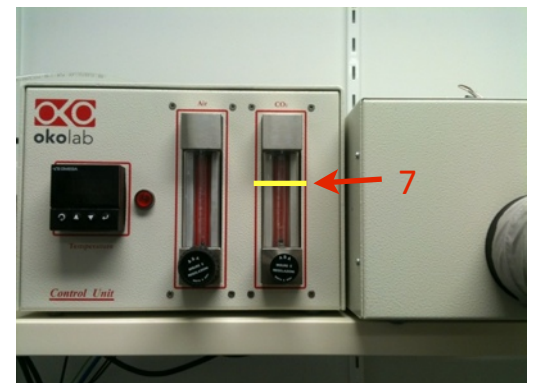
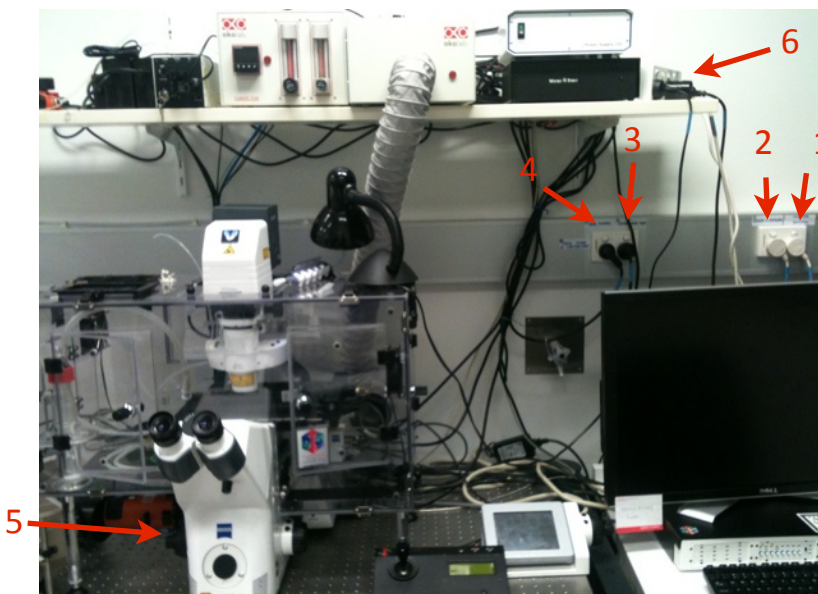


Marinas TIRF/FRET/FRAP Microscope

Getting Started


1. Switch on the four switches along the wall (right to left).
 - Computer + Monitor **(1)**
 - Laser + Cooler **(2)**
 - Xenon Lamp **(3)**
 - Main System **(4)**
2. Switch on the microscope (at the *back left* of the microscope stand). **(5)**
3. *If imaging live cells:*
 - Switch on the incubator - switch located on the powerboard on the shelf above the monitor **(6)**
 - Turn on the CO2 by adjusting the CO2 valve to 2/3rds open **(7)**
 - The heat should be left to equilibrate for ~2 hours before starting imaging.
4. Switch on the computer and log in as **Fred Meunier**
5. Once the microscope has finished starting-up double click the **Slidebook 5.0** icon.
6. On the microscope touch screen **open the reflected light shutter (RL shutter)**

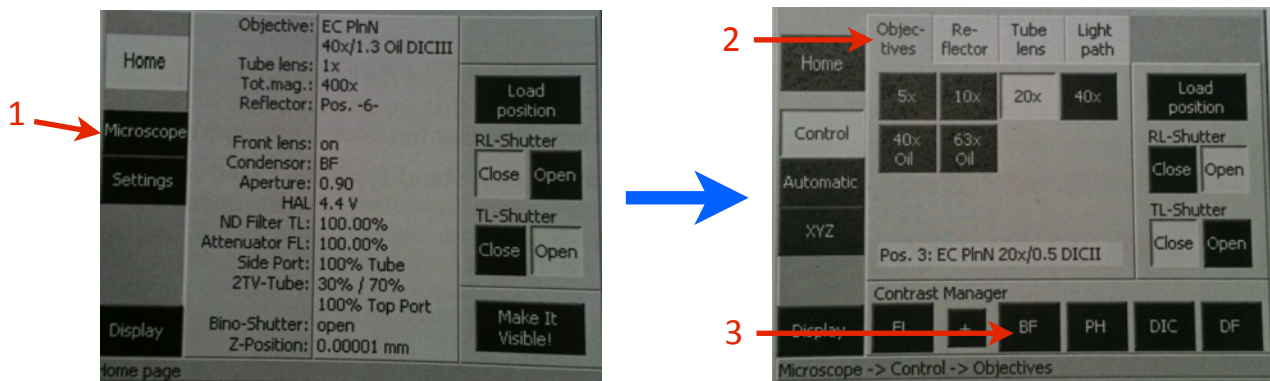


Shutting Down

1. Lower the objective and remove your sample.
2. Gently wipe any oil objectives you have used with lens tissue (**do not use kim wipes to clean objectives**).
3. Exit the software and copy your files to your *home or group network/USB drive*.
4. Turn off the four main switches from left to right.

