

Confocal 5 – Zeiss 710 (BIG)

Quick-start Guide

Location: Room 6.028

(Updated: 25/11/2019)

Acquisition Software:



Available Objective Lenses: 10x Air, 20x Air, 40x Air, 40x Water & 63x Water (more available)

Available Laser Lines: 405, 458, 488, 514, 561, 594 and 633nm

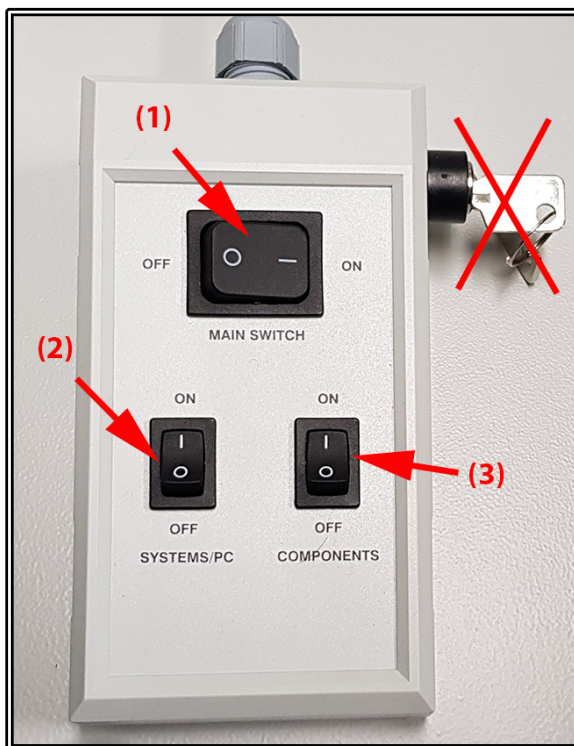
Be mindful of what you are doing at ALL times: The microscope and the lenses are delicate and very expensive and should be treated with care.

Slide Cleaning and Preparation:

- Make sure your slide is scrupulously clean – all dust, old oil and mounting media should be removed with a small amount of 70% ethanol.
- If you can use a hard setting mounting media please do so and DO NOT use glitter nail polish to seal your slides as this will scatter laser light. Be sure to give your mounting media and sealant at least 8 hours to dry properly.

Start-up Procedure

- Turn the Main switch **ON (1)**
- You should not have to touch the key since this should never be turned off
- Turn the Systems/PC switch **ON (2)**
- Turn the PC **ON** and wait for it to boot up completely
- Log in to the PC using your AD login details
- Turn Components **ON (3)**
 - o Ensure the Real-time controller computer is connected by clicking on the network connection button near the clock. It should read imb.uq.edu.au and Unknown Network (the unknown network is the microscope. Don't start Zen until this is present for 60 seconds)
- Double-click the ZEN Black icon on the desktop
- Click the Start System button and wait for the software to load
- If the Argon laser is required, turn the key on the black box (you will hear the fans turn on)



If the system will not boot properly (or you get a lot of errors) do a full restart

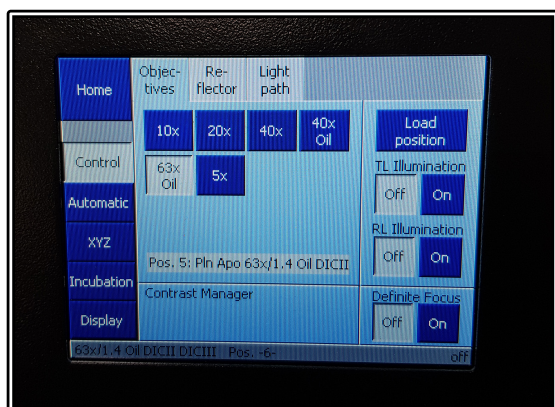
- o Close ZEN.
- o Wait *at least* sixty (60) seconds.
- o Shut down the computer.
- o Shut the confocal down by turning off the right hand bottom switch, waiting two (2) seconds, then turning off the left hand switch, waiting two (2) seconds, and finally turning off the top switch.
- o Wait *at least* sixty (60) seconds, but ideally two (2) minutes or more, to allow the components in the confocal to lose any residual charge and reset themselves.
- o Start the system again as described above.

Incubation

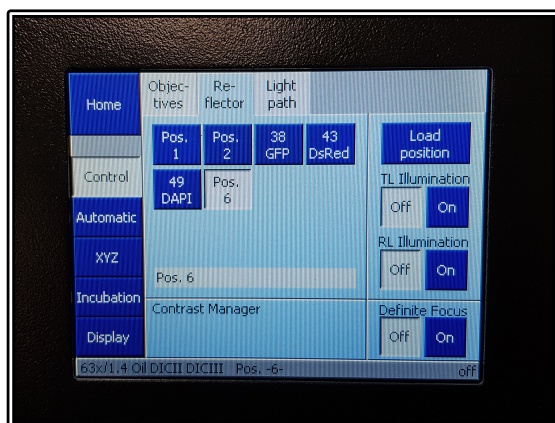
The incubation on Confocal 4 should always be set at 28°C. If you require a different temperature, the cool-down/warm-up times need to be factored into **YOUR** booking time and you are responsible for ensuring the temperature is back at 28°C **AND** stable before the next user is booked on. A booking for a different temperature can be logged while creating your session booking in PPMS.

