

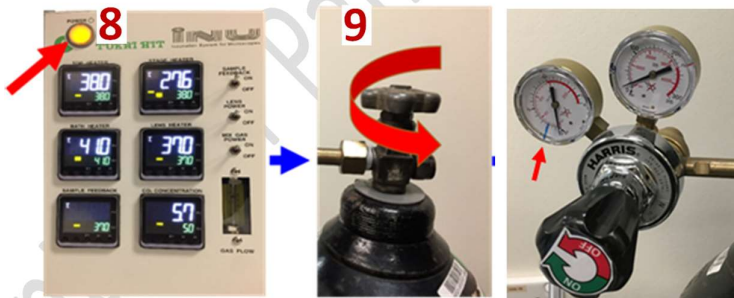
LSM 780 Confocal Microscope Standard Operation Protocol

Turning on the system

1. Sign on log sheet according to the **Actual** start time
2. Check Compressed Air supply for the air table
3. Switch on Main power switch ①
4. Switch on System / PC power button ②
5. Switch on Components power button ③
6. Switch on the power and shutter of HXP fluorescent lamp ④
(Skip this step if 458/488/514nm excitation is not needed)
7. Turn on Standby/On key switch for argon laser ⑤ and Flip the Run-idle-switch to Run for argon laser ⑥
8. Turn On Computer ⑦



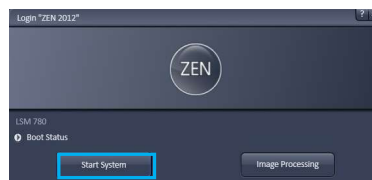
9. (Optional) Live cell incubator procedure as follows:
 - i. Turn ON Tokai HIT controller module ⑧
 - ii. Turn gas tank valve counter-clockwise ⑨Read second regulator gauge to confirm gas supply pressure is at ~17 psi (blue line). Do not adjust the knob if it is at desired pressure.



10. Install Objective heater on the objective to be used

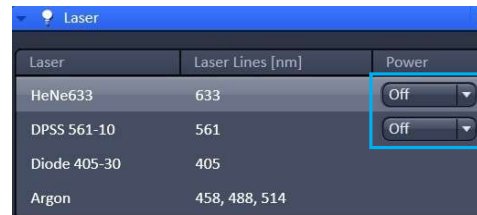
Starting the ZEN software

1. Log on computer with “LSM User”
2. Double click the **ZEN** icon on desktop
3. Choosing **Start System** for acquiring new images



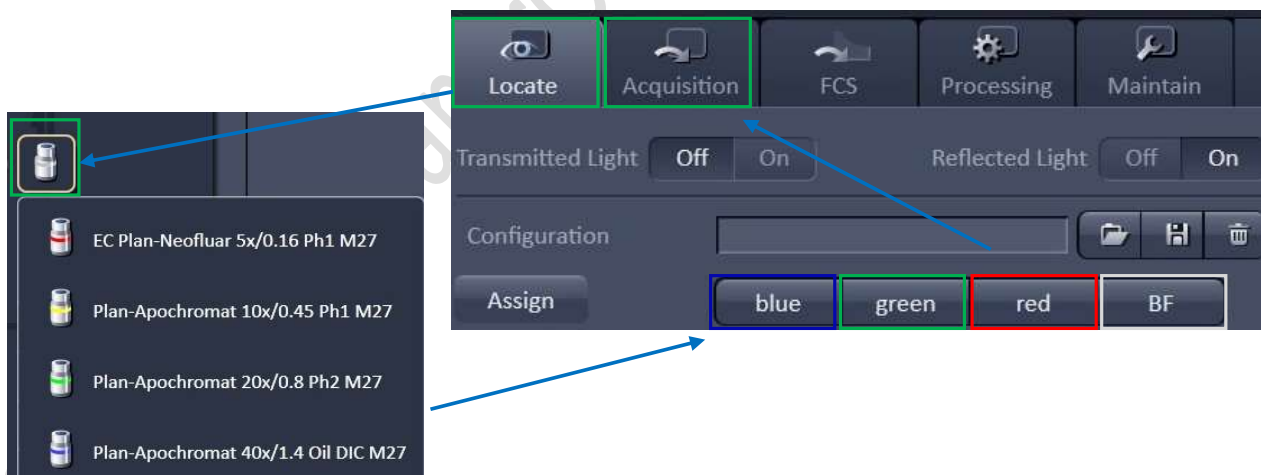
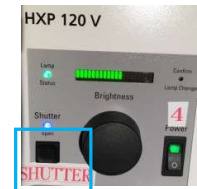
Turning on the 561nm and 633nm lasers (if applicable)

