Zeiss LSM 900 Confocal Training

Neuroscience Imaging Facility
The Ohio State University
Rightmire Hall
614-292-1367

Neuroscience Imaging Core

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Access

Access to the Confocal Microscope

	Supervised	Unsupervised		
	Mon-Thurs 9a-5p	Mon-Fri 9a-5p	Evenings and Weekends	Required Experience
Trainee	X			Seminar & minimum of 2 supervised sessions. Advancement decided on case by case basis
Standard user		X		Seminar & minimum of 10 unsupervised sessions. Advancement decided on case by case basis
Experienced user		X	X	Advancement decided on case by case basis

Access to the room

–Key card access. Contact Paula for access.

Scheduling time

–Use Google Calendar. Provide a gmail address to Paula for access.

Contact numbers

Question about how to use the microscope, i.e. software questions
- –Contact Paula 614-292-1367 9a-5p, M-Th
- –Zeiss Hotline 800-233-2343 10a-6p, M-F

Physical problem with microscope, i.e., it's broken
- –Contact Paula 614-292-1367 9a-5p, M-Th

Problem after hours
- –Contact Paula 614-270-3239

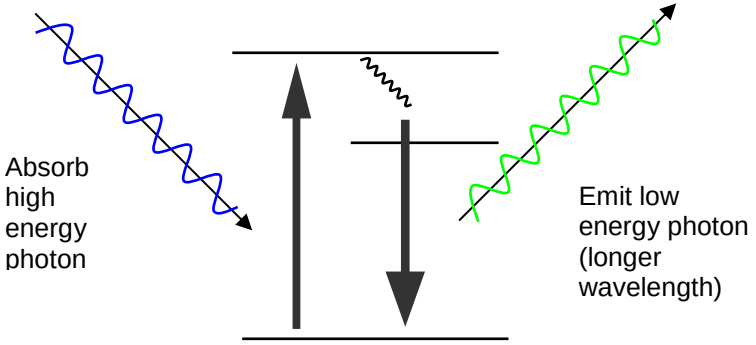
Computer

Data storage
- –Archive to an external hard drive USB ports, including USB 3.0

Users
- –All users will have a user name and password
  - User name – PI name + lab (eg, "McKinney lab")
  - Password – last five digits of the lab phone number

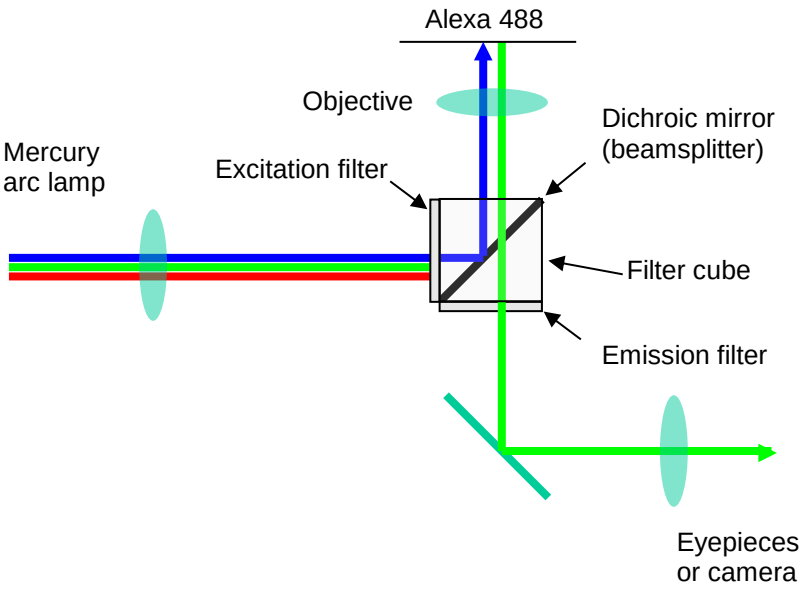
Rules
- – All files must be saved to the user files on the D drive, files stored elsewhere will be deleted
- – Files saved to the D drive will be deleted after one week

What is fluorescence?



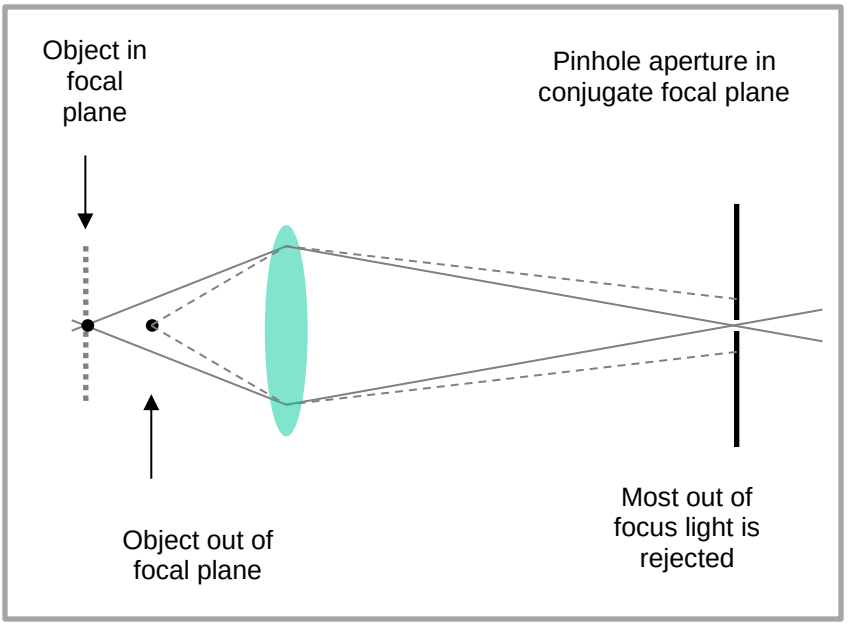
Excite with high energy photon (shorter wavelength)
Emit lower energy photon (higher wavelength)

How does a fluorescence microscope work?



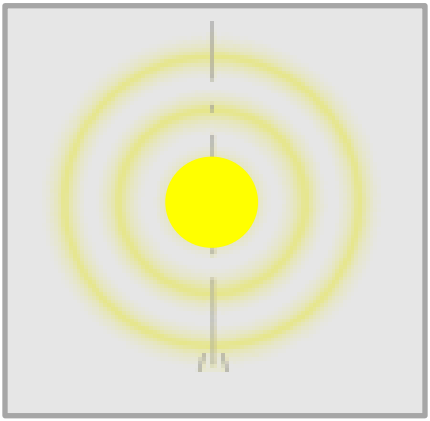
The principal behind confocal microscopy:

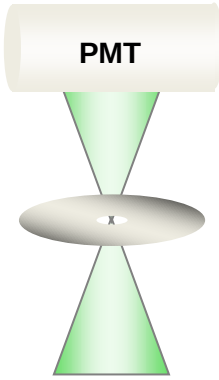
Rejection of out of focus light via the pinhole allows us to capture optical sections.



Limits of Pinhole Rejection

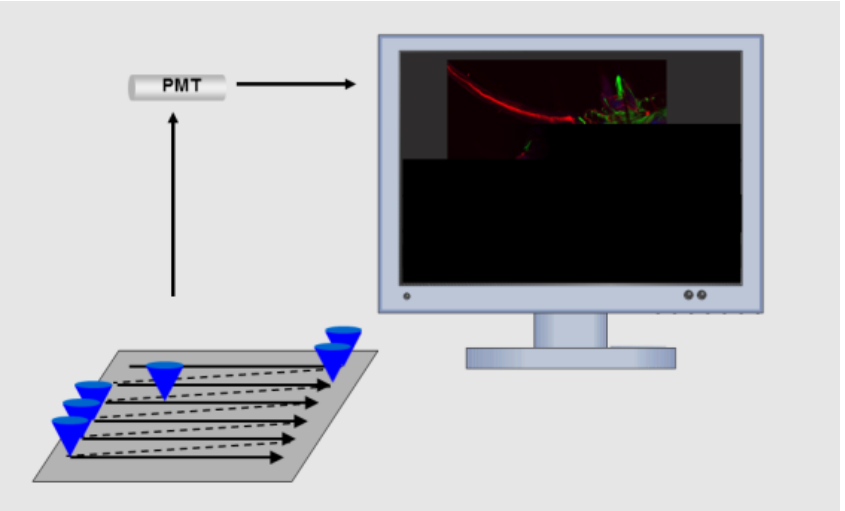
The size of the pinhole is a compromise between thin optical sections and reasonable signal levels.





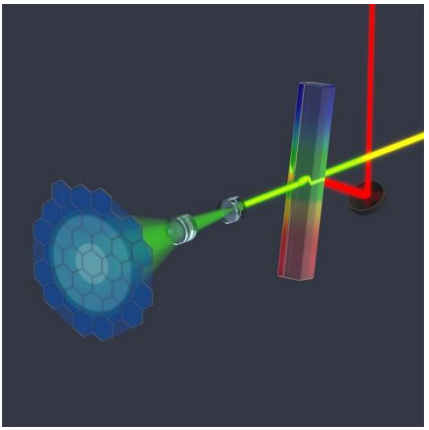
Laser Scanning Microscopy

The laser scans across the sample in a raster pattern, building the image pixel by pixel.



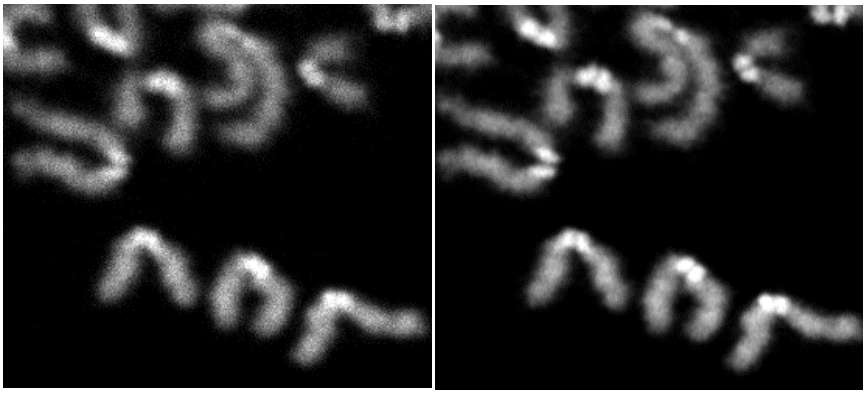
The Airyscan Principle

Instead of rejecting light at the pinhole, a 32-channel detector collects all light of an Airy pattern. During post-processing, the light in the outer rings of the Airy pattern can be reassigned to the center point.

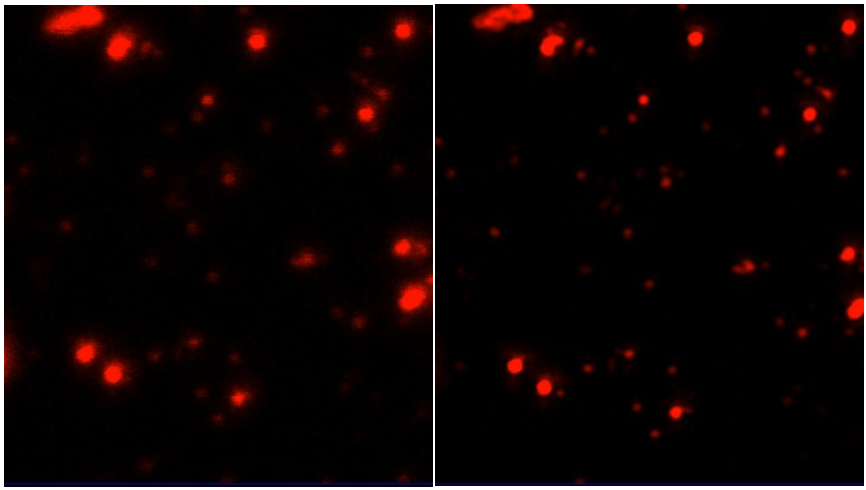


Benefits of Airyscan

Airyscan can provide up to a 1.7 fold increase in resolution, down to 120nm, as compared to ~250nm for conventional confocal.

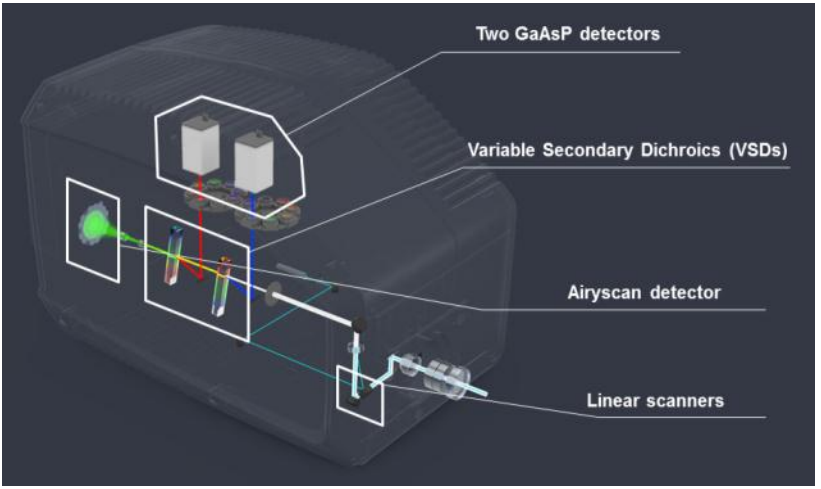


Airyscan can improve the signal to noise (SNR) or contrast in an image. Improvements in SNR make it easier to see dim objects and make the background appear darker.



