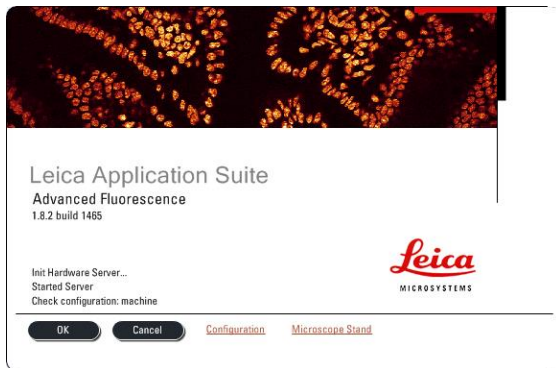


Leica SP5 DM Imaging Protocol

1) System Startup

- ☞ Please note our sign-up policy. You must inform the facility at least 24 hours beforehand if you can't come; otherwise, you will receive a charge for unused time. The facility will allow for extenuating circumstances (cells dying, sick day, etc.) if you inform us in a timely fashion.
- ☞ **Follow each step** of the startup poster.
- ☞ **Log into** the computer with your user account.
- ☞ Open LAS AF, click **OK** on the opening splash screen, and wait for the microscope software to fully load (it usually takes five minutes).
- ☞ Click **No** on the prompt to initialize the stage.



2) Lens Cleaning

- ☞ Please **clean all of the lenses** (used and unused) **before and after** your session. Refer to the lens cleaning poster if you need any help recalling the rules and steps.

3) Microscope control

- ☞ Please note the touchpad at the microscope base—this touchpad controls the illumination and lens hardware in the microscope. The relevant menus in the touchpad are:




Status menu

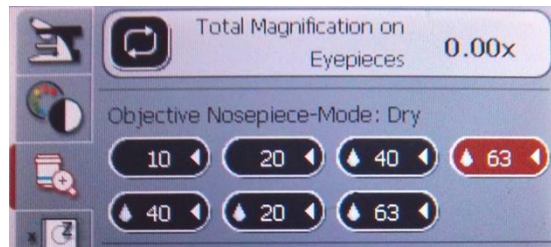


Illumination control menu



Lens control menu

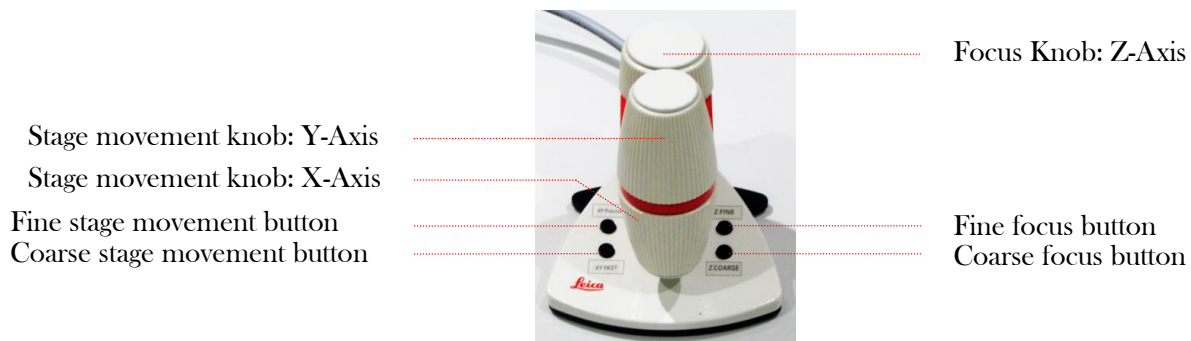
- ☞ First, use the lens control menu  to switch between your microscope lenses. Please note the following lens immersion classifications:



Dry: 10x, 20x (top), Oil: 40x, 63x (top)

Water Immersion: 10x, 20x, 40x (bottom)


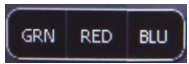
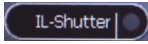
- When you switch lenses, the stage drops while the microscope switches between types of immersions. Please note the following points:
 - First, since the stage recalls the focal position between each lens, **take care to properly set your focal position with a lower power lens first** before loading your slides and before using a higher power lens. For example, start with 10x for most tissue samples, 20x for cells. Proper focusing from low power to high power will help prevent the microscope from breaking your slide.
 - Secondly, when switching between lenses that use different immersions, take the appropriate action needed to move to the next lens: apply oil when switching from a dry lens to oil lens, and remove oil when switching from an oil lens to a dry lens.
- Before you load your slide, **select the 10x or 20x lens.**
- ☞ **Load your slide** and take care for the following issues:
 - **Select and inspect each slide**
 - If it is dirty, gently clean with a KimWipe and/or cotton swab. You should do this with all your slides before you come.
 - Carefully rest the slide onto the universal specimen holders. Be careful not to apply much pressure to the stage due to its delicate mechanical nature.
- ☞ Use the stage movement and focus control joystick to position your specimen under the lens.
 - Note the following controls in this diagram.



