## LSM980 with Airyscan 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. Turn the Main power switch ① 90° clockwise for switch on → wait for 5 seconds
2. Switch on Components power button ② → **wait for 2 mins**
3. Turn the key switch ③ 90° clockwise for laser switch on → wait for 5 seconds
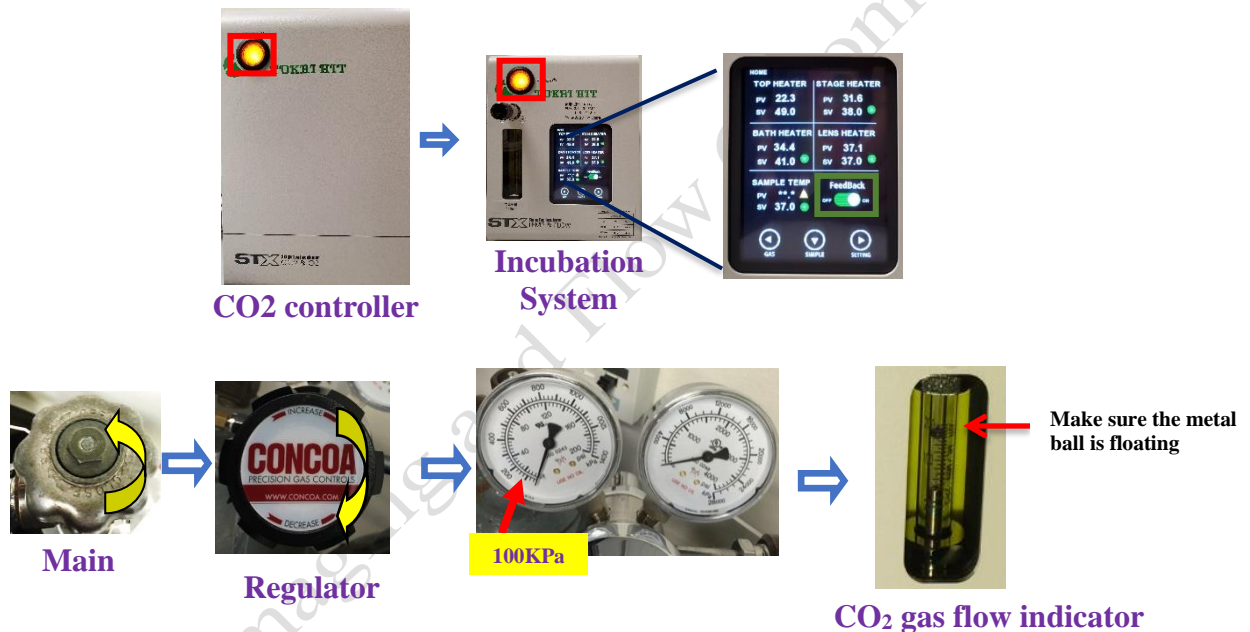4. Turn on the computer power ④
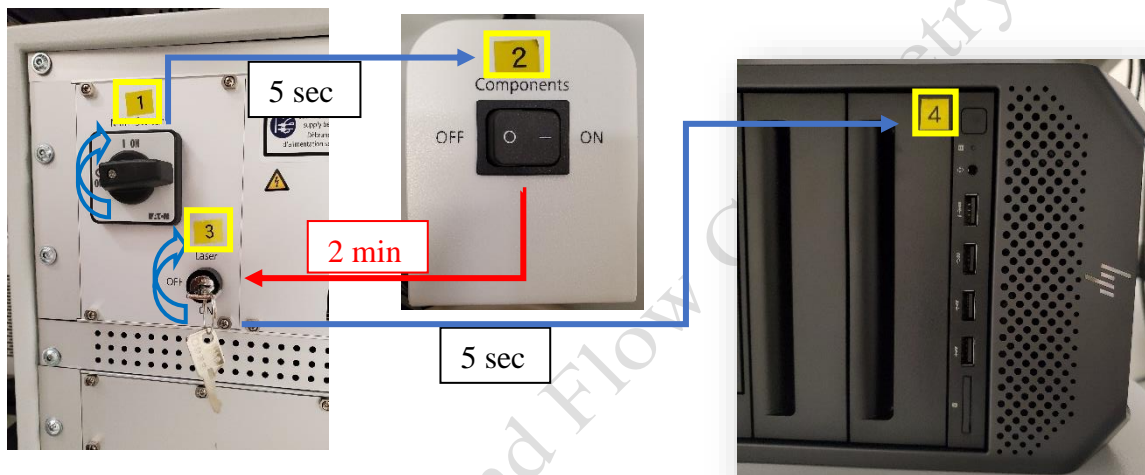
ZEN Software Initialization

1. Log on computer with "**User**" account
   (Please find User account password posted under the bottom of computer screen)
2. Double click the **ZEN 3.1 blue** icon on desktop
3. Click **ZEN system** to run the software

Only applicable for live cell imaging, please skip this step if it is not needed

Set the temperature and CO_2 control for live cell imaging

- Switch on the **Power** of CO2 controller and Tokai Hit incubation system controller.
- Temperature can be altered via pressing the green button of each heating parts on the touch screen (Temperature has been set up for live cell imaging. If other temperature is needed, please contact Imaging core staff for temperature alteration)
- Make sure the CO_2 sliding button is turned **ON**.
- Turn on CO_2 tank by turning the main switch **anticlockwise**.
- Turn on CO_2 regulator by turning regulator **clockwise** to set output pressure at 100kPa.
- Put on objective heater on objective if oil objective is used.
- Metal ball floats is an indication of the presence of CO_2 gas flow.
- Add MilliQ water into the water chamber and covered if overnight(s) acquisition is required.

CO2 controller   **Incubation System**

Main   **Regulator**   **100KPa**   **CO₂ gas flow indicator**

Make sure the metal ball is floating

**Locating Sample and Finding a Region of Interest**

1. Select a **desired objective** from the touch screen. Apply a drop of oil onto the tip of the lens if 40x oil objective is used.
    Never put oil on Air objective!!!(2.5x, 5x, 10x, 20x, 40xoil, 40x)
    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 cause damage to the air objectives)

2. Place the sample (slide/dish) on the stage with coverslip facing down.
3. Move the region of interest to right above 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).
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 **Green**/**Red**/**Blue**/**G+R+B**/**BF** tab for green emission /red emission/blue emission /green+red+blue emission /Bright Field 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**".
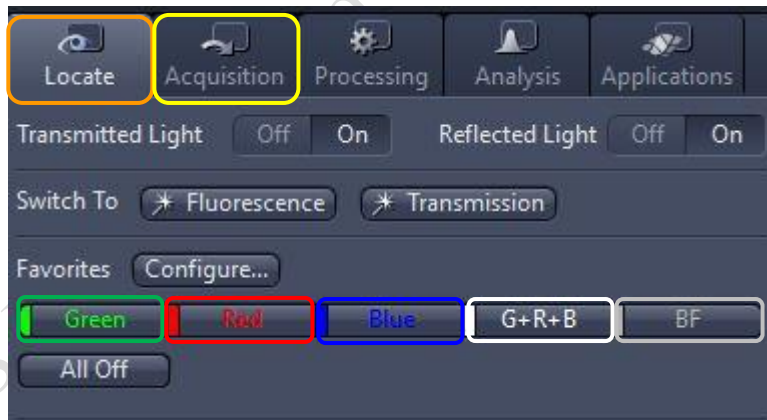
Setting up the Scanning Track/Channel

Method 1

Load a **predefined configuration** of combination dyes from the **Load configuration list**. Select "0IFC_Confocal" if confocal mode is required.

