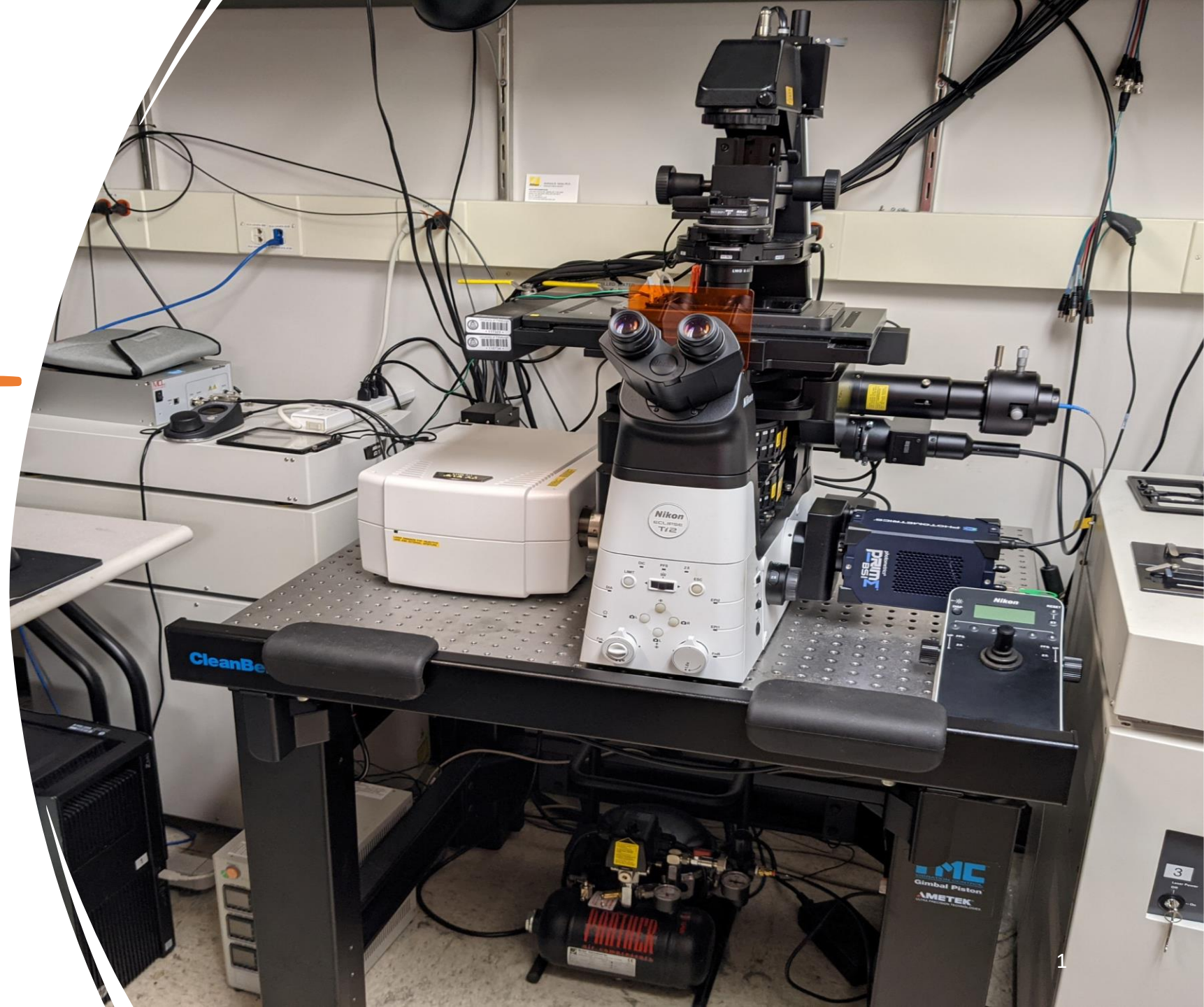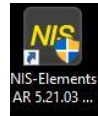# Nikon A1R Confocal & TIRF System

User Manual

Microscopy and Imaging Facility

Shared Research Support Service

Dietrich School of Arts & Sciences
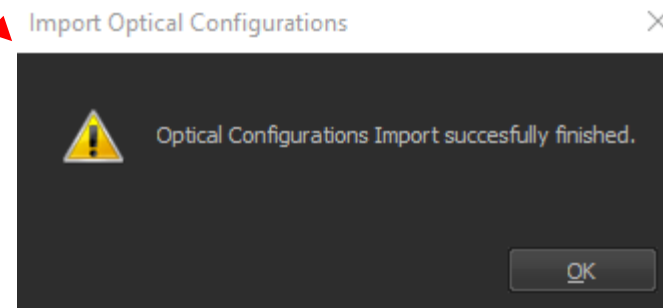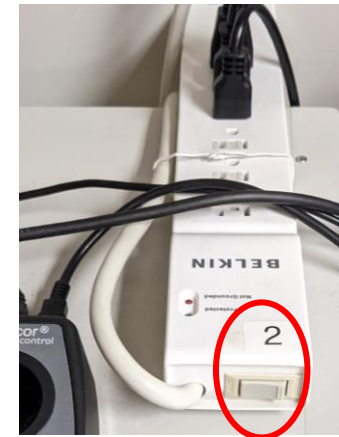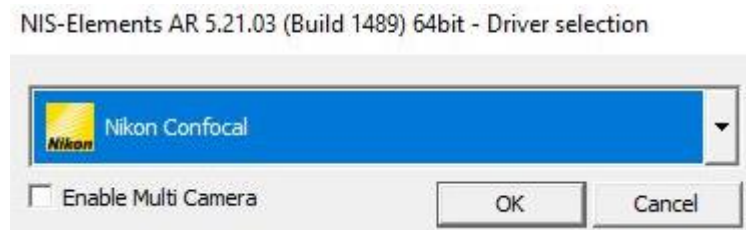
University of Pittsburgh

Pittsburgh, PA, USA

Start up the Nikon System

1. Sign on the log sheet before starting the system.

2. Check if the four corners of the floating table floats freely.

3. Check if the stage is clean/oil free. If not, please clean it before you start.

4. To use the confocal system, turn on the microscope in the order of 1→4 as labeled on the equipment.

5. To use the TIRF system, turn on the microscope in the order of 0-4 as labeled on the equipment. For TIRF imaging, please refer to the separate TIRF user manual.

