

Leica DM 5000B

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Leica DM 5000B Microscope

An upright Leica DM 5000B microscope connected to a Hamamatsu Orca-Flash 4.0 digital camera and Volocity™ imaging software.

Microscope: Upright Leica DM 5000B, equipped with DIC, bright field and epifluorescence optics. Automated ASI xy controlled stage (scanning, stitching, multiple points) and a piezo drive for z-stacks. Additionally, colour imaging of histological slides is possible with the ScopeLED Illuminator B190-RGB.

Camera: Hamamatsu Orca-Flash4.0, monochrome, optimized for high sensitivity and speed.

Objectives: 10x (Dry), 40x (Dry), 63x (Oil, DIC), 20x (water), 40x (water), 63x (water, DIC)

Fluorescence filters: Semrock - DAPI (UV), FITC (blue), Texas Red (green); Chroma - Triple Band Filter Set DAPI/FITC/TXRed

Imaging and image processing software: Volocity™ (version 6.3)

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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.

Leica DM 5000B Microscope

General Information & Care

Objectives

Lenses	Immersion	Refractive Index	Numerical Aperture (NA)	Coverslip μm (#)	Recommended Step Size for Image Stacks (μm)
10 x	Dry	1	0.3	0.17 (1.5)	2.0 - 4.0
40 x	Dry	1	0.85	0.17 (1.5)	0.6 - 0.7
63 x	Oil	1.52	1.4-0.6	0.17 (1.5)	0.3 - 0.4
100 x	Oil	1.52	1.3	0.17 (1.5)	0.3 - 0.4
20 x	Water	1.33	0.5	None	1.5 - 3.0
40 x	Water	1.33	0.8	None	0.7 - 1.0
63 x	Water	1.33	0.9	None	0.4 - 0.5

Immersion oil

Use only the immersion oil (Type F) provided by the facility. Do not mix the immersion oil 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.

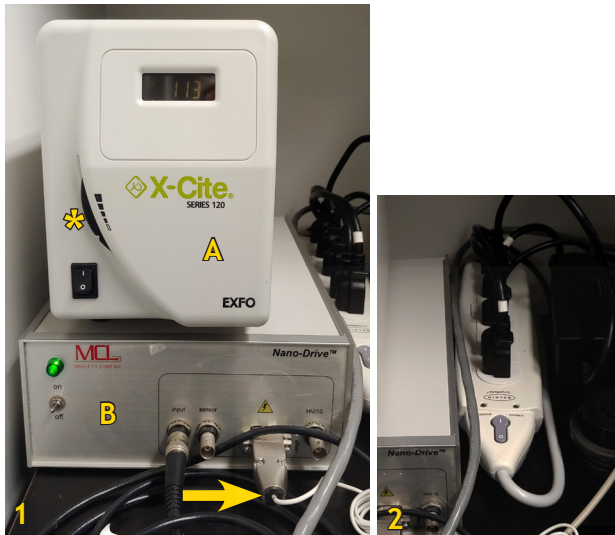
Cover slip

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).

Leica DM 5000B Microscope Startup and Sample Setup



System Startup

1. X-Cite light bulb, wait at least 30 seconds (Fig. 1A, only if needed)
2. Powerbar (Fig. 2, this will start up all accessory equipment), wait 30 seconds
3. Computer
4. Login: .\Scope User, Password: MCIF
5. Volocity, log into your personal account

Accessory Equipment

X-Cite 120: The X-Cite light source has a shutter with tunable transmission (Fig. 1, *; it works like an intensity control) - adjust it while imaging and minimize it when you take a break or during partial shutdown. General rules are the same as for UV mercury lamps!

Camera: The system has a monochrome Hamamatsu Orca Flash 4.0 for fluorescence and transmission imaging with a frame rate of 80 fps at 8bit.

Scope LED: The ScopeLED Illuminator provides the capability to acquire colour images with the monochrome camera. For imaging, it is controlled by Volocity. Light intensity, colour temperature, and the shutter can be adjusted manually (Fig. 3). However, for image acquisition with Volocity the shutter needs to be closed!

Stage: The stage is a fully motorized ASI stage. The motorized stage is needed for acquisition of stitched images or multiple independent positions. For this, it needs to be calibrated in Volocity. XY movement is controlled with the joystick on the ASI controller (Fig. 4A).

NanoDrive: Since the focus drive of the microscope is manual, the piezo drive (Fig. 1B) provides the capability to do Z-Stacks. The thin white cable (Fig. 1, arrow) is very fragile and if it's bent too much, the NanoDrive might not connect during startup. You can check this by looking for the stage symbol in Volocity (Fig. 19A, page 12). If it's not there, you need to shut down Volocity, carefully adjust the cable and restart.

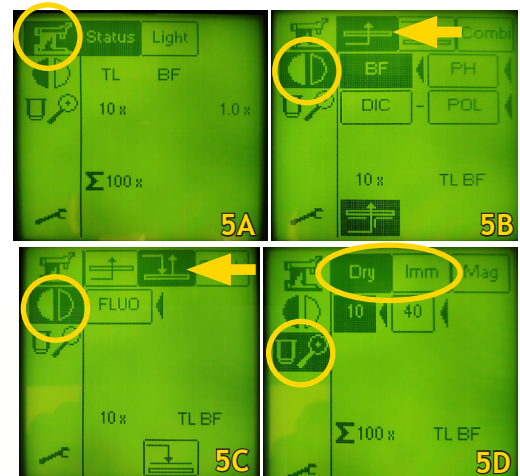


Sample Setup and Observation With the Microscope

The microscope itself is mainly manual (objectives, focus drive) but some features can be controlled either via Volocity or the touchscreen dialog.

On the microscope display (Fig. 5):

- The 'Status' icon gives very general information on the current setup (Fig. 5A).
- The 'Light paths' icon has the possible light paths - either in the transmitted light pathway (Fig. 5B) or fluorescence (Fig. 5C).
- The 'Objectives' icon shows which objective is currently used - both dry and immersion objectives are available (Fig. 5D).



Place your slide, petri dish, or well plate on the stage and - if you use any of the oil immersion objectives - add the immersion oil on the coverslip.

The X/Y of the stage drive is the joystick on the ASI controller box (Fig. 4A). Switching between fast and precise movement is done by a click on the top of the joystick (Fig. 4,*) - the speed is indicated by the letters 'f' (fast) and 's' (slow) on the ASI display (Fig. 4). Centre your sample.

Move the stage up manually until your sample is in focus: the large focus wheel on the left is the coarse focus, the smaller wheel on the left and the wheel on the right are both the fine focus.

Observe through the oculars, find your ROI (region of interest) and change to the objective you will use for imaging.

Adjust the focal plane again in your sample with the fine focus. Switch the joystick to slow movement.

