

Leica Sp5 II Confocal User Guide

Turning on the Confocal System (instructions are posted in the room)

1. Turn on Laser Power Button
2. Turn Key to On position
3. Turn on Scanner Power Button
4. Turn on PC Microscope Button
5. Turn on EL6000 (fluorescent lamp)

You will need a password to log onto the computer. These will be assigned by the confocal support staff.

To Launch the Software

- Click on “LAS AF” icon
- A menu will appear with some selections on it
- If you are using the resonant scanner, make sure the “resonance scanner” box is checked

NOTE - The resonance scanner has a maximum image size of 1k x 1k. The conventional Scanner allows up to 8k x 8k images.

- Stand> make sure “DMI6000” is checked
- Configuration> make sure “Machine” is checked (*this makes sure that all the hardware is recognized to run the system. The other option is a simulation that only runs the software without the hardware*).
- You will then be asked if you want to initialize the stage. This is very important for time-lapse imaging if you are using multiple stage positions and also for tiling (montage) imaging.

Using the Microscope Stand/putting samples onto the stage, etc.

- Samples must be cultured on or mounted under a 0.17mm coverslip. If you are using microscope slides, these need to be inverted so that the coverslip is facing downwards towards the objective.
- If you need to use nail varnish or other sealants to seal your coverslip on, make sure they are COMPLETELY DRY before putting the slide on the microscope stage.
- Use the universal sample holder for most applications. ***If you need a different sample holder, you must ask for assistance of the confocal support staff to change it.***

NOTE –BE VERY GENTLE WHEN LOADING SAMPLES ONTO THE STAGE HOLDER. DO NOT PRESS DOWN ON THE STAGE AS THIS MAY CAUSE THE Z-GALVO TO HAVE AN ERROR AND COULD DAMAGE THE GALVO.

- To see Transmitted light through the eye pieces, press the TL/IL button located on the lower left side of the microscope stand (see fig 1).
- It is better to start by winding up the lens close to the coverslip and then winding the lens downwards to focus (that way you will avoid crashing the lens into the sample).
- To use the fluorescence light, make sure you have selected the appropriate cube using the control panel on the front of the microscope(see fig 2). Then press the “shutter” button to direct the fluorescence light onto the sample
- **IMPORTANT: - Setting the “zero” position for the focus:**
 - After you have focused on your sample, you should set a “zero” point for the microscope. *(this tells the microscope where your sample is in relation to the lens - if you don't set the zero position, you may get an error message later)*
 - On the right side of the microscope stand there are a series of three “set Z buttons” (see fig 3).
 - First you need to clear the current zero position - press and hold the center button and press top button one time.
 - Next, to set the current focus as the zero position - press and hold the center button and press the top button one time.