6. Log in to your Pitt's account on the computer.

7. Click on the Nis-Element software icon on the desktop.

8. Then between Nikon Confocal and Photometrics (TIRF), select Nikon Confocal.

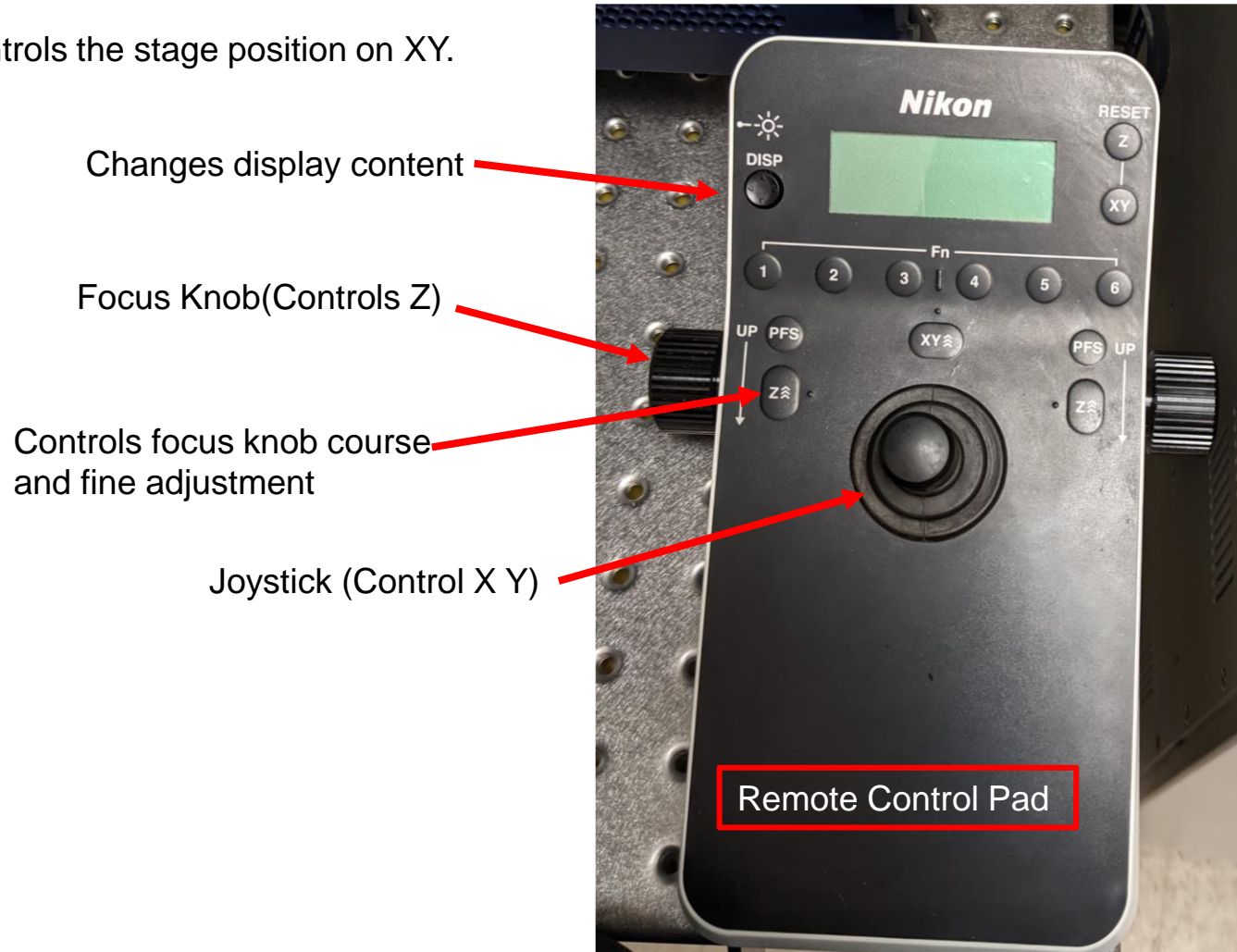9. Wait for the software to load, click OK when this window pops up

Equipment:
0. Camera
1. Computer
2. Power control
3. Lasers
4. Controller

NIS-Elements AR 5.21.03 (Build 1489) 64bit - Driver selection

Nikon Confocal

☐ Enable Multi Camera   OK   Cancel

Import Optical Configurations   ✕

⚠ Optical Configurations Import succesfully finished.

OK

View samples through eyepieces

1. Select the right sample holder according to your sample and place it in the stage. Place your sample coverslip-side down on the stage.

2. The focus knob and stage control/ joystick are on the remote control pad.

3. Turning the focus knob towards you moves the objective up. (Decreases the distance between your sample and the objective.)

4. The joystick controls the stage position on XY.

Changes display content

Focus Knob(Controls Z)

Controls focus knob course and fine adjustment

Joystick (Control X Y)

Remote Control Pad

Sample holders

5. You can change objectives by flipping this button (in front of the microscope) to the left or the right. You can also change objectives through the software under the Ti2 Pad section. The 40x in position 3 is 40x oil. The 40x in position 6 is usually 40x water (switchable).
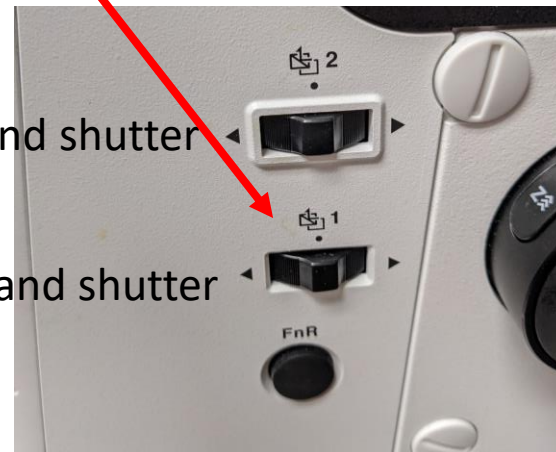
6. On the left side of the software, under the View Sample by Eye section in the OC Panel, you can choose from DIC-EYES, DAPI-EYES, FITC-EYES, and TRITC-EYES to turn on light for eyepiece observation.
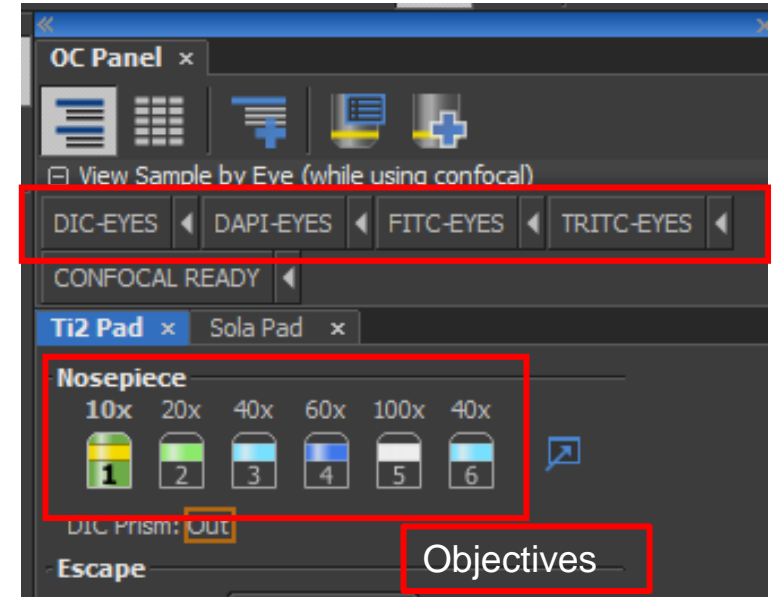
7. Press this button on the right side of the microscope to open and close the shutter. Flipping this button back and forth changes filter turrets.
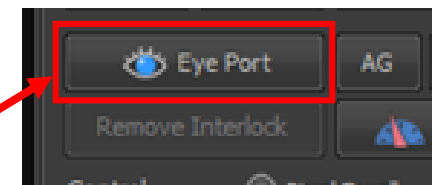
Epi/TIRF filter turrets and shutter

Confocal filter turrets and shutter

An additional step to use DIC-EYES is to click Eye Port (on the right side of the software) after clicking DIC-EYES. When you are ready to switch to the software live view, turn off Eye Port.
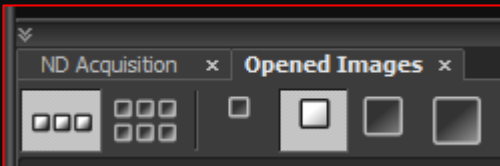
8. After finding and focusing on the sample, switch to live view in the software.

Objectives

NIS-Elements AR [Current user: CHS330]

File Edit Acquire Calibration Image ROI Binary Measure Reference Macro View Devices Window Applications JOBS Addons NIS.ai Deconvolution Help

Search [Ctrl+F3]

OC panel

OC Panel

View Sample by Eye (while using confocal)

DIC-EYES DAPI-EYES FITC-EYES TRITC-EYES

CONFOCAL READY

Ti2 Pad   Sola Pad

Nosepiece
10x 20x 40x 60x 100x 40x
1   2   3   4   5   6

DIC Prism: Out

Escape
Escape Z

Light Path   PFS
EYE   Glass   Dichroic
Bertrand OUT   PFS   IN
Off
L100 R100
AUX   Offset: 8405

Z Drive
Move by step[µm]:   Z[µm]:
0.1 1 10 20.0   483.88
Accuracy[µm]:
0.16

DIA
0.0   100.0   23.8

Filters
Turret-Up   Out
                          Analyzer
Turret-Lo
Analyzer Slider: Out
EM Wheel1

Condenser
6: N2

Polarizer: In

Configure...

