Version 7.0

Application Guide: Dynamic Fluorescence

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Contents

Dynamic Fluorescence Imaging . . . 1
Qualitative Measurements . . . 1
Ratiometric Measurements . . . 1
Fura-2 . . . 1
BCECF . . . 2
Excitation Wavelength Presentation . . . 2
Balancing Emission at Two Excitation Wavelengths . . . 3
Getting Started in Dynamic Fluorescence . . . 3
The Experiment Settings Dialog Box . . . 4
Custom Timing: Discrete Manipulation of the Excitation Components . . . 5
Combining Long and Short Inter-Ratio Intervals . . . 6
Selecting a Filter Wheel . . . 7
Calibrating Ratio Experiments . . . 9
Ratio Timing Setup . . . 9
Refresh and Apply . . . 13
Viewing Limits . . . 13
Ratiometric Imaging . . . 13
Compression (Display Settings) . . . 14
Camera Setup . . . 16
Setting Up an ICCD Camera . . . 16
Setting Up an Integrating Camera . . . 17
Acquisition Trigger . . . 18
Using the Trigger Functions . . . 19
Background Definition . . . 21
Using Advanced Corrections . . . 22
Acquiring Images . . . 23
Channel Management . . . 23
Multiple Ratios and Channel Linking . . . 24
Specifying Component, Ratio, and Concentration Data . . . 25
Simultaneous Reading of Data From Multiple Times . . . 25
Maximizing and Minimizing the Data Display . . . 25
Graphing Ion Kinetics . . . 26
Using Photometer Mode . . . 27
Mixing Photometer and Imaging Mode . . . 28
Using Image Processing Functions . . . 28
Using the Transforms Functions . . . 29
Deconvolution . . . 30
“Confidence” Mapping . . . 32
Alignment . . . 33
Saving and Retrieving Experiments . . . 35

On-Line 35
Saving Data from a Ratio Experiment 35
Exporting Images 35
Saving a Ratio Experiment as a Single Image 36
Cine Loops 36
Fura-2 External Calibration 38
Calibrating to a Series of Calcium Standards 38
An Alternative: Calibrating Within the Cells 40
References 41

Dynamic Fluorescence Imaging

Dynamic imaging refers to the imaging of specimens that change over time. Dynamic fluorescence imaging includes qualitative, single wavelength procedures (such as fluo-3), and ratiometric procedures that can be calibrated to intracellular ion concentration. The most popular ratiometric paradigms are the measurement of cytosolic free Ca^{++} concentrations by using the spectral shift displayed when the calcium chelator fura-2 binds to its target cation, and the measurement of intracellular pH using BCECF. Much less common are single excitation / dual emission procedures (such as indo-1). The list of fluorescent probes suitable for dynamic imaging is growing rapidly. **MCID™ Elite** currently supports any single wavelength procedure, and ratio imaging with fura-2 and BCECF.

Qualitative Measurements

Qualitative single wavelength procedures are straightforward. We simply capture a sequence of images and measure ROD or gray levels as they change over time. A popular single wavelength paradigm uses the Ca^{++} indicator fluo-3. This responds to increased Ca^{++} concentration with an increase in fluorescence intensity, but without changes in excitation or emission wavelengths. It is excited at 503-506 nm, in the visible portion of the spectrum. This allows the use of much less costly fluorescence equipment.

Fluo-3 has a weaker affinity for Ca^{++} (K_D about 400 nM) than do fura-2 or indo-1, permitting measurement of lower Ca^{++} concentrations. It also exhibits very marked changes in fluorescence intensity (about 4 decades) with Ca^{++}binding. Compare this with the tenfold change in fluorescence intensity exhibited by fura-2. Note that the response of fluo-3 to Ca^{++} binding is a simple intensity change across time. There are no spectral emission shifts which would allow ratios to be formed. Therefore, this is a semiquantitative technique, useful for showing that Ca^{++} is binding, but not the actual concentration of Ca^{++}.

Ratiometric Measurements

Fura-2

The saturating calcium form of fura-2 has a maximum absorbance at about 335 nm. The calcium free form absorbs maximally at about 362 nm. The ratio (usually 340 nm: 380 nm) of intensities of fluorescence, changes by about an order of magnitude between saturated and calcium-free solutions. Thus, a relative brightening of the 340 image and a relative darkening of the 380 image reflects an increase in the proportion of fura-2 bound to Ca^{++}.

Discrete 340 and 380 nm images are formed of cells incubated or injected with fura-2. Very low levels of UV light are used to avoid cell damage, so intensified or cooled cameras are necessary for image formation. To avoid differential bleaching during image acquisition, the excitation wavelengths may be rapidly alternated under computer-control. Finally, the 340 and 380 nm images are corrected by the appropriate background, and a ratio image is formed.

The ratio of 340 nm to 380 nm is passed through a simple equation to arrive at an estimate of Ca^{++} concentration.:

$$[Ca] = K_D\left[\frac{R - Rmin}{Rmax - R}\right](Fo/Fs)$$

Rmin is the ratio (340:380) of fluorescence intensity, formed at minimum Ca^{++} concentration. Rmax is the ratio (340:380) formed at saturating Ca^{++}concentration. Fo / Fs is the ratio (380 nm) of fluorescence intensity at minimum and saturated Ca^{++}concentration. K_D is the equilibrium dissociation constant for Ca^{++} and fura-2, usually stated as about 224 (Grynkiewicz, Poenie and Tsien, 1985), though variability has been observed (Williams and Fay, 1990). Each laboratory should determine the K_D under its own calibration conditions.

