

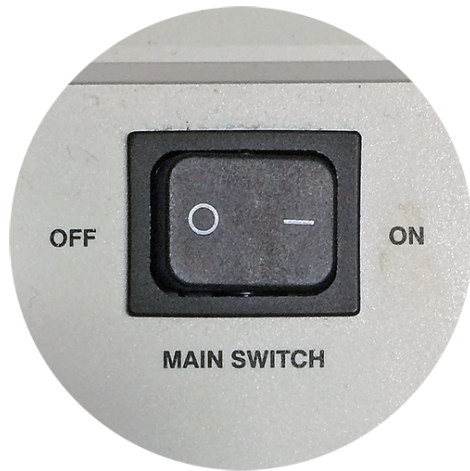


Training Guide for Carl Zeiss LSM 780 Confocal Microscope
ZEN 2012



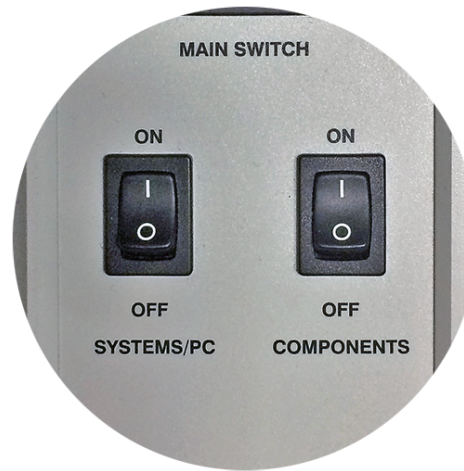
Optical Imaging & Vital Microscopy Core
Baylor College of Medicine (2017)

1



Turn ON 'Main Switch' from the remote control paddle.

2



Turn ON both 'Systems/PC' and 'Components' switches.

By default, the 'Lasers' key located on the side of the remote paddle should always be in the 'ON' state, but please verify.

3



Turn Key from 0 to 1 position to start up Argon laser.



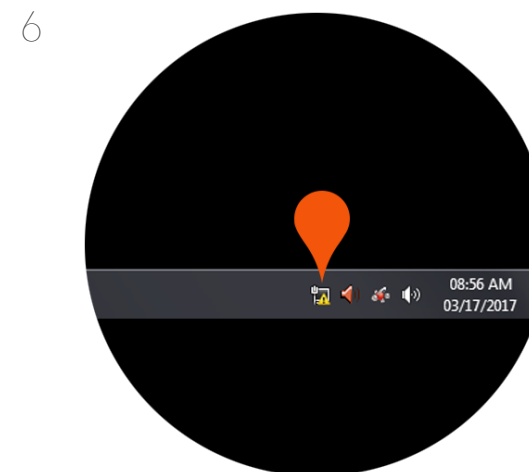
Turn ON X-Cite epi-fluorescence light source.

This light source is required for visualizing fluorescence via the microscope eyepieces.



Turn ON HP Z820 PC.

Wait for PC to boot into Windows OS and login to 'LSM User' account.



Once logged into OS, please check the status of the network connection to the system.

The icon above shows the network is connected and functioning.



If you get an icon with a red X or a busy icon then please wait for the network connection to establish before continuing.

7



Double click ZEN Black icon to start software.

Note: Be sure to use Zen Black instead of Zen Blue. Icon above is for Black version.

8



When software has started, you will be presented with a login screen.

Select 'Start System' to scan new images. If processing existing images, select 'Image Processing'.

9



Wait for initialization of system to complete.

ZEN 2012 is separated into three distinct areas.

Image Acquisition Tools

Represented by a group of blue bars each containing a series of tools for sample observation, image acquisition, image processing and system maintenance.

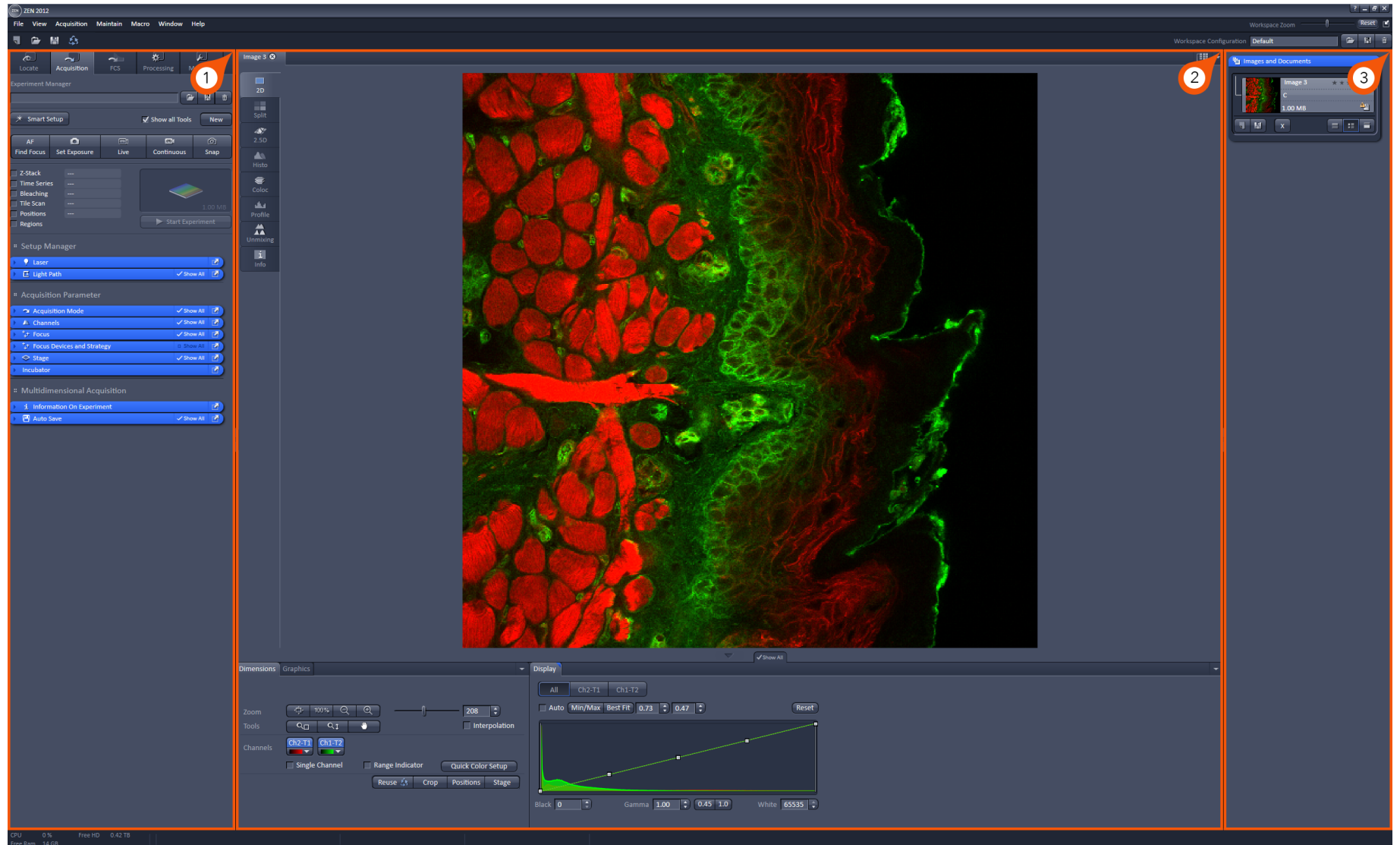
Image Display

This area is where each newly captured image will appear. Settings here allow the user to control how the image is viewed on screen.

Catalog of Open Images

This list displays each image that is currently open within the ZEN software. This area contains tools for saving data sets.

Getting Started with ZEN 2012



Mounting Sample on the Microscope



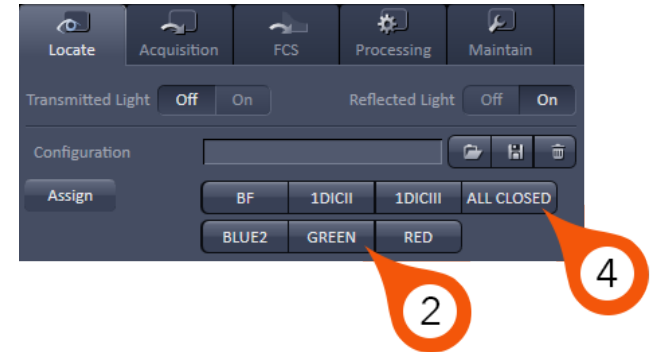
Once the system has been powered on, we can mount our specimen on the stage above the microscope objective.

