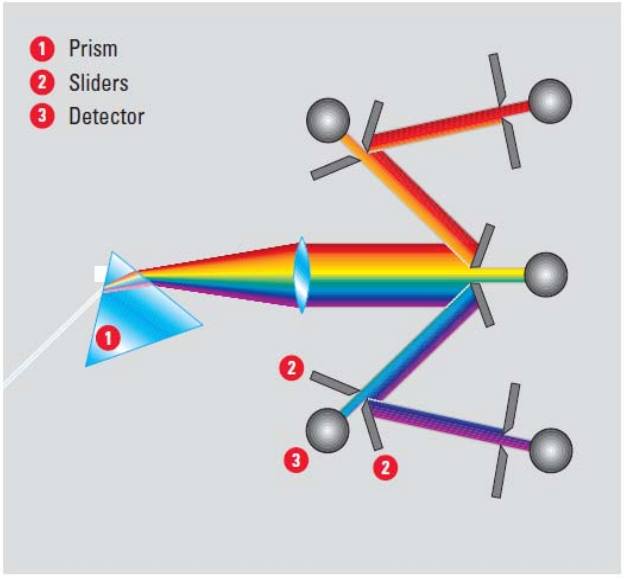
Leica TCS SP8

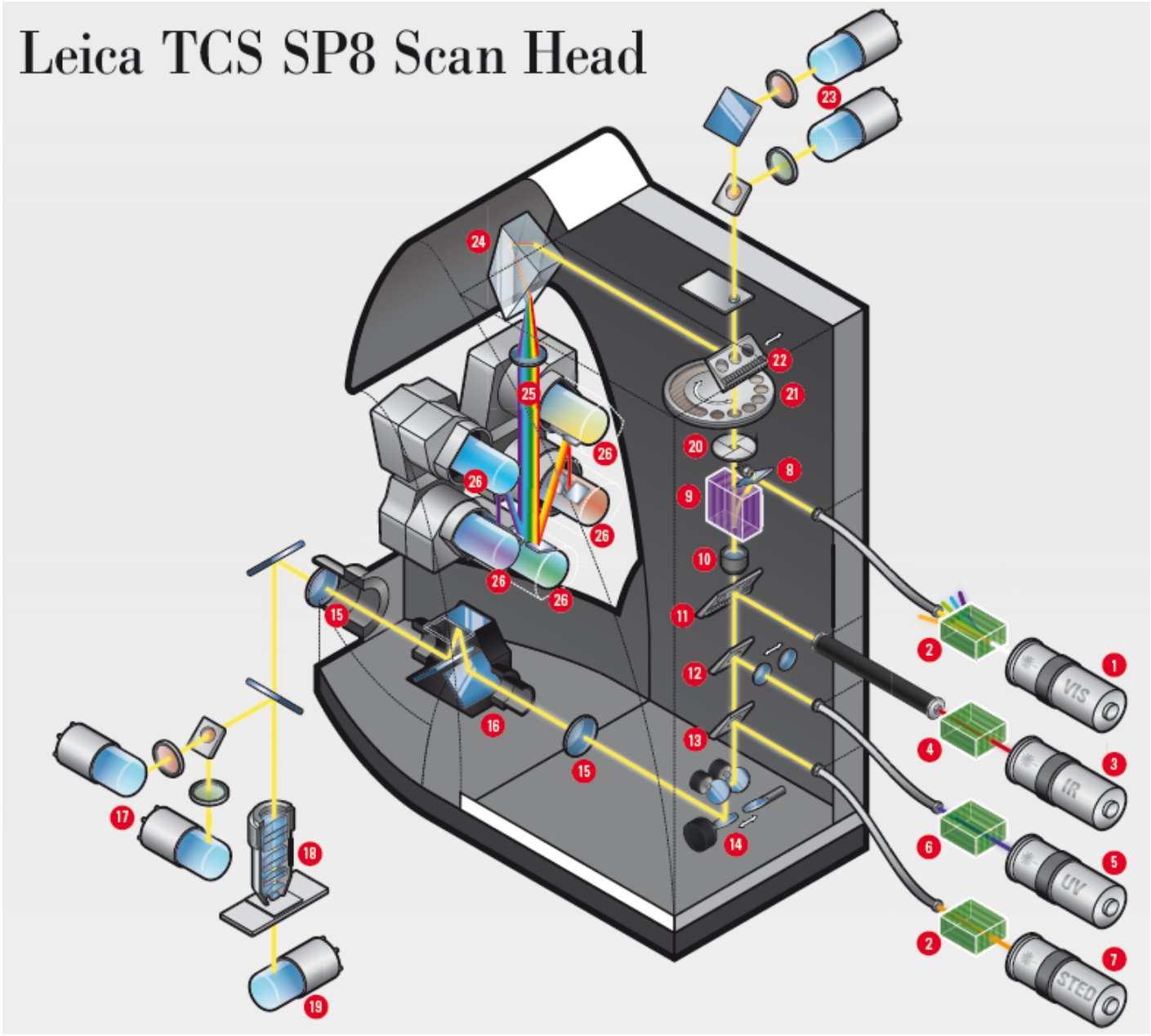
Quick Start Guide

Leica TCS SP8 System Overview



1. Research Microscope
2. Scan Head
3. Anti-vibration Table
4. EL6000 Fluorescence Illumination
5. Microscope Controller
6. Smart Move
7. Monitor
8. Control Panel
9. Keyboard
10. Computer Mouse
11. Computer Table
12. Compact Supply Unit
13. Supply Control
14. Workstation

Leica TCS SP8 Scan Head

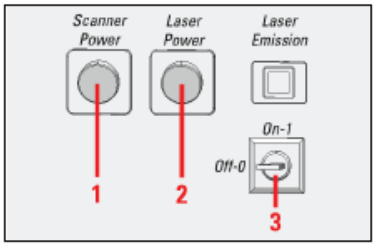


1 Visible line lasers or white light laser
2 Acousto-optical tunable filter (AOTF)
3 Infrared (IR) lasers (optional)
4 Electro-optical modulation (EOM)
5 Ultraviolet (UV) lasers (optional)
6 AOTF or direct modulation (DMOD)
7 STED laser (optional)
8 Monitoring diode for Setlight
9 Acousto-optical beam splitter (AOBS), other options available
10 FRAP Booster (optional)
11 IR laser incoupling
12 UV laser incoupling with CS2 UV optics
13 STED laser incoupling
14 FOV scanner with tandem scanner option
15 Scan optics with alternative UVIS, HIVIS or VISIR coating
16 Scan field rotation (Abbé-König rotator), optional
17 Reflected light detection (RLD) in non-descanned position (optional)
18 Objective lens (different options available)
19 Transmitted light detection (TLD) in non-descanned position (optional)
20 Square confocal pinhole
21 Fluorifier disc (optional)
22 Outcoupling with X1 port (optional)
23 External detection (optional)
24 Prism-based dispersion
25 SP detection with spectrophotometer arrangement
26 Up to five photo-multipliers (PMT) or up to four hybrid photo-detectors (HyD)

Start-Up Procedure

1. Turn on the **CTR Control Box, EL6000** fluorescent light source for the microscope stand.

2. Turn on the **Scanner Power** (1) on the front of the Compact Supply Unit (CSU)



3. Turn on the **Laser Power** (2)

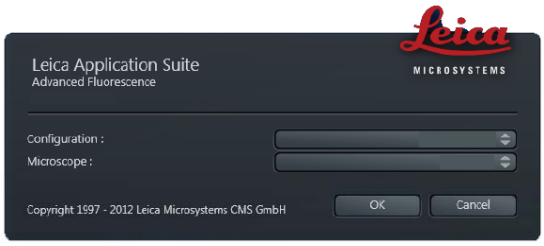
4. Enable the lasers by turning the key from the ***Off*** position to the ***On*** position (3).

5. Turn on **Workstation** and **Monitor**

6. Log into user profile

7. Double click on the **LAS X** icon to start the software.

8. Select **Machine** for the Configuration and **DMi8** for the Microscope. (the simulator is used to view and analyze images and not acquistition)



10. Click ***OK*** to start the initialization of the LAS AF (Leica Application Suite for Advanced Fluorescence)

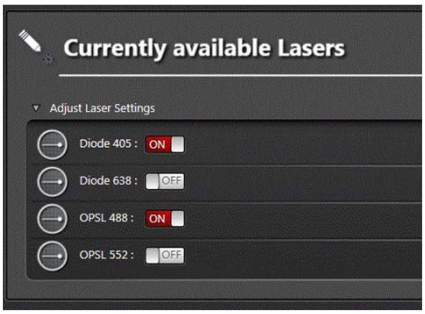
11. A message will appear asking whether you want to initialize the stage. Initializing the stage is required to activate the Tiling and Mark & Find features in the software.

If you select yes, the stage is going to move to all 4 corners to calibrate the position of the stage.

Make sure there is nothing on the stage that will hit the condenser before you select yes!

