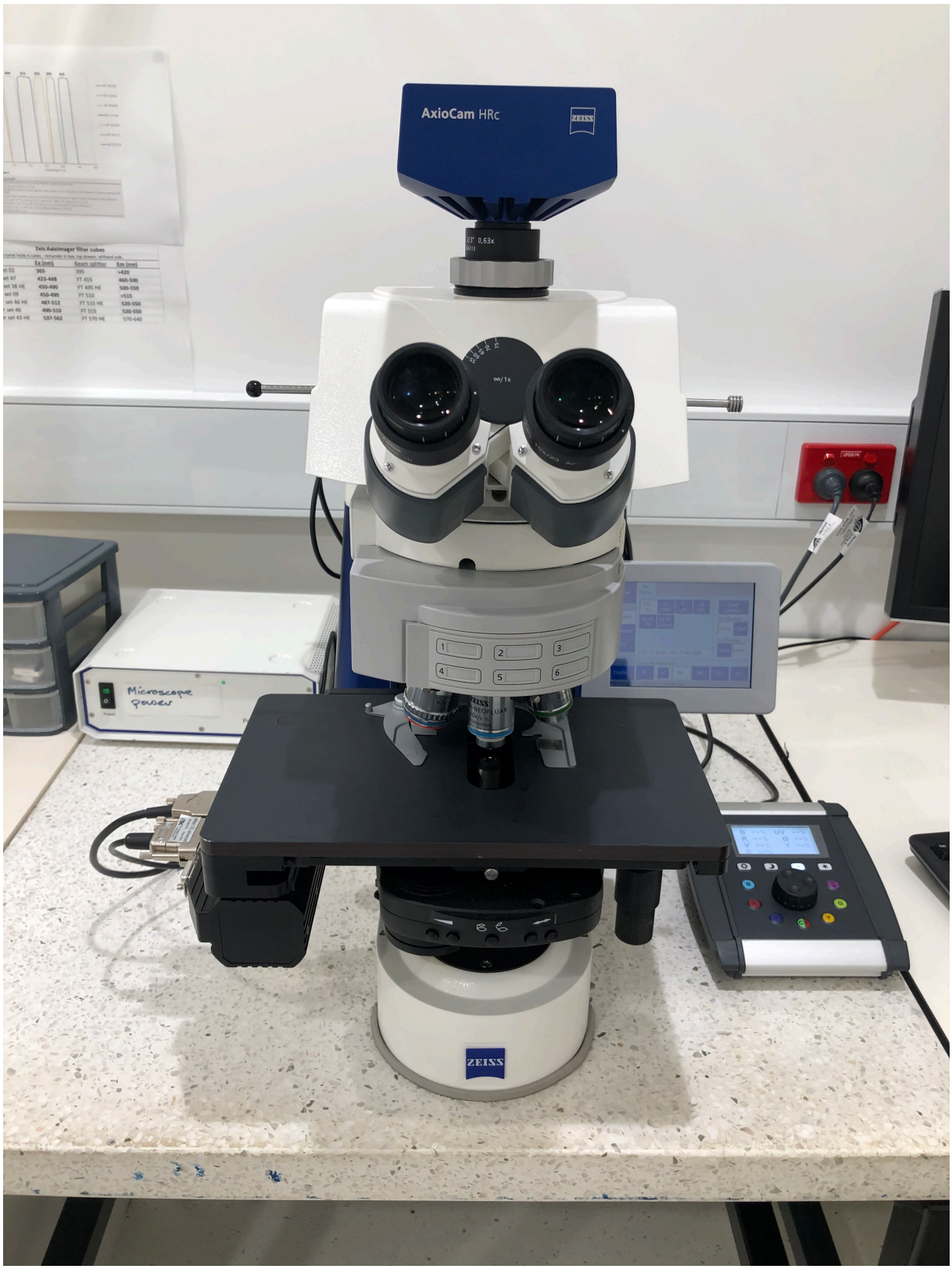
Zeiss AxioImager and Zen Blue instructions – setup for optimal imaging and image collection

Use of this microscope requires an initial training session with Viv Rolland or Phil Hands



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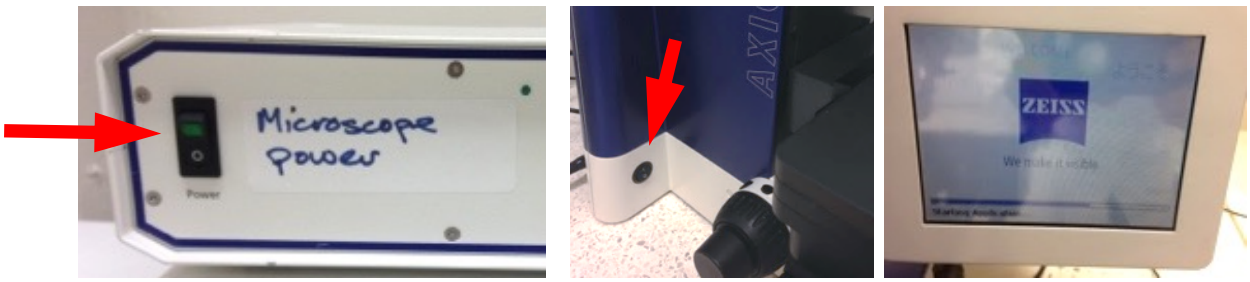
1. Microscope setup

Carefully remove dustcover and rubber eyepiece covers.



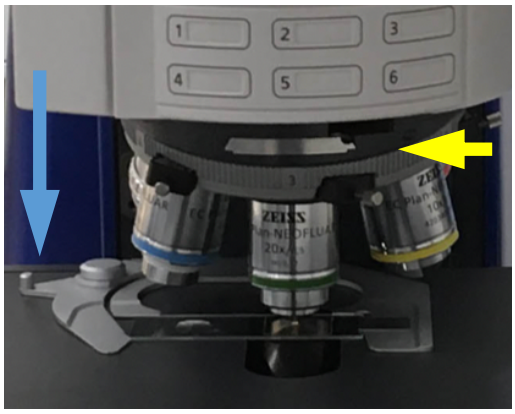
- Turn on the AxioImager microscope power supply
- Turn on the microscope. The power switch is located on the left-hand side of the microscope.
- The blue touch screen on the righthand side of the microscope will light up and the instrument will initialise.
- If required, the Colibri fluorescent light source can also be turned on at this stage by pressing the button on the control box. Initialising and operating the fluorescence light source is further detailed in section 5.

For any microscopy session it is important to 1st ensure you are positioned comfortably and the microscope is properly adjusted for your needs

- Looking into the eyepieces, adjust chair height, positioning, etc so that you are positioned comfortably.
- Adjust eyepiece interpupillary (horizontal) distance and ensure you are able to see an entire field of view using both eyepieces. If one or both eyepieces are blocked move the lever behind them to ensure light is not directed to the camera, as shown below.
- Individual eyepiece focus can be adjusted. 1st ensure the eyepieces are at 0, as shown below. Once you have a sample in focus, close your dominant eye and adjust the eyepiece focusing ring until the image is clear. This is particularly important for long imaging sessions where a difference in focus between the eyepieces can lead to eye strain, headaches etc.
- It's a good idea to note the eyepiece distance and focus positions for reference when setting up in the future

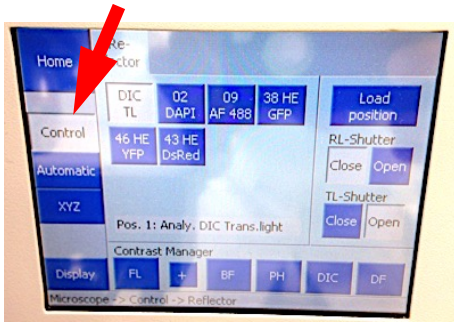
Out = light through eyepieces **Out** = light to camera

2. Basic microscope operation



- To place a slide into the stage, ensure the 5x or 10x objective is in the forward position for good clearance to slide a sample into place. Objectives can be changed by manually rotating the turret (yellow arrow) holding the objective lens body. Move the spring-loaded clamp out using the lever (blue arrow) and gently release to hold the slide in place

Bring your sample into focus using brightfield illumination.

