Imperial College London

Facility for Imaging by Light Microscopy

Observing Life As It Happens

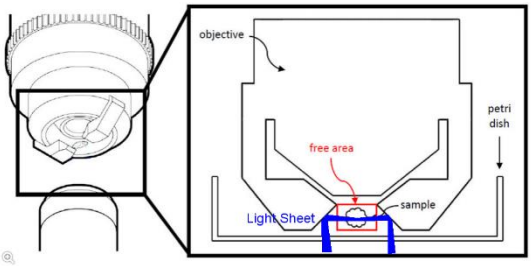
QUICKSTARTGUIDE:

LS1 – LightSheet

LEICA Stellaris 5 DLS

(SAFB 410)

Contents

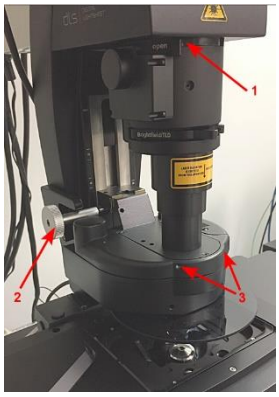


Leica DLS sample preparation guide

System check

Check that the right condenser for LIGHT SHEET microscopy is fitted on the microscope:

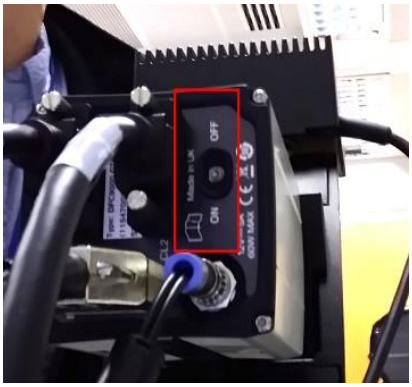
light sheet



confocal microscopy

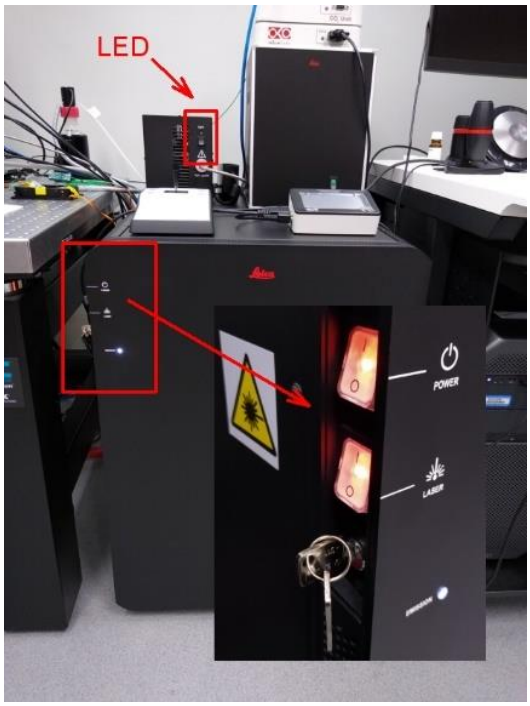
If not, ask FILM staff to change the condenser. Do not change on you own, this can result in serious damage of the microscope. The microscope must be powered down to change the condenser.

- Camera needs to be switched on, the switch is on the back, toggle switch should be in down position. Usually the camera is left on.



Startup Procedure

- Start PC
- On the central power unit - Switch on:
    - Power
    - Laser
    - Turn on the Laser Emission Key ON (Emission LED lights up)
- Login (IC network account)
- Switch the LED on at LED power supply on top of the laser unit
- Wait until the Touchscreen on the front of the microscope has finished booting



Microscope stage

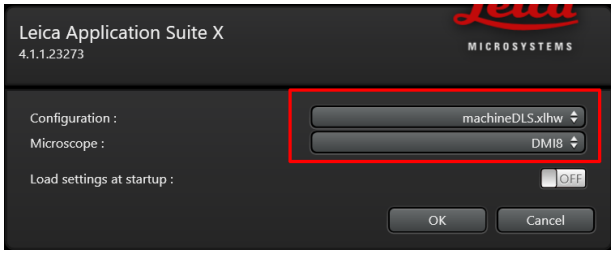
The microscope stage has modes of operation, when the button on the right side is constantly illuminated, the stage is locked and can be moved with the joystick. When the button is flashing, the stage can be moved manually.

- Pressing the button once will switch between these modes of operation.
- When the stage is moved by hand or encounters an obstacle during movement, it will automatically switch to being unlocked and the button will flash.

IMPORTANT: Be very careful when manually moving the stage, as the stage might hit an objective which would cause expensive damage.

- The speed of the stage can be reduced by pressing the F4 button on the stage controller, F2 switches back to fast speed.

- Start LEICA Application Software “LAS X” on desktop



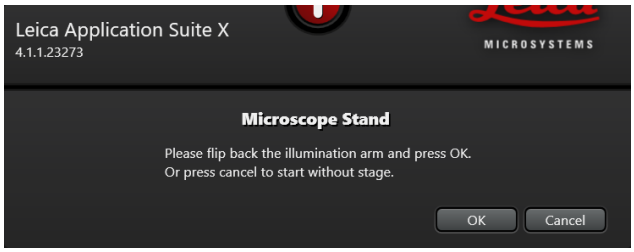
- In the start-up window select the required configuration from the drop-down-menu:

  machineDLS .xlhw – Light Sheet Module without environmental control

  - machineDLS – Okolab.xlhw – Light Sheet Module with environmental control

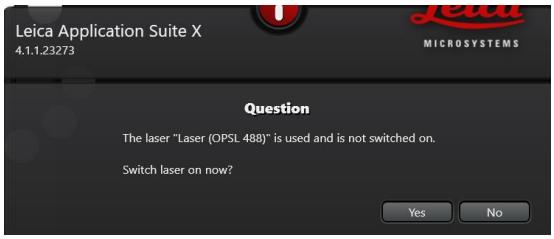
- Calibrate the stage if you want to use multi-position or tiling.
- If the stage has not been calibrated since switching on, then a message window will appear asking you to flip back the illumination arm
- **Check if the microscope stage is locked (see above),** if not, check the stage is in middle position and press the button at the stage once.

