Axio Observer Live Cell Imaging SOP

START UP

Switch on the incubator heater module, the temperature module, and the CO_2 module power supply

There are two plug sockets labelled 'incubator', switch these on.

Switch on Power Supply 232

Switch on SMC 2009

Switch on HXP 120V FL Lamp

This is only required for imaging fluorescence.

- Switch the power switch on.
- Leave the intensity level at 2/3 (you can turn this down intensity if sample is sensitive or bleaches easily).
- Check the blue shutter open light is on, if not press the shutter open switch.

Switch on microscope

Switch on PC

- Switch on the PC and login using your UoG ID and password.
- If you are going to be doing large tile scans in LCI, you may need to use the .\ZEISS login (no password) in order for the computer to be able to process and save large files.
- Open the **ZEN blue** software to run the microscope. Select **ZEN Pro** for image acquisition.

Switch on the incubator

- Finally, turn on the incubator using the touchscreen monitor. Once this has been done, the temperature and level of CO_2 can be set for the following equipment:
    - H Insert P = the holder for a petri dish
    - Inc PM = the lid for a cell culture plate (if using one which is connected by an electrical wire)
    - H unit XL = the heater for the entire incubator
    - H Dev Humid = the humidity water bottle

- All mounts/ lids/ inserts can be found inside the incubator and should be kept here for convenience. It takes up to **4 hours** for all of the microscope components to reach a stable temperature of 37^{o}C.
- The temperature range for the incubator is 10-50^{o}C.
- Check all doors/ movable parts on the incubator (which have blue handles) are closed during warm up.
- Check the humidifier water bottle is filled with dH_2O (there are min/max indicators on the bottle).

Set up

- Fit the appropriate mount to the stage: align the red dots on the mount and the stage, then push into place.
- Fit the lid to the mount. This will help minimise evaporation of media during imaging. Use masking tape to secure the lid if necessary.
- Load the sample onto the microscope: select an appropriate objective, press the 'load position' button on the touchscreen to slightly lower the objectives away from the stage, and place the sample in position. Press the upward arrow on the touchscreen to return the objectives to the work position.

Running software

Locate

- Select **ocular online** to be able to see the sample using the eyepiece.
- Adjust the knob on the microscope to divert the image to the eyepiece.
- Check the lightpath settings are ZEN is directing the light to the eyepiece.
- Select **transmitted light (TL) on** and **reflected light (RL) off** to look at **brightfield** images.
- Select **reflected light (RL) on** and **transmitted light (TL) off** and then select the appropriate filter to look at **fluorescence** images.
- Select the appropriate objective.

- All objectives are dry, except the X63 and X100 which need oil. A message will pop up to prompt you to change the media. Click ‘done’ when you have added/ removed oil and the objective will move back into position for viewing.

Acquire

- Select **ocular offline** to acquire images.

Transmitted light setup

Transmitted light setup

- Select the appropriate objective, condenser, and slider (you only need this for DIC) for your requirements.

Objective	Condenser	Slider
X2.5	H	
X5	H/ Ph1	
X10	H/ Ph1	
X20	H/ Ph2/ Plas DIC*	DIC I/II
X40	H/ Ph2/ Plas DIC*	DIC I/II
X40	H/ Ph2	
X63 (oil)	H/ Ph3/ DIC II	DIC II/III
X100 (oil)	H	

- If using plasDIC, adjust the correction collar on the objective to correspond to the thickness of the surface on which the sample is mounted: 1 = for use with thick plastic surfaces eg petri-dish/ cell culture plate; 0 = for use with thin coverslips.
- If using DIC, you also need to move the polarising discs above the condenser into the light path.
- You must ask before swapping objectives eg the X10 for the X5 and ensure that you return the objectives to their default positions as listed in the table.
- Check the **Active Camera** is the colour camera.
- Adjust the knob on the microscope to divert the image to the camera.
- Alter the lightpath settings in ZEN, to divert the light to the colour camera.
- In **live** mode, **set exposure** and the **white balance**.
- In **live** mode or following acquisition, go to the **Display** tab and select **min/max** (for best contrast) or **best fit** (for best spread of values) or adjust the arrows underneath the histogram to set the contrast (left arrow), gamma (middle arrow), and brightness (right arrow). Gamma correction (relationship between detected brightness and display brightness) may be adjusted. The default, optimum value is 0.45.
- The software automatically opens a new window for each snap (2D and 3D).

Fluorescent light setup

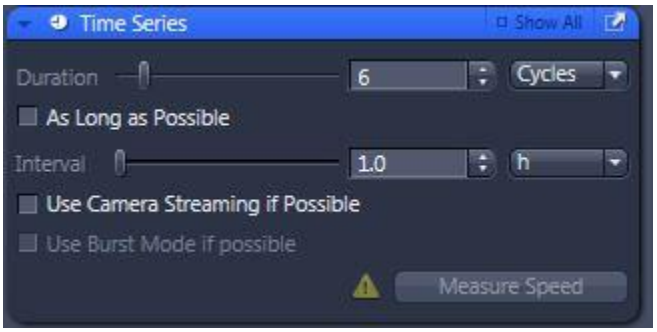
- Select the brightfield (H) condenser.
- Select the FL reflector module with the appropriate fluorescence filter.

Filter	excitation nm BP, emission nm BP
DAPI (49)	ex BP 365, em BP 445/50
GFP (38 HE)	ex BP 470/40, em BP 525/50
DsRed (43 HE)	ex BP 550/25, em BP 605/70
Cy 5 (50)	ex 640/30, em 690/50

Acquire

- Check the **Active Camera** is the monochrome camera.
- Alter the lightpath in ZEN, to divert the light to the monochrome camera.
- Open the **Smart Setup** and add your colour channels by clicking the ‘+’ button/ double-click on a fluorophore or contrasting method from the list. Use the **Automatic ‘P’ motif**.
- Go to **Experiment Manager** and select **All Channels**.
- In **live** mode, adjust the focus and **set exposure** for each channel in turn.

    - When checking the exposure of a channel in live mode, only the highlighted channel will be shown (in grey scale) even when the other channels are ticked. To image all channels sequentially (and in colour) use the continuous button.
    - To acquire the highest quality images with the least bleaching (which is important when acquiring many images over time in LCI) there will be a trade-off between gain and binning in the acquisition mode window (note that binning is nearly always preferred over gain), and also between light intensity and exposure time in the channels mode window. Binning is used to aggregate pixel intensities: the higher the binning, the higher the pixel intensity, the lower the resolution.
- As in the TL setup, images can be further enhanced by adjusting the settings in the display tab. You can also change the pseudocolours applied to each channel, as preferred.
- To perform LCI:
    - Select the time-series option in the acquisition tab.
    - Set the duration of your time lapse in milliseconds, seconds, hours, days etc.
    - Set the interval between each time point.



    - Select the tiles or positions (more detail below).
    - Set the **Focus Strategy** (more details below).

If the data being collected is too large (Error message: “the available disk space at the specified saving path is not sufficient for the image size of this experiment!”) there are various options to reduce the size of the experiment: reduce the duration, the interval, the camera resolution (binning), the number of channels, or the dynamic range of the acquired image.

Setting the Focus Strategy for LCI

You can minimise phototoxicity by focusing on samples using transmitted light during the set up.

Choose an appropriate **Focus Strategy** and change any parameters necessary in **Focus Devices**:

None – This is the default setting for all experiments. The current Z position at the time the experiment is started is set as the reference Z-position and remains unchanged during the experiment. Exception: By default, Z-stacks are acquired at the fixed Reference Z-position that has been defined as the centre in the Z-stack tool.

Absolute Fixed Z Position – Allows you to define a Reference Z-Position. You can choose between two modes: 1) Absolute Z-position allows you to define a reference Z-position that remains unchanged during the entire experiment. 2) Z-position from Tile Setup (only for tile experiments) uses the individual reference Z-positions that can be defined for tile regions and positions in the Tiles tool. See the ZEISS Axio Observer Tile Scan Advanced Setup doc. for more details on how to set this up.

Software Autofocus – The focus position is determined via the contrast calculation or intensity calculation of a series of images (Z-stack) and set as a Reference Z-Position. This automates the focusing before and during acquisition and is particularly useful for LCI.

Local Focus Surface – Local Focus Surfaces are specific to tile regions. They are interpolated from the Z-values of reference points, which can be created for the tile region in the Tiles tool. The interpolation results in an individual Reference Z-Position for each tile. If no reference points are defined, tile regions and positions automatically have a central reference point with the Z-value of the tile region or position. This allows you to ensure that all tiles are in focus on irregular specimens.

Local Focus Surface As Start For Software Autofocus – The Reference Z-Position calculated from the Local Focus Surface is used as the starting point for an additional Software Autofocus search, which updates the Reference Z-Position. This allows you to reduce the search range and step size of Software Autofocus.

Local Focus Surface Updated By Software Autofocus – The Local Focus Surfaces are regularly adjusted by means of a Software Autofocus search, which is performed exclusively at a defined position. A resulting correction of the Reference Z-Position is adopted for all focus areas.

Global Focus Surface – The Global Focus Surface is interpolated on the basis of reference points which are located on the sample carrier (eg slide or multi-well plate). These reference points can be defined by editing the sample carrier template. The interpolation results in an individual Reference Z-Position for each tile. This allows you to compensate for any tilting or bending of the sample carrier and therefore improve the focusing of the tiles.

Global Focus Surface As Start For Software Autofocus - The Reference Z-Position calculated from the Global Focus Surface is used as the starting point for an additional Software Autofocus search, which updates the Reference Z-Position. This allows you to reduce the search range of Software Autofocus.