Inside the LSM 900

Our LSM 900 has 3 detectors – two GaAsP and one Airyscan. The Variable Secondary Dichroics (VSDs) allow us to direct the light to any detector, giving us a lot of flexibility in imaging.



Choices

So many choices

Image Resolution	Switch Mode	Speed	Resolution & Sensitivity	GaAsP 1	Airyscan	GaAsP 2
Confocal	Sequential	++	++	Blue, Green, Red		
Confocal	Semi-Sequential	++++	++	Blue & Red		Green
Confocal	Simultaneous	++++++	++	Blue	Green	Red
SR	Sequential	+	++++		Blue, Green, Red	
SR + Confocal	Sequential	+	++++/++	Blue	Green, Red	

The Microscope

Why use an inverted microscope?

Benefits

-More flexibility, can image both open and closed chambers
-Better suited to imaging living cells than upright microscope
-Can image live cells and organisms in open dishes using high NA objectives
-Can accommodate a variety of different chambers

Cautions

-Location of objective makes it more possible for liquids (oil, water, media, etc) to find their way inside the objective, possibly rendering it unusable
-More care must be taken using the immersion objectives
-Specimens on slides are inverted to take advantage of the best optics and must be sealed to prevent leakage
-More difficult to apply oil when specimen is in place

Objectives

Objective specifications						
Mag	2.5x	10x	20x	40x Water	63x Water	63x Oil
Numerical aperture	0.085	0.3	0.8	1.1	1.2	1.4
Correction	EC Plan Neofluar	EC Plan Neofluar	Plan Apochromat	LD C-Apochromat	C-Apochromat	Plan Apochromat
Working distance	8.8 mm	5.2 mm	0.55 mm	0.62 mm	0.28 mm	0.19 mm
Resolution (520 nm)	1 µm	1.1 µm	0.40 µm	0.29 µm	0.26 µm	0.23 µm

The water immersion objectives have a correction collar for coverslip thickness. A #1.5 coverslip is nominally 0.17mm, but the range of acceptable thickness is 0.16-0.19mm. It is possible to compensate for this variation in thickness empirically.

PMT

Understanding Objectives

Quality

-Objectives are highest quality = expensive
-Image is only as good as the objective

Numerical Aperture (NA)

Measure of the width of the cone of light that the objective can gather

-High NA objectives have:
- •High resolution
- •Brighter image
- •Shorter working distance

-NA > 1.0 – generally are immersion objectives

Care of Objectives

DO NOT REMOVE OBJECTIVES!

Immersion objectives

-Use the CORRECT IMMERSION FLUID
-Use oil on the 63x Oil objective
-Use water on the 63x Water and the 40x Water objectives

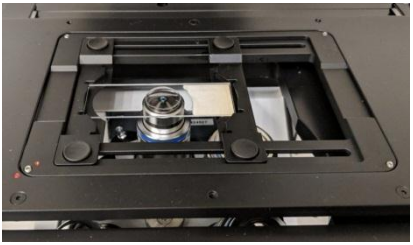
Using Immersion fluid

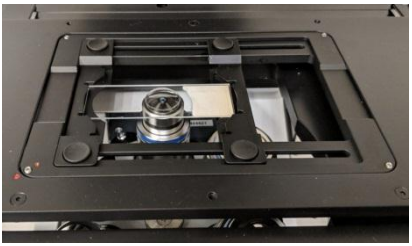
-Use what is supplied in the room – do not mix oils
-Use minimum amount necessary
-Clean slides after use
-Use only lens tissue to clean objectives – blot, don't rub
-Always clean objective after use with oil
-Don't drag dry objectives through the oil on the slide

Specimen Mounting

Fixed Specimen

-Coverslip #1.5 ~0.17mm
-Permanent mounting media
-Mounting media that does not dry should be sealed with nail polish

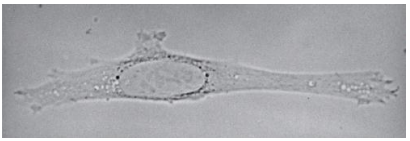




Brightfield & DIC

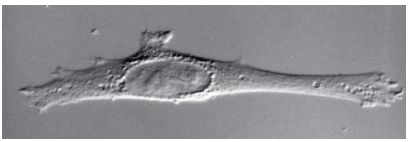
Brightfield

-Available with all objectives
-Very low contrast



DIC

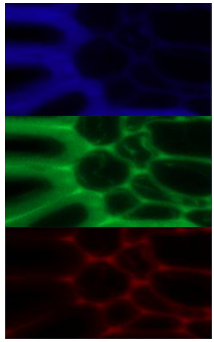
-Available with 20x dry, 40x water, 63x water, and 63x oil objectives
-Reveals gradients in refractive index, so the resulting image tends to show edges
-Generates low contrast
-Better for overlaying with fluorescent images than brightfield



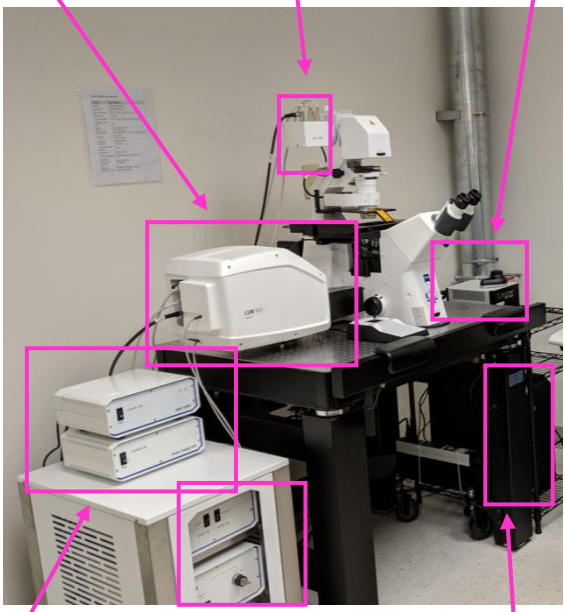
Molecular Biology of the Cell 5/e

Epifluorescence Filter Cubes

Filter Cube	Example Fluorochromes
Blue	Alexa 405, DAPI
Green	Alexa 488, Cy2, Fluoroscein
Red	Alexa 568, Alexa 546, Cy3, Rhodamine



The Confocal Microscope and Workstation Layout



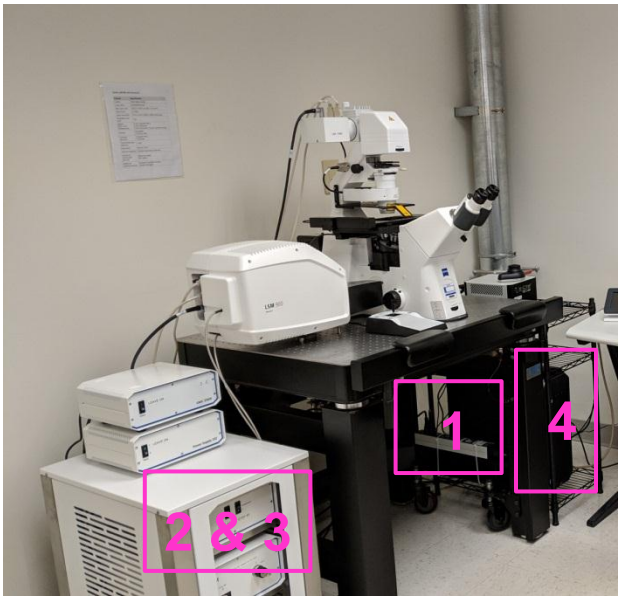
Lasers

Our system has 4 laser lines, allowing us to excite a wide range of fluorochromes.

Laser line	Example fluorochromes
405	Alexa 405, DAPI
488	Alexa 488, Cy2, Fluoroscein
561	Alexa 568,Alexa 546, Cy3, Rhodamine
640	Alexa 647, Cy5, TOTO-3, TO-PRO-3

Getting Started

Start up in numerical order. All of the switches are labeled.



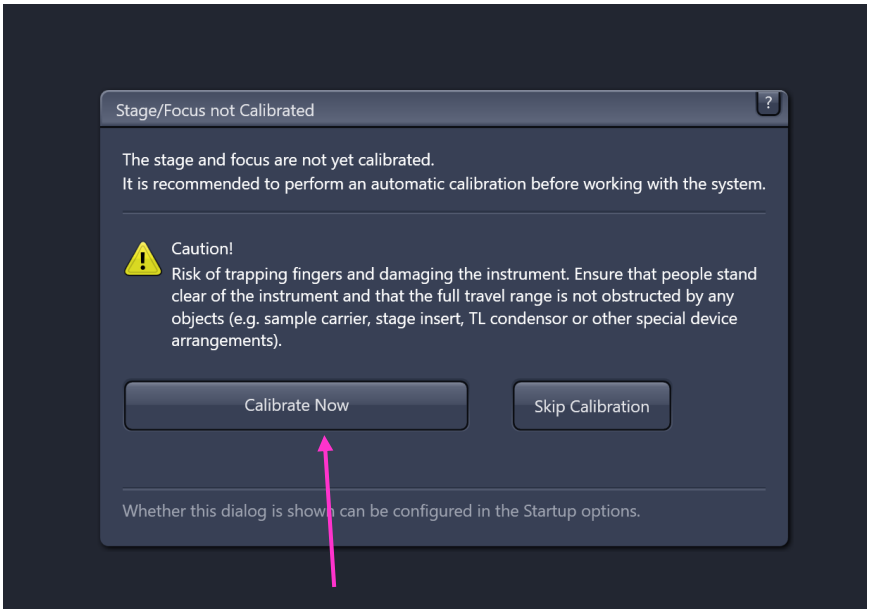
Start the Zeiss Confocal Software

Double click on the Zen 3.0 icon.

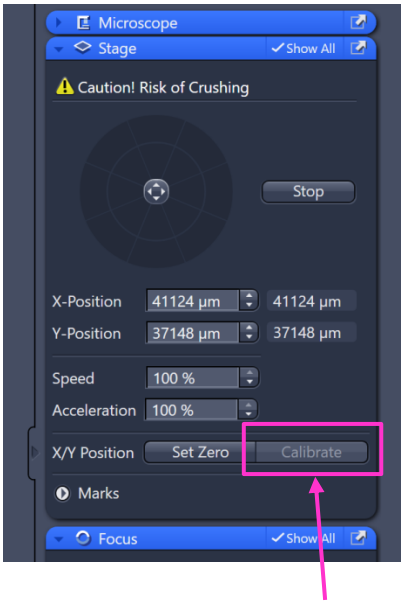
Software Startup

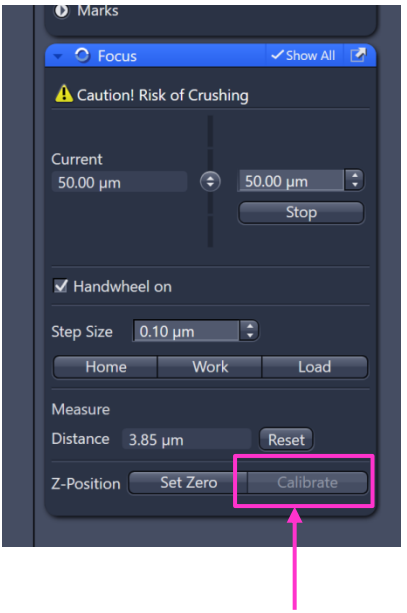
Stage and Focus Calibration

The system will ask to calibrate the stage and the focus upon software startup. This should be done BEFORE you put a sample on the stage. The objectives will move up and down. The stage will move left /right and front/back. If this step is skipped, the software may not be able to remember stage or focus positions.



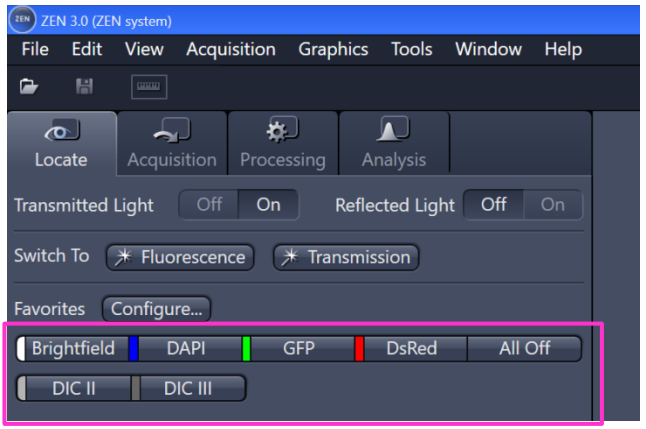
When the stage and focus are calibrated, the “Calibrate” buttons will be greyed out.





The Locate Tab

The Locate tab contains presets for viewing your sample thru the eyepieces. You can shutter the light and change filter cubes here.