Shut-Down Procedure

1. Turn off the lasers in the software

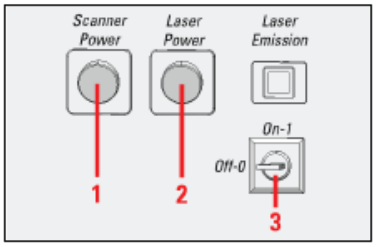


2. Lower the objective to the lowest position, remove specimen and clean all of the objectives

3. Save all unsaved data and exit LAS X.

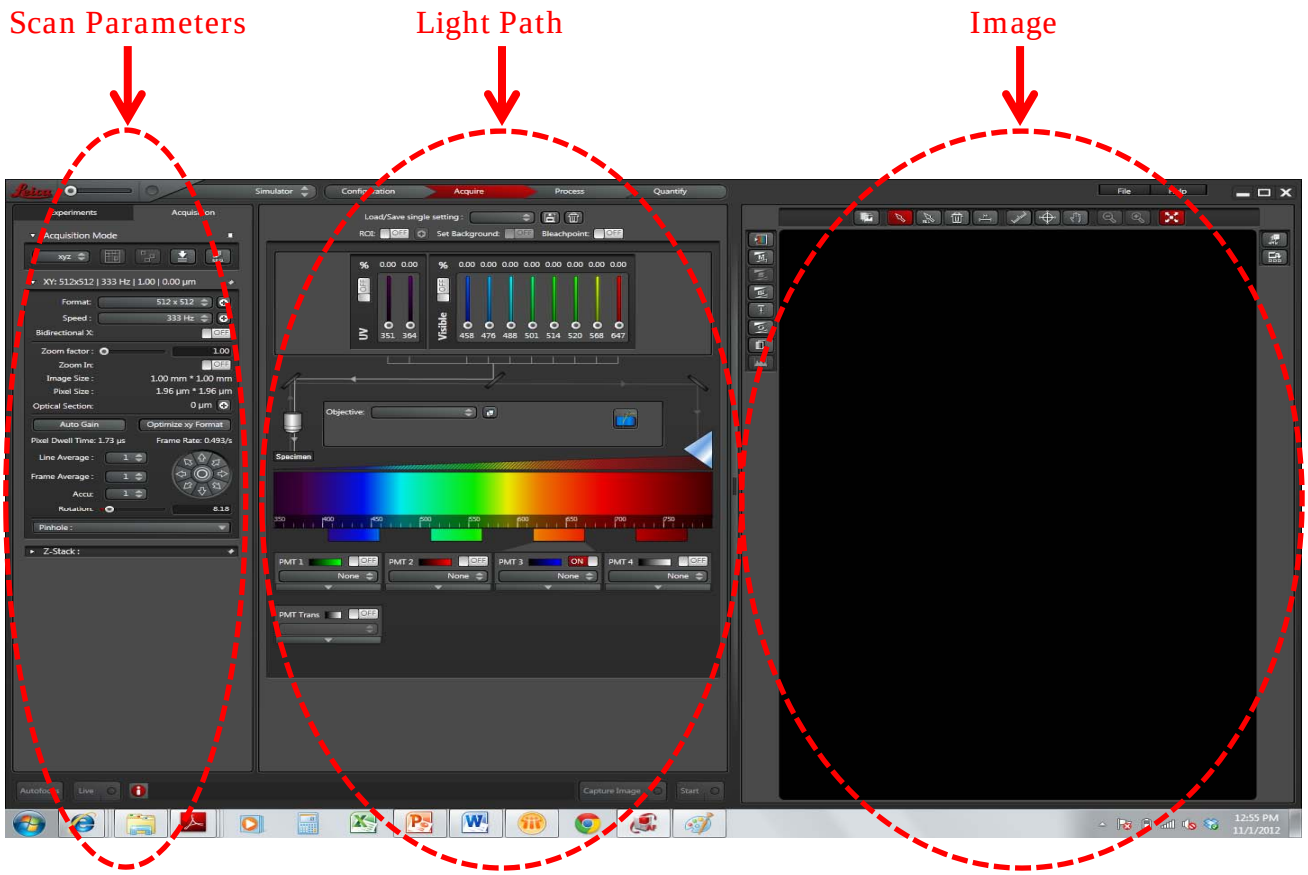
   NOTE: Do not turn off scanner and/or CTR control box for the microscope before the software is closed

4. Turn off the switches on the front of the laser supply unit in the reverse order (#3-#1) of start-up (ie. turn key off, then turn off Laser Power and then turn off Scanner Power)



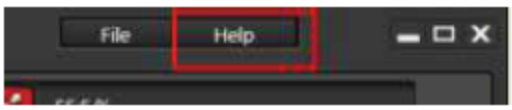
5. Turn off microscope control box

The LAS X software will open in the ***Acquire*** tab. There are 3 portions to this window:



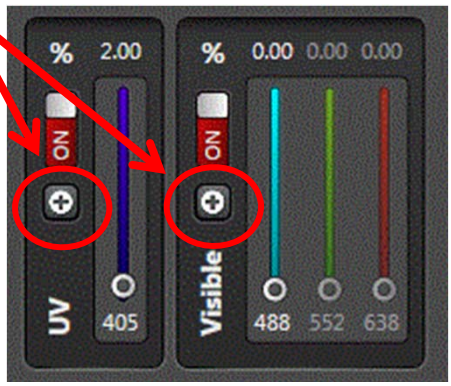
Context sensitive online help can be opened by pressing F1 and clicking on the question mark icon in the respective panel.

A complete version of the online help is found by clicking on Help in the right top corner of the user interface.

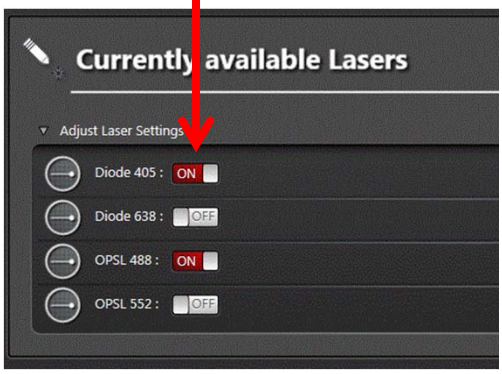


Turning on the Lasers:

1. Click on the "+" in the Laser Lines Box in the Beam Path Settings Window to directly open the main Laser Control Window

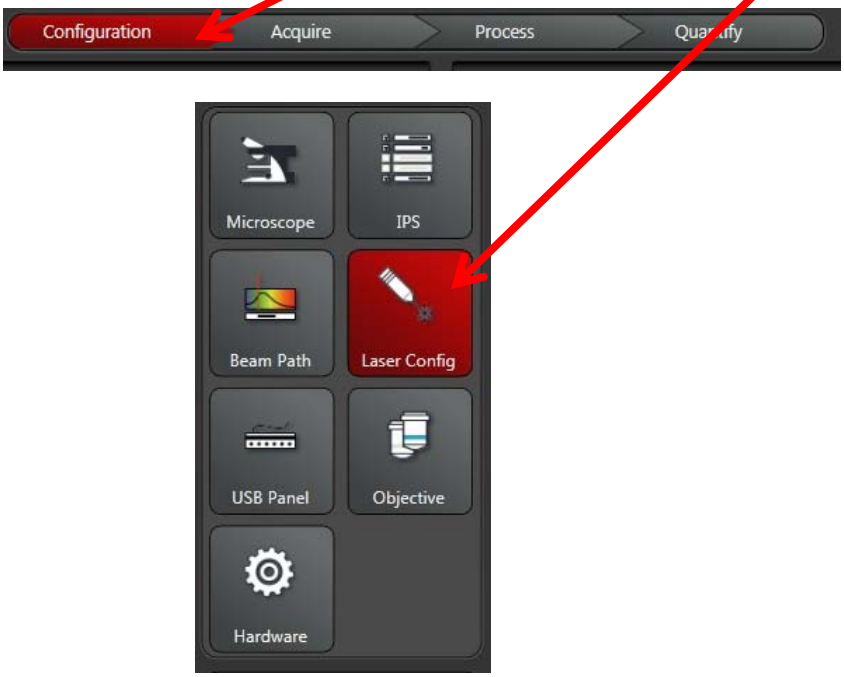


2. Activate the lasers you require



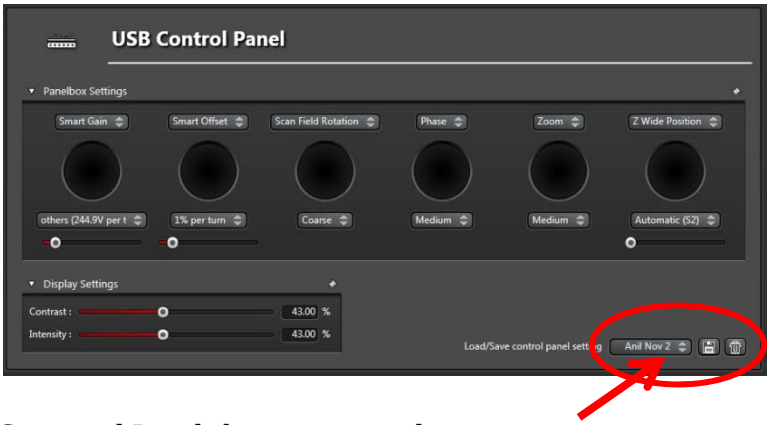
NOTE: Only turn on the laser(s) with the appropriate laser line(s) that will excite the fluorophores you are using

3. Alternatively, click on the **Configuration** tab and the **Laser** icon to open the main Laser Control Window



Additional Tools in the Configuration Tab…

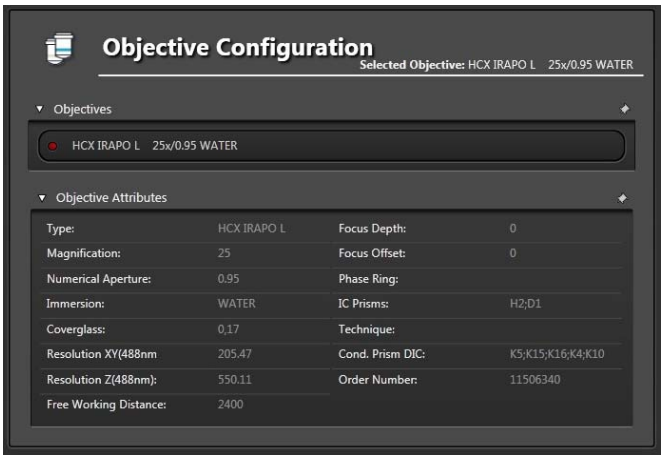
1. Customize the **USB Control Panel -** assign various parameter and sensitivities to the various knobs of the control panel



Save and Load the customized settings

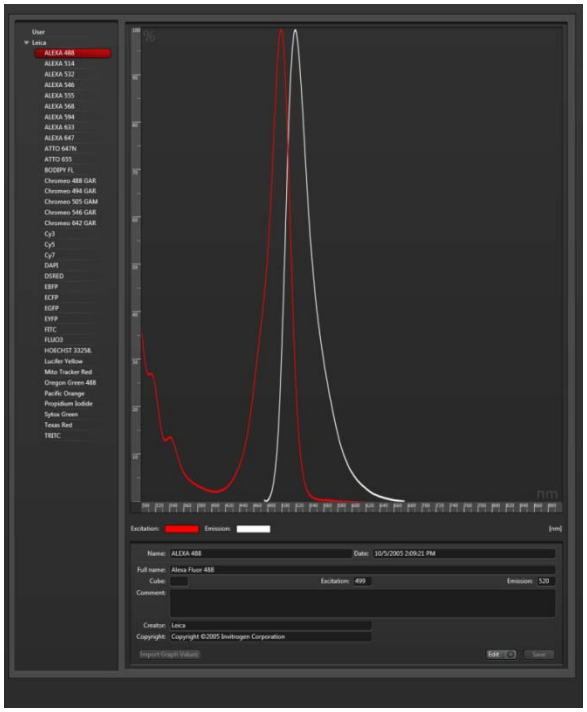
Alternatively , click on the Control Panel Icon in the Beam Path Settings Window as a short cut to the USB Control Panel Window

2. Specifications of the **Objectives** equipped on the microscope



Additional Tools in the Configuration Tab Continued…

3. Dye Database with the excitation and emission spectra of common fluorochromes

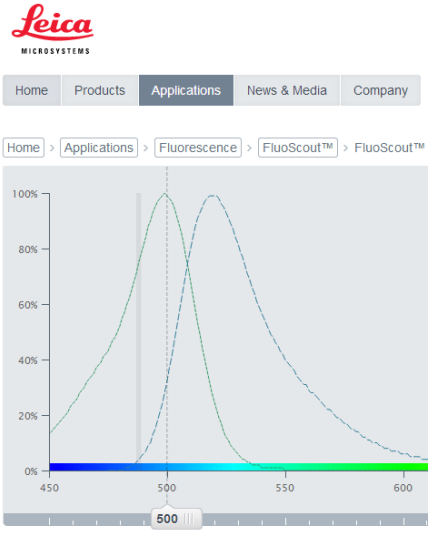


Emission spectrum from a lambda scan can be added to the dye database.

