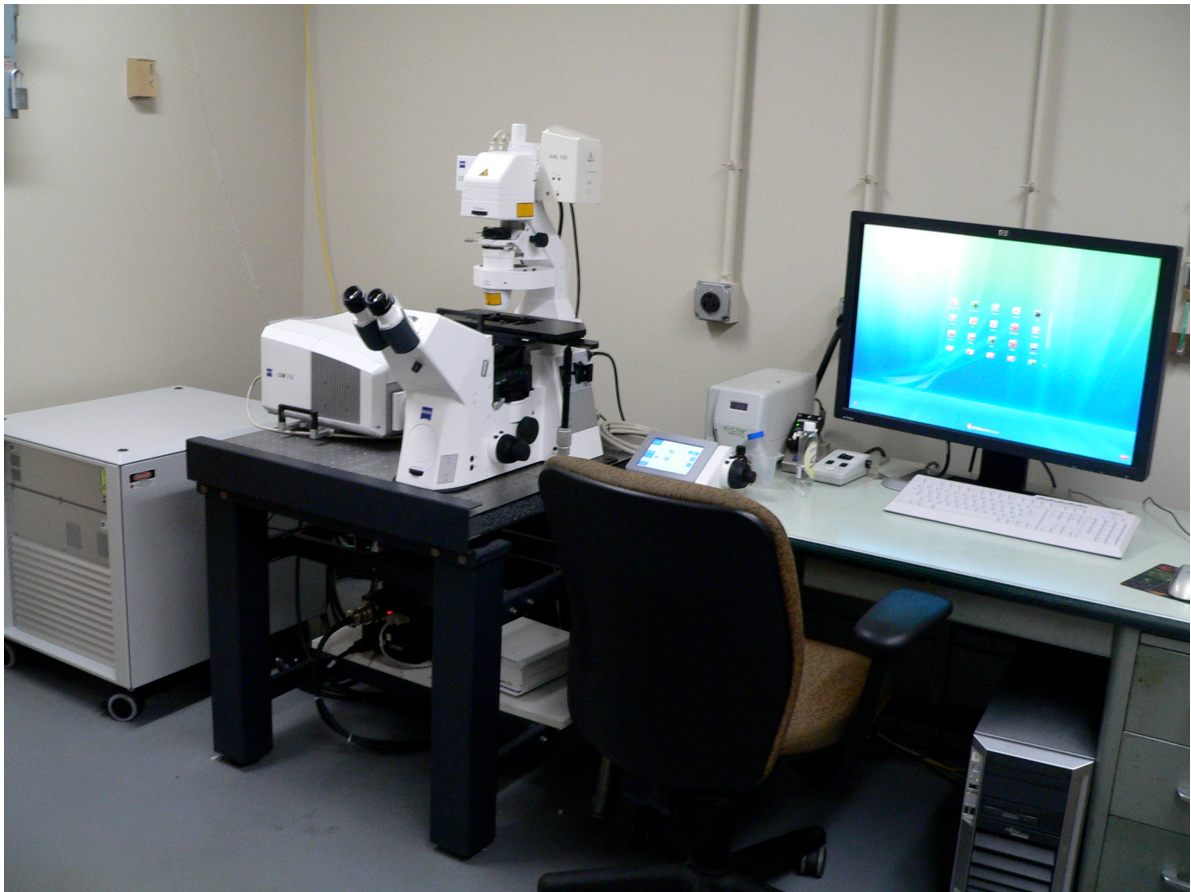


# Quick Start Guide

## *LSM 710*



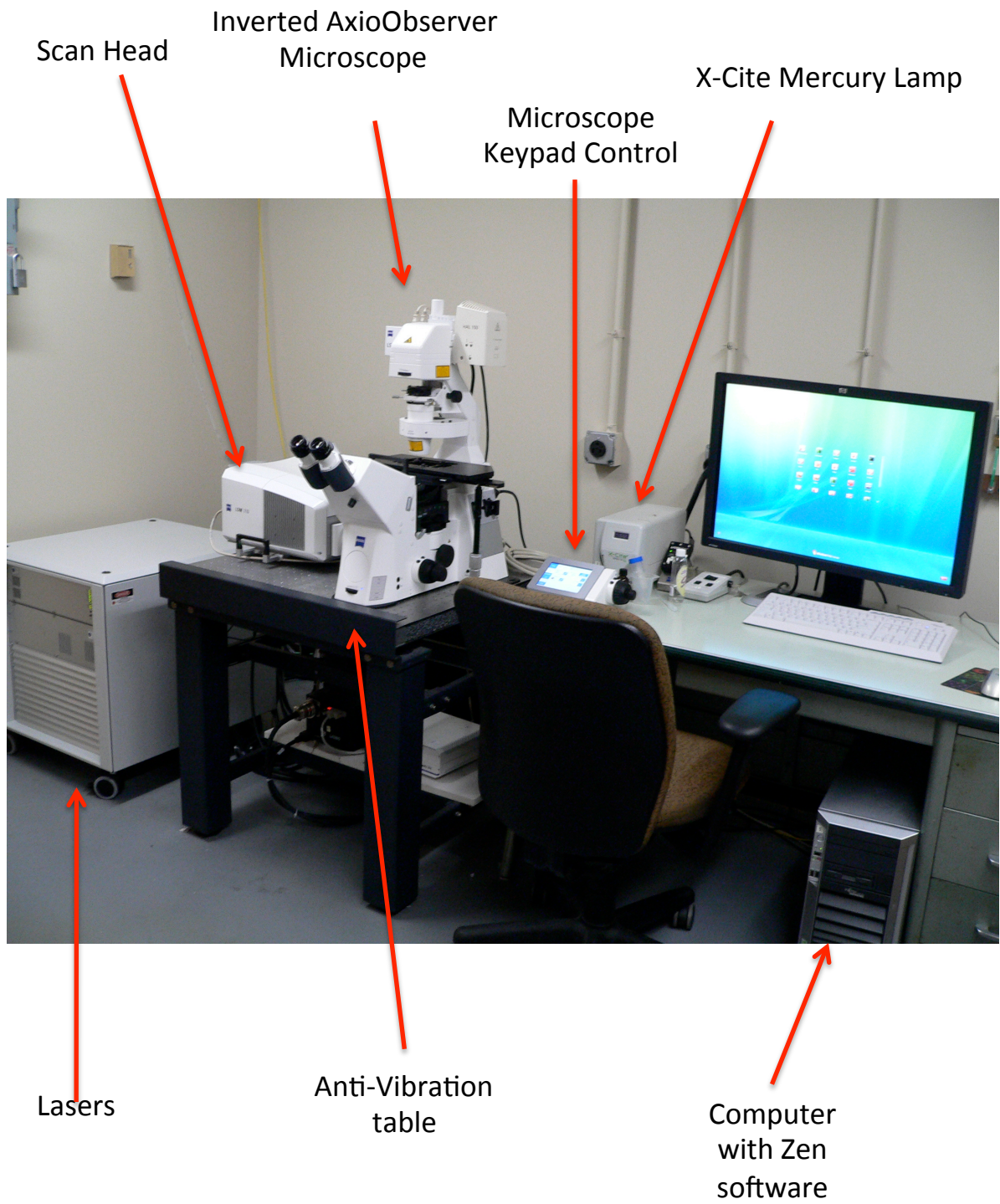
UNIVERSITY OF  
MARYLAND

# Table of Contents

- **System Components**
  - Basic System Components.....1
  - Basic Microscope Components.....2
- **System Start Up**
  - Start the Hardware.....3-4
  - Start the Software.....5-6
- **Find Your Sample**
  - Choose an Objective Lens.....7
  - Transmitted light (brightfield).....8
  - Köhler Illumination.....9
  - DIC.....10
  - Fluorescence Mode.....11
- **Confocal Imaging: Configurations**
  - Configuration Overview.....12
  - Load Single Dye Configuration.....13-15
  - Load Sequential Scan.....16-17
  - Modify Sequential Scans.....18
  - Smart Setup.....19-20
- **Confocal Imaging: Scanning Parameters**
  - Acquisition Mode.....21-23
  - Channels .....24-26
  - Transmitted Light Image.....27
  - Regions of Interest.....28
- **Advanced Techniques**
  - Setup and Collection of Z-Stack.....29
  - Basic Viewing of Z-Stack.....30
  - Time Series with Bleach (FRAP).....31
- **Export Images.....32-33**
- **Shutdown Procedure.....34**
- **Appendix A: Confocal Principle.....35**
- **Appendix B: Frame Size and Bit Depth.....36**
- **Appendix C: Gain and Offset.....37**
- **Appendix D: Optical Zoom Explained.....38-39**

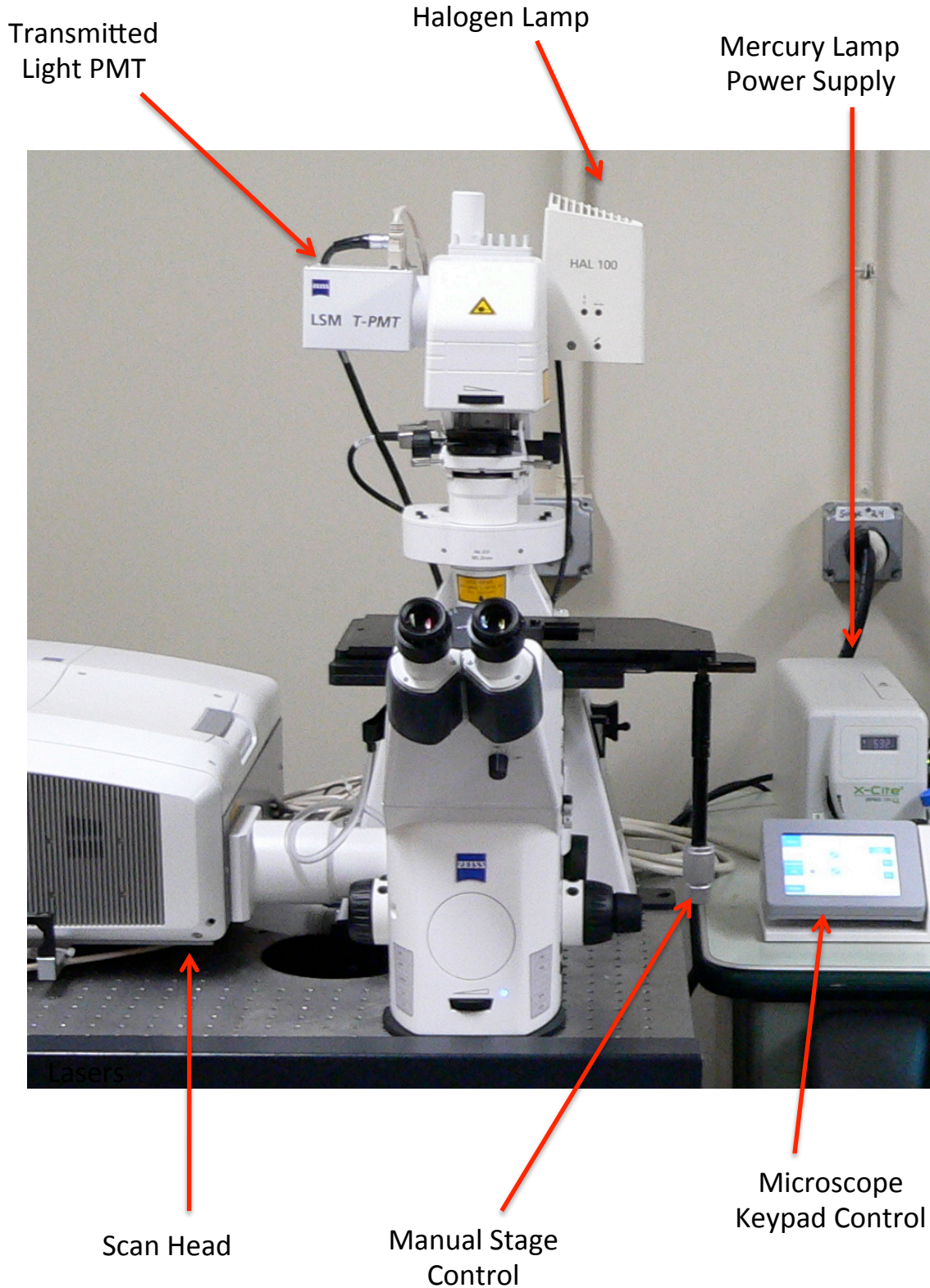


# System Components





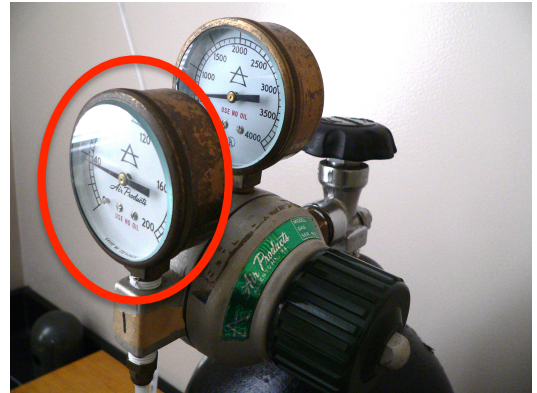
# Microscope Components





# Start the Hardware

1. Make sure the valve on the nitrogen tank is open. Open if needed. The pressure should read ~**30-40 psi**. If it doesn't, contact the facility manager.
2. Turn on the **Main Switch** (#1).
3. Turn on the '**Systems/PC**' and '**Components**' (#2).



4. Turn on the **X-Cite Mercury Lamp** (#3).
  - Note- Once the lamp has been turned on, it should remain on for at least one half an hour
5. Turn the **laser key** (located underneath the microscope) to the right, so that it is in the horizontal 'on' position.



## Start the Hardware

6. Turn on the **computer** (#5) and login to your Windows account.
7. Five minutes after turning the laser key (#4) to the 'on' position, switch the argon laser power from 'Idle' to 'Run' (#6). Note- it is very important to let the laser warm up 5 minutes before switching the power to 'run'. Following this step will help increase the lifetime of the laser.

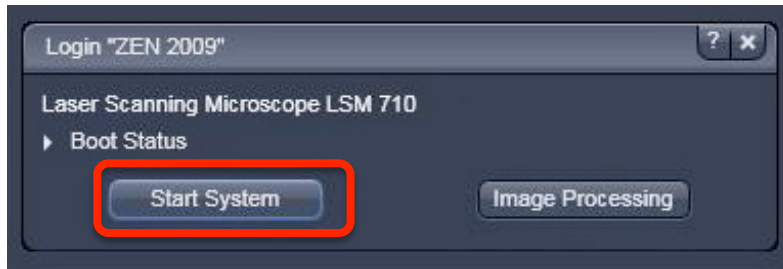
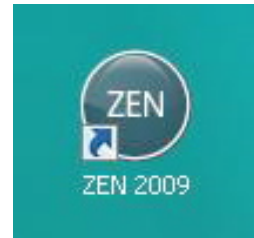


8. Wait approximately 30 seconds and then turn the **light control knob** (#7) clockwise until the red light comes on, then turn the knob back slightly, just until the red light turns off. The Argon laser is now set at 50% power.
9. Make sure to fill out the log book with your name, PI's name, and start time (actual clock time).



