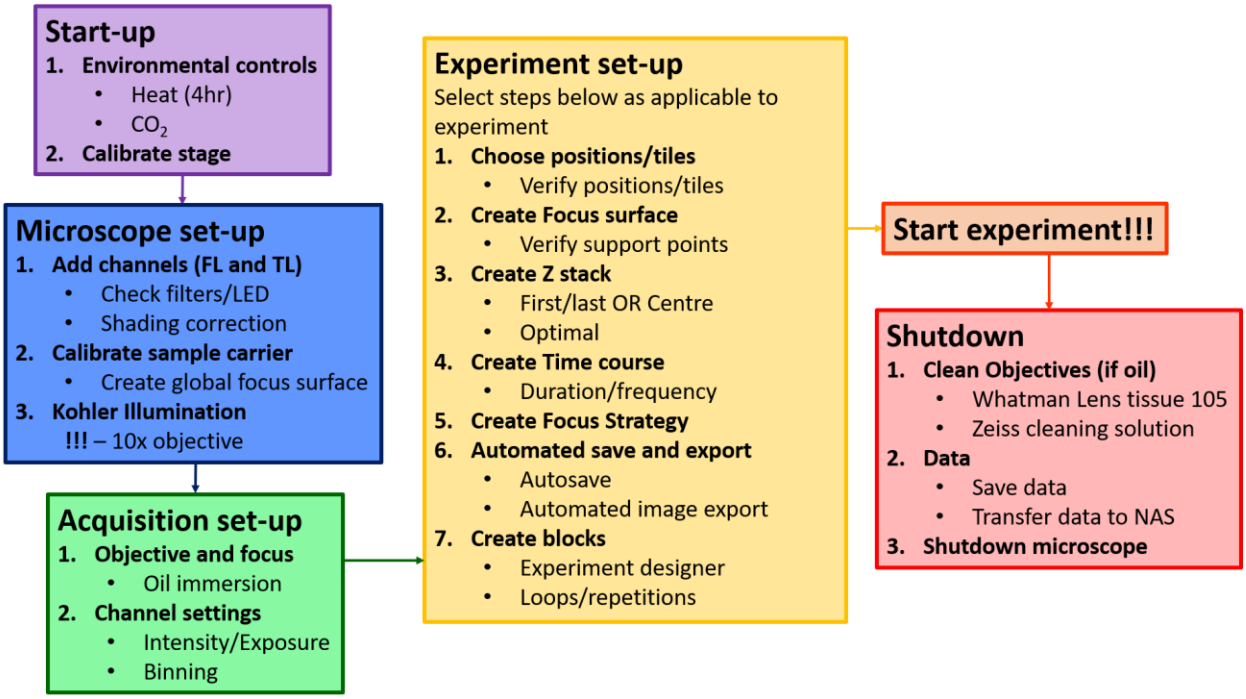
Axio Observer Handbook

Jessica Rowley

HTSCA Facility

Contents

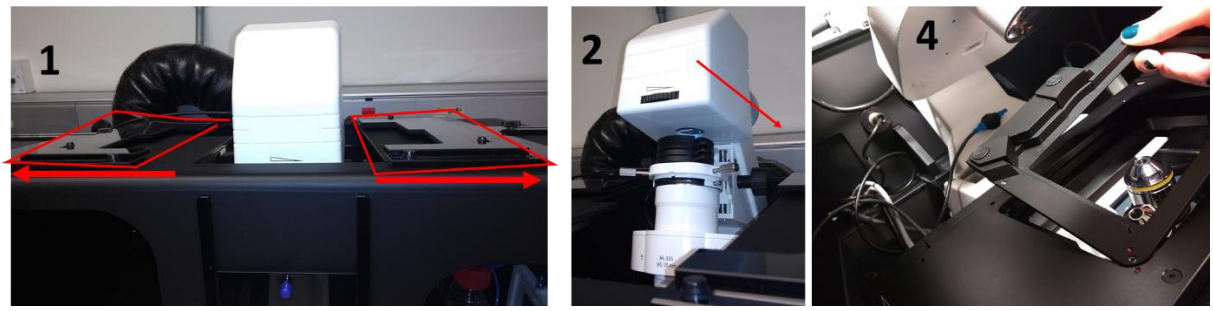
Experiment workflow



Start-up

Change plate insert

1. Open slide *clips* on top of environmental chamber by sliding outwards (A).
2. Push *condenser turret* back (B).
3. Remove slide insert.
4. Align *red dot* on insert with *front-left corner* and ensure clips on left and bottom are depressed. Place insert in and check it lies flush.
   Note: no red dot on CO_2 plate insert.



Insert guide

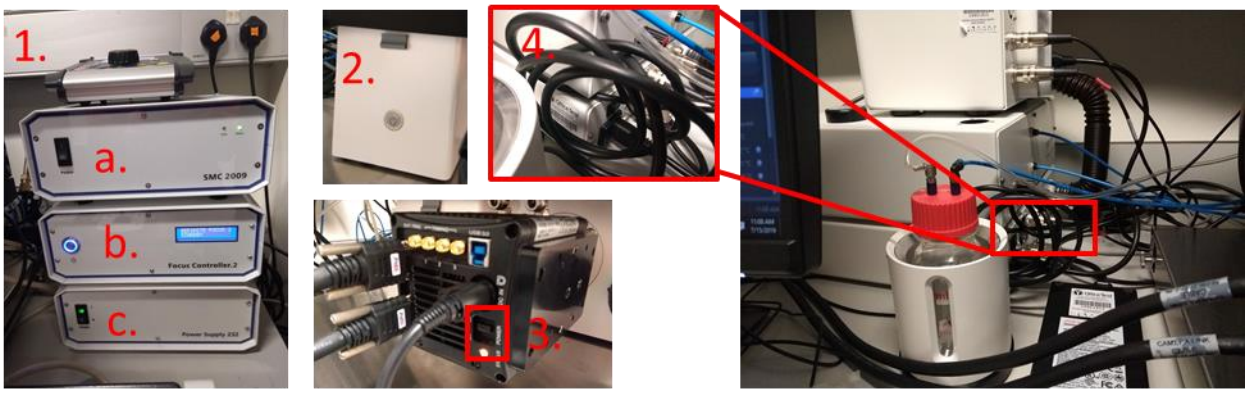
Inserts are kept in *'really useful box'* on bench opposite CD7.

Incubation

- If heating is required switch on at least *30 mins* (*optimal 4hr*) prior to experiment.
- Heating will be left on if microscopes are in use continuously.
- Coordinate with fellow users.
  !!! Ensure correct insert plate is in whilst pre-heating – can warm inside chamber if previous user requires different insert.

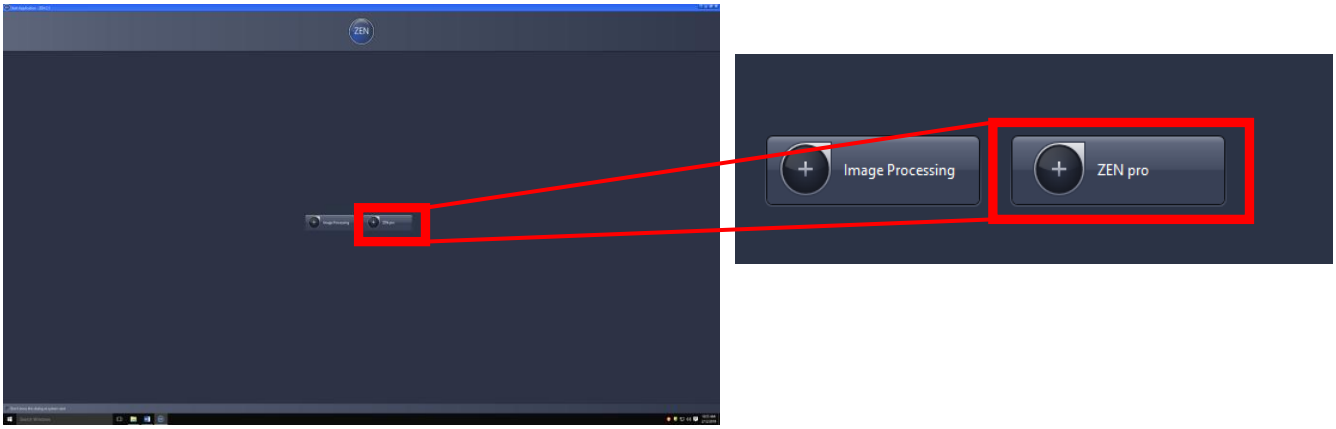
Switch-on microscope

1. Switch on *power boxes* from top to bottom (must be switched on before touchpad).
    a. **'SMC 2009'** = stage control.
    b. **'FOCUS CONTROLLER'** = Definite Focus.
    c. **'POWER SUPPLY 232'** = microscope.
2. Switch on *microscope* (silver power button on left side).
3. Switch on *camera* (slide switch on black box – left of microscope).
4. Switch on *environmental control module.*
    a. Top box = CO_2.
    b. Bottom box = Heat.
5. Switch on *computer* and log-in (college log-in).



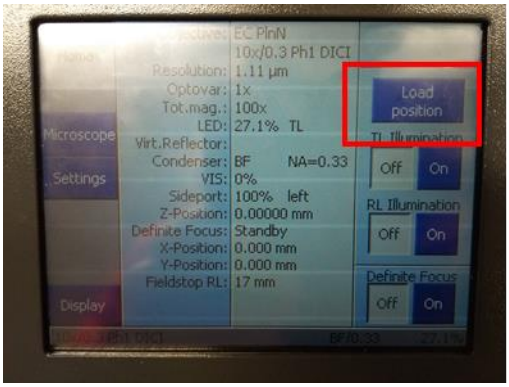
!!! All hardware must be switched on **before** opening software.

6. Open '**ZEN BLUE 2.6**' and log in.
7. Select '**ZEN PRO**'.



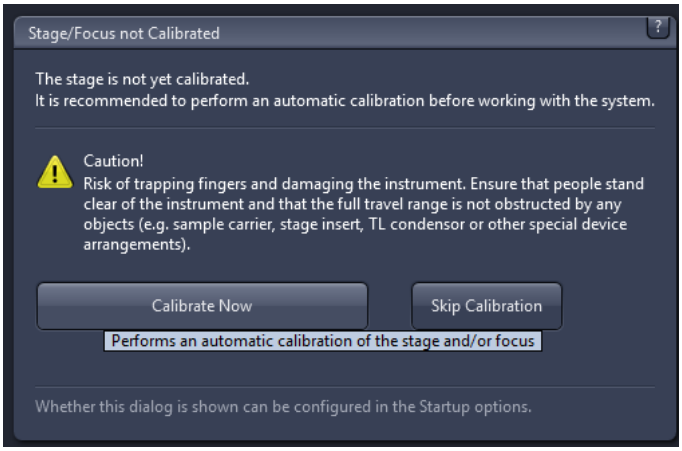
Stage calibration

- **!!!** Ensure objective is on '**LOAD POSITION**'.



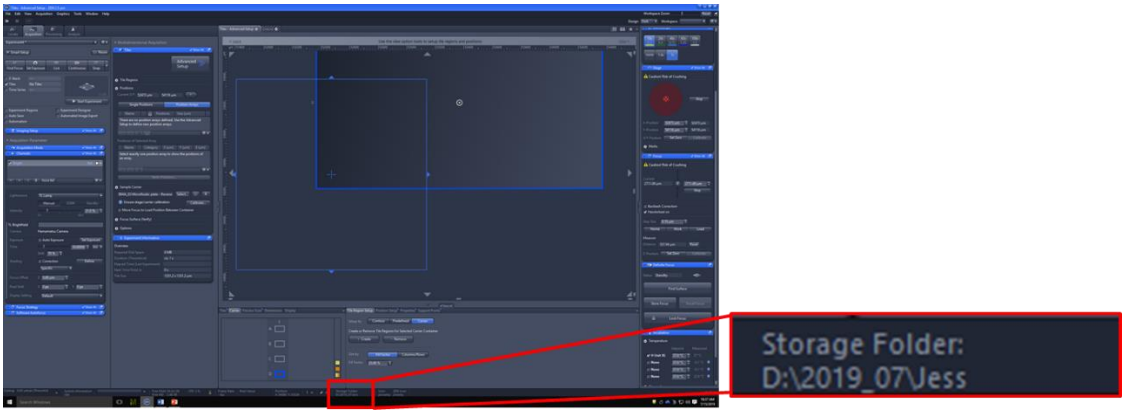
!!! Ensure nothing at side of stage to crash into.

- Click **'CALIBRATE NOW'**.

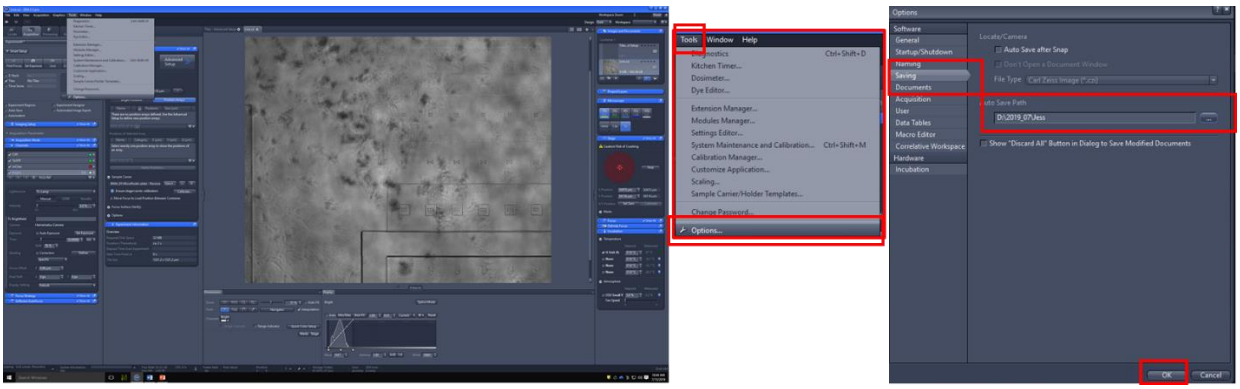


Change auto save file path

- Check storage path
- **!!!** Should be **D:\ USERNAME** e.g. **D:\Jess**



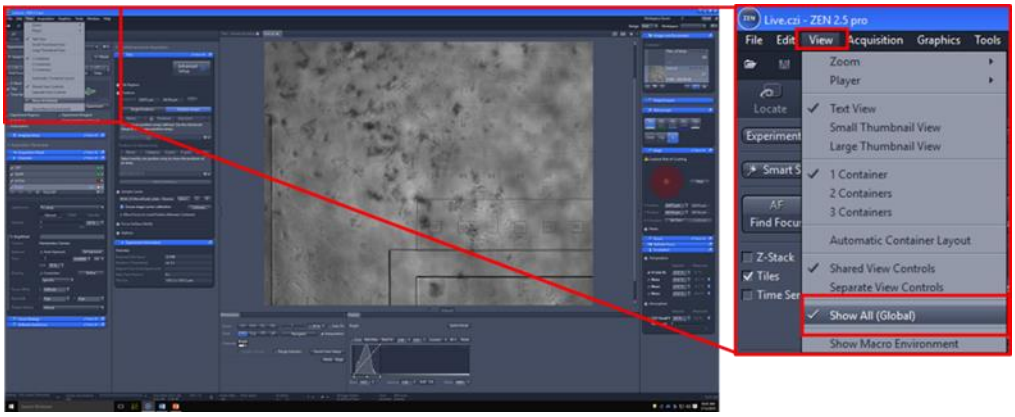
- To change: **TOOLS > OPTIONS > SAVING > AUTO SAVE PATH**



Global 'Show all'

To show all hidden options:

1. Click **'VIEW'**.
2. Tick **'SHOW ALL (GLOBAL)'**.



Environmental controls

- Environmental controls can be accessed via the **'INCUBATION'** tool.

Heating

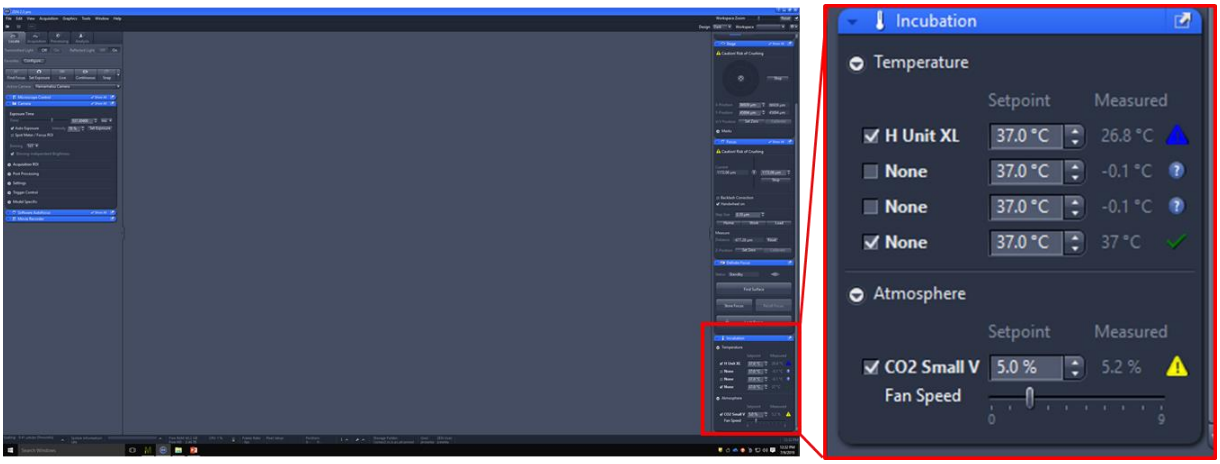
- Under **'TEMPERATURE'** tick **'H UNIT XL'** and input desired temperature in **'SETPOINT'**.

Carbon Dioxide

- Under **'TEMPERATURE'** tick the bottom **'NONE'**. This warms the humidifier jacket for CO_2 water.

- Under **'ATMOSPHERE'** tick **'CO_2 SMALL V'** input desired % (usually 5%). Turn on *fan* (speed 2).

- Check **'MEASURED'** temp/CO_2 is correct before starting experiment.



Humidification

Ensure humidifier bottle contains enough water (*between min and max*). Refill with *sterile dH20*. (On shelf opposite CD7).

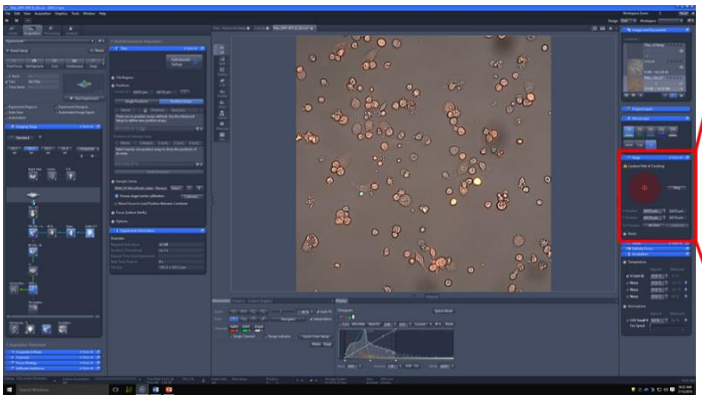
Basics

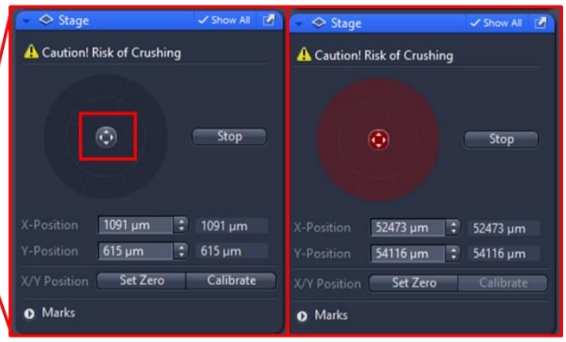
Navigation

Joystick

Stage Tool

- Drag *stage controller button* in direction of travel.
- *Right click* on stage controller button to *increase speed* of movement (turns red).
- OR input x/y coordinates.

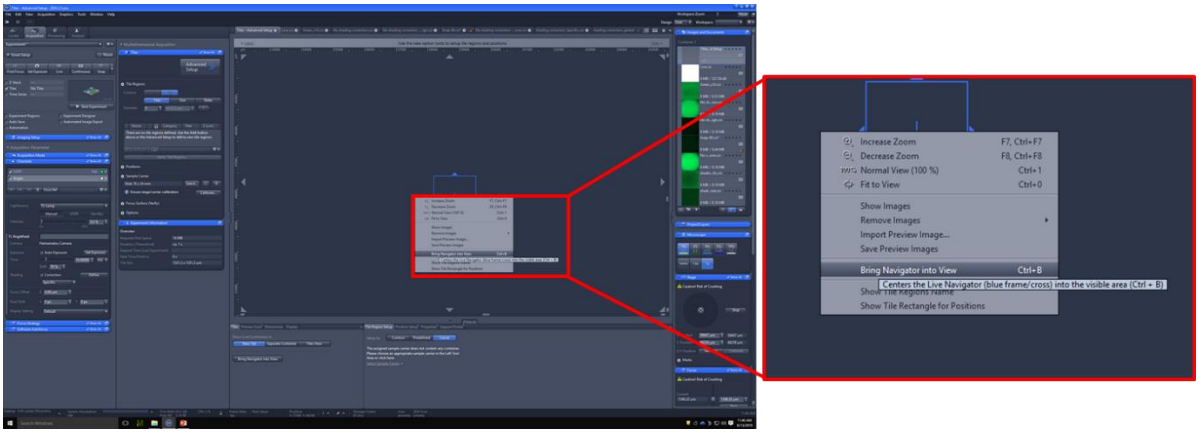




Bring navigator into view

If you lose the *navigator*: 

1. *Right click* in navigation map.
2. Click **'BRING NAVIGATOR INTO VIEW'**.



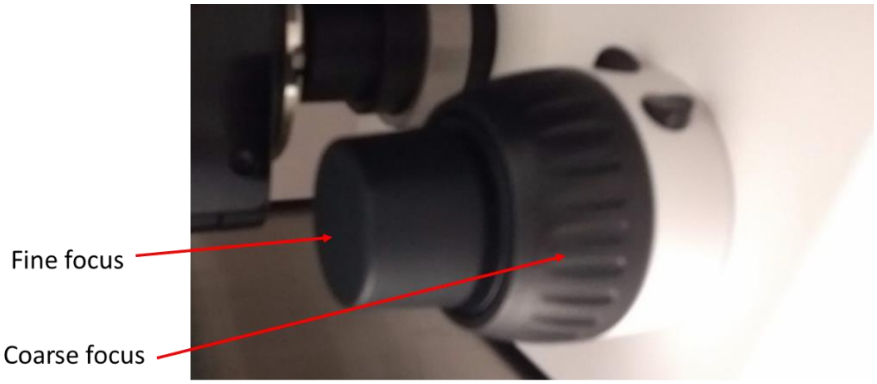
Focusing

!!! Start at low magnification and work up

Manual

Focus knobs

Fine and *coarse* focus knobs on microscope (turn knob away from you to move closer to sample, toward you to move away from sample)

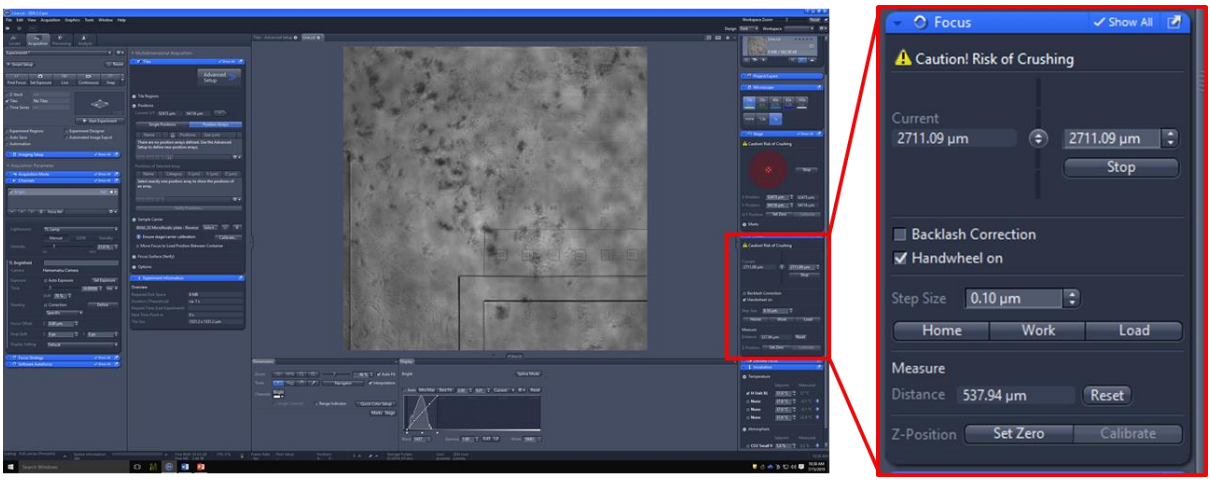


Mouse.

