Image Acquisition Basics for SP8 Confocal Microscope

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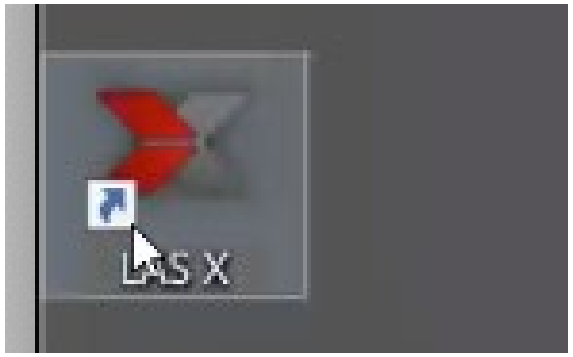
Sample

- U87 Glioma Cells in PEGDA hydrogel
- Stained with Acridine Orange (Green) and Propidium Iodide (Red)
- AO dye is in gel, so gel appears green

Opening LASx and Image Preparation

Opening LASx Software

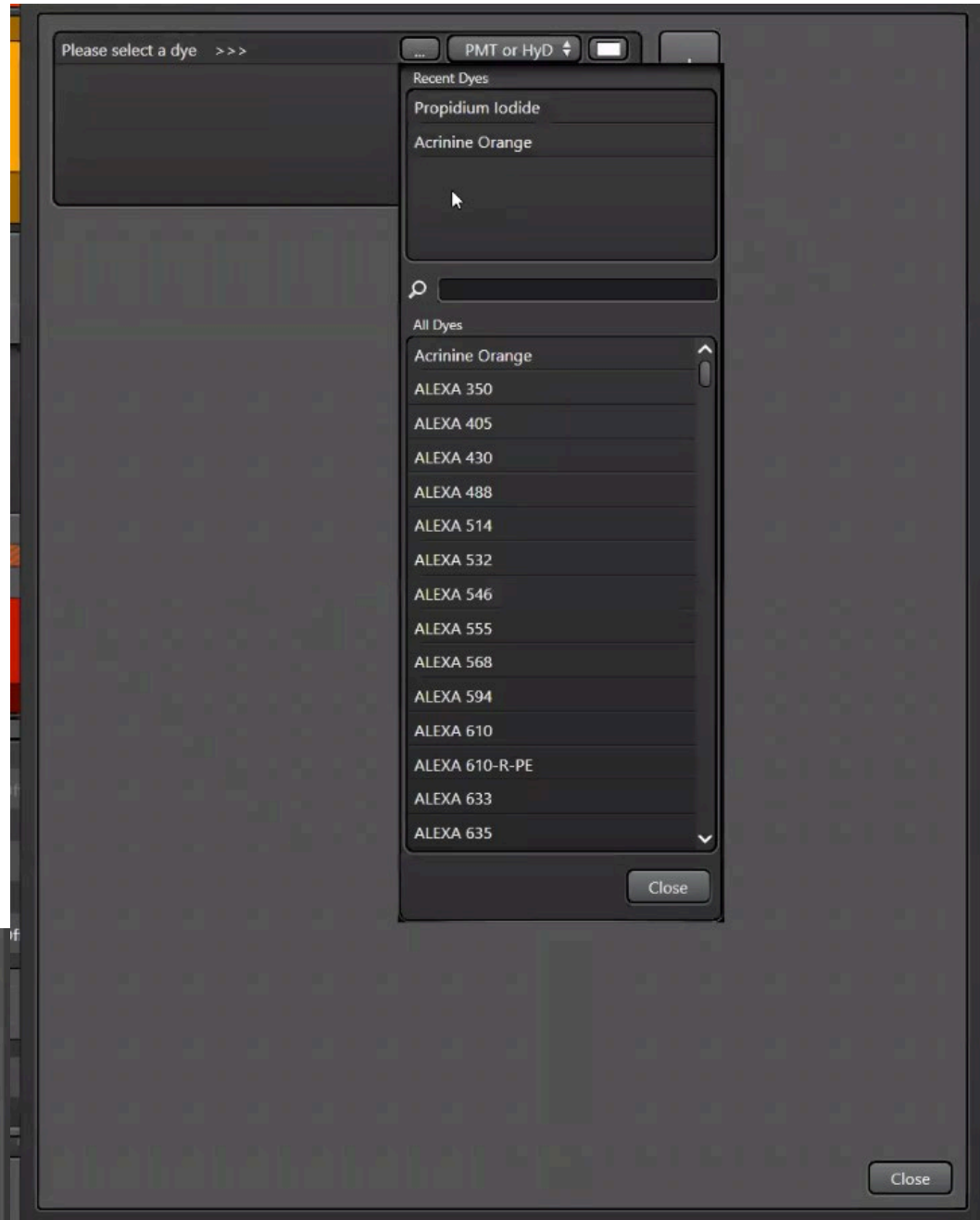
- Open LASx software using icon shown below and with the settings on the right