1. Select the laser and click “On” button to switch on lasers.



Setting the microscope and Locating the specimen

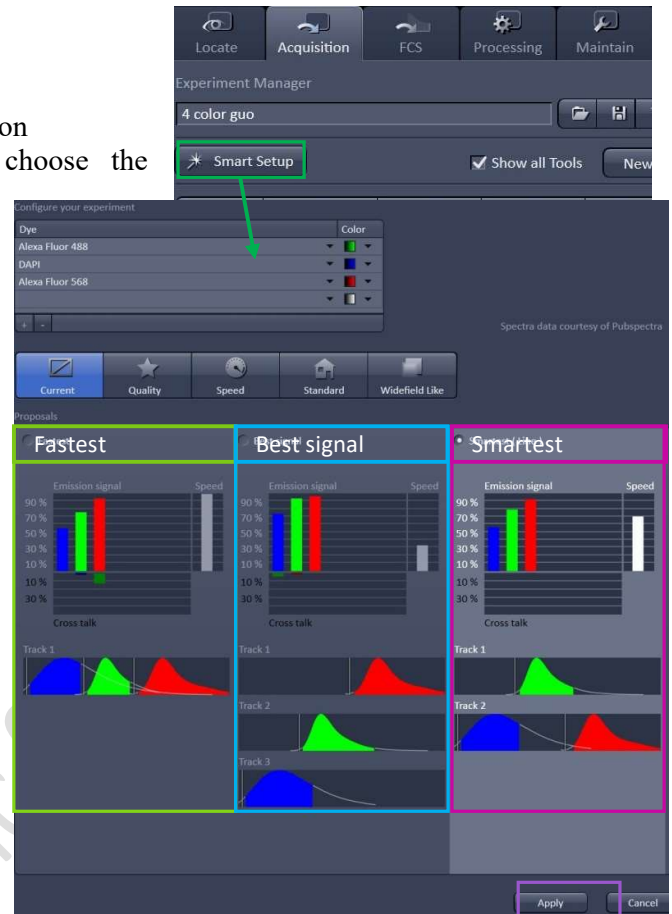
1. Click on **Locate** icon to set the microscope for direct observation via the eyepieces.
2. To select an objective, open the graphical pop-up menu by clicking on the **Objective** button and click on the objective you want to select.
3. Add one drop of oil on objective or your sample if 40X oil objective is in use.
Never put oil on Air objective!!!
Note: Please make sure there is no oil or water at all on your slide when you are using air objective. Any Oil or water will damage air objectives)
4. Fix your sample (slide/dish) on the stage with coverslip facing downwards.
5. Move the area to be observed right above the objective lens.
6. To set the microscope for brightfield observation, click **BF** tab.
7. To set the microscope for fluorescence observation, open the fluorescent lamp (HXP) **Shutter** and click on **Blue/Green/Red** tab for blue emission/green emission/red emission channel observation respectively.
8. Focus the sample with Coarse/fine focus knob.
9. Locate the view of interest through stage control panel and move the desired area to the center of view image acquisition.
10. Click **Acquisition** tab for image scanning.
11. Close the fluorescent lamp (HXP) **Shutter**.



