Short protocol:
CIAN SD3 MICROPOINT PHOTO-MANIPULATION
Version 2, September 2014

Content:

I. SYSTEM COMPONENTS

1. Micropoint controller
2. Dye cells:
   a. 435-440nm (blue dot), strongest
   b. 404nm (yellow dot)
   c. 365nm (green dot)
3. Micropoint 3µs pulsed laser; 337nm/200µJ/4mW
4. "beam splitters", magnetic

5. Micropoint device
   a. Micropoint laser entry
   b. Dye cell position
   c. Dye cell focus ring
   d. Beam splitter
   e. *X-Cite* lamp entry
   f. Entry to the microscope
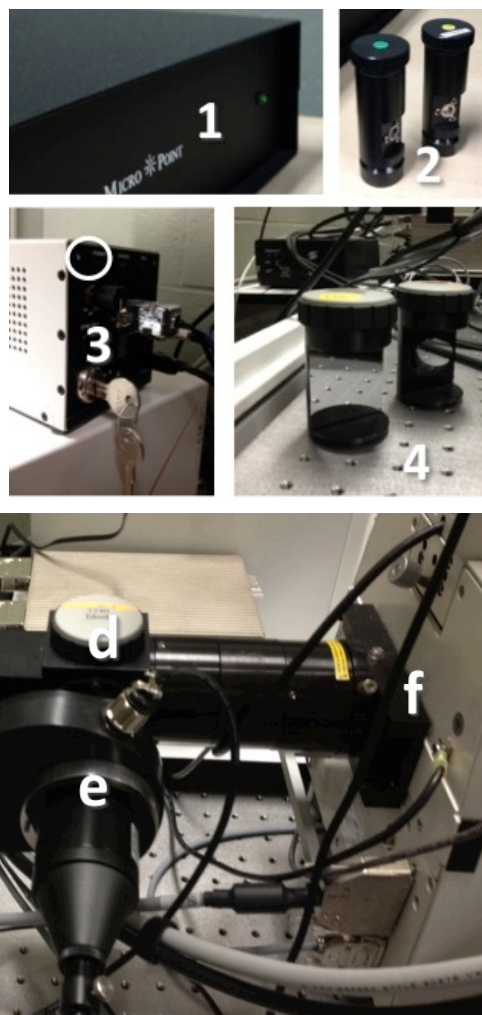
II. START UP AND SHUT DOWN PROCEDURES

Start up procedure:

1. Check that **dye cell/cuvette** (Fig.5 b) has sufficient dye solution inside, with no meniscus or bubble visible. (If different dye cell is installed, adjust as described in section XII.)
2. Arrange the required **beam splitter** configuration (see section III).
3. Turn on the **system** (see separate SD3 SOP).
4. Turn on the **Micropoint laser** by turning the key. The light "power" turns on.
5. [Launch the Metamorph acquisition software.]
6. 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 turned off.
2. Quit **MetaMorph**.
3. Turn the **key** of the mosaic laser (the "off" position is when the key can pulled out completely).
4. Pull out the **eyepieces laser safety interlock**.
5. Return the Micropoint device **beam splitter** to configuration 1 (see below).
6. Proceed with the **shutdown** of the rest of the system (see SD3 SOP).

III. MICROPOINT LIGTH PATH CONFIGURATIONS

The Micropoint device light path can be arranged in two possible configurations by using magnetic beam splitters. There are two beam splitters, an empty one, and a mirror splitter.
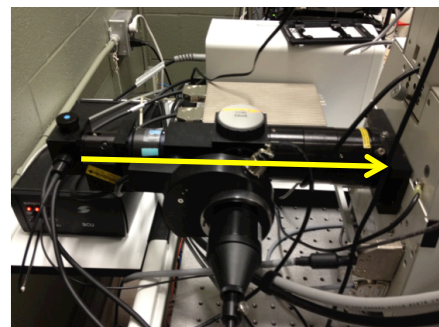
Configuration 1:
For wide-field fluorescence microscopy (no PM possible)
Install the **mirror beam splitter**.
Note: Please return to this position at the end of the session.
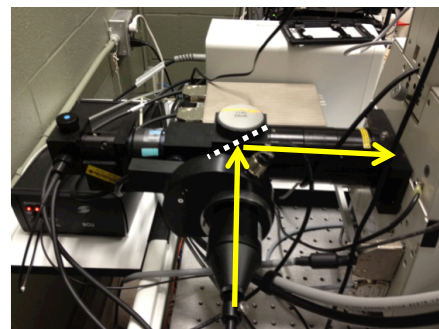
Configuration 2:
For using the Micropoint laser
Install the **empty beam splitter**.
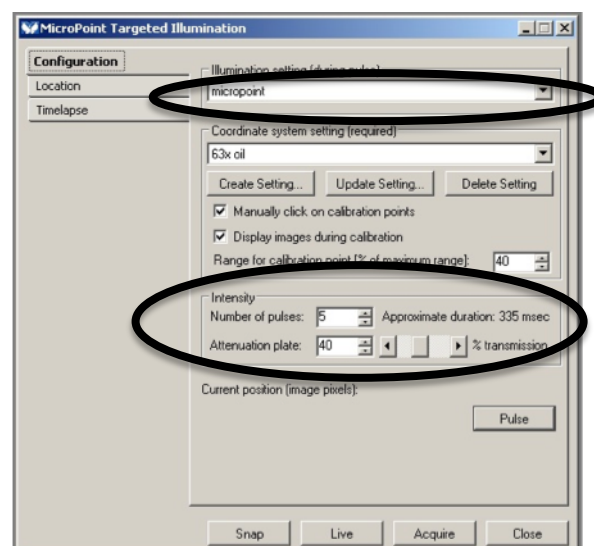
IV. MICROPOINT CALIBRATION