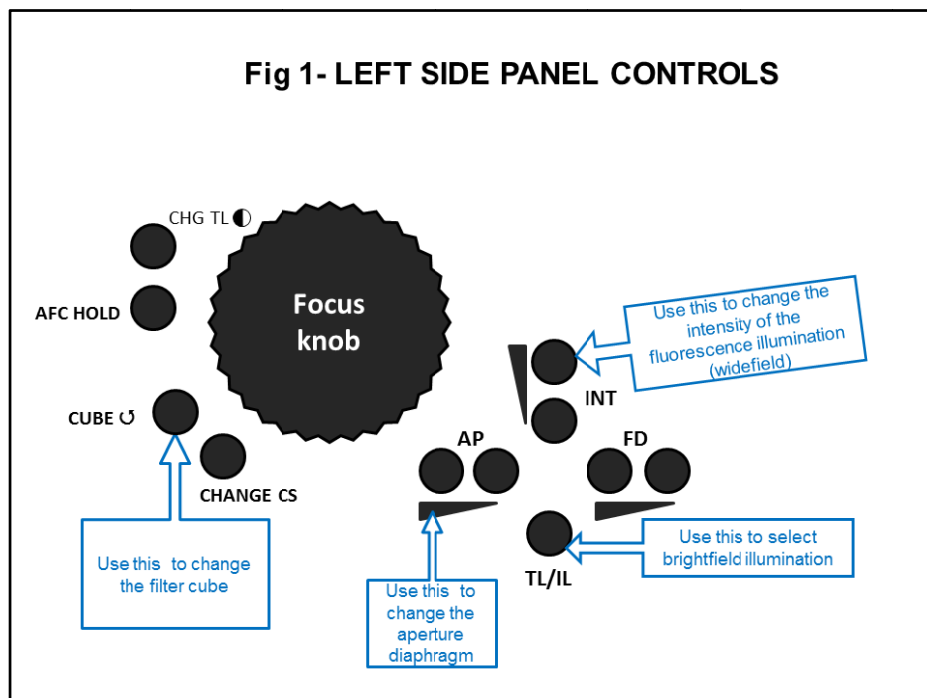


Fig 2: FRONT PANEL CONTROLS AND SCREEN

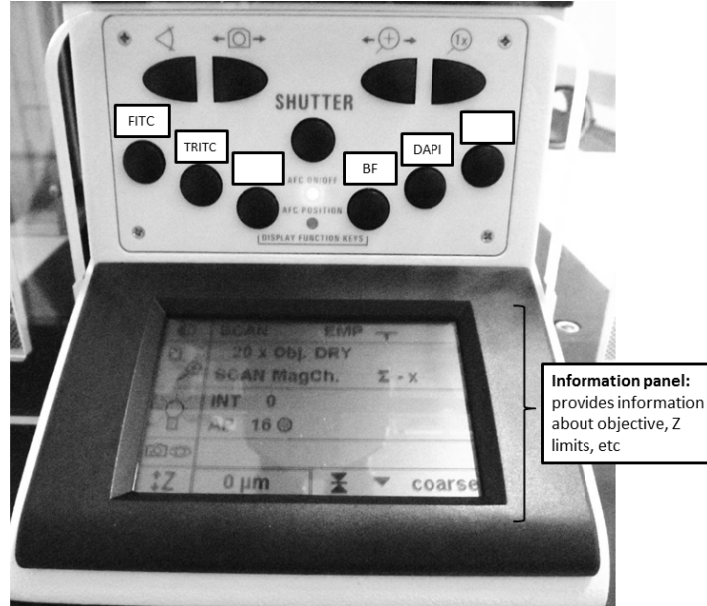
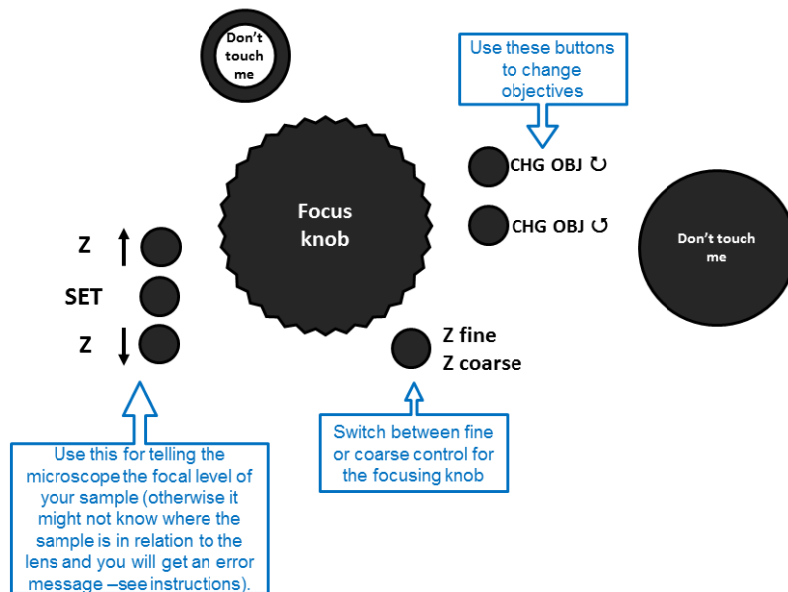


Fig 3- RIGHT SIDE PANEL CONTROLS



Operating the Confocal From Within the Software:

There are two main tabs that you will be working in, called “ACQUISITION” and “EXPERIMENTS”. There are also other tabs that are used for setting up the configuration of your experiment.