Setting up the Scanning Track/Channel

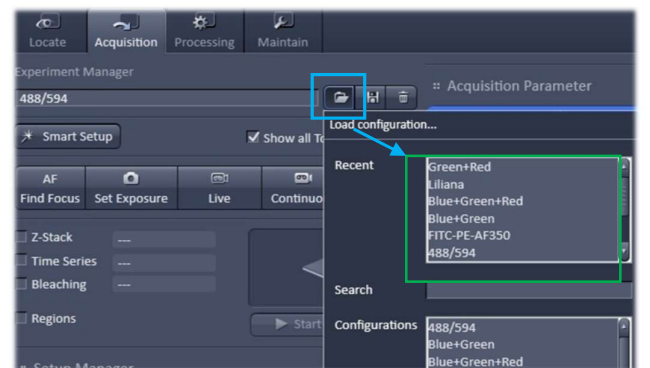
Method 1

1. In Acquisition tab, click on the **Smart Setup** button
2. Click on the **Arrow in the Dye list** and choose the fluorophore in the specimen, add one by one down the rows
3. Choose the Pseudo Color according to you need
4. In Proposal, choose
 - **Fastest** (simultaneous scanning) for fast live image acquisition but with (if any) excitation/emission crosstalk among your fluorophore, or;
 - **Best signal** (sequential scanning) to minimize crosstalk among each fluorophore but slow image acquisition or;
 - **Best Compromise** (combined mode scanning) to simultaneously scan non-cross talking fluorophore to speed up while other cross talking fluorophore are scan sequentially when necessarily
5. Press **Apply**



Method 2

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

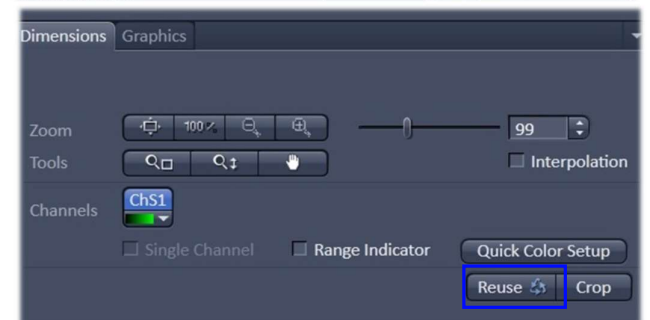


Method 3

Open any previous image with desire setting and click **Reuse** button in Dimension panel.

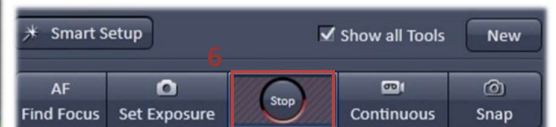
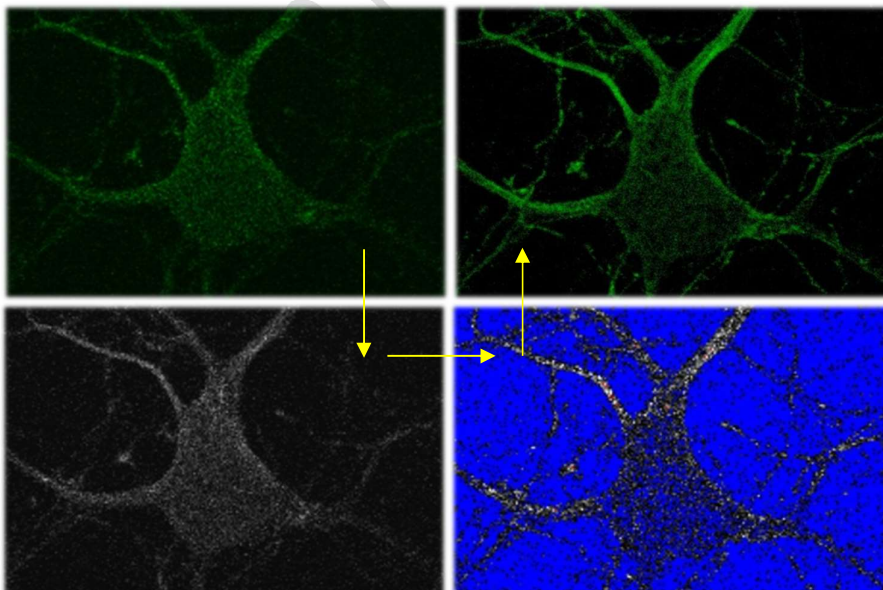
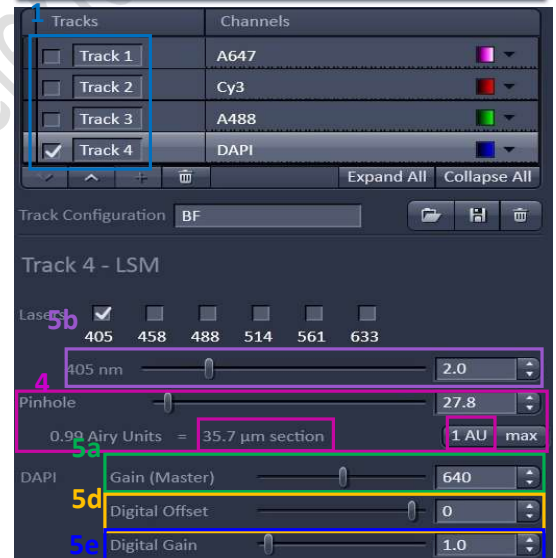
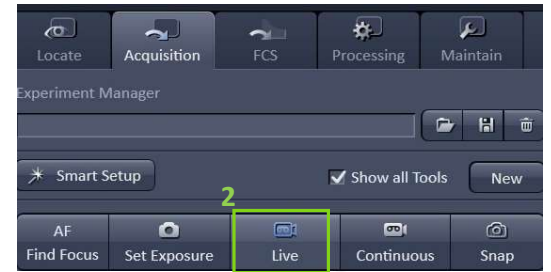
Method 4

