Zeiss LSM 880 with Airyscan - Users' Guide

Author(s): Mariana Ferreira **Last Update - June 2020**

Purpose: Zeiss LSM 880 is an inverted confocal laser scanning microscope with Airyscan, a new detector featuring a 32 channel GaAsP-PMT area detector. The Airyscan detector brings improvements in spatial resolution and signal-to-noise ratio in comparison with conventional confocal microscopy. This allows resolving structures up to 140 nm (XY) and 350 nm (Z) even in thick samples. The fast module for the Airyscan offers a compromise between perfect optical sections and live cell imaging experiments. The Fast Airyscan mode reduces photo-bleaching and allows acquisition speeds of up to 19 frames per second at 512 x 512-pixel image resolution.

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Safety instructions and contacts

Safety instructions and first aid procedures

All lasers are connected via shielded fibres to their respective microscope systems. Lasers are activated by key switches and via software control. Beam paths are shielded except for a fixed open beam between objective and specimen. All systems are equipped with remote safety interlocks and integrated beam-splitters that prevent stray laser light if the user switches from confocal observation to eyepiece observation.

- Never look directly into the laser beam or a reflection of the beam.
- Never interfere with the beam path.
- Never change a specimen during scanning.
- Never change objectives during scanning.
- Never change filters during scanning.

Lens cleaning solution is toxic.

- Use gloves to avoid skin contact (if contact occurs wash thoroughly with water).
- Avoid spillages when cleaning objectives.

Specimens should be sealed before observation under the microscope and handled with care using the appropriate level of protection.

- Leaky or cracked samples must not be examined.
- Any spillages should be cleaned up immediately and the area swabbed with 95% alcohol.
- Glass slides should be disposed of in the contaminated sharps bin – broken fragments of glass should be brushed on to paper and disposed of in a similar fashion.

All biological samples and hazardous waste must be appropriately disposed of by the user. Potential risks associated with the waste material should be addressed by the user in conformity with ITQB-NOVA rules.

Contacts

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Equipment location, description and technical specifications

Location: Room 5.01 (Bacterial Imaging Cluster), 5th floor, Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA)

Manufacturer: Zeiss

Model: Zeiss LSM 880

Year Installed: 2019

Equipment Specifications

- Axio Observer 7 SP Inverted Microscope Stand
- Motorized Stage 130 x 100 STEP
- Stage Controller XY STEP SMC 2009
- Z-Piezo WSB 500
- Motorized Condenser w/ six positions (w/ DIC)
- Definite Focus 2 w/ ACR (Automatic Component Recognition) motorized nosepiece
- Eyepieces: PL 10x/23 Br. Foc. (High eyepoint and Focusing)

Laser Lines

- Diode - 405 nm (maximum power: 30 mW)
- Multi-line Argon - 458/488/514 nm (maximum power: 25 mW)
- DPSS - 561 nm (maximum power: 20 mW)
- 633 nm (maximum power: 5 mW)

Incident Light

- Fluorescence (HXP 120 V illuminator (w/ 200 W mercury short-arc reflector lamp)

Detectors

- 2 Photo-Multiplier Tube (PMT)
- 1 Gallium Arsenide Phosphide (GaAsP)
- 1 Airyscan (32 GaAsP)
- 1 Transmitted Photo-Multiplier Tube (T-PMT)

Transmitted Light Techniques

- Differential interference contrast (DIC)
- Phase Contrast
- Bright Field

Potential Advanced Techniques

- Super-Resolution microscopy
- Photobleaching *
- FRAP *
- FRET *
- Spectral unmixing *
- Lambda scanning *
- FLIM (please contact Mariana Pinho, mgpinho@itqb.unl.pt)

* For instructions on how to use these techniques please refer to the Advanced User's Guide available at the BIC website and contact the responsible people.

Objectives

Magnification[1]	**10x**	**20x**	**63x**
ZEISS System	EC Plan-NEOFLUAR	Plan-APOCHROMAT	Plan-APOCHROMAT
Class	Colour and Chromatic Correction	Colour and Chromatic Correction	Colour and Chromatic Correction
Numerical Aperture (NA)[2]	0.3	0.8	1.4
Immersion	Air	Air	Oil
Free Working Distance (mm)	5.2	0.55	0.19
Cover Glass (mm)	0.17	0.17	0.17
Contrasting Methods	-	Differential Interference Contrast (DIC II)	Differential Interference Contrast (DIC III)
Lateral Resolution (XY) @525 nm (µm)[3]	0.7	0.2625	0.15
Nyquist Sampling (XY) @525 nm (µm)[4]	0.28	0.105	0.06
Zoom to Nyquist Sampling (XY) @525 nm with 512x512 scan format	Number of field/(magnification*zoom)	=	Nyquist sampling
Axial Resolution (Z) @525 nm (µm)[3]	$\frac{0.735}{0.09}$ = 8.16	$\frac{1.4\times0.525\times1.338}{0.64}$= 1.53	$\frac{1.4\times0.525\times1.515}{1.96}$ = 0.56
Nyquist Sampling (Z) @525 nm (µm)[4]	3.2667	0.6146	0.2273

[1]Notice that the microscope is equipped with two Eyepieces: HC Plan S 10x/23 Br. M

[2] NA - Numerical Aperture. For more information, follow this link.

[3] $r_{lateral} = \frac{0.4\,\lambda}{NA}$ and $r_{axial} = \frac{1.4\,\lambda\times\eta}{NA^2}$ from Olympus website: Resolution and Contrast in Confocal Microscopy

[4] $Nyquist\ Sampling = \frac{r_{lateral\ or\ axial}}{2.5}$

- Remember to choose the proper calibration if doing montages/size measurements.

- The pixel size in microns of your picture is saved automatically (if you've chosen the correct calibration). Use LOCI to recognize it with ImageJ.

- The pixel size in microns of your picture is saved automatically when using Zen. Use Bio-formats to recognize it with ImageJ/Fiji (already installed in the Fiji package).

