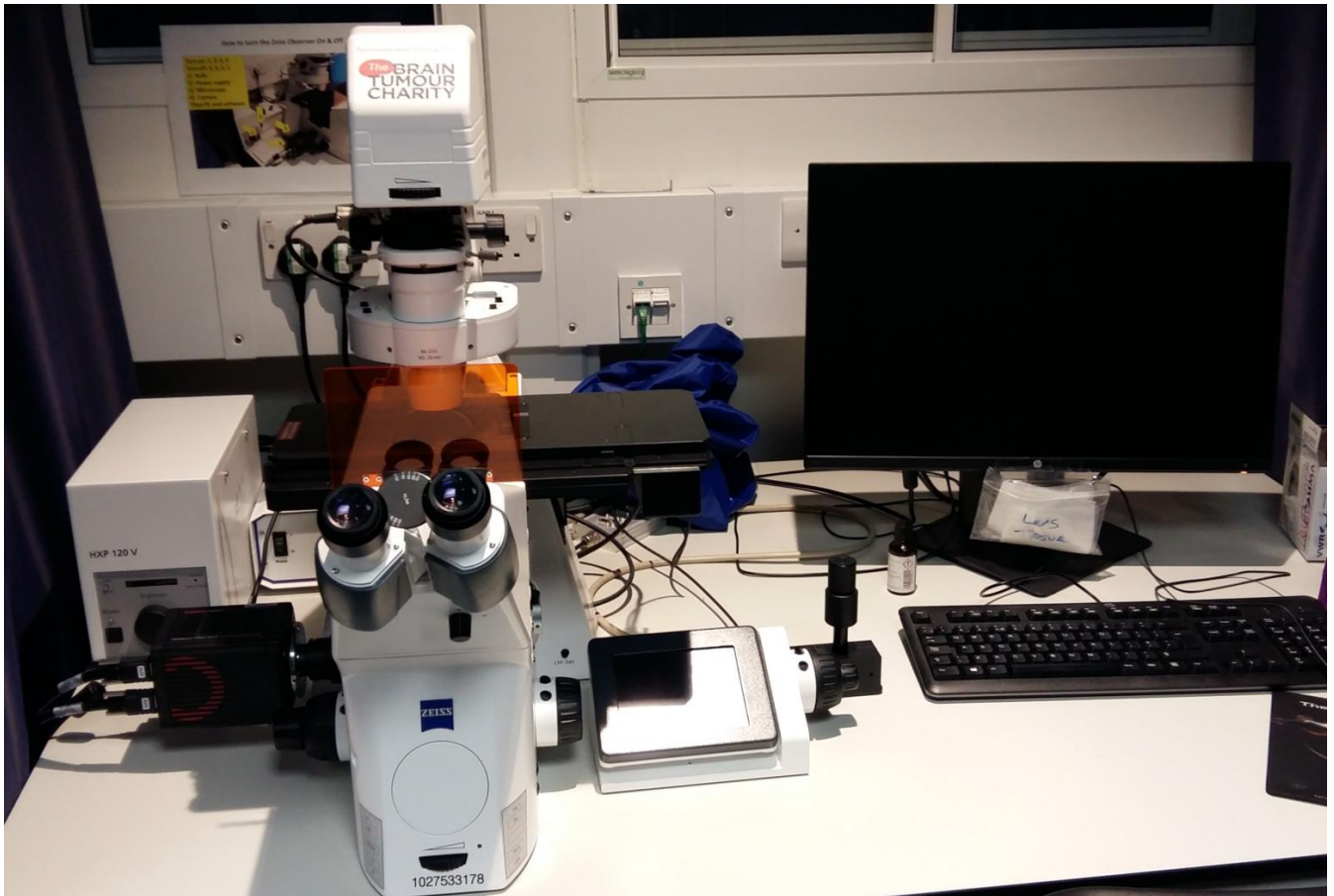


Zeiss Observer 4 Colour Fluorescence Hamamatsu Flash 4.0v3 camera

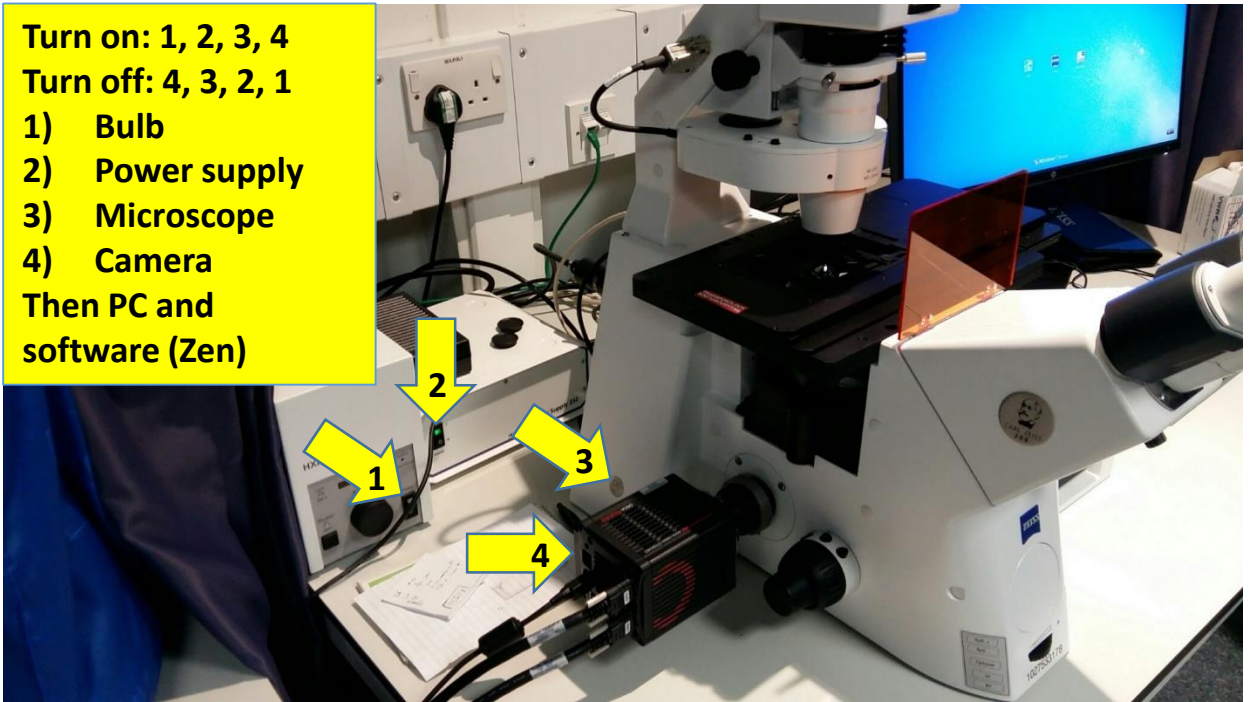
Instructions for Brightfield (black and white camera) Fluorescence (4 colours), Z stacks and Tile scans.

Other functions available, such as scanning multiwell TC plates, adjusting focus offset for different channels, time series, high speed imaging (100-1000+ fps), deconvolution.
Ask Dale for details.

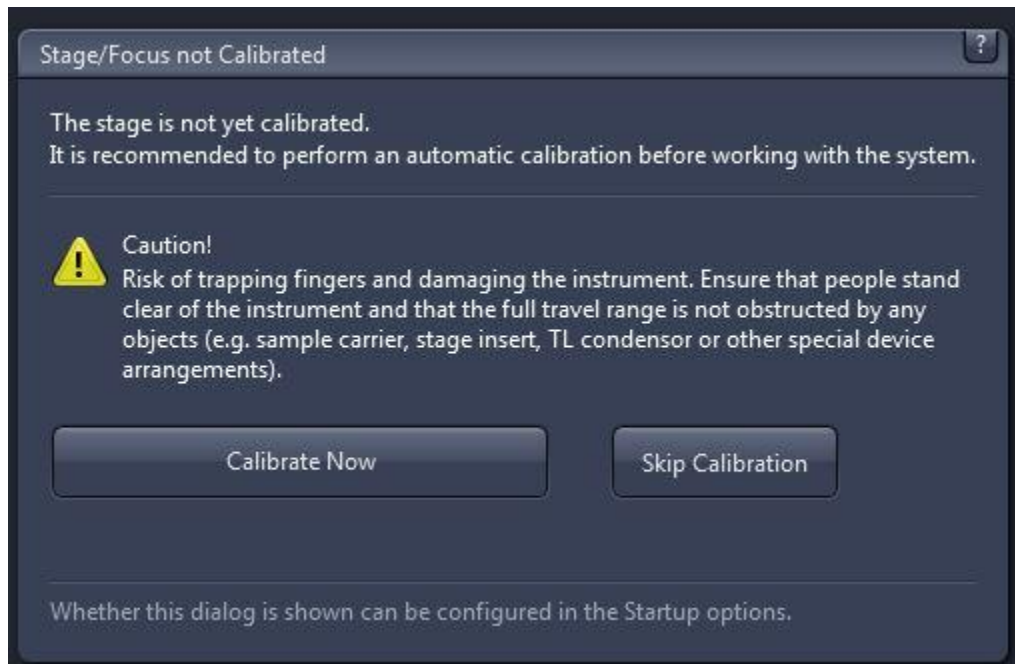


Turn on the system:

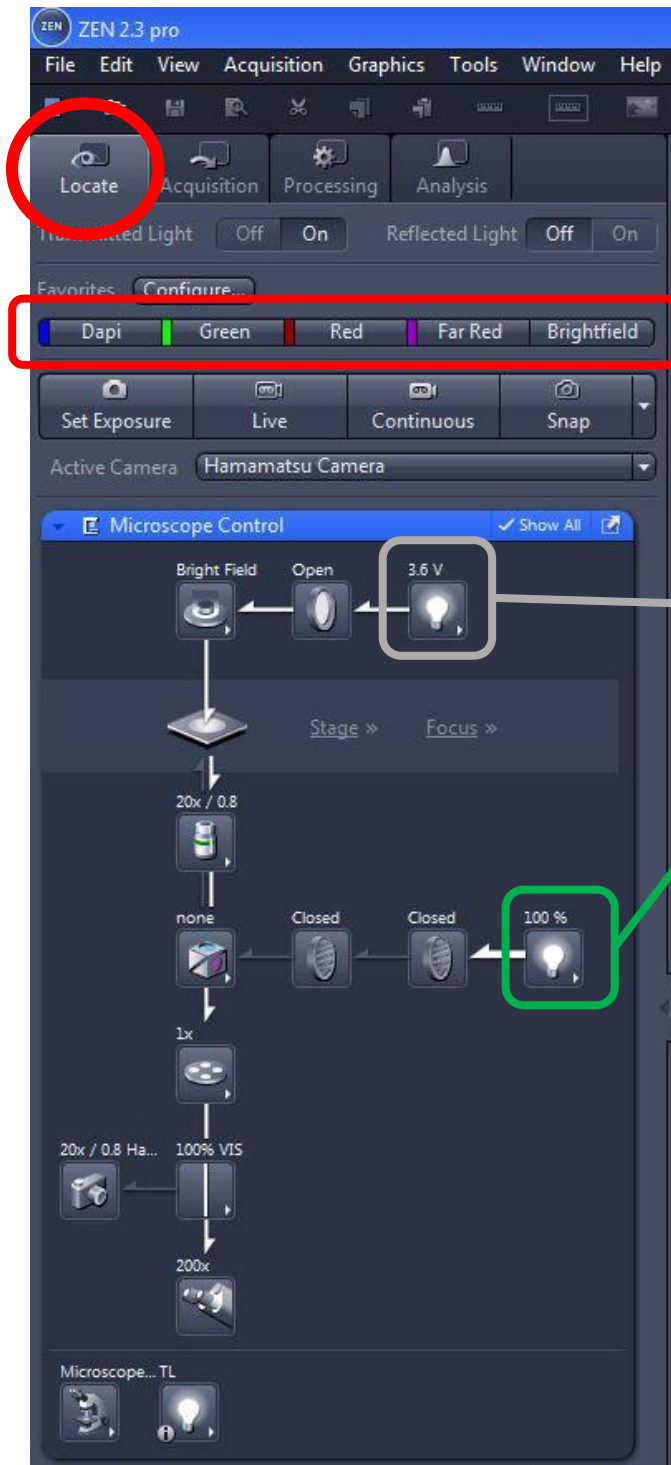
Turn on: 1, 2, 3, 4
Turn off: 4, 3, 2, 1
1) Bulb
2) Power supply
3) Microscope
4) Camera
Then PC and software (Zen)



After you start the Zen software, if you are the first user it will ask to calibrate the stage...
Press 'Calibrate Now'



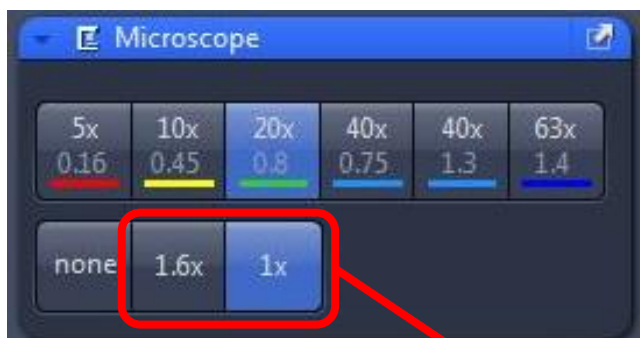
Use the Locate Tab for looking down the eyepieces...



Choose the **colour** to see:
Dapi, Green, Red, Far Red
or Brightfield.

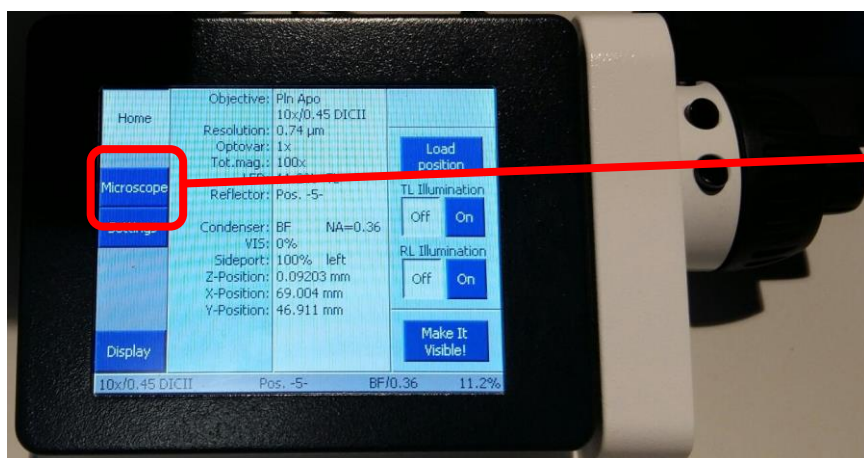
You can adjust bulb power
Brightfield
or
Fluorescence

Set magnification from the software or the touchscreen next to the scope



5x, 10x 20x & 40x dry objectives. The last two (40x 1.3 & 63x 1.4) are oil objectives.

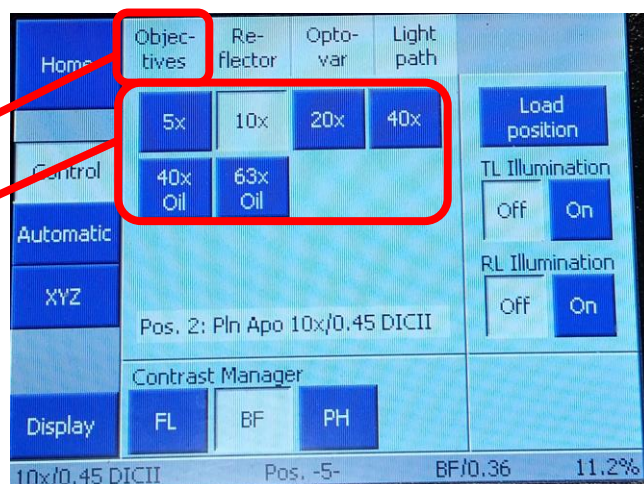
Additional magnification with the optovar.
Leave this on 1x, unless you plan to do deconvolution.



