UNIVERSITY of GUELPH
Advanced Analysis Centre

Molecular & Cellular Imaging Facility

Zeiss Axiozoom.V16

Contents

April 2020

Zeiss Axiozoom.V16

The Zeiss Axiozoom.V16 is a stereoscope that combines a 16x zoom with high numerical aperture lenses. It is capable of bright field colour imaging and fluorescence imaging of large fields or multiple samples.

Cameras: Hamamatsu Orca Flash4.0 v3 (monochrome) and Zeiss Axiocam305 (colour)

Stage: fully motorized Märzhäuser Stage

Fluorescence light source: HXP 200 C

Fluorescence filter blocks: DAPI, FITC, RFP

Transmitted light modes: Brightfield, Dark Field, Relief Contrast

Objectives: 1x and 2.3x

Optical sectioning: Apotome.2 (Structured Illumination)

Software: Zen 2.6 Blue

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User Guidelines

1. When you begin to use these facilities 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 book 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 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.

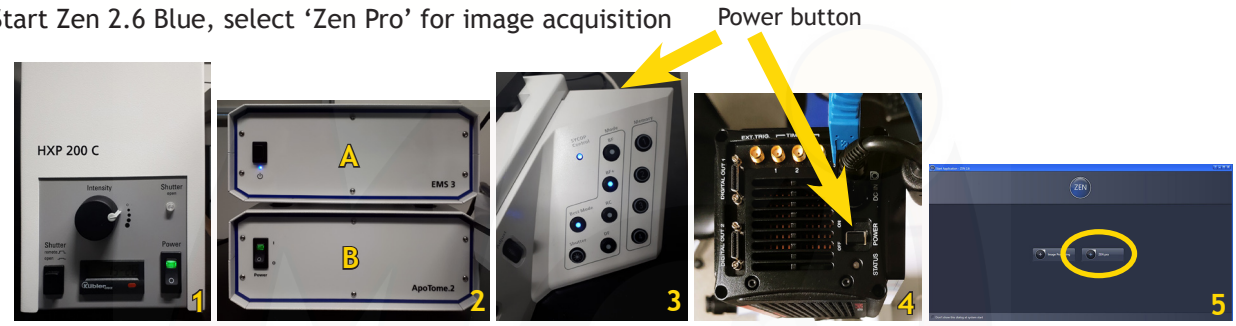
6. Files saved to computer hard drives must be removed as soon as possible. All computer hard drives will be cleared on a regular basis - it is the users' responsibility to manage their own image files. USB keys are not allowed on any of the instrument computers! The facility offers a variety of other 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.

Zeiss Axiozoom
Startup and Sample Setup

System Startup

1) HXP 200 C (Fig. 1)
2) EM3 (Fig. 2A)
3) Apotome.2 (Fig. 2B)
4) Stage Controls at back of base (if not switched on when EM3 started) (Fig. 3, arrow)
5) Hamamatsu camera (for fluorescence imaging) (Fig. 4)
5) Computer
6) Login to Bitlocker, no other login required
7) Start Zen 2.6 Blue, select 'Zen Pro' for image acquisition

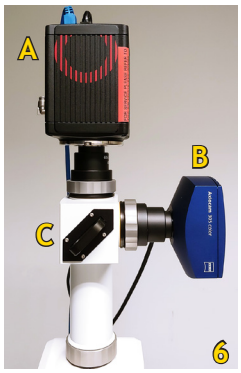


Accessory Equipment

HXP 200 C: The HXP 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. Setting the intensity to the second notch might be enough, except for imaging at low magnifications. General rules are the same as for UV mercury lamps!

EMS 3: Core Power Supply – it starts, in theory, the stereoscope and stage controls; but the stage and light controls often need to be started up separately (round button at right back side of stand)

ApoTome.2: Power Supply for the apotome – SIM (= structured illumination microscopy, see page 4); if you use the Hamamatsu camera, start the ApoTome.2 in any case, even if you don't need it since the camera is calibrated with the apotome activated.

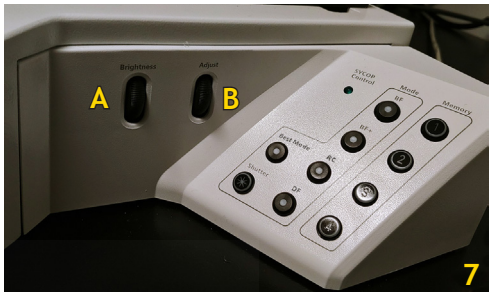


Cameras: The system has two cameras (Fig. 6): a monochrome Hamamatsu Orca Flash 4.0 v3 for fluorescence or black/white transmission imaging with a frame rate of 80 fps at 8bit (Fig. 6A); a Zeiss Axiocam 305 colour camera for transmission colour imaging (Fig. 6B).
A manual slider (Fig. 6C) switches between them: in = Axiocam, out = Hamamatsu

Stage: The stage is a fully motorized Märzhäuser stage and the software receives its coordinates automatically. Stage calibration is only needed for repeated imaging of the same area (e.g. well-plate scans) during one session. The specific calibration is not held from one session to the next, therefore, the function is generally deselected in the software.

Zeiss Axiozoom Controls

The Axiozoom is fully automated and all functions can be controlled through the software. Therefore, you will likely not need to use the buttons on the stereoscope. Nevertheless, a quick overview of the controls available is given here.



Transmitted light base capabilities:

Bright Field (BF) - Dark Field (DF) - Relief Contrast (RC)

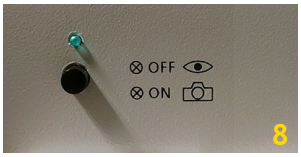
The transmitted light optics are adjusted by a mirror.

Controls at the right side of the base (Fig. 7):

Dials ‘Brightness’ for intensity (Fig. 7A)

‘Adjust’ for the angle of the mirror (Fig. 7B)

Buttons for different transmitted light paths (BF, DF, RC, Best Mode) and TL shutter



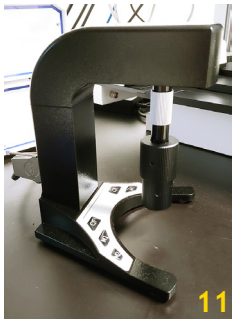
At the top right side of the stereoscope is a button to manually switch between eye piece (blue light off) and camera (blue light on) observation (Fig. 8).

However: pretty much all controls will be operated through the software!

The Axiozoom has 2 objectives - 1x and 2.3x (Fig. 9), which need to be switched manually!
Additionally, it has continuous zoom capability from 0.7-11.2 - the zoom is selected only in ZEN Blue.
The 1x objective has a nice long working distance but the 2.3x objective comes pretty close to your sample.

Red STOP button (Fig. 10): press if automatic focusing might run the objectives into the sample

Moving the stage in XY is done manually (Fig. 11) or through the software (see next chapter)



Focus is only possible with the ZEN Blue software! (see next chapter)

Fluorescence capabilities:

DAPI - GFP - Texas Red (TR)

The fluorescence filters are switched with the buttons 1 (DAPI), 2 (GFP), 3 (RFP), and the FluoShutter is opened and closed with the control button ‘*’ (Fig. 12A) on the left top side of the Axiozoom (Fig. 12).

