Version 1.2 2025

Live-SR Super Resolution/TIRF Standard Operation Protocol

IMAGING AND FLOW CYTOMETRY CORE
CENTRE FOR PANOROMIC SCIENCES
THE UNIVERSITY OF HONG KONG

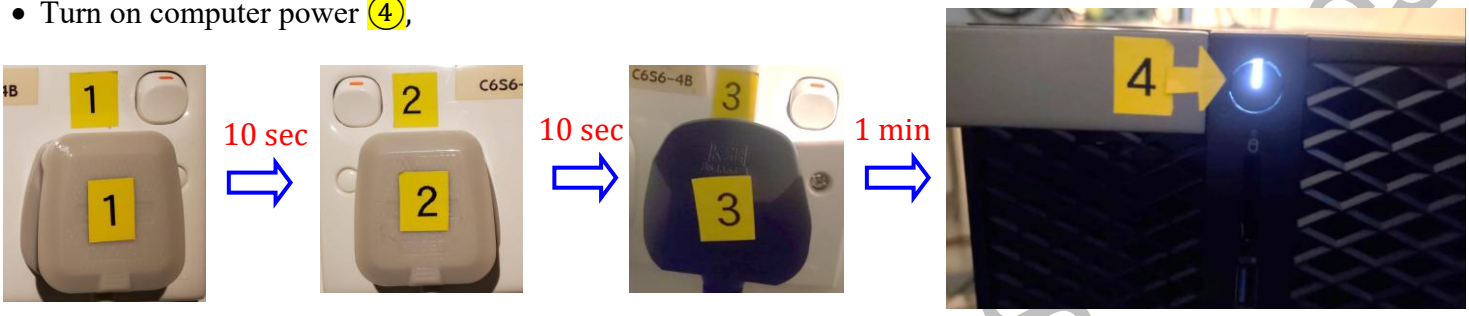
Imaging and Flow Cytometry Core

Contents

Turn on system

Please sign on the log sheet before switching on system.

- Switch on main power control on the wall ①, leave 10 seconds before next step
- Switch on main power control on the wall ②, leave 10 seconds before next step
- Switch on main power control on the wall ③, leave 1mins before next step
- Turn on computer power ④,

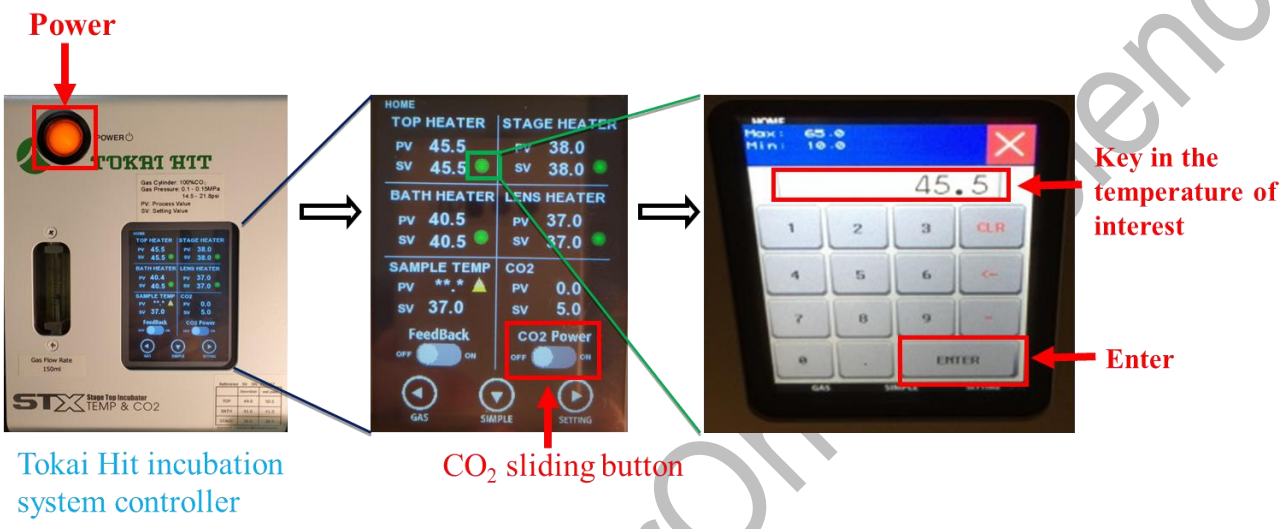


- Click to log into **USER** account at the startup screen. The password is below the PC screen. leave 1mins after Windows startup.
- Start the MetaMorph software

Set the temperature and CO2 control for live cell imaging

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

- 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_2 sliding button is turned **ON**.
- Key in the temperature of interest, then press Enter. (Please do change temperature back to default setting if any modification is made)



- 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 gas supply.
- Add MilliQ water into the water chamber and covered if overnight(s) acquisition is required.

Imaging and Flow Cytometry Core

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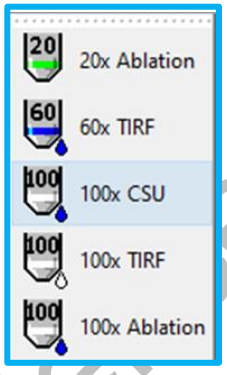
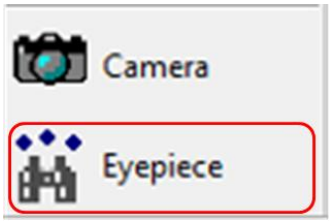
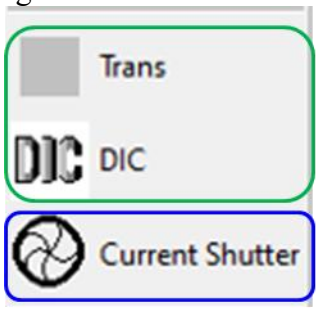
Sample locating and focusing

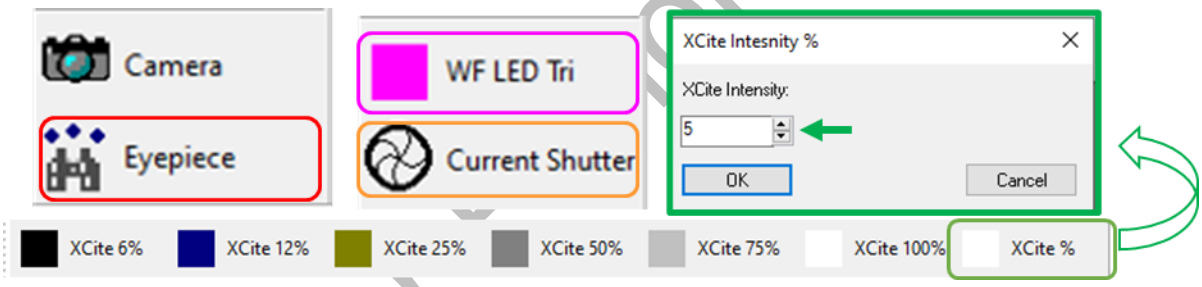
- Select objective
  - select 100×TIRF or 60×TIRF for TIRF imaging
  - select 100×ablation or 20×ablation for ablation
  - select 100×CSU for super-resolution imaging
- Apply a drop of immersion oil if oil objective is used
- Place your sample, make sure the coverslip of the slide is facing down
- To view Brightfield under the eyepiece, click Eyepieces →select Trans or DIC select Current Shutter → push the Bright Field knob up/down to increase/decrease the light brightness.







- To view Fluorescence under the eyepiece, click Eyepieces →select WF LED Tri → select Current Shutter→to adjust intensity, choose XCite 6%, XCite 12%, XCite 25%, XCite 50%, XCite 75% or XCite 100%. Or click XCite%, input number into the space and click OK.



- Press the right arrow of the DISP button 4 times to find X: Y: and Z: position panel.
- Move the Stage Controller to adjust XY position (XY speed can be adjusted by XY button:)
- Focus the sample with the focusing knob → Clockwise_Down; Anti-clockwise_Up (Focusing speed can be adjusted by Z button:)
- Switch on the "PFS" and adjust the focus with focus knob to lock the focal plane of interest.

