Quick Start
Zeiss LSM 510

Wright
Cell Imaging Facility
Toronto Western Research Institute
University Health Network

Table of Contents

1. START HARDWARE 3
2. START SOFTWARE 4
3. START LASERS 5
4. FIND THE SPECIMEN (AXIOPLAN 2) 6
4. FIND THE SPECIMEN (AXIOVERT 200M) 7
5. CONFOCAL FILTER SET CONFIGURATION 8
6. ACQUIRE PRELIMINARY CONFOCAL IMAGE 9
7. OPTIMIZING THE SETTINGS 10
   - *7.1. GETTING THE DISPLAY READY 10*
   - *7.2. SCAN CONTROL: CHANNELS WINDOW 12*
   - *7.3. ACQUIRING YOUR FINAL IMAGE 15*
8. SAVING YOUR FINAL IMAGE 17
9. ACQUIRING A *Z*-SERIES 18
10. ADVANCED OPTIONS 20
    - *10.1. SINGLE-TRACK – SIMULTANEOUS ACQUISITION 20*
      - *10.1.1. Getting the display ready 20*
      - *10.1.2. Optimise settings 20*
    - *10.2. Z-ATTENUATION COMPENSATION 21*
    - *10.3. TRANSMITTED LIGHT IMAGE 22*
    - *10.4. FRAP 23*
11. SHUTTING DOWN THE SYSTEM 25
    - *11.1. TURN OFF LASERS 25*
    - *11.2. REMOVE SPECIMEN AND CLEAN MICROSCOPE 25*
    - *11.3. EXIT THE SOFTWARE 25*
    - *11.4. POWER DOWN THE SYSTEM 25*
12. OPENING YOUR IMAGES OFFLINE 26
    - *12.1. ZEISS IMAGE BROWSER 26*
    - *12.2. IMAGEJ 26*

1. Start Hardware

- Turn on the mercury short arc lamp light switch.

Note: Whenever the mercury lamp is turned on, it should be left on for at least 30 minutes. Once the lamp has been turned off, it should not be turned on again for 30 minutes.

- Remote control

- PC power

- IF REQUIRED: UV laser power (black switch in white frame)
  Do not touch the cooling unit; it has been set to "automatic".

2. Start software

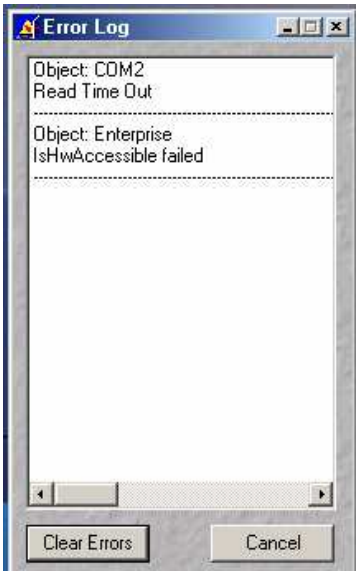
- Log on to Win2000 (you will be issued with a username and password during training). Change your password now if you have not already done so.

- If you are using the UV laser, start this no before starting the LSM software.

- Double click the LSM 510 desktop icon.

- The Zeiss LSM 510 switchboard window will appear

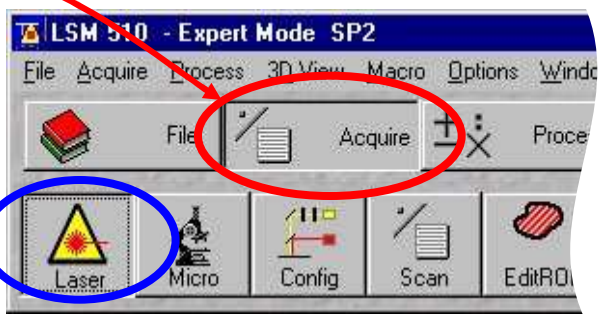
- Make sure ***Scan New Images*** is pressed and then click the ***Start expert mode*** button. If ***Scan New Images*** is not pressed, the software will start but no initialize the hardware.



- You will get an error message. Ignore it. It's just telling you the UV laser is not switched on. Contact facility staff if you require the UV laser. Press ***Cancel*** to close the window.

3. Start Lasers

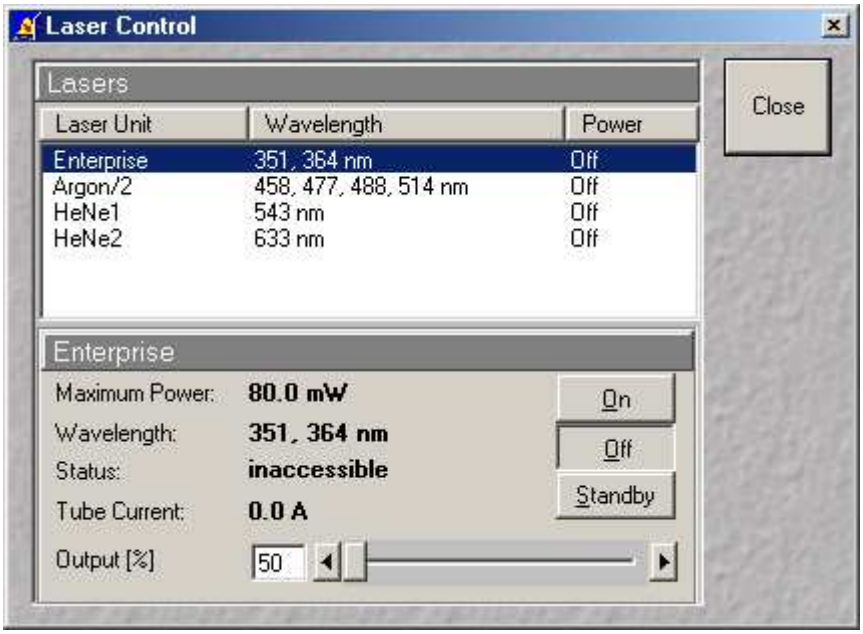
Click the ***Acquire*** button on the toolbar. The lower toolbar will now change to the Acquire sub-toolbar and show the acquisition controls.



Click the ***Laser*** button.

Turn on the desired lasers position: HeNe's to ***ON***, Argon to S***tandby.*** When the ***Status*** reads ***Ready***, click the ***On*** buttons.

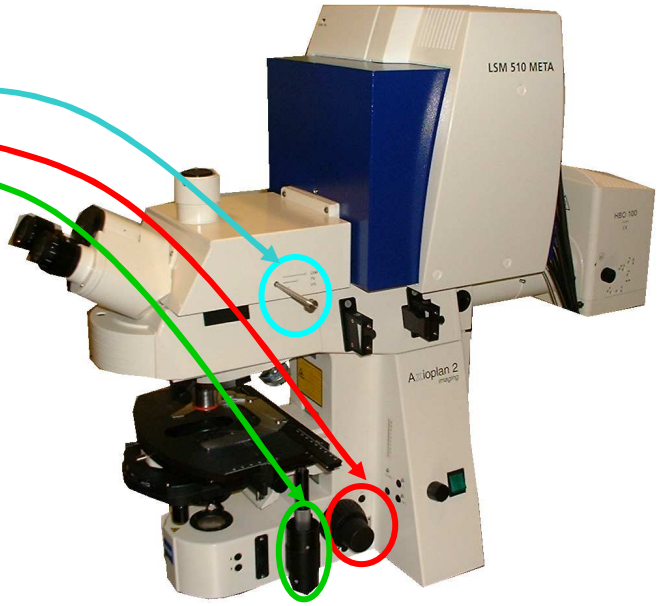
Also turn ***OFF*** any lasers that have been left on for you, but you will not be using.
If the previous user has left the lasers on for you, Argon and Enterprise may already be in standby mode and ready to be tuned on.



When all the ***Status*** reads ***Ready***, close the Laser Control window.

4. Find the specimen (Axioplan 2)

The ***light path select***,
focus knob
and ***stage***
are manual controls. The rest of the microscope is controlled by the software.



