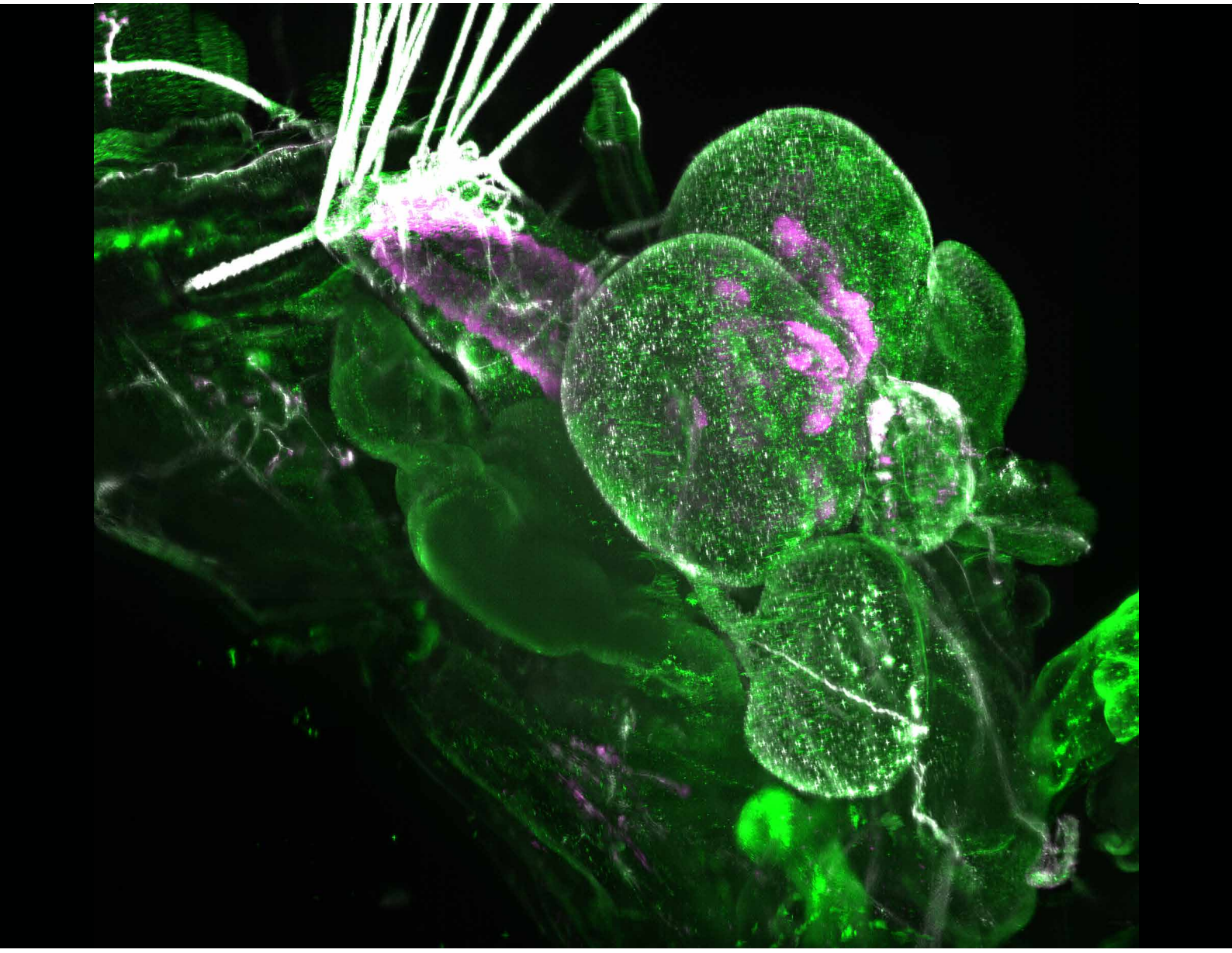
From Eye to Insight

Application Note

IMAGING WITH THE DIGITAL LIGHT SHEET MICROSCOPE STELLARIS DLS

Step by step guide



Cover image: Fixed Drosophila larvae brain. Green: myo10A-GFP (motor neurons), white: Futsch (neurons), magenta: Bruchpilot (presynaptic active zone). Sample courtesy of Sigrist Lab, FU Berlin.

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Before starting LAS X, make sure that the Hardware components you need for your experiment are taught in. Here is how you can do it by starting with the Hardware Configurator.

Hardware Configurator

Teach in the detection objectives and mirror caps in pairs

> Open the Leica Hardware Configurator

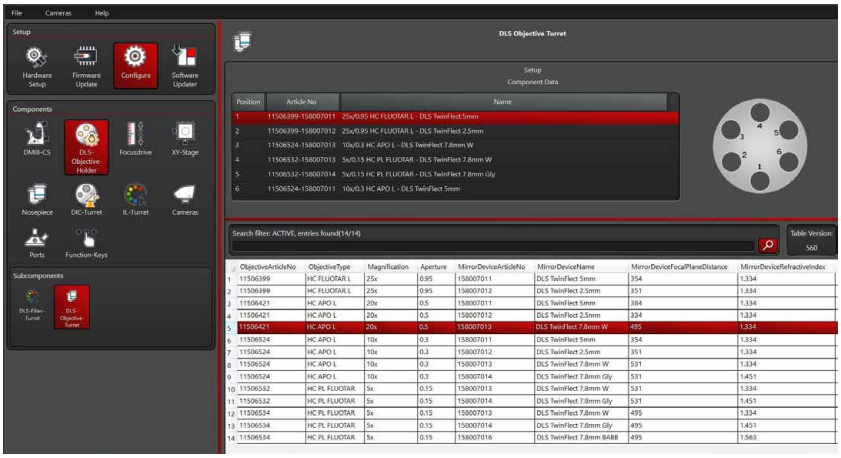
> Select Configure -> DLS-Objective-Holder -> DLS-Objective-Turret

> Teach in the respective DLS detection objective(s) together with the mirror cap(s):

Detection Objective	Article Number	Mirror Cap	Article Number
5x/0.15 IMM	15506534	5 mm	158007011
		7.8 mm W	158007013
		7.8 mm Gly*	158007014
		7.8 mm BABB	158007016
		7.8 mm Gly/BABB	158007020
10x/0.30 W	15506524	2.5 mm	158007012
		5 mm	158007011
		7.8 mm W	158007013
		7.8 mm Gly*	158007014
		7.8 mm Gly/BABB	158007020
16x/0.6	15506536	2.5 mm	158007012
		5 mm	158007011
		7.8 mm W	158007013
		7.8 mm Gly/BABB	158007020
20x/0.5 W	15506421	2.5 mm	158007012
		5 mm	158007011
		7.8 mm W	158007013
25x/0.95 W	15506399	2.5 mm	158007012
		5 mm	158007011

Possible combinations of detection objectives and suitable mirror caps.

*Legacy



Teach in DLS detection objectives with mirror caps.

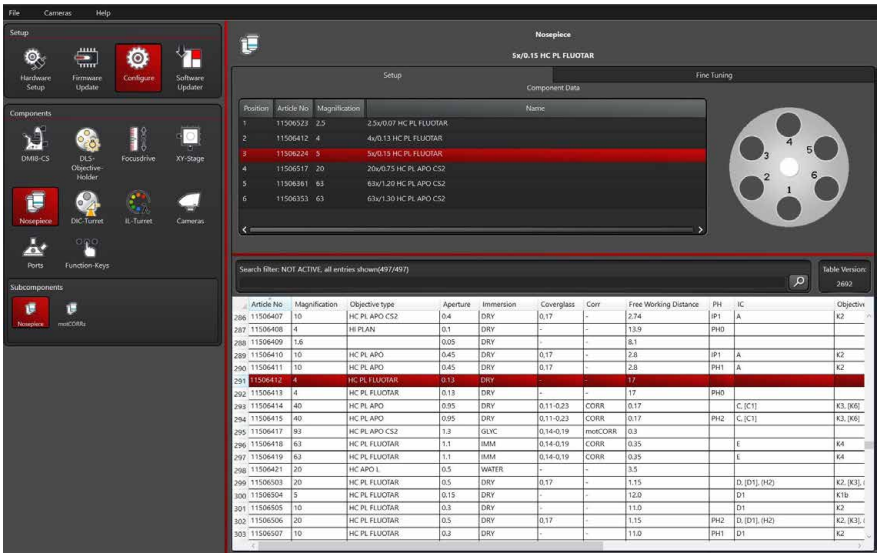
Teach in the illumination objectives

> Select Configure -> Nosepiece -> Setup

> Teach in the DLS illumination objective(s)

Illumination Objectives	Article Number
1.6x/0.05	15506409
2.5/0.07	15506523
4x/0.13	15506412

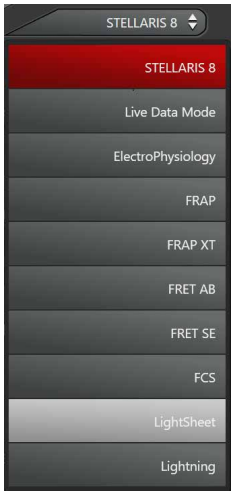
Available DLS Illumination objectives (see also page 28)



Teach in DLS illumination objectives and, if needed, further objectives.

