

Zeiss 880 (Inverted) Confocal/Multiphoton Microscope Detailed User Guide

Starting the Zen Software

- Double click the ZEN icon on the WINDOWS desktop
- The ZEN Main Application window and the LSM 880 Startup window appear on the screen

- In the small startup window, either choose to *Start System* for image acquisition or go to *Image Processing* mode to edit already existing images. Optional: Toggle the little arrow symbol to view the Boot Status display.



Zen Main Application window

- After Startup, the ZEN Main Application Window opens.
- On the Left Tool Area, the user finds the tools for sample observation, image acquisition, image processing and system maintenance, easily accessible via the five Main Tabs (*Locate*, *Acquisition*, *FCS*, *Processing*, and *Maintain*).
- All functions needed to control the microscope can be found on the **Locate** Tab; to acquire images use the **Acquisition** Tab.

Locate

View sample through microscope eyepieces using either a halogen lamp or metal halide lamp source; focus, and locate region to image.

- Click on the objective icon to select objective lens
- Click on either the Brightfield or Fluorescence buttons to turn on lamps and view sample thru eyepieces.
- For Fluorescence, click on filter icon to select filter to view specimen fluorescence.
- To turn light source off, close appropriate shutter, or for Brightfield, click Off button next to Transmitted Light, and for Fluorescence, click Off button next to Reflected Light.
- Incubator blue tool bar – for temperature and CO₂ control



Light path to view sample thru eyepieces: L - Brightfield, R - Fluorescence

- When ready to do confocal, click on *Acquisition* Tab

Acquisition

- *Make sure Show all Tools box is checked.*
- *Click on blue tool panels to view/hide items.*
- *Activate Show all by hitting View on the main menu, then click on Show all (global).*

Tools in Main Acquisition Tab:

Laser – manual turn on/off of lasers; view Properties of highlighted laser

Light Path – Imaging modes; diagram of excitation and emission light path of fluorophores

Acquisition Mode – imaging parameters

Channels – laser power, detector and pinhole settings

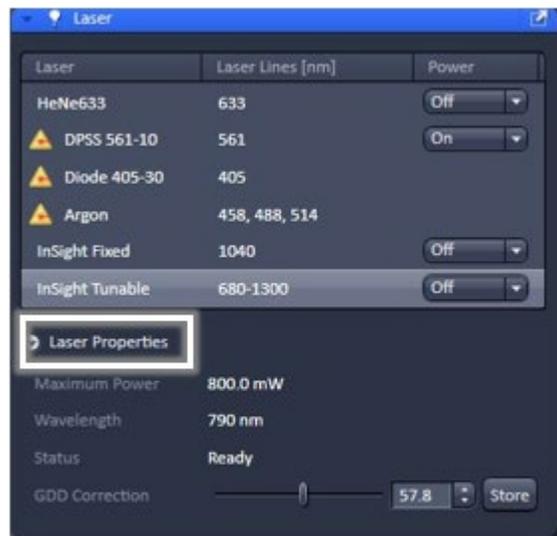
Focus and Stage – adjustments through software

Focus Devices and Strategy – autofocus, definite focus

Laser

If needed, turn on the Argon multi-line laser. The laser is first highlighted in red (turning on), then goes on Standby (warming up ~5 min). It turns ON when ready.

The multiphoton lasers (Insight) include a fixed line at 1040 nm and a tunable line from 680-1300. Highlighting the Tunable laser and clicking on *Laser Properties* shows the current wavelength of the tunable laser.