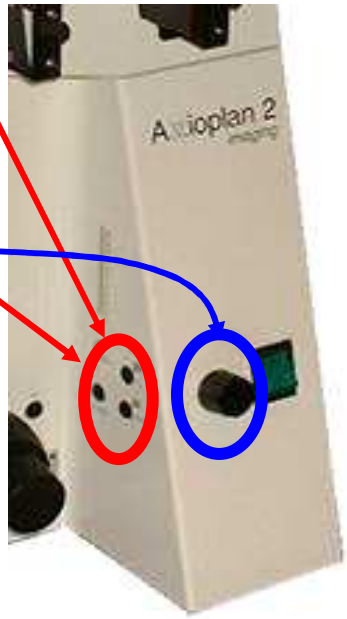
To set up the Axiovert microscope to locate your specimen first move the ***light path selector*** to ***VIS.***

Move the correct objective in to place.
Do not use air-objectives after an immersion objective without wiping the immersion liquid (water or oil) from the specimen and objective.

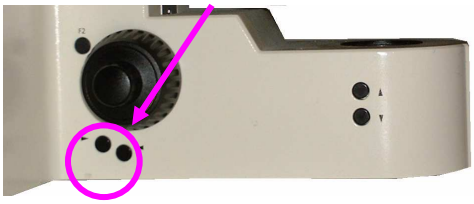
Brightfield, halogen light can be switched on and off ***HAL*** button on the body of the microscope.

The intensity of brightfield light is most easily adjusted with the ***Intensity control knob*** on the body of the microscope

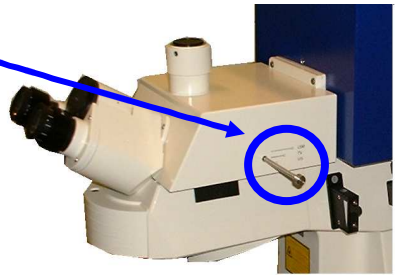
Arc lamp light can be shuttered on and off by either the ***FL*** button on the body of the microscope on the right hand-side.



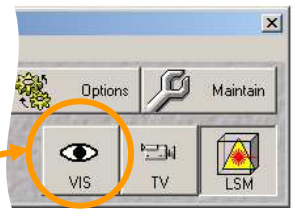
The ***fluorescence filter*** block carousel can be switched back and forward using the two buttons on the body of the microscope, underneath the left hand focus knob.



Once you have found your specimen, pull the ***light path selector*** out to the LSM position. The microscope will automatically change the filter-position and shutter the light sources. If you fail to do this you will receive an error when you try to acquire a confocal image.



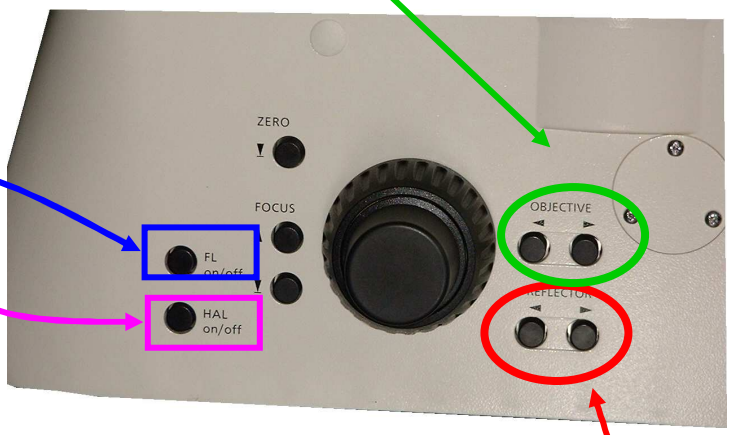
4. Find the specimen (Axiovert 200M)



- Change the microscope-light path to direct the emitted fluorescence is sent to the eyepieces by clicking on the ***Eye*** button in the LSM software toolbar.

- Move the correct objective into place using the ***Objective*** buttons on the microscope button.

Do not use air-objectives after an immersion objective without wiping the immersion liquid (water or oil) from the specimen and objective.



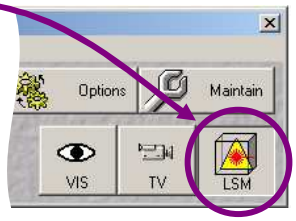
- The arc-lamp light can be shuttered on and off by the ***FL*** button on the body of the microscope on the right hand-side.

- Brightfield, halogen light can be switched on and off ***HAL*** button on the body of the microscope.

- The fluorescence filter block (a.k.a. *reflector*) carousel can be switched back and forward using the two buttons marked ***Reflector*** on the body of the microscope, behind the right hand focus knob.

- Once you have found your specimen, redirect the light-path to button in the confocal by clicking the ***LSM button*** in the LSM software

The microscope will automatically change the filter-position and shutter the light sources

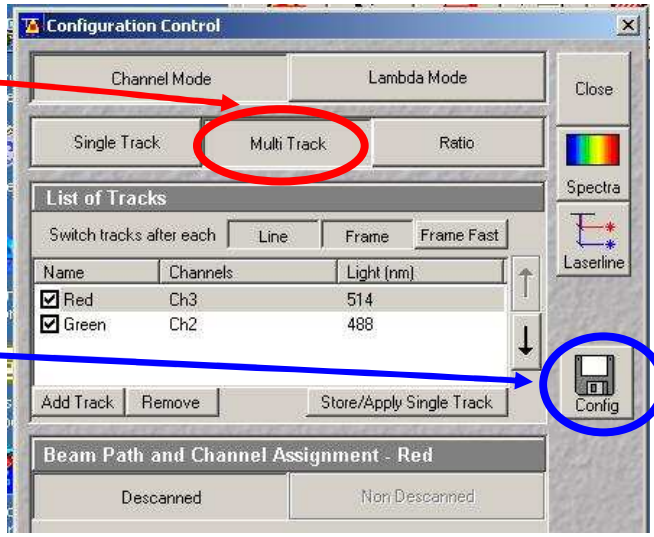


5. Confocal filter set configuration

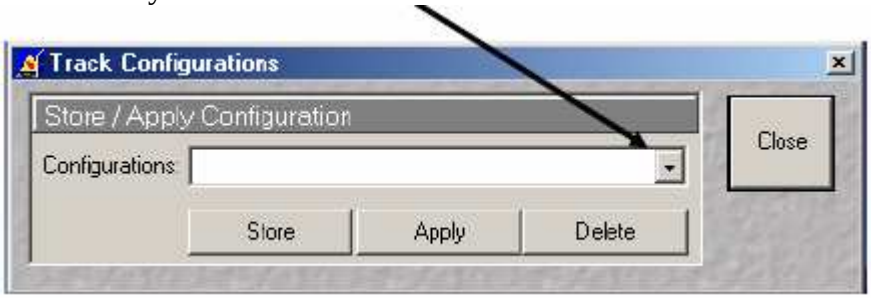
Click the ***Config*** button (in the ***Acquire*** sub-toolbar).

To activate multi-tracking simply chose the ***MultiTrack*** button in the ***Configuration Control*** window.

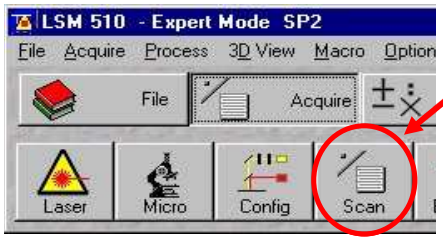
Load the configuration that matches your fluorophores (referred to as 'tracks') by clicking the ***Config*** button.



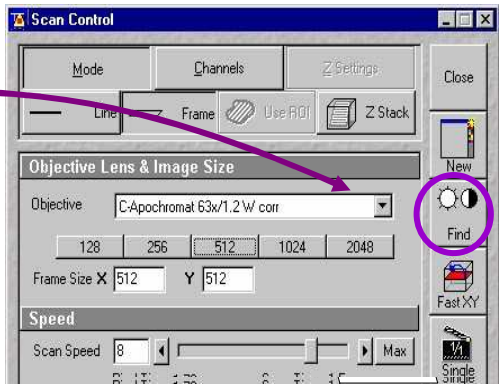
- Click on the ***drop down box*** and select a desired configuration, apply then close. If you cannot see the appropriate configuration for you fluorophores, contact Facility staff.



