

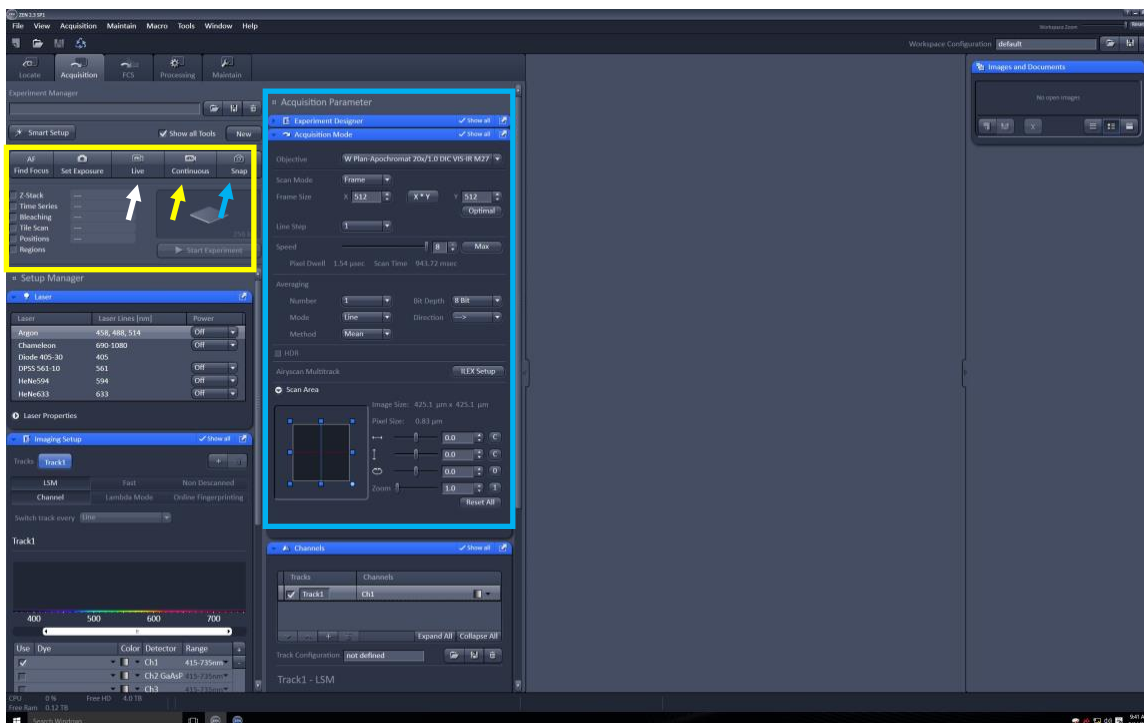
SOP for Acquiring Confocal Images with Zeiss LSM 880 and Zen Black Software

1. First Locate, View and Focus your sample through the eyepieces as outlined previously with controls in the Locate Tab window. (See SOP for turning on system)
2. Click on the Acquisition Tab in Zen



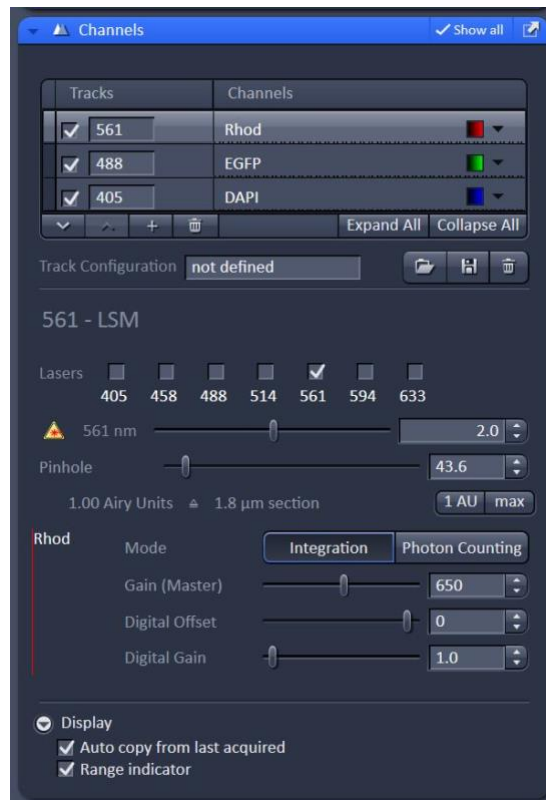
3. The Acquisition Window is divided into several parts
 - a. Experiment manager (White Box) has buttons for acquiring
 - i. a Live Image (Live – white arrow) (a 512X512 image with no averaging) – allows for focusing and setting Laser intensity and detector gains and offset.
 - ii. a Continuous image (Yellow arrow) – includes averaging and shows what an acquired image will look like. Use primarily for Airyscan imaging.
 - iii. Snap image (blue arrow) – acquires an image with averaging, etc.
 - iv. Z-stacks and Time-series stacks using the Start Experiment Button (Green Arrow)
 - b. Experiment manager also has check boxes to enable acquisition of:
 - i. Z-stacks
 - ii. Time-series
 - iii. Bleaching
 - iv. Tile Scans
 - v. Positions
 - vi. Regions
 - c. Acquisition Parameter panels (Yellow box) controls the
 - i. Acquisition Mode
 1. The microscope objective being used