# Start the Software

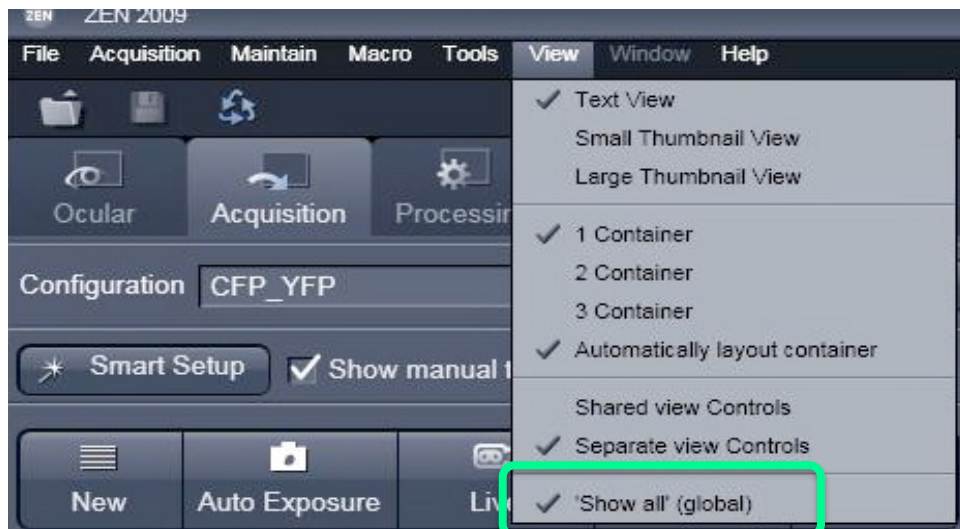
1. Double click on the Zen icon to start the software.
2. Click on **Start System**. It will take ~30 seconds for the software to start up.



3. In the Ocular tab, select 'Online' to connect the software to the microscope.
4. If needed, adjust the workspace zoom (located in the upper right quadrant of the screen).



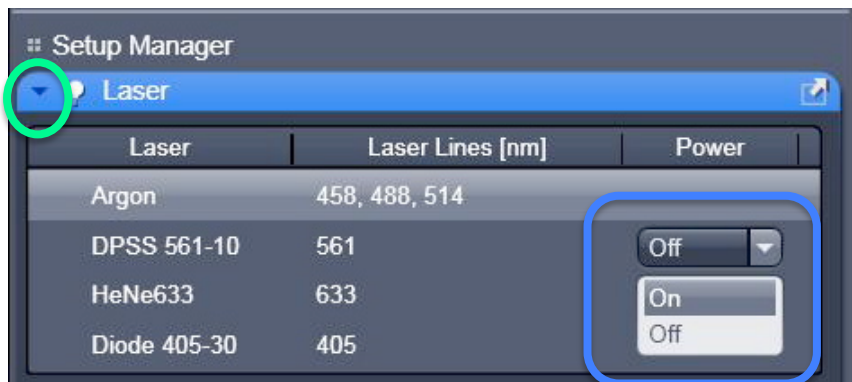
5. Click on the Acquisition tab, select View from the top menu and make sure 'Show All' (global) is checked. This will ensure that you can see all tool options in each window.





## Start the Software

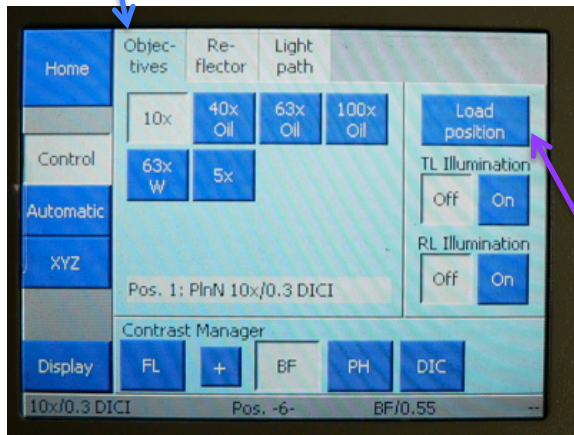
6. Check the box next to “**Show manual tools**”. This will open the Laser, Imaging Setup and Light Path Windows.
7. Expand the laser window by clicking on the **arrow**. If you plan to use the 561 laser, turn it **on**. The 633 HeNe and 405 diode lasers are automatically turned on during software initialization. Note- it is best to start the lasers as soon as possible to allow them to warm up before beginning confocal imaging.




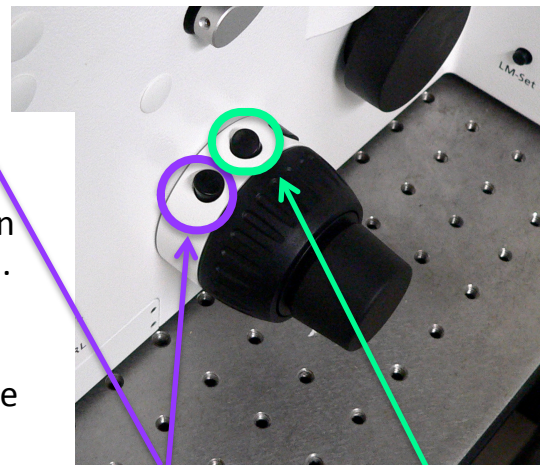
# Find Your Sample:

## Choose an Objective Lens

1. Click on the 'Ocular' tab.
2. Choose your objective lens in the **software window**, or by using the **microscope keypad**.
3. Available objective lenses include:
  1. 10x dry lens
  2. 40x/1.3 NA oil lens
  3. 63x/1.4 NA oil lens
  4. 100x/1.4 NA oil lens
  5. 63x/1.2 NA water lens



4. Put the microscope objective in the 'Load' position by pressing the **front button** on the right focus knob or the 'Load position' button on the keypad. This will lower the lens down.
5. Add a small drop of the correct immersion media (oil or water) to the coverslip of your slide or to the objective lens. Place your slide on the universal stage coverslip-side down.
6. If needed, adjust the stage to position the objective lens underneath your sample.
7. Bring the objective lens up by pressing the **back button** on the right focus knob or by pressing the  symbol on the keypad.

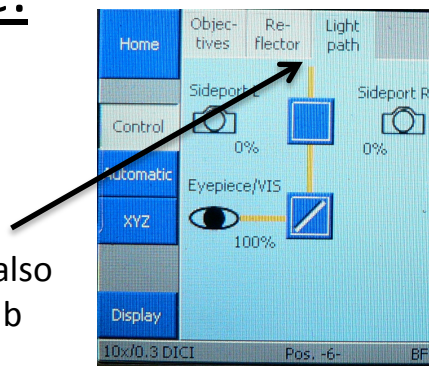


Raises objective lenses

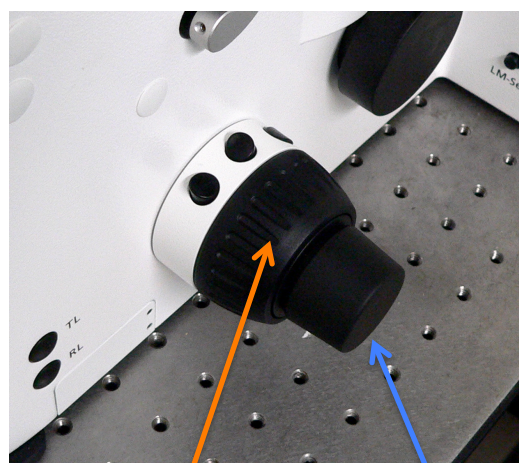
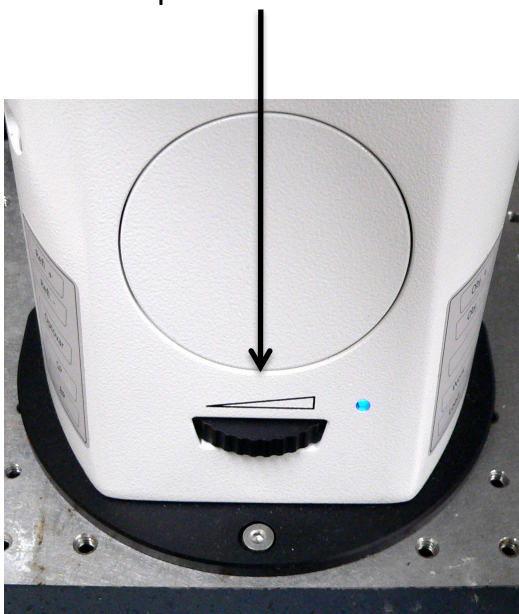
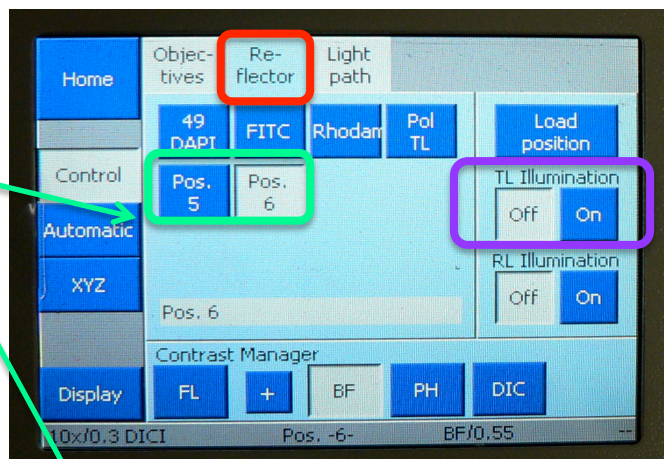
Lowers objective lenses

# Find Your Sample: Transmitted light

1. Make sure light is being directed to the eyepieces. Clicking on the Ocular tab in the software will automatically send light to the eyepieces. You can also choose the light path by selecting the 'Light Path' tab on the microscope keypad.



2. Select 'Reflector' on the microscope keypad.
3. Select Pos. 5 or Pos. 6 (both can be used for brightfield). Or, select 'Transmitted light' in the Ocular tab of the software.
4. Open the transmitted light shutter by selecting 'On' under TL Illumination on the keypad.
5. Look through the eyepieces and focus on your sample using Coarse or Fine focus.
6. If needed, adjust the transmitted light intensity using the dial on the front of the microscope.



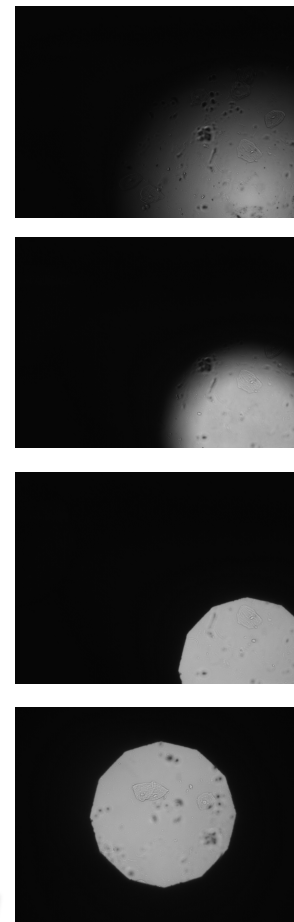
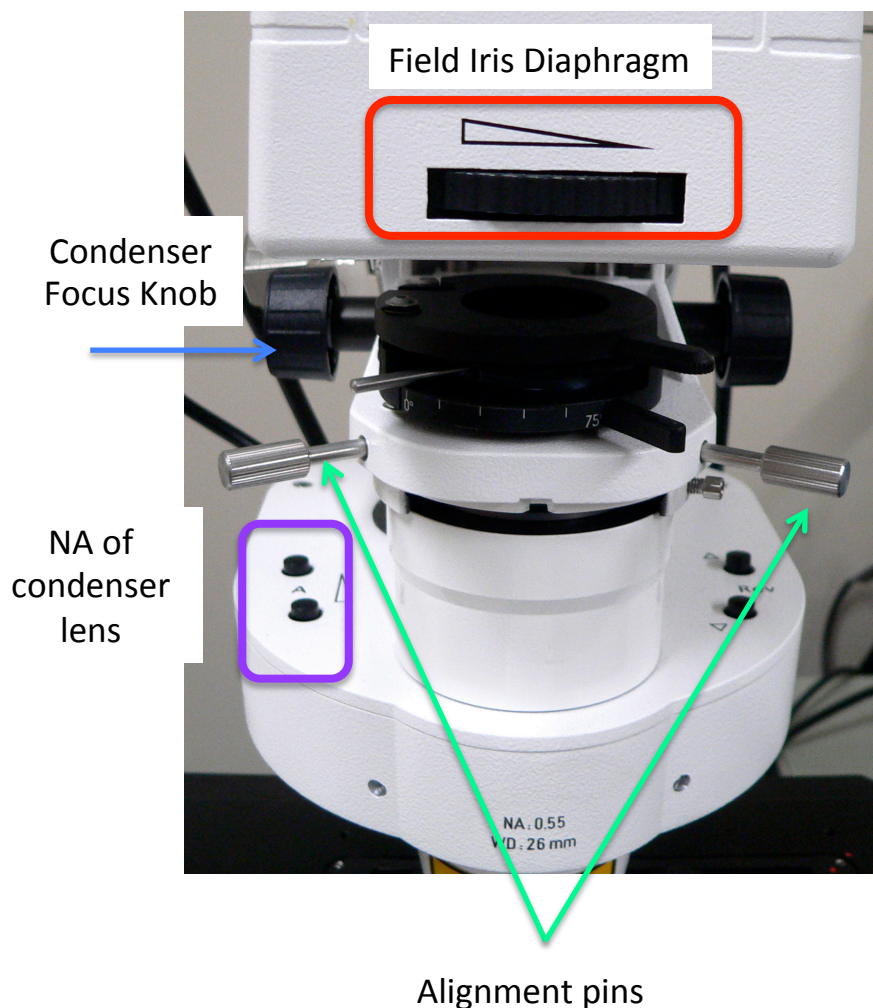
Coarse focus knob      Fine focus knob



# Find Your Sample:

## Köhler Illumination

1. If you plan to take a transmitted light (brightfield) image of your sample using the lasers, you should focus and align the condenser lens for Köhler illumination.
2. Köhler Illumination steps:
  - Make sure you are focused on your sample.
  - Close down the **field iris diaphragm**.
  - Look through the eyepieces and **focus the condenser** lens until you see an octagon of light. It may or may not be centered.
  - Center the spot of light using the **alignment pins**.
  - Open the field iris diaphragm back up until you can see the whole field of view again.
  - Adjust the **numerical aperture (NA)** of the condenser if necessary. Increasing the NA will result in higher resolution, lower contrast images.

