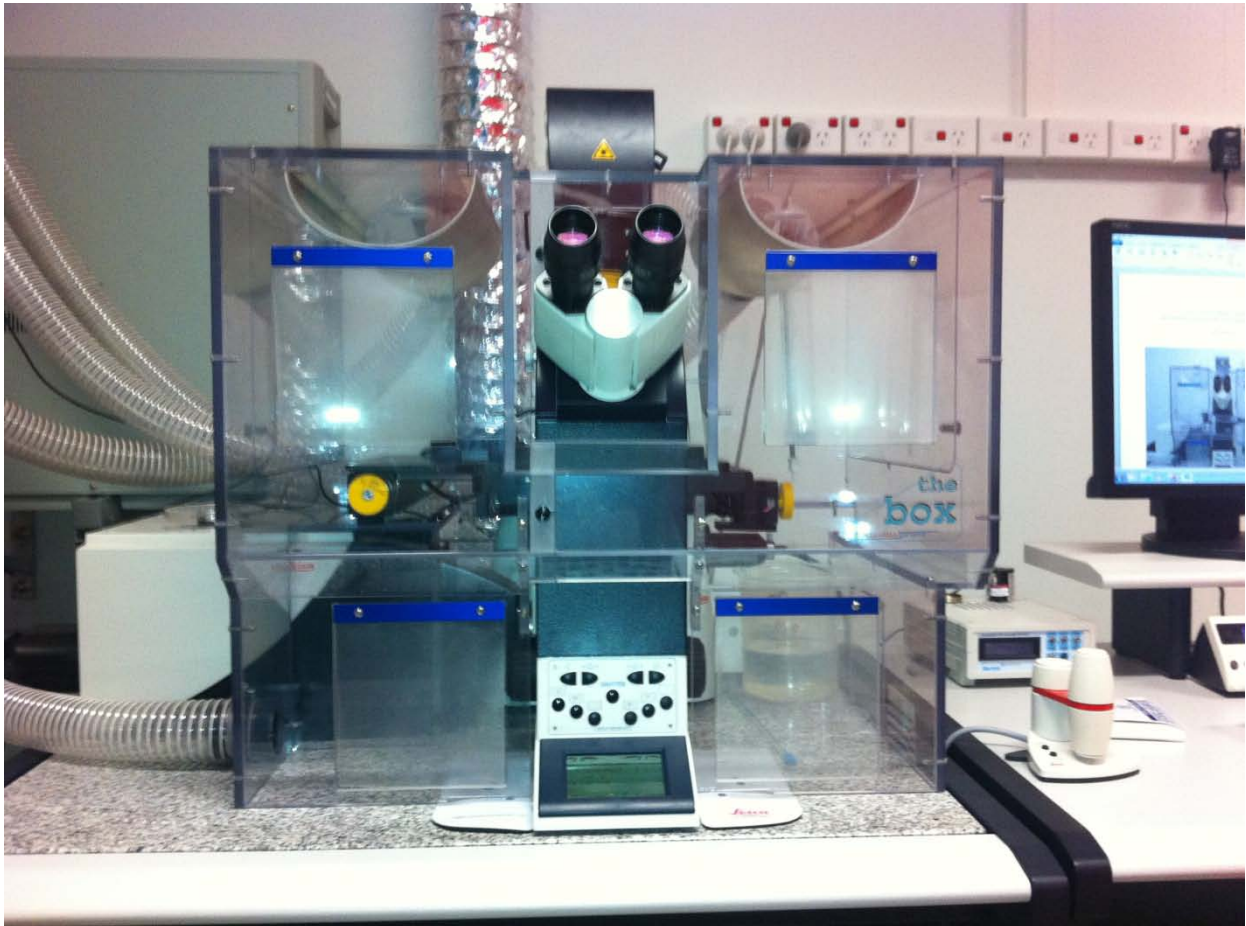# LEICA TCS SP5 CONFOCAL MICROSCOPE
WITH INVERTED MICROSCOPE AND ENVIRONMENTAL CHAMBER

USER MANUAL

This user manual was compiled from PDFs sourced from the internet ("SP5INVERTED MC_UK date.pdf " and "SP5 confocal microscope user manual Y.KREMPP CHUV 2006"). We acknowledge and appreciate the generosity of both authors for sharing this information with the Confocal community. The manual has been updated with details relevant to the Leica TCS SP5 hosted at the Kids Research Institute for the University of Sydney on behalf of the Westmead Research Hub.

Laurence Cantrill and Dongwei Wang, Kids Research Institute, 2013: Revised 2015

I. Starting up the Leica SP5 confocal microscope

Switch on the buttons in following order:
(1) PC/Microscope.
(2) Scanner power.
(3) Laser power.

(4) Turn the laser key clockwise, from *OFF* to *ON*. The yellow light should become illuminated.

(5) Turn on the fluorescent light source to observe fluorescence through the eyepieces

Check that
The "**Intensity"**
knob is turned up to allow enough
light onto your specimen (or turned
down if it is too bright).

Check that the "**shutter** "
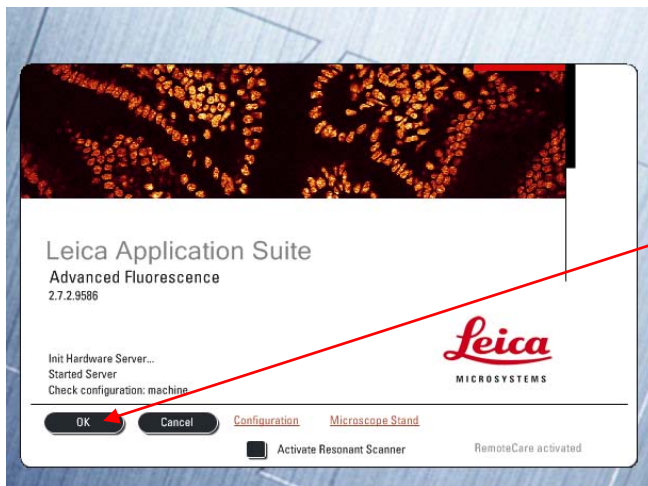button is pushed in for the
"**open** " position.

