Widefield Zeiss Axio Imager.Z2
Manual/Quick guide

CENTER FOR NEUROSCIENCE
AND CELL BIOLOGY
UNIVERSITY OF COIMBRA
PORTUGAL

Microscopy
Imaging
Center
Coimbra

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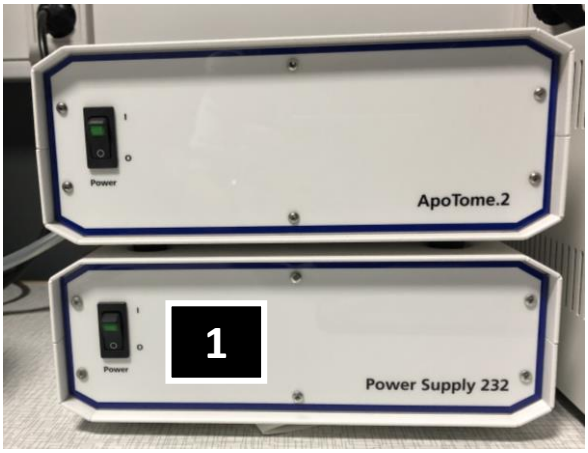
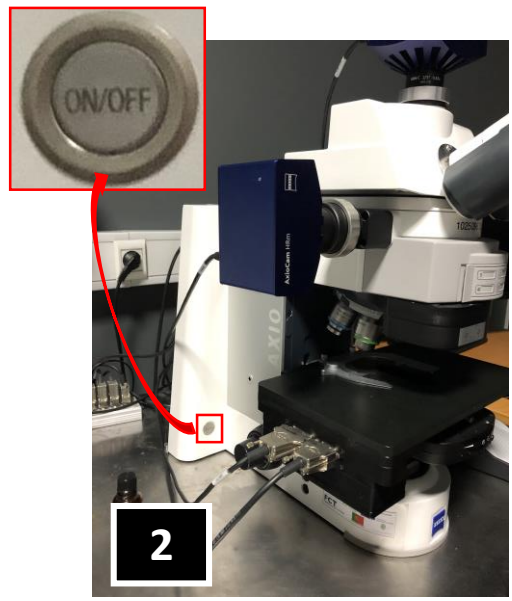
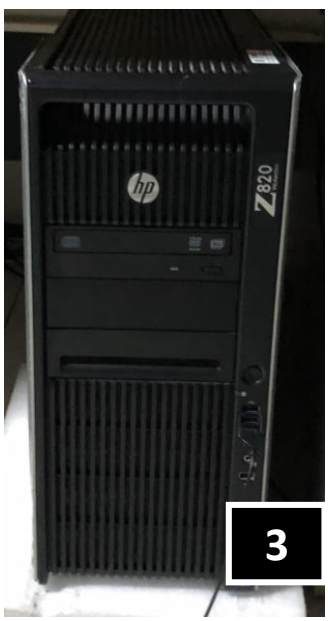
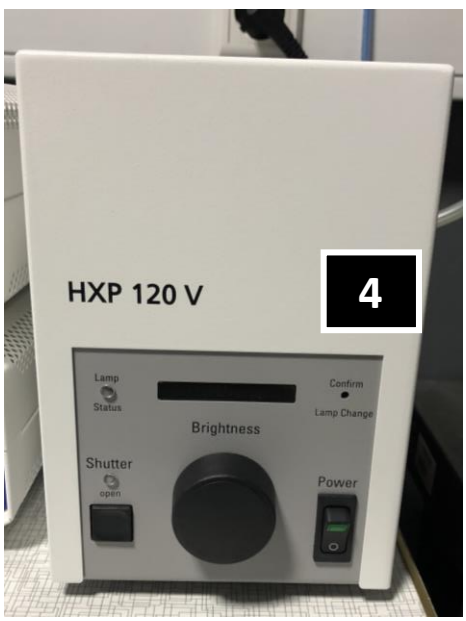
Starting the system

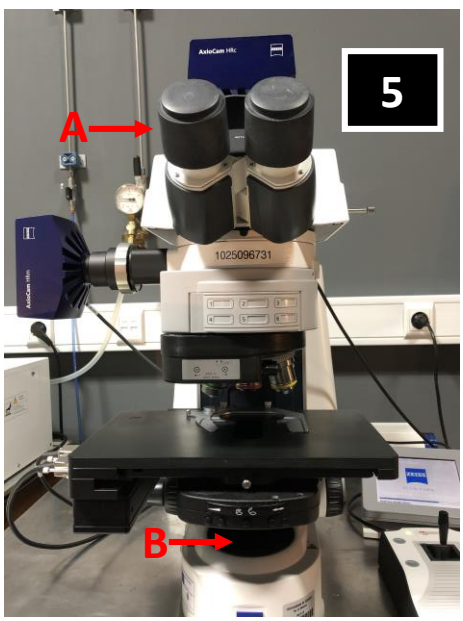




- Press the Power Supply 232 switch button **[1]** to ON.
- Switch ON the microscope by pushing the 'ON/OFF' button on the left side of the system. **[2]**







- Turn on the PC workstation **[3]**
- Turn on the fluorescence lamp (**only if you need it**) **[4]**
- Remove the "protections": eyepiece (**A**) and transmitted light (**B**) **[5]**

Switching off the system

1. Save your data on D:/Users/PI folder/”your folder”.

2. Clean the objectives (x40, x63 and x100) with LENS TISSUE ONLY and cleaning solution.

3. Check if the Axio Imager.Z2 is in use in less than 1 hour after the end of your session. Leave the fluorescence lamp ON **only** if the next user comes in less than 45/60 minutes.

If there is no one after you:

5. Exit Zen software.

6. Turn the fluorescence lamp off.

7. Burn your data onto a CD, DVD or MICC external drive. Do not use USB keys to transfer data as it put the computer at risk of viruses.

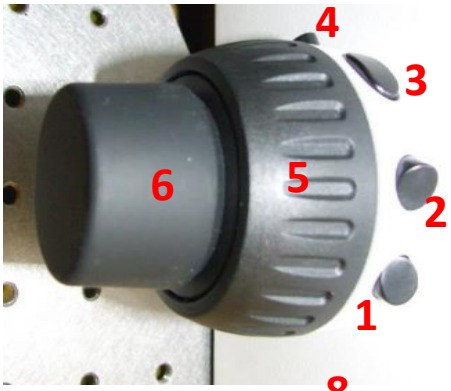
8. Shut down the computer.

9. Turn off the Power Supply 232 **[1]** switch button.

10. Place the ”protections” in the right places: eyepiece and transmitted light.

11. Write the information in the logbook.

Microscope stand presentation



Left side knob

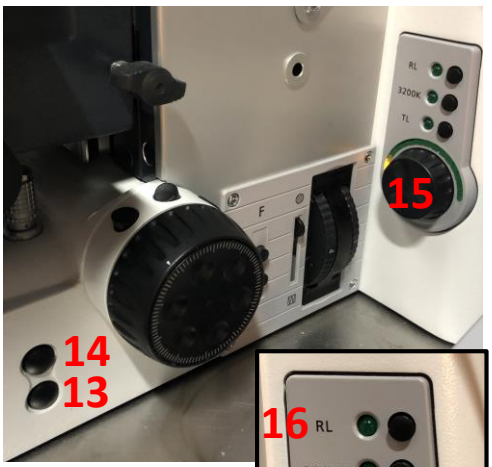
- Change contrast methods **[1 & 2]**.
- Change light path (binocular or left camera) **[3]**.
- Filter cube change for fluorescence (2 back buttons) **[4]**.
- Macrometric knob **[5]**.
- Micrometric knob **[6]**.

Right side knob

- **No action [7 & 8].**
- Open/Close the binocular shutter **[9]**.
- Change objectives (2 back buttons) **[10]**.
- Macrometric knob **[11]**.
- Micrometric knob **[12]**.

