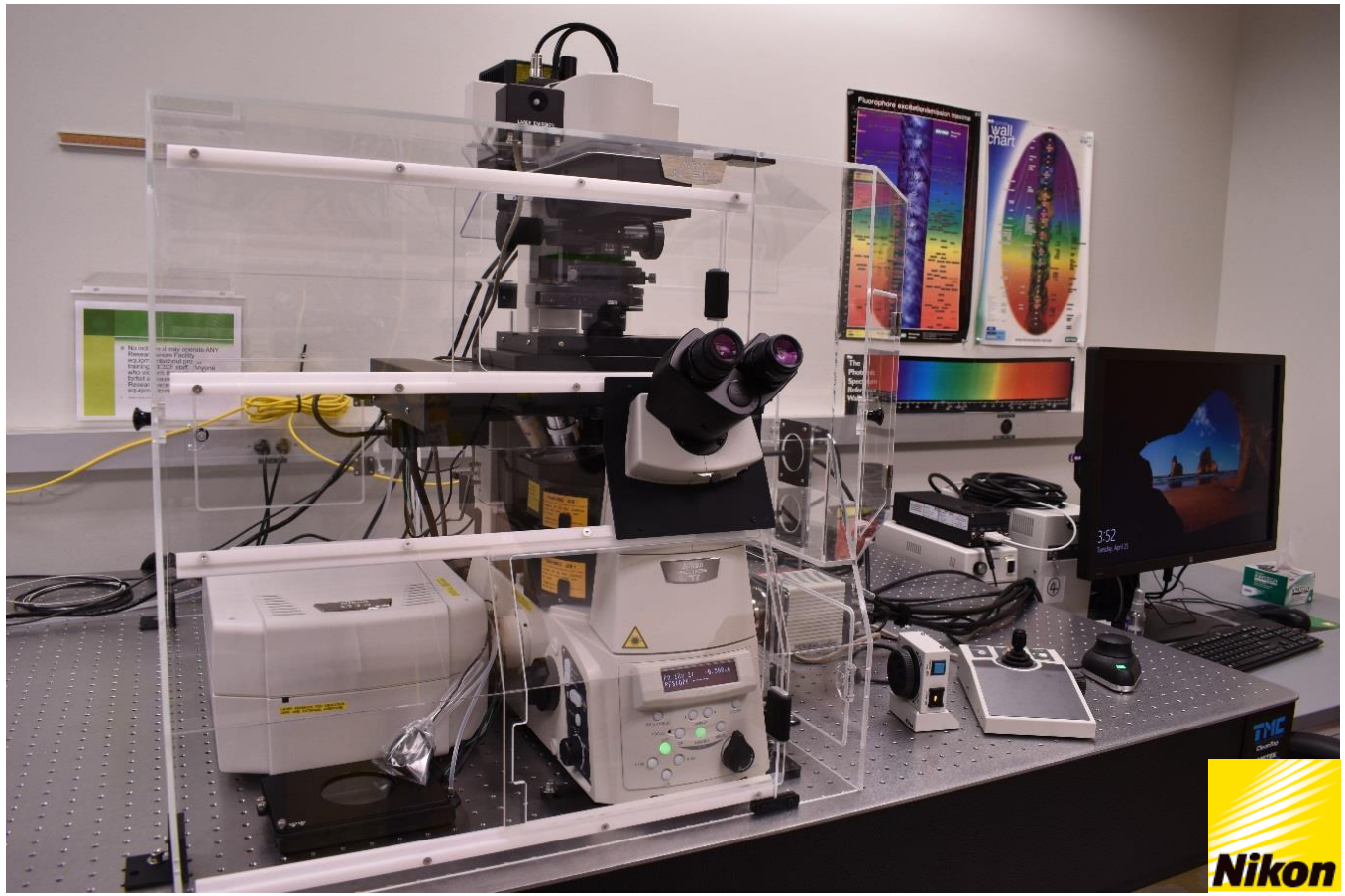
Nikon SIM-E & A1-R System

USER GUIDE



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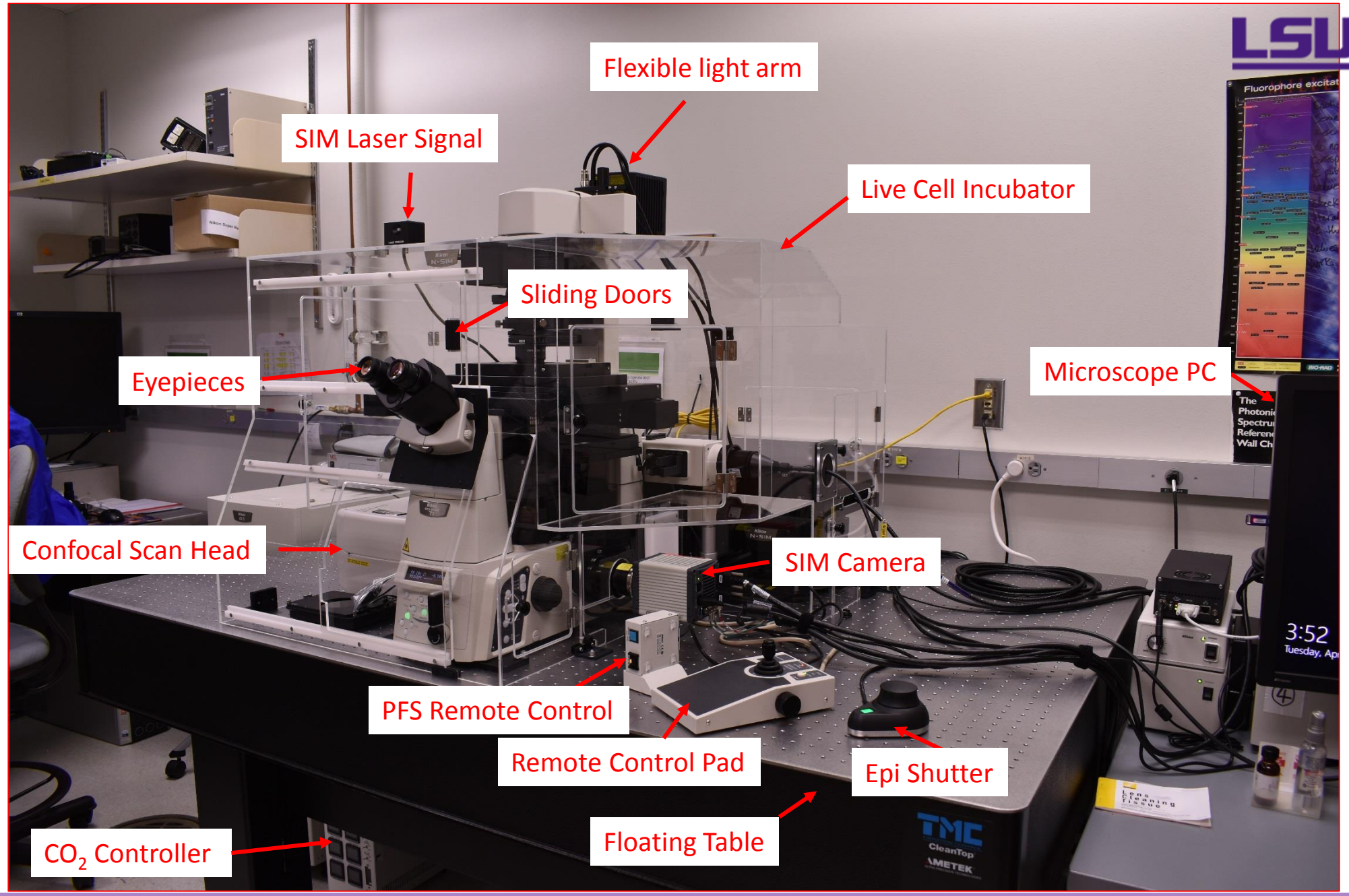
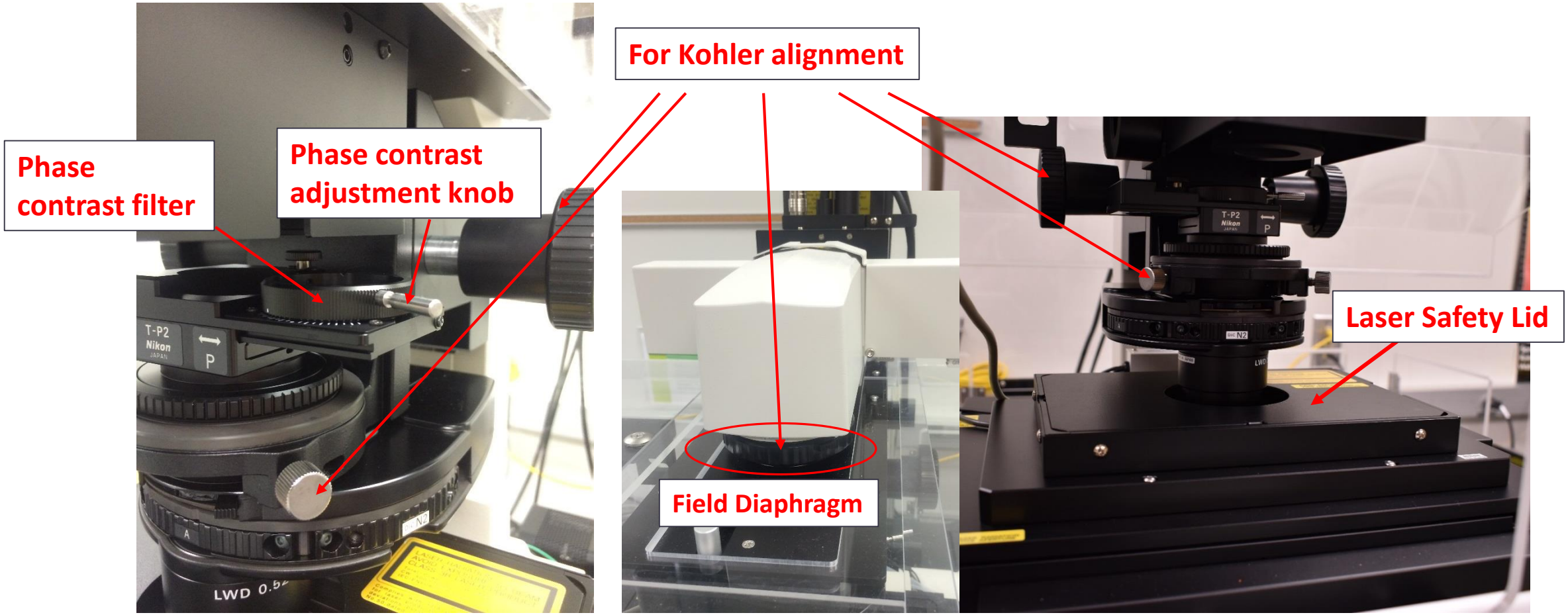
Hardware and microscope introduction


Flexible light arm
SIM Laser Signal
Live Cell Incubator
Sliding Doors
Eyepieces
Microscope PC
Confocal Scan Head
SIM Camera
PFS Remote Control
Remote Control Pad
Epi Shutter
Floating Table
CO_2 Controller

Kohler alignment is needed for transmitted light/bright field imaging. You will be trained on how to do Kohler alignment by staff if you need transmitted light most of the times. If you seldomly use transmitted light, you do not need to learn Kohler alignment. Staff will periodically check the alignment for all users.



1: Focus knob (Z-position wheel)

2: Z-focus speed adjustment

3: Objective selection

4: Transmitted light shutter

5: Transmitted light intensity adjustment wheel

1: Focus knob (Z-position wheel)

2: Focus speed adjustment

6 FL Block: Fluorescence selection

7 Refocus: Set the Z position to the previous position

8 Escape: Set Z to the lowest position. **When Escape is pressed, focus adjustment is disabled.** To enable focus adjustment, press **Refocus** and **Escape** buttons simultaneously, or release Escape through the software.

9: Epi shutter button is inactivated

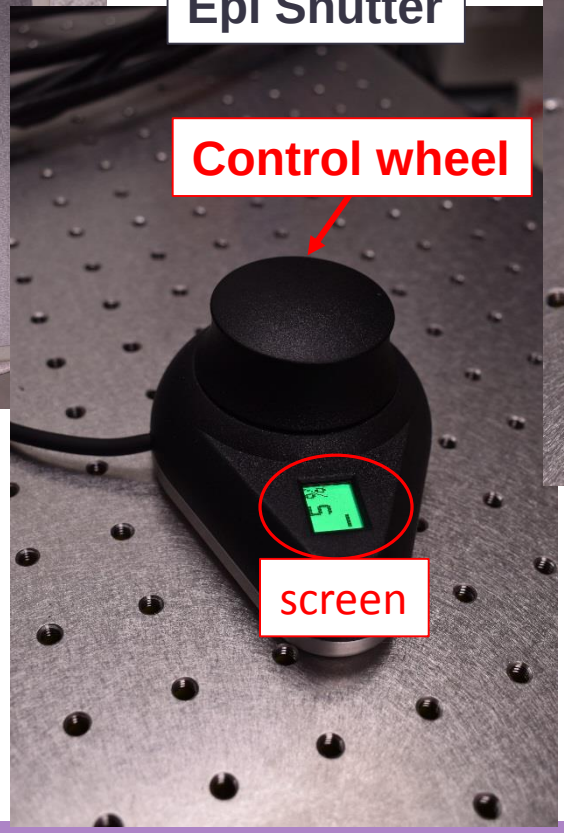
Remote Control Panel:
Controls the XY position of the stage by pushing the joystick. Twist the joystick to change the speed of XY movement (coarse, fine and extra fine).

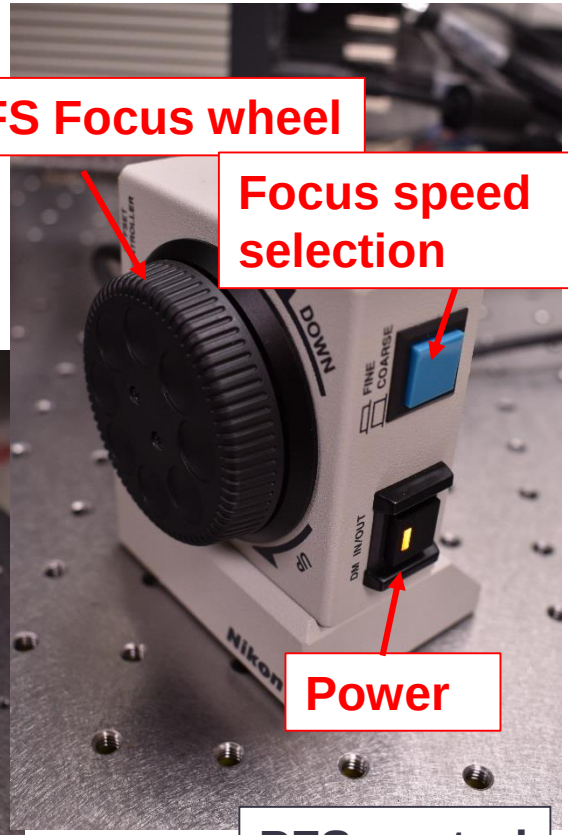
Focus wheel:
Changes the Z-position of objectives. Adjust the speed by pressing the **Z-speed** button (coarse, fine and extra fine).

Epi Shutter:

- Press the Control Wheel: Opens or closes the shutter.
- Turn the Control wheel: Changes the intensity of epi fluorescence light.
- Changes will be displayed on the screen.