Global Focus Surface Updated By Software Autofocus – The Global Focus Surfaces are regularly adjusted by means of a Software Autofocus search, which is performed exclusively at a defined position. A resulting correction of the Reference Z-Position is adopted for the entire focus area. This strategy is only relevant if tiles and time series are combined.

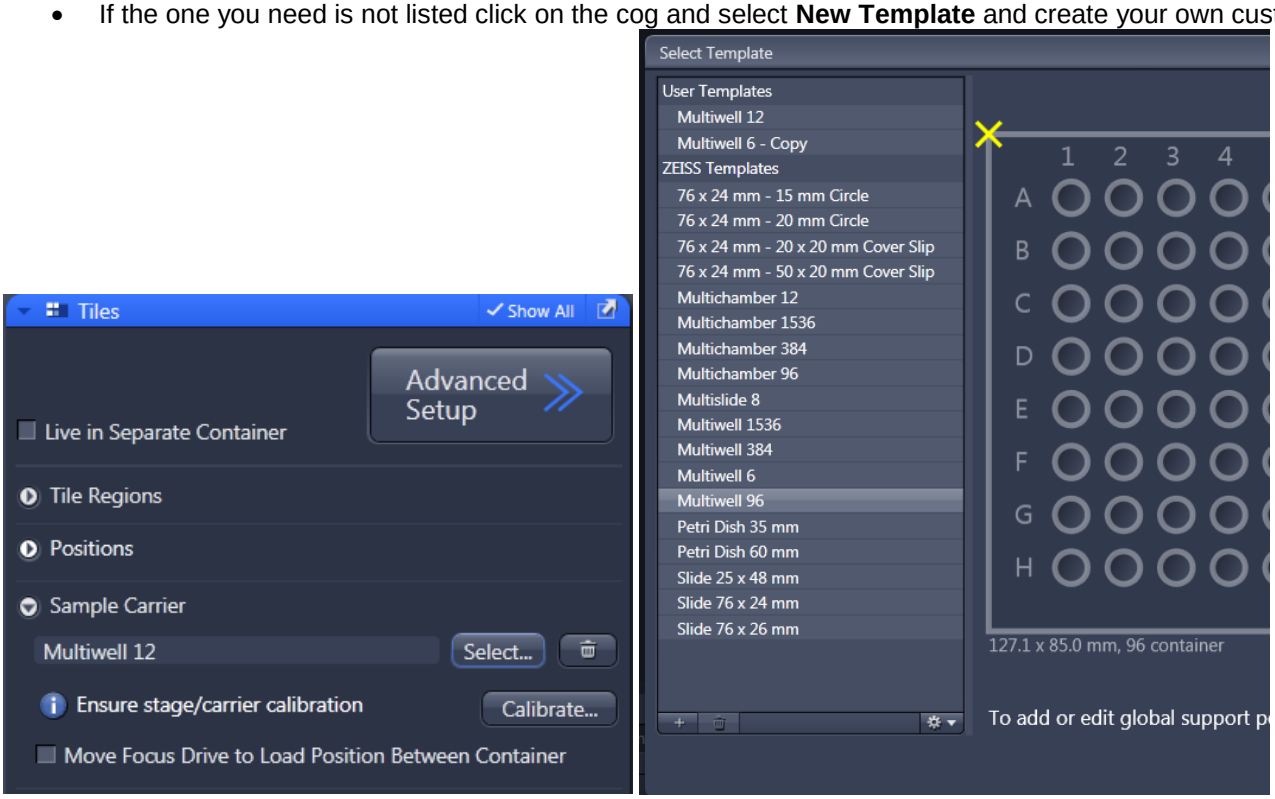
We do not have **Definite Focus**. Definite Focus is a focus strategy designed to minimise drift in the Z plane over time by maintaining a certain distance between the sample carrier and the objective. Drift can occur when there are fluctuations in temperature and parts of the microscope expand or contract. This is why it is important that you allow sufficient time for all components to warm up prior to LCI.

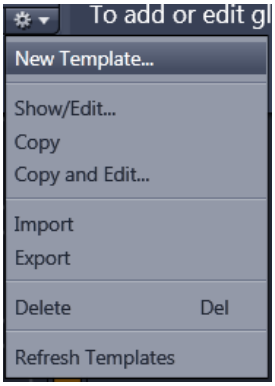
Acquiring tiled images in LCI

Tiling Part 1- Calibration of a multi-well plate

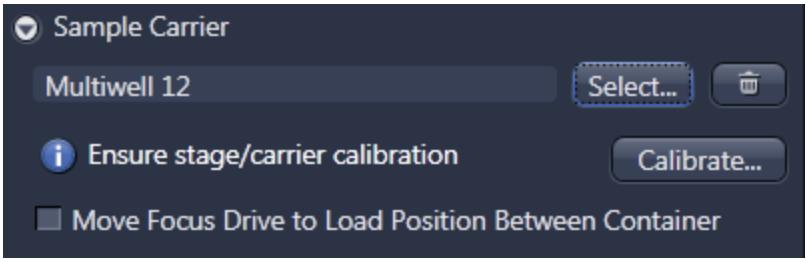
If you have already calibrated your multi-well plates and have saved the experiment you can move on to Part 2.
If not:

- Inside **Acquisition** go to the **Tiles** tab and click the **Show All** option.
- Go to the **Sample Carrier** drop down and click Select to choose your Sample Carrier…
- If the one you need is not listed click on the cog and select **New Template** and create your own custom one.

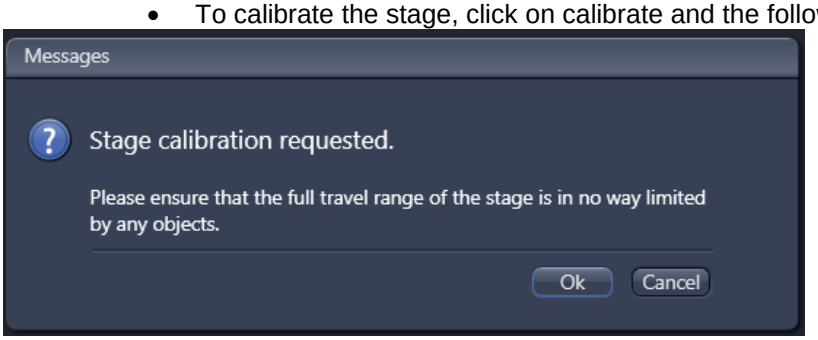




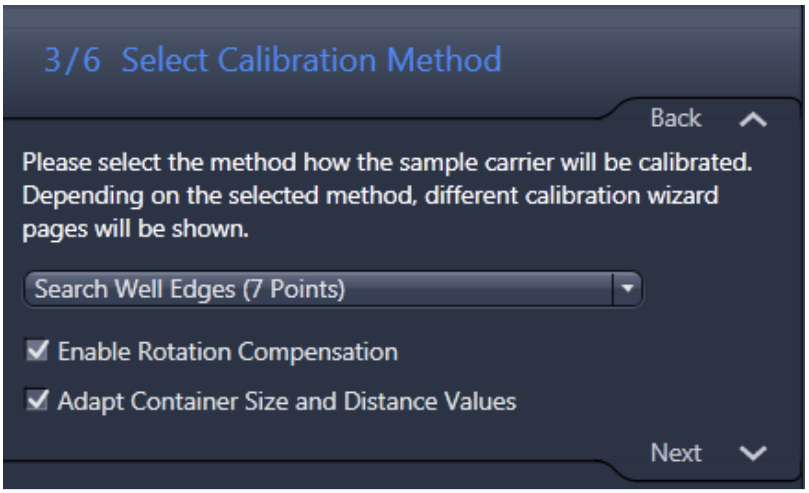
- After you have selected your sample carrier click **Calibrate** (note, use a brightfield or phase channel for this calibration and disable the fluorescence channels).



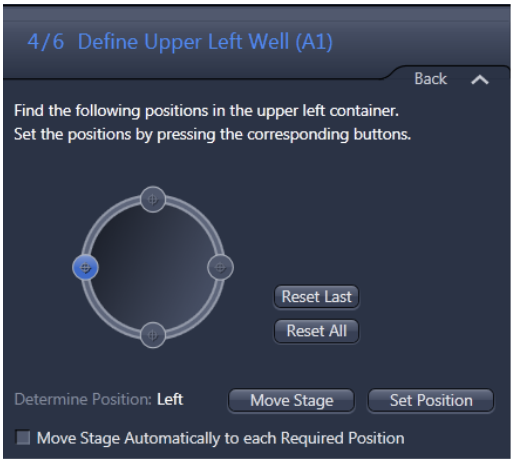
- This is a multi-step wizard:
  - Set your exposure and get an image on the live view.
  - To calibrate the stage, click on calibrate and the following message will appear.