☞ Use the illumination control menu  to view and focus your sample.

- First, note the BF  & Fluorescence  sub menu buttons:



- Select the Fluorescence sub-menu.
- Within the  menu, you can toggle between three basic filters to view your sample: 
- Use , the reflected light shutter control, to turn your fluorescence light on or off.

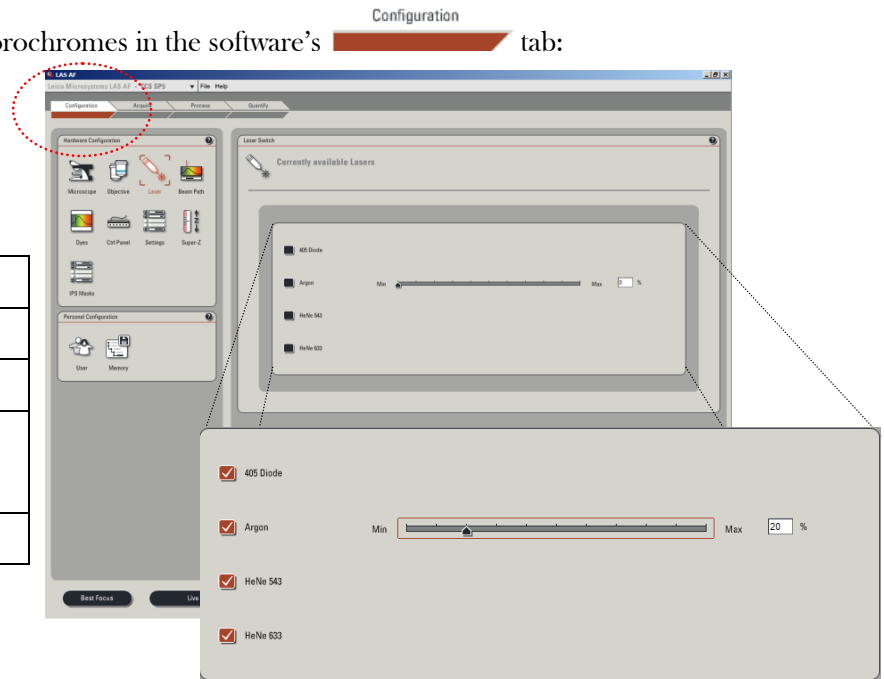
☞ Focus on your sample and center your region of interest.

☞ **Check all of your fluorophores** in your specimen. You need to have an idea of what to expect before you start your scan.

☞ Close the reflected light shutter to prevent photobleaching of your sample.

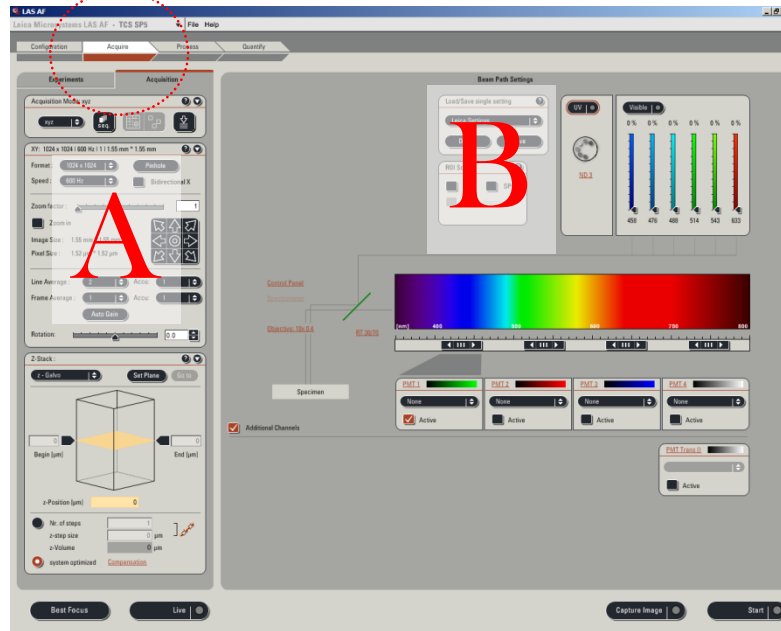
4) *Setting up your experiment*

☞ Turn on the relevant lasers for your fluorochromes in the software's  tab:



Laser	Common Labels
405 Diode	DAPI, Hoescht
Argon	Alexa 488, FITC, GFP
HeNe 543	Alexa 543/568/594, Cy3, Rhodamine, Texas Red
HeNe 633	Alexa 633/647, Cy5

Return to the **Acquire** tab:

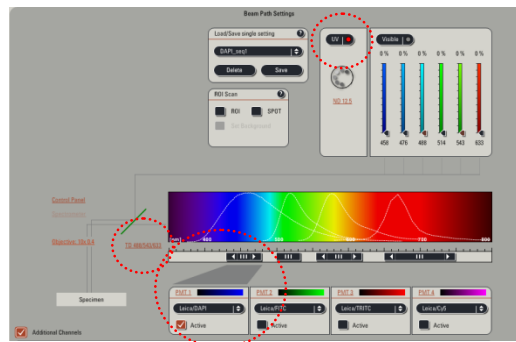
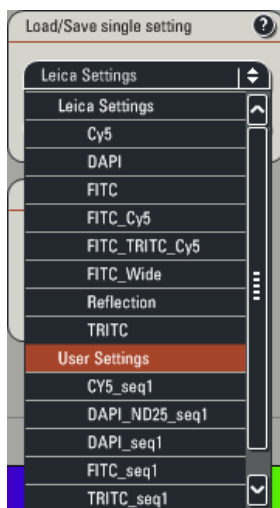
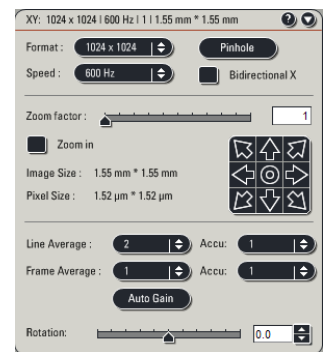


Set up the following imaging parameters (in panel labeled **A** in above image):

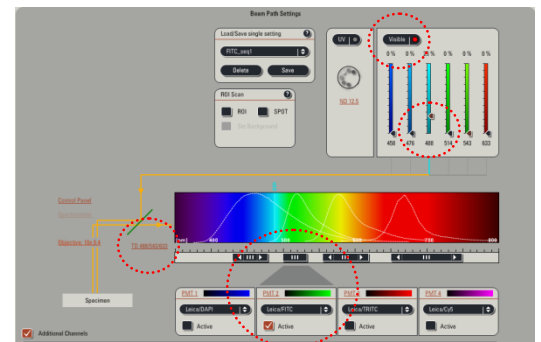
- Format: 1024 x 1024
- Scan speed: 600 Hz
- Line Average: of 2x
- These parameters are a good starting point, but later on you can change them to suit your needs

Load a single track setting for one of your fluorochromes (in panel labeled **B** in above image):

- You can choose these from User settings, not Leica settings
- Note the beam path to ensure you have the correct settings. Please consult us before making any changes to this part of the screen!