Visualising a Sample Through the Oculars

1. On the touch screen attached to the microscope press “**load position**” and position the slide/dish on stage. Pressing  the button will return you to the working position.
2. Press **microscope** (1) on the far left of the touch screen.
3. Here you will be able to change the *objectives* and adjust the *light path* via the tabs at the top. (2)
4. It is only possible to view a sample with brightfield before starting the software.
 - To do this press either the BF or DIC quicks links at the bottom of the touch screen. (3)
5. Check the light path is allowing the sample to be seen via the oculars (under the “**light path**” tab).
 - To do this press the eye icon.
6. An image will now be visible down the oculars.
 - Adjust the brightness of the lamp via the dial at the very front of the microscope.
 - Adjust the stage position and focus as necessary.



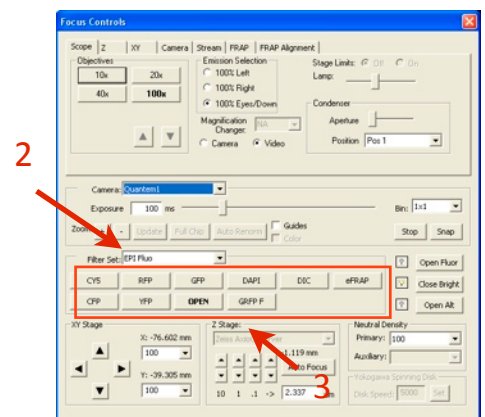
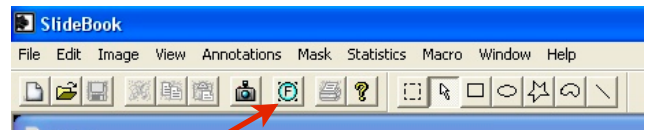
Preparing to Image

Live View

1. Click on the **focus control** button. (1) The focus control and live view window will appear.
2. From the dropdown list (2) choose the type of imaging you wish to do:
 - **EPI Fluo**
 - For visualisation through oculars and standard epi-fluorescence imaging
 - **TIRF**
 - For single channel TIRF imaging and high speed TIRF to epifluorescence imaging
 - **Dual TIRF - EPI**
 - Use these settings for simultaneous imaging of dual colour TIRF and/or epi-fluorescence
 - **Dual FRET**
 - For imaging CFP/YFP or GFP/RFP FRET in TIRF or epifluorescence - 3 colour sensitised emission fret non 1:1 stoichiometry
 - **Ratio TIRF FRET**
 - CFP/YFP or GFP/RFP FRET - 1:1 stoichiometry
3. In the buttons below, click on the marker you wish to see. (3)

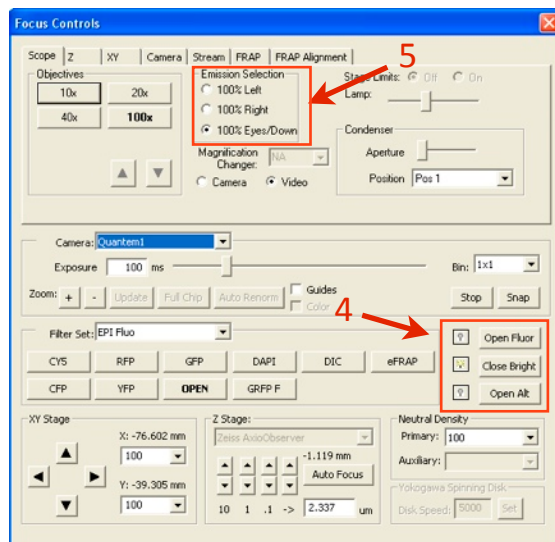
To see **GFP as standard epifluorescence** - Choose **EPI Fluo** in the drop down list then click the **GFP** button.

To see **GFP as TIRF** - Choose **TIRF** in the drop down list then click the **tGFP** button



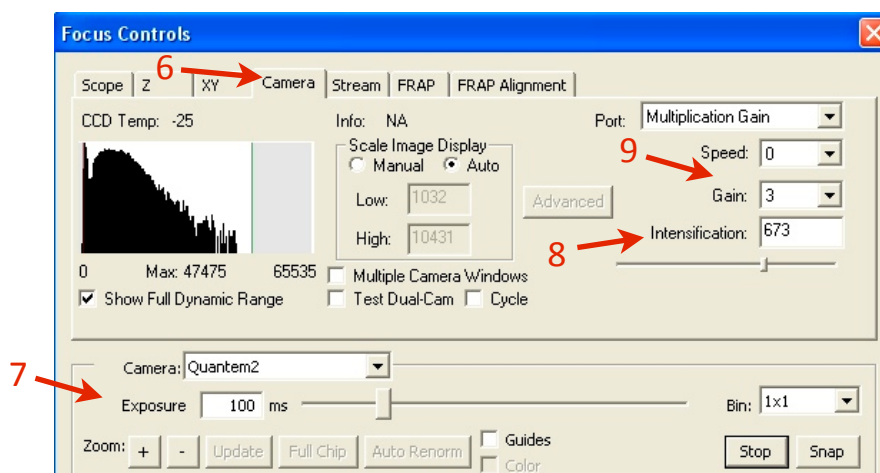
Preparing to Image

- Open the fluorescent light shutter to start getting a preview image in the live window. (4)
 - **Open Fluor** - opens the fluorescent light shutter
 - **Open Bright** - turns on the transmitted light (for brightfield/DIC)
 - **Open Alt** - opens the laser shutter (for TIRF)
- If you want to see down the oculars click the **100% eyes/down** button. (5)
 - Clicking **100% left** sends the light back to the camera.