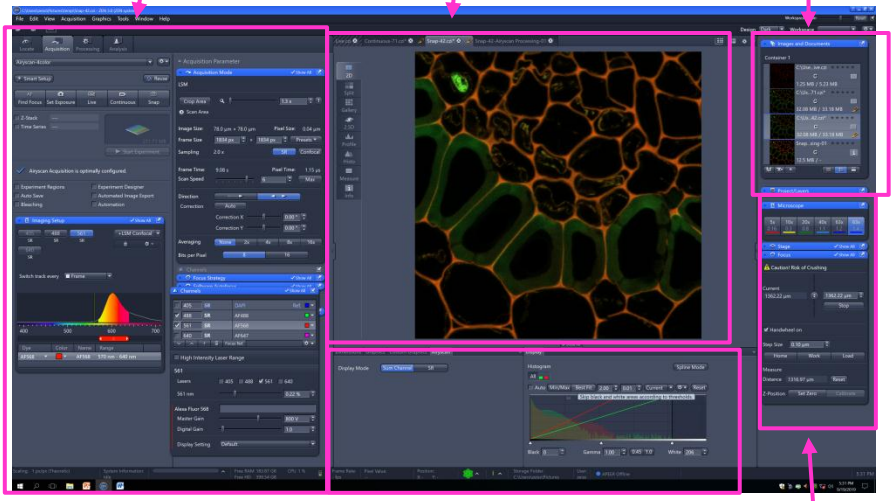


Acquisition Tab

Image Capture Settings

Preview Window

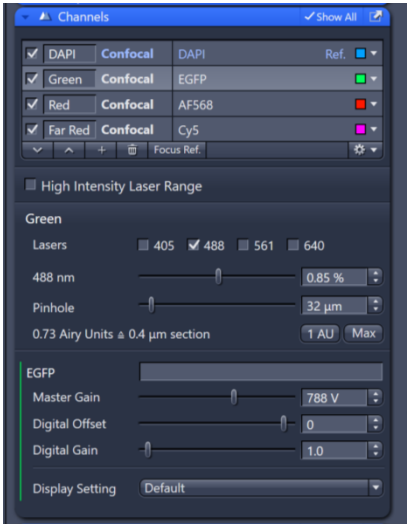
File Manager



Display Controls

Microscope Controls

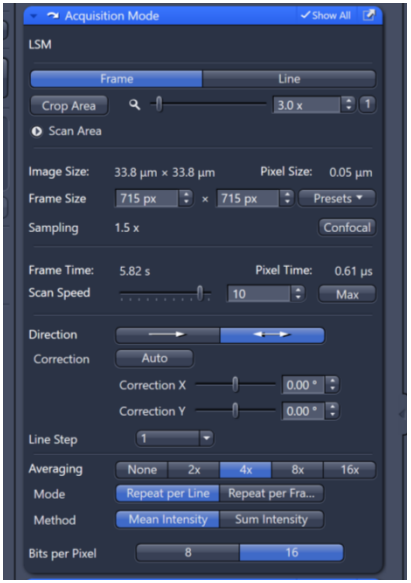
Image Capture Settings - Channels



Choose which channels to capture.

Adjust the laser power, master gain, digital offset and pinhole size.

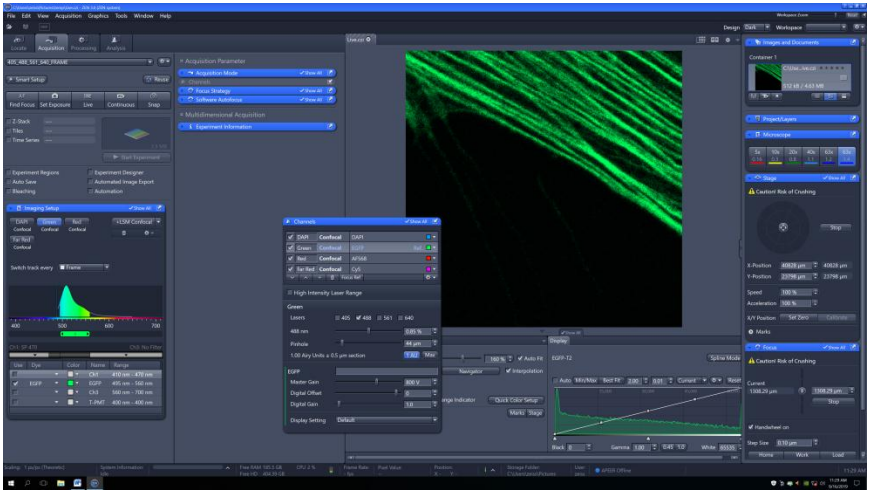
Image Capture Settings – Acquisition Mode



Here is where you can set the imaging parameters – zoom, number of pixels, scan speed, averaging, and bits per pixel.

Flexible Layout

Zen has a flexible layout. Dialogs can be “popped” out and moved around. Whole sections of the software can be hidden. The entire workspace can be zoomed in or out.



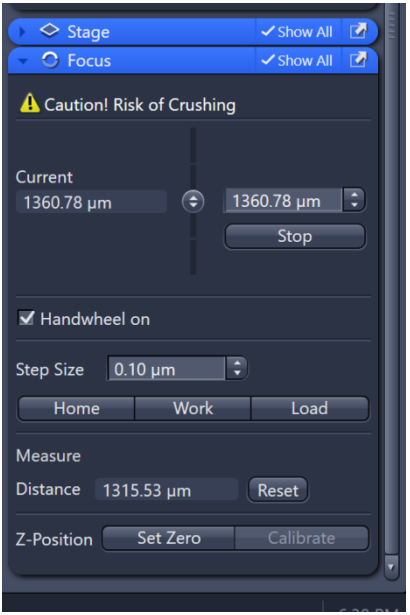
Microscope Controls - Objectives

The objectives can be changed from the software. Upon selecting an objective button, the objectives will lower, change position, and raise up to the working position.

Microscope Controls - Stage

The stage can be moved in this menu. This is also where stage positions can be memorized and revisited.

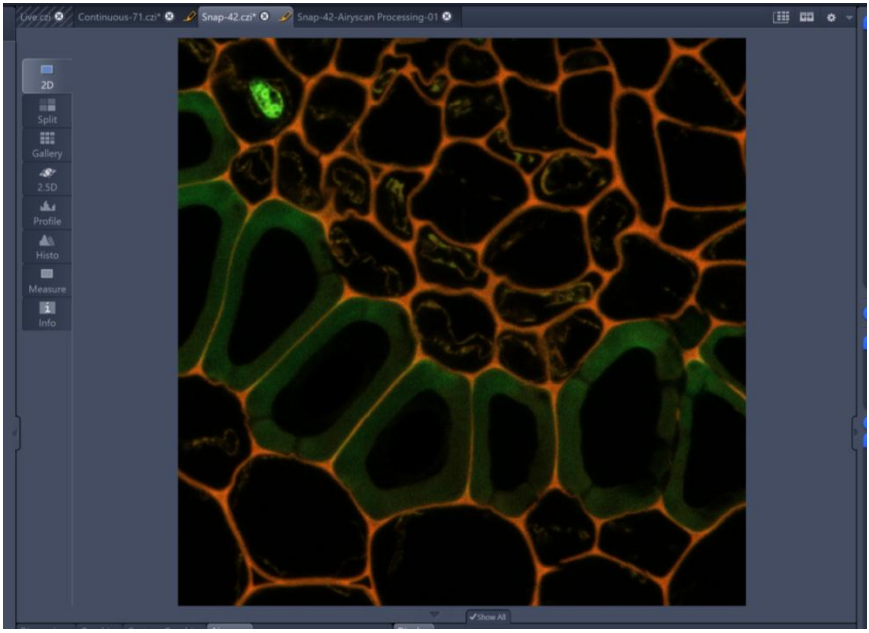
Microscope Controls - Focus



The focus can be changed in the Focus dialog. The objectives can be completely lowered with the “Load” button

Preview Window

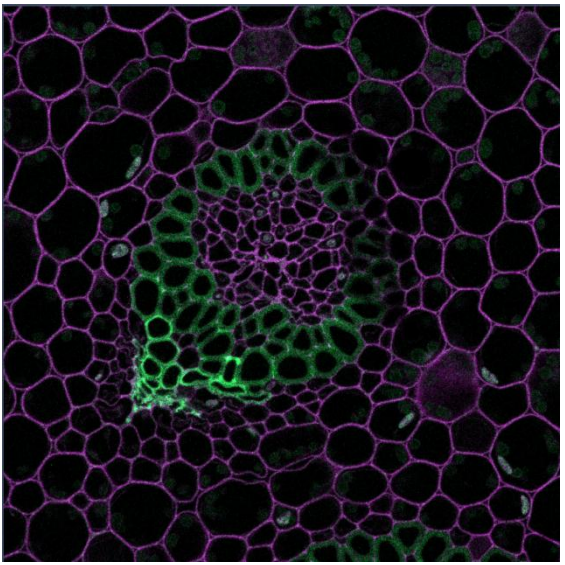
Your images show up in this window. Tabs on the side of window show different views, including single channels, orthogonal views, and image information.



Display Settings

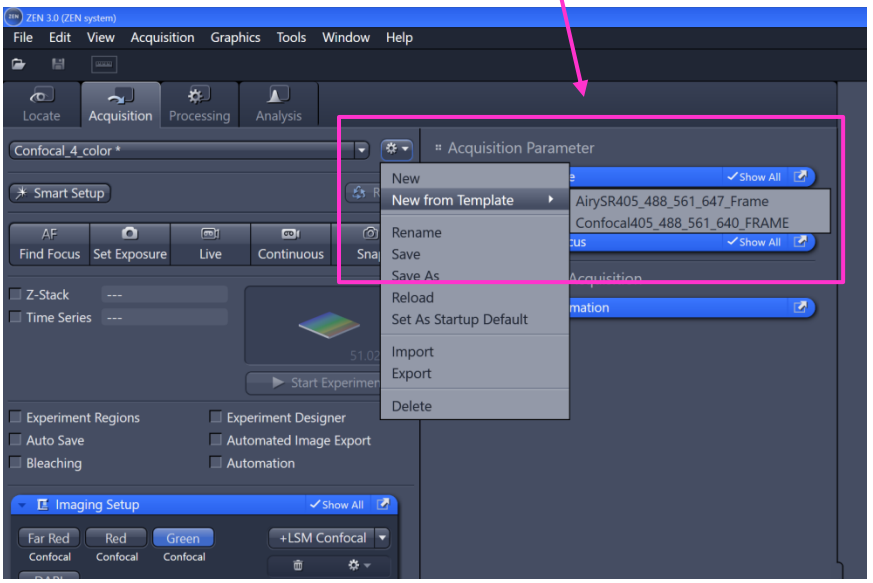
The histogram of pixel intensities is located here. You can change how the image looks on the screen – brightness, zoom, z-slices.

Capture a Confocal Image



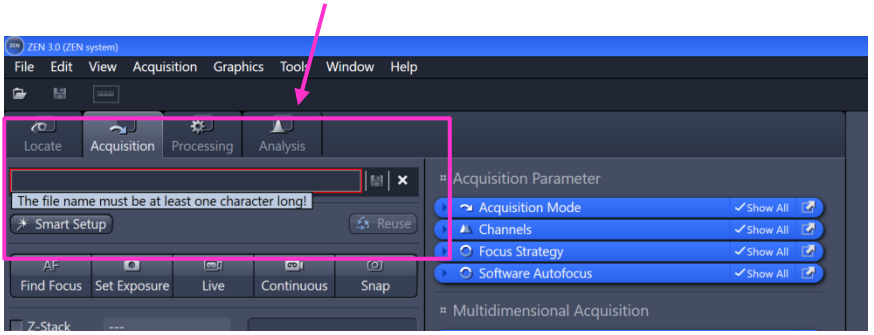
Load settings from template

Choose the gear icon and “New from Template”. Load the “Confocal405_488_561_640_Frame” setting.

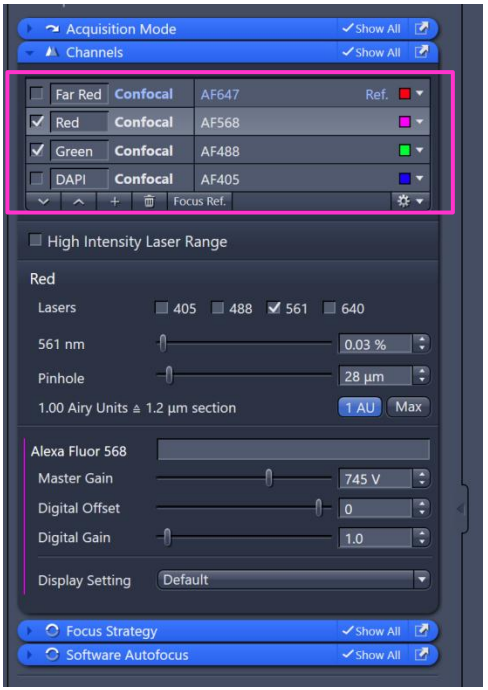


You will need to save the setting with a new name. This will only show up under your login.

If you have previous images with settings that you want to use again, you can load the image and choose the “Reuse” button.



Select channels to image



Open the “Channels” dialog and check the boxes next to the fluorochromes you want to image. I’ve chosen red and green here.

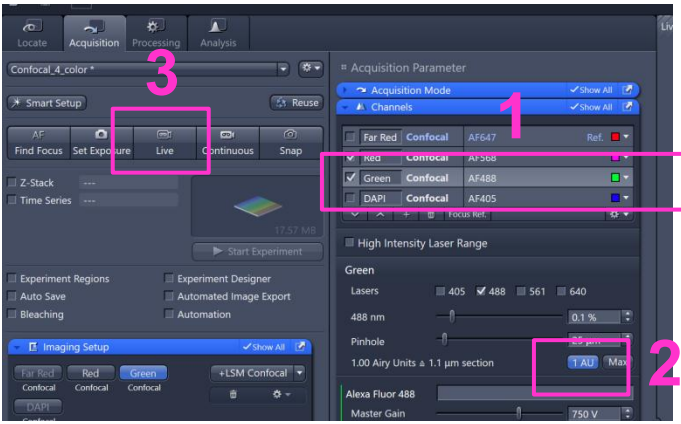
Optimize the channel settings

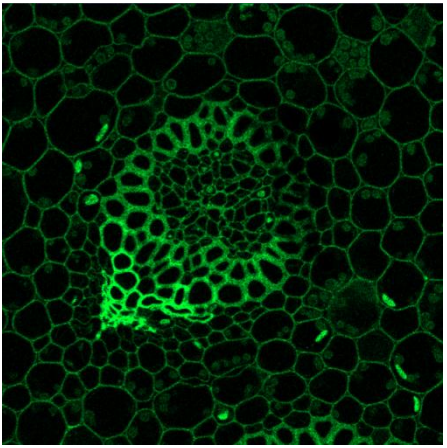
You will need to adjust the gain and laser power for each channel. To start:

1 – Choose a channel. Green is selected here. It appears as a lighter grey than the other channels.

