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**Imaging and Flow Cytometry Core**

# **LSM880 with Airyscan Confocal Microscope Standard Operation Protocol**



## Imaging and Flow Cytometry Core

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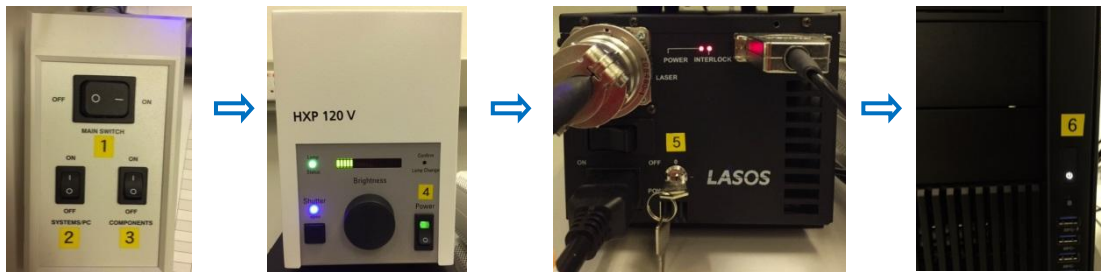


## Imaging and Flow Cytometry Core

### System Start Up

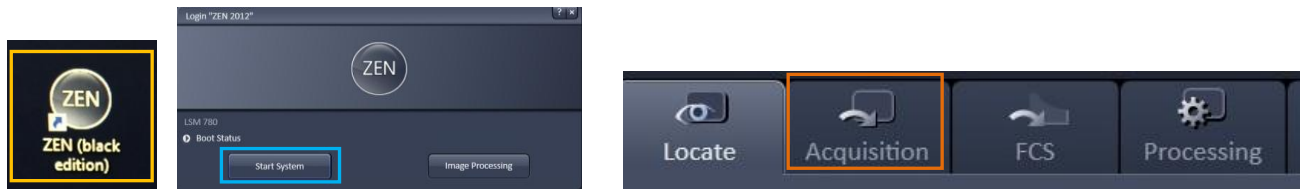
Sign on log sheet according to **Actual** start time

1. Switch on Main power switch ①
2. Switch on System / PC power button ②
3. Switch on Components power button ③
4. Switch on HXP fluorescent lamp ④ and turn on the shutter button.
5. Turn on Standby/On key switch for argon laser ⑤ from vertical to horizontal direction.
6. Turn on the computer power ⑥

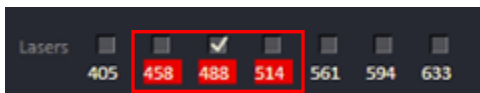


### ZEN Software Initialization

1. Log on computer with “LSM User”
2. Double click the **ZEN** icon on desktop
3. Click **Start System** to run the software.
4. Go to **Acquisition** tab.



5. Warm up time is needed for 458 nm, 488 nm and 514 nm lasers. Once anyone of the box of these 3 channels is checked, a pop up message will ask whether the selected laser is agreed to be turned on → click “Yes” → the background will turn to **RED**.



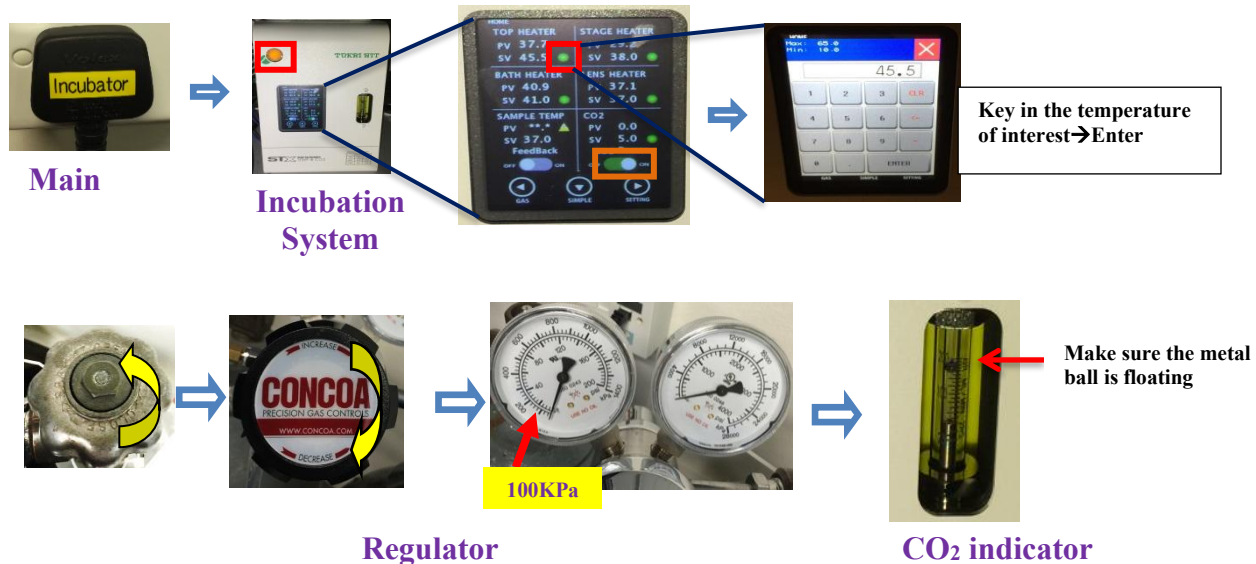


## Imaging and Flow Cytometry Core

### Set the temperature and CO<sub>2</sub> control for live cell imaging

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

- Switch on **“Incubator”** for temperature and CO<sub>2</sub> control.
- Switch on the **Power** of Tokai Hit incubation system controller. Temperature can be altered via pressing the green button of each heating parts on the touch screen.
- Make sure the CO<sub>2</sub> sliding button is turned **ON**.
- Turn on CO<sub>2</sub> tank by turning the main switch **anticlockwise**.
- Turn on CO<sub>2</sub> 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<sub>2</sub> gas.
- Add MilliQ water into the water chamber and covered if overnight(s) acquisition is required.