Power switch.
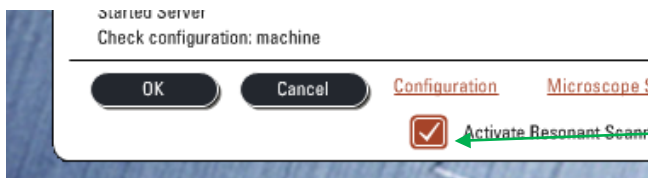
II. Starting up the LAS AF software

To start the program double-click on the LAS AF icon on the desktop.
After the main menu window appears you will have to choose which scanner you want to use.
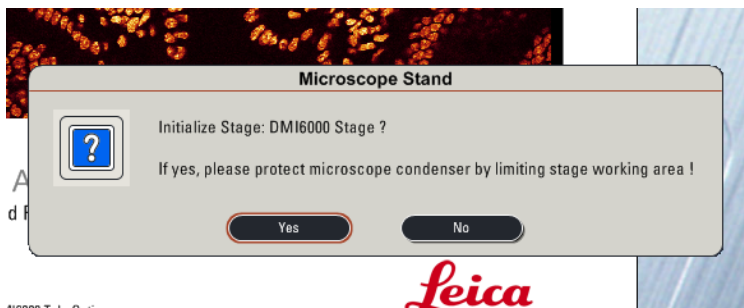
For the **conventional scanner** (high definition, slow speed, 1x default zoom) **click "OK"** (check that the resonant scanner box is unticked).
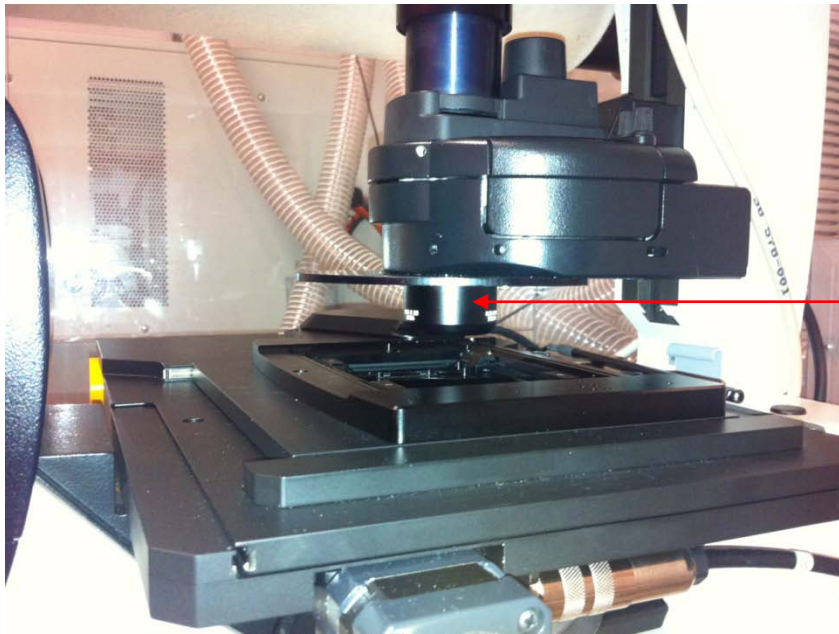

For the **resonant scanner** (high speed, live cell, rapid Z-stacks, 1.7x default zoom) **tick the resonant scanner box**, then click "**OK**".
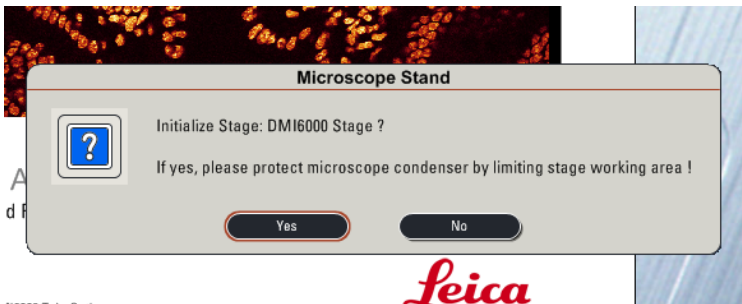
When asked if you want to initialise the stage,

Please check that the objectives are lowered as far as they will go and that there are no obstacles that might run into the microscope condenser tower (see below).

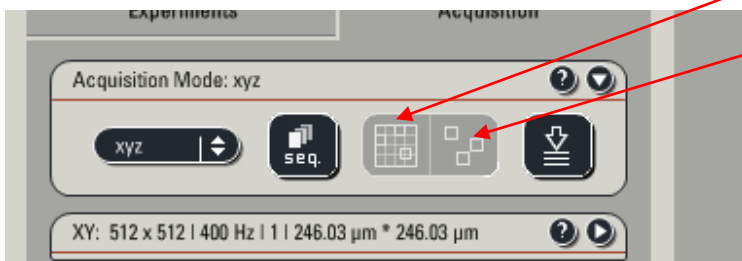Condenser tower

If all is clear click "**Yes**", and the stage will calibrate itself.

It is ok to click "**No**", but the stage will not calibrate itself and the **"Tile Scan"** and **"Mark and Find"** functions will not be active in the program.

The main window should open.
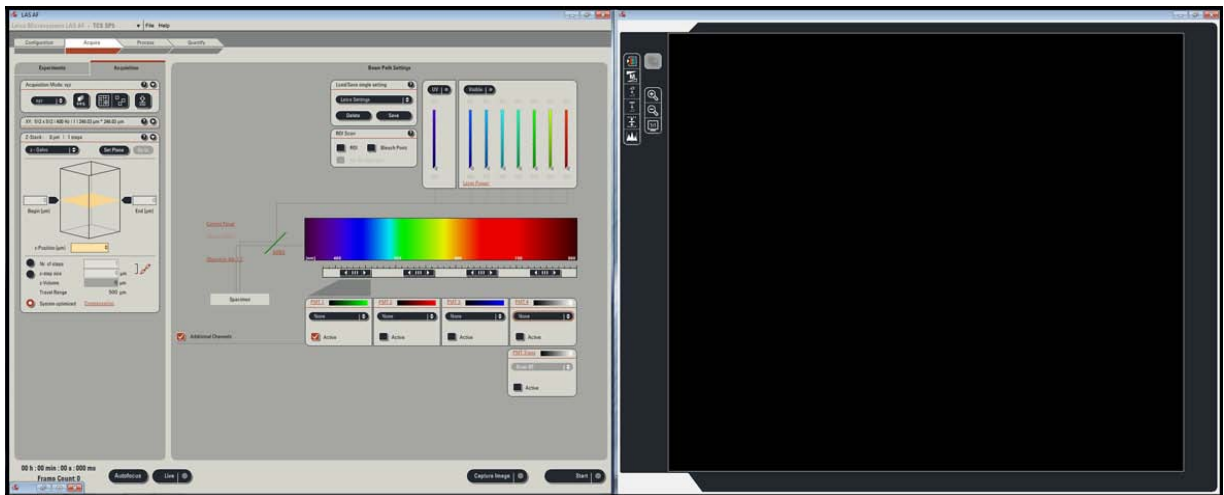
Go into the "**Configuration**" menu, and select the "**Laser**" icon.
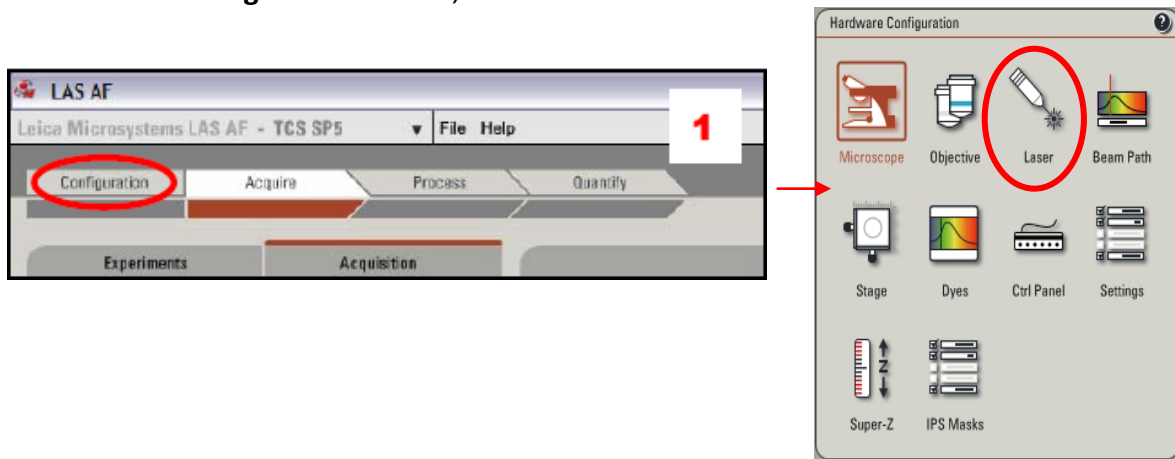
Turn on the lasers that you will be using by ticking the box beside the appropriate laser. Set the slider for the argon laser to ~**20%**.

