SOP: Confocal Microscope (Nikon A1)

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Equipment Overview

The confocal microscope distinguishes itself from conventional microscopes because of it's pinhole that rejects out-of-focus light to produce sharp images of thin cross-sections from a sample. By scanning many sections of the sample, a clean 3D image can be obtained. In other types of microscopy where the entire sample is excited, confocal microscopy only scans one point at a time – never a complete image of the sample.

There are 4 lasers for the Nikon confocal microscope. Each laser provides an excitation light that reaches the sample, and emits light through a dichroic mirror and a pinhole. The light passes through a pinhole and is captured by the detector.

Below are position and details of the available lens for the microscope:

1 = 40x/0.6, ∞/0-2 WD 3.6-2.8, DIC N1. Plan Fluor ELWD. Recommended for petri dishes, microplates, and microfluidic chips.

2 = 60x/1.4, ∞/0.17, WD 140 um, DIC N2, Apo. Recommended for petri dishes, microplates, and microfluidic chips. Recommended for coverslips. *This oil immersion lens needs to be wiped with 70% ethanol and lens paper after each use. Failing to do so will result to debris build-up and ruin the lens.*

3 = 4x/0.20, ∞/- WD 20, Plan Apo.

4 = 10x/0.3, ∞/1.2 WD 15.2, Ph1. Plan Fluor.

5 = 40x/1.30, ∞/0.17 WD 0.24, DIC N2, Plan Fluor. *This oil immersion lens needs to be wiped with 70% ethanol and lens paper after each use. Failing to do so will result to debris build-up and ruin the lens.*

6 = 20x/0.45, ∞/0-2 WD 7.4, DIC L/N1, Plan Fluor ELWD. Recommended for petri dishes, microplates, and microfluidic chips. DIC

The filter cubes available for the instrument include: **DAPI, FITC, TRITC, ANAL.**

*Note: the ANAL cube is for DIC and polarization, please see section **Optional Method – DIC and Polarization** if interested.*

Notes before use:

- Log into the instrument on LMACs to start using it, and sign-out before leaving.
- During system start-up, wait 30 seconds between instrument units to prevent communication issues.
- Distance the objective lens about 0.5 com and then increase to focus in on the sample. Never start by getting super close to the sample.

Supplies and Reagents

Provided by CRAFT:

- RTU disinfectant
- 70% Ethanol
- Kimwipe
- PPE (lab coat and safety glasses)
- Stage insert for holding samples (dish, microplates, slides, etc)

Method – Confocal Setup

Hardware Startup:

* Please wait 30 seconds between components during start-up, otherwise communication issues may occur.

1) Under the table, turn the keys clockwise for the 4 laser units.
2) Turn on the microscope by pressing the switch on the right side.
3) Switch ON the Transmitted Light & Stage Controller.
4) Turn ON the A1 Confocal Controller by pressing the button to the left of the unit.
5) Place the sample onto the microscope stage using the appropriate platform (microscope slide holder, well-plate holder, etc).
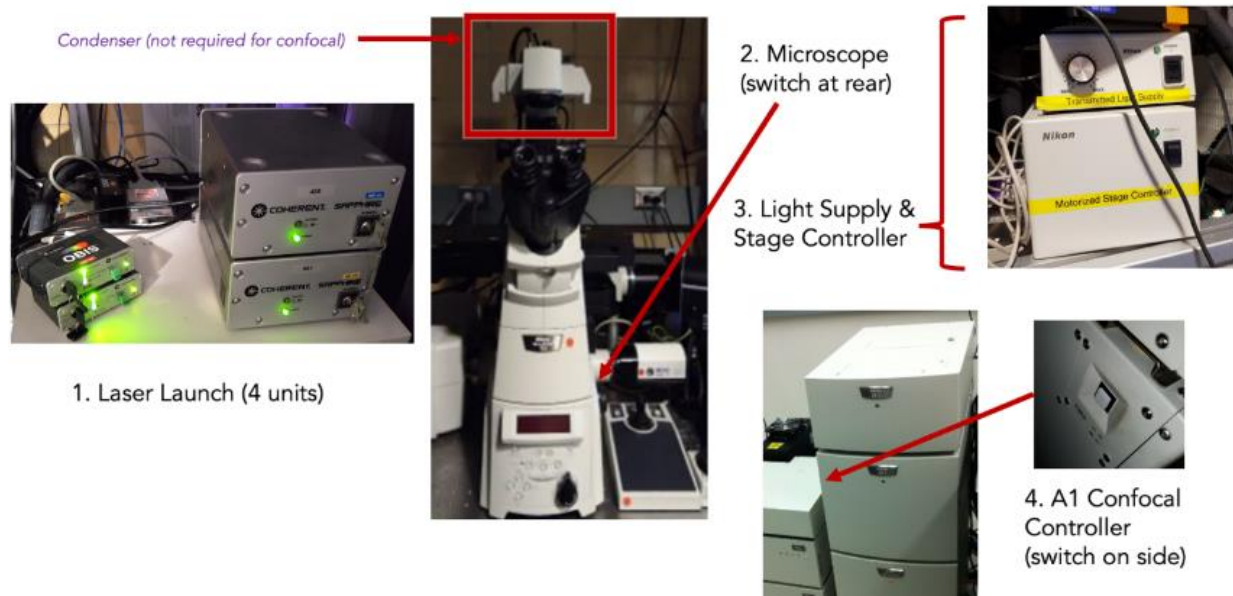
Software Setup