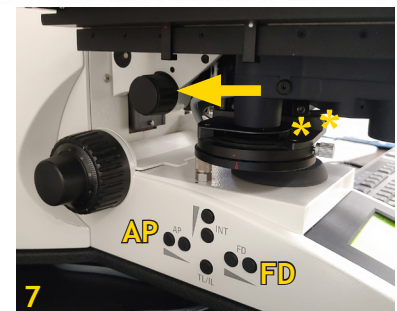
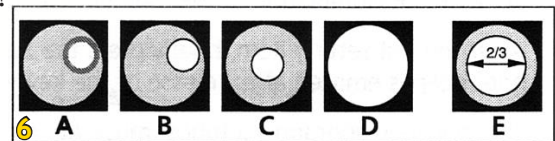
Köhler Illumination

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. 6, 7):

Focus the image

- Close the field diaphragm (FD), adjust the aperture diaphragm (AP) if necessary
- Adjust the condenser height with the condenser focus wheel (Fig. 7, arrow) until the edge of the field diaphragm is in focus
- Centre the field diaphragm with the screws on the condenser (Fig. 7, *)
- Open the field diaphragm (FD) until the edge just disappears from the field of view
- Adjust the aperture diaphragm (AP) so that it is two-thirds open



Once you have adjusted these settings, you can continue with Volocity if you want to do transmitted light imaging.

If you have fluorescently labeled samples, switch to epifluorescence (Fig. 5C or Fig. 8A), select the filter for your dye/fluorescent protein (Fig. 8B) and open the FluoShutter (Fig. 8C). The fluorescence filters generally installed are from Semrock - DAPI (UV), FITC (blue), Texas Red (green). Turn up the intensity on the X-Cite at least one notch. Check whether the signal strength is sufficient, find your region of interest and adjust the focal plane if necessary. Close the FluoShutter and continue with Volocity.

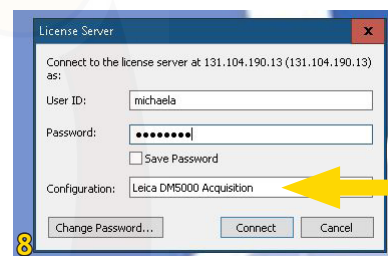
The microscope has 2 sets of objectives: the combination of dry and immersion oil objectives (10x, 40x, 63x, 100x) and a set of water immersion objectives (20x, 40x, 63x). Since they are not compatible and could be damaged, only one set will be on the microscope for your session.

Volocity Acquisition Setup

Volocity Startup

Double-click the Volocity icon. In the pop-up window (Fig. 8), enter your user name and password.

Make sure the correct module is selected: **Leica DM5000 Acquisition**

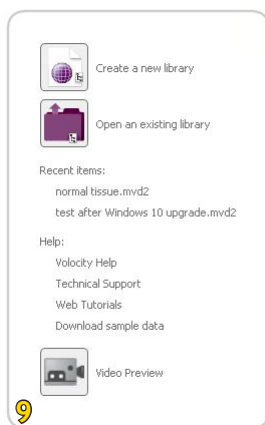


Volocity files are called libraries. What is a Library?

A library is the term given to the data storage format used by Volocity. You have to create a library for Volocity to acquire data. If you forget this, you will end your session and no images will be saved!!!

Libraries are based on a database structure and will store changes as they are made.

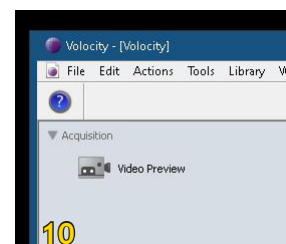
Creating libraries



You can select 'Create a new library' or 'Open an existing library' from the prompt shown when Volocity starts (Fig. 9).

Or, once you are running the application, select 'New Library' from the File menu. You can only have one library open at a time. If you already have a library open, you will be prompted to close it first. Use the dialog to name the library and choose the location to store it.

Next, select the icon 'Video Preview' to call up the control interface and the live stream (Fig. 10).



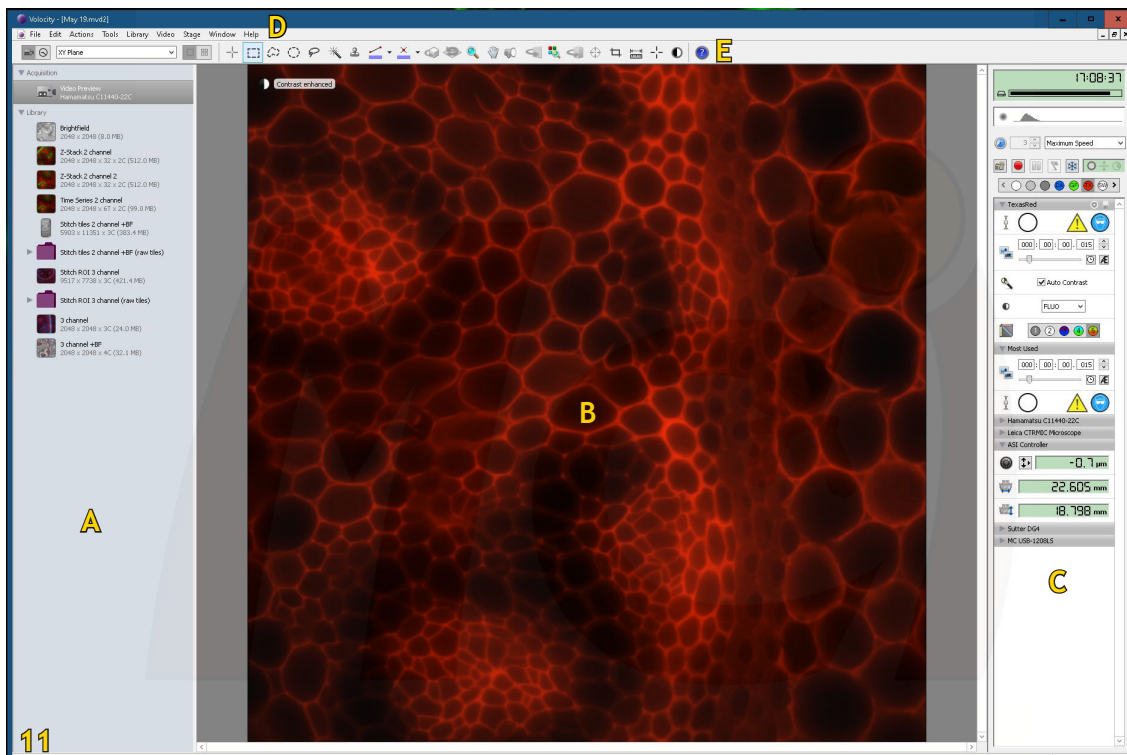
Software Interface

The Volocity program interface is divided into 3 main areas.

The left panel shows the 'Video Preview' icon that you need to select for live streaming and the current library with all images and image series (Fig. 11A).

The centre screen area (Fig. 11B) is used to display your live image or selected images/image series.

The right panel (Fig. 11C) provides you with all imaging parameter controls like light paths, shutters, exposure times etc.



