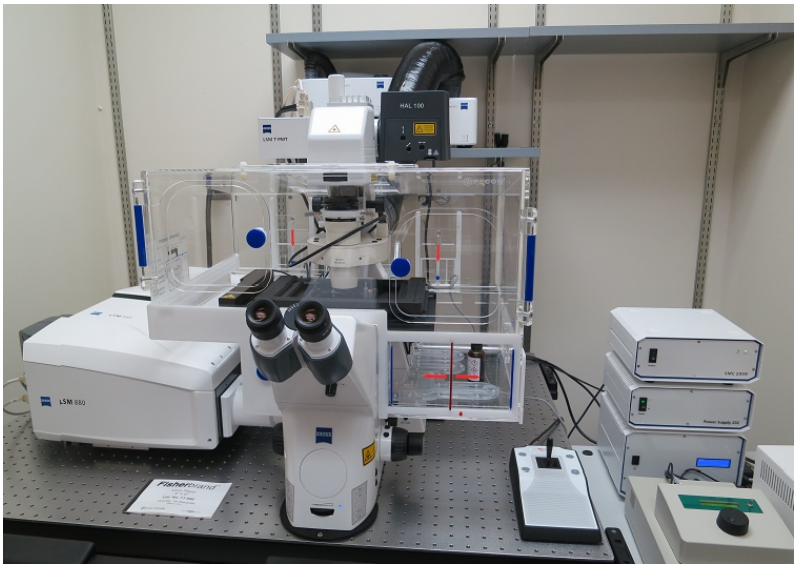
Zeiss LSM 880 Quick Guide

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 LSM880

If the system is OFF, start here:

1. If you are doing a live cell experiment and need the incubator system, turn on the power strip located on the right table leg first (A). Refer to appendix A for instructions on how to use the incubator system.

2. Turn on the main power switch (1), located to the right of the monitor, followed by the systems/PC switch (2), and the components switch (3) in numbered order. The "laser" key inside of the plastic cover should always be left on.

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

If the system is ON, start here:

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

5. A black DOS window may open indicating registry items are loading: it will close automatically.

6. Double click the ZEN icon with the grey shading (5).

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

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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 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. The F1 button on the joystick body (arrow) changes the speed of movement from fast to slow and back to fast with each press.

NOTE: This scope has an additional Piezo stage for super fine movements in the Z plane. Please ask a CISR staff member for the extra training needed to use this feature.

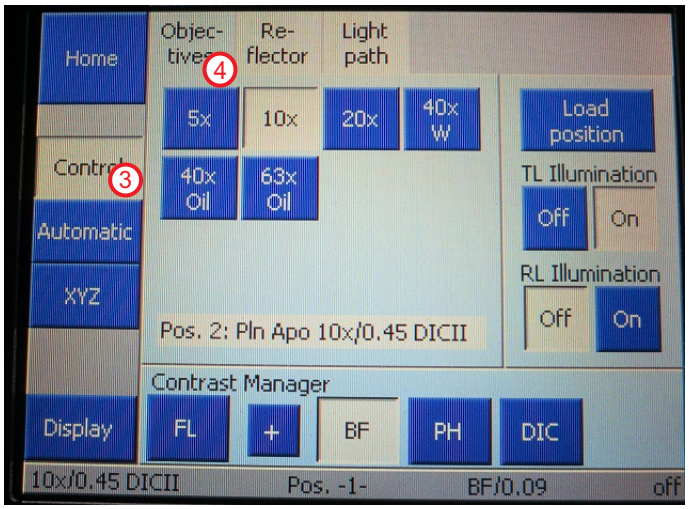
4. Changing lenses and viewing your specimen through the oculars (eye pieces) requires the Touch Pad and/or the ZEN software. Please refer to the appropriate sections for instructions.

C. USING THE TOUCH PAD

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

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”).

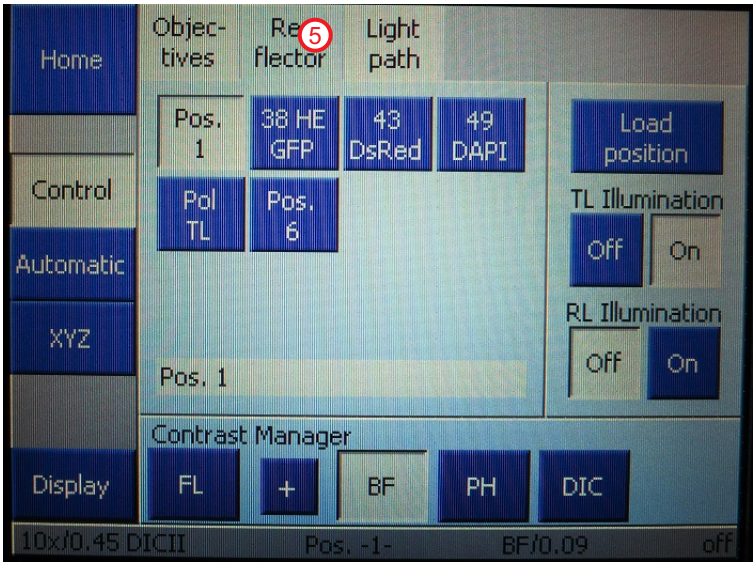
3. The “microscope” screen② can be used to change objectives, choose fluorescent filter cubes (”reflectors”) for viewing fluorescence through the oculars, and controlling other aspects of the light path, **with the exception of the HXP fluorescent light source which can only be turned on through the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

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 reflectors⑤. Three colors are available along the top row, with DIC white light (Pol TL) in the second row.

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, choose "BF" or "DICIII"③ for a white light view or one of the fluorophore buttons, for example the blue nucleic acid marker, DAPI④ .

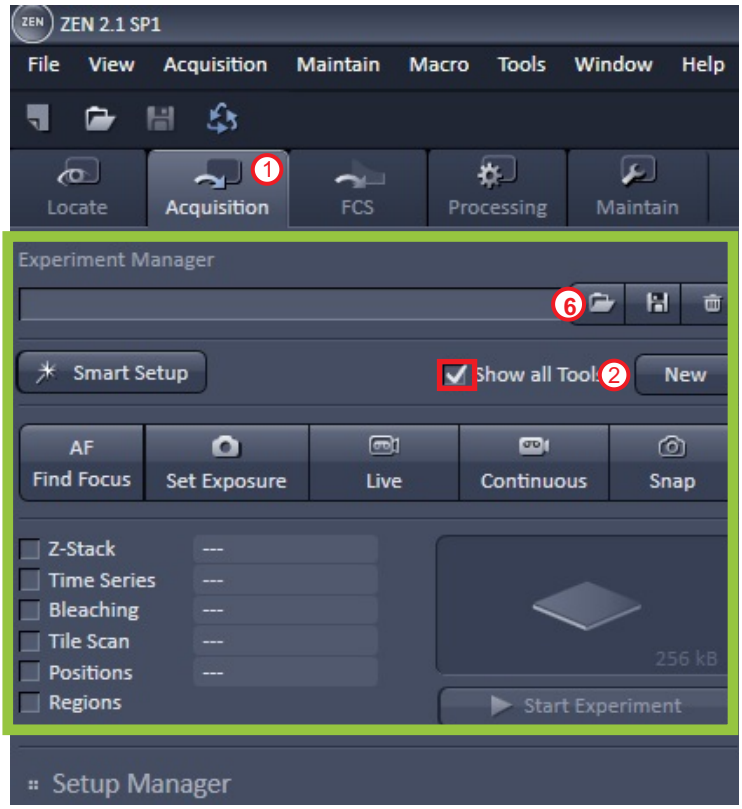
3. In the "Ocular" portion of the screen,⑤ changes can be made in light source intensity, open or closed state of the white light or fluorescent excitation light shutters, etc., by clicking on the appropriate icon. Do not use higher than 25% power with a 50% filter⑥ on the HXP light (fluorescence) unless you want to bleach your sample on purpose.

4. After focusing and visually setting up your sample for image capture, immediately turn off the HXP 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 using either BF or fluorescence mode.

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.

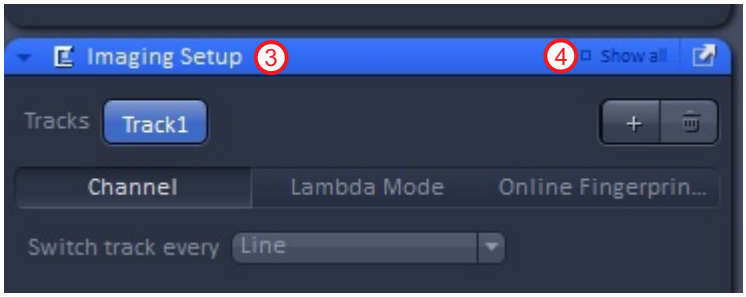


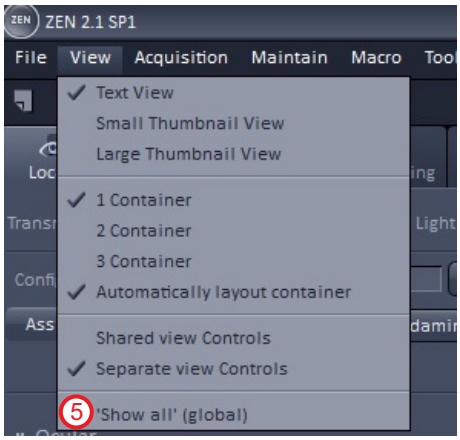
2. The upper left quadrant (outlined **here** in green) contains the main control buttons for opening scanning configurations, starting and stopping scans, and opening advanced setup windows for z-stacks, time series and others.

