

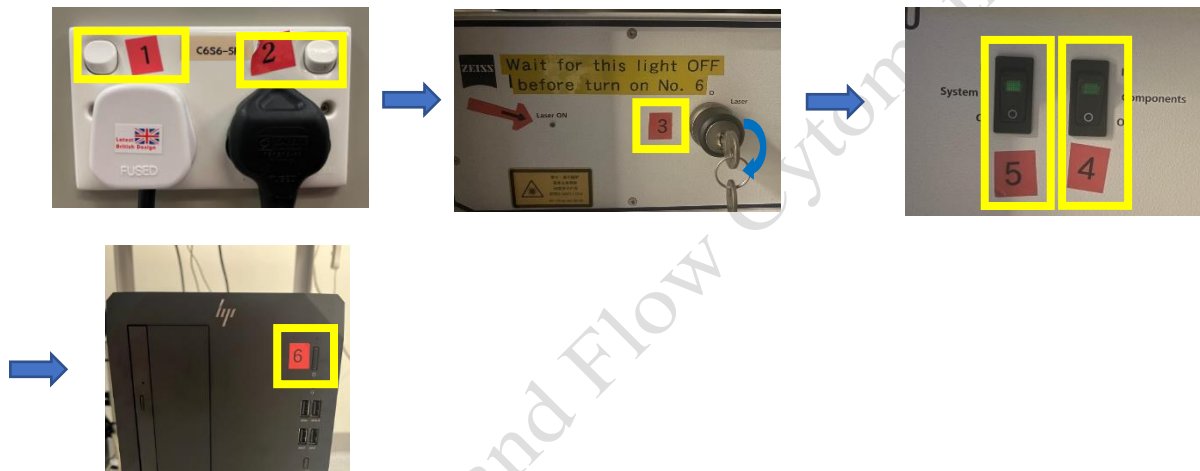
## Upright LSM900 Confocal Microscope

### Standard Operation Protocol

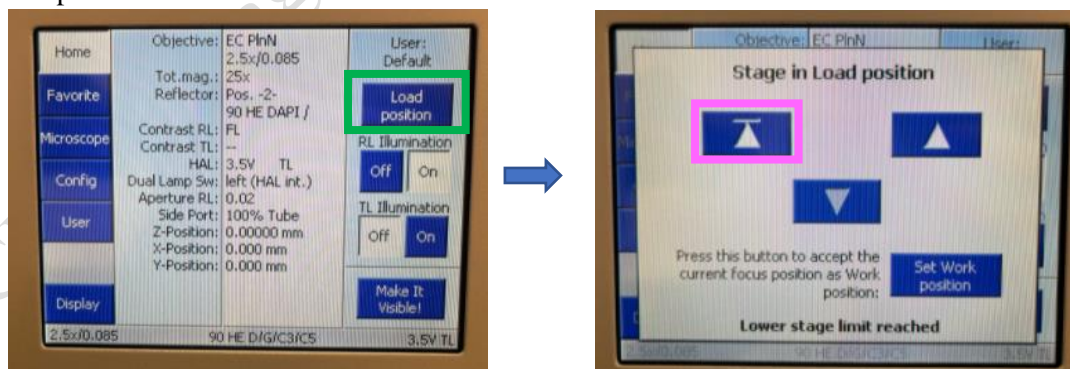
#### System Start Up

Sign on log sheet according to **Actual** start time (**Please leave at least 5 seconds gap in between each step of machine Start Up process**)

1. Switch on Main power switch ① → wait for 5 seconds
2. Switch on power switch ② on power socket → wait for 5 seconds
3. Turn the key switch ③ 90° clockwise for laser switch on → wait for 5 seconds
4. Switch on power switch ④ on power unit → wait for 5 seconds
5. Switch on power switch ⑤ on power unit → wait for the light (near step 3) to be off
6. Turn on the computer power switch ⑥

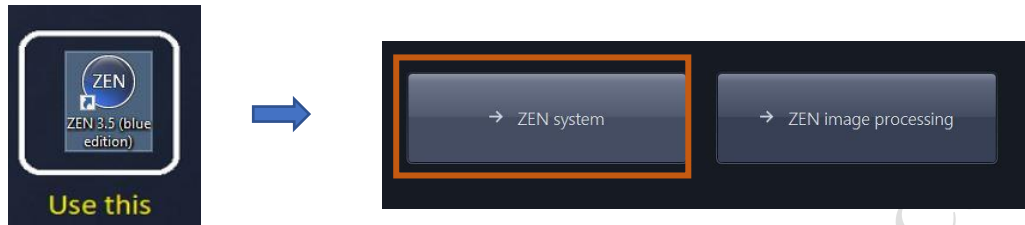


7. Press the “load position” button to move the stage to the preset sample loading position.
8. Slide in your sample slide with coverslip facing up onto the sample adapter. Then press the ▲ button to move the stage back to the original position. Repeat steps 7-8 whenever you change sample slides.



## ZEN Software Initialization

1. Log on computer with “**USER**” account  
(Please find **USER account password** posted under the bottom of computer screen)
2. Double click the **ZEN 3.5 blue** icon on desktop
3. Click **ZEN system** to run the software

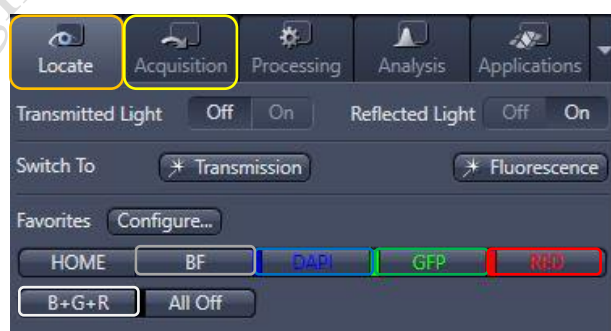


## Locating Sample and Finding a Region of Interest

1. Select a **desired objective** by switching the objective lens **Manually**. (2.5X, 10X, 20X)



2. Move the region of interest to right below the objective lens with the **joystick**. Press the **top right button** to change the speed of stage movement (from fast to slow mode or in reverse).
3. Push the “**Push-pull rod**” to the end to switch to **eye-piece mode**.

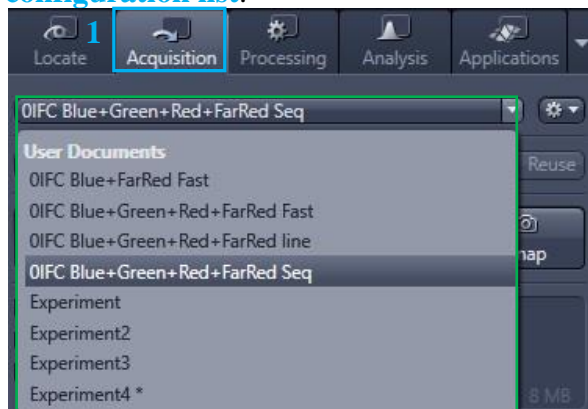


4. Click “**Locate**” tab in the software and select a fluorescent channel of interest for direct observation via the eyepiece.
5. Click **BF** tab to set the microscope for brightfield observation.
6. Click on **BF/DAPI/GFP/RED/B+G+R** tab for Bright Field/ blue emission /green emission/red emission/red+green+blue emission observation respectively.
7. Focus the sample with Coarse/Fine focusing knob.
8. Once the region of interest is found and focused, switch the tab to “**Acquisition**”. Pull the “**Push-pull rod**” out to switch to **acquisition mode**.