Hold *CTRL* and *scroll* (up to move closer to sample, down to move away from sample). Change increments to alter speed of focus.

Focus Tool.

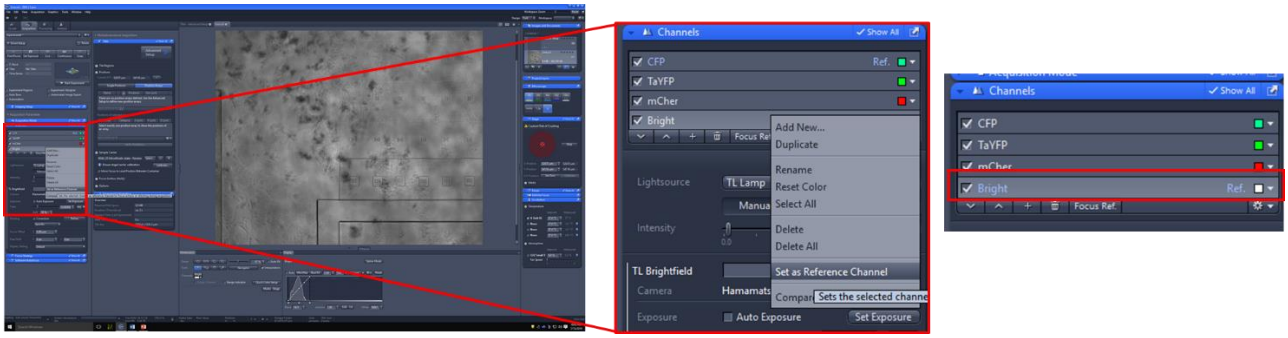
Focus tool in ZenBlue (input value or use slider bar).

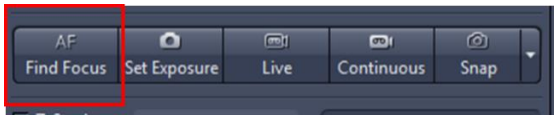


Software Autofocus

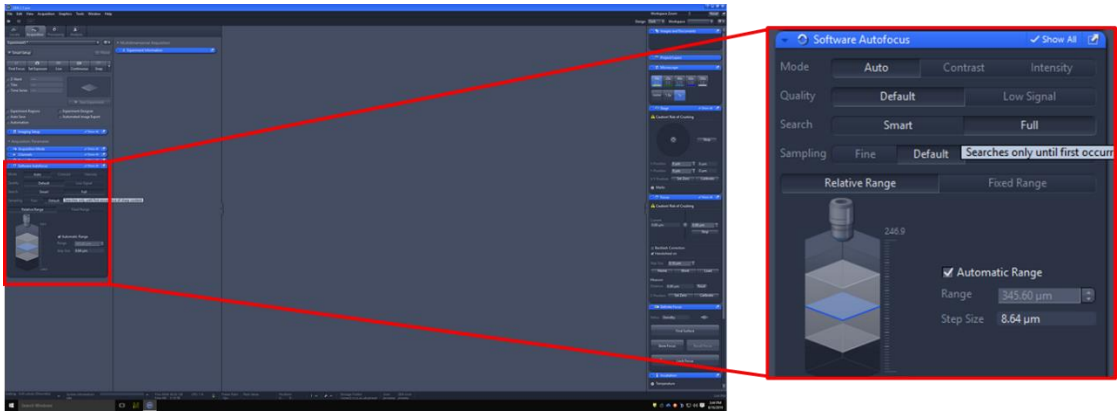
Note: Software Autofocus will use whichever channel is set as the *reference*. A signal will be required in each position if this method is to be used as a focus strategy. The software will move through z-planes until *maximum image sharpness* is found. Although *contrast* is default sharpness parameter – *intensity* can be used if desired.

1. Change *reference* channel, under **'CHANNELS'** tool
    I. Right-click on channel to be used as reference
    II. Click **'SET AS REFERENCE CHANNEL'**





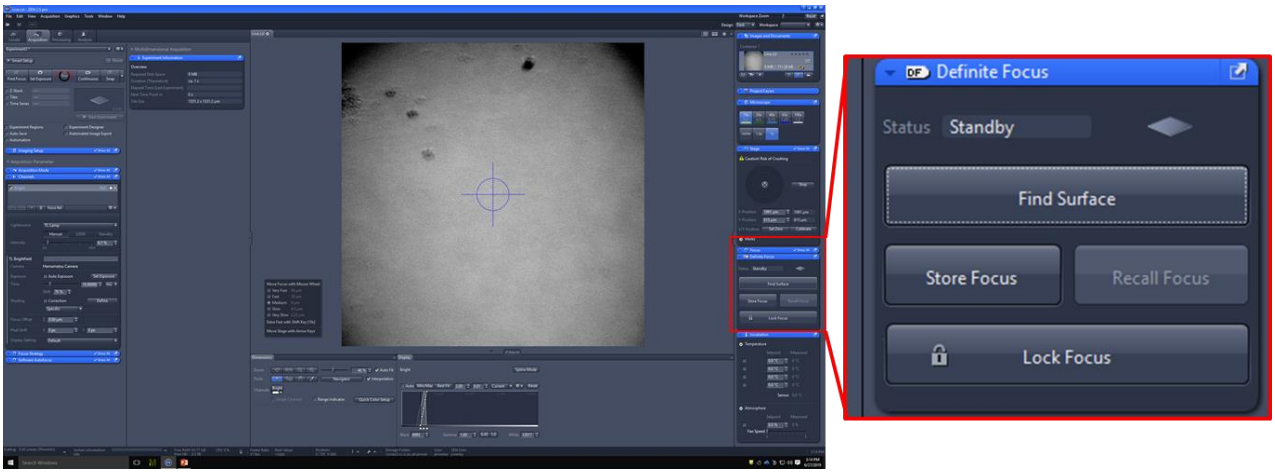
2. Click **'FIND FOCUS AF'**.
3. When autofocus finishes, the light may be turned off. Click **'STOP'** then **'LIVE'** (i.e. switch on and off) to switch light back on.
4. Adjustments:
    - *Mode:* choose to use **'CONTRAST'** (default) or **'INTENSITY'** as image sharpness.
    - *Quality:* can change to **'LOW SIGNAL'** if few objects/image.
    - *Search:* **'SMART'** – will stop at first sharp contrast/intensity, **'FULL'** will check full range.
    - *Sampling*: thickness of intervals/optical slices.



Definite Focus

Definite Focus projects a *far-red grid* onto the plane of focus. When the grid is projected onto the sample carrier surface it is reflected into the Definite Focus detector. This method can therefore be used to find the *surface of the sample carrier*.

1. Click '**FIND SURFACE**'.
2. If sample is not in focus on the sample carrier surface, adjust focus manually or using Software Autofocus.
3. Click **'STORE FOCUS'**. Definite Focus will calculate *offset* between sample carrier surface and focus plane.
4. Click **'RECALL FOCUS'** to find stored focal plane in relation to surface.



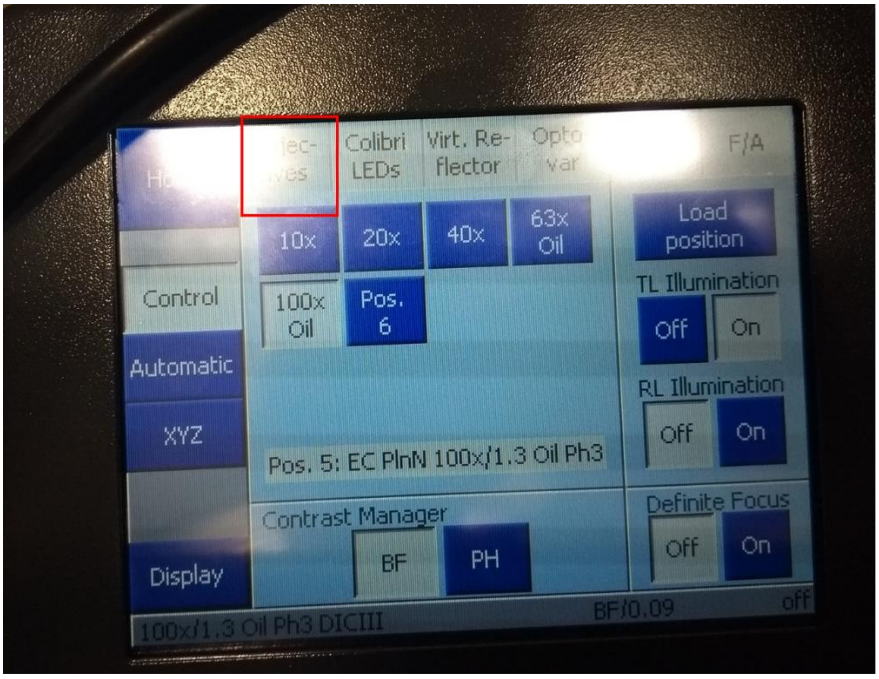
!!! Definite Focus requires reflective surface (works well with culture media in culture well). Will not work with media/immersion with high refractive index (>1.4).

Objectives

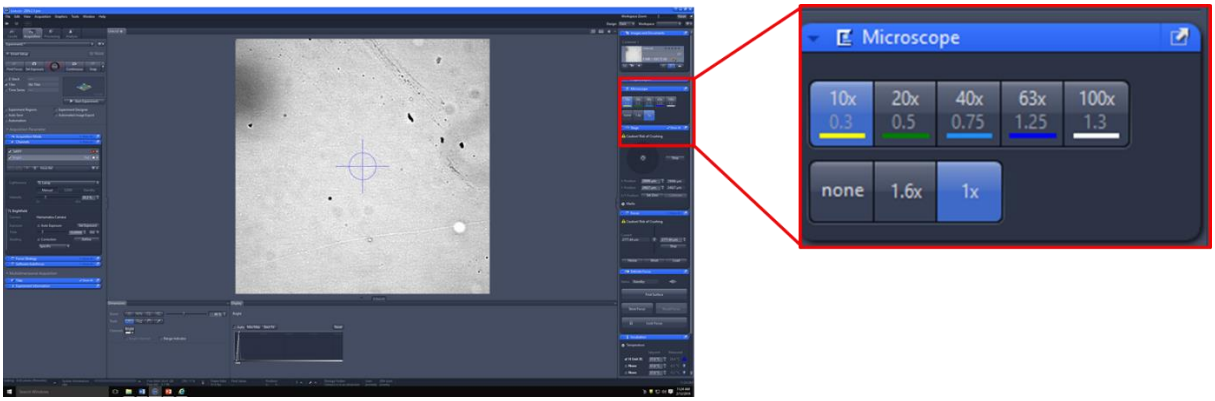
[Objective information]

Selection

TFT



Software



Numerical Aperture

The number displayed beneath the magnification for each objective is the numerical aperture (NA) – the objective's light capturing capability. The *higher the NA, the better the resolution*. For an *NA higher than 1.0* (the refractive index of air), *immersion oil* must be used. High NA is suitable for *#1.5 coverslip* (glass or polymer) thickness only (not plastic).

Working Distance

The working distance is the *distance from the lens to the surface of the coverslip*. A longer working distance must be used with plastic (e.g. tissue culture plates). Short working distance, such as that of oil immersion objectives are not suitable for use with plastic.

Oil Immersion

Checklist:

- A mode of transmitted light has been selected under channels.
- Kohler illumination and sample carrier calibration have been performed on **10x objective**.

!!! **63x** and **100x** objectives require *oil immersion* (Immersol 514f)

1. *Change objective* to 63x or 100x.
2. Objective will *lower to load position*.

3. *Remove* sample.
4. Add *drop of oil* to objective.
5. *Remove oil* using *Whatman Lens Tissue 105* **before** changing to another objective.
6. *Clean coverslip* thoroughly to remove oil **before** placing on non-immersion objective.

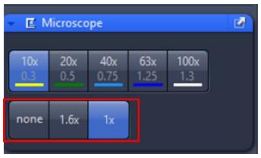
!!! Only use *Whatman Lens Cleaning Tissue 105* on objectives

!!! Clean oil before changing objective – excess oil may fly off.

!!! *Clean coverslip* thoroughly with Kimwipes or lens tissue to remove oil before placing sample on non-immersion objective.

Optovar

Optovar is a *magnification changer* that can introduce factors of **1x** or **1.6x**.



Coverslips/sample carriers

For oil immersion, coverslips and sample carriers **must** have #1.5 thickness. This is usually *glass* but can be *polymer (COC).*

For lower magnification/NA (40x, 20x, 10x) coverslip thickness is less important, and *plastic tissue culture dishes* can be used.

For best quality images, use *#1.5 thickness* for all magnification/NA.

Sample carriers for live-cell imaging

IBIDI chambers

Nunc Glass Bottom dishes

Mat Tek

CELL View – single or quarters

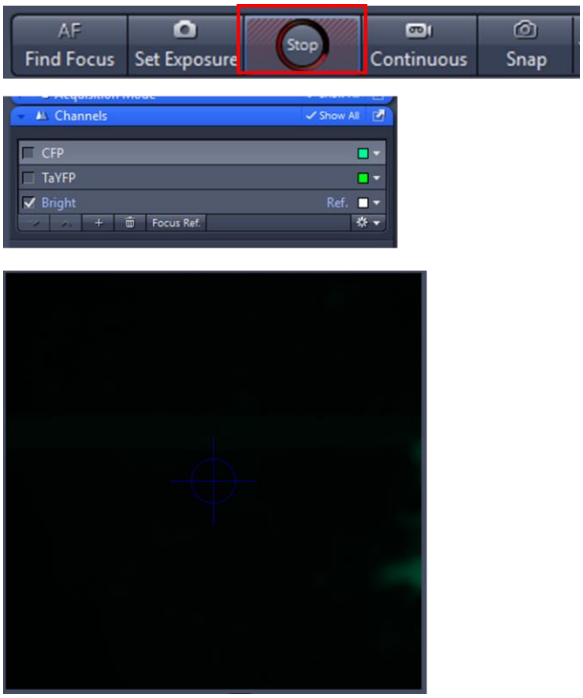
Cellvis

!!! *Autofluorescence* of sample carrier (and media) should also be considered if performing fluorescence imaging.

Image view

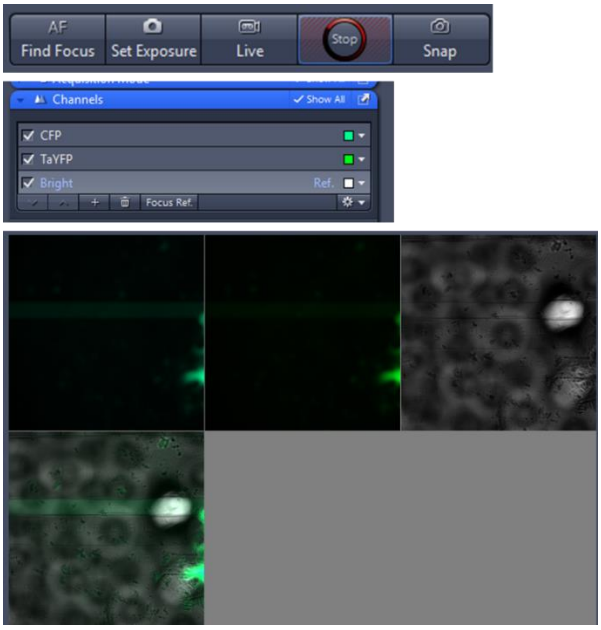
Live

- Live image of channel that is *highlighted*.



Continuous

- Cycles through live images of each channel that is *ticked*.



Snap

- Takes an image of all channels that are *ticked*.

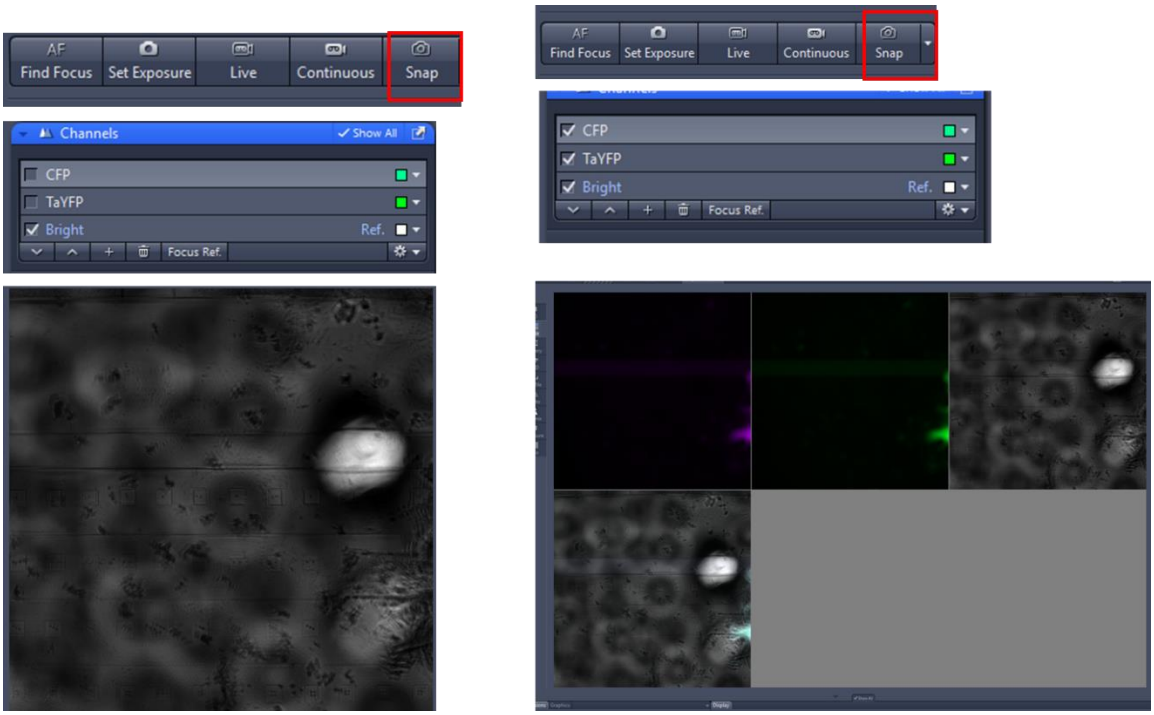


Image Display

2D

Shows current position/time/z for all activated channels as an *overlay*.

Split view

Displays position/time/z for all activated channels as *individual channels and an overlay*.

Gallery

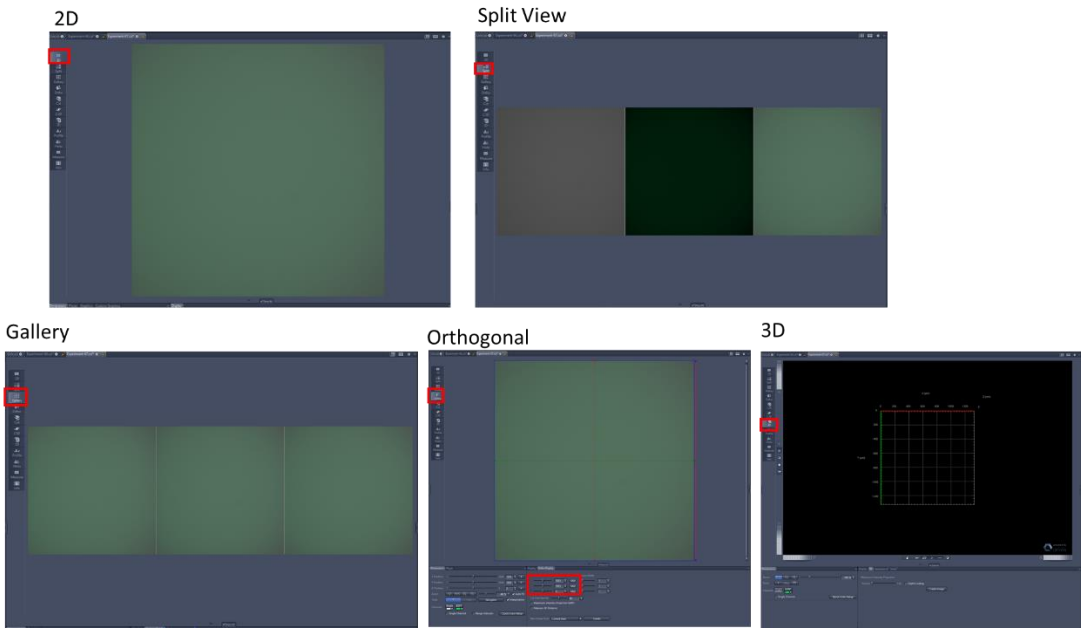
Displays *each z/tile/position* as an overlay, or each individual channel.

Orthoganol

For current scene/time, displays *entire Z stack*, with current optical slices in *XY*, and all optical slices in *XZ* and *YZ*. Use slider bars to move through stack.

3D

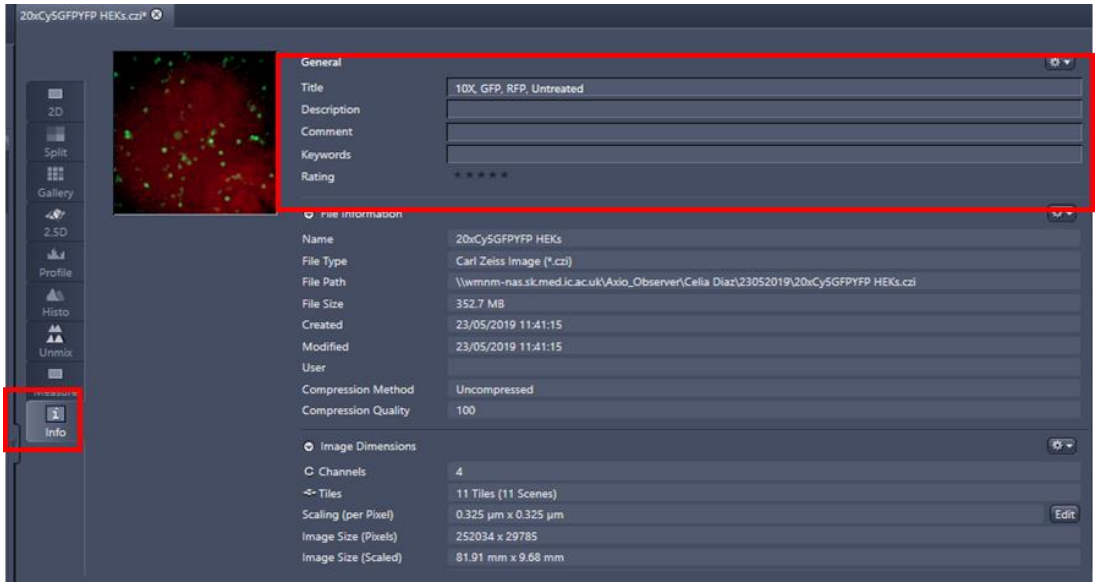
Creates a *3D view* of Z-stack for current time/position, for all activated channels.



Info

Displays information of image acquisition including LED intensity, exposure time, image path (filters/LED), number of positions/tiles/timepoints/channels etc.

Can be used to *annotate* images.



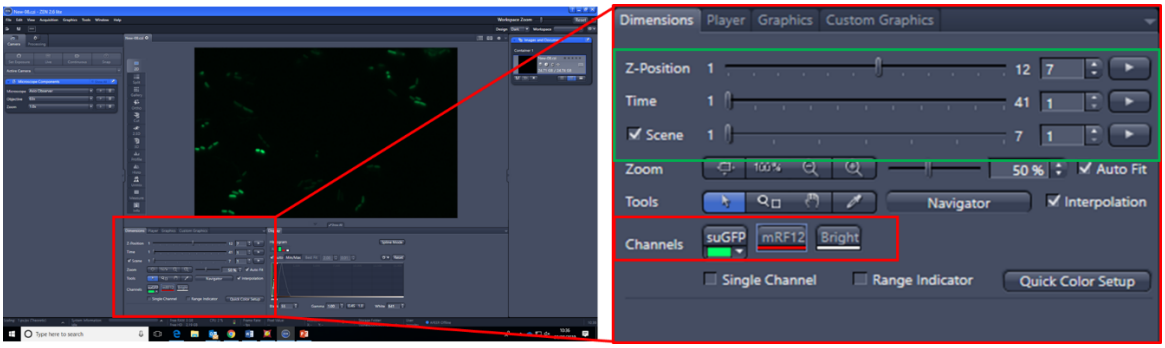
Change dimensions

Z/time/scene

1. Toggle to **'DIMENSIONS'** tab.
2. Adjust *slider bars/input number* to change *z/time/scene* (position).
3. *Hover over slider* to see *current* z/time/scene (position).

