**Multiphoton imaging on the Leica TCS SP8**

Notes: There is a choice between the Clarity objective and "normal objectives" – if the one you want is not on, let Ingrid know and she will change it. We have a few options for the detector filters, let Ingrid know what you need and she will change filters.

1) Turn the system on:
    i)   Turn the computer on.
    ii)   Turn the power for the detectors (one box on the left, one on the right)
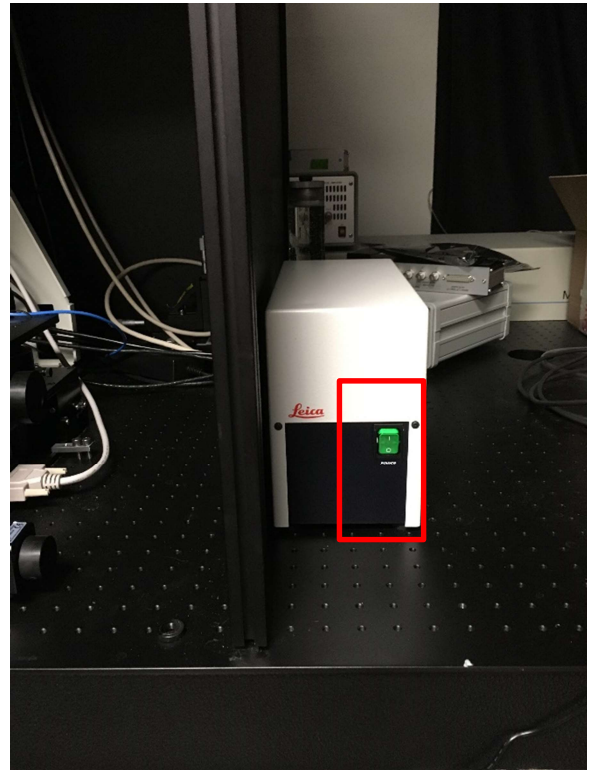
    iii)   Make sure nothing is blocking the stage. Turn the power strip on (under the cover to the left of the microscope).   Turn the piezo on if you are using the 25X water dipping objective.
    iv)   Turn the Mercury arc lamp on if you want to use epifluorescence to find your sample.

2) Log into computer. Use "TCS-user" account (password: Confocal).
3) Start LAS X software:

 The software starts initializing and opens a new window.

4) Choose your options:

   Configuration: machine_MP-Laser_ON

To use the fast scanner make sure resonant is on. For the normal scanner resonant needs to be off.

5) Once the software is up, choose the type of acquisition you want in the top left. xyz is the default.

If you are using the resonant scanner the speed of acquisition is 8000. If you are not using the resonant scanner, the speed can be varied from 10 to 1800Hz; the default is 400, you can increase to 600 and turn the bidirectional mode on for faster scanning without a loss of pixels. If you choose a higher speed the number of available pixels decreases and the system automatically zooms in.

6) Turn MP laser on. The system has a 488 line for one photon confocal imaging and a tunable laser (690 to 1040nm) for two-photon imaging.   The MP laser needs time to warm up and start pulsing, so turn it on at the beginning.
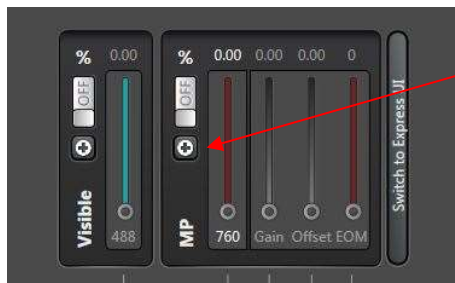
Click on the plus sign above MP

This opens a new window. At the bottom of this window, click on the arrow next to Laser to expand that window. Click the MP laser on.

After a few minutes there will be output power and the mode will change from CW to pulsing (as shown in the picture).

You can continue setup while the laser warms up.