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,

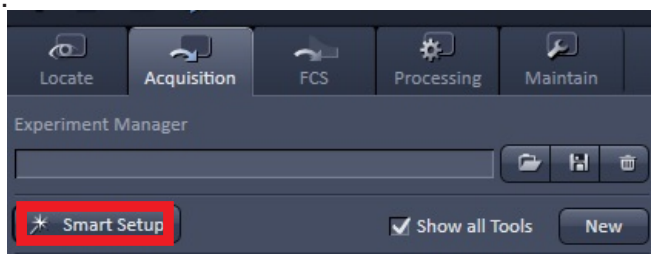
With the menu maximized, for example the “Imaging Setup”, ③ look whether the “show all” box ④ is checked. If not, click the box in order to see all of the menu functions. Or click “Show all” (global) ⑤ under the “View” drop down.



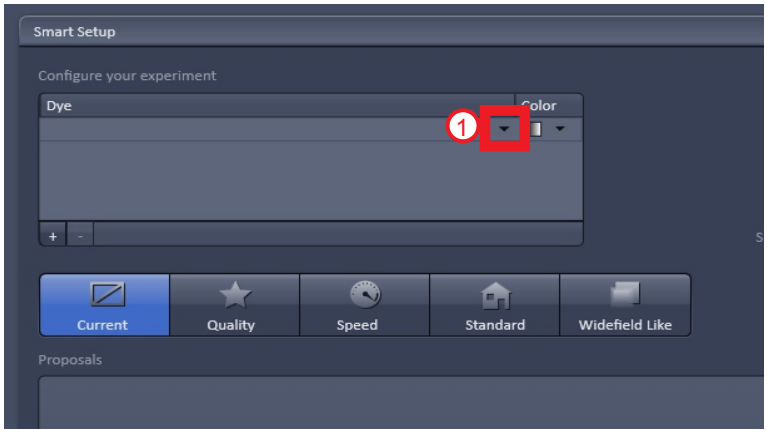


5. Create an imaging configuration that includes all of the excitation/emission settings needed for your fluorescent molecules. Use the “Smart setup” tool discussed in the next section or **load the same configuration used in a previously saved image. To use an old config, open the image and click the “reuse” button ⑥ near the bottom of the screen below the opened image.**

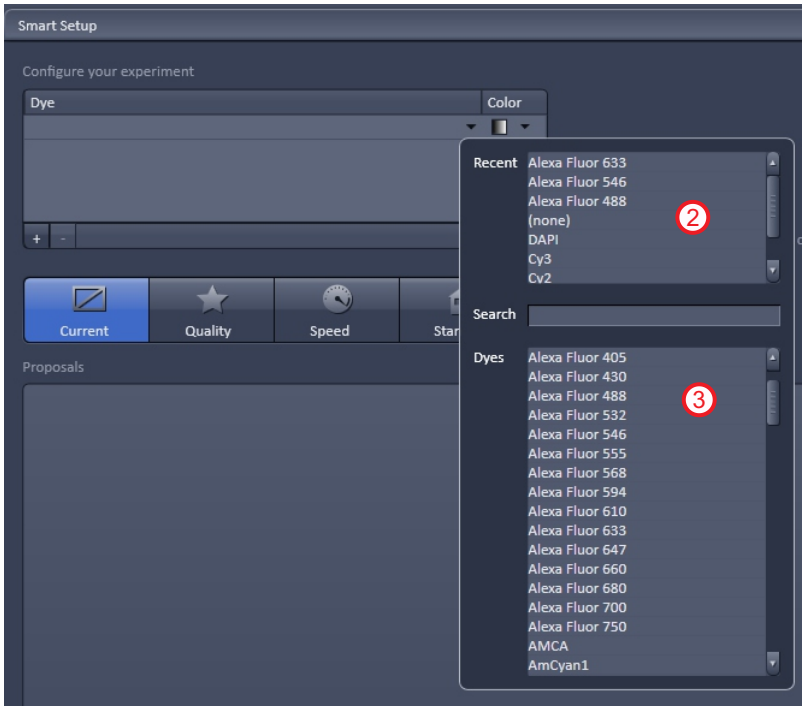
6. If none of the pre-made configs will work for your colors, another way to create a non-Airyscan configuration is to use “Smart Setup”. Click this button to use it.



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.



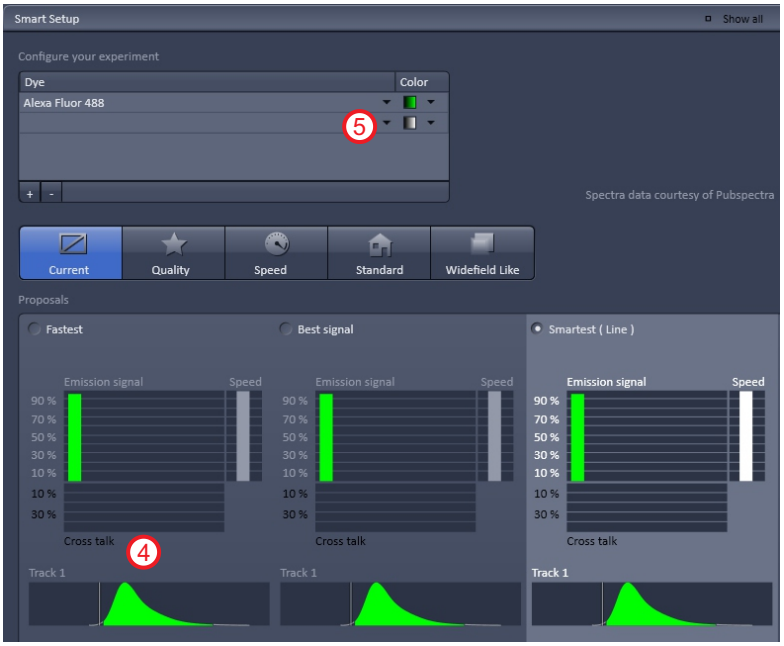
Previously used dyes are listed in the “recent” box② and a more extensive list in the “dyes” box③. Click on the first dye that you want to include in your image.

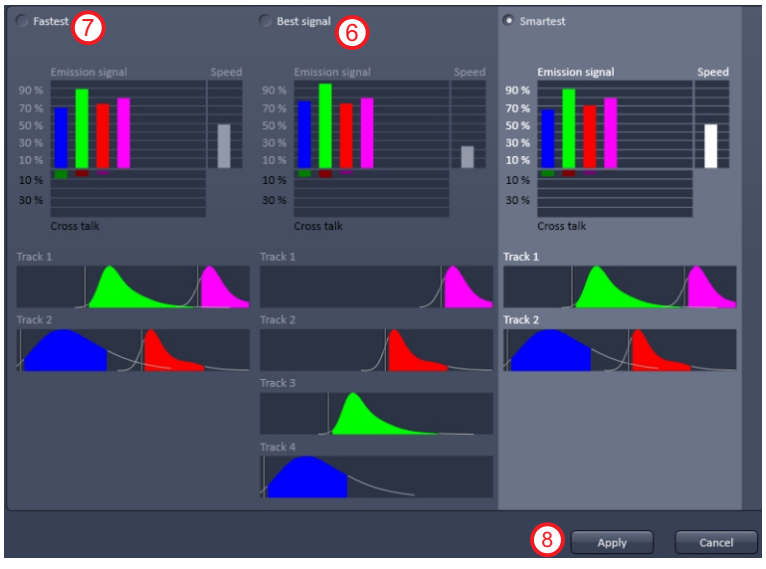


8. A new window will open that shows the emission bandwidth that will be collected④. A new blank track appears in the color column so that a second dye configuration can be added⑤.

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.

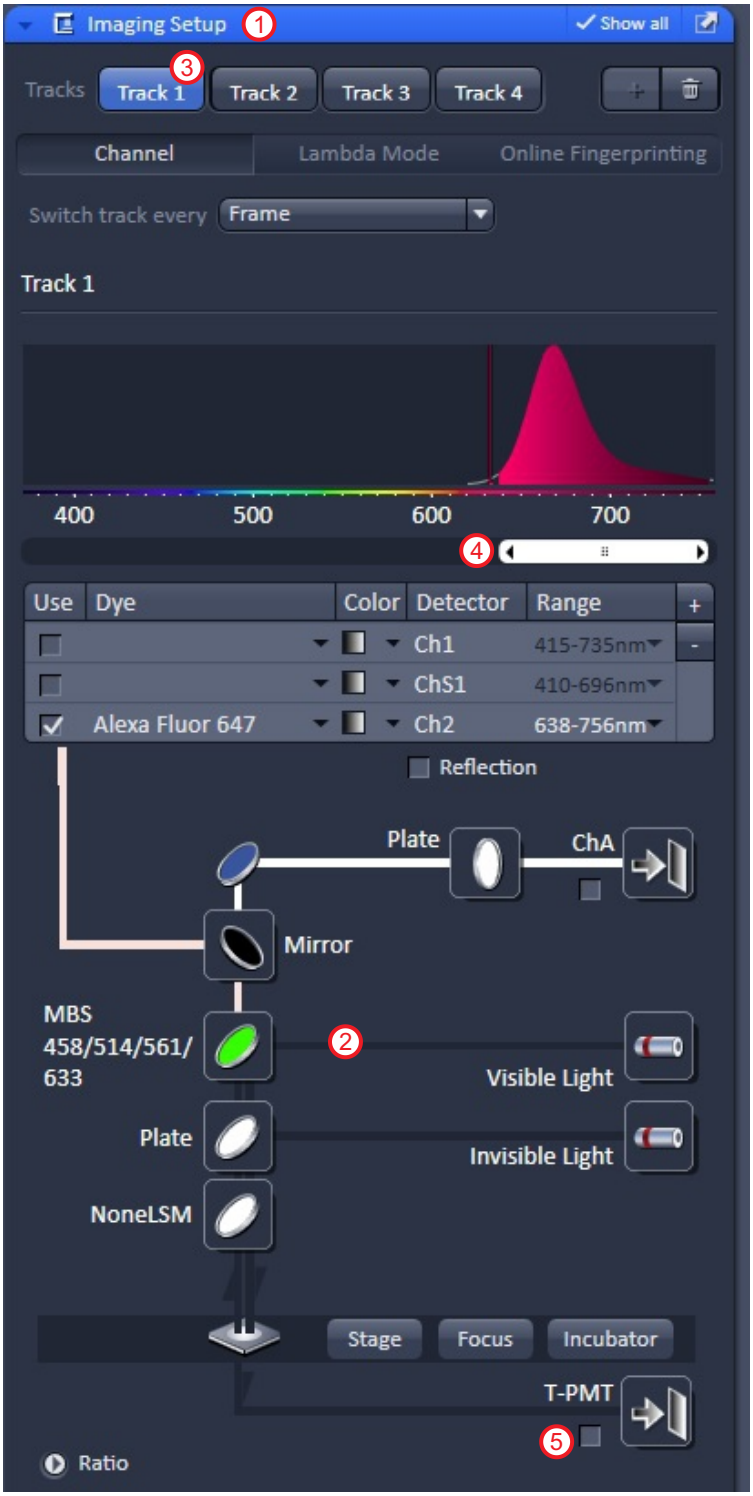
12. After the configuration is loaded, the menus within the setup manager are updated to show which track has which color(s) in "Imaging Setup"(1) as well as the pre-configured light path for each track(2). The highlighted (blue bar) track is the one shown in the light path(3). In the example shown here, "best signal" configuration was chosen in smart setup so there are four tracks with one color in each track.