- A - Left Panel
- B - Centre Screen Area
- C - Right Panel (Control Panel)
- D - Menu Bar
- E - Tool Bar

Basic Image Acquisition

Volocity has the following setting short cuts (if it's not labeled, you won't need it!)

Light Paths

All light paths you will need are arranged in the 'Light Path Manager' (Fig. 12A):

- DA - UV Fluorescence (387/11; 447/60)
- GF - Blue Fluorescence (482/35; 536/40)
- TX - Green Fluorescence (562/40; 624/40)
- SW - ScopeLED monochrome (Bright Field)
- SR - ScopeLED red filter
- SB - ScopeLED blue filter
- SG - ScopeLED green filter

Whenever you make changes to a light path setting (e.g., exposure time!), save these changes by clicking on the small disk symbol in the light path header (Fig. 12B)!

Single Image Acquisition

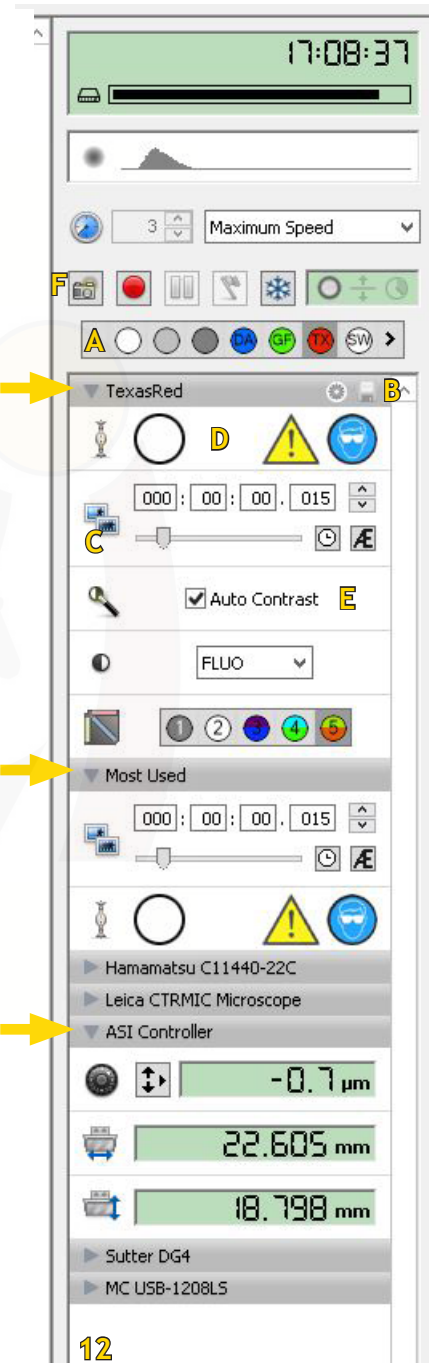
To start the live stream, select one of the light paths. Adjust the exposure time (Fig. 12C) if necessary.

The right panel seems to have a large number of controls, but most of them are duplicated or automatically changed by the software. In practice, you will only need the 'Light path', 'Most used', and 'ASI Controller' submenus open (see Fig. 12, arrows).

The shutters (Fig. 12D) are automatically opened and closed when you change light paths and after image acquisition. To see your live image after acquisition, you will need to open the shutter again. Click directly on the shutter icon to change its state. Gray indicates shutter closed. White indicates shutter open.

If you select any of the fluorescence light paths, make sure the checkbox for 'Auto Contrast' is marked active (Fig. 12E) - otherwise you will not see any signal.

Once you have adjusted all parameters, click on the 'Camera' icon (Fig. 12F) or tap on the space bar to take a single-channel image.



Advanced Image Acquisition

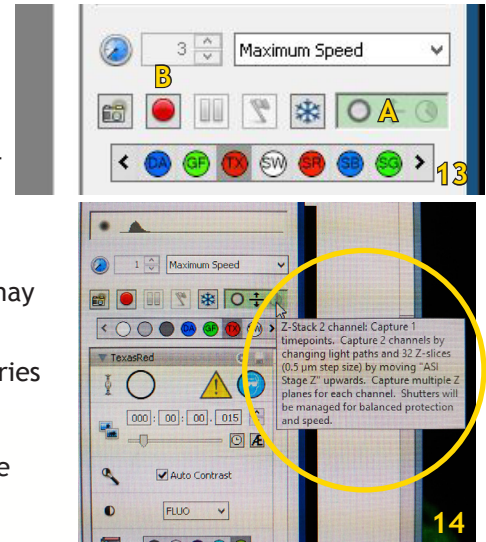
If you want to take a multichannel image or an image series like a Z-stack, time series, or stitched image, you need to define these actions in the 'Acquisition Setup Window' (Fig. 13A). Double-click on the acquisition panel to open the 'Acquisition Setup Window'.

The dialog is divided into tabs according to the type of experiment being performed (details see below). Configure only the tabs needed and you may ignore the rest. Check the summary to make sure a previous user has not configured a step you do not want! You can rename your image/image series under the Channels/Z tab (Fig. 15E).

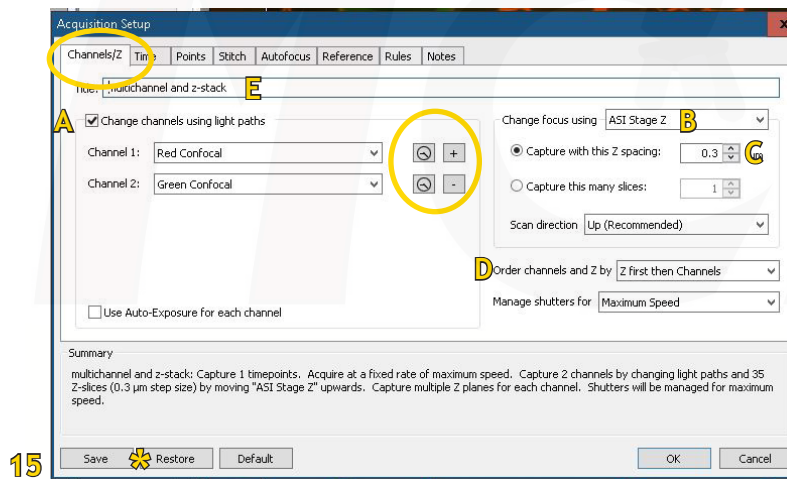
You can see the summary at the bottom of the window or if you hover the mouse cursor over the acquisition setup panel (Fig. 14).

Image acquisition is started by clicking on the red button (Fig. 13B).

The acquisition protocols most often used are: Channel/Z - Time - Stitch. The stage needs to be calibrated for 'Stitch' and 'Points' (see page 13).



Channels / Z (Fig. 15)



If you wish to acquire a multichannel sequence check the 'Change channels using light paths' box (Fig. 15A). Add those channels you want to acquire ('+' and drop-down menu). Select the fluorescence channels in the order from longest to shortest wavelengths to minimize interferences!

The ScopeLED colour image acquisition also uses the 'Change channels' option. More details on colour image acquisition can be found under 'ScopeLED Image Acquisition' on page 12.

