# THOR

## Nikon Inverted Structured Illumination (N-SIM) & A1 Laser Scanning Confocal Microscope

User Guide

v. 1.0 (12/2015)

**Objectives**

10x/0.45NA (WD 4 mm) Plan Apo λ
20x/0.75NA (WD 1 mm) Plan Apo λ
40x/0.95NA (WD 0.25-0.16 mm) Plan Apo λ with correction collar
40x/0.6NA (WD 3.6-2.8 mm) ELWD Super Plan Fluor with correction collar

60x/1.27 (WD 0.17 mm) SR Plan Apo **water immersion** with correction collar
100x/1.49 (WD 0.12 mm) SR Plan Apo TIRF **oil immersion** with correction collar

Standard coverslip thickness 0.17 (#1.5 coverslip)

DIC sliders for 20x, 40x, 60x, 100x

Figure 1

**Lumencor Sola light engine (oculars)**

Brightfield, Dapi, Fitc, Texas Red

**Nikon LU-NV Solid State Lasers**

**405, 445, 488, 515, 561, 647 nm**

**Detectors**

SIM - Andor iXon3 (DU-897) EM-CCD camera - 16 μm x 16 μm pixel (with 2.5x relay lens)
A1 – 2x PMT, 2x GaAsP (gallium arsenide phosphide) detectors

**→ Please use the following statement in the acknowledgements of any paper utilizing the SIM system:**

"SIM in MCDB was made possible by equipment supplements to R01 GM79097 (D. Xue, PI) and P01 GM105537 (M. Winey, PI)."

**Contents:**

**Quick Guide:**

1) Turn on components in order. Only turn on lasers you will be using.
2) Start NIS Elements Acquisition software, choosing appropriate shortcut (confocal or SIM) for your experiment.
3) Put sample on scope coverslip down. If using oil immersion, do not switch back to air objective. No oil on air objectives!
4) Find and focus your sample through the oculars.
5) Acquire images, timelapses, Z-series, etc.
6) Lower stage away from sample, and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!
7) Leave the microscope on 10x objective for next user.
8) Save and copy your data. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB Server.
9) Close NIS Elements.
10) Log use in Excel sheet, save and close.

**CHECK THE MCDB CALENDAR:**

➔ **If someone is signed up within the next two hours**

11) Leave it all as is.

➔ **If no one is signed up within the next two hours or you are the last of the day:**

11) Shut down system: Start → Shut down PC, turn off A1 confocal if it is on, turn the key on the laser launch to off position.

12) Close all the plexiglass doors around the microscope and close the doors to the room when you finish.

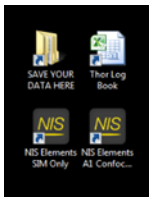
## Starting Up the System:

1) Turn on PC and let it boot into Windows. NOTE – IT IS VERY IMPORTANT THAT THE PC BE FULLY BOOTED UP BEFORE POWERING ON THE A1 CONFOCAL CONTROLLER!

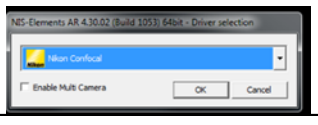
2) Turn on the A1 confocal controller (side button) if you will be acquiring confocal images. For SIM only work, skip this step.

3) Turn the key on laser launch to the "On" position. Colored lights indicate that the lasers are on and at temperature. Blinking colored lights means the lasers are still warming up. Press the colored buttons to turn off lasers you will not be using to preserve laser life span.

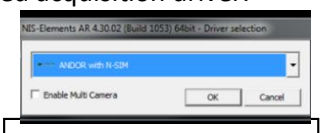
4) Open NIS Elements Acquisition software, selecting either the SIM or A1 Confocal Only option in the middle of the desktop.




5) Click OK to launch with the selected acquisition driver.




Go to Page 22 for Confocal




Go to Page 5 for SIM


http://www.nikon.com/products/instruments/lineup/bioscience/s-resolution/nsim/index.htm

N-SIM can yield up to 2 fold improvement in resolution, 85-120 nm in X,Y and 300-400 nm in Z via the virtual extension of the microscope's Fourier (frequency) space. The striped excitation pattern projected onto your sample adds new, high frequency (thus high resolution) components to the original lower resolution components. The reconstruction software restores these high frequency components to their true position.

| Modality | Lateral Resolution | Axial Resolution |
|---|---|---|
| Conventional | 220 nm | 500-700 nm |
| SIM | 110 nm | 300 nm |

## N-SIM Structured Illumination Super-Resolution Microscopy:

**To optimize resolution improvement:**

- BRIGHT sample with low background, high labelling density, high concentration
- If sample is live, the dynamics must be SLOW
- Clean slide with #1.5 coverslip, recommend using high tolerance #1.5 for greater precision (e.g., made by Schott and Marienfield)
- At most two coverslips per slide, do not mount coverslips too close to the edge of the slide (preferably one coverslip per slide mounted in middle)
- Use the correct mounting media – you want to match the refractive index of the coverslip glass and immersion oil. Most will work, Nikon recommends using Prolong and avoiding Vectashield. Avoid mounting media with DAPI mixed in since these can result in higher background fluorescence. Allow mounting media to fully harden/cure for optimal refractive index. For non-hardening mounting media, completely seal the coverslip.
- Sample mounted as close to coverslip as possible (see below for maximum sample thickness guidelines)

- High density of fluorophore/information in FOV – more is better for SIM
- Adjust correction collar (more on this later)
- Keep acquisition intensity values at or below 4000-6000 counts (use the LUT) to avoid saturation in the reconstructed image
- Less camera gain is better
- Minimize photobleaching over the course of the acquisition (this will reduce efficiency of the reconstruction)

- Do not rely on the auto reconstruction parameters
- Consider applying chromatic shift correction after reconstruction

100x/1.49 oil immersion – keep front lens on 1x

3D SIM – <7 μm sample thickness at 1 sec/frame
2D SIM – <3 μm sample thickness at 0.6 sec/frame
TIRF SIM – <200 nm at 0.6 sec/frame

60x/1.27 water immersion – switch front lens to 1.5x and change in software

➔ It is more challenging to achieve optimal SIM resolution improvement with this objective; suggest starting with the 100x/1.49 oil immersion objective.

3D SIM – <20 μm sample thickness at 1 sec/frame
2D SIM – <6 μm sample thickness at 0.6 sec/frame
TIRF SIM – at 0.6 sec/frame

**SIM Modes:**

Switching between SIM modes or objectives requires aligning the laser, switching the SIM cubes and adjusting the grating focus. If you need a mode other than 2D or 3D SIM with 100x, you must let Jolien know ahead of time to allow for cube alignment.

| | |
|---|---|
| 3D Ex V-R 100x/1.49 | |
| 3D Ex V-G 100x/1.49 | 3D Ex V-R 60x/1.27 |
| TIRF 488 100x/1.49 | TIRF 561 100x/1.49 |

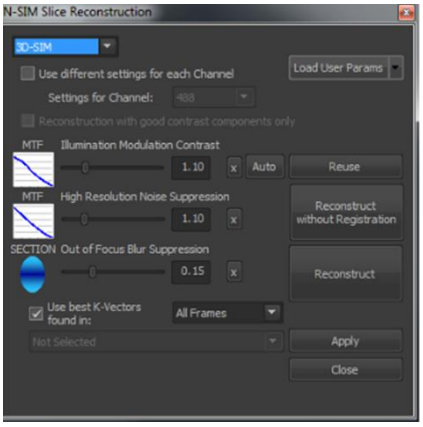
➔ SIM is incompatible with DIC, do not use DIC sliders in conjunction with SIM imaging.

Nikon N-SIM dichroic cubes

N-SIM 405, N-SIM 445, N-SIM 514, N-SIM 640

N-SIM 488/561 Dual pass dichroic with emission filter options: 605/70, 525/50 or 523/610-25 Dual pass emission (this will result in some bleedthrough, so should only be used for high speed acquisition)

**SIM Reconstruction Parameters:**




Suggested starting points:

IMC balance for artifacts, HRNS balance for high frequency data and artifacts

TIRF SIM – IMC @ 1.0, HRNS @ 1.0, OFBS n/a

2D SIM – IMC @ 1.0, HRNS @ 1.0, OFBS n/a

3D SIM – IMC @ 0.5-1.0, HRNS @0.50-1.0, OFBS @0.1-0.2

"Deconvolve when illumination contrast is low" check box – compares high frequency diffraction components against image noise and (when checked) will perform a Weiner deconvolution instead of full SIM processing on Z planes where contrast is low

IMC - Illumination Modulation Contrast: Balances the contributions of the original and high frequency, moiré diffraction components

- Auto setting automatically adjusts weight of frequency components.
- Setting of 1 results in no adjustment to the weight of frequency components.
- For 2D-SIM, larger values decrease weight of the xy high spatial frequency components.
- For 3D-SIM, larger values will decrease the weight of xy high frequency components and increase the weight of z high frequency components.

HRNS - High Resolution Noise Suppression: De-emphasizes or emphasizes the contrast of high spatial frequencies in a non-linear fashion. Can be used to suppress image noise.

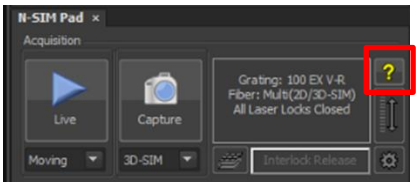
- Setting of 1 is linear suppression
- Settings <1 will increase the contrast of highest spatial frequencies
- Settings >1 will lower the contrast of highest spatial frequencies

OFBS - Out of Focus Blur Suppression: This option is active for data collected in 3D-SIM mode. The filter adjusts the strength of out of focus light removal to provide an optical sectioning effect.

- Larger values remove more out of focus light, yielding a thinner optical section.
- Values > 0.2 should be avoided to prevent possible removal of high frequency information and loss of resolution.

Use Best Reconstruction Parameter Found In: The software identifies the best reconstruction calculation from all planes in a data set and applies it to the entire z stack or time lapse.

➔ **Note**: The built-in SIM help is very well written and provides a wealth of information regarding SIM acquisition and reconstruction. Please consider looking through this.




## Sample Preparation Guide

Nikon's N-SIM system can provide a 2x increase in the resolving power of a diffraction limited fluorescence microscope. It can provide up to 85 - 120nm lateral resolution and 300 – 400nm axial resolution.

It does not require z stacks, and can get rid of out of focus light with one single image when doing 3D SIM. The fastest acquisition times are 0.6 sec for 2D/TIRF SIM and 1.0 sec for 3D SIM.

It has 6 lasers and emission channels: 405nm, 445nm, 488nm, 515nm, 561nm and 647nm and will work with all fluorophores that can be excited with these lasers as long as they are bright enough.

## Cover Glass

For the optimal results samples must be prepared correctly. All samples must be mounted on #1.5 coverslips (170um thickness). Maximum sample thickness recommendations: 60x 3D SIM < 20μm, 100x 3D SIM < 7μm, 100x 2D SIM < 3μm.

## Slides & Dishes

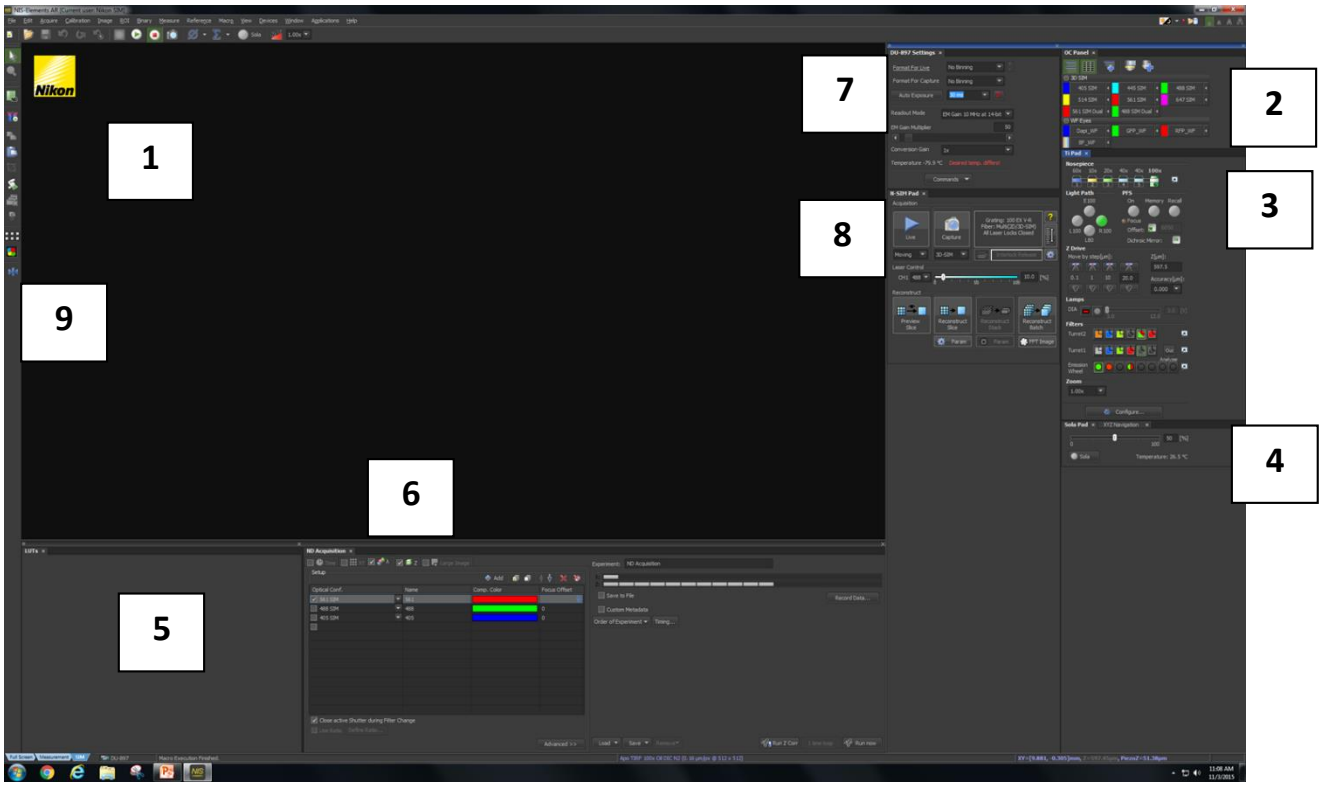
We recommend that samples are mounted on regular sized slides for fixed tissue and 35mm dishes with cover slip bottoms or Nunc LabTek II Chambered Cover glass with #1.5 cover glass.