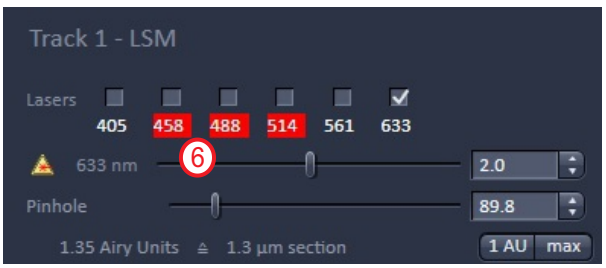
Note that the Argon laser that gives rise to the 458, 488 and 514 nm laser lines needs a few minutes to warm up after the configuration is loaded. During warmup, the laser lines are boxed in red.(6) Once the laser is ready to use, the red boxes will disappear.

13 There are many options within the Imaging Setup 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 spectrum (4). 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.

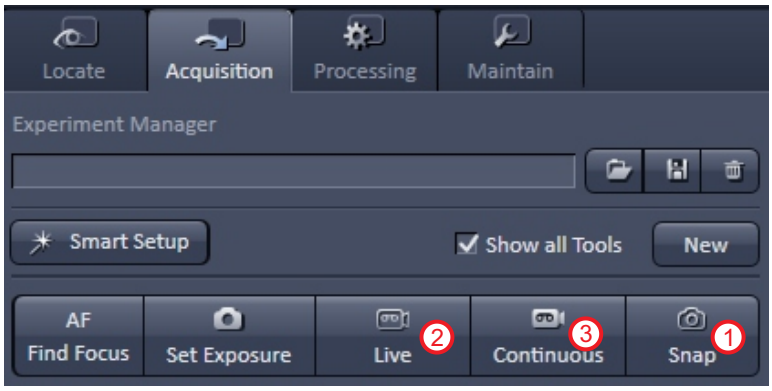
15. Second, a grey scale, DIC image can be added as an additional channel by clicking the box marked "T-PMT" at the bottom of the menu (5). This can be done on any of the tracks (only need one), the choice based upon which laser you prefer to use to collect the DIC image. For optimal DIC images, Kohler alignment is recommended (see page 14 for instructions).



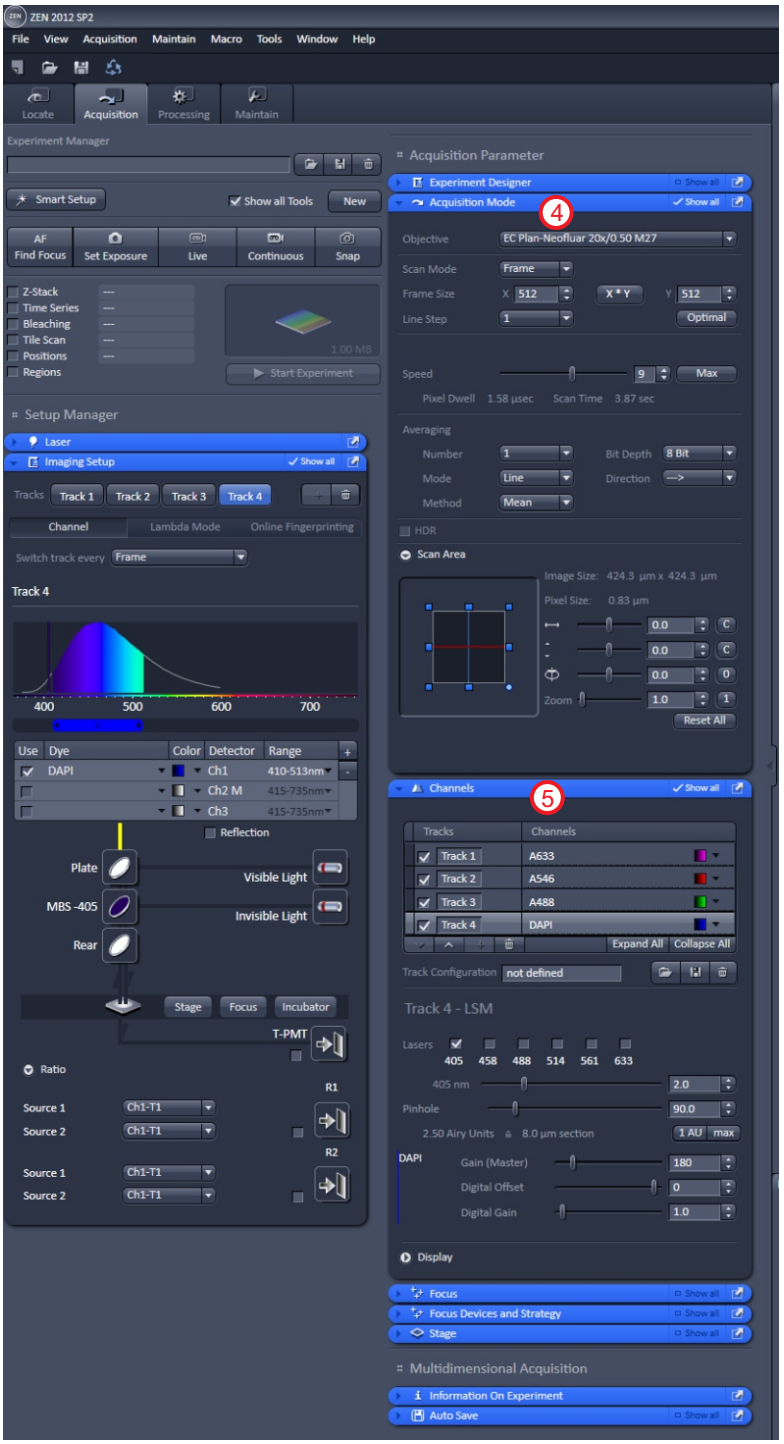


F. LASER SCANNING: OPTIMIZING THE IMAGE

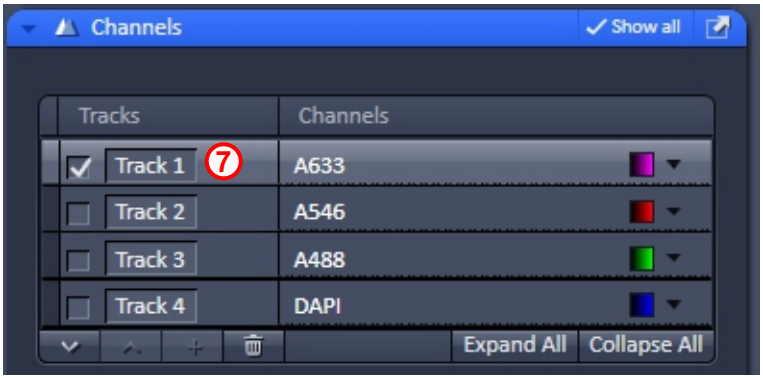
1. Once a configuration is in place, single image scans are initiated with the "snap" button①. To scan repeatedly so that the effects of changing laser power, signal amplification, etc. can be seen quickly, use the "live" button ②. The "continuous" button③ also scans repeatedly but scan rate, frame size 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"④ and the "Channels"⑤ 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 track⑦. Choose the track with the dimmest signal, as seen previously through the eye pieces. Or start with the track with the longest wavelength. Click in the blank part of the track title in order to activate its controls (turns it lighter grey⑦). 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 output ⑧. Keep default output of 2% or less initially to prevent bleaching.

