Zeiss LSM 780 Quick Guide

BEFORE YOU START

- **RECORD YOUR USE IN THE LOG BOOK.**
- **FOLLOW THIS MANUAL STEP-BY-STEP UNTIL YOU ARE FAMILIAR WITH THE SYSTEM OPERATION.**

A. STARTING THE LSM780

If the system is OFF, start here:

1. If you are planning to use the Chameleon laser for 2-photon imaging, turn on the laser with the key (A) on the control box located under the left side of the air table.

1. Turn on the main power switch (1) located to the left of the monitor, followed by the key to "on" (2), the systems/PC switch (4), and the components switch (5) in numbered order.

2. Turn on the switch and the key on the Argon laser power supply located on the floor under the scope (3). Set the toggle switch on the Argon laser control module to "run" (6).

3. Turn on the Excite Light (7).

4. Turn on the computer to the left of the monitor (8).

If the system is ON, start here:

5. Find the pGina log-in window and when it says "connected", enter your VUnetID and e-password.

6. Double click the ZEN icon with the grey shading.

7. In the center of the first Zen window, click "start system".

B. SETTING UP YOUR SPECIMEN

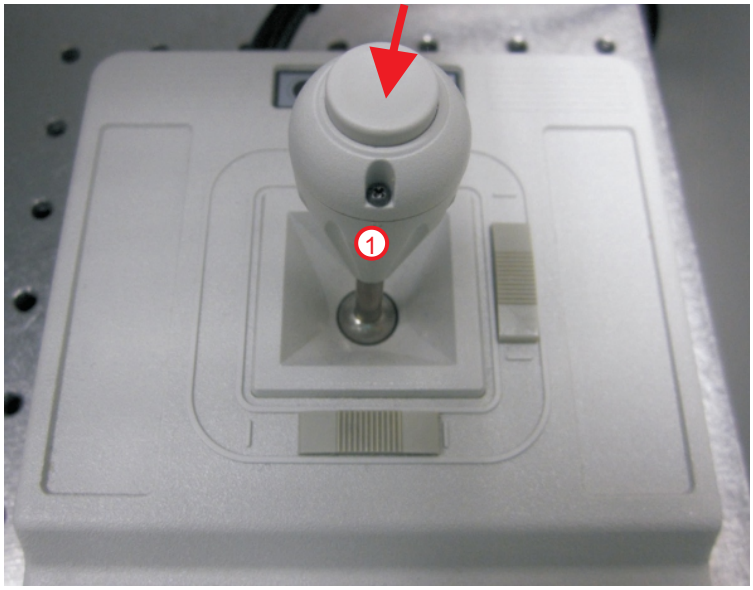
1. Check the objectives before proceeding.

A. Clean off any oil found on the stage with a Kimwipe.

B. Clean the oil/water objectives with **lens paper**.

C. If you find a significant amount of oil or any other substance on any lens or the stage or the objective turret, please inform a CISR staff member.

Do not adjust or remove the stage insert unless a CISR staff has shown you how. The insert and stage are precision aligned and leveled and must be handled carefully.



2 .**Make sure the sample cover glass is clean, dry and sealed.** Mount the specimen on the stage. Remember that for *inverted* microscopes - those with the objectives *below* the stage - microscope slides must be placed upside-down.

3. Move the stage with the joystick(1). The button on the top of the joystick (arrow) changes the speed of movement from fast to slow and back to fast with each press.

4. Changing lenses and viewing your specimen through the oculars (eye pieces) requires the Touch Pad and/or the ZEN software. Please refer to the appropriate sections for instructions.

Contact information

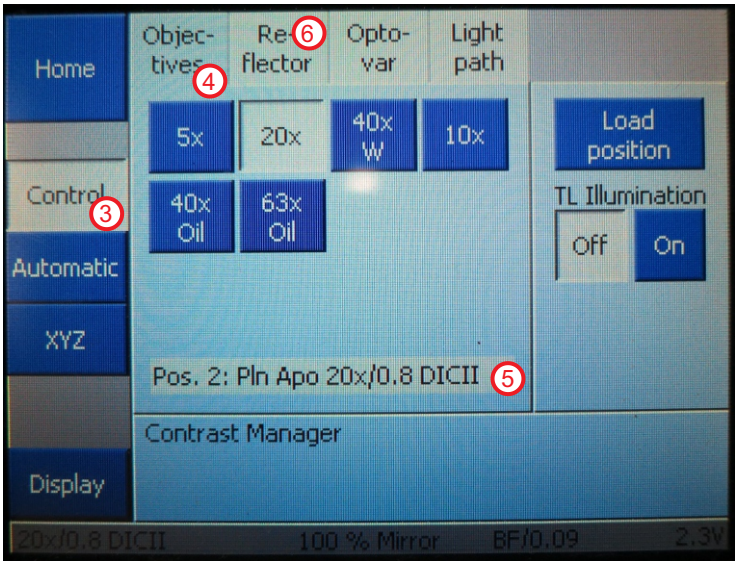
Sam Wells 322-6687, sam.wells@vanderbilt.edu
Jenny Schafer 343-3750, jenny.c.schafer@vanderbilt.edu
Bob Matthews 343-3750, robert.matthews@vanderbilt.edu
Sean Schaffer 936-3706, sean.schaffer@vanderbilt.edu

C. USING THE TOUCH PAD

1.The touch pad is a convenient way to control the motorized mechanics of the microscope. These controls are duplicated in the on-screen ZEN software. You also can focus the microscope with the knobs on the right side of the touchpad.

2. The “Home” screen will open on the touch pad as the default①. The home screen shows details about the state of the microscope and allows turning the white light on for a bright field view of your sample (TL illumination “on” or “off”).

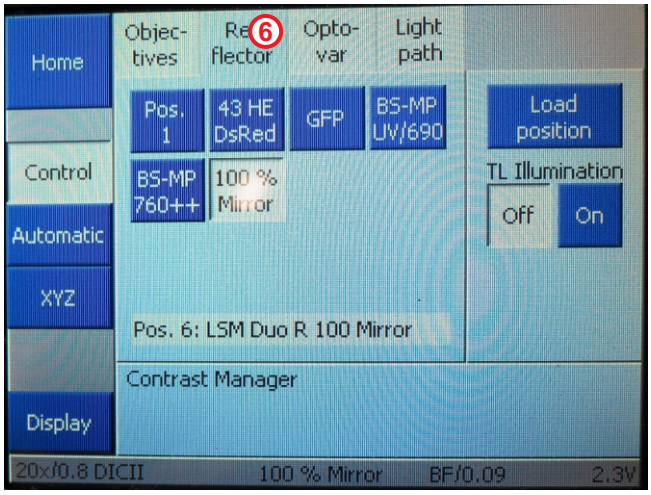
3. The “microscope” screen② can be used to change objectives, choose fluorescent filter cubes (”reflectors”) for viewing fluorescence through the oculars, and controlling other aspects of the light path, **with the exception of the Excite Light fluorescent light source which can only be turned on through the software.**



4. Click the “control” screen③ and then the “objectives” tab④. The available objectives (5x, 10x, 20x, 40x W [W=water immersion], 40x O, 63x O [O=oil immersion]) can be moved under the sample by clicking the appropriate button. Information about the chosen objective is displayed below the buttons⑤ .

Caution: If you are using an oil or water lens, do not switch between immersion and dry lenses without first removing and cleaning the slide surface! If you accidently get oil or water on an air lens, or in the case of a spill, please notify a CISR staff member immediately in order to prevent permanent damage.