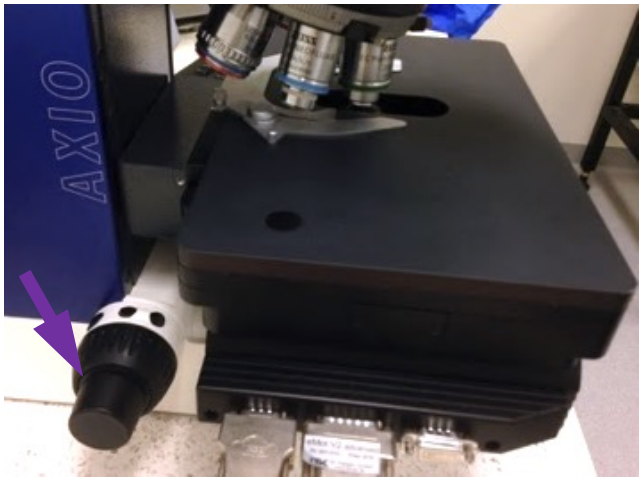


- Using the touchscreen select the **Control** menu on the left-hand side.
- Ensure the **Transmitted Light (TL) Shutter** is open (selected = white)
- Select **DIC TL** filter under reflector.
- You may also need to select **BF** in the **Contrast Manager**

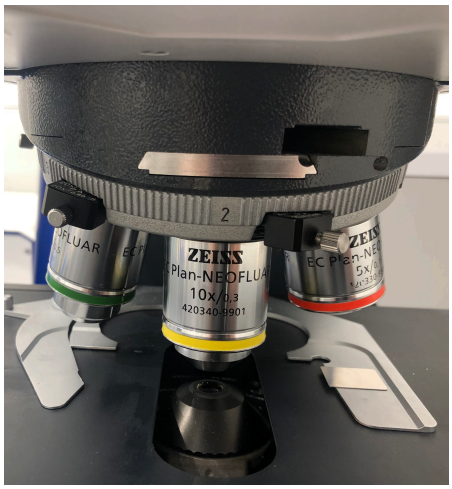
Light should now be visible on your slide.

- Adjust **transmitted light intensity** using the control located behind and below the touchscreen.
- Adjust **Stage Position** using the x-y controls at the righthand side of the stage (green arrow)
- Adjust **Focus** using the controls located on the left or right-hand side of the microscope behind the stage to bring your sample into focus (purple arrows). Exterior dial provides coarse focus adjustment and the interior fine adjustment.

You should now have a satisfactory Brightfield view of your sample.
Note this is an overview of the basic, physical operation. For most users it will be preferable to use the Zen software rather than touchscreen to control the microscope, detailed in section 6 & 7.



3. Changing objectives



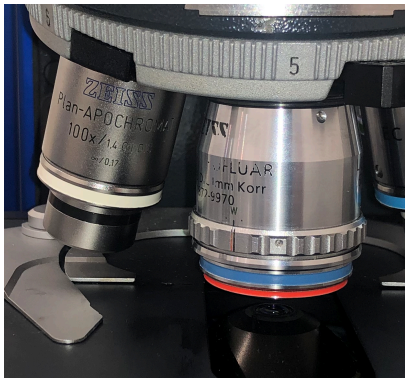
To move between objective lenses the turret must be moved manually. When moving between the 5, 10, 20 and 40x magnification lenses the stage will automatically lower and return to an appropriate working distance for the selected lens.

Note: The 5-40x magnification lenses are all **dry** - immersion liquid is **not** required.

Note: To use the 5x magnification objective the condenser must be removed from the transmitted light path using the button located on the front of the stage.

4. Immersion objectives.

The AxioImager is also equipped two high magnification immersion objectives. A 63x **water** and 100x **oil** immersion, shown opposite.



When selecting these lenses, the stage automatically moves to the loading position. The touch screen will display a prompt to apply immersion media to the slide.

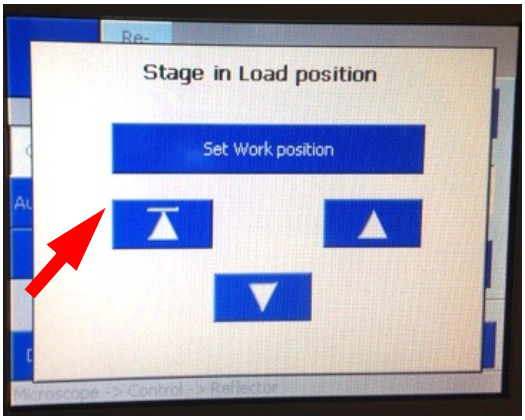
Note: the 63x = water immersion.
the 100x = oil immersion

Only clean water should be used. Ensure the collar on the lens is lined up with the W, as shown in the image.

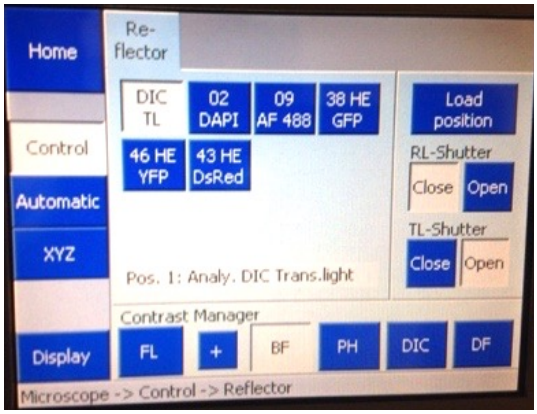
It is IMPORTANT to ensure oil is used with the 100x lens ONLY. Once oil has been used do not switch back to any other objectives!

If you are at all unsure about using immersion lenses please seek advice from BMIC staff before attempting to use them.

Press “return to focus” to return the stage to the working position (red arrow).



You can also use **Load position** located on the right-hand side of the touch screen to automatically lower the stage to the loading position, shown below.



5. Koehler illumination

These steps provide optimal transmitted illumination of your sample and should be performed whenever 1st using the microscope and for each objective used.

After focusing on your sample, move to an area of clear background on your slide.

I. Raise the condenser to its uppermost position
   - Control is via the black knob located underneath the stage on the righthand side.

II. Fully close the field of view diaphragm.
   - Control is via the thumbwheel located on the righthand side of the microscope body, marked "F". In the fully closed position only a small patch of light will be visible on your slide.

III. Lower the condenser slowly until the patch of light is in focus and diaphragm leaves can be seen clearly, forming an octagonal shape.

