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Operation Of The Leica SP8 Multiphoton Confocal System Using Single Or Multiple Fluorochromes

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OPERATION OF THE LEICA SP8 MULTIPHOTON CONFOCAL SYSTEM USING SINGLE
OR MULTIPLE FLUOROCHROMES

by

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ABSTRACT

The goal of this thesis is to systematically examine each decision made in obtaining images on the Leica SP8 Multiphoton Confocal Microscope and discuss the basic operating parameters in order to generate publication quality images. The capabilities of the LAS X software are discussed along with an in depth discussion of each icon in order, beginning with the Configuration icon, moving then to Acquire, and a brief overview of the possible imaging processing functions within the Process icon. At the conclusion of this thesis, the reader should feel comfortable with the operation of a confocal system and understand the tradeoffs that occur between obtaining high quality images, specimen damage, and the time it takes to collect the image. Although this paper discusses the operation of the Leica SP8 Multiphoton Confocal Microscope specifically, the principles of confocal microscopy hold true regardless of the system hardware or software used.

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LIST OF ABBREVIATIONS

AOBS	Acousto-Optical Beam Splitter
AU	Airy Unit
FCS	Fluorescence Correlation Spectroscopy
IL	Incident Light
IPS	Instrument Parameter Settings
IR	Infrared
LUT	Look Up Table
LAS X	Leica Application Suite X
MP	Multiphoton
NA	Numerical Aperture
OPSL	Optically Pumped Semiconductor Lasers
PMT	Photomultiplier Tube
SNR	Signal to Noise Ratio
STED	Stimulated Emission Depletion Microscopy
TL	Trasmitted Light

CHAPTER 1

INTRODUCTION

The goal of this thesis is to systematically look at each decision made in obtaining images from a sample stained with either single or multiple fluorochromes on a Leica SP8 Multiphoton Confocal Microscope equipped with an STP8000 upright microscope. This specific system, available at the University of South Carolina School of Medicine's Instrument Research Facility, has a 10X, 20X, 25X water, 40X oil, 63X oil, and 25X Clarity objective as well as 488 and 552 nm optically pumped semiconductor lasers (OPSLs), a 638 nm diode laser, and a Coherent tunable IR laser (680 to 1080 nm) for Multiphoton imaging. While this paper details the setting of instrument parameters in order to generate an image on this specific system, the basic choices made are the same regardless of the manufacturer of the system being used and the available hardware. When operating various confocal instruments, it is often simply a matter of identifying the terminology used, for example, pinhole versus iris, the name of a specific look up table (LUT), or finding where a particular software function is hidden in the multiple menus necessary to operate the system. Once the basic principles are understood, it is possible to quickly learn to operate any confocal microscope and to obtain publication quality images (Price and Jerome, 2011).

The goal of this paper is to cover the basics of image collection and the compromises made to improve image quality, while minimizing specimen damage that results from laser exposure. Primary components of the hardware and software system and their effect on image quality and specimen damage will also be discussed.

Figure 1.1 shows the layout of the Leica SP8 system and the flowchart below illustrates the procedure for starting the instrument and opening an imaging session. It should be noted that for ease of operation there is considerable redundancy within the operating system. For example, the lasers can be started and operated from several different windows and the objectives can be changed from several locations within the software. In some cases these points of redundancy are discussed, while in other cases, only the most common method for performing a function is discussed.

Flowchart for starting an imaging session on the Leica SP8 Multiphoton system

1. If the IR laser will be used during the imaging session verify the coolant is $\sim 20^{\circ}\text{C}$; if greater than 25°C call for maintenance (Fig 1.2). If coolant is operating appropriately change the IR laser from “Standby” to “On”
2. Most ancillary equipment such as the halogen bulb, microscope, computer etc are started through a common power strip(s)
3. Using the toggle switches turn on the scanner and laser power and turn the key to “on” to allow laser entry into the system (Fig 1.3). Note that this laser control box is separate from and has no effect on operation of the IR laser.
4. When appropriately logged into the system start the LASX confocal microscope operating software
5. Select “Machine” from the Configuration drop down menu, and either “machine_MP_ON” for multiphoton imaging or “machine_MP_OFF” for imaging with only the OPAL or Diode lasers. (Fig 1.4)

6. If a tiling experiment will be performed make sure the condenser lens is protected by lowering it to the lowest position. This prevents the stage from striking the edge of the condenser when it is initialized. Stage initialization is only required when tiling with a XY motorized stage. If tiling is not required, select “No.” (Fig 1.5)
7. The LASX software will open in the “Acquire” menu (Figure 1.6). Visible lasers can be turned on in this window by clicking on the “+” which will display the available lasers (Figure 1.7). Each laser can then be activated and the percent transmission set with the vertical slider. Note that this system has 488nm, 552nm and 638 nm lasers. Lasers may also be activated through the “Configuration” window as discussed below. Only the lasers needed should be activated to preserve hours on the unused lasers.
8. Alternatively, in the “Acquire” window, a saved project can be selected. The header information in the saved file contains all settings including laser and detector settings, scanning parameters such as speed, resolution, bit depth etc and the collection parameters such as pinhole diameter, and Z-series parameters.
9. Obtain a wide-field fluorescence image by setting up the light microscope operating parameters.

1.2 The Leica SP8 Smart Control and Smart Touch Panels

As previously mentioned, there are often several ways to change the setting of an operating parameter in the SP8 software. An additional feature of the Leica SP8 Multiphoton system is the Smart Control Panel (Fig 1.8). This control panel allows increased ease of use when adjusting the detector Gain and Offset, Field Rotation, Pinhole Diameter, Image Zoom, and Specimen Z position with control knobs, rather than setting each parameter within the software.

Another supplementary feature of the STP8000 upright microscope is the Smart Touch Panel (Fig 1.9) which provides controls of all of the microscope parameters. The

X, Y, and Z positions of the microscope stage can be adjusted with the control knobs on the right side of the panel. Icons located on the left side of the touch pad window provide separate controls for the operation of the microscope platform. For example, when the microscope icon on the top left of the window is selected (Fig 1.11) adjustments in light intensity, condenser aperture, and field of view can be made. The shutter can also be opened or closed as needed. The color wheel (Fig 1.12) allows for viewing of the specimen in brightfield (BF), fluorescence (FLUO), or a combination of the two (CS) modes. The TL shutter is used for imaging in the TL mode and the IL shutter is used for imaging in the fluorescence mode. The objective icon (Fig 1.13) allows for the selection of available objectives on the system by simply touching the desired objective. While it is still possible to manually change the objectives, by doing so on the Smart Touch Panel there is an increase ease of use as well as convenience. The X, Y, and Z icon provides stored positions for the objective focus (X), and the X, Y stage position. Coarse and fine adjustments of the manual controls on the right of the unit can also be set in this window (Fig 1.14). The two bottom icons (Figs 1.14 and 1.15) provide settings for each of the black buttons on the bottom of the unit including language of use, opening of specific shutters etc. for ease of use. These are adjustable settings and will not be used often, if at all, by the everyday user. One such setting icon can be seen in Fig 1.15. The red button on the bottom provides information for setting function key options.

1.3 Test Specimens and System Performance

As discussed in his article “The 39 Steps: A Cautionary Tale of Quantitative 3-D Fluorescence Microscopy,” Pawley posed the question, does a fluorescent micrograph reveal the actual location and number of labeled molecules in a cell or tissue, to members

of his well-known confocal microscopy workshop. Based on the several factors that can affect the numerical values stored in a computer that represent a fluorescent micrograph the conclusion was that all we “can really be sure of measuring with most confocal microscopes in the fluorescence mode is some feature of the number of photons collected at a particular time” (Pawley, 2000).

In this thesis, the setup of microscope operating parameters for image acquisition and how each affects the final image, is discussed. Primary considerations when determining the setting of each of the adjustable parameters on an instrument include X, Y, and Z resolution, signal to noise ratio (SNR), appropriate spectral resolution so no bleed through or spectral overlap exist, specimen damage, bit depth, and file size. Even with all of the variables present while imaging with a confocal microscope, it is possible for an investigator to obtain images on confocal instruments that are superior to wide field fluorescent images with relatively little training. However, minimally trained investigators seldom take advantage of the superior imaging capabilities of confocal microscopes and rarely do they realize the compromises (Table 1.1) required to minimize specimen damage while optimizing image quality when each instrument setting is altered. Improper setup of the system may also result in the collection of data artifacts that may not be recognized. It is only through a thorough understanding of the operation of each component of the confocal system that informed decisions can be made and optimal images obtained on a routine basis (Price, 2011).

Zucker and Price (1999) published an excellent article, “Practical Confocal Microscopy and the Evaluation of System Performance,” on methods of instrument maintenance and quality control to ensure an instrument is operating at optimal

specifications. While these topics are important and it is essential that attention is paid to these details, extensive discussions concerning instrument maintenance and specifications will not be discussed here. Rather, this discussion focuses on the proper setup of an optimally functioning instrument for everyday imaging. Inherent in these discussions is the assumption that a core director or other personnel extensively trained in instrument maintenance are routinely checking and correcting instrument specifications and alignment (Zucker).

1.4 Definition of a Good Confocal Image

Even though a number of operating parameters on a confocal microscope can be adjusted, the goal should always be to set these operating parameters so that the digital image which is collected accurately represents the image as seen through the microscope (Price, 2011). A large number of operator decisions and compromises affect image resolution, SNR, and qualitative and quantitative assessment of colocalization and structural information in a confocal image. Before discussing the changes that can be made in various operating parameters that affect specimen damage and image quality, reviewing the definition of a good confocal digital image is essential. An ideal confocal image is one that has appropriate resolution, a good SNR so that little or no noise is apparent, good balance between contrast and brightness, and use of the full dynamic range of the available pixel values. For an 8-bit image, this would represent pixel values between 0 and 255 and for a 12-bit image pixel values between 0 and 4,095. It may be desirable to leave 5-10 pixel values open on each end of the range for the application of histogram stretch functions for contrast enhancement. However, if any type of

quantitative or semi quantitative imaging is the goal of the study analysis must be performed prior to the histogram stretch operation (Price, 2011).

With the large number of specimen, hardware, software, and user definable factors that determine the quality of a confocal micrograph, it is essential that a mechanism be in place to ensure routine collection of quality images that include all available data without the introduction of artifacts. Within the confocal software LUTs are available that take advantage of the ability of the human eye to detect colors more efficiently than gray tones. These LUTs set pixel values of zero to a specific color, typically blue or green, and pixel values of 255 in an 8-bit image to a contrasting color, such as red. All other pixel values are a shade of gray. This allows the operator to quickly determine that the full range of pixel values is being used and that no detail is being lost on low or high ends of the image histogram. (Price, 2011) .The histogram function specific to the Leica SP8 LAS X software is further discussed in Chapter 3.

Table 1.1 Advantages, disadvantages, and imaging compromises associated with the adjustment of confocal microscope operating parameters.

Parameter	Advantage	Disadvantage
Increased laser output	Improved S/N ratio	Increased specimen damage
Decreased scan speed	Improved S/N ratio	Increased scan time and specimen damage
Line or frame averaging	Improved S/N ratio	Increased scan time and specimen damage
Increased pinhole size	Improved S/N ratio	Decreased contrast and resolution in x, y, and z
Increased number of pixels (smaller pixel size)	Improved resolution	Increased scan time; large file size
Increased amplifier gain	Fewer photons required	Poor S/N ratio
Increased amplifier offset	Improved gamma	Decreased contrast
Narrow band pass filter	Minimal bleedthrough	Increased scan speed time and specimen damage
Sequential imaging	Eliminates bleedthrough	Increased scan time and specimen damage
Simultaneous imaging	Decreased scan time and specimen damage	Increased bleedthrough of fluorochromes with overlapping emission spectra
Long wavelength fluorochrome	Improved depth of imaging	Decreased resolution
Short wavelength fluorochrome	Improved resolution	Decreased depth of imaging

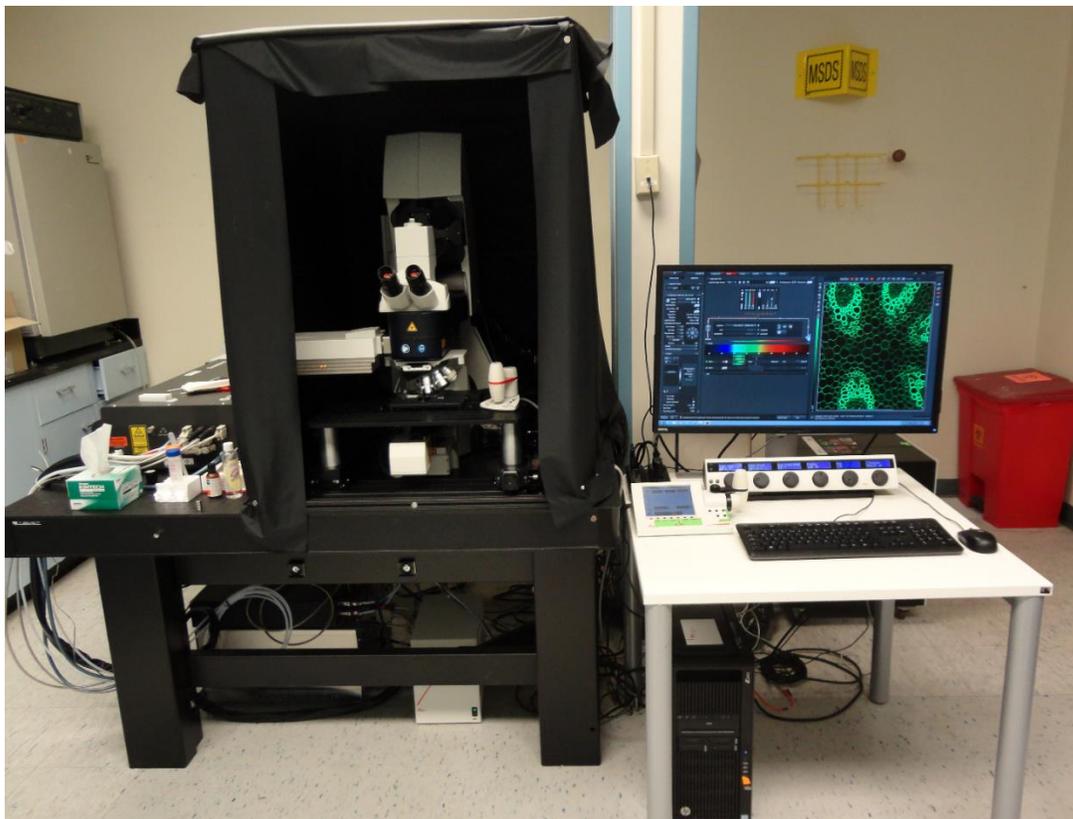


Figure 1.1 Overview of the Leica SP8 Confocal Microscope System. This system consists of (from left to right) a STP8000 Upright Microscope, a Smart Touch Panel, a Smart Control Panel, and the computer/monitor system. Although out of view from this image, the system also includes a condenser, a control panel to turn on the scanner and laser power, and a control box to turn on the Multiphoton Coherent Tunable IR laser.



Figure 1.2 Coolant should be verified and read no greater than 20.00° C before operation of the system.



Figure 1.3 Scanner and Laser power can be turned on here. The key should be switched to “on” to allow laser access.