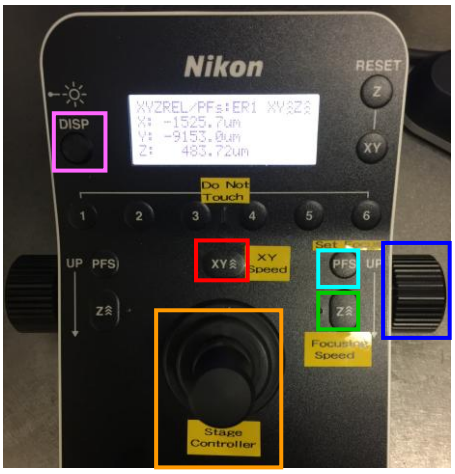
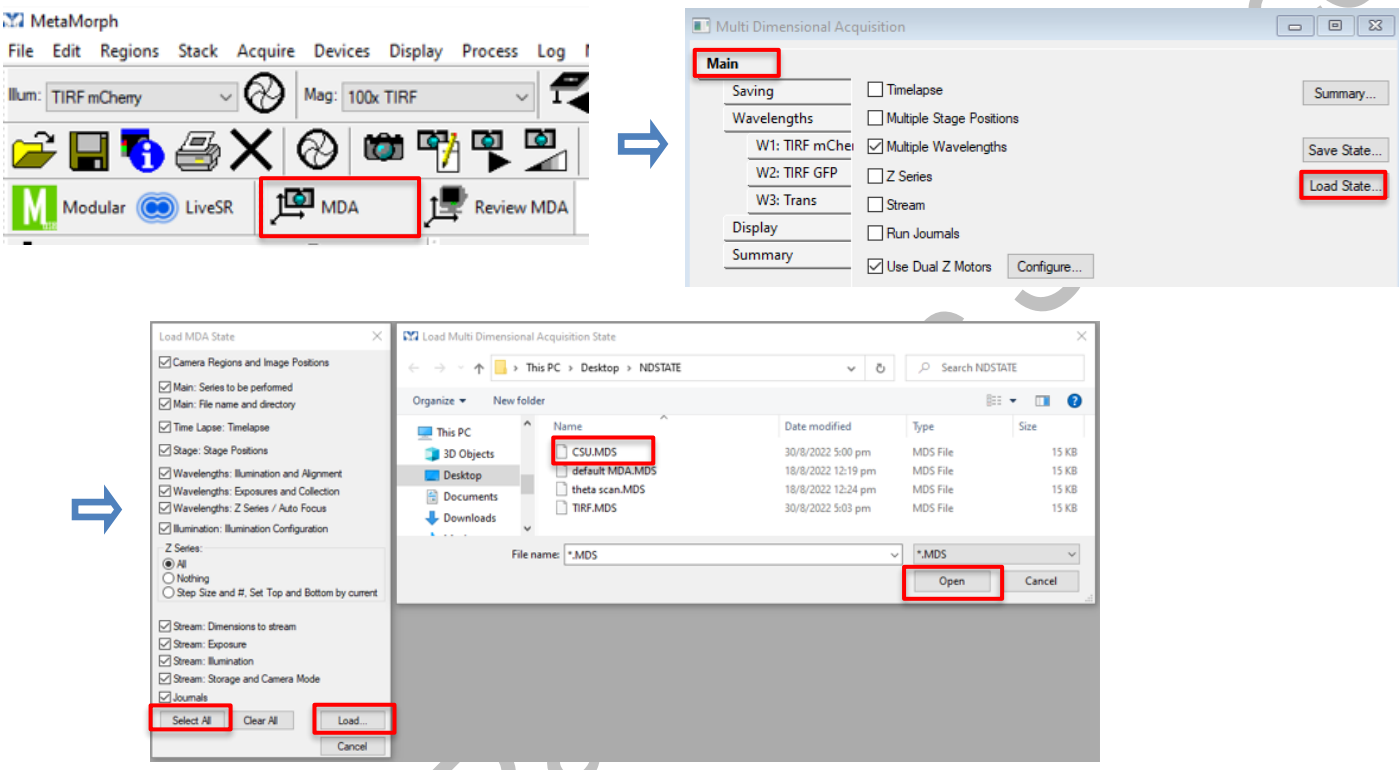


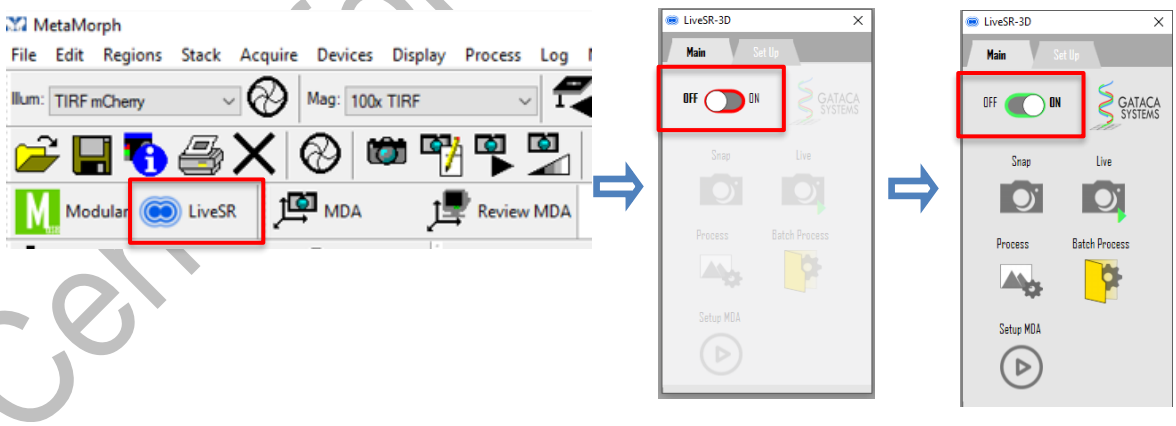
Image Acquisition

Live SR(Super-resolution) Imaging

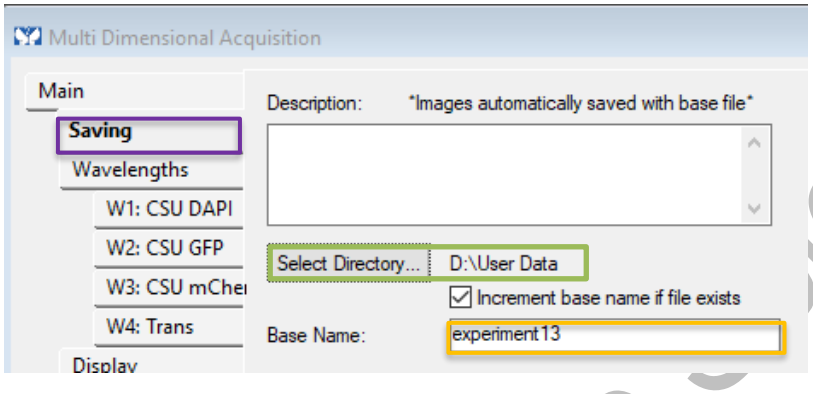
- Select 100xCSU objective. Apply one drop of immersion oil on the objective.
- Click MDA on the task bar
- Go to Main>check Load State
- Click Select All>check Load > choose CSU.MDS from Desktop>NDSTATE folder> check Open



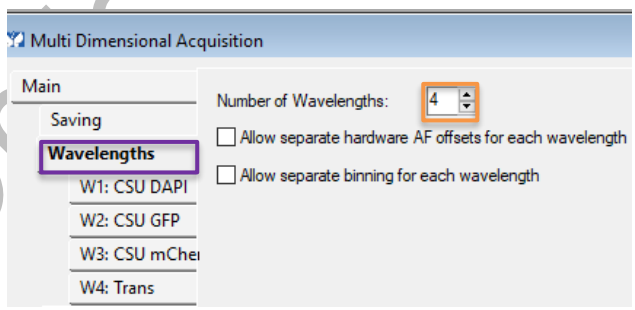
- Select **CSU cube**.
- Click Live SR and turn it on.