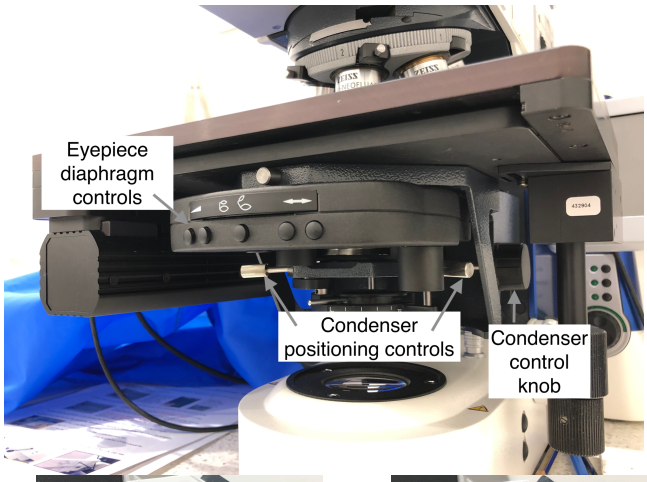
IV. A crosshair is visible in the field of view. Use the two silver positioning controls underneath the stage to adjust the condenser until the light is centred on the crosshairs.

V. Open the field of view diaphragm until the edges move just beyond the visible area.

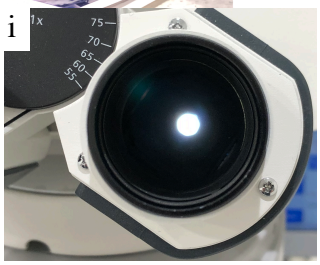
VI. Finally, remove the right eyepiece and, using the controls located on the front of the stage, close the eyepiece diaphragm all the way, as in picture i.

VII. Looking down the eyepiece tube, open the eyepiece diaphragm into a position where ~ two thirds of the area visible down the eyepiece tube is revealed, as in picture ii.

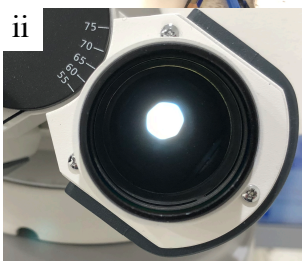
VIII. Replace the eyepiece.



i



ii



Optimal Koehler illumination is now set.

6. Fluorescence illumination, filters and imaging.

The microscope is equipped with a Colibri 7, a LED light source providing 7 discreet light wavelengths detailed below which can be used along with one of 5 fluorescence filters housed in the microscope turret.

You will need to know your sample's fluorescent excitation and emission properties to properly select the required LED and filter combinations. If unsure, please speak to BMIC staff before your experiment.

Available filters and their specifications are listed below and on the wall behind the microscope. By default the microscope is set up with filter sets 02, 47, 38 HE, 46 HE and 43 HE. Acquisition settings profile for these filters DEFAULT 1. Settings profile DEFAULT 2 replaces filter CFP (47) with the alternative YFP filter set (09)

If filters other than the DEFAULT 1 set are required please contact BMIC staff, before your session, to install them. Do not exchange filters yourself as this can have impacts upon all other users.

Colibri LED light Source

	Name	Available Excitation Bands
1	UV	**370 - 400 nm**
2	V	**401 - 445 nm**
3	B	**450 - 488 nm**
4	C	**489 - 533 nm**
5	G	**540 - 570 nm**
6	Y	**576 - 603 nm**
7	R	**614 - 647 nm**

Zeiss AxioImager filter cubes

Name	Ex (nm)	Beam splitter	Em (nm)
Filter set 02 (DAPI)	**365**	395	**>420**
Filter set 47 (CFP)	**423-448**	FT 455	**460-500**
Filter set 38 HE	**450-490**	FT 495 HE	**500-550**
Filter set 09	**450-490**	FT 510	**>515**
Filter set 46 HE	**487-512**	FT 515 HE	**520-550**
Filter set 46	**490-510**	FT 515	**520-550**
Filter set 43 HE	**537-562**	FT 570 HE	570-640

Microscope turret holds 6 cubes - remainder in box, top drawer, lefthand side.

*Remember when working with fluorescence to close the RL-Shutter whenever you are not directly viewing your sample, to limit the extent of photobleaching.

ZEN software provides the best method for fluorescent light and filters selections and provides combined control of both light source and filters for fluorescence imaging. From now on you will be dealing with the Zen Blue 2.6 software to control both the microscope and camera.

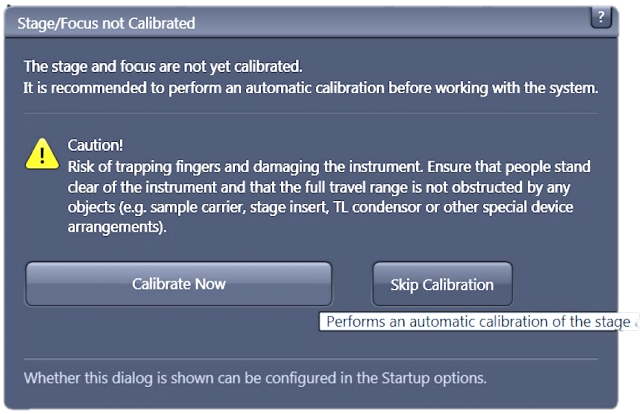
7. Starting **Zen** software.

- Open imaging software on by double clicking the **ZEN** icon on the desktop.
- At the start-up screen select **ZEN Pro**

- The start-up window will appear and display two warnings

  a. confirm the light-path is directed 100% to the camera i.e. the sliding lever on the microscope turret, detailed in section 1, is positioned all the way to the right and click **OK**.

  b. select **Calibrate Now** for this one:



The motorised stage will move to it x-y and focus calibration positions. Don't click on anything while this is going on or touch the microscope!

The software is now ready to be configured and used for imaging

8. Initial sample analysis and single-channel imaging

Zen starts in the **Locate** tab which provides convenient controls for initial examination of your sample, and for collecting single channel images.

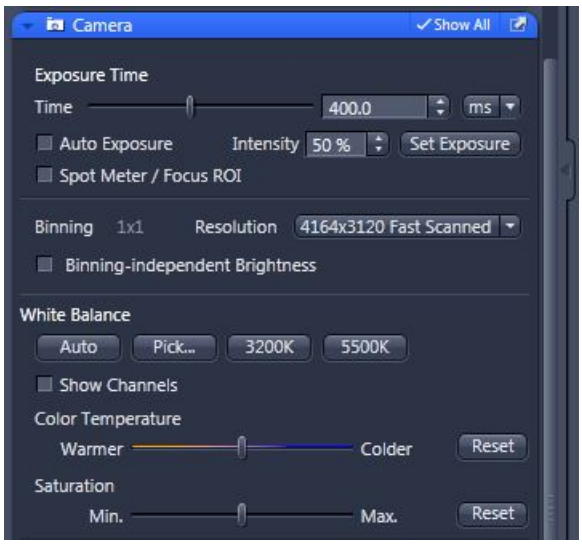
It is best to use the eyepieces, rather than the screen, to 1st examine your slides as they offer wider and clearer field of view for locating your sample and regions of interest

Eight shortcut buttons located close to the top of the locate tab, shown opposite, automatically change both activated wavelength and filter combinations. For DIC the required condenser is also set. These controls are the best way to quickly change between light and filter combinations.

To view an image on the screen, click the live button to display the live feed from the camera and move the eyepiece sliding lever on the microscope turret, detailed in section 1, all the way to the right.