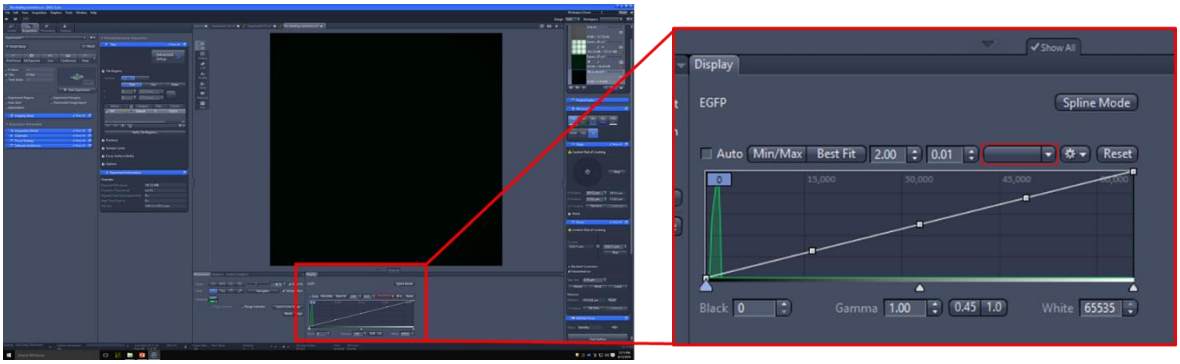
Channel

1. Toggle to **'DIMENSIONS'** tab.
2. Click/unclick *channel boxes* to turn channels on/off.



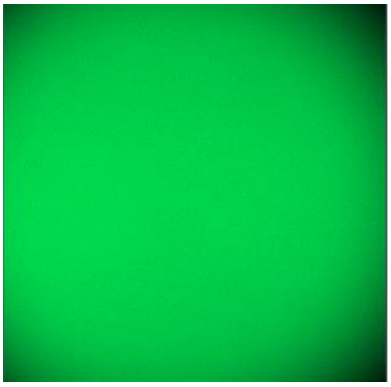
Data display

If you are not using the full range of pixel intensities, you will need to adjust how your data are displayed to visualize your image. This changes how data are displayed and has *no effect on pixel values.*

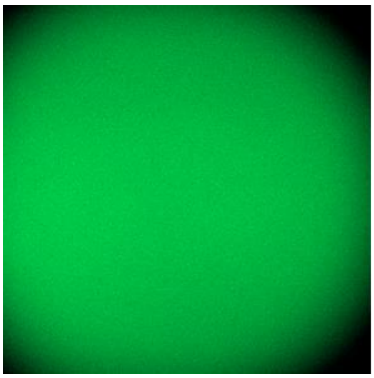


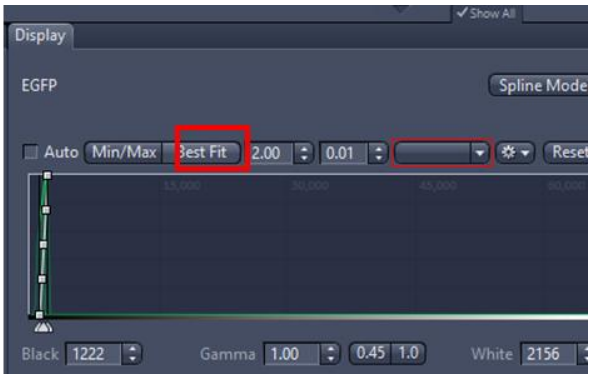
Full pixel range

Min/Max



Best Fit



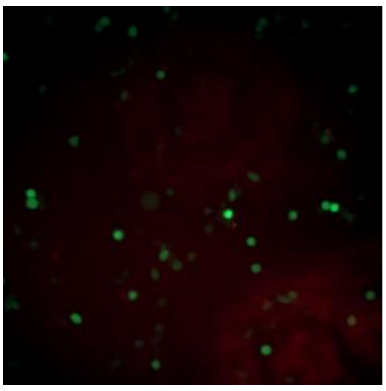


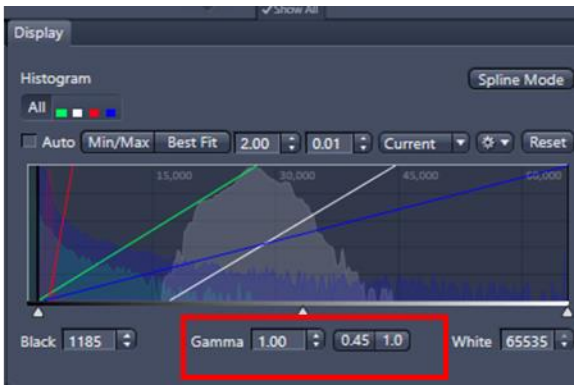
Gamma

Gamma correction stretches the histogram at one end, to make *darker* or *lighter* areas more visible to human eye. *Gamma* correction alters linearity of data and therefore should only be used for *visualizing* data.

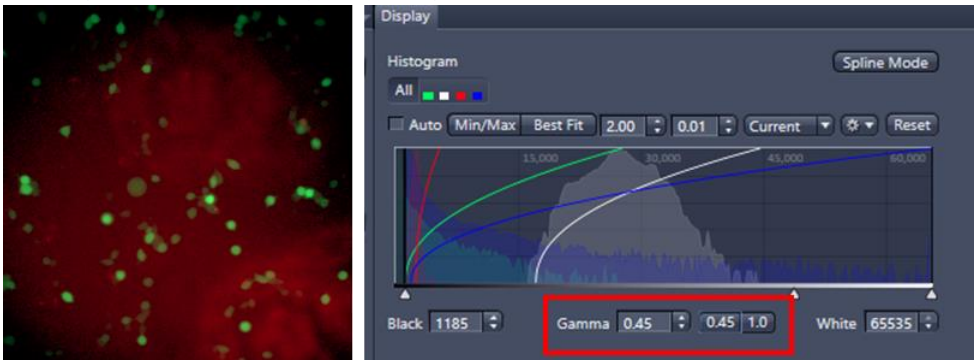
!!! All image processing/analysis should be performed on *linear* (gamma 1.00) data.

1.0 – *linear*





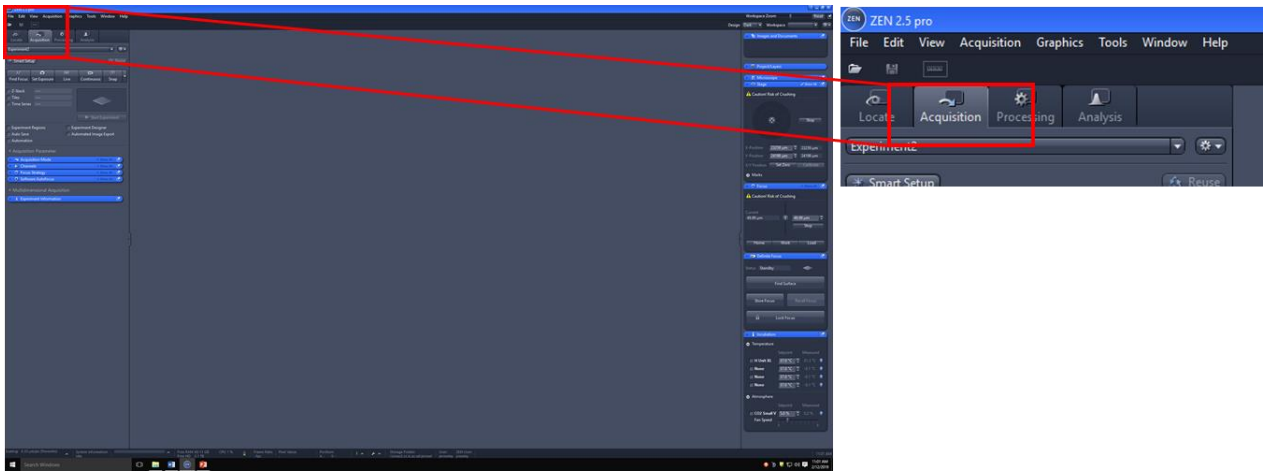
0.45 – gamma correction (non-linear)



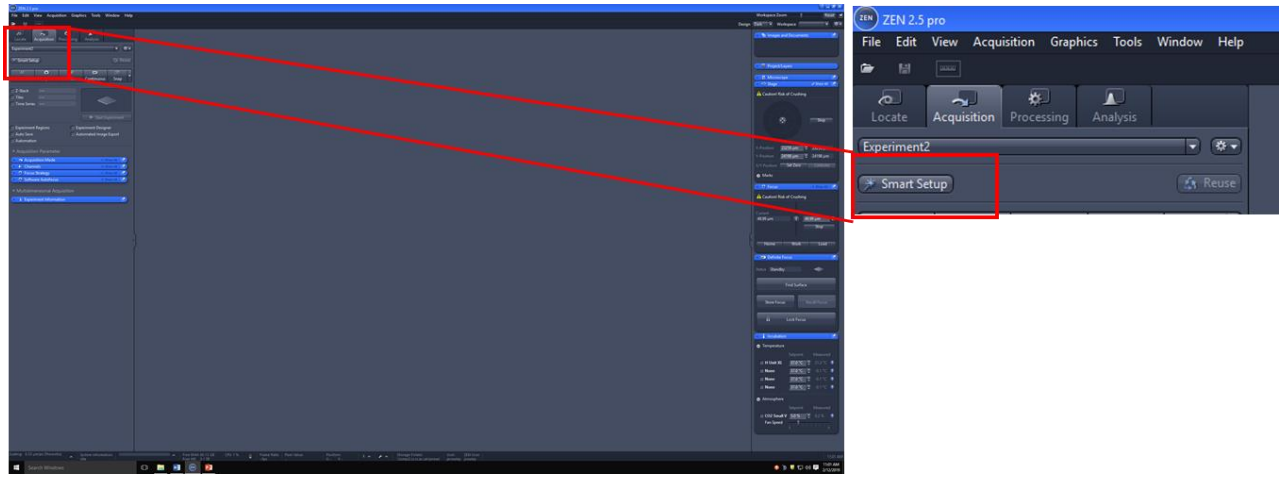
Microscope set-up

Add channels

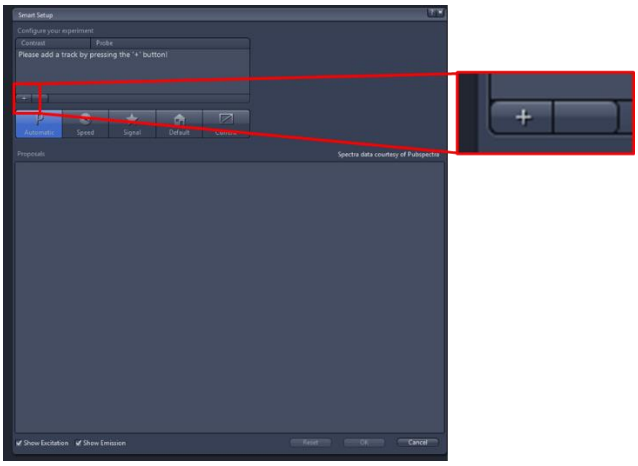
1. Select **'ACQUISITION TAB'**



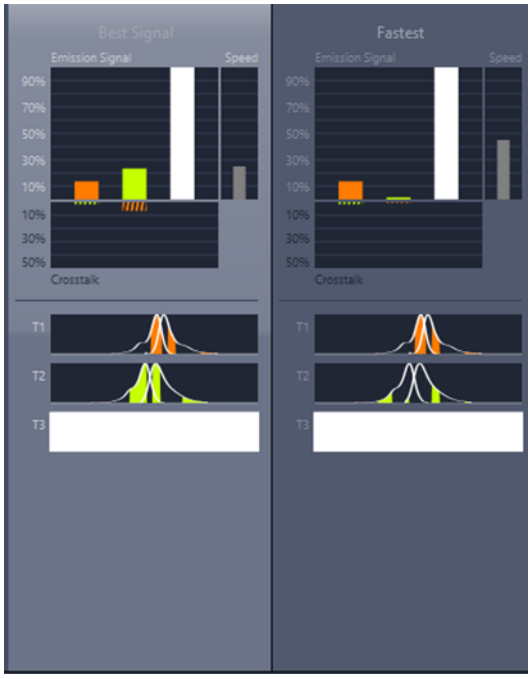
2. Click **'SMART SET UP'**



3. Click **'+'**.

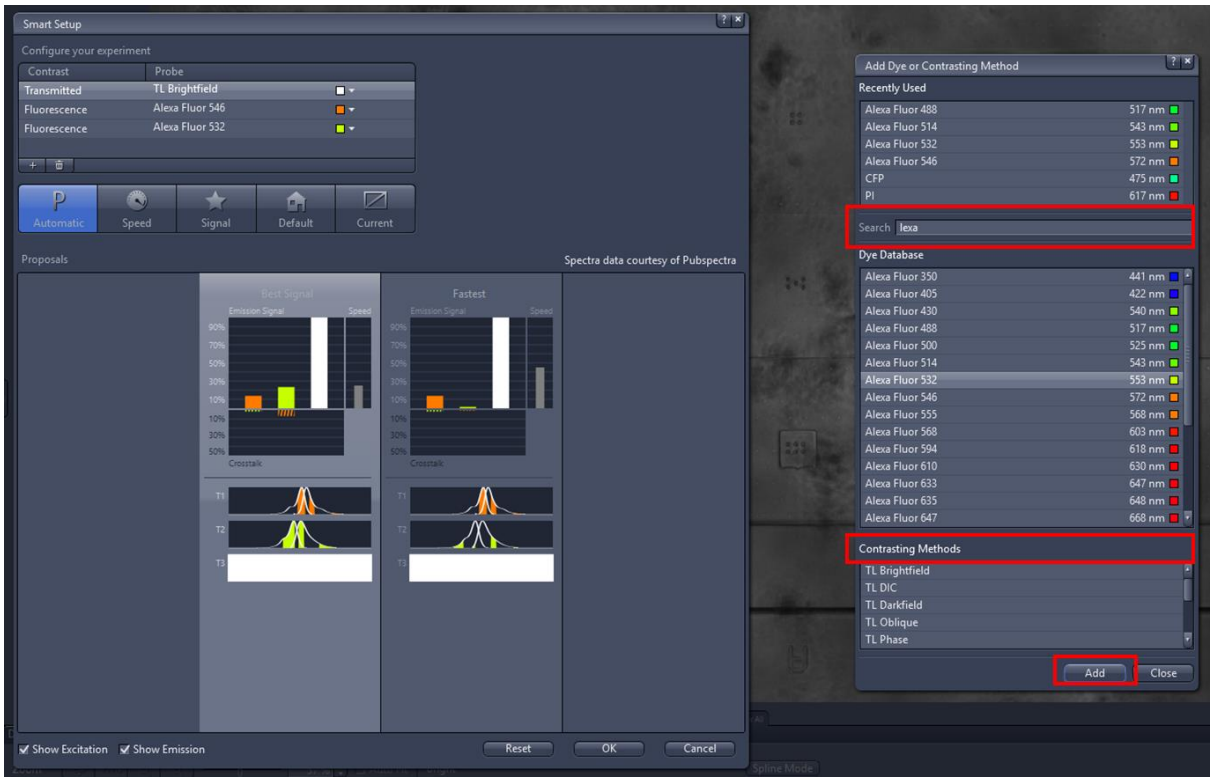


4. Use search bar to select fluorophores. Click **'ADD'**.
5. Check *cross-talk* and *signal*. If performing multi-colour imaging decide if speed (**FASTEST**) or **BEST SIGNAL** is more important (for quantitative choose Best Signal, if drift may occur between images select Fastest).



!!! Always check filters and led are correct under Imaging set up tool

6. Choose a **CONTRASTING METHOD**.
    - TL Brightfield
    - TL Phase contrast
7. Click **'OK'**

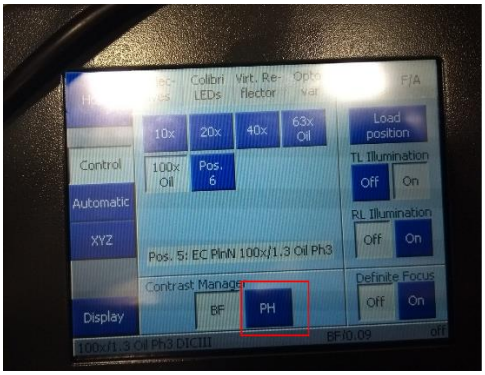


TL Phase Contrast

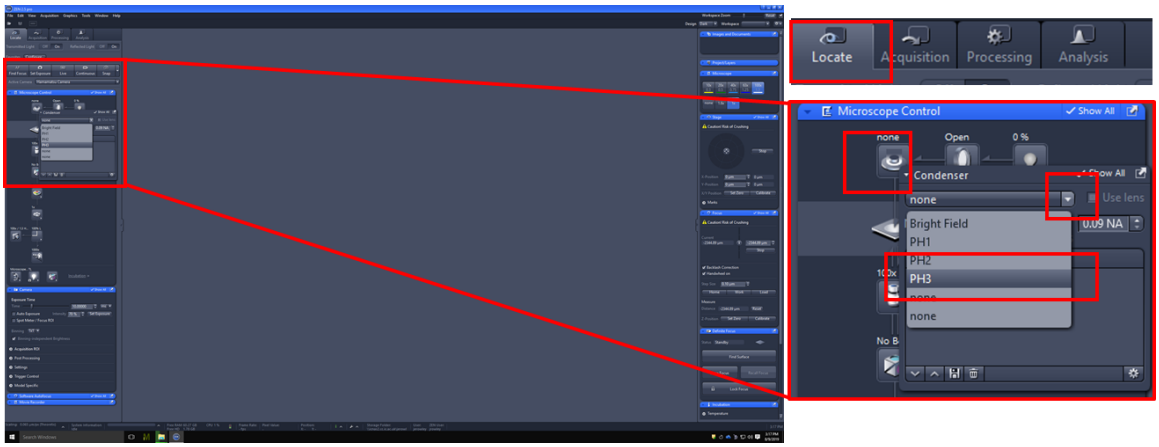
Phase is a little glitchy and must be added in a specific order.

!!! Change to phase **after** setting up Kohler illumination and calibrating sample carrier as these processes require low magnification.

1. Ensure *correct objective* is in place.
2. Add phase contrast (**'PH'**) from *TFT*.



3. Toggle to **'LOCATE'** tab and ensure *correct phase ring* is in place.
    - **10x** = Ph 1
    - **20x** and **40x** = Ph 2
    - **63x** and **100x** = Ph 3



4. **'TL PHASE CONTRAST'** can now be added under **'CHANNELS'** or **'SMART SETUP'**.

Objectives - **HTSCA SHAREPOINT > DOCUMENTS > USER GUIDES AND TECHNICAL SPECS**

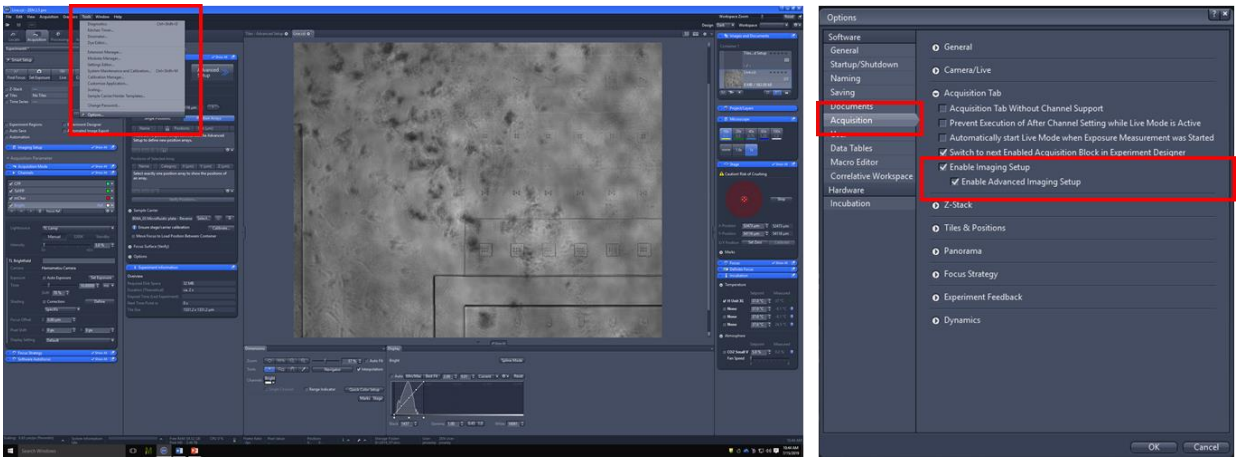
Select filters

Although Smart Setup will create light paths for specified fluorophore, it will invariably select the quad- or tri-band emission filters. If performing multi-colour imaging, *cross-talk* should be eliminated by selecting *single band emission filters*.

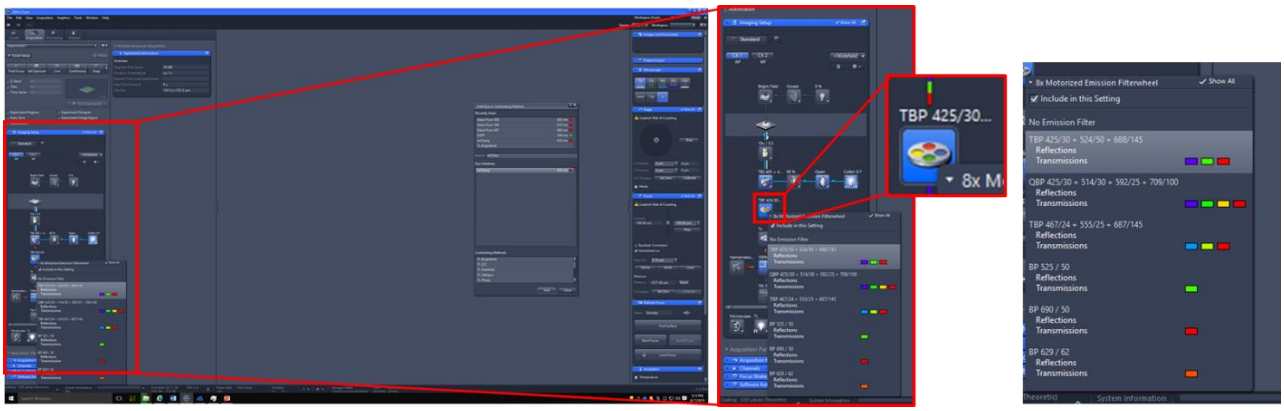
Filters and LEDs – **HTSCA SHAREPOINT > DOCUMENTS > USER GUIDES AND TECHNICAL SPECS**

Thermofisher Spectra Viewer

1. Enable imaging setup tab (**TOOLS > OPTIONS > ACQUISITION>ENABLE IMAGING SETUP**)



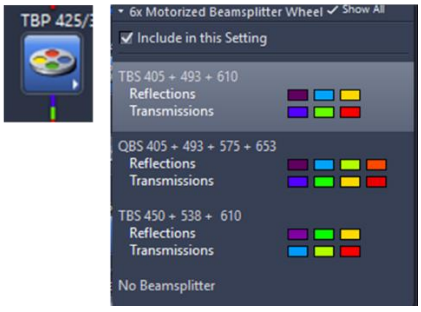
2. Expand **'IMAGING SETUP'** tool.
3. Click on the *'emission filter button'* in the *light path*.



