

Short protocol:
CIAN SD2 MOSAIC PHOTO-MANIPULATION
Version 2, September 2014



The Mosaic device can be used for several Photo-**Manipulation** (PM) applications, including but not limited to:

- Fluorescence Recovery after **Photobleaching** (FRAP)
- Fluorescence Photo-**Activation** (PA): a light-induced process by which the fluorescence emission of a molecule is activated.
- Photo-**Conversion** (PC): a light-induced process by which a molecule fluorescence emission is modified (e.g. from green to red fluorescent emission).
- Photo-**Uncaging**: a light-induced process of releasing a "caged" molecule from a caging group to proclude an actively fluorescent molecule.

Note that effective photobleaching will occur with the 40x objective or higher. The 10x and 20x objectives are less suitable for these applications.

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I. SYSTEM COMPONENTS



- a. Randomizer
- b. Mosaic laser shutter
- c. 405nm/475mW Mosaic laser
- d. Mosaic laser intensity controller (DAC converter)
- e. Mosaic Master Switch
- f. Mosaic device

II. START UP AND SHUT DOWN PROCEDURES

Start up procedure:

1. Turn on the **system** (see SD2 SOP).
2. Turn on the **Mosaic Master Switch** (e).
3. [Activate the **Mosaic laser** by turning the key, then pushing the "start" button (circle on c); will not be needed during calibration.]
4. Launch the **Metamorph** acquisition software.
5. Proceed with the **calibration** (section IV) and the PM applications.

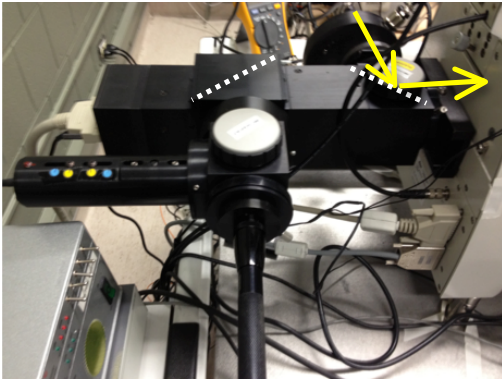
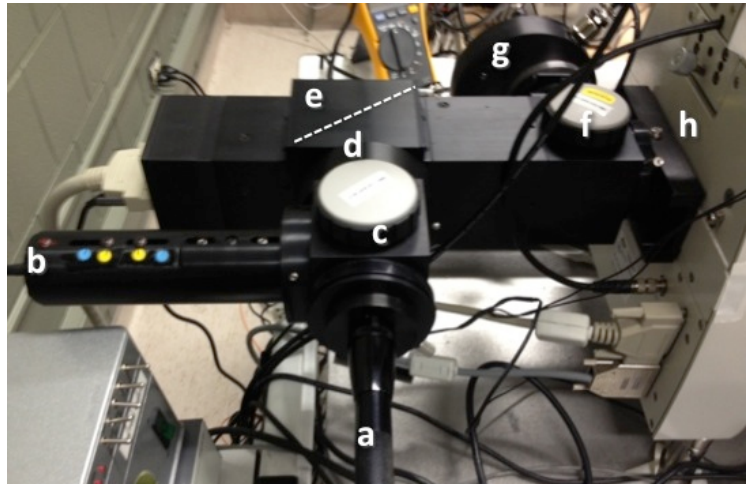
Shut down procedure:

1. In the Metamorph laser intensity window, ensure all **laser shutters** are closed.
2. Quit **Metamorph**, log off or shut down the **computer**.
3. Turn the key of the **Mosaic laser** (the "off" position is when the key can pulled out completely).
4. Turn off the **Mosaic Master Switch**.
5. Pull out the **eyepieces laser safety interlock**.
6. Put the Mosaic device light path in **configuration 1**.
7. Proceed with the **shutdown** of the rest of the system (see SD2 SOP).

