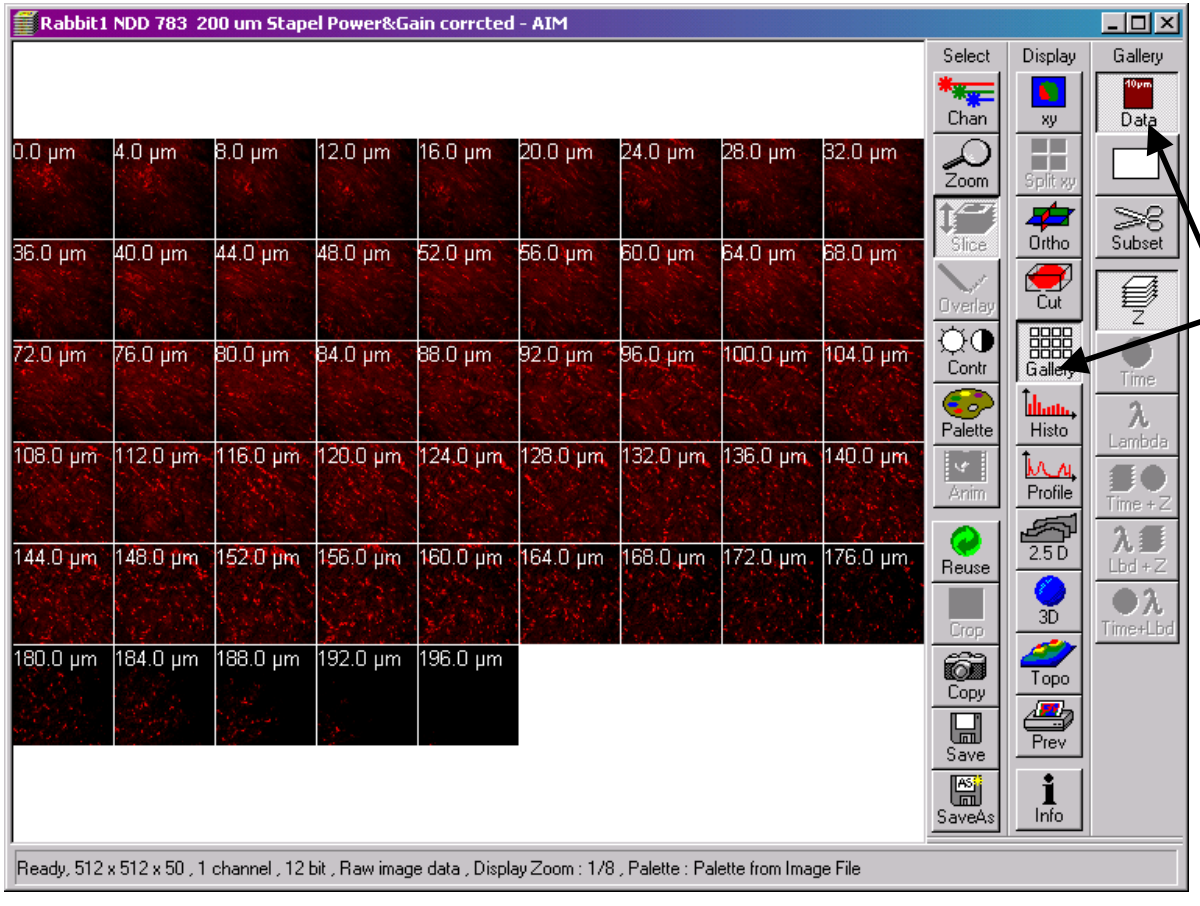
In the image window

1) Select *xy*

2) Select *Slice*

3) Use scroll bar to view individual sections

Viewing a Z - Series using *Gallery*

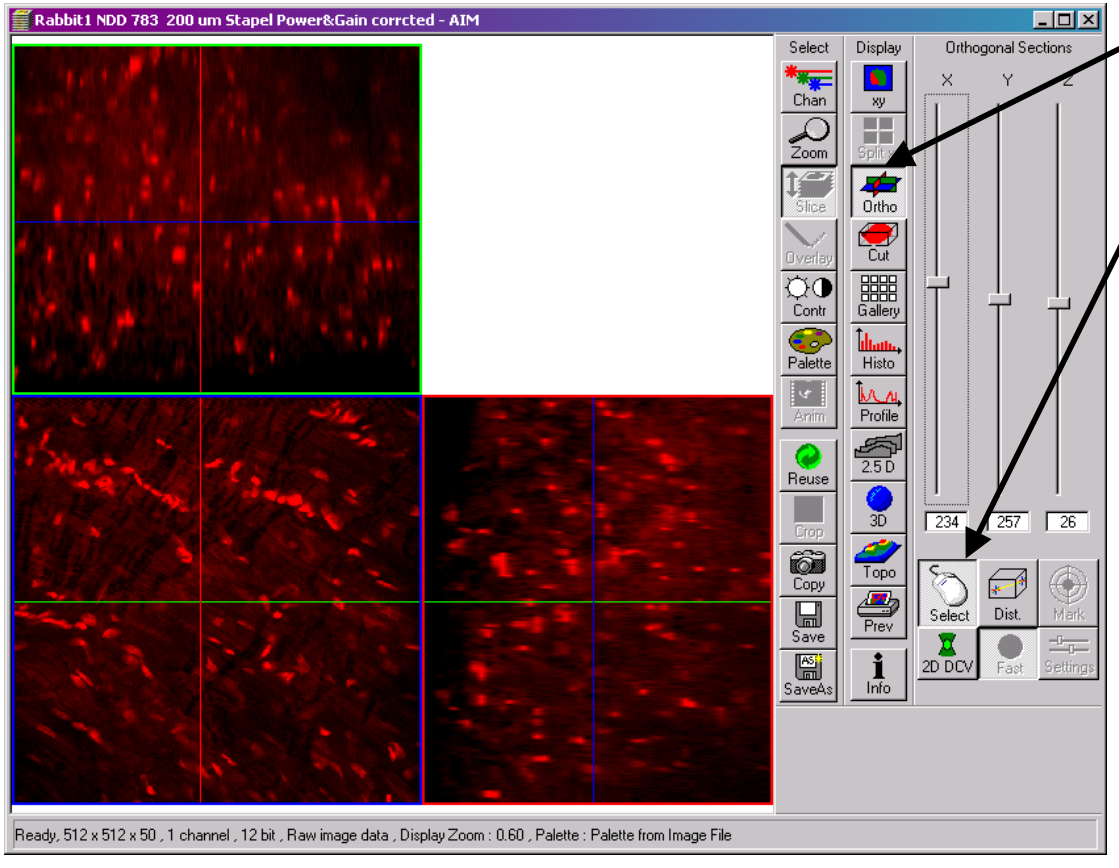


1) Select *Gallery*