Click on the **Acquire** tab to return to the imaging screen.

Changing objectives

The SP5 has seven objectives available for general use, with 6 of them on the microscope at any one time.

To change objective, click on the word **"Objective"** in the middle area between the "**Acquisition**" windows and the excitation/emission spectrum to obtain the dropdown list.

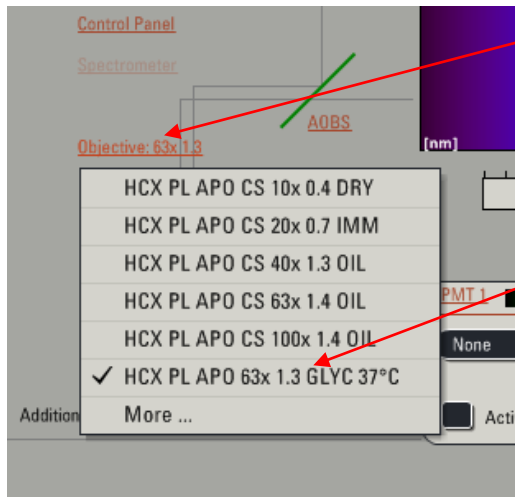**Always double check which objective is in place. This is the glycerol 63x objective and should only be used with glycerol!**

Choose the objective by clicking on it and it will automatically rotate into place.

Sometimes you will get a warning message asking you to check that you are not incorrectly changing from one immersion type to another without cleaning the bottom of your slide or dish. Please double check before clicking yes.
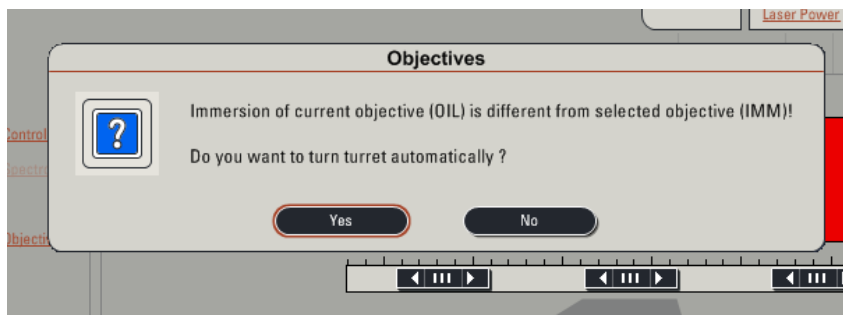
The available objectives are:

Permanently on the turret
Dry	10X,
Multi-immersion	20X
OIL	40X
OIL	63X
OIL	100 X
Glycerol	63X

Not on the turret so please ask if you want to use

Water	63X

Also available: 5x objective and long working distance water dipping objectives (10x, 20x, 40x, 63x). These will allow imaging through standard thickness dishes, but they have lower numerical apertures and are therefore not as efficient at collecting light as the standard objectives.

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III. Specimen observation via eyepieces

Tilt condenser back to access the stage. Apply the appropriate immersion fluid on the slide or objective. Raise the objective until it touches the immersion fluid.

Place slide with **coverslip side down**, or culture dish with glass coverslip bottom on the stage, return condenser to normal position.
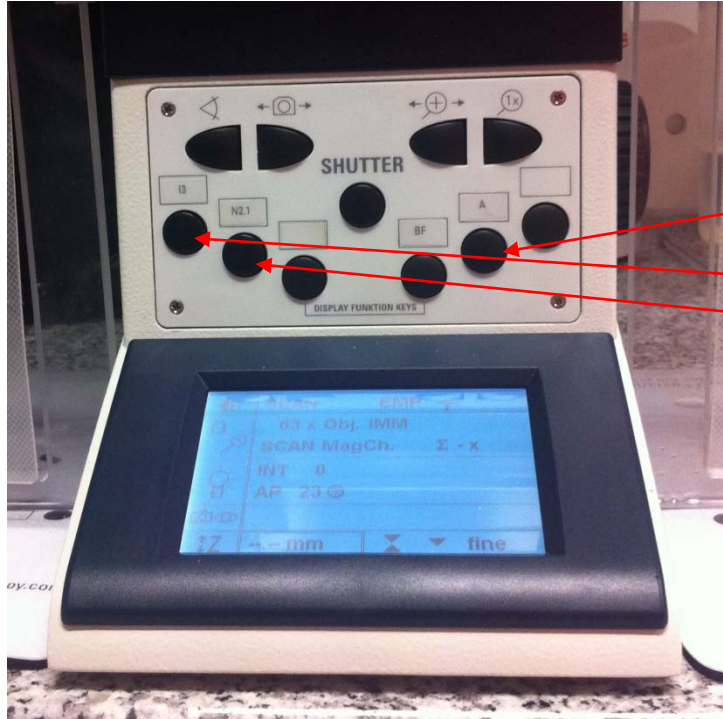
Push the appropriate button for your fluorophor located on the front of the scope.
"A" is **for the blue (DAPI) channel**
"I3" is **green**
"N2.1" is **red**
Push the **"Shutter"** button in the centre of the scope.
You should see light coming from the objective.

The controls for the stage and focus are all controlled by the remote located to the right of the scope. The X & Y knobs located on the front of the remote control the movement of the stage. This movement can be adjusted from fast to precise by the two small buttons on the left side of the remote.

The smaller Z knob at the back controls the focus/Z position of the microscope.

The focus can be adjusted from fine to coarse with the two small buttons on the right side of the remote. Turning the Z knob clockwise brings the objective closer to the specimen.