- Once program is open, load sample onto microscope and focus

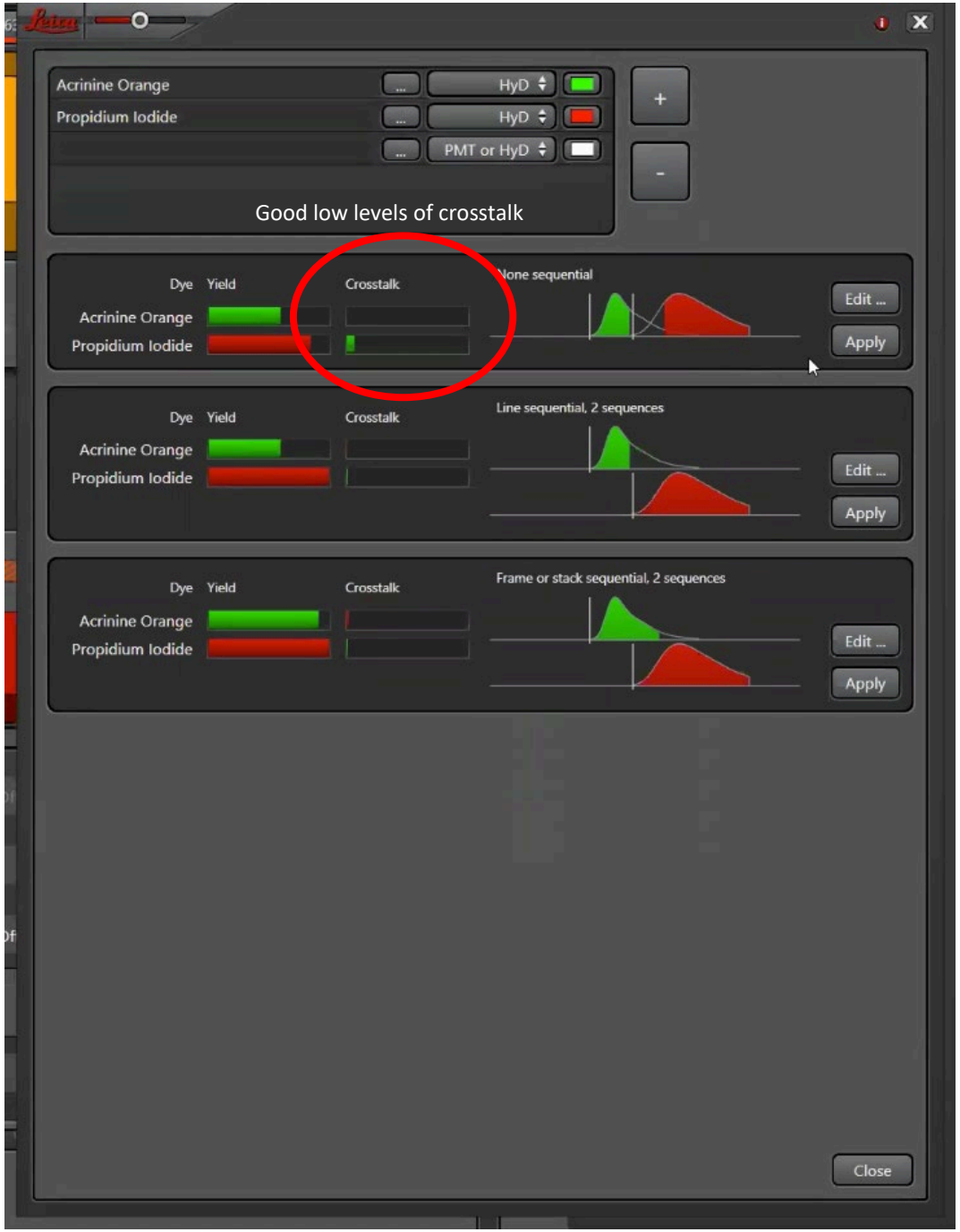
Setting Up Laser Lines

- Use the dye assistant as shown below to select the proper dyes to image with
- Common dyes can be searched in bar. If the dye is not present, you can select a dye with similar emission spectra



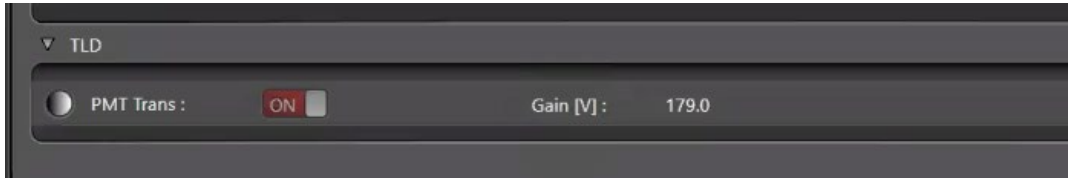
Set-up Scan Sequence

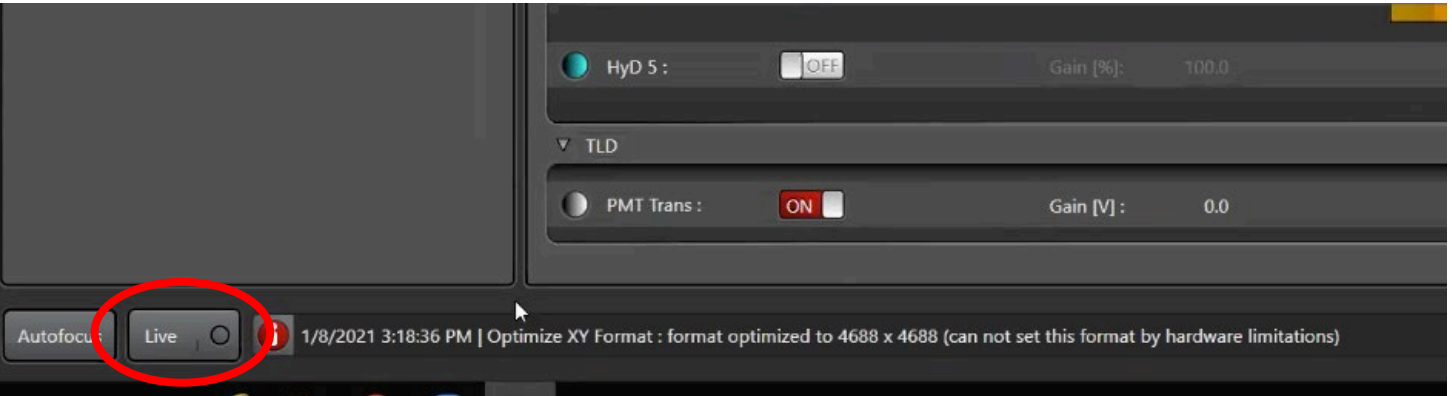
- Scan can be run either sequentially or non-sequentially
- Running non-sequentially, allows for speed, but causes crosstalk
  - Crosstalk results in emission from one channel to show up under a different sample
- Running sequential scans eliminates crosstalk, but slows down scan speed
- Use hybrid (HyD) detectors
- Note: if lasers are not on, turn them on when prompted



Adjust laser power/gain

- Turn on transmitted light detector for phase contrast images (optional)
- Go to “Live” to begin laser scan
- Adjust gain on TLD to see sample
- Gain can be adjusted on smart dashboard or on LASx software



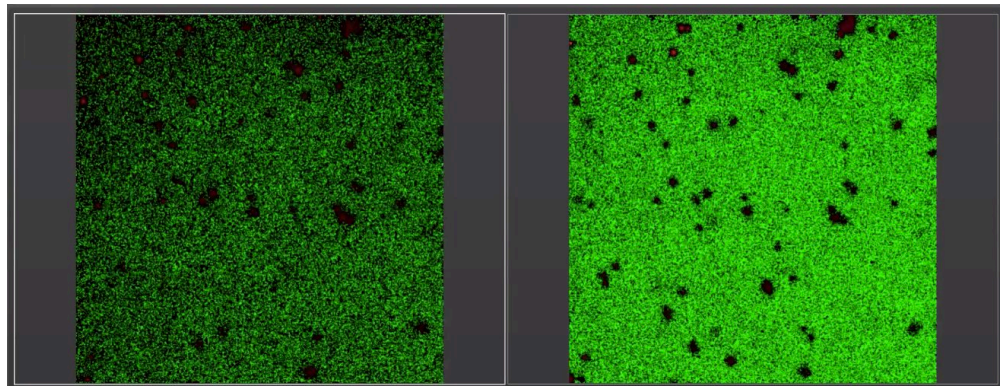


Adjust laser power/gain

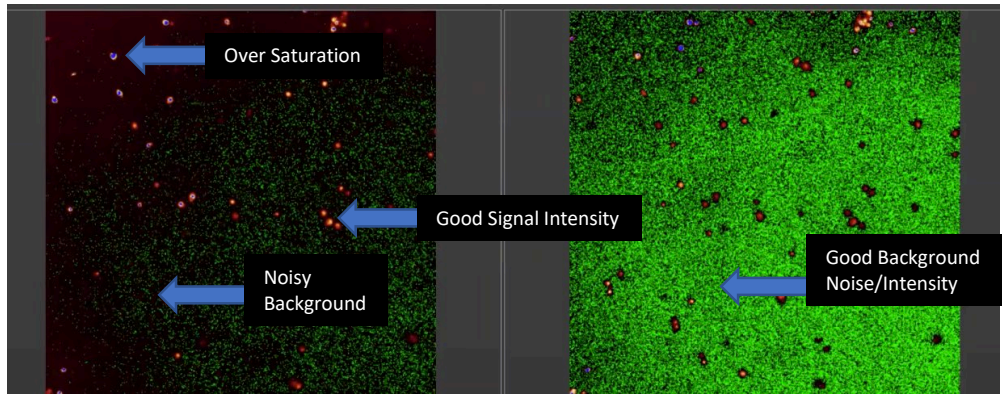
- Go to over underexpose to get appropriate laser power/gain
  - Top left of the images