Zen Blue Acquisition Setup

Program Interface

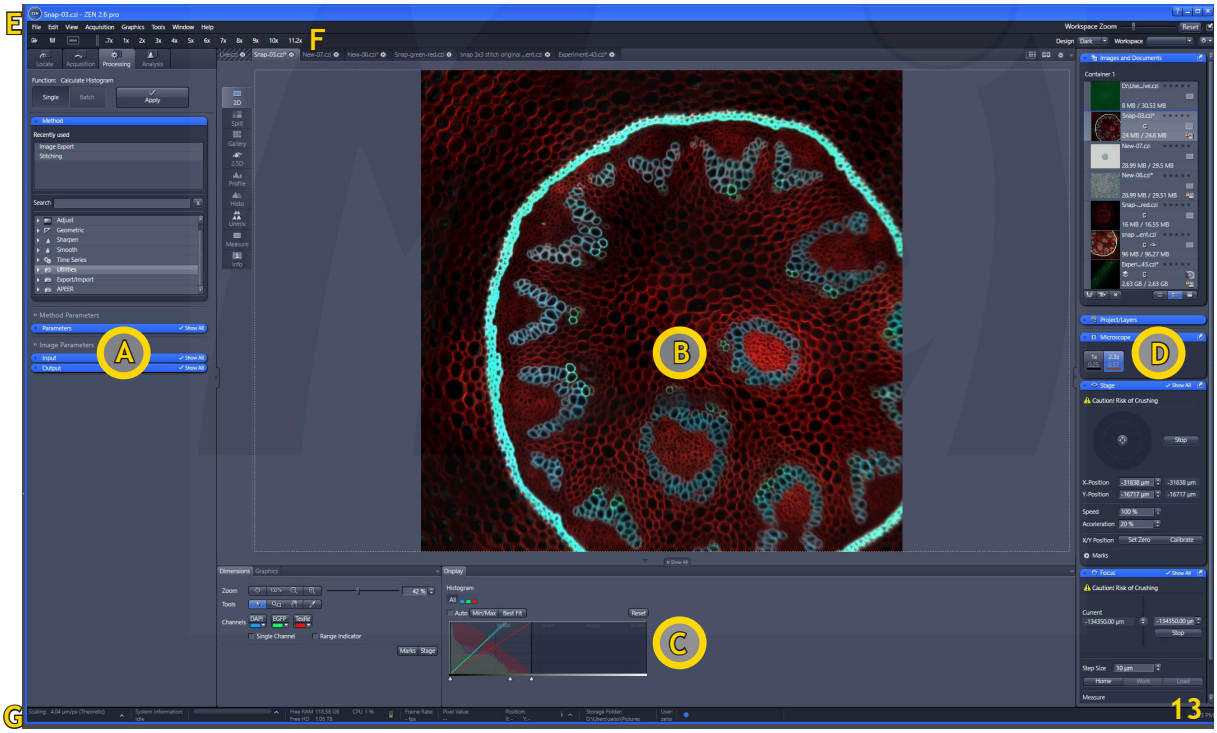
The Zen program interface is divided into 4 main areas.

Via the tabs in the left tool area (Fig. 13A) you can access all the main tools for microscope and camera settings (‘Locate’ tab), image acquisition (‘Acquisition’ tab), image processing (‘Processing’ tab), and image analysis (‘Analysis’ tab).

The top centre screen area (Fig. 13B) is used to display your images and is divided into 3 sections: Document Bar, Image Data View, and Image Area.

The bottom centre screen area (Fig. 13C) shows specific view and post-acquisition modification tools.

The right tool area (Fig. 13D) provides you with an overview of all open documents and has the controls for stage movement and coarse focus.



A - Left Tool Area
B - Top Centre Screen Area
C - Bottom Centre Screen Area
D - Right Tool Area
E - Menu Bar
F - Tool Bar (includes preferred zoom settings)
G - Status Bar

Basic Image Acquisition

Once the software is started, select the tab ‘Locate’ (Fig. 14A)

Locate

Open ‘Microscope Control’ and check the configuration of the microscope (Fig. 14): if there’s a red X on any of the components, it hasn’t been switched on – the software needs to be restarted after switch on.
However, remember there are two different configurations available: if you want to image with transmitted light and the HPX200 and/or the Hamamatsu camera are not switched on, a red X and ‘Broken Camera’ message for the Hamamatsu is fine, just ignore it.

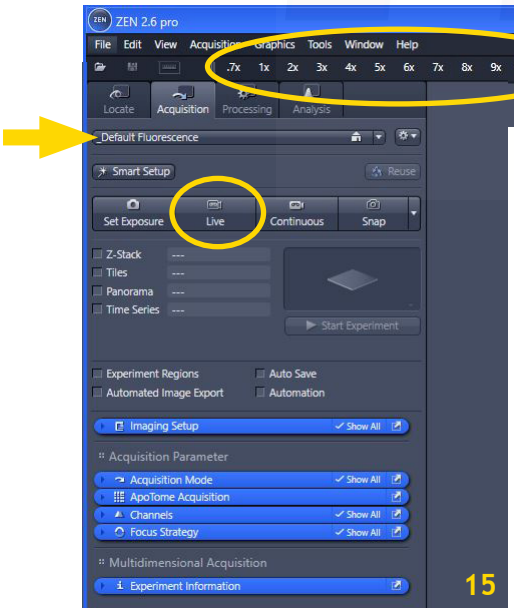
You will also see the 4 pre-configured light paths (Fig. 14, arrow):

DAPI – GFP – RFP – Brightfield

Never use ‘Continuous Snap’ or ‘Live’ under the ‘Locate’ tab - move to ‘Acquisition’ (Fig. 14B)!

Acquisition

Once you’re certain all components are ready, switch to the ‘Acquisition’ tab (Fig. 14B).
Select one of the 3 predetermined configurations in the drop-down menu (Fig. 15, arrow). These configurations are read only and can’t be permanently modified.



Default Fluorescence (default camera: Hamamatsu Orca Flash 4)

Default Brightfield (default camera: Axiocam 305)

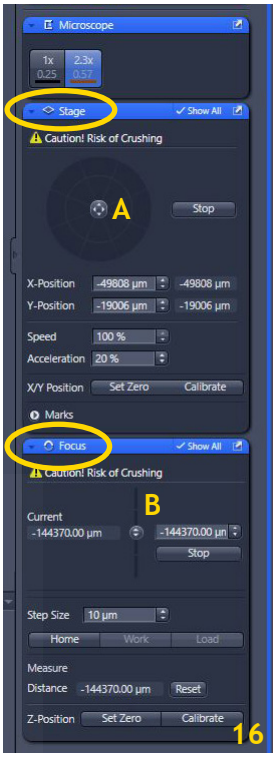
Default Relief Contrast (default camera: Axiocam 305)

Select the configuration you need

Place your sample on the stage

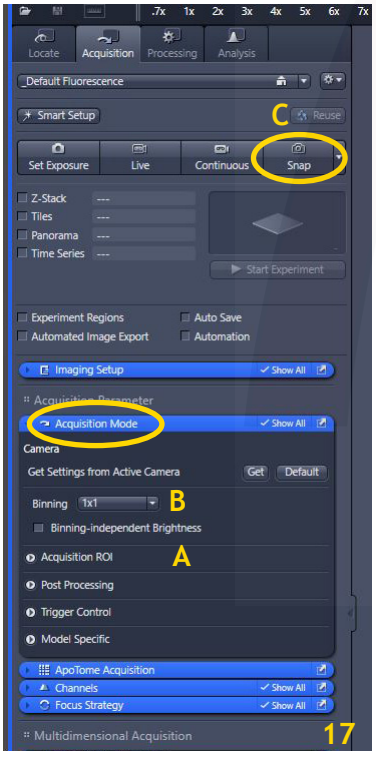
Select the objective manually and select the light path under the ‘Locate’ tab (Fig. 14, arrow). Make sure the manual slider for the cameras (Fig. 6) is in the correct position. Select ‘Live’ (Fig. 15), focus your sample (see next page), find the visual location (i.e., ROI, region of interest), and select a higher zoom if required. The preferred zoom options are in the top tool bar (Fig. 15). You will need to refocus for each zoom!

To move the stage, you can use either the manual control (Fig. 11), or the software: in the right panel (Fig. 16) open ‘Stage’, and move the button (Fig. 16A) in any direction. You can also move one frame at a time with the key combination ‘Ctrl + arrows’.



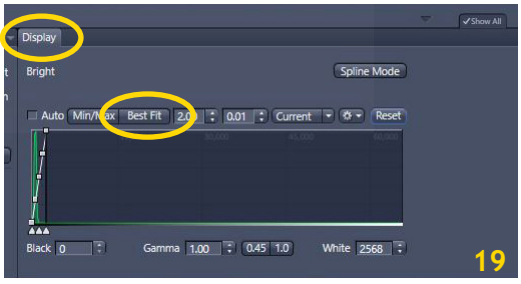
Focus is possible only through the software:
In the right panel (Fig. 16) open ‘Focus’ and move the slider (Fig. 16B) up and down. This is your coarse focus. To use fine focus, use the combination ‘Ctrl + mouse wheel’ while hovering the mouse over the image; a small pop up window will appear in which you can change the degree of fine focus.

Select one of the three default configurations and modify the parameters in ‘Acquisition Mode’ (Fig. 17) and ‘Channels’ (Fig. 18) according to your imaging needs. Since the cameras (especially the Hamamatsu) are highly sensitive and have a huge range of intensity values, you may need to select ‘Best Fit’ in the ‘Display’ window (Fig. 19) in order to actually see your live image on the screen.
This will only change the display of your image on the screen, the camera settings will not be changed.