A read out of the focus setting is also located on the lower right side of the microscope control panel window.
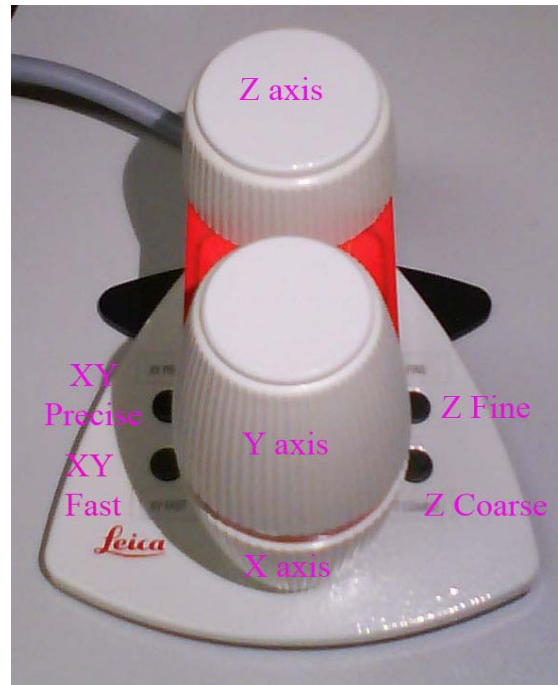
IV. Choose or create a configuration for acquisition

If you want to use a premade configuration, just use the drop-down menu **"Load/Save single settings"**:

Click here to select a premade configuration

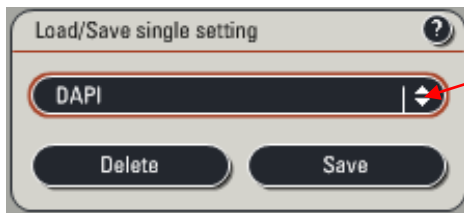**If you want to create your own configuration, follow this step-by-step:**
1) Activate the laser lines you need ("**UV**" and/or "**Visible**").
2) Tune their output power (usually between **15 and 50 %** depending on the line and the sample that is used). A good starting point for this setting is 15-20%.
Settings that are much higher than this can cause photobleaching of your samples!
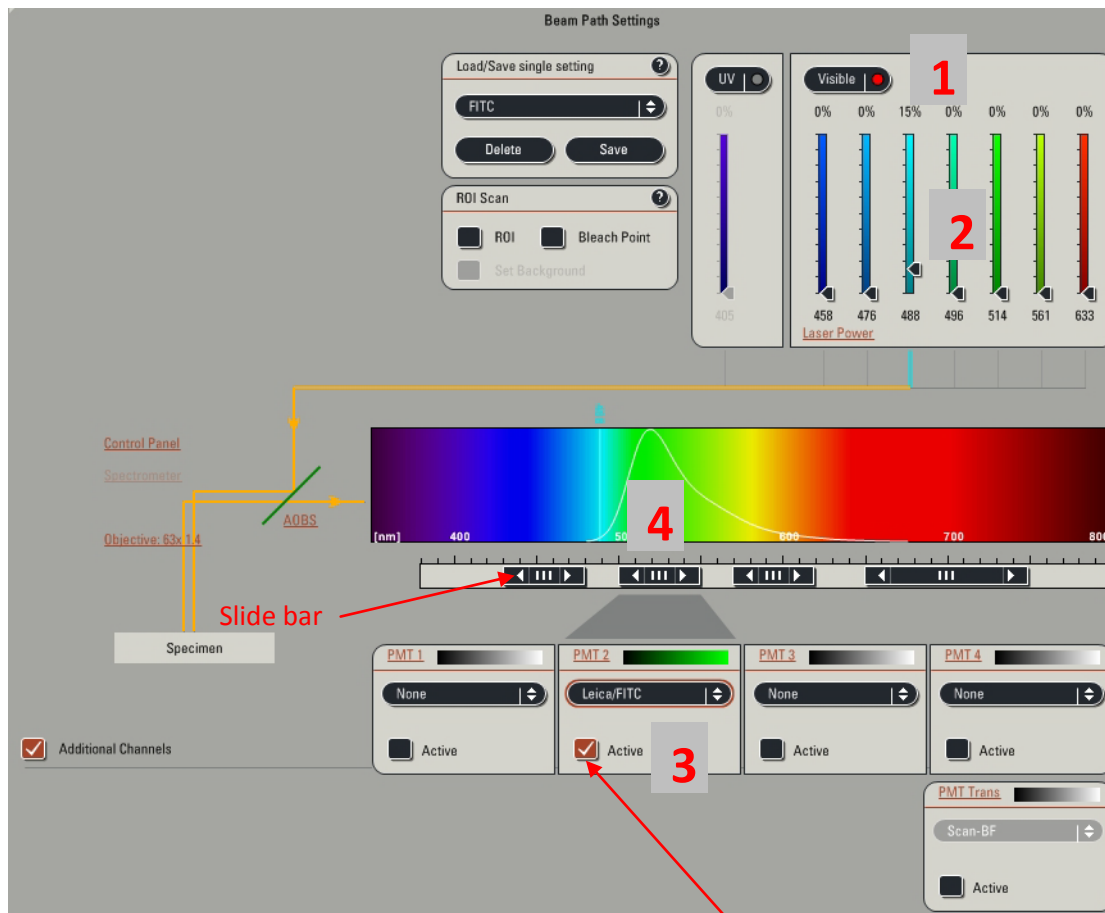
3) Activate the PMTs (detectors) that you need (tick "**Active**"). Use the PMT located just under the spectral band you want to detect. The colour of your image can be changed by double clicking on the PMT colour bar for each PMT.
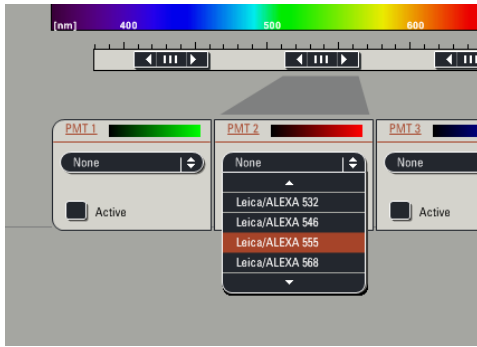
If you wish to see the emission spectrum of a particular dye click on the drop down menu underneath the colour bar. This will place an emission spectrum curve on the spectrum in the middle of the screen. Note that this curve has no influence on the imaging settings, it is just a visual guide for the user so that the appropriate bandwidth can be selected

4) Choose the bandwidth according to your staining for each PMT.
The emission wavelength can be adjusted in two ways:

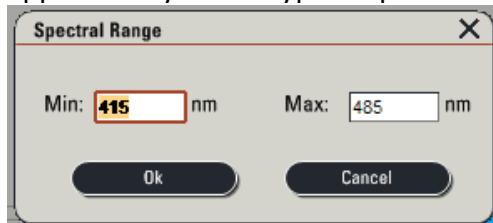Drag the edges of the horizontal black slide bar on the emission band bar to widen or narrow the emission band. Alternatively, double click on the appropriate black slide bar; a dialogue box will appear and you can type in specific limits.

V. Preview images
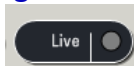
1. Click on the button at the bottom of the left monitor screen to begin scanning. The button will be highlighted and will now say "**Stop**". On the far right side of the underline control box (below), turn the **"Z POS"** knob slightly in both directions to choose the brightest portion of the sample.

