

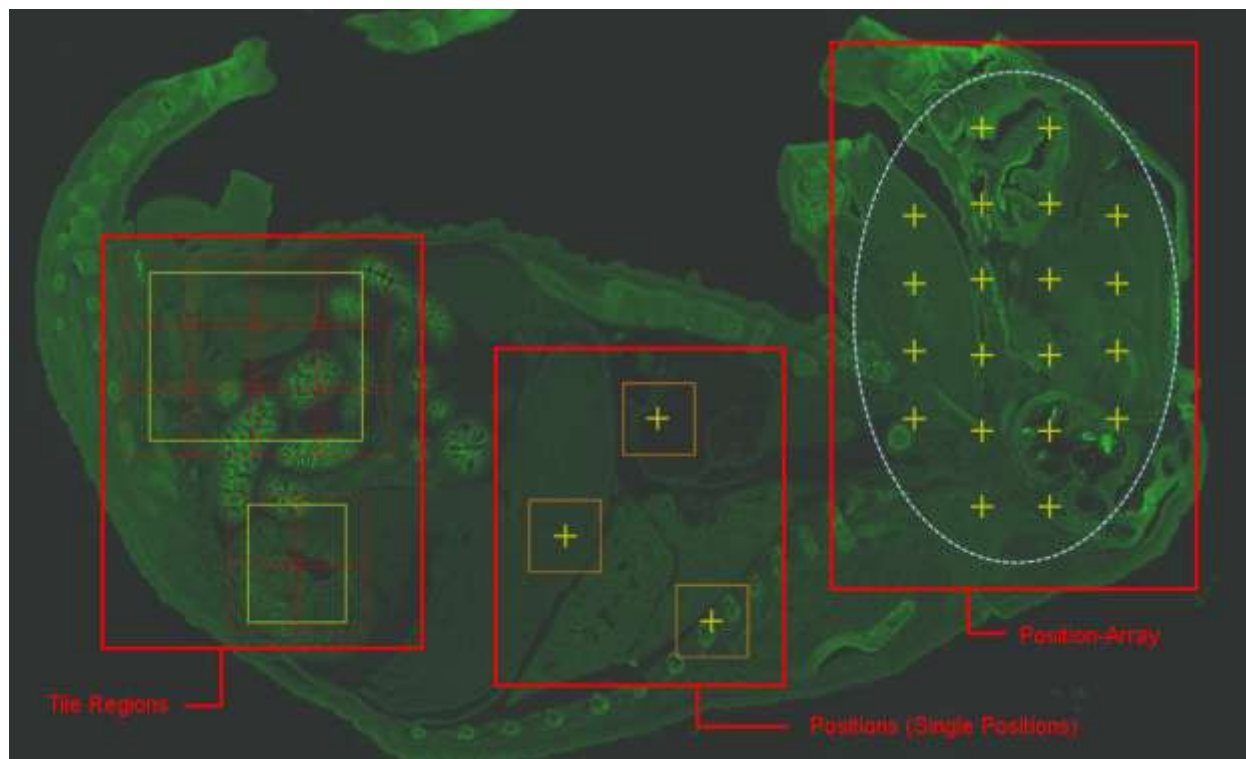


How to Do Tiles Imaging

in ZEN Blue 2012 SP2 DVD 52?

Using the Tiles tool you can acquire images that are made up of a number of individual images (tiles). To do this, it is possible to define tile regions and positions. The Tiles module supplements the functions of the Tiles tool with the Advanced Setup feature. This allows you to set up Tiles experiments more easily and also to use sample carriers and focus surfaces. Please refer to ZEN help file in the software for how to set up a simple tiles or a simple positions experiment. This how-to guide introduces common focus strategies for tiles experiment, examples and simple trouble shooting advices. Any questions or concerns can be directed to the ZEISS Product and Applications Support group at 1-800-509-3905 or Support@zeiss.com

Glossary



Tiles: Each captured picture frame from single positions in a sample is a tile. To set up Tiles experiments, a motorized stage is required. This stage must be configured and calibrated correctly in accordance with the camera orientation.

Tile Region(s): In a tile experiment a tile region refers to a group of individual image fields (tiles) that belong together and are arranged in the form of a grid. With the help of tile regions it is possible to acquire areas with dimensions that exceed the size of an individual image field. Within an experiment a number of tile regions can be acquired at various positions on the sample. Each tile region is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Tile regions are defined using the Tiles tool. After acquisition the individual tile regions are displayed as scenes.

Z-stack: Acquired serial Z-plane frames at various Z depths with the same XY position. In ZEN a Z-stack can be acquired by either defining first and last Z position of the Z-stack or just defining the center Z position of the Z-stack. In ZEN, a Z-stack experiment can be multiple single tile Z-stacks or multiple tile regions Z-stacks combined with time series. Z-stack imaging requires a motorized focus drive.

Time Series: Acquired time lapse serial images. They can be Z-stacks, Tiles, and Multiple Single Positions images.

Position(s): In a tile experiment positions refer to independent individual image fields (tiles) that are localized at various places on the sample. A position corresponds to a tile region consisting of just one tile. Each position is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Individual positions or position arrays (grouped individual positions) are defined using the Tiles tool. After acquisition the individual positions are displayed as scenes.

Focus Strategy: Focus strategy is a tool in ZEN to help imaging at the best focal plane in the sample that the system can reach. Unless the imaging is a single position or the sample is pretty flat (i.e., all portions of the sample are in the same focal plane with the same Z position), a Focus Strategy is needed. A motorized focus drive is required to use focus strategies.

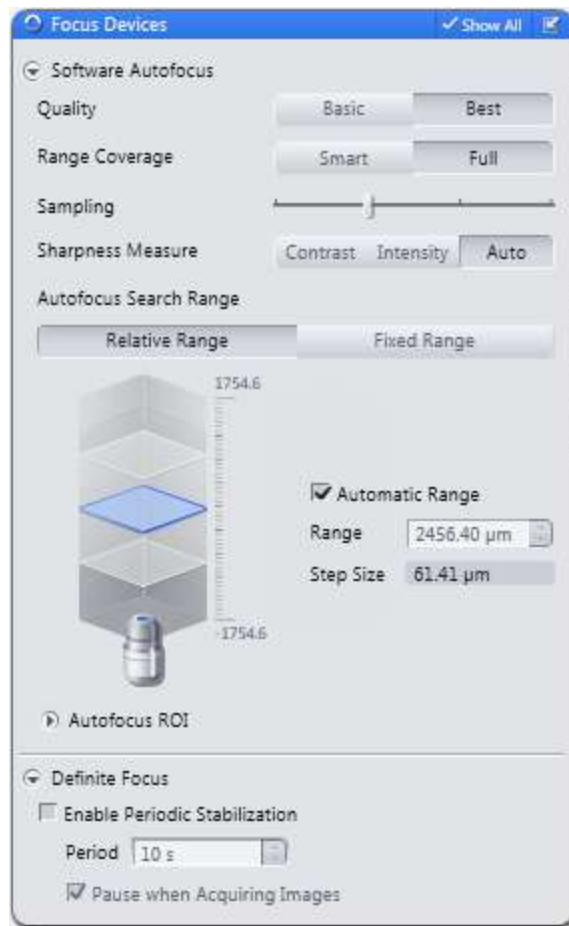
Sample Carrier: Slide, dish, well plate or customized container that carries the sample are sample carriers. If the image position(s) need to be precisely recorded and re-found in the sample carrier, every time when a sample carrier is placed on a stage it needs to be calibrated so that ZEN knows where the sample carrier is on the stage and where the imaged positions are.

Local Focus Surface: Local Focus Surface is specific to tile regions. They are interpolated from the Z-values of reference points, which can be created for the tile region. 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 reference point defines a horizontal focus map.

Global Focus Surface: The Global Focus Surface is interpolated on the basis of reference points which are located on the sample carrier. These reference points can be defined by editing the sample carrier template. The interpolation results in an individual Reference Z-Position for each tile.

Software Autofocus: The focus position is determined via the sharpness calculation or intensity calculation of a series of images (Z-stack) and set as the Reference Z-Position. The autofocus Z-range can be defined through the Focus Devices tool.

Definite Focus: Equipment from Zeiss that attempts to maintain a certain distance between the cover glass of the sample and objective, in order to compensate for mechanical and thermal movements. The Definite Focus is initialized at the start of the experiment by setting the current distance as the reference distance. When the focus is stabilized during the experiment, the current distance is adjusted to the reference distance. This is achieved by moving the focus drive accordingly. The new Z-position resulting from this is used as the Reference Z-Position for acquisition. Two modes are available, which you can use in parallel: **A:** Stabilization at certain time points /certain Tiles/Positions. This mode is set up in the Loop sections (Time Series, Tiles, etc.) of the Focus Strategy tool. **B:** Periodic Stabilization, available for Time Series experiments only, runs a regular stabilization with a defined time interval (e.g. every minute). This mode is set up in the Definite Focus section of the Focus Devices tool.



Common focus strategies in ZEN 2012 SP2 DVD 52



General steps shall be done under Acquisition Tab that includes light path settings, camera settings, channels selection, exposure time for each channel, etc. The 'Tiles' option shall be checked. If the experiment is to include 'Z-Stack' or 'Time Series' imaging, these two options shall be checked also.

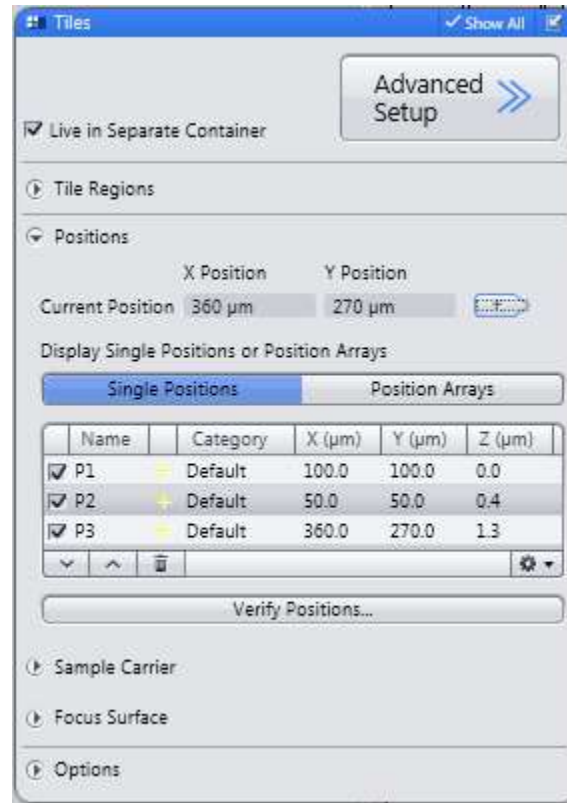
Then choose a focus strategy and define it according to different imaging needs. General check list for a tile imaging experiment is as follows.