"Right" side of microcope stand

- Take the objectives down at the lowest position **[13]**.
- Take the objective back up to its working position **[14]**.
- Light intensity adjustment (wrench adjuster) **[15]**.
- RL - Open/Close the fluorescence shutter **[16]**
- TL - Open/Close the brightfield shutter **[17]**



The Joystick

Move the motorized stage in X and Y.
Press the joystick button F1 **[18]** to move faster/slower.

TFT screen (touch screen)

- Open/Close the fluorescence shutter **[19]**
- Open/Close the brightfield shutter **[20]**
- In '*Microscope*', in the '*Turret*' menu*, there are several tabs allowing you to:
  - Choose the objective '*Objectives*' **[21]**
  - Choose the contrast method '*Contrast*' **[22]**
  - Choose the filter cube for fluorescence '*Reflector*' **[23]**

Push-pull rod for camera path deflection **[24]**

- Push-pull rod pushed in: 100% observation through eyepieces
- Push-pull rod pushed out: 30% : 70% Eyepiece/camera path or 100% camera path

Start Zen / Observation with the oculars

- Start the '*ZEN*' software (Icon on the desktop).

- When Zen opens you will be under the '*Locate*' tab **[1]**, this is where you can view your sample through the eyepiece;

- Lower down the stage a bit before loading a slide; Position the sample right below the objective (**coverslip facing the objective**) and adjust the microscope to see a focused image through the eyepiece;

- Choose your objective by clicking on the objective button **[2]** (or touch screen); this will bring up a list of objectives currently on the microscope; please remember to add oil when appropriate;

- To check your sample using Transmitted Light, switch to the desired lamp **[3]** and turn on respective shutter: using the software (shortcut buttons TL **[4]),** touch screen or shortcuts on the microscope;

- To check sample using fluorescence turn on respective shutters using the software **[5]** and choose filter using:

  - Touchscreen (Microscope / Control / Reflectors)
  - filter button **[6]**; this will bring up a list of filters currently on the microscope;
  - quick access buttons **[7]**

    Green
    Red
    Far-red
    Blue
    '*TL open*', open the shutter for the transmission
    '*TL off*', close the shutter for the transmission.

- Once you focus, close the fluorescence shutter and go to acquisition tab.

NOTE: For transmitted light acquisitions, phase contrast or DIC, it is important to make adjustments on the microscope (set the Khöller illumination...) Please ask for help.

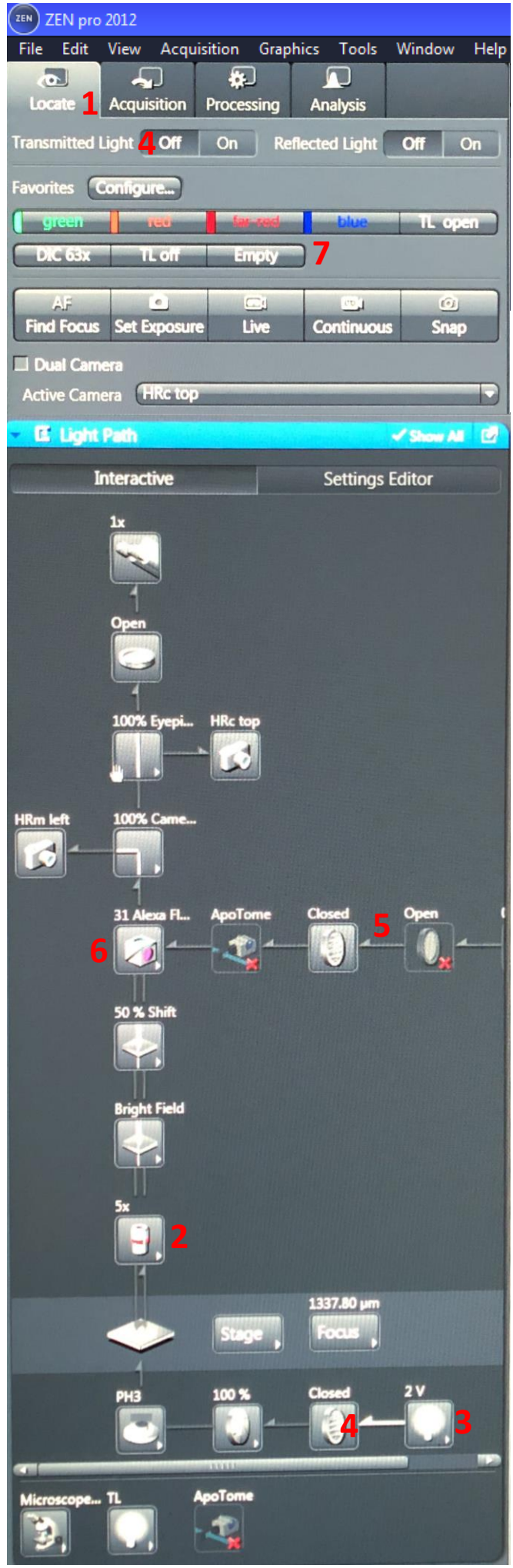
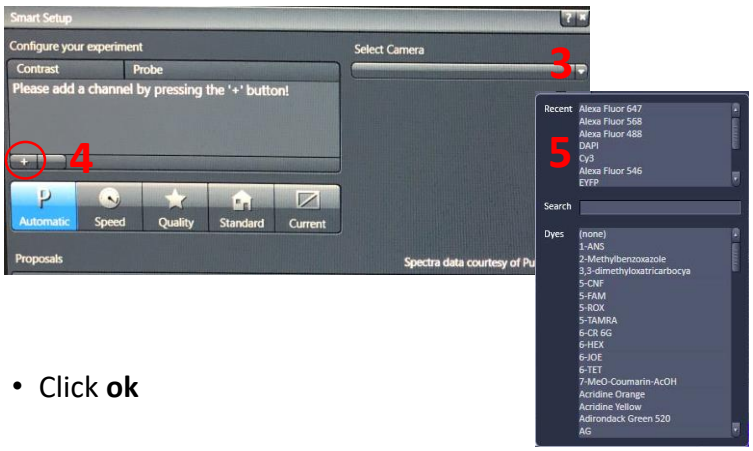


Image Acquisition - transmission/fluorescence with the black/white camera

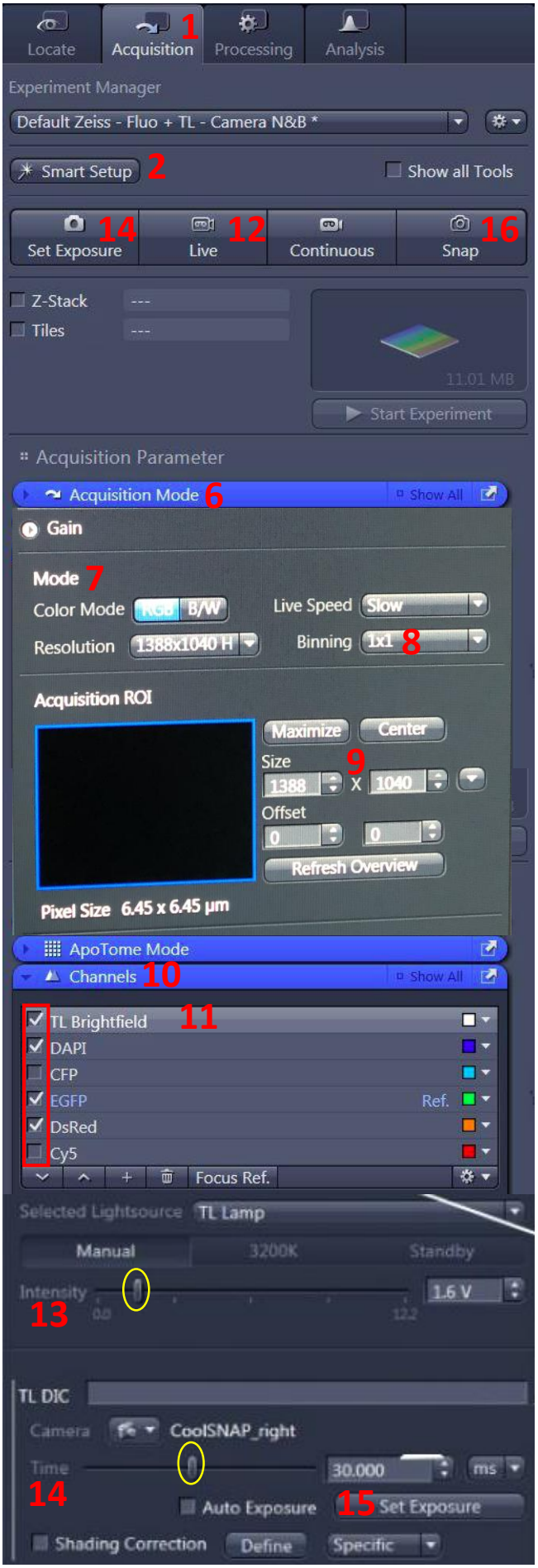
- Go to Acquisition tab **[1]**;
- Use **Smart setup** to define channels: Click on '*Smart Setup*' **[2]**
- Choose the camera **[3]**: **HRm** (fluorescence) or **HRc** (transmission)
- To add a channel click on the [+] button at the '**Configure your experiment'** section **[4]**. The **Add dye or contrasting method** list appears **[5]**. Double-click on the desired fluorophore or TL Brightfield under Contrast methods for transmitted. Repeat for further channels.