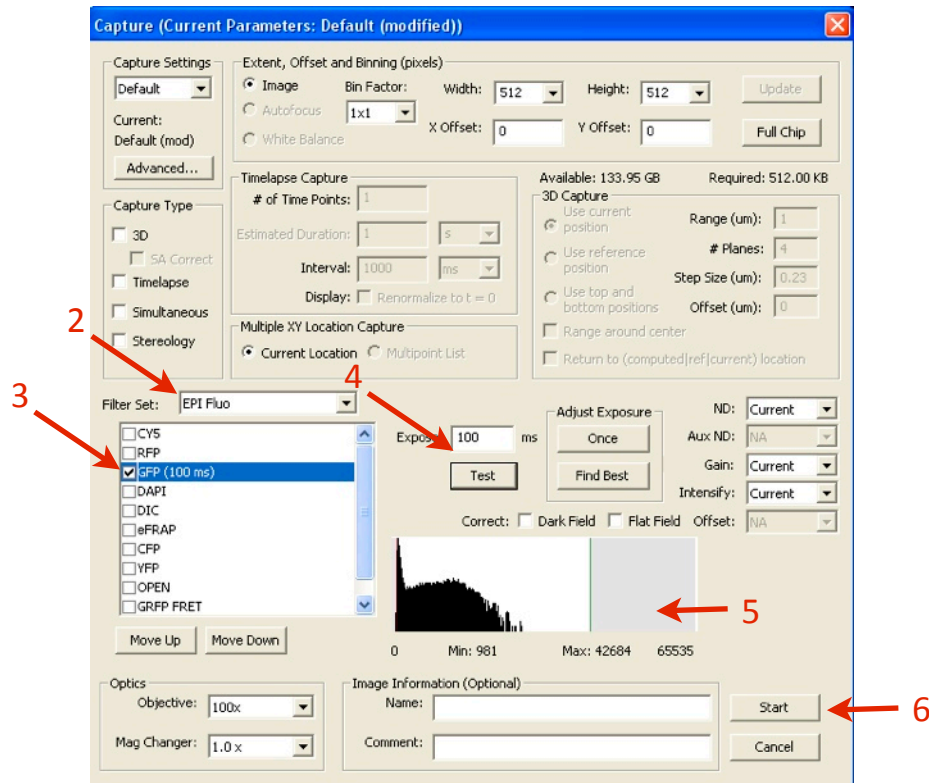
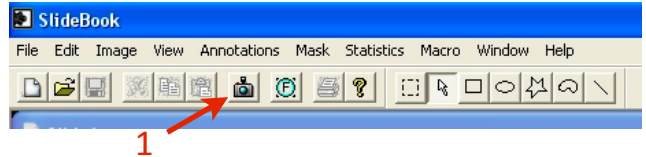
Adjusting the Camera

- To adjust the camera settings click on the camera tab. (6)
- In the top left is the histogram.
 - Use this to judge how bright your sample is, as the software always autoscales to make the image look bright.
 - Aim to have the histogram 2/3rds full (unless there are time constraints or the sample bleaches easily)
- To adjust the brightness of your image you can:
 - Adjust exposure (7)
 - Adjust intensification (8)
 - Adjust gain (9)
- Use intensification and gain to get a bright image whilst keeping your exposure time short.
- Preview all the channels you wish to image with the focus control (and ensure both cameras have had their intensification and gain adjusted).



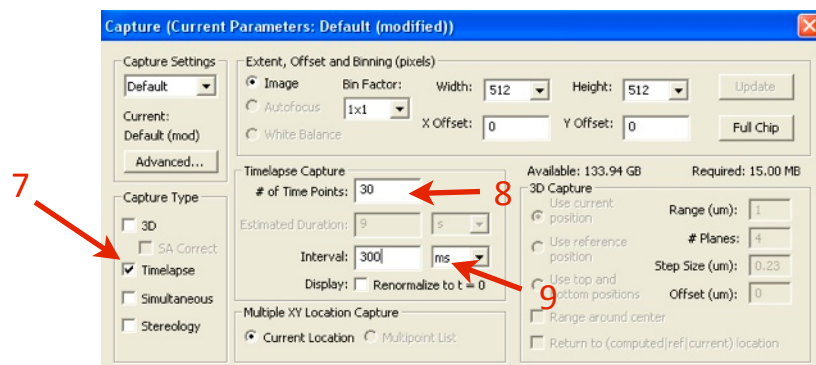
Capturing an Image

1. Click the **capture image** icon. (1) The focus control and live view window will appear.
2. In the drop down list choose the imaging type. (2)
3. Tick the box next to each channel you wish to capture. (3)
4. Check the exposure time of each channel.
 - Do this by highlighting the channel by clicking on it, then pressing test. (4)
 - Adjust the exposure time as necessary to get a good range of intensities in the histogram box. (5)
 - Each time you press test it will take only one image - prevents bleaching
5. Check the exposure on each channel you wish to image then click **Start** (6) to take an image.



Timelapse Imaging

1. Enable timelapse. (7)
2. Enter # of timepoints (8) and interval. (9)
3. Click **Start** to capture movie.



Capturing an Image

TIRF Imaging

TIRF uses an angled laser to illuminate the bottom **100nm** of a cell and can only be achieved using the **100x objective**.

When the angle or focus of the laser is not set correctly it will not be possible to get a TIRF image.

If you have trouble getting an image - **do not attempt to adjust the laser yourself**. Instead, call Luke on 0402 586 647.

