ZEISS LSM 710
Confocal microscope
Manual/Quick guide

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Imaging
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Index

Starting the system 3

Switching off the system 5

Starting Up ZEN black 6

Viewing your sample – **Locate** 6

Experiment Manager **- Acquisition** 7

Setup Manager

Laser 7

Light path 7

Acquisition Parameters

Acquisition mode 8

Channels 9

Transmitted Light (Brightfield) 9

Optimizing the dynamic range (saturated pixels) 10

Zoom – Crop Box 10

Scale Bar 10

Multidimensional Acquisition

Z-stack 11

Time Series 11

Bleaching 12

Tile scan 12

Stitching a tile scan 13

Positions 14

Regions of Interest/ROIs 14

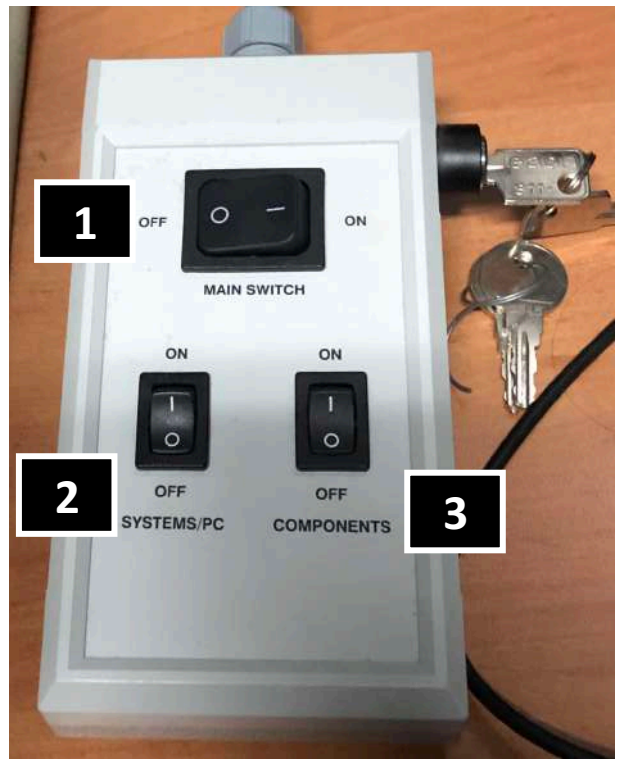
Saving Images 14

Opening .czi files later 14

Troubleshooting

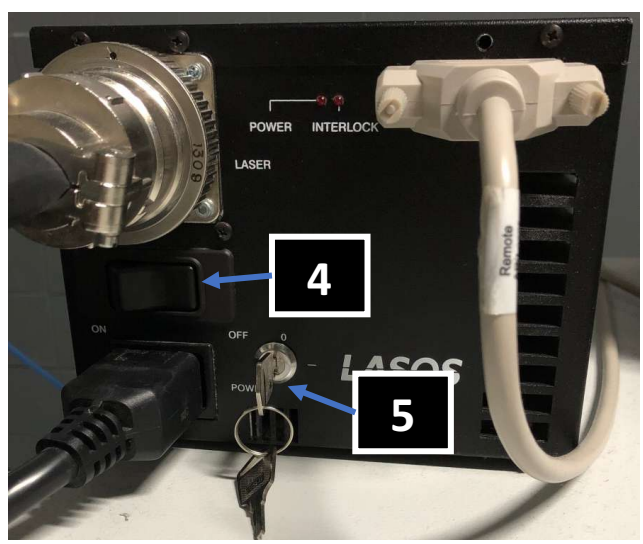
Zen software stalled at 2% 15

Starting the system



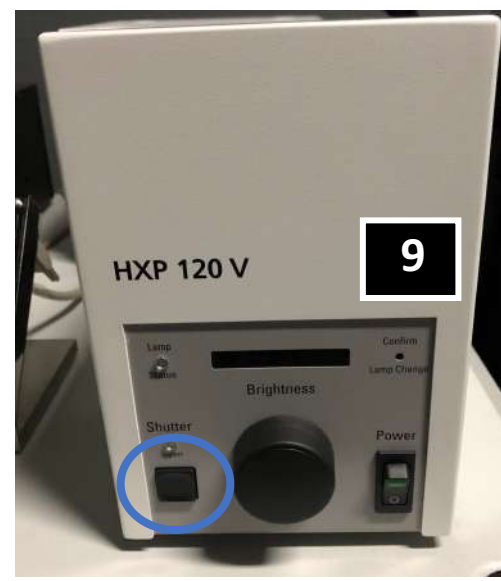
- Press main switch to ON **[1]**
- Press System/PC switch to ON **[2]**
- Press Components switch to ON **[3]**

NOTE: If you only need to retrieve data from the computer you just need to turn the computer ON (no switches)



- If the Argon Laser (black box) is required (458, 488, 514 nm) press the switch button to ON **[4]** and turn the key (horizontal= ON) **[5]**

REMEMBER : After 5 minutes, switch the Argon laser from standby (idle power) to run (UP) with the toggle switch **[6]**. LED should be green **[7]**



- Turn the PC workstation On **[8]**
- Turn the fluorescence lamp On **[9]** (don't forget to open the shutter)

Switching off the system

1. Save your data on D:/Users/PI folder/"your folder".

2. Check if the confocal is in use in less than 2 hours after the end of your session. If **yes** leave the system and lasers ON (check which lasers will be used). Leave the fluorescence lamp ON **only** if the next user comes in less than 45 minutes.

