

# Guide to Confocal 8

## Starting session

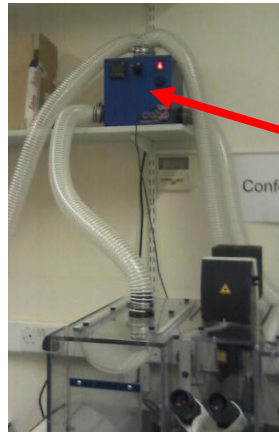
1. Switch on microscope – box under microscope table.
2. Switch on fluorescent lamp – check that power dial is turned on.
3. Controls to the right of monitors: switch on scan electronics button, switch on fan for lasers and turn key to vertical position to switch lasers on. Check that the orange light above the key comes on.

The PC is always left on.



1. Microscope power

2. Fluorescent lamp



3.

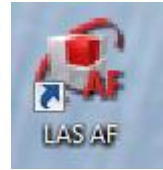


Heater – if required press orange switch to turn on. Heater is on when the green numbers are illuminated. Change desired temperature with controls on front. Confocal 8 is generally kept close to room temperature (22-27°C). Allow time for the temperature to stabilize if warming. If you want to run an experiment at higher temperatures such as 37°C please use Confocal 9 or one of the other confocals.

## Starting software:

Remember to make sure scan electronics is switched on **before** opening software.

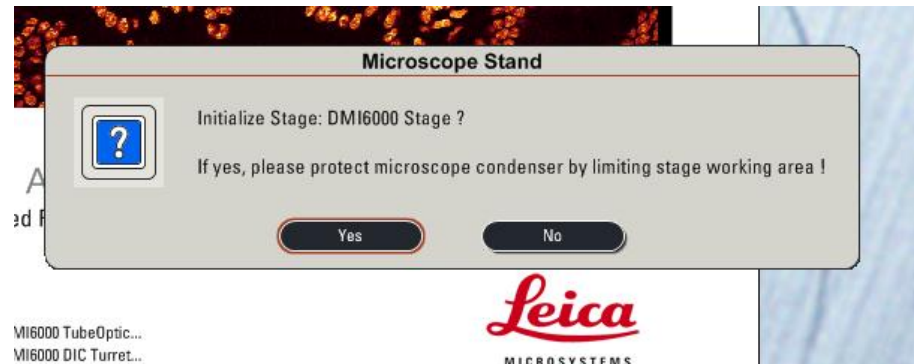
To open software click on LAS AF icon on desktop



When prompted **do not** select “Activate Resonant Scanner” unless you require extremely high speed imaging..



If you want to do a tile scan or “mark and find” multisite imaging you will need to initialise the stage. Before clicking “Yes”, make sure nothing is on the microscope stage that can come into contact with the condenser as the stage moves during the initialisation.

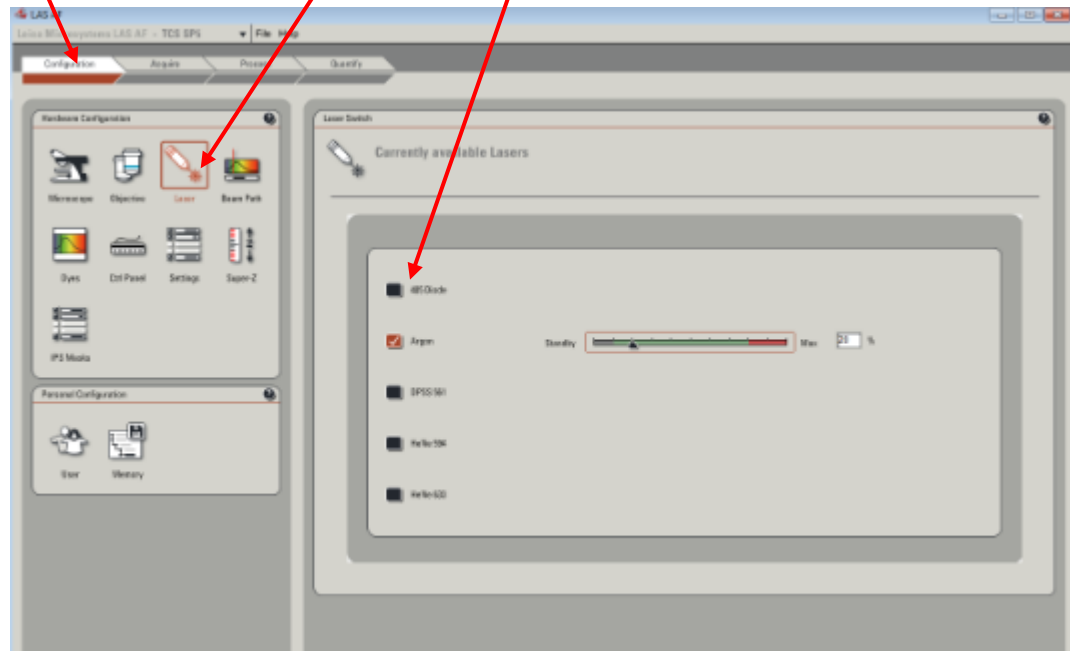


## Leica LAS AF software

- Once software is started, go to **configuration**, then **lasers**. Turn on the lasers you need by ticking the appropriate boxes.
- Turn up the power on the Argon laser (488nm) to 20% after ignition