Dual TIRF / Epi

These settings are used to get an image of the TIRF layer and the epifluorescent layer in the same focal plane. This technique is used to determine whether carriers are fusing to the plasma membrane or moving upwards into the cell.

Choose **Dual TIRF / Epi** in the dropdown menu and then select the appropriate channels

- For example in the case of GFP choose **dtGFP** and **deGFP**

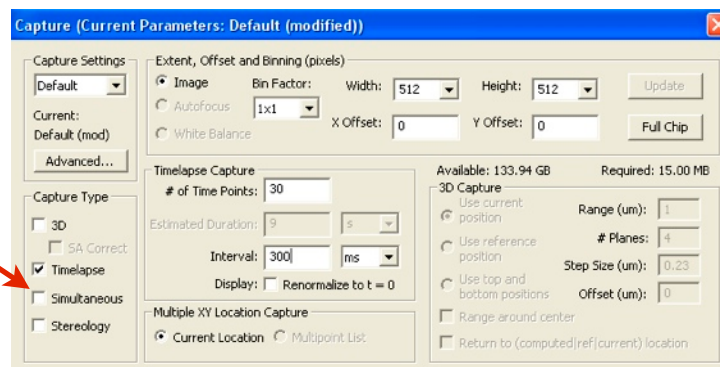
Simultaneous Imaging

The Marinas microscope has two cameras so that two channels can be captured simultaneously.

To image simultaneously use the **Dual TIRF / Epi** settings.

It is important to ensure that the two channels that are captured simultaneously (e.g. **dtGFP + dtRFP**) have the same the same exposure time.

In the capture type, enable **simultaneous** imaging.



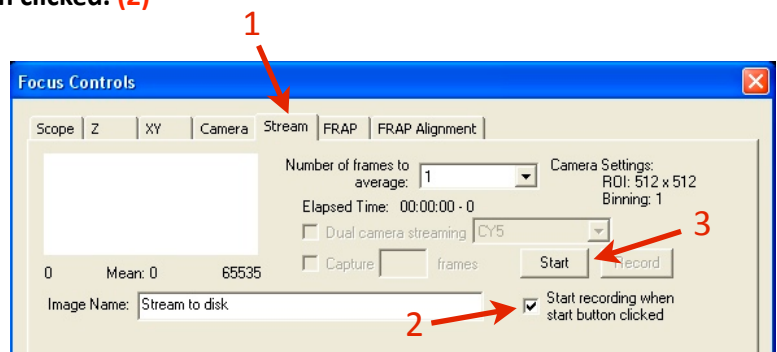
High-speed Timelapse / Streaming

Using timelapse in the **capture** window the maximum speed is limited to **20 Hz** (full frame/chip). If you need to go faster than this you will need to use **Streaming** in the **focus control window**.

Using **Streaming** you will be able to image at **30Hz full frame**, **60Hz at 256x256**, **105Hz at 128x128**

To use streaming:

1. Set-up channel in focus control (exposure, intensification, gain).
2. Click on the **Stream** tab. (1)
3. Enable **Start recording when start button clicked**. (2)
4. Open the **FI** or **Alt** shutter to illuminate sample.
5. Click **start**. (3)



FRET Imaging (Forster/Fluorescence Resonance Energy Transfer)

$$F_{\text{image}} = F_{\text{real}} + \text{Donor bleed-through} + \text{Cross excitation}$$

therefore

$$F_{\text{real}} = F_{\text{image}} - \text{Donor bleed-through} - \text{Cross excitation}$$

Capture

1. Donor only sample - Donor excitation and Donor emission
2. Acceptor only sample - Donor excitation and Acceptor emission
3. Donor + Acceptor Emission

1. Donor ONLY sample: Donor Excitation

- Look at donor emission
- Look at acceptor emission
 - Image several fields figure out bleed-through for each and get mean
 - Use same settings (e.g. 100ms exposure, same ND filter etc)
- Create segment mask based on donor (mask button above captured image)
- FRET > compute FRET bleed-through
 - Have background ROI (draw ROI, select, set as background)
 - Set up FRET pairs (e.g. GFP, RFP, RFP as Transfer)
 - Factor 0.64 means 64% of image is bleed-through
 - Make default

2. Acceptor ONLY sample: Donor Excitation

- Segment mask on acceptor (look at donor)
- Draw ROI - background
- FRET

3. Donor + Acceptor Emission FRET settings

- Image sample

4. FRET settings

- Click use default (imports values from steps 1 and 2)
- Different options for calculating FRET
 - 1st is basic calculation
 - 2nd and 3rd ratiometric for visualisation BUT ratio of molecules has to be 1:1
 - 4th has correction factor "g" to allow for different ratios of donor/acceptor
 - 6th Efret also calculates for photobleaching

5. Run calculation and create virtual FRET channel

- Segment out cells
 - Menu statistics > mask statistics
 - Run statistics to normalise for different brightnesses in donor

Notes:

- Make sure your background ROI is in a clear spot --> view image with high contrast (manual scale low)
- ALWAYS use the same method - don't change algorithm
- Donor and acceptor alone can have different exposure times
 - But for actual experiment exposure times for all needs to be the same
 - Calibrate on brightest channel - usually GFP
- in 1:1 cross excitation irrelevant
 - only option is to FRET or not FRET (only excite with donor excitation)
- Ratio - FRET > add donor and transfer

