UNIVERSITY of GUELPH
Advanced Analysis Centre

Molecular & Cellular Imaging Facility

Leica TCS SP5

Contents

July 2020

Confocal Laser Scanning Microscope
- Leica TCS SP5 -

Filter free spectral detector: a spectrophotometer for each detector channel enables the user to design filters, maximize sensitivity, minimize crosstalk and record emission spectra as required, AOBS (acousto-optic beam splitter) for dynamic beam splitting.

Microscope: Upright Leica DM 6000B microscope, fully automated, equipped with DIC, bright field and polarisation optics.

Objectives: 20x (Multi-immersion: Oil or Glycerol), 40x (Oil), 63x (Glycerol), 63x (Water)

Laser system: Radius: 405 nm;
AR (50 mW): 458 nm, 476 nm, 488 nm, 496 nm, 514 nm;
GreenHeNe (1.2 mW): 543 nm;
Orange: 594 nm;
RedHeNe (10 mW): 633 nm

4 detection channels for fluorescence, 1 detection channel for transmitted light.

Leica Application Suite Advanced Fluorescence (LAS AF) software (version 2.7) for 2D and 3D imaging, Region of Interest (ROI) scanning, and time-lapse imaging, FRAP and FRET applications.

Image Analysis and Processing workstation

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User Guidelines

1. When you begin to use any system in the facility please provide a Billing Authorization Sheet including the supervisor's signature and the Trust Fund Account number.
2. Before any unsupervised access is granted, users must enroll in supervised training sessions during which they will review with the confocal manager how to operate the equipment properly and safely. The time required for the training sessions will vary depending upon the user's demonstrated competency with the equipment. Billing will be at the "Training" fee rate.
3. When using these facilities you must clearly write in the sign-up sheet the date, your name, department, log on and log off time, and total number of hours you used the equipment.
4. For any planned after hours use of the confocal system, please make arrangements with the confocal manager for access to the hallways.
5. Users are expected to bring all their own supplies including pipets, slides, coverslips, computer disks, etc. However, the facility will provide immersion oil and lens paper.
6. Files saved to computer hard drives must be removed as soon as possible. All computer hard drives will be cleared on a regular basis - it is the users' responsibility to manage their own image files. USB keys are not allowed on any of the instrument computers! The facility offers a variety of other options for data transfer.
7. Please notify the confocal manager immediately of any problems that you encounter with the equipment - it is essential that we work together in taking care of the facility. Improper care of the equipment will result in rejection of access to the facility.

Laser Safety

Please be aware that the confocal microscope is Class 3B laser equipment!
This laser equipment may be operated only by persons who have been trained in the use of the system and the potential dangers of laser radiation.
The University of Guelph offers a Radiation Safety Course, please visit the Occupational Health & Wellness webpage for details.

As it is not possible to anticipate every potential hazard, please take care and apply common sense to the operation of the confocal microscope.

- Observe all safety precautions relevant to Class 3b lasers.
- **Avoid exposing eyes or skin to direct radiation!**
- Do not deviate from the operating instructions provided.

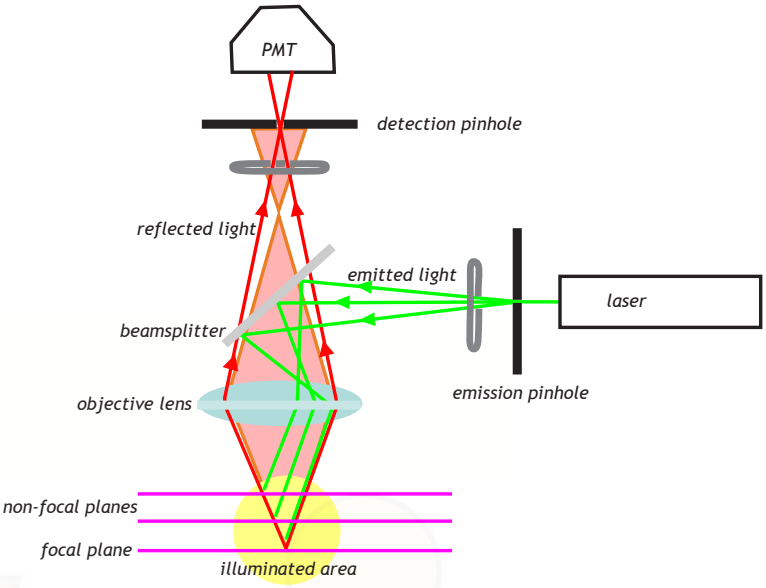
The failure to observe these instructions shall be exclusively at the user's own risk!

Confocal Basics

Basic principle of a confocal microscope

The principle of confocal imaging was advanced by Marvin Minsky and patented in 1957, and is employed in all modern confocal microscopes.

In a conventional widefield microscope, the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly by eye or an image can be taken. The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole and forms extended Airy disks in the aperture plane, usually experienced as a blurry image.



In contrast, the illumination in a confocal laser scanning microscope is achieved by scanning one or more focused laser beams across the specimen. The emitted light only from the focal plane in the specimen is detected by a photomultiplier tube (PMT) through a detection pinhole, and transformed into electrical signals which are converted to images by the specific software and displayed on the computer screen.

The detection pinhole suppresses signal from structures that are out of focus.

The depth of the focal plane is determined by the excitation wavelength, the numerical aperture of the objective, and the diameter of the detection pinhole.

Although unstained specimens can be viewed using light reflected back from the specimen, samples are usually labeled with one or more fluorescent probes/proteins.

Major improvements of a confocal microscope over a conventional microscope

1. Signal not originating from the focal plane will not be captured
2. Optical sectioning: Change of the focal plane does not create blurring, but gradually cuts out parts of the object as they move away from the focal plane. Thus, these parts become darker and eventually disappear.
3. Three-dimensional data sets can be recorded
4. Scanning the object in x/y-direction as well as in z-direction (along the optical axis) allows viewing the objects from all sides
5. Due to the small dimension of the illuminating light spot in the focal plane, stray light is minimized
6. Image processing allows superimposition of many optical slices, giving an extended focus image which can only be achieved in conventional microscopy by reduction of the aperture and thus sacrificing resolution

Leica DM 6000B Microscope: General Information & Care

Objectives

Lenses	Immersion	Refractive Index	Numerical Aperture (NA)	Coverslip µm (#)	Recommended Step Size for Image Stacks (µm)
20 x	Oil / Glycerol	1.52 / 1.43	0.7	0.17 (1.5)	1.2-1.3
40 x	Oil	1.52	1.25	0.17 (1.5)	0.4-0.5
63 x	Glycerol	1.43	1.3	0.17 (1.5)	0.3-0.4
63 x	Water	1.333	0.9	without	0.4-0.5

Immersion oil

Use only the immersion oil (Type F) and Glycerol (Type G) provided by the facility. Do not mix the immersion oil and glycerol with each other or with other substances including immersion oil from another source. If you used any other immersion oil during previous observations with a different microscope, remove all traces with Windex. Likewise, it is not practical to use the 40x and 63x in one session, since the sample will need to be cleaned with Windex and the immersion fluid changed between imaging with those objectives.