Note: For calibration at least one illumination objective < 5x needs to be installed.

Start your experiment



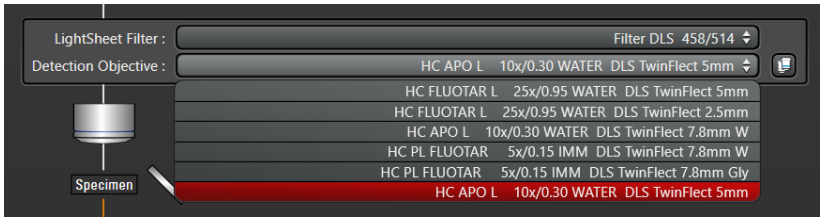
> Start LAS X

> Open the LightSheet Wizard in LAS X

Before you start imaging your sample, you have to calibrate the light sheet in 3 steps

1. Calibration-Detection
2. Calibration-Illumination
3. Calibration-Optimization

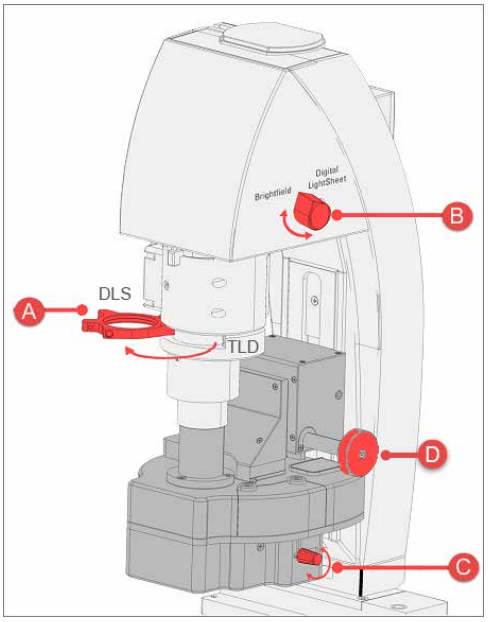
→ The LightSheet Wizard opens directly in the DLS Calibration Step1 “Calibration-Detection”. If you have not yet switched on the laser(s), the wizard asks you to do so.



> Select the correct detection objective with mirror cap from the list.

> Check that the mirror cap and illumination objective are compatible with each other (see the table on page 4. Possible combinations of detection objectives and suitable mirror caps).

> Orientate the mirrors parallel to your sample by turning the screw set (C). If the objective with the mirrors is very strongly twisted, press the screw set backwards to unlock. Hold it in this position and turn the objective by hand until the mirror and sample are parallel. Exact fine alignment is performed later.



A: Lens for TLD (Transmitted Light Detector)

B: Knob to change to BF (Brightfield)

C: Setscrew to align the mirror caps

D: Z-actuator of the DLS objective holder

Focus your sample

> Make sure that the TLD lens (A) is swung out.

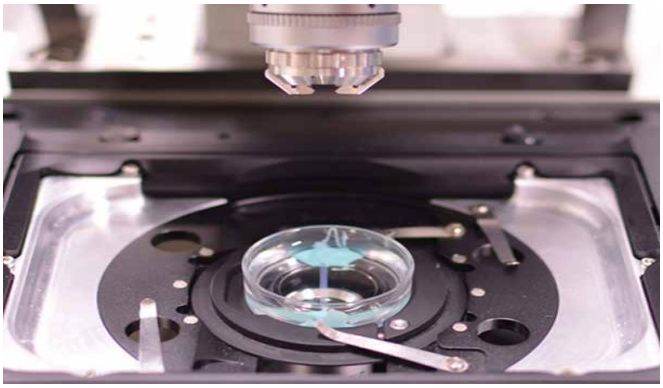
> Move the DLS objective holder (D) upwards to a safe height to prevent a collision with the sample when tilting the transmitted light arm.

> Fasten the sample on the stage. The sample (e.g. FEP tube with beads) in the Petri dish has to be centered between the two reflecting surfaces of the mirror cap.

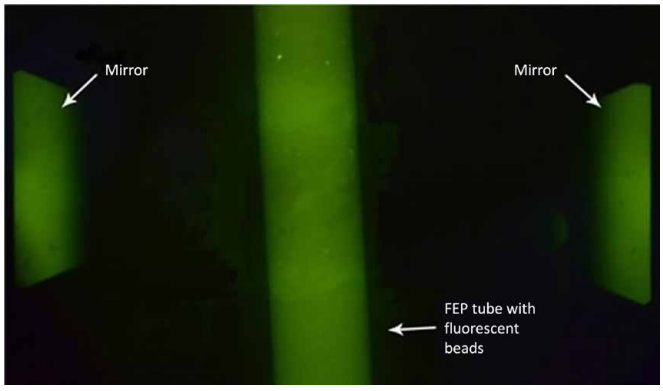
> Fill the Petri dish at least 2/3 full with distilled water/medium. Ultimately, the detection objective needs to be immersed in the medium.

> Set the EL6000 fluorescence lamp / LED to the lowest level (on the EL6000 intensity regulator and microscope display).

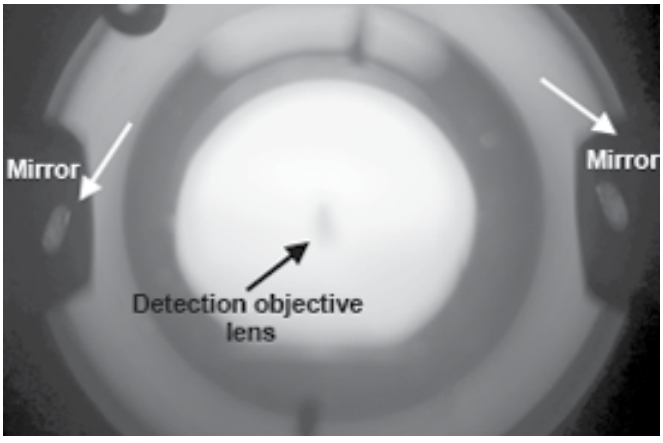
Note: There is no difference between using fluorescence or transmitted light to focus the sample. It is always advisable for beginners to check both.



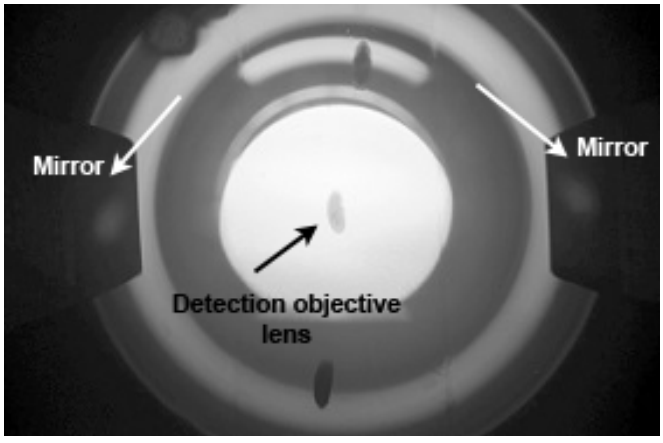
Orientation of the sample (here beads in FEP tube)



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



Transmitted light image (focus on mirrors)



Transmitted light image (focus directly on sample)

> Tilt the transmitted light arm down.

