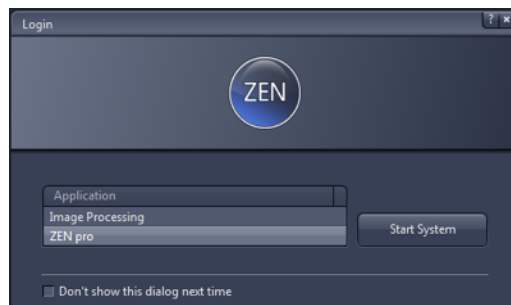


Zeiss Axio Imager/Observer Guide

Getting Started


1. Switch on the white *Power supply* box.
 - If you are using **fluorescence** switch on the *HXP120* and *Colibri* control box.
 - If you are using **ApoTome** switch on the *ApoTome* box.
2. Switch on the microscope (at back left of microscope stand).
3. Switch on the computer and log on with your UQ username and password.
4. Once the microscope has finished starting up double click the **Zen** icon.
 - When the menu appears, choose **Zen Pro**

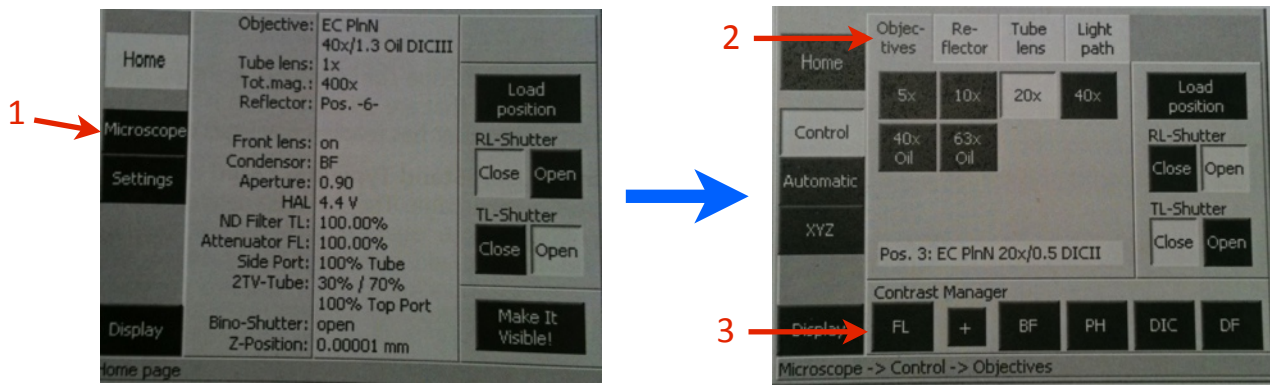


Shutting Down

1. Lower the stage and remove your sample.
2. *Gently wipe* any oil objectives you have used with *lens tissue* (**do not use kim wipes to clean objectives**).
3. Exit the software and copy your files to your *home or group network/USB drive*.
4. Turn off the microscope power supply box and the HXP120/Colibri/Apotome modules.

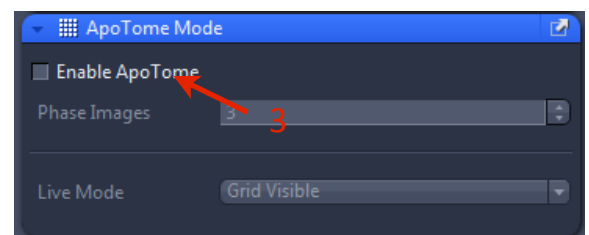
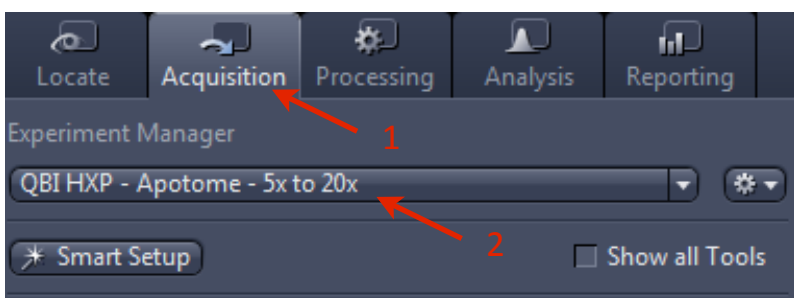
Visualising a Sample Through the Oculars