- Below are laser power/gain examples

Low Signal – increasing either laser power or gain



- Green is no signal, red is low signal, yellow is high signal, black is noise, and blue is overexposure



Adjust laser power/gain

- Generally, we do not want a high laser power because it could damage sample, but gain could cause increased noise
  - Using gain over laser power is usually preferred, but too much gain will be noisy
  - Takes practice to understand appropriate power/gain for each specific sample
- Over exposure loses information because any signal over saturation is registered as same value
  - i.e. signal with intensity of 255, 300 and 350 would be recorded as an intensity of 255
- Good practice is finding power/gain where there is slight oversaturation and go just below that level
- Noisy Background is not necessarily bad, it is important to focus on acquiring the data you are interested in
  - i.e. if you cannot see your signal without background noise, that is ok

Image Capture

Decide on Image Settings

- Choose image quality
  - Higher quality means slower acquisition
- Choose imaging speed
  - Increased speed limits frame size and increases noise
- Choose averaging
  - Increased averaging lowers noise
- Choose accumulation
  - Increases intensity and signal to noise ratio
- Use Start or Capture Image to take picture once setting are determined

How to Decide on Image Settings

- If you are taking many images for data acquisition, speed might be more crucial rather than image quality
- If you want publication ready image, quality would be more important
- Slower imaging causes the sample to be exposed to lasers longer, thus might damage sample

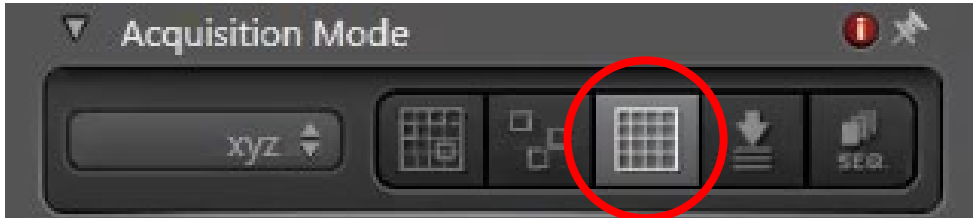
How to make a Z-Stack

- Set boundaries using beginning and end by scanning through sample
  - Bar in LASx software (arrow) can be used to scan through sample or depth knob on dashboard
  - End and beginning can be selected and changed by dragging the planes up and down or clicking begin and end
- Choose how to section Z-Stack
  - Number of steps automatically chooses step size
  - Step size automatically chooses number of steps
  - System optimizes gives best step size for 3D reconstructions
- Press start to being Z-Stack

Navigator for Large Area Imaging

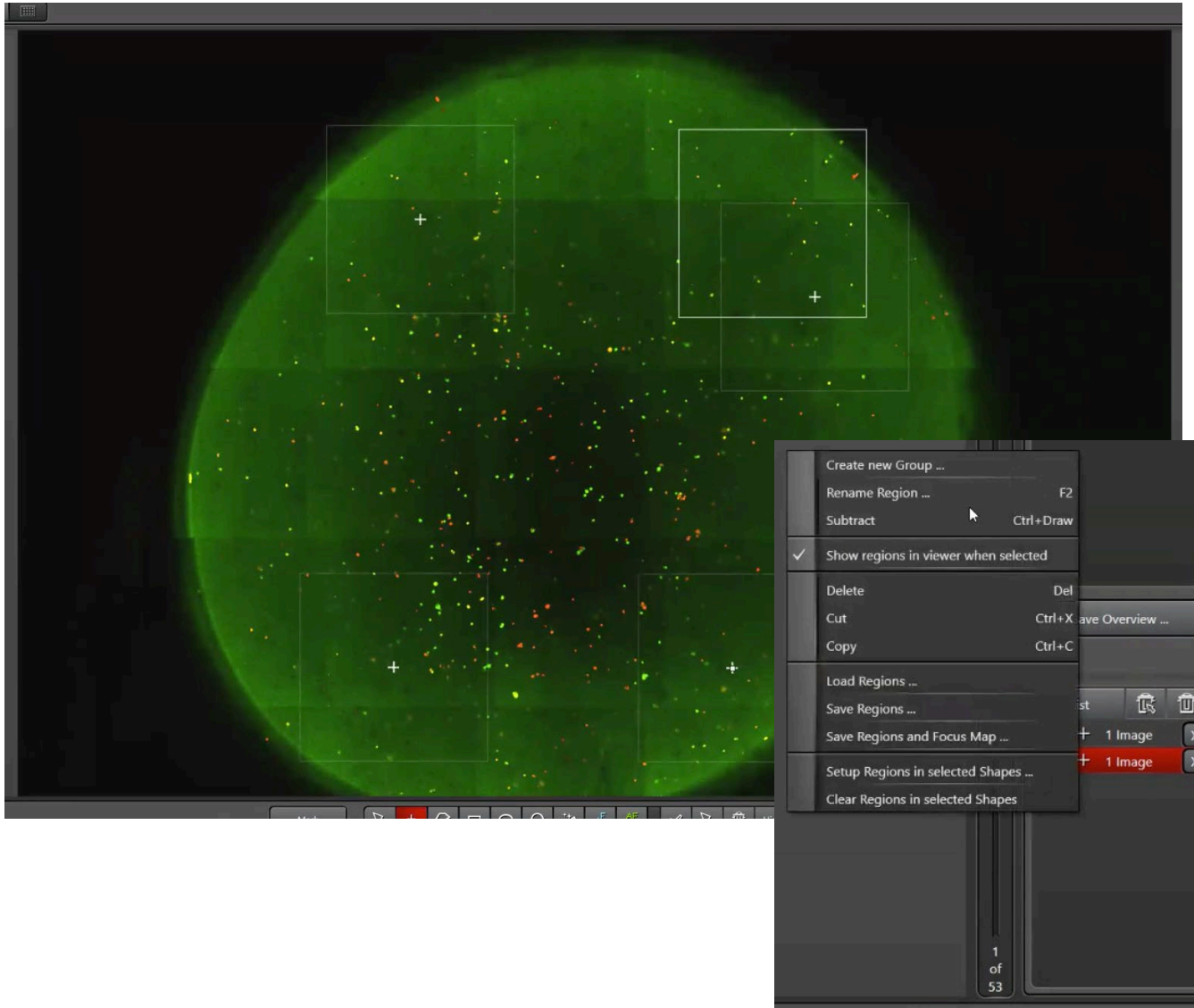
Opening Navigator and Using Spiral to Obtain Preview