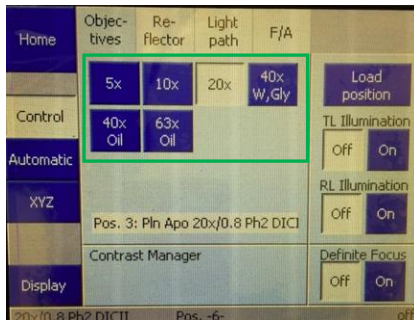
## Imaging and Flow Cytometry Core

### Locating Sample and Finding a Region of Interest

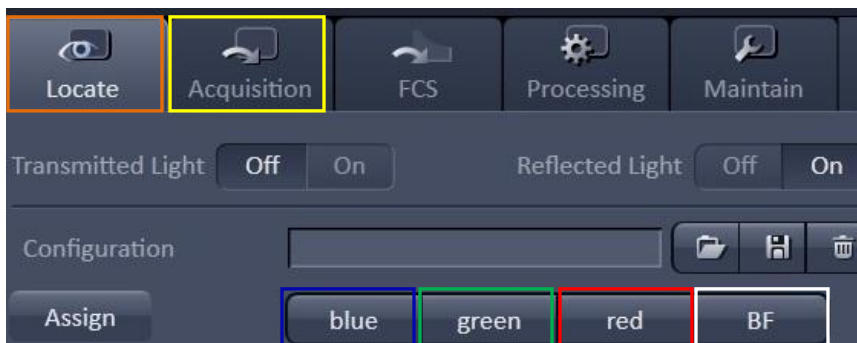
1. Select a **desired objective** from the touch screen. Apply a drop of oil onto the tip of the lens if 40x oil or 63x oil objective is used. Apply a drop of gliserol if 40x W/Gly objective is used.

**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)



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 **Blue/Green/Red** tab for blue emission /green emission /red emission channel observation respectively.
7. Focus the sample with Corse/fine focusing knob.
8. Once the region of interest is found and focused, switch the tab to “**Acquisition**”.

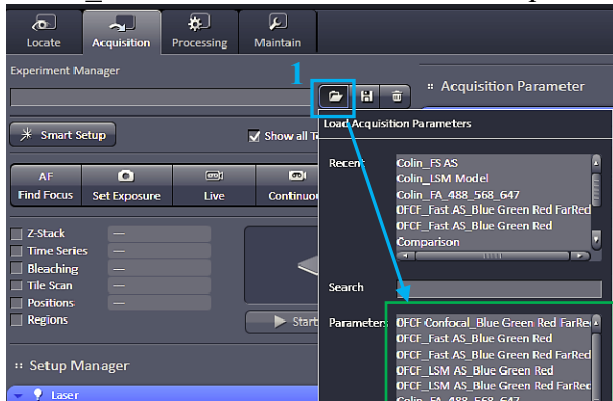


## Imaging and Flow Cytometry Core

### Setting up the Scanning Track/Channel

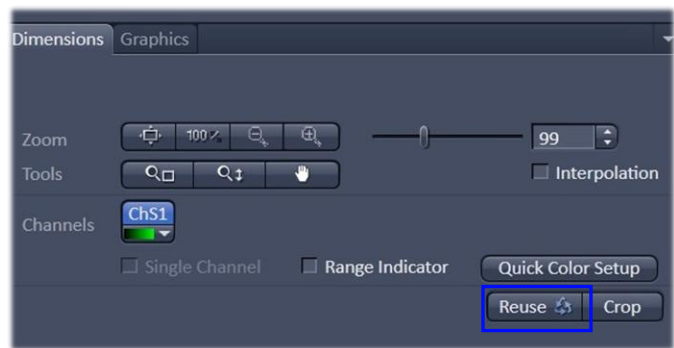
#### Method 1

Load a **predefined configuration** of combination dyes from the **Load configuration list**. Select “0IFC\_Confocal” if confocal mode is required.



