Leica Digital Light Sheet (DLS) ==LIGHT SHEET== Operation Guide

Brief overview of this Light Sheet system: https://youtu.be/ne9xNT1OwEU

SP8 Microscope guide from web (not light sheet mode): 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 guide is for ==LIGHT SHEET MODE== (not confocal mode).

Use "LightSheet" setting at the top of LAS-X software.

==AVOID THESE DAMAGING EVENTS:==

- Scratching / damaging the lens and twinflect mirrors
- 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)
- 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 5% if possible. Slow scanning can also send too much light to the detectors.
- **Reboot 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.

==System Startup==

Turn on:

1. Power Strip, wait 2 seconds (orange switch, far left)
2. (if needed) Computer (button on PC)
3. LASX (LEICA) Software
   Don't use the microscope controls until you start LASX software.
   Choose **"machineDLS"** and **"DMi8"** as indicated in the image below.
4. "Do you want to init the stage?" (Initialize Stage). Answer yes. Condenser arm must be titled back.

Be patient, startup is slow.

System Shut down

- Close LASX software, allow software time to close (can take ~5 min).
- Transfer your data onto USB drive : This make take a long time with large light sheet files. You can allow this to run overnight. The Powerstrip can be shut down while the computer runs.
- Raise and remove the detection objective
- Remove the twinFlect mirror
- Clean everything. Use mostly water, or mild detergents like "Splash" (ethanol can be used at the end).
- Use cotton swabs and lens paper and very gentle touch: **no strong physical force to lenses or mirrors**
- Sign Out of system (Control Alt-Delete > Sign Out)
- Turn off power strip (AFTER LASX HAS 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 (bright field / transmitted light does not seem to work with the light sheet condenser in place)
- Focus up using standard microscope "Z-wide" dial knob, or use the "joystick"
      Joystick has course and fine buttons.

The touchscreen looks like this:
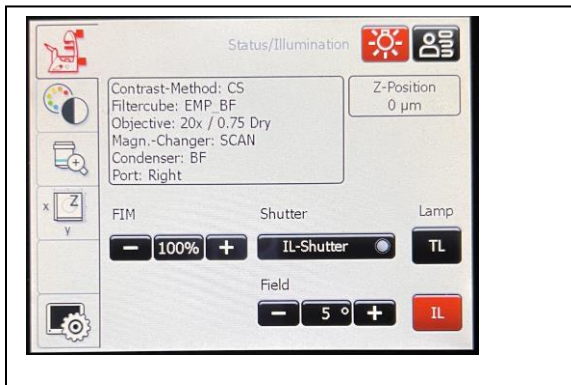
If you can't see by-eye fluorescence, go to objective tab and press the "eye" button.
You can also go to the color wheel tab and press the FL (fluorescence wide field mode) button. The CS button sends controls to the confocal.

Leica SP8 Digital Light Sheet operation

Sample prep

- Use a glass bottomed dish with minimum 20 mm diameter (e.g. MatTek p35G-1.5-2-C) otherwise mirrors cannot reach imaging depth.
- Twinflect mirrors have an X direction gap of 7.8, 5, or 2.5 mm, so your sample needs to be narrower than the one used and should be raised from the coverslip surface ~2 mm (not too high or detection lens won't fully immerse during imaging). Z-range (Z-galvo Z-stack depth) is about 1.5 mm (see image).
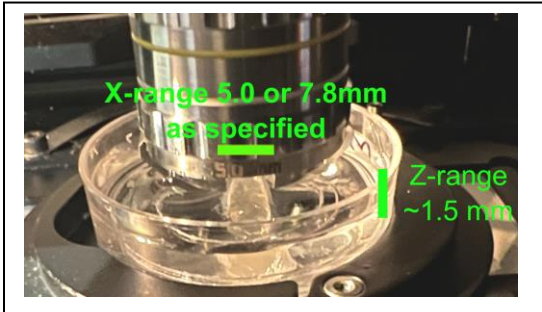
- Samples can be glued to the dish using silicone or superglue (or possibly nail polish or others). Immersion can include Histodenz, Glycerol/H2O (80% glycerol has ~1.46 RI and can be a replacement for Histodenz) , PBS or water.

