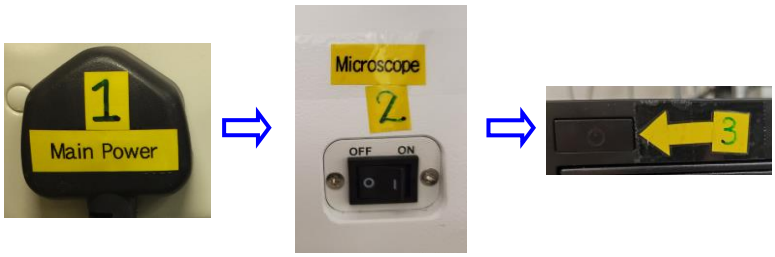
TIRF Microscope Standard Operation Protocol
Basic Operation

Turn on system

Please sign on the log sheet before switching on system.

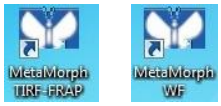
- Switch on main power control ①
- Switch on microscope power ②
- Turn on computer power ③



The following steps A to E are for TIRF users, please skip A –E if widefield will be used.

- Switch on "A Laser" (For TIRF and laser imaging)
- Laser power B1(488nm), wait for ~30 sec to for laser warm up
- Once laser status is at "standby" mode, turn on laser key B2(488nm) to horizontal
- Laser power C1(561nm), wait for ~30 sec to for laser warm up
- Once laser status is at "standby" mode, turn on laser key C2(561nm) to horizontal
- Laser power D (405nm)
- Laser power E (642nm)

- Click to log into **USER** at the startup screen
- Start the MetaMorph software
  - For TIRF users, please click **MetaMorph TIRF FRAP** icon
  - For widefield users, please click **MetaMorph WF** icon



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):

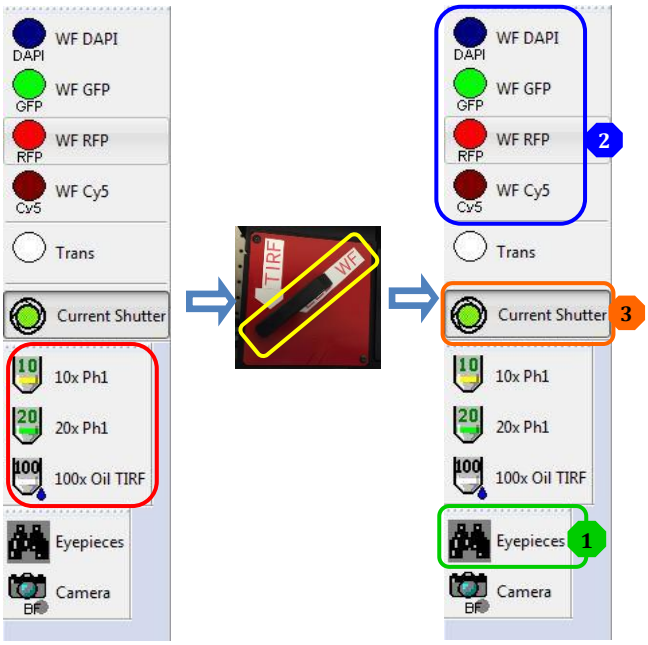
- Switch on "Incubator" for temperature and CO_2 control.
- Turn on CO_2 tank by turning the main switch anticlockwise
- Turn on CO_2 regulator by turning regulator clockwise to set output pressure at 100kPa
- Turn on tube switch for TIRF
- Put on objective heater on objective if oil objective is used
-

Main Switch LCI Regulator Tube Switch

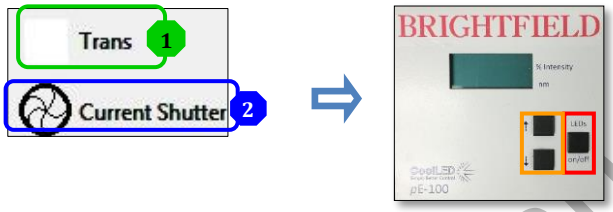
Sample locating and focusing

- Select objective
- Apply a drop of immersion oil if 100x oil objective is used
- Place your sample, make sure the coverslip bottom is facing down (slide/dish/chamber slide)

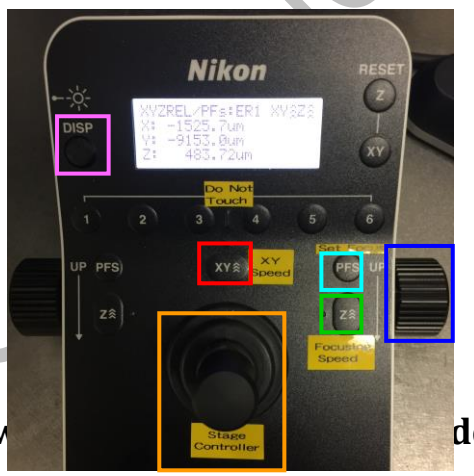
- Turn the light path switch to "LED WF" if necessary
- To view under the microscope, go to Eyepieces →select fluorescence channel → Current Shutter