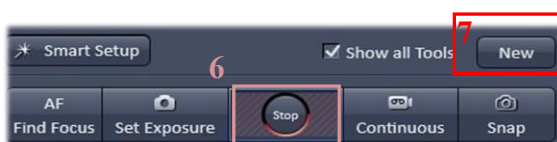
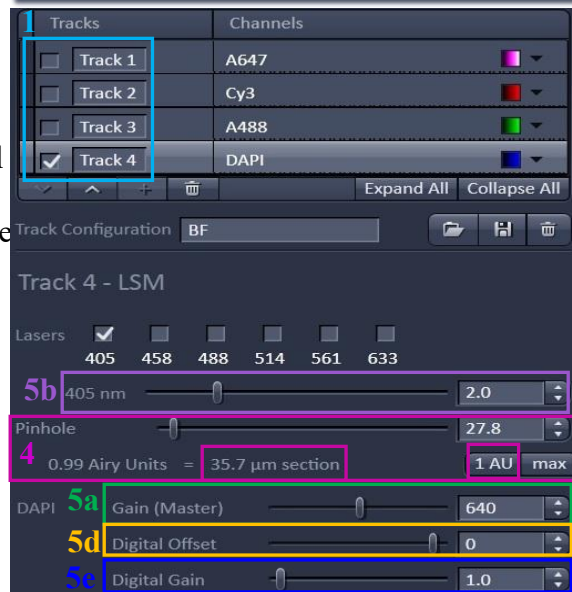
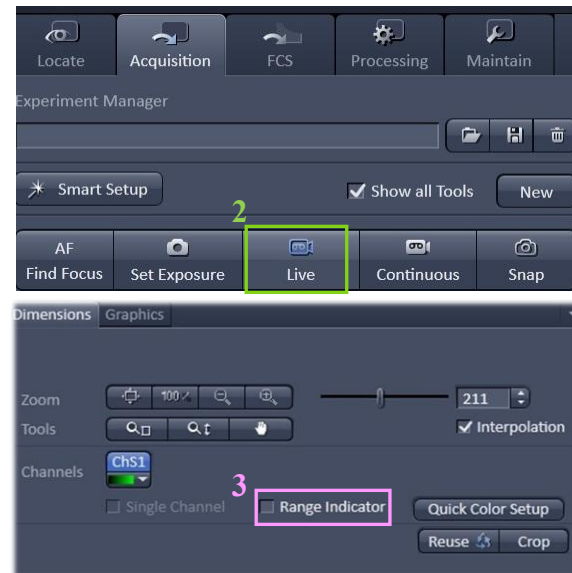
#### Method 2

Open any previous image with desire setting and 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 a false-color presentation. Red pixel = saturation (max. intensity). Blue pixel = 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.
8. Check all channel boxes → click “**Snap**” when finish adjusting the other channels.

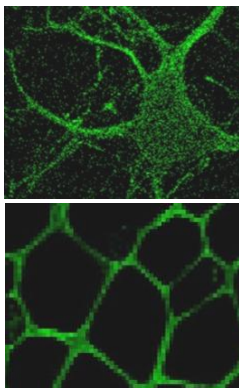




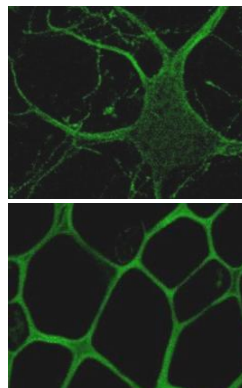
## Imaging and Flow Cytometry Core

### Scanning Parameters Set Up

1. Go to Acquisition Mode.
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 of **Pixel size**, thus change of resolution). 1024x1024 usually produces good results for general purpose. Click on the **Optimal** button for best resolution which depending on objective N.A. and  $\lambda$ .
4. Adjust the scan speed with the **Speed** slider. Speed 6 or 7 usually produce good results. For samples with high background noise, use Speed 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 proposes.
8. Click **Snap** to acquire a single frame (multi-channel) image.



Lower scan speed;  
or More averaging



Larger frame size  
(Higher resolution)

The screenshot shows the software interface for microscope acquisition. Key settings are highlighted with colored boxes and arrows:

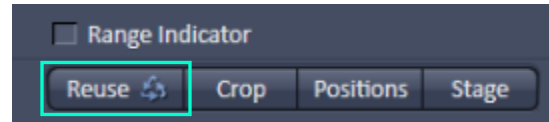
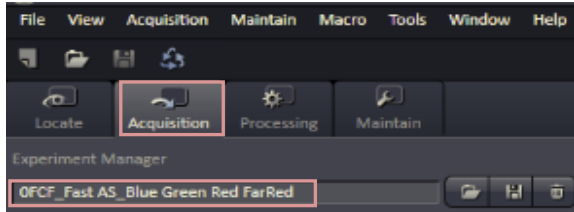
- Dimensions/Graphics:** Zoom is set to 99. The **Crop** button is highlighted with a green box.
- Acquisition Mode:** Objective is Plan-Apochromat 40x/1.4 Oil DIC M27. Scan Mode is Frame. **Frame Size** is X 1024 and Y 1024. Line Step is 1. **Speed** is set to 5. **Averaging** Number is 1 and Bit Depth is 16 Bit. Mode is Line and Method is Mean.
- Scan Area:** Image Size is 212.3 μm x 212.3 μm. Pixel Size is 0.21 μm. Zoom is 1.0.
- Bottom Panel:** The **Snap** button is highlighted with a purple box.



## Imaging and Flow Cytometry Core

### Fast-Airyscan Imaging Set Up

- Once a region of interest is found under the eyepiece → click **Acquisition** → Select a predefined Fast-AiryScan configuration (e.g. **0FCF\_Fast AS\_Blue Green Red FarRed**) of combination from the list. Alternatively, click “**Reuse**” the previous settings by loading an old image file.



- Click on Fast Scan (**FS**) → set the speed to maximum (**Max**) for image preview → adjust the settings for a channel of interest (one channel at a time, **check the box and highlight**) → click on **Continuous** → **Airyscan** → **Display** → **Processing Preview** → adjust focus to the best focal plane (**brightest hexagon in the middle**) → adjust the **laser power and Gain (Master)** to get the best range of signals → click **Min/Max** → **White range** >6000 → repeat same approach for other channels.

