Laser scanning confocal microscope Leica TCS SPE

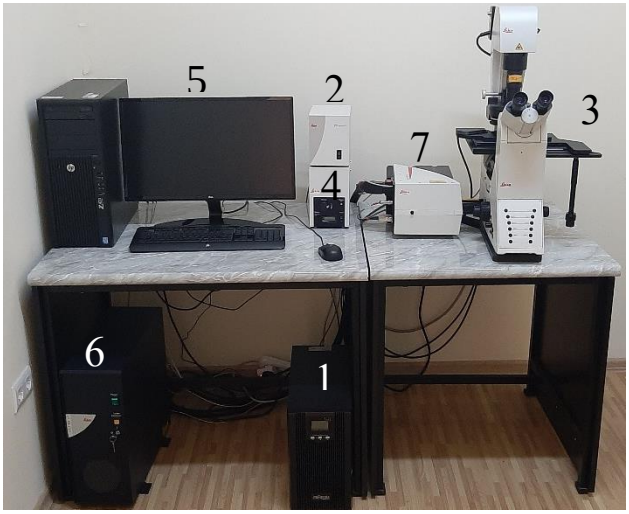
Introduction

Confocal Imaging Concept The primary functions of a confocal microscope are to produce a point source of light and reject out-of-focus light, which provides the ability to image deep into tissues with high resolution, and optical sectioning for 3D reconstructions of imaged samples. The basic principle of confocal microscopy is that the illumination and detection optics are focused on the same diffraction-limited spot, which is moved over the sample to build the complete image on the detector. While the entire field of view is illuminated during confocal imaging, anything outside the focal plane contributes little to the image, lessening the haze observed in standard light microscopy with thick and highly-scattering samples, and providing optical sectioning.

The Leica TCS SPE is mounted on a Leica DMi8 inverted microscope with four objectives (dry 10x, 20x, 40x, and oil immersion 63x). The system consists of three low noise solid-state lasers with a wavelength of 488, 532, and 635 nm. And there is the Leica EL6000 an external light source designed for enhanced fluorescence imaging. There are two filter cubes blue and green. System is equipped with LAS X software that allows to process and quantify the acquired images.

General Parts of the microscope

1. UPS unit
2. Power supply of microscope
3. Microscope
4. Fluorescent lamp power source
5. Computer
6. Laser power unit
7. Optical unit



UPS

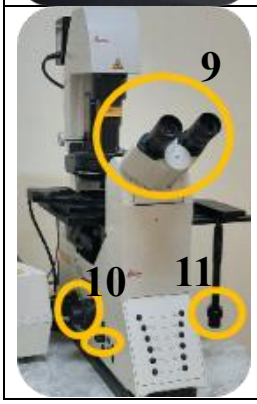
The device must always be turned on.
Check if it is off before use, then turn it on.

If you see green light it is turned on.
To turn on, press and hold both keys until you hear the device beep.
If you see yellow light it is turned off.
To turn off, press and hold both keys until you hear the device beep.

Brightfield Microscopy

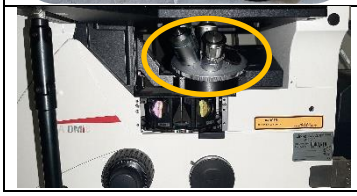
Before turning on the microscope note the date, time and your name into the logbook.

	1. Turn on the microscope by pressing the power button.
	2. Pull back the microscope head
	3. Place the specimen on the stage and retract the microscope head.
	4. Open the diaphragms in the directions shown in the picture.
	5. Press the bright light mode key.
	6. After pressing the bright light mode key, you will see these 2 light bulbs. The bottom one means you've turned on bright light mode, the top one means you've turned on the light bulb. If the top one is not lit, press the key to turn on the lamp
	7.Turn the filter disc until you see a light on the indicated key

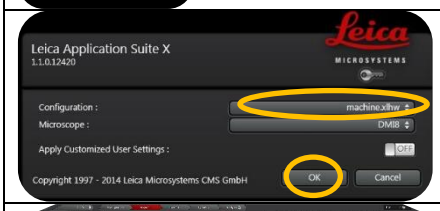
	8. Turn the light intensity adjustment knob and observe the light illuminates the sample
	9. Look through the eyepieces
	10. Adjust the focus. Rotating the macro and micro knobs will up or down the objectives
	11. By turning these knobs, you will bring the sample into the field of view

Fluorescence Microscopy

Before turning on the Leica EL6000 note the lamp hours (format is HH-MM) from the digital screen into the logbook.