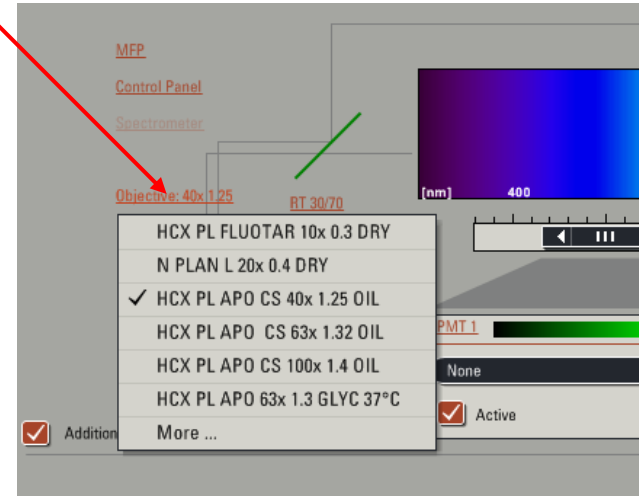
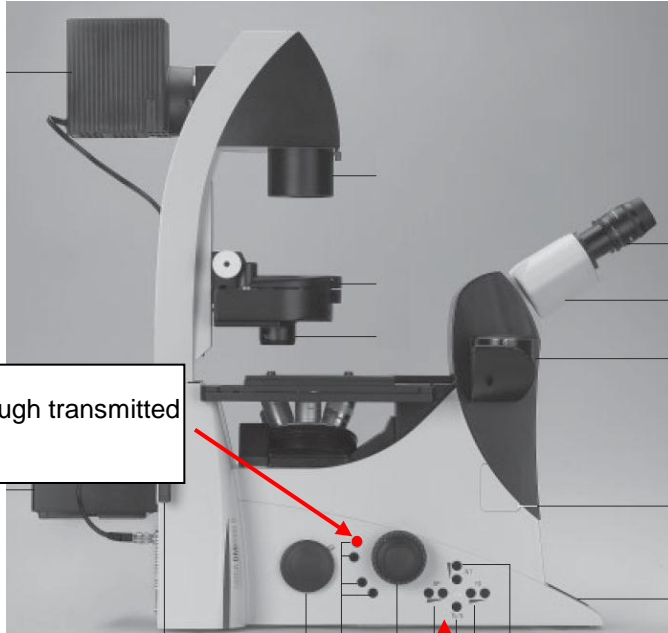
To switch on/off lasers, go to **Configuration** and select **Lasers**

Tick the boxes of the lasers that you need



## Using the microscope

- **Very Important** – always select or change objective lens through the software. Do not change lens by hand as this can damage them by scraping the underside of the stage.



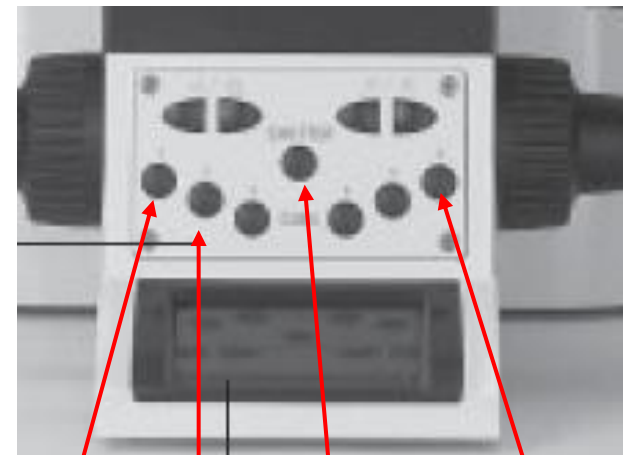
• Toggles through transmitted light modes.