2 – Choose the “1 AU” button. This will set the pinhole size to 1 Airy Unit for optimal confocality. If the button is blue, that setting will be maintained until you change it.

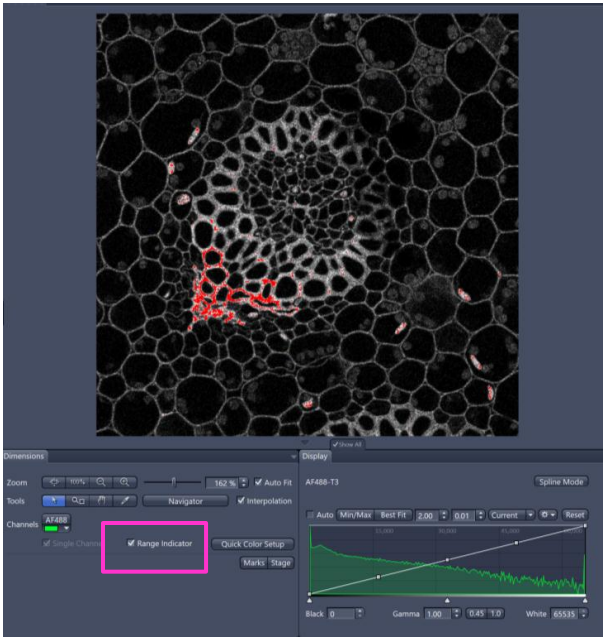
3 – Choose the “Live” button. This will scan with the selected channel (green in this case) with a 512x512 pixel resolution and at the highest scan speed.





The live image. It will be in the same orientation as thru the eyepieces.

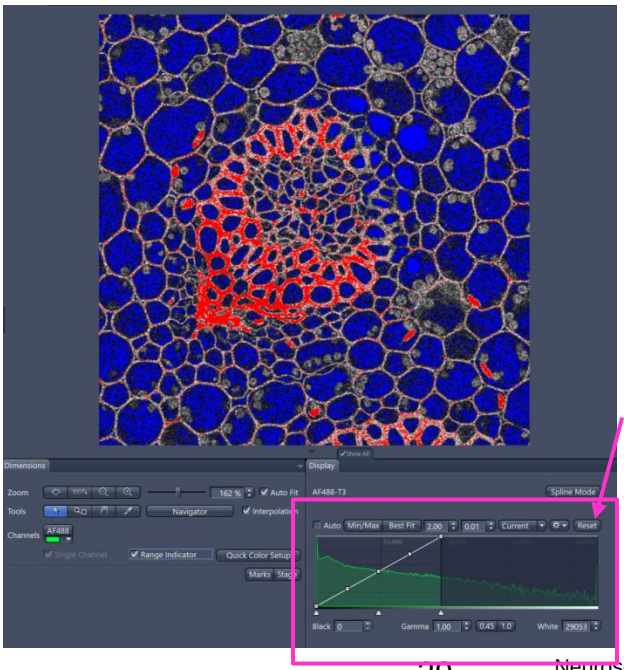
Range Indicator



Click the “Range Indicator” checkbox to change the display so that pixels of maximum value are red and pixels of minimum value are blue.

A good target is to use the full range – have a few pixels of blue and and few of red.

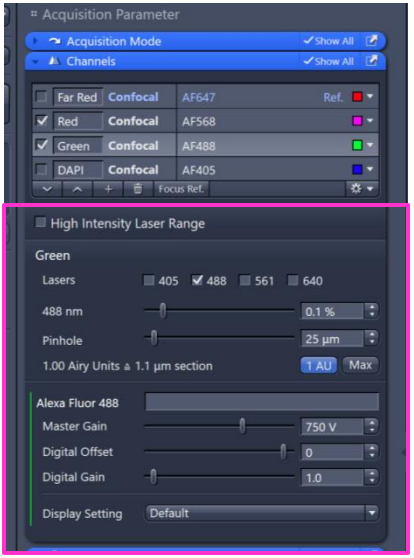
If you will be measuring pixel intensities, there should be no red pixels in areas that will be analyzed.



!!! Be careful !!!

Make sure to choose the “Reset” button to display the full range. Adjusting the brightness will give a false sense of saturation!

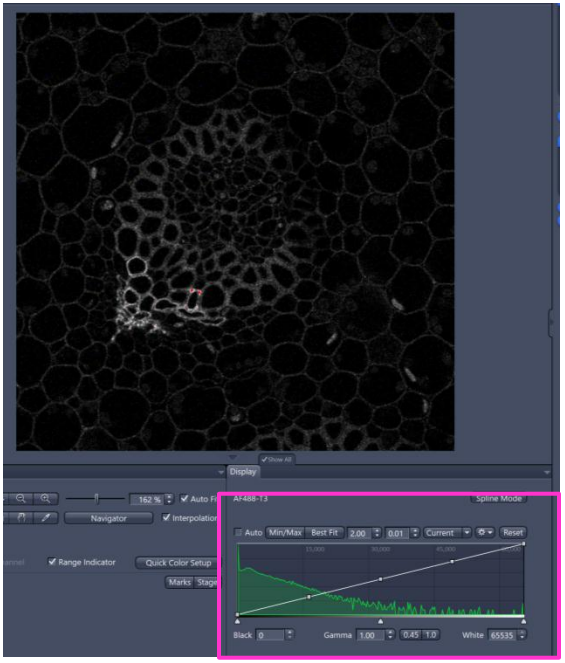
Adjust the Master Gain and Laser Power



Adjust the Master Gain up to 800, then adjust the laser power so that histogram fills up the full 16 bit range and there are a few red pixels in the “Range Indicator” view.

If you will be measuring pixel intensities, there should no red pixels.

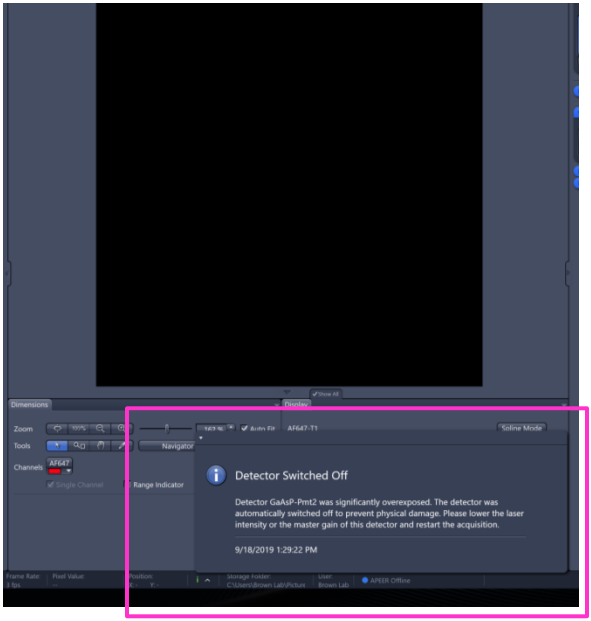
If you need more laser power, click the “High Intensity Laser Range”. This allows you to use higher laser powers. It changes ALL the channels to the higher laser range.



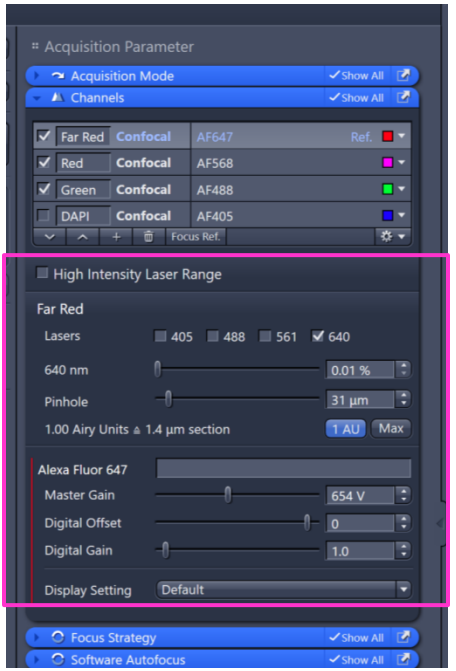
The adjusted image. The histogram is filling the range without a peak at the maximum values (right hand side).

Repeat these steps for all the channels.

Detectors are Sensitive!



The detectors can be damaged by high intensity light. They will shut off if the brightness of you sample exceeds a certain range. The image will be black and there will be a message that the detectors are switched off.



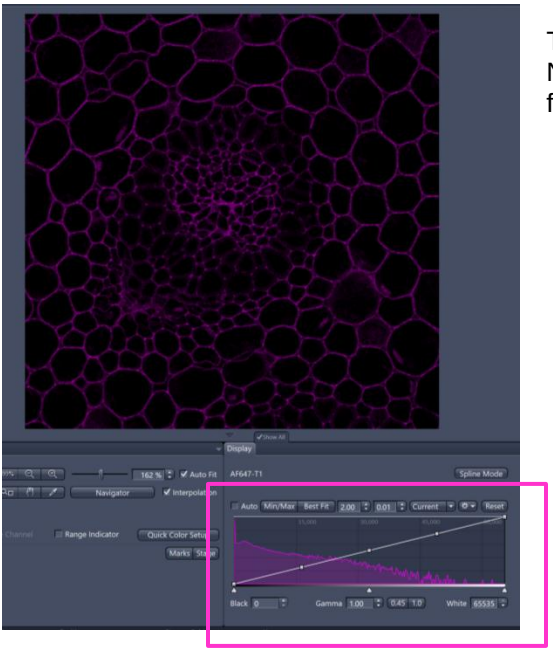
To adjust the setting so that the image is not too bright:

1. Stop the scanning.
2. Reduce the Master Gain and Laser Power to minimum levels
3. Choose Live to start scanning

While watching the histogram:

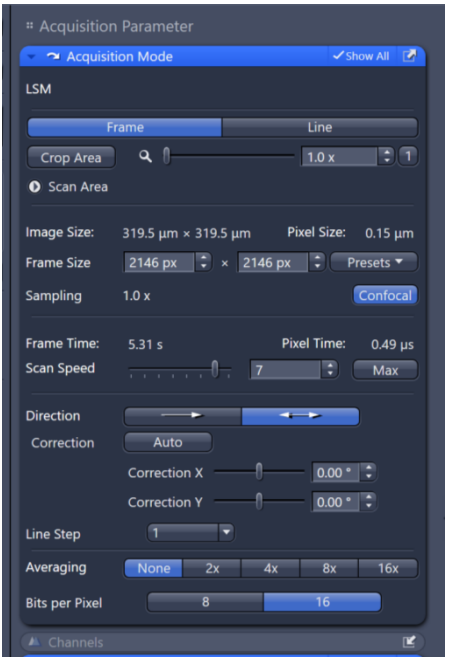
4. Slowly bring up the Master Gain (up to 800) and adjust the Laser Power

so the histogram is full and there are a few red pixels and a few blue pixels in the Range Indicator image.



The adjusted image. Notice how the histogram fills the full range.

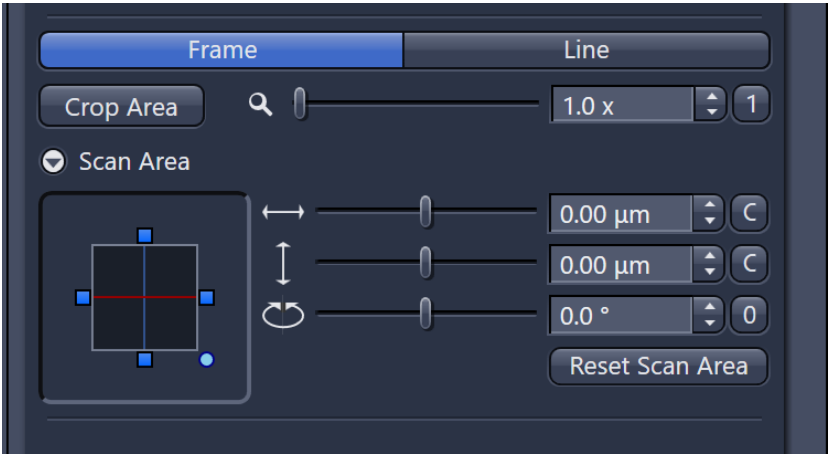
Acquisition Mode



These are the scanning parameters for your image.

Scan Area

Choosing “Scan Area” allows you to rotate the scanning field. “Crop Area” is zoom. Choose the “1” button to go back to a zoom of 1.



Averaging and Bits per Pixel

You can choose to average several frames or several lines. This will result in a smoother looking image.

The detectors are monochrome detectors. The confocal settings default to 16 bits per pixel. This is the number of levels of grey. 8-bit images measure black to white as 0-255 and 16-bit images measure the same black to white as 0-65,535. A 16-bit will give you more control over brightness & contrast. If you are measuring intensities, a 16-bit image will allow you measure smaller differences. 16-bit images use a little more hard drive space, but are worth it.

