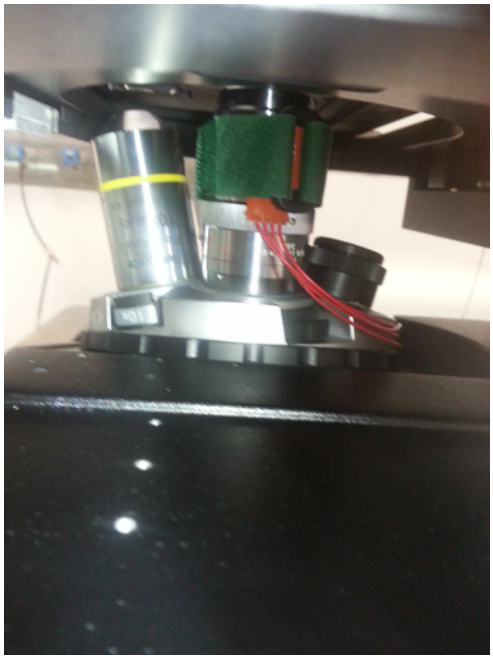
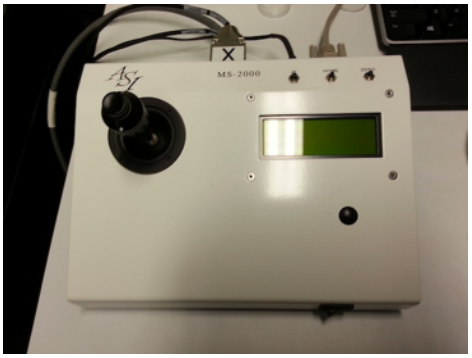
Roper Guide

Getting Started

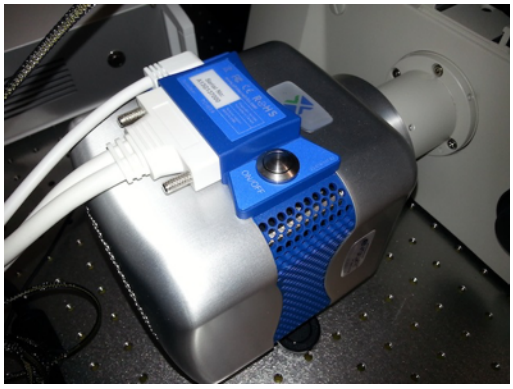
IMPORTANT

The microscope is equipped with a heated stage insert and objective lens heater which is to be left permanently switched on and operating at 37C. In addition the microscope stand and XY stage are to be left permanently switched on. This is to ensure mechanical stability for single molecule imaging experiments.





Other components



Before starting MetaMorph turn on:

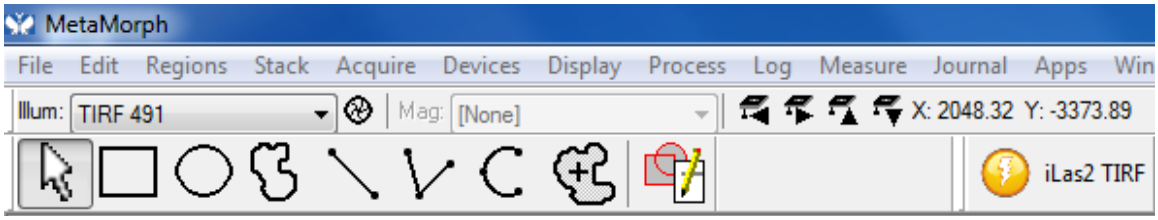
1. The laser bed (if switched off)
2. The Photometrics EMCCD
3. The iLas unit
4. On iLas remote, turn key to 'On' and open shutter (note that the shutter will only open when the interlock plate is in place and the microscope light-path is set to L100 for the camera) and select the desired laser lines (colour coded as: 405nm, 491nm, 561nm, 642nm)

