Standard Operating Protocol for the Leica SP8 5D

Cautions:

This microscope has powerful fluorescent light sources and laser lights. Do not look straight down the objectives when in use as you may irreversibly damage your eyes

This microscope is equipped with an automatic stage. Do keep your fingers away when the stage is moving.

What should I use this microscope for?

This is an inverted confocal microscope equipped with four long- and two short-range lenses and a fully motorised stage. Its heated incubation chamber is available on special request. As a confocal microscope, it will scan your sample point by point -as opposed to a whole field at once- and will only collect the light at the focal point. It is therefore slower but gives neater images than widefield microscopes.

Use this microscope for

- Slides or cell culture dishes
- Cell culture on imaging quality substrate (either coverslips or imaging plates)
- Cell and explants on normal Tissue Culture dishes can be imaged with the 10x, 20x air and 25x water lenses
- Live tissue/embryos on imaging quality substrate (either coverslips or imaging plates)

Data management

*As with every microscope in the facility, you are expected to save your data in the data folder of this computer. Before you leave, you **must** push your data to a NAS server (Mick or Nelly if you are in SCRM) before backing your data up to the cloud as agreed with your lab head. It is good practice to label the folders that have been backed up.*

Users from outside SCRM are encouraged to back up on their university cloud drive. Files up to 2Gb can be sent via an internet site such as WeTransfer.com

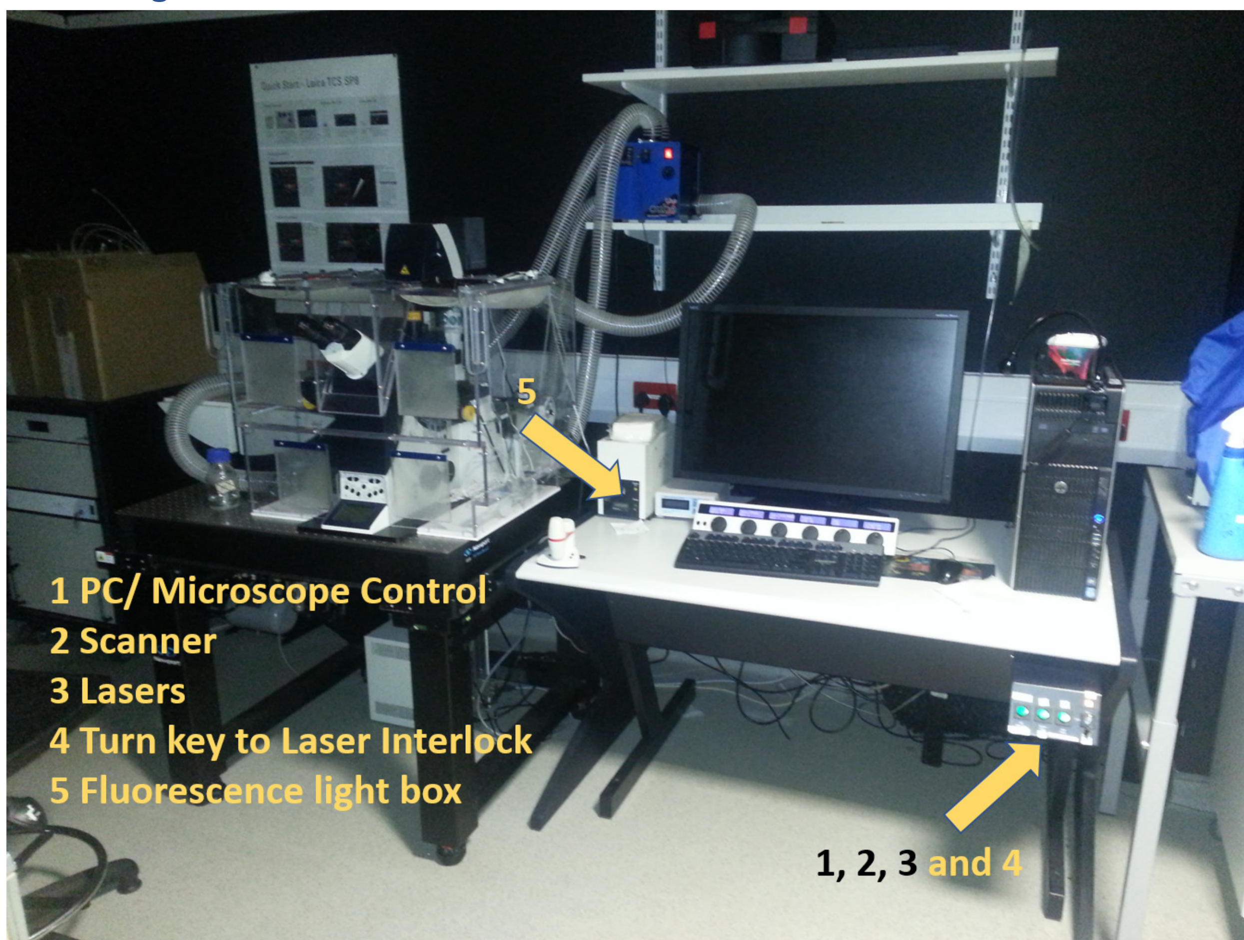
Your data is not safe on this computer. We make space for all users by deleting old data without notice. Please back up.

Lenses

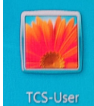
Which Objective Should I use?