## Mounting Media

For the 100x/1.49 NA oil lens we recommend that fixed slides are mounted in Prolong Gold[1], TDE[2] (2,2'-Thiodiethanol) or glycerol (not Vectashield!). Prolong gold takes 48 hours – 1 week to fully cure and increase to the correct refractive index so best results will be obtained after 48 hours. TDE can be mixed with water to match oil exactly.
For the 60x/1.27 NA water lens we recommend water or agarose.

[1] http://tools.lifetechnologies.com/content/sfs/manuals/mp36930.pdf
[2] Staudt T, Lang MC, Medda R, Engelhardt J, Hell SW. 2,2'-Thiodiethanol: A New Water Soluble Mouting Medium for High Resolution Optical Microscopy. *Microsc. Res. Tech.* (2007) 70:1-9 PMID: 17131355

## N-SIM – The NIS Elements User Interface:




1) Open/live images will display here.

2) OC Panel – Preset configurations for SIM imaging using the Andor EM-CCD camera. Use WF Eyes OCs for illumination to the eyepieces.

3) Ti Pad – Use these options to select objective and brightfield lamp settings. Other options should not be changed (e.g., filter turret position or light path position) as these are part of the OC configurations.

4) SOLA Pad and X,Y,Z Navigation Pad – Open/close the EPI illumination shutter and adjust the intensity of the EPI illumination light. The X,Y,Z Navigation Pad can be used to adjust position (if necessary).

5) LUTs – The lookup tables for open images will display here. You can adjust how the image is displayed and toggle autoscaling options. Use the display here to keep an eye on over- and under-saturation.

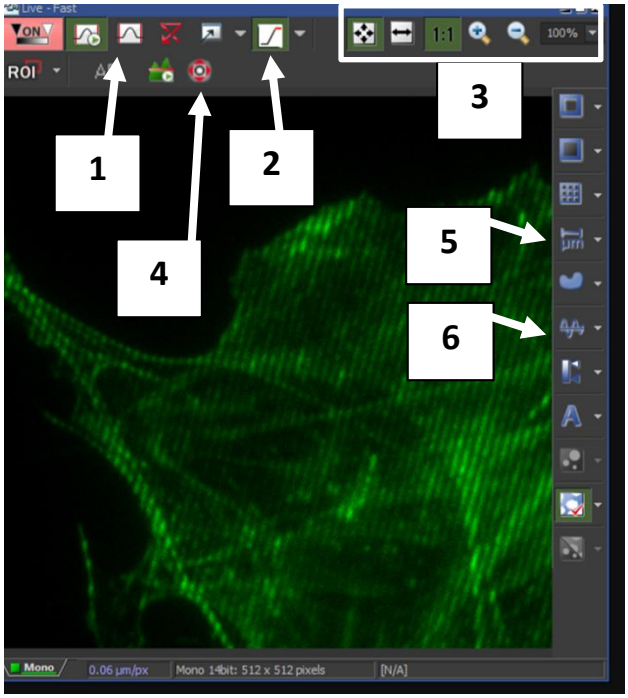
6) ND Acquisition – Options for acquiring multidimensional acquisitions including time lapses, Z-stacks, X,Y montages, multiple X,Y positions, and/or multiple colors (lambdas).

7) Andor DU-897 EM-CCD Camera Settings – Use these options to control the exposure time and gain settings.

8) N-SIM Pad – This panel contains the controls for adjusting and acquiring SIM super-resolution images.

9) Blue dual arrow button – restores default layout and OCs. If you accidentally change something and can't find it, press this to restore the software to defaults.

**N-SIM – The NIS Elements image window (live):**




1) Autoscaling: Press the right autoscale button to scale the image once and press the left autoscale with play button to turn on or off the continuous autoscaling of the image.

2) Pixel saturation indicator: Press this button to display over- and under-saturated pixels in color on your image window. By default, this should be on.

3) Zoom and image window size buttons.

4) Mouse X,Y button: Click here and then click and drag in your image window to move the live image instead of using the joystick.
   ➔ To move a specific spot to the center of the field of view: right-click inside the image window on the spot to move to the center and select "Move this Point to Center."

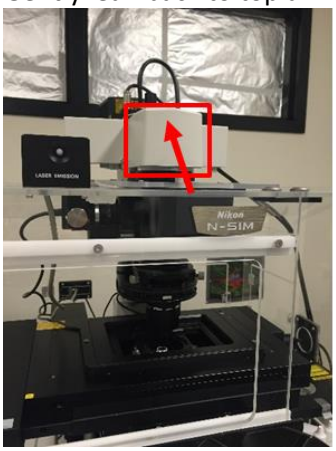
5) Scale bar button and options.

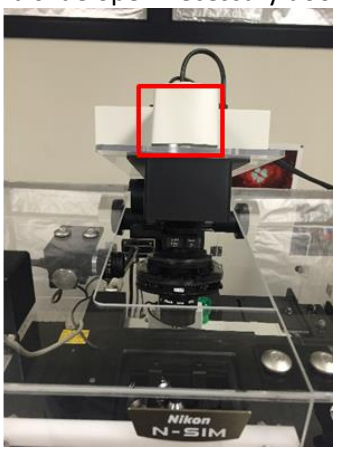
6) Line profile button and options.

➔ Press X to create a non-quantitative snapshot of the image as displayed on the screen

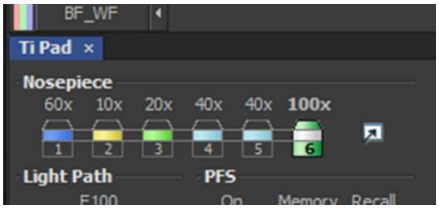
**N-SIM – How to look at sample through oculars:**

1) Gently lean back to top arm and slide open necessary doors for greater access to the stage area.



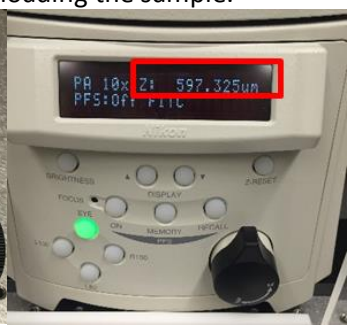




2) Load the correct objective in position.
   - Objectives can be changed by selecting the desired lens in the software.
   - If you are using an air objective, start with 10x at low focus position.
   - If you are using the 100x oil immersion objective lens, place a drop of oil on the objective (don't touch the oil dropper to the lens!). Once you go oil, DO NOT SWITCH BACK!

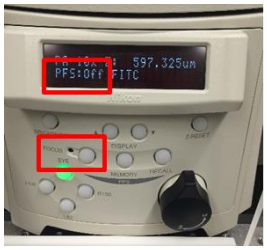


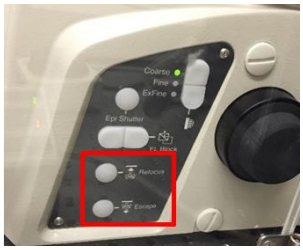

3) Adjust stage position with the joystick if needed. To change speed of joystick, gently rotate the top portion clockwise to reduce speed and counterclockwise to increase. The current speed is denoted by the orange light on the joystick (Coarse – Fine – ExFine). Adjust the Z focus with the knob on the right side of the joystick controller. The current speed of Z focus change is denoted by the green light in Z Speed (Coarse – Fine – ExFine). Make sure the focus position is lowered to below 800 on the front display of the microscope body to avoid damaging the objective when loading the sample.

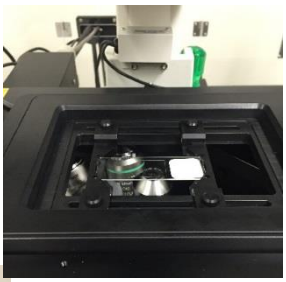




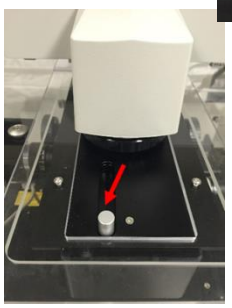

   - <u>If the focus knob is not responding:</u> make sure that it is set at the correct speed (Coarse – Fine – ExFine), that Perfect Focus is not engaged (no green light on the front of the microcope), and that the previous user has not hit the Escape button on the side of the microscope.



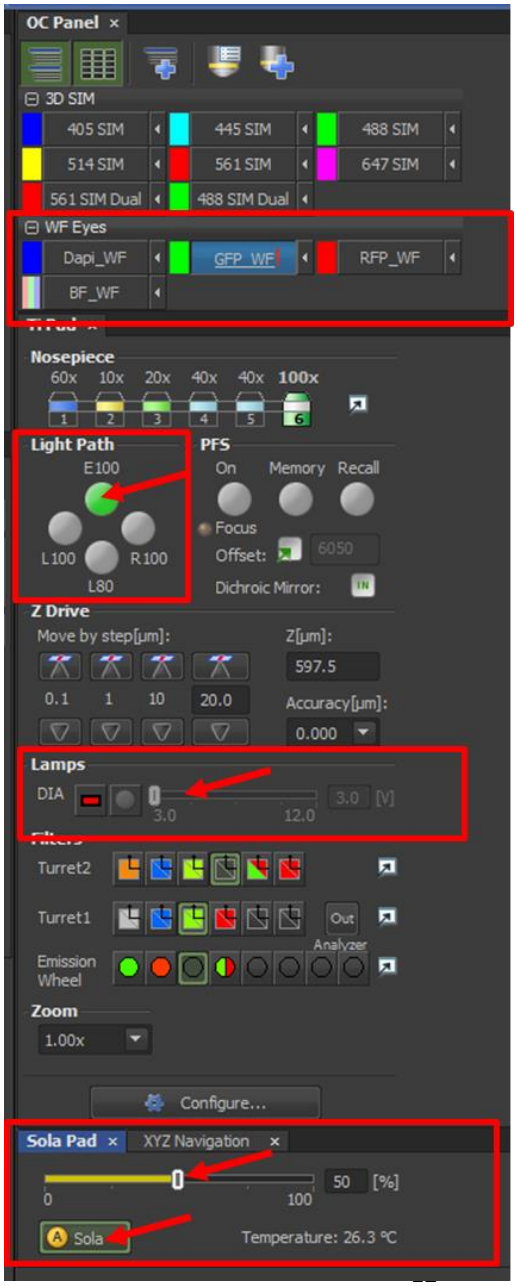





4) Place the slide, coverslip down, onto the slide holders on the stage. Please be sure that your slide is clean and dry – do not get wet mounting media, sealant, or condensation on the stage. If you are using the 100x oil immersion objective, turn the focus knob up until the objective lens is just touching the oil. **Do not switch back to an air objective**.



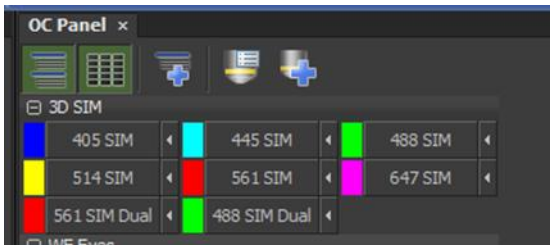
5) After loading the slide, gently lower the top arm back down and close all the doors around the microscope. Make sure the small silver knob at the top of the condenser arm is in the forward position (if not, it will block the transmitted light path).




6) Select appropriate widefield illumination optical configuration (OC) – DAPI_WF, GFP_WF, RFP_WF, or BF_WF. Widefield optical configurations will default by sending fluorescence light to the oculars (E100).
   - To open or close the fluorescence shutter, click on the Sola button at the bottom of the right toolbar. Adjust the intensity of the Sola light with the slider.
   - The Brightfield OC will default with the DIA lamp on. To adjust brightfield lamp intensity, use the DIA slider in the middle of the right toolbar. See Appendix B for instructions on Kohler alignment of the brightfield.
   - Remember: DIC is incompatible with SIM, so do not use the DIC sliders with SIM imaging.

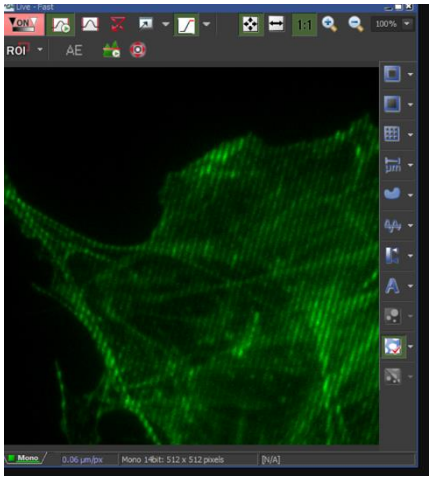
7) Adjust the focus using the focus knob on the joystick or the knobs on the sides of the microscope. To adjust focus speed, press the button on the right side of the joystick to switch from Coarse – Fine – ExFine. Adjust to get the field of view you want.
   - If you are doing SIM, you will need to use the 100x oil immersion lens – once the slide has oil on it **DO NOT SWITCH TO ANY OTHER OBJECTIVE!**
   - Optimal focus for most slides is generally ~1600-1900 on the front display (except when using the ELWD 40x air objective).

