

Leica SP8 Upright confocal microscope 7, 11, 2020

- **System description:**

- DMI6, Upright microscope: BF, Fluorescence (blue, green, red)
- BF TL Detector (transmitted light image)
- Scanning stage with z-Galvo and Navigator (overviews, selection of regions of interest...)
- Scanner with Scan-field Rotation
- 2 PMT detectors (photo multipliers)
- 1 Hybrid Detector normal (more sensitive than PMT and no detector noise nor background)
- 1 Hybrid Detector SMD (fast, for FLIM, FALCON)
- Laser lines: 405 continuous wave, White Light Laser (pulsed) (470-670nm, up to 8 laser lines simultaneously)
- FALCON (FAST Lifetime CONTRast) including Pulse Picker for WLL
- LIGHTNING (Deconvolution) for Super-resolution and super sensitive imaging
- Phasor plot for direct examination of lifetime data

- **Objectives:**

- Objective HC PL FLUOTAR 5x/0.15 Dry , WD=13.3mm (overview) (506224)
- Objective HC PL FLUOTAR 10x/0.3 Dry , WD=11mm (506507)
- Objective HC HC PL APO 20x/0.75 Dry , WD=0.62mm (506517)
- Objective PL APO 20x/0.75 IMM CORR VISIR, WD 0.67 mm (H₂O, Oil, glycerol)- (506343)
- Objective HCX PL APO 40x/1.25 OIL PH 3 CS Oil , WD=0.1mm (506251)
- Objective HC FLUOTAR L 25x/0.95 Water-VISIR, WD=2.5 mm, wide angle (41°) (506374)
- (Not installed)-Objective HCX APO L 63x/0.90 Water , WD=2.2mm (506155)

- **Inserts:**

- z-galvo insert with 0.5mm range (1.5mm in extended range)
- Single slide holder (bracket style)

- **Heating & gas mixer**

- Dark box with temperature control (heating unit)
- No Gas mixer

- **System Start Up**

Turn on heating Unit if needed

- Switch on 'PC-microscope' button (1). This gives power to the computer and microscope controller
- Switch on 'scanner power' (2)- This enables the scanning head
- Switch on 'Laser power' (3)
- Turn the 'Laser ignition key' (4) in position 'on'- enables Laser shutters (-> Only an interlock)
- Switch on fluorescent lamp(5) for visual examination
- **TL/LED** manual switch is at the back of the mic (if you don't see the TL light in the eyepiece, it might be the switch)
- Make sure lowest objective is selected and retracted
- For imaging: start LASX software. Select configuration **Machine.xlhw** or **machine with zgalvo** and **DM6** microscope (You should insert zgalvo stage and fix in place using 2 knobs at the back-> **If you are not using it, place it face down in the heating chamber!**)
- **!!!! Never use "Use last system settings"**