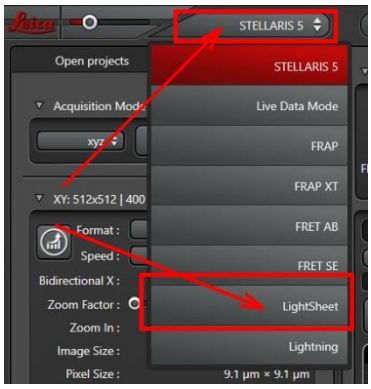
  Choose ‘OK’ on the software message, to calibrate the stage.



On the Stellaris 5 tab:

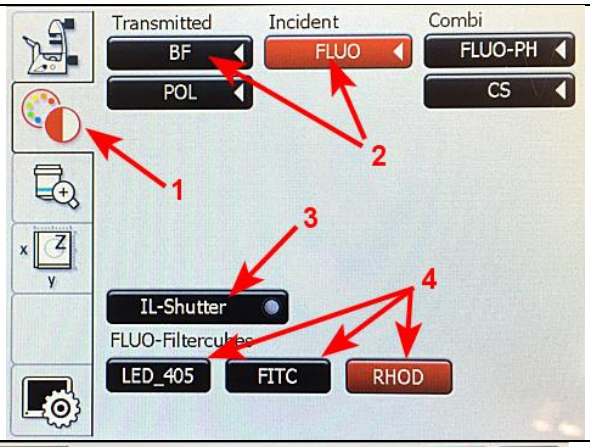
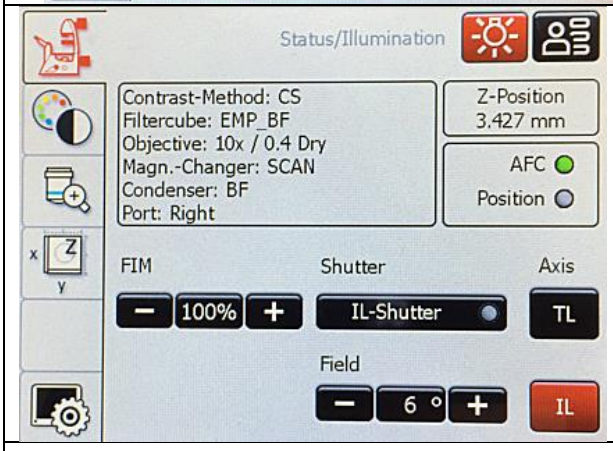
- Select “LightSheet” to enter the Light Sheet wizard
- Switch on laser when asked





The software enters the calibration workflow

Touchpad Controls

	Contrast tab (1) - select viewing method (2): Fluorescence (Fluo) (2). • open IL-Shutter (3) • select required fluorescence channel (4) Brightfield (BF) • see next window
	To Adjust the Brightfield use the microscope tab (1) open the brightfield shutter, and adjust intensity (2) and condenser aperture (3),

Objective and mirror combinations:

The following combinations of available mirror caps (**TwinFlect**) and objectives are ideal for use in a Lightsheet experiment:

Detection Objective

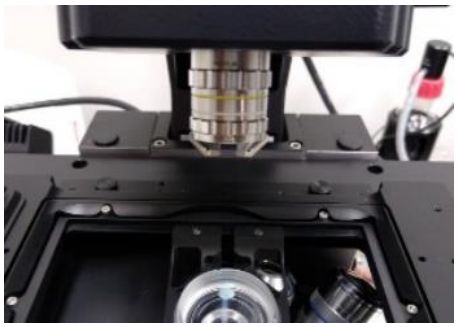
Illumination Objective	25x	20x	16x	10x	5x
4x	2.5 mm	2.5 mm	2.5 mm		
2.5x	2.5 mm 5 mm	2.5 mm 5 mm	2.5 mm 5 mm	5 mm	
1.6x		7.8 mm W	7.8 mm W 7.8 mm Gly/BABB	7.8 mm W 7.8 mm Gly ~~7.8 mm Gly/BABB*~~	7.8 mm W 7.8 mm Gly/~~BABB**~~ 7.8 mm BABB

* Glycerol only (for material-related reasons)
** Glycerol only (for optical reasons)

If you use another combination, a corresponding warning is displayed.

Mounting objective on light sheet condenser

- Select the right imaging objectives for your sample (5x, 10x, 20x or 25x) and mirror (7.8 mm, 5mm, or 2.5 mm).
- Assemble mirror and objective (screw onto top of objective, where the front lens is)
- Move condenser to highest position (knob 1 in figure 1) to make objective is furthest away from the sample.
- Carefully mount objective (2) on the light sheet condenser.
- Turn knob (3) until mirror is aligned horizontally (as in image below)



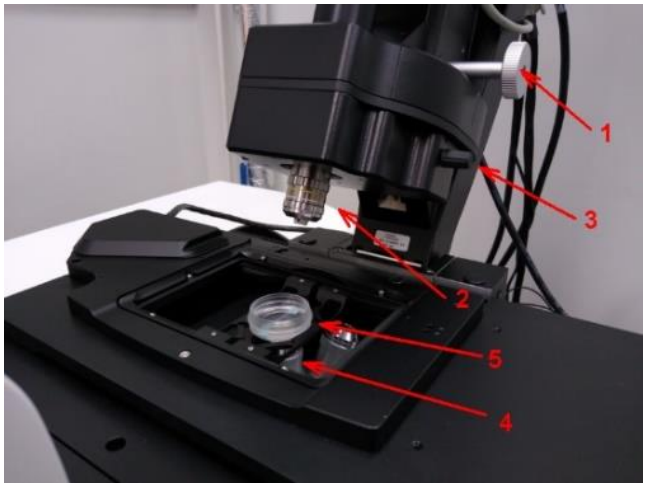


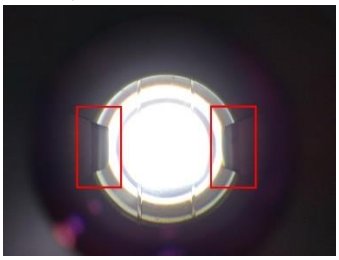
Figure 1

- Select a suitable illumination objective (4) for mirror size (Touchpad or in software), here the 2.5x illumination objective together with 5 mm mirror were chosen together with the 10x 0.3 NA detection objective.
- Put the sample on the stage (water immersion) and carefully move the stage position with the joystick so that the sample is over the illumination objective. The joystick has a slow (F4) and fast setting (F1).