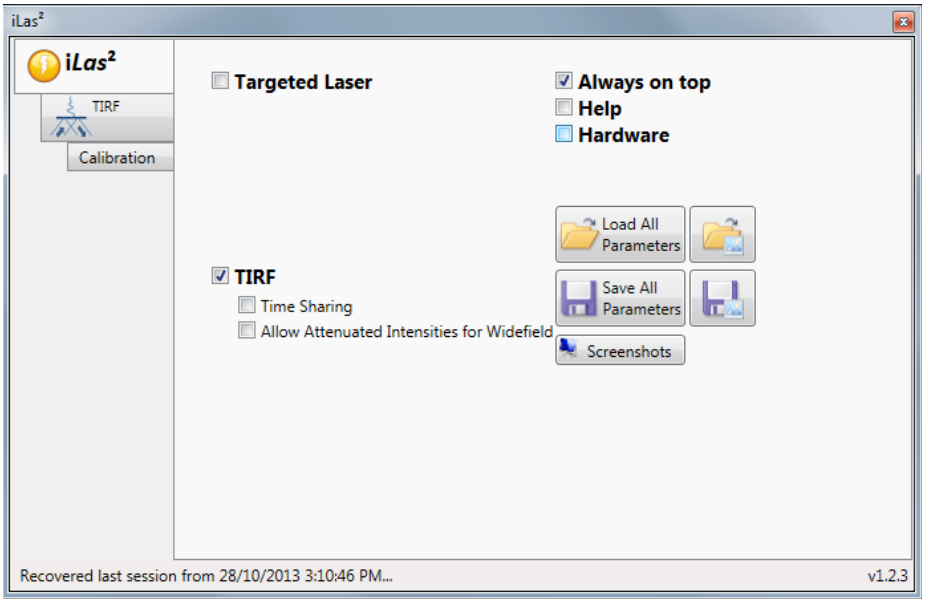
*note about PSF

First steps

1. After starting all the hardware components launch MetaMorph and begin by starting the iLas^2 plugin.
2. Choose an illumination configuration from the MetaMorph toolbar. The illumination configuration does two things:
   (i) It selects a reflector in the microscope
   (ii) It activates a particular laser (or combination of lasers) to be controlled in the iLas^2 plugin

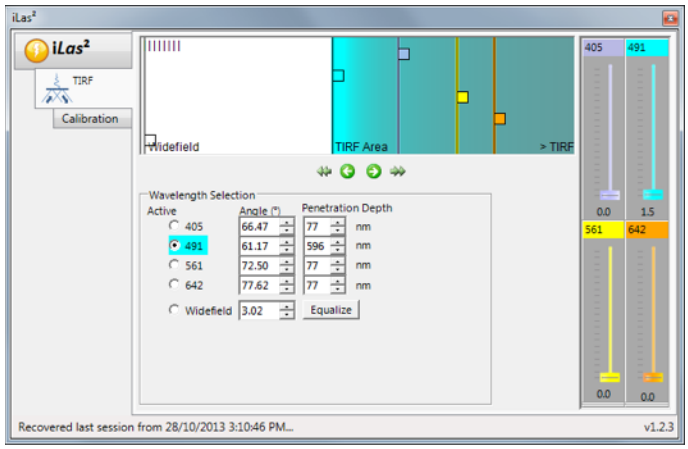
   For example:
   (i) The "TIRF 491" configuration selects the "G/R" reflector cube and allows control of the 491nm laser.
   (ii) The "WF 405+561" configuration selects the "G/R" reflector cube and allows control of both the 405nm and 561nm lasers with the TIRF angle assigned to the "Widefield" control in the iLas^2 plugin





The iLas² plugin

1. Press the TIRF tab to control the TIRF angle and laser power.



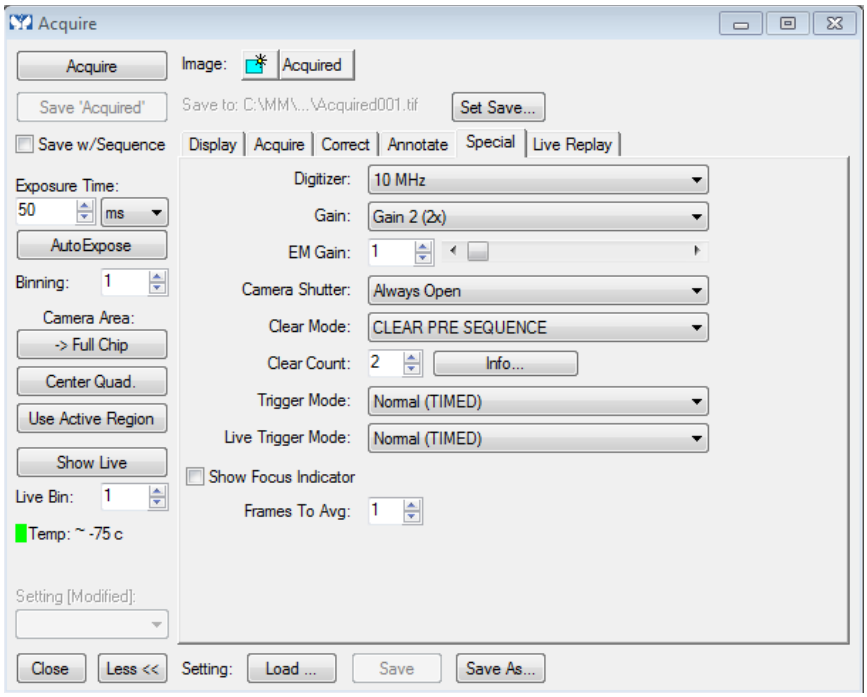
2. The sliders at the top of the window allow adjustment of the TIRF angle for each individual laser line defined in the currently selected **Illumination configuration**. Clicking on each line (colour coded according the laser lines defined on the right of the GUI window) makes that laser line active.

3. The currently active laser, the calculated angle and penetration depth are displayed in the **Wavelength Selection** panel. If multiple laser lines are defined in the Illumination configuration these are controlled using the **Widefield** slider (in white).