1. Place sample on microscope stage above objective (roughly in the center of the field of view).
2. From the 'Locate' tab in the Image Acquisition Tools, use the Shortcut buttons to select filter set for visualization in the eyepieces.

- GREEN - generic green filter set
- RED - generic red filter set
- BLUE - generic blue filter set
- ALL CLOSED - turn off all light sources.

Note: These shortcut buttons will automatically select the filter set and open the light source shutter.

Alternatively, you can turn on the epi-fluorescence light path manually using the 'Ocular' tab located below the configuration shortcuts. Here you can turn on/off the epi-fluorescence light source (A), change your filter set (B) and control transmitted light (C) if applicable.



Mounting Sample on the Microscope



3. Via the microscope eyepieces, visualize your sample and adjust the focus (Z) and stage position (X,Y) accordingly.

4. Once a suitable location is found, click 'ALL CLOSED' from shortcuts (2).



5. Select 'Acquisition' from main toolbar to begin imaging.



Powering On Lasers

Before we begin setting up the software for imaging, we first need to verify that our lasers are powered on. This particular model of LSM 780 has a manual Argon laser power supply and we must put the laser into a run state before we begin imaging.

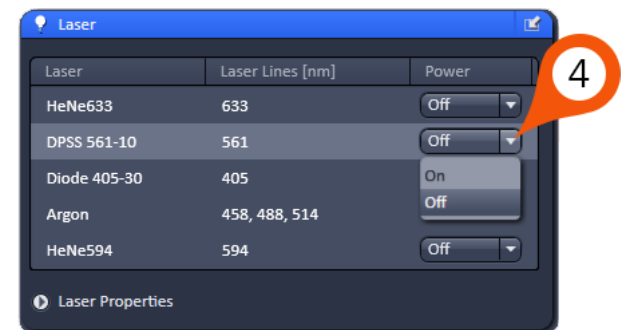
1. Allow the system to warm up for at least 5 minutes.
2. Once the warm up period has passed, switch 'laser' from 'idle power' to 'run'.
3. Verify that 'Power stabilized' light is green (this may take a few minutes).

Note: When the LED has turned green, power is stable to Argon laser. The 'light control' rheostat controls the current to the laser, and this can be adjusted to a point where you are just below triggering the 'High Power mode' with the red LED. If the light changes to red, back off rheostat counter-clockwise until the green 'Power stabilized' LED is illuminated.



4. Turn ON remaining lasers from the software dialog 'Laser'.

Note: The 405 laser is automatically powered on with the system. The 561, 594 and 633 lasers can be powered on by selecting each laser and switching from Off to On state.



Beam Path Configuration

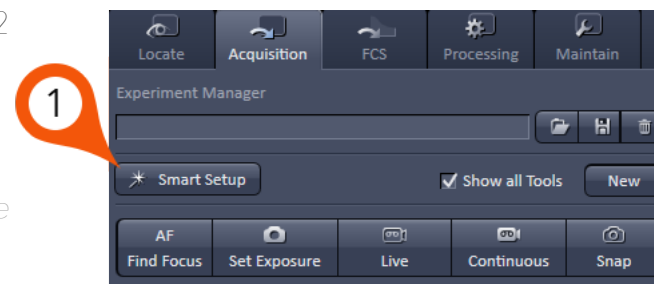


The LSM 780 is equipped with a 34 channel spectral detector plus transmitted light and has 5 lasers with wavelengths 405nm, 458nm, 488nm, 514nm, 561nm, 594nm, and 633nm.

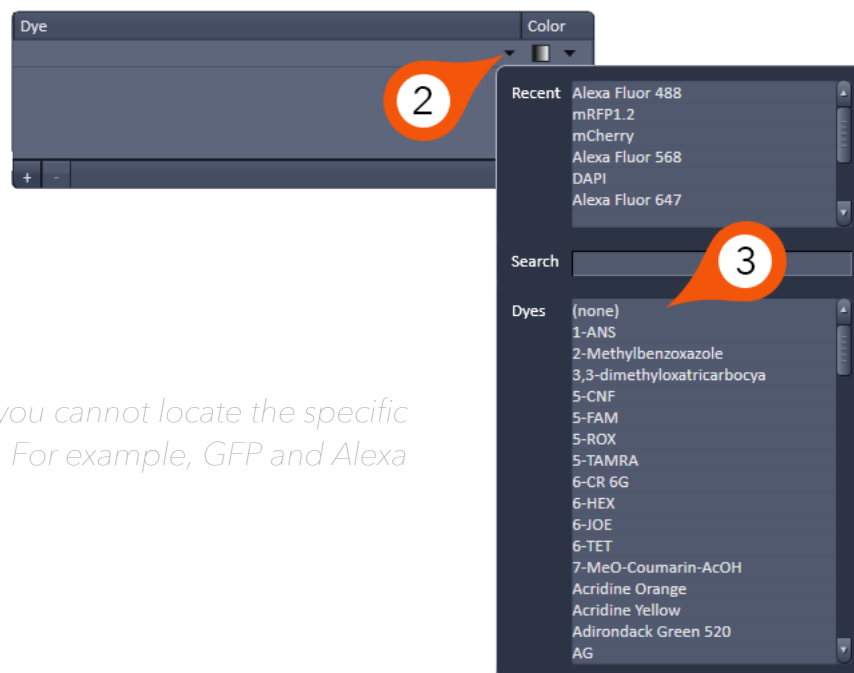
In this example, we are going to walk you through setting up the beam path for a 2 color imaging experiment.

1. From the Acquisition tab, select 'Smart Setup'.

Note: Smart Setup is a software tool that allows us to choose the fluorophore combinations we want to image and have the system intelligently select the beam path configuration.



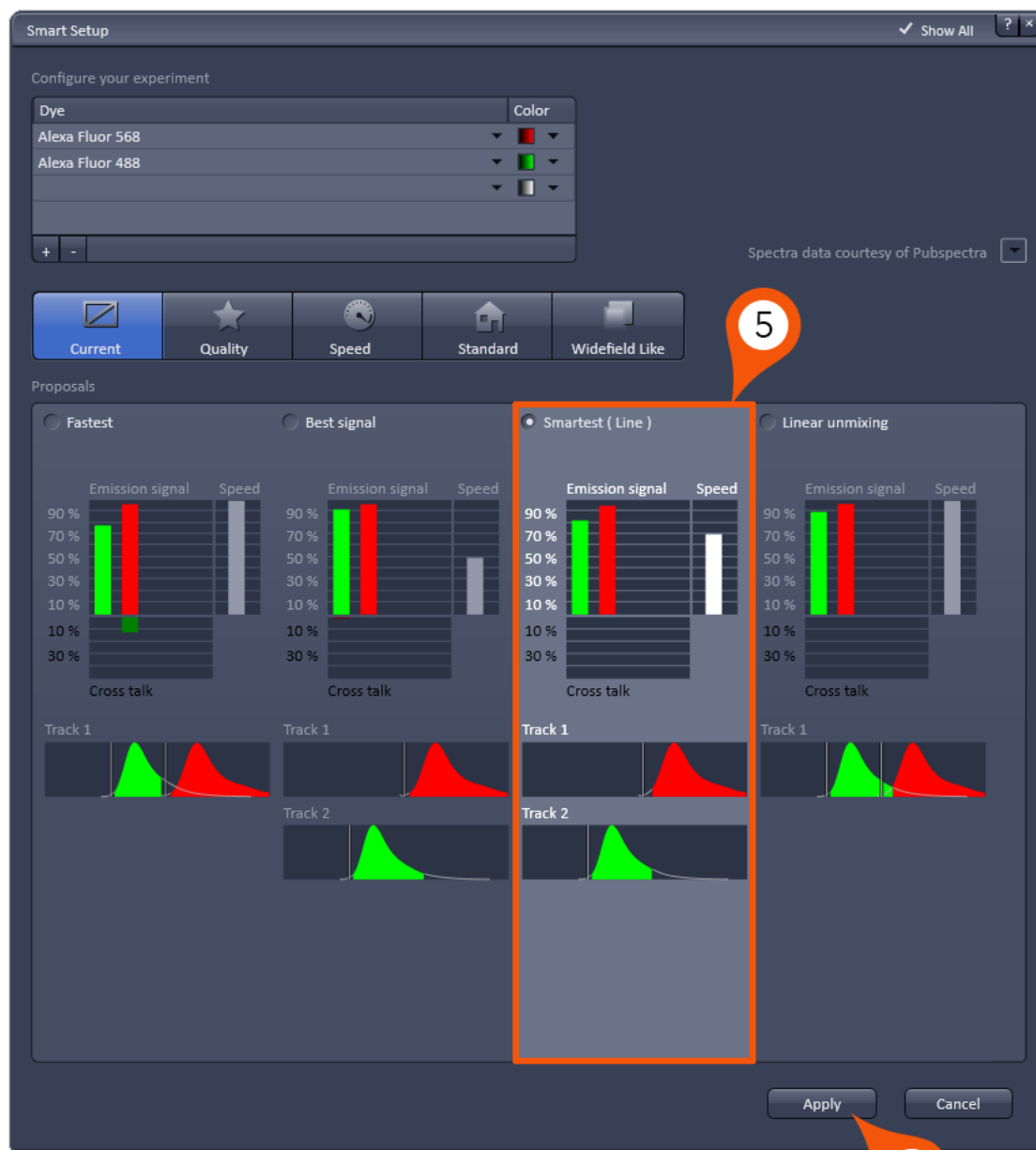
2. From the Smart Setup popup, configure your dye combination by clicking on the dropdown arrow in the 'Dye' tab.
3. Choose your dye either from the 'Recent' list (if you have used it before) or from the more comprehensive 'Dyes' list.
4. Repeat Steps 2 & 3 until all your dyes have been selected.



