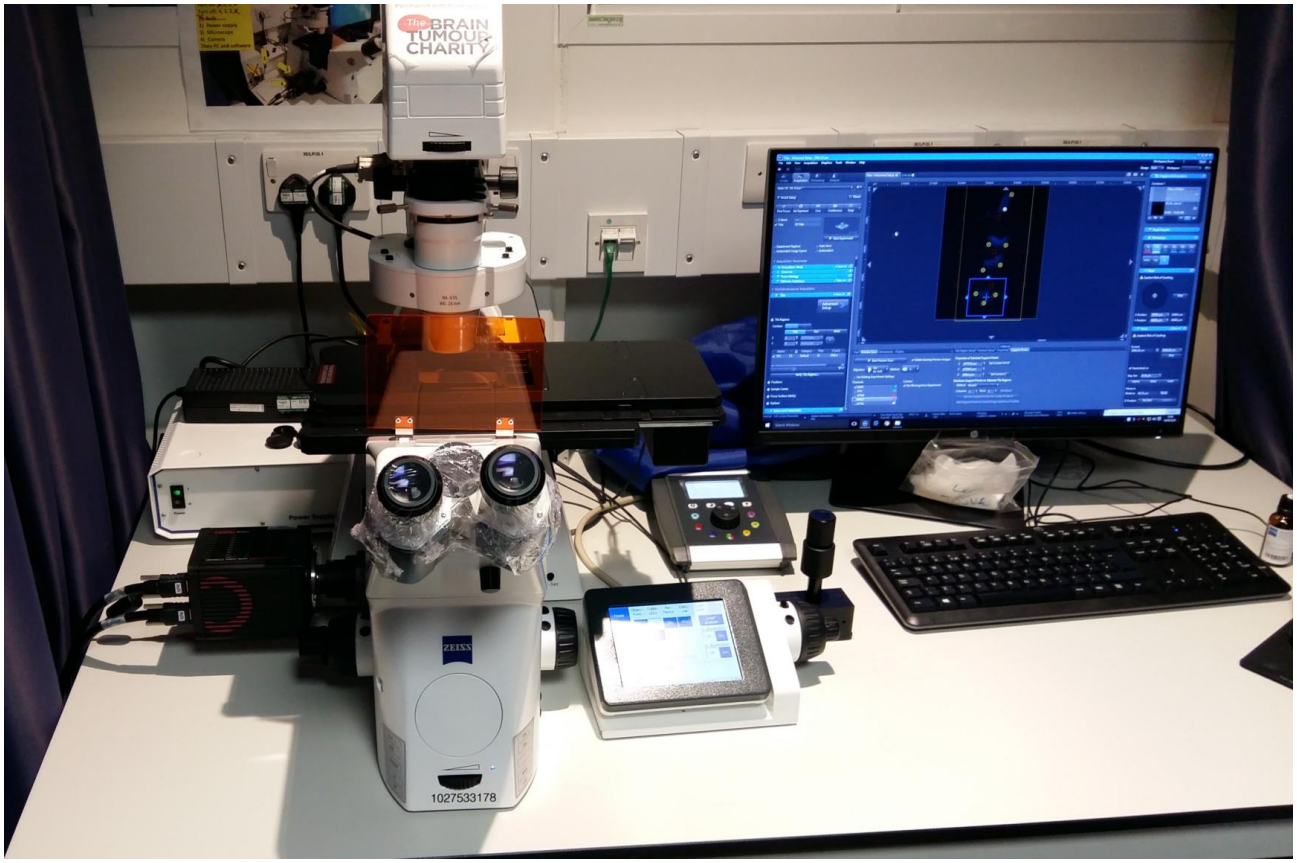
Zeiss Observer 7 Colour Fluorescence
Hamamatsu Flash 4.0v3 camera

Instructions for
Brightfield (black and white camera)
Fluorescence (up to 7 colours),
Z stacks and
Tile scans.

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

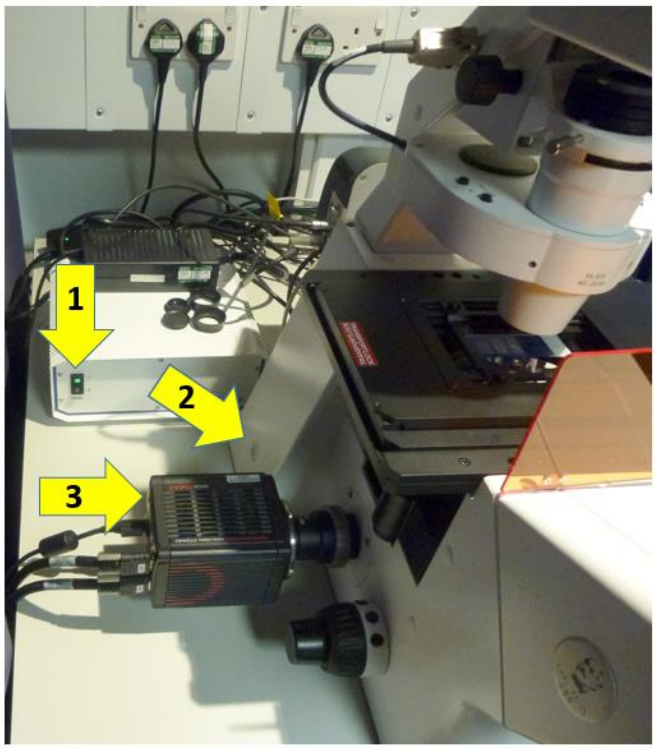


Turn on the system:

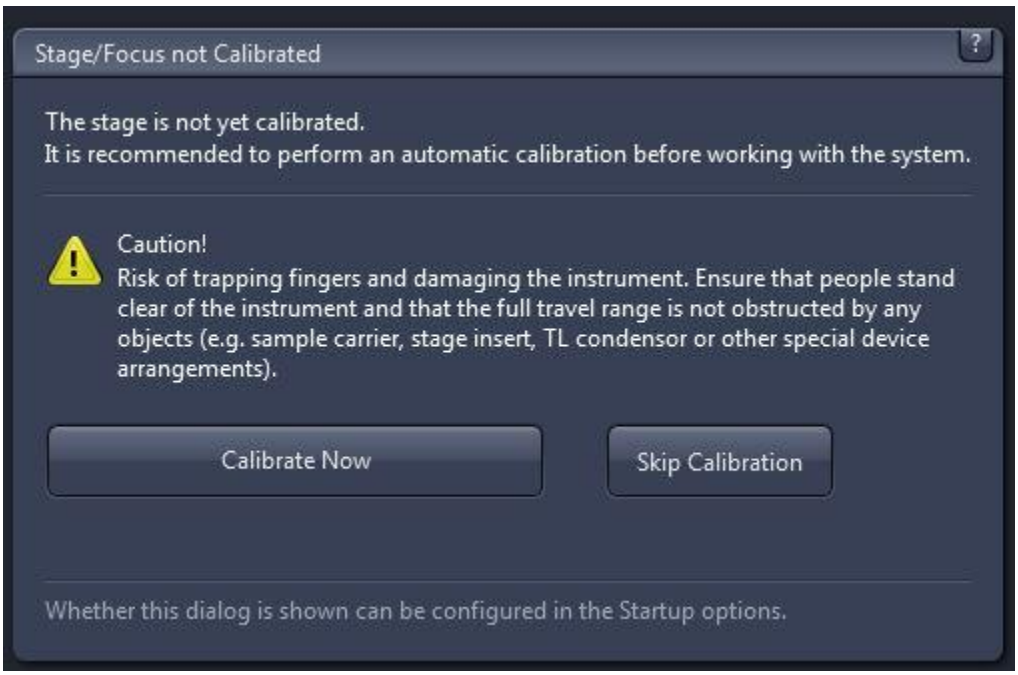
Turn on: 1, 2, 3
Turn off: 3, 2, 1

1) **Power supply**
2) **Silver button on microscope**
3) **Toggle switch in top left of camera**

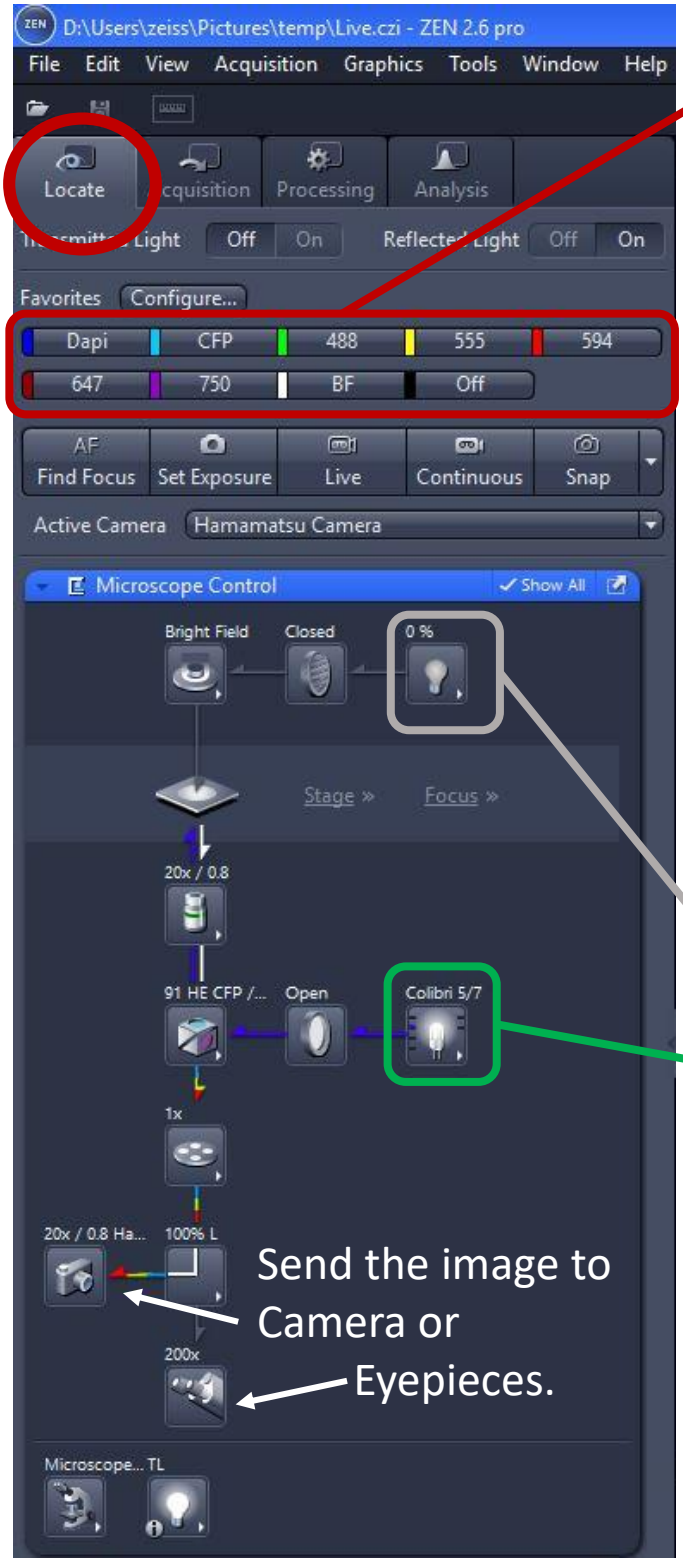
Then start Zen Blue software



After you start the Zen Blue (Pro) 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 (350, 405, BFP) *

CFP (430, TFP)

488 (488, GFP, YFP, Cy2, Fitc, DiO)

555 (546, 555, 568, 594, Cy3, dsRed, DiI)

594 (mCherry, 594, 610)

647 (647, 633, Cy5, APC) **

750 (750, 790, Cy7, IRDye750/800) **

BF: brightfield

Off: turn all illumination off

* All numbers indicate AlexaFluors by excitation wavelength.
** Your eyes can't see these wavelengths but the camera can.