7) Set your objective. Changing between the "normal turret" and the Clarity objective is done by Ingrid. In the normal turret, the 25X objective is attached to a piezo, when you change the objective, please be careful to rotate it so that the piezo cable doesn't get caught.
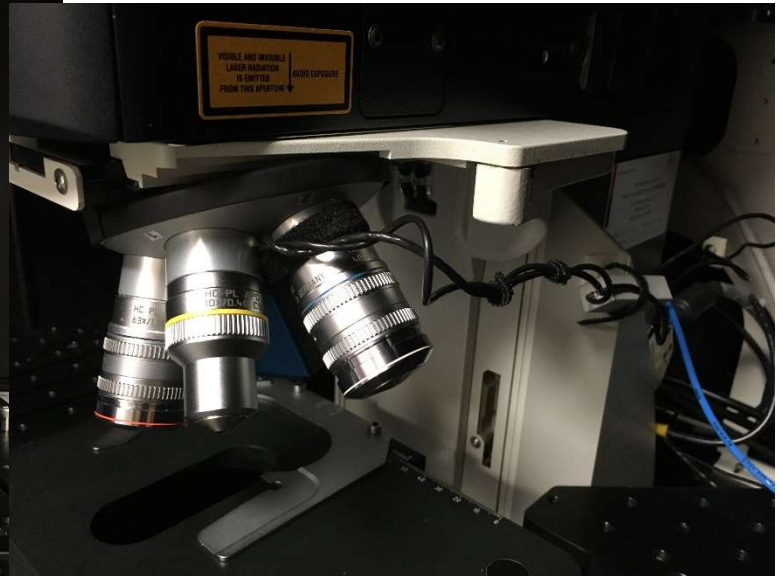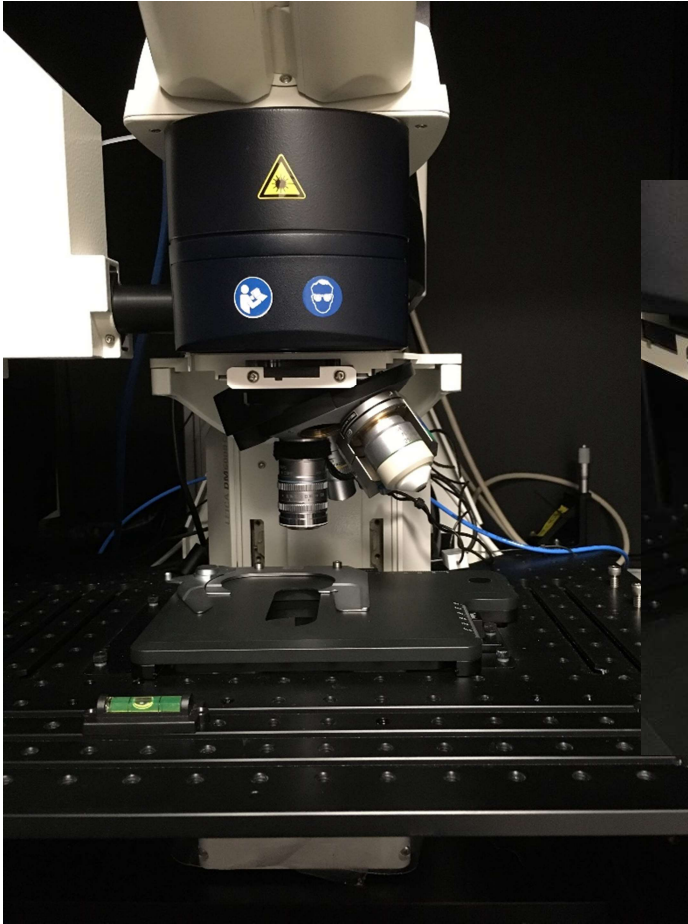