- For Brightfield, click Trans → Current shutter →Switch On the Brightfield LED →adjust brightness



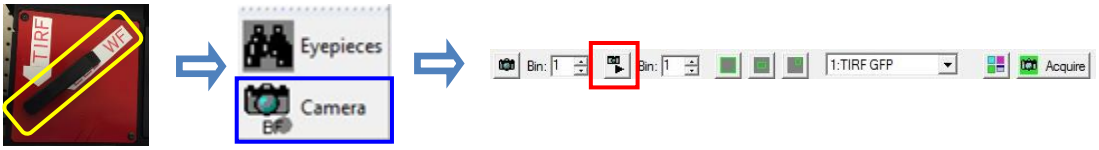
- Press the right arrow of the DISP button.
- Move the Stage Controller to adjust XY position (XY speed can be adjusted: ^^^>^^^>^)
- Focus the sample with the focusing knob → Clockwise_Down; Anti-clockwise_Up (Focusing speed can be adjusted: ^^^>^^^>^)
- Switch on the "PFS" and adjust the focus to lock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



Sw de

For Widefield imaging:

- Make sure the light path switch is pointing to WF → Camera → Click Live in "Multi Dimensional Acquisition"

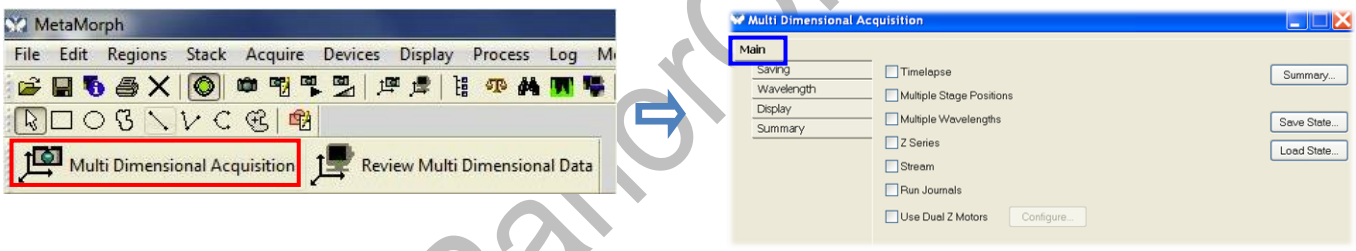


For TIRF imaging:

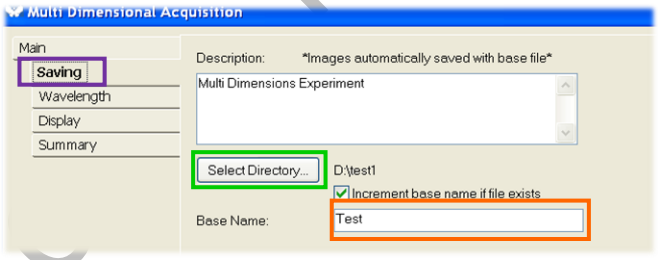
- Turn the light path switch to "TIRF"→ turn on Shutter → Camera→ Click Live in "Multi Dimensional Acquisition"

Image Acquisition

- Click **Multi Dimensional Acquisition** on the task bar
- Go to **Main** tab to set up acquisition configuration step by step. Check the box(es) of the application(s) as required.

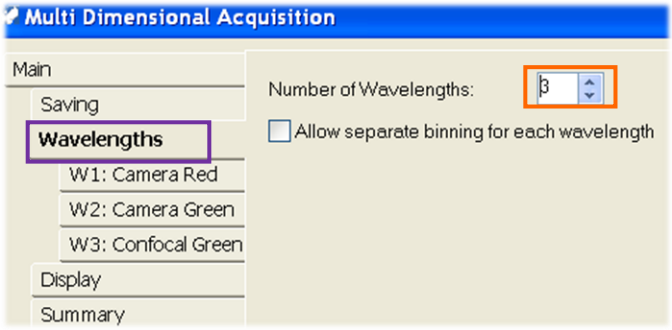


- Click **Saving** → **Select Directory** (all data should be saved in E drive/USER under your name)
  - Type in the base name of your file (experiment or date or etc.) in **Base Name**.
  - Do not use digits at the end of the base name, a digit will be added by the system according to the acquisition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