Cover slip

Sealing the coverslip completely with nail polish or other sealing materials is highly recommended. This prevents mixing of immersion oil and embedding materials, movement of the coverslip when moving the stage, and moving embedded materials during z-sectioning. Be sure that any nail polish is completely dried before you place the slide onto the stage.

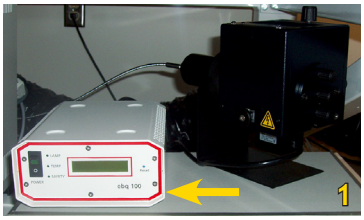
Lens care

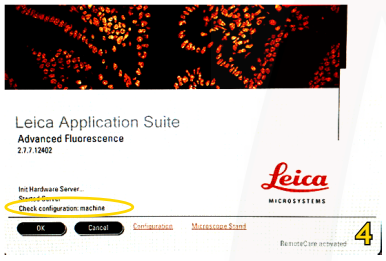
After use, clean the lenses with lens paper only. Fold the paper and hold both sides of the paper, keeping the folded edge straight. Draw the folded edge back and forth over the lens surface. Repeat with another piece of lens paper and continue until there are no more traces of immersion oil on the paper. Use Kim Wipes to remove excess oil on the objective (around the lens).

Confocal Laser Scanning Microscope Startup and Sample Setup

System Startup

1) UV lamp power supply (Fig. 1, arrow)
2) PC/Microscope button (Fig. 2A)
3) Laser Power button (Fig. 2C)
4) Turn Laser Emission key (Fig. 2D)
5) Switch on microscope box (Fig. 3)
6) Press Scanner button (Fig. 2B)
7) Login to your account and start LASAF software





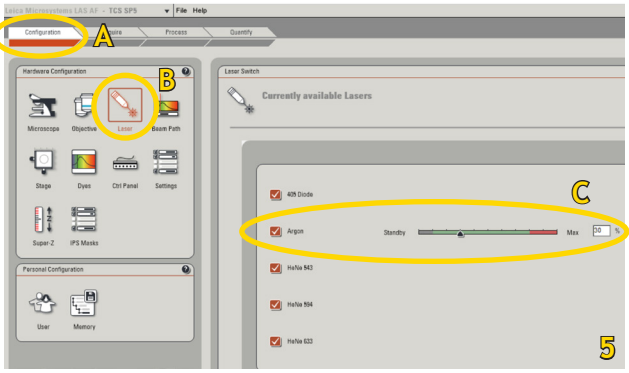
Software Startup Window

Under configuration select or confirm 'machine'

Stage initialization is only needed if you want to use the 'Tile' or 'Points' function in the software. If you do want to initialize the stage, make sure it is lowered and far away enough from the objectives.

Laser Selection and Activation

- Select the 'Configuration' tab (Fig. 5A) and then the 'Laser' icon (Fig. 5B)
- Check all the lasers you require
- If you are using the Argon laser, drag the slider to 30% (Fig. 5C)
- Select the 'Acquire' tab and set up your sample on the microscope



Sample Setup and Observation With the Light Microscope

On the microscope display (Fig. 6A):

- select the 'Light paths' icon and select 'BF' (Fig. 6B)
- select the 'Objectives' icon and select the objective you want to use (Fig. 6C)
  please note that all objectives are immersion objectives (IMM tab)!

Place your slide or petri dish on the stage and add the correct immersion fluid (place a drop of oil or glycerol on the cover slip); if you use the 20x make sure the dot on the correction collar (arrow) is set to the appropriate immersion fluid (Fig. 7).

- Using the Smart Move (Fig. 8), move the stage up until the objective touches the immersion fluid
- Switch to 'fine focus' and 'precise XY' on the Smart Move or the display
- Observe through the oculars and focus your sample; change to the objective you will use for imaging, if needed

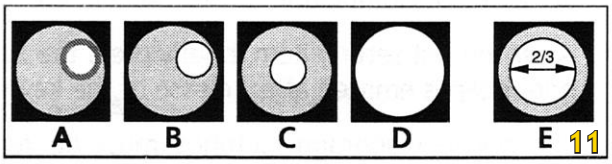
Once your sample is in focus, select the 'Save' icon on the display and set this position as your reference focal plane by pressing 'Set' (Fig. 9).

You now have saved your focal plane as 'upper threshold' and can move the stage quickly between the limits with the buttons at the right side of the microscope (Fig. 10).

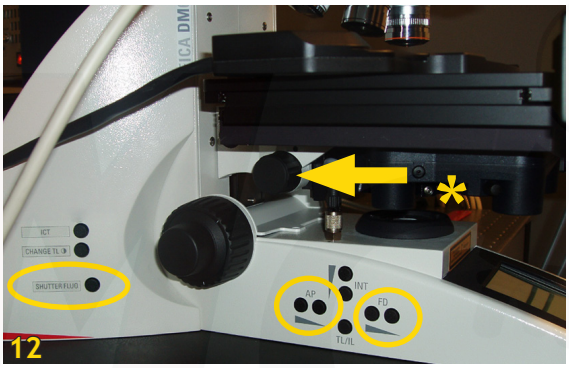
Köhler Illumination

Köhler illumination provides homogeneous illumination of your specimen without stray light. You get images with optimum contrast and resolution. Please do this in the BF setting!

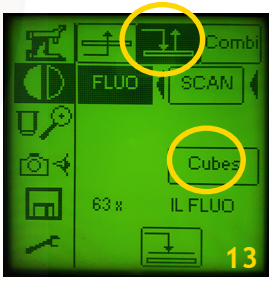
These are the steps (Figs. 11, 12):



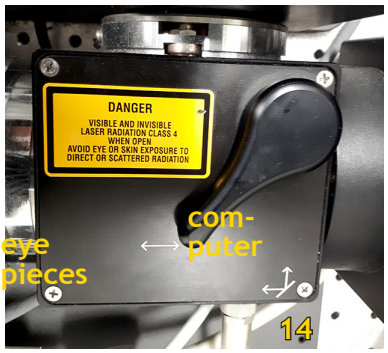
Focus the image
A - Close the field diaphragm (FD), adjust the aperture diaphragm (AP) if necessary
B - Adjust condenser height with focus wheel (arrow) until the edge of the field diaphragm is in focus
C - Centre the field diaphragm with the screws on the condenser (*)
D - Open the field diaphragm (FD) until the edge just disappears from the field of view
E - Adjust the aperture diapragm (AP) so that it is two-thirds open



Once you have adjusted these settings, switch to epifluorescence (Fig. 13), select the filter for your dye/fluorescent protein and open the FluoShutter (Fig. 12). Check if signal strength is sufficient, find your region of interest and adjust the focal plane if necessary. Close the FluoShutter and continue with the LASAF software.

