Confocal microscopy training (Stellaris)

The purpose of confocal microscopy practice is to achieve high-resolution imaging of biological samples by using a laser to illuminate specific points within the specimen. This technique allows for the collection of detailed, layered images by eliminating out-of-focus light, resulting in clearer, three-dimensional representations of cellular structures. Additionally, users will become familiar with the associated software, enabling them to independently develop and execute experimental protocols. This dual focus on both imaging techniques and software proficiency ensures a comprehensive understanding of confocal microscopy for research applications.

Step 1: Operating the Microscope

1. Connect to bookit in your mobile phone(https://lsbiology.ls.biu.ac.il/Bookit/) and in the home menu log on to the microscope
2. Turn on the computer: login using: User: .\LASX-USER Pass: 12345
3. Turn on the lasers by switching on the 2 buttons on the left and then turn on the laser key

4. Turn on to LASX software and press OK to all the popup questions.

Step 2: Familiarization with the Software

1. Open project:

In the open project tab, you can either open an existing project or create a new project and save it. In addition, you can import the data from your saved image or export your image to different format such as tiff in this place.

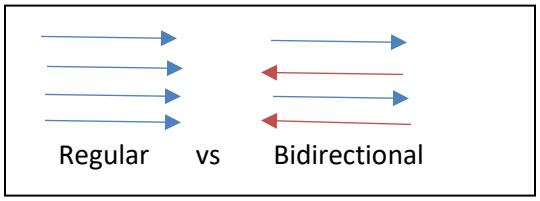
To open a new project press “new project” and then “rename”.

To open an existing program press “open project” and open the image. Then right click and choose “apply image settings” to use the same parameters.

2. Acquisition

In the acquisition tab you can set up the parameters

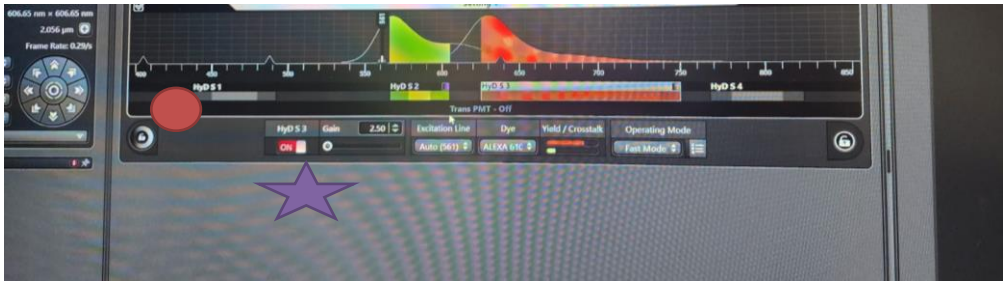
3. You can choose which parameters you are taking X Y Z T
4. Bidirectional: scanning type which allows faster scanning