2. The Scan mode (Frame is the default, but line scans are also possible)
3. The pixel density imaged (1024 X 1024 or 512 X 512 are most common – other densities possible – the low the values the faster the image is acquired)
4. Acquisition Speed (Slider from min to max – shows the pixel dwell time and the Scan Time.
5. Averaging
 - a. Number of images averaged (1 is no averaging, 2 or 4 are common values);
 - b. Mode of averaging - Line is default (fastest);
 - c. Method (mean is default – can also sum)
 - d. Bit depth (8, 12 or 16)
 - e. Direction (<--> = bidirectional (fastest); --> = unidirectional (slower)
6. Scan area
 - a. Provides info on image size (in μm)
 - b. Pixel size and Pixel squareness
 - c. Zoom - from 1X to 5X – this works great such that most imaging can be done with a 20X objective.



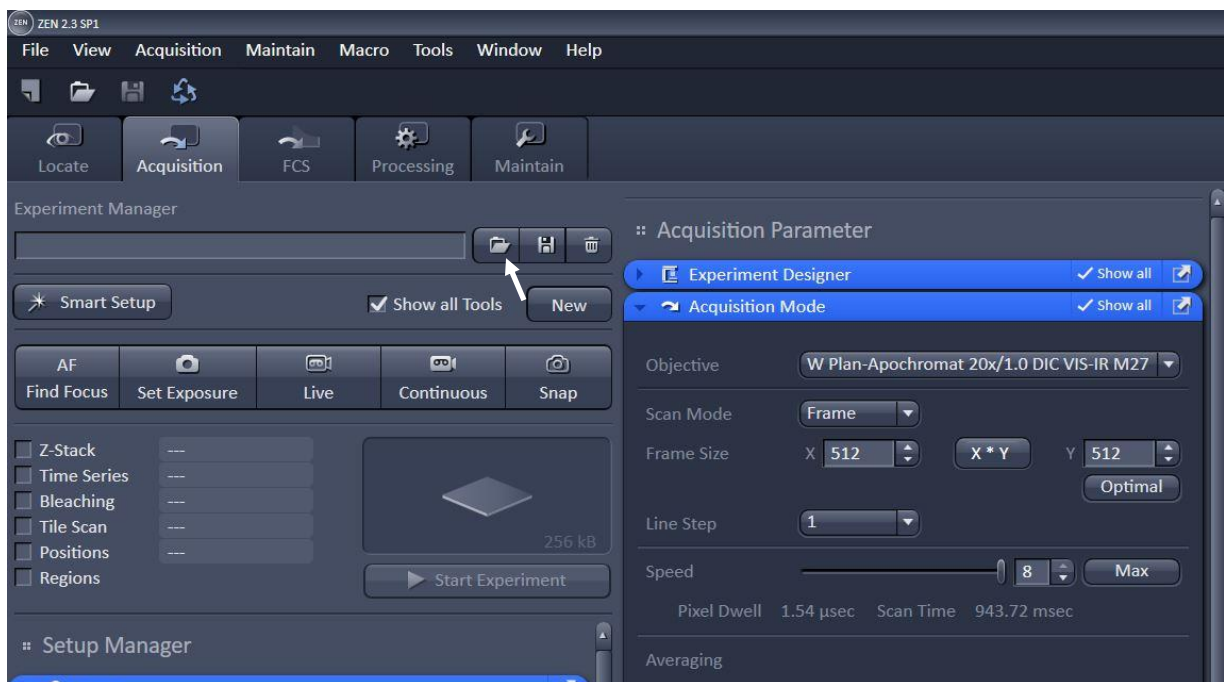
4.

- a. Channels (see image below)
 - i. The number of Tracks: (1 – 4) for line scan mode
 - ii. The Laser Lines for selected tracks
 - iii. The Laser Power (percent of 100%)
 - iv. The detector gain and offset