The screenshot displays the following configuration details:

- Acquisition Parameter:** Objective: Plan-Apochromat 20x/0.8 Ph2 M27; Frame Size: X 716, Y 116; Sampling: SR; Nyquist: Opt; Speed fps: 12; Pixel Dwell: 0.55 µsec; Scan Time: 84.96 msec; Speed: 11.
- Setup Manager - Laser:**

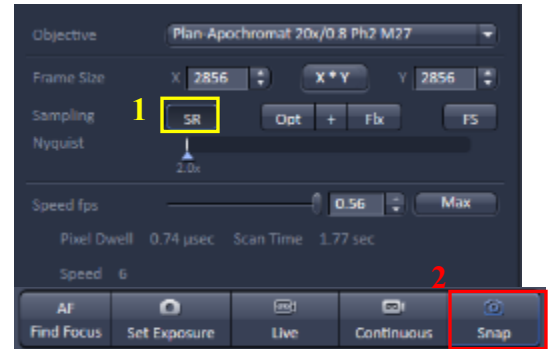
Laser	Laser Lines [nm]	Power
Argon	458, 488, 514	On
Diode 405-30	405	On
DPSS 561-10	561	On
HeNe594	594	Off
HeNe633	633	Off
- Imaging Setup:** Tracks: Track4, Track3, Track1, Track2; LSM: Fast; Switch track every: Frame.
- Online Processing Options:** Channels: Track4 (ChA), Track3 (ChA), Track1 (ChA), Track2 (ChA).
- Track 3 - Fast:** Lasers: 405, 458, 488, 514, 561, 594, 633; 488 nm; Gain (Master): 800; Digital Gain: 1.0.
- Image 1:** Shows a green fluorescence image of cells. A hexagonal ROI is highlighted with a blue border and a scale bar.
- Processing Preview:** Shows a histogram of the ROI with a 'White' range of 25320.



## Imaging and Flow Cytometry Core

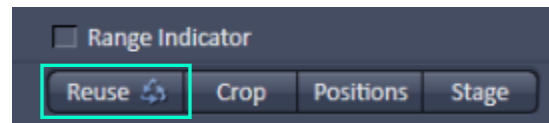
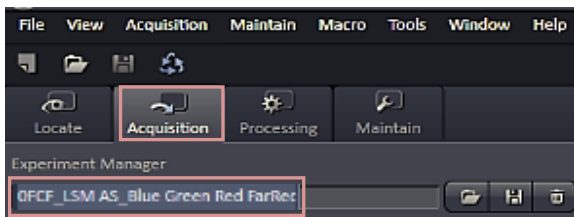
3. When all channels have been set well → click on **SR** (super-resolution) → **Snap**

- **SR** sets the sampling for superresolution imaging with Fast at 2,0x Nyquist sampling. (4x faster than conventional Airyscan SR).
- **Opt** sets the sampling for optimal confocal imaging with Fast at 1,0x Nyquist sampling.
- **Flx** sets the sampling for flexible confocal imaging with Fast at 0,7x Nyquist sampling.
- **FS** sets the sampling for fastest speed imaging with Fast at 0,5x Nyquist sampling. (Allows highest frame rates of up to 200 fps with sufficient resolution for good image quality. Sampling below 0,5x is not possible with Fast Airyscan).

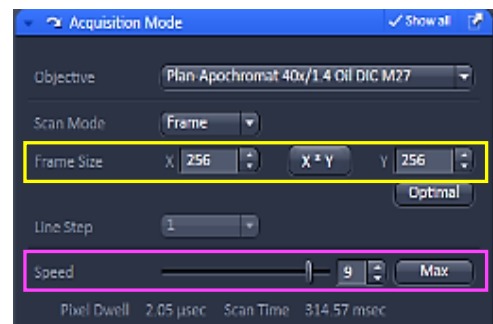
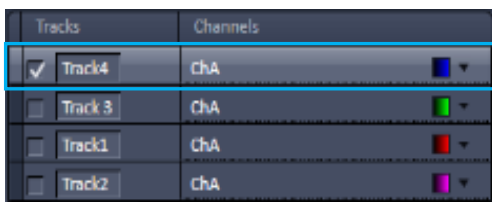


## LSM-Airyscan mode Imaging Set Up

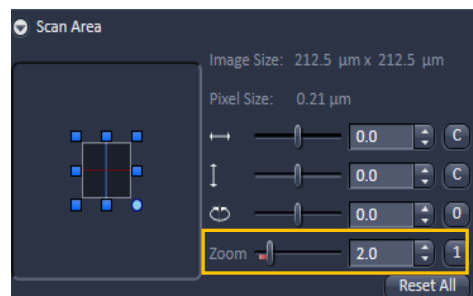
1. Once a region of interest is found under the eyepiece → click **Acquisition** → Select a predefined LSM-AiryScan configuration (e.g. **0FCF\_LSM AS\_Blue Greed Red FarRed**) of combination from the list. Alternatively, click “**Reuse**” the previous settings by loading an old image file.



2. Select **one channel** of interest at a time → set to a smaller **frame size** and adjust to the **highest speed** for a fast scan.



3. Set the Scan Area for a **zoom factor** ≥ 1.8x or click Optimal to set the required Frame Size for optimal resolution.





## Imaging and Flow Cytometry Core

4. Click on **Continuous** → **Airyscan** → **Display** → **Processing Preview** → adjust focus to the best focal plane (**brightest hexagon in the middle**) → adjust the **laser power and Gain (Master)** to trigger the auto adjustment of the alignment from Bad to Good and get the best range of signals → (“**Quality and Status**” can be checked in the Airyscan tab under Maintain panel) → click **Min/Max** → **White range** > 6000 → repeat same approach for other channels → click “**SR**” for Airyscan mode → click **Snap**.