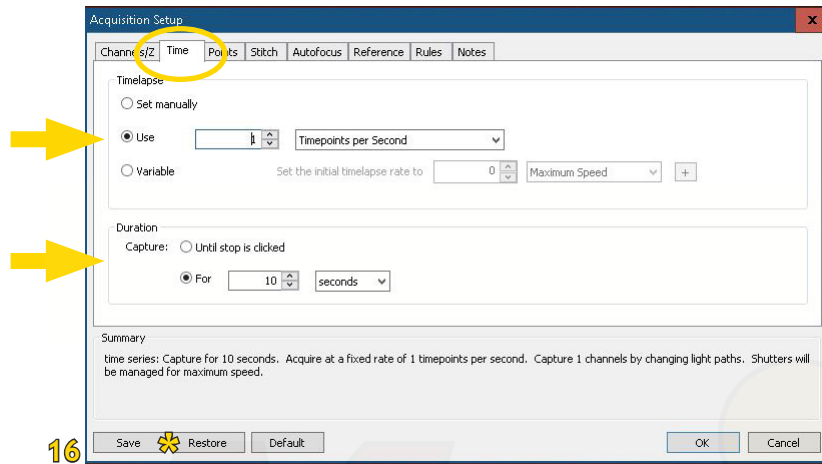
If you wish to acquire a stack of images, select 'ASI stage Z' under 'Change focus using' (Fig. 15B).

The recommended step size (Fig. 15C) depends on the objective. Recommended step sizes are listed on page 4. You can choose (in multichannel images) if you want to acquire first the stack and then switch channels, or if you want to first acquire each channel and then change planes (Fig. 15D). More details on Z-Stack acquisition can be found on pages 12-13.

Time (Fig. 16)

To define a time series, select 'Time'. Under 'Timelapse' you define the interval - maximum speed, x timepoints per ms, per second, per hour, etc.

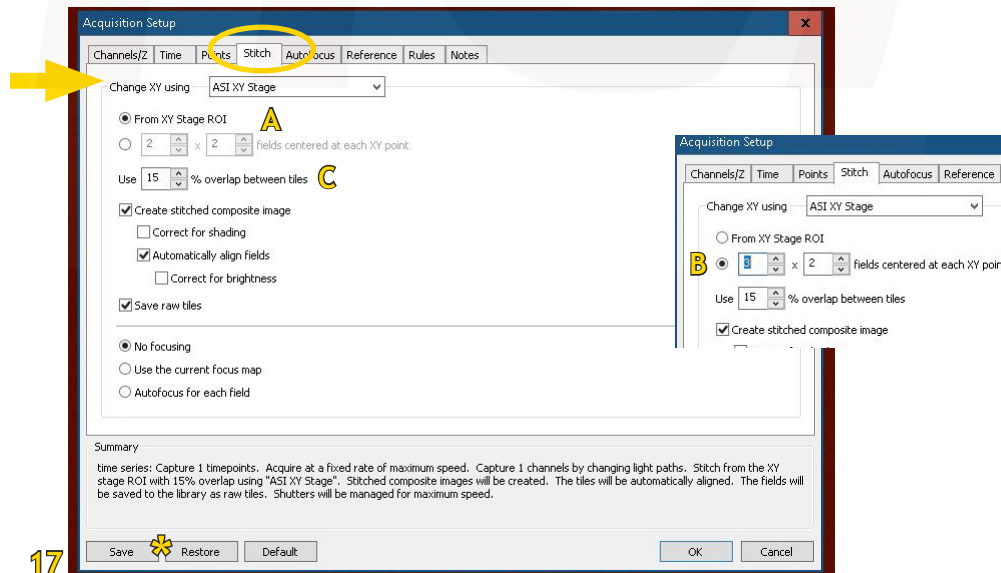
Under 'Duration', you define the length of your time series - 'until stop is clicked' or xx seconds, minutes, hours, etc.



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Stitch (Fig. 17)

To compose a large image from several regular images, select the 'Stitch' tab. Select 'ASI XY Stage' under 'Change XY using'. Then choose either 'From XY Stage ROI' (Fig. 17A) or define the number of fields in X and Y around your current position (Fig. 20B, inset). A 10-15% overlap between the tiles is recommended (Fig. 17C). More details on large image acquisition can be found on pages 13-14.

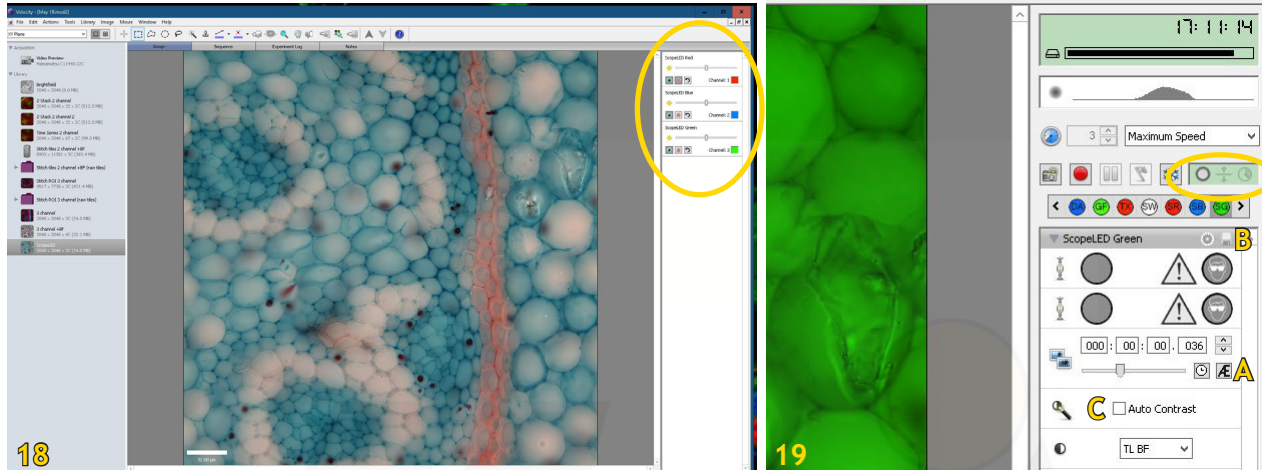


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Points: This is usually used in combination with a time series. Select the 'Points' tab, select 'ASI XY Stage' under 'Change XY using'. Find the points you want to image and mark them on the stage (see page 14).

ScopeLED Colour Image Acquisition (Fig. 18)

Since the Hamamatsu is a monochrome camera, you need to acquire a colour image as overlay from a red, blue, and green image (Fig. 18). Under 'Channels/Z' select 'Change channels using light paths' and select the light paths SR, SB, and SG. For each channel, select 'Auto Exposure' (Fig. 19A) and save the LP settings (Fig. 19B). This ensures realistic colour representation in your image. You should do this for each image acquisition, even if you're working with just one sample.