- Navigator is square button with grid
- Once navigator is opened, used spiral to get preview of sample
  - Center sample before using spiral
- Double clicking on location gets frame centered on that location
- All image acquisition settings for navigator can be accessed on left bar and used same manor as mentioned before



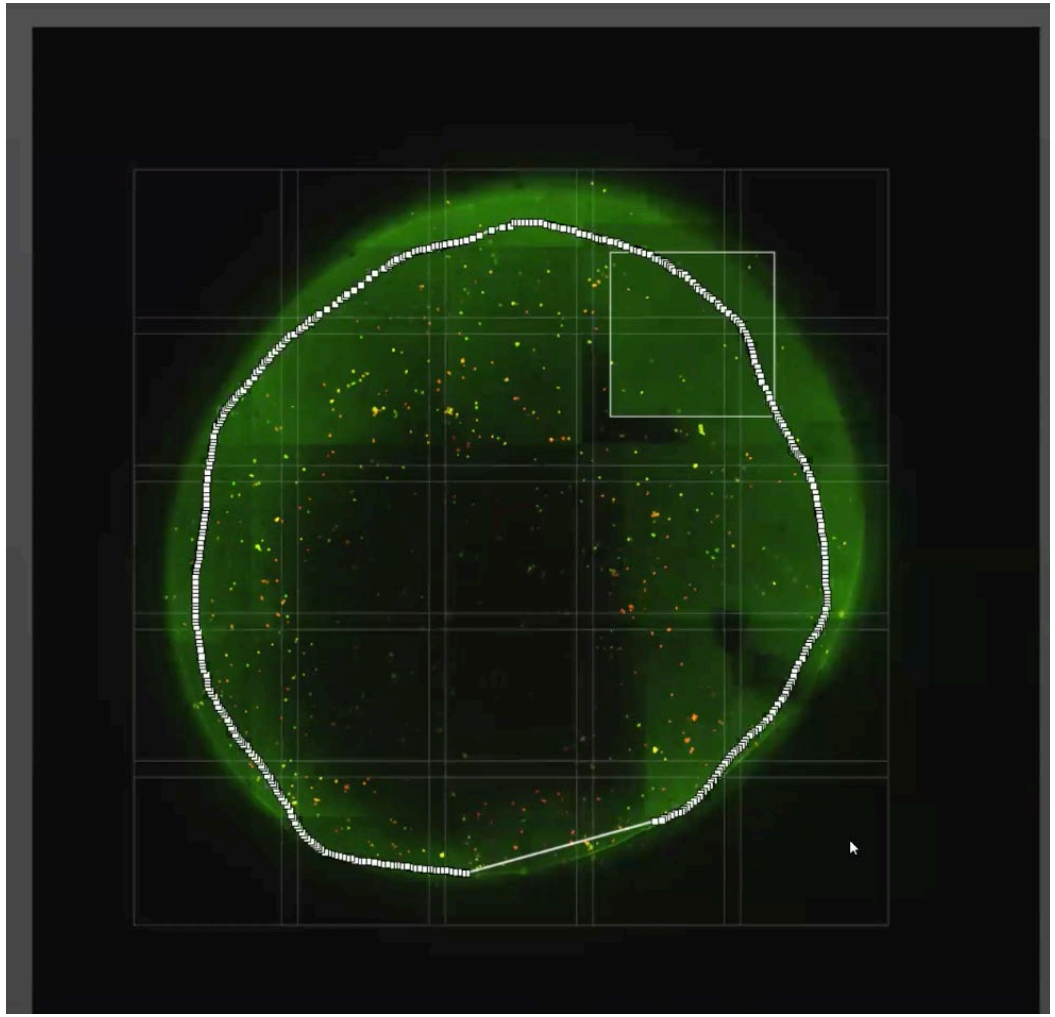
Mark, Group and Define Images

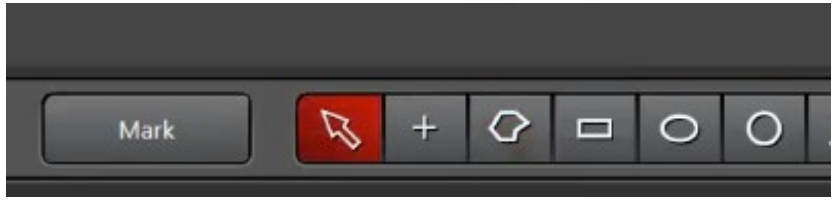
- Use crosshairs in Navigator to mark location of images
- Right click task list in bottom right to organize into groups
  - Optional
- If Z-Stack is being used, stacks for each region can be defined separately
  - Ensure each stack has correct dimensions



Selecting Area on Navigator

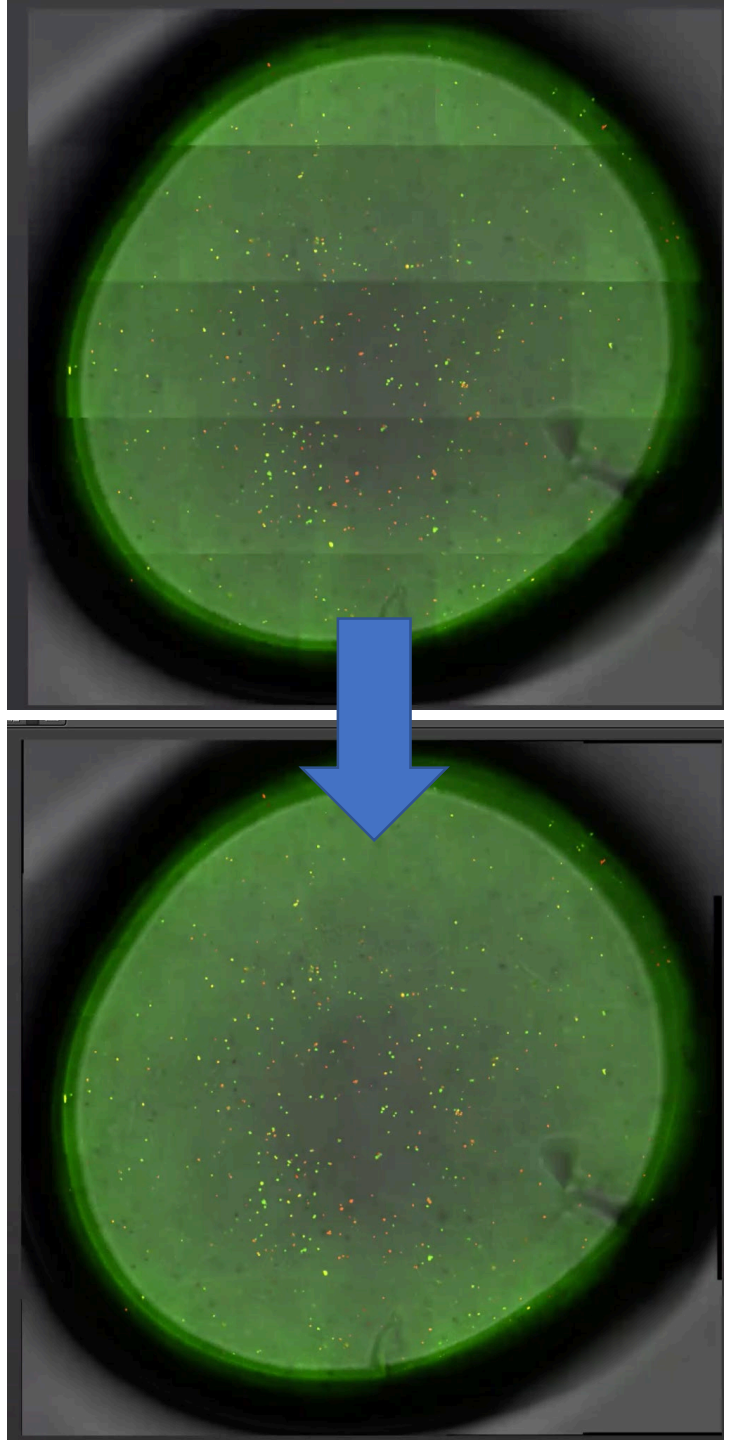
- Use defined shapes (rectangle, circle or oval) or free drawn shape to select area for imaging
- Images of Z-Stacks or single z position can be taken of large area
- Multiple areas can be defined and imaged simultaneously
- Press start to begin imaging area