2) Select *Data* for scale

Use *Subset* to extract sections

Viewing a Z- Series using Orthogonal Sections



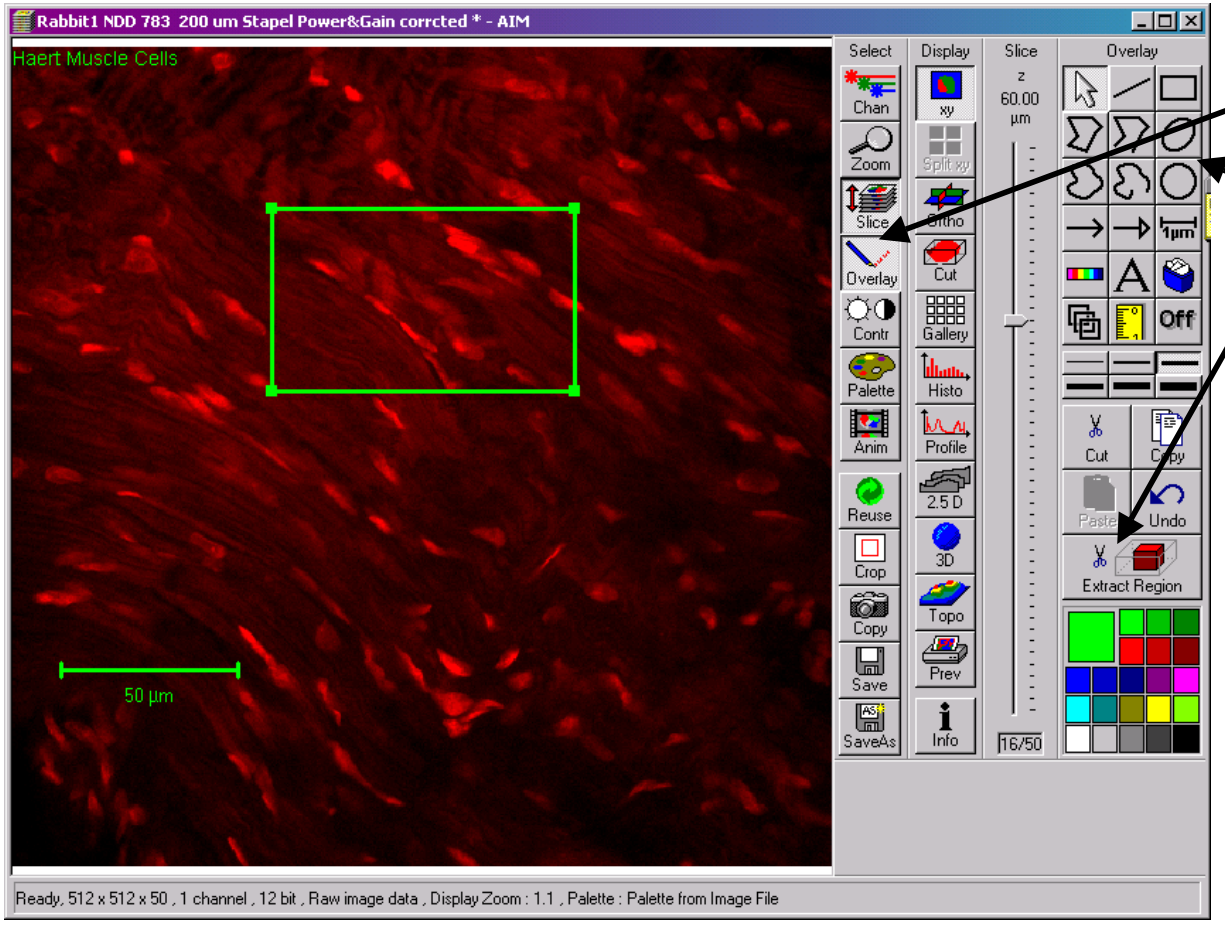
1) Select *Ortho*

2) Select mouse (*Select*)

3) Using the mouse, position the cut lines.