- **Once the software has launched you must first turn on the lasers under the CONFIGURATION tab.**
 - Click on the lasers icon
 - Either turn on all of the lasers by checking them, or if you know that you only need specific ones for your application just check those.
 - Give 25% power to the Argon laser if you need that laser.
- If you want further information about the lenses, you can find that under the “OBJECTIVES” icon (e.g. this gives you information about their optical limits, etc)

WORKING IN THE ACQUISITION TAB

Most of the work in setting up your image acquisition is performed in the ACQUISITION TAB.

- The laser controls are in two panels at the top of the ACQUISITION TAB.
- You need to activate the shutters for both the U.V. and the visible lasers (click on the round dots in the top left corner).
- When you set up your imaging parameters, you will need to adjust the power of the lasers you want to use. The table below gives information on the maximum power settings for each of the laser lines (*these are the maximum powers to use if you want to avoid bleaching the sample*):

LASER	Comments	Recommended Power
405	50 mWatt=very powerful laser line	Not more than 15%
488		Not more than 15%
633	20 mWatt	Not more than 15%
458		Not more than 20%
514		Not more than 20%
476	Weaker laser line	Not more than 30%
496	Weaker laser line	Not more than 30%
543	1 mWatt	50-60% is O.K.
594	2 mWatt	50-60% is O.K.

Imaging Procedure (5 step sequence)

Step 1: - Show the spectrum of the dye(s) you want to image

- Do this by selecting the dye from drop down lists on the PMT tabs (you can make new entries if your dye is not already listed).
- The default view is the emission spectrum only (if you want, you can also display the excitation spectrum by right clicking on the graph – but this can make the display to busy).

Step 2: Turn on Lasers

- The laser you want must always be a wavelength that is to the left of the emission maximum of your dye.
- Click on the laser line that you think is appropriate and move the slider up to give some power to the laser (use the table on the previous page to help you decide how much power to use – you should always start low and then go up only if you need to later when you optimize your live image).
- The laser line will then be illustrated on the spectrum so that you can make sure it is to the left of your emission peak.
- You can try a couple of different ones to determine which looks the best (you may also want to display the excitation spectrum of the dye [see step 1] to help you decide).

Step 3: Turn on PMTs and configure the detection

- There are two HyD detectors (higher sensitivity and lower background compared to standard PMTs) so you want to use these for your weakest dye(s).
- Select the PMTs that you plan to use (maximum of 5) and assign them to the dyes you want to image (they have to go in order blue to red from the left). You can skip a PMT (i.e. not use it), but you can't assign dyes in the wrong left-right order.
- Adjust the spectral collection window for each PMT using the sliders (if you are skipping any PMTs, just make the window as small as possible). You can also double click on the slider to see the wavelength range you have selected and alter the numbers manually.
- DO NOT overlap the collection window for any dye with any of the excitation laser lines, otherwise you will collect a lot of the excitation light rather than the light emitted by the dye (the collection window needs to be at least 5nm shifted from the laser line wavelength)
- If your collection window falls between two excitation laser lines, do not overlap the collection window with either laser line.
- Avoid overlapping your PMT collection window with other dye emissions as this can lead to “bleedthrough” of the dyes from one image into another. If your dyes are very close in wavelength, it may be better to do a sequential acquisition(s) (see later description in the guide).
- Assign a “pseudocolor” to each PMT (this will be the color that will be displayed during the acquisition, but in reality all the images are monochrome greyscale images)