**INT**- changes intensity of fluorescent or brightfield lamp – depending on what mode is in use

**AP**- adjusts field diaphragm of fluorescent lamp

**FD**-adjusts field diaphragm of brightfield lamp

**TL/IL** -toggles between fluorescence and brightfield modes

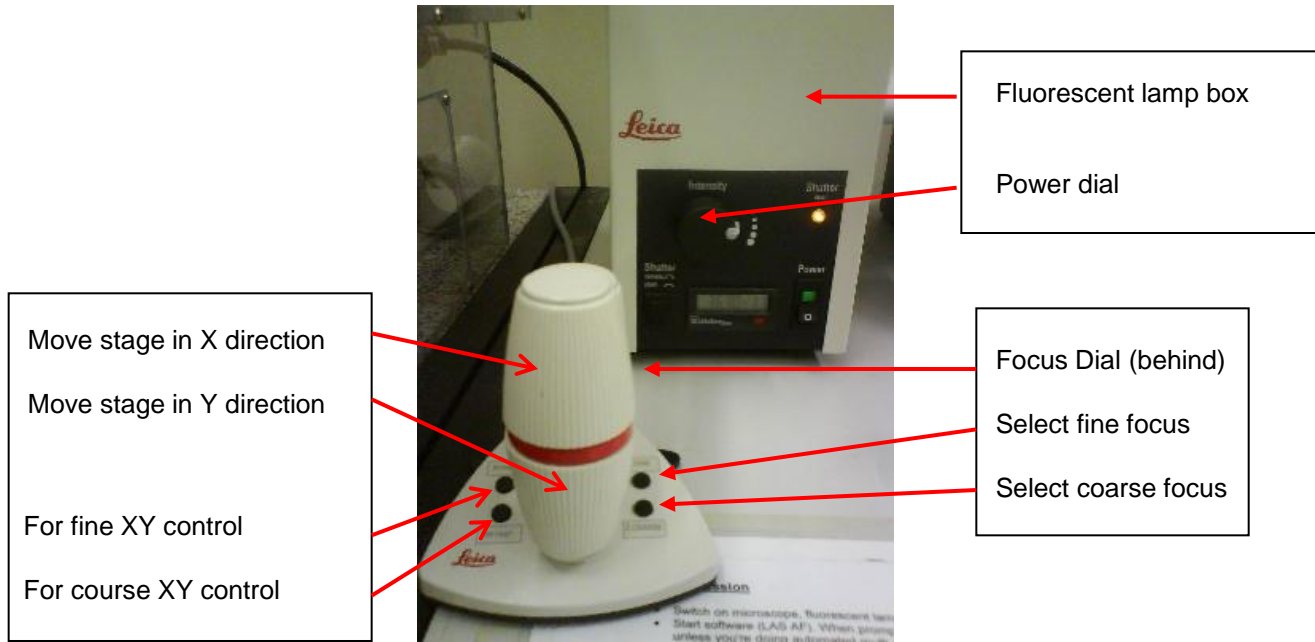


FITC filter cube

TRITC filter cube

Shutter

DAPI filter cube



Move stage in X direction

Move stage in Y direction

For fine XY control

For course XY control

Fluorescent lamp box

Power dial

Focus Dial (behind)

Select fine focus

Select coarse focus

## Notes on using the microscope

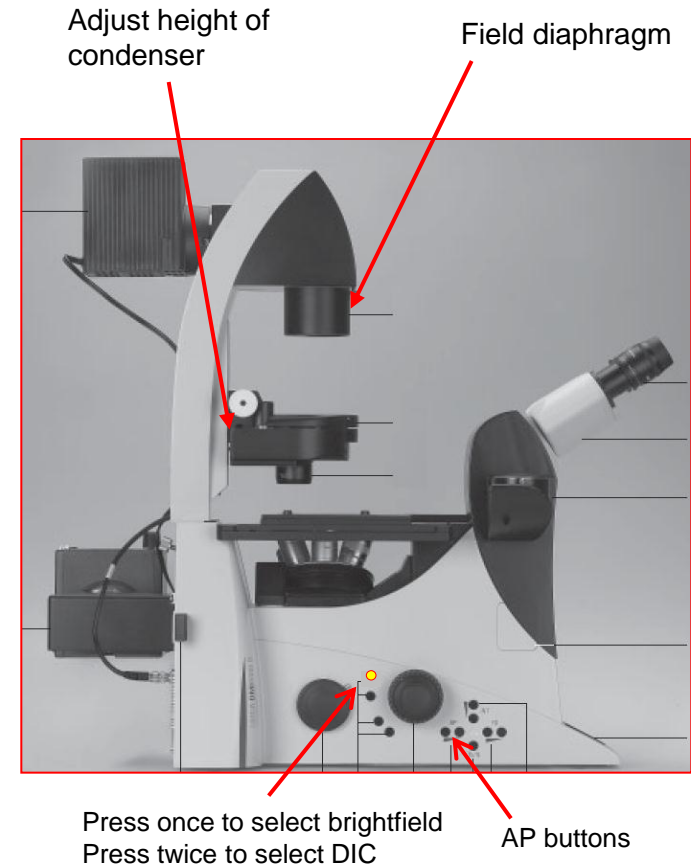
- The microscope stage is fitted with a universal specimen holder – adjust the sliders (gently) to the appropriate width.
- Check sample is clean. Clean with tissue and 75% ethanol if necessary.
- Check lens is clean by wiping with non abrasive lens tissue, check the spring loaded top is fully up, check adjustable aperture dial is fully open (on some lenses).
- Select the correct immersion liquid (type F oil or glycerine) and apply a small drop to lens or sample
- 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.
- 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 box.

## Transmitted light

You can capture transmitted light images (brightfield or DIC) simultaneously with fluorescence images. Note that **none** of the lenses on confocal 8 are phase lenses. Often the microscope will be already set up correctly but if in doubt follow these steps:

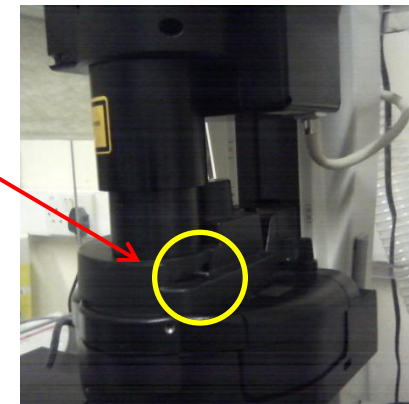
Check that the microscope is set up correctly for viewing transmitted light:

- Get your sample into focus
- Push the top button behind the focus wheel on the left to view sample with brightfield. Press the upper button behind the focus wheel twice for DIC. 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 display will flash.
- Check aperture diaphragm is open 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.
  - Centre 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



### **For DIC:**

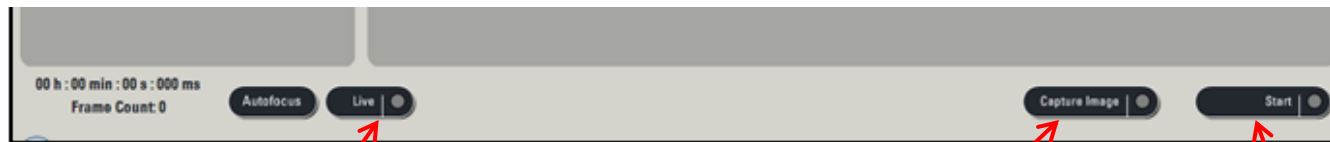
When you select DIC a small metal bar will appear on the right hand side of the condenser. Move this bar from side to side whilst looking down the eyepieces to obtain the best DIC Image  
Ensure that there is no plastic in the light path as this will negatively affect your DIC image.