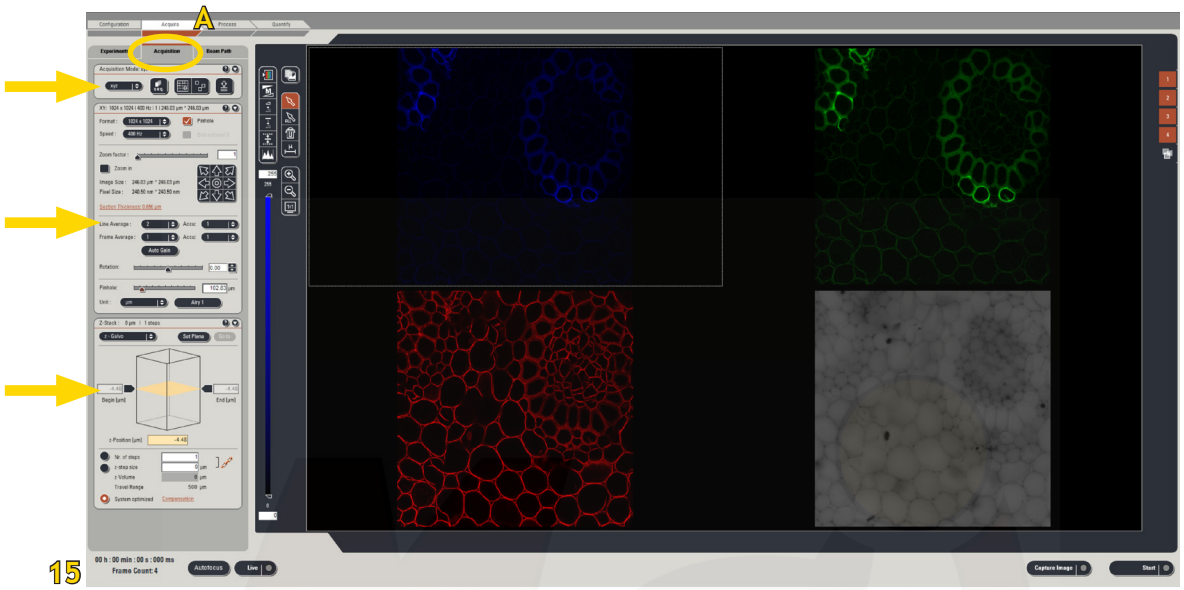


Important: If you want to acquire a transmitted light image, you need to manually flip the transmitted laser detector switch - behind the microscope (Fig. 14).



LASAF Acquisition Setup

Control Interface



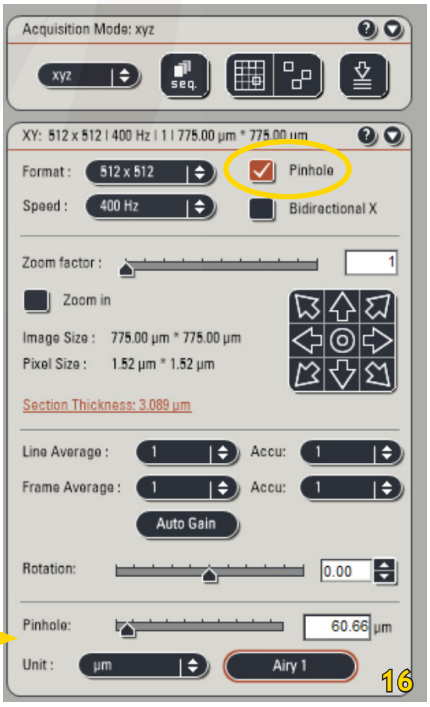
In the control window under the 'Acquire' Tab (Fig 15A), you see 3 containers (arrows) on the left side of the screen under 'Acquisition' (Fig. 15, circle). These are controls for some imaging parameters like acquisition moe, resolution, scan speed, zoom, and Z-stack settings.

Imaging Parameters - 'Acquisition Mode' and 'XY' Windows (Fig. 16)

- The default acquisition mode is xyz
- The default format (pixel size) is 512 x 512 pixels
- The default speed is 400 Hz (lines/second)

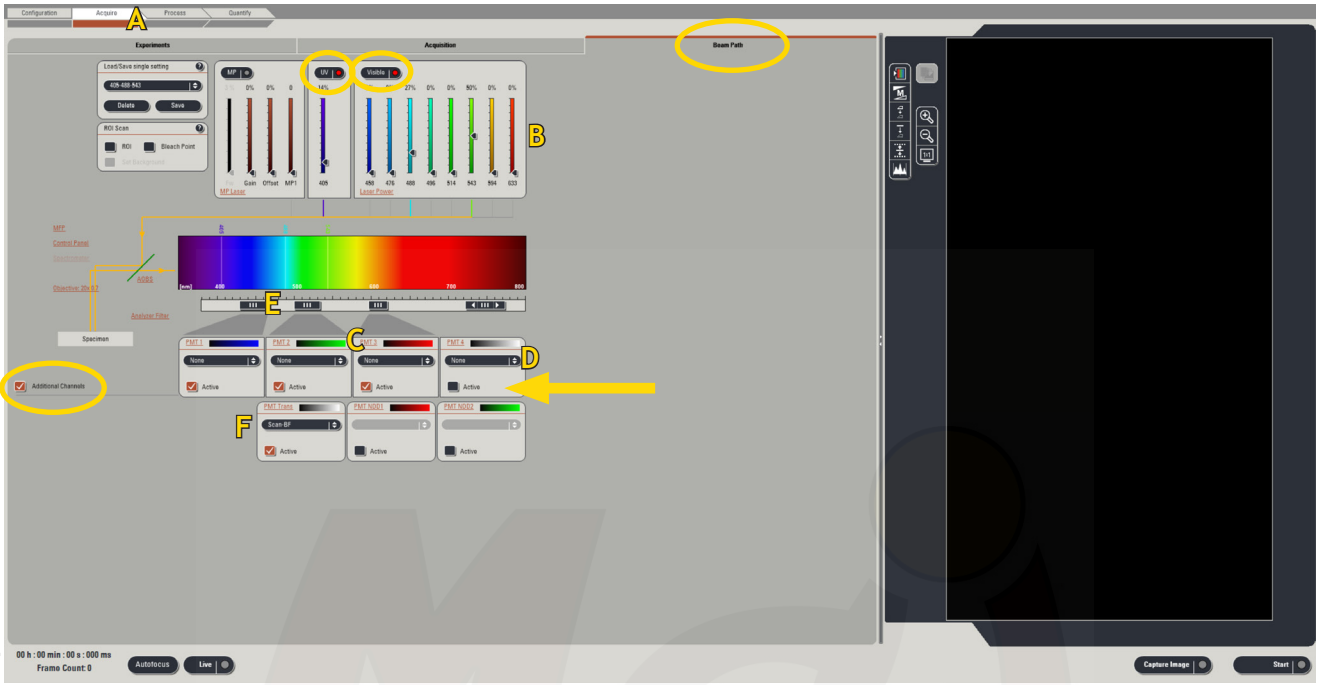
We encourage new users to <u>not</u> change these parameters at first. Optimize the images at this resolution and scan speed and acquire an image. You might then decide to adjust scan speed or format - depending on your question and image quality needed. Image size and pixel size are automatically calculated and displayed on the top line of the XY window.