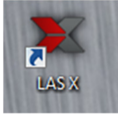
Magnification	Type	Aperture	CS type	Sample carrier	Usage	Working Distance (µm)
10x DRY	HCPLAPO CS	0.40	1	All		2200
20x DRY	HCPLAPO CS2	0.75	2	Coverslips		620
25x WATER	HCXIRAPOL	0.95	1	All	With or without pump	2400
40x WATER	HCXPLAPO	1.10	1	Coverslips	With or without pump	630
40x OIL		1.25	1	Coverslips		100
63x OIL	HCPLAPO CS2	1.40	2	Coverslips		140

Switching the Confocal ON



Please switch the microscope in the order indicated. Once your session completed, check PPMS to see if you are the last user. If you are not, please leave the system on. Instruction to switch off are on page xxx.

Once the computer is on, log in as TCS-User  and enter your PPMS username and password

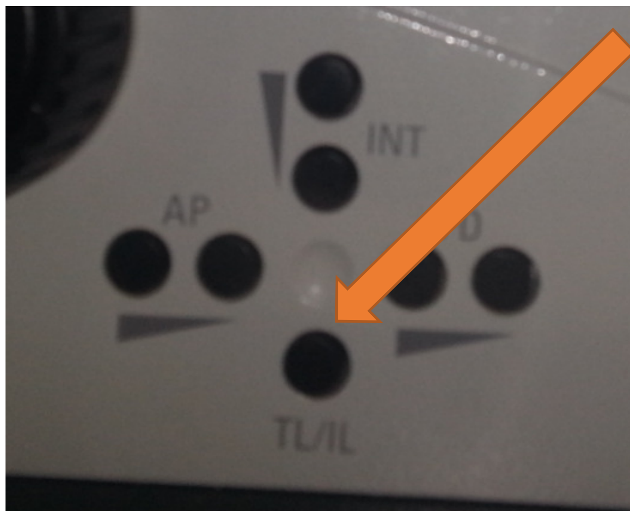
Switch on  and select **Machine_Without_STED_or_Water_Pump** as configuration

Wait for the stage to calibrate and the software to start

Find your sample

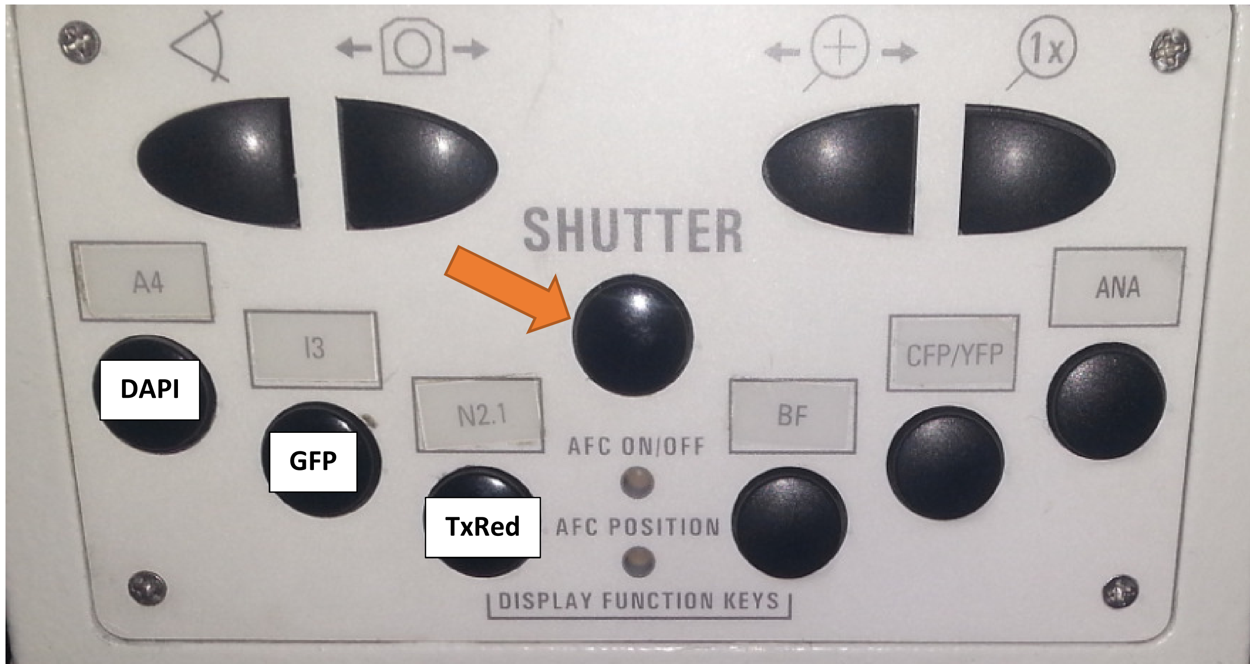
Check that the lens in use on the microscope is the 10x lens, if not change it to 10x

Once the software is on, you need to find your sample. Go to the microscope and select the appropriate light path configuration for either bright field (TL) or fluorescence (IL). You'll find this command on the left-hand side of the microscope.



If you choose Brightfield (TL) and that the light is too dim, you can increase its intensity with the upper INT switch, or decrease it with the lower one.

If you choose fluorescence (IL), you then need to open the shutter and choose the appropriate filter cube for your sample. These controls are at the front of the microscope.

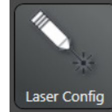


You have a choice of DAPI (A4), GFP (I3) or TxRed (N2.1). Open the shutter and use the joystick to find your sample by looking through the eyepieces.