5. The next option is pinhole ⑨ which determines the optical slice thickness and creates a confocal image. A pinhole setting of 1.00 Airy Units (1 AU) will result in the sharpest image, collecting all in-focus light and rejecting out of focus light. However, a pinhole setting of > 1 AU 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 600-800. Leave the digital offset at default "0" ⑪. Begin scanning by clicking the "live" button. (② on previous page)

If an image appears, skip to step 7

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

A. click the "max" button ⑫ to fully open the pinhole or use the slider to open it enough to get an image.

B. Refocus while scanning.

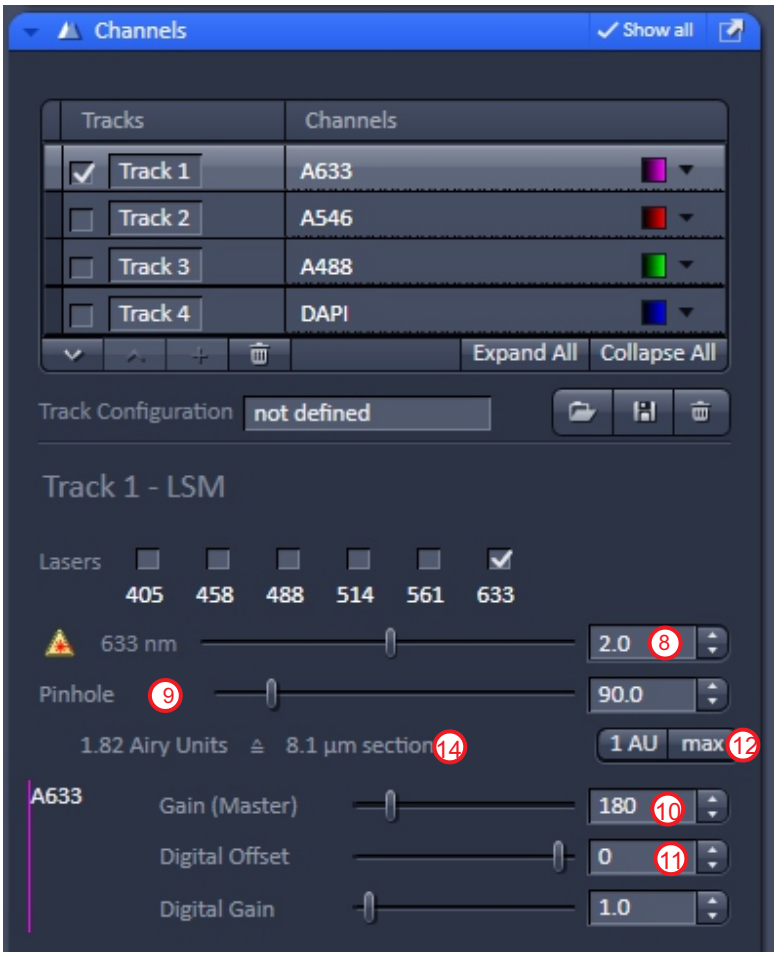
C. Increase laser output to as high as 15 for short wavelength lasers (405, 458, 488 & 514 nm) or 30 for long wavelength lasers (561 & 633 nm).

7. With an image visible, click the box called " Range Indicator" at the bottom of the center screen ⑬. In this palette, red pixels in your image mean the PMT 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 65,536 are red (16 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. Leave the Digital Gain at "1".

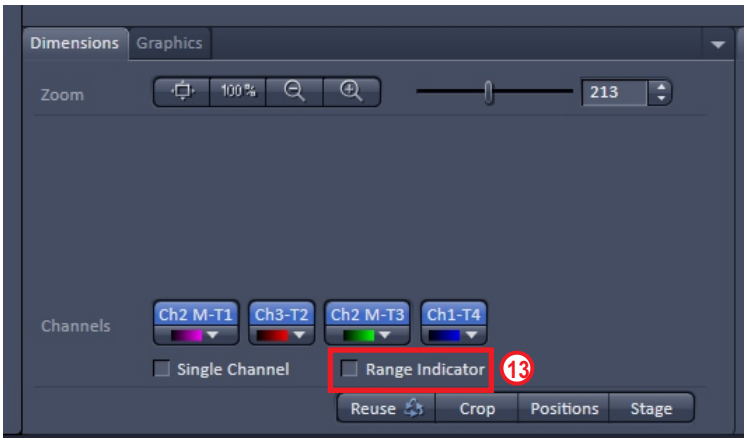
Note: The goal in optimizing is to use the smallest pinhole size (≥1 AU), lowest laser output and a Gain (master) at or below 800 that produces an acceptable image with just a few red pixels visible when using the range indicator palette.

8. After the 1st track is optimized, note the final optical section thickness ⑭. 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. To capture an image, put check marks in all tracks and click "snap".



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 PMT detector. However, a larger pinhole results is a "fuzzier" image.



If you are satisfied with your image and do not plan to do a Z-stack, skip to page 12 for instructions about saving your image.

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 "optimal" (2) for the best resolution possible for a particular objective and field size. Higher than optimal resolution 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. 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.

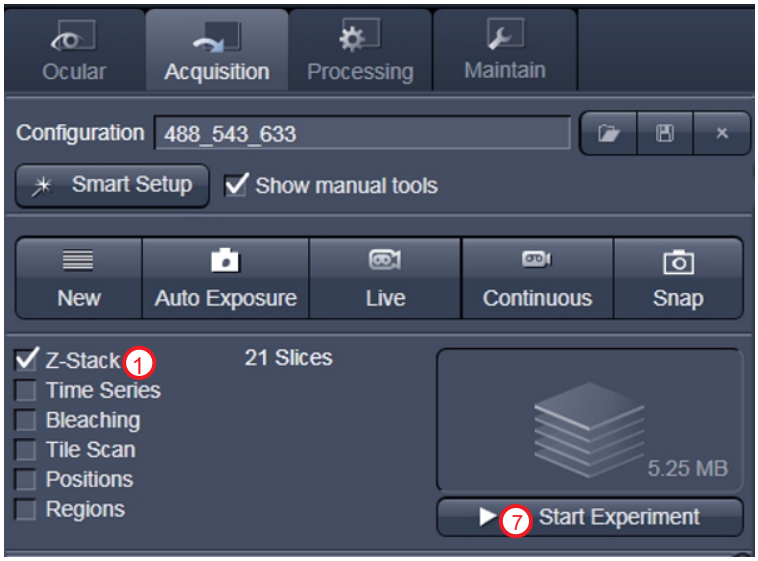
3. Set an optimal overlap between images by clicking the "Optimal" button (5) . This will synchronize the z-stack step size with your chosen optical slice thickness (pinhole size dependent) and the objective for the best software reconstruction of a 3D image. Click "Optimize Sectioning and Step" submenu (6) to reveal additional buttons at the bottom of the z-stack menu, such as Z correction used for very thick stacks.

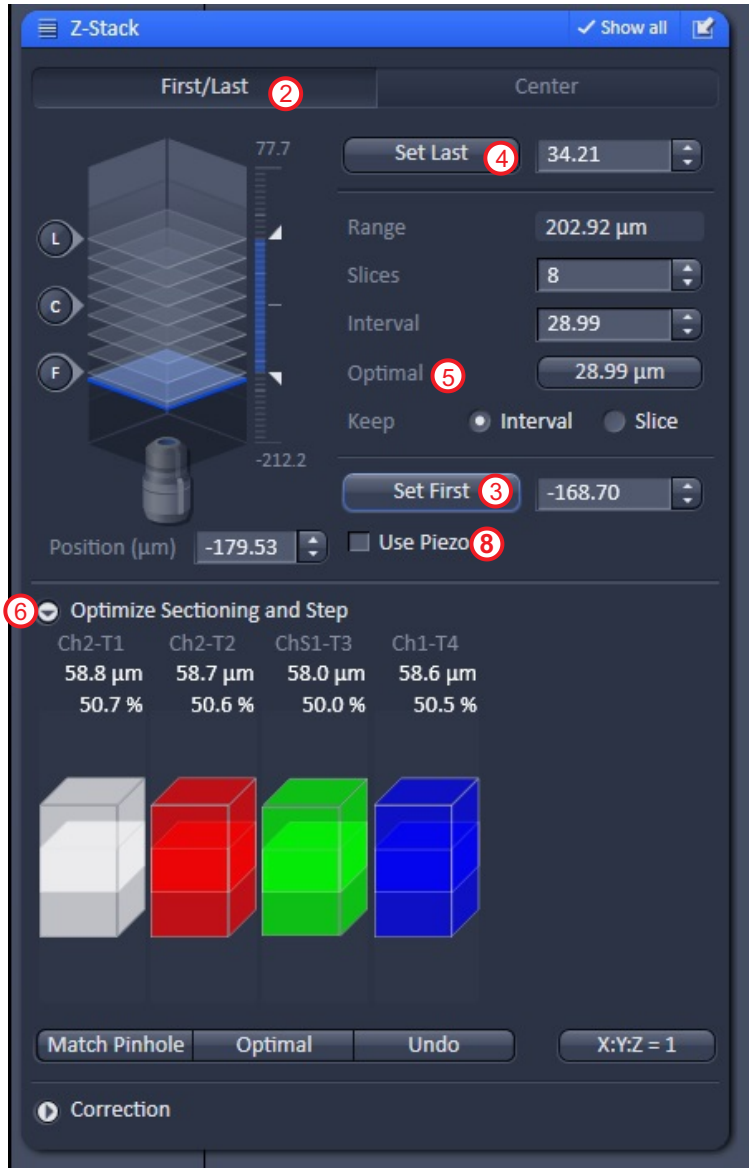
4. Change any Mode settings as discussed previously in order to allow completion of your z-stack in a reasonable amount of time. When ready, turn on all channels and click the "Start Experiment" button (7) (NOT the snap button).

5. If you need to use the Piezo stage, check the box here (8) in order to turn it on (see Appendix for detailed instructions).

6. 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.

