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 CO₂ control.
- Turn on CO₂ tank by turning the main switch anticlockwise
- Turn on CO₂ 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



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)

Sapphi	.re 488	nm 100mW
Laser9	Status:	StandBy
Set:	50.1mW	50%
Act:	0.0mW	0%

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



• For Brightfield, click Trans \rightarrow Current shutter \rightarrow Switch On the Brightfield LED \rightarrow adjust brightness



- Press the right arrow of the DISP button.
- Move the Stage Controller to adjust XY position (XY speed can be adjusted: $\frac{1}{2} > \frac{1}{2} > \frac{1}{2}$
- 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.



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"

🖉 Multi Dimensional Acq	quisition
Main Saving Wavelengths W1: Camera Red W2: Camera Green	Number of Wavelengths: Allow separate binning for each wavelength
W3: Confocal Green	
Display	
Summary	

- 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

Main	- Illumination	Trane
Saving Timelapse	Gain:	[None] Laser WF Cy5
Stage	Digitizer:	Laser WF DAPI Laser WF GFP
Wavelengths	EM Gain:	TIRF Cy5
W1: Trans	Evposure	TIRF DAPI
W2: WF RFP	- Exposure.	TIRF RFP
W3: WF GFP	- Auto Expose:	WF Cy5 WF DAPI
Z Series	- Acquire:	WF GFP WF RFP

un	Illumination:	TIBE GEP
Saving		
Wavelengths	Gain:	Gain 2 (2x)
W1: TIRF GFP	Digitizer:	10 MHz (EM Gain)
W2: TIRF RFP	EM Gain:	300 -
W3: Trans	Emanuel	100
Display	Exposure.	
Summary	Auto Expose:	No Auto Expose
	Auto Focus:	No Auto Focus 💌 🔽
	Alignment Crop	ping X: 0 🛨 Y: 0 🛨 Set Alignment.

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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.



405nm 491nm 561nm 642nm – 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 Dimensional Acc	juisition								
Main	Experiment Length								
Saving	Number of time points:	600	•						
Timelapse			*						
Wavelengths	Duration:	599	min	~					
W1: Camera Green					🛍 Bin: 1 🕂	Bin: 1 📑	1:TIRF GFP	-	Acquire
W2: Camera Red	Time Interval:	1	min	~					
W3: Confocal Phase	Estimated minimum inte	erval: 0.01 min	ms sec						
Display			min						

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

	Multi Dimensional Acc	quisition	
C	tan Saving Timelapse Stage Wavelengths W1: Camera Green W2: Camera Red W3: Confocal Phase	Position Label: position 2 ** 1.52422e • ** 6.08522e • Z: 3143.93 • • Offset 2 for travel to this position	
	Summary	Z travel offset Positions: Position 1 (-1.52422e+006, -6.08522e+006, 3143.93) X Move to Position Load. Use physical stars position/www.elength table accuriting parameters	0 C

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.

	Multi Dimensional Acquisition 🔚 🛄 🔼	
🎽 Multi Dimensional Acquisition 📃 🔲 🔀	Fime Point 3 of 55	
Time Point 2 of 600	Stage Position 1 of 2	
Single Stage Position	Single Z Position	
Single Z Position	√avelength Confocal Green	
Wavelength Camera Phase		
Next acquisition in 00:00:04	- Paused -	
	Time Interval: 10 💲 sec 💌	
	Stage	
	Position Item: a2	
Acquisition hot keys:	Enabled Go to Set to current	
Alt-M: Mark Event dialog	Was planeth 2:Canfacel Croop	
F5: Mark Event- Stimulation	Wavelengur.	
F6: Change event type	Acquisition hot keys:	
	Alt-M: Mark Event dialog Alt-P: pause acquisition	
Pause Mark Event Cancel	F5. Mark Event-Stimulation	
	Fo. Change eventype	
	Live	
	Besume Mark Event Cancel	

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





Main

Version 1.2 2019

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

	 Interactive seturius 						
Saving	Current Position:	3143.93	^	um	Increment	1	1
Timelapse				P			Y
Stage	- Settings for acquisi	tion series-					
Wavelengths	Loop order						
W1: Camera Green	O Acquire wave	length set a	t each	ηΖ			
W2: Camera Red	Acquire Z seri	es for one v	/avele	ength	at a time		
W3: Confocal Phase	Keep shutter	open betwe	en ste	eps			
Z Series	Range:	3	\$	🖌 Ri	ange Around	Current	
Display	_	01.45.40					
Summary	Top:	3145.43	Ţ.	S	et Top To Ci	urrent	
	Bottom:	3142.43	* *	Se	t Bottom To (Current	
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Recommended Step Size: 0.3 um

- 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 Y
- Click on the calibration button 😔 to begin calibration
- When calibration is done, click on the save icon to save the calibration setting



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Duration

Pre Sequence

Perturbation

Post Sequence

150 ÷

300 ÷

1000 ÷

1000 +

160

₩ #1

F #2

F #3

Interval

30 ÷

30 ÷

100 -

1000 -

Click on the Acquire icon to begin acquisition

milliseconds

Setup MDA

inutes

econd

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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.

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- 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.
 Selections [X3] Display Z Projection | Event marks]
 Full Image Active Region
 Color Composite (None> Confoced)
- 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.

Full Image	Active Region						
	Red:		Green:	Blue:		Gray:	
Color Composite	<none></none>	*	Confocal Blı 💌	<none></none>	~	<none></none>	~

Selections [X's] Display Z Projection	Event marks
Z Projection	
Type: Maximum	Border Color: U Create Rotation
Angle: 0	Vertical Calibrated User Specified
Planes: 1 to: 2 C	All Planes Z Dist.; 1.5 💲

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 computer(3).
 - 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 CO₂ 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.