- Choose a focus strategy.
- Use Z-Position from Tiles Setup.
- Define autofocus range or definite focus period from Focus Devices.
- Add Positions or Tile Regions from Tiles setup.
- Select and calibrate sample carrier.
- Define Focus Surface. Local is for Tile Regions and Global is for Sample Carrier.
- Define Software Autofocus or Definite Focus frequency
- Data save method.
- Stitching method.

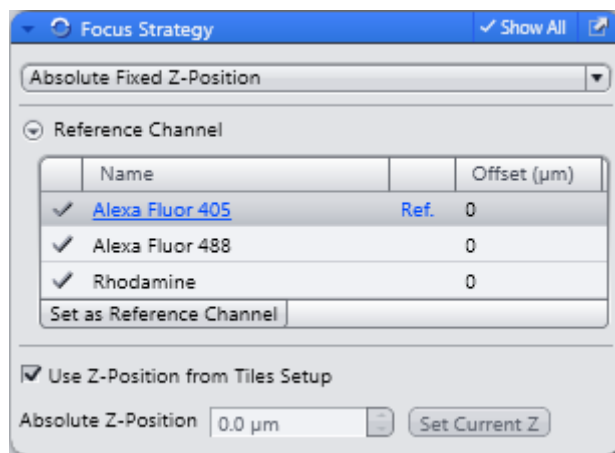
Multiple Single Positions (Tiles)

1. Absolute Fixed Z-Position

To image a few single positions on a sample, add these positions manually. Locate the first position in X and Y, and bring it into focus (Z). Then, in the blue tool bar “Tiles”, go to “Positions” and click the “+” button to add this position to the Single Positions list. Repeat this until all positions of interest are added in. If needed, the positions can be verified through “Verify Positions”.

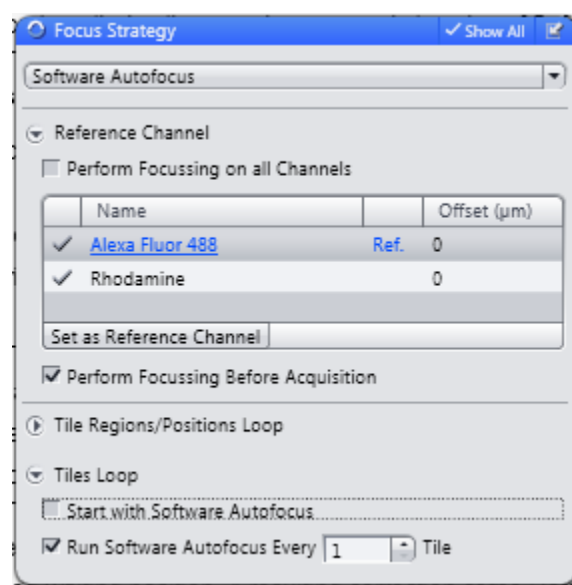


For the Focus Strategy, you need to choose ‘Absolute Fixed Z-Position’ and make sure that the option ‘Use Z-Position from Tiles Setup’ is checked.

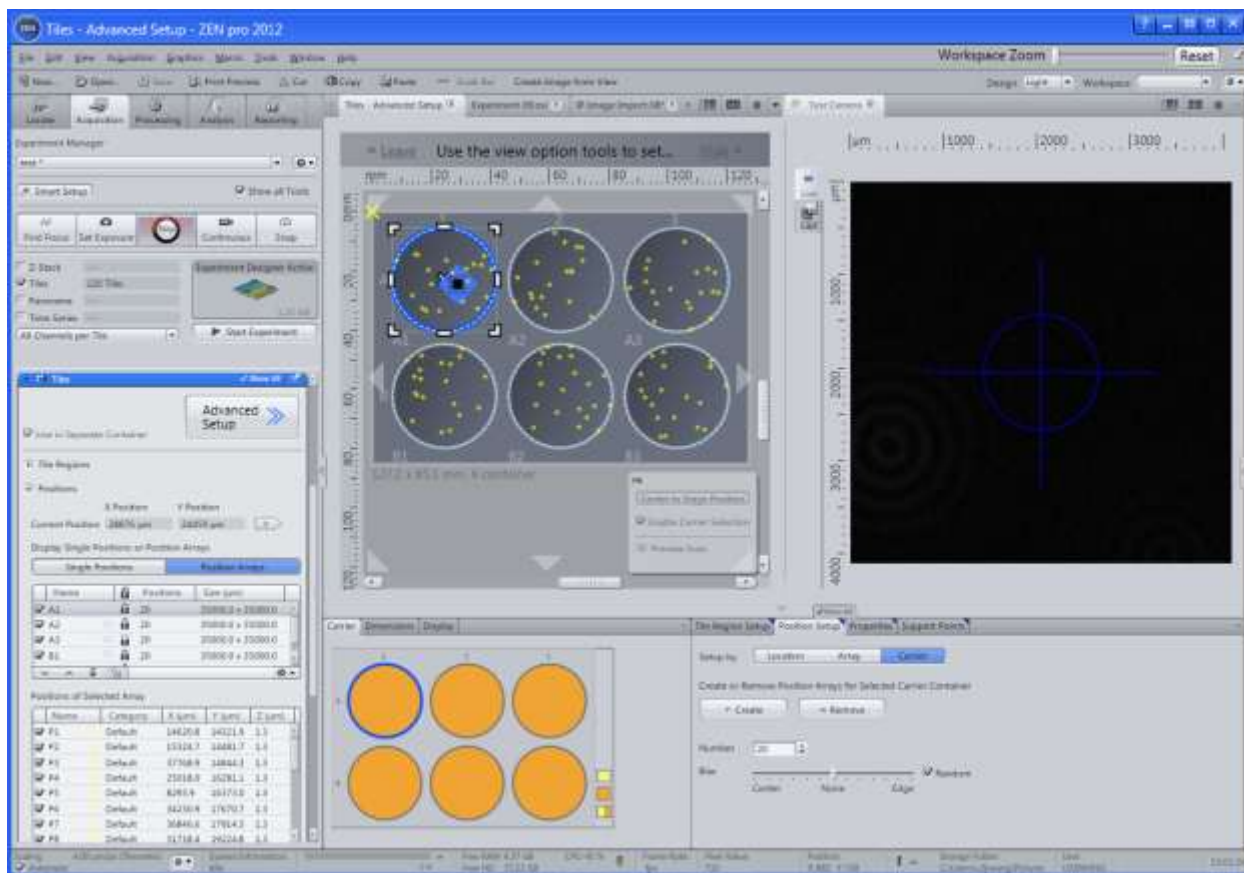


2. Software Autofocus

If there is large number of single positions to be imaged and the sample has strong imaging signal, then Software Focus Strategy could be used and the positions could be added in the positions list with just roughly focusing. You may define the Software Autofocus Reference Channel and Autofocus Search Range (refer to previous screen snapshot Focus Devices) to save some autofocusing time.

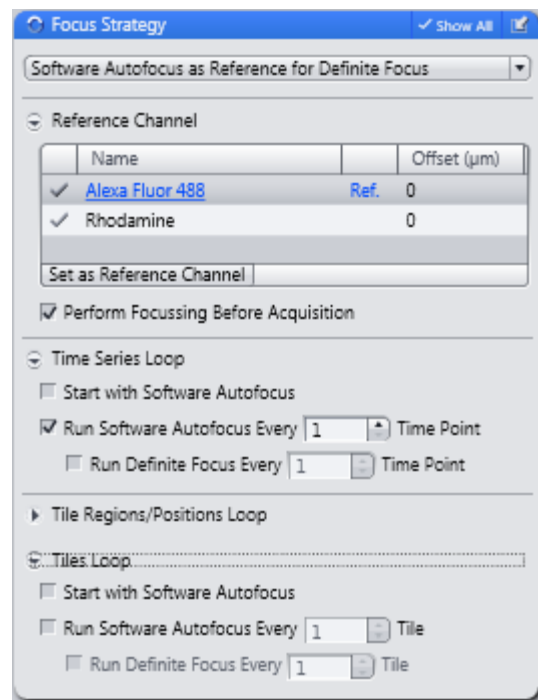
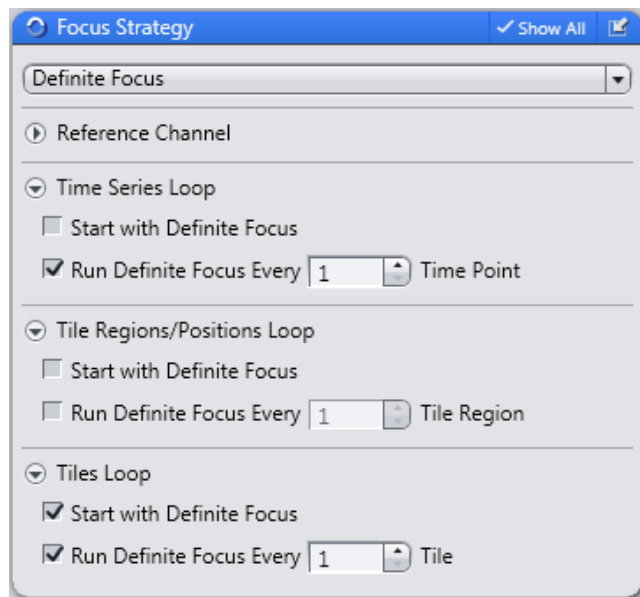


You may use Positions Setup in Sample Carrier with Advanced Setup to help adding in the positions. Within blue tool "Tiles", click "Advanced Setup" then you have "Tiles-Advanced Setup" window. In the example below, twenty random positions were added to each well of a 6-well plate. A calibration of the sample carrier may be needed before the experiment if you want to record the positions' coordinates precisely.



3. Definite Focus (if equipped) / Software Autofocus as Reference for Definite Focus / Definite Focus as a Start for Software Autofocus

When creating a time series experiment, definite focus may help minimize the Z drift over time. Please refer to page 2 for detailed description of definite focus. Below the two screen snapshots presents two examples. The 'Time Series Loop' and 'Tiles Loop' provide options to define how often Software Autofocus and Definite Focus will be utilized.