Step 4: Configure your imaging formats

- This is done using the tabs in the acquisition menu (left side of screen)
- **File format** – the default is 512 x 512. The maximum for the resonant scanner is 1024 x 1024 (conventional scanner can go up to 9600 x 9600).
- If you are using the resonant scanner with more than two dyes, you can only do up to 512 x 512 imaging. If you want a higher resolution, you can split into sequential acquisitions (see description later in this guide)
- Resonance scanner can only do 12 bit imaging
- **Scanner speed** - (default is 8000 Hz – this cannot be adjusted for the resonant scanner)
- **Line averaging** - Use at least 4 to 8 for LIVE imaging/looking at your sample initially and optimizing settings

NOTE – if you want the line averaging to be applied during the LIVE image acquisition, you need to go to the CONFIGURATION tab>settings > check “line averaging during live imaging”. Otherwise, the line averaging for live imaging will default to 1.

- Pinhole – no need to adjust for most applications. The pinhole always defaults to 1 Airy unit (which is optimized for each objective). If you do need to alter the pinhole size for your application it is recommended not to go above 2 airy units.
- When you have configured the PMTs and acquisition parameters, it is now time to get a live image to see what it looks like and then optimize
- Click the “LIVE” button

Step 5 – Optimizing the Image

A lot of the optimizing is done using the dial controls on the control panel that is located behind the computer keyboard

- Adjust the focus using the Z-focus dial (Clockwise = to go from the coverslip into the sample, counter clockwise = to go towards the coverslip).
- You can move around to center the feature of interest using the x,y stage controller (joystick) (you can press “ctrl T” to display a crosshair that you can use to find the center of the image).
- **Gain Adjustment** -To increase the signal intensity, you should adjust the gain settings (dial on control panel) before resorting to increasing the laser power. To do this, click the mouse on the image (dye) that you want to adjust and then increase gain using the smart gain control dial.
- Use the “glow over/under” display setting when adjusting gain (QUICK LUT icon at top left of image screen) which pseudocolors the image to show saturated pixels in blue and zero pixels in green. You should adjust the gain until you can just see a few saturated blue pixels (or none).
- Gain recommendations – not more than 100% for HyD detectors.
- **Smart Offset Adjustment**- Use this to adjust the zero value for the conventional PMTs (don't go below -0.6). Adjust it until most of the background pixels look green (it is resetting the “zero” value of the background). This cannot be used for the HyD detectors, as their background is virtually zero anyway.
- While adjusting the gain and smart offset you can toggle between the “glow over/under” display or the monochrome or pseudocolored images to make sure they look good.

NOTE 1 – remember that your live image only has 4-8 line averaging, so it will look pretty grainy and ugly. The low line averaging is to avoid bleaching your sample and also so that the screen refresh rate is sufficiently fast for you to make the adjustments easily. When you have optimized the live image, you will change the line averaging to a higher value which will give a much higher quality image.

NOTE 2 – A cool feature of the Leica Sp5 II system is that you can set separate gain adjustments for all 5 PMTs. Many conventional LSCM microscopes can only apply a single gain to all the detectors.

NOTE 3 - When you are optimizing the image look out for potential “bleed through” of your dyes into the other channels. For example, DAPI has a very wide emission spectrum and often bleeds through into the GFP or FITC channel and even into the TRITC channel. You should suspect bleedthrough if your images have features that look very similar (e.g. you can see “nuclei” in the GFP channel, but you know your GFP should only be in the cell membrane). If you think a dye is bleeding through into another channel, you can check by turning off its excitation laser. If the other image is not affected, then there is probably no bleedthrough, but if the other image changes this is probably indicating a bleedthrough problem. If this happens you may be able to stop it by reducing the laser power on the dye that is bleeding through. Otherwise, you can set up a sequential acquisition where you image one or more dyes separately from the others.

- When you have finished optimizing the image, you can change the line averaging to a larger number (e.g. 32, 64, 96) and then take a snapshot image by clicking on the “capture image” icon to see if you like the result. If not, you can do more optimizing.