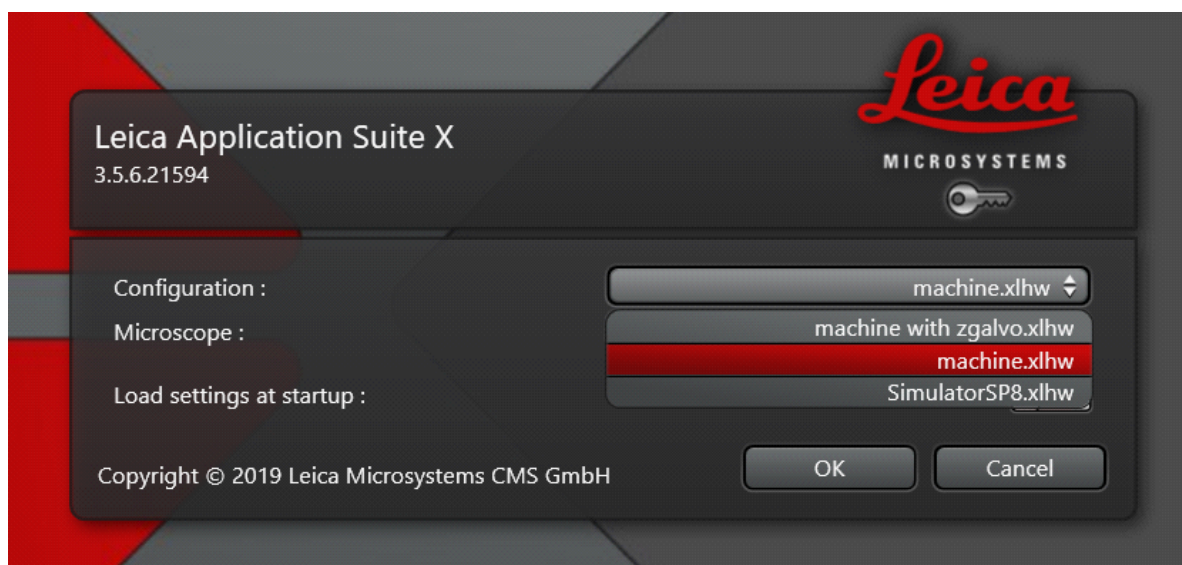


Fig.1

- **Initializing the stage** “clicking yes in the window that pops up”, Otherwise Navigator is not shown-> If not clicking “Yes”, the system will start without initializing the stage after 1min
- In the LASX software, go to ‘**configuration**’ –‘**lasers**’- Turn ON the lasers you want to use. Set the Laser power of the WLL to 85% and 80MHz (usually the default numbers).

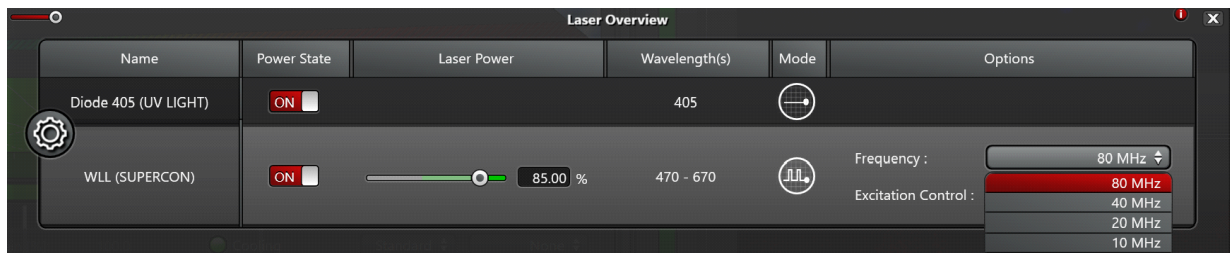


Fig.2

- make sure the pulse picker is set to **80 MHz**, otherwise laser power is significantly lower (Fig.2)

- **Visual examination of the sample**

- On the microscope touchpad choose the illumination icon to open the illumination menu.
- For transmitted light press the **BF/TL** button and operate the TL shutter from touchscreen (if you don't see any light probably the manual shutter "Lever" is closed)
- Intensity buttons are found on the left side of the microscope as well (TL indicator light is next to it)
- For fluorescent illumination, press **FLUO** button in the illumination menu of the microscope touchpad.
- Choose filter cubes on the front panel: (**DAPI, GFP, RHO**). Open the IL-shutter to see fluorescence.
- Intensity buttons (same as for BF) on the left side of the microscope stand lets you change the LED intensity
- **FOCUSing on your sample- To prevent crash, look from the side and raise the sample very close to the objective (to pass the focus point), then look in the eyepiece and always move the stage downward until you see the sample.**
- Visual illumination is disabled during scanning, the system is automatically changing to "CS" mode. (For TL detector you need to manually switch back the "lever" at the back of the microscope).

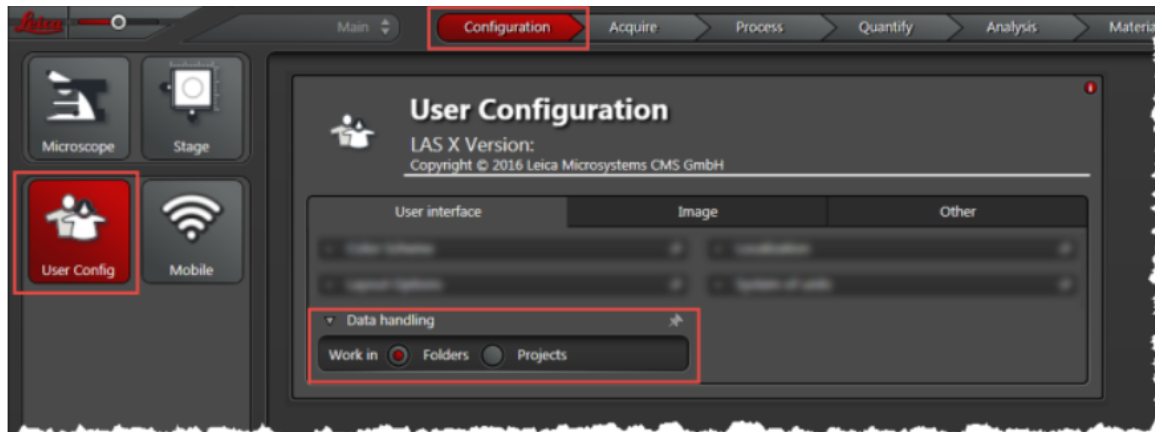
- **Data organization**

In User Config -> Your data can be organized into **Folder** structure or **Project** based structure. In the folder based structure files are saved as TIFF while projects are saved as Lif/Lof. (Due to a software bug do not use Folder base file saving)