4. Beamsplitter and *LED*s can be changed if necessary.

LED

Beamsplitter

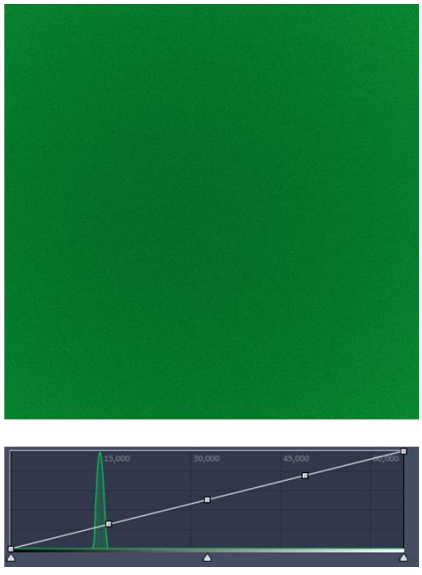


Shading correction

If illumination is uneven, shading *(flatfield) correction* can be performed.

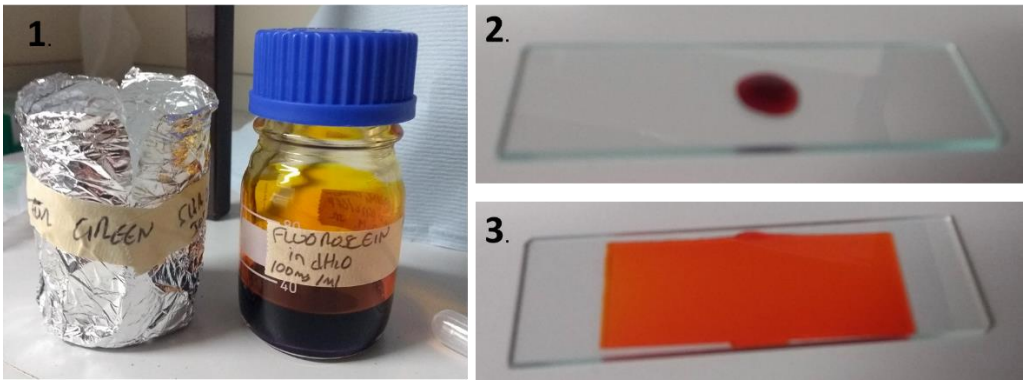
Before shading correction

After shading correction



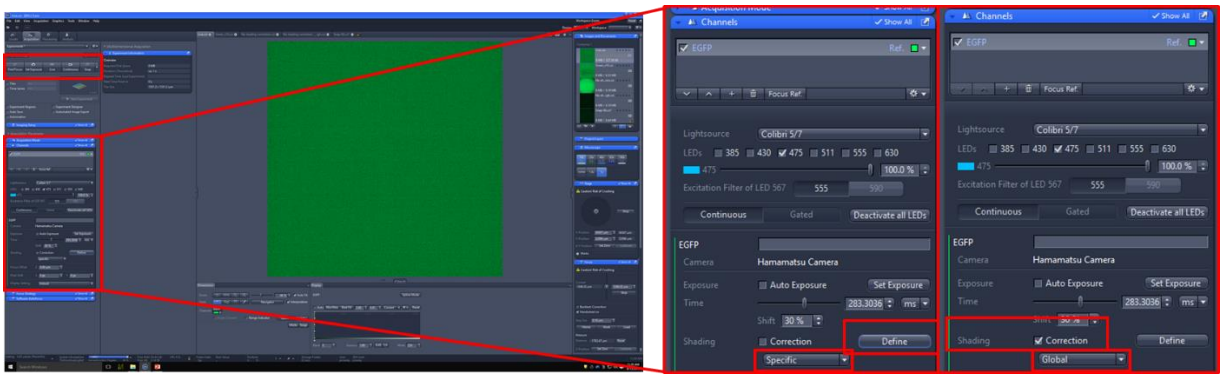
Prepare flat fluorescent field

1. Remove fluorescent dye from fridge on level 3.
    - For *blue* (e.g. DAPI/BFP/CFP) use *7-diethylamino 4-methyl coumarin*.
    - For *green* (e.g. GFP) use *fluorescein*.
    - For *yellow/orange* (e.g. RFP/mCherry) use *rose Bengal.*
    - For *red/far red* (e.g. Cy5/Alexa647) use *acid blue 9*.
2. Filter through *0.2µm syringe filter* to remove particulates.
3. Use disposable transfer pipette to *add drop of dye* to microscope slide or well of culture plate.
4. Place *coverslip* over drop on slide OR *swirl* plate to *spread evenly* over well.



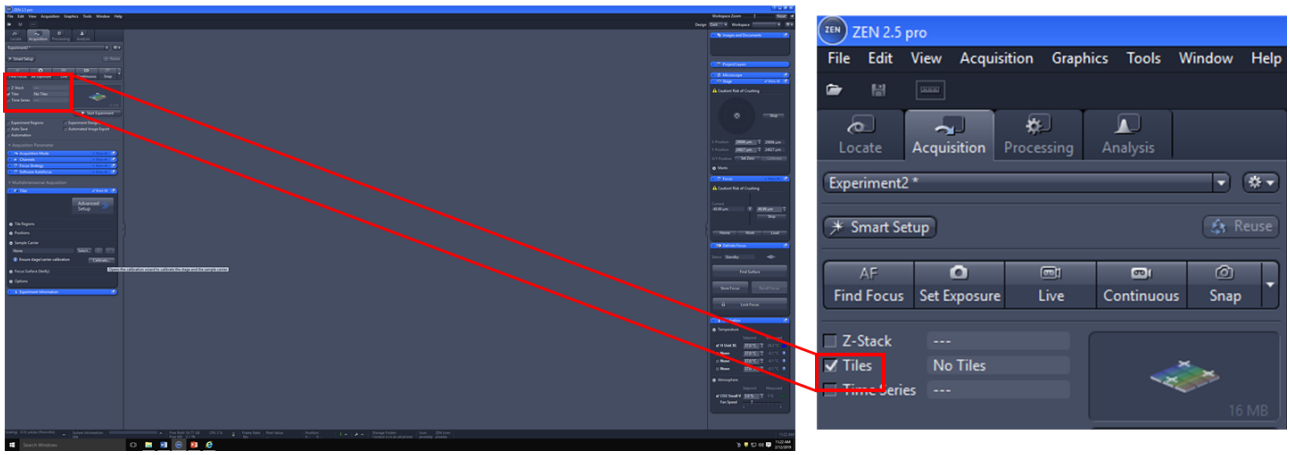
Calculate correction

1. Expand **'CHANNELS'** tool.
2. Use Definite Focus to focus on surface of slide/well.
3. Click **'LIVE'**.
4. Set LED intensity and exposure time to give a bright image.
5. Ensure *no bubbles* in field of view.
6. From dropdown menu select:
    - **'GLOBAL'** for *objective-specific* shading correction. (*Recommended*)
        - Magnification (objective and optovar) is considered.
        - Fluorescent-specific components, e.g filters are not considered'
        - Shading correction is objective-specific in principle.
    - **'SPECIFIC'** for *channel-specific* shading correction.
        - Considers excitation/emission filters and beamsplitters.
7. Click **'DEFINE'** to calculate shading correction.
8. Tick **'CORRECTION'** to apply calculation to images.



Calibrate sample carrier

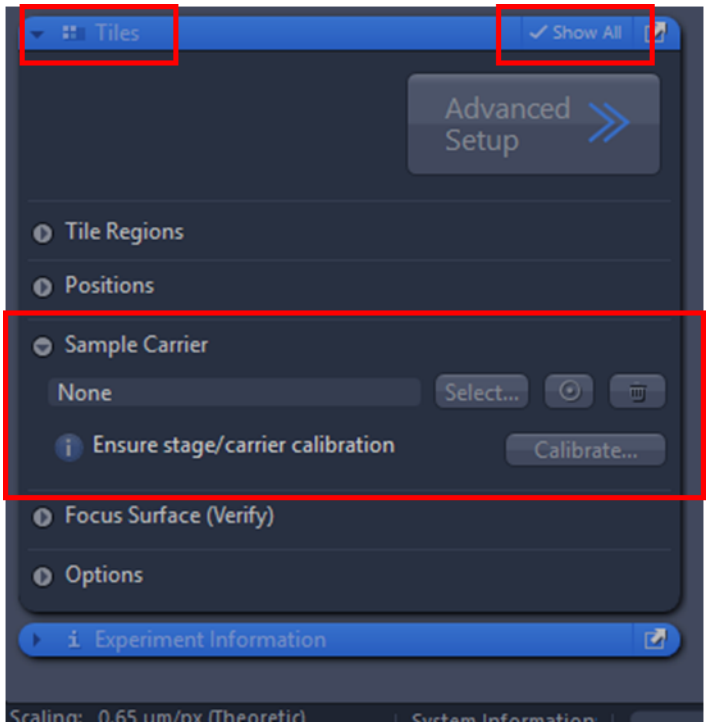
1. Tick **'TILES'**



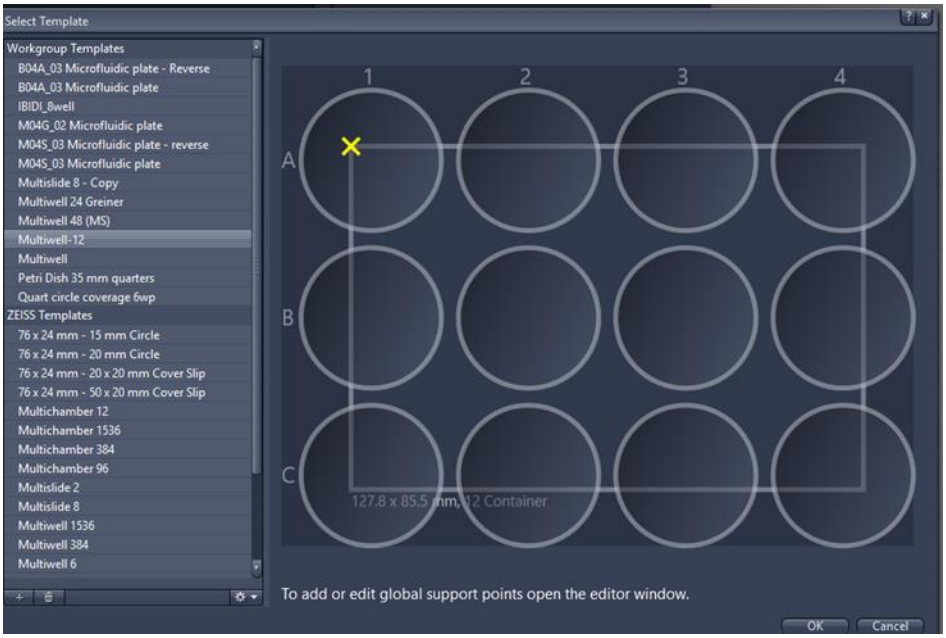
Checklist:

- A mode of transmitted light has been selected under channels.
- Objective is 10x.

2. Open **'TILES'** module and ensure **'SHOW ALL'** is selected.
3. Expand **'SAMPLE CARRIER'**.



4. Click **'SELECT'** and *Select Template* to match carrier type. Click **'OK'**.



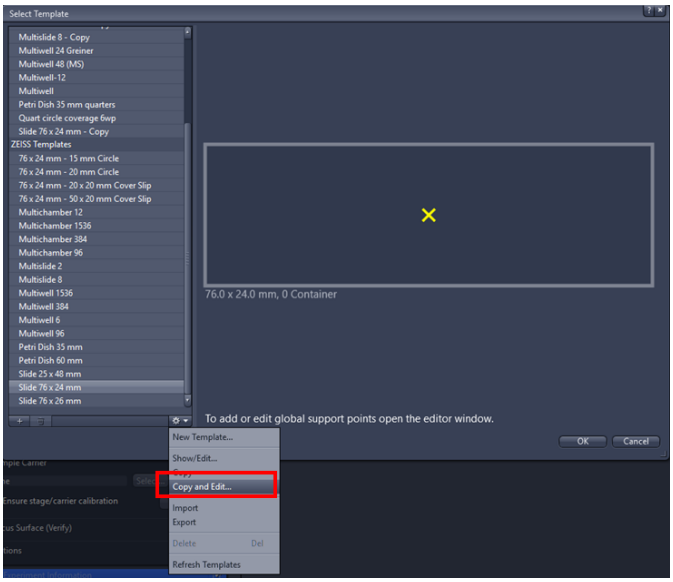
5. Click **'CALIBRATE'.**

Add Global Focus Surface to Sample Carrier

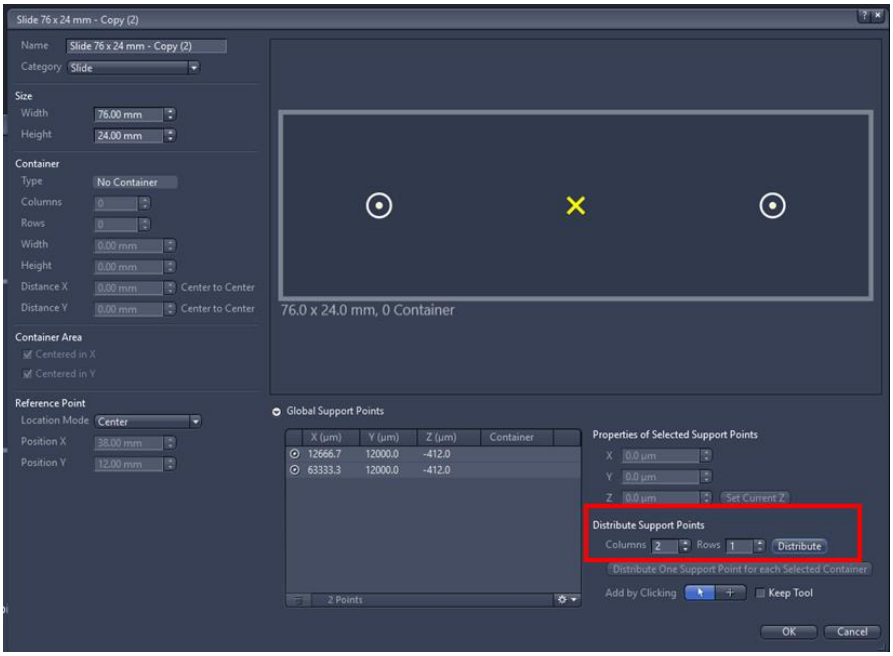
If your sample carrier is uneven, you can create a *global focus surface* by addition of *support points* to the sample carrier template. This will compensate for tilting and bending.

!!! Incompatible with using Definite Focus/Software Autofocus as focus strategy. Use with [Tiles-setup defined Z values.]

1. Select [sample carrier template].
2. *Right click* and click **'COPY AND EDIT'**.



3. Expand **'GLOBAL SUPPORT POINTS'**.
4. Distribute support points by:
    - *Sample carrier*
        I. Enter *number of columns and rows*.
        II. Click **'DISTRIBUTE'**.



- *Container*

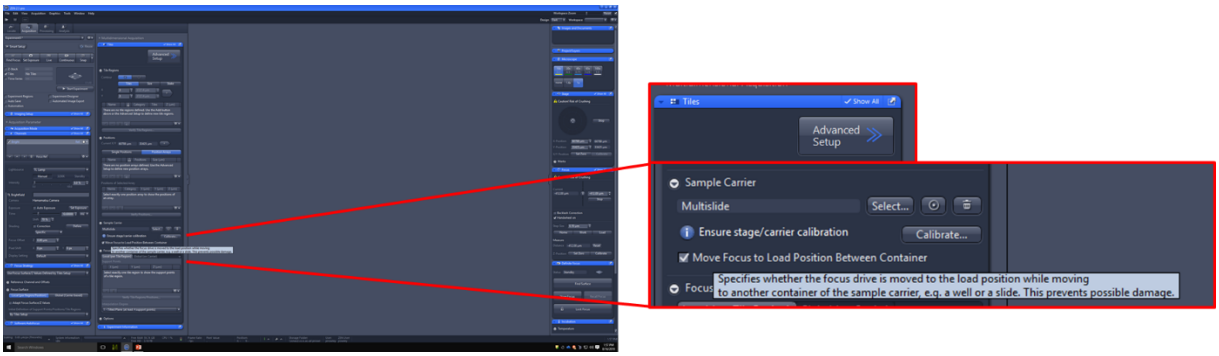
I. *Select containers* (wells) to add support points too.
II. Click **'DISTRIBUTE ONE SUPPORT POINT FOR EACH SELECTED CONTAINER'**.

5. Click **'OK'**.

Move Focus to Load Position Between Container

Protect objective from damage when moving between containers by dropping to load position.

1. Expand **'TILES'** tool.
2. Expand **'SAMPLE CARRIER'**.
3. Tick **'MOVE FOCUS TO LOAD POSITION BETWEEN CONTAINER'**.

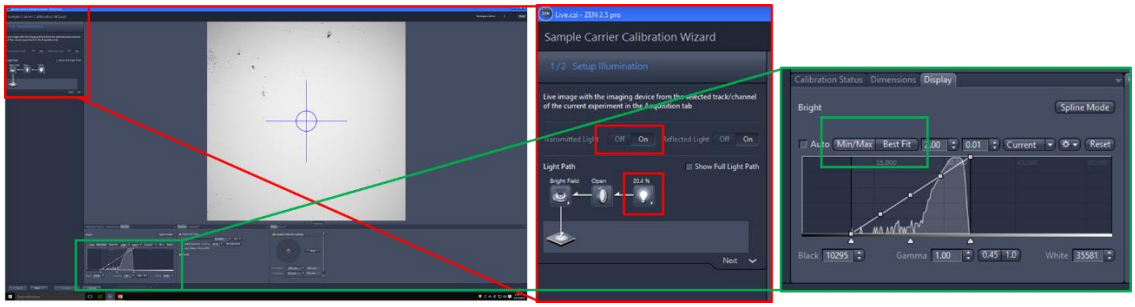


Sample Carrier Calibration Wizard

Setup Illumination

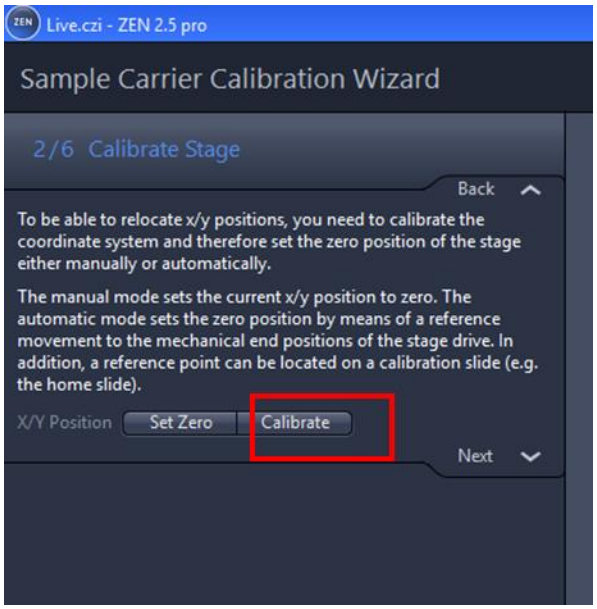
I. Ensure *'Transmitted Light'* is *'On'* and *'Reflected Light'* is *'Off'*.
II. To increase light intensity, click *'light bulb symbol'* under **'LIGHT PATH'** until sample carrier is visible.
III. May need to adjust data display (**'MIN/MAX'** or **'BEST FIT'**).
IV. Click **'NEXT'**.

Tip: Focus on edge of well or use '**DEFINITE FOCUS**' to focus on surface of well.



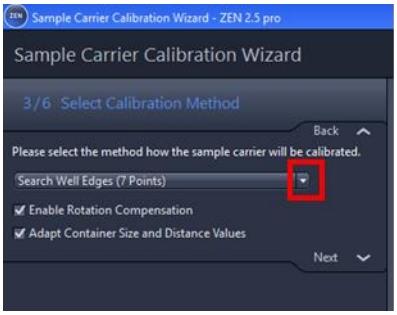
Calibrate Stage

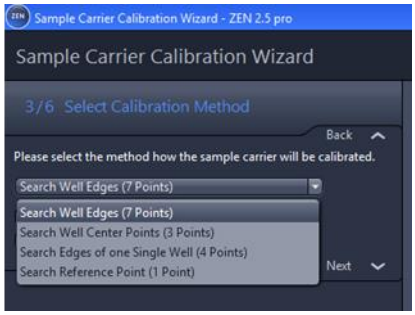
I. If stage has not been calibrated, click **'CALIBRATE'**. Otherwise ignore this step.
II. Click **'NEXT'**.



Choose Calibration Method

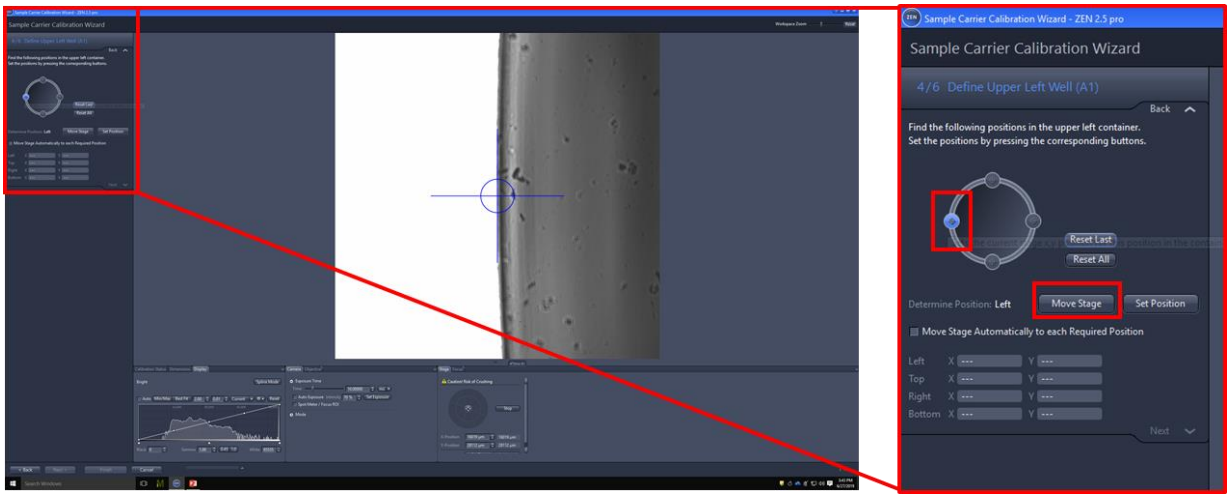
I. Select calibration method from dropdown menu.
II. Click **'NEXT'**.

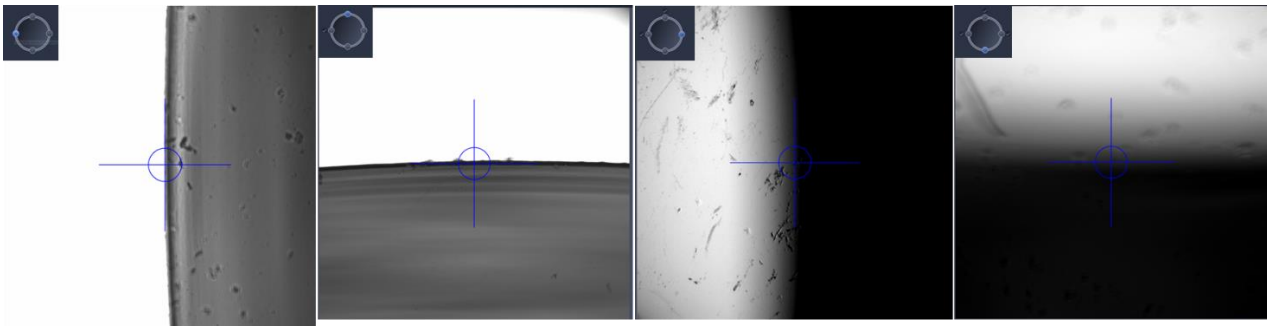




Define Upper Left Well

I. Find edges of well as instructed (depends on calibration method selected – the *7 point method* is detailed here). Click *blue button* to input x/y coordinates.
II. Can click **'MOVE STAGE'** to move automatically to next position. **!!!** Check that has moved to the correct well and position if using '**MOVE STAGE**'.
III. Click **'NEXT'**.



