

STANDARD OPERATING PROCEDURE

Leica Stellaris 5 DLS Microscope – Digital Light Sheet SOP

INTRODUCTION

This document describes the operational setup and procedures for digital light sheet (DLS) imaging using the Leica Stellaris 5 DLS microscope.

DLS imaging capacity, enabled by Leica's TwinFlect mirrors, is a module installed onto the inverted Stellaris 5 confocal imaging system. DLS imaging can take advantage of the system's 405nm, 448nm diode lasers as well as the white light laser (WLL) covering a wide range of excitation from 465nm to 790nm allowing multi-color DLS imaging. A high-performance CMOS camera (Hamamatsu ORCA-Flash 4.0) is installed for fast speed DLS detection.

DLS brings flexibility with the capacity to image thick samples (fixed or live) such as model organisms, organoids or cleared tissue. The light sheet experiments can be performed for multi-positions and using the tilescan function for larger volume imaging.

SCOPE

This procedure only applies to DLS imaging mode (confocal imaging is a separate SOP) covering basic operations including hardware components preparation, DLS calibration & DLS imaging. In addition, the document covers widefield (transmitted light) imaging in DLS mode.

This SOP does not include sample preparation procedures as sample preparation is highly dependent on researchers' sample types. Please refer to the DLS Sample Preparation Guide provided by Leica for more information.

SAFETY

The operator must exercise caution during loading or removing of the sample to/from the stage, never put fingers into the sample loading area while the stage is moving or initialising.

In addition, safe operation of the instrument also requires the awareness of other risks e.g. biological hazard considerations, exposure to lasers and UV light, fatigue and gesture discomfort/injury etc. Refer to the latest Safe Work Procedure (SWP) published on the Westmead Imaging internet site, WIMR-SWP-WHS-GEN, "Using a fluorescence or laser scanning microscope" and the SWP for Stellaris DLS imaging.

Acquisition of samples on the Stellaris 5 DLS microscope must only occur after the approval of an associated project in PPMS. Any projects to be run on the Stellaris 5 involving hazardous chemicals and/or biohazard samples must have appropriate approval. OGTR requirements for safe work in a PC2 laboratory apply. Users performing live DLS imaging must wear proper PPE including lab coats, gloves, face masks, and safety glasses.

TRAINING / COMPETENCIES

All personnel require training prior to independent operation of the instrument. Training is conducted by staff with competency demonstration necessary before authorisation.

Competency is assessed via demonstration of independent instrument operation, in conjunction with verbal explanation of all aspects of operation and troubleshooting common faults. After training is carried out, a training competency quiz is to be completed and passed before instrument access is authorised by imaging staff. All instrument operation is to be conducted by trained operators.

EQUIPMENT & SUPPLIES

The Stellaris 5 DLS microscope is located in J210, Level 2 WIMR.

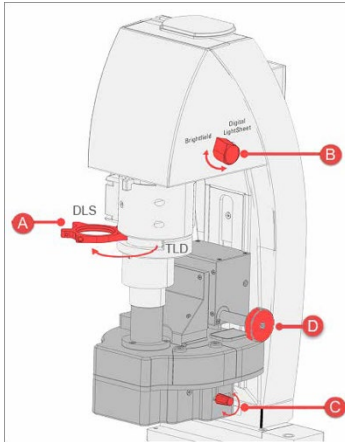
OUTLINE

- **Hardware preparation**
 - Put the DLS components in position
 - Combination of detection & illumination objectives and mirrors
- **Start your experiment**
- **DLS calibration**
 - Step 1: detection
 - Step 2: illumination
 - Step 3: optimisation
- **DLS imaging**
 - DLS imaging
- **Wide-field imaging**
 - Widefield mode with the Lightsheet Wizard
 - Option A: widefield-complex acquisition in widefield mode
 - Option B: widefield-quick overview
- **System shut-down & finish-up**

PROCEDURE

Hardware preparation

- > Ensure the system is off.
- > Address the following components for DLS imaging



A: Lens for TLD (Transmitted Light Detector)

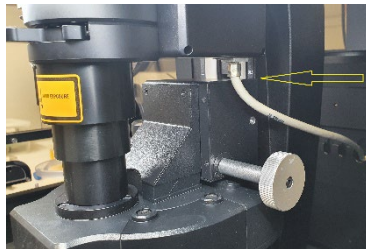
B: Knob to change to BF (Brightfield)

C: Set screw to align the mirror caps

D: Z-actuator of the DLS objective holder

1. Turn on the DLS camera
2. Change the BF knob to DLS position (B)
3. Swing out TLD and block the slot with the DLS magnetic shield (A)
4. Replace the confocal condenser with the DLS objective holder. Once replaced, bring up the DLS objective holder to a safe level (D) to prevent a collision with the sample when tilting the transmission light arm forward to sit straight

Camera switch is at the back



5. Install the right detection lens (capped with the proper mirrors) to the DLS condenser



6. Tilt the transmitted light arm down (towards back of the microscope) very gently.

> Turn on the system

Note: Combinations of illumination and detection objectives

Recommended (application driven) combinations of illumination & detection objectives and mirrors

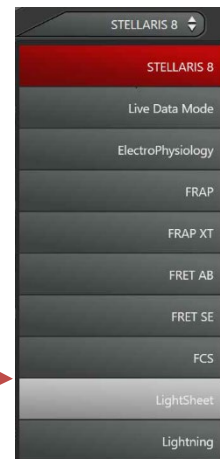
Illumination objectives		Mirrors TwinFlects		Detection objectives	
Objective/NA	Article No.	Mirror cap	Article No.	Objective/NA	Article No.
2.5x/0.07	15506523	5 mm	158007011	25x/0.95 W	15506399
4x/0.13	15506412	2.5 mm	158007012	25x/0.95 W	15506399
				10x/0.3 W	15506524

Start your experiment

- > Login to LASX
- > Start LAS X with the TLD arm tilted down (backwards).