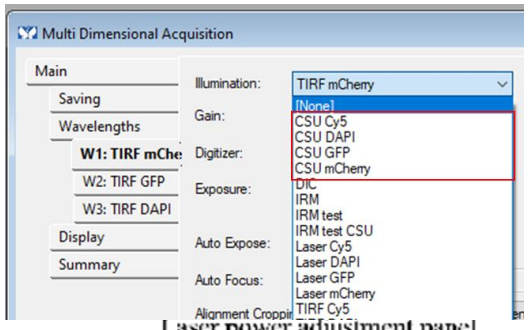


- Click **Saving** → **Select Directory** (all data should be saved in D drive/USER under your name)
  - Type in the base name of your file (experiment or date or etc.) in **Base Name**.
  - Do not use digits at the end of the base name, a digit will be added by the system according to the acquisition sequence.
  - Another suffix will be added for record time series image (t1, t2….) or multi-stage-position image (s1, s2….).
  -

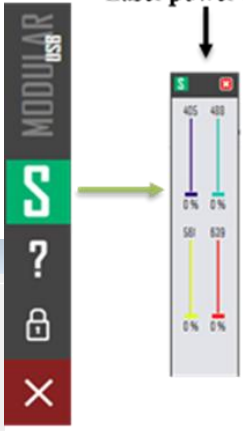


- If multiple fluorescence channels are required,
  - Check the box of "Multiple wavelengths" in the main menu
  - Click **Wavelengths**
- Key in the **number** of channels in "Number of Wavelengths
- Select the proper wavelength to set the required "Illumination" from the illumination dropdown list.
  - Select "CSU DAPI" for Blue emission (e.g. BFP) channel
  - Select "CSU GFP" for Green emission (e.g. GFP) channel
  - Select "CSU mCherry" for Red emission (e.g. mCherry) channel
  - Select "CSU Cy5" for Farred emission (e.e. Cy5) channel.

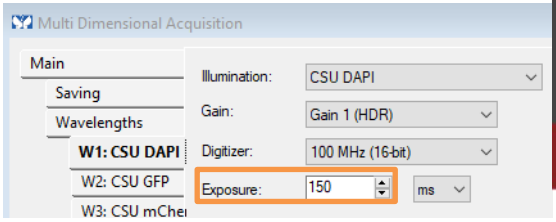




- Preview the image on screen by clicking Live or snap to review the intermedium image (image before processing)
- Adjust below parameter for each channel
  - laser power (Click S button on Modular to open laser power adjustment panel)
  - key in exposure time in Exposure

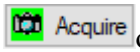


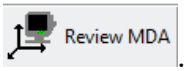
Laser power adjustment panel



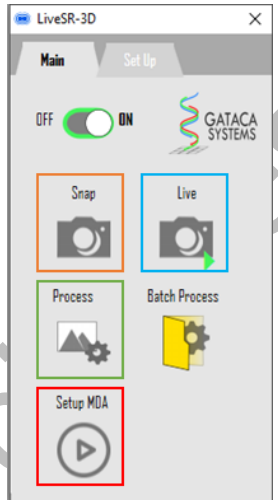
- Two methods as below to acquire SR image.

Method 1

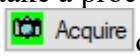
- Click Acquire 

on MDA to get intermedium image
- Open ND file in Review MDA 

.
- Select intermedium images (which has no SR at the end of file name) and click Process to get the SR images.



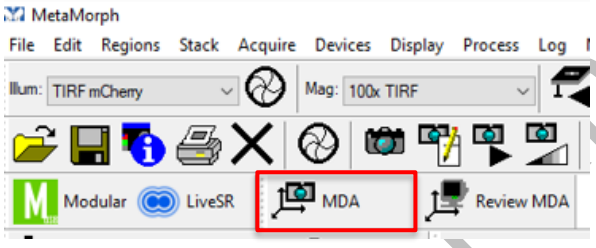
Method 2

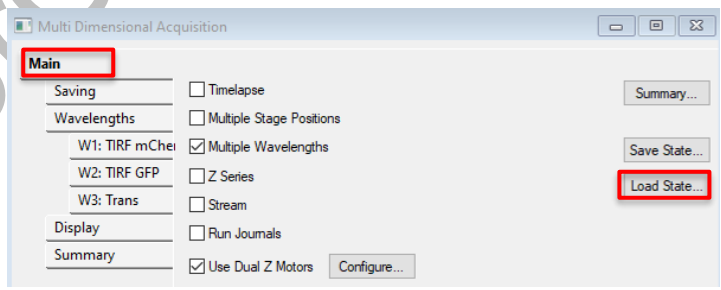
- Click live to review intermedium image for each channel.
- Set up 2.5times higher laser power or longer expose times.
- Click Setup MDA.
- Click Snap to take a processed Live SR image for current channel.
- Click Acquire 

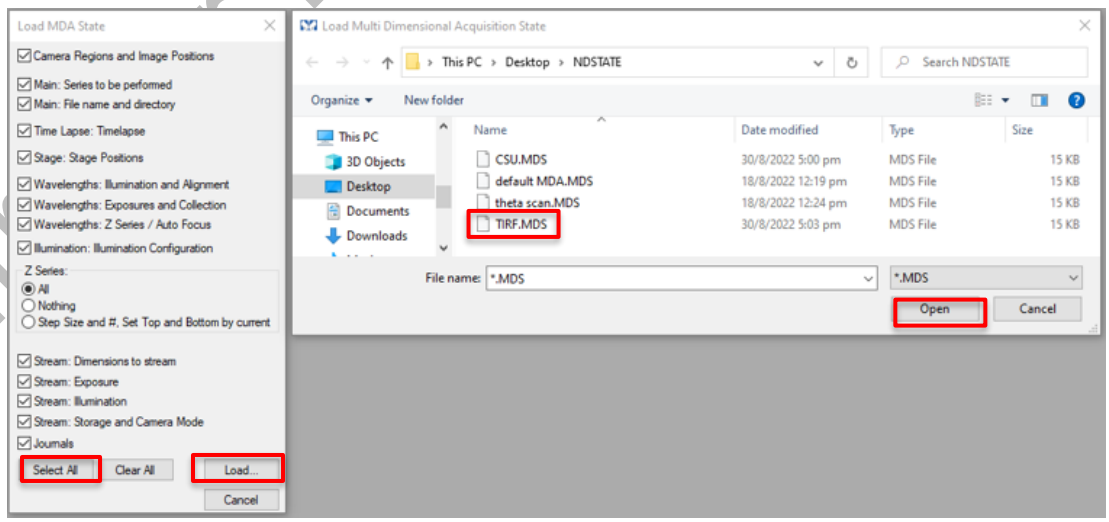
on MDA. Both intermedium image and SR image will be acquired and save to your selected directory.

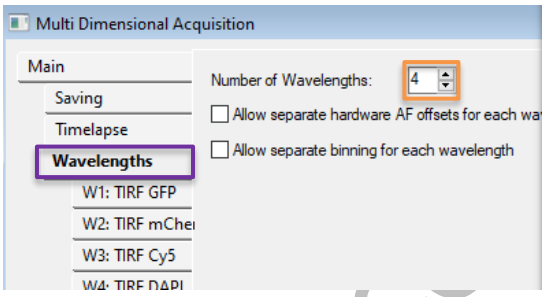
TIRF imaging

- Select 100xTIRF objective. Apply one drop of immersion oil on the objective.
- Click MDA (Multi Dimensional Acquisition) on the task bar
- Go to Main>check Load State
- Click Select All>check Load to choose TIRF.MDS from Desktop>NDSTATE folder> check Open

