LSM 900 CONFOCAL WITH AIRYSCAN

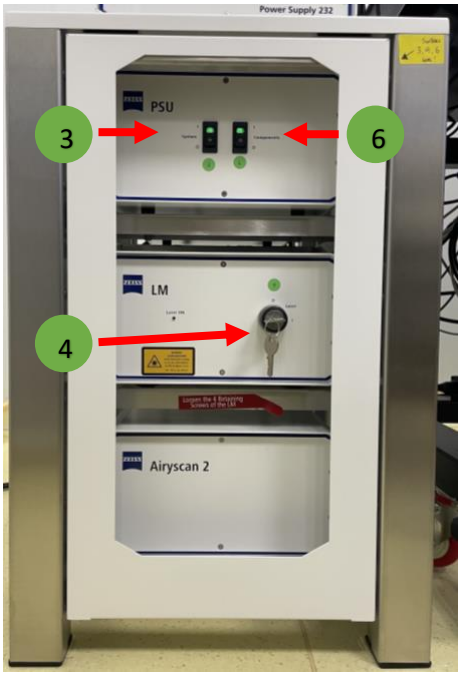
REFERENCE MANUAL

CONTENTS

Enter your sign in information at the log book.

1. Turning on the system

1. Switch on the switches in the following order:
   - Switches 1 and 2 (mounted on the wall)
   - Switches 3 and 4 (rack to the left of microscope)
   - Switch 5 (workstation)

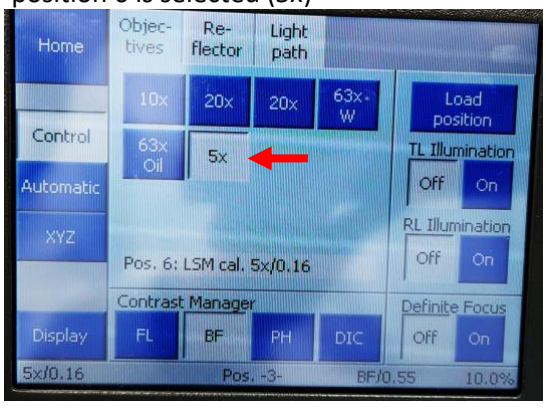


At this point, the Focus Controller 2 box will show the text in the image below

After switching on Switch 6, the display text “DEFINITE FOCUS 2 STANDBY” will be shown instead

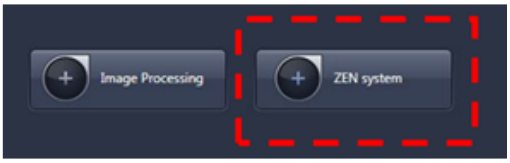
2. Log into the user account “DBS Users”.
   → Password: lsm900
   Sign in with your PPMS account credentials at the PPMS login window (light blue).

3. Check the touch screen of the microscope, “Microscope”-> “Objective”, make sure position 6 is selected (5x)



2. Start Zen Blue 3.1 Software

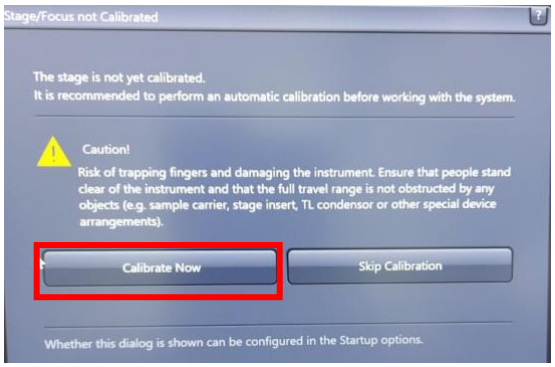
1. Double click the **Zen 3.1 (blue edition)** software icon on the desktop.
2. Select "**Zen System"** to initiate the confocal component



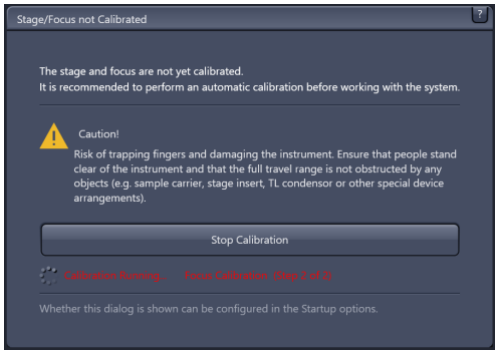
When switching ON the system from step 1, the calibration window will appear after starting the ZEN system software.

If you are **the first user of the day**, you will need to calibrate the stage.

Click 'Calibrate now' in the pop-up window.

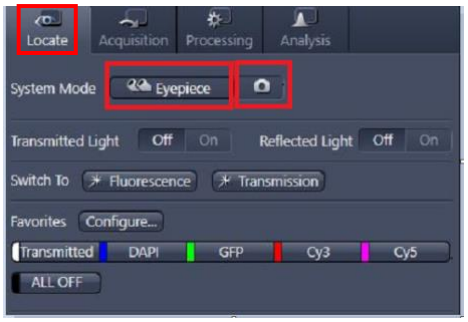


The second pop-up window will appear. Wait until the calibration is completed to proceed.

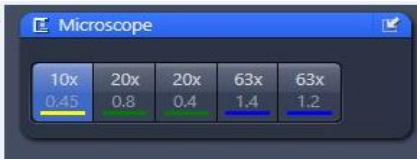


3. Setting the Microscope and Locating the specimen (Bright field and fluorescence illumination)

1. Use '**Locate**' tab to check your sample by viewing through the eye piece or camera.



2. On right side of panel, find "**Microscope**" module and select appropriate objective to examine the slide/dish.



3. Add one drop of appropriate medium (water/oil) onto your sample if objective **(63X)** is to be used.
   - ***Never put oil on Air/Water objective!!!***
   - *Note: Please make sure there is no oil or water at all on your slide when you are using air objective.*
   - *Any incorrect immersion medium /contamination will deteriorate image quality!*

4. To set the microscope for brightfield observation, click "**Transmitted**" button

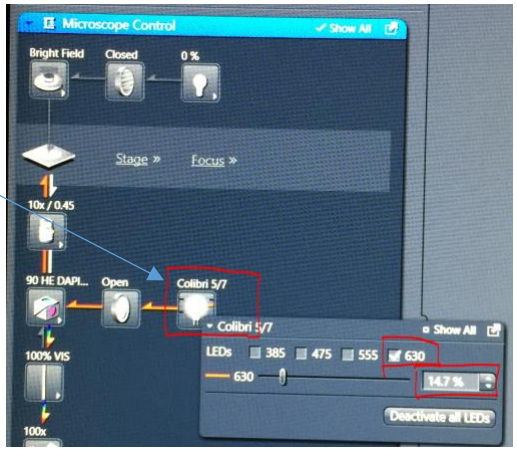
5. For DIC, refer to "**Setting up DIC Image in OCULAR imaging mode"** in this manual

6. To set the microscope for fluorescence observation, click on the following button for their respective emission channels:
   - DAPI → Blue emission
   - GFP → Green emission
   - Rhod → Red emission
   - CY5 → far-red emission

7. Move the area to be observed right above the objective lens using joystick. **F1** button on the joystick to changes the movement speed.

8. To change focus:
   a. Use the handwheel on the microscope
      i. Clockwise → objective up
   b. Hold "Ctrl" on the keyboard while scrolling with mouse (Only use in LSM mode)

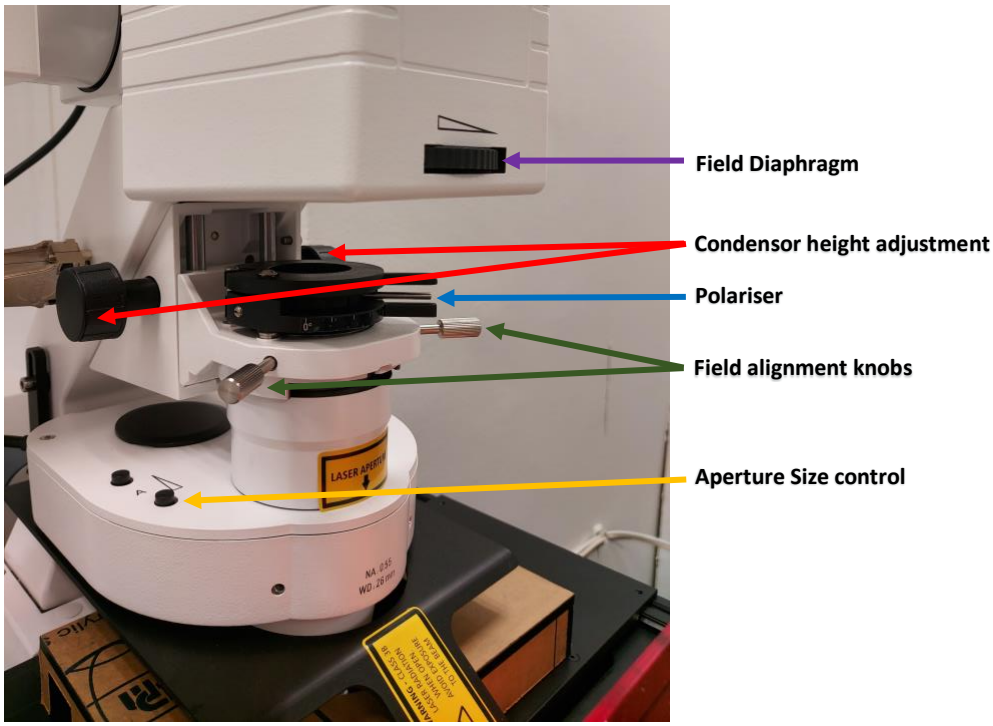
9. To change the LED light intensity, go to "**Colibri 5/7**" to select the LED light and change the output power.



