Contact person: Ronald Breedijk, C2.267 (+7860, r.m.p.breedijk@uva.nl)
2nd contact person: Mark Hink, C2.264 (+ 6211, m.a.hink@uva.nl)
Information microscope: http://www.lcam-fnwi.nl/facilities/leica-stellaris/

Before using the Leica Stellaris8 microscope, the user (and co-workers) should have had the official intake discussion with the LCAM-staff, succeeded the confocal training course & exam and had an individual training at the Leica SP8 by LCAM staff.

Start-up procedure

1. Switch on the computer and wait until the PC has started and the Windows login screen is visible.

2. Switch on the microscope and activate the scanner and laser by turning on the red switches [the key is already switched ON, do not switch it OFF] to unlock emission of the laser.

3. Optional: Turn on the OKOLab incubator for specific environmental conditions (temperature and CO2 set-up). At the moment this is manually controled.

4. Log into the PC-Leica:
   User: **LASX-User**, Password: **imcb**

5. Never store data on the local harddisk, all data should be stored at the dataserver. Data present on the local PC will be deleted without further notice. Contact Mark Hink in order to get access from your office computer. Login using the **green Connect dataserver** icon at the desktop: Type your userID (UvAnetID), group and password. After succesful login a network drive U:\ will be visible where you should store your data.
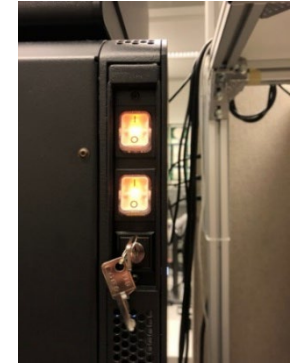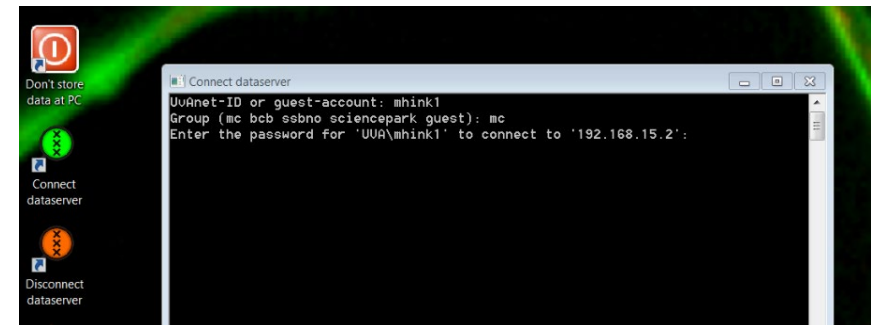
6. Check visually if there are lids or holders placed directly next to the motorized table. Put them to the side of the Okolab box such that the table can move around freely during initialization without crushing a holder!

7. Check if the **blue blinking** starting light has turned into a **green stable** one at the left back of the scanunit. Then start the LAS X software. After some initialization you are asked to select the microscope Configuration. Select for the configuration: **Machine** or when you want to have software control of the temperature and CO2: **Machine +Environmental control (not yet active!)**. Select Microscope: **DMI8**, Load settings: **Off** and press **OK**.

8. Halfway the startup procedure the software will ask you to put the transmission light arm to the back. Now initialize the motorized table only after you have removed any holders/lids next to the table (see point 6), then press **YES**.

Visual inspection of your sample

1. Start by checking the selected objective. In the LasX software or at the front panel of the microscope body one can select the wished objective. Check by visual inspection if the lens is clean. If not, clean it using lens-tissue and some ethanol from the plastic bottle. Do not use other paper types and do not press or rub!

2. To activate the internal light of the Okolab incubator box turn the white knob on top of the Okolab incubator (Note: The non-used black foot pedal should be in the ON mode). Now you have light to place your sample.

3. Put the correct immersion liquid on top of the objective (air, water or oil). A small bottle of MilliQ water can be found inside the incubator as well as a small red-labeled bottle of Leica oil. Note we have two types of Leica oil: for Room Temperature (21°C) and 37°C. Keep the bottles inside the box to prewarm them. In case an oil bottle is empty: **NEVER** use the immersion oil from other brands (Zeiss/Nikon/Olympus) but contact Ronald or Mark for a refill.

4. For a visual inspection of your sample in transmission light mode you have to activate the BrightField mode (**BF)** or **POL** at the microscope front panel and open the light shutter by clicking on **TL-Shutter** button. Also PHase contrast (**PH**) and Differential Interference Contrast (**DIC**) options are available.