Pixel Size and Resolution (Nyquist Principle)

Each objective has a limit of resolution. It is recommended that when you are zooming in on your samples you do not set your pixel size lower than:

$$\frac{\text{objective resolution}}{2.3}$$

If you set it lower than this it will lead to over sampling and can cause artifact in the image. This is the Nyquist Principle.

If you don't know the objective resolution, it can be calculated by:

$$\text{Objective resolution} = \frac{0.61 \times \text{emission wavelength}}{\text{NA of objective lens}}$$

Therefore an overall formula that you can use to calculate the smallest pixel size is:

$$\text{Smallest pixel size for max. resolution} = \frac{\left(\frac{0.61 \times \text{emission wavelength}}{\text{NA of objective lens}} \right)}{2.3}$$

- The resolution can be changed either by changing the pixel dimensions (e.g. 512 x 512 changed to 1024 x 1024) or by changing the zoom.
- Once you reach the maximum resolution, you can increase the line averaging to improve the image quality.

NOTE – below is a table of recommended values for the smallest pixel size of our confocal's objectives with some of the commonly used fluorochromes for your reference.

Recommended Pixel Sizes (nm) for Optimal Resolution

Fluorophore	Objective				
	5x (DRY) NA 0.15	10x (DRY) NA 0.4	20x (DRY) NA 0.7	40x (OIL) NA 1.25	100x (OIL) NA 1.44
DAPI <i>Emission 455</i>	804.49	301.68	172.39	96.54	83.80
GFP <i>Emission 507</i>	896.43	336.16	192.09	107.57	93.38
FITC <i>Emission 519</i>	917.65	344.12	196.64	110.12	95.59
TRITC <i>Emission 572</i>	1011.36	379.26	216.72	121.36	105.35
Cy3 <i>Emission 570</i>	1007.83	377.93	215.96	120.94	104.98
Cy5 <i>Emission 670</i>	1184.64	444.24	253.85	142.16	123.40

Z-stack Imaging

Use the **Z-stack acquisition** menu tab for setting this up.

- On this menu there is a “cube” that shows you what your imaging volume looks like.
- You can set the “begin” and “end” positions of a z-stack by focusing up or down through the sample and defining where you want the acquisition to start and end.

NOTES

- you do not have to set “top” and “Bottom” - the software can start at the bottom and work up if you want or it can start at the top and work down if you prefer.

-If you are imaging deep into the tissue, the signal may be fainter and in that case it may be better to set the start point in the deeper parts first and work down towards the coverslip so as to image the fainter areas before they get bleached by the laser exposure.

- Once you have set the top and bottom, you will be able to see what the total thickness is that you are imaging,
- The system will suggest a default optimum stepsize. You can override that and set your own desired step size if you want.
- The number of slices that will be needed for the z-stack should also be displayed.
- If you are doing Z-stack imaging rather than capturing images from a single plane, you will have to click on the “START” icon to initiate the image acquisition

Compensation

- Click on [compensation](#) at the bottom of the Z-stack panel.
- Select compensation type. AOTF controls the laser intensity, and Gain adjusts the detector sensitivity. Choose both to optimize for signal to noise and photobleaching simultaneously.
- Add beginning and end.
- Go to beginning of stack or whatever position is closest to coverslip.
- Turn on live imaging.
- With the quickLUT view selected begin moving away from the coverslip up the z-stack until the intensity begins to visibly fall off. At this point adjust the gain and laser power to match beginning points.
- Add point in the compensation menu.
- Repeat until you have reached the top of a stack and close out the compensation menu.
 - Note: the more points you select the more uniform the intensity of the stack will be.