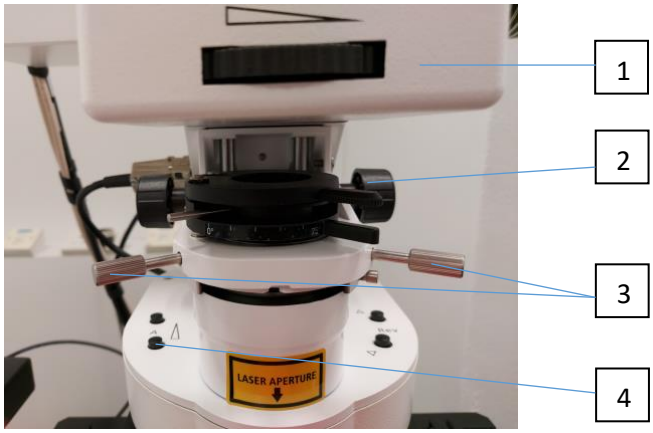
10. When not observing the sample, click on **"ALL OFF"** to prevent photobleaching of the sample

KOHLER ILLUMINATION AND DIC SETTINGS

The following steps are for KOHLER ILLUMINATION that is performed to ensure transmitted light focuses on the specimen, before DIC phase contrast imaging.



Kohler illumination



1. Ensure that the point of interest is in focus
2. Close field stop [1] so that you can observe the field
3. Align the condensor height [2] so as to see a sharp edge in the hexagon field
4. Move the alignment metal knobs [3] until hexagon is in center of the field of view
5. Open field stop [1] until you stop seeing the edge
6. If necessary, adjust the condenser APERTURE STOP [4]. Remove one eyepiece lens and visualise the hexagon

   Note: When the Aperture Stop is fully opened, the Highest Resolution is achieved

   The default value set for the aperture stop (80%) in the Microscope control is such that contrast, and resolution are balanced out for each objective

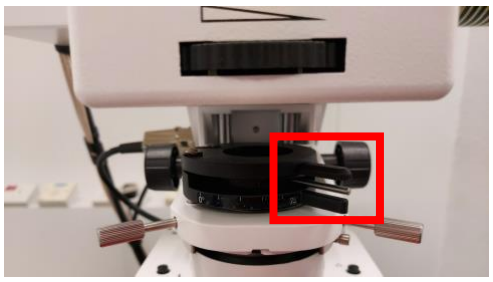
Setting up DIC Image in OCULAR imaging mode

1. Under **"Locate"** tab,
   Select the appropriate settings for DIC based on the objective being used:
   - DIC 2 – 10X, 20X
   - DIC 3 – 63X objectives

2. Engage the polarizer (silver knob) to **0 degree** (left most) when observing through the ocular mode.

3. Turn the knob in the DIC prism slider (Located Right under the objective) to adjust the contrast of the image

When switching to capture DIC image in LSM mode (using transmitted light PMT)



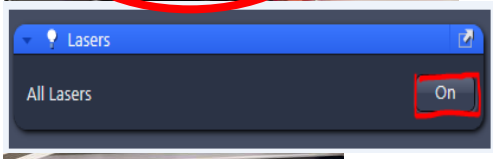
When capturing the DIC image with the T-PMT detector using the laser LSM settings, the light is already polarised.

Therefore, the polariser should be set to **90 degrees** (right most), before acquiring images with LSM mode.

4. Switching to laser scan mode:

1. Before starting laser scan, cover sample with the stage sample cover provided.

2. If you lift up microscope trans illuminator, laser will be cutoff. To resume laser, go to software->**Acquisition**-> **Laser**, click the "**On**" button.



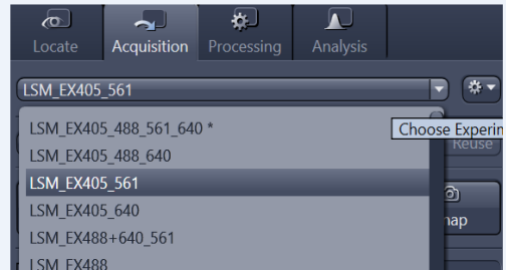
3. Make sure the laser box LED light is lit, indicating laser ON

5. Setting up the Scanning Track/Channel

Method 1

Load a predefined configuration with combination of different dyes from the configuration list box. The prefix of the name indicates:

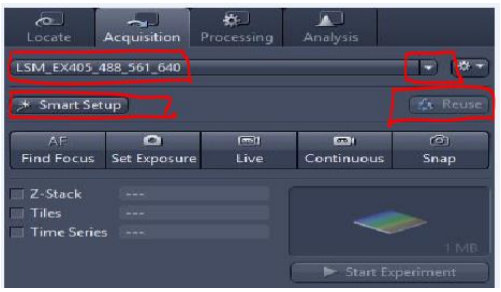
a) LSM: normal confocal scan
b) SR: super-resolution airyscan
c) MPLX: Multiplex



This is followed by the laser to be used. "+"indicates the laser will be used simultaneously. "_" indicates sequential scan

Method 2

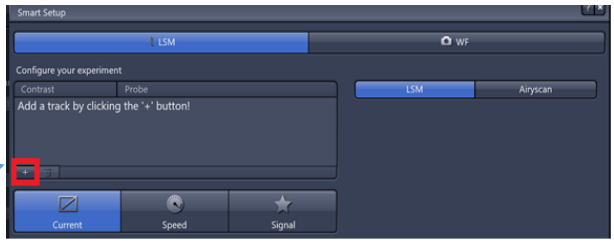
Open any previous image (image captured with LSM800/LSM900) with desired setting and click **Reuse** button in Dimension panel.



Method 3

Smart Setup

1. **Detection mode** – to work with LSM confocal or airyscan mode



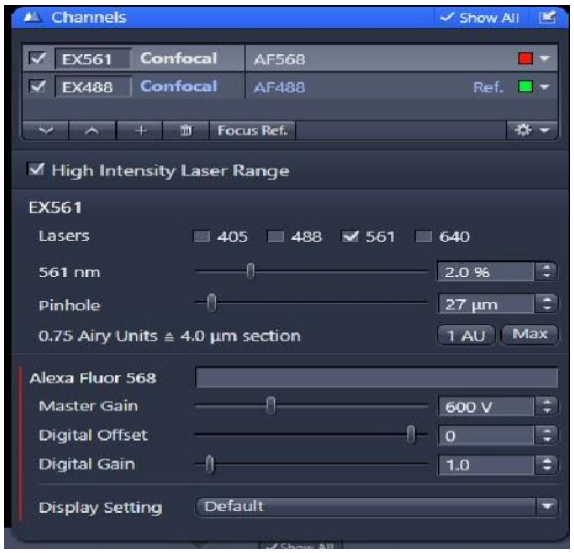
2. "**+**" Select the dye/dyes to image from the list and select the pseudocolors.

3. Select one of the following, and click **APPLY**
   a. Fastest – The fastest solution
   b. Best signal – to get the best signal with low cross-talk
   c. Smartest (line) – changes lasers after each line (good for live cell imaging with multiple colors)

Verify light path and scan settings

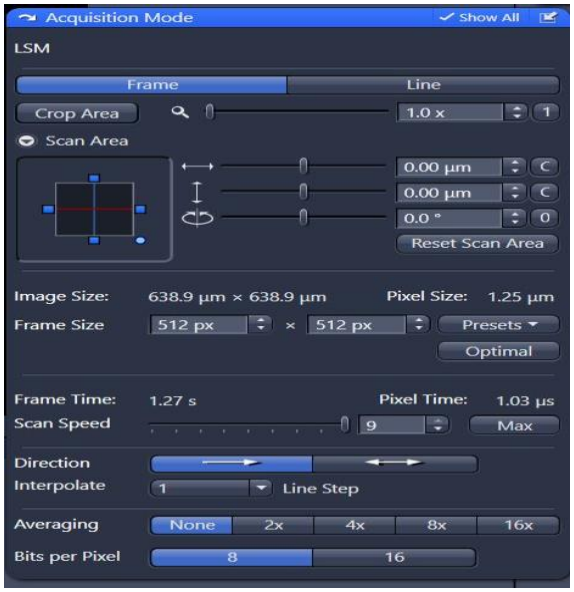
1. In **Imaging setup** window, check:
   a. Emission range.
   b. How many detectors have been engaged?
   c. For simultaneous scan, more than one detector/laser is engage for one track.
   d. Picture on the right shows: one detector is engaged and emission range is 550-617nm.

2. In **Channels** Window, check:
   a. Desired channels boxes if necessary.
   b. **Lasers** selection: select proper laser for excitation
   c. Laser light power: start from low, e.g. 0.05%
   d. With "**High Intensity Laser Range**" box is checked, you may push the laser to high power laser. Otherwise, laser power is limited to 0.01- 5%.
   e. **Pinhole**: Set the Pinhole to 1 AU (Airy Unit).
      - For colocalization studies, adjust pinhole of each channel to the same Optical Slice Thickness (µm section) as the reference channel.
      - If signal is very weak, select **"Max"** to locate the signal, before reverting pinhole to previous size for acquisition
   f. **Master Gain**: start from 600 for normal confocal and 800 for airyscan
   g. **Digital Offset**: start from 0.
   h. **Digital Gain**: leave at 1.



3. In **Acquisition Mode** window, check:
   a. **Pixel size**: Highlight "Confocal" to follow Nyquist Sampling
   b. **Scan speed**: Max, or 1µs - 2µs for pixel time
   c. **Direction**:
      - Bidirection for fast imaging; Uni-direction for co-locolization studies.
      - Use autocorrection before acquiring image if bi-direction scan is preferred.
   d. **Averaging**: increase this value to increase SNR but may introduce photobleaching.
   e. **Bits per pixel**: 16 bit, if file size is not a concern.

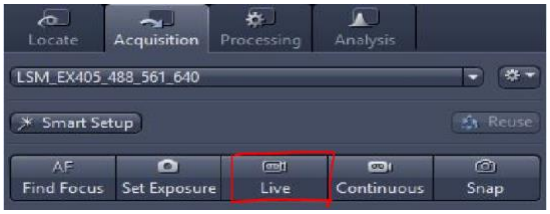
4. Repeat step for other channels.



