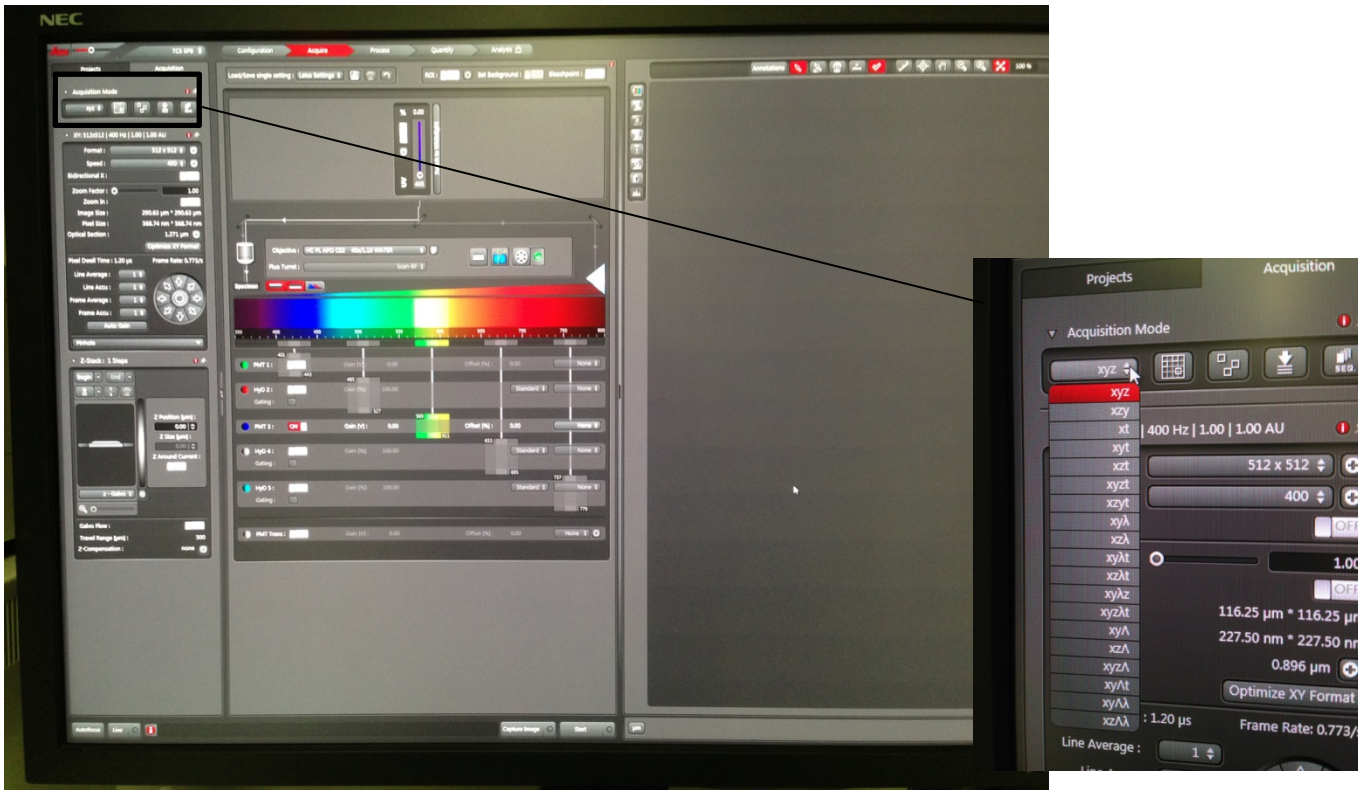
Confocal imaging on the Leica TCS SP8

1) Turn the system on.
2) Use “TCS-user” account.
3) Start LAS X software: 

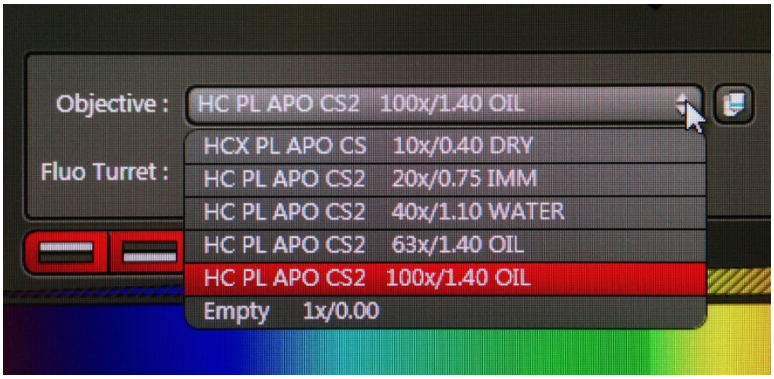
4) Do not touch the microscope while the software is initializing. Choose your options:

Turn Resonant on to use the fast scanner.
Turn STED on for STED imaging.
Turn off any option that is on and you will not use.
The default settings are those of the previous user.

