The University of Queensland
Queensland Brain Institute Microscopy

QBI Zeiss LSM 980 NLO Airyscan 2 User Guide

Getting Started

1) Turn on the black switch to vertical on the main power module.
2) Turn the key to ON (clockwise to 6 o'clock) on the main power module.
3) Turn on the Components switch.
4) Turn on the PC.
5) Log on to the PPMS using your UQ credentials.
6) When Zen asks you to put the 10X in place, select the 10X using the touchpad.
7) Open ZEN Blue (this takes ~2 min).
8) Zen will ask if you want a calibration – click Start Calibration.
9) If you are using the Ti:sapphire two-photon laser, go to the Ti:sapphire laser controller and turn the key to ON. Do NOT touch the black or orange switches.

Shutting Down

1) Remove your sample and *gently wipe off* any oil-immersion objectives you have used with *lens tissue.*

Do not use Kim wipes to clean objectives. Use only lens tissue.

2) If you used the Ti:sapphire two-photon laser, turn it off in Zen.
3) Turn off the Ti:sapphire laser at its controller by turning the key to OFF. Do NOT touch any switches.

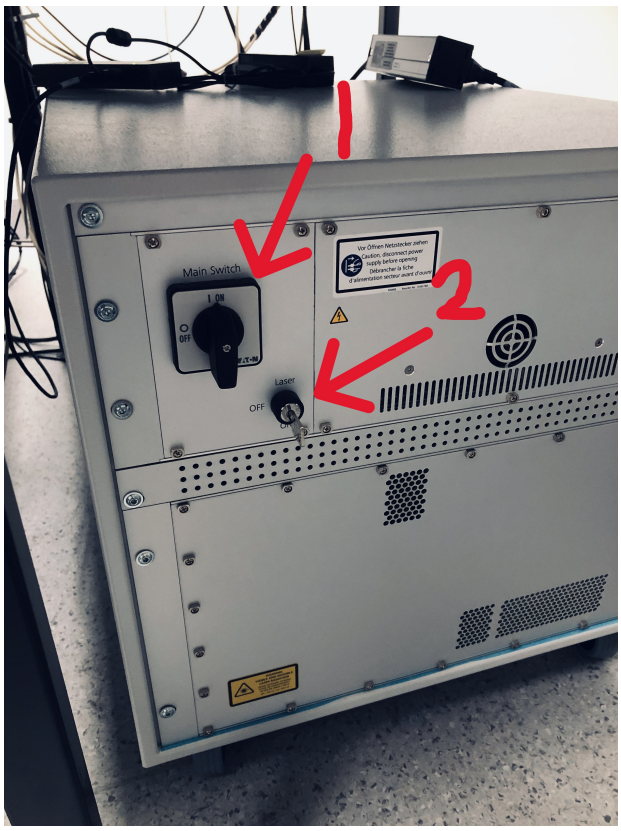
If there is another user after you,

Exit Zen.
Log off PPMS.

If you are the last user,

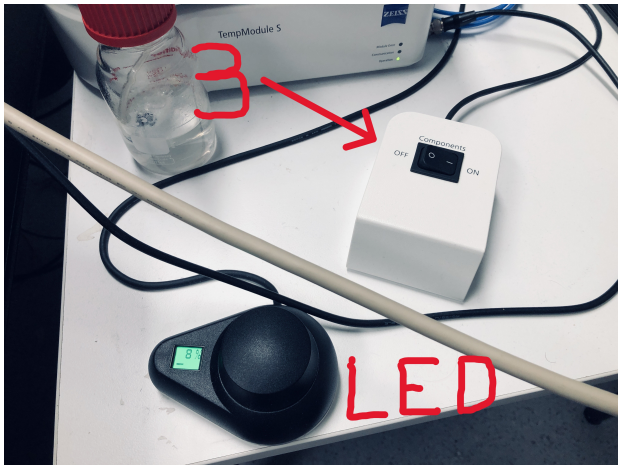
Exit Zen.
Turn off the PC.
Turn off the Components.
Turn the laser key to OFF.
Turn the large black switch to 9 o'clock.
Turn the small key on the Ti:sapphire laser controller to OFF.

If you notice the objectives are not clean or you are concerned you may have spilt oil on an air objective, please contact facility staff immediately.



1 Turn the black knob to ON

2 Turn the key to ON



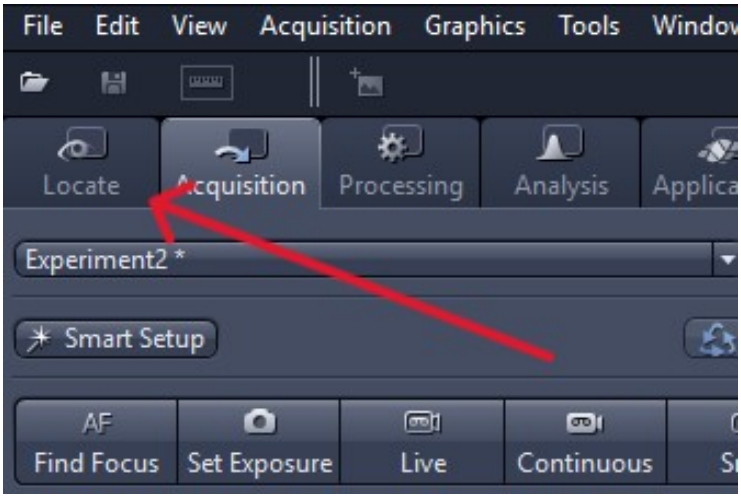
3 Turn on the Components switch

The LED wheel is used to control power to the fluorescence excitation and is an on-off switch (press down on the wheel).

Visualizing a Sample through the Oculars

In Zen, select ***Locate.***
On the touchpad, select the ***10X objective.***
Open the incubation chamber and place your slide in the holder.

Locate for visual use.
Acquisition for imaging.
Processing for Airyscan processing or stitching.



Using the touchpad

Use ***Home / Control / Microscope / Channel*** to select blue, green, or red fluorescence.
Use ***Home / Control / Microscope / Objectives*** to change objectives.

Open the excitation shutter, and LED power, with the black wheel.

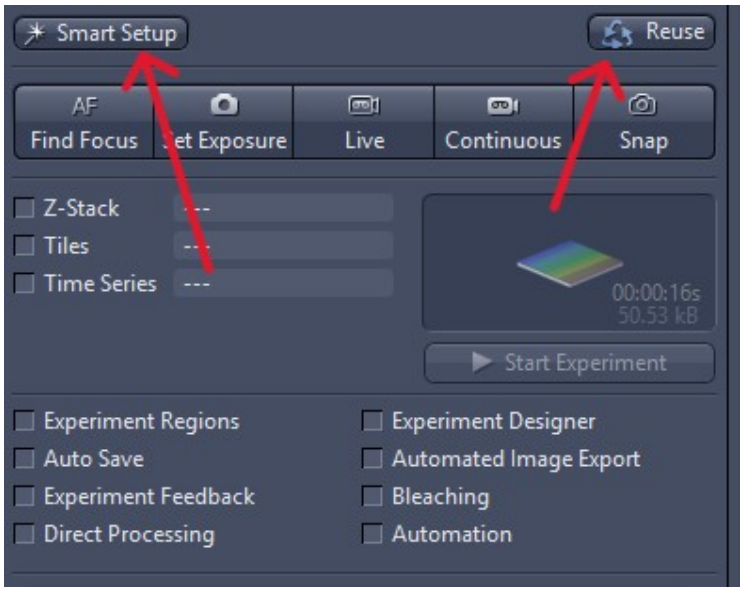
Use the focus knob and joystick to focus on the sample and move it around.

Setting up Zen

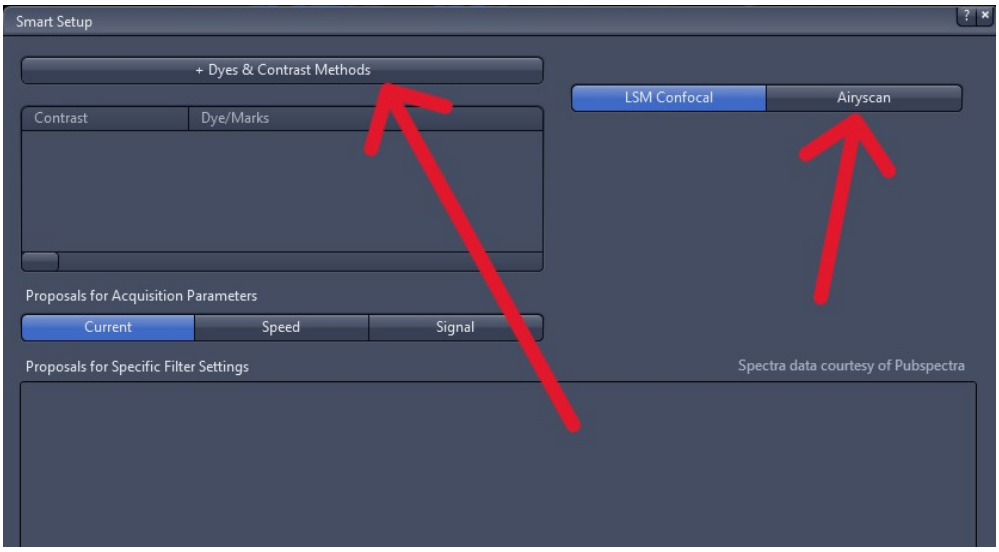
Once in Zen, you have to set up the fluorophore channels and confocal/Airyscan detectors.

Click on ***Smart Setup.***

Alternatively, ***Open*** a previous image and select ***Reuse***. This will reproduce the channels, etc from a previous image.



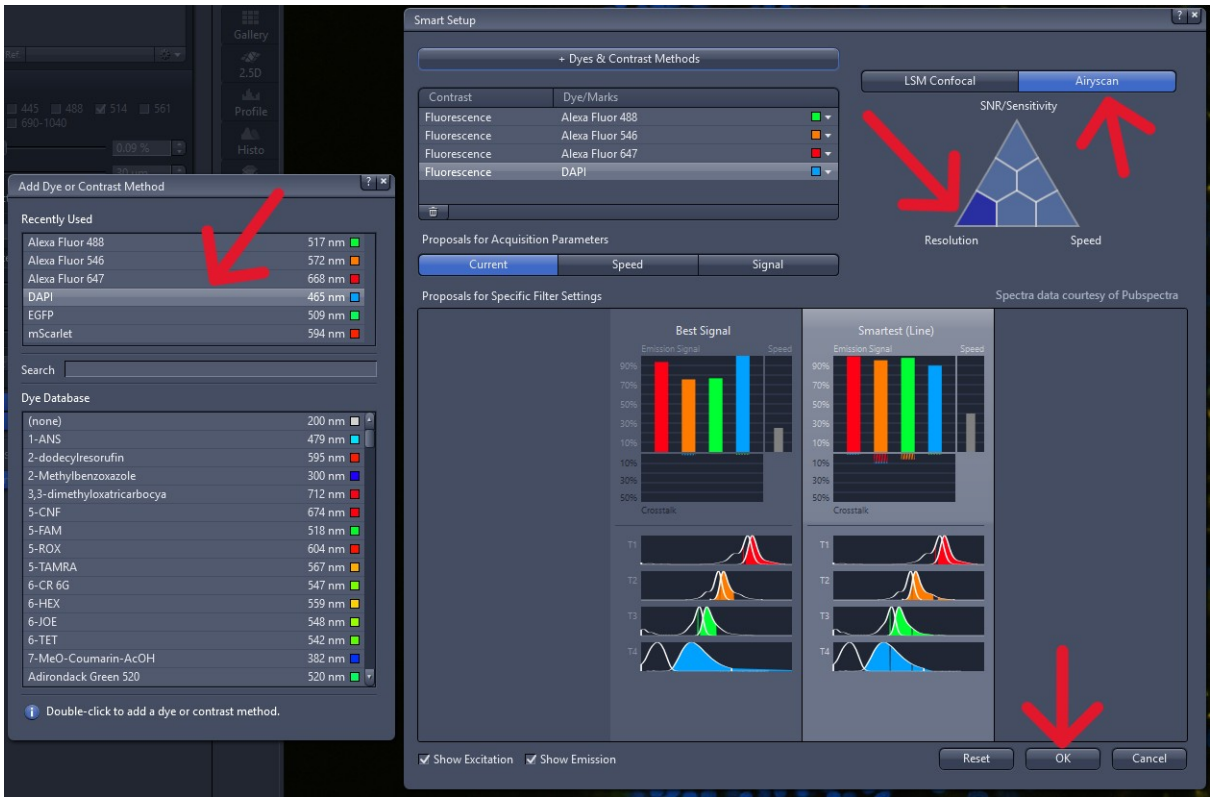
When the Smart Setup box opens, choose either ***LSM Confocal*** or ***Airyscan***.



Airyscan has several modes, shown in the triangle, favouring ***Resolution***, ***Speed*** or ***SNR/Sensitivity***.

For now, choose ***Resolution***.

Click on ***+Dyes & Contrast Methods*** and type in the name of the fluorophore you will use, such as ***DAPI.***
Double-click on it.
Continue until you have all the fluorophores you need.
Click ***OK*** to close the ***Smart Setup*** box.