Imaging parameters:

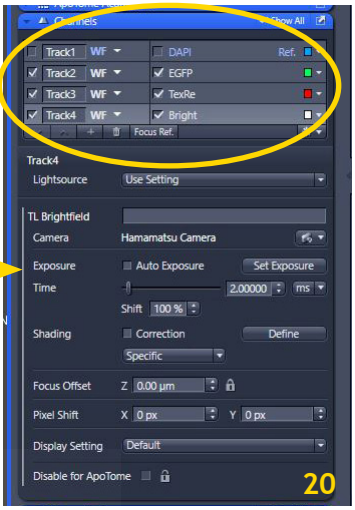
White Balance - Fig. 17A
under ‘Acquisition ROI’,
for transmitted light imaging only
Binning - Fig. 17B
Light path(s) to be acquired - Fig. 18A
Camera - Fig. 18B
Exposure - Fig. 18C



Once your settings are optimized, select ‘Snap’ (Fig. 17) and your image will be displayed in the centre screen area.
IPS (instrument parameter settings, i.e. your imaging parameters) can be recalled from a saved image each time through the ‘Reuse’ option (Fig. 17C).

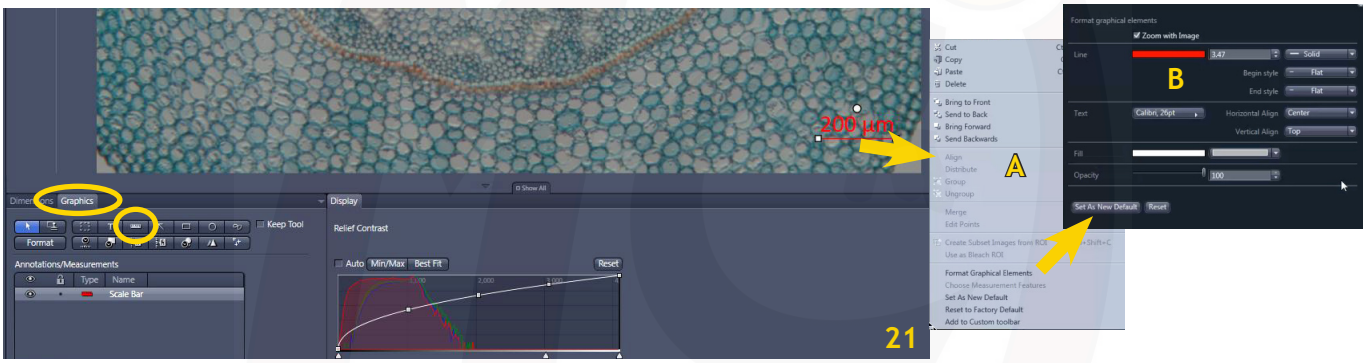
Multichannel acquisition

Besides the three fluorescence channels, the brightfield channel can be included in your acquisition setup. You need to define and select all desired light paths under ‘Channels’ - ‘Track’ (Fig. 20). If you select more than one channel, the software will automatically image all channels sequentially when you select ‘Snap’. Make sure you check each required channel and adjust the imaging parameters (Fig. 20, arrow) under ‘Live’ before you select ‘Snap’.



Adding a scale bar to your image (Fig. 21)

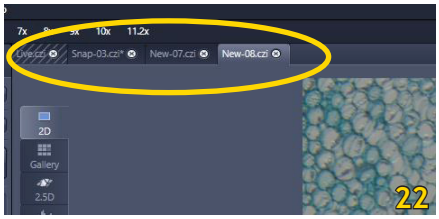
Select the ‘Graphics’ tab in the bottom centre screen area and click on the scale bar icon. The scale bar will appear directly in the image. If you need to format the scale bar (size, colour, line width) right-click on the scale bar. This will open the context menu (Fig. 21A). Select ‘Format Graphical Elements’ - in this dialog (Fig. 21B), you have numerous formatting options. More options are available in the main menu under ‘Graphics’.

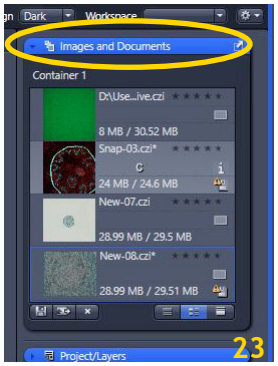


Saving files (Figs. 22, 23):

By default, none of the snapped images is automatically saved!
All snapped images are kept open in the ‘Image Area’ (you see tabs of the acquired images lined up at the top of the centre screen area, Fig. 22) and they can also be accessed in the right panel under ‘Images and Documents’ (Fig. 23).

Images are saved in .czi format either by right-click on the image tab, or in the menu under ‘File’ - ‘Save As’. See ‘Data management’ on page 27 for more options and information.

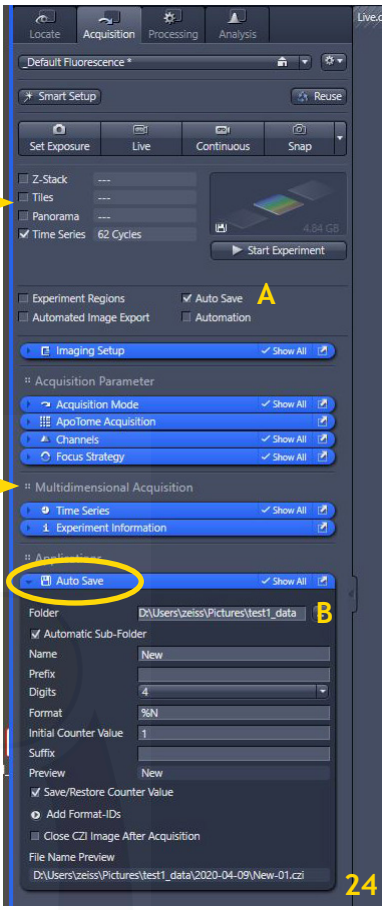




Advanced Image Acquisition Protocols

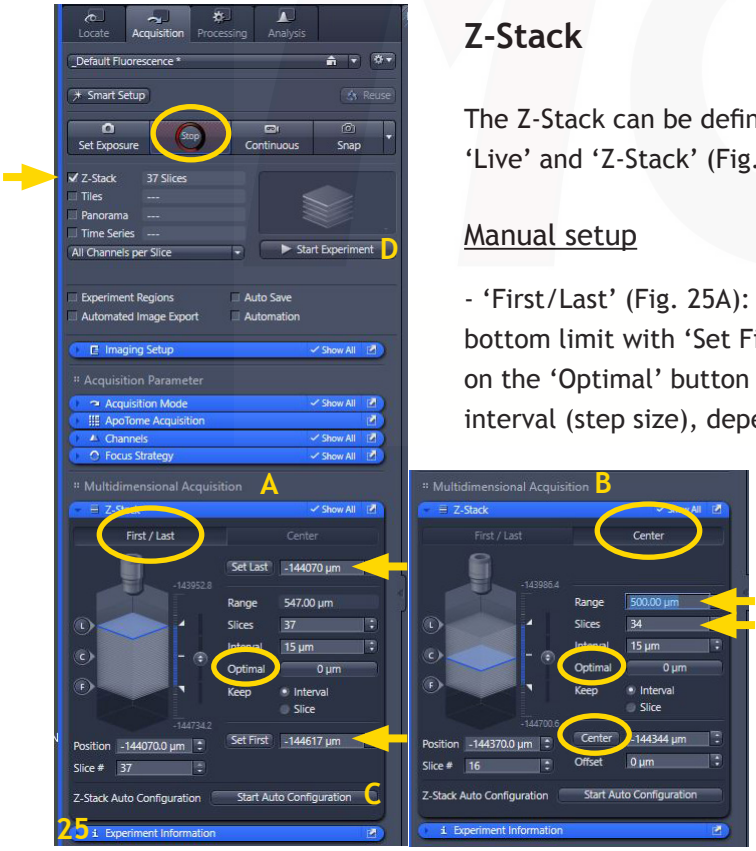
These are:
- Z-Stack
- Tiles
- Panorama
- Time Series

Advanced image acquisition protocols are set up through the MDA (Multi Dimensional Acquisition) window.
The specific parameters for image acquisition and experimental setup are defined under the 'Multidimensional Acquisition' Menu (Fig. 24, lower arrow).
When 'MDA Acquisition' is selected, the application 'Auto Save' is activated (Fig. 24A): the MDA files will be saved automatically - but you need to define the path and name the file (Fig. 24B) under 'Auto Save' before you start your acquisition.



Z-Stack

The Z-Stack can be defined manually, or with 'Auto Configuration'. Select 'Live' and 'Z-Stack' (Fig. 25)



Manual setup

- 'First/Last' (Fig. 25A): Focus through your sample (blue plane), set the bottom limit with 'Set First', then set the top limit with 'Set Last'. Click on the 'Optimal' button - the software automatically selects the optimal interval (step size), depending on objective and zoom.