LUTs

Microscope settings

LUT

No Image

Live view window

ND Acquisition ✕   Opened Images ✕

ND Acquisition and Opened images

ND Acquisition Opened Images

Full Screen HCA Confocal* TIRF/Epi   A1plus

Acquisition settings
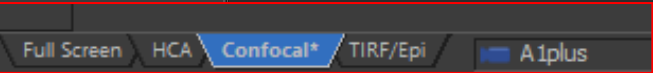
A1plus Compact GUI

Scan   Capture   Find   Galvano
                              Resonant

Eye Port   AG   Skip 2x

Remove Interlock

Pixel Dwell ● Frame/sec
Fast Mode   1/2 1/4 1/8 1/16 1/24 1/32
Keep Pixel Brightness
Size   64 128 256 512 1024 2048 4096

Normal 2x 2x Scan Area

Ch Series Ch.Setup   [1]->[2]->[3,--]->[4]

Fps: 0.237; Frame Time: 4.2 sec   Settings

Pinhole   1.5   1.2 AU
AU calculated for: 640.0   26.8 µm

DU4 CB VB V4

405   Laser 405.0 nm 0.0
HV   70
Offset   0
405   3.00

488   Laser 488.0 nm 0.0
HV(GaAsP)   40
Offset   0
488   2.50

561   Laser 561.0 nm 0.0
HV(GaAsP)   40
Offset   0
561   2.00

640   Laser 640.0 nm 0.0
HV   90
Offset   0
640   7.00

TD

Lasers

A1plus Scan Area

Crop ROI Edit

Scan area

Zoom: 1

Pixel size: 2.49   Nyquist XY
Scan size: 512   Rotation: 0
Width: 512   Height: 512

Plan Apo λ 10x (2.49 µm/px @ 512 x 512)   XY=[27.937, -13.267]mm, Z=483.880µm, PiezoZ=0.000µm

Full Screen HCA Confocal* TIRF/Epi   A1plus

Image Acquisition

1. To switch to live view in the software, first click on CONFOCAL READY in the OC Panel.

2. For live view, keep scanning mode as Normal, click on Ch Series, and **make sure pinhole size is 1.2 AU (important step! Do not forget.)**.

3. By default, DU4 is selected, indicating that you are using filter cubes for the emitted light. For most imaging experiments, this is good enough. In some cases, if you need to use spectral detection, you can use VB or CB (See details on page 14).

4. Next, **select one channel at a time** to adjust image brightness through laser power, gain (HV: Gain), offset, and laser power.

5. Then click on Scan. By default, the image size is set to 512x512 at a faster image speed. This is good for live scan. If you do not see any images at first, click on the auto-scale icon on the top of the live-view window.

6. Turn on the signal intensity saturation indication icon.

7. For signal intensity saturation, we typically set white color for overexposure and yellow color for background (under saturated/ no signal). **DO NOT** overexpose your region of interest. **DO NOT** overfill the background with under saturated signals.
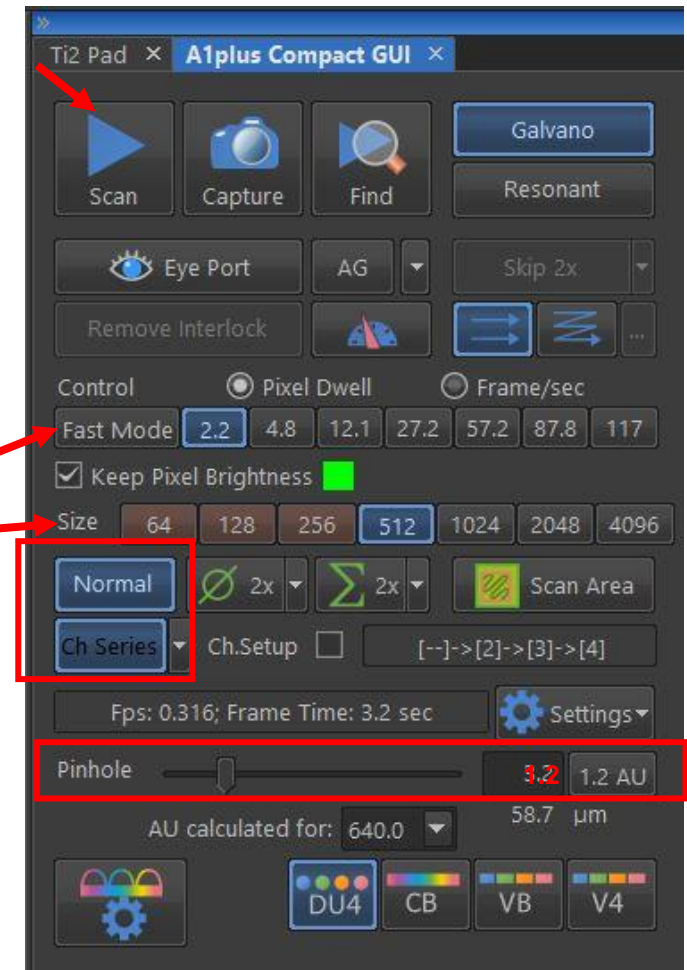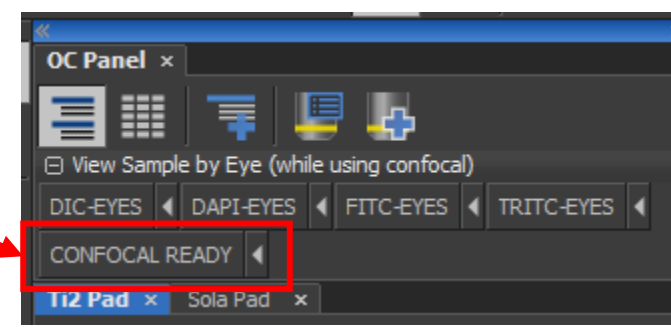
Scan speed

Image size

8. You can improve image quality by adding numbers of averaging, or slower the scanning speed.

Scan speed

Image size

9. Accumulation increases the signal brightness, but also increases scanning time.

Average Accumulation

10. After adjusting image intensity, click on Scan again to stop the live view.

Split channels

11. Then switch to another laser line and do the same adjustment.

12. To start acquisition, click to check all the laser lines you need.

13. Then switch imaging format from 512 to at least 1024x1024 (DO NOT FORGET THIS STEP!). It is also suggested to use a slower scanning speed than 2.2 pixel dwell or ½ frame/sec.

14. Click Capture to acquire the image.

15. To view multi-channel images, clicking on each channel at the bottom of the image allows you to switch between channels. You can also split the channels to view them simultaneously.

