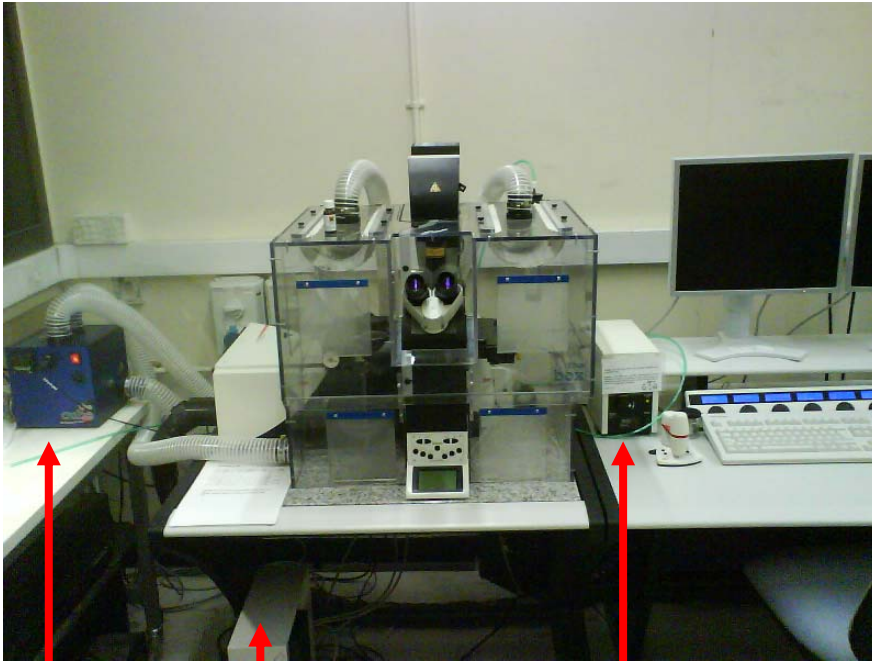


Guide to Confocal 5

Remember that when booking and before starting session you can check for any problems at <https://www.bris.ac.uk/biochemistry/uobonly/cif/index.html>

Starting session

- Switch on microscope – box under the table
- Switch on fluorescent lamp – check that power is on 1st notch (at least)
- Box to the right of monitors: switch on scan electronics button, fan and turn key to vertical position. The PC is always left on.



Heater

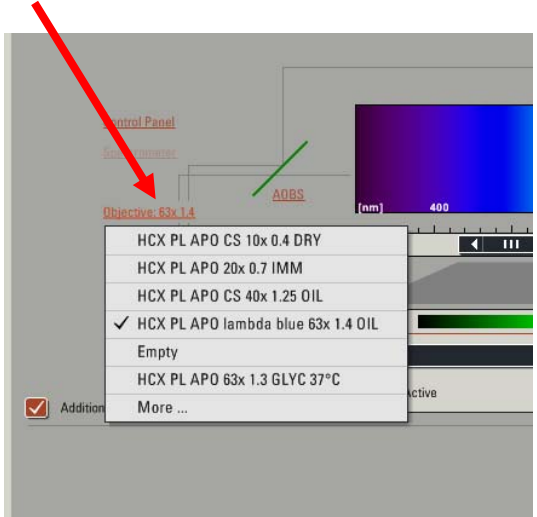
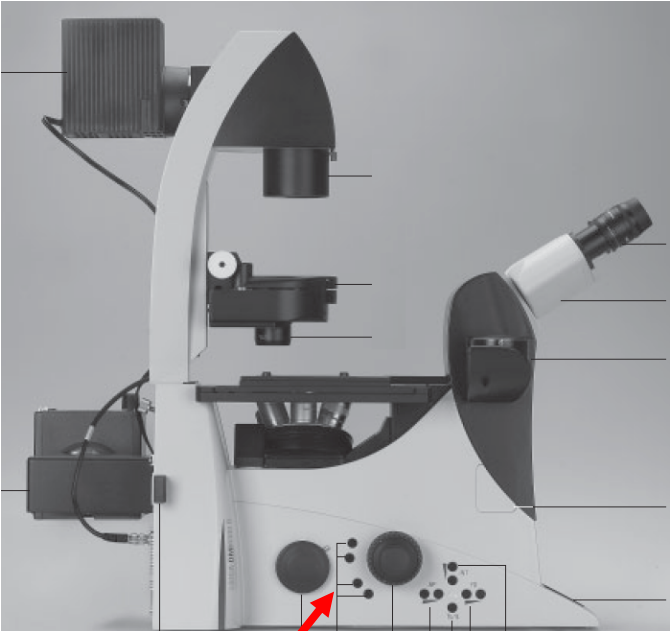
Microscope power