Camera settings for exposure, white balance etc can now be adjusted for the best image before collecting single channel images, as detailed below.

Obtaining single-channel images.



Select the blue camera bar in panel on the left-hand side of the screen to show the camera settings panel.

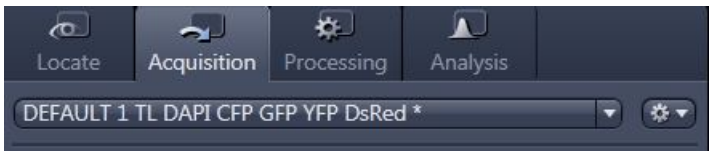
- Exposure time can be manually adjusted using the slider or arrows on the time box. Selecting auto exposure will allow the software to automatically determine the best exposure for the image.
- For brightfield or DIC images the intensity of the light can be adjusted. Adjust lamp intensity using the separate slider. For the 10x and 20x objectives a voltage of between 3-5 V will generally give good illumination of your sample

  *Note: It is important to change the intensity of the TL lamp via the software using this slider and **not** the control wheel on the microscope body. Changes using the control wheel are not recorded by the software and it will revert to the original value during image acquisition.
- For the fluorescence channels light intensity can also be adjusted, but if this is required it is best to collect images using the acquisition tab, as described below.
- Where possible position the camera at an area of clear background on your slide for white balance. Auto white balance button generally provides good results. Alternatively click "Pick…" and select an area of known clear background. White balance can be manually refined using the colour temperature slider. Compare the image displayed on the screen to that you can see through the eyepieces to confirm the white balance is correct.

Once a satisfactory image is obtained, use the snap button to record a single image for the channel displayed on the screen. More detail on naming and saving images is given section 10.

9. Configuring Zen software for multi-channel imaging.

Multichannel imaging setup is configured using the **Acquisition** tab



- Select this tab on the left-hand side of the screen.
- Select a settings profile from the drop-down menu.

Note: For most users the DEFAULT 1 settings profile provide a good starting point for most samples Once configured individual user settings profiles can be saved to this menu for subsequent use. Please use labels that include your ident and date, limit the number of profiles saved and delete old profiles that are no longer needed wherever possible.

The **active channels**, along with respective **illumination intensity**, **white balance** and **exposure** must all be configured to optimise the image before acquisition. This an iterative process that generally takes several minutes to get right, but is important for the best quality images. It's necessary to 1st configure the brightfield image settings and obtain a satisfactory brightfield image before the fluorescence channels. Steps and details for these processes are given in the sections below.

A. Configuring active imaging channels

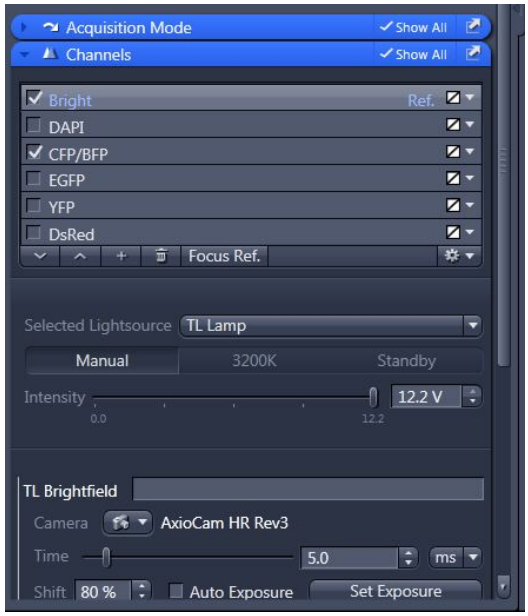
Selecting the channels bar displays the available channels for image acquisition.

A tick in the checkbox indicates which channels will be collected when an image is acquired – in this example brightfield and DAPI images would be collected.

The highlighted bar indicates which channel is currently displayed in live view.

Selecting channels via this panel automatically changes the filter and shutter settings in the same way the shortcut buttons in the locate tab. This the easiest way to change between filters while imaging.

B. Configuring brightfield image settings.

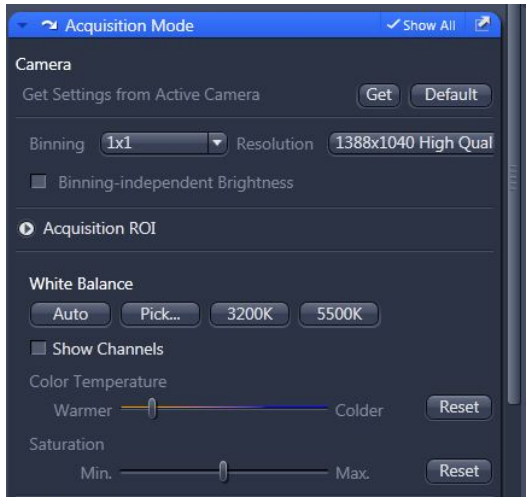


- Select “Bright” in the channels bar to display the brightfield image in the live view.

  It is likely the image at this stage will be too bright. It’s necessary to adjust the lamp intensity and camera exposure for the best image.

- Adjust the Transmitted Light (TL) intensity using the slider below the channel’s bars. For the 10x and 20x objectives a voltage of between 3-5 V will generally give good illumination of your sample

  *Note: It is important to change the intensity of the TL lamp via the software using this slider and **not** the control wheel on the microscope body. Changes using the control wheel are not recorded by the software and it will revert to the original value during image acquisition.



- Exposure time can be automatically set using the set exposure button, and can be manually adjusted using the slider or arrows on the time box.

- White balance can be set under the **Acquisition mode** bar. Where possible position the camera at an area of clear background on your slide for white balance. Auto white balance generally provides good results. Alternatively click “Pick…” and select an area of known white background. White balance can then be manually refined and adjusted using the colour temperature slider. Compare the image displayed on the screen to that through the eyepieces to confirm the white balance is correct

Note: it is important to configure your brightfield image 1st, confirming the sample is in focus, before configuring fluorescent channel illumination and exposure settings.

Brightfield image quality may also be further improved by additional adjustment of illumination intensity and exposure, and with adjustment of the condenser for contrast.

C. Configuring fluorescent image settings.

- Select the checkboxes for the required fluorescent channels and highlight to display the image in the live view.

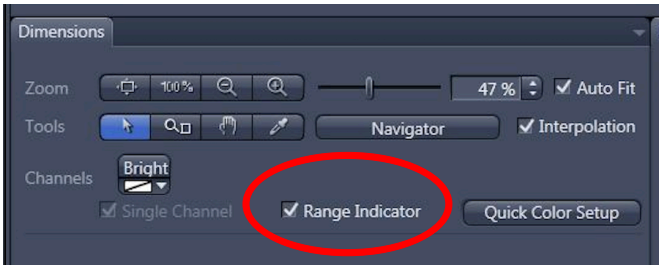
For each channel required;

- Adjust exposure, as before. Set exposure will generally give an OK initial setting, then manually adjust using the exposure slider.
- If the exposure time is high/low, light intensity can also be adjusted. Use the slider under **Lightsource** to set the value and readjust exposure accordingly.

Note; It is generally preferable to use a lower light intensity and increase exposure time to limit photobleaching.

- The range indicator is also a useful tool here, to obtain the best exposure setting. Section D below provides information on this tool and gamma settings to best configure fluorescent imaging setup.