16. On the right side of the image, this button allows you to add a scale bar.
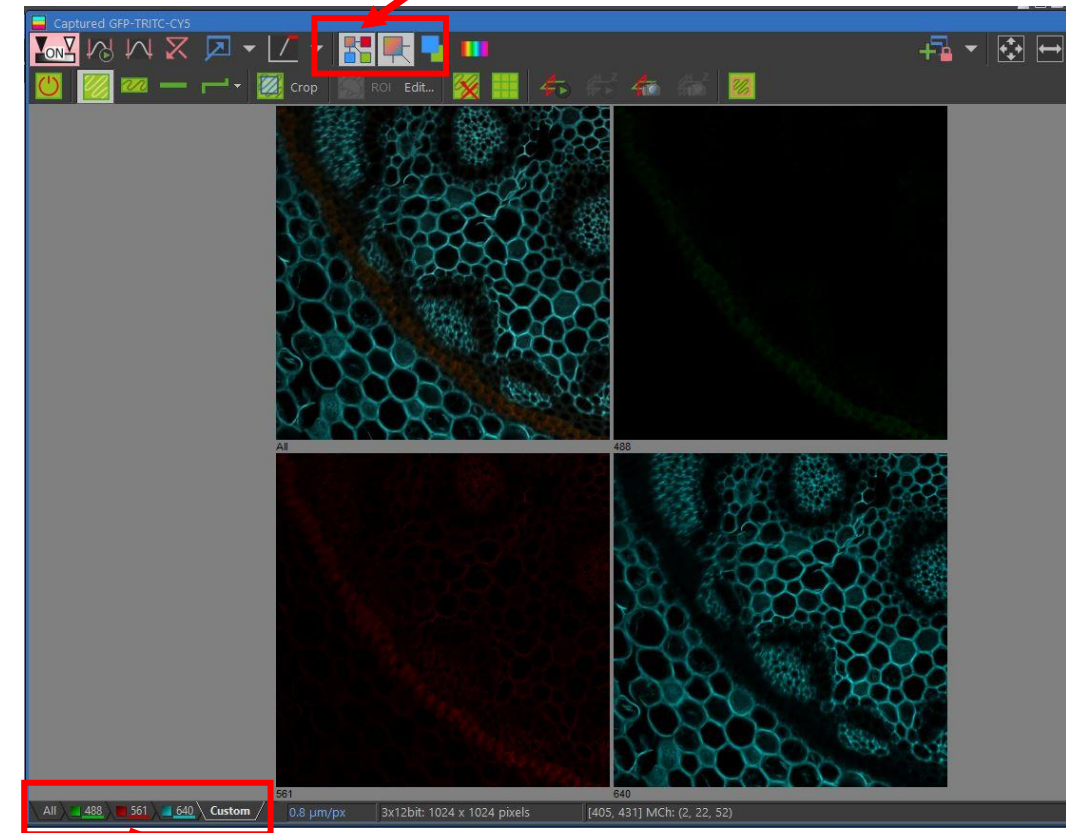
Image Field of View

1. In the A1plus Scan Area, the green frame represents the imaging field of view. You can change the shape of the field of view (square, rectangle, line scan, or free draw).

2. Put the mouse cursor on the frame, left click, then drag it to zoom in or out, or rotate, or move around to your desired region of interest.

3. Once the field of the area has changed, **make sure to right-click** on the frame to turn the red frame to green to activate the change.

4. The Zoom factor indicates how much you have zoomed in.

5. When you click on Nyquist XY, it will show you the maximum zoom factor for the objective selected. Zooming in more than the Nyquist XY suggested number will cause oversampling.

6. To restore to the full field of view, click on .

7. If the software layout is messed up, or you accidentally closed any section, you can click on the restore icon on the left sidebar of the software to restore everything to default.
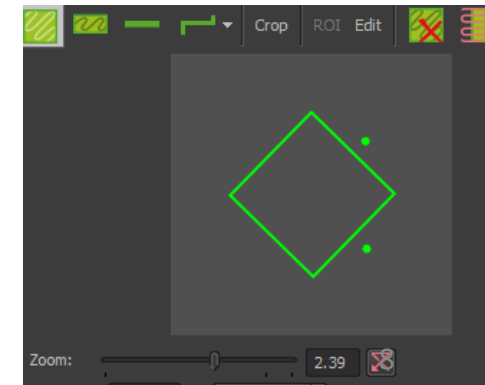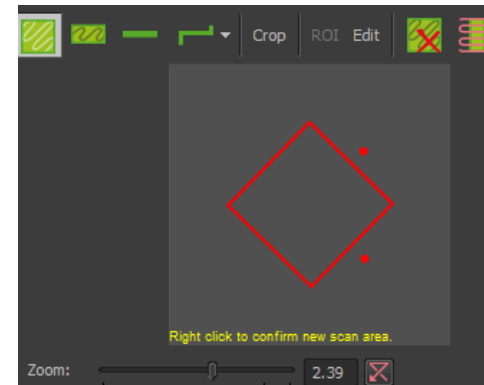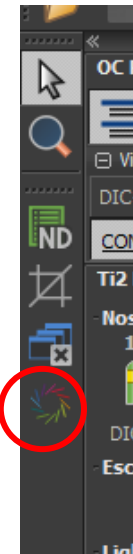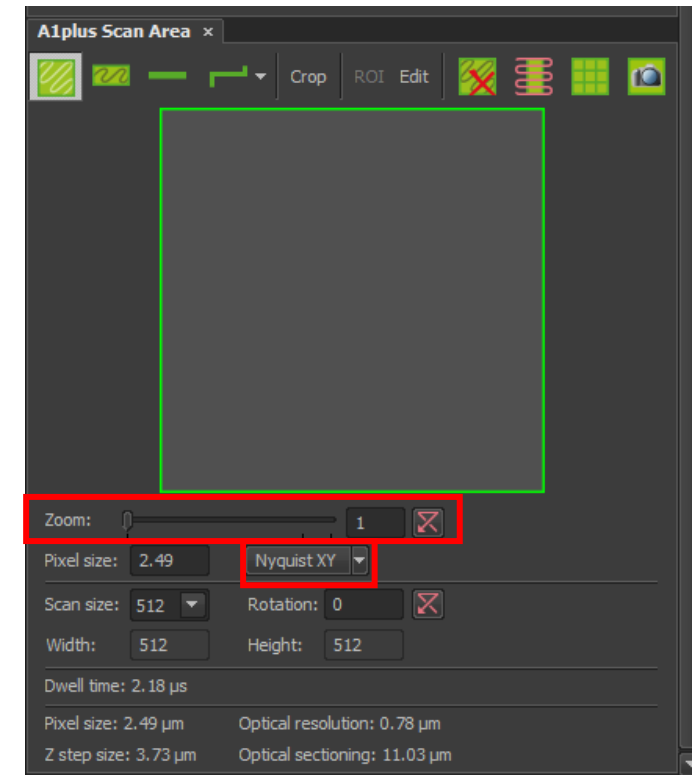
ND-Acquisition

1. Settings for time lapse (Time), multipoints (**XY**), Z-stacks (**Z**), multichannel (**λ**), and large image scan (Large Image) are in the ND-Acquisition tab.
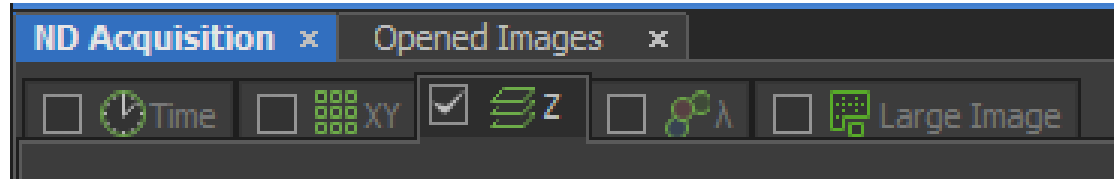
2. To activate any of these functions, check the box in front of the name of that function. Any of the functions under ND-Acquisition can be combined. The Large Image function in the ND Acquisition panel cannot be used alone, it must be combined with other functions. (To use scan large image function alone, please refer to page 13).

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.

