

Leica SP8 TCS Users Manual

Follow the procedure for *start up* and *log on* as posted in the lab. Please log on with your account only and do not share your password with anyone. We track and confirm usage through Windows by your log in and you will be charged for the time spent on the machine as documented through the Windows log in.

Start up:

- 1 Turn computer on by pushing the button on the front of the CPU.
- 2 Log in with your user account and password.
- 3 Turn on the two green switches and turn the key (large box under the table).
- 4 Turn on the microscope box (CTR advanced).
- 5 Turn on metal halide lamp (gives fluorescence on the microscope preview).
- 6 Double click on LAS X software (can take up to two minutes for software to launch).
- 7 “Machine” operates the microscope while “Simulator” is the image processing portion of the software.
- 8 Highlight the *configuration tab* to access the lasers.
- 9 Highlight “*Laser Configuration*”.
- 10 Check each laser box to turn it on.
- 11 Highlight the *Acquire Tab* to get back to the acquisition software.

Initial focusing hints: This is an inverted microscope with the objectives below the specimen. If using a coverslipped slide, you must turn it upside down to image. If using a plate, the bottom thickness of the plate is critical. It must be a specialized glass-bottomed dish, ideally with a thickness of 1 or 1.5mm (same as a coverslip). If you try to use a plastic dish or right-side up slide, you will have too great a working distance with higher NA objectives and will not be able to focus on your sample.

When using oil immersion objectives, use only one small drop of oil, either placed directly on the oil objective or on your coverslip before inverting. **DO NOT USE OIL** on the air or water lenses! You will severely damage them and incur a high repair bill.



Overview of available objectives:

20x: not UV rated (will NOT transmit dyes efficiently in the UV range, i.e. DAPI) You may get some signal but your images may not line up with multi-channel labeling.

40x CS2 oil: chromatically corrected; ideal for use with UV and other spectrum dyes.

63x CS2 oil: chromatically corrected; ideal for use with UV and other spectrum dyes.

100x oil: not UV rated (will NOT transmit dyes efficiently in the UV range, i.e. DAPI) You may get some signal but your images may not line up with multi-channel labeling.

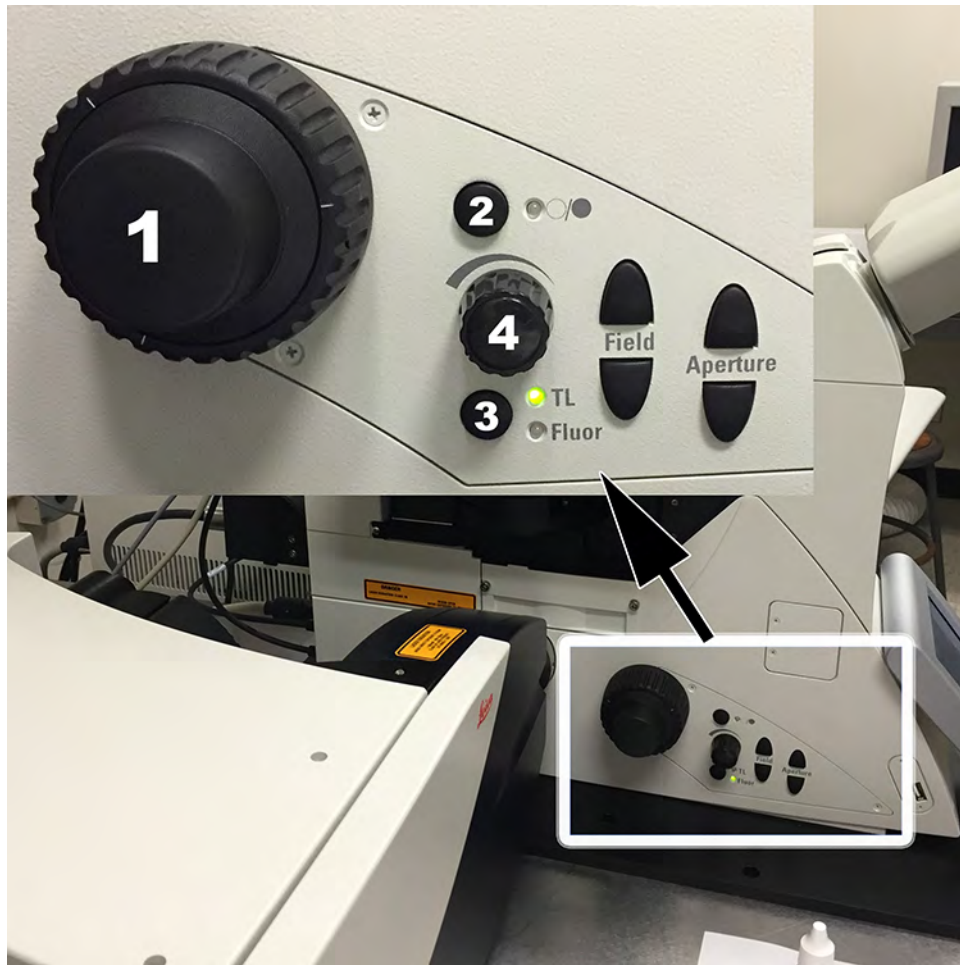


Objectives are selected on the **front touchscreen control panel (1)** by touching the desired button. The turret will turn automatically to the selected lens. Alternatively, you can manually turn the turret to the objective you want and the touchscreen will register the change or you can select your desired objective from the acquisition tab (see page 12).

The Salt and Pepper shaker is the only way to move the stage in the X/Y plane. The top knob (Y) moves in the Y plane and the bottom knob (X) moves in the X plane. You can toggle between *precise* and *fast* by pressing the buttons on the left side of the base (arrow). Similarly, the back knob controls the Z movement as an alternate to the focus knob on the microscope proper. Using the buttons on the base on the right side, you can toggle between *coarse* and *fine* focus in the Z plane. This knob is useful when initially focusing on the sample and bringing the objective up to get within range of focus or to make contact with the oil droplet on the inverted slide or bottom of plate when using an oil objective.

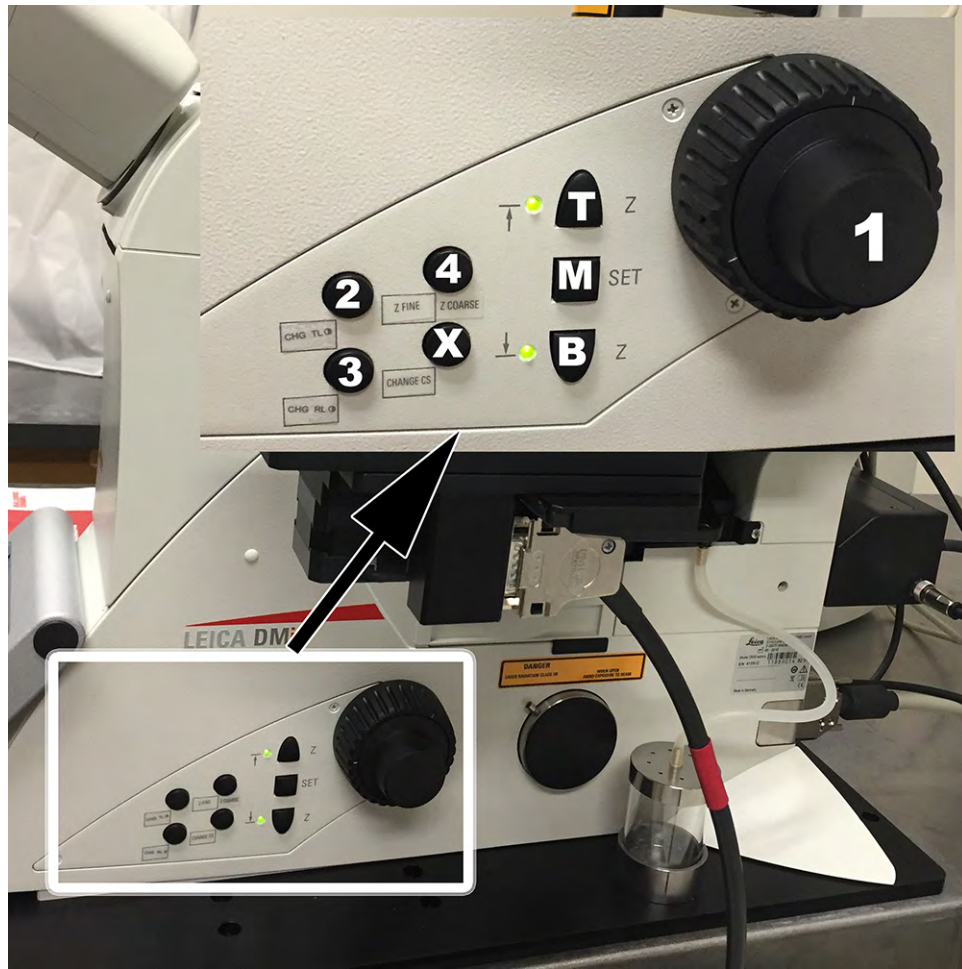


Overview of buttons on both sides of the microscope:



Left side:

- 1) *Focus knob*: outer knob is the fine focus, inner knob is the coarse focus.
- 2) *Shutter button*: this turns on and off the light going to the eyepieces. It is duplicated on the second tab of the touchscreen. If you don't see anything through the eyepieces, try clicking the *shutter button* to open up the shutter.
- 3) This button toggles between brightfield and fluorescence. You can use it to view your sample through the eyepieces and see where the fluorescence is located in relation to your morphology. This method is an alternate way of switching between brightfield and fluorescence which doesn't require the user to look away (ie, at the touchscreen) from the sample.
- 4) Knob that controls the intensity of the light going to the eyepieces (both the white light and the fluorescence). If the eyepieces are dark, try turning it to amp up the light.



Right side:

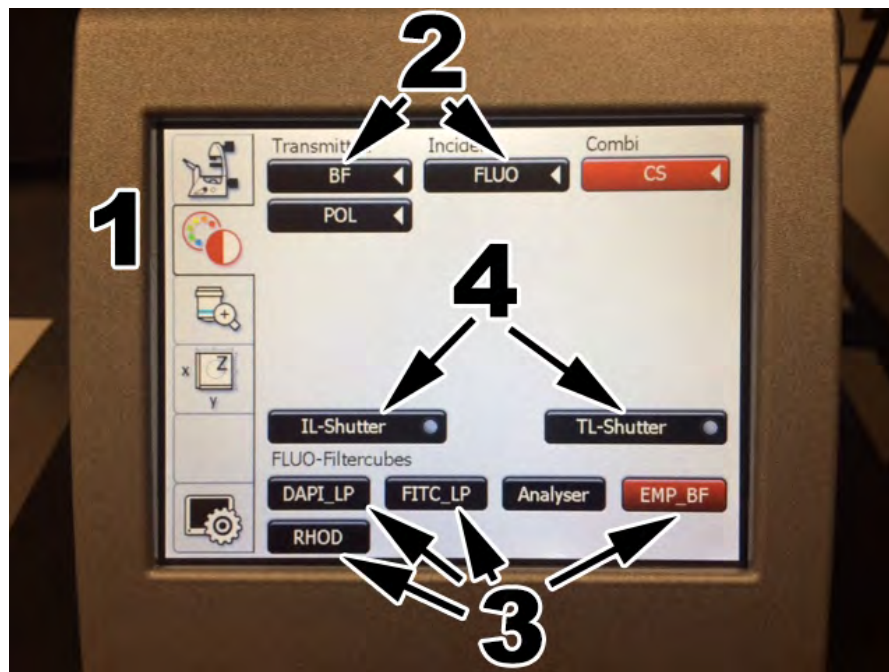
- 1) *Focus knob*: outer knob is the fine focus, inner knob is the coarse focus.
- 2) *Brightfield*: switches on and off the white light.
- 3) *Fluorescence*: switches on and off the fluorescent light; you can toggle between buttons #2 and #3 if you want to see where the fluorescence is in your sample in relation to the overall morphology.
- 4) *Fine/coarse toggle*: toggles between fine and coarse focus speeds for the Z focus.
- x) Does nothing.