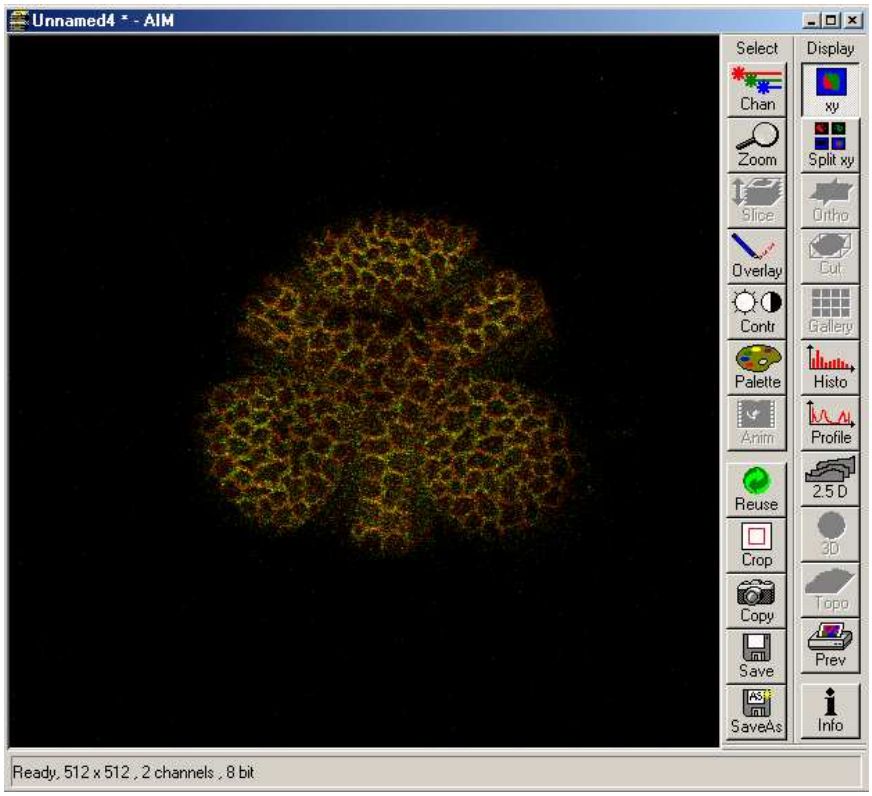
6. Acquire preliminary confocal image



Click the ***Scan*** button in the Acquire sub-toolbar to open then ***Scan control*** window.



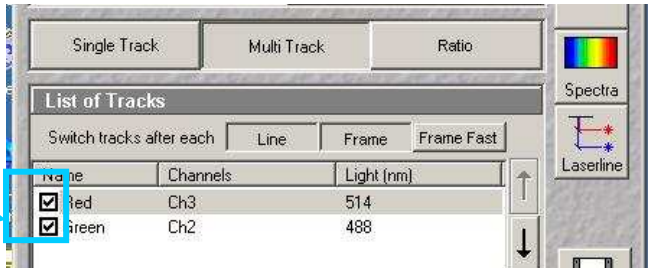
Click the ***Find*** button and the computer will open a new image window and calculate the approximate levels to generate a starting image.



You will see an image looking something like this (left).

7. Optimizing the settings

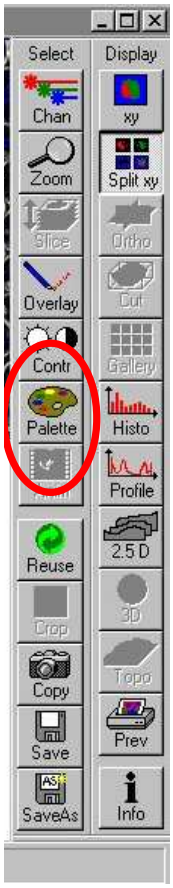
Due to the sequential nature of 'Multi-track' acquisition optimizing the settings in this mode is difficult. It is easier to set the imaging parameters for each ***Track*** individually. You can do this by switching off each track with the ***checkbox*** along side it.
Turn off all but one channel now.



7.1. Getting the display ready

This current type of display does not allow fine tuning of the imaging parameters and so needs to be adjusted.
Optimizing the confocal settings is best done with the image pseudo-colored to highlight saturated and zero value pixels.

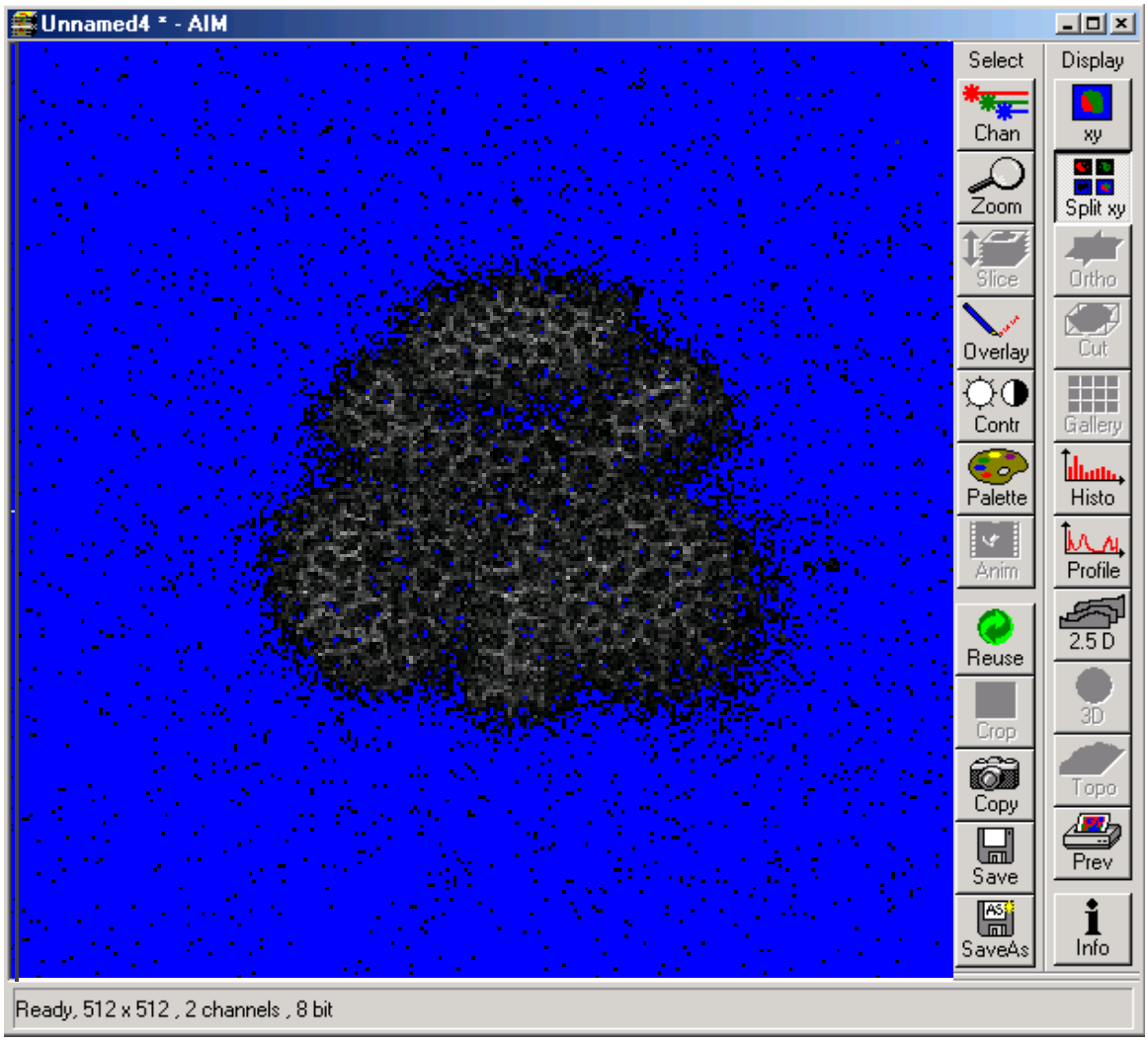
To do this, first click ***Palette*** on the image window tool bar and the ***Color Palette*** window will open.



Click ***Range Indicator*** and close the window.

Now you will have an image where red represents the saturated pixels (i.e. =255) and the blue represents the black pixels (i.e. zero).