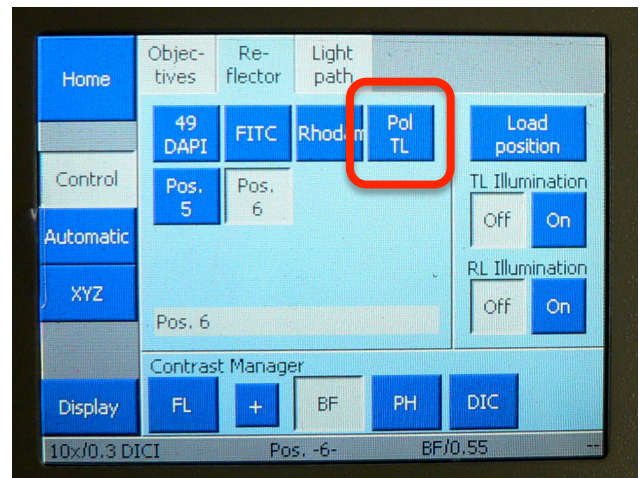


What you should see if the microscope is properly aligned.

# Find Your Sample:

## Differential Interference Contrast (DIC)

1. Some of the objective lenses on the microscope are capable of differential interference contrast imaging (DIC). For samples that are very transparent, DIC imaging is a good way to increase contrast in the sample.
2. To use DIC, select **DIC III** in the software window or **Pol TL** from the microscope keypad.



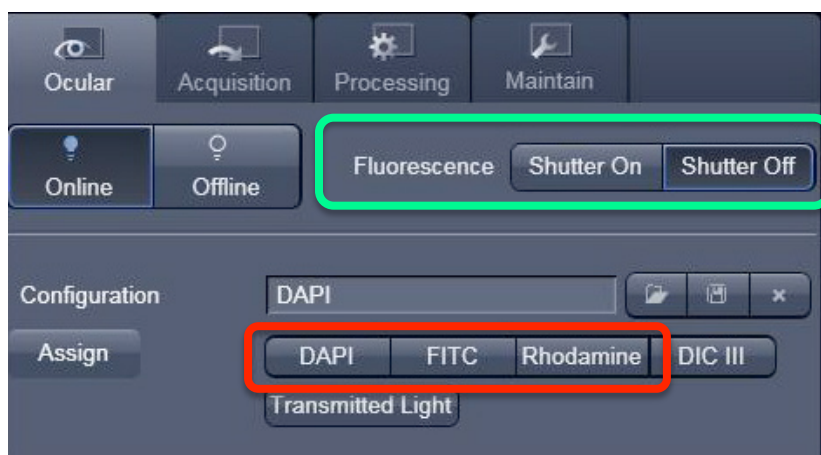
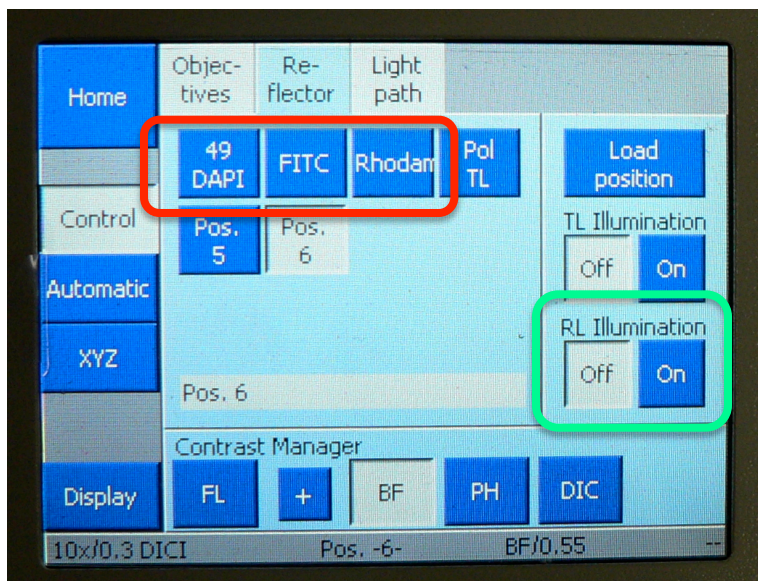
3. Adjust the **rotation** of the polarizer if necessary. Setting the polarizer to 0 degrees results in the highest contrast image.





# Find Your Sample: Fluorescence

1. If the transmitted light shutter is open, turn it off using the microscope keypad.
2. Select a **fluorescence filter** in the ocular tab of the software or using the microscope keypad. The microscope is equipped with standard DAPI, Fluorescein (FITC or GFP) and Rhodamine fluorescence filters.
3. Open the **fluorescence shutter** in the software or by using the microscope keypad (called RL Illumination here).
4. View your sample through the eyepieces.
5. Lamp intensity can be adjusted using the **dial** on the side of the X-Cite mercury lamp.
6. When finished viewing the sample, turn the fluorescence shutter off to prevent photobleaching.



Lamp intensity adjustment





# Confocal Imaging:

## Configurations

1. Now that you've found your specimen, it's time to set up the microscope to take confocal images.
2. The first step is to set up a configuration based on the fluorescent dye(s) you are using.
3. If your sample is labeled with multiple dyes, you can choose simultaneous or sequential scanning.

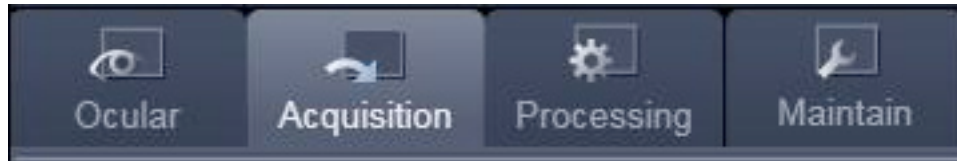
Simultaneous Scanning	Sequential Scanning
Used for single labeling and **multiple labeling	Used for multiple labeling
Advantage: Faster image acquisition	Advantage: Reduces emission crosstalk
Disadvantage: Cross talk (bleedthrough) between fluorophores	Disadvantage: Slower image acquisition

\*\* Recommendation: Unless you need to acquire images quickly (such as for live cell imaging), choose sequential acquisition when your sample is dual or triple labeled. This will help prevent cross talk between dyes. This is particularly important if you are doing colocalization studies.

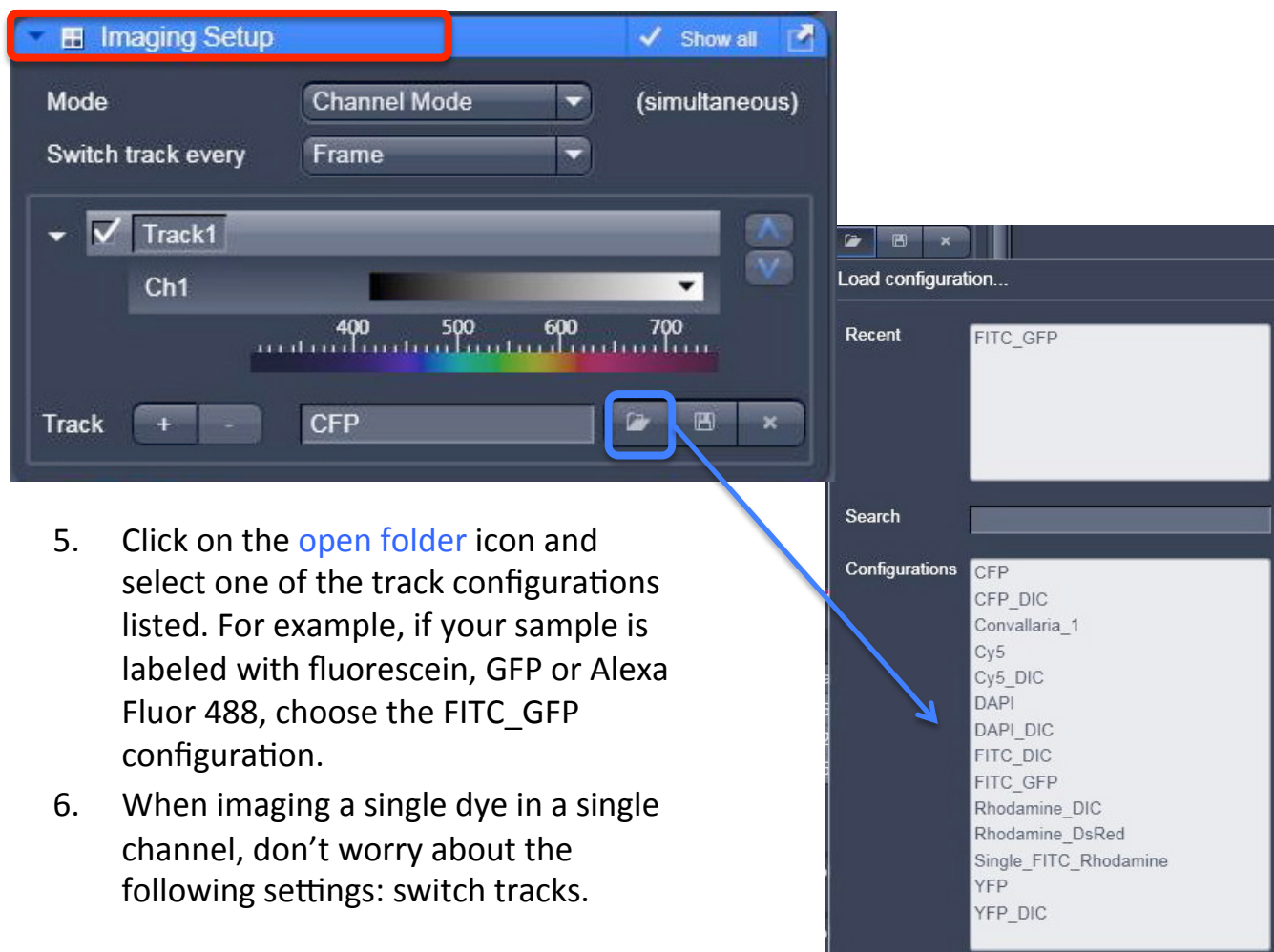
# Confocal Imaging:

## Configurations- Load Single Dye Configuration

1. Select the Acquisition tab in the software.



2. Open the **Imaging Setup** window.
3. Make sure 'Channel Mode' is selected.
4. If your sample is labeled with one fluorescent dye only, you can choose a standard configuration from the Imaging Setup Window. Note- the configurations listed here are used to image single dyes only or to image multiple dyes in simultaneous scan mode.

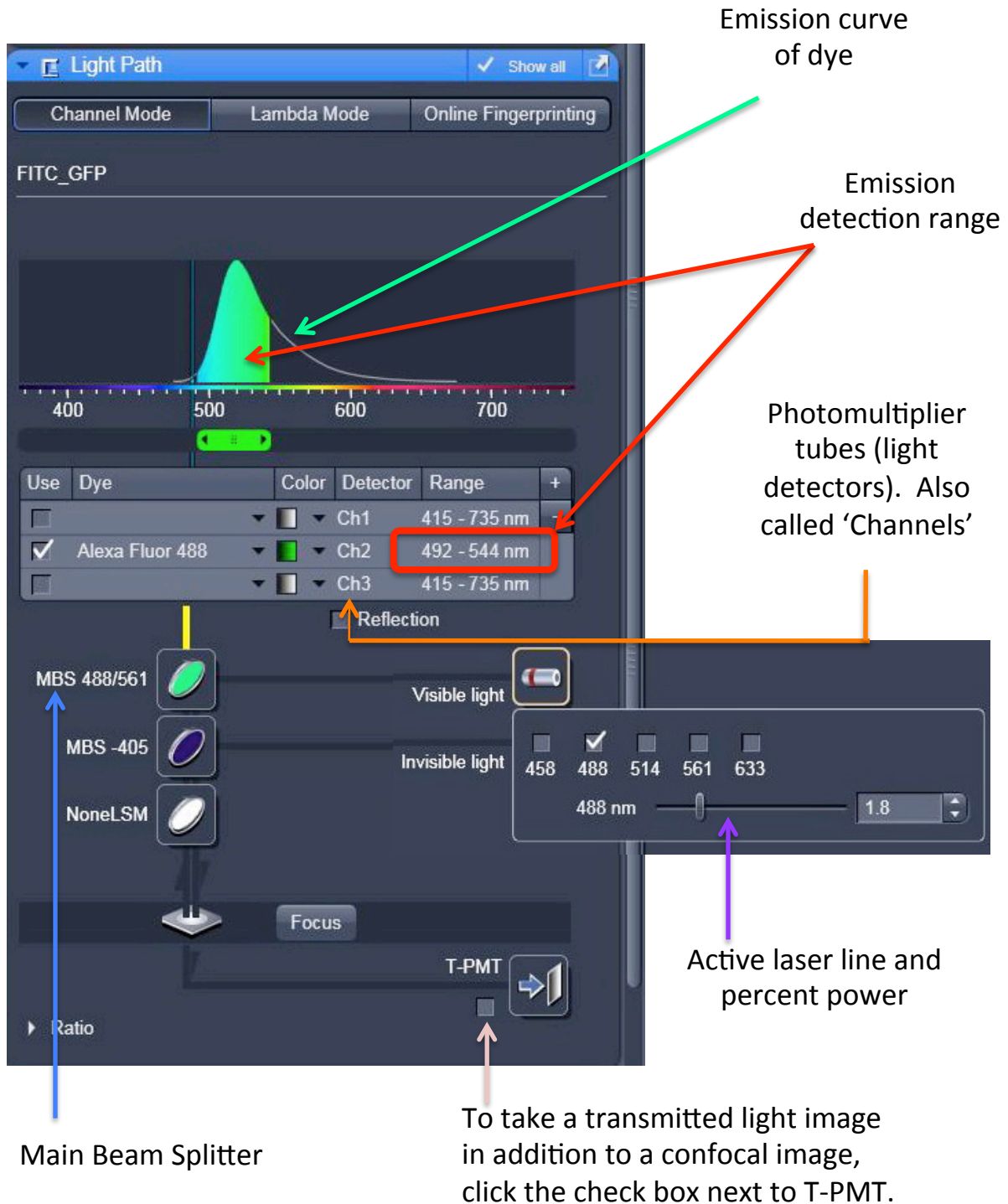


5. Click on the **open folder** icon and select one of the track configurations listed. For example, if your sample is labeled with fluorescein, GFP or Alexa Fluor 488, choose the FITC\_GFP configuration.
6. When imaging a single dye in a single channel, don't worry about the following settings: switch tracks.