The screenshot displays the ZEN 2.3 SP1 software interface with several panels and settings highlighted with numbered callouts (1-10):

- 1:** The **Continuous** button in the **Acquisition** panel is highlighted.
- 2:** The **Airyscan** button in the **Image 1** panel is highlighted.
- 3:** The **Display** button in the **Dimensions** panel is highlighted.
- 4:** The **Processing Preview** button in the **Dimensions** panel is highlighted.
- 5:** A comparison of two hexagonal patterns is shown, with an arrow indicating a transition from a noisy pattern to a cleaner one.
- 6:** The **Track Configuration** panel is highlighted, showing the **Lasers** section.
- 7:** The **Quality and Status** window in the **Airyscan** panel is highlighted, showing "Good (Waiting)" and "Adjust in continuous scans" checked.
- 8:** The **Min/Max** button in the **Processing Preview** panel is highlighted.
- 9:** The **White** value in the **Processing Preview** panel is highlighted, showing a value of 25320.
- 10:** The **Airyscan Mode** dropdown menu in the **Lasers** section is highlighted, showing "SR" selected.

- SR mode:** increased resolution, the Pinhole has no effect and the default position should not be changed.
- R-S mode:** increased signal to noise ratio at the expense of the achievable resolution. Pinhole has no effect, the amount of signal gain is depending on the pixel resolution set for the frame format.



## Imaging and Flow Cytometry Core

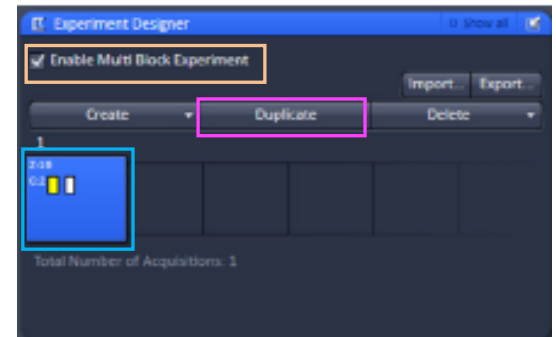
- c. **VP mode:** set the detector to virtual pinhole mode, the hardware pinhole has no effect and the default position should not be changed.
- d. **CO mode:** set the system for conventional confocal mode, the hardware pinhole is effective and can be set at the required value for confocal sectioning.

### 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.
2. Check on the “**Enable Multi Block Experiment**” box → one **blue block** will be activated for various parameters set up:

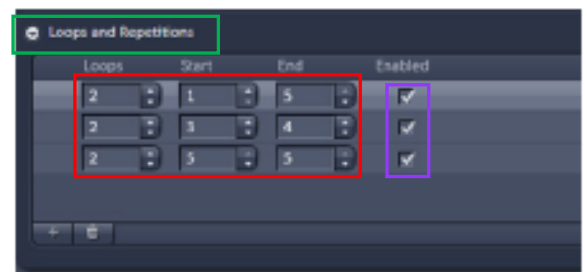
E.g. Acquisition Mode; channels; Z-Stack; Time Series; Tile Scan; Positions; Regions; Bleaching

3. After setting up the acquisition parameters for one block → click “**Duplicate**” to create another block → then change the acquisition parameters accordingly in the second block.
4. You may use the “Loops and Repetitions” function if many rounds of the same sequence of experiment blocks are required.



Expand the “**Loops and Repetitions**” → key in the **required details** → check “**Enabled**” → 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).



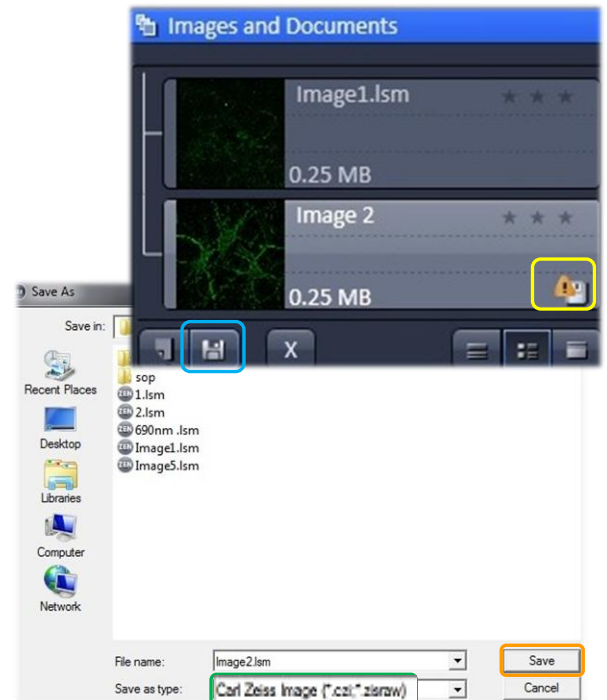
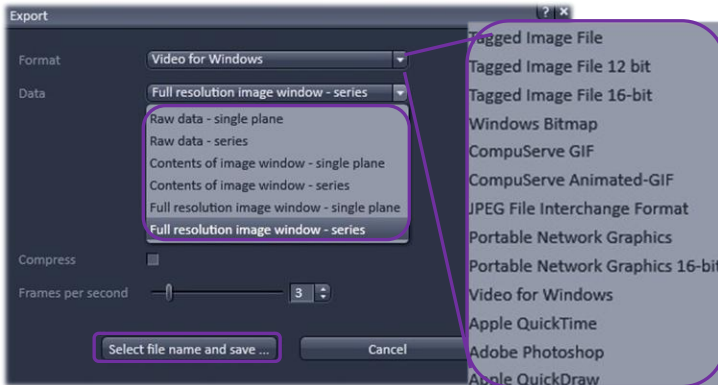
- **Wait Block:** pauses the acquisition until the user actively confirms the message box.
- **Loops:** states how often a sequence of Blocks defined by the number of the Block in Start and End is run. One loop/repetition is set as default and reflects the minimum of running the experiment once.
- **Two 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.