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



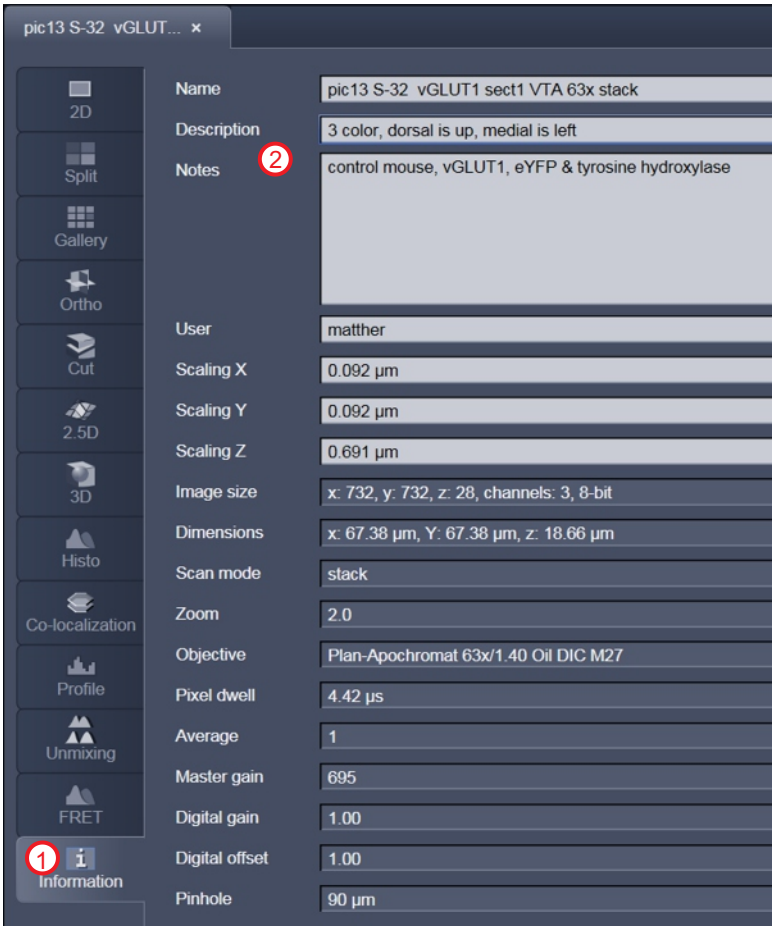


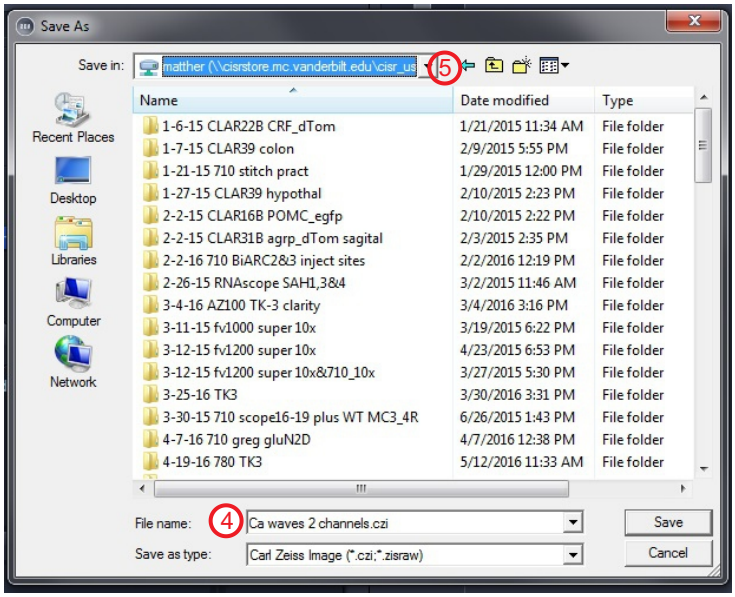
I. SAVING YOUR IMAGES

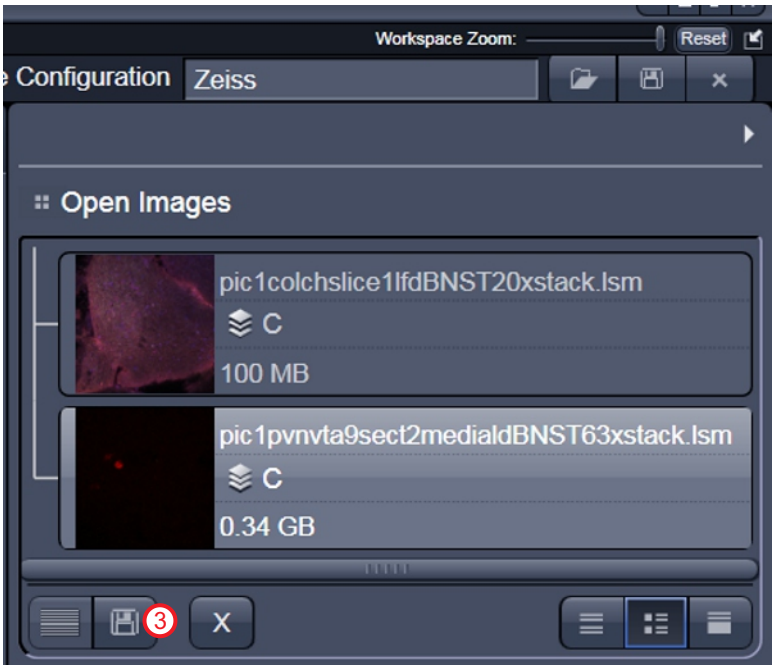
1. Before saving an image, you may want to annotate the image with special information. If so, click the "Information" button to the left of the image ① and add your text to the "Description" and "Notes" boxes ②. Note that other technical info, for example scaling, objective used, etc., are already filled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

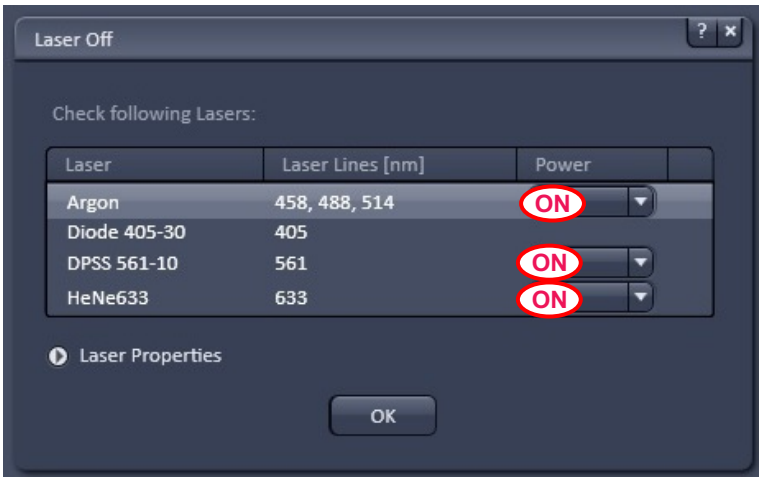






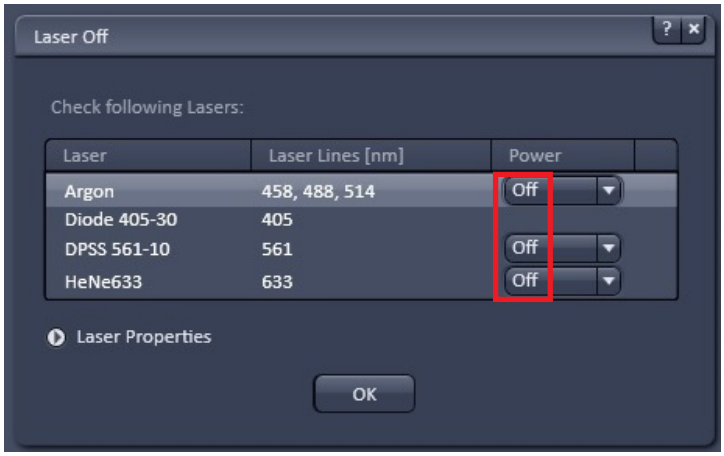
J. SHUT DOWN

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 ON, 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. The argon laser power supply must be allowed to cool off with the fan running before turning off the rest of the switches (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.

6. When the cooling fan stops, turn off switches 3, 2 & 1.

7. 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 VUMC Cell Imaging Shared Resource, (supported by NIH grants CA68485, DK20593, DK58404, DK59637 and Ey08126).

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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.**

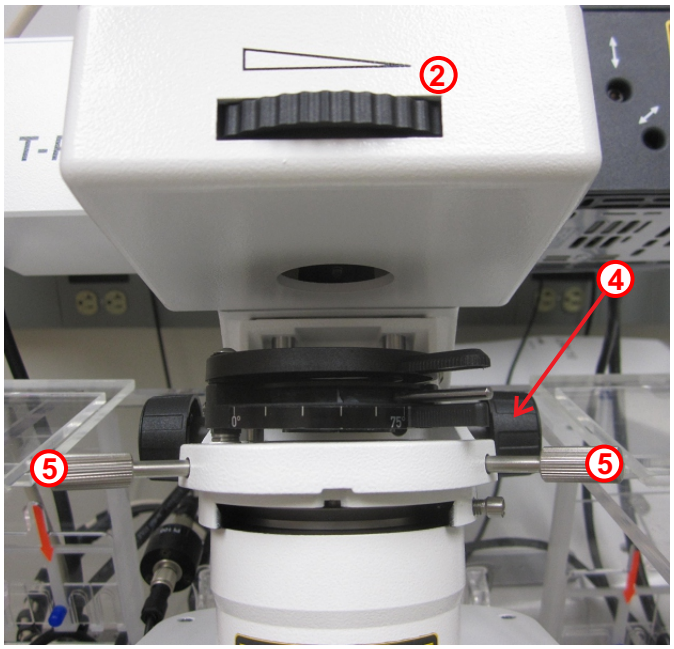
1. Focus the specimen using transmitted light activated by the DIC button ①.