Note: Choose longest wavelength fluorophores first. Also, if you cannot locate the specific name of your dye, select the dye that is closest in wavelength. For example, GFP and Alexa 488 would give identical beam path setups.

- From the list of proposals, choose a suitable imaging mode.
 - Fastest** - collect all images simultaneously.
 - Best Signal** - collect each channel sequentially.
 - Smartest (line)** - hybrid between fastest and best signal. This proposal will attempt to sequentially acquire using the fewest number of tracks possible.
 - Linear Unmixing** - spectral scanning mode.

6. Click 'Apply'.



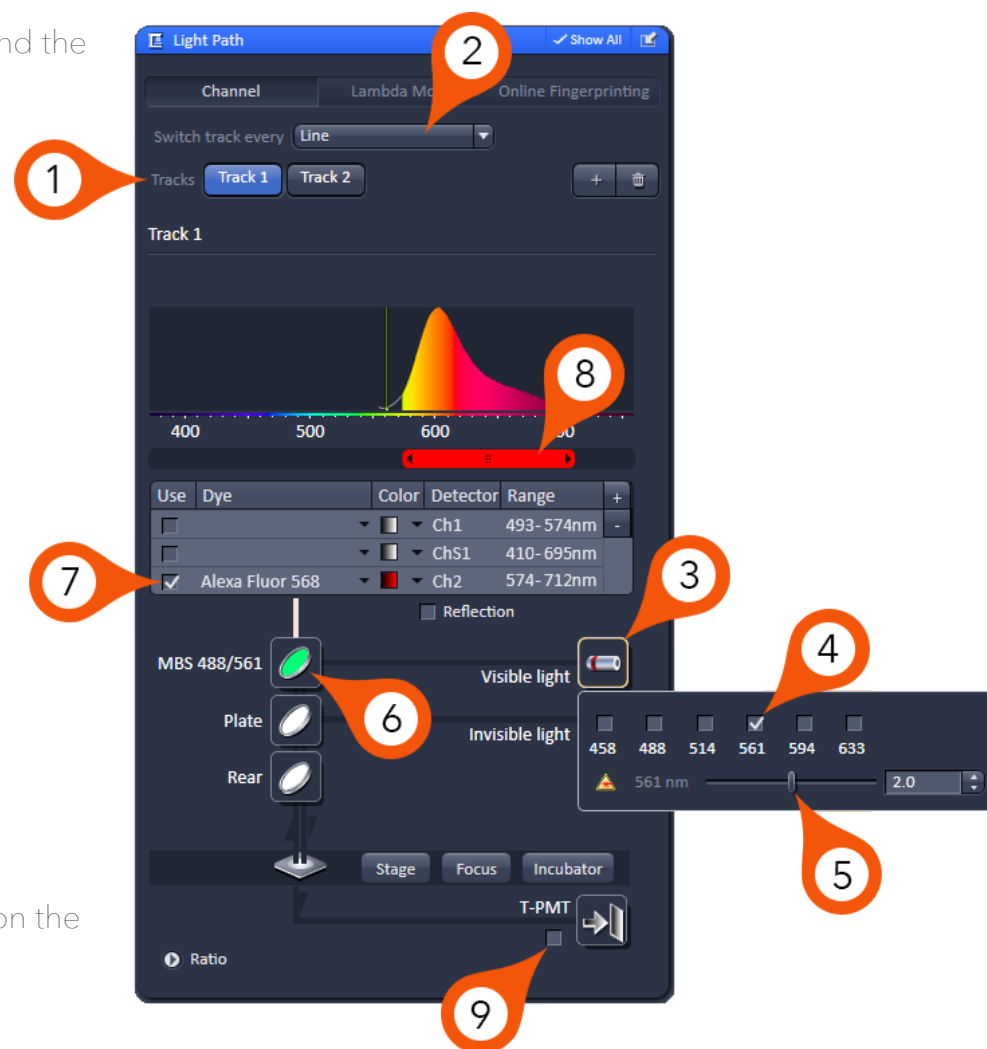
Beam Path Configuration



The Smart Setup provides a good baseline for quickly setting up the system to collect images. It is still important to understand the 'Light Path' dialog should the need arise to fine tune the acquisition settings.

The example above is a sequential (2 track) acquisition of Alexa 568 and Alexa 488. Here we will illustrate how the Alexa 568 track is designed manually.

1. **List of Tracks** - each track corresponds to a particular dye and the group of settings designed for imaging that particular dye.
2. **Switch track every** - here you can choose to change tracks between each X line or each Frame during the scan. Line mode is preferred as it will reduce the potential for movement due to drift.
3. Click 'Visible light' laser icon to open the laser dialog.
4. Place a check mark in the box for the required laser line.
5. Set power slider - in this example, we are using 2% (this is a starting value and can be changed later).
6. Set MBS (Main Beam Splitter) to a filter that matches the combination of laser lines you will need for ALL TRACKS.
7. Choose detector for imaging.
8. Define upper and lower emission band thresholds based on the spectrum for your fluorophore.



Beam Path Configuration



Note: PMT selection will be based on where your fluorophore(s) fall in the linear range of the emission spectrum. Meaning that Ch1 typically corresponds to the lowest wavelength while Ch2 corresponds to the longest wavelength fluorophore. In our example, we will be using a sequential scan with Alexa 568 and Alexa 488, so Ch1 will be used for the Alexa 488 signal and Ch2 will collect the Alexa 568.

9. The system is equipped with a Transmitted Light Detector for imaging DIC or brightfield images. Check to enable.

Once our beam path setup has been completed we can expand the scanning tools to begin collecting an image. From 'Acquisition Parameter' there are 2 blue tabs used for imaging, 'Acquisition Mode' and 'Channels'.

Acquisition Mode

This dialog controls static image settings such as:

Objective - displays currently selected objective as programmed into microscope.