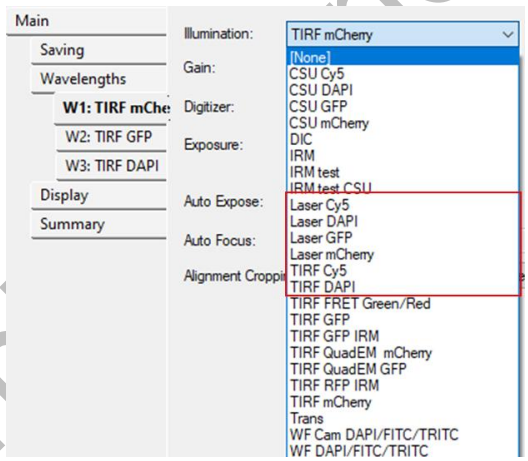




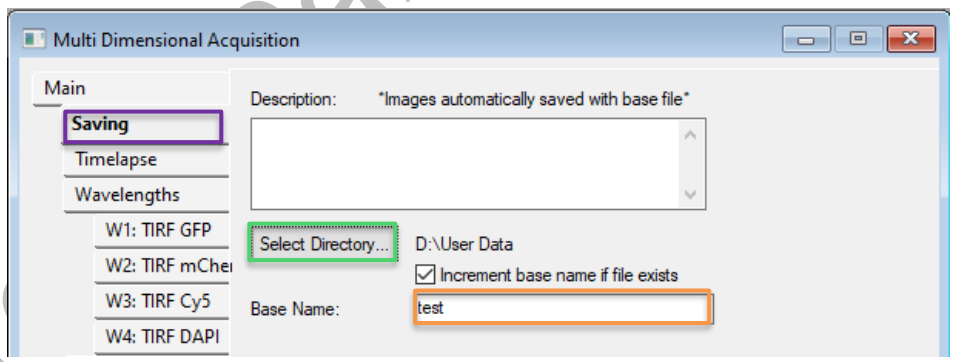




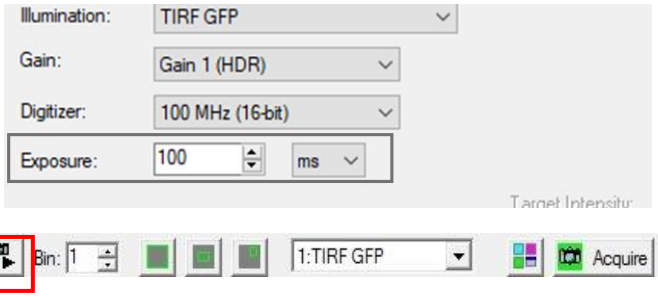
- If multiple fluorescence channels are required,
  - Check the box of "Multiple wavelengths" in the main menu
  - Click **Wavelengths**
  - Key in the **number** of channels in "Number of Wavelengths"
- Select the proper wavelength to set the required "Illumination" from illumination dropdown list.
  - Select "TIRF DAPI" for Blue emission (e.g. BFP) channel
  - Select "TIRF GFP" for Green emission (e.g. GFP) channel
  - Select "TIRF mCherry" for Red emission (e.g. mCherry) channel
  - Select "TIRF Cy5" for Farred emission (e.e. Cy5) channel.



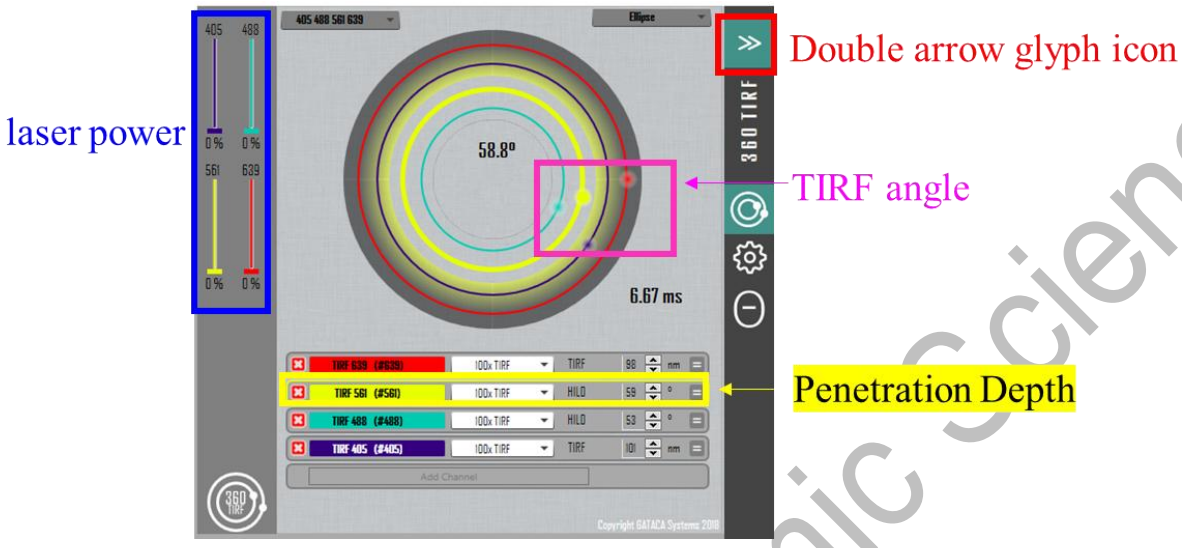
- Click **Saving** → **Select Directory** (all data should be saved in D drive/USER under your name)
  - Type in the base name of your file (experiment or date or etc.) in **Base Name**.
  - Do not use digits at the end of the base name, a digit will be added by the system according to the acquisition sequence.
  - Another suffix will be added for record time series image (t1, t2….) or multi-stage-position image (s1, s2….).



- Preview the image on screen by clicking Live  at the bottom of "multi-dimensional acquisition" and adjust the focus and parameters (Exposure Time, Laser Power, TIRF angle).



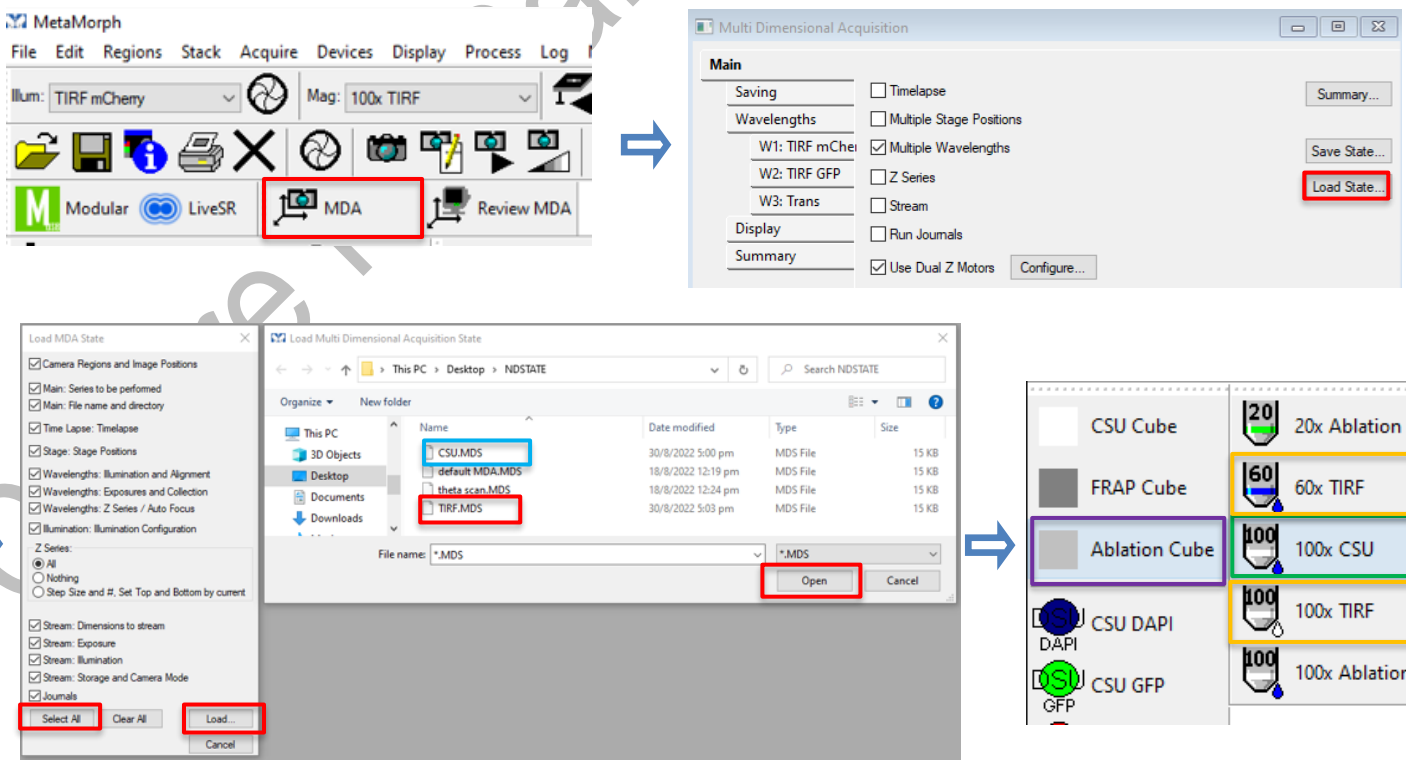
- Click double double arrow glyph icon over the "360 TIRF" on the right panel
- Adjust **laser power** by moving the slider bar for each laser
- Adjust TIRF angle for each laser by dragging the edge of the circle from center to edge. The actual Angle and Penetration Depth are shown below the circle.



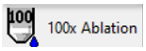
- Click **Acquire** at the bottom of MDA right to start acquisition.

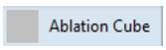
FRAP

- If you are using **TIRF with FRAP**, please load **TIRF configuration** in MDA. Then choose **TIRF objective**.
- If you are using **CSU with FRAP**, please load **CSU configuration** in MDA. Then choose **FRAP cube** and **CSU objective**.