8) Once the chosen objective is in place, place your sample on the stage.
This is a large stage, height controls are on both sides and should be used together, check that the stage is level and make sure you do not move it past the last mark on the right control.
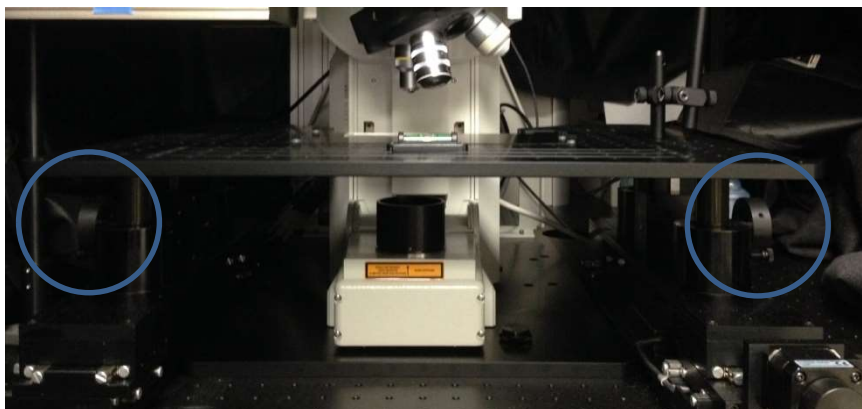
XY controls for the stage are on the microscope controller next to the computer:

XY stage controls

Z controls

Fine

Coarse

This controller has several screens that can be selected on the left column, it is a touch screen.
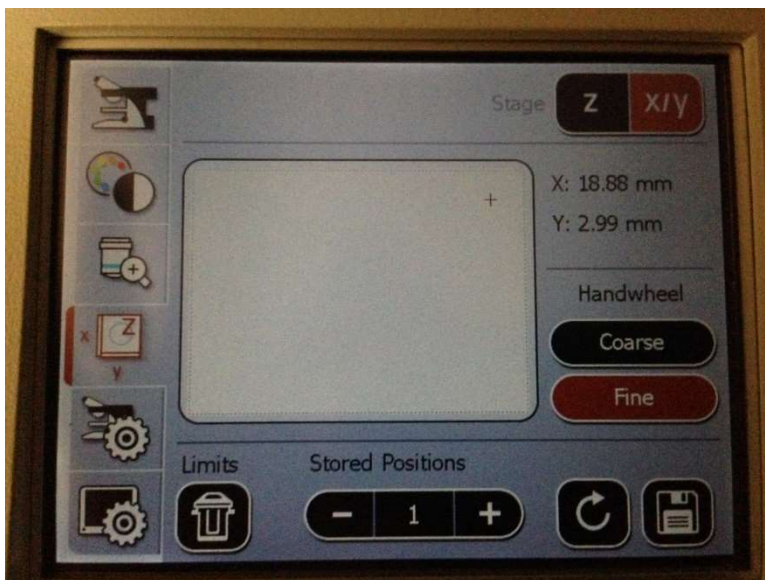
The fourth icon selects the xyz controls. On the top right, select x/y or z to switch between the two controls. The x/y moves the stage, here you can select coarse or fine movements for the wheel, and you can center the stage.

The z control moves the objective nosepiece down (closer to the sample) and up. Home is set to the top position, holding the home button will move the objective up.

This symbol should be set close to but above the focal point. Pressing it will move the objective towards this point.

Note: These positions can be cleared and set on this window, when you use these buttons make sure they are correct.

To find your sample in epifluorescence, choose the second icon on the left column:

Choose **Fluo**, then choose your filter:

I3: 488/ FITC/GFP

N21: 568/TRITC/RFP

A: DAPI/UV

Finally press IL-Shutter to open the shutter (and close it when you are done).

9) Once you have found your sample, lower the black cloth to cover the front of the scope and close the curtain around you.

On the MP laser window, tune the laser to the desired wavelength. Use the slider to select the wavelength or type it in and click tune.

Select the objective you are using from the drop down window to set the GDD curve

Open the MP-shutter by clicking and maintaining the click until the circle is red.