III. MOSAIC LIGHT PATH CONFIGURATIONS

The Mosaic device light path can be arranged in three possible configurations by using magnetic beam splitters. There are three of them, two empty splitters, and one mirror splitter

- a. LED lamp for calibration (used to be flashlight)
- b. Mosaic laser entry
- c. Beam splitter position 1
- d. Mosaic laser shutter
- e. Mosaic mirrors
- f. Beam splitter position 2
- g. X-Cite lamp entry
- h. Entry to the microscope

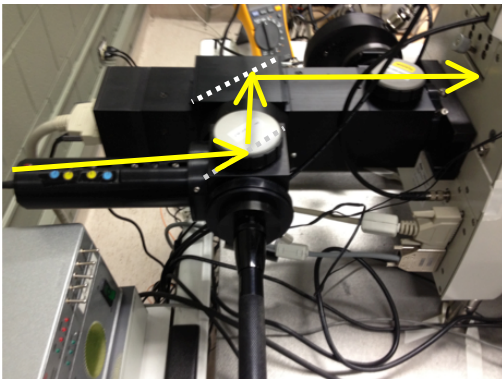


Configuration 1:

For wide-field fluorescence microscopy (no PM possible)

Please return to this position at the end of the session.

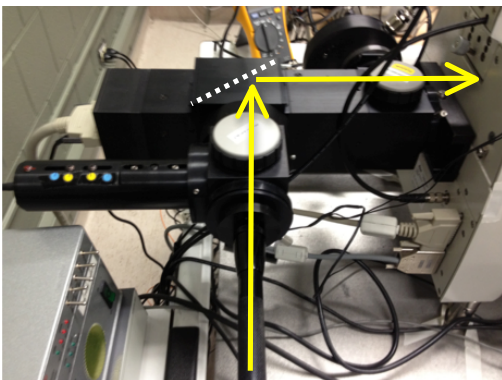
- Put the mirror beam splitter in position 2.
- Put an empty beam splitter in position 1



Configuration 2:

For using the Mosaic laser

- Put the mirror beam splitter in position 1.
- Put an empty beam splitter in position 2.



Configuration 3:

For calibrating the Mosaic device using the LED light source

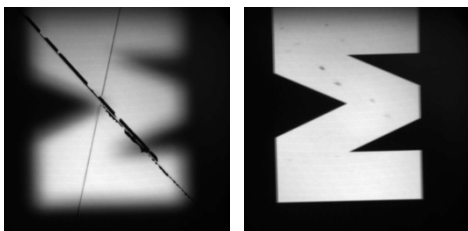
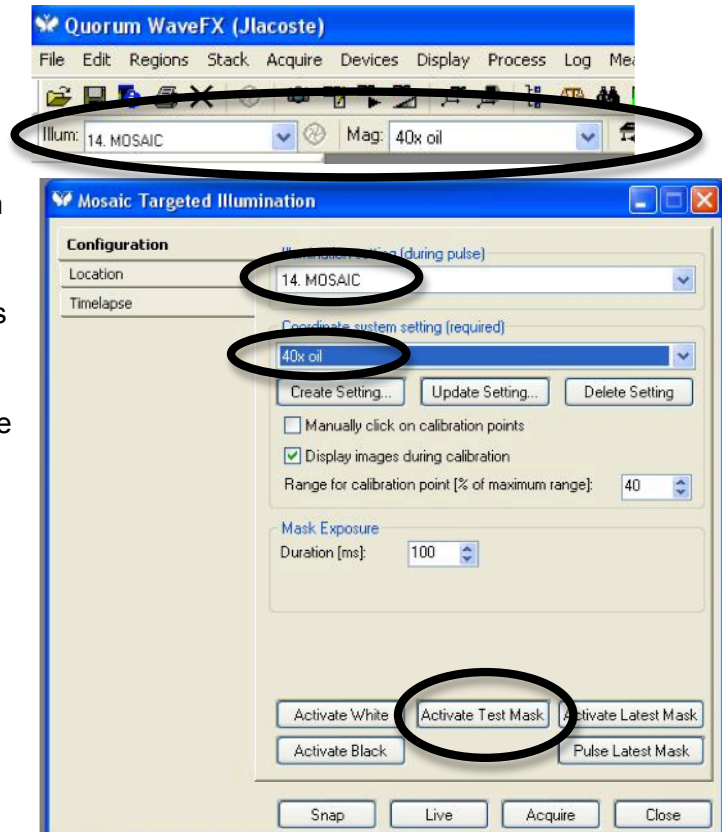
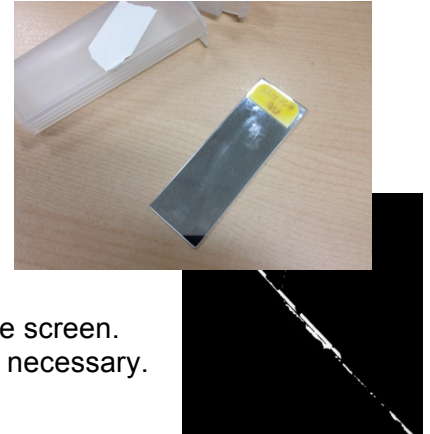
- Put empty beam splitters in positions 1 and 2.