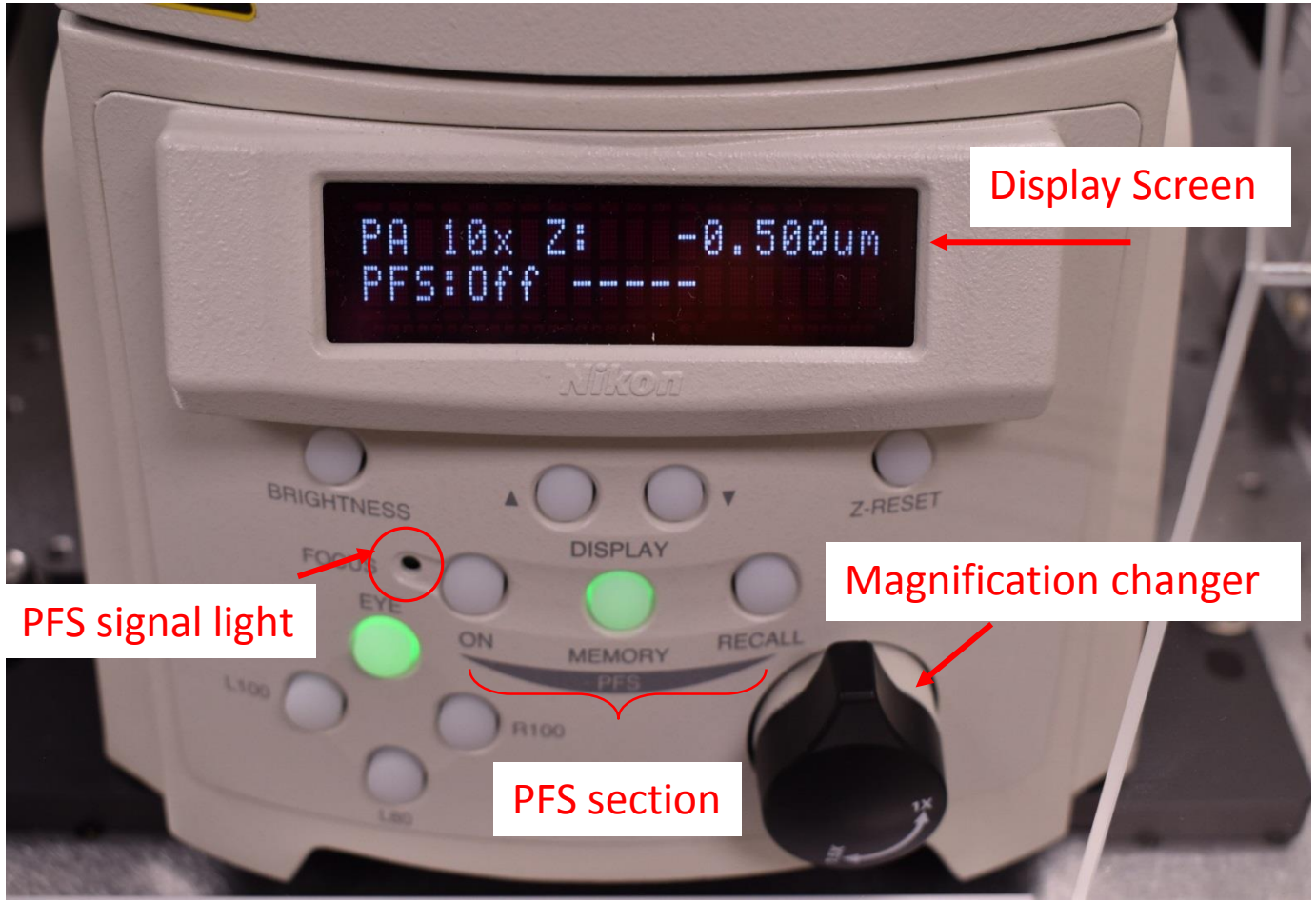
PFS Control: PFS is mostly used for live-cell imaging. It maintains the selected Z-position for longer time.
When PFS is turned on, all other Z-position controls are disabled, only PFS control can be used (See page 8).





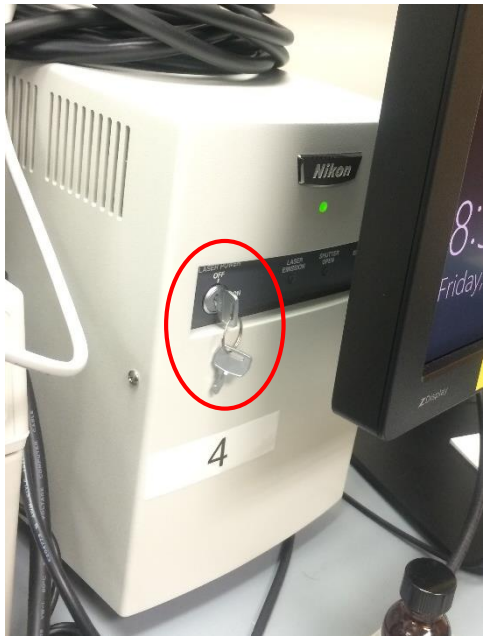
Microscope Display

- The **Brightness** button controls the display screen. Three modes rotate when pressing the **Brightness** button: On/Bright, Dim, and Off.
- **Display**: Chose display info on the screen.
- Light path controls : **EYE**: Eyepiece, **L100**: confocal, and **R100**: N-SIM. **L80**: Inactivated.
- PFS (perfect focus system) section. **On**: turns on the PFS. Once the PFS is turned on, all focus/Z-position adjustments on the remote control pad and on the two sides of the microscope are inactivated, **only the PFS control can be used (Page 7)**. **Memory**: remembers the current Z position. **Recall:** load to the last memorized Z-position. The PFS signal light will blink when the PFS is searching for a focused plane. The light will turn solid once a focused plane is found.
- **Magnification changer:** Default to 1, **DO NOT CHANGE.**
- **Z-RESET**: Sets the current Z-position display as 0 on the display screen. The actually Z-position does not change.



Turn on the Microscope

- When entering the room, pay attention to the signs on the door and do not turn on the light if “scanning in progress” label is on the door. Ask other users in the room for permission before you turn on the light.
- **Number 1 (Power Strip) is <u>always on</u>. Turn on the machines in the order of 2-->3-->4.**
- Number 4 (SIM lasers) does not need to be turned on if only using confocal. (Turn on 2 and 3).
- Number 2 (Confocal lasers) and 3 (Controller) do not need to be turned on if only using SIM (Turn on 4).
- <u>Do not turn on or off any other unlabeled machines</u>.



1. Start Up the System

Load samples and open the software

1. Login to the PC with your personal account: **lsumc-master\username** and your personal password (same with your email password).

2. Tilt open the flexible light arm, and open the sliding doors to load your sample.

3. Make sure the slide holder is clean and in position before placing the samples.

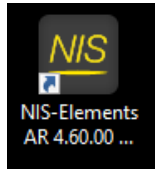
4. If using oil objectives, add one drop of oil on top of the objective or the cover slide. **Be cautious to not drop oil on the microscope body**.

5. Lower the Z-positions of objectives before placing samples.

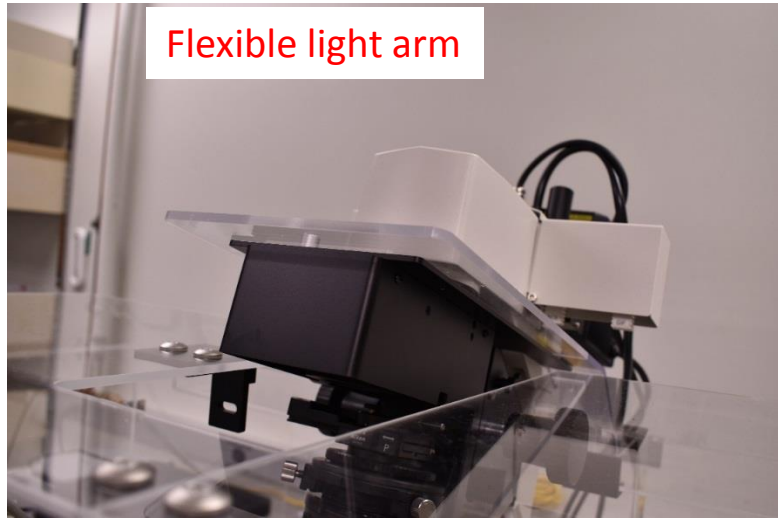
6. Place the sample on the slide holder with the **cover slip-side down**.

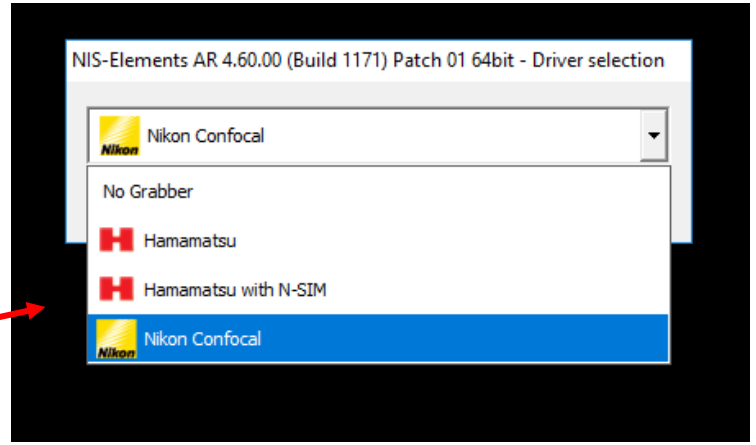
7. You can change the objectives to the desired one through pressing the objective selecting button on the left side of the microscope (See page 6), or you can do it later through the software.

8. Open the software NIS-Elements AR from the PC.



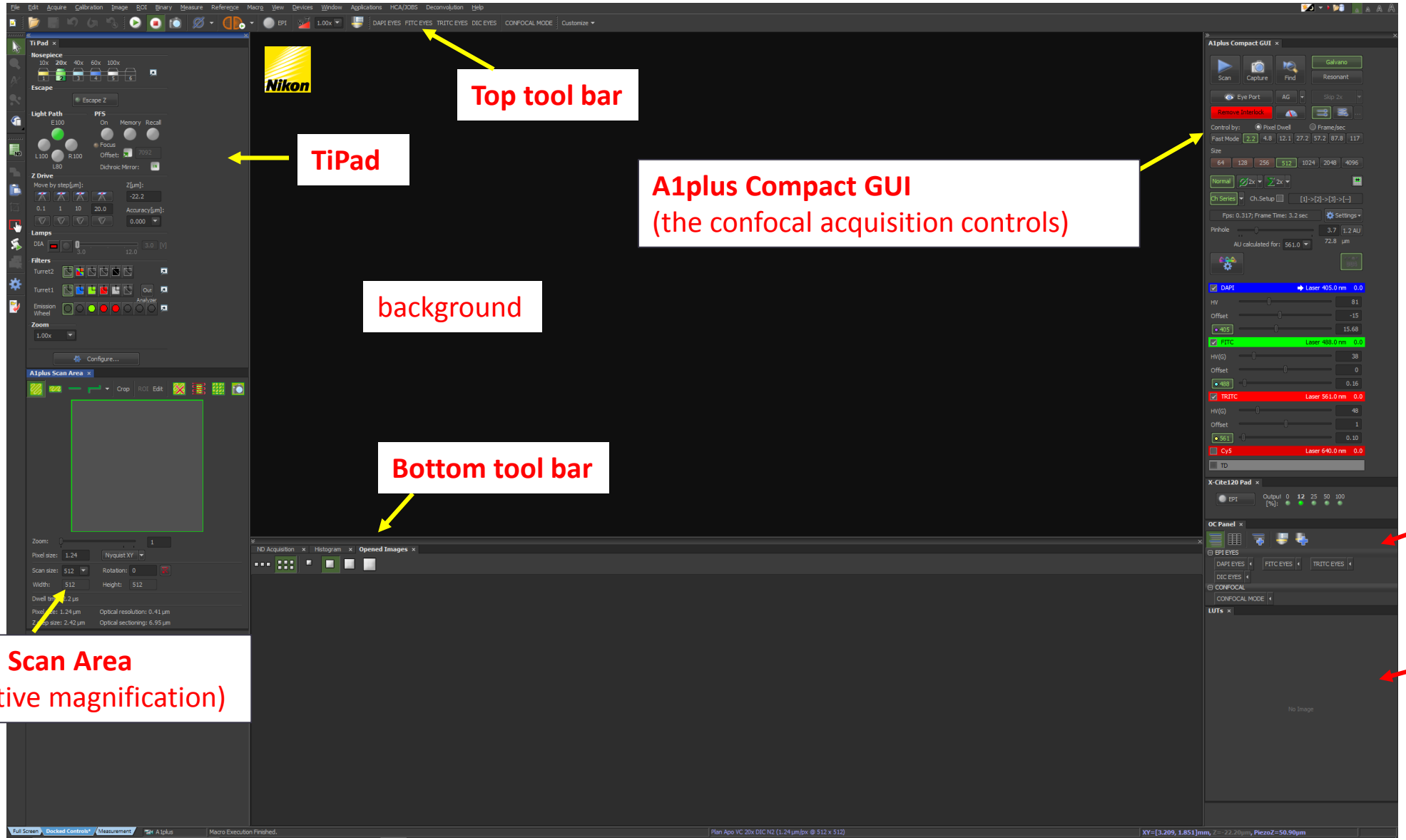
9. Select **Nikon Confocal** among **No Grabber**, **Hamamatsu**, **Hamamatsu with N-SIM**, and **Nikon Confocal** in the drop down menu.





2. Confocal Imaging

NIS-Elements Confocal Interface



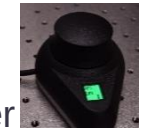
Eyepiece Observation (confocal)

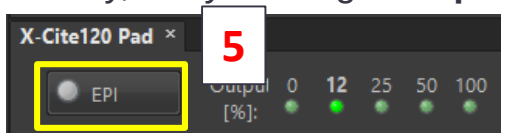
1. If you do not select objective from the microscope (see page 6), you can also select it through buttons at the top of the **TiPad**.

2. Click on the **Escape Z** to release it if it is on. You can also release the Escape Z by simultaneously press **Refocus** and **Escape** buttons on the right side of the microscope (See page 6).

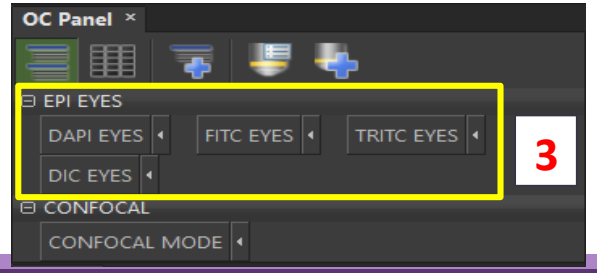
3. To enable fluorescent light to go through eyepieces, under the **EPI EYES** group in the **OC Panel**, select icons among **DAPI EYES, FITC EYES, TRITC EYES, and DIC EYES**. The light pathway (**E100**) and the filter turret in the **TiPad** will automatically switch according to the icons selected.

4. The **Remove Interlock** button in the **A1plus Compact GUI** panel will turn to red color after selecting a fluorescent light from the EPI EYES group. *Keep in mind: There is no Cy-5 option for eyepiece observation, because you cannot see far red through eyes.*

5. Open the Epi Shutter by pressing the Epi shutter  manually, or by clicking the **Epi** shutter button in the **X-Cite 120** Pad.



6. Observe through eyepieces. Find and focus on your sample. Then turn off the Epi shutter.



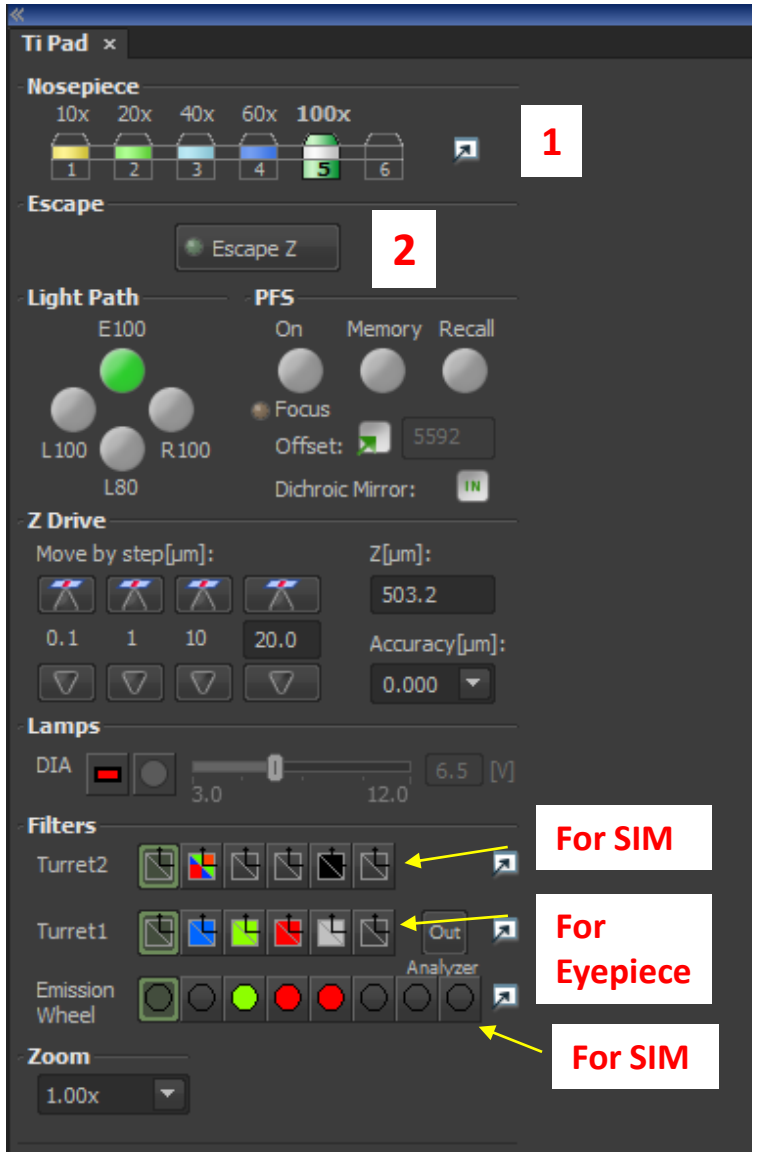


Image live view in software (confocal)

6. After observation through the eyepieces, click on the **CONFOCAL MODE** button in the OC Panel. This will automatically change the light path shown on the **TiPad** from **E100** to **L100**.