## Imaging and Flow Cytometry Core

### Storing and exporting image data

1. To save the acquired image → highlight the desired image → **Save Data** → store the data in a specified folder named “user”. **Yellow icon** will disappear once an image is saved.
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 File > Export, and choose the **Format and Data** as shown below. Adjust Frames per seconds if necessary. Click **Select file name and save** and save in your own folder.





## Imaging and Flow Cytometry Core

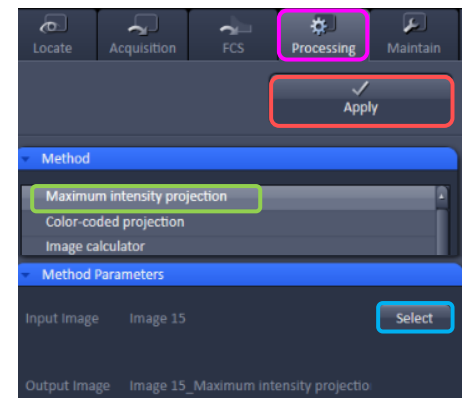
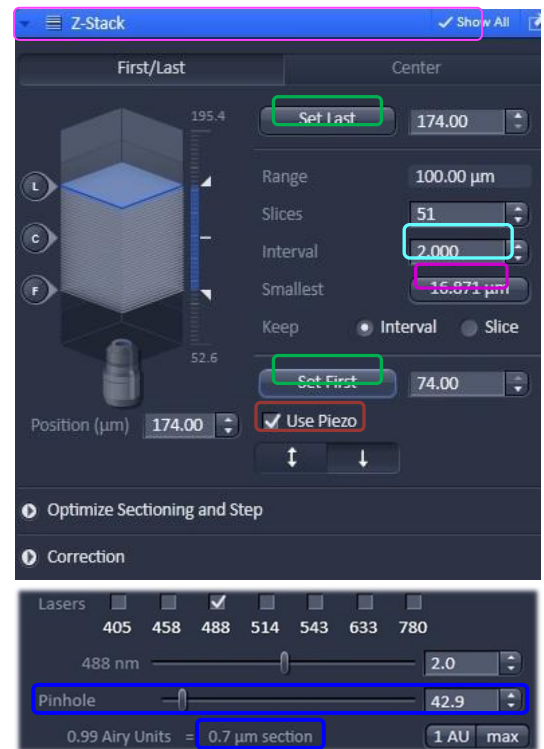
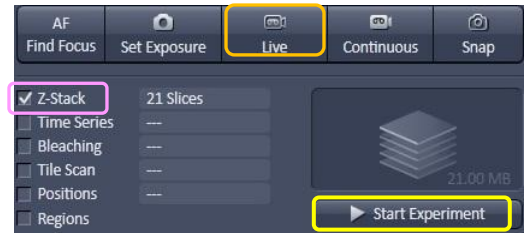
Single Frame	Format	Tagged Image File JPEG File Interchange Format Portable Network Graphics
	Data	Raw data – single plane (without any overlay graphics) Contents of image window – single plane (for analyzed plot of histogram or as displayed as in image container; compressed) Full resolution image window – 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)



## Imaging and Flow Cytometry Core

### 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.  
**Smallest:** 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. Check **Use Piezo** checkbox to perform quicker z focus move if necessary.
7. Click **Start Experiment** to start the recording of Z-Stack images.
8. 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 to visualize all the signal for the specimen in 2D. Go to “**Processing**” tab → click **Maximum Intensity Projection** → **Select** the z-stack image of interest → **Apply**.





## Imaging and Flow Cytometry Core

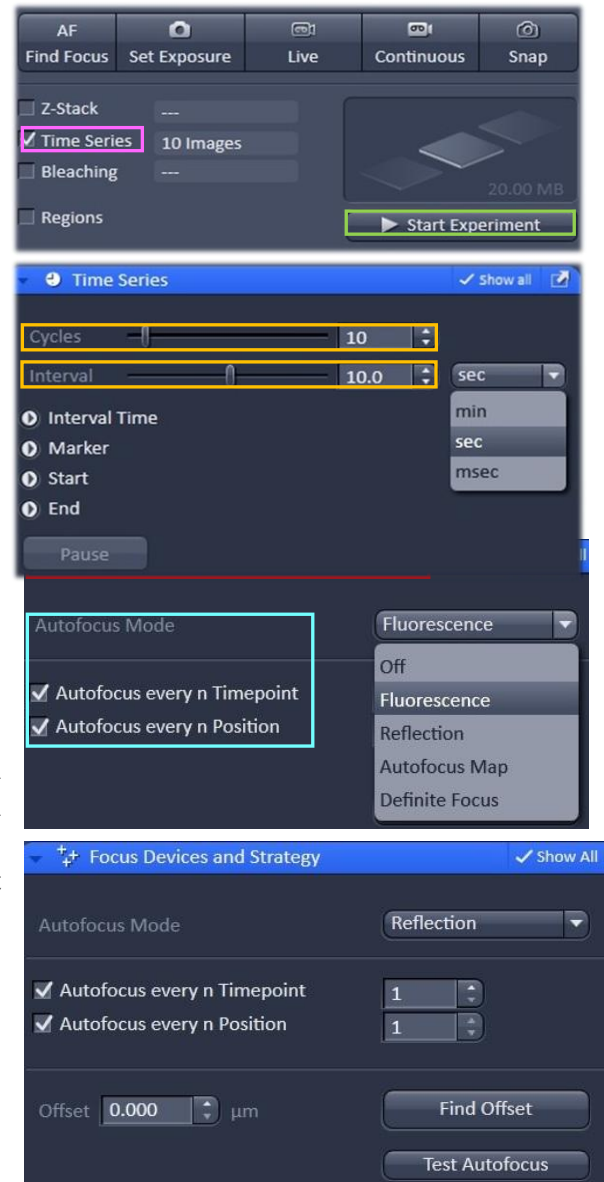
- A video of rotating 3D image can also be made. Activate the **Series** tap, select the **rotating axis**, number of frame and the rotation range (= **Total Frame** × **Difference angle**). Click **Apply** and the series images will be generated in a new tab.