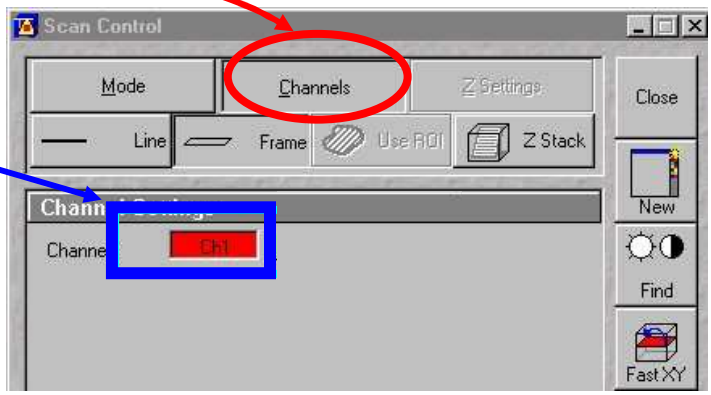
Your image window will now look like this.



7.2. Scan control: Channels window

Click on the ***Channels*** button of the ***Scan control*** window.

The active channel will appear here.

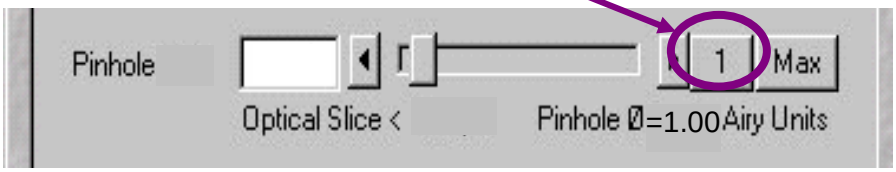


The settings below need to be adjusted for each channel separately.

`

1. Set ***Pinhole of each channel***:

The pinhole diameter determines the thickness of the optical section – i.e. the ***axial resolution***. By increasing the pinhole diameter (and therefore optical section) you are also increasing the amount of out of focus fluorescence being detected. Start with each at the optimum (1 Airy Unit) by clicking the ***1 button***.

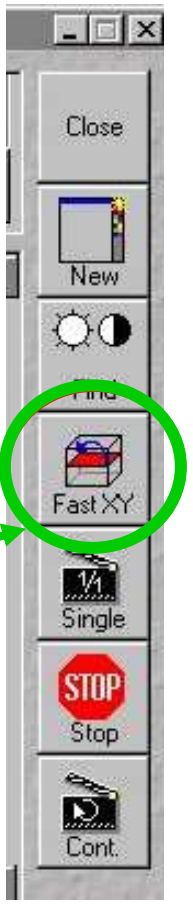


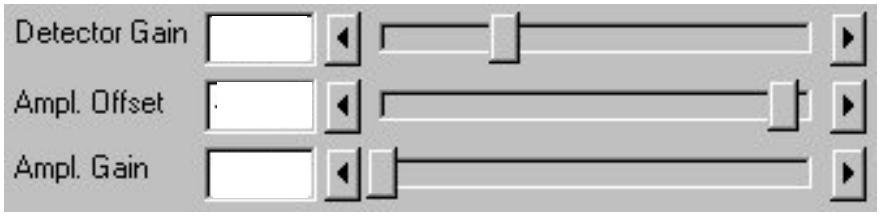
This will give different ***Optical Slice*** values for each channel. Increase the pinholes of the shorter wavelength channels to match the longest wavelength.

Do not go below 1 Airy unit – you will lose signal with no significant improvement in axial resolution. Adjust the Pinhole for each channel so that they have the same optical slice (typically the green channel will be ~ 1.1 Airy units).

2. Check focal plane

Click the ***Fast scan*** button to start acquiring a high-speed, low quality image. Use this mode to focus up and down through the specimen to get the desired focal plane.

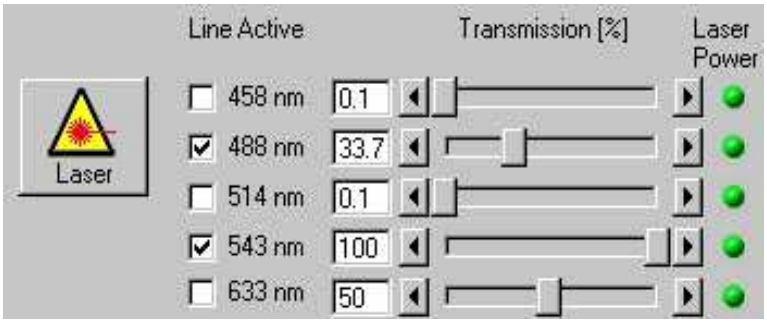




You need to set the detector range to match the dimmest and brightest signal from the specimen. Setting the detector incorrectly results in the loss of information from the specimen.

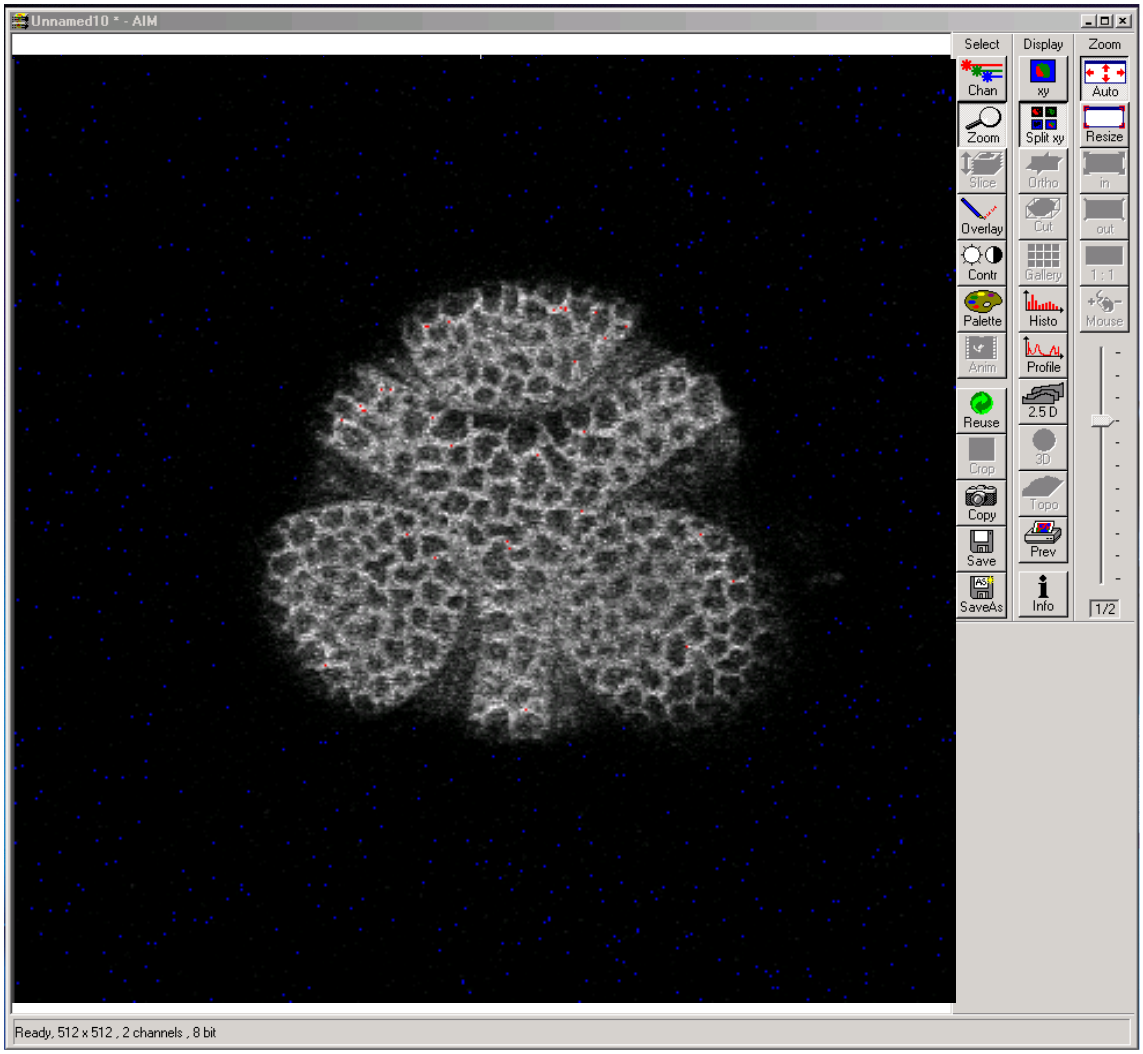
3. Set ***Ampl. Offset*** – setting the 'min' signal
This should be set next, but may need re-setting later. While acquiring with the ***Fast scan*** button, adjust the ***Amp. Offset*** so that only a few of the pixels in the background of the image are blue. If you have a lot of blue colour in the background, move the slider to the right.