## Setting up the Scanning Track/Channel

### Method 1

Load a **predefined configuration** of combination dyes from the **Load configuration list**.



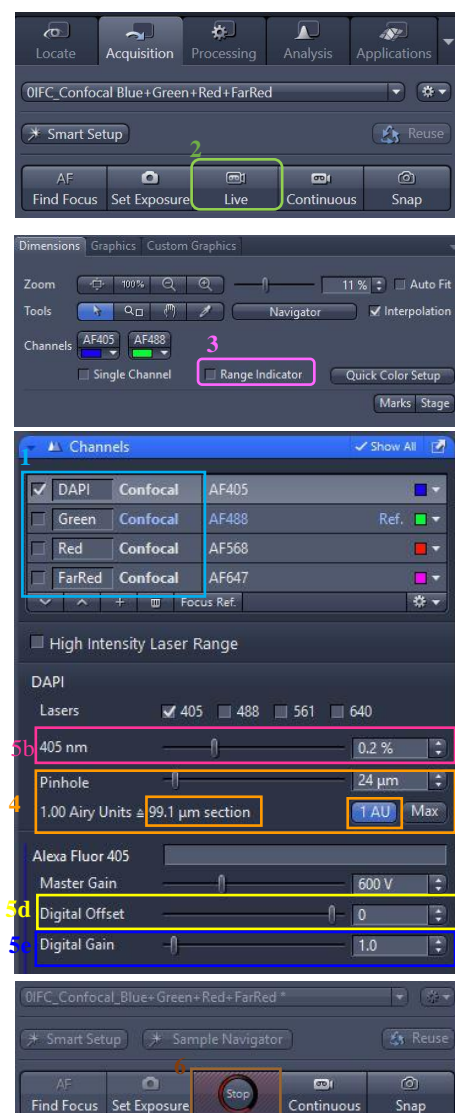
### Method 2

Open any previous image with desire click **“Reuse”** button in Dimension panel.



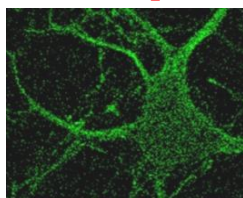
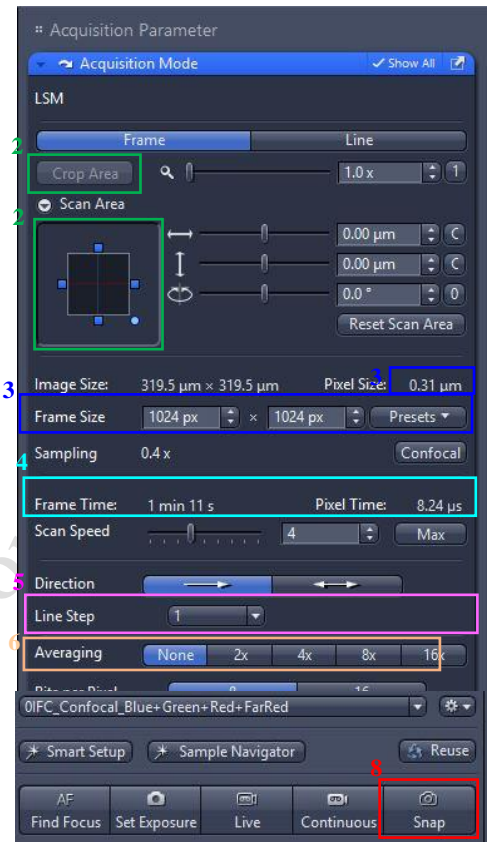
## Confocal Imaging Set Up

1. Select only **one channel** tool for parameter adjustment at a time.
2. Click **Live** for continuous fast scanning.
3. Activate **Range Indicator**. The scanned image appears in greyscale with red pixel indicating saturation (max. intensity) and blue pixel indicating zero (min. intensity). Advise to adjust the laser power and/or gain master until a little bit of red pixel is seen.
4. Set the **Pinhole to 1 AU (Airy Unit)**. For co-localization studies, adjust the pinhole of each channel to the same **Optical Slice Thickness**.
5. To get optimal intensity and background signal,
  - a. Increase **Detector Gain (Maximum of 800)** until a few red pixel (indicating saturation) appear in the image.
  - b. Increase the **Laser Power** (too high cause bleaching), if increasing detector gain cannot achieve the desired intensity.
  - c. Fine-tune the focus with the fine adjustment knob to the brightest or preferred z- position. And then adjust the detector gain and laser power to optimize the signal intensity.
  - d. Decrease the **Digital Offset** to reduce background signal until the desired background region is filled with blue pixel.
  - e. Increase **Digital Gain** to increase signal amplification if needed.
6. **Stop** the Live scan process and uncheck the Range Indicator.
7. Repeat step 1- 6 for other tracks.

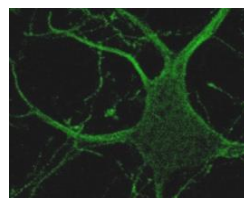


## Scanning Parameters Set Up

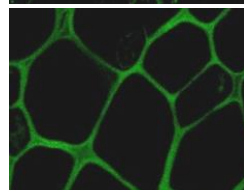
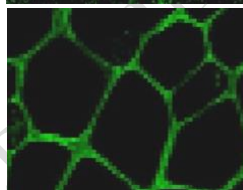
1. Go to Acquisition Mode.
2. Select a suitable **Scan Area** to capture image of interest (or use **Crop Area** function after a Live image).
3. Choose a **Frame Size** (change of **Pixel size**, thus change of resolution). 1024x1024 usually produces good results for general purpose. Click on the **Confocal** button for best resolution which depending on objective N.A. and  $\lambda$ .
4. Adjust the scan speed with the **Speed** slider. Speed 4 or 5 usually produce good results. For samples with high background noise, use Speed 4.
5. Select the number of **Average**. Averaging (usually in 2 or 4) improves the image quality by increasing the signal-to-noise ratio but it also increases the scan time.
6. Select the Bits per Pixel of 8 or 16 Bit in the **Bits per Pixel**. 16 Bit is recommended when doing quantitative measurements or other post-analysis.
7. Other setting can be kept as defaults (as in the right) which will be good enough for general purpose.
8. Click **Snap** to acquire a single frame (multi-channel) image.