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.



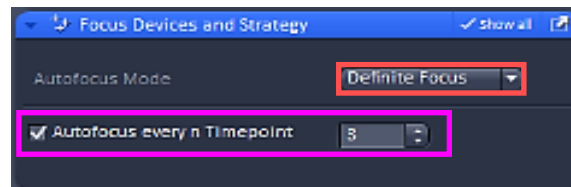
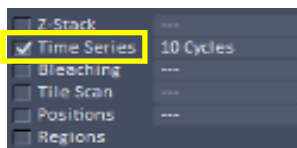
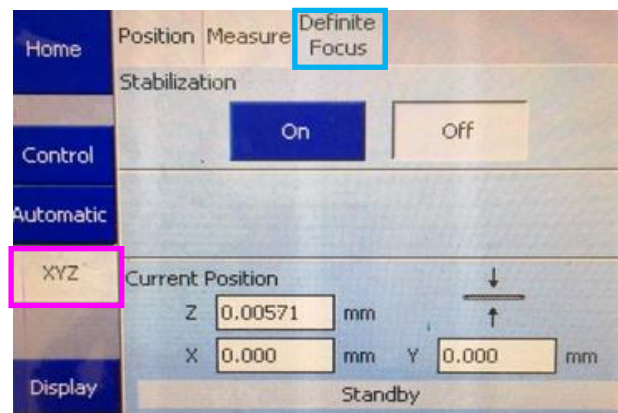
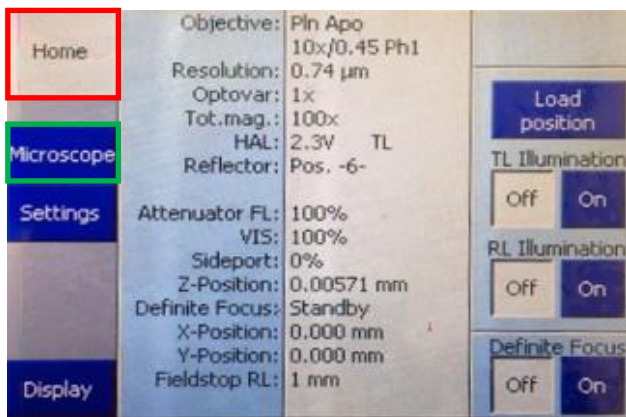
## Time Series Experiment Set Up

- Check the **Box for Time Series** → open the **Time Series** module.
  - 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 be ≥ scanning time of one multi-color frame.)
  - Open the **Focus Devices and Strategy** to maintain focus stability.
  - Select the focus method in **Autofocus Mode**.
    - Fluorescence** mode: Identification of the focus reference plane based on the maximum intensity of a designated 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.



## Imaging and Flow Cytometry Core

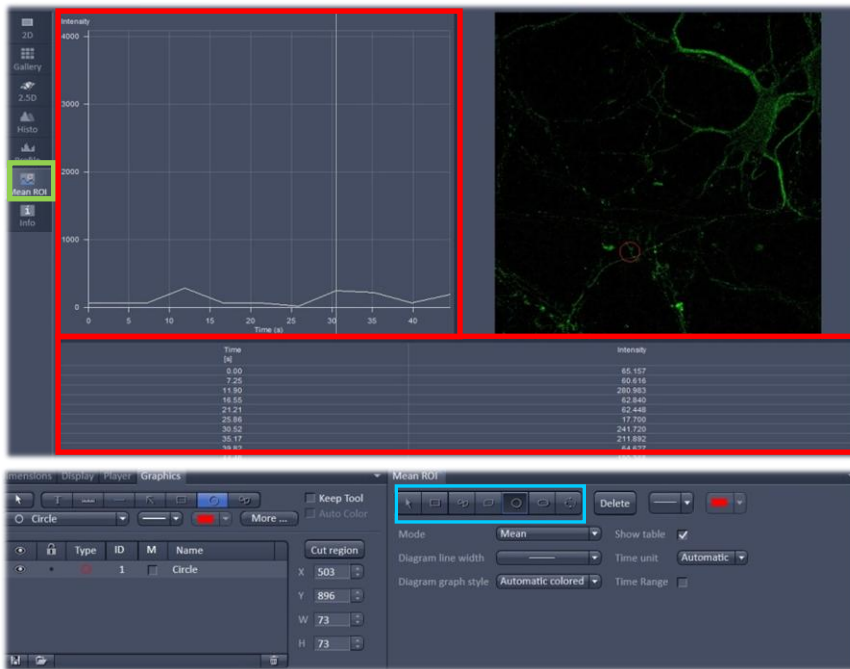
- b. Step 2: Press “**Find Offset**”
- c. Step 3: The software copies the offset into the “**Offset**” spin box of “**Position**” Tool.
  - **Autofocus Map** mode: Only applicable for multi-well plate. One offset will be applied to all wells of one plate.
  - **Definite Focus** mode: The Z level of Petri dish-medium interface will be maintained by the reflection of 835nm LED light. 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 → check the box of **Autofocus every n Time point** → key in **at which time point** interval you would like the autofocus mode to function.