1. Once the hardware is on, open the software by clicking the **NIS-Elements** icon on the desktop.
2. Select **Nikon Confocal** as the driver.
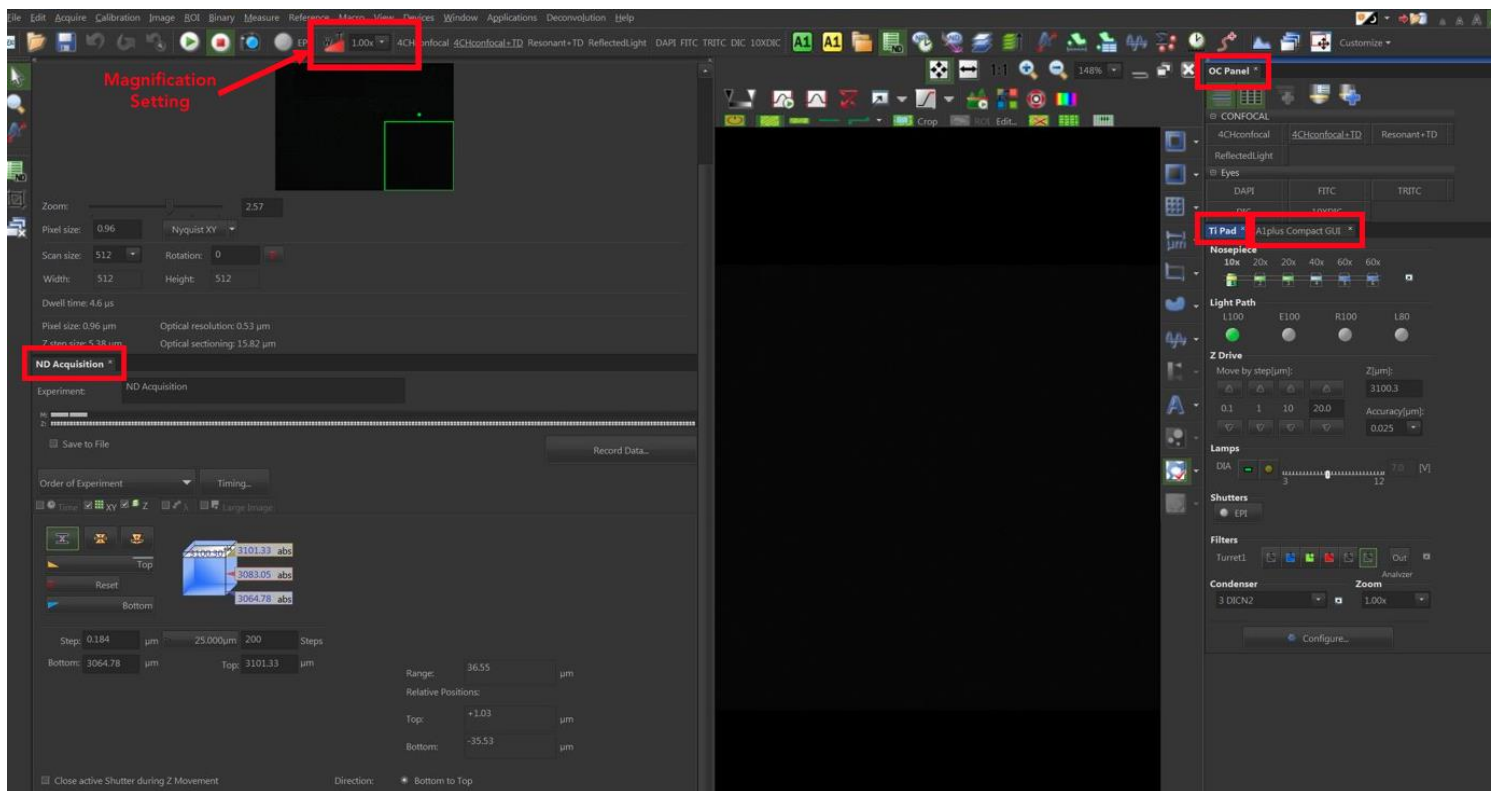
Note:

- *When the 1-1.5x **magnification** knob on the confocal is adjusted, it also needs to be selected in the software (see above photo).*

Below are the standard widgets:

- <u>TI pad</u> → Controls the microscope settings such as brightfield light intensity, switching between eyepiece, filter cubes, etc.
- <u>A1Plus Compact GUI</u> → Controls the confocal including image acquisition, resolution, frame rates, and laser settings.
- <u>A1Plus Scan Area</u> → Shows the microscope field of view.
  - The green box can be dragged and reshaped to zoom in or out of an area. Once the box is modified, right-click to apply changes.
- <u>ND Acquisition</u> → Offers Z stacking, timed experiments, multiple locations, large image stitching, etc.

*Note: The widgets can be rearranged to the user's preference. If a widget is missing, right-click on the screen and select **Acquisitions Controls** to find it.*

<u>Viewing Samples with the Brightfield Lamp:</u>

1. Bring the condenser unit to the down position, above the sample.
2. In **A1plus Compact GUI,** select the settings icon (fireworks) and ensure the transmitted detector (TD) is in the **OUT** position.
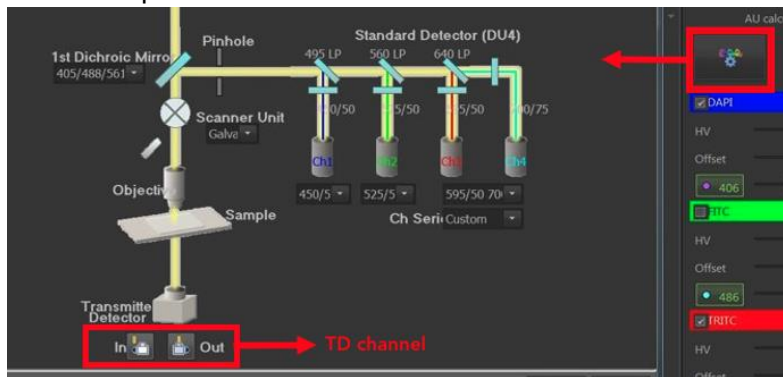
3. Select **Ti Pad,** then **E100** (for the eye piece). Ensure **Lamps (DIA)** has a green bar, and the orb is turned ON (yellow). Adjust the light intensity (V) as needed on the slider.
4. Use the <u>Nikon TI-S-EJOY</u> to control the microscope stage and find your sample.
   There are 2 major components to this controller:

a. The centre joystick: this controls the X/Y movement of the stage. Twist the joystick to adjust the movement speed (indicated by yellow LED).
b. The right focus knob: this controls Z-axis movement of the objective. The real-time Z value is displayed on the bottom right of the software window. As a general guide, at 10x, Z is ~3100 for microscope slides, and ~4700 at 20x. Adjust the speed of Z-axis movement by clicking on the **Z Speed** button (the speed is indicated by the green LED)

*Note: brightfield in this scenario is for visual purposes only using the physical microscope. For actual brightfield images (not using the TD), please refer to **Optional Method: Brightfield Imaging Only**.*

Imaging with the Transmitted Detector (TD) channel:

The TD channel uses the FITC laser to produce **brightfield-like** images using the transmission detector located in the condenser. This mode allows users to capture bright-field like images alongside with fluorescent probes.