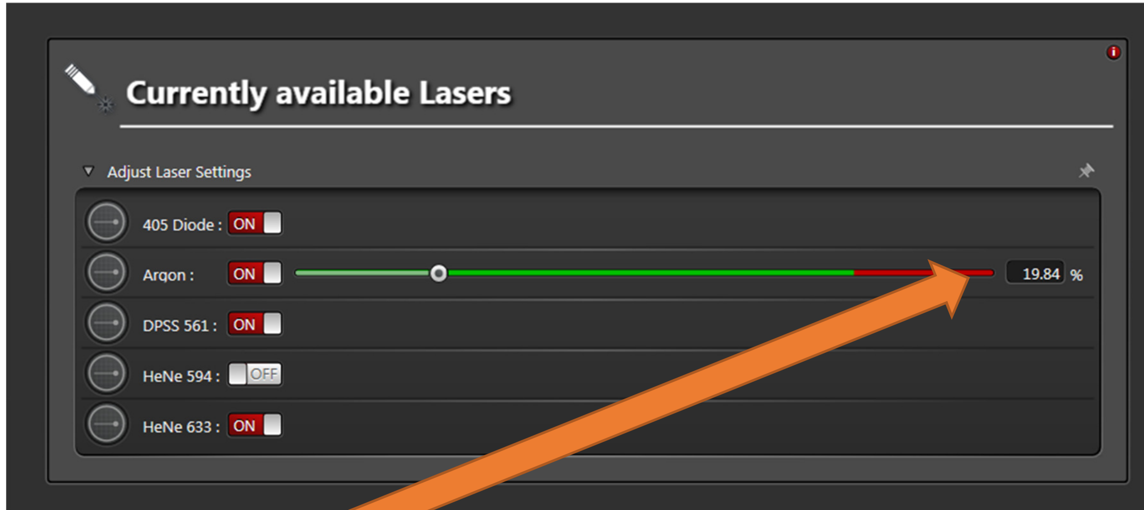
Once you have found your sample, close the shutter and go back to the LASX software on the computer

Switching the lasers on

Go to Settings (Top of screen) and select the Laser Config tab



Switch on the lasers you need for your experiment.



Set the Argon laser to 20% power.

Define bit depth

Most people only need 8-bit depth for their confocal imaging need. However, sometimes you need more depth because your sample has very bright and very dim but equally important signal, or you would like accurate quantitative analysis.

Go to Settings (Top of screen) and select the Hardware tab

Select the appropriate bit depth for your image: 8-, 12- or 16-bits

Do you need the Galvo?

Following a series of incidents, we had to move the Galvo (a device that has a super accurate Z step) off the stage. You need to make sure that the software is aware that the Galvo is away by switching the Z control from Z-Galvo to Z-Wide (left column on screen).

You also need to change the dial control from Z Galvo position to Z wide Position.

Select the icon for the panel box setting (middle of the screen, this icon:

Change the control of the last dial from Z-Galvo to Z Wide and change the sensitivity of the wheel according to your sample.

If you need the Galvo, let us know and we'll remount it on the stage and change the settings for you.

Define your light settings

The Leica SP8 5D is equipped with a series of five tuneable photoreceptors. They are of two kinds:

1) Three **PMT** which are sturdy and good at collecting light
2) Two **HyD** which are sensitive to overload and are very good at collecting light

If you have a very bright sample, like DAPI, you are better off using the PMT. Overexposing the HyD will cause them to shut down. Too many shut down incidents lead to an expensive breakage.

This confocal will also give you choice if you want

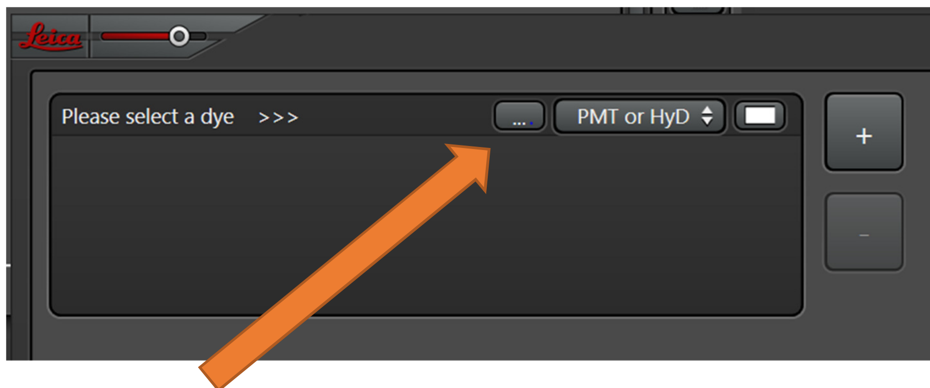
- Line scanning, where each light setting scans a single line of the field of view before passing on the next line. This is the most accurate scanning method and ideal for colocalization studies or very small objects.
- Frame scanning where a single field of view is scanned by one light setting before passing on the next setting. Once all settings have covered the frame, the microscope may move deeper in the sample and start a new frame.
- Stack scanning, where a whole volume (stack) is scanned with one light setting before returning to the beginning and passing on the next light setting. This is the fastest scanning method, but it relies on your sample not moving and the microscope stage being very accurate. Good for large objects such as whole embryos.

Light settings are defined using the Dye Assistant, manually or by copying or loading an existing setting

Dye Assistant

Leica has a Dye Assistant which will define your light settings for you. Whilst it sometimes makes interesting choices, it's a good help if you are a beginner.

Click on the Dye Assistant icon in the middle of the screen, a new window pops up.



Click on the **"…"** icon and find your dye of choice. You then tell the software if you want to use a PMT, a HyD or either. Enter as many dyes as you need. The software gives you a series of combinations of light settings and asks you to select the one you would like.

Settings have three informative sections.