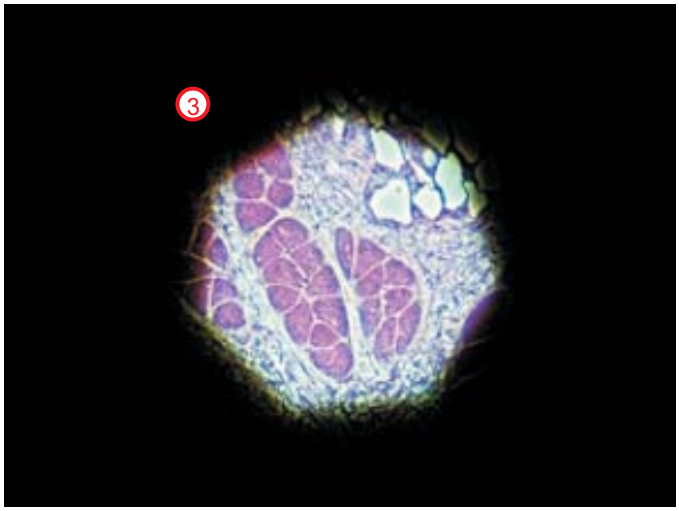
2. Close the field iris ② until only a spot of light remains ③. If you can't see a spot of light, try raising or lowering the condenser with the adjustment knob ④.

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.



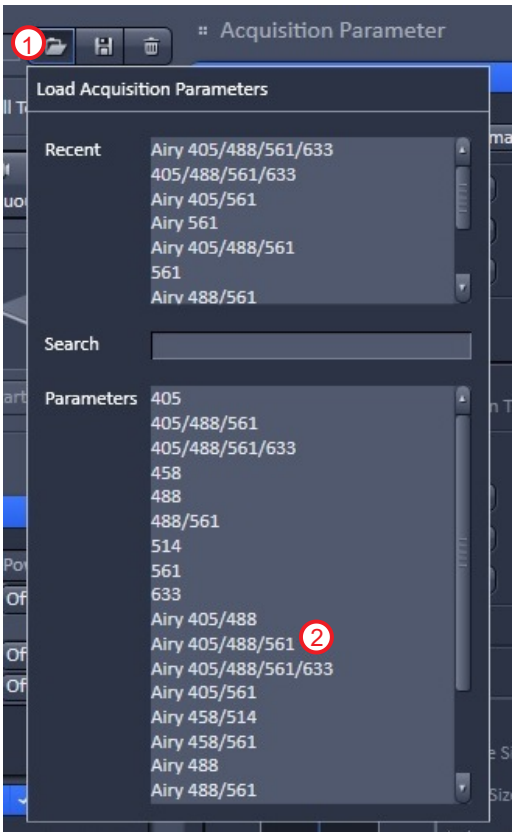


APPENDIX A: Airyscan

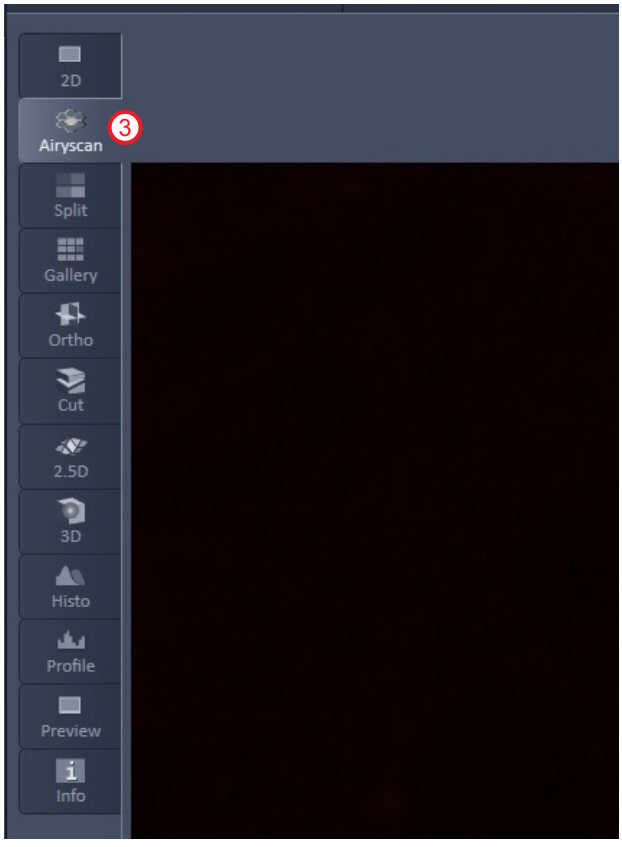
1. Airyscan is a type of super resolution microscopy that can be applied to any fluorescent sample. It requires special attention to selecting the correct objective immersion fluid to match the temperature of the sample, making sure the sample is perpendicular to the light path (ask a CISR staff to explain how to check this) and optimizing the acquisition settings specifically for the airyscan scanning mode.

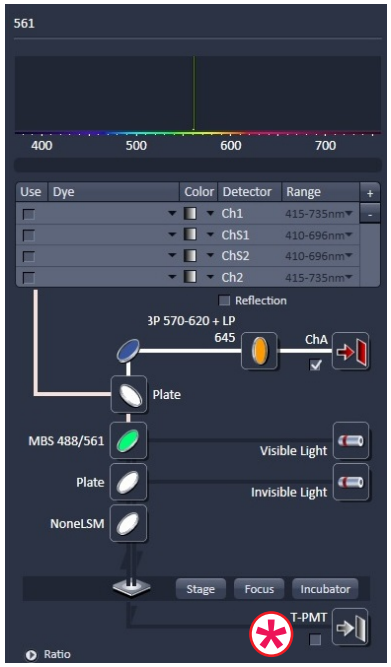
2. The 63x objective is designed to give the best Airyscan image but other lenses, including both 40x lenses and the 20x lens can be used. Be sure that the Wollaston prism beneath the lens has been removed (ask CISR staff to do this).

3.Begin by finding an area of interest in your sample using the locate functions described elsewhere. Under the acquisition tab, open the configurations list (1) and click one of the Airy configurations that has the appropriate excitation wavelengths that you need, for example a blue, green and red set of dyes (2) . You must choose from this list, you can not use smart setup to create an Airy configuration.



4. Optimize one channel at a time starting with longer wavelengths and finishing with the shortest wavelength. Use “continuous” scan mode NOT “live” scan to optimize. While scanning, switch the image view from “2D” to “Airyscan” (3) .





5. The Airyscan light path (shown above) is significantly different from the normal confocal path, i.e. there is no control over the emission bandwidth and no DIC image is possible, DO NOT CHECK THE T-PMT BOX, (*) DOING SO WILL REQUIRE YOU TO RELOAD YOUR CONFIGURATION.

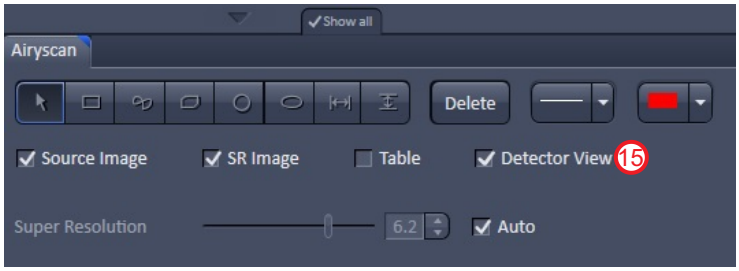
Note: All of the Airy configs have default settings for the 63x objective. If you are using a different objective, you will need to reset the pinhole in each channel.

6. For optimal channel brightness, adjust laser power only (4), leave pinhole between 2 & 3 Airy units (5), Gain (master) should be about 800 (6), digital gain of 1 (7), and there is no offset to adjust. Airyscan mode should be "SR" (8) for most applications.

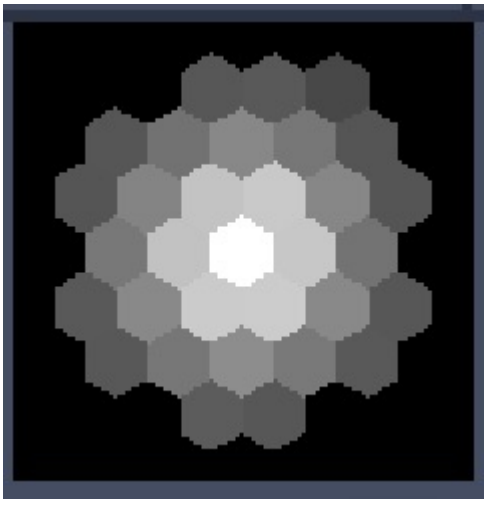
Note: the optical section thickness (9) is **NOT** controlled by the pinhole. The pinhole is used only for blocking stray light. Reducing the pinhole size below 2 Airy units blocks too much light and compromises the resolution of the final processed image.