Many manufacturer's will also provide the data for fluorophores that can added to the database (see LAS AF Help)

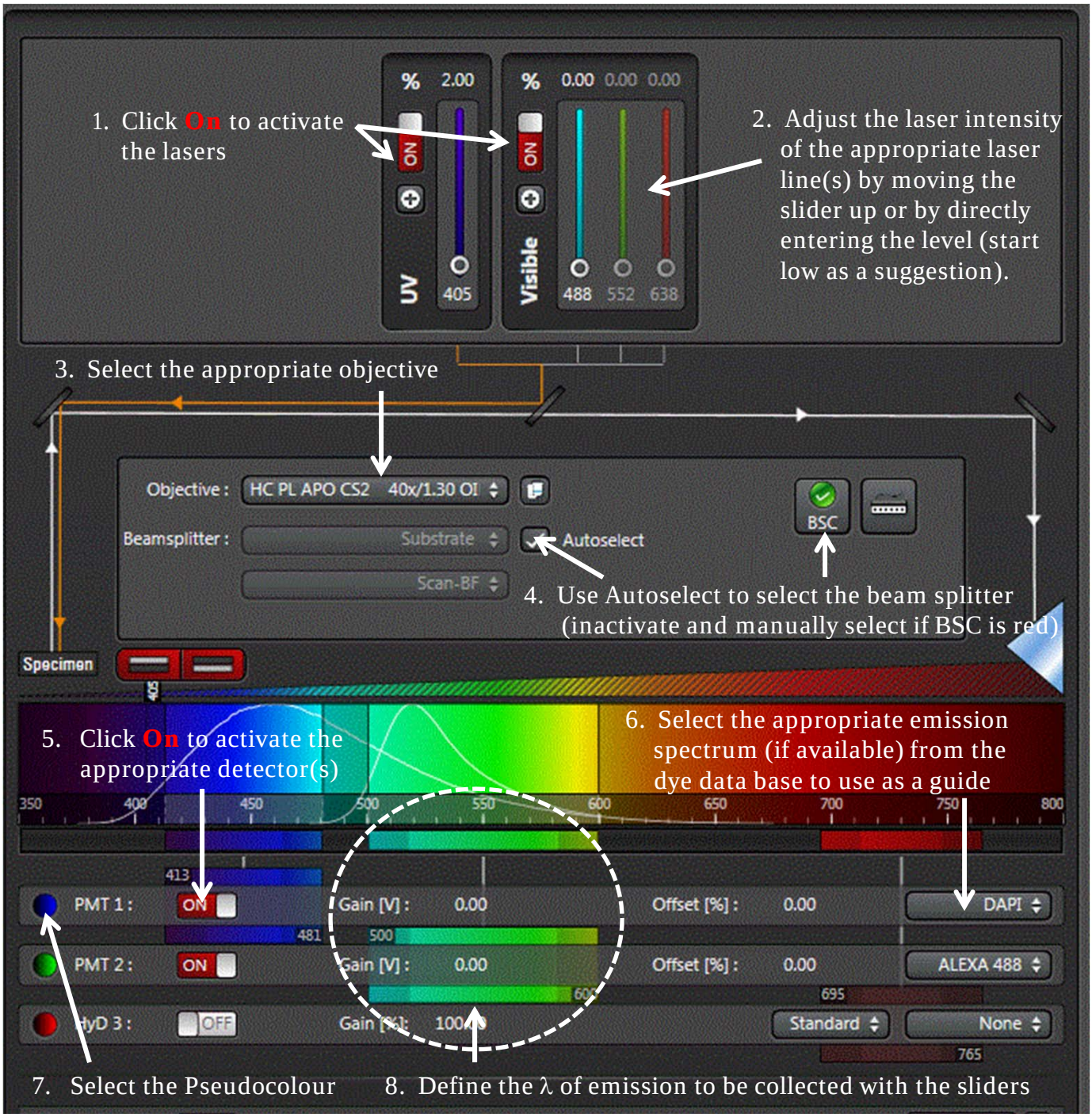
Another great resource is the Leica FluoScout interactive tool

http://www.leica-microsystems.com/fluoscout/



Beam Path Setting

Option #1: Manual setting of the beam path configuration



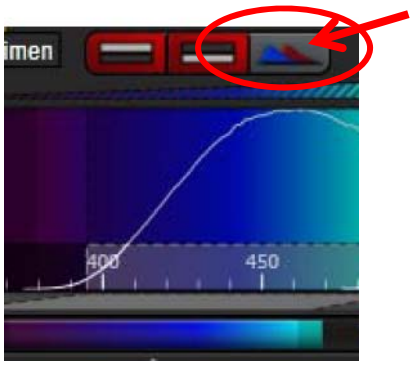
Keep in mind that the emission spectra displayed are to be used as a guide and are not defining the wavelengths collected. The specific wavelengths collected are determined by the position of the gates.

Alternatively, double click on the slider to open a window that allows you to directly enter the start and end position of the gates.

Beam Path Setting

Option #2: Dye Assistant to set beam path configuration

The **Dye Assistant** offers suggestion on system configuration based on the spectral characteristics of the fluorescent dyes being used. The user can select the appropriate option for his or her application



1. Activate the Dye Assistant.

Select the dye from the database

Select the type of detector (if applicable)

Use the "+" or "-" to add or delete a dye

Edit and Apply the settings.

The different configuration options for acquisition are suggested here and the overlap is shown graphically:

- *Yield: Intensity yield of the individual dyes*
- *Crosstalk: Intensity of the crosstalk in other channels*

(see next page)

The **Edit** (4) function opens an additional dialog.

The following settings are mode for image acquisition when clicking Apply:

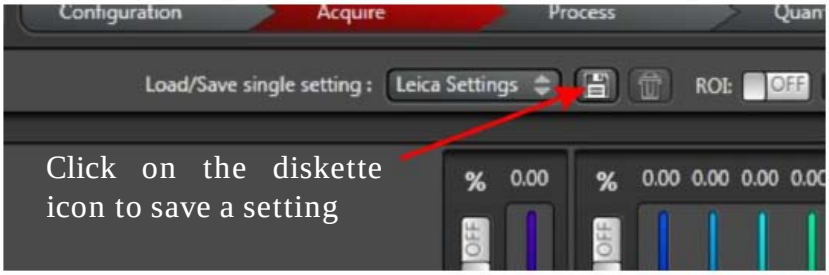
- *Selection of the laser lines*
- *Selection of the detectors*
- *Setting for the detection range*
- *Assignment of the fluorescent dyes to the respective detectors*
- *Assignment of the appropriate colour look-up table (LUT) for the respective fluorescent dyes*

All other settings for image acquisition are made as usual

Beam Path Setting

Option #3: Load/Save specific settings

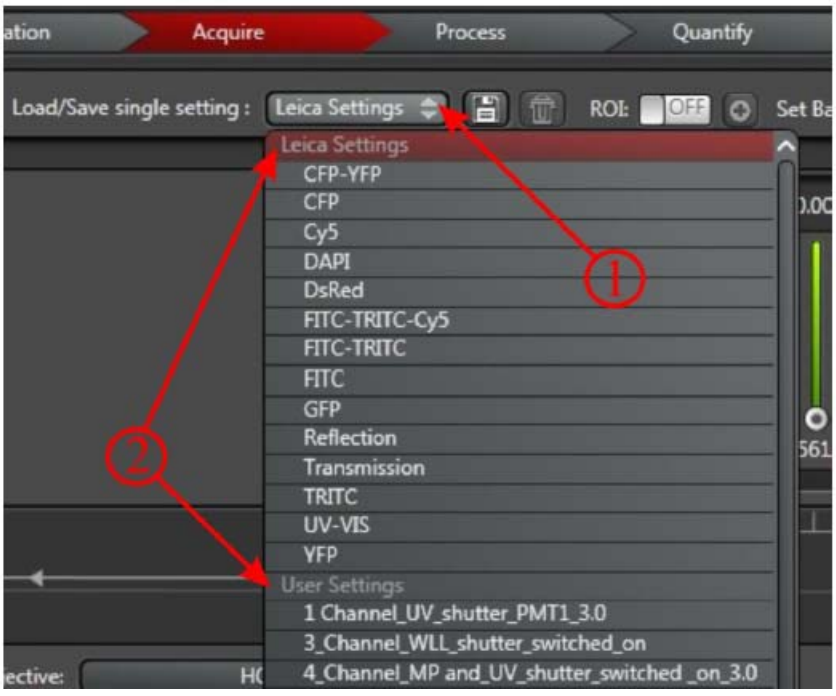
The settings can be saved for subsequent experiments with the same or similar specimens.



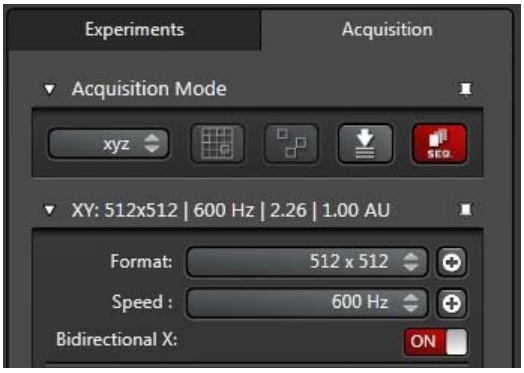
SUGGESTION: Include your name or initials when saving the settings to identify who created the configuration.