4. Laser power for each line defined in the Illumination configuration is controlled using the vertical sliders on the right of the GUI. For example the configuration WF_405+561 allows simultaneous control of the TIRF angle of the 405nm and 561nm lasers using the **Widefield** slider. The power levels of each laser are individually controllable.

Capturing an image

Choose Acquire --> Acquire from the MetaMorph toolbar.



1. Wait until the **camera temperature** reads **-75C**.
2. Set **Exposure Time**
3. On the **Special** tab, set **EM Gain**
4. Start a live acquisition using **Show Live**.
5. Capture a single frame using **Acquire**.
6. Regions of interest on the camera can be set using **Center Quad.** (to use 1/4 of the chip in the center) or set an arbitrary region and press **Use Active Region**.

Notes:

1. Control of the lasers (TIRF angle and power) only becomes active when an acquisition is started using **Show Live**.

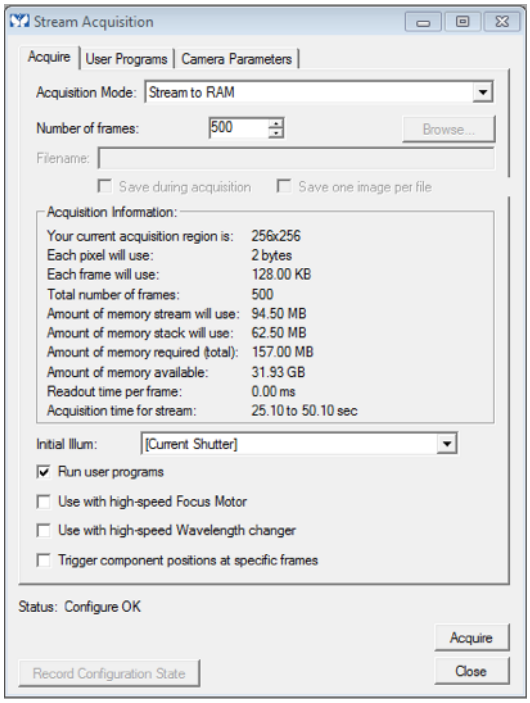
2. For exposure times > 40ms use (these parameters minimise readout noise):

   Digitizer --> 10MHz
   Gain --> 2
   EM Gain --> 40 - 300
   Camera Shutter --> Always Open
   Clear Mode --> CLEAR PRE SEQUENCE
   Clear Count --> 2
   Trigger Mode --> Normal (TIMED)
   Live Trigger Mode --> Normal (TIMED)

3. If using exposure times < 40ms use Digitizer --> 20MHz and keep all other parameters the same (adjusting EMGAIN according to sample SNR). In this situation it is also useful to use a cropped region of the CCD chip in order to minimise frame readout time. Faster readout rates can also be achieved by binning.

Streaming

Streaming is the fastest way to capture timelapse data from the EMCCD. Frames are grabbed from the camera and stored in RAM to minimise the readout time between frames. This type of acquisition is critical for capturing single molecule localisation data. There are two ways to perform a stream acquisition, using **apps --> Stream Acquisition** or **apps --> Multidimensional Acquisition** (described later).



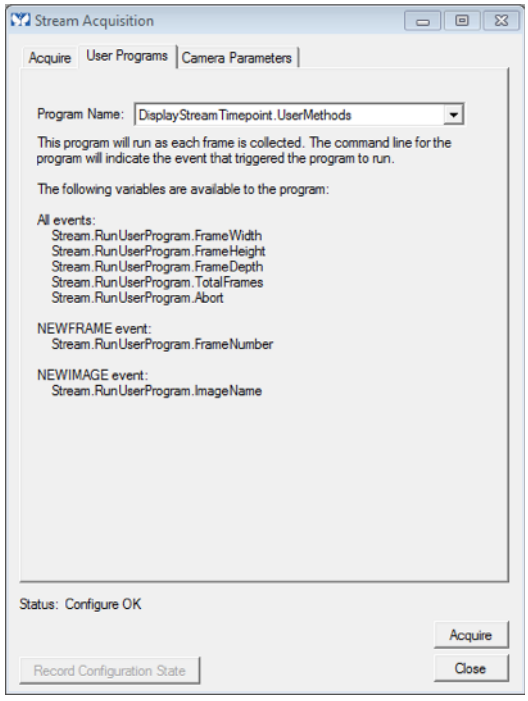
In **Stream Acquisition**:

1. Choose **Stream to RAM** from **Acquisition Mod**

2. Set the number of frames to be acquired.

3. Check **Run user programs** to activate the **User Programs** tab.

4. Select **DisplayStreamTimepoint** in **Program Name** in the **User Programs** tab. This is a Visual Basic program that will run to provide information about the number of time points acquired.



Camera parameters (EMGain and exposure time) will be taken from the Acquire GUI (described previously) used to configure the live acquisition.