Adjusting detection objective

- Switch to brightfield mode on illumination arm

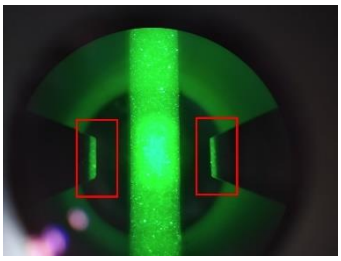
- Switch to BF mode on the touchpad and look through microscope eyepiece
- Focus on Sample, center the sample in the field of view by moving stage.
- Carefully move the illumination arm in normal position, watch if the detection objective fits in the dish, it will still be in the upper position, not touching the water/buffer.
- While looking through the eyepiece, carefully lower the detection objective with knob 1, you will see when the mirrors touch the water surface. Lower the objective further, to see the mirrors in the field of view

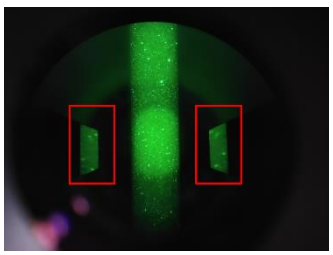


IMPORTANT:

- **The mirrors should end up left and right from your sample, they must not touch the sample.**
- **Do not lower the objective too much, otherwise you might break the dish and flood the microscope with liquid.**

- Switch to fluorescence, you should see reflected fluorescence on the mirrors
- Adjust objective so that you see a reflection of your sample on the mirrors, (not in focus), here you can see the beads.

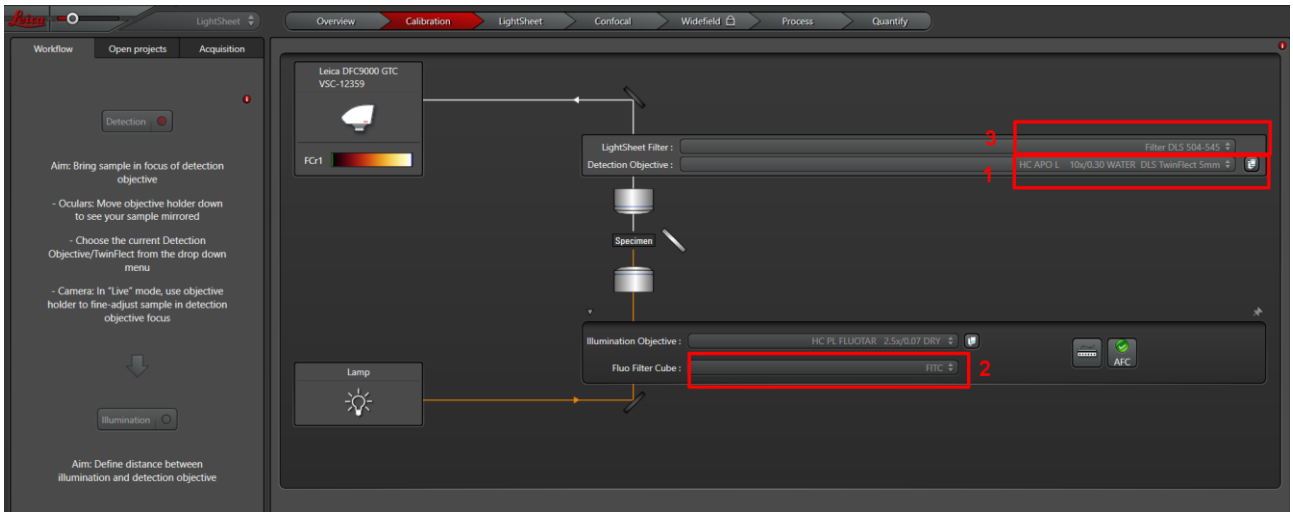




- Switch off fluorescence at touchpad (IL-shutter)
- Switch to DLS Lightsheet (top of illumination arm)
- Swing out illumination condenser lens (1)
- Add cover labelled Digital Lightsheet (2)

Software Calibration process

Step 1: Detection



(1) Select the chosen combination of detection objective and mirror (here 10x/0.3 water, 5 mm)

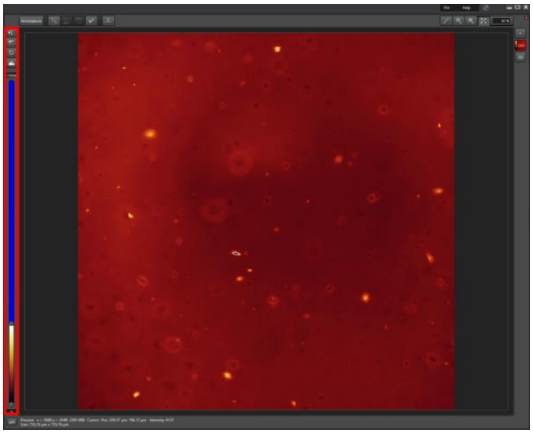
(2) Select the right filter cube to see the fluorescence of the sample (here FITC), also available: 405, Rhodamine

(3) Select the right emission filter for the fluorescence (here 504 – 545)

The standard emission filters are:

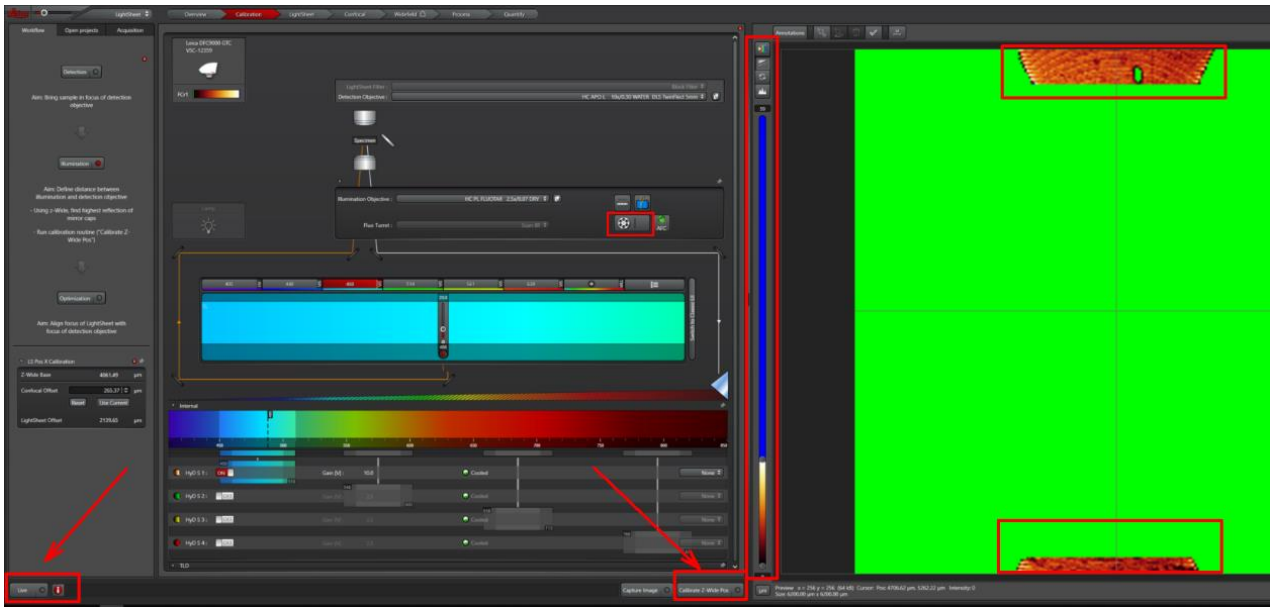
455 – 495 for DAPI, Hoechst, blue fluorescent dyes
504 – 545 for GFP, FITC, Alexa 488 and all other green dyes
557 – 615 for Red fluorescent proteins and red fluorescent dyes such as Alexa 568
In this step, using the LED, it is not possible to excite far red fluorescent dyes efficientl
The 405/488/561/638 laser blocking filter cannot be used with the LED

- Go LIVE at the bottom left of the screen
- Adjust look up table
- Move detection objective with knob 1 in Figure 1 (big silver knob at the illumination arm of the microscope) up and down, so that an interesting part of the sample is shown, **be careful not to move too much down, as you might break the coverslip of the dish or damage the galvo stage.**
- Stop LIVE mode



Step 2: Illumination

- Go to next step in the calibration workflow (Illumination), the aim of this step is to determine the right z-position for the illumination objective which forms the light sheet. This will be different from the focus position when looking through the eyepiece!
-
- The microscope switches automatically to confocal scanning in reflection mode using the 488 laser.

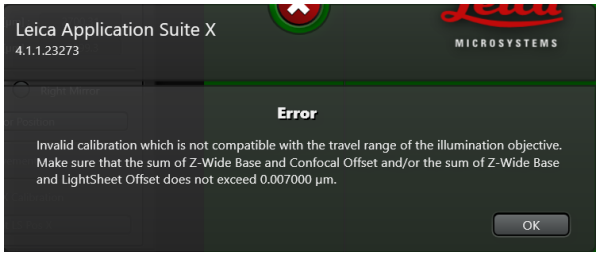


- Go LIVE, the image will be blank (green = zero pixels)
- Move the z-wide position (at the black Leica controller on the computer desk), it moves the illumination beam upwards until it hits the mirrors.
- When the z-wide position is high enough, the reflection of the two mirrors appears at the edge of the image, you might need to adjust the LUT. This can be a high z-wide value e.g. 4000, so you will have to move a lot. You might need to reduce the laser power if the signal is saturated.
- Adjust z-wide position until mirrors appear brightest.
- Turn knob 3 in figure 1 (small black knob at illumination arm of the microscope) until both mirrors are centered with regard to middle line

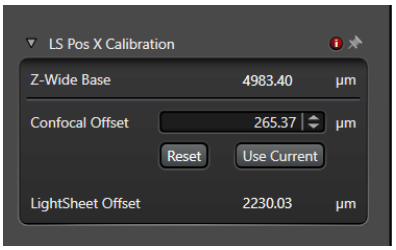
- Stop live, start “Calibrate z-wide position” on the bottom right, the microscope will perform a z-stack to determine the best z-wide position

Step 3: Optimization

You might get an error message “Invalid calibration…” when switching to the Optimization step, this is caused by the sample being mounted to high or the selected imaging plane to high up in a large sample. Either re-mount the sample or lower the detection objective and repeat the Illumination step.