BCECF

The most popular indicator dye for pH is BCECF (Rink, Tsien and Pozzan, 1982; Bright et al., 1987). BCECF fluoresces strongly at visible wavelengths, with an excitation peak at 503 nm and an emission peak at 525 nm. Both peaks are pH dependent, being quenched by acidification and enhanced by more alkaline environments. At 436-439 nm, however, fluorescence is independent of pH. Therefore, a ratio can be constructed between pH-dependent and pH-independent images of BCECF fluorescence. In theory, this ratio will reflect pH independent of irrelevant influences such as dye concentration, illumination intensity, etc.

A filter set for pH measurement with BCECF includes excitation filters at 440 and 495 nm, a 515 nm dichroic mirror and an emission filter at 535 nm. Backgrounds are acquired at 440 and 495 nm. All the procedures are as for Ca^{++} imaging. Ratios are passed through the following equation:

$$pH = pK + \log\frac{(R - Rmin)}{(Rmax - R)}$$

R is the normalized 495 / 440 nm fluorescence ratio, obtained as a ratio of the mean intensity value over any portion of the image, at each wavelength, at a pH of 7.0. We start with a value of 7.17 for pK, and suggest that you calculate values appropriate for your own conditions. BCECF is most commonly calibrated by using the K+/H+ ionophore, nigericin, to expose cells to known internal pHs (Thomas, et al., 1979).

Excitation Wavelength Presentation

Three techniques are commonly used in the presentation of excitation wavelengths. Dual excitation lamps may be used, each with its own filters or grating monochromator. This method offers the advantage that wavelengths may be switched very rapidly using a simple optical chopper. However, a dual monochromator is costly, and obtaining optimum performance requires very careful alignment and calibration of both monochromators. This is a non-trivial procedure. Various devices can be used to tune excitation wavelengths from a

single source. Tunable lasers can be adjusted to required emission, or tunable filters (e.g., acousto-optical filters) may be inserted into the light path.

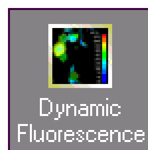
By far the most common method for the alternation of excitation wavelengths is the filter wheel. This contains a shutter, and a multi-position rotatable wheel, into which filters are inserted. Wavelength is alternated by selecting different wheel positions under computer control. The filter wheel is easy to work with, easy to integrate into software, and low in cost. Its disadvantages are that alternation speed is limited (minimum about 30 msec), that vibration is generated by the rapid acceleration and deceleration, and that insertion of a wheel after the lamp collector lens can accentuate optical aberrations. These problems can be minimized (e.g., by decoupling the wheel from the microscope body), and filter wheels are very popular.

Balancing Emission at Two Excitation Wavelengths

In most cases, the emission from our tissues will be greater at one excitation than at another. As an example, we will use a standard fura-2 paradigm. Fluorescence intensity at 380 nm may be much greater than at 340 nm. Mercury lamps, in particular, are peaky and are much stronger at 380 nm than at 340 nm. The situation is helped by use of Xenon illumination, but can continue to present a problem for the limited dynamic range of ICCD cameras. Setting the intensifier so that 340 nm images are not too dim usually results in saturation of 380 nm images (and nonlinear intensifier response). One solution is to decrease fluorescence intensity at 380 nm, either by adjusting the intensifier amplification between wavelengths, or by using a ND filter mounted prior to the filter. We prefer to use the ND filters, feeling that intensifier adjustment could be prone to unpredictable errors. Various ND filters (e.g., 0.2, 0.6, 1.0) may be mounted together with 380 nm filters at different positions in the filter wheel. If you use multiple 380 nm filters, place the 340 nm in position 0, and the 380 nm filters with their associated ND filters on either side. This minimizes the distance the wheel must travel between positions and yields the most rapid alternation of excitation wavelengths.