Lower scan speed;  
or More averaging

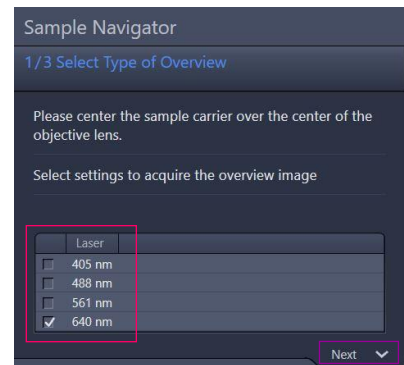
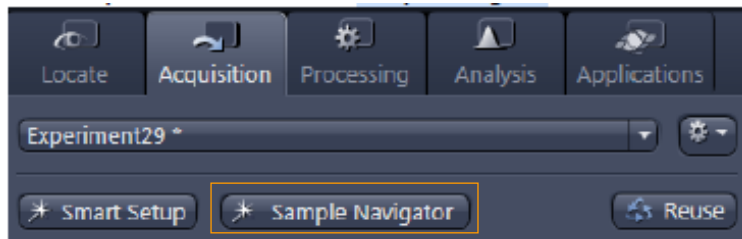


Larger frame size  
(Higher resolution)

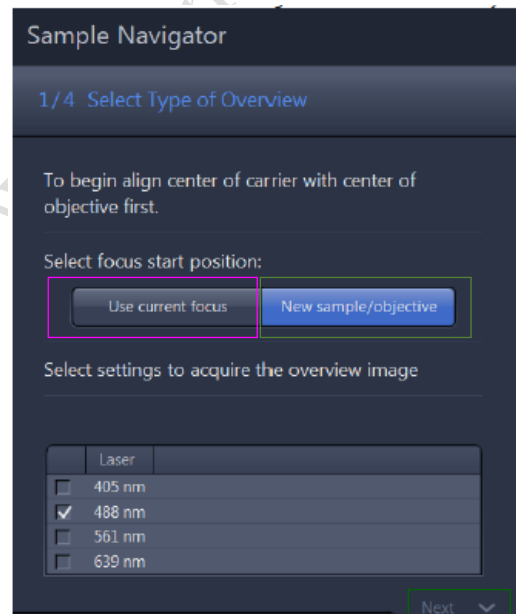


## Sample Navigator

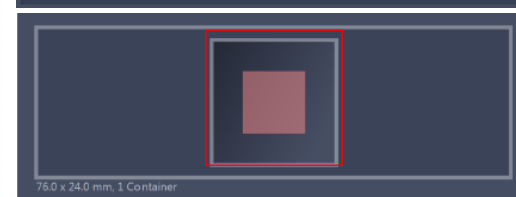
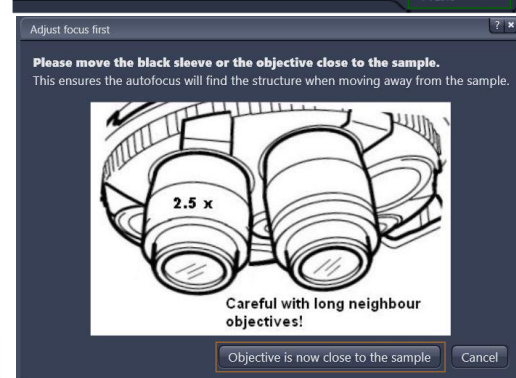
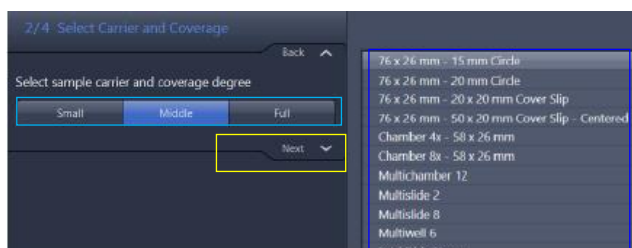
1. Put a sample on the stage and center the sample with some structure over the center of the objective.
2. In the Acquisition tab, click on the **Sample Navigator** button → Select at least one checkbox with the wavelength (405nm/488nm/561nm/640nm) that matches the label of your sample (strongest channel with less photon bleaching is preferred) → Click on **Next**



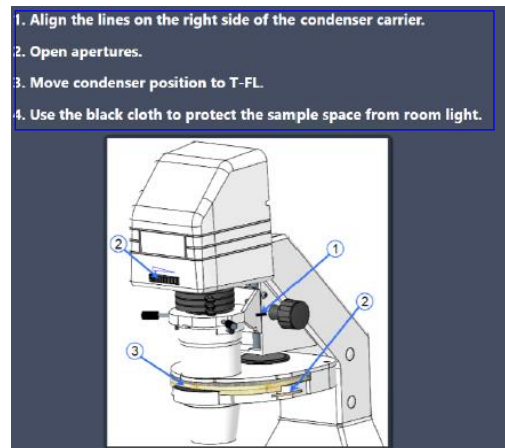
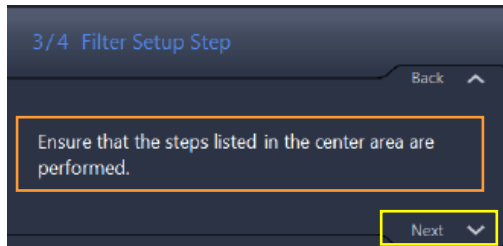
3. Click on **Use current focus** if sample was already focused, or click on **New sample/objective** if a new sample was put on the microscope. With a new sample, you are asked to switch to use 2.5X objective and focus the objective a bit closer than the putative focus plane. This is because the autofocus will move away from the sample when searching for the focus plane. Click on **Objective is now close to the sample** → Click on **Next**, go to step 2.



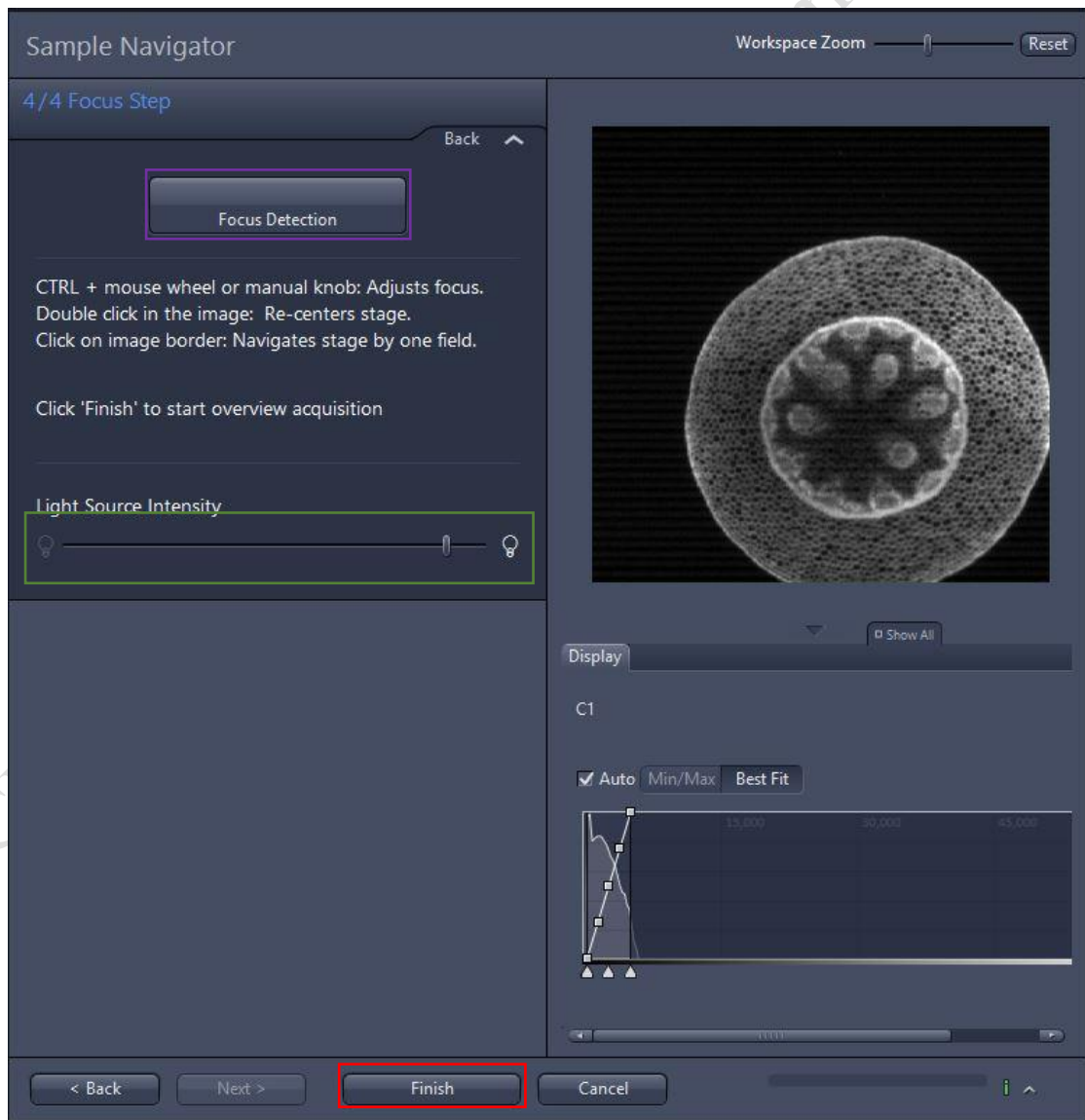
4. Select a matching **sample carrier type** from the selection list in the middle → select the coverage degree **Small**, **Middle** or **Full** (Full means full coverage of the Cover slip area, middle means 1/4 coverage of the Cover slip area, Small means 1/16 coverage of the Cover slip area). The selected coverage is displayed by a **red area** on the sample carrier drawing → Click on **Next** at the bottom of the wizard, go to step 3.