Ablation (Only applicable for ablation, please skip this step if it is not needed):

- Choose 100xablation objective 

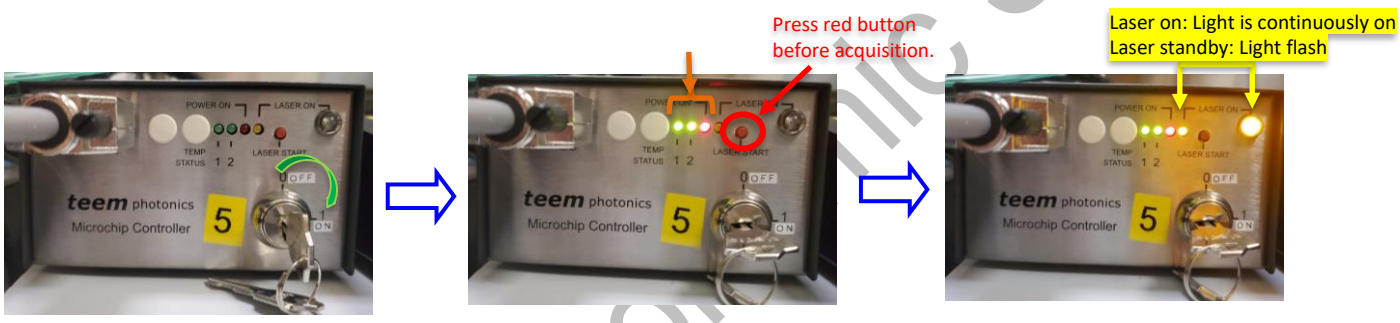
- Choose ablation cube 

- Turn on 355nm laser.

Please be noted that 355nm lasers are hazardous for eye/skin exposure. For safety reason, the 355nm will go standby mode (shutter will switch off) when change to eyepiece mode.

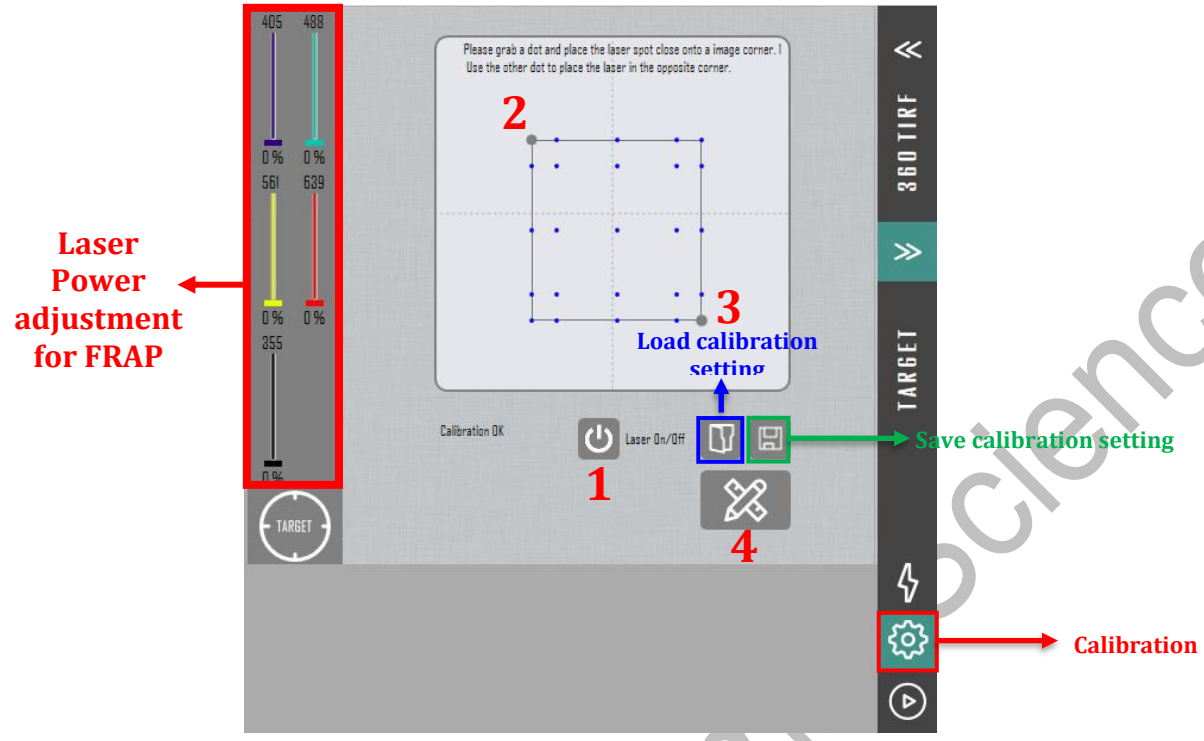
– Switch on laser by turning key clockwise for 90 degrees ⑤
– The lights of POWER ON will flash for a few seconds.
– Press red button before acquisition. *

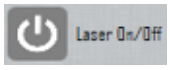
*Two lights of LASER ON will on continuously means 355nm laser is ON. Laser can only be switched ON under camera mode and go standby under eyepiece mode. You need to press red button to start laser every time when change back to camera mode.

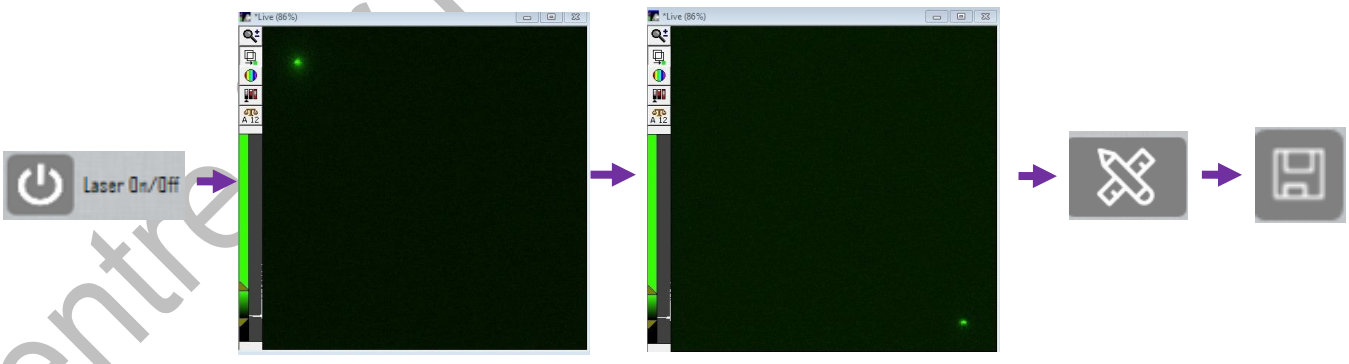


Targeted laser calibration

– Replace with plastic fluorescence slide.
– Preview the focused image on screen by clicking **Live**  at the bottom of MDA
– Select "Calibration"  on the right black bar below the "**TARGET**"



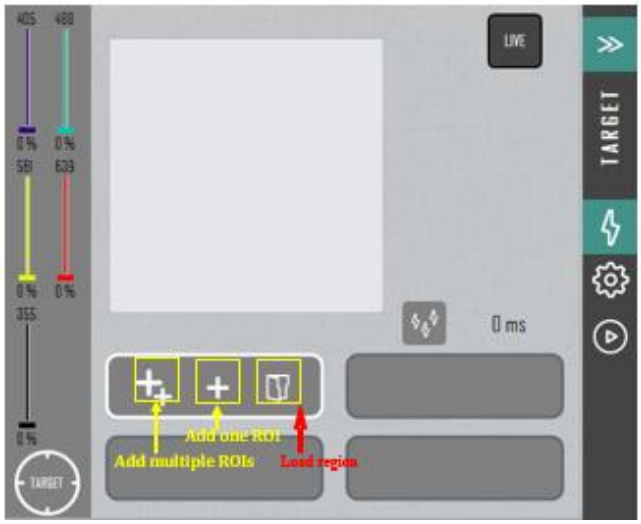
- Set exposure time to 100 ms and laser power of FRAP to 0% or 0.5% (lower than 1%).
- Click on the icon  to activate the targeted laser.
- Through MetaMorph live window, move the light spot in the blue grid calibration area to place the laser spot to the top left corner by dragging the Grey Point 2. Then dragging the Grey Point 3 to bring the laser spot to the bottom right corner.
- Click on the calibration button  to begin calibration.
- When calibration is done, click on the save icon  to save the calibration setting to your folder.