1. Bring the condenser unit to the down position, above the sample.
2. In the **TI Pad**, ensure the orb for the lamp is OFF (not yellow anymore), and select **L100**.
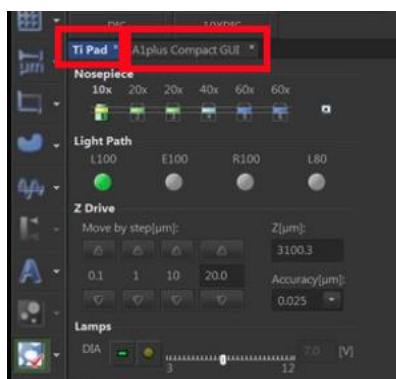
3. In the **A1Plus Compact GUI,** ensure the TD channel is at the **IN** position. Select the **TD channel**, and adjust the gain (HV) as needed.
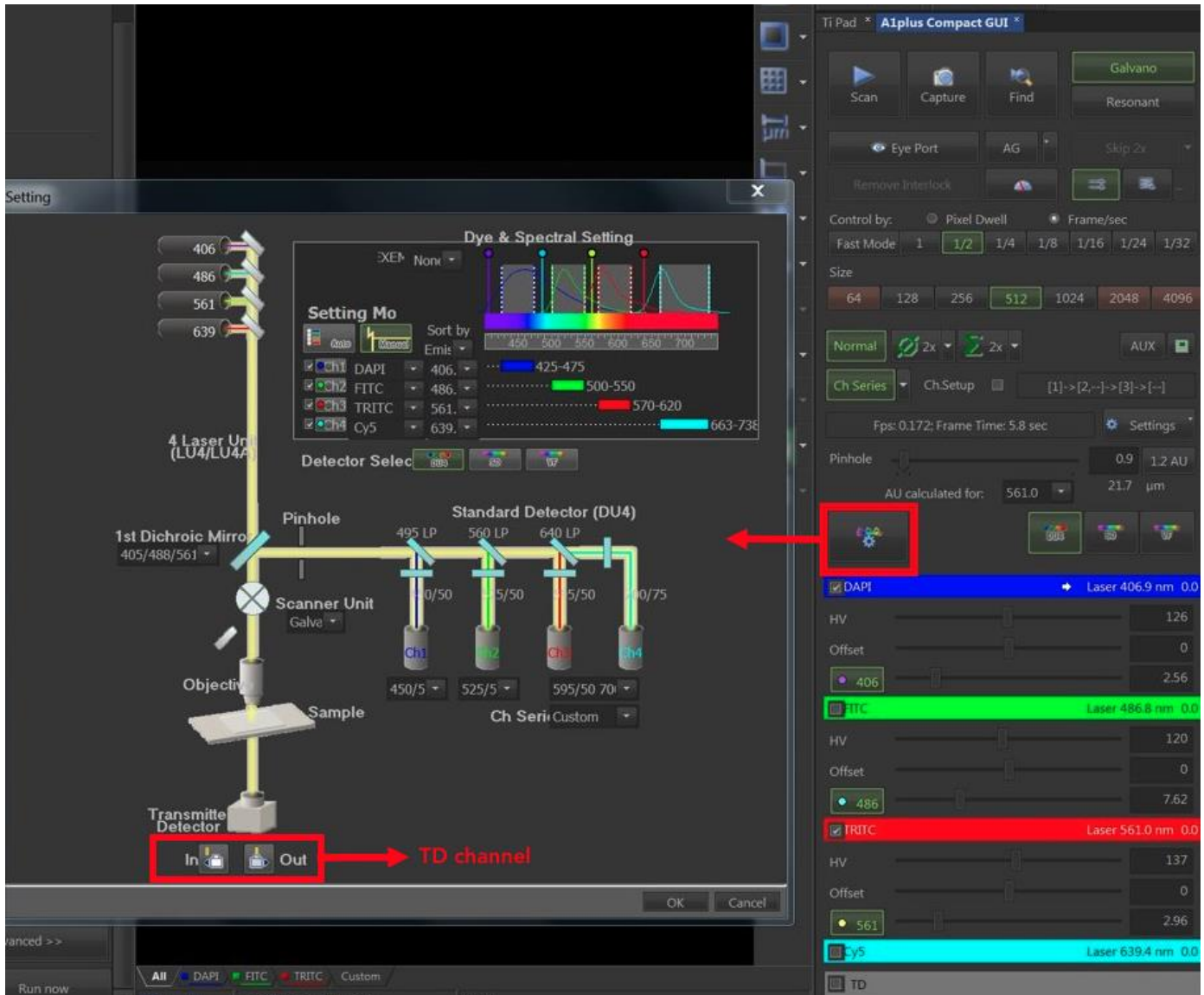
Note: Images from the eye piece cannot be visually seen at this point because the TD is placed in front of the transmitted light in the optical path.

Confocal Fluorescence Setup:

This popular technique provides true 3D images. The light from the laser passes through a pinhole, reflected by a dichroic mirror, and focused by a microscope objective to a small spot in the specimen.

1. Begin by selecting the tab **4CHconfocal+TD** from the **OC Panel.** This turns on all channels (DAPI/FITC/TexasRED/CY5/TD) to get everything working. Go into **A1plus Compact GUI** and select the desired channels.

2. Click **Scan.** If you no visible light is emitted onto the sample, click **Remove Interlock.**
3. Select **Resonant,** a high frame-rate imaging mode at lower resolution that produces a live video-like image on the monitor while you find the sample using the joystick controller for stage and Z adjustment. Below are the recommended, initial laser settings:
    a. HV-Gain: **115**
    b. Offset: **0**
   c. Laser power: **5%**

Note: a sample is in focus when it is the brightest.