3. Turn off the fluorescence lamp. **[9]**

4. Turn off lasers (561, 633) within ZEN .

5. Put the Argon laser on standby mode (toggle switch DOWN).**[6]**

6. Turn the Argon key to OFF. **[5]**

7. Exit Zen software.

8. Burn your data onto a CD, DVD or MICC external drive. Do not use USB keys to transfer data as it put the computer at risk of viruses.

9. Clean the objectives (40x and 63x) with LENS TISSUE ONLY and cleaning solution.

10. Shut down the computer.

11. Cover the microscope with the blue cover to protect from dust.

12. When the Argon fan switches off (after ~5 min), press the **switch button** of the Argon laser (black box) to OFF. **[4]**

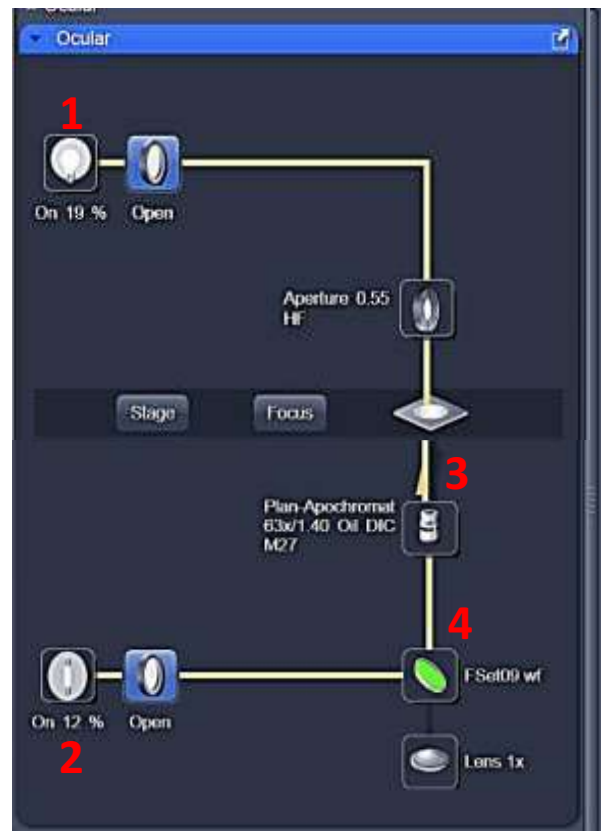
13. Turn off the **Components [3]**, **System/PC [2] and Main switch [1]** switches.

Starting Up ZEN black

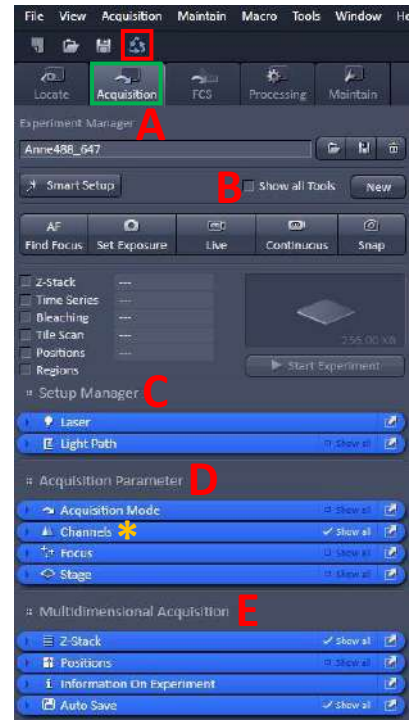
- Follow the directions for Powering up the confocal LSM 710
- Double click Zen black icon 
- Click start system tab

Viewing your sample - Locate

- When Zen opens you will be under the Locate tab, this is where you can view your sample through the eyepiece;
- Set your sample on the stage with the coverslip facing the objective; turn the objective down before you do so (load position). Manually bring the objective up to the slide with care; don't smash the objective into the glass; *Please remember to add oil when appropriate*;
- If you want to locate your sample with transmitted light, turn 'Transmitted Light' on **[1]** , for locating a fluorescent signal, choose 'Reflected Light' **[2]**. Make sure the Light is "On" and the Shutter is "Open" (a fat yellow beam line appears)
- Choose your objective by clicking on the objective button **[3]** (or touchpad); this will bring up a list of objectives currently on the microscope.
- Choose filter (for fluorescence) by clicking on the filter button **[4]**; this will bring up a list of filters currently on the microscope (blue, green and red).
- Find your sample, move it to the center and focus. The stage joystick has two movement modes, slow and fast, which can be switched by pressing the F1 button of the joystick.



Experiment Manager- Acquisition



- Under '*Experiment Manager*'[**A**] you can load previous settings (open "your" folder, open a previous image and Reuse); you can choose different kinds of acquisition modes (z-stack, Tiles, etc);
- *'Show all tools'* should always be checked **[B]**
- There are 3 blocks of blue windows (*setup manager [**C**], Acquisition parameter [**D**] and multidimensional acquisition [**E**]*) which will expand/retract if you click on the blue bar:

Setup Manager

Laser

Switch on the Lasers you want to use **[1]. What laser should I use?** (examples of commonly used dyes and the laser lines used to excite them)

Laser	Dye
405	Dapi, Alexa 405
458	CFP
488	GFP, FITC, Alexa 488
514	YFP
561	Alexa 543, Alexa 568, Alexa 594, Rhodamine, Mitotracker red
633	CY5, Alexa 633, Alexa 647, DRAQ5