## General acquisition controls

1. View experiment folder – and open new experiment
2. In Acquisition Folder - Select scan mode
3. Activate sequential scan
4. Select tile scan or “Mark and Find” – only active if you have initialised stage when starting up software. Not available if greyed out.
5. Bidirectional scanning – effectively doubles scan speed. Make sure phase correction X is set at -33.64
6. General scan settings – minimum recommended pixel size 80nm
7. Zoom control – can either select “Zoom in” and draw ROI on screen or type in required zoom factor.
8. When zoomed can use these controls to position the image.
9. Line/Frame Average – useful for reducing background noise
10. Z-stack controls –see later for detail
11. Sequential scan controls –see later for details.

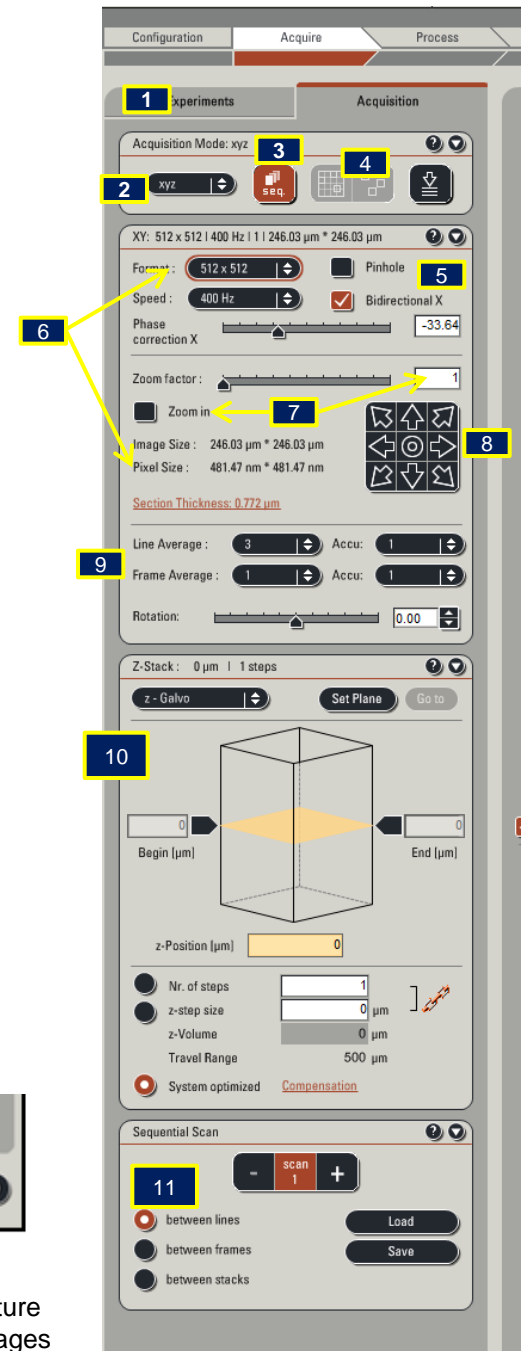
### Controls along the bottom of the screen:



Click to scan continuously while setting PMT gains, laser power, focus etc.....

Click to capture a single image

Click to capture series of images (in Z, T or  $\lambda$ )





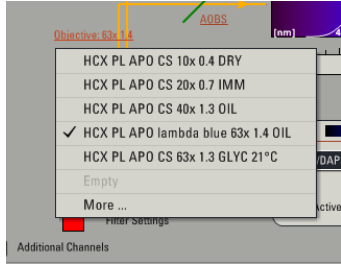
# Beam Path Settings

AOTF sliders control % transmission of lasers. **NB** the hybrid detectors are very sensitive and so need only a **very low** laser power (usually  $\leq 10\%$ ) Setting the laser power too high will cause the detector to shut off and may damage the system.

Select scan setting (non sequential settings)

Select to activate lasers

More important info about hybrid detectors on next page .....



Click here to select and change lens

Notch filters suppress reflection from lasers (automatically selected).

Selecting a fluorophore from this list brings up its emission spectrum for reference

Tick box to capture brightfield images (Scan-BF or Scan TL-DIC)

The interface includes several key sections:

- Load/Save single setting:** Includes a dropdown for 'Leica Settings', 'Delete', and 'Save' buttons.
- ROI Scan:** Includes checkboxes for 'ROI', 'Bleach Point', and 'Set Background'.
- Laser Activation:** 'UV' and 'Visible' sections with red indicator lights and a '10%' power slider.
- Laser Power Sliders:** Seven individual sliders for different laser lines (405, 458, 476, 488, 496, 514, 561, 594, 633 nm) with '0%' and '10%' markers.
- Spectrometer:** A color-coded emission spectrum plot with a wavelength scale from 400 to 800 nm.
- Specimen:** Includes 'Adaptive Focus Control' (AFC) with 'AFC' and 'Pos.' radio buttons, and a 'Filter Settings' section with a red square icon.
- Additional Channels:** A checked checkbox at the bottom left.
- Detector Settings:** Five detector channels (PMT 1-5) with dropdown menus for filter types (e.g., 'Leica/DAPI', 'Leica/ALEXA 4') and 'Active' checkboxes.
- PMT Trans:** A 'Scan-BF' dropdown and an 'Active' checkbox at the bottom right.