When changing between air and immersion lenses, the touch pad display will prompt you to add or remove immersion fluid and will not bring the new lens up to focal position until you close the prompt.



5. Click the “reflector” tab to see the available reflectors⑥. Two colors are available (”DsRed”=red & “GFP”= green), along with DIC white light (”Pos 1”).

D. ZEN SCREEN LAYOUT

1. The left tool area of the opening screen gives you the choice of examining your sample through the oculars (Locate ①) or scanning the image to the monitor (Acquisition ②).

2. To look at your sample through the oculars, click "Oculars Online" ③, then choose "Transmitted"④ for a white light view or one of the fluorophore buttons, green (GFP) or red.

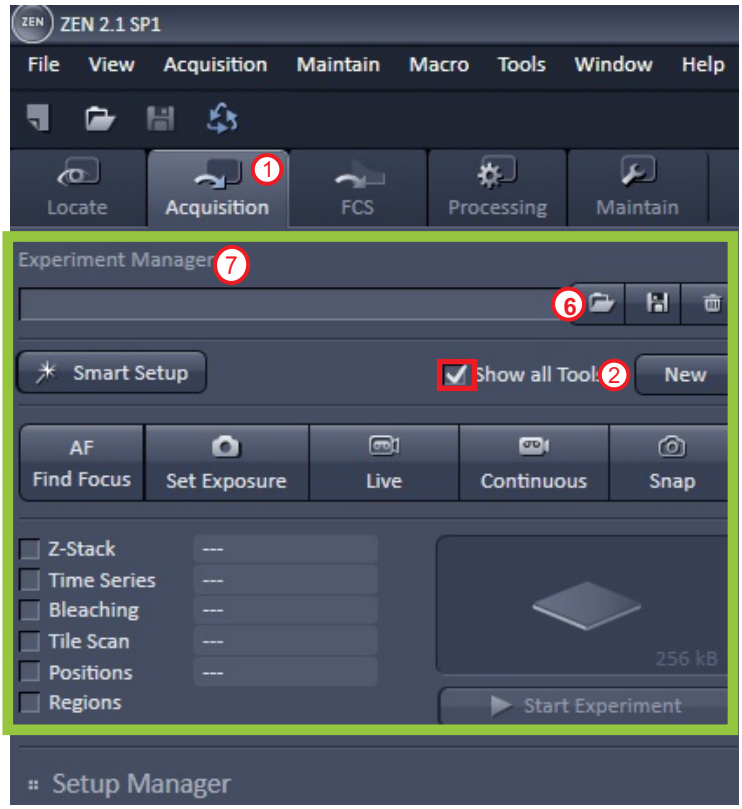
3. In the "Ocular" portion of the screen,⑤ changes can be made in light source intensity, open or closed state of the white light or fluorescent excitation light shutters, etc., by clicking on the appropriate icon. Do not use higher than 12% power on the Excite light ⑥ (fluorescence) unless you want to bleach your sample on purpose.

4. After focusing and visually setting up your sample for image capture, immediately turn off the fluorescent light,"all off"⑦ to prevent bleaching.

5. Click the objectives icon ⑧ to get more detailed information about each lens. If you want to capture images with a different lens than the one you used to get your sample in focus, use this drop down list to change lenses Or use the touch pad as described on a previous page. Re-focus your sample after changing lenses.

E. SETTING UP A CONFIGURATION

1. Click the "Acquisition" button ① in order to open the menus shown in the picture to the right. A series of **setup menus** appear, each headed by a blue bar with white lettering.

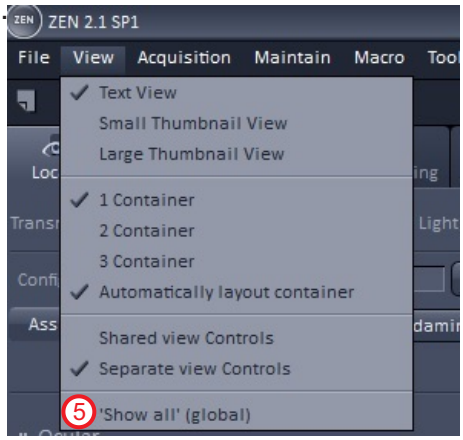


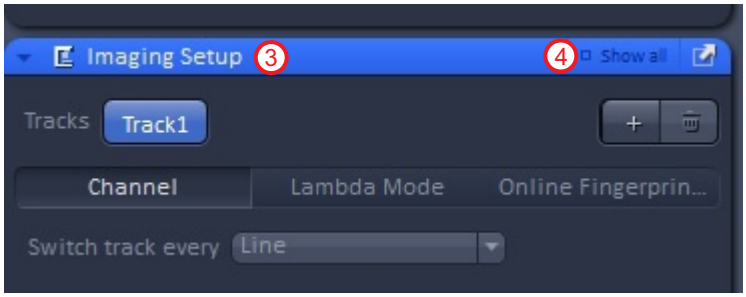
2. The upper left quadrant (outlined **here** in green) contains the main control buttons for opening scanning configurations, starting and stopping scans, and opening advanced setup windows for z-stacks, time series and others.

3. If not already done, click the small box to the left of "**Show all Tools**"②.

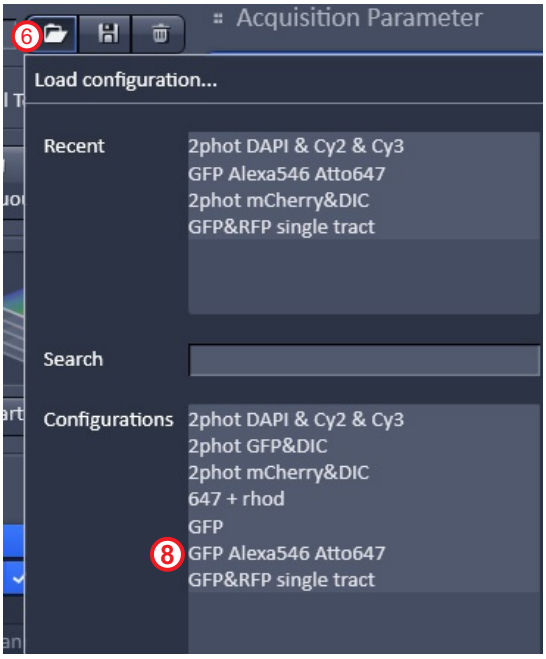
4.To show the items inside of any minimized setup menu, click the small arrowhead at the left end of the blue bar,

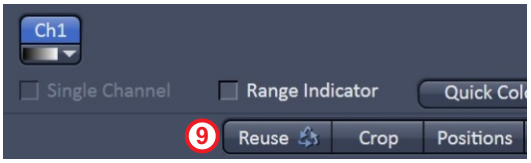
With the menu maximized, for example the "Imaging Setup",③ look whether the "show all" box④ is checked. If not, click the box in order to see all of the menu functions. Or click "Show all" (global)⑤ under the "View" drop down.