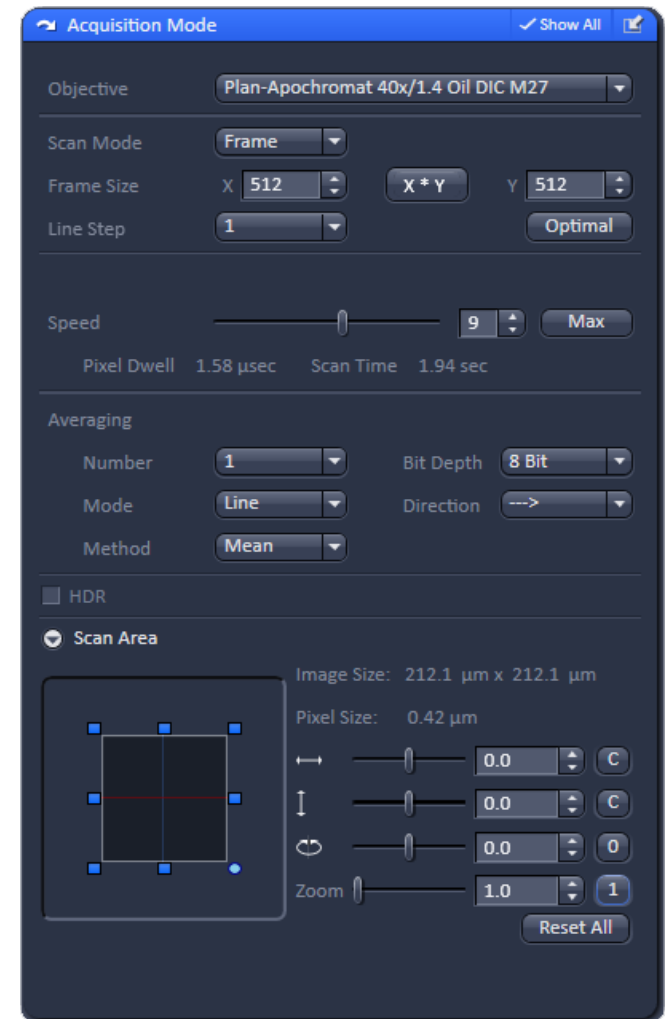
Frame Size - image size in pixels. Default is 512x512, however this can be optimized based on objective and zoom settings. Ideal sampling frequency can be quickly selected with the 'Optimal' button.

Scan Speed - overall speed of the scan during acquisition. Reports pixel dwell time and total scan time. Increasing dwell time (slowing down the scan) will improve signal-to-noise ratio.

Bit Depth - the number of bits used to indicate the range of signal level in the sample. The default A/D conversion is 16 bit.

Averaging - selecting 'Number' >1 will scan each X line the 'number' of times and average the result. Used to reduce image noise.

Scan Area - allows scan area adjustments such as X,Y displacement and rotation of scan in 360 degrees. 'Zoom' can be used to increase magnification without signal loss or objective change.



The 'Channels' menu contains the three components used to dynamically control image quality. They are (1) Laser Power, (2) Detector Sensitivity, and (3) Pinhole or Confocal Aperture Diameter. For further discussion on these parameters and how they affect image quality please see 'Understanding Image Quality' starting on page 20.

Channels

Functions available in the Channels dialog:

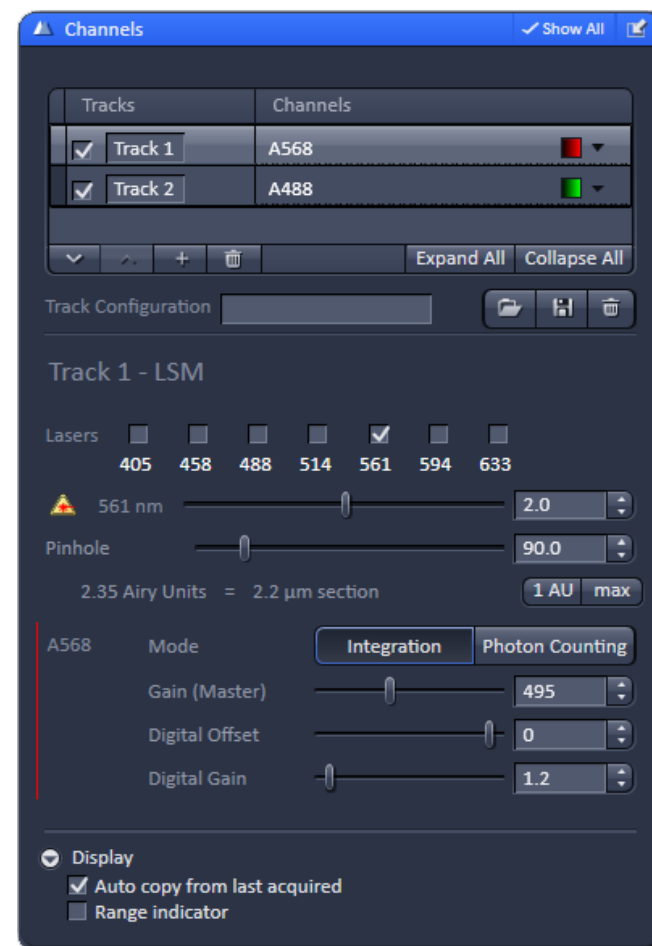
Lasers – controls laser power (%) and wavelength selection.

Pinhole – confocal aperture diameter that controls optical section thickness. Reports section thickness and pinhole diameter.

Gain (Master) – controls the analog amplification voltage for the PMT (in Volts). The higher this value the more sensitive the detector becomes to the signal. Noise is also amplified by gain, albeit at a slower rate.

Digital Offset – controls the dark current offset for the imaging system. When scanning an image with all lasers off this value set to '0' should report background grey values close to/at 0 grey levels.

Digital Gain – is a digital amplification of signal that is applied post A/D conversion. This amplifies signal at the same rate as it amplifies noise. Leave this value set to 1 – there are other locations to control image brightness that are more efficient and less destructive.



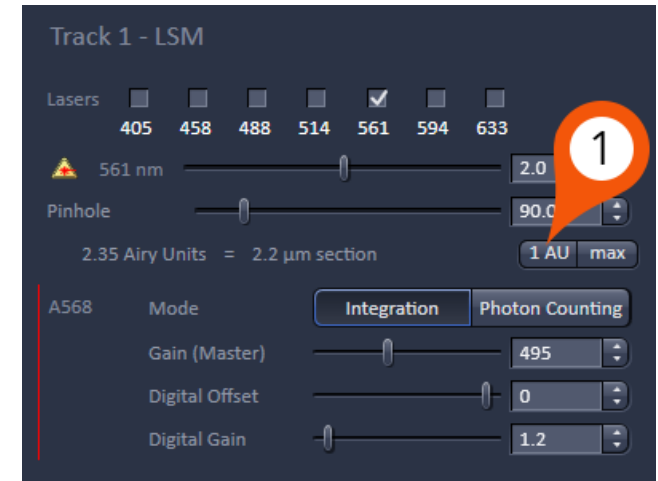
Scanning an Image



Once the laser(s) have been turned on and the detector(s) have been selected, we can scan an image and begin adjusting our signal level.

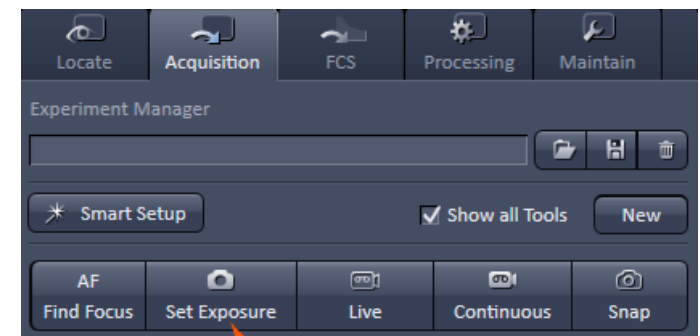
1. From the 'Channels' dialog, set the 'Pinhole' to 1 AU.

Note: Setting the pinhole to 1 AU is a compromise between axial resolution and signal level. When you start with an open pinhole, you are at a point where your signal is the highest but your axial resolution is at its lowest. As you reduce the aperture diameter you are increasing your Z resolution but reducing your signal level. This is a reasonable trade off until you reach 1 AU. Reducing the pinhole lower than 1 AU will still linearly increase your Z resolution but now signal will start to drop exponentially.



2. Click 'Set Exposure' to have the system scan the image and automatically adjust the gain and offset.

Note: The automatic exposure setting works reasonably well with very bright signals. It is not intended as a final optimization, just as a baseline for fine tuning.