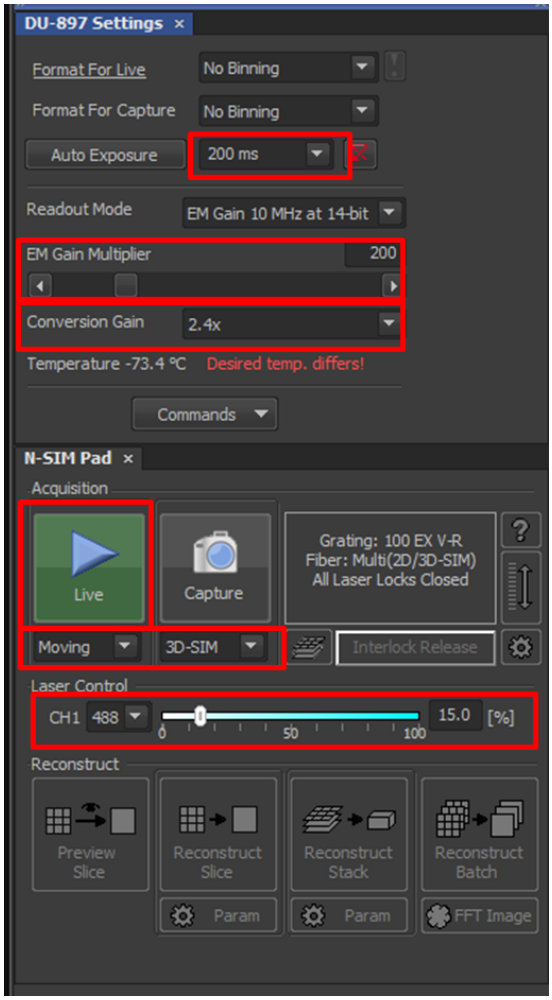
## N-SIM – Adjusting the Optical Configurations (OCs):



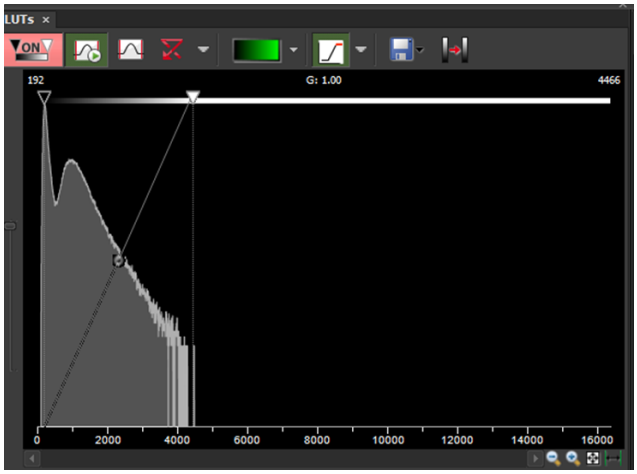

1) Once the sample is centered in generally in focus, select the appropriate 3D SIM OC shortcut from the top right. You can't look at SIM OCs through the eyepieces.

2) Turn on live mode (Moving 3D SIM) on the N-SIM Pad. You should see a live image with moving lines (as below). Adjust autoscaling options as needed to see the image. The better you can see these lines, the better your SIM reconstruction will be. See page 15 for the adjustment of the correction collar.

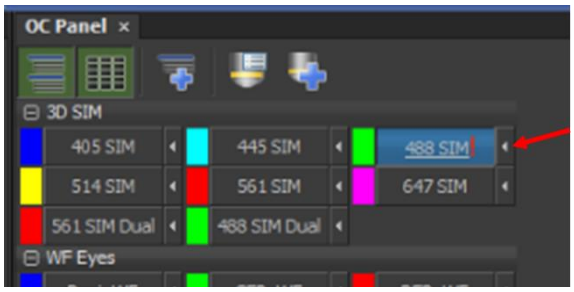





3) Adjust camera settings to get maximum intensity counts ~4000-6000 (use LUT). Adjust camera exposure time, EM Gain, Conversion Gain, and Laser power. SIM generally does better with shorter exposure times and as low as gain setting as possible. Balance exposure time and gain with laser power to avoid photobleaching over the course of acquiring the 15 frames needed for the SIM reconstruction. Do not use binning at all and don't change the readout mode (it should be set to the fastest EM Gain mode).



4) Once you are happy with the settings, click the white arrow next to the OC to lock in the settings.




5) Repeat this process for all colors you want to image. Be efficient to minimize time spent in live mode (this photobleaches your sample!). To stop live mode, click the same Live button. The red and green OCs with Dual in the name go through the Dual emission filter – these are the fastest for imaging green and red in live samples but will introduce bleedthrough/spectral overlap in your images. For routine imaging of fixed samples, do not use the Dual OCs.

**N-SIM – Adjusting the Correction Collar:**

- Carefully slide your hand underneath the stage to access the correction collar. Start with the correction collar in the middle position 23 C – 0.17 by aligning the white line with the appropriate position on the upper part.


Approach A:

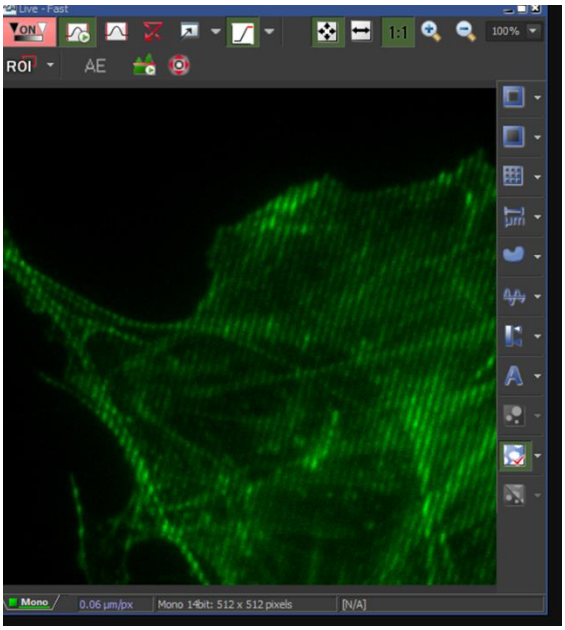
- Looking at a fluorescent signal through the eyepieces, place your right hand on the correction collar and the left hand on the focus knob.

- Adjust the correction collar a little while turning the focus up and down until the out of focus blurring looks the same above and below the focal plane. This is most easily accomplished by looking at diffraction limited dots.

Approach B:

- Turn on the live image on the screen using a SIM OC with a short exposure time (helps to have autoscaling on). Place your right hand on the correction collar and the left hand on the focus knob.

- Adjust the correction collar a little while turning the focus up and down until the out of focus blurring looks the same above and below the focal plane. You are looking for the sharpest stripe pattern on your sample at the most in-focus plane.

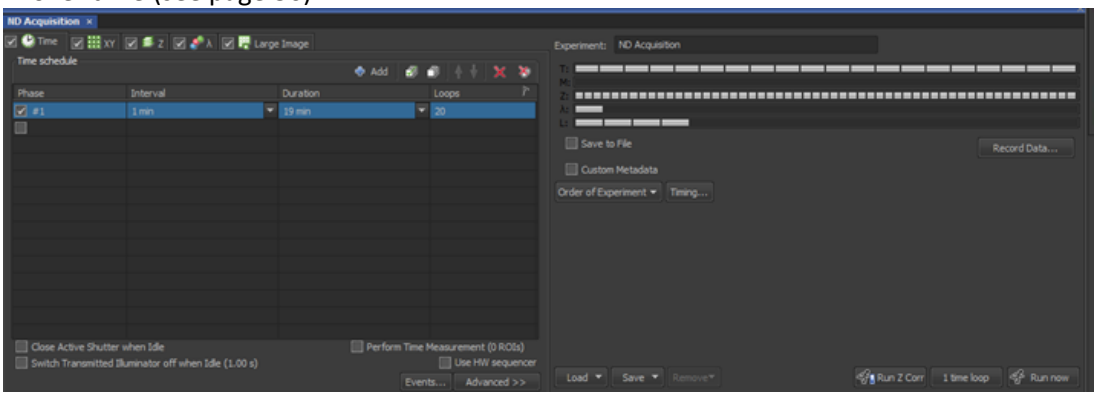


## N-SIM – Setting up and Acquiring an ND Acquisition:

At the bottom of the screen is the ND Acquisition window that is used to capture images in multiple dimensions.

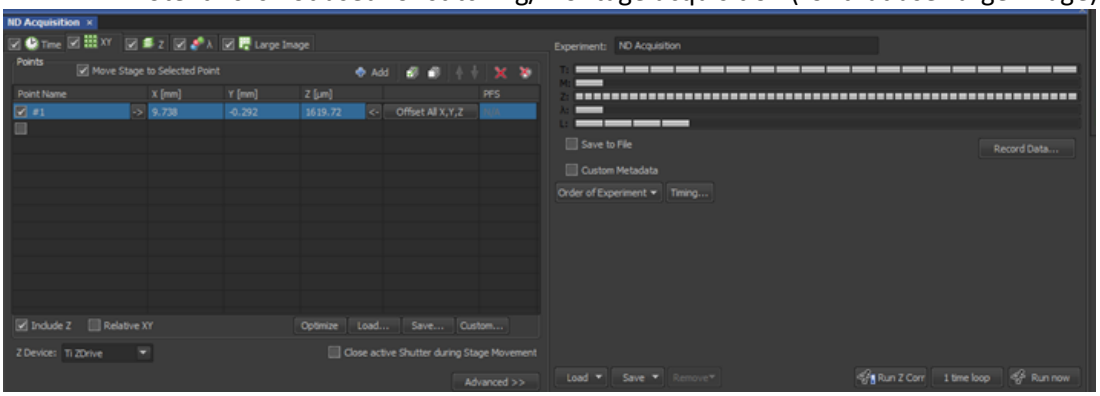
Options (uncheck those you don't want):

1) Time – choose the interval and duration of the desired time course. Multiple phases with different settings may be added to run consecutively. Make sure to check on the "Close Active Shutter when Idle" option. If you're performing a timelapse, use Perfect Focus to maintain focal position over time (see page 36).

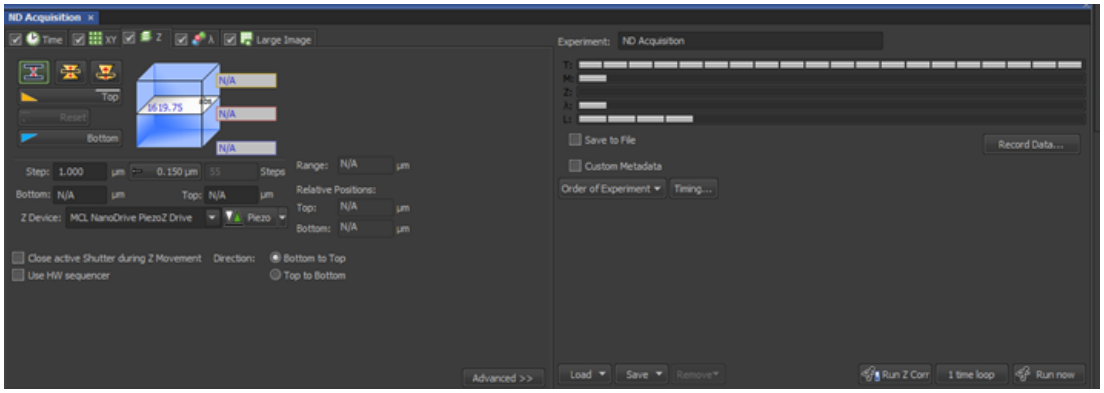



2) XY positions – Once the sample is positioned correctly, click "+Add" button. Check the "Include Z" box to also lock in the Z position. Find a different field of view, and click "+Add" button to add. To delete a position, click the red X button. Continue until all desired positions are locked in. Check on the "Close active shutter during stage movement" to eliminate any spurious excitation light from illuminating the sample while the stage moves. Use Perfect Focus to maintain focal position (see page 36).

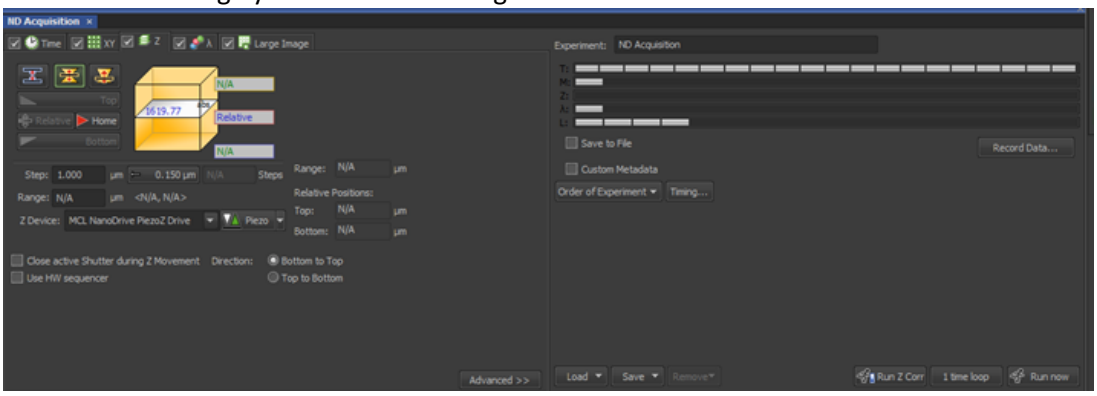
   ➔ Note: this is not used for stitching/montage acquisition (for that use Large Image).



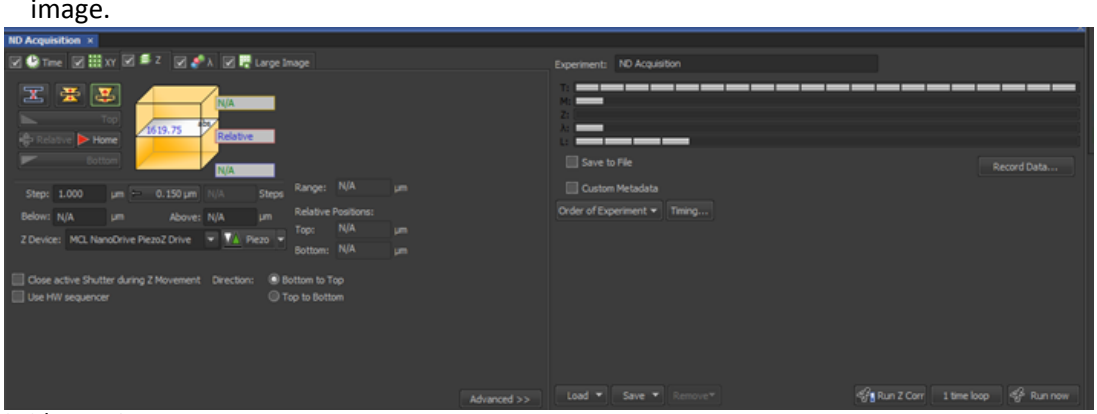
3) Z – Set top and bottom – With the image window live, adjust focus knob to go to one extreme, click Top, go to other extreme, click Bottom. This will set the range to acquire. Then set the step size or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size. For SIM, consider using a step size of 150-200 nm, depending on the total range you will want to image.