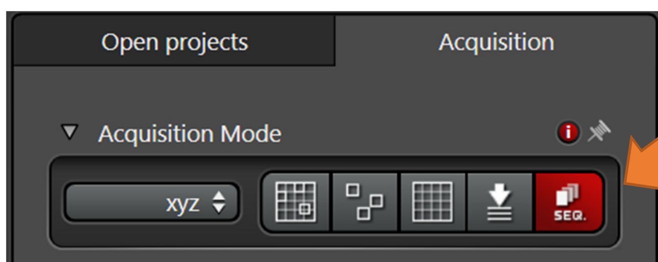
- On the left is the yield for each dye, it tells you how much light from your dye is collected. The more yield is better for dim samples whilst you can get away with a lower yield for very bright sample.
- In the middle is the crosstalk, which tells you how much light from a dye will be detected when collecting light from a different dye. As an example, if you look simultaneously at DAPI and Alexa 488, you'll find a lot of light from the DAPI detected in the Alexa 488. It is a good idea to minimise crosstalk as it may lead to misinterpretation.
- On the right are the emission spectra of your dyes where the colour shaded parts represent the collected light. The white vertical bars represent the lasers. Dye Assistant tells you if you have sequences, of what kind and how many.

Select the settings that are appropriate for your experiment and click "Apply".

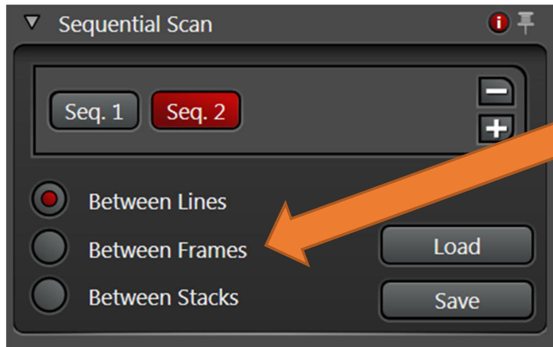
Dye Assistant give you a series of sequences which you must review one by one (see below).

Manual definition

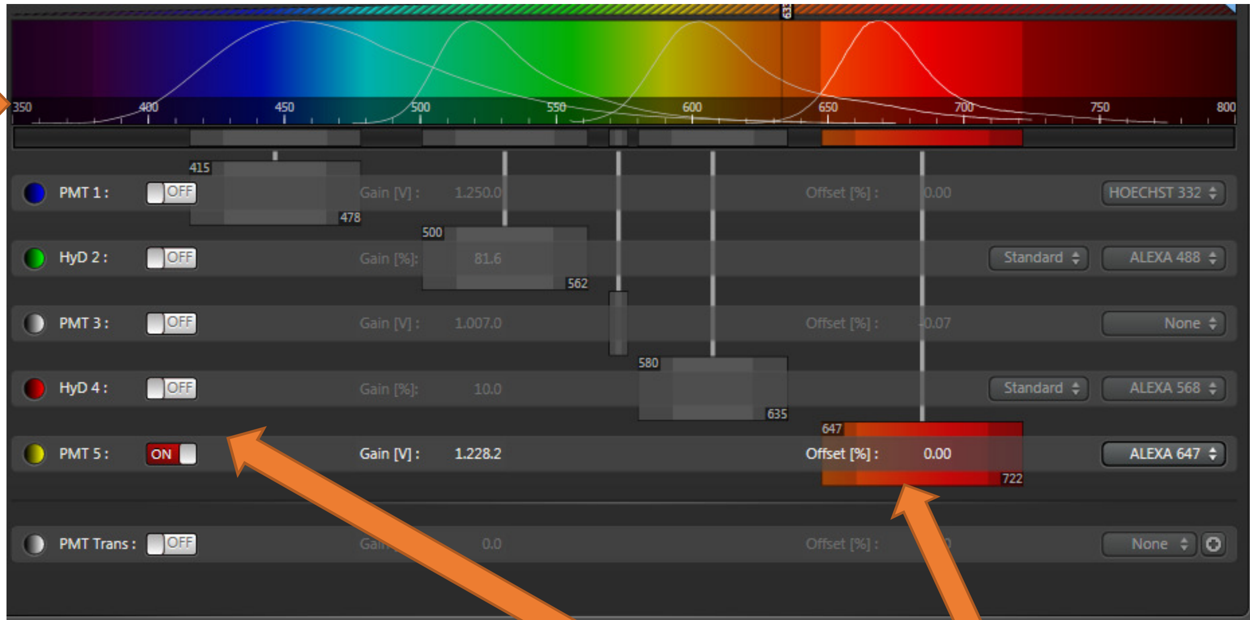
If you feel confident enough, you can manually define your light settings. If you have more than one dye and that their emission spectra overlap, select the "Seq." button under Acquisition



A new panel appears bottom left of your screen

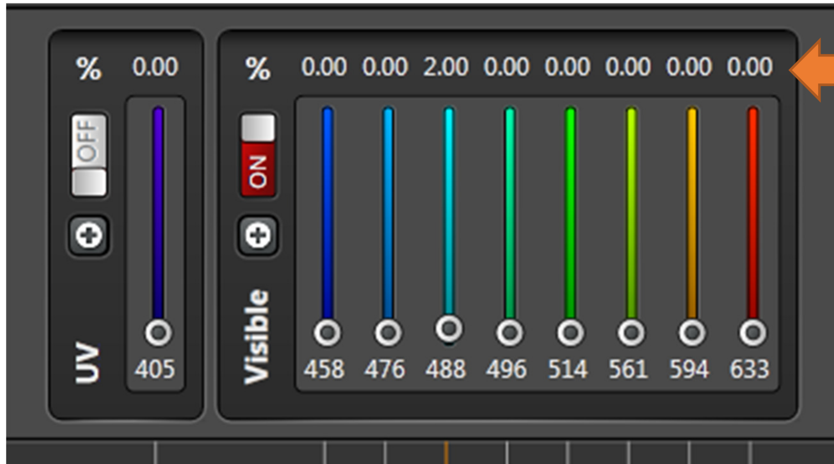


At first, you only have a single sequence. You choose if you want to scan sequentially between lines, frames or stacks.