A calibration procedure is required to ensure that the region of interest selected in the camera preview matches the region to be illuminated on the actual sample. The calibration procedure should be run before each session.

Calibration procedure:

1. Put the **40x dry objective** in place.
2. Set the Micropoint device for **configuration 2**.
3. Install the **mirror slide** on the stage specimen holder. Make sure the mirror surface is facing down.
4. 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.
5. In the "**Acquire**" menu, set the camera **exposure time** to 350ms.
6. 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.
7. Push in the **eyepieces laser safety interlock**. This turns on the "Charge" light on the laser box.
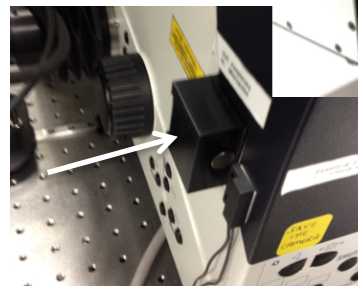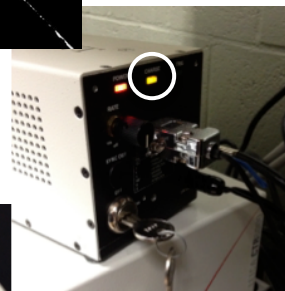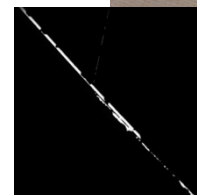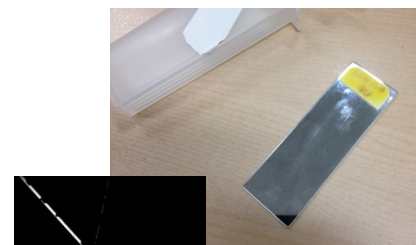
8. In the "**Micropoint targeted illumination**" menu:
   a) Select "micropoint" as the illumination setting.
   b) Select the objective to be used for the experiment (even though the 40x dry is actually used for the procedure).
   c) Set the number of pulses to 5 (for an approximate duration of 335ms).
   d) Set the attenuation plate between 20-40% (see p. 6, 4a and b for details).
   e) Click on "Pulse" to evaluate the strength of the laser power. Reduce laser power if the illumination dot is larger than necessary.
   f) Move to a field of view with no scratches or dots.
   g) Select "Manual click on calibration points", and "Display images during calibration".

9. In the "**Mosaic Targeted Illumination**" window, click "**Update Setting**…" (or "Create Setting" if creating new calibration):

    a) "Place a fluorescent calibration slide…", click OK.

    b) It draws 9 points within a square region, one after the other.
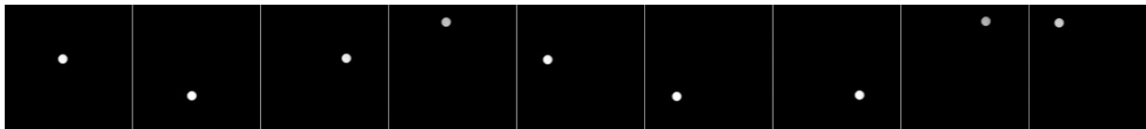
    c) Manually click on each point being illuminated. The more precisely this is done, the better the resulting calibration will be. (If "manual click" is not checked, the system will attempt to find the points itself.)

    d) "Update?", click Yes.

    e) "Calibration successful", click OK.
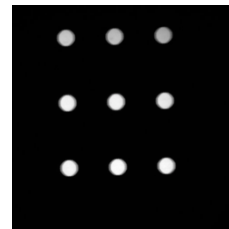
10. Further **check the alignment**:

    a) In a live window, draw a few regions of interest (select tool in top menu bar)

    b) Click "FRAP.JNL" (bottom left). The laser will pulse one spot at the center of the region of interest, clearing the mirror.

    c) Evaluate if the illuminated area matches the center of the region of interest that was drawn. If not, try to redo the calibration. Keep in mind that targeting will be best in the center of the accessible area.