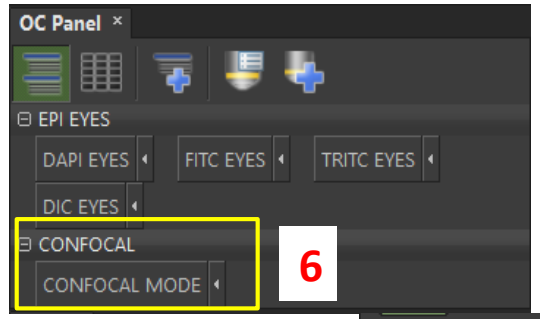
7. Then click on the red **Remove Interlock** button in the **A1plus Compact GUI** panel and wait for the red color to turn to grey color.

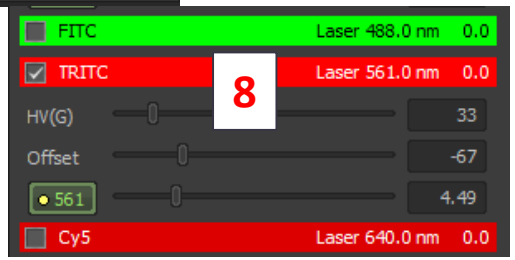
8. Select the PMTs (lasers) that you want to use and click on **Scan** in the **A1plus Compact GUI** panel. A live view window will pop up. Live view can also be selected alternatively by clicking  in the top tool bar (See page 13).

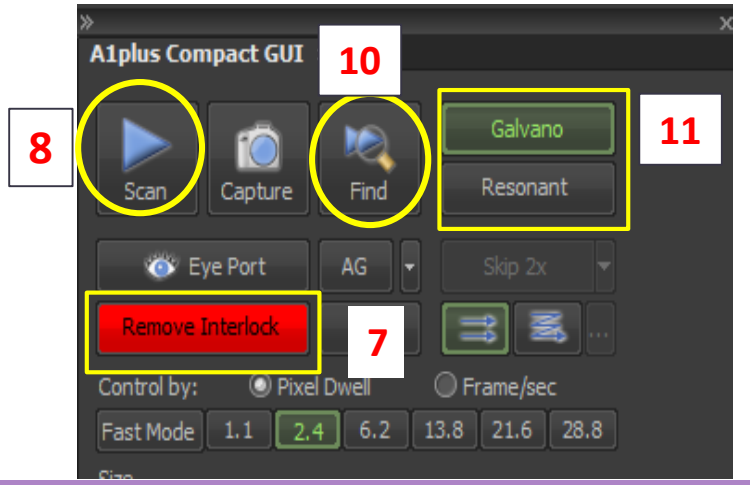
9. Then click on the auto scale  icon in the top tool box <u>of the live view window</u>. This will give you a better looking image.

10. Alternative to using the **Scan** function to view your sample in the live view window, You can use the **Find** function. This allows for fast scan (lower resolution, smaller image size, and scan of a small portion of the entire image field), saves your time, and decrease sample bleaching.

11. **Galvano** is default for confocal scanning and image acquisition**. Resonant** gives you fast scan, but with low resolution (It is useful when selecting z-stack positions). <u>**Galvano** is used most of the time</u>.

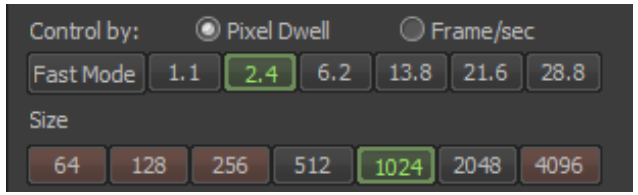




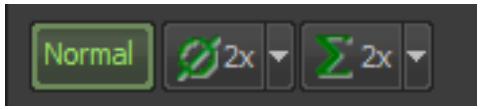


Adjust image quality through the A1plus Compact GUI panel.

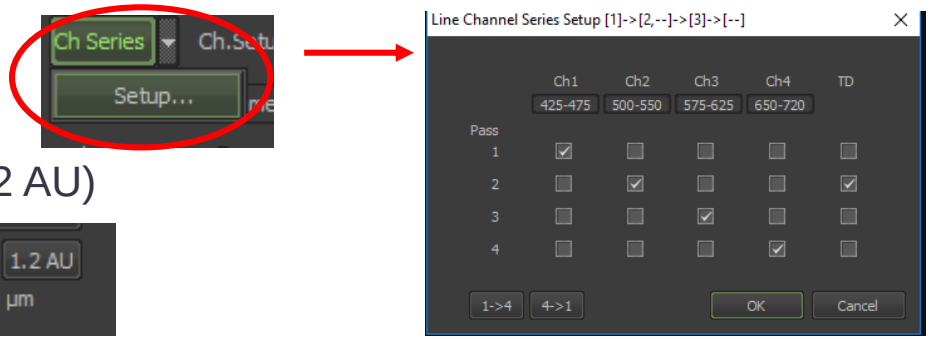
1. Scan speed and image size



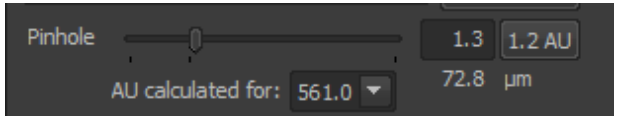
2. Averaging and accumulation.



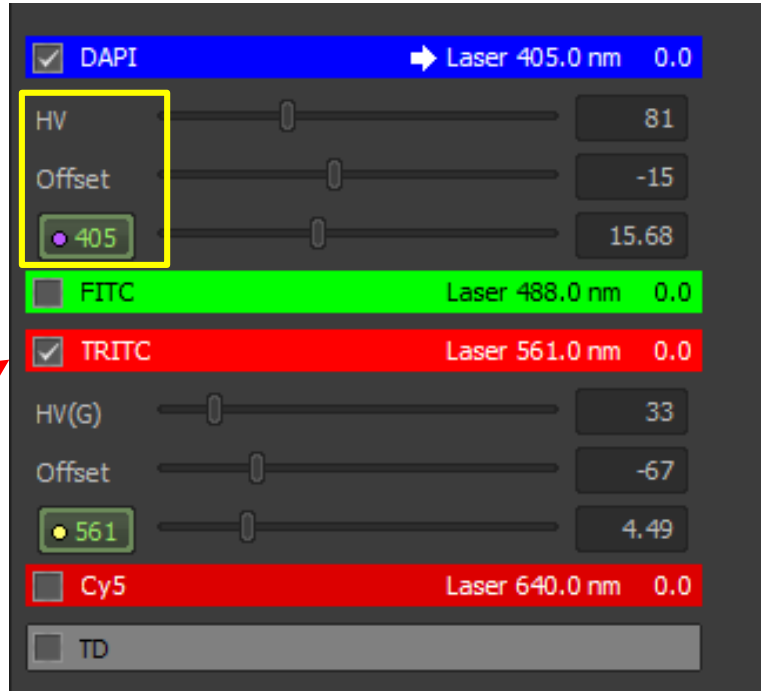
3. Scan mode: simultaneous or sequential. The lasers and the orders of sequential scan can be selected in the **Channel Series Setup** window. **Important**: Select the **Ch Series** button to enable sequential scan if you have multi channels in one image. (See page 47 for detailed description of simultaneous or sequential scan.)



4. Pinhole size (Default is 1.2 AU)



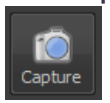
5. Four PMTs (**DAPI, FITC, TRITC**, and **Cy5**) can be selected. **HV(G)**: Gain. **Offset**: background adjustment.



6. Laser power strength scroll bar:

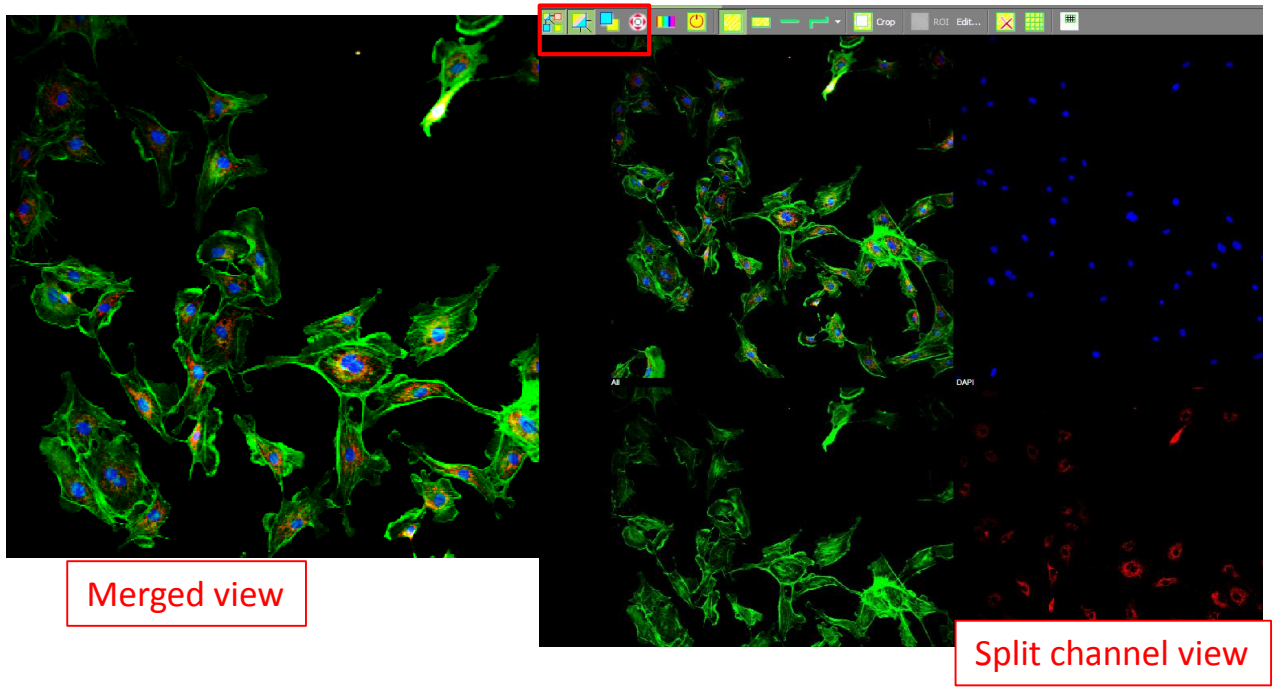
7. Click to turn on the **saturation indication** button  in the tool bar at the top of the live view window. This allows you to visualize pixel saturation.

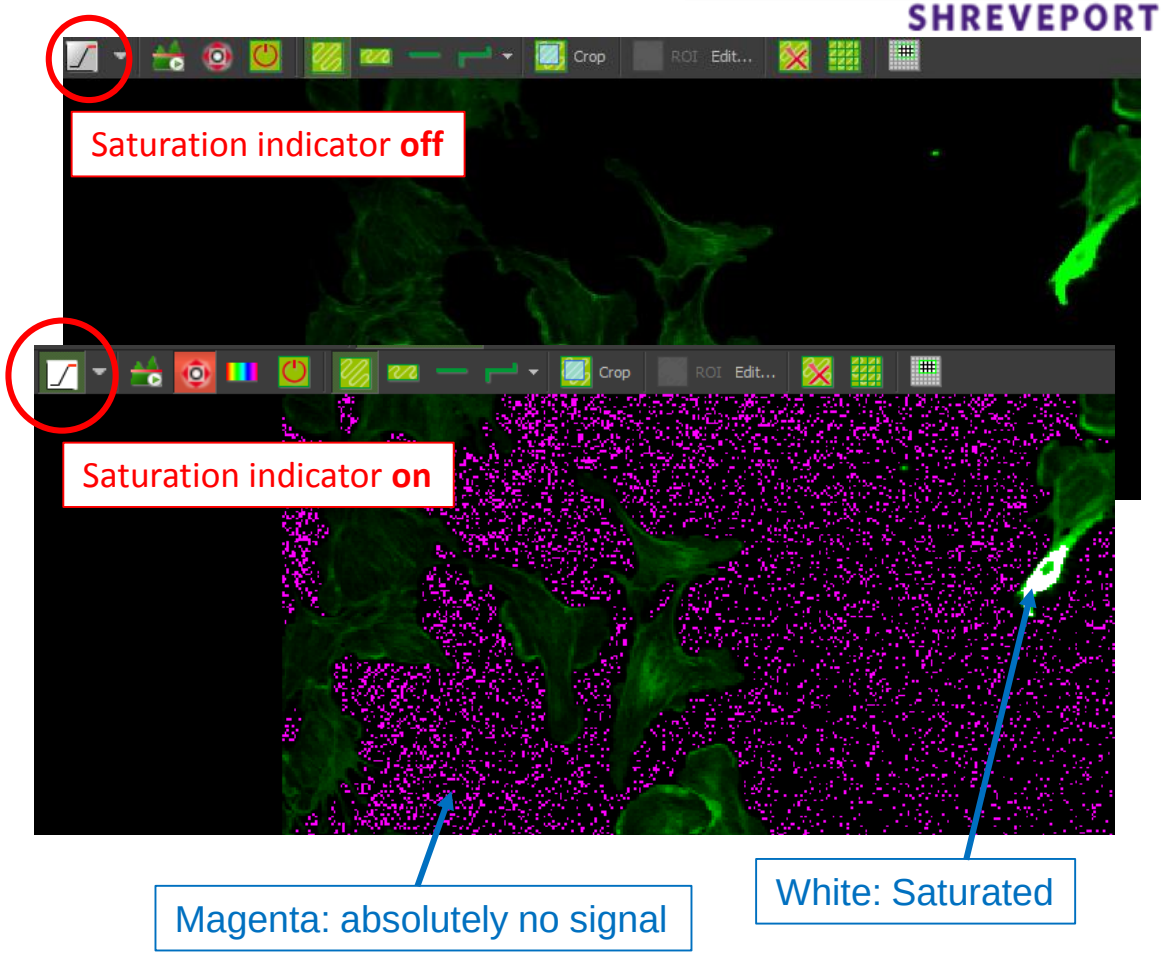
8. Then check individual PMTs (lasers) and adjust the laser power, Gain and Offset for each channel according to the pixel saturation status.

9. After adjustment, click on **Capture** 

in the **A1plus Compact Gui** panel to acquire a scanned image.

10. For multichannel images, you can click the split image icons  to view split channel images.





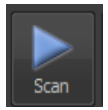
11. In an acquired image, a sale bar can be added through clicking the icon  at the right side bar of an opened image.

The **A1plus Scan Area** section is a useful magnification tool. This function zooms in on the image objectively (not digitally), yet do not change the actual objective. It is especially useful when a certain region of interest needs to be magnified and the sample is viewed at a lower magnification objective. The drawback is that stronger laser power will be used for the magnified region and bleach that area more than the surrounding area.

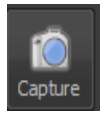
1. Select among icons  . These icons are tools to choose a region of interest (ROI).

2. Drag the cursor to change the size and position of the red-bordered square. The zoom in factor and other parameters will change according to the ROI selected.