Setting up the configuration manually.



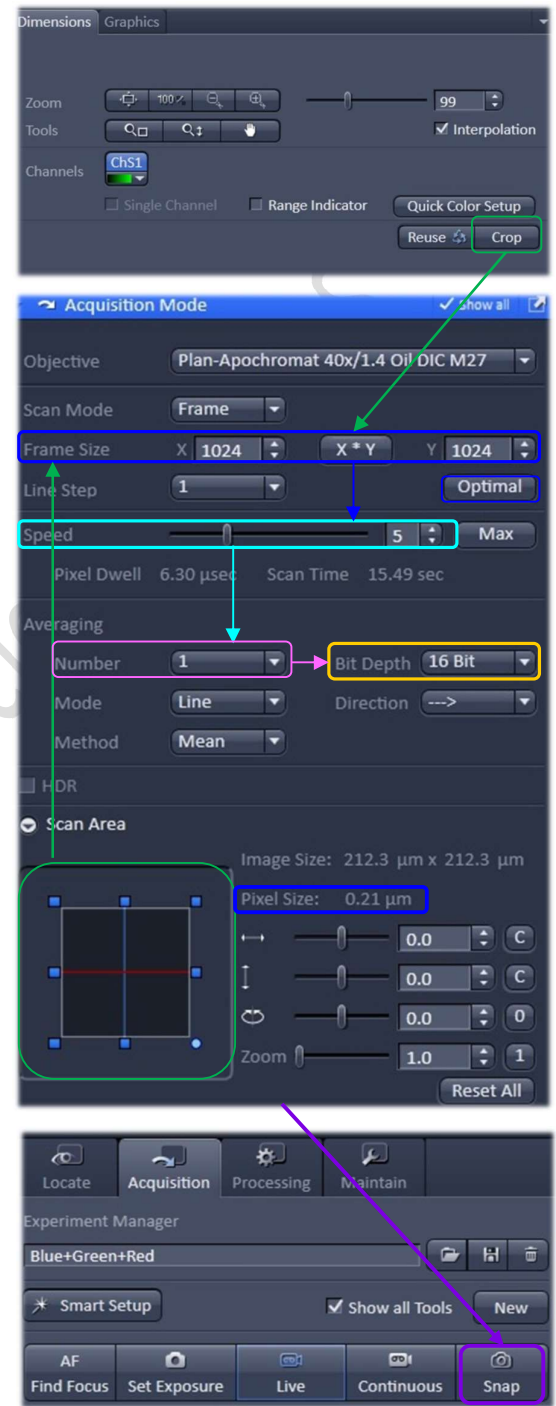
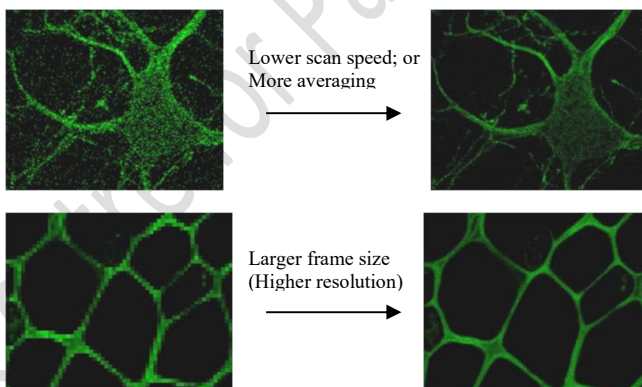
Optimizing scanning parameters