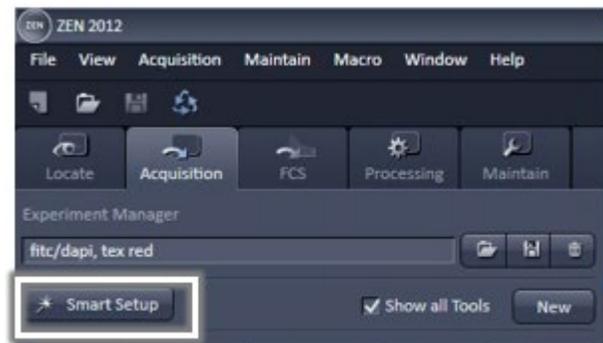


Laser tool for software control of lasers

Smart Setup

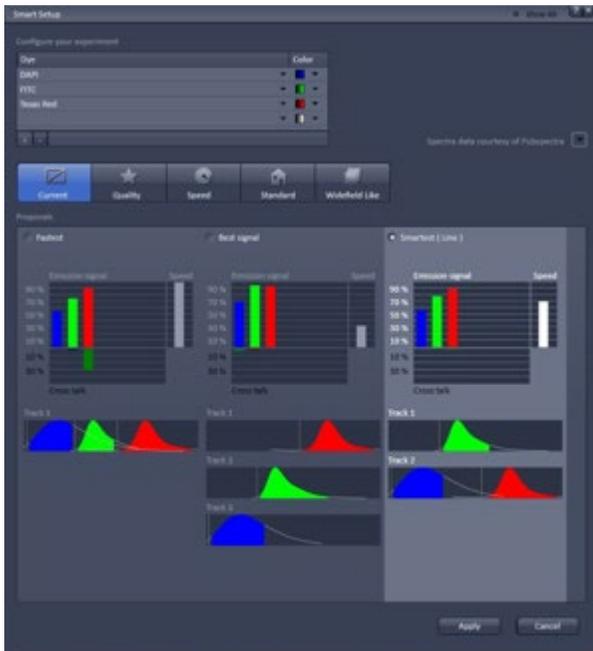
Smart Setup can be used for almost all standard confocal applications. It configures all the system hardware for a chosen set of dyes.

- Click on the *Smart Setup* button to open the Smart Setup window. This window can be accessed any time from the software to change dye combinations.
- Click on the arrow in the Dye list and choose the dye(s) you want to use. Dyes can also be searched by typing the name in the search field.



Smart Setup for configuration of dyes

- Smart Setup suggests alternative considerations:
 - a. *Fastest* imaging
 - b. *Best signal*
 - c. *Smartest* (compromise between both speed and best signal)
 - d. *Linear Unmixing* of the dyes is sometimes displayed.
- Pressing **Apply** automatically sets the hardware parameters in the Light Path. If the option Linear Unmixing is selected, the system is set in the lambda mode automatically.



Smart Setup with suggested acquisition configurations for a given set of fluorophores

The graphs display relative values for the expected emission signals and cross-talk. The resulting imaging scheme (single or multitrack) is shown below the graphs.

Light Path

The Light Path tool displays the selected track configuration that is used for the scan procedure. You can change the settings of this panel using the following function elements:

- Main dichroic beam splitter (MBS) for visible and invisible light from the relevant list box
- Spectral emission range detected: adjust horizontal sliders underneath displayed spectra
- Activate/deactivate (via check box) selected channel for the scanning procedure; assign a color to the channel.
- Laser icon: to select the laser lines and set the attenuation values (transmission in %) in the displayed window
- Detection Bands & Laser Lines are displayed in a spectral panel to visualize the activated laser lines for excitation (vertical lines) and activated detection channels (colored horizontal bars).

Ch1 and Ch2 are standard alkali PMTs.

ChS1 is an array of 32 high-sensitivity GaAsP PMTs.

This can use up to 8 channels in Channel mode (but they have to have the same gain at any time, but different digital gain is allowed).

- If you have created a configuration, either manually or via Smart Setup, you can save this in the Experiment Manager for easy reloading in the future.



Tool for saving acquisition configurations

Setting up a configuration manually: Tracks

- *Simultaneous* scanning of single, double and triple labeling (uses 1 Track): faster image acquisition, but potential cross-talk between channels.
- *Sequential* scanning of double and triple labeling; line-by-line or frame-by-frame (uses 2 or more Tracks): only one detector and one laser are switched on at any one time. This reduces crosstalk, but image acquisition is slower.
- *A maximum of 4 tracks with up to 8 channels can be defined simultaneously and then scanned one after the other. Each track is a separate unit and can be configured independently from the other tracks with regard to channels, spectral emission, dichroic beam splitters and pinhole settings.*
- Switch track every *Line*: Tracks are switched during scanning line-by-line. The following settings can be changed between tracks: Laser line, laser intensity and channels.
- Switch track every *Frame*: Tracks are switched during scanning frame-by-frame.
- The following can be changed between tracks: Laser line and intensity, all filters and beam splitters, the channels settings for gain and offset and the pinhole position and diameter.
- Add Track (+): An additional track is added to the configuration list in the Imaging Setup Tool. A maximum of 4 tracks can be used.
- Remove Track (trash): The track marked in the List of Tracks panel is deleted.



Tool for scanning sequential acquisition configuration: by Line or by Frame

Acquisition Mode: Setting scanning parameters

- Scan Mode: Frame or Line
- Frame Size: default is 512 x 512 pixels; Click on **X*Y** button to select a different frame size; Click on the **Optimal** button for calculation of appropriate number of pixels depending on objective N.A. and wavelength.

The number of pixels influences the image resolution!

- Speed: Use the scan speed slider to adjust the scan speed.
- *A higher speed with averaging results in the best signal-to-noise ratio. Scan speed 8 usually produces good results. Use speed 6 or 7 for superior images.*
- Bit Depth: Select the dynamic range 8, 12, or 16 Bit (per pixel)
 - 8-Bit: 256 gray levels*
 - 12-Bit: 4096 gray levels*
- *Publication quality images should be acquired using 12-Bit data depth. 12-Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.*



Acquisition tool for setting up scanning parameters

- Averaging: improves the image by increasing the signal-to-noise ratio. Select the Line or Frame mode in the Acquisition Mode tool.
- Select the number of lines or frames to average.
 - Frame averaging helps to reduce photobleaching, but does not give quite as smooth of an image.*
- Direction: Bi-directional scanning makes imaging faster, but may cause color alignment artifacts when using high NA objectives.