	1. Turn on the power **N.B.** Once the lamp is on it must be left on for at least 30 minutes before switching off. After switching off it cannot be switched on again for at least 30 minutes.
	2. Turn on the shutter
	3. Turn the light intensity adjustment knob
	4. Press FLUO to turn on the fluorescent mode
	5. Here you will see the bright light bulb go off and the mode change. If the bright light has not gone out, turn it off by pressing the key
	6. Turn the disc clockwise to get a blue light. Turn one more to see green light.
	7. Change magnification by rotating the objectives disc. There are 10x, 20x, 40x, 63x objectives. Only 63x needs immersion oil.

Laser Scanning Confocal Microscopy

	1.Turn on Laser power
	2. Turn the key clockwise
	3. Push the button FLUO
	4. Turn on the computer
	5. Double-click the LAS X icon
	6. Choose in configuration “machine.xlhw” and press OK
	7. LAS X interface

LAS X Software

The LAS X software is separated into four distinct areas.

1. **Image Acquisition & Control Settings**

These functions control the static acquisition settings for image collection. Here you can find settings such as Frame Size, Scan Speed, Averaging, Zoom, etc.

2. **Beam Path Configuration**

This area is where the user sets the beam path configuration and control detector settings to optimize the fluorescence signal.

3. **Image Display**

This area is where the image will be displayed. Settings here allow the user to control how the image is viewed on screen.

4. **Scan Action Functions**

These functions start/stop scanning and initiate an experiment acquisition.