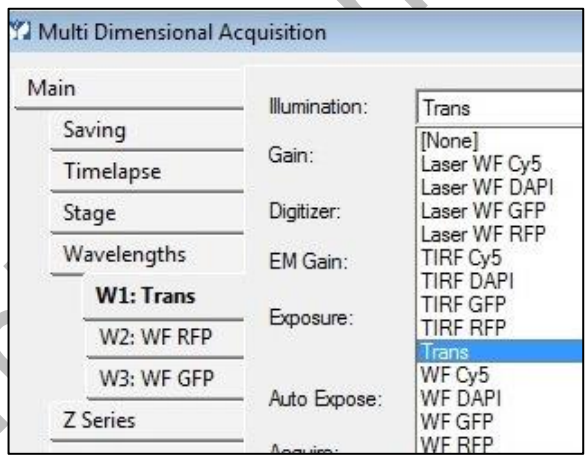


- If multiple fluorescence channels are required,
  - Check the box of "Multiple wavelengths" in the main menu
  - Click **Wavelengths**

– Key in the **number** of channels in "Number of Wavelengths"



• Select each wavelength to set the required "Illumination".



For Widefield Imaging:

– Select "WF DAPI" for Blue emission (such as DAPI)
– Select "WF GFP" for Green emission (such as GFP)
– Select "WF RFP" for Red emission (such as mCherry)
– Select "WF Cy5" for FarRed emission (such as mCherry)
– Select "Trans" for brightfield channel

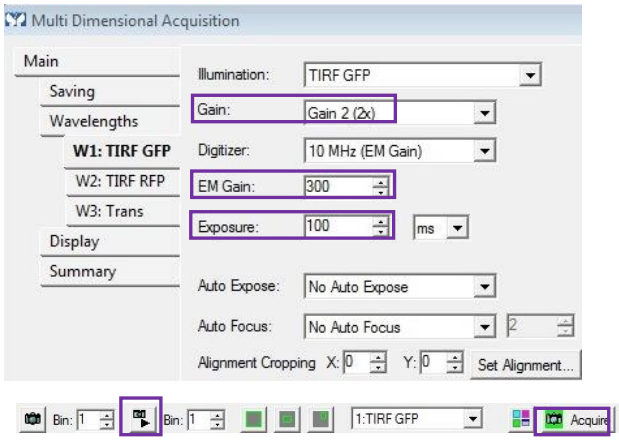
For TIRF Imaging:

– Select "TIRF DAPI" for Blue emission (e.g. BFP) channel
– Select "TIRF GFP" for Green emission (e.g. GFP) channel
– Select "TIRF RFP" for Red emission (e.g. mCherry) channel
– Select "TIRF CY5" for Farred emission (e.e. Cy5) channel
– Select "Trans" for brightfield channel

Image Adjustment

For Widefiled Imaging:

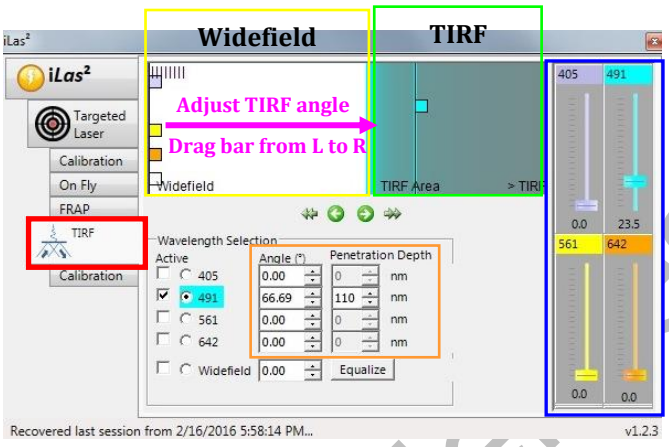
– Select "W1" to adjust the first channel
– Click **Live**  at the bottom of "multi-dimensional acquisition" panel to have real time image
– Adjust **EM Gain** and **Exposure** time to have optimal signal intensity
– Adjust **Gain** if necessary (1x, 2x or 4x)
– Select "W2" and repeat the same procedure to adjust the second channel



Live

For TIRF Imaging:

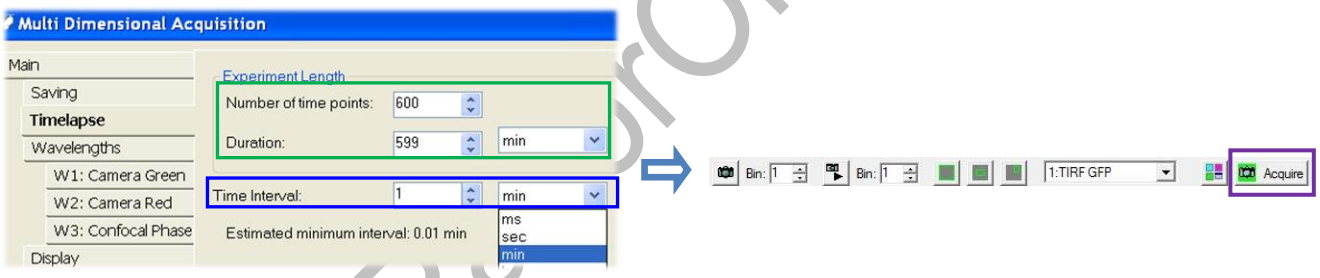
- Preview the image on screen by clicking Live  at the bottom of "multi-dimensional acquisition" and adjust the focus and parameters (EM Gain, Exposure Time and Laser Power) to achieve the best focus.
- Click "TIRF" in iLas2 software panel
- Adjust laser power by move the slider bar for each laser
- Adjust TIRF angle for each laser by moving the slider from Widefield to TIRF area. The actual Angle and Penetration Depth are shown on the panel.