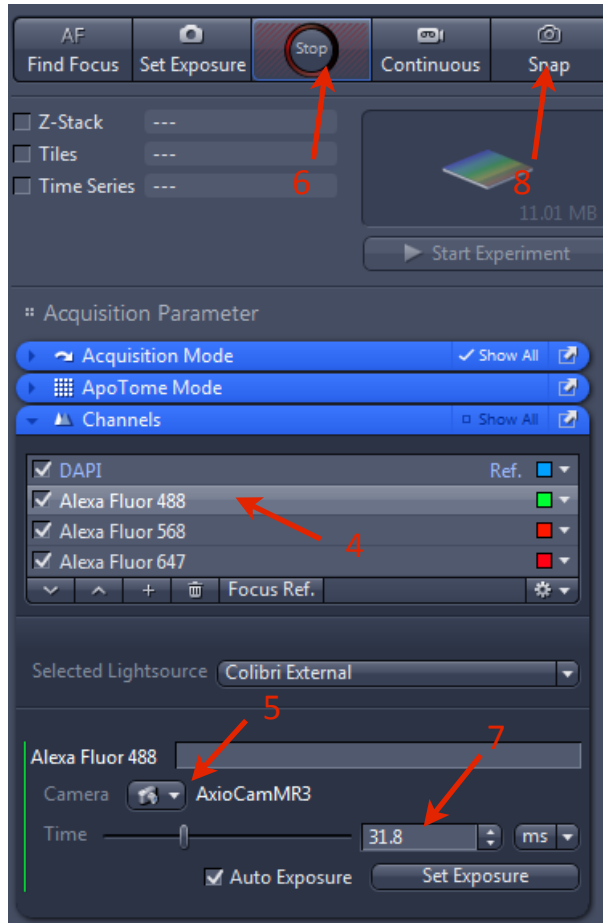
1. On the touch screen attached to the microscope press “**load position**” and position the slide on stage. Pressing the  button will return you to the working position.
2. Press **microscope (1)** on the far left of the touch screen.
3. You will be able to change **objectives, reflectors (filtersets)** and adjust the light path via the tabs at the top. **(2)**
4. You can use one of the quick buttons at the bottom of the screen to get the microscope ready. **(3)**
 - Press **FL** for fluorescence, **BF** for brightfield, **PH** for phase and **DIC** for DIC/Normaski Imaging modes.
 - If imaging fluorescence ensure you have the appropriate fluorescent light switched on
5. Check the light path is allowing the sample to be seen via the oculars (under the “**light path**” tab).
 - **Axio Imager:** 100% tube (100% side port is for the colour camera only) and ensure the push-pull rod is all the way in.
 - **Axio Observer:** 50 or 100% eyepiece/vis
6. An image will now be visible down the oculars - adjust stage position and focus as necessary.



Imaging a Sample - Using the *Live Window*

- Select an imaging mode on the touch screen.
 - **FL** at the bottom left for **fluorescence**.
 - **BF/PH/DIC/DF** for transmitted light imaging.
- Adjust the light path so light reaches the camera.
 - **Axio Imager**: Pull the push-pull rod on the side of the microscope out to the camera position.
 - **Axio Observer**: In the **light path** menu on the touch screen – adjust the settings to 100% camera.
- Ensure the shutters are open and illuminating the specimen.
 - **Fluorescence** - both the reflected light shutter (**RL-shutter** via the touch screen) and the HXP lamp shutter (via the Colibri control panel – press the **Ext. button** (external light source) then press the **shutter button**).
 - **Brightfield** – the transmitted light shutter (**TL-shutter**) (via the touch screen).
- Change to the acquisition tab **(1)** and choose a hardware configuration from the **Experiment Manager (2)** (start with a configuration prefixed with “QBI” if a personalised setting does not exist).
- Open the **Apotome Mode** tool and uncheck **Enable Apotome (3)** if not using the Apotome module (if this option remains checked the software will attempt to calculate an optical section).
- Open the **Channels** tool and highlight the channel to be visualised **(4)** (the generic “QBI” configuration has definitions for the four main classes of fluorophores), make sure the correct camera is selected **(5)** and click the **Exposure** button **(6)**.
- Adjust exposure time by **(7)** pressing **Set Exposure** or using the **Auto Exposure** option. The **Set Exposure** button applies the hardware settings and as such closes off the **RL** and **External** shutters after the exposure time has been calculated. Using the **Auto Exposure** checkbox will dynamically calculate the exposure time such that this is adjusted each time the stage position etc. changes.
- Click **Snap** to create an image. Note: the images are captured but not saved at this point and are with an * to indicate as such.



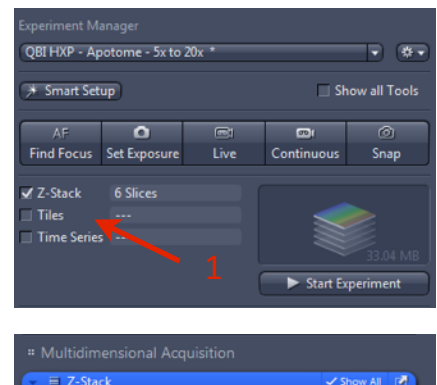


Multidimensional Acquisition

Multidimensional acquisitions can be achieved by choosing an Experiment(s) from the **Experiment Manager (1)**. Checking the box next to the desired experiment adds a tool to the Multidimensional Acquisition tab. Combining these experiments with channels selected in the **Channels** tool it is possible to create automated experimental protocols for single and multiple channel imaging, Z-series, time-lapse and mosaic imaging or any combination of these.

The multidimensional acquisition options:

- **Z (Z-series):** Image multiple planes through tissue/cells (*page 6*).
- **Tiles:** Controls tiled image settings for imaging across numerous fields of view. This tool also includes an option for automated imaging a selected locations on your slide/dish in a similar way to **Mark and Find** in Axiovision (available on Green and Indigo).
- **Time Series:** Controls time-lapse settings for live imaging of living cells/tissues.



Hardware settings for Axio Imagers / Observers

Each microscope has the option of using Colibri LEDs or the external HXP Xenon lamp for fluorescence.

Colibri, when used with filterset 62, gives comparable and in some cases better results than using the HXP lamp, doesn't change intensity as the LEDs age (unlike the HXP) and turns on instantly (no warm up or cool down time).