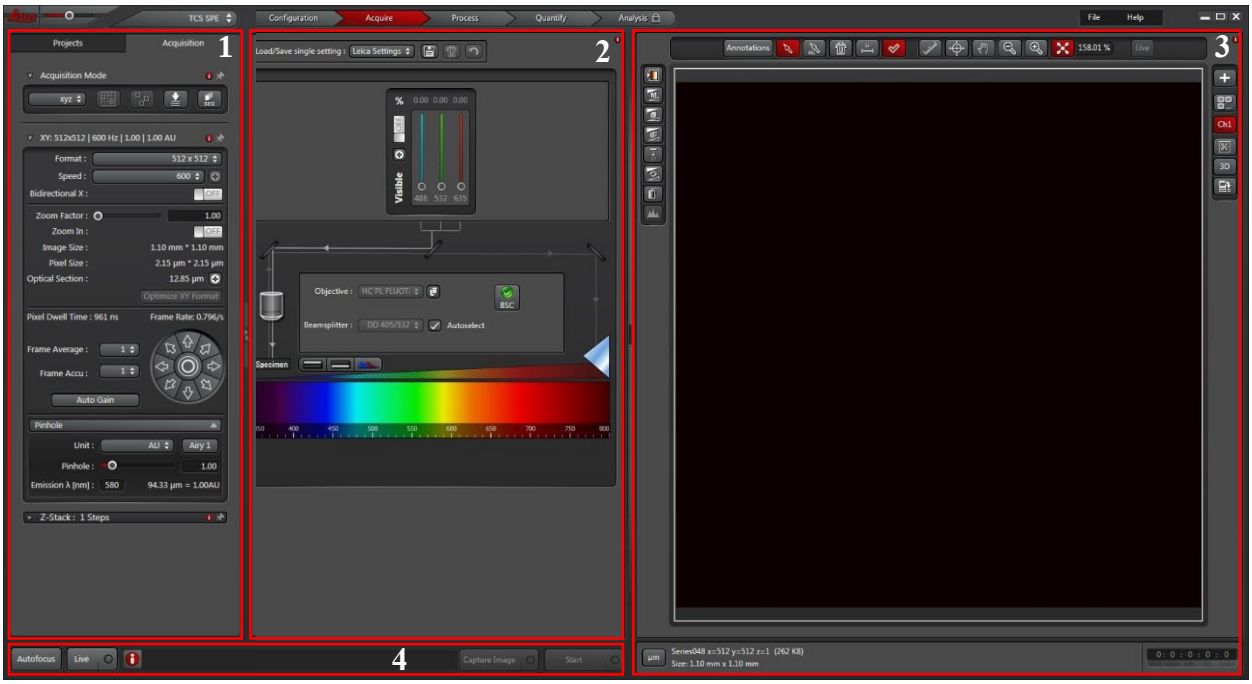


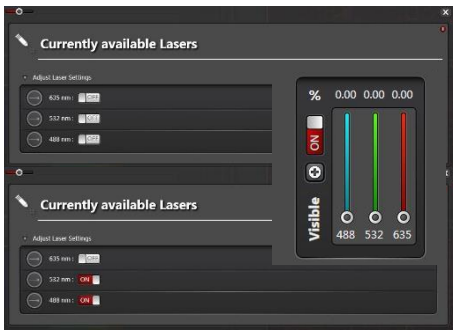
Image Acquisition & Control Settings

The Acquisition Mode parameters located on the left-hand side of the software display contain all the settings available for image optimization and collection.

From the dropdown, choose the Acquisition Mode. For example, if you want to collect 3d image stacks, select 'xyz' from the dropdown.
The remainder of the settings in the acquisition mode control dialog can remain at their defaults to start. Depending on sample morphology and signal quality these settings will need to be adjusted later.
Format – image size in pixels. Default is 512x512 however this can be optimized based on zoom and objective settings. The ideal Nyquist sampling frequency can be achieved by calculating the optical resolution and dividing this value by 3. The resulting value will correspond to "Pixel Size".
Speed – overall speed of scan during acquisition. Generally, try to run this at the default 400Hz to start and change depending on noise levels. Bidirectional scanning will double the speed as pixels are recorded in both directions.
Zoom Factor – change magnification without changing objective lens.
Frame Average – selection >1 will scan the same image multiple times and average the result to produce an image that has an improved signal-to-noise ratio.
Frame Accumulation - selection >1 will scan the same image multiple times and sum the result to produce brighter image. In this case the user has to decrease the laser intensity to avoid oversaturation.
Pinhole – this controls the optical section thickness. To start – select 'Airy 1' button to set optimal pinhole diameter. Airy depends on the emission wavelength, so set the wavelength of the maximum of the fluorophore emission. This can be increased or decreased to adjust image quality.

Before we configure the beam path for the particular fluorophores being imaged we need to turn on the individual lasers we require.

1. Click on the + icon for the Visible Laser dialog.
2. Toggle on each laser required.
3. Close Available Lasers dialog.
4. To enable Visible Lasers for use – toggle the ON/OFF switch above + to ON state.

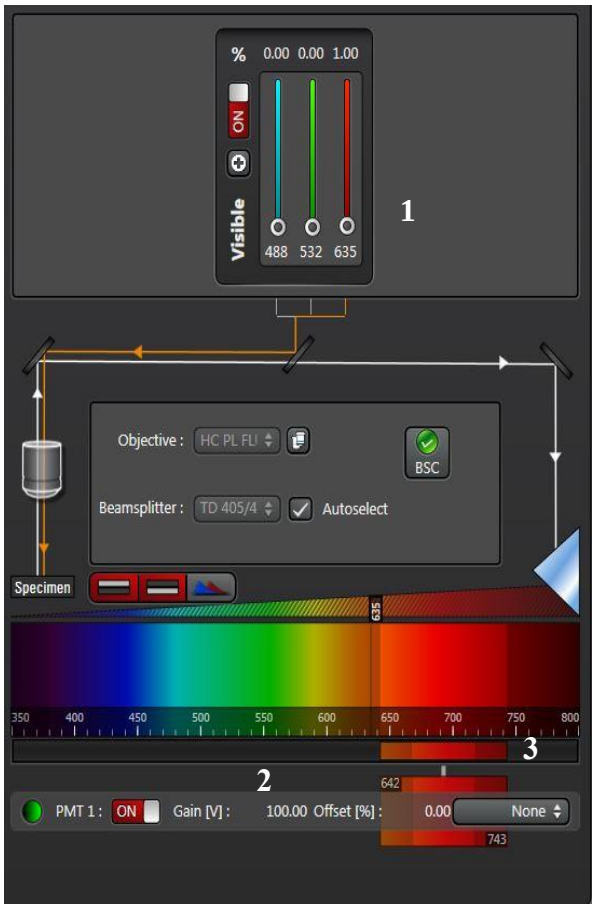


Once the lasers are powered on we want to configure the beam path to collect a fluorescence signal.