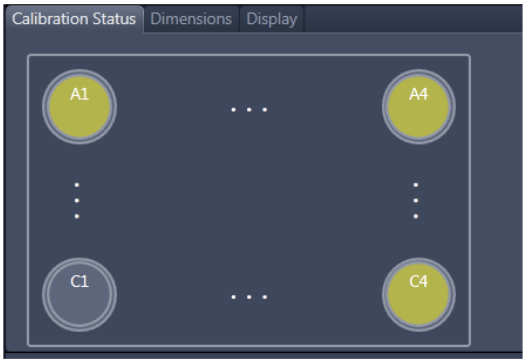


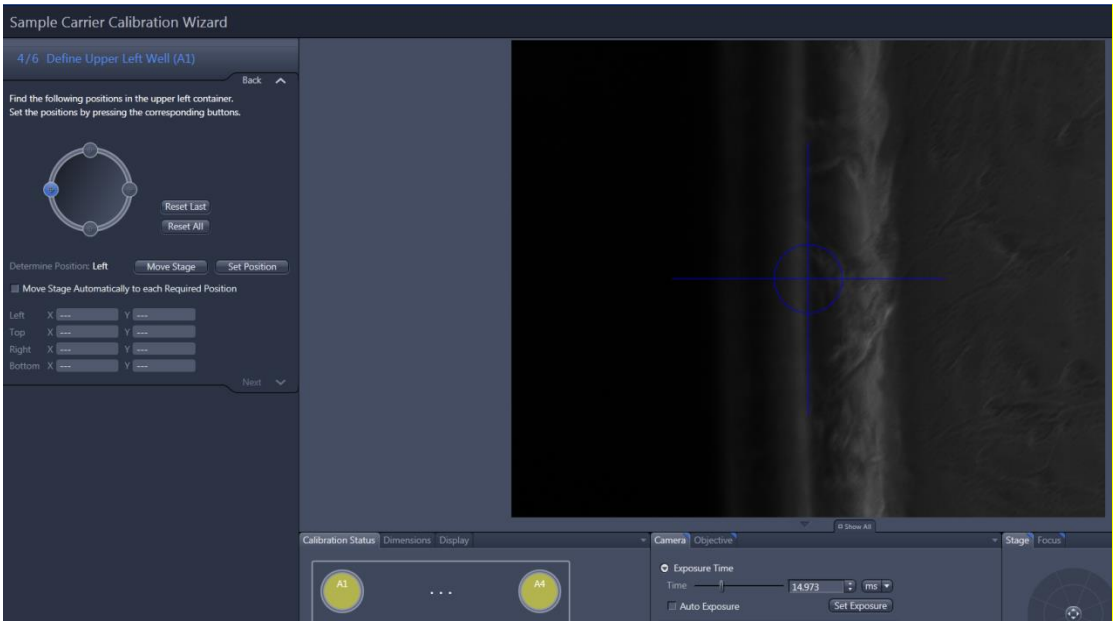
  - Click OK and the objective will move away from the sample and the stage will move to its end positions for calibration.
  - Select the 'Search Well Edges 7 points' for the most accurate calibration.



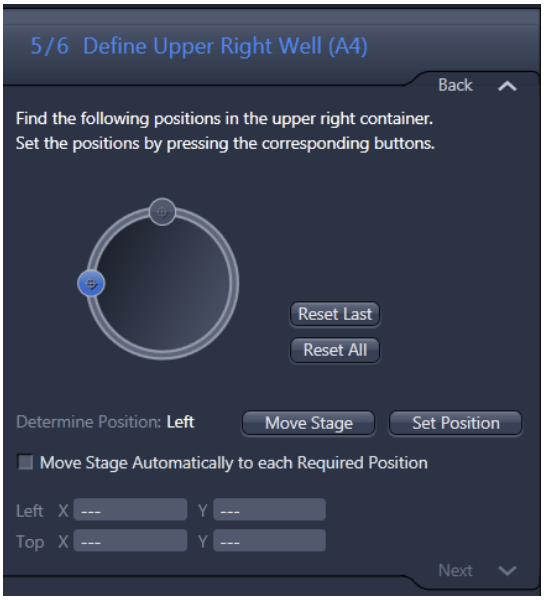
  - Move to well A1 (top left) and position the cross on the live preview over the left hand edge of the well and click on the blue round button.





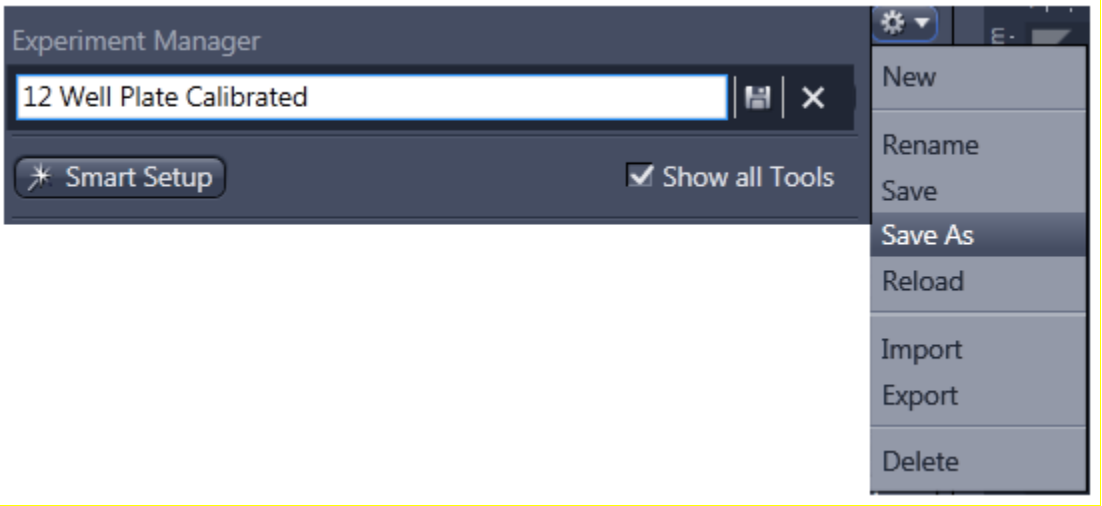


- Click on move stage and it will move to the second calibration point at the top to allow focusing and moving in to position.
- Repeat for the right hand and bottom points.
- Press next to then define the upper right well – only the left hand and top of the well are defined for calibration.



- Again press next and define the single calibration point for the lower right well.

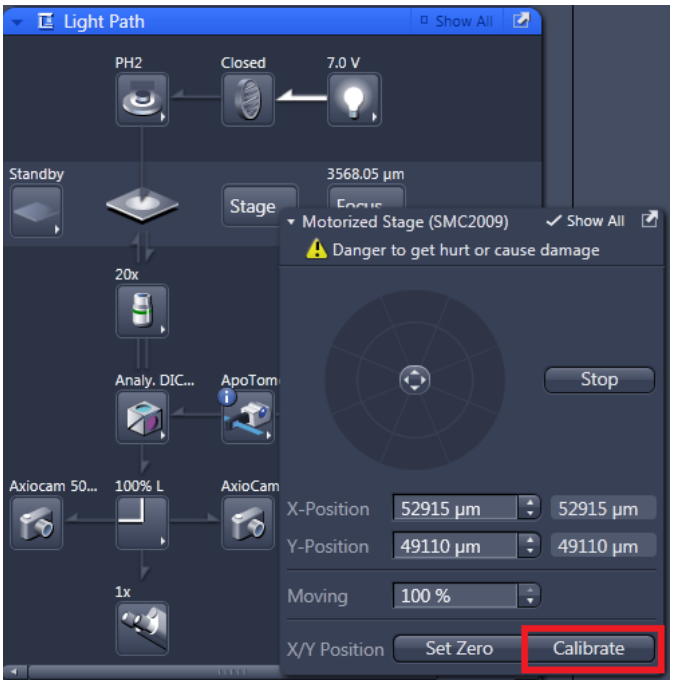
- Now save the experiment as a default multi-well plate experiment which can be used in the future.



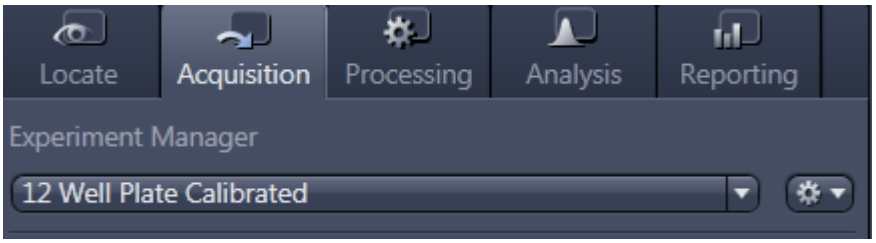
Calibration does not need to be repeated unless the dimensions change for the number of wells, for example if you buy from a different supplier.

<u>Tiling Part 2- Tiling with calibrated multi-well plates</u>

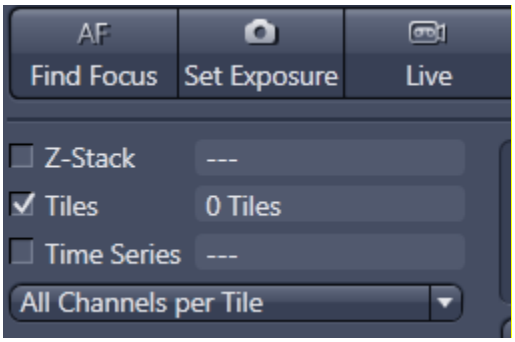
- In the **Locate** tab click **Stage** in the **Light Path** window and make sure **Show All** is ticked in the stage window.