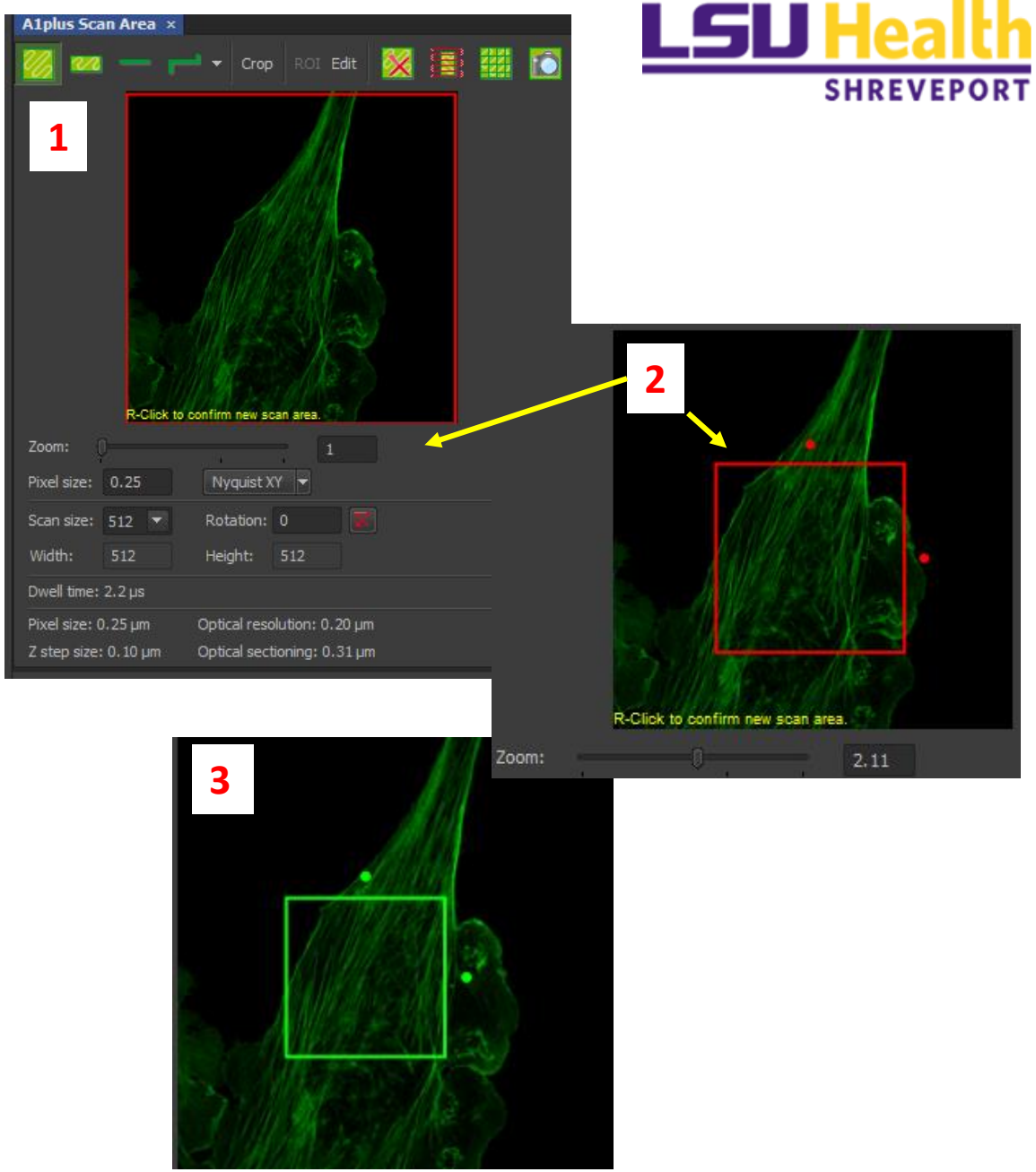
3. Right click on the new ROI border and it will turn green.

4. Click on **Scan** 

for live view of the newly selected ROI.

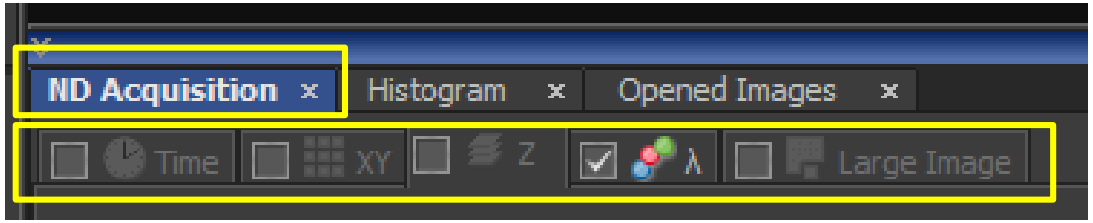
5. Adjust the image quality if needed and click on **Capture** 

to acquire an image.



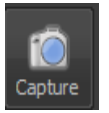
3. ND Acquisition

Settings for time lapse (**Time**), multipoints (**XY**), Z-stacks (**Z**), and multichannel (**λ**) images are in the **ND Acquisition** tab located in the bottom tool bar (See page 13). The **Large Image** function in the **ND Acquisition** panel cannot be used alone, it has to be combined with other functions. (To use scan large image function alone, please refer to page25).

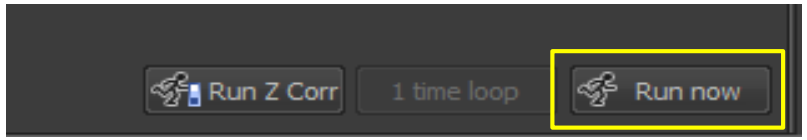


1. Select a desired option under **ND Acquisition** by checking the box in front of the name. This will open corresponding windows to set up the experiment (Detailed introductions from page 21 to 24).

2. All options under ND Acquisition can be combined to take an image.

3. To capture images through **ND Acquisition**, instead of clicking the **Capture** 

button in the **A1plus Compact Gui** panel, click on the **Run now** icon at the lower bottom of the ND Acquisition tab.

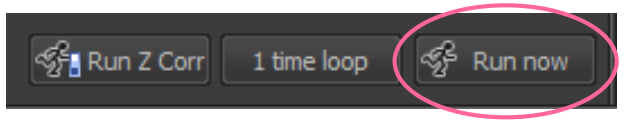


Time lapse

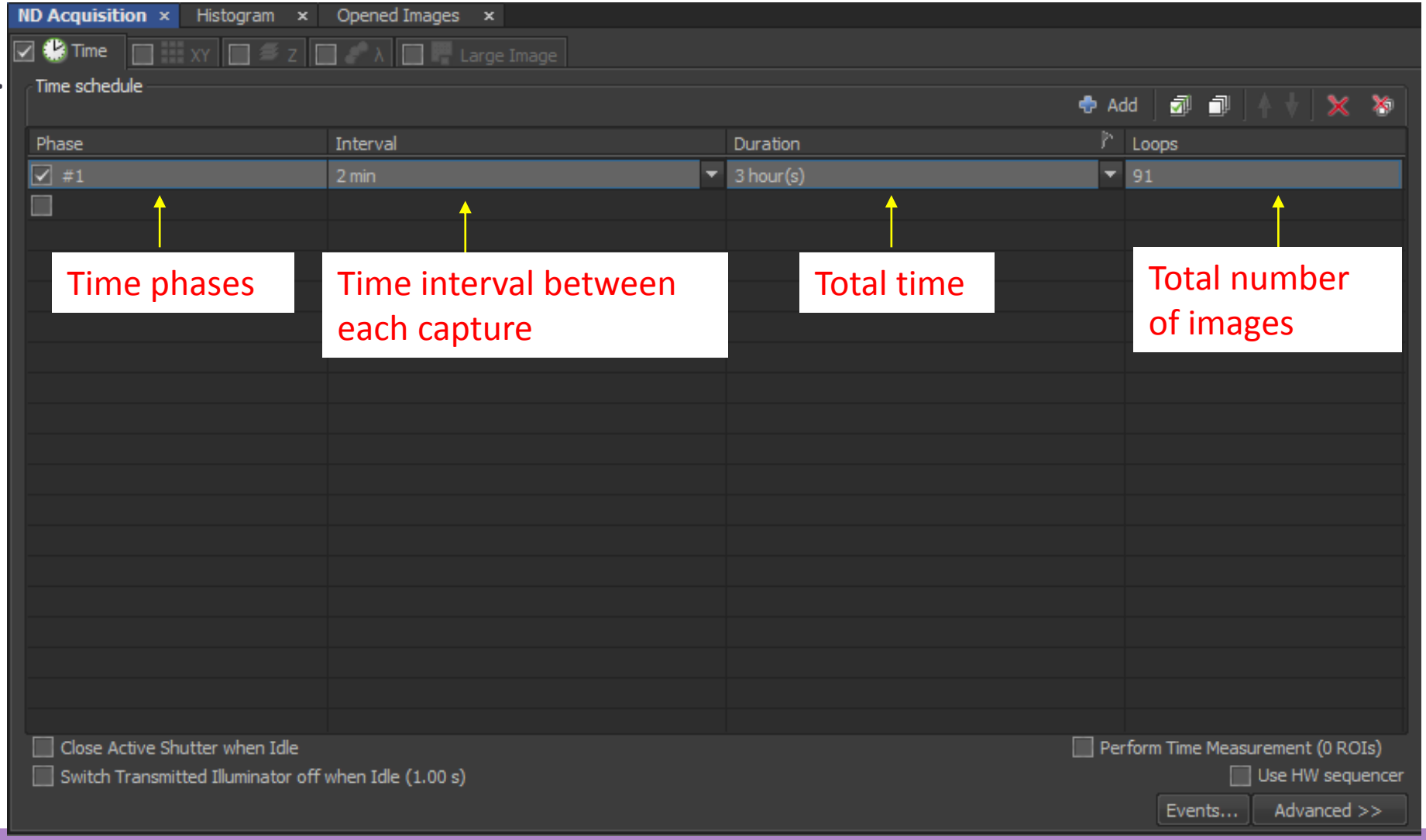
1. Check the **Time** icon 

2. Set up the experiments as indicated in the figure.

3. Run the experiments by clicking the **Run now** button at the lower right corner of the layout.



Time lapse imaging is mainly used for live cell imaging. It allows you to capture images at certain time points, and with in a set time duration.



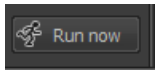
Multipoints

1. Check the **XY** icon 

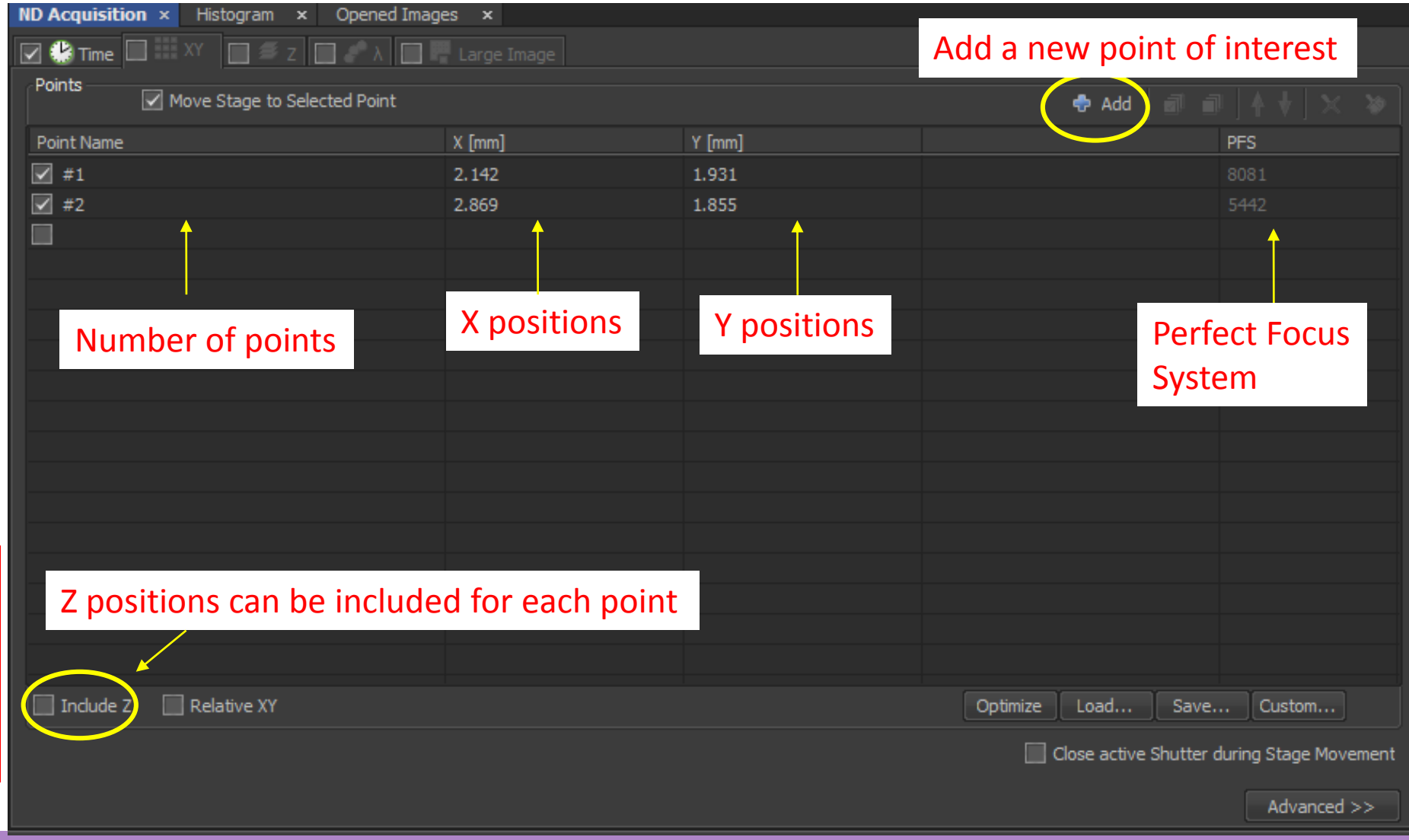
2. Find a position in the sample through the live view window and click on **Add**, or click on a new row, the current position will be added. Z-positions can be included if the **Include Z** is checked.

3. Repeat step 2 to add more points.

4. If a point is not selected (tick in front of number), even though it is in the list, no image will be taken at that point.

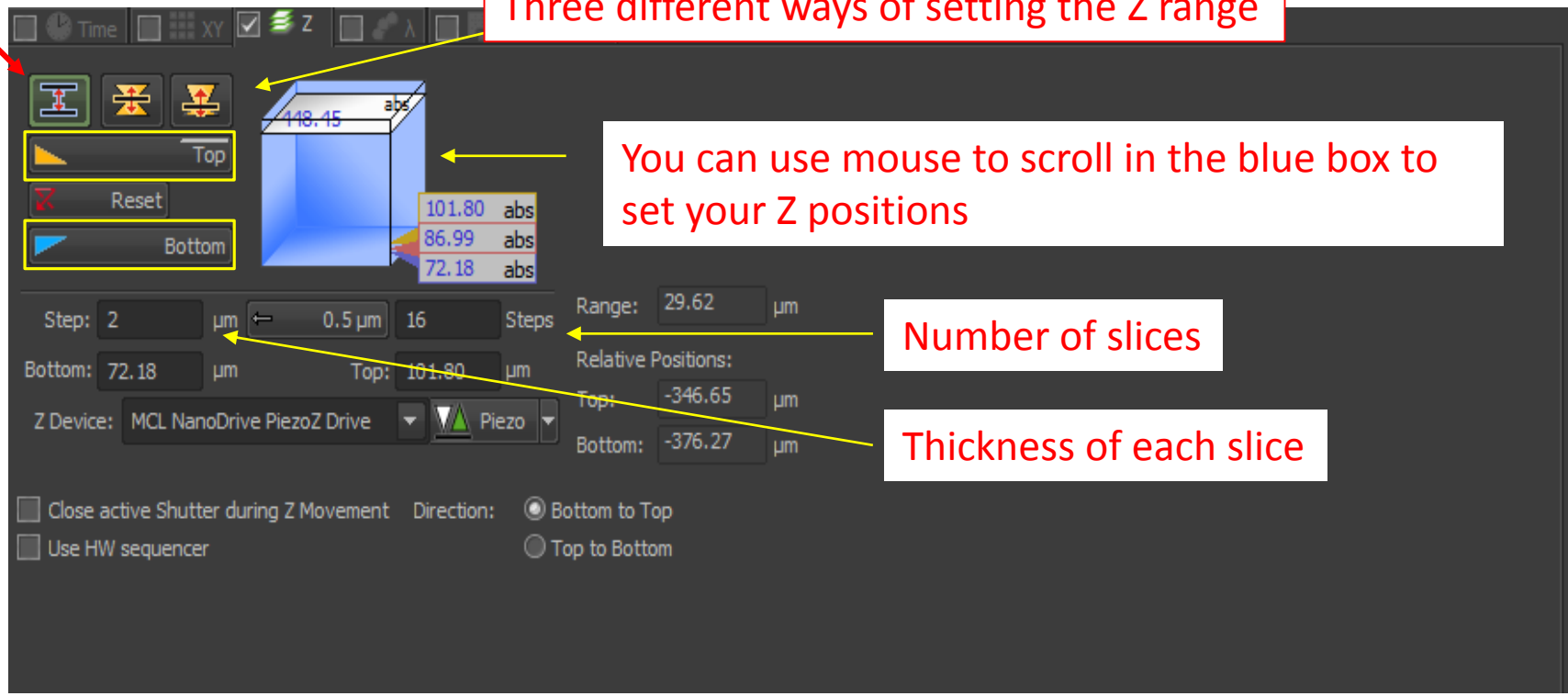
5. Click on the **Run now** icon at the bottom right of the interface.