T,M,B Limit buttons- set upper and lower stage limits as follows: To clear the upper limit, hit the *T* and the *M* together. The top green light will turn off. Focus the stage to where you want to set the top limit; hit the *T* and *M* together again. Similarly, to clear the bottom stage limit, hit the *M* and *B* together and the bottom green light will turn off. Focus the stage manually to your lower limit and hit the *M* and *B* together to reset the bottom stage limit.

Touchscreen overview:

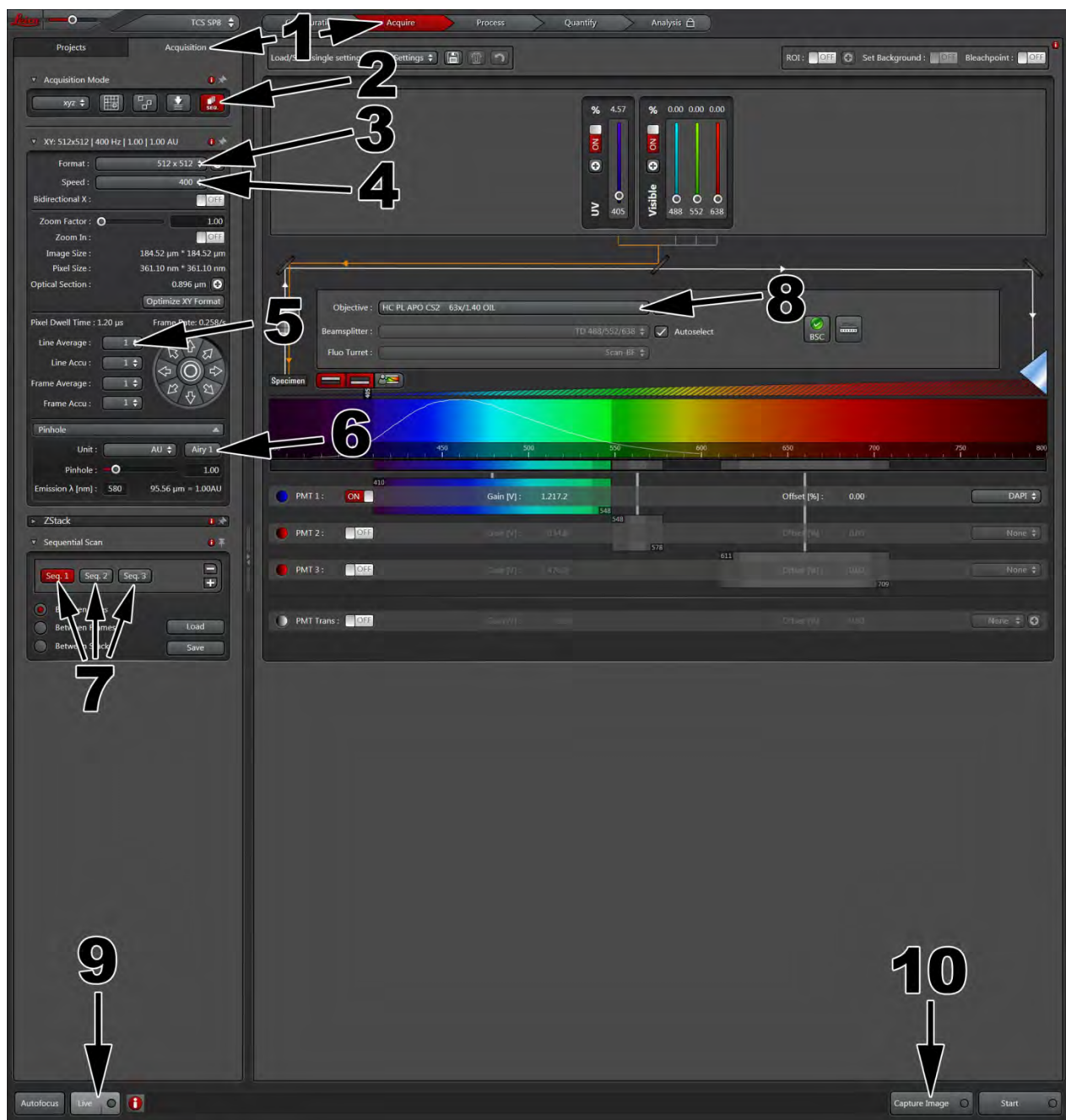


Microscope tab (1): This screen highlights the aperture opening and the intensity of the transmitted light. TL=transmitted light (2) toggles the shutter between open and closed for brightfield illumination. *Intensity* and *Aperture* (3) adjustment.



Dichroic color tab (1) Allows the user to switch between brightfield (BF) or fluorescence (FLUO) (2). CS and POL do nothing. Below, you can preview DAPI (blue), FITC (green) or RHOD (red) through the eyepieces by touching the appropriate buttons (3). IL-Shutter opens and closes the fluorescence shutter and the TL-shutter (4) opens and closes the shutter for transmitted light.

Familiarizing yourself with the Acquisition Tab:



1) To acquire an image, make sure you have selected the *Acquire* tab in the middle portion of the screen and the *Acquisition* tab selected in the left portion of the screen.

2) If you have multiple colors you want to image, it is recommended to use a sequential scan. To perform a sequential scan, hit the *seq* button. For help in setting up your light paths, please see pages 10 and 14.

3) *Format* refers to how many pixels will be captured when you acquire an image. A **512 x 512 pixel** format will give you good resolution at a reasonable speed. If you select **1024 x 1024**, you will have better resolution but it will take longer to image and be a much bigger file size.

4) *Speed* refers to the speed of the scanhead as the lasers pass over each pixel. The slower the scan *speed*, the better your signal-to-noise ratio becomes. However, slower scan speeds increase the pixel dwell time which can increase photo bleaching of your sample. The scan speed defaults to 400 Hz, but by increasing the speed to 600 Hz, you limit bleaching and the image quality is usually comparable. **Either 400 or 600 Hz are good scan speeds to select.**

5) *Line Averaging* is another means to clean up the signal-to-noise in your image. You want to have *line averaging* at at least 4 (**8 is recommended**) to acquire the best image with minimal background. The higher the averaging, the longer your scan will take but the image quality should improve significantly. Keep in mind that when scanning in *Live* mode, the line averaging is not incorporated. Only when you *Capture* an image does the line averaging become active. Note: *Frame averaging* is an option and averages each scanned frame separately, accumulating the averaging as it adds the number of scans indicated. It's a matter of preference as to which averaging technique you use, but we generally recommend *line averaging*.

6) *Pinhole*- this is the size of the opening in which the laser(s) pass through. For true confocal microscopy, you always want to choose *Airy 1*. When you hit *Airy 1*, the red slider will adjust the pinhole to the appropriate diameter based on the objective and laser wavelengths you are using. However, if your signal is weak, you can open up the *pinhole* to allow more laser light through to the sample and you will be able to detect a stronger signal. Keep in mind that the more open a *pinhole* is, the closer you are approaching basic fluorescence microscopy and losing the “confocalness” of your scan. If your goal is to determine co-localization of multiple fluorophores, all your channels need to be set to ***Airy 1***.

7) If you selected *seq* (the sequential button, see #2) you can see your separate light path sequences here. Each sequence corresponds with the proper excitation settings and emission gating to visualize a single dye in your sample.

8) Allows you to change objectives from the software screen instead of the touchpad (see page 12).

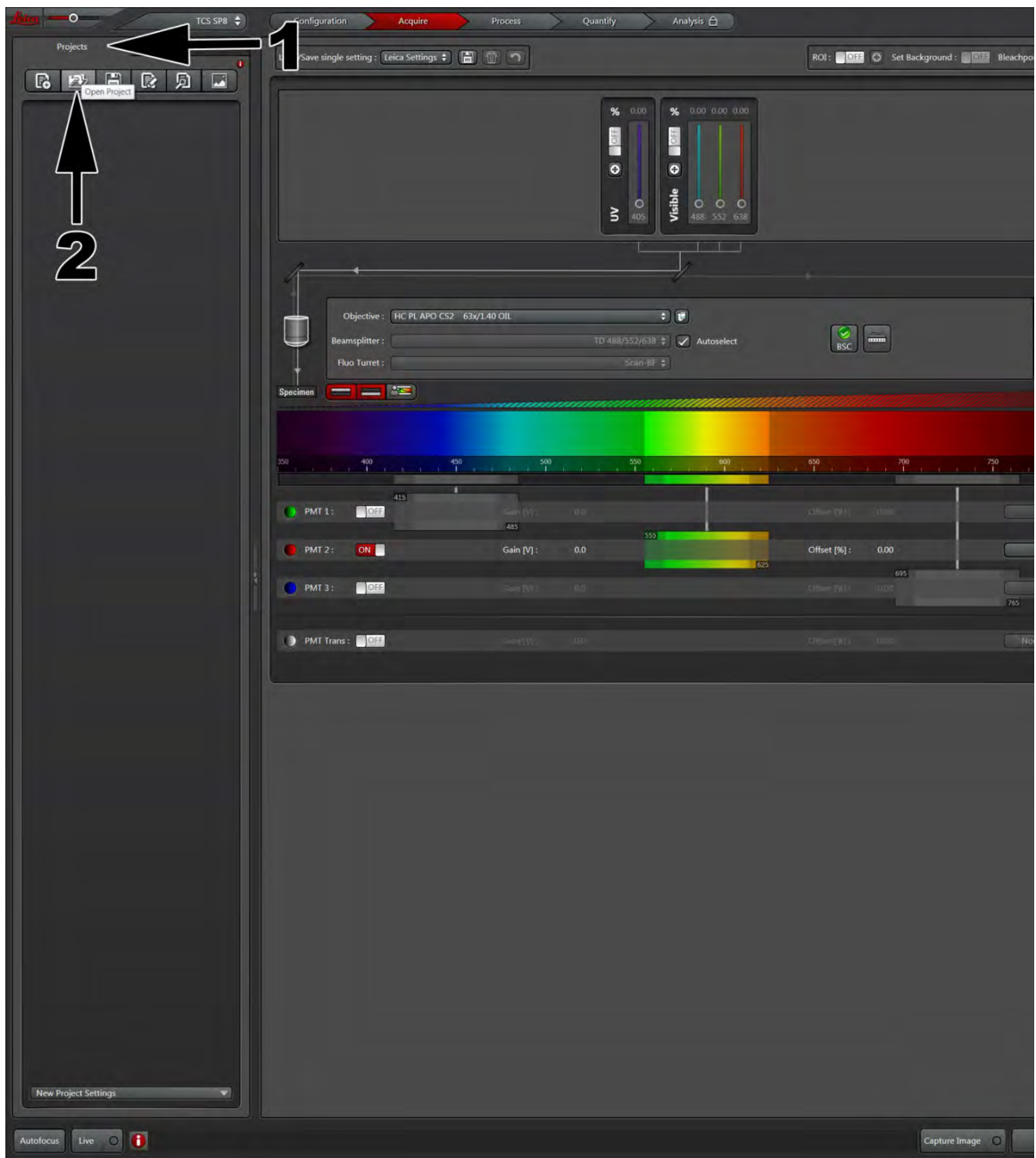
9) Initiate a *Live scan* in order to preview your image and set the brightness (Gain) and contrast (Offset) levels accordingly (see page 17).

10) After you have set your imaging settings appropriately, hit *Capture Image* to actually collect the picture. By *capturing* the image, you are incorporating all the averaging settings into the final picture. All *captured* images are stored under the “Projects” tab. If you are merely doing a *Live* scan, those images are not stored.