4. Set ***Detector Gain and Excitation power*** – setting the 'max' signal
The 'max' signal is set by adjusting the ***detector gain*** and ***laser transmission*** simultaneously. You need to empirically work out the best laser power settings – low laser power causes less bleaching but requires the detector gain to be set high; which introduces noise.



Whist acquiring with the ***Fast scan*** button, adjust increase the ***Detector Gain*** so that you get a bright image, but not too many red (saturated) pixels and not too much noise. You may need to adjust the ***Ampl. Offset*** if you increase the ***Detector Gain*** a lot. Around 600 is a good start. This should be set in conjunction with the excitation intensity.

5. Set ***Ampl. Gain***
Leave as 1 unless you have a very dim signal and nothing else works. This will amplify noise as well as signal.



A few red speckles; a few blue speckles

After you have set both channels, stop scanning by pressing the ***Stop*** button in the ***Scan Control*** window. Your image will look like this (above).

Turn off the optimised channel in the ***Configuration Control*** window. Turn on the next channel and optimise these settings for this channel.

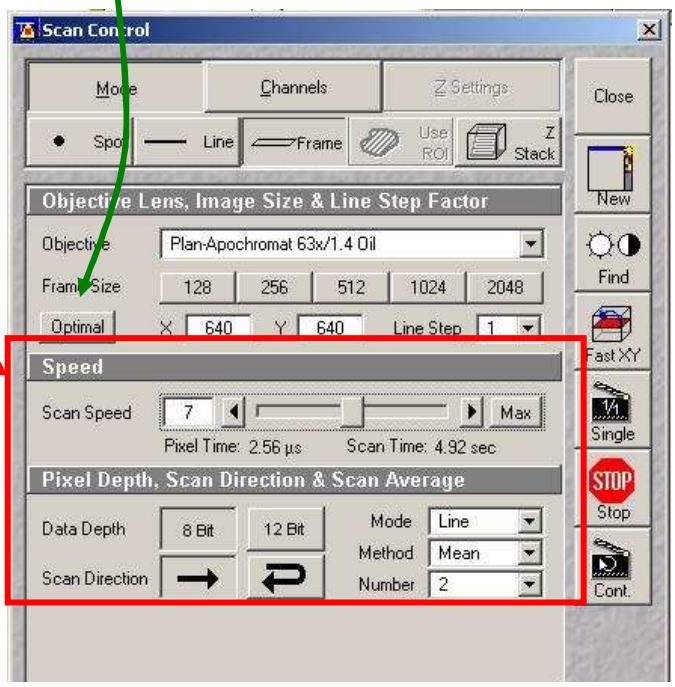
7.3. *Acquiring your final image*

Once you are satisfied that each channels detector is set optimally to the range of the image, you can create your final image. Ensure that all ***Tracks*** are checked when acquiring the final image.

Go to the ***Scan control*** window and click the ***Mode*** button.

Change the ***Frame size*** by clicking on the ***Optimal*** button (see Appendix 1 for an explanation of this).

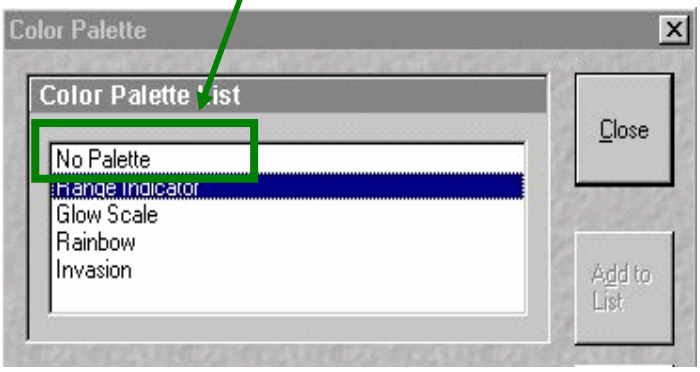
Noise can be reduced by averaging a number of frames and slowing the scan speed.
In the first instance, try the following values:
Mode: line
Method: mean
Number: 2
Scan speed: 6



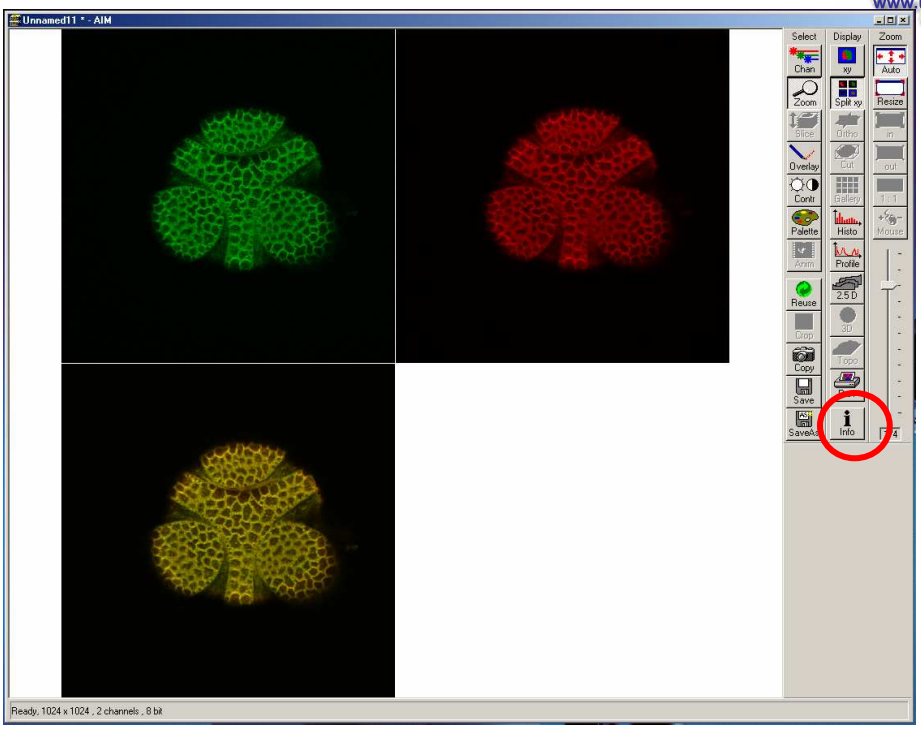
Change the image Palette back to ***No Palette***.

Click ***Palette*** on the image window tool bar and the ***Color Palette*** window will open.

Click ***No Palette*** and close the window.



Click on the ***Single*** button in the scan control window to collect your final image (bottom).