Select the 'Pinhole' box (Fig. 16) and in the submenu that appears (Fig. 16, arrow), check 'Airy 1' to make sure the system is optimized.



Beampath Settings

In the control window under the ‘Acquire’ Tab (Fig 17A), select ‘Beam Path’ to set all parameters relating to laser excitation and signal detection.



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- Click on ‘Visible’ and/or ‘UV’ to activate the laser(s)
- Select the lasers and their intensity by moving the sliders (Fig. 17B) up to the required power (if you don’t know, start with AOTF 20-30%)
- An active laser line will be expressed as a line on the spectrum
- The choice of the laser line(s) depends on your fluorophore/fluorescent protein (for example: FITC and GFP require the laser line 488 nm of the Argon laser, Texas Red the 543 nm or 594 nm laser line)
- Activate the PMTs (= detection channels, Fig. 17, arrow) by selecting the ‘Active’ box and choose the colour for your fluorophore emission image (Fig. 17C); a gray shadow will appear underneath the PMT bar, confirming that the PMT is active
- If desired, click on ‘None’ to open the drop down menu and select the emission spectrum of your fluorophore/fluorescent protein (Fig. 17D)
- Place the PMT bars (Fig 17E) in accordance to the spectrum - you can move the bars freely left and right, increase or decrease the size or define a specific range numerically (double-click on the bar)
- Under ‘Additional Channels’, you can select to include the transmitted light channel (‘PMT Trans’, Fig. 17F)

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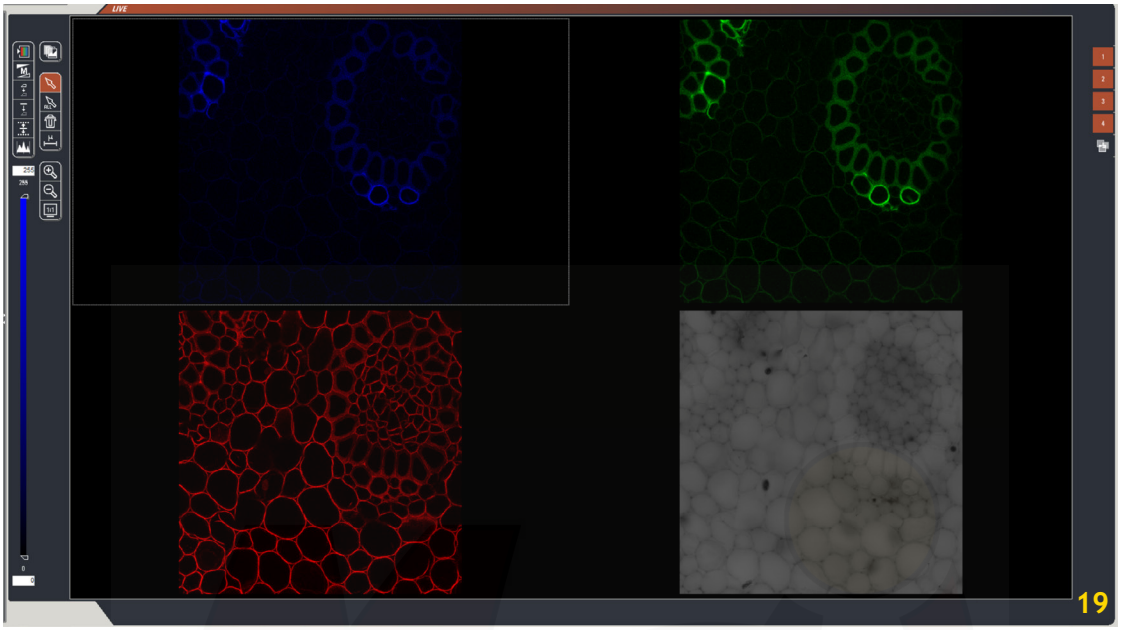
At the bottom of the control interface you see 3 laser scanning buttons

- Click on ‘Live’ to start a continuous scan and see a live image of your sample
- If you have more than one fluorescent marker and more than one PMT activated, your viewer screen will be separated in two halves, or 3 quarters, etc.

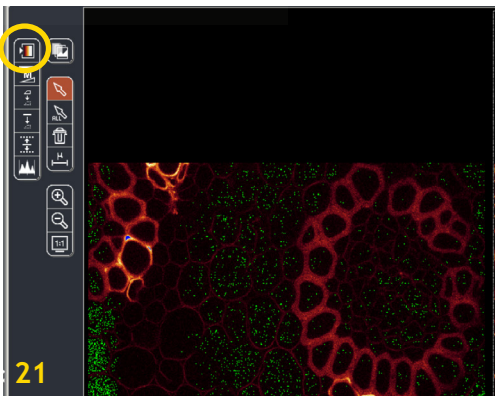
Optimizing Image Quality

Viewer Screen



- Select a channel on the viewer screen by clicking on the image and turn the ‘Smart Gain’ dial (Fig. 20A) until you can visualize your signal
- Repeat for additional channels
- Focus with the Z Position dial (Fig. 20D) to find the optimal focal plane
- Select the QLUT (= Quick Look Up Table) button (Fig. 21, circle) to change your image colour to a display of intensity values with the Smart Gain and Smart Offset dials (Figs. 20A, B). Set up your image intensity as shown in Fig. 21 with few blue (= saturated) pixels, and your background as mostly black or green (= zero intensity) pixels.
- Adjust the intensity (= BLUE pixels) with the ‘Gain’ dial and the background (= GREEN pixels) with the ‘Offset’ dial.
- Clicking on the QLUT button will cycle through the following settings: Colour - QLut - Grayscale

BLUE = saturated signal = 255
BLACK to ORANGE to WHITE = signal between 1 - 254
GREEN = no signal = 0



During this process, you might also consider adjusting the laser power intensities and the PMT bar positions to optimize signal strength and background.
The ultimate goal is to minimize necessary laser intensity, since high laser power means high laser radiation, and this will bleach your sample faster. Enhancing the gain will not affect your sample, on the contrary, you can decrease laser power and protect your sample to some extent.