Reuse Button:

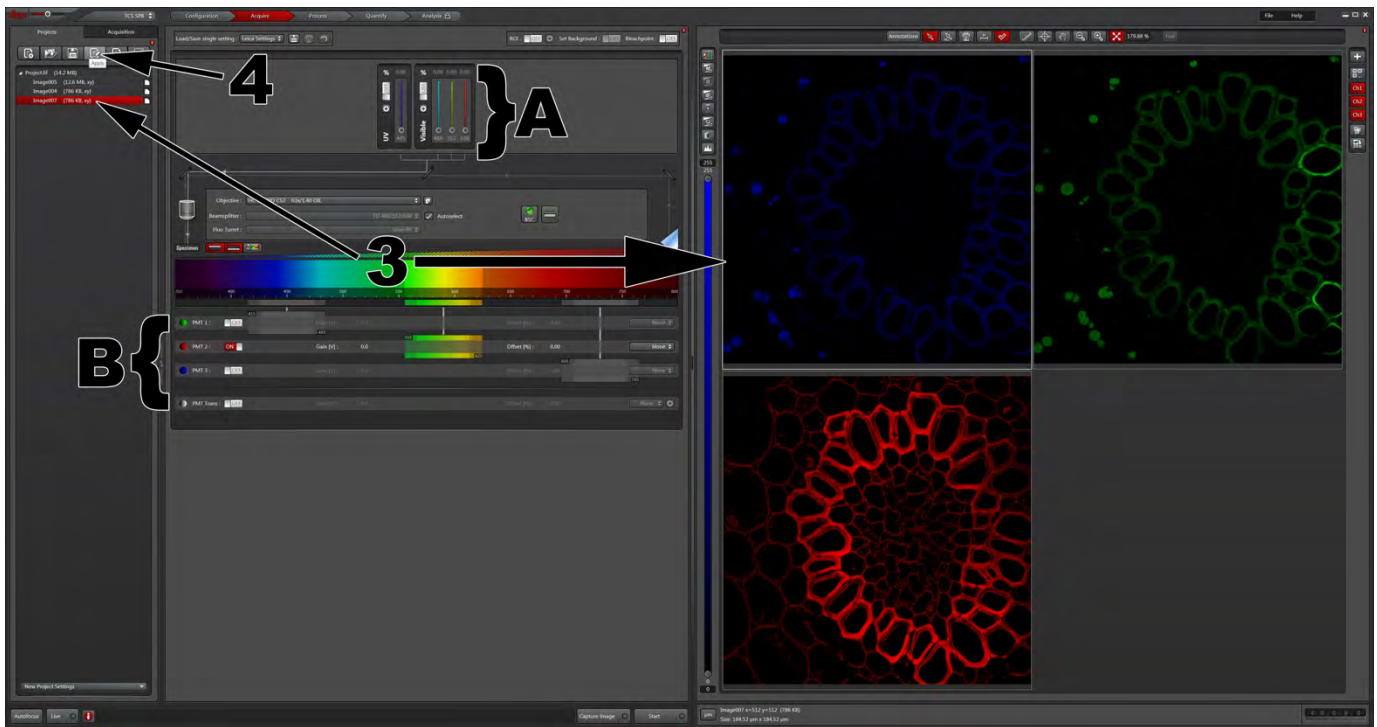
The easiest way to set up a light path is to reuse the settings from a previous session. You will need to load in a previously collected file.

- 1) To Reuse the settings from a previous picture, go to the *Projects Tab* (Labeled 1).
- 2) Click the *Open* button (Labeled 2). Find your file containing a picture you have already captured and hit open.



3) Once you have opened one of your previous experiments, you can preview the pictures by clicking through the list. The preview of the picture can be seen on the right side of the screen (labeled 3).

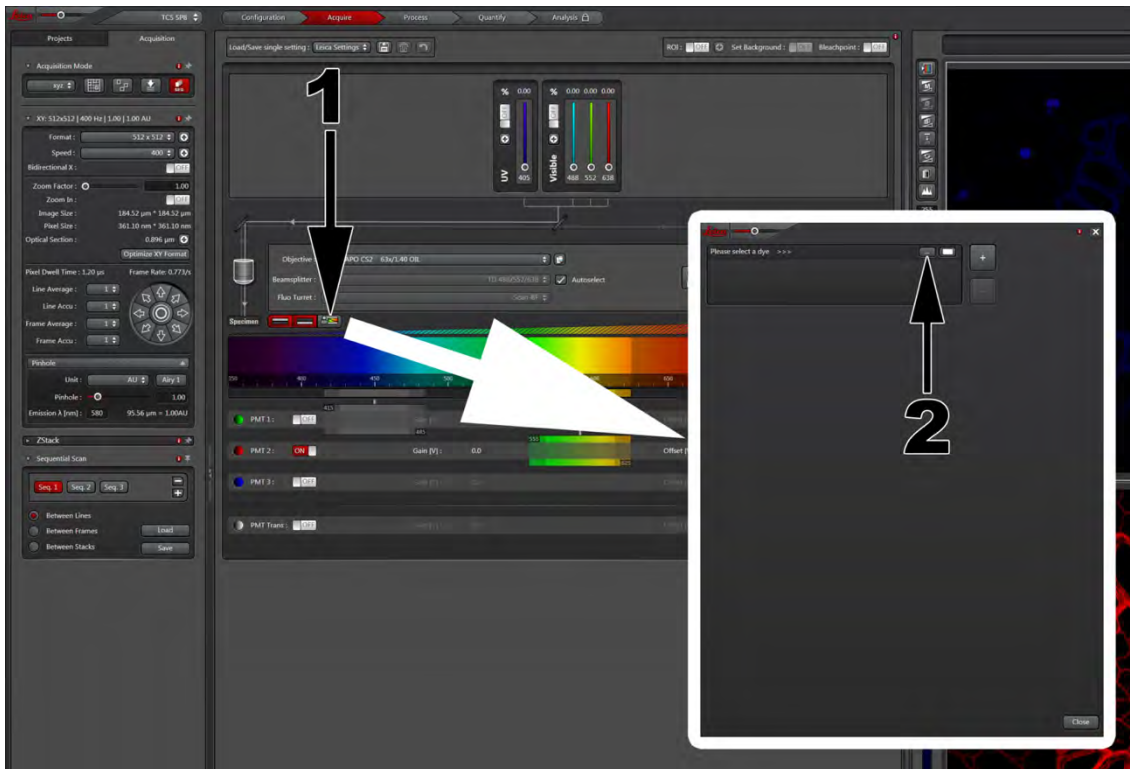
4) When you find the picture that you want to reuse the settings for, highlight that picture so it's red then hit the *apply* button on top (labeled 4). Hitting the *apply* button will set the **Laser Power** (labeled A) and **Detection Gating** (labeled B) to the same configuration as was used for your previous image. The reuse function also calls up the gain, offset, pinhole and zoom factors used in your previously stored image acquisition setup.



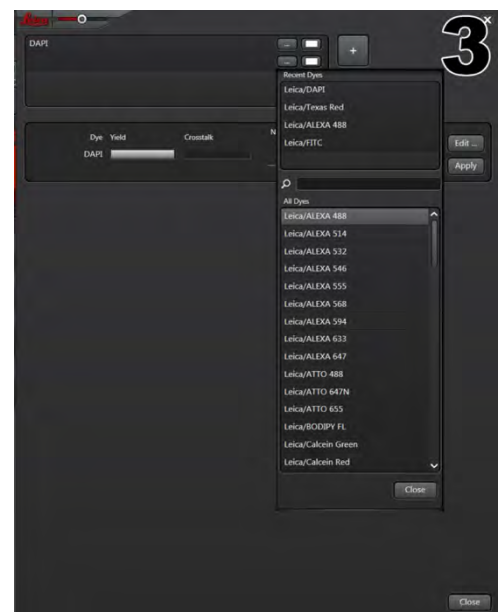
Dye Assistant:

The *Dye Assistant* can help you select the appropriate light paths and channel setup based solely on the dyes you have used in your experiment. If it's your first time with a particular imaging setup, the dye assistant is a great place to start.

- 1) Click on the *Dye Assistant* button near the center of the page labeled #1.
After clicking the *Dye Assistant* button, the window highlighted in white should appear.
- 2) Click on the ... button to select your desired dyes.



- 3) After Clicking the ... button, a list of dyes will appear. Select your dyes or a dye that is similar in emission and excitation as your own.



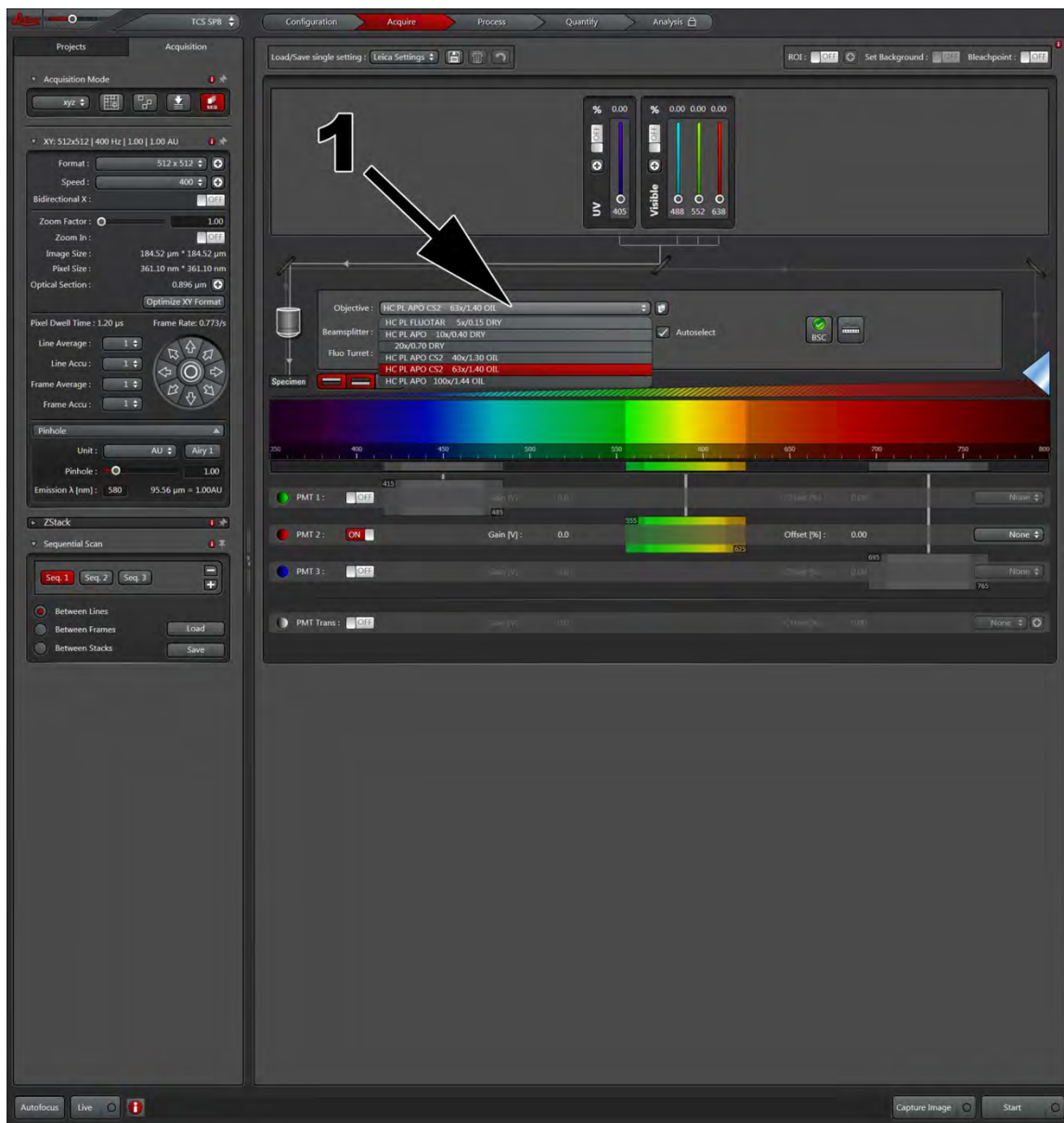
4) After your dyes have been selected, you will be given several experimental setups to choose from. Choose the setup with the least amount of cross talk then hit the *Apply* button for that particular setup.