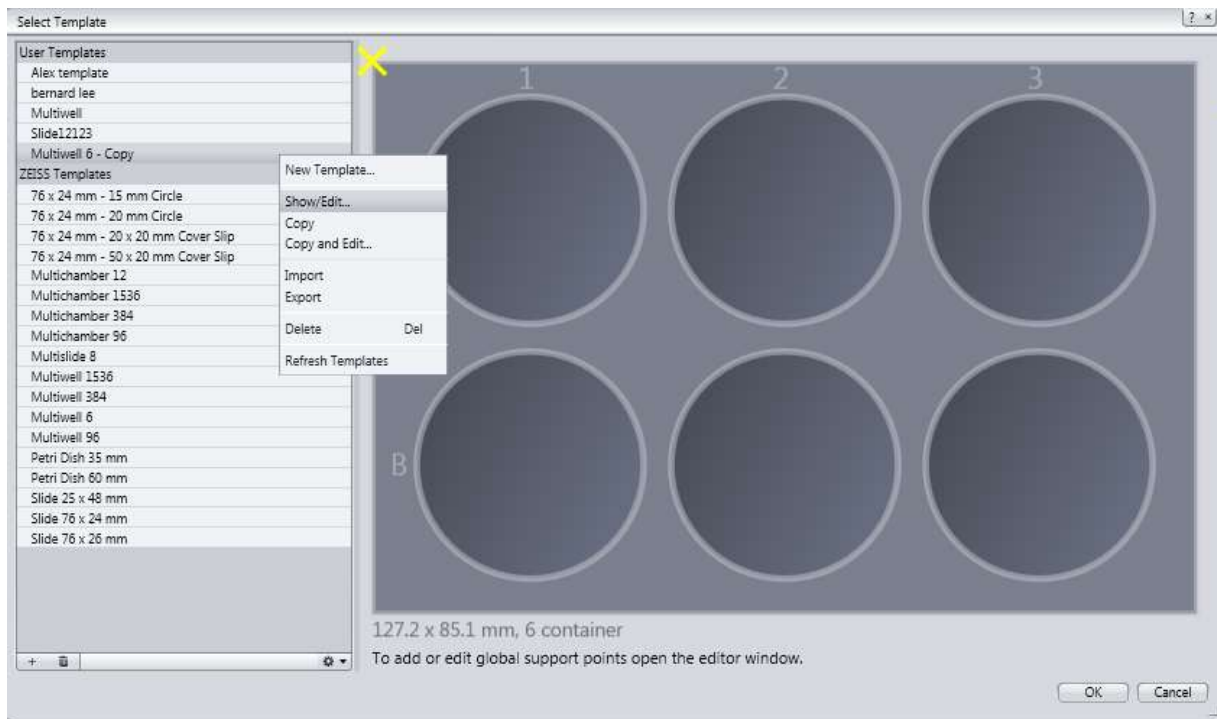
4. Global Focus Surface / Global Focus Surface as Start for Software Autofocus / Global Focus Surface Updated by Software Autofocus / Global Focus Surface Updated by Definite Focus

A Global Focus surface should be used when imaging single positions on a sample that is held within a sample carrier. First, the desired sample carrier should be selected in the Tiles tab by clicking Select.

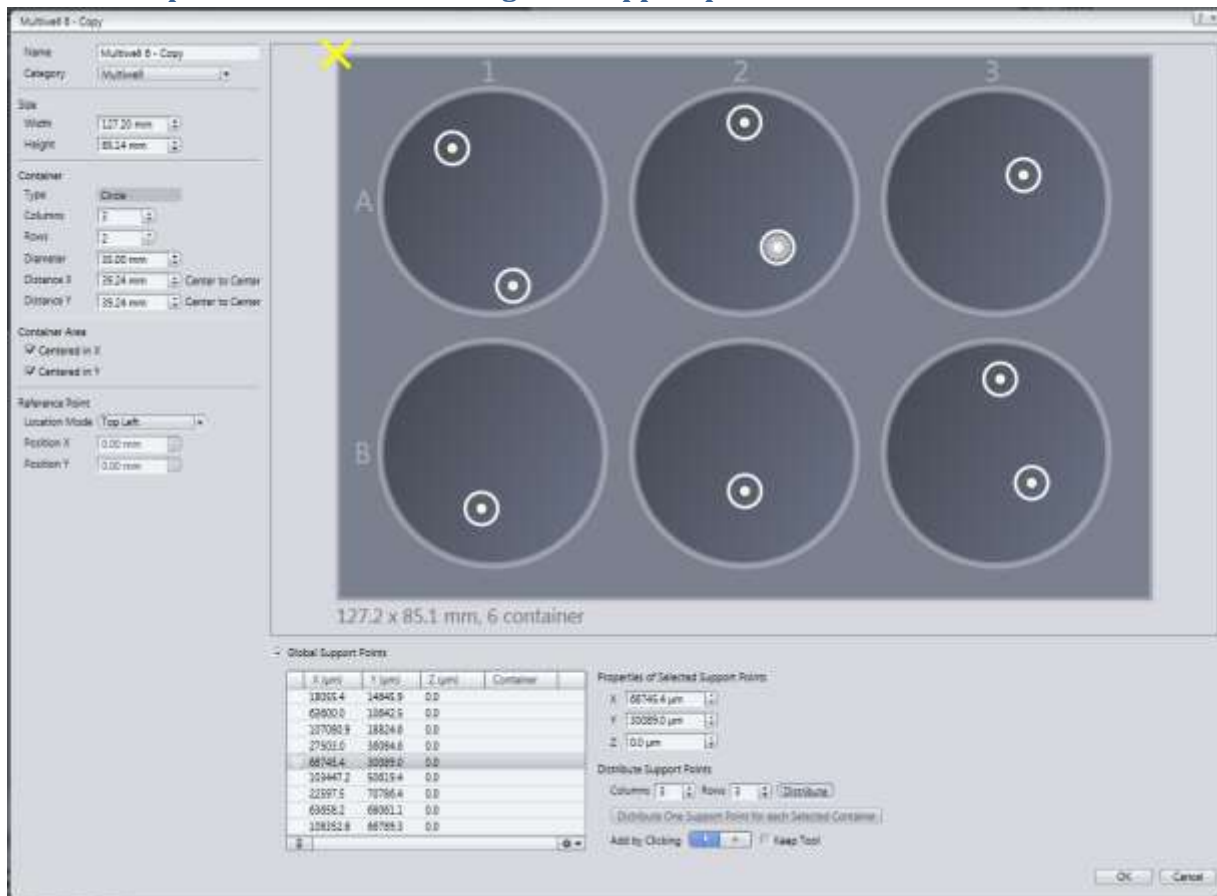


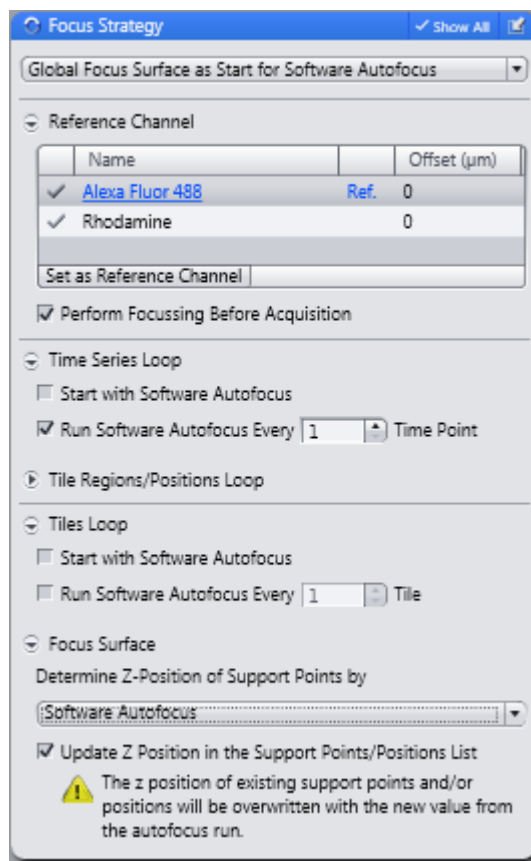
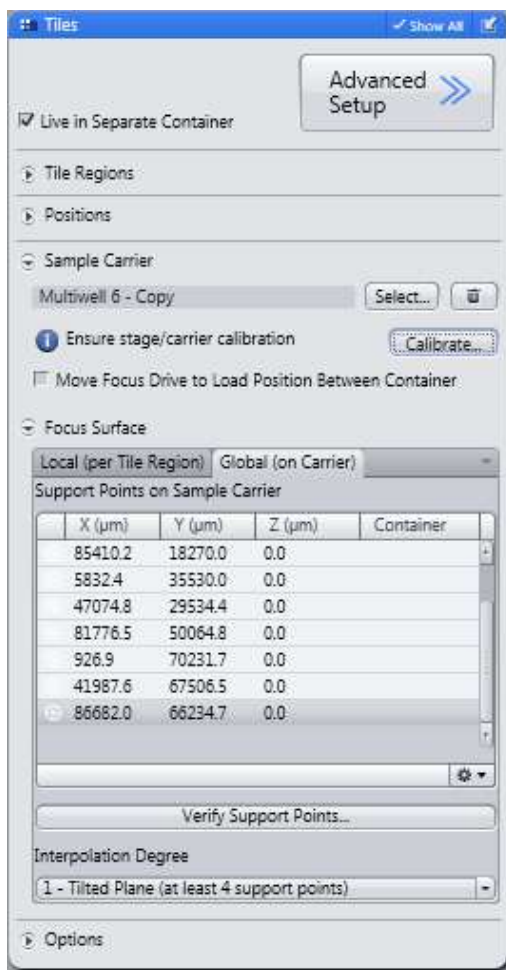
You may use a customized carrier template or copy an existing carrier template to edit. Right click on the sample carrier template and click Edit to add global support points.

The global support points can then be verified in Focus Surface section of the Tiles tab. Furthermore, the interpolation method that is used to estimate the focus position in between the global support points can be selected here.