# Confocal Imaging:

## Configurations- Load Single Dye Configuration

- Open the Light Path window to view details about the configuration. The window will look something like this:

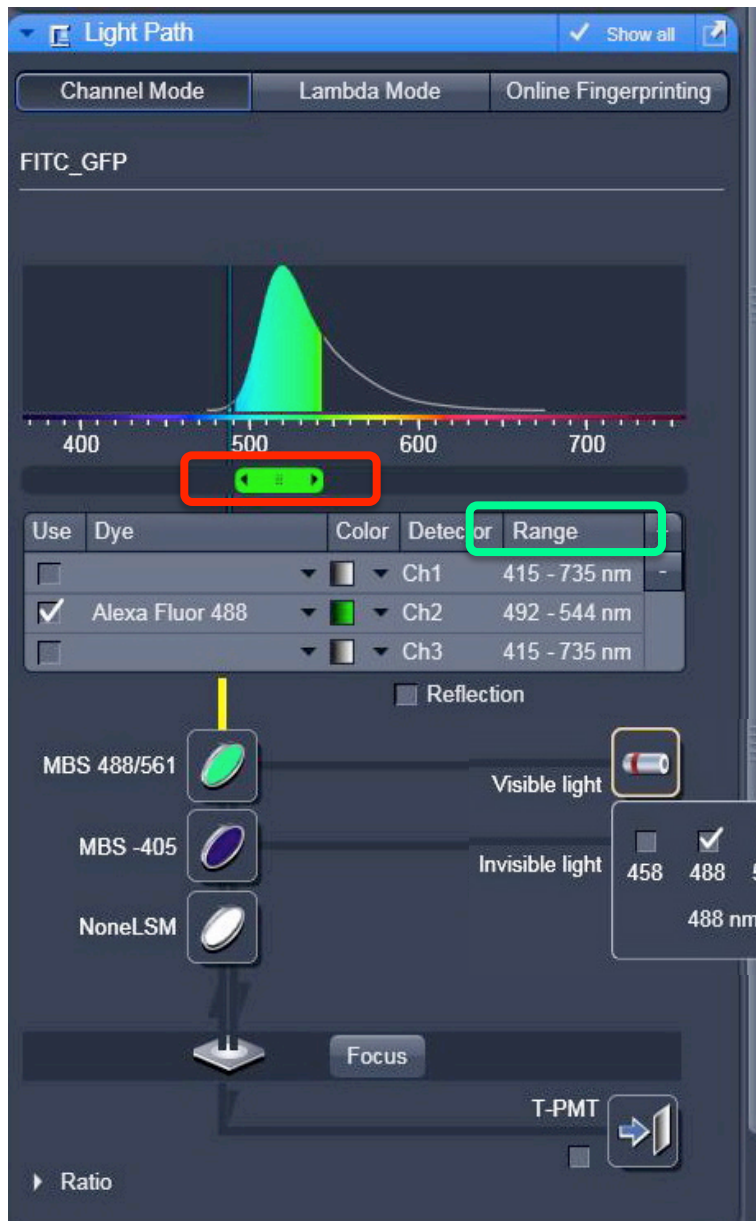




# Confocal Imaging:

## Configurations- Load Single Dye Configuration

- The **emission detection range** can be modified by moving the bar to the right or left, or by grabbing the arrows to increase or decrease the range. The exact spectral range will be displayed in the **Range column**.

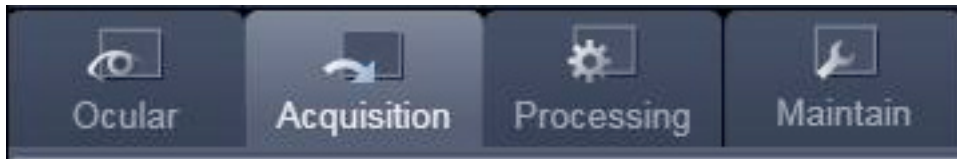


- Laser power** can be increased or decreased from this window, or changed in the 'Channels' window.

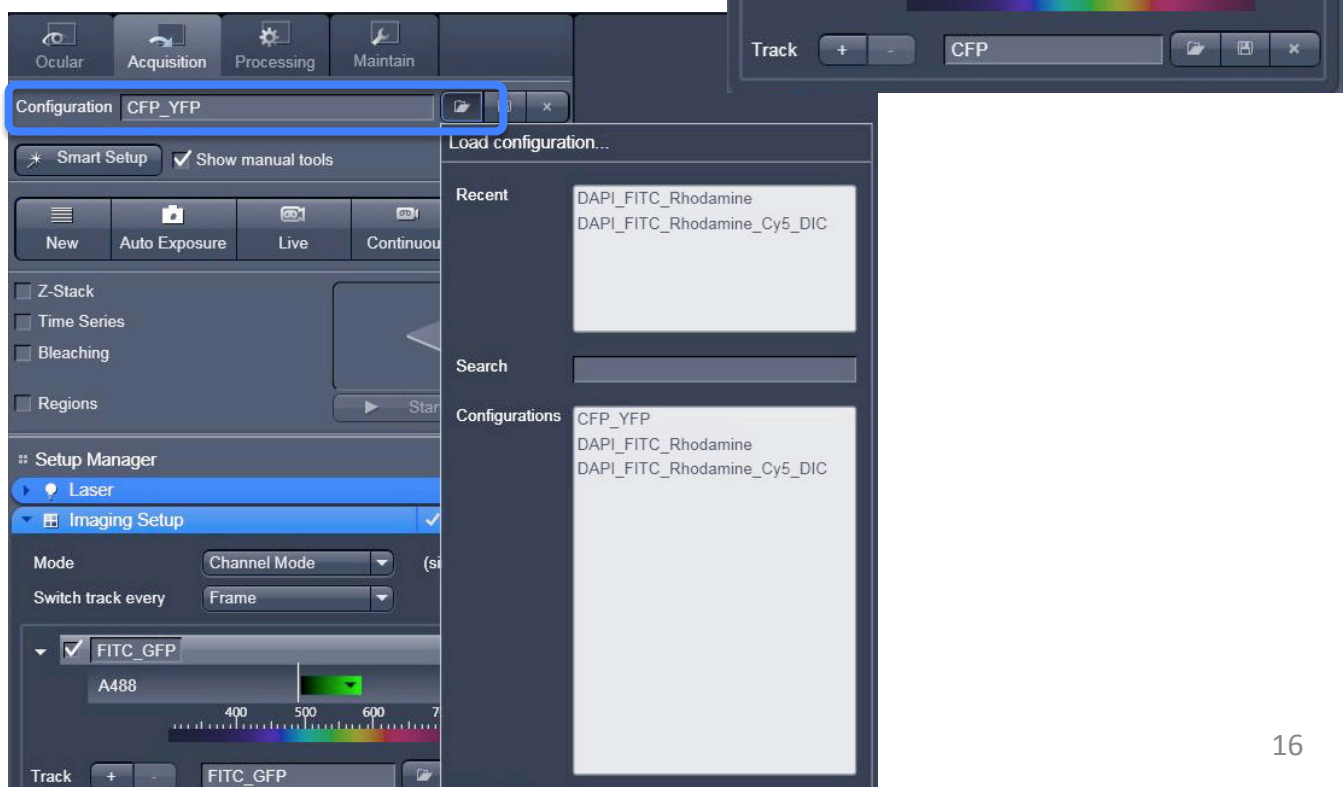
# Confocal Imaging:

## Configurations- Load Sequential Scan

1. Select the Acquisition tab in the software.



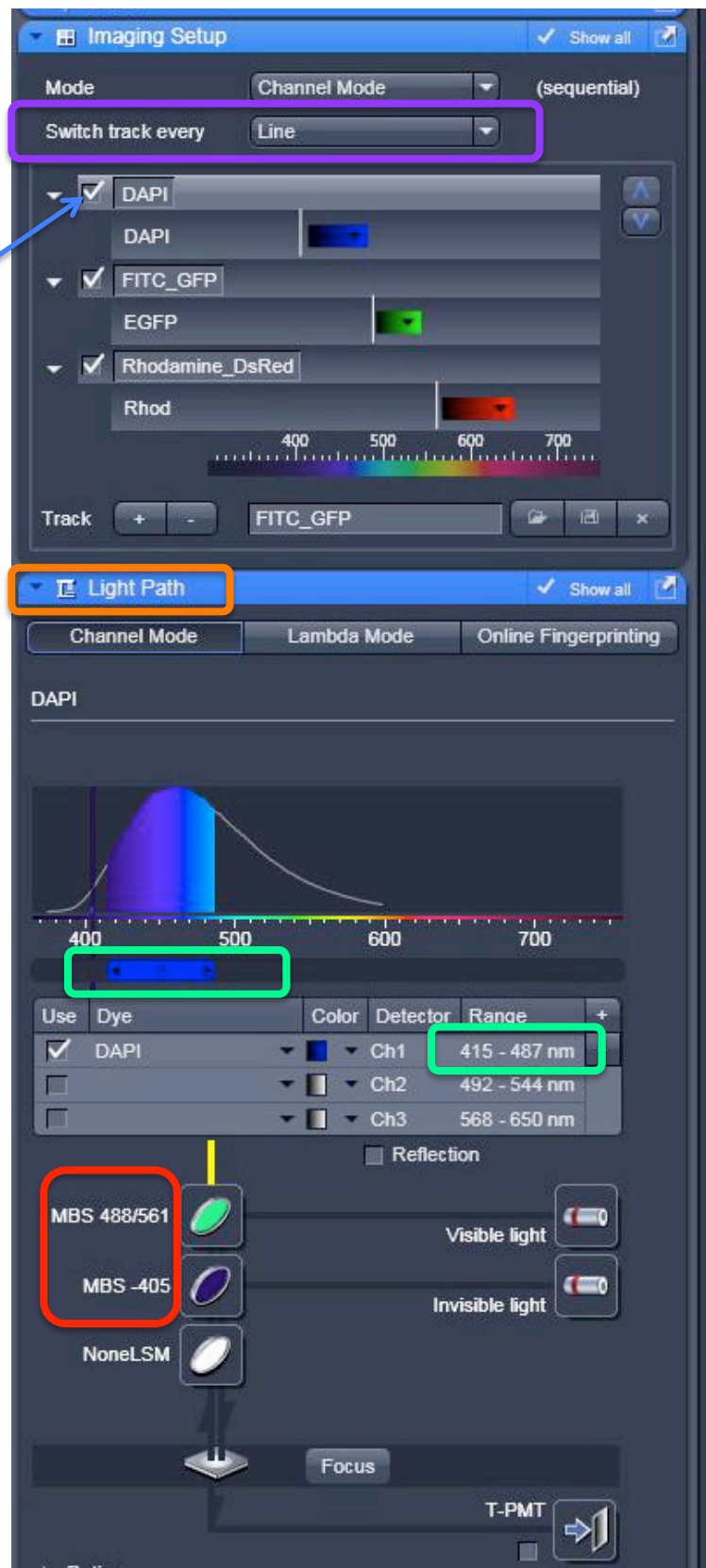
2. Open the **Imaging Setup** window.
3. Make sure 'Channel Mode' is selected under Mode.
4. If your sample is labeled with more than one dye, you can choose a standard sequential configuration. Sequential scans (rather than simultaneous scans) will help reduce crosstalk between fluorophores. However, it will increase the time needed to scan the image.
5. For example, if your sample is labeled with DAPI, GFP and Rhodamine (or a similar combinations of dyes), select this configuration by clicking on the **open folder icon** under **Configuration**.



# Confocal Imaging:

## Configurations- Load Sequential Scan

6. Open the **Light Path** window.
7. To view the details of a particular track in the Light Path window, **highlight the track** in the Imaging Setup window.
8. The **emission detection range** can be changed by moving the bar to the right or left, or by grabbing the arrows to increase or decrease the range.
9. In sequential scanning mode, you have the option to **switch tracks** after each line or frame. Switching track after each line means that the laser will scan one line of track #1 (i.e. DAPI), then one line of track #2 (i.e. FITC), then one line of track #3 (i.e. Rhodamine). This will repeat rapidly until the whole image is scanned. In frame switching, the laser will scan the entire track #1 image, then track #2 and then track #3.
10. In order to switch tracks every line, the **MBS** must be the same for all three tracks. The standard configurations available are set up so that line scanning and frame scanning are both possible.

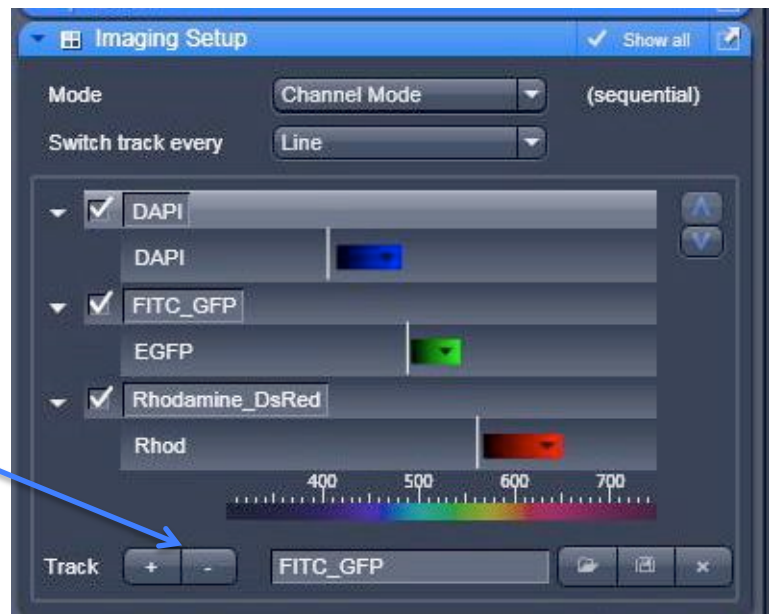




# Confocal Imaging:

## Configurations- Modify Sequential Scan

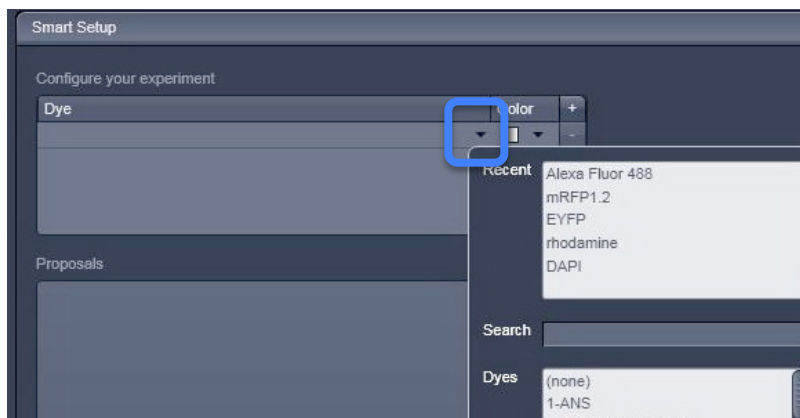
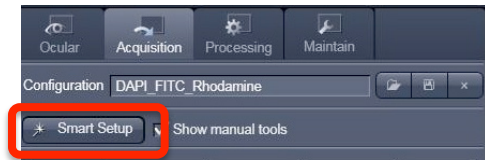
1. Sequential scans can be modified.
2. For example, if your sample is labeled with a green and red dye only, load the 'DAPI\_FITC\_Rhodamine' sequential scan. Highlight the DAPI track and delete it by clicking on Track (-).
3. The DAPI\_FITC\_Rhodamine configuration can be used for most combinations of blue, green and red dyes. The emission curves shown in the Light Path window are specific for DAPI, FITC and Rhodamine. To display the emission curve of another dye, click on the arrow to the right of the Dye field and select a fluorophore from the drop down window. This will aid you in adjusting the emission detection window if needed.