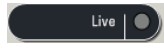
Example 1: DAPI_seq1

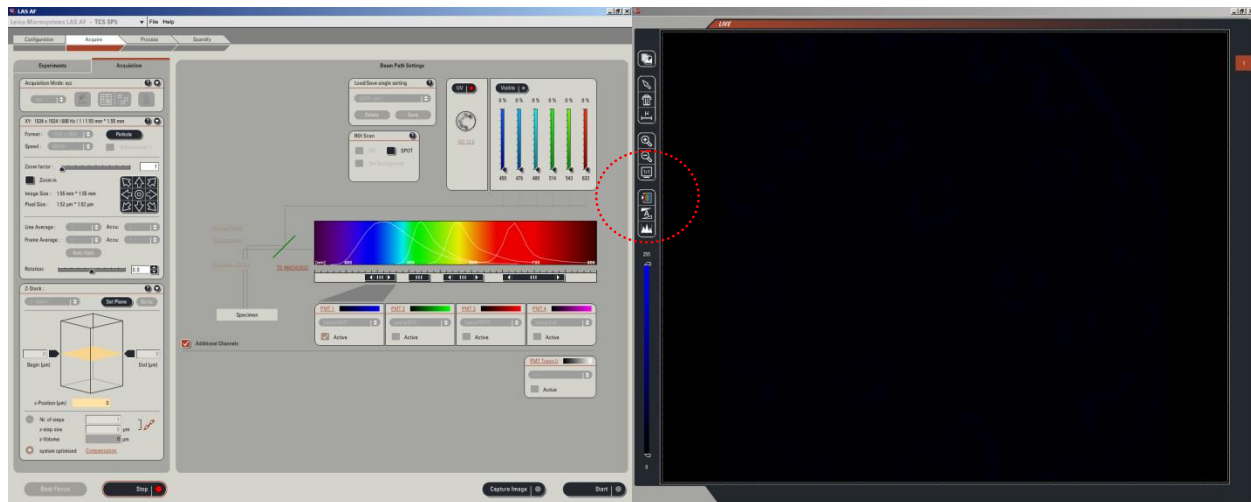
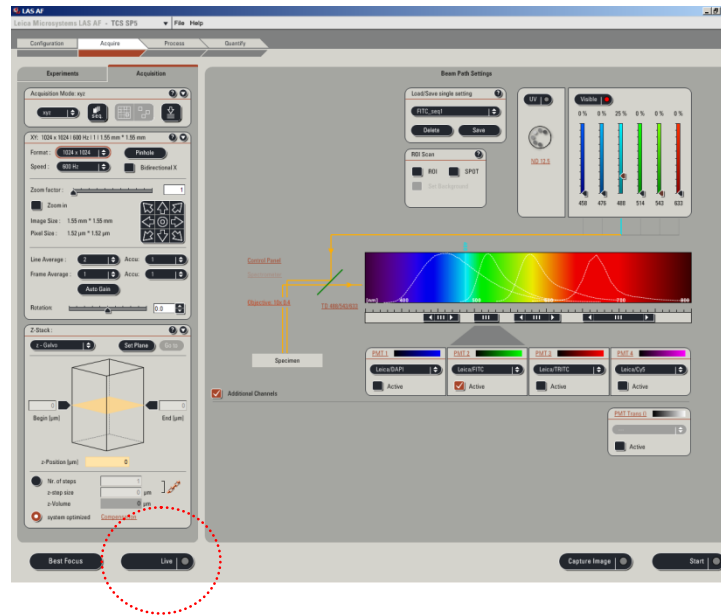



Example 2: FITC_seq1

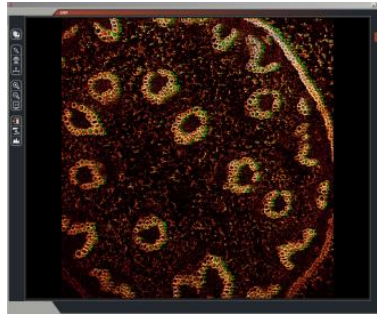
5) *The imaging process*

☞ Set the gain and offset settings for the currently loaded settings. This process optimizes the system to capture as much an intensity range of your sample's signal as possible.

- In the bottom left of the main  screen, click  to start your preview scan:

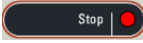
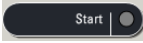



- Click the Quick LUT button  to toggle through the three available pseudocolor options: the current channel's color, the Glow Scale, and the grayscale.
- Select the Glow Scale; the image color will be red/orange/yellow and may have patches of blue or green:

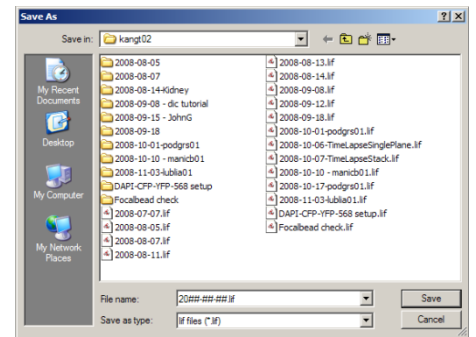
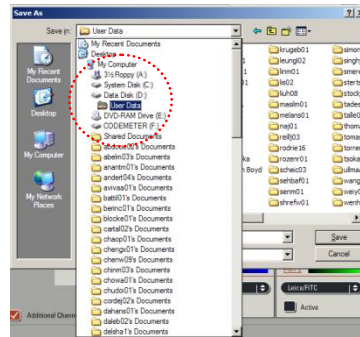
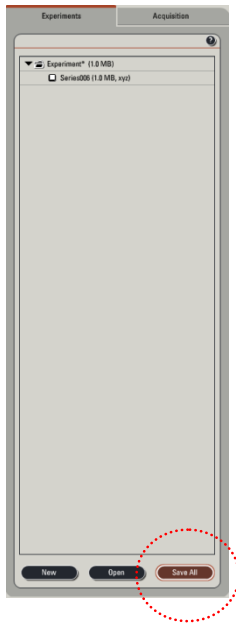