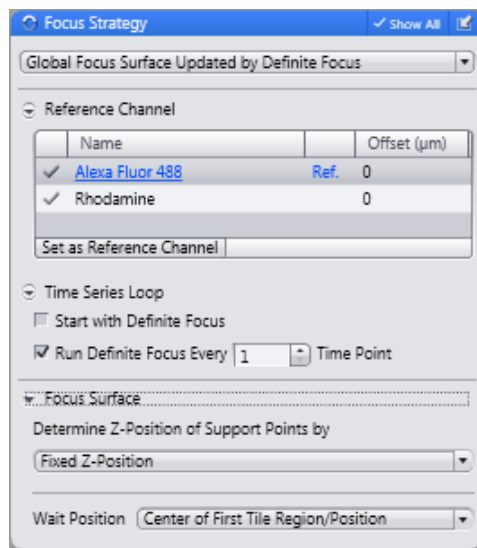
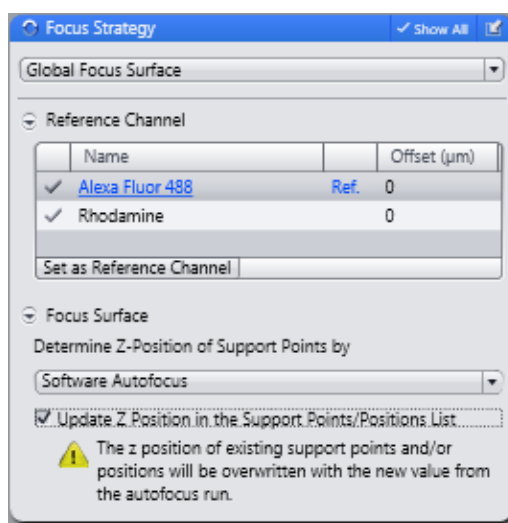


Use the sample carrier edit to add in global support points.





“Move focus drive to load position between container” will avoid oil drag if oil lens is used.

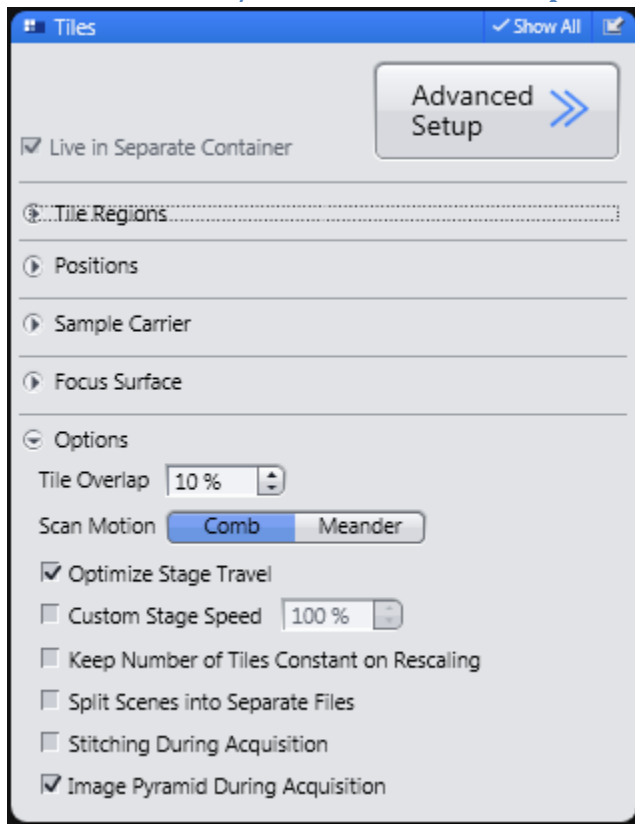


The above screen snapshots show several combinations of the focus strategies. If the sample has very good contrast, Autofocus is recommended for the focus strategy. When using a low magnification lens

and the sample carrier is a little bit tilted then Global Focus Surface is recommended. The Definite Focus option is recommended for long time series imaging.

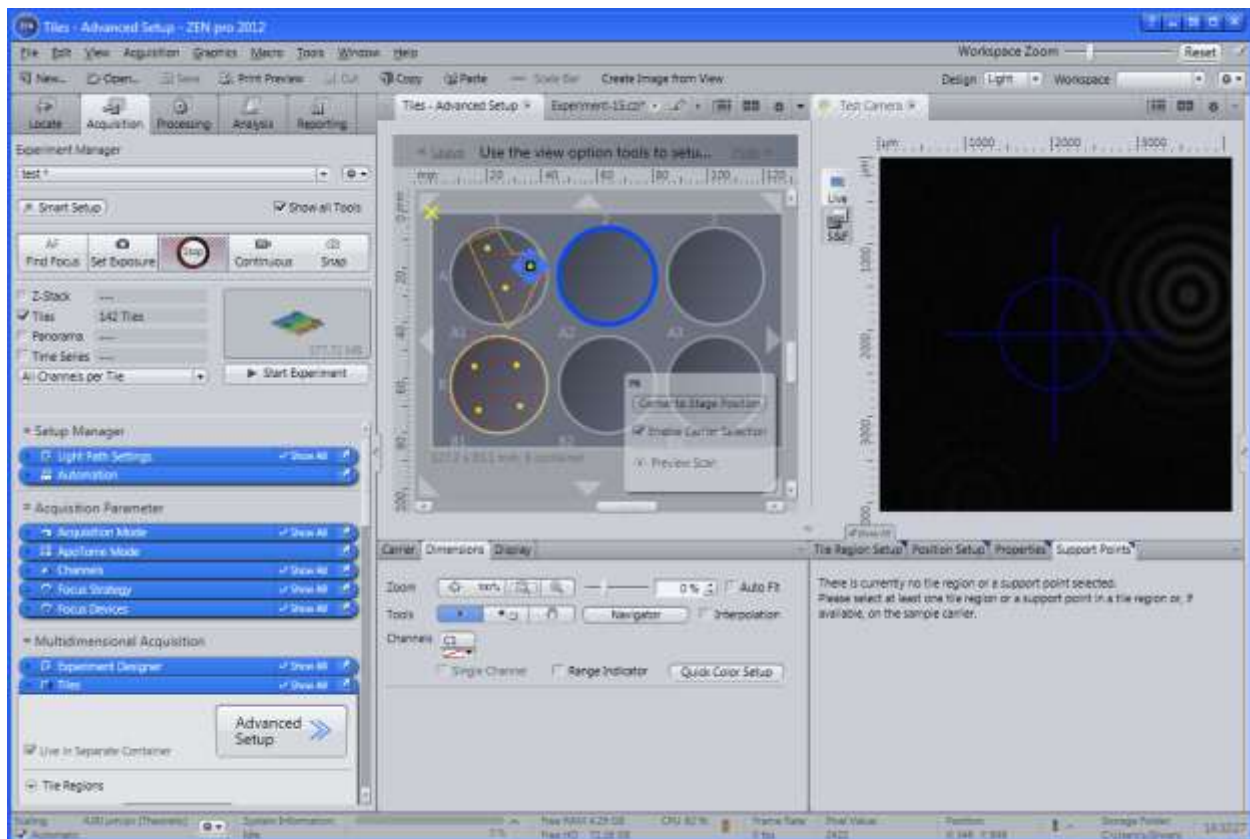
Multiple Tile Regions

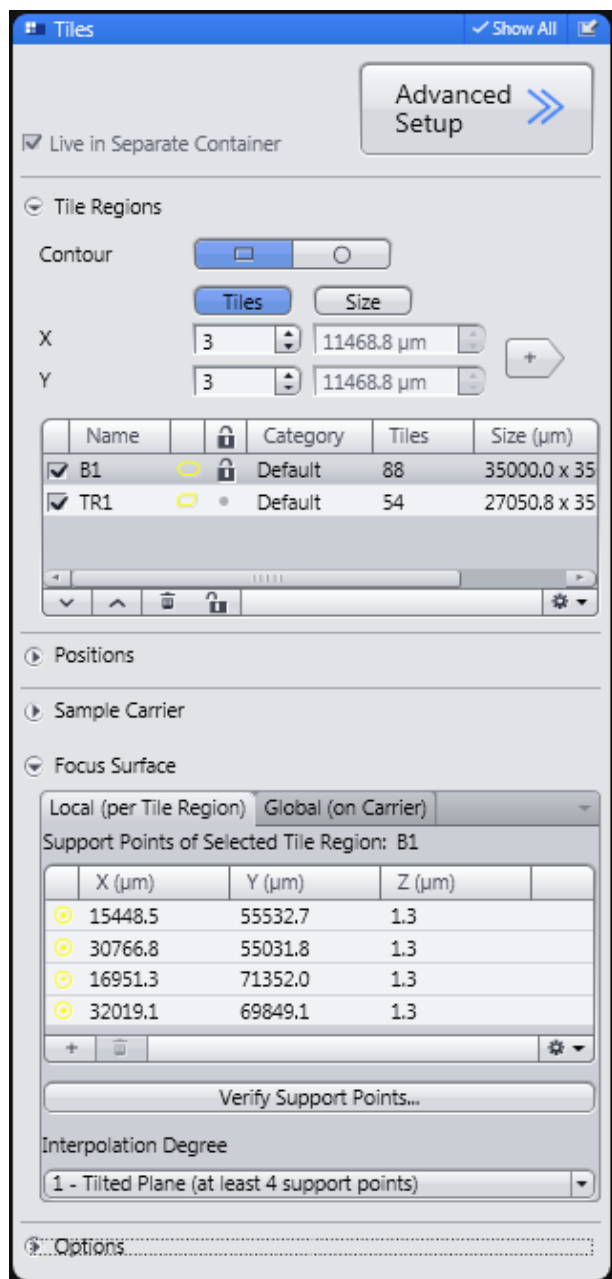
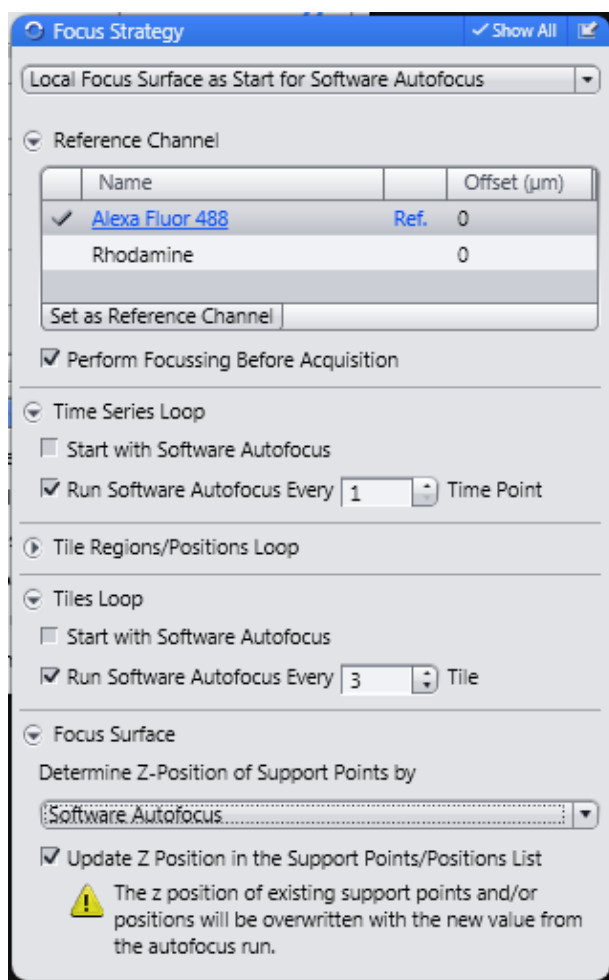
1. Local Focus Surface / Local Focus Surface as Start for Software Autofocus / Local Focus Surface Updated by Software Autofocus / Local Focus Surface Updated by Definite Focus



To get decent stitching effect, 10% or more Tile Overlap is recommended. If you check 'Stitching During Acquisition', usually it will slow down the experimental speed.