Alternatively, save an image as a sample "configuration" image and apply those settings from the Experiment tab (ie. Save an image and open it to apply the settings)

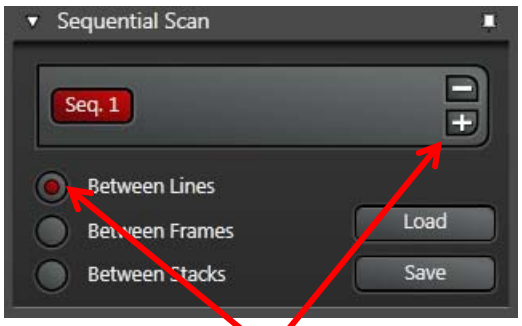
To Load the instrument parameter settings, click on the arrow keys beside "Load/Save single settings" and select the appropriate setting



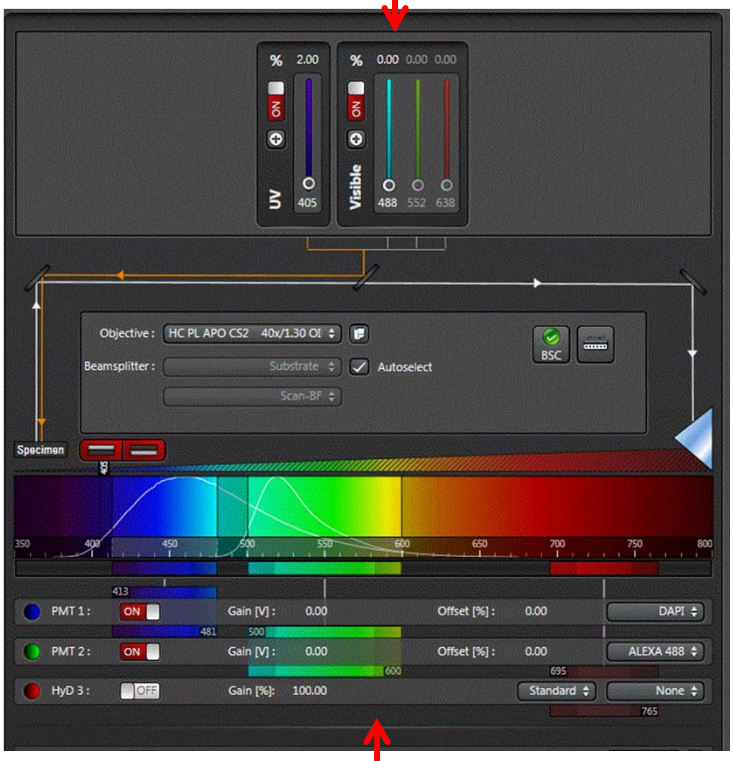
Beam Path Setting - Sequential Acquisition



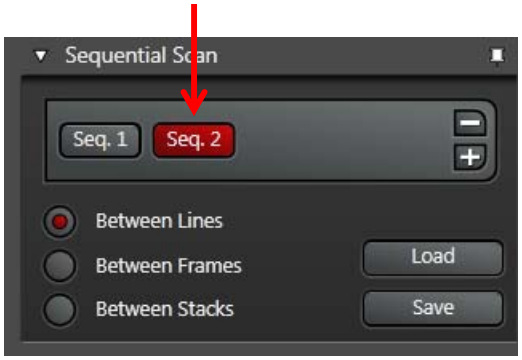
1. Click **SEQ** to open the Sequential Scan control window



2. Set the light path configuration for the first sequence (ie laser, beam splitter, detector, emission window, etc,)



3. Select switching mode and click **+** to add a sequential scan



4. Set the light path configuration for the new scan

5. Repeat if necessary

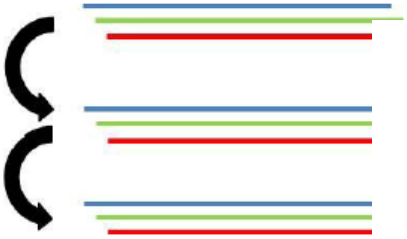
NOTE: Sequential scan settings can be saved and loaded as an alternative.

Turning on or off lasers and detectors is very fast whereas changing the beam splitter and/or position of the detection gates is much slower.

Therefore, in order to switch image acquisition after each line, these components must be the same for each of the scans

1. "Between lines"

"Between lines" is the fastest acquisition mode of the sequential scan. In this method, each line is scanned one after the other with the different settings of the individual sequences.



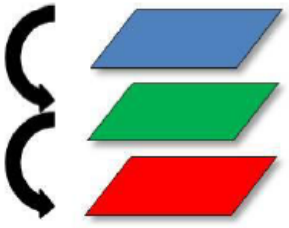
The individual images are created more or less simultaneously. The method has advantages, above all, for specimens that change their position (e.g. living cells).

In the case of overlapping detection ranges or filter changes, a "Between Lines" sequential scan is not possible, since the mechanical componants can not be changed at this speed.

Please note when configuring the sequences that hybrid detectors are not shut down electronically between the individual sequences. You should therefore avoid selecting a detection range for the HyD™ that is below the excitation line of another sequence.

"Between Frames"

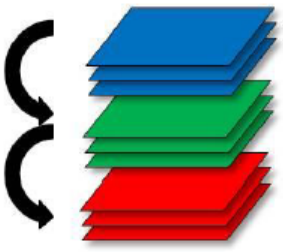
In this mode, the acquisition for each sequence is made frame by frame, i.e. with the settings for Seq. 1, a complete frame is acquired before the settings are adapted to Seq. 2 to acquire the next frame and so on.



In this mode, a sequential scan with overlapping detection ranges or filter changes can be carried out because the mechanical components have sufficient time to change their positions.

"Between Stacks"

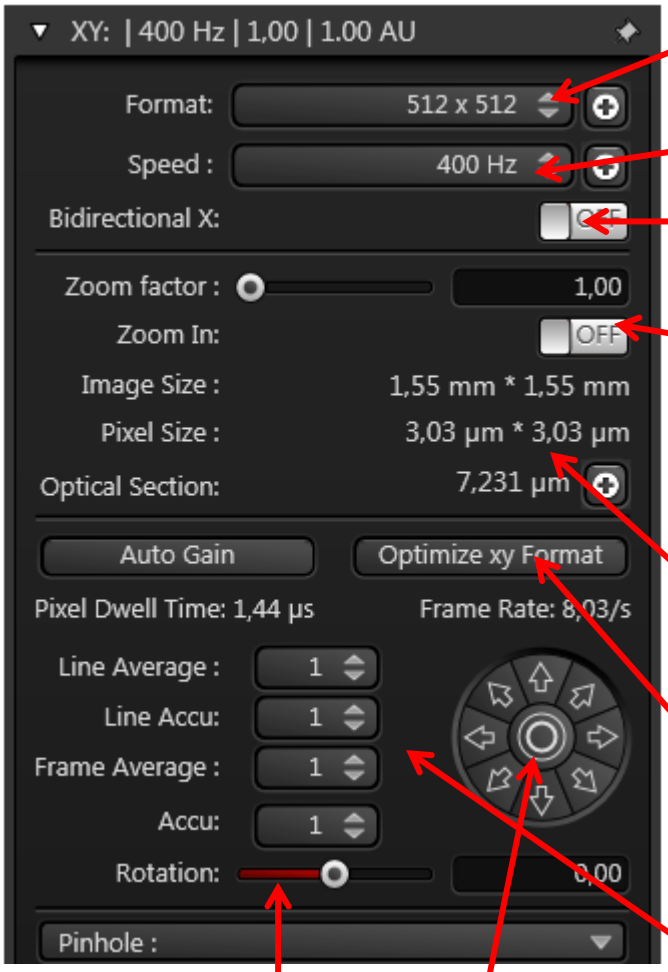
In this mode, the image acquisition takes place between the individual sequences stack by stack. For Seq. 1, the complete stack is acquired before the settings are adapted to Seq. 2 and the acquisition of the next stack takes place.



This method is suitable e.g. for fast bleaching dyes. Such a dye can be acquired with the first sequence. A subsequent bleaching of that dye during the remaining sequential image acquisition is then no longer relevant to the resulting image.

In "Between Lines" mode it is possible to switch between the individual sequences during live scan to optimize image settings. This is not possible in "Between Frames" or "Between Stacks" mode for the above-mentioned technical reasons. The live scan must be interrupted in this case in order to change to another sequence.

Acquisition Parameters



Format or # of pixels in the image (start with 512 x 512)

Scan **Speed** (start with 400-600 Hz)

Option for **bidirectional** for ~2x faster acquisition (may need to adjust phase)

Adjust zoom factor various ways by:

- Adjusting slider
- Entering specific zoom factor
- Activating the **Zoom In** and drawing a ROI in image
- Zoom knob on Control Panel

The Image Size and Pixel Size will change accordingly as the Format and/or Zoom Factor is adjusted

Select **Optimize xy Format** to set the optimal # of pixels correctly over-sample (resolution depends on NA of objective)

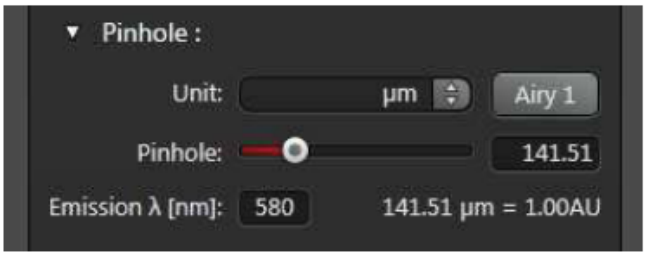
Set **Average** and/or **Accumulation**

NOTE: Averaging removes noise and in general, less averaging is required with a lower gain setting for the detectors.

The HyDs have very little, if any, noise and therefore requires less averaging than the PMTs.

Rotate image if necessary (Optical rotation)

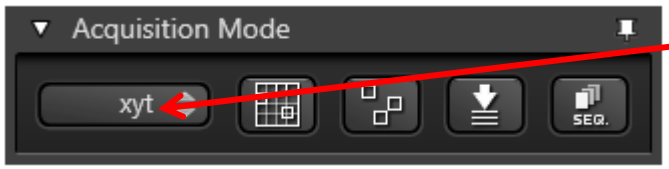
Panning to position specimen in image window



The pinhole will automatically default to 1 Airy unit (optimal) and will adjust accordingly with the different objectives.