Double click on the detector's slider to define exact emission wavelengths to detect

**Fluorescent PMTs**  
**Hybrid Detectors**  
**Brightfield PMT**

Tick box to activate detector (PMT or HyD)



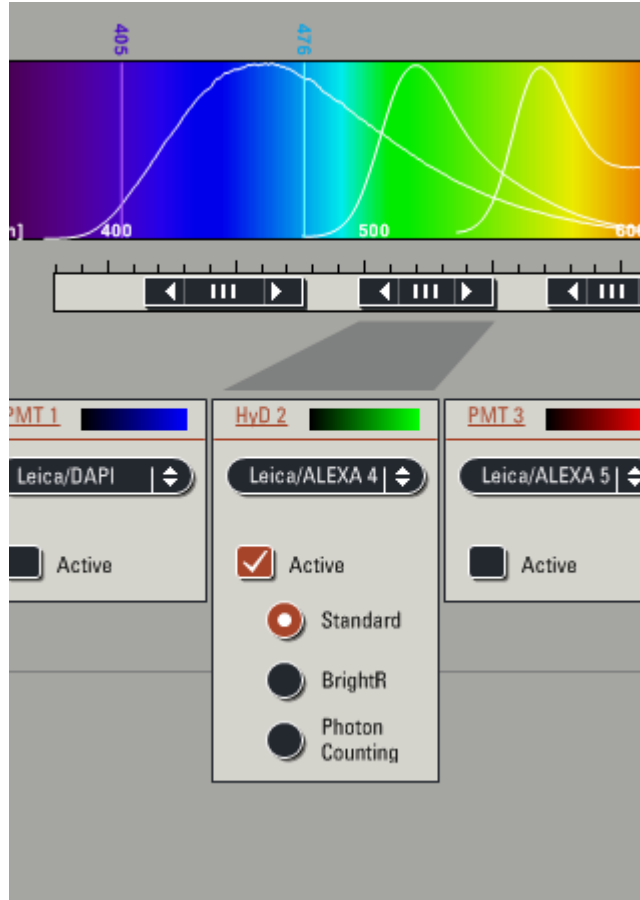
## Hybrid detectors – greater sensitivity and lower noise

There are 3 options when a hybrid detector is selected:

Standard – for most applications

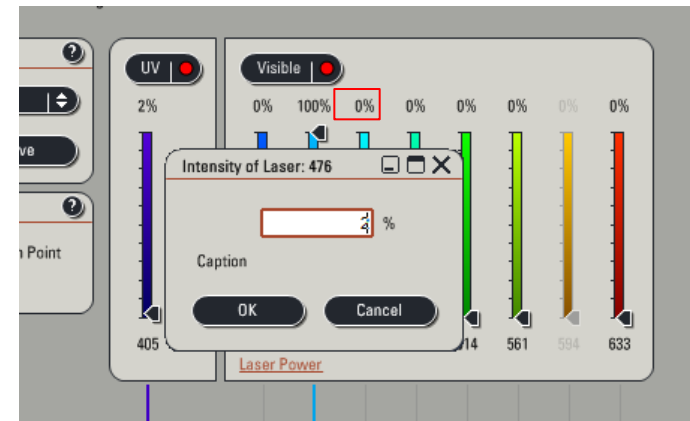
Bright R – can increase dynamic range by amplifying dim signals more than bright ones.

Photon Counting – allows quantitative analysis of photons



Always start scanning with a low laser power (<5%) to avoid hitting detector with too much light. If there is a lot of saturation, the detector can be damaged and the software will shut it off.

It is easier to set the laser power by clicking on % power and then typing in required value in box.



Hybrid detector gain is given as a percentage and smart offset is unavailable.



## Basic image optimisation and capture

- Ensure the top of the microscope is in the upright position (90° vertical).
- Select or define a scan setting.
- Check general scan settings – in ‘Acquisition’ and ‘XY’ panels which should be :
  - Scan mode (default is xyz), image format (default is 512x512), scan speed (default is 400hz), zoom = 1.
  - Usually advantageous to tick [Bidirectional X] which doubles scan speed. (default position is **-33.64** and should not be altered)
  - Check pinhole size – 1 Airy Unit is optimum
  - Click “Live” – sample is scanned continuously allowing you to see changes to the image as you adjust the following main controls:
    - Z position (focus) dial
    - Gain for PMTs - max 1250v, ideally about 700v to reduce noise  
for HyDs - max 500%, ideally  $\leq 100\%$
    - Laser power (using AOTF sliders, or type in required value after right clicking on % power. For the 488 laser can also use the power slider in the laser configuration window)
    - Zoom (either type factor required, move slide, use soft zoom or tick “Zoom in” then 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 2-4. (NB select either line or frame average – the result will be almost the same for fixed cells but line averaging (or no averaging) preferable for most live cell imaging.
- Click [Capture Image] to capture single image.

### Notes:

- Increase format ( $> 512 \times 512$ ) and/or zoom to optimise XY sampling (pixels 70-100nm, no point going smaller) for best resolution.
- When using PMTs you can decrease the smart offset to make the background blacker, but use carefully as negative offset will subtract from 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). NB pinhole adjustment in software needs pinhole box to be ticked.
- Use “QLUT” next to image window to help judge optimum brightness – pixels will turn blue on saturation and green at zero.
- Double click the PMT or HyD slider to specify the emission wavelengths that you want to detect (emission range should start at least 10nm higher than the excitation wavelength to avoid reflection). Selecting an appropriate notch filter will suppress reflection and allow you to go closer to the excitation line.
- If you have more than 1 fluorophore it’s often advisable to use the sequential scanning mode to avoid “crosstalk” (see next page)