You will need to use the HXP if you are using **Alexa 546/555/568 or Cy3**

A recommended setup if you want to observe multiple proteins is:

DAPI + Alexa488/GFP + Alexa555 + Alexa647
(Look out for **crosstalk** between **Green** and **Red** channels)

Another 4 colour combination is:

DAPI + Alexa488/GFP + Alexa594 + Alexa647
(Look out for **crosstalk** between **Red** and **Far Red** channels)

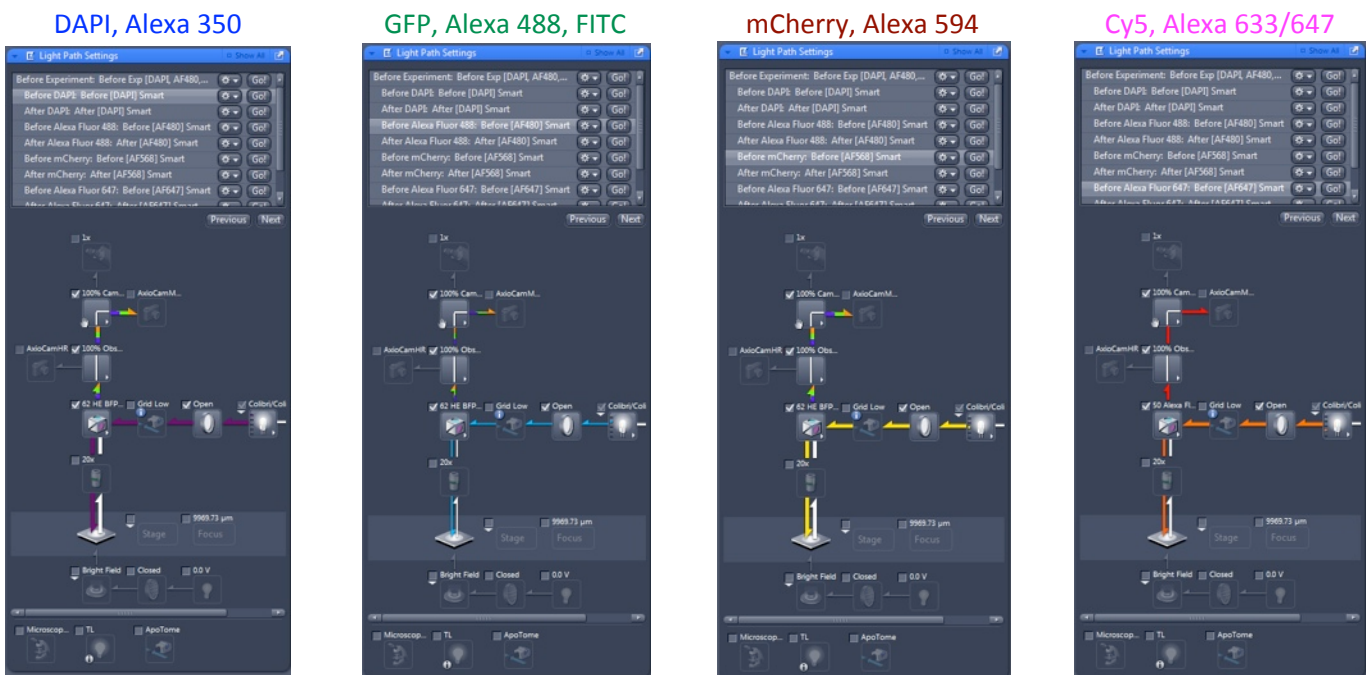
Experiment Settings:

There are four generic predefined experiment configurations which you can adapt to your particular needs:

1. QBI HXP (one for 5x to 20x ApoTome and one for 40x, 63x and 100x ApoTome)
2. QBI Colibri (one for 5x to 20x ApoTome and one for 40x, 63x and 100x ApoTome)
3. QBI Brightfield BW Camera
4. QBI Brightfield Colour Camera

Each channel has an associated configuration which can be accessed in the **LightPath Settings** tool (see illustrations below). Each configuration contains the hardware settings which are automatically applied before any experiment and after the experiment has completed. These settings can be defined using the **Smart Setup** tool.

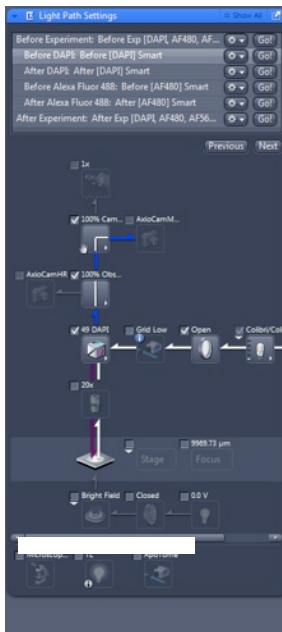
Colibri Settings:



Hardware settings for Axio Imagers / Observers

HXP Settings:

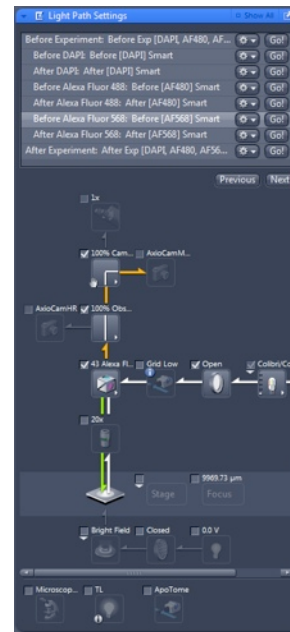
DAPI, Alexa 350



GFP, Alexa 488, FITC

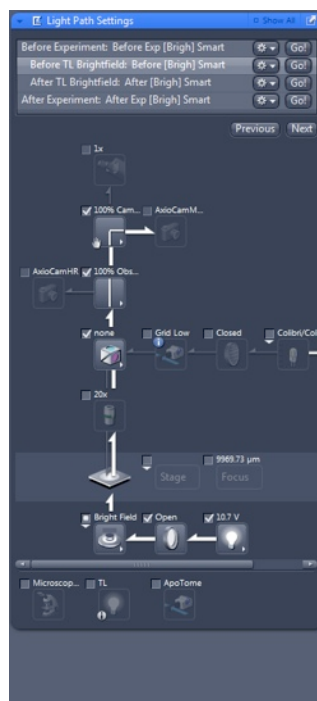


Cy3, Alexa546/555/568



DIC and Brightfield Settings:

Monochrome camera

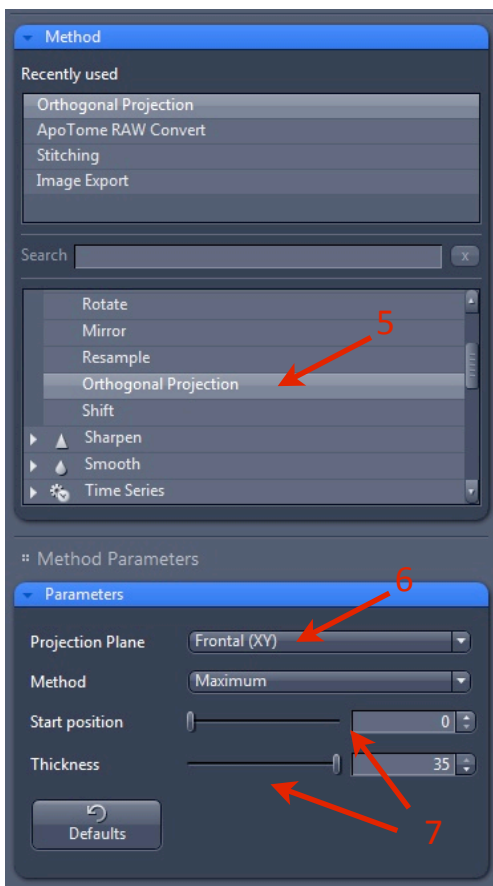
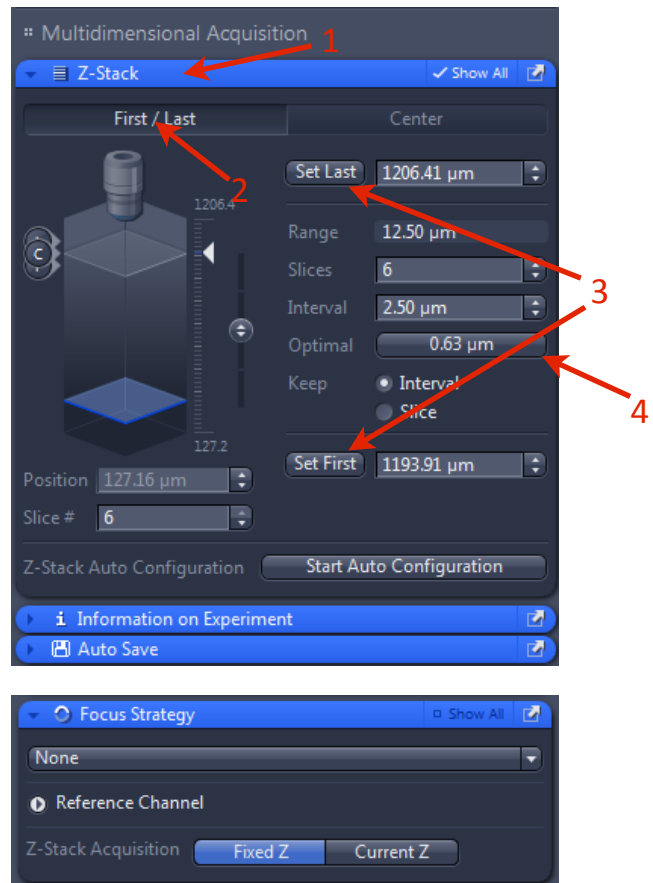


RGB camera



Creating a Z-Series Experiment

1. Highlight your channel of choice in the **Channels** tool and run the camera live and set the exposure time as described above. Manually focus to the brightest plane – set the exposure time for this plane to prevent over-exposure in the rest of the Z-series.
 - Repeat this step for all channels required in the experiment.
2. Tick the Z-Stack option in the experiment manager and open the Z-Stack tool in **Multidimensional Acquisition**. (1)
3. There are two modes of operation **First/Last** and **Center**. The first option allows the user to set the start and stop Z-positions for the volume being recorded. The second sets the current Z-position as the center position for the stack and allows the user to set a number of slices above and below this center point. The **First/Last** option is described here. (2)
4. With the camera running live adjust the fine focus control to set the Z-position to the start position for the stack and press **Set First**. Move the fine focus in the opposite direction and adjust the position for the stop position for the stack and press **Set Last**. (3)



5. For good Z resolution click the **Optimal** button (4). 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.

6. Under **Focus Strategy** make to select **Fixed Z** 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.

7. Click **Start Experiment** to begin a Z-series experiment with the channels you have activated under **Channels**.

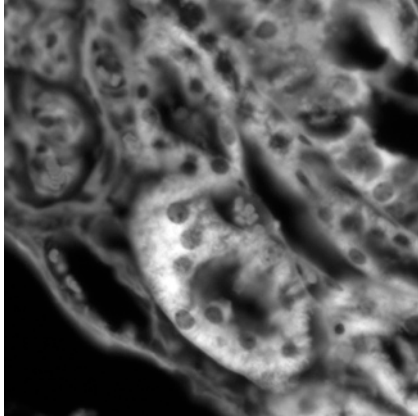
8. Once captured you can turn a Z-series into single flattened image called a **Maximum Intensity Projection**

- To do this click on the **Processing** tab and choose **Orthogonal Projection** as the **Method**. (5)
- Choose **Frontal (XY)** for **Projection Plane**. (6)
- Choose the **Start position**. Typically this will be the first slice in the Z-Stack.
- Choose the **Thickness**. Typically this will be the last slice in the Z-Stack. (7)
- Click **Apply**.