Fine Tuning Image Quality

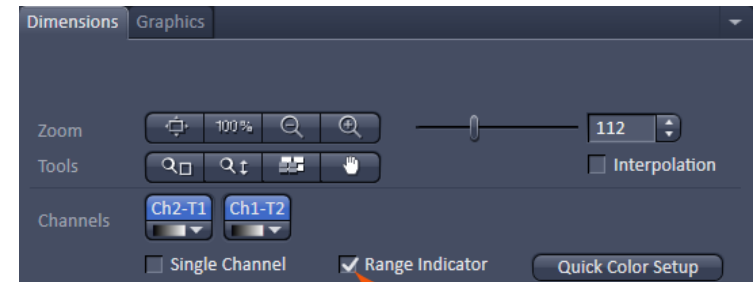


In order to find the optimal settings for a particular condition, you must first be able to identify the thresholds of the signal level in the image.

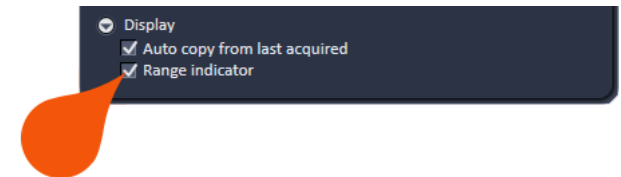
From the 'Dimensions' tab of the Image Display toolbar located below the image being scanned, you will find a checkbox labeled 'Range Indicator'. To activate the Range Indicator, place a check in the box.

The Range Indicator LUT assigns **red** pixels to areas in the image that are overexposed and **blue** pixels to background areas that are underexposed.

Note: The 'Range Indicator' checkbox in the Dimensions tab is a temporary setting, meaning it will only activate when you check the box during the live scan and will automatically turn itself off at the completion of the live scan.



If you want to have the Range Indicator displayed at all times you can turn it on from 'Display' options located at the bottom of the Channels dialog.



Fine Tuning Image Quality

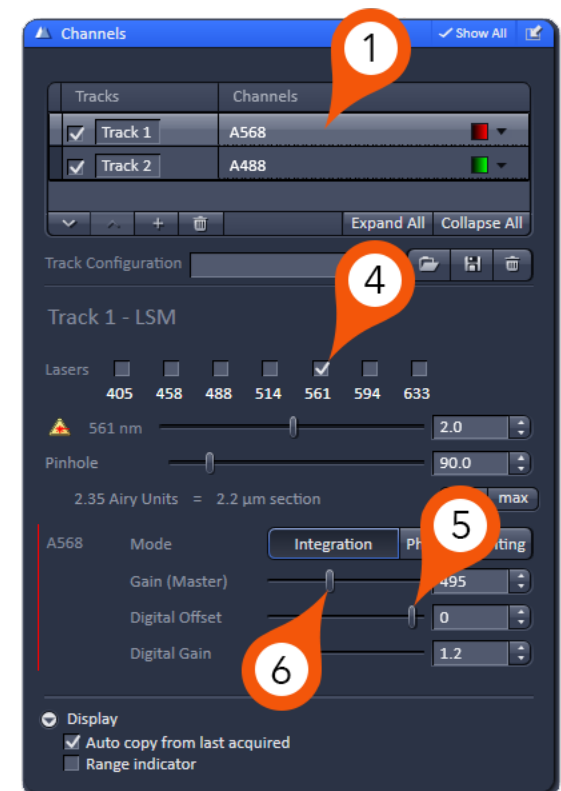
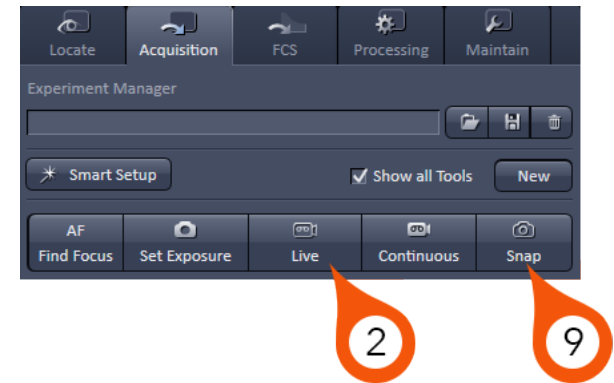


To fine tune the image intensity to ideal levels for final image acquisition:

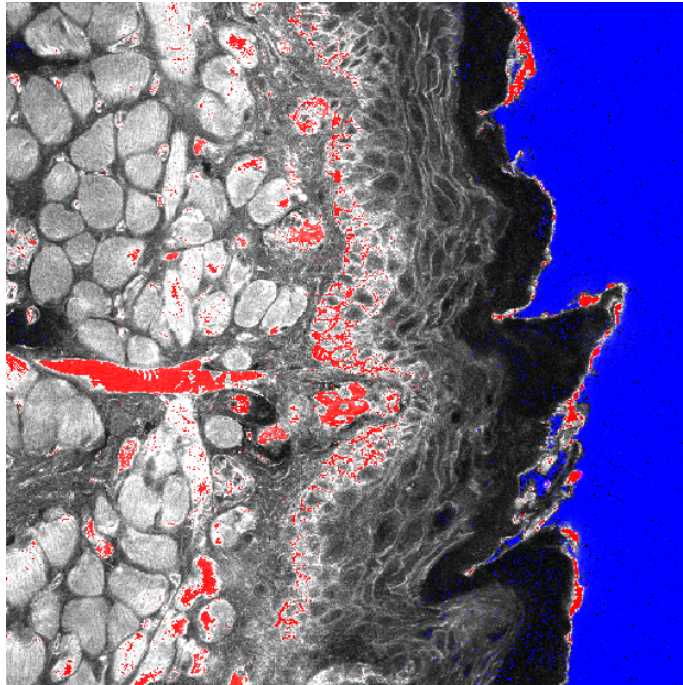
1. Select one Track at a time - begin by highlighting the first Track in your list.

Note: Make sure to uncheck all other tracks during this process so you are only scanning one color at a time. This will save time and ultimately prevent any unnecessary photobleaching of other channels.

2. Click 'Live' to start scanning.
3. Turn on 'Range Indicator' LUT as shown on the previous page.
4. Deselect laser checkbox for the active track.
5. Adjust Digital Offset until background areas are just above the display of any blue pixels.
6. While still scanning, reselect the laser line (4) and increase Gain (Master) until the brightest areas in the image are just below the display of any red pixels.
7. Stop Scan.
8. Repeat this process for each track independently.
9. Click 'Snap' to collect final image.



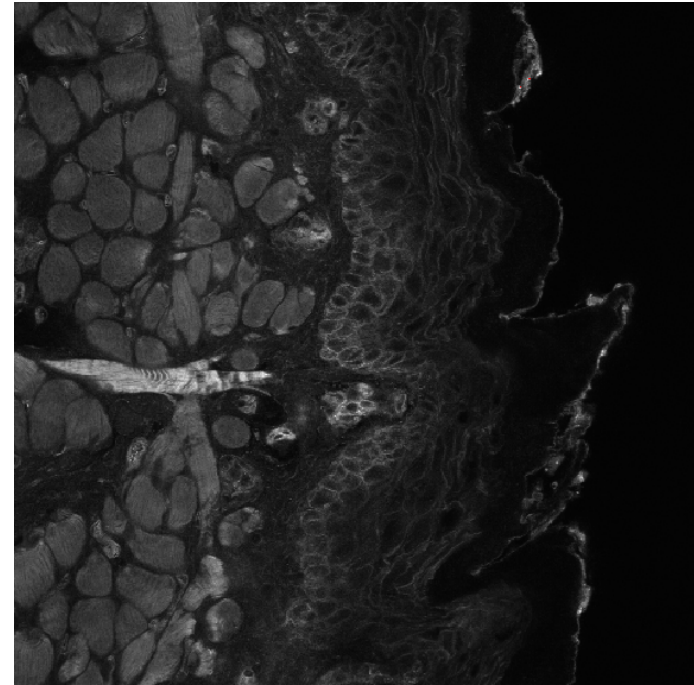
Ideally, you do not want any area in the final image to contain **red** or **blue** pixels with this LUT. For maximum contrast, have the brightest areas fall just below overexposure (**red**) and the background areas fall just above underexposure limit (**blue**). In a properly balanced image you should see no **red** or **blue** pixels with this LUT.