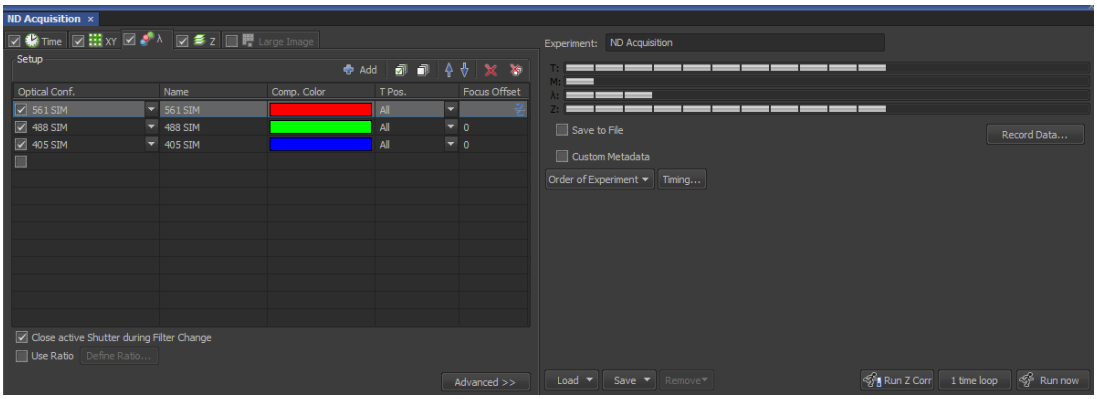

4) Z – Range around center – With the sample focused in the center plane, click the "Home" button to set the center home position. (If you're using Perfect Focus, click Relative – PFS won't work with an absolute Home position.) Then set the step size, total range, and/or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size. For SIM, consider using a step size of 150-200 nm, depending on the total range you will want to image.



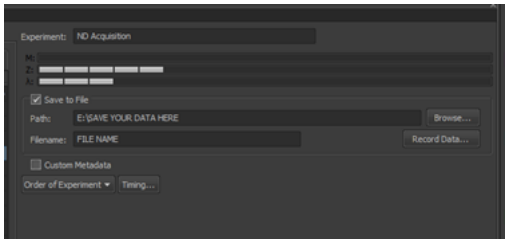

5) Z – Asymmetric range around center – With the sample focused in the center plane, click the "Home" button to set the center home position. Then set the step size, range to acquire above, range to acquire below, and/or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size. For SIM, consider using a step size of 150-200 nm, depending on the total range you will want to image.



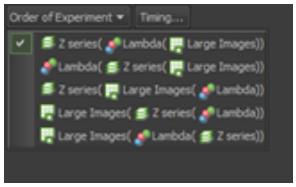
6) Lambda – Select the desired optical configuration (OC) from the drop down. Select if you want to acquire this wavelength at all Z positions or just a subset of the total planes. You should already have dialed in settings for these OCs (see page 13).




7) Large Image – Incompatible with SIM imaging, do not use. See section on N-SIM large image acquisition on page 19.



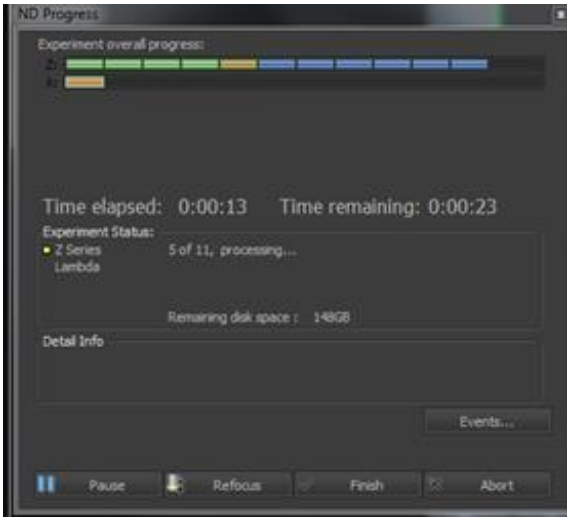

8) Right side – Name your experiment. Check or uncheck save to file. If you check save to file, select the correct file path and file name. File path must go to the E:\SAVE YOUR DATA HERE\ drive (NOT C:\).




9) Order of Experiment – Adjust the order in which the various parameters will be acquired. Read this from right to left, the parameter inside the parenthesis will be acquired first. The Z drive is relatively fast as is switching colors. Multiple X,Y positions (either Large Image montage or XY) will be a bit slower. Put the slower components more to the left to speed up overall acquisition time.

10) When you're ready, click "Run now" at bottom left to start the acquisition.

11) The ND Progress window will show the progress of your imaging experiment. Green squares are done, orange is in progress, and blue has not yet been done. Time remaining can sometimes be off, depending on the order of experiment. Click Abort to stop the acquisition and discard all data. Click Finish to stop the acquisition after the current round is completed. Based on the order of experiment it is possible that the image window will not update until all images are done being acquired.



**N-SIM – Large Image Stitching:**

1) After configuring the desired OCs, in ND Acquisition, under the XY tab, in the bottom right corner, click on “Custom.”

2) Set the number of rows/columns desired. Set Manual XY distance to 0.025mm.

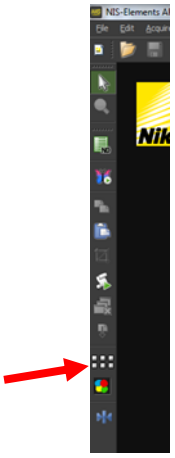
3) Multipoints will be automatically populated, centered around your current position. To recenter around a new position, go to the “middle” point (B2 in the 3x3 example below), and click “Offset all X, Y.”

4) You may want to use either PFS or Focus Surface to maintain sharp focus across the large field of view. Click on the “Advanced button at the bottom of the XY tab, then check the box marked “Use Focus Surface.” Make sure that the “Include Z” box is NOT checked, or the Focus Surface option will be grayed out. To set up Focus Surface, open XYZ overview (from the Right Click menu → Acquisition → XYZ overview). Pick several points from your XY point list, focus, then click the + button in the Focus Surface tab. You need at least three points, but the more points you use, the more accurate it will be.

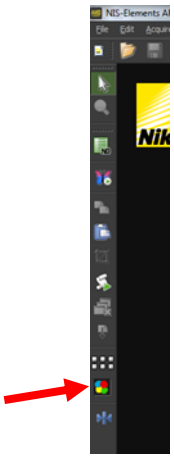
5) Run the ND Acquisition. This will produce a single, multipoint SIM file.

6) Reconstruct SIM file.

7) Click on SIM Stitch macro button to automatically crop and stitch together the multipoint, reconstructed image file.



## N-SIM – Color Shift Alignment:



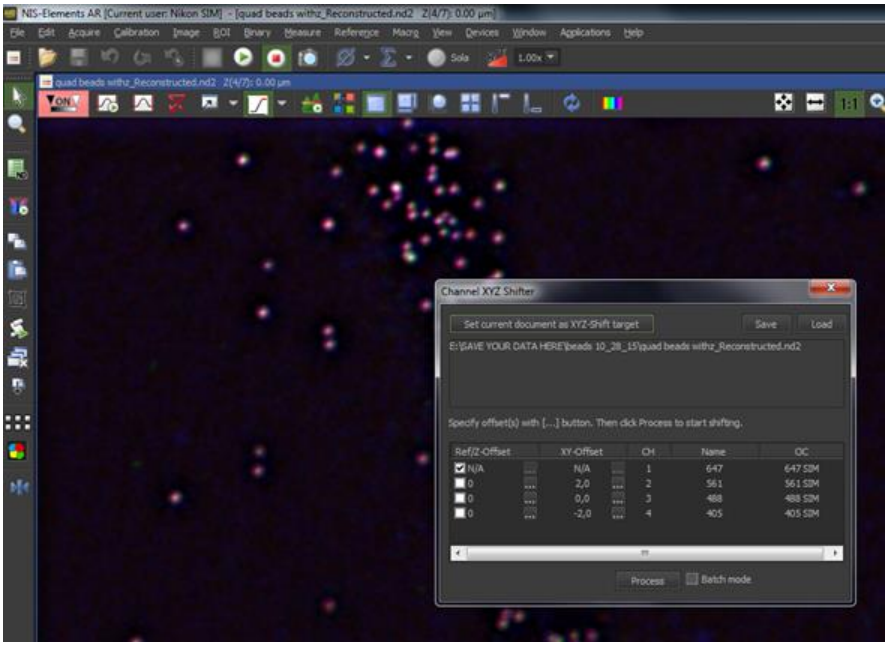
1) After images are acquired and reconstructed, click on four color dot button on left hand menu to open the “Channel XYZ Shifter.”

2) Set current document as target should be your saved, reconstructed SIM image.

3) We have measured the offset using four color 100 nm beads. Specify the offset as shown below. Adjust based on what channels you have available. The ref channel will likely always be 647 or 488.

| | |
|---|---|
| 647 | REF N/A |
| 561 | 2,0 |
| 488 | 0,0 |
| 405 | -2,0 |

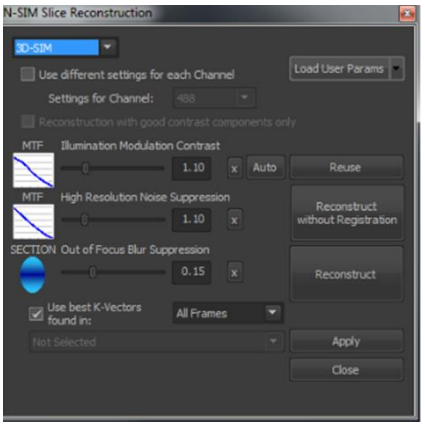
4) Click Process. Save as a separate file.



## N-SIM – Reconstruction:

**This matters as much as the sample prep and image acquisition – DO NOT OVERLOOK!**

**SIM Reconstruction Parameters:**




"Deconvolve when illumination contrast is low" check box – compares high frequency diffraction components against image noise and (when checked) will perform a Weiner deconvolution instead of full SIM processing on Z planes where contrast is low

IMC - Illumination Modulation Contrast: Balances the contributions of the original and high frequency, moiré diffraction components

- Auto setting automatically adjusts weight of frequency components.
- Setting of 1 results in no adjustment to the weight of frequency components.
- For 2D-SIM, larger values decrease weight of the xy high spatial frequency components.
- For 3D-SIM, larger values will decrease the weight of xy high frequency components and increase the weight of z high frequency components.

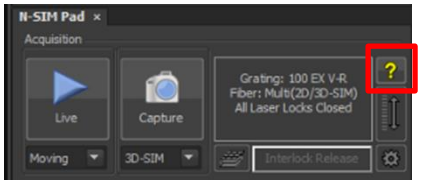
HRNS - High Resolution Noise Suppression: De-emphasizes or emphasizes the contrast of high spatial frequencies in a non-linear fashion. Can be used to suppress image noise.

- Setting of 1 is linear suppression
- Settings <1 will increase the contrast of highest spatial frequencies
- Settings >1 will lower the contrast of highest spatial frequencies

OFBS - Out of Focus Blur Suppression: This option is active for data collected in 3D-SIM mode. The filter adjusts the strength of out of focus light removal to provide an optical sectioning effect.

- Larger values remove more out of focus light, yielding a thinner optical section.
- Values > 0.2 should be avoided to prevent possible removal of high frequency information and loss of resolution.

Use Best Reconstruction Parameter Found In: The software identifies the best reconstruction calculation from all planes in a data set and applies it to the entire z stack or time lapse.




➔ **Note**: The built-in SIM help is very well written and provides a wealth of information regarding SIM acquisition and reconstruction. Please consider looking through this.

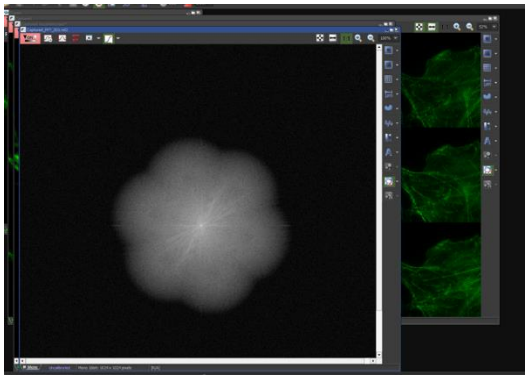
Suggested starting points:

IMC balance for artifacts, HRNS balance for high frequency data and artifacts

TIRF SIM – IMC @ 1.0, HRNS @ 1.0, OFBS n/a

2D SIM – IMC @ 1.0, HRNS @ 1.0, OFBS n/a

3D SIM – IMC @ 0.5-1.0, HRNS @0.50-1.0, OFBS @0.1-0.2



You're looking for maximum resolution improvement as visualized by the FFT (larger "petals") while minimizing artifacts. This is something of an art, and it is highly recommended that you try multiple settings.


http://www.nikon.com/products/instruments/lineup/bioscience/confocal/singlephoton/a1/index.htm

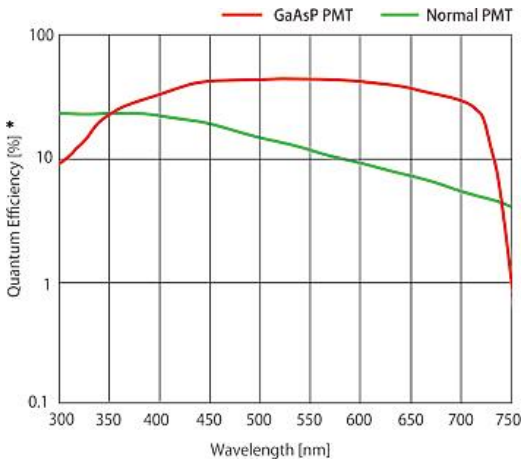
"Nikon confocal microscope system A1$^{+}$ provides high-resolution imaging of up to 4096 x 4096 pixels with a galvano (non-resonant) scanner. With diverse innovative optical and electronic technologies, superior image quality has been achieved.

The A1$^{+}$'s galvano scanner enables high-resolution imaging of up to 4096 x 4096 pixels. In addition, with the newly developed scanner driving and sampling systems, plus image correction technology, high-speed acquisition of 10 fps (512 x 512 pixels) is also possible.