Merge Areas

- Select image of region you want to merge in navigator
- Go to Mosaic Merge
- Merge images in bottom right
- This merges all frames into a single image



Important Notes for Navigator

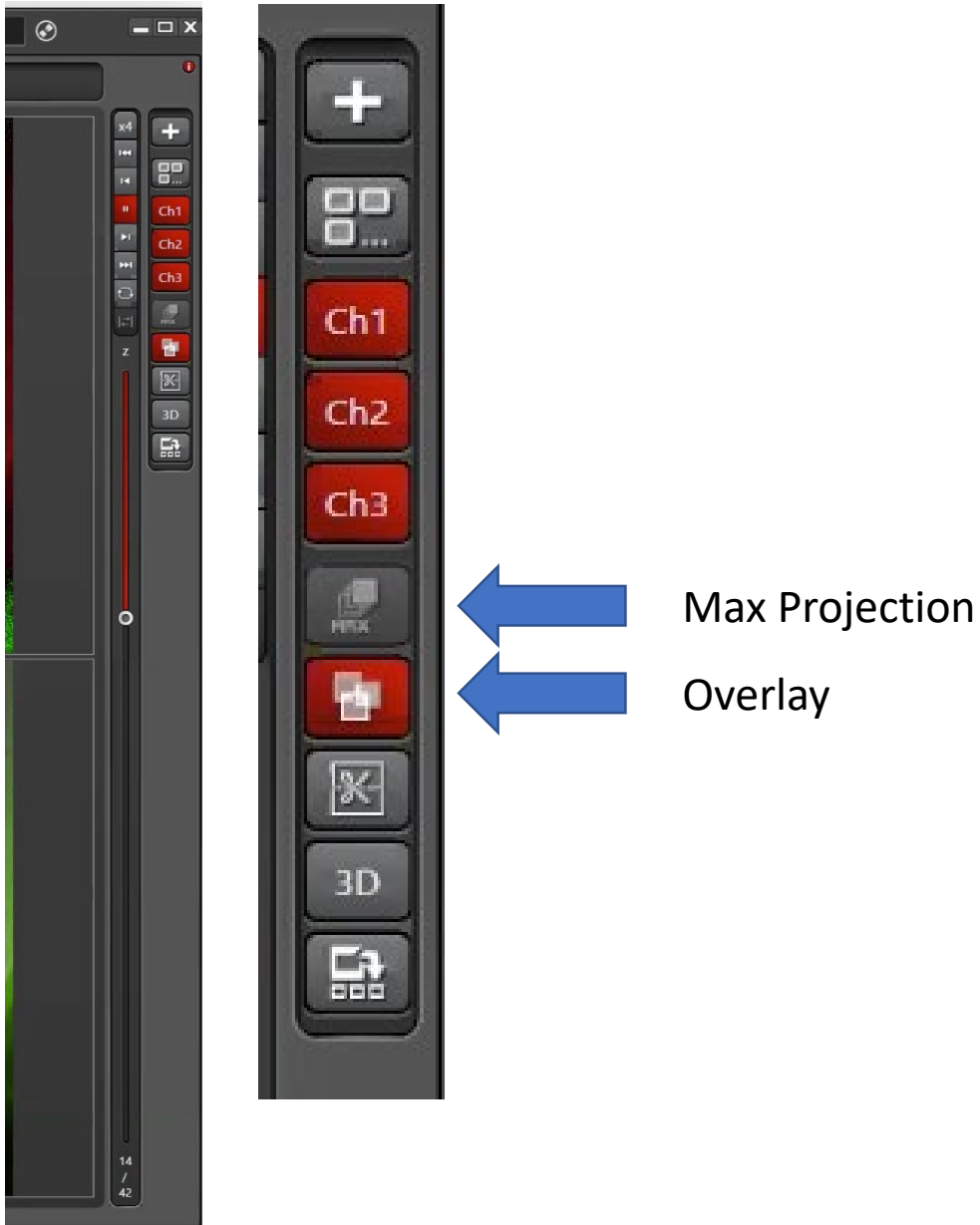
- Be careful using an immersion objective navigator when imaging a large area because immersion media will be spread and could be dry

- When using Z-Stack in navigator make sure all stacks are appropriate size and step size
  - Good to delete previous Z-Stack before marking then redefine each stack
  - With large areas, any tilt on slide will be very noticeable and could result in not imaging sample due to being in wrong z-location