Additional info @:

<file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm?Highlight=user>

configuration#DLG/Configuration/DLG_configuration_user_configuration.htm?Highlight=user_configuration



- **Acquiring confocal images**

*Select PMT, choosing FLIM Automatically activates the FLIM mode.
Setup all channels as PMT the system automatically choose PMT or
HyD (if the signal is good avoid using the HyD-> manually change it to a
PMT).*

Before you start:

Make sure that the Autosave option is switched off in Open Project!!!

***CHECK IF THE HyD SMD 1 DETECTOR COOLING CONTROL LAMP IS GREEN. IF IT'S RED
DON'T USE IT, CONTACT BIF TEAM IMMEDIATELY!!!***

- ***Turn on the lasers from the configurations***
- ***Acquisition mode: xyz***
- A simple way to plan your confocal imaging is using the dye assistant. Open the menu and enter your fluorophores. Choose PMT. Then choose the imaging methods (simultaneous and/or sequential scanning according to your needs considering cross talk and imaging speed. Press "Apply").

Additional info @:

file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm#INS/INS_initial_image_acquisition.htm%3FTocPath%3DStep-by-step%2520Instructions%7CImage%2520Acquisition%7C 5



Fig.3

- The system is turning on laser lines “or ask for it if you didn’t turn them on from config” (tunes to the excitation maxima of selected dyes), activates detectors with appropriate bandwidth a, sets the gain and shows the emission spectra of selected dyes.
- Switch on T-PMT detector if needed for transmitted light imaging-> Electronic switch (tied to the frame)
- Press “Live” to get an image preview
- Laser power, detector bandwidths and Detector gain may be adjusted
- Check for over exposure using pseudo color or histogram. Overexposing the detectors damage them

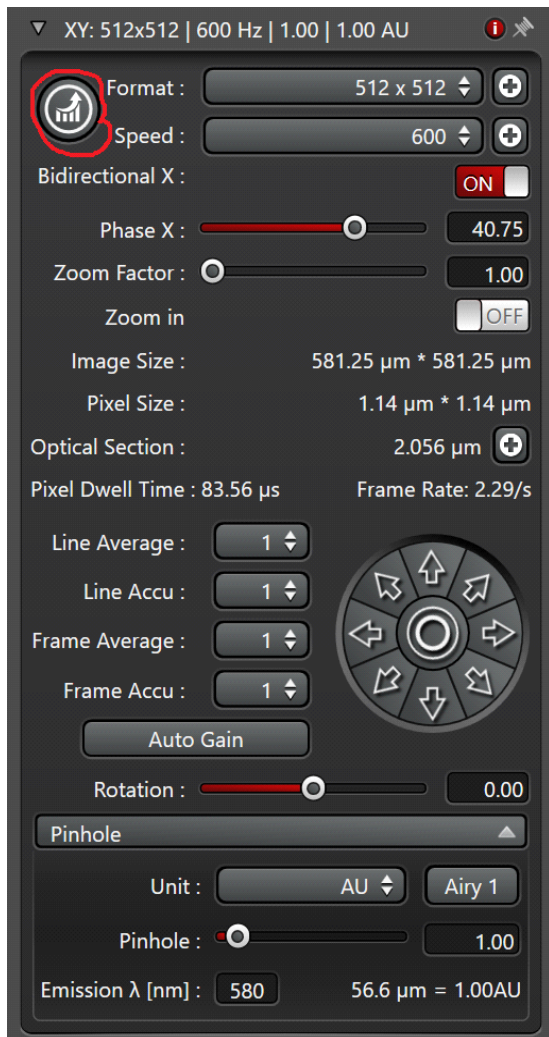


Fig.4

Set your confocal image:

!!! Note: In addition to the software (Fig4), gain, offset, scan field rotation, pinhole, zoom, and z-position settings can be changed via the control knobs in front of you

Position your sample:

Change the zoom or rotate your image -> Changing scan speed (>700 Hz) will change the minimum zoom. **Up to 600 Hz the minimum Zoom is 0.75.**

Adjust the image format:

Press [Nyquist \(optimal\)](#) button for proper sampling

Adjust the pinhole:

Changes will alter the confocal section thickness (**Reference wavelength is set to 580nm**, you can change it to your need)

And the SNR of the image; default is AU 1

Adjust your Signal to Noise ratio (SNR) by:

Applying frame or line averaging reduces the imaging speed.