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.

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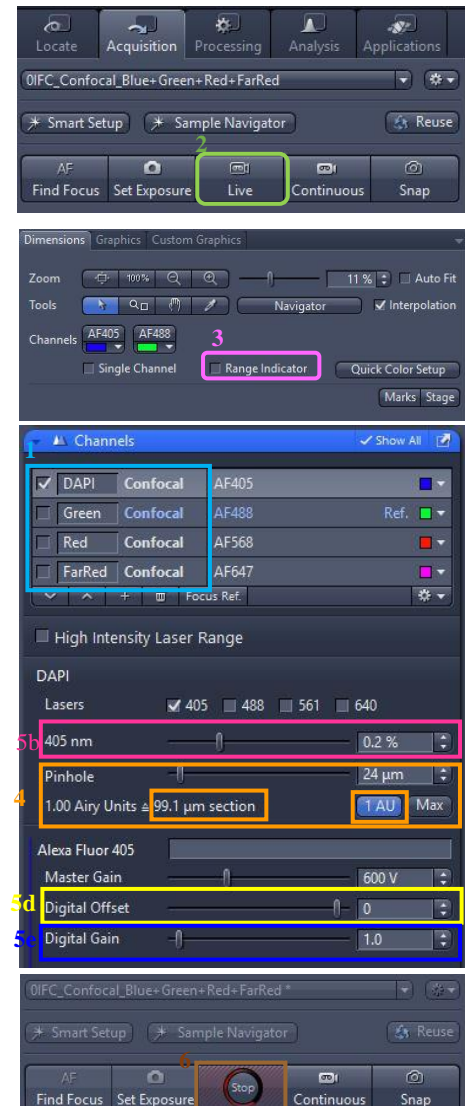
Scanning Parameters Set Up

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 λ.

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.

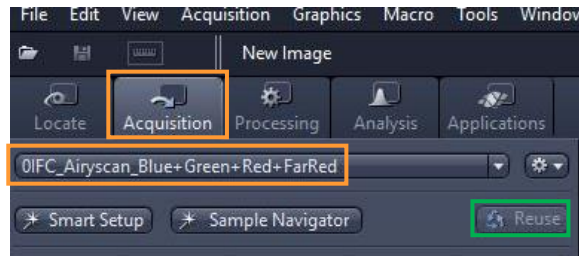8. Click **Snap** to acquire a single frame (multi-channel) image.
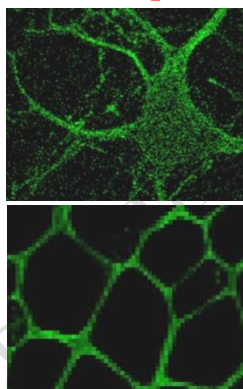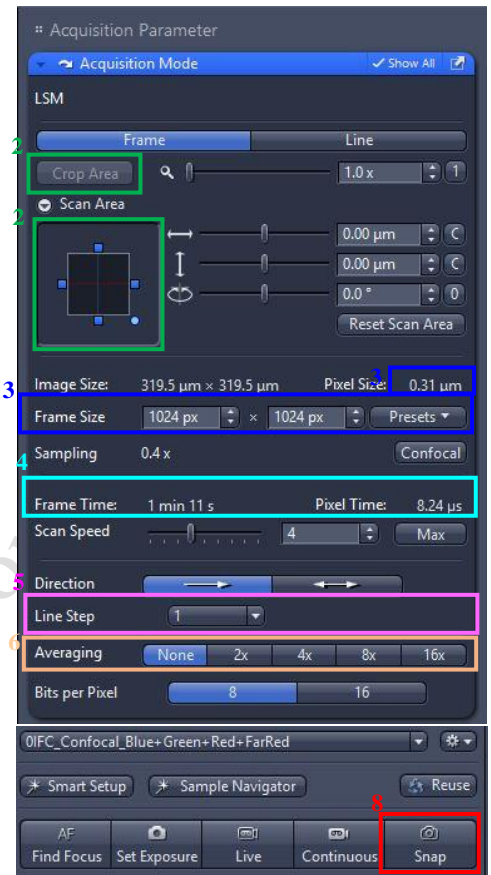
Lower scan speed; or More averaging
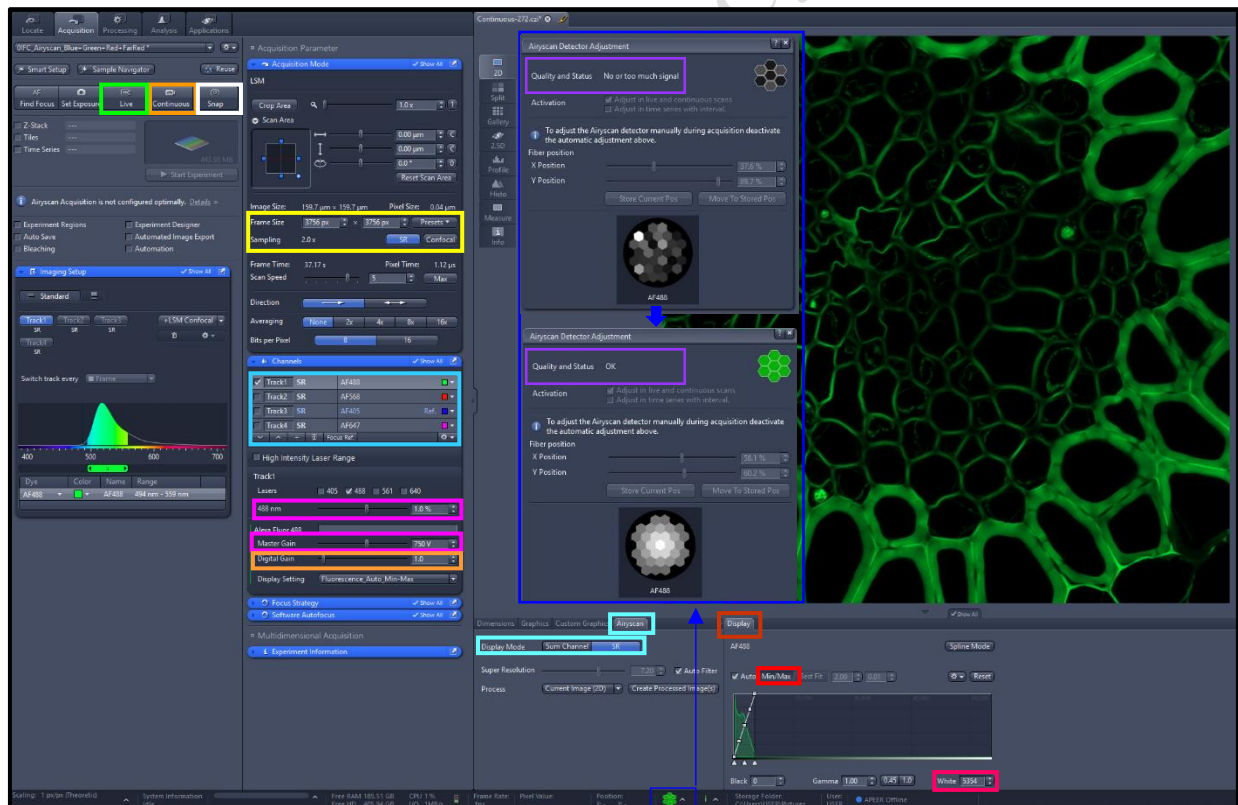
Larger frame size (Higher resolution)
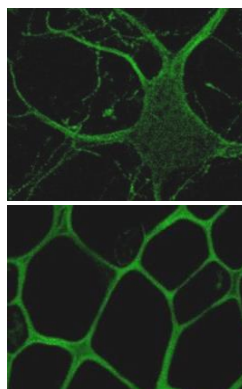
LSM-Airyscan mode Imaging Set Up