5. Ensure that the steps listed in the center area are performed: 1. Open apertures 2. Move condenser filter to T-FL. 3. Use the black cloth to protect the sample space from room light. → Click on **Next** at the bottom of the wizard, go to step 4.



6. Click on **Focus Detection** to start the autofocus, and observe the image in the middle → adjust the light intensity with the slider **Light Source Intensity** if needed → manually focus the sample with the focus drives if needed → Click on **Finish** to start the automatic overview tile scan.



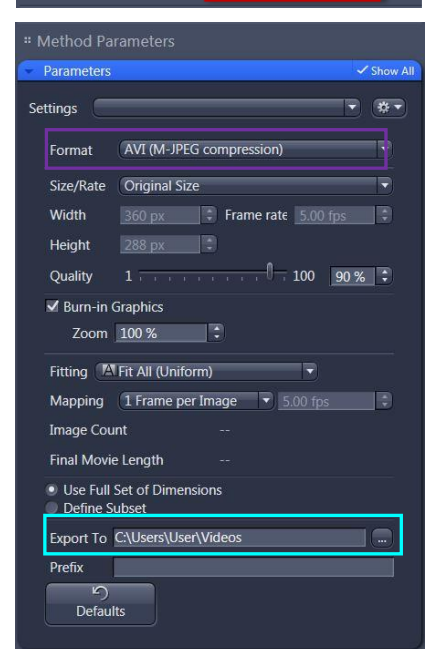
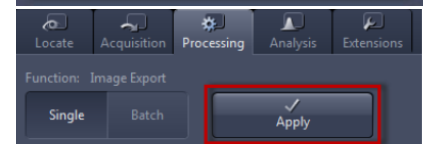
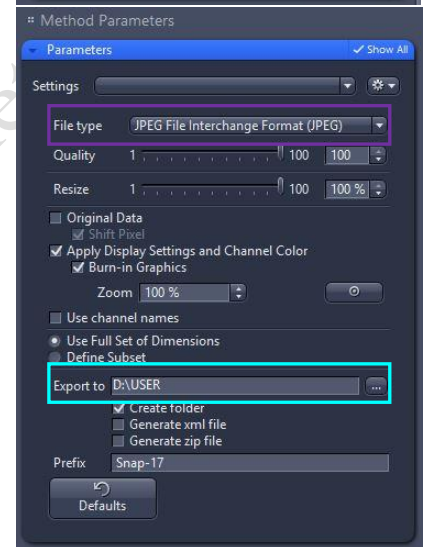
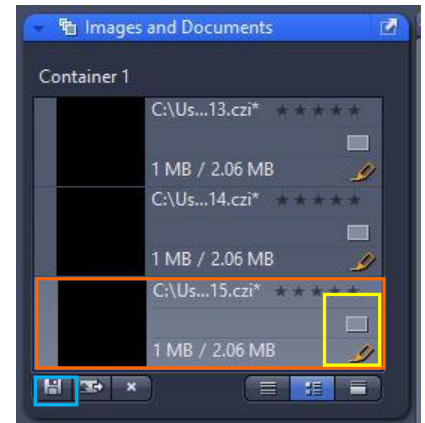
### Storing and exporting image data

- To save the acquired image → **highlight** the desired image → click on **Save** icon button or Save As button in the File menu → Create or choose your own folder in the D Drive (D:/user). **Warning Icon** indicated unsaved.
- Create or choose your own folder in D:\user, enter a file name, select format as **CZI** and click on the **Save** button.
- After saving the raw data as CZI format, Image can be exported as various Image format and series images can be export as video. Go to processing (or Go to File > Export) → Image Export → choose the **File type** as shown below → Choose **export to** → select the image you want to export → click on **Apply**.

File type	JPEG File Interchange Format (JPEG) Windows Bitmap (BMP) Tagged Image File Format (TIFF) Tiff Format(64Bit) (Big Tiff) Portable Network Graphics (PNG) JPEG XR (WDP) DigitalSurf SUR (SUR)
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- For export as video → Go to processing (or Go to File > Export) → Movie Export → choose the **Format** as shown below → Choose **export to** → click on **Apply**.

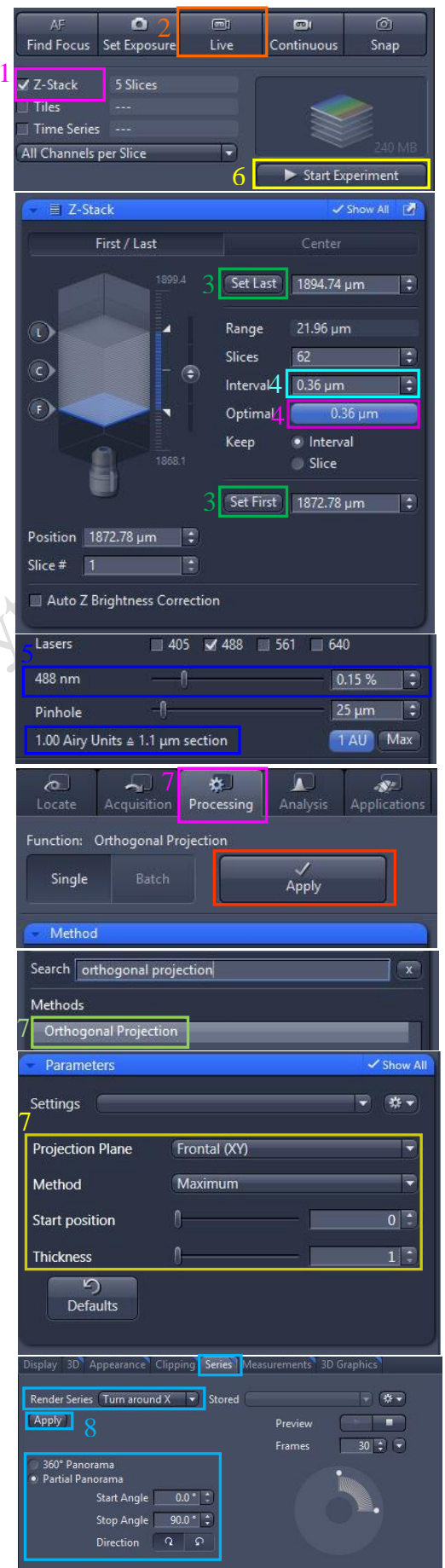
Series (Gallery of images; Time series movie or 3D animation)	AVI (M-JPEG compression) AVI (uncompressed) AVI (DV) WMF (Windows Media) MOV (H.264) MOV (MPEG4) AVI (MS-Video1)
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## Z-Stack Experiment Set Up