Acceptor photobleaching FRET

- Image donor > bleach acceptor > image donor again
- Donor brightness should increase
- Quantitative technique (other FRET techniques are QUALITATIVE - Yes/No - won't tell you how much FRET is occurring)

Imaging a Sample: FRET

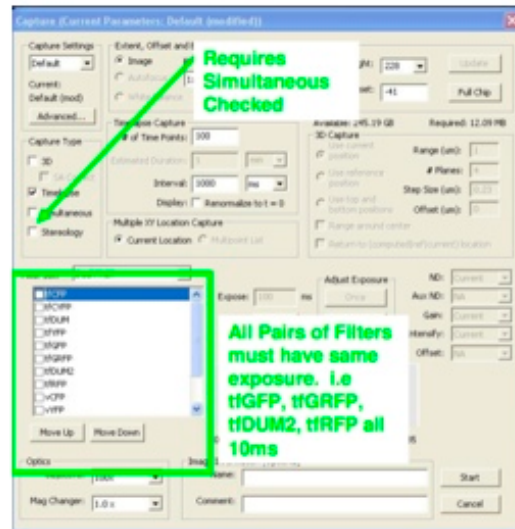
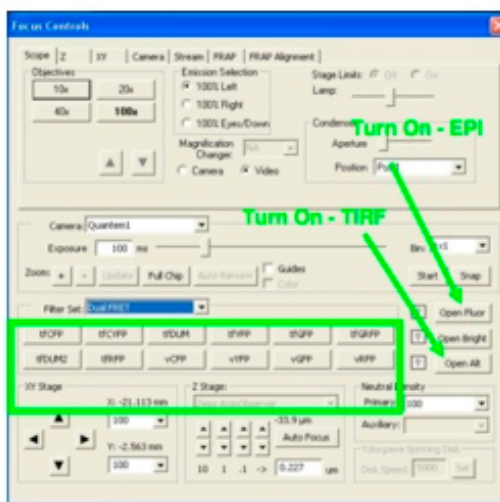
Dual FRET

For high speed FRET experiments for 3 channel FRET capturing Donor, FRET, Acceptor.

Each subset of filters must be captured using the same exposure time - requires simultaneous imaging.

Filters: GFP/RFP = tGFP + tGRFP + tDUM2 + tRFP

CFP/YFP = tCFP + tCYFP + tDUM + tYFP



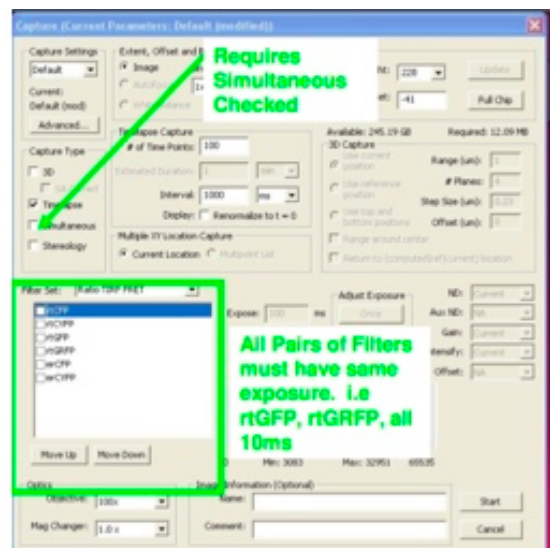
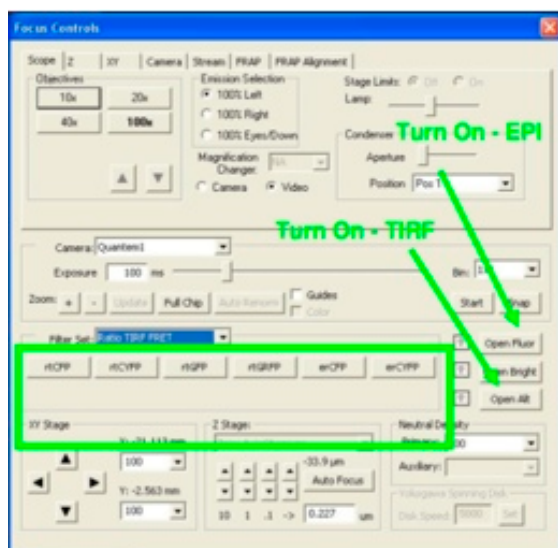
Ratio FRET

For high speed FRET experiments for 2 channel FRET capturing Donor, FRET, Acceptor.

Only when you have bound Donor and Acceptor in a 1:1 stoichiometry.

Data will be automatically displayed in pseudocolour as FRET/Donor

Filters: rtGFP + rtGRFP or rtCFP + rtCYFP or erCFP + erCYFP



FRAP - Performing FRAP experiments and analysis

FRAP will work in epi, TIRF, FRET and Ratio modes

FRAP system uses a 337nm laser to excite cumarin dye - which then emits a 440nm beam able to bleach GFP.