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

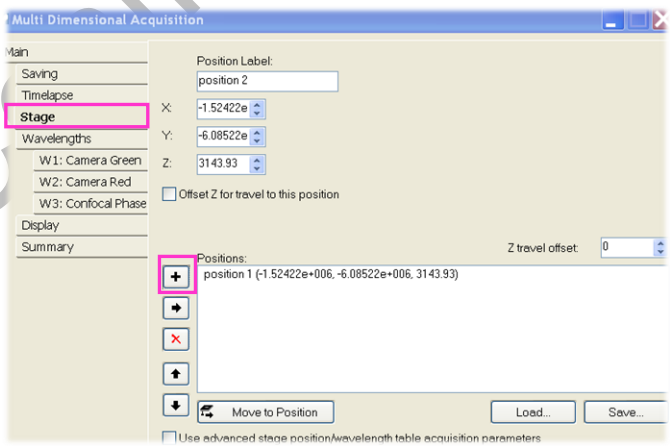
Timelapse

- 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 → Click **Acquire** to start the acquisition.



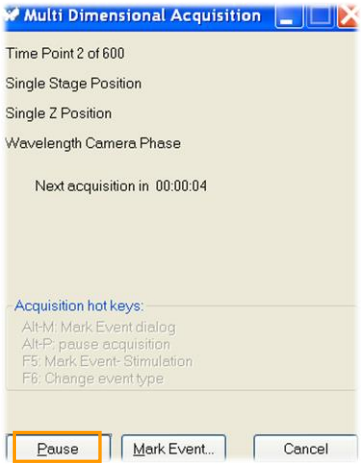
Multi stage positions

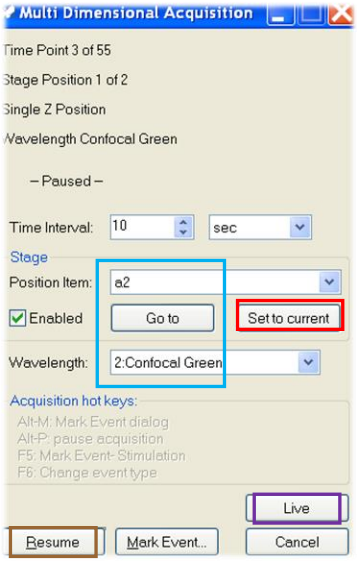
- Give a Label for your stage positions; (Label name should be ended with digit "1". The number will be automatically updated to record the subsequence position.)
- Use "Live" mode to find the right position (x, y) and focus level (z)
- Click "+" to add the position (x, y, z) in position list
- To overwrite recorded stage position, highlight the one to be overwrote and click "+".
- Click **Acquire** in the bottom to start acquisition of necessary



Adjust Focus during Time Lapse Acquisition

– If amendment is needed halfway through the acquisition process, click "**Pause**" →"**Live**"→ choose a **Position of interest** → **select wavelength**→ click "**Go to**". Adjust the position and focus followed by clicking "**Set to current**"→ click "**Stop**" (initially it is "Live") and then "**Resume**" to continue the acquisition.



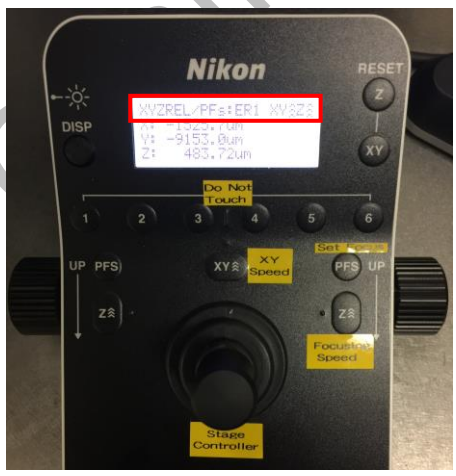


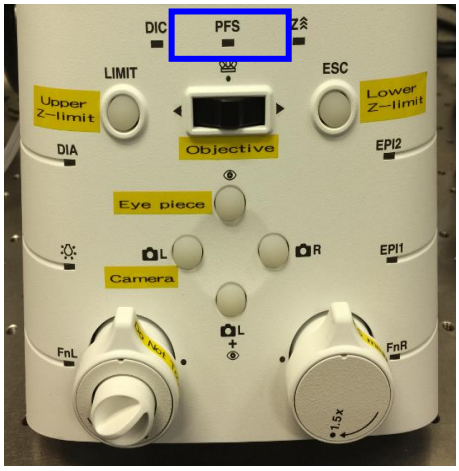
Perfect Focus System (PFS)

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.