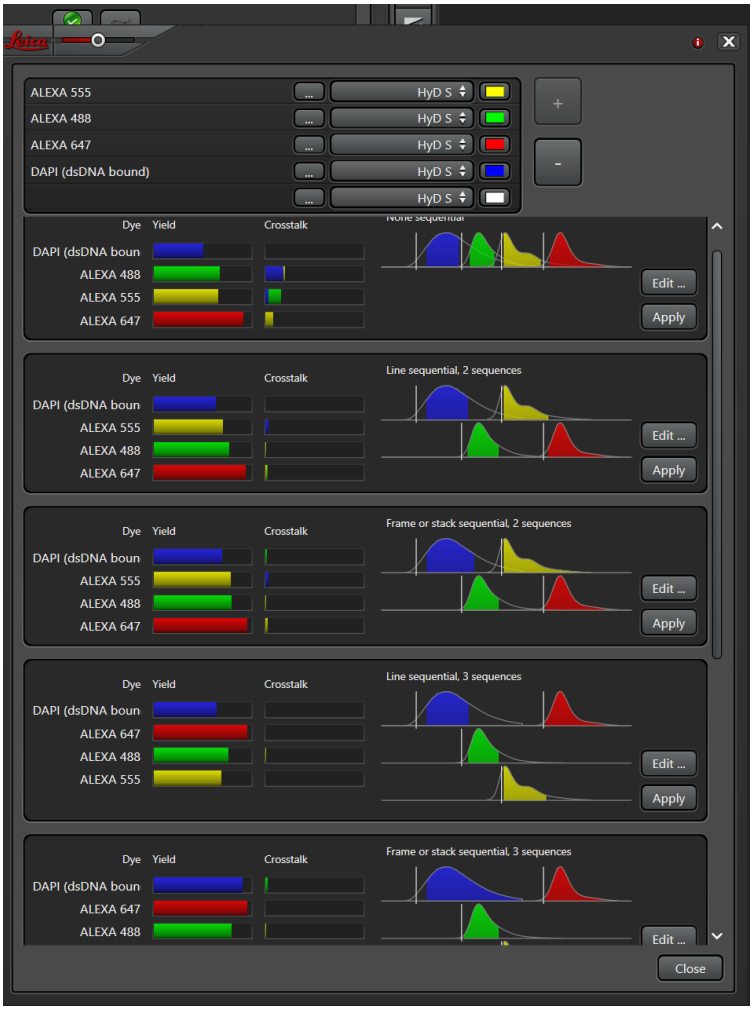
5. Averaging – In this option, the laser scans each row or frame several times and average the signal on each pixel. This smoothens the signal and improves signal to noise ratio.
6. Accumulation – in this option, the laser scans each row or frame several times and accumulate the signal. This option also improves signal to noise ratio and is chosen when the fluorescent signal is relatively low.
7. Format – number of pixels in the image - determines the resolution of the image. Increasing the resolution (more pixels→ smaller pixels) will increase the quality of the image but will take longer time to scan and more storage space.
8. Magic button: determines the optimal resolution, thus choosing higher format than the optimal will not further improve the quality of the image.
9. Speed – pixel dwell time -the duration that the detector spends on each pixel. The longer the time, the higher the quality of the image→ more time and more bleaching.
10. Zoom – you can either choose a specific area to zoom in by turning the zoom in bottom on and marking the area of interest or you can choose a zoom factor that will apply the whole image.
11. Pinhole – determines the size of the pinhole (amount of light that goes through the sample), large pinhole → large optical section size. The standard size is 1 AU, and as we close the pinhole, we get a smaller optical

section and increased resolution but the decreased intensity, as less light in coming through.

New settings:



There are several options to start a new setting. You can drag the lasers or you can use the button (see image) and the system will guide you (recommended).



First, you need to choose the fluorophores that you are using and the scanning program (between lines, frames, sequential) that is most suited to your colors and allows minimal overlapping. You can change the wavelength manually to minimize overlapping.

Between lines – scanning each line with all channels and moving to the next line. Enables us to see all channels simultaneously in live mode.

Between frames- scanning all frame with one channels then moving on to the next channel. Allows flexibility in each channel separately (order of channel, diff averaging and accumulation).

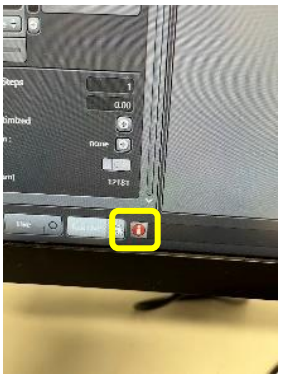
You can change the presented color by double clicking (see orange circle above) and choose the desired color.

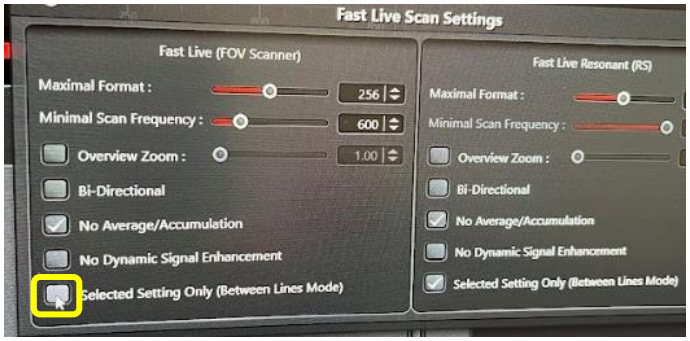
You can add transmitted light by turning on the button near the yellow star.