Setting up the imaging area

Crop area = The area being imaged. This zooms into the sample. Start with 1.x

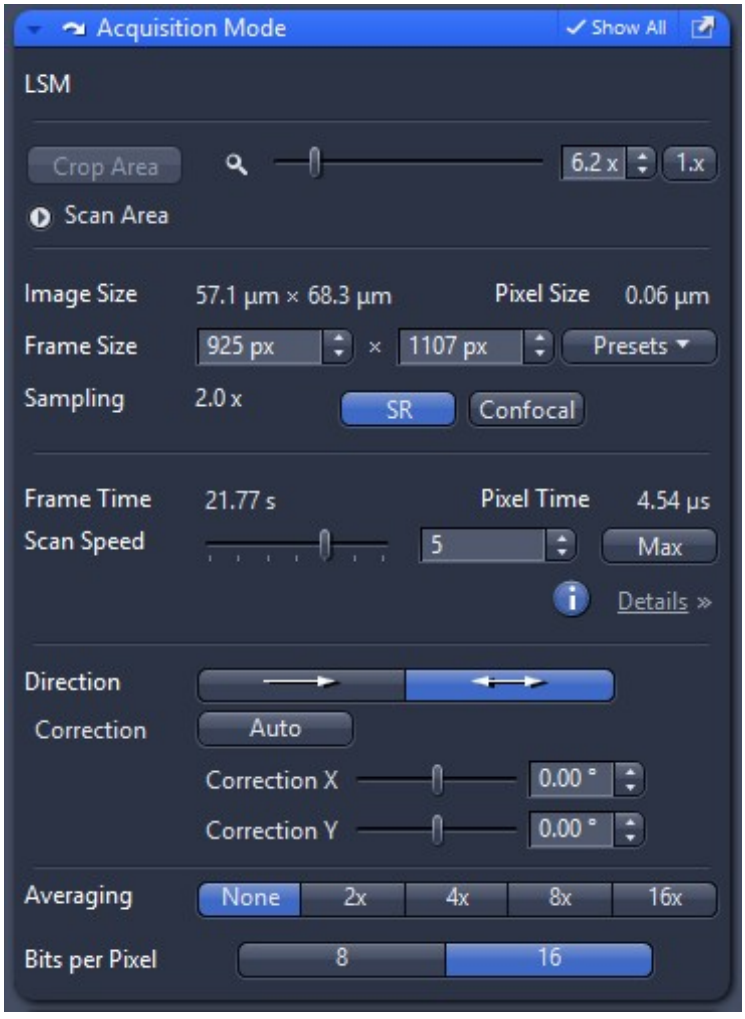
Image size = The X and Y pixel values. Don't use a value that is too high as scan times will be long. 1024 x 1024 is a good start. Click on Presets to get predefined values such as 512 x 512. The frame size and sampling rate are dictated by the mode selected, for example, "SR" (super-resolution) will use a larger frame size than "Confocal".

Scan Speed = The time spent collecting light per pixel *(Pixel Time*) and time to acquire an image (*Frame Time*). Fast scan times can lead to noisier images. Slow scan times result in cleaner images but also cause you to sit and wait for a long time for an image to form, wishing for a better life. Start with a higher value.

Direction = The direction of the scanning. Bi-directional scanning (double arrowheads) is ~2 times faster but may show a phase defect. Use **Auto** to rectify the phase defect or rectify it using the Correction X slider. Uni-directional scanning is slower but more reliable.

Averaging = Increasing this will cause the software to take e.g., 4 images and then display the average. Higher values take longer to image but produce less noisy images. Don't use 8 or 16.

Bits = Always make this 16 bits. This will represent brightness over 65,535 values (2^16). 8 bits represents brightness over only 256 values (2^8), so has a narrower dynamic range.



Setting the laser power(s)

After setting up the channels using Smart Setup, you should see the dyes (channels) you selected for imaging in ***Channels***.

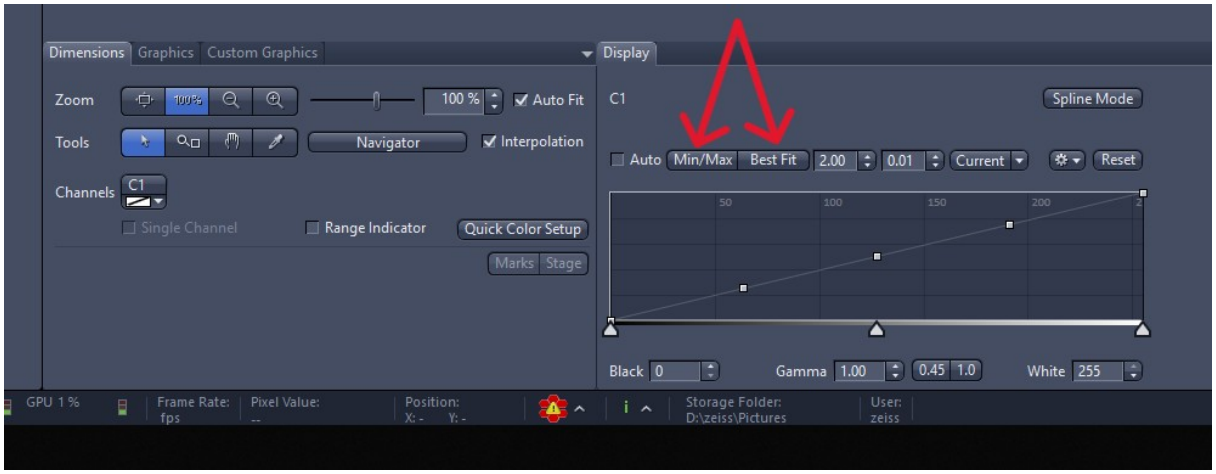
The ticks indicate which dyes will be imaged. Tick all of them.

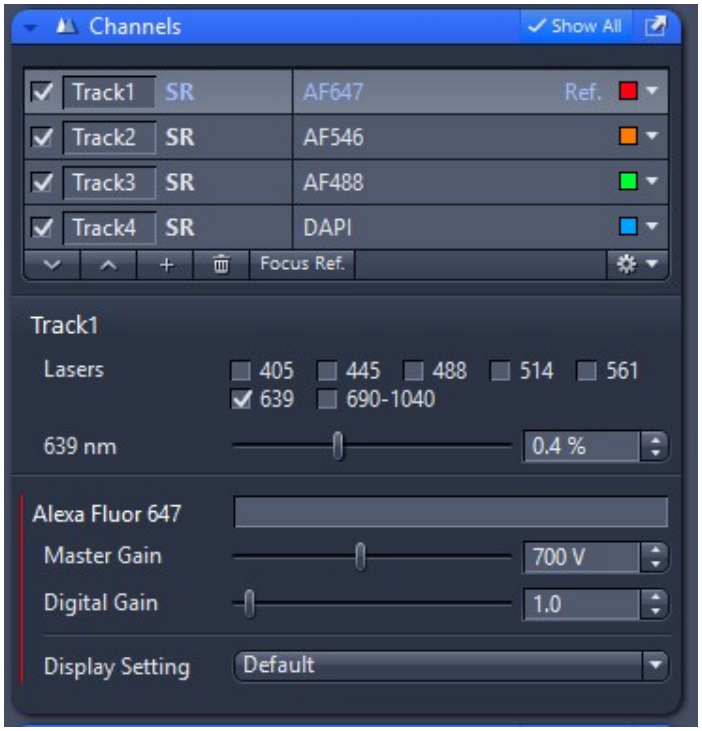
Next, set the power and ***Master Gain*** values for each laser. Do this by selecting a dye such as AF 647 and then clicking on ***Live*** (not ***Continuous***).

While looking at the ***Live*** image, use the slider to change the laser power used to excite the fluorophore. Low values (0.1-5 %) will rarely photobleach your specimen, but images may be noisy.
Higher laser values will lead to a higher signal-to-noise ratio in your images but will also cause photobleaching.

Change ***Master Gain*** if the image is too dim or too bright. Use 600 - 850 V.
Don't change ***Digital Gain*** (leave it at 1.0).

Adjust the image display by going to the histogram and clicking ***Min / Max*** or ***Best Fit***.





⇦ Tick all the channels you want to image. Click on the one you want to see in ***Live***.

⇦ Set the laser power here.

⇦ Set the ***Master Gain*** here.

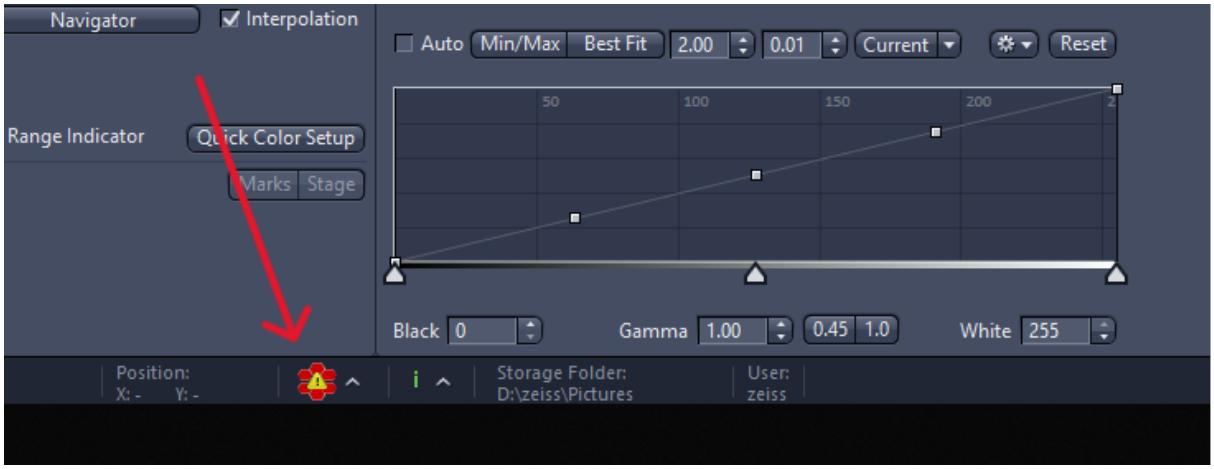
⇦ Don't touch ***Digital Gain***.

Once you have set the laser power and gain for each dye, stop ***Live***. Click on ***Snap*** to take an image.

Calibrating the Airyscan Detector Array

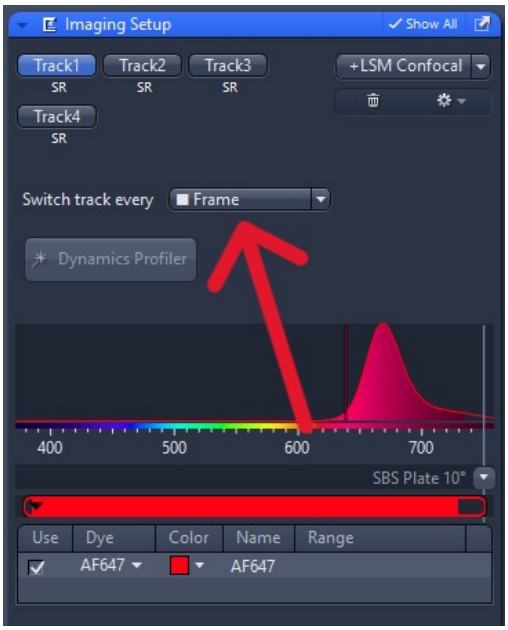
For Airyscan to work optimally, the Airy disk and interference pattern produced by the objective must be calibrated to fall precisely on the 32-detector array. If it isn't properly aligned, images will be dim and blurry.

Look along the bottom of Zen. The red/yellow icon indicates Airyscan is not calibrated. If it is calibrated, it will be green.

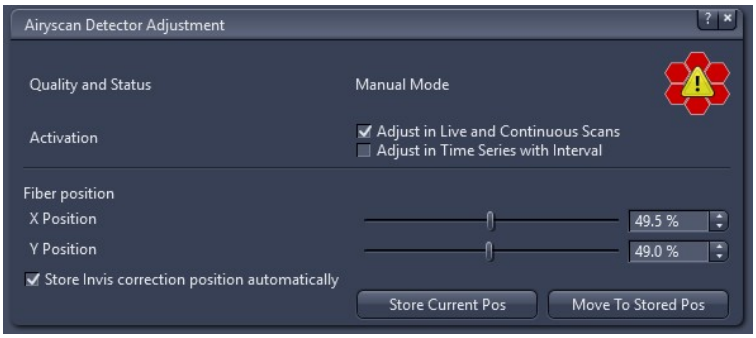


To calibrate the Airyscan array,

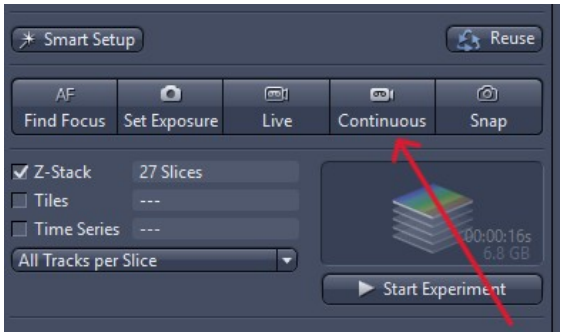
1) Set the laser power and gain for each channel (dye) to be optimal (i.e., bright but not overexposed). It should cover about 30-50 % of the histogram. If your sample is not bright enough for the Airyscan calibration to complete, you can use the *Convallaria* slide on the desktop.
2) In ***Imaging Setup***, change the "***Switch track every***" to ***Frame***.



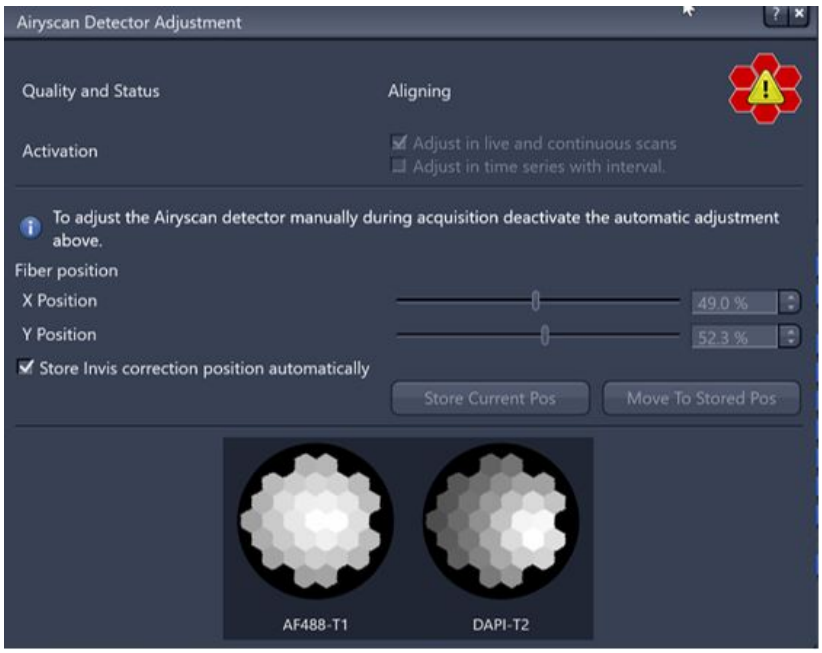
3) Click on the Airyscan icon along the bottom of Zen (i.e., the red/yellow octagon). It will open the ***Airyscan Detector Adjustment*** box. Make sure "***Adjust in Live and Continuous Scans***" is ticked.