2. Click the button located on the left side of the right monitor's screen. This is an aid in choosing the proper PMT settings for gain (brightness) and offset (contrast).

3. If you have more than one colour, click on the image that you want to adjust. A dotted line should appear around that box.

4. Turn the **Gain** knob until bright blue pixels just begin to appear in the image. These pixels are saturated. You only want a few bright blue pixels in the structure of interest.

5. Turn the **Offset** knob until green pixels begin to appear in areas where there should be no fluorescence or tissue. **Note:** The **Offset** knob operates in reverse compared to most controls. Turning it to the right will give you less green, and turning it to the left will give you more green in the background.

6. Click the [image] button again to get a grey-scale image. Click the button one more time to get back to the original colour image.

7. Click **"Stop"** to stop the scan when all adjustments have been made.

8. After adjustment, you can **save** the configuration setting into the setting system. See "**Sequential Scanning**" below.

VI. Capturing images

-Select the proper **Mode**. xyz is the normal mode for scanning.

-For setting up images use 512 X 512 format, subsequently change **Format** to 1024 x 1024 or higher depending on the resolution you want (bigger files but better resolution). See the notes on XY Resolution below.

-Acquisition **Speed,** standard speed is **400 Hz**. The speed may be slowed down to improve image definition (the longer the scanner dwells on each point, the more information is collected). The effect is similar to averaging
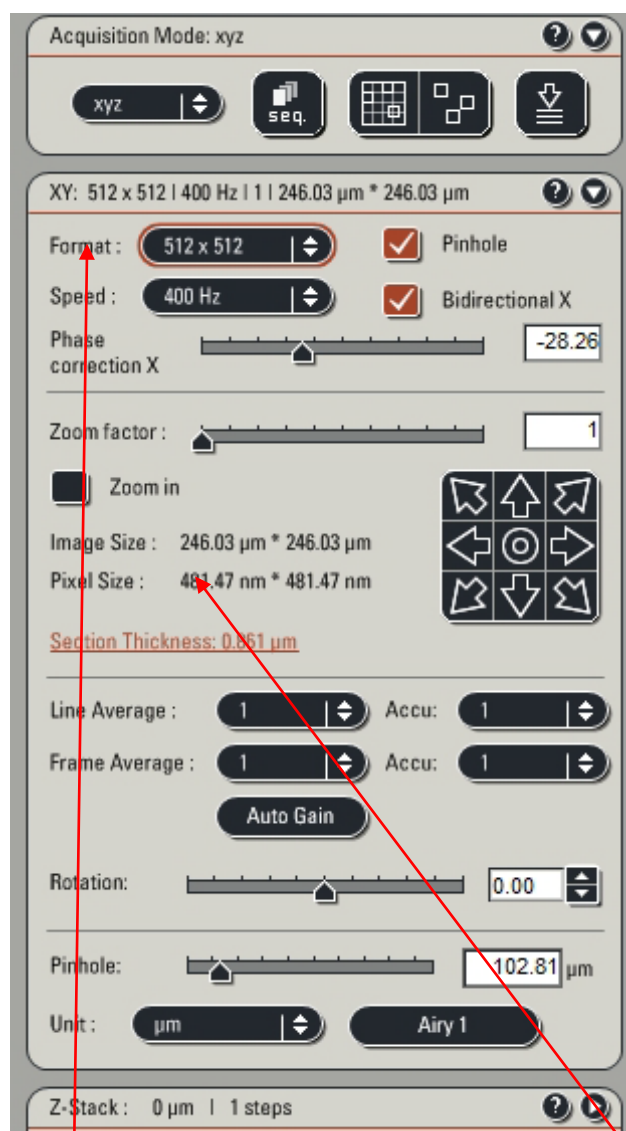
Click on **"Line Average"** or **"Frame Average"** buttons to choose the number of sweeps by the laser to average out background noise and fill in missing image data. The greater the background the more that averaging can clean up the image. However, sometimes this can be overdone (the more sweeps, the more bleaching occurs).

XY Resolution

There are two components to resolution: the image format and the resolution of the microscope. These components interact to produce the resolution in your digital image. In the window above "**Format**" allows you to change the resolution of the image (the number of pixels in the image, which influences the size of the pixels represented by "**Pixel Size**" above). The resolution limit in the microscope can be defined as the smallest distance that two objects can be separated and still appear as separate objects. This is determined by the wavelength of light being used, the NA (numerical aperture) of the objective, and the refractive index of the material that the light passes through. For a confocal microscope the resolution is around 200-300nm in the XY direction. With high magnification and high NA objectives (63x, 100x), you can take full advantage of the resolution in the XY direction by oversampling your image 2-3 times. By changing the "**Format**" you can change the "**Pixel Size**" to around 100-200nm to achieve the optimum image quality.

VII. Z Stack Operation:

Z stack allows for multiple optical sections to be cut through the structure of interest.

Open the **Z Stack** window on the left side of the screen. You should see a representation of a cube with two boxes labelled "Begin" and "End".
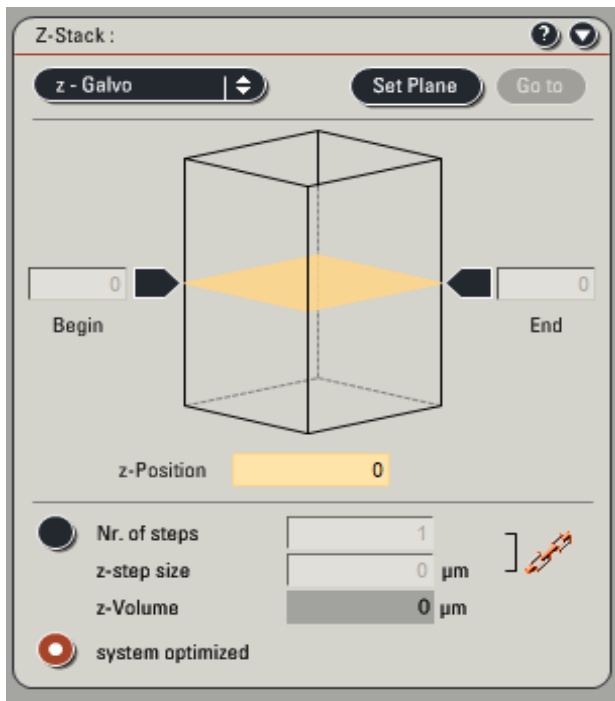
Make sure you are in 512X512 format. Click the **"Live"** button.

The **Z POS** knob on the control box is used to set the beginning and end positions of the area to be sectioned.
Turn the **Z POS** knob to the right until the fluorescence starts to disappear. This is the part of the sample closest to the coverslip. Click the arrow labelled **Begin** so that it changes to a brown colour.

Turn **Z POS** knob to the left to find the end of the sample, when fluorescence begins to fade again. This is the part of the sample closest to the slide. Click the **End** box.
Click the **"STOP"** button to stop the scan

The **"System Optimized" button is normally active, and the** software suggests a number of sections for your chosen volume of sample as well as the z-step size. **THIS IS NOT CORRECT!** The resolution of the microscope is not good enough for the numbers suggested here.