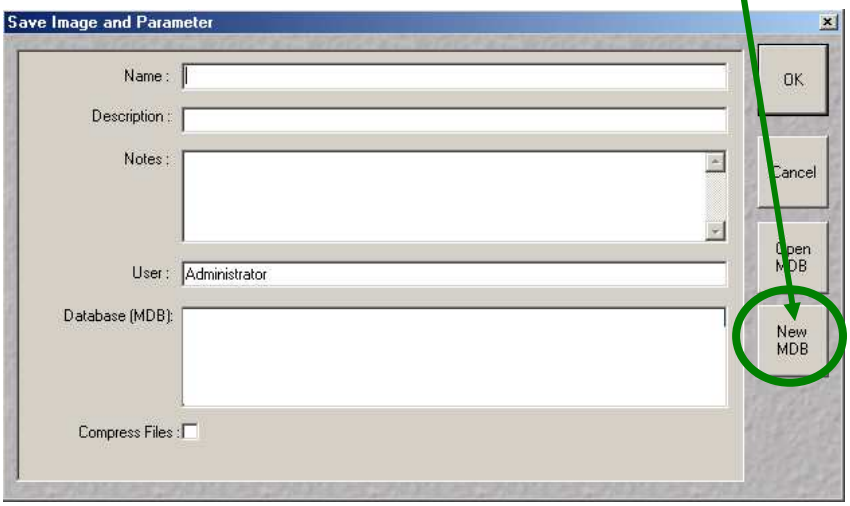


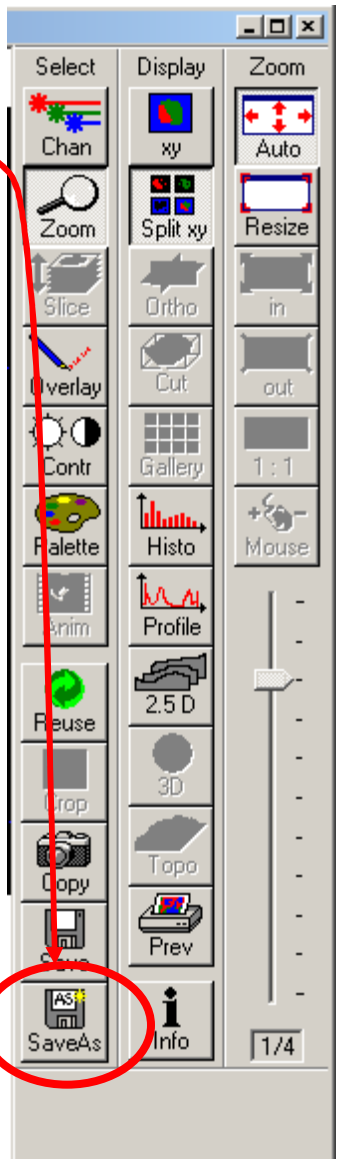
Click on the image window ***Info*** button. This will bring up a bar on the left of the image window with the image information on it.

8. Saving your final image

When you are satisfied with your image you need to save it to your database. Images are saved to Databases. A database can be single or multiple images or stacks.

Press ***Save as***
in the image window and the ***Save Image and Parameter*** window will open.
You can now chose to add your image to an existing database (***Open MDB*** button), or create a new database (***New MDB*** button)
Name the file and include any other information in the description of notes sections.
Do not select ***Compress file*** option.





9. Acquiring a *Z*-series

Having set the system to acquire a satisfactory image, you can acquire a z-series. It may be worthwhile changing the frame size to 512×512 to minimize file size and to speed acquisition.

Click on the ***Z-Stack*** button in the ***Scan control*** window.

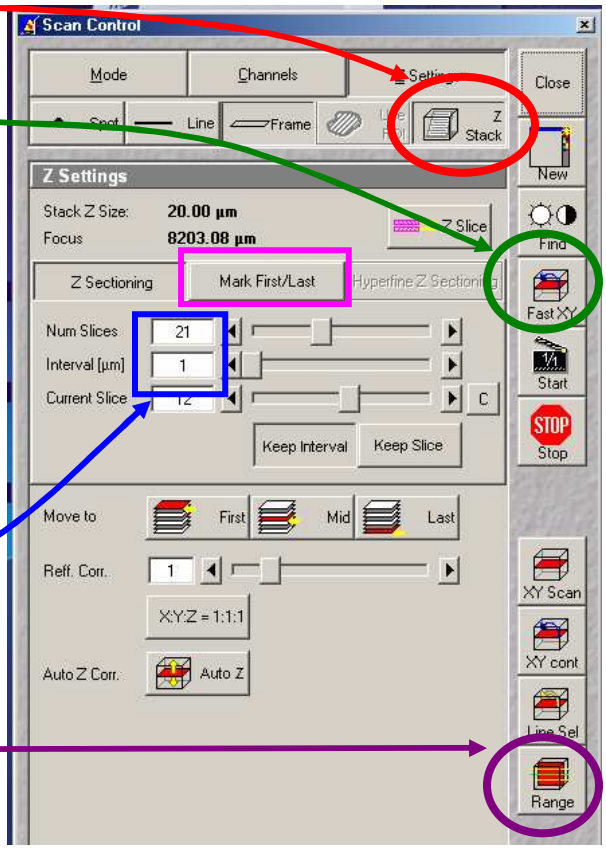
Using ***Fast scan*** mode to acquire a continuous image of the specimen. Focus up and down with the microscope focus wheel to ensure your specimen is in the centre of the field of view and the microscope is focused in the middle of the specimen.

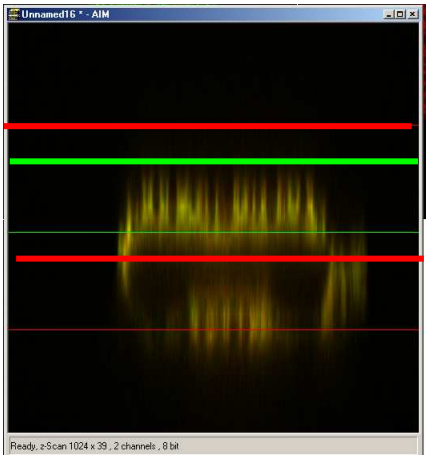
Stop acquisition.

You can now precisely define the middle and depth of you z-series.

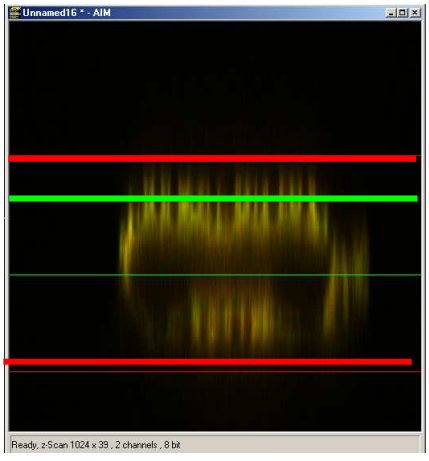
Ensure the ***Num Slices*** = 20 and the ***Interval*** = 1 µm at this point to ensure the top and bottom of the specimen is located.

Click the ***Range*** button from the ***Scan control*** window – this will generate a side view of you specimen. This button is not available if the ***MarkFirst/Last*** button is depressed.

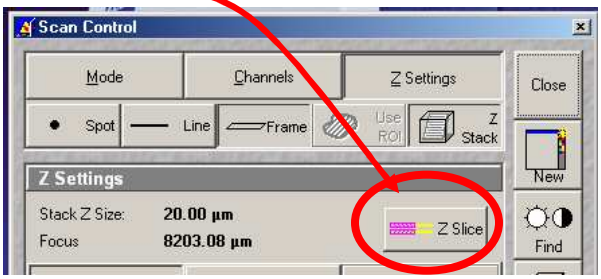




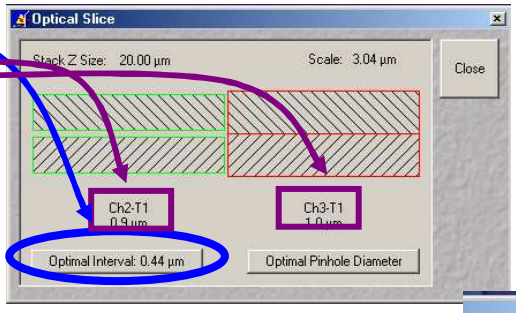
(LEFT) The green line represents the middle of your z-series. The upper red line represents the top and the lower red line the bottom of the z-series. (RIGHT) Drag the green line to the centre of the specimen and the red lines to just outside the top and bottom of the specimen.



In the ***Scan*** control window, click the ***Z-slice*** button to bring up the ***Optical Slice*** dialog



Click the ***Optimal Interval*** button. Check the ***optical section*** for each channel is the same and close. If you optical sections are different for each wavelength go to ***Mode/Channels*** and adjust the ***Pinhole*** so that each channel has the same optical section – around 1 Airy unit, but one channel will have to be slightly larger.



Readjust the red lines that indicate the top and bottom of the specimen (Step 7 above).

Reduce the ***Frame Size*** also if possible to speed stack-acquisition.

To acquire your z-series stack click the ***Start*** button.

The system will begin scanning the specimen. You can check progress by selecting ***Gallery*** button on the image window sidebar.

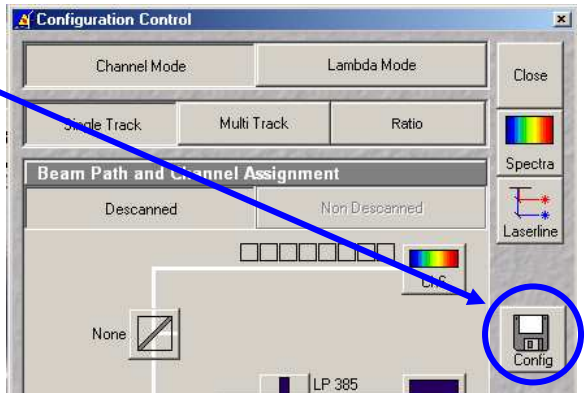
10. Advanced Options

10.1. Single-Track – simultaneous acquisition

Multi-track can solve the problem of cross-talk. Typically this occurs with bleed through of green fluorescence in to the red channel. Multi Track avoids this by acquiring the fluorescent channels sequentially, not simultaneously as with ***Single Track*** acquisition. In ***Multi Track*** mode the red channel detector is turned on, the green one off. Then the red fluorophore is imaged.
The red channel detector is then turned off, the green one on and the green fluorophore imaged. Any bleed through of fluorescence from the green fluorophore to the red detector does not register.