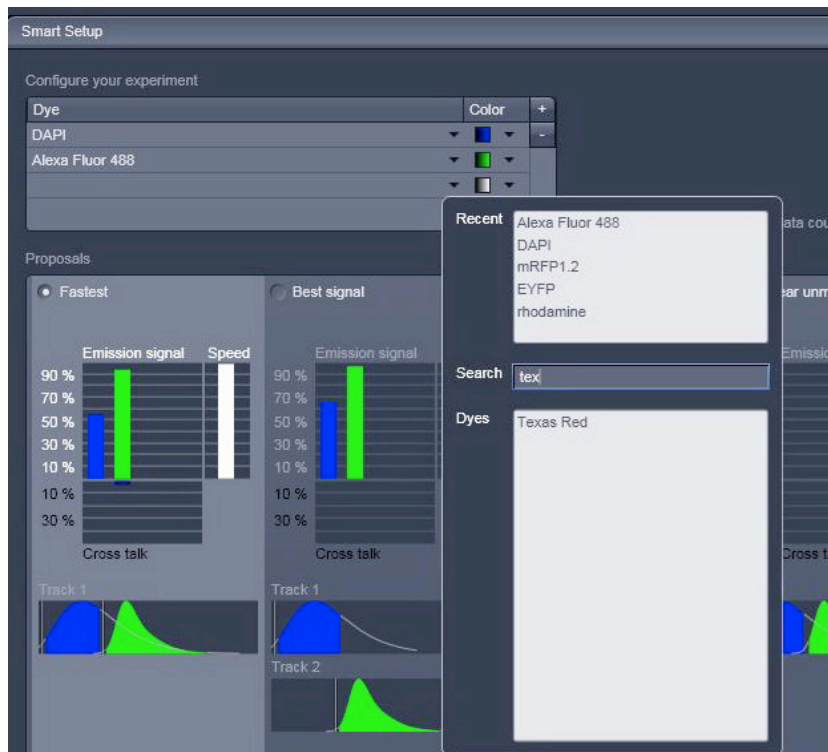
# Confocal Imaging:

## Configurations- Using Smart Setup

1. Select the Acquisition tab in the software.
2. Select **Smart Setup**.
3. In the new Window, click on the **arrow to the left** and type in the name of the dye your sample is labeled with in the search field. If the dye is in the database, it will show in the field under Dyes. Select the dye from the list.



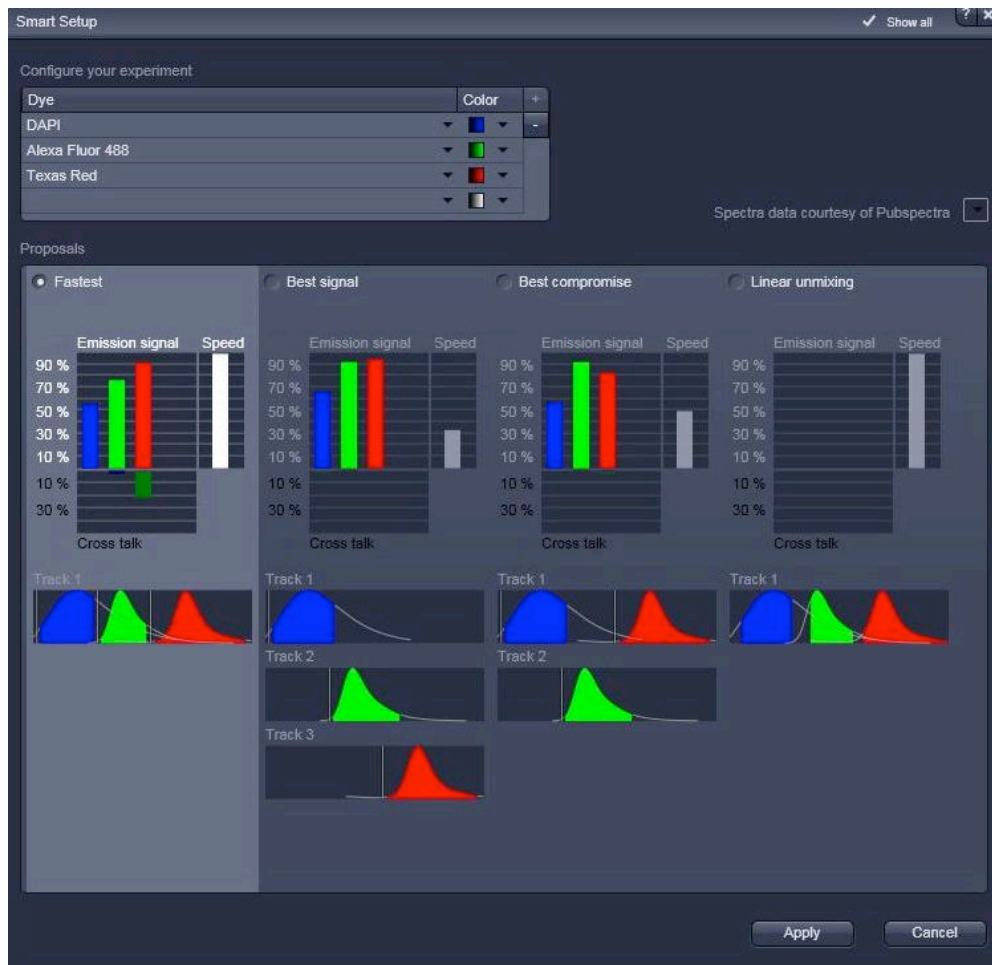
4. Repeat the process with your other dyes.



# Confocal Imaging:

## Configurations- Using Smart Setup

1. The software will give you 3-4 configurations to choose from. The 'Fastest' option is a simultaneous scan. It is the fastest option but results in the most crosstalk. The 'Best Signal' option is a sequential scan. It is the slowest option but results in the least crosstalk. Depending on your dye selection, the software may suggest a 'Best Compromise', as in the example below. In this case, the blue and red dyes are scanned simultaneously and the green dye is scanned separately (sequentially) from the others.
2. Select the desired configuration and click on Apply. In the current version of the software, any sequential scans will be collected by switching frames, rather than by line switching, because the MBS (beamsplitters) are different for each channel. Do not change this setting.





# Scanning Parameters: Acquisition Mode

1. Open the Acquisition Mode window.

2. The objective lens you are currently using is shown at the top of the window.

3. To scan a full frame (i.e. a full image), make sure 'Frame' is selected under Scan Mode. To scan a single line of your sample, select 'Line'. Note- line scan is for specialized applications only.

4. Select the Frame Size. You may choose a predefined value (for example, 512 x 512) or enter your own values. A larger frame size means you will collect more pixels per unit area scanned, effectively increasing the resolution of the image. However, larger frame sizes take longer to scan (which can cause increased photobleaching) and will result in larger file sizes. A good starting point is 512 x 512.

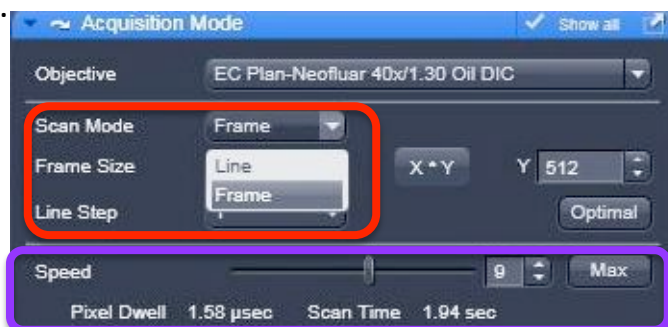
Publication quality images should be taken at 1024 x 1024 or greater at Zoom 1. For more information on Frame size, see Appendix B.

5. If you select 'Optimal', the software will calculate the highest frame size (image resolution) possible based on zoom level and the numerical aperture of the objective lens you are using.

6. Select a scan speed. A higher scan speed will reduce the possibility of photobleaching your sample but will result in a noisier image.

7. A slower scan speed will increase the time the laser illuminates the specimen (increasing the possibility of photobleaching), but will result in a less noisy image.

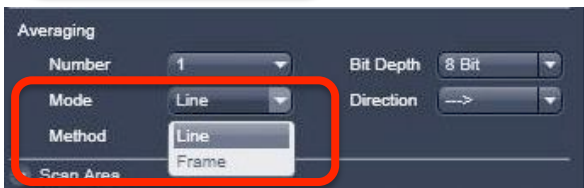
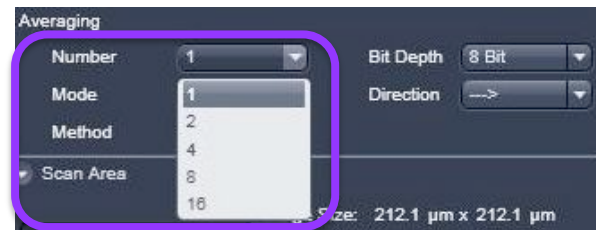
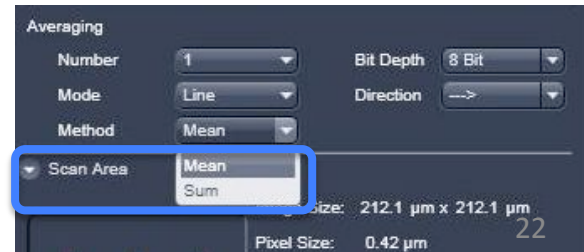
8. A good starting point is a scan speed of 7-8.



# Scanning Parameters:

## Acquisition Mode

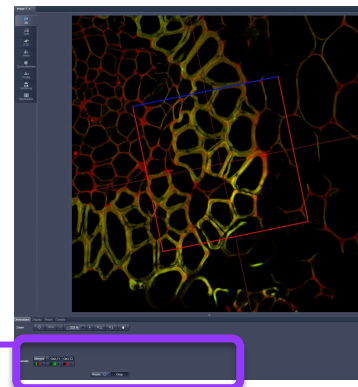
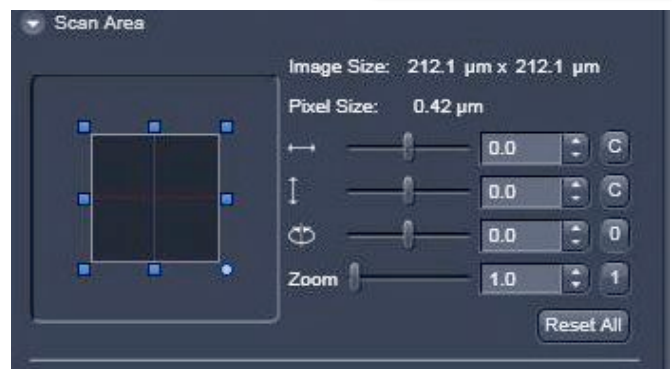
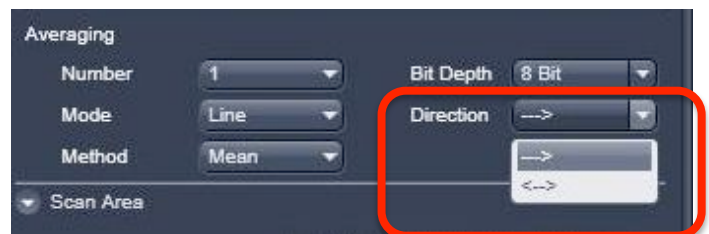
9. Averaging improves image quality by increasing the signal to noise ratio. It is particularly useful for samples that have low fluorescence emission and/or have low signal to noise ratios. Not all images will be appreciably improved by averaging, though, so it is best to start with a single scan then try averaging to see if the image is improved or not. Keep in mind that the more you average, the more times the sample is scanned and exposed to laser light. This increases the potential for photobleaching.



10. To set up averaging, under Method, choose **Mean**.
11. Under Averaging and **Number**, choose the number of times you want the image to be averaged.
12. Under **Mode**, choose line or frame averaging. This determines how the image is scanned and averaged.
13. For example, if you choose Line Mode, 2, each line of the image will be scanned two times and the average pixel intensity will be calculated and displayed as each line is scanned.
14. If you select Frame Mode, 2, the entire image will be scanned once from top to bottom. Then, the image will be scanned again from top to bottom and the average of the 2 images will be displayed.
15. Note- It is best to use line averaging with live samples that are moving.
16. Note- Frame averaging helps reduce photobleaching, but will take longer when scanning multiple channels sequentially and also does not give quite as smooth of an image.

# Scanning Parameters: Acquisition Mode

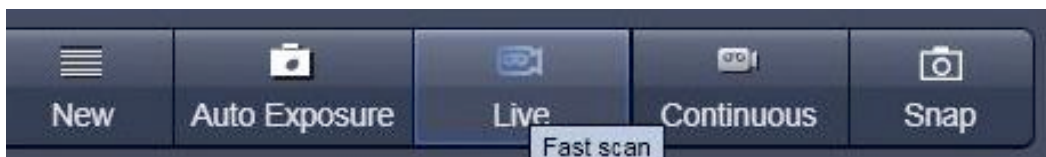
16. Select your bit depth (data depth).
17. 8 bit data depth will give 256 gray levels. Each pixel in your image will be scaled between 0 (black) and 255 (white). 12 bit data depth will give 4096 gray levels. 16 bit data depth will give 65,536 gray levels. Note- the greater the bit depth, the greater the file size. Publication quality images should be taken with 12 bit data depth. For more information about bit depth, see Appendix B.
18. The default scan direction is **unidirectional**.
19. If additional scan speed is needed, bidirectional scan mode may be selected. To correct for phase differences in x and y, select 'Auto'. Note- unless increased speed is very important for your experiment, I recommend keeping the default unidirectional scan mode.
20. In the Scan Area window, the scan area can be shifted in x and y, the image can be rotated. The default zoom level is one. Zooming can be accomplished in this window, or by selecting the 'Crop' button located below the image window. Zooming using the 'Crop' button allows you to choose the zoom area by placing a box directly over the image window).





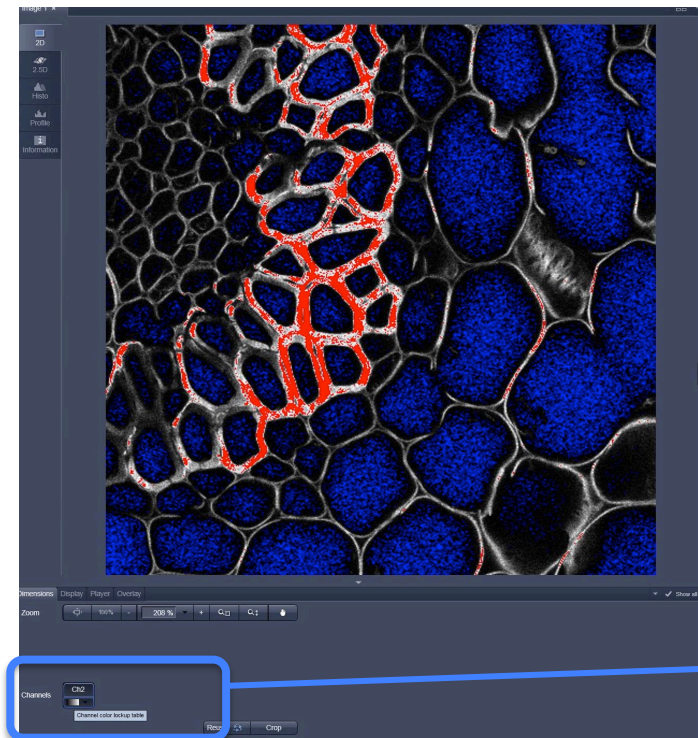
# Scanning Parameters: Channels

1. In the Channels window, you can set laser power, detector gain, detector offset and pinhole size for each channel.
2. Select a channel by clicking on the channel to highlight it. The parameters of that channel will be displayed below.
3. Set the pinhole for each channel by highlighting each channel and selecting **1 AU**. This will set the pinhole at one Airy Unit, which is the best compromise between depth discrimination and detection efficiency. The **optical slice thickness** will be displayed under the slider bar.
4. Note- opening up the pinhole will let more light through to the detector, but will also increase the optical slice thickness and decrease resolution in the z-axis. For more information on confocal pinhole principle, see Appendix A.
5. If you have multiple channels, uncheck one of them and highlight the checked channel, as in the sample above (FITCGFP is highlighted and checked). This way you can work with one channel at a time, without the risk of photobleaching the other channel.
6. Set the **Gain** relatively high (around 800). The gain is the voltage on the photomultiplier tube, which detects the emitted light from your sample. Low gain = low PMT sensitivity and less noise. High gain = high PMT sensitivity and more noise.
7. Start a Live scan by clicking on the Live button. Live scan will scan the sample continuously at a high rate of speed until you click on the stop button.