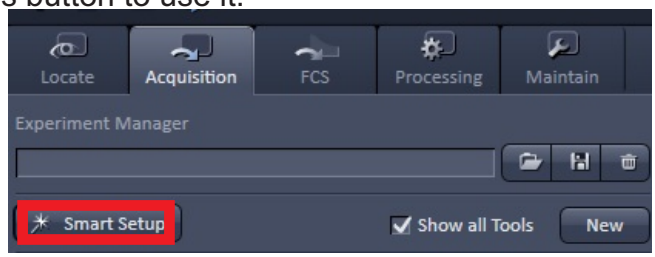
5. To create a new track configuration that will acquire images of your fluorophores, click the "open file" icon⑥ under the "Experiment Manager"⑦ . Choose the most appropriate configuration for your image acquisition. Note that some configs are specifically for using the Chameleon laser and are prefixed by "2phot" followed by the single photon excitation wavelengths and/or dye names. For single photon, i.e. normal confocal imaging, choose the set of wavelengths without 2phot, for example the common green (488 nm laser line), red (561 nm laser line) and far red (633 nm laser line) configuration ⑧ .



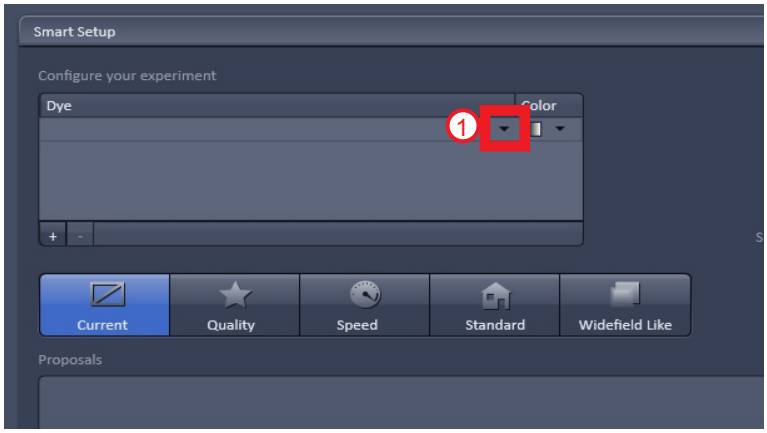


To load the same configuration used in a previously saved image, open the image and click the "reuse" button near the bottom of the screen below the opened image. ⑨

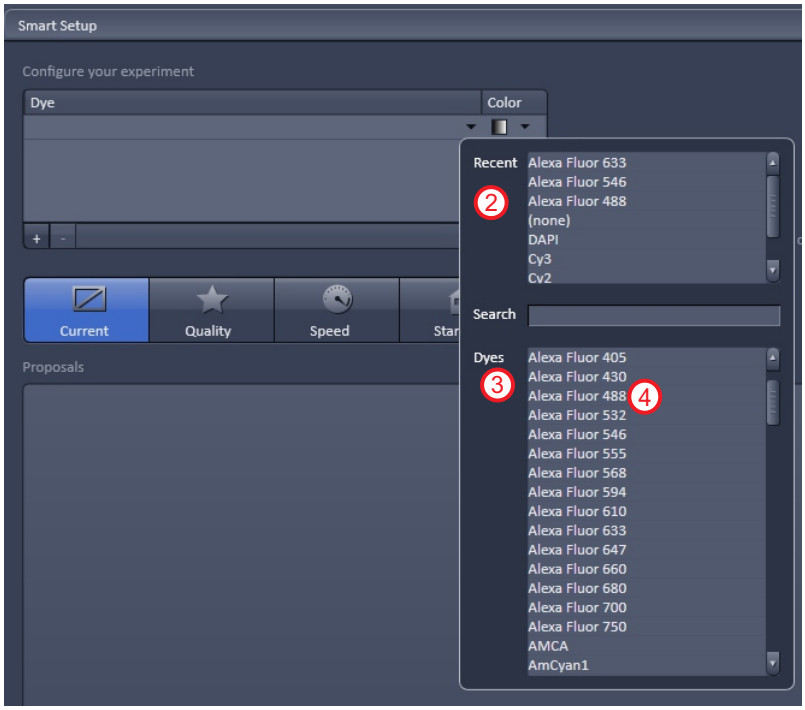
6. If none of the pre-made configs will work for your colors, another way to create a configuration that does not use the 2-photon laser, is to use “Smart Setup”. Click this button to use it.



7.The smart setup menu has a drop-down list of dyes/ fluorophores to choose from. Click the small arrowhead to the left of the first symbol in the “color” column①.



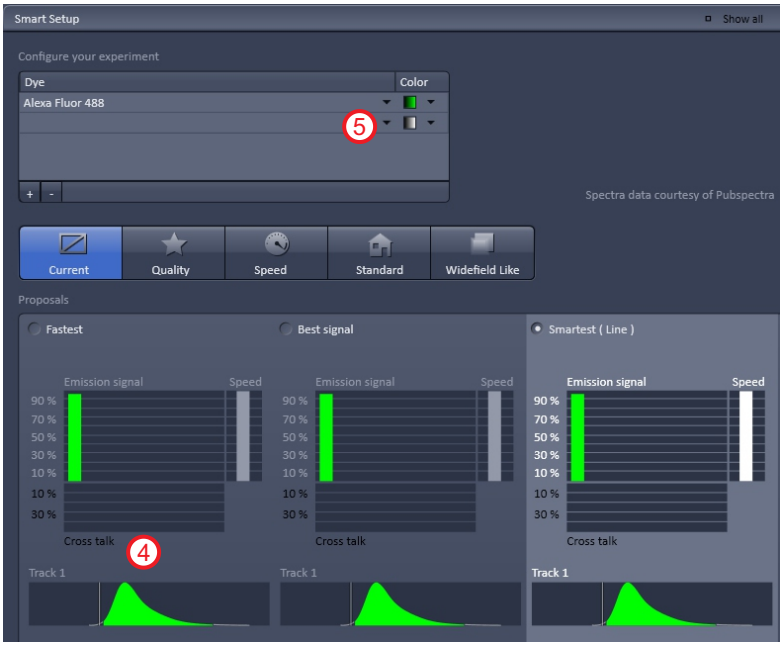
Previously used dyes are listed in the “recent” box② and a more extensive list in the “dyes” box③. This list includes some dyes that can not be imaged on this scope without modifications to the light path. Ask a CISR staff for help if needed. Click on the first dye that you want to include in your image, for example Alexa 488④.



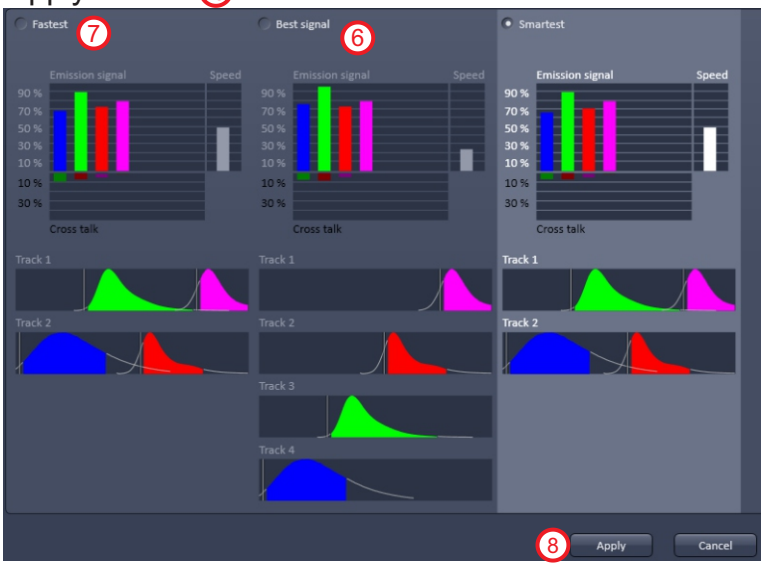
8. A new window will open that shows the emission bandwidth that will be collected④. A new blank track appears in the color column so that a second dye configuration can be added⑤.