Light Path

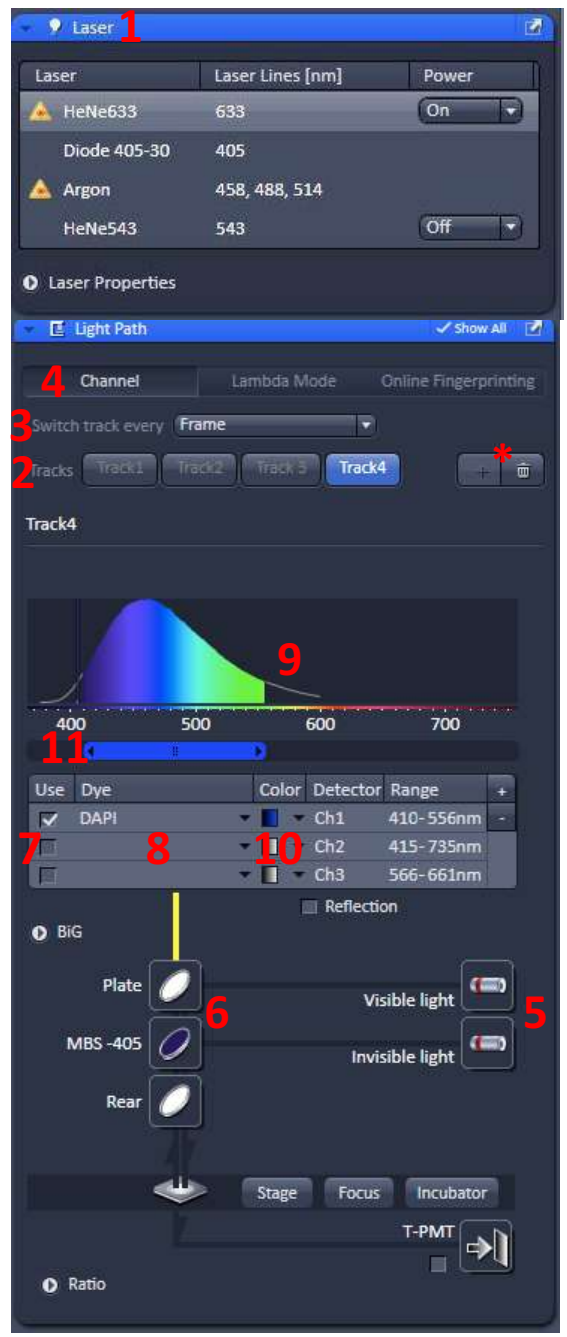
Here you setup each light path you will be using to capture your images.

- Each light path configuration is known as a **track [2]** (the detector settings appear under **Channels window***).
- You can capture multiple channels (using different detectors) within the same track (*Single-Track/Simultaneous*) OR
- You can capture multiple channels (using the same or different detectors) with different light paths, using separate tracks (*Multi-Track/Sequential*). Tracks can be added/deleted by clicking on "+"/trash bin icon*
- In Multi-track mode, you can choose how the microscope should switch between different tracks: *'Switch track every'* **Frame** or **Line [3]**.
- Configure a track under the **Channel [4]** tab:

I. Choose the required laser line **[5]** and primary dichroic mirror **[6]**. The laser line will appear on the track definition as a vertical line at its emission wavelength.

II. Check one or more detection channels **[7]** and choose the fluorophore **[8]** being imaged from the drop-down menu. The emission spectrum for the fluorophore (from PubSpectra) is displayed on the track definition **[9]**. Assign a colour map to the track **[10]**.

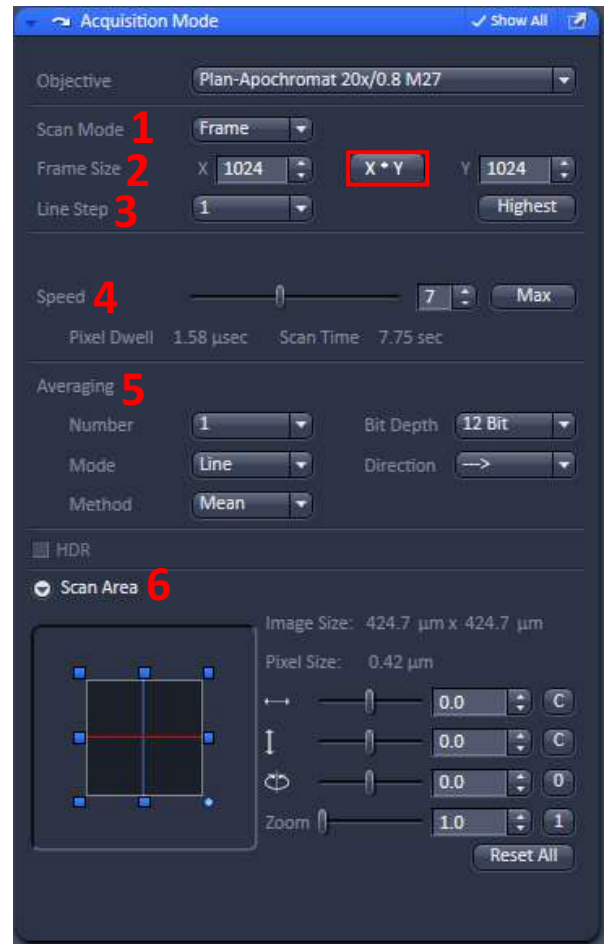
III. Choose the detection bandwidth using the slider **[11]** covering the required emission wavelengths.
(*Repeat steps I-III for the other tracks*)



