Procedure for operating Confocal Microscope Nikon *Eclipse Ti2* in Compact view.

This procedure does not replace in person training, provided by Lab manager.

Always obtain in person training by the Lab manager.

APPLICATION 2: Three confocal channel IMAGING

1. Every user will have a separated login and password. This will assure that your application setting is not changed by another user or another lab.
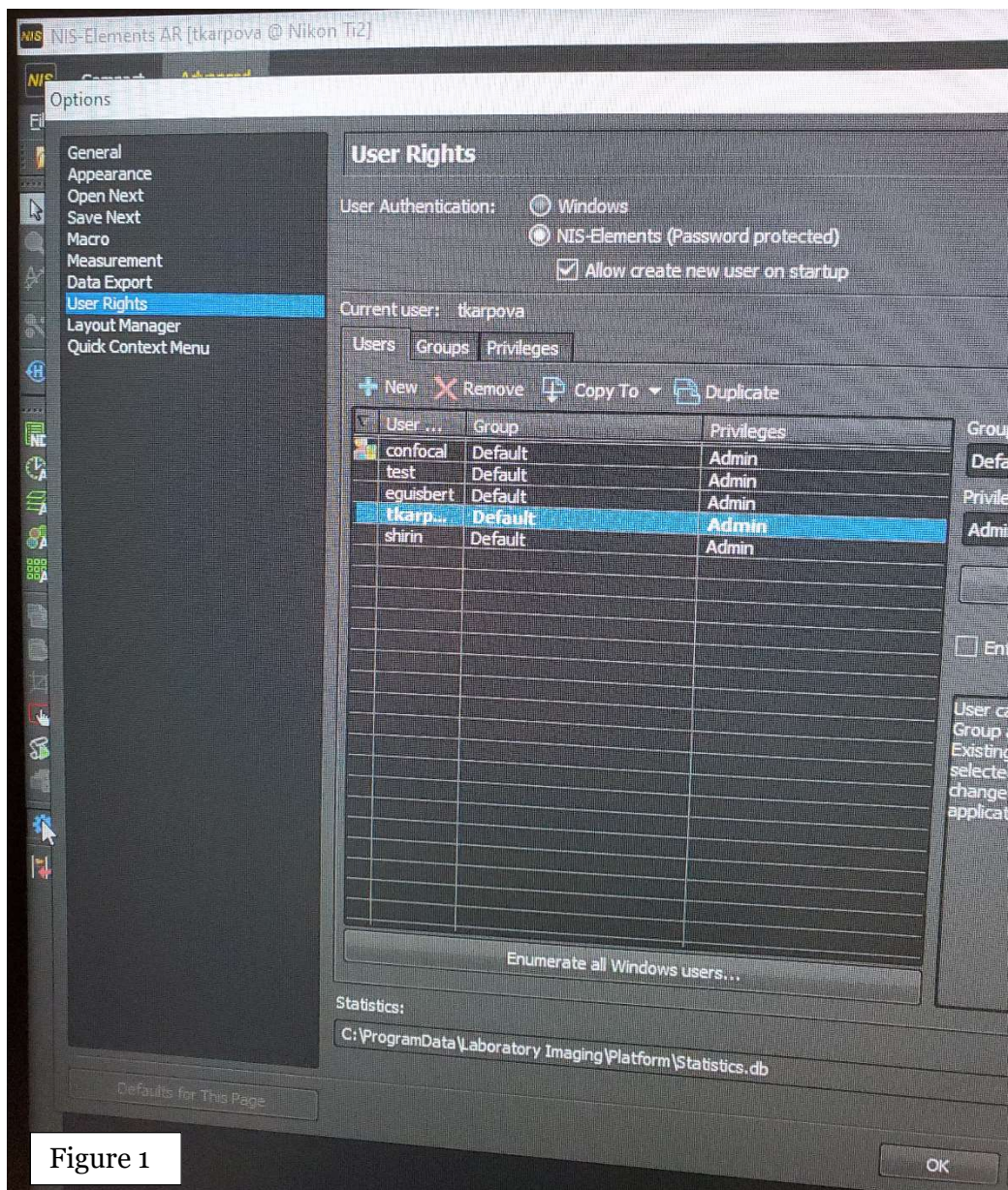

Figure 1

To create a new user login contact lab manager. From lab manager's account open "Option" box, choose "User Rights", then in the table highlight "confocal Default Admin" row, click on "Duplicate" button and in the next window type in a login name and a password.