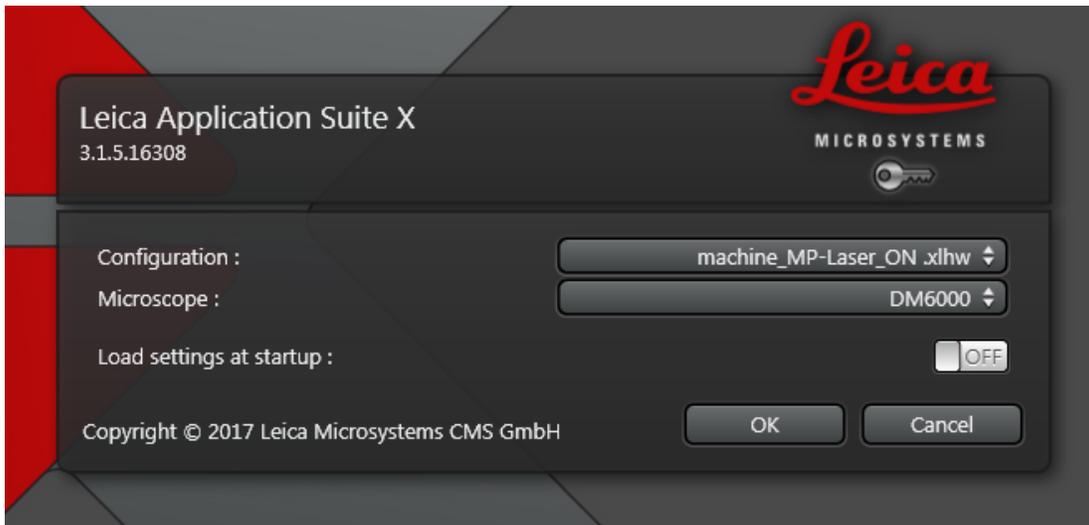


Figure 1.4 Upon startup of the LAS X program, two pop up screens will prompt the user to set up various aspects of the Leica SP8 system. The first of these calls for the setup of the Configuration and Microscope. Under the Configuration drop down, either “machine_MP-Laser_ON” or “machine_MP-Laser_OFF” should be selected based on if Multiphoton imaging will be used for image generation. The Microscope will always be set to DM6000.



Figure 1.5 Upon startup of the LAS X software, two pop up screens will prompt the user to set up various aspects of the Leica SP8 system. The second of these asks if the stage should be initialized. The stage only needs to be initialized if a tiling experiment will take place. Otherwise, “No” can be selected from this window.

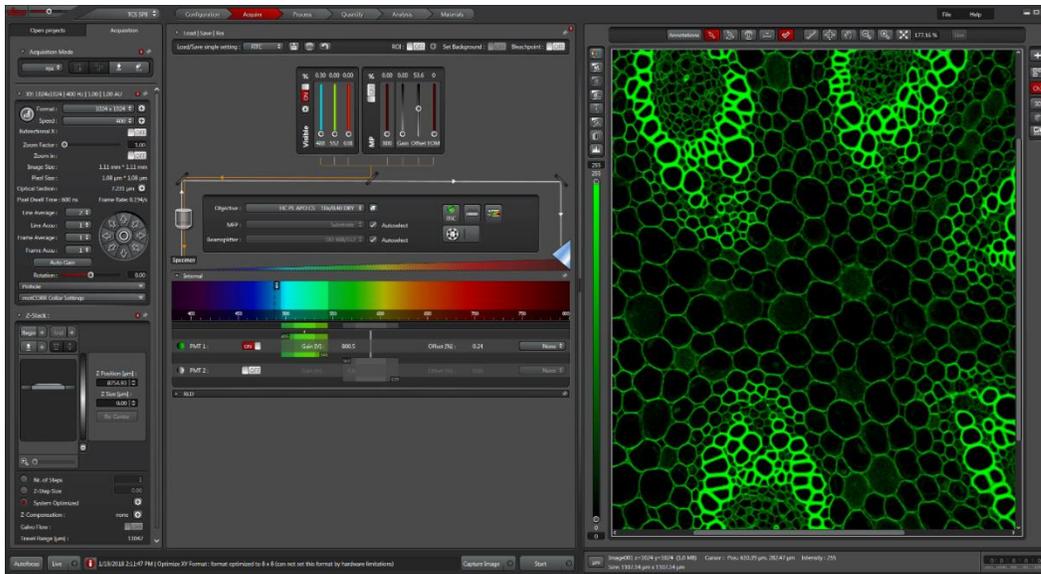


Figure 1.6 Opening screen of the LAS X software. From this window it is possible to access the various icons in the system (left to right): Configuration, Acquire (the icon being shown), Process, Quantify, Analysis, and Materials.



Figure 1.7 Lasers can be turned on under the LAS X Acquire screen in the center panel (the Dye Database Panel). By selecting the “+” as indicated by the red arrow the currently available lasers can be seen and turned on. Only the lasers that will excite the fluorophore should be turned on as to preserve laser life of lasers not in use. There is redundancy in the system so it should be noted this is not the only screen the lasers can be turned on from.

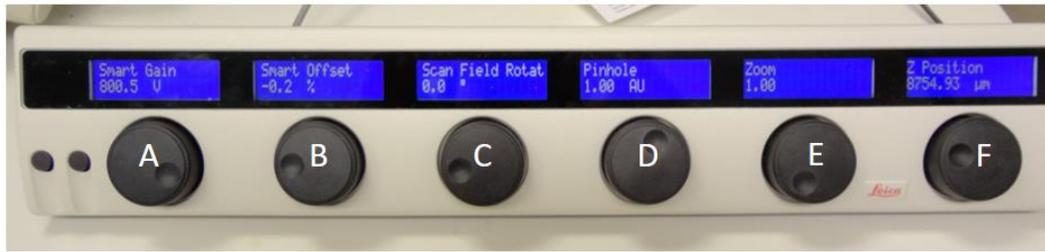


Figure 1.8 Leica Smart Control Panel provides easy access to (A) Smart Gain, (B) Smart Offset, (C) Scan Field Rotation, (D) Pinhole, (E) Zoom, and (F) Z position. These functions can also be adjusted in the LAS X software, but this control panel makes adjustments easier.



Figure 1.9 Leica Smart Touch Panel overview. This panel provides access to various icons including the a) Microscope icon, b) Color Wheel icon, c) Objective icon, d) X, Y, and Z Stage icon, and e) two system setting icons. The arrows indicate knobs that allow the user to move the stage in the X and Y direction, as well as in the Z direction.

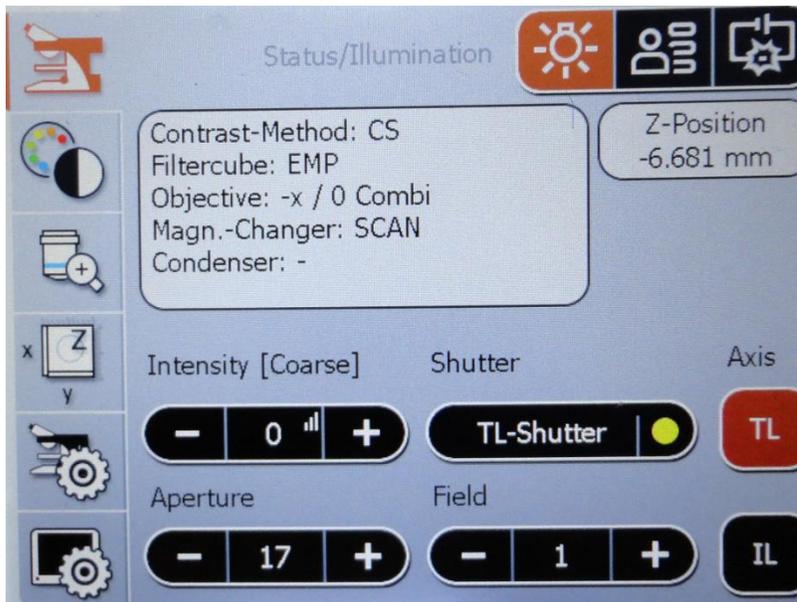


Figure 1.10 Leica Smart Touch Panel overview. Under this panel, the intensity, aperture and field can be adjusted. The shutter (either TL or IL) can be turned on.



Figure 1.11 Leica Smart Touch Panel Color Wheel icon overview. Under this icon, Brightfield (BF), Fluorescent (FLUO) or a combination of the two (CS) can be selected. The IL and TL shutters can be turned on and off by selecting the corresponding icon and various filters can be applied.

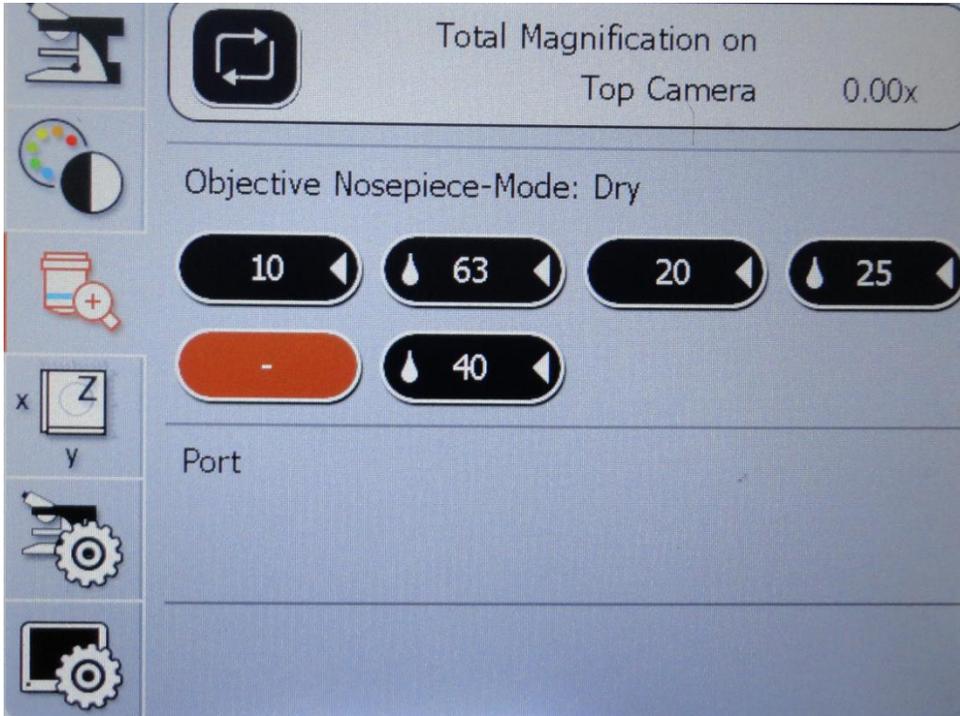


Figure 1.12 Leica Smart Touch Panel Objective icon overview. The objectives available on this system, that can be selected from this screen, are the 10X, 20X, 25X Water, 40X oil, 63X oil, and a 25X Clarity objective.

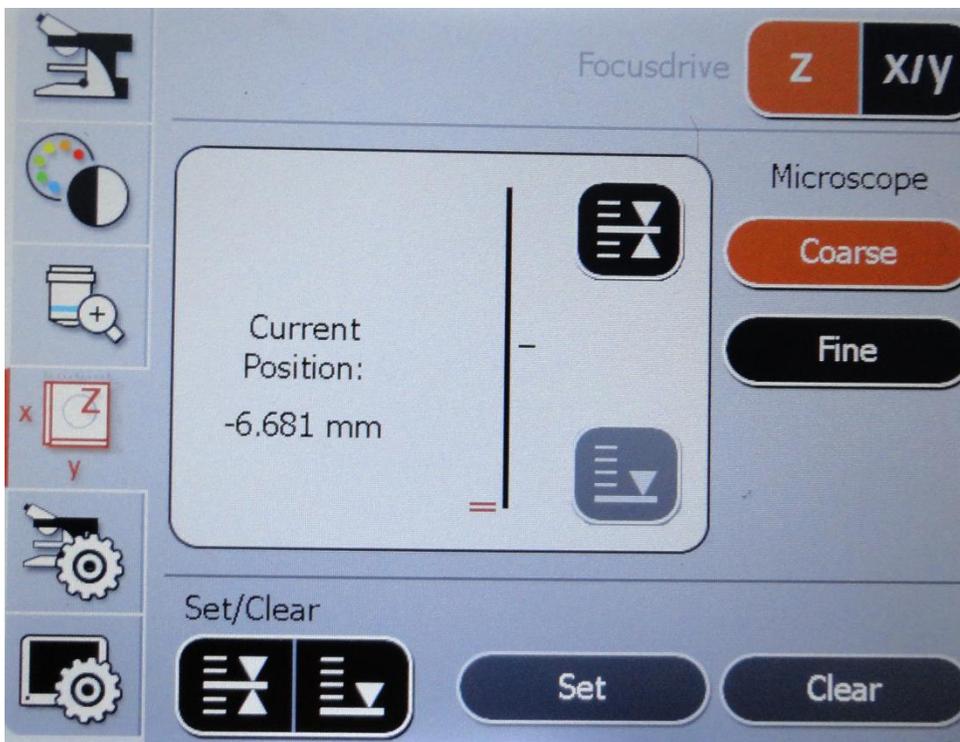


Figure 1.13 Leica Smart Touch Panel X, Y, and Z icon overview. The current stage position can be viewed here and set with the X, Y, and Z knobs from Fig 1.9.

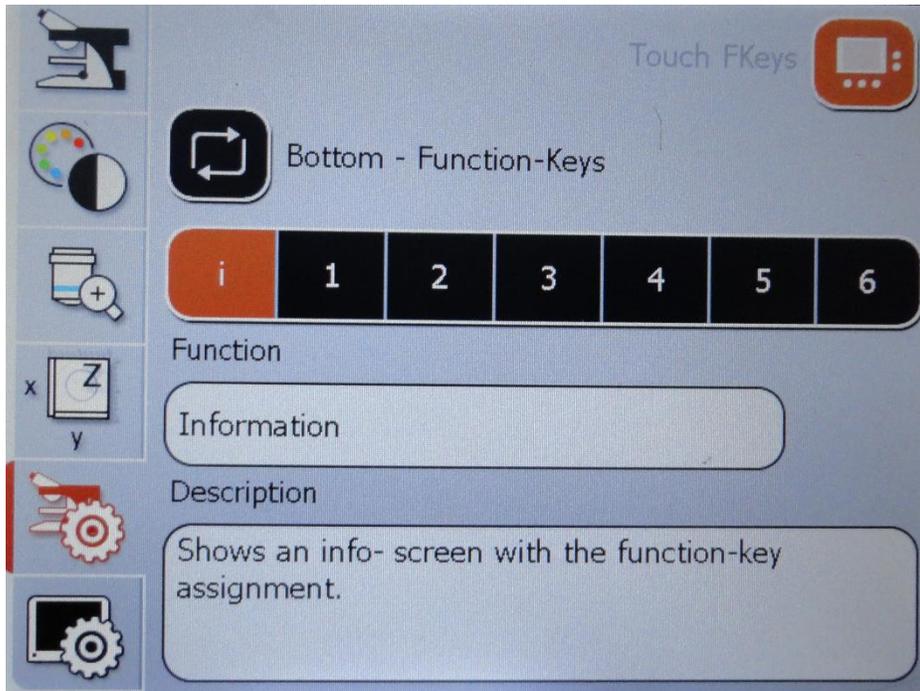


Figure 1.14 Leica Smart Touch Panel Microscope Setting icon overview. This icon will rarely, if ever, be used by anyone other than the manager of the system.



Figure 1.15 Leica Smart Touch Panel System Setting icon overview. This icon will rarely, if ever, be used by anyone other than the manager of the system.

CHAPTER 2

CONFIGURATION

2.1 The Opening Screen

An often daunting task for many inexperienced confocal microscopists, as well as those with considerable experience, is navigating the extensive software necessary to operate a confocal microscope. This task is often made more intimidating because optional functions that are not available on a system are still present in the software creating a need for additional pages and icons. Also, there is often extensive redundancy, as previously discussed. This redundancy that seeks to make the use of a system easier, may make the initial use appear more complicated than it actually is. The goal of this chapter is to discuss the main software applications for the SP8 and to discuss the primary functions necessary for basic operation of the instrument.