Acquisition Parameters

Acquisition mode – Recommended settings

Here you select the resolution and quality of imaging – essentially you need to find a balance between image quality and speed while considering how much your sample will bleach:



Scan Mode [1]: would be usually 'Frame'

Frame size [2]: how many pixels in the image; 512x512 will give you a fast image but a resolution of at least 1024x1024 is recommended (if you need to change your Frame Size click on the X*Y; this has an impact on your pixel size

Line Step [3]: you can choose to skip every nth (series of numbers) horizontal scan line: makes the scan faster, but the horizontal resolution decreases

Speed [4]: how fast the laser line travels over your sample; a slower speed will give you a better image but will take more time (a scan speed of 7/8 is recommended)

- Pixel Dwell indicates how long the laser illuminates one pixel (depends on both Frame Size and Speed),
- Scan Time indicates how long it takes to scan a frame with your chosen settings.

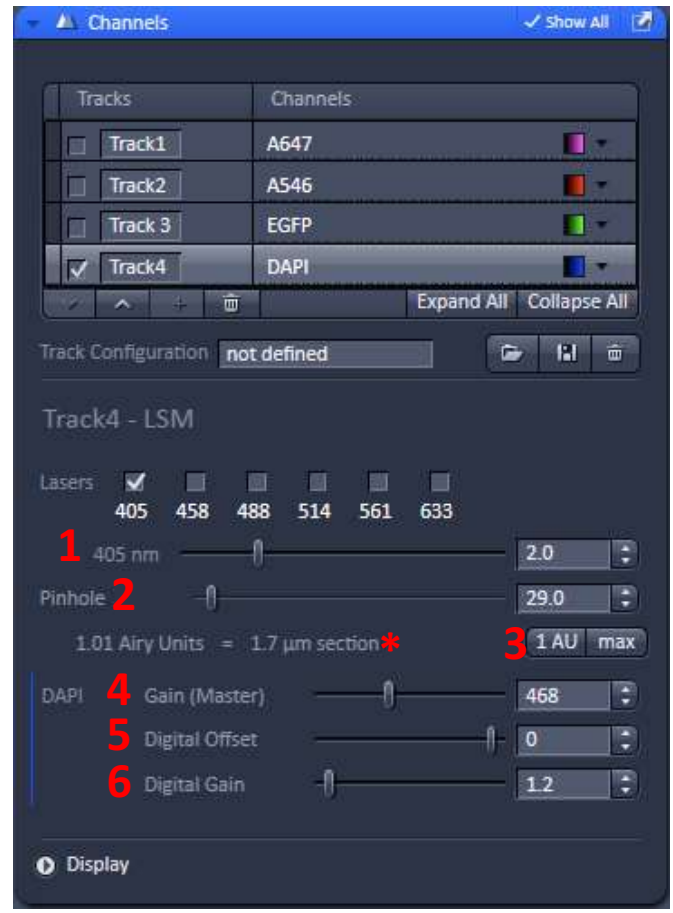
Averaging [5] Averaging will remove noise from the image. A slower scan speed will often require less averaging

- Number: how many times is each pixel scanned; generally 2 – 4x averaging will produce a good image
- Mode: if the repetition of the scan should happen after each line or after each frame;
- Method: if the Mean or the Sum of the repeated scans should be taken (choose **Mean**);
- Bit Depth: standard - 8 Bit uses 0-256 levels of grey. 12 Bit uses 0-4096 levels of grey, producing a smoother image with more information for intensity analysis but will generate a larger file;
- Direction**:** Choose between --> and <--> for mono- and bi-directional scanning. Bi-directional scanning will significantly reduce the acquisition time for each frame

Scan Area [6]: If you would like to see more detail in an area you can increase the zoom

Channels

Here you see again your track configuration. This is the control window for adjusting the detector sensitivity and pinhole size and needs to be set *for each channel.*



Laser [1]: laser power can be adjusted from 0-100%. The higher the laser power, the stronger the signal and the stronger the bleach. Don't crank the laser to max before trying adjusting the detector settings!

Pinhole [2]: *For each channel you need to press the **1 AU button*** **[3]** (1 airy unit) to ensure you are taking a confocal slice. You should ensure that all channels have the same *optical section thickness*.* This will mean adjusting the pinhole settings slightly for each channel.

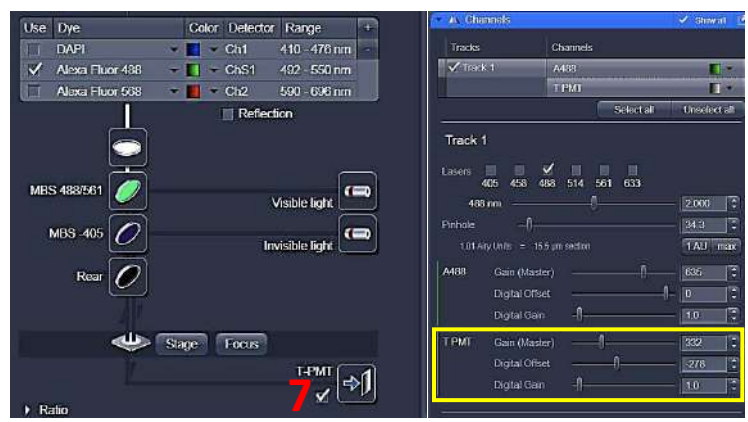
Gain (master) [4]: Controls the sensitivity of the detector. The higher the setting the brighter the white. Drag the controller to about halfway (It is recommended you stay between 600-700), any higher than this and you begin to introduce noise. If it is too bright when you take an image you can drag it back towards zero or decrease the laser power (if the image is still too dim you may need to increase your laser power).