1. From the Visible Laser dialog, choose excitation wavelength that matches your fluorophore.

Note: Set the transmission % to a reasonable starting value (this may be changed dynamically depending on signal strength). A starting value between 5-10% is recommended.

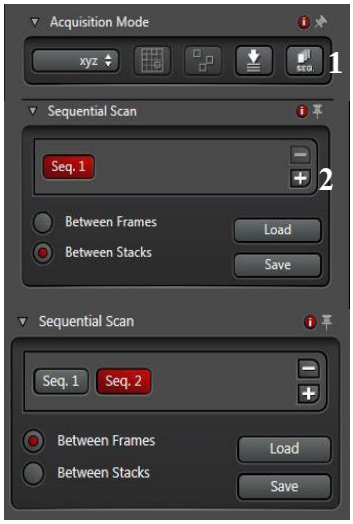
2. Set the gain on PMT at 1000 for detection and select color for that particular channel.
3. Define upper and lower emission band thresholds based on the spectrum for your fluorophore.



Sequential Imaging for more than one color

1. Once setup for initial single color is complete, ensure the ‘Seq’ button is selected in your Acquisition control settings. This will activate the Sequential Scan dialog. By default, your previously designed single beam path will be Seq 1.
2. Once these options are selected you can click + to add ‘Seq. 2’ and repeat steps 1-3 on the previous page to change the beam path settings to match your second fluorophore.
3. Repeat process for total number of channels required.

Note: The ‘Start’ button on the lower right is used to acquire the image/stack. ‘Capture Image’ will acquire just the preview image, not the whole stack or all sequences.



Scanning an Image

Once you have completed the beam path configuration for your fluorophore(s) it is time to begin scanning to optimize the image quality.

1. Press ‘LIVE’ to begin scanning.
2. Image will appear in Image Display area.
3. From the Acquisition Mode dialog, set the Pinhole to ‘Airy 1’.

Note: Setting the pinhole to 1 AU is a compromise between axial resolution and signal level. When you start with an open pinhole, you are at a point where your signal is the highest but your axial resolution is at its lowest. As you reduce the aperture diameter you are increasing your Z resolution but reducing your signal level. This is a reasonable trade off until you reach 1 AU. Reducing the pinhole lower than 1 AU will still linearly increase your Z resolution but now signal will start to drop exponentially.

4. Beginning with one channel, adjust ‘Gain’ in under PMT for selected channel until you can see a reasonable image on screen.

Note: Increasing Gain will effectively increase PMT sensitivity to signal, making your image brighter. This will also increase noise which can be reduced with scan speed/averaging adjustments.

5. Repeat for remaining channels.
6. Stop scan and move to ‘Fine Tuning Image Quality’ section.

Fine Tuning Image Quality

In order to find the optimal gain settings for a particular condition, you must first be able to identify the thresholds of the signal level in the image.

1. While scanning a live image, click Quick LUT.

Note: LUT assigns a green color to underexposed areas in the image with a grey value of 0 (or less). It assigns a blue color to overexposed areas in the image with grey values >255 (for 8 bit).

2. Adjust the ‘Gain [V]’ so that your brightest areas fall just below the overexposed limit (blue pixels).
3. Adjust ‘Offset [%]’ (negative value) for PMT to the minimum possible value so the image is just above the display of any green pixels (mostly it’s -1%).
4. Click ‘Capture Image’ to collect final scan.

Note: Capture Image will scan with same scan speed and frame size as LIVE, and in addition it will apply any Frame Averaging you have set to control image noise in the Scan Control settings.

Pinhole (Confocal Aperture)

Ideally, the confocal aperture should be set to the size of the structure(s) you are trying to resolve. However, for example, some sub-cellular structures of interest may be beyond Abbe’s diffraction limit and therefore beyond the microscope’s capability to resolve. The confocal aperture has some practical limits that can be used to guide the usage of this setting.

Start by closing down the confocal aperture to ‘1 Airy’. You can certainly close the confocal aperture below this value and continue to improve resolution, just understand that below ‘1 Airy’ you will lose signal level at an exponential rate. Conversely, you can increase the confocal aperture diameter to improve the detected signal level if you can sacrifice axial resolution.