Filter-cubes for Eye-piece visualization (Locate Tab)

Filter Cube	Excitation Filter	Dichromatic Mirror	Emission Filter	Emission Colour
DAPI	G 365	395	BP 445/50	Blue
GFP	BP 470/40	495	BP 525/50	Green
RFP	BP 565/30	585	BP 620/60	Red

BP – Band Pass filter

Confocal Main Beam Splitter (MBS) Dichroic Mirrors

Visible Light

Position	Wavelength(s)	Example Fluorophore	Notes
1	458	CFP	
2	458/514	CFP/YFP	
3	458/561	CFP/RFP	
4	None	-	-
5	488	GFP	
6	488/561	GFP/RFP	
7	None	-	-
8	488/561/633	GFP/RFP/Cy5	
9	T80/R20	-	For confocal reflection microscopy
10	Plate	-	Use when not using Visible Light lasers

Invisible Light

Position	Wavelength(s)	Example Fluorophore	Notes
1	Plate	-	Use when not using invisible Light lasers
2	None	-	-
3	405	DAPI	
4	445	-	No corresponding Laser
5	440/532	-	Used for FLIM system
6	None	-	-
7	None	-	-
8	None	-	-
9	T80/R20	-	For confocal reflection microscopy
10	None	-	-

Emission Filters for Airyscan Modes

Position	Wavelengths	Emission Colors	Optimal Fluorophores
2	BP 420-480 + BP 495-550	Blue + Green to Yellow	DAPI + GFP, FITC, YFP
3	BP 420-480 + BP 495-620	Blue + Green to Red	DAPI + GFP, FITC, YFP, Rhodamine, RFP
4	BP 465-505 + LP 525	Cyan + From Yellow	CFP + YFP, Rhodamine, Cy5
5	BP 495-550 + LP 570	Green to Yellow + From Red	GFP, FITC, YFP + RFP, Cy5
6	BP 570-620 + LP 645	Red + Far Red	RFP + Cy5

BP – Band Pass filter

LP – Long Pass filter

Additional Components Specifications

Computer

- Type: Desktop
- Brand: HP®
- Model: HP 840
- Features: 2x Intel® Xeon® E5-2623 v3 3.0 GHz (quad-core); 64GB DDR4-2133 MHz ECC RAM (12 free slots); 4x 2TB 4 TB RAID 10; NVIDIA Quadro K2200 4GB
- OS: Windows 7 Ultimate Embedded x64
- Available Software: **Zeiss Zen 2.3**

Monitor

- Brand: HP®
- Model: DreamColor Z32x
- Diagonal: 32”
- Native Resolution: 4K UHD (3840 x 2160 @ 60 Hz)

Zen 2.3 (black edition)

- The ZEN 2.3 (black edition) can be equipped with several modules, applications and licenses. This guideline is strictly specific for the **ZEN 2.3 (black edition)** version available on the microscope’s computer.

Airyscan vs Confocal

What is Airyscan?

Airyscan is a special detector module added to the out-coupling port of LSM 880. Its design enables to be more light efficient than would be possible with a standard confocal point detector. The extra photons can be used to increase the sensitivity of the image, to scan faster or to improve the resolution.

What is the principle of the Airyscan detector?

The Airyscan detector consists of a Gallium Arsenide Phosphide (GaAsP) photomultiplier tube (PMT) array of 32 elements, which are arranged in a compound eye fashion. In this way each detector element receives light that is displaced from the optical axis by defined distances.

How efficient is the Airyscan detector?

The GaAsP detector is ideal for confocal laser scanning microscopy due to its high quantum efficiency (QE), extreme low dark counts and fast read out times.

What is the resolution gain in Airyscan?

A resolution gain of 1.7-fold is obtained in all spatial directions.

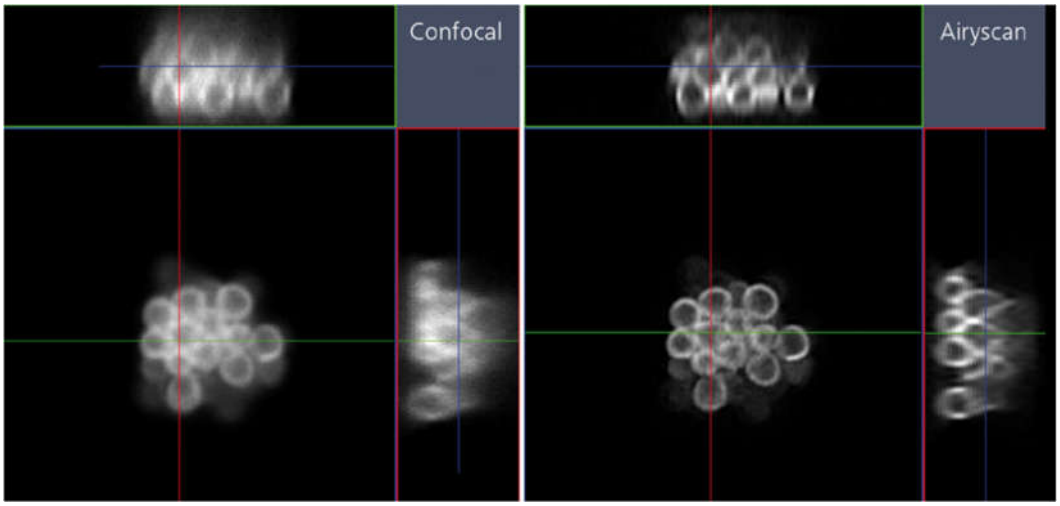
How is the resolution increased by Airyscan?

The majority of resolution improvement stems from the significant increase in SNR. In the super-resolution (SR) mode 1.25 Airy units (AU) will be imaged on the detector array. Since along the diagonal there is a maximum of 6 elements, each of them acts as a pinhole of 1.25 AU / 6 ≈ 0.2 AU. In other words, one obtains a resolution comparable to a confocal microscope with the pinhole set to 0.2 AU, but with a much better SNR. A pinhole set to 0.2 AU in a standard confocal will capture only 5% of the light compared to Airyscan.

What is the advantage of Airyscan compared to other technologies based on pixel reassignment?

First of all, a resolution gain in Airyscan is obtained for all lateral and axial directions. All other methods show only lateral resolution gain; axial resolution will stay confocal. Second, compared to hardware solutions, you have all the flexibility to analyze data post acquisition in different ways.

Airyscan and conventional confocal images and information courtesy of Zeiss.



Please note that Airyscan images also include some deconvolution so care has to be taken not to introduce artefacts into the image.

It is more important than ever to keep all the raw unprocessed data!

Keywords: *confocal, Airyscan, live imaging, inverted microscope, super-resolution, FRET, FRAP*

Suggestion for description in "Materials and Methods".

Confocal Z-series stacks (step size 200 nm) were acquired on a Zeiss LSM 880 point scanning confocal microscope using the Airyscan detector, a 63x Plan-Apochromat 1.4NA DIC oil immersion objective (Zeiss) and the 405 nm, 488 nm and 561 nm laser lines. The Zeiss Zen 2.3 (black edition) software was used to control the microscope, adjust spectral detection for the emission of 4′,6-diamidino-2-phenylindole (DAPI), Green Fluorescent Protein (GFP) and tetramethylrhodamine (TMR) SNAP-tag fluorochromes and for processing of the Airyscan raw images.