4. After finding the sample, switch to **Galvano** mode to capture high resolution images.

Acquiring Confocal Images:

1. Look at each individual laser channel in **A1plus Compact GUI** by selecting the laser tabs on the bottom of the live preview window and adjust the settings (HV, offset, intensity) to produce a good image. Use the mouse scroll for fine tuning, or values can be entered manually.
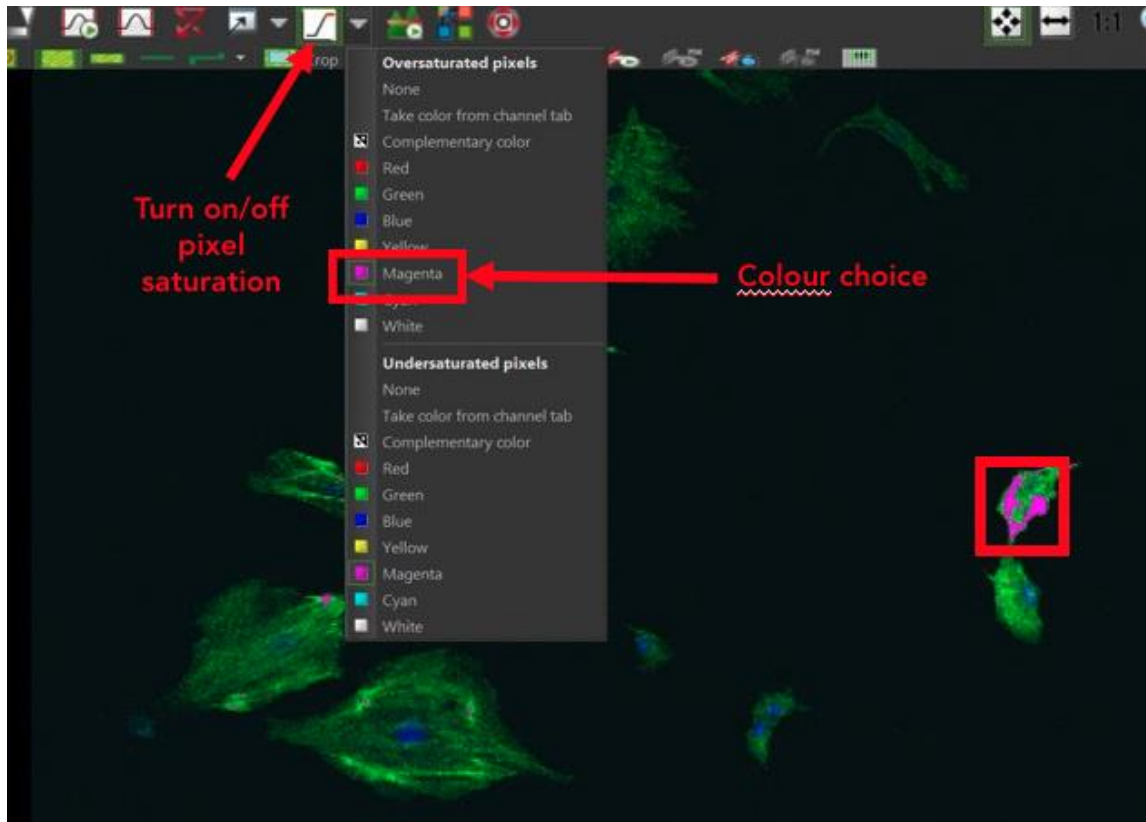
Pixel saturation:
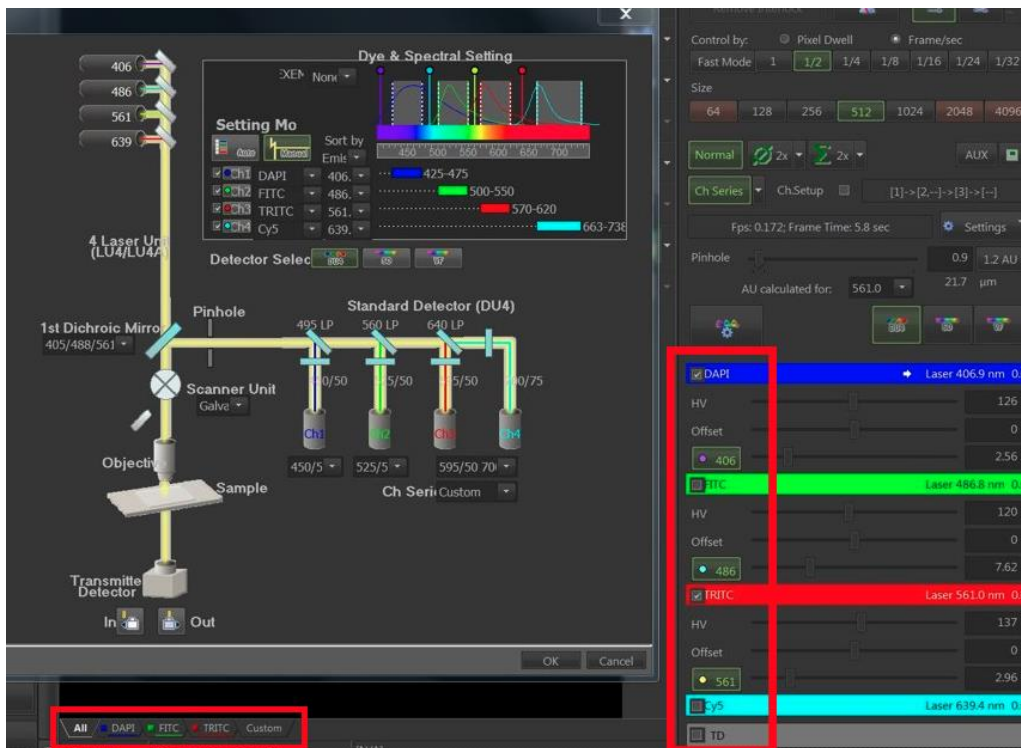
Enabling this feature allows users identify if pixels of the image will be over or under saturated. This helps bring out definition and details of the image.

1. Click on the **pixel saturation icon** on the top menu bar to turn it **ON,** and adjust each channel setting (HV, offset, laser intensity) to reduce over or under-saturation. Any choice of colour can be selected by dragging down the arrow next to the icon (see image below).

2. When satisfied with the settings, click the pixel saturation icon again to deactivate it.

Look-Up Tables (LUT):

The LUT table display pixel population (X) vs. pixel intensity (Y). These tables can be enabled during live-preview or after an image has been acquired, allow modification of the brightest and dimmest pixels for each laser channel to improve contrast and sample definition.

Select the LUT icon and drag the left and right outer limits (representing 0% intensity and 100% intensity, respectively) of each laser channel until the background doesn't change colour and the sample's definition improves.