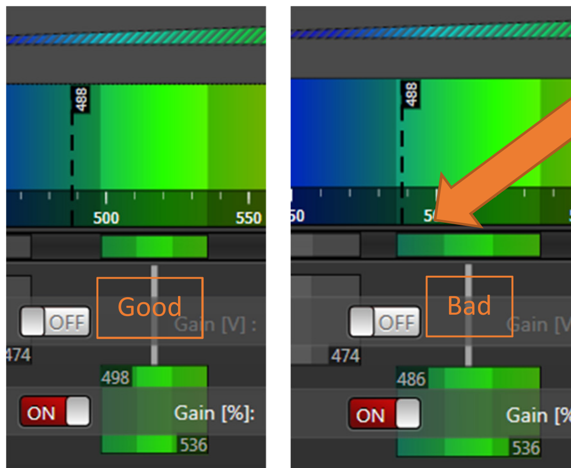
Select the photoreceptor you want to use by switching it on. A grey bar turns colourful. On the right-hand side is a drop-down menu where you can select a dye of interest. If selected, you will see the emission spectrum of this dye appearing as a white curve on the spectrum on top. Note that this information has no bearing on which light is collected by the photoreceptor, but it will help you tune the photoreceptor.

If you click on the coloured disk next to the HyD/PMT, you will be given the choice of colour displayed on screen. Remember that the receptors are colour blind and only see in levels of grey.



On top of the screen, click on "Classic UI" and select the laser you will be using to excite your dye by switching it on and setting its power to 0.1%. The wavelength of this laser will appear as a vertical dashed black line on the spectrum where the PMT/HyD setting are defined.

Double click on the colourful bar, it defines the collection window of the receptor. A small window pops up and asks you to define the wavelengths of light the receptor needs to collect. You need to match collection window as much as possible with the emission spectrum.

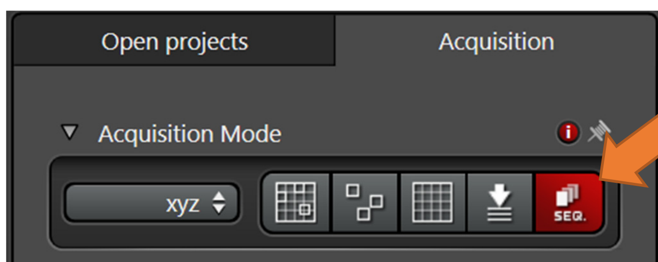


IT IS CRITICAL THAT YOU DO NOT COLLECT LIGHT WHERE A LASER IS SWITCHED ON. COLLECT AT LEAST 5 NANOMETRES AWAY FROM LASER LIGHT.

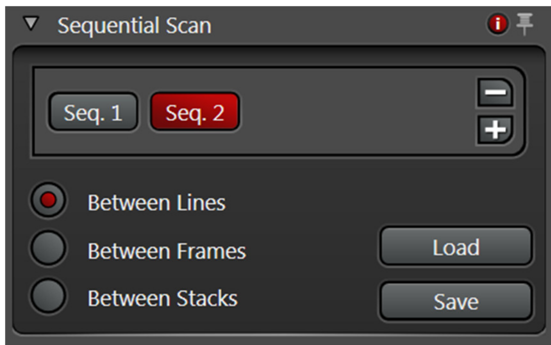
It is sometimes a good idea to define all the settings in a single sequence, duplicate this sequence and shut down lasers and photoreceptors accordingly. This way, you minimise mirror movements between sequences and accelerate your scanning. This is compulsory for line scanning.

Copying or loading an existing setting

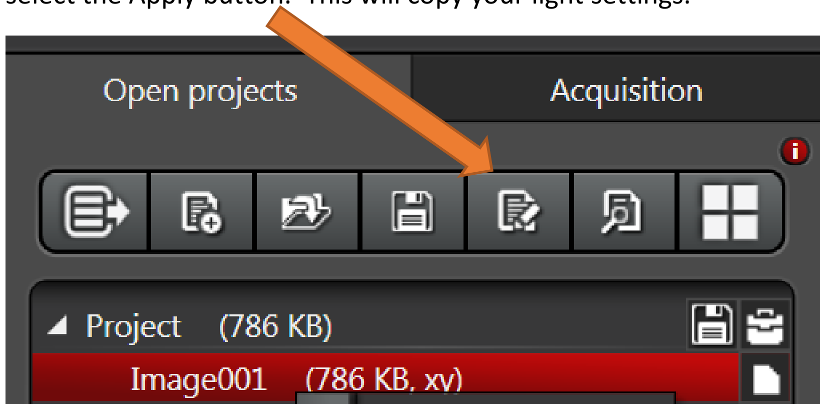
If you know of a saved setting that works for you, select the "Seq." button under Acquisition



Look at the bottom left of the screen and click "Load" to select your setting.



Alternatively, if you haven't saved your settings, open a file from a previously successful experiment and select the Apply button. This will copy your light settings.

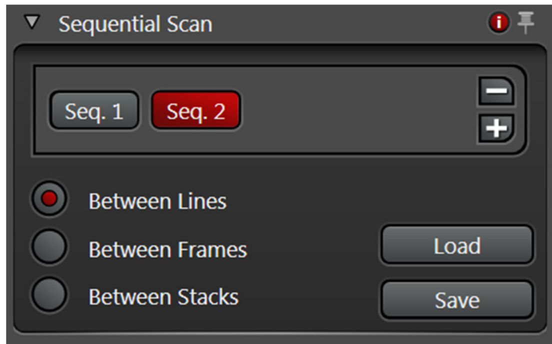


BEWARE! ***Apply*** only copies light settings and laser power. It does NOT copy any other information like image format, bidirectionality, average… You may want to check them.