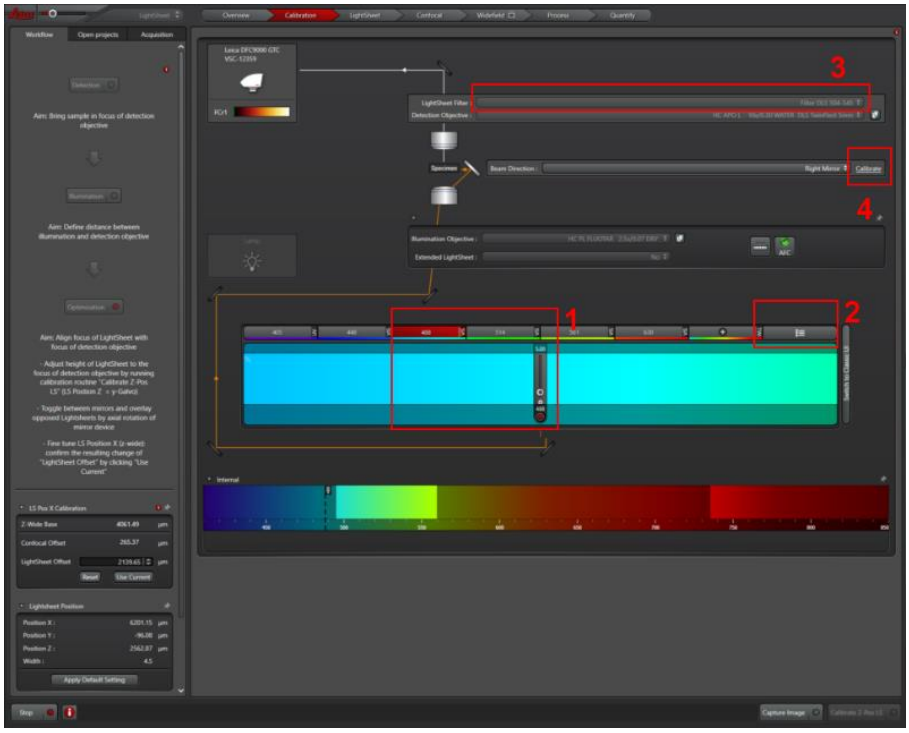


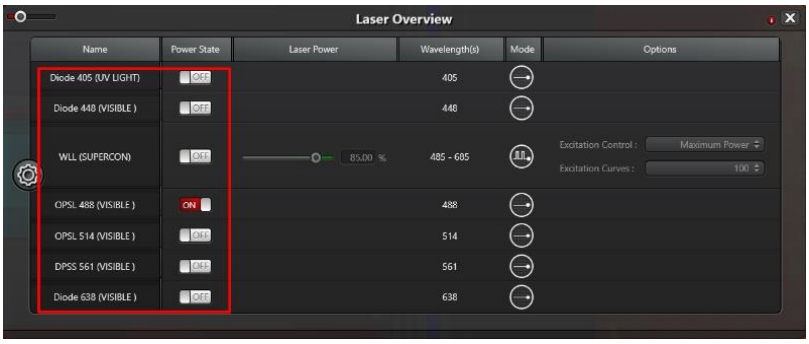
The sum of Z-Wide Base and LightSheet Offset cannot exceed 7000 µm.



The system switches automatically to Light Sheet mode

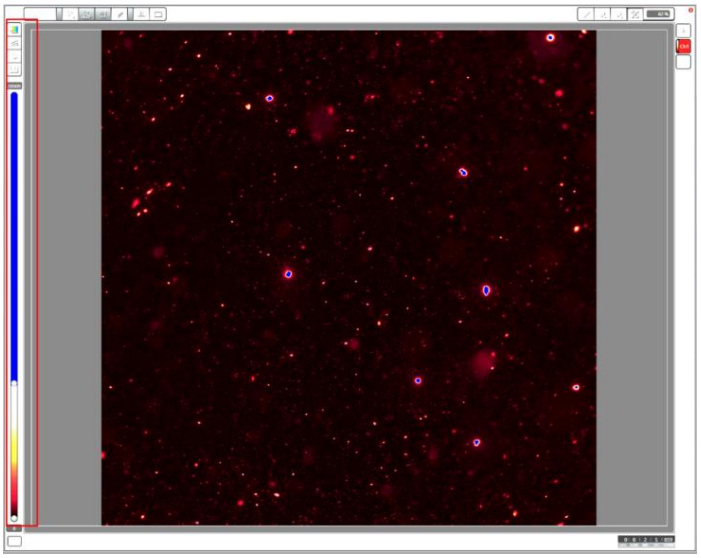
- Select a suitable laser wavelength for your sample (1), you might need to switch on additional lasers (2)
- Select a suitable detection filter
- Go live



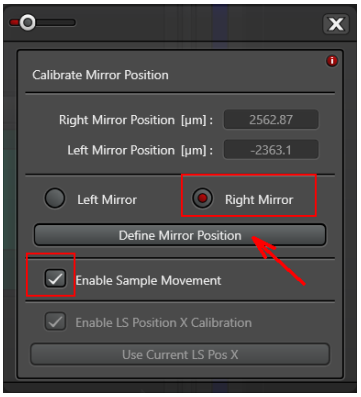


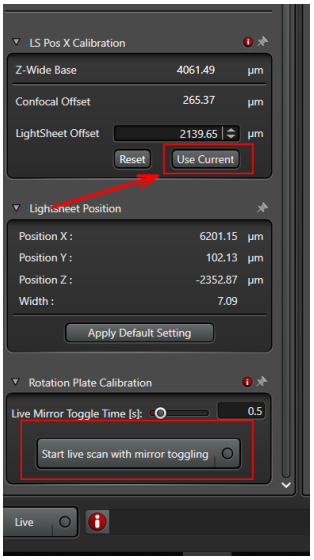
The following controls on the panel below the monitor are available:

You should see an image of the sample (here beads), otherwise adjust LUT or "Z Pos Sample", if the light sheet is not positioned correctly, you might not see anything yet.



- Select calibrate (4 on previous page) in the main window
- Choose one mirror and enable sample movement.
- Focus on one part of the sample with "Z Pos Sample".
- Now watch a prominent small feature in the centre of the sample and adjust "LS Position Z" until the feature appears sharp and bright, now the light sheet is positioned in the centre of the detection objective focus.
  IMPORTANT: The position of the light sheet can be quite a way off!
- **"Define Mirror position" to save this position** for the selected mirror
- Select the other mirror.
- Adjust "LS Position Z" until you see the same feature and it is sharp and bright.
  IMPORTANT: The position of the light sheet can be quite a way off!
- Define Mirror position
- Adjust "LS width" so that only a stripe is illuminated
- Position the stripe with "LS Position Y" so that the stripe is centered
- Adjust "LS width" so that the whole sample is illuminated





Optional: Control the adjustment by using live scan with mirror toggling. You will see alternating images from both mirrors.
If the light sheet focus (waist) is not in the center of the viewer, fine tune with "LS Position X" and confirm any change by clicking "Use Current" LightSheet Offset.

