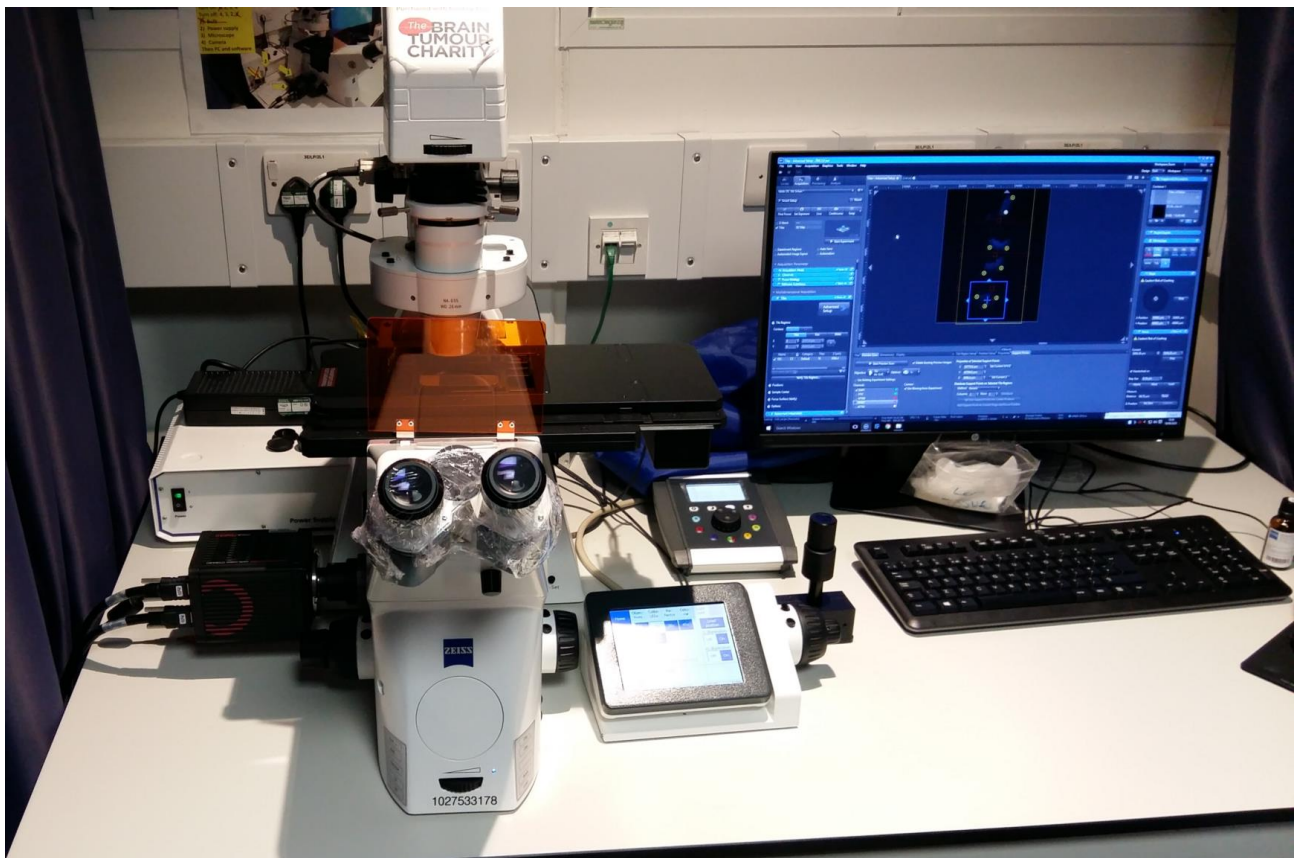


# Zeiss Observer 7 Colour Fluorescence Hamamatsu Flash 4.0v3 camera

## 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.

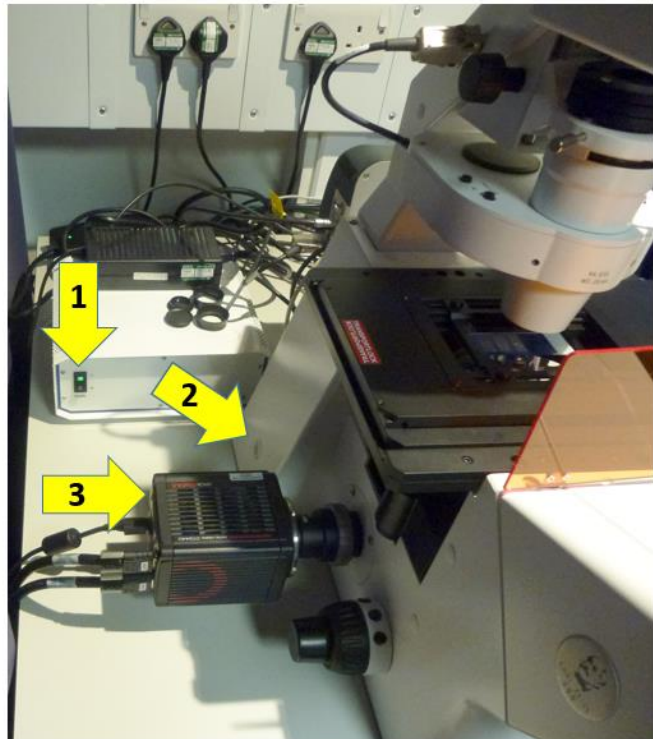
Ask Dale for details.



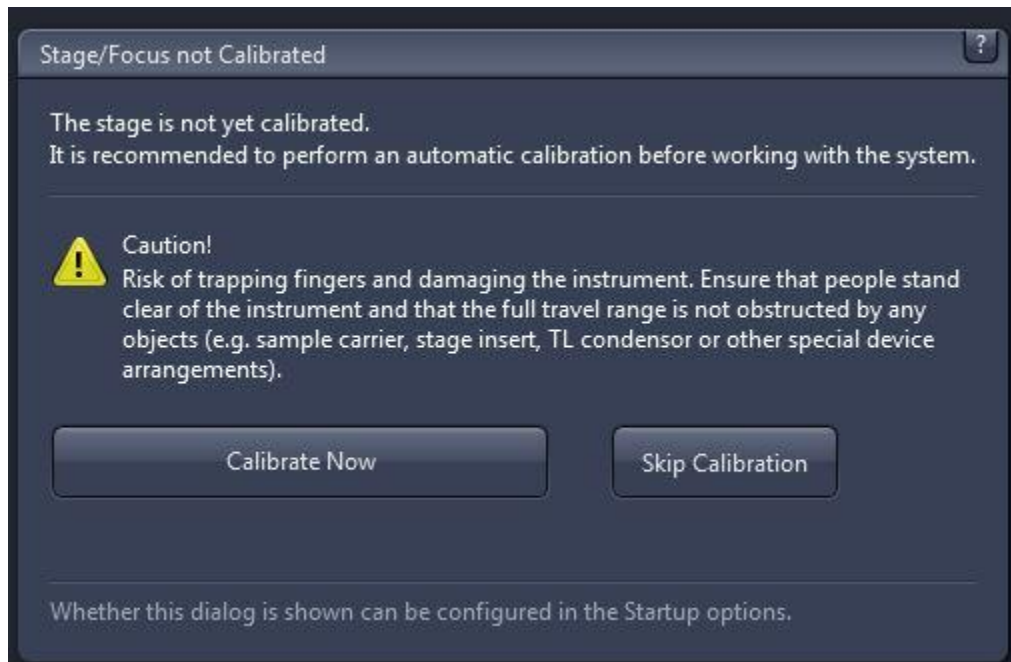
## Turn on the system:

- 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, Dil)
- 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.

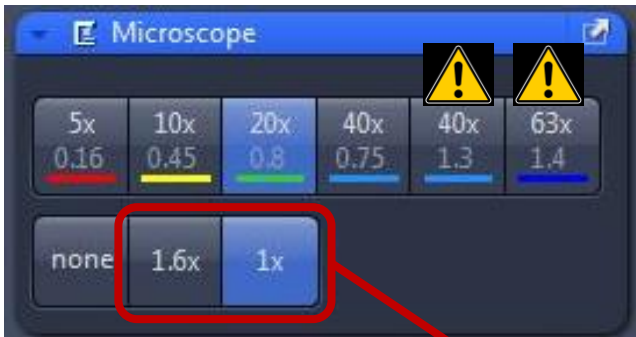
You can adjust bulb power  
**Brightfield**  
 or  
**Fluorescence**  
 This is the Colibri LED system.  
 Adjust power with the dial, or in the software.



Send the image to  
 Camera or  
 Eyepieces.