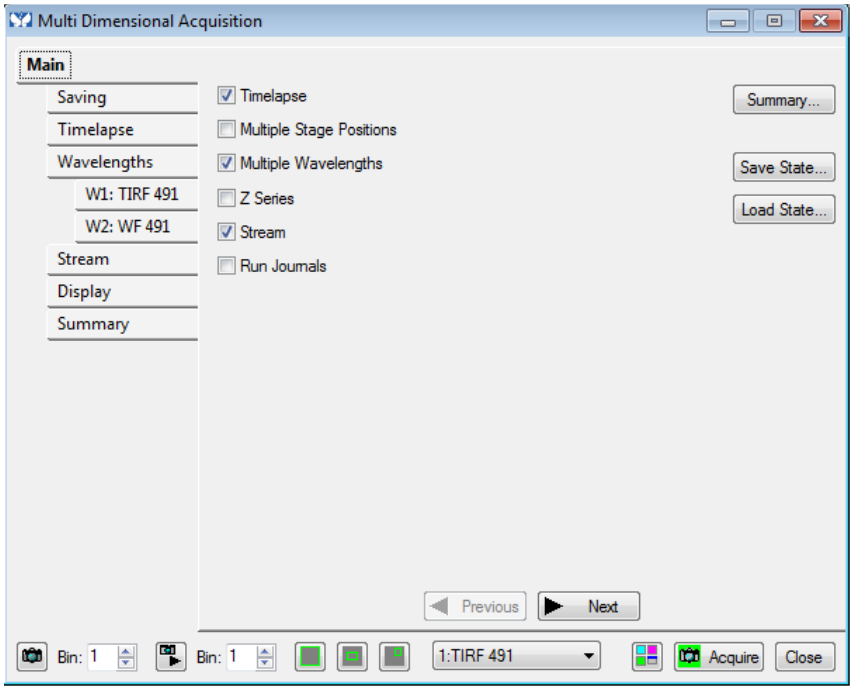
Note A bug in MetaMorph currently prevents the camera running in live mode when the Stream Acquisition is open.

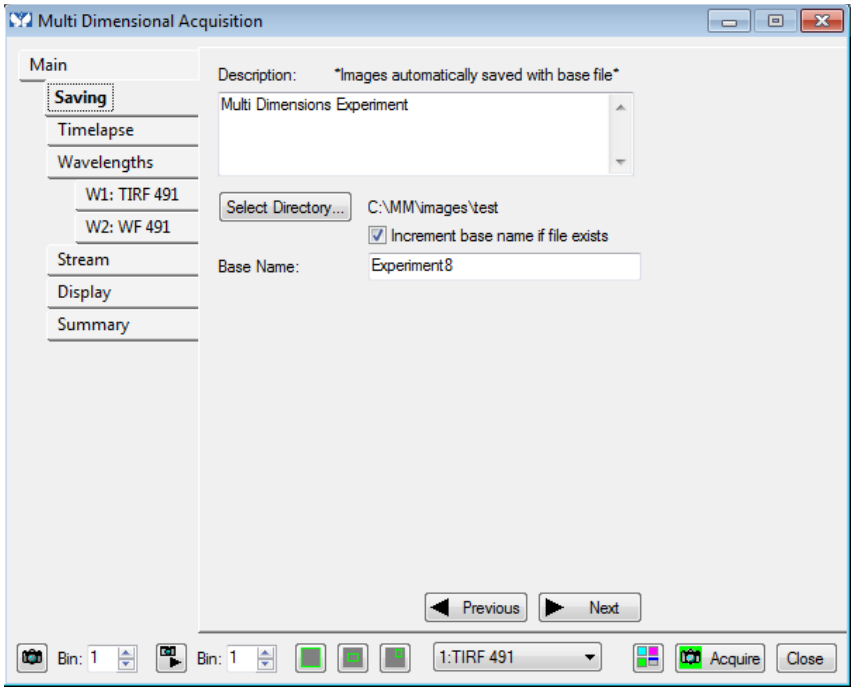
Multidimensional Acquisition

Multidimensional acquisitions are configured from **apps --> Multi Dimensional Acquisition** and allow complex experiments to be performed. Choices are:

Timelapse
Multiple Stage Positions
Multiple Wavelengths
Z series
Stream
Run Journals

The options can be combined from the main tab using the checkboxes. When configuration is complete a status box provides information on whether the selected configuration parameters are compatible.