Important:

- Make sure 'Auto Contrast' is deselected (Fig. 19C)!
- Make sure the ScopeLED shutter on the controller (Fig. 3A) is closed (light off)!

Then proceed to take the multichannel image as described on page 10.

Restoring Acquisition Protocol Settings

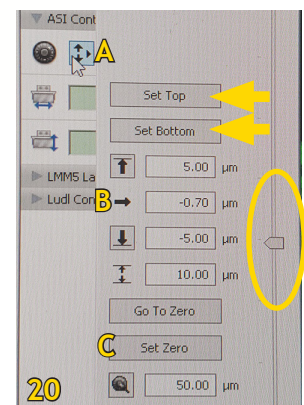
If you always use the same acquisition protocol, you can save it and easily recall it in your next session. In the 'Acquisition Setup Window', click on 'Save' to save your protocol in a designated folder and on 'Restore', to display a list of saved acquisition protocols (Figs. 15-17, *). Select the protocol that you wish to restore and click 'Restore'.

Z-Stacks Details

Define the range of your Z-stack

Once you have set the parameters for Z-stack imaging in the 'Acquisition Setup Window' (Fig. 15B-D), you need to define the beginning and end points of your stack.

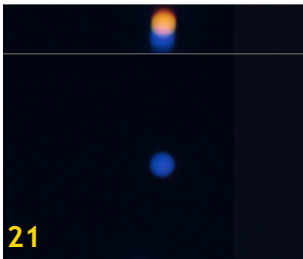
- To do this, close the 'Acquisition Setup Window', open the 'ASI Controller' submenu in the right control panel and click on the icon with the 3 arrows (Fig. 20A)
- Use the slider on the right side to focus through your sample (do not use the Leica microscope focus!!!)
- Set the bottom limit of your stack by clicking 'Set Bottom' and the top by clicking 'Set Top' (Fig. 19, arrows)



Given the recommended step size, the software will automatically calculate the number of images in your stack. It is important to remember that the positions here refer to settings of the ASI controller and not the Leica microscope! Therefore, ensure that your centre focal plane is close to '0' (Fig. 19B) before you define the beginning and end of your stack. If the stage position value is much different from zero, select 'Set Zero' to define your focal plane (Fig. 19C).

Registration correction

If you take Z-stacks of multiple channels including the 'UV Fluorescence' path, you need to perform a 'Registration Correction' on your Z-stack. The UV channel has a mismatch in the Z-plane (chromatic shift) from the other light paths, see Fig. 21. While this is more crucial in confocal microscopy, you might consider applying this correction as well.



Volocity can generate a 'Registration Correction' that can be applied for 3D data to shift pixels in all channels to their correct positions. This is done routinely twice a year and the current Registration Corrections are saved on the computer desktop, but it may be necessary that you repeat this procedure.

Please contact Michaela and she will show you how to do the correction.

Working With the Motorized Stage (Stitch and Points)

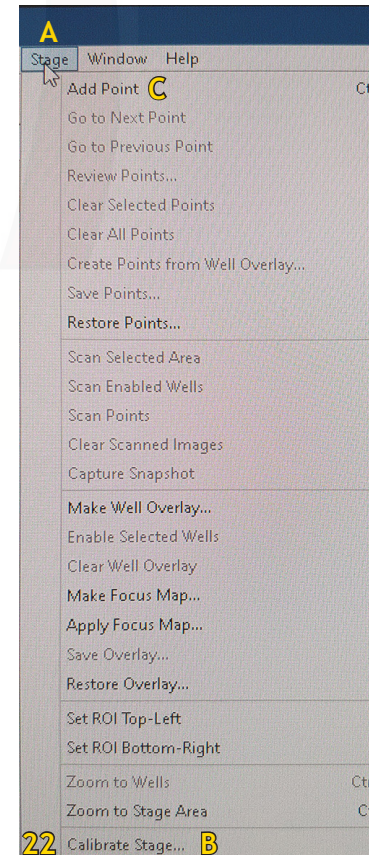
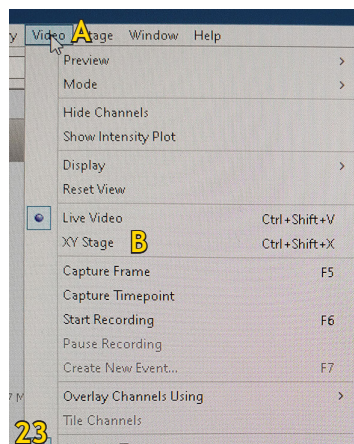
Stage calibration

Before you can define ROIs and points on the stage, it needs to be calibrated in Volocity.

- In the main menu, select 'Stage' (Fig. 22A)
- Select 'Calibrate Stage ...' (Fig. 22B)
- The software will prompt you to remove your sample and lower the stage - so this is best done at the very start of your session!
- Wait until the calibration process is finished. Then place your sample on the stage.

Define ROIs for stitching on the stage screen

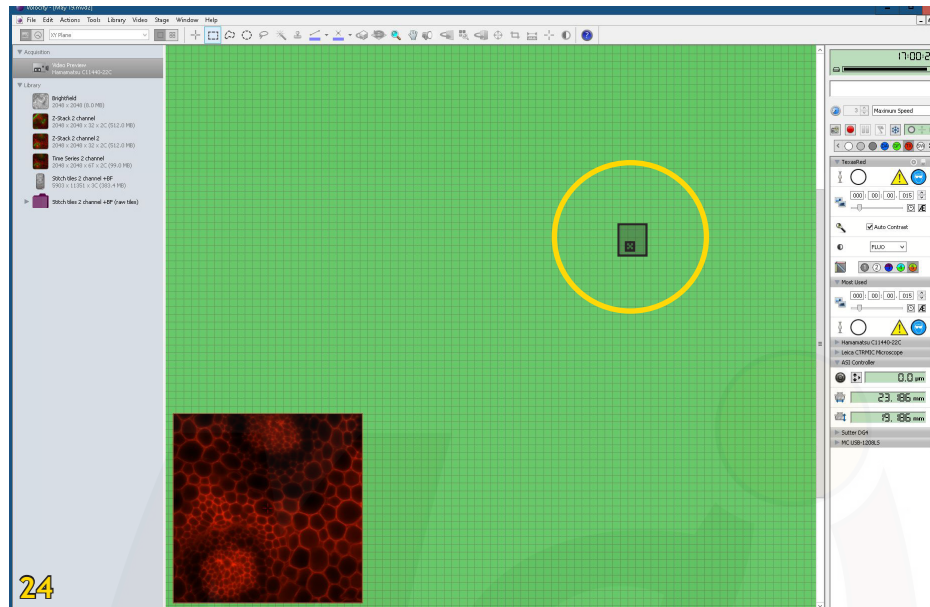
- In the main menu, select 'Video' (Fig. 23A)
- Select 'XY Stage' (Fig. 23B)



You will now see the stage area in green on your screen and a small live image in the corner. Your current position is marked with a crosshair.

You can define the ROI by drawing a rectangle around your current position (Fig. 24).