1. Check the **Box for Z-Stack** → Open the **Z-Stack module**.
2. Choose a channel that has signal throughout the interested volume → start **Live**.
3. Use the focus knob to locate one end of the specimen → **Set First**. Focus to locate another end of the specimen → **Set Last** → stop **Live**.
4. Set the Z-stack **Interval** between each frame equal to to/or less than the thickness of the thinnest **Optical Section** of the selected channel wavelength (so that you can scan all the space within the specimen). **Optical Section (pinhole)** of the channel is calculated according to the emission wavelength, objective lens, and the pinhole diameter.  
**Optimal:** double sampling with interval equal to half of the thinnest optical section and thus provide better 3D image reconstruction.
5. For colocalization studies, adjust the **Pinhole** of each channel in the Channels panel to the same **Optical Slice thickness** (so that each voxel contains data from the same volume of the specimen).
6. Click **Start Experiment** to start the recording of Z-Stack images.
7. After saving the CZI format, a 2D stack up image can be generated, in which image in all frames will be projected onto a single plane. Go to “**Processing**” tab → choose **Orthogonal Projection** → select **projection plane**, **Method**, set **Start position** and **Thickness** → **Apply**.
8. A video of rotating 3D image can also be made. Activate the **Series** tap, select the **Render Series**, number of frame and the rotation range (= **Total Frame** × **Difference angle**). Click **Apply** and the series images will be generated in a new tab.

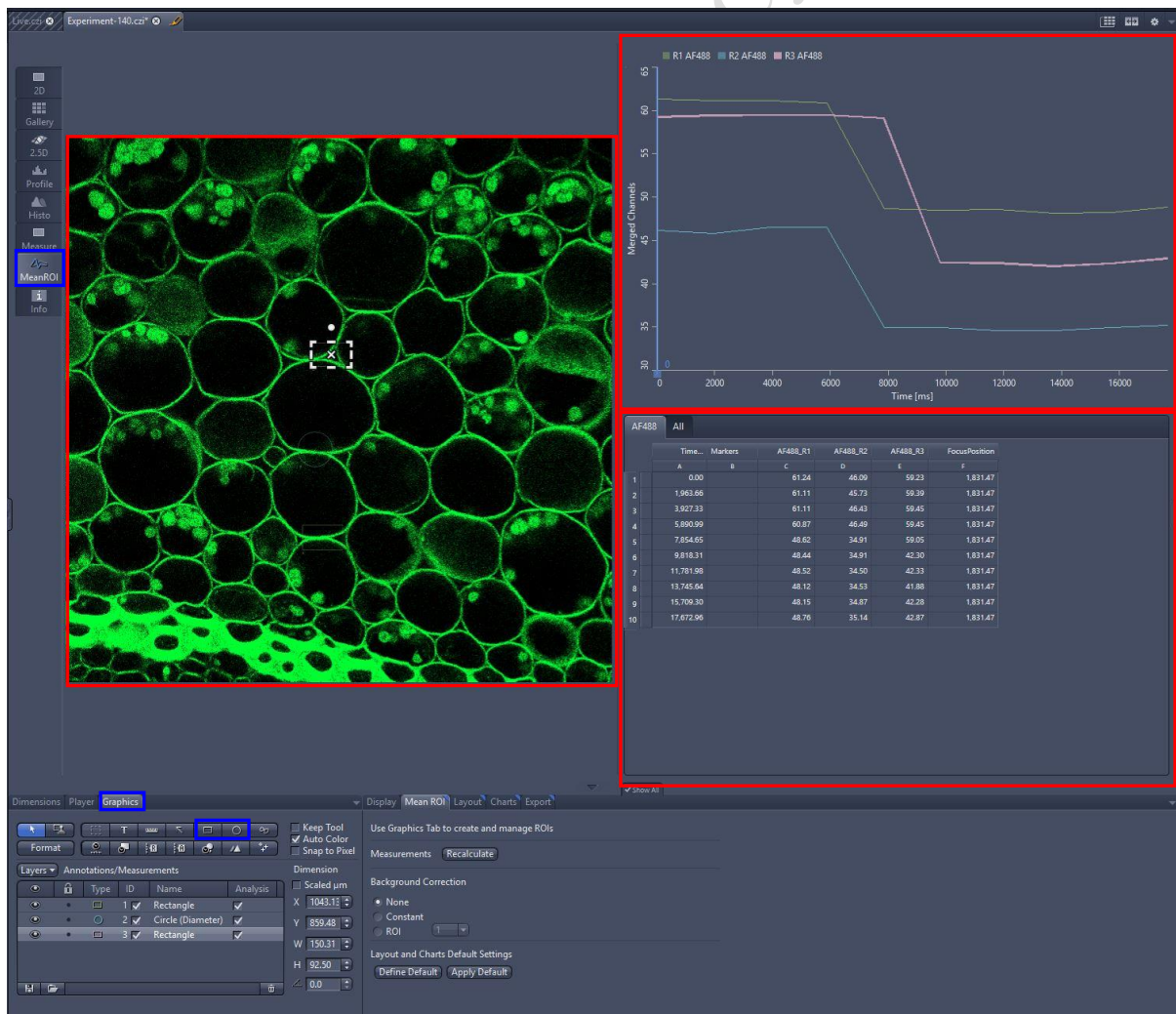
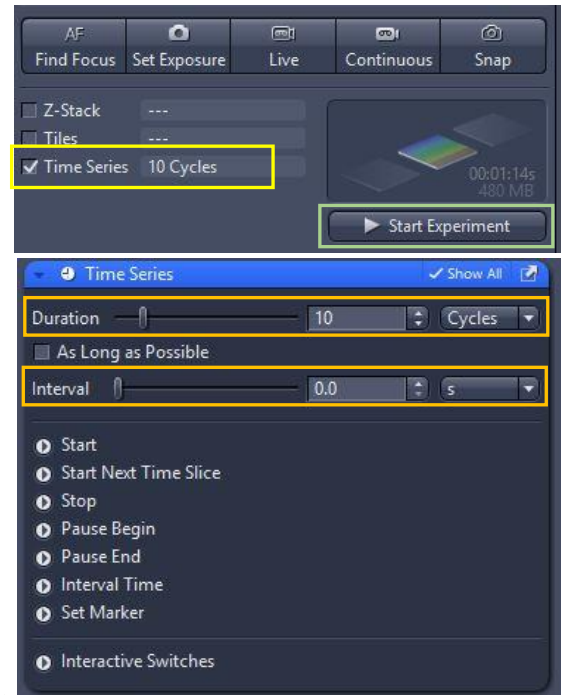
Go to File > Export, choose **movie export** and then choose a suitable **series** Adjust the playing speed by **Frame per Second** and **save** in your own folder.