Channels

- Image optimization via adjustments of laser power, pinhole size, detector gain and offset.
- Set the Pinhole size to 1 AU (Airy unit) for best compromise between depth discrimination and detection efficiency.

Pinhole adjustment changes the Optical Slice thickness. When collecting multi-track, multi-channel images, adjust the pinholes so that each channel has the same Optical Slice thickness. This is important for colocalization studies.

- Ch2 and ChS detectors can be used to image samples via *Integration* or *Photon Counting* Mode. The latter is only recommended for very weak fluorescence signals.
- To view all channels under various tracks, click on *Expand All*.



Channels tool for image optimization

Image Acquisition

Use one of the Set Exposure, Live, Continuous or Snap buttons to start the scanning procedure to acquire an image. A clicked button changes to a Stop button when scanning.

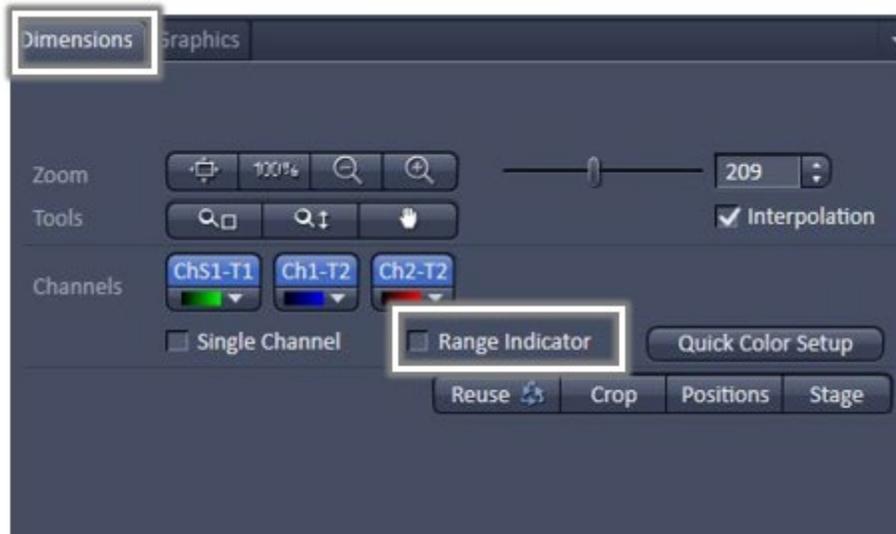
Scanned images are shown in separate windows.

- *Set Exposure*: automatic pre-adjustment of detector gain and offset
- *Live*: continuous fast scanning; useful for finding and changing the focus
- *Continuous*: continuous scanning with the selected scan speed and averaging
- *Snap*: records a single image.
- *Stop*: stops the current scan procedure.



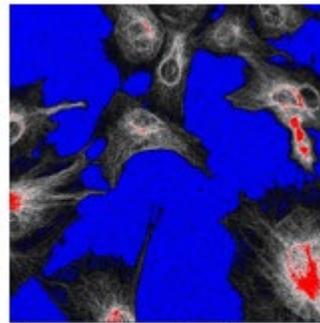
Tools for starting a scan

Image Optimization: Range Indicator



Range Indicator Tool to check image brightness saturation

- In the Dimensions tab located below the image field, check Range Indicator.
- The scanned image appears in a false-color presentation.
- If the image is too bright, it appears red on the screen.
- If the image is not bright enough, it appears blue on the screen.
- Red = saturation (maximum); Blue = zero (minimum).



Range Indicator shows saturated pixels in red and undersaturated background pixels in blue

Image Optimization: Adjust Laser Intensity

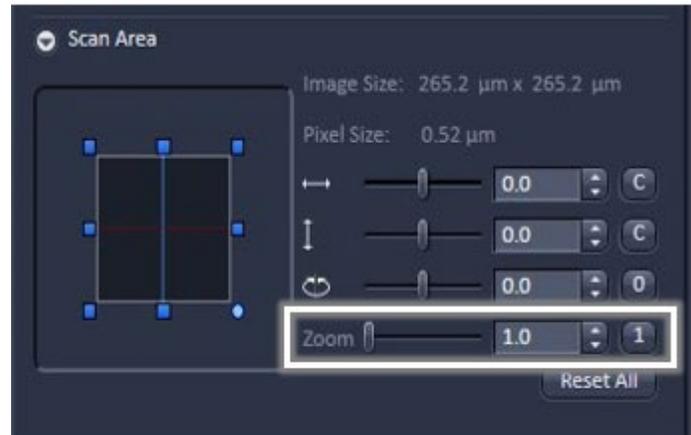
- Set the Pinhole to 1 Airy Unit.
- Set the Gain (Master) high.
- When the image is saturated, reduce AOTF transmission in the Laser control section of the Channels Tool using the slider to reduce the intensity of the laser light to the specimen.

