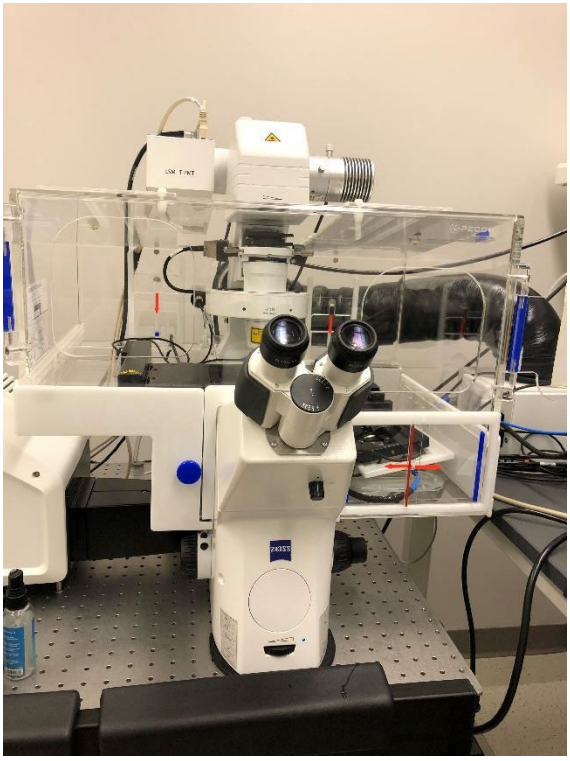
UMBC

Keith R. Porter Imaging Facility



Zeiss LSM 900

With Airyscan 2

Standard Operating Procedure

Interdisciplinary Life Sciences Building

Room 324

Text Dr. deCarvalho for time-sensitive issues: 301-529-5008

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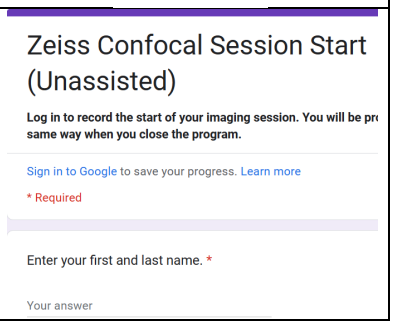
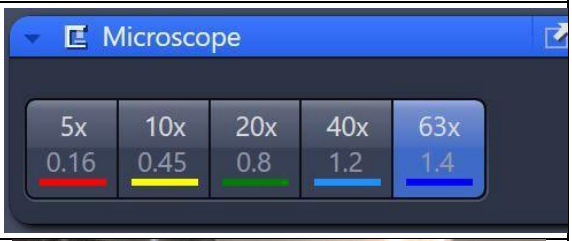
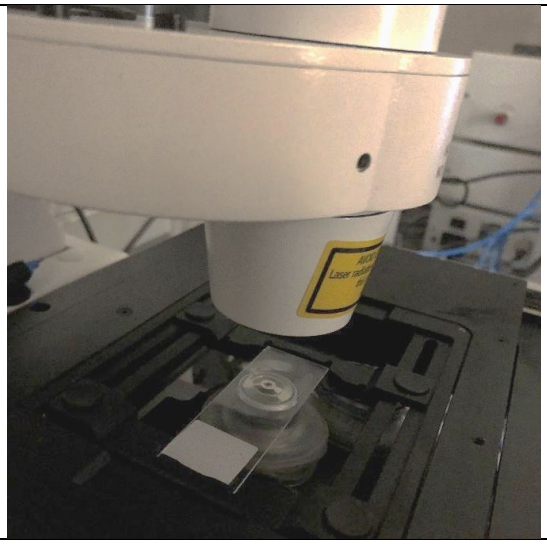
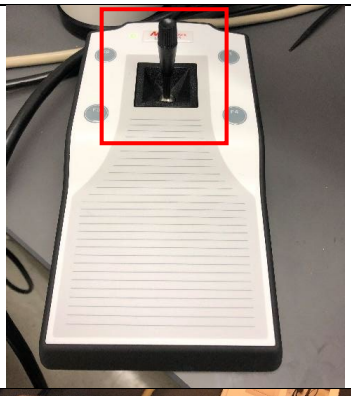
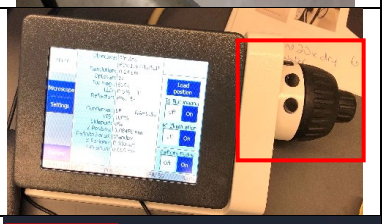
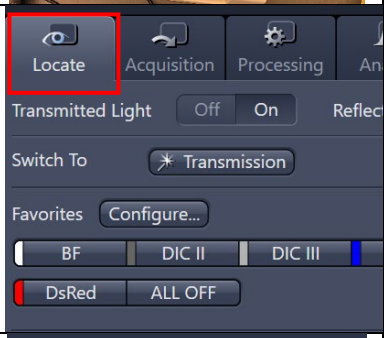
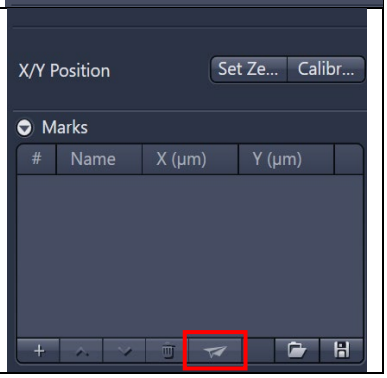
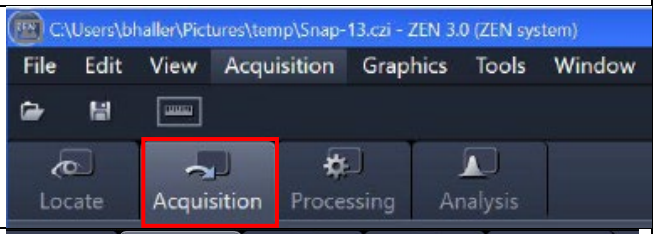
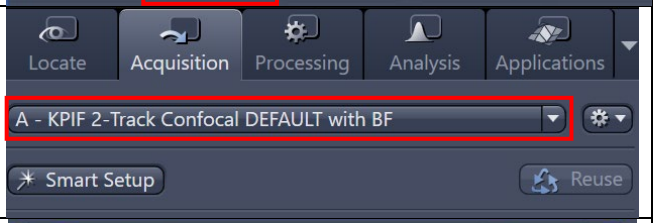
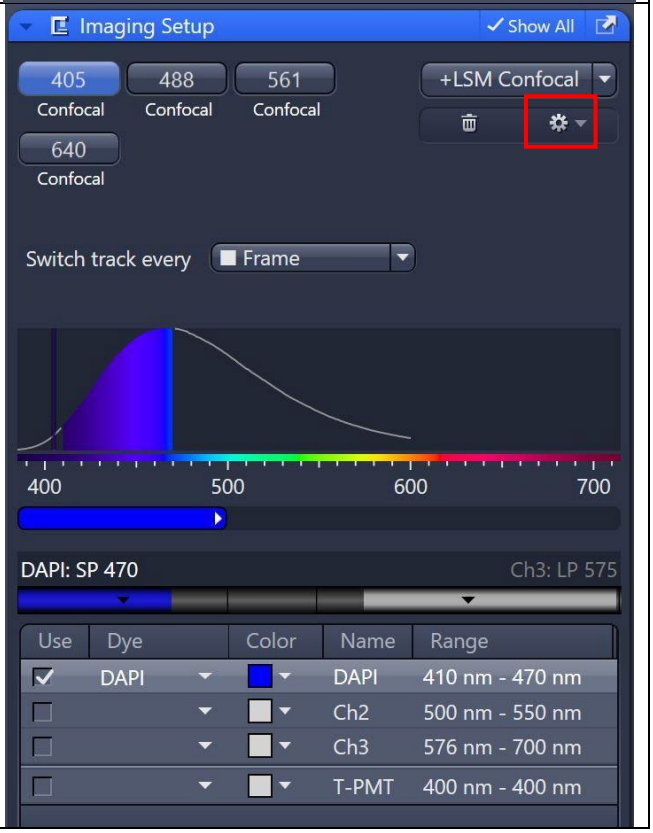
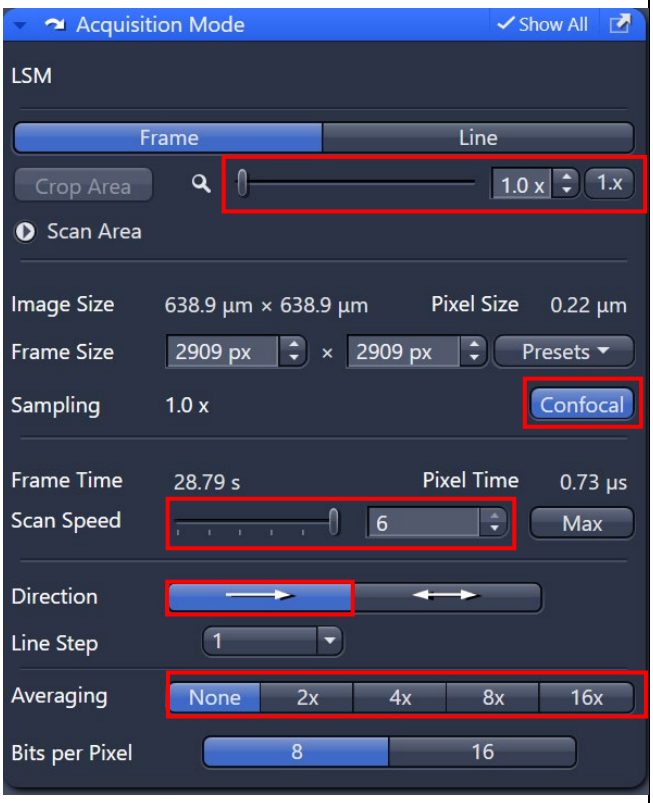
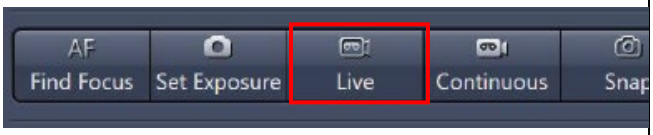
POWERING ON		
1.	Turn on the main power switch (left).	
2.	Turn on components switch (right).	
3 & 4.	Turn on the power strip. Wait a few seconds to turn on the PC.	
5.	Open **ZEN 3.5** from the desktop.	
6.	A web browser will pop up with the Google Session Start Form that you must fill out. The acquisition software will not load until the form is submitted.	