You can adjust bulb power

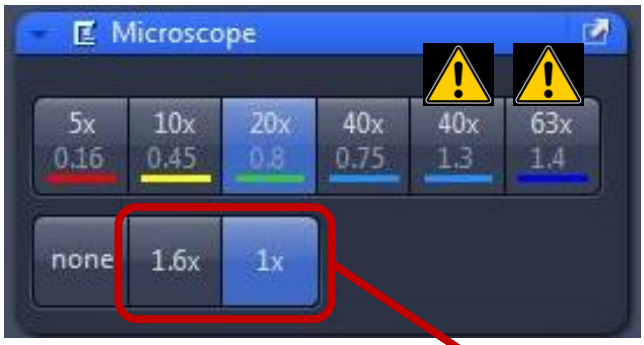
Brightfield

or

Fluorescence

This is the Colibri LED system. Adjust power with the dial, or in the software.

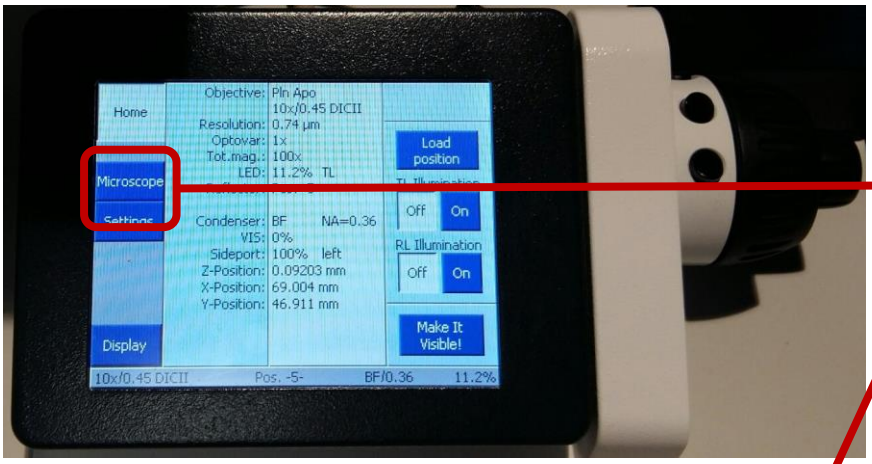
Set magnification from the software or the touchscreen next to the scope



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

See warning below about using Oil

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



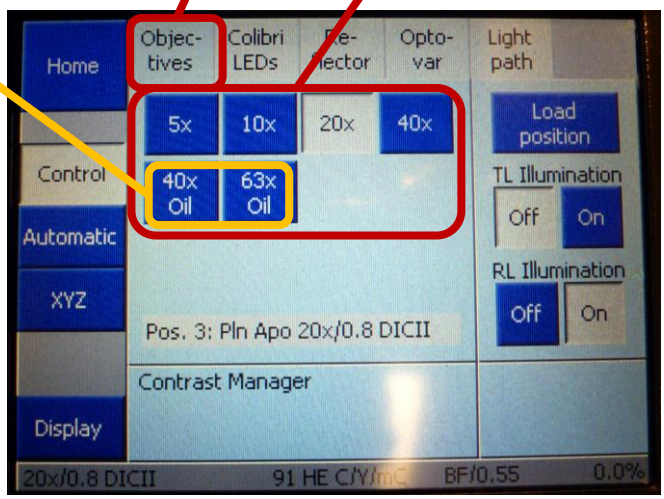
Touchscreen controls
Select: Microscope

Go to Objectives tab

Choose objective

WARNING

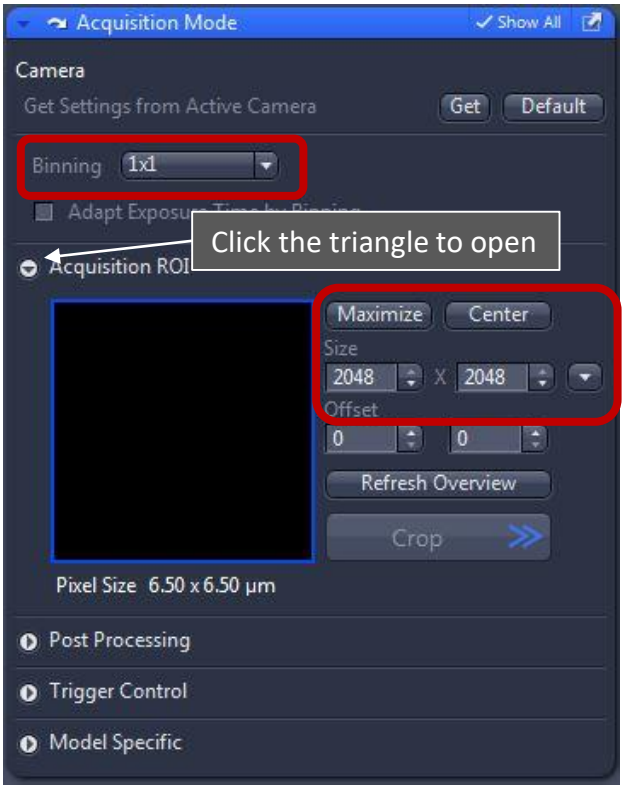
Only use oil with the **40x Oil** and **63x Oil** objectives.
Note: there are two 40x objectives: one dry, one oil. The 40x oil is much better quality.
It is **essential** to clean all oil from slides when using 5, 10, 20, or 40x dry objectives.
Clean oil from slides with a clean tissue (not lens tissue), then wipe residual oil away with a tissue wet with 70% IMS.



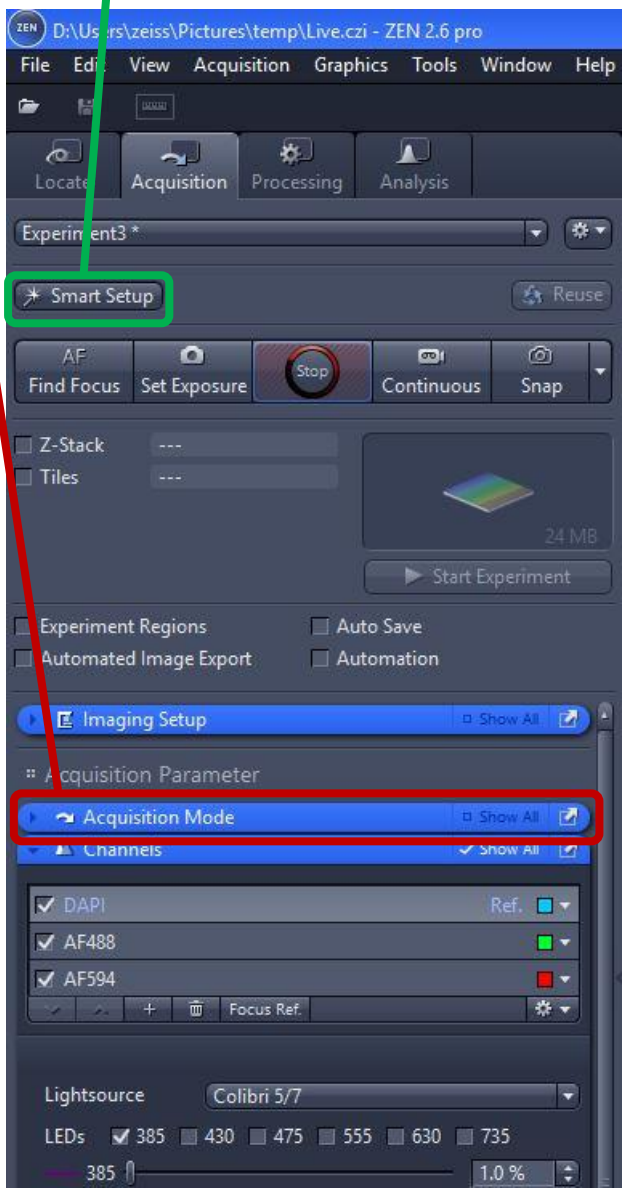
Acquisition tab for capturing images

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. You will need to have done smart set up or have some channels activated before checking the Acquisition Mode settings.

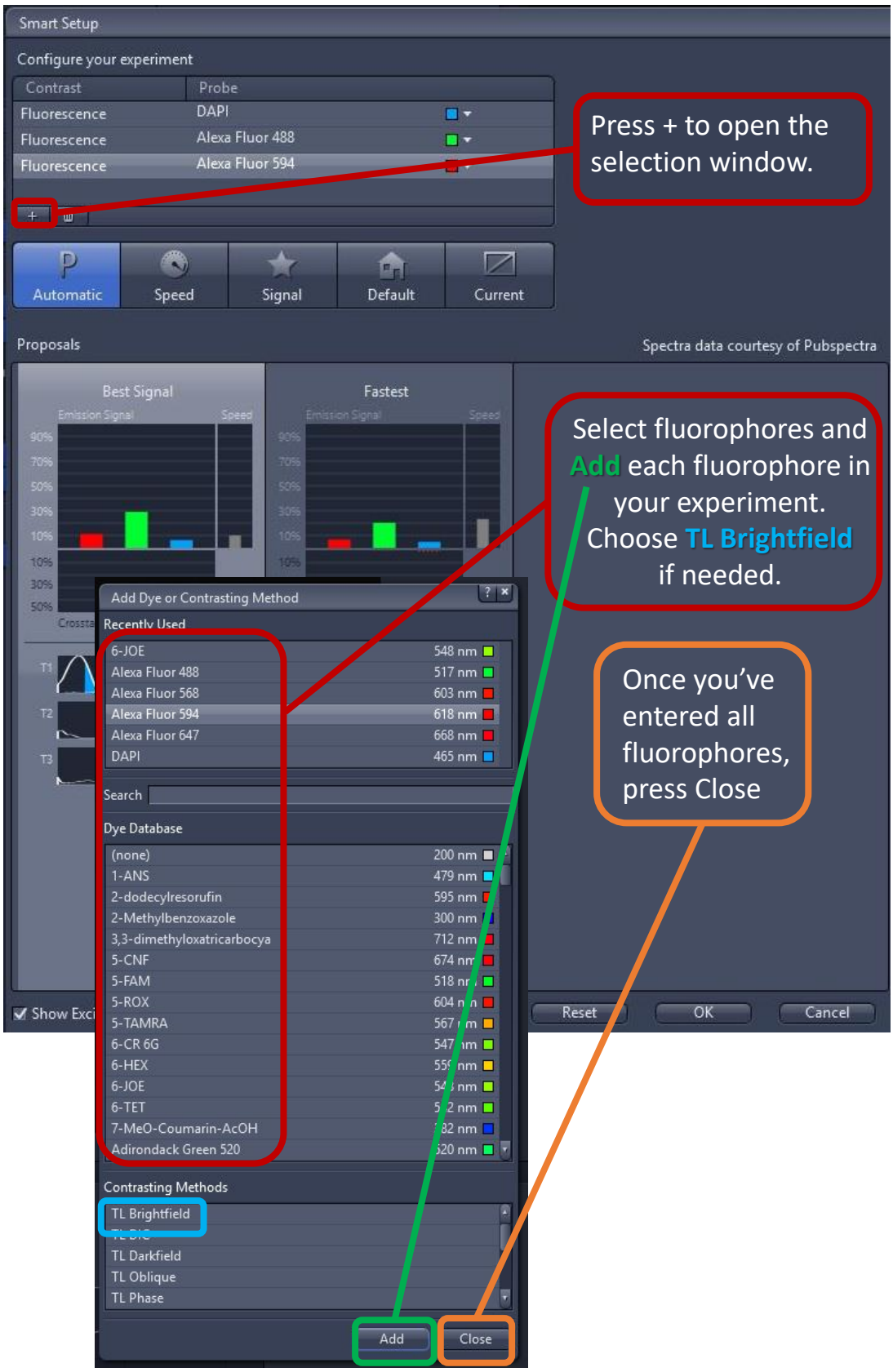


Set up the fluorescence channels (and Brightfield if needed) by pressing **Smart Setup**.