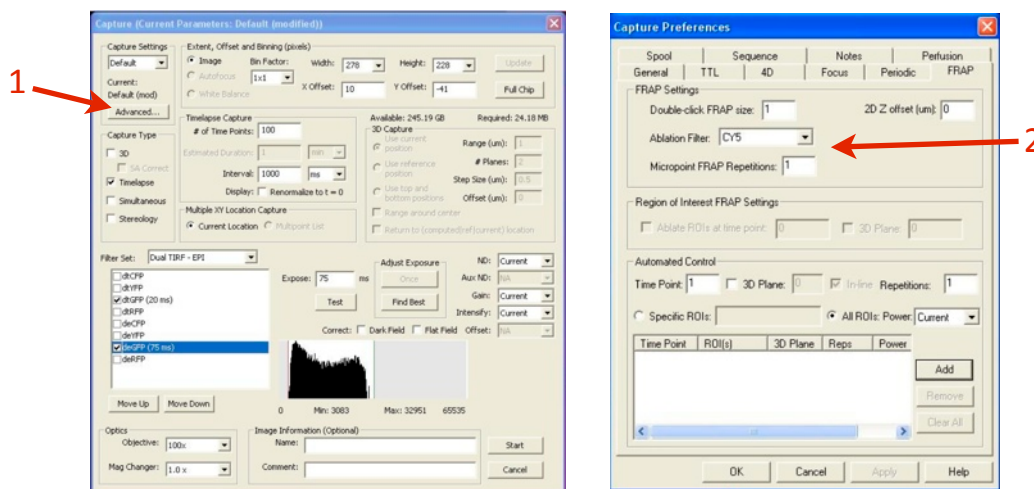
You must have an emission filter in place when performing FRAP. This will prevent damage to the CCD.

Setting up a FRAP experiment

1. Insert the correct Mirror at the back of the microscope - **50/50 Mirror**
2. Switch on the FRAP module via the key.



3. Ensure FRAP laser has been aligned and calibrated.
4. Set up your normal imaging preferences in the focus window
5. In the Image Capture window click on **Advanced...** (1) and go to the **FRAP tab**



6. Select the filter you will be using from the **ablation filter** drop down menu (e.g. tGFP or dtGFP) (2)
7. Adjust the radius of the **double-click FRAP size** (1 = 1 pixel / default) or edit the settings for a FRAP ROI (draw an ROI on the live image via the region select tools in the top toolbar)

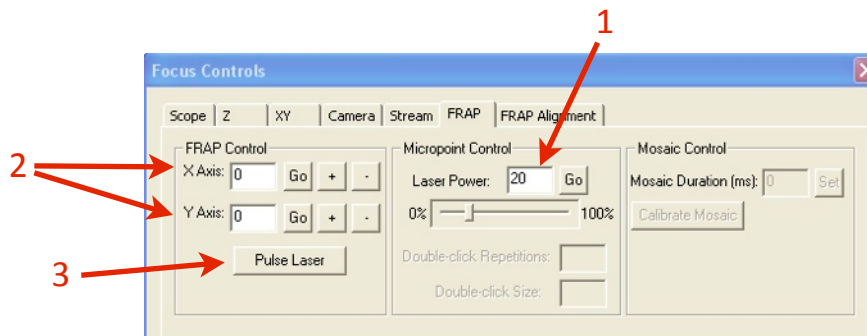
ROI FRAP and Monitoring Intensity

1. Set up your imaging preferences. Click **Test** in the Capture window and draw the ROI for FRAP
2. Choose your ablation filter (as above) and number iterations required
3. In the **FRAP tab** under Automated control add in the ROI you have drawn.
4. You may also draw a background ROI, by drawing a ROI in an empty space (right click on ROI and select "make background")
5. While capturing click **Show** (in the lower right corner of the capture controls, e.g. Show: dtGFP)

FRAP - Calibration and Alignment

Alignment of the FRAP laser

1. Insert the correct Mirror at the back of the microscope - **50/50 Mirror**
2. Switch on the FRAP module via the key.
3. Choose the objective you wish to use and open the **Focus Window**
4. Place the mirror slide on the stage (mirrored surface face down)
5. Use the dual TIRF GFP/RFP filter cube
6. Direct the light-path to the camera (*do not look down eyepieces*)
7. Under the **FRAP tab in the Focus window** set the **FRAP laser power to 30-60% (1)**



8. Centre the laser by entering "**128**" into both the X and Y Axis **(2)** and clicking **Go**.
9. Turn **on** the FRAP laser and turn the repetition rate knob until it is at **3-5 Hz** (you will hear a click every time the laser fires).
 - A hole should appear when the laser has repeatedly struck the mirror
 - **If a hole does not appear - call Luke (0402 586 647)**
 - Pivot the galvo heads with repetition rate at 0 - do not loosen hex screws that hold galvos
 - Locate the OFF switch for the galvos - galvos must be turned off immediately if they make any noise during pivoting.
10. Turn the repetition rate knob back to **0 Hz** (external pulse mode) and move to a *new position* on the slide then press **Pulse Laser (3)**
11. If a hole appears - reduce the laser and **repeat step 8** - keep going until one pulse does not produce a hole.
12. Now repeat step 8 but instead of adjusting the power, adjust the focus of the laser (telescoping lens)
 - The aim is to get the maximum laser power at the plane of focus