Touchscreen at start up.
Press:
Microscope

Go to Objectives tab

Choose objective



Acquisition tab for capturing images

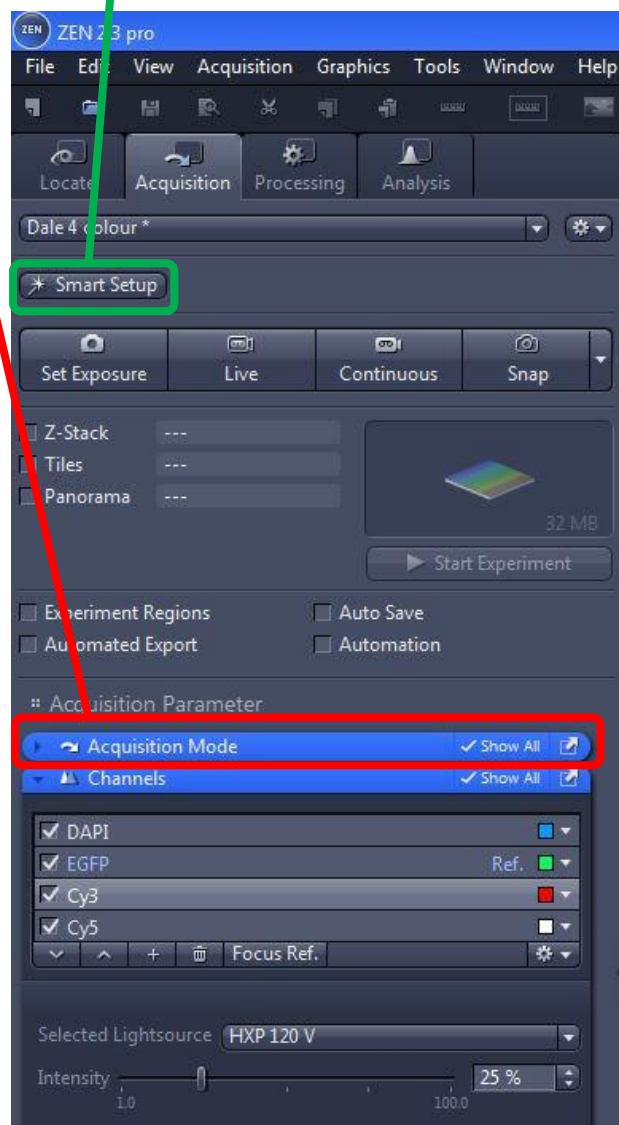
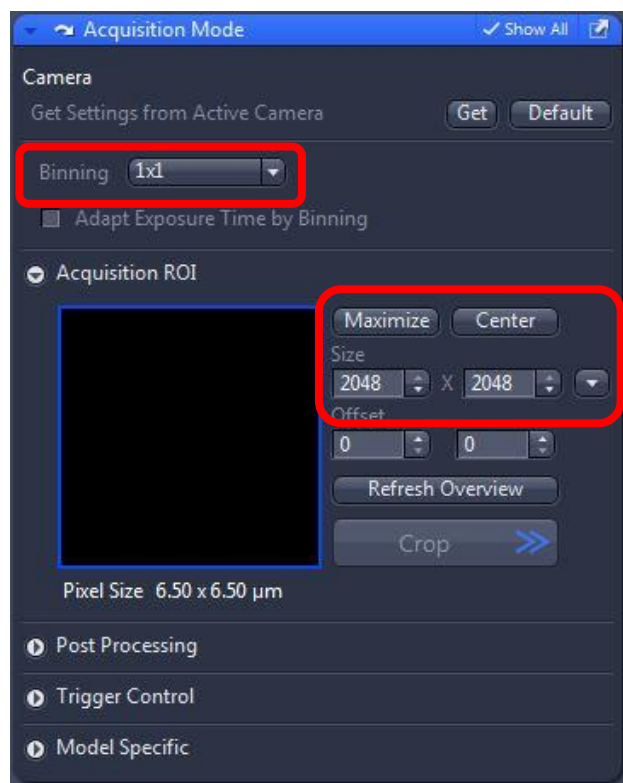
Check the **Acquisition mode**, open the control window and check **Binning** is 1x1, **Acquisition ROI** is 2048 x 2048, (Press Maximize).

You only need to check this at the start of your session, to be sure it hasn't be changed by the previous user.

Binning at 2x2 (or more) increases sensitivity and speed for fast live imaging.

Reducing the acquisition ROI can help with producing an evenly illuminated tiled image.

Set up the fluorescence channels (and Brightfield if used) by pressing **Smart Setup**.



Smart Setup – What colours are you imaging?

The screenshot shows the 'Smart Setup' window with the following configuration:

| Contrast | Probe |
|--------------|-----------------|
| Fluorescence | DAPI |
| Fluorescence | Alexa Fluor 488 |

Below the configuration table are buttons for 'Automatic', 'Speed', 'Signal', 'Default', and 'Current'. A red box highlights a '+' button to the left of the 'Automatic' button, with a callout: 'Press + to open the selection window.'

The 'Add Dye or Contrasting Method' dialog is open, showing a list of dyes and contrast methods. A red box highlights the 'Recently Used' list, which includes:

- Alexa Fluor 488 (517 nm)
- Alexa Fluor 568 (603 nm)
- Alexa Fluor 594 (618 nm)
- Alexa Fluor 647 (668 nm)
- DAPI (465 nm)
- TL Brightfield

A green box highlights the 'Add' button at the bottom of the dialog. A callout points to the 'Add' button: 'Select colours and Add each fluorophore in your experiment. Choose TL Brightfield if also needed.'

A yellow box highlights the 'Close' button at the bottom of the dialog. A callout points to the 'Close' button: 'Then press Close and OK.'

The 'OK' button at the bottom right of the dialog is also highlighted with a yellow box. A callout points to the 'OK' button: 'Then press Close and OK.'

The 'Dye Database' section lists various dyes with their excitation/emission wavelengths and color indicators. The 'Contrasting Methods' section lists various contrast methods, with 'TL Brightfield' highlighted by a blue box.

The 'Best Signal' section shows a bar chart comparing 'Emission Signal' and 'Speed' for different dyes. The 'Crosstalk' section shows two spectral plots, T1 and T2, with overlapping emission peaks.

Channels set up – Adjust bulb intensity and exposure time for each channel. You can also give each channel a specific name.



Channels. Highlight to adjust, or see it live, TICK to include in the captured image

Bulb power (Intensity). I typically use <50% for Dapi, > 50% for all other colours

You can modify the channel name

Exposure time. Longer = brighter

Typically the initial short (150 ms default) exposure time will be too dim. The image will look dark, and the histogram will be at the left edge (low values only). Increase bulb power and exposure time to make the image visible, without saturating any pixels...

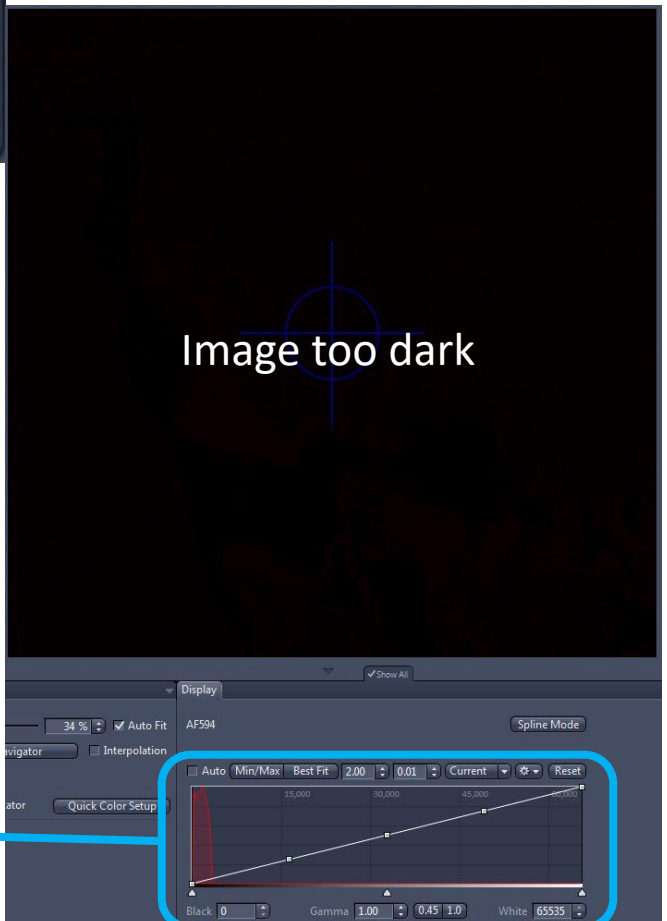
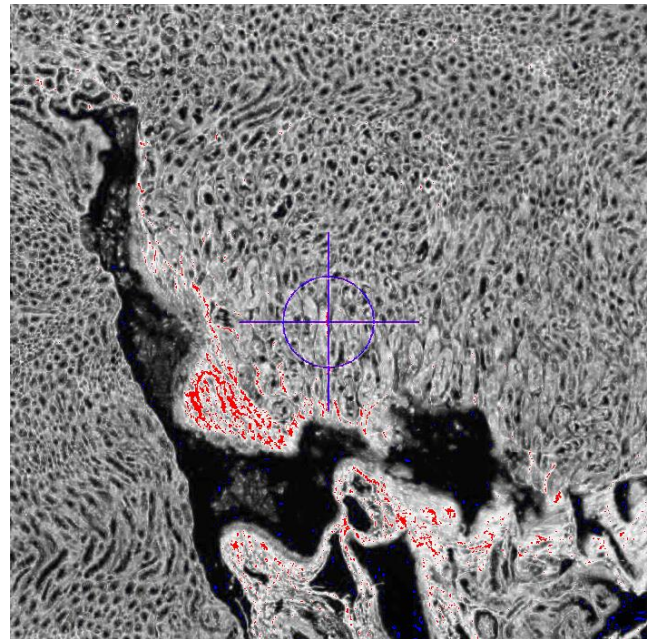
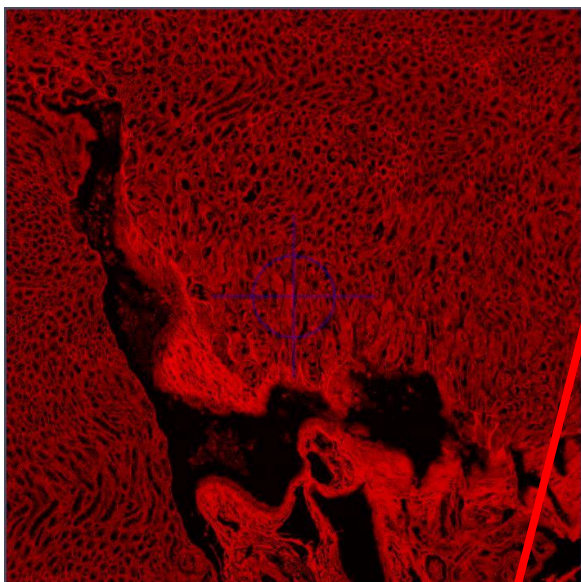
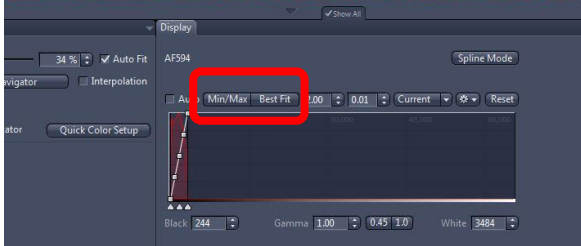