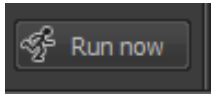
Multipoints allows you to select several positions of interest and build a list for the microscope to remember and capture images at these points automatically.

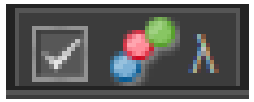


Z-stack acquisition

1. Check the **Z** icon 
2. Select one mode to set the Z range
3. Find your plane of interest using the live view window.
4. Turn the focus wheel to change the Z-position to a desired starting point and click the **Bottom** icon. Then this position is recorded.
5. Turn the focus wheel again to find an ending Z-position and click the **Top** icon. This position is recorded.
6. You can change the number of slices or the slice thickness by typing in your desired numbers in the indicated boxes.
7. After setting the experiment, click the **Run now** icon to run the experiment.







The **Multichannel (λ) image** function is used to capture multichannel fluorescence images.

Under Confocal mode, it is not necessary to use the **(λ)** function. Multichannel options for confocal images are selected from the PMT (laser) properties in the **A1plus Compact Gui** panel (See page 16).

For description of the **(λ)** function under SIM (super resolution) mode, please refer to page 37.

Large image function is used to scan several images, each of them having 10-15% overlapping, for the software to automatically stitch them together to form a large image. (Also called:Mosaix)

The Large image function under **ND Acquisition** can not be used alone. It can be used when combined with other ND acquisition choices. To scan a large image alone, please refer to page 25.

Scan Large Image

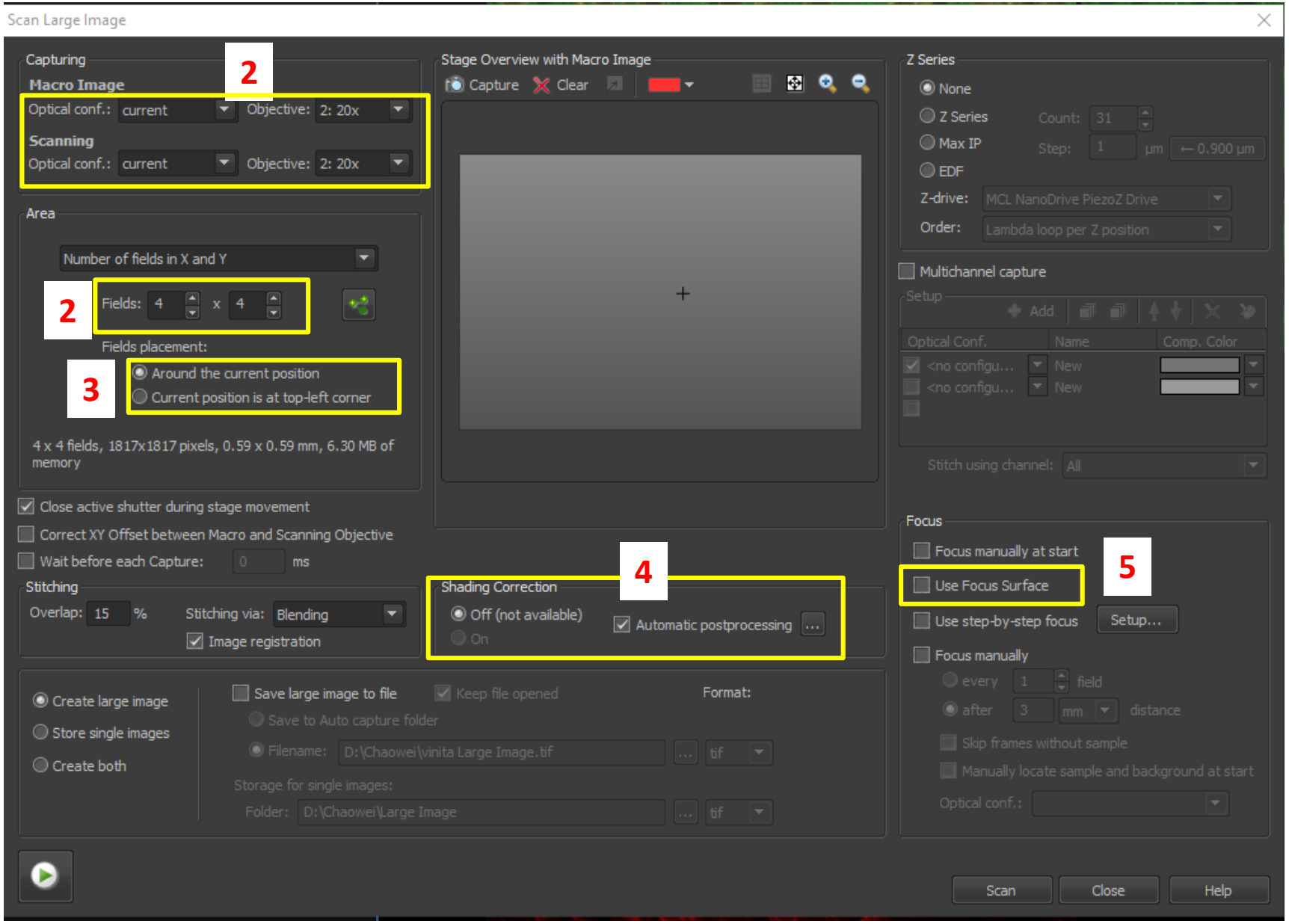
1. In the top tool bar, under **Acquire,** Select **Scan Large Image.**

2. Select the correct objectives and number of fields in X and Y (number of images in each column and row).

3. The current position can be placed in the middle of the entire stitched image or at the top left corner of the entire large image.

4. Check "**Automatic postprocessing**", and select Shading Correction **Off** for fluorescence image stitching. Shading correction **On** when performing bright-field imaging.

5. If the surface of your tissue sample is not even, this can be compensated through the **Use Focus Surface** option.



Scan Large Image

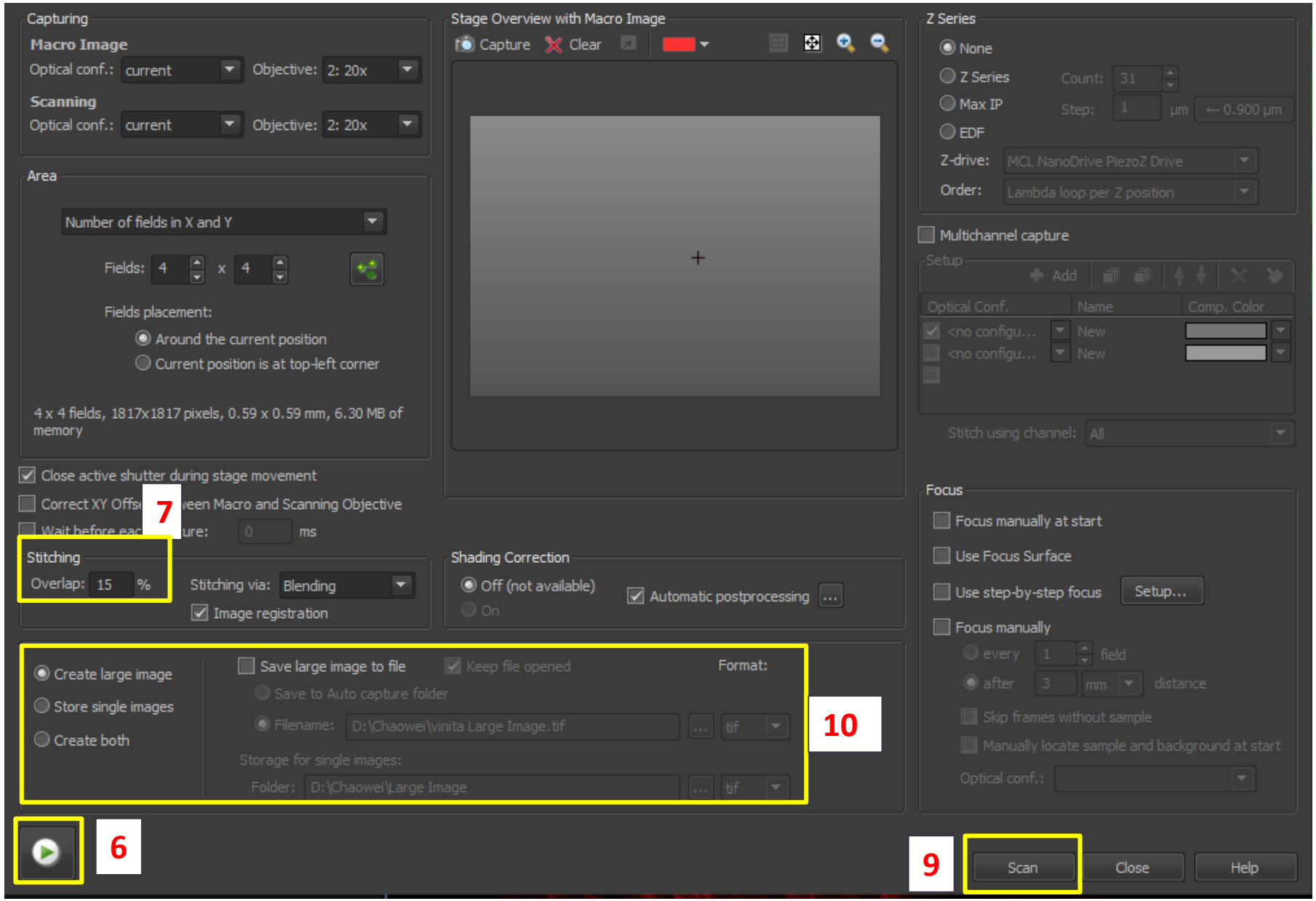
6. For live view, click on  at the lower left bottom.

7. For stitching, the default overlap area is **15%**, overlapping between 10-15% is good.

8. Z-stacks and multichannel images can be combined to scan large images.

9. To start scan, click on **Scan** at the lower right corner. The **Capture** icon do not perform large image scan. It only captures the image at current position.

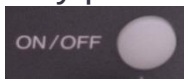
10. You can choose to save only the stitched large image or save individual images in the save settings section.



4. Bright Field Imaging

1. Pull out the knob on the flexible light arm for transmitted light to pass through.

2. Click on **DIC EYES** from the **OC Panel**. Then you should see that the light path goes to **E100**, and the **Remove Interlock** button turns into red (See page 14).

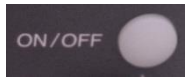
3. Turn on the transmitted light by pressing the transmitted light shutter on the left side of the microscope 

(See page 6).

4. Adjust the transmitted light intensity by turning the intensity wheel on the left side of the microscope.

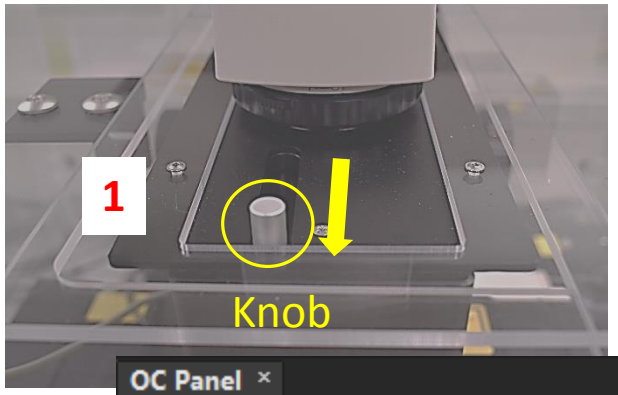
5. Find and focus on your sample through eyepiece.

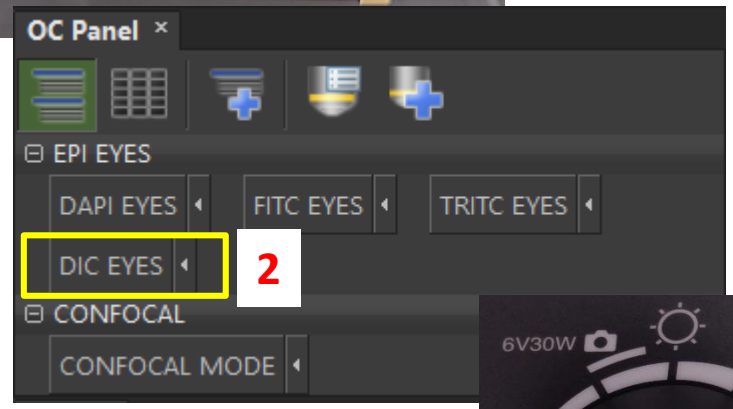
6. The phase contrast effect can be adjusted through inserting the phase contrast filter and tuning the metal knob shown on page 5.

7. After Eyepiece observation, turn off the transmitted light by pressing the ON/OFF shutter again. 

8. Go back to confocal mode and activate the **FITC** and **TD** PMT for imaging. Use the FITC laser for bright-field imaging.

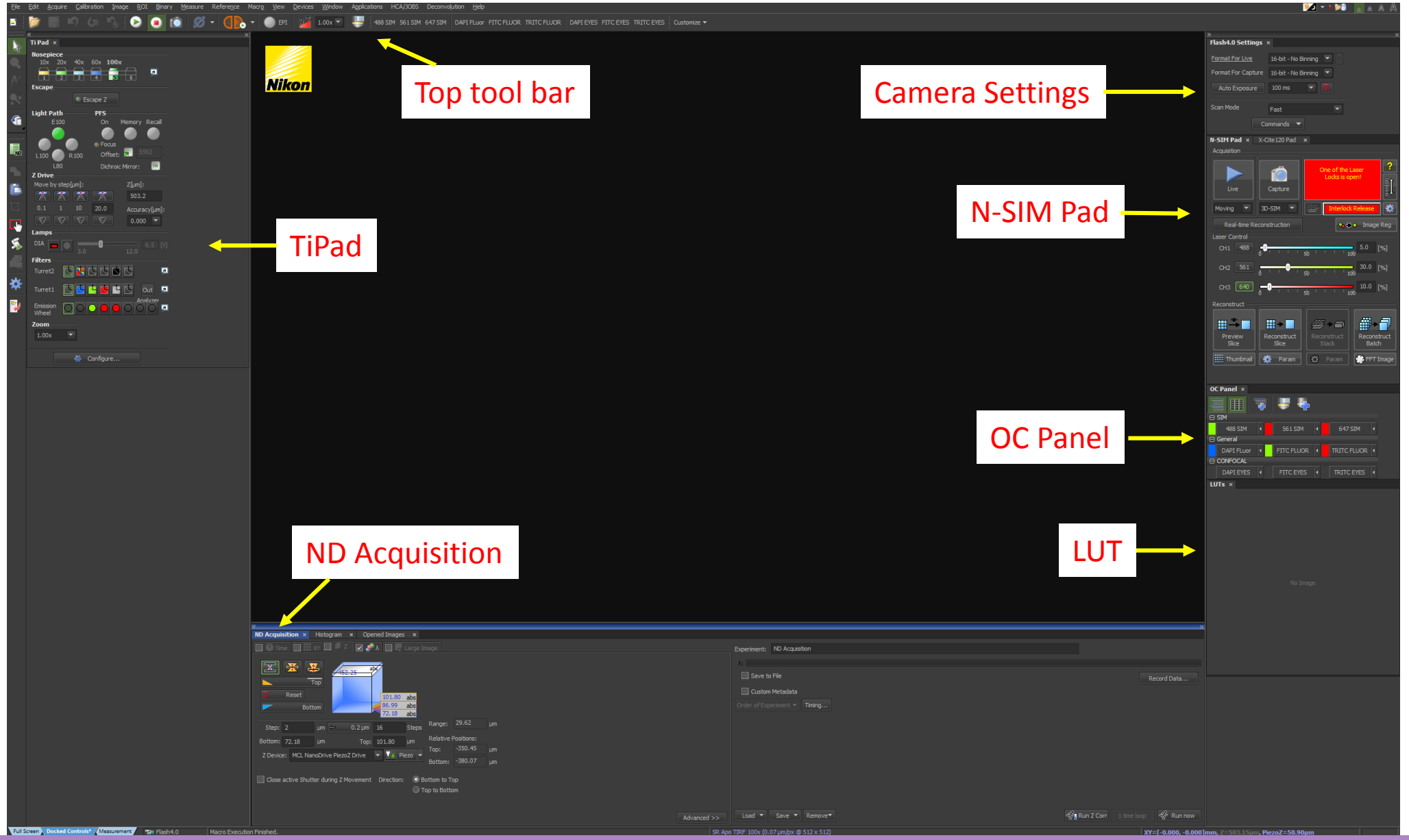
9. The live view, adjustment, and capture process is same as for fluorescent imaging (See page 15-17).