1. Once a region of interest is found under the eyepiece →click **Acquisition** → Select a predefined AiryScan configuration (e.g. **0IFC_Ariyscan_Blue Greed Red FarRed**) of combination from the list. Alternatively, click "**Reuse**" the previous settings by loading an old image file.
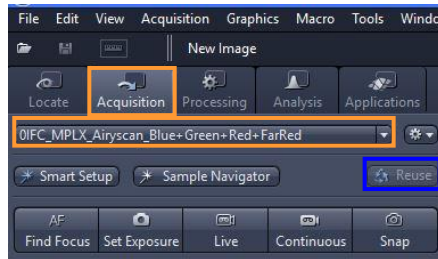

2. Select one channel of interest at a time→Click Live→Click on **Airyscan tab** under Maintain panel→Adjust focus to the best focal plane (**brightest hexagon in the middle**) →Adjust the **laser power and Master Gain**→Check **Quality and Status** in the **Airyscan tab** from **No or too much signal** to **OK**→Click on **Continuous**→Click Airyscan→ Choose SR in the Display Mode →Click display→Click **Min/Max**→**White range** between 3000 to 8000

3. Repeat step 2 for other channels → Choose a **Frame Size** or click SR→Click **Snap**.


a. **SR mode:** increased resolution, the Pinhole has no effect and the default position should not be changed.

b. **Cofocal mode:** set the system for conventional confocal mode, the hardware pinhole is effective and can be set at the required value for confocal sectioning.

MPLX-Airyscan mode Imaging Set Up

1. Once a region of interest is found under the eyepiece →Click **Acquisition** → Select a predefined MPLX-AiryScan configuration (e.g. **0IFC_MPLX_Blue Greed Red FarRed**) of combination from the list.


2. Select one channel of interest at a time→Click Live→Click on **Airyscan tab** under Maintain panel→Adjust focus to the best focal plane (**brightest hexagon in the middle**) →Adjust the **laser power and Master Gain**→Check **Quality and Status** in the **Airyscan tab** from **No or too much signal** to **OK**→Click on **Continuous**→Click Airyscan→ Choose SR in the Display Mode →Click display→Click **Min/Max**→**White range** between 3000 to 8000→ Repeat same approach for other channels→Click on **SY-4Y/SR-8Y/CO-8Y**→Snap
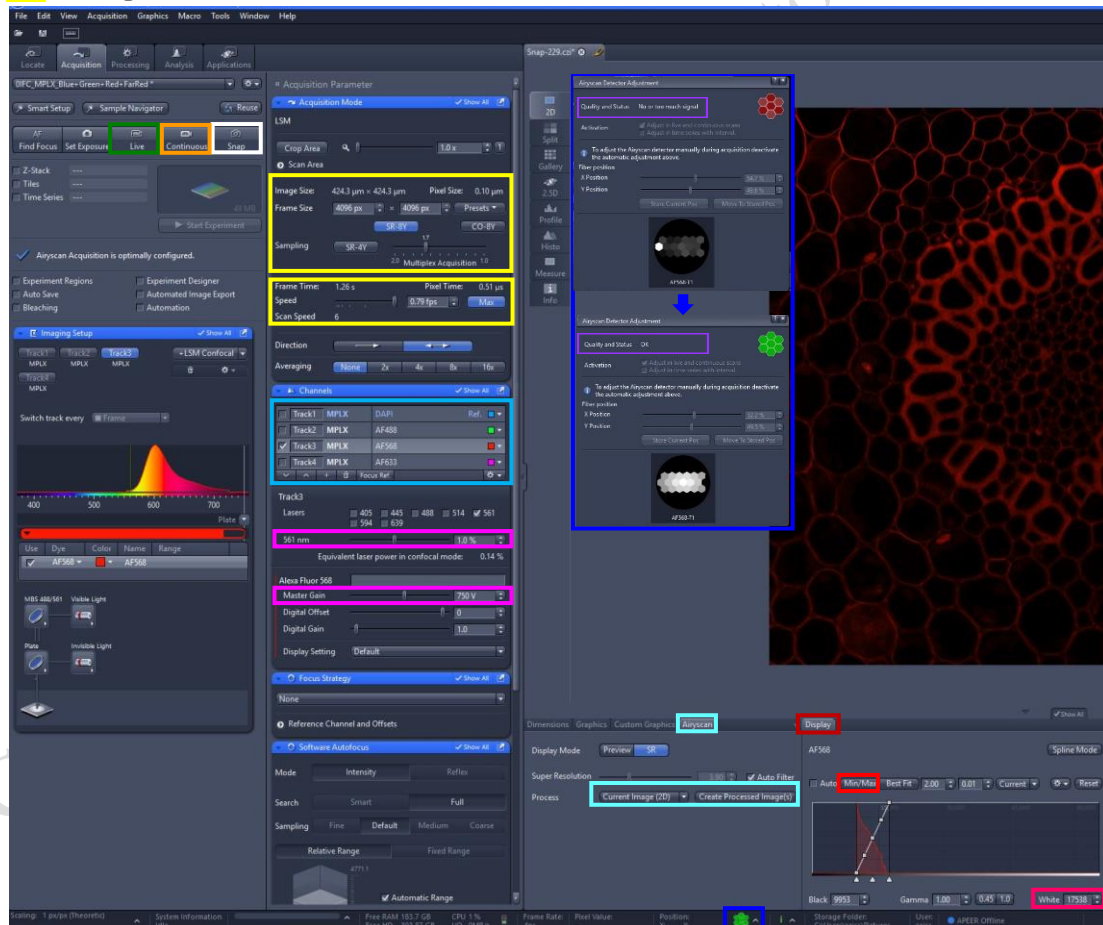

> **SR-8Y** sets the sampling for superresolution imaging with Fast at 2,0x Nyquist sampling. (8xfaster than conventional Airyscan SR).
> **SR-4Y** sets the sampling for superresolution imaging with Fast at 2,0x . (4x faster than conventional Airyscan SR).
> **CO-8Y** sets the sampling for flexible confocal resolution imaging with Fast at 1.0x Nyquist sampling. (with increased signal-to-noise with Airyscan detector.)