Image Optimization: Adjust Gain and Offset

- Increase the Digital Offset until all blue pixels disappear, and then make it slightly positive (*stay as close as possible to zero*)
- Reduce the Gain (Master) until the red pixels only just disappear.

Zoom: Live

- Active while live scanning
- Adjust zoom value
- Move, resize, rotate using graphic, values, slider, or the small up/down arrows
- *Reset All* to return to zoom 1 and center of Scan Area
- You can zoom out to 0.6 but the corners and edges may be less bright.
- Zoom: Real optical zoom, resolution increases, image area decreases. To image a larger field with small pixels, switch to a larger frame size (e.g. 1024 x 1024 pix).



Tool to adjust scan area, including zooming in or out

Zoom: Crop Box

- Stop scanning to use
- Under the *Dimensions* control block, select **Crop**
- Position box that appears in image display on object of interest
- Resize, move, rotate
- Scan *Live* to see zoomed image



Crop tool to allow selection of a smaller image area

Reuse

- *Reuse* transfers ALL acquisition parameters (except objective and collimator) from stored image data to the Microscope Hardware Settings / Control Tools and applies those parameters directly to the system.



Reuse tool opens up previously used acquisition and scanning configurations

Image Display

To the left of the image container, one can select how the image acquired is displayed:

- **2D** – displays a single image in frame mode, or a multiple channel image in superimposed mode.
- **Split** – displays the individual channels of a multi-channel image as well as the superimposed image.
- **2.5D** – displays the two-dimensional intensity distribution of an image in a pseudo 3D mode, shows the intensity values in profile, grid or filled mode, as well as separate distribution for each channel in a multi-channel image.
- **Histogram** – displays a histogram (distribution of pixel intensities) of an image or Region of Interest; shows the histogram values in table form; allows one to copy table to clipboard or save as text file, and to measure area and mean gray value and standard distribution in an area.
- **Profile** – displays the intensity distribution of an image along a straight or curved line; shows the intensity values in table form and copy table to clipboard or save as text file; shows separate profiles for each channel in a multi-channel image.

Saving Images

- To save acquired or processed images, click on the Save or Save As button in *File* Menu, or click the *Save* icon in the Main Toolbar, or click on the *Save* icon at the bottom of the File Handling Area (located just below the image thumbnail).
- To export image display data, a single optical section in raw data format or the contents of the image display window including analysis and overlays, choose *Export* from the File menu.

In the Export window, you can select from a number of options and proceed to the Save As window to save the exported data to disk.



Saving images using main toolbar (Top) or via file handling area (Bottom)

- *NOTE: an unsaved 2D image in the active image tab will be overwritten by a new scan. Multi-dimensional scans (z-stack, time series, etc.) or saved images will never be over-written and a new scan will then automatically create a new image document.*
- Save files as .czi. This ensures that all hardware settings are saved and can be reused for future imaging sessions.
- *Acquired data is not automatically saved to disc. Make sure you save your data appropriately and back it up regularly. The ZEN software will ask you if you want to save your unsaved images when you try to close the application with unsaved images still open.*
- Copy to or Save images to Z: Imaging Lab FileShare.

Scanning a Z-stack: First/Last Mode

- Select Z-Stack in the main tools area.
- Open the Z Stack tool in the Left Tool Area under Multidimensional Acquisition
- Select *First/Last* on the top of the Z-Stack tool.
- Scan *Live*. Use the focus drive of the microscope (or focus knob on touchscreen monitor) to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the *Set First* button to set the upper position of the Z Stack.
- Focus on the lower specimen area where the recording of the Z Stack is to end. Click on the *Set Last* button to set this lower position.
- The *Smallest* button sets the number of slices to match the optimal Z-interval for the given stack size, objective lens, and the pinhole diameter. You can use this number (or a value close to this) for the *Interval*.
- Click on the **Start Experiment** button to start the recording of the Z-Stack.



Z-stack in main tool area



Tool to setup z stack by First/last focus positions

Center Option for Z-series

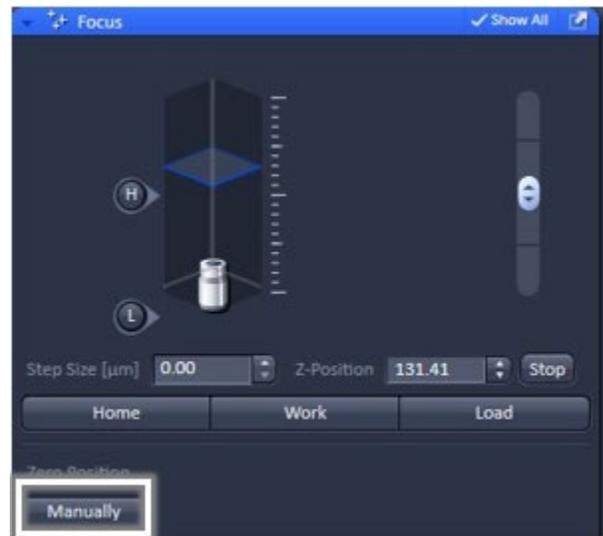
- Choose *Center* vs First/Last
- Focus to the center of sample
- Hit Center button
- Set Interval, then adjust number of Slices to determine range
- Use the focus tab to set the center to zero, if desired, by hitting *Manually*.
- Range Select: Will do an x-z scan; you can change center or top/bottom on this image