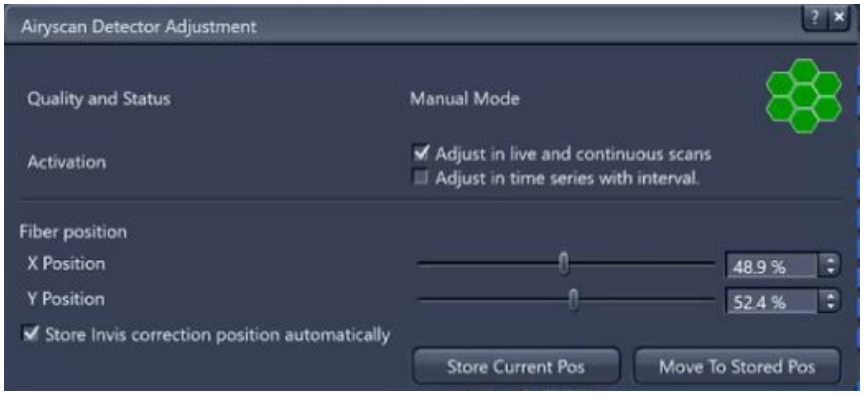
4) Click on ***Continuous***.



5) Once you have clicked on ***Continuous***, the Airyscan detectors will start to be calibrated for each channel you have. This may take a few minutes. When all channels are calibrated, the Airyscan icon will change from red/yellow, to green. Make sure you untick ***"Adjust in Live and Continuous Scans",*** or else Zen will keep on trying to calibrate during the whole of your session. You can then close the ***Airyscan Detector Adjustment*** box.



The Airyscan icon along the bottom of Zen should also be green. If not, repeat the calibration.



⇦ When this icon becomes green, airyscan has completed calibration.

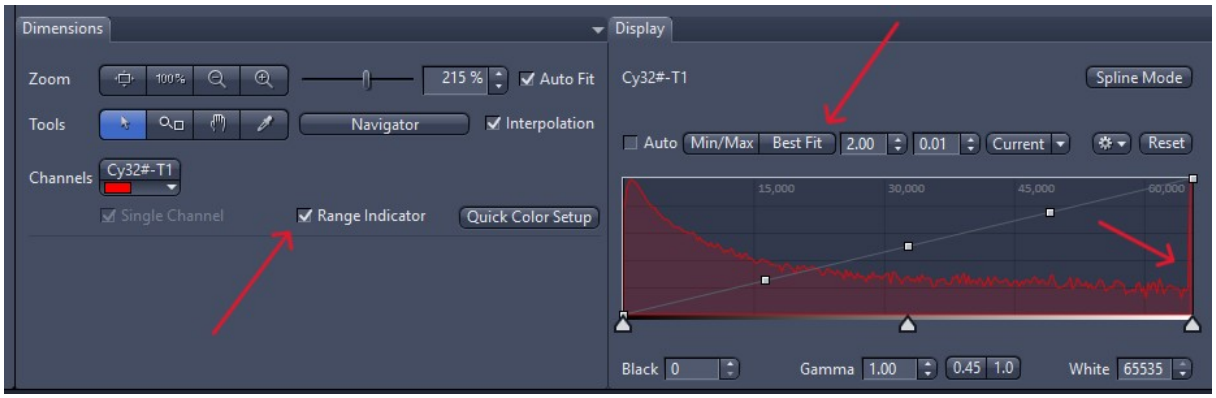
If the Airyscan is not calibrating (especially for the DAPI channel), try increasing the laser power and/or gain. Try moving to a different part of the sample.

Optimising the Dynamic Range

Live mode will configure the scanner to capture images as fast as possible.
Continuous mode uses the settings in ***Acquisition*** mode.

With a sample on the stage, press ***Live***.

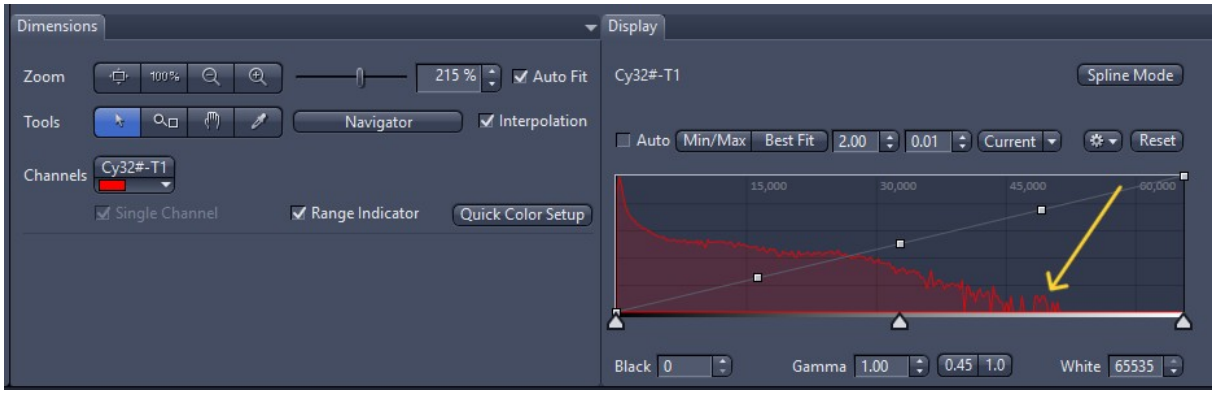
Near the histogram, tick the ***Range Indicator*** box.
On the ***Histogram***, click on ***Best Fit*** (or ***Min/Max***).



Oversaturated pixels in the image are shown in red. Look at the histogram. See how the curve goes to the right side of the plot. That is also due to overexposure.

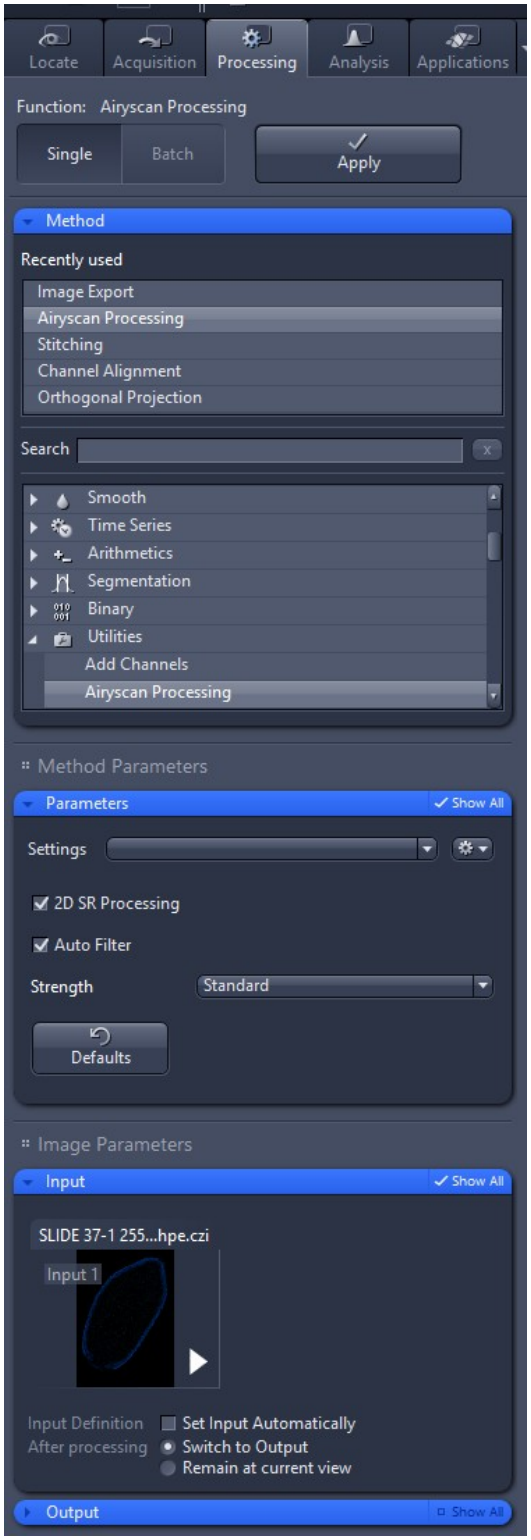
Adjust the laser power and/or gain until little or no red pixels remain.

The histogram should extend about halfway across the brightness range (see below how the curve stops at the yellow arrow). It should never be allowed to reach the right side (this will result in oversaturation).



Processing Airyscan Images

In the ***Processing*** tab, find ***Method / Airyscan Processing*** and fill out the box as shown below and then press ***Apply***. A new image will appear which has been Airyscan-processed.



⇦ The processing tab

⇦ Select Airyscan Processing

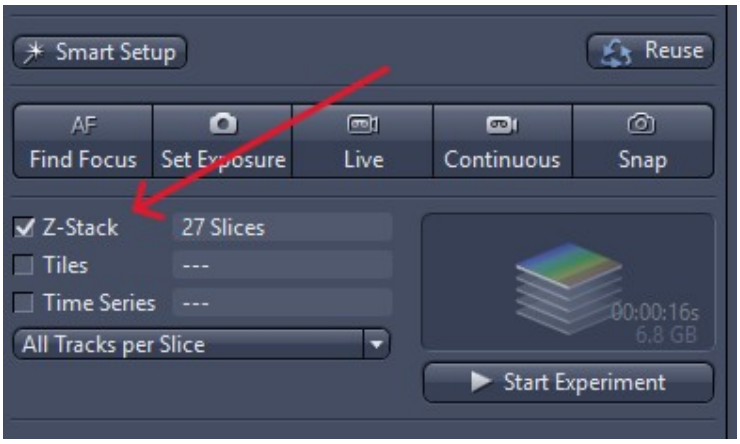
⇦ Tick 2D SR Processing

⇦ Tick Auto Filter

⇦ Select the image you want to process

Taking a Z-stack of Images

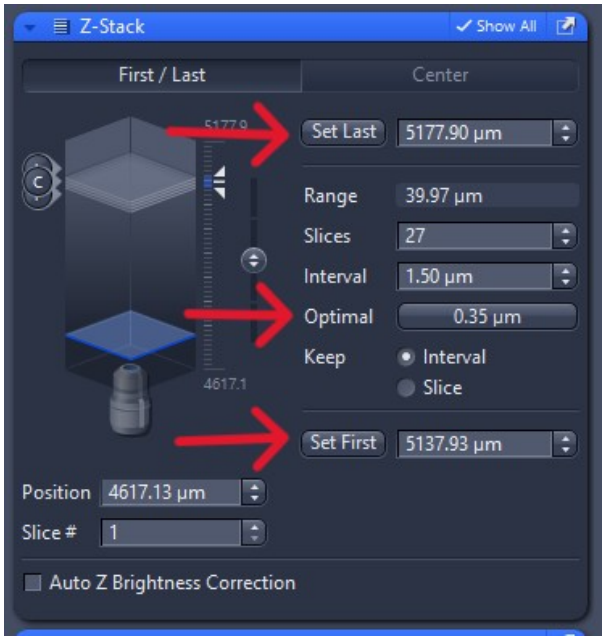
First, set the laser power, gain, and image conditions in ***Channels***.
Select the ***Z-stack*** option.



Find the ***Z-stack*** box.
Ensure that ***Show All*** is selected.
Select ***First/Last***

To set the z-stack,

1) click on ***Live*** and, when you have set the focus on the Z level you want the stack to start at, press ***Set First***.
2) Then focus through the sample and click *on* ***Set Last***.
3) Then click on ***Optimal***. Alternatively, you can also set the interval or number of slices to take.
4) Press ***Start Experiment*** to take the z-stack images.

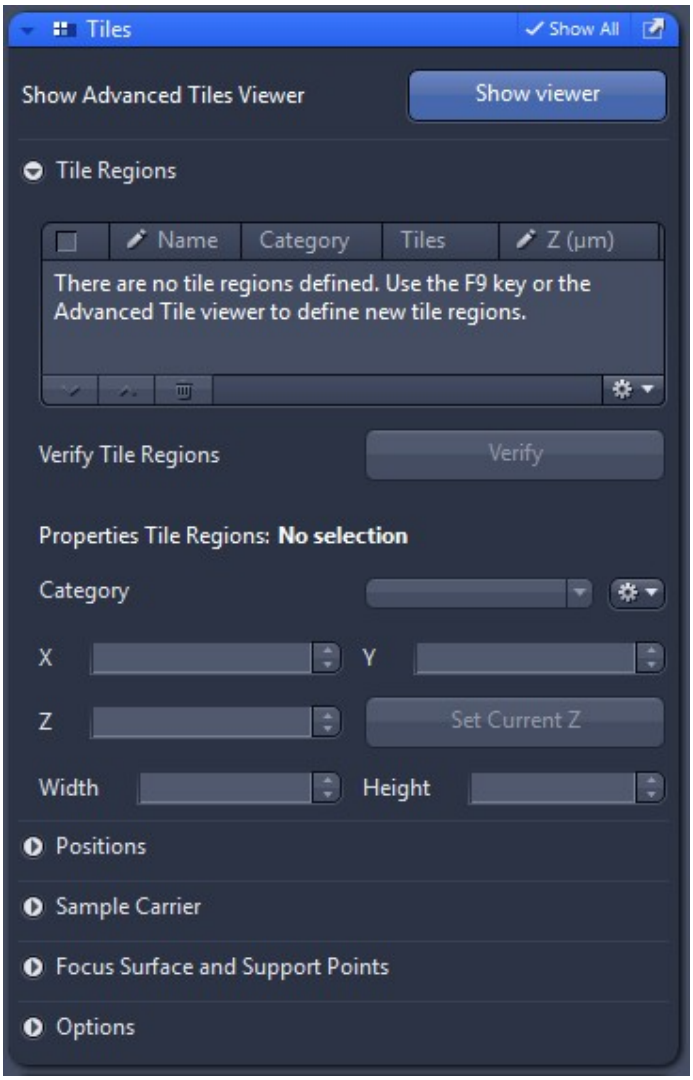


NB: If you are using the Zeiss LSM 980 in confocal mode, make sure the pinholes for all channels are the same (or at least very similar), otherwise the image thickness will be different for each channel, resulting in channel image mismatch.