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
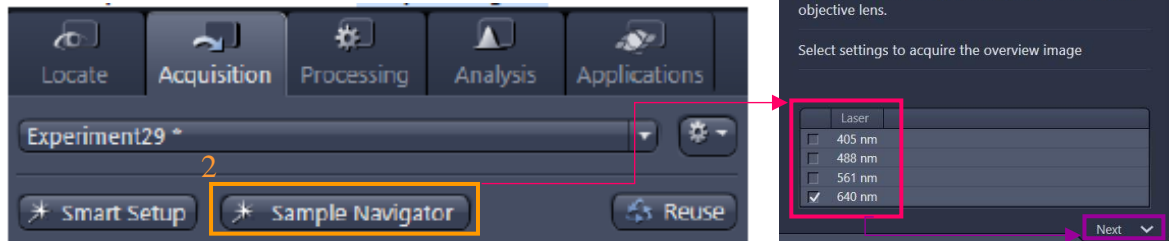

3. Click on Use current focus if sample was already focused, or click on New sample/objective if a new sample was put on the microscope. With a new sample, you are asked to focus the objective a bit closer than the putative focus plane. This is because the autofocus will move away from the sample when searching for the focus plane. Click on Objective is now close to the sample→Click on Next, go to step 2.
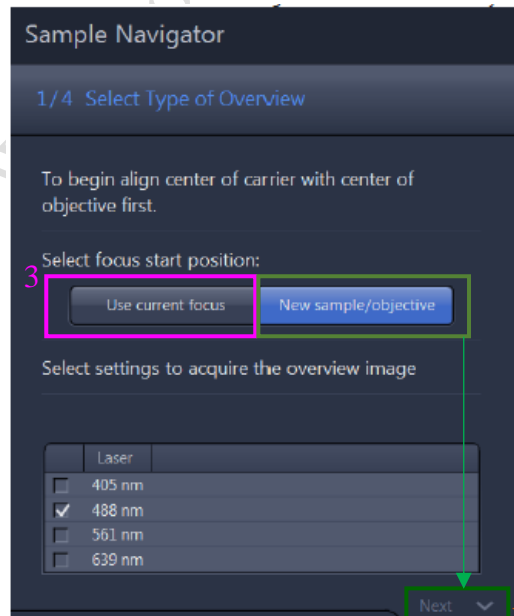
4. Select a matching sample carrier type from the selection list in the middle→select the coverage degree Small, Middle or Full (Full means full coverage of the Cover slip area, middle means 1/4 coverage of the Cover slip area, Small means 1/16 coverage of the Cover slip area). The selected coverage is displayed by a red area on the sample carrier drawing→ Click on Next at the bottom of the wizard, go to step 3.
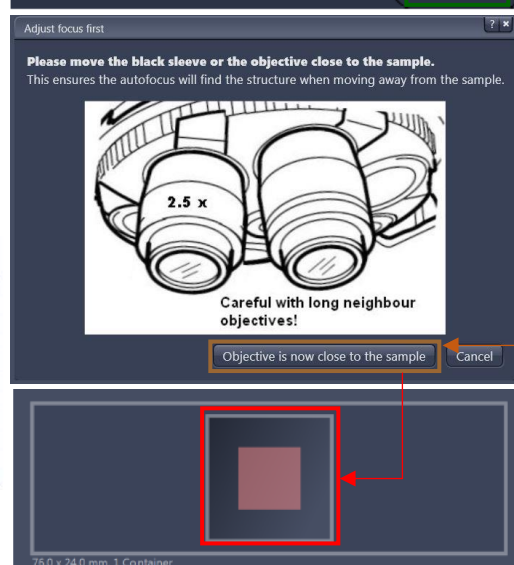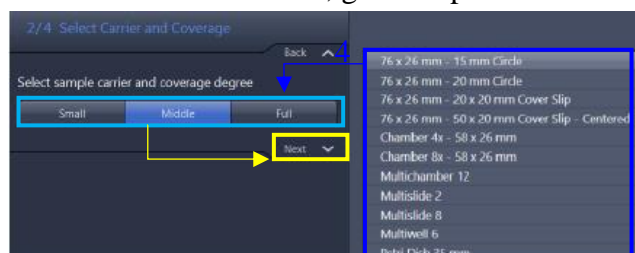
5. Ensure that the steps listed in the center area are performed: 1. Align the lines on the right side of the condenser carrier. 2. Open apertures. 3. Use the black cloth to protect the sample space from room light. → Click on Next at the bottom of the wizard, go to step 4.
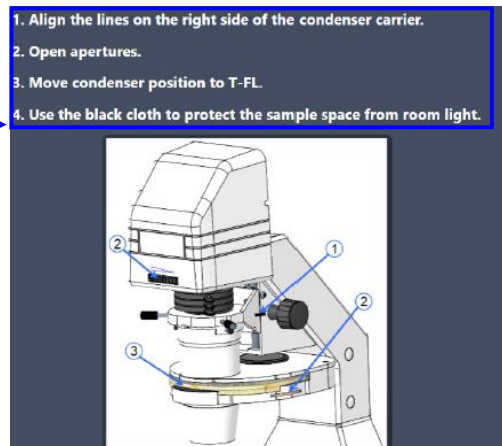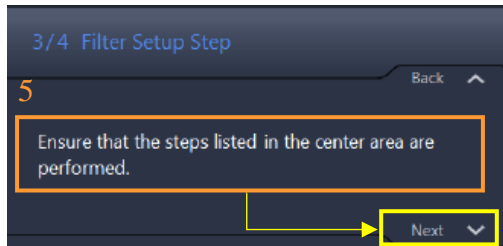
6. Click on Focus Detection to start the autofocus, and observe the image in the middle→ adjust the light intensity with the slider Light Source Intensity if needed→ manually focus the sample with the focus drives if needed→ Click on Finish to start the automatic overview tile scan.
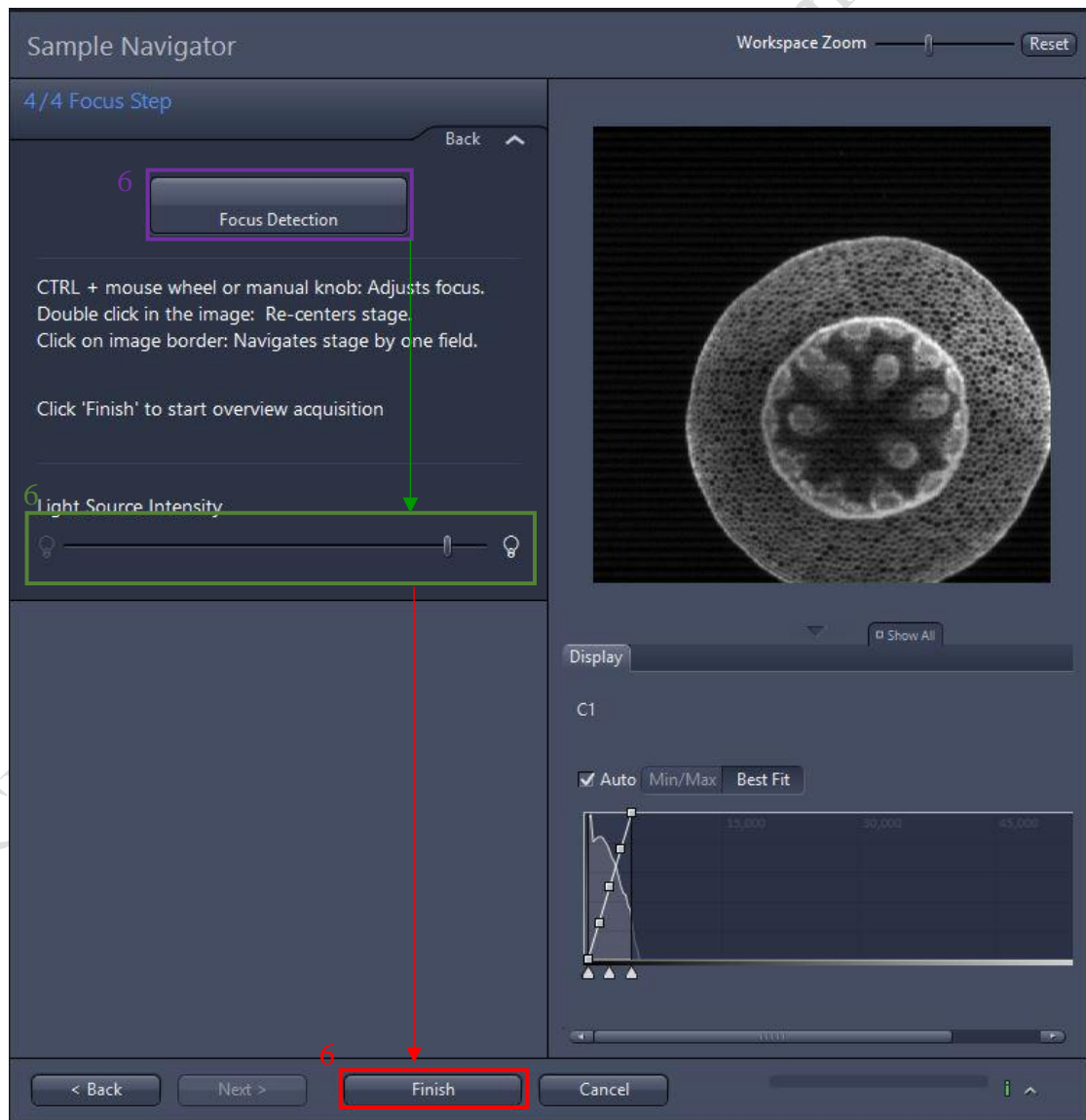
Experimental Designer