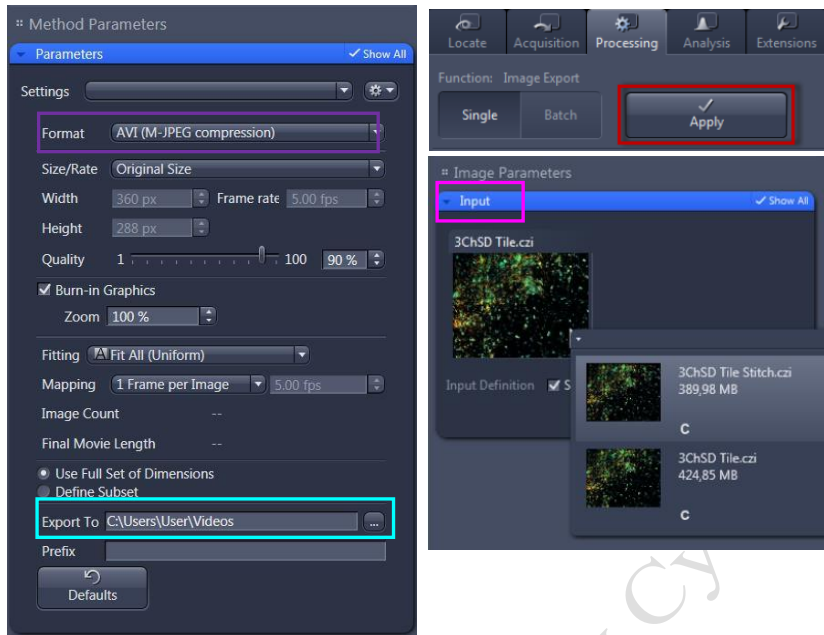


## Time Series Experiment Set Up

1. Check the **Box for Time Series** → open the **Time Series** module.
2. Set the number of **Cycles** and time **Interval** between each frame. (The scanning of each frame is included in the countdown of the Interval, therefore Interval time should be  $\geq$  scanning time of one multi-color frame.)
3. Set the channel and acquisition parameter if necessary and then click **Start Experiment**.
4. To quantifying changes in signal intensity after acquisition, click **Mean ROI** tab and create a **ROI region with the drawing tool** for the region of interest.
5. The **intensity profile** along the experiment duration will be shown in the graph while corresponding **data** will be shown in the table right.

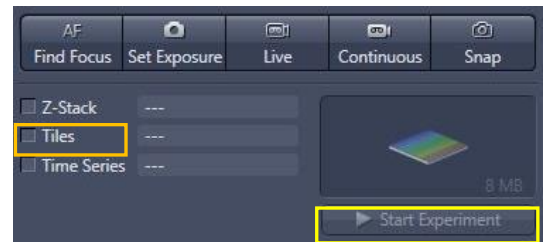


6. After saving the CZI format, video for the time series images can be exported. For export as video → Go to processing (or Go to File > Export) → Movie Export → choose the **Format** as shown below → Choose **export to** → click on **Apply**. Adjust the playing speed by **Frame per Second** and **Select file name and save** in your own folder.

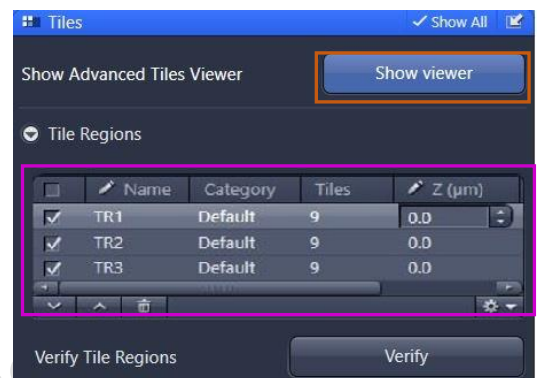


## Tile Scan Experiment Set Up

1. Open the Tiles tool in the Multidimensional Acquisition module and activate the Show all mode.  
 → Check the Box for **Tiles** Scan → open **Tile scan** module by clicking the **Tile Regions**



2. Click “**Show Viewer**” to enable advanced setup.  
 Start **live** mode to locate approximately the center of the region.

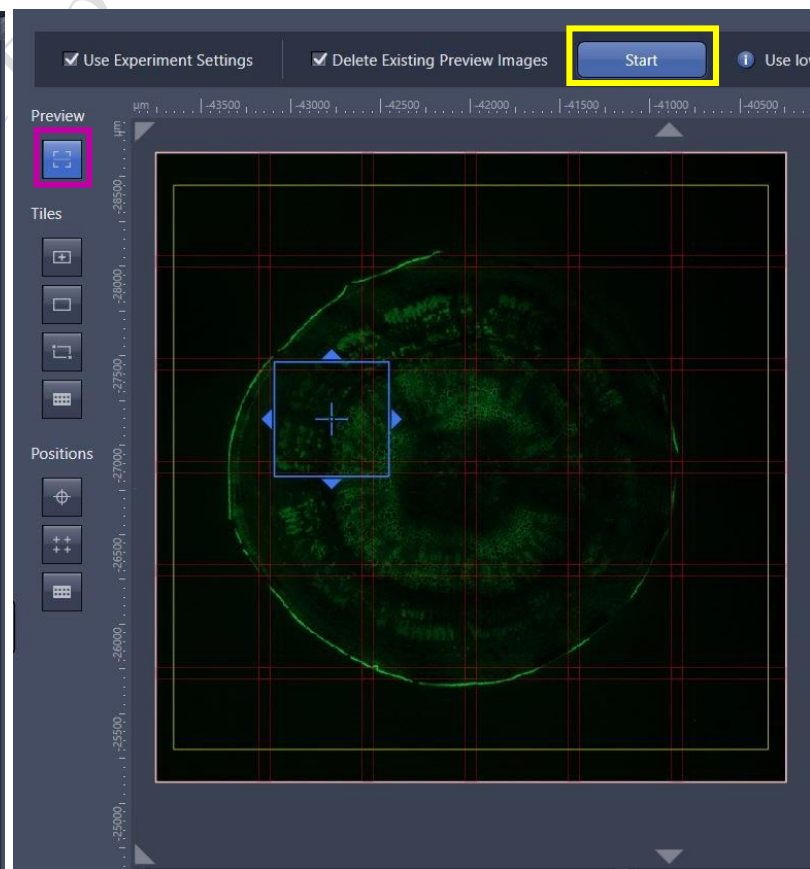
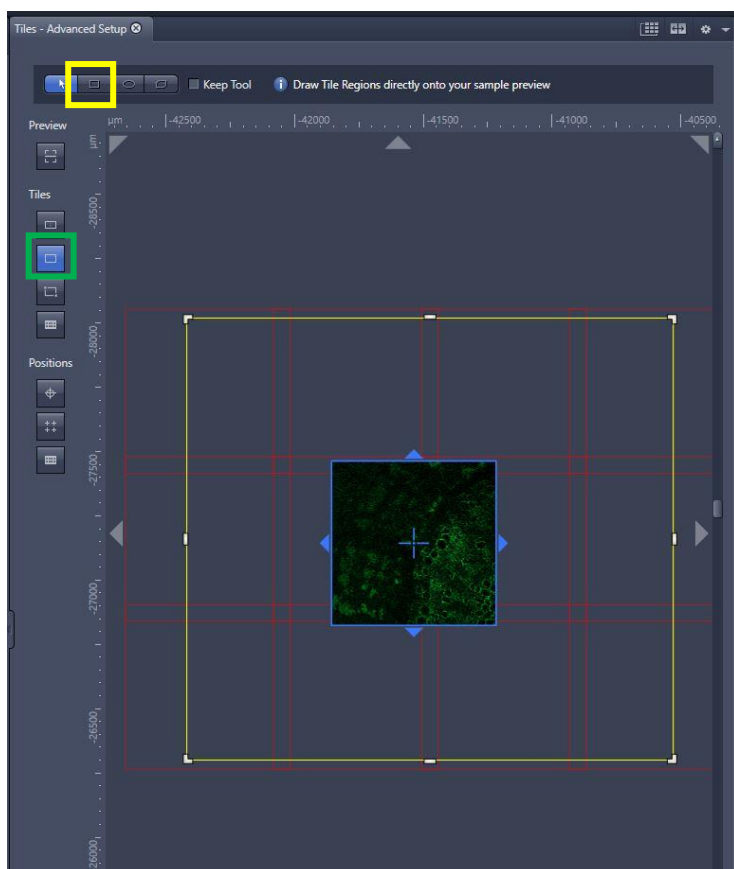


