Leica Digital Light Sheet (DLS) Confocal Users Guide

Additional guide from the web:
https://micr.med.wayne.edu/pdfs/leica_sp8_users_manual.pdf
Video tutorial of "SP8" confocal microscope:
https://www.youtube.com/watch?v=_TH86da5Nuw

This is a guide is for **CONFOCAL MODE**, not light sheet mode.
Use "TCS SP8" setting at the top of LAS-X software.

AVOID THESE DAMAGING EVENTS:

- Bumping Objective Lenses with the stage Insert or your sample (glass slide)
- Getting nail polish or other damaging substances on the objectives, or on any microscope components (e.g. Oil on Dry lenses)
- Using very bright laser intensities
- Any rough handling of any part of the instrument
- Frequently turning on and off the lasers
- Turning off the power strip switch before software indicates it is okay

Note:

- **If you see a <u>warning</u>, turn down excitation lasers (warning appears at bottom of screen)** Detectors should not be exposed to excessive signal. Keep laser excitation below 10% if possible. Slow scanning can also send too much light to the detectors.
- **This reboot procedure can solve many issues:** close LASX software **WAIT UNTIL THE SOFTWARE FULLY CLOSES** (laser cool-down), shut down the computer, turn off microscope control power (power strip). Wait 5 seconds and turn these back on in the reverse order. In general, however, please avoid turning on and off the lasers.

System Startup

Look over the microscope area. Find me (room 8-801F), email jwm2175@, or Slack message if things look unusual.
Turn on:

1. Power Strip, wait 2 seconds (orange switch, far left)
2. (if needed) Computer (button on PC)
3. LASX (LEICA) Software
   Avoid using microscope controls until LASX software is running.
   Configuration: **"machineDLS", "DMi8"** as indicated in the image below.
   Initialize stage: Chose "yes" to "Do you want to init the stage" (**Initialize Stage**)
   <u>You must tilt the microscope condenser arm back</u> to initialize the stage (stage will move across range).
   Be patient, startup can take a few minutes.
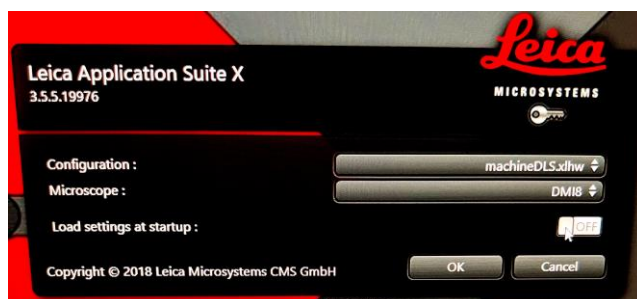
System Shut down

- **If using oil or water immersion: gently wipe lens with lens tissue**
- **Lower the objective lens to lowest point**
- **Remove your slide**
- **Set to 10X objective lens**
- **Close LASX software**
- **Transfer your data to an external hard drive.**
  - **Data can be temporarily saved to the E: drive under your lab group**
  - **All data > 2 weeks old may be deleted.**
- **Sign Out of system (Control Alt-Delete > Sign Out)**
- **Shutdown the computer.**
- **Turn off power strip (if LASX HAS FULLY CLOSED).**
- **Put dust cover over microscope**

Locating your sample by-eye (once LASX software has started)

- Use **Leica touchscreen** to find your sample by:
- FLUOR, IL-shutter (=Incident Light (fluorescent))
- BF TL-shutter (=Transmitted Light, i.e. bright field, or phase) => BF is Currently Disabled
- Focus should initially be at zero or negative numbers.
- Carefully focus up using microscope knob or the "joystick"
  Joystick has course and fine buttons
- Look at the Z position, touch screen x-y-z to orient your focusing. "In focus" seems to be around **1.1 mm**.

The touchscreen looks like this:
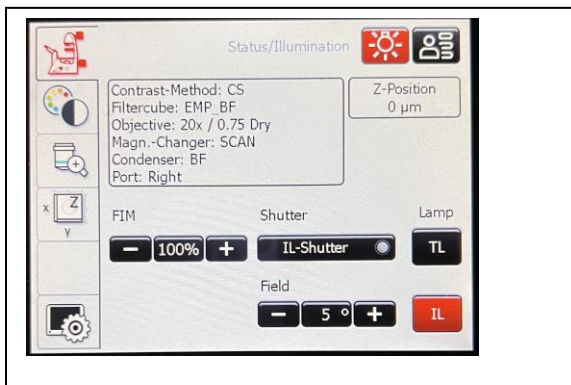
If by-eye fluorescence does not turn on: go to objective tab and press the "eye" button.
You can also go to the color wheel tab and press the FL button. The CS button sends controls to the confocal.