But please note: If you increase the gain too much, you might run into problems optimizing for saturated pixels sometime during imaging. A smart gain around 800 V is the 'rule of thumb': A smart gain value lower than 400V would mean that you can lower the laser power and increase your smart gain to ~800 V. A smart gain value between 1,100-1,250 would suggest increasing laser power (AOTF%, Fig. 17A).

If the image is still too dim or not visible at all:

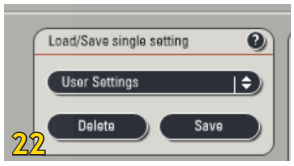
Increase laser power by using the vertical slider (AOTF%) until you can see an image

Adjust the PMT Smart Gain (maximum value is 1,250)

Once you have optimized the intensity settings:

Click on the 'Stop' button to stop the live scan

Click on the 'Capture Image' button to acquire an image

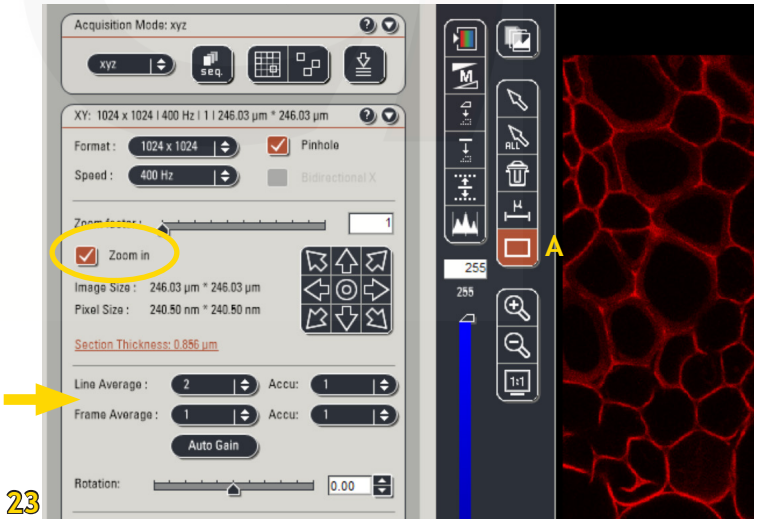


You can save the optimized beam path settings for each fluorophore/fluorescent protein (or any combination) in the 'Settings' menu and recall them from 'User Settings' in your future sessions (Fig. 22). Remember to check the gain and offset values each time, as these change from sample to sample!

Changing Other Parameters of Your Image Acquisition

Based on the acquired first image you can decide whether you want/need to change specific image acquisition settings like 'Format' or 'Speed'

In any case you should select to average the line number and/or the frame number (arrow) of your image acquisition: this often enhances the quality of your image, sometimes daramatically.
The averaging improves the Signal-to-Noise ratio and it is recommended to capture images with at least a 2x line average.
The command is found in the 'XY' window under the 'Acquisition' tab.



Zoom

You can zoom into your image without sacrificing resolution. There are 2 options:

- Zoom in with the 'Zoom' dial (Fig. 20C) - using the image centre as reference point
- Zoom in the 'XY' window: check the box 'Zoom in' (Fig. 23) - a rectangle function is automatically active in the Viewer Window (Fig. 23A) and you can draw a box around the region of interest (ROI) you would like to enlarge

Acquisition of a Z-stack

To acquire a Z-Stack, you need to open the third container ('Z-Stack') under the 'Acquisition' tab (Fig. 24).

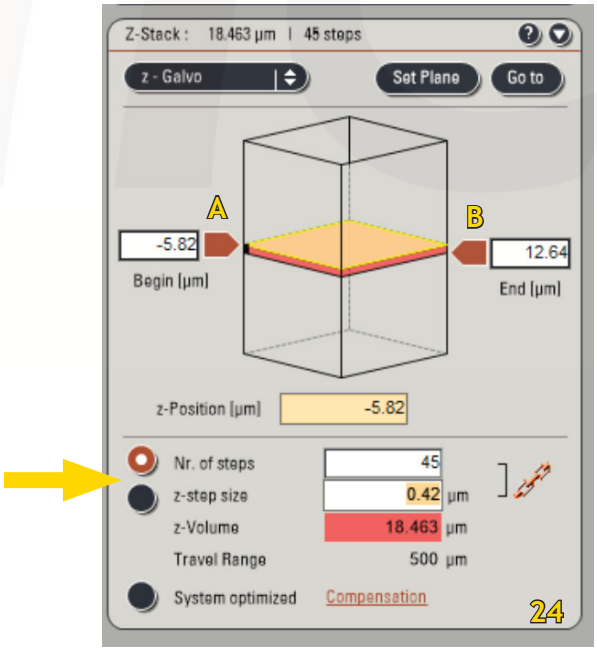
You will see the range of the Galvo stage and the current focal plane highlighted

- Select 'Live'
- Move to the top of your sample (only use the 'Z Position' dial for this, not the microscope focus wheel or the Smart Move!!!) and set the position of your Z-Stack by checking the 'Begin' arrowhead (Fig. 24A, fill colour changes)
- Move to the bottom of your sample and set this as bottom position by checking the 'End' arrowhead (Fig. 24B)
- Select 'Stop'
- To set the number of z-slices, you can select 'System Optimized' (Warning: this setting takes the minimal step size and you end up with a lot of images, which will take a long time)
- You can also select (Fig. 24, arrow)

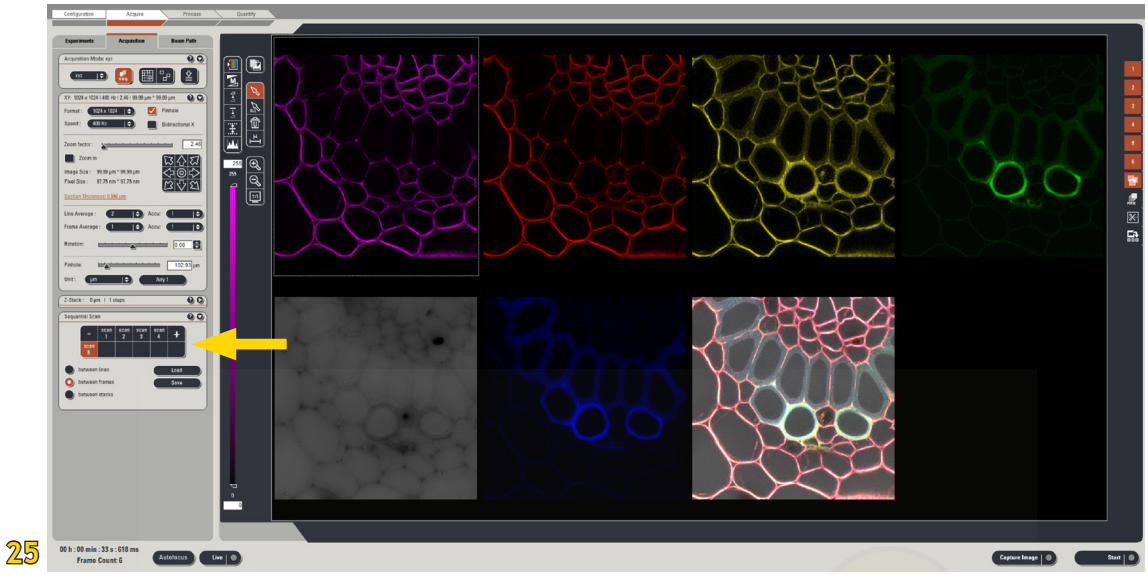
  Nr. of steps: a specific number of images (e.g., if you only want some representative images from the stack)