Image Histogram

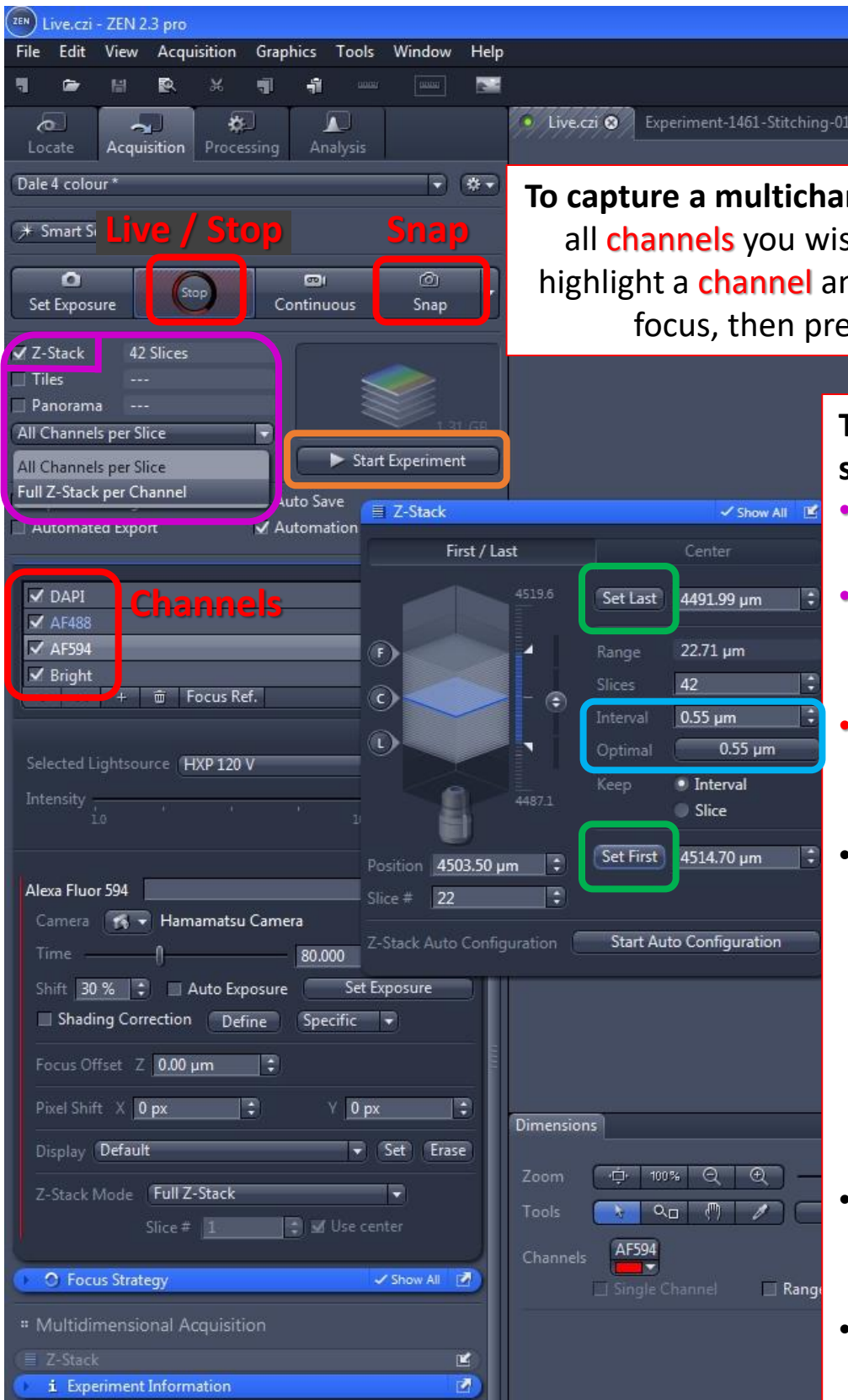
Channels set up – set exposures via Histogram and Range Indicator



Reset the Display histogram, then adjust the bulb power and exposure time to approximately half fill the histogram, and avoid any saturated pixels.



Capture an image, or a Z-stack



To capture a multichannel image: tick all **channels** you wish to capture, highlight a **channel** and press **Live** to focus, then press **Snap**.

- To capture a Z-stack:
- Tick the Z-stack option
 - Choose Full Z-stack per channel
 - Highlight a channel and press LIVE
 - Manually focus to the top and bottom of the stack to **Set First** and **Set Last** for the stack
 - Make sure **Interval = Optimal**
 - Press – **Start Experiment**