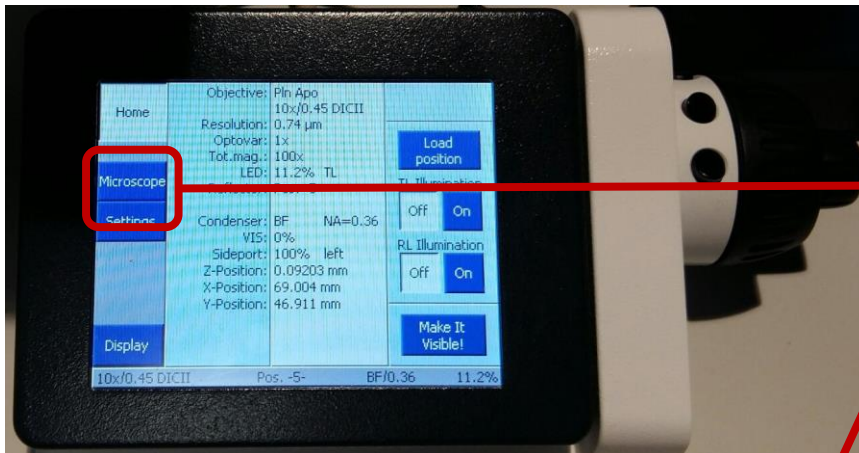
## Set magnification from the software or the touchscreen next to the scope



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

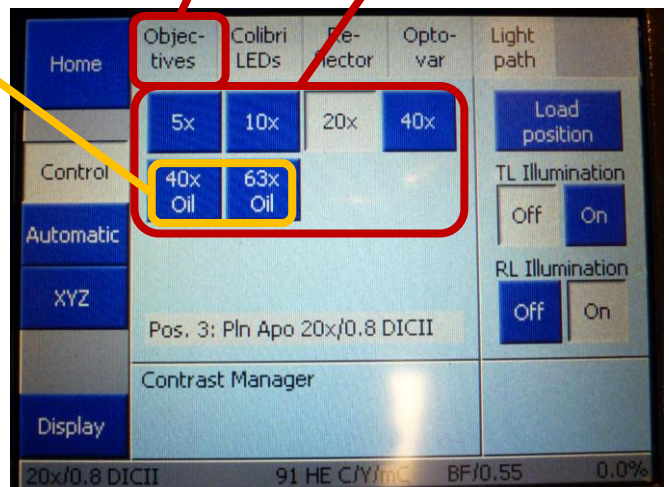


Only use oil with the **40x Oil** and **63x Oil** objectives.

**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.

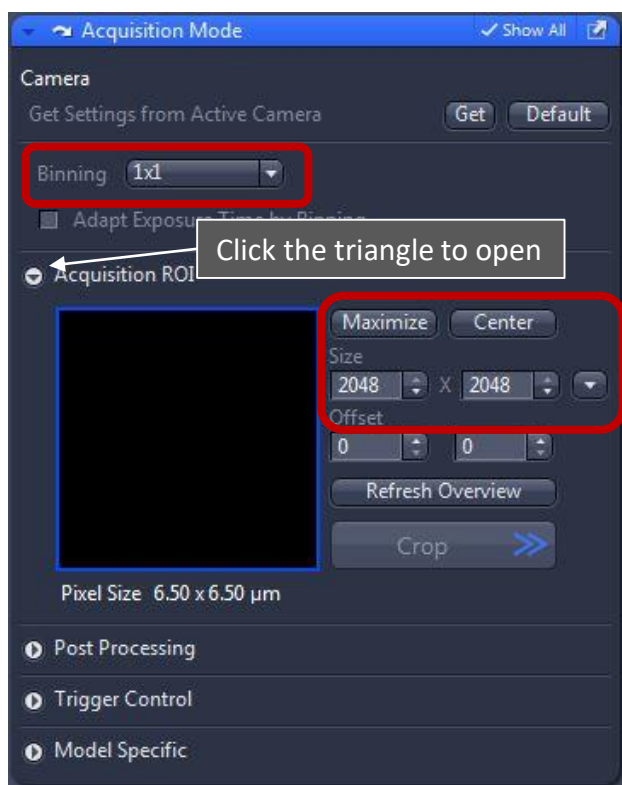
Clean oil from slides with a clean tissue (not lens tissue), then wipe residual oil away with a tissue wet with 70% IMS.



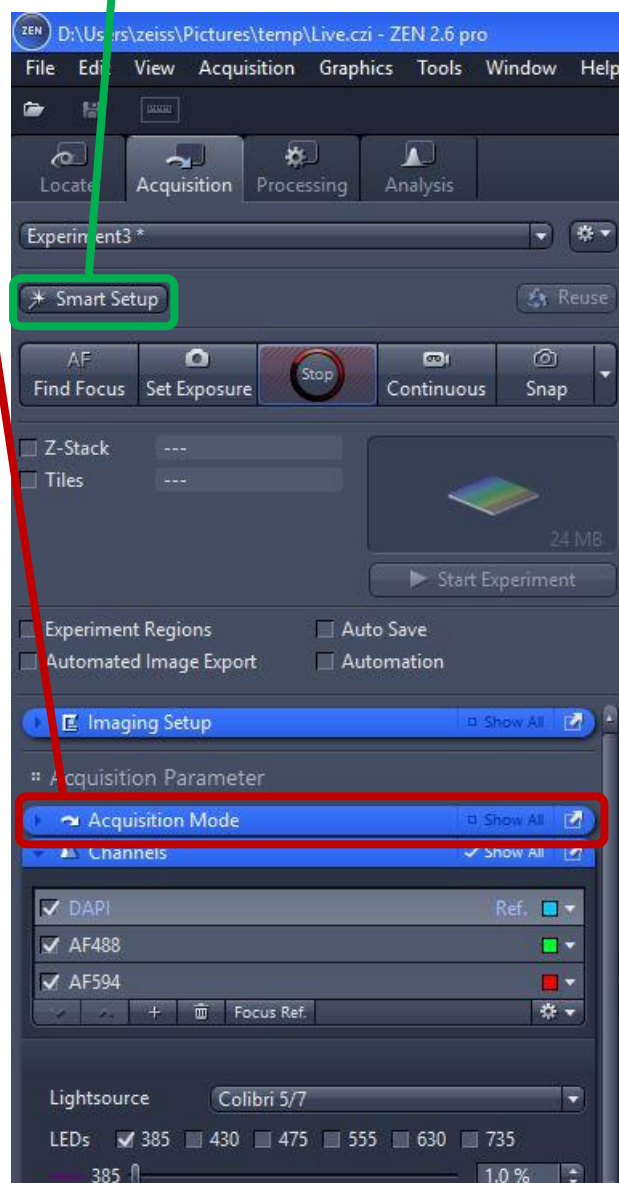
## Acquisition tab for capturing images

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?

The screenshot shows the 'Smart Setup' interface. At the top, there's a 'Configure your experiment' section with a table of channels. Below it are buttons for 'Automatic', 'Speed', 'Signal', 'Default', and 'Current'. The 'Proposals' section shows two bar charts: 'Best Signal' and 'Fastest'. A 'Dye Database' window is open, showing a list of dyes and their emission wavelengths. The 'Contrasting Methods' section is also visible.

Channel	Probe	Color
Fluorescence	DAPI	Blue
Fluorescence	Alexa Fluor 488	Green
Fluorescence	Alexa Fluor 594	Red

Dye	Emission Wavelength (nm)	Color
6-JOE	548	Green
Alexa Fluor 488	517	Green
Alexa Fluor 568	603	Red
Alexa Fluor 594	618	Red
Alexa Fluor 647	668	Red
DAPI	465	Blue

Contrasting Method
TL Brightfield
TL DIC
TL Darkfield
TL Oblique
TL Phase

**Callout 1:** Press + to open the selection window.

**Callout 2:** Select fluorophores and Add each fluorophore in your experiment. Choose TL Brightfield if needed.

**Callout 3:** Once you've entered all fluorophores, press Close.

## Smart Setup – What colours are you imaging?

Smart Setup

Configure your experiment

Contrast	Probe
Fluorescence	DAPI <span style="color: blue;">■</span>
Fluorescence	Alexa Fluor 488 <span style="color: green;">■</span>
Fluorescence	Alexa Fluor 594 <span style="color: red;">■</span>

Automatic Speed Signal Default Current

Proposals Spectra data courtesy of Pubspectra

**Best Signal** **Fastest**

Emission Signal Speed Emission Signal Speed

Crosstalk Crosstalk

T1 T2 T3 T1 T2 T3

Now you choose:  
Best Signal  
or  
Fastest  
& press **OK**

Recommended:  
Fastest  
(but see notes below)

Show Excitation  Show Emission Reset **OK** Cancel

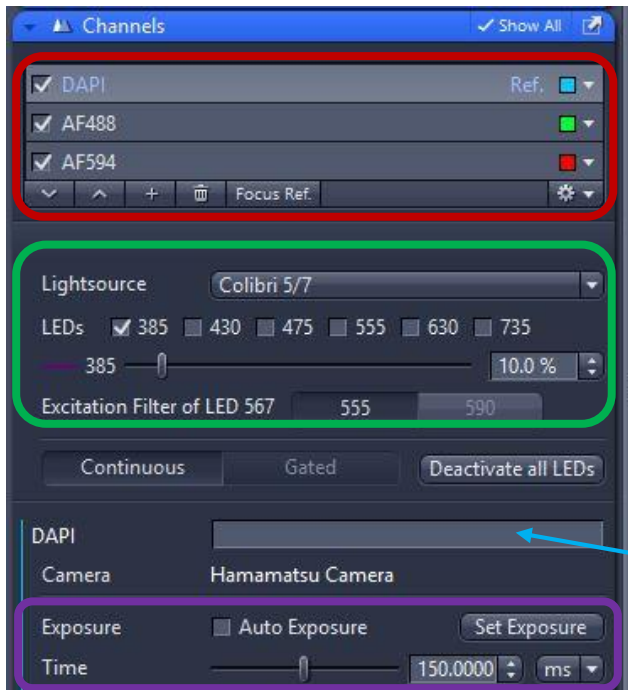
**Best signal** will use a different filter for each fluorophore wherever possible. This should result in the brightest signal, and the least possible bleed-through from other channels. It is the safest but slowest mode.