  z-step size: a specific step size (e.g., for 3D image projection but not in increments as small as the system would suggest) - this is our recommended setting, the step size depends on the objective

  Recommended step sizes can be found in the objectives table on page 5
- Select 'Start' and your Z-stack acquisition will begin and end automatically when finished



Sequential Scan



In the ‘Acquisition Mode’ window select ‘Seq’ (Fig. 26)

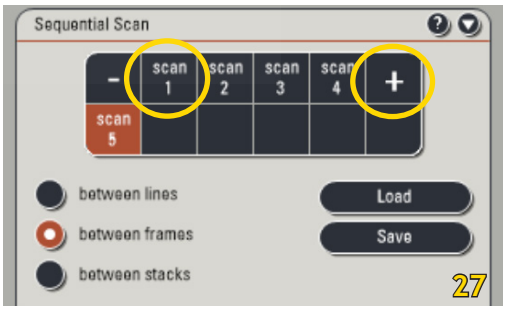
A new window will appear in the ‘Acquisition’ tab which lets you set up your sequential scan series (Fig. 25, arrow)
To set up the sequential scan, you need to have saved beam path settings!

In a sequential scan, the order in which we scan is from right to left = from longest to shortest wavelength!!

Since interferences usually only happen from a shorter to a longer wavelength, we can thus minimize any disadvantageous and damaging effects.

You can choose to switch between the beam path settings either line by line, frame by frame, or stack by stack. Recommended is frame by frame, but feel free to experiment and see what works best for your sample.

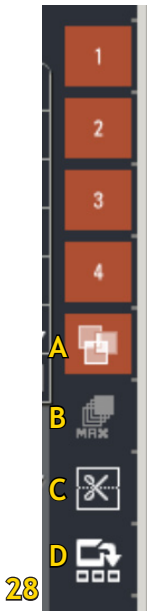
- ‘scan 1’ is automatically highlighted; select the first beam path you need and optimize
- Click once on the ‘+’ icon: ‘scan 2’ will appear; select the second beam path and optimize
- Repeat until you have your entire sequence defined
- Select ‘Start’ to take the sequential series of images



To switch between the different scan channels for live optimization, click once on the ‘scan #’ icon.
Please click only once on the ‘scan #’ icon and give the software enough time to change all settings (you can see the changes on the spectrum)!!
You can save your sequential scan setup to load it during future sessions.

Viewing Options

Display of Images and Image Series



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Overlay image

Select or hide the channels for an overlay image: When any channel (#1-4 in Fig. 28) is not used for an overlay, hide it by clicking the corresponding button. Click the button ‘Overlay’ (Fig. 28A).

Single image and tiled screen

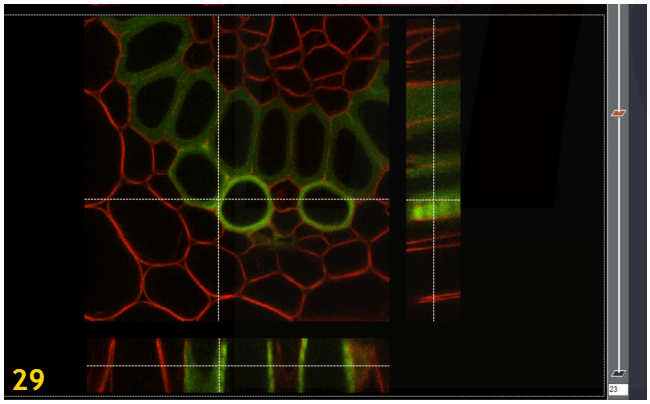
If you want to look at just one of the images or at only the overlay, simply double-click the image on the viewing screen; double-click again to revert to tiled view.

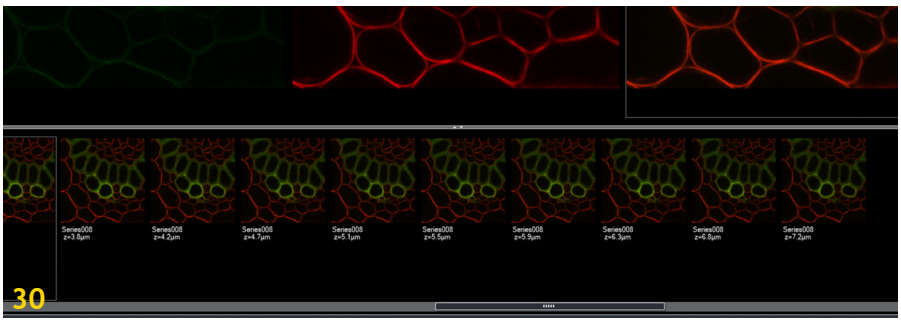
Maximum projection

A very quick 3D view of all optical slices of a Z-Stack is created if you select the ‘MAX’ icon (Fig. 28B). It will not create a 3D projection file as described under Data Processing (page 18)!

Orthogonal view

The orthogonal view (Fig. 28C, Fig. 29) is also available for an image stack only. It shows the XY image in one focal plane in the centre, and the XZ and YZ view on the bottom and the right side, respectively. Navigation of your sample in XY is done with a crosshair and in Z with a slider.


29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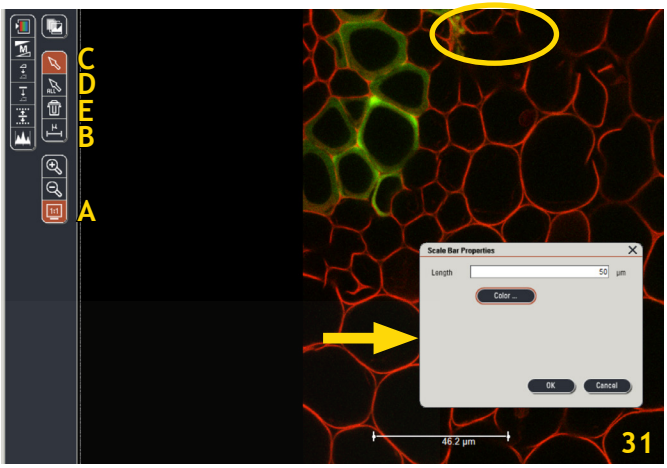
Gallery view

The individual images of any image series are shown if the gallery icon is selected (Fig. 28D, Fig. 30).