2. The order of switching "on" the components of confocal system is very important. If you do not follow the right sequence of switching "on" all of the components, the microscope will not work and we will have to call the technical support. The order is indicated by the numbers attached to each unit.
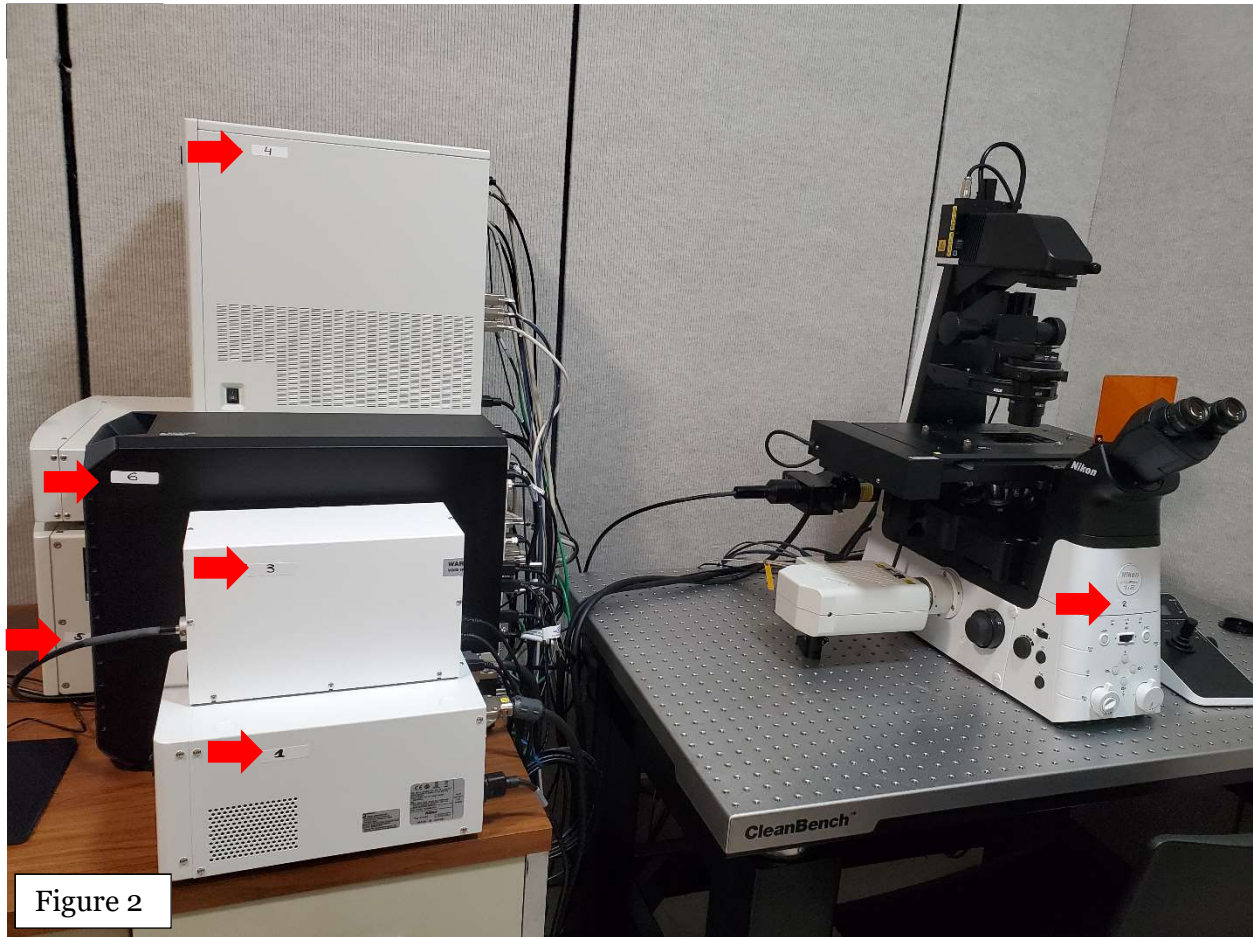

Figure 2

- (1) turn on the Nikon Power unit, **very important** – go to step (2) immediately
- (2) **very important!** Count 10 seconds after switching the Nikon Power "on" and turn on the microscope within these 10 seconds
- (3) turn "on" sola light engine box by pressing on a round ⏻ button ,
- (4) turn "on" Nikon C2 *Si* by pressing on a round button ⏻ (there is a power switch on the side of the unit

, do not switch this off, it always remain in the "on" position)

Turn on this button

Don't touch this power switch

- (5) turn "on" laser power unit with the key.

3. Place the sample on the microscope table. <u>Important for imaging with 20x objective or higher:</u> place microscope glass slide upside down (coverslip down).
4. Double mouse click on the NIS elements software icon and next login window will be opened. Type in your user name and a password.
5. Next window will have a highlighted row C2 Acquisition as shown in the picture below:

 Click "ok".

6. Next, the NIS software window is open, which has two options: "**Compact**" view and "**Advanced**" view (Figure 3). Choose "**Compact**" and the 3 panels will be opened: the left panel has commands to work with the image, middle panel will store and organize all of your captured images or videos for easy navigation through them, right panel has commands for the microscope. On the right panel there are two pads *C2plus* Pad and *Ti2* Pad (Figure 4). *C2plus* Pad controls confocal scanning and imaging, while *Ti2* Pad controls epi-fluoresent light for observing your sample through the eyepiece of the microscope. *Ti2 Pad* has several tabs: *Lightpath, Nosepiece, Filters, Condenser*.

7. *Condenser* options: most of the imaging require N2 condenser. Only one experiment will need N1 condenser, when 10x objective will be used for white transmitted light imaging.

8. In the *Lightpath* menu: choose the "**Eyepiece-EPI**". This will send fluorescence light to the microscope's eyepiece and you can locate the area of interest within the sample on the microscope slide. In the Ti2 Pad adjust following settings (Figure 4):

   - for white transmitted light imaging: visible first message Turret-Lo, first cube has **X**, selected white cube, activated Analyzer (you will see **In** inside the related cube)