**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.

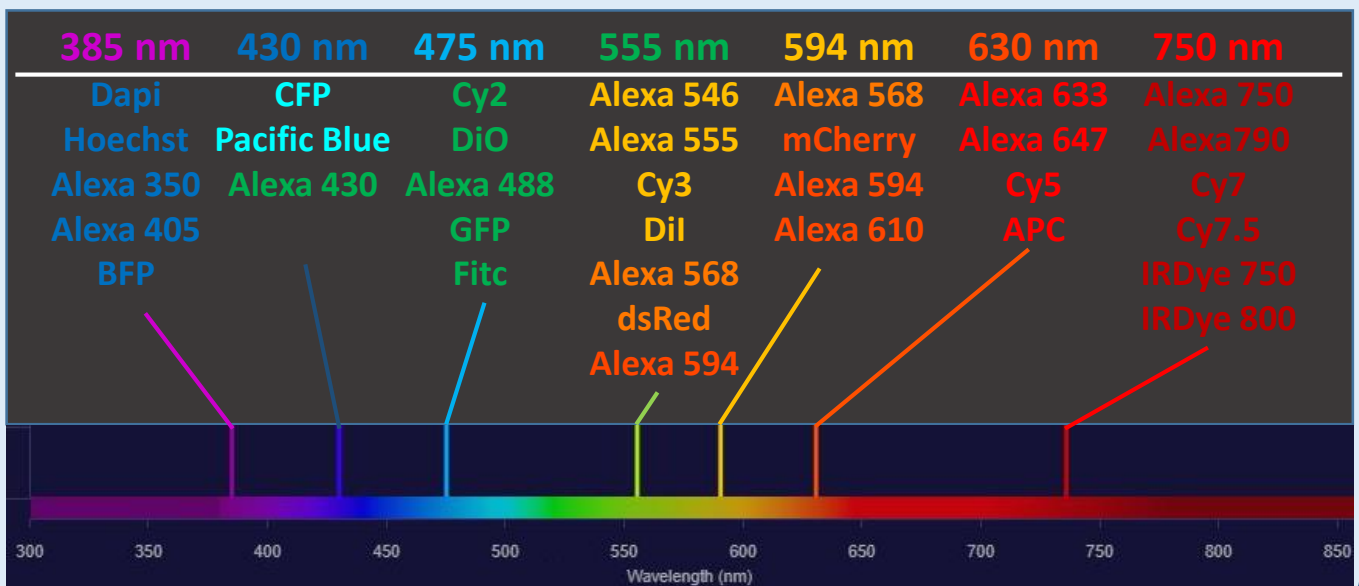
You can modify the channel name

**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.



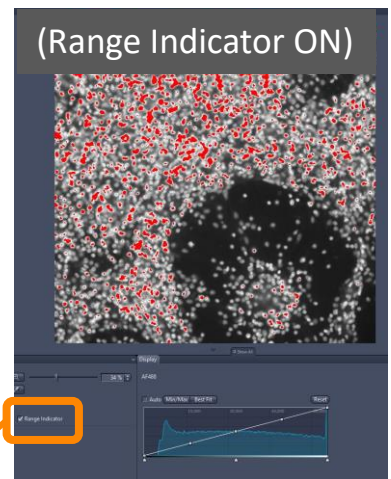
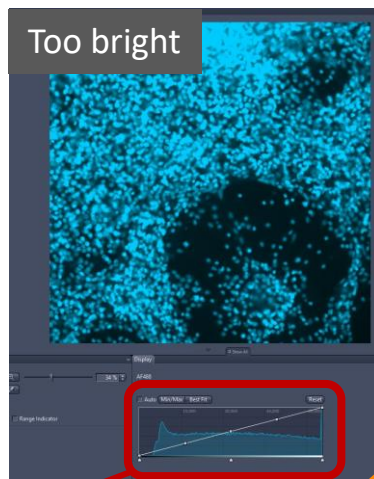
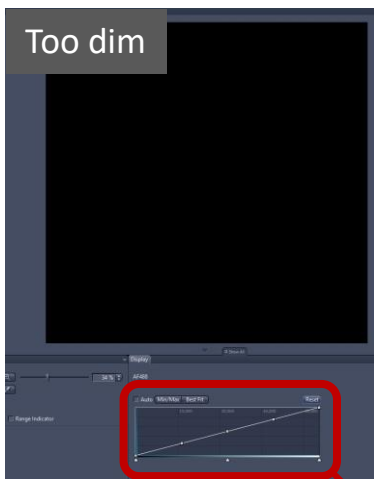


# Channels set up – Set the LED power and camera exposure time to give a visible image.



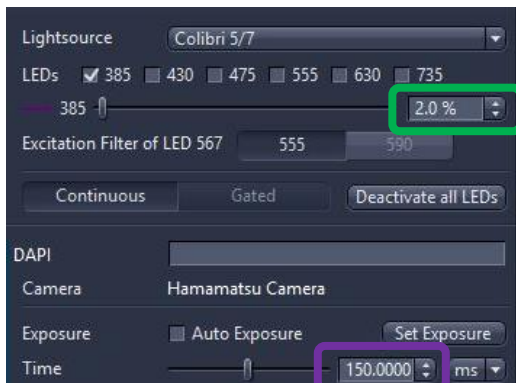
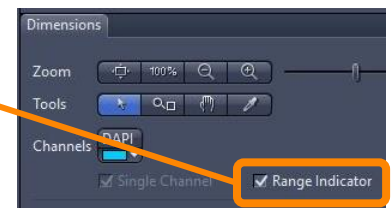
Press **Live / Stop** to see a live image.  
**Tip:** Always press Stop as soon as practical so you don't bleach your samples.

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

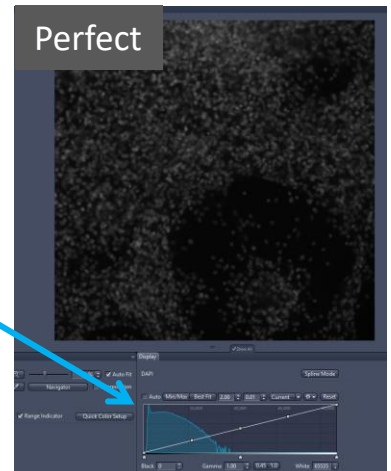


Use the image **histogram** & **Range Indicator** to set image brightness.

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

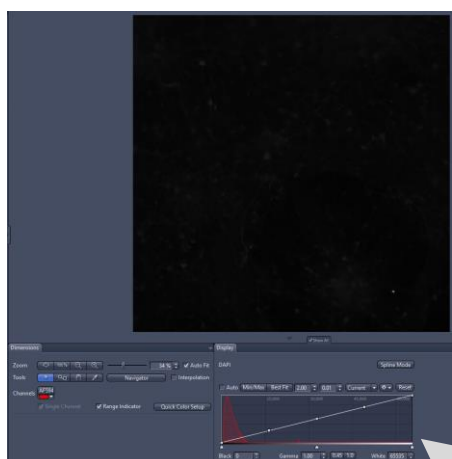


Adjust **% Power** and **Exposure Time** to adjust the brightness until the histogram covers about 1/2 of the full range.



See next page for advice on Power and Exposure Time

## Channels set up – adjust the histogram to see dim images

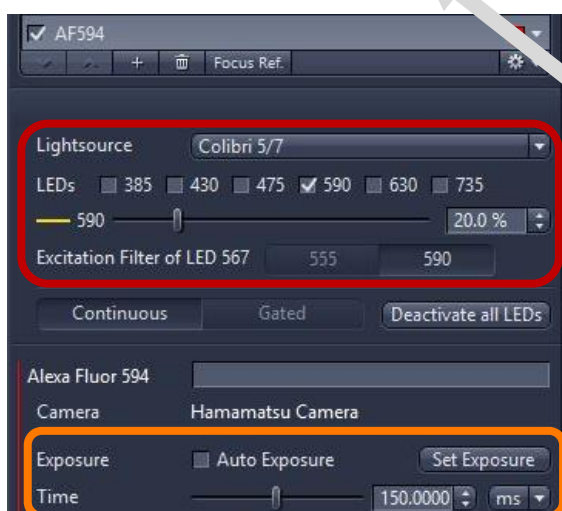
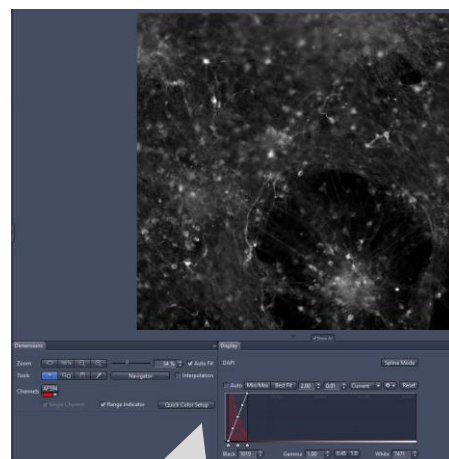


These images are taken with identical settings.