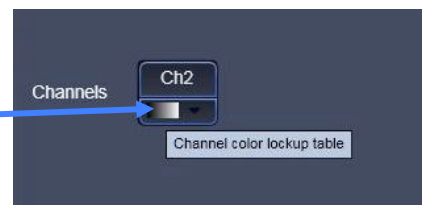


# Scanning Parameters:

## Channels



9. While scanning, click on the **range indicator** button below the image window.
10. The image will now appear in gray scale mode, with pixels that are too bright (saturated) colored in red, and pixels that are at 0 (minimum) in blue.

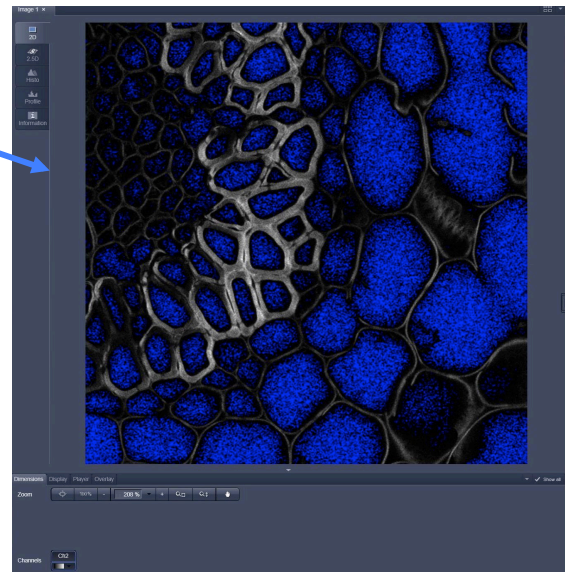


11. Depending on how bright the image is, you may need to increase or decrease the laser power or the detector gain. Note- for more information about Gain and Offset, see Appendix C.
12. If the image is very saturated (many red pixels) at a gain of 700-800, then I suggest lowering the laser power. Image brightness can also be decreased by lowering the gain, (rather than lowering laser power), but keep in mind that higher laser means increased risk of photobleaching.
13. If the image is not saturated (no red pixels), you may want to increase both laser power and gain until one or two pixels are highlighted in red. Always keep in mind that the intensity of any pixels in red cannot be quantified (red pixels have an intensity value of 255 or greater).
14. Increase the amplifier offset until all blue pixels disappear, and then make it slightly positive.
15. Stop the Live scan and start a Continuous scan. A continuous scan will scan at the speed you selected in the software. Because scan speed can effect image intensity, it is a good idea to check the image using the final selected scan speed. Make any necessary adjustments to laser power and gain.
16. Repeat these steps for all image channels.

# Scanning Parameters: Channels

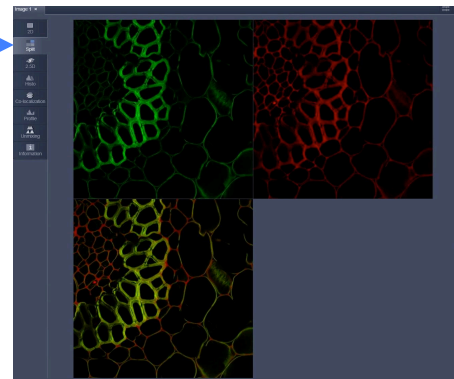
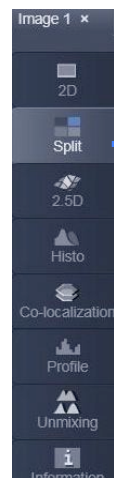
17. The final image for each channel should have few or no red pixels and a few blue pixels in the background.

18. When you are ready to take the final image, check all channels to activate them, and click **Snap** to take the final image. Save the image immediately, as it will be overwritten unless saved. All Images should be saved on Data drive D: under User Data. Each image is saved individually (and not part of a database file, like the old LSM510 or the Leica SP5X).



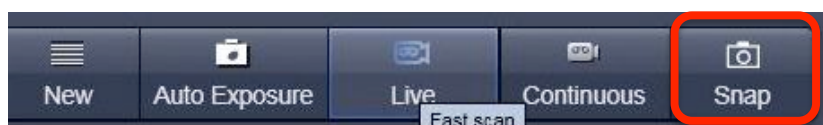
19. To display the overlaid image only, select 2D from the menu to the left of the image

20. To see each channel individually and with the overlaid image, select Split from the menu next to the image window.



21. A note on scanning options:

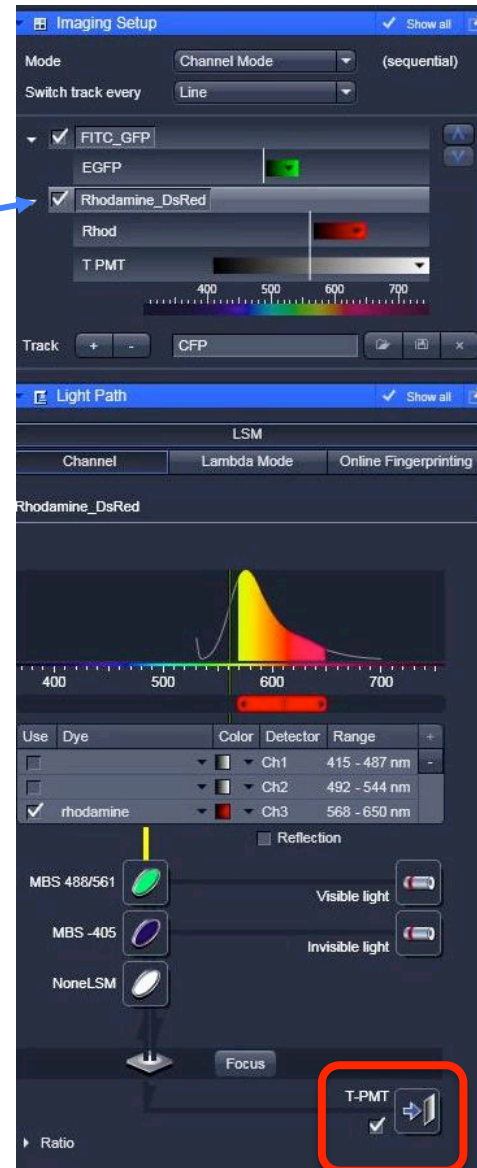
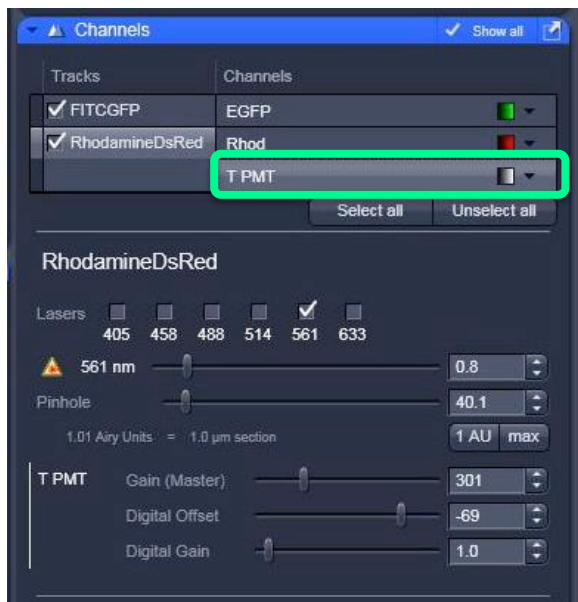
- Autoexposure will automatically adjust the detector gain and offset based on the chosen laser power.
- Live scan will result in a continuous fast scan. This is useful for finding the sample and changing the focus, and to get a quick idea of image intensity.
- Continuous scan will scan continuously at the selected scan speed. Final image modifications should be done using continuous scan.
- Snap will snap a single image. The final image should be taken with Snap.



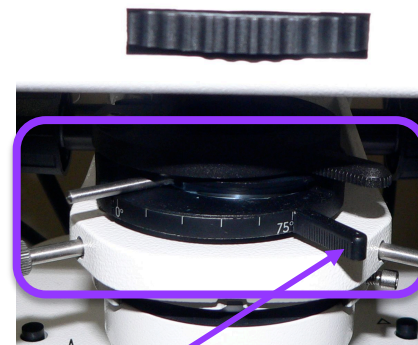


# Scanning Options: Transmitted Light Image

1. To take a transmitted light image, highlight the longest wavelength channel in the Channels Window. For example, the Rhodamine channel in the configuration shown to the right.
2. Check the Box next to **T-PMT** to activate the transmitted light photomultiplier tube.
3. In the Channels Window, highlight **TPMT**.
4. Start a Live scan and increase the Gain on of the T PMT until you see an image.
5. If additional contrast is desired, try decreasing the Digital Offset.



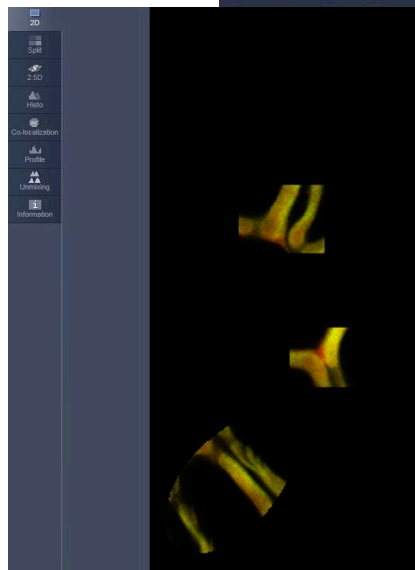
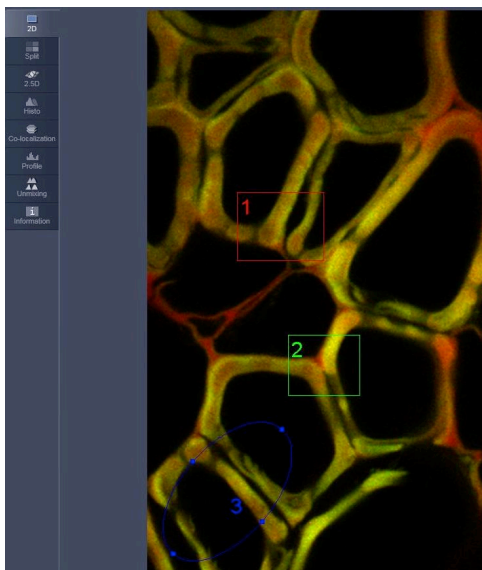
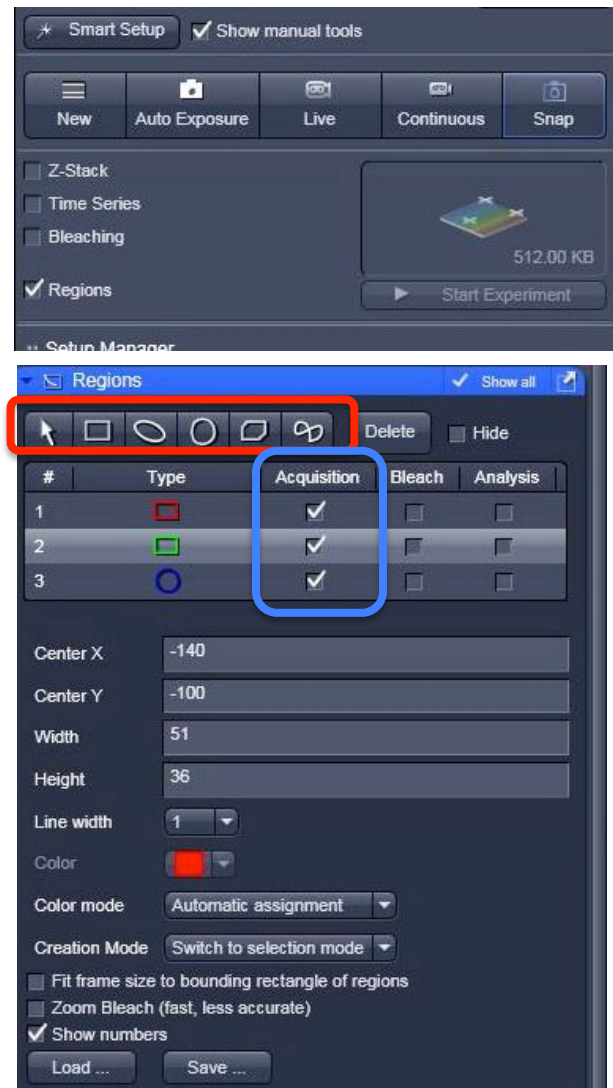
6. Unless the **polarizer** is removed from the light path, the transmitted light image may be a DIC image (depending on whether or not the objective lens is DIC capable).
7. If a regular transmitted light (non DIC) image is desired, remove the **polarizer** from the light path.