1. Select only **one Track in the Channels** tool for parameter adjustment at one time (untick other tracks if multiple tracks involved).
2. Select **Live** for continuous fast scanning
3. Activate **Range Indicator**. The scanned image appears in a false-color presentation. Red pixel = saturation (maximum intensity). Blue pixel = zero (minimum intensity).
4. Set the **Pinhole to 1 AU (Airy Unit)**. For colocalization studies, adjust the pinhole of each channel to the same **Optical Slice Thickness**.
5. To get optimal intensity and background signal, adjust
 - 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 saturation or 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 necessary.
6. **Stop** the Live scan process and uncheck the Range Indicator.
7. Repeat step 1- 6 for other tracks.
8. Check all the channel boxes when finish adjusting.



Setting the scanning parameters

1. Select the Acquisition Mode tab in the middle column.
2. Select a suitable **Area** and **Zoom Power** to capture image of interest (or use **Crop** function in Dimension under the image container after a Live image).
3. Choose a **Frame Size** (change with **Pixel size**, thus resolution). 1024x1024 usually produces good results for general propose. Click on the **Optimal** button for best resolution which depending on objective N.A. and λ .
4. Adjust the scan speed with the **Speed** slider. 6 or 7 usually produce good results. For samples with high background noise, choose Speed 4 or 5.
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 dynamic range of 8 or 12 or 16 Bit (per pixel) in the **Bit Depth**. 12 or 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 propose.
8. Click **Snap** to acquire a single frame (multi-channel) image.

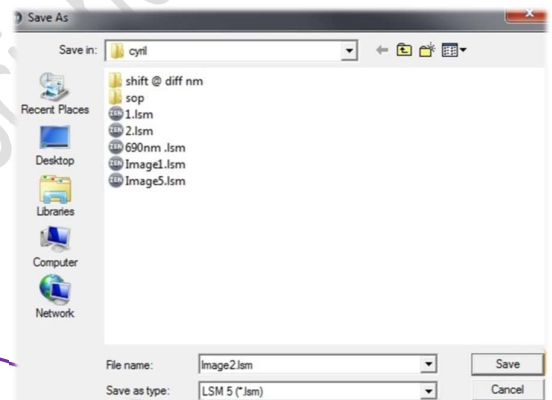
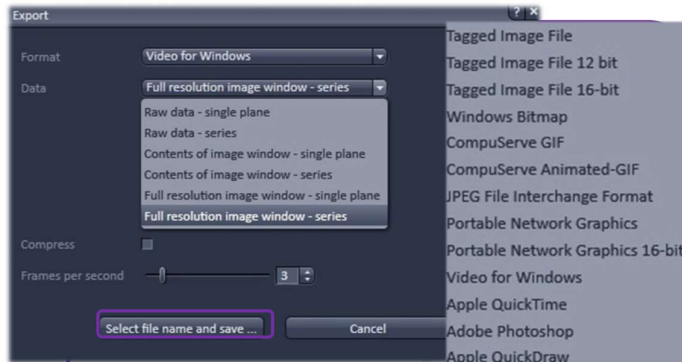


Storing and exporting image data

- To save the an acquired or processed images, click on that image to highlight it and then click on the **Save Data** button in the Images and Documents panel in the right or Click **Save** or **Save As** button in File menu.