Once a Tile Region is defined, you may choose the region and add in local focus surface support points if the sample is not flat enough. To add in the Local Surface Support Point, usually you will need the Live Window to help. You may go to either blue tool Tiles and click Focal Surface and Local (per Tile Region) to add them in. Or you can go to the Tiles advanced setup window to set up tile region and support points. If needed, the added support points can be verified through "Verify Support Points". If the experiment also contains time series, Definite Focus is an option if equipped. If software autofocus does not give reliable results, you may choose fixed Z position for focus surface support points, the logic applies to a global focus surface strategy.





If it is a time series experiment, then Definite focus becomes an available option.

2. Global Focus Surface / Global Focus Surface as Start for Software Autofocus / Global Focus Surface Updated by Software Autofocus / Global Focus Surface Updated by Definite Focus

This is similar to that for multiple single positions global focus strategy.

Multiple Single Positions / Tile Regions Z-Stack

Usually center mode is recommended for the Z-stack imaging for multiple positions or tile regions. Focus surface support points or definite focus can be used for the each one of the center Z.

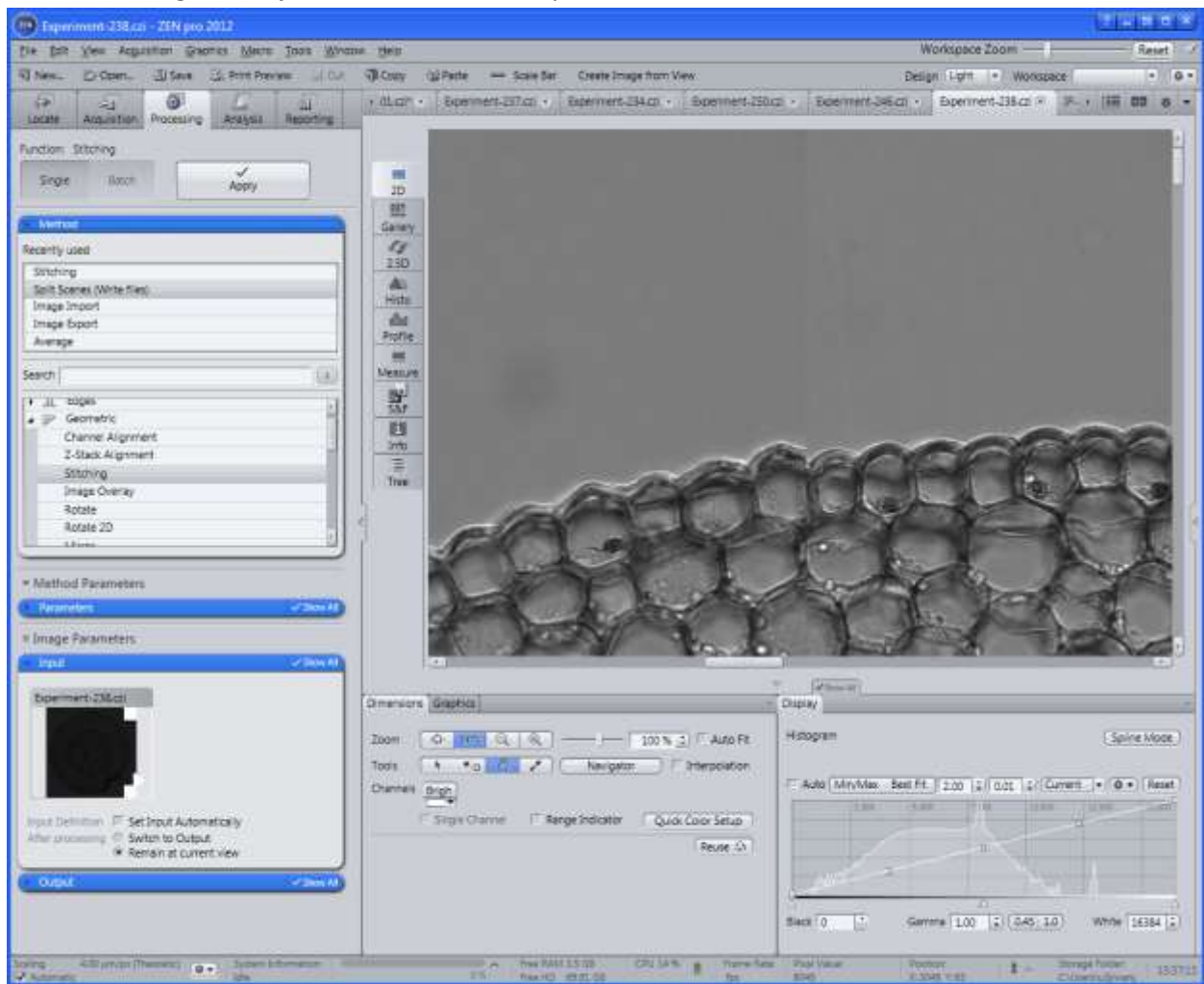
If first/last Z stack is preferred or different Z-slice numbers is desired, then use Experiment Designer to set each Z-stack from each position as a block and define first and last Z position for each block.

In the Experiment Designer tool (The Experiment Designer tool is only available if the Show All Tools checkbox is activated in the Experiment Manager.) you can create experiments for multidimensional acquisition. The experiments can consist of any number of components. A component is referred to as an experiment block. Each experiment block has a distinct number, which is shown above the block. Special actions that influence the course of an experiment are performed by means of a special block. In the Show All mode you can define repetitions and specify the number of image files. Please refer to ZEN help file for further information.

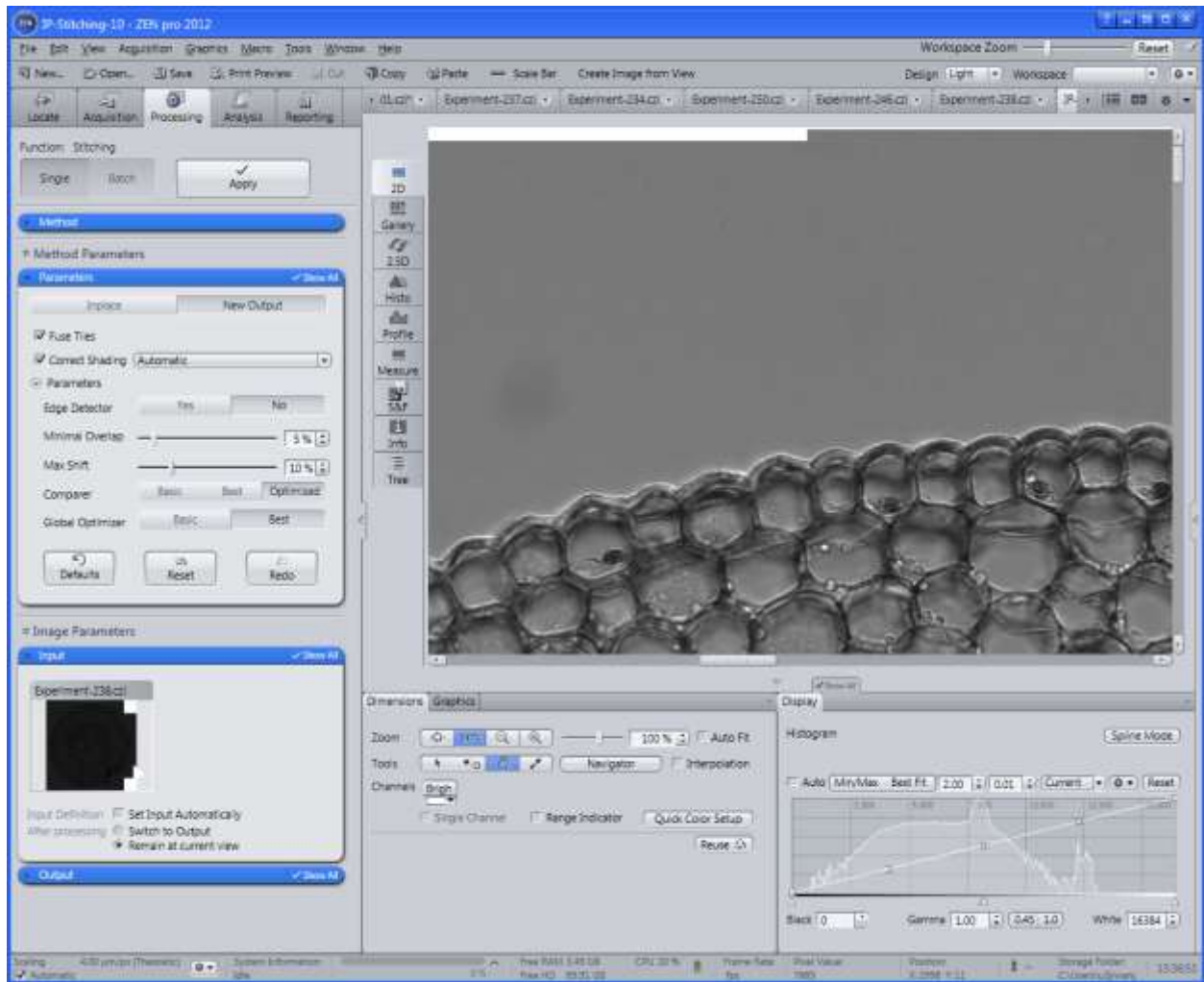
Stitching

A stitching process is needed to stitch the tiles in a tile region together to get an overall image of the sample. Stitching the tiles together can be done during acquisition via the Tiles tab or after acquisition using the Processing tab. The latter option allows the user to choose many different parameters for optimal stitching.