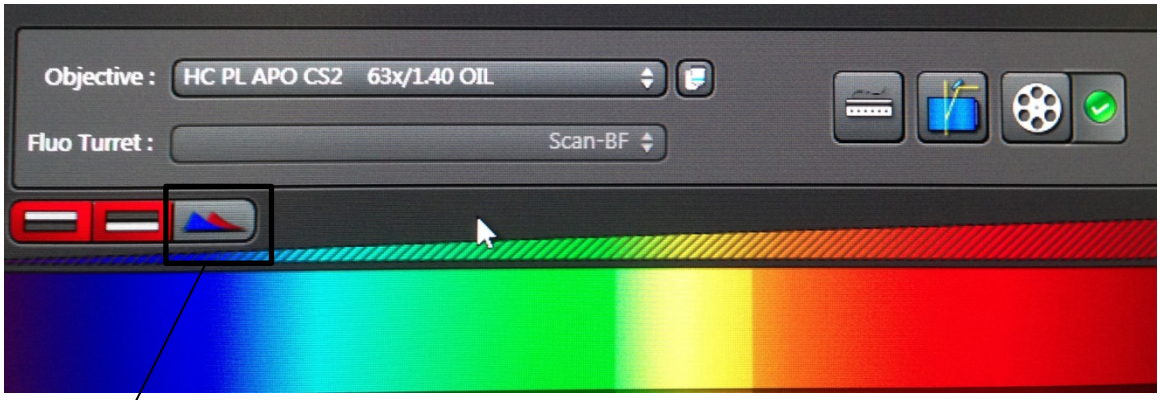
5) In the top left choose type of acquisition



6) In the middle, choose your objective:



7) Once the chosen objective is in place, place your sample using the correct immersion liquid. Find the area you want to image
8) Configure the excitation and emission settings. The easy way to do this is to use the dye assistant.