Do not manipulate the diagonal line crossing the tables. This relationship, called gamma, is considered artifical manipulation of the image and is not permitted for images that will be published.
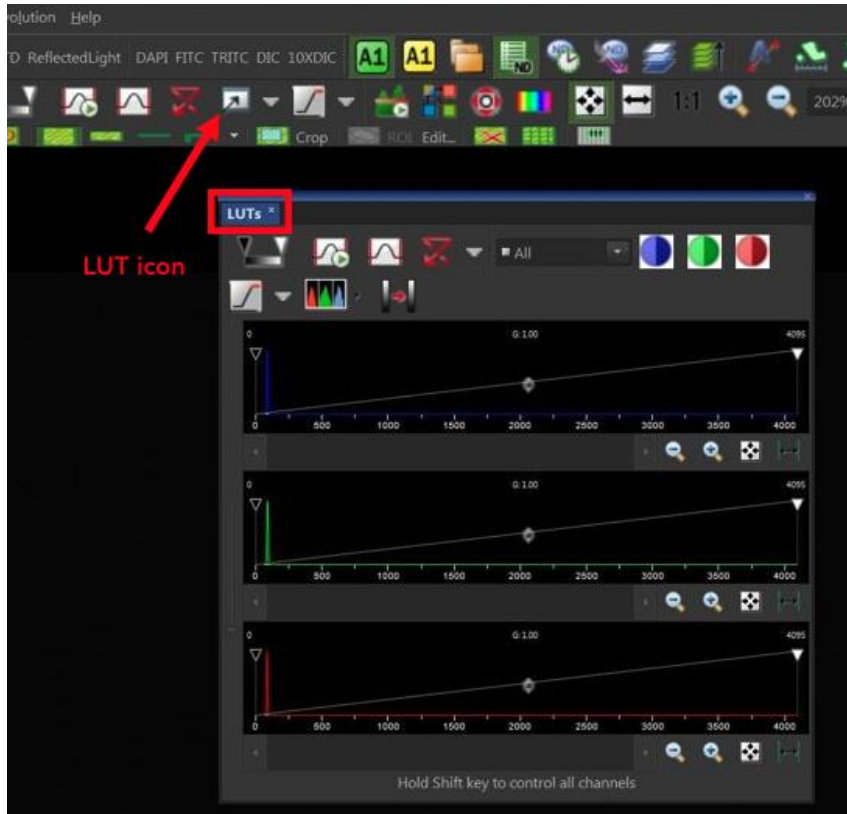
Annotations:

Found on the right toolbar, users can <u>add lines, symbols, scales, writing, etc</u>. These annotations can be burned into pictures as a JPEG or TIFF file, meaning the photo will display as it is (see **Saving & Exporting Images** section below):
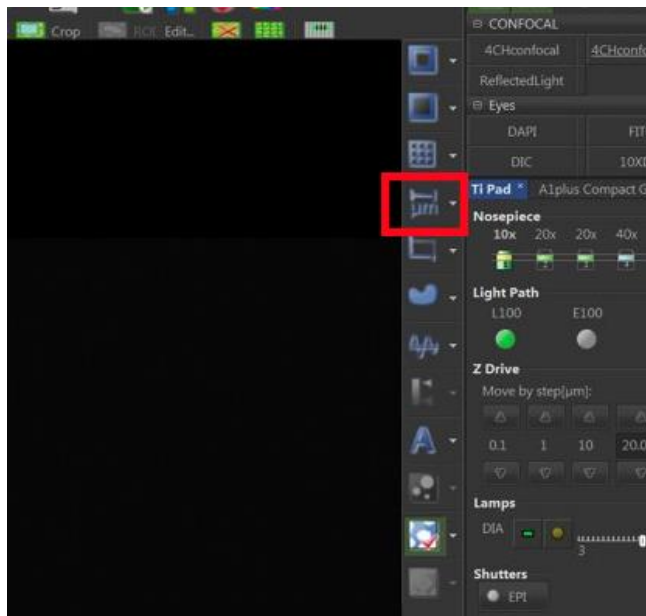
Saving & Exporting Images:

Note: Users can open previous ND2 files to reuse the same camera/device settings.

1. To acquire an image press **Capture.**
2. To <u>save an image</u>, select **File → Save As.** (use either **.tif** or **.jp2** for raw data) . Users are to save data in their personal folders. All data saved must immediately be backed up or exported from the computer. The confocal computer is regularly wiped clean to prevent the disk from getting full. CRAFT does not take responsibility for lost files so please make a duplicated of all data you generate on this computer.
3. To <u>convert </u>into viewable image, click **Image → Convert to 8bit RGB** and save a copy.
   To save the experiment (data + settings) choose **nd2**. This can be opened at a later date, and in several other software (ImageJ, Fiji, etc).

Advance Imaging Using ND Acquisition:
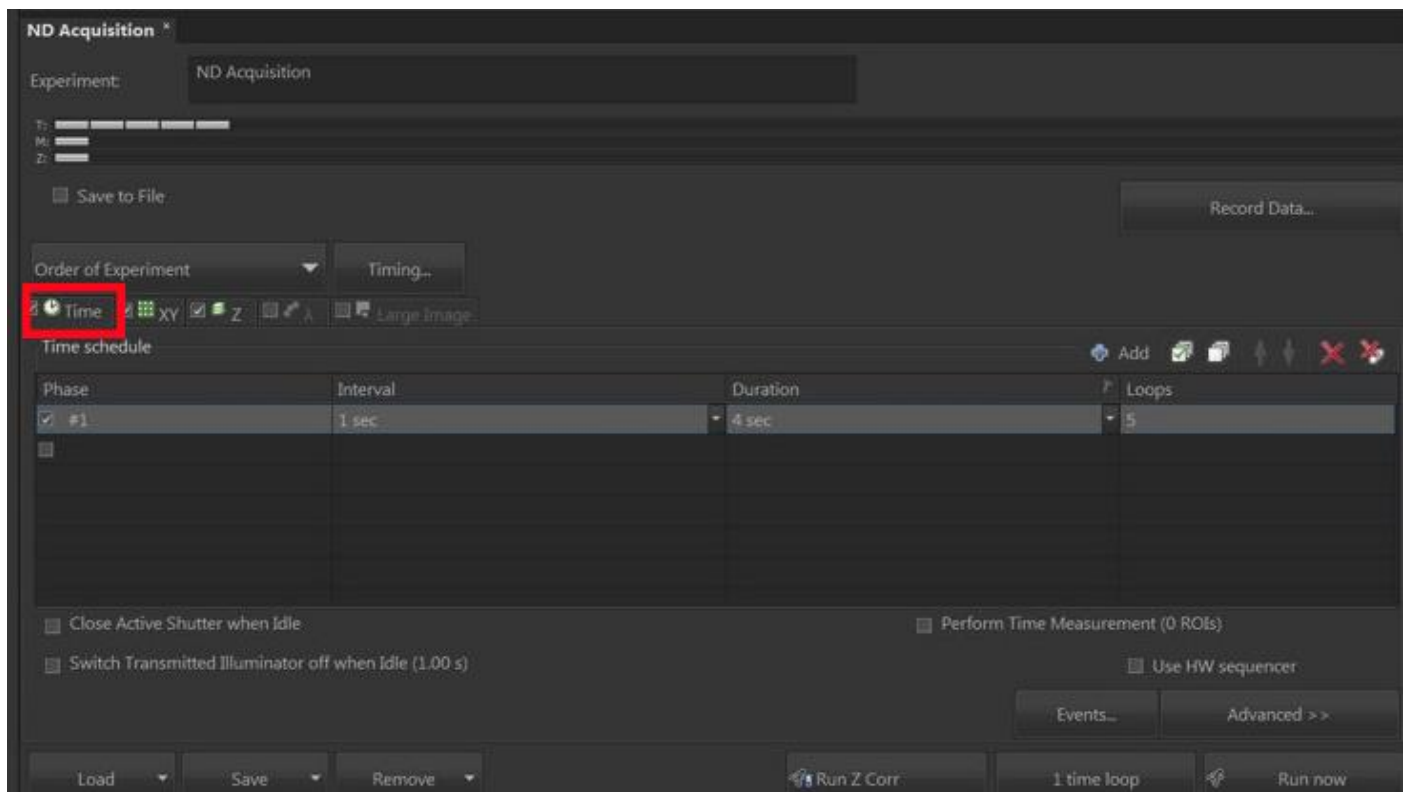
<u>Time tab</u>: this allows the user to capture photos at set intervals for time lapse.