LOADING THE SLIDE		
1.	Select your desired objective in the **Microscope** menu on the right side of the program.	
2.	This is an inverted scope, so place the coverslip facing downwards. Note: the 40x objective requires Immersol and the 63x objective requires immersion oil. Place immersion media directly onto the objective before slide placement.	
Before* changing objectives from oil to dry, clean your slide AND the objective		

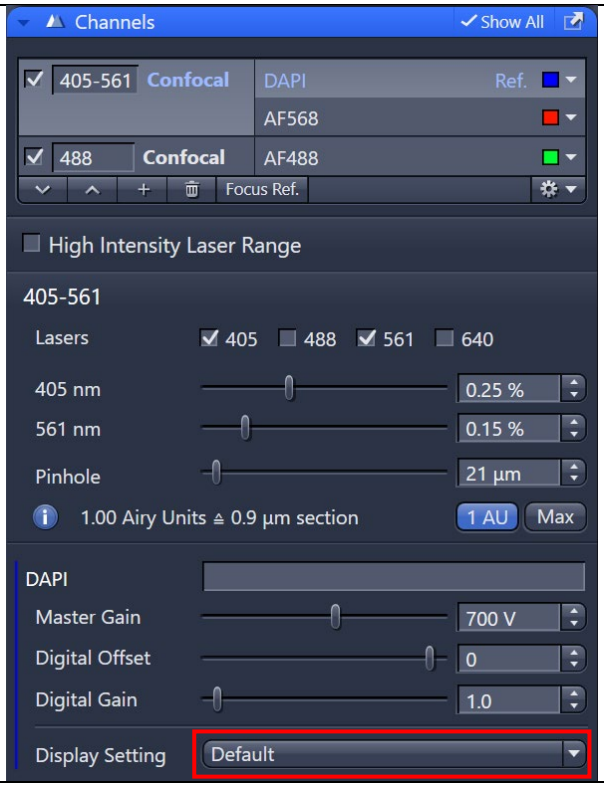
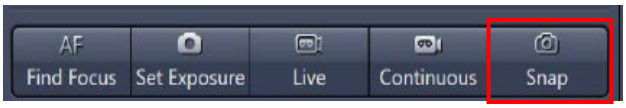
LOCATING THE SPECIMEN		
1.	Use the joystick for XY movement.	
2.	Use the mini display for focusing (Z movement).	
3.	Use the **Locate** tab to view your sample. Select transmitted light, DAPI, GFP or DsRed.	
4.	If you'd like to save a position to return to later, it can be specified with a 'Mark' under the **Stage** menu on the right-hand side of the screen. You can return to this exact position using the 'airplane' button.	