Processing and Saving Images

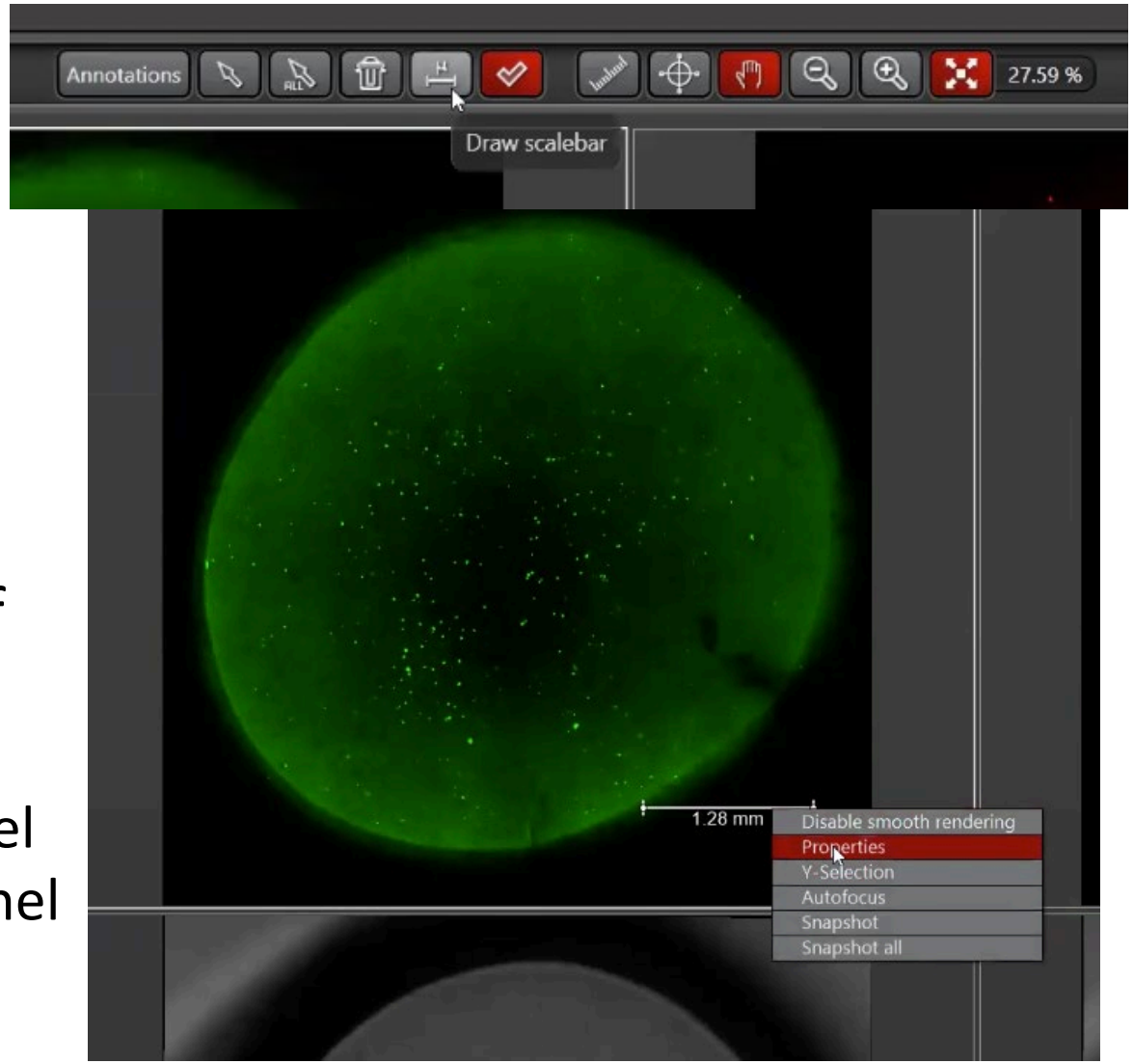
Scrolling Through Stacks and Regions, Max Projection and Overlay

- Use scroll bar to the right to scroll through Z-Stack
- Use scroll bar on bottom to scroll through unmerged region or time points
- Choose visible channels and channel overlay on right
- Create max projection on right



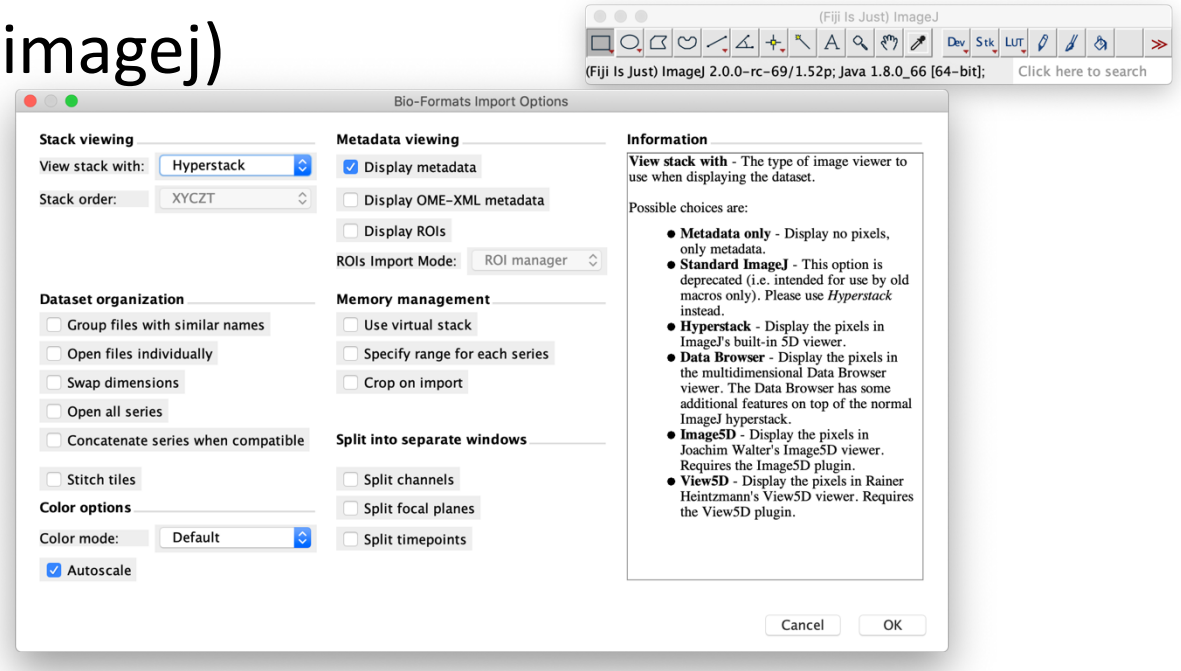
Adding Scale Bar and Saving Image

- Click on Draw Scalebar
- Draw scale bar where appropriate
  - Can be moved after placement
- Select arrow and right click on the scale bar to change properties like angle and size
- Right click on image to take snapshot of either single image or all images on screen
  - Snapshot take snapshot of selected channel
  - Snapshot all takes snapshot of every channel



Saving, Exporting and Opening .lif files at home

- Save .lif file in correct location and save on google, drop box or another cloud-based file storage
- To export single image or snapshot right click and export files in desired file format
- .lif files can be opened using fiji (just imagej)
  - https://imagej.net/Fiji
- Open .lif using bioformats importer and manipulation can be performed as usual on imagej
  - Use settings on right and choose images you want to view



SP8 Confocal Resonant Scanner

Resonant Scanner Similarities

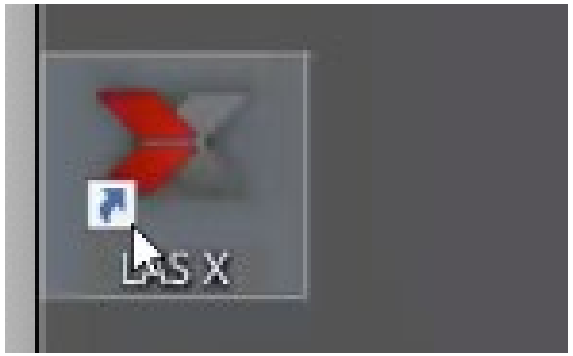
- Image acquisition steps for the resonant scanner is very similar to that of the galvanometer scanner
- Selecting beam path with dye assistant
- Laser power and gain
- Image quality
  - Resolution, averaging, gain, zoom
- Z-stack
- Navigator
- Post image acquisition analysis