IV. MOSAIC MIRROR CALIBRATION

A calibration procedure is required to ensure that the region of interest to be illuminated and imaged on the camera matches the micro-mirrors in the Mosaic device.

The calibration procedure should be run before each session. It uses a mirror slide, and the LED swan-neck lamp as a light source. A calibration will also be required for each objective used, or when either the camera or the Mosaic unit is moved.

1. Install the **mirror slide** on the stage specimen holder. Make sure the mirror surface is facing down.
2. Select **bright field** in Metamorph and focus (through the eyepieces) on **scratches** located roughly in the middle of the slide. Scratches will look like white lines.
Important: On the 40x and 63x objectives, make sure that the **variable NA ring is all the way open**, i.e. set to the highest NA.
3. Send the image of the scratch to the **camera** and re-focus on the screen. Adjust the halogen lamp intensity, exposure time and EM gain if necessary.
4. Set the **Mosaic light path to configuration 3**.
5. Turn on the **LED lamp**. Lift the empty beam splitter in position 1 up to check if the lamp is on or off.
6. On top left of the **Metamorph** desk top, select:
 - i. Illumination path (Illum.): **14. Mosaic**
 - ii. Ensure the right **objective is selected**.
7. Go to "**Targeted Illumination.JNL**" (bottom left):
 - a. Location tab: ensure the right objective is selected.
 - b. Configuration tab: set the "Coordinate system setting (required)" to the objective to be used.
 - c. Configuration tab: set the "Illumination setting (during pulse):" to "14. Mosaic".
 - d. Click "Activate Test Mask".
 - e. Stop live/show live from the "Acquire" window is necessary



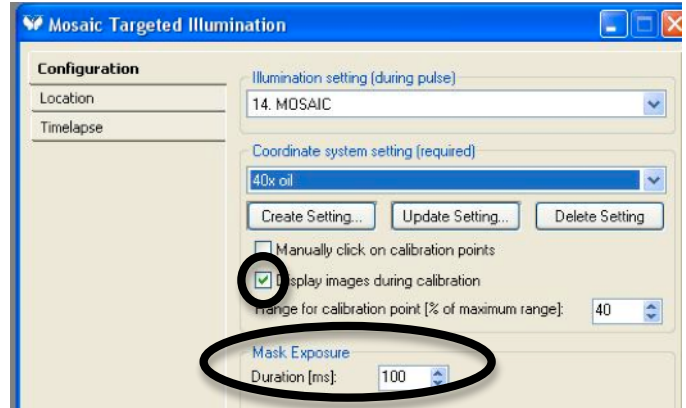
8. The "**M**" pattern is seen.
 - a. Move the scratch out the field of view
 - b. Focus on the "**M**" pattern

9. If needed, move the **LED lamp** to get an even illumination on the screen.
10. Adjust the exposure time (EM gain should be set to zero). If the LED lamp is too bright, make sure binning is 1, not 2, and/or put in a confocal emission filter in the light path. Values below are suggestions:
 - i. 450ms for the 40x objective.
 - ii. 250ms for the 63x objective.
 - iii. 200ms for the 100x objective.