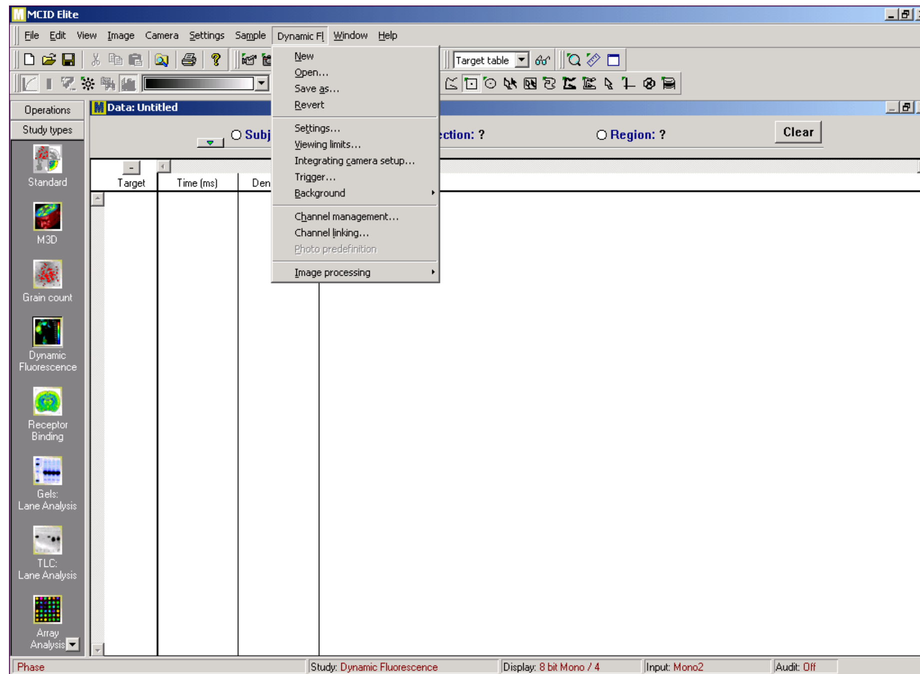
Getting Started in Dynamic Fluorescence

To enable the **Dynamic Fluorescence** study type, select *Settings > Study > Dynamic Fluorescence* or press the **Dynamic Fluorescence** shortcut icon in the study type Shortcut Bar:



In the Dynamic Fluorescence study type, the *Dynamic Fl* menu becomes available (Figure 1). Use this menu to set up experiments, calibrate the system, and for most other functions specific to ratio imaging.

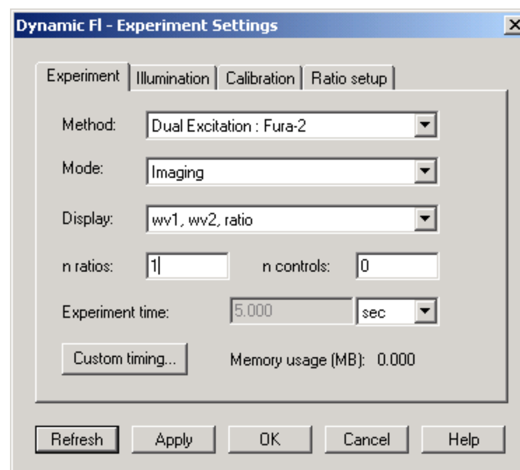
Figure 1: *MCID Elite in the Dynamic Fluorescence study type. The Dynamic Fl menu gives access to many application-specific procedures.*



The Experiment Settings Dialog Box

Select *Dynamic Fl > Settings* to display the *Dynamic Fl - Experiment Settings* dialog box (Figure 2). This dialog box contains many of the controls that dictate how ratio imaging is performed. It allows selection of a filter wheel, definition of filters at each location in the selected wheel, selection of filters for each of the two wavelengths, type of operation, timing, etc.

Figure 2: *Dynamic fluorescence experiments are set up using the 'Dynamic Fl > Settings' menu option, which brings up the Dynamic Fl - Experiment Settings dialog box.*



Selecting Single Wavelength or Ratiometric Imaging

Under the **Experiment** tab the **Method** drop-down menu allows selection between single (e.g., fluo-3) and dual (e.g., fura-2) wavelength procedures. Selecting **Single wavelength** clears the calibration portion of the *Dynamic Fl - Settings* dialog box, and changes some of the timing settings (e.g., there is no inter-wave interval). However, in most respects the single wavelength method is run in the same way as dual excitation.

The Mode Option

Under the **Mode** drop down menu three different modes are available, **Photometer**, **Imaging** and a combination of the two (**Photometer/Imaging**).

The Display Option

All **MCID Elite** systems display the component images, as they are acquired. In some cases, you may also want to view the ratios as an experiment progresses. Displaying the ratios in real time adds about 80 msec to the minimum inter-ratio-interval (IRI).

Although it is convenient to see ratios throughout the course of an experiment (on-line display), you can usually tell what is happening by watching the two component images. Lack of an on-line ratio display may not be critical. The choice is yours. If short IRIs are required, and if lack of on-line ratio display is not critical, select **wv1**, **wv2** from the **Display** drop down menu. With **Display** set to **wv1**, **wv2**, IRIs will be shorter, but ratios will be formed at the end of the experiment, instead of during acquisition.

Ratios

Enter the total number of ratios to be acquired in the **n ratios** entry field.

Controls

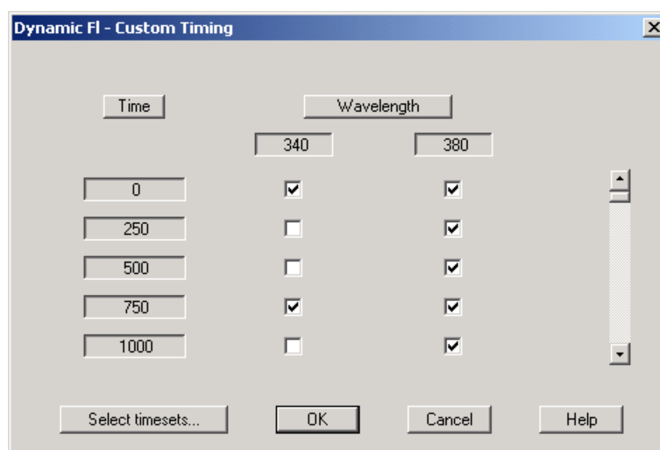
Control images are taken before administering an experimental treatment. Enter the number of control acquisitions in the "n controls" entry field. The controls will be part of the total number of ratios specified in the **n ratios** entry field. During acquisition, **MCID Elite** will take the specified number of controls and then pause. It will display a dialog box instructing you to press any key to continue with the experiment. When you press a key, **MCID Elite** will immediately continue with the experiment, designating the first ratio acquired after the last control at the time zero point.