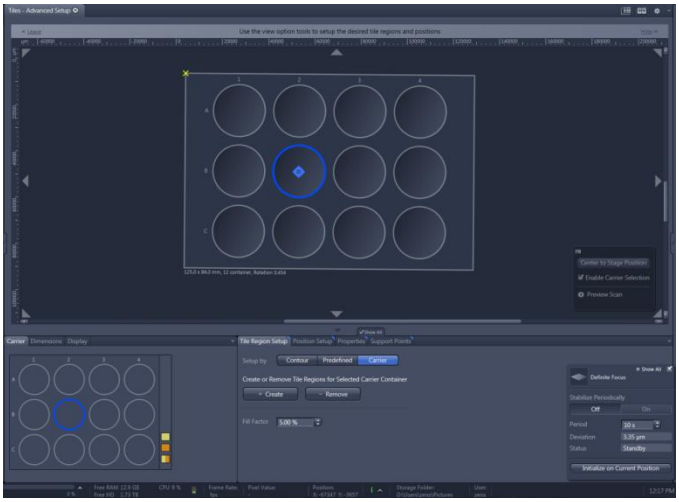
- Move to the **Acquisition** tab and load your pre-saved calibrated multi-well plate experiment in experiment manager.



- If your channels have been set up and focused already, go to the tiles window
    - (If the tiles window is not displayed click the checkbox near the top left of Zen).



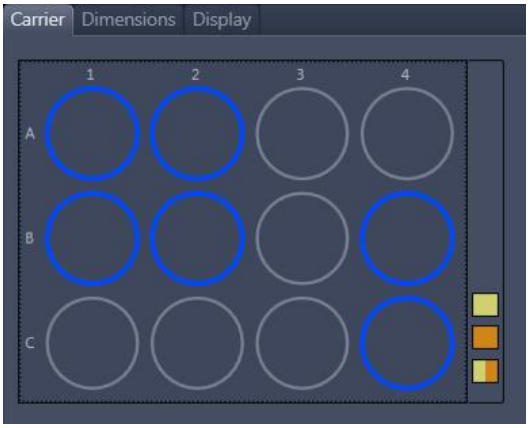
- Inside the tiles window click ‘advanced setup’.
    - You can zoom in and out using the mouse wheel over the main screen to see the whole multi-well plate graphic interface. Keep pressing ‘centre to stage position’ on the floating toolbar to home in on the area selected.

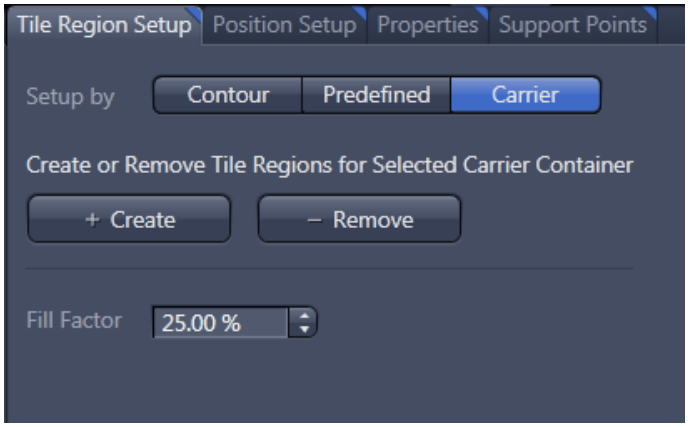


- There are now 3 choices:
    - Section 1: Adding tiles to each well.
    - Section 2: Adding single positions to each well (arranged or random).
    - Section 3: Adding tiles and single positions to the same well.

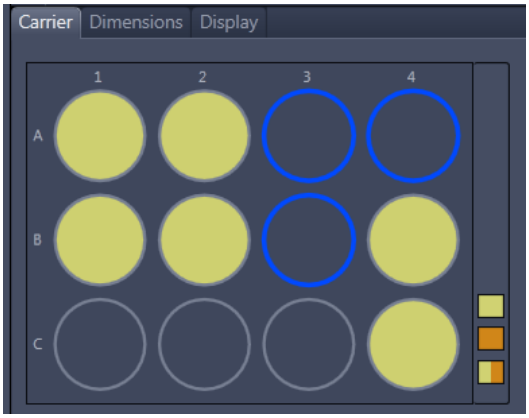
<u>Section 1: Adding tiles to each well</u>

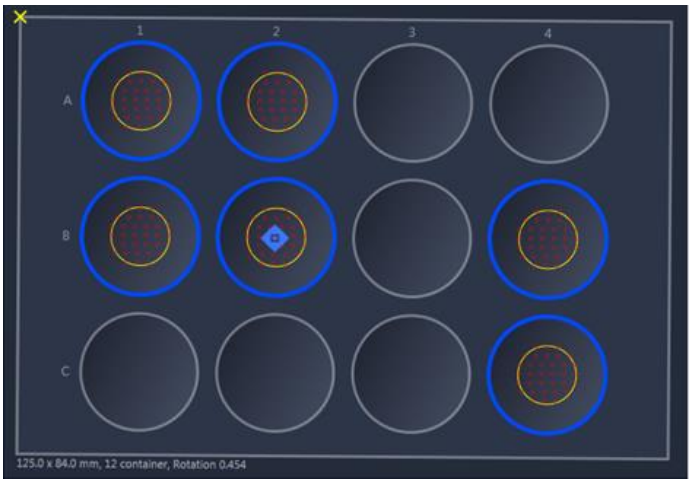
- In the **Carrier** tab select the wells you want to image by clicking on the first well and then with Shift pressed down click on the last well. This will fill in all the wells in between. If you want to select specific wells click on each of the ones required with the CTRL key pressed down.
- Inside the **Tile Region Setup** make sure it’s **Setup by Carrier** as shown below.



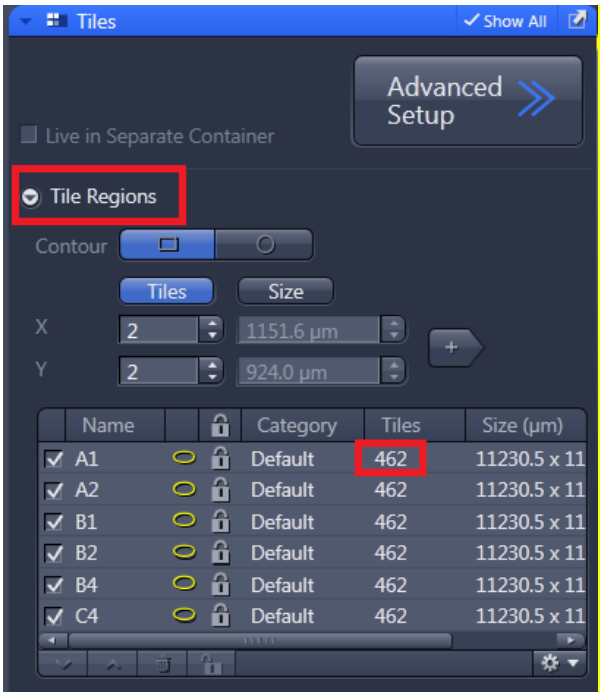


- To add a tile region to the selected wells choose how much of the well will be tiled, in this case 25%, and then click on the create button.

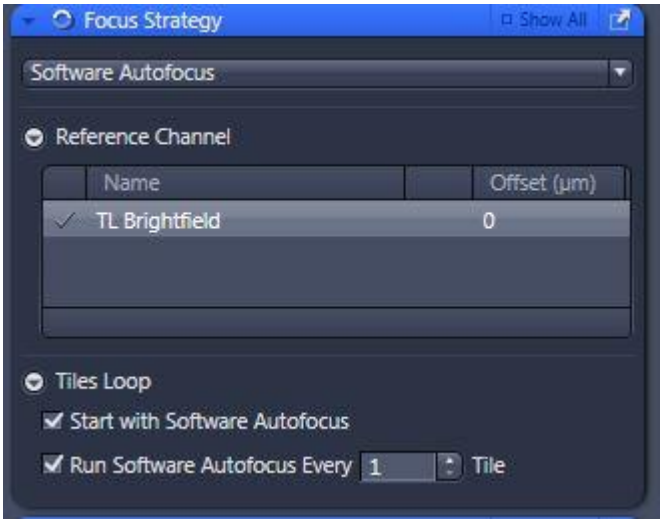




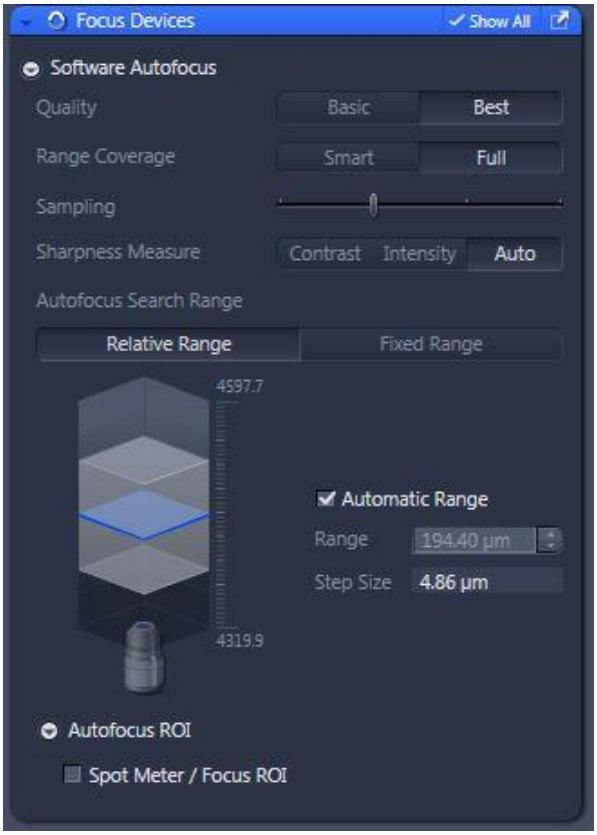
- You will now see that the selected wells are yellow in the carrier window signifying that they have tile regions inside them. On the main image window you will be able to see the tiles in each well. If you want to see how many tiles are in each well go back to the tiles tab and expand **Tile Regions** and you will see how many tiles are in each well.