> Carefully move the DLS objective holder downwards with the setting wheel (D) until the mirror cap with the 2 mirrors is in the medium.

> Focus on the sample by using Z-Wide as usual. Check focus via the eyepieces.

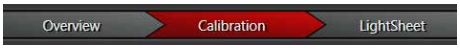
> Do not use Z-Galvo!

> Move the detection objective with mirror cap further down, check via the eyepieces until you can see both the right and left mirror.
The process is finished 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.

> Align the mirrors by turning the rotation plate (C).

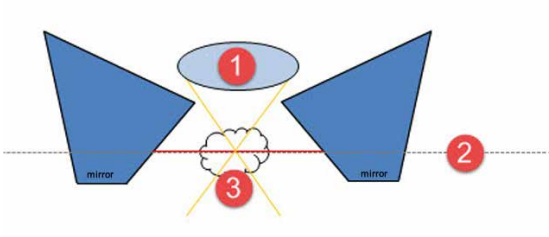
Note: You cannot focus on both the mirror image and the sample at the same time.It is only possible to see either the reflection or the sample sharply in the mirror. Either one can be used.

DLS Calibration



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.

> Use the rotary switch (B) to change from the Brightfield to Digital LightSheet mirror position.

> Click "Detection" in LAS X.

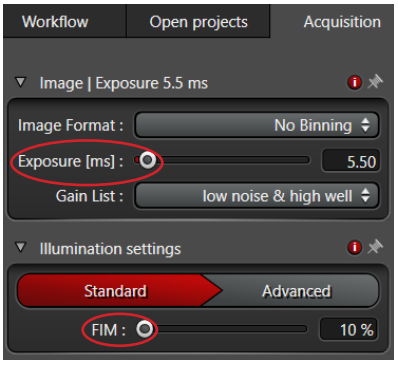
> Select a suitable filter cube, such as GFP, for "Fluo Filter Cube". For "LightSheet Filter" select a compatible fluorescence filter, e.g. filter cube I3 in combination with a 505–545 band pass filter, or the Empty position. The Empty position on the side of the LightSheet filter is recommended, because this allows you to see the sample in a quasi-brightfield mode.

> Click the "Live" button.

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

> If necessary, rescale the image using the slider of the color look-up table.

> If necessary, reduce the exposure time ("Exposure" slider) and the illumination of the microscope ("FIM" slider) in LAS X.



LAS X LightSheet Wizard: Camera Settings

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

Calibration step 2: Illumination

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 is used, the Illumination part of the calibration has to be performed with the illumination objective < 5x. Otherwise, the bottom edges of the mirrors might not be hit by the illumination beam (see below).

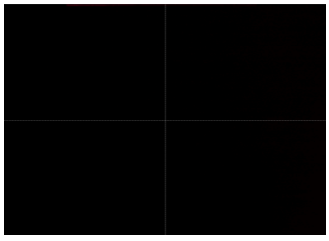
> Click the button “Illumination”

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.

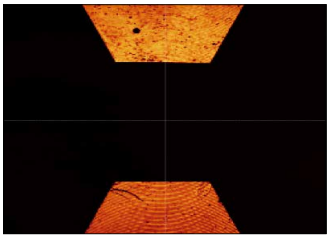
> Click "Live".

> Use Z-Wide to focus on the mirror caps until you see the reflection of the bottom mirror edge of the detection objective. Make sure that you have found the correct maximum during calibration.

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



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



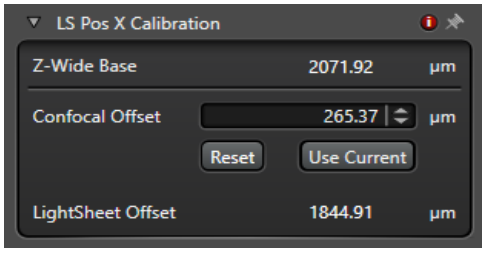
TwinFlect 2.5 mm with 2.5x illumination objective, 25x detection objective

The lower surfaces of the TwinFlect 2.5 mm 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".

The automatic calibration is carried out by means of a z-stack. The Z-Wide position of the illumination objective, where the highest reflection is measured, is automatically stored as the "Z-Wide Base" value. The values are displayed in the "LS Pos X Calibration".



For additional information see page 26.

Calibration step 3: Optimization

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

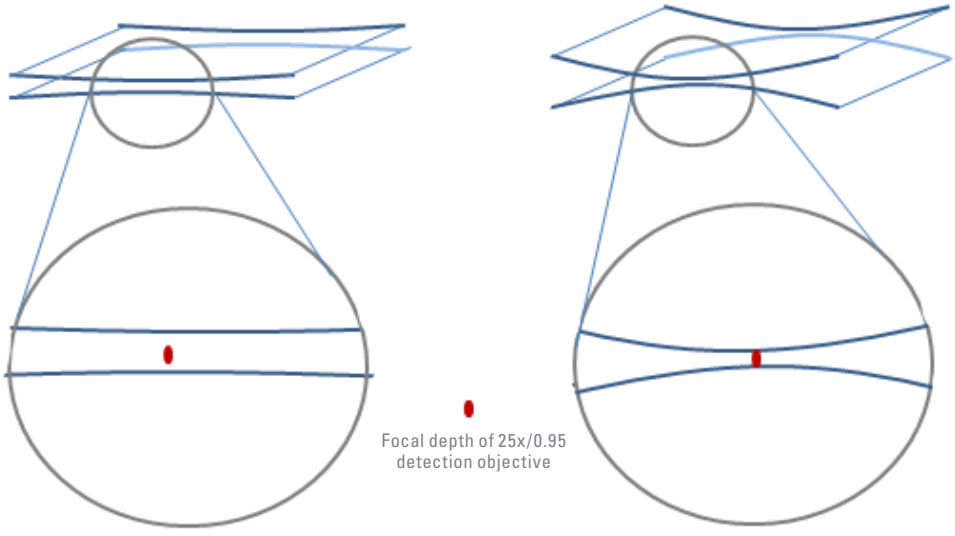
> (Z Pos Sample)

For the next steps, use the control panel. This figure shows you the drives which are in the light sheet system (for detailed information see page 27).

Why do we call it the "beam waist"?

The light sheet is created by a line scan of the illumination objective (=virtual light sheet). Therefore, it has the shape of the point spread function of this objective.

An illumination objective with a higher NA creates a thinner and shorter light sheet than an objective with a lower NA (see page 29, table showing the light sheet properties). For the recommended combinations, the geometry of the light sheet fits to the z-resolution and field of view of the detection objective. The thinnest part of the virtual light sheet is the "beam waist".



The light sheet is generated with two different illumination objectives 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

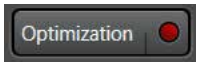
B. Focus the beads – Adjust LS Position Z

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

D. Fine Adjustment of the mirror orientation – Turn objective holder manually

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

A. Preparation – First steps



> Click "Optimization". The mode is changed to LightSheet.

> For "LightSheet Filter" select the empty position (this is suitable for samples in agarose which generate scattered light).

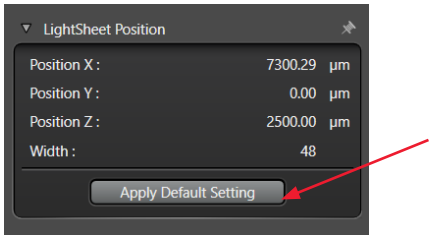
> Set "LS Width" to 48 to make the light sheet narrower and brighter.

> 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 via "LS Position Z" on the control panel.

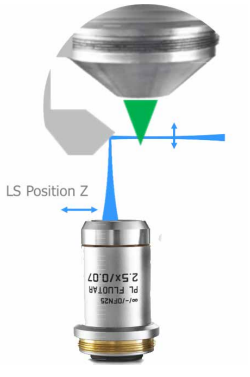
20 LS Width

Note: If you don't see any light sheet at this point, click "Apply Default Settings". This button only works correctly, if the current detection objective/TwinFlect combination has been selected from the drop-down menu.