For CO₂, this can be switched **ON** at the start of your session and set to your desired concentration through both the software and the touch-pad controls. Please make sure to turn the CO₂ **OFF** again once you have finished your session.

Microscope Controls



- Controls for the hardware can then be accessed through the software
- The touchpad controls can be used to change between the objective lenses automatically
 - o Warning messages will pop up if changing between lenses of differing immersion media (e.g. between oil and water lenses)



- The touchpad also contains Reflector turret options allowing to rotate between fluorescent filter cubes such as DAPI, GFP or DsRed
- Incubation controls can also be accessed on the left hand column of options
- Press the Load Position to lower the objectives away from your sample at any time

Locate Mode

This is used to focus on and find the sample through the eyepieces.

1. Click on the *Locate* tab.

Ensure you remove any remaining water from the objective or sample before changing objectives.

2. Select the objective you wish to use by clicking on the objective icon in the *Ocular* menu and apply any immersion oil if required and place your slide on the stage.

3. Select the dye you wish to image, by pressing one of the dye configuration buttons that most closely matches your own dyes emission colour.

4. Ensure that the fluorescence lamp is turned *On* and the shutter is open. Set the lamp to an appropriate power level (about 12% - 50%).

Set the eyepieces for your comfort.

Using the joystick, locate a region of interest in your sample.

5. You may also wish to adjust the temperature of the incubation chamber and sample insert, as well as the CO₂ concentration. It may take up to 60 minutes for the temperature/CO₂ to stabilise.

Applying Water for a Slide

- Open the incubation chamber and tilt the microscope turret back
- Remove the slide and rotate the water objective into position with the software
- Place a small drop of the correct water immersion onto the middle of the slide or very carefully place a small drop onto the centre of the objective lens
- Replace the slide, coverslip side down, lower the turret and close the chamber back up
- **DO NOT** over water the objective as this will cause immersion fluid to run down the sides and into the housing of both the objective AND the objective turret
- To remove the slide simply open the incubation chamber and remove it from the stage insert

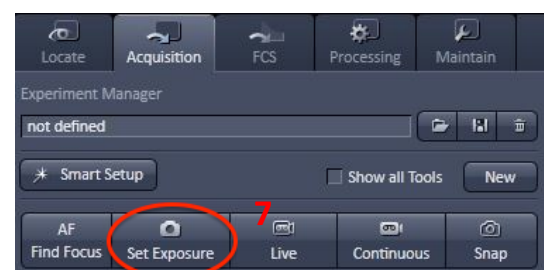
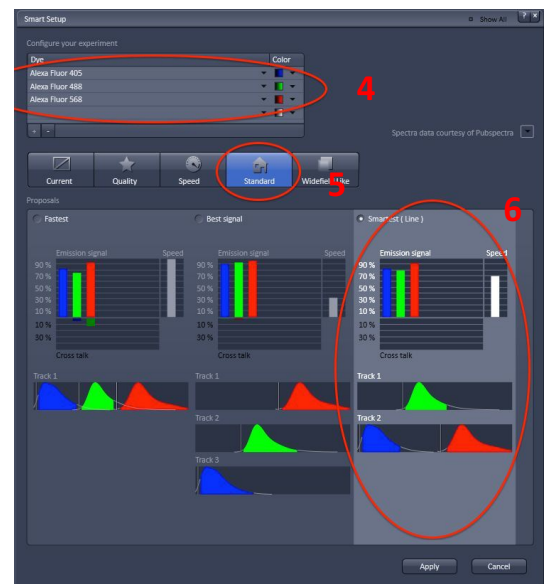
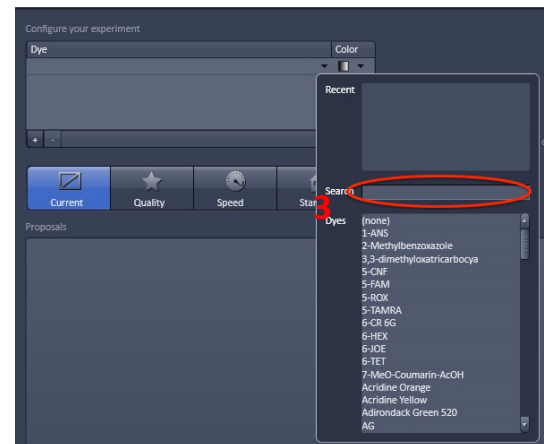
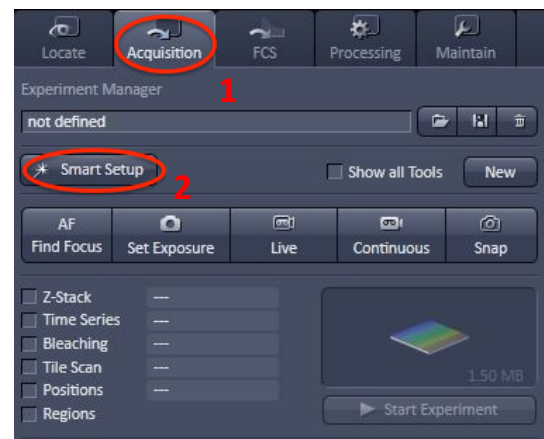