Resonant Scanner Differences

- Scan speed is set at 8 kHz
- Because scan speed is much higher, pixel dwell time is lower, thus signal and photobleaching is lower
- With increased speed more averaging is needed

Opening LASx Software

- Open LASx software using icon shown below and with the settings on the right



- Once program is open, load sample onto microscope and focus
- Same as Galvometer, but turn on Resonant

Image Capture

- After loading sample adjust power and gain as done with galvanometer
- Due to faster scan speed, signal may be a little more difficult to find
  - Be sure sample is focus in through eye piece

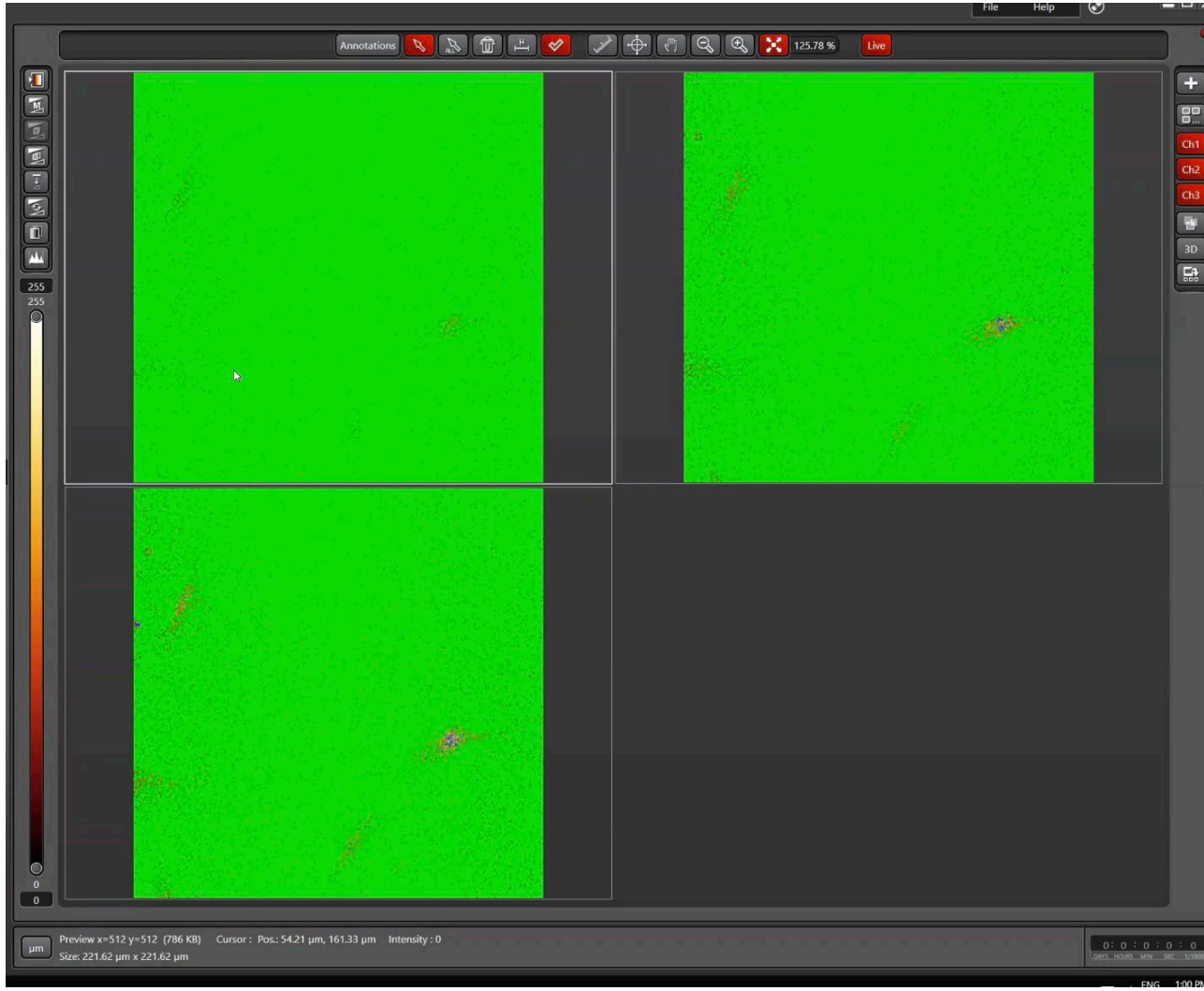
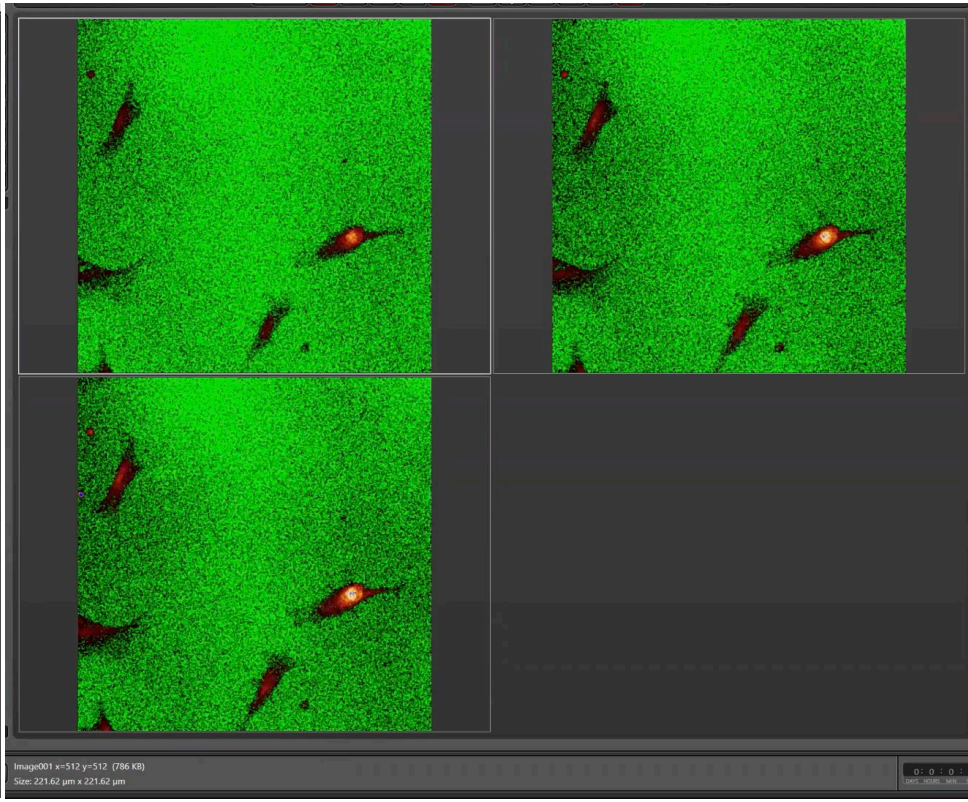