9. In the *Nosepiece* tab, choose objective (as an example 20x)

Compact   Advanced   Eyepiece-EPI   Objective 20x   White filter
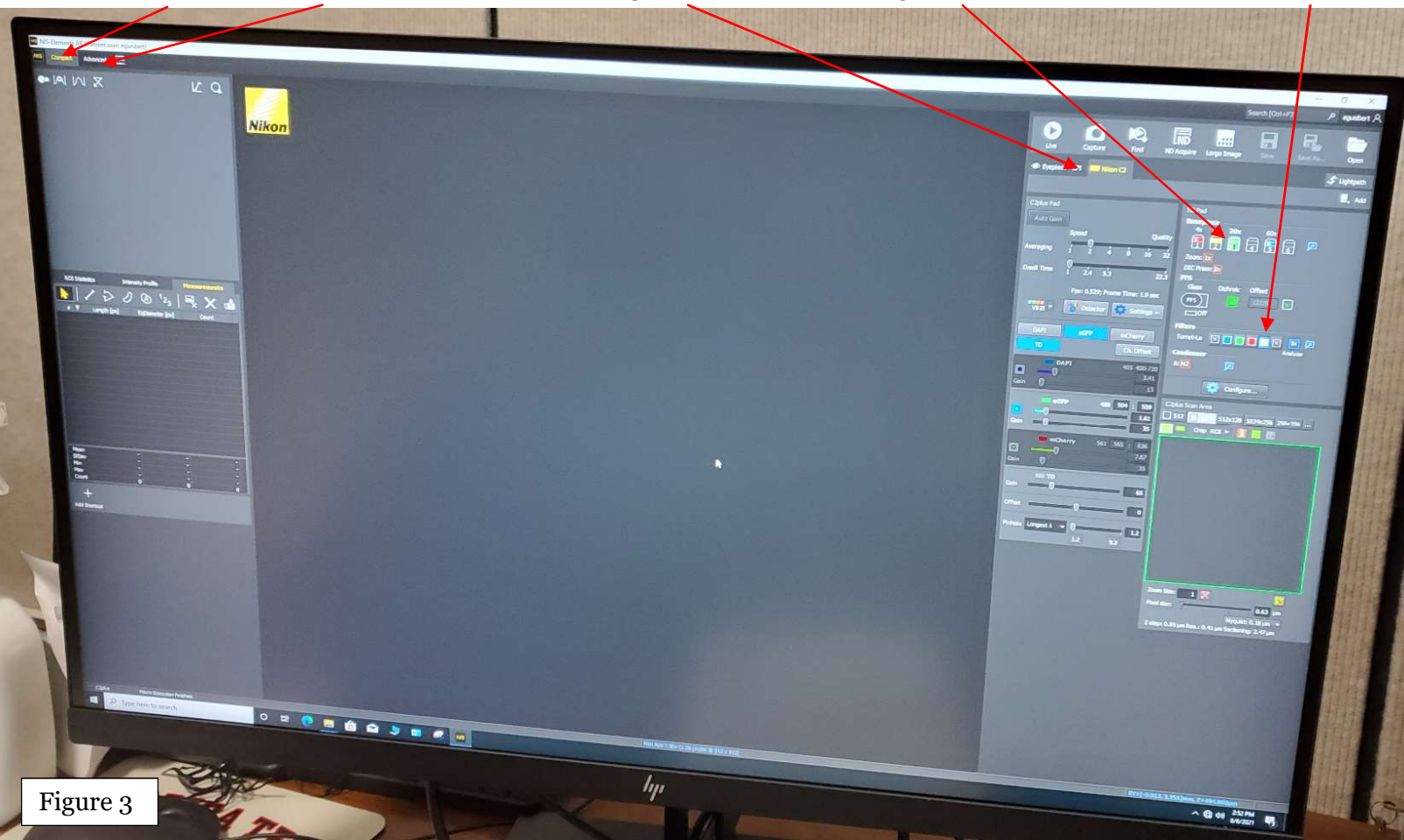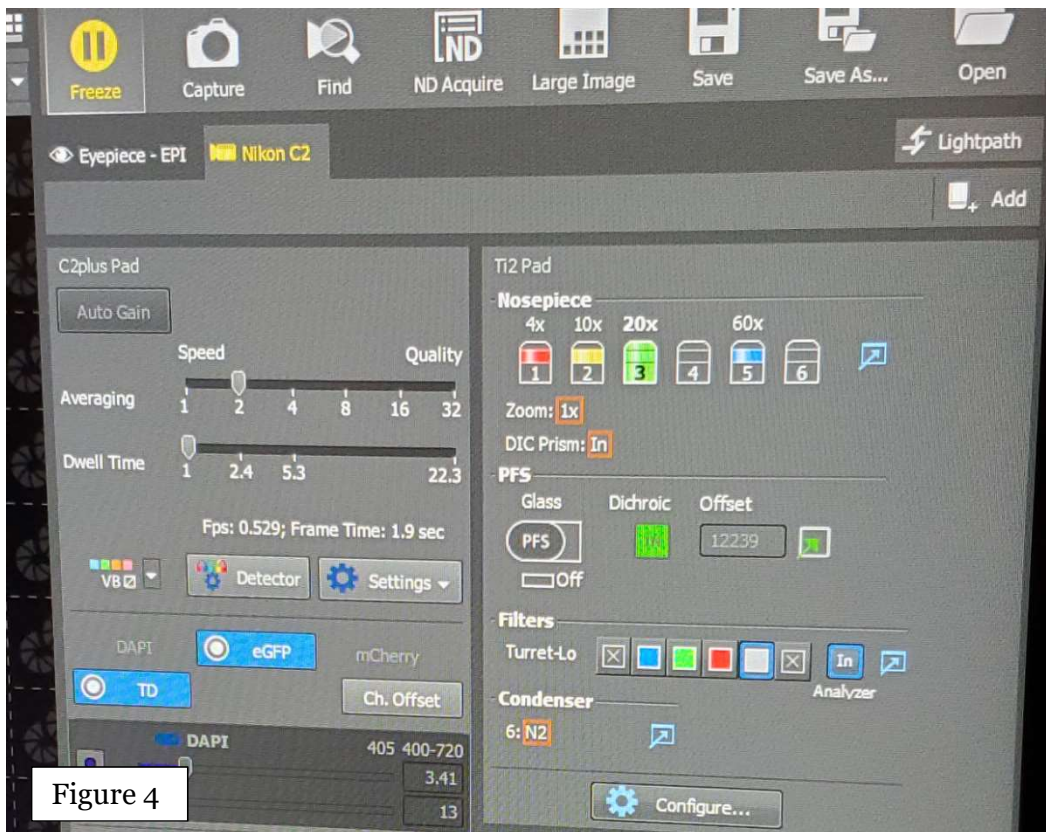

Figure 3

Figure 4

10. Now, look in the microscope's eyepiece and find the sample. You can move the sample slide in X and Y directions using joystick on a manual pad. Movement across X and Y axis will be faster if the button XY⚡ is depressed.