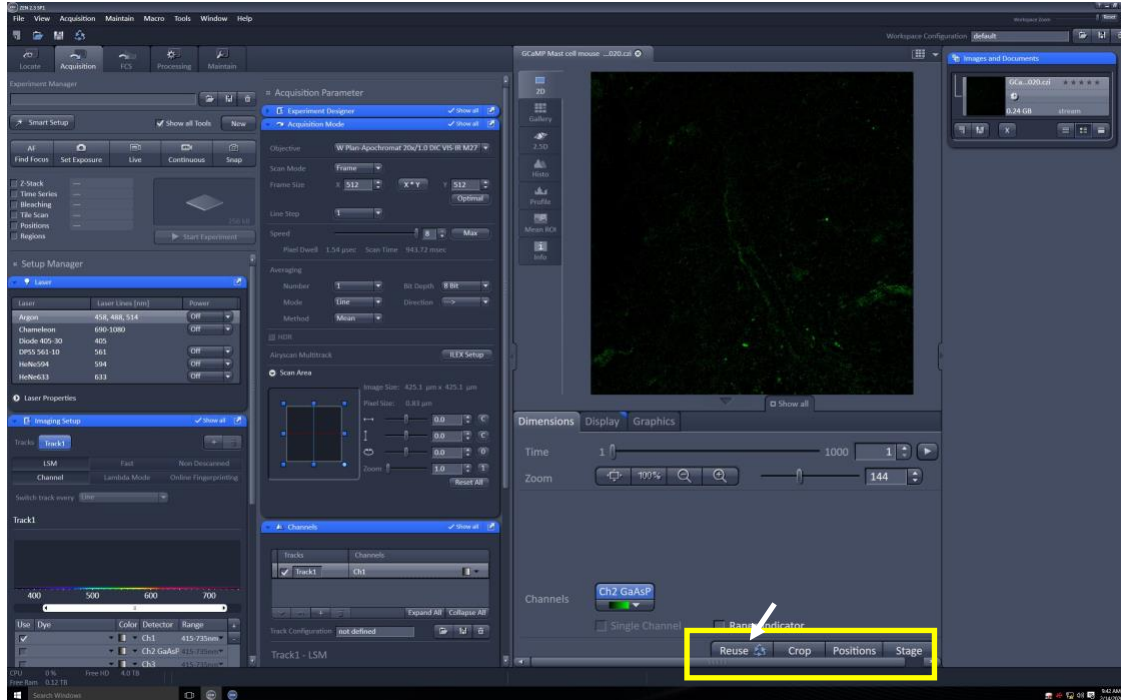
v.
vi.

b.



- 5.
6. Load an Acquisition Parameter file by clicking on the file folder icon (white arrow in image above) and selecting a parameter file from the drop-down list.
7. There are files for 4-color, 3-color, 2-color and separate files for 1-color images for red, green or blue emitting fluorophores – it is suggested that you use one of these Settings files as a starting point.
8. Alternatively, you can load a previously saved image and click on the Reuse Parameters icon (see yellow box and white arrow in the image below). When loaded, these turn on the lasers that you need for those types of images and adjust all other settings. Please note that the Argon laser used for 458, 488 and 514 nm excitation requires about 5 min or so to warm-up after it is turned on. The diode laser (405 nm), DPSS (561 nm) and the HeNe laser (594 and 633 nm) require less time. Please do not alter

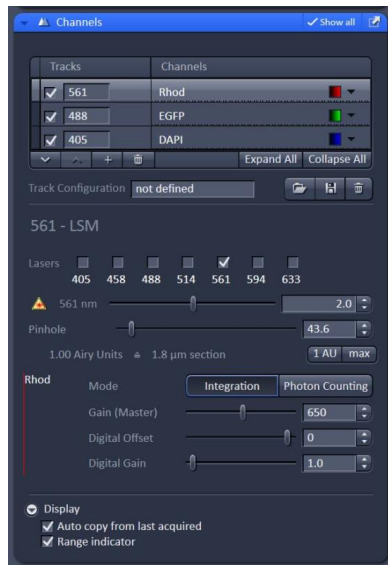
settings and then save the Acquisition Parameter file as you will overwrite the default files. If you want to create a tailored settings file, please save under a new name – Please talk to Dr. Jackson before doing this.