1. Set the **Interval.** For example, setting to 1 second will tell the instrument to take a photo after 1 second has passed.
2. Set the **Duration.** For example, the entire experiment should be captured for 4 seconds.
3. **Loops** is the number of photos. In this example, there are 5 loops because each photo is captured at 1 second intervals for a total of 4 seconds, plus one default photo at t = 0 seconds (initial).
4. If additional time lapse scans are needed, click the **Add** button and set the parameters. For example, the first experiment could be to take a photo every min for 20 minutes while the second experiment is to take a photo every hour for 5 hours.

<u>XY tab</u>: users can capture images at different locations of their sample on the stage.

1. First, remove all previous points.
2. Click **Add** to add the current location of the sample.
3. Check-off **Include Z** if moving between two distinct points (ie. for different elevation levels in cell culture). When Z is included, the user will need to manually adjust the focus at every new location added. Double-clicking on previous lines will go to that location.

   Note: Z-stacking is not possible at this point.

4. If satisfied with all the settings, click **Run now**.

<u>Z tab</u>: this allows users to define the stack size, step settings, top and bottom layers for Z stacking. Z
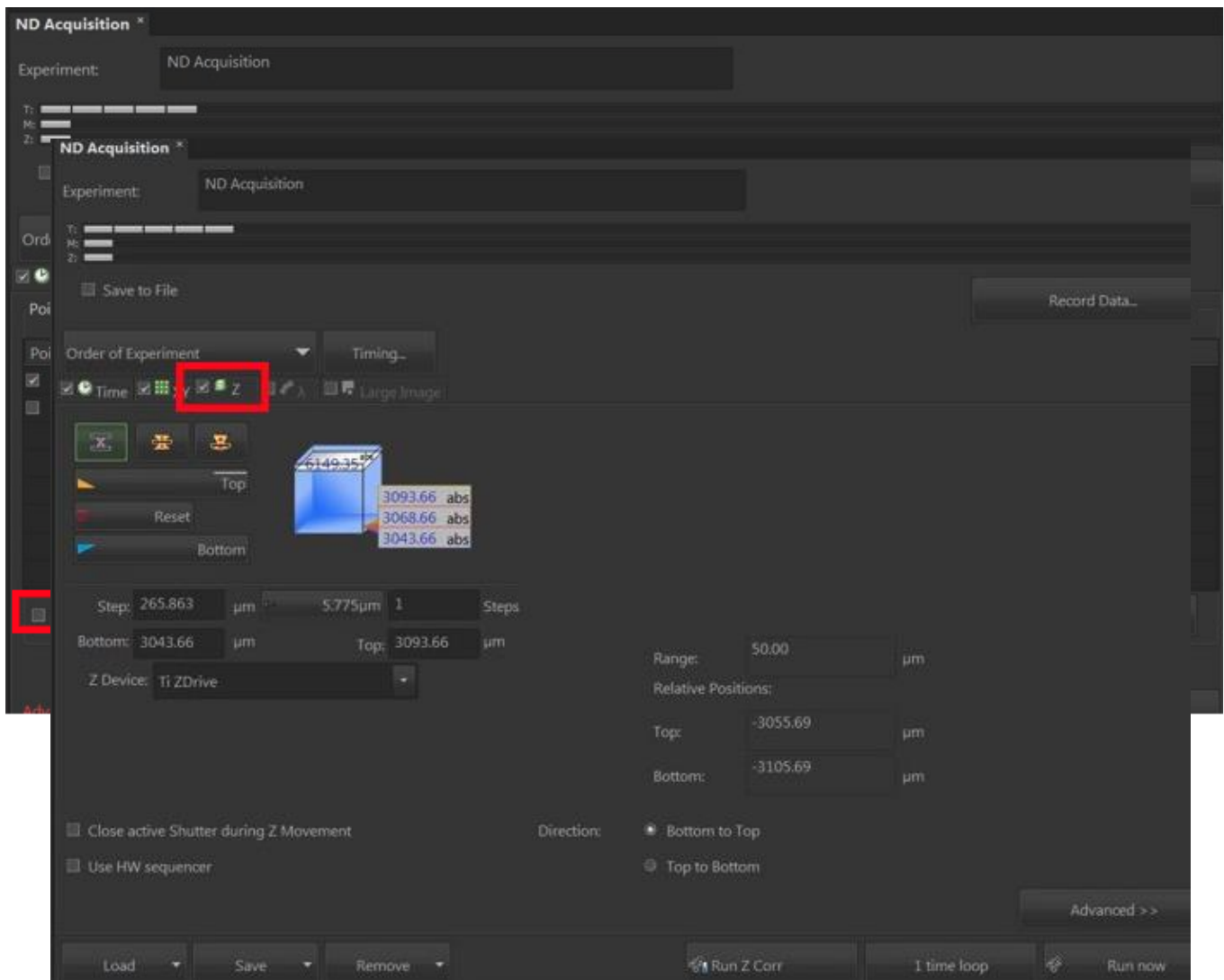
stacking is a method that combines images taken at different focal planes to create a composite 3D image with greater depth of field.