CONFOCAL ACQUISITION

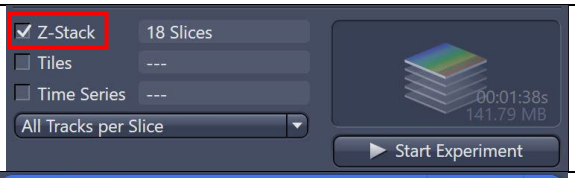
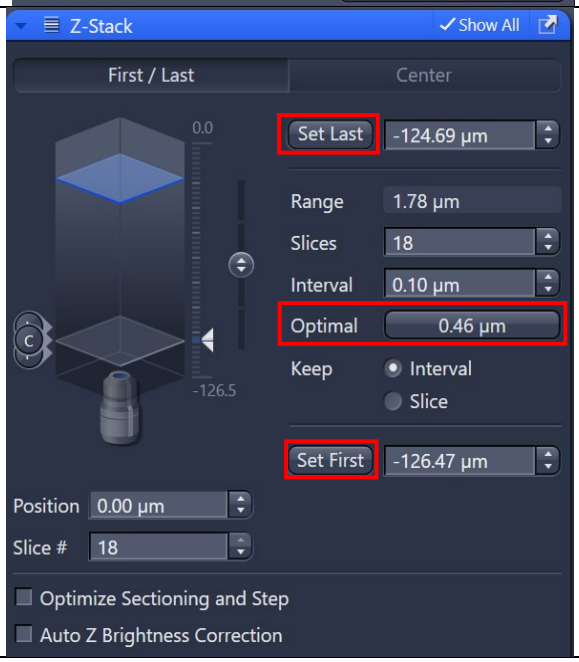
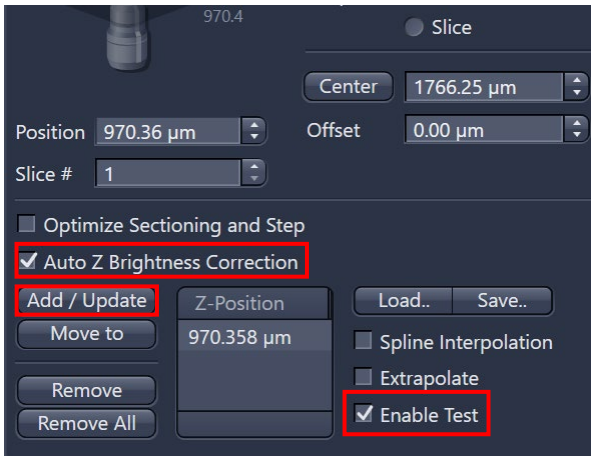
1.	Open the **Acquisition** tab.	
2.	Select your customized experiment that was set up during training or a KPIF DEFAULT program.	
3.	**Imaging Setup** menu If you need to alter the experiment settings for a new sample type, please see the appendix (pg. 19) on how to configure tracks and dye settings. Save modifications as a new experiment using the 'gear' button.	

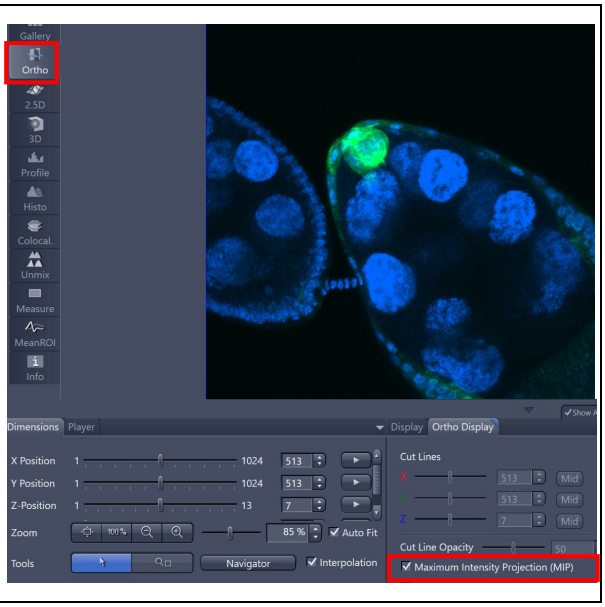
4.	**Acquisition Mode** menu **Crop area**: Set your region of interest by either moving the slider to increase the crop area or adjusting the scan area. **Frame size**: Press the 'Confocal' button to have the frame size optimized for spatial resolution. **Scan speed:** Can slow down for better signal-to-noise (instead of line averaging) or speed up for live samples (but reduced image quality). **Direction**: Bidirectional scanning reduces scan time. Click on 'Auto' to correct scanning resignation if you see artifacts. Use unidirectional scanning for publication quality images. **Averaging:** Select optimal line averaging to reduce noise.	
5.	View your specimen with the 'Live' button. Safety note: Do not look directly into the laser light while scanning.	

6.	**Channels** menu If you want to maximize image signal to noise quality, increase laser power and lower Master Gain (< 800 V). If you want to minimize photobleaching, increase Master Gain and lower laser power. Ensure the pinhole size is optimal by selecting the '1 AU' button.	
7.	Optimize image brightness via the histogram in the **Display** tab. Adjust laser power/master gain until the brightest pixels just stretch to the right without clipping (see pg. 25).	

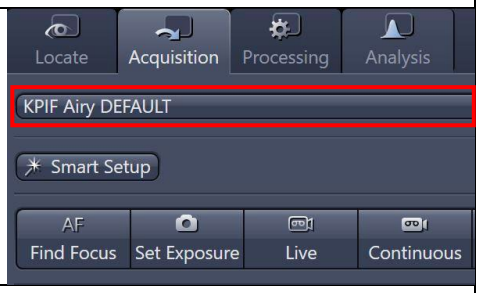
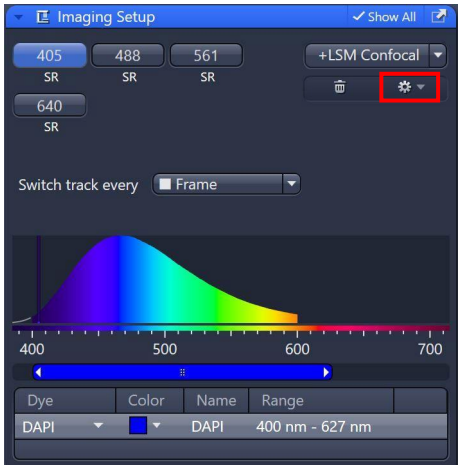
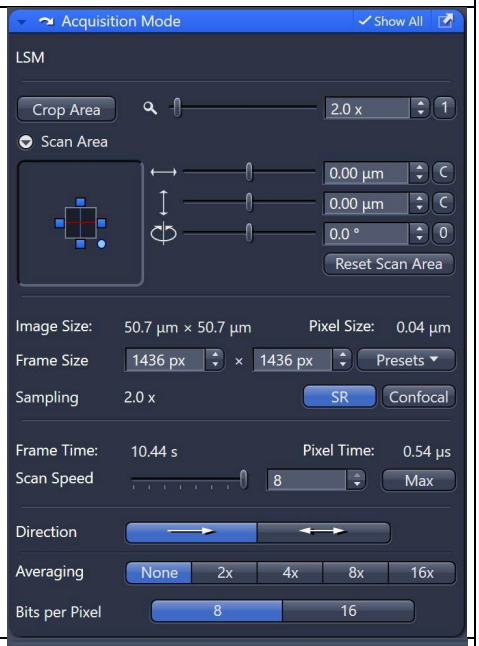
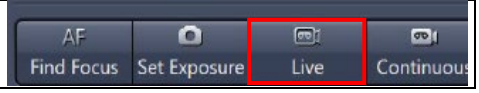
8.	Note: Ensure that the 'Display Settings' is set to 'Default' in the **Channels** menu to visualize the full dynamic range.	
9.	Use the 'Snap' button to capture an image. Save the file by selecting File > Save as.	
10.	Please review "Essential Checklist" on pg. 25 for obtaining optimal images.	