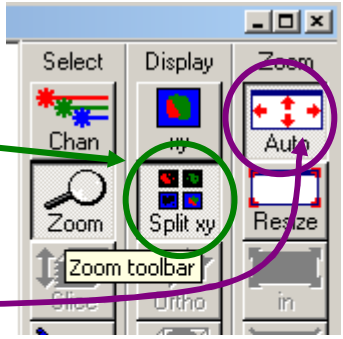
This switching between tracks means that there is a delay between channels. Sometimes this is unacceptable, in particular for live cell imaging where the cell can move between channels creating artefacts. In this case ***Single track*** mode should be used.

- To load pre-designated configurations click the ***Config*** button in the ***Configuration control*** window.



10.1.1. Getting the display ready

For multi-label experiments, it is best to have both channels displayed at once.
To do this:
In the Live image window's ***Display*** side-bar, click the ***Split xy*** button
This will make your image small so resize the image window (usual Windows mouse-drag). Then click on the Zoom button to bring up the Zoom sidebar and select ***Auto*** button.



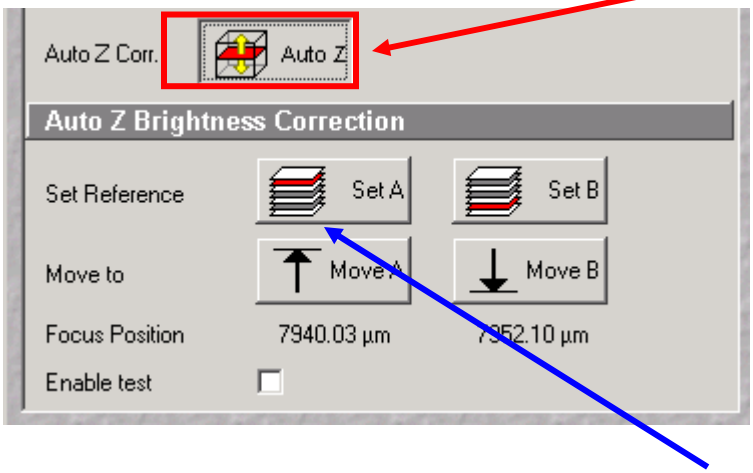
10.1.2. Optimise settings

Unlike Sequential acquisition (Multi-Track), each channel is acquired simultaneously during optimization – so be quick!
Refer to section 7.2 for instructions on how to optimize the channel settings.

10.2. Z-attenuation compensation

As images are collected deeper in to the sample there can be significant loss of signal. This can be caused but refractive index mismatch, light scattering and absorption.
This signal loss can be compensated for collecting the attenuated slices with higher gains and/or with higher laser intensity.
Two reference points are set, one near the top where the signal is the brightest, and one near the bottom where there is still significant signal but it has been attenuated.
The laser intensity and gains are set for each reference slice (the bottom reference slice having higher gain/laser). As the software acquires the z-series, it gradually steps up the gain and laser as it approaches the bottom reference slice.

1. In the ***Scan Control: Z-settings*** window activate the ***Auto Z.Corr***.



2. While in ***Fast XY*** scan mode, focus to ***near*** the top, of the sample where it is brightest. You should have already set the detector and laser settings for this slice.
If not go the ***Scan Control: Channels*** window and set the detector gains and laser intensity to get adequate signal at this focus position.

3. In the ***Scan Control: Z-settings*** window click ***Set A.***

4. While in ***Fast XY*** scan, manually focus to the ***near*** the bottom of the z-series which still has significant signal.

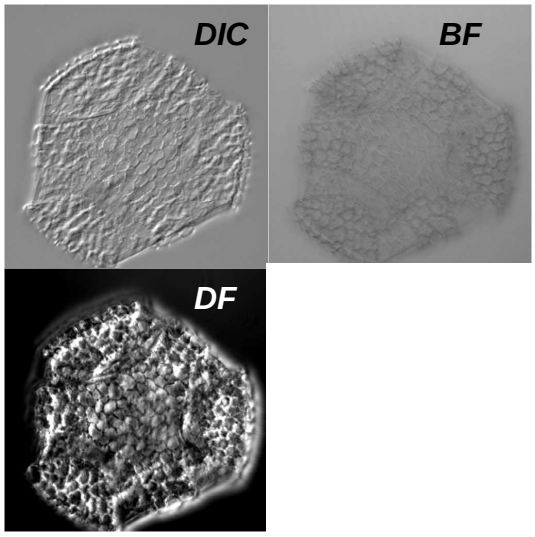
5. Go to the ***Scan Control: Channels*** window and set the detector gains and laser intensity to get adequate signal at this focus position.

6. Go back to the ***Scan Control: Z-settings*** window and click the ***Set B*** button.

7. Now start the Z-series acquisition by clicking ***Start***.

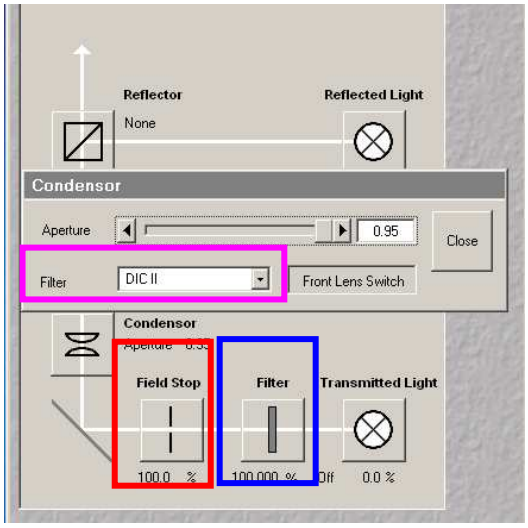
10.3. Transmitted light image

Whilst the laser is scanning the field of view a certain amount of the excitation laser light passes through the specimen. This light can be detected and its intensity in each part of the field of view corresponds to the transmitted light optical properties of the specimen. In this way a "brightfield" transmitted image can be reconstructed. It is not a "confocal" image in that the light does not come from a single plane.



DIC – differential interference contrast
BF – bright field
DF – dark field

For this, the microscope transmitted path needs to be set correctly. Open the ***Microscope Control*** panel (Toolbar ***Acquire*** then ***Micro***)

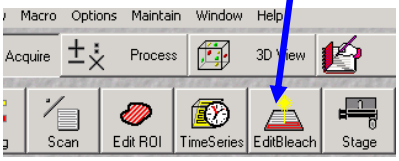


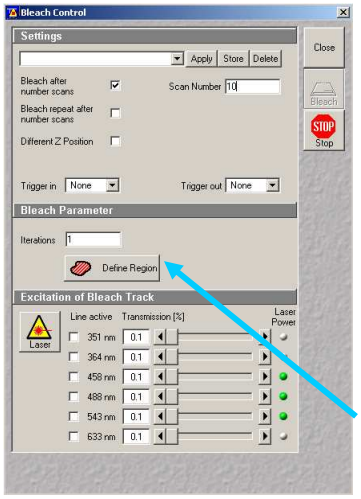
The ***Field Stop*** iris needs to be fully opened – 100%.
The ***Filter*** at 100%
The transmitted light to be at 0%.
Click o the ***Condensor*** button to reveal the condensor options
The ***Condensor Filter*** needs to be set to the appropriate type of brightfield i.e. BF, DIC, DF or Ph.
NOTE: The software keeps changing the condensor settings back to "BF". Keep checking that it is set correctly.

Then, whilst in fast scan mode, adjust the transmitted light channel's (ChD) detector gain and offset so that the background of the image is mid-grey and the brightest part of the image is near saturation (i.e. shows red with the ***Range Indicator*** palette).