Confocal Z-series stacks and tile regions with multiple positions were acquired on a Zeiss LSM 880 point scanning confocal microscope using photomultiplier tube detectors (PMTs) and a gallium arsenide phosphide (GaAsP) detector, a 63x Plan-Apochromat 1.4NA DIC oil immersion objective (Zeiss) and the 405 nm, 488 nm and 561 nm laser lines. The Zeiss Zen 2.3 (black edition) software was used to control the microscope and adjust spectral detection for the emission of 4′,6-diamidino-2-phenylindole (DAPI), Green Fluorescent Protein (GFP) and tetramethylrhodamine (TMR) SNAP-tag fluorochromes.

Images were acquired on a Zeiss LSM 880 point scanning confocal microscope controlled with the Zeiss Zen 2.3 (black edition) software, using a 63x Plan-Apochromat 1.4NA DIC oil immersion objective (Zeiss), the fluorescence filter sets GFP + TX2 and differential interference contrast (DIC) optics.

Information to be added to the "acknowledgements" section.

This work was partially supported by PPBI - Portuguese Platform of BioImaging (PPBI-POCI-01-0145-FEDER-022122) co-funded by national funds from OE - "Orçamento de Estado" and by european funds from FEDER - "Fundo Europeu de Desenvolvimento Regional".

User pre-requisites, booking and general rules.

- The equipment cannot be used without official training.
- Before your first use contact Mariana Ferreira to schedule training.
- New users must have a clear understanding of the principles of light microscopy, how fluorescence and more specifically confocal microscopy work.
- This equipment operates on class III lasers
- Save all data into your own storage device as we routinely clean the computer's data
- Register your utilization in the logbook
- Airyscan raw data reconstruction must be done in the microscope computer (book slots accordingly). For all downstream image analysis image analysis a free software license is available for ZEN lite at ZEISS website. Alternatively, you can use FIJI.
- Contact the responsible people in case of any doubt or any issue with the equipment.

Booking is done online at the ITQB intranet. Please mind the following rules when booking the microscope:

- Priority booking of afternoon slots is given to users with live cell experiments that require growth in the same day. If you have fixed or time insensitive samples, please try to book other times;
- Maximum 2 slots per user per week (one slot = one morning or one afternoon);
- These rules are applicable until the previous Friday. If you need more than two slots in a week and they are still available on the previous Friday, at 4pm, feel free to book them.

Instrument startup procedure

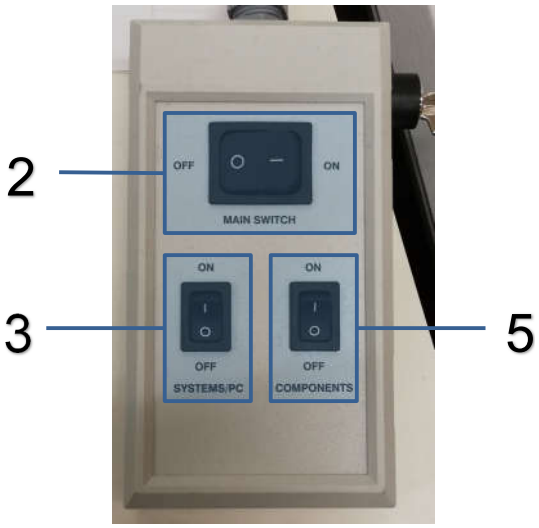
System Overview



1- Confocal PC
2- Confocal Monitor
3- Microscope Body
4- Confocal Module
5- Airyscan Module
6- Confocal Lasers
7- FLIM PC
8- FLIM Monitor
9- FLIM Laser
10- FLIM Detectors

Step by step startup

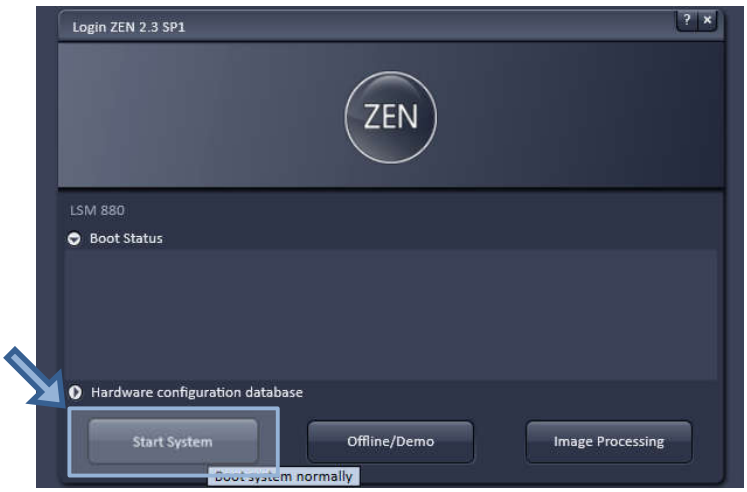
1. Take the dust cover off;
2. Turn on the **Main Switch** and wait a few seconds (button 2);
3. Turn on the **Systems/PC Switch** (button 3);
4. Start up the **Computer** and wait until you see the Windows start up screen;
5. Turn on the **Components Switch** (button 5);



6. Wait for everything to finish initializing;
7. Open **Zen Black** on the computer;



8. Choose **Start System** and wait for the initialization to finish.



In case of any errors follow the shutdown procedure and then start it back up.

If this does not fix the errors, please contact the people responsible for the equipment!

Mounting a sample and acquiring images:

Sample Preparation Notes

- You can use resources such as the FPBase or the Thermofisher Spectra Viewers to explore your fluorophores. Learn their excitation and emission wavelengths, and what are their optimal laser lines and filters! This is necessary to use the confocal to its full potential.
- Use #1.5 high precision glass coverslips if using super-resolution Airyscan.
- Leave the edges of your slide free of mounting medium so they can fit into the sample older;
- Take care that your sample and mounting media are not too thick, less than 1 mm in height is best;
- Confirm the coverslip is secure as this is an inverted microscope.

Zen black General User Interface

The user interface is divided into three main sections, the **Controller Section (1)**, the **Image Display Section (2)**, and the **Image and Document Manager Section (3)**, with a menu bar above and a system information bar below.



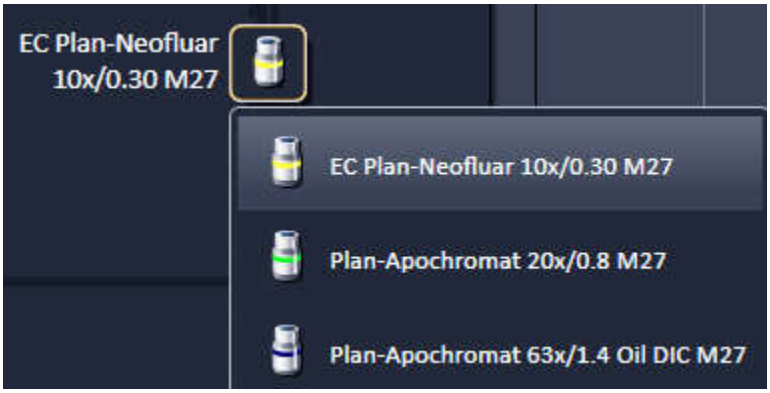
The **Image and Document Manager Section** shows all the currently open files and allows you to save and close the currently selected image.