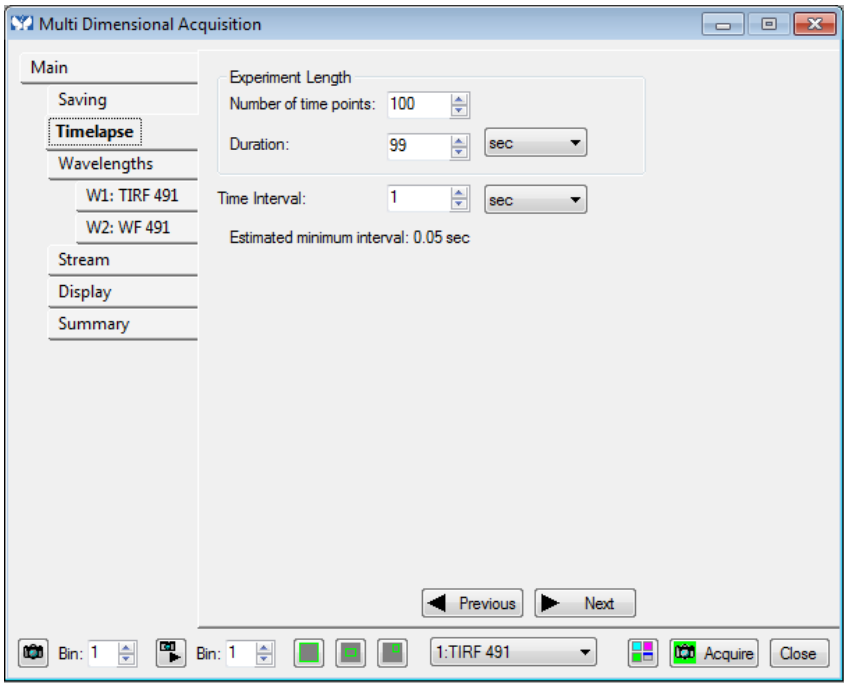


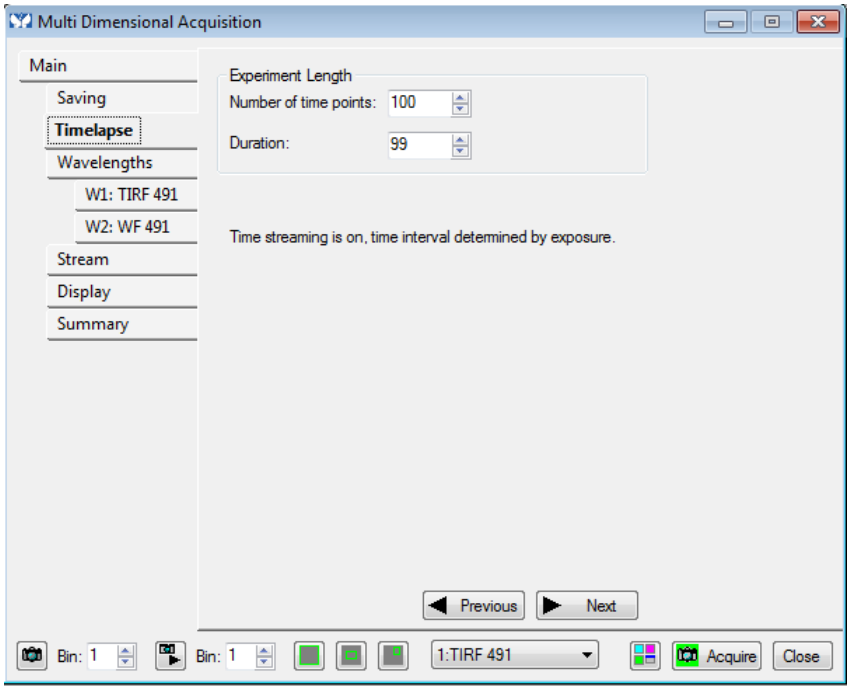


Saving
The saving tab allows the user to provide a description, directory and filename for the acquired dataset.

Timelapse

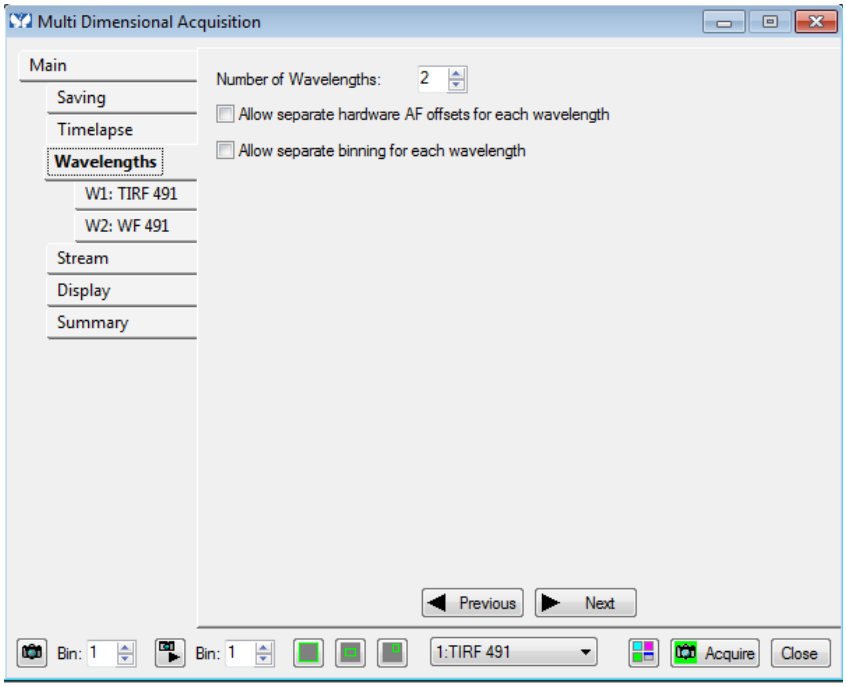
The number of time points and duration can be set here. When combined with **Stream** the time interval between frames is determined by the exposure time that has been set in order to read out frames as fast as possible.