Example of Overexposed (**red**) and Underexposed (**blue**) Areas in Image

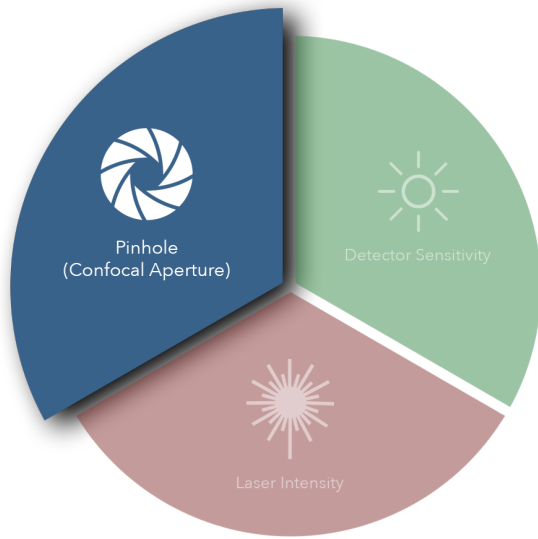
Solution?

Reduce Gain (Master) and increase Digital Offset



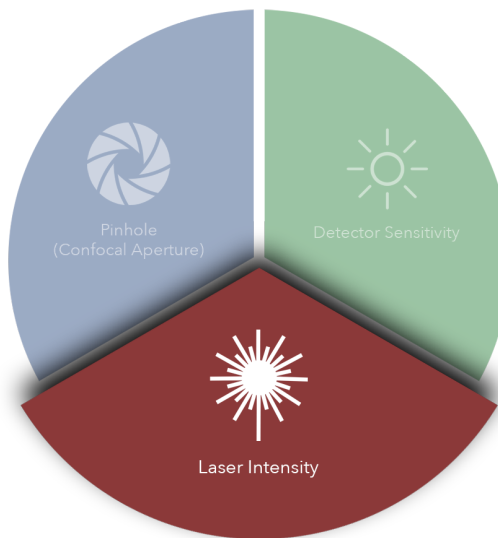
Example of Properly Exposed Image

The result of the final scan above may or may not produce acceptable image quality. Therefore, it is important to have an understanding of the three key factors that play a role in image quality.



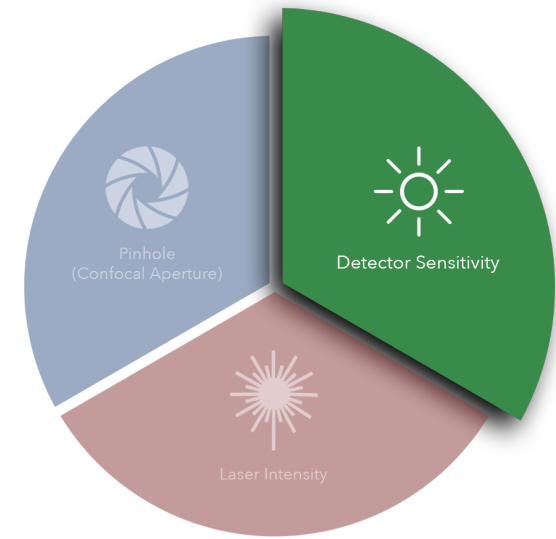
Pinhole (Confocal Aperture)

The confocal aperture controls both axial resolution and signal level. Opening the aperture reduces axial (Z) resolution but increases signal level reported to the detector. Closing this aperture will reduce the signal level but increase axial resolution.



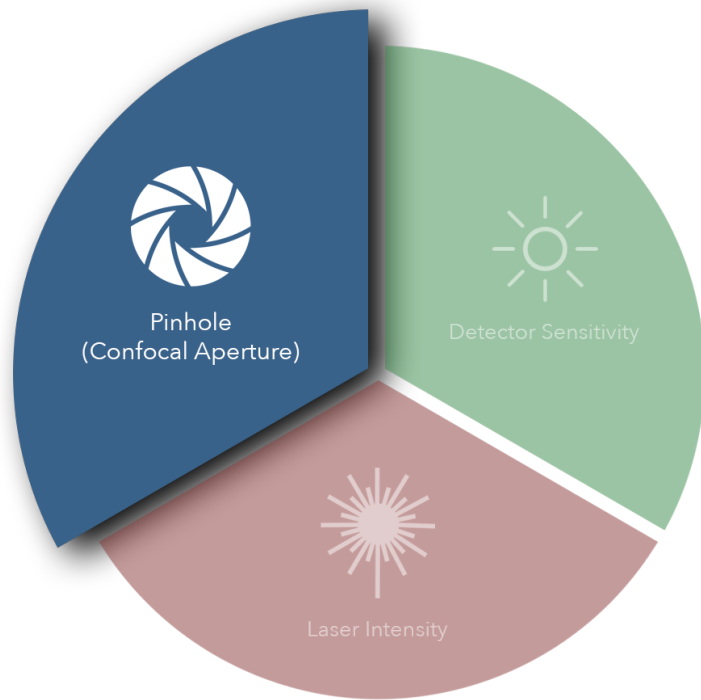
Laser Intensity

The intensity of the laser illumination source has obvious effects on the signal level. Increasing laser power will increase signal levels but may also introduce nonlinear effects such as phototoxicity and photobleaching.



Detector Sensitivity

The detector sensitivity is regulated by the high voltage gain applied to the detector. Increasing the gain directly increases detected signal but also amplifies the inherent noise in the system.



Pinhole (Confocal Aperture)

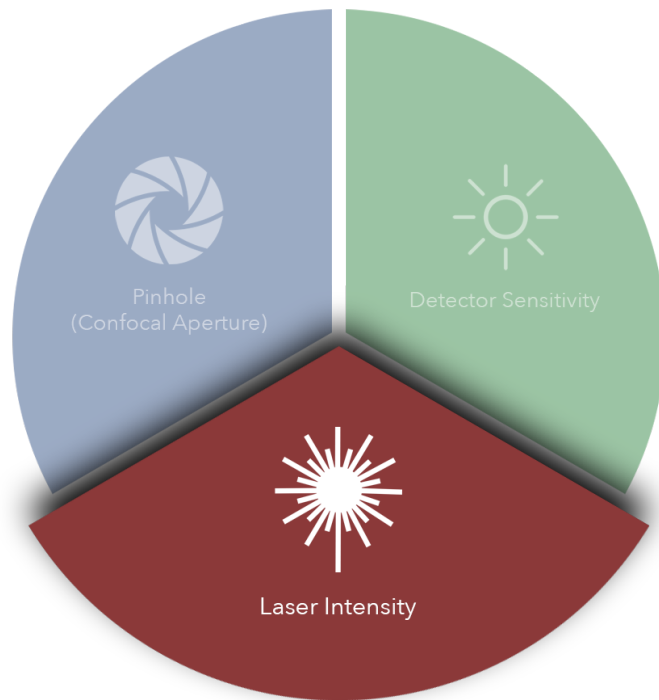
Ideally, the confocal aperture should be set to the size of the structure(s) you are trying to resolve. However, for example, some sub-cellular structures of interest may be beyond Abbe's diffraction limit and therefore beyond the microscope's capability to resolve. The confocal aperture has some practical limits that can be used to guide the usage of this setting.

Start by closing down the confocal aperture to 1 AU. You can certainly close the confocal aperture below this value and continue to improve resolution, just understand that below 1 AU you will lose signal level at an exponential rate.

Conversely, you can increase the confocal aperture diameter to improve the detected signal level if you can sacrifice axial resolution.

In some cases, the signal level may be so low that increasing the aperture diameter is the only way to lower the gain enough to get a usable image.

In other cases, if the gain is too high (causing excess noise) and the laser power cannot be increased due to photo effects then increasing the confocal aperture is the only option to improve image quality.