To save orthogonal sections, select *Export* and save as *contents of image window*.

Selecting and Saving a Region of Interest (ROI)



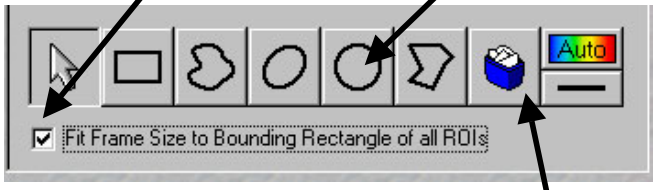
1) Select *Overlay* and define shape of ROI

2) *Extract region* creates a Z-Stack from the ROI

3) Save data

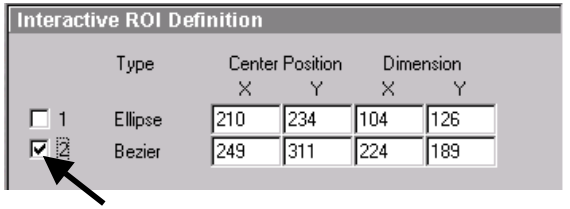
Using a ROI for faster image acquisition and data saving

1) Select *EditROI* from the LSM menu bar
2) Select *Fit Frame Size to bounding Rectangle*
3) Choose shape of ROI

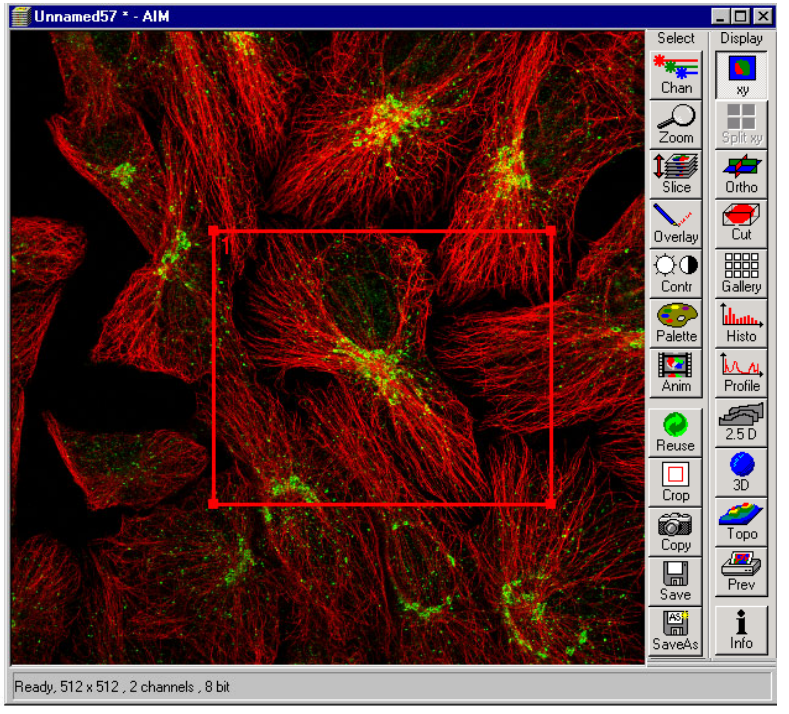


4) Position and size the ROI in the image with the mouse

5) Start Scan



To remove ROI and overlay select blue bin or deactivate ROI. Closing the window only removes overlay, ROI is still active. Deactivate *Use ROI* in the LSM menu.