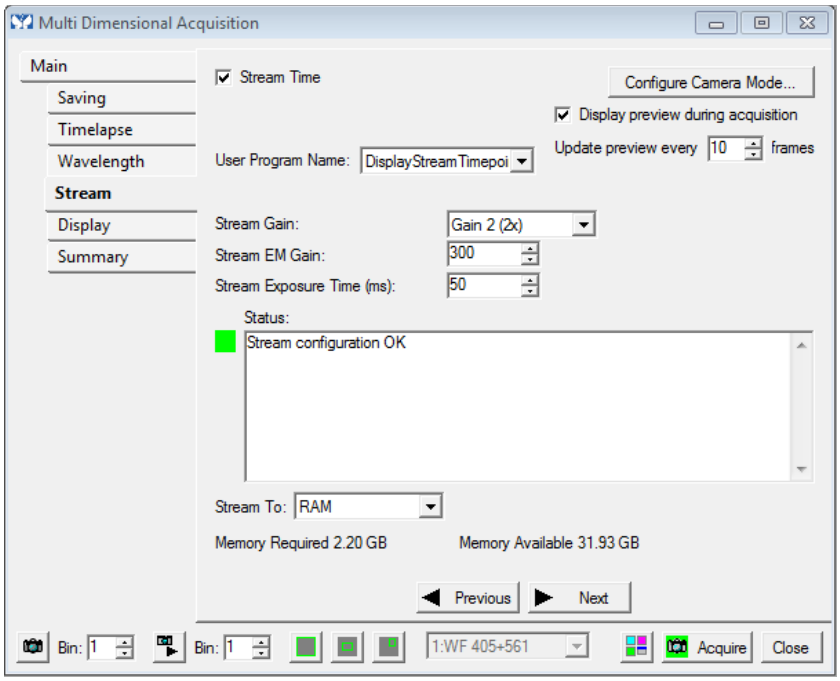
Wavelengths

This tab is used to define which **Illumination configuration** will be used for the acquisition. A separate is used to configure of the wavelengths.

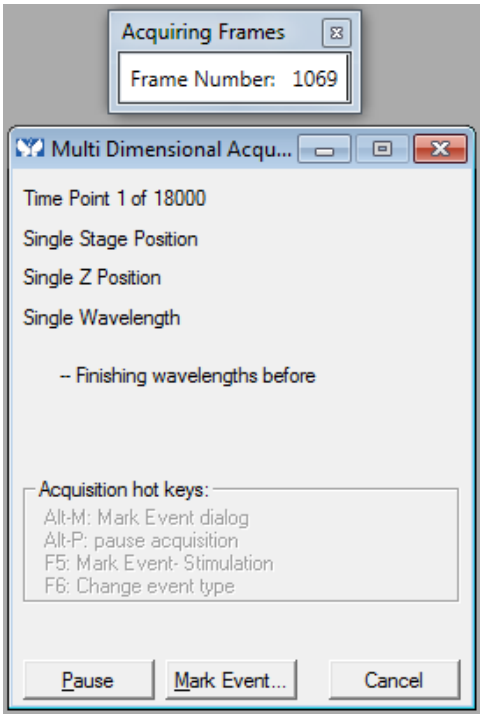


Stream

This tab is used to configure the camera for streaming.



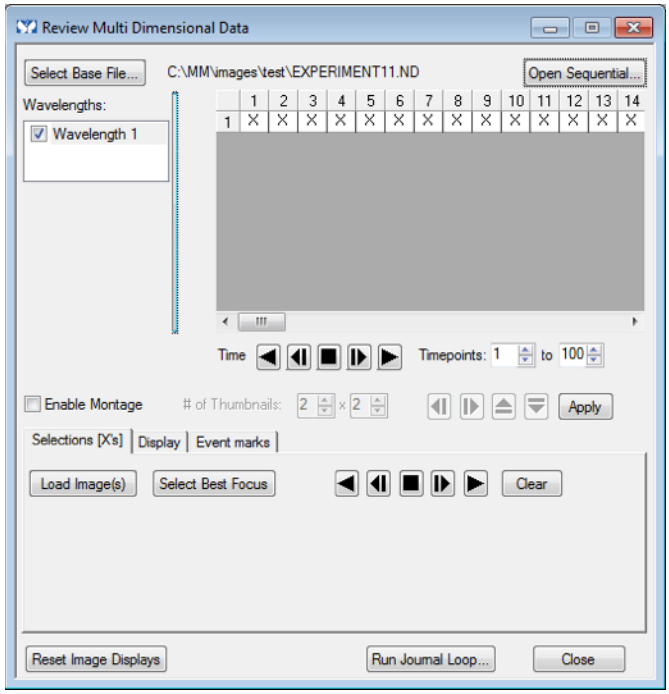
1. Configure the camera properties (EMGain and Exposure time)
2. In order to display time point progress information choose the **DisplayStreamTimepoint** from **User Program Name**. This executes a small Visual Basic program which tracks the number of frames that have been acquired so far.
3. Select **RAM** from **Stream To**.
4. Check the camera parameters using the live and snap buttons at the bottom of the window to acquire data from the camera.
5. Choose either, **full chip**, **centre quad** or **region of interest** to set the region of the camera chip to used during the recording.
6. Push **Acquire** to start. This will launch the **User Program** and also display a progress window. Note that the **Time Point** in this window will never increment beyond "1" to avoid slowing down the stream acquisition. Events can be marked during the acquisition by pressing "F5". The type of event can be changed using "F6".