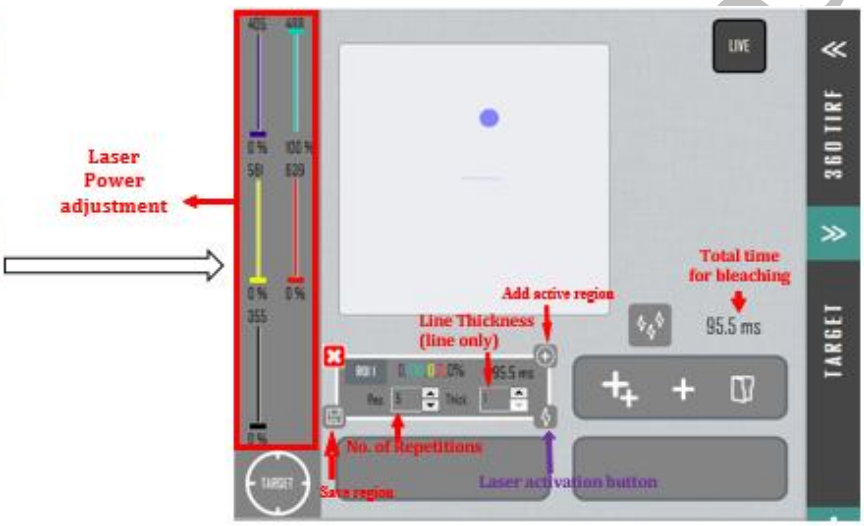


FRAP/FLIP/Ablation Experimental Protocol

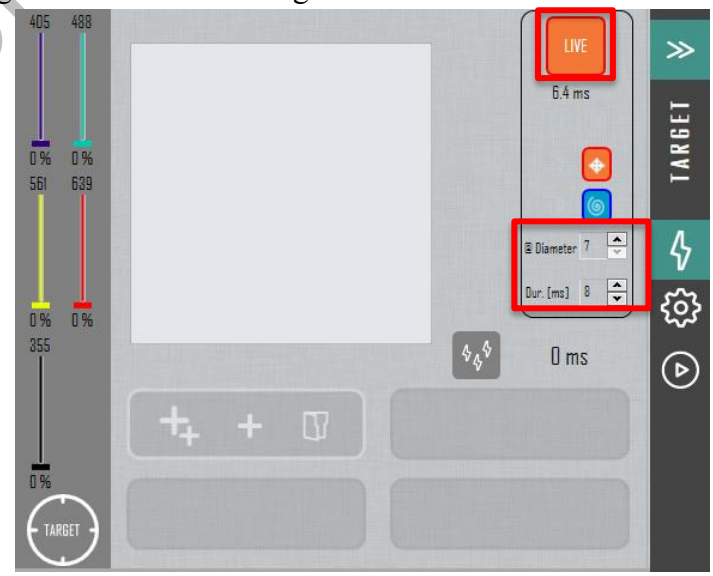
- Preview the image on screen by clicking **Live**  at the bottom of MDA and adjust the focus and parameters (Exposure Time and Laser Power).
- Mark the region of interest (ROI) through using the region tools  at the top of MetaMorph, then add one ROI by click  button or add multiple ROIs by click  button.

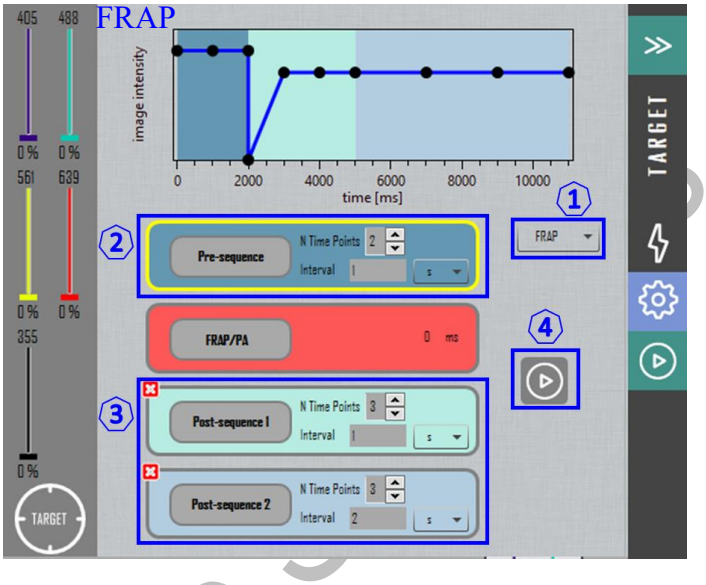
- Adjust bleaching parameters.
  - No. of repetitions
  - Laser power (405nm/488m/561nm/639nm for FRAP, **355nm** for ablation)
  - Line Thickness (for Line only)
- The testing bleaching could be done by click laser activation button 



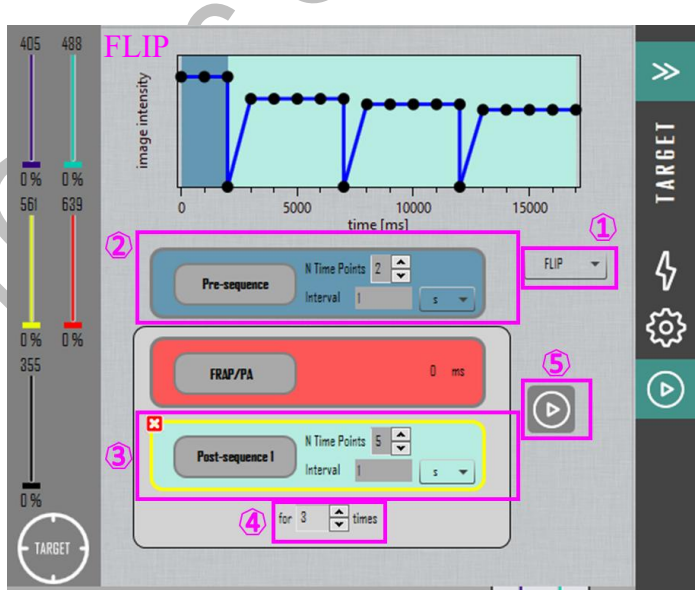


-  is a tool which enables to interactively target the laser while being in live mode.
- Click and expand the function of  .
- Click on the certain position in the MetaMorph live window to activate the laser.
- Select  to define the duration length of targeted laser.
- Select  to define the replication times of targeted laser. Each time takes 0.65 ms. If you set replication times as 2, the total duration shown under LIVE will be 1.30 ms.
- Set the proper diameter of laser spot and duration.





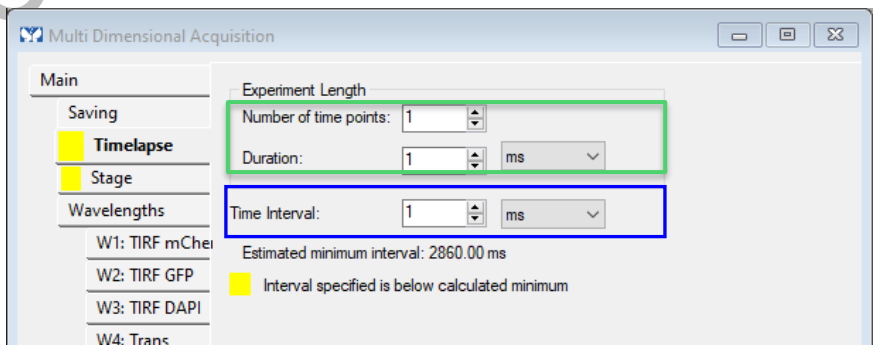
- To setup FRAP, choose FRAP from the dropdown list①, set up Interval (time interval between two time points) and N Time Points (number of time points) ②for Pre-sequence and Post-sequence acquisition (maximum 2 post-sequence are allowed) ③.
- Click Setup MDA  ④to import the parameters into MDA window in metaMorph
- Click on the Acquire icon on MDA to begin acquisition.



- To set up FLIP, choose FLIP from the dropdown list ①, set up Interval (time interval between two time points) and N Time Points (number of time points) for Pre-sequence② and Post-sequence acquisition ③, and repetition time④.
- Click Setup MDA  ⑤to import the parameters into MDA window in metaMorph
- Click on the Acquire icon on MDA to begin acquisition.