Check the pixel dwell time

- **Gated detection (Useful for removing Reflection or background auto fluorescence)**

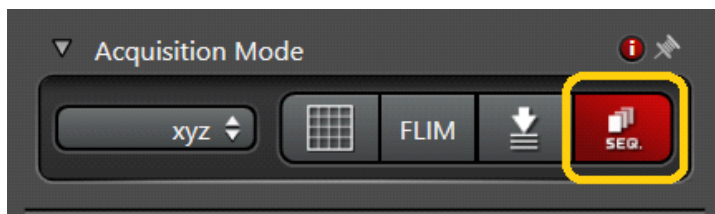
Note: Only possible using HyD detectors!!!

- Used to reduce auto fluorescence or reflection with shorter lifetimes than actual fluorescence

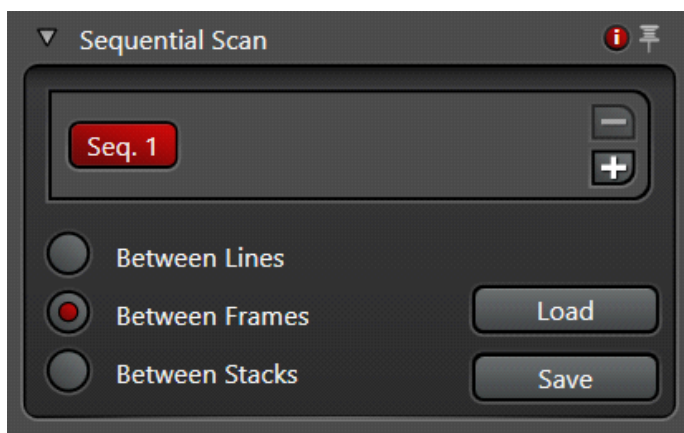


- activate gating
- choose the detection window “in ns” for your fluorophore, adjust during live scan (reflection and Auto-fluorescence should have very short lifetimes)

- **Setting up sequential scan**



- Select 'SEQ' symbol in the 'acquisition mode' menu



- Select **Line**, **Frame** or **Stack** sequential
- Set up first configuration

- **ATTENTION!!! In *line sequential* only PMTs and Lasers are switched on/off between the lines (laser shutters & detector bandwidth remain the same, NO HARDWARE MOVEMENT between lines is possible!!!)**
- Press '+'. Set up next configuration, and so on. Set optimal imaging conditions for each configuration.
TIP-> As an alternative you can optimize your **detection/ crosstalk** and channels in line **sequential** mode and then duplicate the setting (press the +) and deactivate the lasers that are not needed. This way you can reduce the crosstalk and increase the speed of imaging.
- You can save "sequential files" in your folder and load them again.

Additional info @:

[file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm?Highlight=lineseq#INS/INS_sequential_scan.htm?Highlight=line seq](file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm?Highlight=lineseq#INS/INS_sequential_scan.htm?Highlight=line%20seq)

- **Setting up a Z-stack**
 - Joystick is always set to **z-wide**, if zgalvo is selected '**Z position**' knob on the control panel should be used for setting up the z-stack!!! Changing the microscope focus knob/joystick will move the whole stage (z-wide)-> **therefore the z-stack will be shifted**
 - **Acquisition mode xyz**



Fig.6

Select '**Z-galvo**' or **z-Wide** in the '**Z-stack**' menu

Start scanning with 'live'.

Use '**Z position**' knob on the control panel, select the first slice.

Press '**begin**' in the Z-stack menu. Move focus up with 'Z position', select the most superficial slice, press '**End**'.

Set up Z-step size according to your needs or activate system optimized for Nyquist settings according to the objective and pinhole settings used

Additional info @:

file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm#DLG/Acquire/DLG_acquire_z_stack.htm

Setting up time series:

- Acquisition mode xyt or xyzt

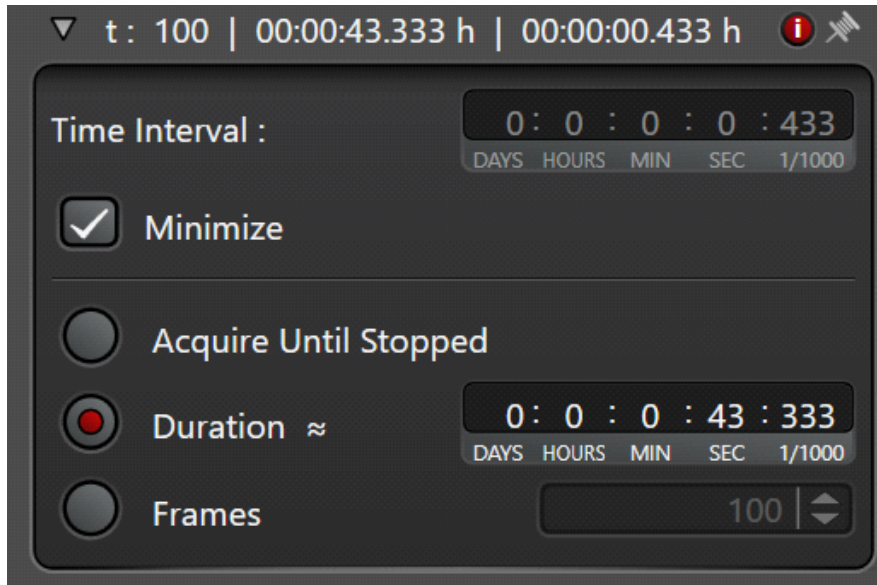
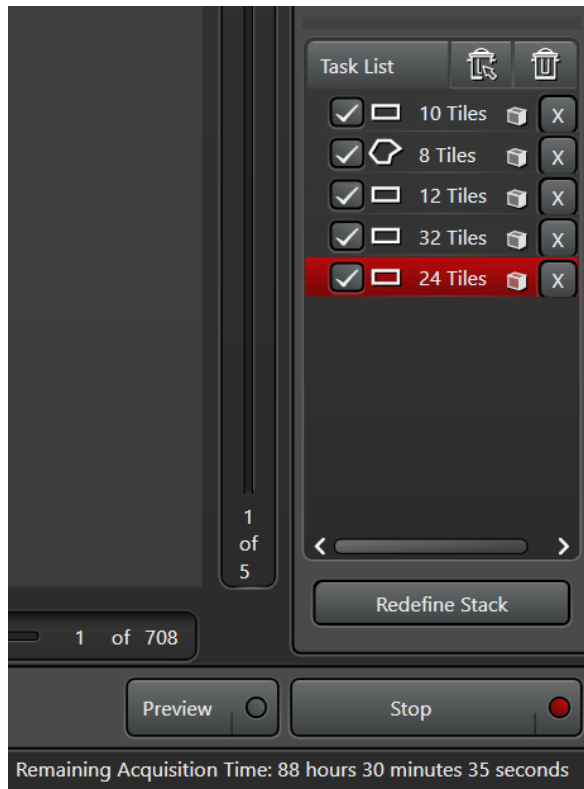


Fig.7

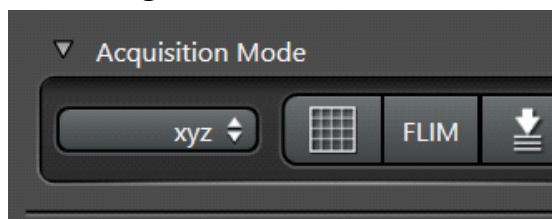
- To estimate minimal interval, activate '**minimize**' in '**t**' menu
- Or set your own time-lapse interval (should exceed the minimal time needed for a stack)
- Select experiment duration or number of frames
- Press '**Apply**'
- To start time series, press '**start**'
- In the **Navigator**, selecting multiple marks with z-stacks and time will not show the correct estimated time.

NOTE: Time estimate is not precise. The time estimate shown usually for the last position or the active position