Custom Timing: Discrete Manipulation of the Excitation Components

We can specify a series of image acquisitions, without taking every image in the set. For example, we use the various **Timing** settings to specify 100 fura-2 ratios. However, we are concerned about UV exposure, so we want to acquire 340 nm images less often than 380 nm images. We also want to acquire images rapidly during the first part of the experiment, and then more slowly later on. These types of manipulation are controlled via **Custom timing**. First set up the overall timing for the experiment. **MCID Elite** will assume an equal number of images at each excitation (for dual excitation procedures), and an even spacing between images (single wavelength or dual excitation). Once the general experiment timing has been set, press the **[Custom timing]** button in the *Dynamic Fl - Experiment Settings* dialog box. For example, in Figure 3 we set up a rapid acquisition sequence calling for 200 ratios at intervals of 250 msec. We will use 2 x 2 compression, but this sequence would still require

the saving of a lot of images (200 at 340 nm and 200 at 380 nm), and would also expose the specimen to a lot of UV. We can decrease both image storage and exposure to UV by reducing the number of 340 nm exposures. In this case, **MCID Elite** can use a single 340 nm image to create a number of ratios.

Figure 3: *An experiment in which 340 nm and 380 nm images are acquired every 250 msec. We use Select timesets to enable only every third 340 nm acquisition, reducing memory requirements and decreasing specimen exposure to UV.*



We could simply turn off the check boxes for any of the 340 nm images. However, with a large number of ratios this would be very tedious. Instead, we press **[Select timesets]** and **MCID Elite** allows us to specify a skip factor for either of the wavelengths. For example, in the above figure we have chosen a skip factor of 3 for the 340 nm images. The first acquisition time point would contain a 340 nm and a 380 nm component. The next two time points would be at the 380 nm component only. During ratio image formation, each of the 380 nm images will be ratioed to the nearest 340 nm image. For measurement purposes, linear interpolation is used if no image exists for a given timeset.

Combining Long and Short Inter-Ratio Intervals

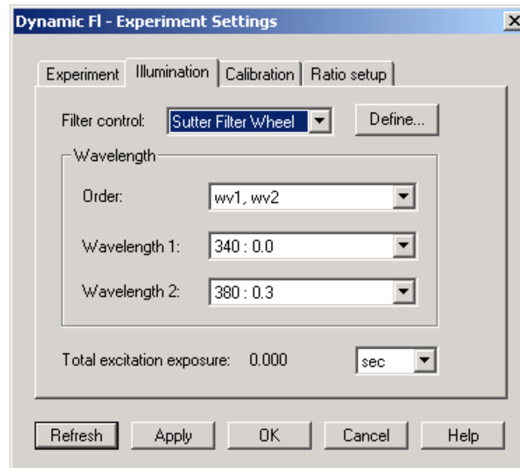
A common paradigm is to observe events at long intervals (e.g., a ratio every 5 sec.) while cells are under stable conditions, and to observe more frequently following experimental manipulation. **MCID Elite** allows multiple IRIs to be specified. Use the **Custom timing** function to turn on or off specific ratio acquisitions. As an example:

Observation	Number of ratios	Spacing (msec)
Control	4	5000
Initial post-treatment	20	250
Continued post-treatment	10	5000

Selecting a Filter Wheel

MCID Elite supports a large number of different filter wheels and filter wheel selection is defined under the **Illumination** tab of the *Experiment Settings* dialog box (Figure 4).

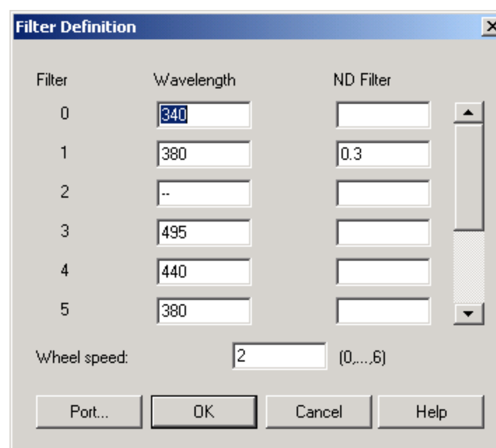
Figure 4: *Selecting the filter wheel type from the Illumination tab of the Experiment Settings dialog box.*



The **Filter control** drop down menu allows selection of a filter wheel. Our supported filter wheels can carry from three to ten filters. The **No filter wheel** setting allows testing of program operation. The **Manual filter wheel** option allows the setting up of timed experiments in which you flip filters manually.

To define the filters in the wheel, press **[Define]**. This displays the *Filter Definition* dialog box for the currently selected filter wheel, (Figure 5).