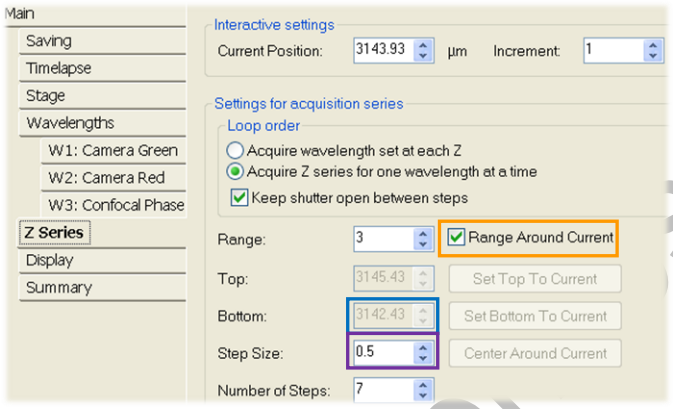


Z Series

a. Select " Z Series" in main menu

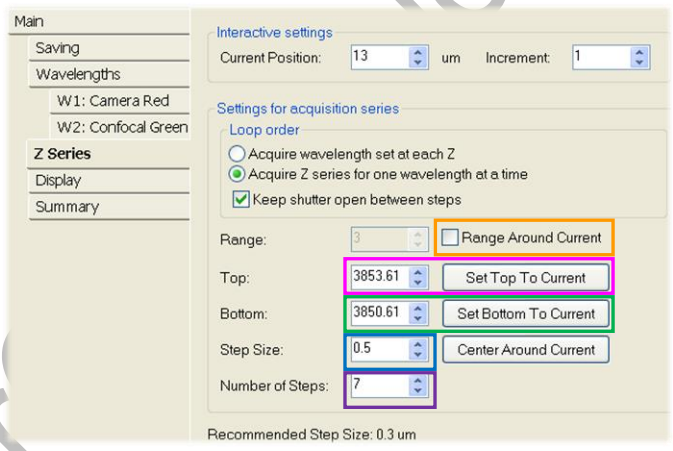
For Spherical object, use "Range around current" mode:

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



Otherwise, use "Top" and "Bottom" mode:

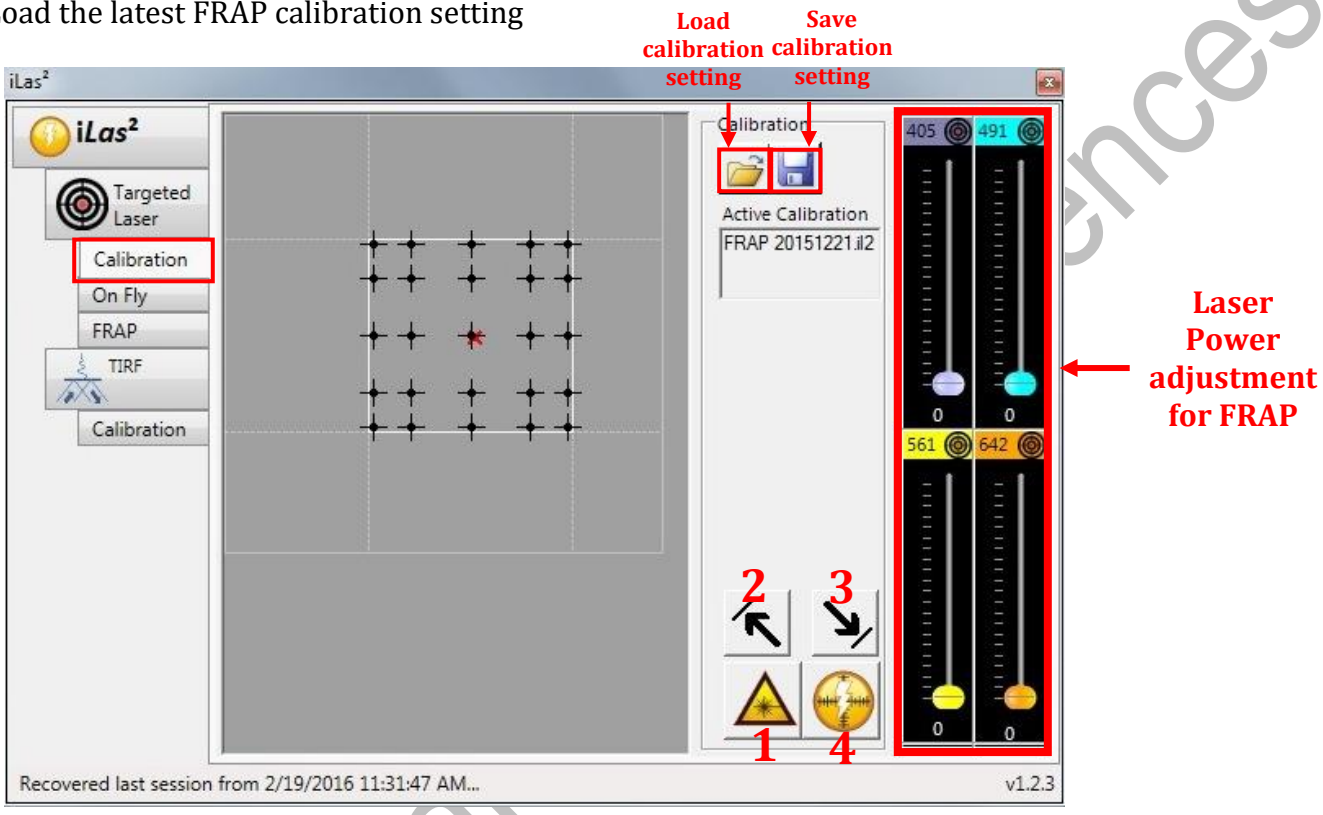
- Tick off "**Range around current**"
- Find any one end of your sample with fine focus, click "**Set Top To Current**"
- Find the other end of your sample with fine focus, click "**Set Bottom To Current**"
- Set up "**Step Size**" or "**Number of Steps**" for distance between each focus plane