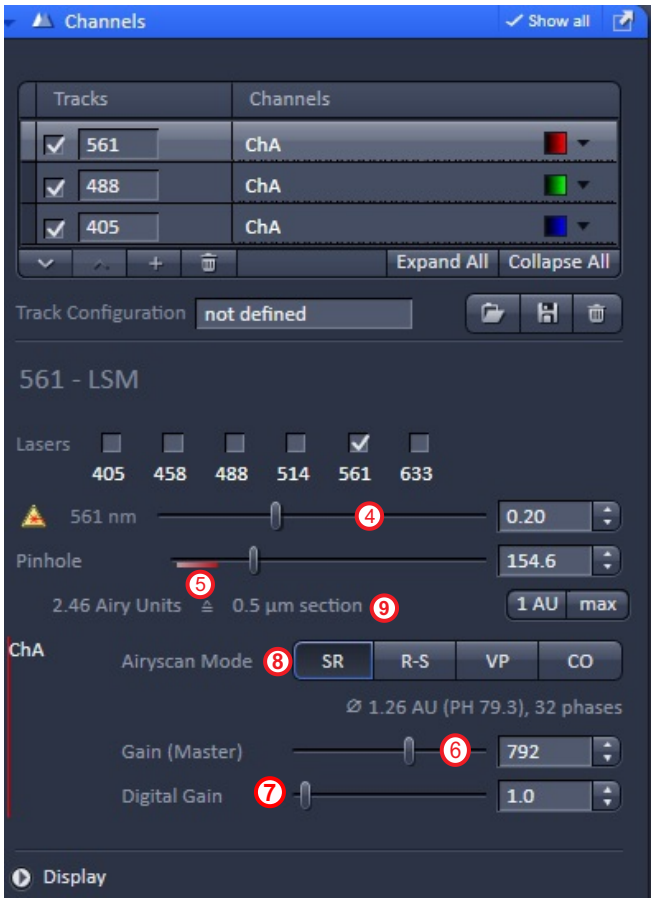
7. Stop scanning and continue optimization by choosing a single channel, green or red channels are good choices, DO NOT choose the 405 nm channel for this part. In the "Acquisition Mode" screen, click the "Optimal" frame size (10). Scan speeds of 6 or less (11) give the best images. 8 bit images (12) scanned bi-directionally (13) are recommended. Finally, the scan zoom must be set at 1.8 or higher (14).

8. After all of these optimizing steps are set, check the "Alignment" graphic (15) one color at a time, while scanning, to be sure that it has realigned itself to give a symetrical honeycomb. If the honeycomb graphic is not visible, check the "Detector View" box (15).

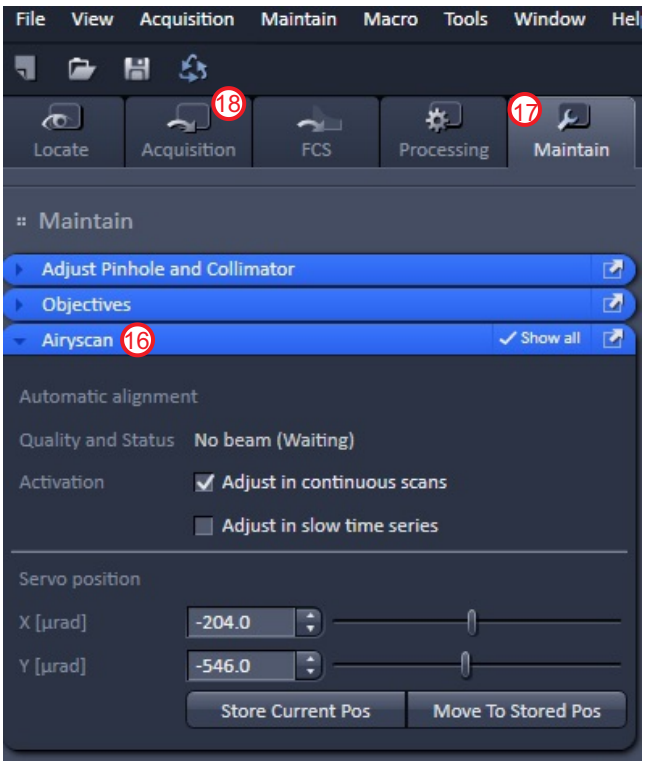


EXAMPLE of an ALIGNMENT (HONEYCOMB) GRAPHIC

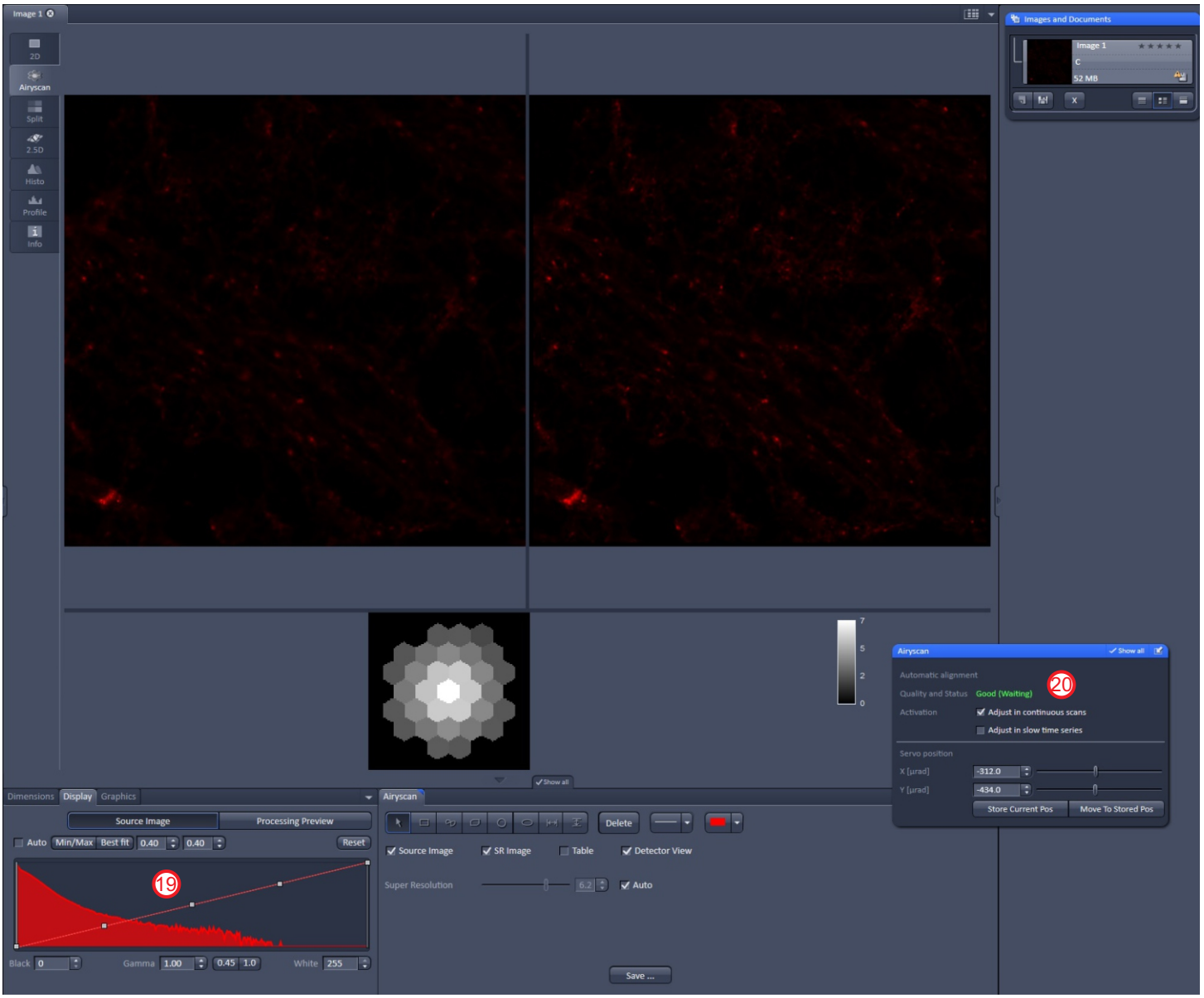




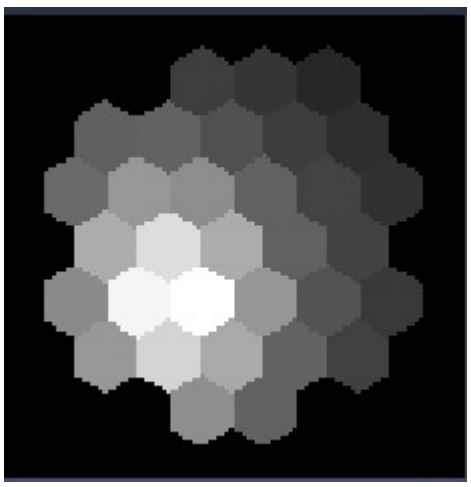
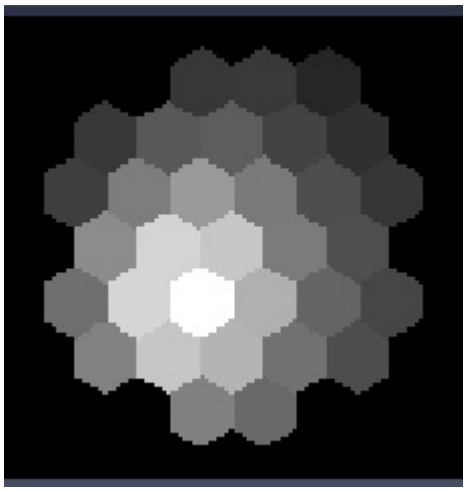
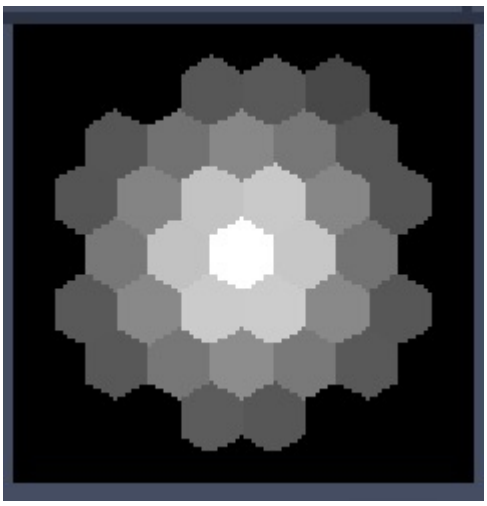
9. Along with the honeycomb graphic, open the Airyscan Quality and Status window (16). Find this window under the "Maintain" tab (17). Drag this window to the far right side of the monitor and go back to "Acquisition" (18).