Pull to the left

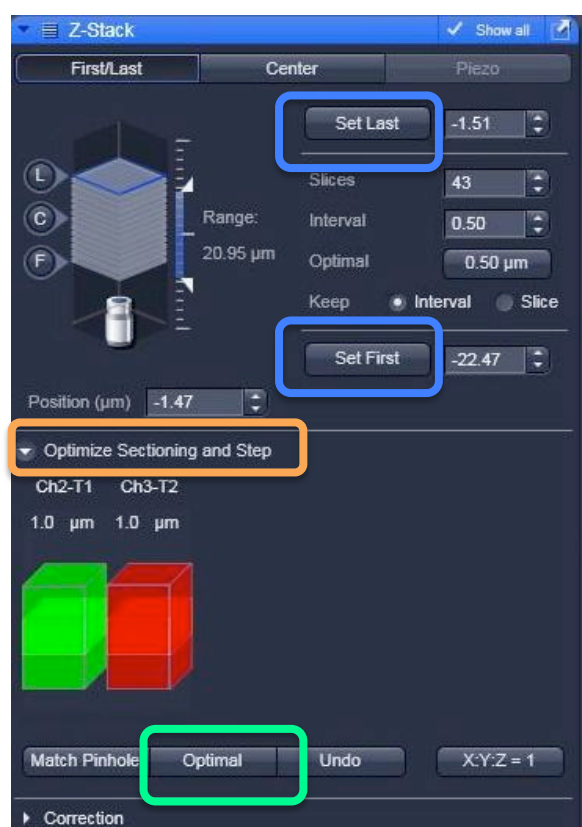
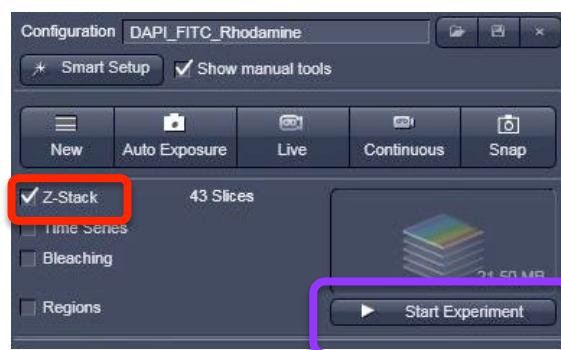
# Scanning Options: Regions of Interest

1. For faster image acquisition, specific regions of interest can be scanned by checking the box next to Regions. Note- when you define an ROI, the laser scans only that particular region. Image resolution and magnification remain unchanged (unlike with the Zoom/Crop feature).
2. In the Regions window, select a **shape**. Position the mouse pointer over your image and draw the shape around the portion of the image that you want to scan. Multiple ROIs can be defined.
3. Check the **Acquisition** box next to each ROI.
4. Snap the image. Note- you may have to close the previous image in order to see the specific ROIs that were scanned.



# Collecting a Z-Stack

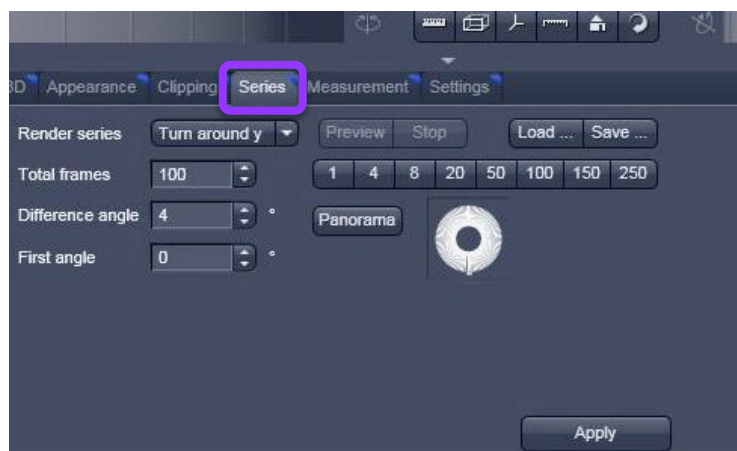
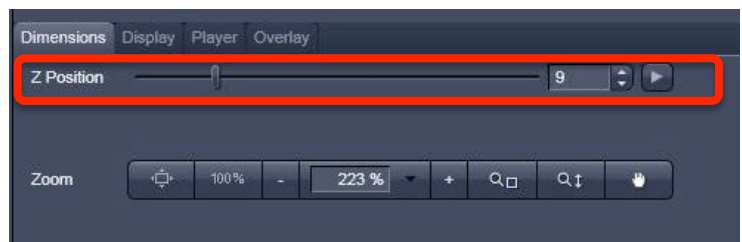
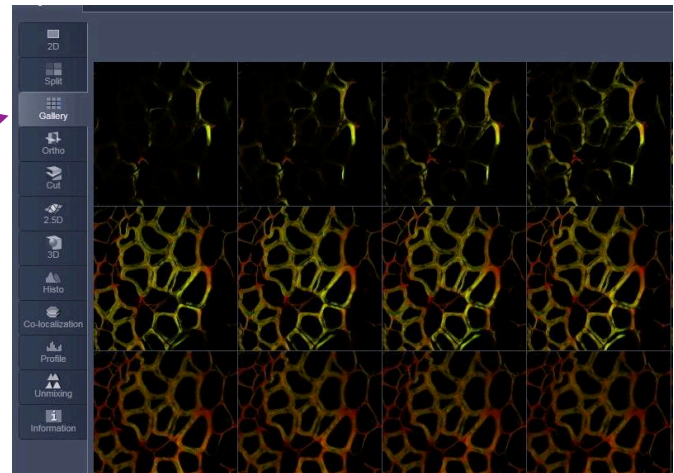
1. Start a live scan. Focus up and down through your sample to find the brightest focal plane.
2. At the brightest focal plane in the sample, use the range indicator and adjust the gain and offset until you see very few (or no) red pixels and few blue pixels. Stop scanning.
3. Check the box next to **Z-Stack**.
4. Start a Live scan again and focus in one direction until you identify where you want to start the Z-Stack. Note- if you need to take a Z-Stack of the whole sample in Z, focus until you don't see any fluorescence. Click on **Set First**.
5. Focus the other direction until you identify where you want to stop the Z-Stack. Again, if you need a stack of the whole sample in Z, focus until you don't see any fluorescence. Click on **Set Last**.
6. Note- turning the focus knob counterclockwise focuses toward the coverslip side of the sample. Turning the knob clockwise will focus deeper into the sample (away from the coverslip).
7. Click the arrow next to **Optimize Sectioning and Step** to open up the window.
8. Click on **Optimal**. The computer will calculate the optimal number of slices and ensure correct Nyquist sampling (slices will overlap by half their thickness). Also check here to make sure that the optical slice thickness is the same for all channels. If it isn't, adjust the pinhole in the channels window so that all channels match.
9. Click on **Start Experiment** to start the Z Stack.





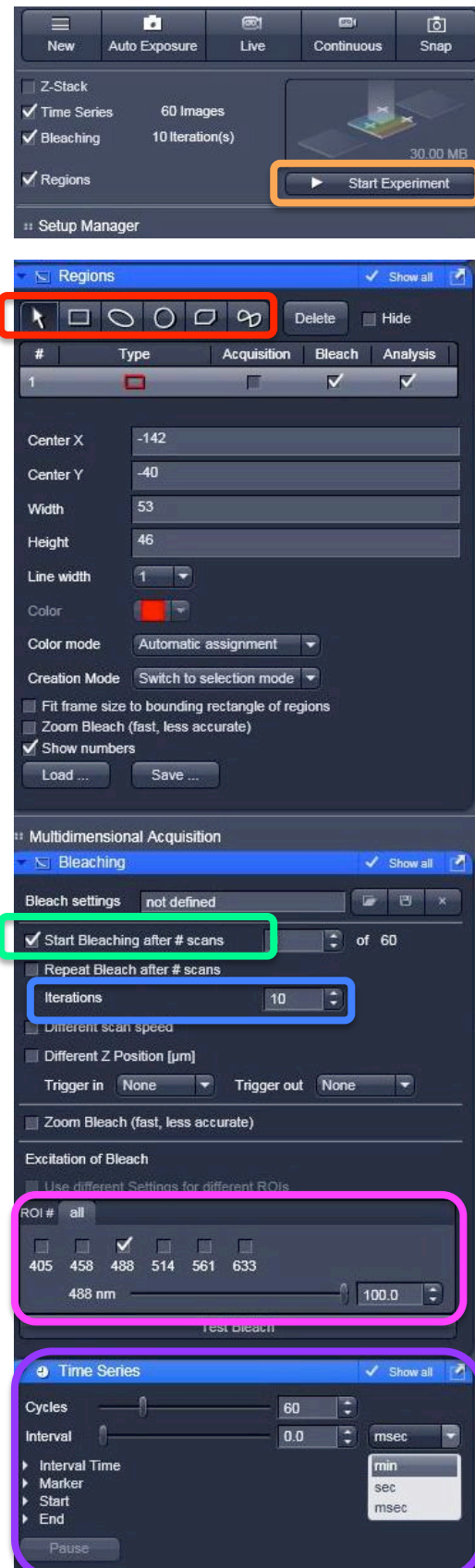
# Basic Viewing of a Z-Stack

1. You can view a Z-Stack in gallery mode by selecting Gallery next to the image window.
2. To scroll up and down through the series, click on 2D or Split, and move the **Z position** slider bar under the Dimensions tab below the image window. The series can be animated automatically under the Player tab.
3. To view the stack as a Maximum Intensity Projection, click on 3-D and **Maximum**. To create the image, click on Create Image.
4. To render the series, click on the **Series Tab**. Choose your turning axis, number of frames and difference angle. Note- the smaller the difference angle, the greater the number of frames (projections). Selecting Panorama will result in a 360 degree projection. Click Apply to create the projection.
5. The image may also be projected at different angles within the Image Window. Click and drag on the image view the stack at different angles.



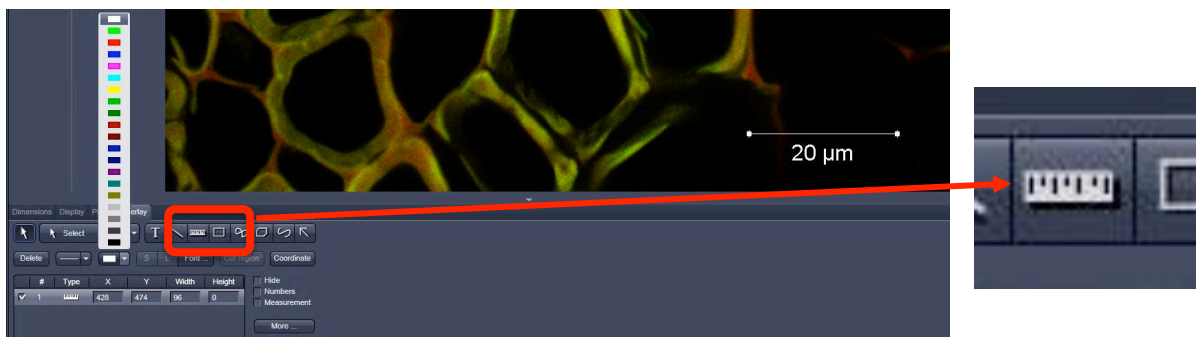
# Time Series with Bleach (FRAP setup)

1. Check the boxes next to Time Series, Bleaching and Regions.
2. Define the regions you would like to bleach by selecting a **shape**. Position the mouse pointer over your image and draw the shape around the portion of the image that you want to bleach. Multiple ROIs can be defined. Make sure Bleach and Analysis are checked next to the ROI.
3. Define the bleaching parameters. For example, to take pre-bleach images, check the box next to 'Start Bleaching after # of scans' and enter a number into the box.
4. Define the number of **iterations** (number of times the region will be scanned). Sometimes, it is necessary to scan an ROI multiple times in order to bleach it. The number of iterations will depend on how easy it is to bleach the ROI.
5. Define the **bleach laser** (the laser line you are using to excite the sample should be the laser you use to bleach it). Increase the laser power to 100%
6. Define the post-bleach scanning parameters in the **Time Series Window**. The number of Cycles is the number of scans taken after bleaching. The interval is the time between scans.
7. Click on **Start Experiment** to start the bleach series.

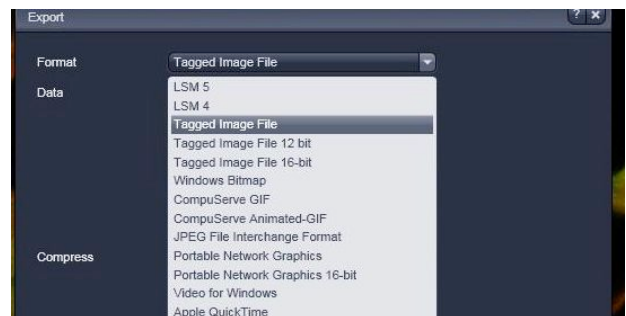
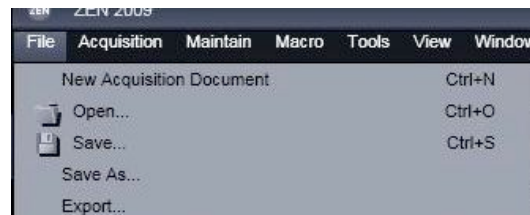


# Exporting Images

1. All LSM 710 images files are saved in a proprietary format. If you would like to open them in Photoshop or put them in a PowerPoint presentation, you will need to export them.
2. It is possible to overlay data onto the image before exporting it. For example, a scale bar can be placed on the image and exported with the image. To put overlays on the image, click on Overlay below the image window and select the **scale bar**. Click and drag on the image to create the scale bar. The color, line weight, font, etc., can be adjusted from the menu options.



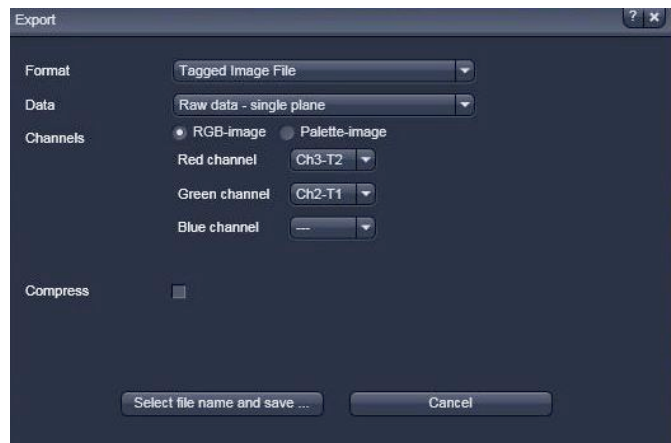
3. Choose File, Export from the top menu.
4. In the Export Window, select the file format.
  - Select a Tagged Image File (TIF) if you need to preserve the format of your image (if you need to analyze it).
  - Choose JPEG to compress the file and make it smaller (a JPEG cannot be analyzed).
  - Choose Video for Windows to export an animation (time series, z-series, 3-D render series etc.)





# Exporting Images

5. In the Export Window, select the data format.
6. Raw data- Single or series will export the raw image data only (no overlays). Single will export a single image only, and series will export all images within a series (time series, z-stack etc.). Select the channels you would like to export and click on 'Select file name and save'. To export the overlaid image, make sure all channels are selected.
7. To save the image with overlays added to it, select Full resolution image window single (for a single image) or Full resolution image window series (for a series of images). However the image appears in the image window is how the image will be exported. To export single channels of a multicolor image, hide the channel by clicking on the **channel** below the image window. Click on 'Select file name and save'.