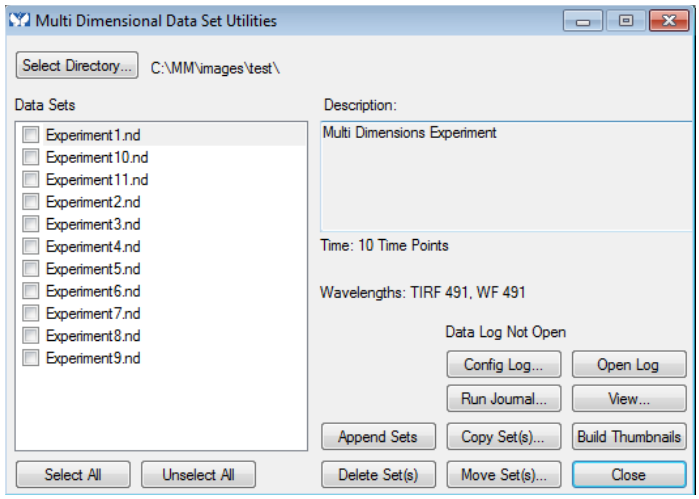


Review MDA data

In order to save recorded MDA data, it must be reviewed:

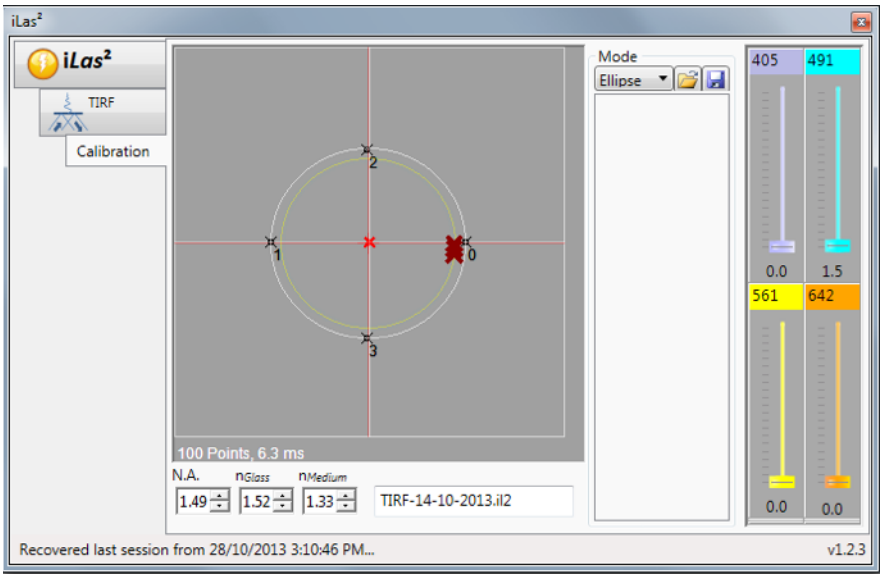
1. Go to Apps --> **Review Multi Dimensional Data**
2. Select the *.ND file named in the MDA app using **Select Base File**.
3. Check the boxes under **Wavelengths** to load the appropriate channel.
4. Click the **right mouse button** at the **top left corner** to select all images to be loaded (selected images are marked with a **X**).
5. Press **Load Images** to build a stack of images which can then be saved as a MetaMorph stack (*.stk) or a multipage tiff.