To define the ROI more precisely, you can move the stage and add position markers by selecting 'Add point' under the 'Stage' menu (Fig. 22C). You can then create an ROI limited by those reference points.

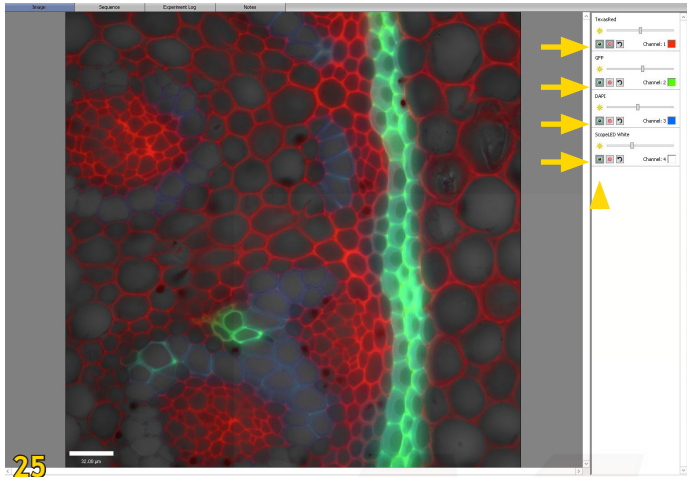


Define multiple points on the stage screen

This is done when 'Points' is selected in the 'Acquisition Setup Window'. Move the stage to the position you want to image and add a marker by selecting 'Add point' under the 'Stage' menu (Fig. 22C). Proceed to the next position and repeat.

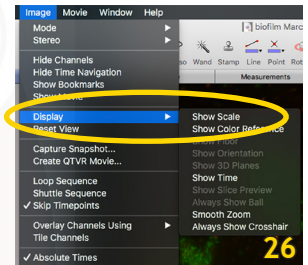
Viewing Options

Once the image is acquired, a thumbnail will appear in the left panel. To view it, simply click once on the thumbnail (double-click will open it in a new window).



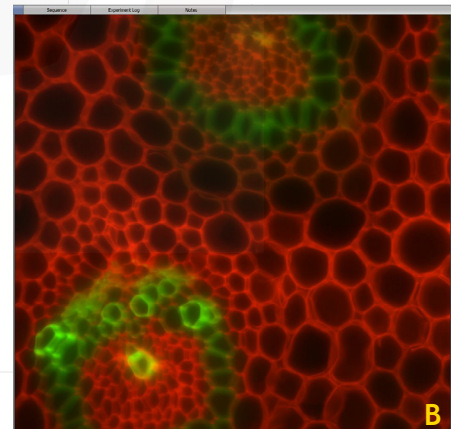
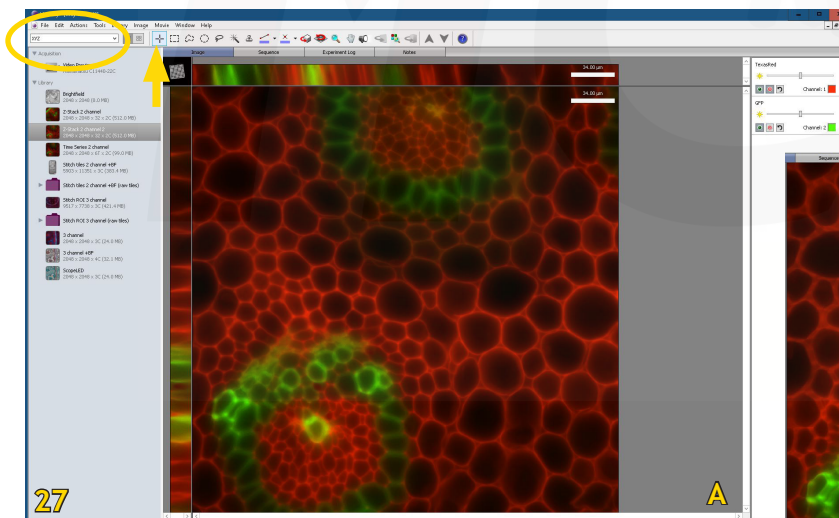
Overlay Image

Multichannel images will automatically be displayed as overlay. To hide one channel, you can click on the eye icon in the right viewing pane (Fig. 25, arrowhead) - here, you can also change the intensity of your channels (Fig. 25 arrows).



Scale Bars (Fig. 26)

The scale bars have been calibrated and you can select to see the scale bar under 'Image' - 'Display' - 'Show Scale'



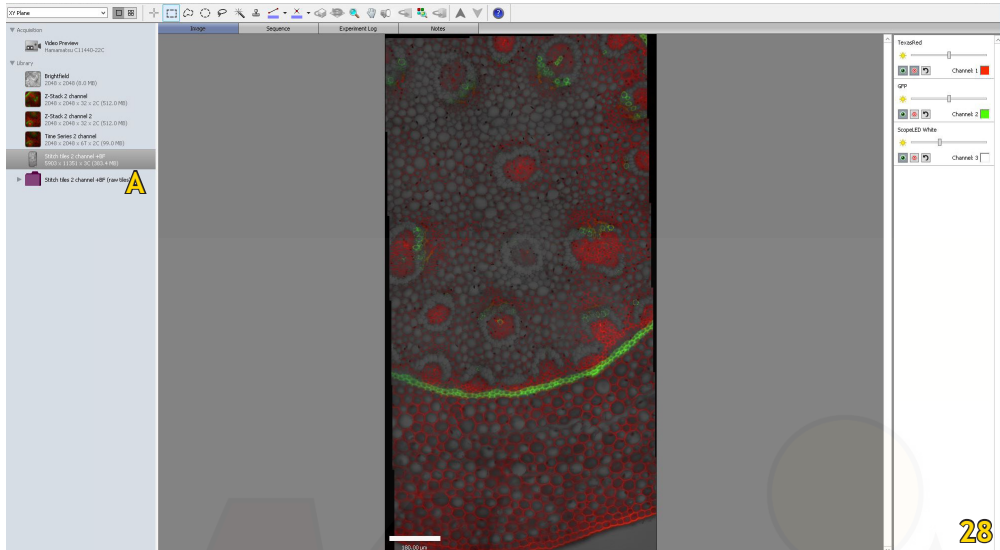
Z-Stack Image

Multichannel stack images will automatically be displayed as overlay in 'XYZ' view (Fig. 27A). The XY plane is the large centre image and the XZ and YZ planes along 2 crosshair lines are shown top and left of it. To navigate within you image, make sure the 'Crosshair' icon is selected (Fig. 27A, arrow).

You can switch to 'Extended Focus' view (Fig. 27B) in the top left drop- down menu (Fig. 27A, circle). This will merge all Z-planes into one 2D image.