Note: stage initialisation is recommended at the start of the LASX.

- > Choose Light sheet mode
- > Focus your sample under the microscope

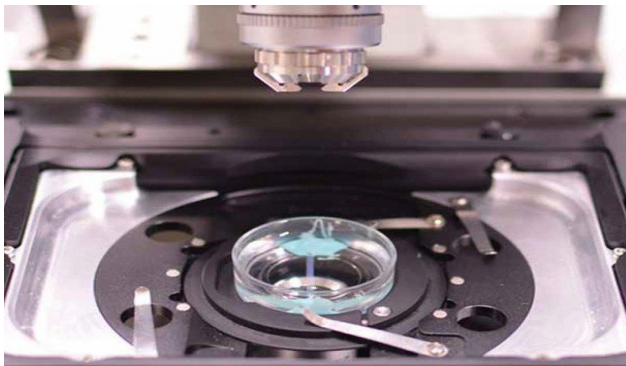


1. With the TLD arm tilted down (backwards), place the sample onto the stage (use the proper stage insert/adaptor).
2. Bring your sample in focus with fluo illumination by adjusting the Z-wide.
3. Fill the Petri dish at least 2/3 full with distilled water/medium.
4. VERY GENTLY tilt the TLD arm forward to sit straight ensuring no damage will occur due to excessive force. Carefully move the DLS objective holder downwards (D) until the mirror cap is in the water/medium. Position the sample in between the twinfect mirrors.
5. Focus on the sample by adjusting Z-wide as usual (do not use Z-Galvo!). Check focus via the eyepiece.
6. Finely adjust the detection objective (D) till you can see both the right and left mirrors in

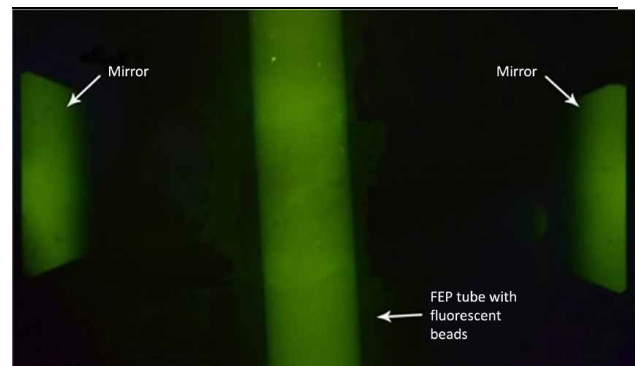
the eyepieces. This step is complete once the sample is reflected in the mirror surfaces and either the sample or its reflection in the mirrors can be seen sharply through the eyepieces.

7. Align the mirrors by turning the rotation plate (C in diagram shown above) if needed. The sample (e.g. FEP tube with beads) in the Petri dish has to be centred between the two reflecting surfaces of the mirror cap.

Note: You cannot focus on both the mirror image and the sample at the same time. It is only possible to see either the reflection or the sample sharply in the mirror. Either one can be used.



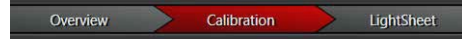
Orientation of the sample



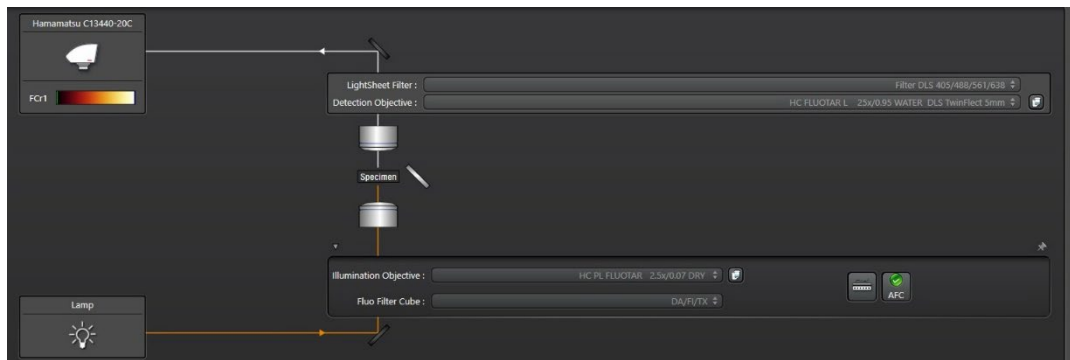
Fluorescence image of FEP tube with beads (center) and two mirrors of the mirror cap (left and right)

DLS calibration

Click "Calibration" to open the Light Sheet Wizard in LAS X

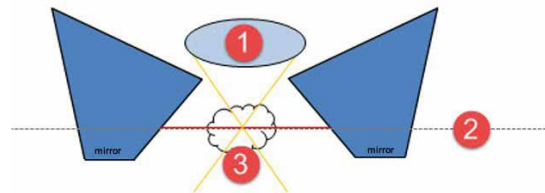


- > Select the correct detection objective with mirror cap from the list.
- > Check whether or not the mirrors and illumination objective are compatible.
- > Select a suitable filter cube. For "Light Sheet Filter" select the "405/488/561/638" filter for fluorescence illumination or the "Empty" position if you want to see the sample in a quasi-brightfield mode.



Calibration step 1: Detection

Aim: Bring your sample into the focal plane of the detection objective by using the camera.



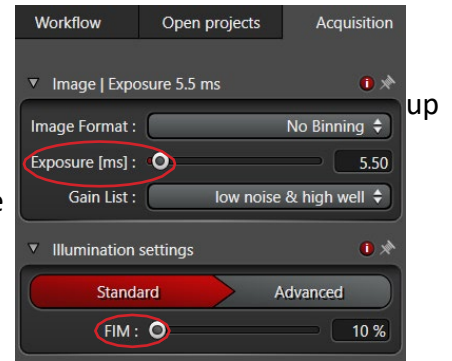
Detection schematic: 1 = Detection objective, 2 = Focal plane of the detection objective, 3 = Sample.