Optimizing imaging parameters and image acquisition

1. Activate **Range Indicator**. The scanned image appears in a false-color presentation.
   - Red pixel = saturation (maximum intensity)
   - Blue pixel = zero (minimum intensity).

2. Select one channel and click on "**Live**" for continuous fast scanning and observe the intensity curve present in Histogram window.
   - **AF Find Focus** – To find the best focus automatically based on intensity profile
   - **Set Exposure** – automatic pre-adjustment of the detector gain and offset
   - **Live** – continuous fast scanning (for finding and focusing on the sample) of the selected channel. Frame size will be 512px x 512px
   - **Continuous** – continuous scanning with selected scan speed. Scans all channels and displays as overlay. Select the different displayed channels under "Dimensions" tab, then Channels
   - **Snap** – for recording a single image
   - **Stop** – for stopping the current scan procedure

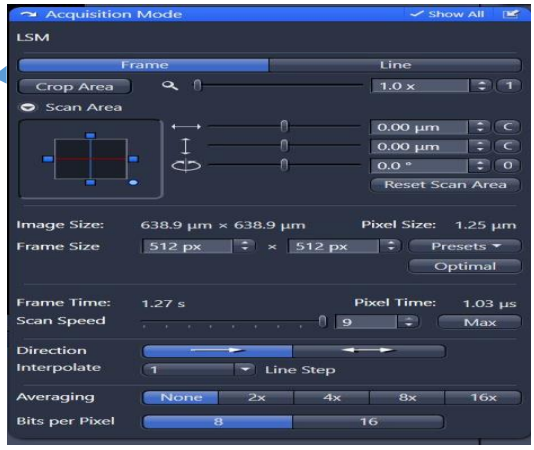


By changing the area of the graph under the histogram, the signal is brighter on the screen. There is no change in signal value, and only causes the signal to appear brighter in the image. This is especially useful for specimens prone to photobleaching. Reset the graph to show true signal level

3. Optimize image as below:
   - Fine-tune the focus with the fine adjustment knob to the brightest or preferred z- position.
   - Increase **Master Gain (**Maximum of 800**)** until a few red pixel (indicating saturation) appear in the image**;** Increase the **Laser Power**, if increasing detector gain cannot achieve the saturation or desired intensity;
   - Decrease the **Digital Offset** to reduce background signal until the desired background region is filled with some blue pixels

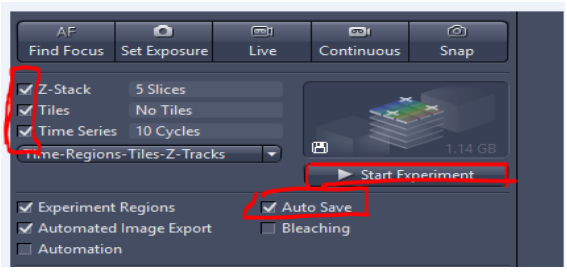
4. Go to next **channel** to repeat step 1-3.

5. **Acquisition Mode**-> If necessary, change **Crop Area**
   - When cropping area, there is a higher chance of photobleaching as the sample will be exposed to laser for a longer time. However, scan speed will be faster



6. **"Snap"** to capture image. If **Time series, Z-stack**, or **Tiles** is required, click "**Start Experiment**" instead of "Snap".

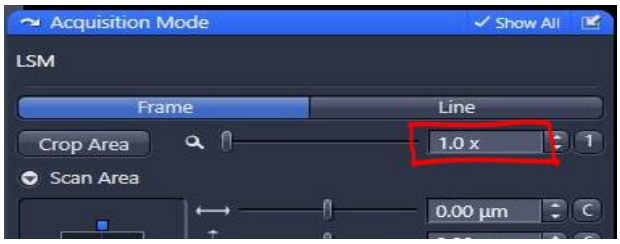
7. You may activate "**Autosave**" function so acquired images will be saved automatically to the destination you have defined in the "**Autosave**" window.
   - If not, right click on the desired image in right column "Images and documents", and select "Save as"



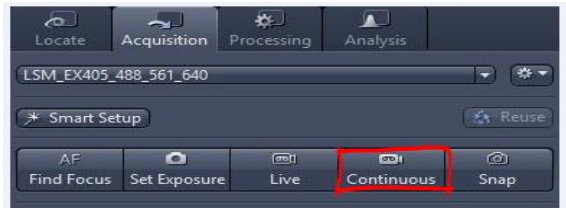
6. Setting up AiryScan Scanning

1) Make sure **63x oil lens or 63x water lens** is in use
2) Refer to the following to setup light path and fine-tune scanning parameters:
3) 4. Setting up the Scanning Track/Channel
4) 5. Verify light path and scan settings

5) **Acquisition Mode→ Crop Area**: set to x0.5 to have a larger view. **"Live"** to find interested area. Move the interested area into the center of the view.
6) **Acquisition Mode → Crop Area**: set the **area of scan ≥ 1.3x** (the larger area will require longer scanning time).
7) **Acquisition Mode → Sampling**: Highlight "**SR**"



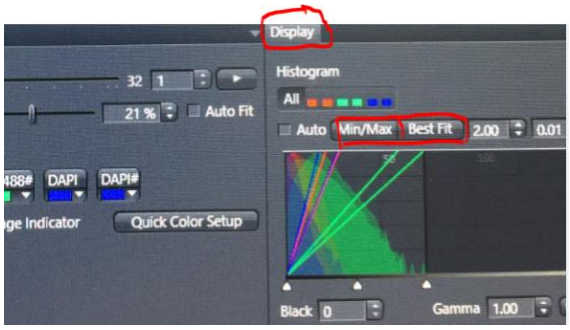
8) **Channel Window**: Select only one **channel** and go to **live** scan



9) Click on **Display** tool tab and click on "**max/min**" or "**best fit**" to measure the signal intensity range.

10) Adjust the **master gain or laser power** to set the signal range spanning 1/3 to 2/3 of the histogram range (Don't use Range Indicator).

11) Set the other channel by repeating steps 6 – 8

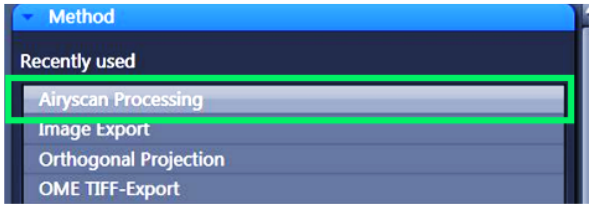


12) Check the **optimal setting** (frame size, speed, lens and scan area) following the warning notice details.

13) **Channel Window**: check all the channels.

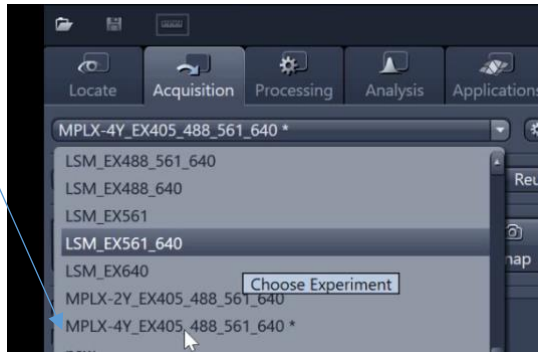
14) **Snap/Start Experiment** and wait for the scanning complete before you preview with **Airyscan** during the scanning)

15) After the scanning, go to **Processing** and choose "**Airyscan Processing**" in method, select the input image on the bottom panel window, set the parameter for filter in the middle panel window, click "apply" to generate processed image with deconvolution.



7. Multiplex Mode

1. Under **Acquisition tab,** in the drop down list choose **MPLX_4Y_EX405_488_561_640**



Note: This is the setting for multiplex acquisition to activate super resolution scan with parallelization of 4 lines in the Y-direction.

Note: To use other multiplex modes 2Y (in case you need slightly better resolution in multiplex with super-resolution) MPLX_2Y and SR-2Y settings will need to be chosen.

2. Under **Acquisition mode**, set **Sampling** as **SR-4Yz**

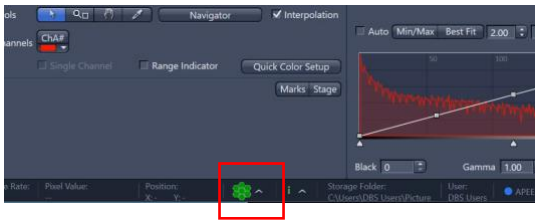
Note: This sets the xy sampling set for 2x Nyquist to achieve 4 parallelization lines in super-resolution imaging

3. Go "**LIVE"**

4. Refer to the following to setup light path and fine-tune scanning parameters:
   - Setting up the Scanning Track/Channel
   - Verify light path and scan settings

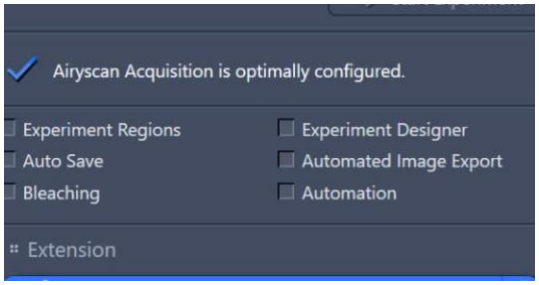
5. Focus on the Region of Interest

6. Ensure that the detector is Green in color (Status:ON) if not wait until it is turned ON.
   - If the issue persists, the laser power may have been too low for the detector, and so adjust the laser power /gain accordingly.



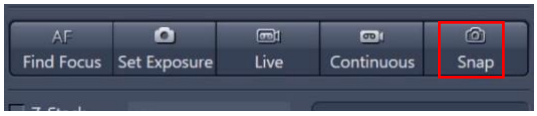
7. Check that the message '***Airyscan Acquisition is optimally configured'*** appears.

