# 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

		-
Please select a dye >>>	Porent Depr	
	Propidium Iodide	
	Acrinine Orange	
	- N	
	All Dyes	
	Acrinine Orange	
	ALEXA 350	
	ALEXA 405	
	ALEXA 430	
	ALEXA 488	
	ALEXA 514	
	ALEXA 532	
a second a second a second	ALEXA 546	
	ALEXA 555	
	ALEXA 568	
	ALEXA 594	
	ALEXA 610	
	ALEXA 610-R-PE	
	ALEXA 633	
	ALEXA 635	
	Close	



# 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





PMT Trans :	ON	Gain [V] : 179.0	
PMT Trans :		Gain [V] : 179.0	

# 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)
  - <u>https://imagej.net/Fiji</u>
- 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

	<b>Bio-Formats Import Options</b>	
Stack viewing	Metadata viewing	Information
View stack with: Hyperstack 🗘	Display metadata	View stack with - The type of image viewer to use when displaying the dataset.
Stack order: XYCZT 🗘	Display OME-XML metadata	Possible choices are:
	Display ROIs	• Metadata only - Display no pixels,
	ROIs Import Mode: ROI manager 🗘	only metadata. • Standard ImageJ - This option is
Dataset organization	Memory management	deprecated (i.e. intended for use by old macros only). Please use <i>Hyperstack</i>
Group files with similar names	Use virtual stack	• Hyperstack - Display the pixels in
Open files individually	Specify range for each series	ImageJ's built-in 5D viewer. • Data Browser - Display the pixels in
Swap dimensions	Crop on import	the multidimensional Data Browser viewer. The Data Browser has some
Open all series		additional features on top of the normal ImageJ hyperstack.
Concatenate series when compatible	Split into separate windows	• Image5D - Display the pixels in Joachim Walter's Image5D viewer.
Stitch tiles	Split channels	Requires the Image5D plugin. • View5D - Display the pixels in Rainer
Color options	Split focal planes	Heintzmann's View5D viewer. Requires the View5D plugin.
Color mode: Default ᅌ	Split timepoints	
✓ Autoscale		
		Cancel OK

# 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

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Open projects	8	TCS SP8	▼ Load   Save   Roi
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Pinhole			405

# 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

Speed O	Resolutio
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Resolution

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Speed

Lightning Settings

# 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

open projects Acquisition	<b>MI</b>
<ul> <li>Project (36.1 MB)         <ul> <li>Image001 (786 KB, xy)</li> <li>Image002 (786 KB, xy)</li> <li>Image003 (3.1 MB, xy)</li> <li>Image004 (7.8 MB, xy)</li> </ul> </li> <li>Lightning 001 (23.5 MB)         <ul> <li>Series005 (7.8 MB, xyz)</li> </ul> </li> </ul>	
Series005_Lng (15.7 MB, xyz)	Ø

#### Questions and Issues

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