10.4. FRAP

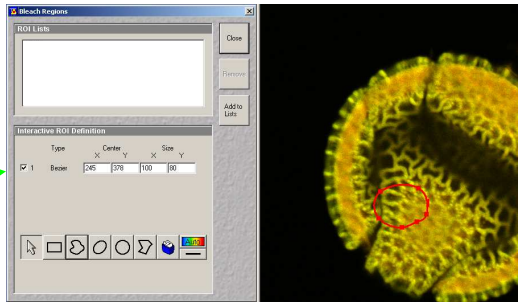
1. Click on ***Edit Bleach*** toolbar button to open up ***Bleach Control*** dialog.



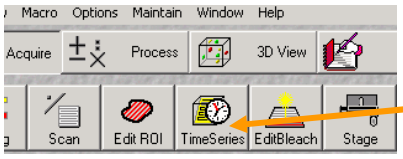


2. Set Bleach parameters in ***Bleach Control*** dialog
Check ***Bleach after Number of Scans***
Set "***Scan Number***" to for pre-bleach imaging
Set "***Iterations***" value – this may require empirical determination. It needs to be enough to allow complete bleaching but few enough to allow quick return to imaging. Try 10 as a first attempt.
In "***Excitation of Bleach Track"*** set bleaching laser to 100%

3. Click ***Define Region*** button to select bleach area



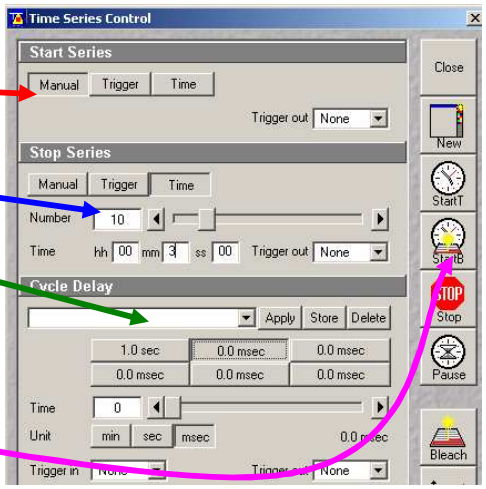
4. Define area to bleach then select it by ticking the **checkbox** along side the ROI.
Close dialog.

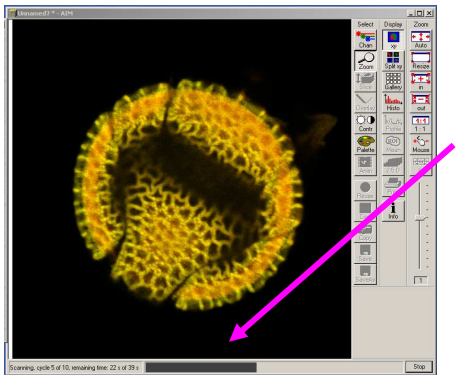


5. Open ***Time Series Control*** dialog by clicking the ***TimeSeries*** button in the main toolbar.

5. Set FRAP time-course in ***Time Series Control*** Dialog
Start Series = ***Manual.***
Stop Series = select ***Time*** then enter the duration of the experiment.
Enter *Cycle Delay* (time between frames).

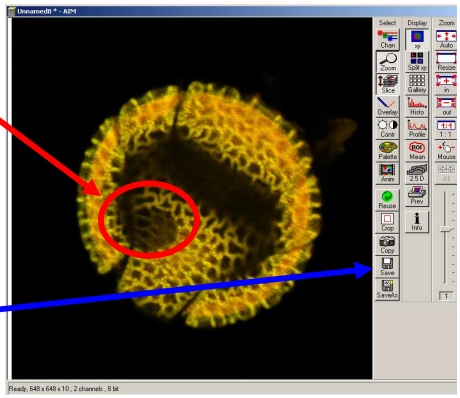
7. Click "***StartB"*** to start experiment.





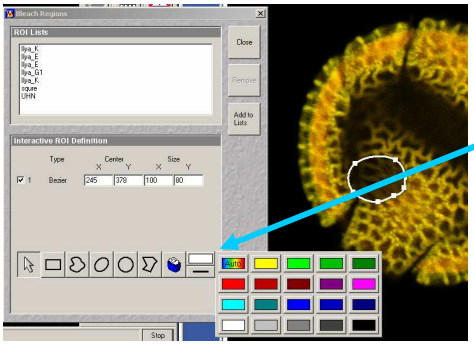
Experimental progress shown here.
Progress bar will pause during the bleach process.

Bleached area

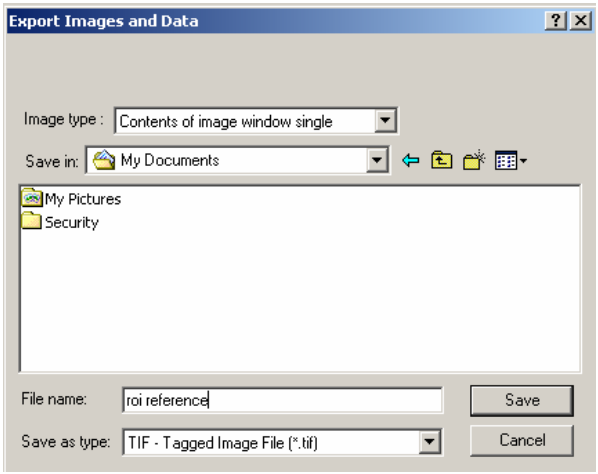


7. ***Save*** experiment

8. Create ROI reference image



Turn ROI white by clicking the ROI colour button and selecting **white**.

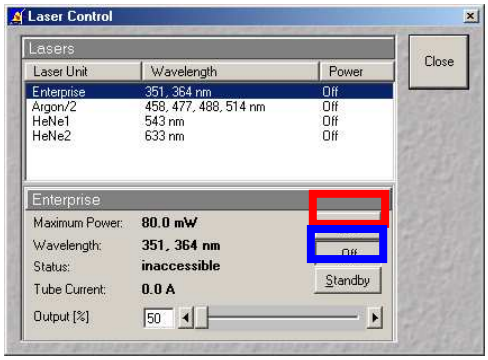


Export this image for reference via the menu command ***File/Export.***
Image Type = "***Contents of Image Window Single***".
Save in MBD folder.
Image type = TIF.

11. Shutting down the system

11.1. Turn off lasers

Click the ***Acquire*** toolbar button, the ***Laser*** button in the sub-toolbar.
In the ***Laser control*** window turn each laser ***Off*** or to ***Standby*** if somebody is using the system within the next hour.



If you have used the UV laser, switch off the black switch in the white frame located on the front of the UV laser power supply. Do not touch any of the cooling unit settings.

11.2. Remove Specimen and clean microscope

Wipe off water from objective and specimen.
Move to a low power objective (5× or 10×) objective. Raise the stage using the buttons on the left had side of the microscope base.
If you switch off the system while the stage is lowered the ***'top'*** of the focus range will be reset to that position when the microscope is next turned on. This will mean the 'top' will need resetting and could result in damage to the $12,000 63× objective.
Turn off the epifluorescence lamp.
Cover the microscope avoiding the hot lamp housing.

11.3. Exit the software

Exit the Zeiss LSM .
A message will come up reminding you not to power down the system until the laser is cool. Click ***OK***.
If you have left the lasers on for the next user, you will also be asked whether you want the lasers switched off. Click ***No***.
Read then close the WCIF Exit screen.
Burn your data to CD or copy across network (once installed).
Once you have finished with the computer, LOGOUT. If you do not logout, the system will continue to charge time to your account.

11.4. Power down the system

If nobody has booked for the next hour, please shutdown the system If the next person is the last booking of the day, please call them and confirm that they will be using it. This requires that the ***remote control*** be switched off and the compressed air shut down.

12. Opening your images offline

12.1. Zeiss image browser

http://www.zeiss.de/lsm
Follow link in lower right hand corner:
"Free LSM Image Browser"

Requires a registration form to be completed and also the download and installation of an extra DAO file if you have Microsoft Office 2000.

12.2. ImageJ

http://rsb.info.nih.gov/ij/

Plus Zeiss plugin
http://rsb.info.nih.gov/ij/plugins/lsm-reader.html
Requires LSM images to be saved "uncompressed".

WCIF ImageJ is a bundle of plugins along with the core ImageJ files. This can be freely downloaded from the WCIF website downloads section
http://www.uhnresearch.ca/wcif.