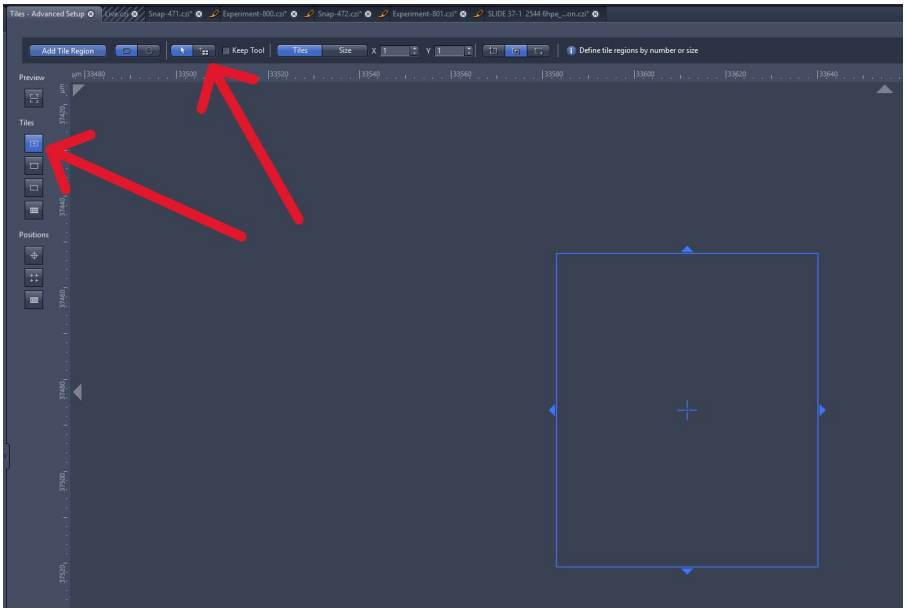
Taking a Tiled Image

Select the ***Tiles*** option (same place where you select the Z-stack option).
Find the ***Tiles*** box.
Ensure that ***Show All*** is selected.

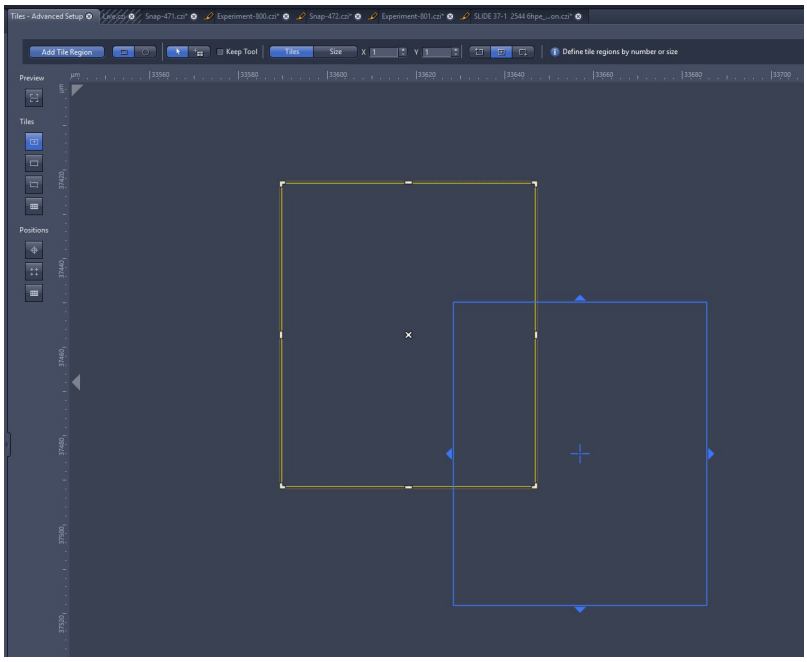
Click ***Show Viewer***



Another window will open.
Click on the icon on the left, and then on the icon along the top.
Now **click near the blue square.**
The blue square shows where your microscope image will be.
The yellow square is a starter tile.



The yellow square is a single tile which is a bit useless. However, you can make it a multi-tile region by resizing the yellow square.

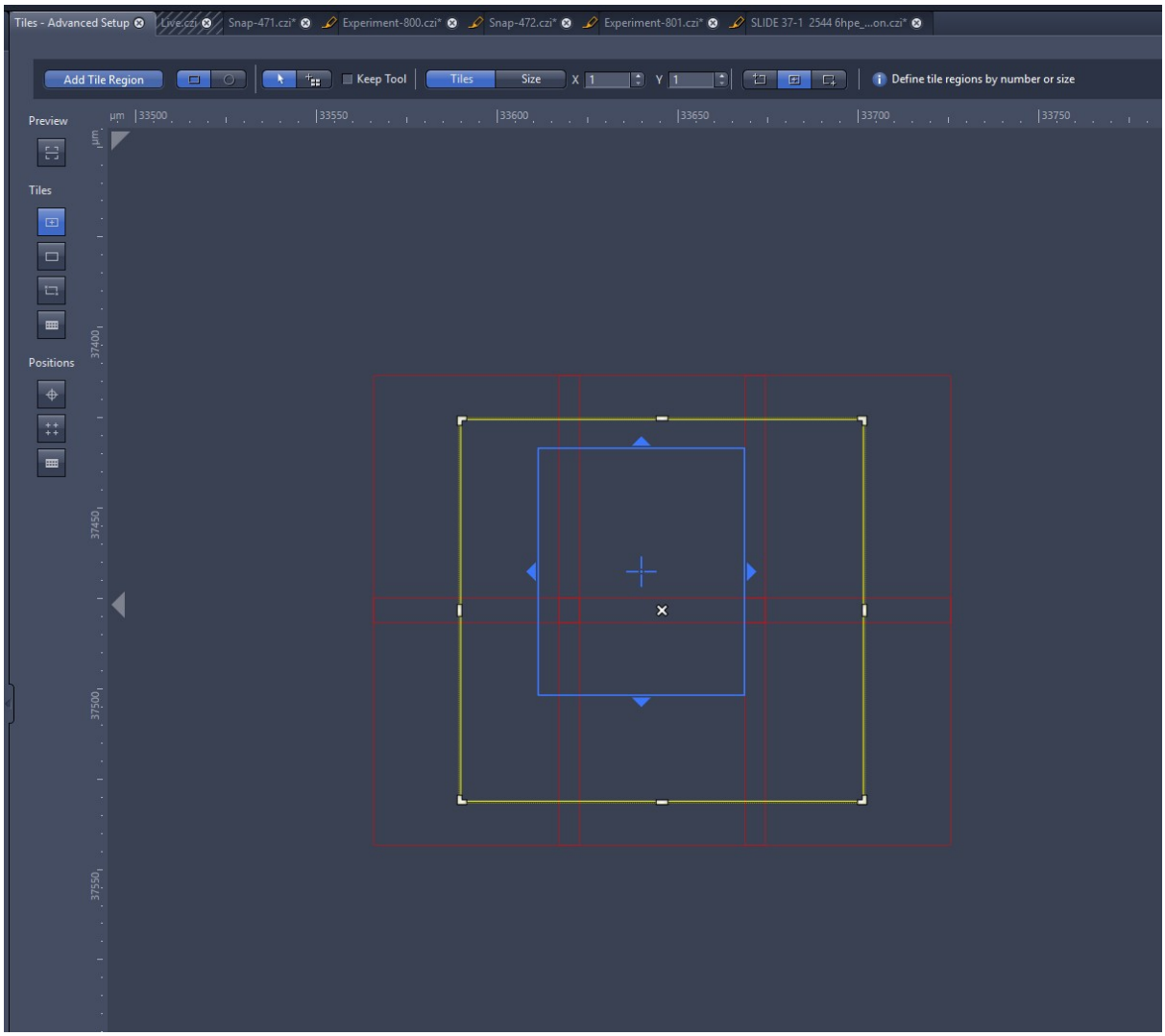


Grab the corners of the yellow square to resize it. This will define your tiled area.

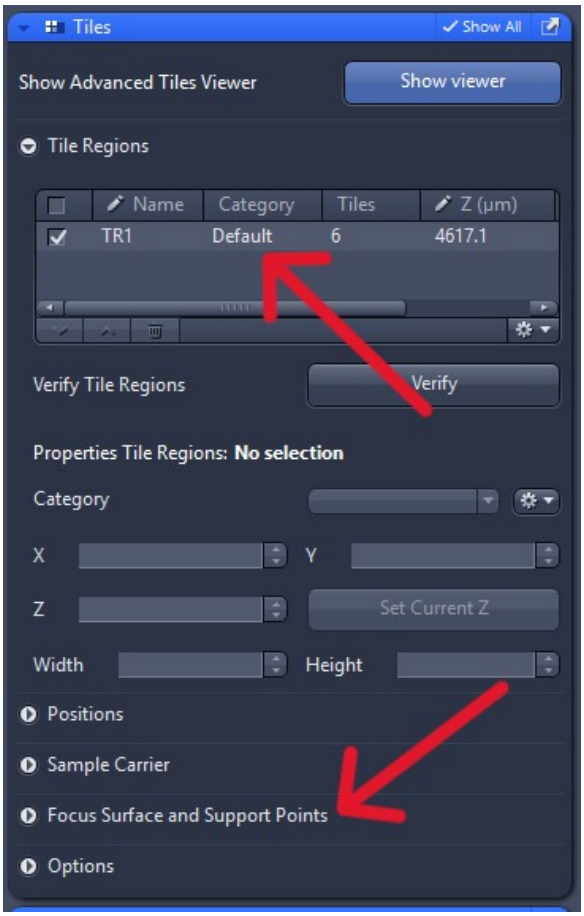
The image below shows a 3 X 2 tile of 6 red tiles.

Click on ***Live*** to see an image. Continue resizing the yellow box until it covers your target.

You can move the blue box, the yellow box, or resize the yellow box.



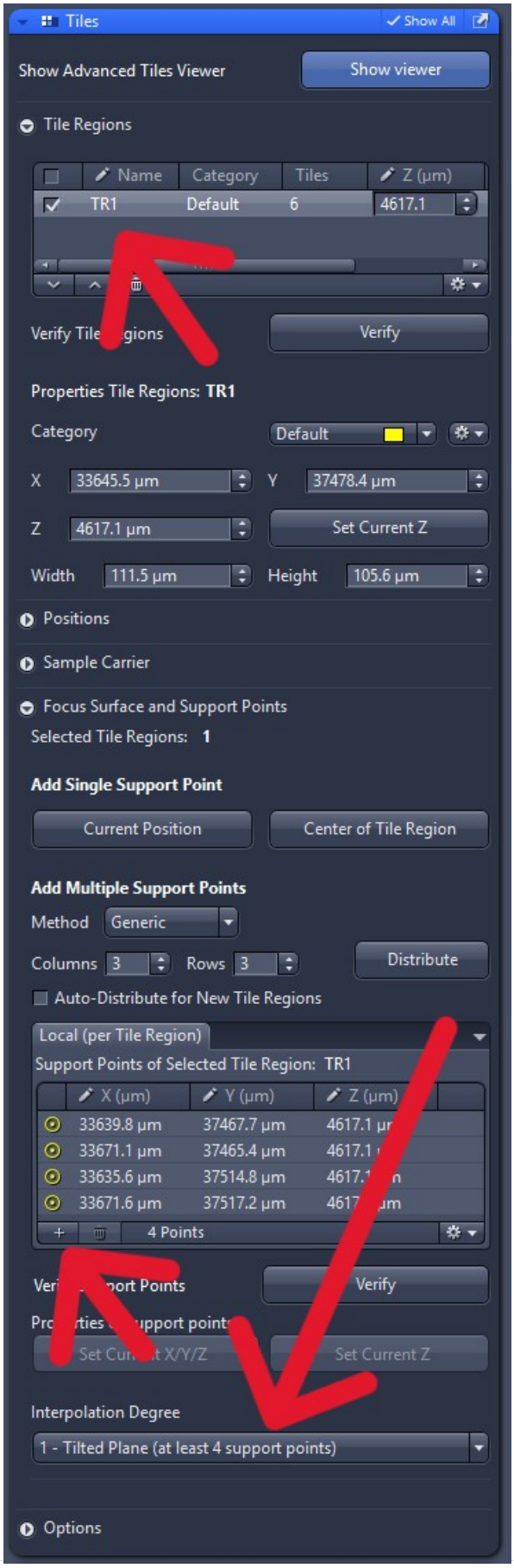
Look again at the ***Tiles*** box. It will show your new Tile Region as ***TR1***.
Since large tiled areas suffer from focus mismatch, you should set a Focus Surface.
To do this, click on TR1 to select it, then select ***Focus Surface and Support Points.***



Move the blue square around the tile, and (with TR1 selected), click on the small **+** in ***Focus Surface…*** This will add a Focus Surface point.