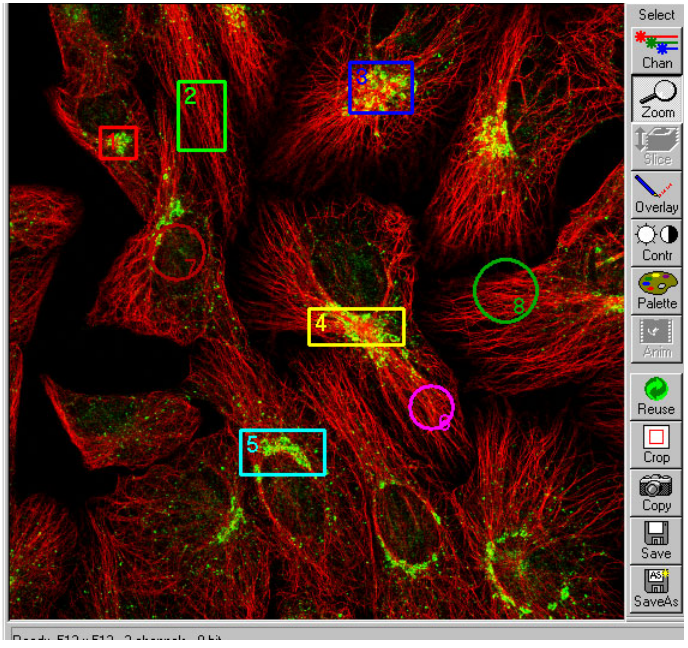
Multiple Regions of Interest

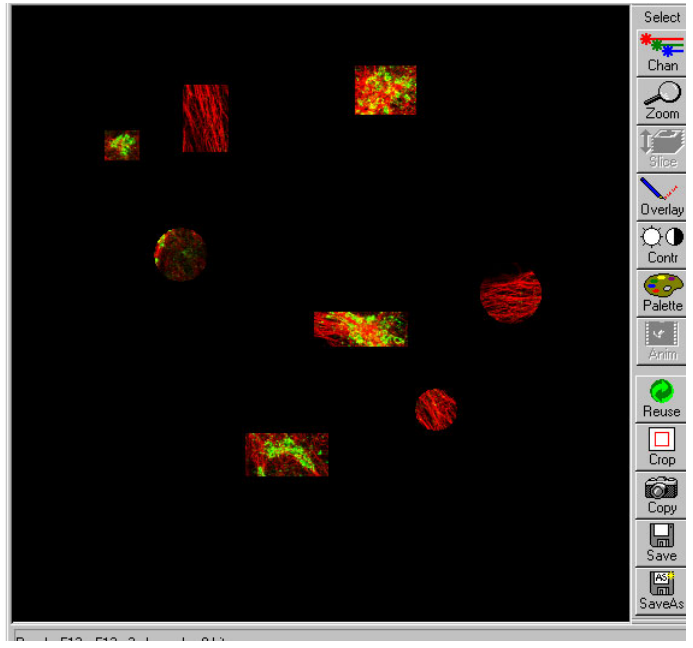
1) Un-select *Fit Frame Size to bounding Rectangle,* Choose shapes of ROIs

4) Position and size the ROIs with mouse

5) Start Scan

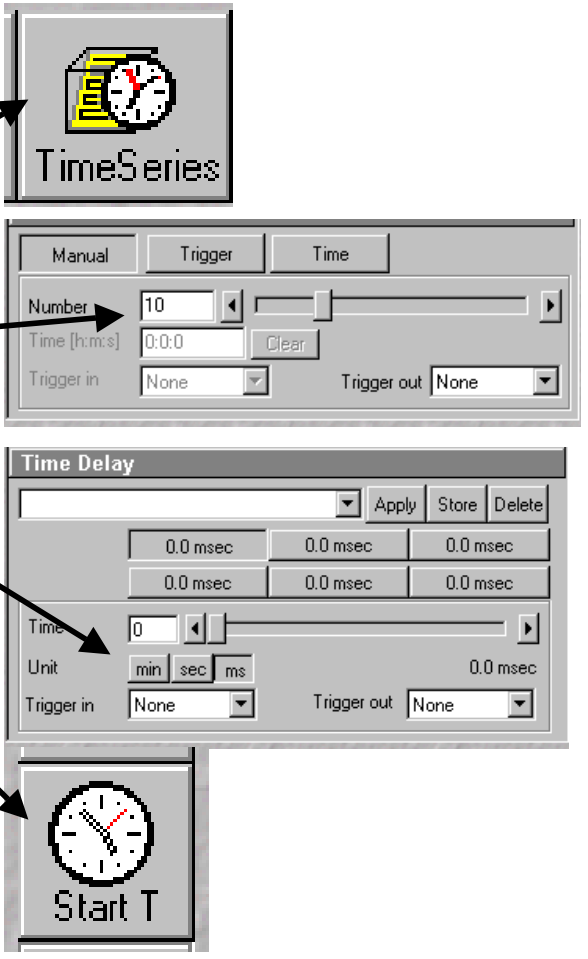
To remove ROIs and overlay select blue bin or deactivate ROIs. Closing the window only removes overlay, ROIs are still active. Deactivate *Use ROI* in the LSM menu.





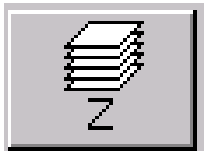
Time Series

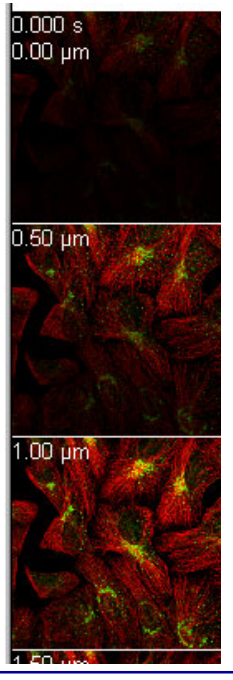
- Set up scanning parameters for image acquisition as described in previous slides
- Select *TimeSeries* from the LSM menu
- Enter the *Number* of cycles
- For a Time Delay between image acquisition select *min, sec* or *ms* and set time with the slider
- Select *Start T to* start image acquisition
- Instead of using *Manual* you can select *Time* to start and stop the series at a certain system time!