- > Click "Detection" in in the DLS calibration work flow.
- > Click the "Live" button.

> Adjust the DLS objective holder (D) carefully until the sample is in focus.

- > If necessary, rescale the image using the slider of the colour look-table.
- > If necessary, reduce the exposure time ("Exposure" slider) and the illumination of the microscope ("FIM" slider) in LAS X.

Note: From this point on, the DLS objective holder (D) should no longer be moved



Illumination calibration

Aim: Measure the Z Position of the detection objective to be able to ultimately place the illumination objective at the optimal position at later steps.

Note: If the TwinFlect 2.5mm mirror is used, the Illumination part of the calibration must be performed with the illumination objective < 5x. Otherwise, the bottom edges of the mirrors might not be hit by the illumination beam (see below two images for comparison).

- > Click the button "Illumination" in the DLS calibration workflow.

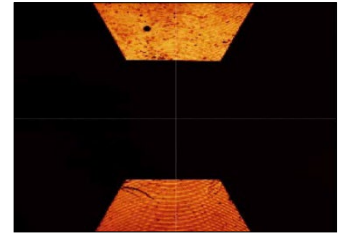
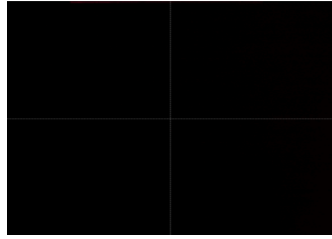
The following measurement is done using the confocal capabilities of the system: PMT is automatically placed under the laser line (488nm) and AOBS/beam splitter (depending on your system configuration) is set to reflection.

- > Click "Live".
- > Use Z-Wide to focus on the mirrors until you see the reflection of the bottom mirror edge of the detection objective. Try to find the maximum reflection during this step.

Note: The first reflection comes from the coverslip and not from the mirrors. Find the reflection maximum closest to the reflection of the coverslip!

The lower surfaces of the TwinFlect 2.5 mm mirrors are only visible by using the 2.5x or 4x illumination objective. If the 5x illumination objective is used, the lower surfaces are outside the field of view and, hence, no base position can be determined.

- > Adjust the two mirrors of the cap to be as parallel and straight as possible by turning the setscrew on the objective holder (C).
- > Click "Calibrate Z-Wide Position".



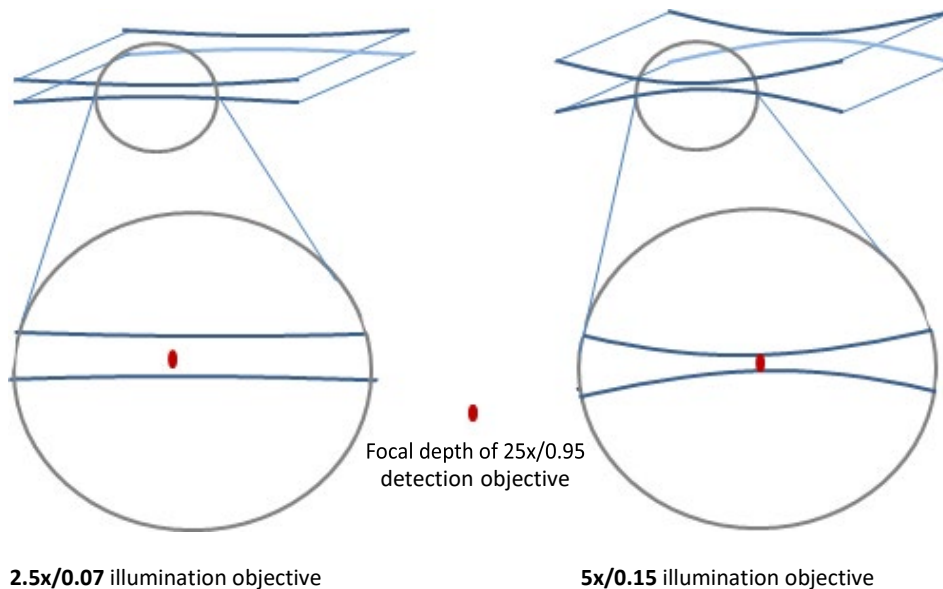
TwinFlect 2.5 mm with 5x illumination objective, 25x detection objective TwinFlect 2.5 mm with 2.5x illumination objective, 25x detection

Aim: Focus the “beam waist” of the light sheet in the focal plane of the detection objective.

Now we start to work with the light sheet. As this is the first time, the next steps are calibration of some parameters that depend on each other. The most important parameters are “LS Position Z” and “LS Position X”.

- > LS Position Z
- > LS Position X
- > LS Position Y
- > LS Width
- > Mirror Cap Orientation
- > Z Pos Sample

For the next steps, use the control panel. This figure shows you the drives which are in the light sheet system.

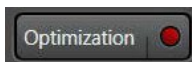


The light sheet is generated with the illumination objective and detected with the 25x detection objective. The shape of the light sheet (focus = “beam waist”) is determined by the illumination objective. The higher NA of the 5x/0.15 illumination objective compared to the 2.5x/0.07 illumination objective makes it possible to generate a shorter, but also thinner light sheet. In this example, 5x illumination with a 25x detection objective gives you much better contrast and signal-to-noise ratio (SNR).