Tool to setup z stack by manual selection of center slice

Center Mode: Z Stack at Several Positions

- Use the center option to define the z-stack at the first position
- Then just add each location (Positions) when in the center of the object.
- You must do the same range and step size for each position
- X-Y-Z coordinate is recorded
- Software sets an offset to relate to center of the first position



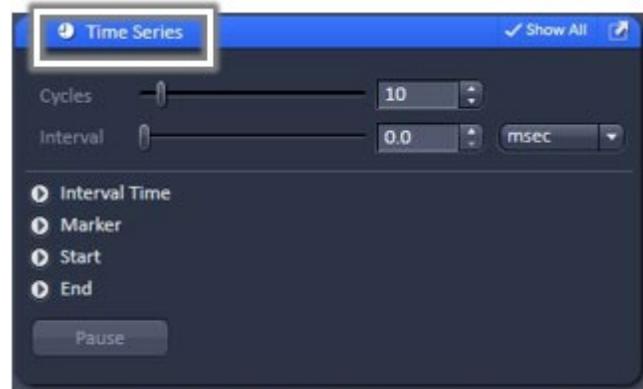
Tool to setup zero focus position

Scanning a Time Series

- Select *Time series* in main tools area.
- Open the Time series tool in the Left Tool Area under Multidimensional Acquisition
- Set *Interval* and total number of scans (*Cycles*).

An interval time shorter than the scan time for a frame or line is not effective.

- Click on the **Start Experiment** button to start the recording of the time series.
- If you also have Z-Stack checked, you will get an xyzt series.
- Definite Focus will keep sample in focus over time.



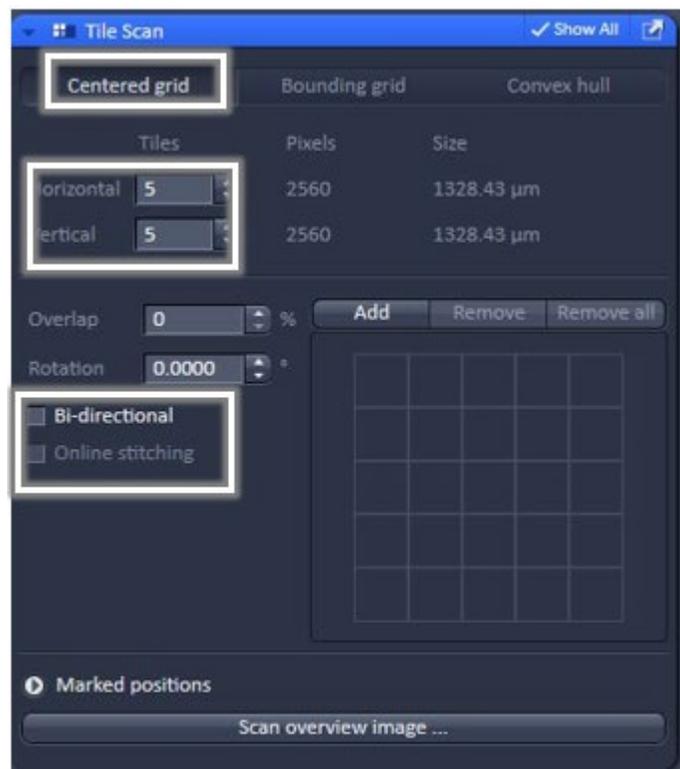
Time series tool to setup number of cycles and acquisition interval

Tile Scan

- Select Tile Scan in main tools area.
- Open the Tile Scan tool in the Left Tool Area under Multidimensional Acquisition

Centered grid:

- *The current position will be the center of the tile scan.*
- Set number of tiles in horizontal and vertical direction
- Scan overview image for quick view
- You can do a z-stack at each tile, setup as usual
- When *Bi-directional* is active, the image acquisition is performed also on the horizontal backwards movement of the stage.
- Click on Start Experiment to start the tile scan



Centered grid option for setting up a tile scan

- Online stitching is possible when a value is put in the Overlap box. A good value to start with is 10%.