Binning at 2x2 (or more) increases sensitivity and speed for fast live imaging. But you lose resolution.
Reducing the acquisition ROI can help with producing an evenly illuminated tiled image.

Smart Setup – What colours are you imaging?



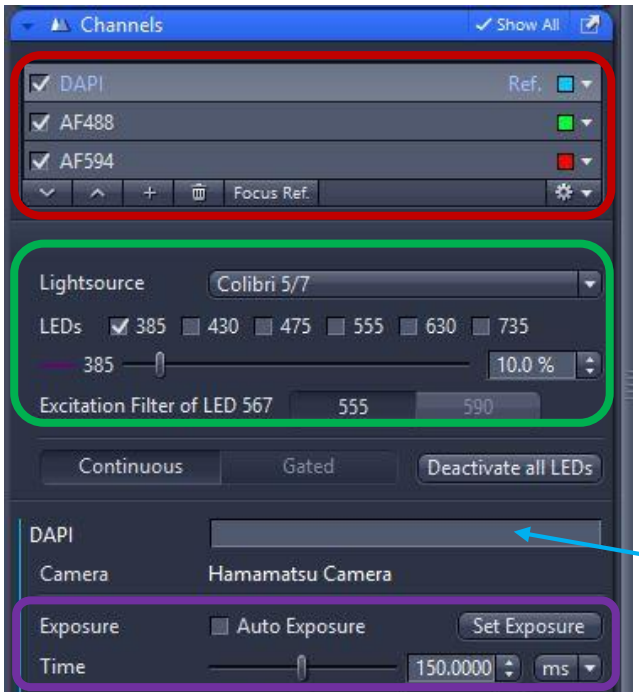
Smart Setup – What colours are you imaging?

Best signal will use a different filter for each fluorophore wherever possible. This should result in the brightest signal, and the least possible bleed-through from other channels. It is the safest but slowest mode.

Fastest will use the LEDs to excite fluorophores one at a time, and if possible use a single 4 band filter to image much faster. You may find some (slight) bleed-through between some fluorophores in this mode. Test by comparing a 'Best Signal' image to a 'Fastest' image if concerned.

See separate appendix for more details of excitation and emission of fluorophores.

Channels set up – Highlight one at a time to set up each channel



Channels

Highlight a channel to view and make adjustments.

Tick to include in the captured image.

Colibri LED power (%)

Recommend 2-20% for Dapi (385)

2-100% for all other colours.

Do not change the auto-selected (ticked) LED for any of the channels. See notes below & appendix for details.

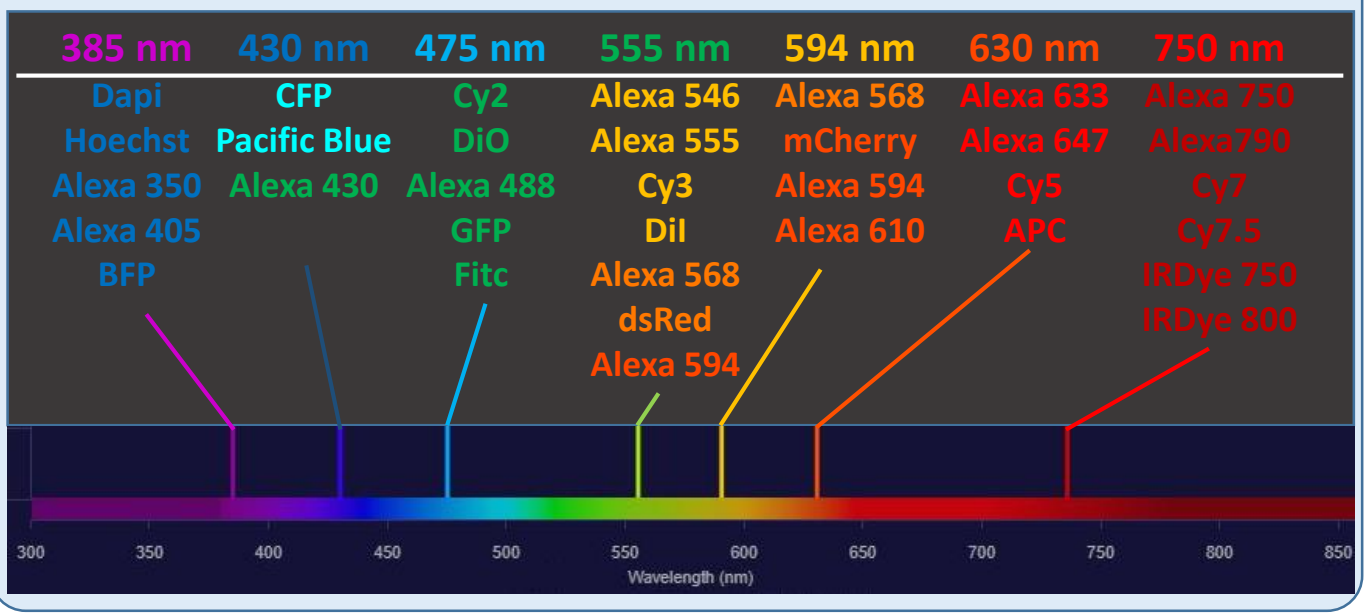
You can modify the channel name

Exposure time Longer = brighter

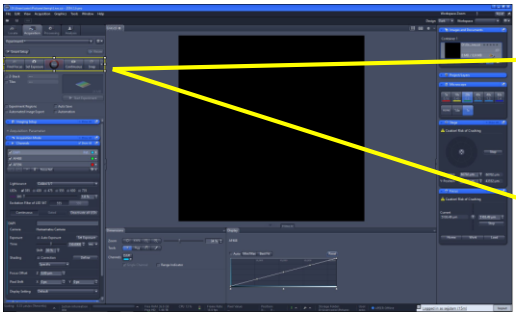
Notes on Colibri LEDs

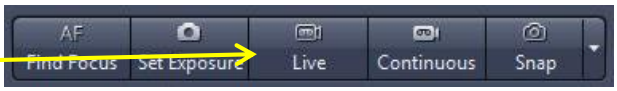
The fluorescence light source consists of 6 LEDs each with a specific excitation filter. The 555 LED is actually a green & yellow LED, that then uses a filter for 555 nm excitation or 590 nm excitation. LED excitation colours are shown below as is the approx. colour of fluorescence of fluorophores.

LED excitation wavelength (nm) and suitable fluorophores.



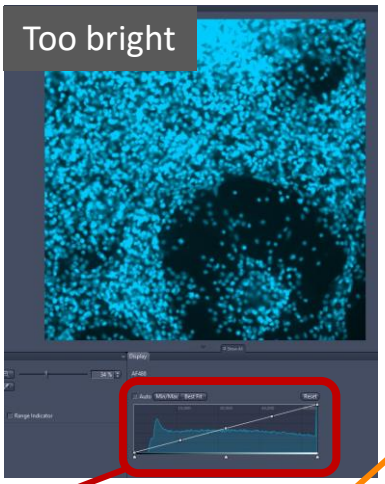
Channels set up – Set the LED power and camera exposure time to give a visible image.

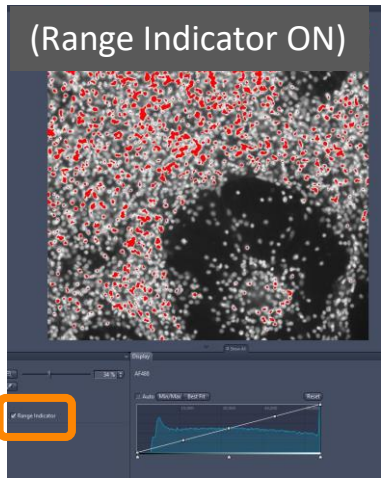




Press **Live / Stop** to see a live image.
Tip: Always press Stop as soon as practical so you don't bleach your samples.

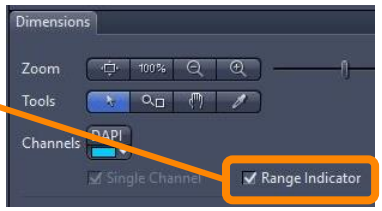
Unless you've already set up or re-loaded your settings, the live image is likely to be too dim or too bright.

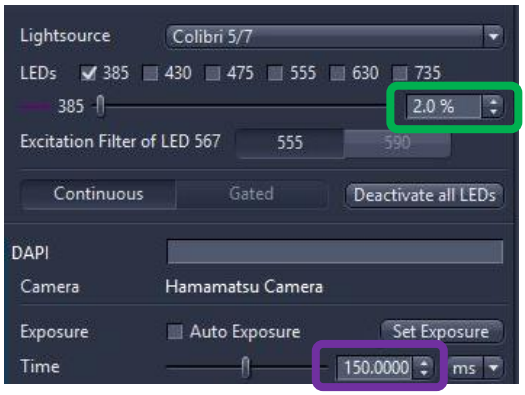




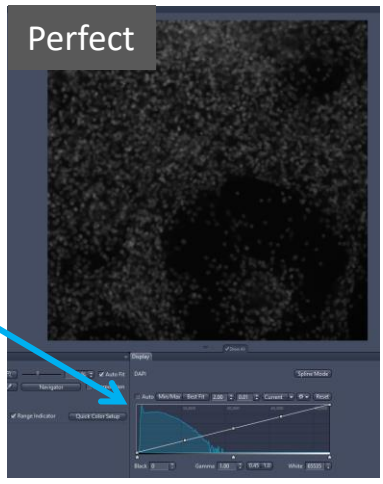
Use the image **histogram** & **Range Indicator** to set image brightness.

The Range indicator switches to a black & white image, highlighting pixels that are too bright as red, too dark as blue



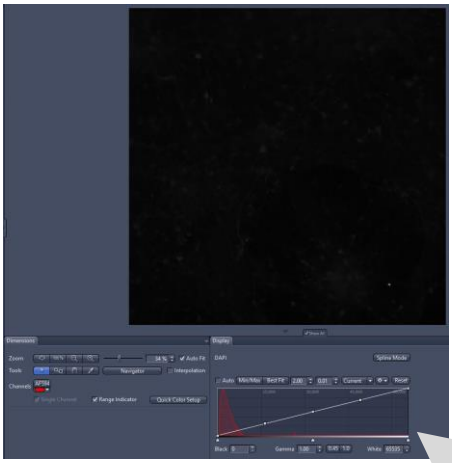


Adjust **% Power** and **Exposure Time** to adjust the brightness until the histogram covers about ½ of the full range.



See next page for advice on Power and Exposure Time

Channels set up – adjust the histogram to see dim images

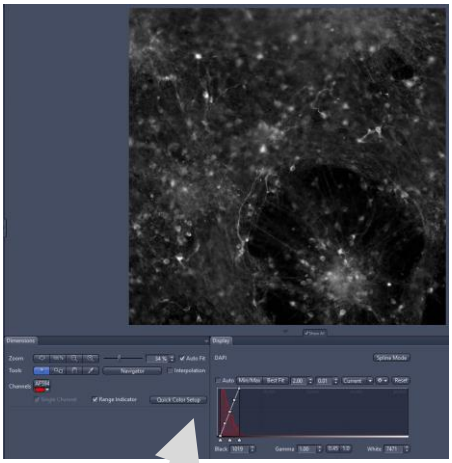


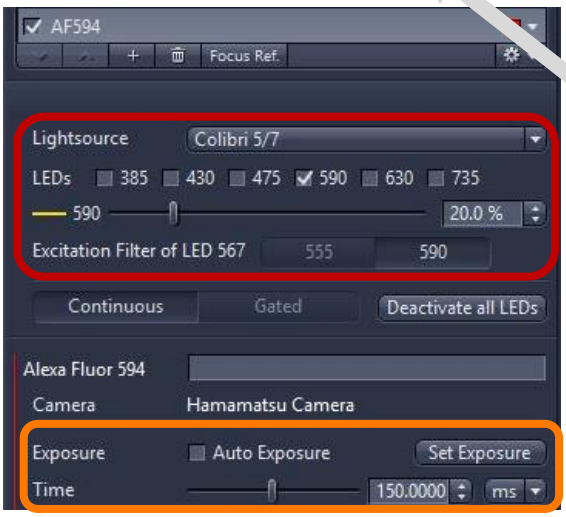
These images are taken with identical settings.