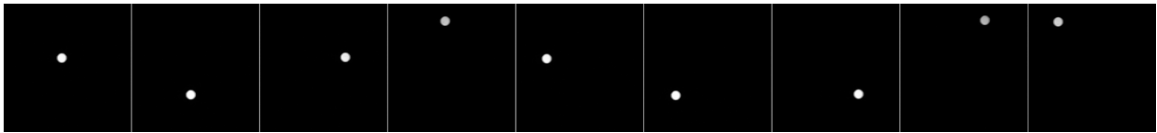
11. If the “M” pattern is not looking good, ensure the Mosaic light path beam splitters are well positioned.

12. In the “Mosaic Targeted Illumination” window, set “**Mask Exposure**”:

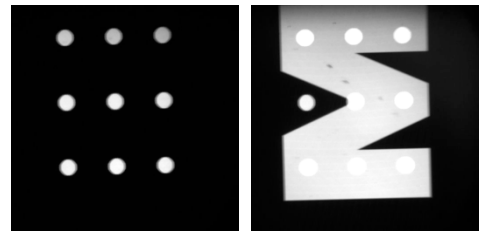
- a. Match it with the above exposure time
- b. Always use “Display image during calibration”



13. In the “Mosaic Targeted Illumination” window, click “**Update Setting...**”:
 - a. “Place a fluorescent calibration slide...”, click OK. It draws 9 points over the “M” within a square. Zoom in the resulting stack to see the results. Make sure all 9 points are seen.

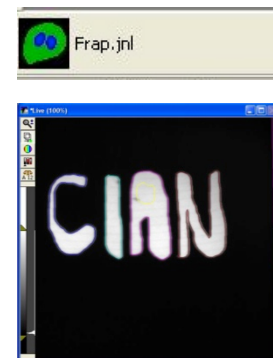


- b. “Update?”, click YES
- c. “Calibration successful”, click OK



14. Further **check the alignment**:

- a. Draw a few regions of interest (see top menu bar).
- b. Click “FRAP.JNL” (bottom left).
- c. Click “Activate Latest Mask”.
- d. Open Mosaic shutter to get an overlay. Evaluate the precision of the FRAP

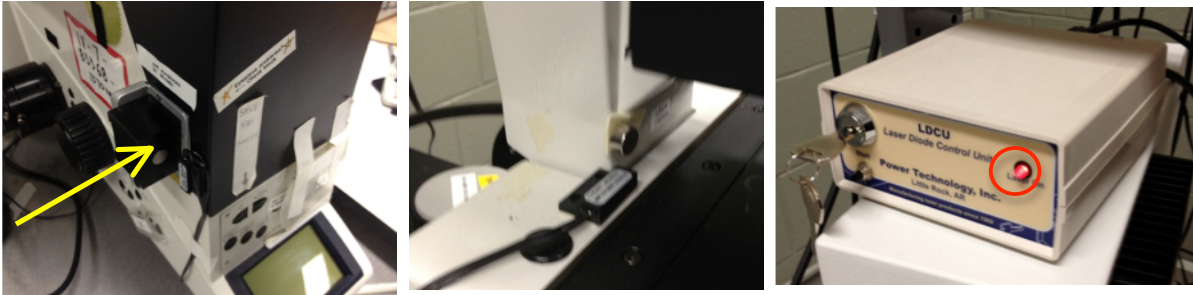


15. **Turn off the LED lamp** and set the Mosaic light path to **configuration 2** for PM operations.

V. FRAP SET UP

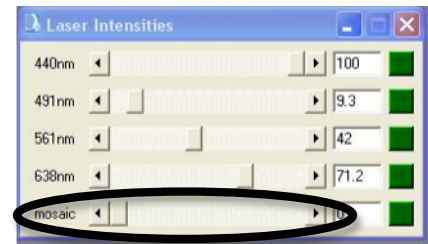
This section deals with the adjustments of the Mosaic laser intensity, and the time length of the Mosaic laser pulse.

1. Activate one of the **confocal imaging light paths**.
2. Adjust the confocal laser intensity level, the camera exposure time and EM gain to obtain an image with a **good signal-to-noise ratio** and **minimal photo-bleaching**.