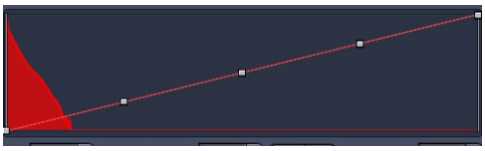
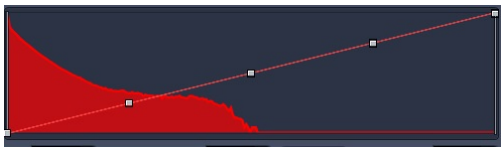
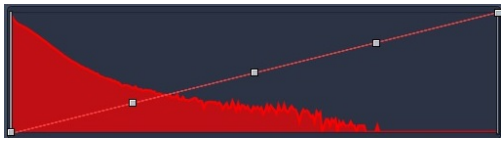


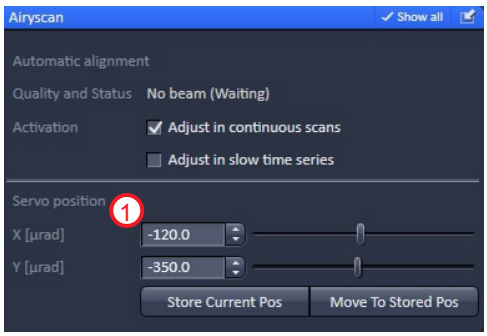
10. While scanning one color, the screen should look like the example shown below. Note that the "Graphic Display" shows that the signal intensity reaches about 2/3 of max (19). For best results, adjust the laser power and gain (master) so that the intensity range is between 1/3 and 2/3 of max. In the example below, the Alignment and intensity are optimal and therefore the "Quality and Status" (20) turns **green "Good (waiting).** Do this for all colors.

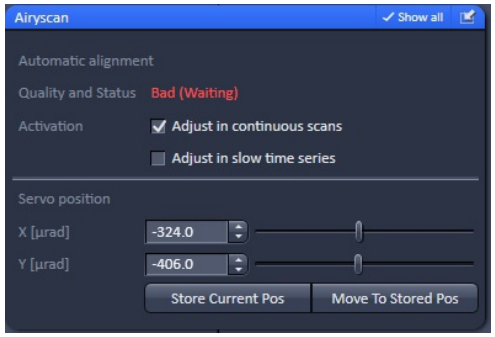


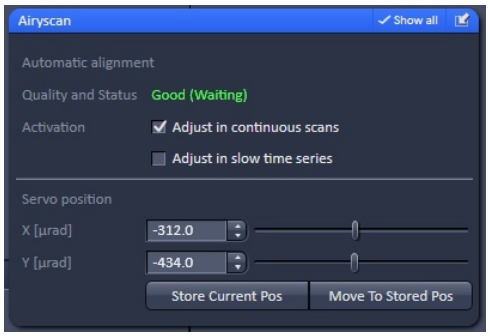
Bad optimization | Poor optimization | Good optimization

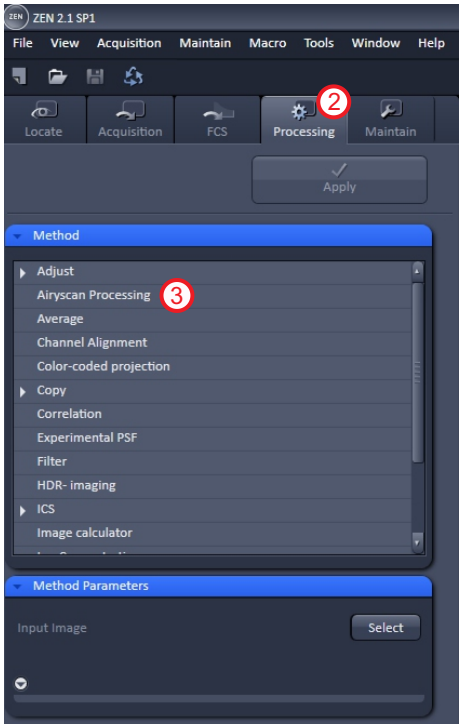






11. If one or more colors cannot be optimized enough to get the best Quality and Status setting, try manually changing the "Servo Position" in X and Y①. When the Honeycomb looks as close to ideal as possible, turn on all channels and click "Snap" for a single 2D image.

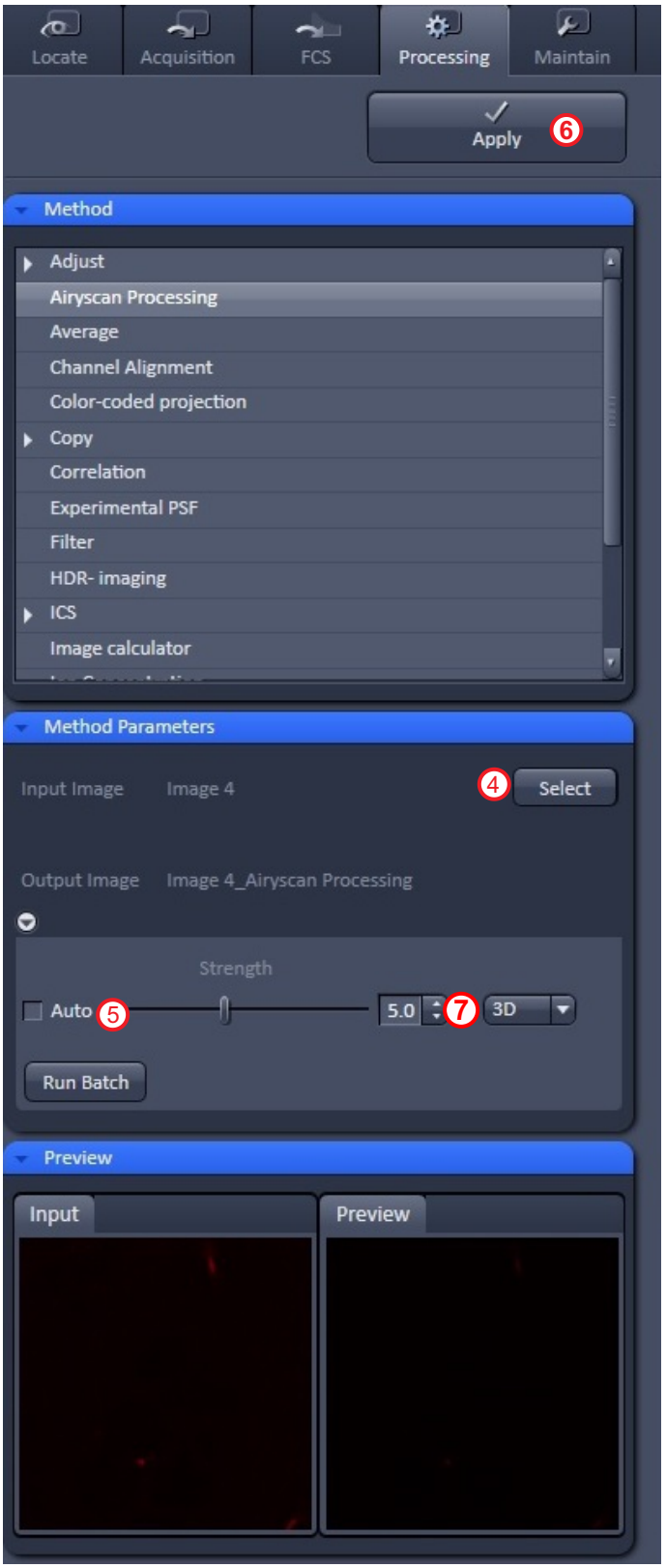
12. Next the raw image needs to be processed. The right hand image is a preview of the processed image. Open the processing tab ②, click on "Airyscan Processing"③ .



13. Click “Select” ④ and choose the image to be processed. Check the “Auto” box ⑤ for an initial image processing strength. Click “Apply” ⑥ .

14. Processing will take several minutes. When it is finished, save the original unprocessed image and the processed image. You will likely need to adjust the display to get a bright image, do so in the “Display” tab below the image, click on “Best Fit”.

15. Auto strength processing is done at a conservative “strength”. More aggressive processing can sometimes increase the image resolution further. Do this by first finding out what strength was used by the auto function. On the left side of the processed image, click on the “Info” button, the strength used is on the last line of the table. Add between 0.5 and 1.0 to this number and type it into the “Strength” box ⑦ after removing the check mark from the “Auto” box ⑤. Rerun processing and save the new image as needed. Note that if the processing is too aggressive, the image will be grossly distorted.



3D Airyscan

1. Z-stacks, time series, and most other special functions are available in Airyscan mode, EXCEPT for tile scans which are not possible.

2. For Z-stacks, optimize all of the channels as previously described for 2D Airyscan. Turn off all but the shortest wavelength channel and open the Z-stack menu. Scan using "Live " mode and set the beginning and end of the Z series as described previously for setting up Z-stacks. Try to limit a multicolor Z-stack to 20 um or less. Stop scanning and click the "Optimal" button (8). Note that the Z step size is smaller than the step size used for standard confocal Z-stacks, resulting in many more images, more time needed to finish the stack and therefore more risk of bleaching if the stack is too large.

3. If the number of "Slices exceeds about 30, try using the Piezo stage (9) in order to speed up the image acquisition.

4. Turn on all of the channels and click "Start Experiment".

5. When acquisition is complete, process the image as described for 2D images.

6. Because these are large files, save them initially to a folder with your name on the D drive, under "Users".