Stitched Image

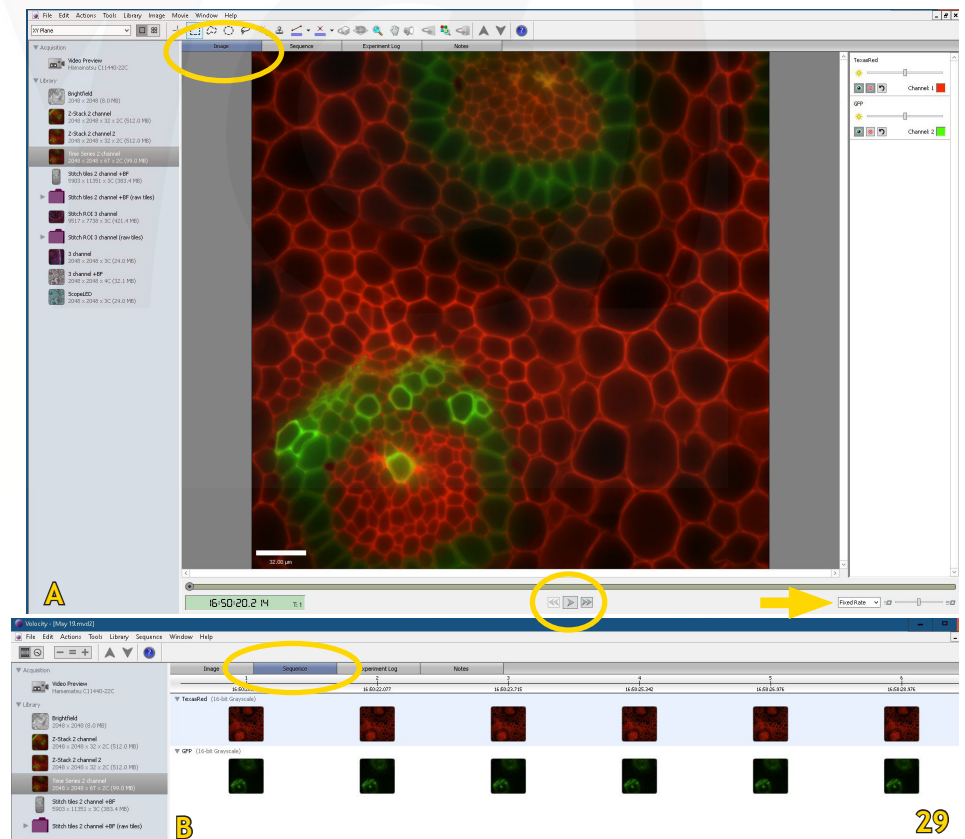
If you use the default setting in the 'Acquisition Setup Window', the tiles will automatically be stitched to a large image and this image is shown (Fig. 28) - a folder with your individual tile images is also saved (Fig. 28A).



Time Series

After acquisition, you can watch your time series as movie if you select 'Image' in the image view menu of the centre screen area (Fig. 29A) or, you can look at the individual images if you select 'Sequence' (Fig. 29B).

To view the movie, define the replay speed at the bottom right (Fig. 29, arrow), just below your image and then press the 'Play' button.



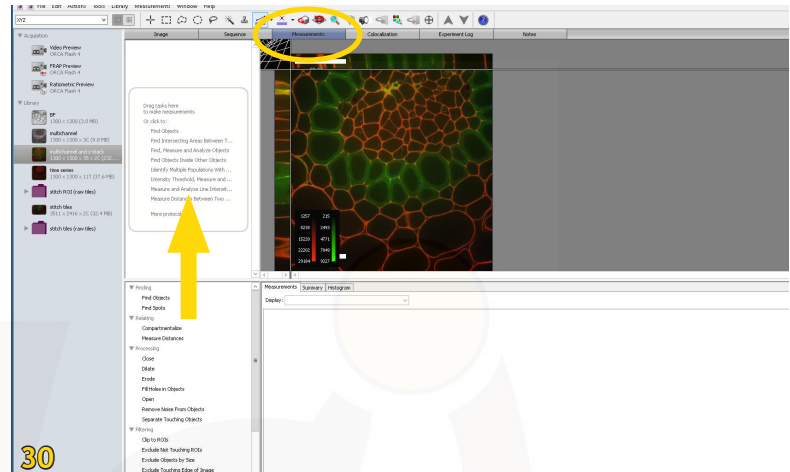
Data Processing

You will do the processing likely not on the imaging computer but with the offline modules of Volocity that are available. The three modules are:

- Measurements (Quantitation)
- 3D (Visualization)
- Decon (Restoration)

Measurements

When you select the 'Measurements' tab in the centre viewing area (Fig. 30), you will see a list of options for quantitation. You will need to select the function you want to use (i.e., 'Measure distances') and drag it into the top area (Fig. 30, arrow). The Volocity Help function will guide you through setup and processing.



3D Reconstruction and Visualization

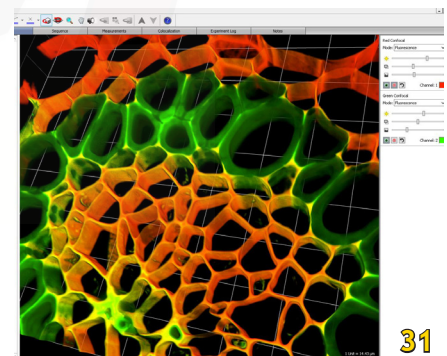
There are several options to reconstruct and view your Z-Stack images as 3D data.

3D Opacity (Fig. 31) is an interactive high resolution renderer with options for how the data is displayed. Resolution is managed to maintain interactivity. If Volocity Visualization is used in conjunction with Volocity Quantitation measurements, feedback can be shown in the 3D Opacity mode. Control over brightness, density and black level is given on a per channel basis. You can also create and export a video of your rendering.

3D Ray tracing is a processor intensive rendering that requires more time to calculate the image and is therefore not interactive. Different effects may be generated with the ray tracer but it does not support measurements feedback or scale shown on the view.

3D Slice allows 3D or 4D data to be viewed at a different orientation of slicing and, if required, the data may be re-sliced to create another version of the data at the new orientation.

Please refer to the Volocity User Manual or Online Help for more detailed instructions and examples.