Then go to the laser window and turn the MP on.
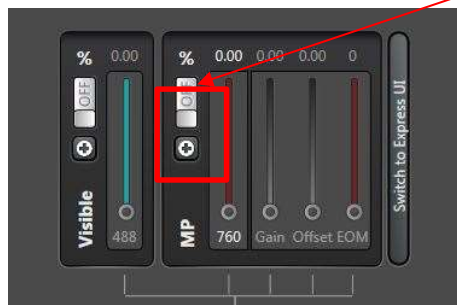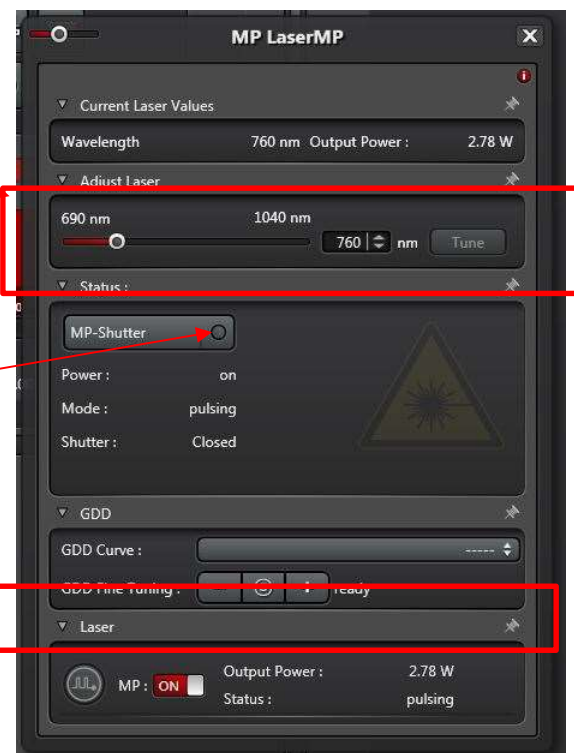

Next move the EOM slider all the way up.

The three sliders ("wavelength", gain, offset) control how much laser power reaches your sample. The offset is set for each wavelength, so that when the first two sliders are at 0, no light reaches the sample. You can adjust this if needed (in step 11).

10) Select detectors. For two photon imaging you can use the external Super HyD detectors (there are 4) OR the internal PMT. The default one on is the PMT, do not turn any external HyD's on before the curtains are closed!
Make your selection by turning the correct one on. The Super HyD have physical filters (see note at start), you can only set the gain on the detector –start with a low gain, they are very sensitive. For the PMT you can select the detected bandwidth, the gain and the offset. Also, open the pinhole if you are using this detector, two photon excitation is confined to the focal volume and does not need a pinhole.

Default detector is the PMT, if you want to use this set the gain and offset.

If you want to use the external detectors, click on the arrow next to RLD.
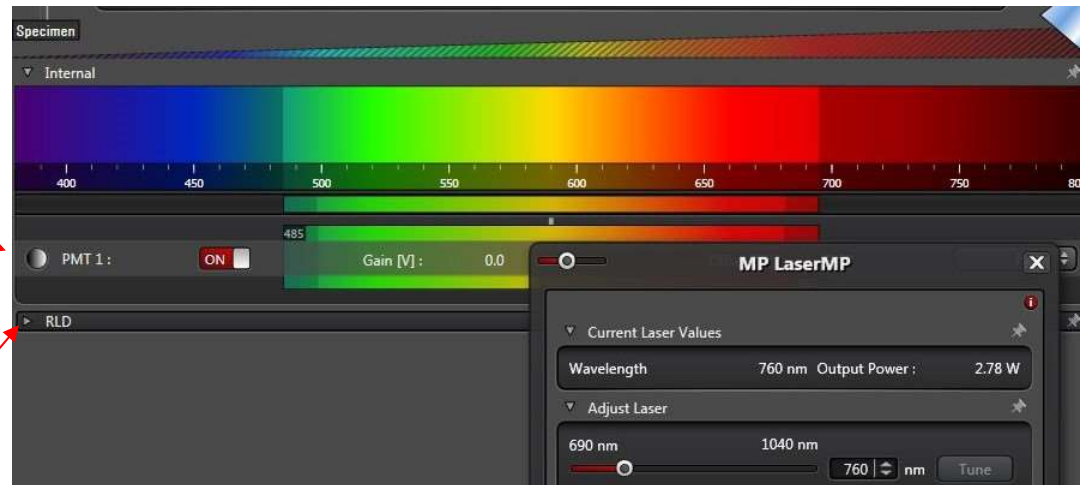
This opens the list of HyD's. Turn on the ones you want to use. Check the diagram on the wall to see which filter is in, ask Ingrid if you want a different one. Filters are:

1: 529/24 or 525/50   - green

2: 585/40 or 587/35 or 618/26 - red

3: 440/20 or 433/24 – DAPI or SHG – blue

4: 482/25 or 483/32 or 466/40 - cyan

Start the detectors with a low gain.

11) Find image: Click **live** on bottom left to see the image. Increase the gain on the MP laser (left two sliders as explained above) and the gain on the detectors to get a good image. You can set up a z-stack, a time lapse, and/or regions to image.
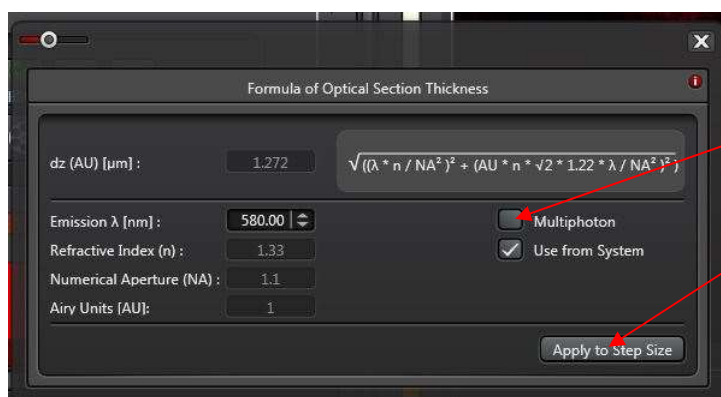
12) To acquire a z-stack, go to the Z-stack window in the left column. The default selection is z-galvo, this is only active for the 25X water lens, if you are using any other lens, change this to z-wide.

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, set the Z size to the desired thickness. If you change the mid focal plane, hit re-center.

ii)   Focus to the top of your sample and click the begin button; then focus at the other end of your sample and click the end button.

If you want the optimized step size, click on the plus sign next to it, this opens a new window:

Formula of Optical Section Thickness

dz (AU) [µm] : 1.272 $\sqrt{(\lambda * n / NA^2)^2 + (AU * n * \sqrt{2} * 1.22 * \lambda / NA^2)^2}$

Emission λ [nm] : 580.00
Refractive Index (n) : 1.33
Numerical Aperture (NA) : 1.1
Airy Units [AU]: 1

Multiphoton
Use from System

Apply to Step Size

Select Multiphoton and Apply to Step Size.

Close window.

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

13) Optimize acquisition settings:
   i)   To select a good number of averages, 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.
   ii)   Set number of pixels: If you are not using the resonant scanner 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

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

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.

14) When you are finished, save your file, remove your sample and clean the objective and the stage if necessary. Leave the objective at a high position and the stage at a low position.
15) Close the LAS X software, when the software asks if it should put the laser to standby say yes, unless the next user is already here. Transfer your files to the server.
16) If there is no other signup within one hour, turn off the epifluorescence lamp, the power strip and the two detectors. Shut down the computer.

Notes:

- During acquisition, don't be tempted to do something else on the computer!
- Warning: phone signals may be picked up by hyd detectors