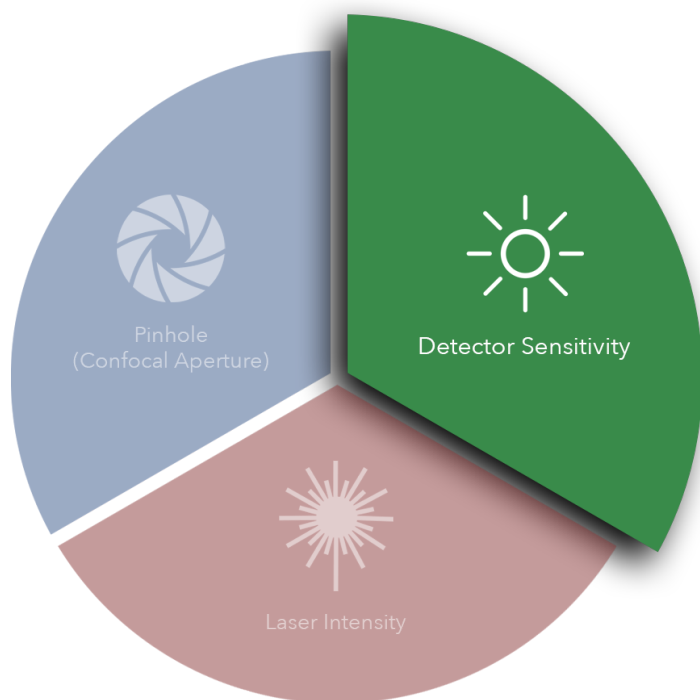


Laser Intensity

The overall power of the laser will directly affect image quality but most importantly it will have the greatest impact on the health of your sample and fluorophore(s). Increasing laser power will yield more signal but it will also induce negative photo effects such as phototoxicity and photobleaching that can harm the sample. Lasers also generate considerable heat when used at higher powers and that may have unforeseen effects on the sample.

For most lasers on this microscope, we recommend laser power settings between 1% and 5% to start. It is recommended to start as low as you can possibly go and still get an image. Then it can be increased as necessary to help balance image quality.

To discover your fluorophore(s) saturation level (i.e. how much laser power you can use before your fluorophore stops absorbing additional photons) you can start imaging at a low laser power and gradually increase the power slider until you reach a point where your image stops getting brighter. Continual increases in laser power will stop yielding more signal. That point is the practical laser power limit.



Detector Sensitivity

The sensitivity of the PMT is a function of the high voltage gain applied to it. The gain setting (as discussed elsewhere in this manual) controls the sensitivity of the detector to photons of light emitted from the sample fluorescence.

Increasing gain provides the most flexible and impactful way of improving image quality, but it has some drawbacks. The most obvious is when you increase the gain you also increase the noise in the resulting image. There are various forms of noise that we won't discuss here, but they are readily apparent when using a gain value $> 500V$.

Below 500V it is very difficult to detect much noise at faster scan speeds, however when using the detector above this value you will find that you will need to make some adjustments to reduce this.

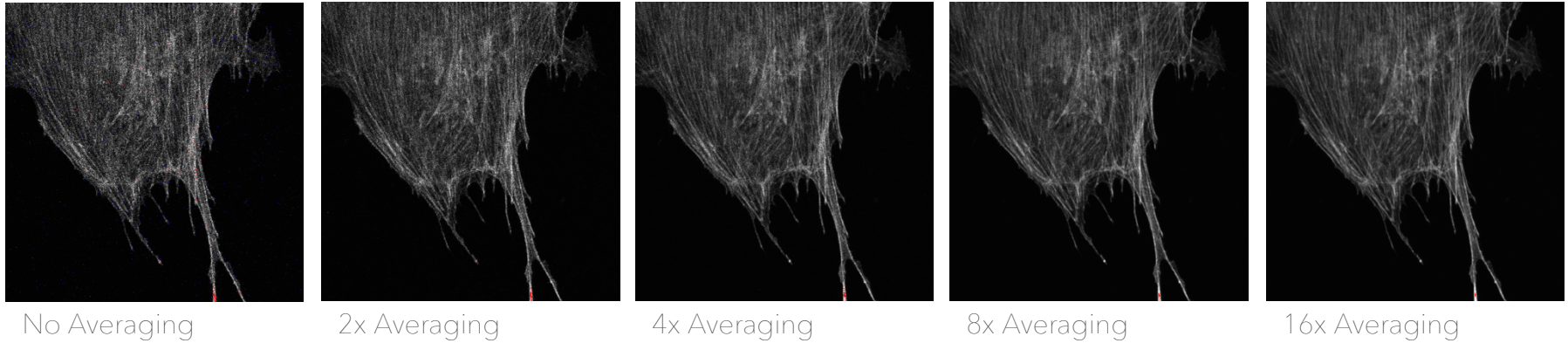
If laser power is high and the confocal aperture cannot be compromised there are a few methods to dealing with noisy signal due to high detector gain.

There are 2 main methods for dealing with high image noise. The first is reducing the scan speed to a slower rate. This will increase the pixel dwell time which will improve the signal-to-noise ratio. The second is signal averaging which will scan the same image multiple times and average the result to produce an image that has an improved signal-to-noise ratio.

Understanding Image Quality



Depending on the quality of the signal level in the final optimized image, you may find that the image is somewhat noisy. To compensate for this, the system has averaging functions in the 'Acquisition Mode' tab (page 14) that can be used to clean up the image. Simply select the rate of averaging (1, 2, 4, 8, or 16x) and rescan.



Note: Averaging signal will increase the acquisition time by the factor of averaging. For example, a 4x average will increase the time to scan the image by a factor of 4.

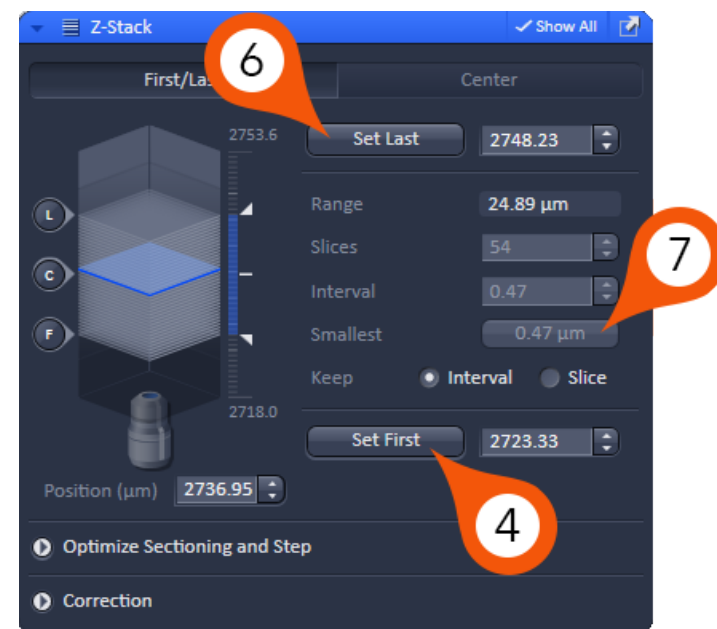
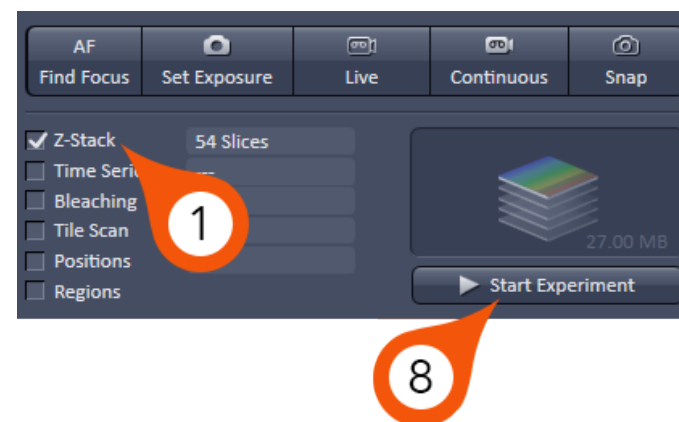
Collecting a Z-Stack



To collect serial sections (Z) we need to first activate the Z-Stack tools in ZEN which are hidden by default.