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).

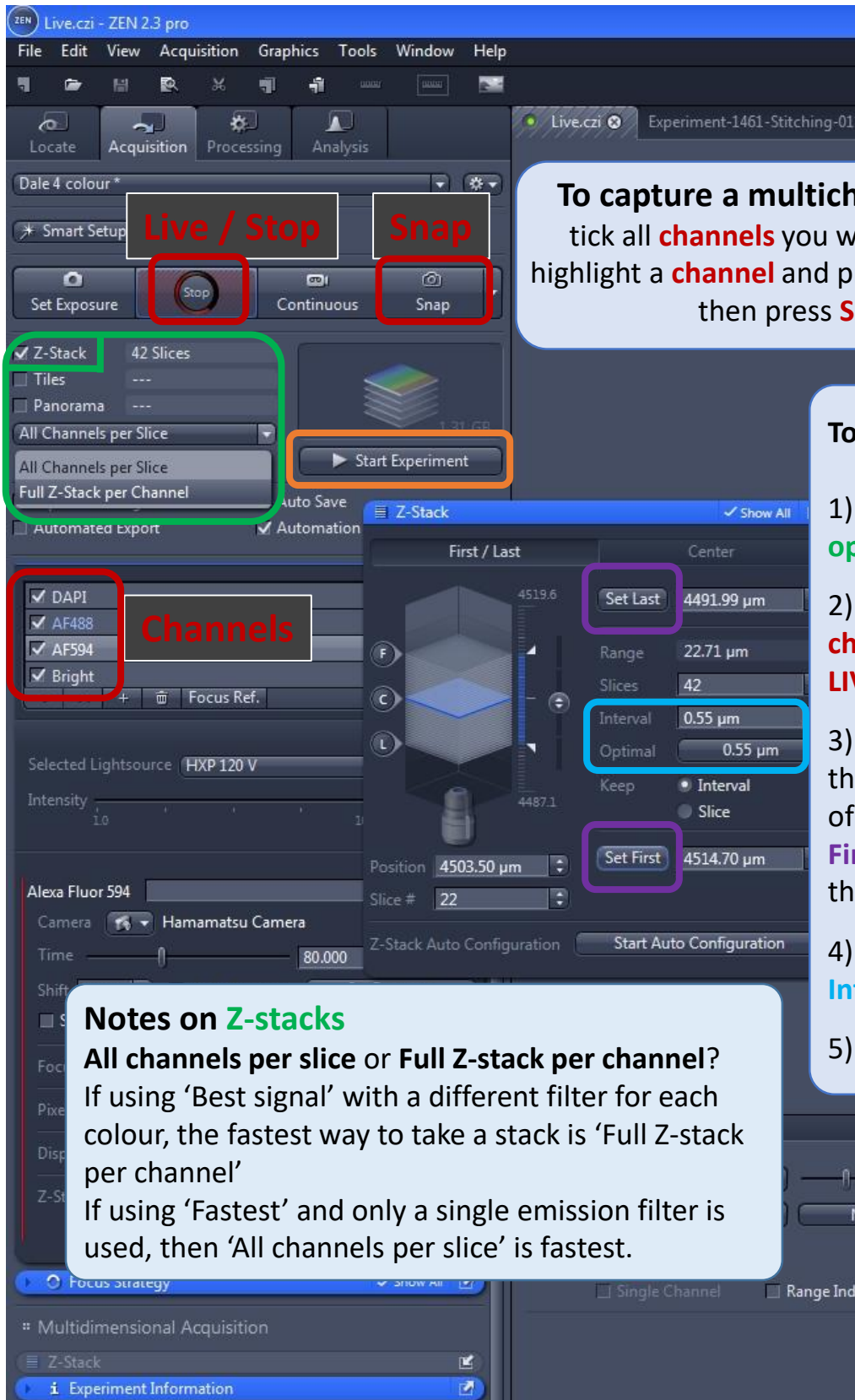
You can make an image LOOK brighter by pressing **Min/Max** or **Best Fit**.

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.

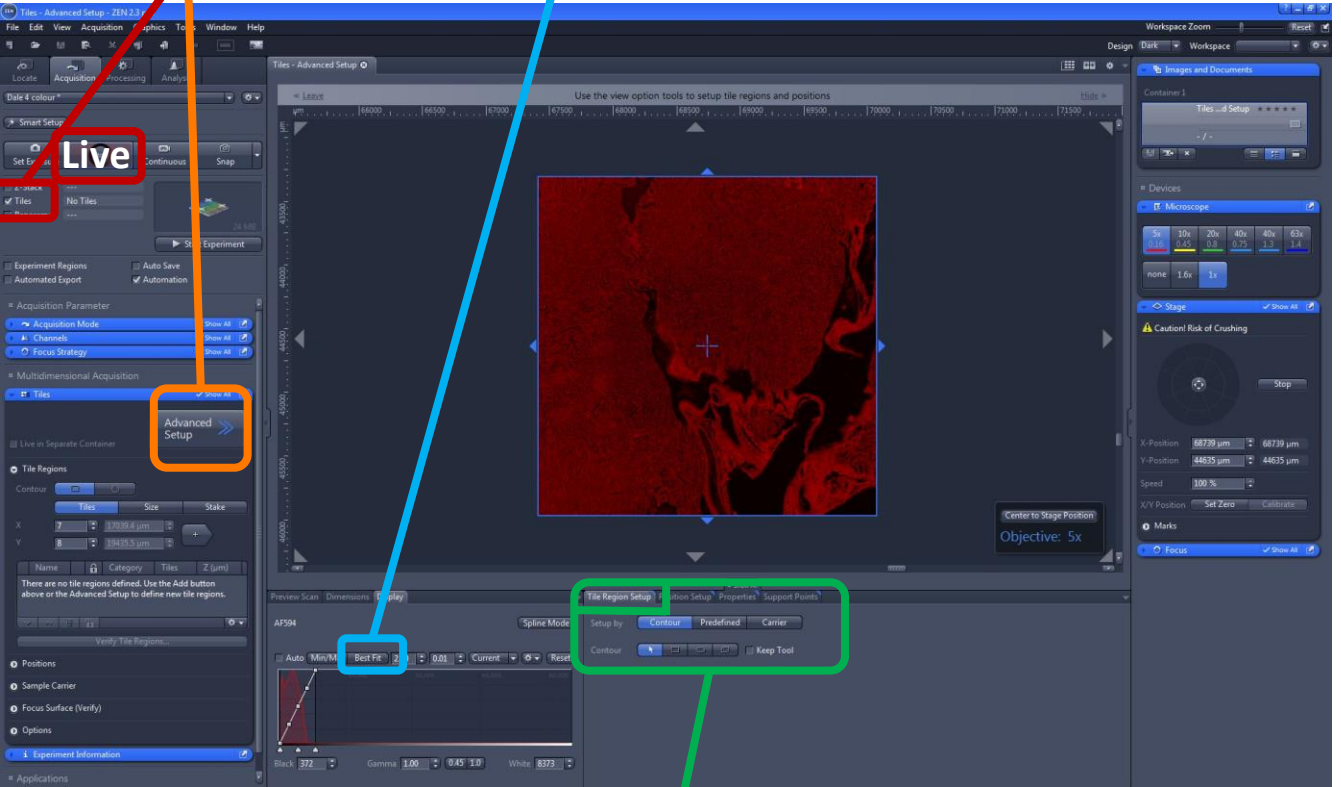
## Tile scan (9 pages)

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

Choose a tool to draw with (rectangle, oval or freehand).

Zoom out and draw a rough region to scan. Select a single channel to scan and start the preview scan.

(Mouse wheel to zoom)

Center to Stage Position  
Objective: 5x

Start Preview Scan  Delete Existing Preview Images

Objective: 5x Air 0.16 Optovar: 1x

Use Existing Experiment Settings

Channels: AF488  AF594  Bright

Camera:  Use Binning from Experiment

Tile Region Setup: Contour  Predefined  Carrier

Setup by: Contour

Keep Tool

Preview Scan: Dimensions Display

Tile Region Setup: Position Setup Properties Support Points

Setup by: Contour Predefined Carrier

Keep Tool

If the first scan is not quite right. Draw a new region and try again...  
...see next page.

Press **Best Fit** if too dark to see.

Center to Stage Position  
Objective: 5x

Use the view option tools to setup tile regions and positions

Preview Scan: Dimensions Display

Tile Region Setup: Position Setup Properties Support Points

Setup by: Contour Predefined Carrier

Keep Tool

Best Fit

Auto Min/Max Best Fit 00 0.01 Current Reset

Gamma: 1.00 0.45 1.0 White: 11756

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

1<sup>st</sup> guess

New region

Regions are called TR1, TR2 etc. Highlight the ones you don't want to scan and press the trash to delete them. Only Ticked regions are scanned.