Scale Bars

- On the viewing screen, select '1:1' (Fig. 31A) (ideally, unless you want to snapshot all images on the screen)
- Select the scale bar icon (Fig. 31B)
- Draw a line (as straight as possible!) to the approximate desired length
- Select the arrow (Fig. 31C) and click on the created line
- Right-click on the line and select 'Properties'
- In the pop-up window (Fig. 31, arrow) set the length of your scale bar and click 'OK'
- While the scale bar is selected, you can also move it around



- Select the arrow for marking all (Fig. 31D) - the scale bar is now set and at a fixed place in the image
- You can now snapshot the image (see below)
- After taking the snapshot, delete the scale bar from view by selecting the garbage bin (Fig. 31E)

Save Annotated Images

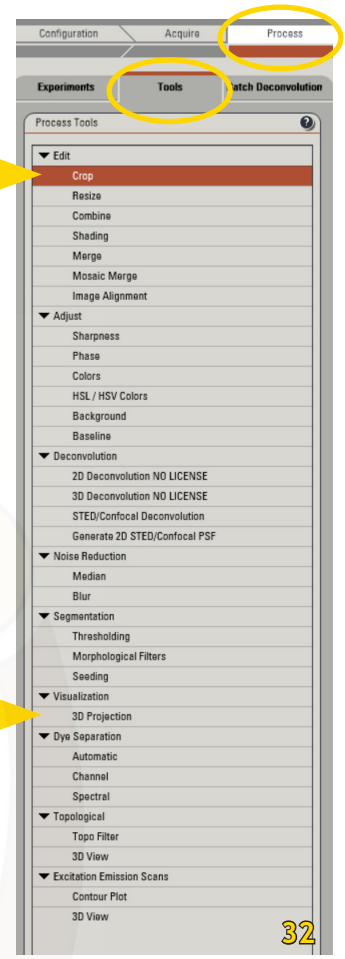
Annotated images are overlay images, images with scale bars, Profile, Graphs, etc.

- Select the button '1:1' (Fig. 31A; if it hasn't been selected yet; the image is then displayed in its actual size) - ideally, you want to snapshot in original size - unless you want to snapshot all images on the screen
- Right-click on the annotated image
- Choose 'Send to', then 'Experiment', then 'Selection (Snapshot)'

The annotated image will be saved as separate file in your experiment, called 'Snapshot'. You will need to export this snapshot file as Tiff. 'Selection (all)' will save a snapshot of all channels visible in the viewing window.

Data Processing

There are several options for image processing under the Tab ‘Process’ (Fig. 32). Generally, we recommend to do image analysis not on the SP5 imaging system but with the LASAF Simulator or another image processing software on the workstation in the facility or a computer in your lab. However, it is fairly quick and easy to create a 3D Projection with LASAF, following the steps described below.



Merge or Crop Images

If you have acquired more channels or z-slices than you want to include in your 3D Projection (e.g. transmitted light channel), you need to crop your image file before processing. Likewise, you might need to merge image files or series that were taken separately.
Each editing creates a new file and will not alter your original file!

You can use ‘Merge’ to:

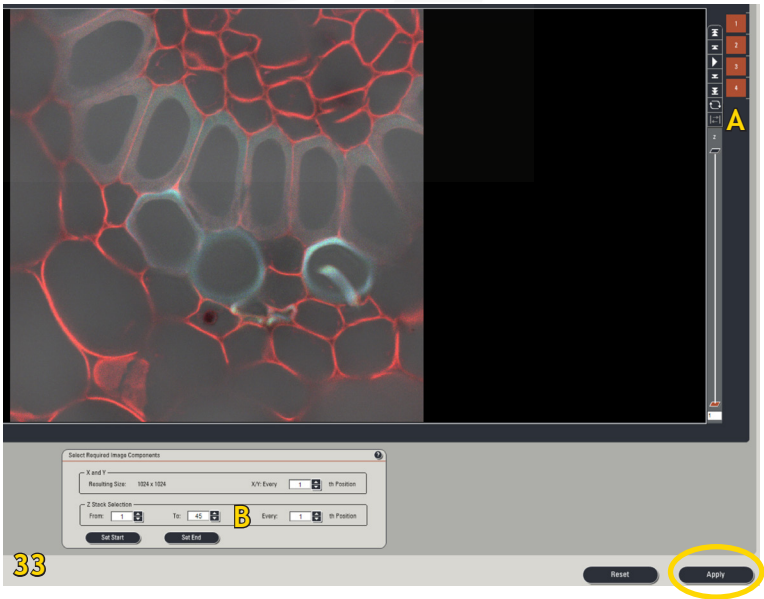
- merge two or more channels of images taken separately (the number of sections, the distance between the slices, and the format have to be the same in all the channels)
- merge several time series into a single file

You can use ‘Crop’ to:

- delete selected channels from a file that contains several channels
- extract optical slices from a stack of images
- crop images of channels in the xy plane

<u>How to crop your image file (Fig. 33)</u>

- Select your file
- Select ‘Process’ - ‘Tools’
- Within the process tools, find ‘Edit’, then ‘Crop’ (Fig. 32, arrow)
- Deselect the channel(s) you don’t want to include (Fig. 33A), possibly deselect the first and/or last z-slices if they are of poor quality (Fig. 33B)
- Select ‘Apply’