Fluorescent lamp



Using the microscope

- Very Important** – to avoid scratching lenses on the stage when changing lens position, lower the lenses down to bottom with the focus knob before turning the turret. Alternatively you can change the lens through the software which will lower and change the lens position for you



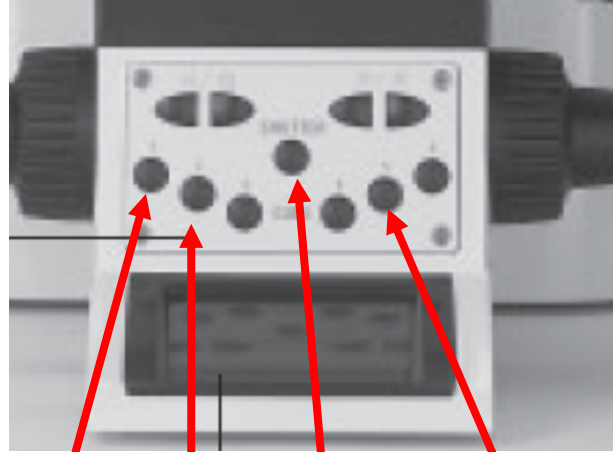
- Switch to brightfield. Press again for Phase Contrast then DIC.
- Switch to fluorescence
- Toggles between fluorescent filter cubes
- Toggles between microscope and scan

Change intensity of fluorescence or brightfield lamp – depending on what mode is in use

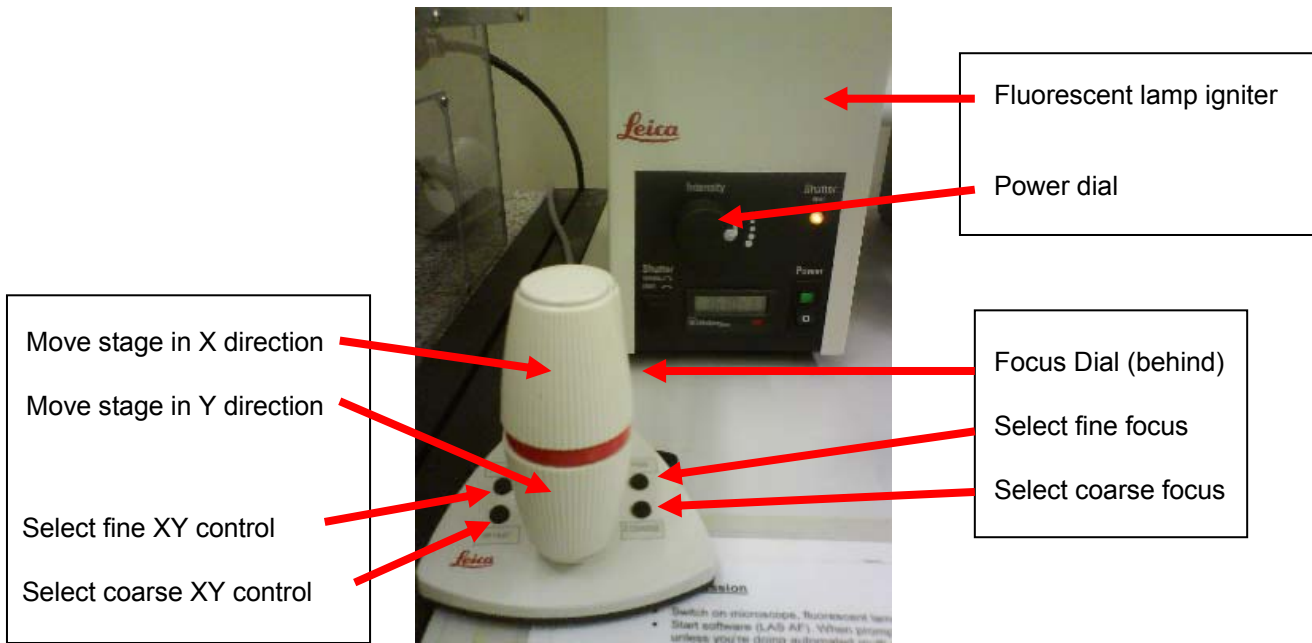
Adjust field diaphragm of brightfield lamp