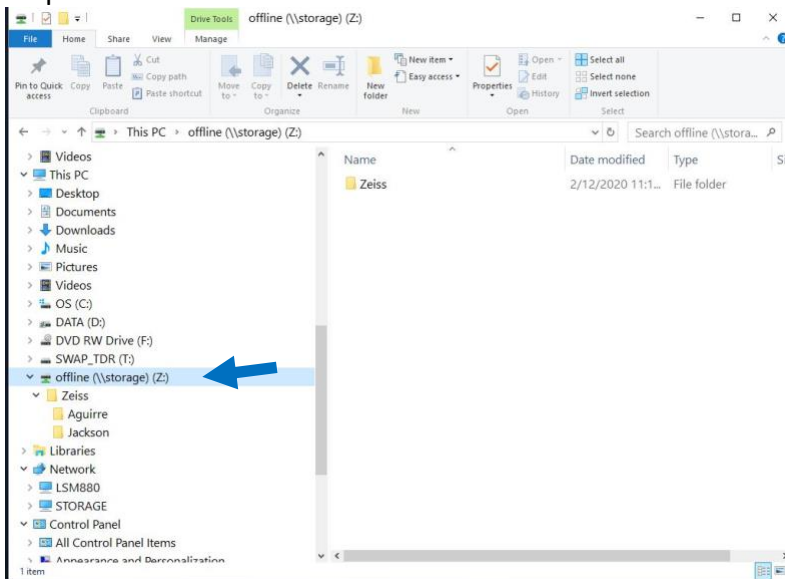
- 9.
10. You can ignore all of the info under the Imaging Setup – this shows the light paths and the specifics for the tuning of the excitation and emission. You should not need to touch any of the settings in this window for normal use.
11. To set up to acquire a confocal image:
 - a. Acquisition Mode Tab – This shows how the image will be acquired and includes info on:
 - i. The objective that is currently in place.
 - ii. How the sample will be scanned by the laser (Frame is the usual)
 - iii. The Frame size and pixel count (the default is 1024X1024 – optimal will increase the pixel count but will also increase imaging time and file size)
 - iv. Line step = 1 is the usual.
 - v. Speed – 6 is the default
 - vi. Averaging – 2 is a good place to start – if you need to increase acquisition speed reduce to 1 (no averaging).
 - vii. Bit depth – the default is 8 bit – may be set to 16 bit for more resolution.
 - viii. Method – Mean is the default for the average.
 - ix. You can also set the scan area here to a smaller region or to zoom in. Default should be set to 1.



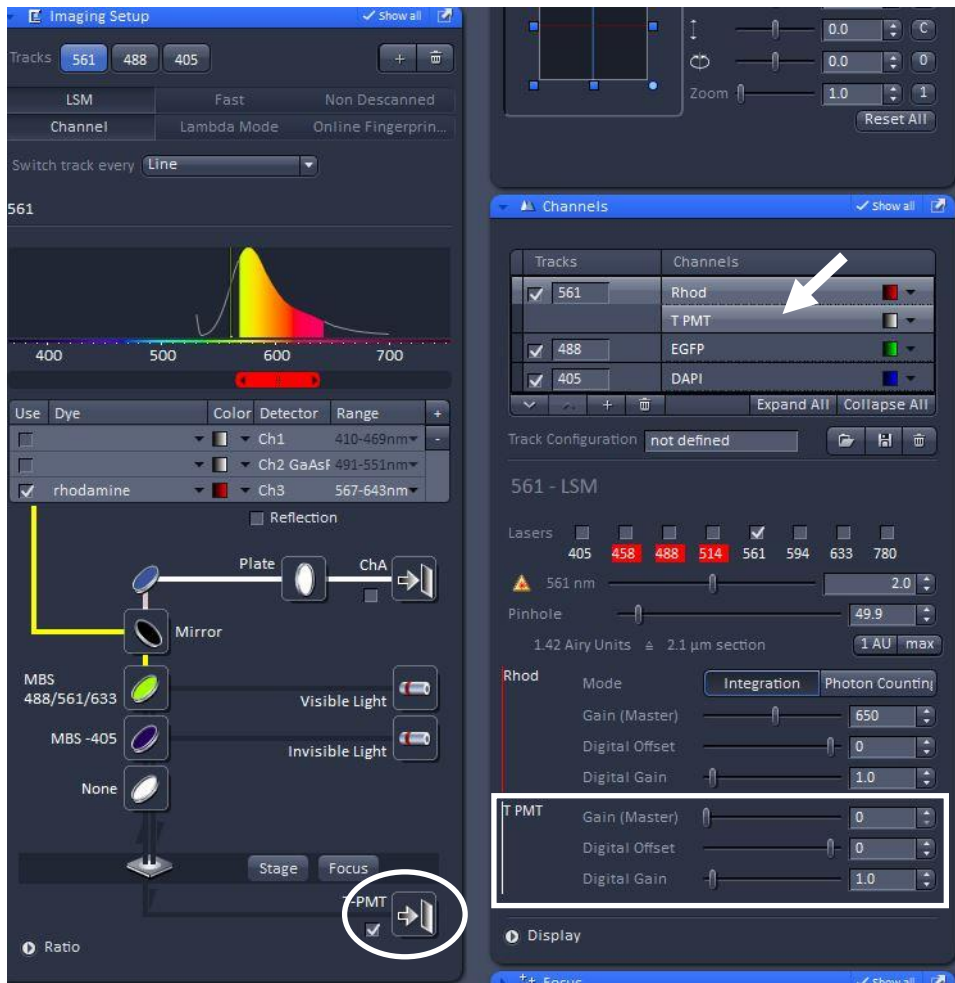
- b.
- c. Adjust the Confocal pinhole for optimal imaging.
 - i. If imaging more than one color, set the pinhole size for the longest wavelength to 1 AU. This will adjust the pinhole size across the other wavelengths to give you about the best images and equivalent optical sections.
- d. Adjust the Digital Offset (Black Level), the photomultiplier Gain and Laser Power under the Channels Tab.
 - i. Turn on the appropriate excitation wavelength by clicking the check box and clicking in the center of that channels row to “select” and turn on that channel.
 - ii. Click Live to give a Fast 512X512 image and focus using the electronic focus controls on the remote-control panel.
 - iii. Click the Range Indicator in the Display panel that shows pixels that are below (blue-colored in the image) and above (red-colored) the level of detection of the photomultipliers.
 - iv. Adjust the Digital Offset (click the up or down arrows rather than dragging the slider as this control is quite sensitive) to “just” eliminate all blue pixels in the image. This should be set no higher than about 3 or 4. Something is likely wrong if higher values are required. See Dr. Jackson.
 - v. Adjust the Gain and the Laser Level to just fill the detectors histogram, but without any red pixels. Use the lowest laser power and Gain possible. Lower Laser power will reduce bleaching and phototoxicity. Lower Gain reduces noise.
 1. Typically, the laser power level required should be no higher than 3 (3%) for most fluorescent dyes.
 2. The default gain settings should work for most situations (the bold values below are default).
 - a. The working range of settings for the gain on the 405 channel is from **650**-750.
 - b. The working range of settings for the gain on the 448 channel is from **550**-750.
 - c. The working range of settings for the gain on the 561 and 594 channels is from **650**-750.
 - d. The working range for the 633 channel is from **650**-850.
 - vi. Leave the Digital Gain setting alone (set at 1).