D. Exposure and gamma settings



Exposure time must be adjusted to ensure that most pixels are not over or under exposed. In the **Dimensions** panel at the bottom of the screen the **Range Indicator** provides a useful guide. When selected overexposed regions of the image will display red pixels under exposed pixels blue.

Adjust Exposure **Time** by either clicking and typing in the ms, or by using the slider. Auto Exposure generally gives OK results, but it is best to adjust manually before collecting the image.

You will need to adjust the exposure for each active channel before collecting images.

Gamma settings are shown in the **Display** panel below the live image.

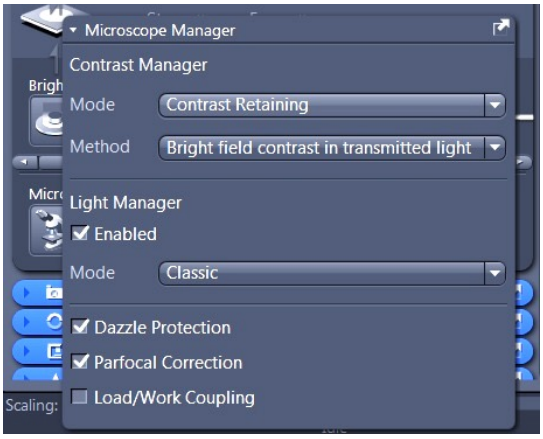
Gamma = 0.45 is optimal for this camera for brightfield images, which will typically look best with this setting. Fluoresent images are generally better using gamma = 1.0 which is neutral for the camera.

Note, by default the software is set to collect and display images with 0.45 gamma for the brightfield channel and 1.0 for fluorescent images. Therefore, it is best to use these settings when adjusting exposure for the different channels. You will need to manually change these settings in live view.

E. Activating the contrast manager.

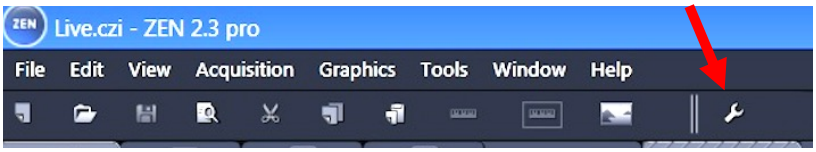
If the contrast manager does not appear on the microscope touch screen the following steps will reactivate it;

- Select the **locate** tab
- Select **Microscope control bar**
- Select the **microscope manager** at the bottom of this box.
- Select the **Mode** drop-down menu and select **Contrast retaining**



10. Image capture, naming and autosaving

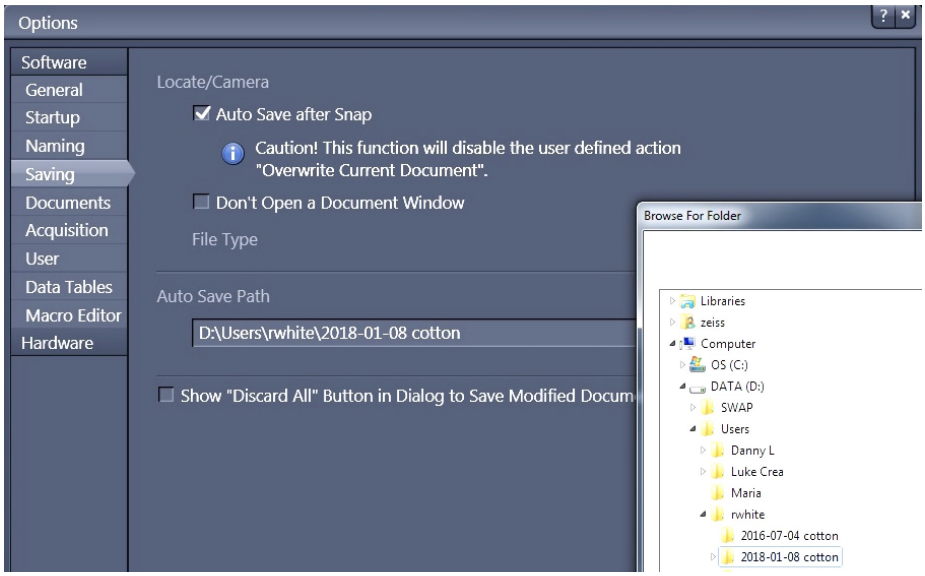
Before starting image capture, you need to set up how and where you will save your images. To do this, select the spanner icon at the top of the page to open the **Options** menu.



A. AutoSaving

- 1st select saving from the list at the left
- Ensure that Auto Save after Snap is selected.
- In Auto Save Path, select your folder from the list at D:\users and create subfolders if required.

This is the folder you will have created during your training session. **Note changing this setting is important to ensure images are not saved into the last user's folder!**

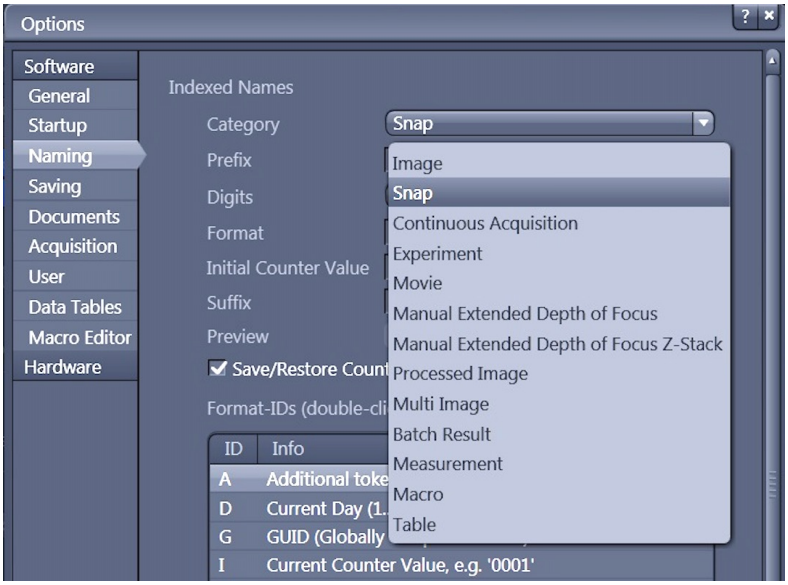


B. Naming

- Next select Naming
- In **Category**, select the type of image to be collected.

Most commonly this will be **Snap**, for single images using the Snap button.

Tiled images and z-stacks are **Experiment**



The image name comprises several components configurable to your own requirements

Prefix = the specific name you assign to the image.

Digits = the number of digits assigned to the image number associated with this name.