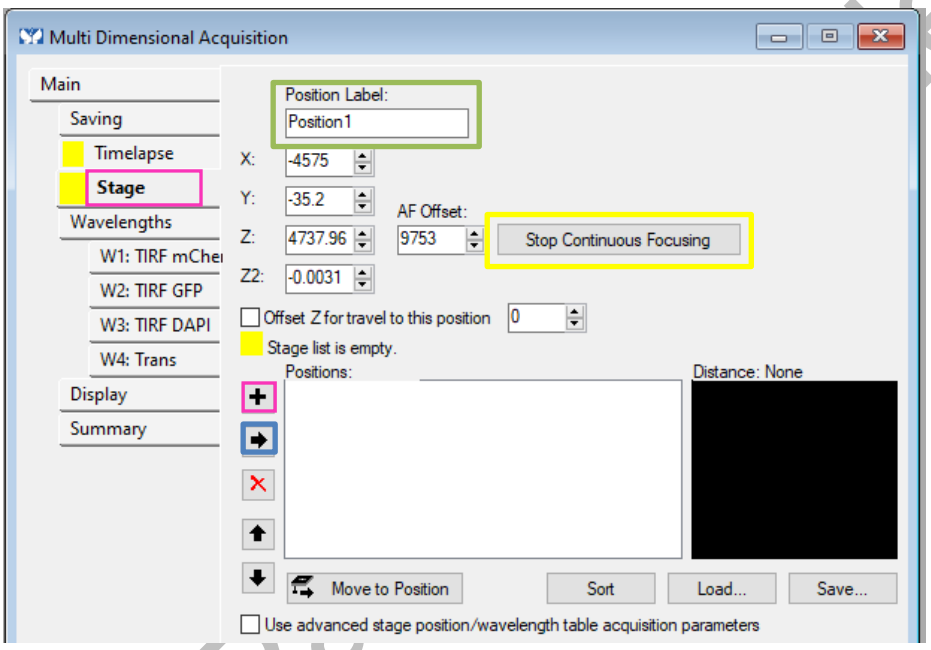
Timelapse

- Set up "**Time interval**" between each acquisition time point→ Set the **Duration** of the entire experiment or **Number of time points**, either one will do



Multi positions

- Choose Stage
- Give a Position Label for your stage positions; (Label name should be ended with digit "1". The number will be automatically updated to record the subsequence position.)
- Click Start Contiguous Focusing tab or press PFS button on the control panel to start perfect focus system.
- Use "Live" mode to find the position (x, y) and focus level (z)
- Click **"+"** to add the position (x, y, z) in position list.
- To overwrite recorded position, highlight the one to be overwrote and click "→". To delete recorded position, highlight the one to be deleted and click "✕".
- Click **Acquire** in the bottom to start acquisition of necessary.

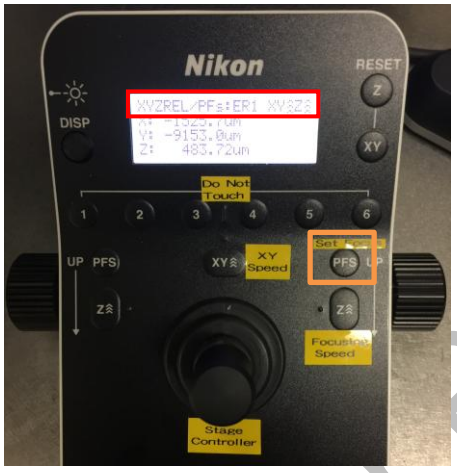


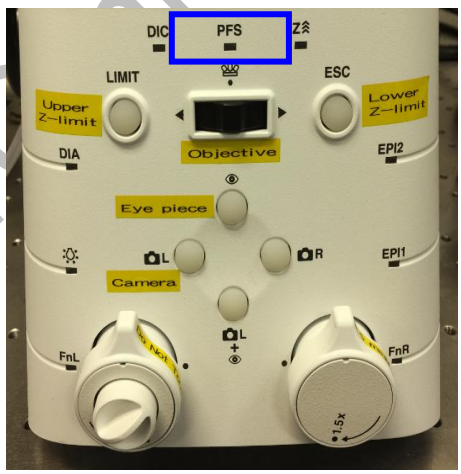
Perfect Focus System (PFS)

The allowable PFS focusing range refers to the range defined for each objective (where PFS is usable).

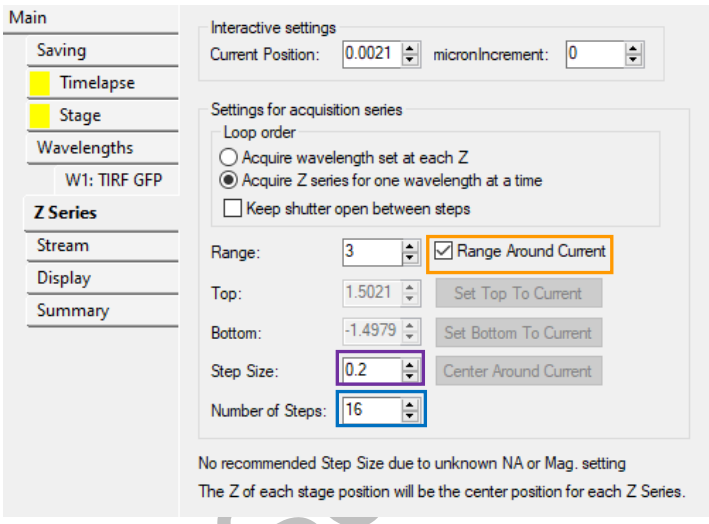
- For glass bottom dish, focus on the sample near to the bottom surface of the sample vessel
- For plastic dish, focus on the sample near to the bottom surface of the sample vessel, and then move the objective down by about 1000um.
- The status of the PFS is displayed on the PFS indicator on the front panel and the LCD of the joystick:

PFS indicator	PFS on/off	Shown on the display	PFS operating status	Details
On	PFS on	PFS: ON	Perfect focusing is in progress	The PFS is maintaining the focal point.
Blinking at slow intervals	PFS on	PFS: DIS	Waiting for interface detection	When the interface is detected within the allowable focusing range by moving the focusing position, the PFS is automatically turned on to start perfect focusing.
Blinking at fast intervals	PFS off	PFS: OFF	Perfect focusing is off.	The interface is detected within the allowable focusing range. Turn on the PFS to start perfect focusing.
Off	PFS off	PFS: OFF	Perfect focusing is off.	The interface is not detected within the allowable focusing range. In this case, turning on the PFS places it in an interface detection waiting state.





Z Series



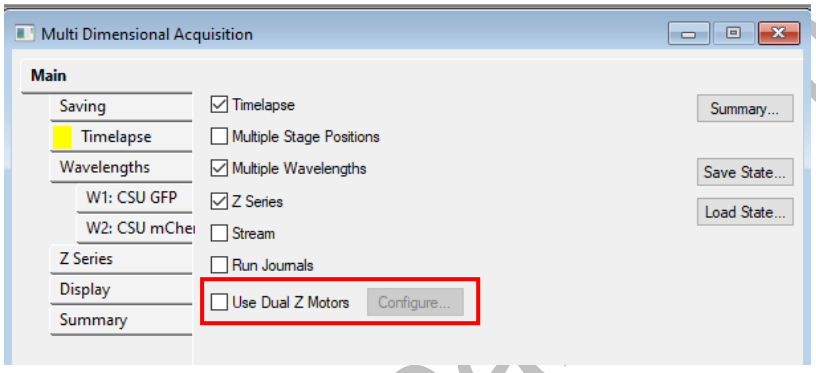
- Select " Z Series" in main menu

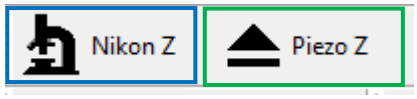
Method 1: use "Range around current" mode:

- Tick "**Range around current**"
- Focus the center of your object
- Set up "**Step Size**" for distance between each focus plane
- Set up "**Number of Steps**" for the total number of planes

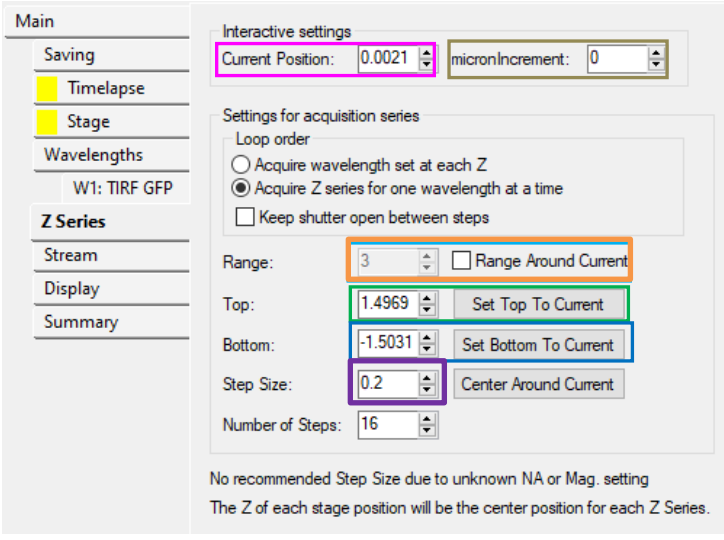
Method 2: use "Top" and "Bottom" mode, there are two different methods: use Nikon Z or use Piezo Z.