- Next set your **Focus Strategy**: eg **Software Autofocus**
- Select a channel then ‘set as reference channel’ (you want to choose the channel which will have the strongest signal).
- In the **Tiles Loop** dropdown you can define when focus actions should be performed during the course of the experiment eg if it is set to **Run Software Autofocus Focus Every 1 Tile** it will run it at the start of each tile.



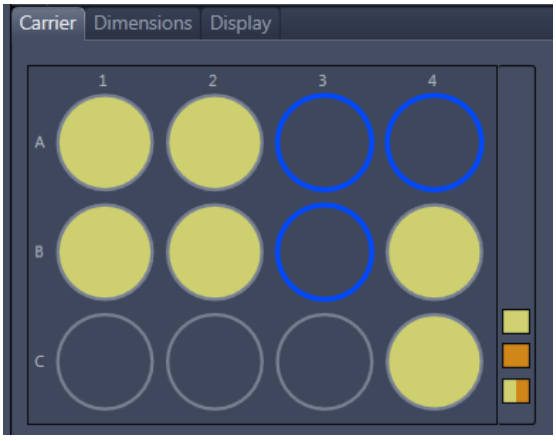
- In the **Focus Devices** tab, you can select the Quality (Basic/ Best), Range Coverage (Smart/ Full), Sampling, Sharpness measure (Contrast/ Intensity/ Auto), and Autofocus Search Range for the Software Autofocus.
- Software Autofocus will be faster if the Search Range is smaller.

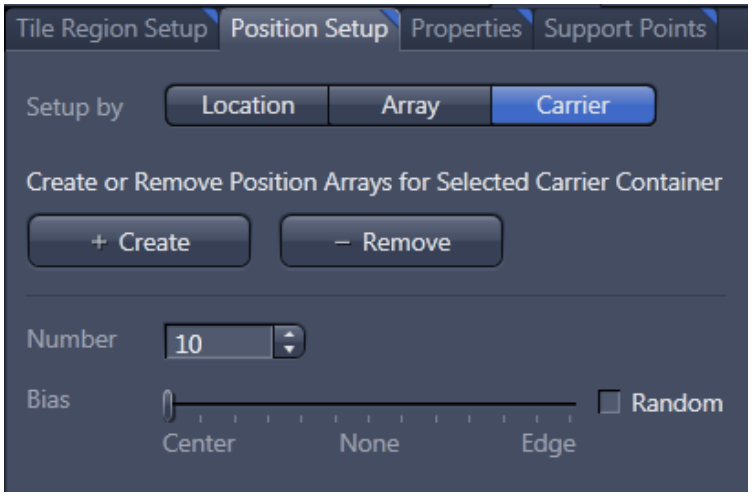


- A 10% overlap is recommended for tile scans.
- Tiles can be stitched together during acquisition (this will increase the acquisition time) or after, using the **Processing** tab (this may be preferable as it gives you more control over different parameters to optimise the stitching).

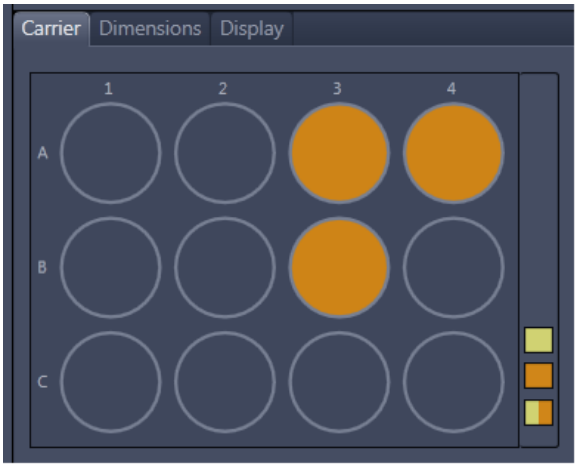
Section 2: Adding single positions to a multi-well plate (arranged or random)

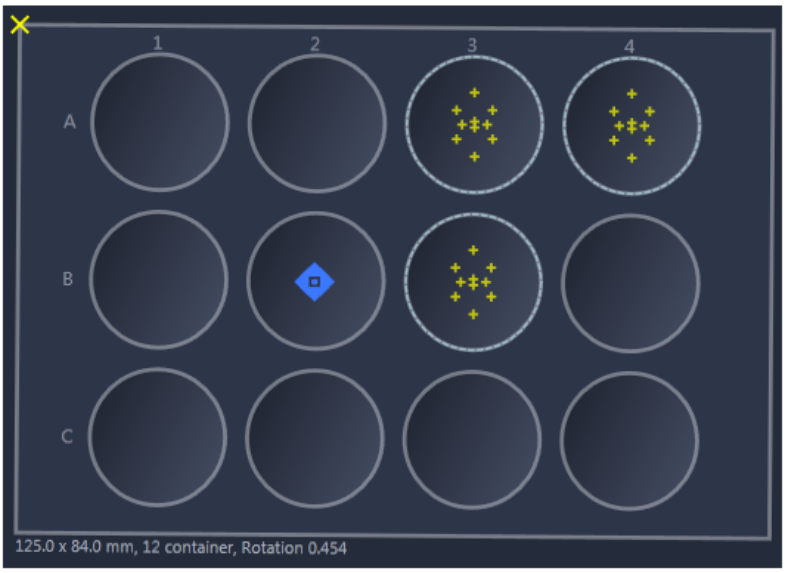
- In the **Carrier** tab select the wells you want to image by clicking the first well and with Shift pressed down click on the last well. This will fill in all the wells in between. If you want to select specific wells click on each of the ones required with the CTRL key pressed down.
- Go in to the **Positions Setup** and make sure Carrier is selected.





- Choose the number of positions you would like in each well, move the bias slider so that the positions are weighted toward the centre, edge or none. Click the **Random** button if you would like the positions to be random. Once all positions are defined click on **Create** to add the positions.



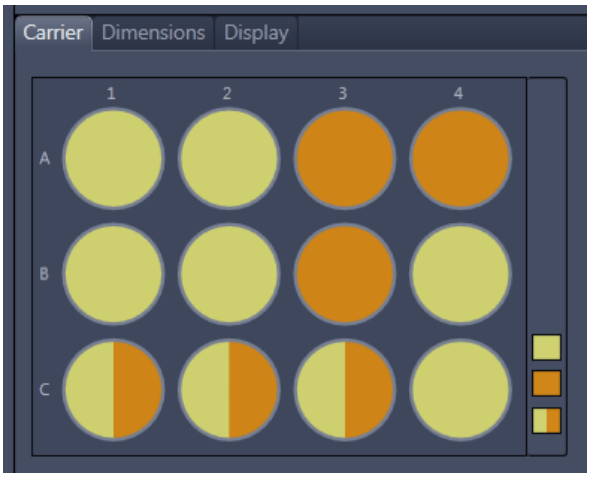


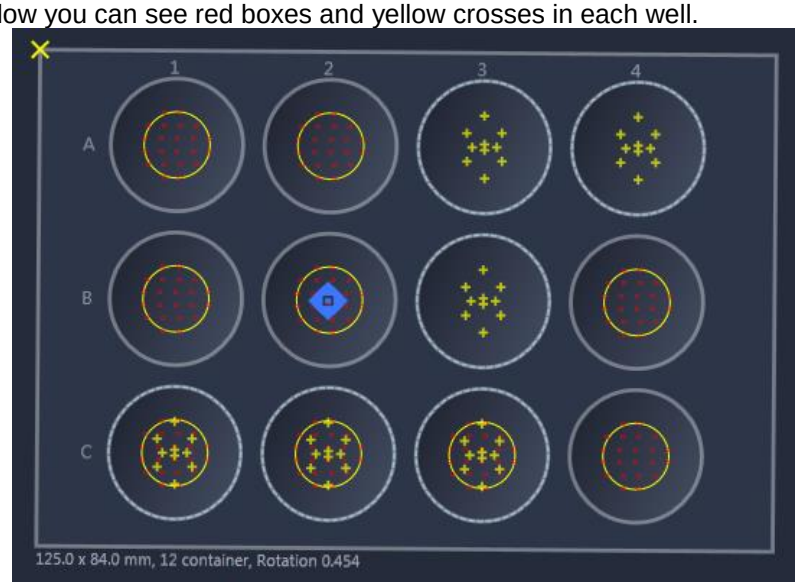
Wells have turned orange in the small window signifying that they contain defined positions. The main window shows the positions in the selected wells.

- Next set your focus strategy.
- If you are jut imaging a few single positions, you may prefer to use **Absolute Fixed Z position** as your focus strategy and select **Use Z Position from Tiles Setup** (this will then use the Z position you have set when you have manually focused each position during the Tiles Setup).
- If there are a large number of positions and you think your sample has a strong imaging signal, then the **Software Autofocus** can be used (as in Section 1).

Section 3: Adding tiles and single positions to a multi-well plate

- Combine the instructions from Section 1 and 2 in the same experiment. You can also combine Tiles and Positions in the same well. When you have both of these in the same well, they turn half yellow, half orange in the small Carrier window. In the main window you can see red boxes and yellow crosses in each well.