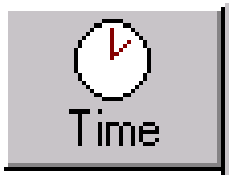
Viewing a Time Series of a Z Stack

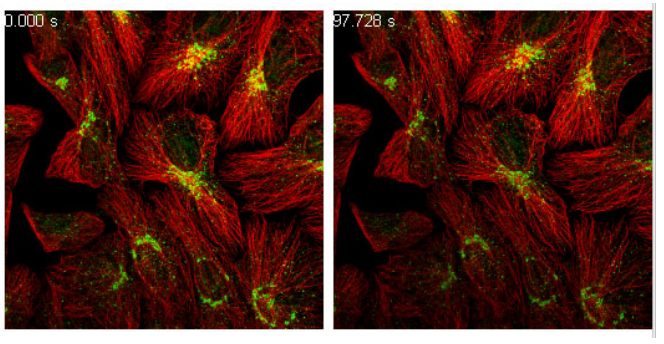
Z Sections for any time



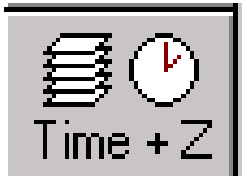


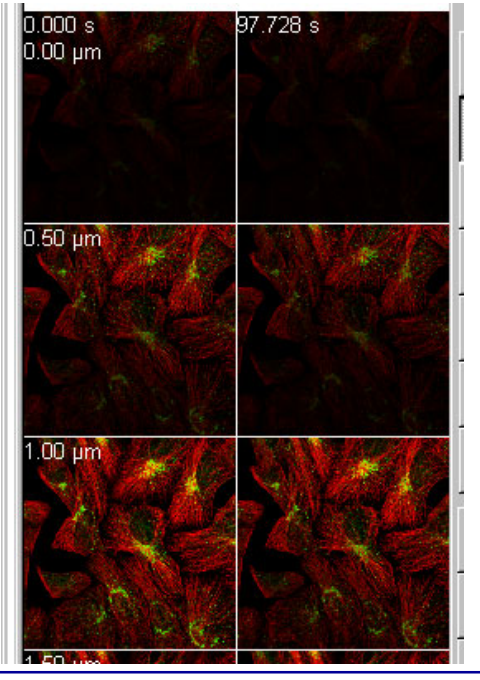
Time points for any Z Section





Both Z sections and time series



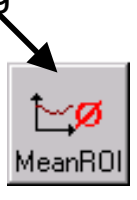


Time Series – Physiology Experiments

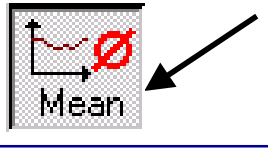
1) If required, use multiple regions of interest