Before stitching, two adjacent tiles are obviously unmatched to each other.

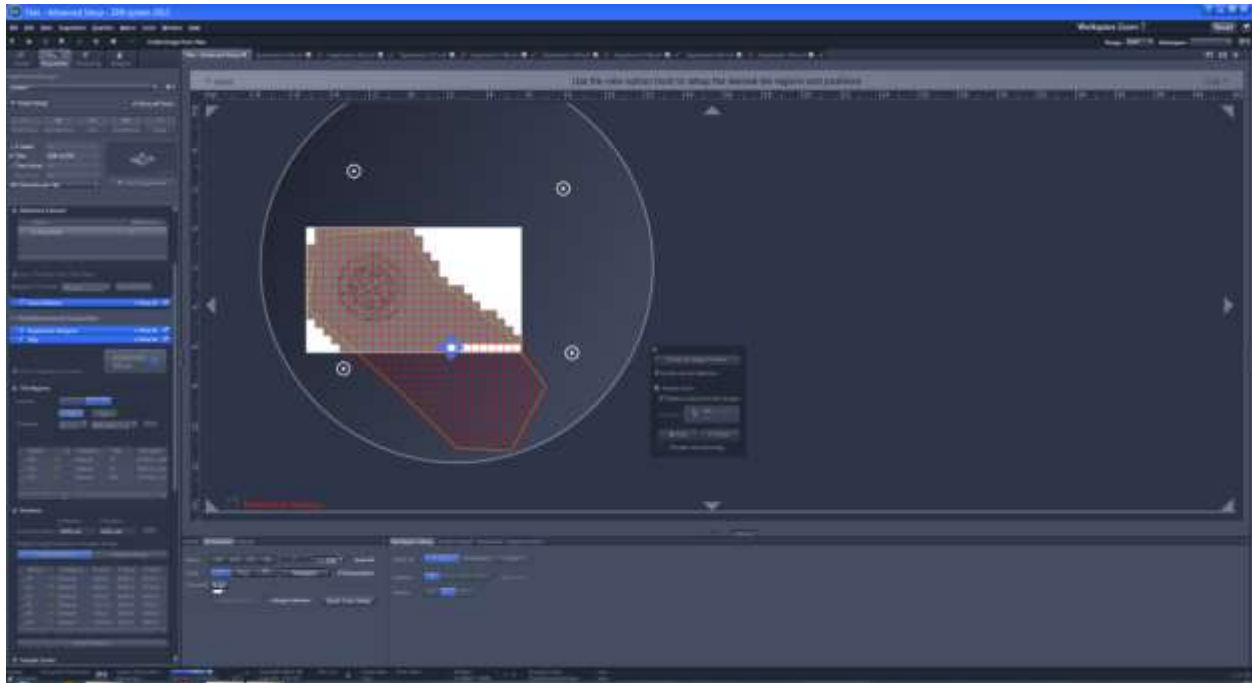


After stitching

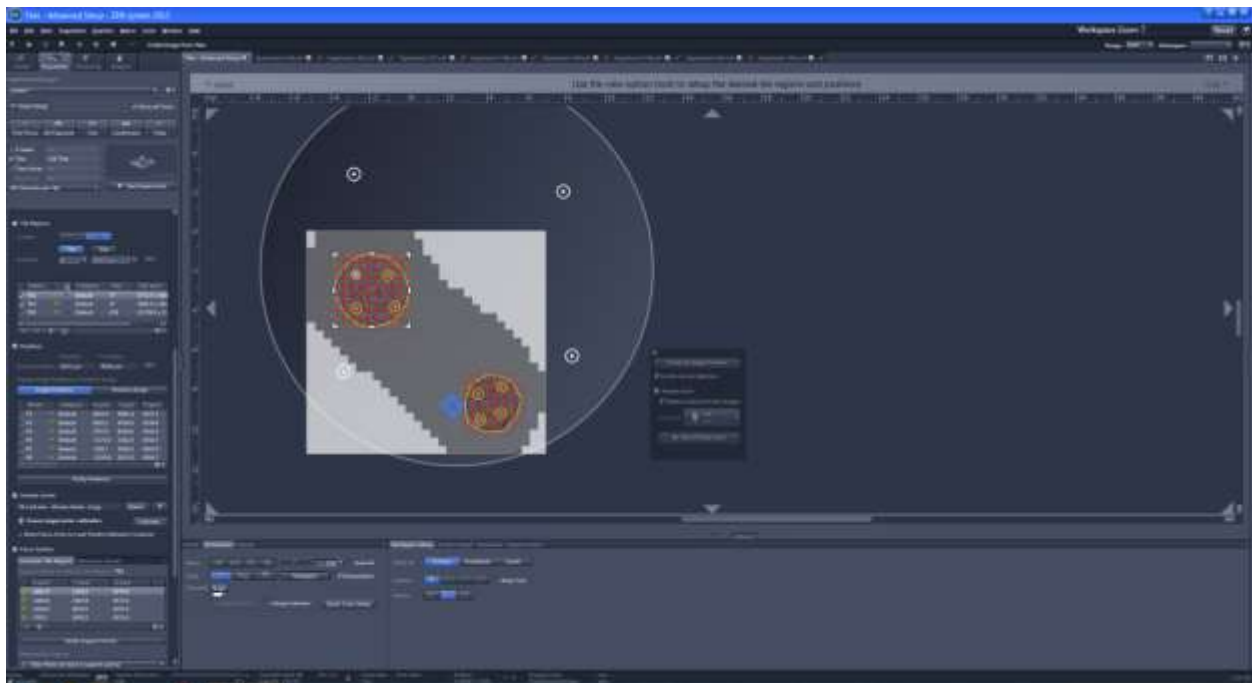


Examples #1: Multiple Tile Regions with Local Focus Surface as Start for Software Autofocus

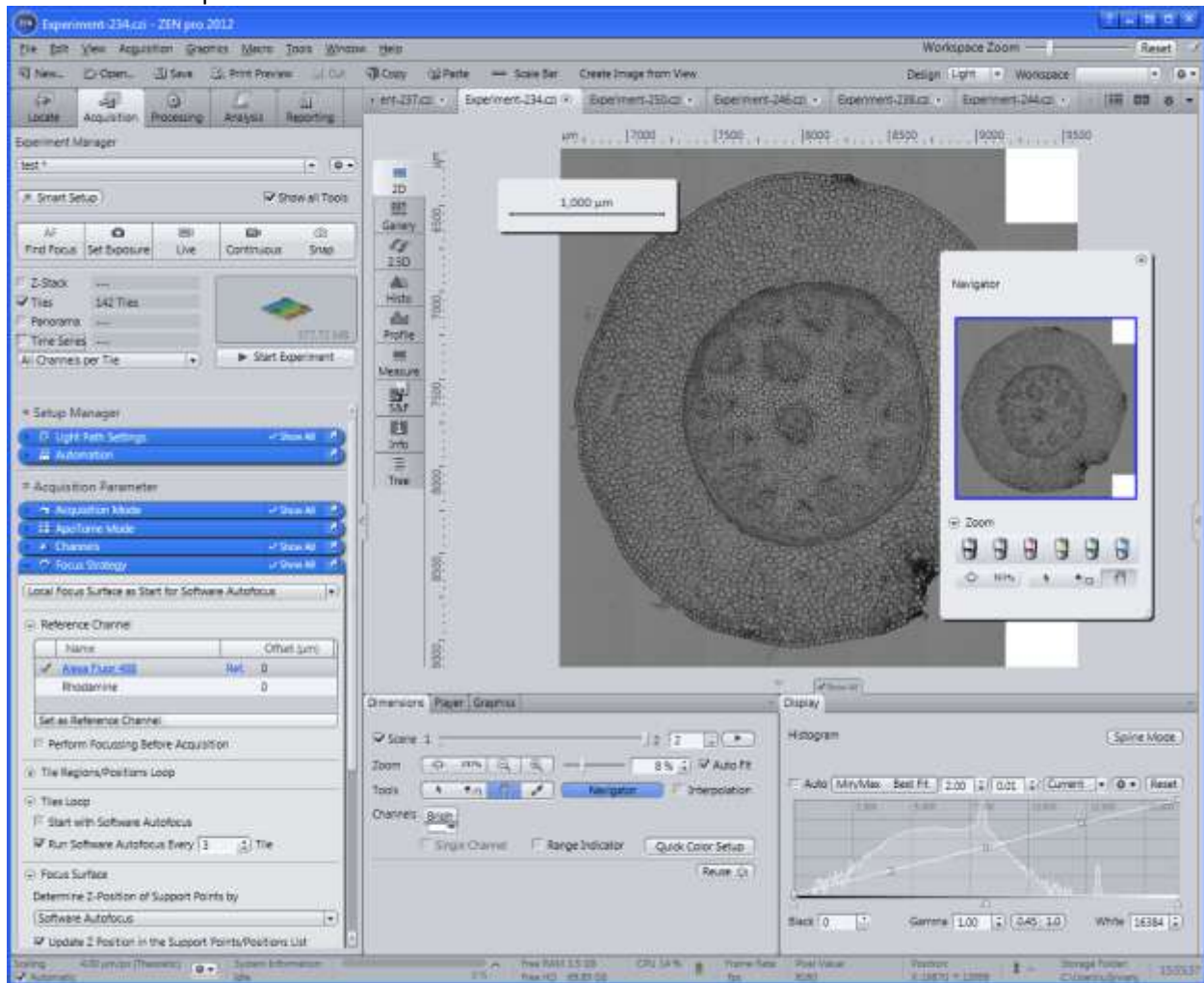
The sample is a slide with two pieces of convallaria stem sections. First we want have a preview before we set up tile regions of the two pieces. So we use low mag lens, e.g. 2.5X, 5x or 10x to scan an area and get an overview in Tiles Advanced Setup.