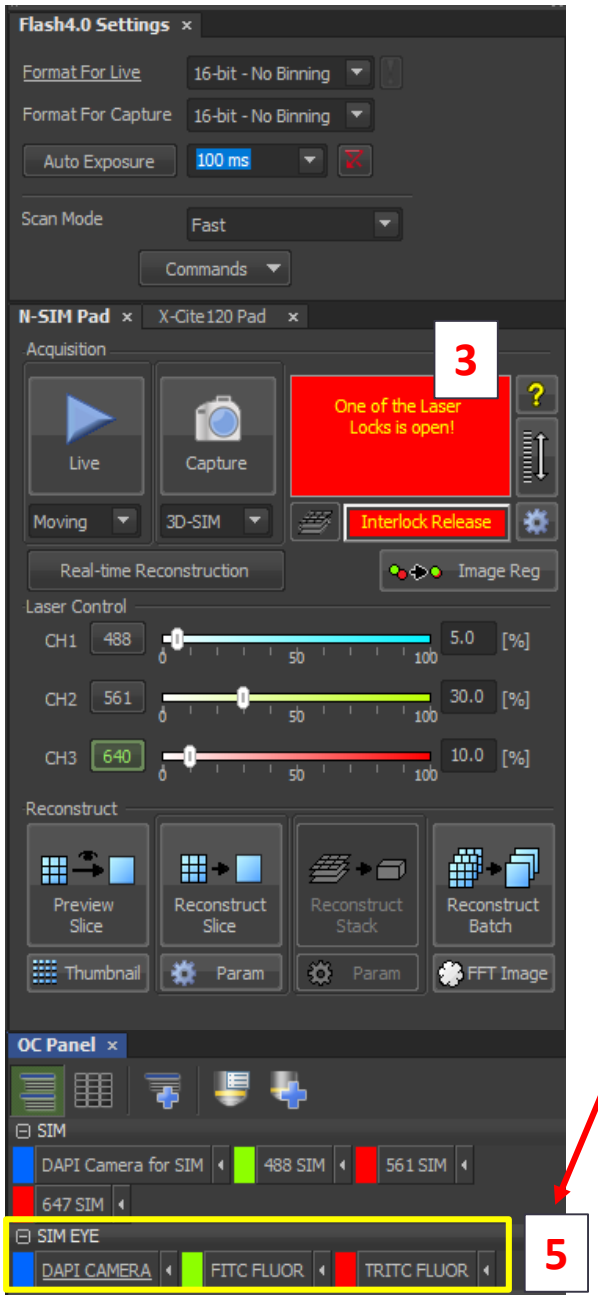


5. SIM (Super Resolution)
(Structured Illumination Microscopy)

NIS-Elements SIM Interface (Select **Hamamatsu with N-SIM** when opening the software)



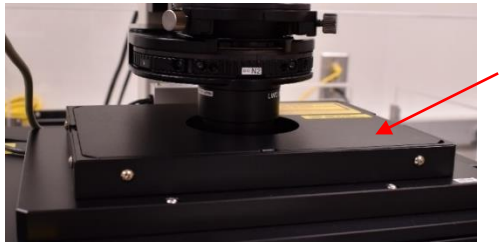
Eyepiece observation

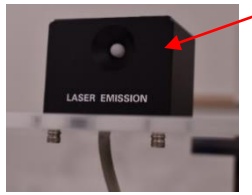


1. Chose **Hamamatsu with N-SIM** when opening the NIS-elements software to enter the SIM mode. If you want to convert from the confocal mode to the SIM mode, close the software and wait for 10 seconds, then open the software again and select **Hamamatsu with N-SIM** .

2. Load your sample with cover slip side down.

3. For SIM visualization, the laser safety lid **has to be in place.** The laser emission light signal **has to be on**.





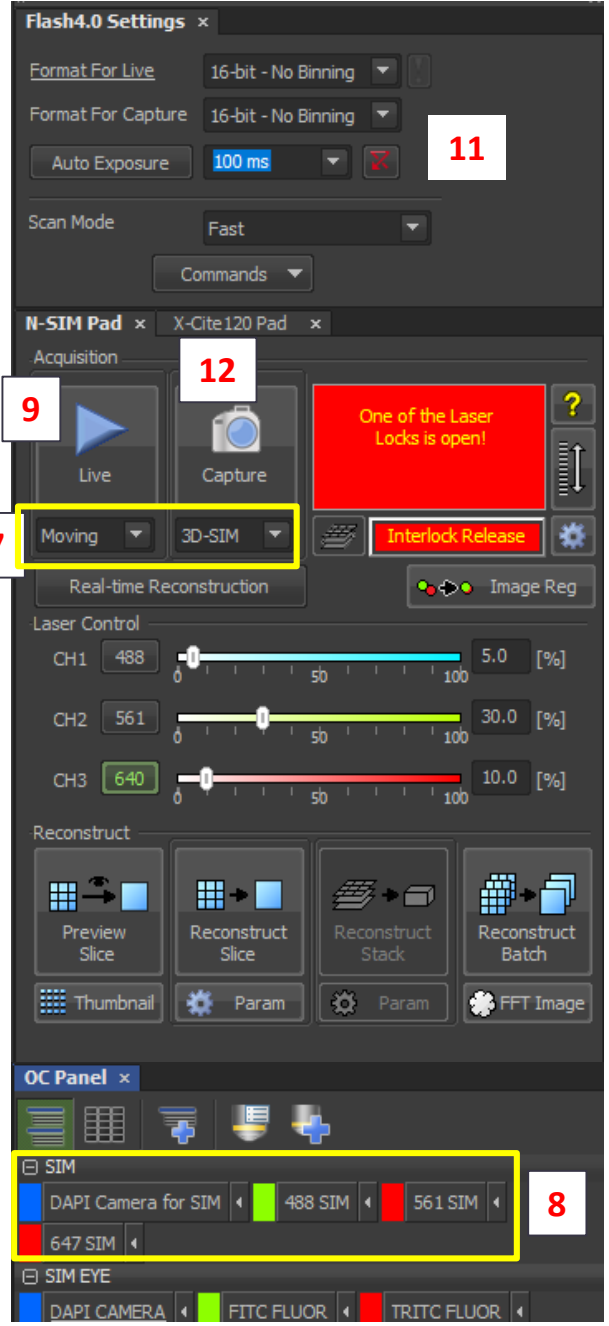
[In the **N-SIM Pad**, the **Interlock** will be in red when the laser safety lid is not in place, or the light path goes through eyes.]

4. Select the 100X objective. **Only the 100X oil objective can be used for SIM acquisition**.

5. Select an icon among **DAPI Camera**, **FITC FLUOR**, and **TRITC FLUOR** in the **SIM EYE** group in the **OC Panel**. This selects a light for eyepiece observation.

6. Then turn on the Epi shutter. 

7. Find and focus on your sample from the eyepiece. Then turn off the Epi shutter.



Adjustment and Acquisition

7. Set the options under **Live** as **Moving**, and **3D-SIM** under **Capture**. This will allow you to visualize the grid in live view mode, and acquire a SIM image instead of a simple wide-field image when capturing.

8. In the **OC Panel**, icons in the SIM group (**DAPI Camera for SIM**, **488 SIM**, **561 SIM**, **647 SIM**) control the lasers for SIM image acquisition. Click on one of the icons, and the red interlock button will turn grey. Only one laser can be selected at a time, and the laser power can be adjusted. *Reminder: There is no DAPI laser for SIM in our system. The DAPI option uses the FITC laser instead.*

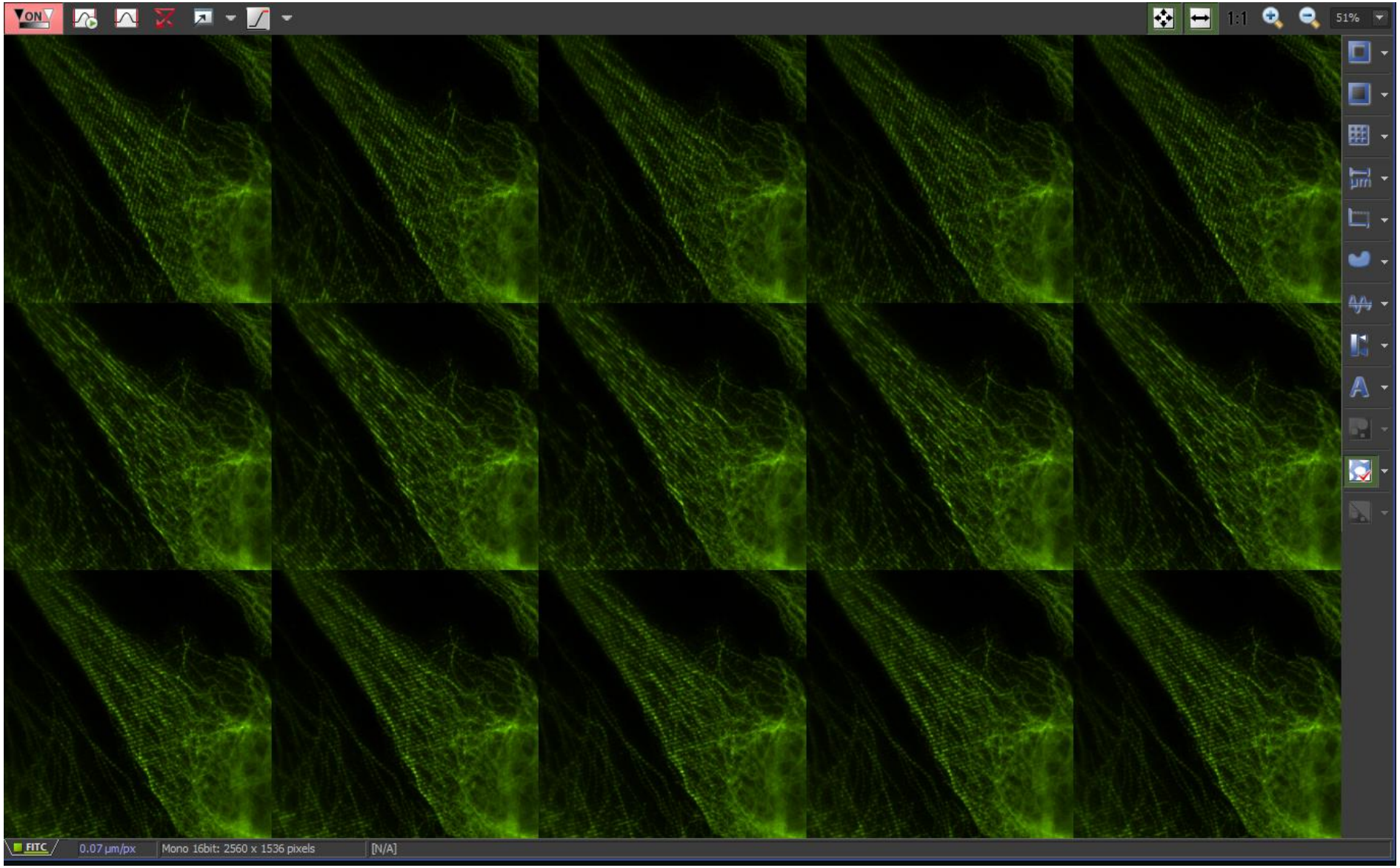
9. Next, turn on the live view window by pressing the **Live**  button.

10. Then click the auto scale  icon in the top tool box of the live view window. This will give you a better looking image. Refocus on your sample using the live view window.

11. In the **Flash4.0 Settings** panel (Camera Settings), the **Auto Exposure** time can be changed to adjust the image brightness. Default settings for Binning is **16-bit-No Binning**.

12. To acquire an image, click on **Capture**  .

[To acquire multichannel images, add channels from the **(λ)** tab in the **ND Acquisition** section (See page 37)].



13. After each capture, 15 raw images for each channel will be acquired and reconstruction is needed after the raw images are acquired.

Raw SIM image reconstruction

1. There is a Reconstruction section in the **N-SIM pad**. When clicking the **Preview Slice icon,** the **N-SIM Slice Reconstruction** window will appear, and this window allows you to setup the parameters for reconstruction.

2. Click on the **Reconstruct Slice** icon, raw images will be directly reconstructed without showing the N-SIM slice Reconstruction window.

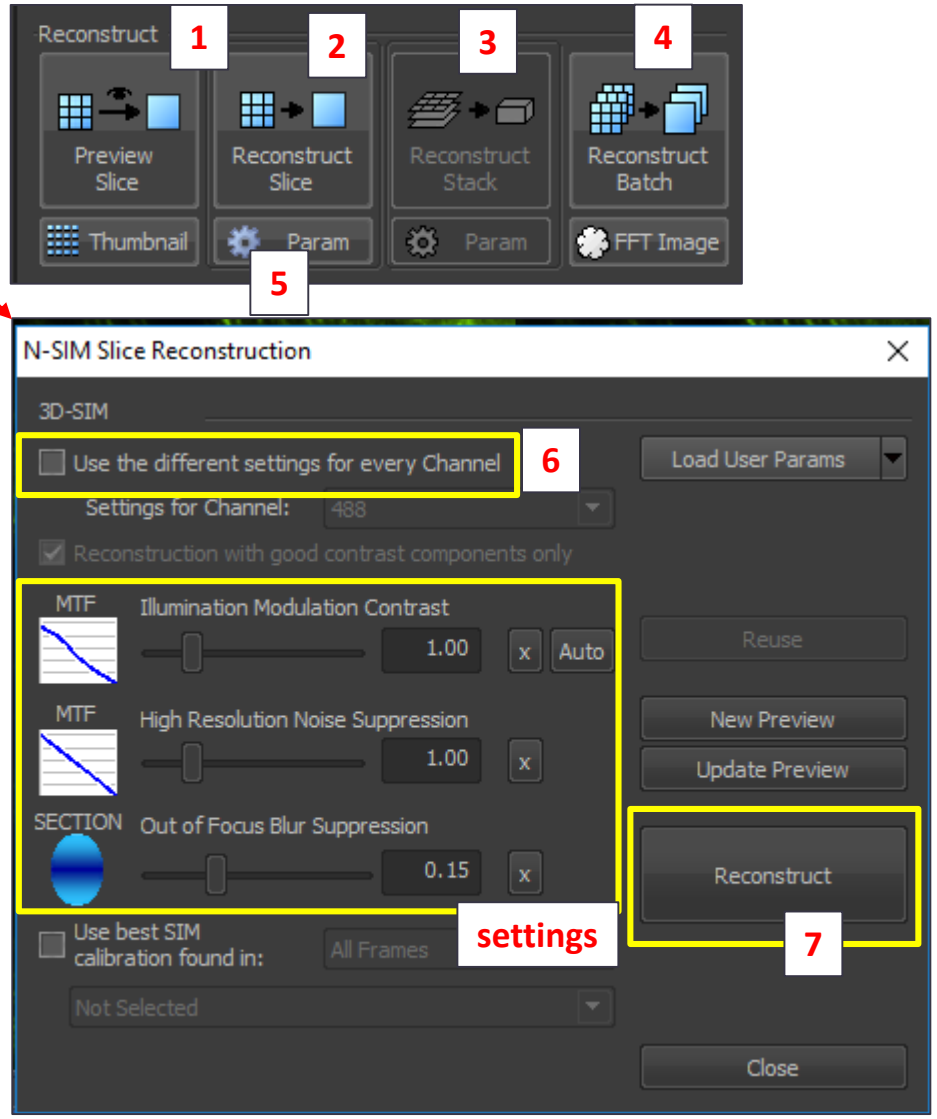
3. The **Reconstruct Stack** icon is for Z-stack images.