Tile scan

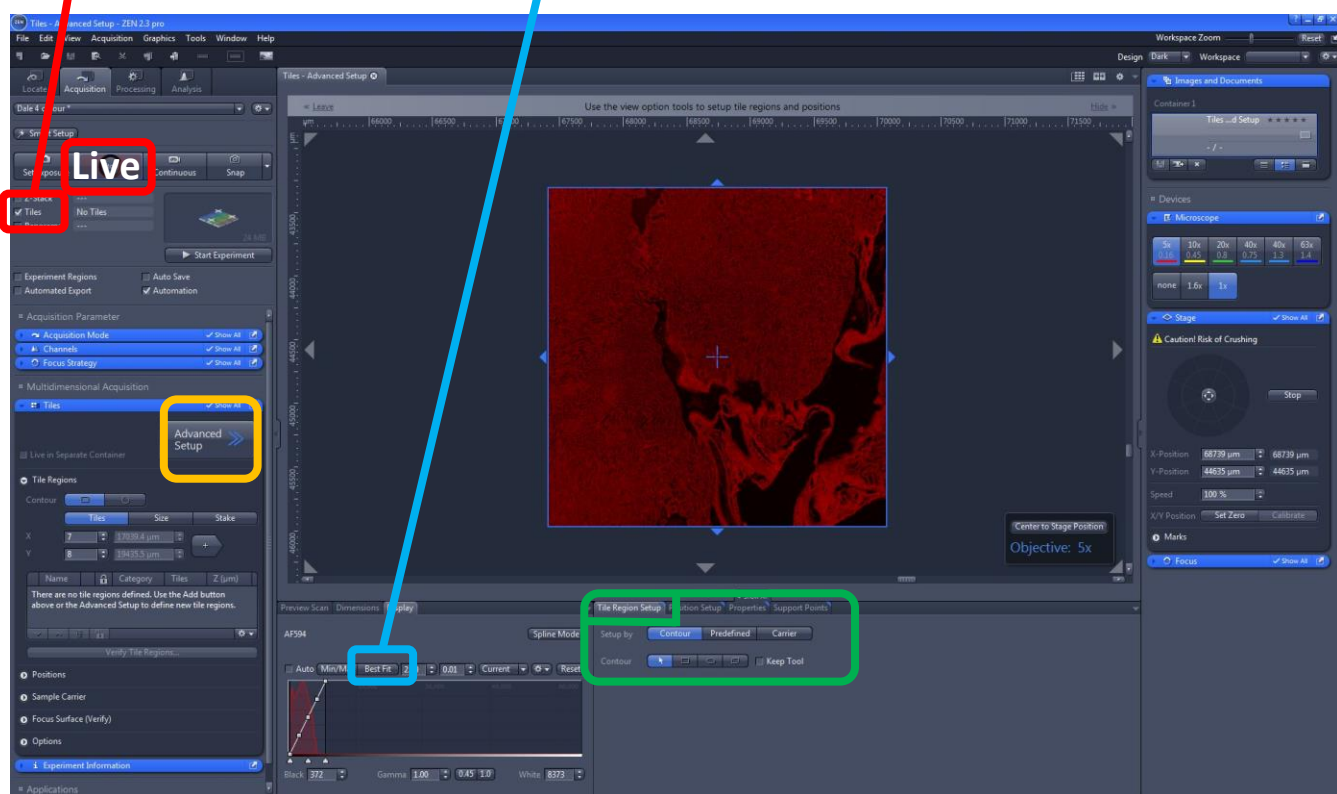
Using the Tiles option you can use a low mag objective (5x or 10x) to quickly scan the slide and draw a region that you would like to image with any objective. Once you've drawn your region (or regions) at low mag, switch to your imaging objective, and make a focus map using support points. Then select all the channels you'd like to image (and also do a z-stack if you wish), and start the experiment.

Select the **Tiles** option

Press **Advanced Set up**

Scan at a low mag

(**Best Fit** on histogram so it is bright enough)



Press **Live** and zoom out the view (wheel on mouse).

Use the tools in **Tile Region Set up** to draw your first guess at the ROI...

Continued next page...

Tile scan (2)

Zoomed out and drawn a region to scan.
Select a channel to scan and start the preview scan.
(Mouse wheel to zoom)

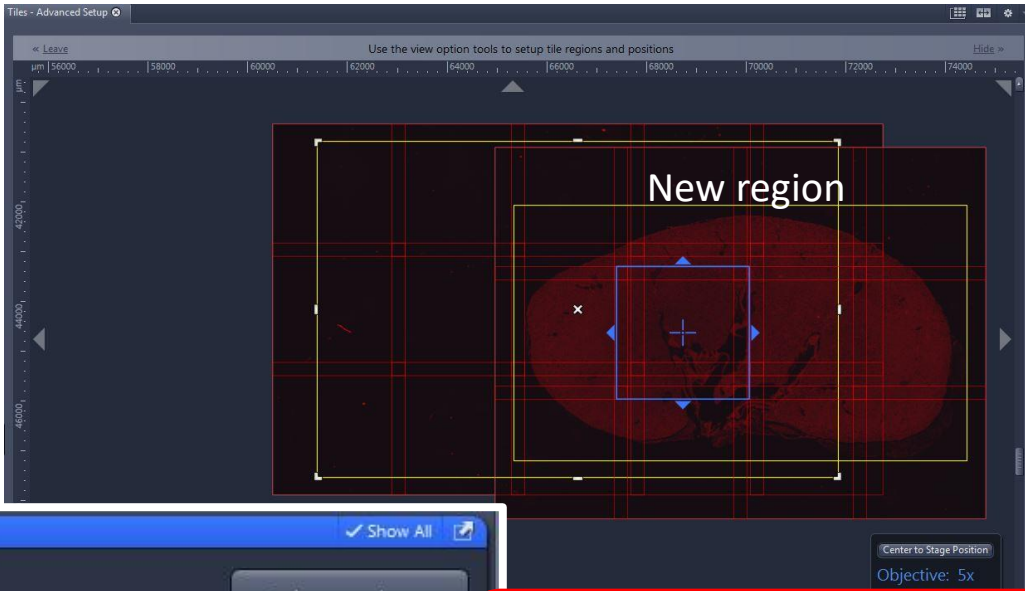
The screenshot shows the 'Tiles - Advanced Setup' window. The main view displays a grid of red lines over a dark field. A small red square is centered within the grid. A red arrow points from the text to the 'Preview Scan' panel at the bottom. The panel includes a 'Start Preview Scan' button, a 'Delete Existing Preview Images' checkbox, and a 'Channels' list with 'AF594' selected. The 'Objective' is set to '5x Air 0.16'. The 'Camera' section has 'Use Binning from Experiment' checked.

First scan not quite right.
Draw a new region and try again...

Press Best Fit if it is too dark to see.

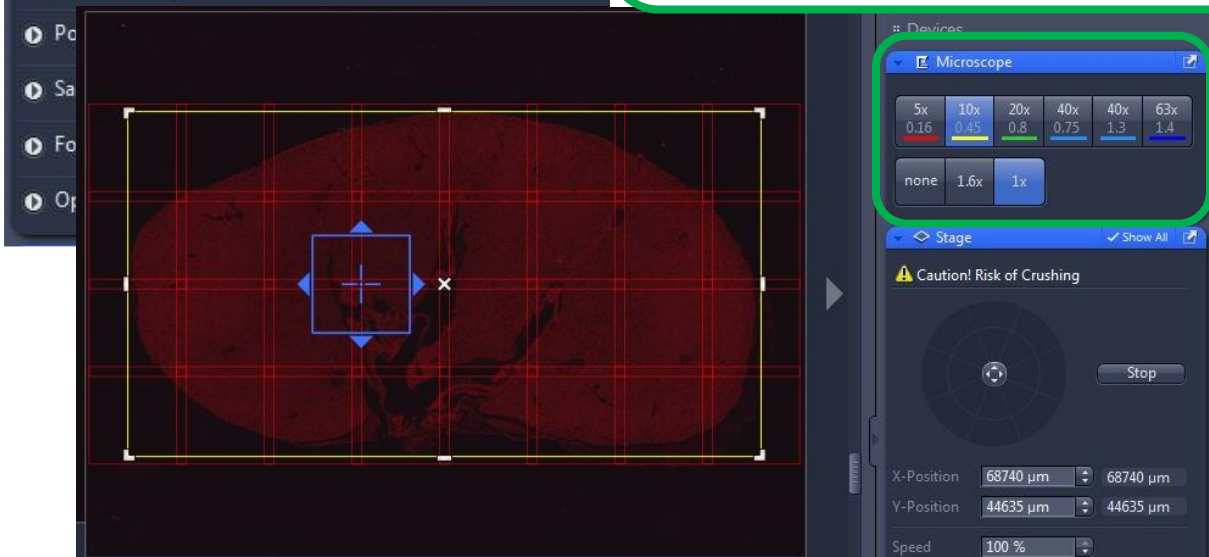
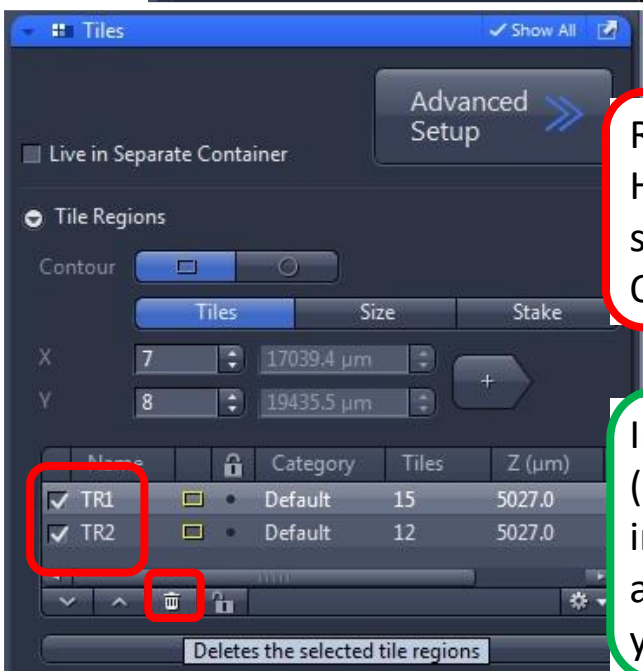
The screenshot shows the 'Tiles - Advanced Setup' window. The main view displays a grid of red lines over a dark field. A larger red square is centered within the grid. The 'Preview Scan' panel at the bottom is highlighted with a red box. The 'Best Fit' button is highlighted with a red arrow. The 'Channels' list shows 'AF594' selected. The 'Gamma' is set to '1.00' and 'White' is set to '11756'.