Figure 2.1 shows the top portion of the opening screen of the LAS X software, from which it is possible to access multiple menus for operation of the system and for data processing. For example, with the TCS SP8 dropdown menu, options such as Live Data Mode, Electrophysiology, FRAP and FRET functions, and HyVolution super resolution software, if available, can be accessed. Since the goal of this paper is to discuss the basics of confocal microscope operation, these advanced imaging functions will not be discussed. The Configuration tab, discussed in detail within this chapter, provides

several separate windows for setting user profiles including laser settings, choices of fluorochromes, objective choices, etc. The Acquire icon, discussed in Chapter 3, provides access to all functions necessary for image generation. The Process, Quantify, Analysis, and Materials icons are all image processing functions that go beyond the image acquisition scope of this thesis and will therefore not be discussed in detail, if at all.

2.2 The Configuration Screen

Selection of the Configuration button (Figure 2.2) provides access to several icons explained in detail below. As noted above, there is redundancy in the software and many operating parameters that can be set in the Configuration menu can also be accessed, set, and adjusted in the Acquire menu. Areas of redundancy will be mentioned in both sections since they often provide user friendly access to various imaging functions. Several icons such as USB, Hardware, and Memory pages are specific to the instrument set up and are not used in routine confocal imaging. These icons are infrequently accessed by individual investigators and therefore, are not discussed here.

2.2.1 Stage Configuration

The Stage Configuration icon (Figure 2.3) adjusts the settings for the motorized XY stage, if available, and should only be adjusted by the manager of the system. Adjustment of the stage settings affects image orientation during tiling and stitching experiments and if not adjusted correctly the final stitched image may have overlap artifacts present. If a tiling experiment will not be performed then it is not necessary to configure the stage during instrument set up.

2.2.2 Instrument Parameter Settings (IPS)

The IPS icon (Figure 2.4) provides access to specific software masks that can be seen in the Acquire window and saved with a file, if desired. These are typically set upon instrument installation by the instrument manufacturer, in this case, Leica, but can be adjusted depending on the file parameters an individual user may need. Typically, most users will use the default settings.

2.2.3 Beam Path

The Beam Path icon, specifically the Fluorifier Disc Settings (Figure 2.5), are used for the setting of barrier or notch filters for use with stimulated emission depletion microscopy (STED), fluorescence correlation spectroscopy (FCS), or white tunable lasers. Since this specific SP8 system has none of these functions there are no available filters seen in Figure 2.5. However, it is possible to image polarized light with this system and at the bottom of the screen the polarized light settings, if used in an experiment, can be adjusted by using the slide bar.

2.2.4 Laser Configuration

Activation of the lasers is a primary example of software redundancy. Lasers can be turned on in the Configuration menu as shown here (Figure 2.6) and also in the Acquire menu as discussed in the next chapter. By selecting the Laser Configuration icon (Figure 2.6), it is possible to access the on/off controls for the OPSL 488, OPSL 552, and the Diode 638 lasers available on this instrument. Awareness of the lasers present on an instrument is important prior to designing an experiment. This prevents designing an experiment and purchasing fluorochromes only to realize that an optimum laser is not

available for the specific fluorochromes used. This should become less of a problem as the availability of tunable Multiphoton/IR, and white light lasers increases, but nonetheless it is always wise to know the system hardware prior to the design of an experiment.

While the MP laser can be turned on in the Configuration window, the MP laser shutter can only be accessed under the Acquire menu. In practice, only the lasers needed for a specific operating session should be turned on in order to save life time on the lasers not needed for a specific experiment.

2.2.5. USB Control Panel

The USB Control Panel icon (Figure 2.7) provides adjustment controls for the functions on the Leica Smart Control Panel shown in Figure 1.8. Each knob is assigned a parameter by selecting a function from the drop down menu directly above each knob. The sensitivity of each turn is then determined by another drop down menu only this time it is directly below each knob. For example, the second knob controls the Smart Offset and is set to increase or decrease in 1% increments when turned to the right or left. As previously mentioned, it is often advisable to allow access to these settings only by the manager of the equipment.

2.2.6 Objective Configuration

The Objective Configuration icon lists the specific objectives and their specifications available on the system. As seen in Figure 2.8 the objectives available on this system are a 10X/0.40 DRY, 63X/1.40 OIL, 20x/0.75 DRY, 25X/0.95 WATER, and a 40x/1.10 WATER. The Clarity objective available on this SP8 is available through a

separate nosepiece (not shown here). By selecting each objective, the attributes including type, magnification, numerical aperture, immersion type, and resolution can be seen. For example, the 25X water objective is selected here, and each of its attributes can be seen in the box below.

2.2.7 Hardware Settings

Hardware Settings (Figure 2.9) provide access to many of the functions active during collection of an image such as line averaging during live acquisition, image orientation, which detector combinations are used, and bit depth of the image (see Section 6.4). While many of these settings as shown here are default settings established when the instrument is installed, the advanced user may make changes in the way live images are collected and data transferred, should they choose to. However, prior to making changes it is wise to understand the ramifications of each change (Table 1.1) and to always reset the operating parameters to the default settings for ease of use by less experienced investigators.

2.2.8 User Configuration and Memory Management

As with many functions in the Configuration menu the User Configuration (Figure 2.10) icon is best used only by experienced investigators and then reset to the system defined (default) functions following an operating session. Functions in this window, such as using the last system settings and working in folders or projects when storing data, are self-explanatory. However, for those less familiar with these software pages, selection of various icons can drastically affect the appearance of the viewing window, how data is stored, and even the unit of measurement. To avoid potential

confusion it is best to use the default functions. The User Configuration screen is also accessible through the “Help” button on the upper right side of the screen under “Options” on the dropdown menu.

The Memory Management icon is a standard Browser for setting the storage tree for where data sets will automatically be saved and an indication of available memory can be accessed.

2.2.9 Dye Database

The Dye Database (Figure 2.11) provides a list of excitation and emission spectra for many fluorochromes that can be imaged with the SP8. By selecting a fluorochrome from the pull down menu the absorbance (excitation) (red) and emission (white) curves and the peak values are shown. For example in Figure 2.11 Alexa 532 is selected showing the maximum excitation wavelength is 534 nm and emission wavelength 553 nm. Excitation and emission curves are also shown which is important when selecting fluorochromes to minimize bleed through. While data for specific fluorochromes are available in the Configuration window there is redundancy in the Acquire menu where the actual protocol for imaging the fluorochrome is set up by use of the Dye Assistant. This will be discussed in Chapter 3.

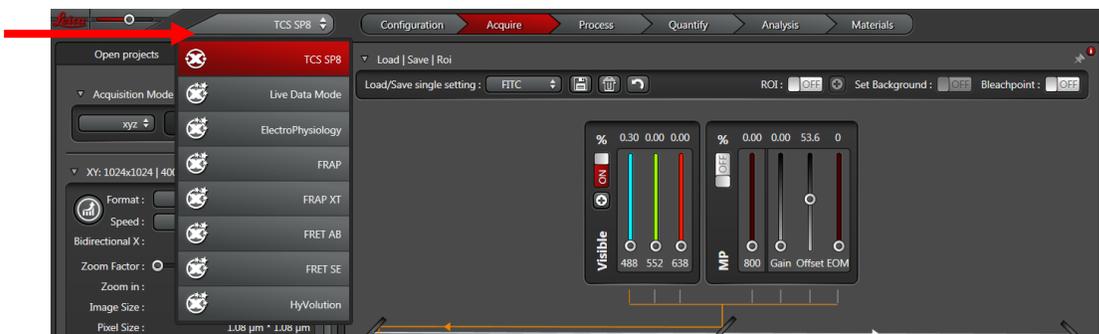


Figure 2.1 Top portion of the opening screen. As seen in the dropdown menu various functions can be selected including Live Data Mode, ElectroPhysiology, FRAP, and FRET. It is also possible to go between the various screens available in the LAS X software including (left to right): Configuration, Acquire, Process, Quantify, Analysis, and Materials. These are indicated by the red arrow.

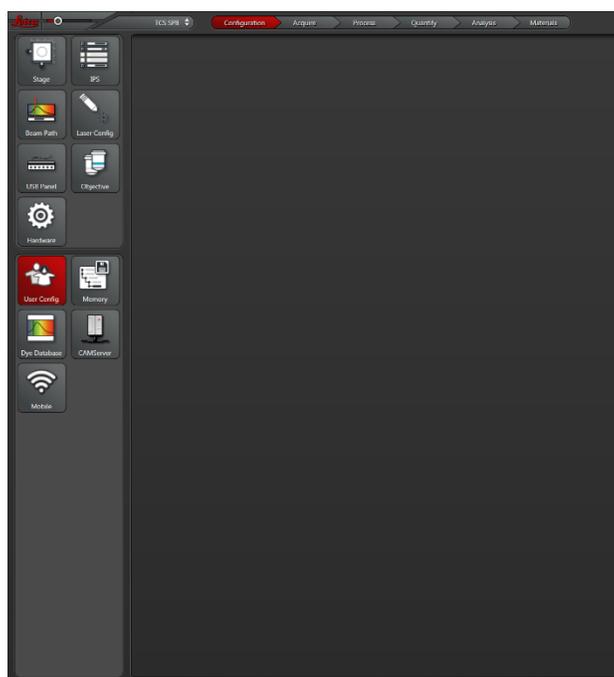


Figure 2.2 Overview of the LAS X Configuration Window. From this screen the following icons can be selected (top to bottom; left to right): Stage, IPS, Beam Path, Laser Configuration, USB Panel, Objective, Hardware, User Configurations, Memory Management, Dye Database, CAMServer, and Mobile. The most commonly accessed icons by the everyday user are the Laser Configuration and the Dye Database.

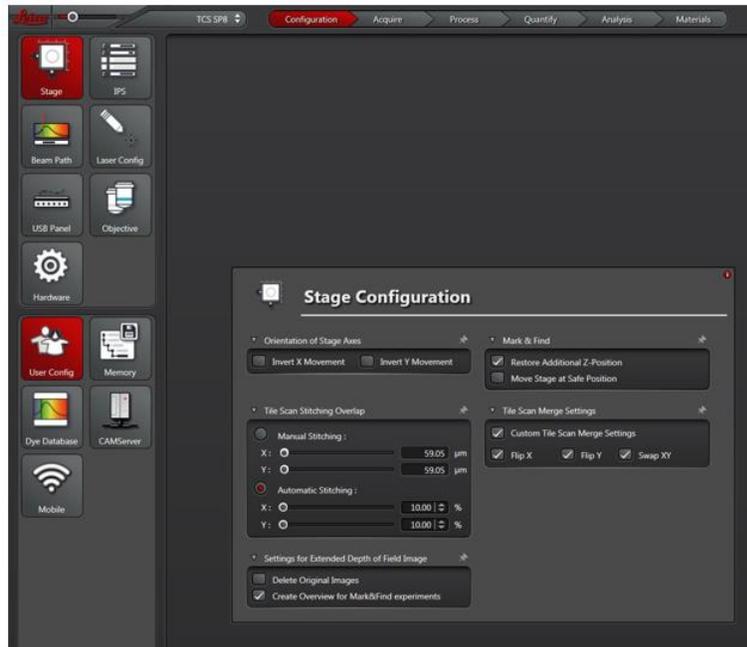


Figure 2.3 LAS X Overview of the Stage Configuration icon. Here settings for the motorized stage can be changed, which affects the image orientation during tiling and stitching experiments. These settings should only be changed by the manager of the system.



Figure 2.4 LAS X Overview of the Instrument Parameter Settings. This screen provides access to specific software masks that are typically set upon instrument installation.

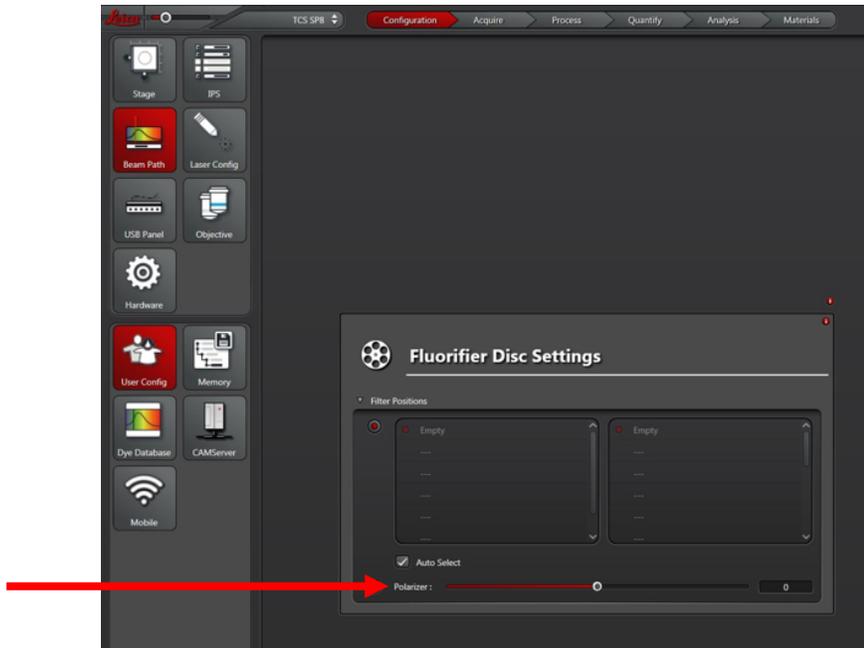


Figure 2.5 LAS X Overview of the Beam Path icon. The Fluorifier Disc Settings are used for setting barrier or notch filters for STED, FCS, or white tunable lasers. This specific system has none of these functions. However the slide bar, as indicated by the arrow, can adjust the amount of polarized light.

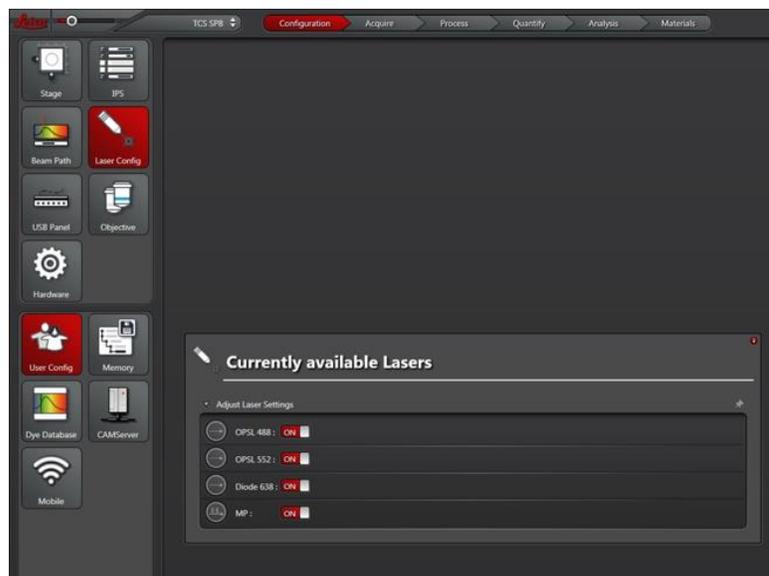


Figure 2.6 LAS X Overview of the Laser Configuration icon. The available lasers: OPSL 488, OPSL 552, Diode 638 and the Multiphoton Coherent Tunable IR Laser can be turned on/off here. Only the lasers that excite the specific fluorophore should be turned on to preserve laser life of those not in use.



Figure 2.7 LAS X Overview of the USB Control Panel Icon. Here the functions on the Leica Smart Control Panel can be adjusted. Each knob is assigned a parameter by selecting from the top drop down menu and the sensitivity of each turn is set with the bottom dropdown menu.



Figure 2.8 LAS X Overview of the Objective Configuration icon. The objectives available are the 10X, 20X, 25X water, 40x oil, 63X oil, and a 25X Clarity objective that is installed on a need-be basis. In this figure the 25X Objective is selected and the corresponding specifications can be seen.