B. Focus on Beads – Adjust LS Position Z

Depending on your sample, there are two alternatives to adjust "LS Position Z": a manual and an automatic calibration.



LS Position Z

Manual adjustment of LS Position Z

> Click "Calibrate" to open the "Calibrate Mirror Position" dialog.

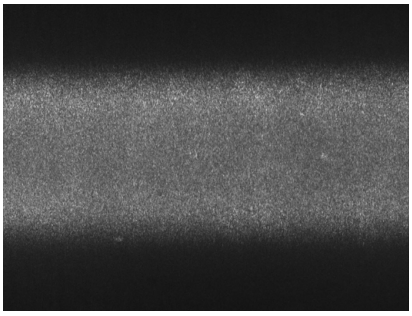
> Select any mirror (left or right): Focus the beads / find the highest contrast via "LS Position Z" on the control panel.

> Click "Define Mirror Position".

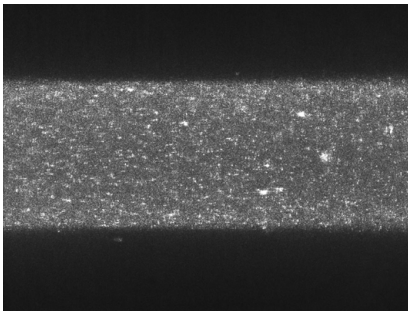
> Enable the other mirror: Focus the beads like before.

> Click "Define Mirror Position".

> If necessary, repeat the steps.

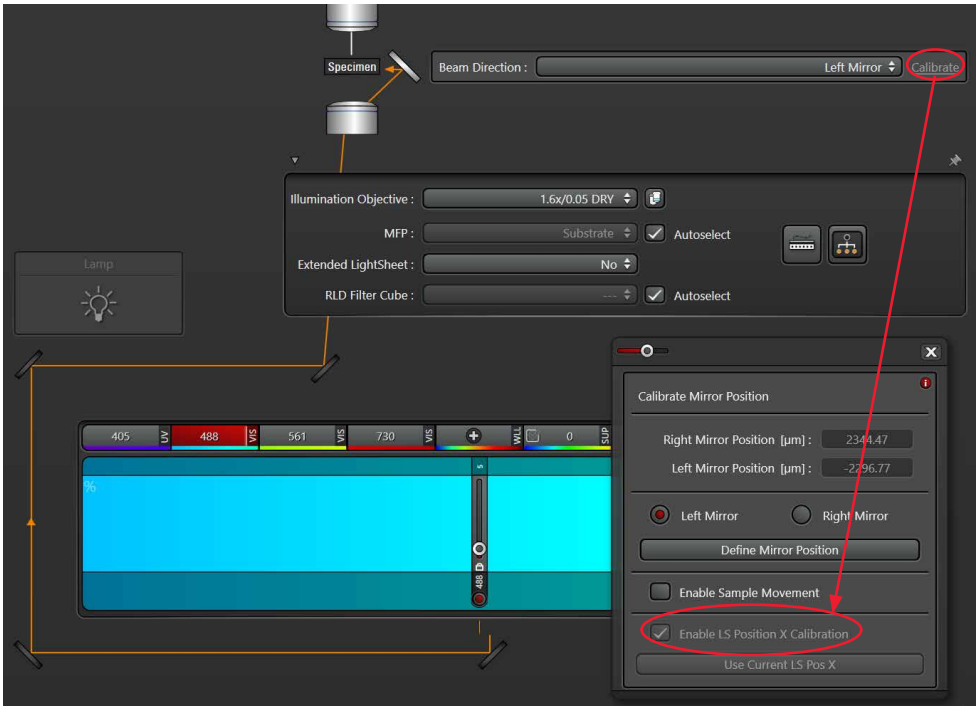


Beads out of focus



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 calibration.



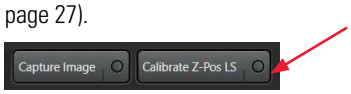
Adjust „LS Position Z" in the "Calibrate Mirror Position Dialog"

Alternatively, an automatic adjustment of LS Position Z can be done

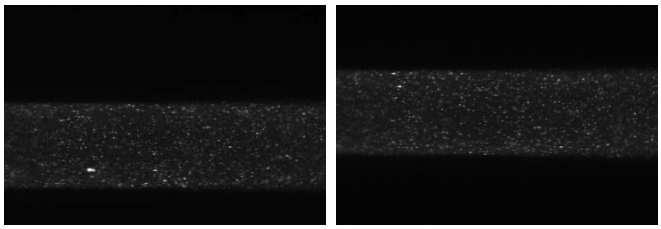
Go to a position of your sample, where you can either get a homogenous fluorescent signal or a homogenous signal in reflection (the latter is shown in our beads sample, reflection of agarose used for embedding).

> Click “Calibrate Z-Pos LS” for automatic calibration.

Note: For each combination of detection objective, TwinFlect, and illumination objective, you also need to find the proper „LS Position Z“ (see also page 27).



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 pure manual adjustment on the DLS objective holder.

Fine adjustment of the mirror orientation

> Click "Start live scan with mirror toggling" (light flickering is normal).

> During the “Toggle" Scan, use the setscrew on the DLS objective holder (see page 6, C) 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.

Fine adjustment of the mirror cap

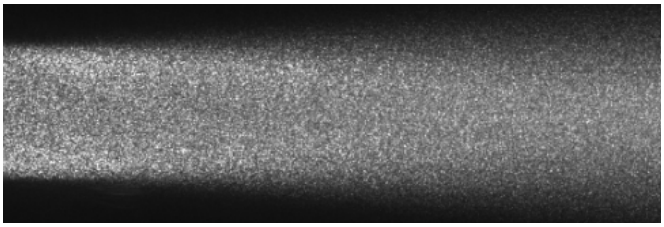
E. "Center the light sheet in horizontal direction" – Adjust LS Position X

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

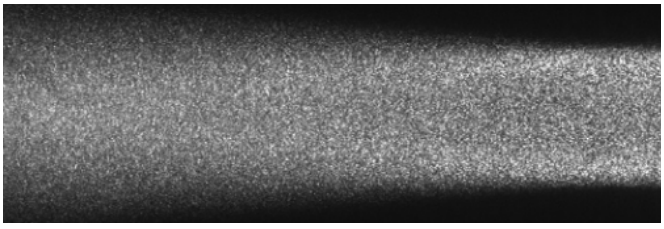
> Click "Live".

> The „LS Position X“ value is displayed on the control panel.

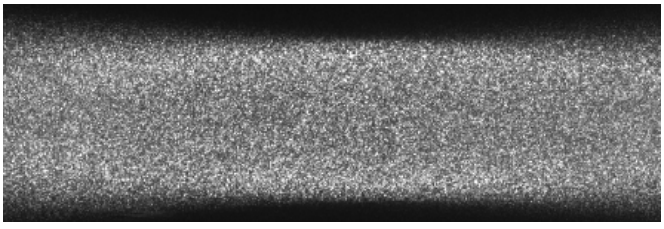
> If now the „LS Position X“ is changed during Live Scan, you will see how the focus of the illumination objective moves either to the left or to the right.



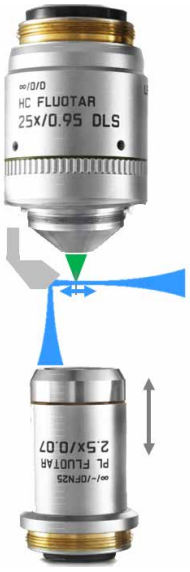
To the left or ...



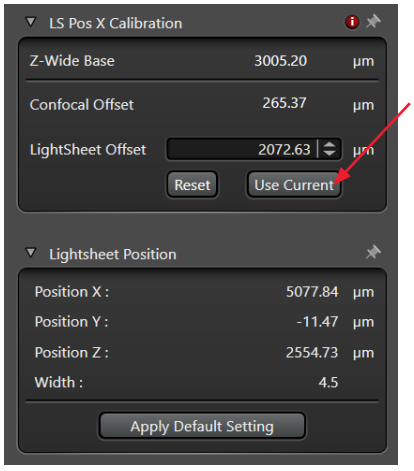
... to the right.



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



LS Position X

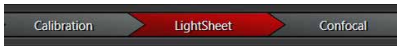


Click "Use Current" to confirm the position of “LS Position X”.