1. Users can use this application if their study involves different multi-dimensional acquisition parameters. Each parameter setting can be assigned to one imaging block.

    Tile Scan; Positions; Regions; Bleaching

2. Tick experiment designer→After setting up the acquisition parameters for one block→ click "**Create Acquisition block**" to create another block→ then change the acquisition parameters accordingly in the second block.

3. You may use the "Loops and Repetitions" function if many rounds of the same sequence of experiment blocks are required.

    Expend the "**Loops and Repetitions**"→ key in the **required details**→ check "**Active**" → click "**Start Experiment**".

    - **Acquisition Block:** image acquisition performed according
      to the acquisition parameter settings.

    - **Delay Block:** sets a time delay between two blocks. The delay time is set using the Delay slider which shows up when selecting a Delay Block (time unit ranging from milliseconds to days).

    - **Wait Block:** pauses the acquisition until the user actively confirms the message box.

    - **Loops**: states how often a sequence of Blocks defined by the number of the Block in Start and End is run. One loop/repetition is set as default and reflects the minimum of running the experiment once.

    - **Two loops**: perform the defined sequence twice. Repeat one Block by choosing the same number for Start and End.

    - Whenever a block being part of a sequence is reached, the defined number of loops is performed. Thus outer loops also contribute to the number of total runs of an inner loop.
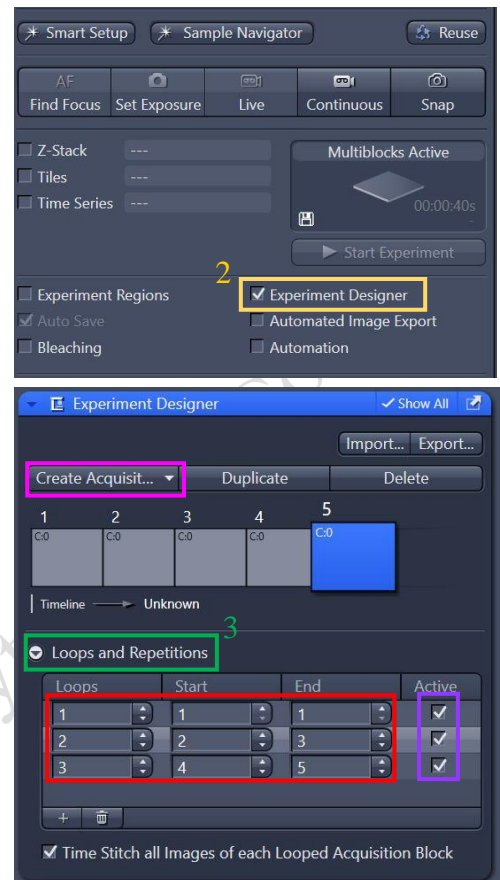
Storing and exporting image data

1. 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.

2. Create or choose your own folder in D:\user, enter a file name, select format as **CZI** and click on the **Save** button.

3. 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.

	JPEG File Interchange Format (JPEG)
	Windows Bitmap (BMP)
	Tagged Image File Format (TIFF)
File type	Tiff Format(64Bit) (Big Tiff)
	Portable Network Graphics (PNG)
	JPEG XR (WDP)
	DigitalSurf SUR (SUR)

4. 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.