- Click **ok**
- Open *'Acquisition Mode'* **[6]**. In **'***Mode'* section **[7]** select camera binning (1x1) **[8]** and acquisition ROI (maximum acquired = Resolution/Size 1388x1040) **[9]**

Most of the time leave binning at '1x1'. If you wish to increase the sensitivity of the camera, you can choose other binning options, but at the cost of lower resolution in the image

- Open the menu *'Channels'* **[10].** The line(s) with your chosen fluorophores of interest (done with '*smart setup*') should be checked (✓)
- Select one channel (light grey) **[11]** and make '*Live*' **[12]**. This will open a Live image tab in the center section. Use the ***tabs below the image (next page)**
- Set the '*selected lightsource*' illumination intensity **[13]** manually by entering the wanted number or moving the corresponding cursor. Do it for every channel of interest
- Set the exposure '*time*' manually by entering the wanted number or moving the corresponding cursor **[14]** or automatically by clicking '*Set Exposure'* **[15]**. Do it for every channel of interest.
- Make a '*Snap'* to image the checked ✓channels **[16]**.



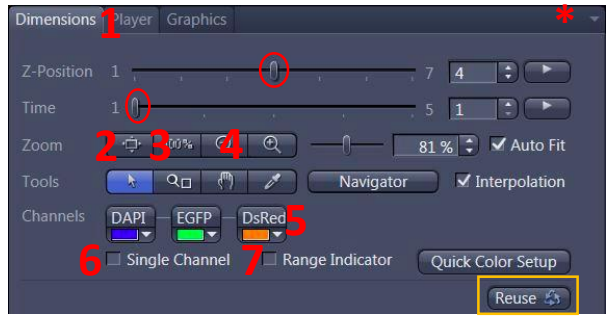
NOTE:
Alternatively (instead of using *'smart setup'*) you can load a saved image which includes the desired channels and acquisition settings:

- Go to File, open "your" folder and open a previous image;
- In the '*dimensions*' **[1]** tab below the image you just opened, click the *'Reuse'* button.

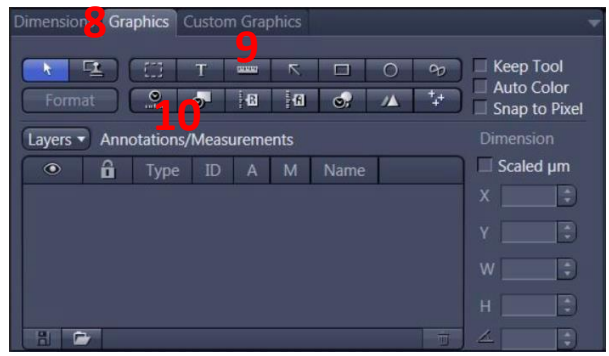
*Tabs below the image:

Dimensions Tab [1]

- Display a plane in the z-stack, or a time position by entering the wanted number or moving the corresponding cursor.
- Adjust the image to the screen size **[2]**.
- Adjust the pixel size of the image to the pixel size of the screen **[3]**.
- Enlarge or reduce the image **[4]**.
- Show/Hide the color (channel) on the screen **[5]**.
- See only one channel at a time **[6]**.
- Show the levels of gray and the saturation **[7]**. ✓'*Range indicator*' to monitor saturation (avoid red pixels which are saturated)



Graphics Tab [8].
Use the graphics tab to add annotations, e.g., scale bar **[9]** or relative time **[10]**. Press format or right click on the annotation to change format



Display Tab [11]
In this tab are the contrast options:

- Change image brightness and contrast by selecting one or All channel(s) (color) **[12]** and moving the line defining the limits of gray levels.
- Adjust the contrast automatically (Min/Max, Best Fit) **[13]**.
- Reset the contrast **[14].**

Note that in order to compare images it is absolutely imperative to change the parameters to exactly the same levels, i.e., the values of Black, Gamma, White should be the same.

Image Acquisition - transmission mode with the color camera

Acquisition in transmission mode

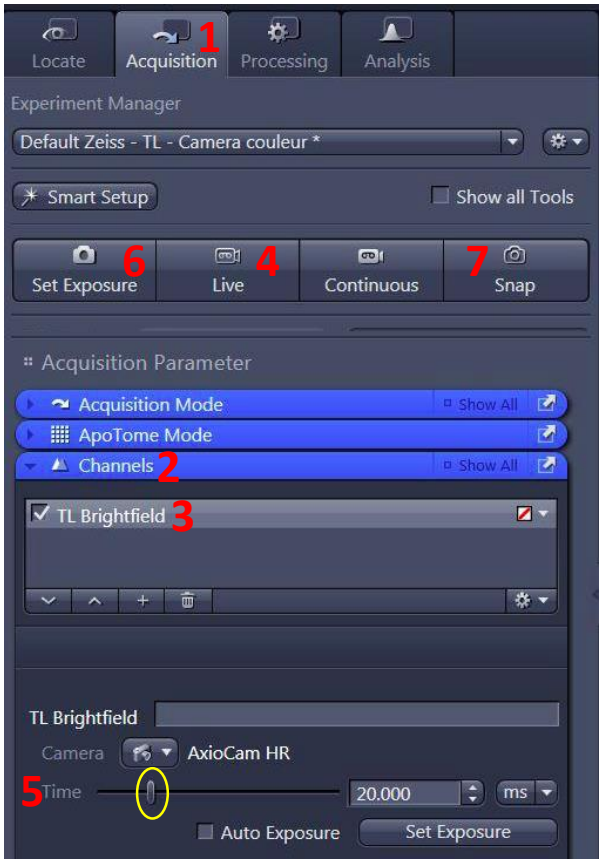
Before observing in brightfield, if you need to use phase contrast or DIC call MICC staff.

Select '*BF*', '*PH*' ou '*DIC*' on the TFT screen.

- Select the tab '*Acquisition*' **[1]**.
- Open the menu '*Channels*' **[2]**.
- Choose '*TL Brightfield*' and check it **[3]** .
- Press '*Live*' **[4]**.
- Adjust manually the exposure time by entering the wanted number or moving the corresponding cursor **[5]**.