The **Image Display Section** is where your current image is shown. When an image is open it has a tab on the bottom that allows the adjustment of brightness and contrast.

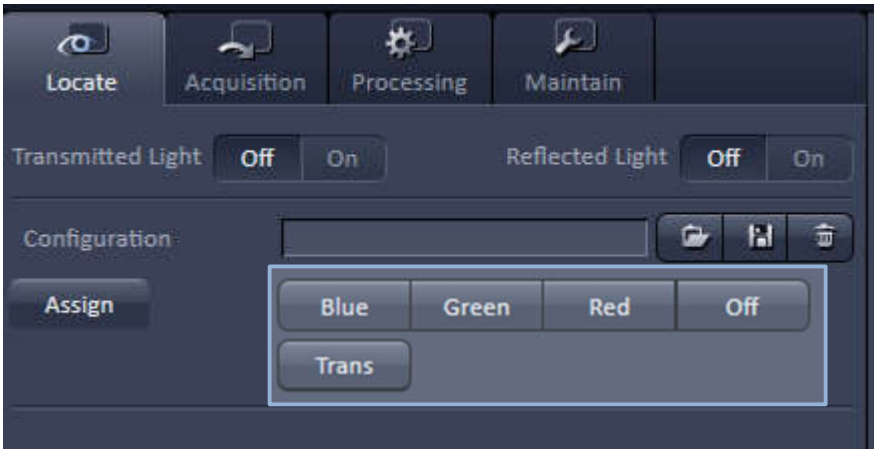
The **Controller Section** is the most important of the three as it allows the user to control the microscope to locate, acquire and process their samples' images, further detailed bellow. The maintain tab is only for system maintenance purposes only, do not alter any settings.

Sample Localization

In the **Locate Tab** you can find and inspect your sample using the eye pieces.
First choose the objective you want to use either through this tab or using the touchscreen to the left of the microscope.



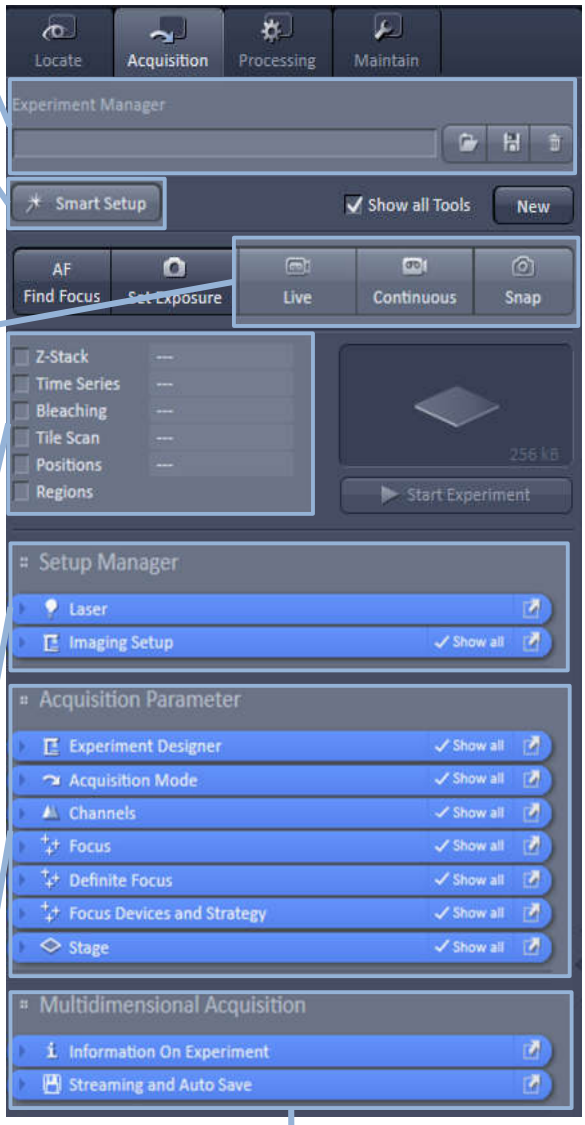
Use the preset buttons to quickly change between the configurations for red, green, blue fluorescence, transmitted light and off. The transmitted light setup is used for every brightfield configuration, with the type (brightfield, phase contrast or DIC) being dependent on the condenser setting and prisms on the light path.



The joystick on the right of the microscope controls the stage and can be used to move the field of view around the sample. On both sides of the microscope body you can find course and fine focus screws to find and focus the sample.
Once you have found your sample using Locate move to the **Acquisition Tab**.

Image Acquisition

Below is a general overview of each section, in the next few pages you can find more details on how to use each section.



Save and Load your Experiment profile.

The software quickly creates a new acquisition setup for your fluorophores.

Live – Low settings live feed; Continuous – Your settings live feed; Snap – Acquire current plane with your settings.

Additional available acquisition options, check to show options tab. To acquire images with these extra options you must use **Start Experiment** instead of Snap.

Control the lasers On/Off, acquisition light path and mode (Confocal, Airyscan, etc.).

The most important are the **Channels** tab and **Acquisition Mode**, which allow fine tuning of the acquisition parameters.

Setup autosave options. Additional tabs may appear here depending on selected acquisition options.