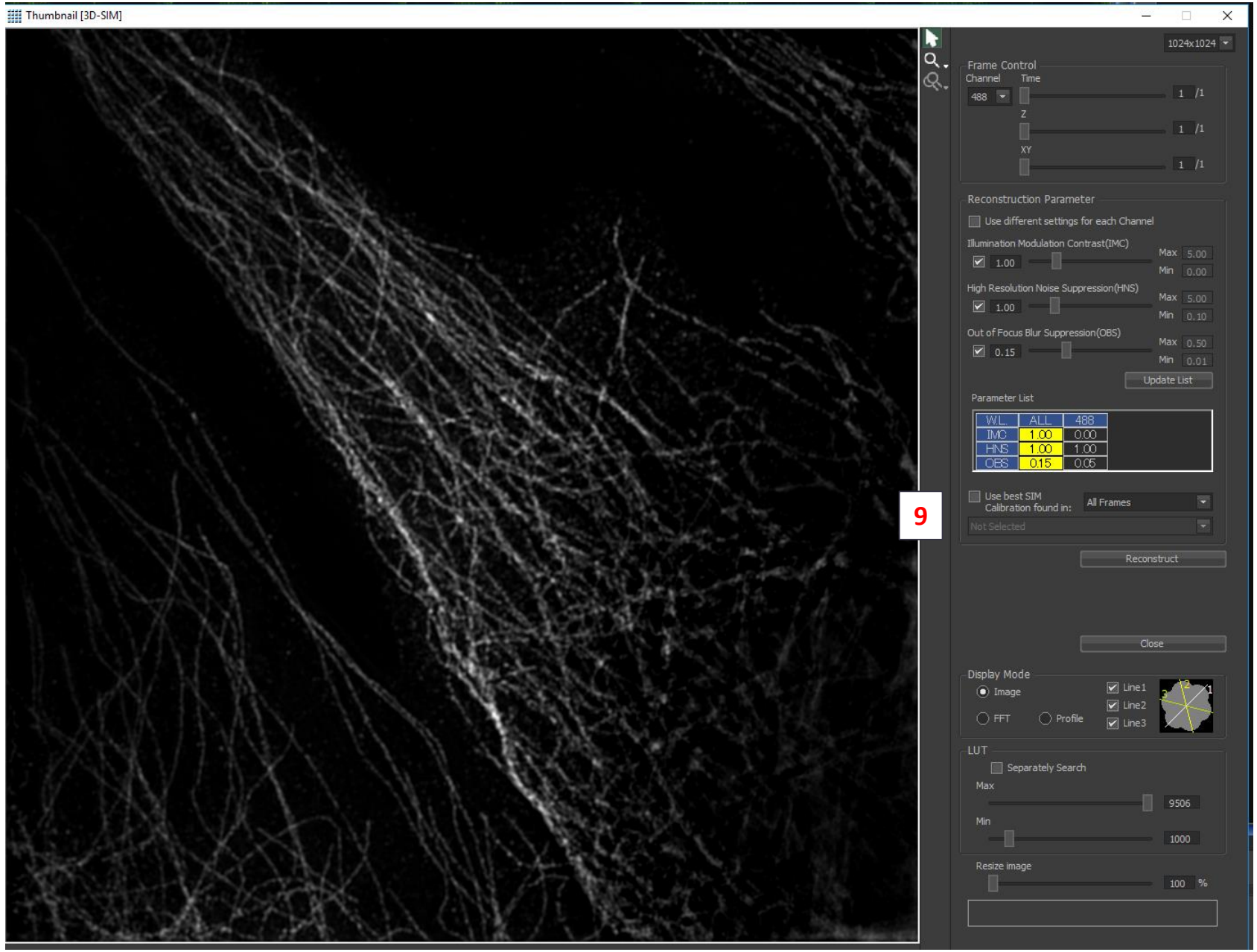
4. The **Reconstruct Batch** icon reconstruct slices for all the images with raw data.

5. The **Param** icon allows you to setup the parameters for reconstruction. The **N-SIM Slice Reconstruction** window will also appear after clicking the **Param** icon.

6. In the **N-SIM Slice Reconstruction** window, different settings can be used for every channel when this option is selected.

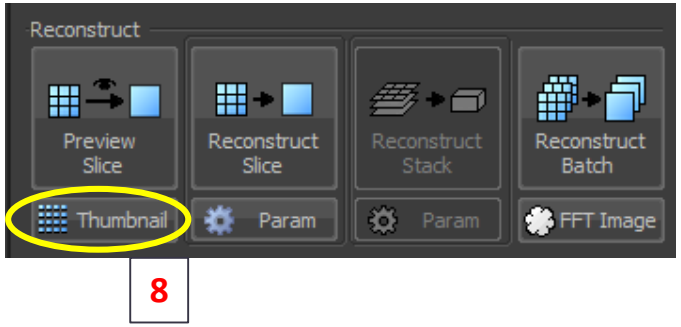
7. After adjusting the settings, click **Reconstruct** or **Preview** to apply the settings.



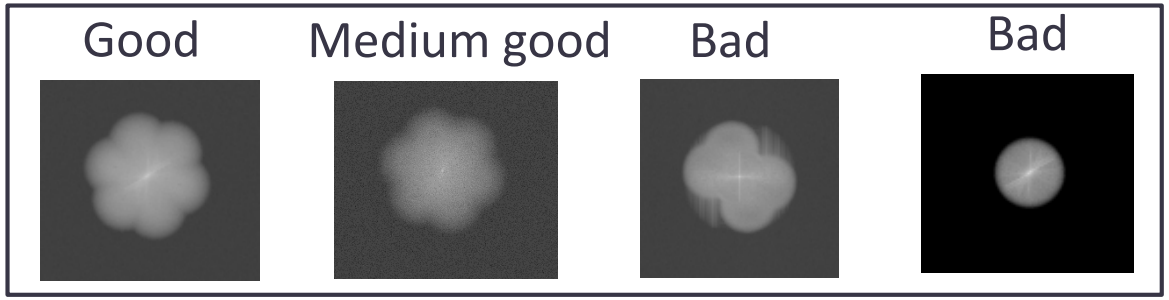


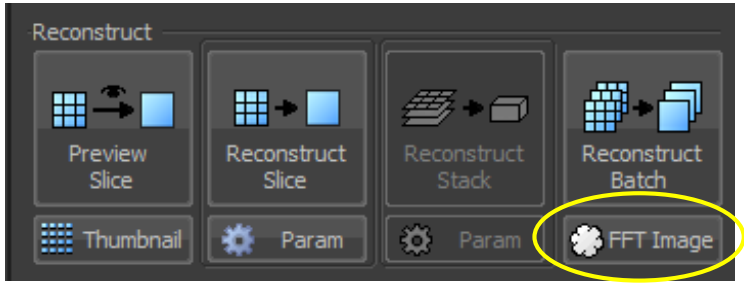
8. Click the **Thumbnail** button and a Thumbnail window will open.

9. In the Thumbnail window, real time view of a reconstructed image is shown. Reconstruction parameter settings, FFT, LUT are in the right side bar of the window.

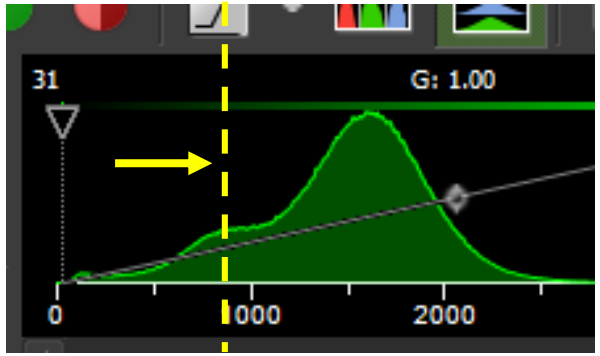


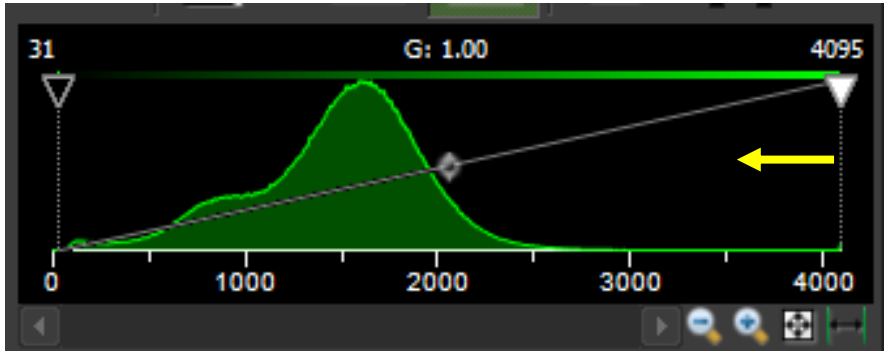
10. Click on the **FFT Image** icon, and a image of white flower shape (diffraction component) will appear, indicating whether the reconstructed image is good or bad.



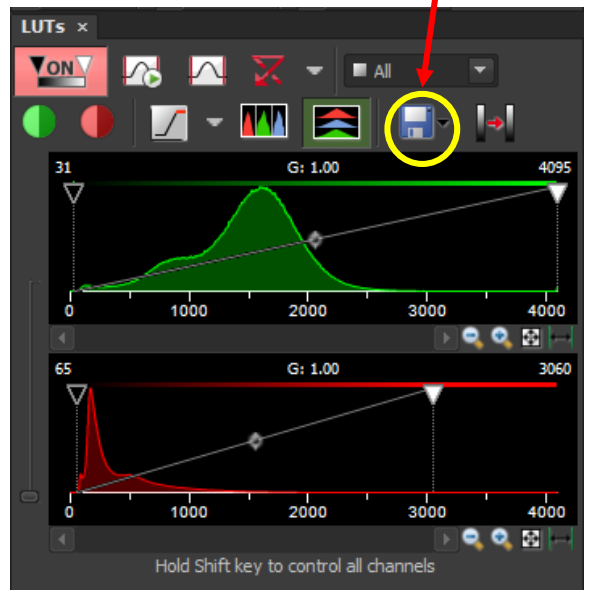


11. Look Up Table (**LUTs**): After an image is acquired, the LUT of each channel is displayed in the **LUTs**. To decrease background, drag the longitudinal black bar from left to right. *Keep in mind that the black bar should not pass the peak of the histogram. You may lose data if passing the peak*. To increase the brightness, drag the white bar from right to left. **The LUT also applies to adjusting confocal images**.





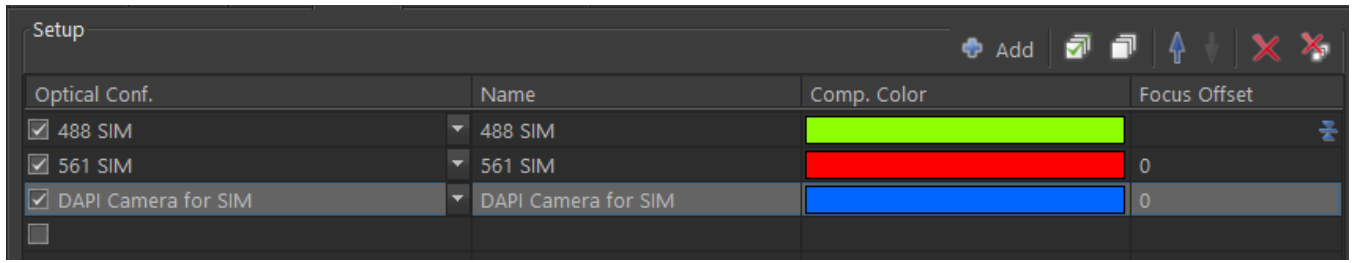
You can save the LUT data of one image and apply it to other images.

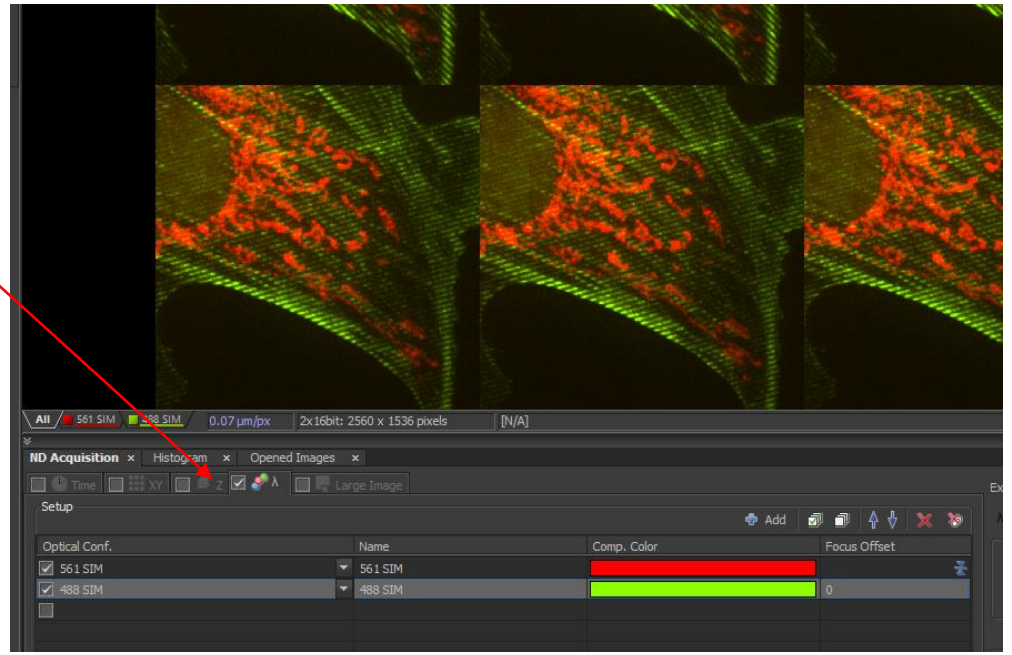


Multichannel (λ) acquisition

1. To take multichannel SIM images, Check the **(λ)** acquisition box under **ND Acquisition**.

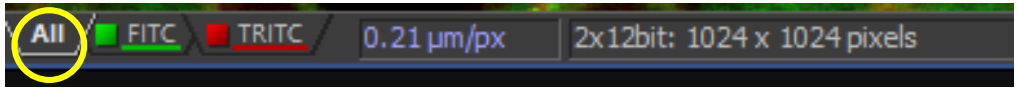
2. Select the channels needed for the image.





3. Click the **Start Run** button from the bottom right of the **ND Acquisition** box.

4. An alternative way to capture multichannel images is to capture and reconstruct single channel images first, as described on page 32-36. Then click on the **All** button at the bottom of one image and drag it into another image to create a merged image.