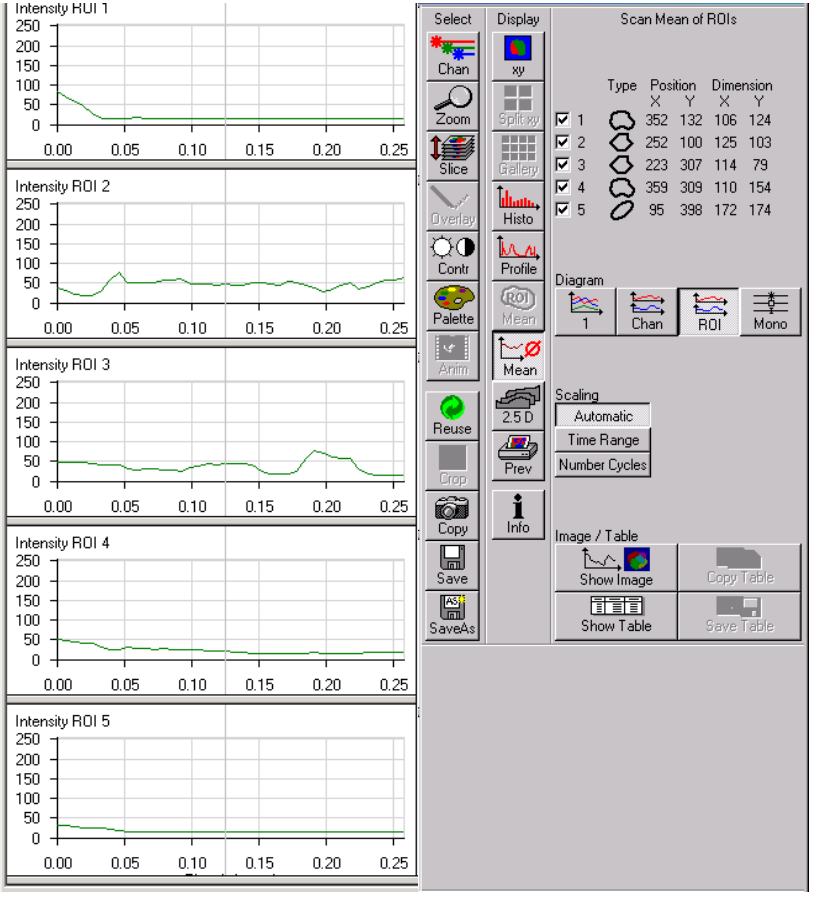
2) Set up Time Series as before

3) Instead of using StartT select MeanROI to start scanning



View and save data by selecting *Mean* in the image window





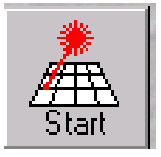
Imaging a large area using *Tile Scan*

This function is only available with a motorized stage

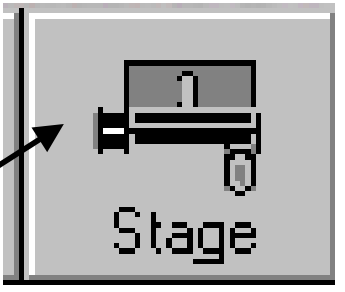
1) Select *Stage* on LSM menu

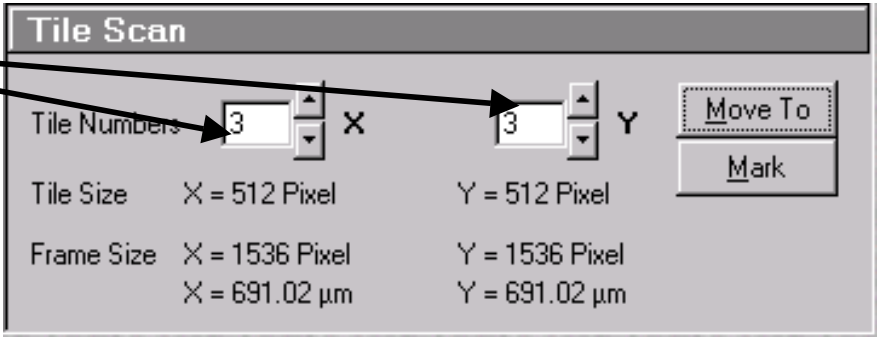
2) Enter the *Tile Numbers*

3) Select *Start*



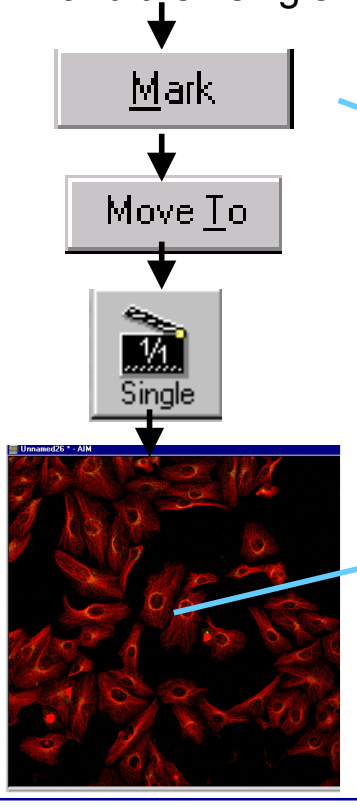
The maximum size is 4096 x4096 pixels

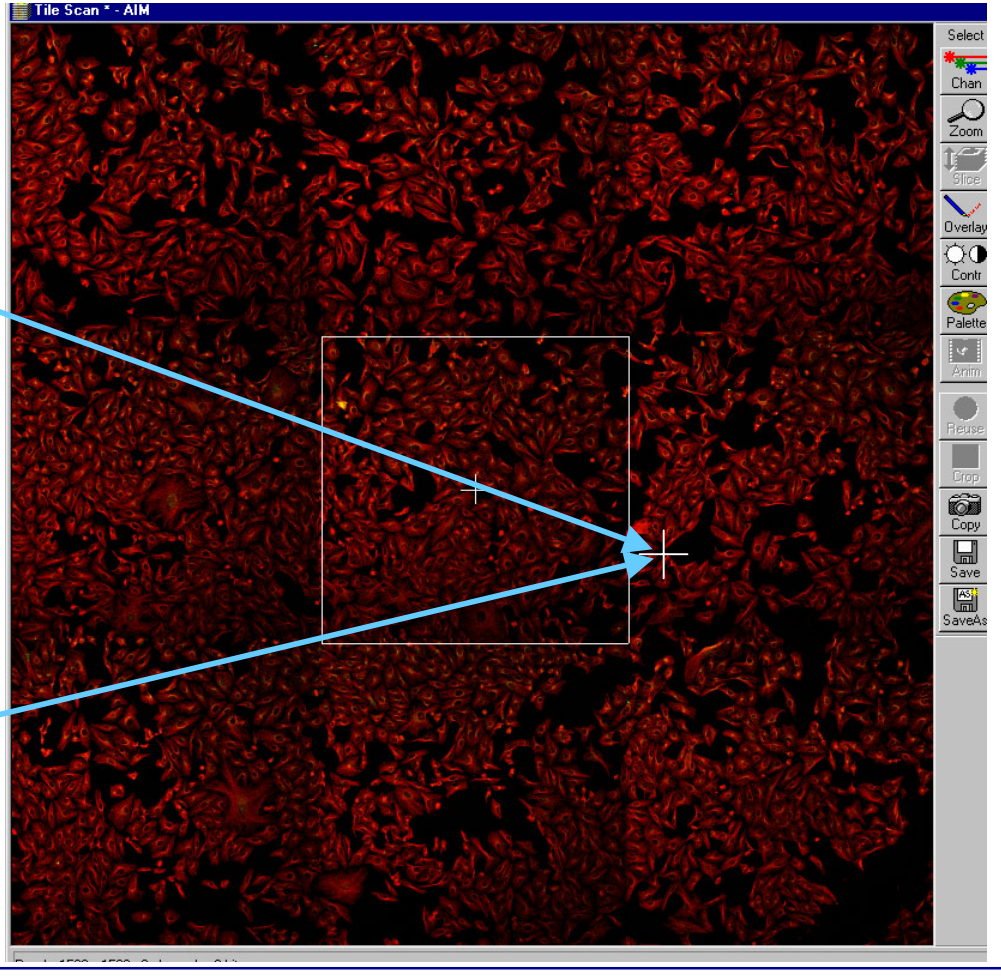




Tiled Image

Any position can then be marked and a single image acquired by selecting *Move to* and then single