Smart Setup

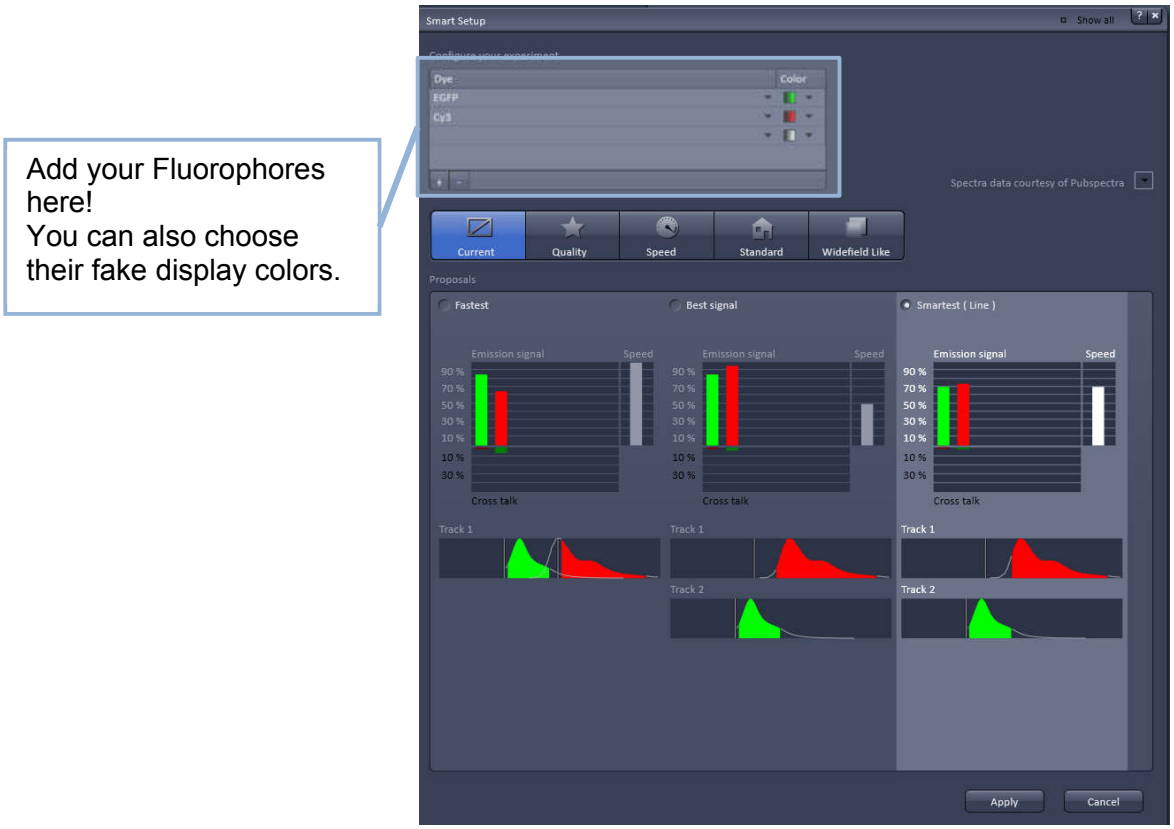
Clicking this opens a new window (shown below) where you can select your fluorophores from a list. If your fluorophores aren't in the list check their data sheets for the excitation/emission wavelengths and find an equivalent fluorophore from the list to select. To each fluorophore you can also assign a display colour to distinguish the different fluorophores. All images are in black and white, the display colour is for visualization only! Eg: GFP can be shown as purple.

Three possible **regular confocal** configurations are generated with optimization for speed (Fastest), for Best Signal and a compromise between both (Smartest). Each configuration will display the speed, percentage of emission signal collected and crosstalk, the fluorophores emission spectrum overlap. We recommend using the **Smartest** configuration.

Before acquiring you should confirm the light path parameters are correct and the proper lasers were turned on before moving to the Channels and Acquisition Mode tabs.

BEWARE pressing apply will OVERWRITE any current Experiment Configuration!

Do not overwrite other users' settings!



Lasers

Confocal uses laser lines instead of lamps to acquire the images, so you must turn on the lasers you need for your fluorophores. If you used Smart Setup, it should turn the needed lasers on automatically. The lasers are not instantly ready to use and may require warming up, during this the laser line numbers are highlighted in red. Once the yellow triangle appears the laser is ready for use. The 405 Diode laser is fully controlled by the software, you can't turn it on yourself.

Do NOT turn on lasers you don't need.

The Argon laser takes a few minutes to warm up.



Warming up



Ready to

Imaging Setup

At the top of this tab you have Tracks. There can be up to four tracks simultaneously, each with their own imaging mode and light path setup. The fewer optical components are different between tracks the faster the acquisition of multiple tracks can be. **(1)**

Select the imaging mode you want to use and then set up the light path. The available modes are:

- LSM + Channel for Classic Confocal and Airyscan;
- LSM + Lambda for Linear Unmixing;
- LSM + Online Fingerprinting for Fluorophore profiles;
- Fast for Fast Airyscan.

To make sure you don't forget anything follow this order when setting up the light path:

Laser -> Optic Components -> Detector

1. First select the laser line(s) you will need, if you haven't turned them on yet you will get a prompt to do so; **(2)**
2. Following the beam, you will then have to choose the appropriate Main Beam Splitter for your laser line(s). Eg: Laser Line 488 uses MBS 488; **(3)**
3. Following the light path UP, you then must choose between Mirror for Classic Confocal and Plate for Airyscan (ignore the other options and never select None). **(4)**

Here the path splits, follow the beam to the Left for Confocal and Up for Airyscan.

Confocal

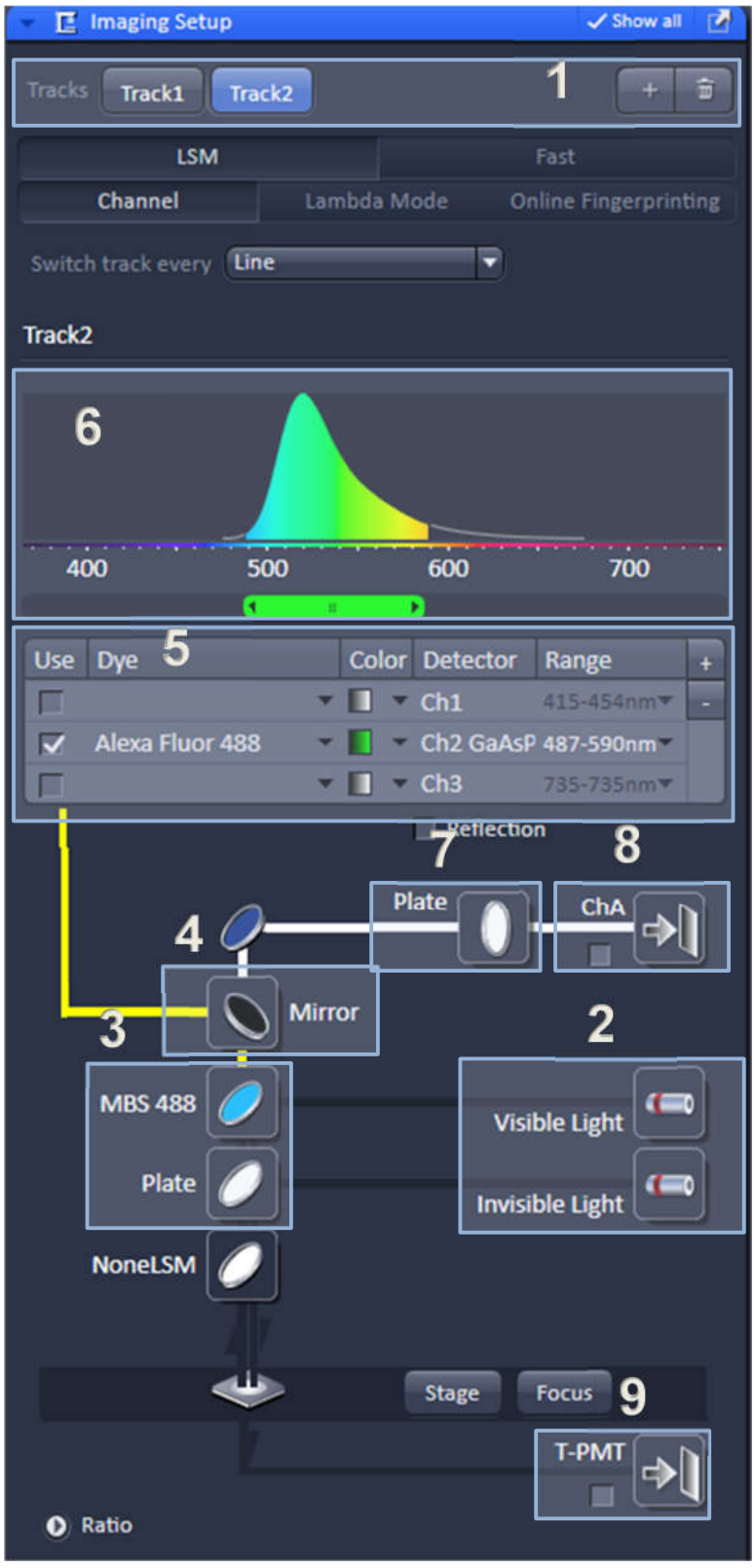
4. Follow the beam (yellow) to select the detector(s) you want for this track. The Ch2 GaAsP is the most sensitive detector, ideal for sensitive or weak fluorophores. The other two are optimal for strong fluorophores, with Ch3 detector having an extra sensitivity to the red part of the spectrum; **(5)**
5. You can also identify the fluorophore from the dropdown list (like in Smart Setup) and assign its display colour. If a fluorophore is selected its emission spectrum will be shown in the graph above; **(5/6)**
6. Adjust the range of detection, either by wavelength numbers or by adjusting the bar below the spectrum. **(5/6)**