A word on selecting light paths: Your choice will always depend on a trade off between time invested in taking your images and the quality of your images. The individual light paths (separate channels per fluorophore) will always give you the cleanest images but does take the most time. If your fluorophores are significantly separate (ie, Dapi and Texas Red) you can save time by having both of those fluorophores collected on the same channel simultaneously. In order to reduce or eliminate cross talk, you can adjust and truncate your gating within the *Acquire* tab (see page 14).

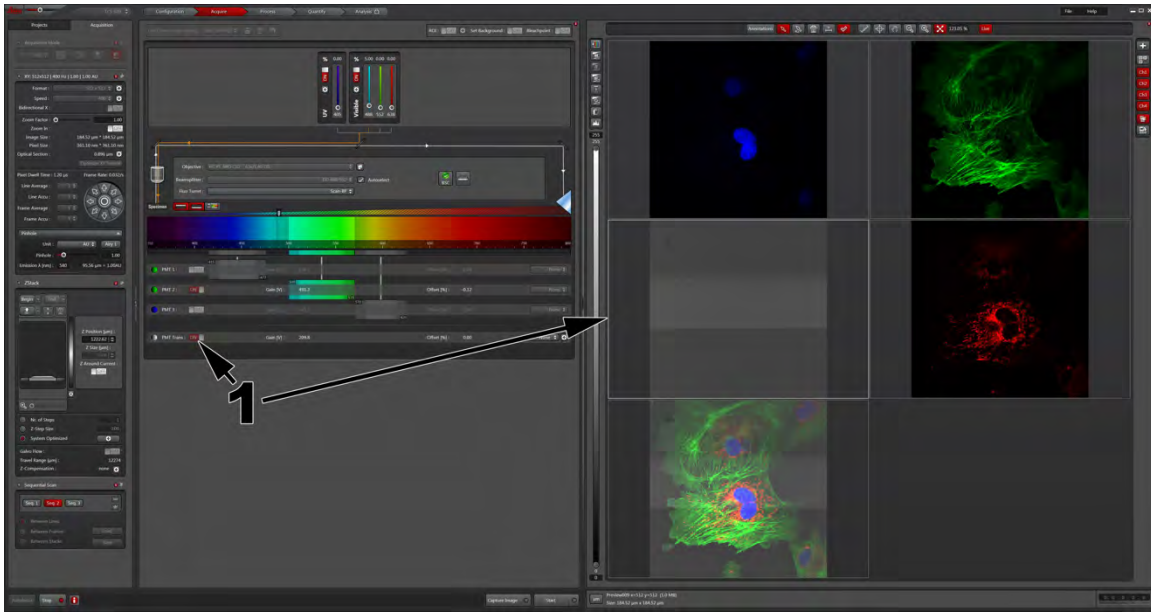
Changing the Objective:

- 1) From the *Acquire* tab, click the objective pull down menu and select your desired objective.
- 2) Alternatively you can also change the objective manually by **CAREFULLY** rotating the objective turret or using the touchpad screen on the front of the microscope (see page 2).



Adding a DIC or Brightfield Image:

1) Click on the *PMT Trans*. It is recommended to turn on the *PMT Trans* in the sequence that is using the 488 nm laser. Once *PMT Trans* is turned on, when you begin a *LIVE* scan, you should see an additional image box appear on the right with your other colors. This image box is for the brightfield image. Adjust the *Gain* and *Offset* the same way you would the other channels, using the control knobs below the screen on the long instrument bar.



Acquiring Basics:

- 1) These *seq* buttons represent 3 separate sequences, or channels. An easy way to think of this is consider each sequence its own image capture for a single dye. When you have multiple dyes, it is best to image sequentially to minimize bleed-through and cross talk. Each sequence allows you to adjust excitation laser wavelength, power, gating parameters and pseudo-color choice independently from the other sequences. This customization allows you to fully optimize the settings for each individual fluorophore. It does not matter which dye is in which sequence; the only effect sequence number has is in the order the picture is displayed on the right. Seq 1 will always be in the top left and proceed through in ascending order.
- 2) This laser control panel is where you choose which laser you would like to use (ie, 405, 488, 552, 638). Click the *ON* button (Labeled big arrow for 405 nm laser- *ON* button for other lasers is slightly more to the right for the 488, 552, 638). The little arrows show the two ways you can adjust laser power, either by using the little slider (bottom arrow) or double clicking the number on top and manually typing in the laser power (top arrow). **LASERS ARE VERY STRONG!!! PHOTBLEACH CAUTION WHEN USING LASERS AT POWERS ABOVE 5%.**
- 3) Shows where to click to turn on the PMT for collecting your image. For more information regarding which PMT should be used, please refer to page 15.
- 4) The big arrow from #4 shows where you can select a dye. After selecting the dye that you used, it will form the *Excitation Spectrum* (little arrow) of that specific dye. This spectrum may be useful to help choose appropriate laser and setting up proper gating for imaging.



- 5) Shows you where you can assign pseudo color within your selected sequence, ie, blue for DAPI, green for FITC, etc. You can assign any color to any dye you desire.

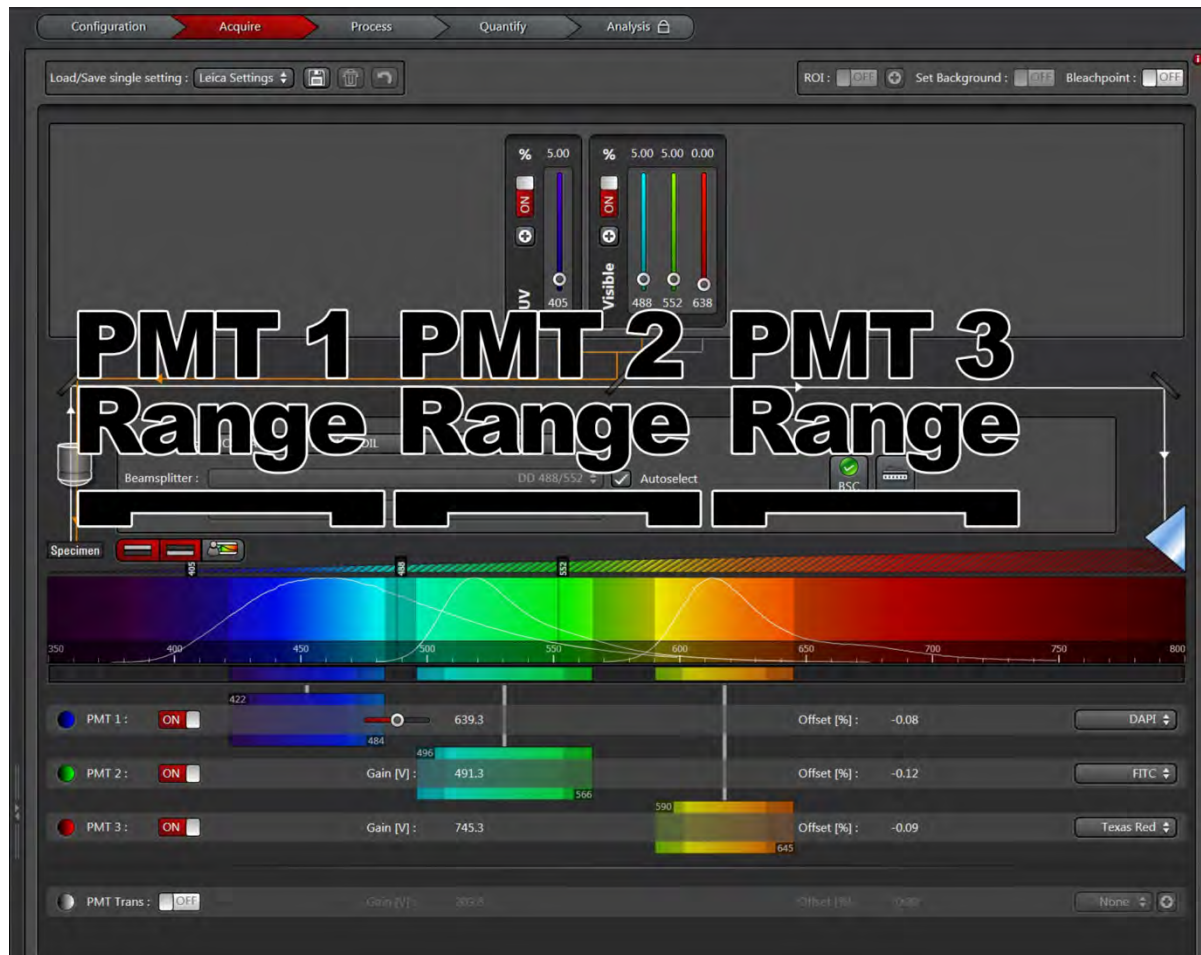
PMT Ranges:

PMT 1 should always be used for your Blue-range dye.

PMT 2 should always be used for your Green-range dye.

PMT 3 should always be used for your Red-range dye.

PMT Trans is only for brightfield images.

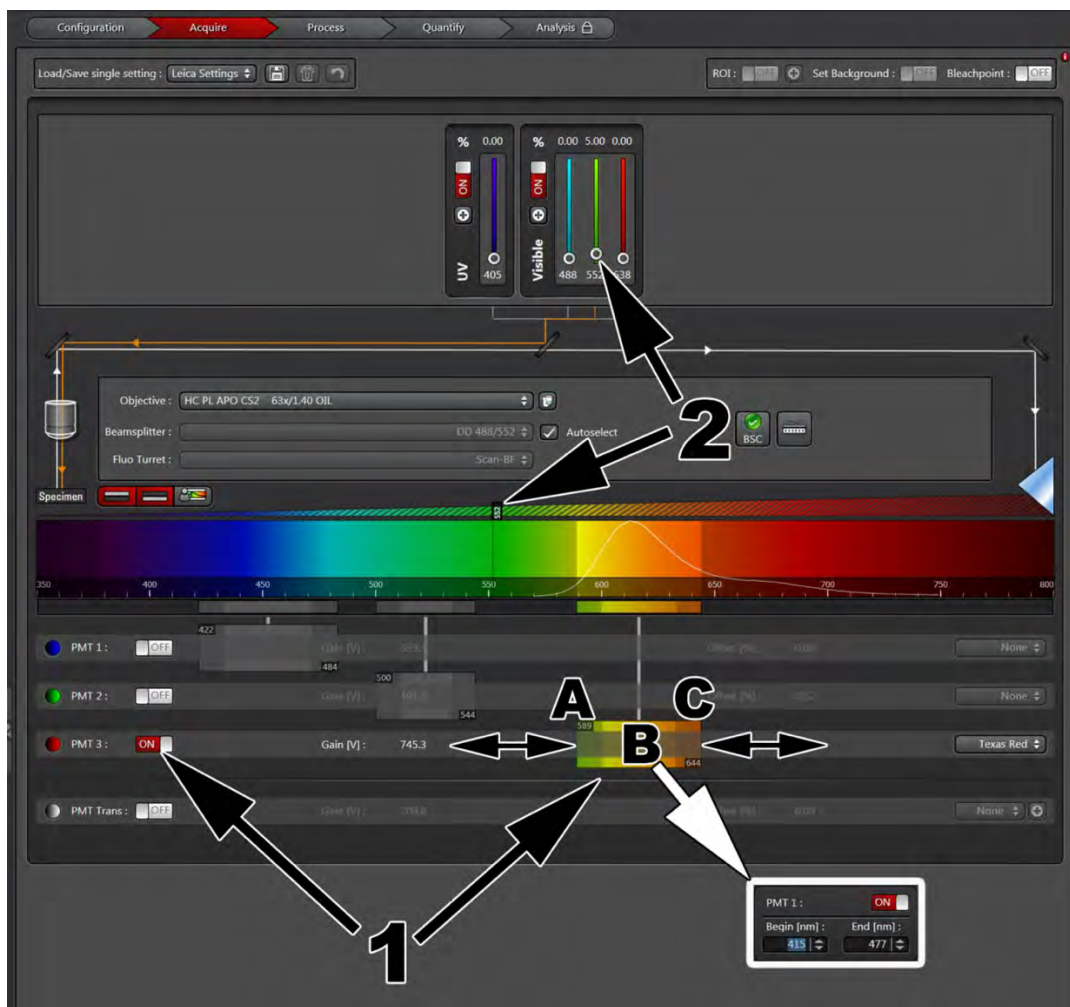


Setting Up Gating:

- 1) Turn on the appropriate PMT for the color that you wish to image. Make sure you are adjusting the appropriate gate for that PMT (each PMT has its own gating).
- 2) Turn on the appropriate laser line and adjust the power. The bottom arrow shows where your laser falls on the spectrum, which will help you with your gating.