The low-angle incidence method utilized on the dichroic mirrors increases fluorescence efficiency by 30%. By employing the hexagonal pinhole, higher brightness equivalent to that of a circular pinhole is achieved.

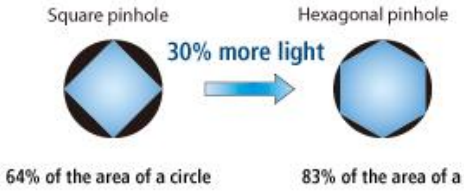
Nikon developed the GaAsP multi-detector unit equipped with two GaAsP PMTs and two normal PMTs.
A GaAsP PMT has much higher sensitivity than a normal PMT, thus acquisition of brighter signals with minimal background noise is possible with a GaAsP PMT, even with weak fluorescence, which, until now, has been difficult to detect."

## A1 Laser Scanning Confocal:




➔ The A1 laser scanning confocal has four detectors – two enhanced PMTs for blue and far red detection and high sensitivity GaAsP detectors for green and red. These are quite sensitive and must be used with caution (do not over-saturate!).

➔ This system has hexagonal pinholes for more optimal light throughput. The setting of 1.2 AU in the Nikon NIS Software is comparable 1 AU for more conventional square pinholes.

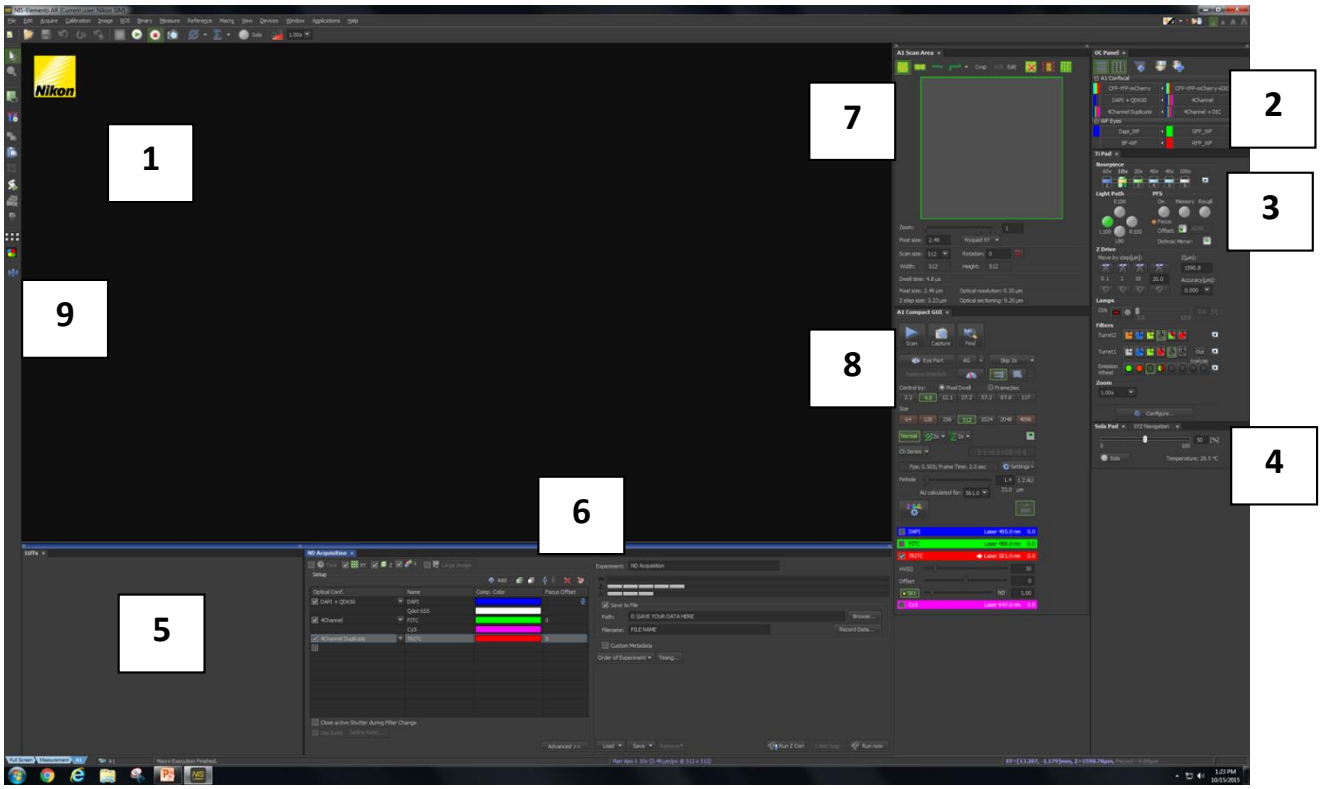



➔ Laser lines and filters in the A1. You will not need to edit these at all and the numbers shown in the software may be off slightly. Go by the table here over the software. The premade Optical Configurations (OCs) have the appropriate filters in place.

| | **Excitation** | **Emission** |
|---|---|---|
| **DAPI** | **405 nm** | 425-475 nm (PMT) |
| **CFP** | **445 nm** | 467.5-502.5 nm (GaAsP) |
| **Green** | **488 nm** | 500-550 nm (GaAsP) |
| **YFP** | **515 nm** | 521.5-554.5 nm (GaAsP) |
| **Red** | **561 nm** | 575-625 nm (GaAsP) |
| **Red (shifted, with CFP/YFP)** | **561 nm** | 627-693 nm (PMT) |
| **Far Red** | **647 nm** | 650-720 nm (PMT) |

| | **1st Dichroic** | **1st Filter Wheel** | **2nd Filter Wheel** | **3rd Filter Wheel** |
|---|---|---|---|---|
| **1** | **405/488**<br>**DAPI/Green** | **450/50 DM480 [405]** | **525/50 DM560 [488]** | **600/50 DM640 685/70**<br>**[561/640]** |
| **2** | **405/488/561**<br>**DAPI/Green/Red** | EMPTY | EMPTY | EMPTY |
| **3** | **405/488/561/640**<br>**DAPI/Green/Red/Far Red** | EMPTY | EMPTY | EMPTY |
| **4** | **400-457/514**<br>**DAPI-CFP/YFP** | EMPTY | **485/35 DM520 [457]** | **538/33 DM585**<br>**660/66**<br>**[514/561]** |
| **5** | DO NOT USE<br>405/488/543/640 | EMPTY | EMPTY | EMPTY |
| **6** | BS 20/80 | EMPTY | EMPTY | EMPTY |
| **7** | EMPTY | | | |
| **8** | **457/514/561**<br>**CFP/YFP/Red** | | | |

## A1 – The NIS Elements User Interface:




1) Open/live images will display here.

2) OC Panel – Preset configurations for confocal imaging using the A1 confocal. Use WF Eyes OCs for illumination to the eyepieces.

3) Ti Pad – Use these options to select objective and brightfield lamp settings. Other options should not be changed (e.g., filter turret position or light path position) as these are part of the OC configurations.

4) SOLA Pad and X,Y,Z Navigation Pad – Open/close the EPI illumination shutter and adjust the intensity of the EPI illumination light. The X,Y,Z Navigation Pad can be used to adjust position (if necessary).

5) LUTs – The lookup tables for open images will display here. You can adjust how the image is displayed and toggle autoscaling options. Use the display here to keep an eye on over- and under-saturation of your images.

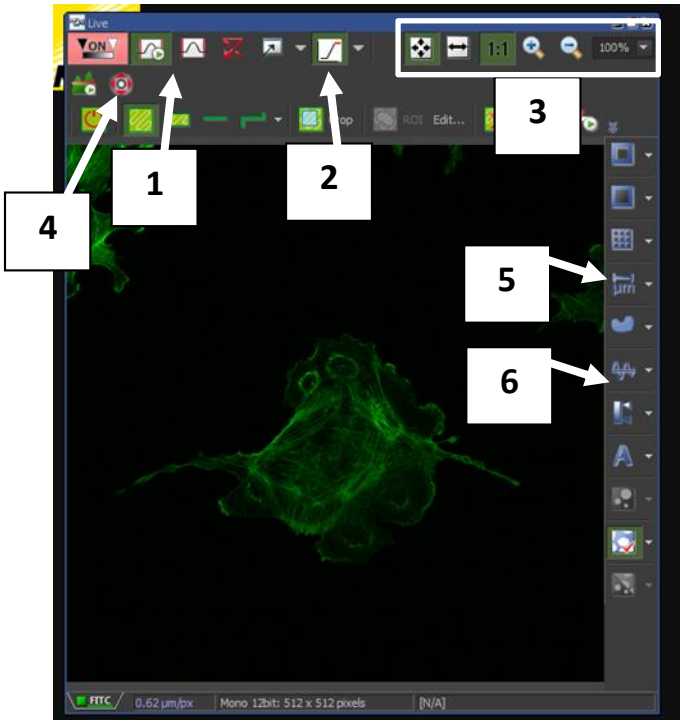
6) ND Acquisition – Options for acquiring multidimensional acquisitions including time lapses, Z-stacks, X,Y montages, multiple X,Y positions, and/or multiple colors (lambdas).

7) A1 Scan Area – Use these options to control the scan area and pixel size.

8) A1 Compact GUI – This panel contains the controls for adjusting and acquiring confocal images.

9) Blue dual arrow button – restores default layout and OCs. If you accidentally change something and can't find it, press this to restore the software to defaults.

**A1 – The NIS Elements image window (live):**




1) Autoscaling: Press the right autoscale button to scale the image once and press the left autoscale with play button to turn on or off the continuous autoscaling of the image (it is on in this example).

2) Pixel saturation indicator: Press this button to display over- and under-saturated pixels in color on your image window. By default, this should be on (it is on in this example).

3) Zoom and image window size buttons.

4) Mouse X,Y button: Click here and then click and drag in your image window to move the live image instead of using the joystick.
   ➔ To move a specific spot to the center of the field of view: right-click inside the image window on the spot to move to the center and select "Move this Point to Center."

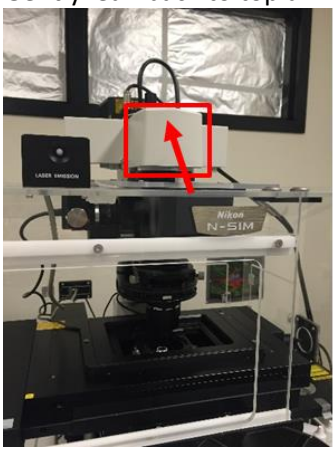
5) Scale bar button and options.

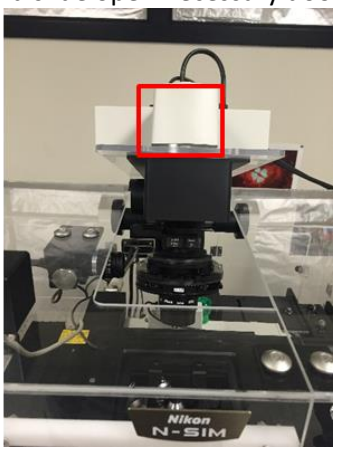
6) Line profile button and options.

➔ Press X to create a non-quantitative snapshot of the image as displayed on the screen

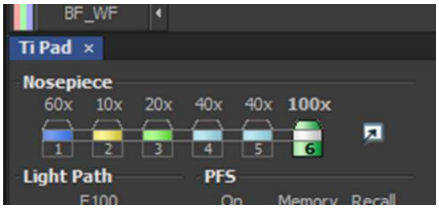
## A1 – How to look at sample through oculars:

1) Gently lean back to top arm and slide open necessary doors for greater access to the stage area.



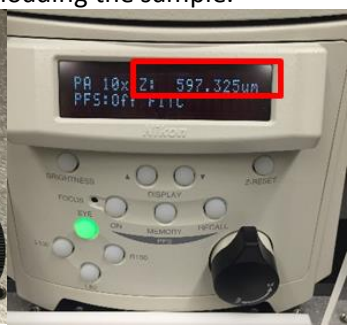




2) Load the correct objective in position.
   - Objectives can be changed by selecting the desired lens in the software.
   - If you are using an air objective, start with 10x at low focus position.
   - If you are using the 100x oil immersion objective lens, place a drop of oil on the objective (don't touch the oil dropper to the lens!). Once you go oil, DO NOT SWITCH BACK!

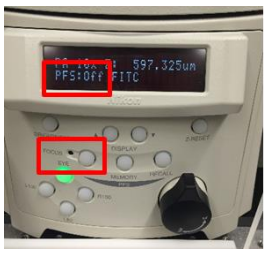


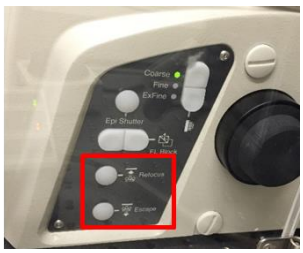

3) Adjust stage position with the joystick if needed. To change speed of joystick, gently rotate the top portion clockwise to reduce speed and counterclockwise to increase. The current speed is denoted by the orange light on the joystick (Coarse – Fine – ExFine). Adjust the Z focus with the knob on the right side of the joystick controller. The current speed of Z focus change is denoted by the green light in Z Speed (Coarse – Fine – ExFine). Make sure the focus position is lowered to below 800 on the front display of the microscope body to avoid damaging the objective when loading the sample.



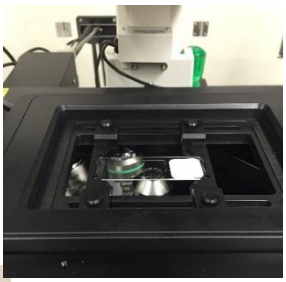


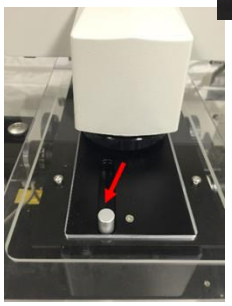

   - <u>If the focus knob is not responding:</u> make sure that it is set at the correct speed (Coarse – Fine – ExFine), that Perfect Focus is not engaged (no green light on the front of the microcope), and that the previous user has not hit the Escape button on the side of the microscope.