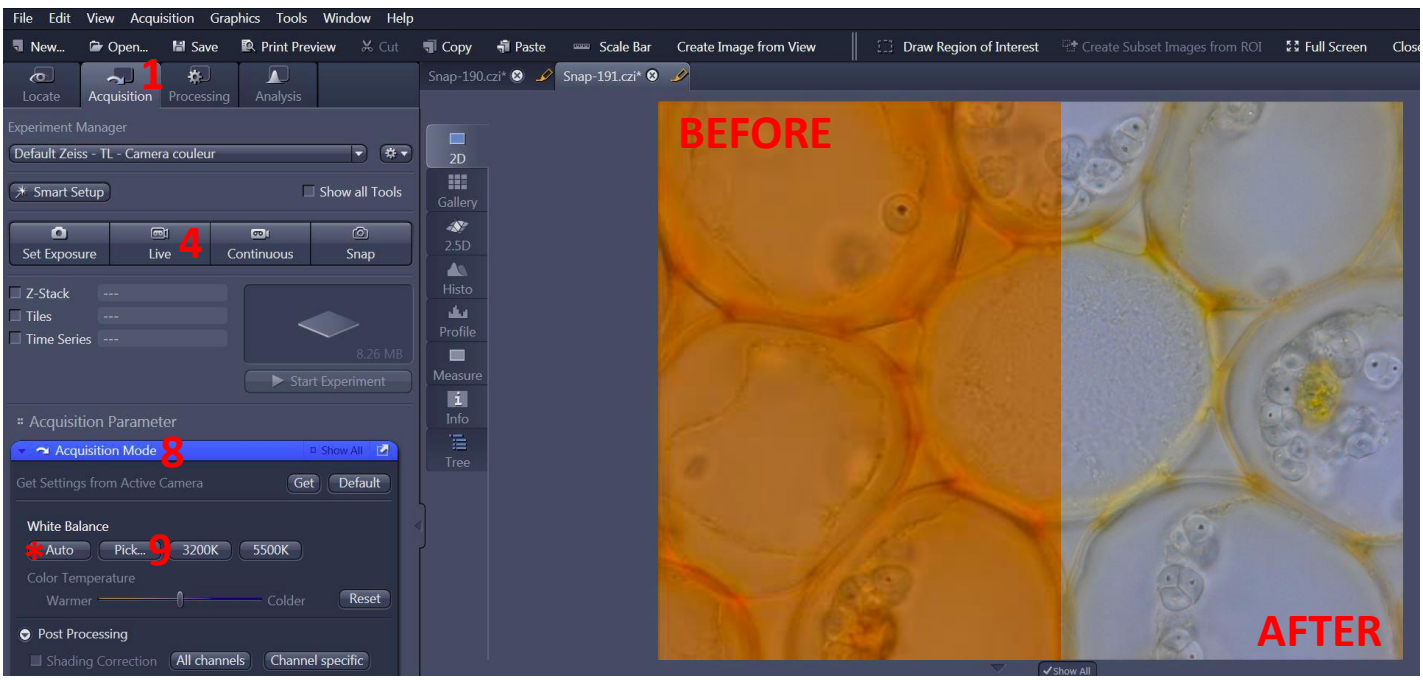
or

- Adjust automatically the exposure time by clicking on '*Set Exposure*' **[6]**
- Make a '*Snap*' to acquire your image **[7]**.



Adjust the white balance

Adjusting the '*White Balance*' allows to define a white reference in the image that you are acquiring.



- Under the tab '*Acquisition*' **[1]**, open the menu '*Acquisition Mode*' **[8]**.
- Make '*Live*' **[4]**.
- Click on '*Pick. . .* '**[9]**, a little pipette will appear instead of the mouse arrow.
- Choose an area that is suppose to be completely white in your image and click on it. The correction is done automatically. (The '*Auto*'* option allows to correct the white balance, but preferably in a region without any sample.)

Multidimensional Acquisition

Z-Stacks

- To make a z-stack first tick Z-Stack (under the experiment manager); scroll down on the left until you see a Z-Stack box (ensure Show All is enabled **1**).

- For multicolor acquisitions, choose the way you want to switch channels*: make the entire z-stack of one channel, then switch to the other one '*Full Z-Stack per Channel*' or all channels per plane '*All Channels per Slices*'.

- There are two ways of setting a z-stack:

First/Last

- Select '*First/Last*' imaging Mode **[2].**

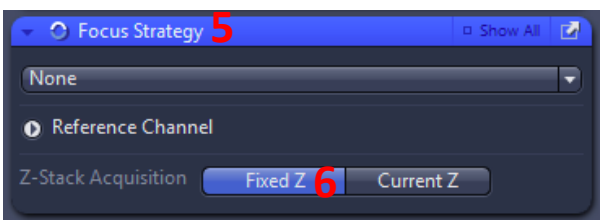
- While fast scanning using Live, move the focus to the top of your sample and click '*Set Last*' **[3]**.

- Now move the focus to the bottom of your sample and click '*Set First*' **[4].**

NOTE: Under **Focus Strategy [5]** make to select **Fixed Z [6]** when recording the Z- Stack. This option makes sure that the center Z-position calculated when selecting the first and last slices is used rather than whatever the current stage position happens to be.

- Click on '*Start Experiment*' **[7].**

- Range: thickness of the z-stack in µm
- Slices: number of planes in the z-stack
- Interval: step (in µm) in between planes
- Optimal/Smallest: Optimal interval in between planes to adapt to the Nyquist criterion according to channels and microscope configuration



NOTE: For good Z resolution click the **Optimal/Smallest** button. However, in many cases you can use a larger step size, especially if you are not using the Z-stack to create 3D images. The number of slices in the stack will be automatically calculated.

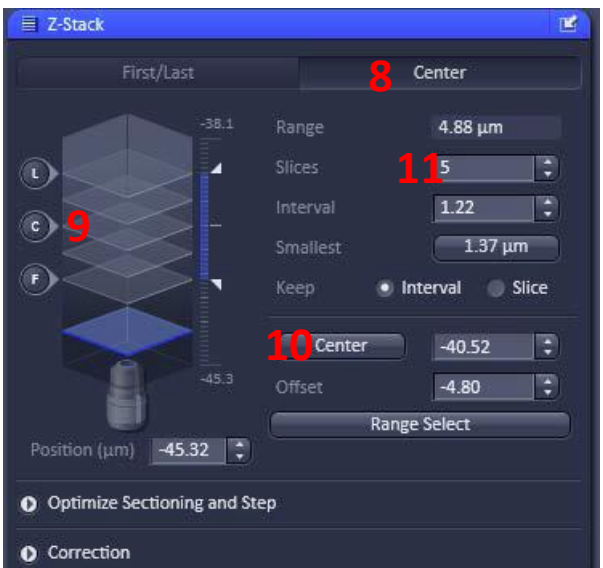
Center

- Select '*Center*' imaging Mode **[8].**

- Click on Center then find the center of the sample you would like to image. Click on '***C***' **[9]** to take a Snap of the center image to make sure you are in the right spot. Click '*Center*' **[10]** to confirm this is the slice in the middle of the stack.

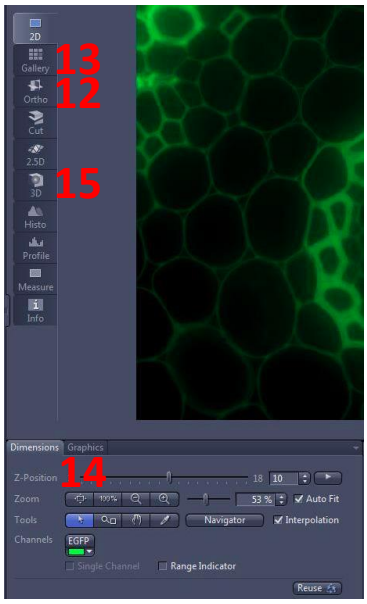
- Set your interval and check your number of slices **[11].** Make sure you have an odd number of slices

- Click on '*Start Experiment*' **[7].**



Display the stack

Your stack is displayed in the ZEN interface at the end of the acquisition. In the tab '*Dimensions*':