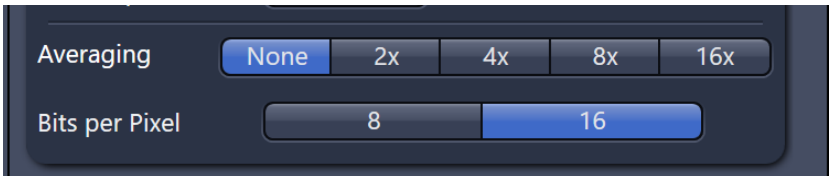
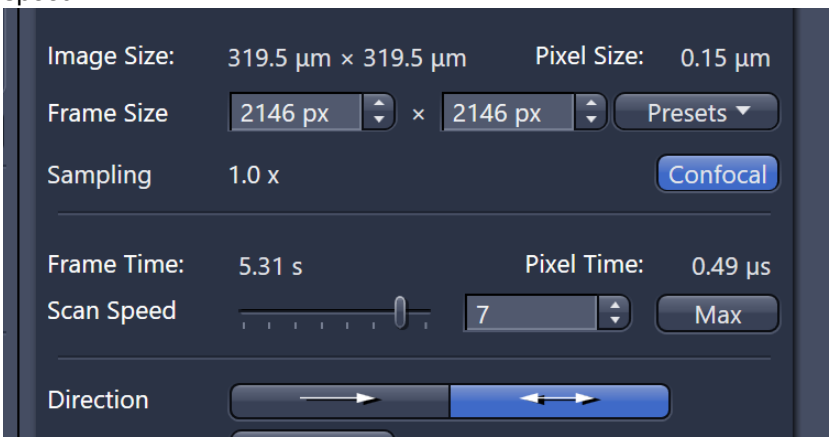


Image Size, Scan Speed & Direction

You can choose the number of pixels in your image. There are presets (up to 6144x6144), but you are not limited to those. Selecting the “Confocal” button will fill in a frame size with optimal pixel sampling for confocal resolution.

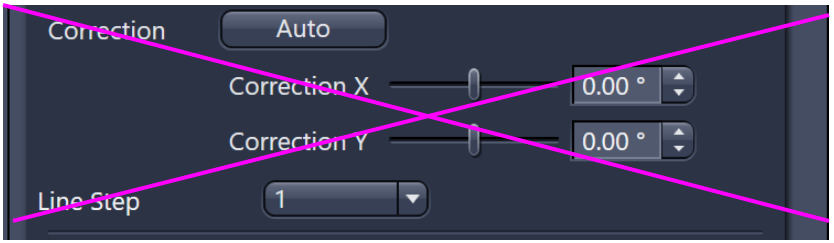
You can change the Scan Speed. It will indicate how long a frame will take. Reducing the scan speed will increase the signa,l as well as increase photobleaching, but can have the same smoothing effect as averaging.

Direction is how the scanner captures signal. It can either capture signal unidirectionally (only from left to right on the scan) or bidirectionally (from both left to right and right to left). Bidirectionally is recommended for speed.



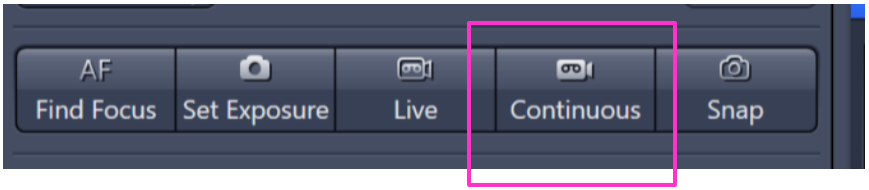
Correction & Line Step

Please don’t use Correction or Line Step. We’ve chosen settings that automatically do these functions. Adjusting them in this dialog will fight against the automatic processes.



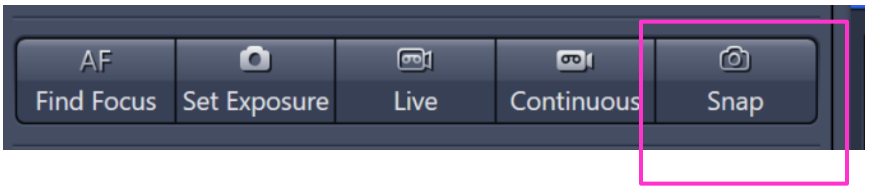
Continuous Button

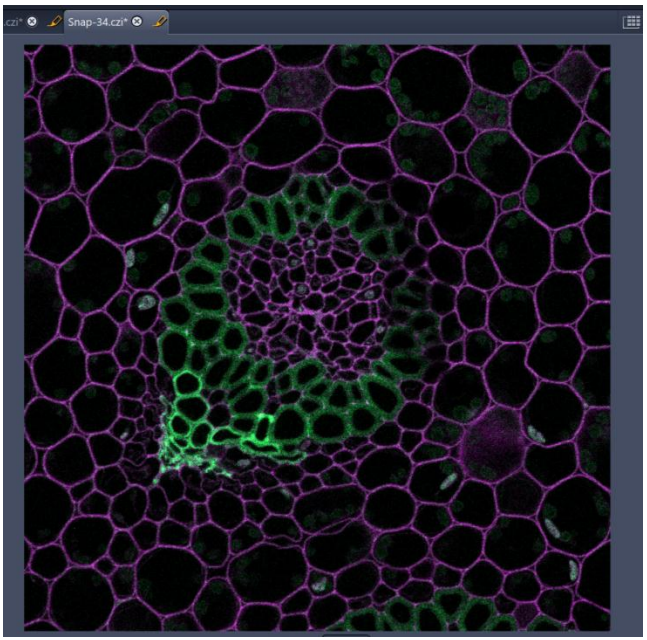
Choosing the “Continuous” button will scan all of the channels with the number of pixels in the final image. It will continue scanning until it is stopped.



Snap Button

Choosing the “Snap” button will capture one image with all of the channels with all of the pixels in the final image. This is your final image.

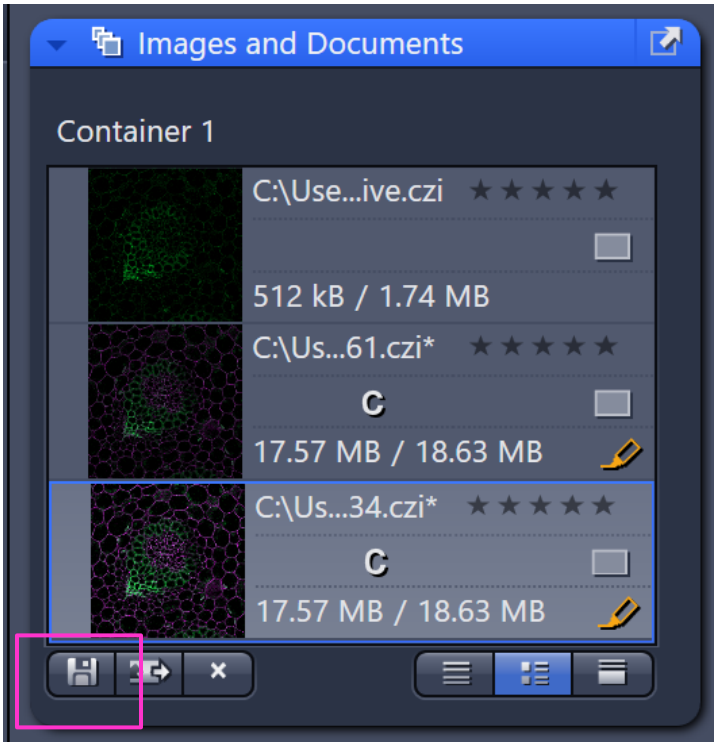




Saving Images

Images will show up in the “Images and Documents” dialog.Right-clicking on the image name will give many options. Choosing the diskette icon will allow you to save the file.

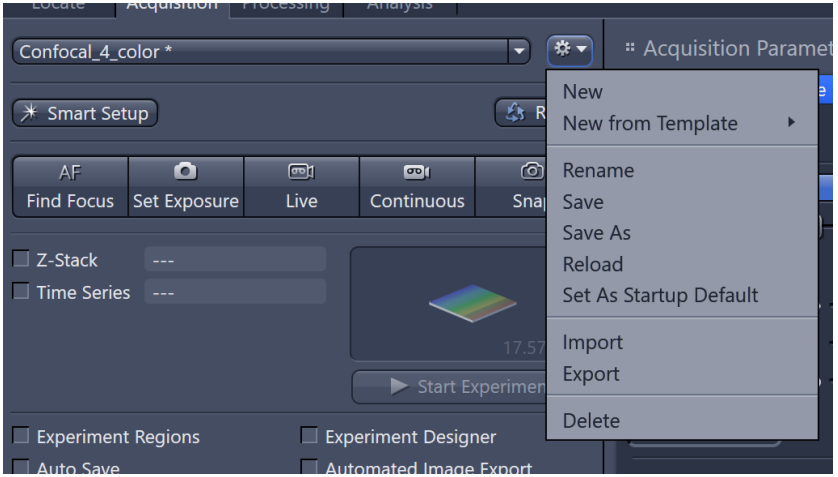
Images are saved as .czi files. These are Zeiss proprietary files. They can be opened in Zen and ImageJ (with the Bio-Formats Importer). Zeiss offers a light version of Zen for free. Images can be converted to .tiff files (or other formats) in Zen, Zen Lite and ImageJ/FIJI.



Save Acquistion Settings

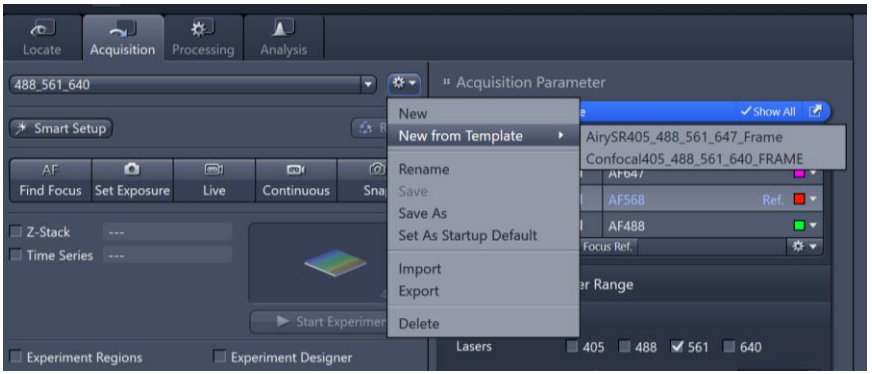
The settings used to acquire your image can be saved. Click on the gear icon to see the menu. These settings will only be seen under your login, but it very simple to share them with another user.

If anything has changed about the setting since last saved, there will be an asterisk next to the name of the setting.



Capture an Airyscan Image

Capturing an Airyscan image starts by loading Airyscan settings. Load the “AirySR405_488_561_640_Frame” setting and save it.



Choose Airyscan Channels

The steps for optimizing the settings are the similar, but the dialogs look a little different. You will still choose which channels to capture in the “Channels” dialog.

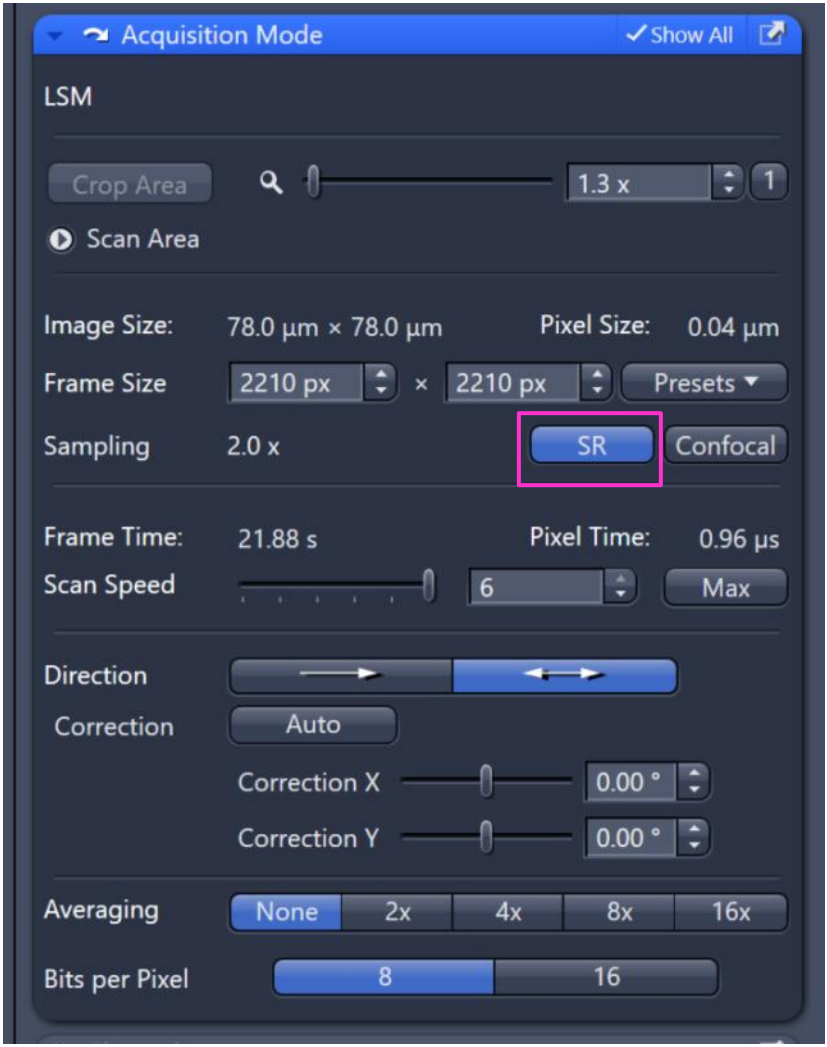
Notice that there is no option for the size of the pinhole. Digital Offset is also gone.