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

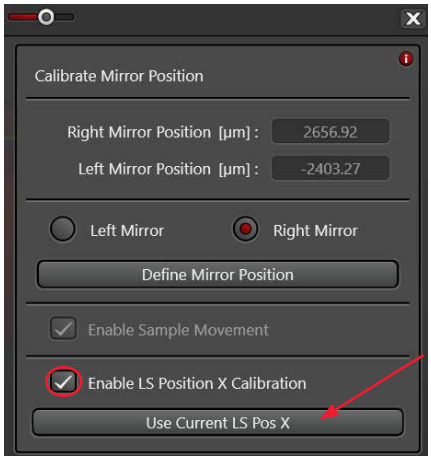
Note: Your current „LS Position X“ is now stored for this combination of detection objective, TwinFlect mirror, and illumination objective. Only by clicking “Use Current”, will changes to the focus of the illumination objective be taken into account once you switch to the LightSheet tab!

DLS LightSheet



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 recognize that a correction of the light sheet parameters might be necessary, you can perform the mirror calibration (see page 12, Step B, 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 (see page 15, step E, Adjust „LS Position X“), where changes of the „LS Position X“ has to be confirmed by clicking “Use current”.

What about moving the sample in the z-direction?

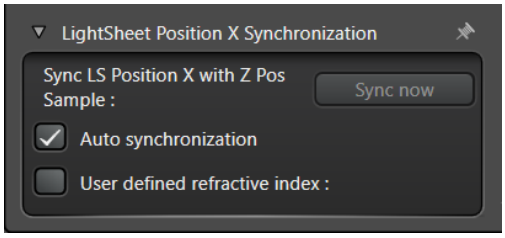
With the control panel drive “Z Pos Sample”, you can move your sample in the z-direction using the super z galvo as usual. In contrast, the Z-Wide drive is blocked by default to prevent unintended changes of the light sheet focus which may interfere with your previous calibration. Z-Wide is blocked both with SmartMove and the control panel (here Z-Wide is called „LS Position X“).

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“).

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

Something to keep in mind

If you move the sample through the light sheet using Z-Galvo, the relative lengths of the illumination beam inside and outside of the medium differ. As a result, the focus position of the light sheet in the x-direction („LS Position X“) also changes, because the lateral focus position of the light sheet is determined by the combination of the two refractive indices (inside and outside of the medium). This lateral focal displacement can be compensated for by a continuous adjustment of Z-Wide during the image acquisition.



This is done with the "LightSheet Position X Synchronization" function. As a result, the lateral x-position of the light sheet is readjusted depending on the axial z-position via the Z-Wide actuator *(focus tracking)*.

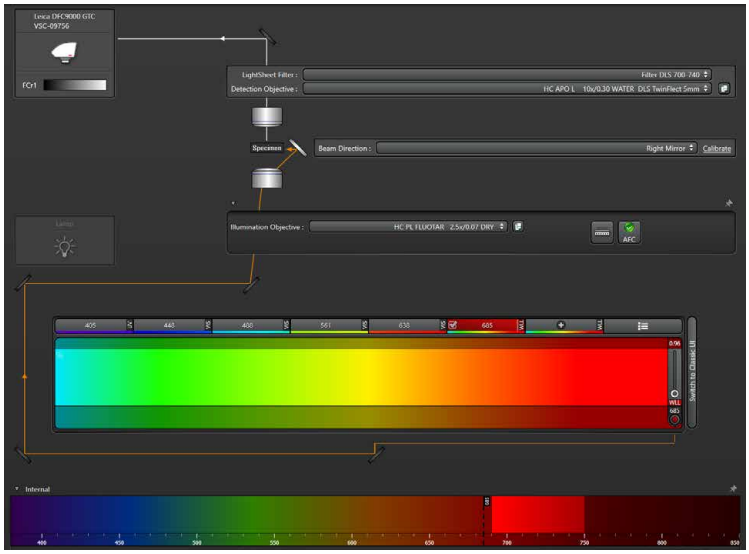
For detailed information, see the LAS X Online Help.

Should I change the illumination objective during the session?

If you want to switch between different illumination objectives within the LightSheet tab, you need to calibrate these different combinations (for example 25x detection objective/TwinFlex 2.5mm with 5x illumination objective and 25x detection objective/TwinFlex 2.5mm with 2.5x illumination objective) separately in the chapter Calibraion step 3: Optimization, page 11.

DLS goes far-red

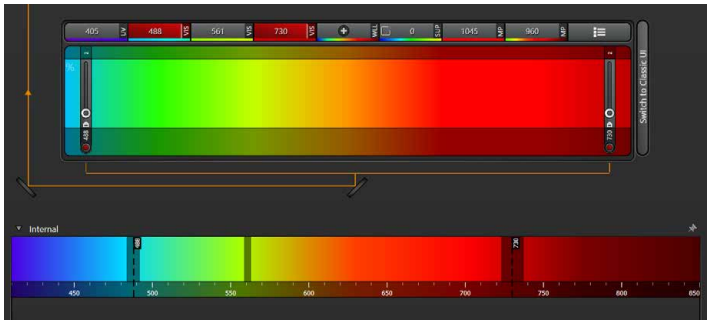
The STELLARIS next-generation White Light Laser (WLL) offers an extended wavelength range of up to 790 nm for excitation (STELLARIS 8: 440-790 nm, STELLARIS 5: 485-685 nm). In addition, a deep red solid-state laser (730 nm) is available. These possibilities allow multi-color capabilities to be further extended for DLS.



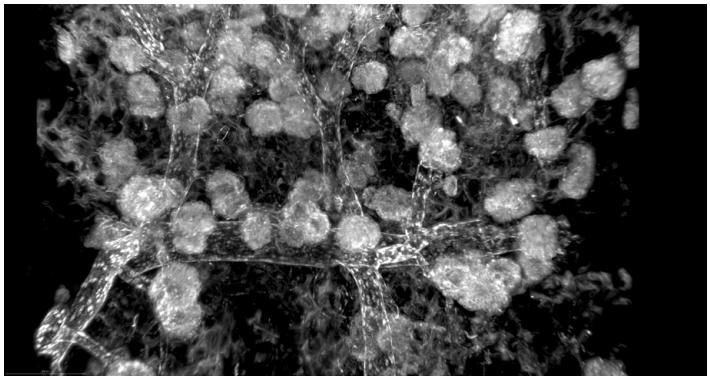
The figure shows a WLL excitation at 685 nm (STELLARIS 5) with a suitable DLS filter 700-740.

Note: For applications with far-red dyes, please use an appropriate filter, i.e. DLS 488/561/730, DLS 700-740 or DLS 745-795 and the multi-immersion objective 15506536 HC FLUOTAR L 16x/0.60 IMM CORR DLS, which is color corrected from VIS to the far-red range.

This objective is designed for live cell applications using water immersion up to the imaging of glycerol-mounted or BABB (Benzyl Alcohol, Benzyl Benzoate) -cleared samples. For recommended combinations of this detection objective with an illumination objective and a Twinflect mirror, see page 28.



Visible solid state lasers 488 nm and 730 nm for excitation in combination with the DLS-488/561/730 filter.



Cleared mouse kidney recorded at 730nm illumination, detection with HC FLUOTAR L 16x / 0.6 IMM CORR DLS.
Sample courtesy of Prof. Gretz, University Mannheim.

Channel specific calibration

The position of the light sheet depends on physical factors such as the optical path length. Due to the offset of the light sheet during acquisition with multiple wavelengths, this can lead to blurred images and image series.