Adjust field diaphragm of fluo lamp

Toggles between Fluorescence And brightfield modes



- FITC filter cube
- TRITC filter cube
- Shutter
- DAPI filter cube



Notes on using the microscope

- The stage of the microscope is fitted with a universal specimen holder – adjust the sliders (gently) to the appropriate width.
- The 63x lens has an adjustable aperture on its collar. It's generally best to have this fully open (turned clockwise).
- Clean oil lenses with lens tissue before you start, then apply a small drop of immersion oil, preferably to coverslip than to lens to avoid over-doing it.
- If looking at microscope slides, invert the slide so the coverslip is on the underside.
- If using an oil lens, bring lens up until the lens touches oil (or oil touches coverslip if oil on lens).
- Get desired cells in focus and in the middle of the field of view before scanning.
- Avoid exposing your cells unnecessarily to fluorescent light (close shutter asap). Photodamage can also be reduced by reducing lamp intensity.
- Fluorescent lamp intensity can be increased if fluorescence is weak using the dial on the lamp igniter box.
- Heating control – cube next to microscope. Controls on front can change desired temperature within plastic box. Heater should be switched on at least an hour (preferably much more) before experiment to allow temperature to stabilize.

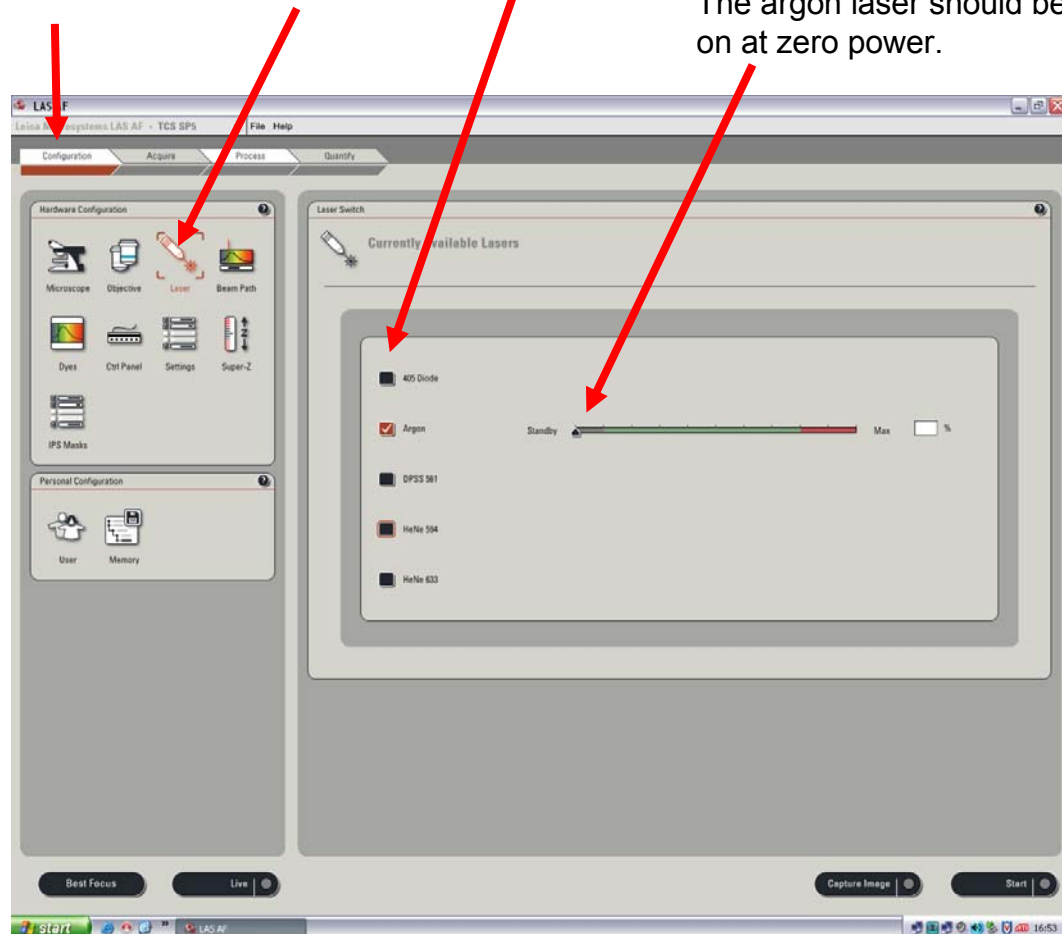
Leica LAS AF software

- Double click Leica LAS AF icon on desktop to start.
- When prompted to initialize stage, click no (unless you're doing automated multi-position imaging)
- Once software is started, go to configuration, then lasers. Ignite the lasers you need by ticking the boxes.
- Note that the Argon laser (488 & 458/476/514nm) should be ignited at zero power and is then generally set to 20%.