Airyscan Acquisition Mode

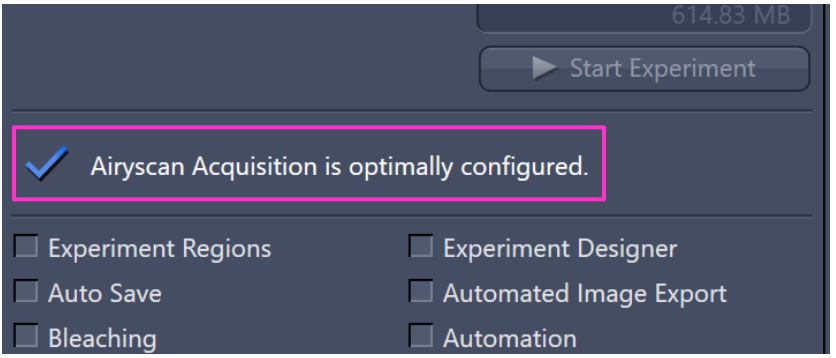
The minimum zoom for Airyscan images is 1.3 x.

Choose the “SR” button to choose a number of pixels that is optimal for Airyscan. There is still the option to use few pixels, however, you will sacrifice the increase in resolution.

Airyscan setting default to 8 bit. This is due to how the images are processed. Using 8 bit reduces the chances of having a saturated image.

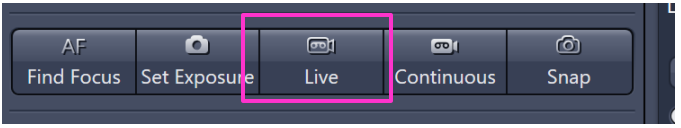


The software will let you know if Airyscan is configured optimally. If it is not, it will tell you what is missing. Airyscan is optimized for the 63x, but it is possible to capture Airyscan images on lower magnifications, however, the gains in resolution will be less.



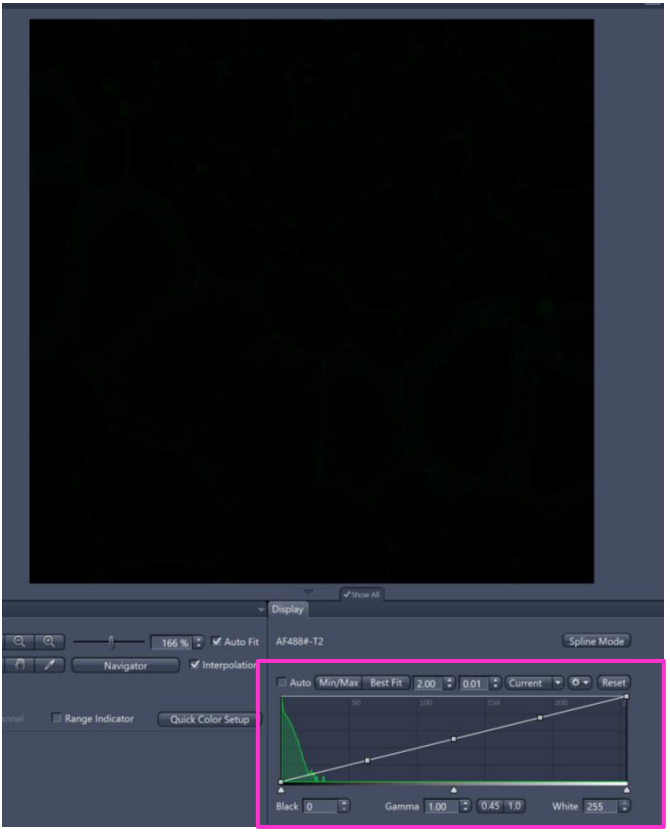
Optimize the Channel Settings

Just like for a confocal image, you'll choose the "Live" button to see an image of your sample.

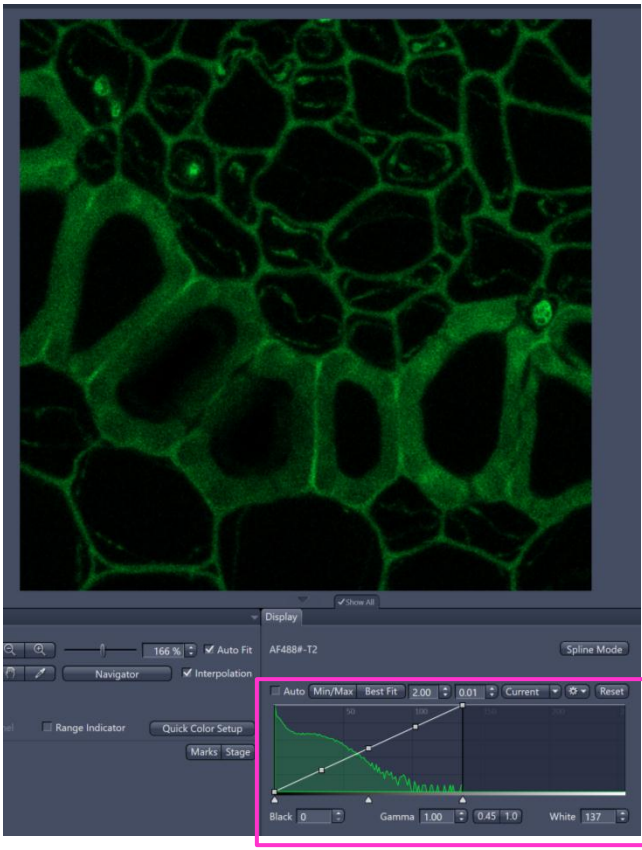


Adjust the Master Gain (up to 900) and the laser power until the pixel intensities fill approximately half of the 8 bit range.

Sometimes an image will show up as black on the screen. Remember to check the histogram. Here there is signal in the histogram, so the image only looks black because the brightness is set incorrectly.



Use the “Best Fit” or “Min/Max” buttons to adjust the scaling in order to see the image.



This image has been adjusted so that the histogram takes up about half of the range

Airyscan Detector Adjustment

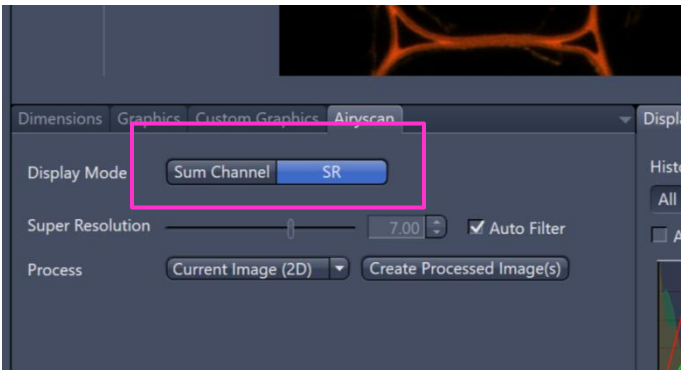
When using an Airyscan setting there will be a honeycomb icon at the bottom of the software.

Open the dialog and focus on your sample. If the brightest spot is not in the middle hexagon, use the adjustments to make sure it is in the middle. The sample must be in focus for this to be effective.

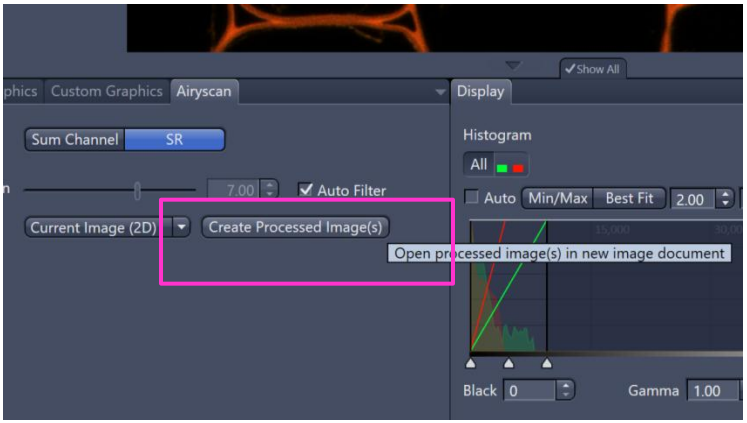
Process the Airyscan Image

Airyscan images require post-processing to reassign the pixel values.

After capturing an Airyscan image, the software will automatically do this processing and display the image as a super resolution (“SR”) image. If you would like to see the raw data, choose the “Sum Channel” button.

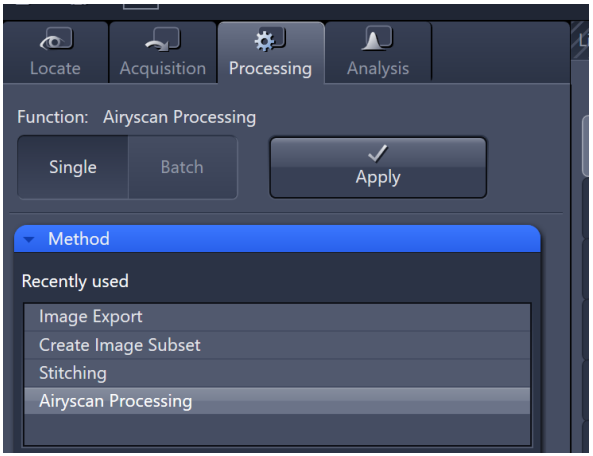


The processed image is only displayed on the screen. To save the processed image, you can choose the “Create Processed Image” button. This will create a new file in the file manager.

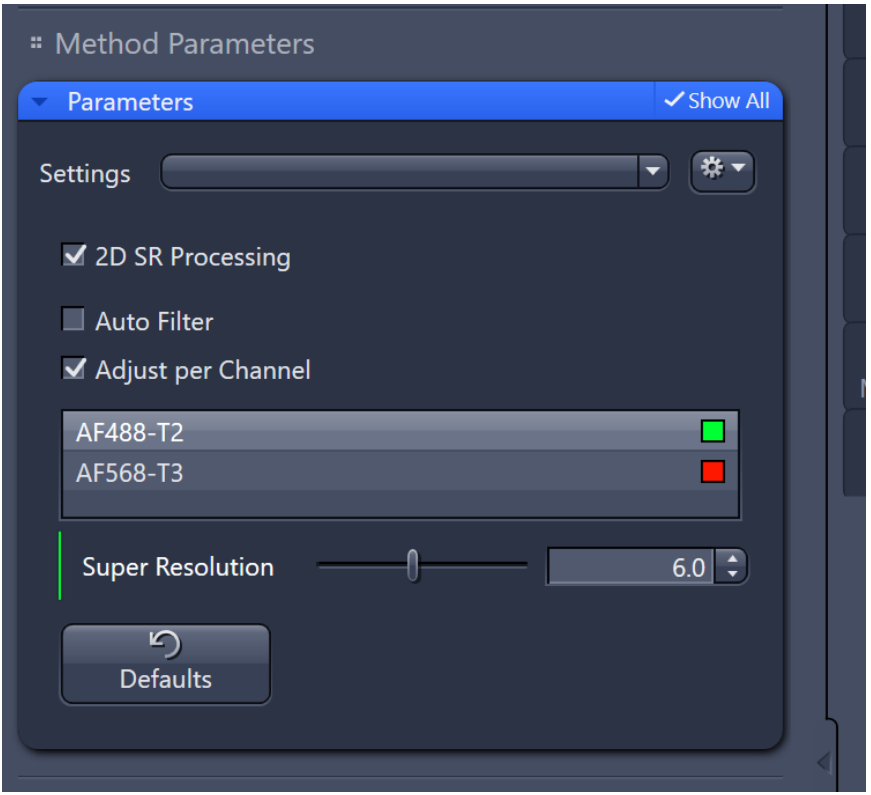


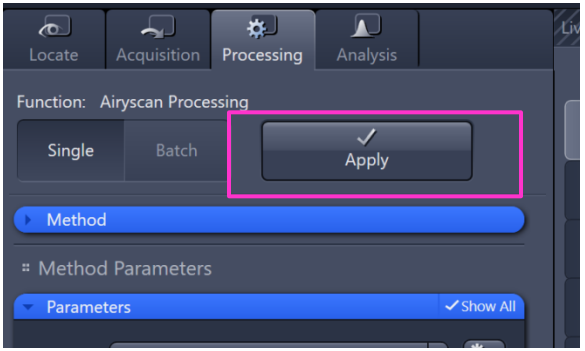
Airyscan Processing Tab

The processing that happens in Display Controls uses auto settings. If you would like to have more control over the settings that are used for the processing, go to the Processing Tab and choose “Airyscan Processing” for the Method.



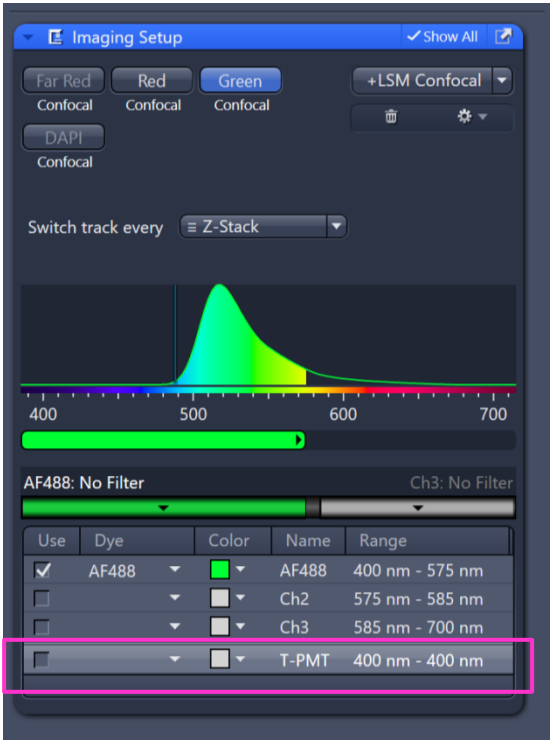
In the Parameters dialog, unclick “Auto Filter” and you will have lots of options. You can adjust the strength of the processing based on the channel. Stronger processing might give a more artifactual look to the image, while weaker processing will give a “softer” image.