Setting Up Kohler Illumination

- Kohler Illumination is an important setup procedure for optical microscopy giving even illumination and reduced sample heating/bleaching
 - To start with, select the BF button on the Locate tab to get a brightfield image
 - Fully close the Field Aperture (at the top of the turret head)
 - Focus the light source using the Condenser Focus knobs until you can see a sharp outside ring of the field aperture while looking down the eyepieces
 - Ensure the light is in the middle of the field of view by adjusting the two silver centring screws at the front of the condenser
 - Once centred, open the Field Aperture until it just fills the whole field of view but no more
- This should be repeated when you change objective magnification as it is often different between lenses

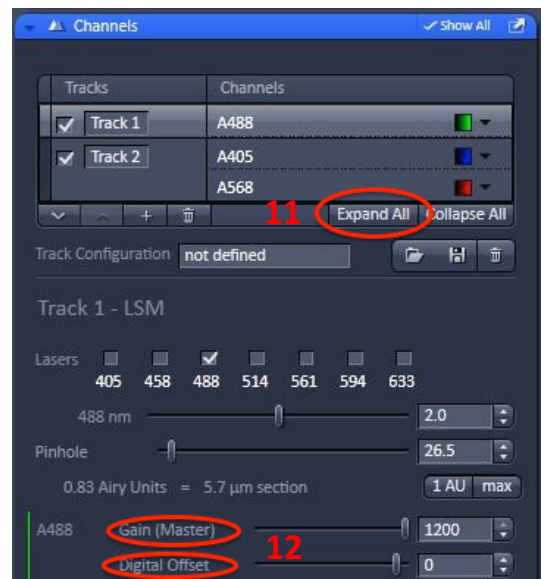
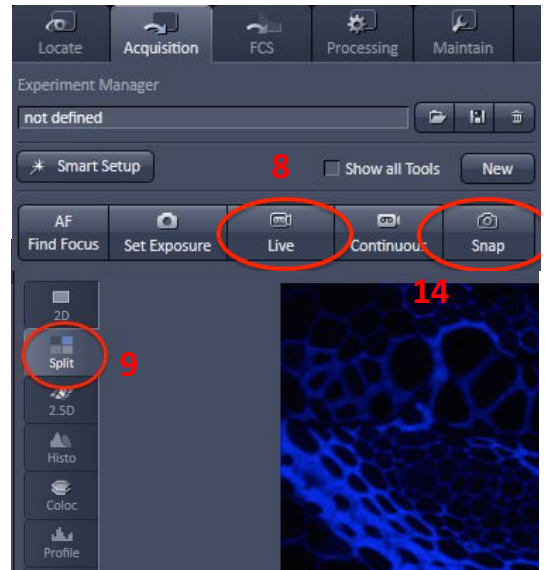
Acquisition Mode

- This is used to capture and save images.
- 1. Click on the *Acquisition* tab
- Working your way down the menus is a good way to be sure all steps are covered.
- How to determine which lasers and filters you need:
 - Make sure that the available laser lines excite your dyes at or near their peak absorption.
 - Helpful websites
- Smart Setup**
 - 2. Click the *Smart Setup* button at the top of the *Acquisition* menu. This will display the *Smart Setup* interface.
 - Configure your experiment.
 - 3. From the drop-down menu, select each of the dyes or fluorescent proteins present in your sample.
 - 4. You may assign various lookup tables (colour values) to your dyes here as well.
 - 5. *Smart Setup* will display 5 Motifs: *Current*, *Quality*, *Speed*, *Standard* or *Widefield Like*. Choose the one that best matches your sample, or choose *Standard* as this suits most samples.
 - 6. *Smart Setup* will display three proposed configurations
 - Fastest* – all of the dyes are imaged simultaneously, which is ideal for fast moving live samples, however there may be crosstalk or bleedthrough issues.
 - Best Signal* – all of the dyes are imaged sequentially, this is ideal for fixed samples as it is slower, but there is less chance of crosstalk or bleedthrough
 - Smartest* – scans dyes that are spectrally close together sequentially; while scanning dyes that are far apart simultaneously (e.g. the system will scan AF405 and AF568 simultaneously and then scan AF488 separately).
 - Select the proposal that suits your needs via the radio buttons and click *Apply*.
 - 7. The system will now scan the sample and attempt to set the best autoexposure settings to obtain an image, unless you choose the *Current* Motif in *Smart Setup*, in which case press the *Set Exposure* button.