4. Images taken under ND Acquisition can be autosaved by checking the Save to File option.
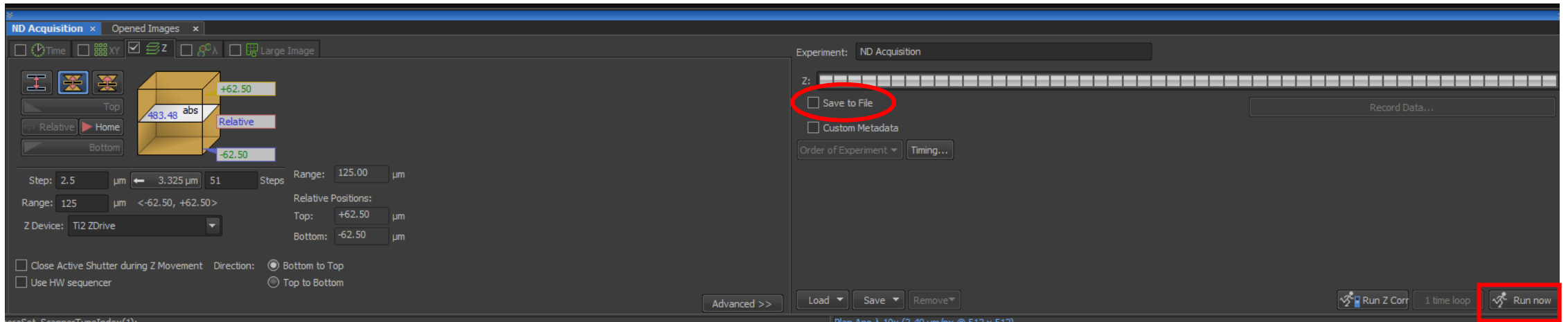
Z-stack acquisition

1. Z stack imaging set up is under ND Acquisition. Check the Z box to activate Z stack acquisition.

    Define top and bottom (Most commonly used)

    Define the z plane in the center

    Define a z plane within the z range

2. To define the top and bottom of the z stack, select only one laser line to reduce photobleaching. Double-check that you are scanning at a faster speed, then click on Scan to start a live scan session. While looking at the live view window, change the focal plane to the bottom of your interested region, then click on Bottom to memorize this Z position. Next change the focal plane to the top, click on Top to memorize the top position.

3. Range shows your overall z range. Step size and the number of steps can be changed within the defined z range. You can click and use the recommended step size.

4. After defining the z range, check all the laser lines you need, and switch to a slower scanning speed and at least 1024 image size.

5. Then click on Run now at the bottom right corner in the ND Acquisition section to start a Z stack acquisition.

Things to keep in mind when setting up a Z scan.
- Use faster live scan speed to avoid photobleaching.
- Make sure that signals from the best focal plane do not overexpose.
- If deconvolution is needed in image analysis, do not use step sizes bigger than recommended. If no deconvolution is needed, 2x the recommended step size is a good start to reduce your overall scanning time.
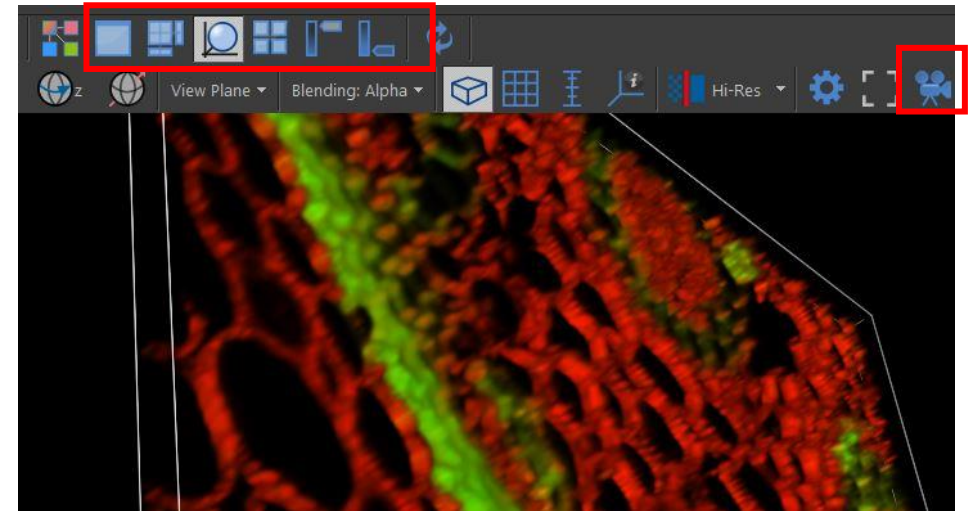

Recommended Z step size
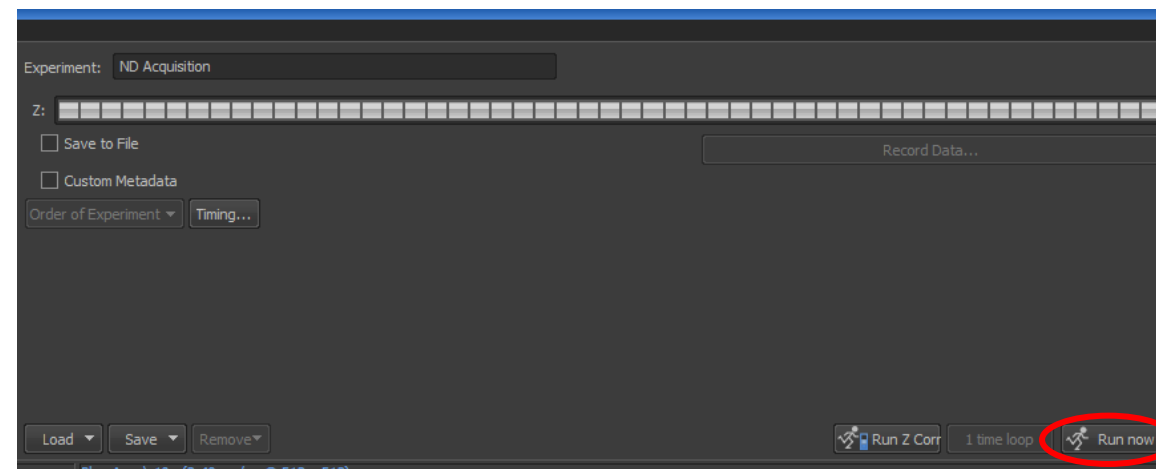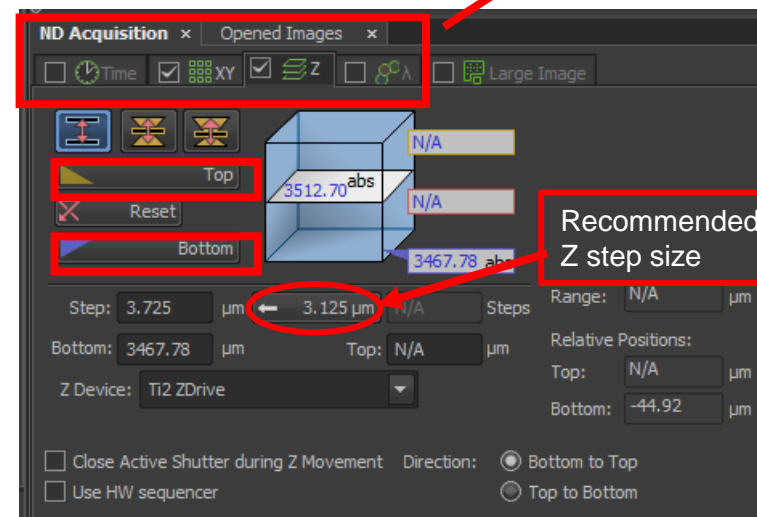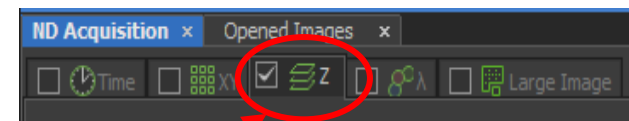
6. There are several option to view your Z stack images.

7. On the top of the image window. Options1-6.

1- 2D view
2- Z view/orthogonal view
3- 3D volume view
4- Z slices display view
5- Maximum intensity projection
6- Minimum intensity projection

8. You can make a video by clicking on the video button.

Resonant Scan

- The default scan is Galvano scan. Resonant scan significantly increases the scanning speed but compromises the resolution. Simply click on Resonant to switch the scanning mode.

- To increase the resolution using Resonant scan, you can choose image size 1024x1024 plus a 8x or 16x averaging.

- Resonant scan is often used for live-cell imaging, also to decrease the time of a huge Z stack image. You can switch to Resonant mode when you are trying to define the Z range, this can minimize photobleaching.