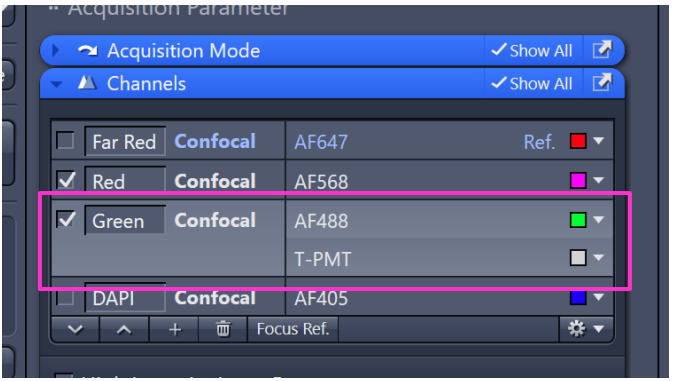
When you have chosen all the settings, choose the “Apply” button to create new files.

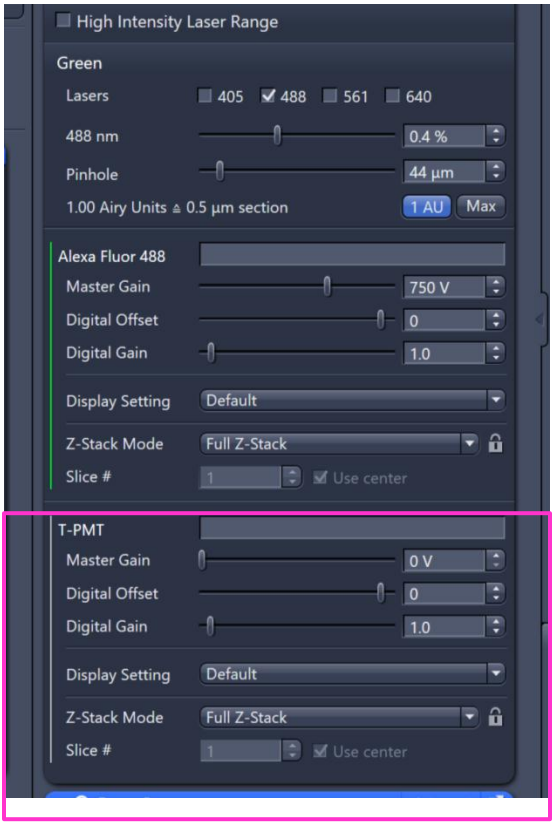
Capture a transmitted light image



To capture a transmitted light image, first make sure all of the settings on the microscope are correct. Then choose a channel in the channels dialog (here I've chosen green). At the bottom of the Imaging Setup dialog, you will see a "T-PMT" option. Click the checkbox next to it to make it active.

Now there is an extra line in the green channel, labelled "T-PMT". This is the transmitted light PMT.





The T-PMT also has independent Master Gain, Digital Offset and Digital Gain. Adjust the Master Gain of the transmitted light channel until the image looks good.

Make sure that the image is not saturated by checking the histogram.

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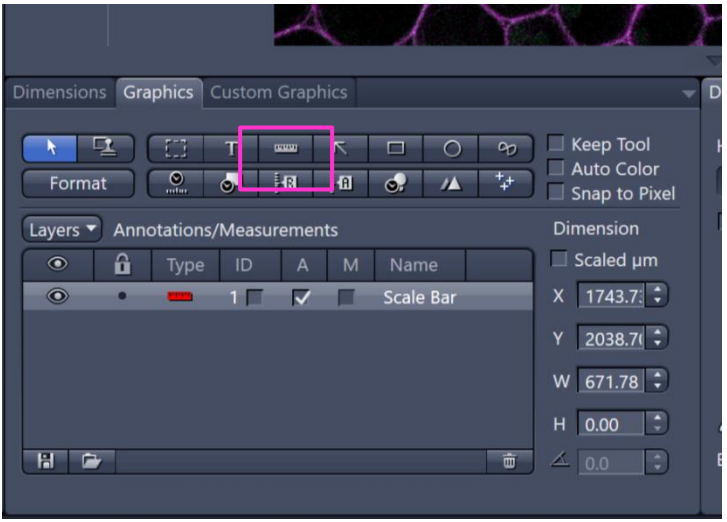
Adding a Scale Bar

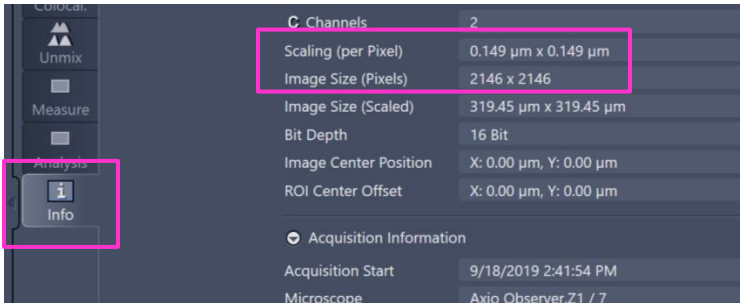
Once you have captured an image, you can add a scale bar, by choosing the “Graphics” tab in the Display Controls area.

Choose the ruler icon and a scale bar will be displayed on your image. It is possible to change the length, font, color, etc.

Zen saves this scale bar as a different layer, rather than making it a permanent part of the image. If you would like to show it on exported images, there is an option to “burn in graphics” in the Processing Tab

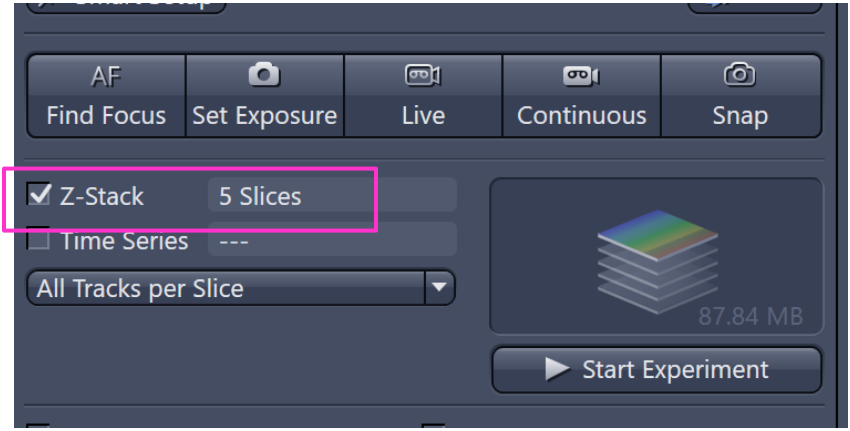


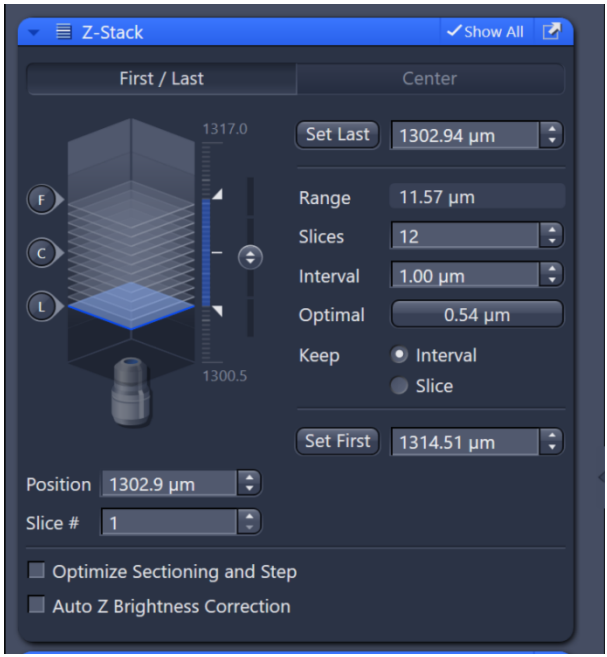
The size of pixel can be found in the Info tab of the image. You can use this information to draw your own scale bar.



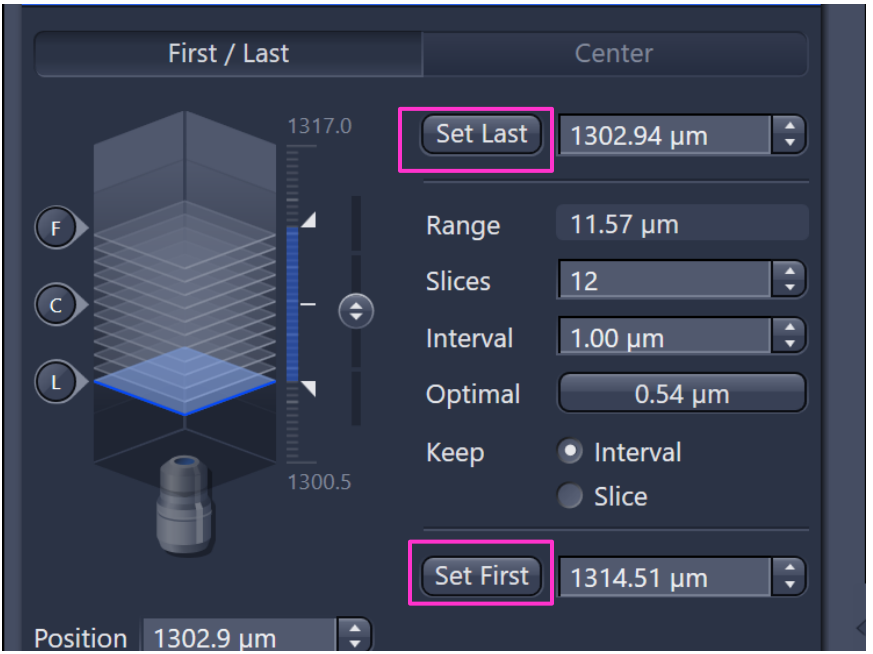
Capture a Z-stack

To capture a Z-stack, click the “Z-stack” checkbox. This will open a Z-stack dialog

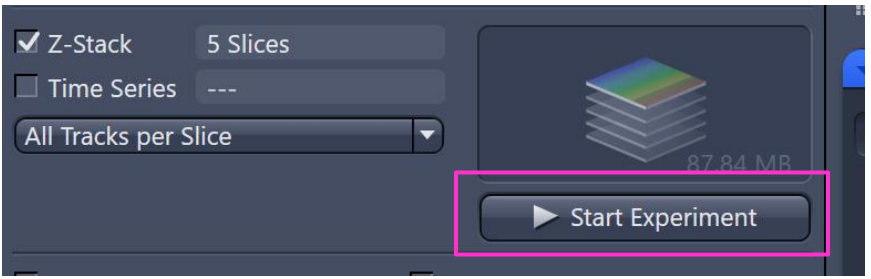




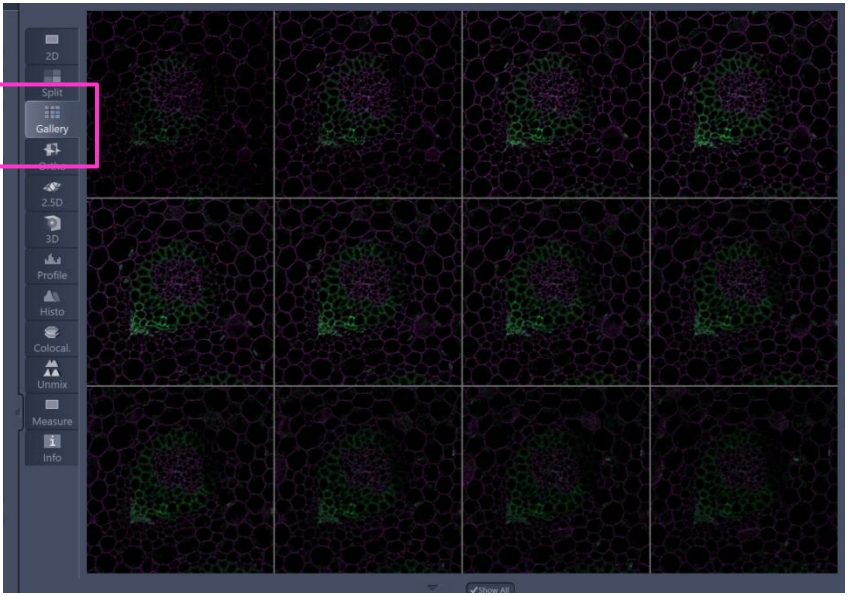
Focus to the top of your sample and choose “Set First”, focus to the bottom of your sample and choose “Set Last”. The software will suggest an optimal Interval (step size). The optimal value oversamples (takes more slices than is really necessary), so it is safe to choose a bigger value for the Interval.