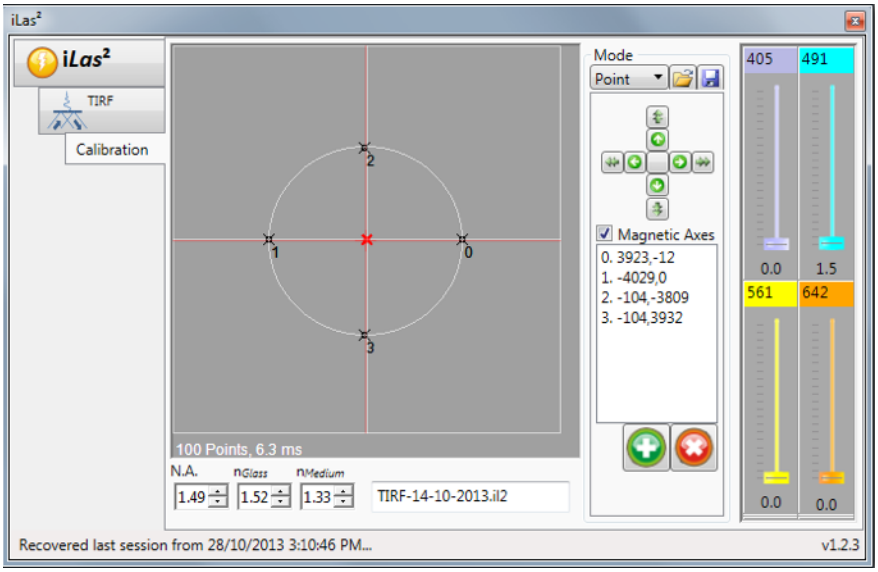




Advanced: TIRF Calibration

The iLas module uses galvonmeter scanning mirrors to control the position of the laser excitation in the back focal plane of the microscope objective. Due to the speed of the scanners an annular illumination pattern is produced which allows even illumination when operating in TIRF mode. For each type of sample used on the microscope (and additionally for each filter cube used in the microscope) the position of the scan mirrors needs to be calibrated .





Mount a sample onto the stage and optimise a live acquisition until a signal is clearly observed.
Choose the **Calibration** tab then:

1. Select **Point** mode from the drop down list.
2. Click on the target area at the due-west position to create **position 0** and observe the sample with a live acquisition running. Adjust **position 0** using the course adjustment until the sample is no longer visible. At this point we are beyond the critical angle. Use the fine adjustment to bring the sample back into view and stop at the point where it is just visible.
3. Click on the target area at the due east position to create **position 1** and repeat the procedure described above in 2.
4. Repeat step 2 at the north and south positions in the target area to complete the calibration. A circle should be visible in the target area and the image of the sample should remain the same when changing between each of the four positions in the list.
5. Select the **TIRF** tab and make sure that TIRF is achieved at the expected angle (~79°).

WORKING WITH CONFOCAL AND TIRF MICROSCOPES

Ergonomics: Use of mouse and keyboard / viewing computer screen – Prolonged use of the microscope and microscope computer without breaks can increase the risk of muscular strain.

Eye strain and fatigue – Viewing samples through microscope eye piece or computer monitor over lengthy periods of time can result in eyestrain and headaches.

Exposure to sharps – Exposure to razor blades, scalpels, forceps, cover slips, glass slides could result in cuts or puncture wounds to hands or other areas of the body. Any microscope slide shards or glass debris must be disposed of in the appropriate shapes disposable bin in accordance with PC2 regulations.

Exposure to intense fluorescent and laser light – Lasers and a xenon light source are attached to this microscope and are the source of intense and potentially dangerous light. Under no circumstances should any optical elements be removed from the microscope light path or fail-safe switches be circumvented. Do not attempt to adjust the lasers, laser light path, or laser modules in any way. Avoid direct exposure to the light.

Scope

This procedure details the method for using the microscopes equipped with laser light sources.

Safety Considerations

Personal Protective Equipment (PPE):

Laboratory coat, **latex gloves** and **closed in shoes** should be worn to prevent injury.

Ergonomics and Risk Exposure:

Appropriate ergonomics, including adjustment of the seat, computer screen and microscope oculars should be undertaken to reduce risk of strain injuries.

Emergency Procedures:

First aid may be required for:

Exposure to sharps – Contact the nearest first aid officer from the list that is beside all first aid kits and on safety notice board.

Exposure to intense fluorescent and laser light – Seek immediate medical assistance if you have been exposed to intense direct light or laser light.

In the event of a laser accident, do the following:

1. **Shut down the laser system.**
2. **Provide for the safety of personnel (first aid, evacuation, etc). If needed, provide further medical assistance for Eye Injuries by:**
   Proceed directly to: **Royal Brisbane and Women's Hospital at**
   Cnr Butterfield St and Bowen Bridge Rd
   HERSTON, QUEENSLAND AUSTRALIA 4029
   (07) 3636 8222

   Note: If a laser eye injury is suspected, have the injured person keep still and looking straight up to restrict bleeding in the eye. Laser eye injuries should be evaluated by a physician as soon as possible.
3. **Contact UQ Security Emergency on 336 5333.**
4. **Inform QBI's Laser Safety Officer, Rumelo Amor on 04 4907 8485, of the accident as soon as possible.**
5. **A UQ online incident report must be completed as soon as possible after the incident.**

All incidents must be reported to the OH&S Manager and on UQs online incident reporting system.

Contacts: Security x53333 or OH&S Manager Ross Dixon 0401 673 654