- vii.
- e. You can view how your image will actually look by clicking the Continuous button – this applies all of the settings that you have in place from the Acquisition Mode Tab (averaging, bit depth, etc.) but does this continuously so that you can focus or move the stage to get the optimal positioning of your sample.
 - f. Once you are ready acquire a confocal image, click the Snap button and a single scan will be performed.
 - g. If the image looks OK and you are ready to save it, click on Files and Save As. Files should be saved to the D: drive on the analysis computer in an appropriate folder. This is a networked drive called: offline ([\\storage](#)) (Z:) (see blue arrow in image below) in the folder labeled Zeiss, in a subfolder named for your lab as indicated in the figure below. Please see Dr. Jackson for help and details.



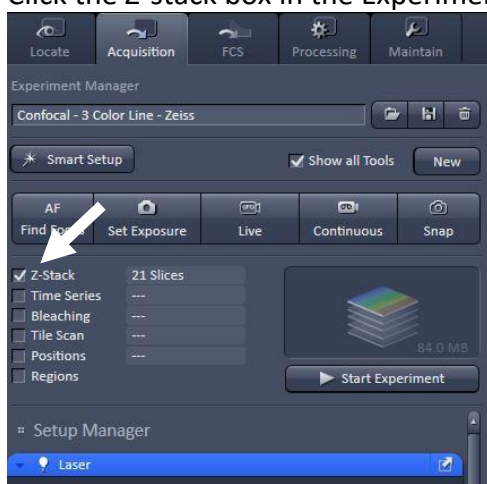
- h.
12. To add a transmitted light channel to your acquisition.
 - a. Find an appropriate site in your sample and set up the Kohler illumination and DIC using the controls under the Locate Tab, then move to the Acquisition Tab.
 - b. Load at least a two color Acquisition Set up (488 and 561 nm for example).
 - c. Click the check box - T PMT (transmitted light PMT) at the bottom of the Imaging Setup Tab for the longest wavelength used (circled in the image shown below).



