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
- 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 iLas² 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 iLas² 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 iLas² plugin

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File	Edit	Regions	Stack	Acquire	Devices	Display	Process	Log	Measure	Journal	Apps	Win
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R	;][\mathbb{C}	$ \setminus l$	∠ C	Æ	4			9	iLas2	TIRF

iLas²		
iLas ²	Targeted Laser	☑ Always on top □ Help □ Hardware
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The iLas² plugin

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

VI A souties		
Acquire		
Acquire	Image: 📑 Acquired	
Save 'Acquired'	Save to: C:\MM\\Acquire	ed001.tif Set Save
Save w/Sequence	Display Acquire Correc	t Annotate Special Live Replay
Exposure Time:	Digitizer:	10 MHz 👻
50 🖨 ms 🔻	Gain:	Gain 2 (2x) 🔹
AutoExpose	EM Gain:	1 🔹 🖌 🕨
Binning: 1	Camera Shutter:	Always Open 🔹
Camera Area:	Clear Mode:	CLEAR PRE SEQUENCE
-> Full Chip	Clear Count:	2 🔹 Info
Line Active Region	Trigger Mode:	Nomal (TIMED)
Use Active Region	Live Trigger Mode:	Nomal (TIMED)
Show Live	Show Focus Indicator	
Live Bin: 1 🚔	Frames To Avg:	1
Temp: ~ -75 c		
0		
Setting [Modified]:		
Close Less <<	Setting: Load	Save Save As

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)

 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.

Course User Programs	Camera Pa	arameters	
Acquisition Mode: Stre	am to RAM		-
Number of frames:	500	÷	Browse
Filename:			
🗖 Save du	ring acquisitio	on 🔲 Save one imag	je per file
Acquisition Information	n:		
Your current acquisition	on region is:	256x256	
Each frame will use:		2 bytes 128.00 KB	
Total number of frame	is:	500	
Amount of memory str	eam will use:	94.50 MB	
Amount of memory sta Amount of memory rec	sck will use:	62.50 MB 157.00 MB	
Amount of memory av	ailable:	31.93 GB	
Pandaut time profess	ne:	0.00 ms	
neadout ume per fram			
Acquisition time for str	ream:	25.10 to 50.10 sec	
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Acquisition time for str nitial Illum: [Cur Run user programs	ream: rent Shutter]	25.10 to 50.10 sec	•
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M Stream Acquisition
Acquire User Programs Camera Parameters
Program Name: DisplayStreamTimepoint.UserMethods
This program will run as each frame is collected. The command line for the program will indicate the event that triggered the program to run.
The following variables are available to the program:
All events: Stream Run UserProgram Frame Width Stream Run UserProgram TrameDepth Stream Run UserProgram TotalTeames Stream Run UserProgram Abort NEWFRAME event: Stream Run UserProgram FrameNumber NEWIMAGE event: Stream Run UserProgram ImageName
Status: Configure OK
Status, configure on
Acquire
Record Configuration State Close

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.

Multi Dimensional Ac	quisition			
/lain				
Saving	Timelapse			Summary
Timelapse	Multiple Stage Positions			
Wavelengths	Multiple Wavelengths			Save Stat
W1: TIRF 491	Z Series			
W2: WF 491	V Stream			Load Stat
Stream	Run Journals			
Display				
Summary				
		Previous	Next	

/lain	Description: *Im:	ages automatically saved with base f	file*
Saving	Multi Dimensions Exc	periment	
Timelapse			
Wavelengths	•		*
W1: TIRF 491	Colort Directory	C/ MM/immers/test	
W2: WF 491	Select Directory	C: WM Mages vesi	te
Stream	Base Name	Experiment8	
Display	baco namo.		
Summary			

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.