Next, choose the intensities of the laser and the detector. The recommended detector gain is 1-100% (HyD), pay attention, as high detector gain can lead to a noisy image, and the laser intensity is max 20 (try lower as possible to avoid bleaching).

Fast live:

In order to see all the colors together **uncheck** selected setting only





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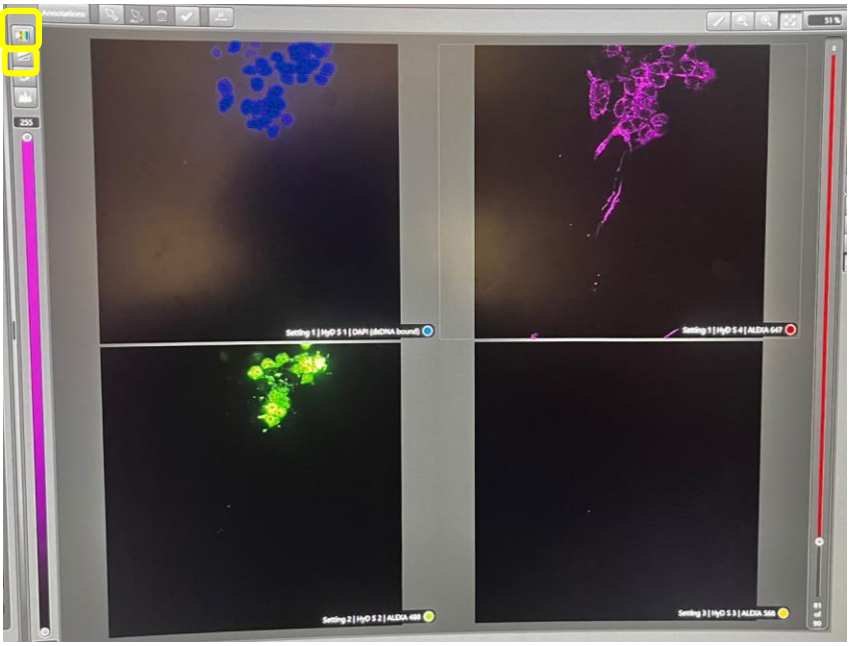
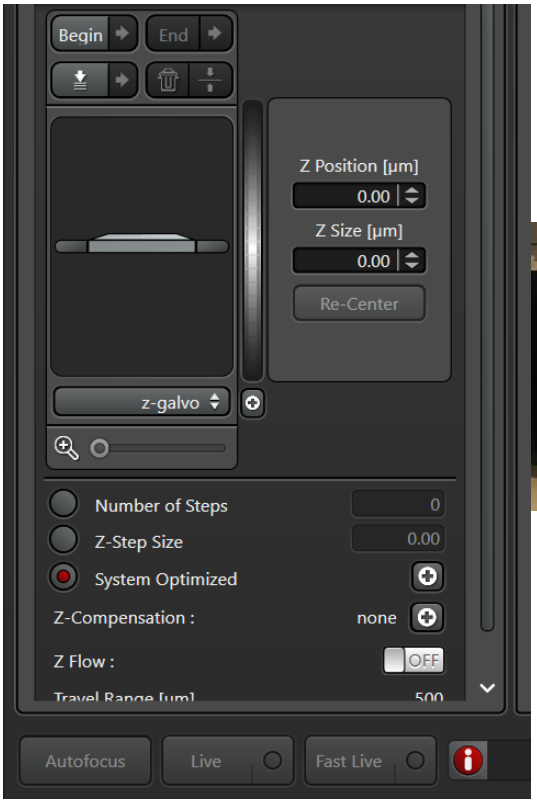
Press the **saturation** button to make sure that the colors are not saturated, blue pixel are saturated. Adjust the detector and the laser accordingly. (1)

Autoscale – adjust Lut to fit the optimal contrast min-max.(2)



LUT- look up table: changes the color of the channels.(3)

Z stack:

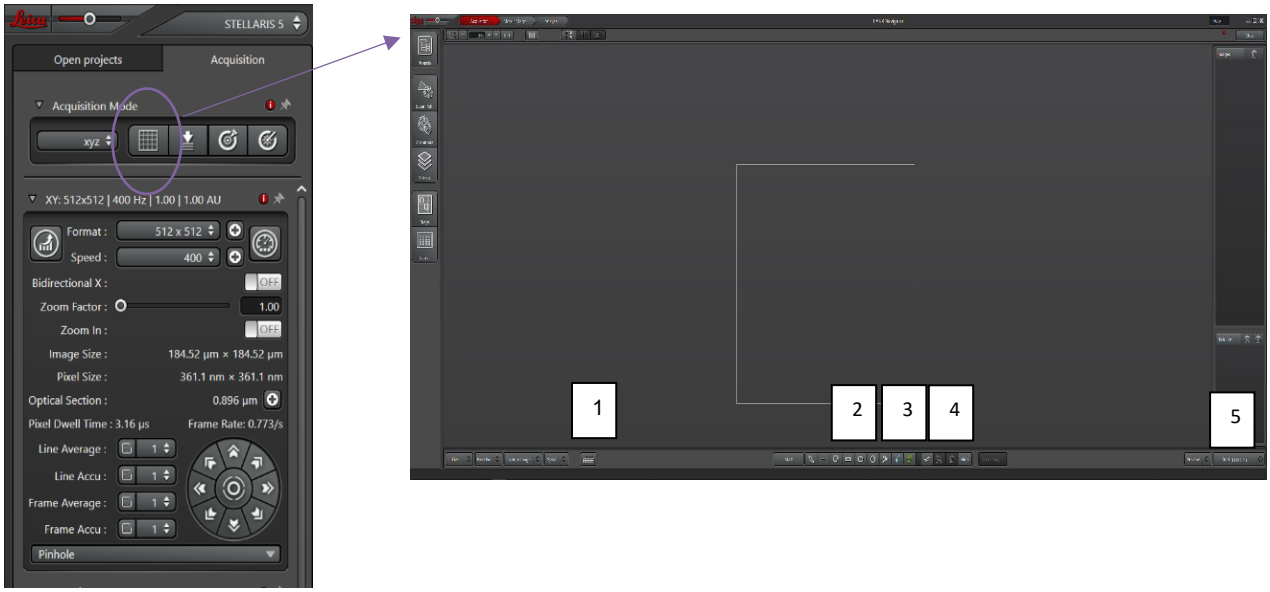


To perform Z imaging: In the left window, select a starting point (Begin) and an endpoint (End), which can be adjusted using the fine focus button on the black panel (purple)

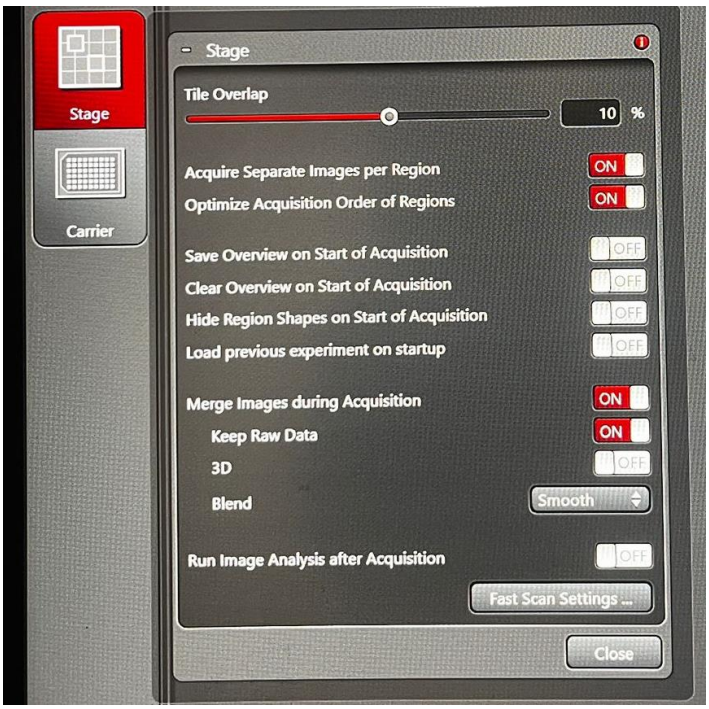
You get a Z volume – the whole depth to image. Next, determine whether the number of steps will follow the system's recommendation (optimal for best sampling) or if you want to change the number of steps (Z step/number of steps). Finally, click "Start Experiment."