B is your actual gate. If you double click on **B**, the window boxed in white will appear that will allow you to manually type in your gating range **OR** alternately, you can click on either side **A** and/or **C** and drag the gate more open or closed as needed.

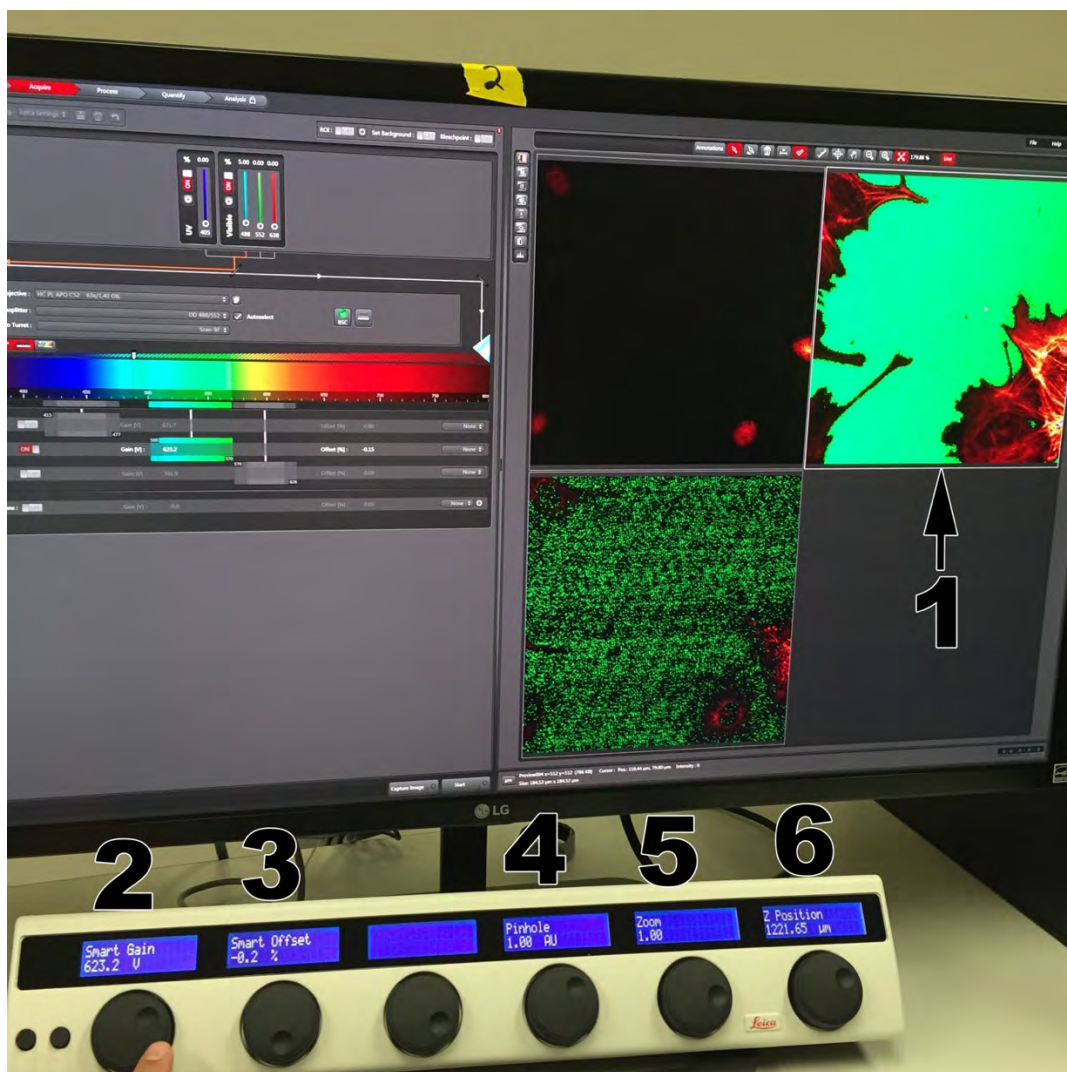
Things to consider: the left side of your gate should always be at least 10 nm away from the excitation laser's wave length. Example: If using a 552 nm laser, your left gate edge should be no lower that 562 nm. Gating allows you to control the amount of potential cross-talk that may occur if you have two fluorophores too close together on the emission spectrum. But creating a narrower gate, you have the ability to select only the peak emission curve if noise is an issue between adjacent channels. You can also widen the gate if your signal is weak and you are trying to amplify it.



Adjusting Brightness and Contrast:

Make sure you are in an active live scan before making any adjustments. The *LIVE* button will be illuminated.

- 1) Select the color you want to adjust by clicking on that color box on the right side of screen. Only the highlighted square will be affected at the time of knob adjustment.
- 2) The first knob, *Smart Gain*, is the control wheel for how bright the image will be. The higher the *smart gain*, the brighter your signal will appear. Optimally, you would like your gain in the 700-1100 range depending on the strength of your signal. The *Smart Gain* maxes out at 1250 and you will hear a loud beeping to alert you that you have reached the maximum *Smart Gain*. If you still have low signal, you can try to bring up the laser power or open the pinhole to find some signal. Remember that laser power above 5% runs the risk of bleaching.
- 3) The second knob is the control wheel for *Smart Offset*. *Smart Offset* can be thought of as contrast, controlling mostly the blackness (or background) of the image. You should NOT have to adjust this very much. Between -.8 and .5 is an appropriate range for *Smart Offset* or you run the risk of distorting the signal to noise ratio of your image.



4) *Pinhole* controls how thick of an optical section you are collecting. Making the pinhole smaller will create a thinner section but will greatly reduce the signal. Alternatively, a larger pinhole will give you a thicker section with a greater signal. Ideally, you should leave the pinhole at 1.00 AU. This is the optimum pinhole diameter that best balances section thickness to signal attained and is critical for colocalization studies.

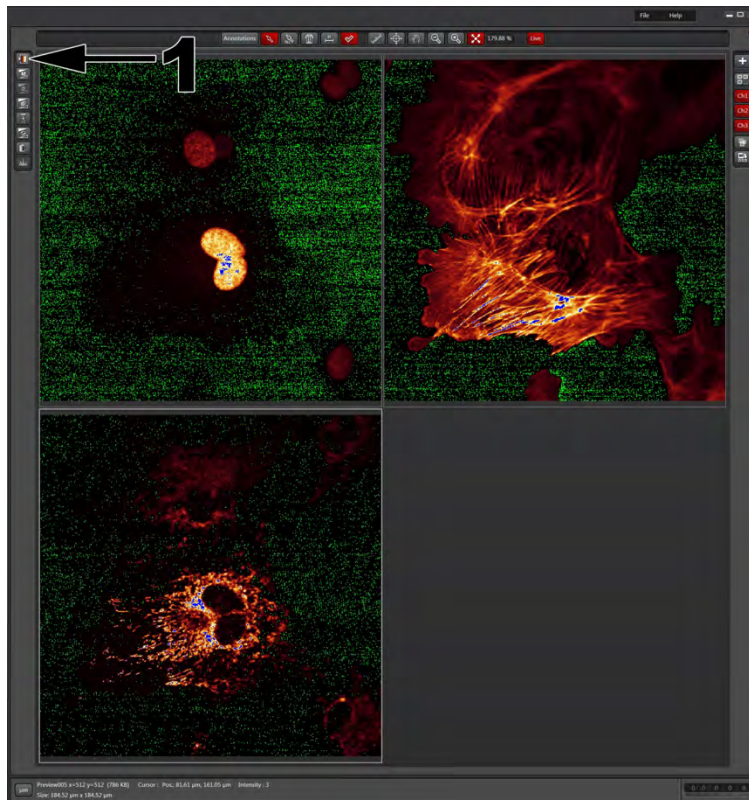
5) *Zoom* allows you to zoom in your field of view. This is pre-acquisition zoom so the image you collect will be captured with more detail compared to a digital zoom (imaging zoom after acquisition). It works by collecting the same amount of pixels but over a smaller area. It is not recommended zooming beyond a factor of 3.5 because any further zoom would be empty magnification with compromised resolution.

6) *Z position* is a duplication of the focus knob. It is helpful to tweak the focus here while looking at the screen as you optimize your *Gain* and *Offset* settings.

Range Indicator/ Quick LUT:

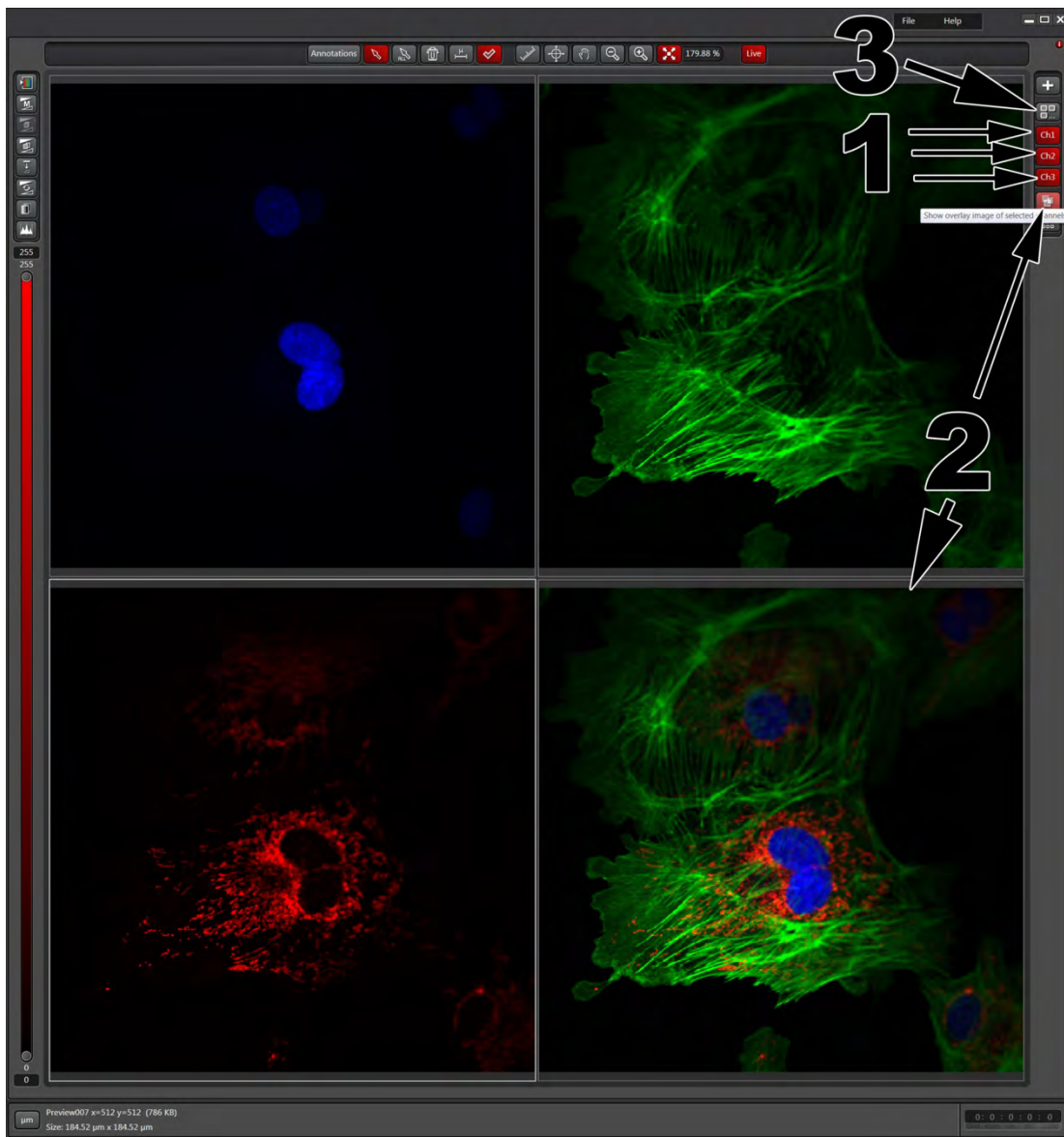
1) Clicking on the *Quick LUT* button provides you with an easier way to visualize whether you have any oversaturated (way too bright) pixels after setting your *Gain* and *Offset*. The oversaturated pixels are represented as blue while the true black pixels are represented in green. The image below shows an example of what your settings should look like in the *Quick LUT* view. Your brightest area should contain a speckle of blue pixels while your background should be a peppering of green. Make your adjustments with the *Gain* and *Offset* knobs while in the *Quick LUT* mode.