Lasers Available on the DLS

405, 488, 552, 638

Detectors Available on the DLS

2 HyD detectors (more sensitive), 2 PMT detectors.

The camera mounted on the condenser arm is for Light Sheet acquisition

Image Acquisition Tile Scanning

Default file names: I suggest setting the default project name for each day and start with the date in the form: **YYYY-MM-DD**. Your files will then sort nicely by date: **Projects (upper left) > New Project Settings (lower left, <u>see image</u>)**
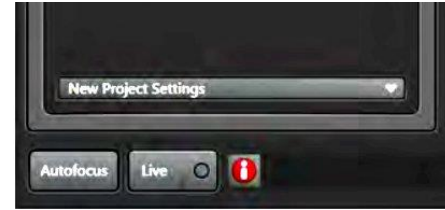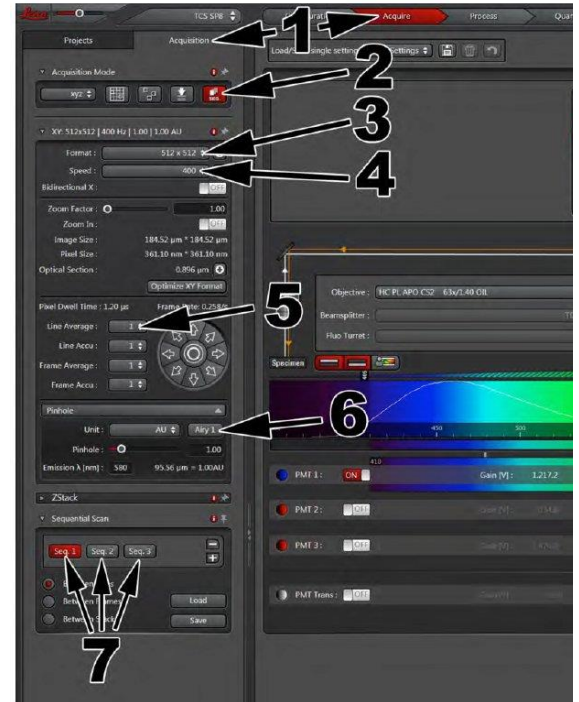
Set up Acquisition settings

In Acquisition menu <mark>(1)</mark>

If you have a **previous experiment,** open that image and choose **"apply"** (under 'Open Projects' tab) to use the settings of the open image. (*Note, old images are subject to deletion, save your "settings" images to your usb drive)

You can also save and open ".seq" settings files.

*Note that this may not save scan speed zoom and image averaging.

First time setting up your acquisition

- Set Objective to 10X dry.
- Set scan speed to 700.
- Set pixel size to 512 x 512 (or 1024 x 1024). Note pixel size and zoom.
- Set to Sequential Scan <mark>(2)</mark> **you will see your different channel settings under "Sequential scan"** <mark>(7)</mark>
- Set frame average to 1 during setup. In final acquisition you may want to average 3 or more (averaging improves image quality, slows acquisition). Frame averaging allows more control over between channel settings (e.g. different pinhole size, different detector ranges).
- You may want to use the **dye assistant** (black arrow) to set your dyes/laser detector setup.
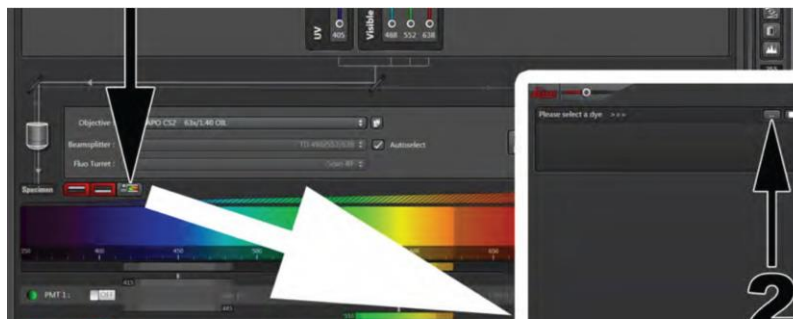
- **Dye Assistant**: type in the name of your dyes: e.g. DAPI, GFP, Alexa 647...