8. **STOP** the live scan

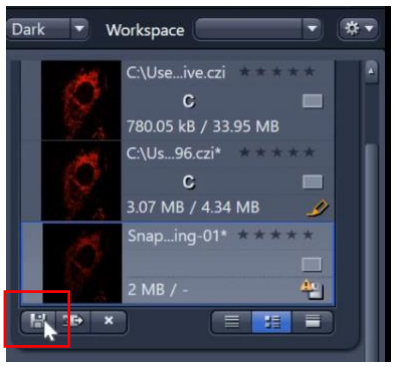


9. Click **Snap** button

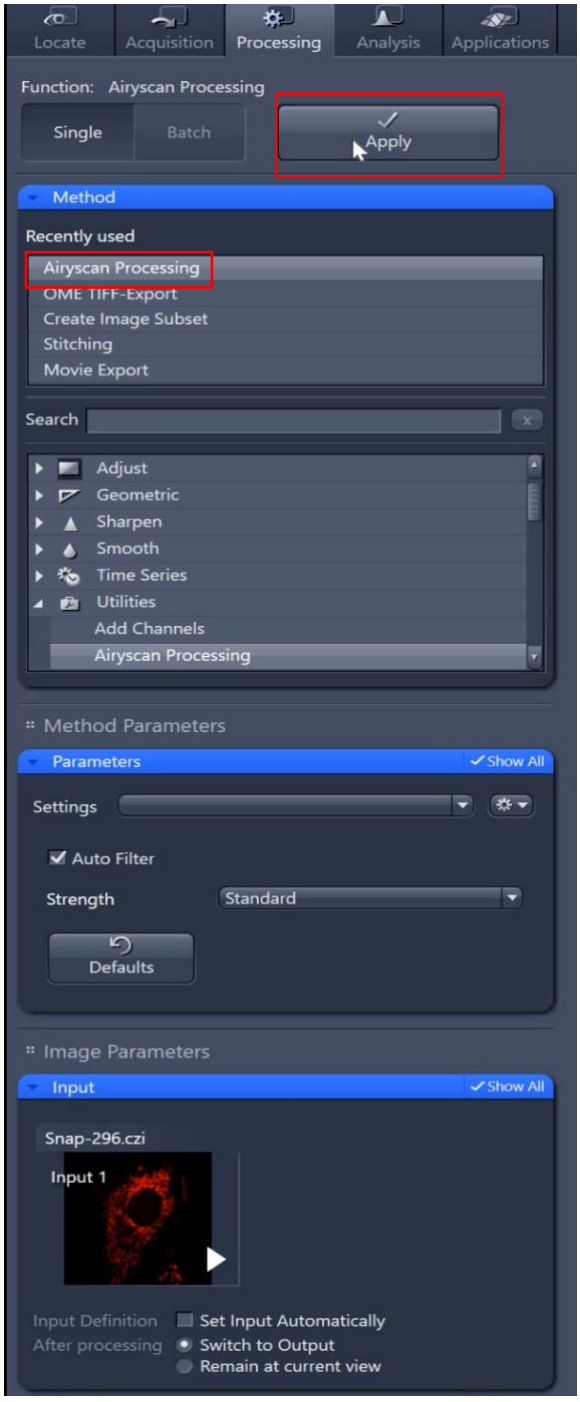
10. Image is acquired



11. To save the raw data file,
    - If autosave in enabled, under "**workspace tab**", select 'save' icon.
    - Or not, right click the image and select **"Save as"**

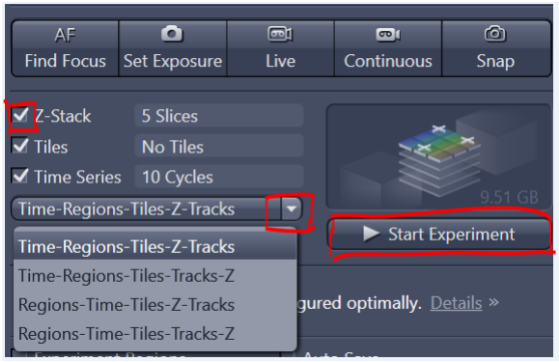


12. To save image, under **Processing tab**
    - Under **Method**
    - Select **"Airyscan Processing"**
    - Select your image under **Input**
    - Click "**Apply**"
    - Save the image under **Workspace tab**



8. 3D volume scan

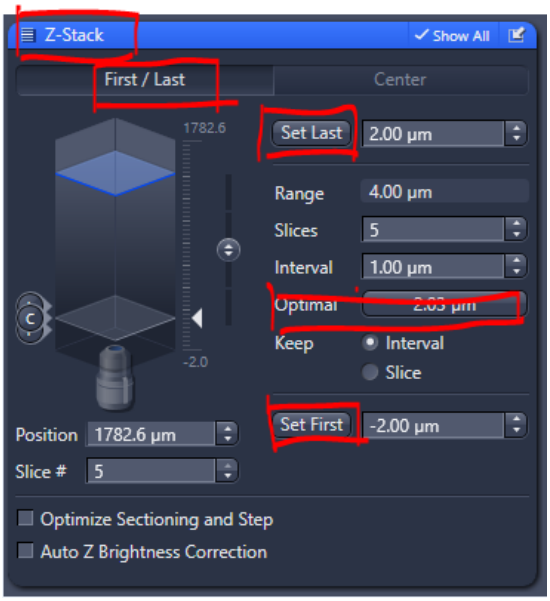
1) Check the **Z-Stack box** in the main tools area



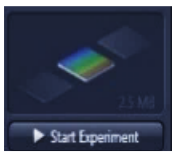
2) Choose preference scanning mode:
   a. All tracks per slice: images one slice for each channel before next slice. Good for co-localization.
   b. Full Z-stack per Track: images one channel for all slices before moving to next channel. Fast imaging.

3) Open the Z-Stack tool panel by clicking on the Z-Stack tab

4) Choose a channel that have signal throughout the interest volume and then click on live
   a. Move to the best focus plane, then under "Focus" tab select "Set zero". This helps to define the zero position of the plane of interest. This **should not be done** if the tile scan has been set up
   b. Use the **focus knob** to locate one end of the specimen and click **Set First** button. The Z value should be negative
   c. Then use the focus to locate another end of the specimen and click **Set Last** button. The Z value should be positive
   d. Stop the "**Live**".
   e. Set the Interval
      i. Keep "**Interval**"
      ii. Select **"Optimal"**: This sets interval that gives 50% overlapping between each frame thus provide better 3D image reconstruction.



5) Click on the **Start Experiment** button to start the recording of the Z-stack.

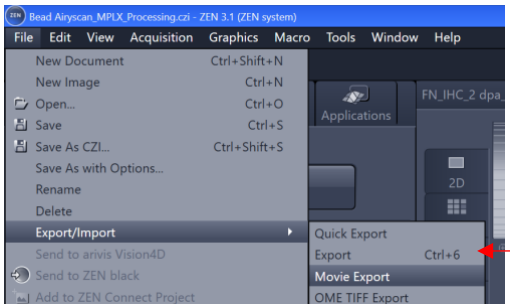


6) 3D image data presentation:
   a. Maximum Intensity Projection:
   Go to **Processing** and choose the **Orthogonal Projection** in the methods. Choose the **Projection Plane and methods** then **Apply**.

   b. 3D image video:
   Click the **3D** tab and activate **Series** tab, select the rotating axis at **Render Series**, number of frame and the **angle**. Select "**360 Panorama**" or "**Partial Panorama**". Click **Apply** and the series images will be generated in a new tab.

Exporting the 3D data (after 3D volume scan)

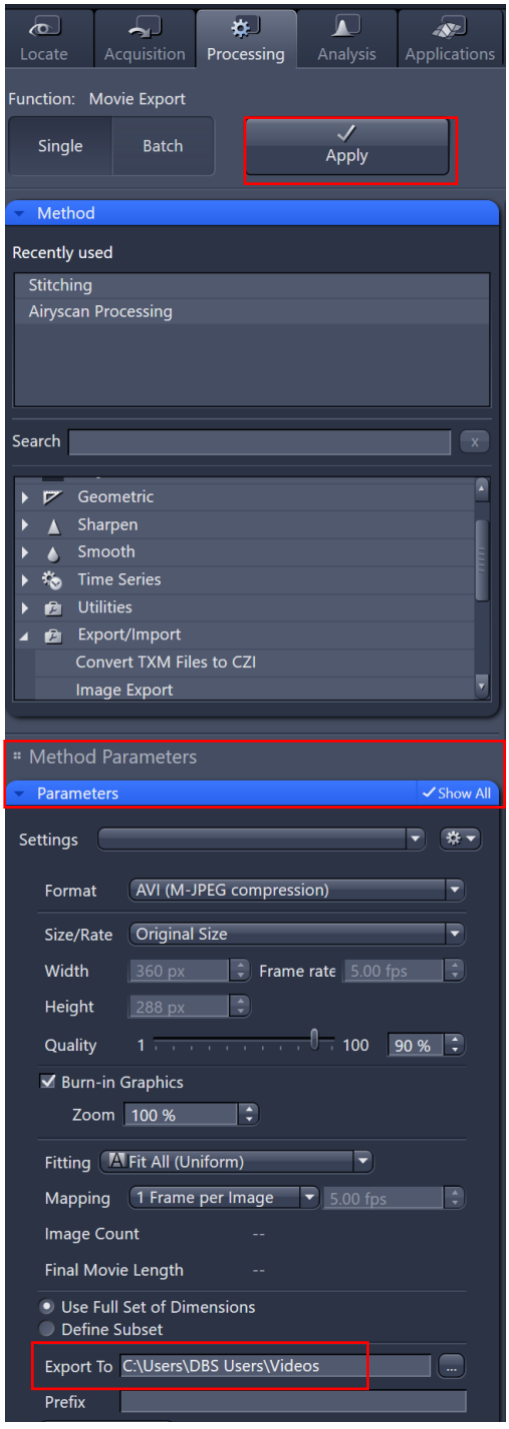
1. After acquiring the 3D volume data:
   a. File → Export/Import → Export



   b. Under Processing → Method Parameters, select the format and other parameters to be set for the export.

   c. Specify the destination folder you would like to export to

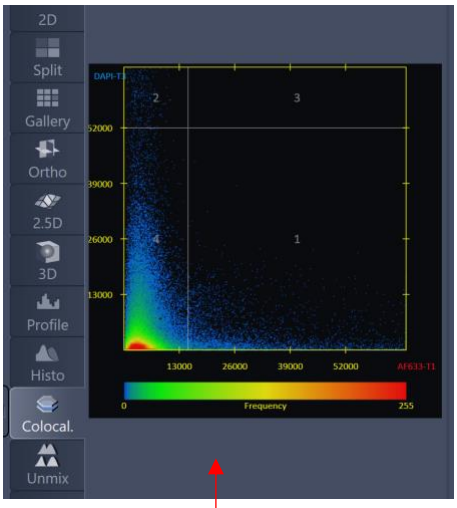
   d. Click 'Apply'



2. For 3D image video:
   a. Go to **File → Export → Movie Export** and then choose a suitable **mode (file type)** and adjust the playing speed **in Mapping (Frame per Second)** and **save** in your own folder.