- **Acquiring an image and basic fine tuning.**

- 8. Click the *Live* button at the top of the *Acquisition* tab.
- 9. Click the *Split* button on the left-hand side of the *Image* window
- 10. Click the *Range Indicator* checkbox below the image. This will use red to indicate pixels that are too bright (white level), and blue to indicate pixels that are too weak (black level) for the current detector settings. These red and blue pixels have been thresholded hence losing the intensity information contained within.
- 11. Click *Expand All* in the *Channels* control to show all of the *Channel* settings at once.
- Tweak the focus to find the object of interest in your sample.
- 12. If there are more than a few red pixels, reduce the *Laser Power* and/or *Gain* until the red pixels are randomly located, but keep the gain between 400 and 850.
- Otherwise, increase the *Laser Power* and/or *Gain* until randomly located red pixels appear, but keep the gain between 400 and 850.
- **Do not** set the *Gain* above 1000 as this can damage the detector.
- If blue pixels remain, increase the *Offset* until just a few blue pixels remain.
- Otherwise, decrease the *Offset* until just a few randomly distributed blue pixels appear.
- 13. Set *Averaging* to between 2 and 4: signal-to-noise ratio (SNR) increases as the square of the *Number* of images averaged.
- 14. Click *Snap* to acquire a high-quality image.



Saving Your Data

15. How to tell if an image has been saved: unsaved images have an exclamation mark in a yellow triangle appearing next to their thumbnails in the *Open Images* menu.

16. There are three (3) different places from which to save:
 At the bottom of the *Images and Documents* control.
 from the diskette icon above the main menu tabs.
 from *Menu > File > Save*.
 And by right clicking on the image file and selecting save

Set up a local folder in My Computer / Data (Drive D:).

Save all acquired data here initially.

Never perform initial save over the network: ZEN isn't built for it and may crash.

When you are finished imaging, transfer images to your network folder.

Data may be cleared from the computers as frequently as once a week. **Back up your data.**

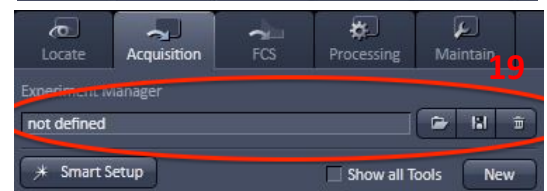
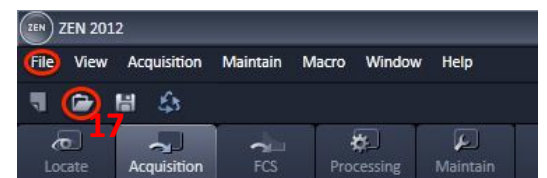
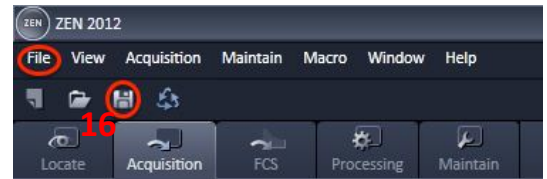
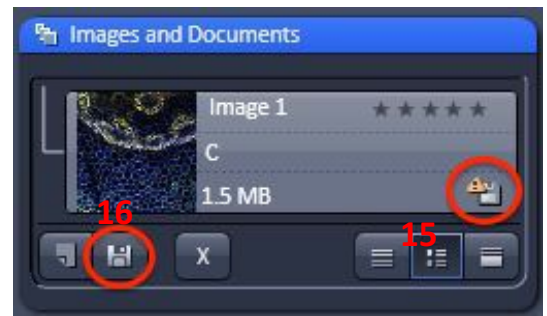
- **Reusing previous settings**

17. Load a previously captured image (*Menu > File > Open...* and select the image you wish to open).

18. Click the *Reuse* button – it has a symbol that looks like a recycling symbol on it and can be found below the current image and above the *Locate/Acquisition* tabs.

19. Alternately, you can save imaging configurations at the top of the *Acquisition* tab in *Experiment Manager*. Click on the *Disk* icon (middle button) to save your current experiment settings. Click on the *Folder* icon (first button) to load a saved experiment setting.

You can only *Reuse* images that have been saved on the same system. Older images may not work due to configuration changes. If this happens you will need to restart the hardware and software, then setup your experiment from scratch using *Smart Setup*.



Connecting to Network attached Storage

- Data you acquire on this device should be backed up to a server for future retrieval and use.
- The IMB has access to a number of servers both internally managed, as well as UQ-wide resources, think carefully where you would like to store your data.
- Internal IMB Server address include:
 - **IMB Share**
 - Backed up
 - Group shared server
 - \imbshare.imb.uq.edu.au
 - **IMB Transfers**
 - Not backed up
 - Deleted every 7 days
 - \transfers.imb.uq.edu.au
 - **Objective Server**
 - Microscopy only
 - Not backed up
 - \objective.imb.uq.edu.au
- UQ-wide resources:
 - **UQ RDM**
 - Project based shared drive
 - Can be cached locally (\data.imb.uq.edu.au)
 - For more information see rdm.uq.edu.au
 - Note this uses UQ-SSO credentials (type `uq\username`)

Shut down

- **Turning off the lasers**
 - Argon ion laser
 - Turn the key on the front of the control box to stop the laser and wait for the fans to stop
- **Exit ZEN software.**
- Transfer files from local computer to network storage.
- **Shut down computer**
 - Wait sixty (60) seconds after quitting ZEN.
- **Cleaning up the scope**
 - Carefully remove water from the objective using lens tissues provided (**not** Kimwipes).
 - Wipe up *any* spills you may have made.
 - Also, wipe up any shards of glass you might have produced: nobody likes being jabbed by little slivers of glass in a biology lab.
- **Turn off the microscope** by switching off the remote control. However, you must first allow the argon ion laser to cool down until the fan shuts off.