Digital Offset [5]: detector black level (keep at 0)

Digital Gain [6]: Simply put, Digital Gain adjusts the brightness of your image; leave at 1.0 (or 1.5 if the image is still to dim after laser power and master gain optimizations)

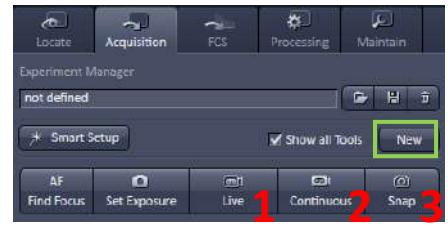
Transmitted Light (Bright Field)

If you would like to add a transmitted light image click the T-PMT in one of the already set tracks:



- Under the Acquisition tab, in Light Path window Click on **T-PMT [7]**. It uses whatever lasers are active (changing any laser power affects this image)
- Under the Acquisition tab, in channels window, adjust gain and offset

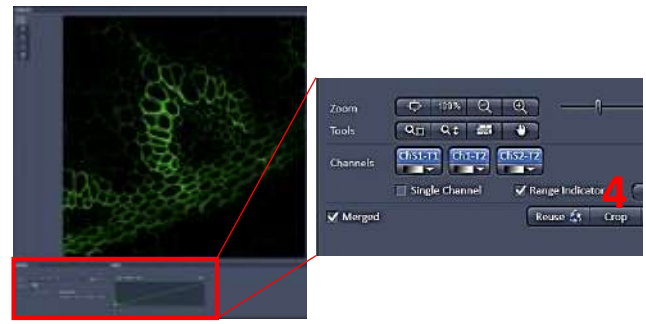
Optimizing the dynamic range (saturated pixels)



- Use **continuous** or **live** imaging so the image is constantly updated. **Live [1]** mode will configure the scanner to capture images as fast as possible whereas **continuous [2]** uses the scanner settings from Acquisition mode.

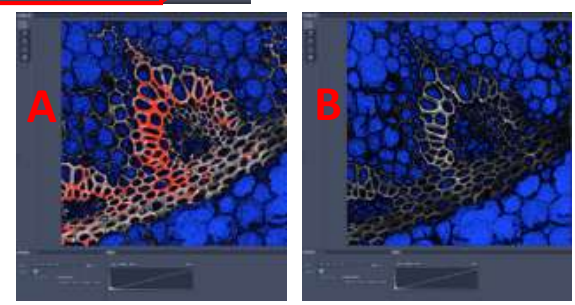
- In the bottom left, below the image, check the '*range indicator*' **[4]** box.

  - The range indicator shows over-saturated pixels as red and true black pixels as blue **[A].**



- Adjust the ***master gain*** until no/little red is seen and the ***digital offset*** until small amount of blue is visible (usually 0.0) **(B).**

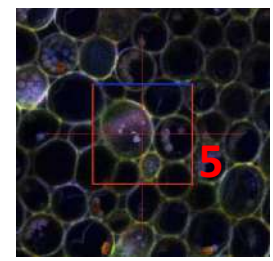
- Use **Snap [3]** to take images for all selected tracks.



NOTE: After taking an image **Save** it immediately or open a new window **(New)** to take a new one.

Zoom-Crop Box

- You must stop scanning to use
  - Click on **CROP**
  - Position box **[5]** on object of interest
  - Resize, move, rotate



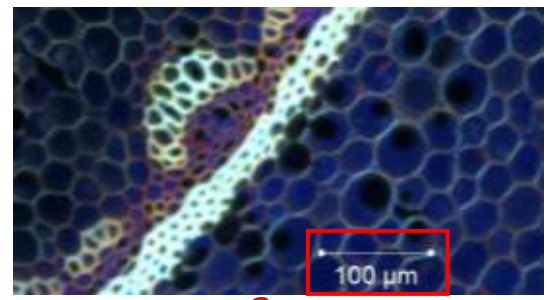
- **Live** to see zoomed image

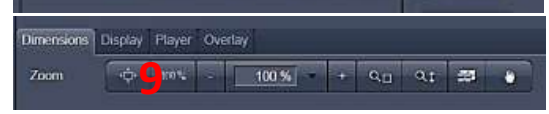
Scale Bar

- Go to Overlay **[6]**
- Click on ruler icon **[7]**
- Click on image and drag to set length.
- Change color **[8]**, etc

To save scale bar on image:

- Change to single image (2D vs Split)
- Use 100% for screen zoom **[9]**
- File-Export
  - Choose High resolution contents of image window
  - Choose channels
  - Save as tif

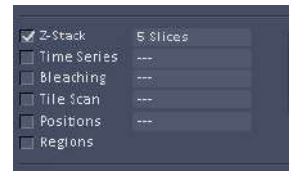




Multidimensional Acquisition

Z-Stack

- To make a z-stack first click on Z-Stack (under the experiment manager); scroll down on the left until you see a Z-Stack box (ensure Show All is enabled 1).