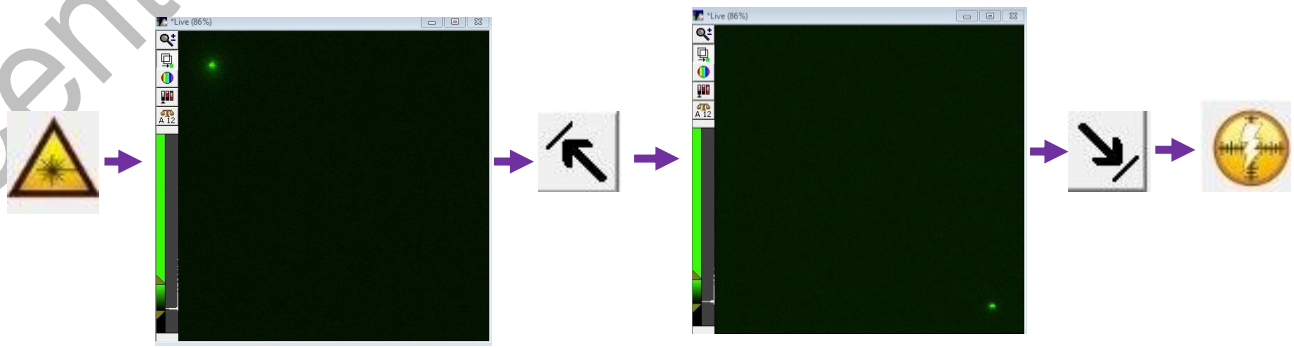
FRAP

b. Targeted laser calibration

- Preview the image on screen by clicking **Live** at the bottom of "multi-dimensional acquisition"
- Select "Calibration" in "Targeted Laser" on iLas panel
- Load the latest FRAP calibration setting



- Click on the icon to activate the targeted laser. Adjust the focus and parameters (EM Gain, Exposure Time and laser power) to achieve a highly contrasted laser spot image in MetaMorph Live window.
- Move the red cross in the grey calibration area to bring the laser spot to the top left corner and press
- Bring the laser spot to the bottom right corner and press
- Click on the calibration button to begin calibration
- When calibration is done, click on the save icon to save the calibration setting