In some cases, the signal level may be so low that increasing the aperture diameter is the only way to lower the gain enough to get a usable image.

In other cases, if the gain is too high (causing excess noise) and the laser power cannot be increased due to photo effects then increasing the confocal aperture is the only option to improve image quality.

Laser Intensity

The overall power of the laser will directly affect image quality but most importantly it will have the greatest impact on the health of your sample and fluorophore(s). Increasing laser power will yield more signal but it will also induce negative photo effects such as phototoxicity and photobleaching that can harm the sample. Lasers also generate considerable heat when used at higher powers and that may have unforeseen effects on the sample.

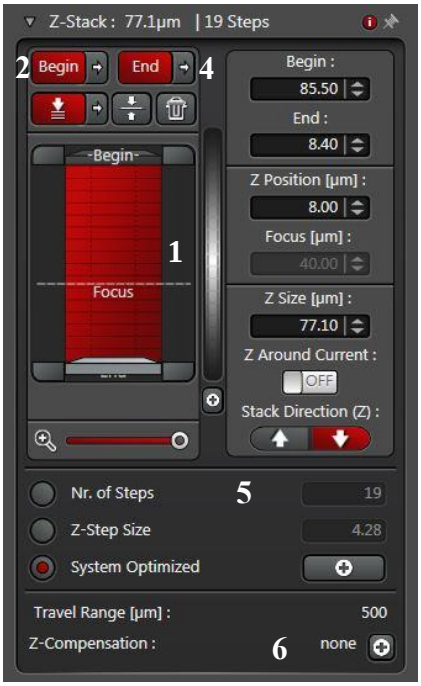
For most lasers on this microscope, we recommend laser power settings between 1% and 5% to start. It is recommended to start as low as you can possibly go and still get an image. Then it can be increased as necessary to help balance image quality.
To discover your fluorophore(s) saturation level (i.e. how much laser power you can use before your fluorophore stops absorbing additional photons) you can start imaging at a low laser power and gradually increase the power slider until you reach a point where your image stops getting brighter. Continual increases in laser power will stop yielding more signal. That point is the practical laser power limit.

Detector Sensitivity

The sensitivity of the PMT is a function of the high voltage gain applied to it. The gain setting (as discussed elsewhere in this manual) controls the sensitivity of the detector to photons of light emitted from the sample fluorescence.
Increasing gain provides the most flexible and impactful way of improving image quality, but it has some drawbacks. The most obvious is when you increase the gain you also increase the noise in the resulting image.
If laser power is high and the confocal aperture cannot be compromised there are a few methods to dealing with noisy signal due to high detector gain.
There are 2 main methods for dealing with high image noise. The first is reducing the scan speed to a slower rate. This will increase the pixel dwell time which will improve the signal-to-noise ratio. The second is signal averaging which will scan the same image multiple times and average the result to produce an image that has an improved signal-to-noise ratio.

Collecting a Z-Stack

When selected on 'xyz' in the Acquisition Mode, you have access to the Z-Stack utility. Here we can define the settings required for 3d imaging in the Z (or focus) dimension.