Image Capture

- Select appropriate averaging
- Because of the speed of the scanner averaging is very important
- Line averages of 8+ are recommended
  - With line average of 16, image was taken at 1 frame/s



Why use resonant scanner

- Allows for much faster acquisition speeds
  - Great for samples for fast moving dynamic samples
- Faster acquisition speeds would be better for easily photobleached samples, live cell imaging
- Allows for a higher zoom aspect ratio
- Lower resolution compared to galvonometer, but can be accounted for and offset by increasing the number of line or frame averages

Lightning Deconvolution

Lightning Deconvolution

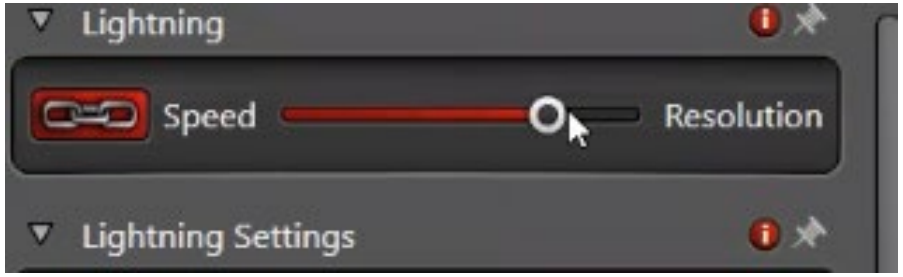
- Deconvolution is an image processing tool that can decrease the blurriness of the image and increase the sample contrast and resolution
- Leica Lightning deconvolution decides the parameters for deconvolution while acquiring the image
- Deconvolution can increase image quality and help isolate smaller features within an image
- **Lightning can be used with both the galvanometer and the resonant scanner**

How to access Lightning

- In the upper left-hand corner, open the drop-down list and select lightning
- Wait for Software to switch to lightning

How to use Lightning

- Adjust the refractive index to appropriate value
  - Refractive index of oil is on bottle
    - Need to double check value
  - Refractive index of water is 1.3333
  - Refractive index of air is 1
- Lightning automatically sets up the optimized parameters for image acquisition
- Scroll using the speed vs. resolution to determine parameters

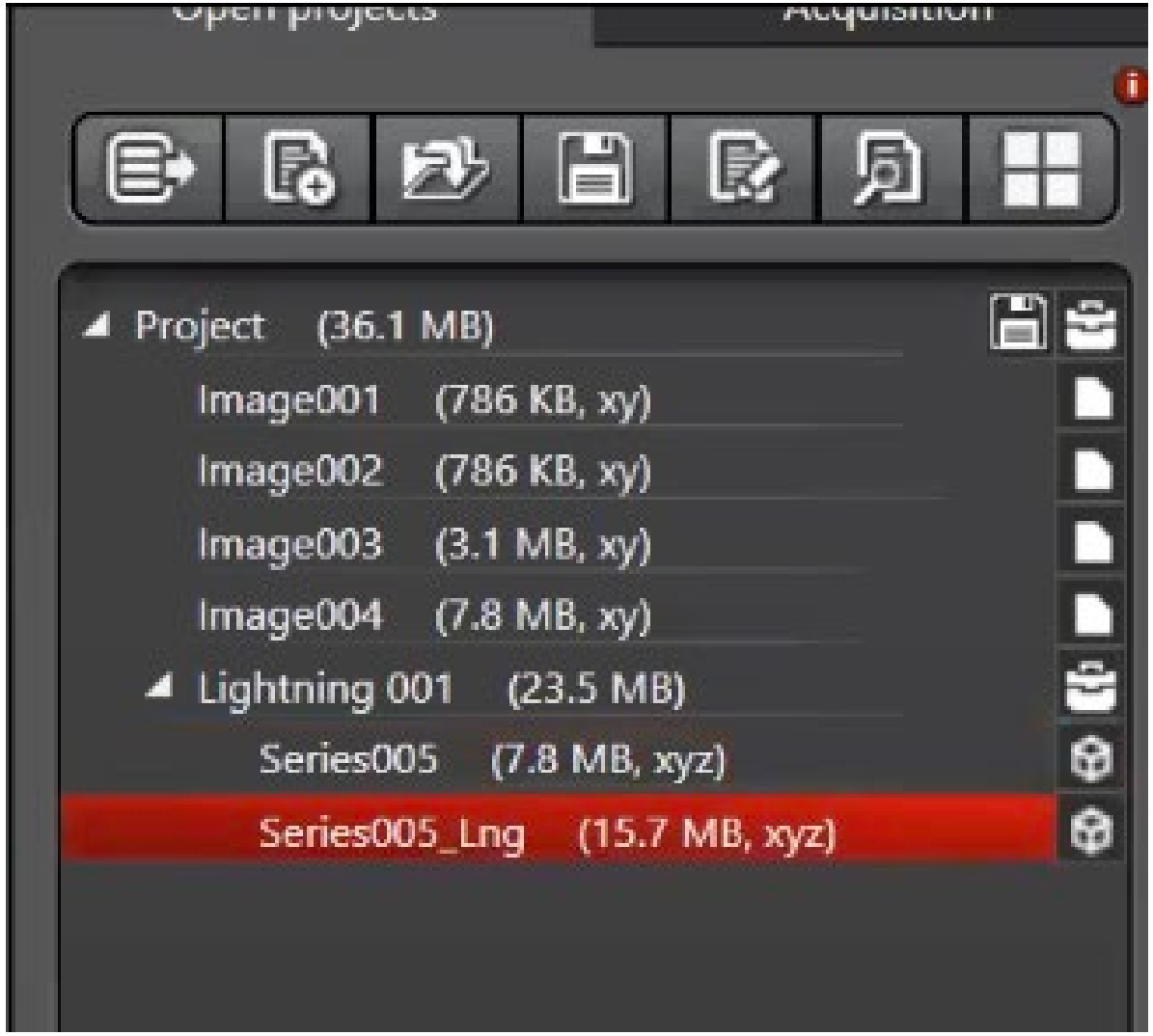


Customized Lightning parameters

- To customize lightning parameters, unclick the chain
  - This uncouples the scroll bar from the parameters
- Now you can choose, averaging number, scan speed, zoom, resolution etc.
- Typically, is it best to keep the lightning setting coupled and optimized

Image Acquisition

- Once parameters are set, click ”Start Experiment”
- Image acquisition will start and Lightning Deconvolution will start
- Deconvolution instantly starts occurring
- Deconvolved image has “_Lng” at the end on name



Questions and Issues

- Any questions or issues can be directed toward the Confocal Manager, Joey Bruns
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