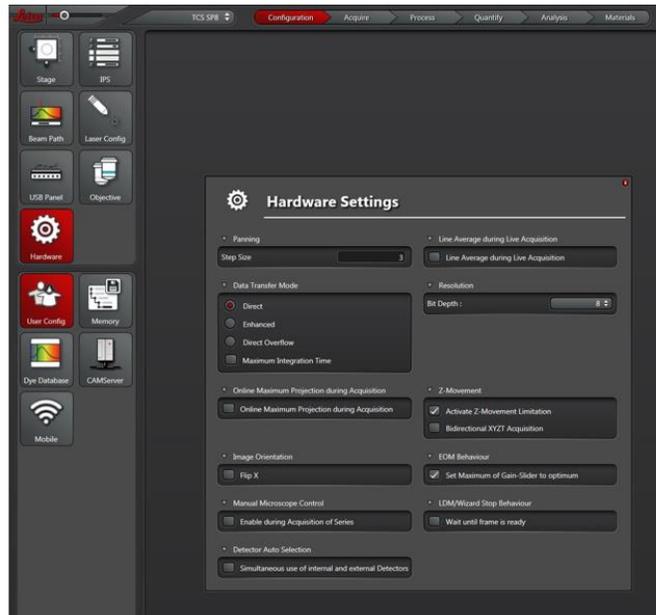


Figure 2.9 LAS X Overview Hardware Settings. This icon provides access to functions active during the collection of an image including line averaging during live acquisition, image orientation, which detector combinations are used and bit depth. This will primarily be used by the manager of the system.



Figure 2.10 LAS X Overview of the User Configuration Icon. Under this window, the last system settings and various system defined functions can be accessed. This icon is primarily used by the manager of the system.

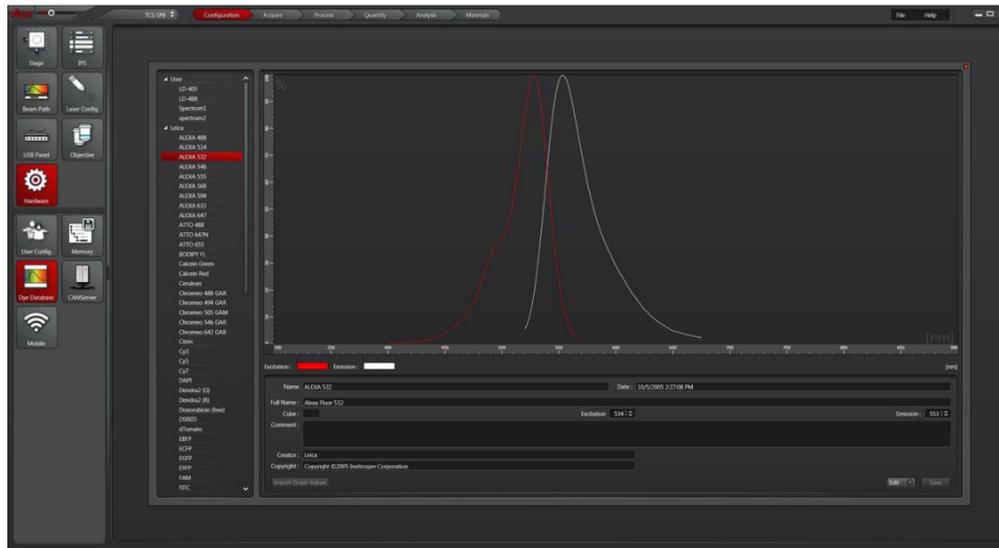


Figure 2.11 LAS X Overview of the Dye Database Icon with Alexa 532 nm selected. The emission and excitation wavelengths of each available fluorochrome can be viewed here. It is important to be aware of these wavelengths when mimizing crosstalk and bleedthrough.

CHAPTER 3

ACQUIRE

3.1 The Acquire Menu

Most user interaction with the instrument software will occur under the Acquire window. In this window, sample fluorochrome excitation and emission spectra are used to establish laser and AOBS settings, pinhole and detector settings, scan speed, z-series and tiling considerations, and acquisition parameters. At this point in the decision-making process it is advantageous to collect images with knowledge concerning the end point of the experiment and how the data will be presented. It will save considerable time and effort if high quality images using the full dynamic range of the microscope are collected as opposed to working in Photoshop, Image J, or other post acquisition image enhancement programs (Price, 2011).

If decisions are not possible concerning the end point of the experiment at the time of image collection, then a general rule of thumb is to collect big and work small. Once an image is collected, it is not possible to improve the resolution or increase the bit-depth. However, if images are collected at high resolution and bit depth, it is possible to selectively reduce the data while working with images in enhancement and analysis programs. It is important that the original files be archived to ensure no significant loss of information occurs (Price, 2011).

Under the Acquire window, settings for components in the optical path, including laser and detector settings, are user definable and can be easily stored for future use. This provides quick access to established protocols for commonly used fluorochromes. Individualized protocols for imaging can also be written, stored, and accessed. A precaution here is to realize that since no two specimens are identical, fine adjustments in settings are usually required. Caution must also be used in comparison of images collected during different operating sessions as laser conditions and other operating parameters can vary over time as components in the system age or are replaced. However, established protocols are very useful in rapidly establishing instrument settings to obtain an image when starting a session.

Figure 3.1 shows an overview image of the Acquire window in the Leica LAS X software. The left panel provides access to setting necessary specimen scanning parameters, the center panel access to laser and detector settings with regard to wavelengths, and the right panel shows the collected images (in this case *Convallaria*) and image annotation functions. The subsequent sections discuss relevant parameters in these three windows.

Along the bottom of the Acquire window are “Autofocus,” “Live,” “Capture Image,” and “Start” buttons. The “Autofocus” button is useful in obtaining a ballpark focused image but many experienced users avoid “auto” functions and prefer setting operating parameters based on experience rather than allowing the instrument to do so. The “Live” button will start a continuous scan for viewing an image in the right panel while establishing operating parameters to obtain a good SNR image. The “Capture Image” button will scan only the channel that is currently active. The “Start” button

should be used to collect the final image that will be saved. To begin collection of multi-channel images, Z-series, or tiled images, “Start” must be selected.

3.1.1 Acquire Window Left Panel: Open Projects

The Open Projects icon (Fig. 3.2) provides access to the various drives and files present in the system. The right icons in the Open Project window allows opening, saving and browsing of files. From the left the icons provide access to the Explorer folder tree, Open New Projects, Open an Existing Project, Save All, Apply, Browse, and Gallery View. When a file is open and the Apply icon is selected it is possible to reuse the parameters set in the data collection of the image displayed in the right window on the monitor (not shown here).

3.1.2 Acquire Window Acquisition Mode: Resolution

As shown in the overview image of the Acquire window (Figure 3.1) several image acquisition functions can be accessed through separate regions of the Acquisition Mode panel (Fig 3.1 panel A). These include various scanning methods such as the default XYZ scan mode shown here, XYt for time mode scanning, and XY λ mode for spectral imaging. Each of the XYZ Acquisition functions present on the SP8 system shown here will be described in the subsequent paragraphs. The XYt and XY λ modes include more advanced techniques and will not be discussed.

Image Format defines the resolution and shape of the image that will be collected by determining the number of pixels present in a set frame size. By selecting the Format dropdown menu a range of pre-set format sizes can be chosen (Fig. 3.3 left panel). It is often advantageous to select a rectangular format such as 1024 X 256 for framing a

region of interest. If the desired format is not listed, the “+” arrow to the right allows for custom selection (Fig. 3.3 right panel). As an example, Figure 3.4 A (left) shows an image format of 2488 pixels X 2488 pixels that would represent a square scanning format with an image size of 175.74 μ m X 175.74 μ m and XY pixel dimensions of 70.66nm X 70.66nm. In Figure 3.4 B (right) the format has been changed to a higher resolution of 4472 X 4472. While the image size remains the same the number of pixels has doubled and XY pixel dimensions are now 39.31nm.

Frame size and the number of pixels per frame in the X and Y directions are selected by default based on the objective lens selected, but can be manually changed. With respect to confocal imaging several choices must be made in the selection of the appropriate resolution to minimize specimen damage and optimize image quality. The Leica SP8 confocal provides several choices of pixel resolution that range from low resolution images that include relatively few pixels (128X128) to high resolution images (8192X8192). The effect of pixel size on image quality can be seen in Figure 3.5. Ideally selection of pixel resolution should be based on the Nyquist Theorem which states that “in order to adequately reproduce a signal it should be periodically sampled at a rate that is 2X the highest frequency you wish to record” (Ruzin, 2009).

While increasing the number of pixels in an image will improve resolution beyond that required by the Nyquist theorem, the time required to scan a specimen will also increase which may result in increased specimen damage. The increased number of pixels will also significantly increase the file size. For example, in the Leica SP8 system scanning at a resolution of 512 x 512 at a scan speed of 700Hz results in a pixel dwell time (the time the laser is in contact with that spot in the specimen) of 0675ns and a

frame rate 1.35 frames/s. At the same scan speed an increase in image resolution to 2,048 x 2,048 results in the same pixel dwell time, but since many more pixels are present the frame rate slows to 0.34 frames/s. Although resolution is improved significantly, the increased time may result in significant photobleaching.

File size and image processing capabilities must also be considered when selecting image resolution. While confocal systems are typically equipped with computers containing large data storage and processing capabilities, many offline processing systems do not have the storage and RAM capabilities to work with the large data sets collected with confocal microscopes. For example, a single channel image collected at a pixel resolution of 1024X1024 will be slightly over 1Mb in size. In comparison, file size for an image collected at a resolution of 8192X8192 is 67.1 Mb for a single optical section of one channel. However, most confocal data sets are multi-channel. A four-channel image collected at a resolution of 1024X1024 is over four megabytes in size and a four-channel image at 8192 X 8192 would be approximately 269 Mb. It is easy to see that a large four-color Z-series collected at high resolution would exceed several gigabytes in size. This often challenges the image processing capability of standard computers and it is now common for collection of data sets with some imaging systems to exceed a terabyte in size. Data collections of this magnitude quickly overwhelms standard computer processors demanding most imaging systems to house multiple hard drives exceeding terabytes in size or the use of cloud storage.

3.1.3 Acquire Window Acquisition Mode: Scan Speed

As noted above the pixel dwell time represents the amount of time the laser remains in contact with the region of the specimen (pixel or voxel) being imaged. If the laser remains in contact with the region for a longer period of time the potential for emission of a greater number of photons (signal) increases. Thus, the selection of scan speed is a critical component in determining the SNR with a slow scan speed resulting in the generation of an increased number of photons. The selection of scan speed can be made directly below the image Format button (Figure 3.6). Decreasing the scan rate will improve the SNR, however this is at the expense of increased photobleaching, which could be a problem with some samples. In Figure 3.7 A, a scan speed of 400 Hz was selected with a frame size of 1024x1024. With these operating conditions, the pixel dwell time was 600 ns and frame rate 0.388 frames/s. If the scan speed is increased to 700 Hz (Figure 3.7 B) the pixel dwell time is decreased to 325 ns and the frame rate is 0.678 frames/s. While the difference in actual time to collect an image may seem small, when imaging events in live cells or preserved samples that are rapidly photobleaching the increased scan speed will reduce the exposure of a sample to high intensity lasers. This may mean the difference between being able to collect meaningful data, although at reduced SNR, and the inability to collect data necessary to finish an experiment.

It should be noted that as the scan speed is increased passed 700 Hz, an image zoom is forced due to limited movement of the galvanometer mirrors above a certain speed. This zoom can be seen in Fig. 3.7 C that compares images collected at 400, 700 and 1400 Hz. When scan speed is increased to 1400Hz pixel dwell time is decreased to 150 ns and the frame rate is 1.35 frames/s. Note the increased noise in Figures 3.7 B

(middle) and C (bottom) in comparison with the slower scan speed used for Figure 3.7 A (top).

As described above, in single photon confocal imaging the laser interacts with a large volume of the specimen during each scan. This may result in excessive bleaching and loss of signal in optical planes above and below the focal plane of the specimen if the sample is not protected against photobleaching with mounting media. While the time necessary to collect a data set should not be a major consideration in most imaging protocols, by knowing the options available on an instrument and compromises made in specimen damage and image quality, both instrument access and operator time can be used efficiently. For example, for a 4 color 1024 X 1024 Z-series of 50 optical sections collected at 400Hz total scan time will be 13.5m. If sufficient signal is present to scan the same data set at 700Hz total scan time can be reduced to 5.25 minutes. If photon emission from the sample is sufficient to collect an image with a good SNR at a fast scan rate this option should be used saving both operator and instrument time. This allows optimal use of the specimen and instrument (Price).

3.1.4 Acquire Window Acquisition Mode: Bidirectional Scanning

The default scan pattern for most confocal systems is unidirectional where the beam scans in the X-direction, is blanked on return, and again scans in the x-direction for the set number of lines. Bidirectional X scanning patterns do not blank the beam while returning resulting in a “U” shaped scan. This may reduce scan time, but may introduce some scanning artefacts that must be corrected (Price, 2011). For example, in the LAS X software if a unidirectional scan speed of 400Hz at a resolution of 1024 X 1024 is chosen

the frame scan rate is 0.093 frames/s. If the bidirectional X function is turned on the frame rate changes to 0.177 frames/s. In the LAS X software the bidirectional X scan pattern can be turned on just below the scan speed button (Figure 3.8). This also activates the Phase X control that can be used to correct the bidirectional scan artefacts seen in Figure 3.9 B by sliding the bar until the edges of the image do not show artefacts (Fig 3.9 C). Care must be taken when using bidirectional X scanning that unintended artefacts are not introduced by over-correction along the edge structures in an image.

3.1.5 Acquire Window Acquisition Mode: Zoom and Rotation

Zoom and Rotation provide a mechanism to frame an image and to digitally increase magnification by scanning a smaller region of the specimen and projecting it to the detector which has a set size. For example, Figure 3.10 A shows an image of *Convallaria* at 10X with a zoom factor of 1. The pixel size at 512 X 512 resolution is $2.17\mu^2$. Without changing objectives and increasing the zoom factor to 2 (Fig 3.10 B) the pixel dimensions are changed to $1.08\mu^2$ and the region is magnified. Similarly, if the Zoom Factor is decreased to 0.75 (Fig 3.10 C) the image magnification is reduced and pixel dimensions are now $2.89\mu^2$. This is an effective mechanism to change magnification to a certain degree without changing the objective. For instance, it may be desirable to continue imaging with a 20X dry objective rather than changing to a 40X oil objective to avoid adding oil to a slide. However, since resolution is set by the objective, imaging with a lower NA 20X objective will not provide the resolution that a higher NA 40X oil objective will provide. A large increase in magnification will result in blurring of the image and this should be performed by using an objective of higher magnification rather than doing so digitally.

Once the zoom factor is applied images can also be moved laterally with the arrows and rotated with the Rotation function (Fig. 3.11) or with the scan field rotation on the USB panel box. Figure 3.12 shows the rotation function with a rotation of 0°, 45°, and 90°. These factors assist with framing an image and are very useful if repeating structures such as myofibrils are present in a series of images. With the rotation function all images can be aligned identically at this stage rather than with image processing programs at a later time.