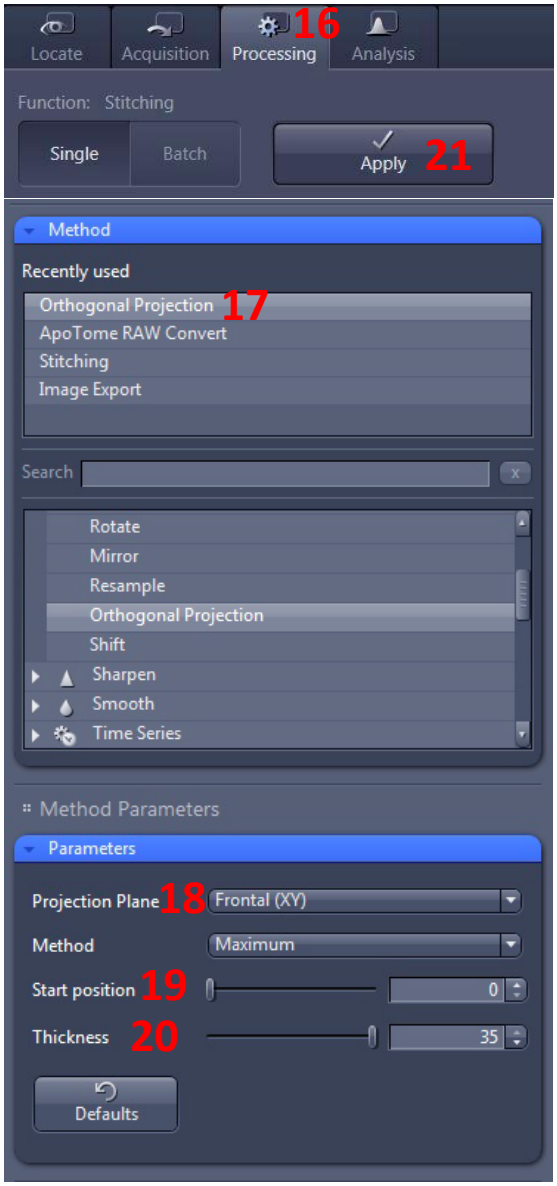


- '*Z-Position*' allows you to see the different planes of the stack **[12]**.
- '*Gallery*' displays a set of all the images composing the stack **[13]**.
- '*Ortho*' allows an orthogonal view of your stack **[14].**
- '*3D*' allows a 3D reconstruction of your stack **[15]**.

Processing the stack

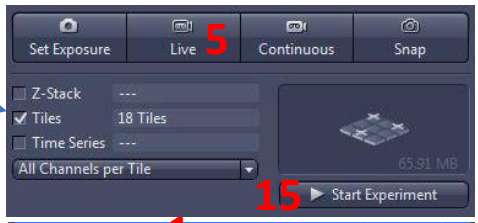
Once captured you can turn a Z-series into single flattened image called a **Maximum Intensity Projection**

To do this click on the **Processing** tab **[16]** and choose '*Orthogonal Projection*' **[17]** as the **Method**.



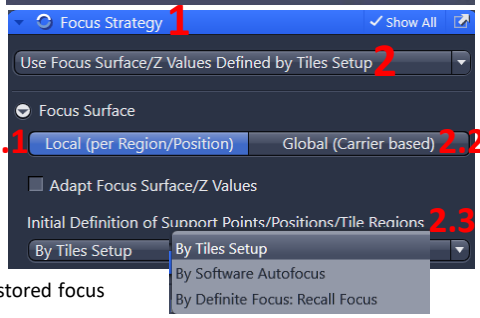
- Choose '***Frontal (XY)***' for **Projection Plane [18].**
- Choose the '*Start position*' **[19]**. Typically this will be the first slice in the Z-Stack.
- Choose the '*Thickness*' **[20]**. Typically this will be the last slice in the Z-Stack.
- Click *'Apply'* **[21]**.

Tile Scan



- To create mosaic images of large areas of your sample select **Tile Scan** from the **Experiment Manager**.

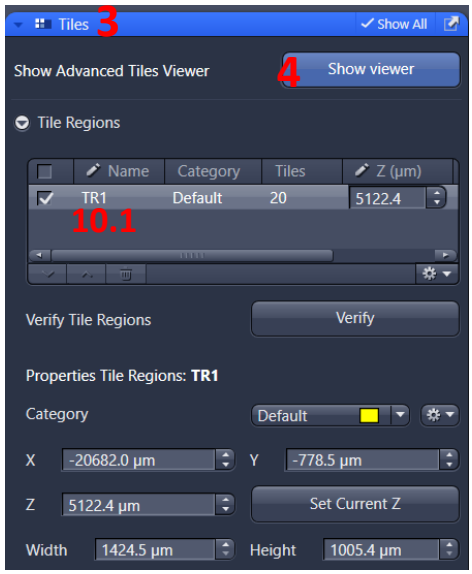
- Open the menu Focus Strategy **[1]**; Select '*Use Focus Surface/Z Values Defined by Tiles Setup*' **[2]**.
  - **Local [2.1]** – For positions: The Z value of each position is used; For tile regions: The Z values are interpolated from a local focus surface
  - **Global [2.2]** – The Z values of tiles and positions are interpolated from a global carrier based focus

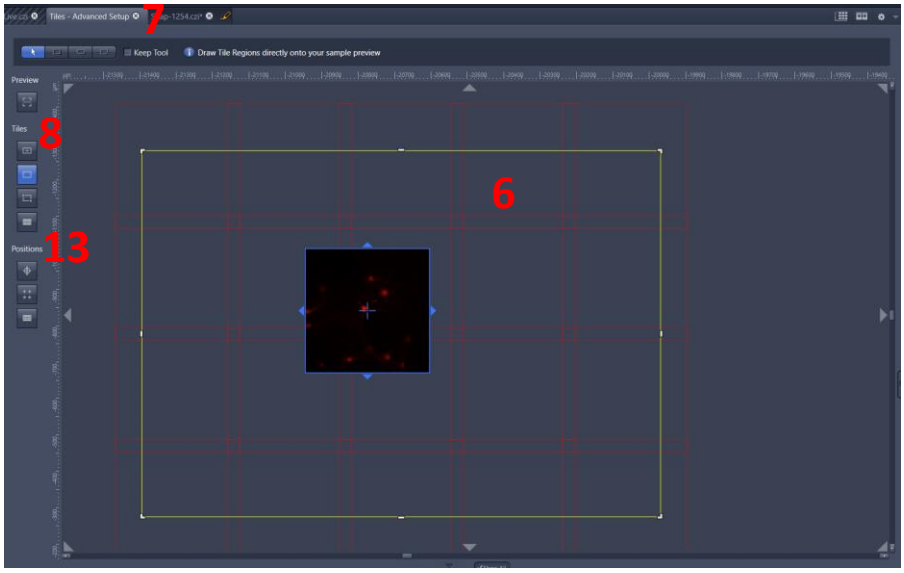


NOTE: Initial Definition of Support Points/Positions/Tile regions **[2.3]**
- **By tiles setup** – The fixed z values from tiles setup are used as the initial values
- **By Software Autofocus** - The initial z values are determined by a separate software autofocus run on each support point/position/tile region
- **By Definite Focus: Recall Focus** – The initial z values are determined by recalling the previously stored focus (distance to coverglass surface) with the definite focus on each support point/position/tile region.

- Open the window Tiles **[3]**. Click on '*Show Viewer*' **[4]**. Make '*Live*' **[5]**.

- The navigation space is displayed in the center of the software interface **[6]**.