590 nm LED @ 20%

Exposure Time = 150 ms

Only the display has been adjusted, to make the image look brighter.





The **Display** tab shows the image histogram, from 0 (no signal) to 65535 (the brightest).

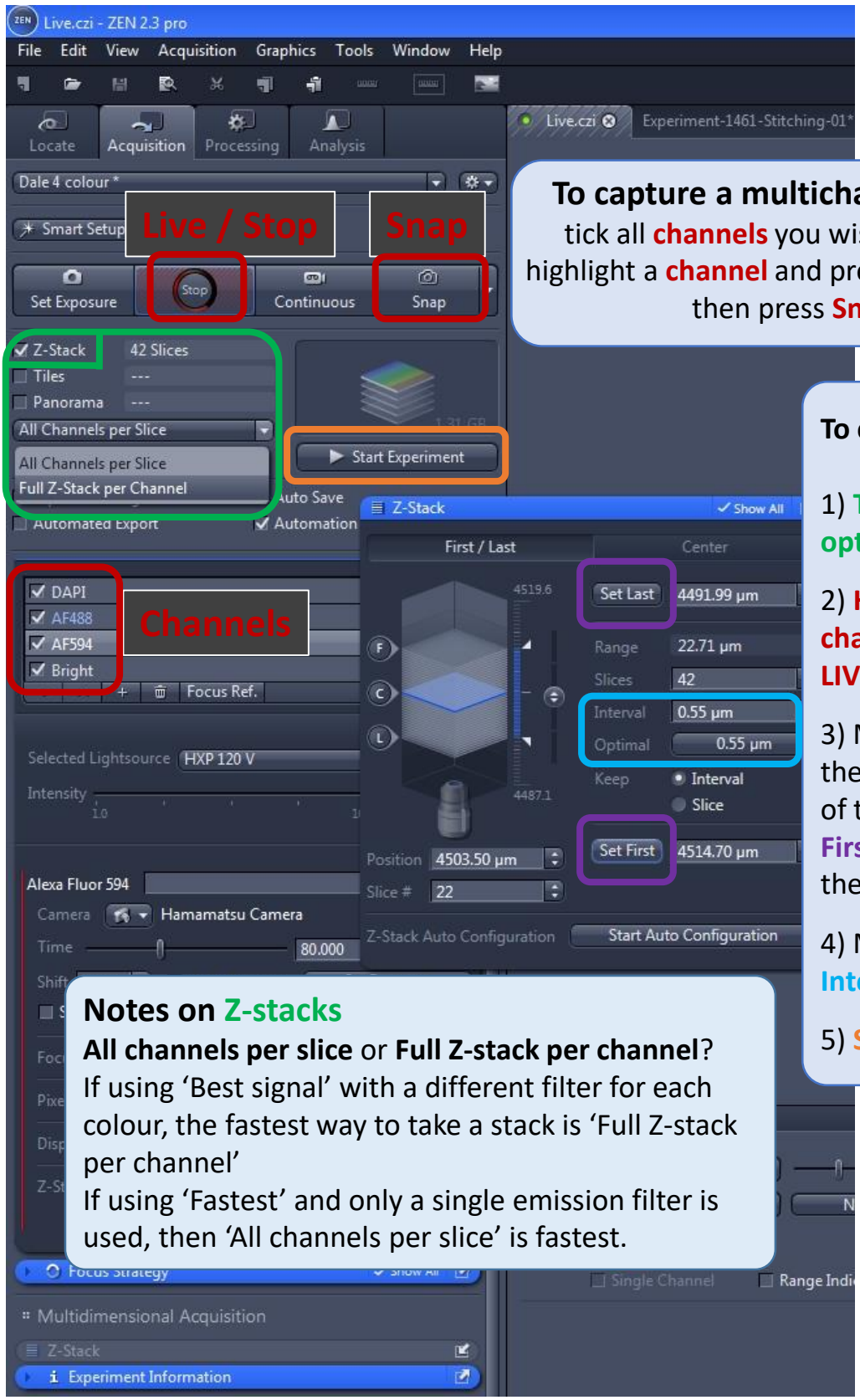
You can make an image LOOK brighter by pressing Min/Max or Best Fit.

This is useful for focusing images when they are very dim without having to increase exposure time or LED power. Remember to reset the display to the full range (0-65535) to see how the image will look without increasing the brightness.

Notes on Setting LED % power and Camera exposure time

Increasing LED power increases illumination intensity and fluorescence. Increasing exposure time increases image brightness. High power for a long time will bleach samples.
Aim for short exposure times (~ 100ms) if possible, adjusting LED power to make the image visible. Aim for 2-20% LED for Dapi (385nm), 2-100% for all other colours. Remember, even a very dim image (as above) gives great data. Here the image maximum brightness is only 7471. The camera is so good that even very dim images are perfect for analysis and can always be turned up to look brighter.

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:

1) **Tick the Z-stack option**

2) **Highlight a channel and press LIVE**

3) Manually focus to the top and bottom of the stack to **Set First** and **Set Last** for the stack

4) Make sure **Interval = Optimal**

5) **Start Experiment**

Notes on Z-stacks

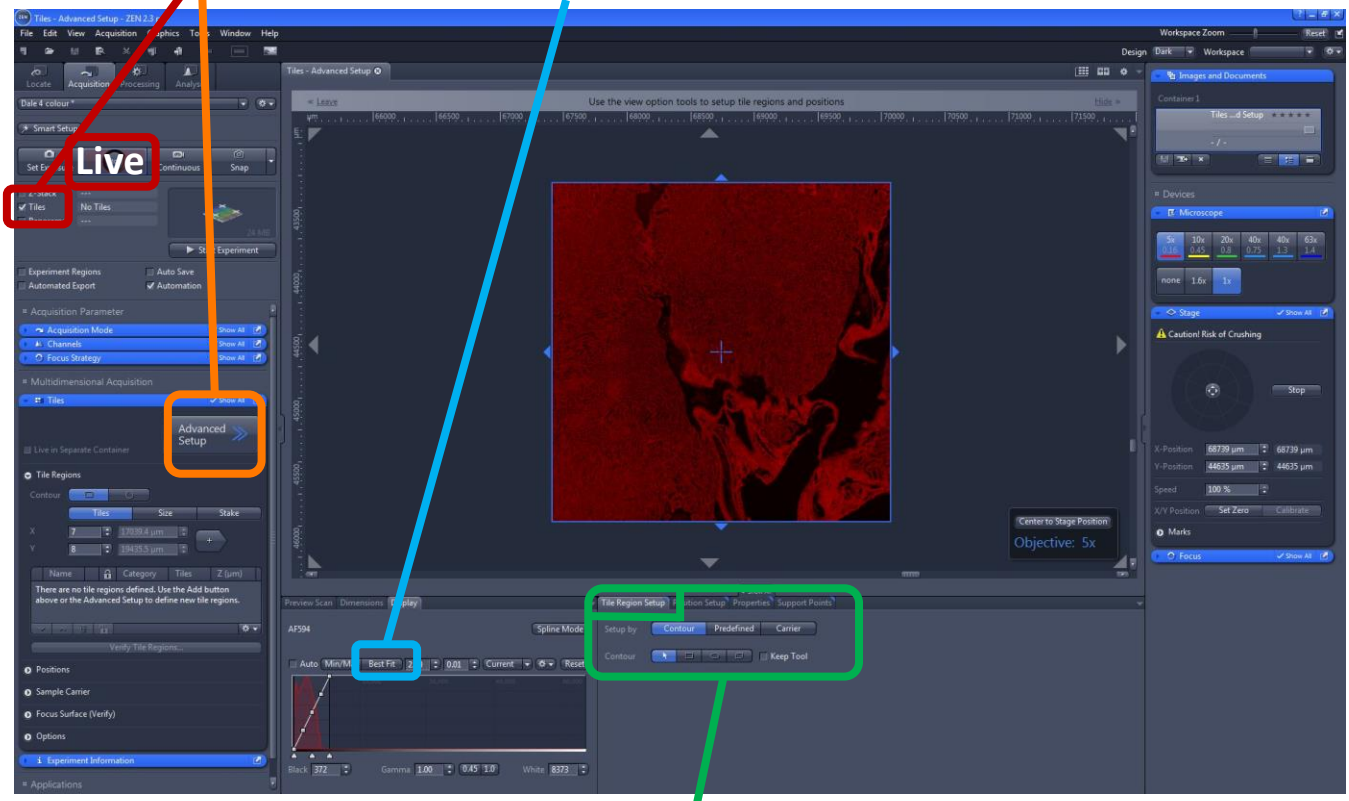
All channels per slice or **Full Z-stack per channel**?
If using 'Best signal' with a different filter for each colour, the fastest way to take a stack is 'Full Z-stack per channel'
If using 'Fastest' and only a single emission filter is used, then 'All channels per slice' is fastest.

Tile scan (9 pages)

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 (higher mag) 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.

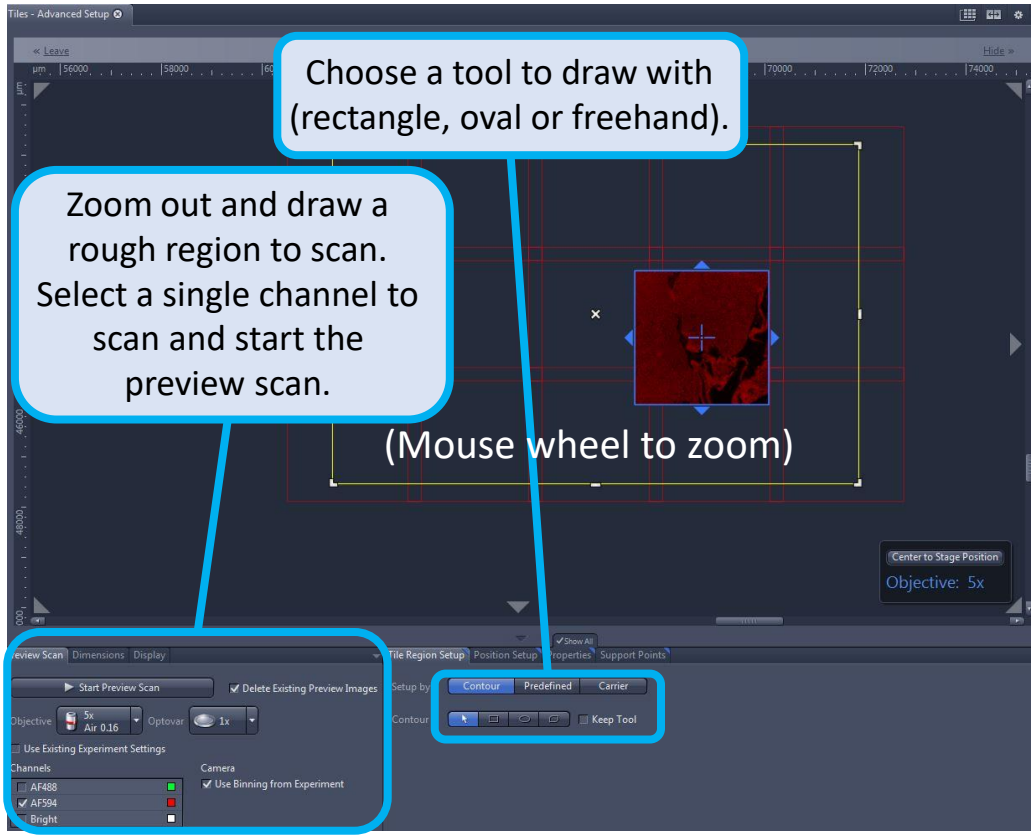
Select the **Tiles** option
Press **Advanced Set up**