Z Resolution and optimising the number of sections
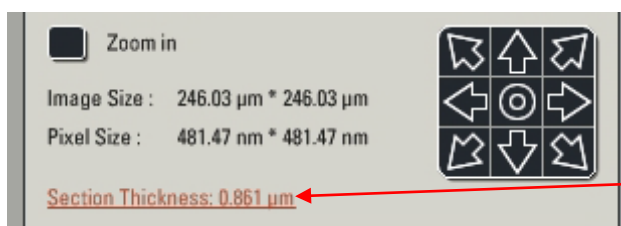
The resolution in the Z direction (axial resolution) is much less than in the XY direction due the blurring of light being scattered through the sample. You can find the Z direction resolution for a particular objective and wavelength in the XY window listed as "**Section Thickness**".

The optimal number of sections to take in a Z stack to enable a complete 3D reconstruction is half this value. This can be loaded automatically into the Z stack function by clicking on **"Section Thickness"** above and then clicking on "Apply to step size" as shown in the image below.
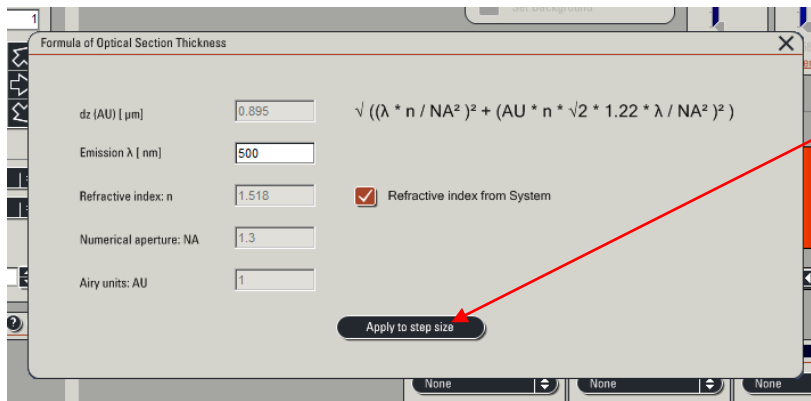
Clicking on "**Apply to step size**" from the "**Section Thickness**" window will send the correct optimised Z section thickness and number of sections to a Z stack.

If you want fewer sections than the optimum:

You can of course click the button, **"Nr. of Steps"**, and enter the number of sections that you want to scan or enter the desired section size in **"z-step size"** and the software will calculate the number of sections needed.

Click on the **"Start"** button to begin a Z stack series.

To view the series of sections click on the button on the right side of the working window (right monitor).
Z stack scanning is finished when the **"Start"** button reappears.

Z sectioning and multiple channels—tricks to speed up the scan

If you are scanning multiple channels (see Sequential Scanning below), choose "between stacks". This is quicker than between lines when acquiring Z-stacks. You can also use "**Bidirectional X**", where the scanner action zigzags rather than running left to right on each line. This speeds up the scanner, but you need to adjust the "**Phase**" of the scan on the control box to ensure that you don't end up with a blurry or doubled image.
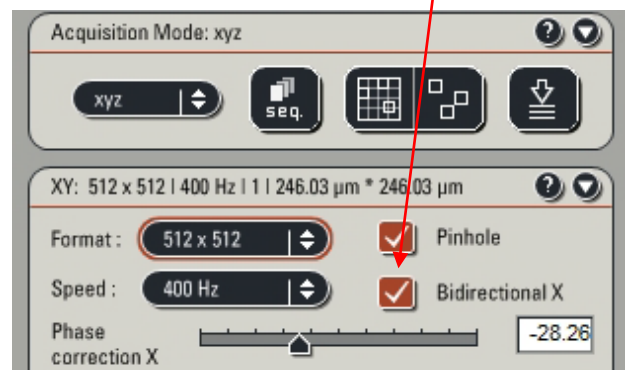
VIII. Sequential Scanning

With most multicolour imaging there is a high risk of exciting two or more fluorescent markers when trying to capture the signal from only one of them. This problem is called cross-talk or bleed through, and it is important to avoid as it can give you false positive staining in one or more of your detection channels. It is particularly important to be aware of this when undertaking colocalisation studies. The best way to avoid this is to not do simultaneous scanning, but to do sequential scanning (one colour at a time) and to also narrow down the bandwidth of each channel to the specific colour of interest to greatly reduce the risk that other colours will bleed through. You can check for overlap of emission bands of different dyes by selecting dyes from the PMT dropdown menus.

To Scan Sequentially (By Lines)
The simplest way to do sequential scanning can be set up through the LAS-SF software using the "Sequence Scan" feature.

1. Click the [seq.] button. This will open the "Sequence Scan" window (below). Scan 1 should be highlighted.

2. Select "between frames" (or "between stacks" if you are doing Z-stacks).

3. Set up Scan 1 by making sure that the **UV** or **Visible** button is active, the correct **PMT** is active, and the slider for laser intensity is raised to 20%. Alternatively, select preset parameters from the "Load/Save single setting" box.
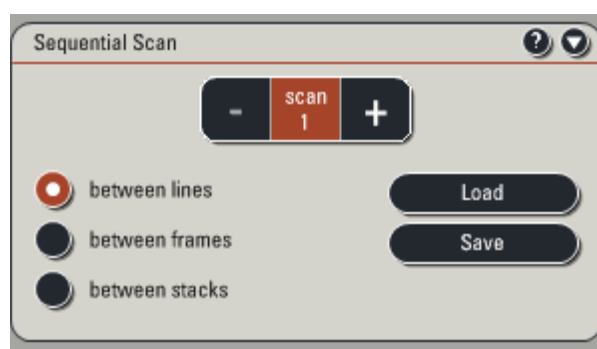
5. Click the **"+"** button in the **Sequential Scan** box to add Scan 2 and so on. **Do not touch any buttons after setting scan 1 before you highlight the next channel.**
6. Set up parameters for Scan 2 etc as in step 5.
7. Click the **"+"** in the **Sequential Scan** box to set up the next scan (Scan 2) for the next channel if necessary and adjust settings as before.
8. When you have successfully made all of the adjustments to do the number of scans you wish to execute, leave the **Sequential Scan** box expanded. If you need to go back to a previous scan channel click on the corresponding number. **Do not click the "minus (-)" button unless you wish to delete the last created scan settings!!**
9. **NOTE:** Line averaging needs to be adjusted separately for each scan channel.
10. Sequential scanning can be combined with Z-stacks as well as other automated functions.
11. When you are satisfied by your settings click on "**capture image**" to acquire one image, or click **"Start"** to begin the entire sequential scan (plus other automated features).
12. Scanning will be finished when the **"Start"** button reappears.
This sequential setting can be saved by clicking **"Save"** in the sequential scan box. When needed for future use, click **"Load"** to recall the setting.