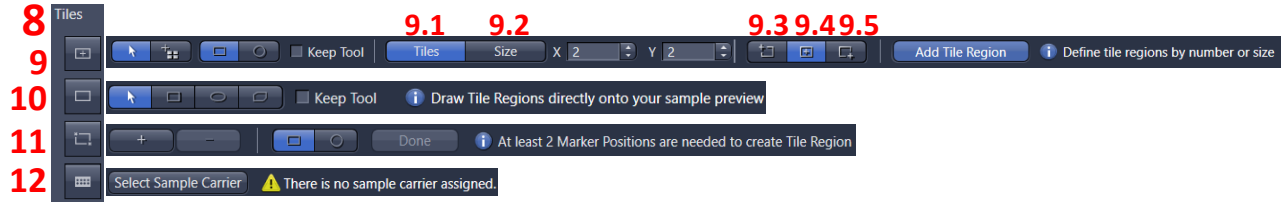
- In '*Tiles-Advanced Setup*' **[7]**, choose a desired '*Tile option*' **[8]**:

- "Setup new tile regions from a predefined size" **[9]**
  - The predefined tile regions are defined by specifying a number of tiles (columns and rows). The absolute size depends on the current tile size. 9.1
  - The predefined tile regions are defined by specifying an absolute area on the stage. The columns and rows are calculated to cover that area. 9.2
  - The tile regions are defined by the top left anchor position. 9.3
  - The tile regions are defined by the top center anchor position.9.4
  - The tile regions are defined by the bottom right anchor position. 9.5

- "Setup new tile regions by drawing a contour" **[10]**(e.g. rectangle, oval, free-drawing and draw a region in the image; the marked area will show up as a new tile region in the region list **[10.1]**);

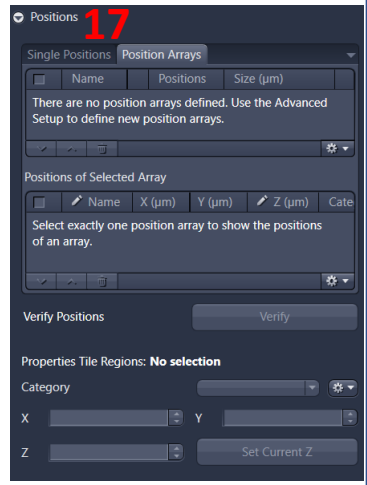
- "Setup new tile regions by specifying two ore more marker positions, e.g, the top-left and bottom-right center of the desired region" **[11]**;

- "Setup new tile regions from a underlying sample carrier" **[12]**.

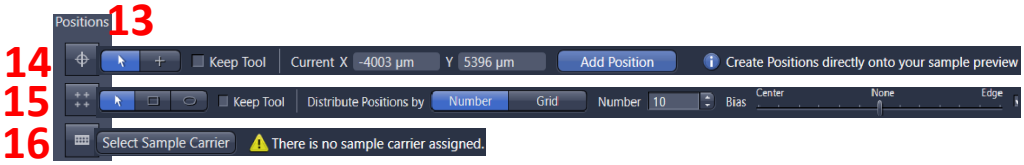


• In *'Tile Advanced Setup'* **[7]**, you can also choose the *'Positions'* option **[13]**:

-"Setup new position by selecting the location with the mouse cursor" **[14]**,
-"Setup new position by defining an array of positions within a drawing shape **[15]**;
-"Setup new positions from an underlying sample carrier" **[16]**.

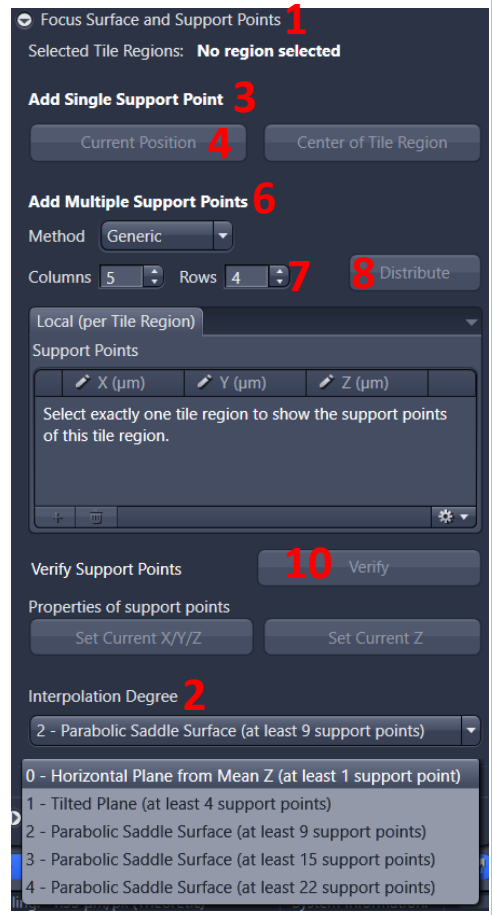


•The chosen positions will show up in the positions list (menu under the tile tab – left **[17]**.)



Focus correction (A) - Support Points

If your sample carrier does not consist of an even flatness on the imaging surface (which most likely will be the case), you need to tell the software reference points, so-called Global Support Points, in order to approximate for an even surface flatness. The necessary amount of Global Support Points is thereby determined by the degree of interpolation the software is supposed to calculate. In order to know how many support points you should add in the next step, please first go to **Focus Surface and support points [1]** *(menu below positions)* in the Tiles section and choose the degree of interpolation **[2].** As a general rule of thumb, you choose more points if your area of interest is quite large.

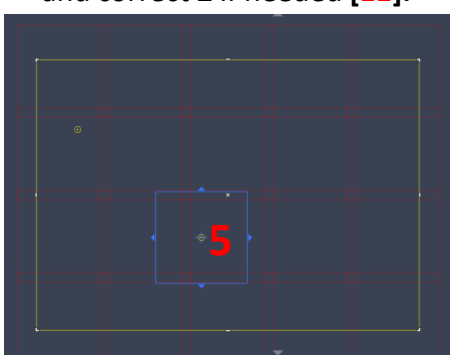


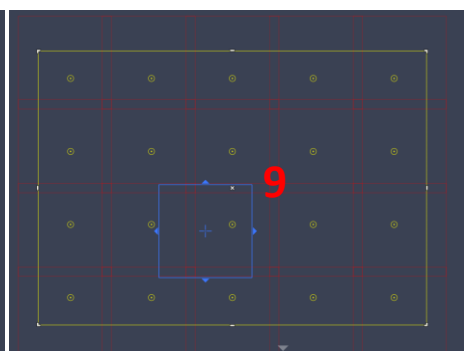
• Correct the focus on the entire tile. It has to be done before any acquisition in *'Focus Surface and Support points'* (menu below positions) **[1]**. You have 2 options:

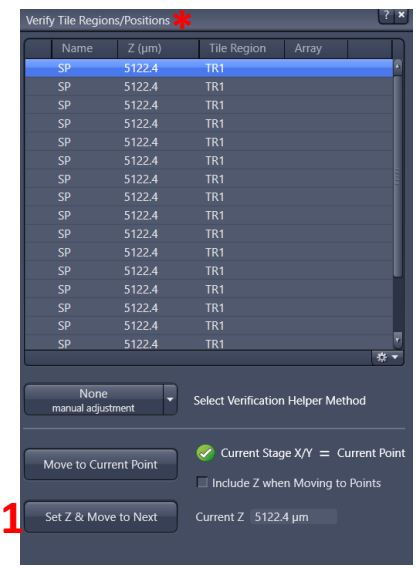
- **Add single Support Point [3]** - Double click on the position where you wish to correct the focus. Do *'live'* and adjust the focus. Click on *'Current Position'* **[4]** in the position menu**,** a yellow circle will appear **[5].** Repeat the process on at least 5 positions.

- **Add Multiple Support Points [6]** – Choose the number of columns and rows **[7]** of the tile area and click distribute **[8]**. Several yellow circles will appear according to the chosen parameters **[9]** (e.g image - tile 5x4 choose 5 columns and 4 rows and you'll have a support point in each tile).

•After adding all the support points, you can/should verify all the positions. Click 'verify' **[10]** (a window appears*) and correct **Z** if needed **[11].**







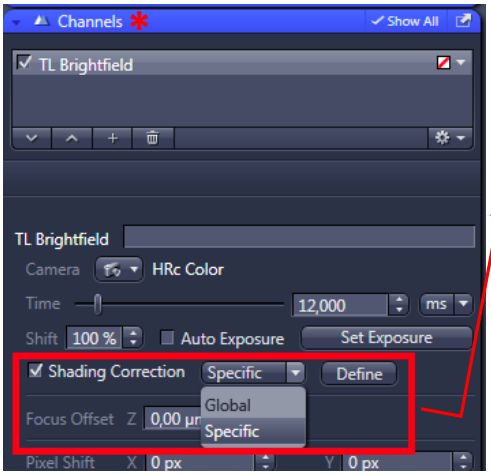
• Click on *'Star Experiment'* to start the acquisition.

• After acquisition click **Processing** and use the **stitching** tool to merge the image tiles together into a single image (see next page).