3.1.6 Acquire Window Acquisition Mode: Optical Section Thickness and Pinhole Setting

When using standard lasers the optical section thickness is determined by the pinhole diameter, NA of the objective, and the emission wavelength as shown in the formula for the Optical Section Thickness in Figure 3.13. For example, when using an emission wavelength of 580nm, a 63X oil objective with a 1.4NA, and a pinhole diameter of 1AU the optical section thickness is 0.896 μ m (Fig. 3.13 top panel). Since the emission wavelength and NA of the objective are set when collecting an image the optical section thickness can be changed by adjusting the pinhole diameter/AU. In Figure 3.13 (middle panel) the pinhole diameter has been increased to 3 AU and the optical slice thickness is increased to 2.368 μ m. If using a multiphoton laser to obtain the correct calculation of section thickness the “+” button next to the optical section information and the multiphoton box must be checked as shown in Figure 3.13 (bottom panel) for the system to correctly calculate the optical slice thickness. Note that when using the multiphoton laser the pinhole diameter is not a factor as only fluorochromes in the focal plane are excited.

Setting the pinhole diameter is one of the most important decisions made when imaging a sample. Adjustments to the pinhole can directly affect image resolution, SNR, quantitative analysis of structure size, and interpretation of colocalization data. Since the effects of pinhole diameter adjustment occurs after interaction with the specimen, effects on specimen damage are indirect. For example, increasing the size of the pinhole improves the amount of signal at the detector which allows reduced laser intensity and/or scan time to saturate the detector. This can protect the specimen from laser damage since laser intensity and scan time can be decreased.

If all other factors are held constant, opening the pinhole will increase the signal (brightness) of a confocal image as noted above, but this also decreases contrast and Z-resolution due to inclusion of information in the image from out of focus areas of the sample. This is illustrated in Fig 3.14 which shows images collected at pinhole diameters of 1 AU (left) and 3 AU (right). Note that in this example all detector and laser settings were held constant. At 1 AU, the signal is relatively low and the image appears dim. When the pinhole is opened to 3AU increased signal is present resulting in a brighter image. While increasing the optical slice thickness will increase the signal (Fig. 3.14), the improved brightness is due to collection of photons from above and below the focal plane and this option should be avoided if possible. By increasing the pinhole, the signal is improved and it is possible to decrease laser intensity and scan speed to protect a specimen from photobleaching. These three factors, scan speed, laser intensity, and pinhole diameter must always be considered when establishing image collection parameters as they will determine image quality versus specimen damage compromises as shown in Table 1.1

3.1.7 Acquire Window Acquisition Mode: Line and Frame Average and Accumulation

Even with the best of specimens and operating conditions many confocal images have the potential to contain noise in an image as a result of the electronics present in the hardware. This is most easily observed in the dark areas of an image that contain little to no fluorochrome. In a single pass of the laser, some pixels in these areas may have a bright appearance that is above the zero value set for black. In subsequent scans the same pixel may have values that range from zero to high enough to show a bright spot in the image background. Shifting of the value of these pixels during subsequent scans indicates electronic noise in the image. If noise becomes a severe problem, it adversely affects image quality, and if a pixel is bright enough it may affect quantitative assessment of the data (Price, 2011).

Since electronic noise is random, it is unlikely that the same pixel experiences a noise event in subsequent scans. Imaging several lines or frames and averaging each pixel value reduces the background level by decreasing pixel values where noise has occurred. Two methods, line or frame, are available for averaging during collection of an image (Fig. 3.15). Note that each box has a pull down menu for selection of the number of scans averaged. During line averaging each line is scanned the set number of times and the average value obtained for each pixel position assigned to the final image. As an example, if an averaging function of 8 is selected and one of the scans has a pixel noise value of 24 while the remaining scans have a pixel value of 0, the average pixel value assigned to that location in the image is 3. In the Frame Average mode, the averaging of pixel values occurs after the completion of each frame scan. Averaging should result in noise values falling at the end of the image histogram reserved for histogram stretch

functions and result in a black image pixel not detectable by eye. The effects of line averaging are shown in Figure 3.16 by comparing an image collected without averaging (top) with those collected by averaging four (middle) and eight (bottom) times.

A common question is if averaging improves image quality why shouldn't several more frames be included while collecting images? In the above example, an averaging function of eight scans was selected which reduced the noise in the pixel from a value of 24 to 3. If the number of scans was doubled to 16 under the same operating conditions, the average pixel value for the location would have only dropped to 1.5. Both values are at the low end of pixel values in the image and would be detected by the eye as a black pixel. Several factors enter into the decision on the number of scans to average. The improvement in an image is directly proportional to the square root of the number of scans averaged. Thus, if an image is averaged 16 times, the image is improved by a factor of 4. If the image is scanned four times, it is improved by a factor of 2. However, to get the improvement in SNR ratio from 4 to 2, it takes four times as long to scan the sample which significantly adds to the total number of scans and time the specimen is exposed to the laser, which may result in photobleaching. For example, if a sample is scanned with a line average of 1 and a speed of 400Hz as shown in Figure 9.33 the pixel dwell time is 125ns. If the same sample is averaged 4 times the cumulative pixel dwell time would increase to 600ns. While this difference may seem small, when imaging specimens that are rapidly photobleaching this may result in loss of signal after only a few scans or the death of cells during live cell imaging (Price, 2011).

As a rule of thumb, if possible images should be averaged at least by a factor of 2 to reduce noise that may not be apparent to the eye, but will be present if an image is

analyzed by computer. In samples where SNR is a significant problem and the fluorochromes are stable, averaging of more frames improves the image quality, but the compromise of SNR versus sample stability and time should always be a primary consideration when establishing the number of lines or frames averaged (Table 1.1).

3.1.9 Acquire Window Acquisition Mode: Z-Series Collection

One of the largest advantages of confocal microscopy is the ability to collect data stacks through a sample in which every optical section is aligned and in focus. This is accomplished by collecting a section, moving the stage an established distance in the Z-direction, and collecting another section. This process is repeated until the required number of sections are collected. Several parameters such as optical slice thickness when collecting sections and the depth that images can be collected must be considered during Z-series generation.

With most modern instruments, the process of collecting a Z-series is fairly easy. Figure 3.17 shows the Z-stack control menu within the LAS X software. To begin the Z-stack collection, the beginning and the end of the series must be determined. This is done by scrolling to the desired beginning and clicking “Begin” and then scrolling through the section until the desired end is reached and clicking “End.” The “End” (8763 μm) and “Begin” (8693 μm) values shown in the boxes to the right of Figure 3.17 represent the stage position at each point in the Z-series and in this case a Z-stack of 70 μm will be collected. Based on the pinhole diameter selected earlier the step size between each section will be 0.57 μm when the system is optimized to meet the Nyquist considerations. In the example shown, to collect 70 μm of tissue at 0.57 μm intervals the Z-series will

consist of 123 sections. Often when sampling thick specimens system optimization may require several hundred sections to meet the Nyquist requirements. This may result in very long data collection times, photobleaching, and excessively large data files. It is possible to reduce the number of sections, for example by undersampling, but there are ramifications related to 3D reconstructions and factors such as missing data and/or data interpolation.

When imaging thick specimens a choice is often necessary on where to set the SNR for optimum image quality throughout the Z-series. If it is set at the mid-point of the series, the signal from the first section is often too bright and the signal from the last section is often minimal making reconstructions difficult. This low signal is often a result of a phenomenon termed Z-drop. Z-drop can be due to excitation and emission wavelengths, photobleaching, and/or refractive index mismatch. Selection of the Z-compensation button provides an adjustment mechanism to minimize these effects when reconstructing data sets. If Z-drop is a problem the optimal SNR should be set using an optical section near the top of the sample and the system will compensate for Z-drop as deeper images are collected (Price, 2011).

Another parameter that must be set during Z-series generation is the scan sequence. The LAS X software provides options for scanning between lines, frames, or stacks. Scanning between lines will increase the time for stack generation as the laser will be switched after each line is scanned. On the other hand, scanning between stacks will drastically decrease the time for image generation because one channel is scanned through the entire region of interest and then the stage is moved back to the “Begin” position for scanning of the second channel. Scanning between frames results in less

switching of the lasers as all channels will be scanned prior to the stage being moved to the next position. Scanning between frames is the optimal scanning pattern because it decreases reliance on the repositioning of the motor and stage to the same exact “Begin” position each time.

3.1.9 Acquire Window Acquisition Mode: Presentation of Z-Stack Data

Once the Z-stack is generated, it is possible to scroll through each of the sections using the vertical scroll bar to the right of the generated image. It is also possible to view all of the sections in the Z-stack at the same time by selecting the “Show Gallery” icon located on the right panel of the Acquire window (Fig 3.18). Projection of the data set in several formats can be accomplished by using the menu bar on the right side of the image. Not only is it possible to view the image in Gallery Mode, but it is also possible to view the Maximum Projection, Orthogonal Sectioning, and open up the image in the 3D Viewer. Since these functions are outside the scope of basic confocal microscopy, they will not be discussed further. However, it is important to know that these options exist when operating the system.

3.2 Acquire Mode Center Window

The center window (Figure 3.1 panel B) in the Acquire mode provides access to stored protocols and information for creating new protocols, control of the lasers, objectives, dye database and the detector settings. Each component will be discussed in detail in the following sections. At the top of the window a row of icons is provided to load existing protocols, save newly written protocols, select a XY region of interest (ROI) for scanning, and to set the Background. The orange line exiting from below the lasers, going to the left through the image of the objective, and reaching the specimen

represents the excitation photons. The spectrum of light represents the emitted light shown as the white line around the area with the objective, Multifunction port (MFP) used to move filters into the microscope beam path, Beamsplitter, Dye Assistant, USB Control Panel, and Fluorifier Disc.

3.2.1 Acquire Mode Center Window Load/Save Single Setting

The Center Window under the Acquire tab is useful in setting laser and detector settings. The following sections will correspond with the order of this window, from top to bottom, and discuss the different parameters that can be accessed and set here, with the first being loading/saving single settings. This allows rapid implementation of protocols saved in the software, or allows a user to save a protocol to return to during different operating sessions. As always, it is important to use caution when selecting these protocols, as certain components in the hardware may have aged or been replaced, as previously discussed. Also, since not all specimens are the same, it is important to fine tune the appropriate settings when selecting a saved protocol.

3.2.2 Acquire Mode Center Window Visible Lasers

As previously discussed, there are many areas of redundancy within the software, including the different windows where the lasers can be turned on and off. Although this can be done under the Configuration tab, due to accessibility, lasers are most often accessed under this window as shown in Figure 3.19, where arrows indicate the panel for visible light and the panel for Multiphoton. Setting the intensity of these lasers remains the same, as discussed momentarily, but the steps to turn on the Multiphoton laser will be discussed in the next section. Once the lasers necessary for image generation are turned

on, by selecting the “+” on the laser panel, the intensity can then be adjusted by hovering the mouse over the desired laser line and scrolling up to increase intensity or down to decrease intensity. Through this method, values between 0 and 100 can be selected in increments of .1. However, by double clicking the intensity value above the laser line, a specific value as low as .0.1% can be typed in. It is important to remember that by increasing laser intensity, specimen damage and photobleaching may result. These effects are further discussed in Table 1.1. Therefore, other parameters, like Gain and Offset, should be adjusted to keep laser intensity to a minimum to preserve laser life and decrease specimen damage.

3.2.3 Acquire Mode Center Window MP Laser

Although the Multiphoton laser can be turned on under the Configuration window, the MP shutter can only be accessed under the Acquire Mode window. Figure 3.20 can be referred to for the following steps. Once the Multiphoton laser has been selected, the shutter can be turned on by holding “MP-Shutter” until a red circle appears on the right of the icon. This will cause the yellow hazard signal to illuminate and the shutter will read “open” (Figure 3.21). The power should still read on, and the mode should read “pulsing.” The laser must then be adjusted by being set to the appropriate wavelength and then the “tune” button should be selected. It is important to retune the laser anytime a new wavelength or objective is used in Multiphoton imaging. Finally, under GDD curve, the objective in the dropdown must match the objective being used to image the specimen sample. After all of this has been performed, the sample is ready to image.

3.2.4 Acquire Mode Center Window Objectives

Many operating programs provide some redundancy for ease of operations, as mentioned numerous times. For example, even though it is possible to select the objective to be used in the Configuration window, as well as on the Leica STP8000 Smart Touch Panel, it is also possible to set the objective in the Acquire Mode Panel, seen in Figure 3.22. Of course, the objectives can be set manually, and it is important to make sure that the automated objectives are routinely and properly serviced and calibrated since scale bars and other image measurement functions are dependent upon calibration of the objective magnification with the data readout for these measurements (Price, 2011).

3.2.5 Acquire Mode Center Light Path

Figure 3.23 shows further parameters that can be set in this panel, including the Beam Splitter Current (arrow A), the Leica Smart Control Panel (arrow B), the Dye Assistant (arrow C), and the Fluorifier Disc Settings (arrow D). Since the Leica Smart Control Panel and the Fluorifier Disc settings have been discussed previously in Chapter 2 (Configuration), details regarding these options can be referred back to in their respective Chapter 2 sections.

The Beam Splitter Current (BSC) can be activated (green) or inactivated (red). In regards to this specific Leica system the BSC is an Acousto-Optical Beam Splitter, abbreviated AOBS. This separates the excitation and emission wavelengths which allows for maximum detection sensitivity while decreasing photobleaching. A beam splitter is necessary because in fluorescence microscopy the excitation is entering the specimen on the same side that the emission wavelength is leaving the specimen. Originally beam

splitters used to operate via dichroic and multichroic mirrors, but the AOBS replaces these as a “single programmable optical element” and operates off of the diffraction in a TeO₂ crystal which creates a refractive index grid as acoustic waves are applied. Since the dichroic or multichroic mirrors do not need to be changed, as they previously were, the AOBS allows for both single and multichannel imaging (Leica). In order to generate an image, a laser is emitted which reflects off, in our case, an AOBS, which scans the laser across the specimen sample. The dye within the sample absorbs that laser wavelength and emits a corresponding photon which then reflects off of the AOBS TeO₂ crystal with varying sound frequencies. The AOBS provides spectral separation of emission wavelengths that are close together. This emitted light then passes through the pinhole and is measured by corresponding photomultiplier tubes. The PMTs are sensitive light detectors that multiply the current of light in order to generate the specimen image (Prasad, 2007).

The Dye Assistant built into the Leica LAS X software offers detailed configurations on several fluorescent dyes. This allows for the appropriate selection of dyes for each experiment. By selecting the Dye Assistant icon, the following screen (Figure 3.24) appears which allows for the selection of a dye, as well as the addition or deletion of dyes. Figure 3.25 shows the follow up screen when a dye is added which shows most available fluorochromes. Once a fluorochrome is selected the amount of yield and crosstalk, depending on other fluorochromes selected in conjunction, will be displayed. It is important to eliminate as much crosstalk as possible. If one dye is selected then it can be applied, but if two or more fluorochromes are selected than the scan setting

must be determined. The dyes can either scan line by line or by frames/stacks, the consequences of both have been previously described.

3.3 Acquire Right Window

At this point, a basic understanding of how to collect an image has been presented. The remaining portion of this chapter will discuss the available editing and analysis tools in the LAS X software. Some of these editing tools are available in different windows and will be discussed briefly in the next chapter. In regards to the tools located in the Acquire window (Figure 3.1 Panel C), only the commonly used ones will be described. There are editing tools that allow the user to draw various shapes and add different annotations to emphasize certain regions of the specimen, but these will not be discussed as most of these functions are commonly done using other programs.

All confocal programs include a Look Up Table (LUT) which takes advantage of our ability to distinguish color. LUTs assign a color (in this case red) to all pixel values above a certain value, a different color (in this case blue) to all pixels below a certain value, and a gray tone to all pixels that fall between the upper and lower limits. In Figure 3.26 a single –channel image of *Convallaria* is shown with the LUT icon selected. It is important to use the LUTs on all channels in an image to ensure they are all collected correctly. Levels of blue and red pixels can be adjusted by adjustments in laser intensity, PMT sensitivity, and pinhole detector settings. Choices that increase signal increases the number of red pixels and choices that decrease signal increases the number of blue pixels while decreasing the number of red pixels. All compromises in setting up the system for

image quality versus specimen damage (Table 1.1) come into play at this point when establishing the operating parameters (Price, 2011).