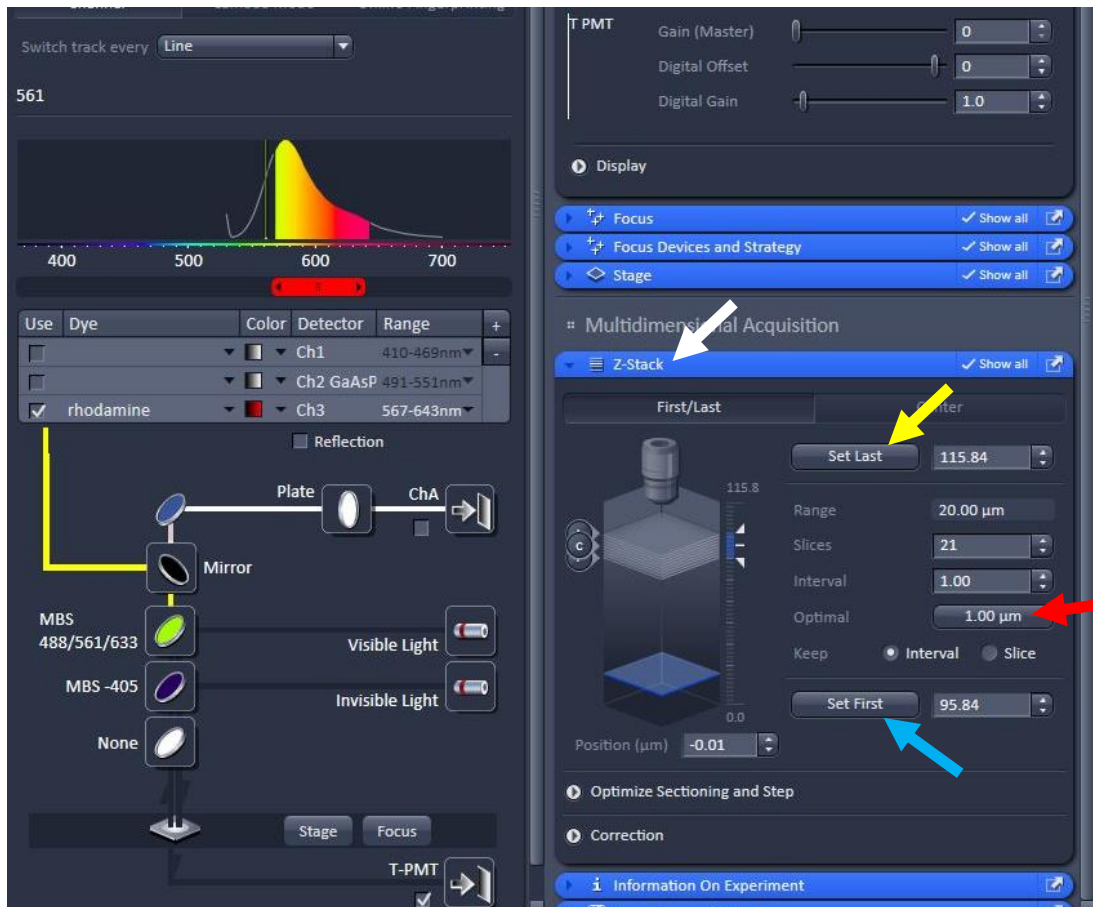
- d.
- e. Click on the T PMT line under the Channels Window (see arrow in figure above).
- f. Click Live to view your sample and adjust the Gain for the T PMT to give a good image (suggest that you look at the Histogram and center the histogram – this really depends on the optical characteristics of the sample) (See rectangular box in image above).
- g. Adjust the other channels as usual.
- h. You are then ready to Acquire an Image.

13. Acquiring a Z-stack.

- a. Adjust the Off Set and Gain for each channel as usual (select the brightest position in the tissue volume for each channel).
- b. Click the Z-stack box in the Experiment Manager



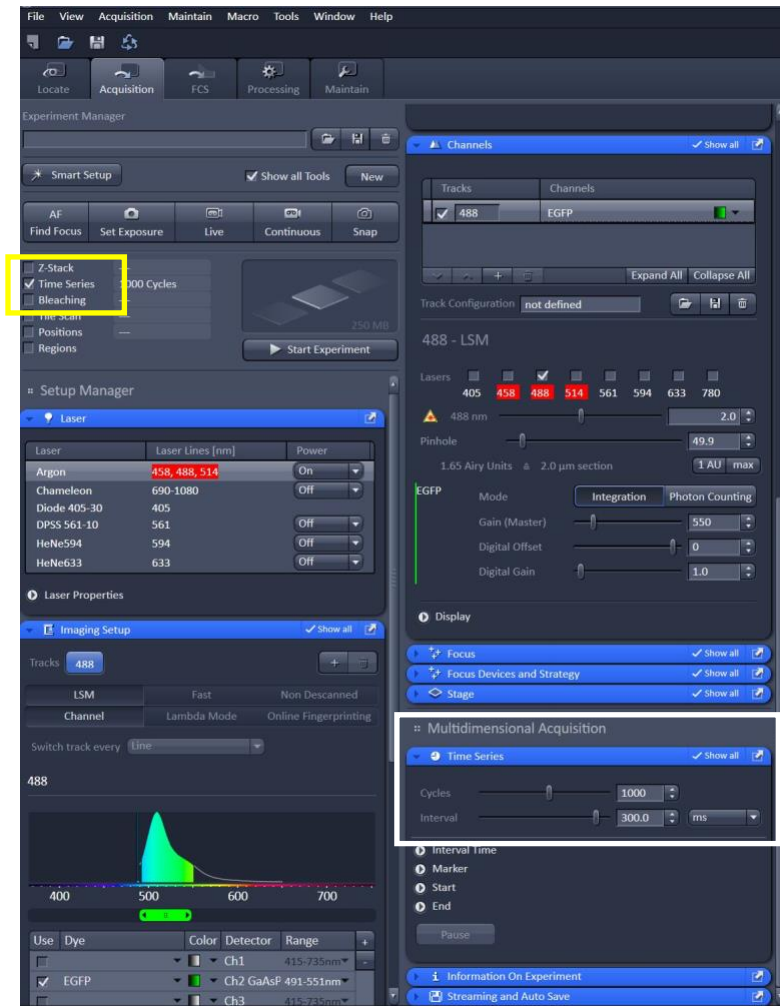
- c.
- d. Open the Z-stack panel under Multidimensional Acquisition (see white arrow below).