Shading correction [brightfield] (B)

When adjusting your acquisition parameters, you can also correct the shadow effects:

• In 'Live' mode, find a position in your slide without your sample or dust (or simply remove the slide).



• In the channels* toolbox select the '*specific*' correction mode in the list and click on *'Define'*.

• The *'shading correction'* option is automatically selected (✓). Stop *'Live'.*

• Re-position the slide (sample) and continue the tile process.

NOTE: The shadow effects can also be corrected after acquisition and under the **Processing** menu (post-shading correction B1).

• After acquisition click **Processing** and use the **stitching** tool to merge the image tiles together into a single image (see next).

Post-Shading correction (B1)

After acquisition, under the **processing** tab and *'stitching'* method# you have two different ways to correct the shading.

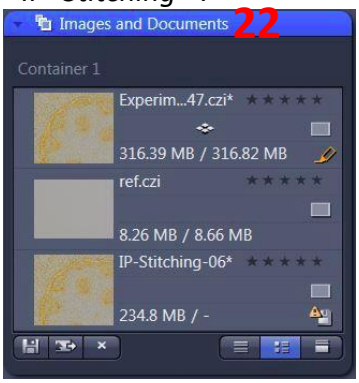
To make an automatic correction by computation:

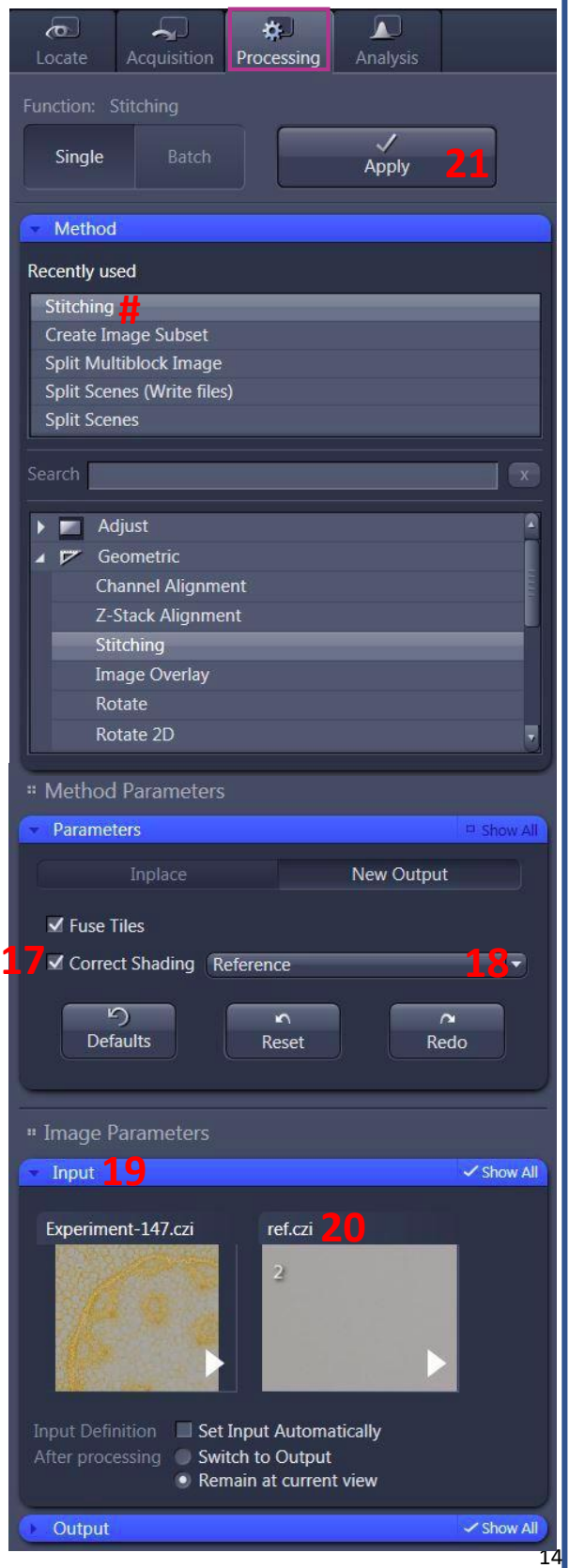
- Check *'Correct Shading'* **[17]** to homogeneize the background
- Select the mode *'Automatic'* **[18]** for an automatic correction.

Or

Select *'Reference'* **[18]** to make a correction with a reference (more reliable):

- In *'Input'* **[19]**, a second window opens
- Select a reference **[20]**, it must be a *'Snap'* of your slide without anything.
- Click on *'Apply'* **[21]**.
- The final image appear in *'Images and Documents'* **[22]** and is named « *IP- Stitching* ».





Stitching a Tile Scan

- Click on the Processing tab

- Under Method **[1]** click on '*stitching*' **[2]** (if it is not in your recently used list then it can be found under Geometric heading **[3]**).

- Under '*Input*' (Image Parameters) **[4]** , select the image you would like to stitch (click on white arrow and select from the list **[5]**) and then click on '*Select*'.

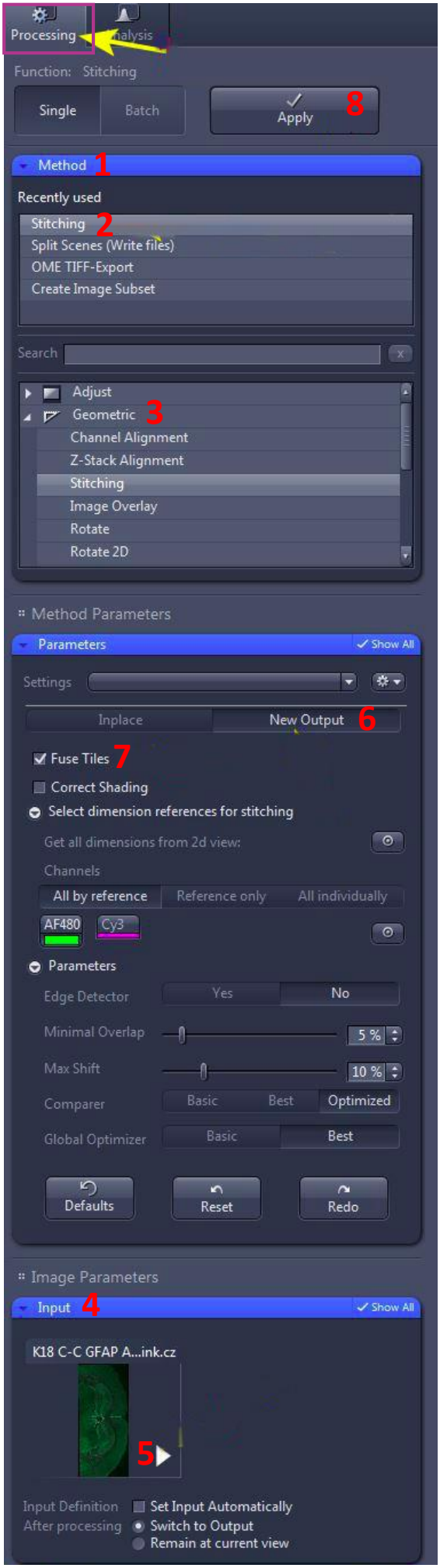
- Set output type in the Parameters tab to:
  A. New Output **[6]**
  B. Fuse Tiles **[7]**
  (This only needs to be done at the start of each session)

- Other parameters are mostly default, but can be adjusted for best alignment and stitching.

- Next click on '*Apply*' **[8]**, this will stitch your image for you. The stitched and fused images will be displayed as a separate images.

- Finally save your file, it will have the original image name with an "_Stitch" at the end.

NOTE: it is worth keeping the original raw version as well as the fused stitched in case you want to re-use your settings (you can't re-use a fused file) and also re-stitch.