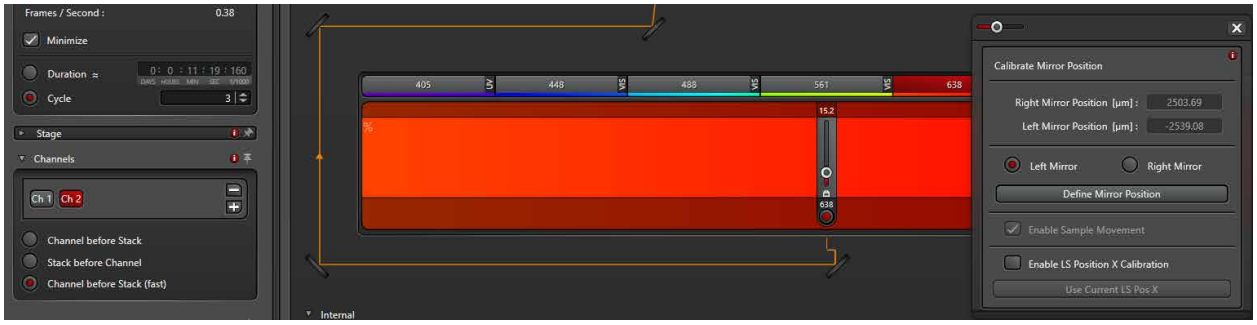
Therefore, we recommend performing a channel-specific calibration before starting the light sheet experiment with multiple excitation wavelengths, especially if the wavelengths of the laser lines are far apart.

This means that for multicolor data acquisition, the calibration values for the "LS positions Z" of both the left and right mirrors are selectively applied to the data acquisition of all defined channels. This channel-specific calibration corrects for lateral chromatic aberrations on the side of the illumination objective and leads to crisp and in focus images for all the acquired channel in a multi color acquisition.

> If not yet completed, perform the general DLS Calibration for one channel as described in the "DLS Calibration" chapter on page 9.

> Go to the LightSheet tab.

> Click on „Calibrate" to open the „Calibrate mirror position" dialog. Here, the „LS Position Z" can be calibrated again or recalibrated if necessary. Apply this by clicking on „Define Mirror Position" (more information in the „DLS LightSheet" chapter on page 17).

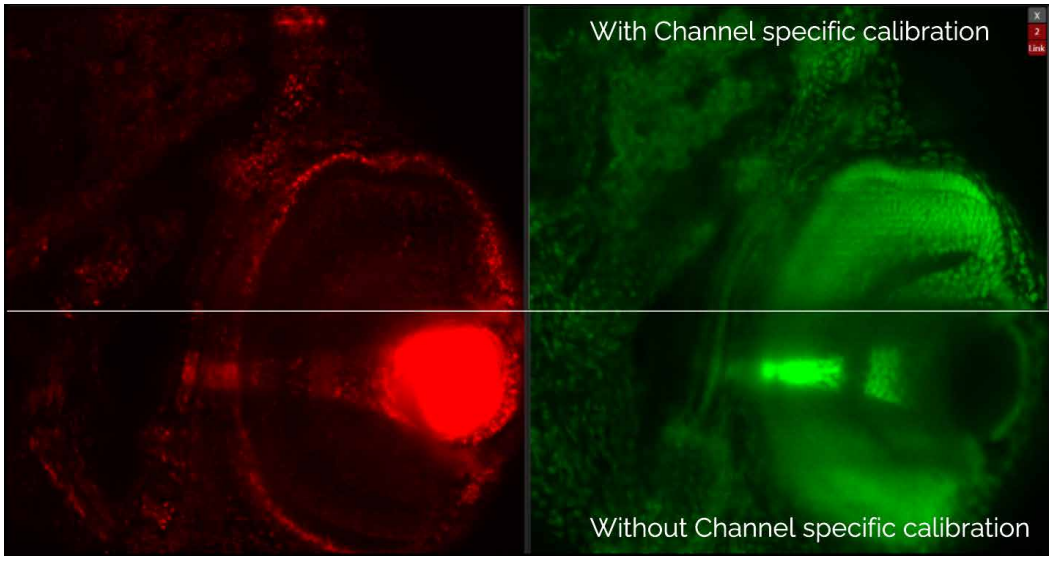


> Select a second channel in the "Channels" dialog by clicking "+" and adjust the imaging parameters needed (excitation laser line, filter, camera settings, etc.).

> Calibrate the mirror positions for the second channel in the same way as for the first channel by defining the „Mirror position" (more information in the "DLS Light Sheet" chapter on page 17).

> Repeat this channel-specific calibration for additional channels.

The "LS Position Z" for every channel is always defined by clicking "Define Mirror Position" and these adjusted calibration values will be kept for every channel specifically.



Zebrafish eye: In the image below, acquired without channel specific calibration, the structures in the red channel are well focused, while the green channel looks rather blurred. In the image above, taken with channel specific calibration, the green channel is also in focus. Sample courtesy of Prof. Jochen Wittbrodt, COS Heidelberg.

Note: For fast image acquisition with multiple channels, use a triple or quadruple filter. This is especially important when you select „Channel before stack (fast)". Depending on the scan mode (xyt, xyzt and xymzt) the existing channels are prioritized over the other scan dimensions: Channel before mirror, z-plane and point in time.

Widefield

Widefield mode within the LightSheet Wizard

Direct access to the widefield imaging mode allows you to rapidly acquire an overview of your sample.

The advantage of widefield mode (transmitted light) is that you can get a quick overview of the sample independently of a fluorescence signal. With transmitted light, navigation is easier, Tile Scan definition is more straightforward and multiple positioning is usually possible.

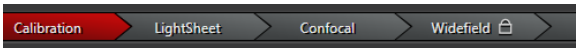
Often, these capabilities can help you to identify interesting areas within your sample (particularly with large samples) and can also simplify the definition of a z-stack for multicolor images.

An additional advantage is that the 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 Widefiled 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.



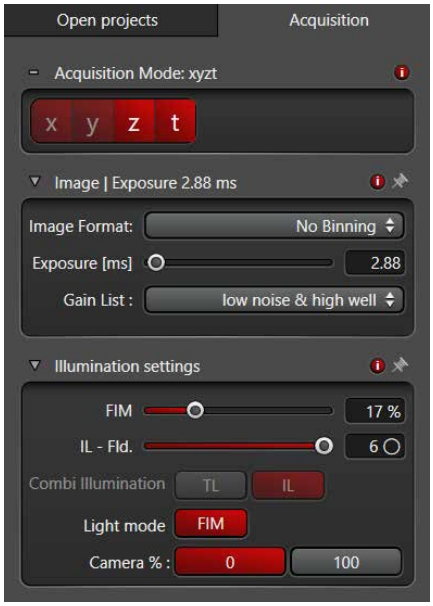
Note:

1. It is prerequisite to perform the general “DLS calibration first”.
2. The Widefield tab is locked (see arrow) 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” (see page 17).

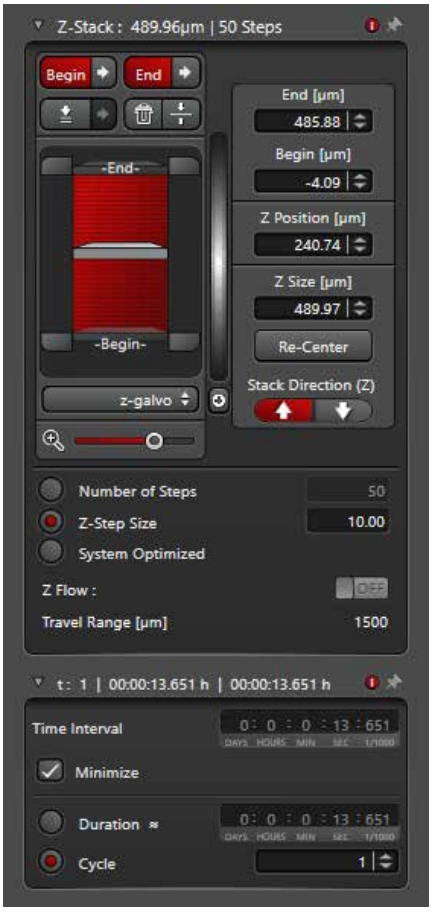


> Under "LightSheet Filter", remove the filter by selecting the "Empty" entry.

> Select the „Acquisition Mode" x,y,z,t to define the dimensions for the image acquisition and optimize camera parameters and illumination strength for widefield acquisition.