Tile scan (3)



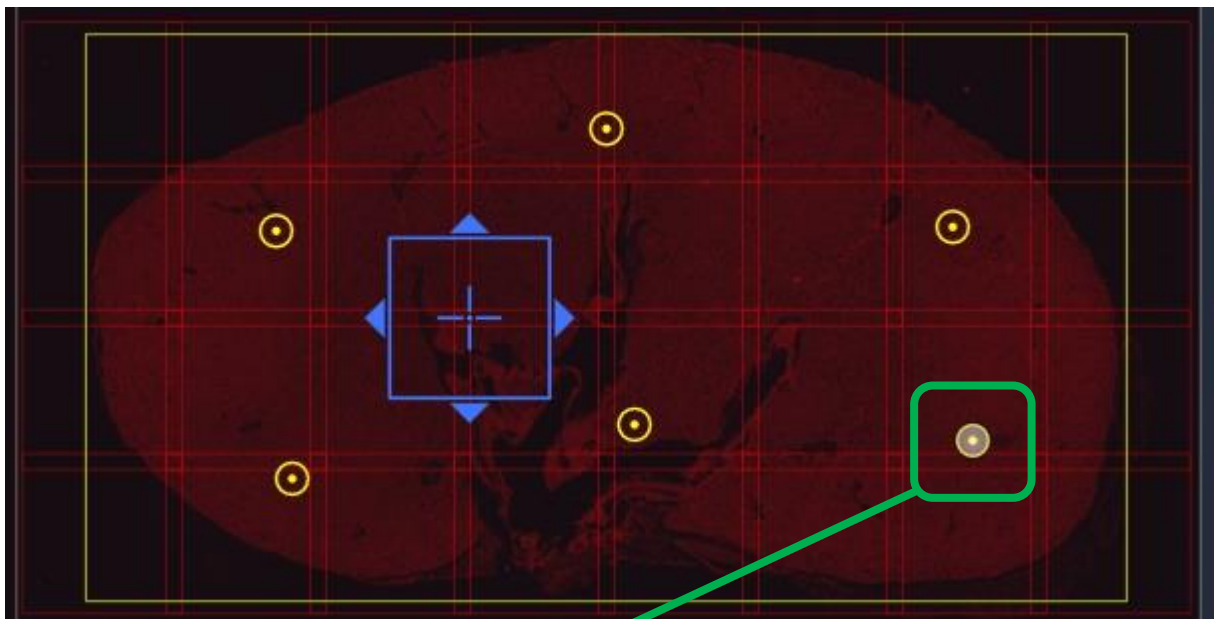
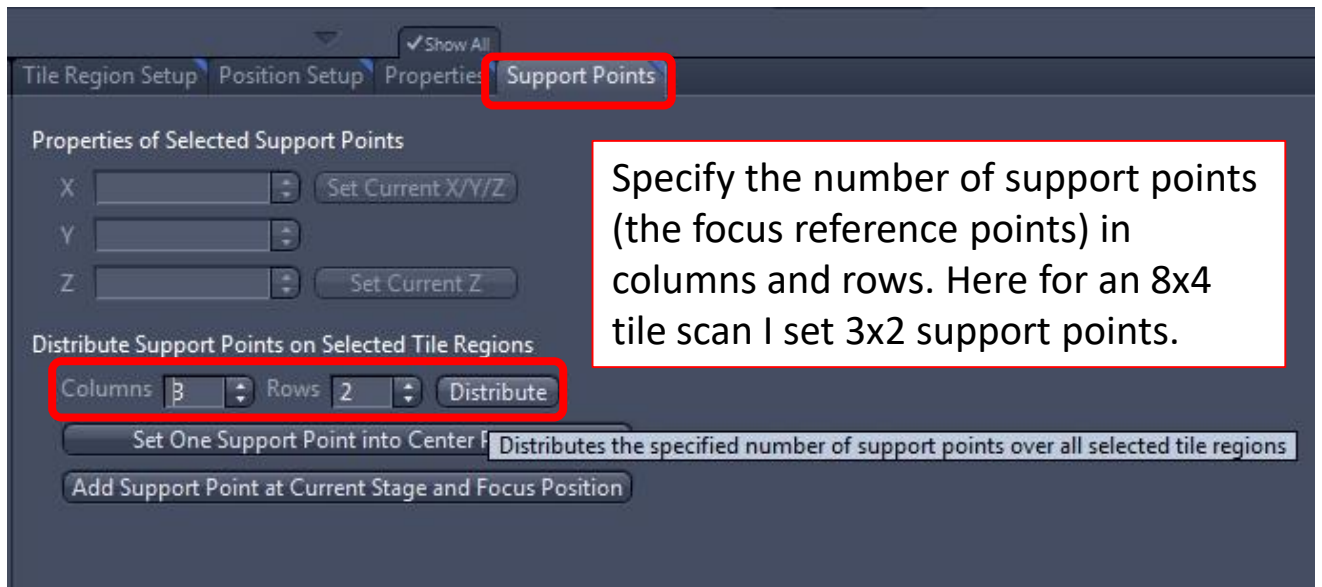
Regions are called TR1, 2 etc. Highlight the ones you don't want to scan and press the trash to delete them. Only Ticked regions are scanned.

Increase magnification if needed (here to 10x). The number of tiles is increased. You can drag the edges of a region to just cover your sample. So you take as few tiles as possible.



Tile scan (4)...make a focus map – set support points

First add support points, choose the Support Points tab below your image



The points are initially aligned as a grid, you can select each point with the mouse and drag them anywhere you like

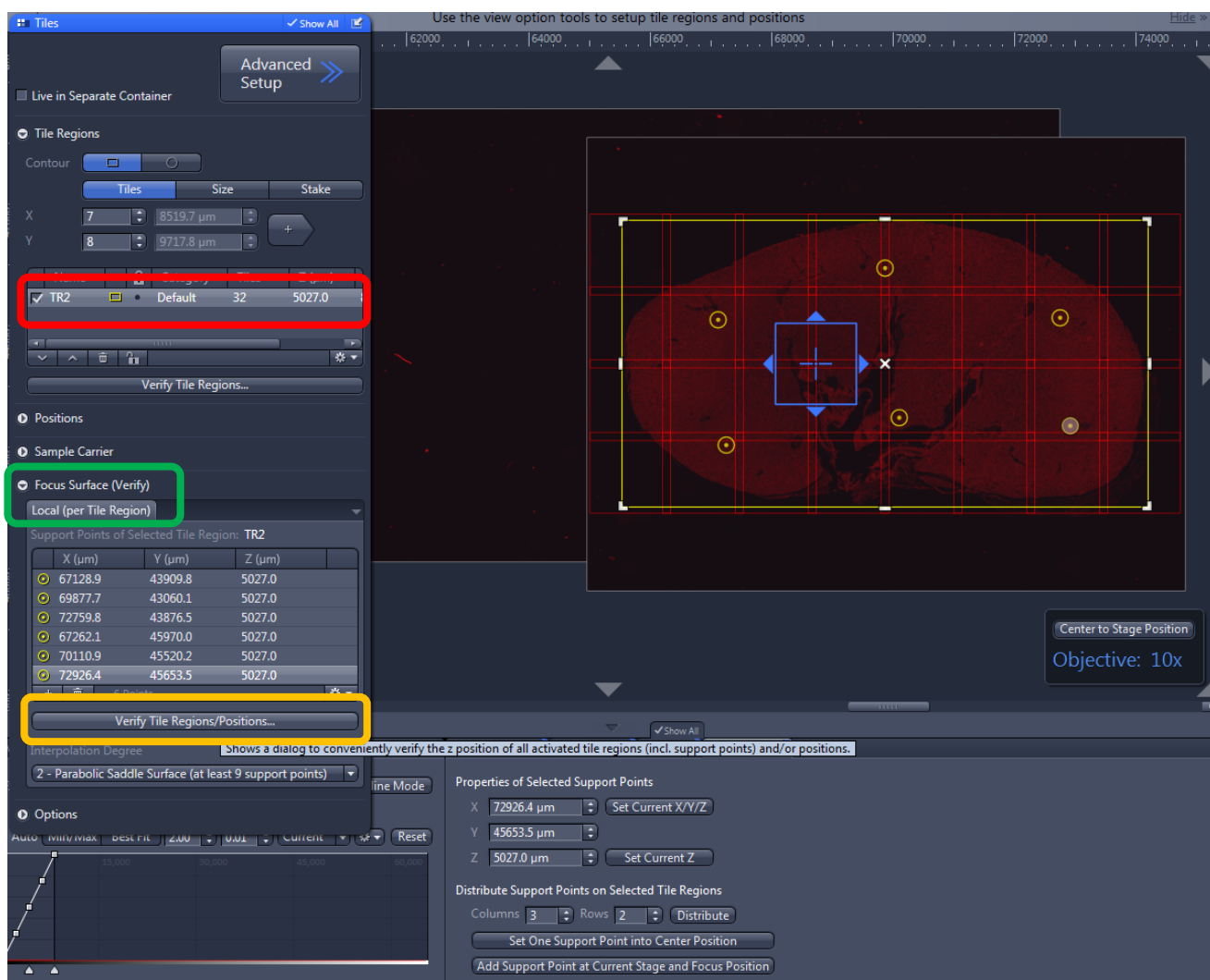
Tile scan (5)...make a focus map – verify support points

In the Tiles Advanced Setup window:

Make sure your **Tile region (TR)** is ticked.