- There are two options for Z-Stack: **First/Last** and **Center**

First/Last

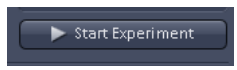
- Select 'First/Last' imaging Mode **[2].**

- While fast scanning using Live, move the focus to the top of your sample and click Set Last **[3]**

- Now move the focus to the bottom of your sample and click Set First **[4]**

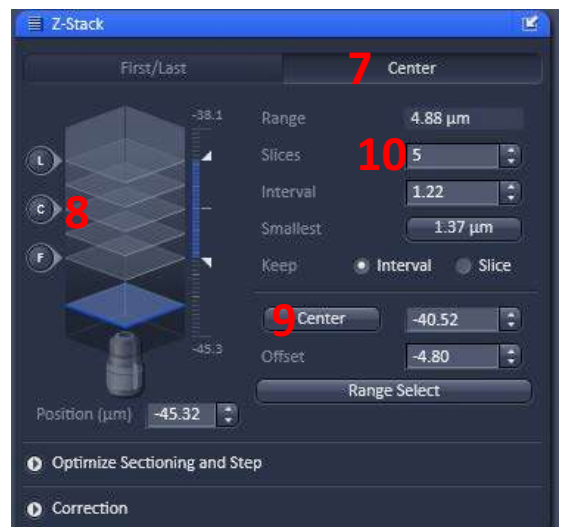
- Click Smallest Interval **[5]**
  - For 3D, the optimal interval is half the slice thickness (determined by the pinhole settings and objective). **note if you are not doing 3D you don't need to oversample; set your interval **[6]** and check your number of slices.

- Click on Start Experiment



Center

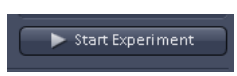
- Select 'Center' imaging Mode **[7].**



- Click on Center then find the center of the sample you would like to image. Click on **C** **[8]** to take a Snap of the center image to make sure you are in the right spot. Click Center **[9]** to confirm this is the slice in the middle of the stack;

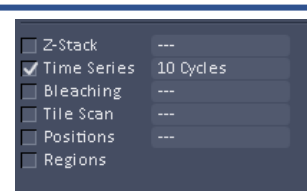
- Set your interval and check your number of slices **[10].** Make sure you have an odd number of slices **note if you are doing 3D you need to oversample

- Click on Start Experiment



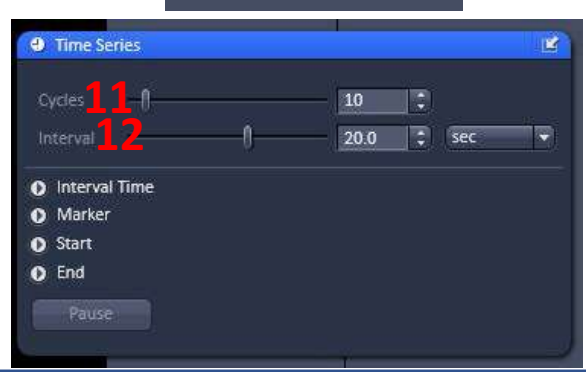
Time Series

- To do a time series, first click on Time Series. The Time Series tab will appear under the Multidimensional Acquisitions on the left side.

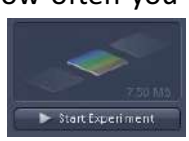


- Set the number of cycles **[11]** for how many images you would like to take.

- Set the interval **[12]** for how often you would like to take an image.



- Click on start experiment



Bleaching

- Click on Bleaching (under the experiment manager); Time series and Regions should also activate

- Define region(s) **[1]** *(see regions menu, pag13)*
  - Select the shape and draw your regions

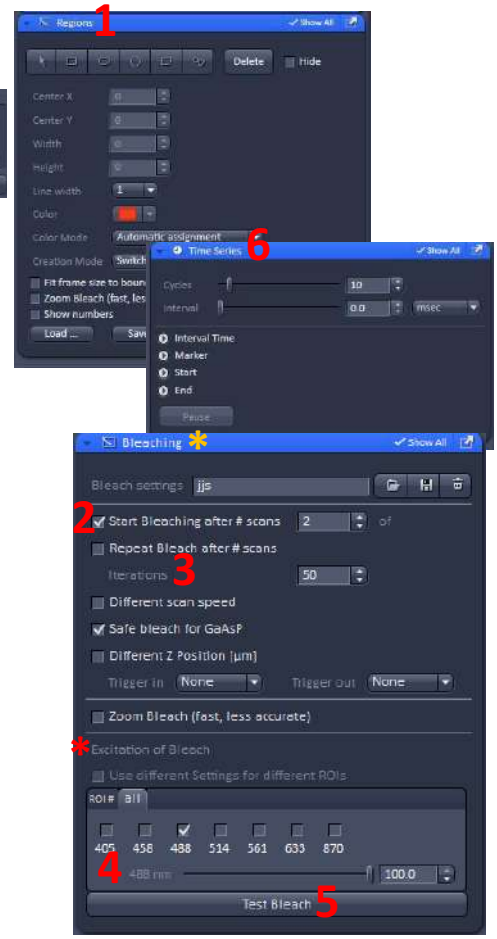
- Under the 'Bleaching' window* check '*start bleaching after # scans*' and select a number **[2]**

- Determine the number of iterations **[3]**; Iterations is the number of scans during the bleaching; more iterations will bleach longer/more

- In Excitation of Bleach* select the laser and laser power **[4]**
  - 405nm at high percent will give fastest bleaching
  - Slower scan speed will give better bleaching, use fewer iterations
  - Click '*Test bleach*' to test iterations and laser power **[5]**

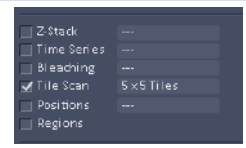
- Set up Time series; in the time Series window **[6]** set the number of cycles and the interval between the cycles;

- Start Experiment



Tile Scan

- To create mosaic images of large areas of your sample select **Tile Scan** from the **Experiment Manager**.