Define tile regions around the two pieces and then add in 4 local focus surface support points for each tile region. In the Focus Strategy tab choose Local Focus Surface as Start for Software Autofocus. In the Focus Devices tool define the autofocus range and verify each support point if needed.



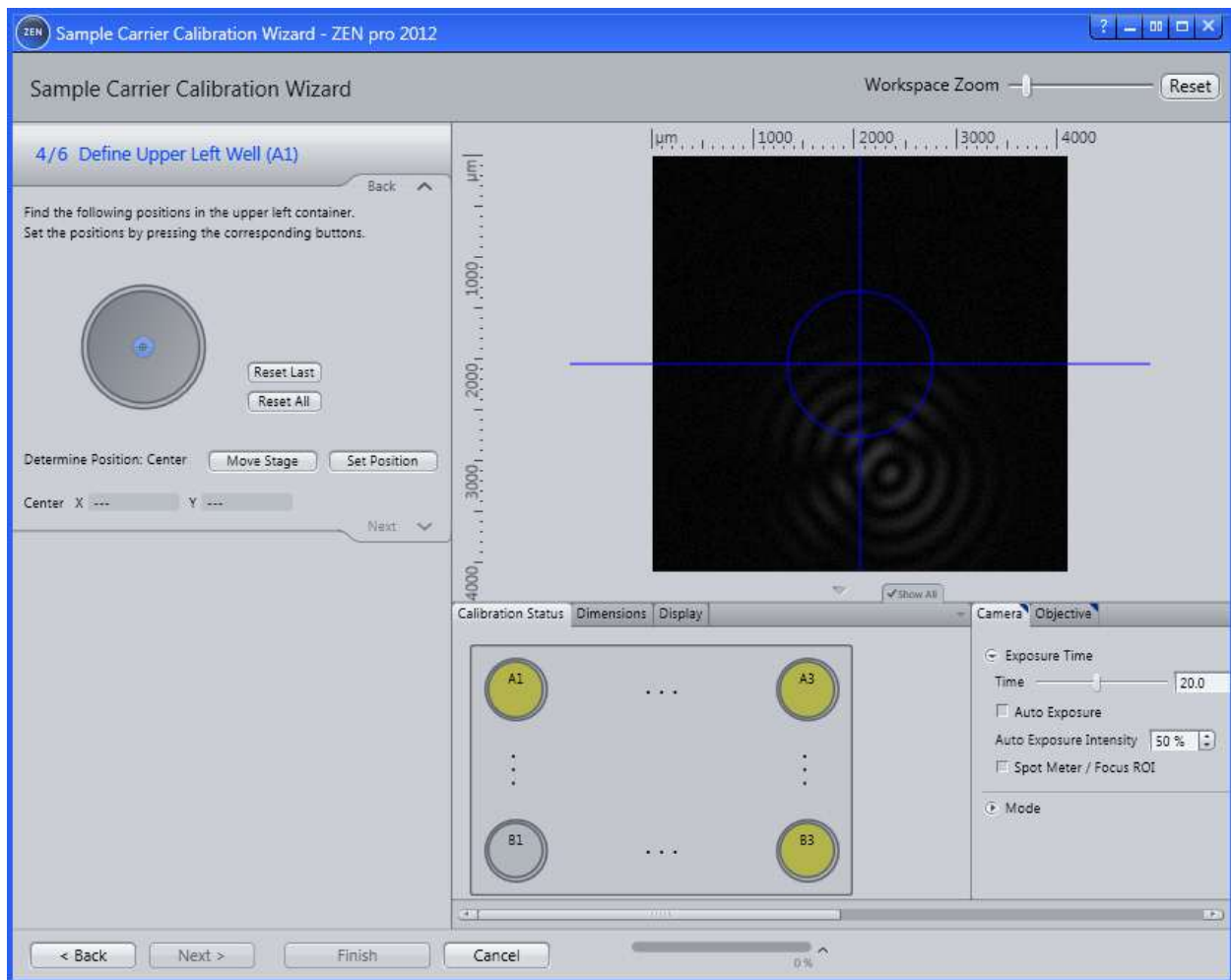
Then start the experiment.



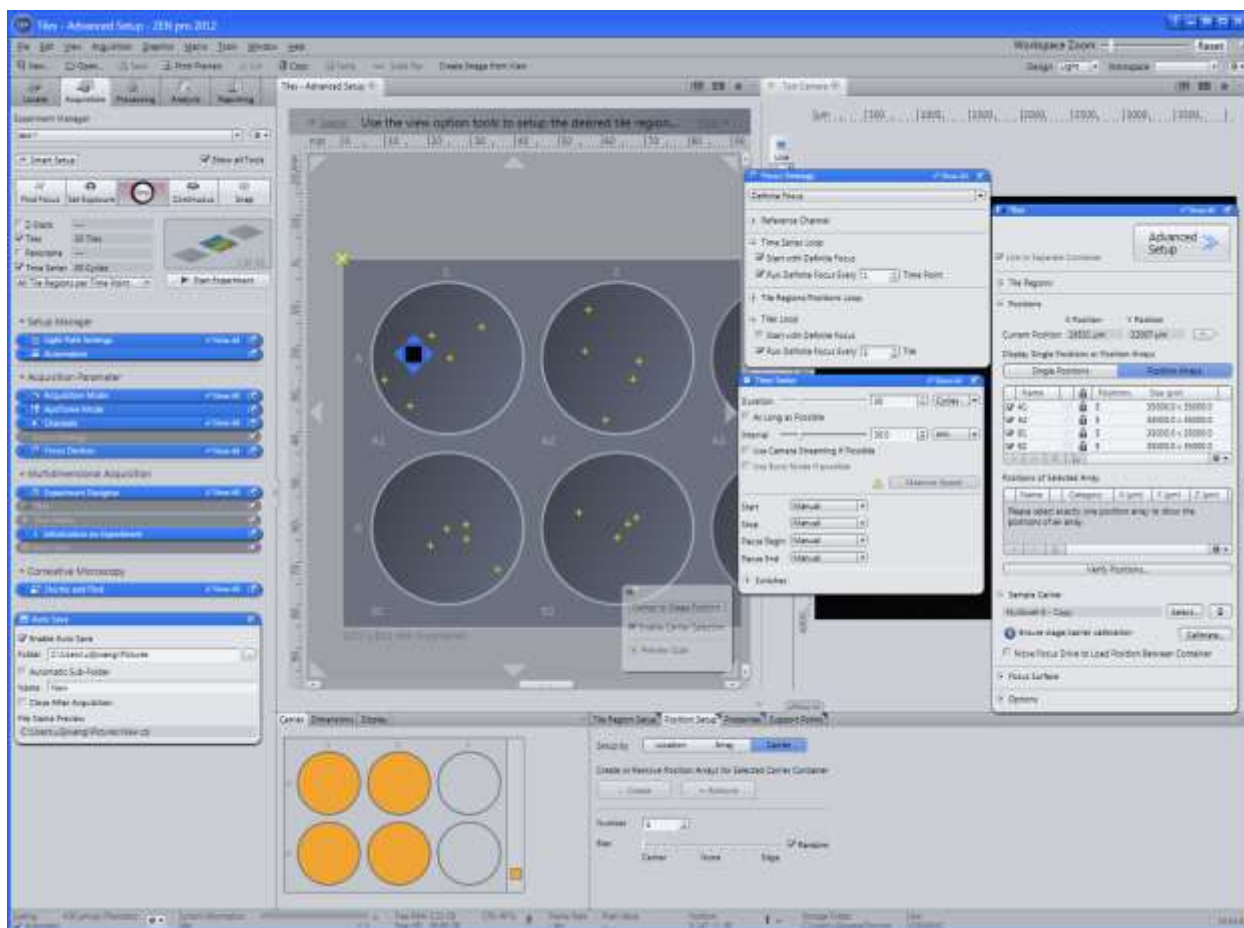
Perform Tiles Stitching if the images were not stitched during acquisition.

Examples #2: Multiple Well Plate Positions Time Series with Definite Focus

First set up the sample carrier. Calibration is needed if we want the positions recorded precisely so that we can later find the positions again. In order to do this, open the Tiles tab, select a sample carrier and then click on Calibrate. Follow the calibration sample carrier instructions below.



After the sample carrier calibration, you may see the sample carrier on the screen. Then you may define the single positions and focus strategy.



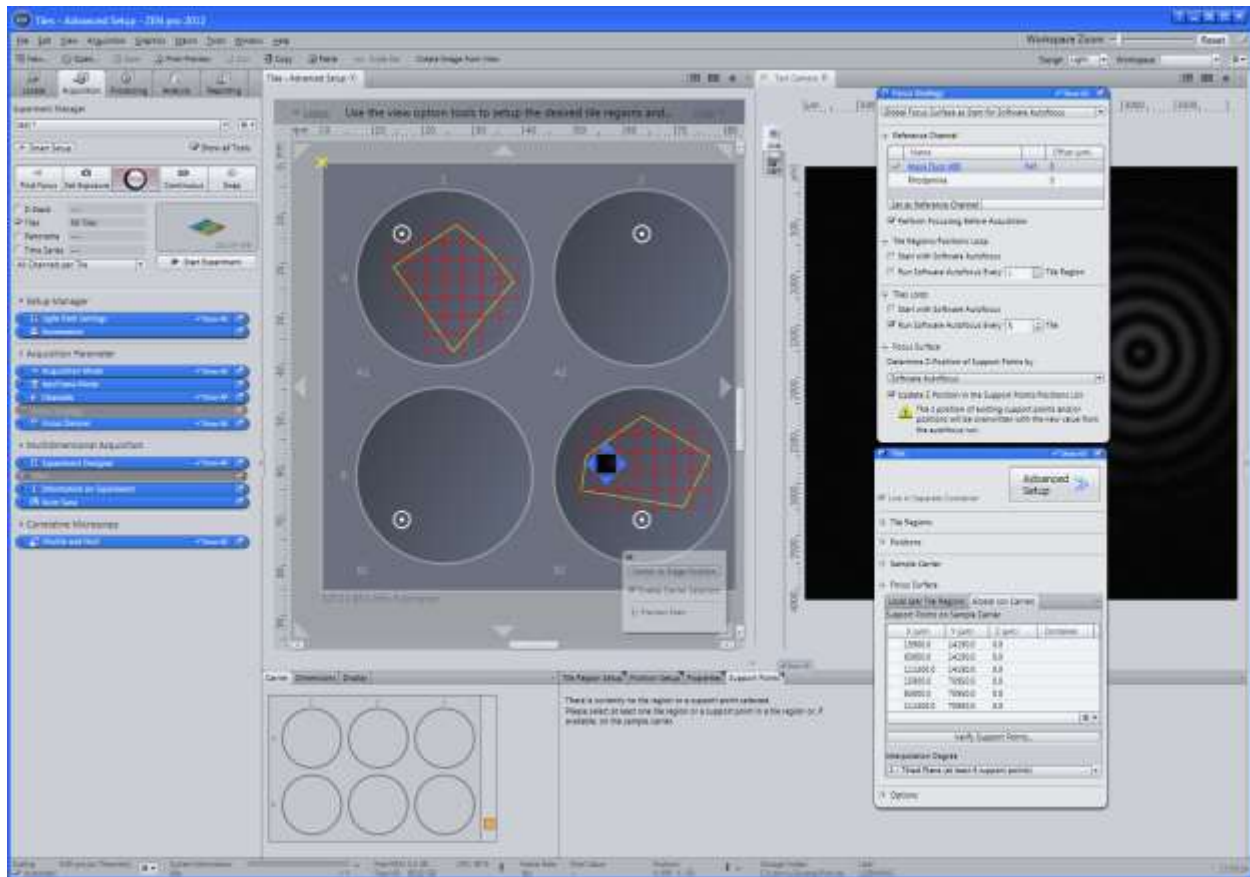
During a long time series experiment, it is recommended to enable Autosave.