Reviewing the light settings

It is important to review your light settings one by one. To ensure that, for example, they do not interfere with one another and minimise mirror movements.

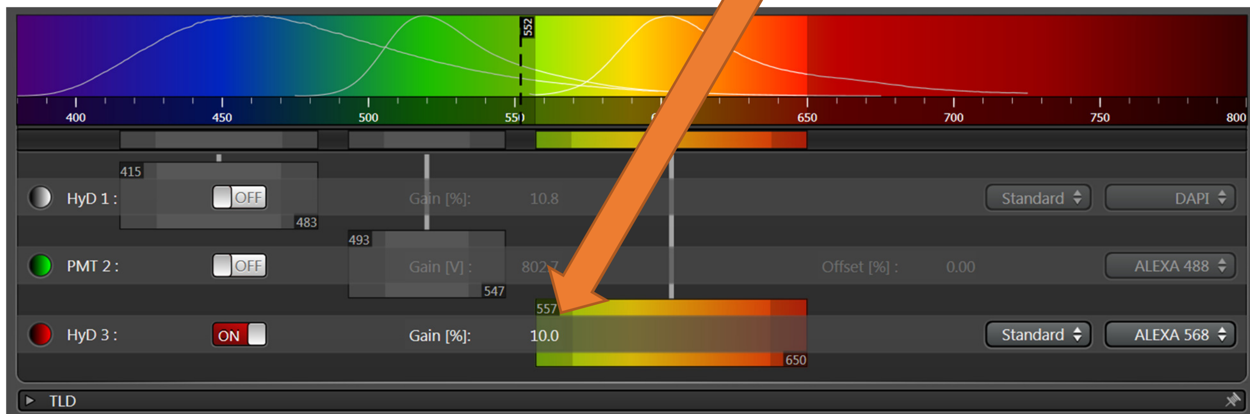
Select your first light setting by clicking on “Seq.1”.



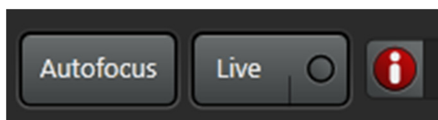
Ensure that the relevant laser is set at 0.1% power.

If you are using a HyD, set the gain at 10%

If you are using a PMT, set the gain at 600



Click on “Live” at the bottom left of the screen.



You will see an image on the right-hand side of the screen and on its top left a series of controls.

The top control switches the display between levels of greys, chosen colour and High/Low. High/Low is the setting that you must use first: it will show you absence of light in green and saturation in blue. THERE MUST NOT BE ANY SATURATION WITH THE HyD RECEPTORS.

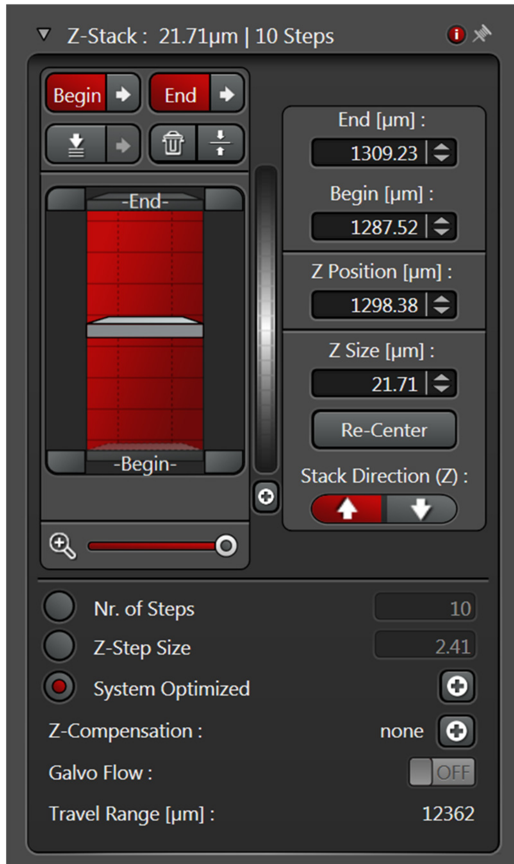
Increase laser power and gain gradually until you get an image with as little background as possible and no saturation. It’s worth considering testing your settings on both your positive and negative controls to avoid wasting time taking irrelevant pictures. Beware, your experimental sample may be brighter than your positive control…

If you are detecting several dyes at once, always test one receptor at time with its single laser on then with all lasers of its sequence on, this will ensure absence of crosstalk.

Repeat this for all settings.

Defining your volume

Once your settings are defined, you may want to define a volume to be scanned – a stack. This is found if you are scanning in XYZ and the controls are on the middle left hand side of your screen.



To do this, go live with the sequence that is brightest and use the right-hand wheel dial.

Turn in clockwise until your signal of interest disappears. Click on "Begin", it will turn from grey to red.