Warning Icon indicated unsaved or modified image

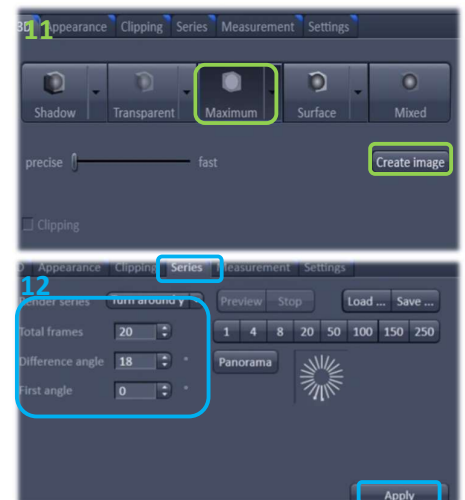
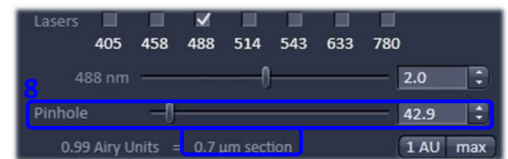
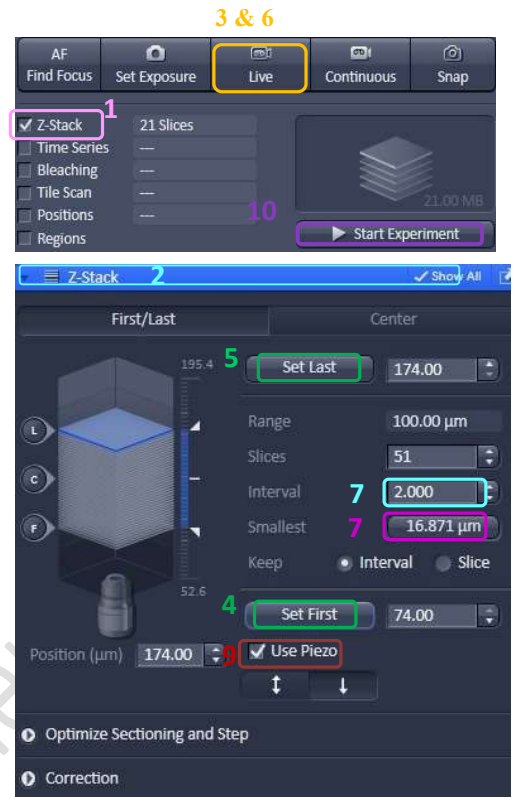
- Create or choose your own folder in D:/user, enter a file name, select format as **LSM 5** and click on the **Save** button
- After saving the raw data as LSM5 format, Image can be export as various Image format and series images can be export as video. Go to File > Export, and choose the **Format and Data** as followed. Adjust Frames per seconds if necessary. Click **Select file name and save** and save in your own folder.



Single Frame	Format	Tagged Image File JPEG File Interchange Format Portable Network Graphics
	Data	Raw data – single plane (without any overlay graphics) Contents of image window – single plane (for analyzed plot of histogram or as displayed as in image container; compressed) Full resolution image window – single plane (with overlay; as displayed as in image container; uncompressed) Full resolution image window – Series plane (for exporting gallery of images to separated graphic files)
Series (Gallery of images; Time series movie or 3D animation)	Format	Video of Window Apple Quick Time
	Data	Raw data – series (without any overlay graphics) Full resolution image window – series (with overlay; as displayed as in image container; uncompressed)

Scanning a Z-Stack

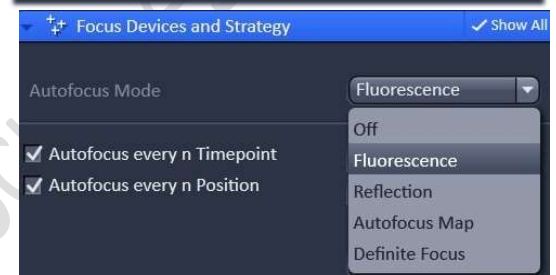
1. Check the **Box for Z-Stack** in the main tools area.
2. Open the Z-Stack tool panel by clicking on the **Z-stack tab**.
3. Choose a channel that have signal throughout the interested volume and then Click on **Live**.
4. Use the focus knob to locate one end of the specimen and click on the **Set First** button.
5. Then use the focus to locate another end of the specimen and click on the **Set Last** button
6. **Stop the Live**.
7. Set the Z-stack **Interval** between each frame equal to or less than the thickness of the thinnest **Optical Section** of the selected channel wavelength (so that you can scan all the space in your specimen). **Optical Section** (see pinhole in P.4) of the channel is calculated according to the emission wavelength, objective lens, and the pinhole diameter.
Smallest mean double sampling with interval equal to half of the thinnest optical section and thus provide better 3D image reconstruction.
8. For colocalization studies, adjust the **Pinhole** of each channel in the Channels panel to the same **Optical Slice thickness** (so that each voxel contain data from the same volume of the specimen).
9. Tick the **Use Piezo** checkbox to perform quicker z focus move if necessary.
10. Click on the **Start Experiment** button to start the recording of the Z-Stack.
11. After saving the LSM 5 format, a **Maximum Projection** images can be made. Image in all frames will be projected onto a single plane to visualize all the signal for the specimen in 2D. Click **Create image** to generate the projection.
12. A video of rotating 3D image can also be made. Activate the **Series** tap, select the **rotating axis**, 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 **video for Windows** and then choose a suitable **series**. Adjust the playing speed by **Frame per Second** and **save** in your own folder.