Overview of the next steps

- A. Preparation – First steps
- B. Focus the beads/sample – Adjust LS Position Z
- C. "Center the light sheet in vertical direction" – Adjust LS Position Y
- D. Fine adjustment of the mirror orientation – Turn objective holder manually (C)
- E. "Center the light sheet in horizontal direction" – Adjust LS Position X

A. Preparation – First steps



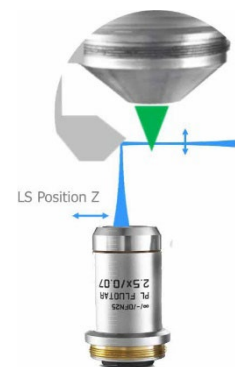
- > Click "Optimization" in the DLS calibration workflow. The mode is changed to LightSheet.
- > For "LightSheet Filter" select the "405/488/561/638" filter or the "Empty" position for samples in agarose which generate scattered light.
- > Set "LS Width" to 48 to make the light sheet narrower and brighter.
- > Use the LUT (look up table) slider to scale the image (pull it down to make the image in LAS X brighter).
- > Click "Live" to focus the beads/sample via "LS Position Z" on the control panel.



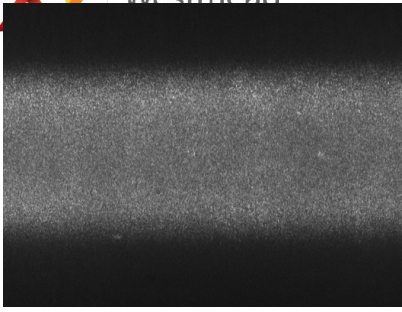
2D LS width

B. Focus on Beads – Adjust LS Position Z

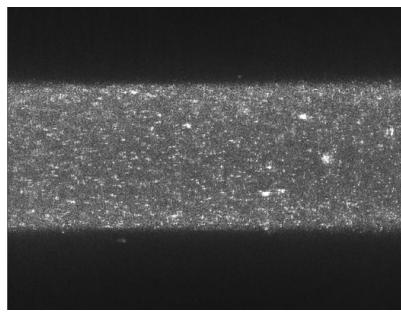
- > Click "Calibrate" to open the "Calibrate Mirror Position" dialog.
- > Select any mirror (left or right): Focus the beads / find the highest contrast via "LS Position Z" on the control panel.
- > Click "Define Mirror Position".
- > Enable the other mirror: Focus the beads like before.
- > Click "Define Mirror Position".
- > If necessary, repeat the steps.



LS Position Z

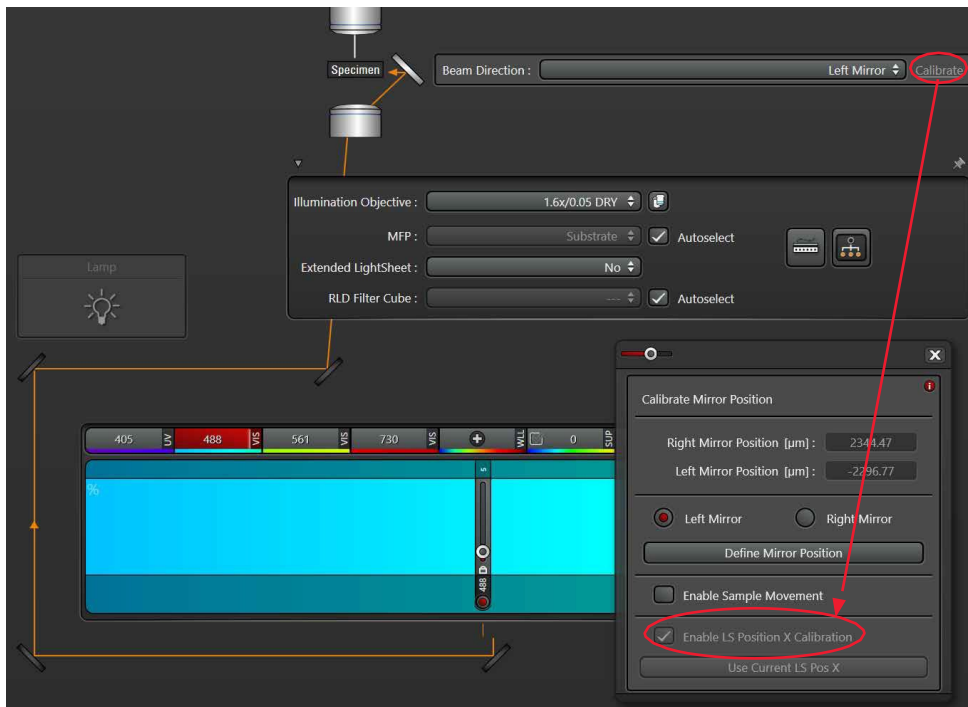


Beads out of focus



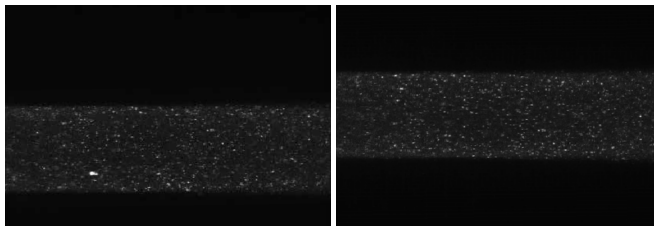
Beads in focus

Note: Z-Galvo (sample movement) is blocked in the Calibration tab by default. On the control panel, Z-Galvo is called "Z Pos Sample". It can be activated in the "Calibrate Mirror Position" dialog. Don't touch Z-Wide at this point during the Optimization calibration step.



Adjust "LS Position Z" in the "Calibrate Mirror Position Dialog"

c. "Center the light sheet in vertical direction"– Adjust LS Position Y



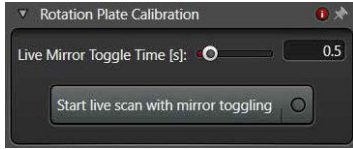
To center the light sheet in the middle of the viewer, use the "LS Position Y" on the control panel.