9. To add more dye colors, repeat the above process.

10. Next a choice of fastest scanning, best quality, or their suggestion for “smartest” scanning must be made. The example shown below is for four dyes that have some theoretical bleed thru in all configurations but in this case the “best signal” choice has the least and would also be the brightest⑥. “Fastest” scan is best for live cell imaging when all colors should be captured simultaneously.⑦



11. When satisfied with the configuration, click the “apply” button⑧.



E. SETTING UP A CONFIGURATION

12. After the configuration is loaded, the menus within the setup manager, including the “Light Path” window are updated to show which track has which color(s)①. The highlighted (blue bar) track is the one shown in the light path②. In the example shown first, a 3 color single photon configuration was chosen from the list of pre-made configs which has one color in each track. For an example of a 2-photon config, skip to the next page.

13 There are many options within the Light Path menu for changing the optical configuration, including advanced features such as Lambda Mode. For routine imaging, only two of the options will be discussed here.

14. First, the emission frequencies collected for the image, both the bandwidth and the wavelengths of the beginning and end of the band can be controlled by dragging the ends of the colored bar located below the spectrum③. Care should be taken when changing the preset bandwidth so as not to create crosstalk between colors or reduce signal intensity unnecessarily. Ask a staff member for help if needed.

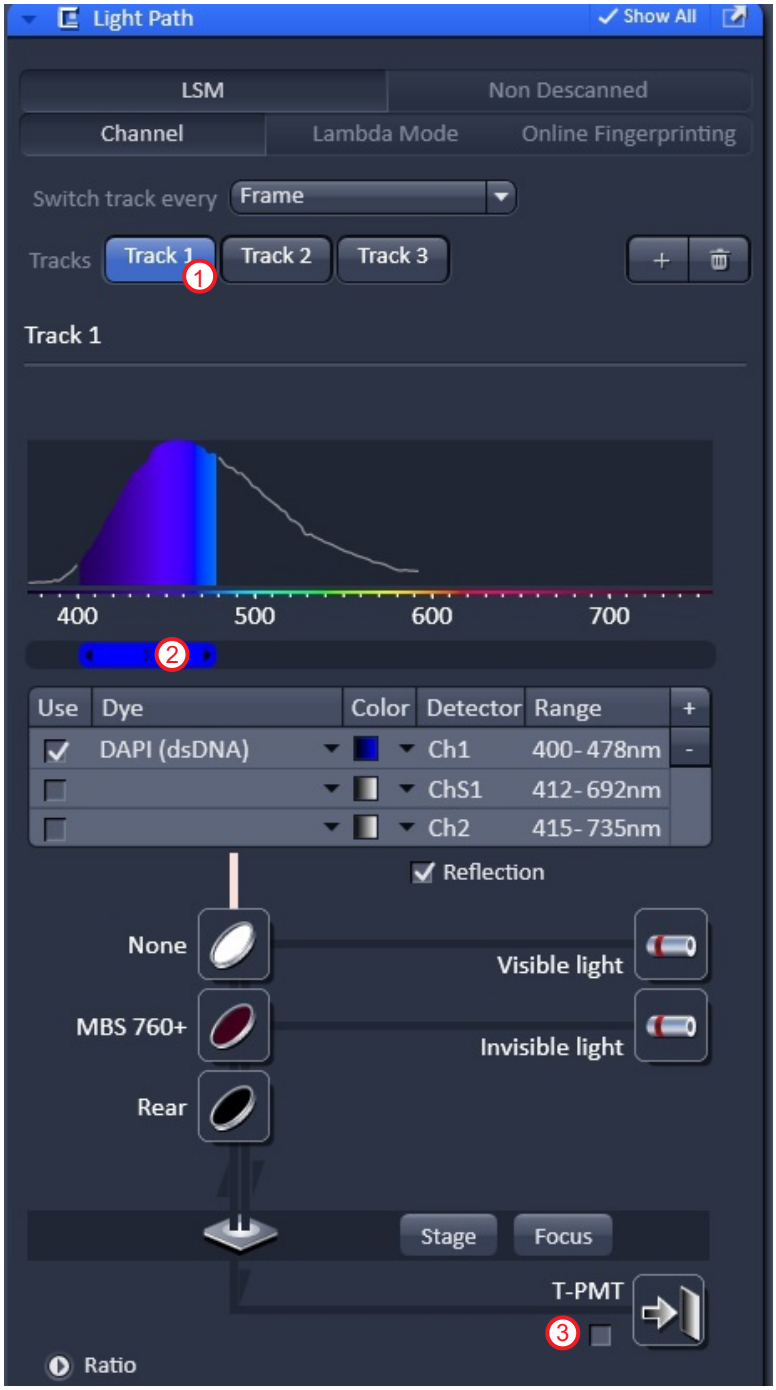
15. Second, a grey scale, DIC image can be added as an additional channel by clicking the box marked “T-PMT” at the bottom of the menu④. This can be done on any of the tracks (only need one), the choice based upon which laser you prefer to use to collect the DIC image. For optimal DIC images, Kohler alignment is recommended (see last page for instructions).

E. SETTING UP A CONFIGURATION

16. An example of the Light Path menu for a 2-photon config is shown on the right. This config is for 3 colors, blue (DAPI) in track 1 (1) is shown. As in single photon imaging, the emission band can be adjusted (2). Both the bandwidth and the wavelengths of the beginning and end of the band can be controlled by dragging the ends of the colored bar located below the spectrum. Care should be taken when changing the preset bandwidth so as not to create crosstalk between colors or reduce signal intensity unnecessarily.

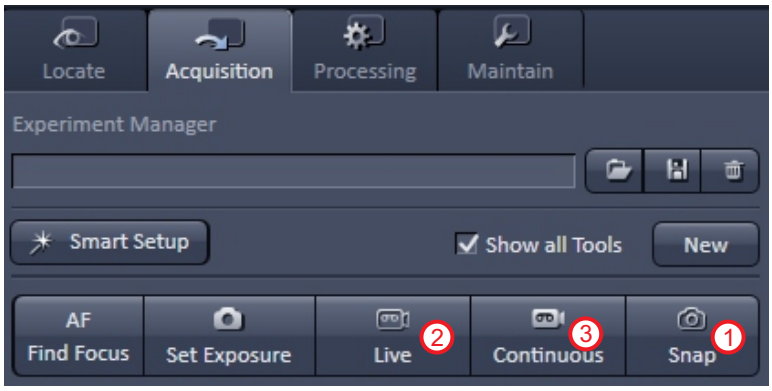
NOTE: On this scope, DAPI can only be imaged in 2-photon mode. In order to image 4 non-overlaping colors, a 2phot 4 color config is available, or a **hybrid** config of 2-photon and single photon excitation can be done. The latter choice will result in a higher resolution image in the channels using single photon excitation. Ask a CISR staff to help you to set this up if needed.