Bounding Grid or Convex Hull:

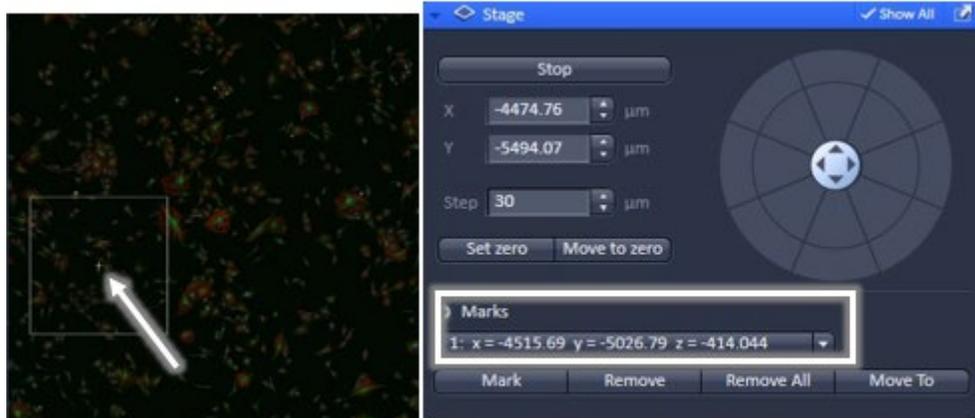
- Positions that should be part of the tile scan have to be marked using *Add*.
- Using marked positions, a bounding grid or convex hull is created, which finally defines the dimensions of the tile scan.

You can select Positions on a tiled image and scan selected position.



Positions option for manually selecting different positions from a tiled image

- Click the **Positions** button in the Dimensions tab located below the Image display.
- Click on the area(s) of the tiled image you want to go to; areas are marked with a white cross and a number.
- Go to the Stage tab (blue tab). Positions marked on your tiled image appear under *Marks*.



Selected positions (xyz coordinates) selected from a tiled image are marked in the Stage tool

- Select the marked position and click the *Move to* button. Scan.

You can also use the Stage button to select stage positions (areas) in tiled image.

- A white box with a number appears on the selected position
- Scan Live to move to this position



Stage option for manually selecting different stage positions from a tiled image

Bleaching (Photobleaching, FRAP/ Photoactivation)

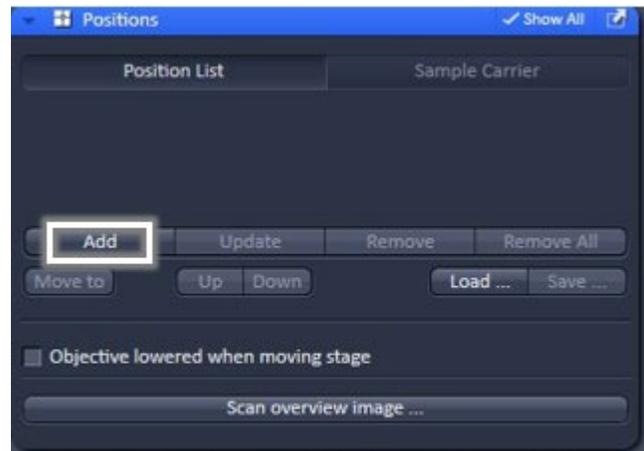
- Select *Bleaching* in the main tools area.
 - Time series* and *Regions* are automatically selected as well..
- Expand these 3 tools in the left tool area under Multidimensional Acquisition.
- Under *Bleaching* tool, check *Start Bleaching after # scans*, and indicate number of frames after which bleaching of the sample happens.
- Under *Iterations*, indicate the total no. of scans for bleaching the selected region during each bleach process.
- In the *Regions* tool, choose a shape to define Regions of Interest (ROI) to bleach
- The bleach process can be accelerated by checking the box next to *Zoom Bleach* (fast, less accurate).
 - *scanner movement will be restricted to the bleach region zooming in onto this region.*
 - *may result in a less accurate positioning of the region as the definition of the region has been made in a different zoom in the image.*
- In the *Excitation of Bleach* area, choose the laser line and the laser intensity to use for bleaching.
- Indicate Cycles and Interval in the *Time Series* tool.
- Click on the *Start Experiment* button to start the bleaching experiment.



Tool areas required for setting up photobleaching, photoactivation or FRAP experiment: Regions (top), Bleaching (center) and Time series (bottom)

Positions or Multi X-Y-Z

- Click on Positions in the main tools area
- Open Positions in the left tool area under Multidimensional Acquisition
- Move stage to desired X-Y-Z location
- Click on *Add* (position is added under Position List); repeat for more stage positions
- Click on *Move to* to go to desired position
- Click on the *Start Experiment* button to start the multi-position scan.