LS Position Y

D. Fine adjustment of the mirror orientation

Use the "Rotation Plate Calibration" dialog to align the mirror surfaces with the direction of the line scanner. This is a purely manual adjustment (C) on the DLS objective holder.



Fine adjustment of the mirror orientation

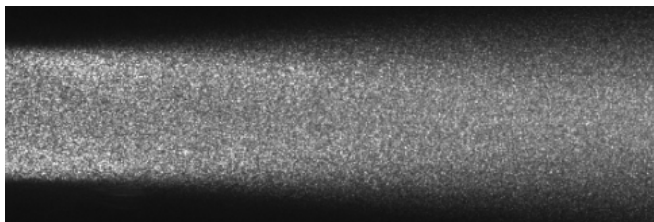


- > Click "Start live scan with mirror toggling" (light flickering is normal).
- > Fine adjustment of the mirror gap
- > During the "Toggle" Scan, use the set screw (C) on the DLS objective holder to turn the rotation plate far enough until the overlap in the horizontal direction is complete.
- > The adjustment is finished once the images from both light sheets are completely superimposed.

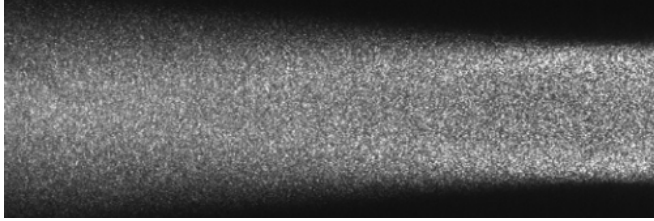
E. "Center the light sheet in horizontal direction" – Adjust LS Position X

Adjust the focus position of the light sheet (= the "waist" of the illumination beam) in the center by using "LS Position X".

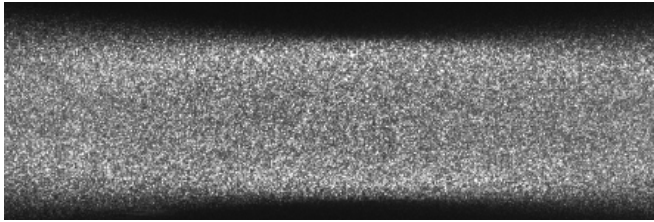
- > Click "Live".
- > The "LS Position X" value is displayed on the control panel.
- > If now the "LS Position X" is changed during Live Scan, you will see how the focus of the illumination objective moves either to the left or to the right.



Too much to the left or ...



... too much to the right



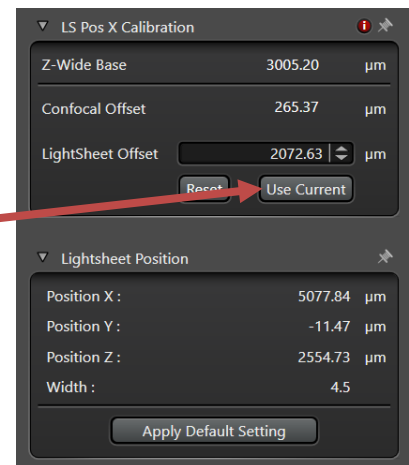
Goal: The “waist of the beam” is in the center.



LS Position X

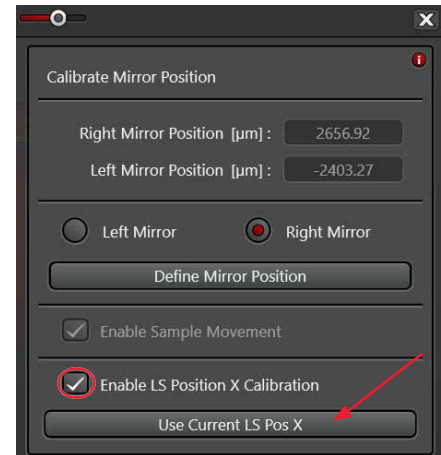
- > Click "Use Current" under “LightSheet Offset” to confirm the position of “LS Position X”. Otherwise, the changes will not be applied for LightSheet imaging.

Note: Your current “LS Position X” is now stored for this combination of detection objective, TwinFlect mirrors, and illumination objective.



When the calibration is finished and the light sheet image looks good in terms of focus and contrast, change the LS Width to use the complete light sheet for imaging. Now you can run your experiments.

