Leica Stellaris 8 Users Guide

Additional guide from the web:
https://www.dbs.nus.edu.sg/wp-content/uploads/sites/7/2021/09/CBIS-Leica-Stellaris-User-Manual.pdf
Leica Tutorials
https://www.youtube.com/@LeicaMicrosystemsTutorials/videos

AVOID THESE DAMAGING EVENTS:

- Oil dripping down objective lenses. Use oil sparingly, do not add extra when changing slides or gently wipe off oil when changing slides. Wipe the top and sides of objective when you are finished. Use only lens paper
- Bumping Objective lens with the stage Insert or your sample (glass slide), avoid any rough handling of any part of the instrument
- Getting nail polish or other damaging substances on the objectives, or on any microscope components (e.g. Oil on Dry lenses)
- Detector beep and high laser intensities (less 10% is recommended, higher can be used for dim samples or far-red wavelengths).
    - Do Use **"frame mode" to avoid detector overload beep.**
- Frequently turning on and off the lasers

Other Important information:

- **All data must be saved to the E: drive** under your lab group, download your data to external hard drive, backup to cloud or multiple drives. After 2 weeks please delete your data
- **4-hour time slot maximum per day per user** so that others have chance to use the system (with exceptions, email me)
- **If you hear a beeping sound, turn down excitation lasers/try to fix it.** Beeping means the detectors are getting too much light. WLL (White Light Laser) intensity is generally kept below 20% (It can be set higher), 405 laser is generally kept below 5% (recommend 0.5% for DAPI). Slow scanning can also send too much light to the detectors. Use frame mode to avoid detector overload.
- **Sometimes the system misbehaves / settings do not work properly. These can often be solved by:** closing out LASX software, shutting down computer, turn off microscope control power, waiting 5 seconds and turn these back on in the reverse order. If this doesn't work you may have to shut the entire system down and re-start. But In general, try to avoid turning on and off the lasers (switch 1 and 2).

System Startup

First, look over the microscope area. Find me if things look unusual or might be broken (also email jwm2175@).

Turn on:

1. Main power, wait 2 seconds (orange switch, far left)
2. Laser power (orange switch, beneath 1)
    a. Laser emission key is always “on” (vertical)
3. Computer

    The following should automatically turn on if they don’t:

    Turn on (these switches **should be left on,** main power turns them on and off):

    a) LED lamp (for viewing bye-eye)

    b) Microscope Control power

    (Turning on the microscope control box causes the stage test movement for its entire area. Make sure the area is clear (nothing on stage)).

    c) Lightbox light (white box, right of microscope)

4. LASX (LEICA) Software

    Don’t use the microscope controls until you start LASX software.

    Chose “machine” and “DMi8” as indicated in the image:

System Shut down

Are you the last user of the day?

Is there someone coming in 30 min or less?

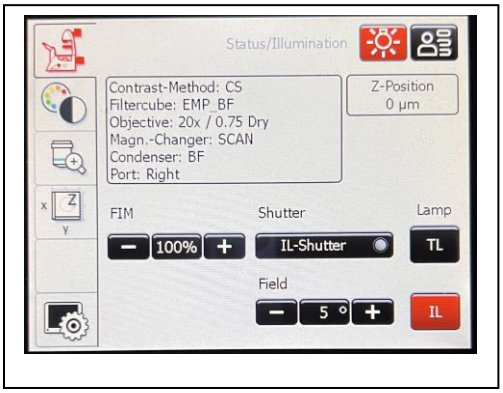
- **If using oil or water immersion: gently wipe lens with lens tissue**
- **Lower the objective lens to lowest point**
- **Set to 20X objective lens**
- **Close LASX software**
- **Download your data to an external hard drive. All data > 2 weeks old may be deleted.**
- **Logout of system**
- **If no one will use it within 30 min:** Close LASX and logout (this places lasers on standby).
- **If you are the last user of the day:**
    - Turn off computer, Turn off switches 2, and 1.
    - In general, avoid turning on and off the system / lasers.

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)
- 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 about 1.400 mm from the lowest point in the focal adjustment.

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.

Image Acquisition Tile Scanning

* Note: sometimes the LASX settings or Touch Screen Controls get messed up.
==> Close LASX software > Turn off then on again microscope power supply > restart LASX software. Avoid turning off and on the main and laser power, if possible.

Set up Acquisition settings

In Acquisition menu

If you have standard imaging settings from previous experiments: Open the previous image and choose **“apply”** to set the settings to those of the open image.
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 20X dry.
- Set scan speed to 700.
- Set pixel size to 512 x 512 (or 1024 x 1024). Note pixel size and zoom.
- Set line (or frame) average to 1 for now. In final acquisition you probably want to average for 3 (averaging this will improve image quality but will slow the acquisition, so I wait to add this after other things are set up).

- Use the **dye assistant** (circled in red) to set your dyes/laser detector setup.

- Type in the name of your dyes: e.g. DAPI, GFP, Alexa 647...

Dye Assistant will try to choose the best options for you. Generally, use no more than 2 lasers, and 2 detectors per "Channel" (=track). 1 channel per 1 dye gives the least cross-talk/bleed through but is slower. Don't image DAPI and A488/GFP in the same channel as DAPI has a broad emission peak that extends to green. Line scanning acquisition is faster than frame.
Accept the dye assistant settings.
For 4 dyes, I generally recommend 2 channels, 2 dyes per channel, but this depends on how bright each dye is / whether there might be cross talk.

Notice the channel layout. There are a lot of settings!

- Control the **laser** by clicking on the **dropped pin.**
- Control the **detector** by clicking on the colored **line below the spectrum** of each channel.

There are 5 detectors that can all be used at once! 2 -HyD S, 2 HyD X, 1 HyD R (best for Near InfraRed). Detection range is ~440 to 825 nm.

Lasers: There is a White light laser (WLL) that acts like up to 8 lasers at any wavelength between 440 nm and 790 nm. There is a separate 405nm laser (for DAPI). The WLL and the detectors can all be tuned for emission and excitation. The dye assistant has chosen the theoretical best, brightest settings but your samples may need specific tuning.
For initial imaging:

- Set the 405 laser intensity to ~0.5
- Leave the other lasers intensities at 2.

Set intensity and gain for each dye (i.e. each laser intensity and detector gain)
Check a single channel / uncheck other channels. The check box is at the upper left of each channel. Using one channel at a time lets you see a bigger image and limits bleaching/excess excitation light hitting your sample.
Click **"LIVE" (live viewing)**. ("Fast live" will allow you to view faster but the settings / brightness will be different than during acquisition).
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". These can also be adjusted by clicking the laser dropped pin or the detector. Click in the window to selects which image the Smart Dial controls. Look at histogram and Saturation and appearance to assess good signal.

Make sure you are in focus, out of focus light is much dimmer.
Repeat gain and intensity adjustments for all your dyes/images. Activation of Smart Dials is tricky, sometimes the settings don't seem to work. Click the window to activate, but you must have all the dyes of the channel showing.
In general, try to limit the amount of excitation light hitting your samples. Samples can bleach easily.

*Tip: If your image has bright signal across the glass that looks like shearing / scattering / 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 and assess saturated image 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".

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 menu you can **"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 and allow it to spiral scan through your slide. Stop any time by clicking again.
Use the Region of Interest (ROI) tools at the bottom of the screen to outline the portion of your slide that you want to image. Click a second time to set the ROI. Acquisition fields will be shown as a gray grid.

Set tile merge settings 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 **line 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.
Good luck!

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(This guide will be routinely updated and may contain errors)