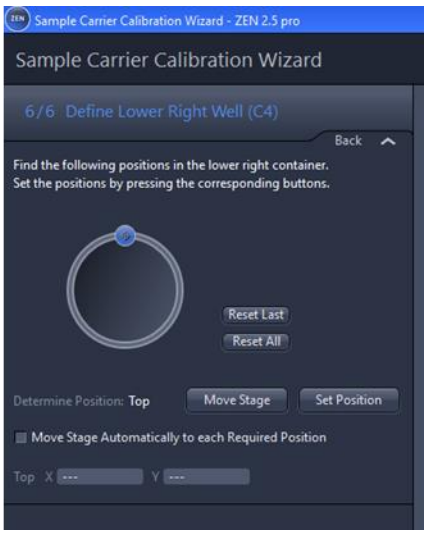


Define Upper Right well

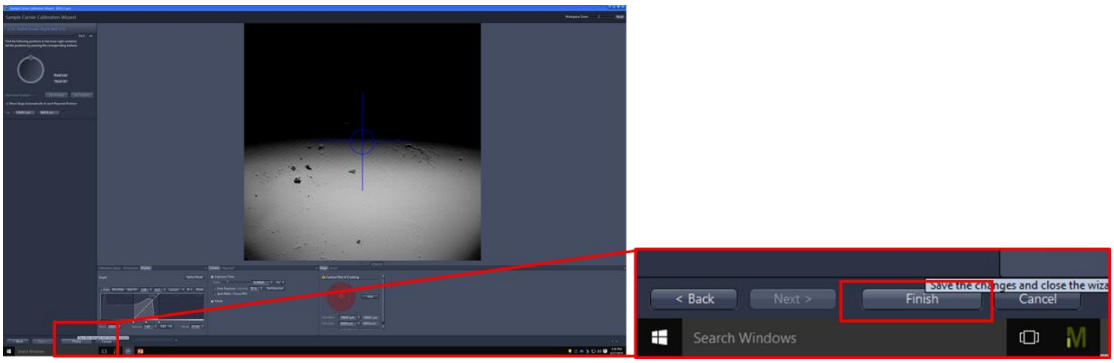
I. Find positions on next well as instructed and click **BLUE BUTTON** as in *Step 4*.
II. Click **'NEXT'**.

Define Lower Right Well

I. Find positions on next well as instructed and click **BLUE BUTTON** as in *Step 4*.
II. Click **'NEXT'**.



Click **'FINISH'**.



- Go to '**ADVANCED SET UP**' in **TILES** tool to see *navigation plate map.*
- *Save experiment* to reuse settings.

Focus on sample

Tip: Start at low magnification and work up

Focus on sample manually or using Software Autofocus or Definite Focus.

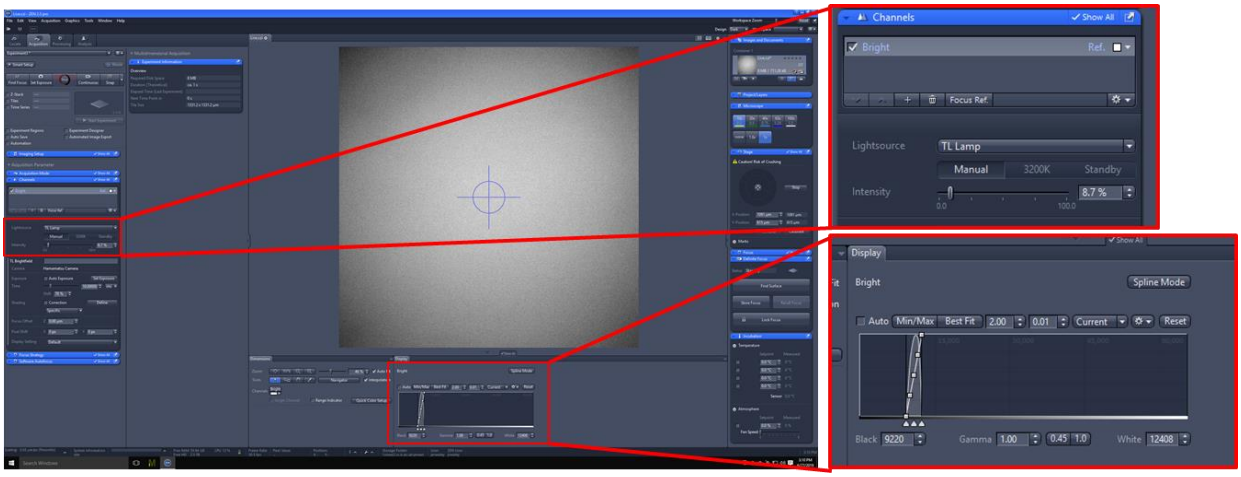
Kohler illumination

Tip: Set Kohler Illumination on *lowest objective*, and illumination will be correct for higher objectives. Sample should be *in focus*.

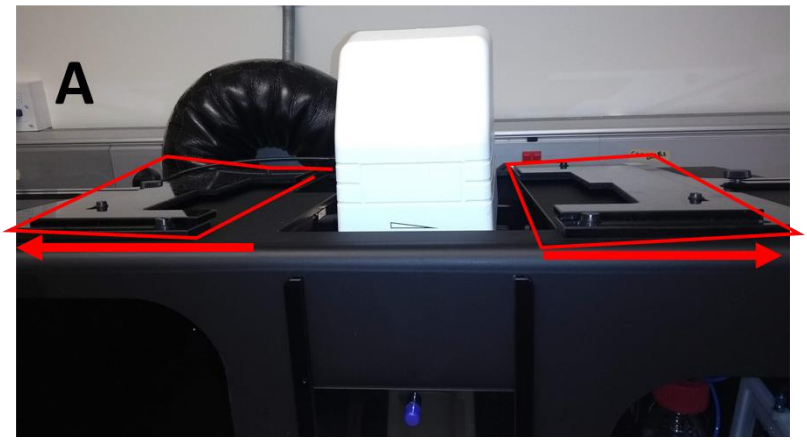
Checklist:

- A mode of **transmitted light** has been selected under channels tool.
- Objective is **10x.**

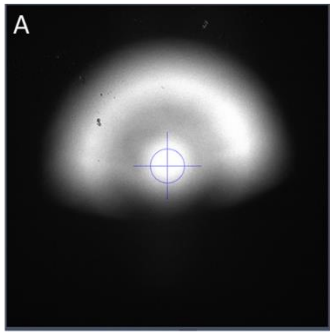
1. Navigate to well.
2. May need to *increase lamp intensity* and *min/max* to display data.

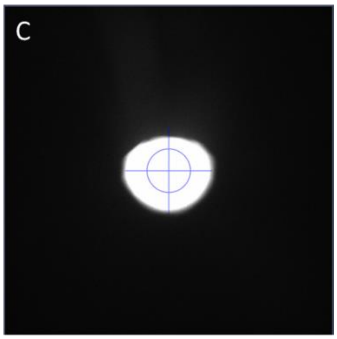


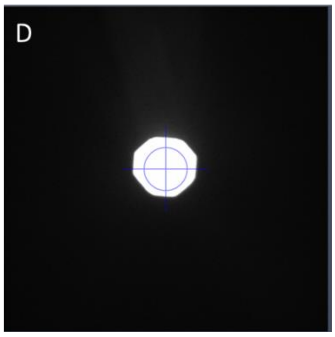
3. Focus on sample (manually or use autofocus/Definite Focus).
4. Slide open clips on environmental chamber (A) to reveal *field aperture dial* (B).

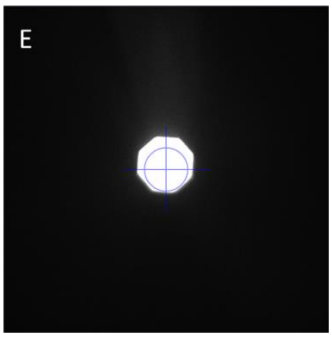


5. Turn dial to the right to fully *close* field aperture.
6. Adjust height of condenser by turning *black knob* (Condenser), until a crisp hexagon is formed (D).

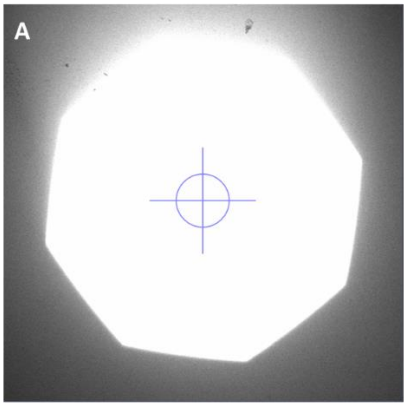


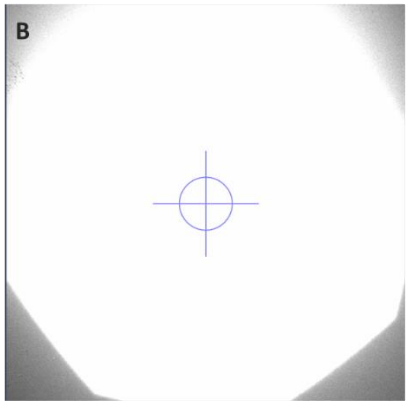


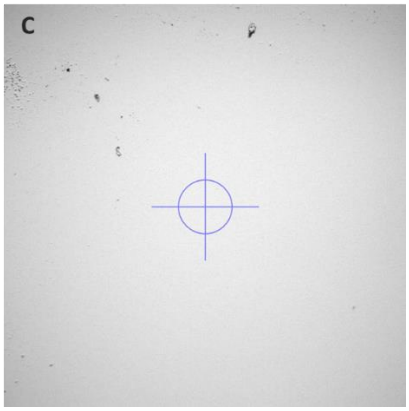




7. Adjust metal *centering knobs* until hexagon is in centre of the image.
8. *Open field aperture* until hexagon just disappears out of field of view







Acquisition set-up

Checklist:

- Channels have been selected under channels tool and correct filters placed in light path
- Correct objective is in place
- Sample is in focus
- Kohler Illumination is set (for transmitted light)

Tips:

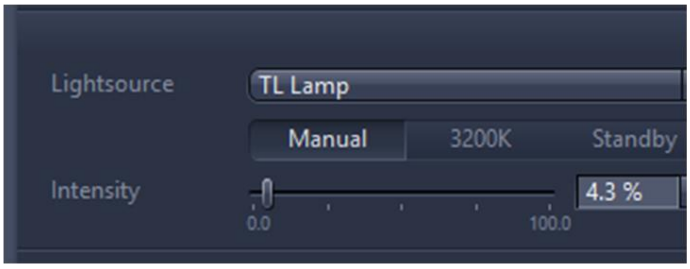
- Sacrifice signal and use *short exposure/low intensity* to maintain cell health.
- Use *longer exposure time* and *low intensity* to prevent photobleaching.
- If poor signal, can increase binning to *increase light capture capacity.*

Intensity

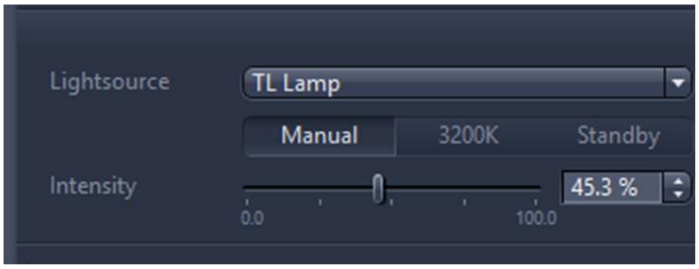
1. Expand **'CHANNELS'** tool.
2. Adjust *lamp/LED intensity* such that there is a decent signal, but no pixel saturation.

Transmitted light

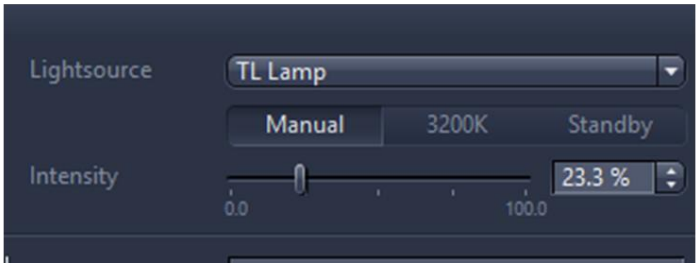
Lamp intensity too low



Lamp intensity too high



Lamp intensity just right



!!! If variation in brightness expected – check intensity on dimmest and brightest samples.

Exposure Time

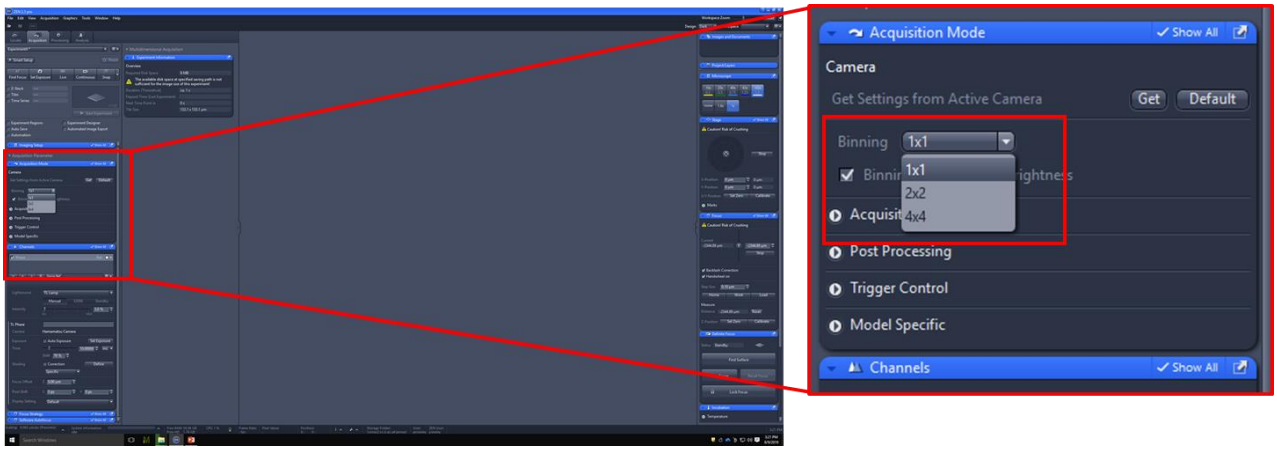
- Can use **'SET EXPOSURE'** function, although sometimes sets too high exposure time.

!!! Avoid pixel saturation.

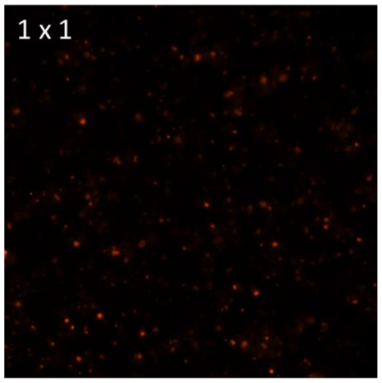
Binning

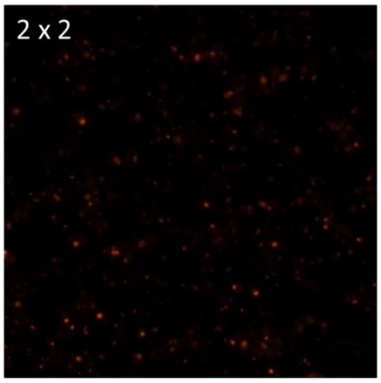
For experiments with low signal, binning can be increased. This will combine a grid of pixels into one, maximizing the light-capturing capacity. Of course, this is at a trade-off with resolution.

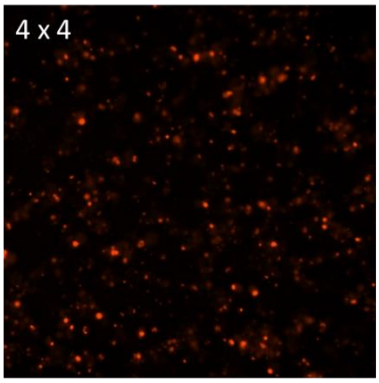
1. Expand **'ACQUISITION MODE'** tool.
2. Select binning pixel value from dropdown menu:



- **1 x 1** = 1 pixel
- **2 x 2** = 4 pixels
- **4 x 4** = 16 pixels







50% LED, 150ms exposure

50% LED, 150ms exposure

50% LED, 150ms exposure

Experiment set-up

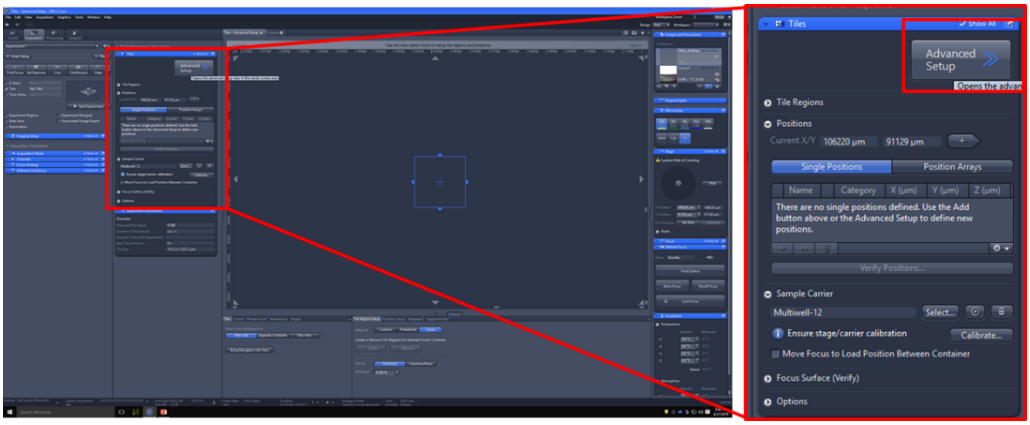
Positions

Checklist:

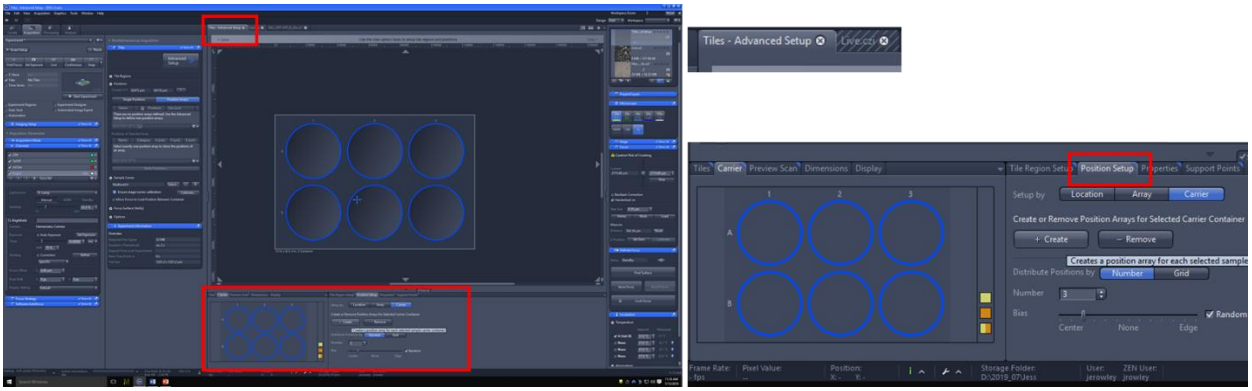
- Stage has been calibrated
- Sample carrier has been calibrated
- At least one channel has been selected.

Add positions

1. Expand **'TILES'** tool.
2. Click **'ADVANCED SETUP'**.



3. Toggle to **'TILES-ADVANCED SETUP'** tab, and **'POSITIONS'** tab.



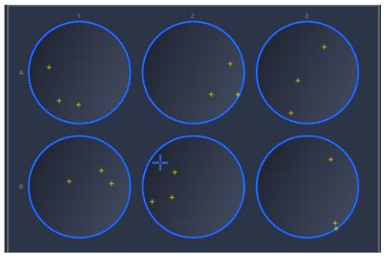
4. Add *positions*. There are three ways to do this;

Carrier.

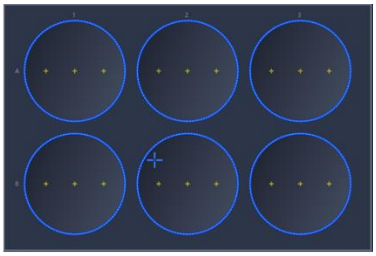
1. Click **'CARRIER'**.
2. Select *well* to add positions to.
3. Choose *number* of positions to add to each well.
4. Option to choose *random,* with *centre* or *edge bias*.

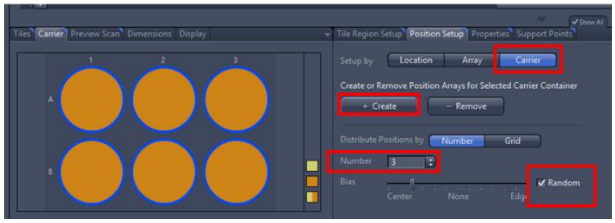
5. Click **'CREATE'**.

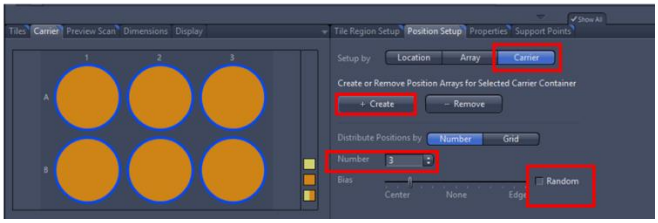
Random



Not-random

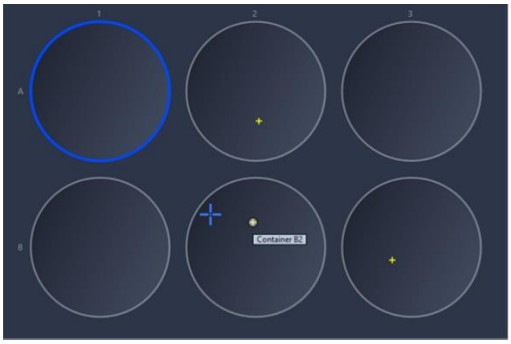


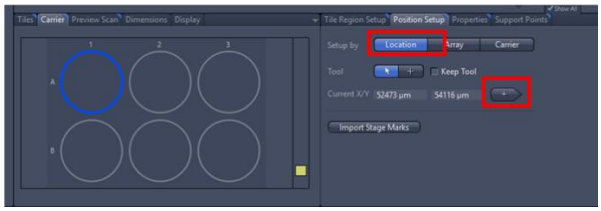




Location.

1. Click **'LOCATION'**.
2. Use **+** Tool to add positions in well.

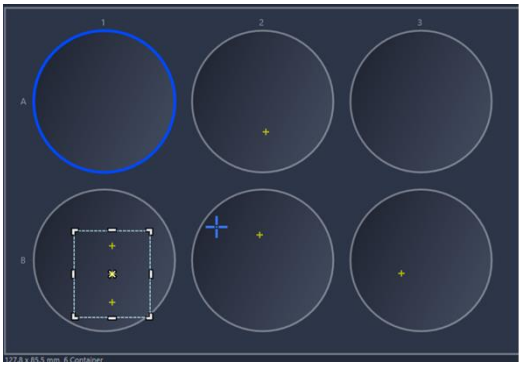


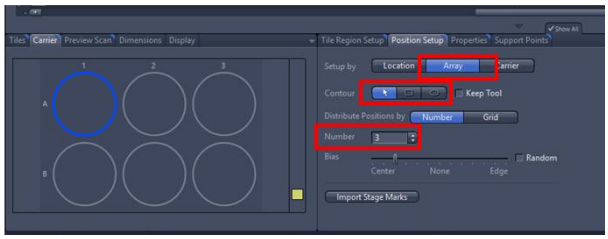


- Move positions by dragging yellow cross

Array.

1. Click **'ARRAY'**.
2. Choose a *Contour* tool, and *Number* of positions per array.
3. Draw shape in well.

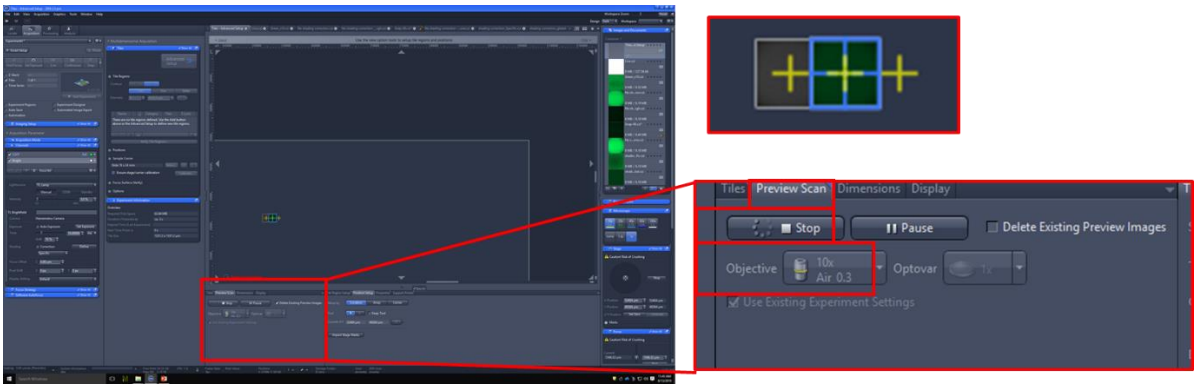




Preview Scan

Creates low resolution scan of each position.