The use of LUTs is an excellent mechanism for setting up qualitative imaging parameters for the overall image. However, at times it may be desirable to also quantify the pixel values in an image and further analyze the data by creating histograms of pixel distributions. In Figure 3.27 the histogram icon is activated and the frequency of each pixel value is shown, ranging from 0 to 255. By hovering the mouse over the area of interest, the intensity of that pixel is displayed in an informational bar below the histogram. All of this data can be exported to spreadsheets, such as Excel, to facilitate quantitative analysis. The histogram can be adjusted using the scroll bar directly below the graph. By eliminating pixels of higher value (sliding the bar to the right), the image begins to darken and vice versa as the pixels of lower value are eliminated (Price, 2011)

Finally, the various channels used and an overlay of those channels can be viewed by using the icons on the far right of this window (Figure 3.28). It is important to note that these channels represent pseudo colors and it is possible to assign a color of choice to each channel. Worth noting is that changing the color of the image here affects only the active image (Price, 2011)

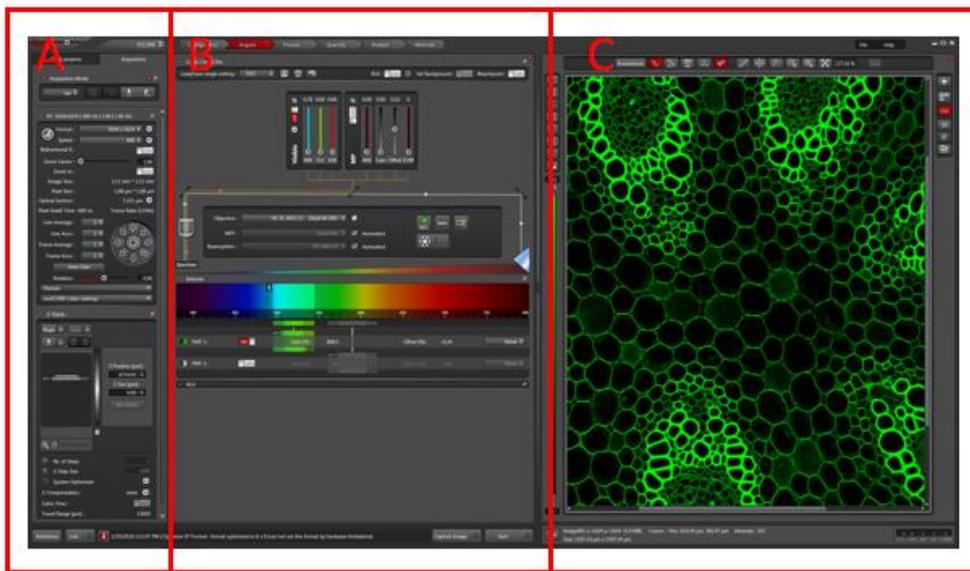


Figure 3.1. Overview of the Acquire screen in the LAS X software. This screen has been broken down into its three components. Panel A represents the Acquisition Mode, Panel B represents the Dye Database, and Panel C is the Image Acquisition Window.



Figure 3.2. The Open Projects icon under the Acquire menu provides access to various drives and files available within the LAS X software.

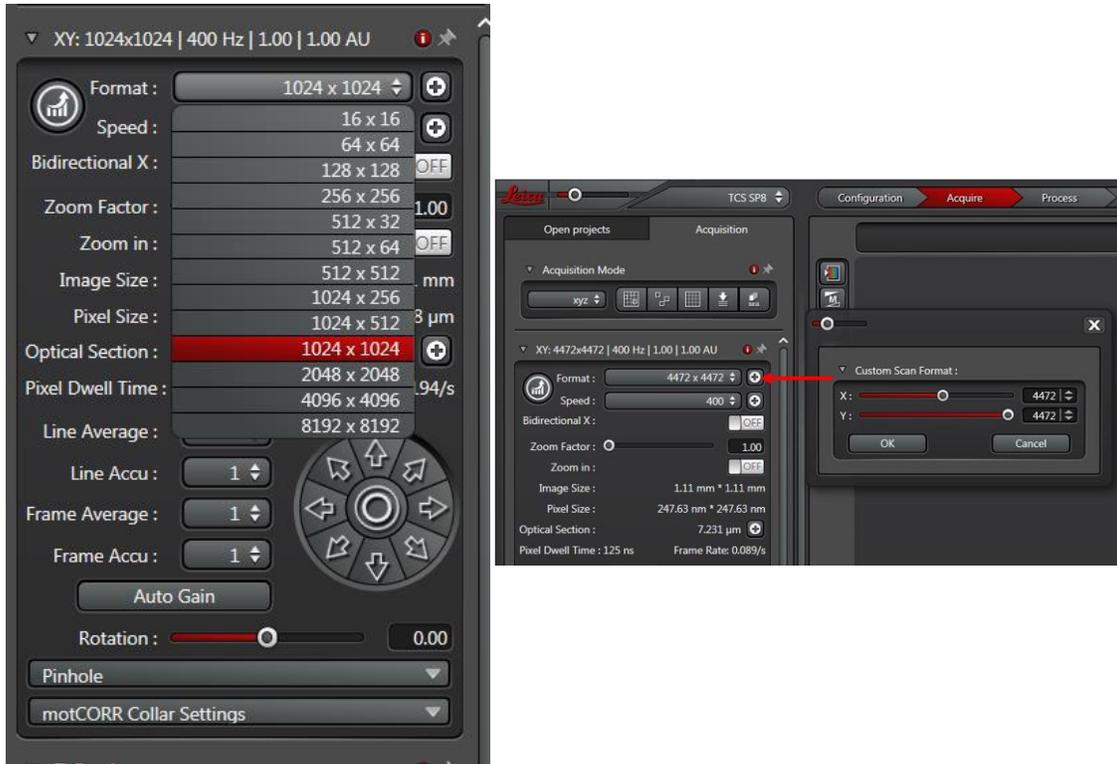


Figure 3.3. There are two ways format sizes can be selected in the LAS X software. On the left image the dropdown menu can be used to select a pre-determined format size. On the right image a custom format size can be specified.

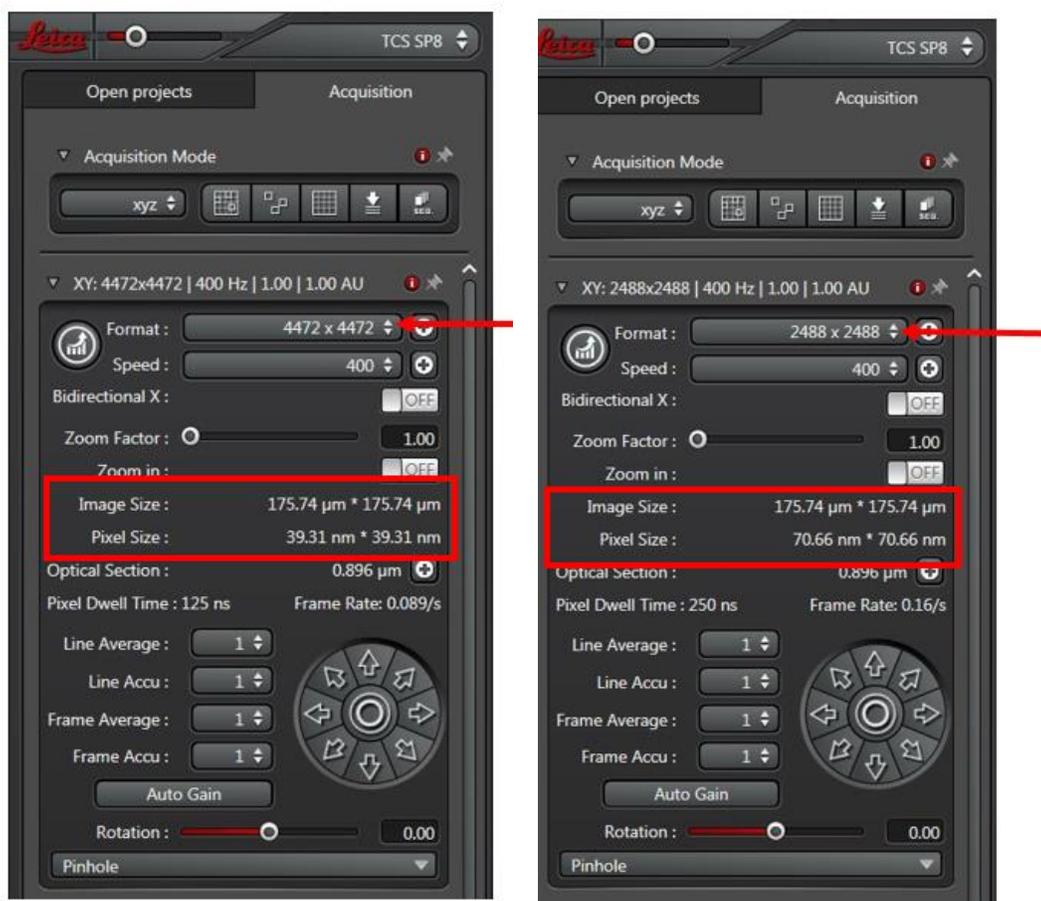


Figure 3.4. Changes in image format results in changes in the number of pixels and the pixel dimensions. These calculations are built into the LAS X software. Panel A (left) shows an image format of 2488 x 2488 with an image size of 175.74 μ m X 175.74 μ m and XY pixel dimensions of 70.66nm X 70.66nm. Panel B (right) shows an image format of 4472 x 4472 with the same image size of 175.74 μ m X 175.74 μ m but a XY pixel dimension of 39.31 nm and the number of pixels has doubled.

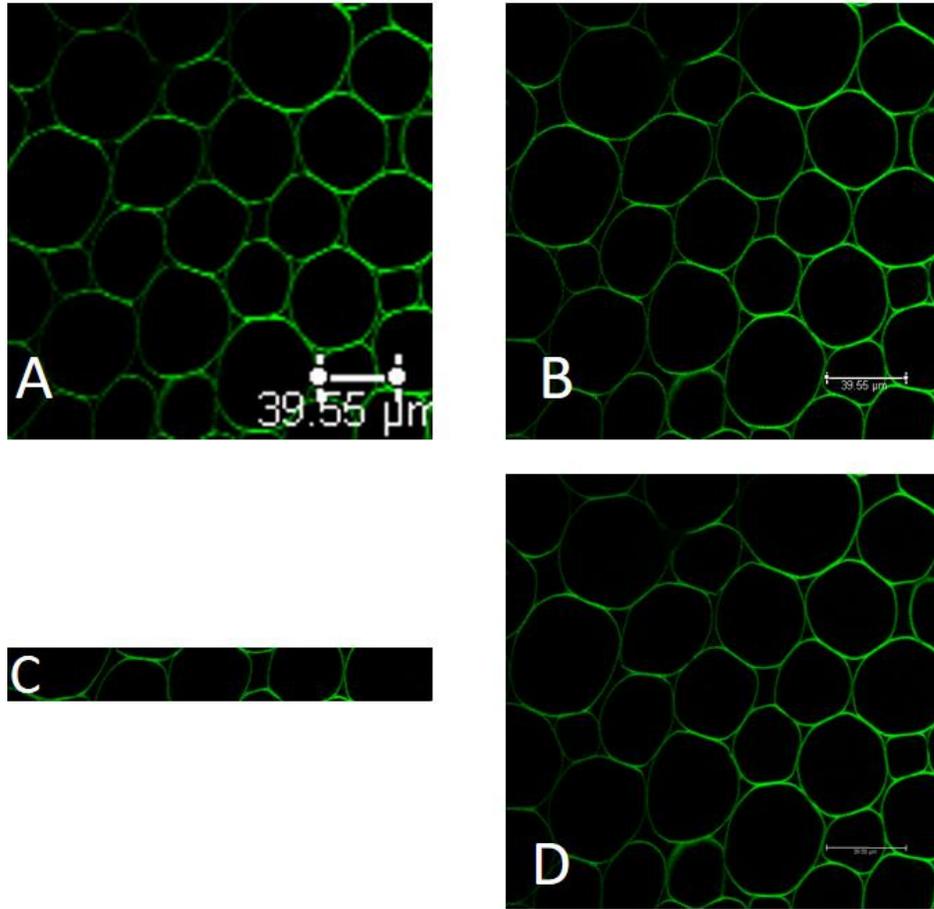


Figure 3.5. Various format sizes are shown here to see the effects of changes in resolution. Panel A is 512 x 512, Panel B is 2488 x 2488, Panel C is 512 x 64 Panel D is 4472 x 4472. Panel C is best used for live cell imaging.

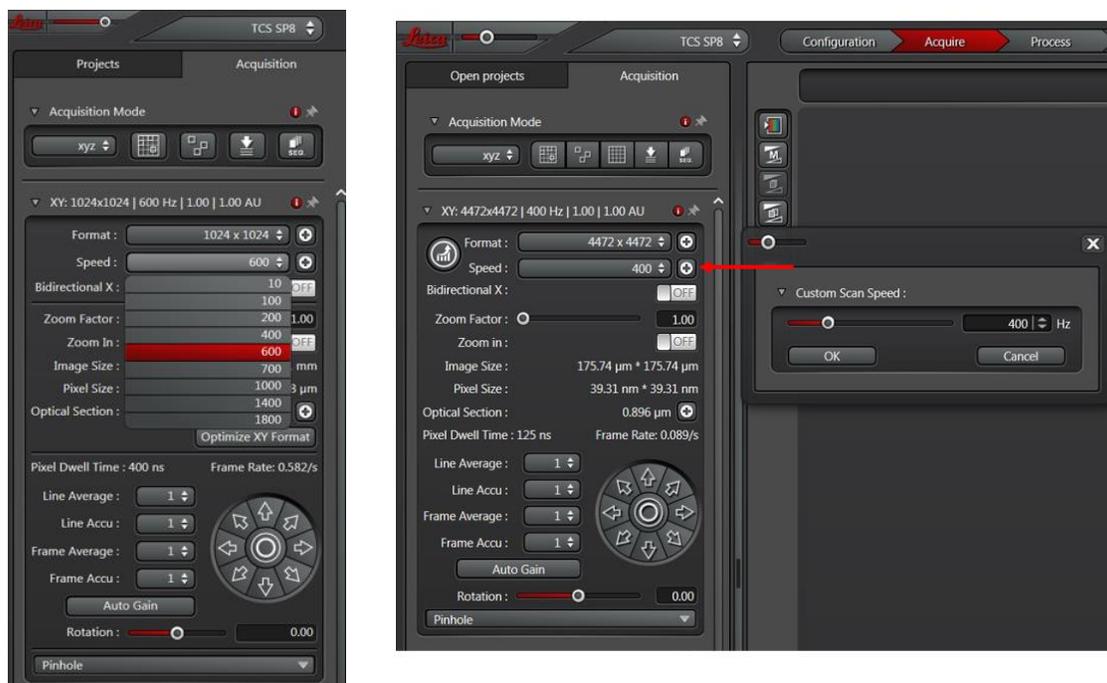


Figure 3.6. There are two ways scan speed can be selected in the LAS X software. In left image the dropdown menu can be used to select a pre-determined format size. In the right image a custom format size can be specified.