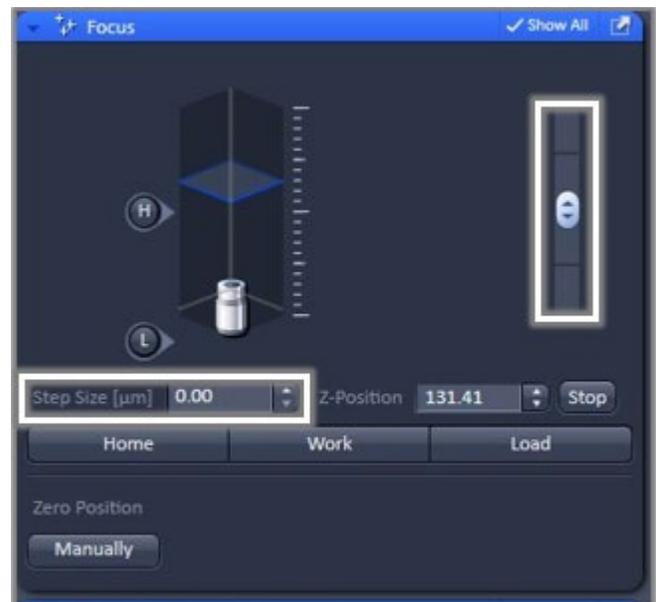


Positions tool for multiple image acquisition at selected coordinates (x,y,z)

Focus

- Set a step size
- Move slider or click arrows in Z-position
- Optional: To set current focus position to zero, click on *Manually* box

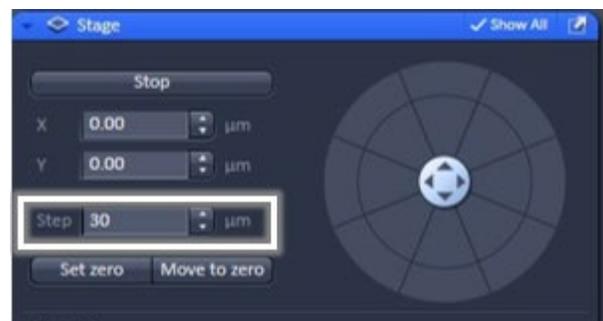
This option can be used for setting up a z series.



Focus tool for step size adjustment of focus

Stage

- Set a step size.
- Click in desired position in wheel or use x/y buttons.
- Optional: To set current stage position to zero, click on *Set zero* box. To return to set zero stage position, click on *Move to zero* box.



Stage tool for x,y adjustment of stage position

Reflected Light Confocal

In *Light Path* window,

- Set Main Beam Splitter to T80/R20
- Check box for Reflection
- Choose detection channel (usually Ch 1 is fine for excitation for 405, 458, 514 or 488 laser; use Ch 2 for 561 or 633 nm excitation)
- Create small region (detector range) covering desired laser line
- Can combine fluorescence and reflected light: Switch track every *Frame*.

Transmitted Light

In *Light Path* window,

- Click on *T-PMT*.
- Adjust gain and offset in Channels tab
- Uses whatever lasers are active
- Changing any laser power affects this image
- **Note:** The image acquired is not an optical section



Sample Light path configuration for reflected light (Ch1) and transmitted light (T-PMT)

Lambda Mode

- Used to create spectral profiles of dyes; used for Spectral Unmixing)
- Under Light Path, choose *Lambda Mode* tab
- Select spectral collection range using the horizontal bar
- Define spectral resolution: *For simultaneous acquisition use resolution of 8.9 nm or greater*
- Select lasers with appropriate MBS
- Adjust Acquisition Settings for balanced Image.

- Use the gallery view of lambda image to balance the detector gain, offset, scan speed and laser powers.
- It is important to eliminate any saturated and under exposed pixels in the image to ensure proper Unmixing.



Sample Light path configuration for spectral acquisition by Lambda mode



Gallery mode of a Lambda scan showing emission at several wavelengths

Multiphoton (LSM or Confocal Mode) using Internal Detectors

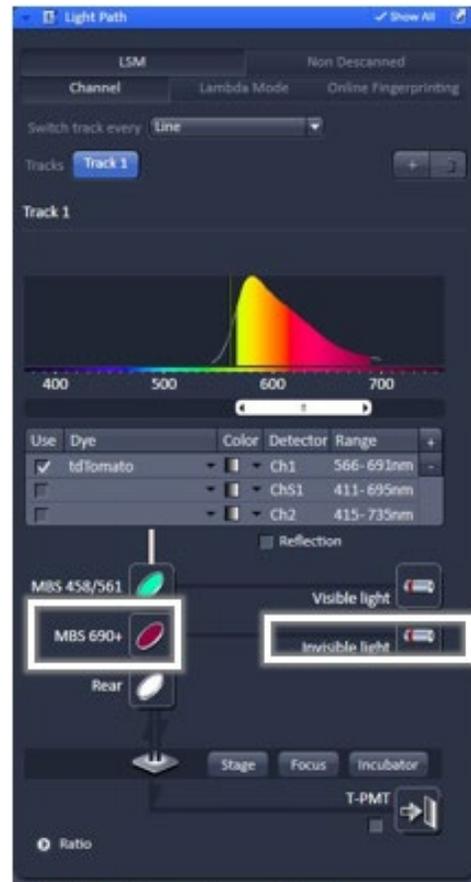
In the Laser tab, Turn on the multiphoton laser and wait until mode-locked and ready.