Dye Assistant will try to choose the best settings for your Sequential Scan. The lasers and detectors are listed above. Manually setting them is not difficult, but you may want to look up dyes on a 'spectral viewer' to set

the detector ranges. Generally, use no more than 2 lasers, and 2 detectors per "Channel" (=Seq = track). 1 channel (Seq) for each dye gives the least crosstalk through but is slower. Don't image DAPI and A488/GFP in the same channel as DAPI emission extends thru green. Line acquisition is faster than frame scanning, frame scanning allows more settings changes between channels.

For 4 dyes, use 2 Seq, 2 laser/detectors per channel. If concerned with crosstalk, image each dye in its own channel.

** Note: The DLS will **sometimes get Detector wavelength ranges wrong**. **Check carefully**. This will especially happen if moving back and forth from Line Mode to Frame Mode

Line Mode vs Frame Mode:
Frame mode allows more setting options, such as changing detector wavelengths between SEQ.
Line Mode allows imaging all SEQ channels in Live imaging (frame mode is one at a time).

Go thru the Seq 1, Seq 2, Seq 3, … and set the laser intensities and detector gains for each channel. For initial imaging:
- Set the 405 laser intensity to ~0.5
- Leave the other lasers intensities at 1 (default).

I prefer the simple "Classic" view of the lasers. Click the ON button for the lasers, if needed.
Adjust laser power by <u>mouse wheel</u>, <u>typing the numbers</u>, <u>or the slider</u> (difficult to control).
<u>**LASERS ARE VERY STRONG!!! USE CAUTION AT POWERS ABOVE 5%. Be careful typing the intensity.**</u> Samples can photo-bleach or photo convert.
 (e.g. DAPI can photo convert to green and red).
Keep the detector range at least 10 nm away from the laser (more may help).
Be mindful of crosstalk (may want to adjust detection away from other dyes).

<u>**Make sure you are in focus during setting adjustments.**</u> Out of focus light is much dimmer.

If you have multiple dyes in a single "Seq" (Track), check for "cross talk" by setting each laser to 0 to see the effect on the other channels. Sometimes you must increase laser excitation for a particular dye so that cross talk into that dye is reduced (particularly for the PMT detectors).
**Click "LIVE" (live viewing) to set your intensity, gain (and offset).**
In the imaging window (the right panel, live images). Focus your image. Click on the image in which you want to set the gain and intensity.
Adjust gain and intensity using the "Smart Dials". <u>**Clicking in the image window to selects which image the Smart Dial controls.**</u> (or you can click lasers or detector and adjust by software).

Use Range/Saturation indicator (1) (saturation=blue color) and **histogram** (bottom of same panel) to assess your signal.
Generally, you want signal that is just less than saturation. Avoid saturated pixels (and use 12-bit acquisition) if you are quantifying your images.

Repeat gain and intensity adjustments for all your dyes/images.
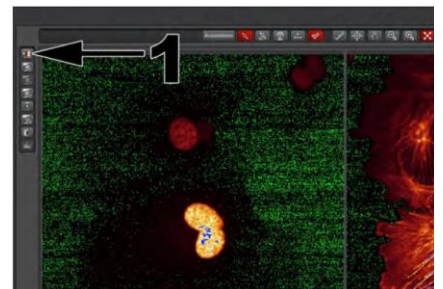
*Tip: If your image has bright signal across the glass that looks like shearing / diffraction rings, this might be light scattering:

Solution: Drag the left side of the detector wavelength further away from the laser (or, you can move the laser further away from the detector). Grab at the shaded region on the left of the activated detector to move it.

Save this adjustment setting so that it is not lost (either save a .seq file or capture and image), the detector and laser lines can easily be reset.

*Tip: view saturated pixels by clicking the box on upper left in the image viewing window (toggles thru viewing options). Leica calls the saturation view, "glow over" and "glow under" (pix=zero intensity). Glow under is very bright green. To view only the "glow over [=saturated]" and avoid the bright green zeros pixels: > configuration > uncheck "glow under".