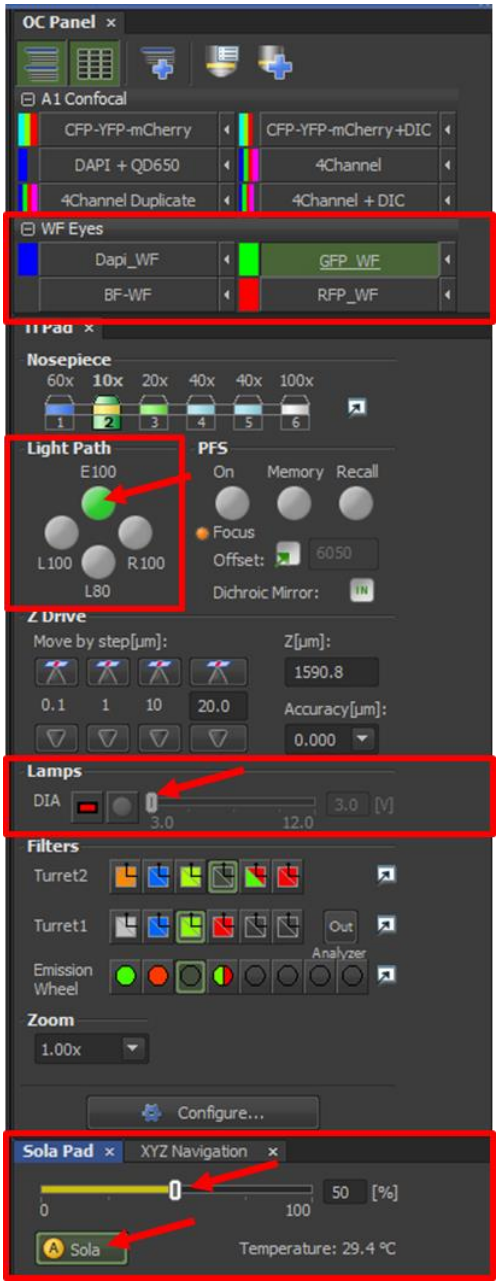
4) Place the slide, coverslip down, onto the slide holders on the stage. Please be sure that your slide is clean and dry – do not get wet mounting media, sealant, or condensation on the stage or objectives. If you are using the 100x oil immersion objective, turn the focus knob up until the objective lens is just touching the oil. **Do not switch back to an air objective**.





5) After loading the slide, gently lower the top arm back down and close all the doors around the microscope. Make sure the small silver knob at the top of the condenser arm is in the forward position (if not, it will block the transmitted light path).

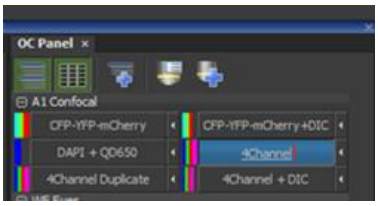
6) Select appropriate widefield illumination optical configuration (OC) – DAPI_WF, GFP_WF, RFP_WF, or BF_WF. Widefield optical configurations will default by sending fluorescence light to the oculars (E100).
   - To open or close the fluorescence shutter, click on the Sola button at the bottom of the right toolbar. Adjust the intensity of the Sola light with the slider.
   - The Brightfield OC will default with the DIA lamp on. To adjust brightfield lamp intensity, use the DIA slider in the middle of the right toolbar. See Appendix B for instructions on Kohler alignment of the brightfield.




7) Adjust the focus using the focus knob on the joystick or the knobs on the sides of the microscope. To adjust focus speed, press the button on the right side of the joystick to switch from Coarse – Fine – ExFine. Adjust to get the field of view you want.
   - Once the slide has oil on it **DO NOT SWITCH TO ANY OTHER OBJECTIVE!**
   - Optimal focus for most slides is generally ~1600-1900 on the front display (except when using the ELWD 40x air objective).

## A1 – Adjusting the Optical Configurations (OCs) for your sample:

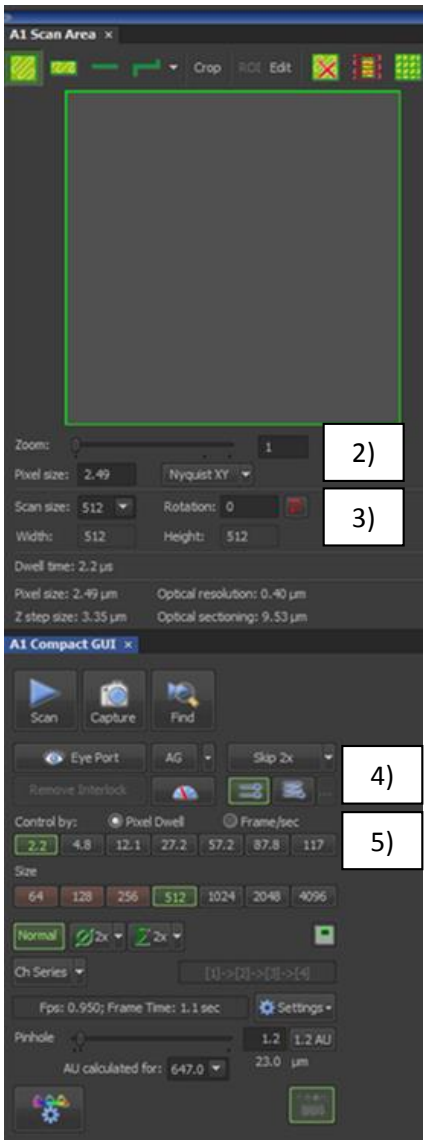
1) Once the sample is centered in generally in focus, select appropriate A1 Confocal OC shortcut from the top right for your combination of fluorophores.




2) In the A1 Scan Area window (top left column), select the desired zoom factor. Alternatively, just click Nyquist XY and the software will calculate the required zoom factor to maintain Nyquist sampling based on the selected objective magnification, objective numerical aperture, and number of pixels.

➔ Nyquist Sampling: "The Nyquist criterion requires a sampling interval equal to twice the highest specimen spatial frequency to accurately preserve the spatial resolution in the resulting digital image." This is determined based on the Abbe limit of resolution (=wavelength/ 2*NA). If resolution limit is 0.22 micron, Nyquist XY pixels are ~0.11 micron. Undersampling (i.e., too large a pixel or Z step size) will lead to loss of high spatial frequency details. Undersampling is faster (fewer pixels to collect) and generally brighter if pixels are larger, but there is a loss of resolution. Oversampling (i.e., a very small pixel or Z step size) theoretically does not improve the spatial resolution of the image but can help improve quantitative accuracy of measurements. Oversampling can lead to improved image quality (not spatial resolution), but smaller pixels require more laser power/gain (more bleaching) and acquisition of more pixels is more time consuming. Optimally, it is suggested to sample at 2.5-3 fold for high-resolution imaging, so your pixels should be 2.5-3 times smaller than the smallest feature to resolve within reason (don't forget this doesn't improve spatial resolution).

http://microscopyu.com/tutorials/java/digitalimaging/spatialresolution/index.html




3) Select the scan size – number of pixels per side of the image. More pixels will improve spatial information (up to the resolution limit) but will require more time to collect and generally require higher laser power/gain for sufficient SNR (see above discussion on Nyquist sampling). Generally, 512x512 or 1024x1024 are good starting points.

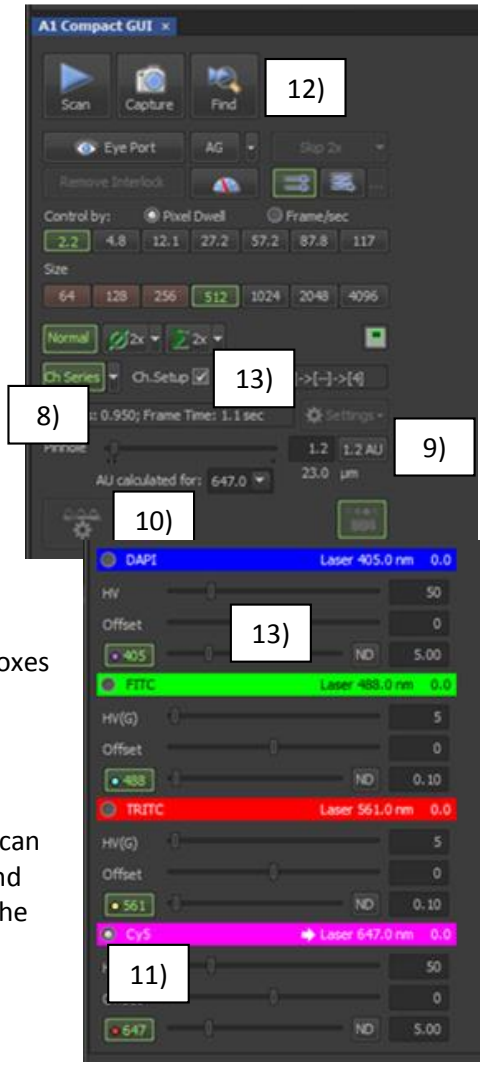
4) In the A1 Compact GUI (middle left), select mono or bi-directional scanning. Mono-directional is cleaner and recommended unless you are doing high-speed live cell work. Bi-directional scanning will require a bit of alignment before using. The Skip option above these buttons will skip collecting lines of pixels – not recommended.

5) Select imaging speed – pixel dwell time or frames per second. Imaging with faster scan speeds may be noisier/ reduced SNR, but also result in less photobleaching and faster imaging times. Generally, faster scan speeds will work on this instrument given its very sensitive detectors.


6) You can change scan size here (as well as in the A1 scan area, step 3 above).

7) Choose Normal, Line Averaging (∅ mean average), or Line Integration (∑ summation) options, as needed. These options can be useful to average out noise in the image and boost signal but require multiple scans (reducing speed and increasing bleaching).

➔ **NOTE**: If you will be imaging your sample using multiple OCs (for example, blue and red in one OC and green and far red in a separate OC), you must use the sample zoom, pixel size, scan size, and scan speed for all OCs. If not, the images will not align correctly.




8) Toggle on or off "Ch Series." If this is on (green background, as is shown here), the system will acquire each color sequentially. If it is off, the system will acquire all colors simultaneously. If you are worried about cross-talk, run the system with Ch Series on.

9) Select a pinhole size. The pinhole will be the same size for all colors, so generally you will select AU calculated for a wavelength in the middle of the range or the one of greatest interest. Pinhole size will need to be adjusted if you switch objectives and if you image different wavelengths. To use the default 1.2 AU pinhole size, click 1.2 AU button.

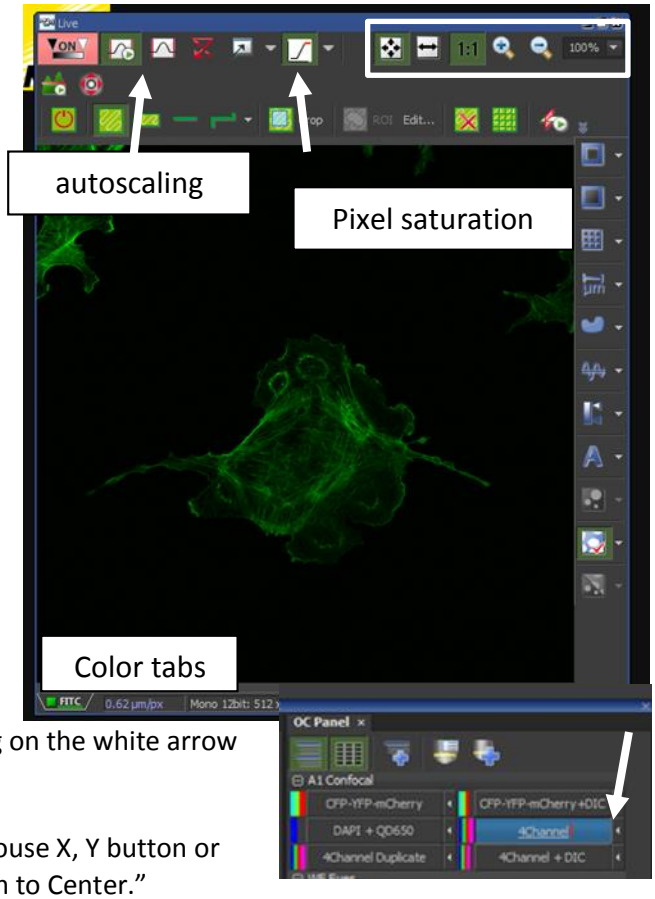
10) Ignore the wavelength configuration button. You should never need to alter any of these options as they are programmed in with each optical configuration.

11) Check on or off the wavelengths you will need with the checkboxes next to each name.

➔ Finally time to go to live scanning!

12) The Find button at the top of the A1 Compact GUI will rapidly scan a smaller horizontal bar of your image. This can be useful to find the focal plane but may also result in a bleached stripe across the field of view. The Scan button will show a live, continuously updating image on the screen. Capture will snap a single plane image with the selected settings.

13) If you are working in Ch Series mode, check on the Ch. Setup box, click Scan, and select the radio button next to the color you want to work with. Adjust focus if necessary – always adjust settings on the brightest/most in-focus focal plane. Adjust Gain (HV), Offset, and laser power to achieve the desired image quality. Gain will multiply all signal, including noise. Offset will reduce all values and can also cut into your signal of interest if too high. **PMT Gain values (HV, the top and bottom channels) should be kept below 80-100 (absolute max of 120) and GaAsP detector Gain values (HV(G), middle two channels) should be kept below 50. DO NOT OVERSATURATE YOUR IMAGE!!! Look at the LUTs (bottom left of the screen) and pixel saturation indication.** Switch to the next color by clicking on the radio button and repeat adjustment process.
If you are not working in Ch Series mode, click Scan and look at one color at a time of the live image by clicking on the desired tab at the bottom of the live image window. Adjust focus and settings as described above.
If you are having trouble with this process:
    - Try the AG (autogain) button found below the Find button to let the software automatically determine Gain and Offset.
    - Make sure the LUT scaling is appropriate for your intensity range (is the image over- or under-saturated?). Consider turning on the dynamically updating constant autoscaling button.




14) Happy with the settings for each channel? Don't forget to lock them in to the OC by clicking on the white arrow next to the correct OC's name.

15) You can adjust position of the image with the Mouse X, Y button or right click in the image and select "Move position to Center." (Alternatively, you can use the joystick next to the microscope.)