Adaptive Focus

- Turn on the adaptive focus by clicking on the adaptive focus icon (arrow pointing down at three lines) at the top right of the acquisition panel.
- Set to “On demand mode”
- The button with the arrows circling the T is used to set the time points at which you wish the adaptive focus to be used.
- The button with the arrows circling the tiling symbol is used to designate the positions you wish the adaptive focus to be used.
- Select a position using the “Mark and Find” tab
- “Go to” the middle of the z-stack
- In the adaptive focus tab check the “Set Experimental Position” box.
- “Delete AFC positions” to remove any of the previous ones.
- “Store AFC position” to store the new position you’ve selected.
- Go to the “Mark and Find” tab.
- Click the button with the arrow pointing to one of two boxes to redefine the position with the new settings.
- Repeat for each position.

Sequential Acquisitions

This is useful for applications where you are having a “bleedthrough” problem with one or more of the dyes. You can split out your acquisition into one or more sequential acquisitions so that you do not image dyes that bleed into one another at the same time.

- It is often better to set up and optimize the imaging parameters together as a single acquisition initially and then to split it out into sequential acquisitions (that way all the other parameters that you have set will be preserved in the sequential acquisitions).
- Click on the “**Seq**” tab to open up the sequential acquisitions menu
- There is an icon with - scan 1 + on it. To add another scan, click on the + button. This will duplicate the current acquisition you have set up.
- Then you can go into each scan and turn off the excitation laser and PMT that you don’t need. Example:
scan 1 = GFP, mCherry(turn off DAPI laser and PMT due to bleedthrough into GFP)
scan 2 = DAPI (turn off GFP and mCherry lasers and PMTs)
- For sequential acquisitions you can pick one of the following:

Between lines- scans one line of GFP, mCherry, then changes lasers and does one line of DAPI

Between frames- scans a whole frame of GFP and mCherry, then changes lasers and does a whole frame of DAPI

Between stacks – scans a whole Z-stack of GFP and mCherry, then changes lasers and does a whole Z-stack of DAPI

NOTE – SLD’s experience is that the “between stacks” may not be ideal because if there is any slight drift of the sample and if the acquisition time is long, the two sets of acquisitions might be slightly shifted and not properly aligned. This might lead to problems when you try to merge/overlay the channels, etc.

- If you want to save the parameters/settings you have set up and optimized for a sequential image scan, go to the “save” tab on the sequential scan tab. Then you can reload the same acquisition parameters if you have a similar imaging application in the future.

Using HyD Detectors in Different Modes

- You should use the HyD PMTS in “standard mode” for most applications
- If you have samples that have a large dynamic range (e.g. very bright and very faint information on the same image) the HyD can be used in other modes that enhance the signal of the lower intensity pixels (*GREAT for generating beautiful images, but may not be a good option if you want to get quantitative data*)
- **BrightR mode** – this does a nice job of bringing out the lower intensity pixels without affecting the higher intensity pixels.
- **Photon Counting mode** – this mode counts all the photons that hit the PMT in a particular location as a function of time and builds up the image by accumulating photon counts.
- If you want to use this mode, go to: configuration>settings> then uncheck “max integration time”. This will make the photon counting mode available.
- Then you can select this mode on the PMT tab
- When using the HyD in photon counting mode you do not want to have any line averaging, so this should be changed to zero.
- You can alter the number of accumulations by changing the line accumulation and frame accumulations (more accumulations gives a better sampling).

Tiling and Mark and Find

- For tiling (montage imaging) click on the grid icon

Spectral Imaging

- Select x,y, λ mode (or similar)
- Set the range of wavelengths by moving the edge of the gray area or click and type in the range.
- In the spectral imaging panel set the bandwidth (Recommended is 10 nm)
- Set the step size.
- Acquire image
- Use of the “Processing” features will allow you to determine spectra of various ROIs as well as doing spectral unmixing etc.

Turning off the Confocal System (instructions are posted in the room)

1. Exit the LAS-AF software
2. Shut down the computer
3. Turn laser key to “off position”
4. When the computer is shut down, turn scanner power off
5. Turn off PC/microscope button
6. Turn off fluorescence lamp
7. Wait about 20 min (or until you hear the laser fan shut off) before you shut off the laser power.