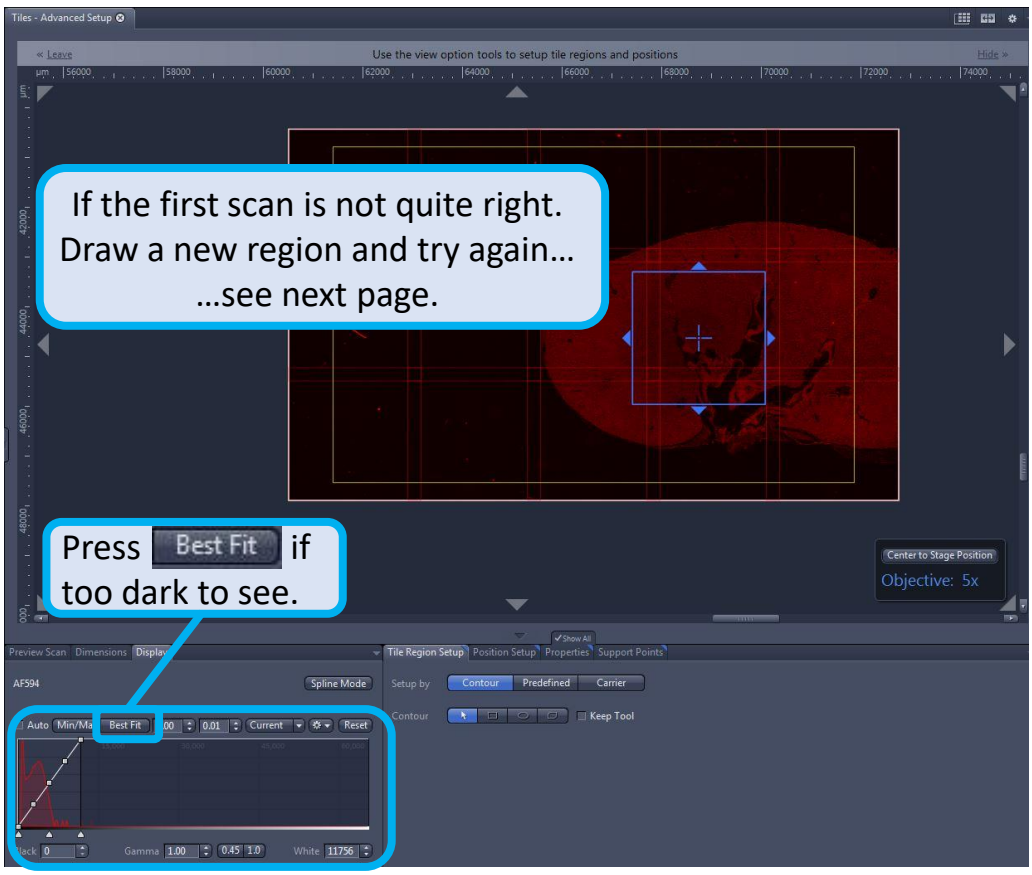
View **Live** image at a low mag.
Press **Best Fit** on histogram to increase image brightness of even very dark images



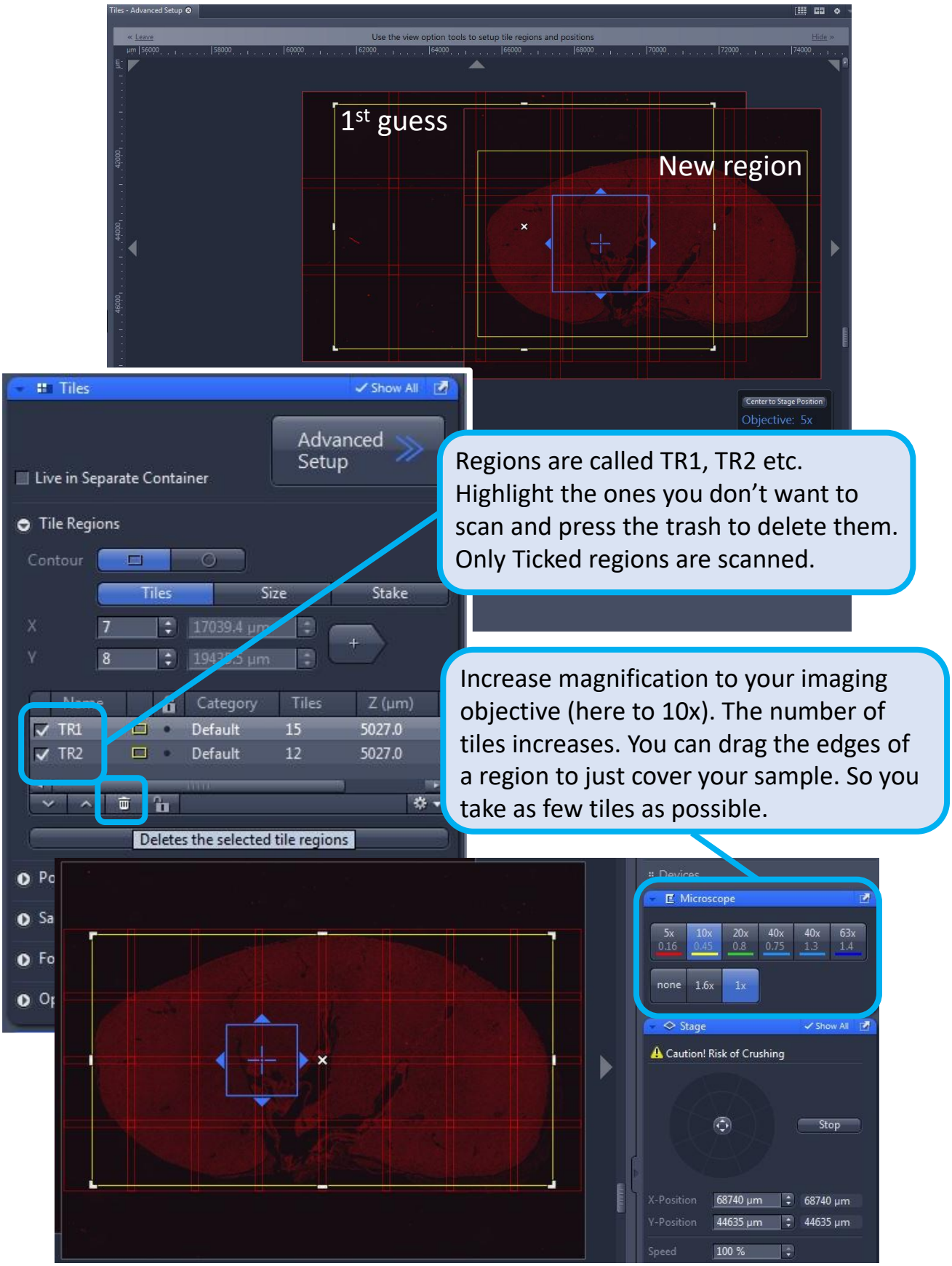
Zoom out the view (wheel on mouse).
Use the tools in **Tile Region Set up** to draw your first guess at the ROI...
Continued next page…

Tile scan (2/9) **… Draw a region that covers the area to be scanned**





Tile scan (3/9) **… Fine tune the size of the region to be scanned**



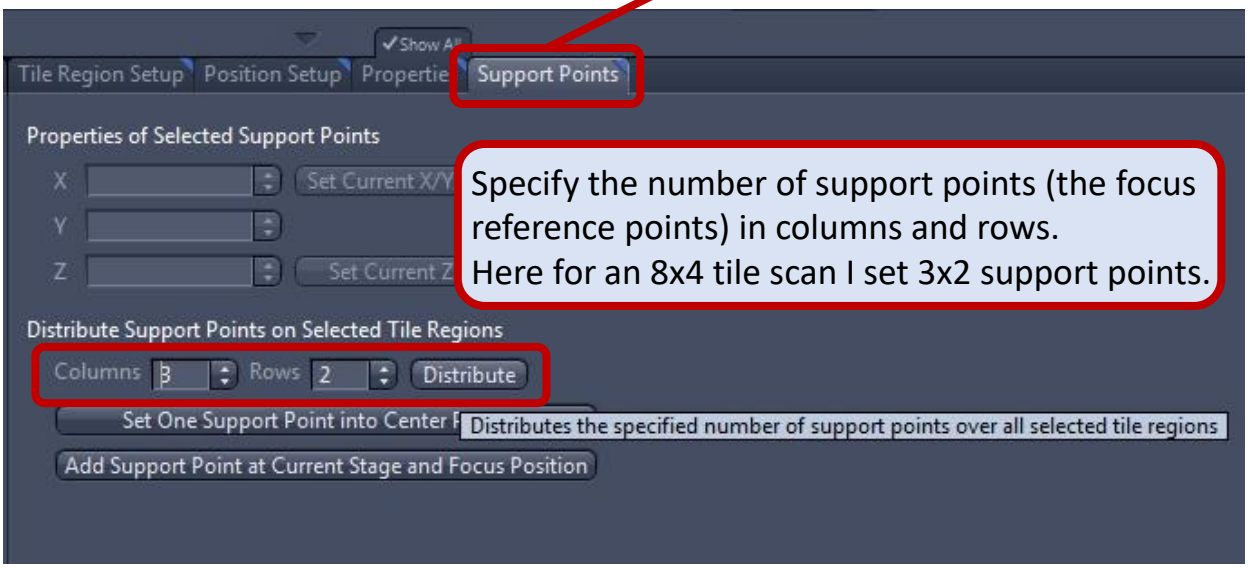
Tile scan (4/9) … make a focus map – set support points

Note: You can optionally use AUTOFOCUS instead of making a manual focus map

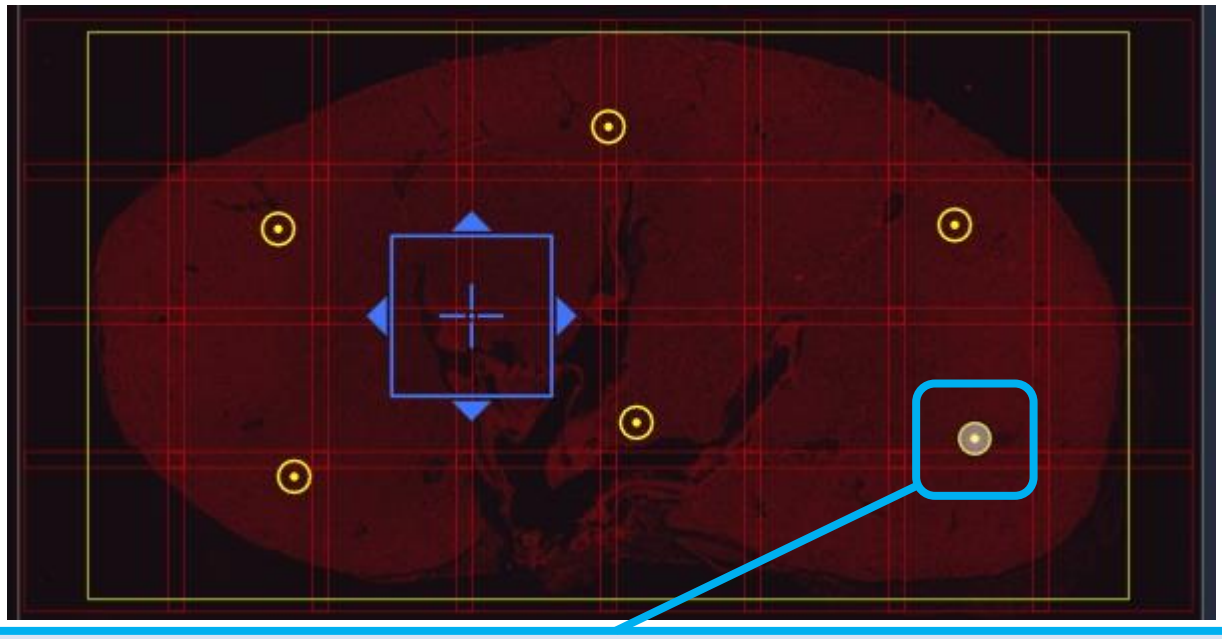
If using autofocus, do not make a focus map here. Skip to Tilescan (7/9) & see autofocus section (from page 21).