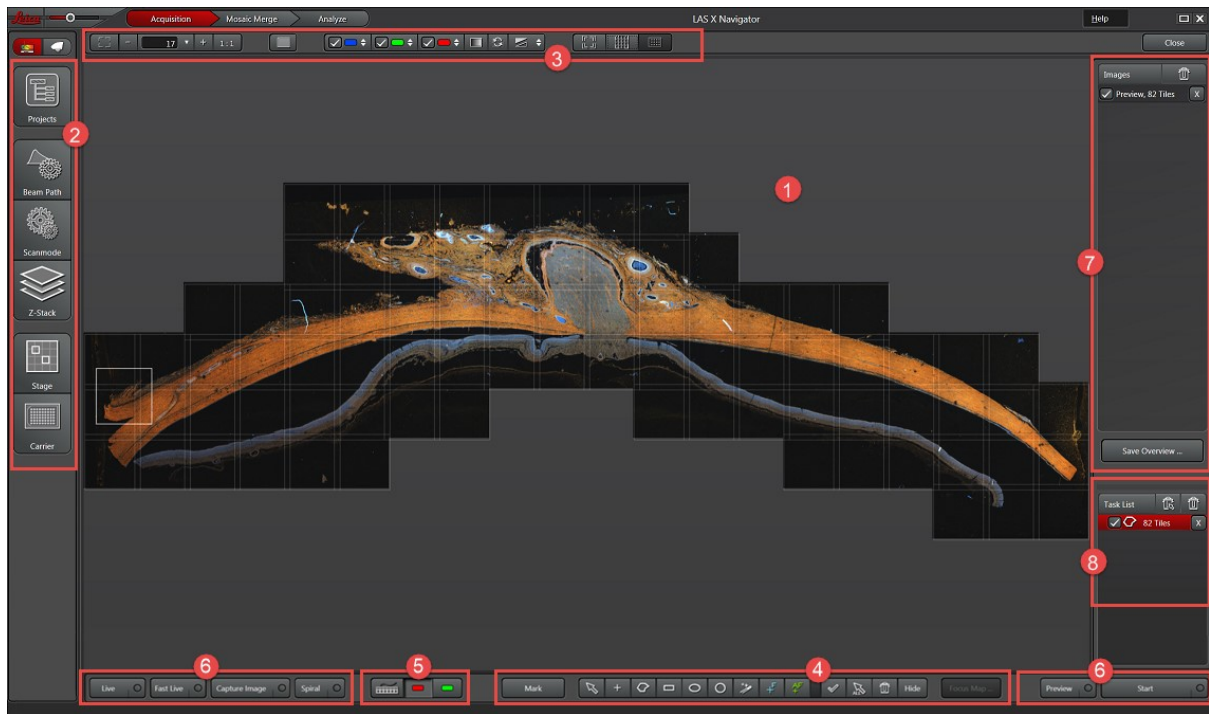
- **Navigator**

The Navigator module can be activate by clicking on the grid logo



Live and Fast live show the current location of the scanner (no.6 in the next image).

Spiral scan can give you an overview of the sample (no.6)-> spiral scan and fast live settings can be changed by hovering over the button, right click and going to the fast scan setting



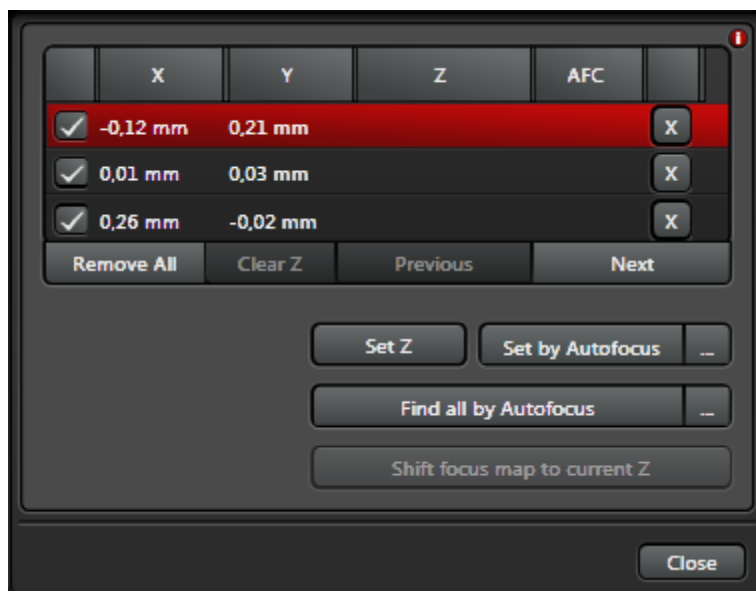
- 1) Shows the images, marked positions and tiles
- 2) All the imaging settings are available via this tool panels
- 3) You can change you channel visualizations settings here
- 4) Scan regions (MARKS, TILE)

At the lower edge of the screen, you find the following buttons for creating [scan regions](#), voxel points and voxel maps:



- | | |
|----------------|--|
| 1 Mark | Marks the current position (x, y, z) on your specimen and creates an image. You use this function for Mark & Find experiments. |
| 2 Select | Selection tool. It allows you to select one or more plotted regions. |
| 3 Single image | Marks the current position (x, y) and creates an image. |
| 4 Polygon | Polygon tool. The polygon tool can be used like a free-hand tool. |
| 5 Rectangle | Rectangle tool. |
| 6 Ellipse | Ellipsis tool. |
| 7 Circle | Circle tool. |