c. FRAP Experimental Protocol

– Select FRAP MDA template in main iLas window

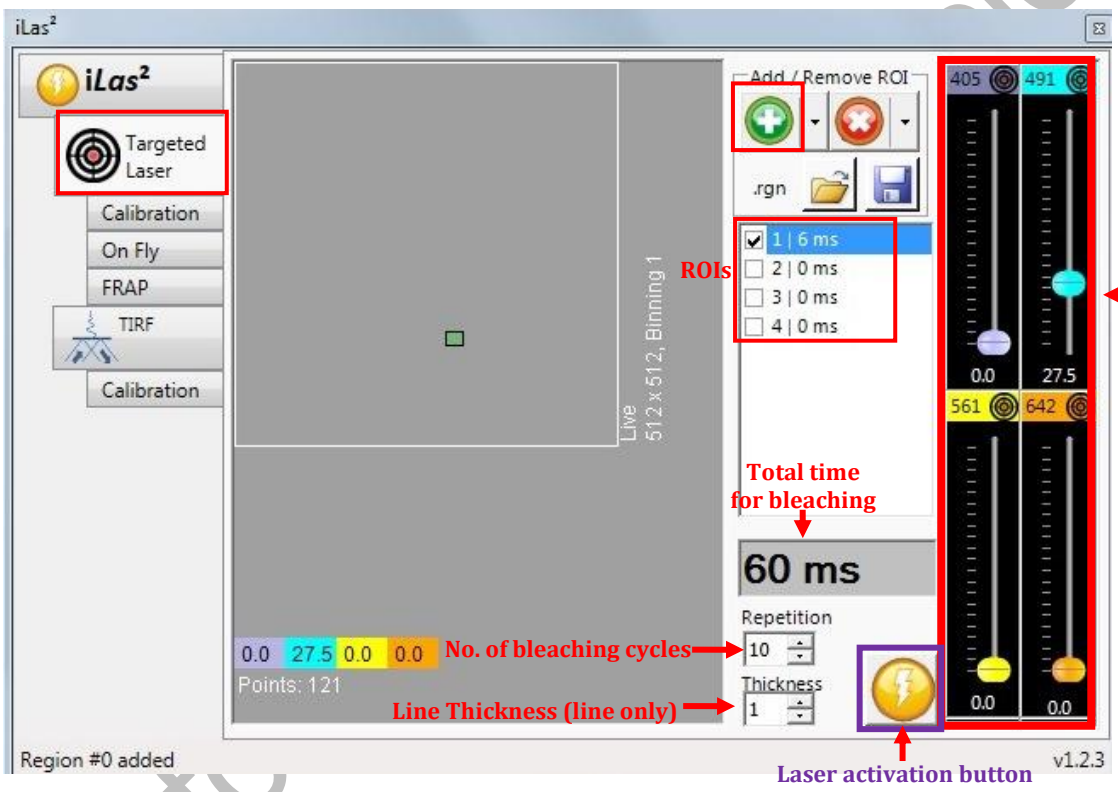
– Preview the image on screen by clicking **Live**  at the bottom of "multi-dimensional acquisition" and adjust the focus and parameters (EM Gain, Exposure Time and Laser Power) to achieve a well-focused and properly illuminated image.

– Click "Targeted Laser" in iLas window

– Mark the region of interest (ROI) using the region tools  in MetaMorph and ROI(s) by click 

– Adjust bleaching parameters (No of repetitions, laser power)

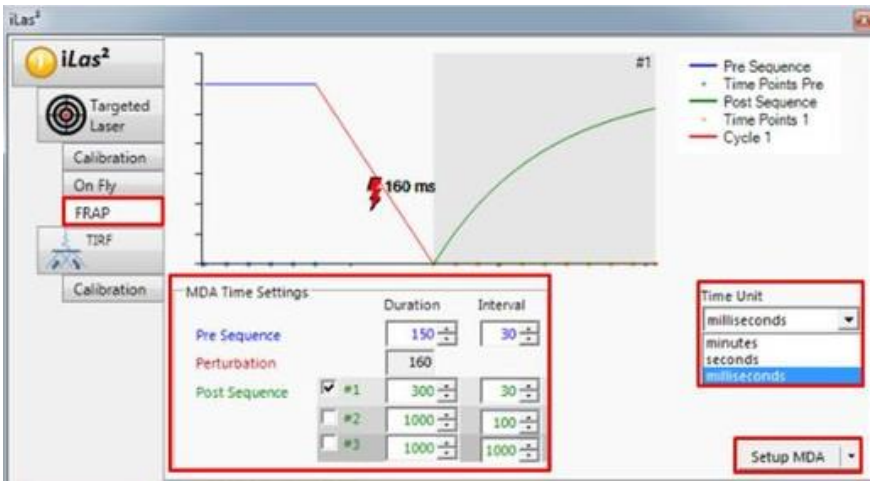
– The testing bleaching could be done by click laser activation button