17. Turn off the room lights before beginning your imaging.



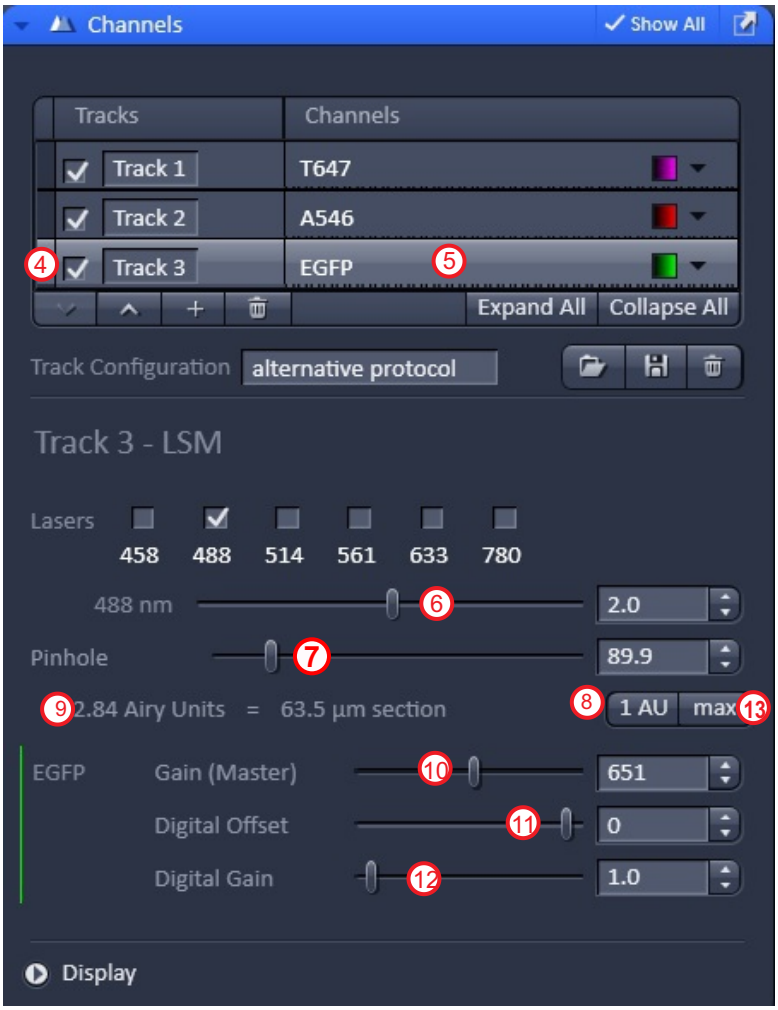
F. LASER SCANNING: OPTIMIZING THE IMAGE in SINGLE PHOTON MODE

1. Once a configuration is in place, single image scans are initiated with the "snap" button①. To scan repeatedly so that the effects of changing laser power, signal amplification, etc. can be seen quickly, use the "live" button②. The "continuous" button③ also scans repeatedly but scan rate, frame size and other parameters are controlled by what you set in the "Acquisition Mode" window④ (see later section) and will be the same parameters used for your final image.



2. The settings in the "Acquisition Mode" and the "Channels" menus must be changed in order to achieve the best possible representation of your sample.

3. Within the "Channels" menu, un-click all but one track④. Choose the track with the dimmest signal, as seen previously through the eye pieces. Or start with the track with the longest excitation wavelength. Click in the blank part of the track title in order to activate its controls (turns it lighter grey⑤). Now set the pinhole diameter, laser power, master gain and offset for the chosen track, step by step to achieve an optimized image.



4. The first option is laser output⑥. Keep default output of 2% or less initially to prevent bleaching.

5. The next option is pinhole⑦ which determines the optical slice thickness and creates a confocal image. A pinhole setting of 1.00 Airy Units (1 AU⑧) will result in the sharpest image, collecting all in-focus light and rejecting out of focus light. However, a pinhole setting of > 1 AU⑨ may be necessary in order to collect enough light to create an optimal image. If unsure what diameter to start with, leave it at the default setting.

6. Increase the Gain (master)⑩ to 500-700. Set the digital offset at "0" or "1"⑪. Begin scanning by clicking the "live" button②. Leave the digital gain at 1.0⑫.

If an image appears, skip to step 7

If no image appears, try one or all of the following:

A. click the "max" button⑬ to fully open the pinhole or use the slider to open it enough to get an image.

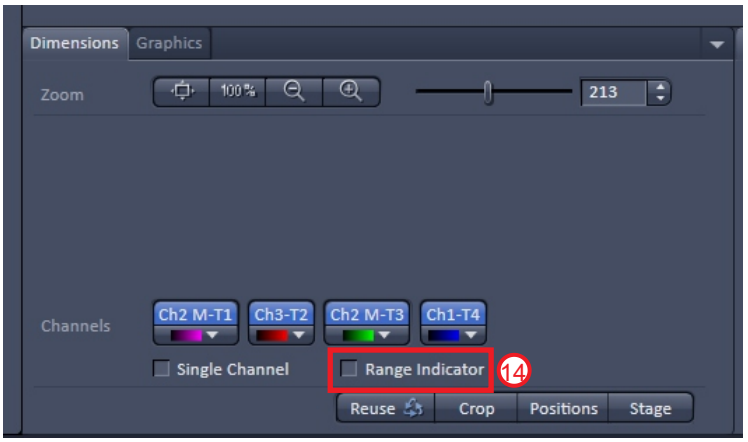
B. Refocus while scanning.

C. Increase laser output to as high as 15 for short wavelength lasers (458, 488 & 514 nm) or 30 for long wavelength lasers (561 & 633 nm).

The pinhole size determines the thickness (Z dimension) of the sample from which light is gathered for the image. Larger pinhole size = more light reaching the detector. However, a larger pinhole results is a "fuzzier" image.

F. LASER SCANNING: OPTIMIZING THE IMAGE in SINGLE PHOTON MODE

7. With an image visible, click the box called " Range Indicator" at the bottom of the center screen (14) . In this palette, red pixels in your image mean the detector is saturated when those locations are scanned (too bright). Blue pixels mean all signal has been removed at those locations (signal lost below threshold). All of the pixels with a value of 0 are blue and all the of the pixels with a value of 255 are red (8 bit image). Use a combination of changes in the pinhole size, laser output and Gain (master) to obtain an image with a few red pixels. Digital offset at "0" will result in an optimal number of blue pixels.



Note: The goal in optimizing is to use the smallest pinhole size (≥1 AU), lowest laser output and a Gain (master) at or below 700 that produces an acceptable image with just a few red pixels visible when using the range indicator palette.

8. After the 1st track is optimized, note the final optical section thickness (15) . T**he pinhole size for all other tracks must be set so that the section thicknesses (um) match across all tracks.** Stop the scan.

9. Uncheck the finished track, check the next track and repeat the above steps for each additional track one at a time, with the limitation about pinhole size mentioned in step 8.

10. To capture a complete image, put check marks in front of all tracks and click the "snap" button.

G. IMPROVING IMAGE QUALITY

Why it's important to optimize your image using the entire intensity range:

In this image the intensity ranges from 30-174 (8 bit but less than full range 0-255). The picture looks flat and dull, and it's difficult to discern detail.

In this image, the intensity ranges from 0-255 (8 bit), but *many* pixels are saturated, and detail is lost in a wash of white. It's not that it's hard to see detail; it's no longer there.

In this image, the intensity also ranges from 0-255, but now very few pixels are saturated. All detail is preserved. This image gives us the most information about the original sample.

1. The "Acquisition Mode" window has a number of controls that may further improve the quality of your image. Change the pixel resolution to make each pixel smaller and enhance the ability to zoom in after acquiring the image. Click the "X*Y" button to see standard resolution sizes (1) or click "Highest" (2) for the best resolution possible for a particular objective and field size; anything higher will result in oversampling. Higher resolution imaging requires longer scan times (3).