Figure 5: *Filter definition for a six-position LEP filter wheel. Positions 0,1 and 5 contain filters for fura-2 imaging of Ca^{++}. Position 2 is empty. Positions 3 and 4 contain a filter set for pH imaging with BCECF.*

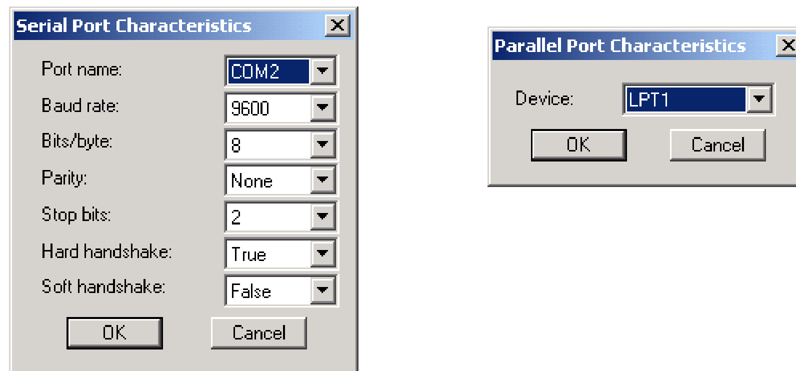


Into the **Wavelength** column, type the filter identification. The **ND Filter** column should contain a comment (usually this will specify an ND filter) to discriminate between similar filters. In the case of dual filter wheels the **ND Filter** column is no longer a comment but

describes the contents of the second filter wheel. Once you have entered a filter definition, that definition will appear in the **Wavelength 1** and **Wavelength 2** drop down menus of the **Wavelength** area. Pressing **[OK]** will return you to the Illumination tab of the *Settings* dialog box. Then press **[OK]** to save the filter and position settings and exit the *Settings* dialog box.

The **[Port]** button is used to set up communication parameters for your filter wheel. The LEP wheel is serial device, the Sutter wheels are parallel devices. Typical setups for the LEP and Sutter wheels are shown in Figure 7.

Figure 7: *Serial port settings for a LEP filter wheel (left) and parallel port setting for the Sutter wheel (right). For a LEP wheel purchased from us, the serial port settings should be used as displayed. The parallel port setting will depend upon which parallel port your Sutter wheel is connected to.*



Wavelength Selections

The **Wavelength** area of the **Illumination** tab (Figure 5) is used to define acquisition order and the excitation conditions.

Acquisition Order

The order in which **Wavelength 1** and **Wavelength 2** are acquired can be adjusted from the **Order** drop down menu. Three choices are available:

- **wv 1, wv 2** – An image is acquired at wave 1, followed by an image at wave 2. This pattern is repeated for the duration of the experiment.
- **wv 2, wv 1** – An image is acquired at wave 2 followed by an image at wave 1. This pattern is repeated for the duration of the experiment.
- **alternate** – An image is acquired at wave 1, followed by an image at wave 2. The next image is acquired at wave 2 followed by an image at wave 1. This alternating order of presentation is repeated for the duration of the experiment. Alternation helps to show the effects of photobleaching. If photobleaching is occurring, you will see a sawtooth effect in the ratio values. Alternate also speeds up the time interval between image acquisitions as it removes one move of the filter wheel per ratio. Alternation of wavelength presentation between ratios is particularly useful with integrating cameras, which cannot cycle wavelength presentations within a ratio.

Wavelength 1 Filter

Select the filter position that corresponds to wavelength 1, in the case of a fura-2 experiment this should be the 340 nm filter.

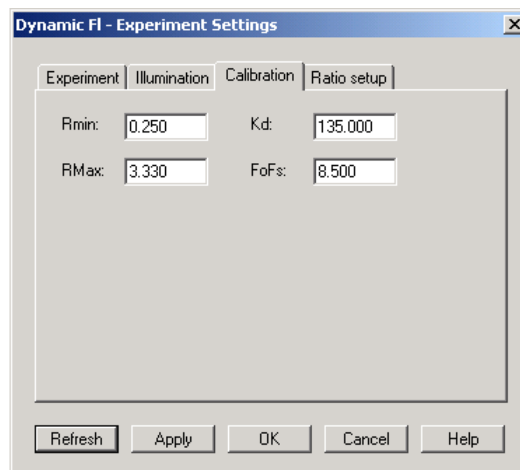
Wavelength 2 Filter

Select the filter position that corresponds to wavelength 2, in the case of a fura-2 experiment this should be the 380 nm filter.

Calibrating Ratio Experiments

The calibration entry fields are relevant to ratiometric experiments. The entry fields in the **Calibration** page of the *Experiment Settings* dialog are used to calibrate the method, using values obtained from the literature and from your own calibration procedures. For example, instructions for typical fura-2 calibrations are included at the end of this chapter. The values entered in the **Calibration** entry fields will dictate the minimum and maximum concentrations that the method is able to measure. These calibration values translate to actual concentrations that are shown in the **Min Conc:** and **Max Conc:** entry fields in the *Viewing Limits* dialog box (see Figure 11). The full calibration limits will probably correspond to concentration values, which exceed those observed in your specimens. Therefore, the **Viewing Limits** area is used to control the portion of the full calibration that is used for specimen display.