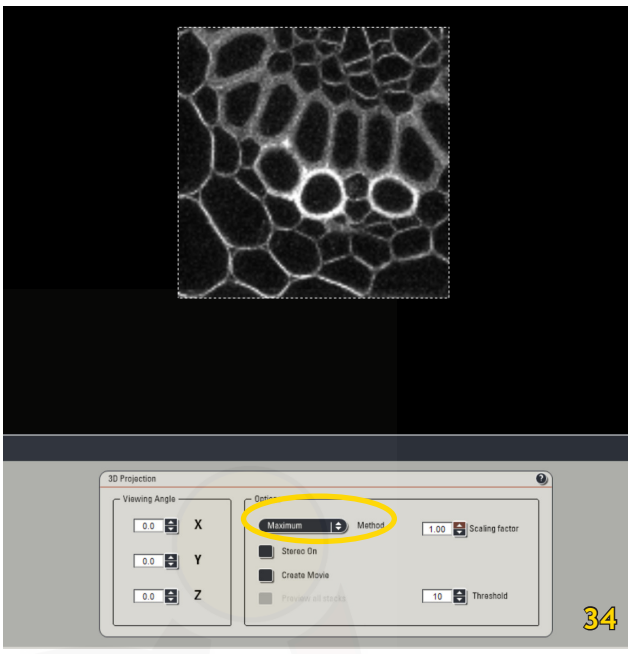


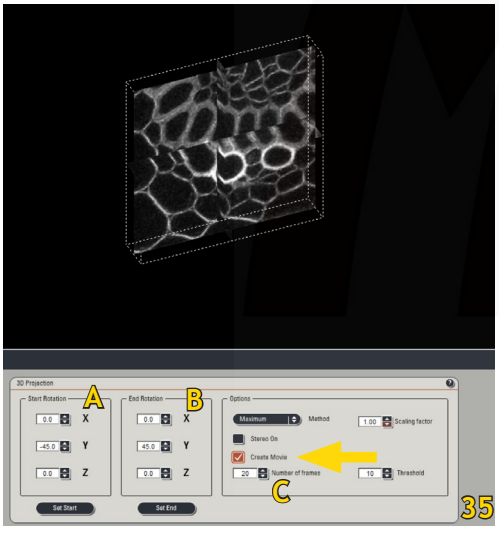
3-Dimensional Projection

After acquisition and editing of a Z-stack, you can process your data and create a 3D projection with or without animation.

Simple 3D Projection (Fig. 34)

- Select your file
- Select ‘Process’ - ‘Tools’
- Within the process tools, find ‘Visualization’, then ‘3D Projection’ (Fig. 32, arrowhead)
- Do not change the X,Y, Z viewing angles if you only want a simple projection
- Default is ‘Maximum’ in the method drop down list (this works in 90% of all cases, ‘Average’ can be used if your fluorescent signal is very strong and a maximum projection would completely saturate the signal).
- Select ‘Apply’



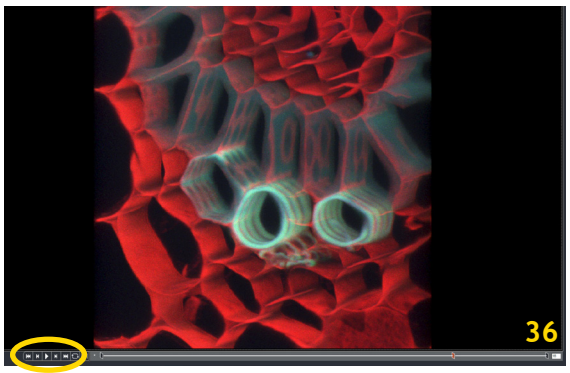


3D Projection with Animation (Fig. 35)

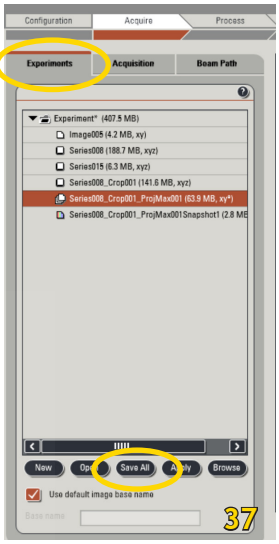
- Select your file
- Proceed as described for simple 3D projection (Fig. 32)
- Select ‘Create a movie’ (Fig. 33, arrow)
- Enter the Start Rotation angle corresponding to the start view of the movie and select ‘Set Start’ (Fig. 33A)
- Enter the End Rotation angle corresponding to the end view of your movie and select ‘Set End’ (Fig. 33B)
- Enter the number of frames for the rotation (Fig. 33C; the higher the frame number, the slower the rotation will be)
- Select ‘Apply’

The movie can be viewed in your Viewer Window by clicking the ‘Play’ button (Fig. 36).

The file needs to be exported in either .avi or .mov format.



Data management



Your image files are temporarily stored on RAM memory and you can see and access them under the 'Experiments' tab on the left side of the screen (Fig. 37).

Please regularly save your files by clicking 'Save all'

The data are saved as .lif file (Leice Image Format) and you will need dedicated image processing programs (e.g., Volocity, Fiji) to open this file.

Naming of the image files:

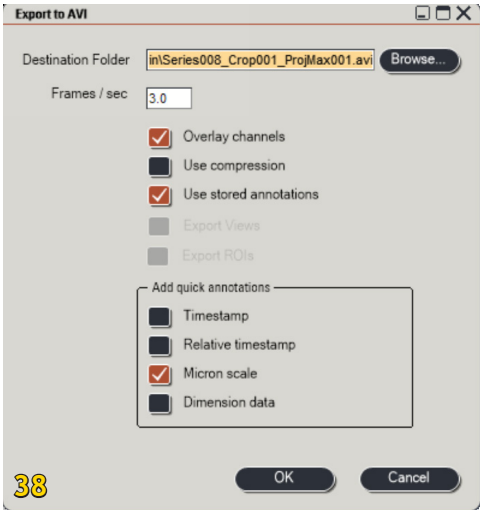
Preview00x: temporary file from the continuous scanning process
This file will be constantly overwritten and disappears after selecting the 'Capture' or 'Start' button.
Image00x: single image with one or more channels
Series00x: Series of images (e.g., Z-stack, sequential, tiles, time series)

If you process your images within LASAF, the original files will remain and additional files with self-explanatory names are created (e.g., Snapshot of Image00x, Cropped Image00x, etc)

You can rename your experiment, or the individual files in an experiment by right-clicking on the name and selecting 'Rename'.

You can delete individual files in an experiment by right-clicking on the name and selecting 'Delete'.

You can export images or image series in your experiment as .tif, .avi, or .mov files by right-clicking on the name and selecting 'Export'



Export of .avi or .mov files (Fig. 38)

- Select Export as .avi or .mov
- Select the folder you want to save your movie in
- Enter a file name
- Select whether you want to overlay channels and add a scale
- Select frames per second (this depends on your file and the number of frames)
- Always save file uncompressed

You will not see the movie file in the 'Experiments' dialog window.

Shutdown

1. Save your data and deselect the lasers in the software ('Configuration')
2. Close LAS AF and transfer your files to OneDrive (preferred) or the server
3. Switch off Laser Emission key
4. Switch off Scanner button
5. Bring microscope stage all the way down, remove your sample, clean objective lenses with lens paper
6. Switch off microscope box
7. Shut down computer
8. Switch off PC/Microscope button
9. Switch off UV lamp
10. Switch off Laser Power button (5-10 min after Laser Emission key)
11. Cover microscope

2-Hour Rule

If someone else has booked the microscope within the next 2 hours, please leave the UV lamp,the laser emission key, and the laser power button on. You can also leave the computer on. Don't cover the microscope.