To move the sample slide up and down in Z axes to get the sample in a focus plane use the focus knob on the manual pad or on the microscope. Similar to the previous step if you press Z⚡ button the movement will be faster.

Basically pressing Z⚡ will initiate coarse option on focusing

11. After you located and focused your area of interest within the sample on the microscope go to the computer and in the *Lightpath* menu change to **"Nikon C2"** tab, click on the warning red bar "overlocked" **.** Next, adjust confocal imaging setting in the *C2plus Pad* (Figure 3):

- Click on **Detector** tab and in the next window that is opened up select **VB** and activate **Transmitted Detector**.
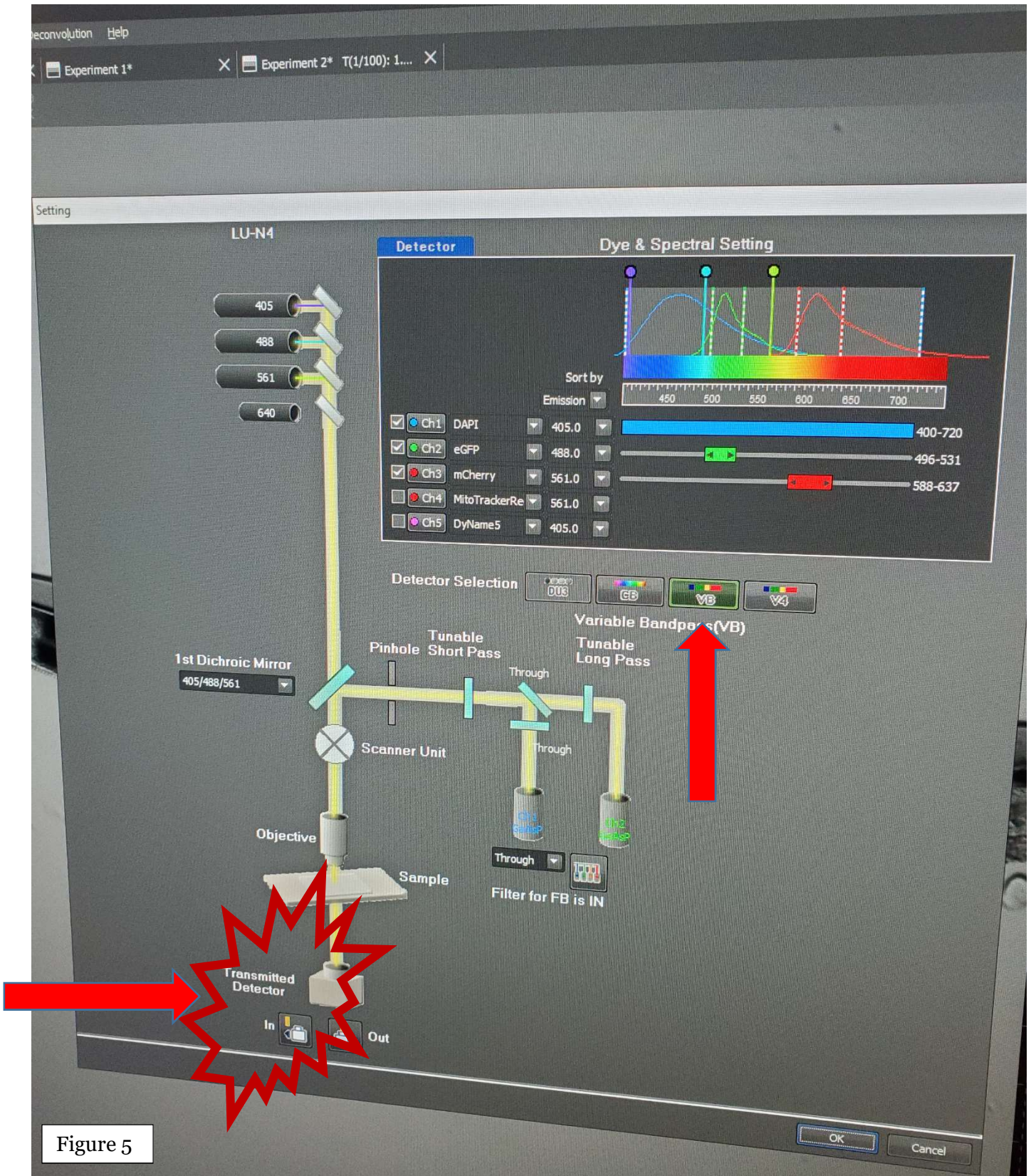- Click Ok.