The system will provide an estimated time after capturing the first image. At the end of the experiment, you can either press the MAX Projection button in the right window or the 3D button. This will take you to a new window. Here you can see the 3d image, you can record and to edit a video. To return to the initial window, press "Close."

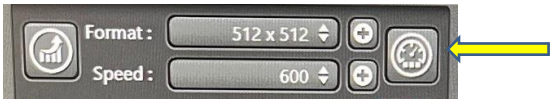
Navigator:



Navigation allows you to navigate in different zones of your sample. Press the navigation button and then press the spiral button (1) this will enable a fast preview of the area. Then you have several options such as imaging a tile image: choose the area (ROI region of interest) that you want to image by pressing one of the buttons (2). Then you need to create a focus map press the add focus button (3) and add several focus points. Then press on the focus map button (4), adjust each focus point and press set Z. When you finish press the start button (5). Before you start check the merge images during acquisition to get a meged image at the end of the acquisition



Live imaging:

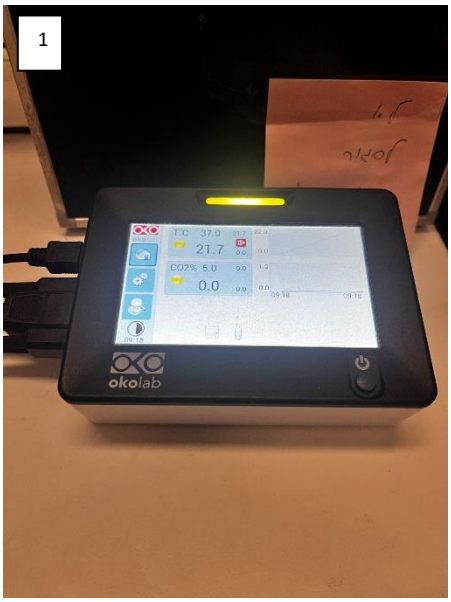


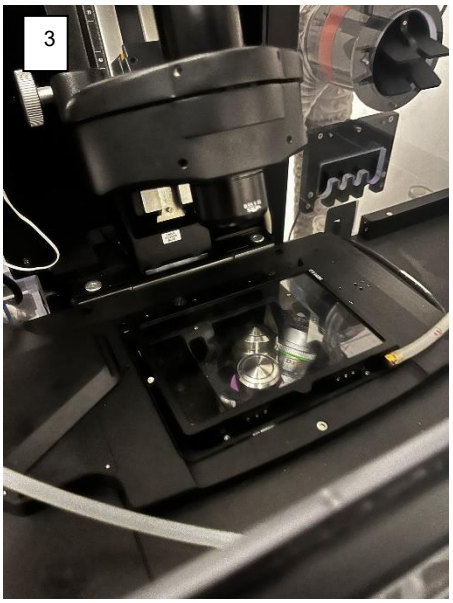
If you want to observe fast live changes, you can choose the resonance option: Resonance – Super fast scanner for fast imaging (moving object or fast dynamics). In the acquisition window switch to resonance (speed 8000). The image will look

nosier so you have to use high averaging to get a smoother imaging (at least 8 avg).

Choose XYZT if you want to use time lapse

<u>Open the incubator module:</u> press the operation system(1) and set the temp and co2 levels, open the CO2 handle on the wall(2) and change to the appropriate





holder with the cover (3). Start the incubator 20 min before the experiment. Please do not forget to close the temp and CO_2 when you finish the experiment, by a long press on the incubator monitor and closing the CO_2 handle on the wall.

Lightning Deconvolution

Deconvolution is an image-processing tool that can decrease the blurriness of the image and increase the sample contrast and resolution. Leica Lightning deconvolution decides the parameters for deconvolution while acquiring the image. Deconvolution can increase image quality and help isolate smaller features within an image.

Before you start set up your basic imaging parameters.

<u>Then activate lightning-</u>

In the upper left-hand corner, open the drop-down list and select lightning. Wait for software to switch to lightning.

<u>How to use Lightning</u>

1. Adjust the mounting medium and the refractive index to appropriate value.

-Refractive index of oil is on bottle (1.4-1.6).

-Refractive index of water is 1.3333 .

-Refractive index of air is 1.