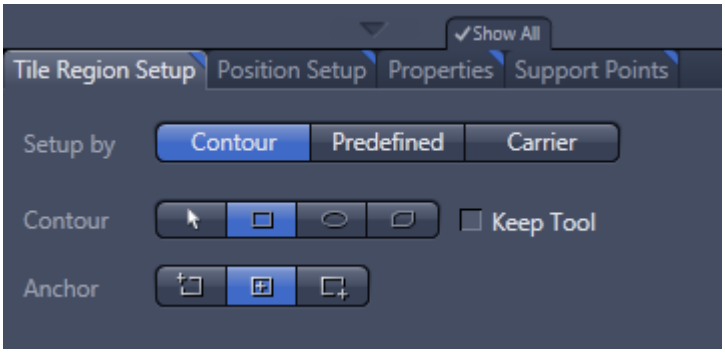


- Next set your focus strategy (as in Section 1 and 2).

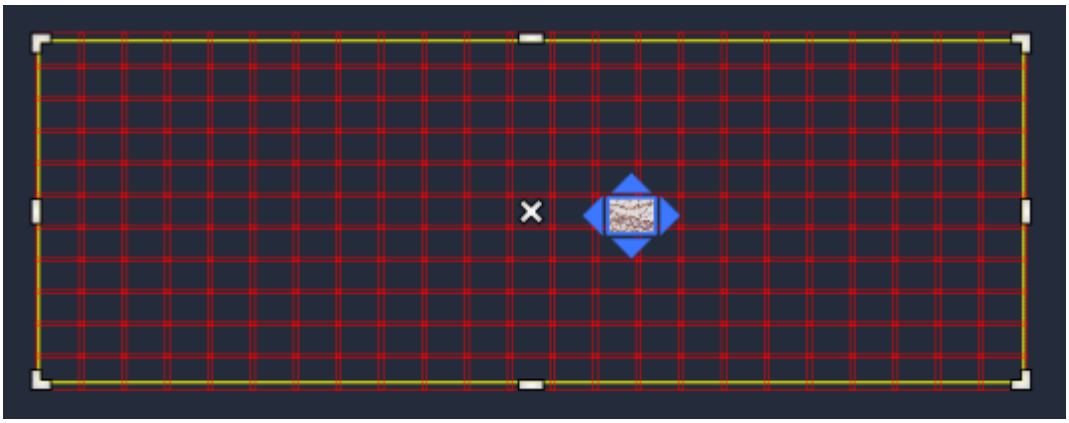
Tiling Part 3 – Capturing a Montage in a single well

*This is likely to be useful if you're working with a single flask or petri-dish…

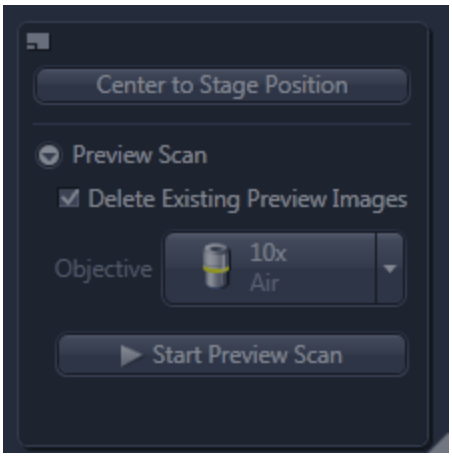
- Go into **Tiles Advanced Setup**.
- Go to the **Tile Region Setup** tab as below.



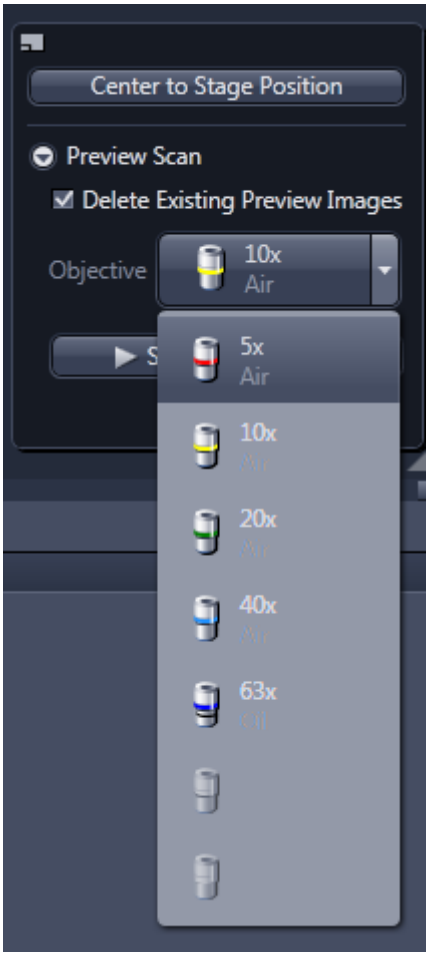
- Ensure **Setup by Contour** is selected.
- Select the **Contour** drawing tool eg rectangle.
- Draw a rectangle around the area that you think your sample is in. To view your sample at any part of the grid, double-click. Adjust your Contour as necessary.



- To create an overview image firstly click on the **Preview Scan** button.



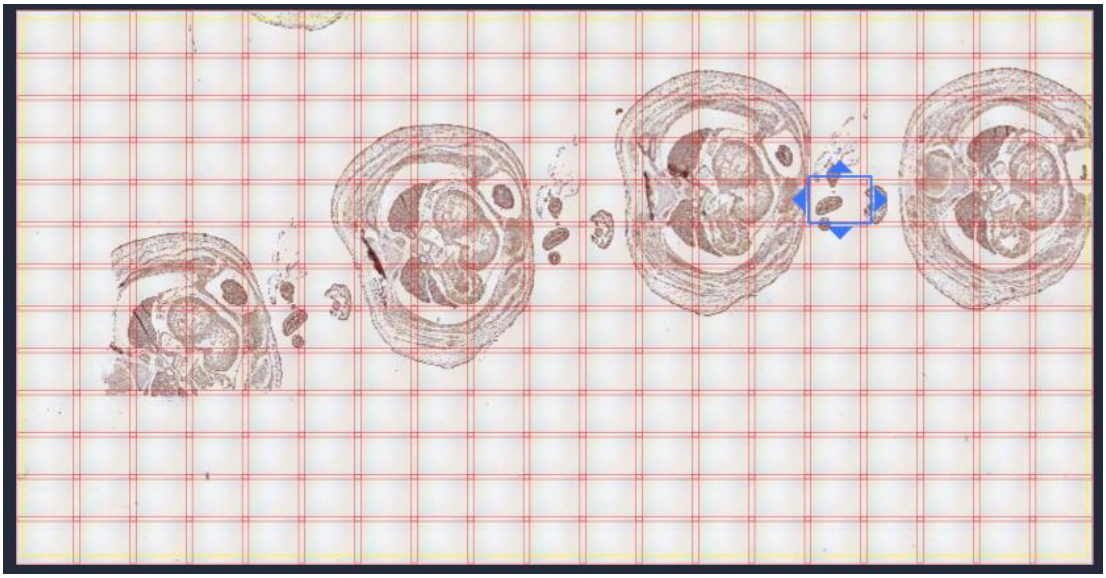
- Click on the Objective button and select a lower magnification.



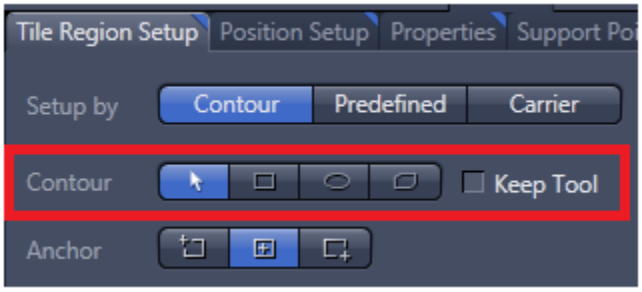
- At low magnification it's easier to move around your specimen and make sure what you want is contained within the tile grid, if not adjust it using the white markers on the grid edges. (If the white markers are not there click inside the grid to select it).

  *For fluorescence the live image will be whatever channel is selected: If all channels are ticked then all channels will be used for the preview scans and for the main tile scan once set up. To only preview scan one channel untick the ones you don't want and then re-tick once ready to do the main scan.

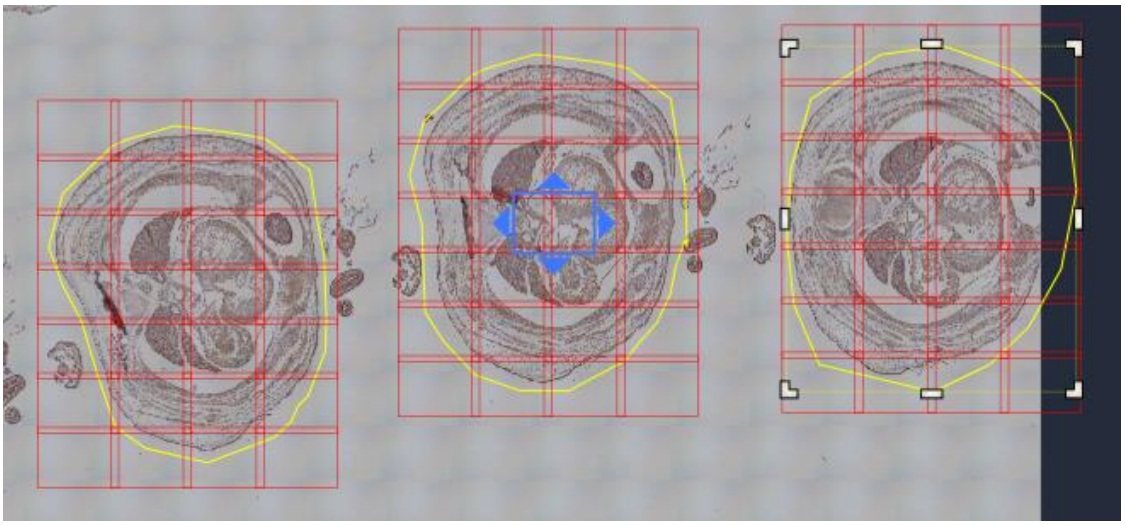
- Once you're happy you have your entire specimen displayed in the grid, click **Start Preview Scan** and your preview scan will be shown.