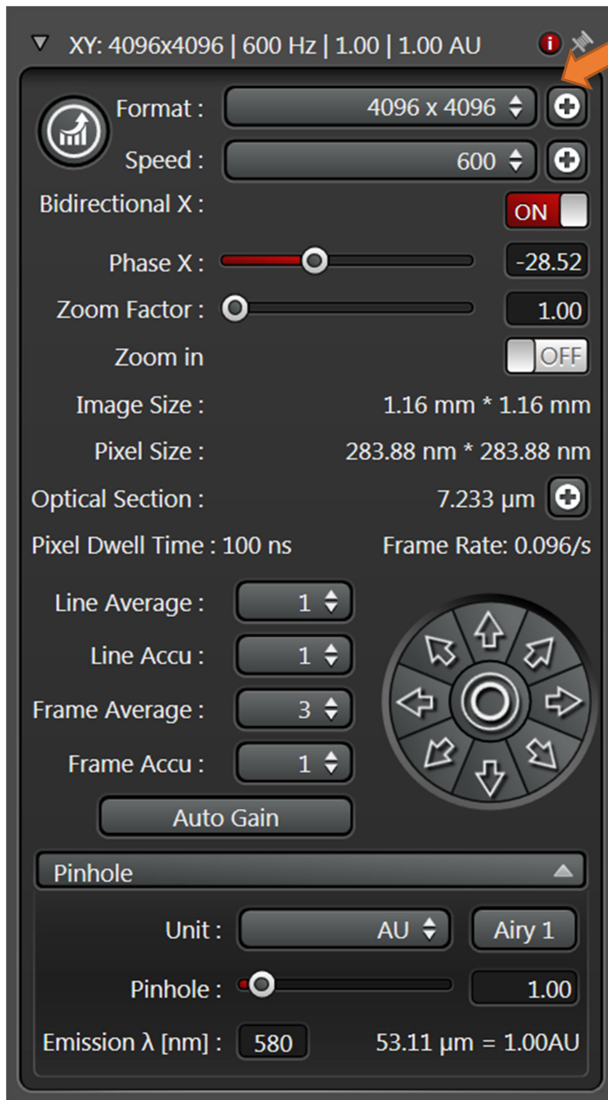
Turn anticlockwise until your signal of interest disappears. Click on "End", it will turn from grey to red.

To go to the beginning or end of your stack, click on either the arrow to the right of Begin or End. To go to the middle of the stack, click on the symbol right of the bin. To delete the stack settings, click on the bin.

You have a choice to define the number of steps, the thickness of steps or to let the system use optimised step size. We recommend that you use the later if you want to accurately reconstruct your volumes.

Defining your image format

Once the light settings and volumes have been defined, you now need to consider the format of your image (Top left of the screen).



Format: this tells you the size of your image in pixels. The dropdown menu gives you a choice of pre-established square formats while clicking on "+" allows you to define rectangles. For prescanning and establishing your settings, you do not need more than 512x512. When it comes to scanning, the format will depend on your sample, the lens that you use and more importantly what you are looking for. Aim for your object of interest to be scanned by at least three pixels. We strongly suggest that you check the Nyquist calculator for optimal pixel size for the lens you are using.

The more pixels, the smaller the pixel size, the more details, the longer your picture takes to be scanned.

Speed: how fast your laser runs. Start at 600. If you are using a zoom, you may be able to go faster.

Bidirectional: on or off. Running bidirectional may be faster but less accurate than normal scanning. It's sample specific. If bidirectional is on, please do not change the value in PhaseX or your confocal may give crenelated structures.

Zoom Factor is self-explanatory.

Note that while the image size remains the same (it's the physical field of view), the size of each pixel changes with format and zoom.

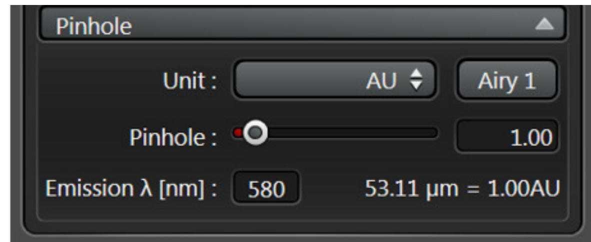
You may choose to average by line or by frame to increase the signal to noise ratio. Don't do both. There is very little reason to average more than three times.

If you are using the "Counting mode" in scanning, you may want to use the line or frame accumulation.

Pinhole

You can change the pinhole size: larger and the confocal behaves like a widefield microscope, smaller and you get a thinner optical section-and less light. We recommend that you keep 1 Airy Unit.

If you are sequentially scanning one dye at time, you can change the pinhole so it is set accurately to its emission spectrum. Simply look at the spectrum, find peak emission and enter this number next to Emission λ[nm]. Click on Airy 1 and the pinhole size will be just right.



If you are scanning several dyes at the same time, you have three options:

1) Use peak emission of the bluest dye: Airy unit is narrower when looking at higher frequency light (i.e. blue vs green vs red). If you use the pinhole for blue light, your red light will be very crisp and you may need to use more laser to see your dye.
2) Use an average peak emission, your blue light may be a bit fuzzy, but your red light will be crisp and you will need a bit more laser
3) Use peak emission of the reddest dye: it will be perfect but the blue dye will be a bit more blurry.

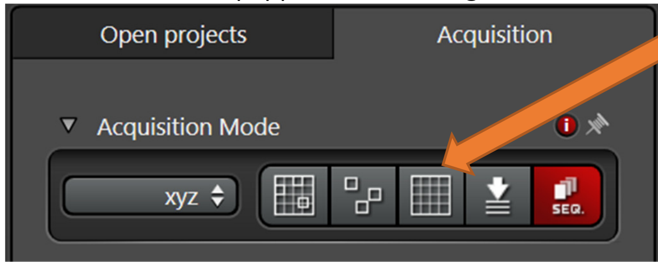
This is sample specific. Please ask for help if you are confused.

Start scanning

Once all these have been defined, click on Start and enjoy your scanning.

The Navigator Module

Both our SP8 are equipped with a Navigator module which you can access by clicking on the square grid under Acquisition. Before using it, it's worth programming your light settings in normal mode, defining your stack, etc… Move to the middle of your stack if you have one.

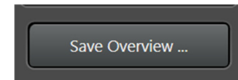


Clicking on the grid opens a new window. All the control panels you accessed previously are arranged in a sweries of tab on the left of the screen. Click on the "scanmode" icon and change the image format to 512x512.

Making a map: Spiral mode

You can quickly see your sample by clicking "Live" and you'll see a single slice taken using a single light setting. For a wider view of your sample, clike on "Spiral" and the software will make a spiral, taking a single slice using all channels at every field of view. Once you have seen enough, click "stop".