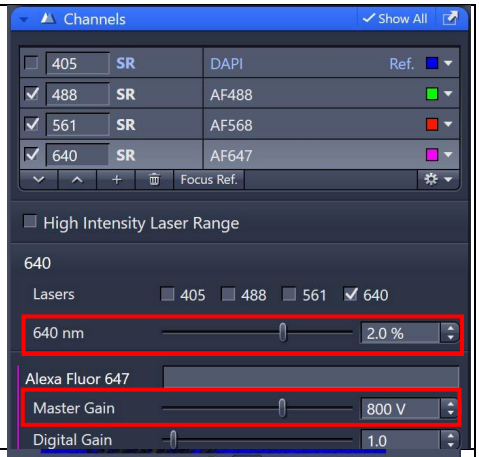
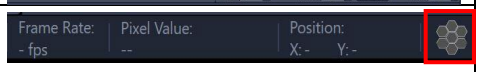
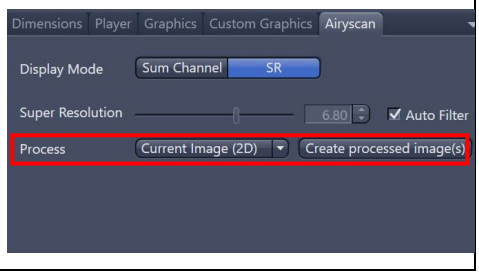
ACQUIRING Z-STACKS

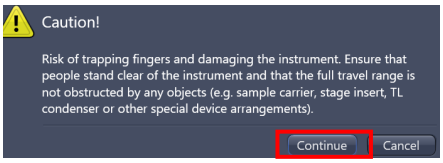
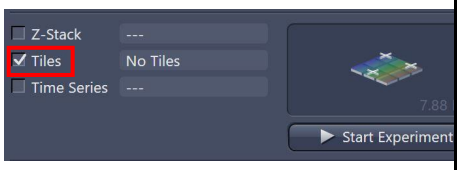
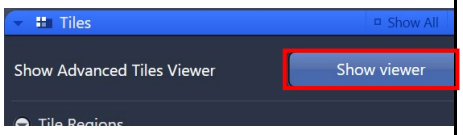
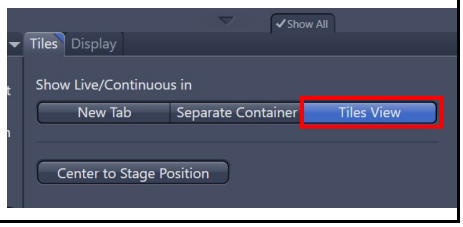
1.	Check the ‘Z-stack’ button.	
3.	**Z stack** menu Focus to one end of the specimen and click 'Set First' and then to the other end of the specimen and click 'Set Last'. Click the button labeled 'Optimal', to automatically set the interval width (step size). Note: You can change the interval if you check 'Slice' under 'Keep' and vice versa.	
	Note: If the signal intensity diminishes through a thick Z-stack, select ‘Auto Z Brightness Correction’. Go to the ‘Last’ Position and set Master Gain. Click ‘Add/Update’. Then go to ‘First’ position and set Master Gain. Click ‘Add/Update’. Select ‘Enable Test’ and choose ‘Continuous’ to preview how the laser power changes at each position in the Z-stack.	
4.	Click 'Start Experiment' instead of ‘Snap’.	

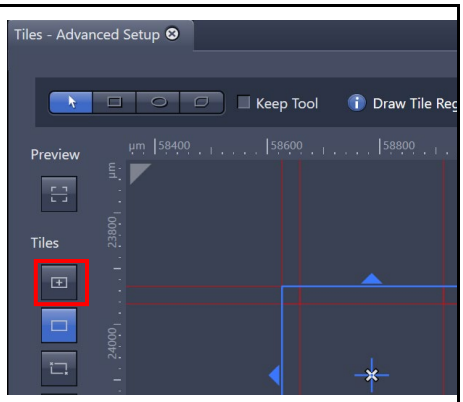
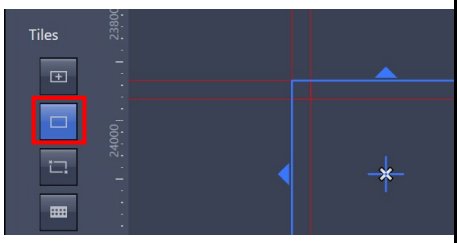
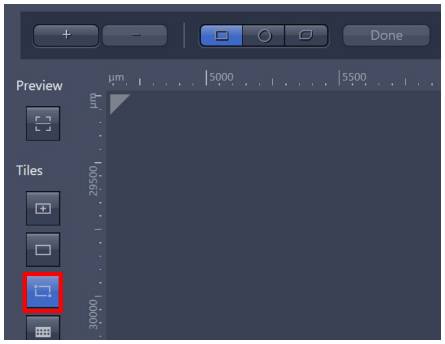
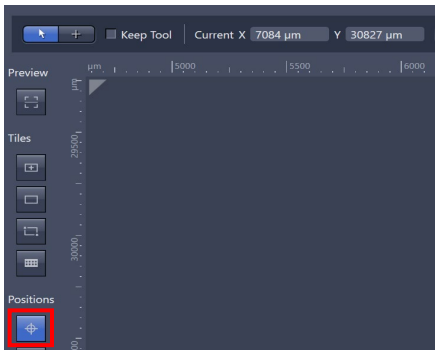
5.	After image acquisition, use the **Ortho Display** tab to view the Z-stack. Check the 'Maximum Intensity Projection (MIP)' button to generate a Z-projection, which is a single image that depicts peak signal intensities from all z-slices. Image will be labeled as an 'Experiment' vs a 'S. Save as a .czi file to include metadata.	

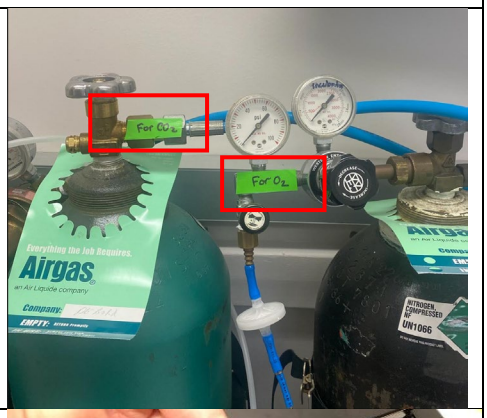
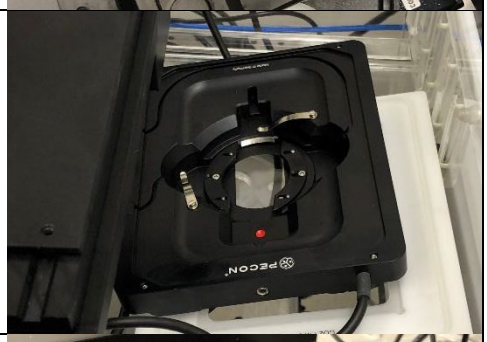
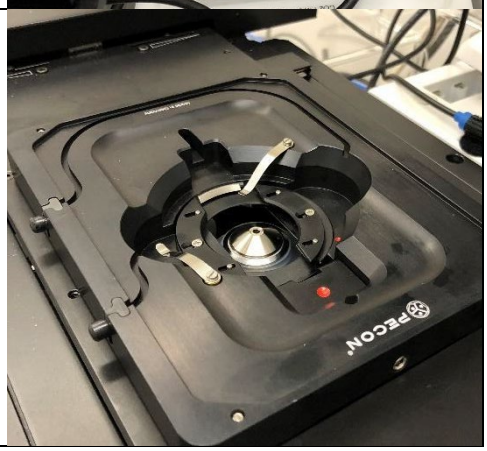
AIRYSCAN ACQUISITION