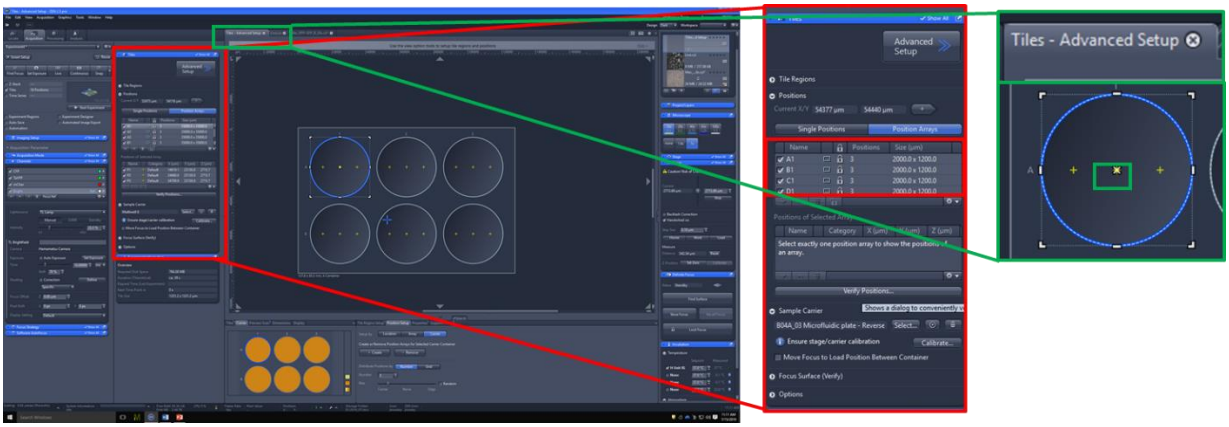
1. Toggle to **'PREVIEW SCAN'** tab.
2. Select *low magnification objective*.
3. Click **'START'** (changes to **'STOP'** when scan is running).



Position navigation

I. Click on *position names* under **'TILES'** tool

II. Click on *yellow crosses* in **'TILES – ADVANCED SETUP'** tab.

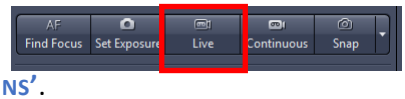


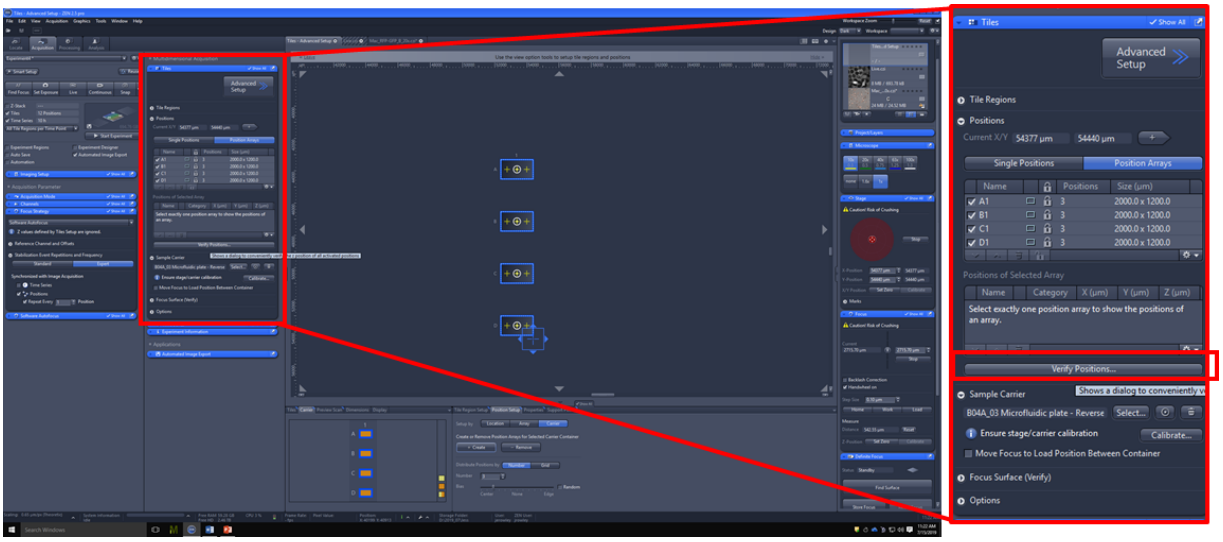
Move Positions

I. *Drag yellow crosses* in **'TILES – ADVANCED SETUP'** tab.

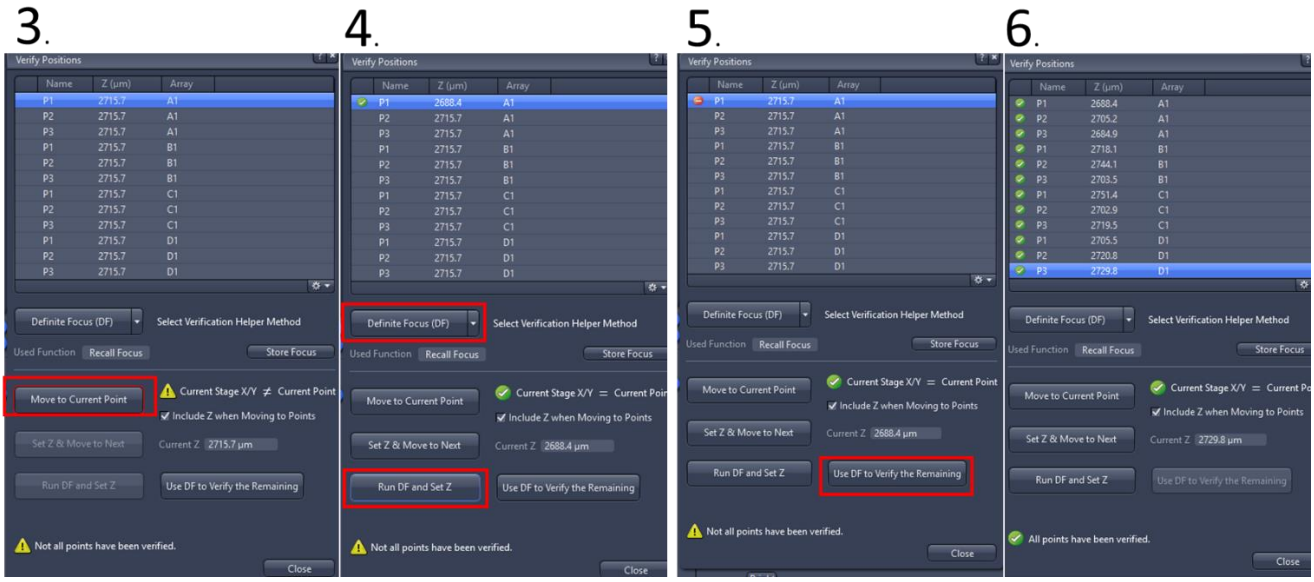
Verify Positions

1. Ensure that **'LIVE'** mode is activated.
2. Under **'TILES'** tool, click **'VERIFY POSITIONS'**.





3. Choose *verification helper method* (Definite Focus recommended, can also choose Software Autofocus or manual).
4. Select position one and click '**MOVE TO CURRENT POINT**'.
5. Click '**RUN DF AND SET Z**' (if Definite Focus selected – otherwise Run AF or adjust manually and set Z).
6. Click '**USE DF TO VERIFY REMAINING**' (or AF/manual).
7. A *green tick* will appear next to each position once verified. A *red cross* if verification was not possible. Ensure that each position is suitably focused by watching verification in Live mode.
8. Click '**CLOSE**'.



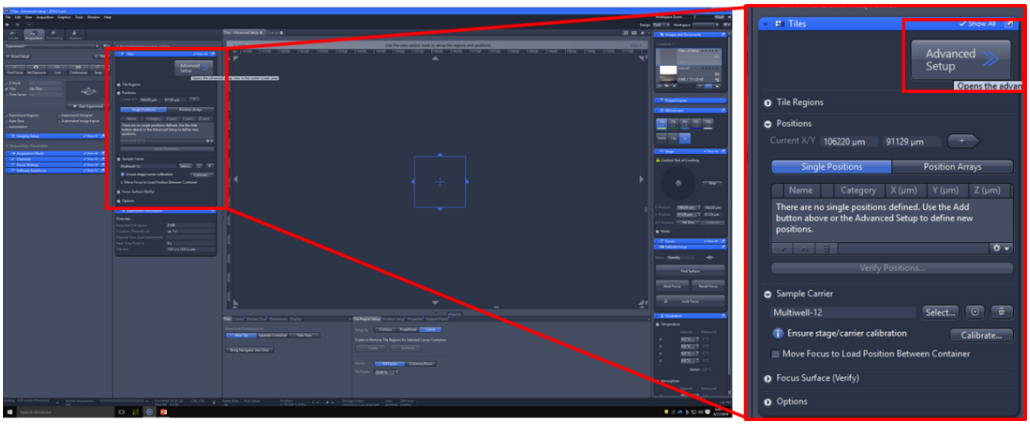
Tiles

Checklist:

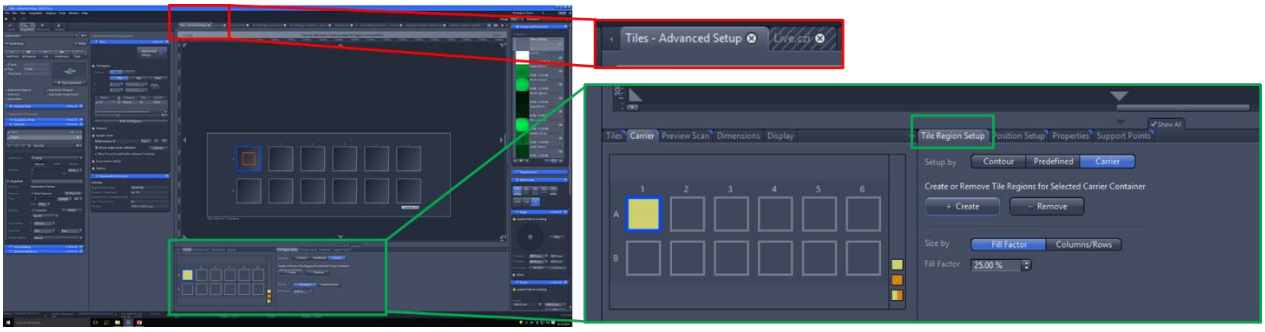
- Stage has been calibrated
- Sample carrier has been calibrated
- At least one channel has been selected.

Add Tile Regions

1. Expand **'TILES'** tool.
2. Click **'ADVANCED SETUP'**.



3. Toggle to **'TILES-ADVANCED SETUP'** tab, and **'TILES'** tab.

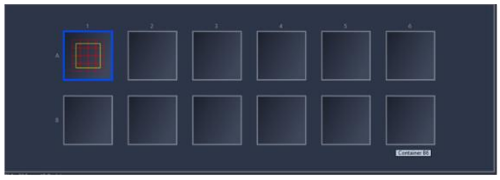


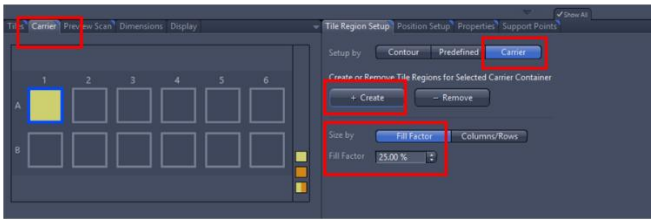
4. Create *tile regions*. There are three ways to do this;

Carrier.

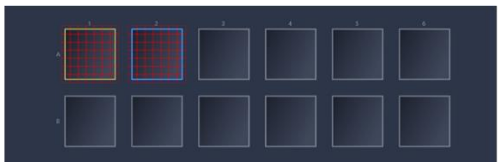
1. Click **'CARRIER'**.
2. Select *well* to add tile region to.
3. Choose tile size by:
    I. **'FILL FACTOR'**: change *percentage* of well to be filled by tile.
    II. **'COLUMNS/ROWS'**: *number* of columns/rows for tile.
4. Click **'CREATE'**.

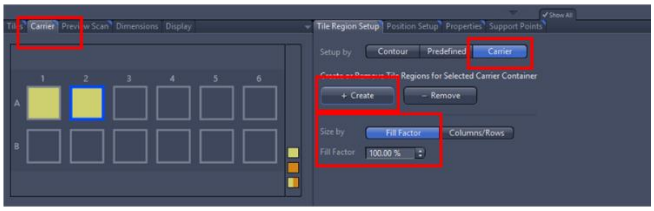
Fill factor 25%



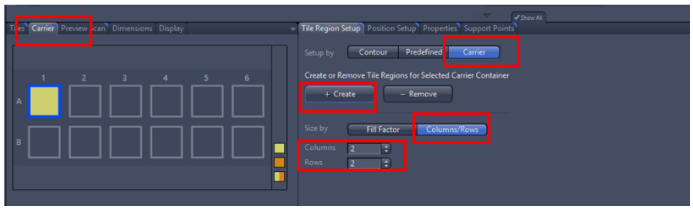


Fill factor 100%





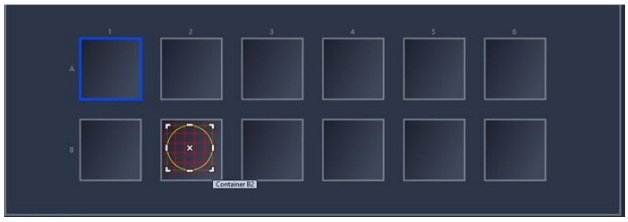
2 x 2 tile

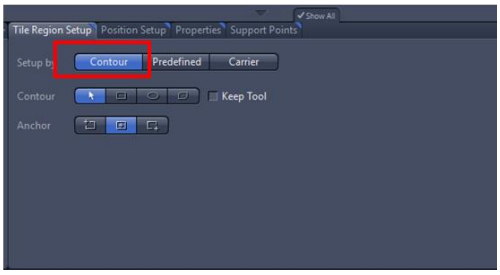


Contour.

1. Click **'CONTOUR'**.
2. Choose *shape* and draw tile region on *navigation map*.

Contour method

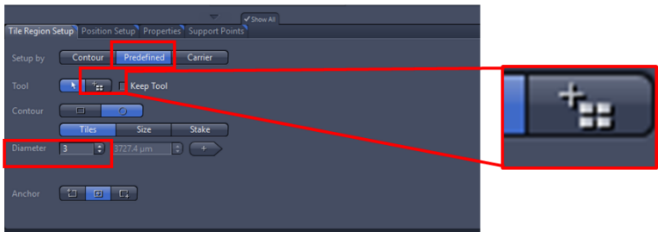




Predefined.

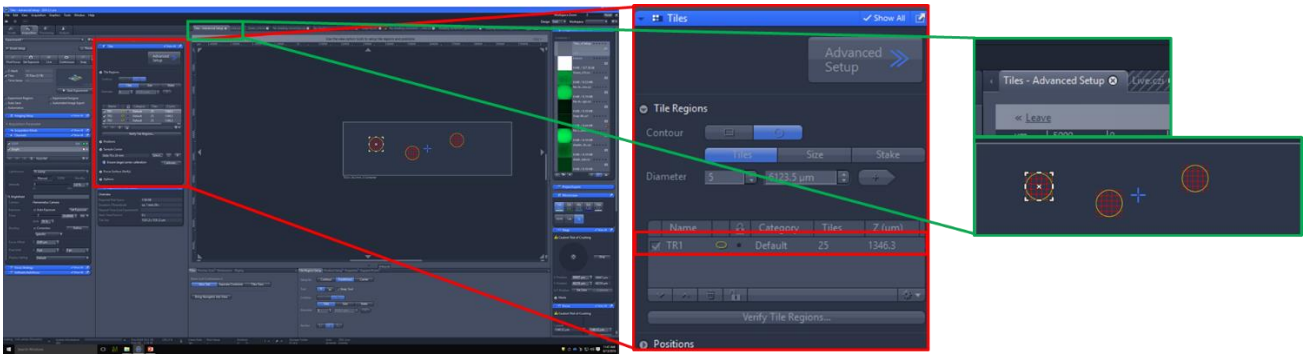
1. Click **'PREDEFINED**.
2. Choose a *Contour* tool, and *diameter of tile region*.
3. Select  tool and click on navigation map to place tile.

Predefined



Tile Region Navigation

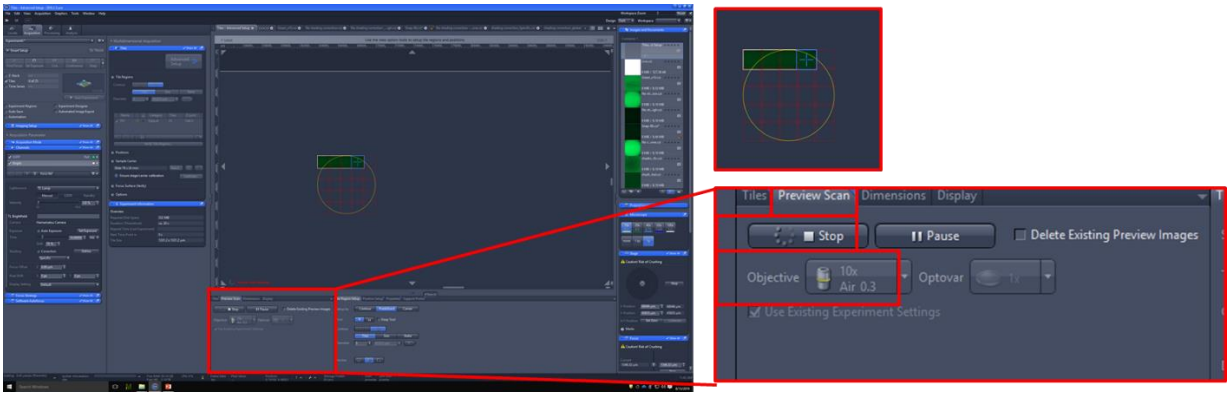
I. Click on *tile region* (e.g. TR1) under **'TILES'** tool.
II. Click on *white crosses* in centre of tile in **'TILES – ADVANCED SETUP'** tab.



Preview Scan

Creates low resolution scan of each tile in each region.

4. Toggle to **'PREVIEW SCAN'** tab.
5. Select *low magnification objective*.
6. Click **'START'** (changes to **'STOP'** when scan is running).



Create Local Focus Surface

If your specimen is tilted/uneven, you can compensate by creating a *local focus surface* by adding *support points*. Local focus surfaces are linked with one tile region, so one must be created for each tile region.

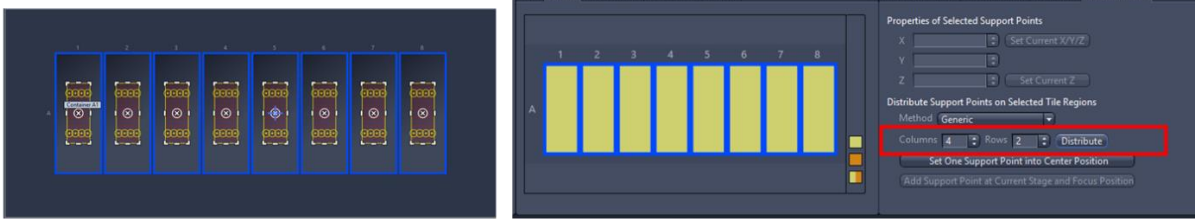
!!! Incompatible with using Definite Focus/Software Autofocus as focus strategy. Use with Tiles-setup defined Z values.

1. *Select* tile regions.
2. *Distribute* by:
    - *Columns/rows*
        I. Input number of columns and rows.
        II. Click **'DISTRIBUTE'**.

- *Centre point*
    I. Click **'SET ONE SUPPORT POINT INTO CENTRE POINT'**.

3. Click **'OK'**.

Columns/rows

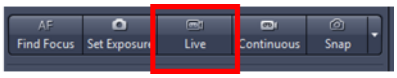


Centre

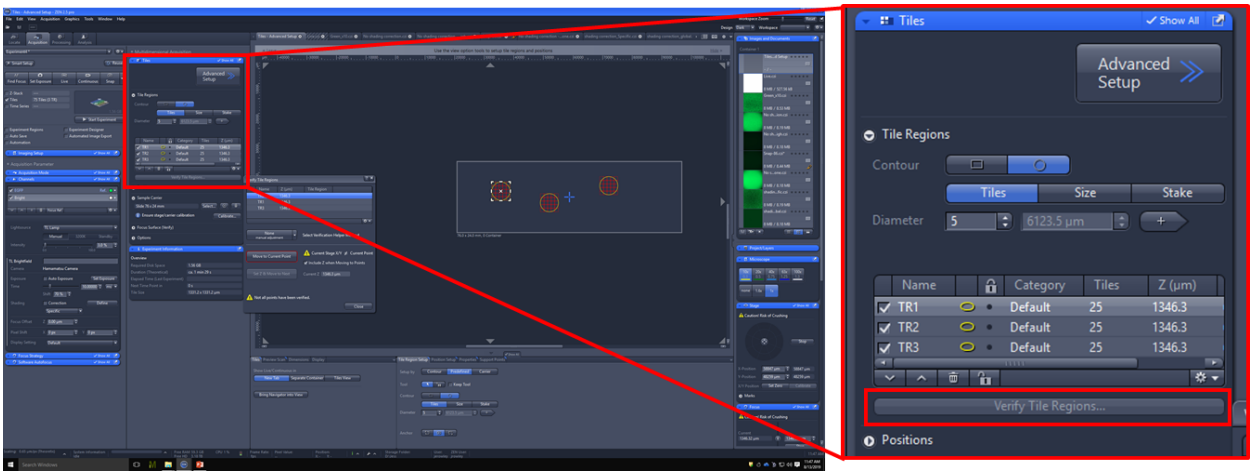
Move Tile Regions

1. *Drag white crosses* in centre of tile in **'TILES – ADVANCED SETUP'** tab.

Verify Tile Regions

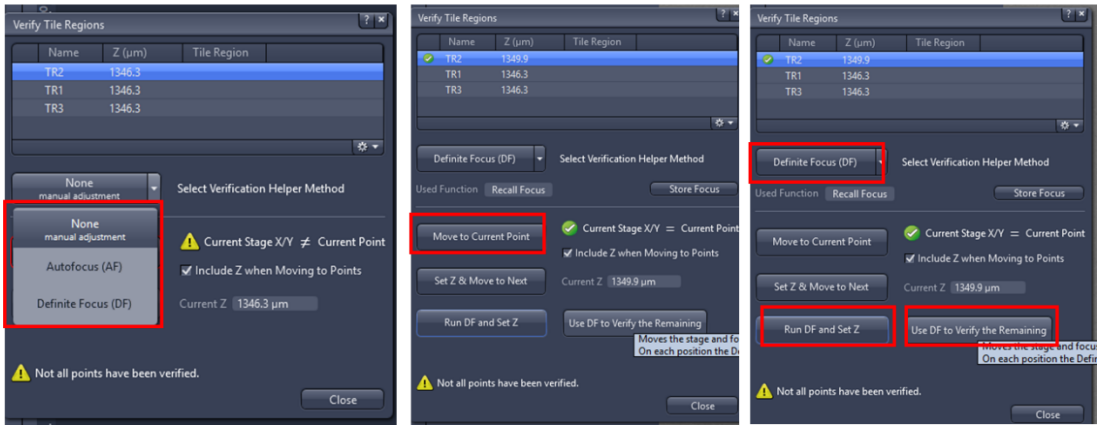
1. Ensure that **'LIVE'** mode is activated. 

2. Under **'TILES'** tool, click **'VERIFY TILES'**.



3. Choose *verification helper method* (Definite Focus recommended, can also choose Software Autofocus or manual).
4. Select first tile region and click '**MOVE TO CURRENT POINT**'.
5. Click '**RUN DF AND SET Z**' (if Definite Focus selected – otherwise Run AF or adjust manually and set Z).
6. Click '**USE DF TO VERIFY REMAINING**' (or AF/manual).
7. A *green tick* will appear next to each tile region once verified. A *red cross* if verification was not possible. Ensure that each tile is suitably focused by watching verification in Live mode.
8. Click '**CLOSE**'.