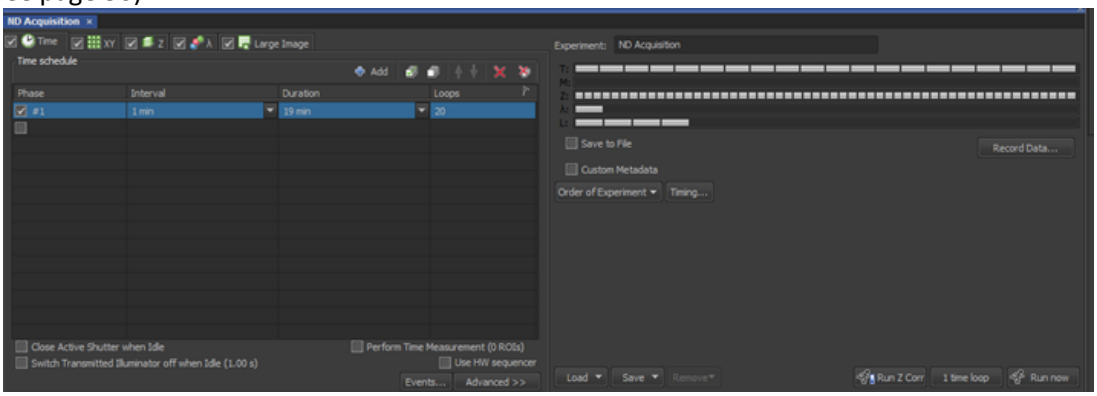
16) Repeat this process for all OCs you will be using. If your sample is labelled with four colors, you can acquire these using the 4Channel OC in either simultaneous mode or Ch. Series mode (all sequential). Alternatively, you can set up the 4Channel OC to acquire blue and red simultaneously and the 4Channel Duplicate to acquire green and far red simultaneously – this may be faster and since these colors are spectrally separated, cross-talk is reduced. If you go the route of using multiple OCs, make sure that all OCs have the same sample zoom, pixel size, scan size, and scan speed for all OCs. If not, the images will not align correctly.

## A1 - Setting up and Acquiring an ND Acquisition:

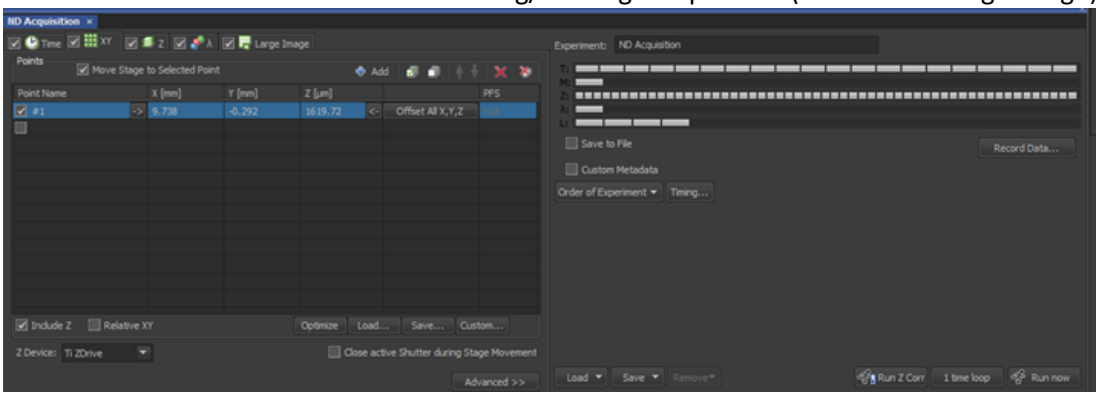
At the bottom of the screen is the ND Acquisition window that is used to capture images in multiple dimensions.

Options (uncheck those you don't want):

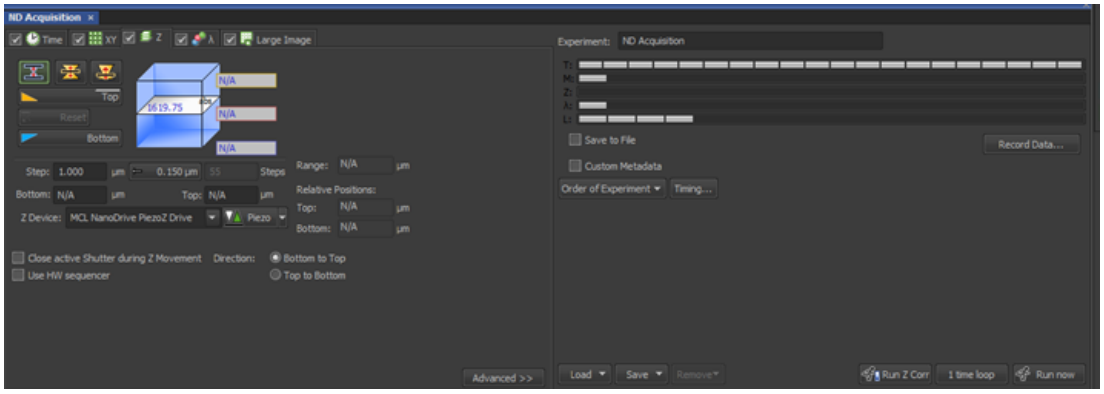
1) Time – choose the interval and duration of the desired time course. Multiple phases with different settings may be added to run consecutively. Make sure to check on the "Close Active Shutter when Idle" option. If you're performing a timelapse, use Perfect Focus to maintain focal position over time (see page 36).



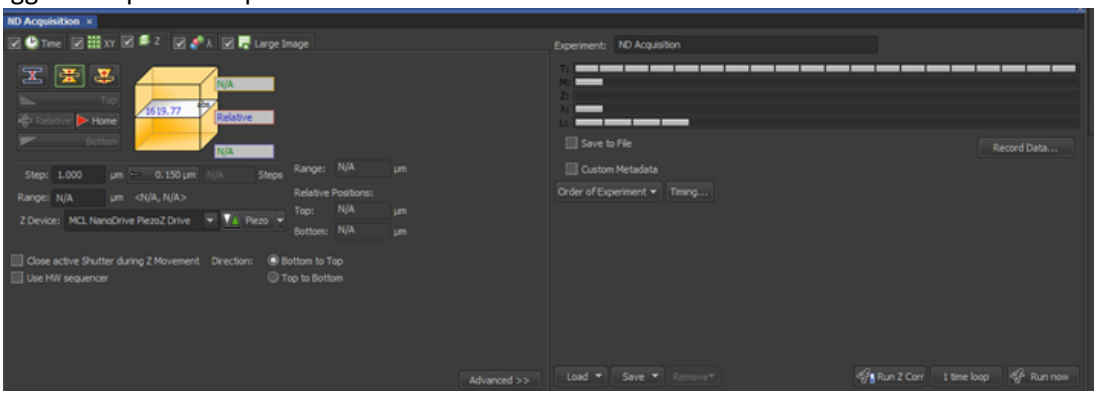

2) XY positions – Once the sample is positioned correctly, click "+Add" button. Check the "Include Z" box to also lock in the Z position. Find a different field of view, and click "+Add" button to add. To delete a position, click the red X button. Continue until all desired positions are locked in. Check on the "Close active shutter during stage movement" to eliminate any spurious excitation light from illuminating the sample while the stage moves. Use Perfect Focus to maintain focal position (see page 36).
   - ➔ Note: this is not used for stitching/montage acquisition (for that use Large Image).



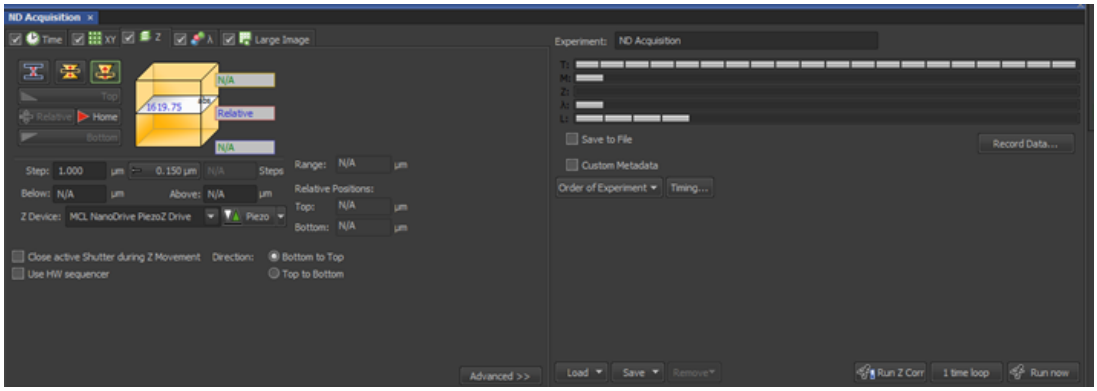
3) Z – Set top and bottom – With the image window live, adjust focus knob to go to one extreme, click Top, go to other extreme, click Bottom. This will set the range to acquire. Then set the step size or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size.



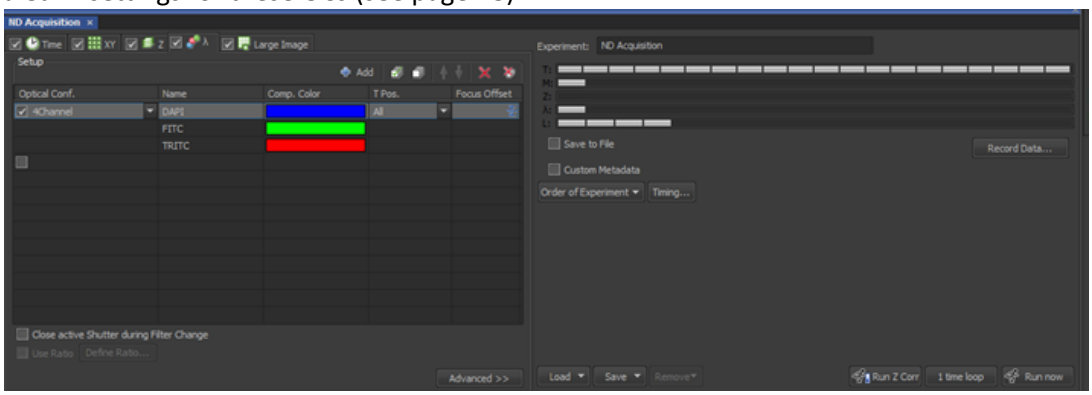

4) Z – Range around center – With the sample focused in the center plane, click the "Home" button to set the center home position. (If you're using Perfect Focus, click Relative – PFS won't work with an absolute Home position.) Then set the step size, total range, and/or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size.



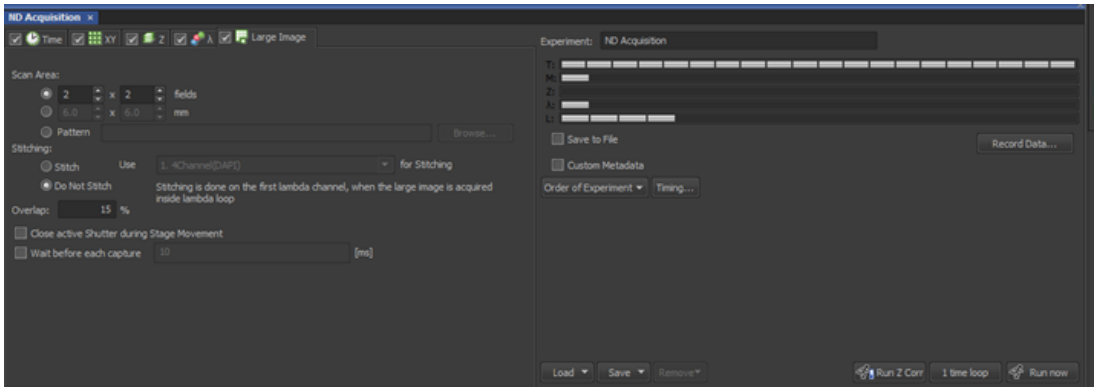

5) Z – Asymmetric range around center – With the sample focused in the center plane, click the "Home" button to set the center home position. Then set the step size, range to acquire above, range to acquire below, and/or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size.



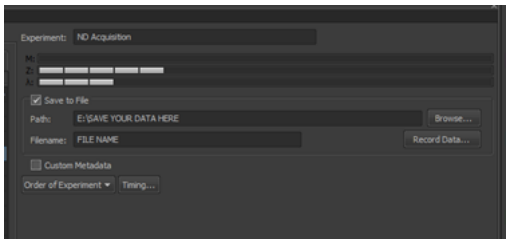
6) Lambda – Select the desired optical configuration (OC) from the drop down. Select if you want to acquire this wavelength at all Z positions or just a subset of the total planes. You should already have dialed in settings for these OCs (see page 28).



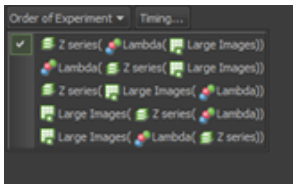

7) Large Image – Use this option to generate montages, with or without stitching. Position your sample in the middle of the region you wish to stitch. Set the number of fields of view you wish to include and check or uncheck Stitch. Select the percent overlap (typically 10-20%). If you do not select stitching, the image can be stitched after acquisition by another program like ImageJ/FIJI. Stitching works best for samples with sufficient detail overlapping the multiple regions to permit the software to determine how to align the images.




8) Right side – Name your experiment. Check or uncheck save to file. If you check save to file, select the correct file path and file name. File path must go to the E:\SAVE YOUR DATA HERE\ drive (NOT C:\).

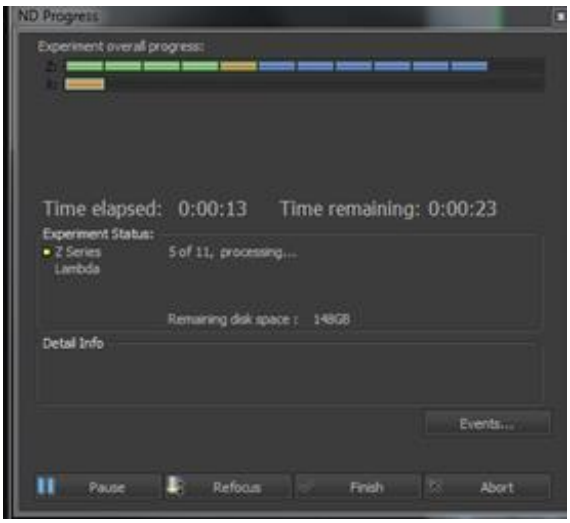



9) Order of Experiment – Adjust the order in which the various parameters will be acquired. Read this from right to left, the parameter inside the parenthesis will be acquired first. The Z drive is relatively fast as is switching colors. Multiple X,Y positions (either Large Image montage or XY) will be a bit slower. Put the slower components more to the left to speed up overall acquisition time.