- | | | |
|----|--------------------------|---|
| 8 | Magic Wand | Magic Wand. Detects the boundary of the object and plots a scan region that completely encloses the object. |
| 9 | Focus Map Point | Sets a voxel point, as the selected focus. |
| 10 | Auto-Focus Point | Sets a focus point for the automatic focus search if the focus search is only to be carried out at one position, see also Autofocus> Stage > First position only . The focus search is performed at the position of the nearest tile. |
| 11 | Keep drawing tool active | The selected plotting tool remains active so that you can plot more regions using the same drawing tool. If this function is disabled, the selection tool will automatically become active again after plotting is done. |
| 12 | Select all Shapes | Selection tool for all plotted scan regions. |
| 13 | Delete Selected Shapes | Removes all plotted scan regions. |
| 14 | Hide | You can hide individual scan regions. These will not be considered when saving using Save Overview.... |
| 15 | Focus Map ... | Opens the dialog for the definition of a focus map-> when clicked the following window opens |



When you plot a [scan region](#), a grid is superimposed over the area of the scan region in the display window, displaying the recording fields for the frames. The double lines (for positive overlap values) highlight the area of overlap.

5)

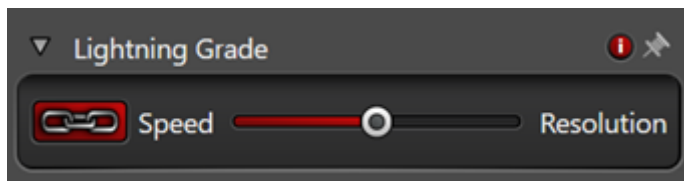
- **Tile Scan**

Tile scan can be done in the Navigator module or the lightning mode (see below)

Additional info @:

file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm#INS/INS_stage_overview_create_overview.htm

in order to do multi position or tiled imaging you can go to the Lightning mode, in the “lightning grade” deactivate the link (chain logo on the left side).



Now you can set your experiment as a normal confocal. In the lightning mode, multi-positions and multi-tiles can be set similar to Sp5 microscopes.

- **Saving your data**

- go to “Open projects” menu

- Save your data directly to **DATAINT (E)** on the Additional info@:

file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm#OVW/Acquire/OVW_acquire_projects.htm

- **Please save the data in the correct group folder the data will automatically updated to the scratch bioimaging:**

Data upload is triggered by Log off/Log on, data will be uploaded to the following folder

\\scratch3.ist.local\scratch-bioimaging\{Your group Name}_ImageDrop\

For manual upload, map the above path. login your username should be inserted as :
IST\username

- **PLEASE NOTE: All data on our E drive are deleted after 30 days automatically!!!**

- ***Acquiring confocal images using the Lightning – Deconvolution environment***

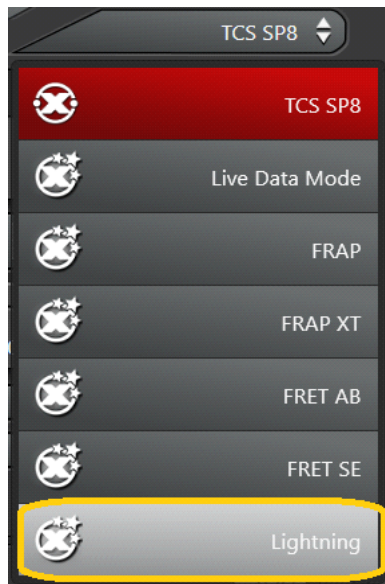


Fig.9

- Choose **Lightning** option from the drop down menu (Fig.9)
- The Lightning slider (Fig.10) controls the deconvolution strength by acting on imaging parameters such as:

Pixel size, scan speed, number of averages, pinhole settings

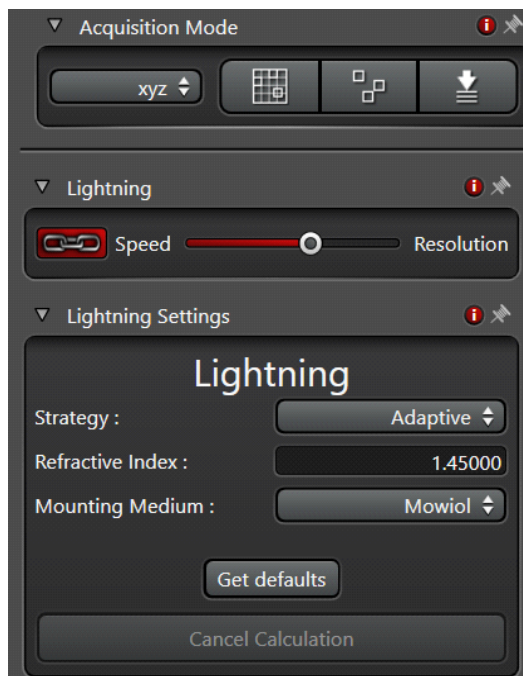


Fig.10

- Select your deconvolution Strategy: **Adaptive** or **Global**

!!! Note: In the **Adaptive** setting Background and bright pixels are treated differently which might lead to non-linearity in your results (may create problems in quantitative measurements). The **Global** method is treating every pixel in the same way!!!