1. Check 'Z-Stack' from the experiment options. This will activate the Z-Stack dialog in the Multidimensional Acquisition settings.
2. Click 'Live' to begin scanning an actively updated image.
3. Focus the drive in one direction (does not matter which direction you start with) until the image of your sample just goes out of focus.
4. From the Z-Stack dialog, click 'Set First' to mark the start position.
5. While continuing to scan, focus the drive in the opposite direction until your sample comes back into focus, then continue focusing until it goes out of focus again. This time on the opposite side of the sample.
6. Click 'Set Last'.
7. Click 'Optimal' to set the interval size to be equal to $\frac{1}{2}$ the optical section thickness.
8. Click 'Start Experiment' to initiate your Z-Stack.

Note: The focus drive will always operate against gravity for maximum reproducibility. On an inverted microscope, if you mark the point furthest from the objective as 'First' the system will automatically start from 'Last' and go to 'First'. The opposite is true for an upright microscope.

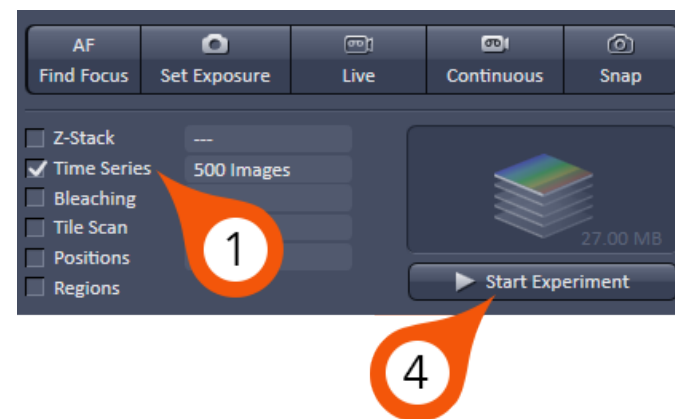


Collecting a Time Series



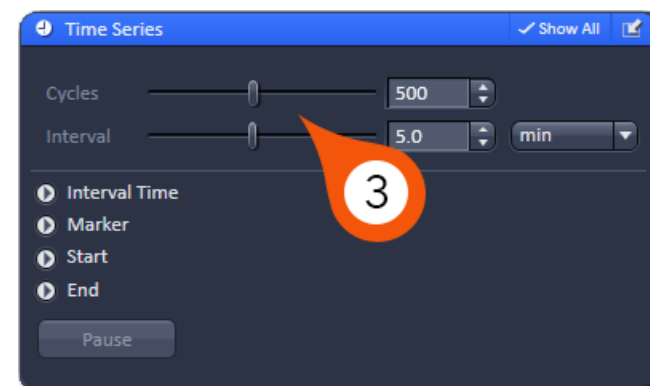
In order to collect a time-lapse (T) we need to first activate the Time Series tools in ZEN which are hidden by default.

1. Check 'Time Series' from the experiment options. This will activate the Time Series dialog in the Multidimensional Acquisition settings.
2. To calculate the required number of cycles, divide the duration you require for the series by the interval.



For example, if we wish to collect for 6 hours in total with an interval of 5 minutes between images we would use the following equation $(6 \times 60) / 5 = 72$ cycles.

3. Adjust the number of Cycles (total # of images) and the Interval (time delay between images) to match your required calculations.
4. Click 'Start Experiment' to begin the Time Series.



Collecting a Tile Scan



To collect tiled images for fields of view larger than a single frame, we need to first activate the Tile Scan tools in ZEN which are hidden by default.

1. Check 'Tile Scan' from the experiment options. This will activate the Tile Scan dialog in the Multidimensional Acquisition settings.

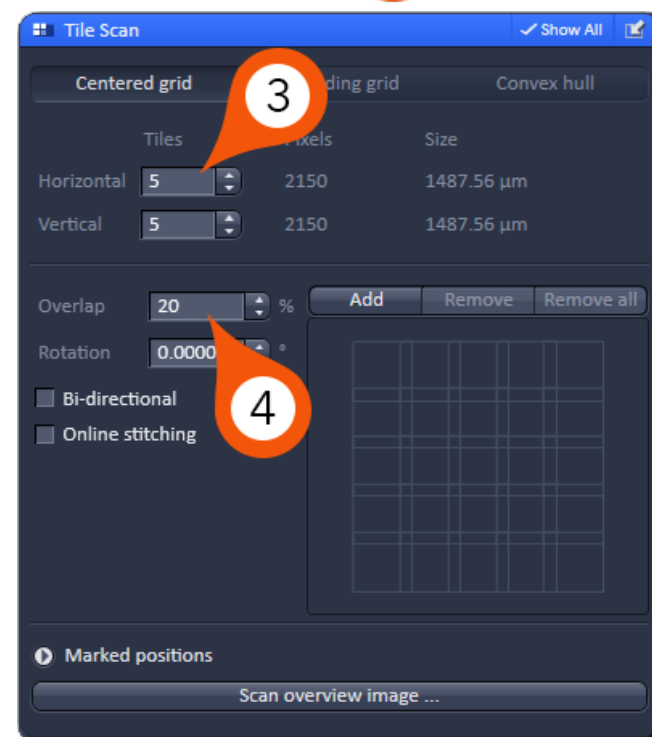
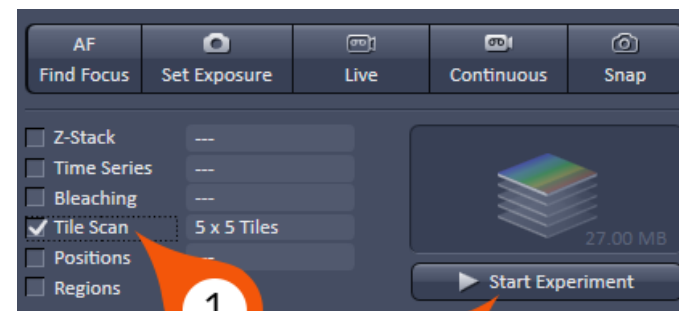
The Tile Scan dialog has 3 modes:

Centered grid - creates a tile scan around the current stage position. Here you can manually define the number of tiles in X and Y along with the Overlap % to define the tile area.

Bounding grid - creates a tile scan by defining the boundaries of the section.


Convex hull - similar to 'Bounding grid', this tool allows the user to define the boundaries of the sample, but along a contour rather than a box.

2. To collect a tile scan using 'Centered grid', position your sample so the current position is located in the center of what will be the final tile.
3. Define the total number of Horizontal and Vertical tiles.
4. Select Overlap equal to at least 10%, ideally 20%.
5. Click 'Start Experiment'.



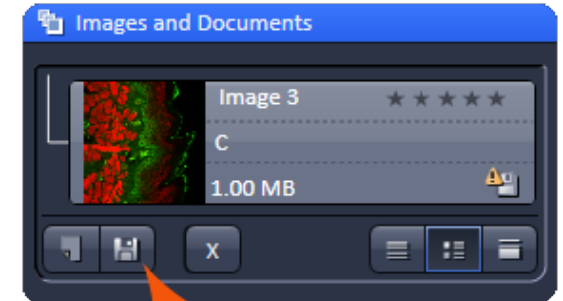
Saving Images

Once your image collection is complete you can save your data to the local hard drive. Along the right-hand side of the ZEN software (Region 3, page 6) you will find the list of images currently opened in ZEN.

1. Select the image you wish to save from the list of open images on the right-hand side of the screen.
2. Click  to save the image.
3. Navigate to the local D:\ and select the folder with your name. If there is not one please create one.
4. Choose the 'Save as Type' to 'CZI'.

Note: The default format for the ZEN system is CZI. This file format is essentially a TIFF file that contains all the requisite system information in the file header. This will allow functions such as 'Reuse' to work correctly.

5. Give your file a name and click Save.



2

Shutting Down the System



1. Close ZEN 2012 application.

Note: When prompted turn off the lasers - change the state of each laser to OFF.

2. Shut down Windows operating system.

Note: Please wait for operating system to shut down completely before proceeding to next steps.

3. Turn off X-Cite epifluorescence lamp (see Power On Routine Step 4).

4. Turn the Argon laser key from 1 to 0 (see Power On Routine Step 3).

Note: Wait 5 minutes before moving on to next steps.

5. Turn off 'Systems PC' and 'Components' power switches (see Power On Routine Step 2).

6. Turn off 'Main Switch' (see Power On Routine Step 1).