*Tip: for very weakly fluorescing dyes, you may want to open the pinhole from 1 (standard) to 2 or 2.5.

Transmitted Light Imaging

(Transmitted light is currently disabled due to light sheet set up)

Click PMT Trans **(1)**. Typically do this in the seq with the 488nm laser. You will see a gray scale image when you click "live". Adjust the gain and offset to get a good image.

Using Navigator for Tile Scanning (very brief)

In Acquisition menu, Click the **Navigator button**, green box below (actual may look somewhat different):

(You can return to the acquisition menu by clicking close or X-out (both are upper right). Navigator settings will be remembered when you click it again)

In the Navigator click **"Spiral"** (lower left) to rapidly scan across your slide. Zoom in or out with mouse wheel, drag with mouse, and control most of the acquisition settings with menus on the left. The white box is your image field.

Click "Spiral" to scan across your slide. Stop by clicking again.

For <mark>FASTER SPIRAL</mark>, spiral in a single track:

You can save an image, then delete the other SEQ (tracks) in your sample, then **spiral in a single track**. When you have defined your scan region, select your saved image image and "apply" to get back all your channel settings
OR
You can turn off "SEQ" in the Scanmode button. It will ask you to save your current SEQ settings. Save them. Then do you spiral scanning and when you want to image all of the channels click SEQ button, then in the Sequential button you can load the settings you just saved (I don't know another way to Spiral as single track).
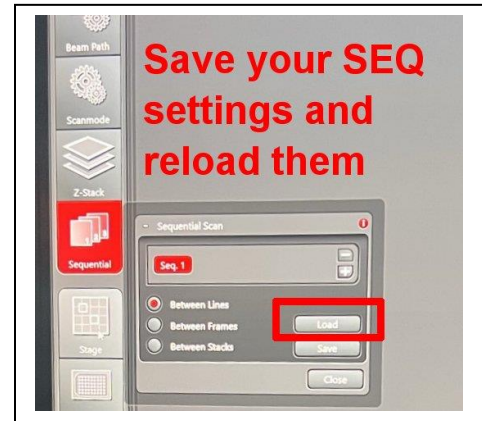
Use the Region of Interest (ROI) tools at the bottom of the screen to outline the region (tiles) to image. Click a second time to set the ROI. Acquisition fields will be shown as a gray grid.

<u>Set tile merge settings</u> using the **Stage Tab** button (lower left):
      Merge images during acquisition: **ON** (or you can merge later in Mosaic Merge tab)
      Set the % overlap to 10 or 15%
After acquisition, new images will appear in the Projects tab. Both Merged and unmerged should be there. Delete un-merged if you are happy with the merge.

Autofocus points (AF+)
Use this to automatically adjust focus at different points in your tile scan.
Click **AF+** points at several places in your large tile image.
Click the **autofocus tab** at the left to see a list of autofocus points and autofocus method. You can delete your autofocus points here.
Set range to something like 30 microns. 80 microns is usually too large and will take too long.
Set the autofocus method (default should be okay).

Manual focus points (F+)
Use this to manually set Z position during the tile scan acquisition.
Manual focus points are set with the "F+" tool at the bottom of the window. Click F+ (circled red), then click on a few tiles of your proposed scan to insert Focus Points.

Click **focus map** (lower right) to view a list of the focus points and their current Z positions. During Live Image, double click on a focus point number and the stage will move to it. Manually focus with the **Smart Dial** or joystick. When in focus, click "**set Z".** Repeat this for multiple focus points.

Additional web guide for setting focus points:

https://cpb-us-e1.wpmucdn.com/sites.northwestern.edu/dist/a/560/files/2020/06/2020_DM6B-Quick-start-guide_LAS-Navigator_done.pdf

A good demonstration of LAS tile scanning is here:

https://www.youtube.com/watch?v=nzYSE468qNQ

Now that your tile scan is set up:

Go to acquisition settings and set **average to 3** (this was set to 1 in order to more rapidly set up acquisition and tile scan settings, you may have better results with different settings). Check one more time that the brightness of each channel is not over saturated and has a nice histogram.

Click Acquire (lower right) to capture your image tiles.

John Murray   2022-07-21
jwm2175@ cumc.columbia.edu
(This guide will be routinely updated and may contain errors)