- There are three options for Tile Scan:
  - **Centered Grid**
  - **Bounding Grid**
  - **Convex Hull**

- You can start with an overview scan with any of the three options - 'Select Scan Overview Image' **[7]** (Select the number of tiles ✛ and the objective and click Scan **[8]**)

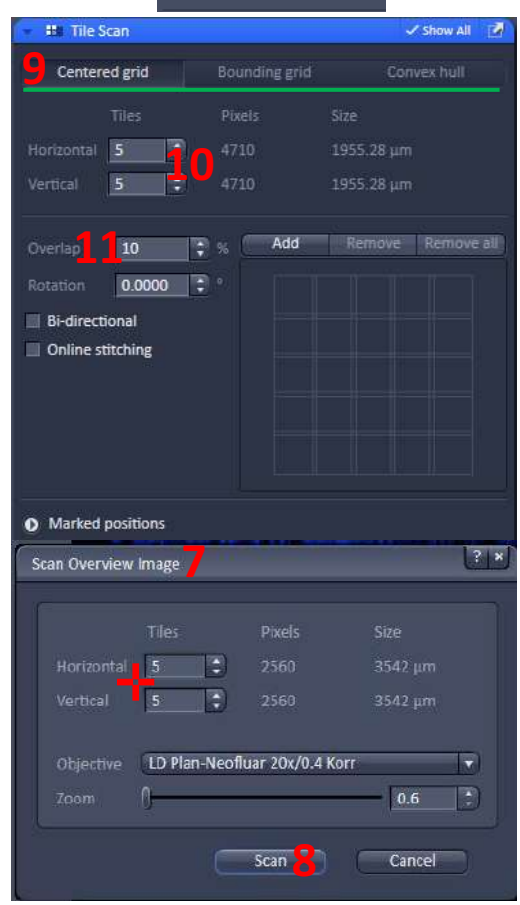
Centered Grid

- Under the Centered grid tab **[9]** choose the number of Horizontal and Vertical **[10]** tiles you would like to use.

- Define an overlap **[11]** (typically 10%) between tiles. This is an important step for stitching the tiles in a post-processing step.

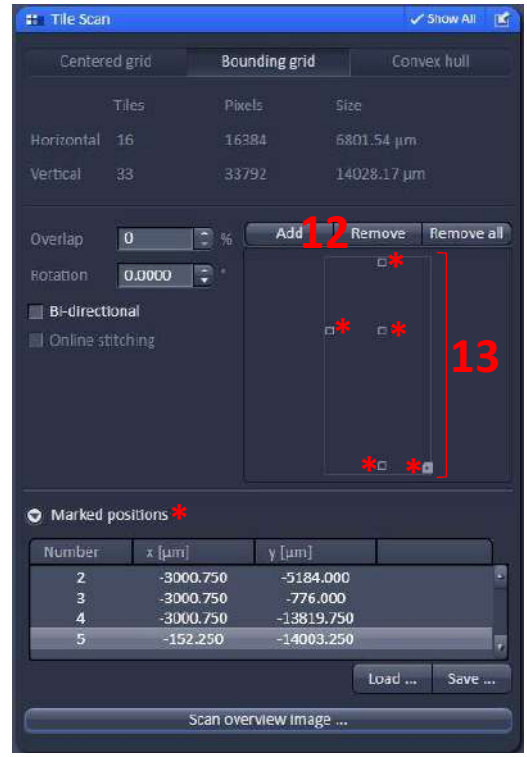
- Click on Start Experiment

- After acquisition click **Processing** and use the **stitching** tool to merge the image tiles together into a single image.



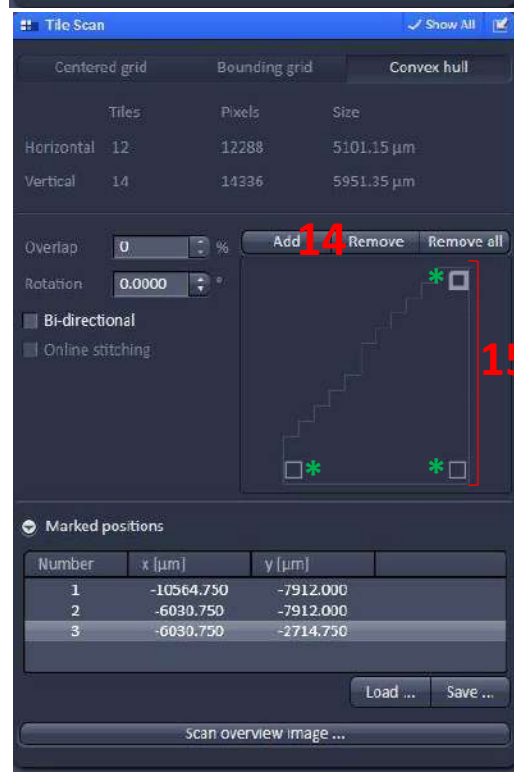
Bounding Grid

- Manually move the stage around to determine the edges of your sample.
- Select Add to add that position**[12]**
- Addition of several positions* creates a large boxed area that will be scanned **[13]**
- Select Star Experiment to begin the scan.
- After acquisition click **Processing** and use the **stitching** tool to merge the image tiles together into a single image.