It is also possible to use a hybrid method where you use autofocus to make your focus map (not recommended, ask Dale for advice).

If you are making a manual focus map, first add support points, in the Support Points tab below your image.



Specify the number of support points (the focus reference points) in columns and rows.
Here for an 8x4 tile scan I set 3x2 support points.



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

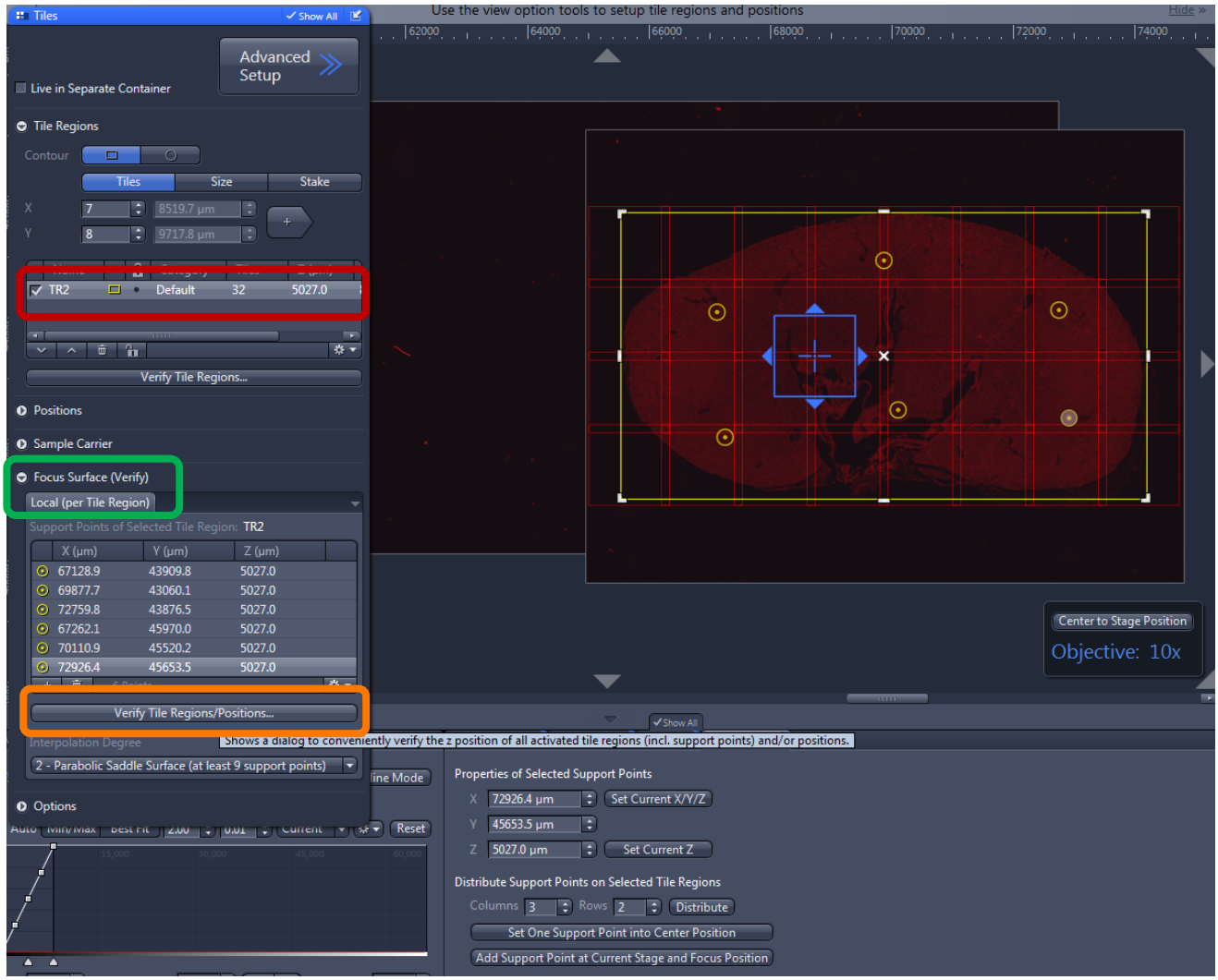
Tile scan (5/9) **… verify support points**

In the Tiles Advanced Setup window:

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

Select: **Focus Surface (Verify)**

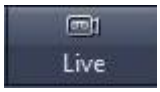
Press **Verify Tile Regions/Positions…**



Tile scan (6/9) **… set focus on all support points**

A new window opens: 

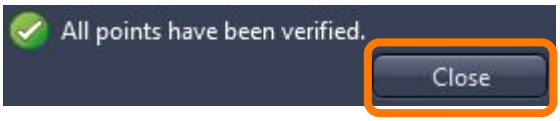
Click **Move to Current Point**

Press 

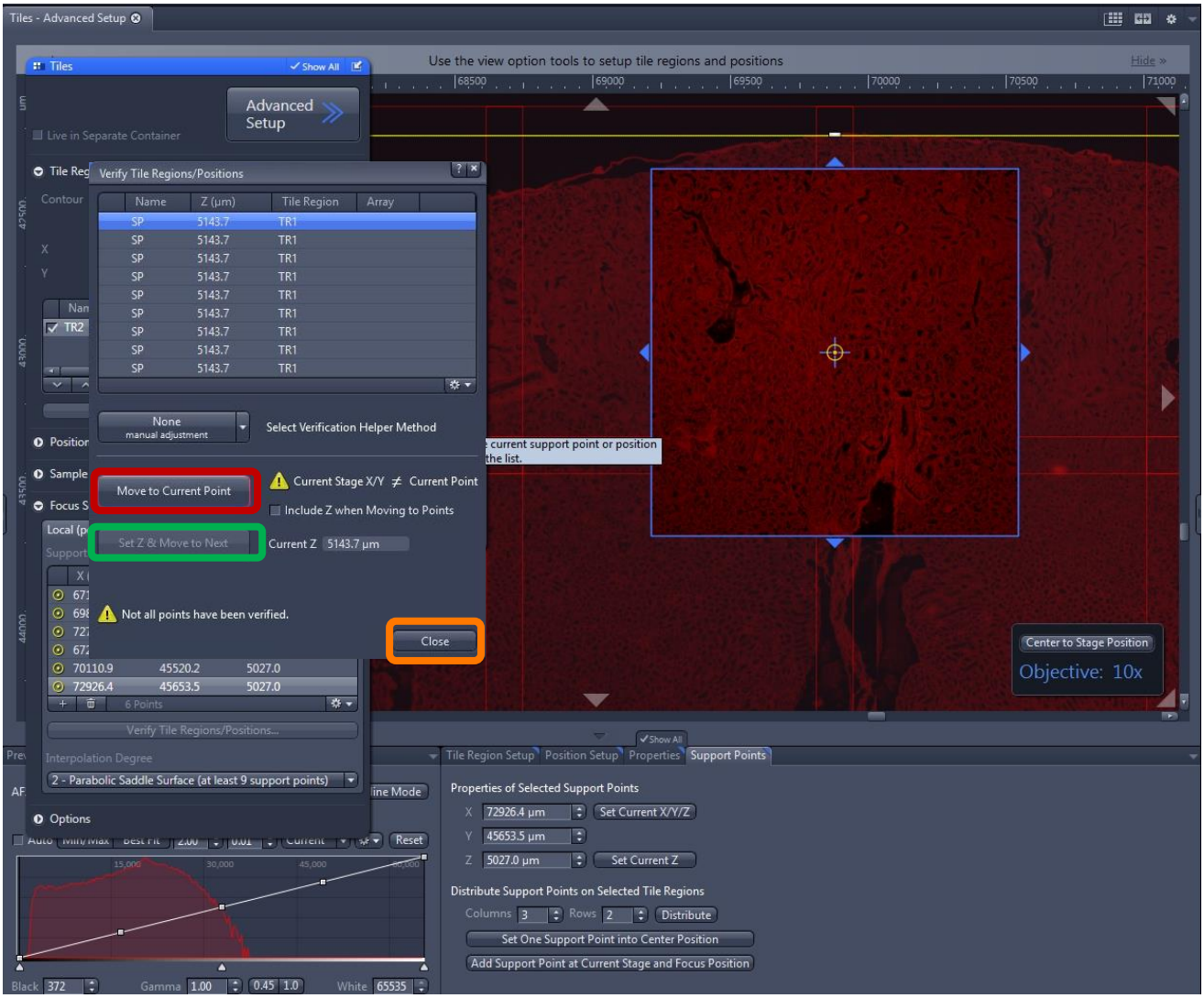
to see the desired channel, adjust the focus, then

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 (7/9) **… Capture the image**

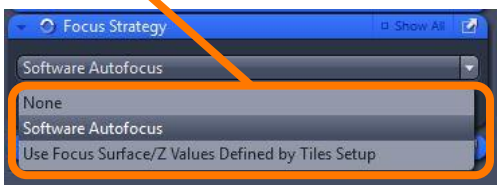
Make sure your imaging **Channels** are ticked.