The diameter can be be adjusted manually with the slider (or control panel)

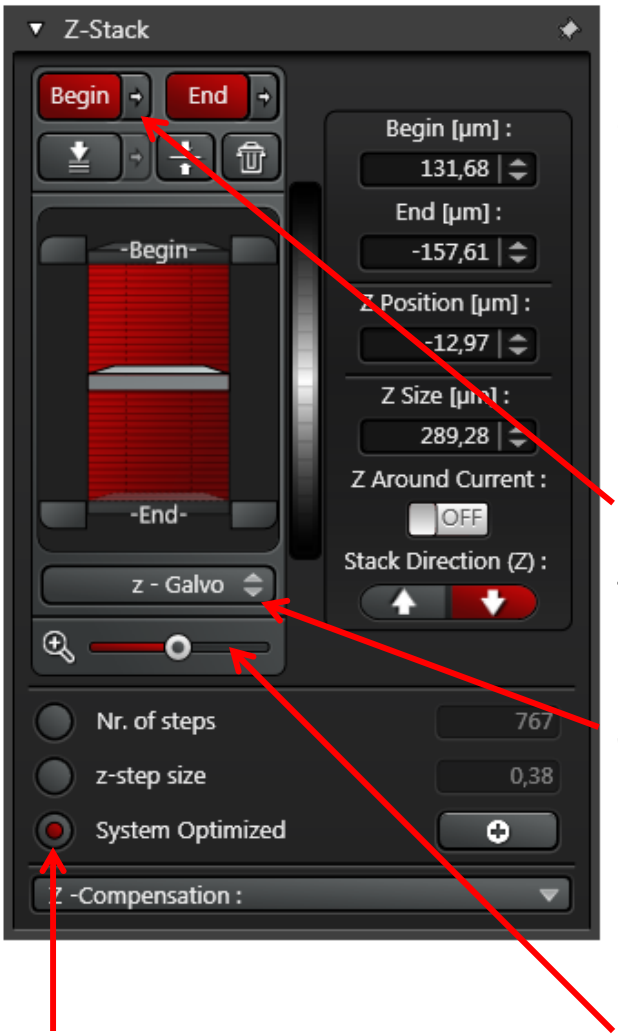
Acquisition Modes:



Select the appropriate **Acquisition Mode**

- xyz – single image or z-stack
- xzy – xz image
- xyt – time series
- xyzt -- z-stack and time series
- xyλ – lambda scan

Z Stack (xyz)



1. Select **xyz** as the Acquisition Mode

2. Use the Control Panel to move the focus position to beginning position of the stack

3. Click **Begin** to set the start position

4. Use the **Control Panel** or the **SmartMove**to move the focus position to the end position of the stack and click **End**

5. Select **Z-Wide** to indicate the focus drive of the microscope stage will be used to control z position during the stack.

   Note: Z-Galvo refers to Z-control through a Super Z Galvo Focusing stage, which in not configured with the particular system.

6. Zoom in to help visualize the schematic representation of the z stack

7. Manually define the number of images of a z-stack (Nr. of steps) and the distance between the images (z-step size), or have them optimized automatically (System Optimized).

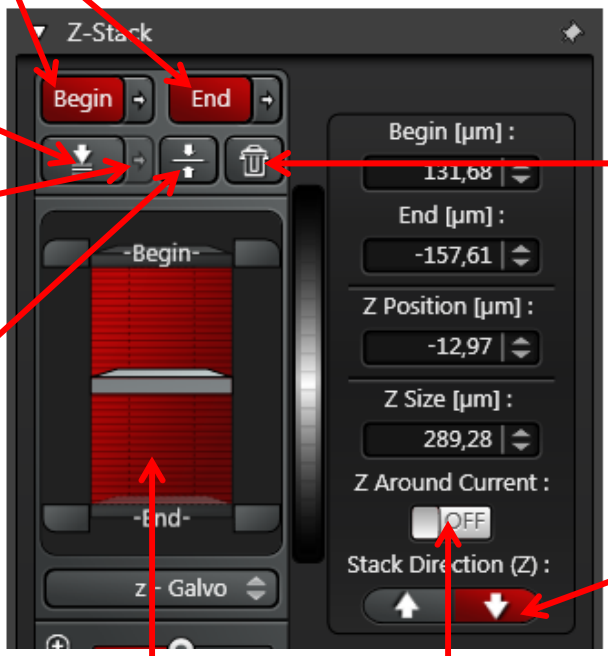
Z Stack (xyz) continued…

Use arrows to move to the set Begin or End position

Use Set Focus to define current position as the focal plane

Move to set focal position

Move to centre position of stack



Delete Begin and End Positions

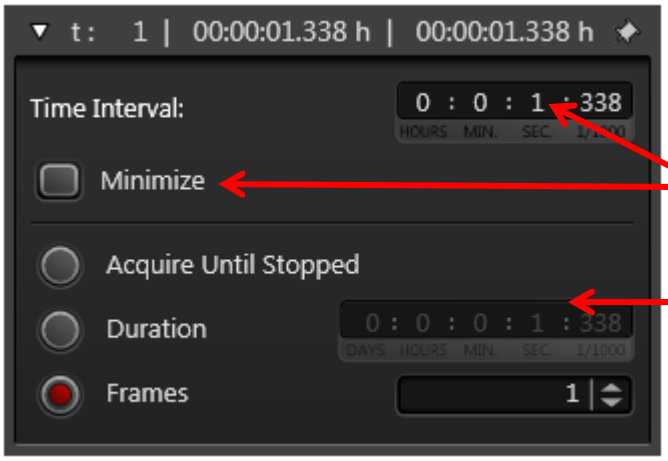
Change direction of acquisition of the stack

Use mouse to move position of objective

Alternative to setting the Begin and End position, a stack can be define around current position.

Activate **Z Around Current** and indicate the size of the stack

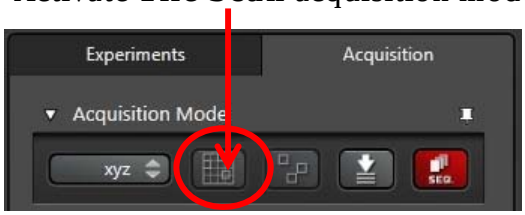
Time Series (xyt, xyzt, etc.)



1. Select the appropriate acquisition mod that includes time (t)
2. Set the Time Interval (for no delay between images, select Minimize)
3. Define the parameter for acquisition to stop

Additional Acquisition Mode – Tile Scan

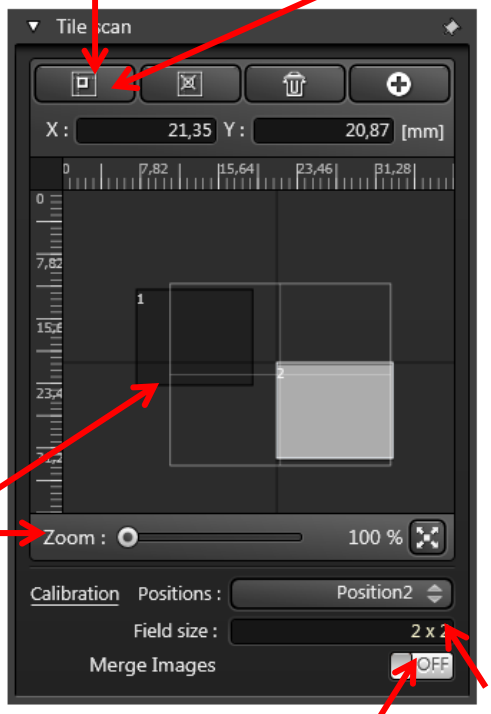
1. Activate **Tile Scan** acquisition mode



NOTE: the stage must be initialized during the start-up

Option 1

1. Move the specimen to the position that will a corner of the tile scan using the SmartMove and **Mark the Position**



2. Move the specimen to the opposite corner of the tile scan and mark the new position.

   The # of tiles will be calculated to accommodate the marked positions.

   Additional positions can be marked if necessary

3. Click on **Start** to begin the acquisition

Adjust **Zoom** slide to visualize better the current stage posistion

Activate **Merge Images** for automatic stitching and smoothing of seams after acquisition is complete.

Both the single images and merged images are added to the data container. The single images can be re-tiled using the Merge tool in the Process Tab.

The % overlap used for the automated stitching can be set under the Configuration Tab in the Stage Control window

Option 2 (easiest):

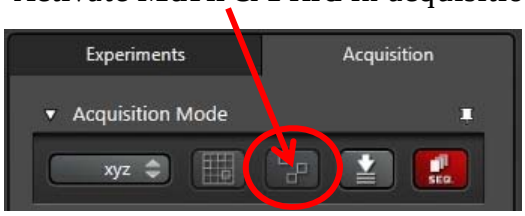
1. Move the specimen to the position that will be the centre of the tile scan using the SmartMove

2. Enter the dimensions of the tile scan (ie. 2 x 2 for a 2 image by 2 image tile scan)

3. Click on **Start** to begin the acquisition

Additional Acquisition Mode – Mark & Find

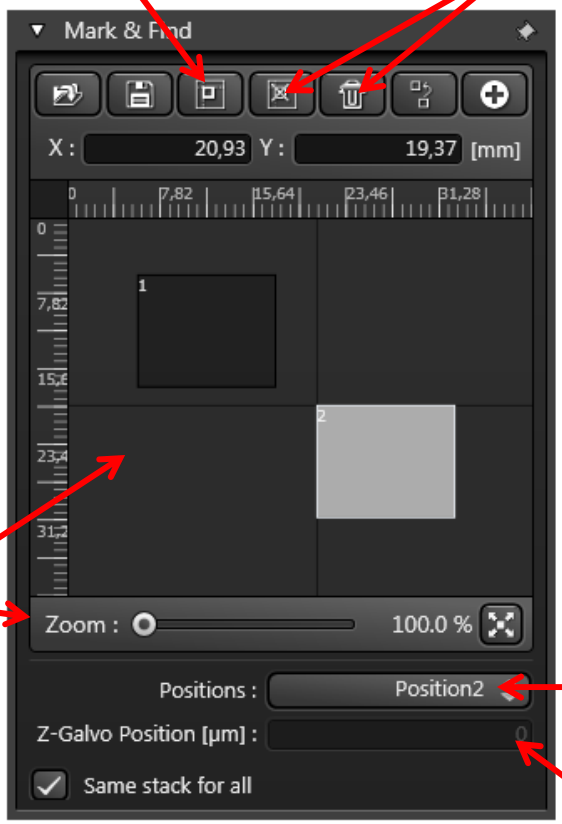
1. Activate **Mark & Find** in acquisition mode