Pushing the *Quick LUT* button a second time will give you a Black and White image and Clicking it a third time will return your image to the normal colors you had assigned. You can toggle through this button to optimize your image and check your settings throughout the acquisition process.



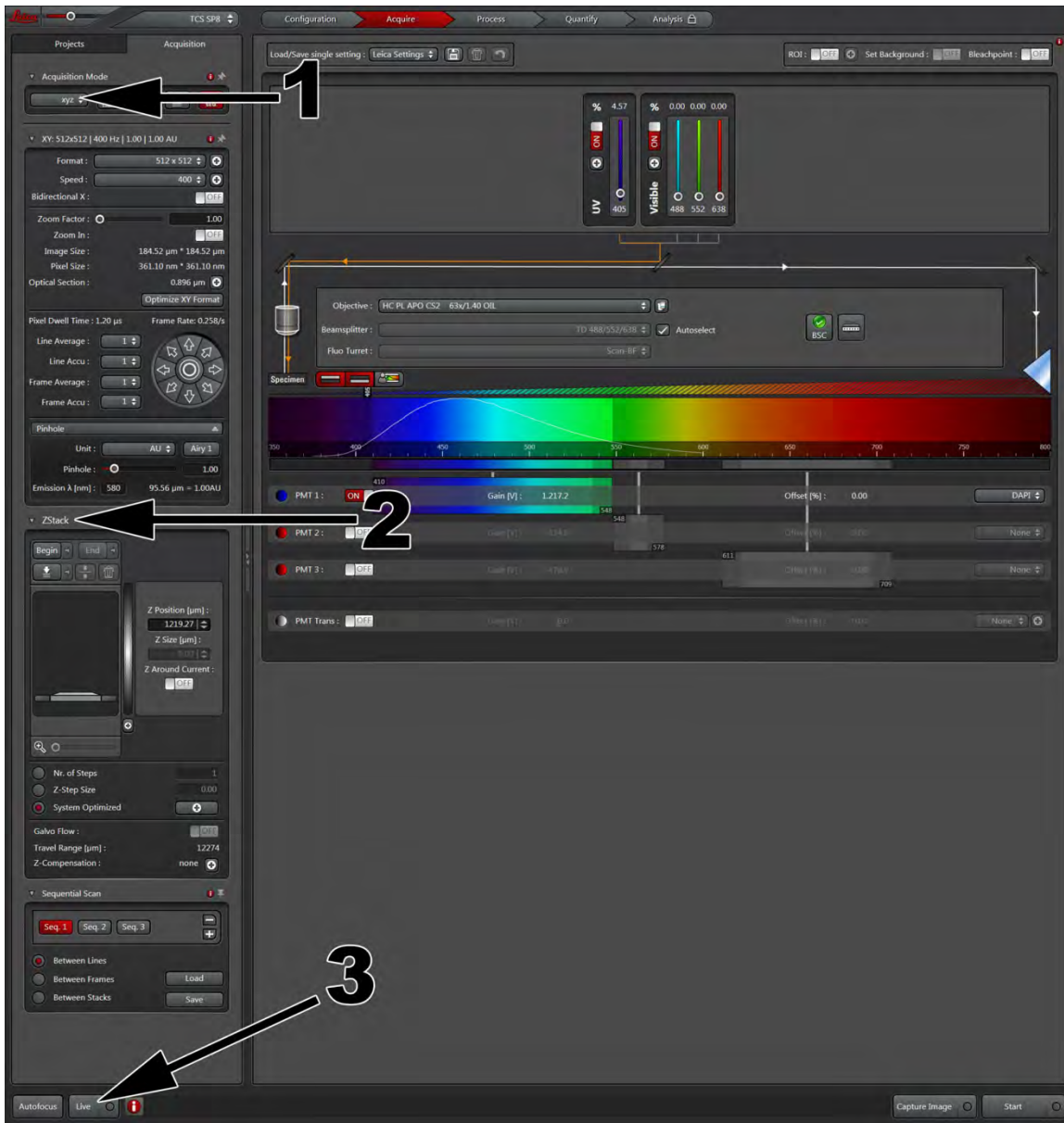
Creating a Merged Overlay and Turning off Channels Post Acquisition:

- 1) You can turn on or off any given color by clicking any of the *Ch #* buttons on the top right. *Ch 1* corresponds to the first square box (upper left), *Ch 2* corresponds to the upper right picture and *Ch 3* corresponds to the lower left image.
- 2) If you took a multi color image, you can create a merged overlay of the colors by clicking on the *Merged* button.
- 3) Click [here](#) if you want a full screen shot of your image.



Setting up a Z stack:

- 1) Make sure that xyz is the selected Acquisition Mode.
- 2) You may need to expand the Z Stack Menu.
- 3) Click the *Live* button to begin to set the parameters for your Z stack.



6) After setting the bottom point, focus back through your sample (in *LIVE* mode) to the other end of where fluorescence is just barely detectable and hit the *End* button. When Z-stacking multiple fluorophores, choose the one most important for your imaging to set your upper and lower limits.

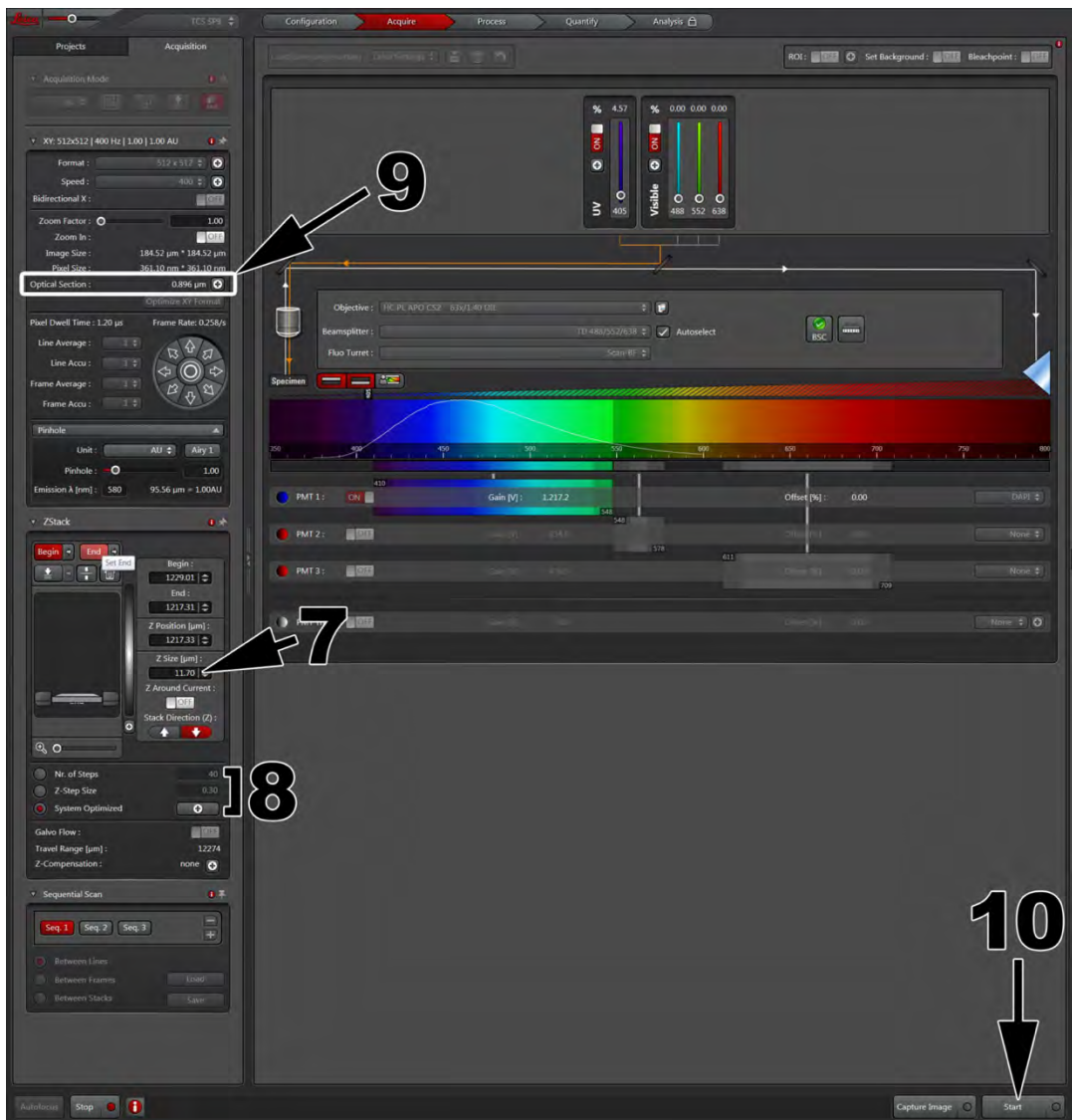


7) Now that you have assigned a *Begin* and *End*, you can see the thickness (Z Range) in μm of the area we have selected to image.

8) *Nr. Of Steps* is the number of images that will be collected in your z stack. The *Z-step size* is the amount the stage will move between images. *System optimized* will set the *Nr. of Steps* and the *Z step size* based on what it deems appropriate based on the objective that is being used as well as the Z range that has been defined.

9) *Optical section* of your sample: If you took a single image, the optical section is the thickness of your sample that is represented in the image. When setting up for a Z stack, you always want to make sure that the *Optical Section* is at least twice as big as your Z-Step Size. By doing so, you are appropriately oversampling your sample to get enough data to reconstruct a 3-dimensional image without distortion in an image analysis software.