Figure 5

- Then, in *C2plus Pad* For imaging with white transmitted light select "**VB**" , highlight **eGFP** bar and **TD** bar (Figure 4), then click on the "LIVE". If your sample is in the right focus plane you will be able to see the image in these two places in the software. If you do not see the image or fluorescence signal is faint, you need to find the right focus plane, the plane that gives you the brightest signal from your sample. In order to achieve that use the mouse scroll button for focusing and moving the sample up and down within Z axes to get to the brightest image. Once you are in the brightest point (or field of view) of your sample, click **Auto LUTs** button (Figure 6), which will adjust image brightness and signal to noise ratio. Rotate mirror above the microscope to adjust the contrast (for white transmitted light).
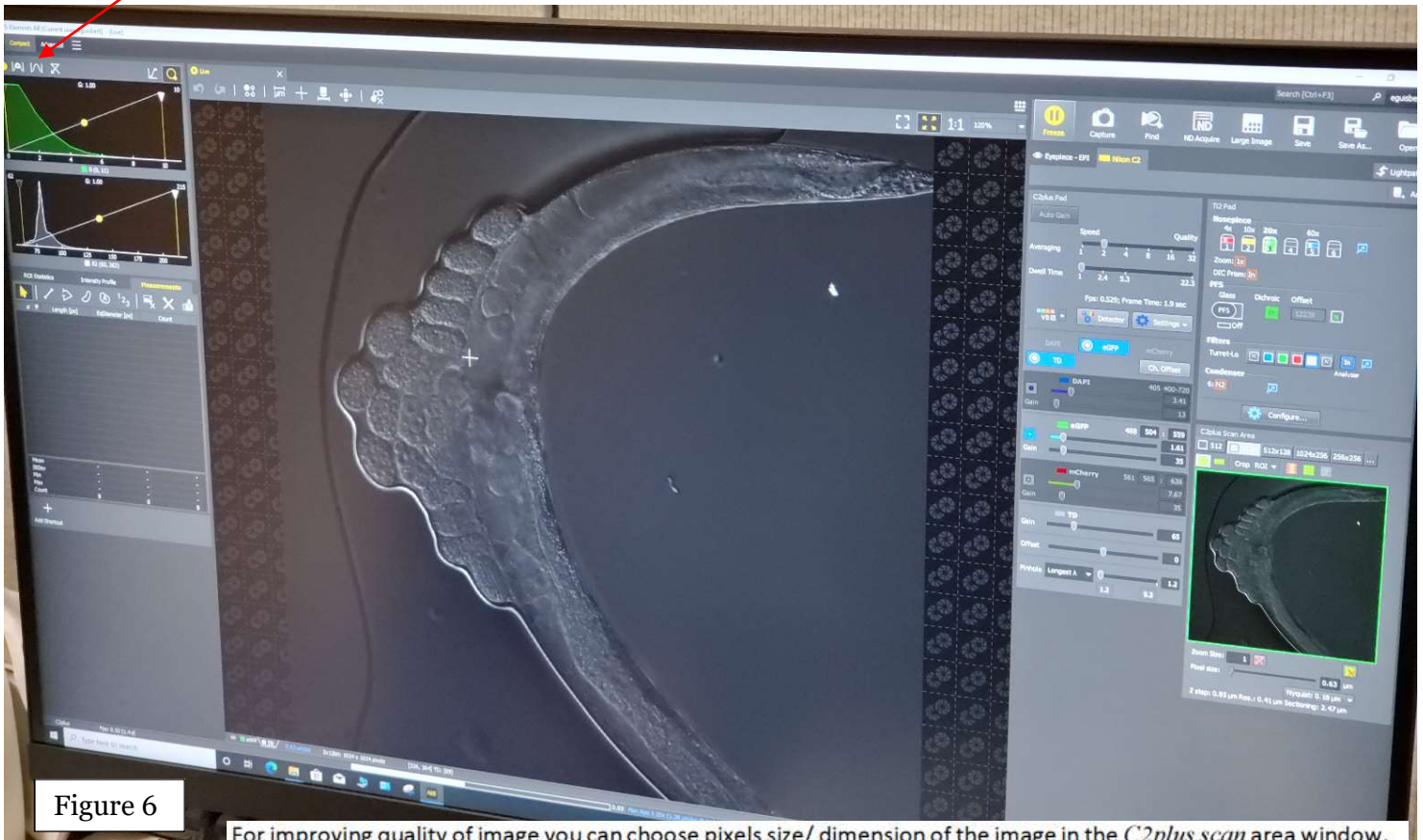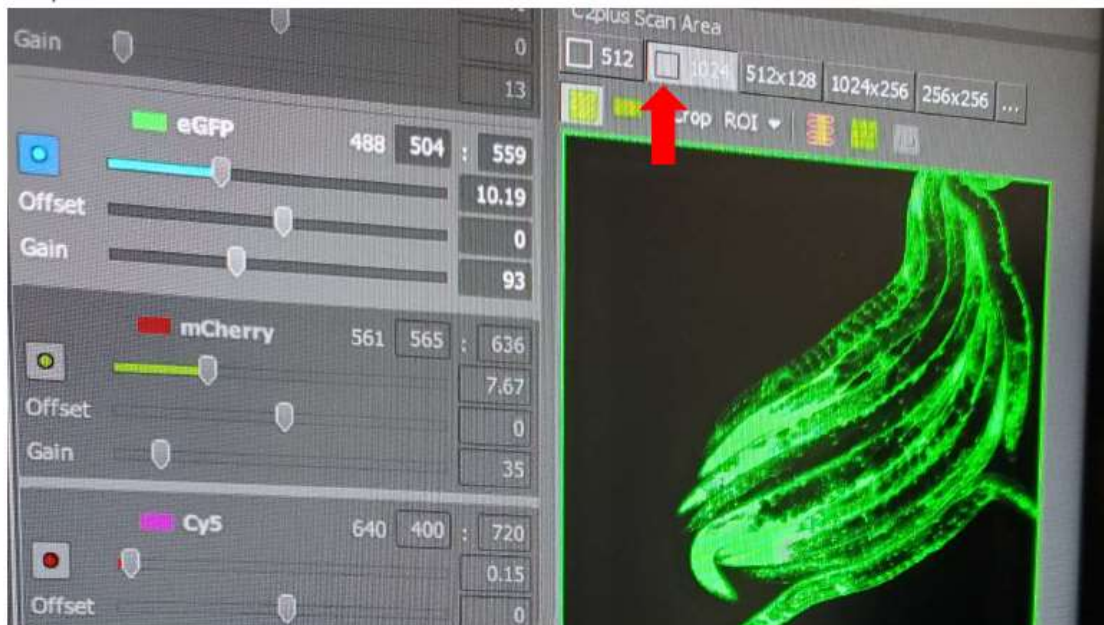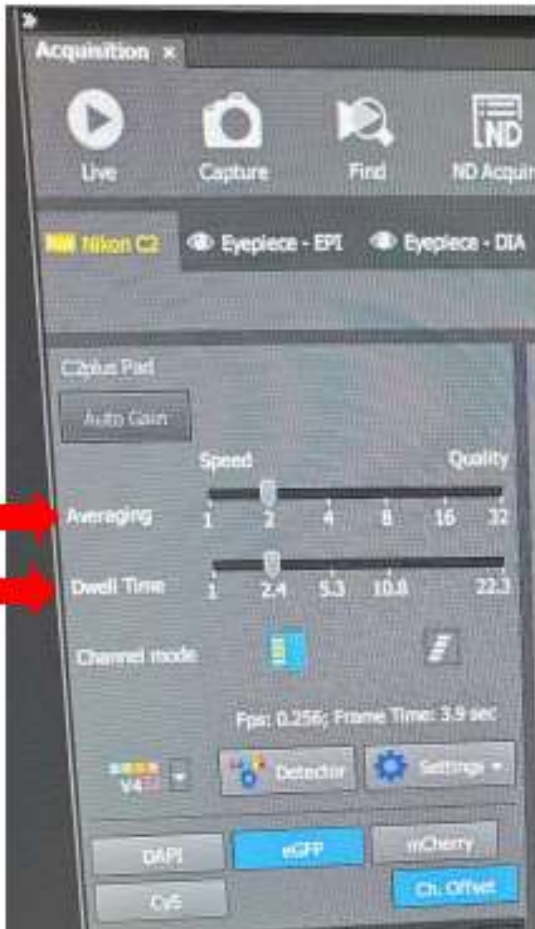
Auto LUTs