Select: **Focus Surface (Verify)**

Press **Verify Tile Regions/Positions...**



Tile scan (6)...make a focus map – adjust focus

A new window opens: Verify Tile Regions / positions...

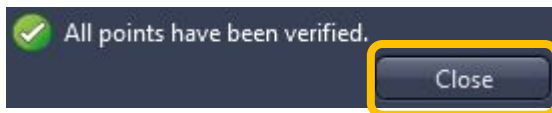
Click **Move to Current Point**

Press Live to see the desired channel, adjust the focus, then

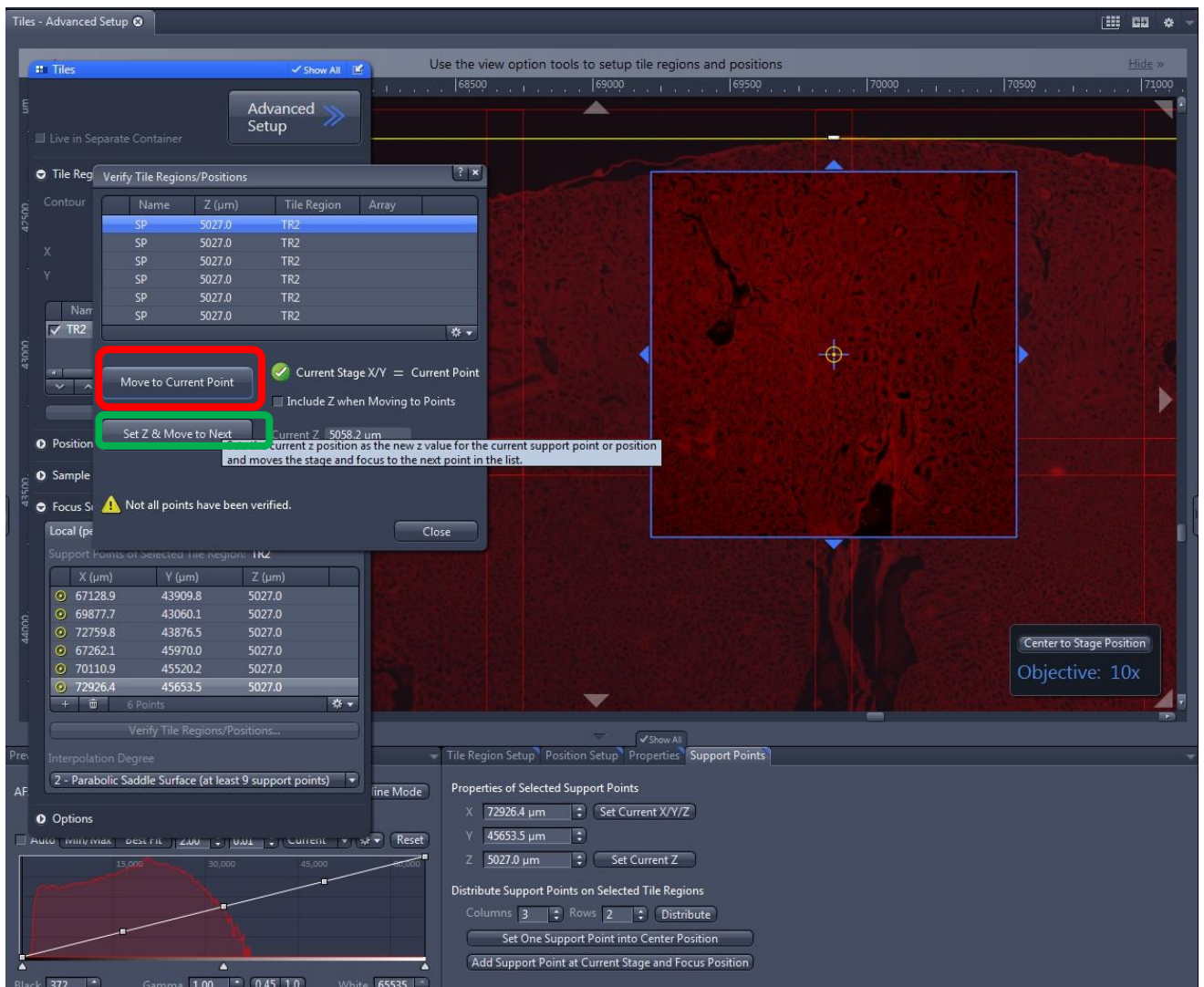
Press: **Set Z & Move to Next** It moves to the next support point.

Keep doing this and setting the focus for each point,

until the window changes to:



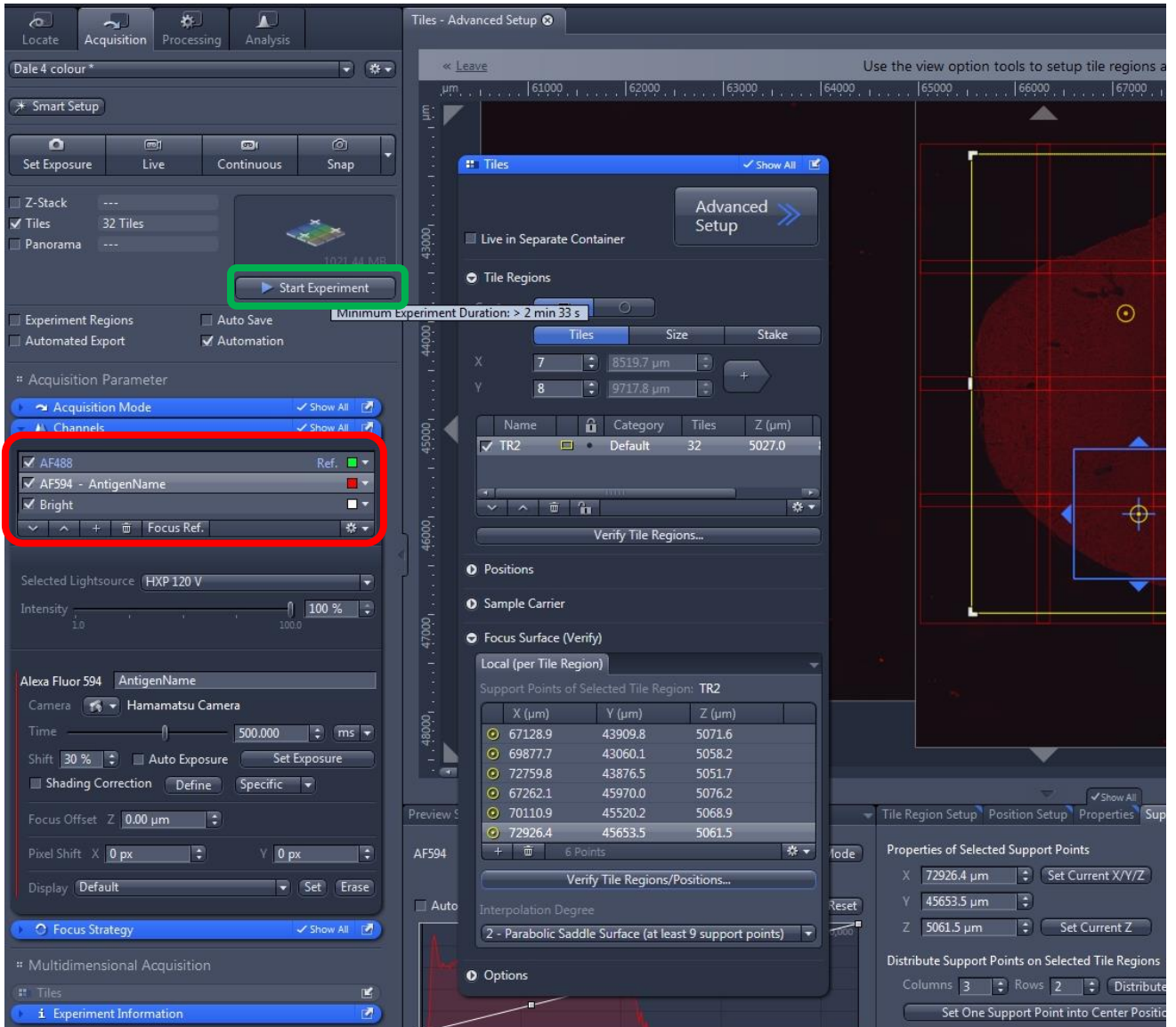
Finally **close** the window.