It can happen that a part of the light sheet is blocked by non-transparent features in the sample and images from both light sheets appear different.
This was the last step of the calibration.

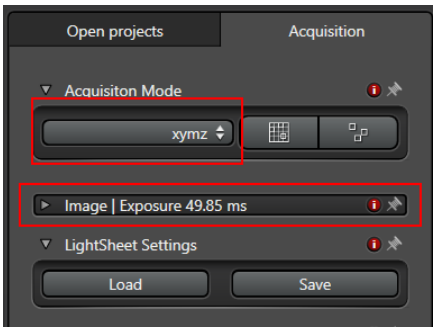
If you cannot adjust the light sheet properly, click Apply default Setting and repeat the optimization step!

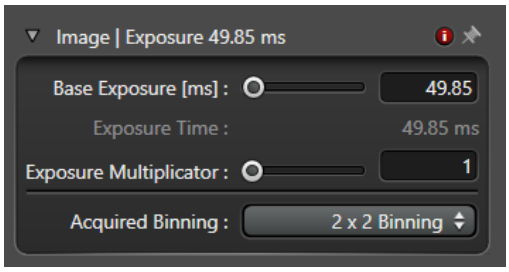
Acquire Light sheet data

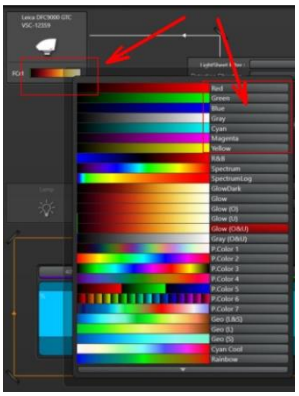
Remember you sample position ‘Z Pos Sample’, then go to the light sheet tab at the top of the software window, when you do this the sample position will jump to a different position. Change it back to the previous one as otherwise you might not see your sample at all.

!!! Help to most features is available by clicking the red (i) next to the function (allow blocked content in browser)

- Select acquisition Mode xymz to automatically merge the images
- Select binning, e.g. 2x2 is sufficient for many experiments, 1x1 is for Nyquist sampling e.g. for deconvolution (not yet available)
- Check laser wavelength and detection filter
- Select LUT for camera (double click)
- Go Live
- Adjust Image exposure time if necessary

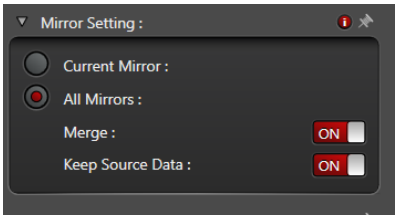






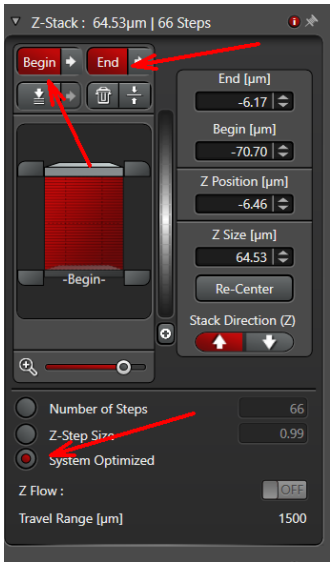
Mirror setting

Select all mirrors if you want to image with both mirrors and merge ‘ON’, this will produce two files, one with the images from the two mirrors in separate channels and another one (named …target) which is a merge between the two mirrors. The second image will be displayed during imaging.



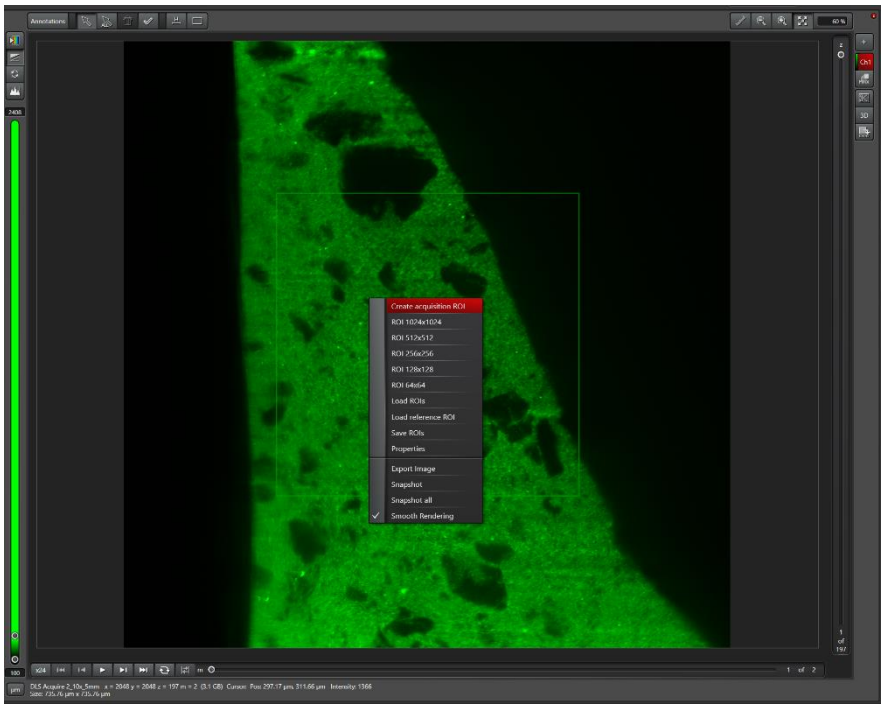
Set up z-stack

- Define Begin and End in Live mode while changing Z Pos Sample
- Use “System Optimized” for z-step size (see below check image size, otherwise the optimized setting is wrong)
- Start the Experiment