Figure 6

For improving quality of image you can choose pixels size/ dimension of the image in the *C2plus scan* area window. "1024" is acceptable dimension for capturing the image. The bigger the dimension the longer the capturing time will proceed.

14.

Another option to improve imaging quality is located in the *C2plus Pad*, it allows to increase **Averaging** numbers from 1 to 2 or 4 – this will increase number of frames that are taken and been averaged from the sample plane or sample spot. Again, remember that the bigger the number the longer capturing time will proceed. Usually 2 (max 4) is an acceptable number.

Increasing a **dwell time** (the length of time that the laser beam remains in the image space corresponding to a single pixel in the image) will also improve image resolution and signal to noise ratio.

Next – setting up your image capturing experiment like Z-stack, timelapse, multipoint or multichannel imaging parameters. You need to be in Live at the *Acquisition* menu. Make sure, that image pixels/dimension is at the minimum (512), **Averaging** is at 1 and **Dwell time** is at 1. This will save you time during finding and adjusting the Z-stack limits or finding

right positions in the microscope slide for multipoint parameters, as an example. Open *ND Acquire* menu and you will see next window, where you have to choose several option to Define/Run your experiment:

- select "save file into folder" and create or find the folder where to save your work,
- select other option (check in the box) for the experiment like timelapse, multipoint, multichannel, Z-stack. For each box checked you will see menu bar appeared in the left portion of the ND Acquisition Wizard window.

14. Open *Z-stack* window from the "Z-stack" menu bar.
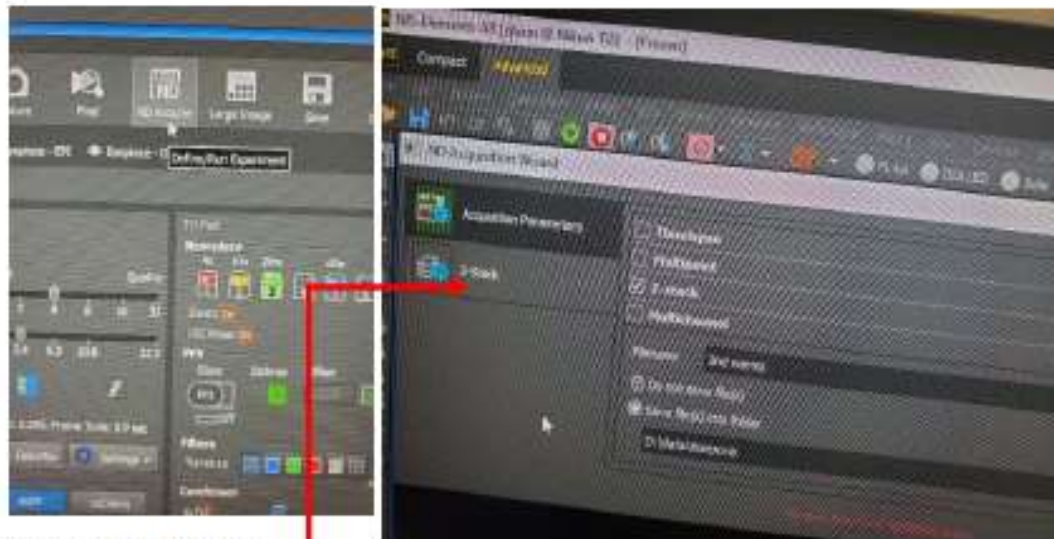
In the Z-stack window first select "Bottom to top", then click "reset" (image 1), then use the mouse scroll button to move down to the bottom of the sample (rotation of the mouse scroll button should be towards you). When you have reached the desirable bottom of your imaging experiment (usually when you do not have any fluorescence signal), click on the bar "Bottom" (image 2). Then rotate the mouse scroll button away from you, during this time the image should be getting brighter, then become the brightest, then getting weaker and will get to no signal again – this will be the top of your imaging experiment. At this point click on the bar "Top"(image 3).