Airyscan

4. Follow the beam (white) and select the emission filter that works for your fluorophore(s). Eg: Fluorophores using Laser line 488 should use BP 495-550 + LP570 filter; **(7)**
5. Check the Airyscan detector. (make sure no other detectors are selected and that Plate is being used.) **(8)**

To take a Transmitted Light image just check the T-PMT at the bottom of the window in any track, this works for both Brightfield and DIC. **(9)**

<u>Empty positions should be left with Plate selected instead of None.</u>


Imaging Setup
Show all
Tracks
Track1
Track2
1
LSM
Fast
Channel
Lambda Mode
Online Fingerprinting
Switch track every
Line
Track2
6
400
500
600
700
Use
Dye
5
Color
Detector
Range
Ch1
415-454nm
Alexa Fluor 488
Ch2 GaAsP
487-590nm
Ch3
735-735nm
Reflection
7
8
Plate
ChA
4
Mirror
3
2
MBS 488
Plate
NoneLSM
Visible Light
Invisible Light
Stage
Focus
9
T-PMT
Ratio

Acquisition Mode

In this tab you can control the dimensions of the image, the speed of acquisition and zoom. You shouldn't have need to change any options in this tab beyond the ones detailed below.

Click (X*Y) to show a list of predefined square frame sizes for Confocal.

Airyscan requires **Optimal** frame size.

Higher speeds give noisier images.

The recommended speed is **4** to **6.**

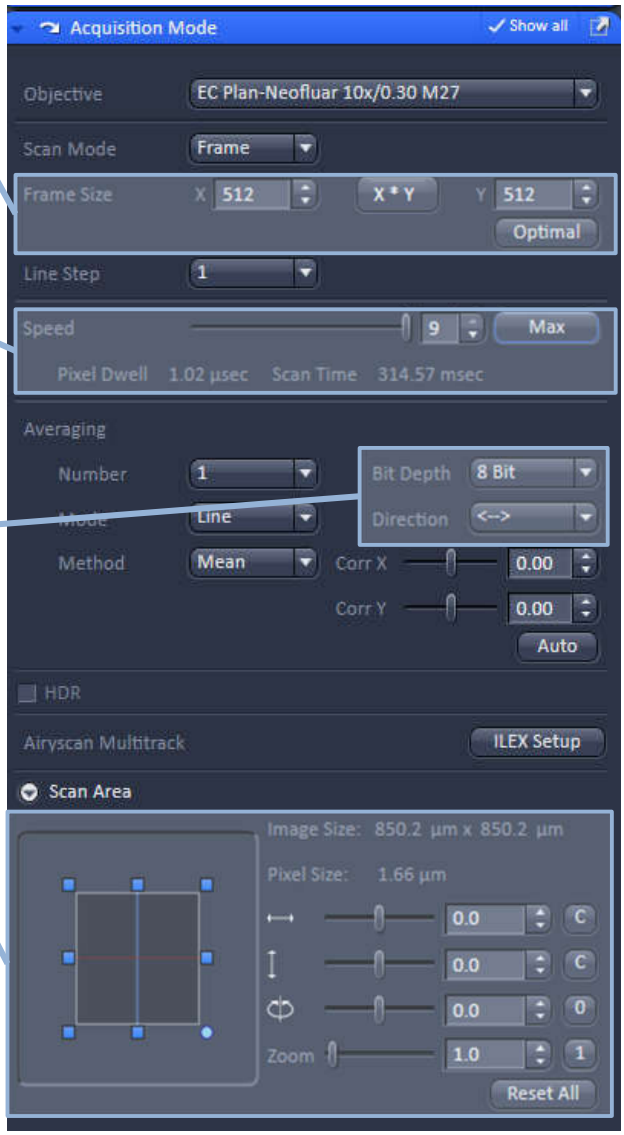
Check the Direction is bi-directional <-->, as it is faster.

Use either 8 Bit or 16 Bit for Bit Depth, preferably 16 Bit.

Calibrated image and pixel size are displayed here.

Zoom, position and rotation controls allow the selection of the area to image.