Before hitting Start Experiment, make sure that the first position is in focus. It is advised to verify each position. The Definite Focus will use the first position as a reference to maintain the focus.

After the image capture, you may use Graphic Elements to add in the image scene name and image scene shape name so you know which position belongs to which well plate. For example, P1 A1 means position one, well plate A1.

Examples #3: Multiple Tile Regions with Global Focus Surface as Start of Software Autofocus

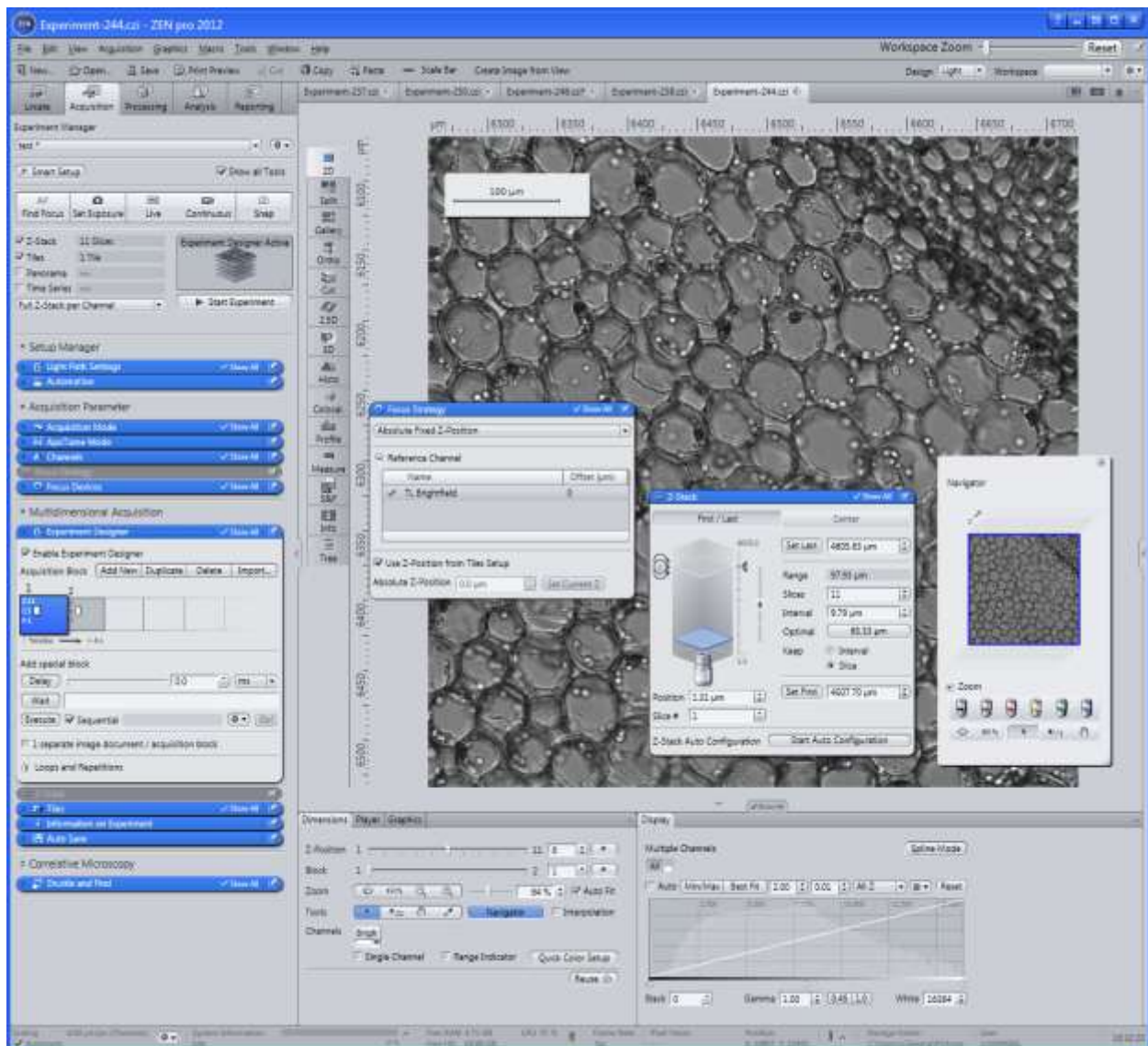
First set up the sample carrier to put in global support points. Calibration may be needed as outlined in Examples #2. Then use Tiles Advanced Setup to setup Tile Regions. Verify the support points and choose the Focus Strategy “Global Focus Surface as Start of Software Autofocus”.



Examples #4: Multiple Single Positions Z-stacks

A: 2 positions or more, first last mode Z-stacks. First position 11 z-slices, second position 7 z-slices. Focus strategy 'Absolute fixed Z-position'.

Define first and last Z-position for each block in experimental designer.



B: multiple positions, center mode Z-stack. Focus strategy can be 'Absolute fixed Z-position' / 'software autofocus' if it works / 'Definite Focus (if equipped)' / Software Autofocus as Reference for Definite Focus / Definite Focus as a Start for Software Autofocus / Global Focus Surface / Global Focus Surface as Start for Software Autofocus / Global Focus Surface Updated by Software Autofocus / Global Focus Surface Updated by Definite Focus.

If it is not time series experiment, usually absolute fixed Z-position or global focus surface will work. For long time series experiment, like overnight experiment, definite focus option is recommended.

C. multiple tile regions Z-stack, center mode is applied. Focus strategy can be 'Absolute fixed Z-position' if the sample is flat; 'local focus surface', 'local focus surface as a start for software autofocus', etc. Finally the Z-stack is a big stitched Z-stack.

Tips for Simple Trouble Shooting

1. I can clearly see my sample image through the live window and I already put in several positions. When I move to each position, the image looks great, but when I hit start experiment, the captured images are blurry. What's wrong?

Probably a focus strategy is not set up properly. 'Absolute Fixed Z-Position' is recommended for the focus strategy and you need to make sure the option '**Use Z-Position from Tiles Setup**' is checked.

2. I acquired two channels (bright field + GFP) images in several positions. The bright field channel looks much dimmer. However when I check it in the live window, it looks OK. Why?

Most of the time it is due to Halogen lamp needs some time to light up. To compensate for that delay, you may use Smart Setup to reset up the bright field channel if a motorized TL shutter is available. In this case, the Halogen lamp is recommended to be always on during the experiment. If it's LED light source, be sure that the voltage is greater than about 3 volts or otherwise the LED won't light up. If there is no TL shutter, then an acquisition delay time, about half second, is recommended to compensate the voltage ramping up time.

3. I acquired several bright field tile regions of sectioned tissue sample. When I tried to stitch them together, I checked 'Fuse Tiles' and 'Correct Shading' but I still got some grid like pictures. What happened?

Probably it is due to uneven illumination to the sample. Re-adjust Kohler illumination of the microscope to make sure the illumination is even and then recapture the tile regions and redo the stitching.

4. I have a sample slide and I took several pictures from several positions on the slide. The other day when I put the same slide on the same position on the stage, I could not find the previous positions. I am sure nobody used the system since then. Why I cannot re-find those positions?

It is possibly the stage was not calibrated before the experiment. Each time when the stage is turned on, stage calibration is needed in order to record the tile x and y coordinates precisely. Also the sample carrier needs to be calibrated every time it is put on the stage. Using Zero for reference point is recommended.

5. I ran a 7-position time series experiment overnight. I set the focus strategy as Definite Focus at every time point and every position. In the morning I found that several positions were good and all others were blurry. What's wrong with the Definite Focus?

The definite focus will use the current position to maintain the objective focus. So once an experiment is set, it is suggested to move the stage to first position and make sure it is in focus.

6. I use position array to set up a more than 300 position experiment and I set Software Autofocus as the focus strategy. During the experiment, I see a red warning that autofocus is out of range. In the final picture, I got several tiles that were quite blurry. What happened?

Check the autofocus range in the Focus Device tool. You may try to use focus support points as a start of autofocus so that the autofocus is likely in range.

7. How can I import a position list created by Excel?

First make sure the stage and sample carrier are both calibrated. Then you may import the tiles experiment. Reselect sample carrier and choose 'No' when asked "Should those be deleted when the sample carrier template is assigned or removed?" Then the imported positions show up in the Tiles-Advanced Setup window. When using Excel to create the position list, just make sure they are not out of the sample carrier. When calibrate the sample carrier, use 'Zero' to set the reference point.

8. How do I know the frame is from which well plate when I captured a multiple positions image from a 6 well plate?

Use Graphic Elements to put in annotations. Right click the Graphic Elements and click the Annotation to expand all options. From the Available Metadata, you need to choose Image.Scene.Name and Image.Scene.Shape.Name to show the position and well plate information.

9. How do I run two time series with the first position having a 5min interval and the second position having a 30min interval?

You will need Experiment Designer. Put the first position time series as block #1 with a 5 minute interval and the second position time series as block #2 with a 30 minute interval.