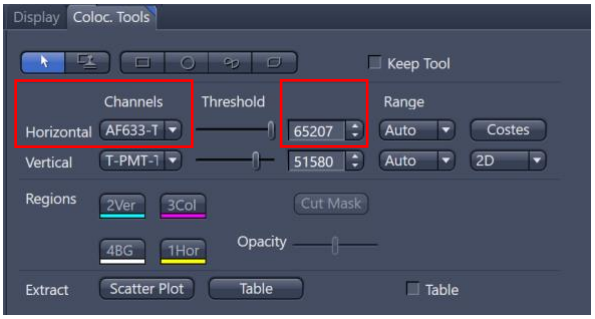
9. Colocalization Study

1. After acquisition, click on the **Colocal.** Tab

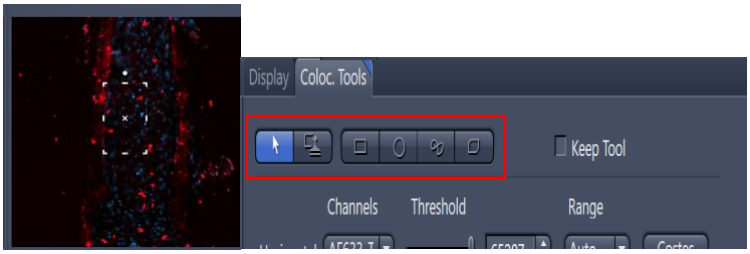


2. Under 'Coloc. Tools' and 'Dimensions', select the frame of capture you would want to analyse

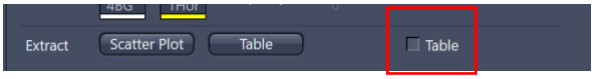
3. The colocalisation map for each frame captured is shown as the graphical representation.
The channels to be analysed for colocalisation can be defined in the horizontal and vertical planes, along with the threshold ranges to be quantified for.

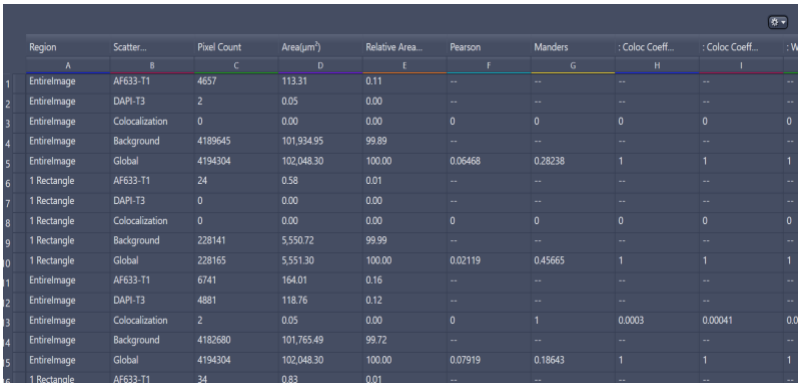


4. Using the ROI draw tools, you may select a region of interest based on the selection and analyse the particular ROI for colocalisation



5. Once the colocalisation tools are set, select **Coloc Tools -> Table** to generate the quantified results from the colocalisation analysis.

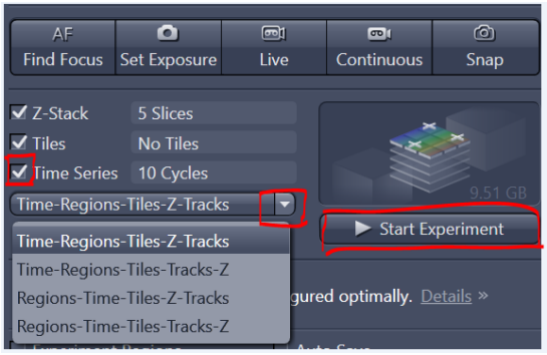




	Region	Scatter...	Pixel Count	Area(µm²)	Relative Area...	Pearson	Manders	: Coloc Coeff...	: Coloc Coeff...
	A	B	C	D	E	F	G	H	I
1	EntireImage	AF633-T1	4657	113.31	0.11	--	--	--	--
2	EntireImage	DAPI-T3	2	0.05	0.00	--	--	--	--
3	EntireImage	Colocalization	0	0.00	0.00	0	0	0	0
4	EntireImage	Background	4189645	101,934.95	99.89	--	--	--	--
5	EntireImage	Global	4194304	102,048.30	100.00	0.06468	0.28238	1	1
6	1 Rectangle	AF633-T1	24	0.58	0.01	--	--	--	--
7	1 Rectangle	DAPI-T3	0	0.00	0.00	--	--	--	--
8	1 Rectangle	Colocalization	0	0.00	0.00	0	0	0	0
9	1 Rectangle	Background	228141	5,550.72	99.99	--	--	--	--
10	1 Rectangle	Global	228165	5,551.30	100.00	0.02119	0.45665	1	1
11	EntireImage	AF633-T1	6741	164.01	0.16	--	--	--	--
12	EntireImage	DAPI-T3	4881	118.76	0.12	--	--	--	--
13	EntireImage	Colocalization	2	0.05	0.00	0	1	0.0003	0.00041
14	EntireImage	Background	4182680	101,765.49	99.72	--	--	--	--
15	EntireImage	Global	4194304	102,048.30	100.00	0.07919	0.18643	1	1
16	1 Rectangle	AF633-T1	34	0.83	0.01	--	--	--	--

10. Setting up Time Series Experiment

1) Check the box for **Time Series** in the main tools area.

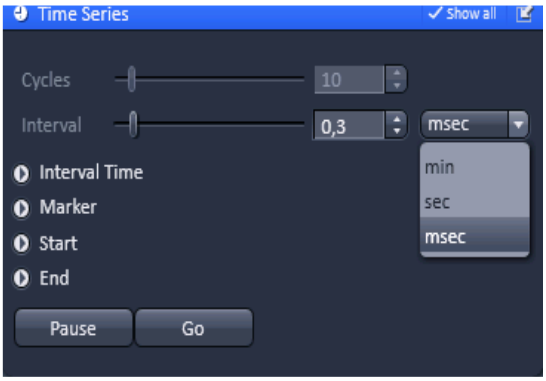


2) Open the **Time Series tool panel** by clicking on the Time Series Tab

3) Set the number of Cycle and Interval between each frame. (The scanning of each frame is included in the countdown of the Interval, therefore **Interval time should ≥ scanning time** of one multi-color frame)

4) Set the channel and acquisition parameter as necessary and then click **Start Experiment** .

5) After saving as CZI file type, video for the time series can be exported. Go to **File →Export →Movie Export**. Then choose **Mode (file type)** and define subset. Adjust the playing speed **(Mapping)** and select the destination and file name for export.

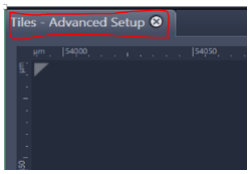


11. Tiles scan Setup: Tile/Stake/Single Position

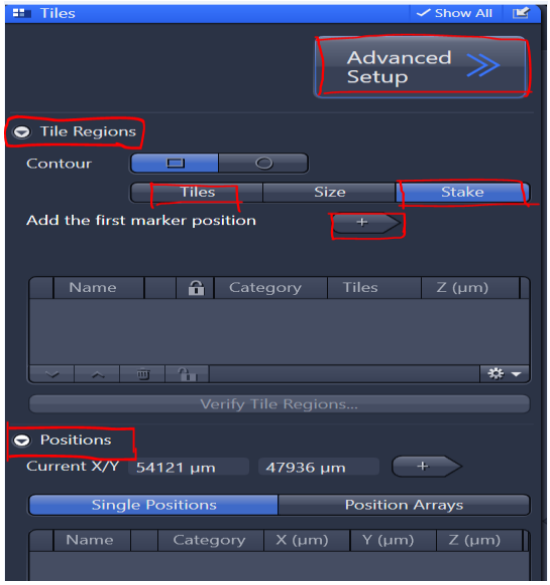
1. Check **Tile Box** in the main tools area.

2. Open the **Tile tool panel** by clicking on the Tiles tab

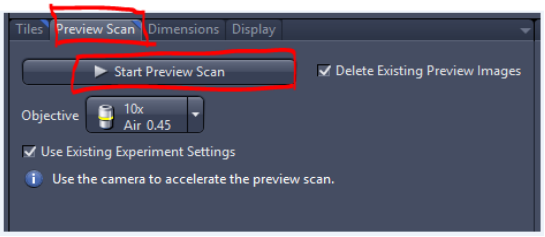
3. Click on "**Advanced Setup**" to view stage navigator.