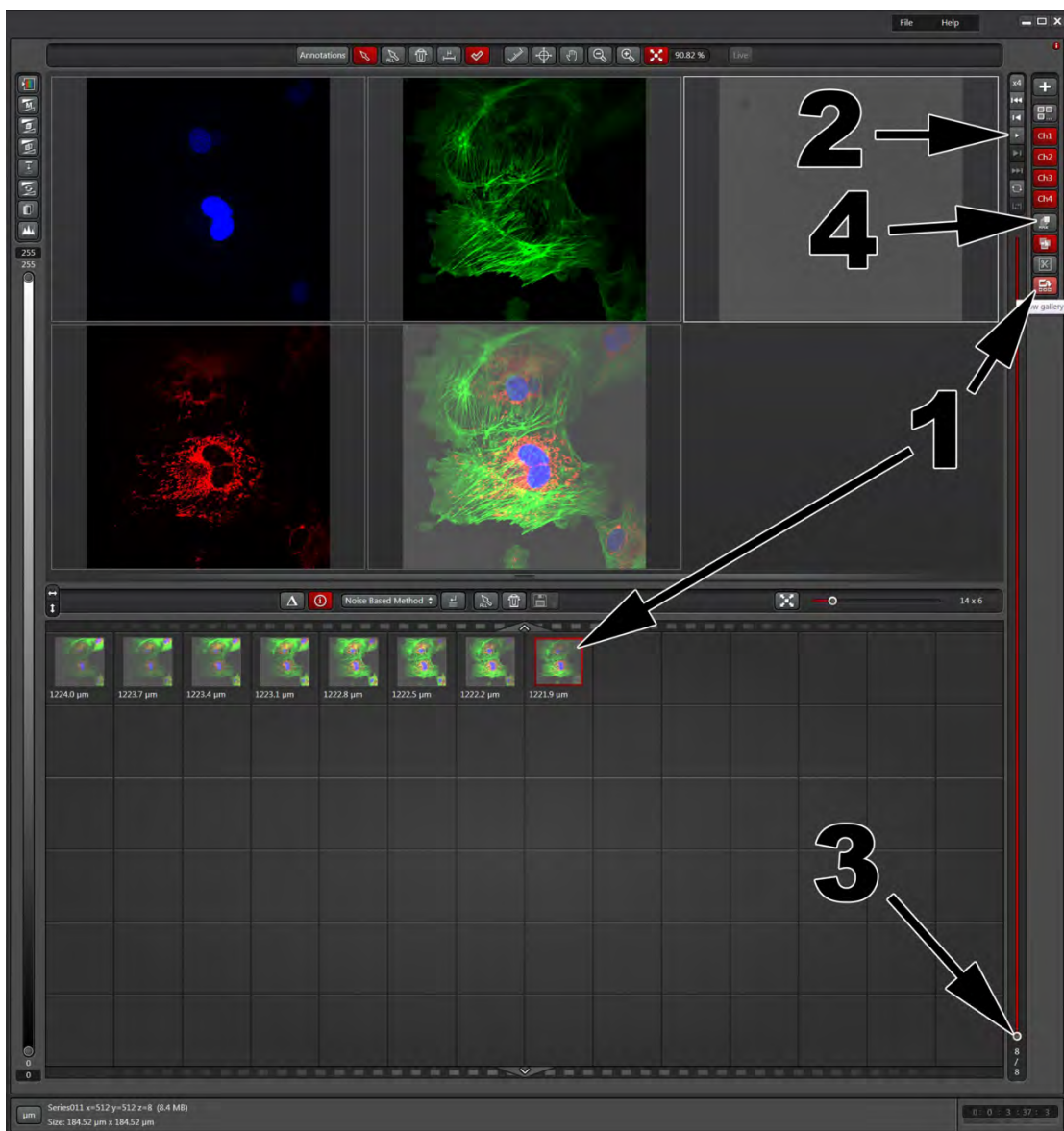
10) Click on *Start* to begin acquiring the Z-stack.



Different Z Stack Views:

These options are only available after collecting a Z-Stack.

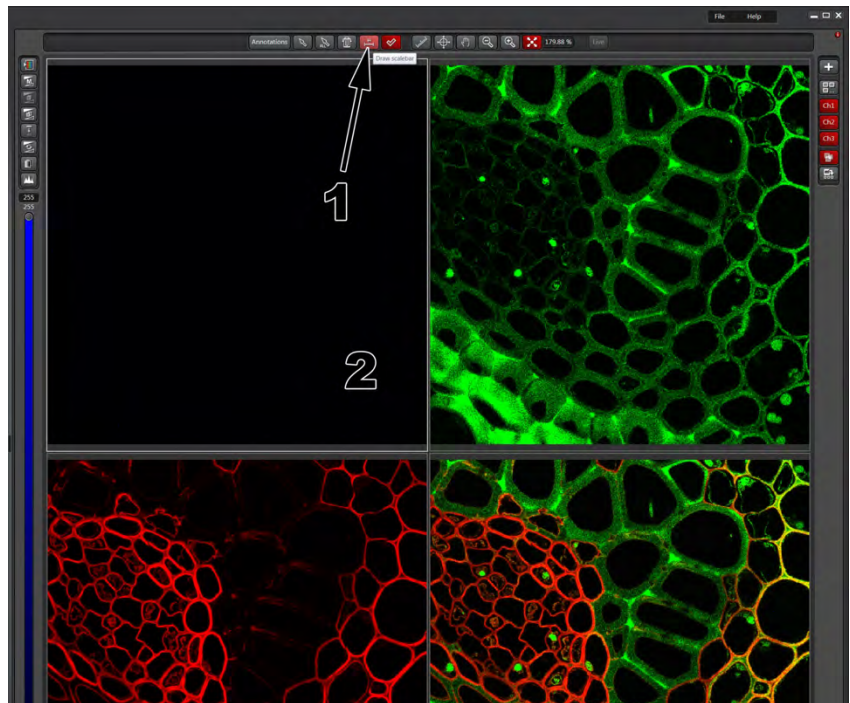
- 1) *Show Gallery* button will allow you to view all the slices of your z-stacks individually.
- 2) By pressing the *Play* button, it creates a movie that will play through each slice of your Z stack.
- 3) If you want just a single slice within your Z-stack, you can sort through each slice by using the slider that runs vertically next to your picture.
- 4) You can *Create a Maximum Intensity Projection*, which uses all the slices from your Z stack and creates a 2D image with a 3D feel to it.



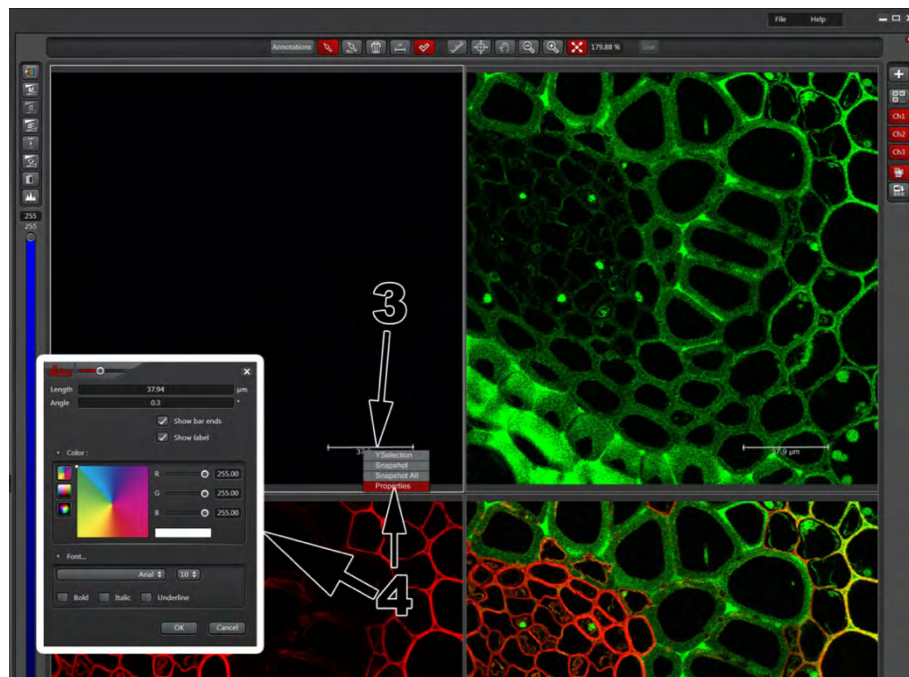
Adding Scale Bar:

1) Click on the *Draw Scale Bar* button.

2) After selecting *draw scale bar*, drag and drop your scale bar wherever you would like your scale bar to appear on your image.



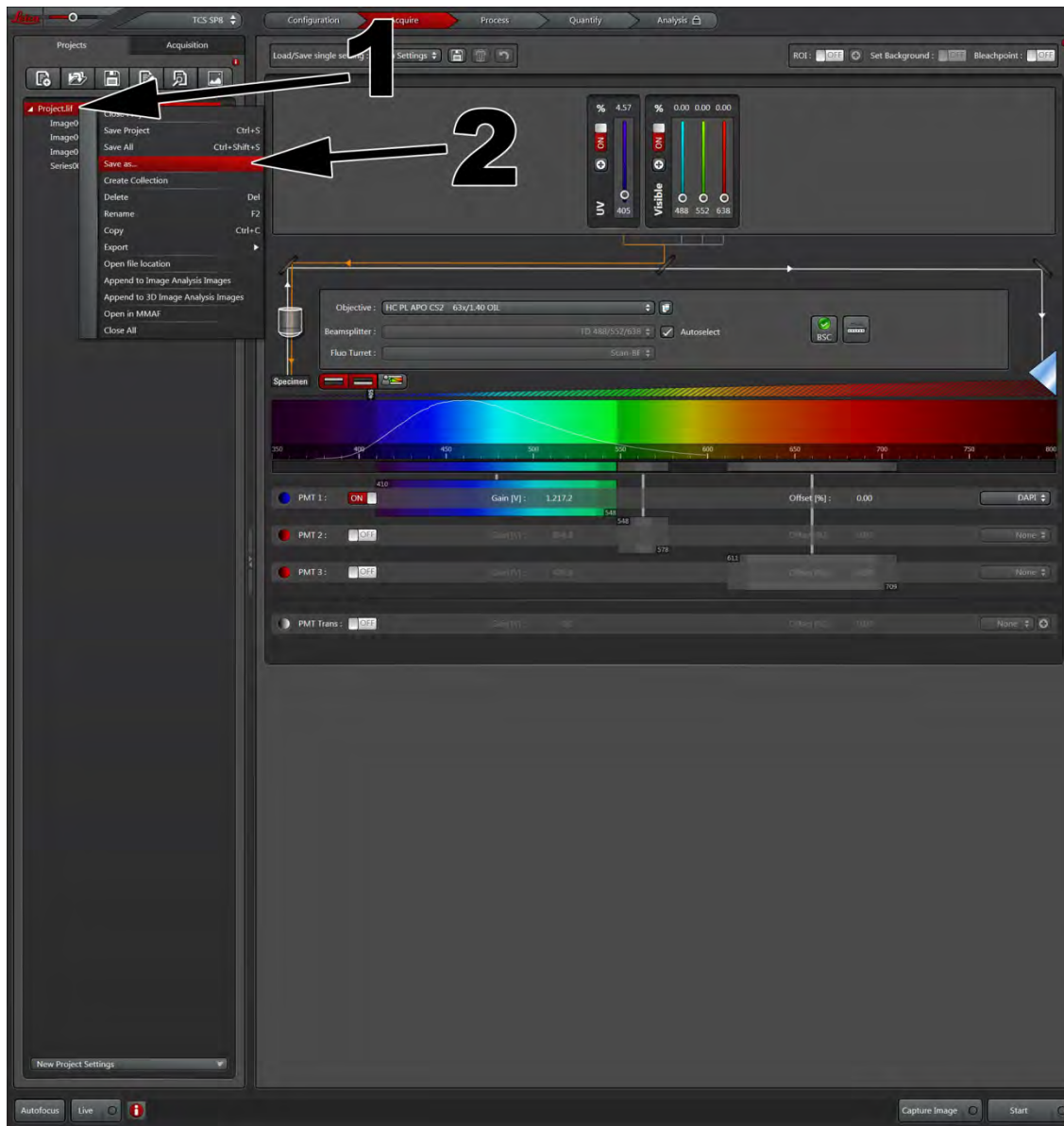
3) To change the properties of your scale bar (ie. color, size, font) Right Click on *Scale Bar*.



4) Select *properties* from pull-down menu. After selecting *properties*, the window boxed in white will appear.

Save as Project:

- 1) Right Click on the *Project* you wish to save (Labeled 1).
- 2) Choose *Save as..* from the pull down menu.