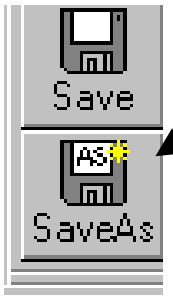


Contents

- Starting the Zeiss LSM 510 microscope, software and laser Selecting an objective and focusing the microscope
- Selecting an objective and focusing the microscope
- Configuring the laser scanning and detection for confocal image acquisition
- Acquiring a Z- and Time - Series
- Data storage

Descriptions also include the LSM 510 META

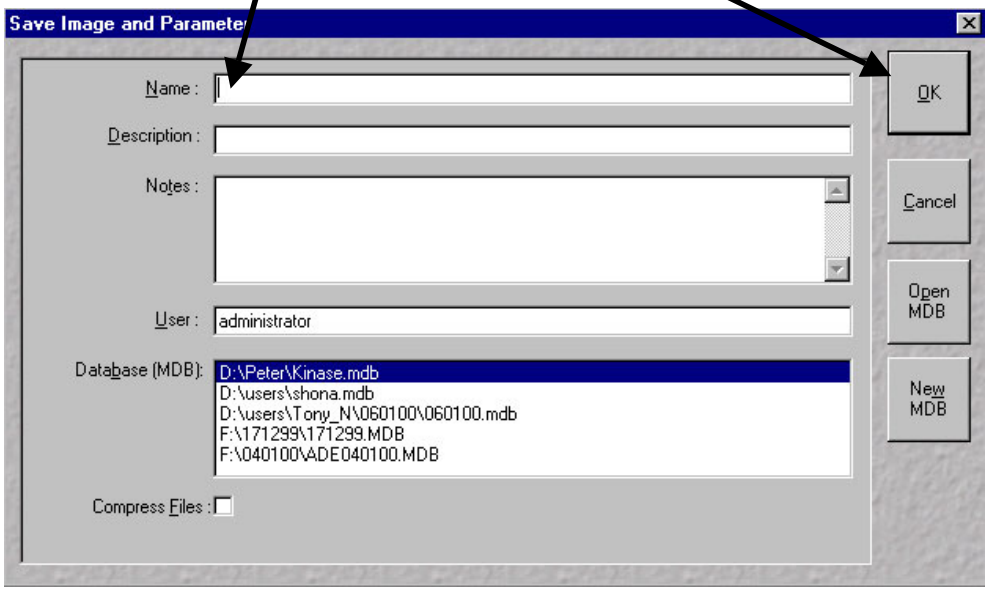
Saving Data - Using Database



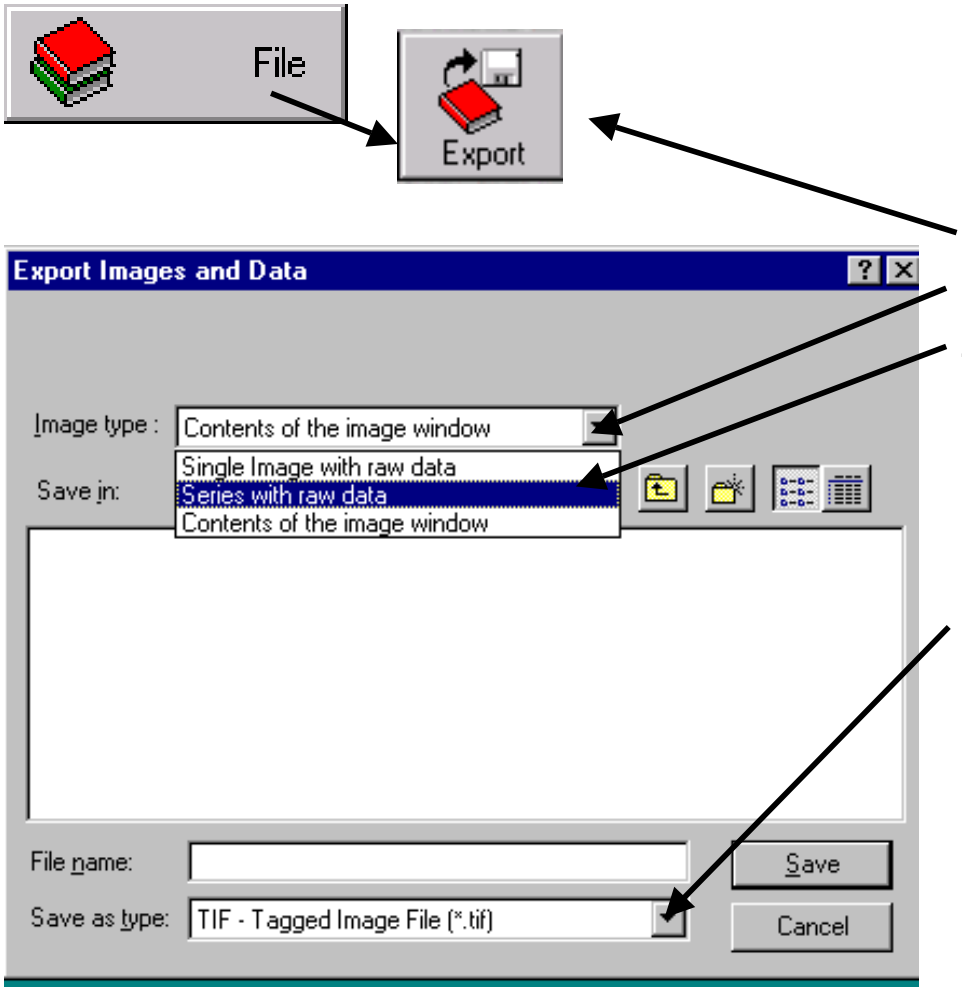
1) Select *Save* or *Save as* on image window or LSM menu bar