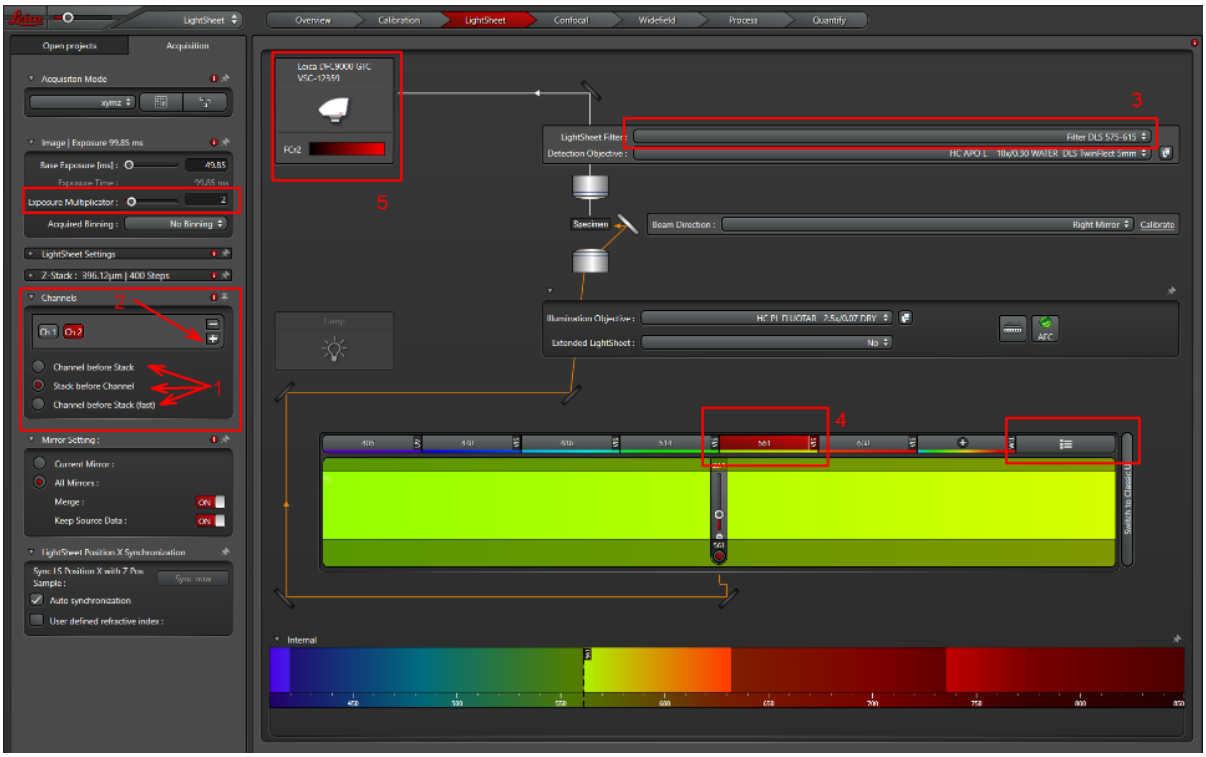
ROI setup

This only works at 1x1 binning, take an image and click with the right mouse onto the image, select an ROI size which will then be displayed in the image. Right click again and select ‘Create acquisition ROI’. The full image will still be displayed in live mode, but when capturing images or stacks, only the ROI will be acquired.



Multi-Channel acquisition

A) Using bandpass filter for low crosstalk and switching channels between stacks (imaging slow dynamics)

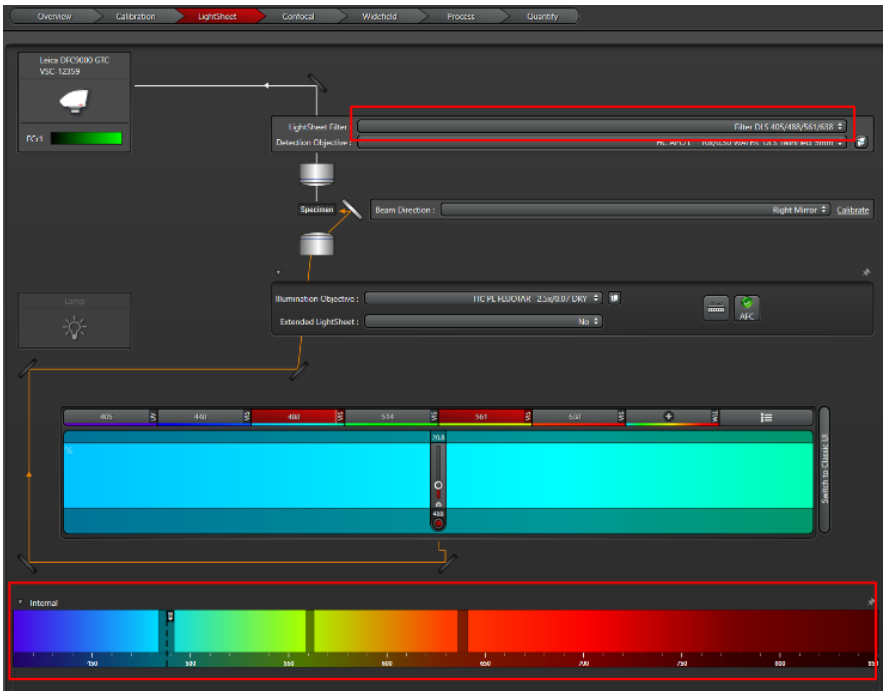


1) In the Channels section select “Stacks before Channels”
2) Add another Channel with “+” button
3) Select right bandpass filter
4) Add suitable laser, you might need to switch on additional lasers
5) Select color map

B) Using laser line filters for switching channels before stacks (imaging fast dynamics)

Same as above but select “Channels before Stack” and laser line filter.