After zooming readjust the optimal frame size in order to have the correct pixel size and consequently the correct sampling

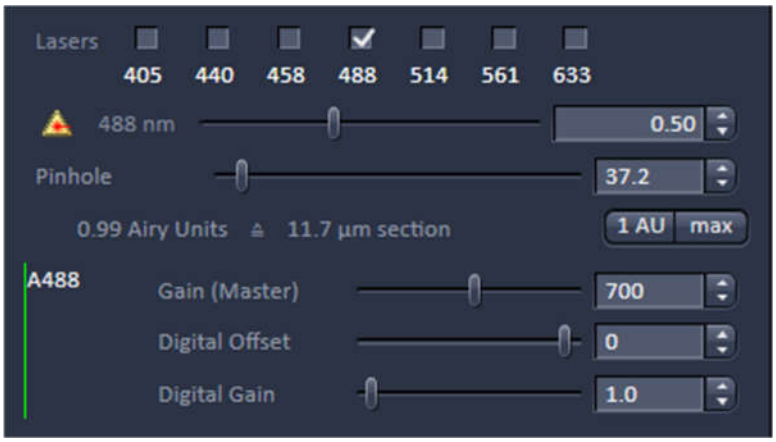


Channels

Here you can select which tracks to acquire, set the pinhole size, laser power and the detector's Master Gain. What you see in this tab varies depending on the Image Setup you made earlier, meaning you could see from one to four channels in a single track.

For Confocal Mode

Each track has a **single pinhole opening** which should be set for 1 Airy Unit (AU) to achieve true confocal images. Therefore, in a multi-channel track not all channels will have an optimal pinhole.
Every channel has its own **laser power**, in percentage, and **master gain**. To start you should set the **Master Gain** to **700** in every confocal channel, then set laser power to 0.2% to 1% and take a snap. You should then use the **Range Indicator**, next to the histogram in the display area, to see if you need to adjust the laser power. If you are using the full dynamic range, a lot of **RED** pixels means you are saturating the detector (all max intensity values will be lost) and you must lower the laser power as you may damage the detector and are losing information about your sample, the background should be mostly **BLUE** if there is no background from the mounting media. You should leave **Digital Offset at 0** and **Digital Gain at 1.0.**



For Airyscan Mode

The pinhole of each Airyscan track **must** be higher than **2 AU**, if the pinhole size is in **RED** it's too small for proper high-resolution imaging.
For Airyscan the **Master Gain** should be set to **800.** Again, you should start at 0.2% to 1% of laser power and adjust as needed based on the Range Indicator.

If you are using live or continuous for focusing check only one track, preferably that of your less sensitive fluorophore.

Airyscan detector alignment

Before starting Airyscan acquisition you should align the light beams and the detector. This process is automatic you just need to set it up.

1. Do a crop with 20x zoom of an area with clear fluorescent object;
2. Set the acquisition speed to Speed 6;
3. Use the **Continuous** mode;
4. Check the alignment is working on the Airyscan tab to the left of the live image, you might have to press Show All (on top of the histogram) for the detector graph to display;
5. Once centered you can stop the continuous mode and the system is ready for acquisition.

If the system is failing to align the detector due to the signal being to scattered try to adjust the focus, select a cleaner area, reduce the acquisition speed, or use a different fluorophore.

Definite Focus 2

Definite Focus uses infrared light to keep your sample focused avoiding thermal and vibrational drift, which will make your field-of-view change over time. It works by measuring the refractive index mismatch between glass and your sample. Hence it only works if the refractive index of your sample is different from glass, for example water-based media, such as agarose pads or cell culture medium will work, whereas curing mounting media such as Mowiol or Prolong Gold for example will NOT work once cured, intermediate mounting media, such as VectaShield will be suboptimal unless diluted with an aqueous buffer.

To use this first select Definite Focus in the **Focus Devices and Strategy** tab, then in the **Definite Focus** tab click Store Focus and Periodic so the current focus is maintained.

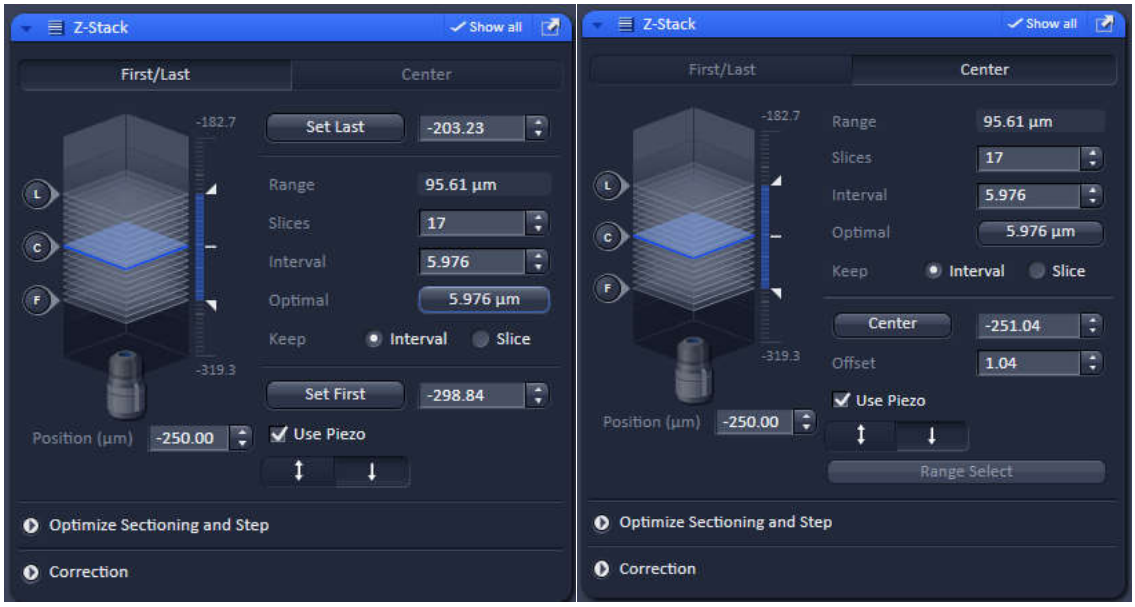
Remember to turn off Definite Focus when changing samples and before shutting down.

Multidimensional Acquisition

All modes under Multidimensional Acquisition can be used simultaneously. Eg: you can do a time-lapse of a z-stack tile region in multiple areas of the sample.
If you are interested in more advanced acquisition techniques available on this system please refer to the Advanced User's Guide available at the BIC website.

Z-Stack

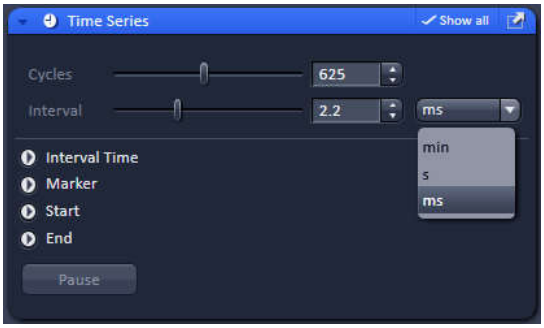
Since confocal imaging gives you optical sectioning acquiring images from different focal planes may yield new information (as you will not collect out-of-focus light from the out-of-focus imaging planes, effectively increasing the signal-to-noise ratio), selecting **Z-Stack** allows you to do so.
Using the optimal distance between slices ensures the best coverage and 3D reconstructions, while avoiding excessive bleaching and photo-toxicity on your sample (see Nyquist sampling).