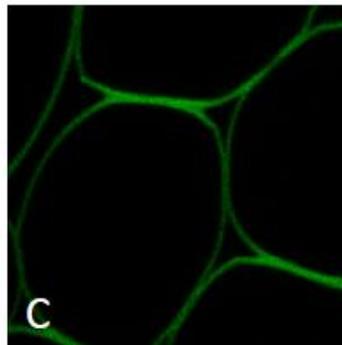
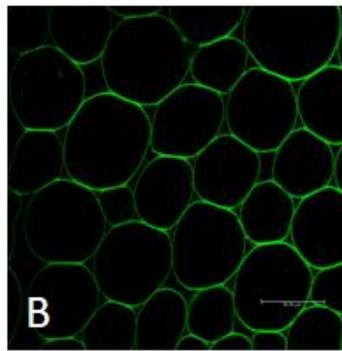
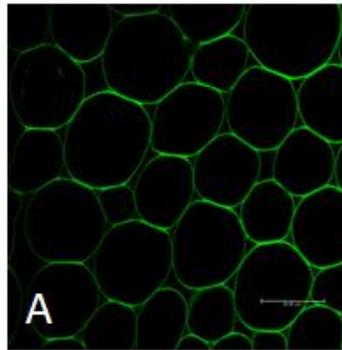


Figure 3.7. Various scan speeds can be selected from within the LAS X software, which will effect both the resolution and time of image acquisition. Panel A shows a scan speed of 400 Hz, Panel B is of 700 Hz, and Panel C is of 1400 Hz. Once the scan speed is increased past 700 Hz, there is a forced zoom due to movement restrictions of the galvanometer mirrors. Notice there is increased noise as the scan speed is increased.



Figure 3.8. An image can be scanned in either a unidirectional (default) or bidirectional pattern. To turn on bidirectional scanning, the bidirectional button is selected as indicated by the right arrow above. Phase X (left arrow) compensates for any distortion created by bidirectional scanning.

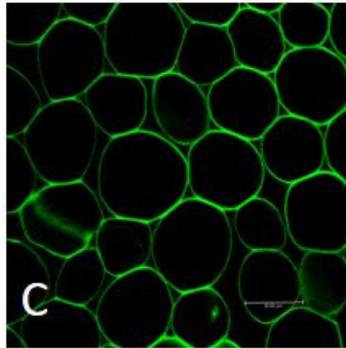
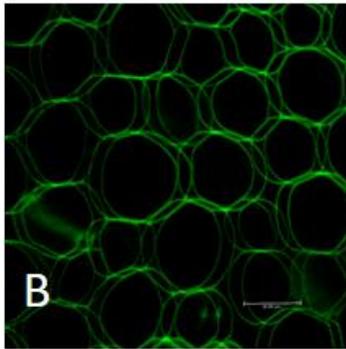
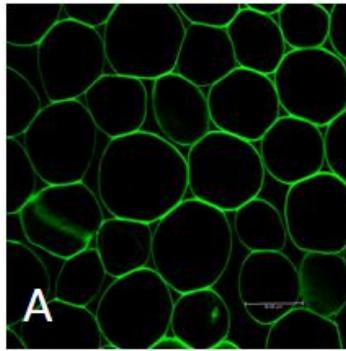


Figure 3.9. An image can be scanned in either a unidirectional (Panel A) or bidirectional pattern (Panel B and C). The system compensates for any distortion created by the bidirectional scanning (Panel C), but when that compensation is turned off via the Phase X scan bar, this distortion can be viewed (Panel B).

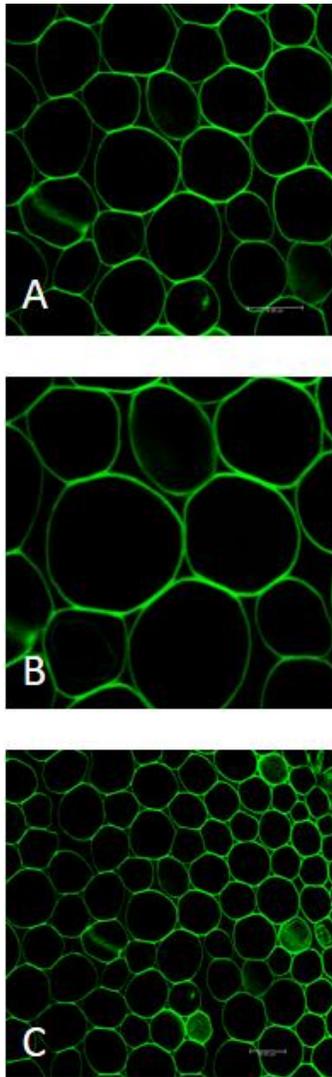


Figure 3.10. In order to magnify specific areas of an image, the zoom function can be used, as opposed to changing the objective magnification. Panel A shows an image with a Zoom of 1 (default), Panel B shows a zoom of 2, and Panel C shows an image with a Zoom of 0.75. Using this method to magnify an image can lead to decreased resolution.



Figure 3.11. Images can be rotated, as indicated by the arrow above, within the LAS X software in order to identically align any desired images.

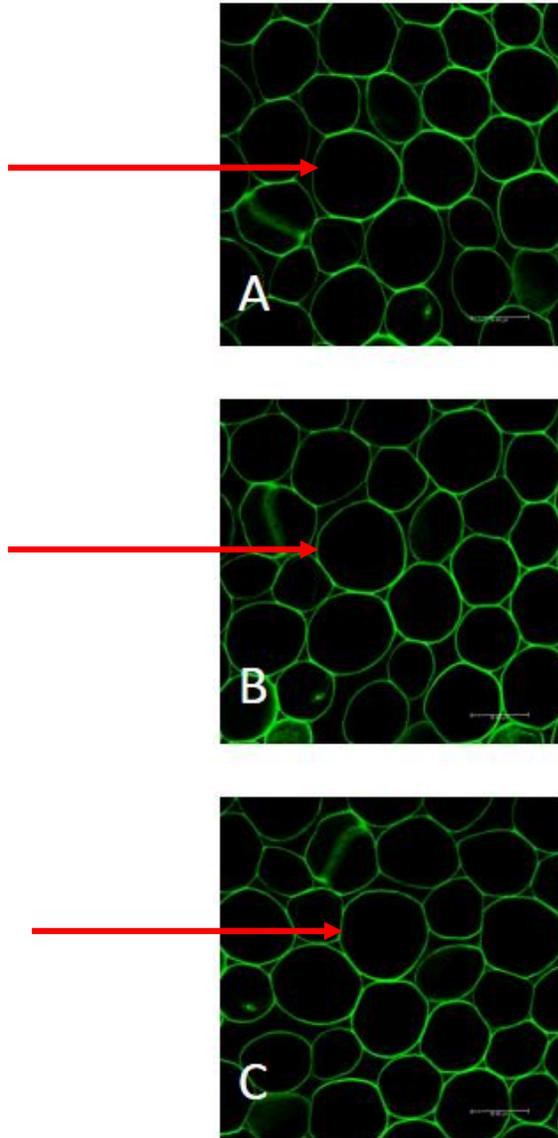


Figure 3.12. The Rotation Function can be seen above with a rotation of 0° (Panel A), 45° (Panel B), and 90° (Panel C). The arrows indicate a rotated structure for reference.



Figure 3.13 shows the formula for Optical Section Thickness. This formula takes into consideration the pinhole diameter, numerical aperture of the objective lens, and the emission wavelength. The top panel shows the formula calculation for an emission wavelength of 580 nm, 63X oil objective, 1.4 NA, and pinhole of 1 AU. The middle panel shows how an increase in pinhole diameter to 3 AU, with the other variables being constant, increases the optical slice thickness from 0.896 μ m to 2.368 μ m. In the bottom panel, the Multiphoton box is checked, which calculates the optical slice without the pinhole diameter, as that is not a factor in MP imaging.

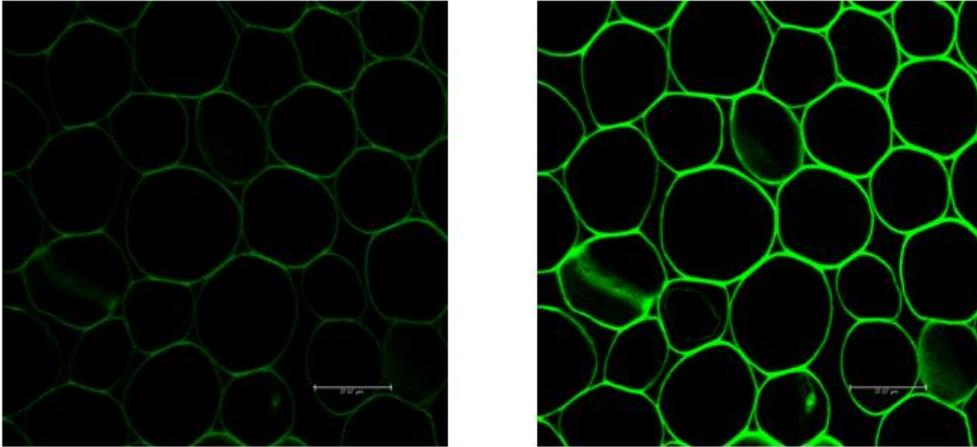


Figure 3.14. The effect of increasing the pinhole diameter from 1 AU (left) to 3 AU (right) can be seen here. As the pinhole increases, there is an increase in signal which results in a brighter image.



Figure 3.15. In order to decrease background noise, image lines and/or frames can be averaged. The number of times the image is scanned can be chosen via the dropdown menus. The dropdown menu for line averaging is indicated by the arrow above, but if it is desired to scan by frame, the frame average dropdown can be selected.

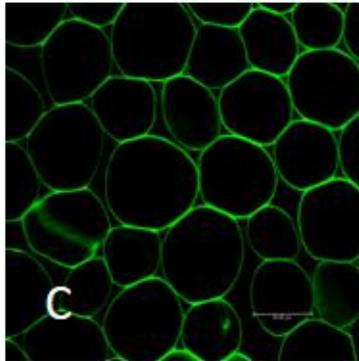
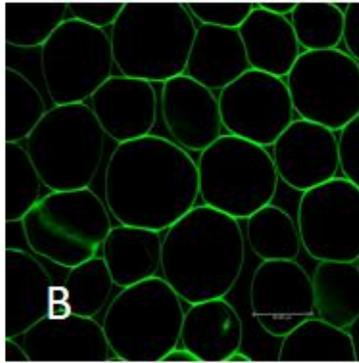
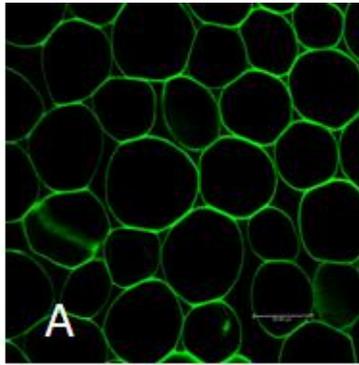


Figure 3.16. A comparison between no line averaging (top), a line averaging of 4 (middle), and a line averaging of 8 (bottom) can be seen in the panels above. Each panel should represent a decrease in subsequent noise.



Figure 3.17. The Z-stack menu under the Acquire Window. For this specific Z-stack the beginning is set to 8693.40 μm and the end is set to 8763.40 μm . With the system optimization setting the optical slice is 70 μm , with 123 steps, and each step size is 0.57 μm . The Z-stack can be scanned between lines, frames (selected), or stacks as seen in the sequential scan box.

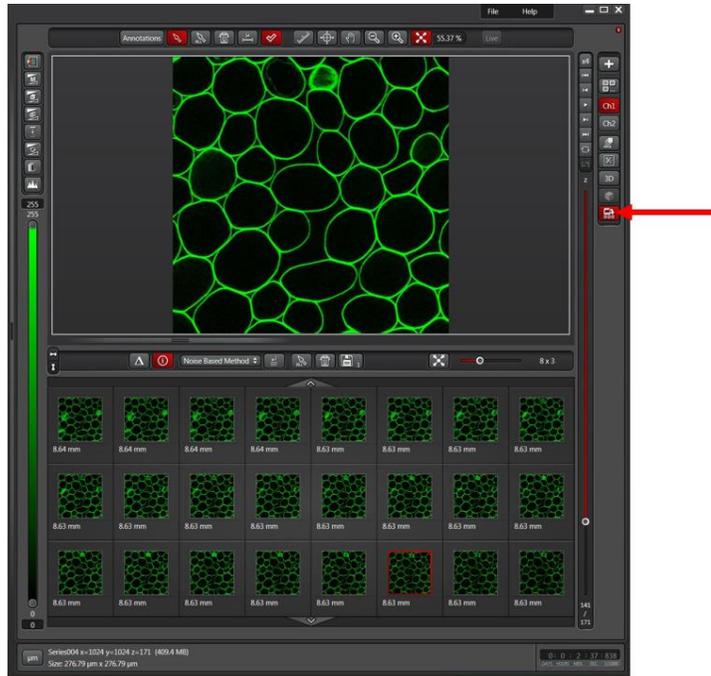


Figure 3.18. Each slice of the Z-series can be viewed by use of the scroll bar and with the selection of the “View Gallery,” icon (indicated by the arrow).

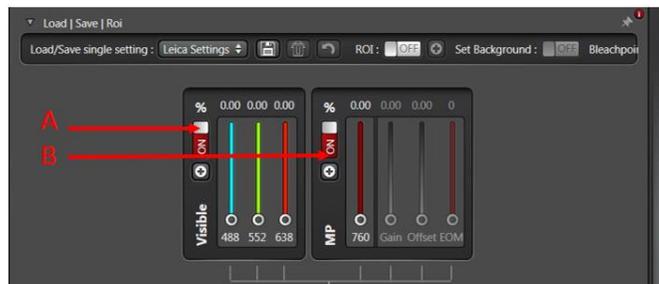


Figure 3.19. The Visible Light and Multiphoton lasers can be turned on here under the Acquire Window. Arrow A and B points to the location of each on/off icon on the laser panels.

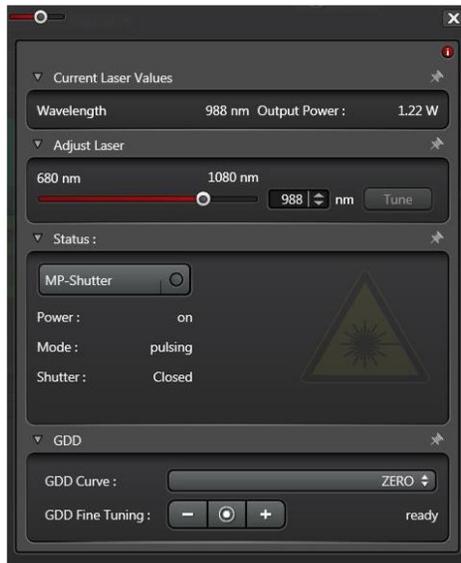


Figure 3.20. Overview of the Multiphoton laser window.

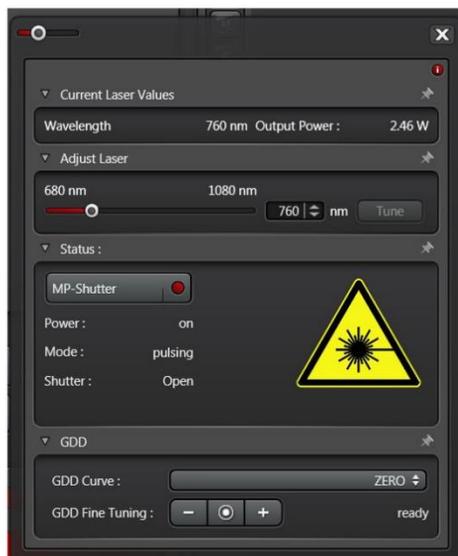


Figure 3.21. Once the MP shutter has been turned on, indicated by the red circle to the right of the button, the MP hazard light will illuminate. From this screen the adjust laser setting and the GDD curve dropdown can be seen.