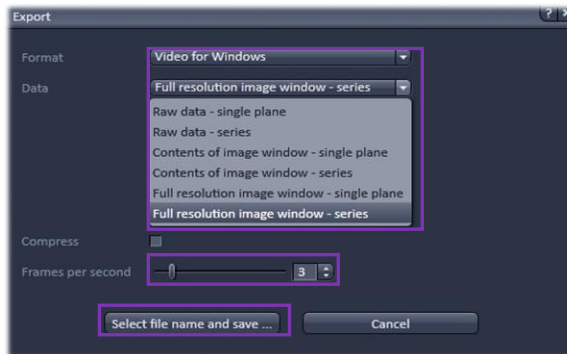
5. Set the channel and acquisition parameter if necessary and then click **Start Experiment**.
6. To quantifying changes in signal intensity after acquisition, click **Mean ROI** tab and **create a ROI region with the drawing tool** for the **region of interest**.
7. The **intensity profile** along the experiment duration will be shown in the graph while corresponding **data** will be shown in the table below.



## Imaging and Flow Cytometry Core



8. After saving the CZI 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.

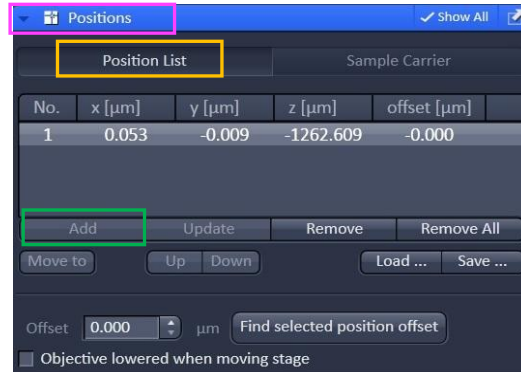




## Imaging and Flow Cytometry Core

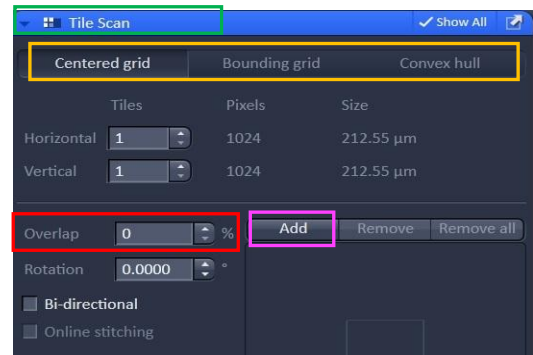
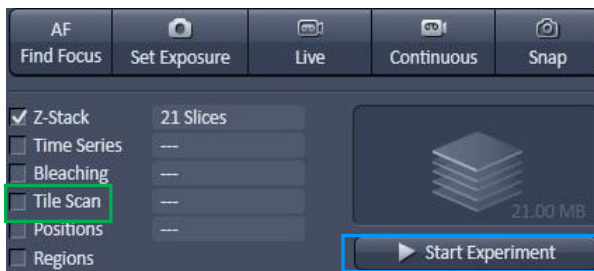
### Multiple Position Experiment Set Up

1. Check the **Box for position** → open the **Position** module.
2. Click **Position List** → **Add** current position to the list.



### Tile Scan Experiment Set Up

1. Check the **Box for Tile Scan** → open **Tile Scan** module
2. Select either **Centered grid** or **Bounding grid** or **Convex hull** → set image **to at least 10% overlap** → **Start Experiment**.
  - a. **Centered grid**: Move the ROI to the center position → set the number of tiles for horizontal and vertical directions to set a rectangle scanning area.
  - b. **Bounding grid**: Move the stage to the upper left and lower right corner of the whole scanning area → click **Add** to set the boundary of the scanning. (Rectangle scanning area)
  - c. **Convex hull**: Move the stage to the edge of the whole scanning area → click **Add** to set the boundary of the scanning for an irregular-shaped specimen.





## Imaging and Flow Cytometry Core

### Turning off the system

1. Please check the schedule and switch off the system if it will not be occupied within 1 hr.
2. Please turn off the Argon laser in the software before exiting ZEN Software, transfer data through the Faculty Core Facility network storage server and then Shut down PC ⑥
3. Turn the key switch of argon laser to Standby mode (horizontal to vertical direction) ⑤  
***Wait for 10 min to let Laser Fan OFF!!!***
4. While waiting, please
  - a. Remove your sample and clean the stage
  - b. Clean the oil objective (with lens cleaning tissue only but NOT Kimwipe)
    - Remove immersion oil from the objective lens with lens cleaning tissue
    - Repeat this step with a new area/piece of the lens cleaning tissue until no oil streaks are seen on the tissue
    - Dry the lens with lens cleaning tissue
  - c. Change objective to lowest magnification
  - d. Sign on log sheet according to **Actual** finishing time
5. Turn off the power of HXP fluorescent lamp ④
6. Components button ③
7. System / PC button ②
8. Main power switch ***(Only after Argon Laser Fan is OFF)*** ①
9. Remove Objective heater from the objective (if applicable)
10. Turn off the power of incubation system controller and CO<sub>2</sub> regulator and terminate the CO<sub>2</sub> supply (if applicable).