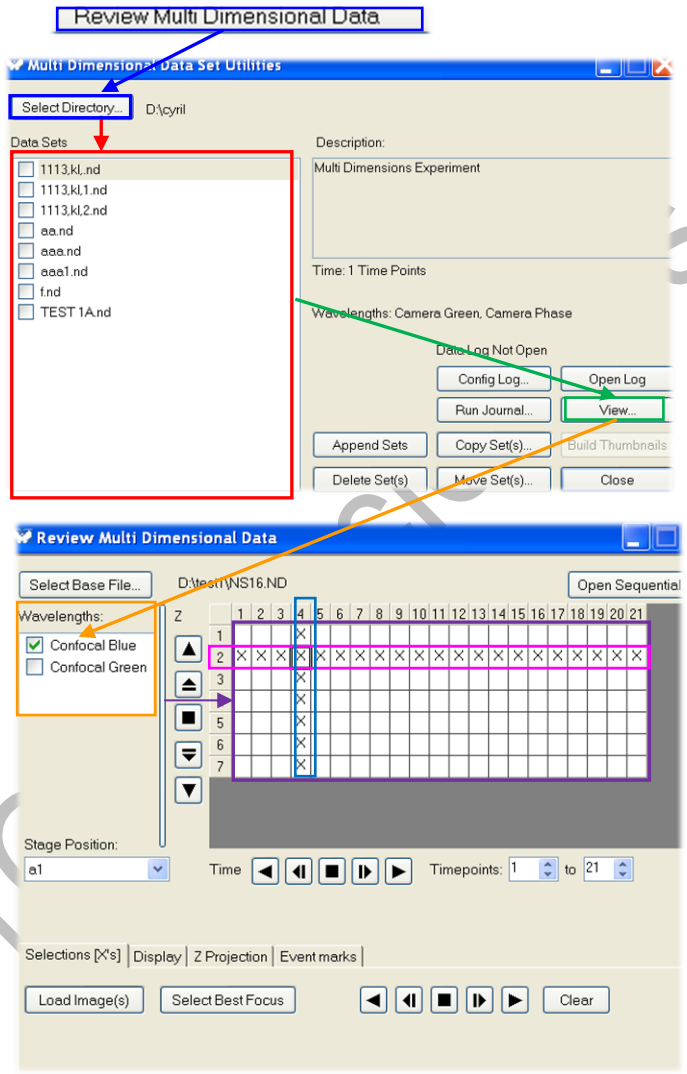
– Click FRAP Tab, set up time interval and duration for Pre & Post sequence acquisition

– Click Setup MDA to import the parameters into Mulit Dimensional Acquisition widow in metaMorph

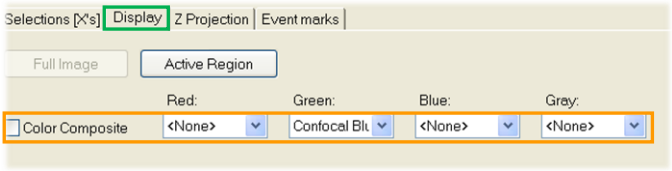
– Click on the Acquire icon to begin acquisition



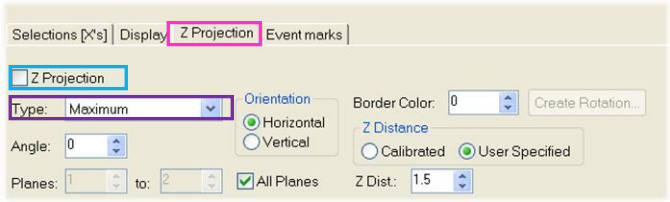
Review Acquired Images



- 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.



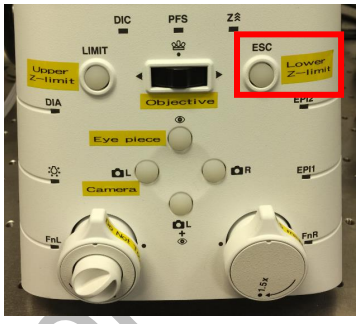
- To Overlay images of different channels, check the **Color Composite** box in the **Display tab** and then assign corresponding channel to the RGB color to composite a overlay image.



- 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

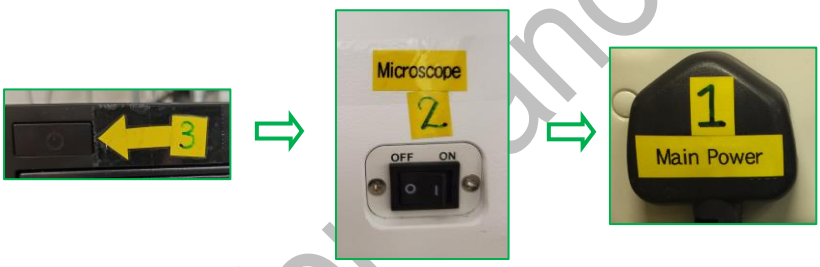
Please check if the equipment will be used by other users. Please switch off system if no one books equipment over two sessions (1h) after you.



- IF 100x 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 10x in the software and set to "**Lower Z-limit**".

a. Exit MetaMorph software
b. Transfer data to Faculty Core Facility storage server and shut down the computer③.
c. Switch off laser power E (642nm)
d. Switch off laser power D (405nm)
e. Switch off C2, wait the laser output decreases to 0, then switch off C1 (561nm)
f. Switch off B2, wait the laser output decreases to 0, then switch off B1 (488nm)
g. Switch off Power switch A (For TIRF)
h. Switch off microscope power②
i. Switch off main power control ①
j. Switch off temperature and CO_2 controller by switch off LCI.
k. Turn off CO_2 tank by turning the main switch clockwise
l. Turn off CO_2 regulator by turning regulator clockwise to the end
m. Turn off tube switch for TIRF
n. Take off objective heater on objective

Those are for TIRF users, please skip E –A if widefield is used.



LCI Main Switch Regulator Tube Switch