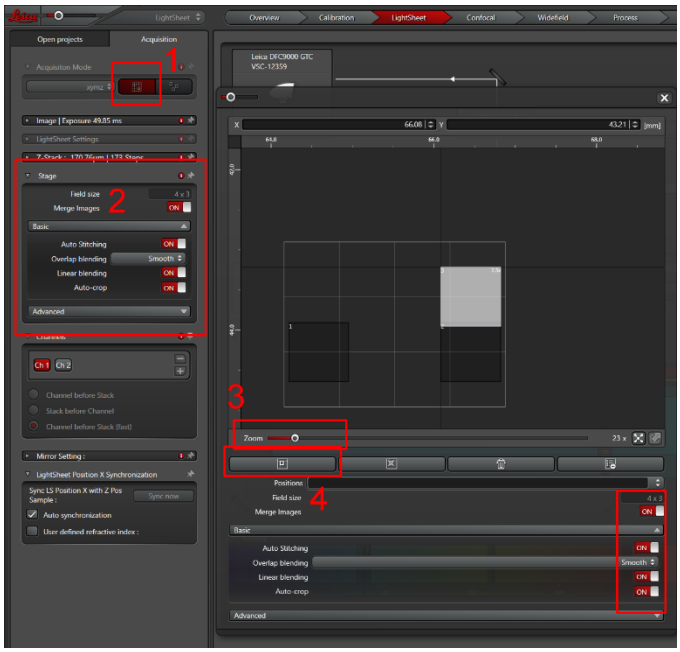
There is only a limited number of filter positions in the turret, so the filter might need to be changed, e.g. for the GFP/YFP filter, ask FILM staff to do this.



Tiling

1) Select Tiling option
2) Expand stage view to get the large window.
   IMPORTANT: do not click into the stage view, the stage will move to the position you clicked on and move a large distance, this can destroy your sample or damage the objective.
3) Zoom in to see current region
4) Add positions to be imaged, the software will create a tiled region.
5) Switch on Auto Stitching
6) Start the Experimant

When z-stacks are set up together with the tiling option, the system will perform both.



Saving Data

Light Sheet data can easily be several 100 GB or even TB in file size, we recommend starting a new .lif file for each data set.

- The data needs to be saved after acquiring the data set, this process can take e.g. 6 minutes for a 300 GB file. Connect to the HIVE data store to save the data:

  In the Windows File Explorer, expand the ribbon
  Select ‘This PC’
  Select ‘Map network drives’
  select a free drive letter (e.g. "Z:")
  for folder use "\\FILM-HIVE1\HIVE1-data"
  tick the box 'Reconnect at logon' and click 'OK'

- The HIVE is a server computer with 70 TB of storage for light sheet data which is connected over a fast 10 Gbit/s network connection directly to the microscope computer. It can also be used for image analysis via Windows remote desktop.

- We recommend saving directly to the HIVE storage. You can then map the HIVE storage from any other computer on the college network and access your data. If you log on directly to the HIVE for data processing, you will find your data on drive E: (RAID6).

- The microscope computer has another mass storage which is drive F: DATAEXT, this is a disk array with 22 TB of disk space and has a fast thunderbolt connection to the microscope computer.

- Do not use the E:\ drive, as saving to this drive is very slow.

IMPORTANT: While the HIVE is a RAID array with UPS and provides some safety regarding hard–disk failure, it is not backed up and data might be deleted when the storage is full. Long term storage of the data and back up is a responsibility of the user!

Shutdown procedure

- **turn off lasers in software**
- Save files
- Close LAS X software
- Move the detection objective to the highest position (knob 1 in Figure 1) and tilt back the illumination arm, then remove your sample
- Clear up the desk
- On the central power – Switch off:

  Laser Key
  Lasers
  Power
- Switch off LED control box

IMPORTANT: Carefully remove the objective from the illumination arm (don't drop it!) and remove excess water/buffer with lens tissue, unscrew mirror unit and clean with ethanol, put back into containers when dry! Tilt arm in normal position.

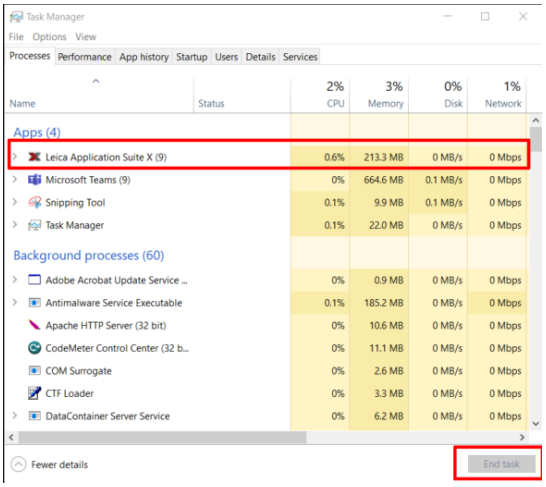
To be done:

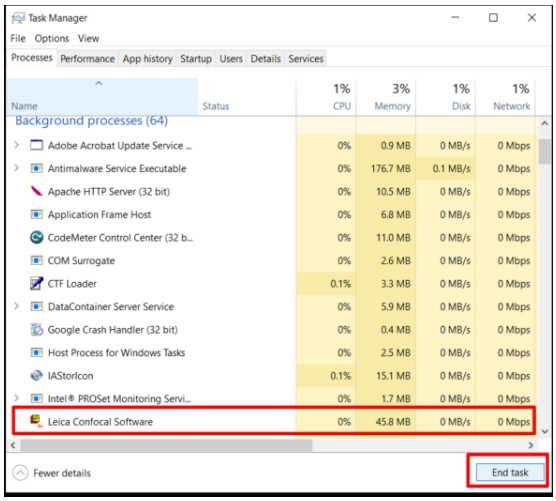
3D visualization, sample holders
Separate manual for incubation and CO2

Stage error

!!! Hardware issue: Soemtimes the stage stops and the button on the stage starts flashing during initialization, this causes the software to crash with the following error message. If this happens, follow the following steps:

- Open the Windows task manager (right click on the task bar at the bottom of the screen), select to see more details, select Leica Application suit X and end the task.
- Then scroll down further in the list until background processes, select 'Leica Confocal Software' and end the task.
- Move the stage in the middle position, press the button and try starting the software again.





If this does not help, try power cycling the computer: Shutdown in Windows, switch off power to computer at wall plug on the right labelled computer, wait 20s, then switch on computer and restart software.