Tile scan (7)...Capture the image

Make sure your **Channels** are ticked.

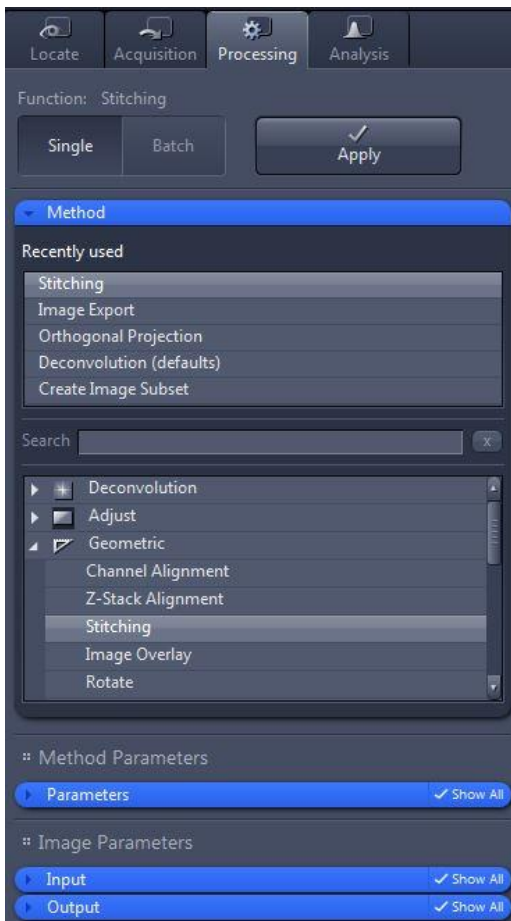
Press: **Start Experiment**



Tile scan (8)...Stitching the image

Go to the processing tab,
choose the method:

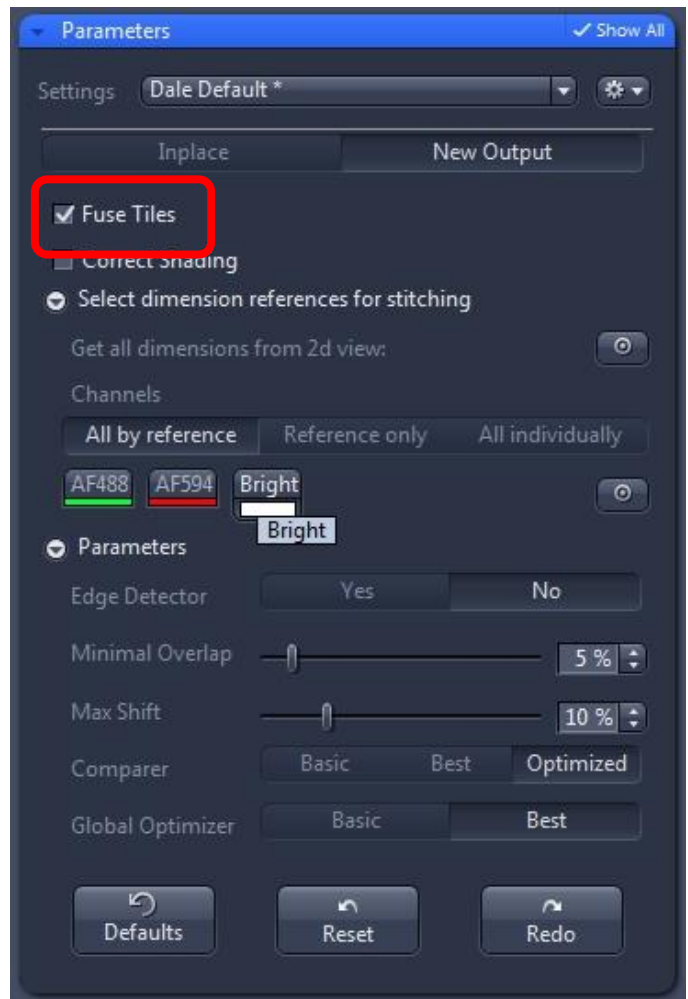
Stitching



Open up the Parameters window,
select the Settings “Dale Default”.

This sets the output to have a single
fused image, in a New Output

window.

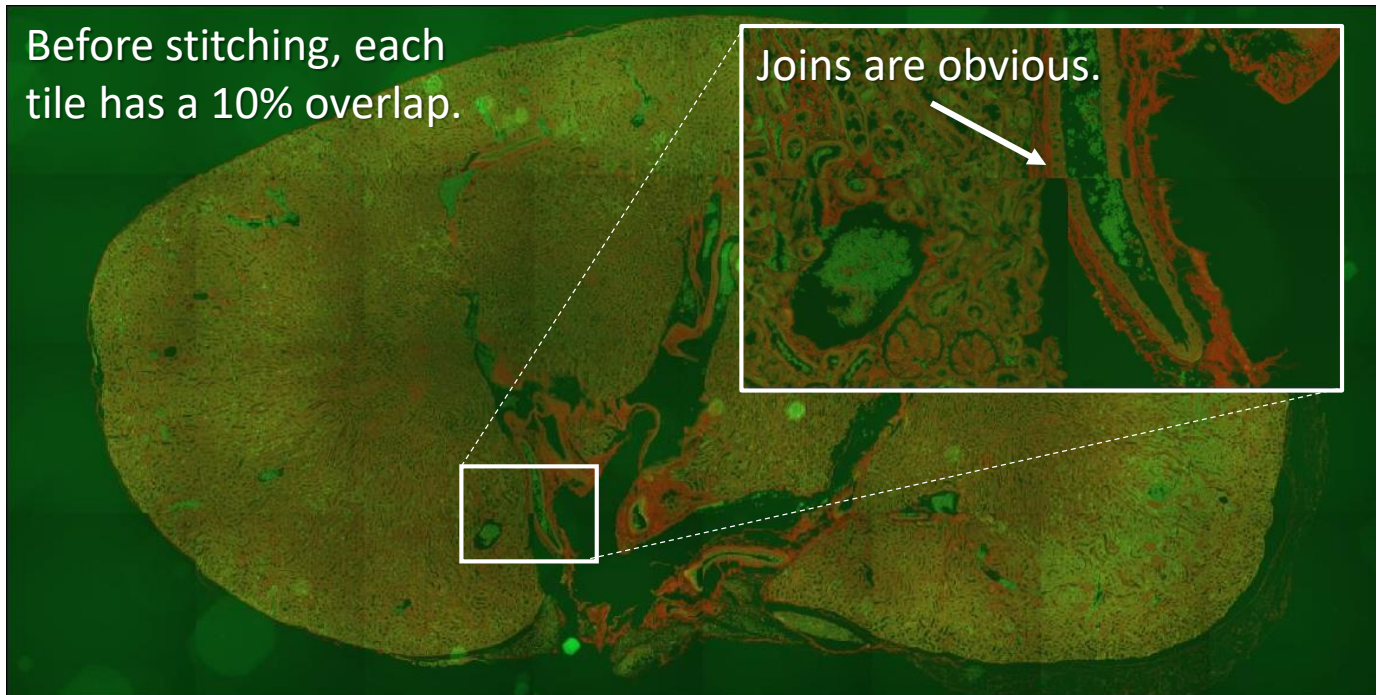


By default it will use the first channel captured to align the image,
(normally Dapi) but you can choose any channel that has sufficient
content to perform the stitching.

Tile scan (9)...Stitched Image

Before stitching, each tile has a 10% overlap.

Joins are obvious.



After stitching, overlaps are still visible due to vignetting. This can be reduced by capturing each tile at less than 2048x2048 pixels.

Joins gone.