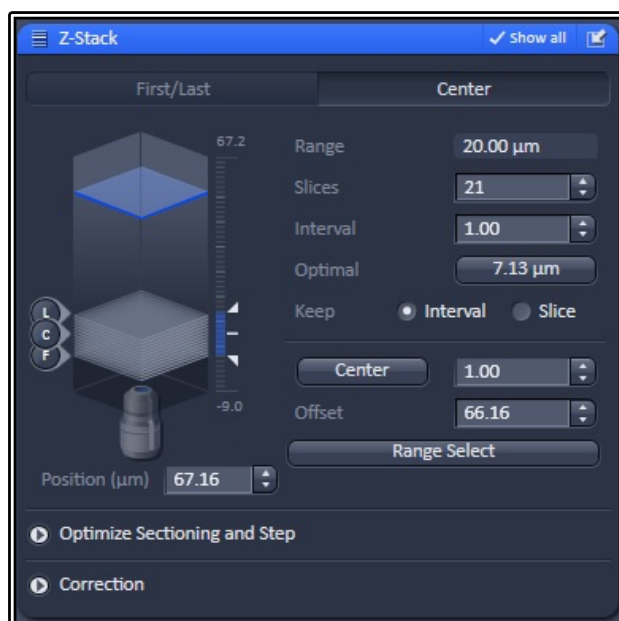
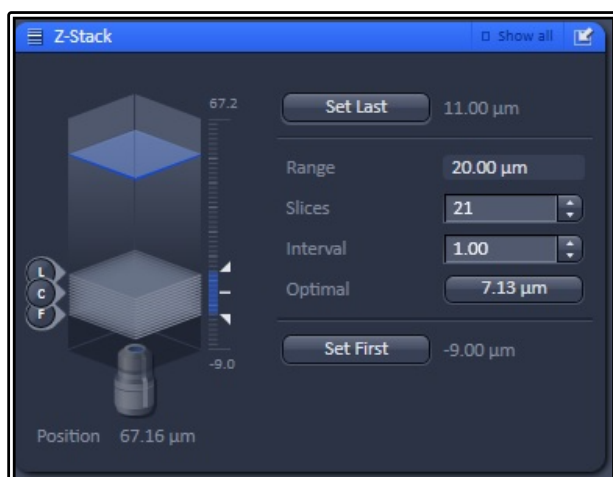
Advanced Imaging Setup

You can combine any of the advanced setup options together, such as Z-Stacks, Time-Series or Tile Scan to create more complex experiments. To activate any of these options simply tick the setting you wish to have added under the Acquisition Tab at the left-hand side.

Z-Stacks

- Click the Live button to get an updating view to assist in focussing
- Select the range of your 3d Z-Stack
 - o For First/Last method, start by focussing to the top of your object and click the Set First button, next focus to the bottom of the object and click Set Last
 - o For Center method, focus to the middle of your object and click the Center button
 - Note that the Center method is only available once the Show All box is ticked
 - Center method is ideal for multi-position acquisition where the Center of your Z-Stack will be taken as the Z co-ordinate of your position
 - Range Select will perform an X-Z scan, showing the sample from a side-view with three lines; two red lines represent the top and bottom of the stack while a green line represents the centre of the scan – these lines can then be moved to adjust the Z-Stack settings
- Click Stop to halt the Live View
- Set the Z slice thickness by either manually entering the Interval size in microns or by clicking on the Optimal Button
 - o In the example shown, the optimal size is set to 7.13um – this is approximately Nyquist sampling, but should you wish high accuracy it is recommended to use the Nyquist calculator to find the exact distance required
 - o You may choose to use the Slice number rather than Interval size
- Click Start Experiment to begin acquisition (**NOT** Snap as before), the experiment will capture all channels set with all 3d slices

NOTE: You can also select to capture all channels for each slice or a more efficient way is to capture a full Z-Stack for a single colour before proceeding to the next track



Time-Series

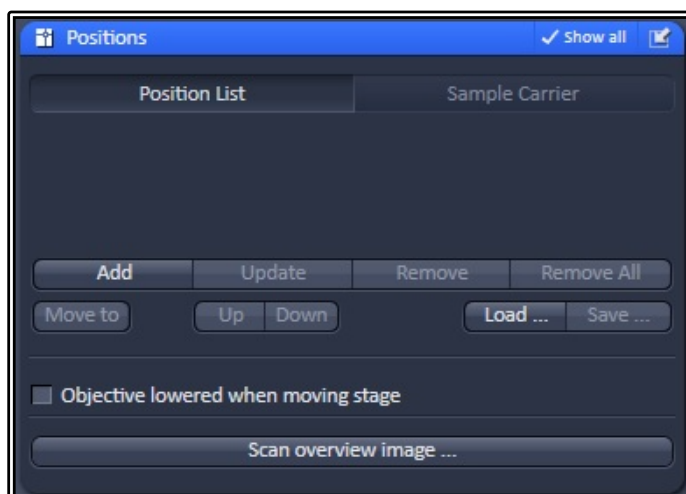
- Activating the Time Series option allows users to set up for time-lapse imaging to capture movies of live events
- Set the Cycle number (this is the number of times the acquisition setup will repeat)
- Set the Interval (this is the time the system will wait between capturing one image and starting the next)
 - o Use 0.0 as your value if you wish to image as fast as possible
- Click Start Experiment to begin the time-lapse capture