3. [Turn on Mosaic laser with key and button, if left off for calibration]
Activate the **Mosaic laser**:
 - a. Push in the eyepieces laser safety interlock.
 - b. Check the microscope head safety interlock
 - c. Ensure the red light on the Mosaic laser is on.

4. Draw a **region of interest** to “FRAP”.
5. Using the laser intensity slider, adjust the **power of the FRAP laser**:
 - FRAP applications tend to require more power.
 - PA, PC, photo-switching, uncaging applications tend to require less power.



6. Click the icon “**FRAP_pulse_time.JNL**” to set length of the FRAP pulse, e.g. 100, 500 or 1000ms. One pulse per click.

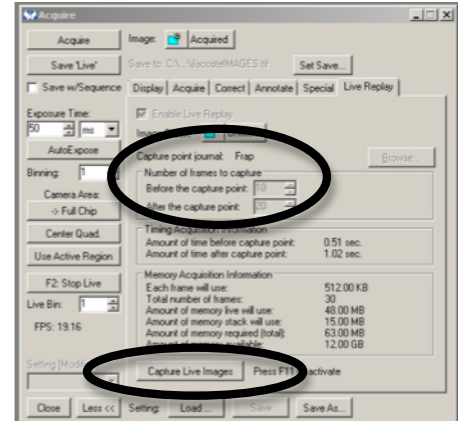
7. Click the icon “**FRAP.JNL**” to activate the FRAP laser for the desired intensity and time.



8. Evaluate the fluorescence/bleaching behavior.
9. If in doubt about the Mosaic laser setup, test by using a large ROI and maximum power; a purple glow should be visible on the stage during the pulse.

VI. FRAP QUICK START UP

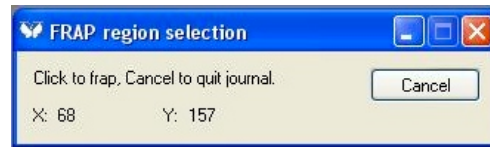
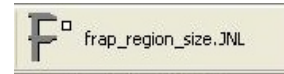
1. Arrange settings to image in one of the **confocal** modes with **minimal photobleaching**.
2. Adjust the **Mosaic laser intensity** and time length of the **FRAP pulse** (as described in the FRAP set up section).
3. In the “**Acquire**” window, click “**F2: Stop Live**” if necessary.
4. In the “**Live Replay**” tab, enable live replay and select:
 - a) Number of frames to capture before the FRAP point.
 - b) Number of frames to capture after the FRAP point.
5. Make sure “**Capture point journal**” is set to “**Frap**”.
6. Click “**Show Live**”.
7. Draw a **region of interest** (ROI) over the region to target.
8. Push in the **eyepieces laser safety interlock**.
9. Click “**Capture Live Images**” (this creates a time lapse of before and after the FRAP).
10. Remember to **close the confocal shutter** at the end of the acquisition (not done automatically). This can also be done by switching from the camera to the eyepieces.
11. The result is a stack of time points. Click on “**Latest_FRAP_regions.JNL**” to overlay the ROIs on the image time sequence. **Save both the stacks and the ROIs** (to reload them later).



VII. FAST FRAP FOR DYNAMIC SAMPLES

When the region to FRAP is moving very rapidly, the “`frap_region_size.JNL`” journal can be used to define a FRAP circle region (in pixels). Then the “`auto_frap_mouseclick.JNL`” journal can be used to FRAP rapidly moving regions in the live preview.

1. Arrange settings to image in one of the **confocal modes** with minimal photobleaching.
2. Adjust the **Mosaic laser** intensity and time length of the FRAP pulse (as described in the FRAP set up, section V.).
3. Click “**frap_region_size.JNL**”.
In the window “FRAP region size” that appears, enter the diameter of the desired FRAP region in pixels. Click “OK”.
4. In the “Acquire” window, click “F2: **Stop Live**” if necessary.
5. In the “**Live Replay**” tab, select:
 - a) Number of frames to capture before the capture point.
 - b) Number of frames to capture after the capture point.
6. Make sure “**Capture point journal**” is set to “**Frap**”.
7. Click “**Show Live**” to start image acquisition.
8. Click “**auto_frap_mouseclick.JNL**” at the bottom of window. A window named “FRAP region selection” appears.