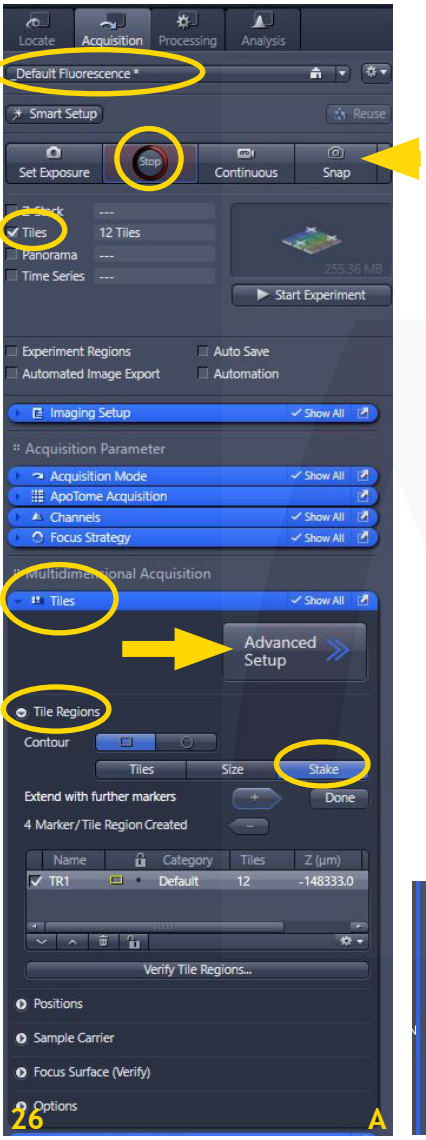
- 'Center' (Fig. 25B): Focus the centre of your sample; select 'Center' to set this position as centre point; define a range or the number of slices; Click on the 'Optimal' button - the software will automatically select the optimal interval and the number of images and range, respectively.

‘Auto Configuration’ setup (Fig. 25C)

- Select ‘Start Auto Configuration’
- Confirm the system message by clicking ‘OK’
- The auto configuration sets the focus position for the first, last, and centre slice of the Z-Stack, the number of optical slices and the interval. This can last from a few seconds up to half a minute, depending on your acquisition settings.

Caution: Depending on your sample this automated approach may not work well!

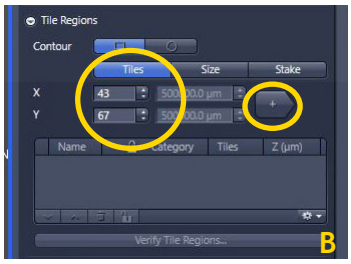
Once you have set up your Z-stack parameters, click on ‘Start Experiment’ (Fig. 25D).

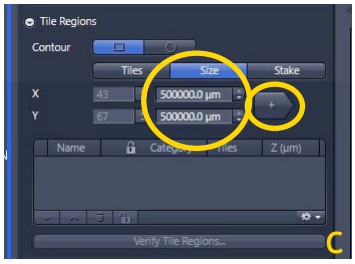


Tiling

With the ‘Tiles’ module you can acquire a number of individual images (tiles) and merge them into a large image. To do this, it is possible to define tile regions and positions. You can also image several, independent single positions with this tool.
The Tiles MDA and image processing for brightfield include shading correction and white balance, which are important to ensure a good quality large image. The white balance may need to be adjusted for each sample. The shading correction needs to be defined for each zoom stage. This has already been done for the main zoom options listed in the menu. If you want to acquire large images at a different zoom stage, please do the following to define your shading correction and white balance:

- Focus your image with the objective and zoom setting you will use for imaging. Select ‘Channels’ and ‘Show All’ - remove your sample (either empty stage or empty area on slide) and select ‘Define’ in ‘Shading Correction’ (Fig. 18).
- For the white balance, you select ‘Auto’ in ‘Acquisition Mode’ - Acquisition ROI’, or pick your own (Fig. 17A).





Setting up your Tiles MDA experiment:

There are 3 ways of setting up the tiling (they are listed from simple to complex)

- First, select ‘Default Brightfield’ or ‘Default Fluorescence’ in the ‘Acquisition’ tab (Fig. 26)
- Select ‘Tiles’ as your MDA
- Start the ‘Live’ mode to find your ROI, zoom in and focus

Option 1 ... uses the 'Snap' button to take your images for the tiling (Fig. 26, arrowhead):

Find the ROI - select centre piece – under the 'Snap' drop-down menu select the number of tiles (2x2 – 5x5) – this is simple, quick, but it is very general and generates only square images.

Option 2 ... is available in the regular MDA window (Fig. 26):

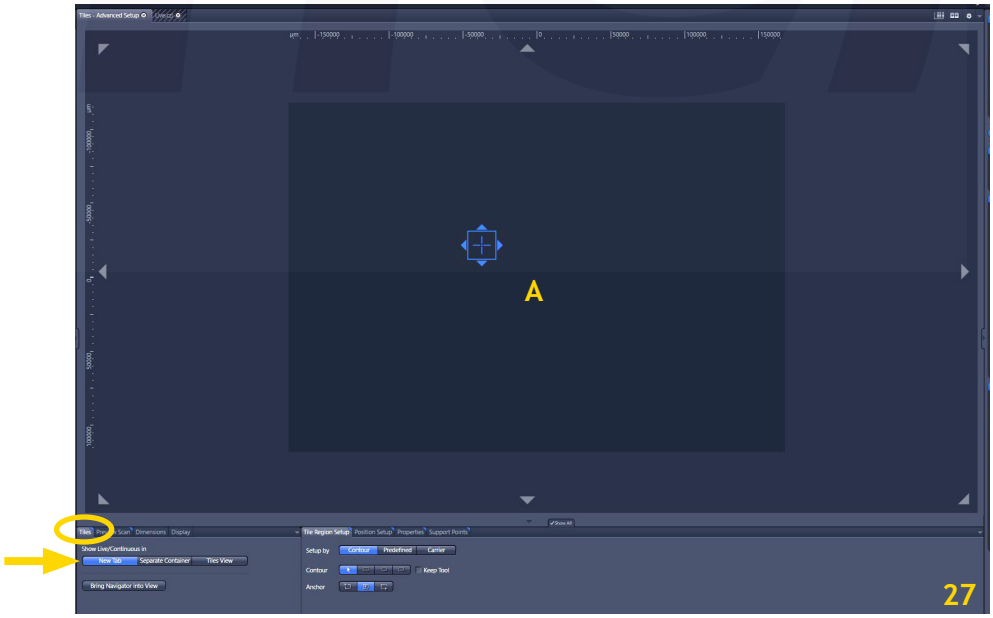
You will define a specific ROI during 'Live' mode. However, all options will still result in a rectagular or elliptical image. Open 'Tile Regions' (Fig. 26). You can define several ROIs in this setting.

- Stake option (Fig. 26A): Select 'Stake'. You can add markers/points to define an ROI based on the shape of your specimen. Move the stage and select '+' at significant positions to define the area of your ROI. Each stake is saved with its XY and Z position, so make sure you focus each position correctly before you add the position!
- Tiles option (Fig 26B): From your ROI, select the starting square, select 'Tiles' and define the number of tiles (e.g., X = 8, Y = 15). Then click on the '+' button to save this region for image acquisition.
- Size option (Fig 26C): From your ROI, select the starting square, select 'Size' and define the area (e.g., X = 50µm, Y = 10µm). Then click on the '+' button to save this region for image acquisition.

Remember to define the folder and path where your image(s) will be saved under 'Auto Save'. Click 'Start Experiment'.

Option 3 ... is available under 'Advanced Setup' (Fig. 26, arrow):

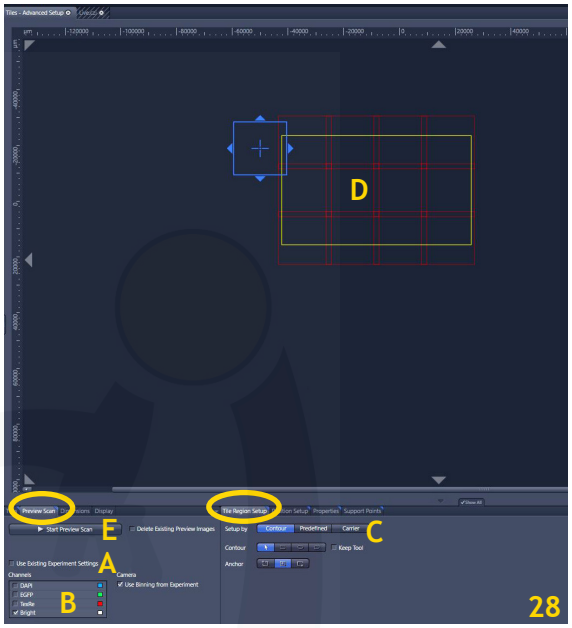
Under 'Advanced Setup', the distribution and dimensions of tile regions and positions are displayed in the travel range of the stage. You can generate a 'Preview Scan' and define tile regions or positions precisely on the basis of this template. You can also define advanced focus. For the preview scan you have the option of using the 1x objective and/or a different channel (e.g. transmitted light).