11
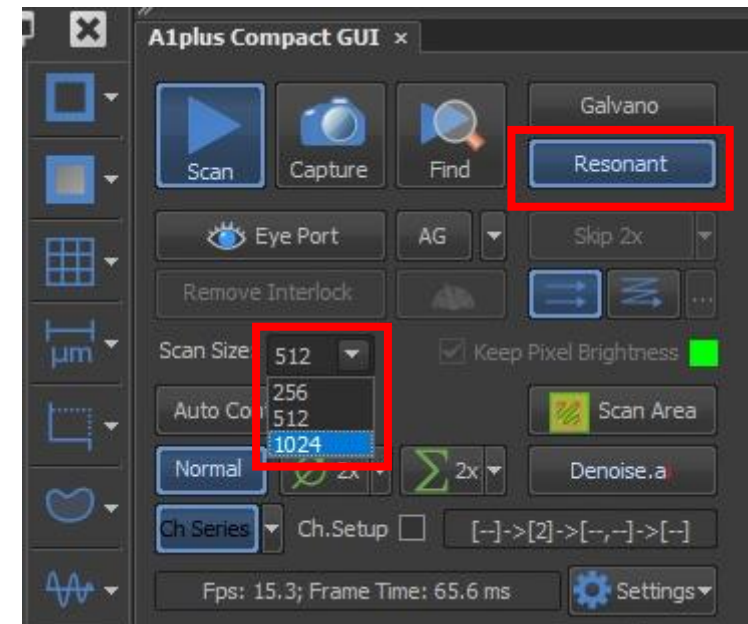
Time-lapse acquisition

1. Check the Time icon for set up time lapse imaging for live cells.
2. Set up a time-lapse setting by defining the interval between image time points, the overall duration of imaging time, and the number of time points (loops).
3. Phase allows you to set up several different settings and will run them sequentially.
4. If you want to image as fast as possible, you can select no delay under Interval.
5. If your time-lapse has a long duration, you must turn on PFS (perfect focus system) to prevent drifting over a long time period.
6. When the experiment is set up, click Run now to start the experiment.

Multi-points acquisition

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 Include Z is checked.
3. Repeat step 2 to add more points.
4. If a point is not selected (tick in front of the 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.

The Multi-points function allows you to select several positions of interest and build a list of positions that the microscope memorizes. Once acquisition starts, images at these points are captured automatically.

Scan Large Image

1. In the top tool bar, under Acquire, Select Scan Large Image.
2. Then in the Scan Large Image window, 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 large area, or at the top left corner of the entire large area.
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.
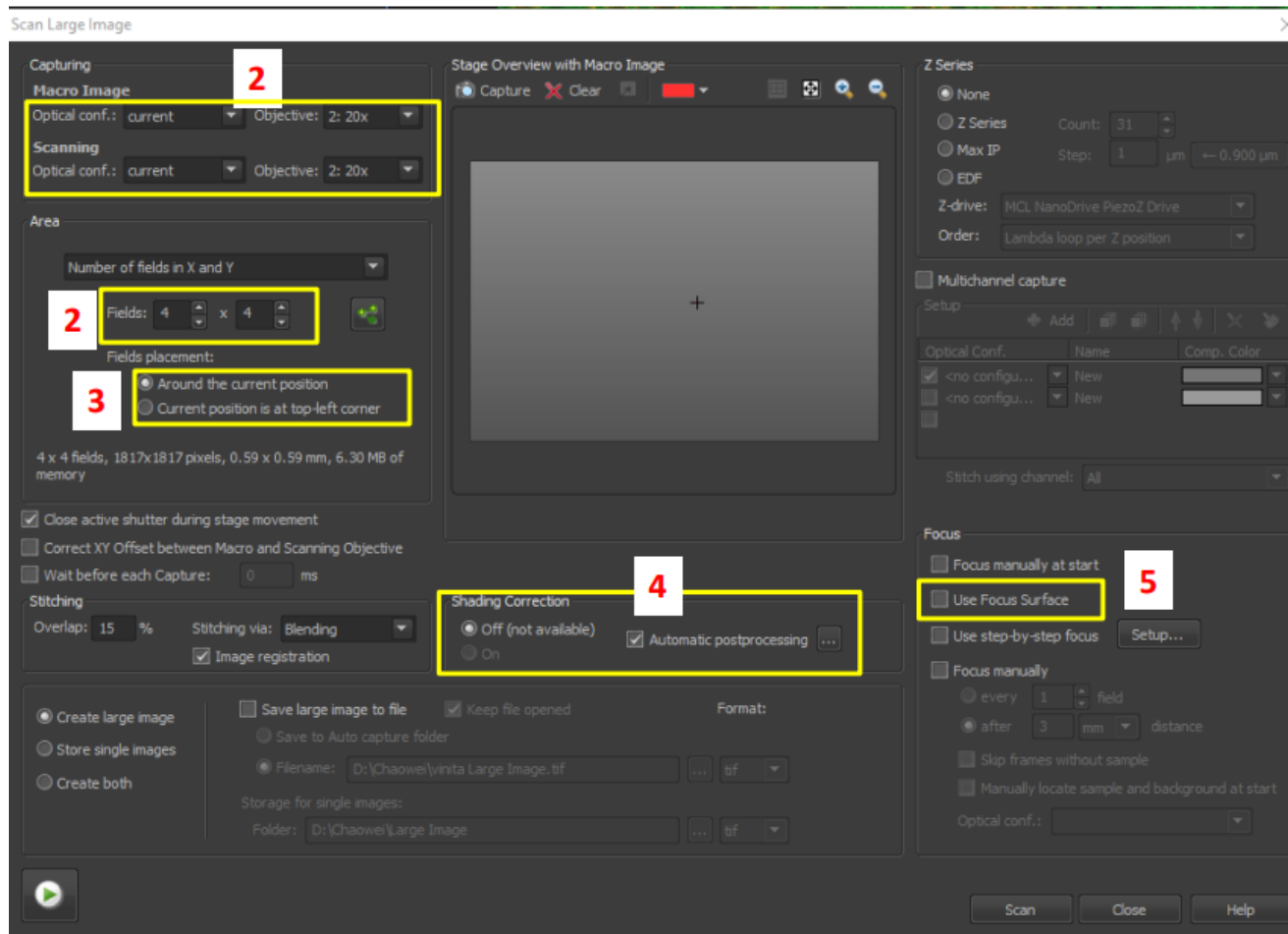

Large image function is used to scan several images to cover up the entire field of view, then the software will automatically stitch them together to form a large image. (Also called: Mosaix)
The Large image function under ND Acquisition can not be used alone. It can be used when combined with other ND acquisition functions. To use the scan large image function alone, you need to open the Scan Large Image window through the top toolbar as indicated in step 1.

Spectral imaging and detectors

1. By default, the microscope is set up to use different bandpass cubes for emission light, which is option DU4. This is good enough for most imaging settings. *(You can ignore this page if you do not have any experience in spectral detection.)*

2. If you prefer to use the spectral detector for custom tuning of emission wavelength to avoid crosstalk, you can select VB. Then click on setting to open the setting window and select laser lines.