1. Begin by changing the Z speed of the Nikon EJOY controller to **Fine**.
2. Turn the focus knob clockwise (Z value decreases) until the sample disappears and click **Bottom**.

3. Turn the focus knob counter-clockwise (Z value increases) until the sample disappears and click **Top**.
4. Adjust the **steps** (# of stacks) or step height (um) as desired. The more steps across the range the better the Z resolution of the final 3D construct.
5. When satisfied, click **Run Now**.

Large Image Acquisition:

This technique stitches image tiles together to form a large image.

1. On the top tool bar, click **Acquire** and select **Scan Large Image**.
2. Ensure **Macro Image** and **Scanning** settings match in the **Capturing** section.
    *   The current settings must match to the microscope settings.
3. Click on **Capture** (middle section) to display the current image.
4. Modify the number of fields in the **Area** section. You'll notice the red tiles arranging around your image.
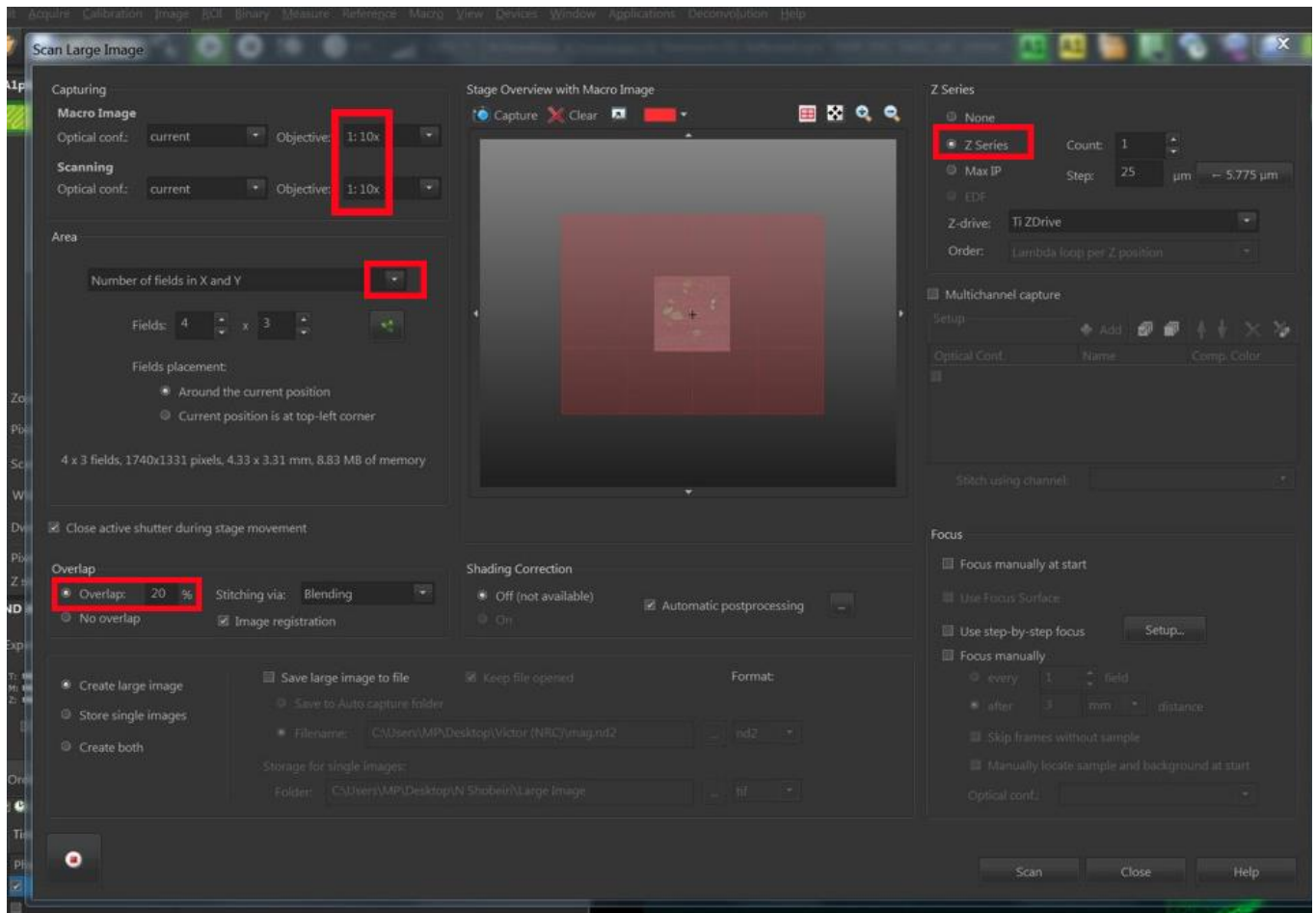

*Note: Increasing the **overlap** % will make the image tiles closer together and allow the software to better stitch the pictures.*

5. Optional: **Z Series** allows the user to combine Z-stacking with large image acquisitions. The **Count** indicates the total number of Z-stacks, and **Step** is the spacing between them. For example, a count of 5 means there is one step at the current focal point with 2 steps above and below it.

Optional Method – DIC and Polarization

Differential interference contrast (DIC) microscopy is a contrast-enhancing method that can produce shadow-like images to indicate gradient intensities for transparent samples. Morphology, details of specimen thickness, and surface components can be seen. Below (left) is a DIC image of cervical cancer cells:
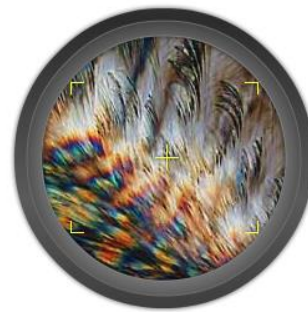
Polarization improves the image quality of birefringent materials (having 2 different refractive indices) and can be seen in this setup. Above (right) is an image of ascorbic acid.