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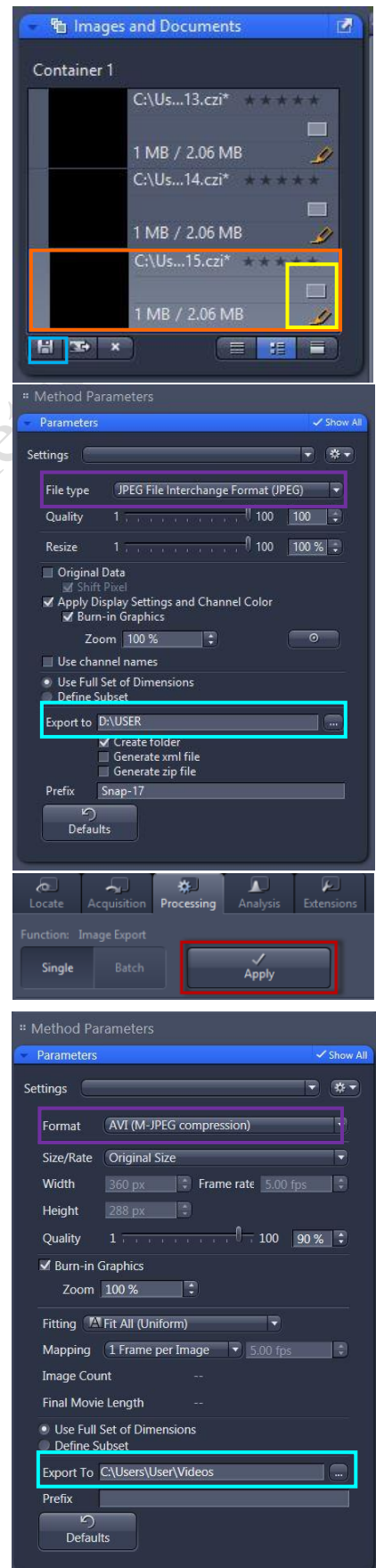
Z-Stack Experiment Set Up

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/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 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.

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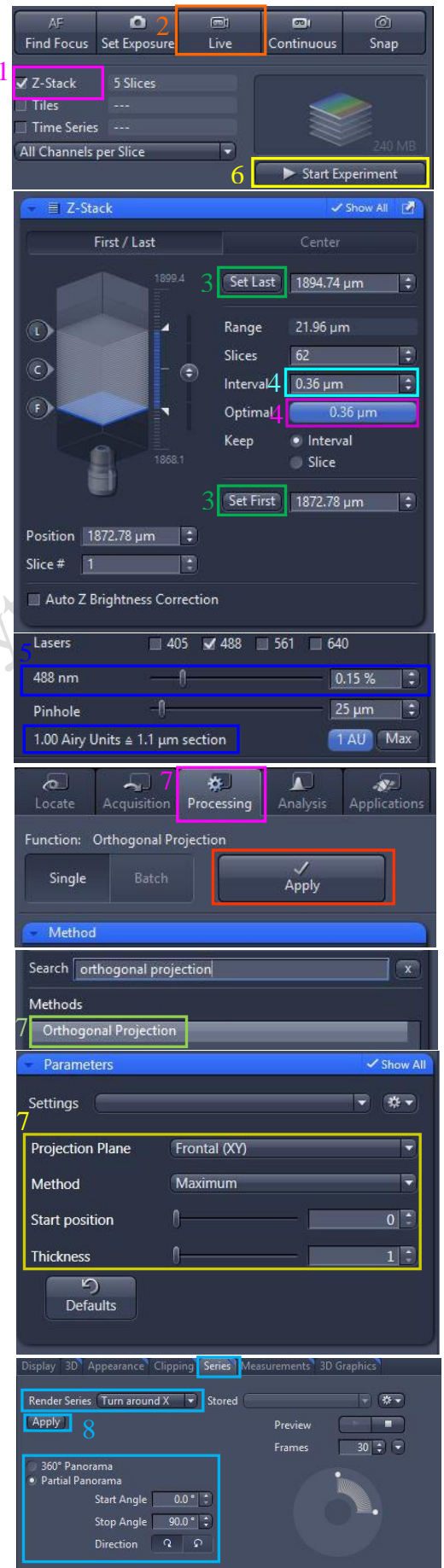
Time Series Experiment Set Up

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 ≥ scanning time of one multi-color frame.)

3. Open the **Focus Strategy** to maintain focus stability.

4. Select the focus method in **Focus Strategy**.

- **None:** No focus strategy has been selected. This is the default setting for all experiments that do not include a tiles dimension. In this case the software automatically selects the Use Focus surface/ Z Values De-fined by Tiles Setup strategy.

- **Software Autofocus** mode: Identification of the focus reference plane based on the sharpness calculation or in-tensity calculation of a series of images (Z-stack). (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)

- **Definite Focus** mode: The Z level of cover glass of sample will be maintained. Applicable to 10x and 20x objective. For 40x objective, only applicable to live cell imaging with medium in petri-dish.

a. Step 1: On the microscope touch screen control panel→ go to **Home** → **Microscope** → **XYZ** → **Definite Focus**.

b. Step 2: Go to ZEN software → Select **Time Series** → select **Definite Focus** mode → go to stabilization Event Repetitions and Frequency→ select Standard or Expert (check the box of Time Series and Repeat **Every n Time point** →key in at which time point interval you would like the Definite Focus mode to function).

- **Combine Software Autofucus and Definite Focus mode:** combine the functions of Definite Focus and Software Autofocus. This can be done in two ways,

a. Software Auto-focus as Reference for Definite Focus. Software Autofocus moves the focus drive to the focus position that has been calculated. Taking this as the starting point, a new reference distance is defined for the next distance stabilization performed by Definite Focus. This can reduce the likely-hood of a stabilization failure when the sample is long and elongated and the carrier possibly tilted.