3. Click “**Show Viewer**” to enable advanced setup.

### Method 1: Setup by contour and creating a preview scan

a. Select **contour shape** (rectangular shape for example) under **tiles** and contour the tile region. Software calculates tile number and size.

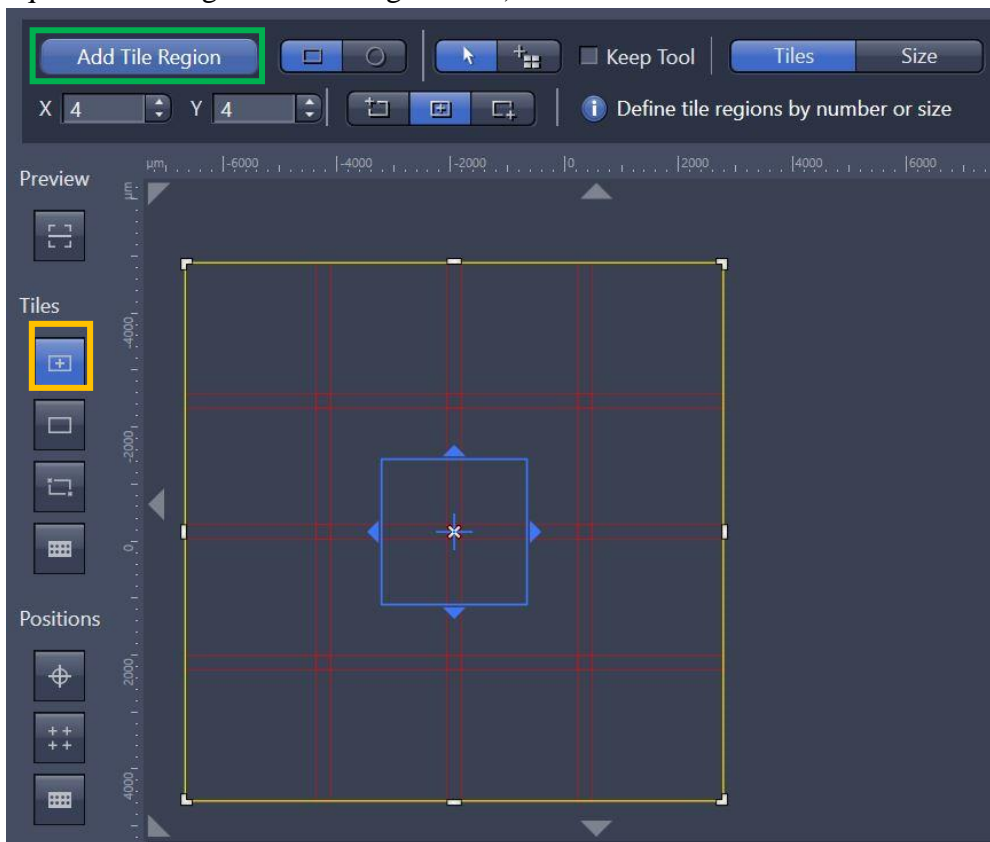
b. Click **preview** and then **start** the preview scan. Check the preview scan if the tile regions include your region of interest completely. Re-contour tile region if you are not satisfied.



**Method 2: creating tile regions by Predefined.**

Click **Predefined button** to set up Tiles and Size.

Tiles: Enter the number of X and Y to define the tile region (Example: X=4 and Y=4 equals to tile region containing 16 tiles)



Size: Define the size of tile region

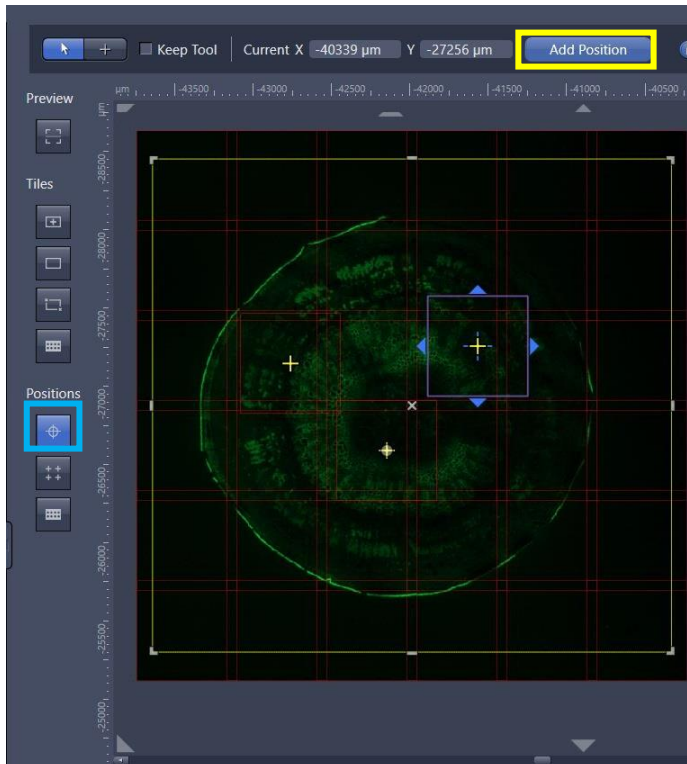


Click **Add Tile Region** to add the respective tile region.

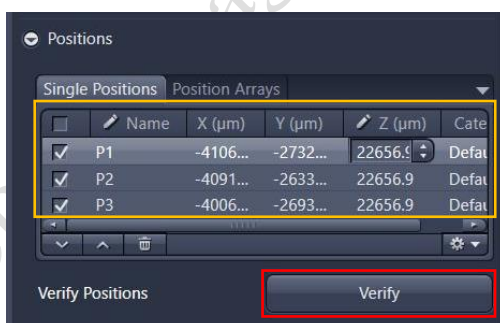
- To add further tile regions, move the stage to another position on the sample and repeat the previous steps. The added tile regions (**TR1, TR2, etc.**) are displayed in the tile regions list.
- Save the experiment. To do this, in the Experiment Manager click on the Options button and select Save As. Enter a name for the experiment in the input field (e.g. SimpleTile Experiment).
- Click on the **Start Experiment button**.