2. Adjust the strategy

- Adaptive for thick samples like tissues

- Global for thin samples like cells

- low signal to noise – for problematic samples with high noise or background.

Lightning automatically sets up the optimized parameters for image acquisition. Scroll using the speed vs. resolution to determine parameters.

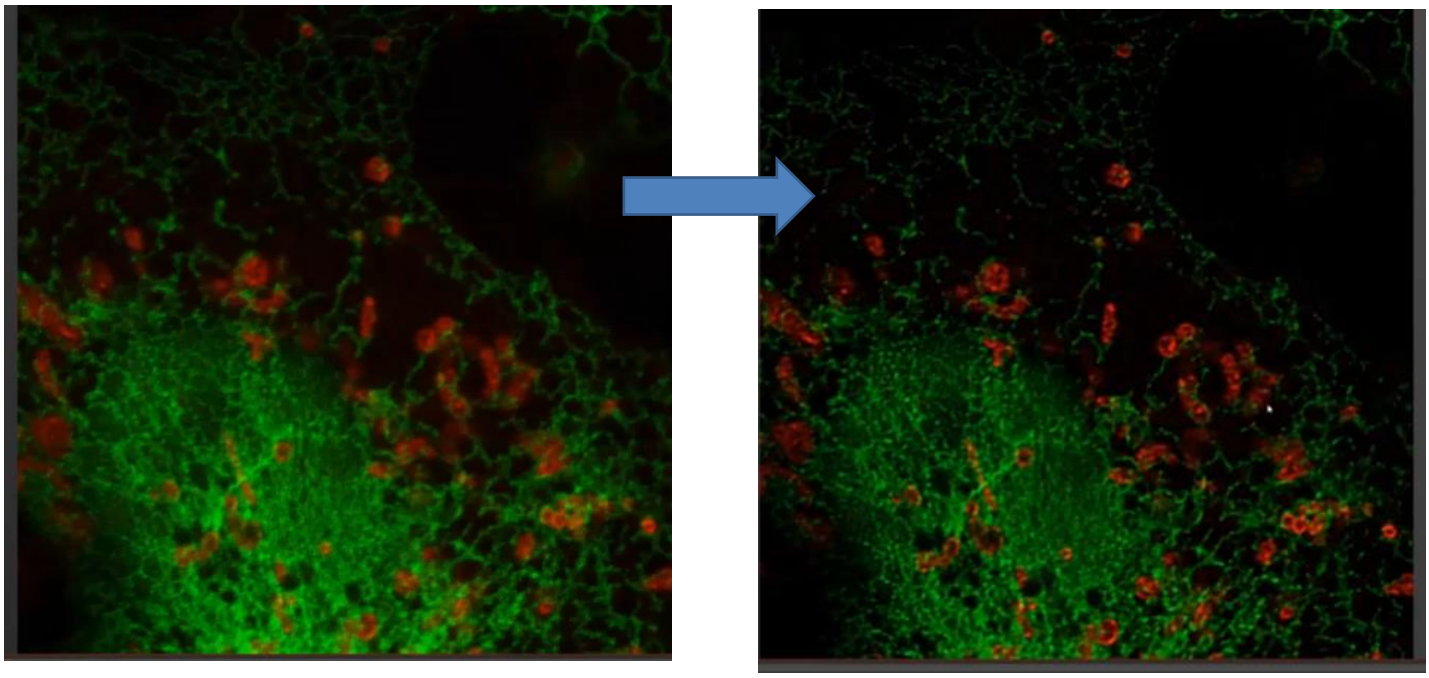
You can choose high speed and low resolution and the system will automatically reduce the format and increase the speed or you can chose low speed and high resolution and the system will choose high format and low speed. Usually its recommended to start in the middle.

Customized Lightning parameters

- To customize lightning parameters, unclick the chain
  - This uncouples the scroll bar from the parameters
- Now you can choose, averaging number, scan speed, zoom, resolution etc.
- Typically, is it best to keep the lightning setting coupled and optimized

Image Acquisition

- Once parameters are set, click ”Start Experiment”
- Image acquisition will start and Lightning Deconvolution will start
- Deconvolution instantly starts occurring
- Deconvolved image has “_Lng” at the end on name



If you want to look at the 2 images together press the lightning tab.