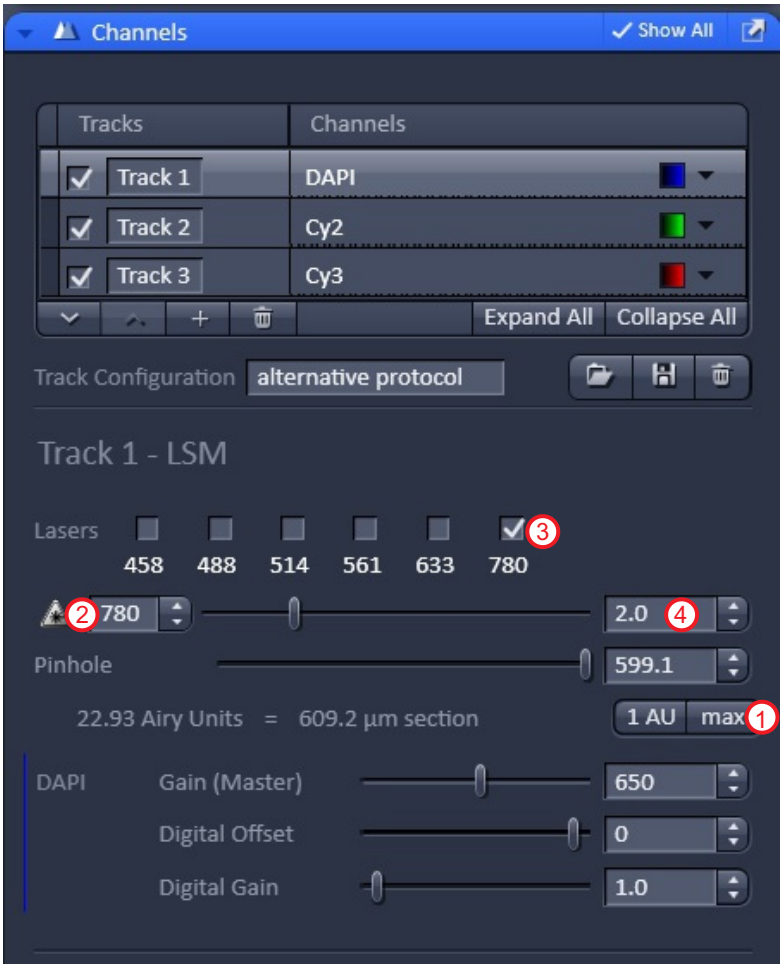
2. Change to a slower scan speed (4) to improve the image, but again at the expense of more time.

3. Increasing the "Averaging" number to >1 (5) may improve image quality, specifically better signal/noise ratio if the noise is random, but again at the expense of more time.

4. Change the "Scan Area" settings as needed to reduce or expand the scanned area (zoom). (6) Or use the "crop" button located at the bottom of the central screen (7) for more precise control of the image contents.

H. LASER SCANNING: OPTIMIZING THE IMAGE in 2- PHOTON MODE

1. Optimizing your image in 2-photon excitation mode follows many of the same principles as for single photon mode. Therefore the following section assumes familiarity with single photon excitation imaging and the focus will be on unique aspects of using 2-photon excitation.



2. In the "Channels" menu, open the pinhole to its maximal size by clicking "max" ① .

3. Set the excitation wavelength appropriate for the fluorophore of the highlighted tract by using the up/down arrows or typing the wavelength in the box ②. When you change the wavelength, the checked laser box ③ will briefly turn red while the laser adjusts and is ready to use when no longer red.

4. The correct excitation wavelength is not usually a simple doubling of the single photon excitation wavelength. You can find published values for your dyes on line or look in the PDF file on your desktop for some common dyes.

Ex_multiphoton_MPE.pdf

5. Laser power ④ should be below 5% if possible and never above 10% except under special conditions designed to minimize heat buildup. Gain (Master) should be kept below 800 and Digital Offset set for a scattered blue background under "Range Indicator", with the laser turned off.

6. Optimize all channels, setting the pinhole to max regardless of the resulting section thickness.

I. Z STACK

1. Creating a z-stack of images refers to collecting a series of overlapping images at different focal planes without changing the x:y stage position. 3D reconstructions of your sample can then be created from a z-stack of images

2. Place a check mark in the z-stack box within the menu control list ① . Maximize the z-stack menu and choose the first/last submenu ② . Go to the channels menu and turn off all but one channel; leave on the channel that will most easily show the beginning and ending focal plane. Start scanning with the "live" button and focus the microscope toward you **(counter clockwise** on right side knob) to lower the objective to the start of your z stack and click "set first" ③ . Turn the focus knob in the opposite direction to reach the desired depth for the end and click "set last" ④ Stop scanning.

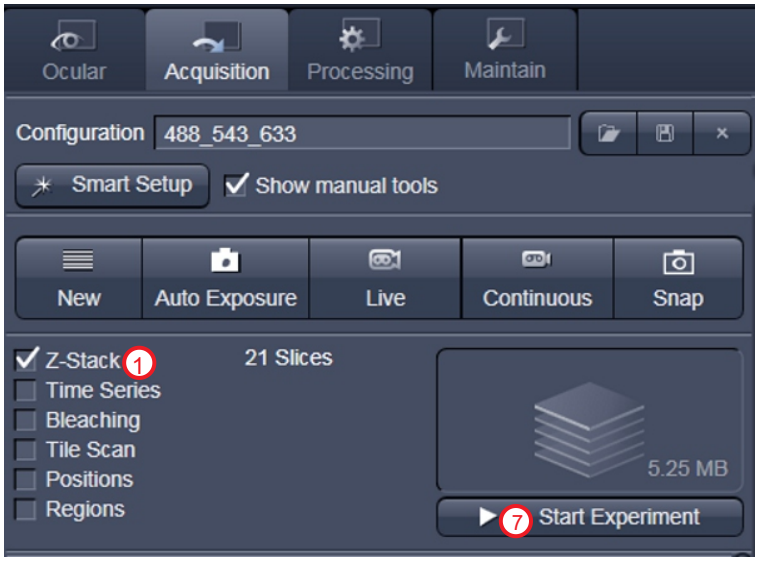
3A. For single photon imaging, set an optimal overlap between images by clicking the "Smallest" button ⑤. This will synchronize the z-stack step size with your chosen optical slice thickness (pinhole size dependent) and the objective for the best software reconstruction of a 3D image.

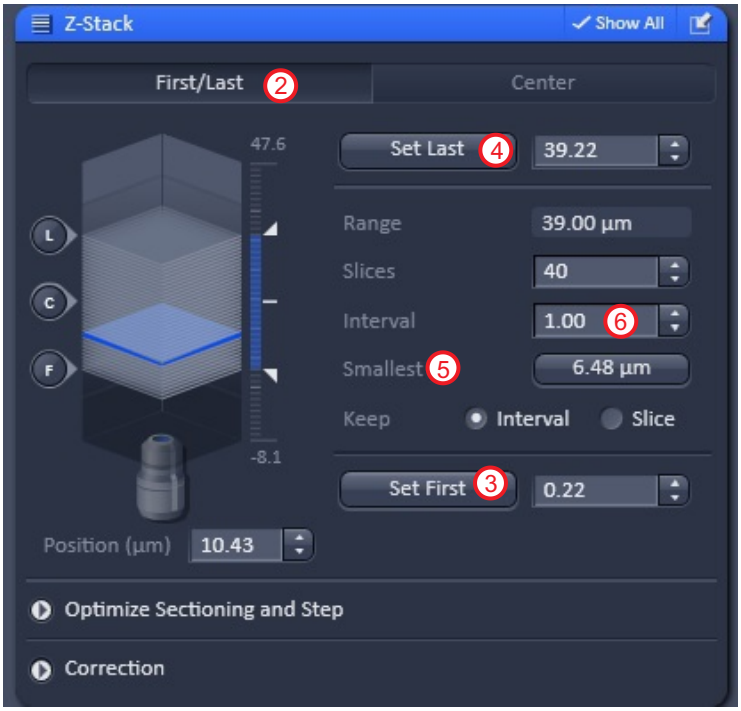
3B. For 2-photon imaging, ignore the recommended :Smallest" step size and set it based upon the smallest z resolution you want to achieve ⑥ . For the 63x lens, 0.5-1 um is good, for the 20x lens, 1-2 um is good.