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

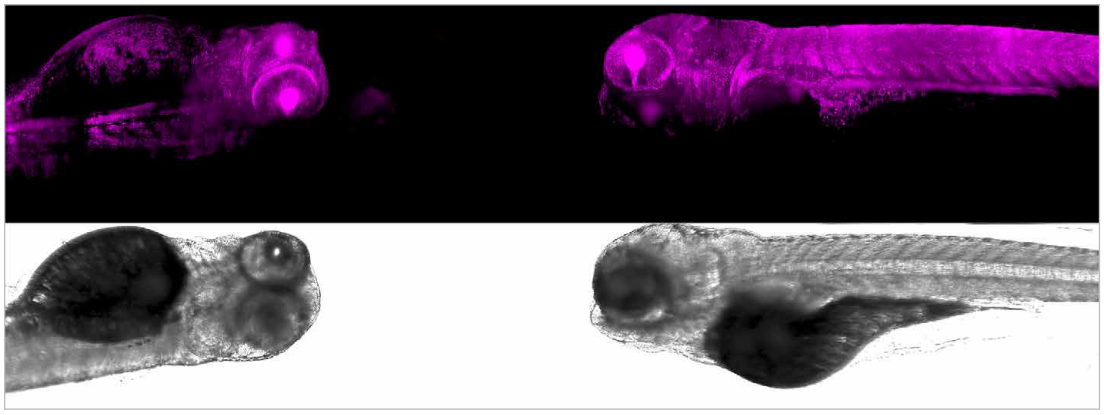
Note: Z-stacks and/or time-series can be defined and performed as well. Both can be combined with Tile Scan/Multi-positioning.



> 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” (see page 17).

> Under “LightSheet Filter”, remove the filter by selecting the “Empty” entry.

> Select the „Acquisition Mode“ x,y,z (t is not available), and adjust camera parameters and the illumination strength for widefield acquisition (see above).

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

> Click “Stop” to stop the live acquisition in Widefield mode.

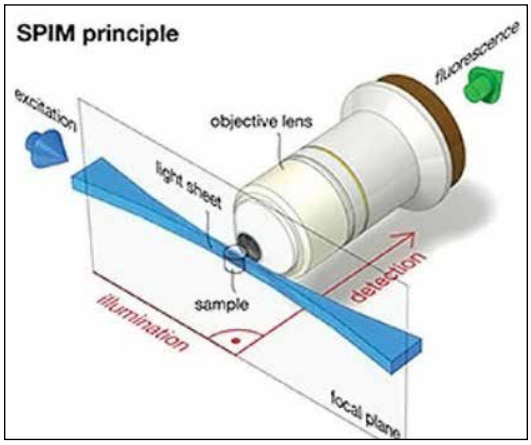
Note: Z-stacks can also be defined in combination with Tile Scan/multipositioning, i.e., define a z-stack in the Widefield tab and go back to the LightSheet tab. The z-stack parameters will be applied automatically, and the light sheet acquisition can be started immediately.

.

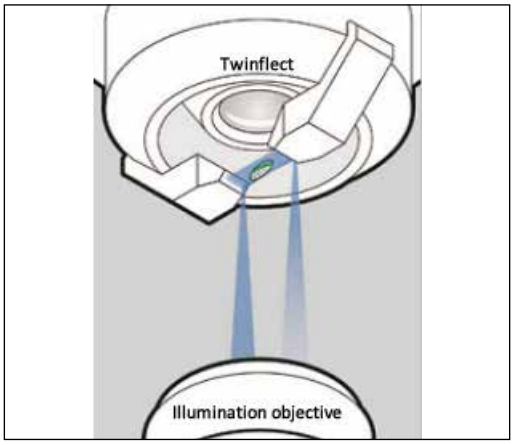
Additional Information

Principle

In light sheet microscopy, the sample is illuminated from the side in a single plane. Conventionally, the generation of a light sheet is made possible by a special optical setup in which the illumination and detection objectives are arranged perpendicularly to each other. Objectives with a low numerical aperture (NA) - which produce a flat focus – are used as illumination objectives.



Conventional generation of a light sheet (Huisken and Stainier, 2009)



Generation of a light sheet with DLS module

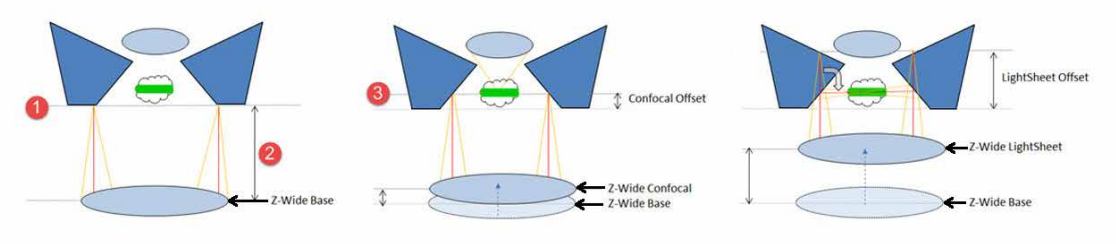
Digital LightSheet Module (DLS Module)

The DLS module consists of an illumination objective and a detection objective along a vertical axis. The two mirrors of the Twinflect deflect the illumination beam at a 90° angle and the sample is positioned between the two mirrors. The light sheet is generated by a laser and a camera records images of the sample area illuminated by this light sheet. To record a 3D- volume, the sample is moved through the light sheet.

The distance between the two objectives has to be calibrated so that the focus of the illumination objective coincides with the focus of the detection objective.

For detailed information see the LAS X Online Help “What is Light Sheet Microscopy?”

Overview

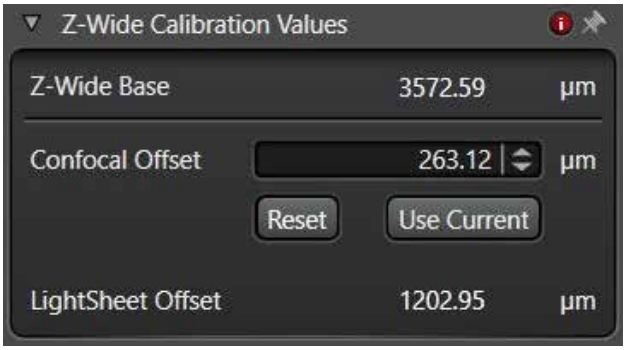


Illumination, schematic: 1 = Bottom edge of mirror. 2 = Distance between illumination objective and bottom edge of mirror.
3 = Focal plane of the illumination objective.

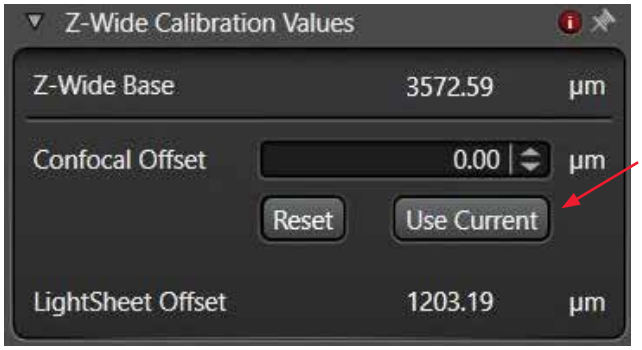
Term	Explanation
Z-Wide Base*	**Z-Position at which the reflection of the mirror's bottom edge is maximum.**
Confocal Offset	**Distance between the mirror's bottom edge and the focal plane of the detection objective** It is a default value, a property of the mirror cap stored in a calibration file.
Z-Wide Confocal*	**Z-Wide Base + Confocal Offset** If you leave the Calibration tab and go to the Confocal tab, the illumination objective will automatically move to this Z-Wide (Confocal) position.
LightSheet Offset	**Confocal Offset + 1/2 Mirror Offset** The distance is required to determine, that the light sheet "beam waist" lies centrally between the two mirrors. Note: A default value for each TwinFlect mirror is stored and can be recalled pressing the "Apply default settings" button.
Z-Wide LightSheet*	**Z-Wide Base + LightSheet Offset** If you go to "Calibration Step 3: Optimization", or to the LightSheet tab, the illumination objective will automatically move to this Z-Wide (LightSheet) position.

*These z-positions are absolute Z-Wide positions.