V. TARGETING LINES OR FILLING SHAPES

Drawing a region of interest will result in one laser spot in the center of the region.

- If you want to direct the laser **along a line**, first draw a line with the line tool, then click on **"CreateSegmentROIs.jnl"** at the bottom of the screen. This will create small regions of interest along the line, which the laser will target when the FRAP journal is activated.
- To **fill a shape** with laser points, draw the desired shape using one of the region tools, then click on **"CreateROIsForLaserApp2.jnl"** at the bottom of the screen. This will create small regions of interest to fill your shape, which the laser will scan through when the FRAP journal is activated.

VI. FRAP SET UP

This section deals with the adjustments of the Micropoint laser intensity.

FRAP set up:

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. If not already done, turn on the **Micropoint laser** and push in the **eyepieces laser safety interlock**.

4. The **Micropoint laser intensity** is controlled in two manners:

   a) **Manually** using the slide neutral density filter located at the back of the laser unit.
   A suggested starting position is to pull it out completely (weakest) and push is back in by ten clicks.
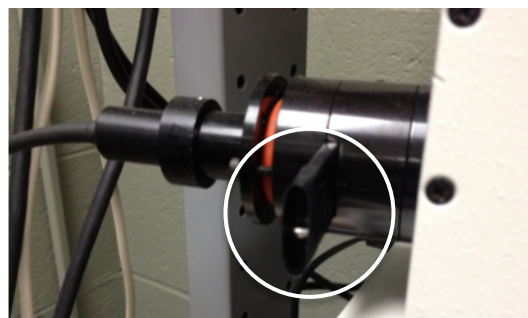
   b) **Electronically**, through the Metamorph acquisition software.
   Activate the Micropoint Targeted Illumination window by clicking on the "Targeted illumination" journal.
   In the configuration tab, the Micropoint laser intensity is set in the section labeled "Attenuation plate".
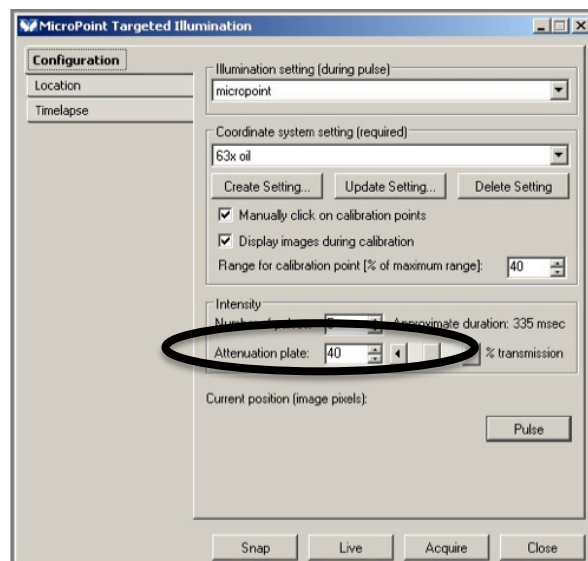
5. Using a region tool, draw a **region of interest** to FRAP and click "**Pulse**".

6. Evaluate the fluorescence/bleaching behavior, adjust settings as needed.

VII. FRAP QUICK START UP

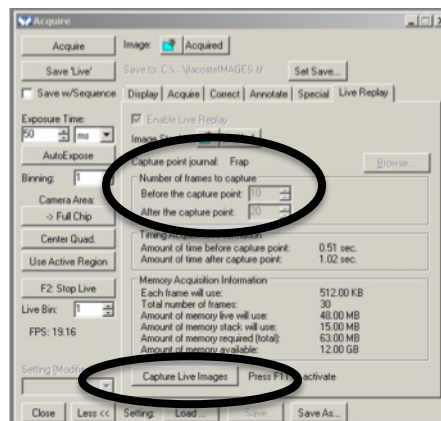This section describes the acquisition of basic FRAP data.

Procedure:
1. Arrange settings to image in one of the **confocal modes** with **minimal photobleaching**.
2. Adjust the **Micropoint laser intensity** and **time length** of the FRAP pulse (as described in the FRAP set up, section VI).
3. In the "**Acquire**" window, click "**F2: Stop Live**" if necessary.

4. In the **"Live Replay" tab**, select:
   a) Number of frames to capture before the FRAP event.
   b) Number of frames to capture after the FRAP event.
   c) Make sure "Capture point journal" is set to "Frap".