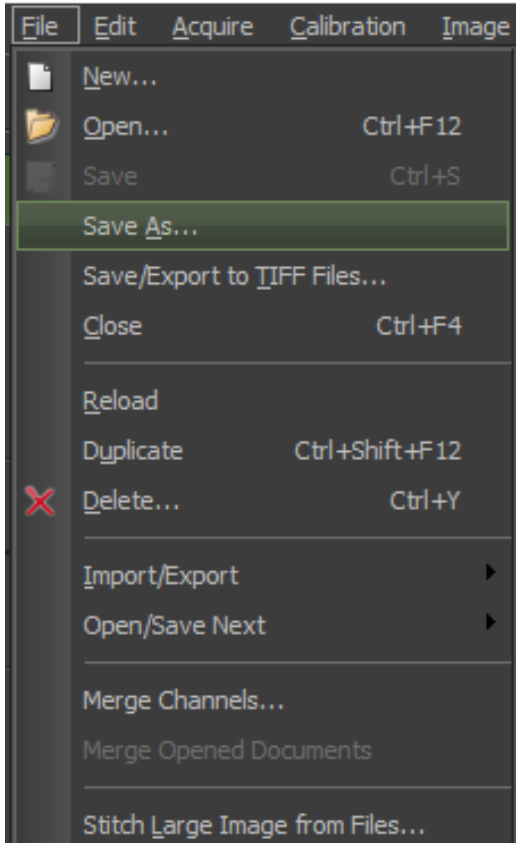


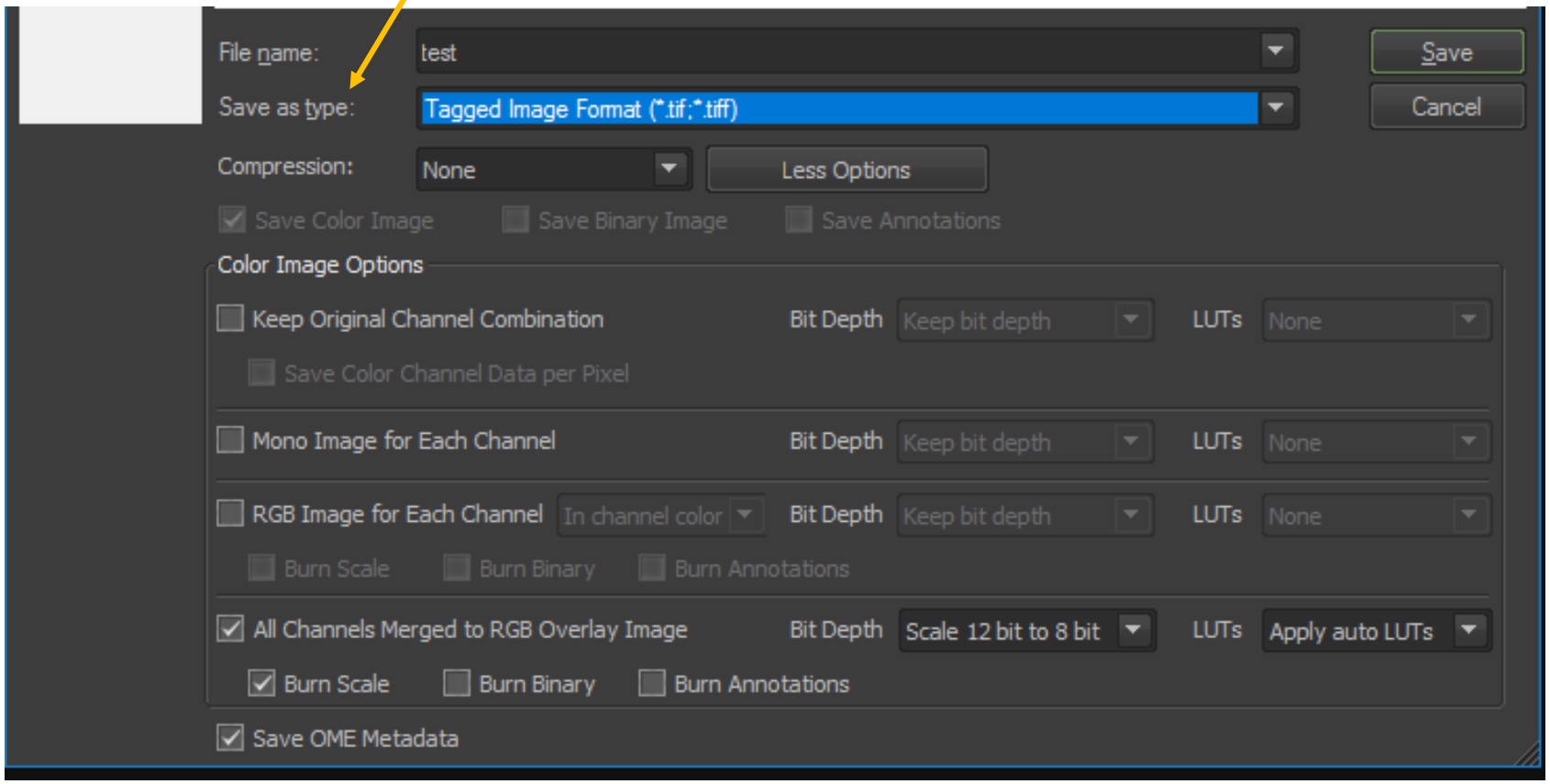
6. Save and Shut Down the System

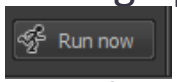
1. To save an image, Go to **File → Save/Save as**.

2. The profile format for the NIS-Elements software is .nd2. **Make sure you always save your images in the .nd2 format before exporting them into TIFF format**.

3. You can select the format of an image through the **Save as type** option.





4. There are saving options in the **ND Acquisition** section that allows you to autosave the images after the **Run now** 

button is being clicked. If you do not want to autosave images captured from ND Acquisition and prefer to manually save them from the "File→Save" option, simply uncheck the **Save to File** option in ND Acquisition.

5. For SIM images, the ND Acquisition **Save to File** option automatically saves the raw images. The reconstructed images have to be saved separately from the "File->Save" option.

6. Close the software through “File->Exit”.

7. Check the calendar (shortcut on the desktop) to see whether any one else will use it after you. If there are users using with in 2 hours, log off the computer and leave the system on. Other wise log off the computer and shut down the system.

8. **To shut down the system, follow the steps 4->3->2**. Always leave #1 on.

9. Clean the oil objectives you used, microscope stage, and the table. Lower the objectives and switch it back to 10x.

10. Sign the ending time on the login sheet.

11. Take off the timed scan notice from the door if you used it.

Notes

1. For fixed sample imaging users, if you noticed on the calendar that the person before you performed live cell imaging, double check that the 100X objective temperature is tuned back to room temperature setting when you use it.

2. When signing up on the calendar, you can only sign up **maximum one week prior to actual use time**.

3. For day-time users, try your best not to use more than 6 hours during work hours per day. For longer time use, do it overnight, or on weekends.

7. Live Cell Imaging

Rules for live cell imaging

1. For live cell imaging, when booking the calendar, **reserve at least 1h extra time for the objective to cool down**. For example: If you need to use the microscope from 8am-3pm, reserve the microscope from 8am to 4:00pm, and stop the experiment at 3:00pm. This will allow the objective to cool down to avoid focus drift for the next user.

2. For live cell imaging, when booking the calendar, **specify "Live Cell".**

3**. For live cell imaging, it is best practice to turn on the temperature controller 30min-45min before you start your scan.** This will avoid focus drift during live cell imaging, especially for objectives that do not have correction collars.

4. For fixed sample imaging users, if you doubt that the objective/microscope has not been cooled down and it may affect your imaging, please inform the core staff immediately.

5. If cell media spills out, **wipe clean the stage and microscope, then clean with ethanol**.

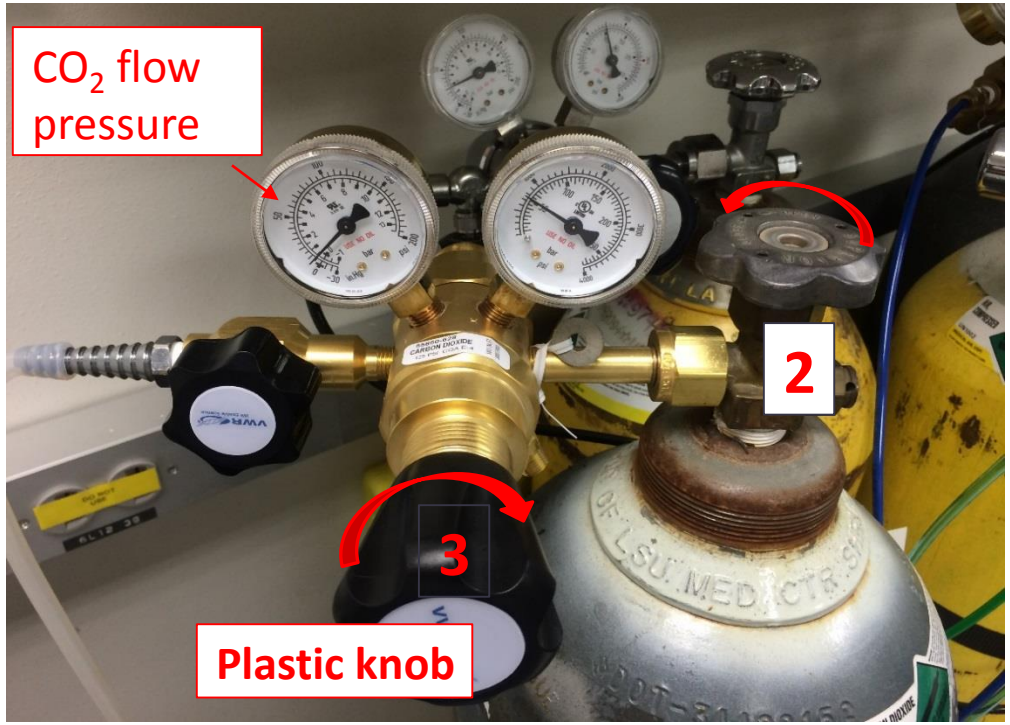
6. If virus infection is involved in live cell imaging, please inform the core staff to evaluate whether it can be brought in to the microscope room or not. Biosafety rules should be followed in the microscope room.

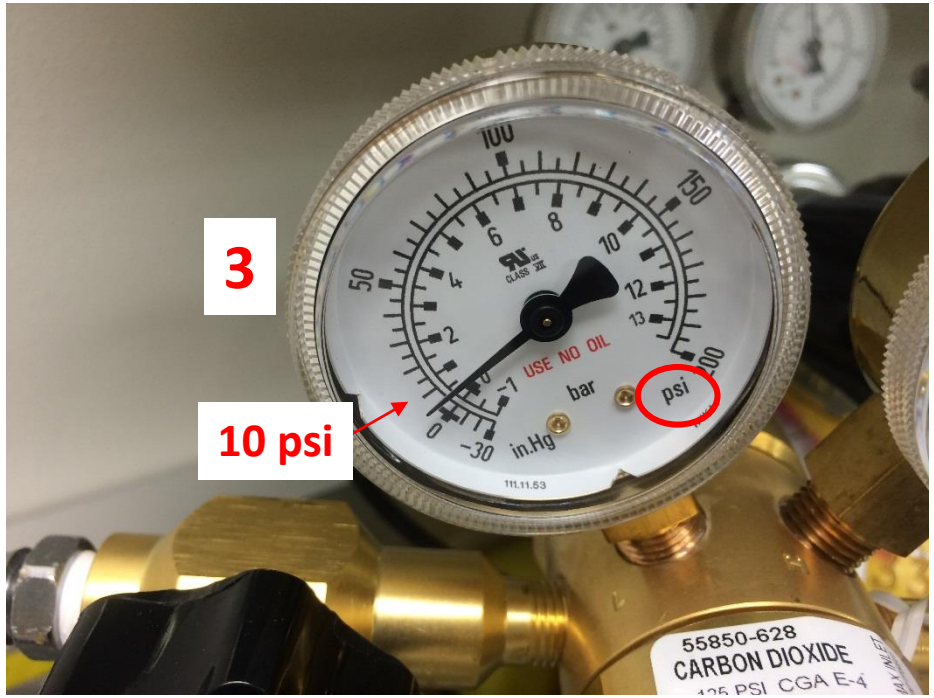
Turn on the live cell imaging system

1. Press the yellow button to turn on the live cell incubator motor. **Do not change any other settings on the motor**.

2. Turn the metal wheel counter clockwise to open the CO_2 tank (Open and Close directions are marked on the wheel).

3. Turn the plastic knob clockwise to increase the CO_2 flow pressure to 10-13 psi. Make sure The CO_2 tank pressure is **below 15** psi. It will take 15 min for the chamber to heat up and for the CO_2 to increase to 5%. When CO2 is filling up the incubator, the CO_2 flow pressure may drop, readjust it to around 10-13 psi. **Attention: Pressure higher than 15 psi is over the limit of CO_2 tubing and will cause damage.**





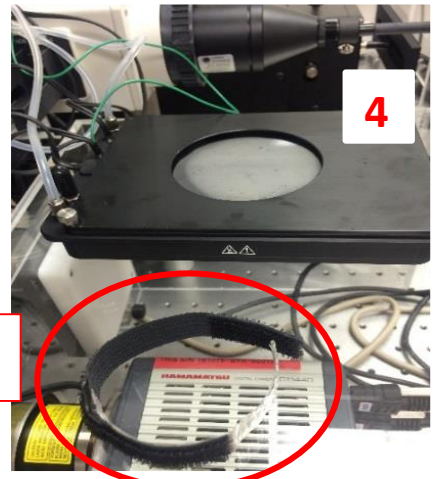
4. Use the live cell dish holder to place samples. There are two choices of dish or plate holders. One for 6 mm dishes, one for slide chambers. <u>The dish or slide chamber that you use has to be glass bottom</u>.

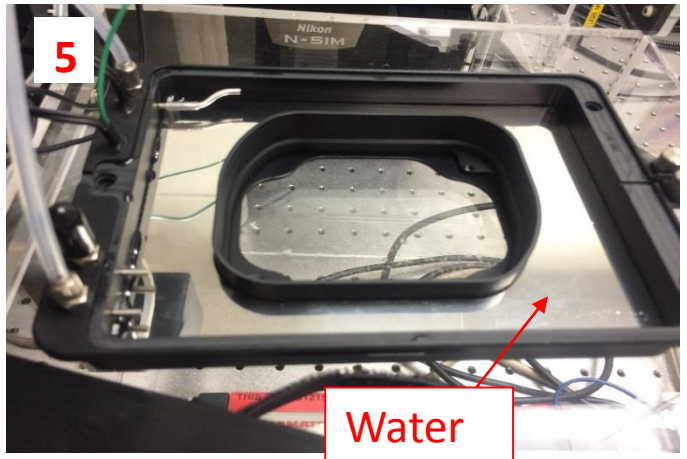
5. Fill distilled water into the surrounding chamber in the sample holder. Be careful not to spill water on to the objectives.

6. Rap the heating band on the objective that you want to use to heat up the objective.

7. For 100X objective, you need to tune the objective for 37 degree environment. Turn the 100X objective to line up the lower vertical red lines with the upper red lines that is labeled with 37ºC (Line up at your coverslip position).

8. Oil is needed when using oil objectives.





Shut downthe live cell imaging system

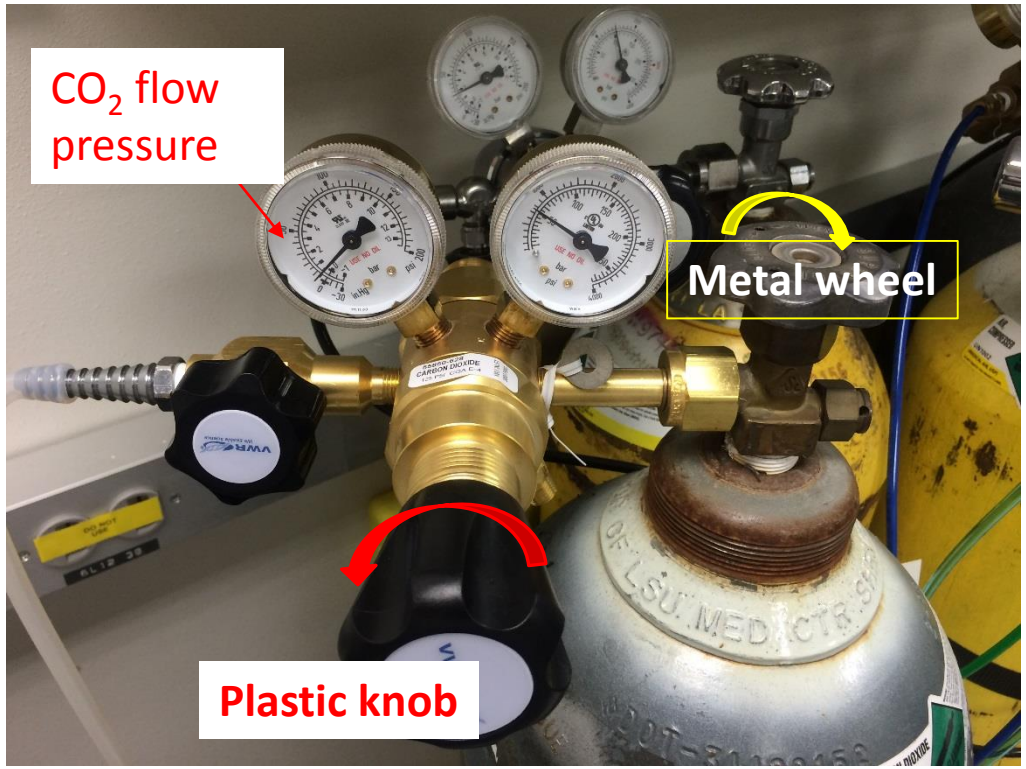
1. After live cell imaging, turn the plastic knob counter clockwise to decrease CO_2 flow pressure (The CO_2 pressure will not drop immediately). **Then, make sure to close the CO_2 tank by turning the metal wheel clockwise**.

   A $50 penalty will be charged if the CO_2 tank is not closed after live cell imaging.

2. If you used 100x for live cell imaging, remember to tune the objective to room temperature settings when you are done.

3. Aspirate distilled water from the live cell stage holder and dry it with paper tower. Put back the regular dish/slide holder.

4. To sign out the computer and shut down the microscope, follow the steps for regular shut down.



Simultaneous and sequential confocal scan

	Simultaneous scan	Sequential scan
Advantage	Fast Good for single channel image acquisition	Prevent multichannel bleach through (cross talk)
Disadvantage	Will create multichannel bleach through or cross talk, especially between DAPI and 488/FITC lasers	Slower
Use	Good for single channel image capture	Good for multichannel image capture. Especially important when analyzing co-localization
Image Compare