Smart  
Gain

Smart  
offset

Field  
Rotation

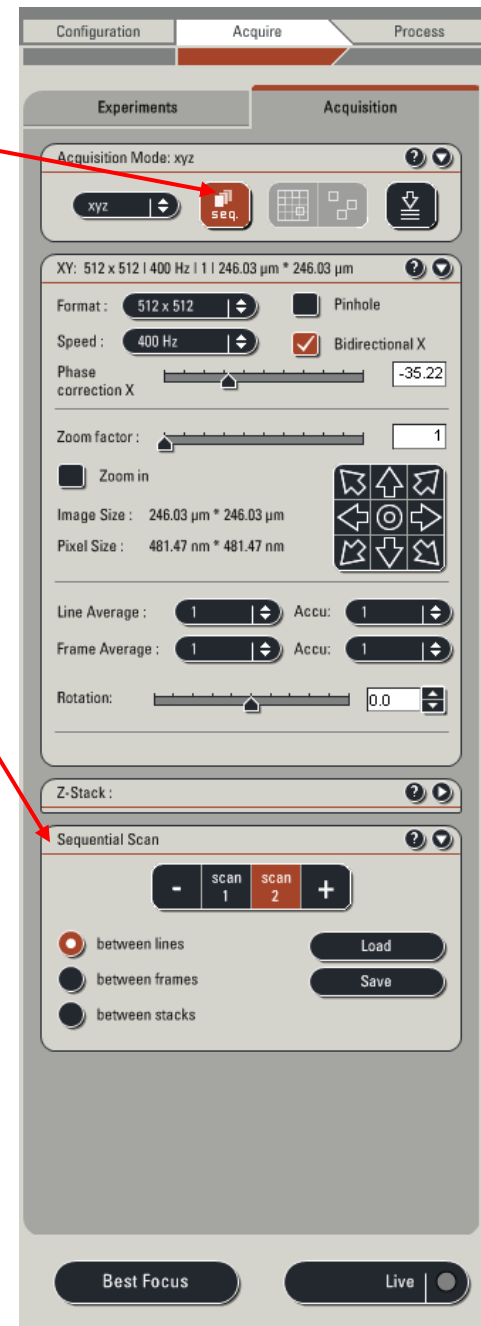
Pinhole

Soft  
Zoom

Z position

## Sequential Scanning set up – for multi-channel imaging

- Click “seq” button and the sequential scan setup window will appear
- The between lines option will be selected by default. This option acquires images with the smallest time delay between channels
- “Scan 1” will be highlighted. Define settings for your 1<sup>st</sup> channel: Select which laser is active, which PMT/HyD is active and the emission wavelengths you want to detect. These settings will be held in “scan 1”.
- Click on the + sign and “Scan 2” will appear. Turn the laser power for your 1<sup>st</sup> channel down to 0 and select the laser for your 2<sup>nd</sup> channel. Deselect the PMT/HyD used in “Scan 1” and activate a new detector and define the emission wavelengths.
- Click + again and repeat if you have 3 or more fluorophores.
- Transmitted light channel can be added to any of the sequential channels – contrast usually better with lower wavelength excitation.
- You can save these settings and then load them up next time by clicking save.
- Click “Live”. The software will start scanning 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, first click on that channel in the sequential scan window, then change the power using the slider (don’t change a laser power before selecting the appropriate channel).
  - Optimise images (see previous page).



## 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. Turning the dial anticlockwise moves to the lowest position.
- Click the Begin arrow to mark this position (arrow will be highlighted).
- Focus through in the other direction until the other extreme. Click the “end” arrow
- Then top the live scan.

3) Click and define Z-step size (better standardisation between stacks) or number of steps.

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.

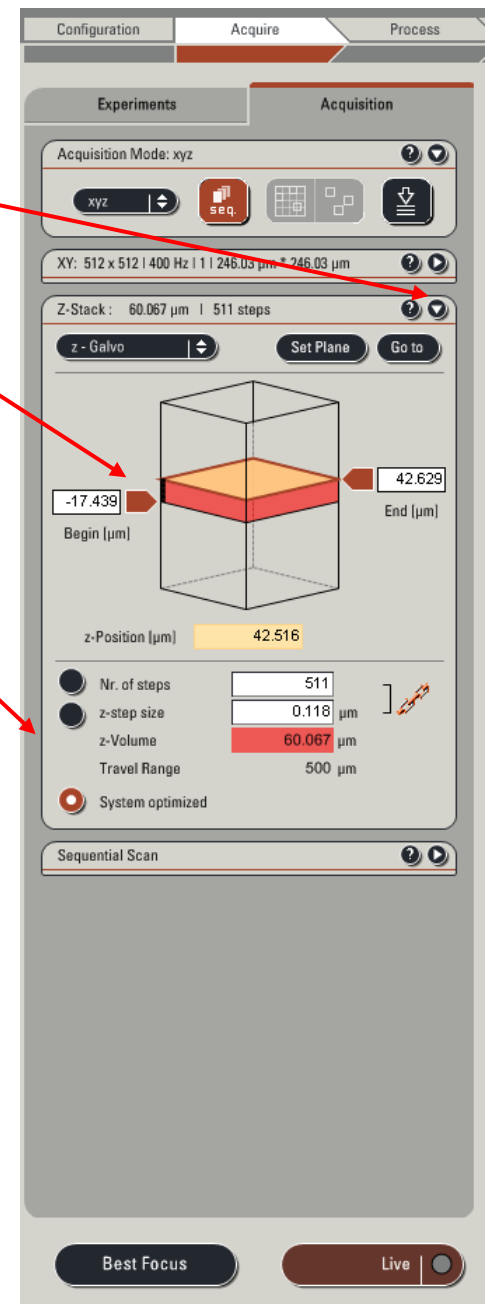
4) Click “Start” to capture the stack

## Notes

- The default focus mechanism is the z-galvo. This is faster than the other option (z-wide) but has a range limit of 500 microns.
- Photobleaching is more likely to occur when capturing large stacks of data compared with single images. If this is a problem, try:

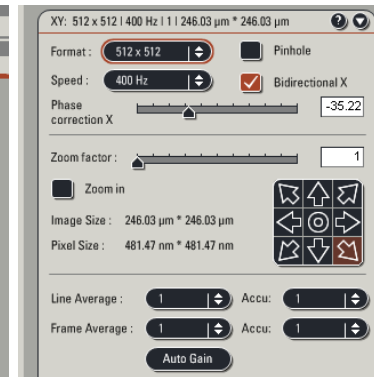
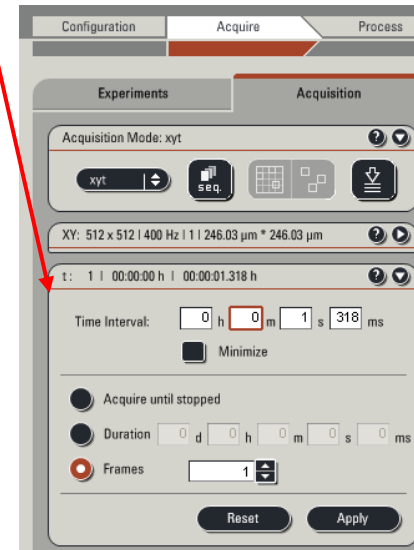
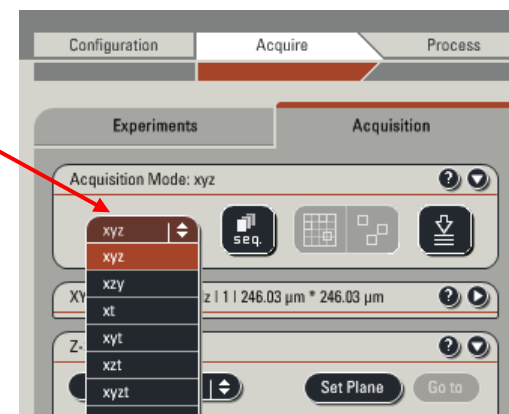
- Using a lower laser power and higher gain
- Taking fewer sections
- Using fewer averages

- 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 smaller 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
  - Time interval – time between acquisition of images
  - Ticking “Minimize” will give the minimum time interval at the current settings taking into account the scan speed, scan resolution, number of averages etc...
  - Define the number of frames, 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 average 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:
  - Bidirectional scanning (doubles speed. No effect on image quality).
  - Use less averages.
  - Change scan format (e.g. 512 x 256 pixel resolution will double speed).
  - Change scan speed (faster speeds will decrease image quality).
- Use the environmental chamber heater if you want your experiments to run at a particular temperature. The heater should be switched on at least 1 hour (preferably much longer) prior to use to allow the system temperature to stabilize.
- Consider using the Adaptive Focus Control to avoid focal drift during your experiment due to thermal expansion / contraction (see next page)


# Adaptive focus control

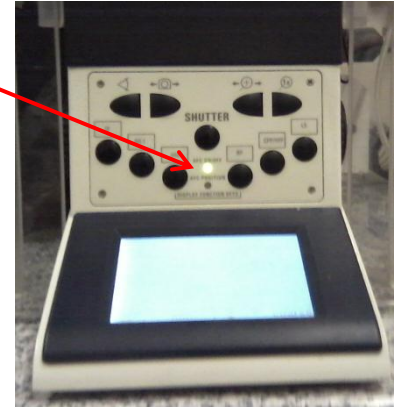
This enables you to select and hold a focus position, which is especially useful during live cell imaging.

Adaptive focus control is only available if the difference between the refractive index of the immersion medium of the objective (oil, glycerol or air) and the medium which the sample is in is great enough.

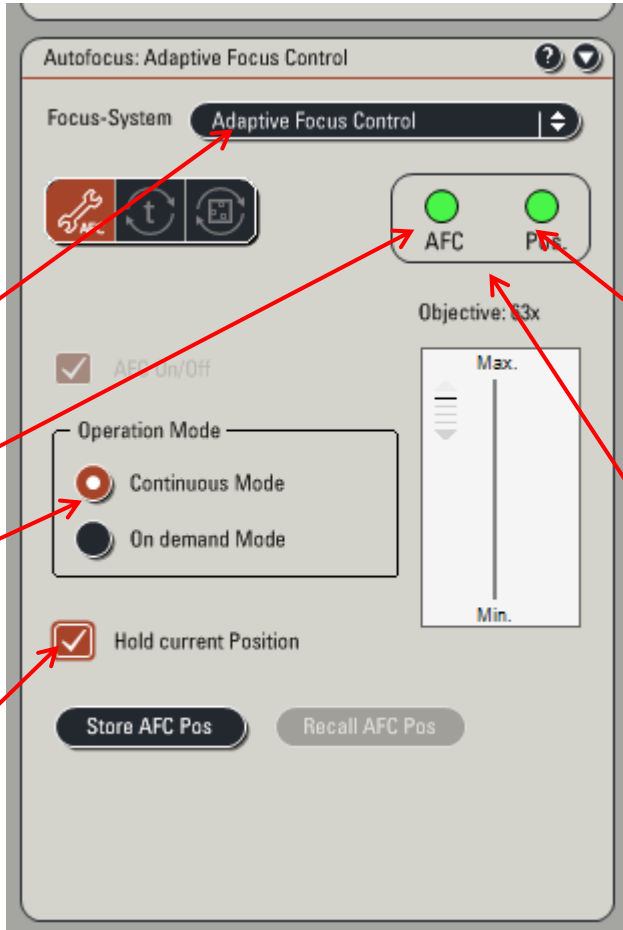
If adaptive focus is available for your sample, the green light on the front of the microscope will be illuminated. If not, the light will be red.