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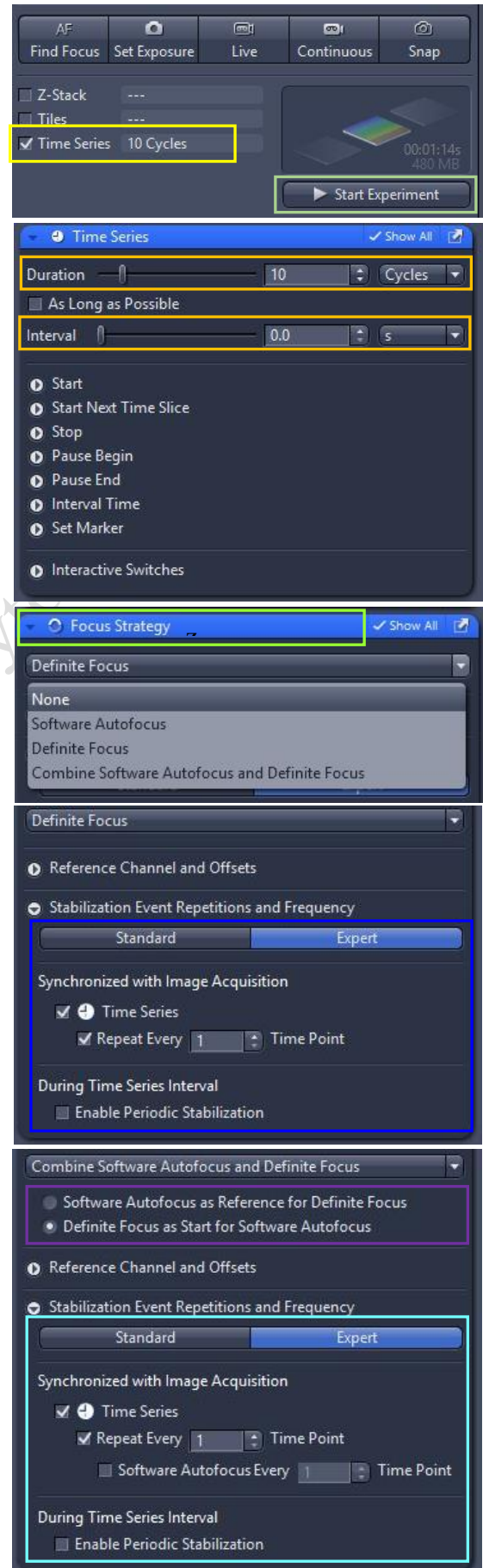
b. Definite Focus as Start for Software Auto-focus. The last valid Reference Z-Position defined by Definite Focus is the starting position for the Software Autofocus search. This allows you to optimize the search range and step size of Software Autofocus.
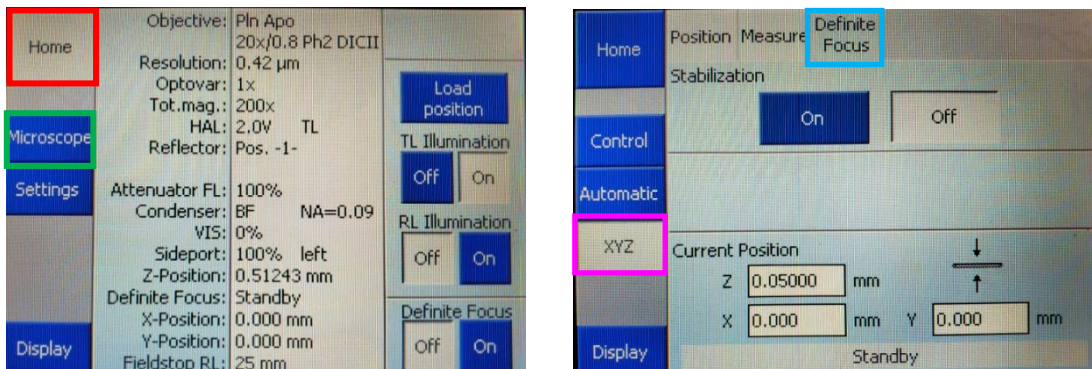
5. Set the channel and acquisition parameter if necessary and then click **Start Experiment**.
6. To quantifying changes in signal intensity after acquisition, click **Mean ROI** tab and create a **ROI** region with the drawing tool for the **r**egion **o**f **i**nterest.
7. The **intensity profile** along the experiment duration will be shown in the graph while corresponding **data** will be shown in the table right.
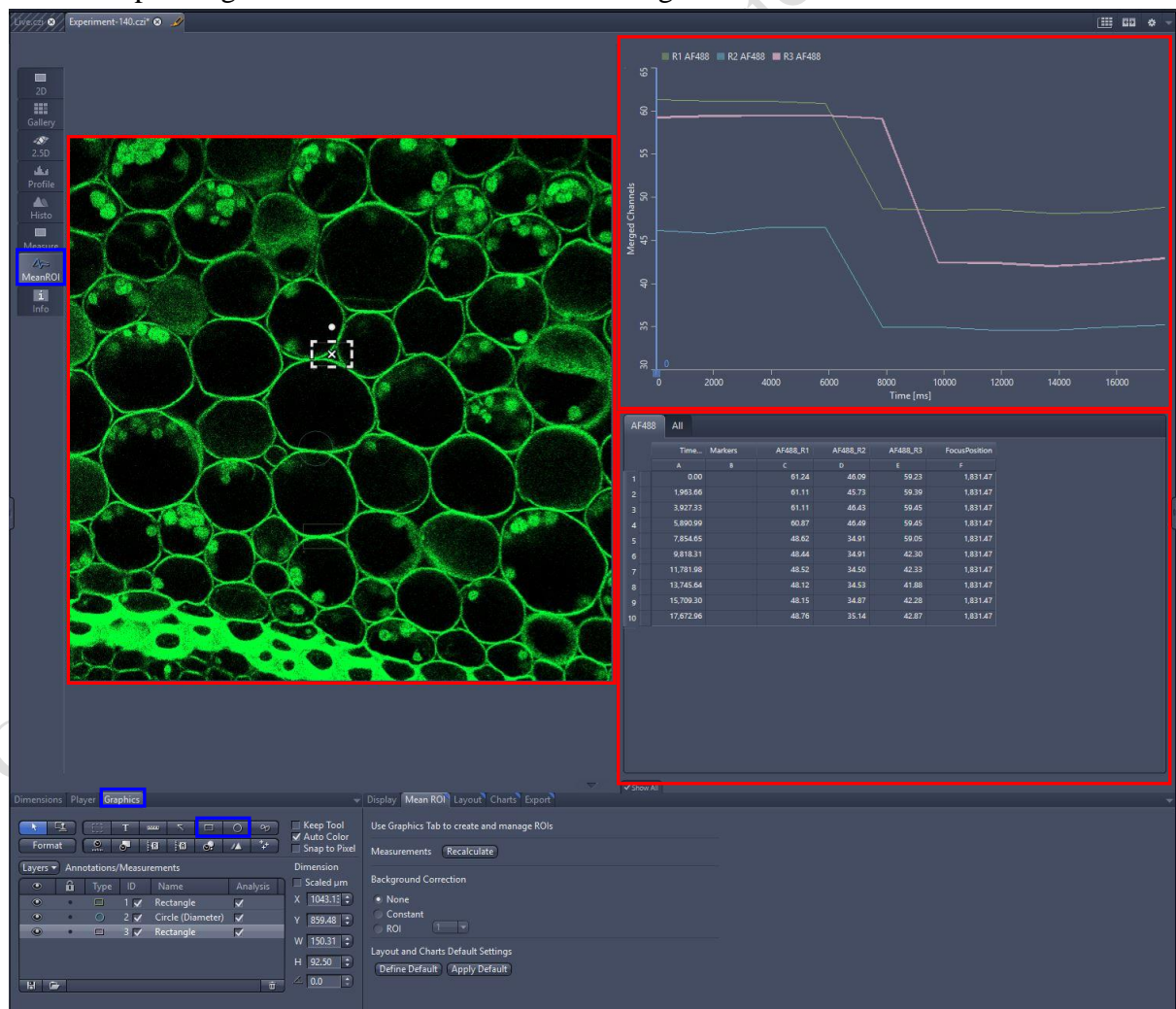
8. After saving the CZI format, video for the time series images can be exported. For export as video→Go to processing (or Go to File > Export) →Movie Export→choose the **Format** as shown below→Choose export to→click on Apply. Adjust the playing speed by **Frame per Second** and **Select file name** and **save** in your own folder.
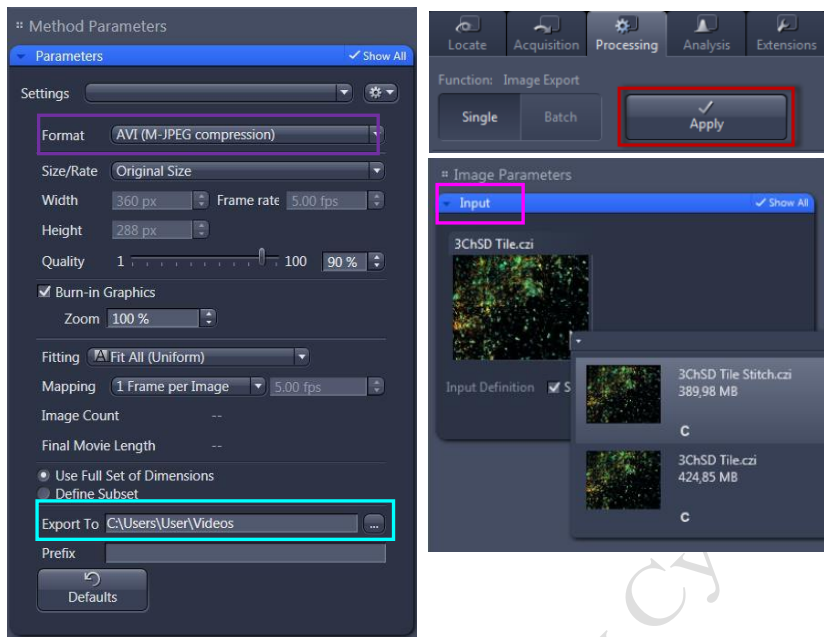