Detection lens, Twinflect (TF) mirrors, Illumination lens
see: DLS-Objective-Mirror_combinations-2017-05-MM.pdf
Detection lens (objective) is mounted on the condenser arm (top or upright imaging). Camera is on the condenser arm.

Detection	5X	10X	25X
Mirrors	7.8 mm	5 mm	2.5 mm
Illumination	1.6X	1.6X or 2.5X (may not work)	2.5X, 5X (may not work)

Remy typically used: 5X detection objective, 7.8 mm mirror, 1.6X illumination

Start up, calibration, and instrument use

- The small black knob on right side of condenser head is used to rotate the mirrors circumferentially.
- **Use a good strong, contrasting sample to set up the LightSheet.** Use a standard if necessary.

1.   Make sure "DLS" stage insert is in place (screw in with smallest hex/allen wrench).

2.   Take out the detection lens. Carefully screw in TF mirror ring. **CAREFULLY** screw the lens on to the condenser using both hands. Sometimes this is hard and threads don't catch. Take a break, reset and try again if you are having trouble. **Don't use force or threads will strip!!!**

3. Align the twinflect mirros so they are perpendicular (so your sample fits between) using the small black knob (see image above).

4.

5. **Use Modeling clay to hold down your dish to the stage. This can really help (may problems occur bc the dish gets bumped / angled).**

6. Select illumination lens. Bring sample to the stage. Add enough fluid to dish.

7. CAREFULLY tilt the condenser arm with the detection lens into the dish containing your sample. **Make sure nothing will hit/crash as it is lowered** (i.e. lens is all the way up (silver knob)), dish in center, sample is between the mirrors). NOTE: Detection Objective lens must be completely immersed into liquid

8. Rough focus the illumination lens to your sample using "Z-Wide" microscope focus knob on scope body. Use side excitation of a headlamp or standard FL shutter excitation. This step is primarily to look at the physical characteristics of your sample and mirrors. Note: illumination is low magnification (1.6, 2.5 or 5X) so <u>fluorescence will be dim</u>. It can help to <u>shine a headlamp on your sample from the side</u> to see your sample and the mirrors.

   <u>Make sure black knob on condenser arm above silver knob is flipped to DLS:</u>

9. **Go to LAS-X software> Calibration tab > Detection (red) button**
   <u>Instructions read:</u>
   Aim: Bring sample in focus of detection objective.
   -Oculars: Move objective holder down to see your sample mirrored
   -Chose the current detection objective/TF mirror
   -Camera: in "Live" Mode, use objective older to fine adjust sample in detection objective focus.

   This means: Click "Live" button (lower left) and focus the detection objective using the silver dial on the condenser arm. Image will be on screen in a kind of "bright field" contrast mode (see below).
   It **uses the bright Mercury Lamp shining into the camera**, so reduce mercury lamp intensity with its dial on the box and reduce exposure time to 5 msec in acquisition tab, upper left.
   <u>Check the excitation and DLS filter cubes (may have to change them).</u>
   Carefully focus with silver knob so to get an image similar to this (i.e. look for contrasting stuff as in image below. It may not look great, but focus on something):

   Don't let the mirrors crash into the coverslip or your sample!

Once in focus, go on to next step:

10.   **LAS-X software> Calibration tab > <mark>Illumination button</mark>**
Instructions read:
Aim: Define distance between illumination and detection objective
-Using Z-Wide, find highest reflection of mirror caps
-Run calibration routine ("Calibrate Z-Wide Pos").