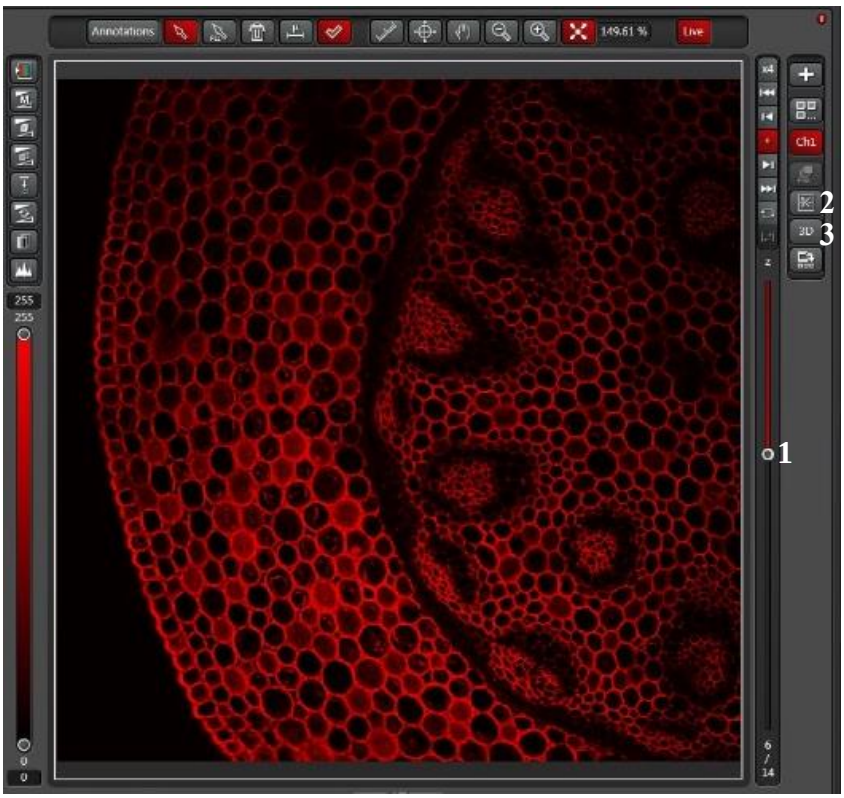
1. While performing a LIVE scan, focus your sample in one direction (does not matter which) using the mouse scroll in the field until it goes almost completely out of focus.
2. Click 'Begin'. This will now mark the starting position for your Z-Stack.
3. Focus back through your sample until you reach a point on the opposite side where the sample begins to go out of focus.
4. Click 'End'. This will mark the end of the stack.
5. By default, the software sets the stack step size to 'System Optimized' using the objective and emission settings from the beam path configuration to calculate the ideal sampling size.

Note: Practical step size will depend on the level of detail required for the stack compared to the signal stability of the fluorescence. If the number of images required from using the optimized settings exceeds the balance between time and exposure, the user can reduce the number of slices manually by toggling to 'Z-Step Size' and increasing the value.

6. If you notice loss of signal in z, this can be compensated with linear AOTF control (there is also the option of voltage compensation).
7. Click Start to being Stack acquisition.

Visualization and handling z-stack

1. A stack can be visualized by moving the z slider.
2. Orthogonal sectioning can be useful to visualize details in all dimensions in a 2D image.
3. The 3D viewer is very useful. Here you can rotate the sample, create surface rendering, 3D orthogonal slicer and generate movies.

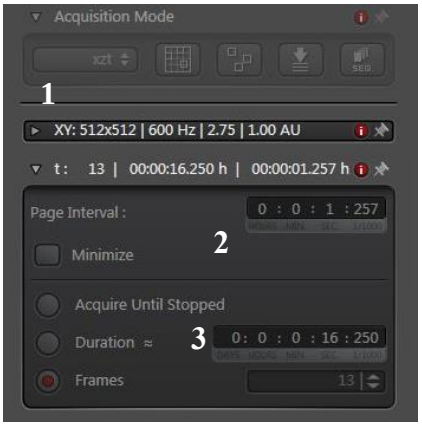


Collecting a Time Series

When selected on ‘xyt’ in the Acquisition Mode, you have access to the Time Series utility. Here we can define the settings required for time-lapse imaging.

1. In the Acquisition Mode – select ‘xyt’ for 2D time-lapse or ‘xyzt’ for 3D time-lapse.
2. Select waiting period between time points under ‘Time Interval’.
3. Select Duration and enter total time for experiment to run.

Note: The system will automatically calculate how many images it needs to collect over the interval/duration you set. You can also manually calculate this and entire the values accordingly.
Press ‘Start’ to initiate Time Series.



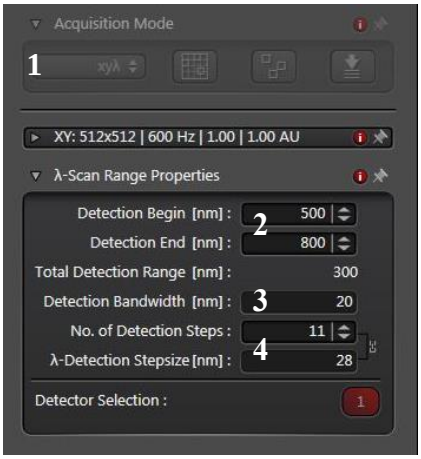
Collecting a Lambda Series

When selected on ‘xyλ’ in the Acquisition Mode, you have access to the Lambda Series utility. Here we can define the settings required for collecting data for intensity in short range to determine emission spectra.