5. Click "**Show Live**".
6. Draw a **region of interest** (ROI) over the region to FRAP.
7. Push in the **eyepieces laser safety interlock**.
8. Click "**Capture Live Images**" (this creates a time lapse of before and after the FRAP).
9. Remember to **close the confocal shutter** at the end of the acquisition (not done automatically).

10. The result is a **stack of time points**.
    Click on "**Latest_FRAP_regions.JNL**", bottom of the window, to overlay the ROIs on the image time sequence. Save both the stacks and the ROIs (to reload them later).

VIII. 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.

Procedure:
1. Arrange settings to image in one of the **confocal modes** with **minimal photobleaching**.
2. Adjust the **Micropoint laser intensity** and **time length** of the FRAP pulse (as described in the FRAP set up, section VI).
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.
    c) Make sure "Capture point journal" is set to "Frap".
6. Click "**Show Live**" to start image acquisition.
7. Click "**auto_frap_mouseclick.JNL**".
    A window named "FRAP region selection" appears.
8. Click on a **point** 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).
9. **Repeat** the FRAP on another region if desired.
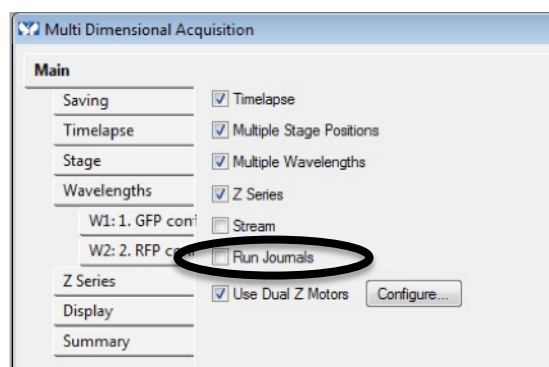10. Click "**Cancel**" in the "FRAP region selection" window to stop.
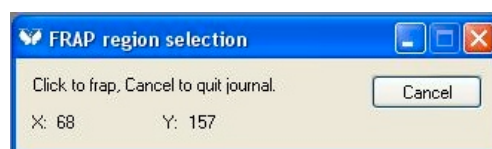
IX. FRAP AND TIME LAPSE FOR LONG TERM IMAGING

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 modes with minimal photobleaching.
2. Adjust the Micropoint laser intensity and time length of the FRAP pulse (as described in the FRAP set up, section IV).
3. Activate "**Multi Dimensional Acquisition**", select the "**Run journals**" and any **other necessary dimension**.

X. 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 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 IV).
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.
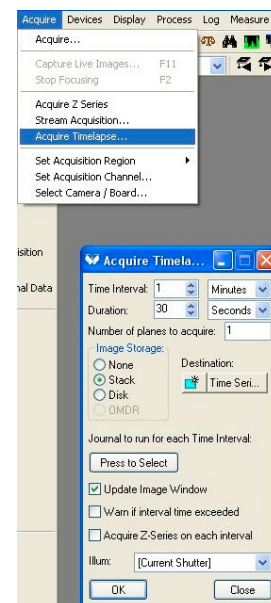
XI. FRAP ANALYSIS

The first step is to generate a graph of signal intensity over the length of time lapse acquired. Further analysis is not covered in this protocol.

Procedure to generate graph:

1. Go to "**Measure/Region measurements**".
2. 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.
3. In the **graph tab**, select the **average intensity** for the Y axis, and **elapsed time** for the X axis.
4. Make 3 ROIs:
   a) Click on "**Latest_FRAP_region**" to load the latest FRAP area (or load saved FRAP ROI if using older data).
   b) Make a ROI in a region that is similar to the treated one but which was not FRAP'ed as **negative control**.
   c) Make an ROI in a **background** region.
5. **Resize** the window as necessary to view the full graph.

Procedure to 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.

XII. Technical specifications and dye cell care

Micropoint laser: 3µs pulsed laser; 337nm/200µJ/4mW

Dye cells: cuvettes containing fluorescent solutions to modulate wavelength

Dot color	Emitted wavelength
Blue	440 nm
Yellow	404 nm
Green	365 nm

Dye cell care (ask CIAN staff to assist):
- Dyes (methanol based) are good for one to two months
- Regular care: dump and refill with appropriate dye solution
- If dye cell looks dirty inside, rinse with methanol (optical grade, at least for last rinse)

If a different dye cell (cuvette) is installed, adjust the Micropoint focus ring (located on the Micropoint device):

1. Loosen the screw on the top (a).
2. Turn against arrow to the tape mark (b), passing it twice, then turn the dye cell focus knob back, passing the tape once, to make it match the coloured dot for dye cell installed.