Positions

Adding multi-point coordinates allows for more efficient time-lapse imaging, granting multiple data points for the same time spent on the microscope

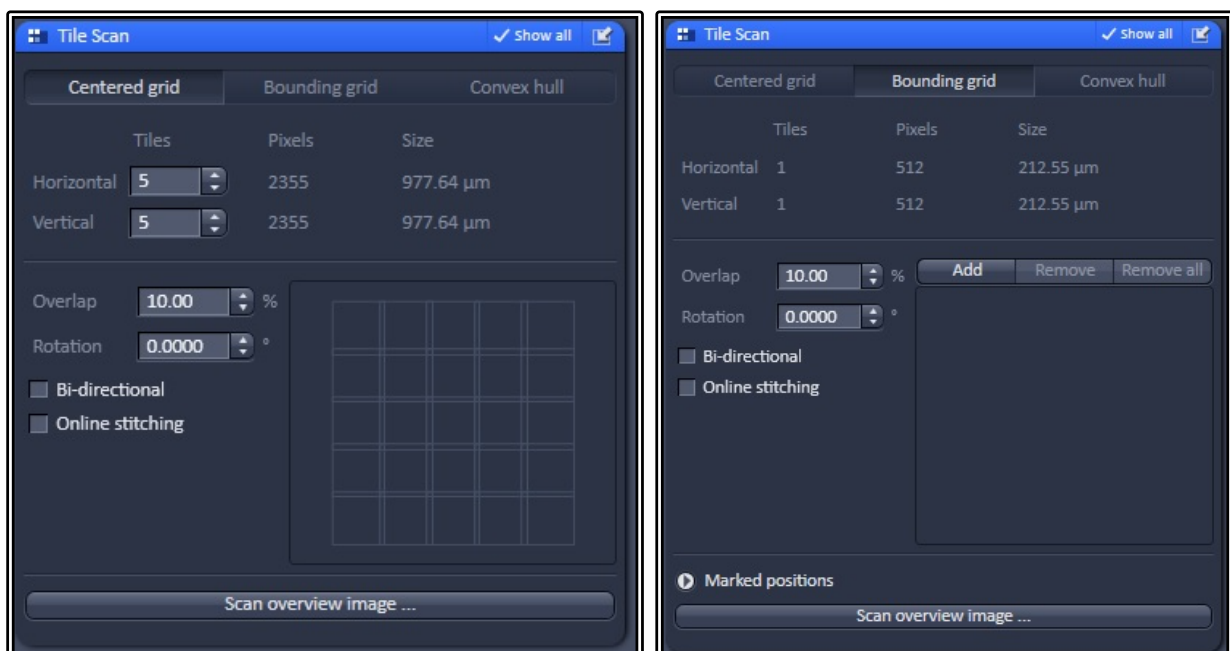
- Activate the Positions option
- Move to the cell or sample area of interest and focus using the Live button
- Click the Add button to add the X, Y and Z coordinates of this area to the Position List
- Repeat the process for any subsequent areas you wish to image
- Should you wish to change any position, click on the point in the list, click the Move To button and readjust the position, click Update to overwrite this position with the new coordinates
- To remove a single position, click on the position in the list and then click the Remove button to delete it from the list
- Remove All will delete the entire list of positions
- Use the Save button to save the coordinate list and Load button to import and reuse a previously saved position list
- Scan Overview Image allows the user to scan a large area at low resolution and utilise this as a quick template scan that can be used to more easily identify positions of interest
- The Sample Carrier section allows templates to be used to assist navigation



Tile Scans

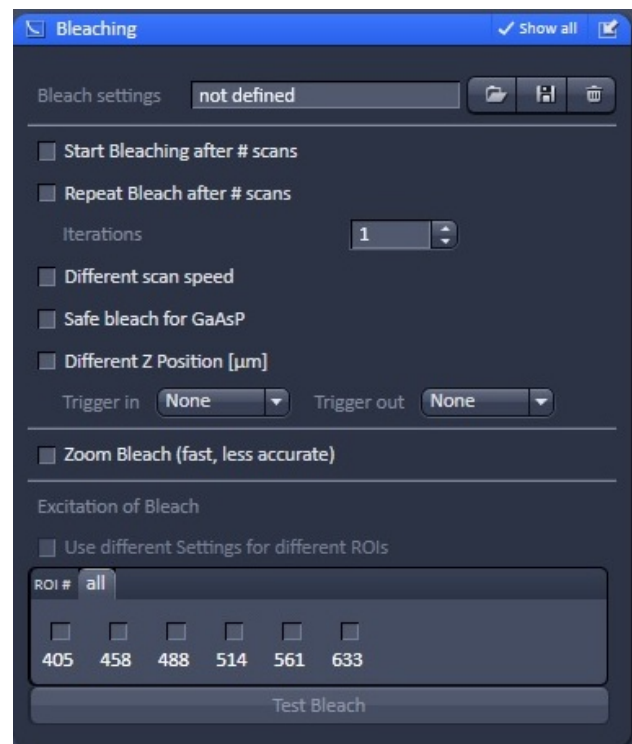
This feature allows multiple smaller fields of view to be stitched together to form one large image, giving users the ability to have a much broader overview of their sample while maintaining the resolution of the current objective.