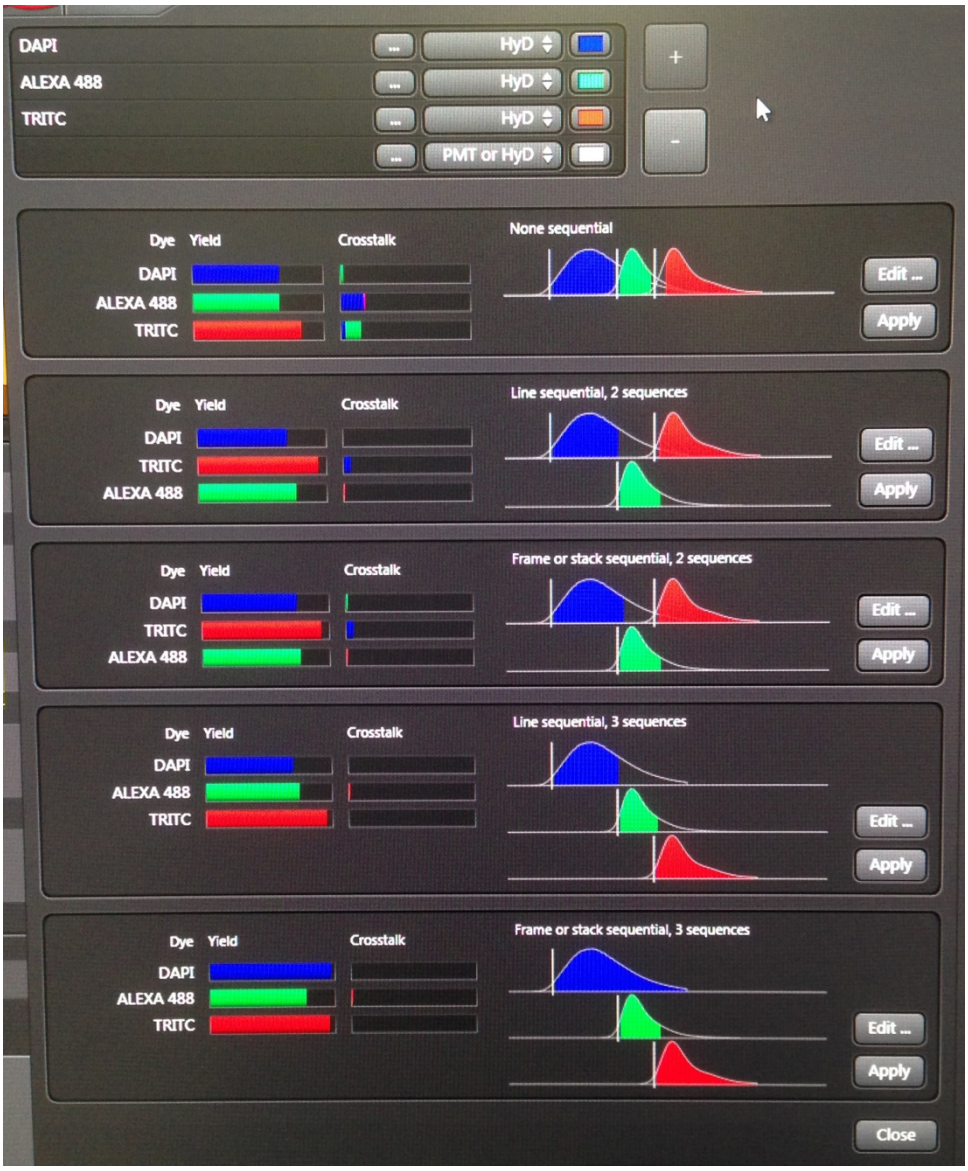


Click on this button to open a new window:

Select the dye you wish to image

And the detector

Select all the dyes you wish to image. In the following example, Dapi, Alexa 488 and TRITC are selected.



The window shows emission spectra and possible options for acquisition: none sequential (= simultaneous), line sequential or frame sequential. Choose how you want to image considering possible cross-talk and speed of acquisition required. Click apply.
If the lasers are not on, the software will query, click yes and wait for everything to be set up – this window will close when it is done.

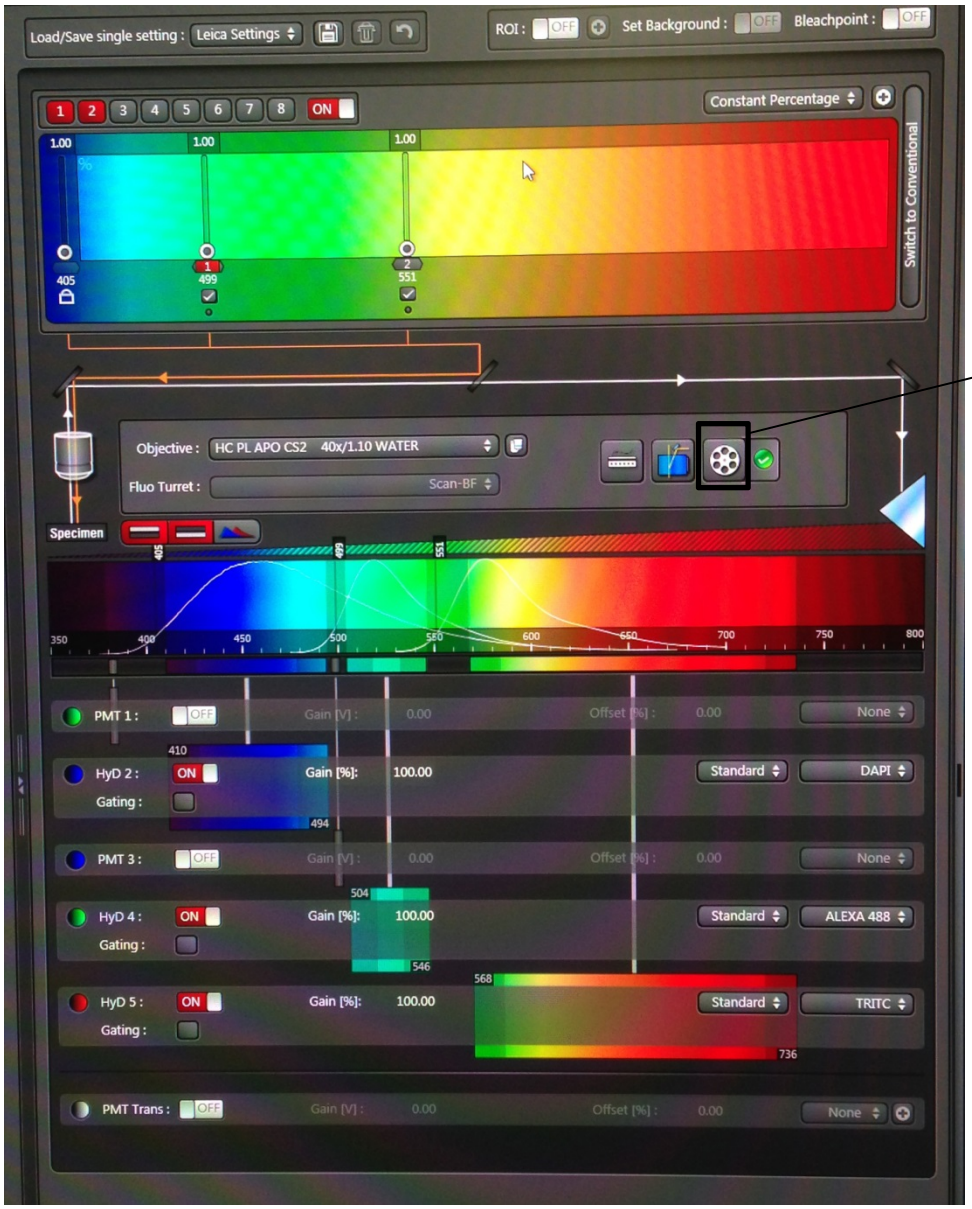
When this window closes, click on switch to whitelight to see the chosen wavelengths. Check the selected excitation wavelengths and the chosen emission bands. Make sure that:

- these match the selected dyes,
- emission bands are at least 8nm away from excitation wavelengths.

To see the excitation and/or emission spectra of the selected dyes, right click on the spectrum below the dye assistant.

Click **live** on bottom left to see the image. If the acquisition is frame sequential, this will only show the selected frame. To acquire faster, click on bidirectional X

Click to turn on.



In this window you can also set

gain and offset for the PMTs

gain for the HyDs

gating

Click on this image (Fluorifier Disc) and check that “auto-select” is selected.

Notes:

If you choose line sequential and then switch to frame sequential, make sure the laser is on for both sequences (the software sometimes turns it off).

In a sequential scan, ensure that wavelengths and detector bands are the same for all sequences (even when for sequences in which they are not used) to maximize acquisition speed.

Recommended strategy: Choose none sequential, check wavelengths and detector bands, check for crosstalk, if crosstalk cannot be eliminated, then switch to sequential by clicking on seq in upper left under acquisition mode. This will activate a window at the bottom left. Add sequences by pressing the + button, then choose which excitation line and detector is active for each sequence by suppressing the others: for the excitation lines, lower the power of the ones you are not using to 0 (do not turn them off) and turn the detectors off.

9) Once you are ready to capture an image, check that the pinhole size is 1 Airy unit, set a line or frame average, and optimize the pixel size:

Should be 1.00 AU. If not, click on Pinhole at the bottom of this window and select 1 Airy Unit.

To select a good number to average, set frame average to 8, click capture image on bottom center and watch the image, count the number of averages that improve the image. Use this number either in line average or frame average.

Click on Optimize XY Format to set the optimum pixel size for the objective used. This will change the format to accommodate the pixel size without changing the zoom. If you want to capture a smaller image, decrease the format and increase the zoom to maintain approximately the same pixel size.

Click on "Start" to capture images in all colors, a z-stack or a time lapse series as explained below.

Note:
When you are searching for a new area to image or adjusting the focus, reduce the format to 512x512 before clicking on "live" so the image refreshes fast. Return to the correct format before capturing the image.

10) If you want to acquire a z-stack, go to the Z-stack window in the left column.

There are two ways to set the stack:

i) If you are focused in the middle of the stack and you know how thick a stack you want, make sure the Z position is 0, turn on the "Z around current" and set the Z size to the desired thickness.

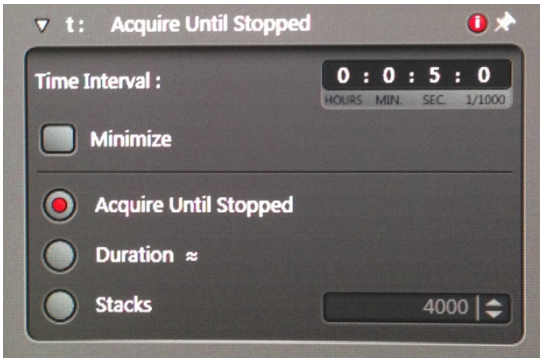
ii) Focus to the top of your sample (either with the focus button on the USB control panel or by hovering the mouse on the picture of the slice and using the mouse wheel) and click the begin button; then focus at the other end of your sample and click the end button.

Make sure that “System Optimized” is selected, this will adjust the z-step size to the optimum for the objective used and select the appropriate number of steps.

If you are acquiring a thick sample, you may want to use Z-compensation. Click on the plus sign to set this up:

Press start to acquire a full stack

11) If you want to acquire a time lapse series
Under acquisition mode (top left) select xyt or xyzt for a time lapse of a single plane or a z-stack respectively.



A new window will open in the left column: Choose minimize to acquire as fast as possible or a time interval if you would like to acquire slower than the minimum time.
Choose the number of time points to acquire (stacks), the duration of the time lapse or acquire until stopped.
Press start to begin time lapse acquisition.

Notes:

- Wipe the immersion liquid off after every slide.
- During acquisition, don’t be tempted to do something else on the computer!
- Warning: phone signals may be picked up by hyd detectors