Should you want, you can save the overview of your sample. By simply clicking on middle right.



Stitching

You can choose to stitch the overview by selecting Mosaic Merge (top left)

This opens a new tab. First , you must select the overview you have just saved. All you then need to do is click on "merge" (bottom right).

Using the map as a reference

Go back to Acquisition.

Defining a region of interest

You can use the map you have made to choose your region of interest by simply clicking on it and the microscope will automatically centre its field of view where you have clicked. You can even change lens and -provided you don't move your sample- the microscope will adapt its field of view.

Remember that you may need to adapt your light settings and image format to the lenses

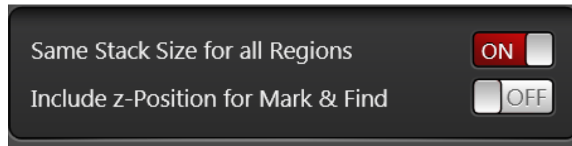
Should your region of interest be larger than the field of view, you can use the navigator buttons to

draw a region and the computer will define the number of fields of view you need.

Defining several regions of interest

It is possible to define several regions on interest at the same time. These may however have different depth.

Click on Z-Stack. You'll find these options available



You can then define the Z range as you define each region of interest

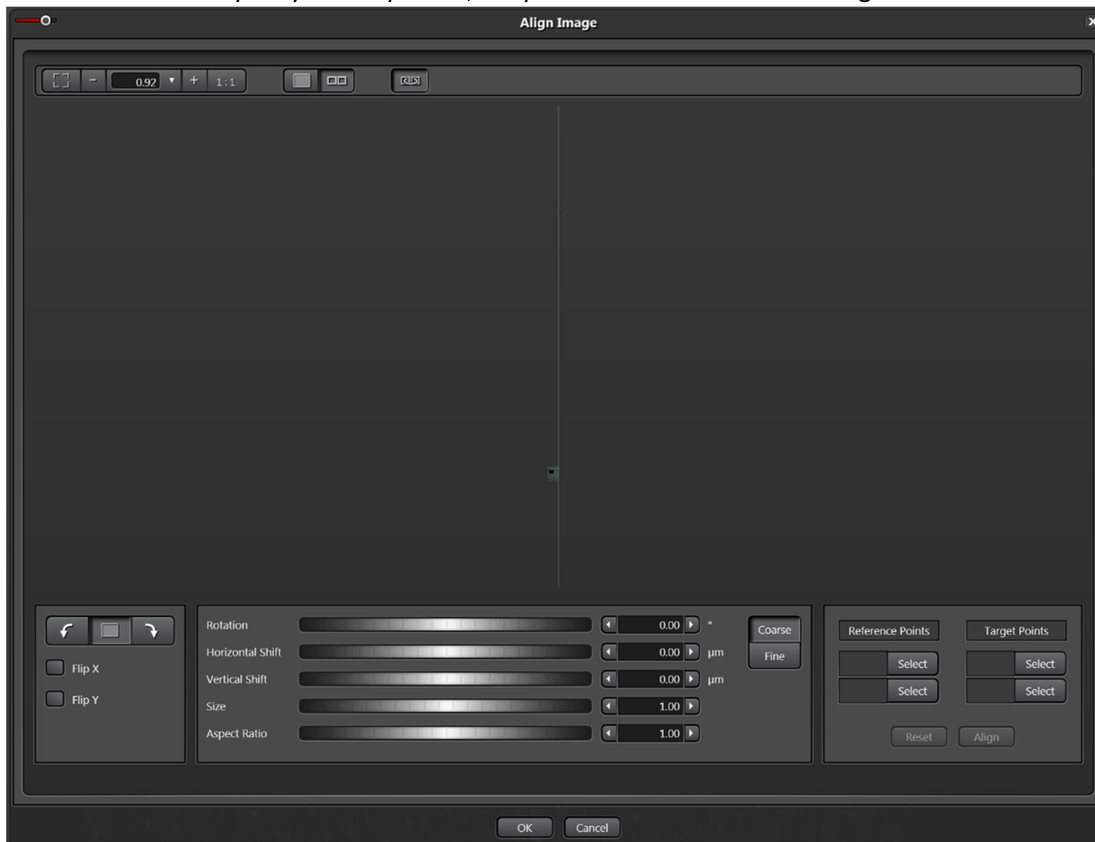
Aligning map with previously taken picture

Navigator allows you to align your map with a previously taken picture -for example on a slide scanner. This is particularly advantageous if you want to compare parallel slides. This picture must be a Tif or a .png file and must be square (use Fiji>Adjust>Canvas size).

To start with, right click on the background of the Acquisition pane and select "Load and align an image"

A window pops open and asks you to find the reference picture

A two-sided window pops up. On the left panel is your map and on the right panel is the reference picture. Note that they may be very small, they tend to be near the dividing line between each side.



At the bottom of the window are controls that move the images (flipping, rotation, horizontal and vertical shift, size). You must try to have a good view of both images such that you recognise landmarks common to your map and the reference image. Ideally, both images will be oriented the same way and have similar magnification. Once you have set both images to your satisfaction, you only need to select two reference and target (on your map) points so that Navigator project the reference image as background to your map.

To switch the system off

Before switching off:

- Clean up your lens, if you have used oil or water, and the stage of any spillage
- Discard used wipes
- Check that you are the last person to use the microscope under PMMS
- Set the lens to 10x and make sure it is away from the stage
- Back up your data

Switching off:

- Computer using the “Switch off” function of Windows
- Fluorescence light box
- Microscope control
- Scanner switch
- WAIT 10 MINUTES FOR LASER TO COOL DOWN
- Laser switch
- Turn key to laser interlock