Select ‘Advanced Setup’ (Fig. 26, arrow), which opens a new window with a navigation pane (Fig 27A): you can see the entire stage and navigate! With the mouse wheel you can zoom in and out on the screen.
In ‘Advanced Setup’ you can do tiling as in option #2, but the advantage is that you can actually see your ROI boundaries; you can also copy, paste, move or resize your ROI in this mode!
Additionally, you can precisely draw the contour of your ROI, or select a template for a carrier (well plates etc.); if you use the carrier template, you need to calibrate the stage!

Select ‘Tiles’- and define whether your live image will be shown in a new tab, a separate container (= split view), or in tiles view (= within view) (Fig. 27, arrow).

How to generate a preview scan



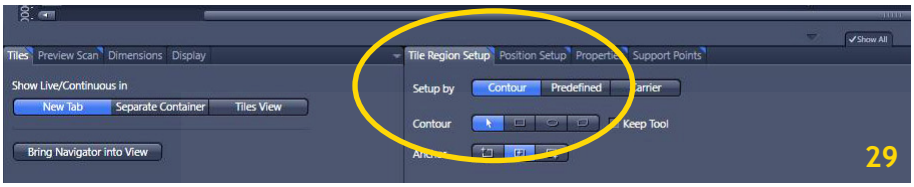
- In the bottom centre screen area under the ‘Specific View’ options open the ‘Preview Scan’ tab (Fig. 28)
- Select the 1x objective
- In the ‘Preview Scan’ tool, uncheck ‘Use Existing Experiment Settings’ (Fig 28A) and select the channel you want to use for the preview scan (Fig 28B). Use the ‘Live’ mode to adjust the focus and exposure following a change of objective or channel.
- To obtain a better overview, slightly zoom out of the advanced setup view (mouse wheel)
- In ‘Live’ mode use the stage to locate approximately the center of the region for which you want to generate a preview scan
- Select the ‘Tile Region Setup’ tab from the ‘Advanced Setup’ view options (Fig. 28)
- Select ‘Setup by’ - ‘Contour’ - ‘Rectangular Contour’ (Fig. 28C)
- In the stage view, use the tool to drag out a rectangle that approximately encloses the region for which you want to generate a preview scan (yellow line, Fig. 28D). The red squares indicate your tile images. With ‘Live’ mode, check whether the desired ROI is covered by the preview region. Use the stage to locate the corners and edges of the tile region and increase or reduce the yellow selection frame as necessary.
- In the ‘Preview Scan’ tab click on the ‘Start Preview Scan’ button (Fig. 28E). A series of snap images is acquired to generate a preview of the marked region.

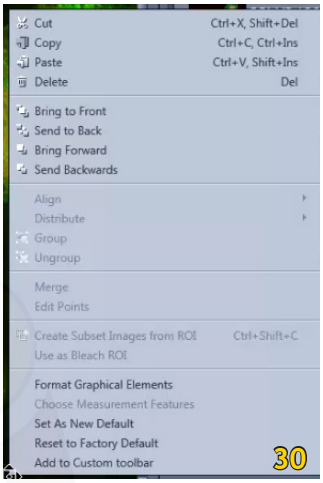
Before you continue with your actual Tiles Experiment,

- deactivate the preview tile region (usually TR1) in the list in the left tool area (Fig. 26), otherwise the entire region will be added to your acquisition when you start the experiment!
- select the objective and zoom you want to use for your actual acquisition. Use the ‘Live’ mode to adjust focus and exposure accordingly.

Define your ROI

Based on this preview scan, you can now define your ROI precisely. When you change to the 2.3x objective and a higher zoom, the number of tiles will be adjusted on the screen.
Select 'Tile Region Setup' - 'Setup by' (Fig. 29)
'Contour': select the desired contour tool (rectangular, elliptical or freeform, Fig. 29) and use the tool in the (preview scan) stage view (Fig. 28D) to mark in the tile regions (ROIs) you want to acquire.
'Predefined' and 'Carrier' relate to specific carrier (e.g., wellplate) templates. Please see 'Working with Carriers' on pages 16-17.



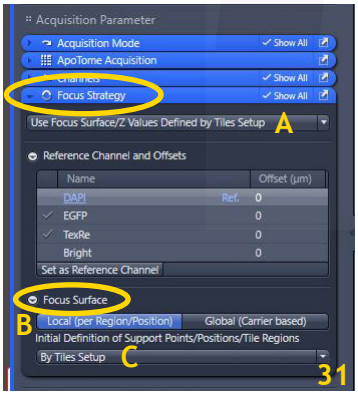


Copy, move and resize a tile region

If you want to copy, move, or resize a defined ROI from a specific area, select and right-click the ROI; this will open the context menu. You can then choose which action you want to do (Fig. 30).

Focus strategies for tiling

When you activate the 'Tiles' tool the software will automatically select the suitable focus strategy 'Use Focus Surface/Z-values Defined by Tiles Setup'. Usually, no change is necessary, but sometimes you may want to acquire tile regions or positions with different Z-positions.
In the left tool area under 'Tiles' - 'Acquisition Parameters'

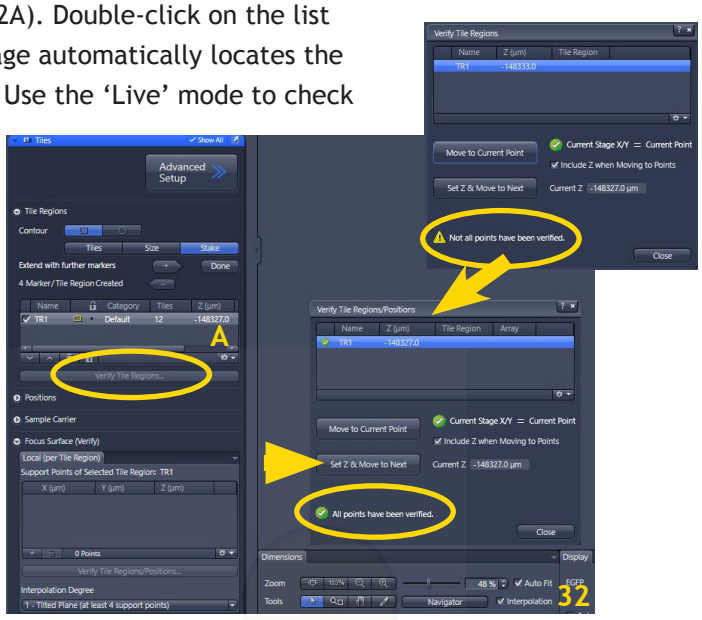


- Open the 'Focus Strategy' tool (Fig. 31)
- Select 'Use Focus Surface/Z-Values Defined by Tiles Setup' from the drop-down list (Fig. 31A, if not selected by default)
- In the 'Focus Surface' section select 'Local (per Region/Position)' (Fig. 31B).
- Under 'Initial Definition of Z-Values for Support Points/Positions' select 'By Tiles Setup' from the dropdown list Fig. 31C)
- Select the (first) tile region (TR) for which you want to create support points. To do this, go to the left tool area, under 'Tiles' - 'Tile Regions' and click on the corresponding tile region in the list (see Fig. 25A: TR1, TR2 ...)

Create a 'Local Focus Surface' for the tile regions by distributing support points across your tile regions:
Navigate your sample and select suitable support points – at each support point you focus manually and then select 'Add Support Point'. When you have added all, select the interpolation degree (depends on sample).
Tile-region-specific focus areas are then interpolated from the values of these support points.
Click on the 'Start Experiment' button.