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IX. Transmission imaging (DIC Imaging):

Differential Interference Contrast Imaging is a light microscopy technique that will enable you to see a bright field (transmission) image of your sample with some contrast of depth depending on the thickness of your sample. This is very useful to see where your cells actually are in relationship to the fluorescent tags that you are imaging with the confocal scanner.
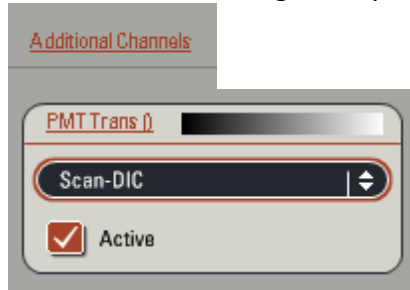
To view a DIC image in single image or sequential scanning format, click on "Additional Channels" on the left screen and choose "Scan-DIC". This image can then be adjusted for **"Gain"** and **"Offset"** in the standard fashion.

The Wollaston prism should also be adjusted to optimise the contrast and pseudo 3D effect of the DIC. This is done by going to the "Configuration" at the top of the LAS window tab, clicking on the "Objectives" icon, and adjusting the slider to the left or right.

The prism is different for each objective, so the slider might need adjusting if you change magnification. The nature of the optical effect of DIC is that the corners of the image can appear brighter than the centre. This is normal, and the bright corners can be avoided by zooming the image.

X. Customising & Manipulating Images:

Right side of the Image screen

-These buttons allow you to turn different channels of a sequential scan off and on.

– Overlays all colours to form one image.

– Takes the multiple images from a **Z Stack** and combines the brightest pixels into one image.

– Shows the intensity along a chosen x & y axis through each section of a stack.

Permits the user to see each image through a stack while the instrument is optically sectioning.

- Advance the images of a stack one at a time, all the way to the beginning or end, or play them in sequence as a movie.

Left side of the Image screen

 – Expands the options so that you have access to all the tools.

 – Highlights items to be moved on the screen or highlight then click the bin to erase from the image.

 – This button highlights all the annotations that have been added to an image. They may then all be removed by clicking

 – Define areas of interest

 – Place arrows.

 – Type in text.

 – Places a micron bar on the image. To determine a bar of a certain length click on , highlight the bar, right click and choose "Properties". You can then enter the required length. The position of the bar on the image can also be changed by highlighting and dragging to the new location.

 – Define numbered ROIs.

 – Zoom in or out of the captured image.

 – Returns the captured image to the default zoom of 1.

XI. Saving Files:

The images in the experiment window are temporary. A software or computer crash could cause you to lose your data. **SAVE FREQUENTLY!!**
Files should be saved to the hard drive as Leica files (.lif) and also exported as tiff files after every session.

1. Open the **"Experiments"** window.
2. Right click on **Experiment** and choose **Save Experiment As**.
3. The **"Save As"** box will open.
4. Find your folder and double click on it so that it appears next to **"save in"**.
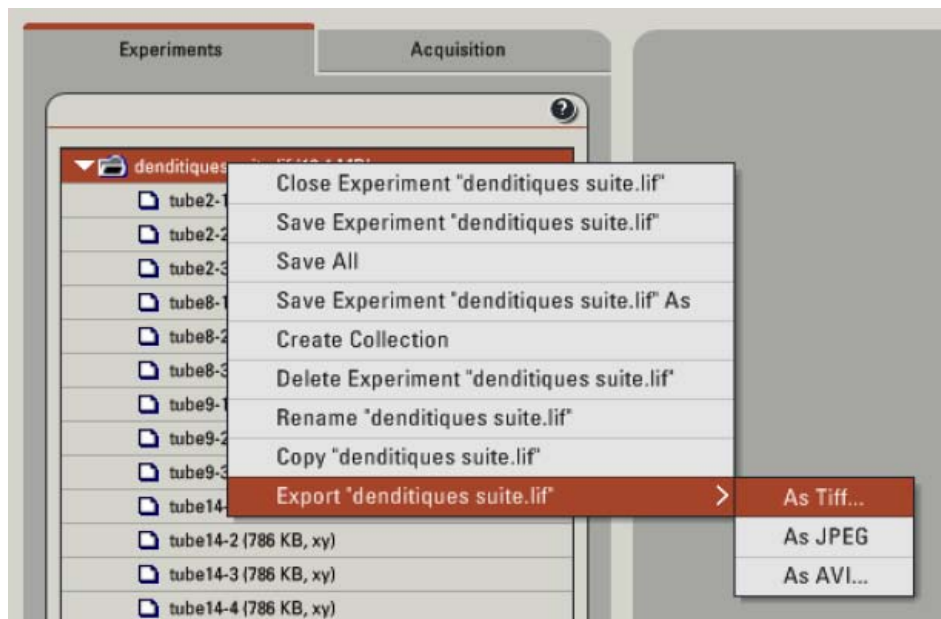5. Next to **"file name"** type in identifying information for your particular experiment. This is the name of the whole experiment and not individual images. Image names can be changed in the Experiments window by right clicking and choosing "rename" for each file separately.
6. Clicking "**save**" to save the whole experiment saves a **.lif file (Leica proprietary format,** but can be read by Image J and other image analysis programs) which contains all the associated parameter data that was used to capture the image, and therefore is your valuable raw data. **It is strongly advised that you keep the .lif files so that you can reload the raw data into LAS for later image analysis or to restore the acquisition parameters for a particular image**. See 10 below.
9. You must also **export** your files as tiff files to open it them Photoshop. Right click on your experiment name, choose export experiment, then export as a tiff image. Save the tiff images in your folder along with the .lif files. You can either save the individual channels or the overlay images per export. The tiff export is always a two-step process if you want to save both. Batch exports of groups of files in an experiment list are also possible if you first highlight multiple files.