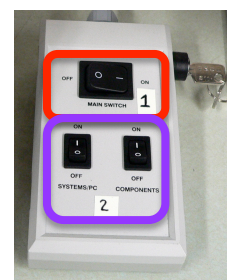
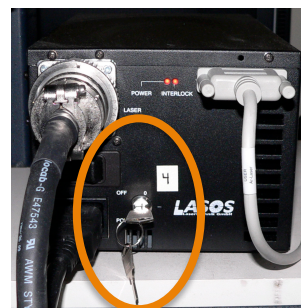
Clicking here will hide the green channel from view

# Shutdown Procedure

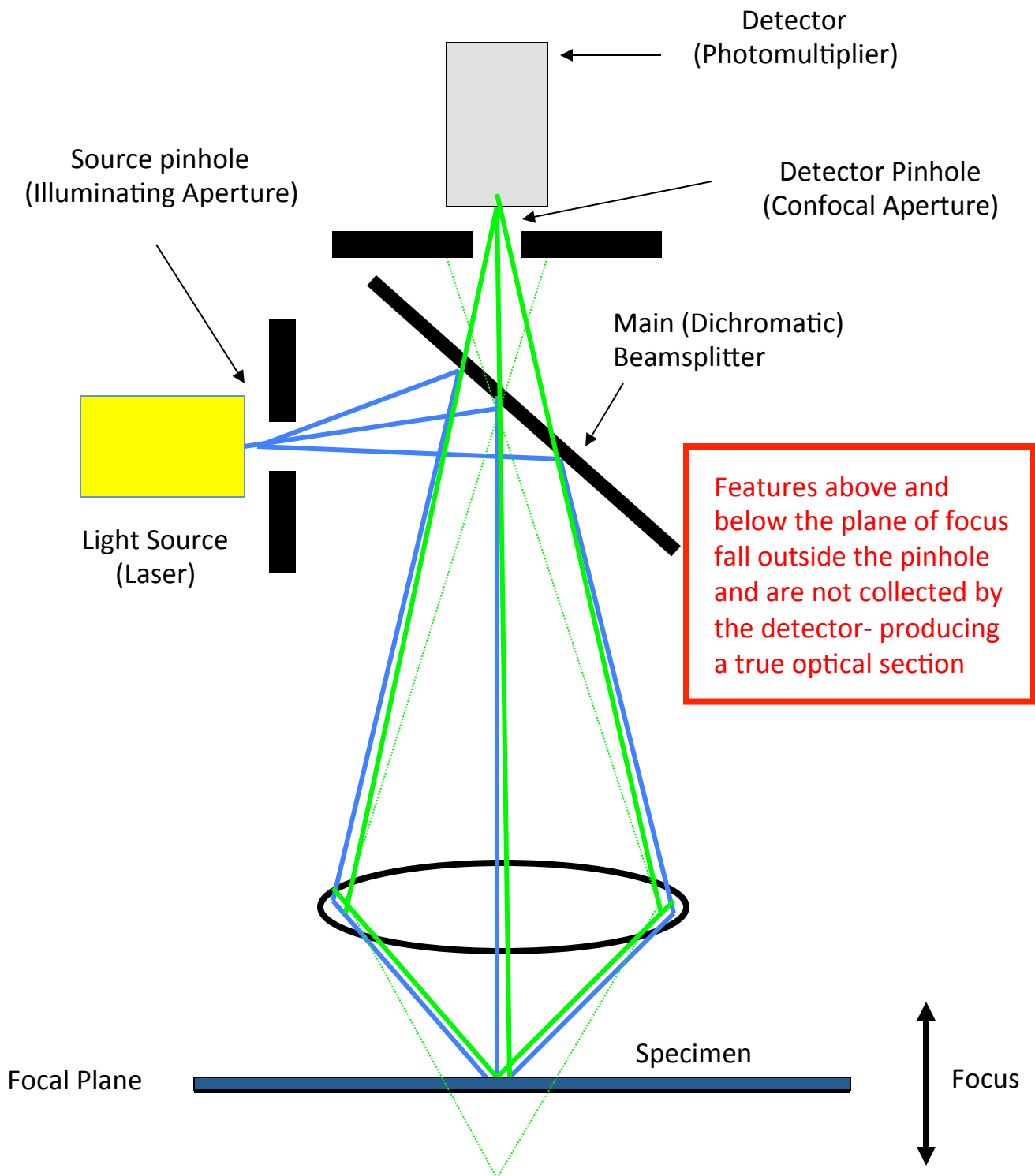
1. If someone is booked within one hour after you are finished with the microscopes, complete the following steps:
  1. Save your images.
  2. Exit the software and log out of your Windows account.
  3. Clean the objective lenses you used with Sparkle and lens paper.
  4. Fill out the logbook. Record the current time in the 'End Time' space on the login sheet.

21. If someone is booked greater than one hour after you are finished with the microscope, complete the following steps:

1. Save your images.
2. Exit the software.
3. Turn the **argon laser dial** (#7) counterclockwise.
4. Turn the **argon laser power** from 'Run' to 'Idle' (#6).
5. Shut down the computer (#5).
6. Turn the **argon key** (located underneath the microscope) to the left, so that it is in the vertical position). Note- you must wait ~2 minutes for the fan to shut down before shutting down the Main Power.
7. Clean the objective lenses with Sparkle and lens paper.
8. Turn off the **X-Cite Mercury lamp** (#3).
9. Turn off the **Systems/PC and Components switches** (#2).
10. After the argon laser fan shuts off (about 2 minutes), turn off the **Main Switch** (#1).
11. Fill out the user log book. Record the current time in the 'End Time' space of the login sheet.
12. Put the cover on the microscope.



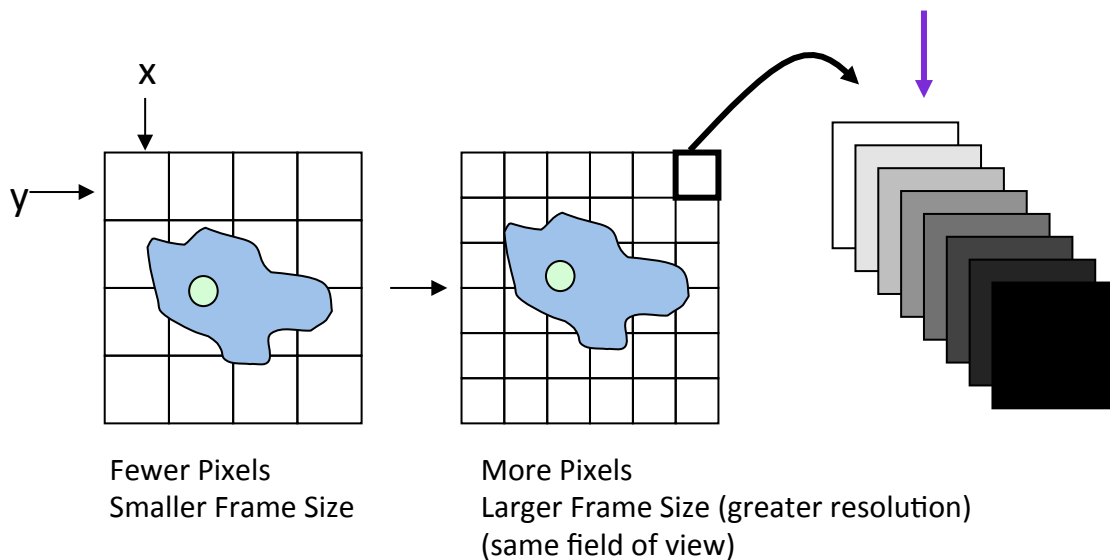
## Appendix A: Confocal Principle Explained





## Appendix B: Frame Size and Bit Depth Explained

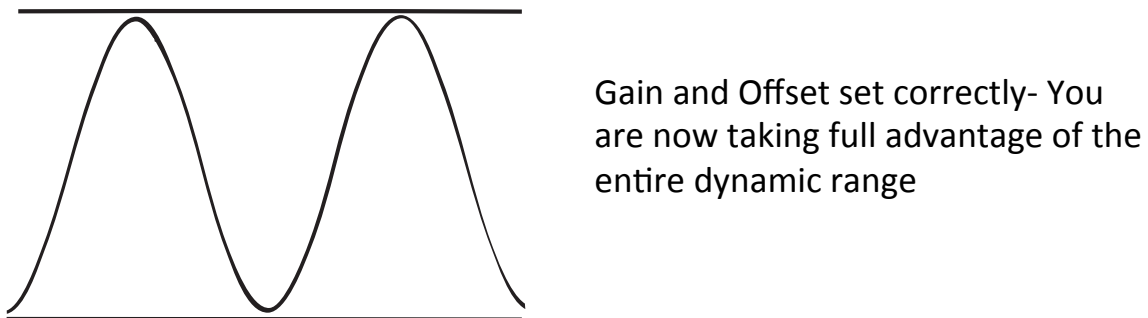
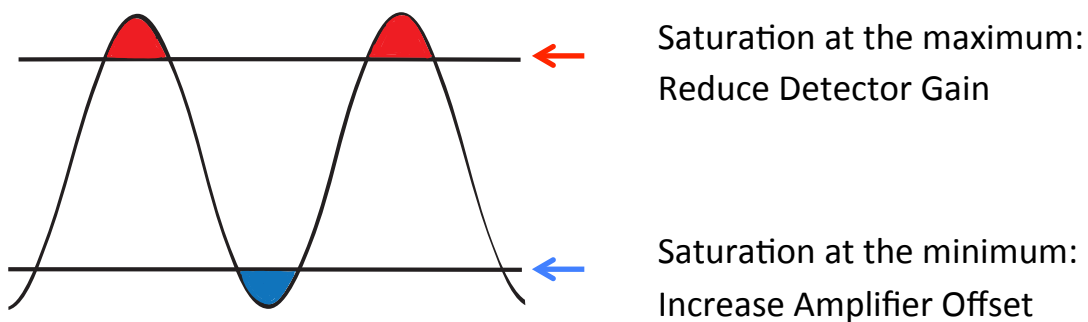
- When your sample is scanned, the emitted fluorescence signal is detected by a PMT (photomultiplier tube) and converted into digital picture elements (pixels).
- Pixels are represented by x and y values. The x value specifies the horizontal position (column) and the y value indicates the vertical position (row). A larger frame size means you will collect more pixels per unit area scanned, effectively increasing the resolution of the image.
- Each pixel in the image has a specific intensity value. The range of intensity values depends on the bit depth. 8 bit data = 256 gray levels. 12 bit = 4096 gray levels. 16 bit = 65,536 gray levels.
- Thus, the quality of the digital image is determined by the number of pixels in the image (frame size) and the bit depth (range of intensity values) for each pixel.



## Appendix C

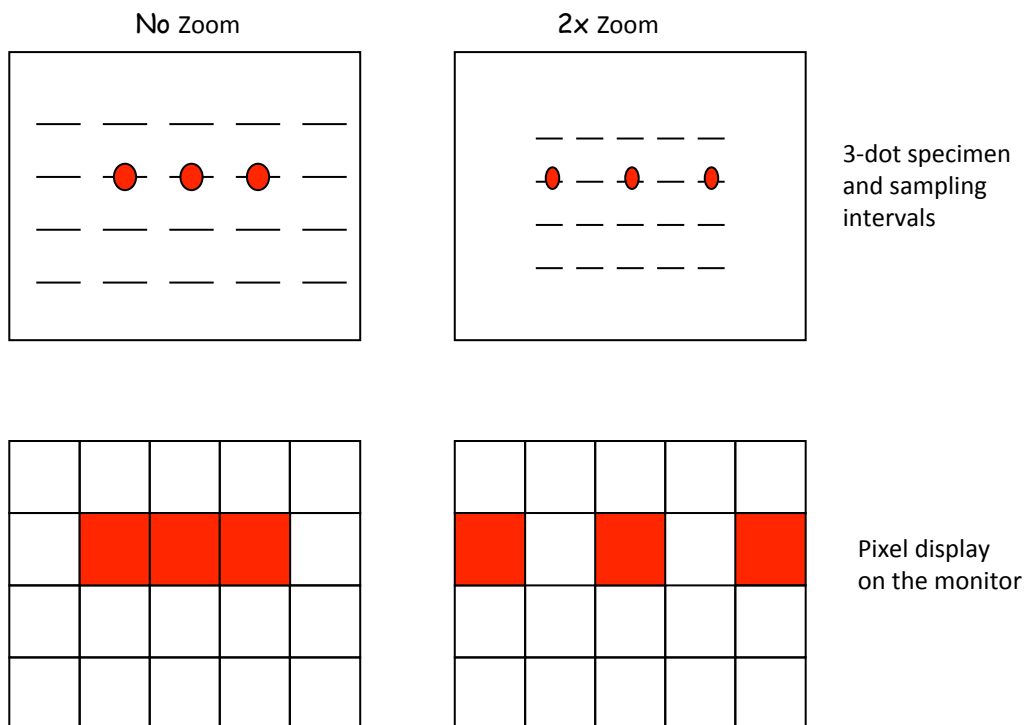
### Gain and Offset Explained

- Detector gain determines the sensitivity of the photomultiplier by setting the maximum limit.
- Amplifier offset determines the minimum intensity limit.



## Appendix D: Optical Zoom Explained

- When you use the zoom feature on a confocal microscope, the laser is scanned over a smaller area of your sample.
- Because the decreased scan area is displayed within the same frame size on the computer screen, the image is magnified.
- For example, if you scan an image at 512x512 pixels and then zoom in on a region of interest, those same 512x512 pixels are now obtained from a smaller area of your sample- effectively increasing the resolution of the image.
- Keep in mind that you are still limited by the theoretical resolution of a light microscope (100-200nm).
- Zooming too much will result in “empty magnification”, that is, magnification without any actual increase in image resolution
- Zooming is also used to match the objective lens resolving power to the image resolution (number of pixels collected) in order to satisfy the Nyquist sampling criteria.
- The Nyquist Sampling Theorem states that, when an analog signal is digitized, in order to retain all of the information contained within the signal the diameter of each pixel collected must be at least 2.3 smaller than the optical resolution of the microscope.



### References-

\* Confocal Microscopy for Biologists by Alan R. Hibbs.

\*\*Fundamentals of Light Microscopy and Electronic Imaging by Douglas B. Murphy.



## Appendix D: Optical Zoom Explained

The optimal zoom factor is determined by the magnification and NA of the objective lens, as well as the wavelength of the excitation light.

### 512x512 Frame Size

Objective Lens	Excitation Wavelength (nm)	Minimum Zoom Factor	Corresponding Pixel Size (nm)
40x, 1.3NA	458	4.9	89.9
40x, 1.3NA	488	4.6	95.8
40x, 1.3NA	543	4.1	106.6
40x, 1.3NA	633	3.5	124.2
63x, 1.4NA	458	3.3	83.5
63x, 1.4NA	488	3.1	89.9
63x, 1.4NA	543	2.8	98.9
63x, 1.4NA	633	2.4	115.3
100x, 1.4NA	458	2.1	83.5
100x, 1.4NA	488	2	88.9
100x, 1.4NA	543	1.8	98.9
100x, 1.4NA	633	1.5	115.3

### 1024x1024 Frame Size

Objective Lens	Excitation Wavelength (nm)	Minimum Zoom Factor	Corresponding Pixel Size (nm)
40x, 1.3NA	458	2.4	89.9
40x, 1.3NA	488	2.3	95.8
40x, 1.3NA	543	2.0	106.6
40x, 1.3NA	633	1.8	124.2
63x, 1.4NA	458	1.7	83.5
63x, 1.4NA	488	1.6	88.9
63x, 1.4NA	543	1.4	98.9
63x, 1.4NA	633	1.2	115.3
100x, 1.4NA	458	1.0	83.5
100x, 1.4NA	488	1.0	88.9
100x, 1.4NA	543	0.9	98.9
100x, 1.4NA	633	0.8	115.3

References-

\*Confocal Laser Scanning Microscopy – Principles, by Carl Zeiss Inc.