Convallaria rhizome test slide, Leica Microsystems



- Set your gain (brightness):
 - Using the Smart Gain knob (A), adjust the gain until you see your signal--any blue pixels that appear signify saturation.
 - Use the Z-position knob (C) to find your brightest focal plane in your specimen of interest.
 - Readjust the gain to minimize the amount of blue pixels until you have a few left in your image.
- Set your offset (background):
 - Using the Smart Offset knob (B), adjust the offset until you see green pixels--any green pixels that appear signify absolute black.
 - Adjust the offset to minimize the amount of green pixels until you have a few left in your image.
- Click  in the lower left hand corner of the screen to stop your live scan.
- Click  to capture your image.
- Please do **NOT** use  -- it does not perform the same task as the Start button.

Click the **Experiments** tab, then **Save All** to save your image:





- The software stores all of its images in a database file, so the first time you save a file, you will first receive a prompt to save the database file for your session. Please save to your folder within the User Data folder.
 - The software needs you to save any change to the database file, including acquiring, renaming, and deleting your images.
- ☞ If you need an image for your other fluorochromes, for each fluorochrome repeat the imaging process: load the relevant single track setting, set the gain & offset, start your scan and save your finished image.

6) *Returning to the microscope*




☞ To return to the microscope to find another field, press the **CHANGE CS** button on the right side of the microscope, then focus & center another region of interest.


☞ If the fluorescent shutter doesn't open, select the  button in the illumination control menu .

☞ You also can perform the following operations after returning to the microscope:


☞ ***Selecting higher magnification***

- If you need to switch to the 40x or 63x oil immersion lens, first select the lens control menu  and tap the immersion objective lens you plan to use; the icon will begin to flash and the stage should drop.
- Apply one drop of oil to the area underneath the lens.
- Raise the stage by selecting your desired lens a second time.
- Focus & center your specimen. Please remember to use the fine focus control!

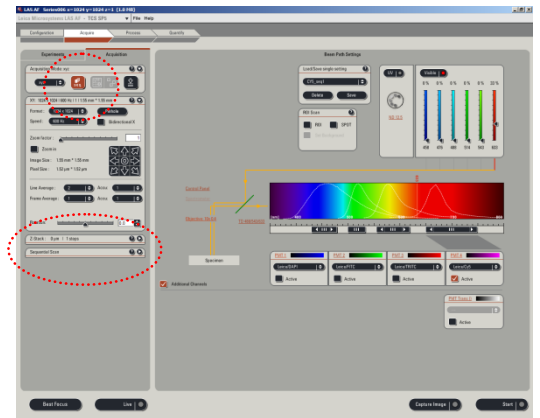
☞ ***Switching slides***


- Lower the stage by pressing the  button on the right base of the microscope or by using the coarse focus.
- Remove your slide.
- Switch the lens to the 10x dry lens with the touchpad.
- Load the new slide.
- Focus and center your specimen.
- Proceed with imaging or switching to a higher power lens

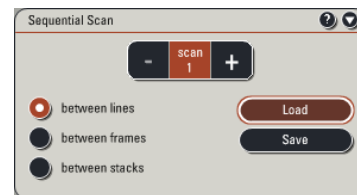
7) ***Sequential Imaging***


☞ In the software Acquisition panel, click the  button:

☞ You should see the Sequential Scan panel appear:




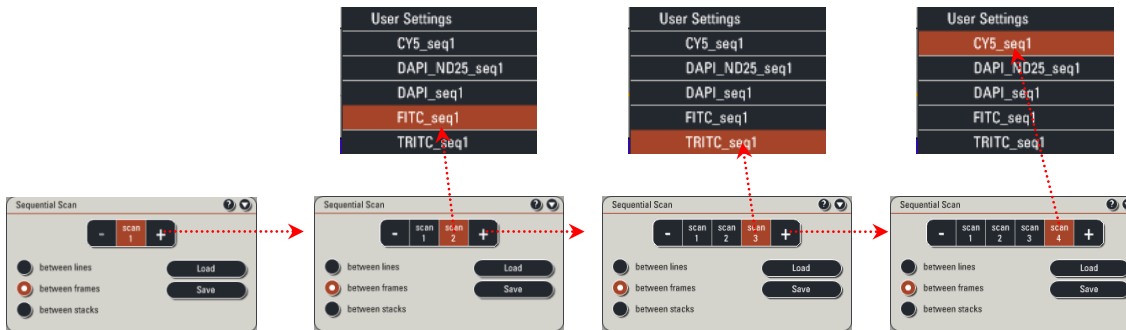
☞ Expand the Sequential Scan panel and click on the  button:






- ☞ Load the single track setting that matches your fluorochrome with the shortest emission wavelength. If you load DAPI, make sure to select the  **between frames** option.