Briefly, if you use a step size smaller than what the Nyquist sampling theorem recommends you will not gain any more information and you will likely increase bleaching and photo-toxicity on your sample by imaging the same "information" more times than needed. You can choose the z-range to acquire by setting the first and last frames of the stack or set the current frame as the central frame of the pile and choosing how many slices you want to acquire. If you are using definite focus using Center might be the best option. Also, when using Center insert an odd number of slices so the current frame is acquired.

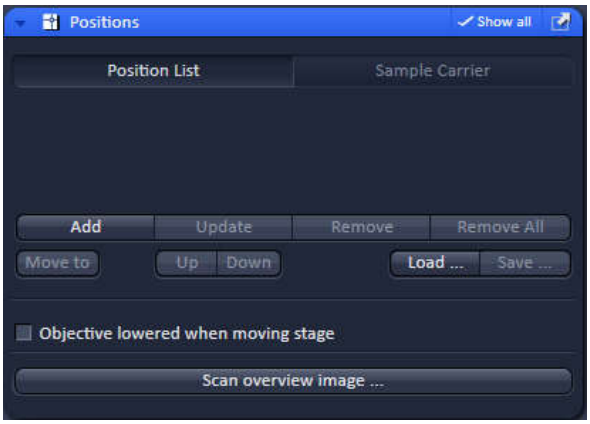
Time Series

This allows you to perform time-lapse experiments, working best with Definite Focus in longer time-lapses to ensure the sample stays focused. Number of cycles is the number of pictures to take, if zero is selected the software will take pictures until manually stopped. Interval is the time between each cycle, note that the interval timer resets when the picture begins being taken, if the interval is shorter than the acquisition time the next cycle begins as soon as the previous ends.



Positions

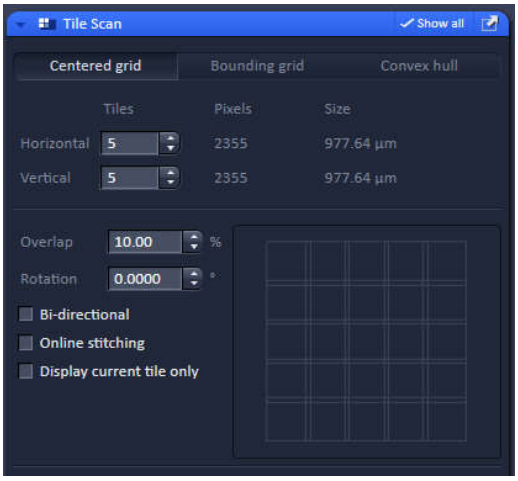
Positions allows you to save the coordinates of frames you may find interesting during sample examination so you can acquire them later. Just click add and the coordinates of the current frame will be saved.

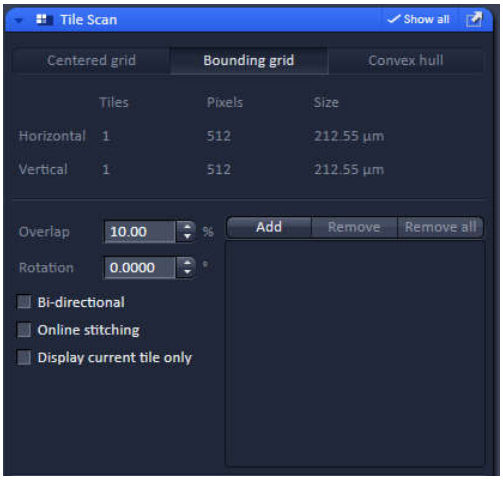


Tile Scan

Using Tile Scan you can have larger samples that do not fit in a single frame in the same image with high magnification. This takes multiple images that overlap each other (at least 10% but optimally 30%) and then they can be stitched together into a single image. There are three modes of tile scanning:

- **Centered Grid** - uses the current frame as a central point and acquires it and the area around it;
- **Bounding Grid** – you set the top left corner and bottom right corner of the area you want to acquire;
- **Convex Hull** – is used for irregular samples where a normal rectangular area would have many blank frames that don't need to be acquired, therefore reducing acquisition time.



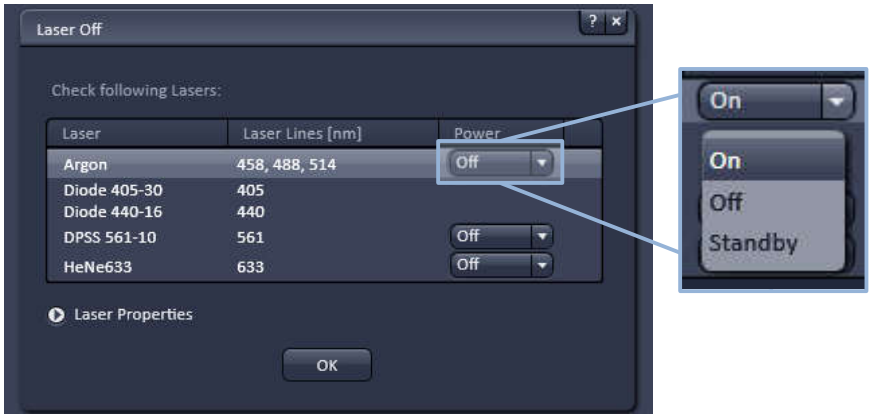


Regions

The pixel by pixel image acquisition of the confocal allows you to acquire only Regions Of interest (ROIs) instead of the whole frame. You can make ROIs using the tools under the Graphics tab in the Image Display section or in the Regions tab itself. Note that the frame will still be the same rectangular size but only the ROIs will have data.

Shutdown procedure

1. Save your images in Users folder in the D drive;
2. Close Zen Black;
3. A window to save any unsaved images/profiles should appear if there are any, **DO NOT overwrite other users' experiment profiles!**
4. A Laser Off window appear:
    a. If someone is using the system in the next **4 hours** leave the Argon laser in **Standby** mode and turn all other lasers off;
    b. Otherwise turn all lasers **Off**.



5. If turned **Off,** it will take the **Argon Laser** at least **5 minutes** to cool (fans turn off);
6. Shutdown the computer and then wait for the **Argon Laser** fans to turn off;
7. **AFTER** the Argon Laser cools down:
    a. Turn off the **Components Switch**;
    b. Turn off the **Systems/PC Switch**;
    c. Turn off the **Main Switch**;
8. Clean the objective you used
9. Put the dust cover back on.
10. Take out everything you brought (pipettes, slides, etc...)

ATTENTION

Turning the switches off before the Argon Laser module is done cooling can damage the laser!