To switch on/off lasers, go to Configuration and select "lasers"

Tick the boxes of the lasers that you need

The argon laser should be switched on at zero power.



General software interface

Fig. 1

The screenshot shows the Leica LAS AF software interface with the following components and annotations:

- View experiment folder:** Points to the 'Experiments' tab in the top left.
- Activates sequential scan setup:** Points to the 'seq.' icon in the 'Acquisition Mode' section.
- Change Scan mode:** Points to the 'xyz' dropdown menu in the 'Acquisition Mode' section.
- General scan settings:** Points to the 'Acquisition' panel on the left, which includes settings for XY, Format, Speed, Phase correction, Zoom factor, Image Size, Pixel Size, Line Average, Frame Average, and Rotation.
- Selecting a fluorphore from the list to brings up an emission spectra for reference only:** Points to the 'Visible' emission spectrum graph in the 'Beam Path Settings' section.
- Select method:** Points to the 'Leica Settings' dropdown menu in the 'Beam Path Settings' section.
- LED indicates that laser is active:** Points to the 'UV' and 'Visible' LED status indicators in the 'Beam Path Settings' section.
- AOTF sliders control % transmission of lasers:** Points to the AOTF sliders in the 'Beam Path Settings' section.
- Click here to select desired lens:** Points to the 'Additional Channels' checkbox in the 'Specimen' section.
- Tick box if capturing brightfield images:** Points to the 'Active' checkbox for the 'Brightfield PMT' in the 'PMT' section.
- To scan sample continuously whilst setting PMT gains, laser power, focus etc...:** Points to the 'Live' button at the bottom of the interface.
- Fluorescence PMTs:** Points to the 'PMT 1' and 'PMT 2' sections in the 'PMT' section.
- Brightfield PMT:** Points to the 'PMT 3' section in the 'PMT' section.
- To capture series of images (in Z, T or λ):** Points to the 'Start' button at the bottom of the interface.

Basic image set up and capture

- Ensure the top of the microscope is in the upright position (90° vertical).
- In the software, select a method from list by double clicking
- Check general scan settings:
- Scan mode (default is xyz), image format (default is 512x512), scan speed (default is 400hz)
- Usually advantageous to tick [Bidirectional X] which doubles scan speed. (default position is 35.22 and should not be altered)
- Click [LIVE] – sample is scanned continuously allowing you to see the changes to the image as you adjust the following main controls:
 - Z position (focus) dial
 - PMT gain (max 1250v, ideally <900v to reduce noise – values given on display above Smart Gain dial)
 - Laser power (using AOTF sliders and for the 488 laser the power slider in the laser configuration window)
 - Zoom (type value required, move slider or tick [Zoom in], select rectangle tool and draw box on image)
- Adjust these controls quickly to reduce sample fading and then click [STOP]
- Select number of averages – typically 3-4. (NB select either line or frame average – the result will be the same for stationary cells but line averaging (or no averaging) preferable for most live cell imaging).
- Click [Capture Image] to capture single image.

Notes

- Increase format (> 512x512) and/or zoom to optimise XY sampling (pixels 70-100nm, no point going smaller) for best resolution.
- You can decrease the Smart offset to make the background blacker. If required – use carefully as negative offset may subtract real signal as well as the background.
- Pinhole should normally be left at its optimum size (1 Airy unit) but can be increased (e.g. to 2-3) to increase signal (with small loss of z-resolution).
- Use [QLUT] next to image window to help judge optimum brightness – pixels will turn blue on saturation and green at zero.
- The standard beam path methods may not be ideal for your sample Double click the PMT slider to specify the emission wavelengths that you want to detect (emission range should start at least 10nm higher than the excitation wavelength).
- If you have more than 1 fluorophore it's usually advisable to use the sequential scanning mode (see next page)



Smart
Gain

Smart
offset

Field
Rotation

Pinhole

Soft
Zoom

Z position

Sequential Scanning set up – for multi channel imaging

- 1) Click [seq] button and the sequential scan setup window will appear
- 2) “Scan 1” is highlighted. Define settings for your 1st fluorophore (which laser is active, which PMT is active and the emission wavelengths you want to detect). These settings will be saved inside “scan 1”.
- 3) Click on the + sign and “scan 2” will appear and will be highlighted. Define settings for your 2nd fluorophore (note you must use a different PMT for each fluorophore if ‘between lines’ selected – the fastest option).
- 4) Click + again and repeat if you have 3 or more fluorophores.
- 5) You can save these settings and then load them up next time by clicking [save]
- 6) Click [Live]. The software will scan your sample sequentially, switching between your scan channels after every line in your image – allowing you to view and optimize all the channels at the same time:

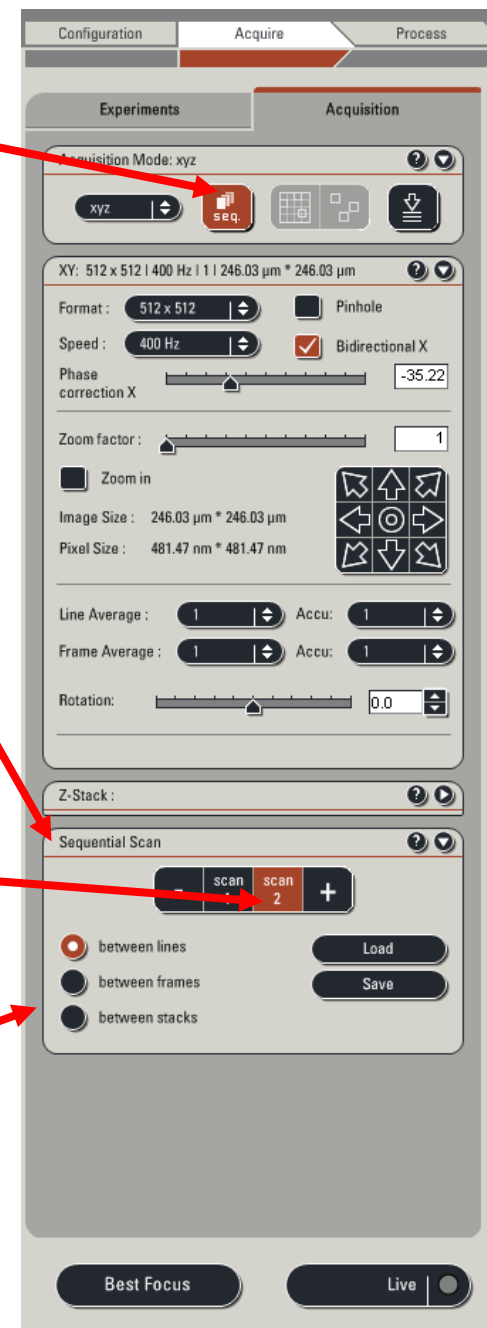
In your image window, click on the channel you want to adjust. The “smart gain” and “smart offset” dials will make their adjustments on the channel selected.

To alter the laser power for a channel, 1st click on the channel in the sequential scan window, then change the power using the slider (don't change a laser power before selecting the appropriate channel)

- 7) When all the channels are set up, stop the live scan then click [Start] to capture your images.

Notes

- Remember to select a method (scan 1, scan 2 etc..) before altering the laser power for that method.
- If using the 405 laser for one of your channels, turn the laser power down to 0 for other channels but leave the laser on (so that the LED is still on [see Fig 1])
- The maximum number of fluorescent channels that can be captured using the “between lines” mode is 3. If more are required, select “between frames” sequential mode. Here you view 1 channel at a time whilst adjusting the settings with the [live] scan. Click [start] to capture all the channels.



Setting up a Z-stack

1) Open up Z-stack window by clicking arrow top right

2) Set upper and lower limits of the z position for your sample:

- Click [Live] and adjust the z position dial until you reach one extreme. Click [Begin].
- The computer records this position and highlights the starting point.
- Then focus through in the other direction until the other extreme. Click [end].
- Then click [Stop]

3) Define the size (better standardisation between stacks) or number of steps you want to take. Note that the “system optimised” value is the optimum step size to maximise Z-resolution with lens you are using – this is likely to be OTT for most applications except for best quality 3D data. Step size 0.5-1.0 micron more commonly used, depending on data required and depth of specimen.