- ☞ Click  to add another track, select the new track and load the single track setting for your next fluorochrome. Repeat this process for each of your fluorochromes in your specimen. For example:

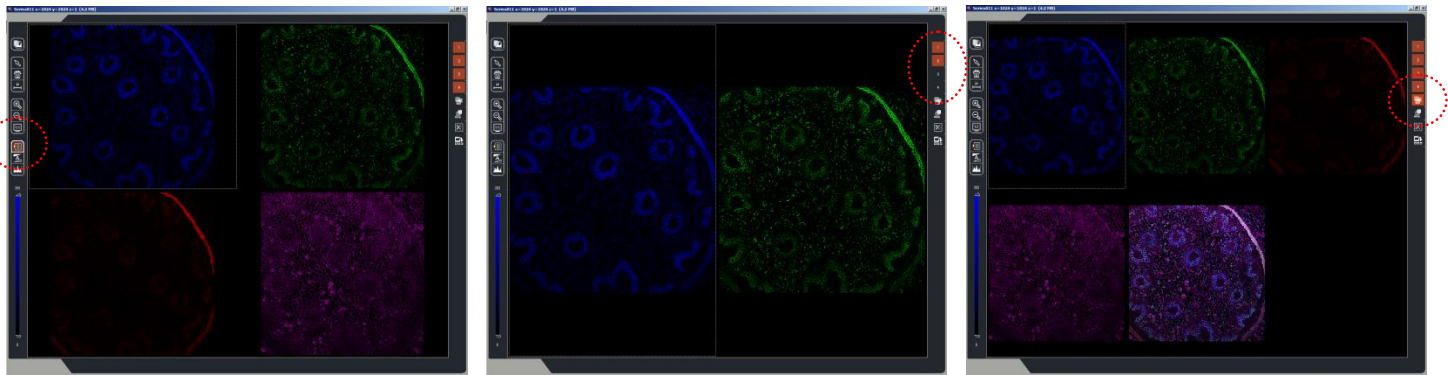


- ☞ Click on the  button again and click . Set your gain, focal plane and offset and click .

Repeat this process for each of the    buttons.

- ☞ Click  and save your image with  after your scan finishes.

☞ Please note the following display options:



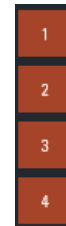
A

B

C

A. To view your channels in the original pseudocolor or in grayscale, toggle the view with the  button.

B. To turn off and on one of your channels, toggle any of the corresponding buttons number in the upper right hand corner of the screen




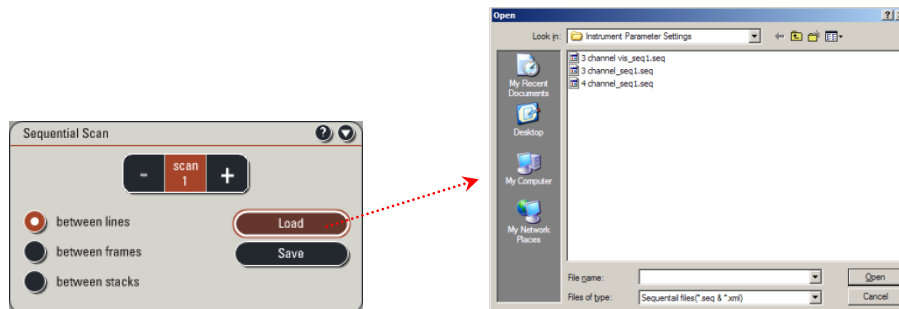
buttons number in the upper



C. To view the channel overlay, click the  button.

- To view one of your channels full screen, double click on that particular panel. To return to the previous view, double click on the image again.

☞ Once you have familiarized yourself with the ins and outs of sequential imaging, you can load a preset that loads the image acquisition settings and sequential tracks for commonly used fluorochromes. You can perform this step from now on instead of loading separate channels.