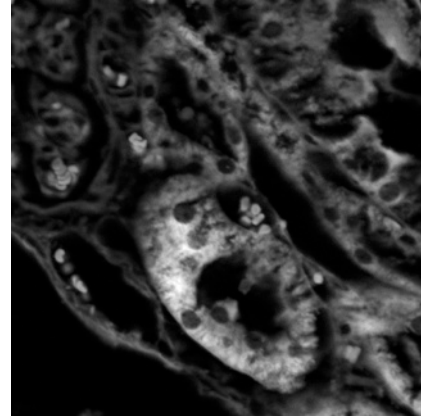
Optical Sectioning with ApoTome

ApoTome 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).



GFP



GFP with ApoTome

Calibrating ApoTome

ApoTome is already calibrated by the administrator - check the correct grid is inserted into the slider (below) then push the slider all the way into the microscope - ApoTome processing will occur when you take images.

Insert the correct grid into the ApoTome slider

- 5x, 10x, and 20x objective = **L-grid**
- 40x, 63x and 100x = **H-grid**

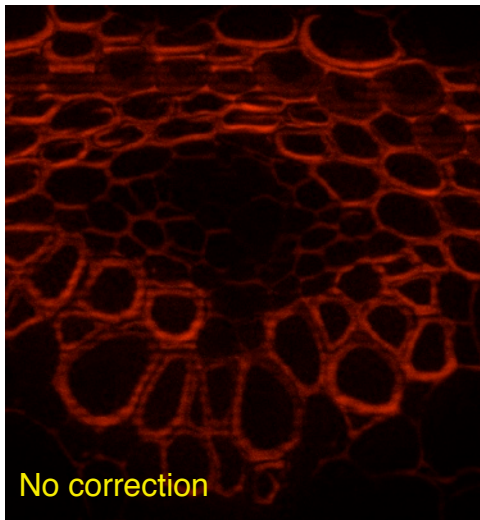
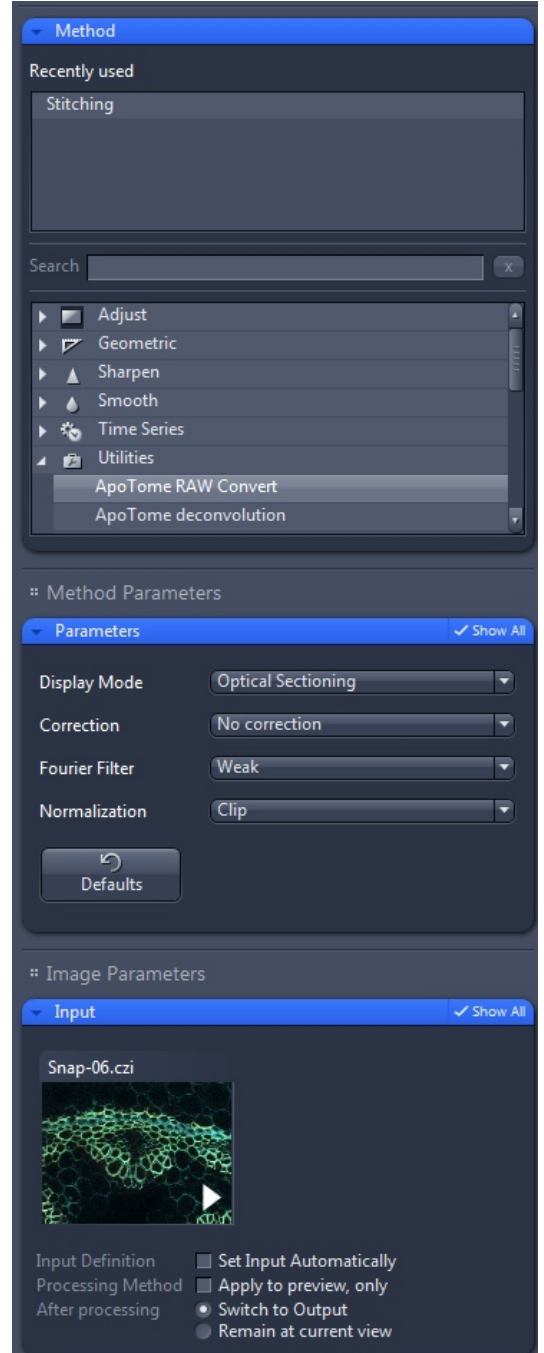
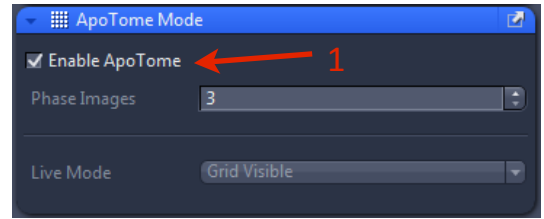
1. Unlock and remove the ApoTome slider from the side of the microscope.
2. Use the tweezers to gently remove and insert the appropriate grid.
 - When inserting, match up the white dot on the grid with the one on the slider.
 - The grid is held in magnetically so if its not sitting level move it around slightly with the tweezers until it clicks into place.
 - Once in place check it is secure by lifting the slider and *gently tapping* it over the palm of your hand.
3. Return the ApoTome slider back into the microscope

Important Settings for ApoTome

ApoTome optical sectioning will occur whenever the ApoTome slider is pushed all the way into the microscope. To return to normal imaging, pull the slider back out to its original position and remember to untick the “Enable ApoTome” check box.