Imaging Setup and Light Path tool

- The multiphoton laser required for excitation will appear under Invisible light in the Light Path (click on laser graphic)
- To detect fluorescence signal from multiphoton excitation, select the dichroic MBS690+ or MBS760+.

MBS 690+ or MBS 760+: main dichroic beam splitter reflecting NIR excitation longer than 690 nm or longer than 760 nm, respectively, and transmitting shorter wavelengths

MBS 405/690+: main dichroic beam splitter reflecting NIR excitation longer than 690 nm, transmitting shorter wavelengths and reflecting 405 nm excitation; for simultaneous detection of signals from NIR and 405-excited fluorochromes.



Light path configuration for multiphoton excitation includes setting up the right dichroic filter (MBS690+ or MBS760+)

Pinhole Settings and Wavelength Selection

- In the Channels tab, set pinhole to max.
- Pinhole for a multiphoton laser can be fully opened for maximum detection efficiency due to the intrinsic optical sectioning capabilities of the non-linear optical effect.
- If the laser wavelength needs to be changed (for the tunable one), type in the desired wavelength. Note that lasers are not ready if highlighted in red.

Bleaching with multiphoton laser

- Follow standard bleaching procedure, but use multiphoton laser (fixed line or tunable) to bleach.



Multiphoton excitation wavelength can be tuned between 680 to 1300 nm. Pinhole aperture is set at Maximum.

Multiphoton (NDD – Non-Descanned Detection) using External Binary GaAsP (BiG) Detectors

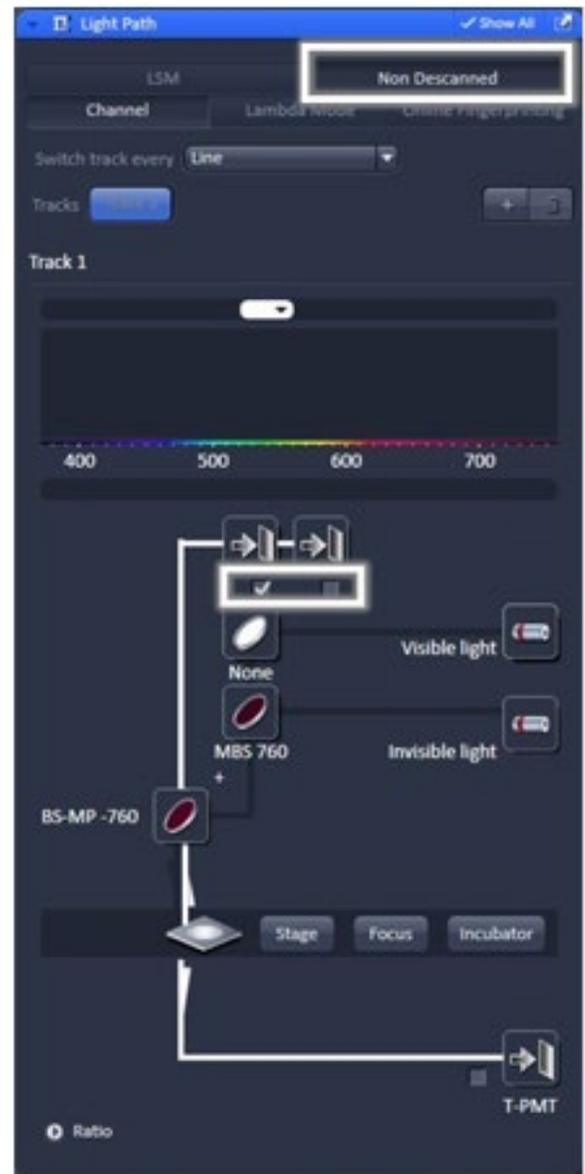
*Important! All confocal lasers and room lights must be turned **off** when using BiG detector for NDD. Make sure multiphoton slider is in position.*

In Non-Descanned Detection, the radiation emitted by the specimen is directly guided onto the BiG detector without passing the scanner mirror again.

- Under *Light Path*, click on the *Non-Descanned* tab to change to Non-Descanned Detection.
- Check the boxes associated to the filters required. Note that the displayed filter range in the software is not necessarily right. (*The computer does not know if the filter modules were replaced*).
- Set up the light path:
 - a. Change main dichroic of visible lasers to *None*. Make sure all visible lasers are unchecked.
 - b. Change main dichroic of invisible laser (check appropriate laser) to MBS 760+ (*reflects wavelengths above 760 nm onto specimen and allows fluorescence emission in the range below 760 to pass thru*) or MBS 690+.
 - c. Change second dichroic to BS-MP-760.
 - d. Check wavelength range of filters and check the NDD channels suitable for your sample.
- Under the *Channels* tab, check the laser to use then set the desired wavelength.

When laser is ready (*red highlight goes away*), make sure to turn power down to lowest setting. Keep detector gain at lowest level.

- Scan by clicking on *Live* button. **Slowly** increase laser power. Adjust focus and gain.



Light Path configuration for Non-descanned detection