- If select Nikon Z,
- Untick "**Use Dual Z motors**" and click "**Nikon Z**" in the tool bar.



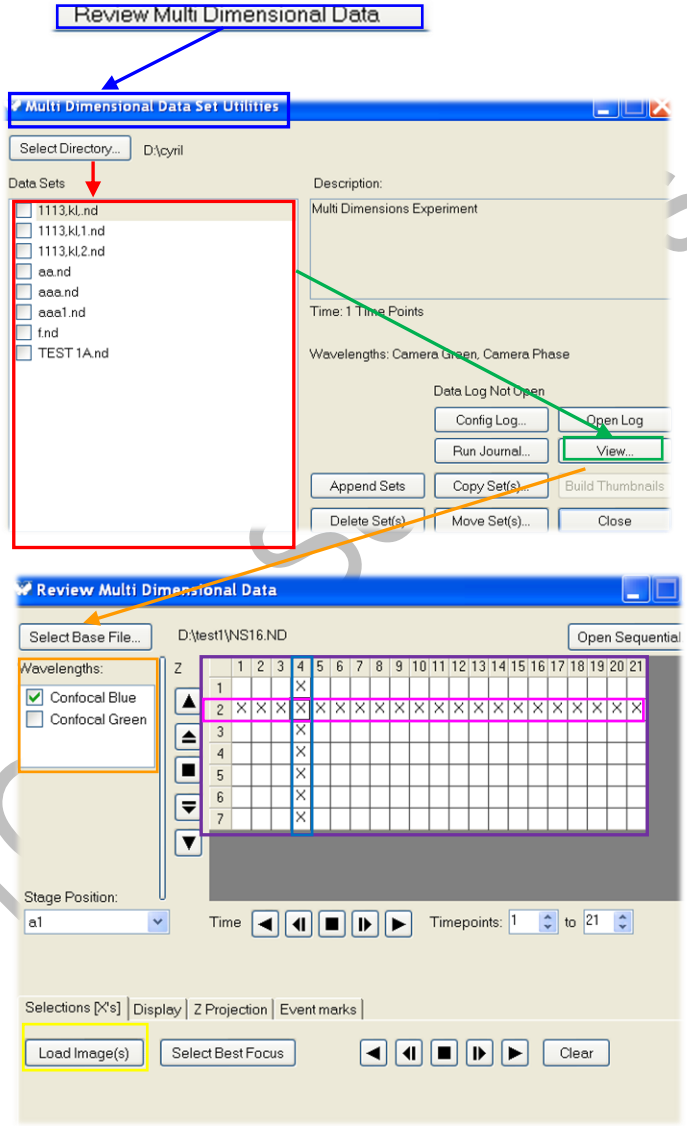


- Tick off "**Range around current**"
- Find any one end of your sample with fine focus, click "**Set Top To Current**"
- Find the other end of your sample with fine focus, click "**Set Bottom To Current**"
- Set up "**Step Size**" or "**Number of Steps**" for distance between each focus plane
- If select Piezo Z,
- Tick "**Use Dual Z motors**" and click "**Piezo Z**" in the tool bar.
- Turn off the PFS if you are using Piezo Z
- If the thickness of sample is over 100 nm, please use Nikon Z instead of Piezo Z
- Set the **micronIncrement** in the interactive setting first.

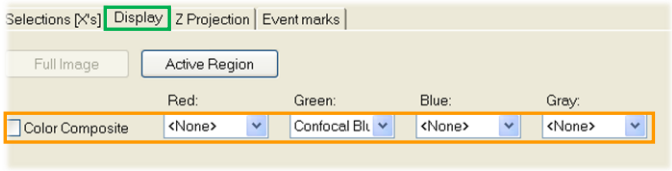


- Adjust the Piezo Z by changing the **current position** in the interactive setting. After that, the current position will be set into **top** and **bottom**.

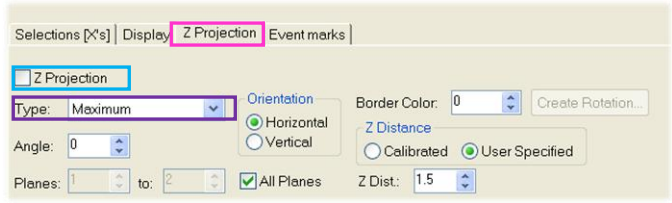
Review Acquired Images



- Click **Review Multi Dimensional Data** in the Task Bar after Images Acquisition
- Choose your folder in **Select Directory** and select an image **Data set (base name +suffix. nd)** and then click **View**
- Select the **Wavelength** acquired to be displayed.
- Display a single image by clicking **any single grid**.
- Select Stage position in the pull down menu.
- To review series images, left click the header number of the **Row** or **Column** for displaying images of **Time series** or **Z-series** respectively.
- Then click Load Image (s)
- To export series images as movie, please refer to MetaMorph analysis software protocol.



- To Overlay images of different channels, check the **Color Composite** box in the **Display tab** and then assign corresponding channel to the RGB color to composite an overlay image.



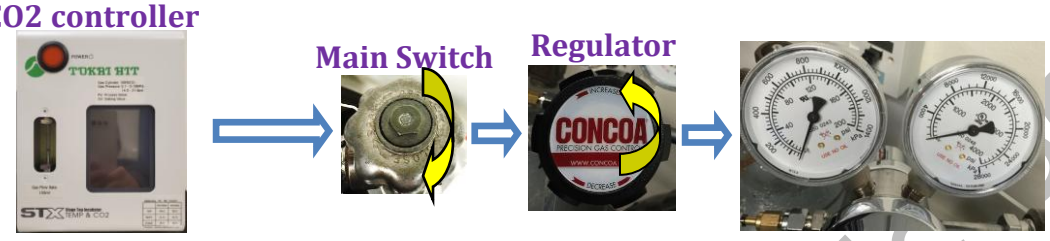
- To stack all plans in a z-series to create a single 2D image, choose **Maximum** projection in **Z Projection** tab and check the **Z Projection** box.

Turn off system

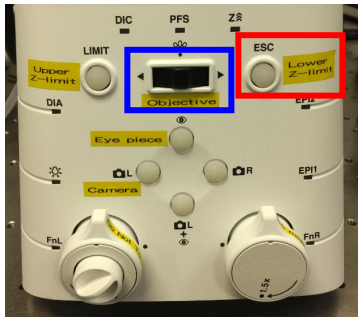
Please check if the equipment will be used by other users. Please switch off system if no one books equipment over two sessions (1h) after you.

- IF you used live cell chamber and CO_2 controller. (Please skip this step if it is not needed)
  - Take off objective heater on objective
  - Switch off temperature and CO2 controller.
  - Turn off CO2 tank by turning the main switch clockwise
  - Turn off CO2 regulator by turning regulator clockwise to the end

Temperature and CO2 controller



- IF oil objective lens is USED, it must be cleaned thoroughly with the **LENS PAPER** instead of Kimwipes.
  - Oil residue from the objective lens should firstly be removed using a DRY lens tissue.
  - Repeat this step with a new area/piece of the lens cleaning tissue until no oil Streaks are seen on the tissue.
  - Switch objective to empty position in the hardware and set to "Lower Z-limit".



- Exit MetaMorph software.
- Transfer data to CPOS imaging and cytometry storage server and shut down the computer.
- Switch off laser power ⑤ by turning key anticlockwise for 90 degrees if you used ablation. (Please skip this step if it is not needed)
- Switch off laser power ③, leave 10 seconds before next step.
- Switch off power ②, leave 10 seconds before next step.
- Switch off main power ①.
- Sign on the log book and put on the microscope cover.