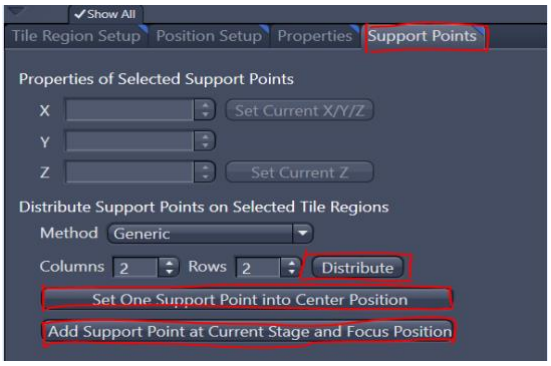
4. To define multiple imaging location by selecting **Tiles**, **Size** and/or **Stake under "Tile Regions"**, and/or **Single Position** under "**Positions**" after you move stage to the area of interest. Click on **"+"** to add tiles to stage navigator
   a. Tiles: Enter the value of X and Y to define the tile region. Eg: X=3 and Y=3 equals to tile region containing 9 tiles.
   b. Size: imaging area will be based on size in µm you defined.
   c. Stake: define an imaging area to cover all the markers you have added by "**+**".
      - Click "+" to add the first position, then move the blue box around on the stage navigator and click "+" to add a new position. The map will be updated with the selected area (shown by yellow box)
   d. Single positions: click "**+**" to add current point into the list. For individual points of interest on the sample



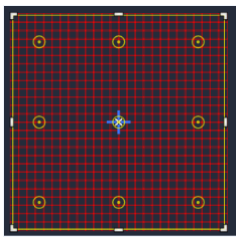
5. In **Advanced Setup** window, select "**Preview Scan**"-> "**Start Preview Scan**".



6. To add focus map into tile: Under "**Support Points**", add focus map by following of the way list below:
   a. Manually add point: click on the Tile, move to one position, "**Set Current X/Y/Z**". Repeat to add few more points if you need.
   b. Automatically add point for one tile: Click "**Distribute**" to add matric of points into the Tile, "**Add support point at current stage and focus position**".
   c. Automatically add points for all tiles: Click **"Set One support point into center position**".

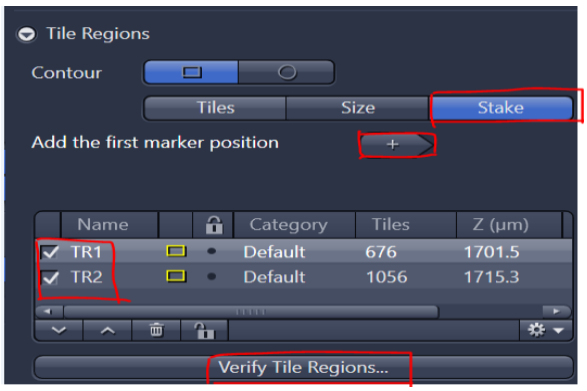


7. An example of using distribute 3x3 support points (yellow circles) into the tile.
   - It is recommended to have 5 support points, ie. at the 4 corners, and 1 in the center of the image
   - If too many support points are added, the generated image might look 'patchy'
   - Support points are not recommended not to be applied for 3D Volume Scan/Z-stack



Tiles Scan: Tile Experiments (acquiring image)

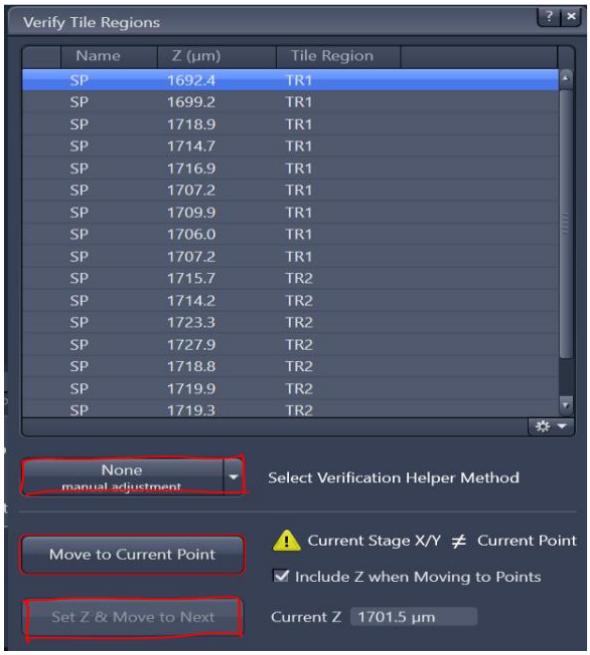
1) Tick the desired position from the Tile region list, such as TR1 and TR2 in the example.



2) Click "**Verify Tile Regions**" to call the corresponding window which shows all the support points (SP) you have defined.

3) Select the first SP and click "**Move to Current Point**", adjust focus if necessary before click "**Set Z & Move to next**". Repeat for all SPs.

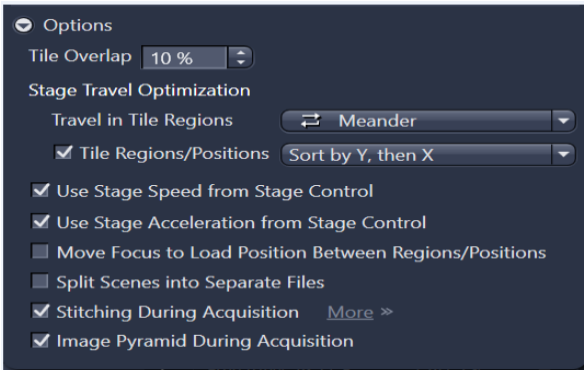
4) Or you may click on "**None manual adjustment**" which will automatically find and update the best focus position for all the SP.



5) In the "Focus Strategy" window, select: "**Use Focus Surface/Z Values Defined by Tiles Setup**".

6) Before you start, under "Tiles" tab, edit "Options" such as "**Image Pyramid During Acquisition**"

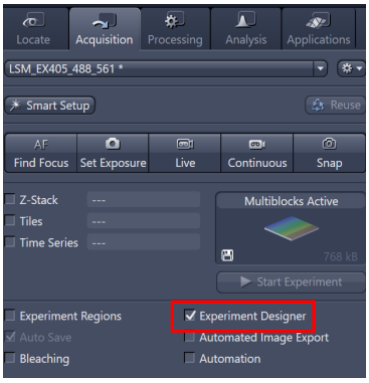
7) Click on **Start Experiment** to begin the experiment



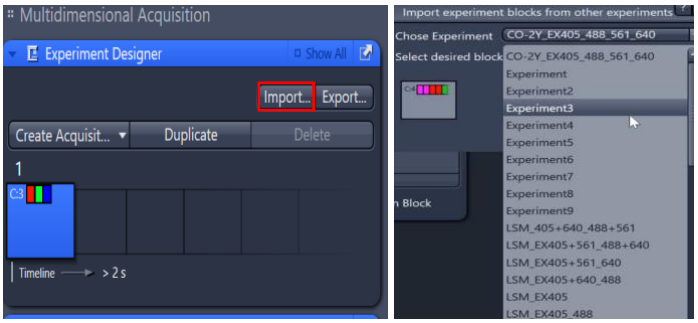
12. Experiment Designer

1. Under **Acquisition tab**, Check **Experiment Designer.**

   The Experiment Designer Tab will appear



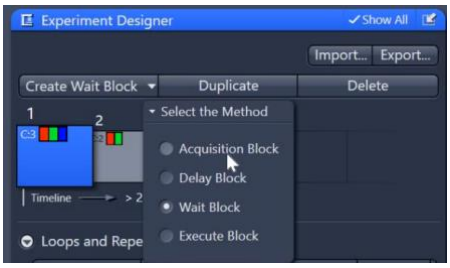
2. Click '**import**' to import the predefined experiment settings you would like to perform at each acquisition block



3. In between, there are a few different blocks that can be added

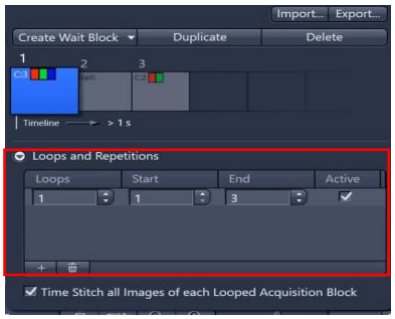
   a. Add a "wait" block to add a block that delays experiment for a specific time and then waits for user prompt to continue with the experiment,

   b. Add "delay" block to add a block that delays experiment for a specific time.

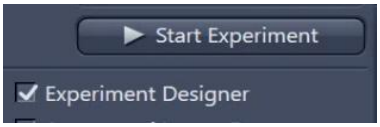


4. For each acquisition block, click and go "live" to set the desired laser parameters and experiment settings you want to perform for that acquisition block

5. Under "Loops and Repetitions" in the "Experiment Designer" you can set the number of cycles that blocks are to be repeated or looped about based on the sequence of experiments required for your design.



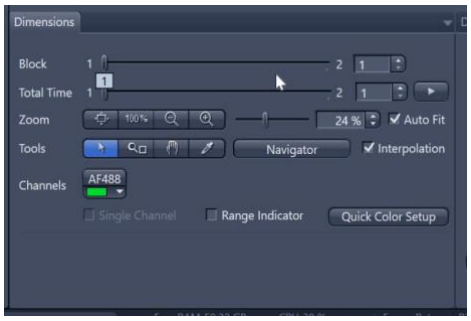
6. After configuring the experiment design, click **"Start Experiment"**



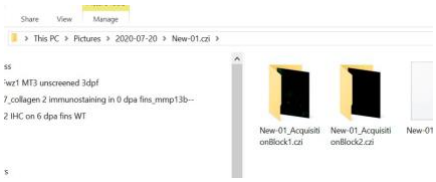
7. The real-time blocks running can be visualized in the Experiment Designer tab

   When the experiment is at the “Wait block”, there is a prompt that says “**Reached Wait block**”. You may choose to “Continue Experiment” or “Stop Experiment”

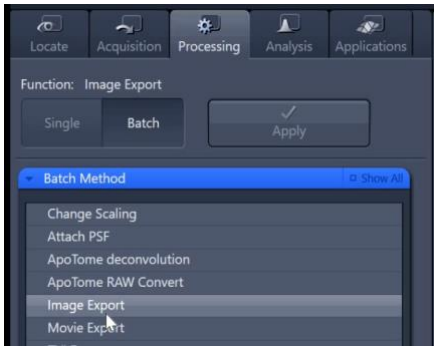
8. After the experiment is complete, under the “Dimensions” tab you can scroll to the different blocks.