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

Name	Category	Tiles	Z (µm)
<input checked="" type="checkbox"/> TR1	Default	15	5027.0
<input checked="" type="checkbox"/> TR2	Default	12	5027.0

Deletes the selected tile regions

Microscope

5x 10x 20x 40x 40x 63x  
0.16 0.45 0.8 0.75 1.3 1.4

none 1.6x 1x

Stage

Caution! Risk of Crushing

Stop

X-Position 68740 µm 68740 µm  
Y-Position 44635 µm 44635 µm  
Speed 100 %

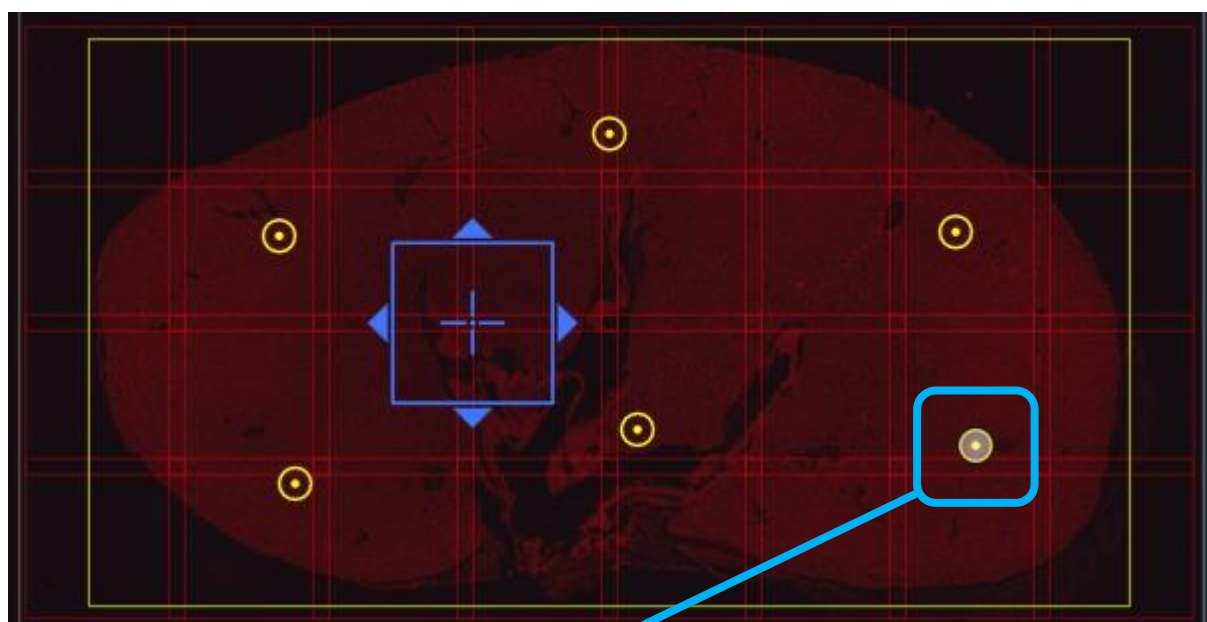
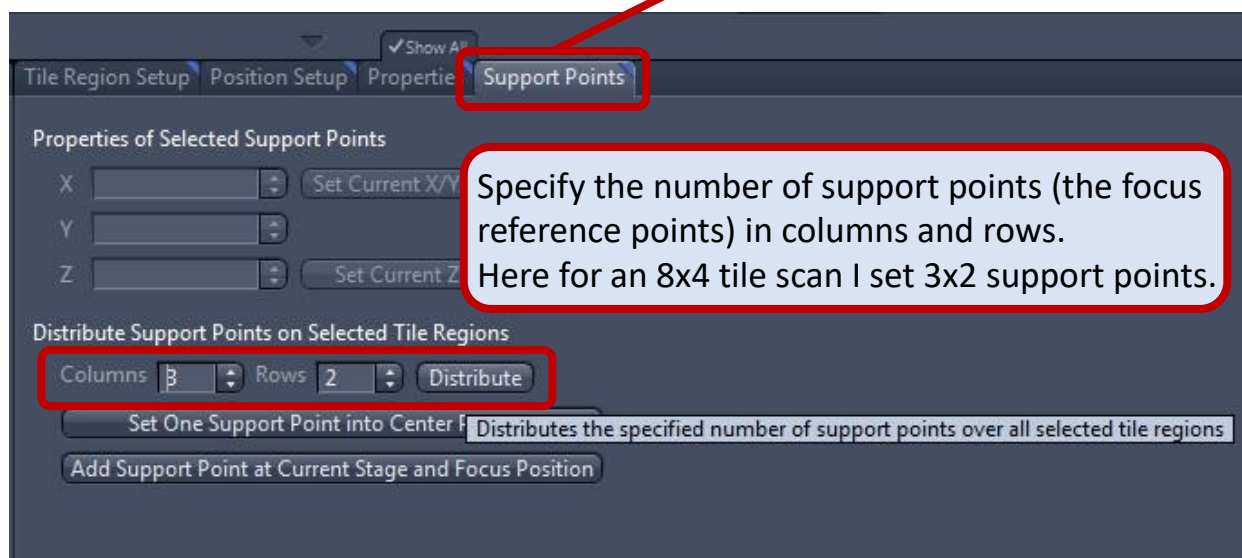
## Tile scan (4/9) ... make a focus map – set support points

**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.



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...**

Use the view option tools to setup tile regions and positions

Advanced Setup

Live in Separate Container

Tile Regions

Contour

Tiles Size Stake

X: 7 8519.7 µm

Y: 8 9717.8 µm

TR2 Default 32 5027.0

Verify Tile Regions...

Positions

Sample Carrier

Focus Surface (Verify)

Local (per Tile Region)

Support Points of Selected Tile Region: TR2

X (µm)	Y (µm)	Z (µm)
67128.9	43909.8	5027.0
69877.7	43060.1	5027.0
72759.8	43876.5	5027.0
67262.1	45970.0	5027.0
70110.9	45520.2	5027.0
72926.4	45653.5	5027.0

Verify Tile Regions/Positions...

Shows a dialog to conveniently verify the z position of all activated tile regions (incl. support points) and/or positions.

Interpolation Degree: 2 - Parabolic Saddle Surface (at least 9 support points)

Options

Properties of Selected Support Points

X: 72926.4 µm Set Current X/Y/Z

Y: 45653.5 µm

Z: 5027.0 µm Set Current Z

Distribute Support Points on Selected Tile Regions

Columns: 3 Rows: 2 Distribute

Set One Support Point into Center Position


Add Support Point at Current Stage and Focus Position

Center to Stage Position

Objective: 10x



## Tile scan (6/9) ... set focus on all support points

A new window opens: 

Click **Move to Current Point**

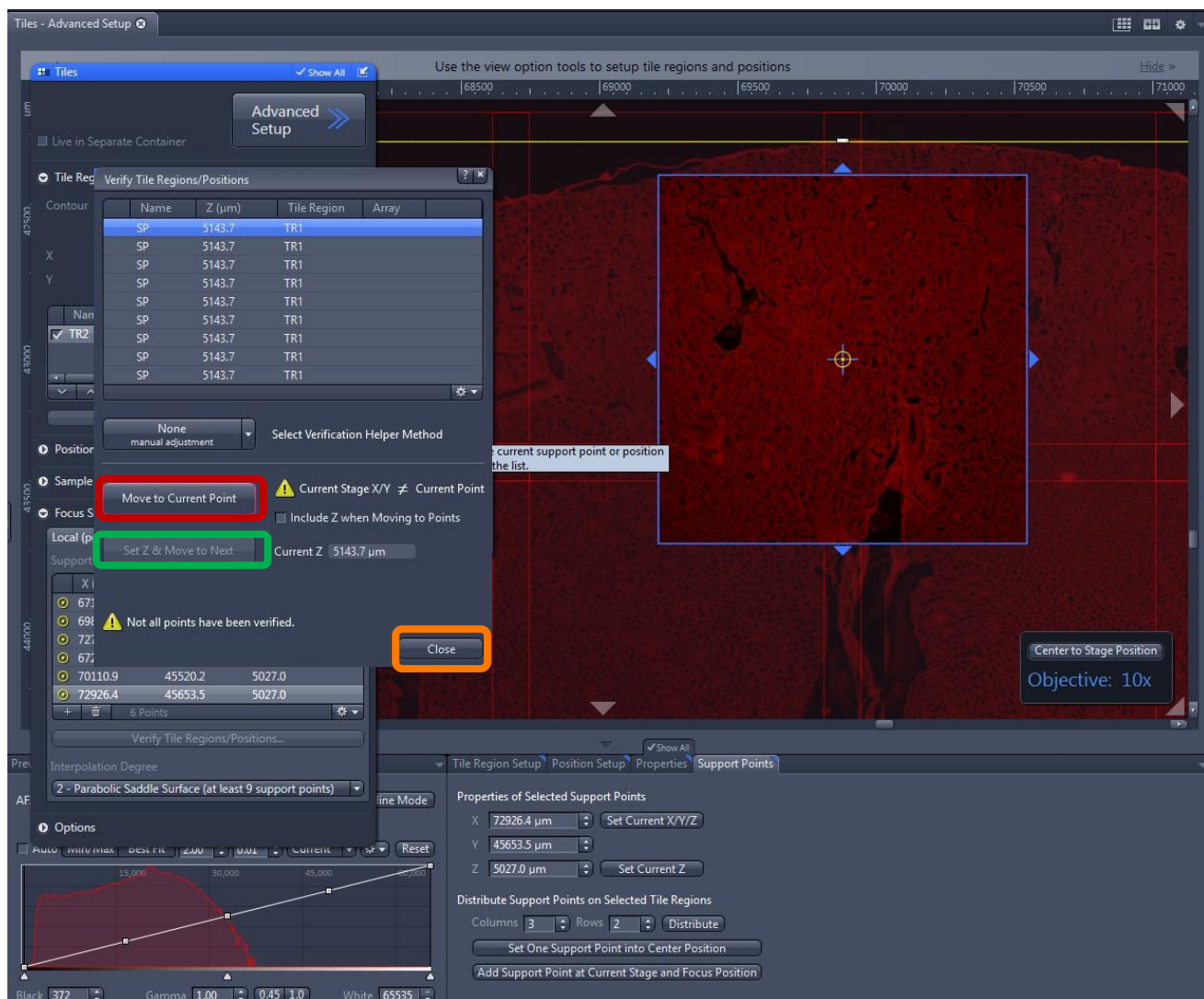
Press  to see the desired channel, adjust the focus, then

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.