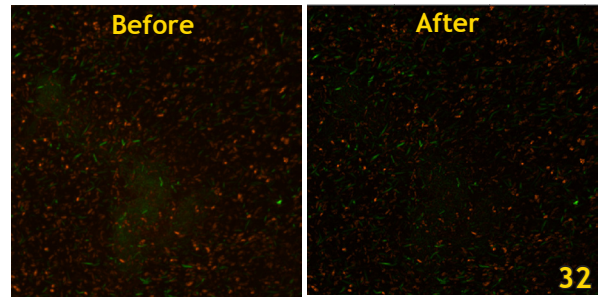


Deconvolution

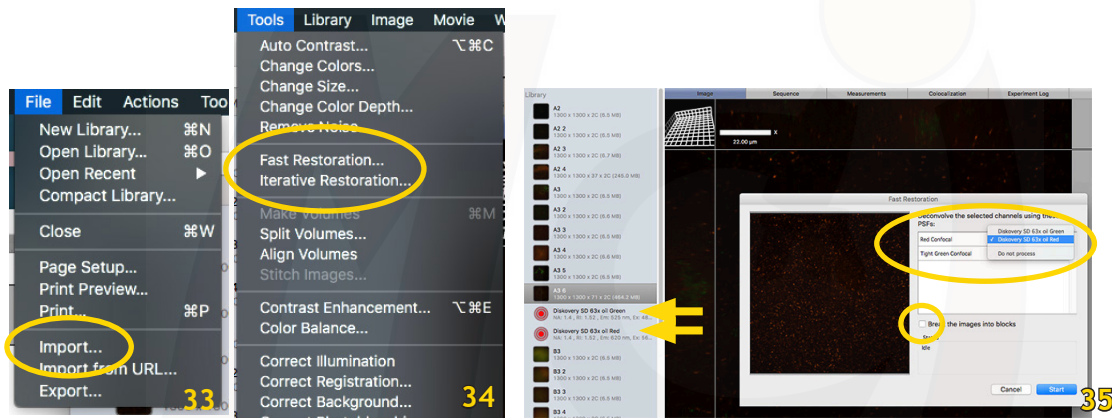
Deconvolution might be necessary for some image stacks (see Fig. 32).

To be able to do this, you need the PSFs (Point Spread Functions) for the corresponding objectives (Fig. 35, arrows). Each objective has its own PSF for each channel. They are available on the computer (folder on desktop) and on the MCIF group page under

‘Documents’ - ‘Epifluorescence Microscopes’

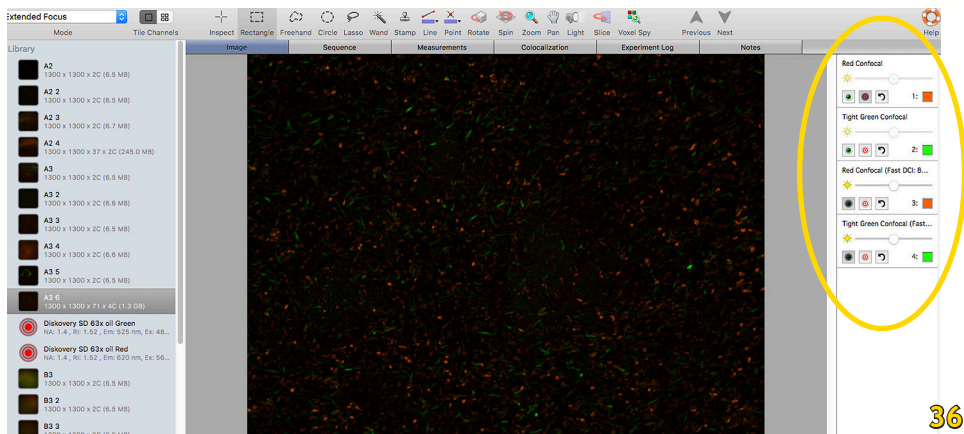


- Import the PSFs you need via ‘File’ - ‘Import’ (Fig. 33)
- Select ‘Tools’ - ‘Fast Restoration’ (Fig. 34)
- In the new window, verify that the correct PSF is assigned to each channel (Fig. 35)
- If your image is very large, select ‘Break the images into blocks’ (Fig. 35)
- Press ‘Start’



Once the deconvolution is finished, all 4 channels (original and deconvolved) will be shown as overlay. Deselect (hide) the original channels with the eye icon to see the deconvolved image only (Fig. 36).

Create a snapshot of the deconvolved image and export the snapshot (see page 19, Fig. 40).



Data management

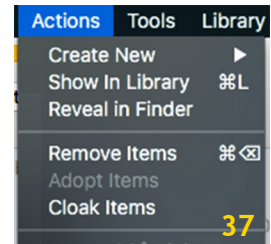
File format

Your saved data will appear in a folder with the experiment name. Within this folder you find another folder called 'Data' and a small file with the extension .mvd2.

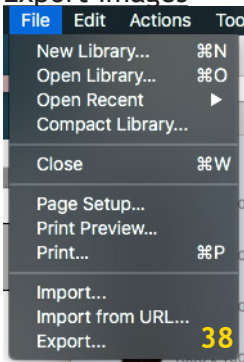
Both items need to stay together in this folder! Volocity or other imaging programs are not able to open your images only from the .mvd2 file.

Delete images from your library (Fig. 37)

- Select the image/image series in the left panel
- In the 'Actions' menu select 'Remove Items'



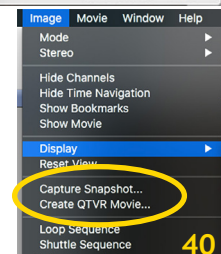
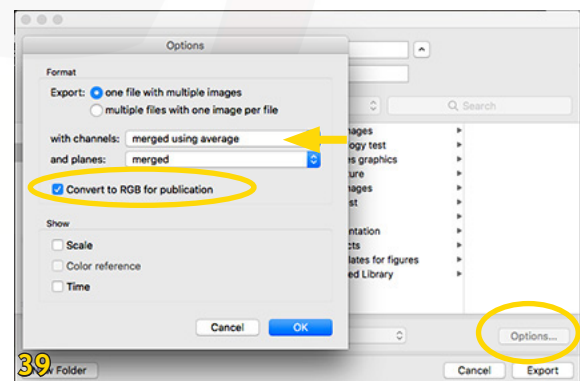
Export images



- Select the image/image series in the left panel
- In the 'File' menu select 'Export ...' (Fig. 38)
- Define/find the folder where you want to save your image
- In the drop-down menu select the format in which you want to save your image. You can choose between 'Item as...' and 'View as ...' - for regular image export without annotations this doesn't make a difference. If you have a multichannel image and you want to export the overlay, select 'Options' (Fig. 39) and under 'with channels' select 'merged using average' (Fig. 39, arrow). You can also select to add a scale bar to your image.

Export annotated images

- Select the image/image series in the left panel
- In the 'File' menu select 'Export Item'
- Define/find the folder where you want to save your image
- In the drop-down menu select the format in which you want to save your image. You can choose between 'Item as ...' and 'View as ...' - if you want to export your image with annotations, you need to select 'View as ...'
- Make sure in the options window the box 'Convert to RGB for publication' is checked.



Sometimes you also have to create a snapshot of your annotated image first:

- Select 'Image' - 'Capture Snapshot' (Fig. 40)
- This snapshot can then be exported as described above.

Shutdown

1. Lower the stage, remove your sample, clean objective lenses with lens paper
2. Close Volocity, and transfer your files to OneDrive (preferred) or the server
3. Switch off main power switch
4. Minimize intensity of X-Cite Light Source **and then switch off**
5. **Shut down the computer**
6. Log your usage into the log sheet and **cover the microscope**

2-Hour Rule

If someone else has booked the microscope within the next 2 hours, please leave the X-Cite switched on, just minimize the intensity! You can also leave the computer on. Don't cover the microscope.