5. For controlling the table position you can use the table controller having 3 knobs for X (top front), Y (top bottom) and Z (top back). All knobs can be operated in *fine* or *course* mode by pressing these buttons at the controller.

6. For a visual fluorescence inspection of your sample go to the fluorescence mode (**FLUO**) and switch ON the IL-Shutter. With the turning knob at the power unit (upper shelf) you can change the mercury lamp intensity.

   You can choose from four different fluorescence filter-blocks on the front of the microscope
   DAPI, Alexa405, … (Blue)
   FITC, GFP, Alexa488, … (Green)
   Rhod, TRITC, Cy3, Alexa543, .. (Yellow/Red)

   Don't forget to close the shutter as soon as possible to prevent sample bleaching.

Basic handling of the confocal microscope

1. Open in the LasX software the **Configuration** tab. Click on the **Laser Config** button and switch on the laser lines you want to use (**ON/OFF** toggle switches) and adjust the main output of these lasers (Typically 85% for our pulsing White Light laser).

2. Via the **USB Panel** button you can define the functions of the knob bar that is present in front of the computer screen.

3. For quantitative measurements: Click at the **Hardware** button and change the **Resolution Bit depth** to *12 bit*

4. Goto the **Acquire** tab. Goto the z-"stack menu (at the left side) and change in the drop down bar below the height image "*Z-piezo*" to "*Z-Wide*". This will allow you to see the real height of your focusing at the knob bar.
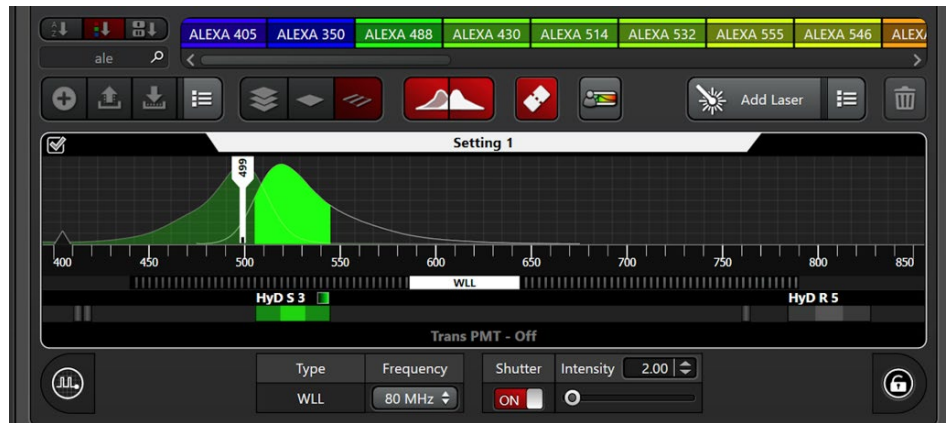
5. Goto the **Acquire** tab. By clicking on the + button, add in the main window the image **Settings** for each individual dye. You can also type in the name of your dye in the magnifying glass database above and drag your favourite dye definition into the **Settings** window. You can couple a Setting with a transmission image by clicking on the Trans PMT OFF text at the bottom of the Setting and turn it ON.

6. Select your favourite excitation wavelength by clicking on the white bar within the graph. By clicking at the Add Laser and button (top right) you can modify the wavelength, frequency and intensity of the laser at the AOBS. In order to have a "clean" excitation line it is recommended to use the wavelengths having a special Notch cleaning filter (these are mentioned in the buttons). For FLIM a laser pulsing frequency of 80 or 40 MHz is NOT advised. Also in the intensity box below the graph, the frequency and AOBS laser throughput percentage can be typed (e.g. 2.00).

7. Select your favourite HyD detector. There are 3 types: S-type (det 1 & 3) are more sensitive in the blue region. X-type HyDs (det 2 &4) have a broad but bit lower sensitivity and are optimized for FLIM and HyD-R (det 5) is mainly red sensitive. Adjust the spectral detection window such that you will detect the fluorescence of the sample but WILL NOT detect the laser line(s). You can do this by moving and reshaping or double click at the colored emission filter box below the detector name and squeeze/move the boxes of non-used detectors away.

8. In case of multiple dyes, thus multiple settings, one can activate/deactivate individual ones by the tickbox at the top left of each Setting. For simultaneous scanning use the Line modus,   for sequential scanning (= low crosstalk) the Frame or Z-stack modus .

9. Goto the **Acquisition** menu at the top left, and adjust the **Acquisition mode** (typically *XYZ*).

10. Adjust Format (*512x512*), Speed (*400 lines/s*), Bidirectional (*OFF*), Zoom factor (*1*), Zoom in (*ON*), Line average (*1*) and Rotation (*0*).