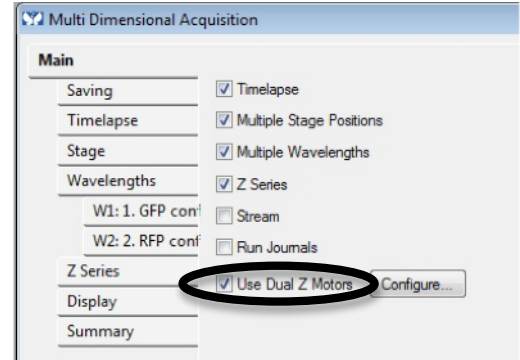
9. Click on a **region of interest** on the live image to FRAP.
A time lapse is acquired with the number of frames captured before and after the capture point (as specified in step 5).
10. Repeat step 9 to redo the FRAP on another region if desired.
11. Click “**Cancel**” in the “FRAP region selection” window to stop.

VIII. FRAP AND TIME LAPSE FOR LONG TERM IMAGING

When a longer time lapse needs to be acquired following the FRAP pulse, the Metamorph “Multi Dimensional Acquisition” (or MDA) can be used.

Procedure:

1. Arrange settings to image in one of the confocal mode with minimal photobleaching.
2. Adjust the Mosaic laser intensity and time length of the FRAP pulse (as described in the FRAP set up section).
3. Activate “Multi Dimensional Acquisition”, select the “Run journals” and any other necessary dimension.

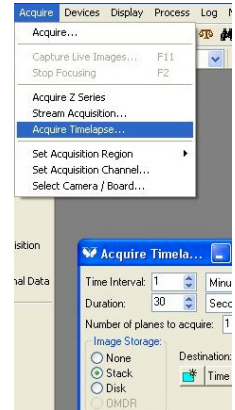


IX. FRAP TWO PHASES TIME LAPSE FOR LONG TERM IMAGING

When a longer time lapse with different duration and interval needs to be acquired following the FRAP pulse, a time lapse acquisition can be appended to the initial live replay.

Procedure:

1. Arrange settings to image in one of the confocal mode with minimal photobleaching.
2. Adjust the Mosaic laser intensity and time length of the FRAP pulse (as described in the FRAP set up section).
3. Set up a live replay as described in the “FRAP Quick Start Up” section.
4. Set up a time lapse from “Acquire/Acquire Timelapse...”
5. When the live replay is done, start the time lapse.
6. When the time lapse is over, append the resulting dataset to the one from the live replay.



X. FRAP ANALYSIS (primer)

The first step is to generate a graph of signal intensity over the length of time lapse acquired. Further steps to extract data are not part of this protocol.

Visual inspection of intensity graphs:

1. **Turn off live** imaging.
2. Go to “**Measure/Region measurements**”.
3. In the window that opens, at top left, set the **sources** for the images: select “**All planes**”, i.e. all the images of the time lapse.
4. In the graph tab, select the **average intensity** for the Y axis, and **elapsed time** for the X axis.
5. Make three regions of interest (ROI):
 - i. Click on “Latest_FRAP_region” to load the latest **FRAP area**, or load saved regions.
 - ii. Make an ROI in a similar region that was not targeted as a **negative control**.
 - iii. Make an ROI in a **background** region.
6. **Resize** the window as necessary to view the full graph.

Log and export numerical intensity data:

1. Set up as described above from recently acquired data, or load images and regions from saved files.
2. In “**configure**” tab, set which data to display and save. Region labels, time stamp, and average intensity are among the most important.
3. In **Region Measurements** window, click “**Open Log**” button. In the little window that opens, select “**a text file**”, then define file location and name in the dialog box. Now, the button has changed to “F9: Log Data”.
4. To write data to log, push “**F9: Log Data**”. This can be repeated, which will append more data at the end of the log.
5. To close a log, go to “**Log/Close Data Log**”.
6. The data log is a text file of comma separated values with the file extension “.LOG”. You can view it in any text browser, or save as plain text. This can be imported to a spreadsheet program like Excel.