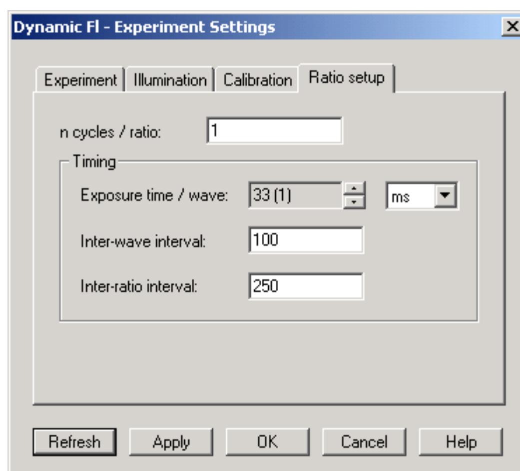
Figure 6: *The Calibration tab from the Experiment Settings dialog box.*



Ratio Timing Setup

The **Ratio setup** page of the *Experiment Settings* dialog box (Figure 7) is used to specify the timing of the acquisitions.

Figure 7: *The Ratio setup tab from the Settings dialog box.*



Timing

The **Timing** controls are used to specify individual ratio acquisition conditions, (with apologies to single wavelength users, we will refer to all acquisitions as ratios). You can select the number of cycles per ratio, an IRI, and so forth. Once the timing parameters have been specified, press **[Refresh]**. The various chosen parameters will update to reflect what the system thinks the minimum times will be.

Frame Averaging

Frame averaging decreases noise. The trade-off for this noise reduction is a lengthening of the IRI, and an increase in the time that specimens are exposed to illumination. That is, **MCID Elite** averages in real time (as fast as the video data are captured by the camera). However, each video image takes 33 msec (RS170-HI systems) or 40 msec (CCIR-HI systems) to acquire, during which time the specimen is illuminated. If you need noise reduction, and short IRIs or low exposure times, consider using compression (see below) instead of frame averaging. Compression sacrifices resolution to achieve noise reduction without an increase in exposure time.

To control frame averaging, press either the up or down tabs, next to the **Exposure time/ wave** box. This will alter the exposure time in frame increments based on the appropriate video frame rate, 33 msec (RS170-HI systems) or 40 msec (CCIR-HI systems). The number in brackets denotes the number of frames averaged. Press **[Refresh]** to have timing entries affect all relevant portions (inter-ratio interval, total specimen exposure time, etc.) of the *Settings* dialog box.

MCID Elite uses iterative averaging, and can take advantage of any number of averages with the following effect:

$$\text{noise remaining} = \frac{1}{\sqrt{\text{n frames}}}$$

The “Inter-wave interval” (IWI) is the period that is required for the filter wheel or other excitation device to switch between positions. The minimum IWI will vary from about 30 msec for the Sutter 3-position wheel, to about 50 msec for the Sutter 10-position wheel and

about 110 msec for the LEP filter wheels. These are minimum times, and longer IWIs can be used to keep filter wheel vibration to a minimum. For example, we might set the following sequence:

1.	Average 8 frames at 340 nm	264 msec
2.	Filter wheel change position	150 msec
3.	Average 8 frames at 380 nm	264 msec
4.	Filter wheel change position	150 msec
5.	Display ratio / Store image	

Cycling Excitation Wavelengths to Minimize Fading Artifacts

The above sequence first completes a period of 340 nm exposure, and then a period of 380 nm exposure. This might result in bleaching during the initial 340 nm exposure, with serious consequences for the accuracy of our ratio values. Therefore, we might wish to cycle between periods of 340 and 380 nm exposure. The “n cycles / ratio” specifies the number of times to switch between the two excitation wavelengths before constructing a ratio.

For example:

1.	Average 4 frames at 340 nm	132 msec
2.	Filter wheel change position	150 msec
3.	Average 4 frames at 380 nm	132 msec
4.	Filter wheel change position	150 msec
5.	Average 4 more frames at 340 nm	132 msec
6.	Filter wheel change position	150 msec
7.	Average 4 more frames at 380 nm	132 msec
8.	Display ratio / Store image	

Make sure that the IWI is not too short for your filter wheel. Problems with short IWIs will be seen as irregular acquisition times.

Wavelength cycling is not possible with integrating cameras, which must build up a complete image on the sensor before transferring the image to the computer. In this case, you have another way to determine if fading is having serious effects. **MCID Elite** allows the presentation of excitation wavelengths in alternating order (e.g., 340:380, 380:340). This option is controlled by the **Order** drop down menu in the *Settings* dialog box (Figure 8). It is probably a good idea to enable alternation of wavelength presentation. Then, examine a graph of your measured values. If there is an obvious sawtooth effect, photobleaching is occurring.

Chaining Ratios in a Sequence

In the examples above, we specified a number of acquisitions prior to ratio construction. Many images were involved, but the result was a single ratio. In practice, it is rare that we are interested in only a single ratio. Observation of Ca^{++} kinetics requires creation of a succession of ratios. Thus, ratio imaging requires that we specify both the excitation wavelength conditions for each ratio (as above), and the sequencing of multiple ratios. We control ratio sequencing using the **n ratios** and **Inter-ratio interval** (IRI) entry fields.