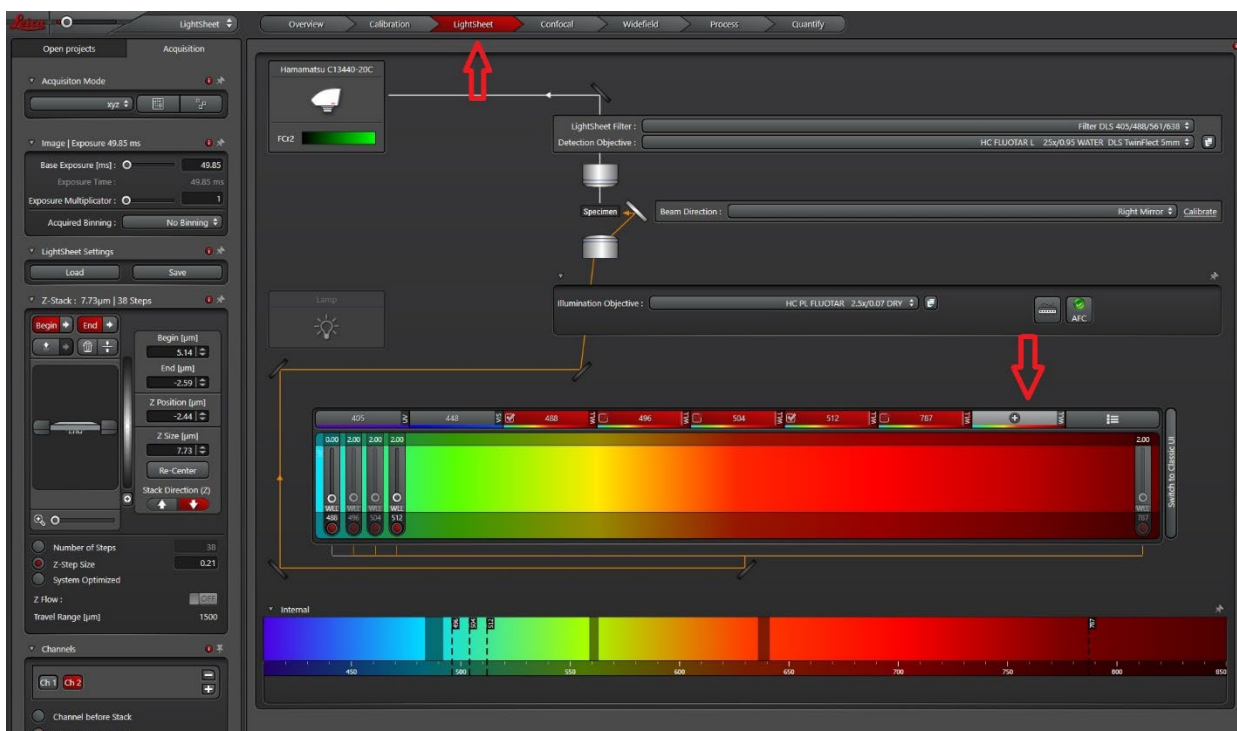
If you recognise that a correction of the light sheet parameters might be necessary, you can perform the mirror calibration (adjust "LS Position Z") once again directly in the "LightSheet" tab. Even a correction in "LS Position X" is possible (red circle). Both can be done in the dialog "Calibrate Mirror Position".



Note: Moving "LS Position X" directly changes the Z-Wide position of the illumination objective. So any correction of the "LS Position X" leads to a change of the "LS Offset" and must be confirmed by clicking "Use Current LS Pos X" (red arrow). If not, the new "LS Position X" (= new adjusted focus of the light sheet) will not be taken into account. It is the same process as in the Calibration step 3 where changes of the "LS Position X" has to be confirmed by clicking "Use current".

A. Set up fluorescence channels

- > Click "Lightsheet" to go to light sheet imaging mode. To add multiple fluorescent channels, keep clicking "+" (see below arrow pointing downwards) till the specific laser line for the to-be-added channel is available.

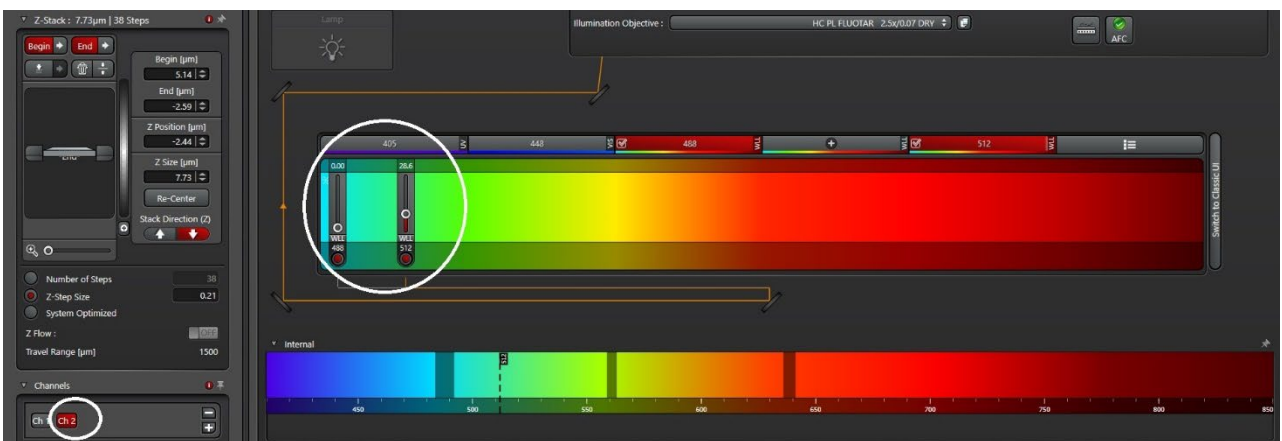
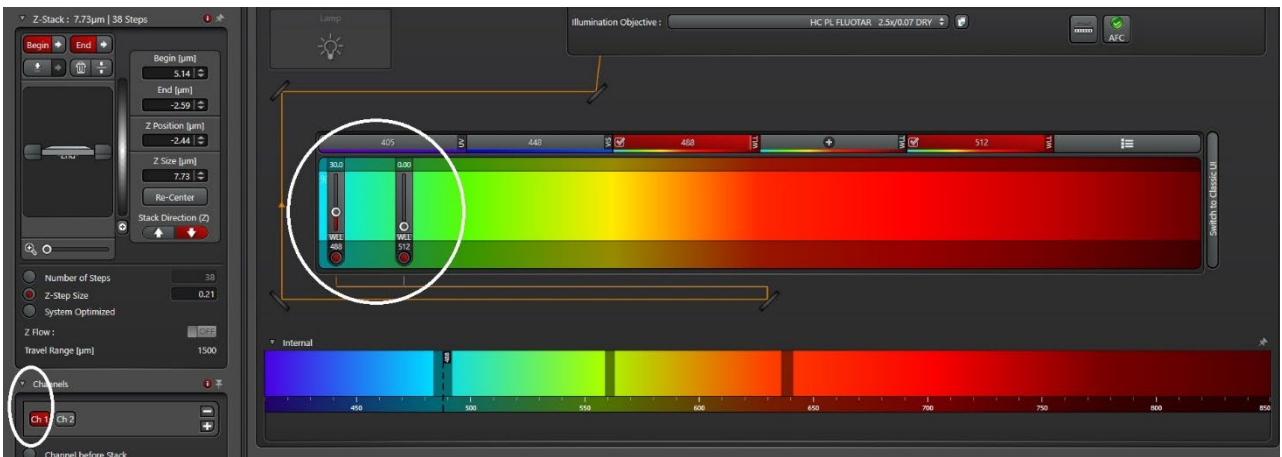


Note: To remove those unwanted channels, just click on the channel, it will disappear. Here we only leave two channels as an example: Channel 488 and Channel 512.