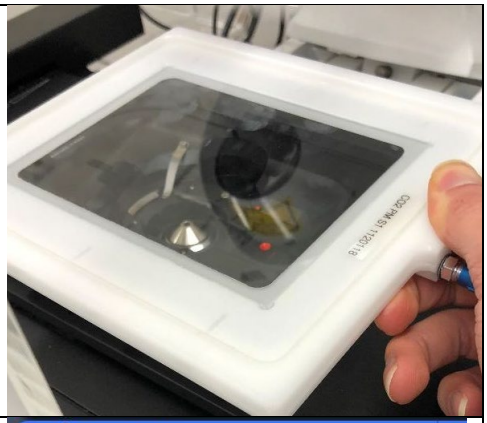
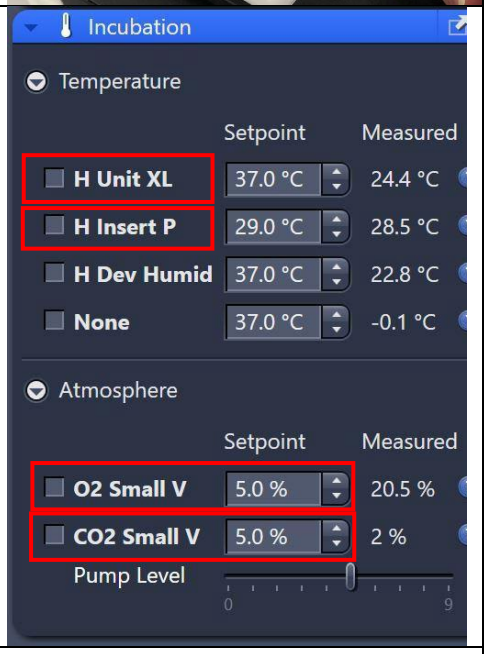
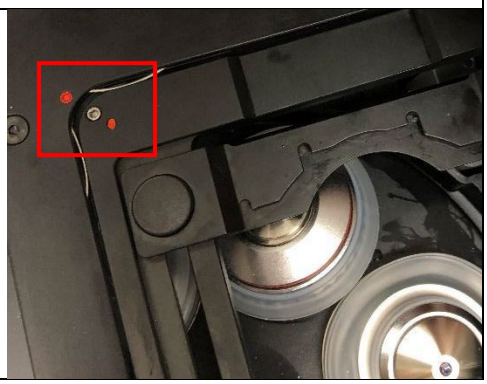
1.	*NOTE: You must use 63x objective for Airyscan* In the **Acquisition** tab, select your customized experiment. Use the 'KPIF Airy DEFAULT' template to set up a new experiment.	
2.	**Imaging Setup** menu If you need to alter the experiment settings for a new sample type, please see the appendix on pg. 19 on how to change tracks, filters and dye settings. Save modifications as a new experiment using the 'gear' button.	
3.	**Acquisition Mode** menu Optimal parameters have already been set for Airyscan experiments. Please refer to this image to double check your experiment settings. Crop or scan area can be reduced to 1.0x, but not below.	
4.	View your specimen with "Live".	

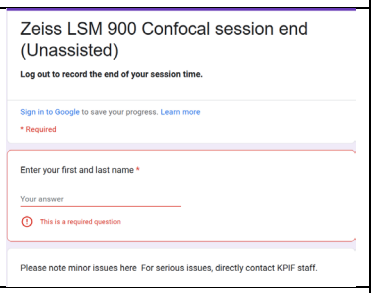
5.	Under the Channels window, adjust each channel's laser power and Master Gain.	
6.	Adjust laser power/Master Gain until the pixels just stretch to the brightest pixels at the right without piling up (i.e., "clipping" see pg. 25).	
7.	Open Airyscan adjustment (gray hexagon) at bottom of screen.	
8.	Select each channel individually to allow adjustments in 'Live' mode, which will be finished when the hexagon turns green.	
9.	Press 'Snap' to capture an image.	
10.	To save the SR image, select the **Airyscan** tab. Click on 'Create Processed Image' which results in a new image labeled 'Snap-Airyscan Processing.czi'. For Z-stacks, choose 'Current image (3D)' on the drop-down menu.	

TILING		
1.	**Stage** menu The stage must be calibrated before proceeding with tiling. Click the 'Calibration' button under the **Stage** menu on the right side of the screen.	
	Click 'Continue' on the pop-up.	
2.	Select 'Tiles' from the experiment menu.	
3.	A new tab will open. Select 'Show Viewer' to open the Advanced Setup Window.	
4.	Select 'Tile View' in the bottom right corner and then turn on 'Live'. There are several options to choose from:	

option 1:	'Set up new tile region from a predefined size'. This option will image a 3x3 tile centered around a specified position. Move the stage to a position of your choice and press 'F9' on the keyboard to specify it under 'Tile Regions'.	
option 2:	'Set up new tile regions by drawing a contour'. This option will image a tile within a boxed region that is drawn. Choose the 'Rectangle' at the top left of the Advanced Setup Window and use your mouse to draw a rectangle directly onto the preview screen.	
option 3:	'Set up new tile regions by specifying two or more marker positions. This option will image a tile that encompasses everything between two positions. Move the stage to your desired starting position and click '+' at the top of the Advanced Setup window. Repeat for the desired end position.	
option 4:	'Single positions'. This option will image only specified positions and nothing in between. Move the stage to your desired position and press 'F10' on the keyboard to specify it under 'Positions'. Repeat for every position you want to image.	
5.	Click 'Start Experiment' to capture the Tiled Image. Save the experiment as a czi.	

LIVE IMAGING WITH INCUBATION		
1.	If using CO_2 and/or nitrogen (for O_2 levels), open the valve(s) of the gas tank(s). They are labeled on the regulators.	
2.	Tilt the neck of the microscope back and remove the slide holder.	
3.	The heated plate holder and lid are located within the microscope chamber on the bottom right.	
4.	Gently place the heated plate holder on the stage.	

5.	Add your sample and place the lid on top of the plate holder.	
6.	In Zen, open the **Incubation** menu and select H Insert P (if only heating stage) and H Unit XL (if heating the entire chamber). Set the desired temperature. If heating the chamber, close the side doors and top. If using CO_2, check CO2 Small V and set the desired percentage. If using nitrogen, check O2 Small V and set the desired percentage.	
7.	When completed, uncheck the temperature and atmosphere settings.	
8.	Remove the plate holder apparatus.	
9.	Replace the slide holder by lining up the two red dots and pushing that corner down first. Close the CO_2/nitrogen gas tank valves.	