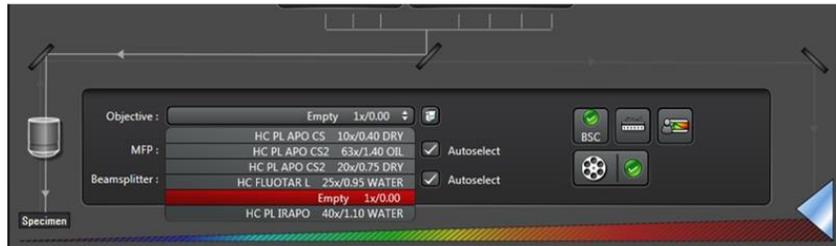


Figure 3.22. Overview of the objective dropdown menu under the Acquire window. Objectives can be selected and changed in this menu for increased ease of use.

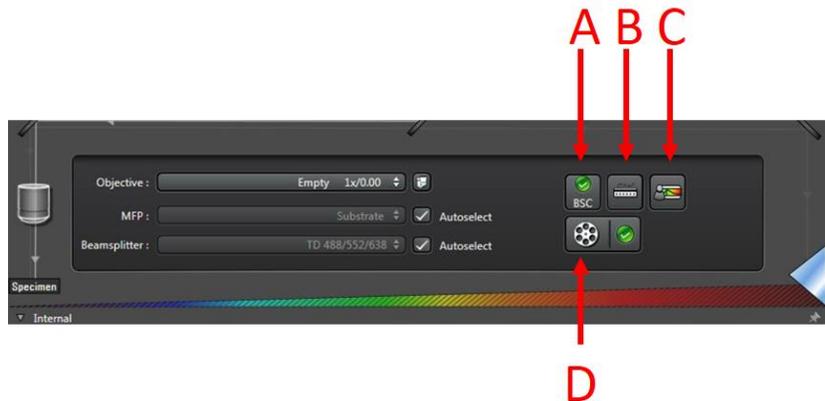


Figure 3.23 Many parameters can be set in the Acquire window including (A) the BSC, (B) the Leica Smart Control Panel, (C) Dye Assistant, and (D) the Fluorifier Disc Settings.

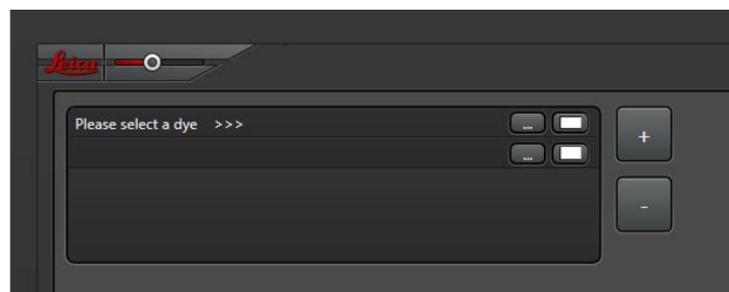


Figure 3.24. Overview of the dye assistant screen. It is possible to add, delete, and select a dye from this menu.

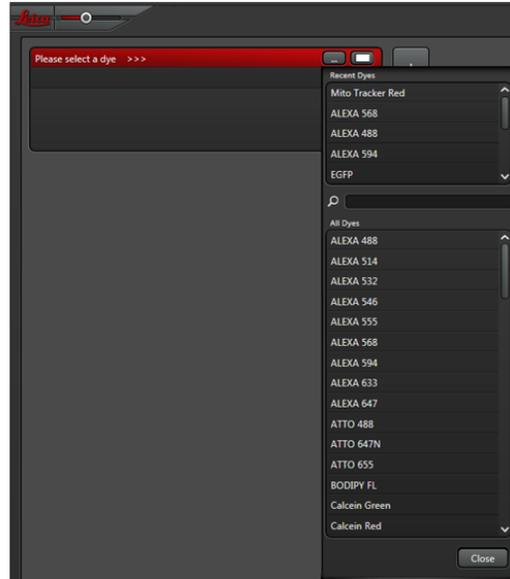


Figure 3.25. Under the dye assistant it is possible to select any fluorochrome available in the software.

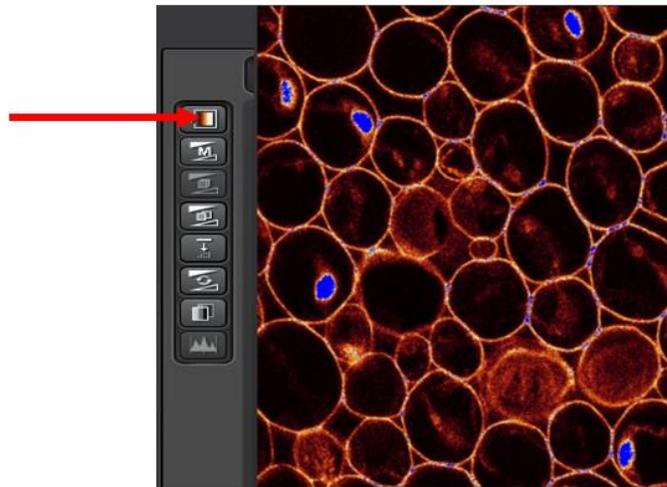


Figure 3.26. The LUT is available under the Acquire window. A look up table makes use of our ability to see color and assigns the color red to all pixel values above a certain color, the color blue to all pixels below a certain value, and an intermediate color to all other pixels that fall between the upper and lower limits.

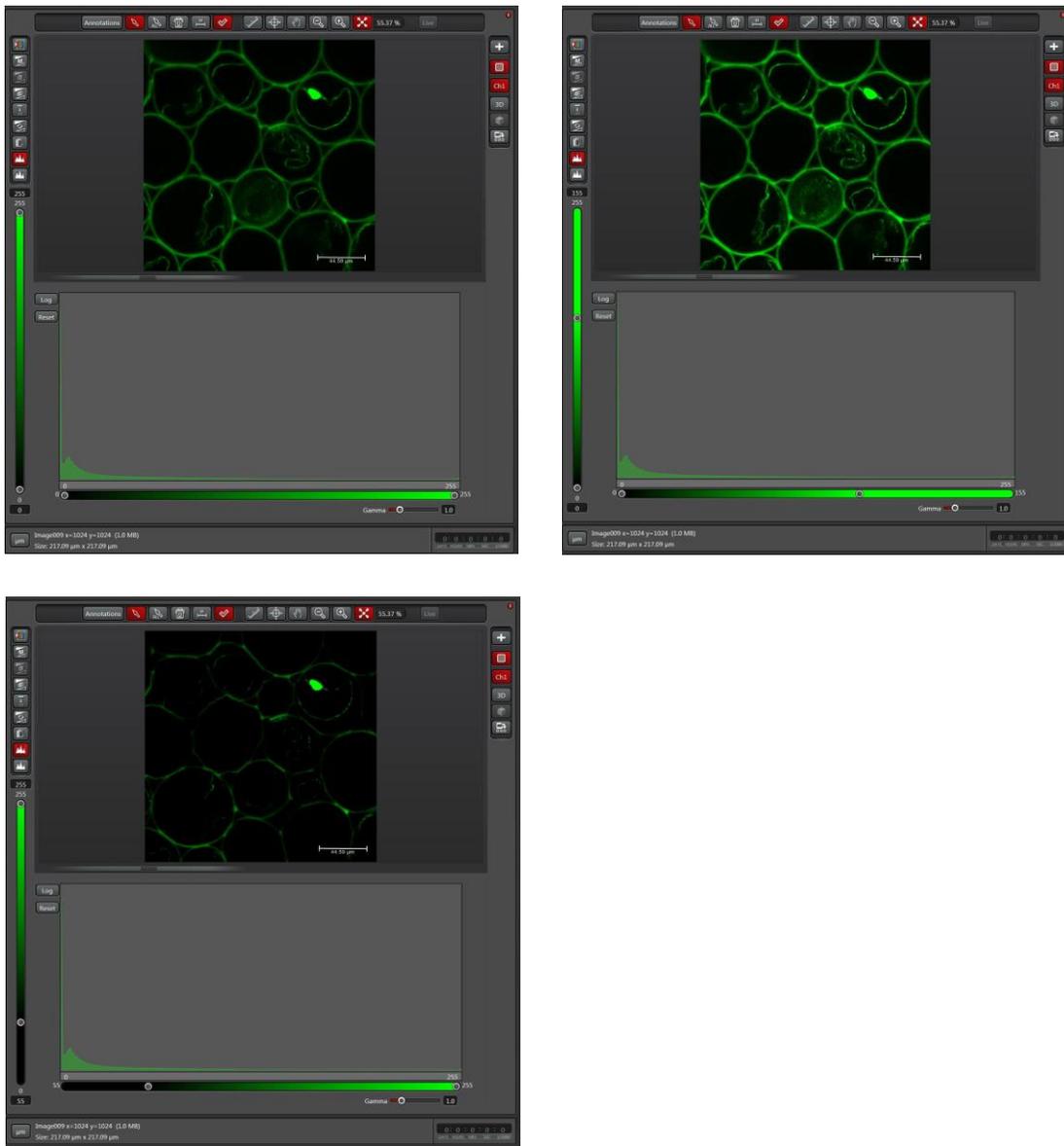


Figure 3.27. The histogram function is available under the Acquire window. The top left panel represents the image without any histogram adjustments. By sliding the histogram to the left, low pixel values are eliminated and the image appears brighter (top right panel). On the other hand, by sliding the histogram to the right, pixels with high values are eliminated and the image appears darker (bottom panel).

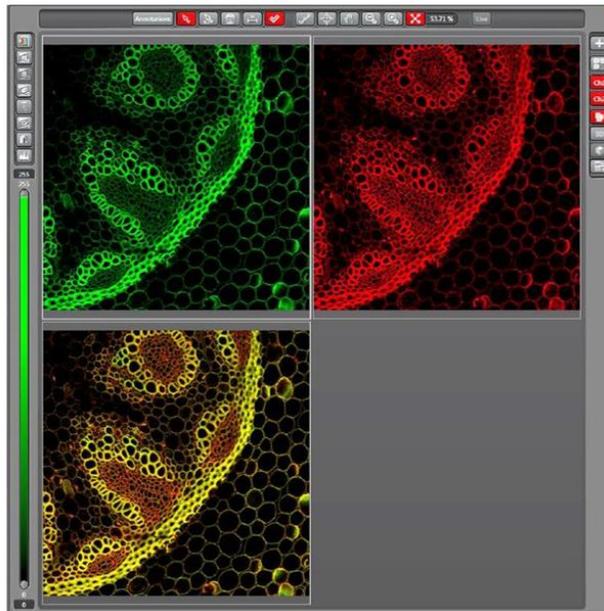


Figure 3.28. Under the Acquire window multiple image channels can be viewed (top panels). The channels can also be overlaid with the selection of the overlay icon as see in the bottom panel.

CHAPTER 4

PROCESSING IMAGES

4.1 THE PROCESS ICON

Built into the LAS X software is the Process icon (Figure 4.1). This icon allows for certain edits and adjustments to be made to the image without exporting it to a post-acquisition editing program like Photoshop or Image J. Since the Process icon is an advanced function and therefore outside the scope of this paper, only a brief overview will be provided. There are eight different editing options within this icon, including “Edit,” “Adjust,” “Deconvolution,” “Noise Reduction,” “Segmentation,” “Dye Separation,” “Topological,” and “Excitation and Emission Scans.”

Under the Edit section of the Process icon, a variety of settings can be applied to the image. These are basic tools including crop, which allows for a region of interest to be excised from the background. However, by cropping an image there is loss of data, so it is important to note that an original image should be saved. The image can also be resized and combined, which allows for the combination of various generated images based on mathematical computations built into the software. Images can also be merged under the “Merge” and “Mosaic Merge” functions, of which the latter combines partial images generated from a tiling experiment to form one whole image. Finally, the image can be aligned, projected, and the field of depth with the use of different focal plane calculations.

The “Adjust” icon also allows for basic edits including adjusting the sharpness, phase, and colors of the image. These are functions that can be performed in other editing software programs, should the user desire, but there is increased ease of use in doing so within the LAS X software. The background function icon allows for an absolute background to be set, which corrects for a background that is too low in which the image may appear noisy, or too high in which there is loss of information due to a dull signal. Although a separate section of the Process icon, “Noise Reduction” applies a median or blurred mask to the image that decreases the noise to increase the resolution (Leica).

The “Deconvolution” icon (either 2D or 3D) gets rid of out of focus light in a method opposite of convolution. Instead of a pinhole removing this out of focus light and eliminating light scattering, a point spread function is used. This reassigns the out-of-focus light to optical sections within the generated Z-Stack thereby increasing the resolution of the image (“Deconvolution.”).

The remaining icons are used infrequently, if at all, by inexperienced confocal microscopists and therefore will not be discussed. It is useful to know what edits, if any, exist within the imaging software but the consequences of editing images should always be considered. While some edits may cause data to be lost, there are many edits that create data artefacts, which creates ethical questions when these images are published and no longer represent the true image.

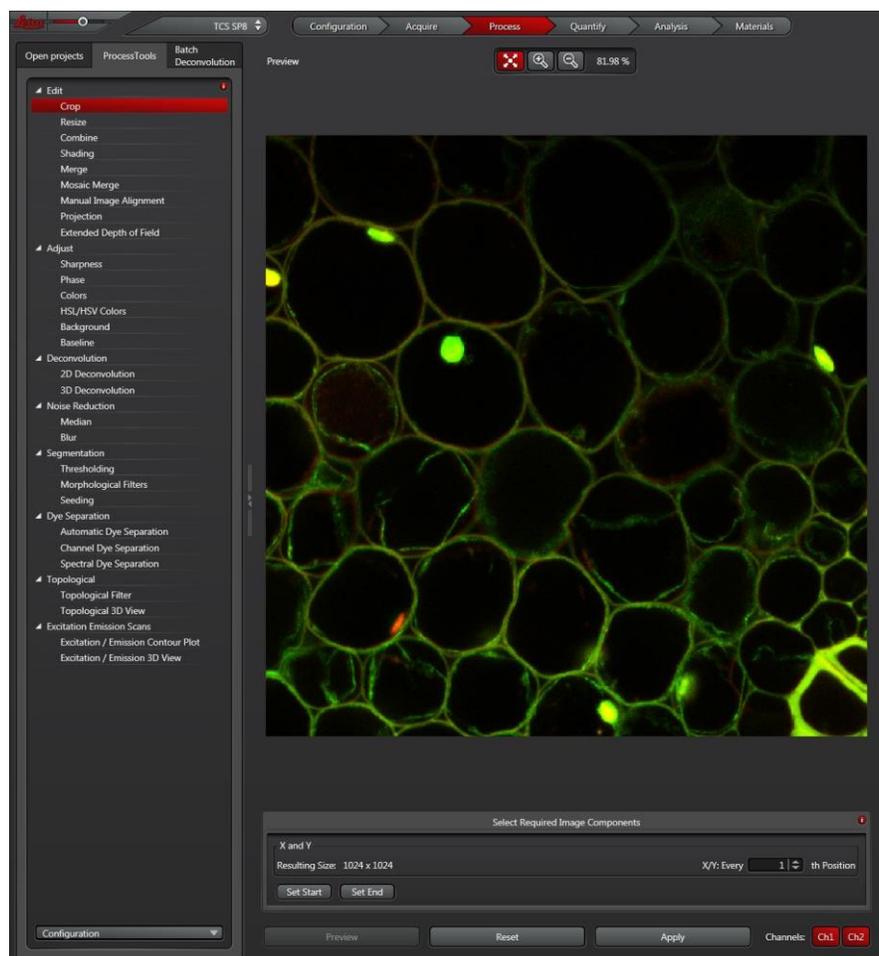


Figure 4.1 Overview of the Process Icon. There are various adjustments that can be made in this screen, which have been broken up into 8 different headings (as seen on the left side of this image). These include, “Edit,” “Adjust,” “Deconvolution,” “Noise Reduction,” “Segmentation,” “Dye Separation,” “Topological,” and “Excitation and Emission Scans.” Most of these edits can be also be performed in Image J, Photoshop, or other post acquisition editing software.

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