> Now we can set up the laser intensity for each channel.

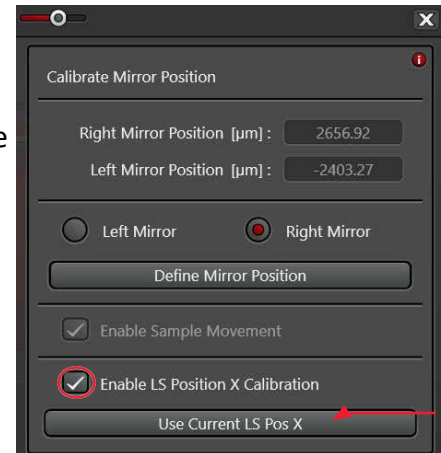
First turn on Channel 1 (Channel 488) (see below left white circle) and reduce Channel 2 (Channel 512) laser to 0. Bring up the laser intensity for Channel 1 (it varies depending on your DLS signal strength). Then set up the laser for Channel 2 in a same way.



> Set up Z-stack as usual by adjusting “Z Pos Sample” in the control panel (this is to adjust Z Galvo not Z-wide). Click “start”.

Note: the Z-Wide drive (here called “LS Position X”) is blocked by default to prevent unintended changes of the light sheet focus which may interfere with your previous calibration. If you want to reposition your sample in the z-direction, but you have reached the end of the Z-Galvo travel range, you can manually readjust the height of the objective holder. In that case, you have to move the detection lens first and then readjust ideally only Z-Wide (“LS Position X”).

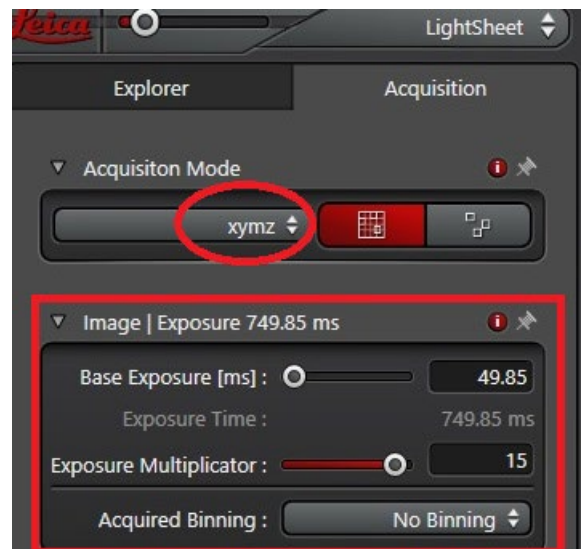
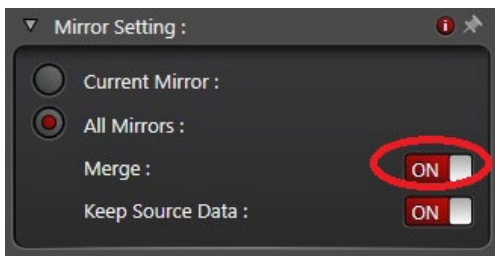
Z-Wide can be activated in the dialog "Calibrate Mirror Position" by checking “Enable LS Position X Calibration” (red circle). This allows you to do fine adjustments without having to go back to the Calibration tab.



B. Set up Z stack as usual using z-galvo.

C. Set up the exposure time (red square) to get the saturation right as usual.

D. Choose “XYMZ” (red circle) to use both left and right mirrors. Note: you can choose to merge left and right images during acquisition (red circle) or manually merge them after acquisition is completed.



Widefield imaging (transmitted light)

Widefield mode within the LightSheet Wizard

Acquisition parameters set in widefield mode are directly transferred to LightSheet mode. Whenever you are ready return to LightSheet mode, the acquisition of the already defined experiment can simply be started and performed here.

Two options to use the Widefield mode

A: Widefield tab at top for defining and recording complex acquisition in Widefield mode.

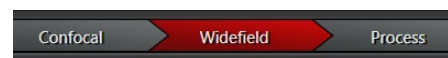
B: Widefield button at the bottom for a quick access to Widefield mode for checking your specimen and / or defining a Tile Scan experiment combined with a z-stack. Widefield data acquisition is not possible here.



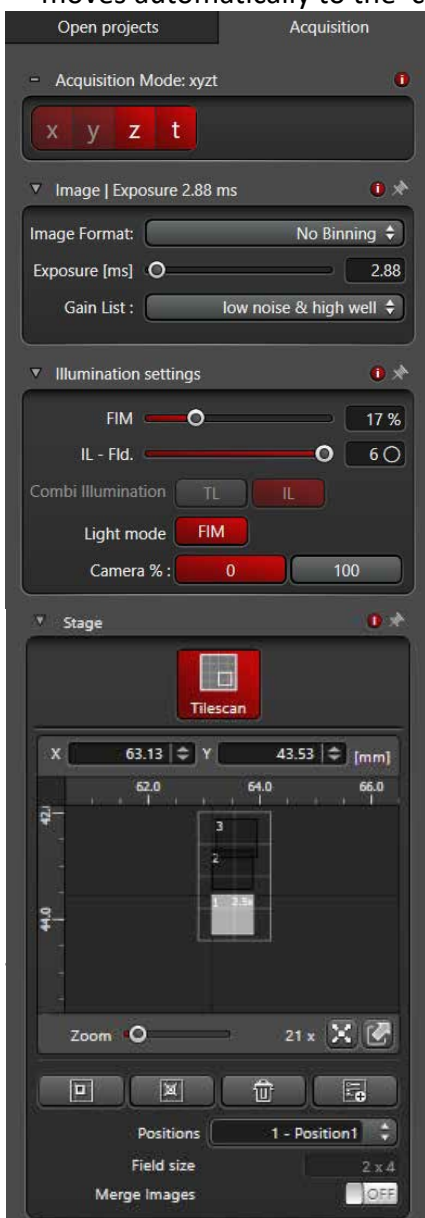
Note:

1. It is prerequisite to perform the general “DLS calibration first”.
2. The Widefield tab is locked (see above) and the “Widefield” button is not visible if you were not previously in the LightSheet tab.
3. If you would like to perform a stage experiment in Widefield mode (Tile Scan or Mark & Find), these functions must first be enabled in the LightSheet tab.

Option A: Widefield – complex acquisition in widefield mode



- > Click on the Widefield tab Transmitted light mode is switched on and the live image of your specimen appears in the viewer. The Z-Wide z-actuator moves automatically to the calibrated position for the “LightSheet Offset”.



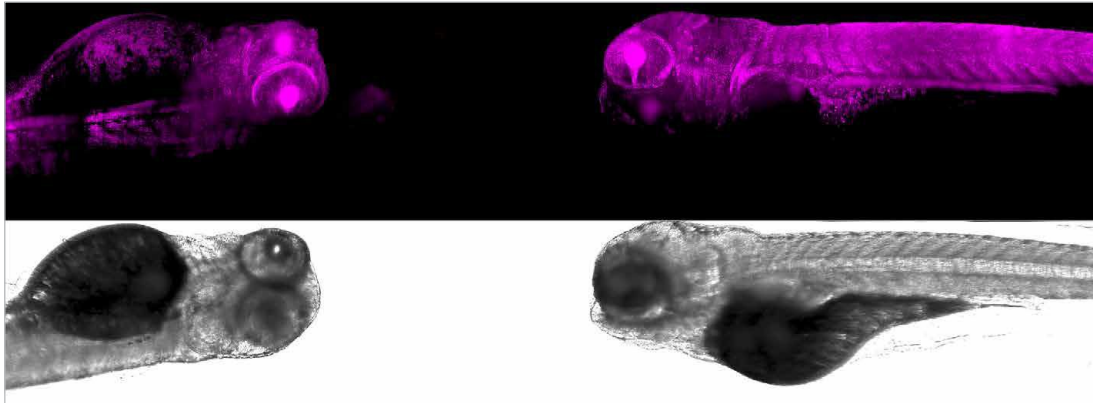
- > Under “LightSheet Filter”, remove the filter by selecting the “Empty” entry
- > Select the “Acquisition Mode” x,y,z,t to define the dimensions for the image acquisition and optimise camera parameters and illumination strength for widefield acquisition

- > Define a Tile Scan (for more instructions see LAS X Online Help)

Note: Z-stacks and/or time-series can be defined and performed as well. Both can be combined with Tile Scan/Multi-positioning



- > Click “Start” to perform your Widefield imaging acquisition
- > Switch to the LightSheet tab. The defined Tile Scan setting is now also used for the LightSheet acquisition of the fluorescence signal
- > Start your experiment by clicking “Start”



Simplified sample navigation with widefield mode: The widefield image and the light sheet image of the zebrafish can be displayed side by side in the viewer.

Option B: Widefield – quick overview

- > Click the “Widefield” button.



Transmitted light mode is switched on and the button is renamed to “Stop”. The Z-Wide z-actuator moves automatically to the calibrated position for the “LightSheet Offset”.

- > Under “LightSheet Filter”, remove the filter by selecting the “Empty” entry
- > Select the “Acquisition Mode” x,y,z (it is not available), and adjust camera parameters and the illumination strength for widefield acquisition (see above)
- > Define a Tile Scan
- > Click “Stop” to stop the live acquisition in Widefield mode

Note: Z-stacks can also be defined in combination with Tile Scan/multi-positioning, i.e., define a z-stack in the Widefield tab and go back to the LightSheet tab. The z-stack parameters will be applied automatically, and the light sheet acquisition can be started immediately.

System shut-down & finish-up

- > Close the LASX
- > Lower the stage to bottom from the touch screen of the microscope
- > Bring up the condenser height to a safe position
- > Remove the sample
- > Take off the detection objective and mirror cap. Clean them with cleaning wipes only.
- > Change the microscope components back from DLS to confocal.
- > Switch the knob B to BF
- > Log off from LASX login
- > Shut-down: Steps 4,3,2 and 1

DATA & RECORDS MANAGEMENT

All data must be saved onto the local computer where it is kept for 7-14 days before automatic deletion.


Save data to E:\users data\“your_folder”. Your data is synchronized to the WIMR Imaging Onedrive account and transferred to the Scientific Platforms server. Scientific Platforms (\\Scientific_Platforms\Cell Imaging\Stellaris\“your_folder” where it will be held for 14 days before deleting without warning. You need to therefore move your data promptly. Staff can share to non-WIMR researchers a onedrive link to access your folder remotely if required.

REFERENCES

1. A PDF file of Step-by-Step Guide for Imaging with Digital Light Sheet provided by Leica is accessible on the desktop of the acquisition computer.
2. More information about what is light sheet microscopy and Tiledscan etc, refer to LAS X Online Help or facility staff.

This DLS SOP is also published on the Westmead Imaging website.

DOCUMENT CONTROL

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Author:	Hong Yu
Policy Owner:	Scientific Platforms
Functional Unit:	Westmead Imaging Facility
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