- Put in the embedding medium that you have used for your sample preparation



Fig.11

- To change factory deconvolution settings, go to **Configuration, Lightning** Tab (Fig. 11)
- We recommend to use pinhole **0.6 AU** and oversampling of **1.7**
- Save your new settings under a different name and load them for imaging
- Set up your experiment, press start
- The deconvolution will start immediately online, when you have started your experiment
- Raw data and deconvolved image will be saved as separate files in Open project

Additional info@:

file:///C:/Program%20Files/Leica%20Microsystems%20CMS%20GmbH/LAS%20X/BIN/UserHelp/UserHelpEnglish/Default.htm#DLG/Wizards/DLG_lightning_lightning_grade.htm

- **Deconvolution processing after imaging in standard mode**
 - Images taken in the regular confocal environment may be deconvolved afterwards

- select the image in Open project, then go to the **Process menu**→ **Lightning**
- put in your parameters and press “**Apply**”

- **System Shut down:**

- Switch off lasers in the LASX software → 'Configuration'-'lasers' (The Laser is not off if you just close the software!!)
- Remove your files from the project list (the next user might write data in your folder or delete your data accidentally)
- Data upload is triggered by Log off/Log on, data will be uploaded to the following folder
\\scratch3.ist.local\scratch-bioimaging\{Your group Name}_ImageDrop\
For manual upload, map the above path. login your username should be inserted as :
IST\username
- Clean the objectives
- Select 10x objective & lower the stage to the minimum
- Remove the non-motorized insert (z-galvo can stay)
- Clean the insert
- Switch off lasers in the LAS AF software- 'Configuration'-'lasers'
- Shut down LAS AF, shut down the computer.
- Turn the 'Laser emission' key (4) to the 'off' position
- Then switch off 'Laser Power' (3), 'Scanner Power' (2) and 'PC/Microscope' (1).
- Switch off the fluorescent lamp (5)
- Disinfect the system by wiping all contact points with EtOH soaked tissue-> optical components and eyepieces has to be cleaned with lens cleaning tissue.
-> never spray ethanol on the equipment

Laser Safety Instructions

- During operation of class 3B and class 4 lasers red warning lights have to be switched on manually
- Red warning light at the door of the room, containing laser-based equipment, prohibits the entrance
- Optical path of the laser beam at all setups has to stay intact and should never be disassembled by a user. Users are never permitted to disconnect optical connections (pipes, fibers etc), remove protective coverings or disassemble any parts of the setups, especially those parts that are labeled with laser-warning signs.
- User has to make sure, that objectives mounts are blocked by objectives or light- blocking plugs, before switching the system on or starting the work
- Any cleaning activities (objectives, stage cleanings) as well as changing of objectives or filters have to be performed only after blocking of the laser light is ensured. This can be ensured by closing the scanhead shutter or switching off the laser.
- Laser class-specific warnings at each setup have to be observed and considered
- Eye contact with direct beam of Class 3B laser, or eye contact with mirror reflection from class 3B laser, should be avoided at all times
- Eye or skin contact with direct or diffuse light of Class 4 laser, should be avoided at all times
- Laser safety goggles are situated at all workspaces and should be used in any situation where potential contact of eyes with Laser light of the classes 3B or 4 is possible, according to the previous two points
- Laser safety goggles have to be worn at all times of operation of Laser Class 4
- Laser safety goggles have to be worn at all times of operation laser Class 3 at the optogenetic setups
- Laser safety goggles are assigned to each setup and matched to the corresponding laser wavelengths. Matching laser safety goggles should be used at all times, and should not be carried over between the setups
- Only one person is allowed to be in the corresponding compartment during Laser Class 4 operation and optogenetic setup operation
- Users are not allowed to wear any reflective objects (rings, watches etc) during laser operation
- Using of the equipment is only allowed after the introduction from a laser safety officer of IST Austria. Introduction has to be done individually for each setup.
- Changing experimental conditions, that involves changes in the laser application, have to be reported to the laser safety officer prior to the start of experiment

Users have to understand that any violations against the instructed rules and also withholding information leading to safety hazards will ultimately result in denial of admission to all laser equipped instruments at the IST Austria.