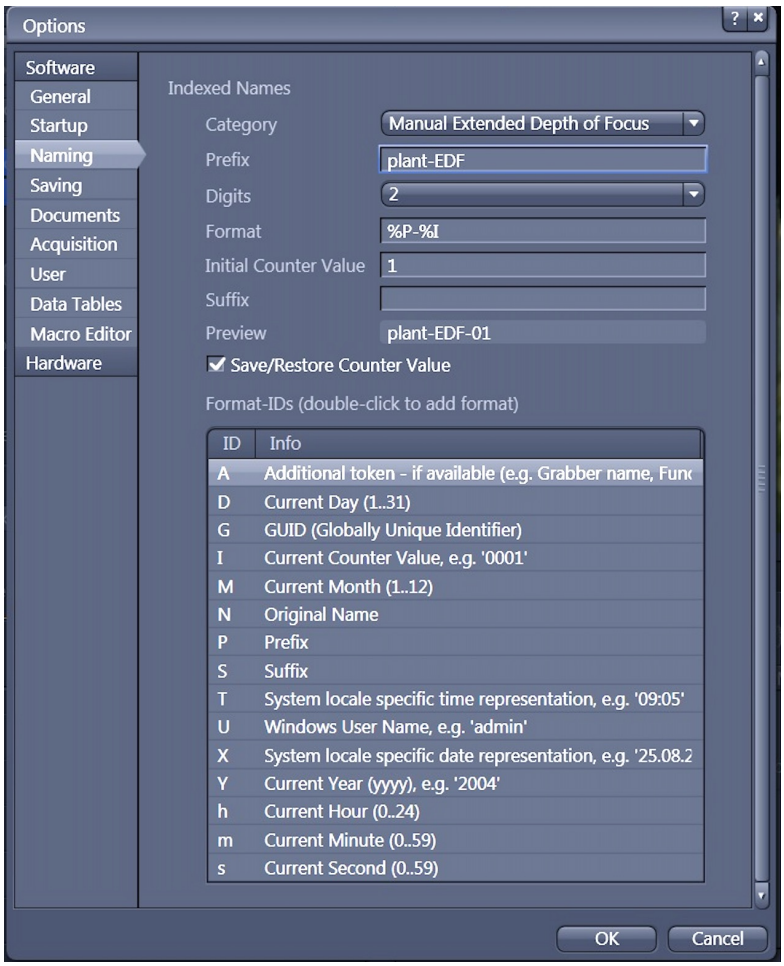
Format = which of the **Format IDs** from the list below you will use. For this image, only the **Prefix %P** and **Current Counter Value %I** are used.

The **Initial Counter Value** is usually 1.

You can also add a **Suffix** if required.

Preview shows you how the image name will appear as a file identifier.

Save/Restore Counter Value is usually selected. When this is checked the software will resume counting from the last recorded counter value.



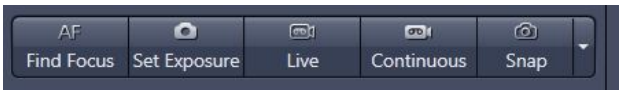
- Once finished in the **Naming** and **Saving** menus, click **OK**.

PLEASE DO NOT ADJUST ANYTHING ELSE IN THE STARTUP PANEL.

The ONLY drop-down menu items you will need to adjust are **Naming** and **Saving**.

C. Multichannel image capture and review

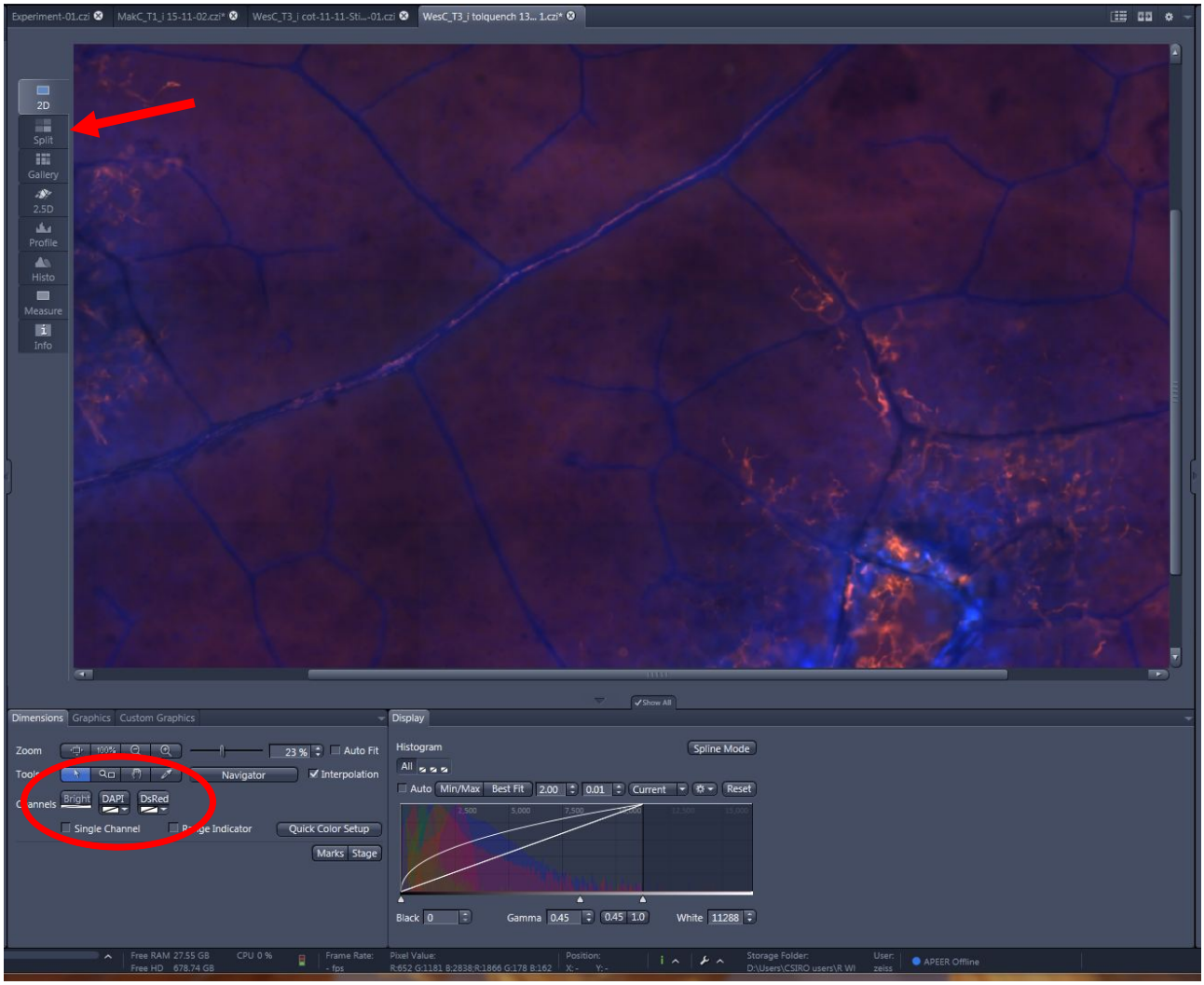
Clicking the **Snap** button in the panel on the left of the screen will record an image for each of the active channels using the configured settings.



*Be sure not to touch the microscope or table during image capture to avoid disrupting the focus.

The recorded image will be displayed in a new tab and live view will be frozen.

By default the displayed image will show all active channels as an overlay. Review your images by deactivating individual channels via the buttons in the dimensions panel (circled) or use the **Split** (arrowed) tab to the left of the image to display the individual channels in separate windows.



If you wish to add scale bars to the image see section 9. To manually save images and export in formats suitable for viewing outside of the Zen software see section 11.

To return to live imaging click the **Live** button again.

Note: if collecting tiled images or z-stacks you will need to use the "**start experiment**" rather than Snap button.