4) Click [Start] to capture the stack

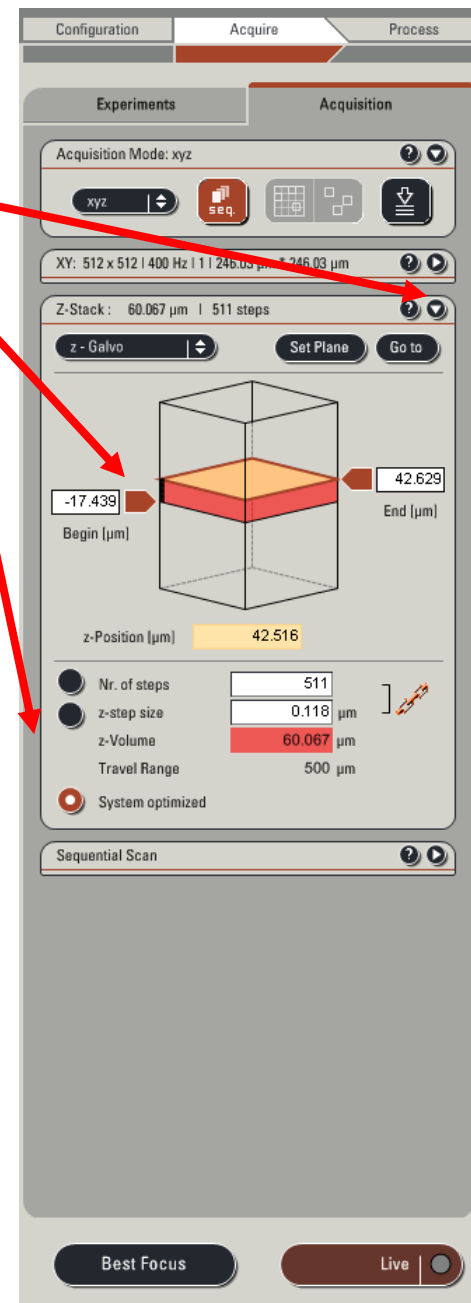
Notes

• Photobleaching is more likely to occur when capturing large stacks of data compared with single images. If this is a problem, try:

- Using a lower laser power and higher gain
- Taking fewer sections
- Using fewer averages

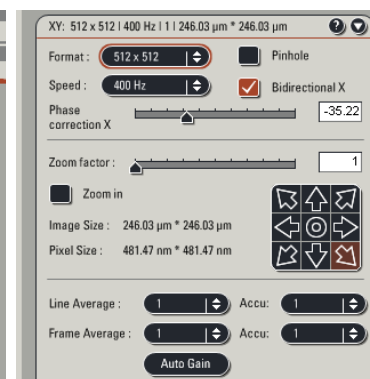
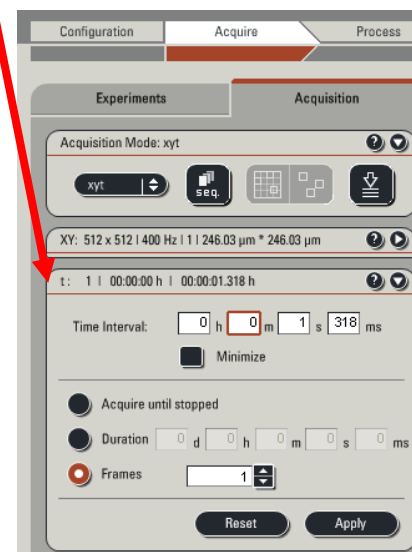
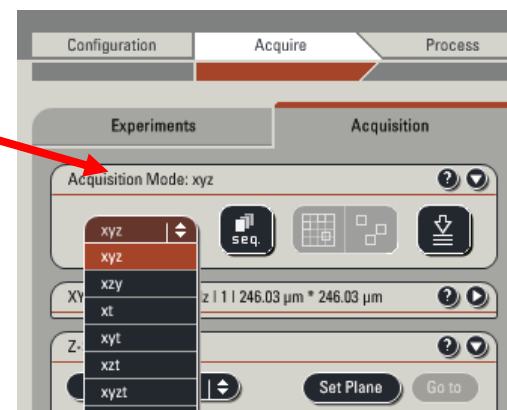
• As a rough guide, to get a decent set of data for 3D reconstruction, you typically need at least 20 sections and 0.5 micron steps or less for relatively thin samples.

• Brightness levels of your fluorophore may be different at different Z positions. When adjusting laser powers and gains, bear this in mind – a good setting at one z position may oversaturate the PMT at another.



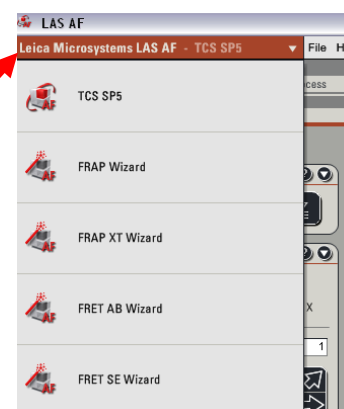
Timecourses

1. Select the appropriate acquisition mode from the drop down list
XYT for capturing images at a single plane of focus over time.
XYZT for capturing a z-stack over time
2. A new dialogue box appears in which you can define the timecourse
 - o Time interval – time between timepoints
 - o Ticking [Minimize] will give the minimum time interval at the current settings taking into account the scan speed, scan resolution, number of averages etc...
 - o Number of frames or duration of experiment or whether to continue until stopped
3. Click [Apply]
4. In the XY dialogue box set the number of averages (best to use Line averages with live cells)
5. Click [Start] to begin capturing timecourse