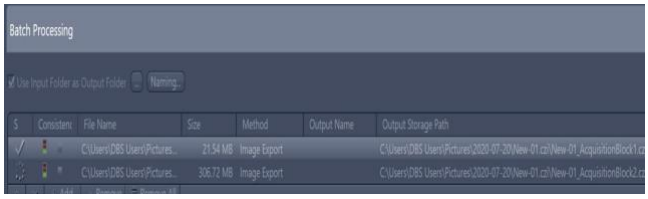
9. The rawdata of the experiment blocks have already been saved as czi files under: ThisPC\Pictures and under the folder of date of acquisition.



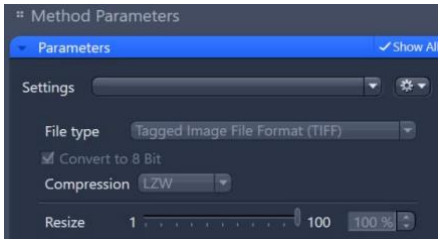
- To export as images, go to “**Processing tab**” → **“Batch” → ” Batch Method**”→ select ”**Image Export**”.



- Under “Batch Processing”, click “+” to select the czi files from which you would want to export.

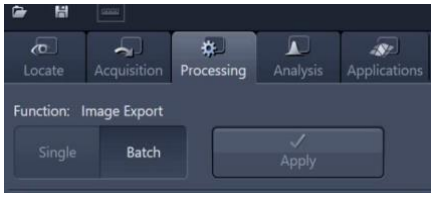


- Under “Parameters” tab select the format and type of the image you would like to export.



- Click “Apply” to start export

- The exported images will be saved under the same folder at which the raw data was saved in.



13. Definite Focus

Definite Focus.2 is a miniaturized optical unit for detecting changes in path lengths resulting from cooling or warming of microscope components or samples (focus drift).

Select this focus strategy to use the definite focus device to stabilize the focus in the Z axis, in the event of temperature fluctuations during your **Time Series** experiments.

Definite Focus.2 can be used in different ways. The software control offers the possibility to use Definite Focus.2 in time-lapse and multi-position experiments and for tile imaging.

Find Surface

The Find Surface function assists the user in locating the sample in the Z direction. First, Definite Focus.2 adjusts the movable lens to a reference value. Subsequently, Definite Focus.2 moves the Z-drive downwards the working distance of the selected objective and then starts a successive search. If Definite Focus.2 finds a signal, the Z-drive is stopped. If no signal can be found, the process is aborted, and the objective is returned to the initial position.

Store Focus / Recall Focus

Definite Focus.2 allows to store and recall a relative Z position.

This allows a sample to be focused or kept in focus, even if the sample container has changed its absolute Z position.

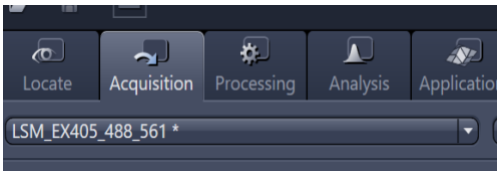
Lock Focus / Interactive Mode

The Lock Focus function is used to continuously monitor and stabilize a set focus position. This function is used to assist the user in locating an appropriate sample position by continuously monitoring and adjusting a preset relative Z position.

If the distance changes due to a temperature deviation or tilting of the sample, it will immediately be compensated. The focus position to be monitored can be changed interactively. The changed focus position will be continuously monitored and adjusted.

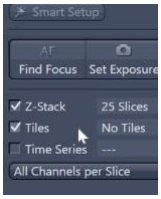
14. 3D multi-position scanning using Definite Focus

1. Under **Acquisition tab,** choose the settings of the LSM scan mode you want to use

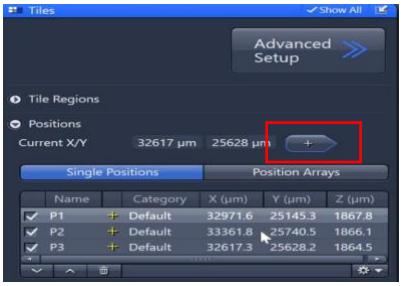


2. Go **Live**
   Refer to the following to setup light path and fine-tune scanning parameters:
   - Setting up the Scanning Track/Channel
   - Verify light path and scan settings

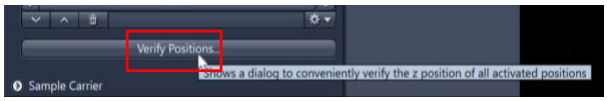
3. Check **Z-stack** (doing 3D volume) and **Tiles** (doing multi-position). You may select **Time Series** if you would want to scan the sample over time as well.



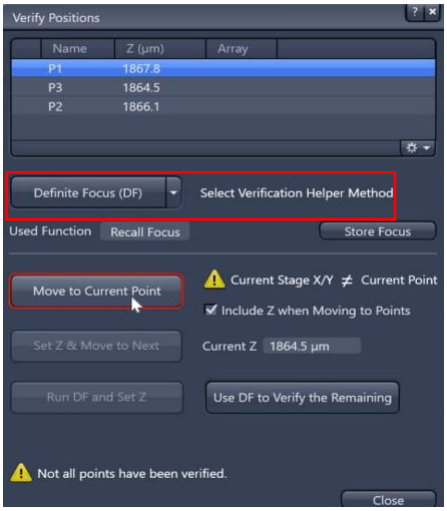
4. Under **Tiles,** under **Positions** add (**+**) positions you would want to scan at the multiple positions.
   - Note: Need not to be in focus when adding the positions.



5. Click **Verify positions**
   Under Verify Positions tab:
   Click **Move to current point**



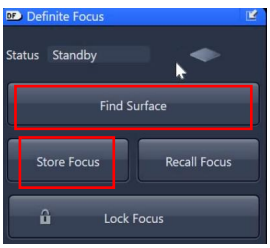
6. In the 'Select Verification Helper Method' click **Definite Focus**



7. Under **Definite Focus** tab, Click **Find Surface.** This will set the focus to the surface of the sample quickly.

8. Adjust focus manually as to focus the sample to the area of interest. Press **Store Focus**

(Note: The software will set the focus of this position you have set as a new reference for other positions as well)



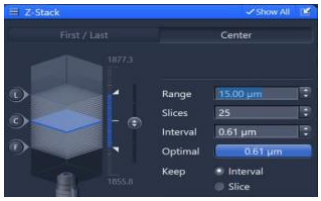
9. Under Verify Positions:

a. Click **Run DF and Set Z.**

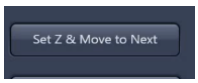
b. **Use DF to verify the remaining** to perform a fast focus along other points based on the set reference focus previously

10. Under **Z-stack tab**

- Set the range and slices parameters you would want to set for the acquisition
- Note: The parameters you set for one position applies to all other positions as well.

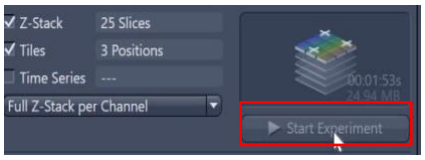


11. For further fine focus adjust, manually adjust the focus of the other positions and **Verify Positions - >** '**Set Z & Move to the Next'** for each position.



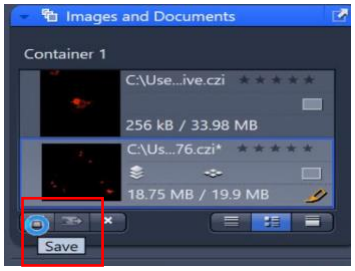
12. After setting all the focus, click **Stop** to discontinue Live position

13. Click **Start experiment** button

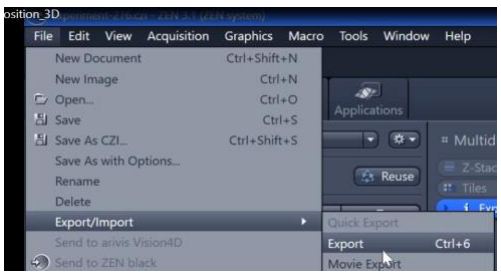


14. Save the raw data for further processing.

- Under workspace, **Images and Documents** tab, select the image file to be saved as raw data



- The raw **.czi** may also be exported as: **File-> Export/Import-> Export.**



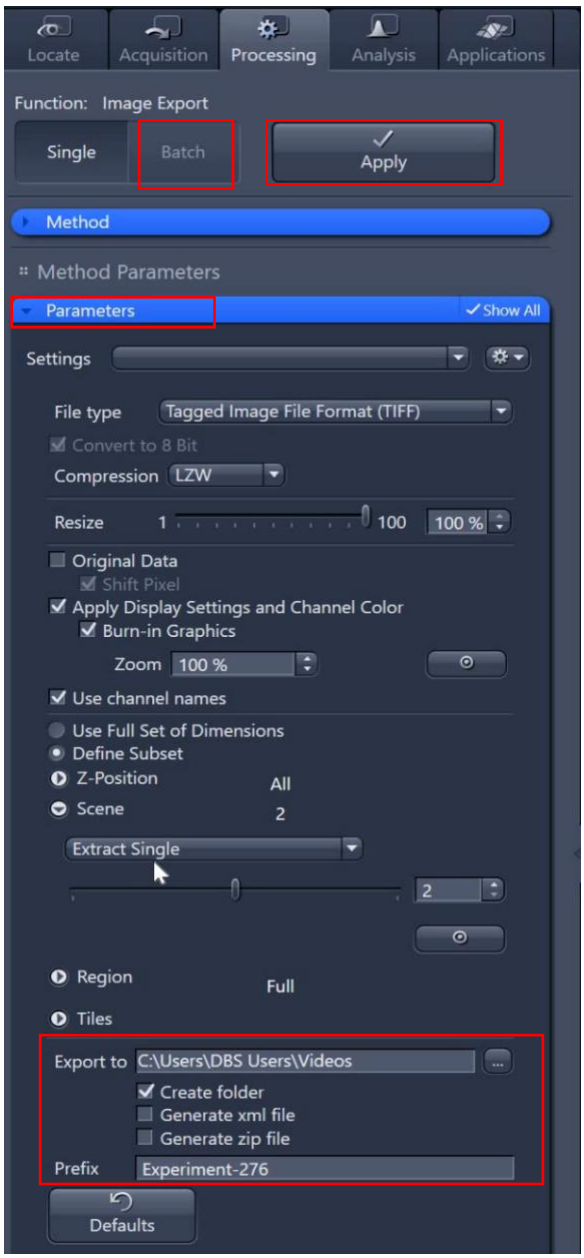
15. To save as specific file type or custom options,

    a. Under **Processing tab -> Batch**

    b. Select raw image data under Input.