MCID Elite will estimate a minimum IRI from the timing settings you have given. That is, the IRI is calculated from the exposure time at each excitation wavelength, the filter wheel change time, and some allowance for overhead. If you want to see an estimate of the minimum IRI for a given configuration of timing settings, enter the value 1 into the **Inter-ratio interval** field and press **[Refresh]**.

Short Inter-Ratio Intervals

The maximum speed of ratio imaging depends upon many factors, including:

1. The speed at which excitation wavelengths may be alternated;
2. Requirement for integration or frame averaging;
3. System power and memory.

MCID Elite creates ratio images about every 200-400 msec. This rate of acquisition can be maintained until memory in the host computer is full (two ratios / MB of RAM). Once host RAM is full, acquisition slows down considerably as images are written to disk. Compression can greatly increase the number of ratios that can be acquired at short intervals (eight ratios / MB of RAM with 2 x 2 compression).

Setting the Inter-Ratio Interval

Any IRI may be entered into the **Inter-ratio interval** entry field. If the specified IRI is relatively long, **MCID Elite** will time image acquisitions to maintain that IRI. However, entering a short IRI (e.g., 100 msec) may not allow enough time for the system to acquire the ratios. In this case, **MCID Elite** will attempt to acquire as rapidly as possible, but the actual IRI will be limited by system overhead, filter wheel response, etc., and may well be slower than your specified rate. With a long IRI the shutter in the filter wheel opens and closes to minimize specimen exposure to illumination. With very short IRIs, the filter wheel shutter will remain open.

The minimum IRI will vary with your timing settings, computer speed, and memory capacity. Enter a value of "1" to select the minimum IRI. With a "1" in the IRI field, press **[Refresh]** to see what **MCID Elite** projects as the minimum IRI. The actual IRI will probably be longer than the minimum shown, as the **MCID Elite** IRI estimate does not include the time it takes your computer to save images.

Short IRIs tend to result in experiments with a lot of images. This can place a real strain on your memory capacity. We would prefer to have sufficient imaging power to conduct our experiments right into the computer. There must be enough free memory in the imaging system and host computer to handle all the images used in constructing the ratios. At least 512 MB of host RAM is recommended for ratio imaging studies.

A potential problem with short IRIs (no averaging and minimum inter-wavelength and inter-ratio intervals) arises from filter wheel mechanics. Vibration is high, as the inertia of the wheel mechanism must be dissipated during rapid swings. Experience must guide you in running maximum speed acquisitions. If vibration is a problem, increase the inter-wavelength and inter-ratio intervals, and/or have **MCID Elite** average two or more frames during acquisition.

A way to check your system for vibration artifacts is to image at maximum speed for a period of time. Check for blurred images and cell displacement. This is easily seen on the ratio

image, as bright edges on one side of cells. It is also wise to keep an eye on the imaging monitor during a study. Make sure that the cells are not wandering away from their initial locations.

Refresh and Apply

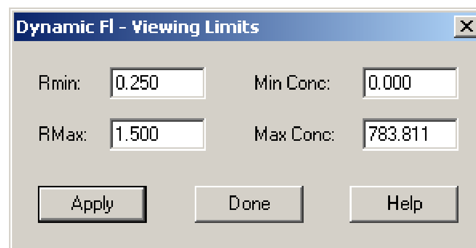
Many of the entry fields in the *Settings* dialog box affect each other. For example, changing the number of frames to average changes the exposure time / wave. Changing **Rmin** or **Rmax** affects the calibration range values. Use **[Refresh]** to have all entry fields updated to reflect the current settings.

Some entry fields, such as **Exposure time / wave** affect only the *Settings* dialog box and the way in which subsequent experiments are conducted. They do not affect an experiment that has already been run. In contrast, entering new values into the **Calibration** areas changes the way in which ratio images are displayed. To initiate the recalculation of ratio images, enter values into the **Calibration** entry fields and then press **[Apply]**. When you press **[OK]** to exit the *Dynamic FL - Experiment Settings* dialog box the current settings are now saved as the default settings.

Viewing Limits

The viewing limits dialog box can be accessed from the *Dynamic Fl* main menu (Figure 2) and when selected will display the *Viewing Limits* dialog box (Figure 8).

Figure 8: *The Viewing Limits dialog box.*



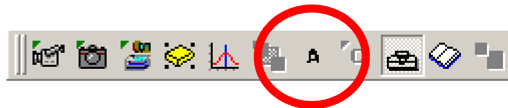
The *Viewing Limits* dialog box is used to control the portion of the full calibration that is used for specimen display. For example, your calibration **Rmin** and **Rmax** values might be equivalent to a range of 15 - 4000 nM Ca^{++}, mapped out across the entire color scale. However, your specimen only contains values from 20 to 1000 nM Ca^{++} and uses only part of the color scale. You could adjust the **Rmin** and **Rmax** values in the *Viewing Limits* dialog box to re-map the colors onto this narrower range of data values. This re-mapping will not affect the values that are obtained from the image. It will only affect the way in which the values are displayed. In effect, the *Viewing Limits* dialog box settings are like using the contrast manipulation in the **Visuals** operational window. However, the *Viewing Limits* controls allow a more precise and subtle color mapping than the visuals functions.