Notes

- Generally use a lower laser power, less averaging and consider opening pinhole for live cell imaging to limit photo-damage.
- To increase the speed of acquisition you can alter these settings in the XY dialogue box:
 - o Bidirectional scanning (doubles speed. No effect on image quality).
 - o Use less averages.
 - o Change resolution (e.g. 512 x 256 pixel resolution will double speed).
 - o Change scan speed (faster speeds will decrease quality).
- Multi stage timecourses can be set up using the FRAP wizard.
- FRAP and FRET wizards are available from a drop down menu.
- Use the Environmental chamber heater if you want your experiments to run at 37C the heater should be switched on at least 1 hour (preferably much longer) prior to use to allow the system to stabilize temperature.



Transmitted light images

You can capture transmitted light images (brightfield, phase contrast or DIC) simultaneously as you capture fluorescence images. Often the microscope will be already set up correctly but if in doubt follow these steps:

1) Check that the microscope is set up correctly for viewing transmitted light.

- Get your sample into focus
- Push the top button behind the focus wheel on the left to view sample with brightfield. Push button again to go to phase contrast mode, again to go to DIC mode. Note that the current mode will be displayed on the front display on the microscope and if the mode is not possible with the current lens the the display will flash.

Note that:

63x lenses can do DIC but can't do phase contrast

40x lens can't do DIC but can do phase contrast.

20x and 10x can only do brightfield

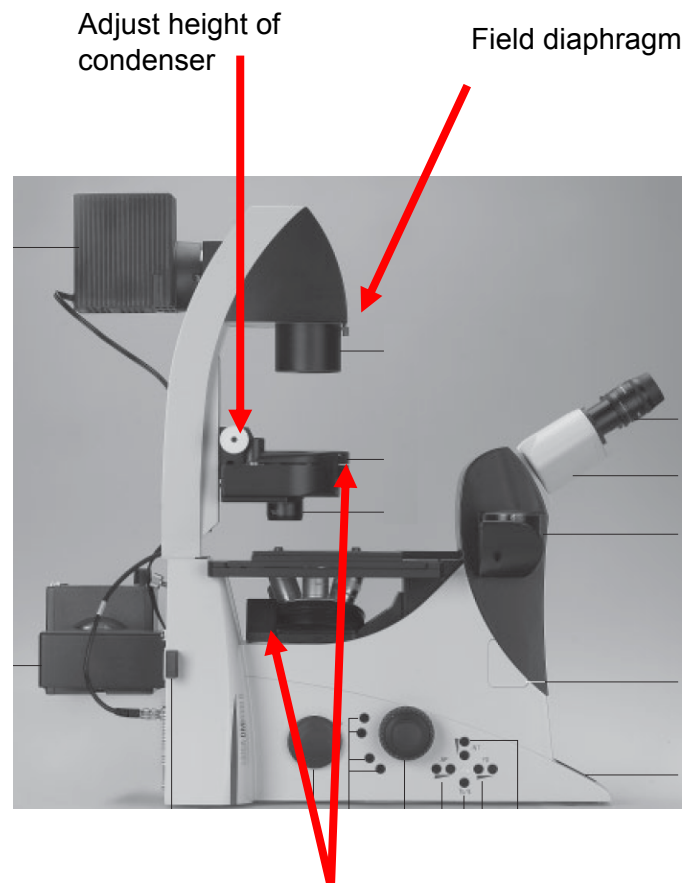
- Check aperture diaphragm is open (above 10 on display) using AP buttons on left side
- Check the condenser position is correct:

Fully close field diaphragm (see figure), look down eyepieces and adjust height of the condenser until octagon of light comes into sharp focus. Center the light if necessary using the pull out screw adjusters (don't use these until you can see the outline of the octagon).

- Open field diaphragm

2) Set up the software for capturing transmitted light images by adjusting one of your fluorescence methods so that transmitted light images will be captured simultaneously using laser light.