Select the appropriate Focus Strategy in the Focus Strategy window

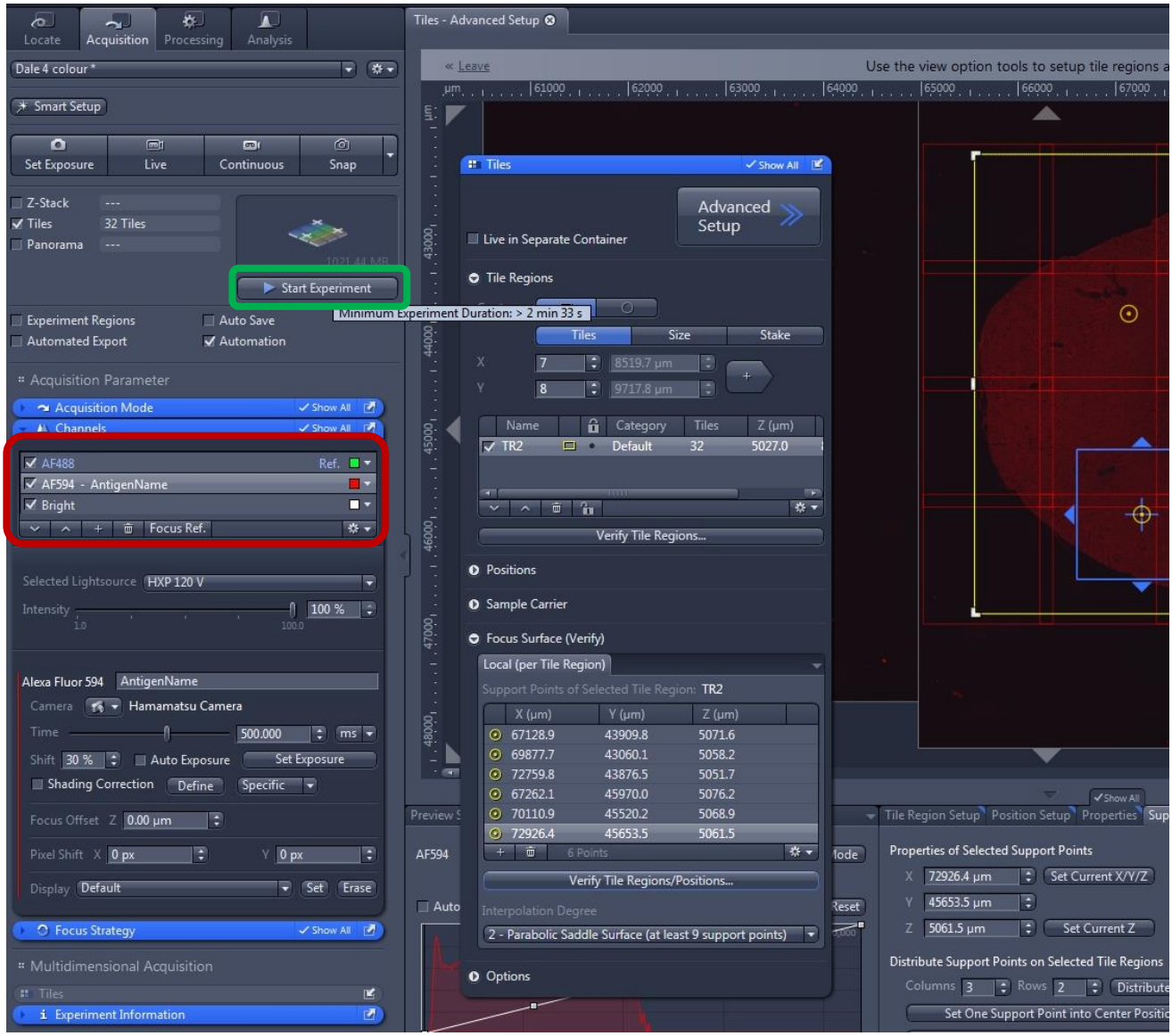
- '**Use Focus Surface/Z Values Defined by Tiles Setup**'

or

- '**Software Autofocus**' if no focus map was made.
  (for software autofocus see page 21)

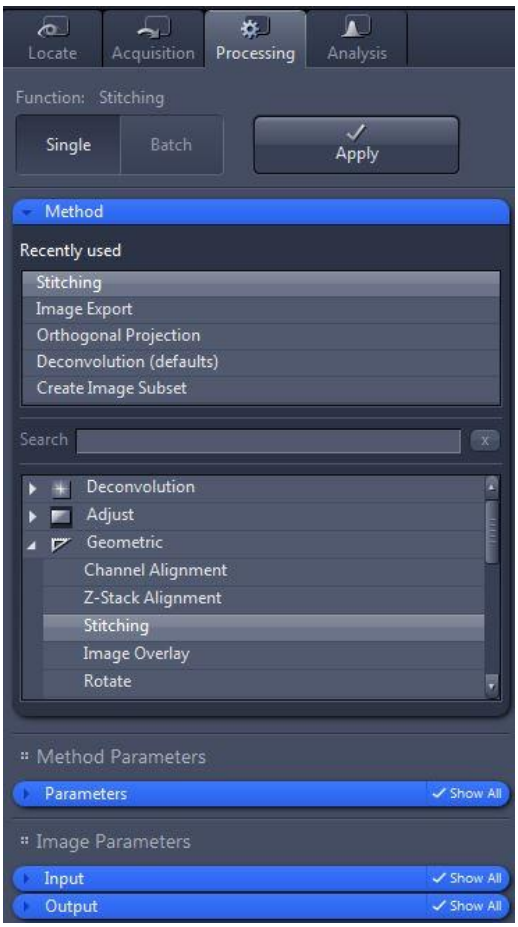


Press: **Start Experiment**

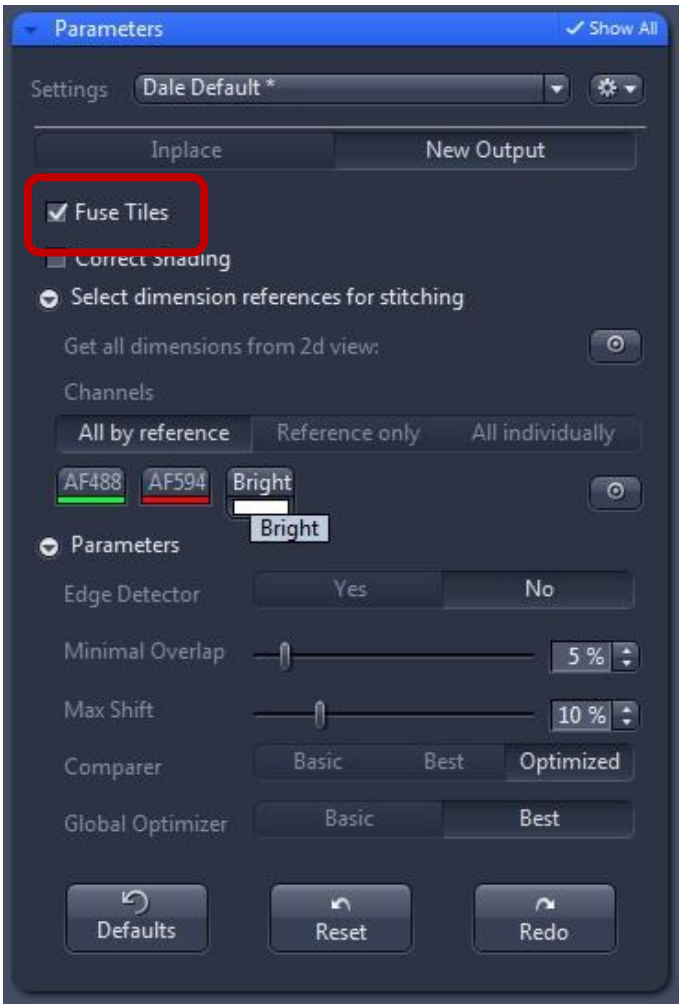


Tile scan (8/9) **… Stitching the image**

Go to the processing tab, choose the method: Stitching

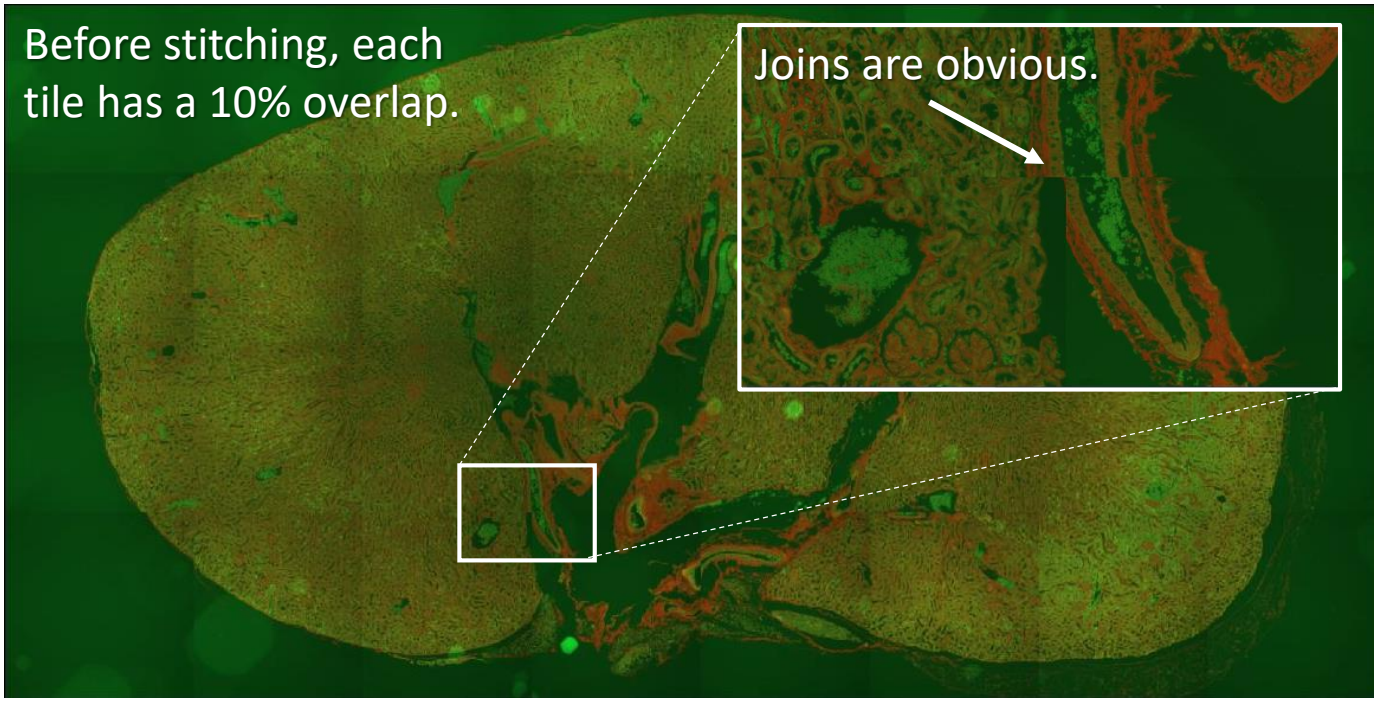


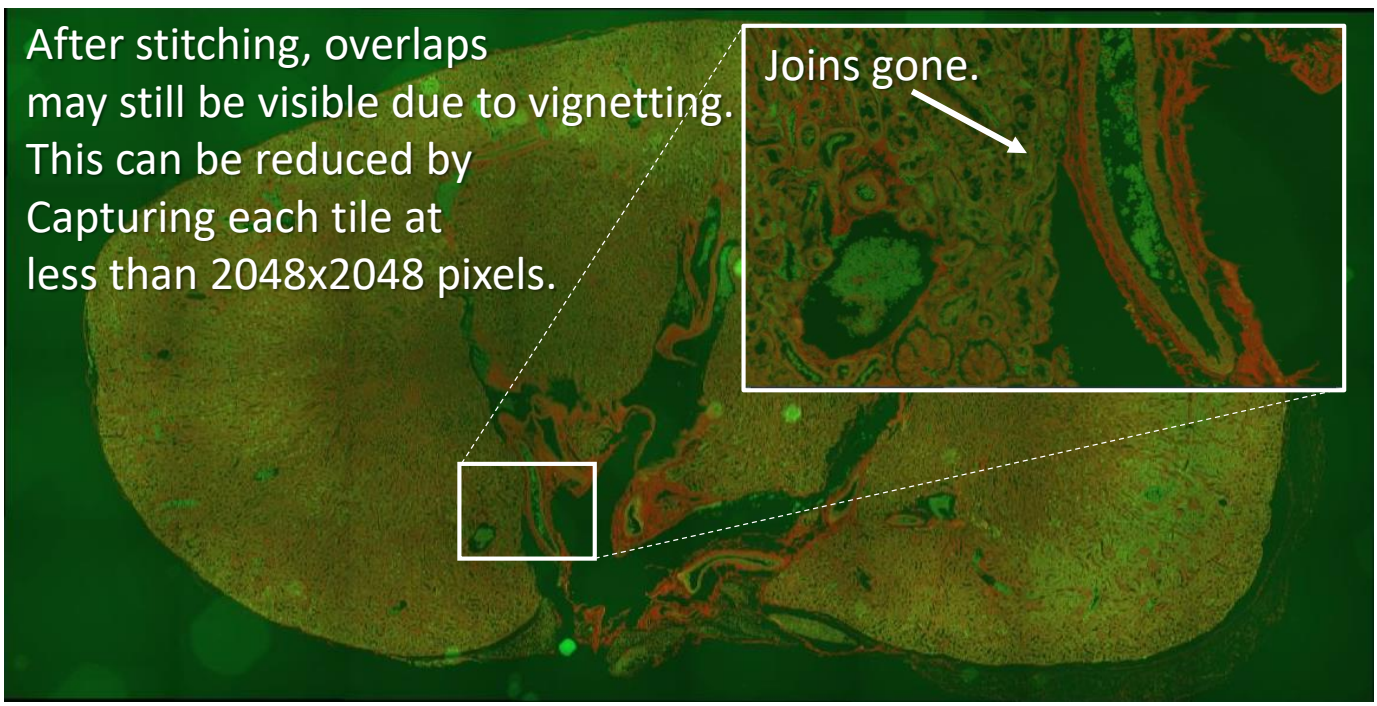
Open up the Parameters window, select the Settings “Dale Default”.
This sets the output to have a single **fused** image, in a New Output window.