2) Enter file name and notes if required

3) Select *OK*



Saving Data – Using *Export*



1) Select *File* from LSM menu

2) Select *Export*

3) Select *Image type*

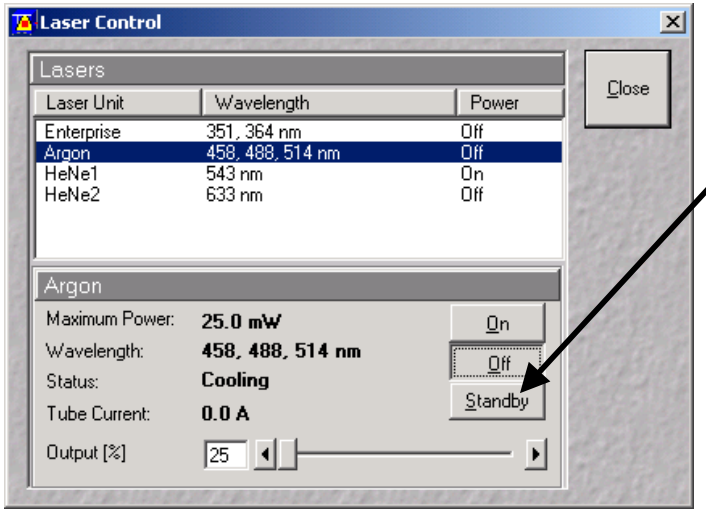
4) Select *Single image with raw data* (No overlay or Look up table etc. is saved), *Series with raw data*, or *Contents of the image window* (Saves the image as shown on the screen)

5) Select *Save as type*

Tif - Tagged image File" is OK for 8 bit - use "Tiff -16 bit" for 12 bit acquired images
(Most other software will not recognize 12 bit)

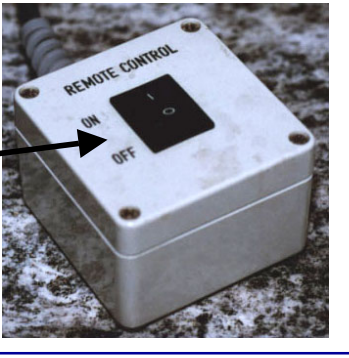
Shut Down Procedure

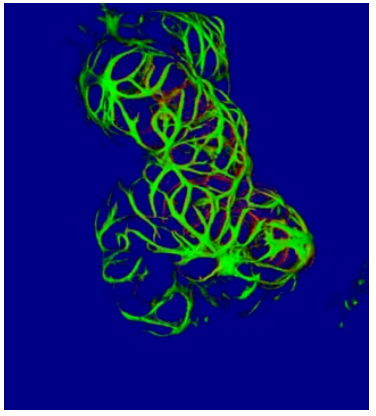
1. Go to: *Acquire* in the LSM menu - *Laser* – and deactivate HeNe Lasers by clicking *Off* to switch off Lasers



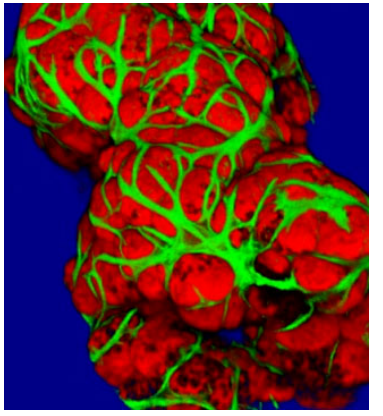
Note: To turn off the Argon laser, first click on *Standby*, then reduce output power to 25%. Select *Off.*

2. Go to *File* - *Exit* to leave LSM 510 program
3. Shut down the computer
4. Wait until fan of Argon laser has switched off.
5. Turn off the remote control box
6. Switch off the mercury vapour lamp.

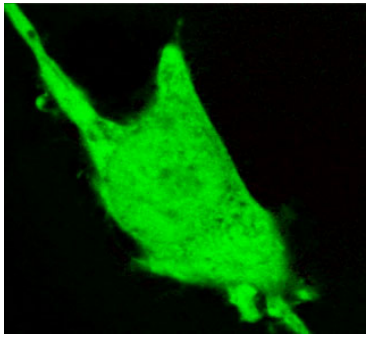


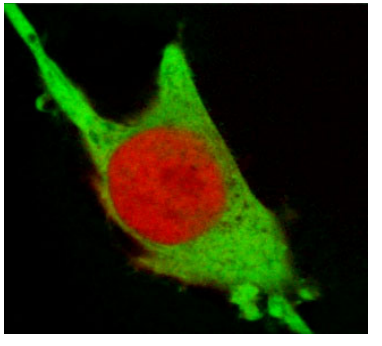


Please note:
This guided tour is intended merely as a quick introduction into the Zeiss LSM 510 software and does not cover all aspects of the system.



Please consult the manual for detailed instructions!





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