- Activate the tick box "additional channels"
- Tick the box for PMT Trans and select the appropriate mode (BF, Phase contrast, DIC)
- Scan sample. Increase gain and decrease the offset to increase image contrast



Dials for adjusting light polarizers for DIC imaging

Saving images

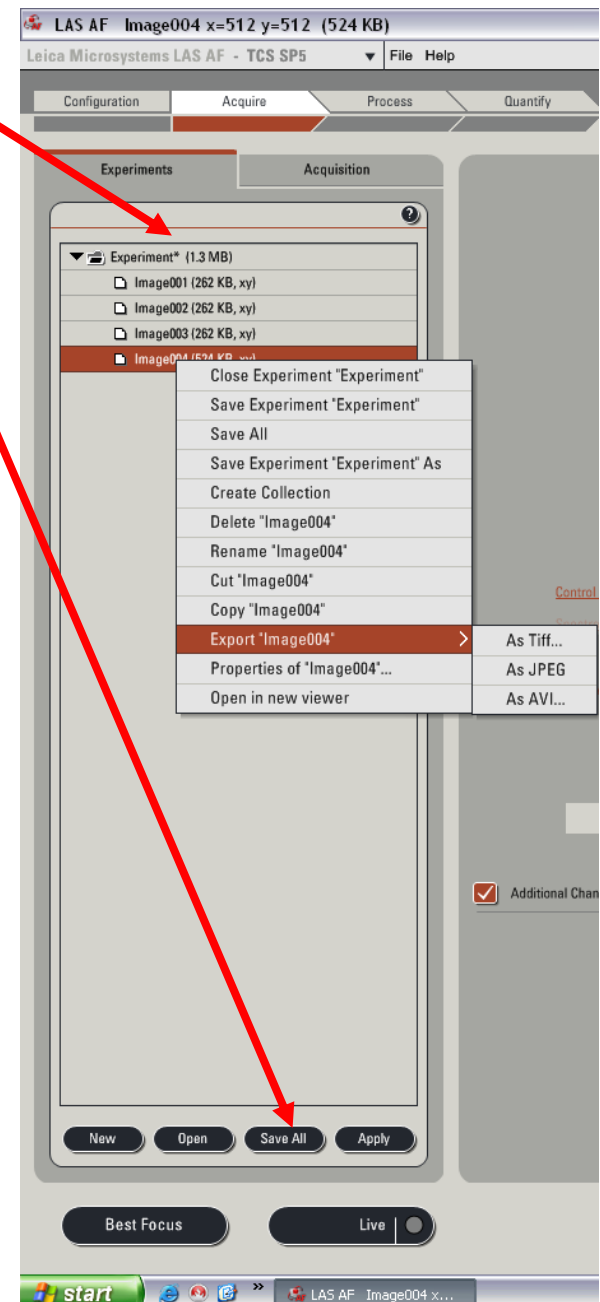
- Each single scan or series scan that you do sends a file to the experiment folder. These files are held in RAM until you save them to the hard disk. Click [Save all] to save the experiment folder and all of the images within it.
- The first time you click save you'll be prompted to give the experiment folder a name and location. Save your images in your own directory within the "users" directory on the C-drive.
- Unlike with the older Leica confocal software, images are not saved as tifs by default. Instead the LAS AF software will save a single .lif file for all the images within an experiment folder. An individual image or series can be exported as tifs by right clicking on the file in the experiment folder....export.
- Overlays and projections need to be saved by right clicking on the image...Snapshot. These can also be exported as tif in the same way as above.

Data on our computers should not be considered secure and should be backed up as soon as possible. Old data is also regularly deleted from our computers at the end of each month.

The users directory is shared and can be contacted over the network from your own computer:

Using a pc: go to the 'start' menu....run
enter "\\ip address of confocal 5"

Using a mac: go to 'go' menu....connect to server
enter smb:"ip address of confocal 5"



Ending session

- Clean oil off lenses with lens tissue.
- If doing live cell imaging, dampen lens tissue with 100% ethanol, clean lens, then dry lens with a dry piece of lens tissue.
- Clean other used surfaces with 75% ethanol
- Fill in log book
- Check Calendar (<http://calendar.bris.ac.uk>) selecting resources then bioc-confocal 5 to see if system is booked after you:

If someone else is booked on within next 3 hours:

- Go to Laser configuration window:
- Turn power on Argon laser down to 0% but leave box ticked
- Untick other lasers
- Fluorescent lamp can be switched off if there is a small gap (>5 minutes) before next booking
- Leave everything else on

If there is no booking within the next 3 hours:

- Go to laser configuration window:
- Turn down power on the Argon laser to 0% and untick box.
- Untick all other laser boxes
- Turn key off (box on right of table) and leave fan running for at least 5 minutes
- Switch off scan electronics button, exit software and switch off microscope.