To set up adaptive focus control, click  In the acquisition menu.



The following menu will then appear:



1. Select "Adaptive Focus Control" from focus-system drop down menu

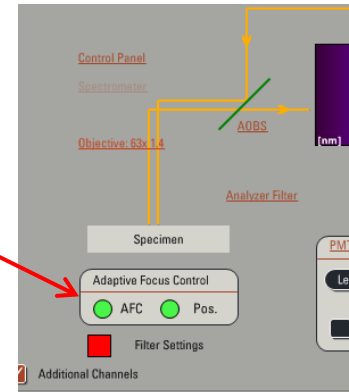
2. If adaptive focus control is available this will be green (if not it will be red)

3. Select "Continuous Mode"

4. When you have focussed at required position click "Hold Current Position" For a z-stack click "Hold Current Position" at any position in the stack.

When focus position is selected this turns green.

This information is also displayed in the beam path setting screen



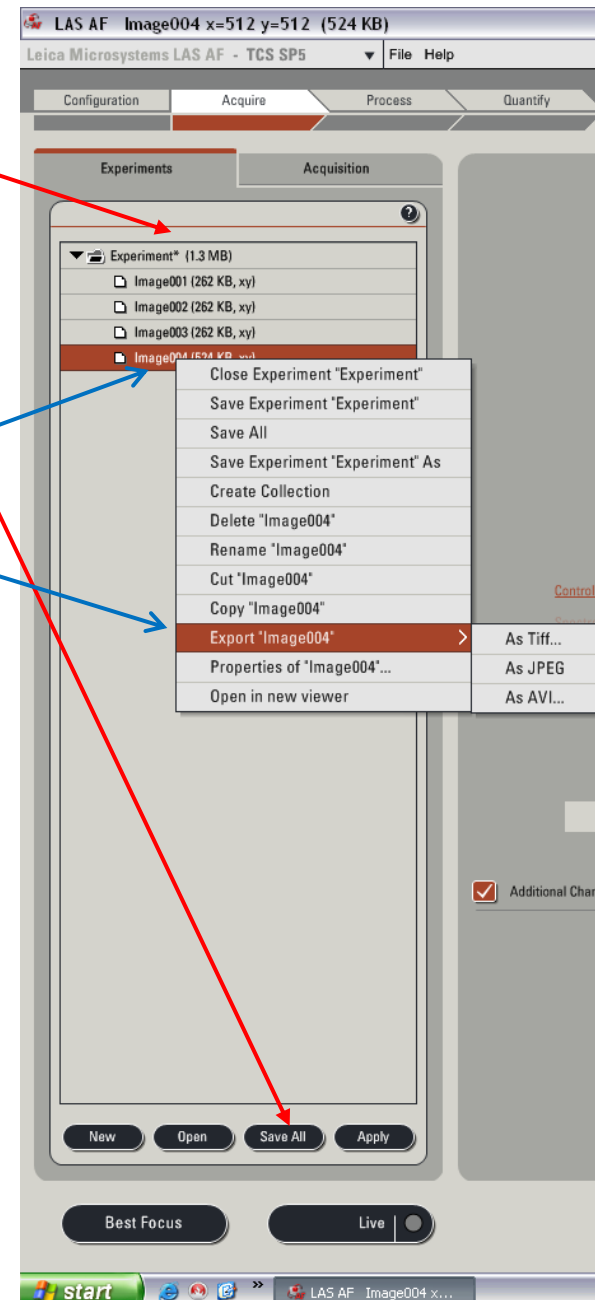
## Saving images

- Each single scan or series scan 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 will be prompted to give the experiment folder a name and location. Save your images in your own folder within the “users” directory on the D-drive.
- A single lif file is saved for all the images within an experiment folder. The lif file contains information about how the images were captured which may be useful post-acquisition.
- Images or series can be exported as tiffs or avi files by right clicking on the file in the experiment folder....export. After selecting a file type you can chose whether to export an overlay of your different channels or separate images for different channels.

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 confocal 8
- Using a mac: goto go menu....connect to server  
enter smb:\\ IP address confocal 8





## **Ending the 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 sheet – shortcut on desktop of computer – you need to enter the date, your UOB username and the time you started and ended your session. Save file after completion.
- Check Google calendar to see if system is booked after you:

### **If someone else is booked on within next 3 hours:**

- Go to Laser configuration window:
- Turn Argon laser power 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
- Exit software. Wait until software is fully closed before switching off scan electronics button.
- Switch off fluorescent lamp
- Switch off microscope.

## **Appendix**

Other features not covered in this guide:

- Resonance scanner mode (scan speed of 8000Hz)
- “Mark and Find” imaging – allows capture of images at multiple sites during a timecourse.
- Tile scanning – allows capture and automatic stitching of adjacent area on a sample.
- Live data mode – allows capture of images at multiple sites, using different lasers, laser powers, detector gains, zoom factor etc.. at each site
- FRAP wizard
- FRET wizard