If other parameters of the experiment need to be identified go to menu bars related to them, for example multichannel or multipoint and set the parameters. Only when all setting for the experiment is identified, then the run experiment green arrow is visible. Before click "*Run*" button, select the **Averaging, Dwell time, Pixel size/Dimension** that you have chosen before adjusting Z-stack or other parameters of the experiment. Now you can hit "Run" button.
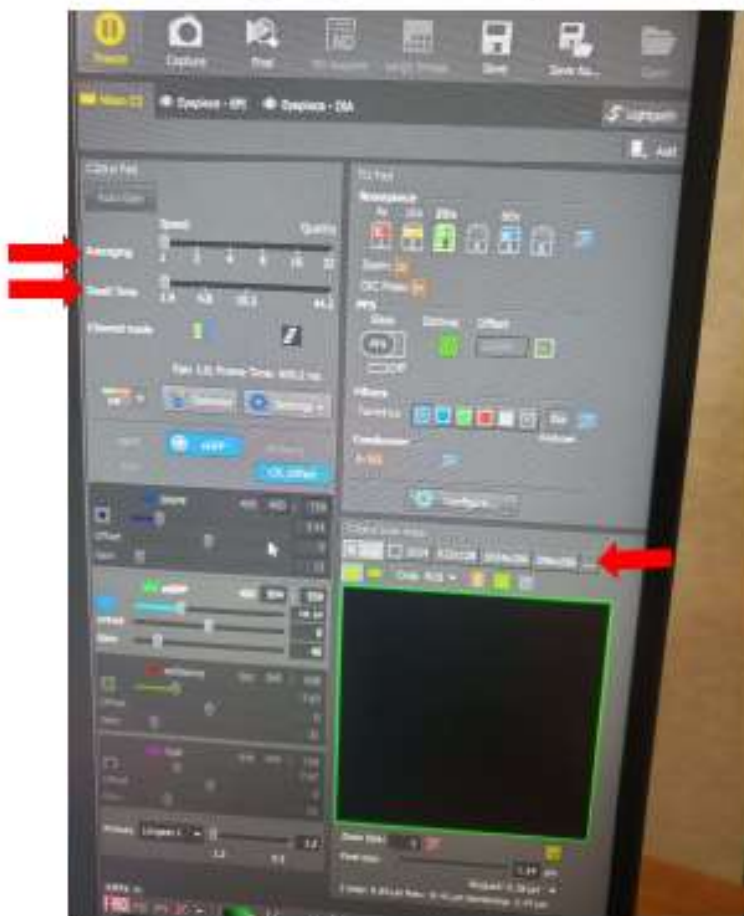

Select **Auto LUTs** when the Z-stack image scanning is in process. Select **Auto LUTs** again after image is taken.

15. In C2Plus scan area you can zoom in and out and/or rotate the image by depicting a square on the area within your image that you want to zoom in. This will increase magnification without changing the objective. Use Nyquest button to control the maximum of zoom or magnification that allowed to keep maximum resolution. You can rotate the image by rotating outlined square. Highlighted square become red and inactive, to activate it do mouse right click, which will change color of outlined square to green.

Figure 7
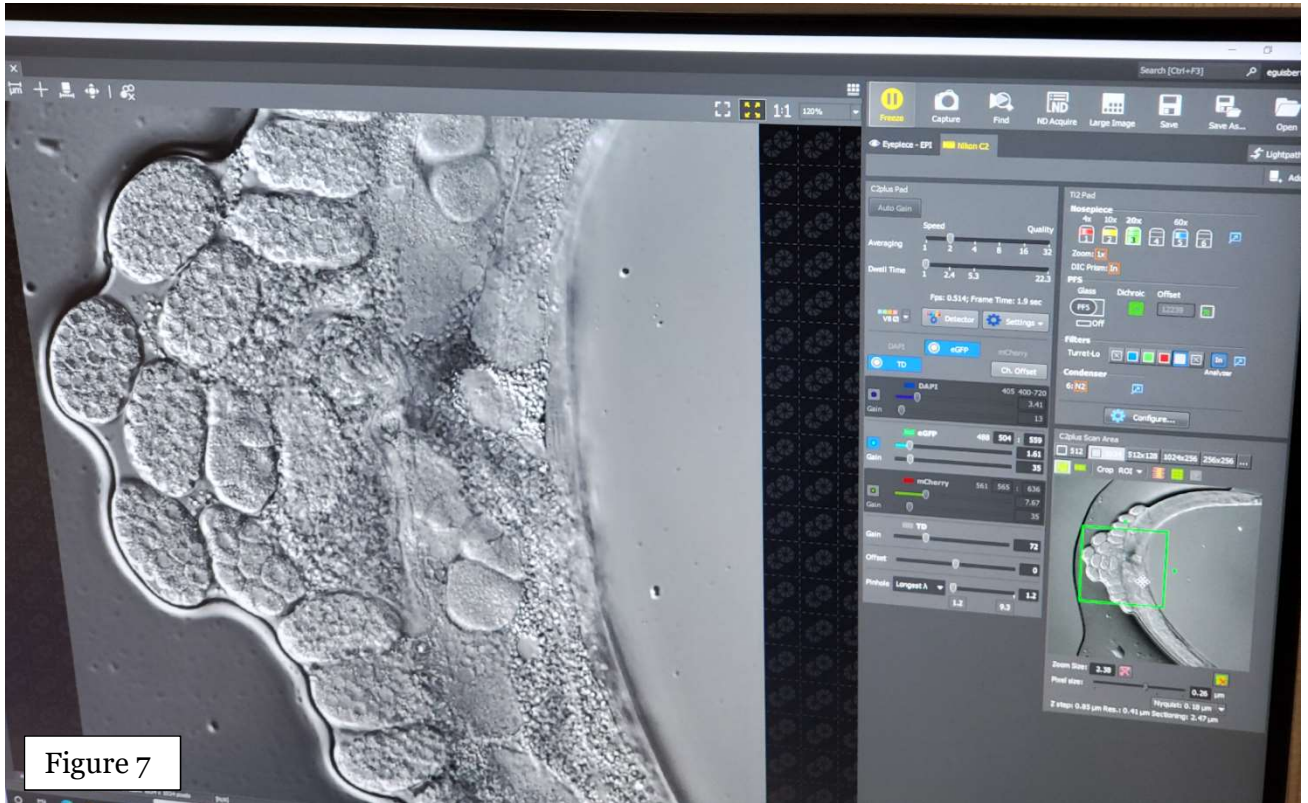
16. If working with two different fluorescence filters, you can choose to see overlapping image or single filter image by selecting tabs on the left bottom corner of the middle image panel in the Compact view.