NOTE: the stage must be initialized during the start-up

Move the stage using the SmartMove and **mark the position** of interest. Repeat as many times as necessary

Click to delete current saved position or all saved positions with the trash can



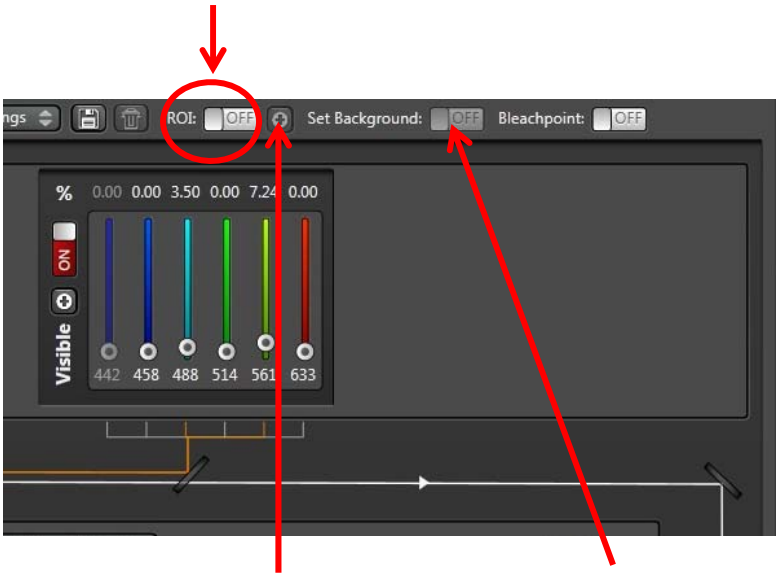
Adjust **Zoom** slide to visualize better the current stage position

Move the stage to a specific saved position

Z-position set with Z-Galvo stage is also save with the xy coordinates

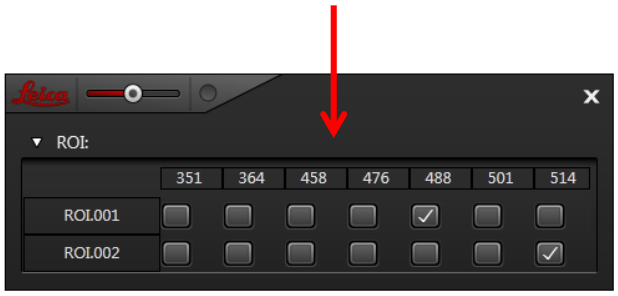
Region-of-Interest (ROI) Scanning

1. Turn on **ROI Scanning** and draw ROI(s) in image (use tools at top of image window)



2. Open the ROI Configuration window by clicking on the "+". Check on/off the laser line(s) for each ROI and adjust the laser intensity with the sliders (in the light pathway window)

3. To adjust the laser intensity for the "background", turn on Set Background and then adjust the laser intensity sliders.



Transmitted Light Image – Brightfield or DIC

Go to the following link for a tutorial on the basic concepts of DIC

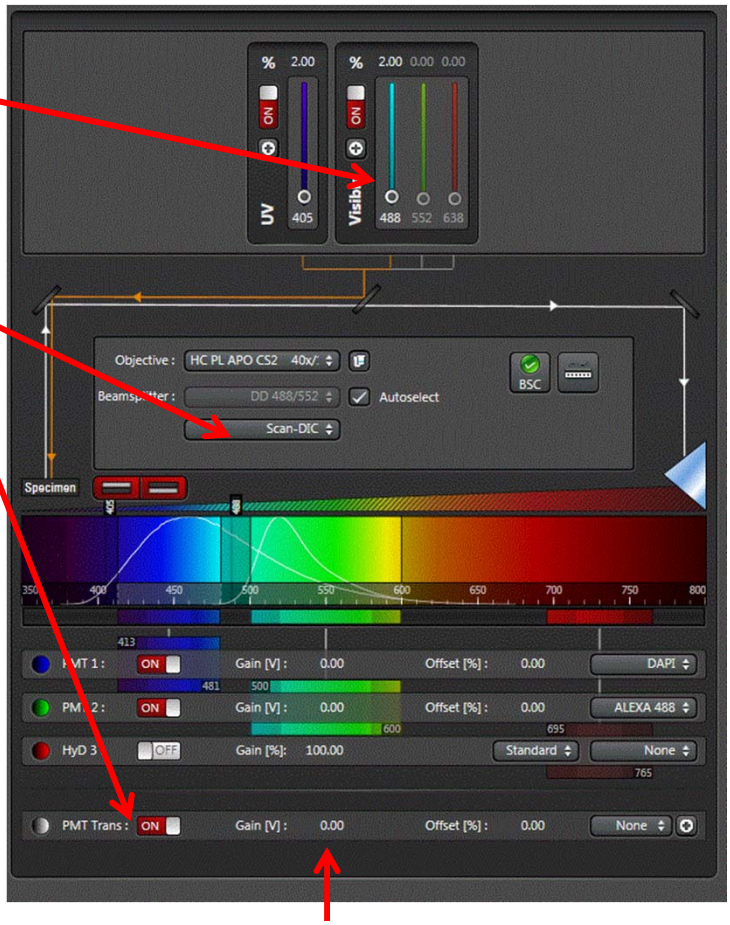
http://www.leica-microsystems.com/science-lab/differential-interference-contrast/

1. Ensure Koehler Illumination of the microscope is set correctly

http://www.leica-microsystems.com/science-lab/koehler-illumination/

2. Activate a laser line

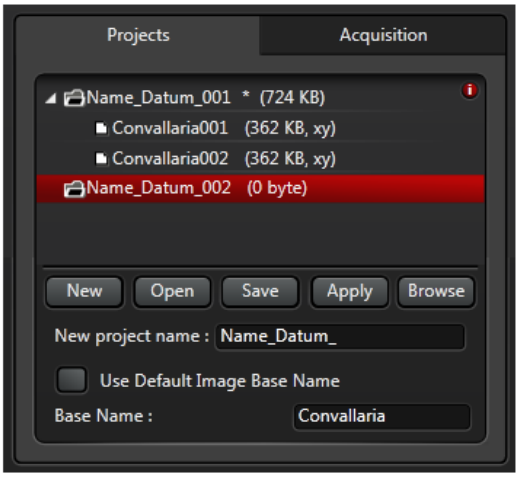
3. Turn on Transmitted PMT and select Scan-BF or Scan-DIC



4. Adjust Gain of detector

5. For DIC, adjust Bias of the objective prism if necessary

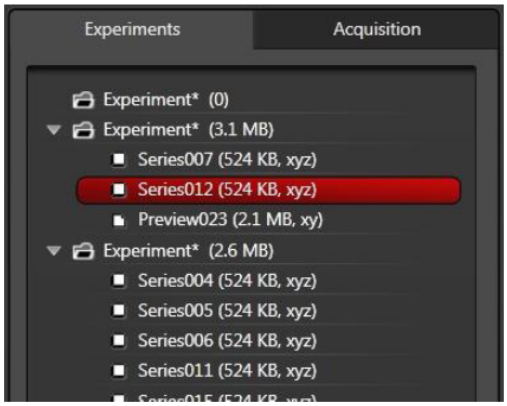
Experiment/Project Tree – Saving Images



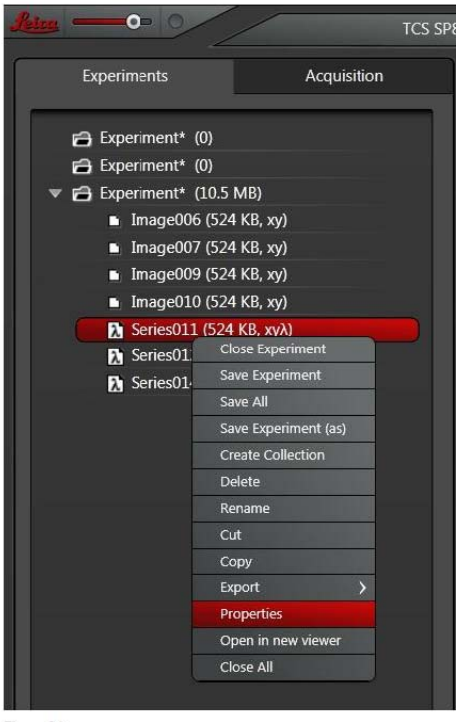
All images and image series you have acquired and (where applicable) edited are collected in **Projects** and shown in a directory structure. Newly created images and image series are always stored in the active project.To display an image or image series in the display window, select it from the directory structure.

You can organize the directory structure according to your requirements. For example, you can copy and move images and image series using drag and drop:

- Copying: Drag an image or image series from one project to another while holding down the left mouse button, then drop it by releasing the mouse button.
- Moving: Drag an image or image series from one project to another while holding down the SHIFT key and left mouse button, then drop it by releasing the mouse button.



Right Click on an image for more options



Cut: Cuts out an image file and copies it onto the clipboard.

Copy: Copies an experiment or image file to the clipboard.

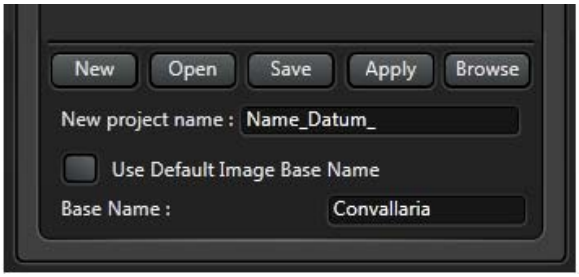
Export: Saves an experiment or image file in a different file format in order to open images or animations in an external application (also refer to section 7.1).

Properties: Opens a display of instrument parameters for a selected image file.

Open in new viewer: Opens a further display window.

Open in MMAF: Opens the experiment in the Leica-MMAF software (if installed).