10) When you're ready, click "Run now" at bottom left to start the acquisition.

11) The ND Progress window will show the progress of your imaging experiment. Green squares are done, orange is in progress, and blue has not yet been done. Time remaining can sometimes be off, depending on the order of experiment. Click Abort to stop the acquisition and discard all data. Click Finish to stop the acquisition after the current round is completed. Based on the order of experiment it is possible that the image window will not update until all images are done being acquired.



**Reusing previous settings:**

To apply settings from a previously acquired image to an OC:

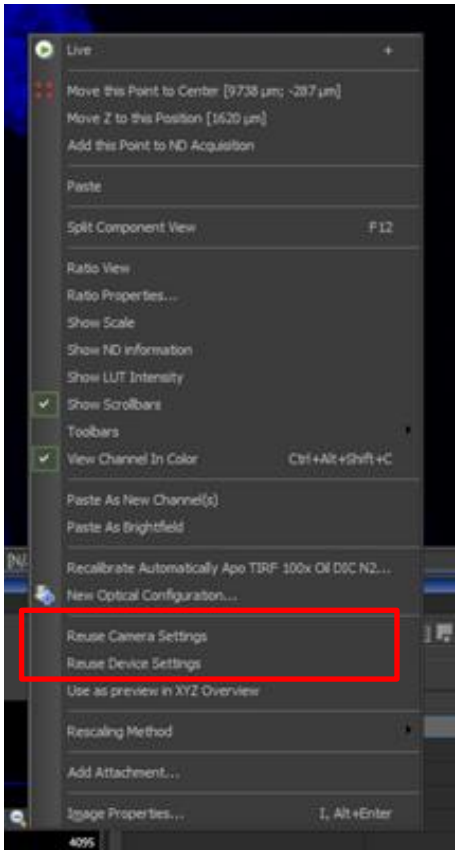
1) Select the appropriate OC to load the settings to.

2) Open a previously saved image.

3) Right click anywhere on the actual image and from the list of options choose “Reuse camera settings” and “Reuse device settings” and select the color.

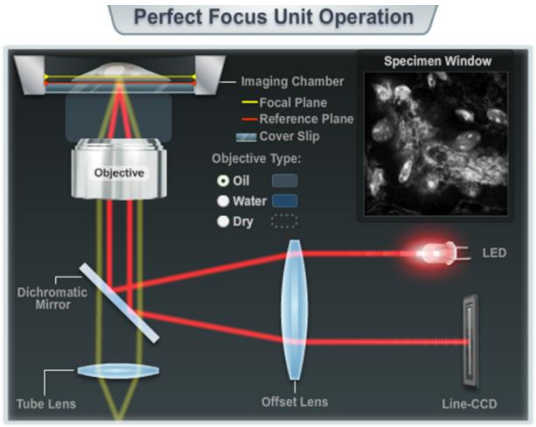
4) After applying all desired colors, don’t forget to lock in changes to the OC by clicking on the white arrow next to the OC name.

5) If you wish to reuse the ND Acquisition parameter settings from the imaging experiment, click “Reuse ND Setup.”



## Perfect Focus System (PFS):

Nikon's Perfect Focus System (PFS) is a very effective method of maintaining sample focus during acquisition parameter setup, timelapse experiments, or across uneven coverslips. PFS uses a far red LED light to find the sample/glass interface.

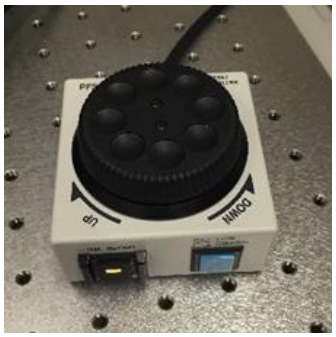



**To use the Perfect Focus:**

1) The sample must already be in focus and the green light next to the PFS ON button must be lit up. If it is not on, the PFS cannot find the appropriate interface. This can be the result of a very out of focus sample or not enough difference in refractive index.

2) Turn on the PFS by pressing the left-most ON button on the front of the microscope. The sample will now go slightly out of focus (the microscope moves to the PFS interface reference plane).

3) The user can then set the "PFS offset" using the PFS focus knob. The knob can be toggled between coarse and fine using the blue button on the front right. The normal focus knobs will not function as long as PFS is on.



4) Z-series ranges, etc can be set using the PFS focus knob, just as you would normally use the focus knob.

5) To turn it off again, just press the left PFS On button on the front of the microscope.

**Note:** The PFS dichroic mirror (DM) can be removed from the light path (disabling PFS) using the left button (black with a light) on the PFS remote to allow for improved detection of far (and really far) red fluorophores.

**When you're finished:**

1) Lower stage away from sample (down to ~500 on the front of the microscope), and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!

2) Please leave the microscope on 10x objective for next user.

3) Save and copy your data. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB Server.

4) Close NIS Elements.

5) Log use in Excel sheet, save and close.

**CHECK THE MCDB CALENDAR:**

➔ **If someone is signed up within the next two hours**

6) Leave it all as is.

➔ **If no one is signed up within the next two hours or you are the last of the day:**

7) Shut down system: Start → Shut down PC, turn off A1 confocal if it is on, turn the key on the laser launch to off position.

8) Close all the plexiglass doors around the microscope and close the doors to the room when you finish.

**APPENDIX A – Saving to your lab's folder on Collie (MCDB Dept Server):**

➔ **Questions regarding MCDB Server should be directed to Erik Hedl.**

https://mcdb.colorado.edu/facilities/computing_resources/faqs/server_connect/windows/

1) Map the network drive. Right click on Computer icon on desktop or Computer in Start menu. Click on "Map network drive…"

2) Drive letter should be Z: and the folder should be \\collie.int.colorado.edu\<your lab name> (for example \\collie.int.colorado.edu\OlwinLab).
Check the box "connect using different credentials."
Uncheck any option to remember the login credentials – this is a shared PC!

3) Click finish.

4) Enter your IdentiKey username preceded by AD\ and password.

5) Click Ok and the drive should now appear. Copy over your files. If you have a lot of files, be sure to allow time for the transfer. Under ideal conditions you will be able to copy close to 42 gigabytes per hour, don't count on ideal conditions. Also, it is safer to copy (not move) your files, then delete after the copy has completed.

➔ You cannot, must not, should not reserve time on the microscopes in MCDB Cal to copy data – reservations are for imaging only.
➔ The LMCF PCs are not long-term data storage places and we are not responsible for lost data.
➔ Do not forget to always safely store and backup your raw data – this represents the ground truth of what you acquired that day and has all the associated metadata. Many journals are now requiring that you submit raw data with your manuscript. For SIM super-resolution data, don't forget to keep the unreconstructed raw data. You may decide at a later date to go back and preform a new reconstruction using different parameters.

## APPENDIX B – Kohler Alignment for Brightfield/DIC

Kohler alignment of the microscope provides optimal performance and contrast of the brightfield diascopic illumination by providing the most uniform illumination of the sample.

1) Focus the sample using Brightfield OC.

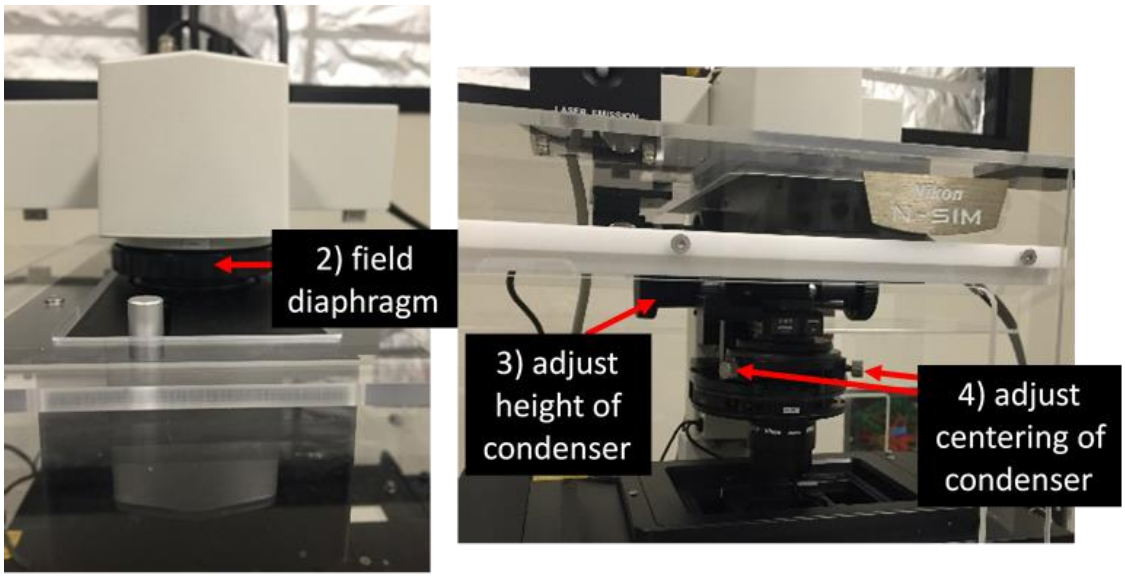
2) Close field diaphragm aperture by rotating as far as possible counter-clockwise.

3) Adjust focus of field diaphragm by changing condenser height until the octagon generated by the field diaphragm aperture is in focus at the sample.

4) Adjust positioning screws to center field diaphragm aperture octagon

5) Open field diaphragm aperture

6) To adjust brightfield image: adjust the opening size of the field diaphragm and/or iris diaphragm, adjust the intensity of DIA lamp on the Ti pad, and/or adjust the camera settings.



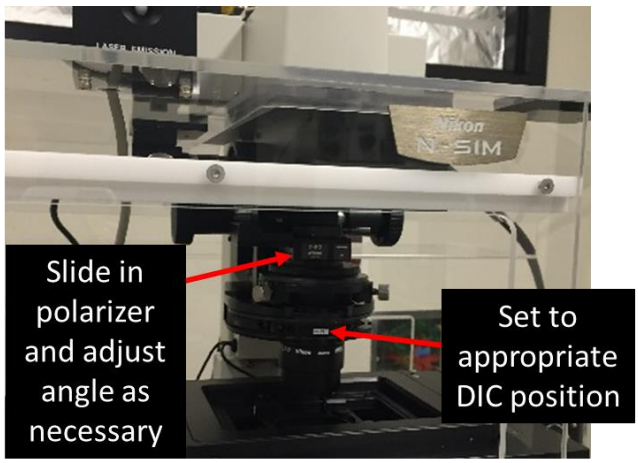

**For DIC imaging:**

1) After Kohler alignment, insert matching slider below objective.

2) Rotate condenser to appropriate position.

3) Slide the polarizer into the light path and adjust angle as necessary.

➔ SIM is incompatible with DIC, do not use DIC sliders in conjunction with SIM imaging.



## APPENDIX C – TokaiHit Incubation chamber for live cell imaging:

- ➔ **The TokaiHit normally resides hooked up to Black Widow. Use on Thor requires a heads-up to Jolien to allow for time to move the equipment.**

- ➔ **Use of the live cell chamber absolutely requires prior authorization from Jolien.**

- ➔ **Each such user must have passed appropriate training for handling of the live cells through their laboratory's (PI's) approved IBC protocol.**

Starting the system:

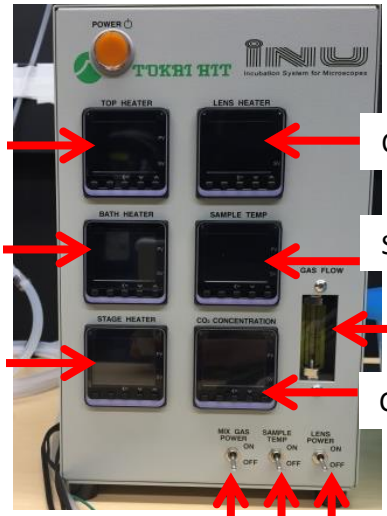
- Install proper stage insert on microscope.
- Remove the chamber from storage box.
- Install proper insert for your sample type – slide/chamber slide or 35 mm dish.
- Make sure all three switches at the bottom of the Tokai Hit unit are off.
- Ensure that the water tube is closed.
- Remove lid and add di $H_2O$ to the chamber to fill the bottom but not to the gas inlet valve.
- Insert a dummy dish or slide.
- Turn on main power switch.
- Turn on appropriate switches at the bottom of the unit – Lens Power (for objective heater), Sample Temp (for sample temperature probe), Mix Gas Power (if using $CO_2$).
- Confirm the unit is set to appropriate temperatures. Default values for maintaining a sample at ~37 C are located on the top of the unit.
- Turn on $CO_2$ cylinder. Ensure that flow coming out of cylinder is between 14.5 psi and 21.7 psi.
- Let unit sit for 30 min to reach equilibrium.
- Carefully place on stage and insert sample.
- Image.
- For long-term imaging, you may need to use a syringe to add water to the unit.

Done:

- Remove sample.
- If you were using a high power objective, switch to 10x. Carefully remove chamber from microscope stage. If there is still a lot of water left, pipet some out before moving the unit.
- Turn off $CO_2$ cylinder.
- Turn off all switches at the bottom of the unit.
- Turn off main power switch.
- Use pipet/syringe to remove any remaining water from chamber.
- Wipe chamber with EtOH or bleach (depending on sample type and PI's protocol).
- Let sit on bench to cool.
- Once cooled, carefully return to storage box.
- Remember to re-install the normal slide stage insert on microscope.

Sample chamber (with slide insert) and objective heater

$CO_2$ gas flow – do not fill water above this level!




Top lid of chamber heater setting

Chamber water bath heater setting

Stage heater setting

Objective lens heater setting

Sample probe readout

$CO_2$ gas flow

$CO_2$ concentration setting

$CO_2$ Mixer ON/OFF

Objective lens heater ON/OFF

Sample temp probe ON/OFF