10. Right click your image files and select "Properties". With the window that is opened (below) you can see the properties pages of the images and restore the acquisition parameters that were used with the "Apply Settings" button. This is an important reason to keep your .lif files.
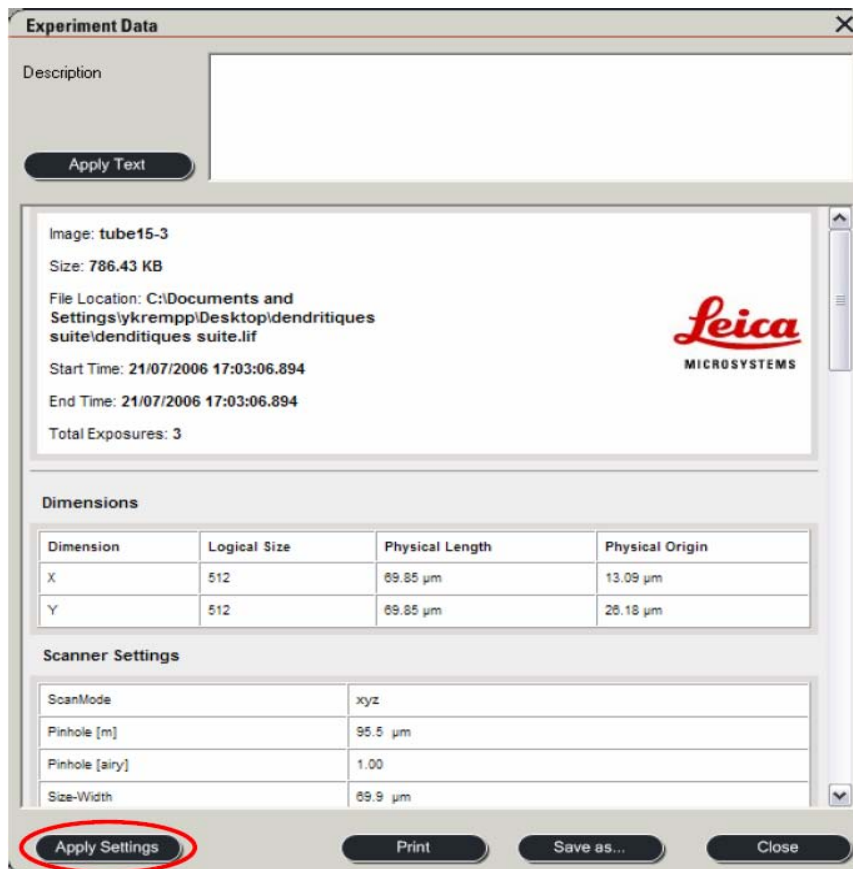
Saving Max Projections, Overlays, Etc:
When you do a stack your **"series"** is automatically placed in the Experiment folder in the Experiments window.

To rename the series right click on the series, and choose "rename series". You may then type in the desired name.

Any image annotations must be saved with a snapshot (screenshot).

1. In the image screen on the right click on the image that you wish to save. The box around it should be highlighted.
2. Double click on the image to make it full screen size.
3. This is the time to add annotations to your image with the tool buttons.
4. Right click on the image, choose "Snapshot".
5. A file named snapshot will be placed in your experiment folder.
6. Rename the file by right clicking on it. Choose rename snapshot and rename it.
7. Being screenshots, these files don't have the same resolution as the originals.

Snapshot All

To use snapshot all, right click on the highlighted image on the screen, and then choose snapshot all. This will save whatever is on the screen at the time, regardless of what is highlighted. This is useful if you want to save tiled images or the whole gallery in one picture.

Note: Nothing that is placed in your Experiment file is saved permanently until you tell the software to save it. You can save the entire file by right clicking on the experiment file and choosing **"Save As"**. Remember that any additional images captured after the initial save process will also have to be saved. You can do this by clicking the **"Save All"** button in the Experiment window.

XII. Ending the session and system shutdown

If someone will use the microscope after you :

-Turn down the power on the Argon laser in the software. Leave the lasers on if someone is using it immediately. You may turn them off if there is a gap of an hour or more.

- Save your images and exit the program.

-Turn off the fluorescence lamp if no-one is using it straight away.

- Clean the objectives you have used with the lens tissue provided.

If no one will use the system after you:

FIRST THING: In the software turn down the argon laser, turn off all lasers by unticking the boxes

1.- Turn the laser key anticlockwise. This starts the 10 minute cool down time.

- Save your images and exit the program.

- Clean the objectives you have used with the lens tissue provided.

- Turn off the computer via the "**Start**" menu of Windows.

- Turn off the fluorescent lamp.

When the 10 minute cool down time is up:

- Check that the blue power light is off on the computer tower

2. Switch off the PC/Microscope switch, **3.** switch off the Scanner switch, **4.** switch off the Laser Power switch.

Turn off the lights and lock the door.

XIII. Connecting to the University of Sydney backed up data store

What it does

This backed up storage system is available for the transfer and short to medium term storage of image data files from the microscope computers, including Spot, DC500, Brightfield/Dissecting microscopes, Leica SP5 and SP2 confocals, and the CCRU Live Cell microscope. This is not meant as a long-term storage for data files, but we will monitor how quickly it fills up. The store is for the transfer of already created files from microscope computer hard drives, it won't work for live dumping of image files microscopes as they are created (eg live cell imaging) because the transfer rates are too slow.

Key points: The data store is only accessible from University of Sydney networked computers. CHW networked computers have no access. All microscope computers are on the University of Sydney network.

Housekeeping rules

1. This store is for the storage of imaging files, and intermediate files created during image processing and analysis.

2. The University specifically requests that only images and related data files be stored on the system. Other types of files will be removed if they are found

3. Feel free to create one personal folder per microscope you use. Please create folders with your full name.

4. For the CLEM Suite alone (SP5 confocal) subfolders are first organised into individual KRI, WMI and CMRI folders.

5. Files stored on the system are automatically backed up overnight by the University of Sydney, thus ensuring the safety of your data

5. As the notes say at the subfolder level, if you create folders outside the already set up institute or microscope folders, they will not receive the automated back-up protection provided by the university.

How to connect from your University of Sydney networked computer

Before connecting to the server for the first time, you should first resynchronise your Unikey password at the address: https://www.auth.usyd.edu.au/extro/pwresync/

Note: Windows server supports file paths of up to 256 characters long (ie. the length of "C:\Path\to\the\folder\and\myfile.txt"). It is therefore possible to run into problems when

copying files over to the server because either the source or destination paths exceed this length. Shorten the folder and file names if this problem occurs.

Connecting with a USyd networked Windows PC

1. Open any folder on your computer. Got to the Tools menu and select "Map Network Drive". A dialog box appears.

2. Pick any drive letter you like from the Drive dropbox. Where it says folder please insert the following text:
\\ssri-fs-pro-5.mcs.usyd.edu.au\Cantrill

WINDOWS XP

3a. (Windows XP). Click the "different user name" text in the line "Connecting using a different user name". Another dialog box appears.

4a. (Windows XP). Enter "mcs\<unikey>" in the user name field (without quotes) and your Unikey password in the password field. For example, "mcs\lcantrill". Click OK to return to the first dialog box.

5a. (Windows XP). Click the "Finish" button. The dialog box disappears and the networked folder should appear, showing the six subfolders.

WINDOWS 7

3b. (Windows 7). Tick the "connection different credentials" option in the dialog box. Another dialog box appears.

4b. (Windows 7). Enter "mcs\<unikey>" in the user name field (without quotes) and your Unikey password in the password field. For example, "mcs\lcantrill".

5b. (Windows 7). Click "Finish" and the networked folder should appear showing the six subfolders.

Connecting with a USyd networked Mac

1. Whilst "in" the Finder, select "Connect to Server" from the Go menu. A dialog box appears.

2. In the text box at the top, type in the following text:

smb://ssri-fs-pro-1.mcs.usyd.edu.au/cantrill

3. Click the "+" button to add this entry to the list for quick "reuse" in the future.

4. Click the Connect button. Another dialog box should appear. Enter the following information:
Workgroup/Domain: MCS

Username: <unikey>
Password: your Unikey password

You may wish to tick the "Add to Keychain" checkbox for easier use in the future.

5. Click OK. The folder should open and be accessible from the desktop with a disk-style icon.