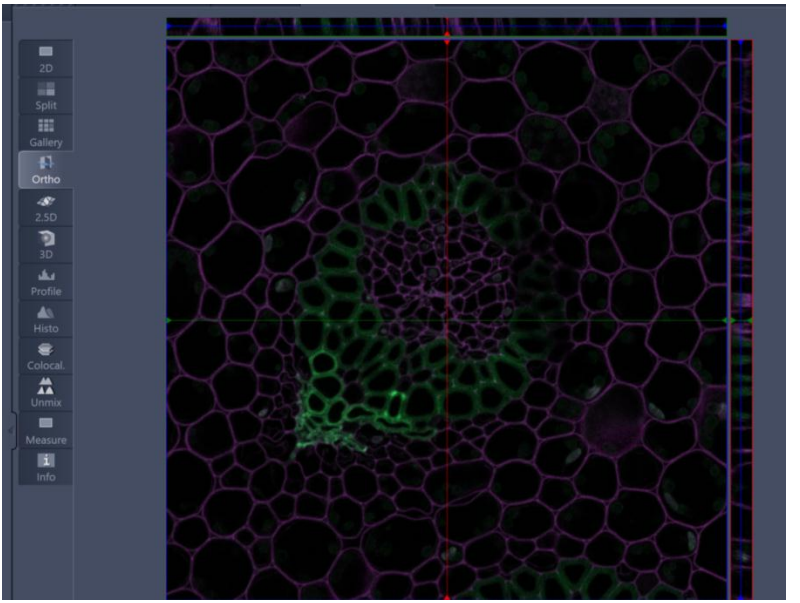
Once you have set the first & last, as well as choosing how many slices, you can start the Z-stack acquisition by clicking “Start Experiment”. The software will give you an idea of how the capture time will be.



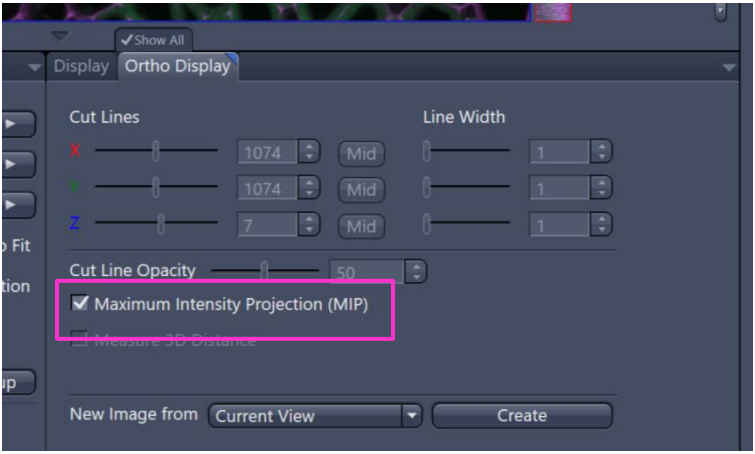
You can see all the slices in your Z-stack by choosing the "Gallery" tab.



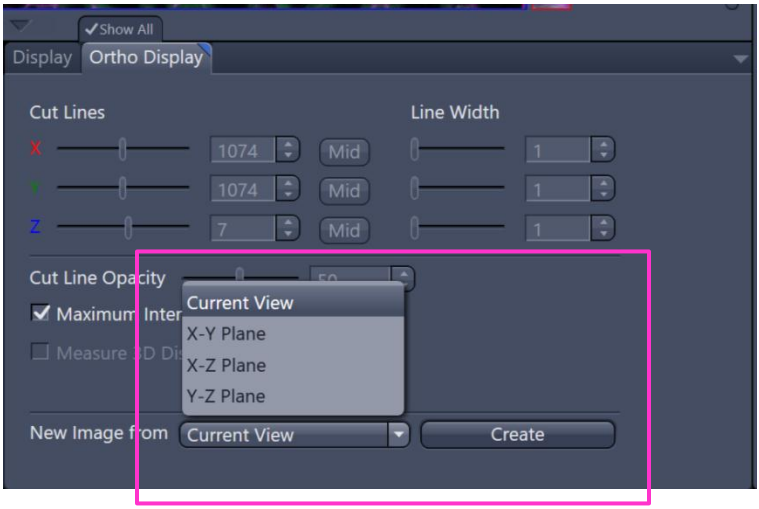
The Ortho tab will display an orthogonal view of the stack.



The Ortho tab will also show a Maximum Intensity Projection.

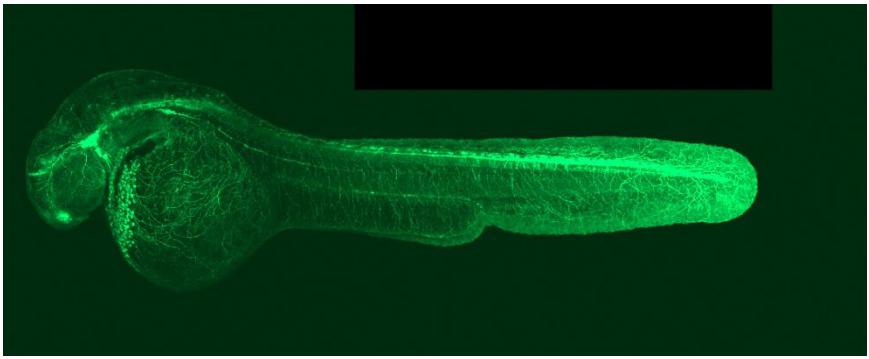


The maximum intensity projection (or orthogonal views) can be saved as a separate file in this dialog as well. Choose what kind of view (Current View, X-Y Plane, etc) and then choose the “Create” button to make a new image file.

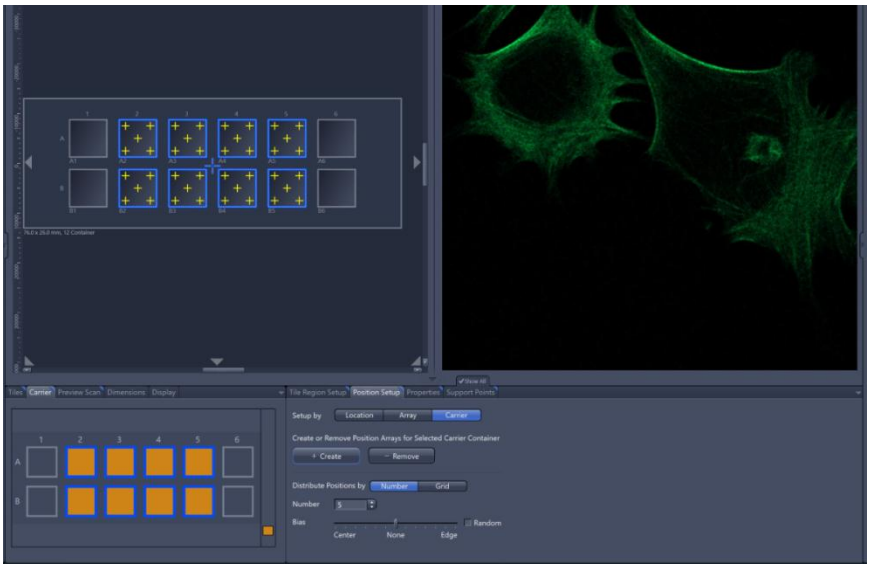


More Features: Tiles

Our system has a motorized stage, which allows us to do all sorts of neat things. We can do montages:

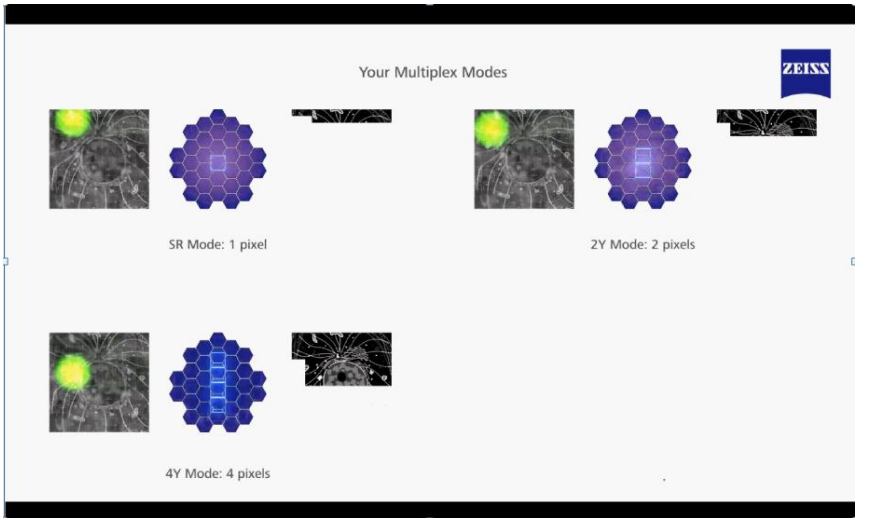


The software knows the dimensions of common sample carriers. We can capture a set of regular or randomized regions in each well. We can ask the software to go to 5 points in each well and capture a Z-stack. There are a multitude of possibilities.



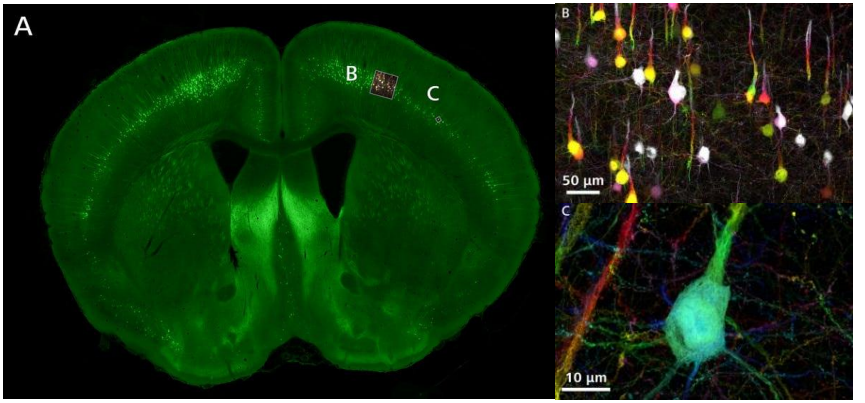
More Features: Multiplex

Multiplex allows us to capture images with improved resolution (down to 140nm resolution) with drastically increased speed. Because the Airy pattern is known, the reassignment algorithm can be altered so that it can determine values for two (or more) rows of pixels for each single scan of the laser.



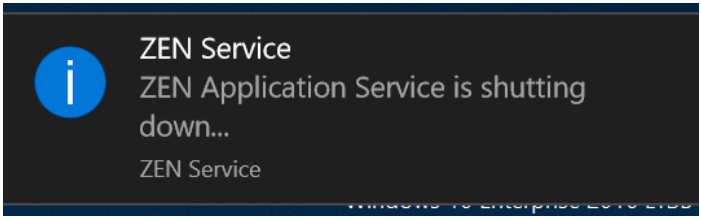
More Features: Sample Navigator

Sample Navigator allows us to capture a very low power (2.5x) image and use that as a “map” for higher magnification images.

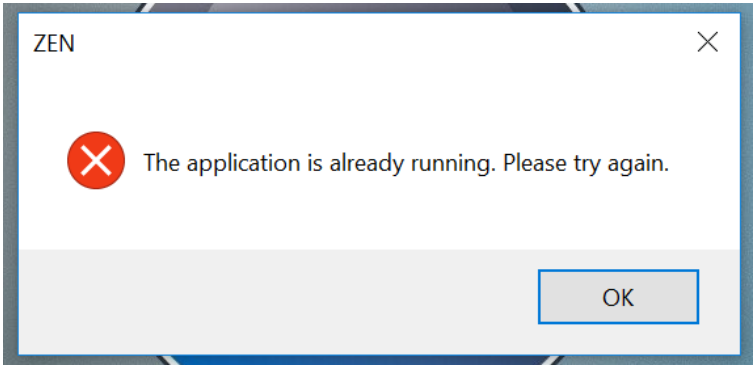


Software Shutdown

The Zen software takes a little while to shutdown.

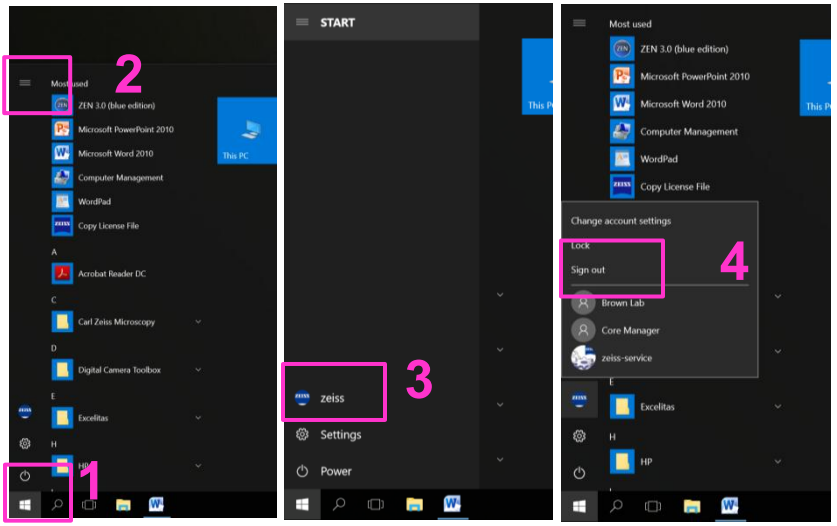


If you try to restart the software too quickly after shutting it down, you might get a message that it is already running. Just wait a little bit and try again.



Signing out of the PC

1. Click on the Windows icon on the lower left of the monitor.
2. Click on the 3 lines icon.
3. Click on your login icon (round Zeiss icon in this example.)
4. Choose “Sign out”.



Shutdown

Shutdown in reverse numerical order. Start at #3 if you want to continue using the pc (to transfer files, export images, etc).All of the switches are labeled.