- Select the Tile Scan option
- Use the Centered Grid tab to create set size mosaics
 - o Set the number of horizontal and vertical tiles required – these do not have to be symmetrical
 - o The software will use the current stage position as the centre of the tile
- Bounding Grid allows the user to set specific X-Y coordinates to include in the final tile scan
 - o Move to the first point of interest to include in the tile and click the Add button
 - o Repeat this move and Add procedure for all remaining points of interest to include
 - o The software automatically creates a larger tile to encompass all of these coordinates
- Convex Hull works in the same way as Bounding Grid but will exclude any tiles the software deems not required, saving time by avoiding the need to scan blank areas or areas of no interest
- Set your Overlap size to 10% minimum, this allows the stitching software to match the repeating patterns to provide a “pixel perfect” final image
- Avoid using Online stitching, this sometimes fails so it is better to manually stitch post acquisition
- Click Start Experiment to begin the tile scan acquisition
- Once completed go to the Processing tab, click on the Stitching method, select the current image and click Apply to create a stitched version
 - o You may wish to also select the parameter to create a New Output for the stitched image rather than replace the raw data



Bleaching (FRAP and FRET)

- Activating the Bleaching module will also activate Regions and Time Series as well since these will be required also
- Use the regions tool to select a specific area of the image, you may select any size or shape you require and even add multiple regions
 - o The region can be used for bleaching a specific area but can also be used for analysis of that area too
- Use the Start Bleaching after # scans to capture a set number of images before the bleach step (a “before” snapshot)
- You may also repeat the bleach after a certain number of images
- Set the number of iterations for the bleach step
 - o More iterations mean a longer bleach step which may be required for samples that you are struggling to bleach effectively
- Use the Different Scan Speed option to slow down the bleach step
 - o Slower speed means longer bleach step and longer pixel dwell time, resulting in a more effective bleach step
- Safe bleach for GaAsP allows for extra protection for the more sensitive detectors
- You may also choose to bleach in a different Z position
- Use Zoom bleach if you want a faster bleach
 - o Note this is a rough scan and therefore won't be as accurate to your ROI of choice
- Tick the laser you wish you use to ON and set the power, typically use 100% for the bleach laser
- Set the Time Series up to a number of repetitions to suit your experiment, this needs to be set to 3 or more for a successful “before” and “after” image experiment
 - o Failure to set this properly will result in a single bleach step but no other imaging
- Click Start Experiment to begin the bleach experiment
- Use the Mean ROI tool in the Image Window to display an analysis of the ROI over the time course of the experiment
 - o For typical FRAP studies you should see an initial level of signal for the “before” images followed by the bleach step causing a decrease in signal, then a recovery of the fluorescence over time
 - o Note the recovery will never reach 100% and instead plateaus after a while at a lower intensity than the original



Using the external BIG (GaAsP) detectors

The external BIG detectors on confocal 5 are GaAsP detectors and are much more sensitive than the internal PMTs making them ideal for use with dim, or not very bright samples. The BIG module comes off the external port of the scanhead and contains two detectors capable of simultaneous sample detection.

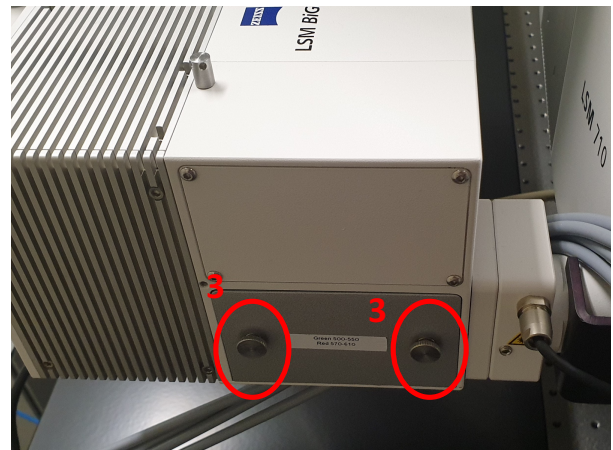
Currently we have two filter cube modules:

- CFP/YFP
- GFP/RFP

When you wish to use the BIG detectors, ensure the correct filter is in place by checking which filter is not in the microscope. (ie. If there is CFP/YFP cube in the container, then the GFP/RFP is in the microscope).

If you need to change the filter and the microscope is on, turn off the room lights before swapping the filter. These detectors can be blown if exposed to too much light.