Additional information on chapter "Calibration step 2: illumination" page 10



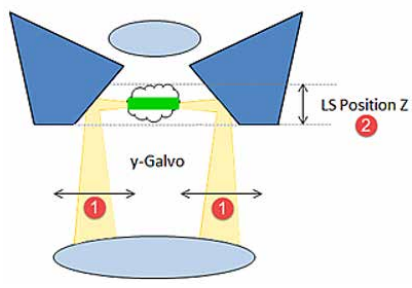
After calibration of the Z-Wide Position (Z-Wide Base)



By clicking "Use Current", the Confocal Offset is set to zero.
This is only relevant if you switch between LightSheet and Confocal Mode.

Light sheet system	Confocal system
LS Position Z	Y-Galvo
LS Position X	Z-Wide
LS Position Y	X-Panning (horizontal panning)
LS Width	Zoom
Z Pos Sample	SuperZ-Stage (set to "0" during the calibration)

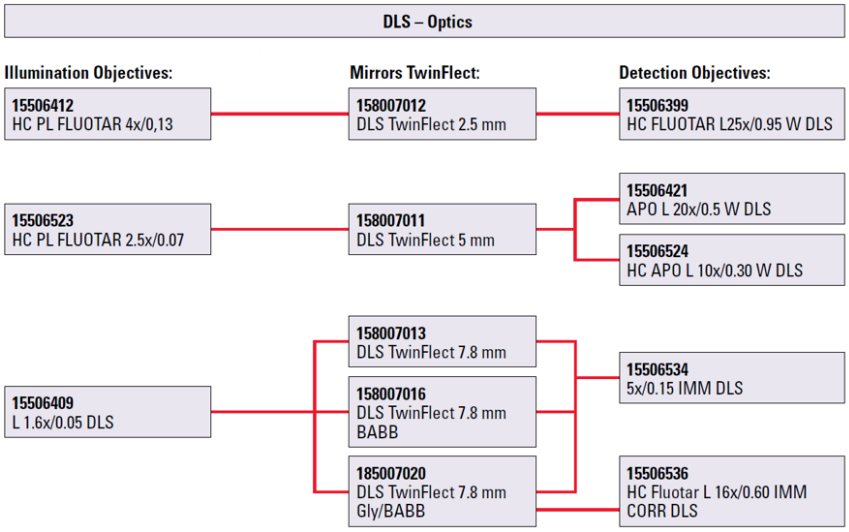
LS Position Z



Illumination objective: 1 = Direction of movement of the y-Galvo. 2 = Direction of movement of the LightSheet Position Z

Combinations of illumination and detection objectives

Recommended (application driven) combinations of illumination and detection objectives



Possible combinations of Illumination objectives and mirror caps

Illumination Objective	Article Number	Mirror Cap	Article Number
1.6x/0.05	15506409	2.5 mm	158007012
		5 mm	158007011
		7.8 mm W	158007013
		7.8 mm Gly*	158007014
		7.8 mm Gly/BABB	158007020
2.5x/0.07	15506523	2.5 mm	158007012
		5 mm	158007011
4x/0.13	15506412	2.5 mm	158007012
5x/0.15	15506224	2.5 mm	158007012

*legacy

Axial and lateral resolutions & light sheet (LS) properties

		Illumination objectives						
	Axial Resolution	1.6x / 0.05	2.5x / 0.07	4x / 0.13	5x / 0.15	**Widefield** Axial Resolution (in comparison)	**Lateral Resolution** @530	**FOV** (full chip)
Detection objectives	5x / 0.15	5.8µm / 13µm	4.0µm / 8.9µm	2.3µm / 5.7µm	1.9µm / 4.5µm	37µm	2.2µm	1470 x 1470µm
	10x / 0.3	4.9µm / 7.8µm	3.7µm / 6.5µm	2.2µm / 4.9µm	1.9µm / 4.1µm	9.3µm	1.1µm	735 x 735µm
	16x / 0.6 (in water)	2.2µm / 2.4µm	2.1µm / 2.3µm	1.7µm / 2.2µm	1.5µm / 2.1µm	2.4µm	540nm	460x460µm
	16x / 0.6 (in glycerol)	3.1µm / 3.5µm				2.6µm	540nm	460x460µm
	16x / 0.6 (in BABB)	2.5µm / 2.8µm				2.8µm	540nm	460x460µm
	20x / 0.5	2.8µm / 3.2µm	2.5µm / 3,1µm	1.9µm / 2.8µm	1.7µm / 2.7µm	3.3µm	650nm	368 x 368µm
	25x / 0.95	1.0µm / 1.0µm	0.9µm / 1.0µm	0.9µm / 1.0µm	0.9µm / 0.9µm	1.0µm	340nm	295 x 295µm
	Lightsheet Properties	1.6x / 0.05	2.5x / 0.07	4x / 0.13	5x / 0.15			
	LS Thickness @ 488nm	5.8µm / 14µm	4.1µm / 9.2µm	2.3µm / 5.7µm	1.9µm / 4.6µm			
	LS Length @ 488nm	~500µm / 3mm	~250µm / 1.2mm	~75µm / 500µm	~60µm / 300µm			
	TwinFlect	7.8mm 5mm 2.5mm	5mm 2.5mm	2.5mm	2.5mm			
		with / wo BE*	with / wo BE*	with / wo BE*	with / wo BE*	*BE=beamexpander		

Light sheet properties

Overview of lateral and axial resolutions

that can be obtained with the different combinations of detection and illumination objectives

Axial Resolution:	Depends on combination of illumination and detection objective	
Axial Resolution for Widefield:	Gives the axial resolution of the detection objective without influence of light sheet illumination	
Lateral Resolution:	Depends on NA of detection objective	
Field of View (FOV):	Depends on detection objective	
Light Sheet Properties:	Depend on NA of illumination objective.	
	Light Sheet Thickness	= **xy resolution** of illumination objective (FWHM)
	Light Sheet Length	= **z resolution** of illumination objective (focal length -> homogenous illumination)
Appropriate TwinFlect:	Depends on FOV of illumination objective	

Troubleshooting

Step 1: Detection:

A. After clicking “Live”, there is a black image in the viewer

> Make sure that the knob on the microscope transmission arm is turned from “Brightfield” to “Digital LightSheet”.

> Make sure that the shutter of the halogen lamp / EL6000 is open (can you see light on the sample?).

> Make sure, that the TLD lens (A) is swung out

> Check that an appropriate “LightSheet Filter” is selected:

  > “Block Filter” does not transmit any light to the camera

  > Bandpass filter might not transmit enough light to the camera (e.g. Filter DLS 575- 615 might block all light if Fluo Filter Cube FI/TRITC is selected)

  > Use the “Empty” position or a Notchfilter (e.g. DLS 405/488/561/633)

B. After clicking “Live”, there is a white (saturated) image in the viewer

> Turn down the intensity of the EL6000 and FIM of the microscope.

> Reduce the exposure time of the camera (hidden in “Acquisition”).

> Move to a “LightSheet Filter” with less transmission (see above).

Step 2: Illumination

A. It is not possible to see the reflection of the lower surfaces when the TwinFlect 2.5 mm is in use.

> Make sure that the 2.5x/0.07 objective is used as “Illumination Objective” when doing the calibration with the TwinFlect 2.5 mm.

B. Advice: Find the reflection maximum closest to the reflection of the cover slip!

> Move to a “LightSheet Filter” with less transmission (see above).

Step 3: Optimization

A. It is not possible to see a light sheet

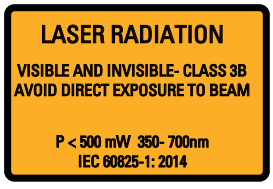
> Rescale the LUT of the Image Viewer. For a standard light sheet image, you normally don’t need more than 5000 gray values for the 16-bit image.

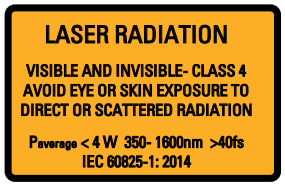
> Use the “Usual suspect” values for the Calibration values („LS Position Z“: half the mirror distance; „LS Position Y“ = 0; LS Width = 48).

> Make sure that the detection objective is fully immersed (add more medium if needed).

B. “LightSheet Offset” is completely out of range, which you are used to seeing, once you have positioned the focus to the center and confirmed with “Use current”.

> Make sure that you have found the correct maximum (the first comes from the coverslip reflection) during the Calibration.





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