3. **4.** **5 + 6.**



Focus Surface

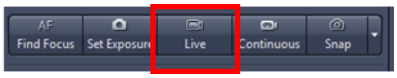
Checklist:

- Stage has been calibrated.
- Sample carrier has been calibrated.
- Selected sample carrier template has support points **OR** support points have been added to tile regions.
- You are not using Definite Focus or Software Autofocus as **Focus Strategy**.

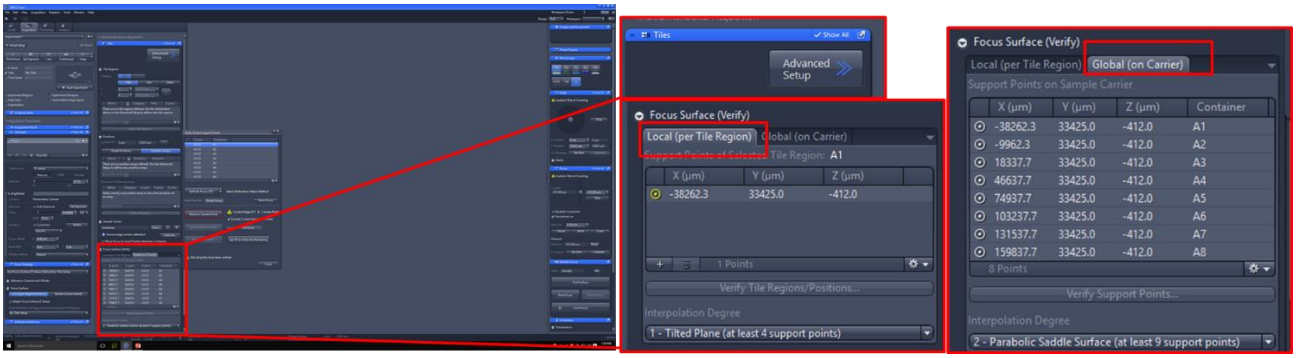
If your sample carrier is uneven, you can add *support points* to a sample carrier template to create a *global focus surface* that will compensate for tilting and bending. When imaging tile regions on an uneven specimen, a *local focus surface* can be created by adding *support points* to each tile region.

!!! Incompatible with using Definite Focus/Software Autofocus as focus strategy. Use with Tiles-setup defined Z values.

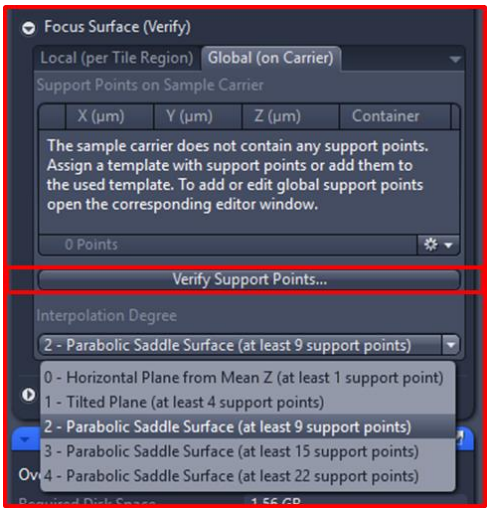
Verify Support Points

1. Ensure that '**LIVE**' mode is activated. 

2. Expand '**TILES**' tool.
3. Expand '**FOCUS SURFACE**'.
4. To verify *local focus surface* for tile regions, toggle to '**LOCAL (PER TILE REGION)**' tab. For verify *global focus surface* for sample carrier, toggle to '**GLOBAL (ON CARRIER)**' tab.

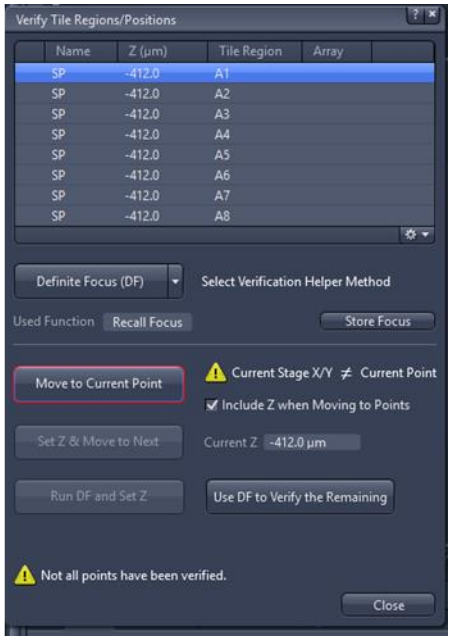


5. Select '**INTERPOLATION DEGREE**' from dropdown menu.
   - The *minimum number of support points* required is indicated next to each entry. For example, for 0 – Horizontal Plane from Mean Z, only 1 support point is required.
   - The calculation will be *more accurate if more support points* than the required minimum are added.
   - Only increase the interpolation degree as far as the sample carrier demands.
   - If fewer support points than the required minimum is added, a lower degree of interpolation will automatically be selected.
6. Click '**VERIFY SUPPORT POINTS**'.



7. Choose *verification helper method* (Definite Focus recommended, can also choose Software Autofocus or manual).

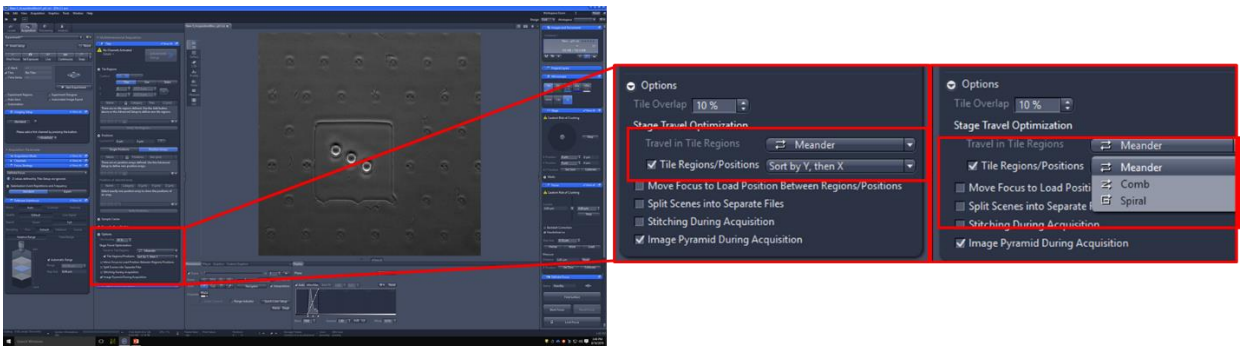
8. Select first support point and click '**MOVE TO CURRENT POINT**'.
9. Click '**RUN DF AND SET Z**' (if Definite Focus selected – otherwise Run AF or adjust manually and set Z).
10. Click '**USE DF TO VERIFY REMAINING**' (or AF/manual).
11. A *green tick* will appear next to each support point once verified. A *red cross* if verification was not possible. Ensure that each support point is suitably focused by watching verification in Live mode.
12. Click '**CLOSE**'.



Options

It might be useful to change the *stage travel direction*.

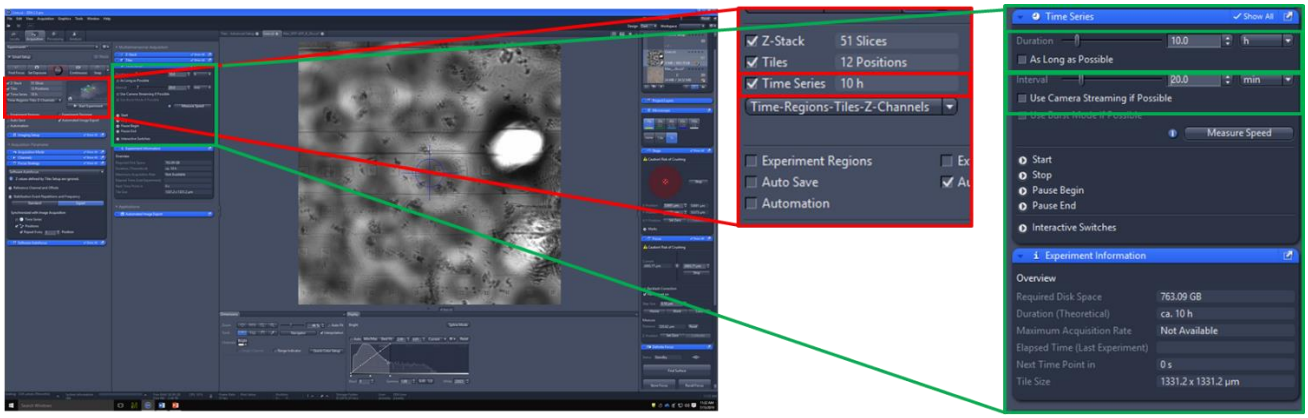
1. Under '**TILES**' tool, expand '**OPTIONS**'.
2. Expand '**TRAVEL IN TILE REGIONS**' and select method.



Time course

1. Tick '**TIME**'.
2. In '**TIME**' tool adjust:
   - '**DURATION**' (how *long* you want to image for)

- **'INTERVALS'** (how *frequent* you want to image)
- *Units* can be selected from *drop down menu* (e.g. cycles/hours/mins).



Z stack

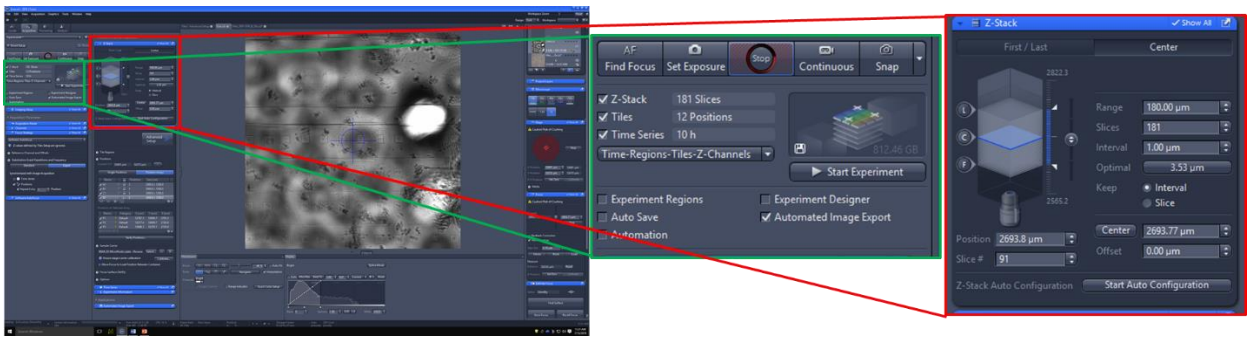
Checklist:

- Z tool has been ticked.
- Positions/tile regions have been setup and verified.
- Sample is in focus at the verified z-position.

Tips:

- Z tool has been tickeUse verified focal plane as Z-stack *centre point*.
- Use **'OPTIMAL'** to ensure adequate number of optical slices to satisfy *Nyquist*.
- For images that will be *deconvolved*, use twice optimal, and extend First and Last optical slices past top and bottom of sample.

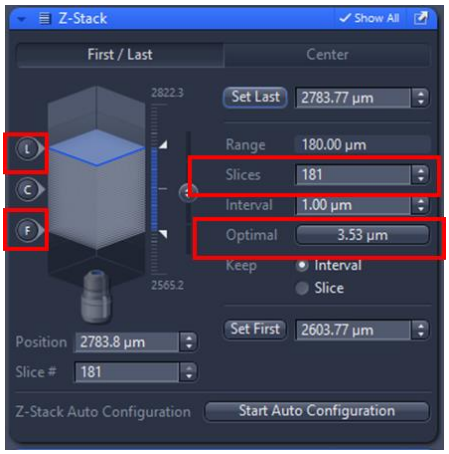
1. Tick **'Z-STACK'**.
2. Use **'FIRST/LAST'** or **'CENTRE'** method of setting up optical slices.



First/Last

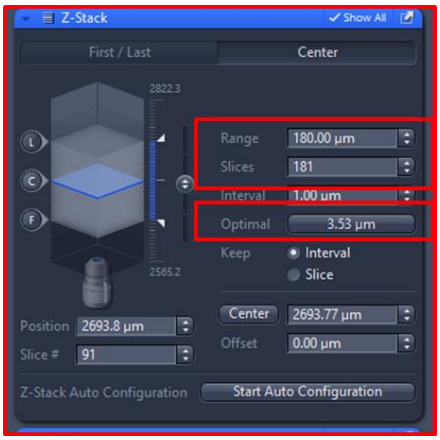
1. Find *first* optical slice by focusing on *bottom* of sample.

2. Click **'F'**.
3. Find last optical slice by focusing on top of sample.
4. Click **'L'**.
5. Choose number of optical slices – click **'OPTIMAL'** (recommended) or input **'SLICE'** number.



Centre

1. Focus on sample (use verified Z position)
2. Click **'C'** to set *centre* plane.
3. Choose **'RANGE'** (can use first/last tab to determine range)
4. Choose number of optical slices – click **'OPTIMAL'** (recommended) or input **'SLICE'** number.

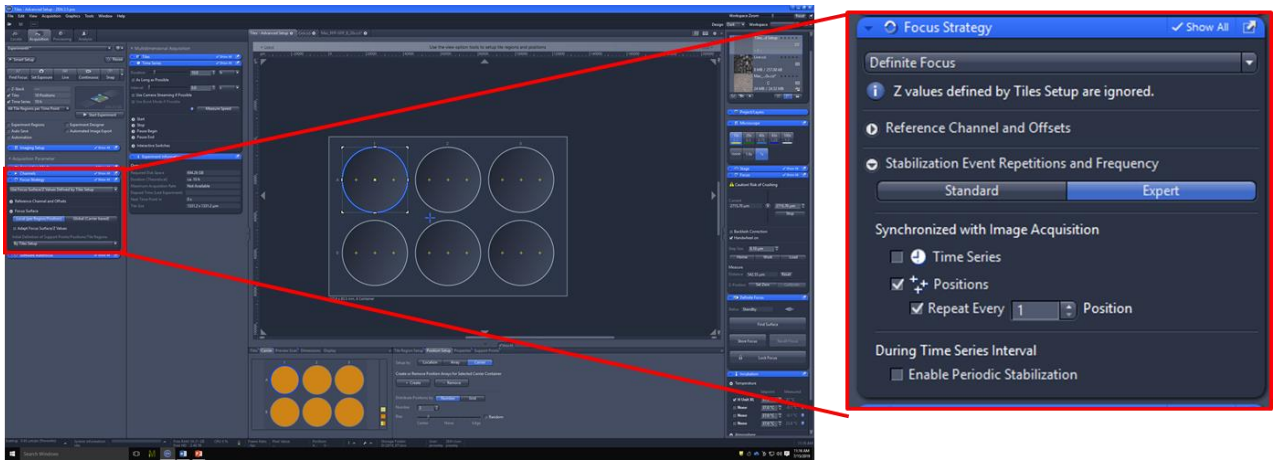


Focus Strategy

Checklist:

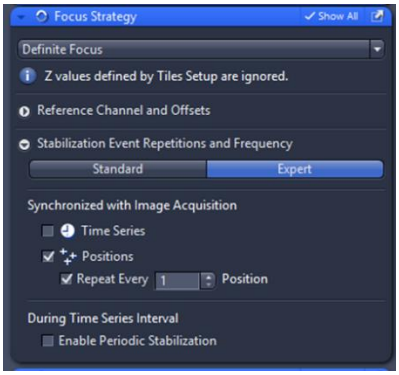
- Positions have been setup and verified.
- Time tool has been ticked.

1. Expand **'FOCUS STRATEGY'** tool.
2. Choose focus strategy:



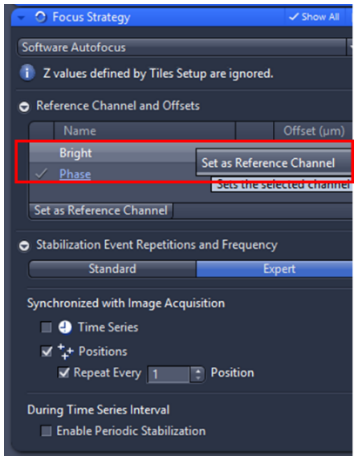
Definite Focus

1. Navigate to **'EXPERT'** tab.
2. Choose *'Time Series'* or *'Positions'* (recommended) to determine how often to stabilize.
3. Can tick **'ENABLE PERIODIC STABILIZATION'** for long intervals between imaging.



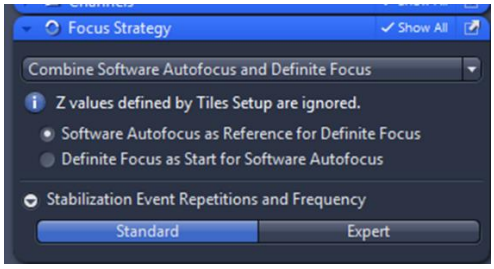
Software Autofocus

1. Expand **'REFERENCE CHANNEL AND OFFSET'**.
2. Right click on channel to use of autofocus and click **'CHOOSE REFERENCE CHANNEL'**.
3. Navigate to **'EXPERT'** tab.
4. Choose *'Time Series'* or *'Positions'* (recommended) to determine how often to stabilize.
5. Can tick **'ENABLE PERIODIC STABILIZATION'** for long intervals between imaging.
6. Check software autofocus settings - e.g. coarse/fine, smart/full.



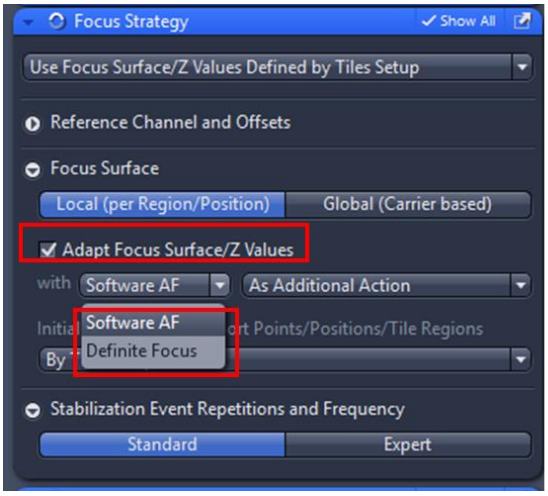
Combine Definite Focus and Software Autofocus

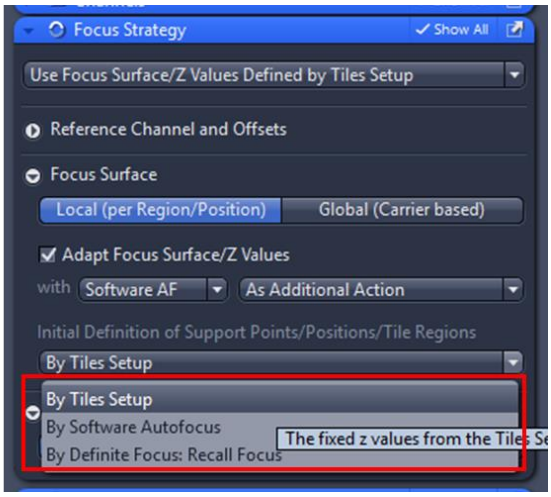
If cells are not well adhered, or have different focal planes in relation to surface, can combine the two methods.



Focus surface/Z values defined during setup

1. Choose Focus Surface (local/global).
2. To *adapt Z values* throughout time-course:
    I. Click **'ADAPT FOCUS SURFACE/Z VALUES'**.
    II. Choose to *adapt* with Definite Focus or Software Autofocus.
    III. Choose to *define initial Z positions* by Tiles Setup, Definite Focus or Software Autofocus.



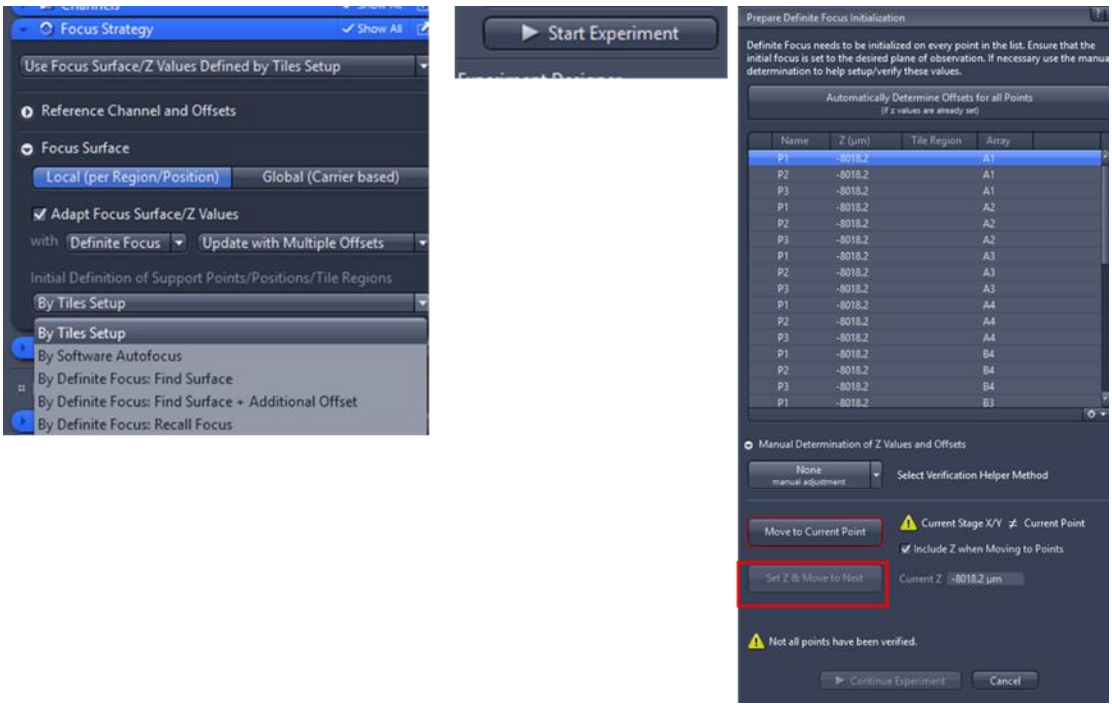


Adjust Focus Surface/Z Values with Definite Focus

This focus strategy is highly effective for samples which have *different offsets* from surface.

1. Select **'USE FOCUS SURFACE/Z VALUES FROM SETUP'**.
2. Tick **'ADAPT FOCUS SURFACE/Z VALUES'** and select *with* **'DEFINITE FOCUS'** and **'UPDATE WITH MULTIPLE OFFSETS'**.
3. Choose *method of initial definition*. **'TILES SETUP'** is usually most useful in this context as Z value can be chosen manually for each position.

After Starting Experiment, you will be prompted to **'PREPARE DEFINITE FOCUS INITIALIZATION'**. This can be done manually (recommended), or by using Software Autofocus or Definite Focus.



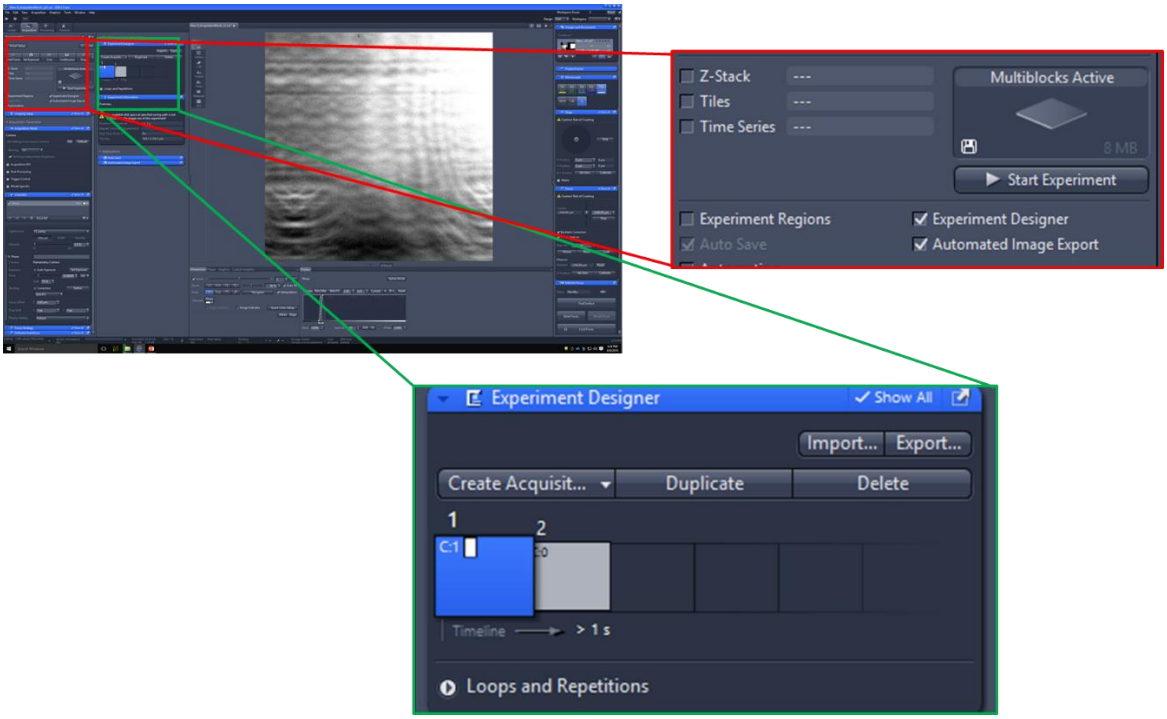
For further information on Focus Strategies see Focus Strategies in Zen , and Focus Strategy Workflow.