11. For optimal confocal imaging set the **Pinhole** to *1 Airy Unit*. One can optimise the pinhole diameter by filling in your favourite emission wavelength and then press the *Airy1* button.

12. Use the **Live** button at the bottom to quickly adjust the position and maybe focus of your sample and take the final image using the **Capture Image** button. For more complicated image series (e.g. time series, z-stacks or multi-point) you should use the **Start** button. To increase the Signal-To-Noise (SNR) of the images **Frame averaging** is advised.

13. Additional tools for Z-stacking (where the top and bottom height should be defined) and Automatic focusing are available.

14. Goto the **Open projects** menu at the top left. Click at an image or project folder you would like to save, Right-click at it and select **Save As**. Now browse to your folder at the dataserver and store the data here (Note: All image data at PC-Stellaris will be deleted).

15. More advanced imaging options will be discussed during your individual Leica Stellaris8 training and can be checked in the digital Leica manual, available via a shortcut at the desktop. Here also some application notes with protocols are available how to use the for applications like FLIM, tau contrast and gating (FALCON), spectral imaging & N&B.

16. To enhance the mercury lamp lifetime: Please turn it off when you are scanning overnight!

17. Always inform the LCAM contact persons of the Leica SP8, Ronald or Mark, in case of microscope or network issues or when more sophisticated imaging is required.
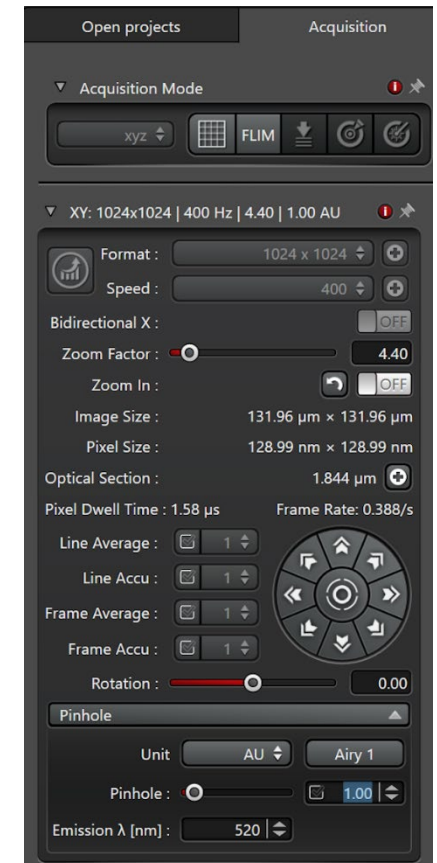
Special features of the Leica Stellaris8

Spectral detection.
Besides standard XYZt scanning the confocal can also measure emission (λ) and excitation (Λ) spectral images.
FLIM, tau-gating and photon counting detectors
The HyD detectors can be operated in various modes.and also tau gating can be applied to reduce f.e. short-lived autofluorescence, e.g. popular to block signals from autofluorescent plant tissue. Using the FLIM button lifetime information can be collected. Most HyD detectors also allow photon counting detection, important to prevent detector noise for e.g. FFS techniques.
Navigator

In order to reduce searching time for the correct cells or make large overviews one can make a quick preview image using the Navigator tool. Select the Navigator button. Then select the spiral button at the bottom to create an overview image. One can add focusing points and store favorite regions in the multi-point list (right bottom). If you want to use a calibrated motorized table press the blinking white button inside of the Oko lab box around the microscope at the right bottom side of the body.

Switching off procedure of the confocal microscope

1. If there is no one using the confocal microscope directly after your session goto the **Configuration** tab and deactivate the active lasers via the **Laser Config** button using the ON/**OFF** toggle switches.
2. **Move the objective manually to the lowest height.**
3. Close the LasX software.
4. Disconnect the link to your folder at the dataserver using the **red Disconnect dataserver** icon at the desktop.
5. Turn off the computer.
6. If you have used the Climate control box: Power off the Oko Touch unit.
7. If there is someone using the confocal microscope directly after your session (check at www.lcam-fnwi.nl -> Bookings) then only clean the lenses with a tissue. First remove the oil, **do not rub**! In a second step clean with a little alcohol, again do not rub and move the paper only in 1 direction!
8. Switch OFF the two main switches (see point 2 at startup).
9. Clean the microscope, the labspace around it and put recombinant waste in the appropriate yellow bins:
   - Sharp/Glass in the yellow containers with white/red caps.
   - Solid material in the larger yellow open cans.
   - Discard fluid recombinant waste (growth and microscopy medium) in the wetlab or special WASTE bottles.
   - Paper should be discarded in the standard black waste bins in the room.