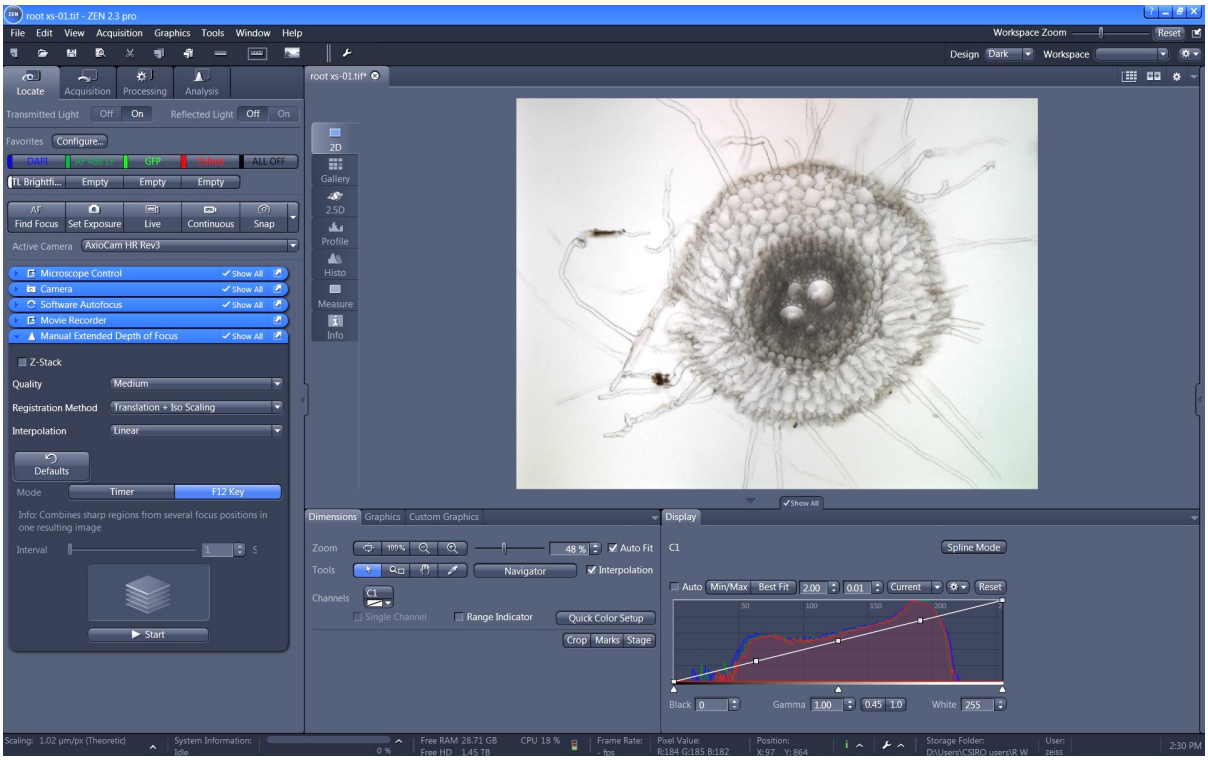
11. Manual Extended Depth of Focus.

This imaging method is useful for capturing all of the in-focus regions of tissues with considerable topological variation, such as a leaf surface, or from thick tissue sections, as in the root section below.

First, open the **Manual Extended Depth of Focus** bar in the **Locate** tab:

You can select the final image **Quality**;

the **Registration Method** used by the software to select the most in-focus regions (i.e., those regions with the highest contrast) and the **Interpolation** method by which the selected in-focus regions are fused into a single image.



You can opt to save the images as a **Z-stack**, but this is usually unnecessary unless you wish to apply other software for 3D reconstruction, etc.

Once you have selected from the options above, the easiest way to collect the image is using the **F12 button** above the main keyboard.

D. Click **Start**
E. Focus on one side of the sample on the slide.
F. Click the **F12 key**
G. Move the focus slightly up or down.
H. Click the **F12 key**
I. Continue until you are satisfied with the image to the right of the live image.

12. Adding a scale bar to an image.

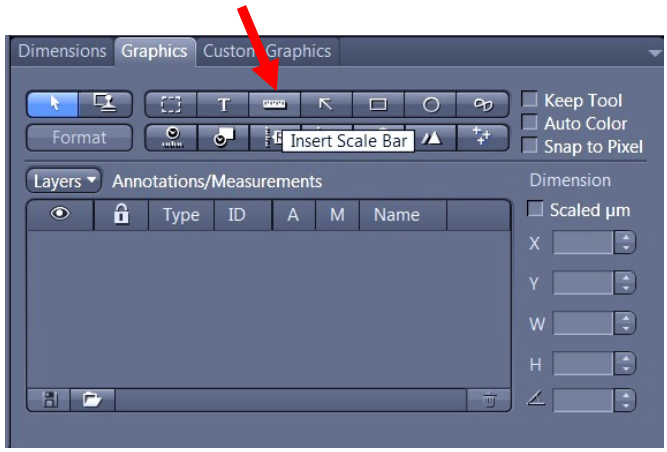
J. 1st select the **Graphics** tab below the image

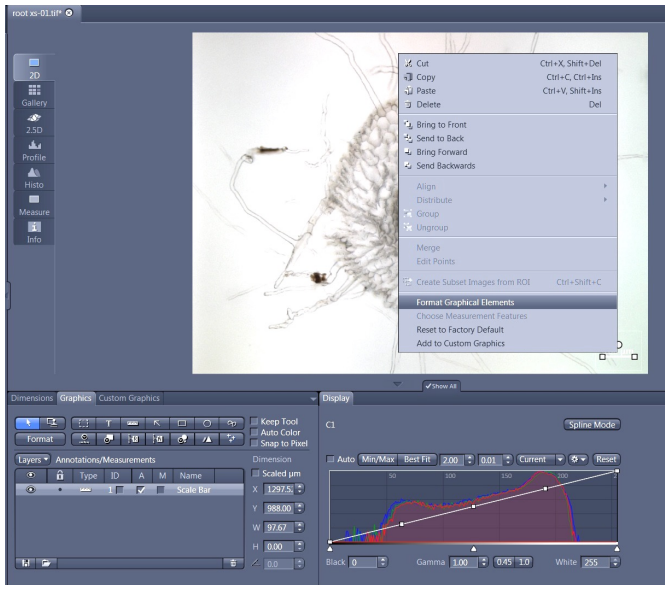
K. Select the small ruler icon

L. By default the scale bar is inserted into the lower right corner of the image, as shown. The software will automatically calibrate the scale bar size.

M. Right-click the scale bar and select **Format Graphical Elements** to change the scale bar properties.

N. Use the various drop-down menus to change the scale bar size, colour, scale, font etc as required.





Scale bars are added onto the image as a layer. To save the scale bar in the image select the **Graphics** menu at the top of the screen and then **Burn in Annotations.** This creates a new image with the scale bar preserving the original without, which is essential for any future image analysis. Remember to save both images and to rename the scaled image with "+ scale", or something similar. Saving images is fully detailed in section 13.

13. Saving and exporting images.

It is recommended to save images in .czi format and to export as .tiff files.

- .czi is the native Carl Zeiss format, allowing you to open, analyse and edit your original files in Zen in the future.
- .tiff is the preferred and best file format to export images, providing high quality images suitable for editing, viewing on computer screens, using in PowerPoints etc. It is possible to export other formats, e.g. .jpeg or .png but these formats are not recommended.

The AxioImager computer is not connected to the network. Please use only **a freshly formatted memory stick** for collecting images to avoid introducing viruses to any BMIC computers.