Click on the **ApoTome Mode** tool:

1. Ensure the correct grid for the objective being used and tick the **Enable ApoTome** checkbox. Choose the number of required **Phase Images** (the default is 3).
2. Use the **Grid Visible** option under **Live Mode** – this will ensure the fastest refresh rate for the camera in live mode
3. After data acquisition (single or multiple Z-planes) Zen presents a preview of the optical section. This preview is a raw unprocessed image and needs to be converted to an ApoTome image before saving. To do this go to the **Processing** tab and under the **Method** tool choose **Utilities** and **ApoTome RAW Convert**.
4. The raw image can also be post-processed to remove noise (averaging) and any grid artifacts (ApoTome filter, **weak** is recommended). These settings are accessed under **Method Parameters** in the **Parameters** tool. Choose **Optical Sectioning** for the **Display Mode**. Use the **Fourier Filter** to remove grid artifacts and choose **Clip** for **Normalization**.
5. Choose **Local Bleaching Correction** for **Correction** if grid artifacts are still a problem.





Tiles Experiment

Before starting check:

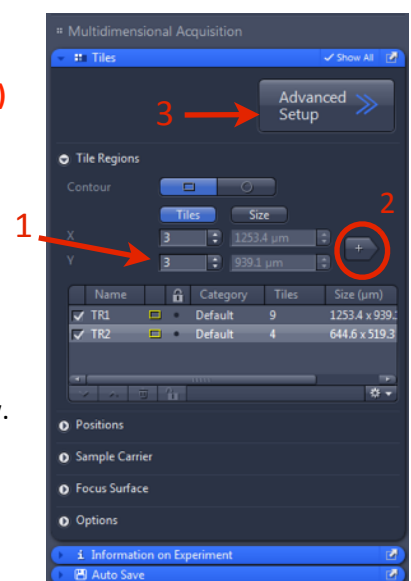
- Under **Options** in the tiles window:
 - ensure that **optimize stage travel** is **off** (if checked on, each region may be imaged and saved in a different order to how you have selected them on the slide)
 - ensure that **split scenes into separate files** is **on**
- For fluorescence mosaics - under the **acquisition mode** window, in **model specific** options make sure the camera is **flipped horizontally**
- If using MosaiX for brightfield imaging you may need to put **Shading Correction** on (under camera settings). Ensure the box is ticked below the **shading correction button**
 - To set up shading corrections go to a blank area of the slide and click the **shading correction button**.
- If imaging multiple tiled regions - enable **Local Focus Surface** and **fixed Z position** in the focus parameters

Setting up a Tiles acquisition


There are two ways to perform a **Tiles** acquisition. The simplest way:

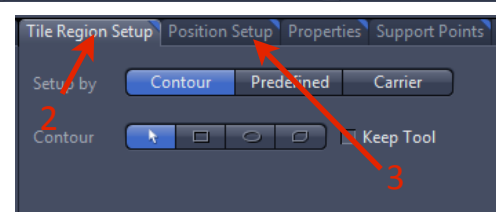
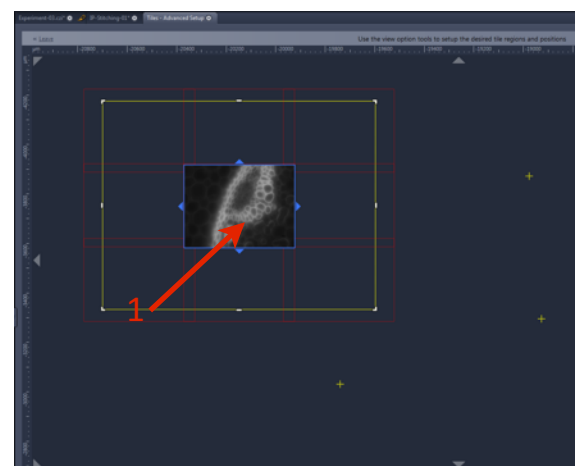
1. Choose rectangle contour  and define the number of X/Y Tiles. **(1)**
2. Click the  button to add this region to the list. **(2)**
3. Set the overlap value at **10%** (too small an overlap will create problems with stitching) in **Options**.
4. Click **Start Experiment** to capture the tiled image. This will use the current stage position as the center of the region and record the specified number of rows and columns.

The second method is to use the Advanced Setup option **(3)** as described below.