If you have added support points but forgot to focus properly:

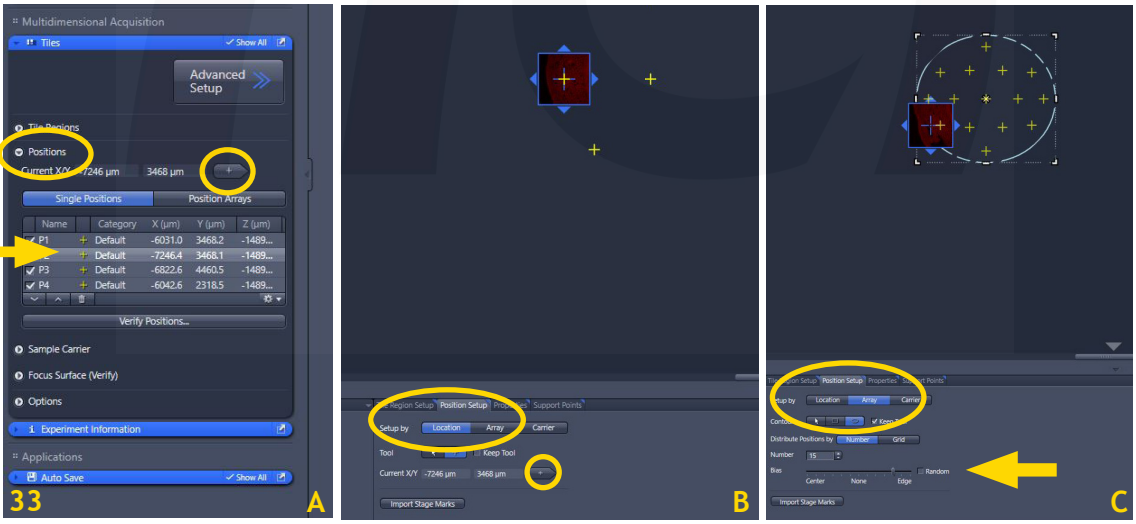
To check the Z-value of tile regions, open the ‘Tile Regions’ section in the ‘Tiles’ tool. The Z-values of the tile regions are displayed in the Z column of the list (Fig. 32A). Double-click on the list entry of the tile region that you want to check. The stage automatically locates the center of the tile region and the associated Z-position. Use the ‘Live’ mode to check the focus of the tile region. To adjust the focus, set the new Z-position using the focus tool. In the ‘Tile Regions’ list click in the bottom right on the ‘Options’ button and select ‘Set Current Z For Selected Tile Regions’.
To check further tile regions, repeat steps 2 to 4.



To check and adjust large number of tile regions, click on the ‘Verify Tile Regions’ button to open the corresponding dialog (Fig. 32). There you find the interface for the verification process of each tile region (Fig. 32, arrowhead). Click on ‘Close’ after you have verified all tile regions.

Multiposition Imaging

If you don’t want to create a large image but instead image several independent positions in your sample (i.e. time-lapse of live samples), you also need to use the ‘Tiles’ tool, but you create ‘Positions’ instead of ‘Tile Regions’.



You can do this in the left tool box under ‘Tiles’ - ‘Positions’ (Fig. 33A):
Select ‘Tiles’ - ‘Positions’ - ‘Single Positions’

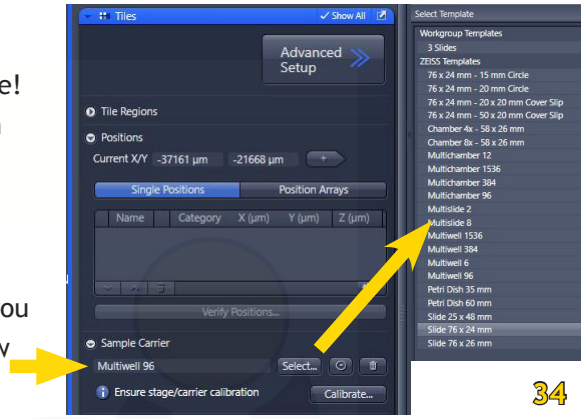
You can also add positions in ‘Advanced Setup’ - in the bottom centre area under ‘Position Setup’ (Fig. 33B:
Select ‘Advanced Setup’ - ‘Position Setup’ - Setup by’ - ‘Location’

In both cases, click in the stage view on the location where you want to add a position and focus your image. Select '+' and the added positions are displayed in the 'Single Positions' list in the 'Positions' section (Fig. 33A, arrow).

If you select 'Position Arrays' or 'Setup by' - 'Array' you need to define a contour of the ROI and you will see parameters to fill this ROI with positions (number, grid, bias) (Fig. 33C).

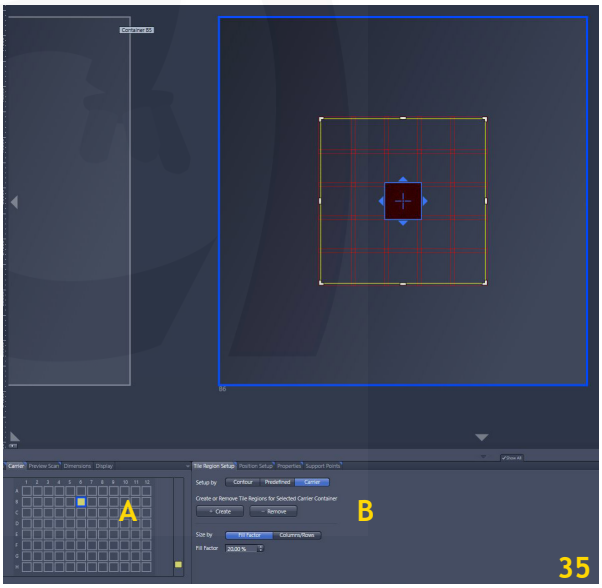
'Carrier' relates to specific carrier (e.g., well plate) templates. Please see 'Working with Carriers' below.

Working with Carriers



The stage needs to be calibrated before you can do anything else! Generally, the workflow is identical to 'Tiling' and 'Multiposition Imaging' described above.

Select the correct carrier type under 'Tiles' - 'Sample Carrier' (Fig. 34). Click on 'Advanced Setup'
In the centre area, the template will be shown on the screen - you can also select the 'Carrier' tab in the lower centre area to show the selected carrier type (Fig. 35).



Select your ROI

In the 'Carrier' tab (lower left side, below the centre screen area, Fig. 35A) select the well(s) of interest by holding 'Ctrl' and clicking on the desired wells. The selected wells are now bordered by a blue circle.

Tiling

Set up your tile region in the lower right area

- 'Tile Region Setup' (Fig. 35B)

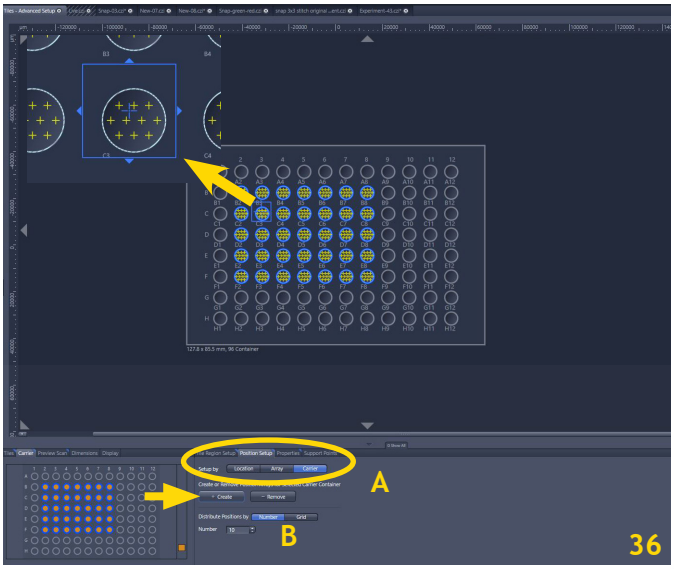
'Predefined':
Select 'Tile Region Setup' - 'Setup by' - 'Predefined'
Choose how many tiles in xy you want to add and click on the '+' button to confirm.

'Carrier':
Under 'Size by' select 'Fill Factor' and enter the desired value (area% of the well) in the 'Fill Factor' input field. Click on the '+ Create' button. According to the selected fill factor, the wells will be filled with a calculated number of tiles that are located around the center (Fig. 35). To create a given size of tile region you can use the 'Columns/Rows' function.

The default focus strategy is 'Use Focus Surface/Z-Values Defined by Tiles Setup' (Fig. 31A). In the 'Focus Surface' section select 'Global (Carrier based)'.

Multiposition imaging

Set up your positions in the lower right area (Fig. 36A) - ‘Position Setup’



Create positions by ‘Array’:

‘Setup by’ - ‘Array’
Choose the contour, adjust the number of required positions and the bias where the positions should be located. The positions will be automatically generated.

Create positions by ‘Carrier’ (Fig. 36):

‘Setup by’ - ‘Carrier’ (Fig. 36A)
Select the containers in which you want to distribute positions. Determine the number of positions per container (Fig. 36B). The selected containers are each filled with a ‘Position Array’ (group of positions, Inset, Fig. 36).