Multiple Position Experiment Set Up

1) Open the **Tiles** tool in the Multidimensional Acquisition module and activate the Show all mode.
2) In the Tiles tool open the Sample Carrier section→Click on the **Select...** button→Choose a predefined **Sample Carrier template** and click **OK** (if applicable).
3) Open the Positions section.
4) Start the Live mode to use the stage to locate a position that you want to acquire.
5) Bring the specimen into focus using the focus drive.
6) Click on the   Add button→The current position is added to your experiment.
7) 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.)
8) 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).
9) Click on the Start Experiment button.

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. Choose the Contour
3. Set the scan mode with Tiles, Size or Stake.

   a. Tiles: Enter the number of X and Y to define the tile region (Example: X=3 and Y=3 equals to tile region containing 9 tiles)
   b. Size: Define the size of tile region
   c. Stake: Adding the area for tile scanning by input the edges of whole tile area. The software will define the total tile number base on the input region.

4. Click on the ＋ Add button
5. 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.
6. Save the experiment. To do this, in the Experiment Manager click on the Optionsbutton and select Save As. Enter a name for the experiment in the input field (e.g. SimpleTile Experiment).
7. Click on the **Start Experiment button**.
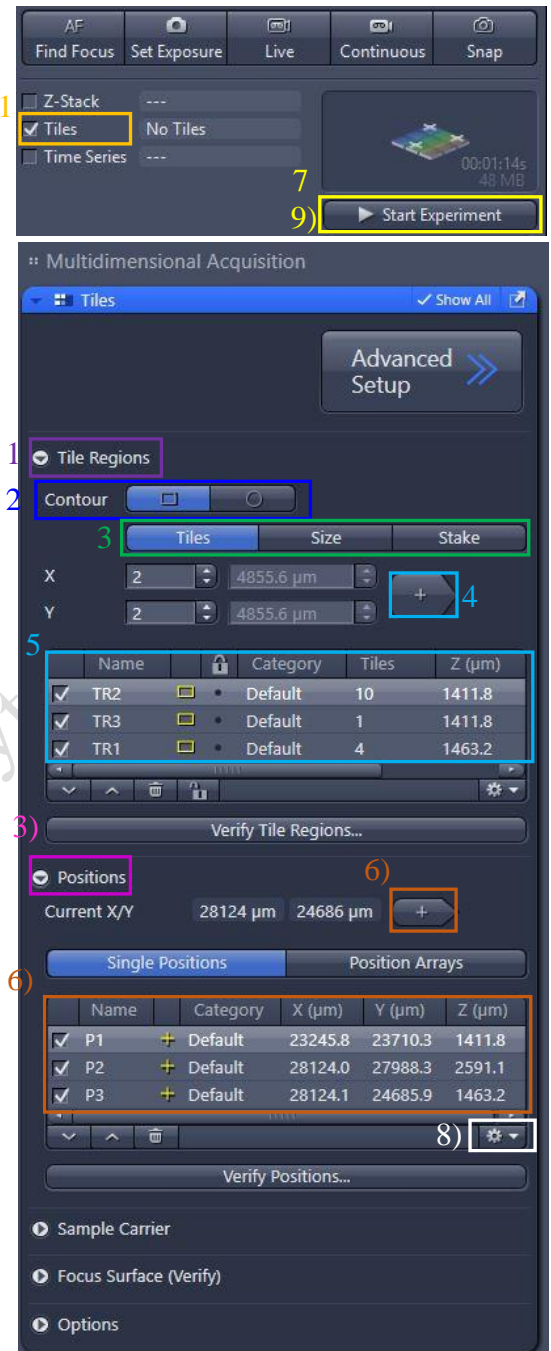
Turning off the system

1. Remove your sample and clean the stage
2. Clean the oil objective (with lens cleaning tissue only but NOT Kimwipe).
   a. Remove immersion oil from the objective lens (40xoil) with lens cleaning tissue.
   b. Repeat this step with a new area/piece of the lens cleaning tissue until no oil streaks are seen on the tissue.

3. Remove Objective heater from the objective (if applicable).
4. Change objective to lowest magnification (2.5x) objective.
5. Click Load Position (on the touch screen)→Click Set Work Position(on the touch screen).
6. Transfer data through the Imaging and Flow Cytometry Core network storage server.
7. 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)
8. Shut down PC④→ 5 second.
9. Turn off the Laser Key (labeled ③) 90° counter clockwise for switch off the laser → wait for 5 second
10. Turn the components button ②→ wait for 5 second.
11. Turn the Main switch① 90° counter clockwise for switch off.
12. Turn off the power of incubation system controller and CO_2 regulator and terminate the CO_2 supply (if applicable).
13. Sign on log sheet according to **Actual** finishing time.