Main			
Saving	Experiment Length		
Saving	Number of time points:	100 🚖	
Timelapse	Duration:	99 🚔 sec 🔻	
Wavelengths			
W1: TIRF 491	Time Interval:	1 🚔 sec 👻	
W2: WF 491	Estimated minimum inte	rval: 0.05 sec	
Stream			
Display			
Summary			
		Previous Next	
🗃 Dias 1 🔺 🛄 I	Pin: 1 🔺 🔳 🔳		

1ain	Experiment Length	
Saving	Number of time points: 100	
Timelapse	Duration: 99	
Wavelengths		
W1: TIRF 491		
W2: WF 491	Time streaming is on time interval determined by exposure	
Stream	and arouning is on, and interval determined by expositio.	
Display		
Summary		
-	Previous Next	
) Bin: 1 🚔 🔍 E	in: 1 🚔 🔳 🔳 📲 1:TIRF 491 👻 📑	Close

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.

Saving Saving Timelapse Wavelengths W1: TIRF 491 W2: WF 491 Stream Display Summary	Main	
Timelapse Wavelengths W1: TIRF 491 W2: WF 491 Stream Display Summary	Saving	Number of Wavelengths: 2
Wavelengths W1: TIRF 491 W2: WF 491 Stream Display Summary	Timelapse	Allow separate hardware AF offsets for each wavelength
WI: TIRF 491 W2: WF 491 Stream Display Summary	Wavelengths	Allow separate binning for each wavelength
W2: WF 491 Stream Display Summary Merevious	W1: TIRF 491	
Stream Display Summary	W2: WF 491	
Display Summary	Stream	
Summary	Display	
Previous Next	Summary	

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".

🎦 Multi Dimensional A	cquisition 🗖 🖼 🛛
Main	Stream Time Configure Camera Mode
Saving	
Timelapse	
Wavelength	User Program Name: DisplayStreamTimepoi 💌 Update preview every 10 📑 trames
Stream	
Display	Stream Gain: Gain 2 (2x)
Summary	Stream EM Gain: 300 🛨
	Stream Exposure Time (ms): 50
	Status:
	Stream configuration OK
	Change Tay DAM
	Memory Required 2.20 GB Memory Available 31.93 GB
	Previous Next
🗰 Bin: 1 📑 🏴	Bin: 1 🛨 📕 🔳 1:WF 405+561 🔽 🔡 Acquire Close



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.

Y Review Multi Dimensional Data	
Select Base File C:\MM\images\test\EXPERIMENT11.ND Wavelengths: 1 2 3 4 5 6 7 8 9 Image: Very Wavelength 1 1 X	Open Sequential 10 11 12 13 14 X X X X X Image: the sequence of
Enable Montage # of Thumbnails: 2 x 2 x	Apply
Selections [X's] Display Event marks	
Load Image(s) Select Best Focus	Clear
Reset Image Displays Run Journal Loop	Close

Multi Dimensional Data Set Utilities	
Select Directory C:\MM\images\test\	
Data Sets	Description:
Experiment 1.nd Experiment 10.nd Experiment 11.nd Experiment2.nd Experiment3.nd	Multi Dimensions Experiment
Experiment4.nd Experiment5.nd	Time: 10 Time Points
Experiment 6.nd	Wavelengths: TIRF 491, WF 491
Experiment8.nd	Data Log Not Open
Experiment9.nd	Config Log Open Log
	Run Journal View
	Append Sets Copy Set(s) Build Thumbnails
Select All Unselect All	Delete Set(s) Move Set(s) Close

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

il ac 🕥 iLas² Mode 405 Ellipse 🔻 💕 🛃 TIRF Calibration 0.0 1.5 N.A. nGlass **N**Mediun 1.49 ÷ 1.52 ÷ 1.33 ÷ TIRF-14-10-2013.il2 0.0 0.0 Recovered last session from 28/10/2013 3:10:46 PM v1.2.3



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°).

QUEENSLAND BRAIN INSTITUTE – STANDARD OPERATING PROCEDURE

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 <u>Eye Injuries</u> 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