Click on the ‘Create’ button (Fig. 36, right arrow)

Copy a tile region or position from one well to others

If you want to copy and paste a ‘Tile Region’ or ‘Position’ setting (e.g. a certain arrangement of tiles, positions or local support points) from one well to other wells or even to all containers of a carrier, apply the following workflow.

- Select the well from where the ‘Tile Region/Position’ setting should be copied.
- Right-click within the selected well in the centre screen area (outside the tile region) to open the context menu and select ‘Copy Container for Replication’.
- If you want to choose specific wells use the left mouse button to select the wells into which you want to paste the copied ‘Tile Region/Position’ setting.
- Right-click in the centre screen area and select the context menu entry ‘Paste Replication to’ and either choose ‘Selected Container’ or ‘All Containers’. The copied ‘Tile Region/Position’ setting is pasted into the selected wells or all wells of the carrier with the same relative coordinates to the center of each well.

Time Series

The time series is defined manually.

Select ‘Live’ and ‘Time Series’ (Fig. 37)

Set the length of your time series with the ‘Duration’ slider (Fig. 37A) and select from the drop-down options (Fig. 37, arrow) either

- time (milliseconds to days)
- number of cycles (1-n)

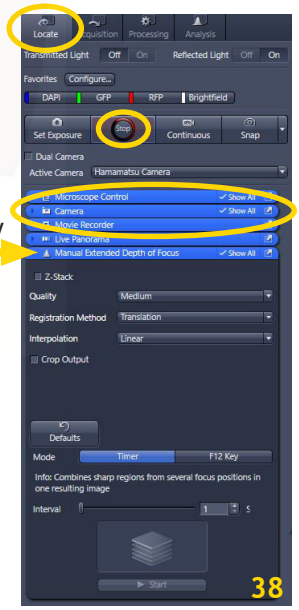
Set the interval of your time series with the ‘Interval’ slider (Fig. 37B)

Once this is set up, click on the ‘Start Experiment’ button

Manual Extended Focus

Extended Focus images are called EDF (= Extended Depth of Focus) in ZEN Blue. The software has the module ‘Manual Extended Focus’.
This function is found under the ‘Locate’ tab (Fig. 38). It allows to create one 2D image from several acquisitions with different focal positions.

- Click on ‘Live’ and find your ROI, then stop the live stream
- Activate ‘Z-Stack’
- Select the mode (see below)
- Press ‘Start’



You can take the images in two different modes: ‘Timer’ or ‘F12 Key’:

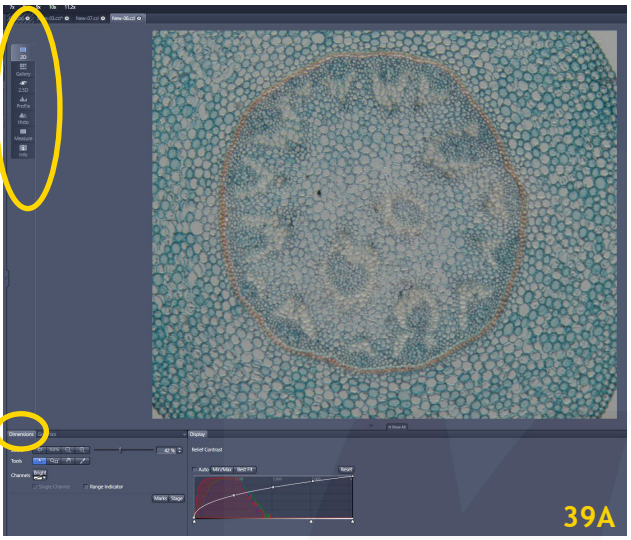
If you select ‘Timer’, the software will take an image at specific time intervals that you define (e.g., 10 seconds), while you manually focus through your sample. After each new recording, a new EDF image is immediately calculated.

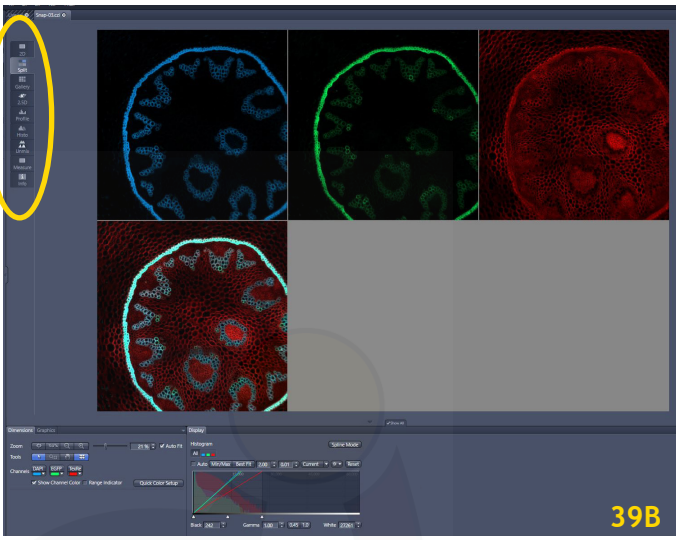
If you select ‘F12 Key’ you will manually record a new image by pressing the F12 key of your keyboard while focussing through your sample.

Press ‘Stop’ when you have acquired recordings from all focal planes.

Viewing Options

Depending on your image (Multichannel, Tiles, Z-stack) you can change how the data of your image are displayed in the left tool bar in the top centre area (Fig. 39). The options will vary depending on your image data.


39A


39B

Some of the options are:

2D - Regular two dimensional display of your image, overlay for a multichannel image (Fig. 39A)

Split - Multichannel image split into the individual channel images plus the overlay (Fig. 39B)

Gallery - All individual images of a MDA are displayed separately

2.5D - Intensity values in a two-dimensional image are converted into a height map. The highest intensity values are represented by the greatest extension in the Z-direction.

Profile - You can create intensity profiles of certain regions in your image. The measured values in the profile are shown in the measurement data table below the original image.

Histo (Histogram) - This shows you the gray value histogram of your image

Measure - You can specify how to draw the graphic elements for measurements into an image and how the measurement data are displayed.

Info - Displays all data saved with your image file

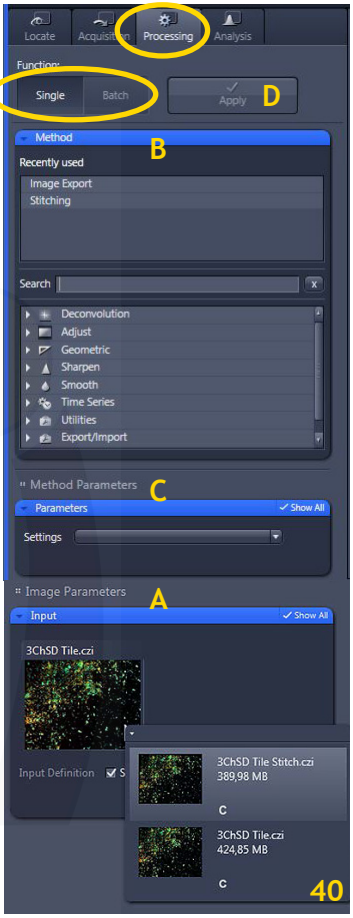
In the bottom centre area, under ‘Dimensions’ you find the option ‘Scenes Slider’. If this box is checked, you will only see one image displayed in the centre area and you can move through all your images of the series by moving the slider. If the box is unchecked, all images will be displayed beside each other.

Image Processing

General Workflow

The ‘Processing’ tab has several image processing functions (IP functions). The basic workflow is quite simple:

- Acquire or open an image that you want to process
- Open the ‘Processing’ tab (Fig. 40). When you open the tab, the last active image (from the ‘Acquisition’ tab) will be used as the input image for the processing function. If you want to select another image, load it under ‘Image Parameters’ - ‘Input’ (Fig. 40A). Note that the image must be open in ZEN Blue.
- Select the desired processing mode (‘Single’/’Batch’, Fig. 40). Per default, single processing mode is selected. Batch processing allows to apply a specific processing function automatically to a batch of images.
- Select the desired processing function under ‘Method’ (Fig. 40B), e.g. Stitching. You can search for processing functions in the ‘Search’ field by just entering the initials of the function(s) you want to search.
- Set the parameters of the function under ‘Method Parameters’ (Fig. 40C). If you need help for a specific function and its parameters press the F1 key. You will find detailed descriptions for each function in the online help.
- To see how the function works you can click on the Preview button under ‘Image Parameters’ - ‘Output’
- Click on the ‘Apply’ button (Fig. 40D) to apply the processing function to the image. This will create a new image in a new image container. The original image will not be changed. Save the processed image under a new name.