Close all: Closes all experiments



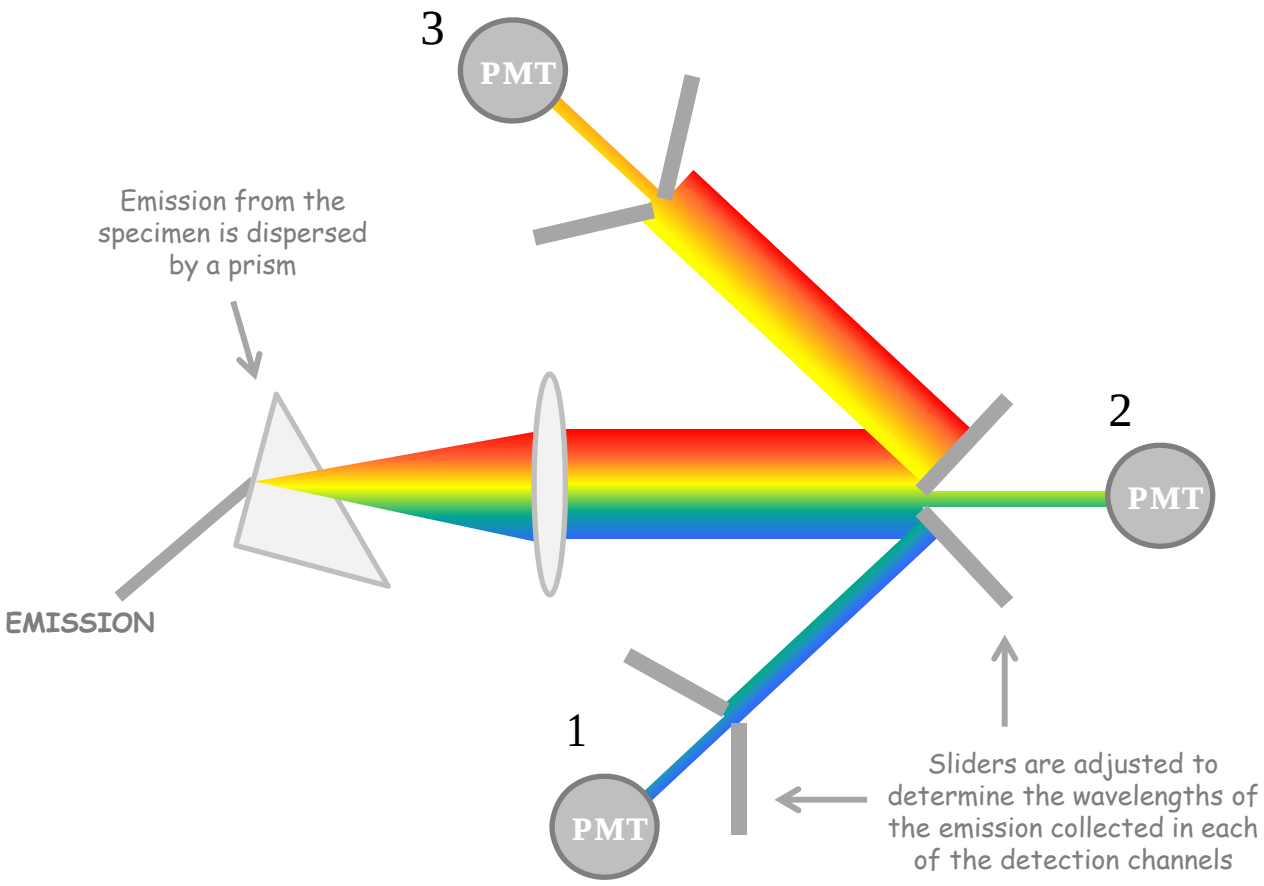
New	This creates a new project with the name specified under **New project name** and a sequential number.
Open	Opens an existing project.
Save	Saves all opened projects.
Apply	Applies the instrument parameters of the highlighted project. Only the instrument parameters that have been selected under **Configuration > Instrument Parameter Settings (IPS)** are applied. See: Instrument Parameter Settings (IPS)
Browse	You can use **Browse** to call up the File Browser dialog. The file browser searches and browses for LIF files created using LAS X and displays a preview of the included images in a display window. The associated metadata can be used as filter criteria, so that you can make a targeted search for certain LIF files, image data sets, or images.
New project name	Here, you can define a default name for your projects. All newly created projects are assigned this name and a sequential number.
Use Default Image Base Name	If this check box is marked, system-specific names are used for your images: "Image" for images, "Series" for image series, etc. If this check box is not marked, you can define the name of your images in the **Base Name** field.
Base Name	Use this function to define the name of your images. All new images are assigned this name and numbered in ascending order. This name can be adapted from one image to the next.

Additional Information - Spectral (SP) Detection System

This particular system has 3 separate internal detectors for confocal imaging

The Leica TCS SP8 does not use emission filters to define the wavelengths of emission collected, but rather utilizes a prism-based approach.

The wavelengths of emission are dispersed across the entire visible spectrum and each detector has a set of gates that are positioned and width adjusted to collect the desired wavelengths.



Optimizing Pixel Format (Resolution)

Optimize xy format will adjust the pixel size to follow the Nyquist-Shannon theorem:
Sampling frequency is at least double that of the resolution. Usually, a factor between 1/2 and 1/3 is chosen.
Accoridng to the Rayleigh criterion the minimal distance of two resolvable point sources is described by

$$R_{x,y} = \frac{0.61\,\lambda}{NA} \qquad R_z = \frac{2\lambda\eta}{(NA)^2}$$

Since the PSF for confocal is ~30% smaller due to the pinhole and the following formula applies

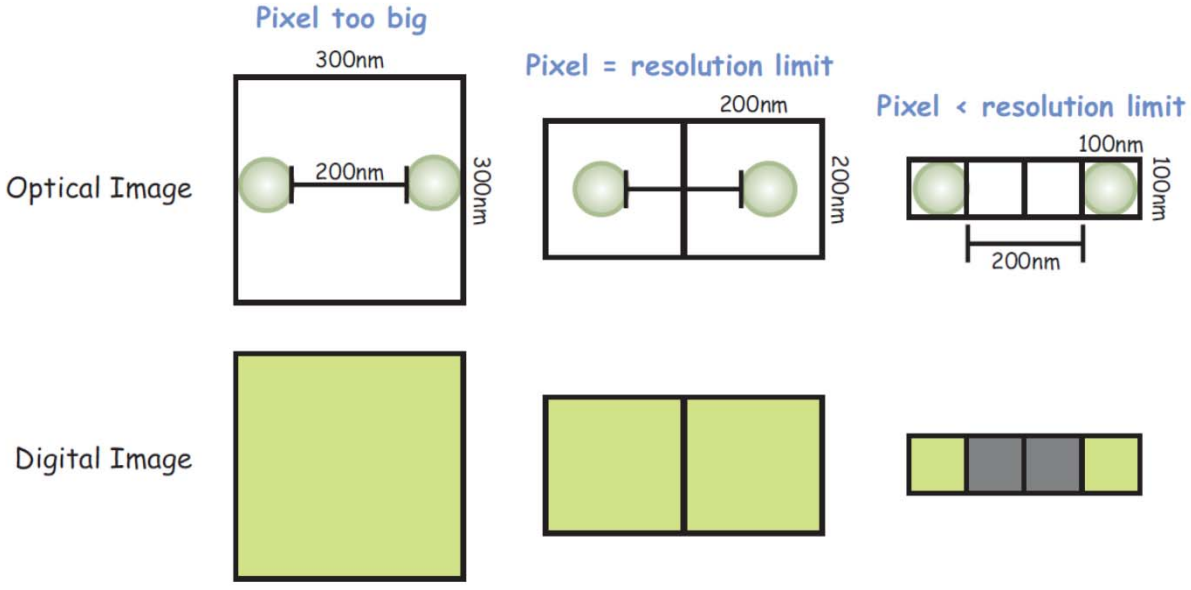
$$R_{x,y} = \frac{0.4\,\lambda}{NA} \qquad R_z = \frac{1.4\lambda\eta}{(NA)^2}$$

When using the above formula to determine the lateral resolution sampling of 3 pixels per Airy disk radius is pratical. The optimal pixel distance is then described by

$$d_{pix} = \frac{1}{3} \times r_{xy} = \frac{1}{3} \times \frac{0.61 \times \lambda ex}{NA}$$

- Requires a sampling frequency of at least 2x-3x the highest spatial frequency to accurately preserve the spatial resolution in the resulting digital image

Too few pixels – pixel size is too large for maximal resolution
Too many pixels – oversampling does not add spatial resolution
Optimal # of pixels – 2-3x the resolution limit



- Optimize the pixel size to for optimal resolution by adjusting the zoom and format (#pixels) of the image

Buttons for further editing of the acquisition are arranged around the display window.
The following functions are available using the buttons at the top edge of the display window:

Expands the list of buttons by means of the image explanation functions (annotation tools).

Plots a rectangular frame by means of holding down the left mouse button and dragging.

Plots a round frame by means of holding down the left mouse button and dragging.

Plots an arrow by means of holding down the left mouse button and dragging.

Inserts a sequential number in the clicked area with each left click in the display window.
A dialog for setting the color, font name and font size opens with each first click. When you click on "OK", the digit "1" is inserted in the area previously clicked on in the display window.

Plots a straight line by means of holding down the left mouse button and dragging.

Inserts a text field in the display window. Within the open dialog, you can set color, font name and font size in the "Format" tab. Choose between text and date/time in the "Value" tab, then you can configure corresponding entries for selection. Click on "OK" to insert the text field in the area previously clicked on in the display window.

For further processing, such as shifting, loading, saving, changing the font type/color, the annotation tools can be treated as ROIs (see section 12.2).

Marks <u>one</u> annotation tool by means of a left click.

Marks <u>all</u> annotation tools.

Deletes all marked annotation tools.

Plots a scalebar by means of holding down the left mouse button and dragging (units in µm). The scalebar is used as an annotation tool for all additional processing steps (e.g. marking or saving).

Inserts a straightedge at the upper left edge of the image. The unit is specified as unit of length or number of pixels. You can switch between the units by clicking on the unit in the upper left corner.

Inserts a crosshair in the display window.

After activating this Hand Tool button, the image in the display window can be moved by clicking within the image and dragging it with the left mouse button held down. For this function to work, the zoom must be greater than the maximum display size in the display window.

Reduces the image in the display window.

Enlarges the image in the display window. If the image size exceeds the display size, gray navigation bars appear at the lower and right-hand edge of the image.

Adjusts the displayed image to the display window size. You can also change the image display size with this function using the mouse wheel, after previously clicking in the display window with the left mouse button.

Maintains any „Annotation Tool" in an active state. This enables to draw e.g. several scalbars in succession, without re-activating the „Draw scalebar"-Button in between.

The following functions can be found at the right-hand edge of the display window:

Displays the acquired channels with consecutive numbering. The channel shown in the display window are shown as active=red; channels that are not shown are inactive=gray. Click on the button to switch between active and inactive (also refer to section 9).