The screenshot displays the 'Verify Tile Regions/Positions' dialog box in the Zeiss software. The dialog contains a table with the following data:

Name	Z (µm)	Tile Region	Array
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	
SP	5143.7	TR1	

Below the table, the 'Position' is set to 'None manual adjustment'. The 'Sample' section has 'Move to Current Point' highlighted with a red box. The 'Focus' section has 'Set Z & Move to Next' highlighted with a green box. The 'Local (p)' section shows 'Current Z: 5143.7 µm'. A 'Close' button is highlighted with an orange box. The background shows a fluorescence image with a grid of support points. A tooltip points to a support point with the text 'current support point or position the list.' The bottom right of the image shows 'Center to Stage Position' and 'Objective: 10x'.

## Tile scan (7/9) ... Capture the image

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

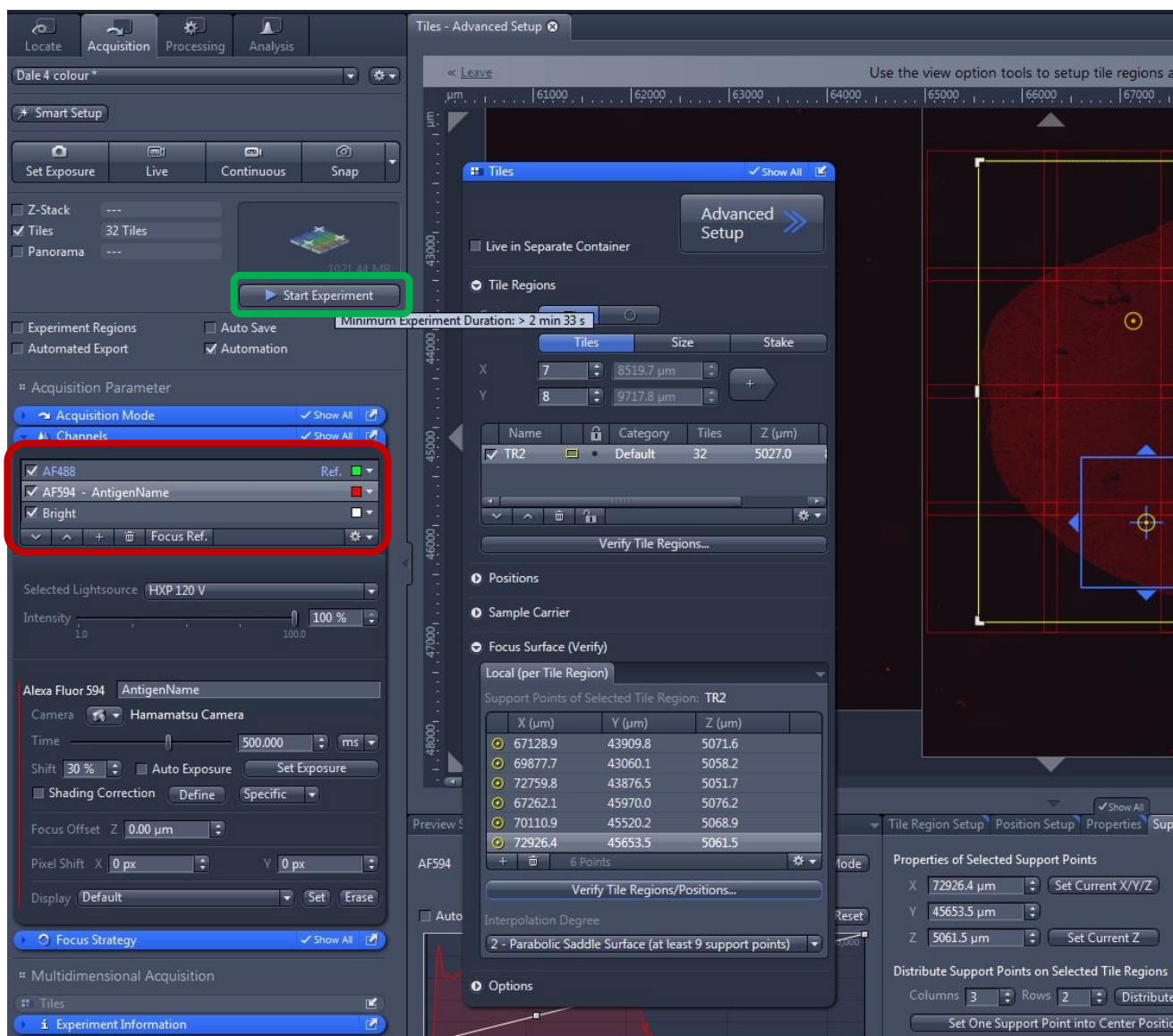
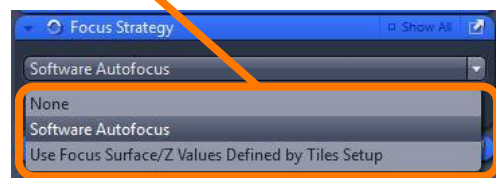
Select the appropriate Focus Strategy in the **Focus Strategy** window

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

or

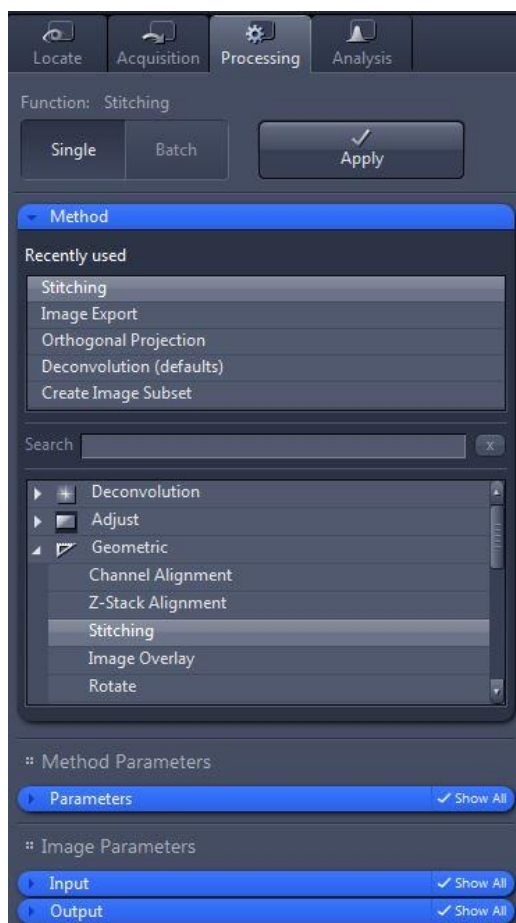
- 'Software Autofocus' if no focus map was made.  
(for software autofocus see page 21)

Press: **Start Experiment**



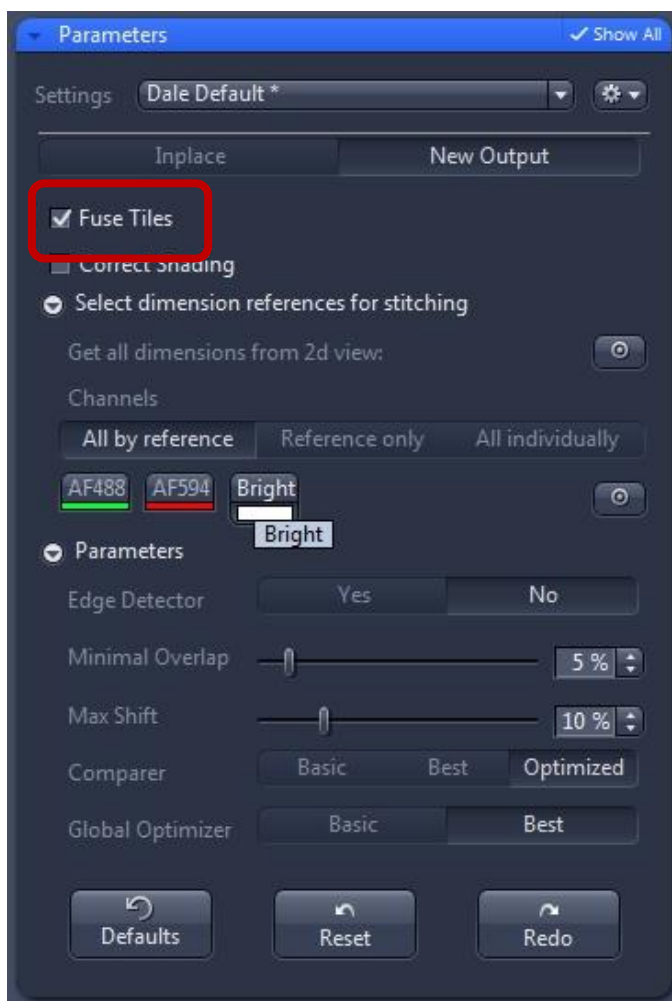
## Tile scan (8/9) ... Stitching the image

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.