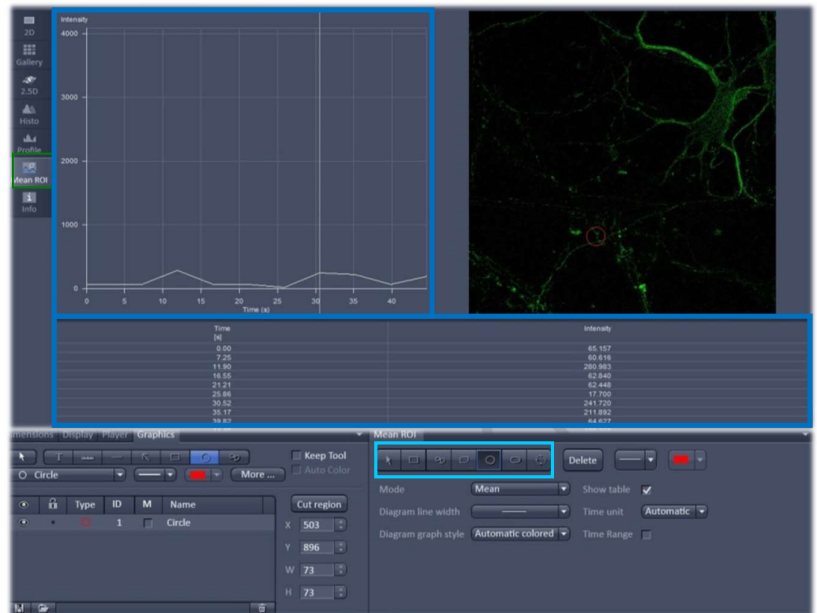
Setting up Time Series Experiment

Check the **Box for Time Series** in the main tools area.

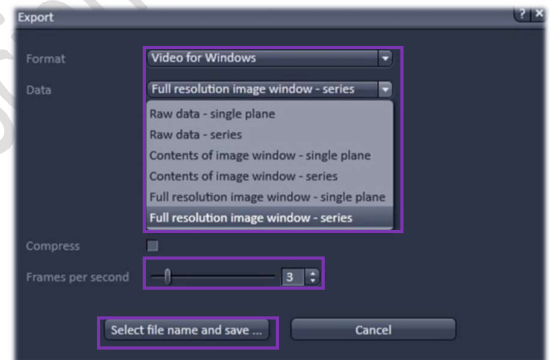
1. Open the **Time Series tool** panel by clicking on the **Time Series tab**.
2. Set the number of **Cycle** and time **Interval** between each frame. (The scanning of each frame is included in the countdown of the Interval, therefore Interval time should \geq scanning time of one multi color frame.)
3. Open the **Focus Devices and Strategy** to maintain focus stability
4. Select the focus method in **Autofocus Mode**.
 - **Fluorescence** mode: Identification of the focus reference plane based on the intensity maximum of the channel. (The sample will be scanned for several times to select the best contrast of image. Bleaching may happen due to the multiple scanning. Applicable to fixed sample and live cell with oil objective)
 - **Reflection** mode: uses the longest wavelength for focusing. 633nm wavelength could be used for minimize the bleaching. The bottom surface of the glass will be reflected and the offset between the Z-level of image recording and the Z-level of the reference plane will be calculated. Applicable for multi-position live cell imaging. Not applicable to fixed sample and live cell with oil objective.
Step 1: Focus your sample on the desired imaging plane.
Step 2: Press “**Find Offset**”
Step 3: The software copies the offset into the “**Offset**” spin box of “**Position**” Tool.
 - **Autofocus Map** mode: Only applicable for multi-well plate. One offset will be applied to all wells of one plate.
 - **Definite Focus** mode: The Z level of Petri dish-medium interface will be maintained by reflection by a 835nm LED light. Only applicable to live cell imaging with medium in petridish.
Step 1: Press the **Home>Microscope>XYZ>Definite Focus>On** to activate Definite Focus.
Step 2: Select **Definite Focus** mode in ZEN software.
5. Set the channel and acquisition parameter if necessary and then click **Start Experiment**.