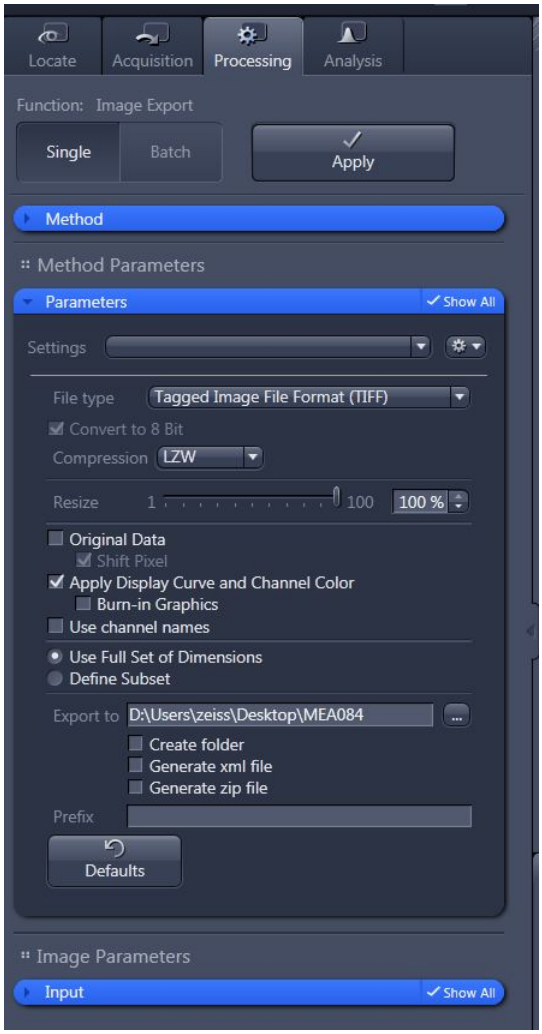
A. Manually saving images in Zen

To save all open images as .czi at the end of your imaging session simply select **File**> **save all**> select the **save location** and click **OK** for each image to be saved.

B. Exporting images

To export an image:

- Select the **Processing** tab
- Select **image export** from the **Method** bar.
- Under **Parameters** configure your requirements
    - By default .JPEG file type is selected, this should be changed to .tiff via the drop-down menu.
    - **Original Data** is best unticked.
    - Select **Apply Display Curve and Channel Colour**
    - if required select **Burn-in Graphics**. Scale bars will be burnt into the exported image.
    - for images with multiple channels select individual and merged as required.
    - For **Export to:** set the required folder.
    - Deselect Create folder unless required. This gives a new folder for each image exported.
- Click **Apply** at the top of the Processing panel to export.



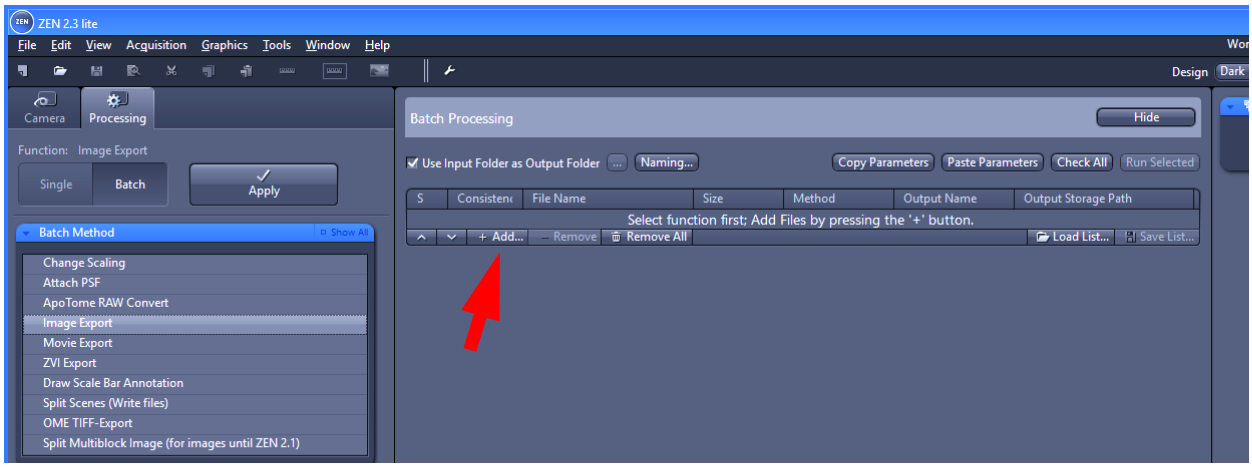
The exported image reflects that which is currently displayed. Be sure to adjust the active channels, gamma settings etc before exporting for the required images. Note that images collected with gamma = 1.0 will have reverted to gamma = 0.45 after they are snapped.

C. Bulk image export

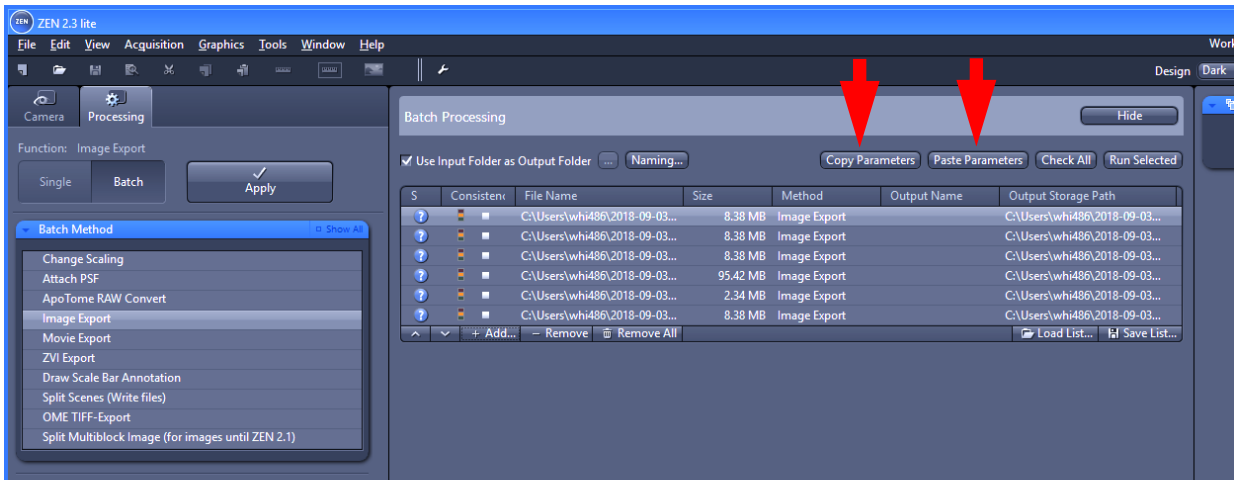
It is also possible to bulk export multiple files at once using **Batch**, as detailed below.

- Select the **Processing** menu and **Batch** convert function.

- Select **Image Export** from the **Batch Method** bar



- Select **Add** (Arrowed above) to select the .czi files to convert.
- Typically it is easiest to check **Use Input Folder as Output Folder** for setting the save location



- Configure the **Method Parameters** as detailed previously. Note that this is applied to the 1st image in the list only and needs to be copied to all in the list as below;
    - select **Copy Parameters**
    - select all images below the 1st
    - Select **Paste Parameters**
- Select **Apply** to export all images. This may take a few moments to complete.

Copies for each image should now have been exported to your folder.