1. Ensure the room is dark
2. Take the container with the filter cube in it behind the microscope (be careful of cables)
3. Unscrew and remove the existing filter (note the screws won't fall out)
4. Swap the filters over and slide the new filter into place gently (note don't touch the glass)
5. Screw the new filter in gently (it only needs to be finger tight)
6. Place the old filter back into the container and return it to the desk

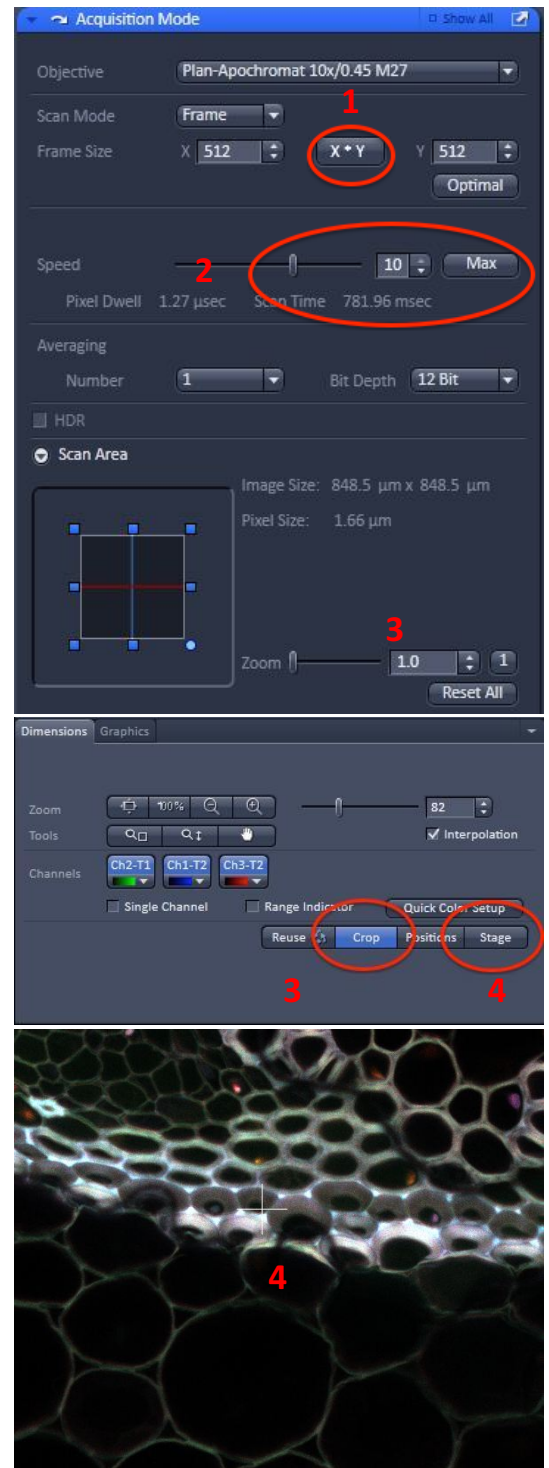


To set up the BIG detector channels for your imaging you need to use the manual light path mode. You will need to change the 'Mirror' dichroic to be either a plate (100% pass) or one of the existing long or short pass filters.

Note: the software doesn't know which filter cube has been put into the detector module, assume CFP/GFP and YFP/RFP go to the same detectors (GaAsP1/GaAsP2)

Additional Settings

- **1. Frame Size (X*Y Image Size and Resolution).**
 - Fewer pixels
 - **Pros** - faster scanning, reduction of photobleaching.
 - **Cons** - lower resolution, decrease in signal-to-noise ratio (SNR).
 - More pixels
 - **Pros** - increase in SNR and resolution (up to a point).
 - **Cons** - slower scanning, increased photobleaching.
 - Matching resolution of the image to that of objective
 - Click *Optimal* button, then
 - Select next larger image size from the X*Y button.
- **2. Scan speeds**
 - Increasing the scan speed will decrease bleaching, signal-to-noise ratio (SNR), and dynamic range of your image; the opposite is also true.
- **3. Zoom and Rotate**
 - **In General, Zoom does not increase the resolution of your image.**
 - Use the *Zoom* slider in the *Acquisition Mode* control, to zoom in on your sample.
 - Alternatively, use the *Crop* tool, which allows interactive control of *Zoom*, *Rotation*, and position with an intuitive interface.
- **4. Stage Tool**
 - Click the *Stage* button to overlay a cross on the centre of the screen.
 - Click on an object of interest in your image to move your sample to the centre of the image.
 - Click *Scan* to capture a new image.



System Hardware Summary

Microscope Stand: Zeiss Axiovert 200 Inverted Microscope

Objective Lens List

- PInApo 10x/0.45 DIC II (WD = 2.0mm)
- PInApo 20x/0.8 DIC II (WD = 0.55mm)
- ECPlnNeoF 40x/0.75 Water (WD = 0.71mm)
- LD C-Apo 40x/1.1 Water (WD = 0.62mm)
- LD C-Apo 63x/1.15 Water (WD = 0.60mm)

Laser Lines Available

- 405nm
- 458nm
- 488nm
- 514nm
- 561nm
- 594nm
- 633nm

Main Beam Splitter Dichroics

- 458
- 458/514
- 458/561
- 488
- 488/561
- 488/561/633
- 80/20
-

Dichroics Beam Splitter for Single v Multi-channel:

- BP420-460 + LP500
- LP525
- LP570
- LP660
- LP460
- SP615
- Mirror (100% internal detectors)
- Plate (100% BIG detector)

BIG Detector Filters

- CFP/YFP
- GFP/RFP