3. If you want to do a fluorescent spectral acquisition, you can select CB.

4. The laser lines setting looks a little bit different when they are in spectral detection mode.
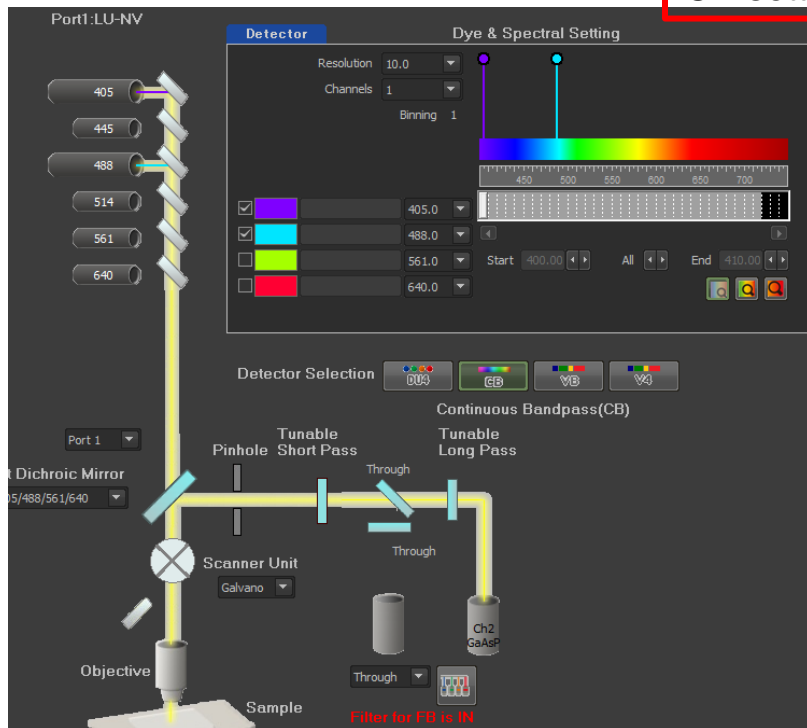
Setting
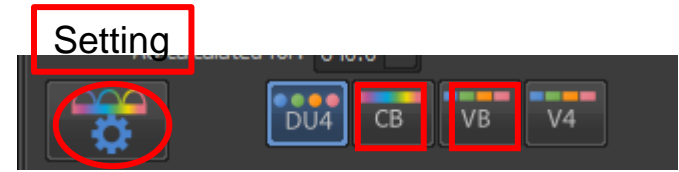
VB setting window
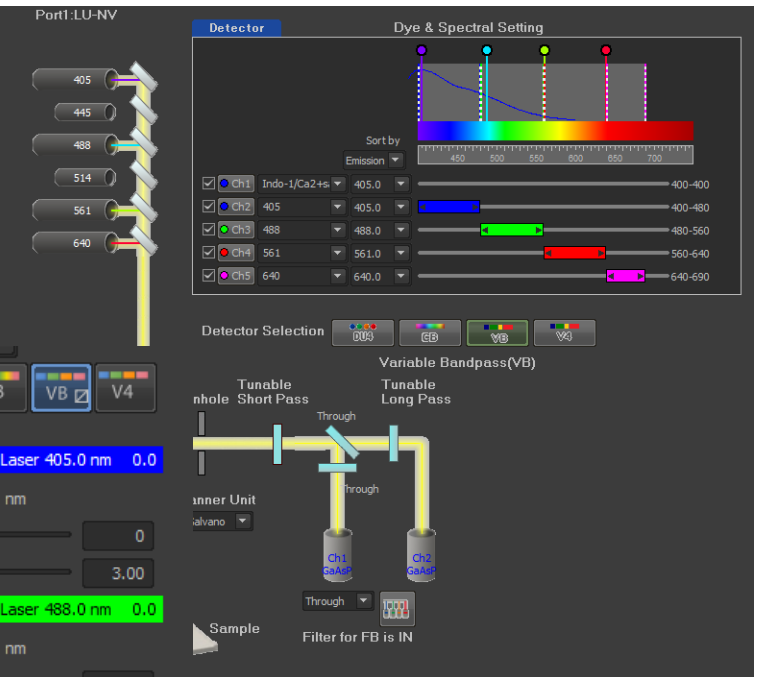
CB setting window

VB laser setting

CB laser setting

15
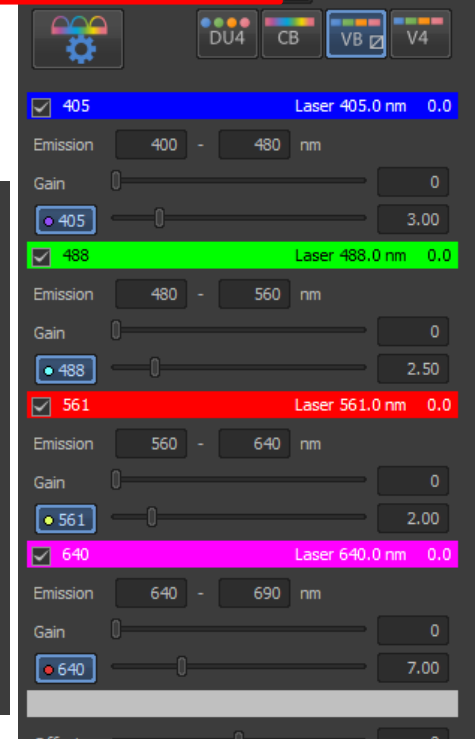
High Content Acquisition

1. At the bottom left corner of the software layout, click on HCA which stands for high content acquisition.

2. Then you will see the Well Plate settings. You can click on to add your plate template. You need to align the plate the first time setting it up.

3. For more complicated experiment setups, you can use JOBS Explorer.

4. The 40x ELWD objective is specifically designed for plastic bottom plate/dish imaging.
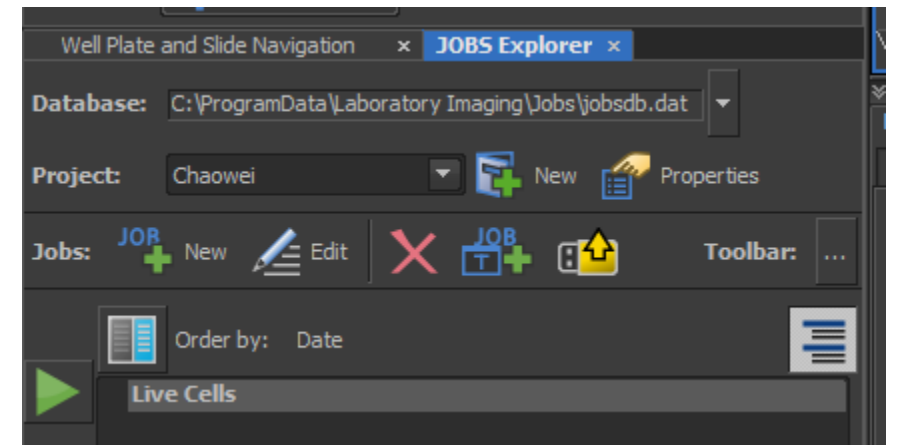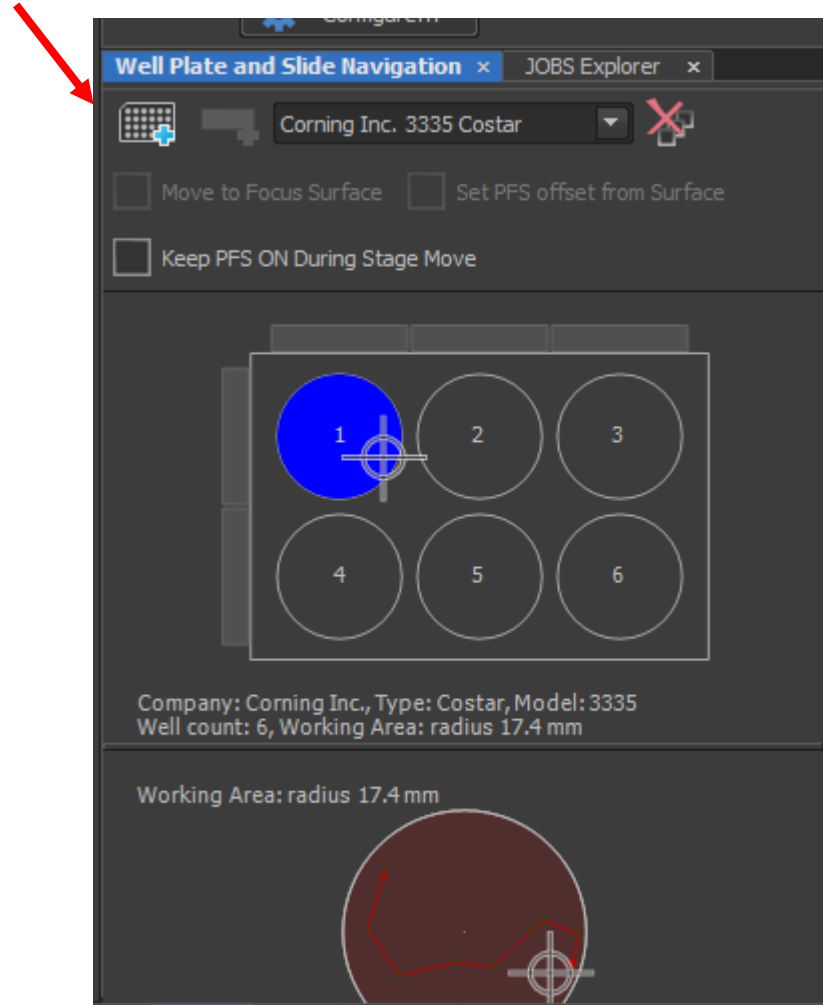
Save and export images