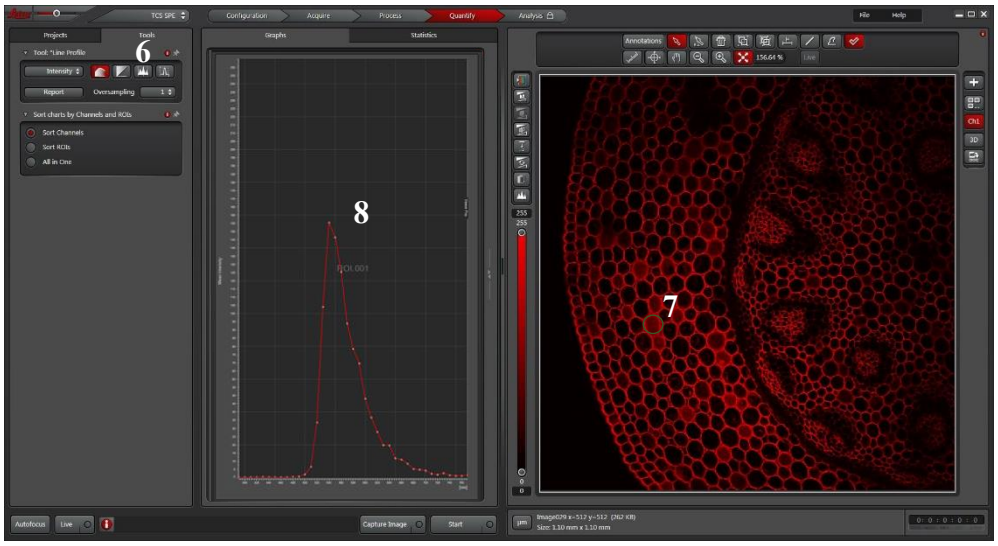
1. In the Acquisition Mode select ‘xyλ’.
2. Set ‘Begin’ and ‘End’ of the range of interest.
3. Set ‘Bandwidth’ (minimum 5 nm)
4. ‘No. of Detection Steps’ sets the division of the ‘Total Detection Range’.

Note: To cover gaps, you can increase the ‘Detection Bandwidth’ and/or increase the ‘No. of Detection Steps’.

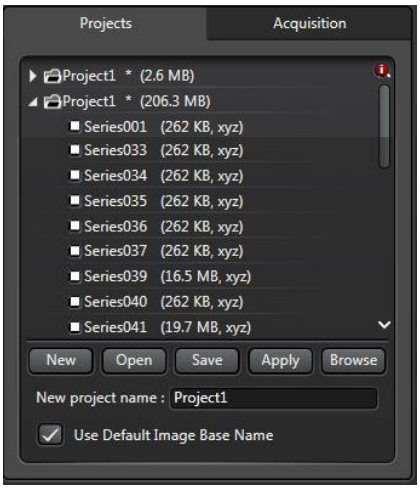
5. Press ‘Start’ to initiate Lambda Series
6. When your scan is finished, click the “Quantify” tab, then choose “Tools” and the “Stack Profile” option.
7. You will need to draw an ROI (region of interest) on your image to generate the emission curve. Select sort ROIs in the Tools panel. Pick a region with a strong signal if you can.



8. Right clicking on the graph gives you options like exporting the data to the dye database, and generating a picture file or spreadsheet of the graph.



Saving the project



When your imaging is complete, you can save your data to the local hard drive in the Projects tab. By default, the LAS X software saves files in the LIF format, however you can choose the format type before storing data.

1. Select 'Projects'.
2. Choose either entire Project or individual image.

Note: Selecting on the 'Project' folder header will save all images into a single LIF file for archiving. Selecting on each individual image will allow saving files separately.

3. Click 'Save' to store data to hard drive.

Shut down procedure

1. Turn off the lasers under Configuration tab / Laser Config. Icon
2. Close the LAS X software
3. Shut down the computer
4. Turn off the laser emission key. **Wait 10 minutes for cooling than turn off the laser power.**
5. Switch off the fluorescence lamp (first the shutter and than the power) and note the hours in the logbook.
6. Fill in the logbook.
7. Take off the sample
8. Close the diaphragms
9. Lower the objectives all the way down
10. Clean the objective lens
11. Put on the plastic hood on the microscope