This means: Click "Live" and on screen in "glow over" mode, focus microscope Z-Wide knob to bring the TF mirrors into focus using bright blue saturated pixels in glow over mode. <u>This is focusing the illumination lens on the mirror caps using a special laser imaging mode.</u> Adjust image normalization (max and min) settings to get the best sensitivity of blue.

Turn off "Live" mode and click and Click "Calibrate Z wide Pos" (bottom middle). System will perform a Z-Scan. **After this step, illumination (Z-wide) and detection (silver condenser arm knob) lenses are calibrated, don't alter them.**

Go on to next step:

11.   **LAS-X software> Calibration tab > <mark>Optimization button</mark>**
Instructions read:
Aim: Align focus of LightSheet with focus of detection objective
-Adjust height of LightSheet to the focus of detection objective by running calibration routine "Calibrate Z-Pos LS" (LS Position Z = y-Galvo)
-Toggle between mirrors and overlay opposed lightsheets by axial rotation of mirror device
- Fine tune LS Position X (z-wide): confirm the resulting change of "LightSheet Offset" by clicking "Use Currrent".

This means: LightSheet imaging mode is used to adjust the Light Sheet as it reflects off of the mirrors. You adjust the three "smart dials" (USB control panel) called LS Pos Z, LS Pos X, LS Pos Y to get the best possible image. "Z" is most important. But you should go back and forth between the dials.
When moving the dials, it helps to set their rotations per turn to highest, maximum values to travel through the entire range of the 3 dials (see image for how to set this):

Set USB Control Panel movement per turn
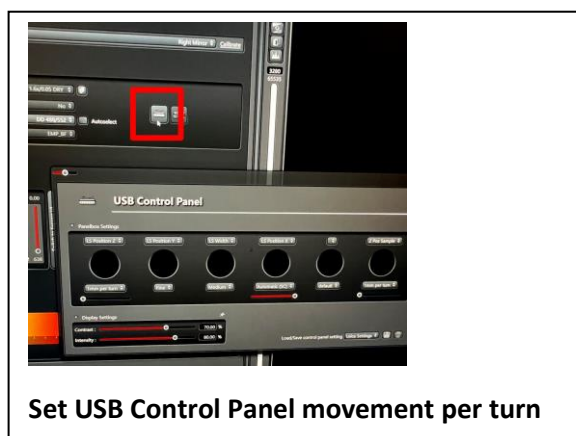
- **<u>I Strongly recommend getting good, contrasting fluorescence in your field, at your focus point</u>** <u>(if better fluorescence is above or below) then you will tend to set Z to the good FL instead of your focus field. To see appearance of sample in light sheet fluorescence: Get a decent Z, then go into Light Sheet mode and move your sample around to find a good region, then go back into optimization.</u>
- Don't worry too much about left and right mirror disagreement unless you plan to image with both mirrors.

- Automatic "Calibrate Z-Pos LS" did not work for me. Instead, manually Define mirror positions.
- <mark>**Note: Defined mirror positions (LS Pos Z) may not be remembered when you do your imaging.**</mark> This seems to be a bug. You may have to re-define the mirror positions (LS Pos Z) when you do your actual imaging.
- I find the "classic" view (switch to classic UI) of the laser settings to be much easier to understand (see image). The image also shows how to turn on the lasers (click the plus sign).

"Classic View" of Lasers is better.
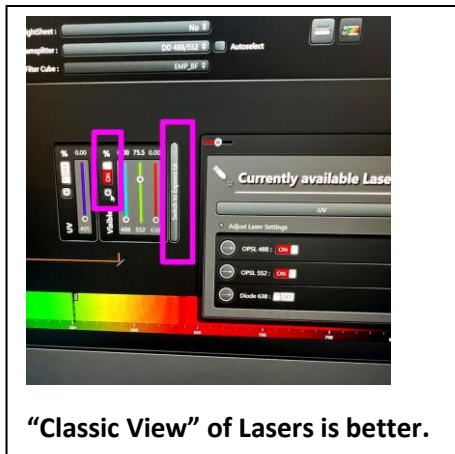
9. **LAS-X software> Calibration tab > <mark>Optimization button</mark> (continued…)**