Figure 8
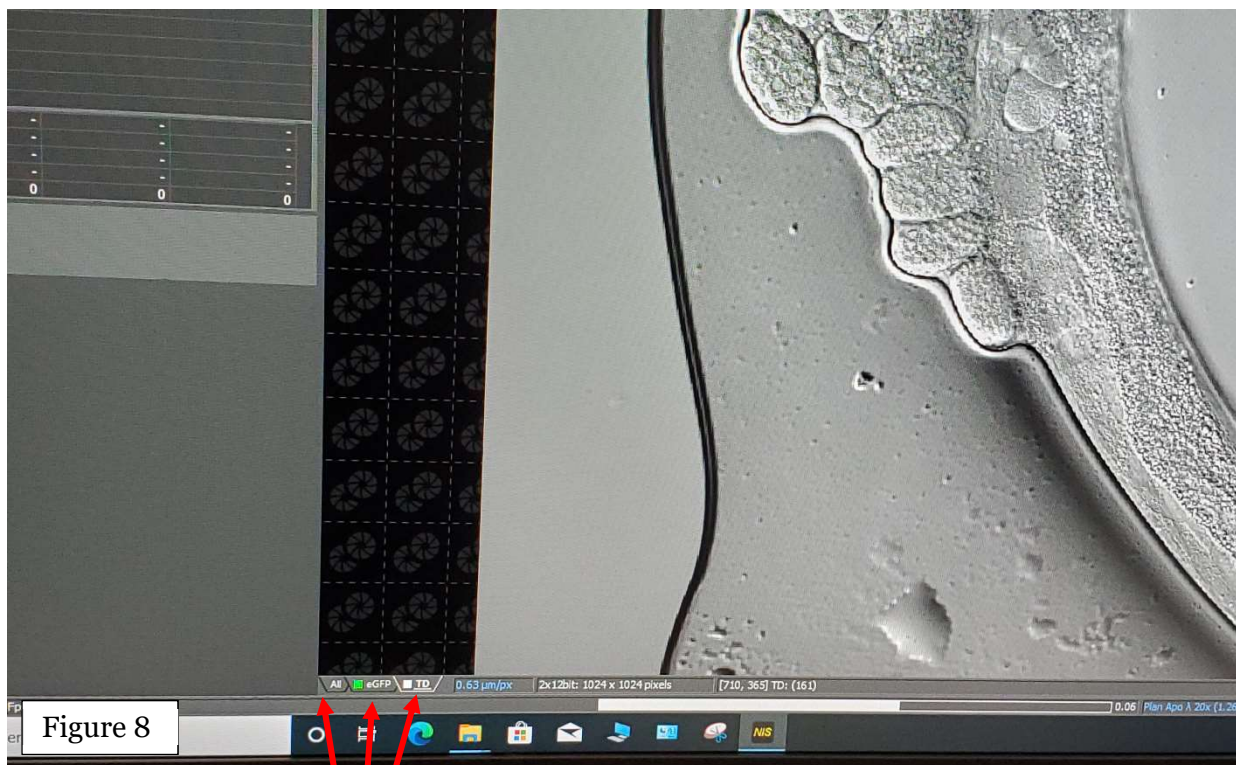
17. To save the image or video you will first need to open additional menu by clicking on ☰ button.
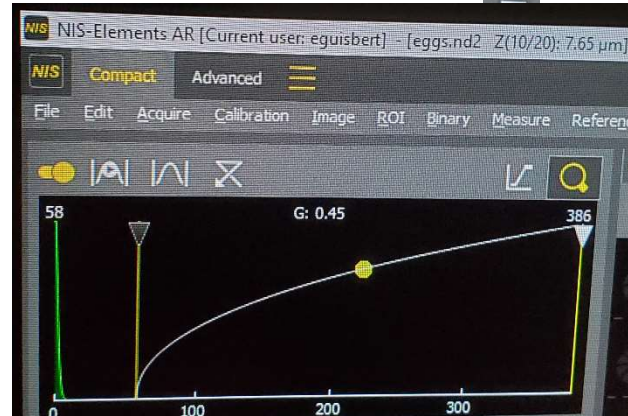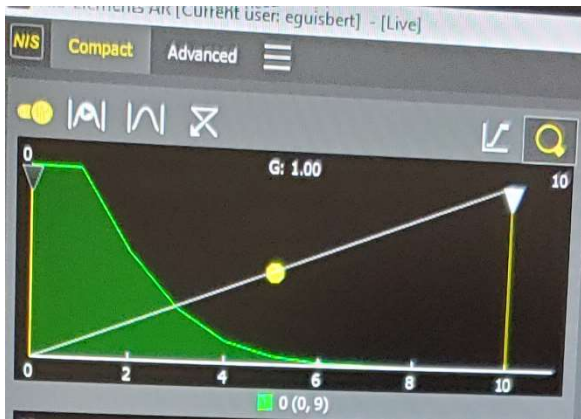
From **File** tab open save as option and choose how to save your work.

Always save file with Nd2 extension, this will keep all information about imaging setting. While saving with Nd2, the notes/table window will open up, where you can list details of the sample or the experiment' condition. This may help you in the future to find samples or images of interest. The files with Nd2 extension can be opened in Image J as well.

There are also other option of saving, for example tiff an etc.

18. If you took the image and want to take image with exactly same setting of the microscope and the software, go to *Advanced* view, make a right mouse click anywhere within the image area and from the opened window and check *Reuse Camera Setting* line.