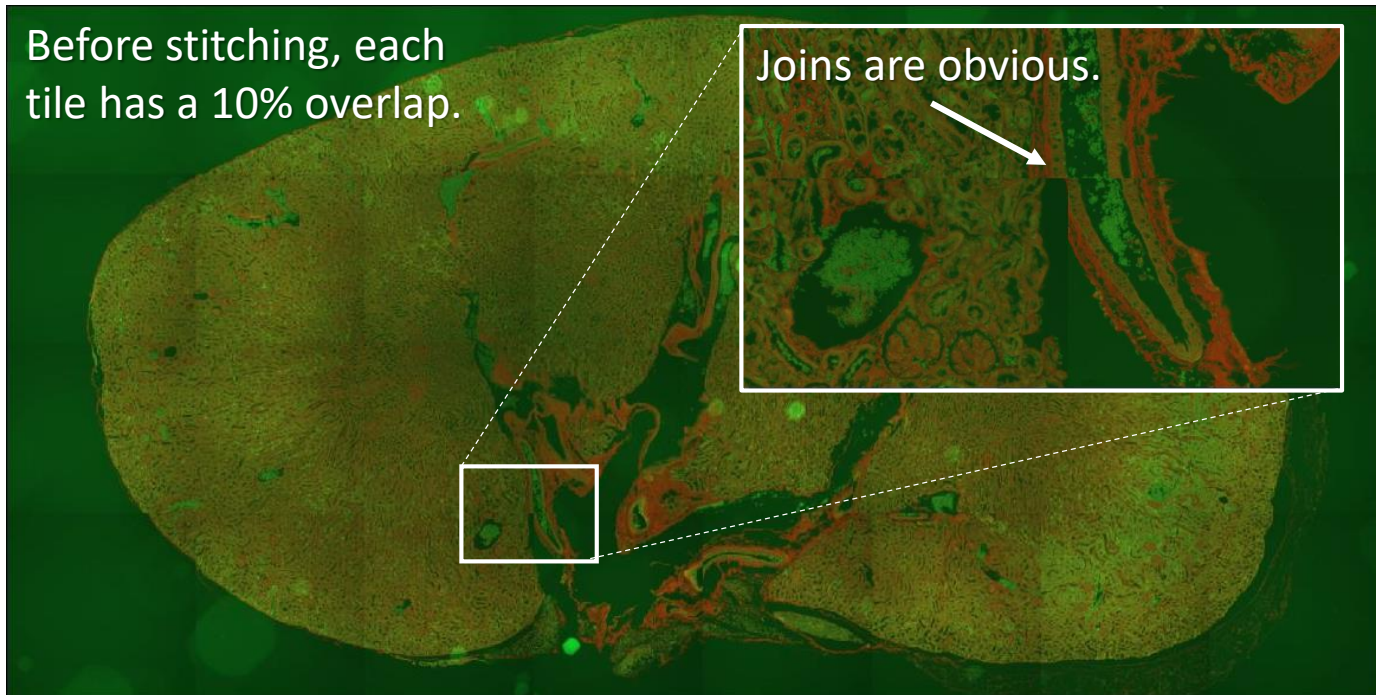
By default it will use the first channel captured to align the image, (normally Dapi, but in this example 488) but you can choose any channel that has sufficient content to perform the stitching.



## Tile scan (9/9) ... Finished, save the raw and stitched Images

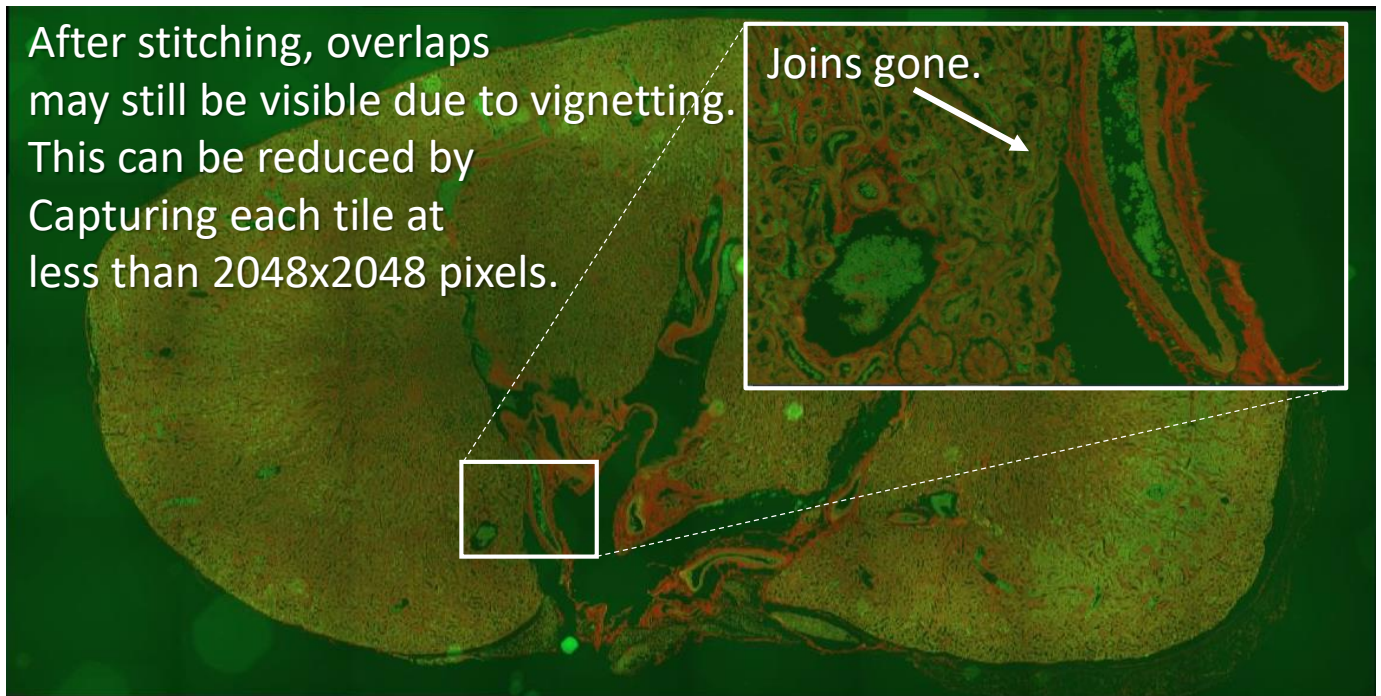
Before stitching, each tile has a 10% overlap.

Joins are obvious.



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

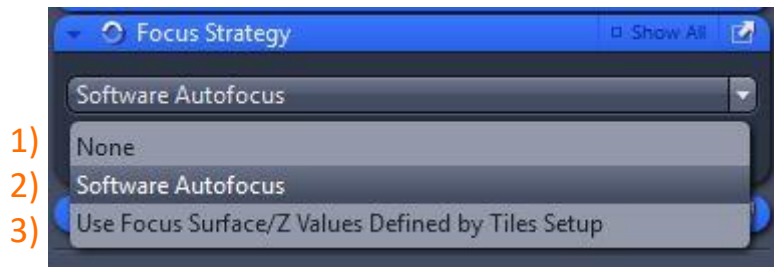
Joins gone.





# 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

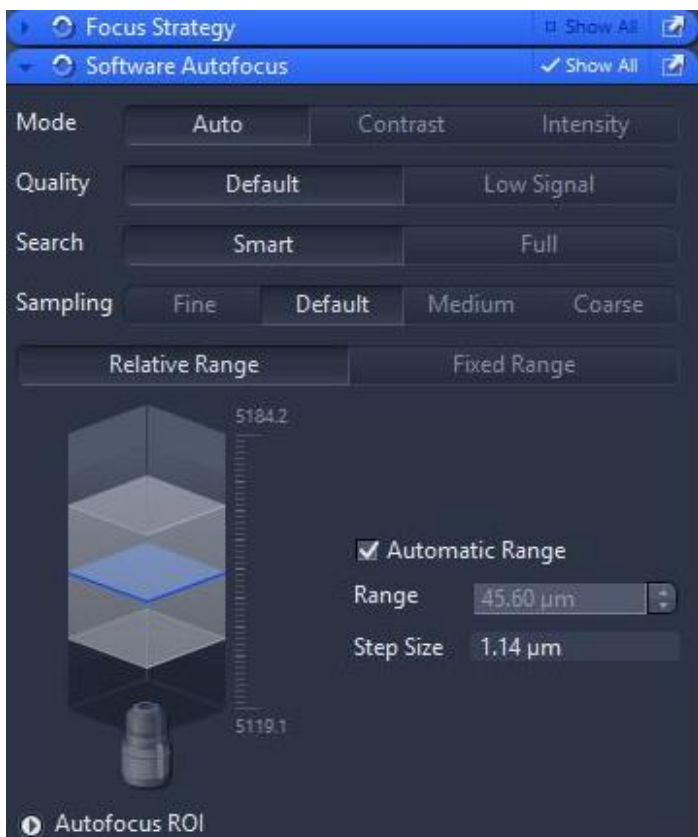
**Sampling:** Default

**Quality:** Default

**Relative Range**

**Search:** Smart

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.