If you cannot see the **+**, it's because you haven't selected TR1.

Try to add at least 5 points. They will appear in Focus Surface.
Under ***Interpolation Degree***, select ***1 – Tilted Plane***. For larger tiles, for example, a 4x4 or 5x5 montage, use more focus points.



⇦ Select TR1 first if you want to add more focus surface points. It should also be ticked. If you cannot add any focus surface points, it is because you haven't selected TR1.

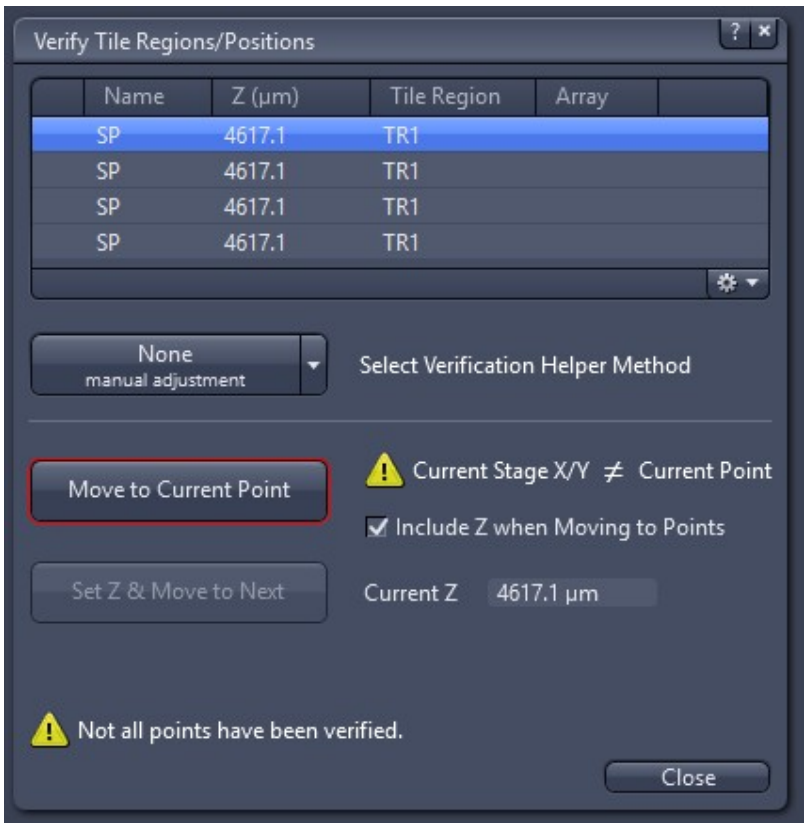
⇦ When you want to add a focus surface point, click on the +. If you cannot see the +, it's because you haven't selected TR1.

⇦ Select *Tilted Plane.*

Lastly, you have to validate that the focus surface points are actually focussed on the sample. To do this, click on ***Verify***.

A box called ***Verify Tile Regions/Positions*** will appear. Click on ***Move to Current Point***, and make sure that the point has the best focus.

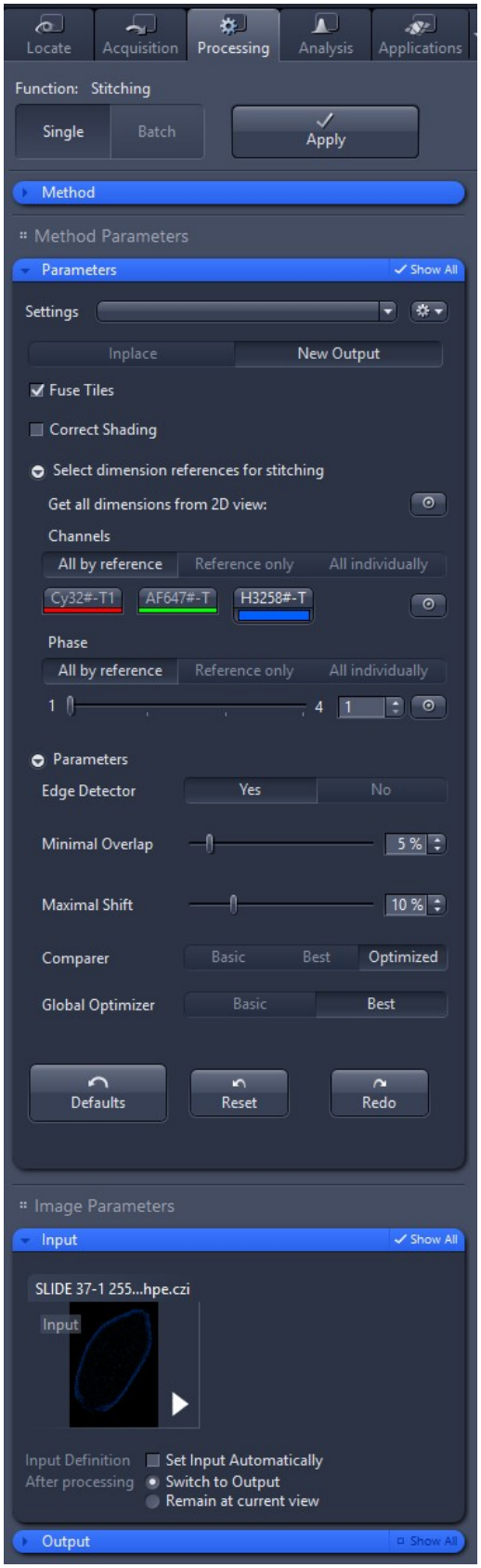
Then click on ***Set Z & Move to Next***.
Once you have verified that all points are focused correctly, click on ***Close.***



You can now click on ***Start Experiment*** to take the tiled image.

Once the tiled image has been taken, you need to do ***Processing / Airyscan Processing*** before you can stitch it using ***Processing / Stitching***.

Stitching the Tiled Images



⇦ Click on Processing.

⇦ Find the ***Stitching*** function in ***Methods.***

⇦ Select ***New Output*** and ***Fuse Tiles.***

⇦ Select ***All by reference***, and then select the channel with the brightest, most extensive staining as the reference channel.

⇦ Select your image here.

Using the Mai Tai Ti:sapphire Laser for Two-photon Imaging

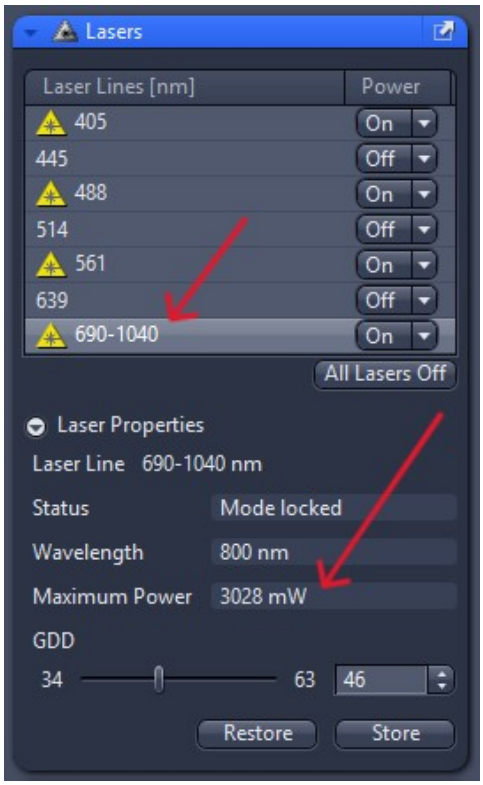
Turning the laser on

Once Zen has opened, go to the Ti:sapphire two-photon power supply and turn the key to ON. DO NOT touch the black or orange switches.

If you want to perform two-photon imaging, turn the key to ON.

Do not touch any other switches.

Now, turn on the Ti:sapphire laser in Zen (labelled 690-1040 nm).



Turning the laser off

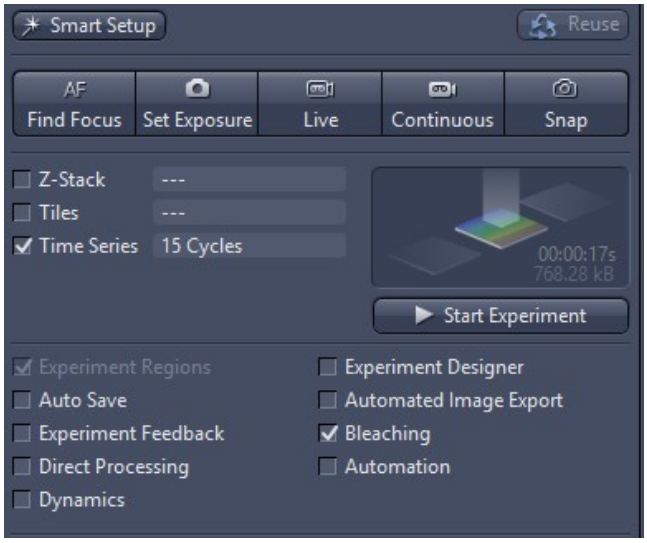
When you have finished using the Ti:sapphire two-photon laser, turn it off in Zen and turn the key to OFF (on the Ti:sapphire laser controller box).

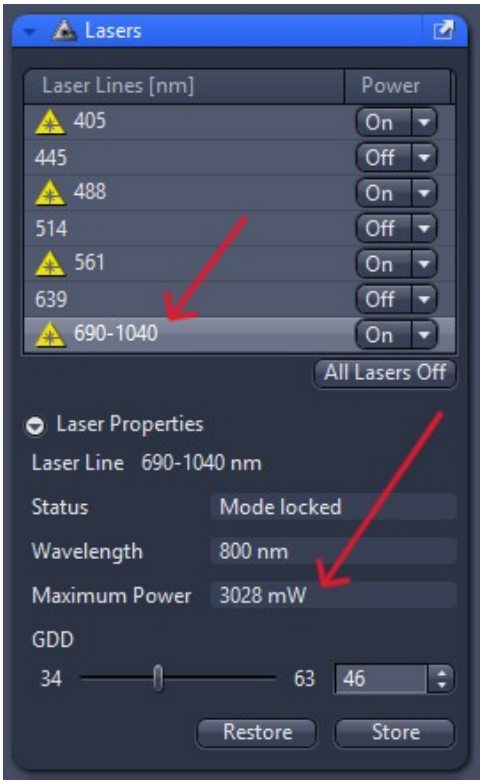
Photo-ablation Using the Mai Tai Ti:sapphire Laser

The Zeiss LSM 980 can be used to photo-ablate tissue by concentrating a laser beam onto a specified region of interest, such as a cell. The visible-light lasers as well as the Ti:sapphire two-photon laser can be used for ablation. An image of the tissue can be taken before and after ablation using either system to record the site of ablation.

Below is a description of using a visible laser to image green fluorescence, with subsequent photo-ablation of cellular material using the Ti:sapphire laser at 800 nm.

Select ***Experimental Regions*** and ***Timed Bleaching***





Look near the right-hand edge of Zen. You will see a box called Lasers.

Find the 690-1040 laser and turn it ON.

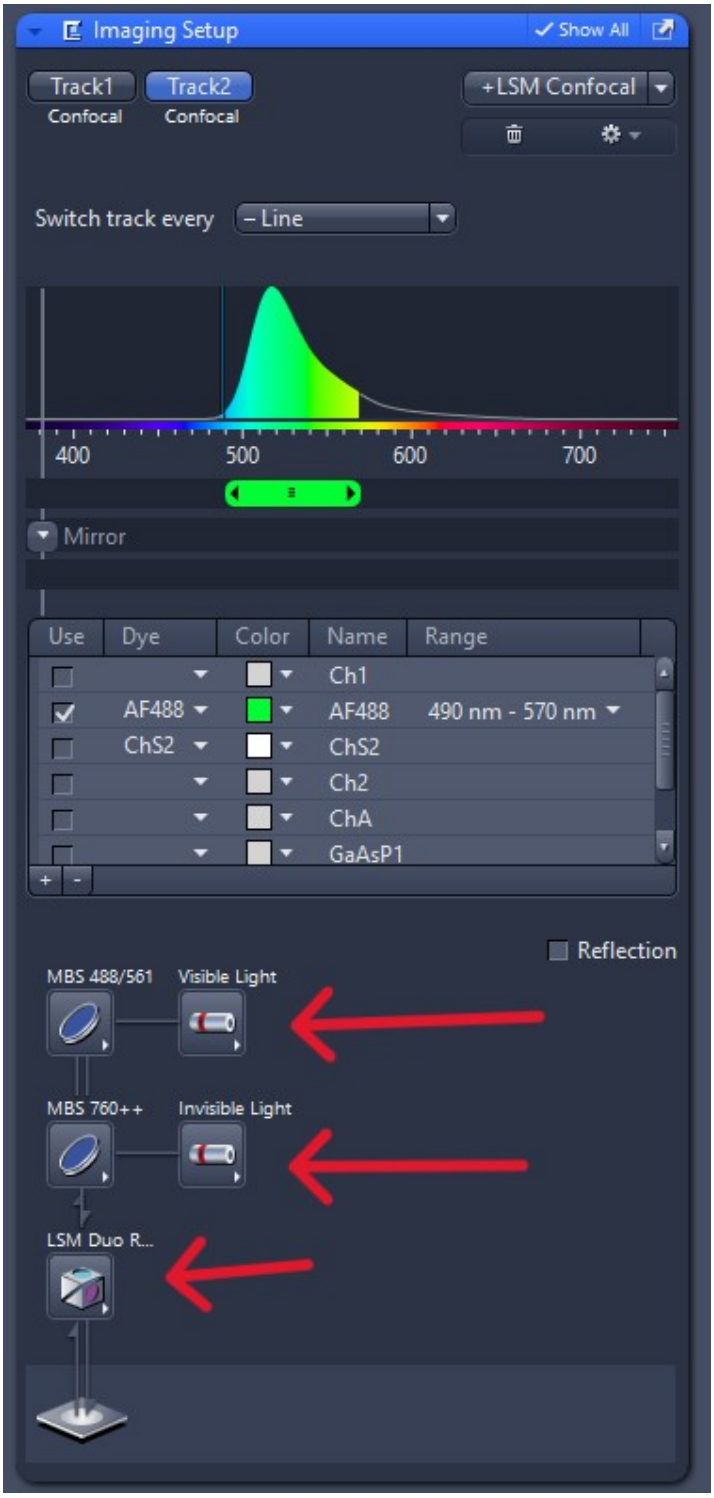
Select it to see its properties. At 800 nm, it should be producing at least 3000 mW.