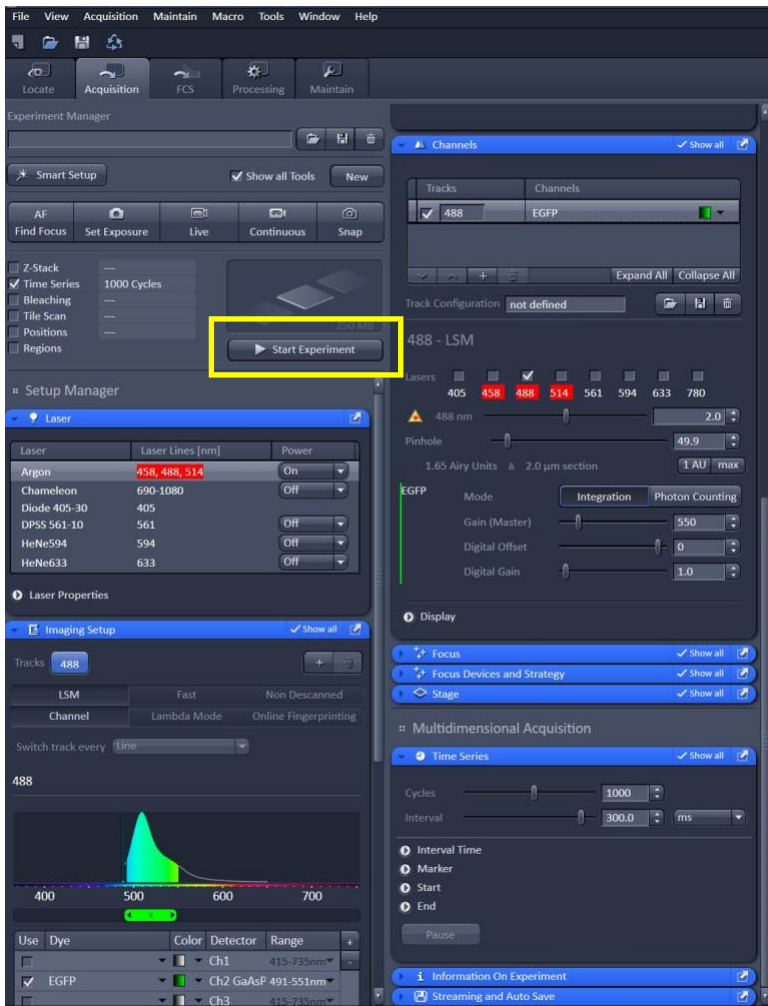
- e.
- f. Click Live and adjust the focus to the top of the sample volume (turn focus knob counter clockwise).
- g. Click the Set Last position button in the Z-stack Window (yellow arrow in image above).
- h. Focus down to the bottom of sample volume you want to image and Click the Set First Position button in the Z-stack panel (Blue arrow in image above).
- i. Stop the Live Image, then click the Optimal button (Red arrow in image above)
- j. It is suggested that you use the optimal slice thickness suggested by the Zen software for the objective you are using. This makes sure you are using Nyquist sampling such that you can do 3D reconstructions if you want.
- k. Click on Start Experiment in the Experiment Manager to Acquire the Z-stack
- l. Save image stack as described for simple images above.

14. Acquiring a Time Series stack of images

- a. Decide what speed of image acquisition that you need.
 - i. This depends on the image size, the imaging mode (Line scan or Frame scan – line scan is fastest), Averaging (1 image, no average is the fastest) and the number of channels you are acquiring.
 1. With conventional confocal with a 512X512 image and 2-3X zoom you can acquire a single wavelength (with or without transmitted light image) at about 3 Hz (<300 ms/image).
 2. You can get up to about 30 Hz acquisition using Airyscan Fast mode (presented in a separate SOP).
- b. Adjust laser intensity and detector gain and offset as usual.
- c. Click on the Time Series box in the Experiment Manager (see yellow box in image below)

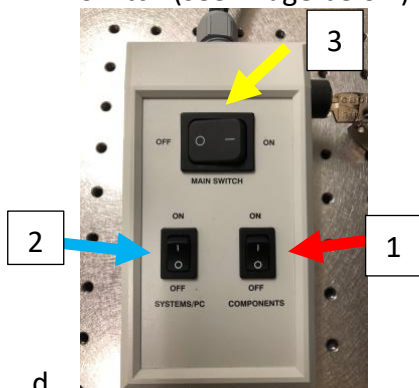


- d.
- e. Open the Time Series panel under Multidimensional Acquisition and select the number of samples that you wish to image (see white box in image above).
- f. Need image here
- g. Set the interval between images in this window as well (Should be greater than the time required to acquire an image – this can be determined by looking in the Acquisition Mode module – see image below). Leaving this blank will set the system to acquire images as fast as possible.
- h. Need image here.
- i. Click on Start Experiment in the Experiment Manager to begin image acquisition.