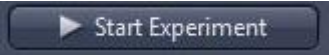
**Quality** can be set to low signal if brightness is exceptionally low.

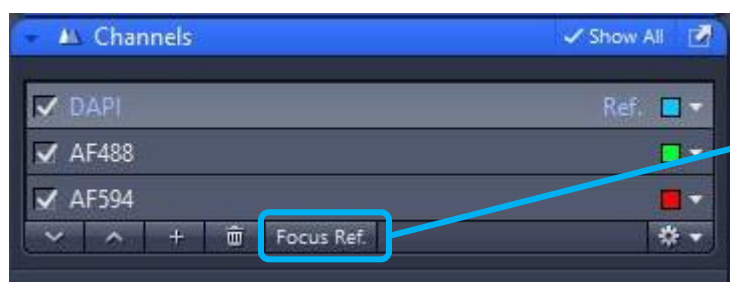
**Search** switches between the slower 'Full' mode, where it moves to the bottom of the set range and scans the whole range to find the best focus. 'Smart' is faster, and moves up and down from the last position until it finds good focus.

**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 your 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) may be bleached if it takes a long time to find the correct focus.

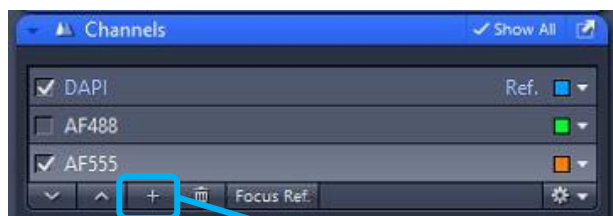
If your exposure time and LED power are low, bleaching is unlikely. You may wish to choose your brightest channel as Focus Ref.

Alternatively, it is possible to set up an extra channel just for autofocusing.

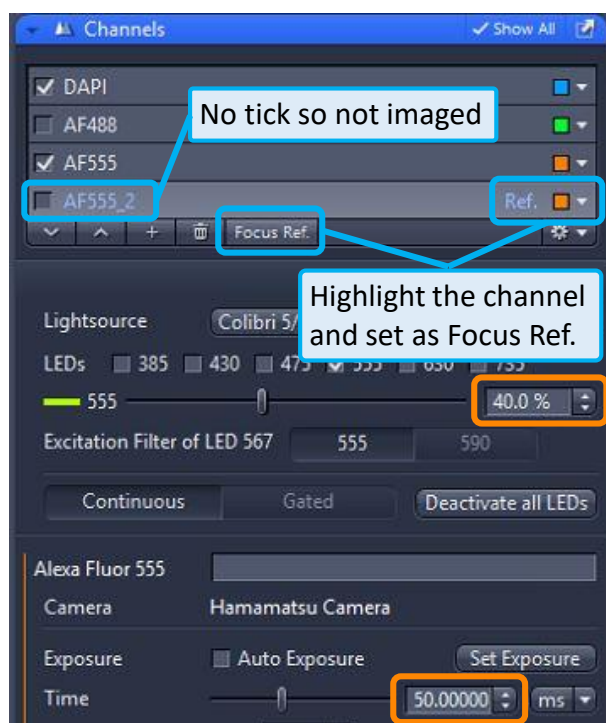
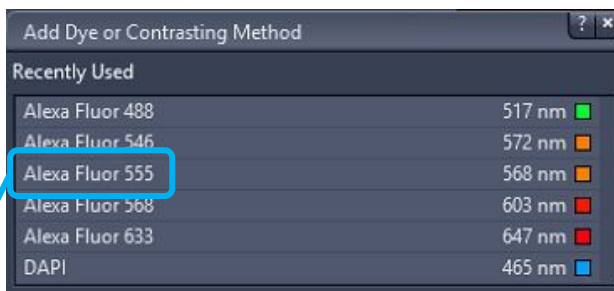
See next page for details. As long as there are >1000 grey levels (image brightness) the autofocus will work. Set this channel as the Focus Ref, but do not tick the channel, this way it won't be saved with your image.

## 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).



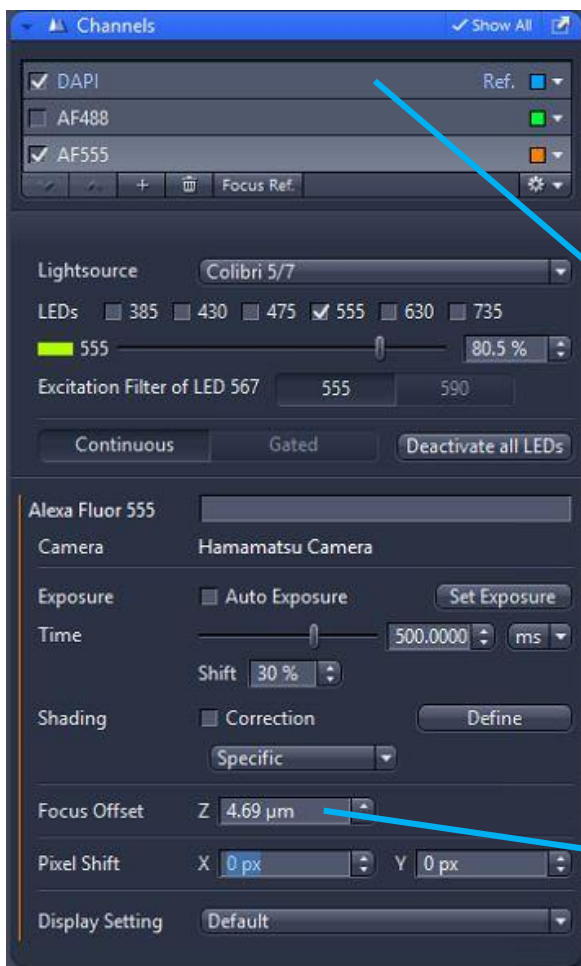
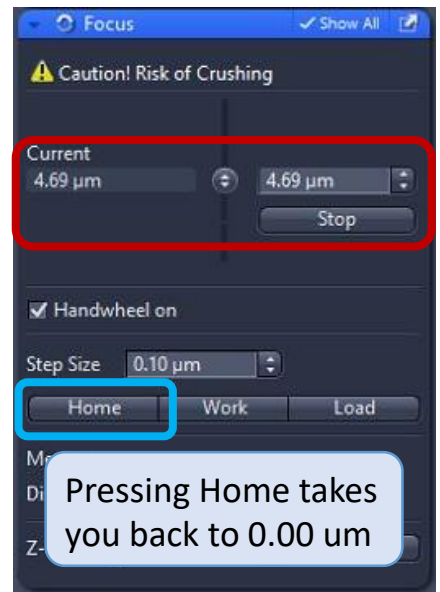
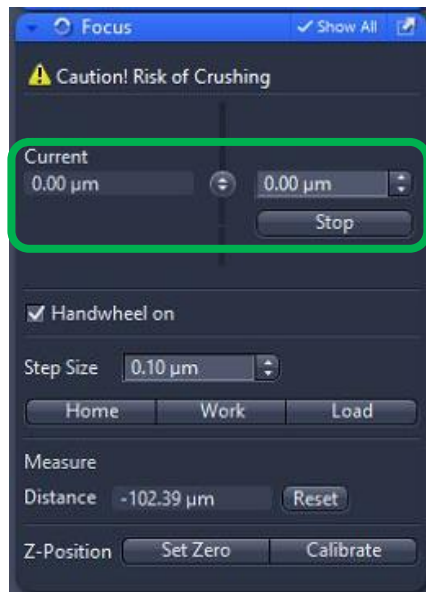
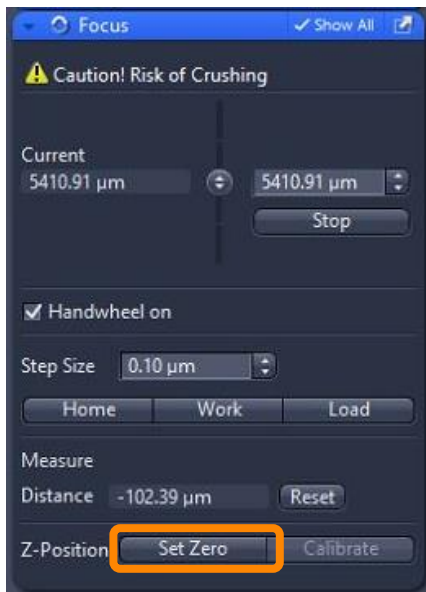
This set up captures Dapi and 555 (the 488 channel is not ticked so not captured).

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 1/20<sup>th</sup> of the light (1/2 power for 1/10<sup>th</sup> 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!