To set the LightSheet Z, X, Y values:
(Note: Z-Galvo (sample movement) is disabled in this calibration step).
- <mark>**Set Fluorescence Excitation and Light Sheet filters appropriate for your sample**</mark> (~~or empty for light scatter~~)
  - "Empty" did not work for me
- Set LS Width to 48 to make a narrow horizontal strip.
- Adjust image normalization (LUT = dynamic range) for a good appearance.
- Click "Live" and focus on your sample using **LS Position Z**, i.e. find an image that looks good and bright. This may need very large change. Set dial settings to easily scroll thru entire range. (If you don't see any light sheet click "apply Default settings" (the current objective mirror combination must be specified) ==> This did not work for me!)
- Go to beam direction (Center of Window), click "calibrate", select right mirror, adjust Light Sheet **"LS Z position"** smart dial on-panel knob to focus on a structure, click define mirror position, select left mirror and find same structure, click define mirror position; narrowing smart dial on-panel "LS width" may help by increasing signal. Repeat multiple times.
  You could also try "Calibrate Z POS LS" to automatically Adjust Z Pos LS (==> didn't work for JWM).
  In a flawless system, the image will be identical from each mirror, but heterogeneity in light scattering molecules throughout an irregularly shaped sample inhibits this.
  Think of this as "focusing the Z". You should see a point where it looks decent, then Z movement either way makes it look blurry with elongated PSF. If it never looks good, there are probably bubbles or something physical interfering with the light sheet. Sometimes it gets better if you go to Light sheet mode, and then switch back to optimization. **If it doesn't look somewhat decent here, then I would lift up the condenser arm and reset the entire imaging.**
- Center the light sheet in vertical direction (up/down on the screen) **"Adjust LS Position Y"**
- Fine adjust mirror circumferential orientation (black knob)
  - Close "LS width" to illuminate a strip, activate mirror toggling, adjust black knob on condenser head so there is no jumping in the y axis. When finished open LS width.
- Center the light sheet in horizontal direction (in / out of the screen)**"Adjust LS Position X"**

- o Click "Live" and adjust the focus position of the light sheet (= the "waist" of the illumination beam) in the center by using „LS Position X".
- o Click "Use Current" under "LightSheet Offset" to confirm the position of "LS Position X".

Calibration is now "Finished" – except that typically you will re-adjust things when you start imaging and realize the image doesn't look great.

  ** As mentioned, the Defined mirror positions (LS Pos Z) may not be remembered. You may have to re-define the mirror positions (LS Pos Z).

If you recognize that a correction of the light sheet parameters is necessary, you can perform the mirror calibration directly in the LightSheet Tab. Even a correction in "LS Pos X" is possible (red circle). Both can be done in the dialog "Calibrate Mirror Position". Any change in "LS Position X" leads to a change of the "LS Offset" and must be confirmed by clicking "Set Light Sheet Offset".

Now you are ready to perform the imaging:
LAS-X software> LightSheet tab
Use smart dial panel "Z position sample" dial from now on (do not use Z-wide microscope stand focus), To use both mirrors, **set xymz (mirror) at** acquisition mode, top of window.
Go through all imaging set up options.
Set the tile positions by clicking the tile box (upper left).
Click merge, don't keep source data
  10. ~50 ms and 2,000 gray scale value are good settings to maximize speed.
  11. Save onto the E drive only.

When Experiment is finished
- Raise detection objective, turning silver knob all the way up when done to not crash mirrors at next use.
- Remove and clean lenses and mirrors (see above)
- Shutdown LASX and Transfer your data
- When LASX has closed, turn off power strip.

Lasers Available on the DLS
  405, 488, 552, 638

John Murray   2023-07-03
jwm2175@ cumc.columbia.edu
(This guide will be updated and may contain errors)