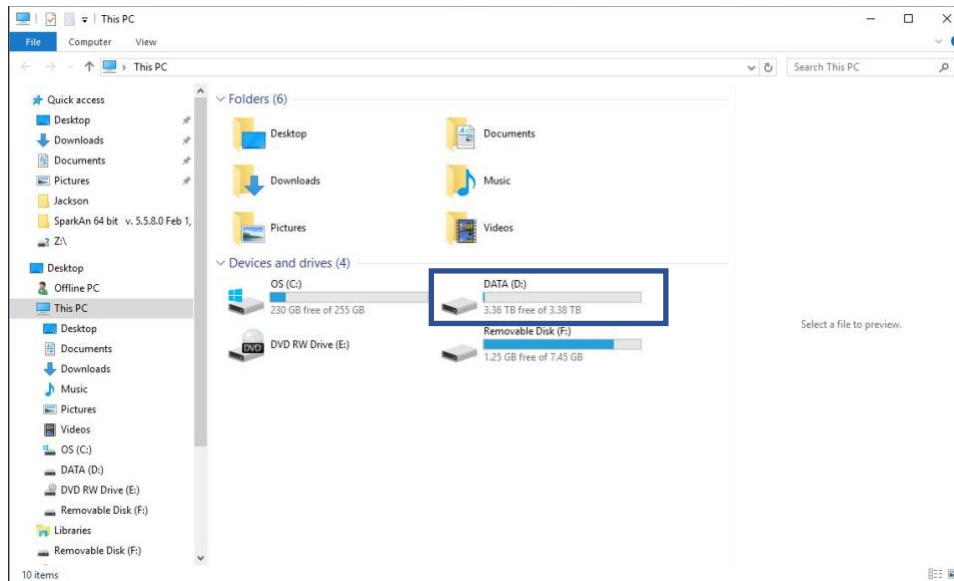


j. 15. Turning off the system.

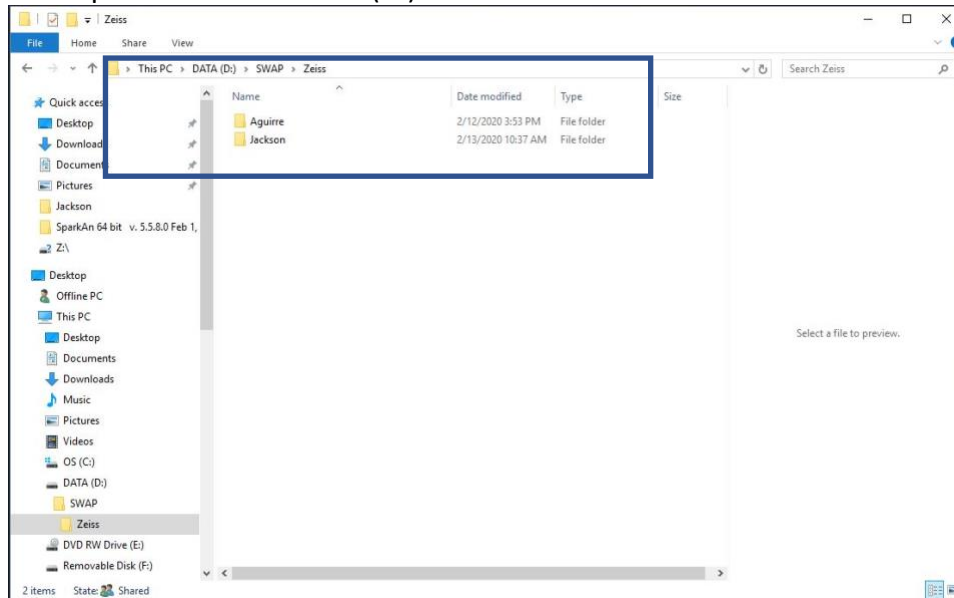
- a. Exit Zen Black – a pop-up window will appear showing which lasers are on. Click all lasers that are on to the off position.
- b. Shut down Acquisition Computer.
- c. Wait about 15 min for the Fan on the HeNe Laser to stop running, then turn off the Main Power Switches in the reverse order to that used during startup (Components, System PC, then Main Switch (see image below)).



- d.
- e. Retrieve data from Analysis Computer
 - i. Image files are located in the DATA (D:) drive,



- ii.
- iii. In the path: This PC > DATA (D:) > SWAP > Zeiss > Your Folder as shown below



- iv.
- v. Where Your Folder is the name of the folder for your lab (see Dr. Jackson if you need help).
- vi. Either use a large capacity Flash Drive (64 GB or larger), external USB drive or
- vii. Send files to your One Drive, Google Drive, or other cloud storage (this is very slow).
- f. When files have transferred, shut down Analysis Computer.