POWERING DOWN		
1.	Save all images or close the ones you did not need.	
2.	Clean immersion media from 40x or 63x objectives.	
3.	Close the ZEN program.	
4.	A web browser will popup with the Google Session End Form that you must fill out BEFORE shutting off the computer.	
5.	Shut down the computer.	
6.	Turn off the microscope by turning off the power strip.	
7.	Turn off the components.	
8.	Turn off the main power.	

APPENDIX - For imaging setup

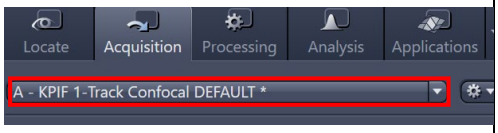
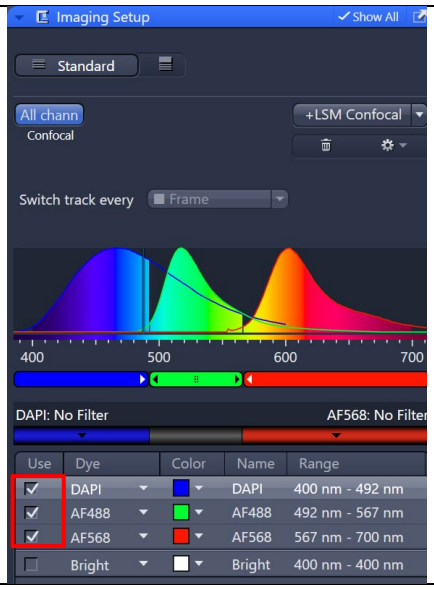
A. Setting Up Fluorescent Channels Without Transmitted Light

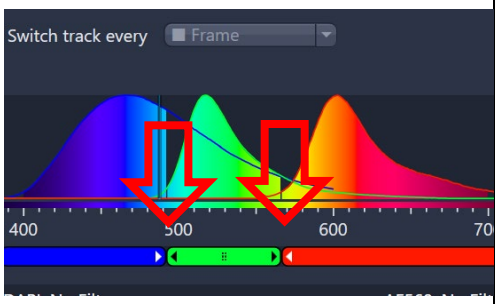
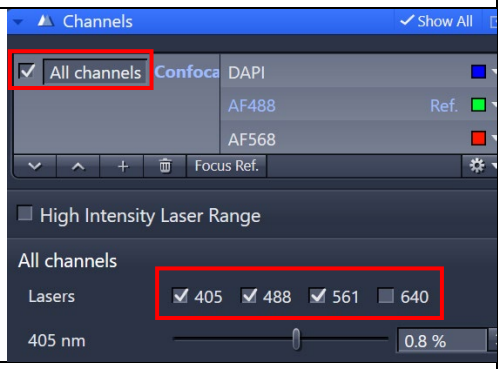
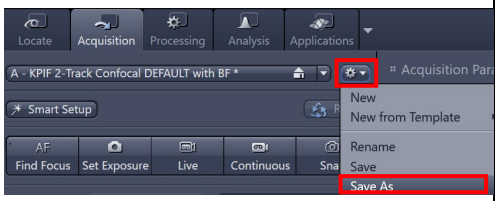
Important Consideration:

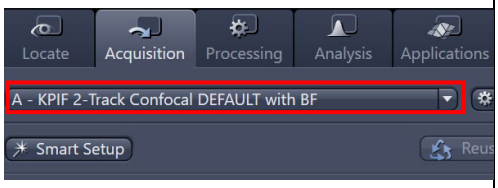
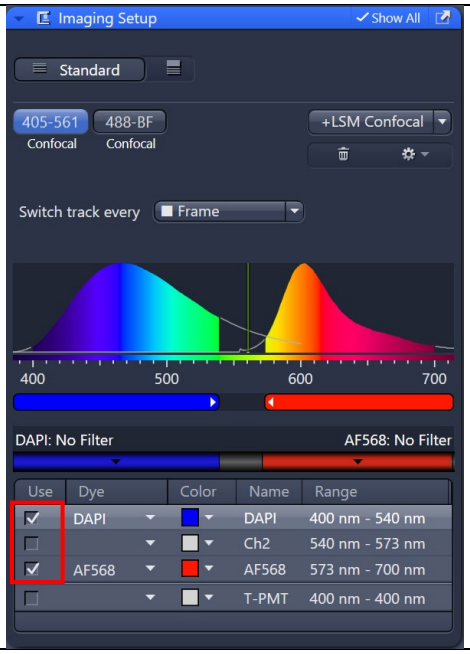
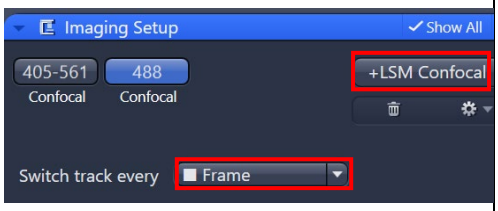
One Track: Fastest acquisition; recommended for ≤ 2 fluorophores without spectral bleed through.

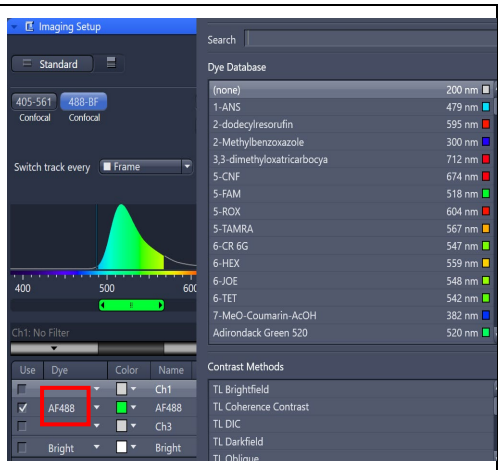
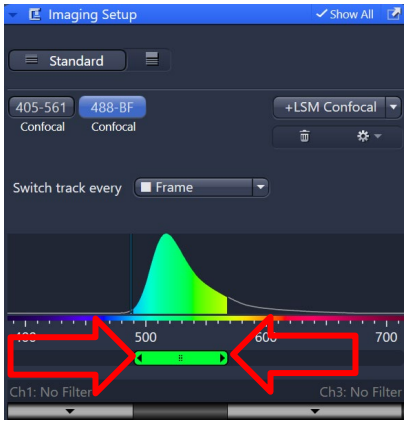
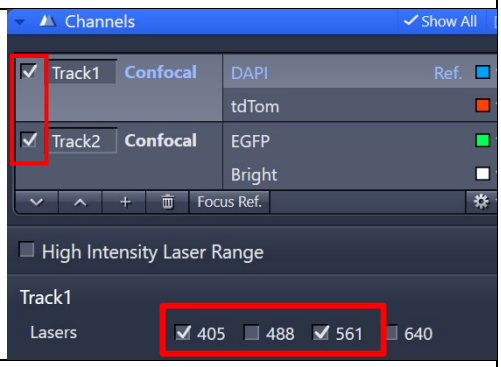
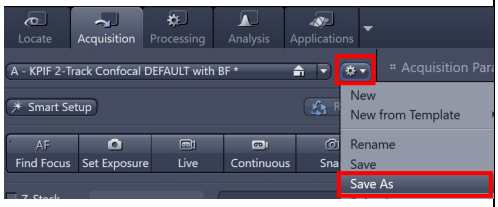
Two or more Tracks (pg 21): Slower acquisition; recommended for multiple fluorophores with emission spectra overlap/bleed through.