4. Change any "Acquisition Mode" settings as discussed previously in order to allow completion of your z-stack in a reasonable amount of time. When ready, turn on all channels and click the "Start Experiment" button ⑦ (NOT the snap button).

5. Once started, the approximate time required to finish will appear at the very bottom of the screen. The scan can be stopped at any time and restarted from the beginning.

6. When finished, save the stack as you would a single image.(see next page)



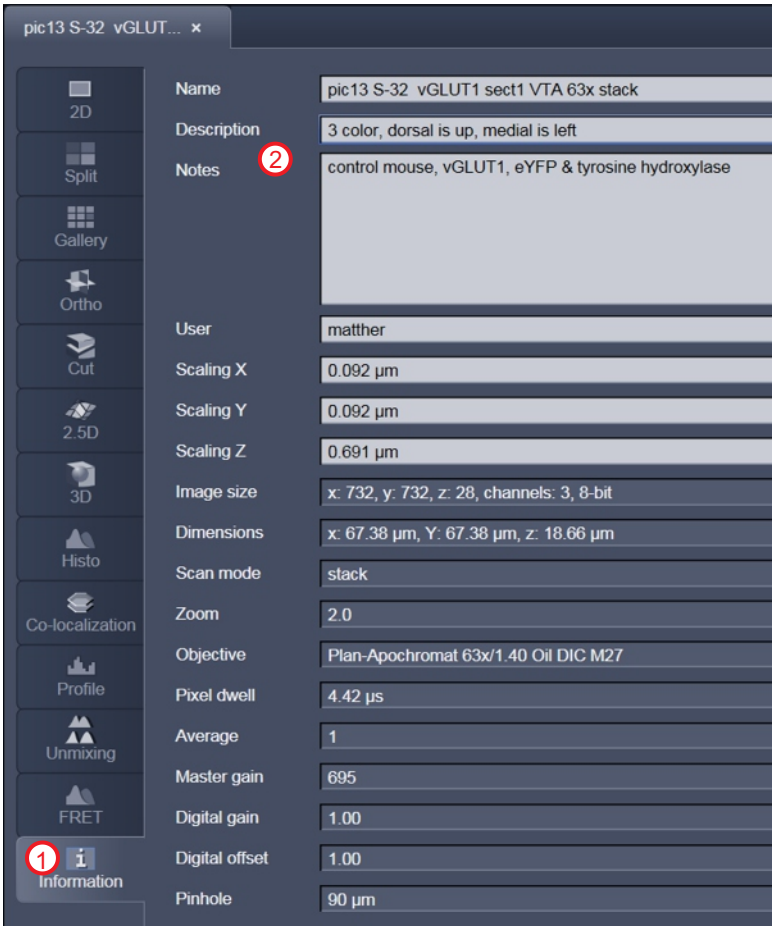


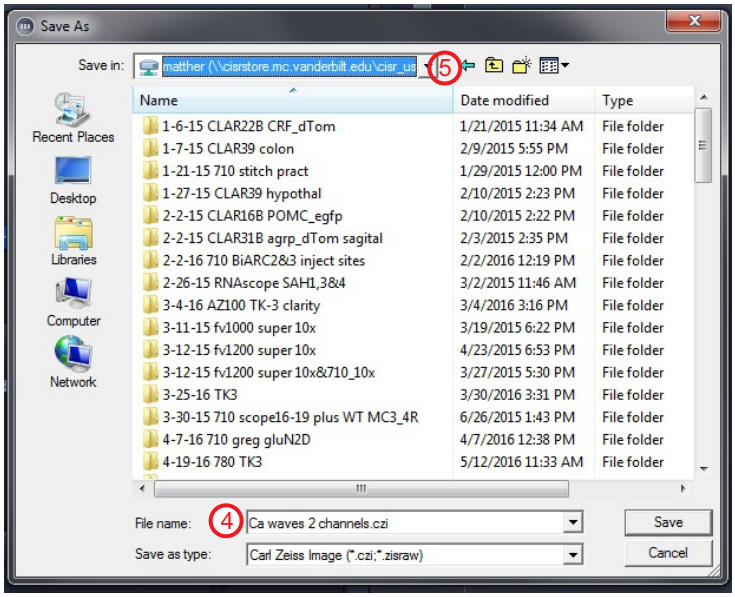
J. SAVING YOUR IMAGES

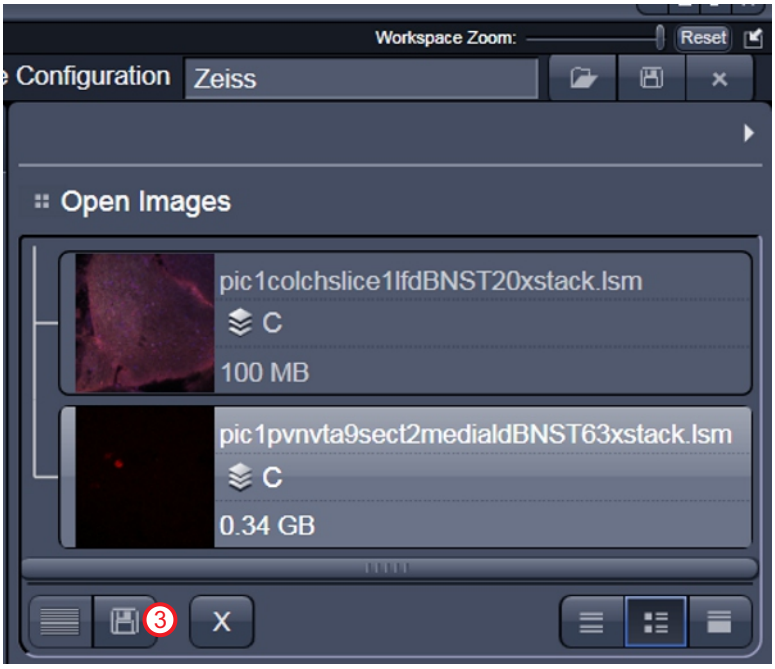
1. Before saving an image, you may want to annotate the image with special information. If so, click the "Information" button to the left of the image ① and add your text to the "Description" and "Notes" boxes ②. Note that other technical info, for example scaling, objective used, etc., are already filled in and will be saved along with the image.

2. Click the save icon under the thumbnails of the open images ③ and fill in the file name in the new window ④. Browse for a destination for where the image will be saved ⑤. Save one copy as a .czi file type.

3. We recommend saving your images to our CISR server called **cisrstore.** Every CISR user is provided a folder with their VUnetID as the folder name, which can be used to store images (up to 50 gigabytes) for free. However, this is not to be use as an archive. Please backup your files elsewhere and erase files older than one year. Our website has instructions for mapping cisrstore on any computer. Find it under the FAQ tab, *how do I save my data*.