Tiles acquisition: Advanced Setup

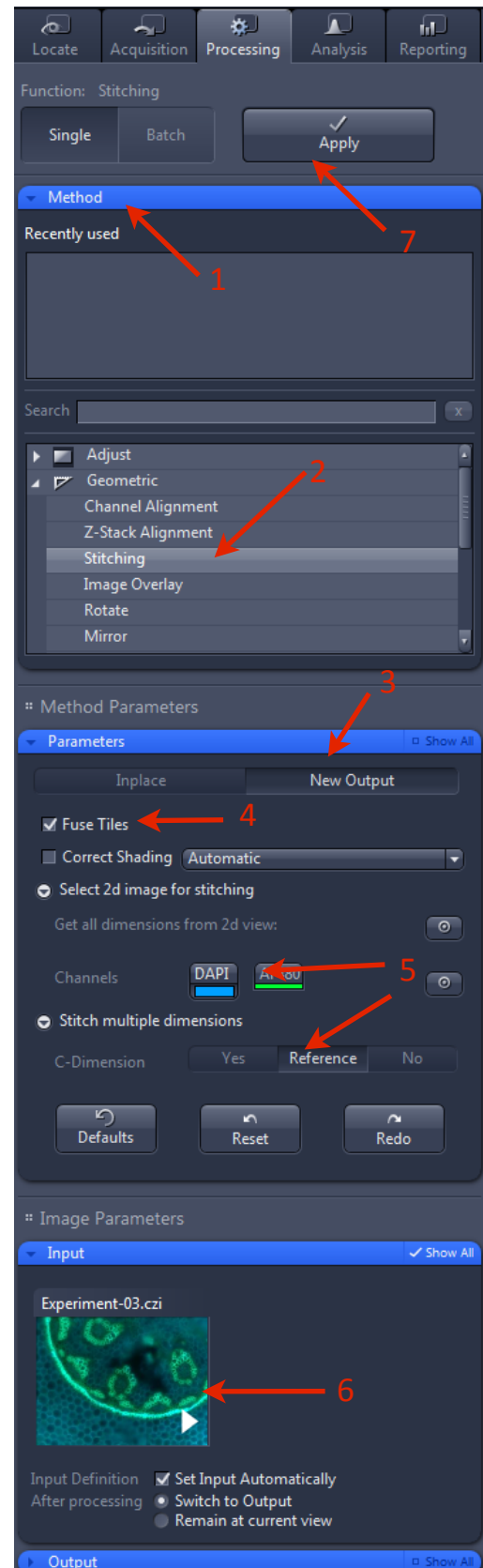
1. Click **Advanced Setup** to access an overview of the selected tiles X/Y positions combined with a live view from the camera. **(1)**
2. Click the **Tile Regions Setup** tab, choose a contour shape and define a region of interest. This region of interest is added to the list of all regions in the **Tiles** tool. **(2)**
3. Multiple regions can be defined and are not confined to rectangle shaped regions.
4. The **Positions Setup** tab can be used to define markers for fields of view at which to record images. With the mouse cursor in the **Advanced Setup** window, click the left mouse button to add to the list of **Positions** in the **Tiles** tool. The selected positions are indicated with a . **(3)**



Tiles: Stitching and Fusing

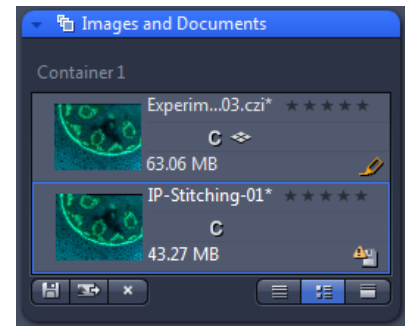
After acquisition, to get the final image, you need to stitch the tiles together and fuse to a single image:

1. Under the **Processing** tab select the **Method** tool (1) and highlight **Stitching** under **Geometric**. (2)
2. Under **Method Parameters** open the **Parameters** tool and choose **New Output**. (3)
3. Choose **Fuse Tiles** (4) to make a single image and, if necessary **Correct Shading** (note that this option will require a shading correction image to be recorded).
4. Choose the reference image for stitching (if multiple channels were recorded) under **Select 2d image for stitching**. (5)
5. Choose **Reference** under **Stitch multiple dimensions** if multiple images were recorded.
6. Under **Image Parameters** open the **Input** tool and make sure the correct input image is selected (6). Either choose the required dataset by clicking in the preview image or choose the correct tab from the image window.
7. Choose **Set Input Automatically** for **Input Definition** and **Switch to Output** for **After Processing** (these are the default settings).
8. Click **Apply** to create a stitched, fused (and shading corrected) image. (7)