One Track

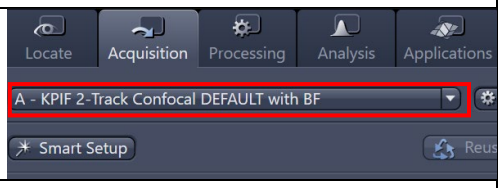
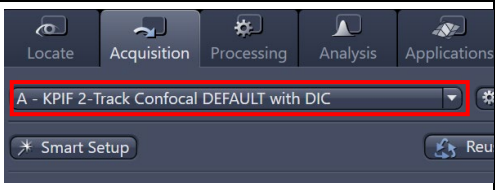
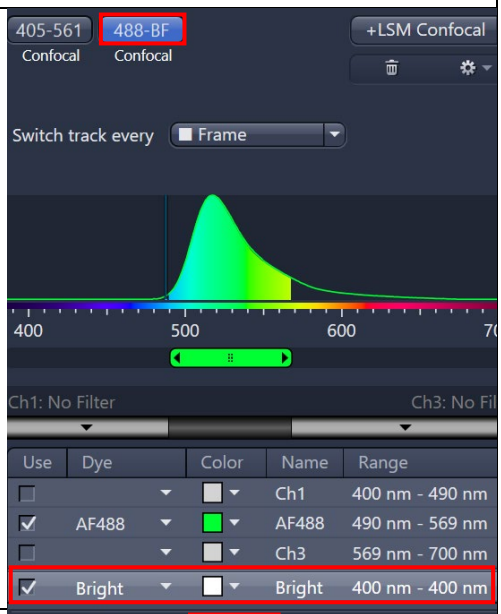
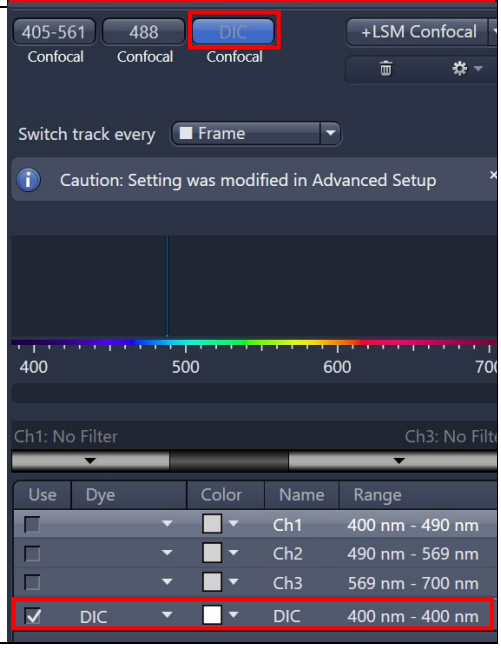
1.	Choose 'A - KPIF 1-Track Confocal DEFAULT' from the **Experiments** menu as a template to modify.	
2.	**Imaging Setup** menu In this default program, the blue, green, and red emission channels are checked. Uncheck any channels you do not require. Channel 1 - Blue emission (400-480nm) Channel 2- Green emission (480-560nm) Channel 3 - Red emission (560-700nm) Channel 4 - Transmitted light	
Note:	If you are concerned about the potential for spectral bleed through, it is recommended to separate channels into multiple tracks (see Two or more Tracks sub-section, pg. 21)	

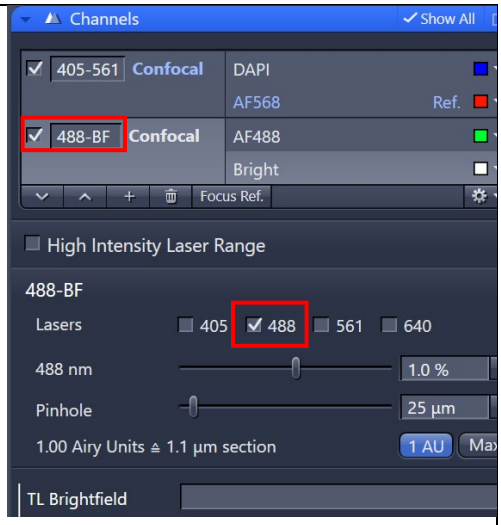
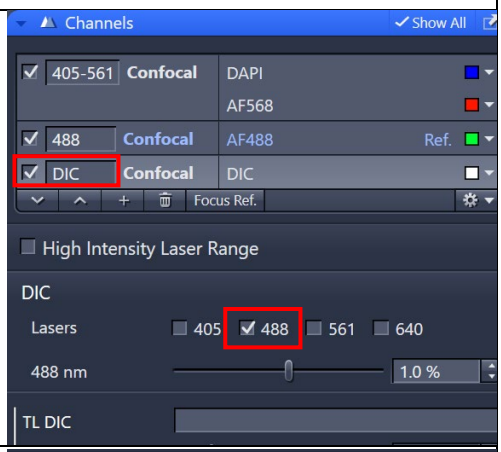
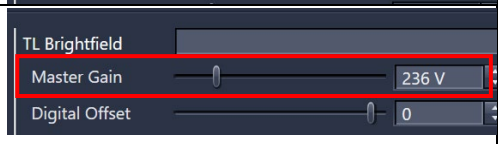
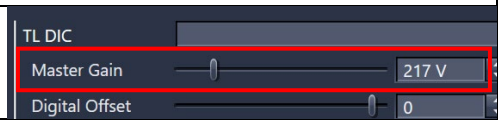
3.	Choose a dye from the dropdown menu for each channel. If you do not find your specific dye, select one with the closest spectral properties (so you can visualize the emission curve).	
4.	Adjust the emission capture window for each channel to a desired range by moving the sliders. Make sure the green emission window doesn't overlap with the 488 laser and/or the red emission window doesn't overlap with the 594 laser (This does not apply to the 405 or 640 laser line).	
Note:	Changing the capture window for each channel will influence the capture window for other channels	
5.	Select your track and lasers from the **Channels** menu under the **Acquisition** tab. If you deleted any emission channels (step 2) uncheck any lasers that you don't require.	
6.	Save your modifications as a new experiment using the 'gear' button and choose 'Save As'. DO NOT CLICK 'SAVE' as this will overwrite the default template.	

Two or more Tracks		
1.	Choose ‘A - KPIF 2-Track Confocal DEFAULT with BF’ from the **Experiments** menu as a template to modify.	
2.	Select channels before specifying a dye. In this default program, the blue and red channels are checked in track ‘405-561’, and the green light channel is checked in track ‘488-BF’. Uncheck any channels you do not require. Channel 1 - Blue emission (400-480nm) Channel 2- Green emission (480-560nm) Channel 3 - Red emission (560-700nm) Channel 4 - Transmitted light	
Note:	Dyes with green fluorescence emission are recommended to be kept in the separate track.	
Note:	If you wish to add a new track: choose either LSM Confocal or Airyscan using the dropdown menu. To delete a track, click the trash can icon. Select switch track every frame.	