In ***Imaging Setup***, choose the correct filters for the ***Visible*** light (the green fluorescence excitation laser) and the ***Invisible light*** (two-photon laser).

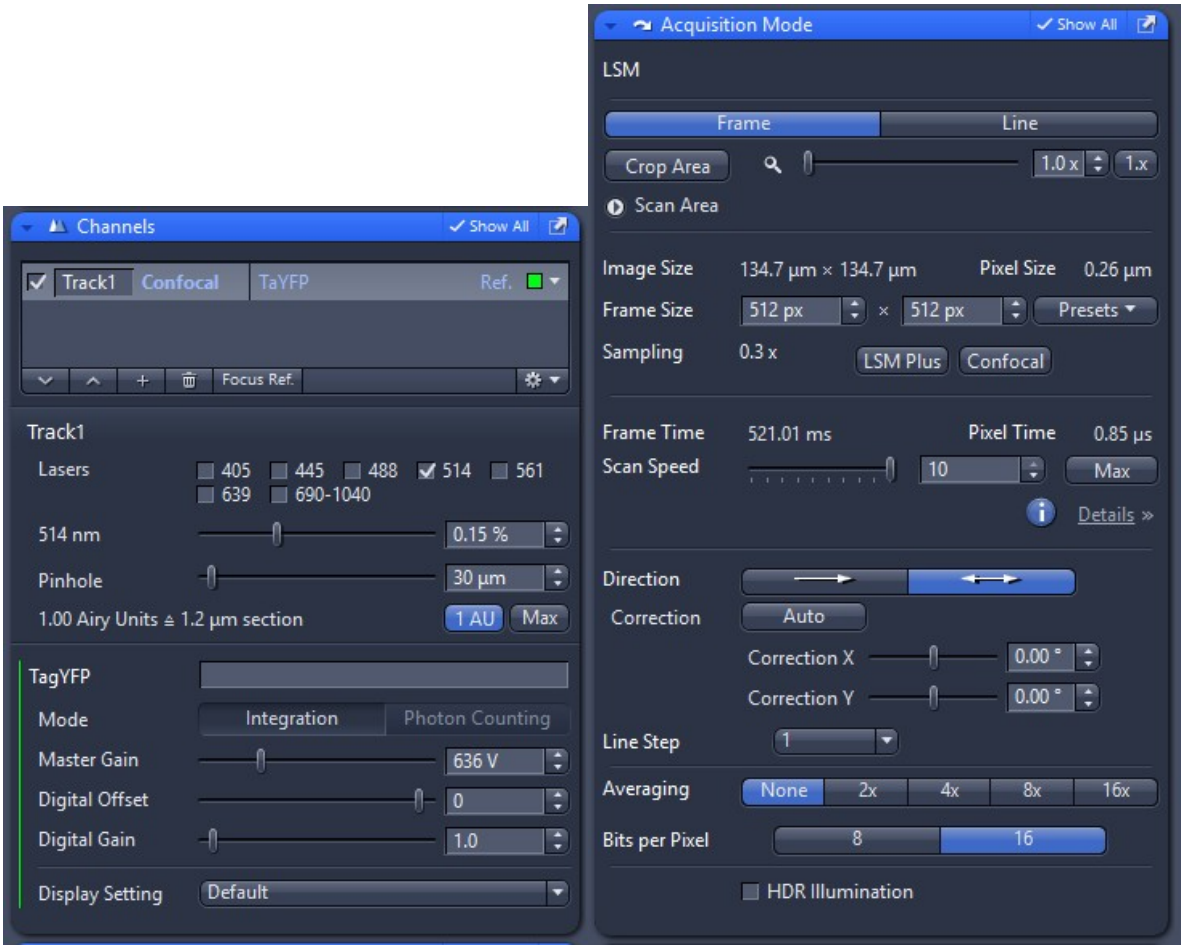
For the visible laser, choose MBS488/561.

For the invisible laser, choose MBS 760++.

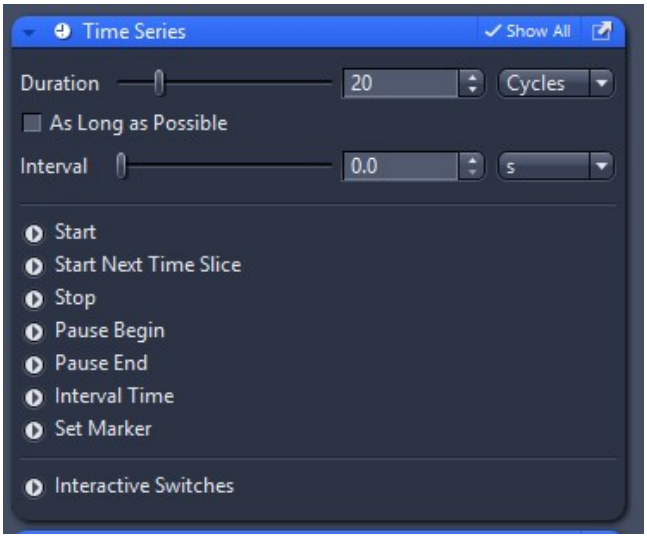
Use the LSM Duo R 100 Mirror.



In ***Channels*** and ***Acquisition Mode***, you only set the laser conditions for imaging for green fluorescence. You need to be able to see your target cells. Image quality is not important. You don't set anything for the two-photon laser. The image below shows typical conditions.



In ***Time Series***, Duration shows the number of images taken **in total**. **Start with 20.** Interval should be 0.



⇦ The ***Duration*** value in ***Time Series*** has to be larger than ***Start after # images*** in ***Timed Bleaching.***

In ***Timed Bleaching***, you set the conditions for the bleaching.

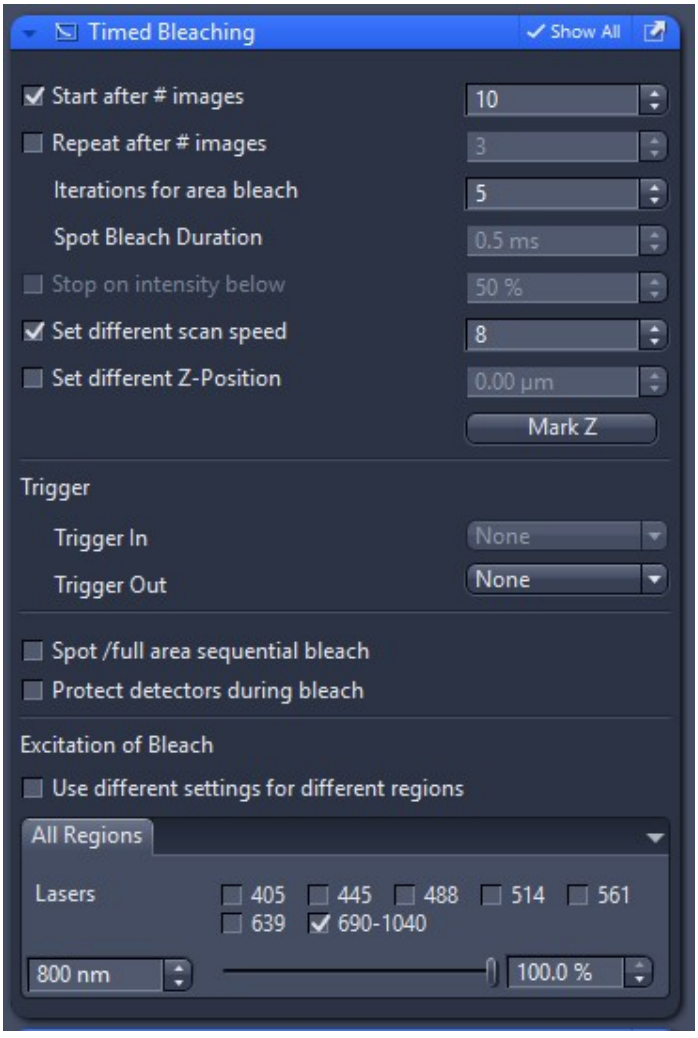
Start after # images – the microscope will take this number of images as a baseline and then it will photo-ablate. Start with 1-10. This number has to be **less** than the number in Duration (in Time Series).

Iterations for area bleach – the number of times the two-photon laser will scan across the area. If the two-photon laser is properly aligned, you only need a few scans. Start with 5.

Set different scan speed – the speed at which the two-photon laser will scan the region that is selected for bleaching. Slow speeds result in stronger bleaching. Start with 8.

All Regions – tick the 690 -1040 box. This will select the two-photon laser for bleaching. You could select visible-light lasers for bleaching, but the damage will be less constrained in Z.

800 nm – select the wavelength for the two-photon laser. The box shows 800 nm but you can select any wavelength between 690 – 1040 nm. The slider can set the power for the two-photon laser. 50 – 100 % is usually enough energy to photo-ablate a cell with 1 - 8 iterations.



Use ***Live*** to find the region of tissue you want to ablate. Use ***Channels*** to get a good image. Now press ***Snap***. This will take a snapshot of the region.

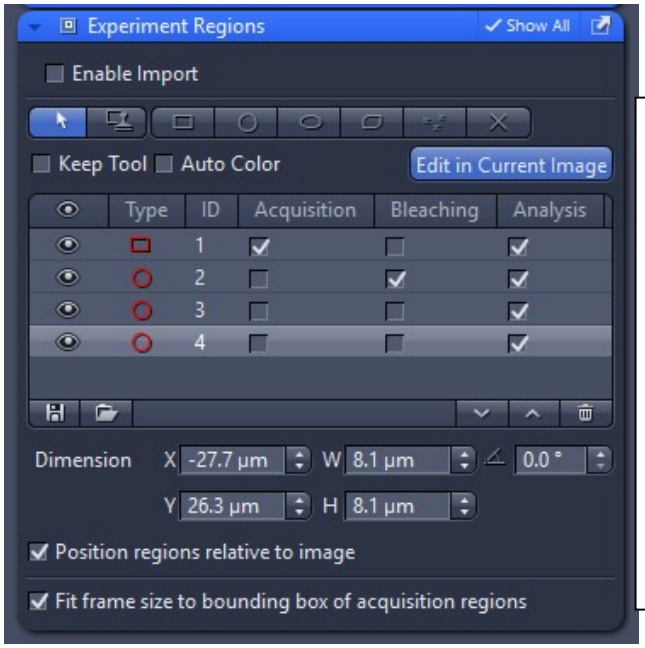
Now open ***Experimental Regions***. Here you will draw the region to image, as well as the objects you want to photo-ablate. You will also include a reference object, and background.

Click on a ***square*** (in ***Experimental Regions***) and draw it around the region you want to image. It should include your target(s), some reference cells and some background.

Now click on the circle icon. Draw circles around the target cells, reference cells, and background.

In the table shown in ***Experimental Regions***, define what you want done with each region by putting a tick against Acquisition, Bleaching, or Analysis.

The ***square*** should be ***Acquired*** but not ***bleached***. It should be ***analysed***.
Cells for ablation should be ***bleached*** and ***analysed***.
Cells for reference should only be ***analysed***.
Background should only be ***analysed***.



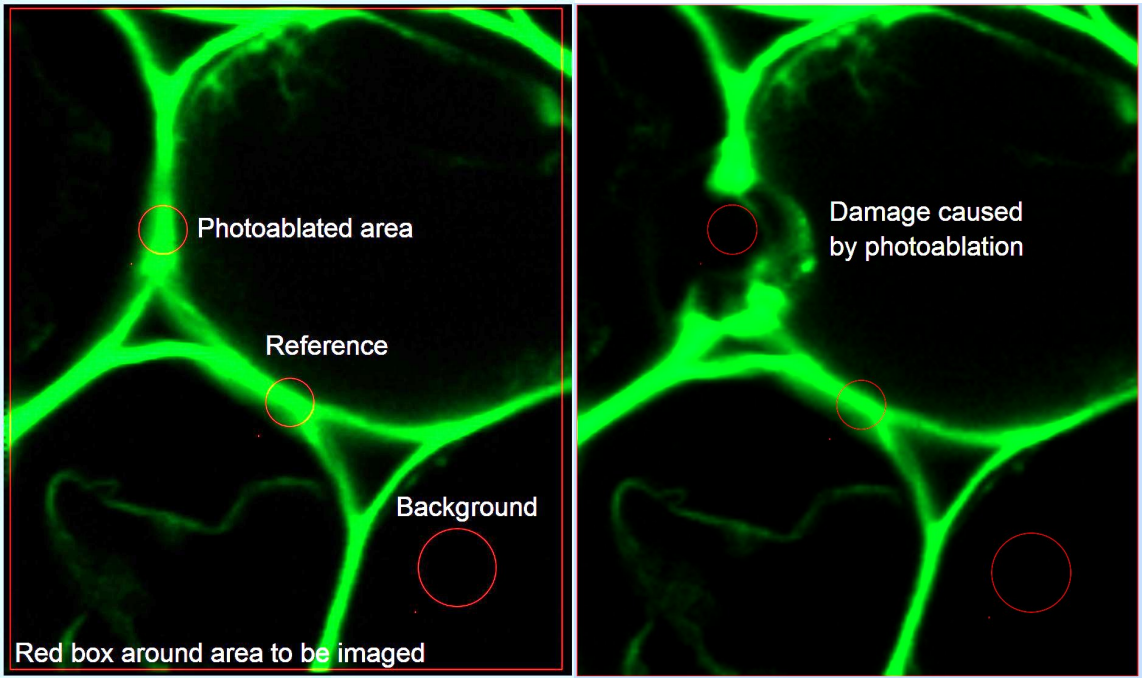
⇦ Select a square or circle.