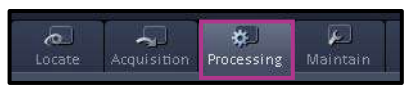
Convex hull

- This tile scan method is similar to bounding grid, but is not square. Addition of positions to the grid will result in a scan pattern with no wasted space
- Manually move the stage around to determine the edges of your sample.
- Select Add to add that position**[14]**
- Addition of several positions* creates a large area that will be scanned (no wasted space) **[15]**
- Select Star Experiment to begin the scan.
- After acquisition click **Processing** and use the **stitching** tool to merge the image tiles together into a single image.

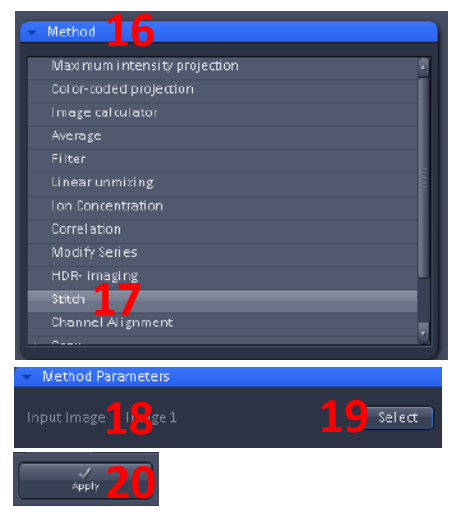


Stitching a Tile Scan

- Click on the Processing tab

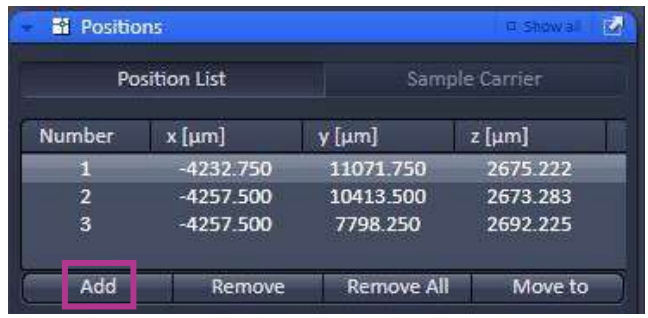


- Under Method **[16]** click on stitch **[17]**
- Under Method Parameters click on the input image **[18]** you would like to stitch and then click on Select **[19]**
- Next click on Apply **[20]**, this will stitch your image for you
- Finally save your file, it will have the original image name with an "_Stitch" at the end.



Positions

- For automated scanning of multiple positions (2D images or Z-Stacks) go to the position you want to scan and then press **Add** The system will save the coordinates.
- For starting the multiple position scan, press "**Start Experiment**" under "**Experiment Manager**"



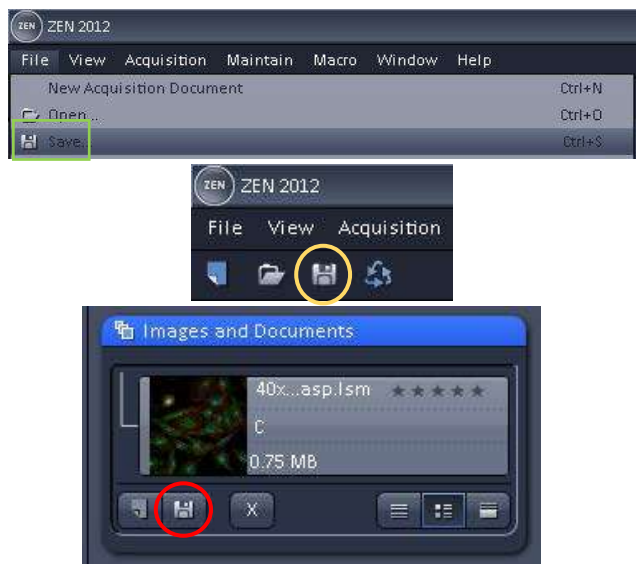
Regions of Interest / ROIs

Can be used to image a small section of the image frame or for bleaching experiments. **The smaller the region in the Y axis (height) the faster the confocal can scan the image.*

- select **Regions** from the **Experiment Manager** and open the regions toolbox **[1].**
- Chose the region shape [**2**]
- Draw the region over the image.
- Tick the "fit frame size to bounding rectangle of regions" box.
- Click single/continuous to image the region of interest.

Saving Image Files

- Single images - **Save Immediately** or open a new document window. You may save your file by using one of the following options:
  - Clicking on File, then Save;
  - Clicking on the disk icon on the top left
  - Clicking on the disk icon on the right, under *'images and documents'*
- Z-stack, Time series, Tile, etc are not overwritten. You can save later.
- Save files to **D:\ALL USERS\ PI folder\"your" folder**
- Save files as .czi.
  - stores all the hardware settings
  - Reuse the settings from any image
  - Information to see settings



Opening .czi files later

- Zen Lite 2012 Blue or Black (free)
  - On our workstations
- FIJI (is Image J)
  - Free software for PC or Mac

Troubleshooting

Zen software stalled at 2%

When the startup screen appears, click on the **BOOT STATUS [1]** drop down menu.

If the **screen stalls at 2%** or an error about the Real Time Controllers pops up:

- Exit ZEN black software, go to the box (PC) near the confocal **[2]**, open the "lid" **[3]** to see the RTC reset button **[4]** and press it for 5 seconds. Wait 30 seconds. Restart the ZEN software.