By default it will use the first channel captured to align the image, (normally Dapi, but in this example 488) but you can choose any channel that has sufficient content to perform the stitching.

Tile scan (9/9) **… Finished, save the raw and stitched Images**





Software Autofocus (4 pages)

You have 3 options for a focus strategy.

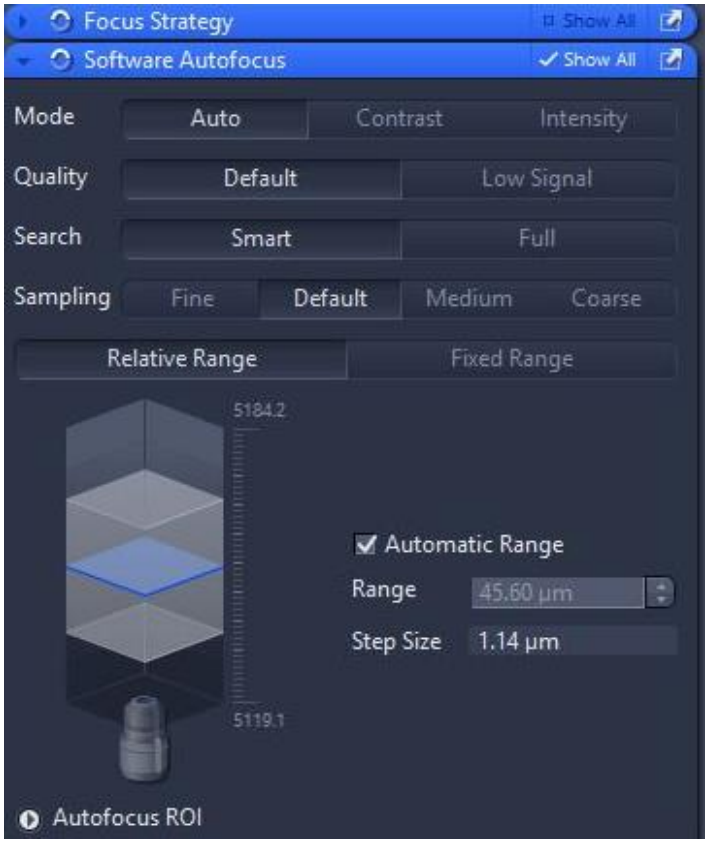
1) Focus manually. None This should be used for standard imaging, unless you are doing multi-position images or tile scans.

2) Software Autofocus The selected Focus Ref. channel is used to automatically adjust the focus. This can be useful for **Tile scans** instead of making a focus map.

3) Use the focus map you previously prepared in Tile scan advanced set up.

Recommended Software autofocus options

Mode: Auto **Quality**: Default **Search**: Smart
Sampling: Default **Relative Range** ☑ Automatic Range



Notes on autofocus options

Mode will use contrast as a default in most situations. Intensity will look for the brightest z position, which may be a bit of fluff on the coverslip. Contrast is normally more reliable.

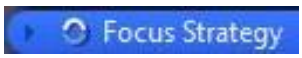
Quality can be set to low signal if brightness is exceptionally low.

Search switches between the slower 'Full' mode, where it moves to the bottom of the set range and scans the whole range to find the best focus. 'Smart' is faster, and moves up and down from the last position until it finds good focus.

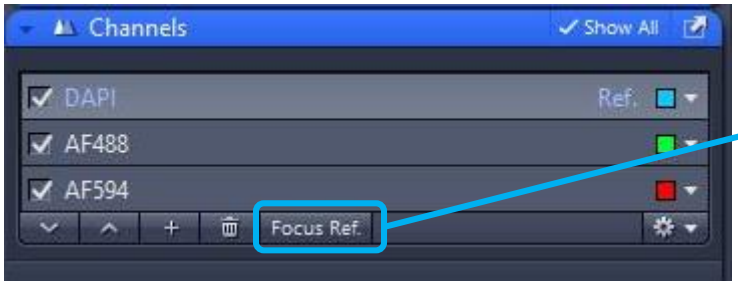
Sampling sets the distance it moves each step to find focus. Fine moves tiny distances (half of Nyquist) is super accurate but slow. Coarse moves large distances (4x Nyquist) is fast but less accurate.

Autofocus (2/4) **Tile scans**

Software Autofocus in a Tile scan is simple to set up.

1) Set up the Software Autofocus control window as suggested on previous page.
2) Define your tile scan region. (Tile Scan sections 1-3, from page 14).
3) Do not make a focus map for your tile scan.
4) Manually focus you sample.
5) Set Software autofocus in the 

window.
6) Press 



Autofocus will use the channel you specify as 'Focus Ref' to adjust the focus. This is set in the Channels control window.

WARNING

Software autofocus should be used carefully, to avoid bleaching.

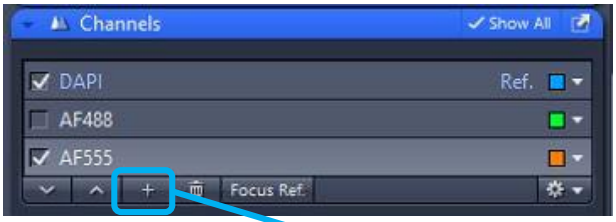
When running Autofocus your chosen channel (Focus Ref) my be bleached if it takes a long time to finds the correct focus.

If your exposure time and LED power are low, bleaching is unlikely. You may wish to choose your brightest channel as Focus Ref.

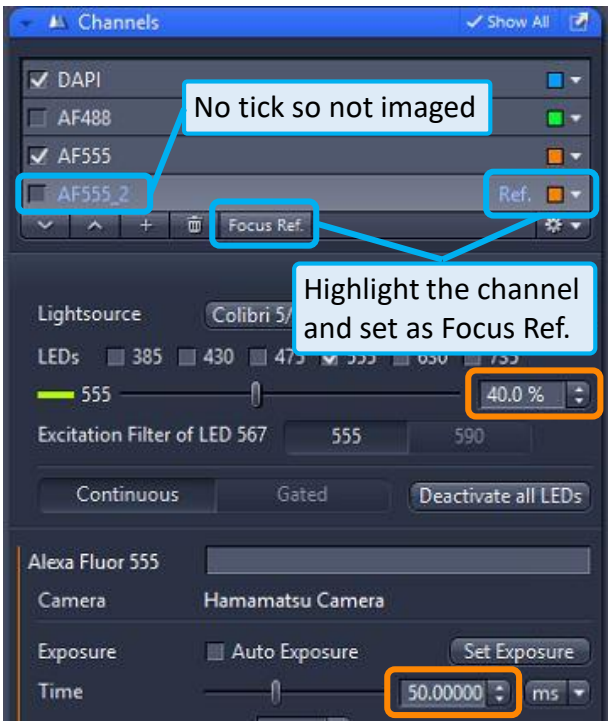
Alternatively, it is possible to set up an extra channel just for autofocusing. See next page for details. As long as there are >1000 grey levels (image brightness) the autofocus will work. Set this channel as the Focus Ref, but do not tick the channel, this way it won't be saved with your image.

Autofocus (3/4) **Avoiding bleaching**

Optional: Add an extra low light channel for autofocus to avoid bleaching



Press + to add a new channel. You can choose the same as an existing channel (here AF555).



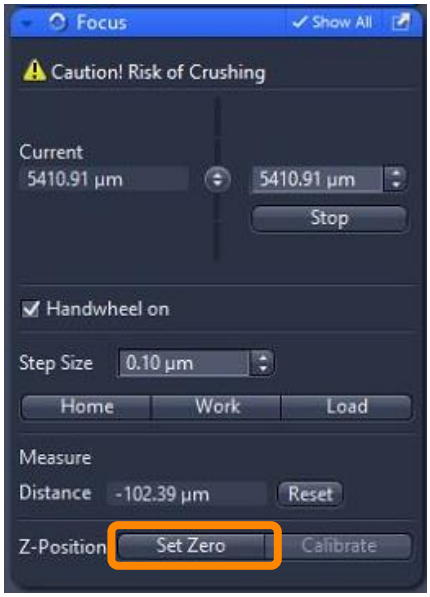
This set up captures Dapi and 555 (the 488 channel is not ticked so not captured).

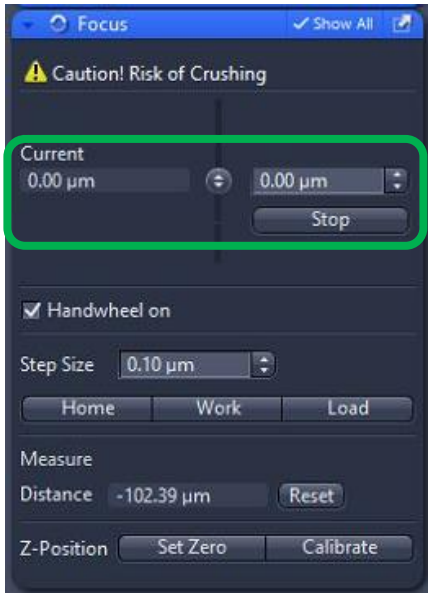
AF555 imaging channel:
80% LED power
500 ms exposure time.

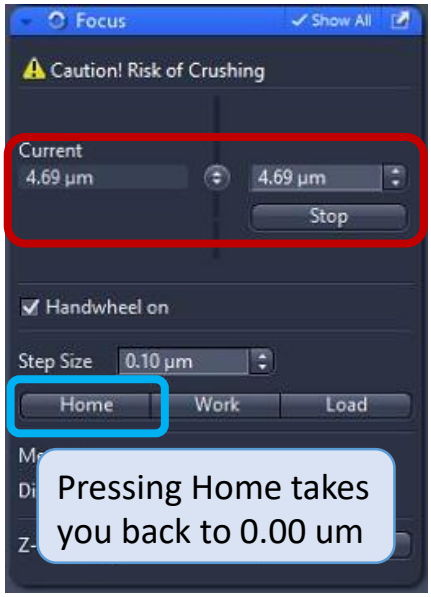
AF555_2 autofocus channel :
40% LED power
50 ms exposure time.

The second AF555 channel uses 1/20th of the light (1/2 power for 1/10th of the time) as the imaging channel, but is fine for autofocus!
Set it as the Focus Ref. channel, and untick the box so it is only used to focus.

Autofocus (4/4) **Offset for different focal planes in different channels.**







You may wish to slightly adjust the focus for different channels.
For example the Dapi may be in focus slightly higher or lower than the other stains.

To do this, select (highlight) your Focus Ref. channel, in this example Dapi.

Focus the sample, then go to the Focus window, press the triangle top left to open it, then tick: Show All

Press **Set Zero** (& OK to the warning window).

The **Current** position is now 0.00 µm.

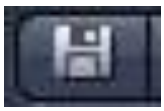
Switch to the channel you wish to have a different focus level. Re-focus. Note the new **Current** position.

Enter this value as the Focus Offset for that channel.

Save your files!

Remember to save your files as you go.

The yellow pen symbol means the file hasn't been saved. 

Press the save button 

and save as a .czi file.

Transfer data via the shared drives.
Never use a USB stick or portable hard drive!