ApoTome (Optical Sectioning)

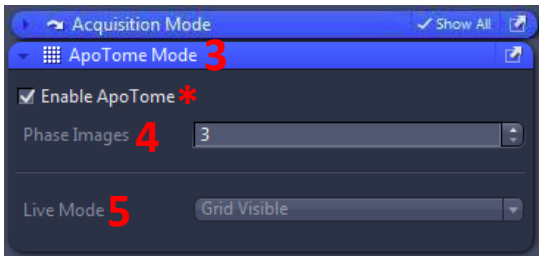
This Zeiss technology uses a moving grid to change how the sample is illuminated. By taking several images, with the grid in different positions (phases), the ApoTome software is able to generate a single image which excludes a large amount of the out-of-focus light typically present in epi-fluorescence images. The resulting images have a higher contrast than standard fluorescence images, which allows finer structures and details to be seen clearly but they are also dimmer (as there is less light reaching the camera). **This technology only works with fluorescent samples.**

A. To use the ApoTome press the switch button **[1]** on the ApoTome.2 module base to **ON** at least 30 min before acquisition.

B. Carefully and gently slide the ApoTome.2 module into the side of the microscope body **[2]**. There is a beep when the module is engaged.

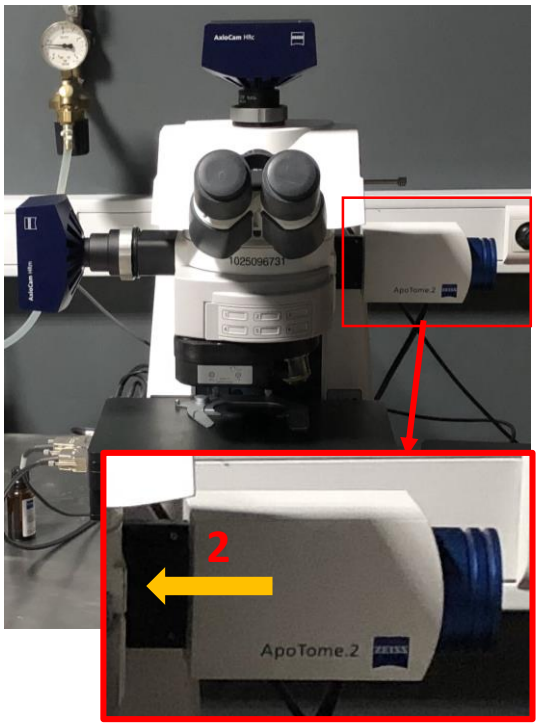
C. Under ZEN acquisition tab, you will now have an '*ApoTome mode*' **[3]**. Tick the '*Enable ApoTome*'* checkbox and choose '*phase images*' (number of frames) **[4]**. Usually 5 image frames are taken (the default is 3).

D. On '*Live Mode*' **[5]** you should choose '*Grid Visible*'; this will ensure the fastest refresh rate for the camera in live mode.



E. Image as usual. However, in comparison to regular imaging, the exposure time/illumination intensity need to be increased by several folds.

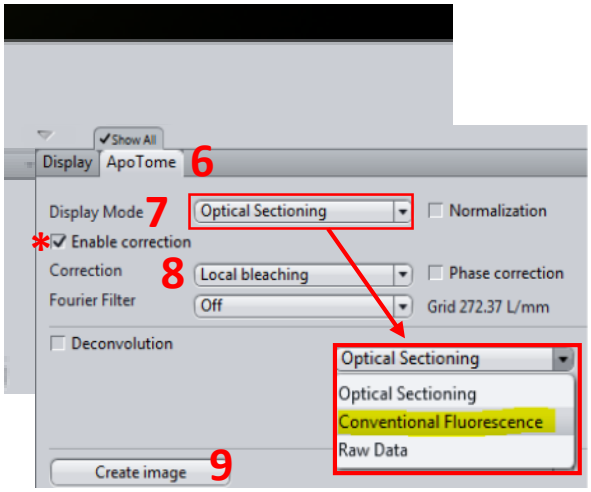
NOTE: To return to normal imaging, pull the slider back out to its original position and remember to untick the *'Enable ApoTome'* checkbox.



Processing of the ApoTome images

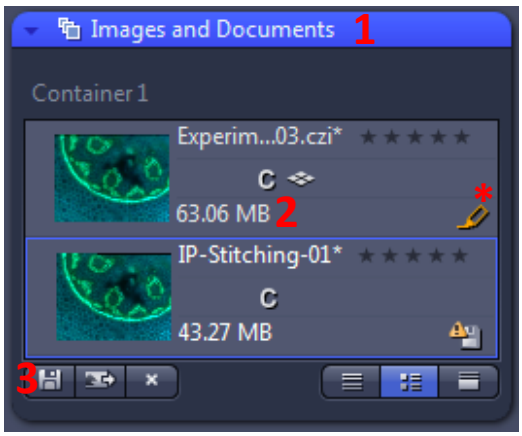
Open the tab 'ApoTome' **[6]** below the display panel:

- The default selection for '*Display Mode*' **[7]** is the '*Optical Sectioning*' view.
- Selecting '*conventional fluorescence*' will display the image as a conventional fluorescence image (widefield)
- To see the difference between a corrected and uncorrected image, deactivate the '*Enable Correction*' **[*]** option. Enable the correction using the '*Local Bleaching*' **[8]** option
- Create a new, processed resulting image by clicking on the '*Create Image*' **[9]** button



Saving Images

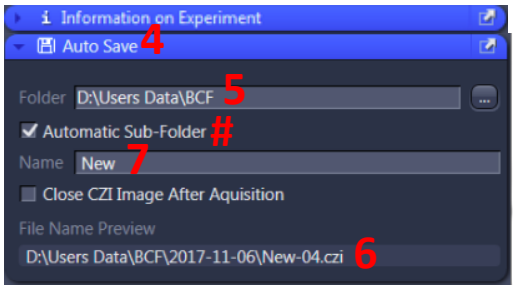
- All documents created in a session are listed in the *'Images and Documents'* **[1]** tool on the right-hand side of the workspace. This list also gives an estimation of the size on disk of each dataset **[2]**. (A file is not saved if it has an "orange pencil"* after its name)



- To save an Image you can click the *'save'* **[3]** button in the '*Images and Documents'* tool: you can save one by one or select all at the same time and 'save selected'. 'File --> Save' can also be used from the software menu.

- Save your files in: **D:\Users data\PI name\User name**
  - This will save the image as a native*.CZI file, which can be opened by Zen, FIJI (ImageJ) or Imaris.
  - It is best to keep the CZI file as your original data because it contains all the experiment parameters and could be requested by any reviewer at time of publication. (Always backup and keep the native file).
  - If you need the image for a powerpoint presentation or want to open it in photoshop you can export your CZI files as TIF files - see next page.

- You can also use '*Auto save*' tool**.** In fact, it is strongly recommended to use it for tile experiments. The *'Auto Save'* tool **[4]** is in the '*Applications*' section



- Choose the folder your files will be saved in and give it a name **[5]**.

- This will be used as a prefix in case more than one files are created before altering the *'Name'* **[6]** field and automatic numbering will be appended.

- Save your files in: **D:\Users data\PI name\User name**

- Checking *'Automatic sub-folder'***#** will create a folder YYYY-MM-DD where your files will be saved **[7]**.

Exporting *.CZI files as TIFs

Images saved as CZI throughout a session can be saved as batch to TIFF as follows:

- Choose the '*Processing*' tab and click the *'Batch'* **[8]** button (single images in the current workspace can be exported by choosing Single).
- In the '*Batch Method*' tool choose *'Image Export'* **[9]**.
- Under '*Method Parameters*' open the '*Parameters*' **[10]** tool. Choose the output file type to be TIFF **[11]**. You can then choose to convert the image to 8-bit **[12]** and choose the compression type (the default is '*LZW*', but usually we choose '*none*') **[13]**. Here you can also choose to apply any display mapping to the final image (note your raw data will be lost!). You can also burn annotations and merge channels.
- Choose the destination directory **[14]** for the exported images and note the '*Create Folder'* **[15]** option. Check this option if you want every new image to have a new folder. This is useful for multiple channels or Z-Stacks.
- Click *'Apply'* **[16]** to convert the batch of images.