Region to image.
Region to bleach.
Region as reference.
Region as background.

Select these two options.

Once you have drawn these regions, press ***Start Experiment***.
You should see the baseline images first (i.e., the 'before' images).
There will then be a brief pause while the two-photon laser ablates.
Finally, the 'after' images will be recorded.

The images below show the damage in *Convallaria* caused by 1 iteration using 70% laser power of the two-photon laser at 800 nm.



Troubleshooting

Cells are imaged but not ablated – Make sure you tick "Bleaching" for the target cells in ***Experiment Regions.***

Cells are imaged but not ablated – Check the two-photon laser power at the objective. When the two-photon laser is properly aligned and working well, it should produce ~3 W at the laser head at 800 nm, and approximately 200 mW at the objective/sample. If you don't get similar values, the two-photon laser may need to be aligned by the Zeiss engineer.

I need to use a larger number of iterations, and a very slow scan speed, to ablate – the two-photon laser probably isn't aligned. Contact the QBI Microscopy team.

Some images are taken but there is no ablation – The ***Duration*** value in ***Time Series*** has to be larger than the value of ***Start after # images*** in ***Timed Bleaching.*** Try 25 for ***Duration***, and 10 for ***Start after # images***.

Some damage is produced by the laser but not much - Do one or all of the following; increase the number of iterations, use a slower scan speed, increase the two-photon laser power.

Heating and CO2 Units

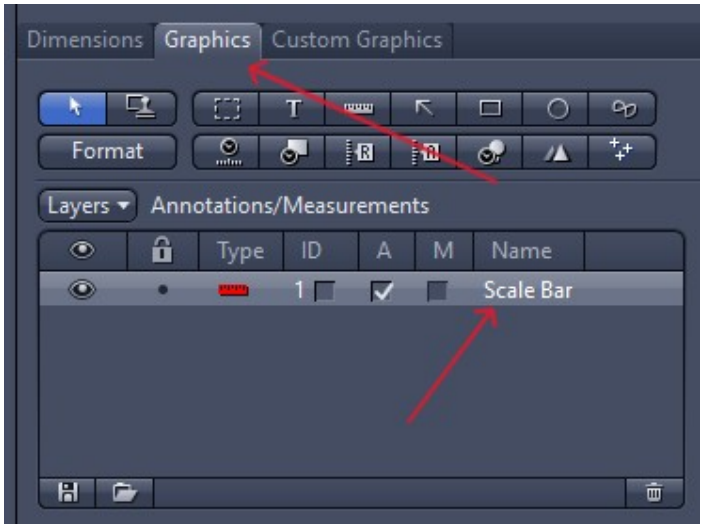
The heating and CO2 are turned on through the ***Components*** switch.
They are activated on the ***Touchpad*** under ***Incubation.***

It is recommended to pre-heat the chamber for at least 30 minutes prior to a booking, which is automatically added when incubation is selected in the PPMS booking system.

1. On the Touchpad,
   a. Select Incubation.
   b. Choose the H Insert P or H Unit XL. Set the temperature, for example, 37 °C.
   c. Choose CO2 Small V. Set the percentage, for example, 5 %.

Where is the scale bar?

Look to the left of the histogram. It will show ***Dimensions*** and ***Graphics***.
Click on ***Graphics*** and select the ***small ruler icon***.
This will show a scale bar on your image.
Click on it and edit it for size or colour.
If you save the data in CZI format, the scale bar isn't permanently added.



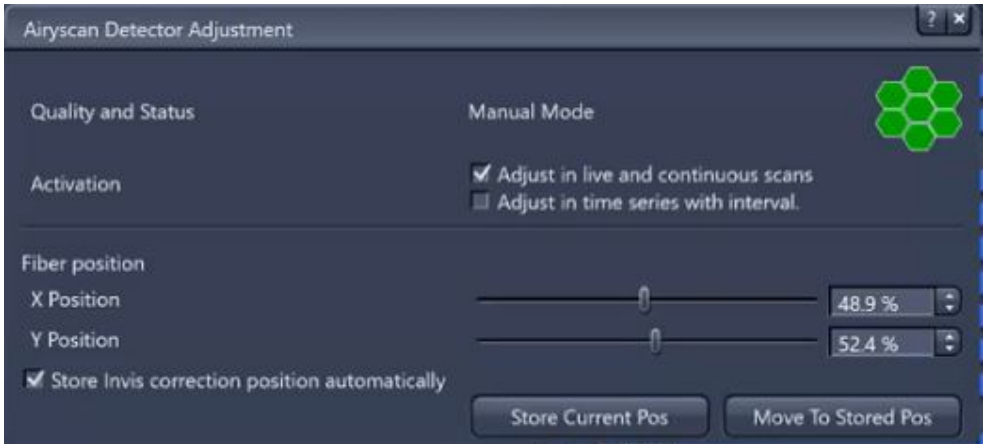
Shutting Down the Mai Tai Ti:sapphire Two-photon Laser Completely

To do a complete shutdown of the two-photon laser,

Turn off the emission in Zen.
Turn the key to OFF.
Wait 15 min for the laser to completely cool.
Turn the orange switch to OFF.
Turn the black switch to OFF (this is the chiller).
Turn off the power at the wall socket for the Mai Tai and the chiller.

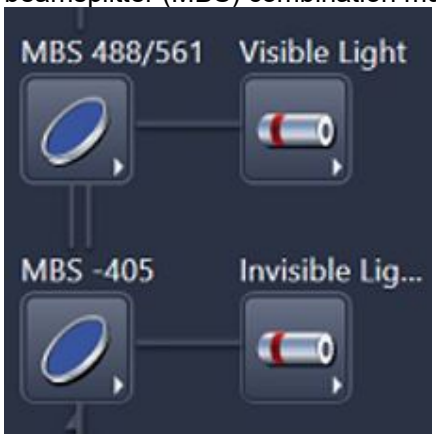
More Airyscan Calibration Instructions

1. If you're using the Airyscan detector, perform the calibration at the beginning of the session by ticking "**Adjust in live and continuous scans**" in the calibration dialogue and once the detector icon goes green, untick this box and don't worry if the icon becomes red later on, because the detector has already been calibrated.

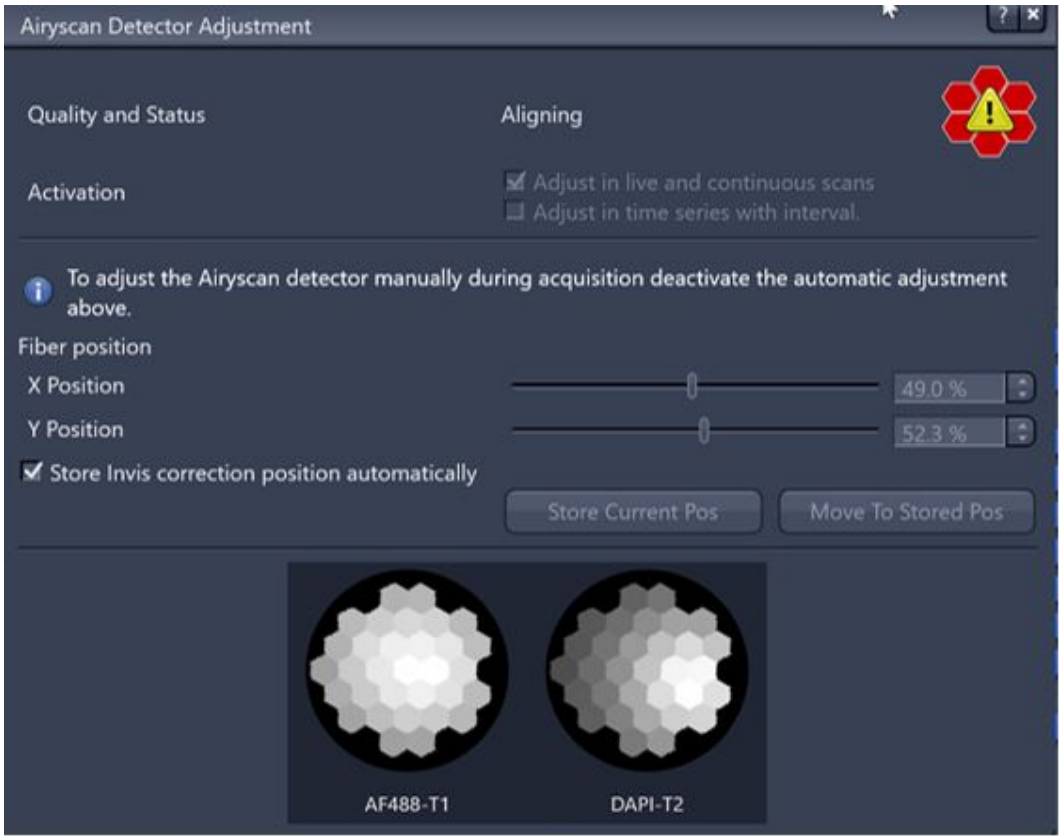


2. When performing a multi-channel experiment, use "**Continuous**" mode for the Airyscan calibration. In "**Live**" mode, the Airyscan alignment is adjusting according to the track that is active, and when toggling between tracks, the alignment in **Live** mode will overwrite the previous track. In "**Continuous**" mode, all of the tracks are taken into account.

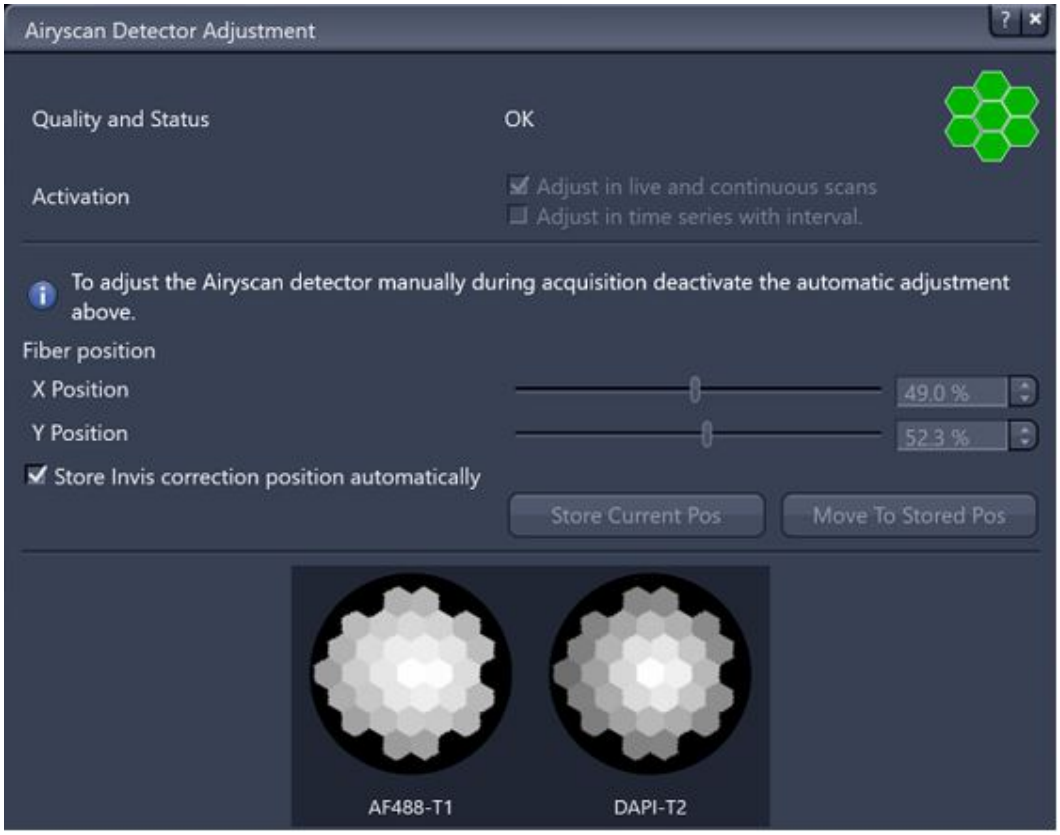
3. If using VIS tracks (639, 561, and 488 nm) in combination with INVIS tracks (405 nm, or if using the 690-1040 nm of the Ti:sapphire laser for Airyscan detection), the main beamsplitter (MBS) combination must be the same for all tracks.