- Click  in the Sequential Scan panel:

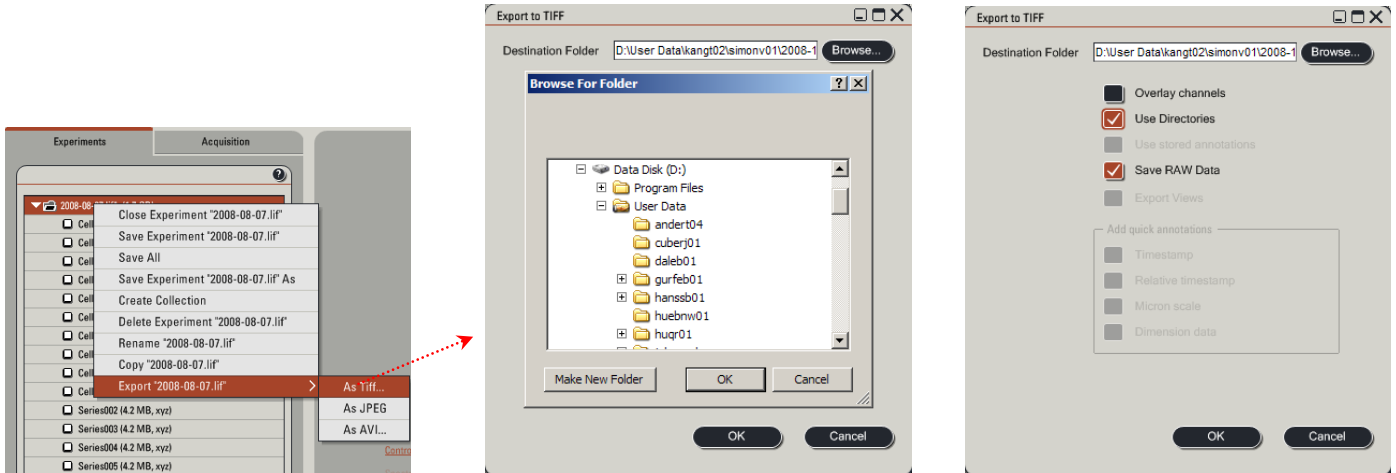


- Select and open the setting that most approximates your specimen.
- If necessary, remove any unused channels by selecting the relevant  button and clicking the  button.

8) *Exporting your data*

☞ Since Leica stores your data in its proprietary LIF format, you'll need to export your data to TIFF for any further use. Thankfully, the process is simple and quick:

- In the Experiments tab, Right click the LIF filename (the top-most name in the list):



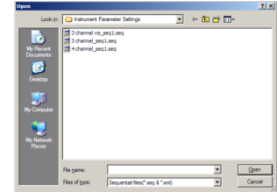
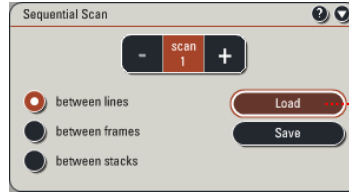
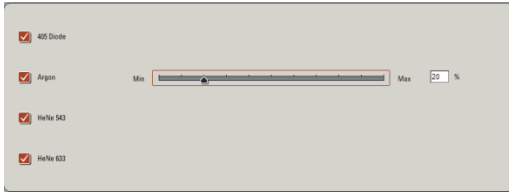
- In the dropdown menu, select **Export**, then **TIFF**.
- Click **Browse...** to choose your directory within the designated User Data folder on D:
- Optional: to export TIFFs into a subdirectory with the experiment database filename, check **Use Directories**
- Select **Save RAW Data**
- Click **OK** to begin your export.

9) *System Shutdown*

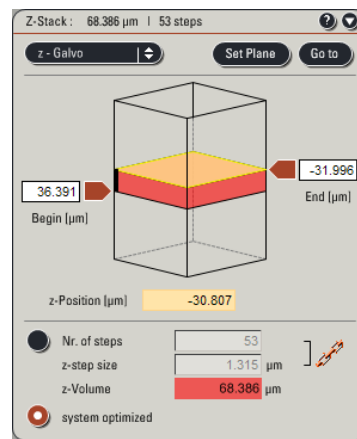
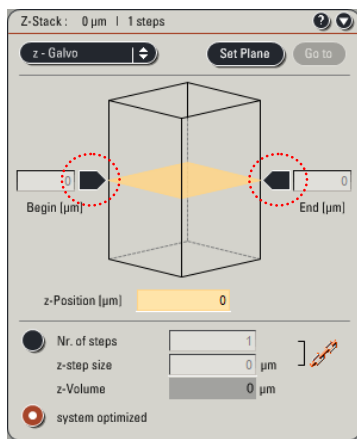
- ☞ Before you proceed with the shutdown sequence, please remember to back up all of your data and to clean the dry and oil immersion lenses.
- ☞ Sign onto the facility calendar and check for users after your session. If you don't see anything scheduled for less than 2 hours, proceed with the full shutdown sequence shown on the Shutdown poster. Otherwise, close the software and log off your account.
- ☞ If you do have to perform a full shutdown, please follow the shutdown poster steps in order. If you used the Argon laser, don't forget to wait for the fan to shut down before you follow the last step.