Shows the active channels as an "Overlay" (also refer to section 9).

Besides the image plane currently shown in the display window, "Show Gallery" allows you to display all other planes of an acquired image series in the lower area of the display window (also refer to section 10.4).

The following functions can be found at the left-hand edge of the display window:

"Quick LUT" allows a fast change of the color look-up table. With left clicks on the button, the previously selected color look-up table, "Glow (O&U)" and a color look-up table in gray tones are displayed in turn (also refer to section 6.5).

Manual/Auto Contrast: In these modes, the display area of the color look-up table can be configured manually or automatically. By clicking on the button, you can switch between "Manual Contrast" and "Auto Contrast". For "Manual Contrast", use the sliders of the color look-up table for configuration (Figure: 111/1).
For "Auto Contrast," the display area of the color look-up table is adapted automatically to the gray-scale values of the acquisition. Gray-scale ranges that are not used are not displayed.

Stack Auto Contrast: In this mode, "Auto Contrast" relates to the image with the largest gray-scale range within an image stack. When navigating through the image stack, the display of the color look-up table is then no longer changed.

Channel Auto Contrast: In this mode, "Auto Contrast" relates to the image currently shown in the display window. When navigating through the image stack, the display of the color look-up table is then no longer changed.

Channel Auto Range: In this mode, only the range of the color look-up table that is contained in the acquisition is displayed. This makes it possible to expand the dynamic range and thus work with the complete bandwidth of the color depth.
This is particularly helpful with very dark acquisitions and acquisition with the HyD™ in "Counting" mode.

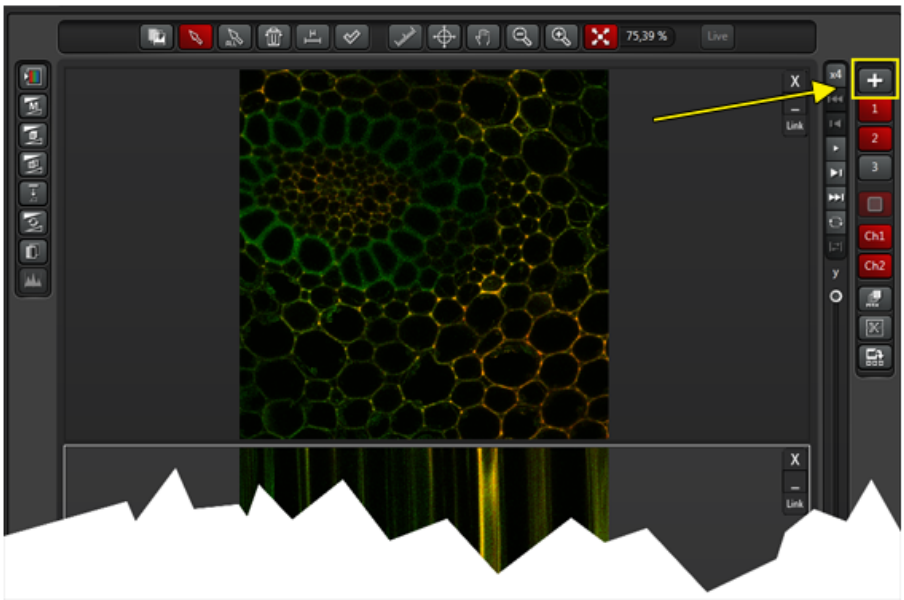
Reset: Resets the display of the color look-up table to the full available color depth.

Use background LUT: Allows you to call up another color look-up table for the image background. To do so, move the sliders in the display of the color look-up table slightly upwards or accordingly downwards. You can now double-click on the area below or above the sliders to select a second or third color look-up table. By shifting the sliders, you can now define up to which gray-scale value of the image pixels the color look-up table for the background is used.

Scaling: Displays in the lower area of the display window a histogram on the gray-scale values of the current display (Figure 110).

Opening the Comparison View

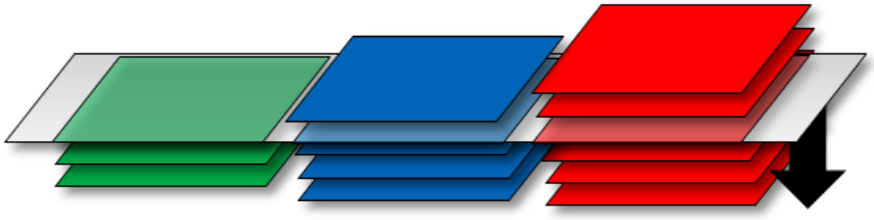
Open the comparison view using the + button on the right edge of the display window.



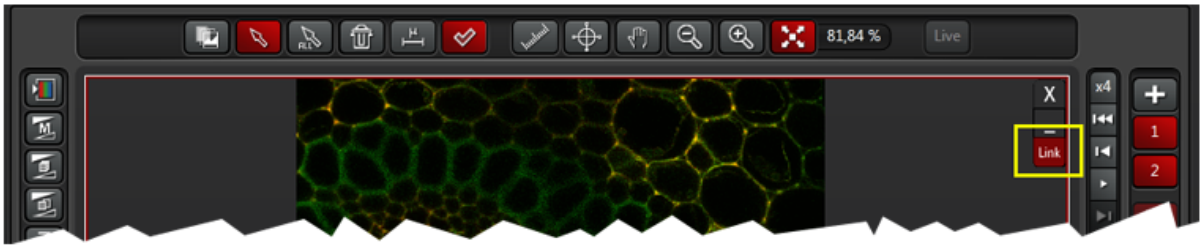
Each additional click on the + button opens a new display window where another acquisition can be displayed. Numbered buttons underneath the + button show the number of open display windows. You can also use these buttons to hide (gray button) and display (red button) the individual display windows.

Linking Displays in the Comparison View

The images and image series shown in the display windows can be linked together to obtain an overview of similar areas and structures within the specimens in all display windows. First, images shown enlarged using the zoom can be linked so that when moving the display in one of the display windows, the linked displays in the other display windows move and follow the same travel path. In addition, image series can be linked so that when you perform tasks such as going through a specimen in the z-direction in one of display windows, the system goes through all of the other linked series in the z-direction. The individual series are coupled at the level displayed in the display window at that moment so that series with a different number of levels can be compared to each other as well.



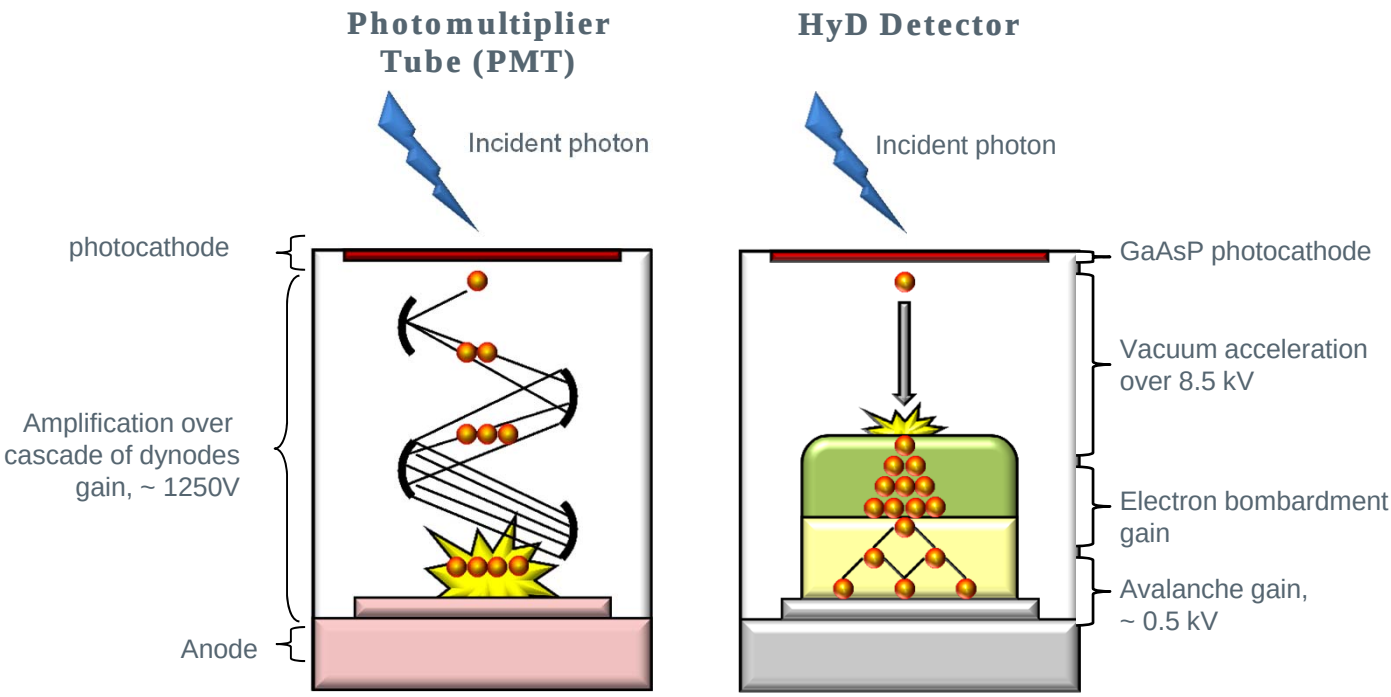
If you have adjusted the displays in the individual display windows accordingly, click on Link in the display windows that have a display that is to be linked. Clicking on the button again removes the linking in the respective display window.



Closing the Comparison View

Click on the **X** in the top right corner to close a display window. Closing the second-to-last display window automatically closes the comparison view. The last remaining image is then displayed again using the standard display in the display window.

Additional Information -- Types of Detectors



The Hybrid Detectors (HyD) are a new type of detector for laser scanning confocal microscopy available only with the TCS SP8.

The HyDs very efficiently detect and amplify the signal from the specimen. This is due to the unique design of the HyD, which which includes a combination of the GaAsP photocathode (45% QE) and the fast, noise-free avalanche amplification rather than the slow and relatively noisy step-wise amplification through a series of dynodes with a standard PMT and a GaAsP-PMT.

NOTE: A GaAsP-PMT (often referred to as a GaAsP detector) is not the same as a HyD.

For more information:

http://www.leica-microsystems.com/science-lab/sensors-for-true-confocal-scanning/

http://www.leica-microsystems.com/science-lab/detectors-for-sensitive-detection-hyd/

http://www.leica-microsystems.com/science-lab/step-by-step-guide-to-hybrid-detection-and-photon-counting/