Arrange the tracks such that the VIS come first and the INVIS last (which will be the case when using Smart Setup). This aligns the VIS tracks first:



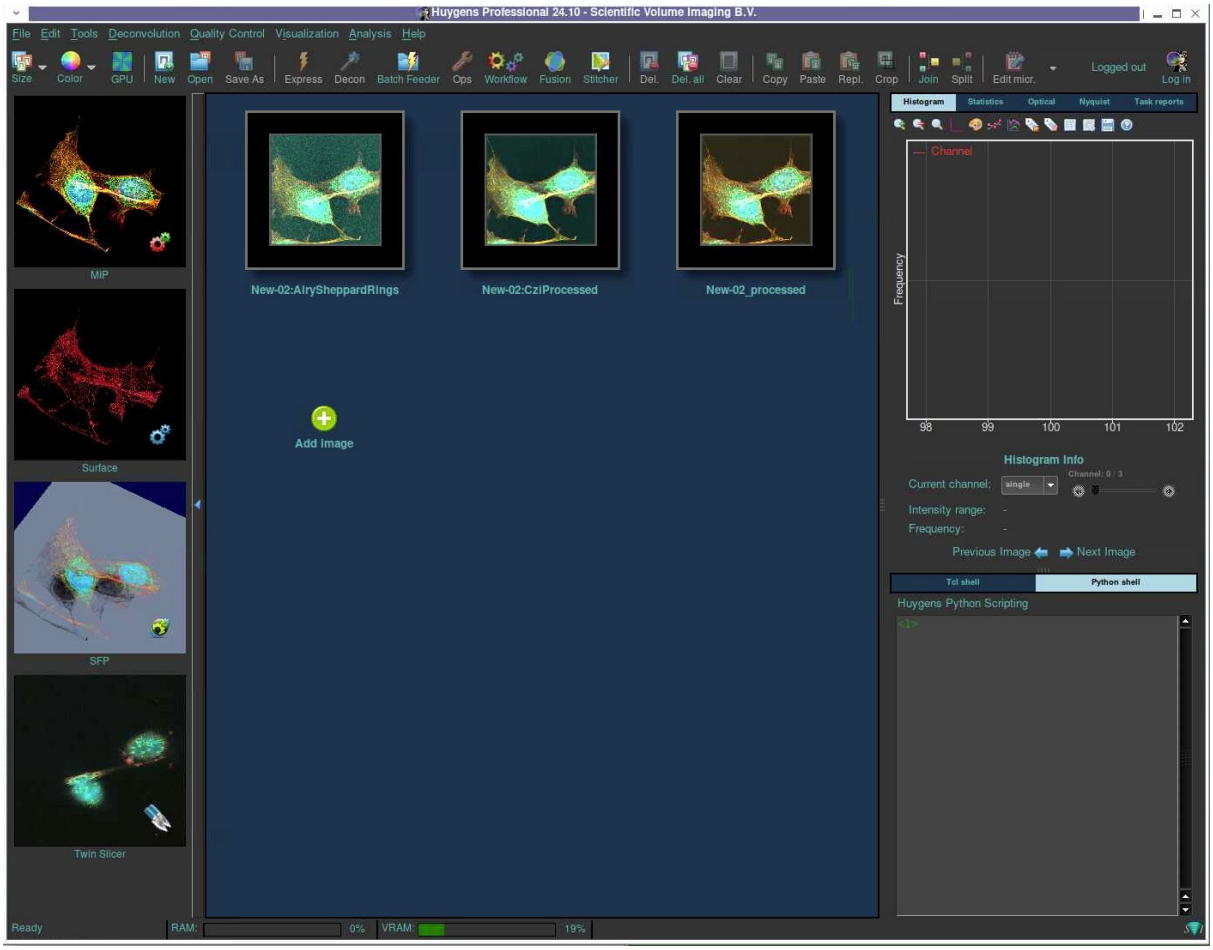
then, by adjusting the beam shift compensator, the INVIS track is aligned.

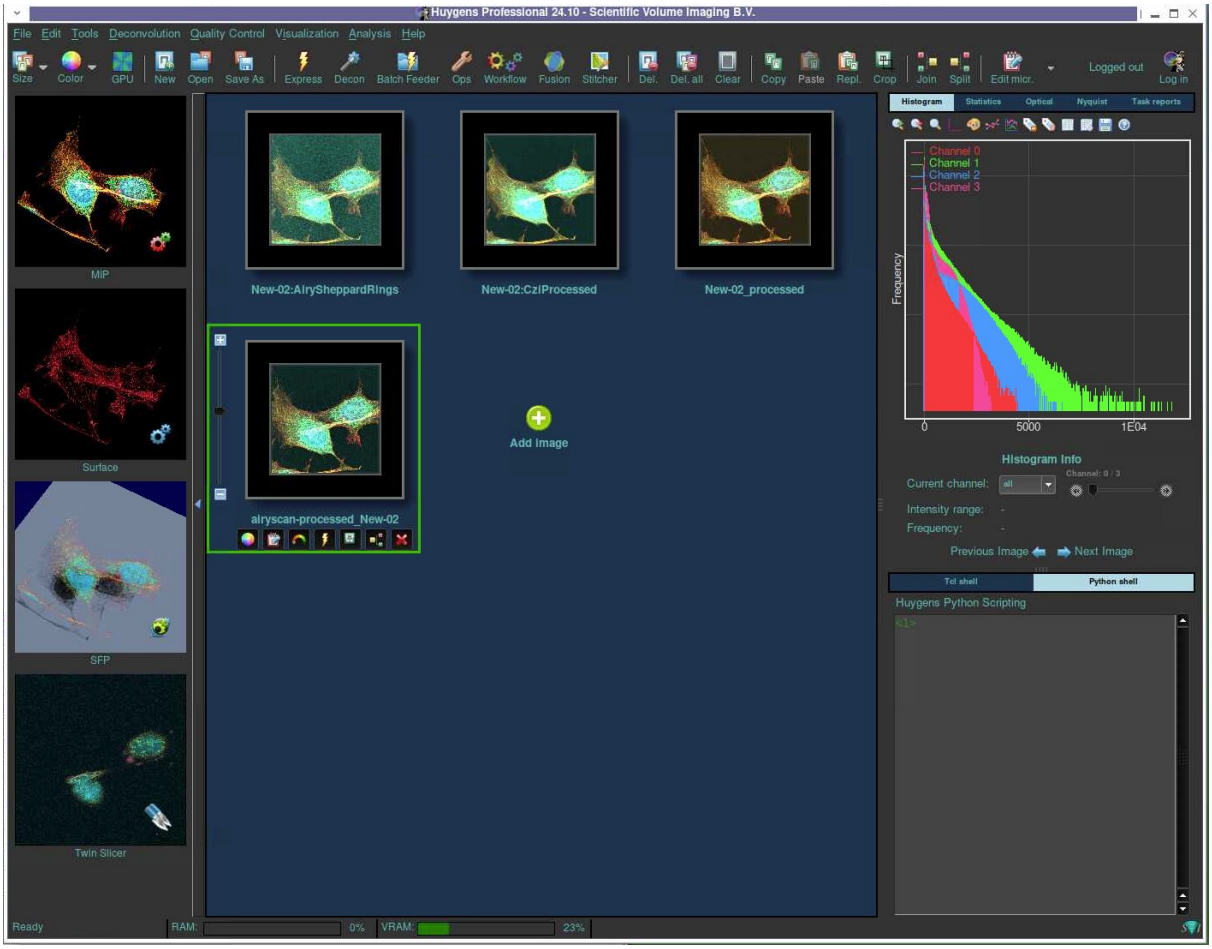


Acquiring proper Airyscan 2 raw data for deconvolution using Huygens Professional

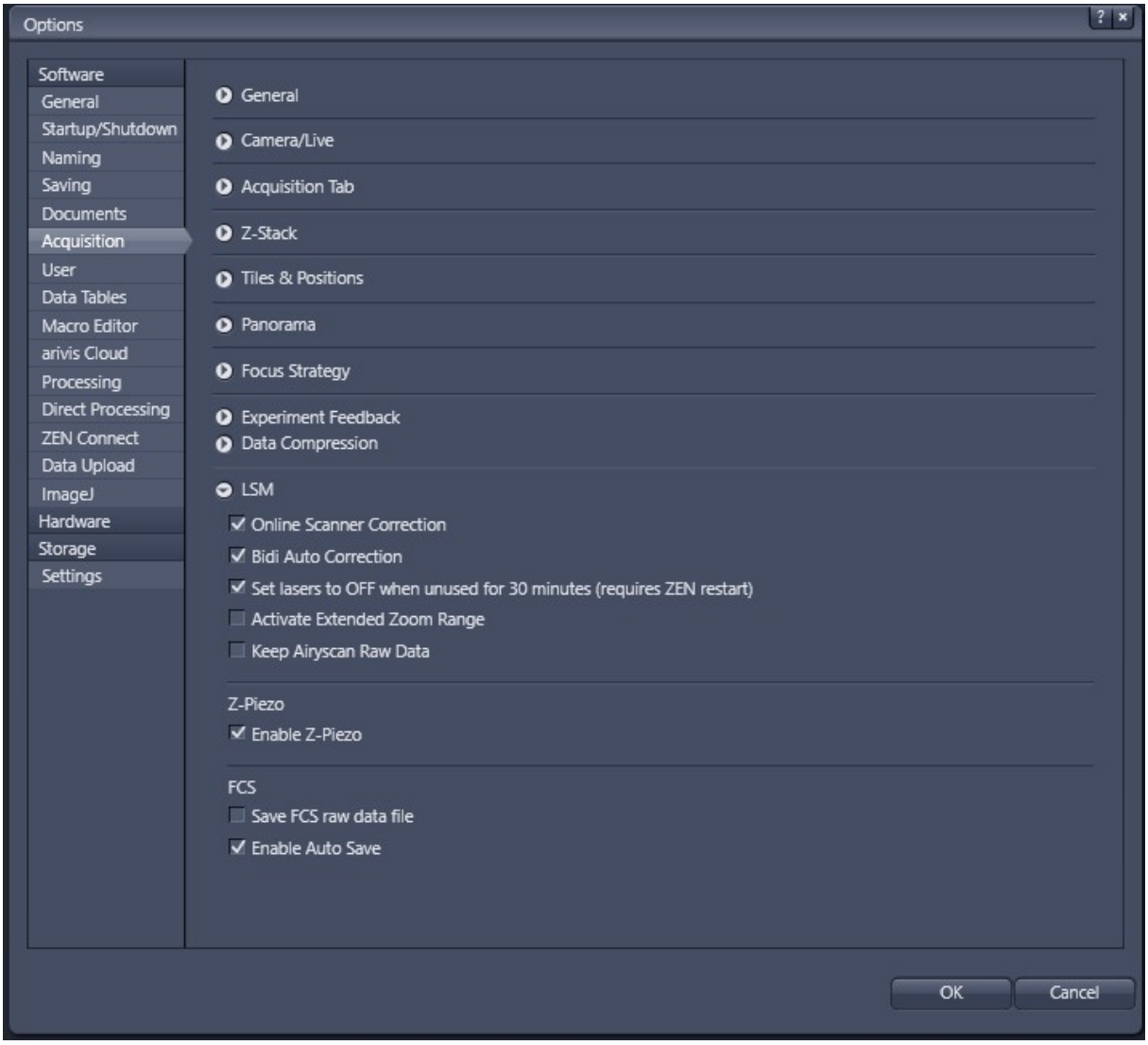
1. When you open Airyscan SR data with Huygens Professional, the software reads that it's actually the partially pre-processed 4-Sheppard Ring mode, and not the raw 32-channel data. See below. Raw 32-channel data is colour-coded in green, 4-Sheppard Ring is yellow, and Zen-processed is red. There is no green option for “Airyscan SR” data.

2. Further examples of Airyscan-processed and Airycan joint deconvolution-processed datasets when these are opened are also attached. These are treated as "normal" files and don't activate the Airyscan dialogue before opening.





3. Deconvolving Airyscan SR data using Huygens Professional does give good results, as stated by Dr Klaus Weisshart, Zeiss Global Product Manager for 3D Systems, at his Airyscan 2 presentation at QBI. However, when deconvolving Airyscan data, we recommend using the raw 32-channel data. To save the raw 32-channel data, go to Tools > Options > Acquisition in Zen, and tick the box that states, "Keep Airyscan Raw Data".



Note that some experiments would actually need the multiplex modes 4Y or 8Y, because of limitations in acquisition speed, but if you're not imaging live cells and have the luxury of taking a long acquisition, we'd recommend using Airyscan SR mode and keeping the raw 32-channel data for deconvolution and analysis.

WORKING WITH CONFOCAL AND TIRF MICROSCOPES

Ergonomics: Use of mouse and keyboard / viewing computer screen – Prolonged use of the microscope and microscope computer without breaks can increase the risk of muscular strain.

Eye strain and fatigue – Viewing samples through microscope eye piece or computer monitor over lengthy periods of time can result in eyestrain and headaches.

Exposure to sharps – Exposure to razor blades, scalpels, forceps, cover slips, glass slides could result in cuts or puncture wounds to hands or other areas of the body. Any microscope slide shards or glass debris must be disposed of in the appropriate shapes disposable bin in accordance with PC2 regulations.

Exposure to intense fluorescent and laser light – Lasers and a xenon light source are attached to this microscope and are the source of intense and potentially dangerous light. Under no circumstances should any optical elements be removed from the microscope light path or fail-safe switches be circumvented. Do not attempt to adjust the lasers, laser light path, or laser modules in any way. Avoid direct exposure to the light.

Scope

This procedure details the method for using the microscopes equipped with laser light sources.

Safety Considerations

Personal Protective Equipment (PPE):

Laboratory coat, **latex gloves** and **closed in shoes** should be worn to prevent injury.

Ergonomics and Risk Exposure:

Appropriate ergonomics, including adjustment of the seat, computer screen and microscope oculars should be undertaken to reduce risk of strain injuries.

Emergency Procedures:

First aid may be required for:

Exposure to sharps – Contact the nearest first aid officer from the list that is beside all first aid kits and on safety notice board.

Exposure to intense fluorescent and laser light – Seek immediate medical assistance if you have been exposed to intense direct light or laser light.

In the event of a laser accident, do the following:

1. **Shut down the laser system.**
2. **Provide for the safety of personnel (first aid, evacuation, etc). If needed, provide further medical assistance for Eye Injuries by:**
   Proceed directly to: **Royal Brisbane and Women's Hospital at**
   Cnr Butterfield St and Bowen Bridge Rd
   HERSTON, QUEENSLAND AUSTRALIA 4029
   (07) 3636 8222

   Note: If a laser eye injury is suspected, have the injured person keep still and looking straight up to restrict bleeding in the eye. Laser eye injuries should be evaluated by a physician as soon as possible.
3. **Contact UQ Security Emergency on 336 5333.**
4. **Inform QBI's Laser Safety Officer, Rumelo Amor on 04 4907 8485, of the accident as soon as possible.**
5. **A UQ online incident report must be completed as soon as possible after the incident.**

All incidents must be reported to the OH&S Manager and on UQs online incident reporting system.

Contacts: Security x53333 or OH&S Manager Ross Dixon 0401 673 654