Experiment Designer

If you want to *change acquisition settings* throughout the experiment, you can create *experiment blocks.* For example, when using microfluidics you may only want to image every 30 mins until addition of a drug, then you may wish to image every 5 mins to observe drug effects.

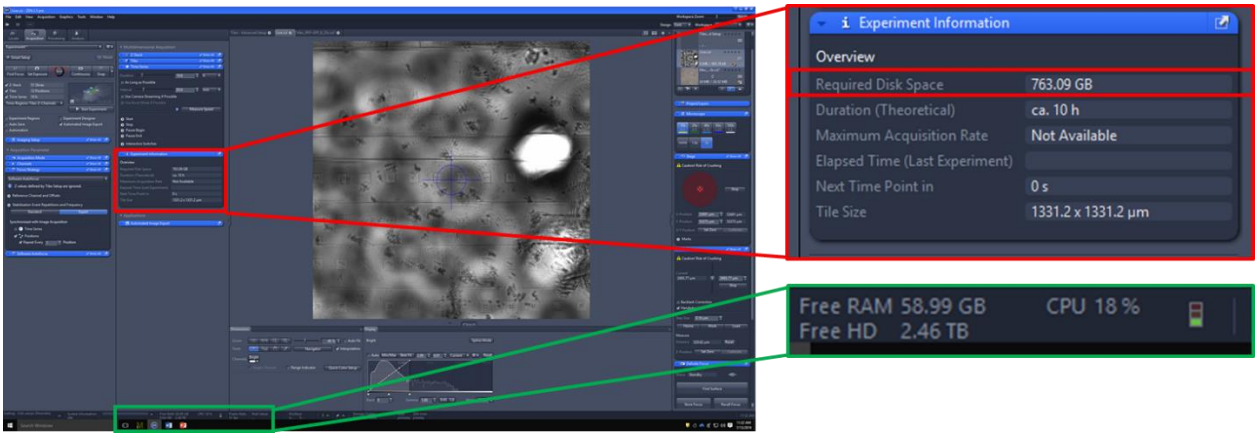
1. Tick **'EXPERIMENT DESIGNER'**.
2. Expand **'EXPERIMENT DESIGNER'** tool.
3. **'DUPLICATE'** experiment block and adjust time (or other parameters), or **'CREATE ACQUISITION BLOCK'** to create a new block from scratch.
4. Blocks can be repeated using **'LOOP AND REPETITIONS'** function.

!!! Any changes made will only be applied to the currently selected block.



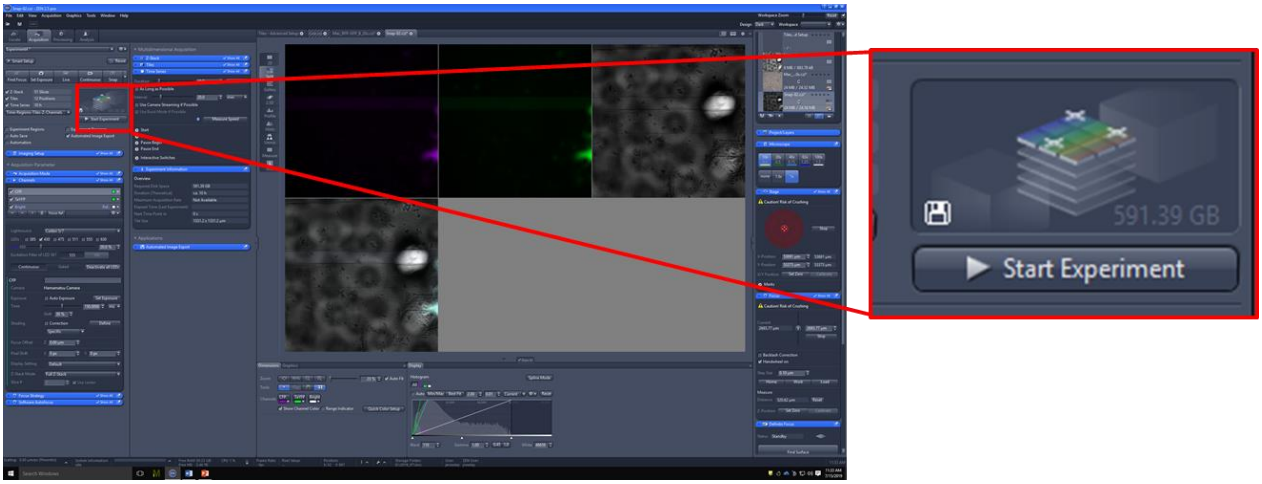
Check expected experiment size

1. Check **'REQUIRED DISK SPACE'** against **'FREE HD'**.
2. Delete old files or reduce experiment size if necessary.



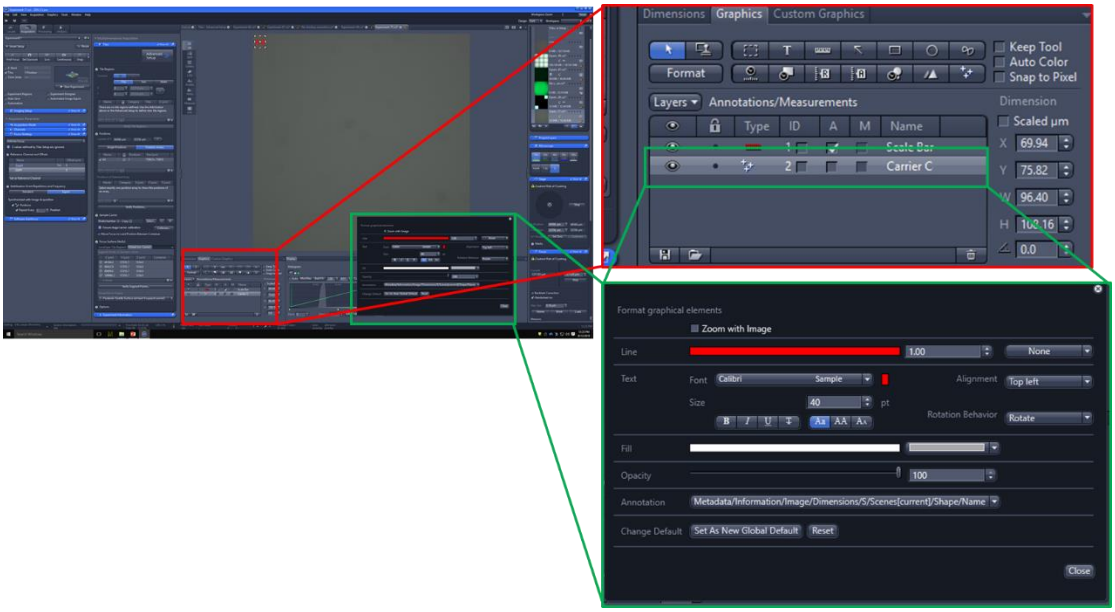
Start experiment

1. Click **'START EXPERIMENT'**.
2. *Watch one run through* (e.g. for first timepoint, watch all channels/z/positions) to ensure focusing strategy is satisfactory.


591.39 GB
Start Experiment

Add graphics

1. Toggle to **'GRAPHICS'** tab.

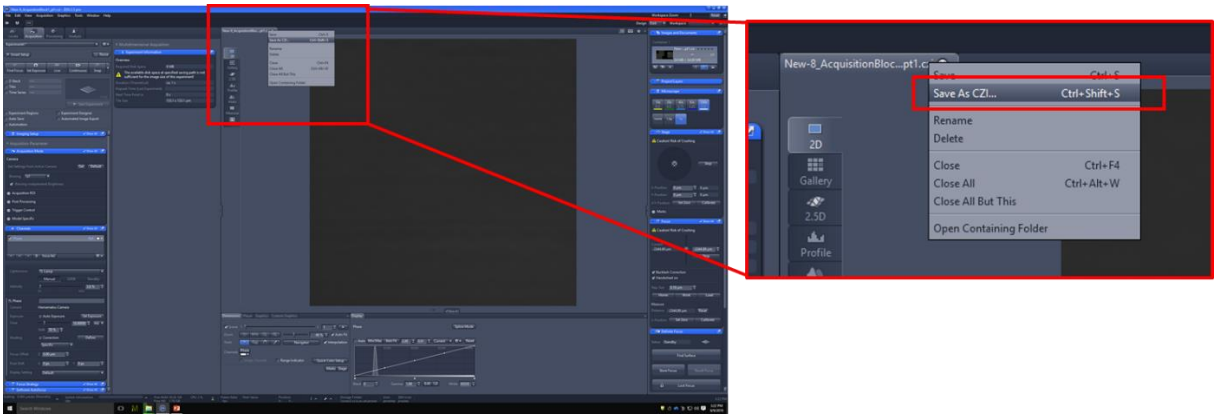


2. Click  to add *scale bar.*
3. Click  to add *well number*.
4. Right click on graphic to *format*.

Save data

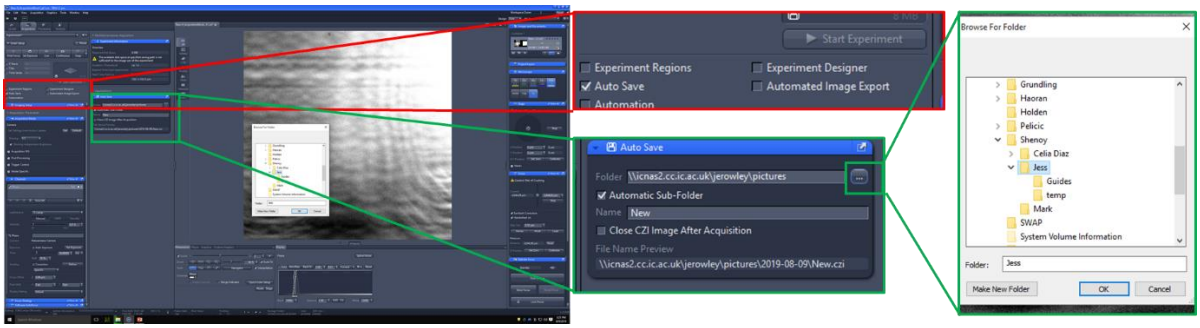
Manual

1. *Right-click* on image tab.
2. Click 'Save As CZI'.
3. Save to **D:\USERNAME** e.g. D:\Jess



Autosave

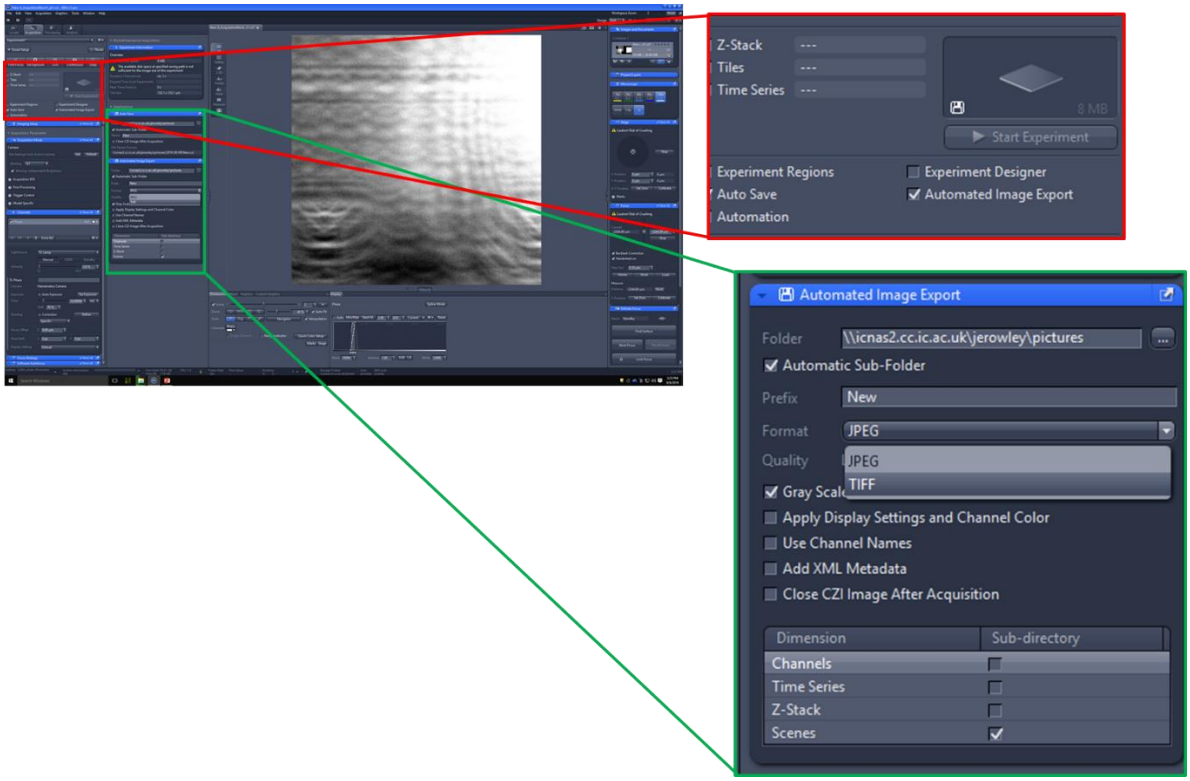
1. Tick **'AUTOSAVE'**.
2. In **'AUTOSAVE'** tool choose folder to save data to.
3. Save to **D:\USERNAME** e.g. D:\Jess



Automated image export

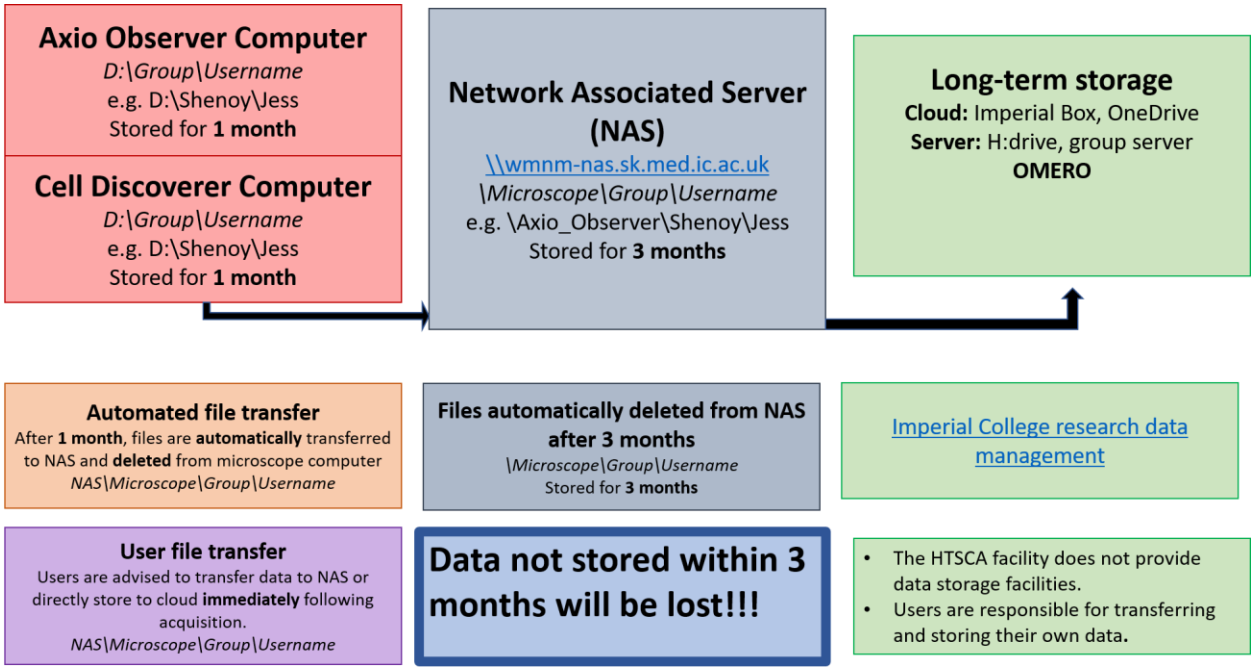
Images can be automatically *split* into *channels*, *scenes* (positions), optical slices (*z*) and *time* series and exported.

1. Click **'AUTOMATED IMAGE EXPORT'.**
2. Expand **'AUTOMATED IMAGE EXPORT'** tool.
3. Choose **FOLDER** to save to.
4. Choose **FORMAT**:
    - **TIFF** – for image *processing/analysis* and data storage
    - **JPEG** – for *display* only
2. Choose *dimension* to split by (e.g. channel, scene, z, time).



Transfer data

- It is recommended that data are transferred immediately after acquisition. Data can be transferred to Network Associated Storage (NAS) or directly to Box/OneDrive.
- Data will be automatically transferred to NAS after *one month* and removed from microscope hard drive.
- The NAS is for *transfer only* and data will be removed after *3 months* (for automatically transferred data) or 1 month (for user transferred data).
- Users are responsible for storing their own data long-term.
- SOP Data Management



Import and export images

File export from Zen

Clean objectives after oil immersion

Objectives requiring oil immersion (63X/100X) should be cleaned with *Whatman lens tissue 105* and *Zeiss cleaning solution*.

!!! Do not use Kimwipes or any other tissue on the objectives.

Shutdown

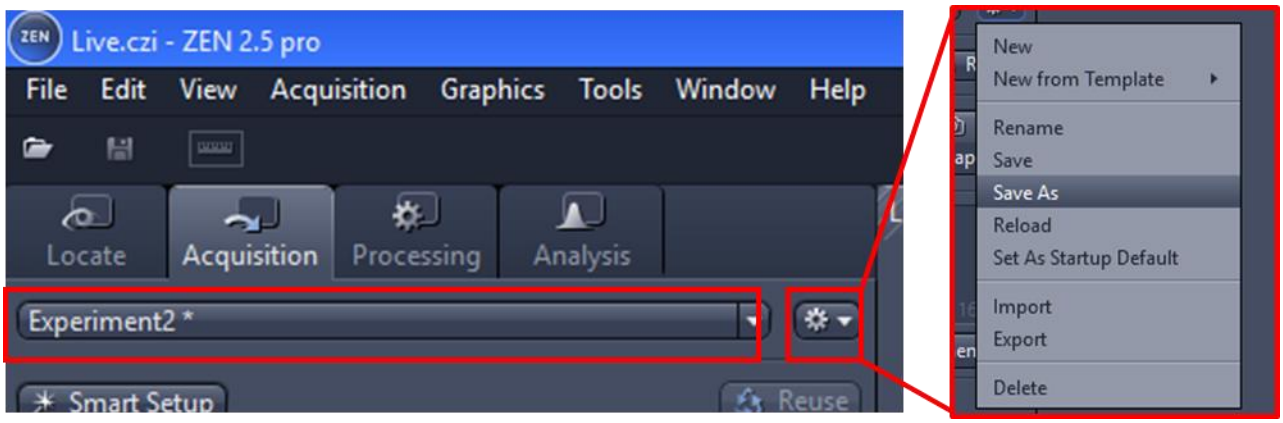
1. Zen blue – click **'X'** in corner
2. Turn off all modules in any order (microscope, power boxes, incubators*, CO_2 and computer)

- * When few people are using temperature control, heat will be switched off after use. It is good practice to switch heat on **4hr** before running experiment to allow acclimatisation. When temperature control is being used daily, heat will be left on at all times.
- Check touchpad (TFT) to ensure heat and CO_2 are at desired level.
- **Turn off CO_2 after use** (in software or on TFT).

Repeating experiments

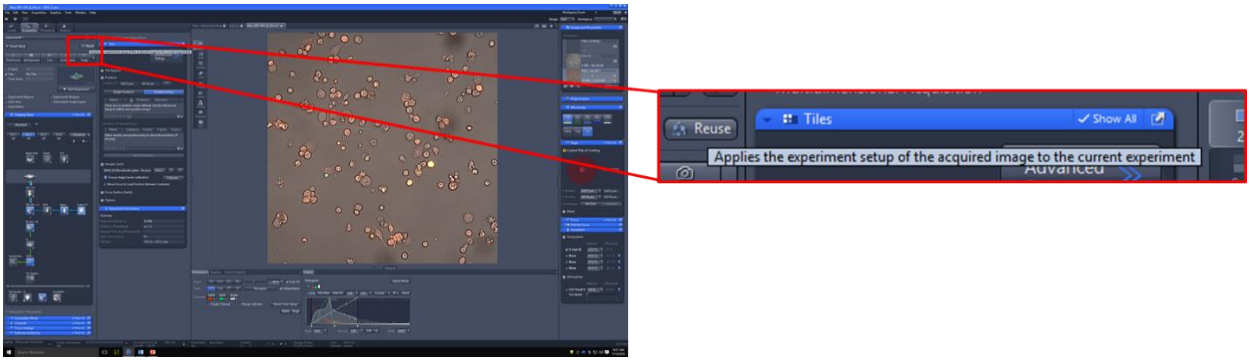
Experiments

1. Expand cog symbol to *rename*, *save*, *load*, *import* and *export* experiments.



Reuse from image settings

1. Acquisition settings can be reused from previous images.
2. Open image (.czi).
3. Click **'REUSE'**.



Further Help

j.rowley@imperial.ac.uk

Slack – **HTSCA_CMBI**

Zen Blue Manual

iBiology Microscopy

HTSCA Sharepoint (Click, ☰ on top left of home page).

ZEISS ONLINE HELP:

1. Hover mouse where help is required
2. Press **F1**

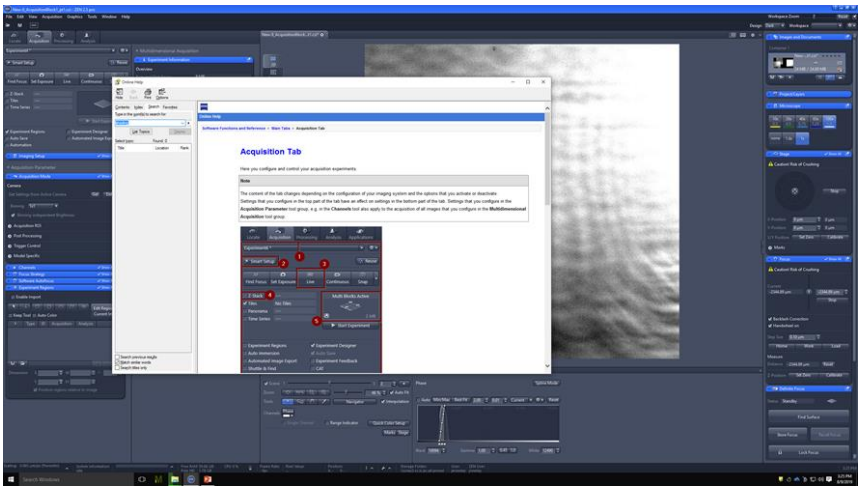


Image processing and analysis

After acquiring your data, you will need to perform processing steps such as *deconvolution*, *background subtraction*, region of interest (*ROI*) cropping and *thresholding*. You can then perform measurements such as *intensity*, *size* and *colocalisation*.

This can be performed in *open access software* such as Fiji, Cell Profiler and Icy. Some processing and analysis tools are available in Zen Lite (freely available).

Full processing and analysis capabilities in *Zen Blue* (including deconvolution, and image analysis) are available on Cell Discoverer computer.

Zen Blue image processing guide – coming soon!!!

Zen Blue image analysis guide – coming soon!!!

Image processing and analysis further help