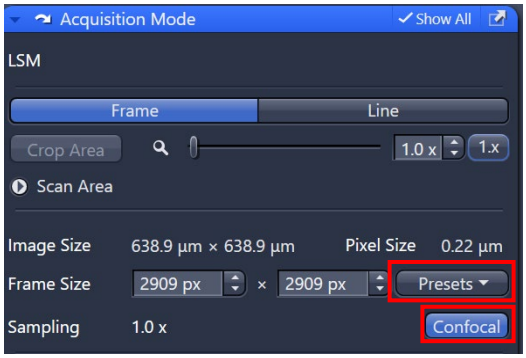
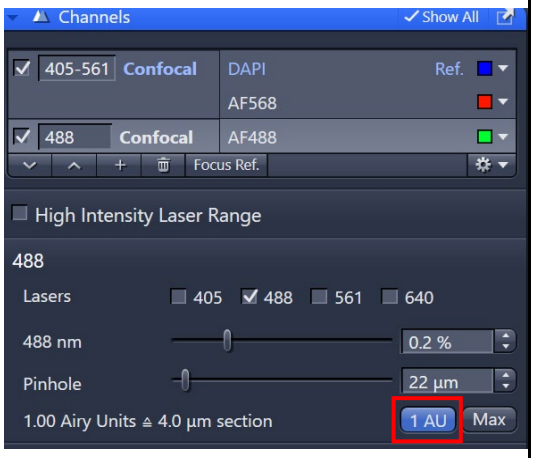
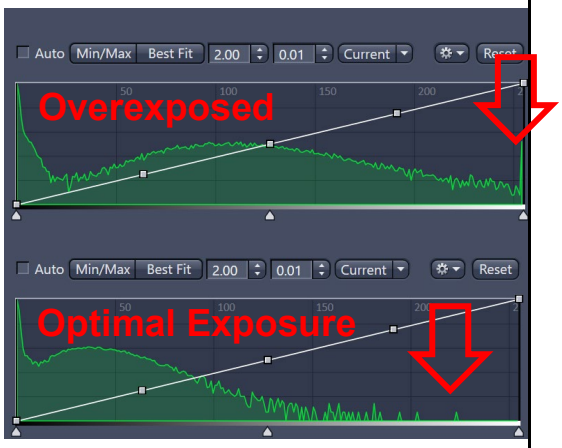
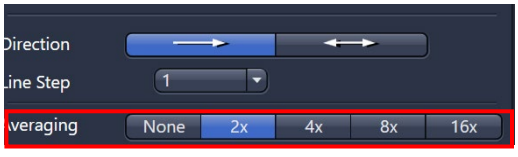
3.	Choose a dye from the dropdown menu for the appropriate channel. If you do not find your specific dye, select one with the closest spectral properties (so you can visualize the emission curve).	
4.	Adjust the emission capture window for each channel to a desired range by moving the sliders. Make sure the green emission window doesn't overlap with the 488 laser and/or the red emission window doesn't overlap with the 594 laser (This does not apply to the 405 or 640 laser line).	
Note:	You can select multiple dyes per track with confocal imaging, as long as their emission curves do not overlap.	
5.	Remember to select your tracks and lasers from the **Channels** menu under the **Acquisition** tab. If you would like to exclude tracks, uncheck the track. If you would like to exclude individual lasers from the track, uncheck the laser from the track.	
6.	If you would like to save your current settings for future use, save your modifications as a new experiment using the 'gear' button and choose 'Save As'. DO NOT CLICK 'SAVE' as this will overwrite the default template.	

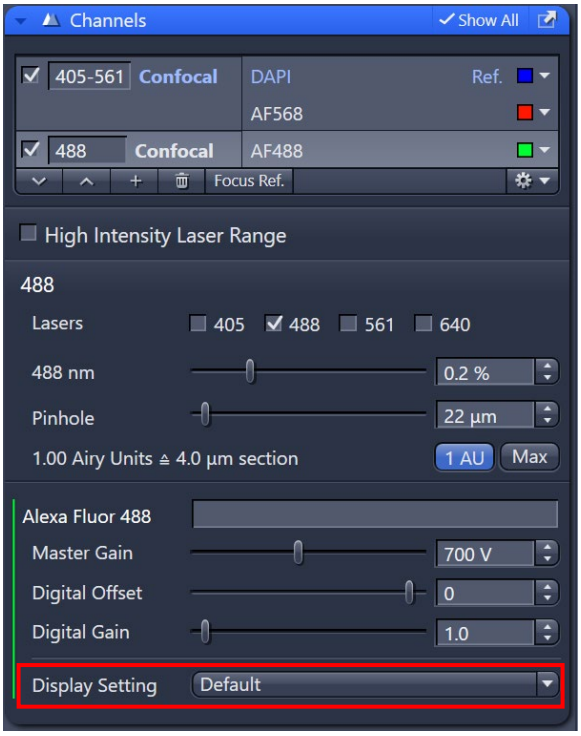
B. Fluorescence with Transmitted Light Channels

1.	For Brightfield (BF) images choose 'A - KPIF 2-Track Confocal DEFAULT with BF' from the **Experiments** menu as a template to modify.	
	For Differential Interference Contrast (DIC) images choose 'A - KPIF 2-Track Confocal Default with DIC' from the **Experiments** menu as a template to modify.	
2.	In the **Imaging Setup** menu: For BF, ensure the transmitted light channel labeled "Bright" is checked in the 488-BF track.	
	For DIC, ensure the transmitted light channel labeled "DIC" is checked on the separate DIC track. Note: DO NOT modify the DIC track. Only DIC should be specified in this track.	

3.	Under the **Channels** menu: For BF, ensure that the 488 laser is checked for the 488-BF track.	
	For DIC, Under the **Channels** menu, ensure the DIC track and the 488 laser are checked.	
4.	To change brightness, adjust master gain. For BF, adjust the master gain under 'TL Brightfield'.	
	For DIC, adjust the master gain under 'TL DIC'.	

ESSENTIAL CHECKLIST - for optimal images

Is 'Confocal' Checked in the **Acquisition Mode** menu? *If not, optimal spatial resolution will not be not attained, However, you can choose lower resolution from the presets for faster image acquisition if optimal spatial resolution isn't required.*	
Is 1 AU checked for each channel in the **Channels** menu? *If unchecked, more out-of-focus light is collected than optimal and is not considered 'confocal'. However, you may want to collect more light if your sample is extremely dim.*	
Is your image properly exposed? *Adjust laser power/gain until dynamic range fits below the end of the histogram and there is no clipping (red arrow on top image).*	
Lots of background noise? *Try adding Line Averaging from the **Acquisition Mode** menu.* Note: This increases acquisition time.	

TROUBLESHOOTING	
Is the whole screen a single color with no visible image during acquisition? *Double check that the 'Display Setting' is set to 'Default'* for each channel.	
Is Zen and/or the computer unresponsive? *Try restarting the computer and opening Zen again. If problems persist - contact Dr. deCarvalho.*