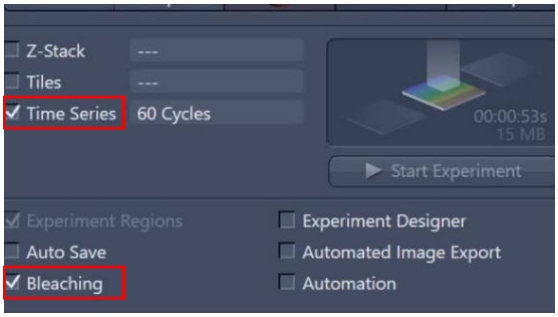
    c. Select the format options you require under the '**Parameters**' tab options.

    d. Specify how the images are to be saved and, Click **Apply** to save.



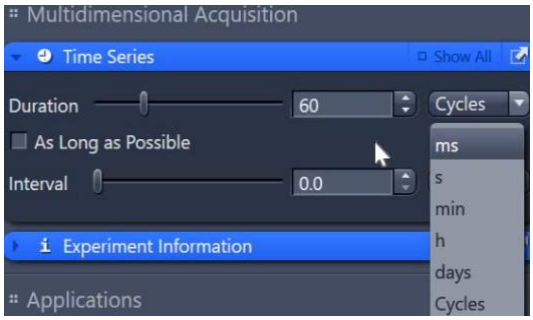
15. FRAP (Fluorescence recovery after photobleaching)

1. Under **Acquisition** tab, check "**Time Series", "Bleaching**"



2. Go **Live**
   Refer to the following to setup light path and fine-tune scanning parameters:
   - Setting up the Scanning Track/Channel
   - Verify light path and scan settings

3. Under "**Time Series**" tab you may define the number of images to be scanned (e.g. total 100 images) and 0 time interval (scan continuously)



4. To set the bleaching, Under " **Timed Bleaching**" tab
   (a) If bleach only one time, click "Start after # images" only. It defines the start of bleaching. In this case, bleach will start after the 10th image.

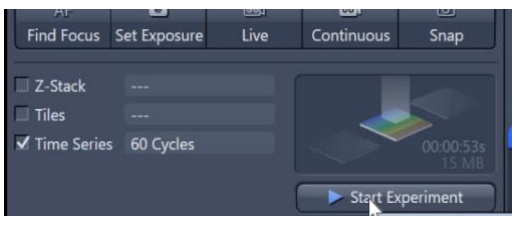
   (b) If bleach continuously, click "Start after # scans" and "Repeat after #images" (e.g. every 30 images). In this case, bleach will start after the 20th image and bleach repeats every 30 images.

   Define "iterations for area bleach" which indicates the number of bleaching repeated.

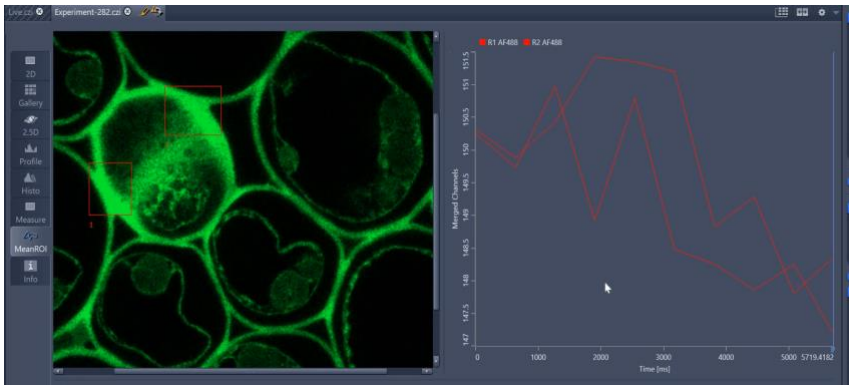
5. Under "**All Regions**" define bleaching power by selecting active laser and its power (e.g. 100% at 488nm)

6. **"Stop"** live scan

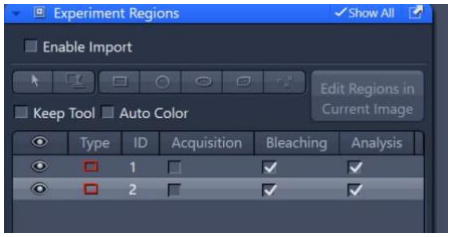
7. After completing the settings, click **"Start Experiment"**

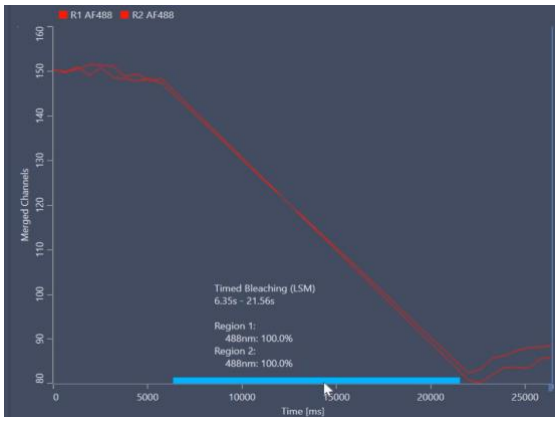


8. For online observation, click "**ROI Mean**" then select "**Mean ROI**"

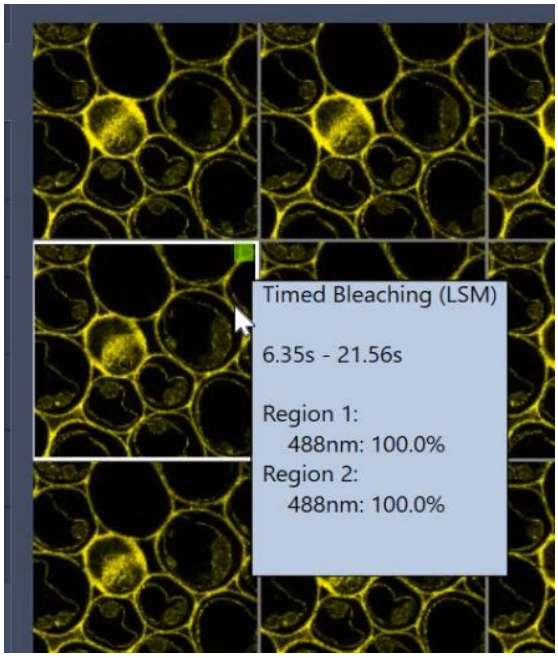


9. Select the region you bleach, Under "**Experiment Regions**" you may note the region you are observing for analysis





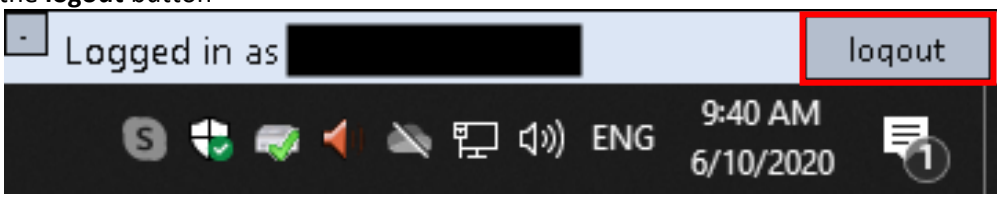
10. In gallery observation, the green square indicates the bleaching time point frame being scanned.



16. Logging out/Turning off the system

a. If there is user after you → Logging out of the system

- Clean oil objective (with **lens cleaning tissue only,** but **NOT Kimwipe**)
  - Remove residue oil from the objective lens with a dry lens cleaning tissue
  - Clean the lens with a new lens cleaning tissue with 100% absolute ethanol
  - Dry the lens with another new lens cleaning tissue
- Change objective to empty position (5x button on touchscreen control panel).
- **Exit** ZEN Software (not just clicking on the cross to close the window), transfer data through CBIS Kraken or directly to shared workstation.
  - Wait for microscope icon at bottom right to disappear
- Click on the **logout** button



- Enter user log book according to **Actual** use time, report any issues faced.

b. If there is no user after you → Turning off the system

- Clean oil objective (with **lens cleaning tissue only** but **NOT Kimwipe**)
  - Remove residue oil from the objective lens with a dry lens cleaning tissue
  - Clean the lens with a new lens cleaning tissue with 100% absolute ethanol
  - Dry the lens with another new lens cleaning tissue
- Change objective to empty position (5x button on touchscreen control panel).
- **Exit** ZEN Software (not just clicking on the cross to close the window), transfer data through CBIS Kraken or directly to shared workstation.
  - **Wait** for microscope icon at bottom right to disappear before shutting down PC
- Do not need to click **logout** button, just shut down desktop (labeled 5) as per normal
- **Switch off the switches 6, 4, 3, 2, 1 in this order**
- Enter user log book according to **Actual** use time, report any issues faced.
- Cover the scope with Zeiss blue scope cover.