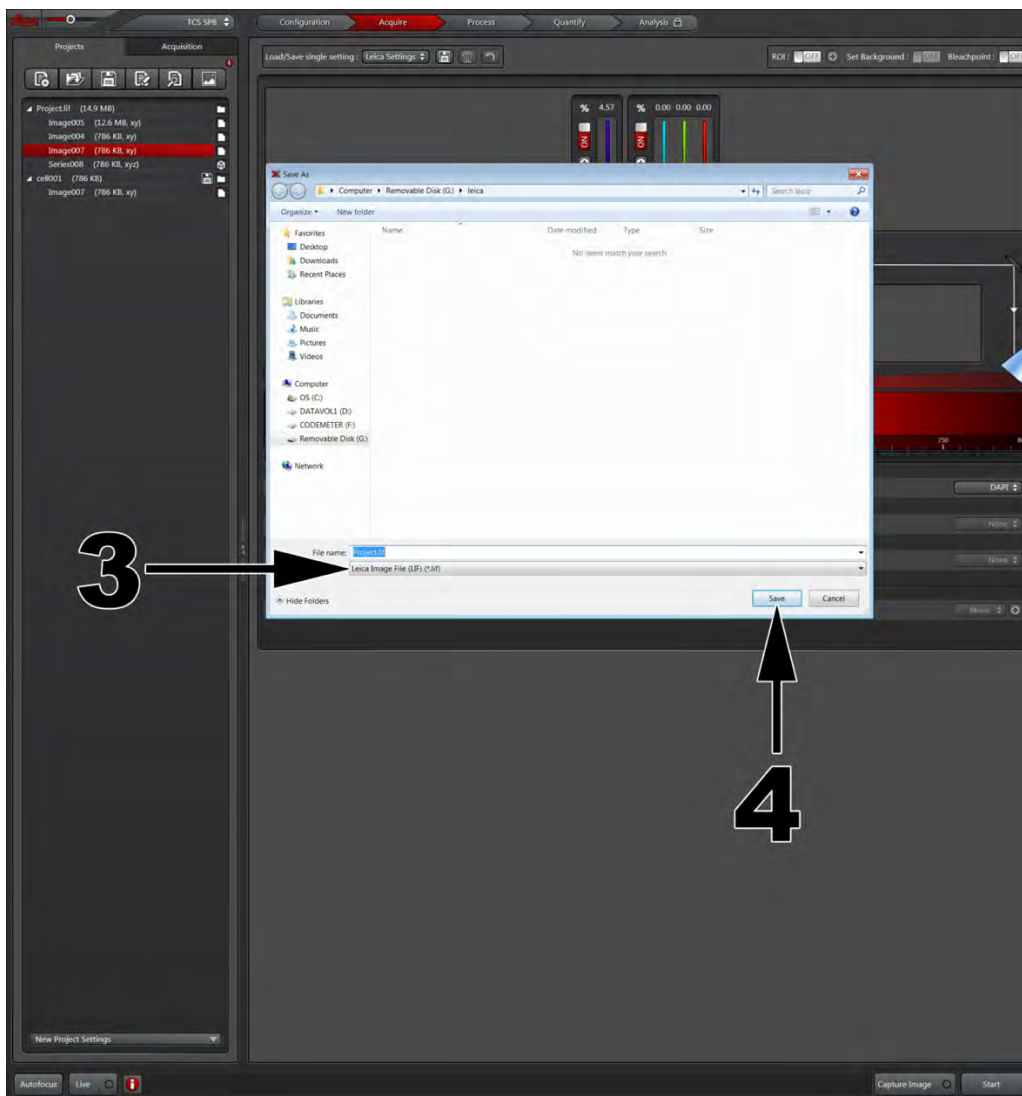


3) After clicking *Save as..*, a window will open asking you what would you like to name your picture. It will also ask what file type would you like to save it as.

ALWAYS SAVE AS A LEICA IMAGE FILE (.lif) By saving as a .lif, you store all meta data necessary to recall your experimental set up, a critical piece when you are repeating the acquisition criteria or writing a paper about the acquisition at a later date. You will need an image analysis software program to view this file type on a different computer, though.

If you want your images in .tiff or .jpeg, please see page 28.

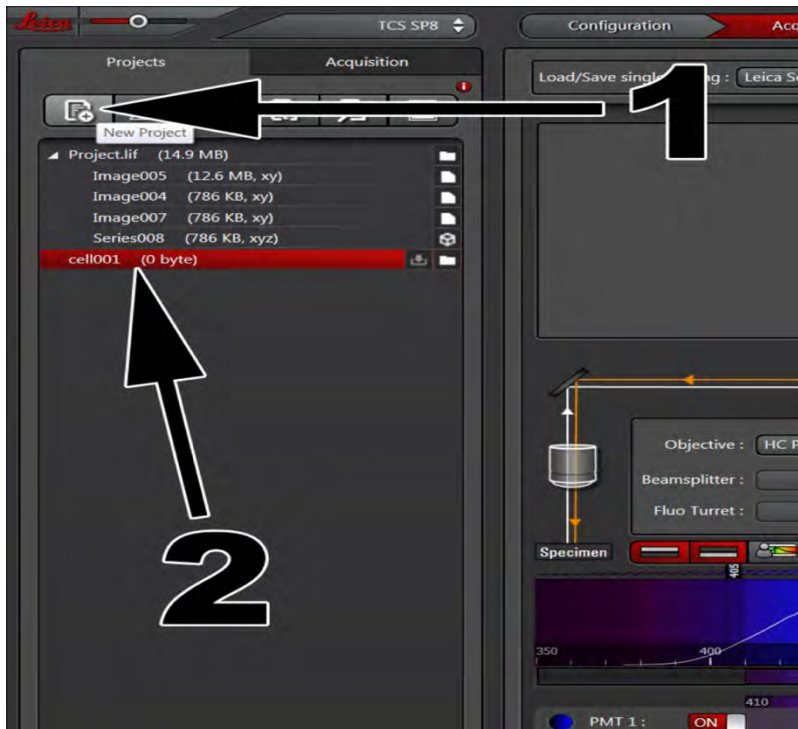
4) Hit the *save* button.



The Saving Method described above will save all the images that you acquired during your imaging session. If you are interested in saving only a single image that you acquired during your session, follow the steps provided below (page 27).

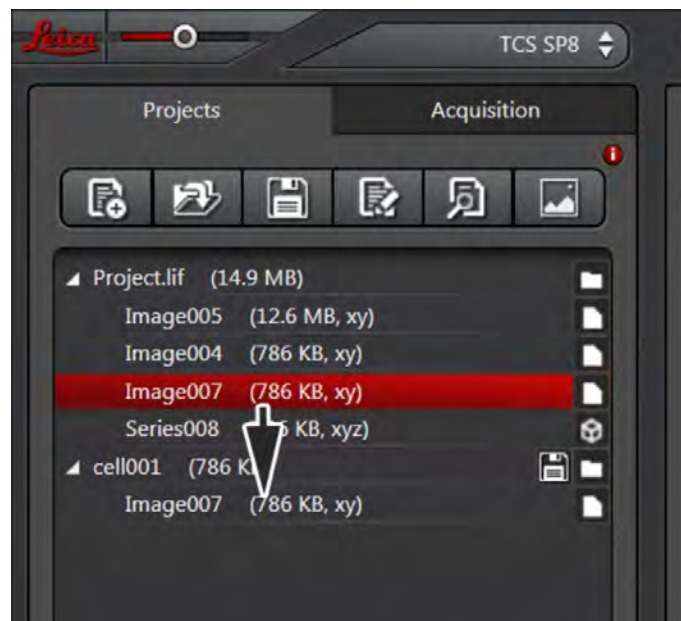
Saving Single Image:

1) Click the new project button in the top left (Labeled 1).



2) Once a new project button has been hit, you should see a new project folder created on your list of images. (Highlighted in red, labeled 2).

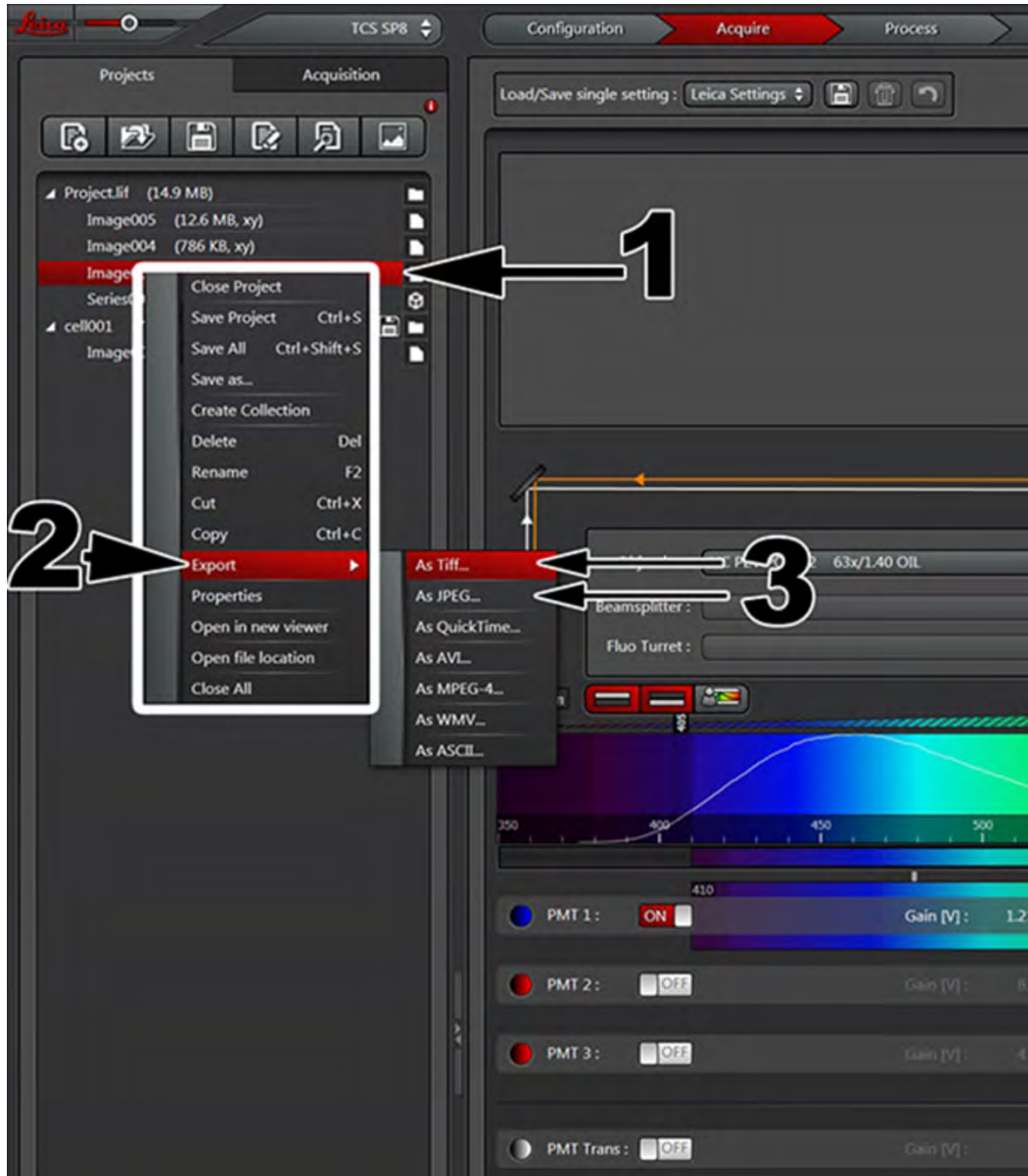
3) You can now click on your desired image and drag it to your newly created project folder (labeled 3).



Once the image of interest is within the new project folder, follow the same *Save as* procedure as described above.

Saving Images as .tiff or .jpeg:

- 1 Select the image you would like to save as a tiff. Once selected, right click on the image.
- 2 After right clicking, you will see a pull-down menu appear (highlighted white box). Select the *Export* option.
- 3 Once you place the mouse over the *export* tab, it will open another drop-down menu that will ask you if you want to save as Tiff or as JPEG. Choose the format that you wish to save in.



To SAVE COLORS SEPERATELY as .tiff and .jpeg:

- 1 Click on *Browse* to choose the folder that you want to save your images into.
- 2 Make sure *Overlay channels* is unchecked.
- 3 Make sure *Export LUT* is checked (otherwise images will only be in black and white).
- 4 Click *Save*.



Save a Merged as .tiff and .jpeg (all colors together):

- 1 Click on *Browse* to choose the folder that you want to save your images into.
- 2 Check the *Overlay channels* box.
- 3 Click the *Save* button.