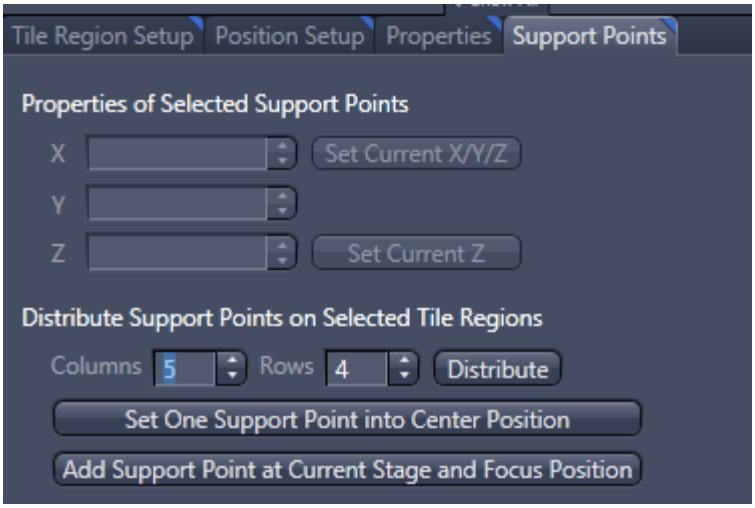
- Now that preview scan is available the **Preview Grid** can be deleted as we will now make smaller more specific grids for tiling. If you think you will need it again, do not delete it.
- Now you can draw specific regions to be tiled - you can use a rectangle, ellipse, or an irregular shape.



- You can draw as many as you like and they will each be set up as individual tiles.

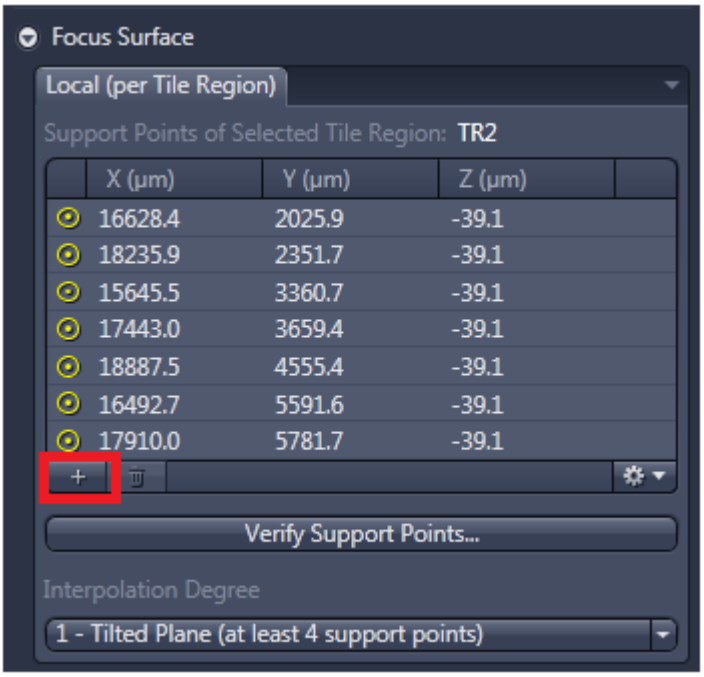


- To set a **Focus Surface Map** go to the **Support Points** tab. To distribute support points select the number of columns and rows that you need and click distribute. Do this for each individual tile.

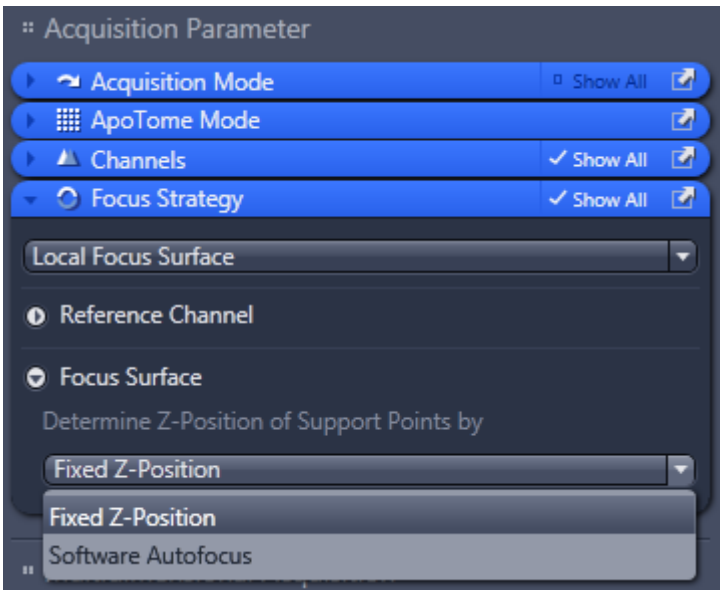


- You can then click on each **Support Point** (small yellow circle with yellow dot in the middle) and move it to an optimal location. Repeat for each tile created.

- If you want to add additional support points without deleting the ones you already have then double click on the area you would like a new support point and click on the + sign under **Focus Surface**.

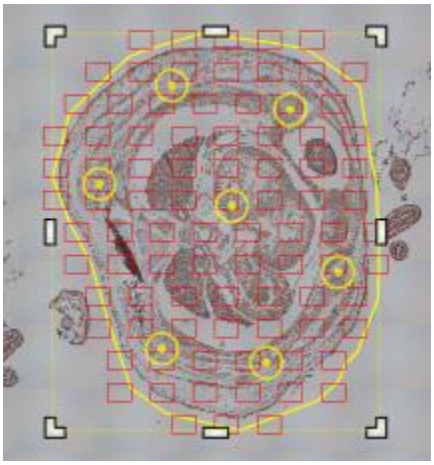


- Now in the preview scan window change your magnification back to the mag you want to scan at eg X20.
- Choose the focus strategy via the focus strategy window.
- Select **Local Focus Surface** as the **Focus Strategy**.
- Under **Focus Surface** we can choose how we want the Z position located:

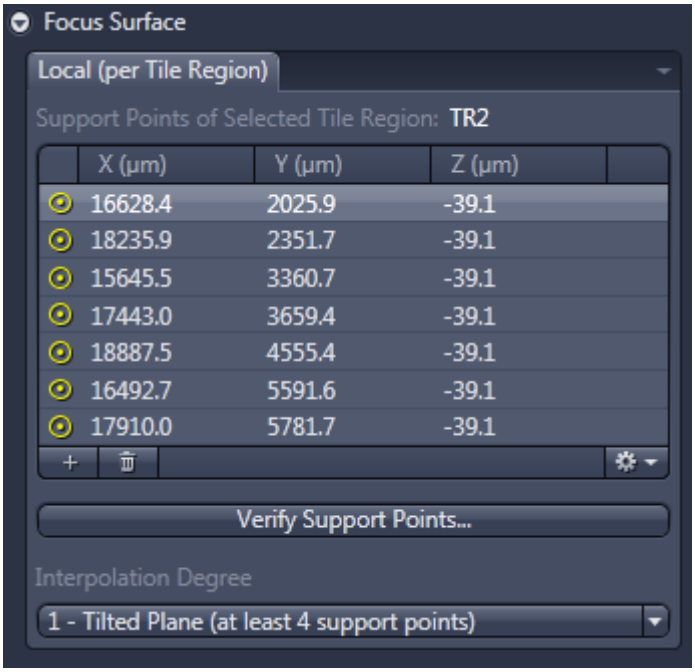


The options are:

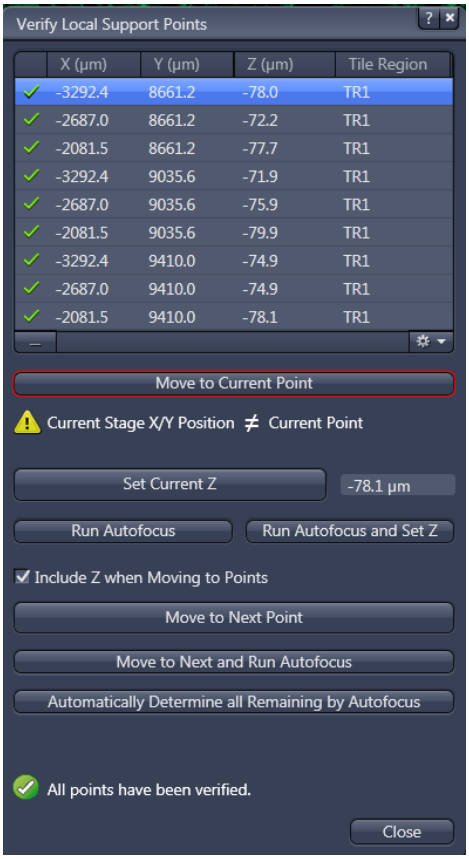
- **Fixed Z-Position** - We choose the focus for each support point manually.
- **Software Autofocus** - With this option when we click Start Experiment the software will go to each support point and run the autofocus to determine the optimal focus position. *This may not be optimal for you as it will look for the best contrast as the best focus. Try it and see and if it's not exactly what you want, go for Fixed Z-Position.
- To focus each Support Point for the Fixed Z Position Focus Strategy, firstly click on **Live** under the acquisition tab.