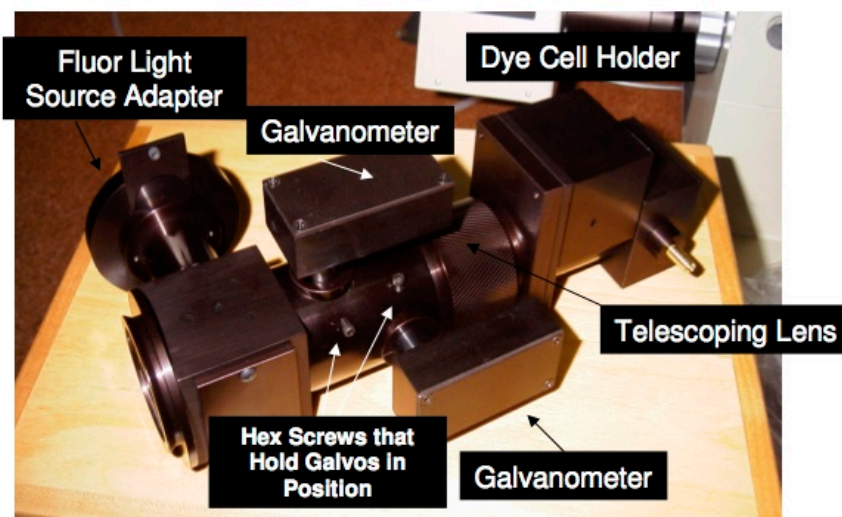


Figure 4: Coarse Focus screw on FRAP Positioning Unit

FRAP - Calibration and Alignment

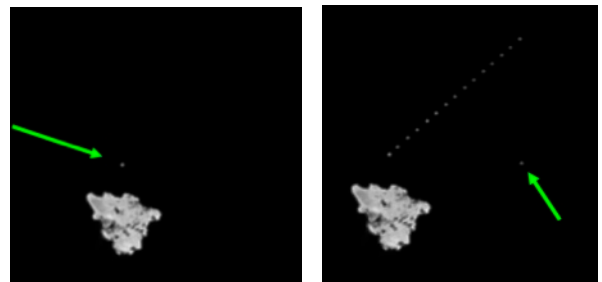
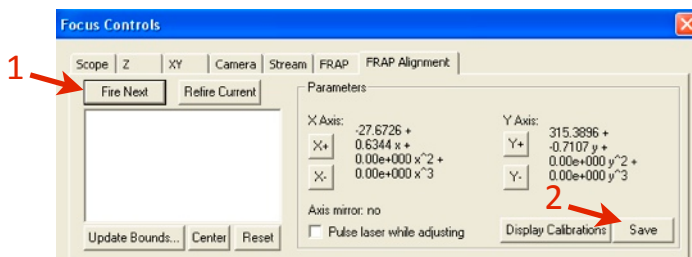
Alignment of Camera with FRAP unit

1. Ensure the Camera view is aligned square with the FRAP module - if you have used the grid slide to ensure both cameras are aligned (*see camera alignment*) this will already be the case. If not, put the grid slide on and make sure the pattern is square.
2. At the bottom of the laser tab of the focus window, select either X or Y axis and enter “25” to “225” step: “20” then **Fire Test Pattern** (on to Mirror Slide)
3. Observe the alignment of the line of holes in the image field. Rotate the FRAP unit slightly and repeat step 2. until the FRAP unit is aligned with the stage.

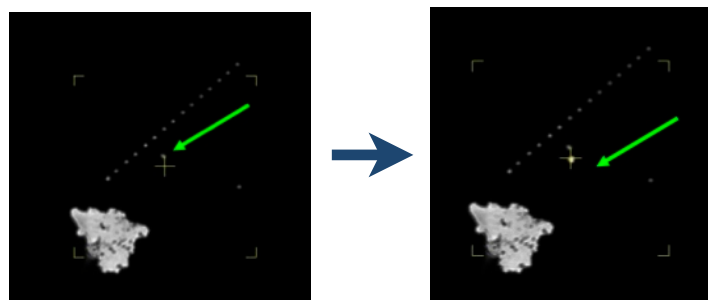
Calibrating the FRAP Positioning Unit

These steps use the **FRAP alignment tab** in the focus control window to calibrate the FRAP galvos.

1. In the FRAP tab set the laser power to 45 or higher
2. Go to the **FRAP alignment tab**
3. Move to a clean section of the mirror and obtain sharp focus on a scratch in the mirror.
4. Click the **Fire Next** button (1), then **double click** the resulting spot that appears (ensure you only double click once)
 - If the point does not appear click **fire next** again (or a few times) until it is visible, then double click the point
5. Continue step 4. till 16 diagonal points have been produced, the final point will be located in the bottom right
6. Click **Save** (2)



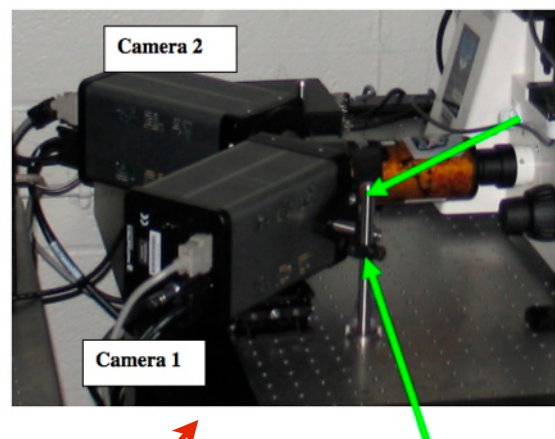
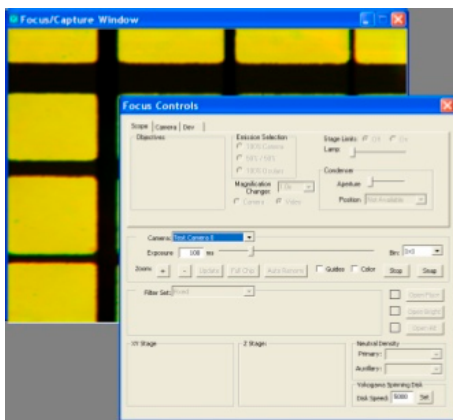
7. Next, click the **centre** button, and check its position on the camera (use the guides - toggled on by a check box found in the centre of the **focus controls window**)
8. Adjust if necessary by using the X+/- and Y+/- buttons in **FRAP alignment** then clicking **save** then firing again.