- To quantifying changes in signal intensity after acquisition, click **Mean ROI** tab and **create a ROI region with the drawing tool** for you region of interest.
- The **intensity profile** along the experiment duration will be shown in the graph while corresponding **data** will be shown in the table below.



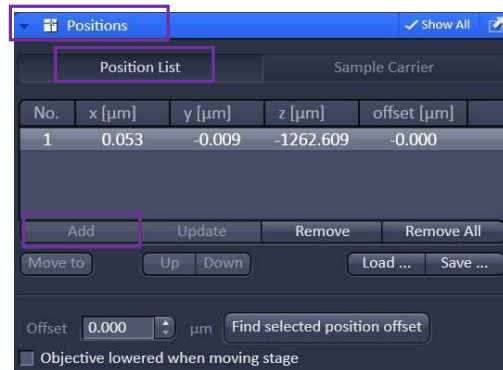
- After saving the LSM 5 format, video for the time series images can be exported. Go to File > **Export**, choose video for Windows and then a suitable **series**. Adjust the playing speed by **Frame per Second** and **Select file name and save** in your own folder.



Setting up Multiple Position Experiment

Check the **Box for position** in the main tools area

- Open the **Position tool** panel by clicking on the **position tab**.
- Press the **Position List** button and click the **Add** button to add the current position to the list



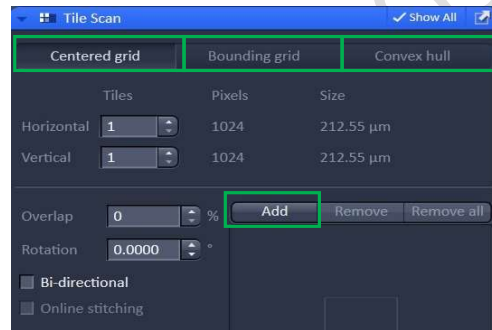
Setting up Tile Scan

Check the **Box for Tile Scan** in the main tools area

Centered grid: Move the stage to the center position of the area to be scanned and set the number of tiles for horizontal and vertical directions. (Rectangle scanning area) It is recommended to set the overlap $\geq 10\%$ for stitching process after acquisition.

Bounding grid: Move the stage to the upper left and lower right corner of the whole scanning area and click “Add” to set the boundary of the scanning. (Rectangle scanning area)

Convex hull: Move the stage to the edge of the whole scanning area and click “Add” to set the boundary of the scanning. The scanning edge is set with irregular shape. Applicable to samples with irregular shape.



Turning off the system

Please check if the equipment will be used / booked by other users. Please switch off the system if no one is using within two sessions (1 hr) afterward.

1. Turn off 561nm and 633nm lasers in software (if they are ON)
2. Flip the toggle of Run-idle-switch of argon laser to Idle ⑥
3. Turn the key switch 90° anti-clockwise for argon laser to Standby ⑤

Wait for 10 min to let *Laser Fan OFF!!!*

While waiting, please

- Remove your sample and clean the stage
 - Clean oil objective (with lens cleaning tissue only but NOT Kimwipe)
 1. Remove residue oil from the objective lens with lens cleaning tissue
 2. Repeat this step with a new area/piece of the lens cleaning tissue until no oil streaks are seen on the tissue
 3. Dry the lens with another new lens cleaning tissue
 - Change objective to 5x;
 - Sign on log sheet according to **Actual** finish time
4. Exiting ZEN Software, transfer data through the Faculty Core Facility network storage server and then Shut down PC ⑦
 5. Turn off HXP fluorescent lamp ④
 6. Components button ③
 7. System / PC button ②
 8. Main power switch **(Only after Argon Laser Fan OFF)** ①
 9. Remove Objective heater from the objective (if applicable)
 10. Turn off temperature and CO₂ control and “CO₂ regulators” to cut down CO₂ supply (if applicable).