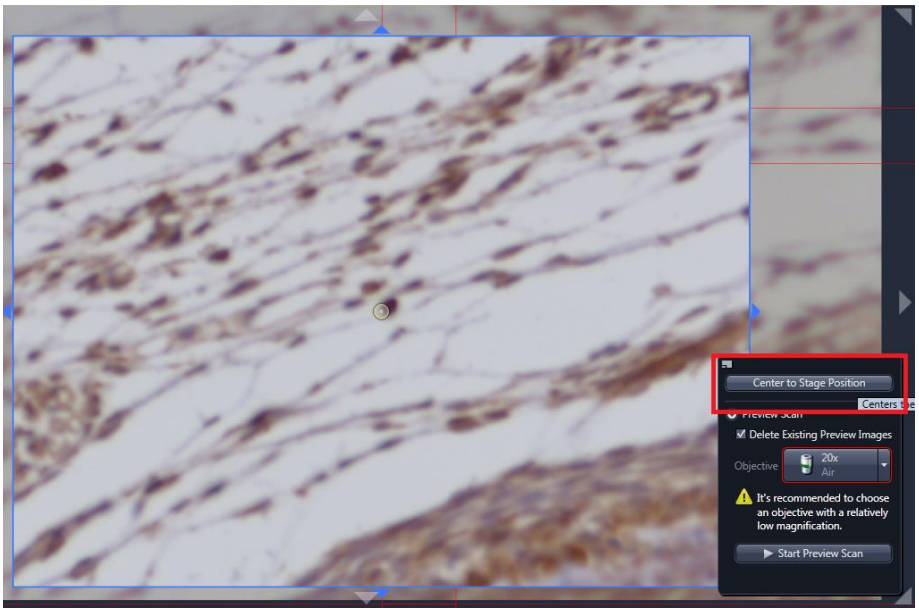


- Select the first tile grid then go to **Tiles** and **Focus Surface** and you will see the list of Support Points. Select **Verify Support Points**.

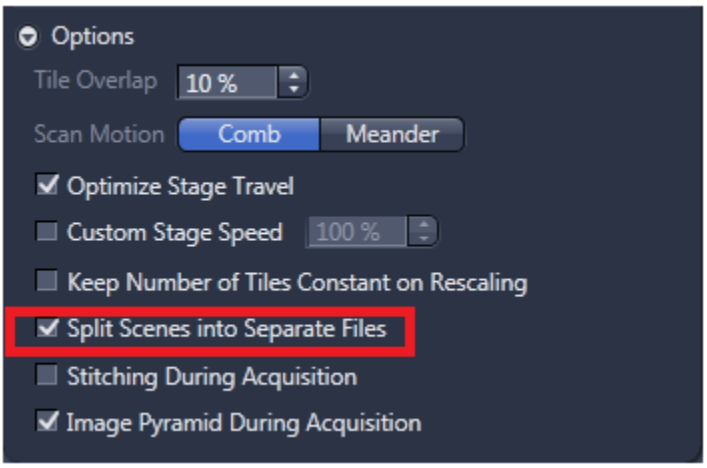


- Now click Move to **Current Point.**
- Focus manually, then click **Set Current Z**.
- Click **move to next point**.
- Repeat until all points are focussed. Each position will end up with its own focus and from this ZEN will build a focus map.

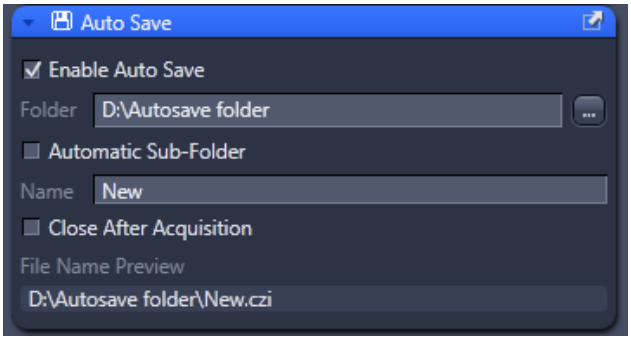




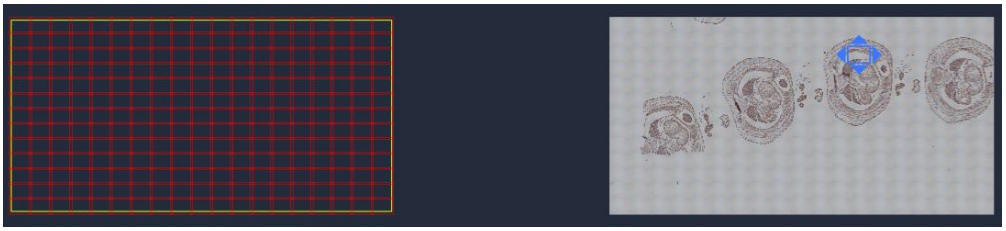
- In the live view, you can use the middle mouse button to zoom in and centre to stage position to maintain a view on the area being zoomed into.
- If you are capturing more than one tile in the experiment (in this demo we have 3) tick the **Split Scenes into Separate Files**. This means you will get one hold-all file for the experiment and three files, one for each tile. This is useful particularly if you have very large tiles to capture as it keeps the file sizes at a manageable size.
- Stitching during acquisition can also be turned on in this window.



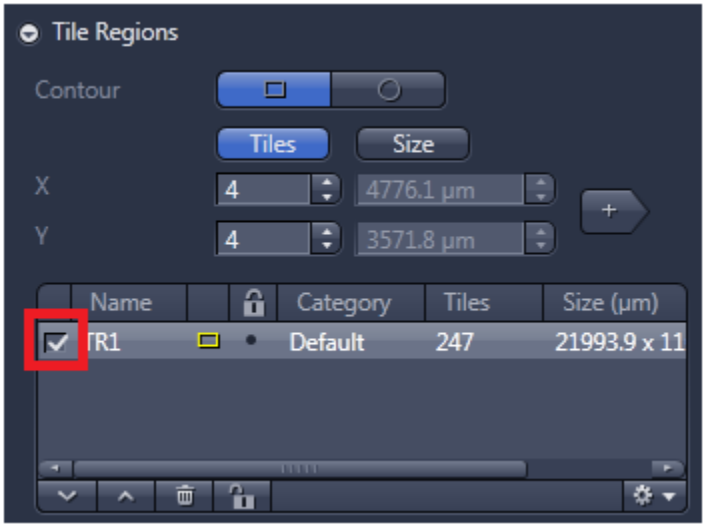
- Finally choose your **Auto Save** options under acquisition and click **Start Experiment**.



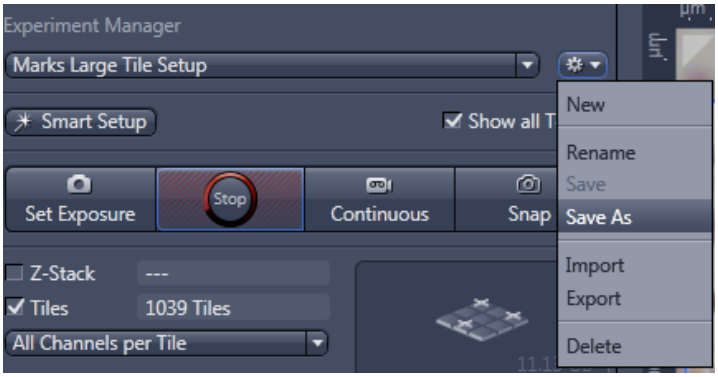
- Instructions for storing your grid:
    - If you want to keep the large **Preview Grid** for your next experiment click it and drag it away from your current slide.



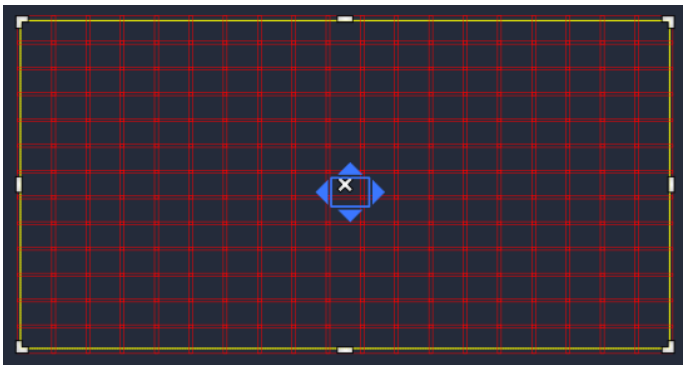
- Once it's moved, in order to make sure we do not scan it at a higher magnification, untick the **Tile Region (TR)** line within the tiles tab under **Acquisition**. The grid will disappear but it will reappear when ticked again.



- Once unticked, it still exists but will not be used until it's ticked again. Now you can save this as your experiment. Click on the cog in the **Experiment Manager** tab > **Save As**.



- When using in the future move your slide to the centre under the objective and then you can grab your old **Preview Grid** and move it so that the blue live window is in the centre of your **Preview Grid**, then you can do a new **Preview Scan**.



Saving images

- When doing tile scans in LCI, you are going to generate images of a large file size. You will therefore need to temporarily save them onto the DATA (D:) drive before transferring them to your personal H: drive or onto an external hard drive.
- Please transfer your files as soon as your experiment has finished to prevent the computer memory being filled up.

SHUT DOWN

= START UP in reverse