K. SHUT DOWN

1. Check the calendar on the CISR website to see whether you are the last user of the day. If you are using the scope during regular work week hours (until 6 PM) and there is another user scheduled before 6:30, please leave all of the equipment turned on. Close the ZEN software, leave the lasers on when prompted, click “OK” and log off.

2. If you are using the scope in the evening or weekend and someone is scheduled to come within 30 minutes, leave the equipment on for them and log off.

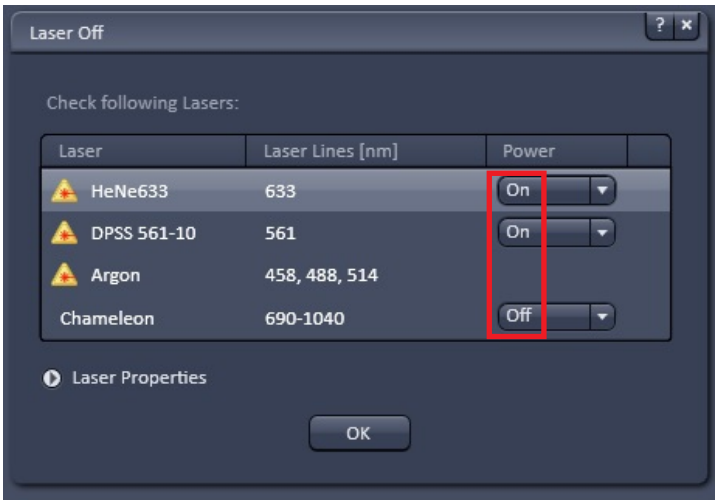
3. If you are the last user of the day or it is an evening or weekend where the next user is not due to arrive for at least 30 minutes, proceed to shut the system down.

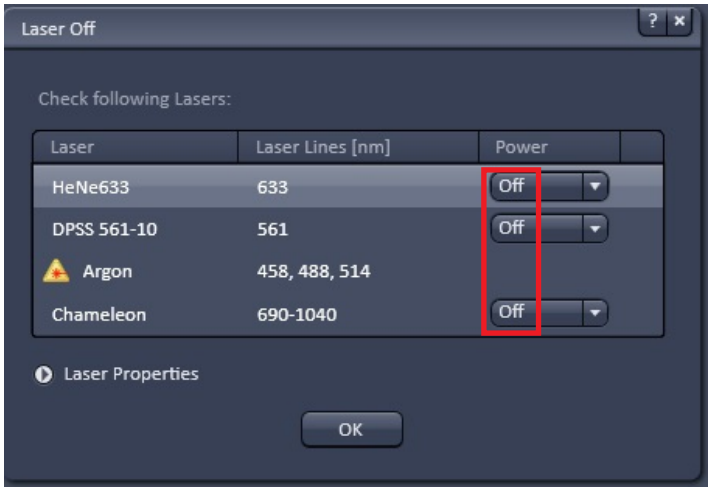
4. Save all images and configurations as appropriate. Close the ZEN software and turn off the lasers when prompted, click “OK”. Log off and when the login screen appears, find the shutdown button in the lower right corner of the screen and shut down the computer.

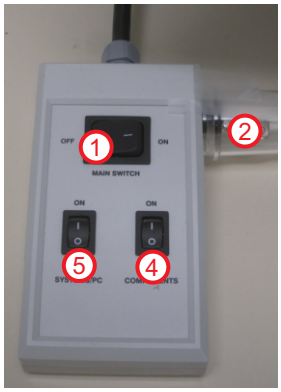
5. Next turn off the key ③ on the argon (Lasos) laser power supply. The power supply must be allowed to cool off with the fan running before turning off the switch next to the key and the rest of the switches on the remote (about 2 minutes). While waiting for the fan to stop, clean off any oil/water from immersion lenses that you used and leave the condensor arm in the down position. Turn off the Excite light.

6. When the cooling fan stops, turn off switches ⑤, ④, ② & ①.

7. If you are using the Chameleon laser, turn the key to “STANDBY” mode.
8. Sign out on the paper log. Come again soon.







Acknowledgment:

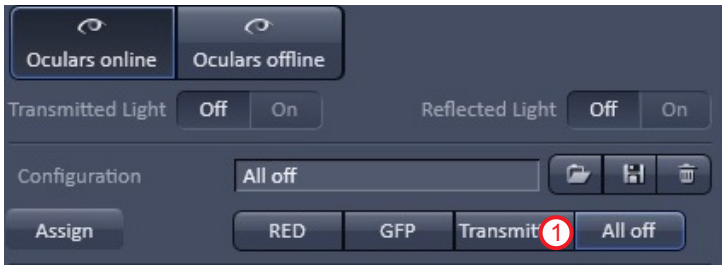
Our funding depends upon your citing the Cell Imaging Shared Resource when you publish data obtained with equipment or services from CISR. This includes images from the microscopes, training in the use of software, consulting on data analysis, etc. The minimum acceptable acknowledgment should read:

“Experiments/data analysis/presentation [include what you use]” were performed in part through the use of the VU Cell Imaging Shared Resource, (supported by NIH grants CA68485, DK20593, DK58404, DK59637 and Ey08126).

Please let us know when you publish and, if possible, send us a reprint of the paper. This is how we justify our existence.

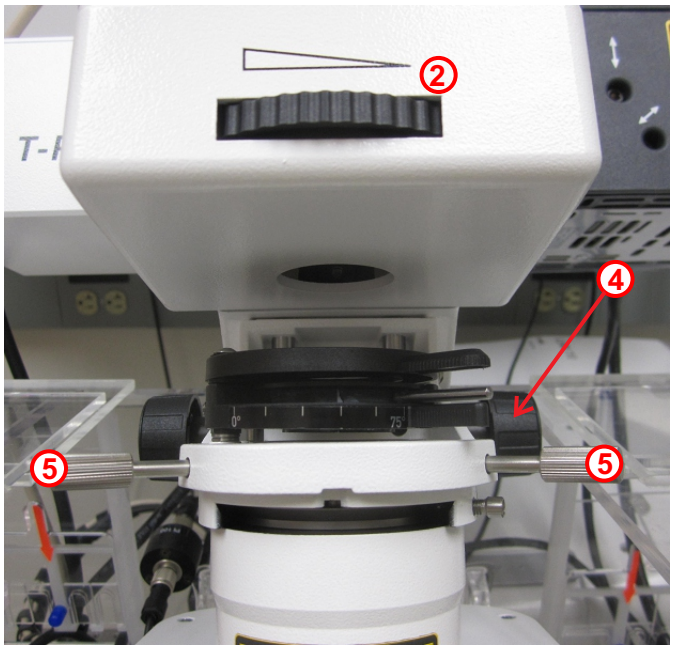
DIC: Ocular Viewing & Kohler Illumination

This microscope can capture brightfield, monochrome images that overlay fluorescent images. These bright field images are DIC (**D**ifferential **I**nterference **C**ontrast) images. For optimal DIC images, light should be focused in the same plane with the sample. This is accomplished by adjusting the **condenser** and is called **Köhler Illumination.**



1. Focus the specimen using transmitted light activated by the "Transmitted" button ①.

2. Close the field iris ② until only a spot of light remains ③. If you can't see a spot of light, try raising or lowering the condenser with the adjustment knob ④.



3. Adjust the condenser knob until the polygon is in focus (edges as sharp as possible.)

4. Turn the "rabbit ear knobs" ⑤ to center the polygon.

5. Open the field iris completely ②.