Saving Images

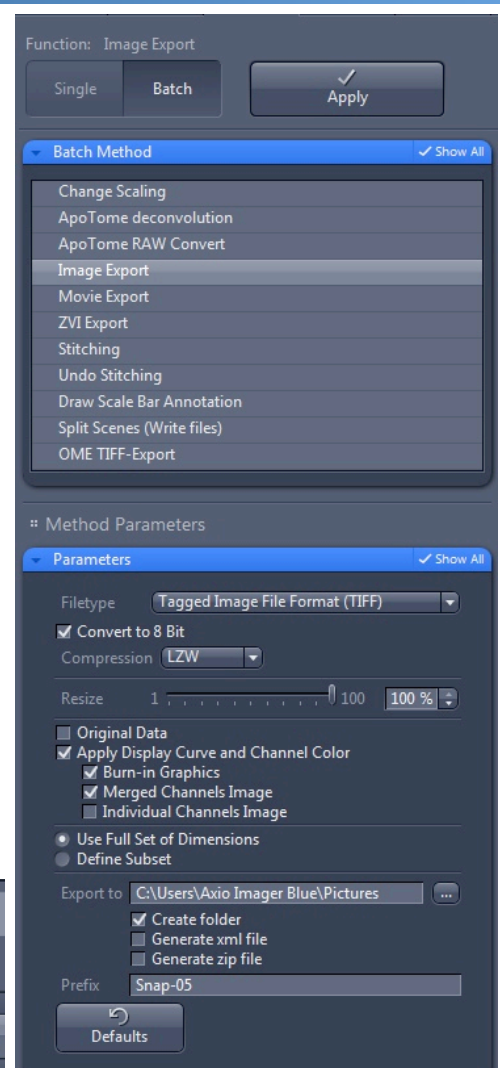
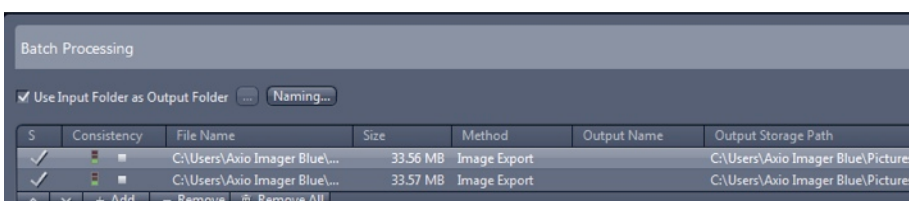
- All documents created in a session are listed in the **Images and Documents** tool on the right-hand side of the workspace. Unsaved documents are marked with a *. This list also gives an estimation of the size on disk of each dataset.
- To save an Image you can click the **save** button in the **Images and Documents** tool or on the top tool bar. (File --> Save can also be used from the menu.)
 - This will save the image as a *.CZI file, which can be opened by Zen, FIJI (ImageJ) or Imaris.
 - It is best to keep the CZI file as your original data but 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 below.
- You can save your images to the desktop - but at the end of each session, either:
 - Move your files to your group share or personal network share.
 - Move your files to a USB stick / portable harddrive
- You can set up automatic prefixes and suffixes for your naming your images under **Tools>Options>Naming**
 - Chose the category you wish to change the settings for as there are separate specifications for each category (for example single acquisition versus multidimensional acquisition).
- You can also set the software to automatically save every image you capture in **Tools>Options>Storage**



Exporting *.CZI files as TIFs

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

1. Choose the **Processing** tab and click the **Batch** button (single images in the current workspace can be exported by choosing **Single**).
2. In the **Batch Method** tool choose **Image Export**.
3. Under **Method Parameters** open the **Parameters** tool. Choose the output file type to be TIFF. You can then choose to convert the image to 8-bit and choose the compression type (the default is LZW). 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.
4. Choose the destination directory for the exported images and note the **Create Folder** option. Check this option if you want every new image to have a new folder. This is useful for multiple channels or Z-Stacks.
5. The workspace allows definition of which images to be exported to TIFF. Choose input and output folders (these can be the same by ticking **Use Input Folder as Output Folder**).
6. Click **Apply** to convert the batch of images.



QBI Fluorescent Marker Guide

Filter Sets to be used with
HXP or **HBO** light source

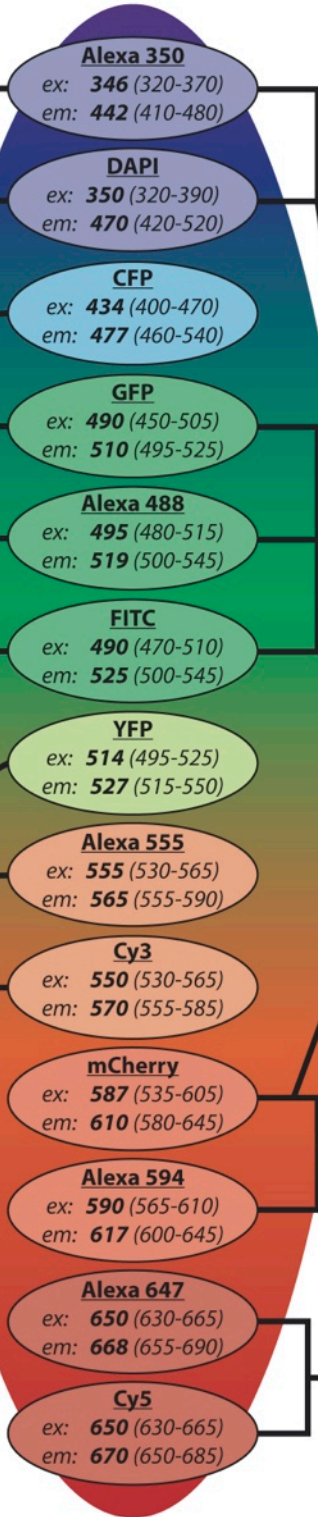
FS#49 : DAPI
Excitation: 300 - 400
Emission: 420 - 470

FS#47 : CFP
Excitation: 426 - 446
Emission: 460 - 500

FS#38 : GFP
Excitation: 450 - 490
Emission: 500 - 550
FS#44 : FITC
Excitation: 455 - 495
Emission: 505 - 555

FS#46 : YFP
Excitation: 490 - 510
Emission: 520 - 550

FS#43 : Cy3
Excitation: 532 - 557
Emission: 570 - 640



Filter Sets to be used
with the **Colibri LEDs**

FS#62 : B/G/HR
for Colibri LEDs
Excitation: 350 - 390
Emission: 400 - 450
(use **LED 365**)
Excitation: 460 - 488
Emission: 500 - 560
(use **LED 470**)
Excitation: 570 - 600
Emission: 615+
(use **LED 590**)

FS#50 : Cy5
Excitation: 625 - 655
Emission: 665 - 715
(use **LED 625**)