## Multiple Position Experiment Set Up

- 1) Open the **Tiles** tool in the Multidimensional Acquisition module.
- 2) Open the **Positions** section.
- 3) Start the Live mode to use the stage to locate a position that you want to acquire.
- 4) **Adding single positions.**
  - a. Click **“Show Viewer”** to enable advanced setup. click **setup by location button**. Click **add position** then current **position** is added to your experiment.

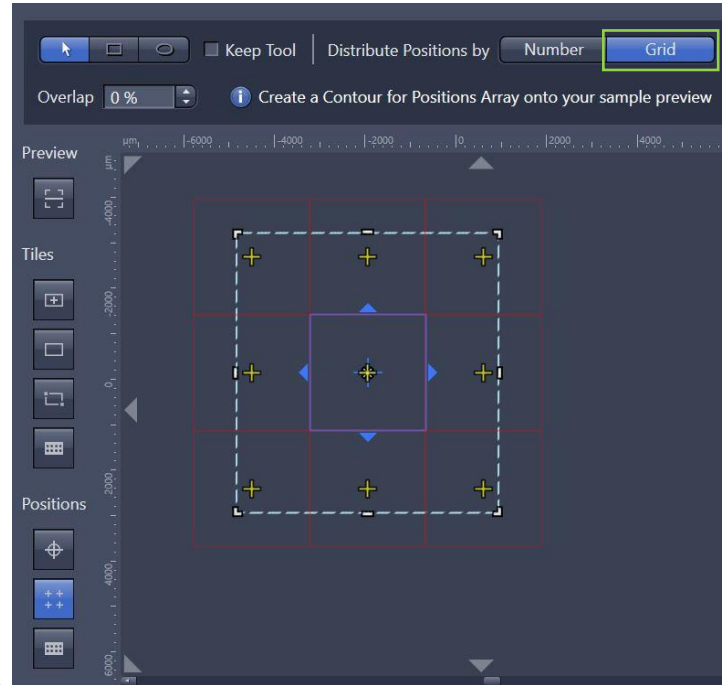
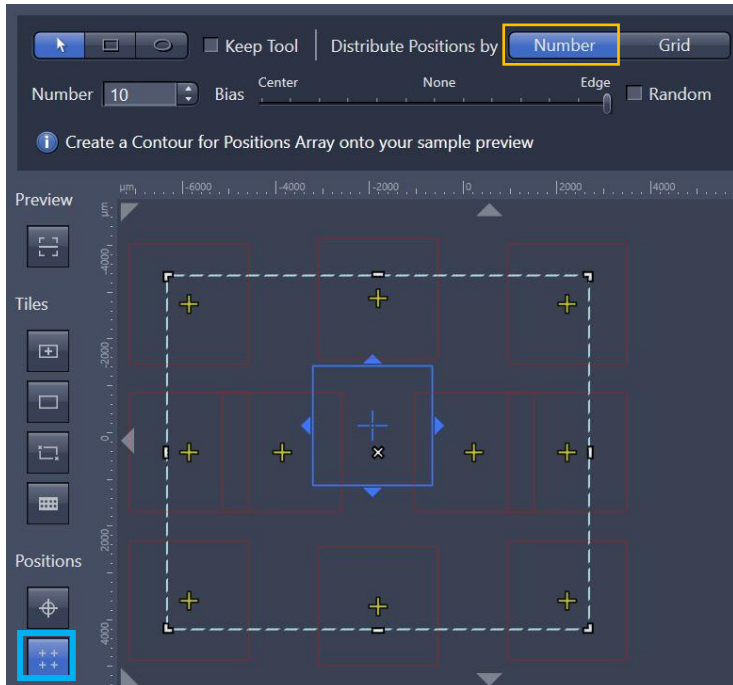


- b. To add further positions, move the stage to another position on the sample and repeat the previous steps. (The added **positions** are shown in the list in the Single Positions section with their X, Y and Z-coordinates.)

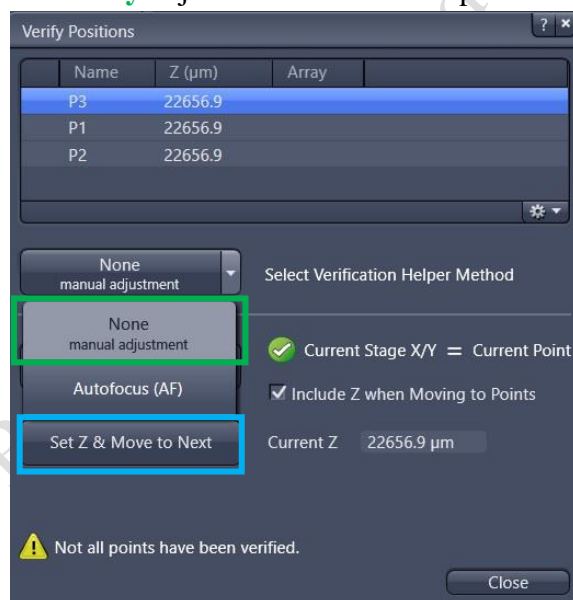


### Position array

- a. Click **position array button**.
- b. Use the contour tools to contour the region to add positions by **number** or **grid**.
- c. Adjust the **Number** of required positions and the **Bias** where the positions should be located.



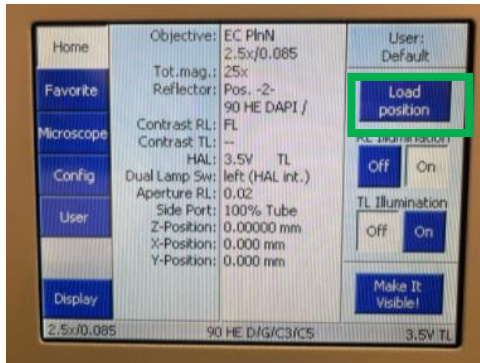
- 5) Verify the defined positions at **live** mode by clicking **verify** button. **Manually** adjust the focus of each position and then click **Set Z & Move to Next**.



- 6) Save the experiment (**if applicable**). To do this, in the Experiment Manager click on the **Options** button and select the Save As entry. Enter a name for the experiment in the input field (e.g. Simple Tile Experiment).
- 7) Click on the **Start Experiment** button.

## Turning off the system

1. Press “load position” button on touch screen. Remove your sample and clean the stage



2. Change objective to lowest magnification (2.5x) objective.
3. Click “Home” button on ZEN software locate mode.
4. Transfer data through the Imaging and Flow Cytometry Core network storage server.
5. Please check the schedule and switch off the system if it will not be occupied within 1 hr.  
(Please leave at least 5 seconds gap in between each step of machine Turn Off process)
6. Shut down PC ⑥ → 5 seconds.
7. Turn the Laser Key (labeled ③) 90° counter clockwise for switch off the laser → wait for 5 seconds.
8. Turn off the components button ④ → wait for 5 seconds
9. Turn off the System button ⑤ → wait for 5 seconds
10. Switch off the Main switch ② mounted on the wall → wait for 5 seconds
11. Switch off the Main switch ① mounted on the wall → wait for 5 seconds
12. Sign on log sheet according to **Actual** finishing time.