Startup:

1. Ensure the objective lens has DIC capability.
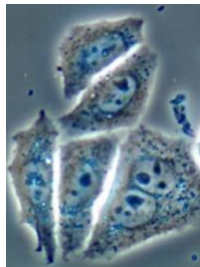2. In the **TI Pad**: select the **DIC** for the condenser and the **ANAL** filter cube.
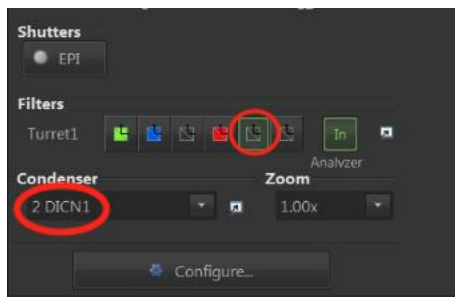
3. Insert the **polarizer wheel** into the light path. Turning it left or right will change the directionality or shadowing of the DIC image. For polarized samples, colour shifts will be seen.

Optional Method – Phase Contrast

Phase contrast microscopy permits visualization of cellular components or internal components this is not possible in brightfield. Unlike DIC, physical structures cannot be determined from phase contrast. However, nuclei can be identified and cells can be counted in phase contrast where this isn't possible with DIC. In the image below, tubular and organelle structures can be seen:

Setup

1. Confirm the objective lens has phase contrast capability (Ph1 or Ph2).
2. Select the phase filter for the condenser component in the **Ti Pad**:
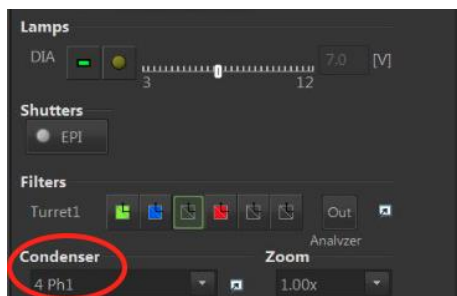
3. Ensure polarizer is removed from the light path on the condenser unit:
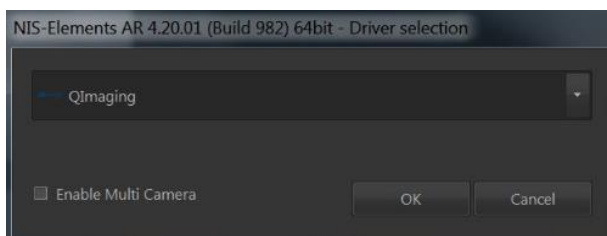
4. Adjust light intensity as needed.

Optional Method – Brightfield Imaging Only

1. Turn on the Qimaging camera (green light indicates it's ON).
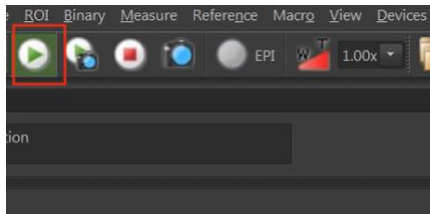

2. Ensure **QImaging** is selected for the Driver.

3. Select **R100** for the light path, and turn ON the lamp (yellow orb).

4. Click the **Live** button on the toolbar to see the sample on the monitor.
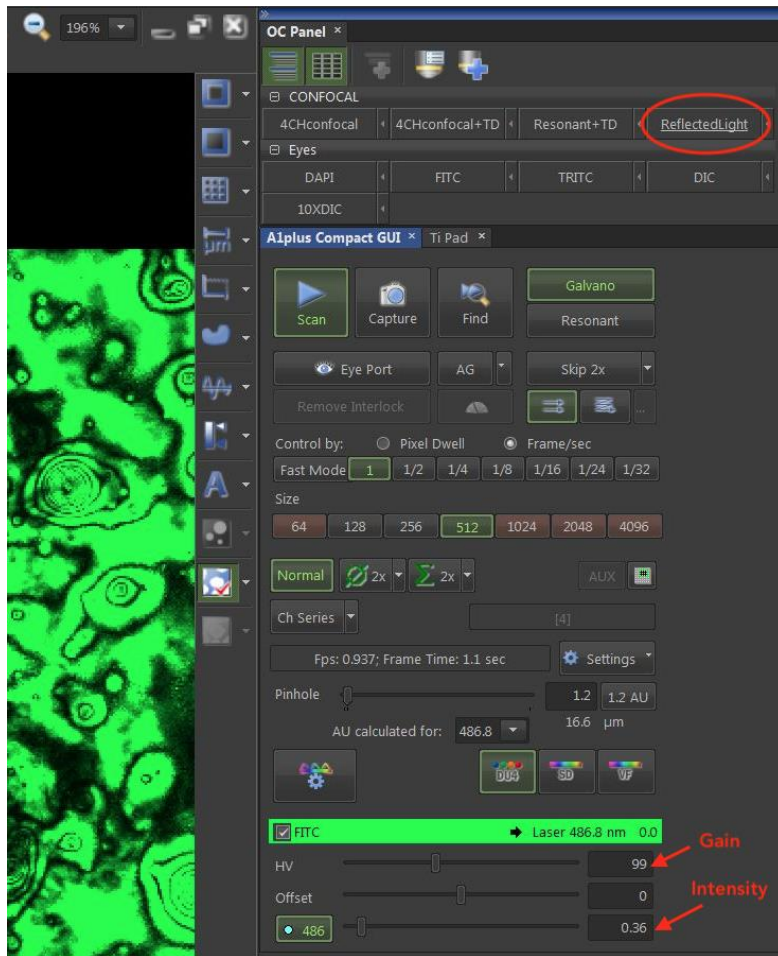

5. Adjust the light intensity as needed.

Note: DIC, polarization, and phase contrast can be performed here as well.

Optional Method – Reflected Light

1. Select the **ReflectedLight** tab from the **OC Panel**.
2. Click **Scan,** and adjust the gain (hv) or laser intensity of the FITC laser.

Cleanup / Troubleshooting

Cleaning:

1. Wipe objective lens with 70% ethanol with lens paper provided.

2. Turn off the system in the reverse sequence it was turned on.

3. Wipe the working area with RTU disinfectant and kimwipe.

Optional: the computer can be shutdown at this step.

4. Sign out of instrument on LMACs.

Troubleshoot Tips:

- If no laser scan is seen on the sample, click **Remove Interlock** on the A1Plus Compact GUI.
- If specific channels aren't responding, select the **Confocal HD+4** (OC Panel) for a soft reset.

- If the lasers are not working after a soft reset, try turning off the system (4 components). A reminder again to wait a minimum of 30 seconds between components.
- If restarting the system doesn't work, restart the computer and confocal system.