10) *Your 2nd session and beyond: Taking a Z-Stack*

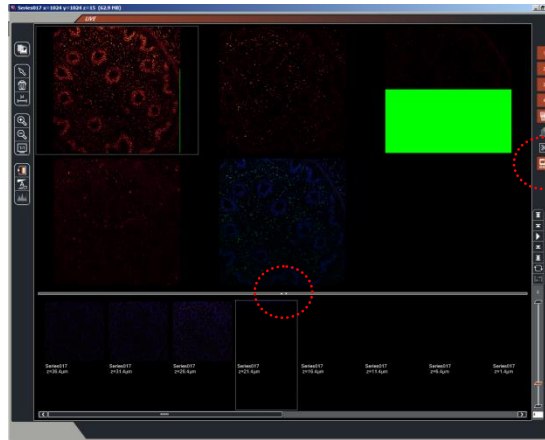
- ☞ When you come in for your second session, proceed with system startup and cleaning the lenses. Load your specimen and remember to focus from low to high power.
- ☞ Turn on your relevant lasers and load your sequential scan settings.



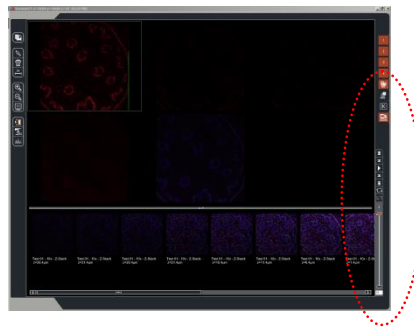
- After selecting a **scan 1** channel and clicking **Live**, use the Z-position knob to search for the brightest focal plane in your specimen. Set the gain & offset for all of your channels and click **Stop**.
- Choose the **scan 1** channel that has the signal to determine your stack limits.
- Expand the Z-Stack menu and ensure that you have selected **z - Galvo**:



- Click **Live** again and set your stack limits:
 - Use the Z-position knob to set the focal plane towards the top of your specimen (counterclockwise) and click the left arrow so that it's orange.
 - Use the Z-position knob to set the focal plane towards bottom of your specimen (clockwise) and click the right arrow so that it's orange.
- Click **Stop**. The Z-stack panel should appear similar to the right image above.
- Note the stack size (z-Volume) and number of steps. The software selects the **system optimized** option by default for post processing techniques like deconvolution or 3D reconstruction.
- If you don't need that many slices or have such small Z slice intervals, select the **Nr. of steps** option and input either your desired amount of steps or slice interval.
- Click **Start** to begin your scan. You can click **Gallery**, the gallery view mode, to see your images acquire in succession. You can also double click the **Maximize** button shown circled on the gray bar to maximize the gallery.






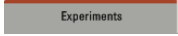

- ☞ After acquisition completes, click on the **Experiments** tab and save your image with **Save All**.
- ☞ To view and explore your Z-stack data, note the following icons on the upper right corner of the image screen:

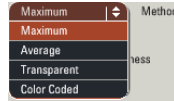



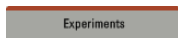
- Use the slice slider controls to scroll through the sections:

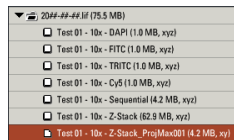


- Use the  to view cross sections of your Z-stack.
- Use the  button to display a Maximum Intensity Projection (MIP) of your entire stack. This tool only functions as a visualization, so the MIP will not be saved.
 - To generate an actual MIP:
 - (a) Click on the  tab at the top of the left window.

- (b) Click on the  tab and select the Z-stack of interest.
- (c) Click on the  tab and select “3D Projection” in the list of operations.
- (d) Ensure that the Method field is set to “Maximum”:



- (e) Click  to create your projection. If you click on the  tab, you should see the projection appear as a new item in your file list:



☞ Save your experiment. To retake a single image, disable the stack limits to a single plane and set slice amount to 1.