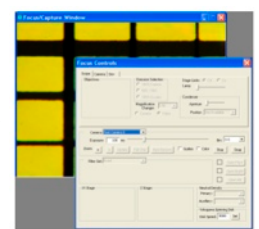
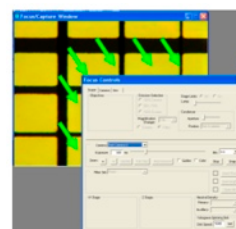
Aligning the Cameras

Whenever the camera splitter is swapped over (e.g. from GFP/RFP to CFP/YFP), or before an important experiment is started, the camera alignment should be checked. Doing so ensures that both cameras are seeing the same image. It will not be possible to get 100% alignment, but at least in the centre of the field of view everything should be overlapping.

1. Using the brightfield light and Camera 1 (Quantem1) focus the grid pattern at the centre of the grid slide provided.
 - The slide should be placed with the blue side facing down (onto the objective) and the silver side facing up.
2. Move the **focus control window** over the live image window and use this as a guide to straighten the grid pattern. **Do not move the focus window / live image window from now on.**

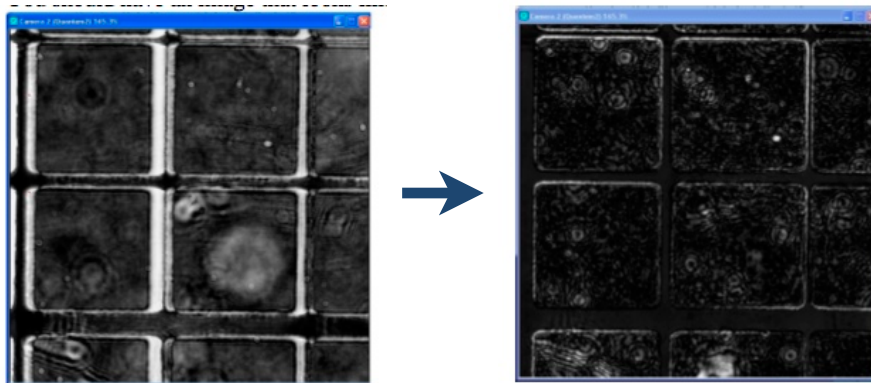
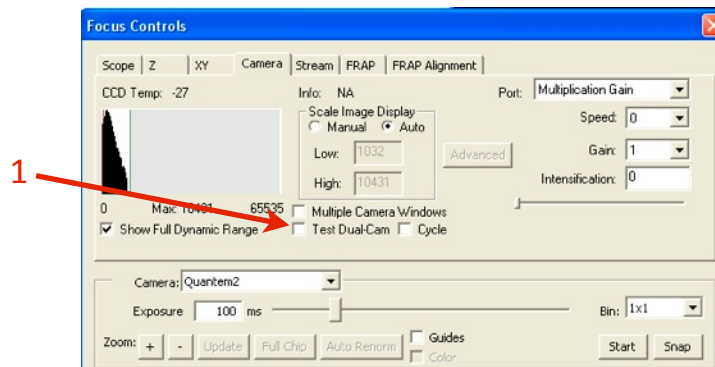


3. Rotate camera 1 (front camera) until the lines appear straight against the focus window.
 - Loosen the two screws attached to the side of the camera.
 - Gently twist the camera until the lines appear straight against the focus window.
 - Retighten the screws.
4. In the software switch view to camera 2 (Quantem2).
5. Now adjust the view of camera 2 to match the view of camera 1 by aligning it with the focus window.
 - If necessary rotate the camera (as described for camera 1).
6. To move the camera view Up/Down or Left/Right use the screws on the dual view.
 - Camera 1 screws are on the front, Camera 2 screws are on the opposite side.



Aligning the Cameras

7. Finally, under the **camera tab** in the **focus window** choose **Test Dual View (1)**
8. The new live image shows overlapping areas as black, and areas where there is no alignment as white.
 - Use this view to make fine adjustments to each camera until you have the best alignment (alignment will never be 100%)



Other Software Features

Deconvolution

Image --> deconvolve

Try various settings for best results

Montages

1. take montage
2. click file
3. image > generate montage > move tiles > ok

Mask Statistics and Object Tracking

- Mask > Statistics > tick on appropriate
- Mask > Advanced operations > Convert Mask to ROIs
- Mask > Particle tracking > Mask Statistics (sort by object available)
- Mask > Statistics > Text File > excel

Delta F/F

- Compares fluorescence to beginning
- Shows the amount of bleaching

Other Software Features

FRAP Analysis Statistics

- > FRAP analysis
- get baseline time points
- asymptote time points (where flattened out)
- photobleach to ROI (make ROI on unbleached region)
- gives half life and mobile fraction

Computing Channel Registration

- Use beads
- Chan 1 > Mask
- Chan2 > Mask
- Image > Advanced operations > Compute Channel registration

Export

Image --> Export --> channel intensities as 16bit tif file

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 sharps 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