Examples

Extract individual fluorescence images from a multichannel image

If you have acquired a multichannel fluorescence image, you can extract the individual channel images with the ‘Create Image Subset’ tool:

Select the ‘Processing’ tab - ‘Method’ – ‘Utilities’ - ‘Create Image Subset’
In the section ‘Image Parameters’ under ‘Input’ select your multichannel image
Under ‘Method Parameters’ - ‘Parameter’ - ‘Channels’ deactivate all but one channel
Click on ‘Apply’

Do this for the other channels as well and save the extracted sub-images as separate .czi files.

Stitch tiles images

After image acquisition of the 'Tiles' experiment, the entire large image will appear on the screen, but you still need to stitch the individual images together before you can save the large image:

Select the 'Processing' tab - 'Method' – 'Geometric' - 'Stitching'
Click on 'Apply'
Save your stitched image as .czi file

Start with the default parameters, which work most of the time. However, if your image does not stitch correctly, you can change the parameters and process again.

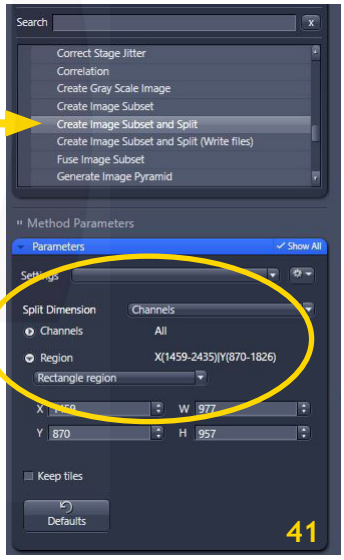
Create an EDF Image (Extended Focus)

If you have acqired a Z-Stack, this function will calculate all images into one 2D EDF image:

Select the 'Processing' tab - 'Method' – 'Sharpen' - 'Extended Depth of Focus'
In the section 'Image Parameters' under 'Input' select your Z-Stack image
Under 'Method Parameters' - 'Parameters' - select 'Wavelets' and 'High' in the dropdown lists
Click on 'Apply'
Save this image as .czi file

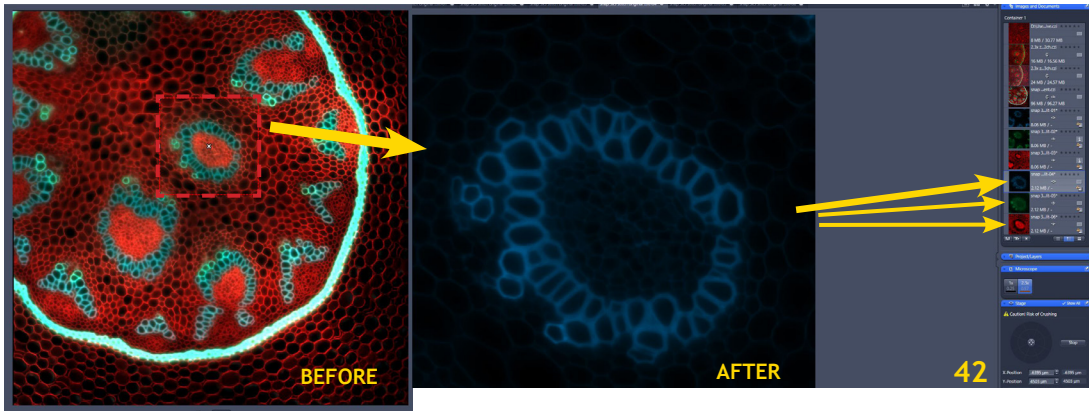
Create an image subset and split dimensions

Depending on your loaded image, you can split certain dimensions, e.g., channels or timepoints, and save them to separate images. You can also define a crop region of interest in your image. First, you select the method, and in the 'Parameters' tool you specify which dimension and/or region (Fig. 41). Each of the dimensions is only visible if it is present in the input image. In the example in Fig. 47 the multichannel image was cropped and split into the separate channels.



'Create Image Subset and Split' opens the resulting images in the ZEN Blue document area (Fig. 42). Therefore, for this image processing function, 'Split Dimension' is limited to a maximum of 20 timepoints, tiles, and slices, respectively. To split images along a dimension with more than 20 elements, use 'Create Image Subset and Split (Write files)', which creates the image files in a folder specified by you.

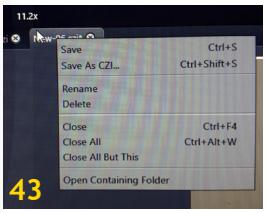
Select the 'Processing' tab - 'Method' – 'Utilities' - 'Create Image Subset and Split'
Define your settings in the sections 'Channels' and/or 'Time' (Fig. 41)
In the section 'Parameters' under 'Split Dimension' select the dimension(s): Channels - Time - Region.
Under 'Input' specify whether you want to set the input automatically and which image you want to display after processing.
In 'Output' specify how to proceed with the new image. The output tool is only available if you selected the 'Create Image Subset and Split (Write Files)' method.
Click on 'Apply'



There are many more Image Processing options - please refer to the online help or contact Michaela if you need a specific function not listed here.

Data Management

By default, none of the snapped images is automatically saved!
All snapped images are kept open in the image window (you see tabs of the acquired images lined up at the top of the window) and can also be accessed in the right panel under ‘Images and Documents’ (Fig. 23).
Images are saved in .czi format (Fig. 43), either by right-click on the image tab, or in the menu under ‘File’ - ‘Save As’.



The .czi image format saves additional data (meta-data) such as image number, date of acquisition, microscope settings, exposure values, size and scale details, contrast procedures used, etc. (Fig. 44). Annotations and measured values are also saved with the image.

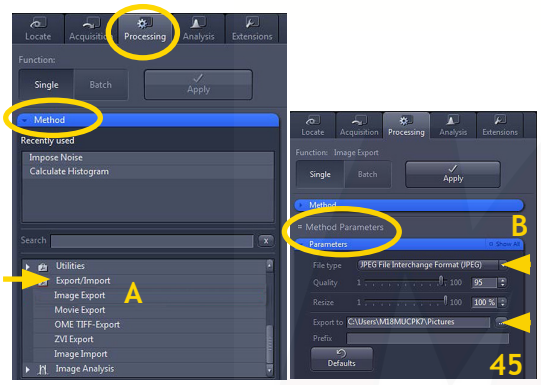
CZI images can be opened and processed by imaging software like Fiji (ImageJ), Volocity, and Imaris.

Export of .czi files:

Saving CZI files as tif, jpg, png via the 'Save As' option described above will not save the annotations (e.g., scale bar). To export a saved .czi image or image series into another format (TIFF, JPEG, AVI, OME, etc.), you need to go into the 'Processing' tab (Fig. 40). The general workflow is the same for image, movie, or OME export.

- Select 'Processing' - 'Method' - 'Export/Import'
- Pick whichever export option you need (Fig. 45A)
- Under 'Method Parameters' open 'Parameters'
- Select the file type and define a path and folder into which the images should be exported (Fig. 45, arrowheads) - start with the default parameters, you can change those if necessary
- Under 'Image Parameters' - 'Input' select the image file you want to export
- Click 'Apply'

Multidimensional images like Z-stack and time lapse are exported as individual images in the 'Image Export' option (Fig. 45A), make sure you select 'Movie Export'!



If you want to export the overlay of a multichannel image, make sure you check the 'Show All' box at the right top of the 'Method Parameters' box (Fig. 45B). Then you will see the option 'Merged Channels Image' which needs to be selected for overlay export. With the 'OME TIFF-Export' function you can export your images into OME (Open Microscopy Environment) TIFF format for further analysis. The images are then available as a multipage TIFF file. If you have saved all your .czi images in a folder, you can easily export all of them through Batch Processing.

Export of 'Tiles' images:

- Under 'Export/Import' select the file type that you want to use.
- Activate the 'Apply Display Settings and Channel Color' checkbox. This means that the images will be exported with the settings you have made, e.g. tonal value corrections or contrast. If you activate the 'Original Data' checkbox, the images are exported unchanged. In this case, the settings from the display curve, e.g. tonal value corrections and contrast, are not adopted.
- Select the 'Define Subset' button.
- The settings for the available dimensions open.
- Open the settings for the Tiles dimension.
- Select 'Existing Tiles'
- Click on 'Apply'

Shutdown

1. Bring the objectives back up, remove your sample
2. Save your data, close ZEN Blue, transfer your files to OneDrive (preferred) or the server
3. Switch off camera (Hamamatsu)
4. Switch off Apotome.2
5. Switch off EMS 3
6. Shut down computer
7. Minimize intensity at HXP 200C Light Source and then switch off
8. Log your usage into the log sheet and cover the stereoscope

2-Hour Rule

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