1. After an image is captured, click on File→ Save or Save as to save it on the data drive (**D drive**). Do not save anything on the desktop or C drive. Your original image format is .nd2.

2. To export into .tif files, click on File→ Save/Export to TIFF Files. In the export window, save the files as merged and individual channels as shown below. Check Burn Scale if you want your exported tiff files to show the scale bar.
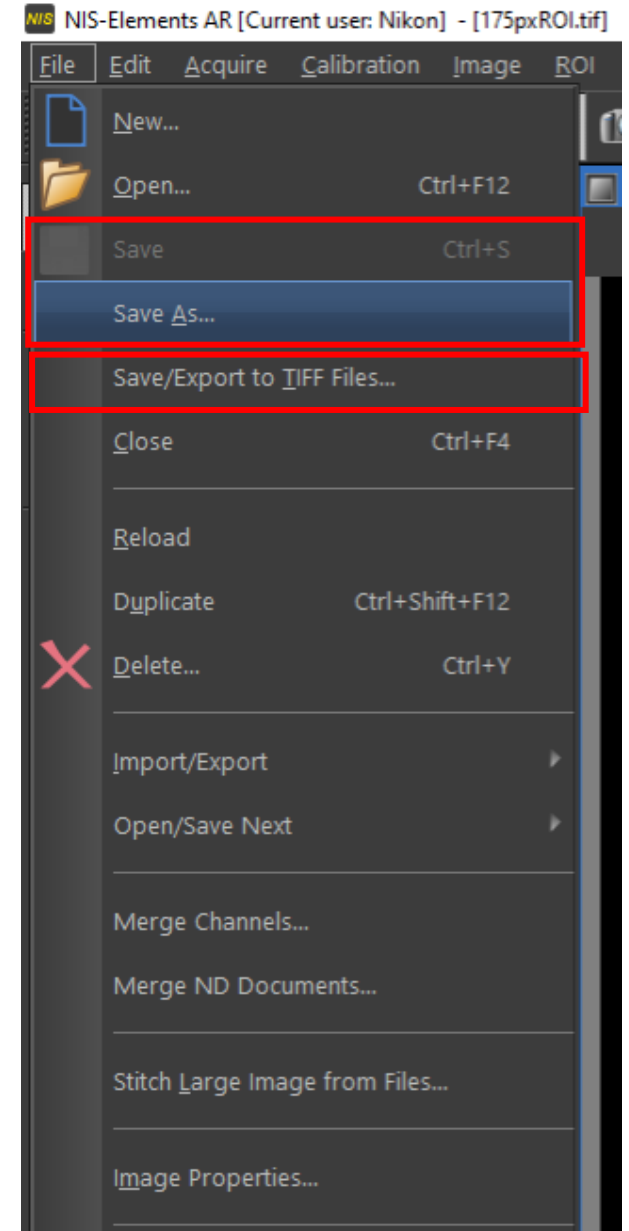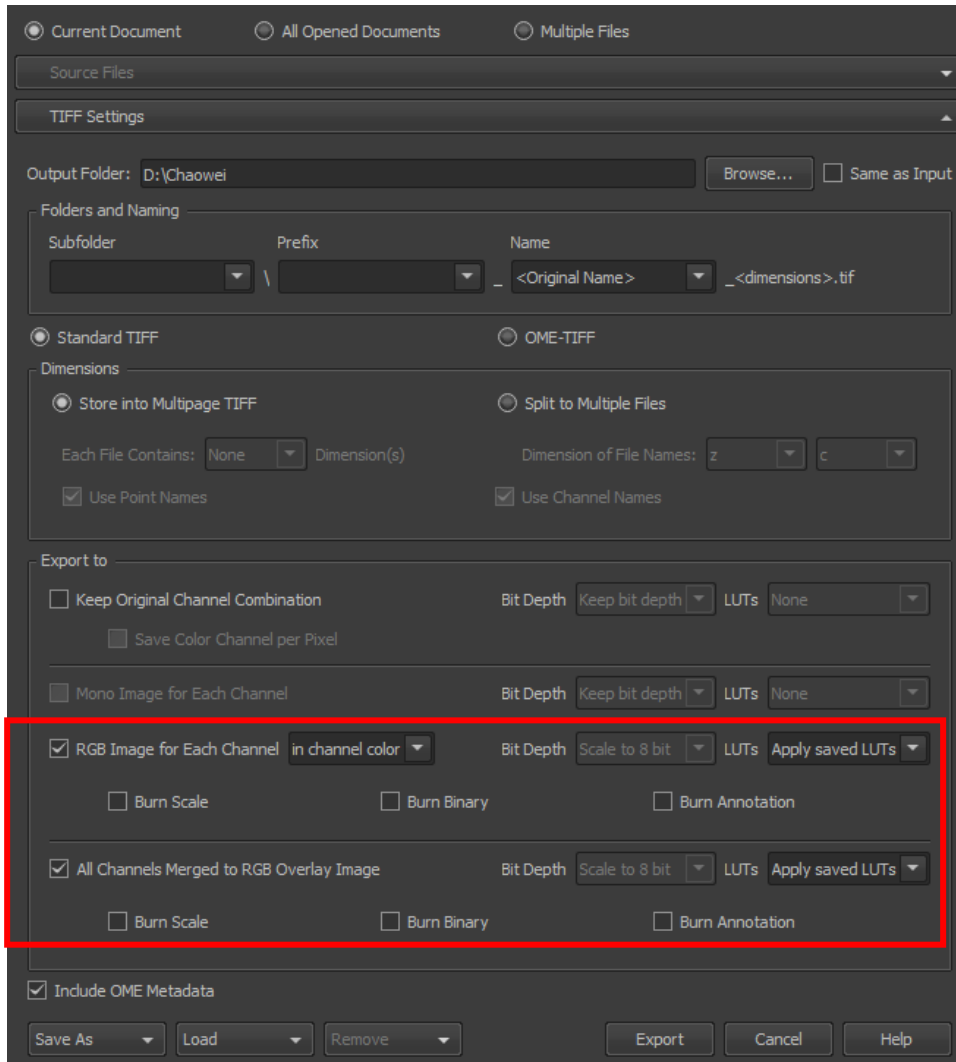
Shut down the Nikon confocal & TIRF system

1. Clean the objectives with lens paper if you used oil objectives. Please also check whether the dry objectives accidentally caught oil if you used both oil and dry objectives.

2. After cleaning, change the objective to 10x and lower the objectives to the bottom.

3. Clean your sample slide if you added oil to it with kimwipes.

4. Clean the sample holder and the stage if oil is caught on stage. Check both sides of the sample holder (Do not skip this step!)

5. Turn off the microscope in the order of 4→1 or 4→0 as labeled on the equipment.

6. Clean up the desk and do not leave any samples behind.

7. Clean up the biohazard bag if you had live samples.

8. Log out on the log sheet.