Ratiometric Imaging

The really difficult part of ratio imaging is getting the cells properly prepared, and calibrating the system for your conditions. After a bit of practice, the imaging is fairly straightforward and it should be possible to run ratio experiments quickly and easily. A step-by-step

procedure is presented, below. Each of these steps is discussed in more detail in subsequent sections.

1. Select *Dynamic Fl > Settings*.
   a) Select the type of filter wheel, calibration and viewing parameters, and filters for wavelength 1 and wavelength 2.
   b) Set the number of frames to be averaged, the inter-wave interval, the inter-ratio interval, the number of ratios to be acquired, and the number of control ratios to be acquired. At first, you may wish to run only one ratio acquisition to test your cells and illumination.
2. Press **[OK]** to finish with the *Dynamic Fluorescence Experiment Settings* (*Settings*) dialog box.
3. Select *Settings > Display format* (optional). Specify 2 x 2 as the # of channels per page if you have enough memory, or 3 x 3 or 4 x 4 to acquire large numbers of images.
4. Click the **Experiment Acquisition** icon (located in the Application toolbar) to start acquisition:



5. Select *Dynamic Fl > Background* to define backgrounds for 340 and 380 nm images.
6. Select *Dynamic Fl > Channel management* to display the 340 nm, 380 nm, or ratio images.
7. Select *Dynamic Fl > Channel linking* to specify which images are to be read from during data sampling.
8. Place any **Sample** tool on any image. The data from all linked images will be reported.

Compression (Display Settings)

The need for compression arises from two factors. First, each full resolution image is about 1/4 MB in size, and occupies 640 x 480 pixels. In a ratio experiment, we save two such images to create each ratio. The ratios themselves are not stored, but are recalculated each time an experiment is conducted or retrieved from disk. Even so, each set of component images used to construct a ratio requires more than half a megabyte of RAM. In a long sequence of ratio images, our computer's storage capacity will be exceeded.

A second problem with working at full resolution is that we can display only four full resolution ratios on the monitor at a time. Compression allows us to show more ratios, and demonstrate the effects of an experimental treatment over time.

We can compress either during or after image acquisition. Compression during acquisition saves memory, as the images are compressed before being stored in the computer. It also allows simultaneous display of multiple images. However, the compressed images cannot be regenerated at full resolution. In contrast, compression after acquisition does not save

memory. The images are stored on the computer at full resolution, and can be viewed compressed or at full resolution.

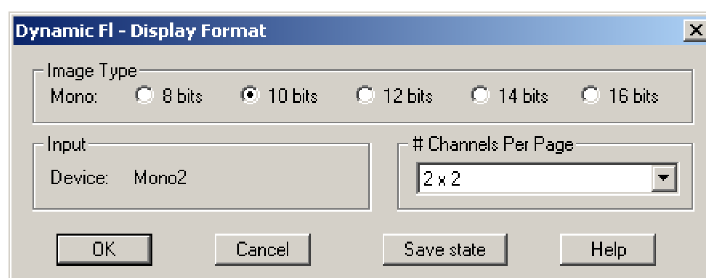
MCID Elite uses *local averaging* compression. This uses all the data as each block of four, nine, or sixteen pixels is averaged to one value. This has the same effect as averaging an equivalent number of frames at each wavelength, decreasing noise.

During Acquisition

In most cases, we want to store the component images to RAM. The alternatives are storing them on the computer hard disk (very slow). We must have enough RAM in the computer to store an entire ratio experiment. The more RAM, the better.

The simplest way to decrease storage requirements is to store smaller images. Use *Settings > Display format* from the main menu to set **MCID Elite** to various compressed modes.

Figure 9: *Use the Display Format - Dynamic Fluorescence dialog box to control compression in dynamic fluorescence.*



Compression factors range from **2 x 2** to **8 x 8**. These can be selected from the **# Channels Per Page** drop-down menu option. Balance the loss of resolution from compression against the gain in image storage capacity. Most ratio images can be acquired satisfactorily at 2 x 2 channels per page. Displays of less than about 160 x 120 pixels often result in too much loss of resolution to be useful. **MCID Elite** can display 64 such images on a single screen.

Table 1: *Number of images which may be displayed simultaneously at various resolutions.*

Image Size (pixels)	# Images Displayed
640 x 480	4
320 x 240	16
212 x 160	36
160 x 120	64

After Acquisition

We have described compression performed in real time, during image acquisition. This type of compression has the benefit that it reduces memory requirements during image acquisition. The size of the disk file made when the experiment is saved will also be smaller. However, if your computer has enough memory and disk space, you can chose to acquire at full resolution, and then to compress later.

Compress images that have been acquired at full resolution by selecting *Settings > Display format* from the main menu after the acquisition process is complete. When a compression factor is selected from the **# Channels Per Page** section, following a full resolution acquisition, **MCID Elite** asks if the existing images are to be compressed. Say **Yes** to see your images in compressed form. Only the displayed images are compressed. The original images stored in RAM or on disk are maintained at full resolution. The experiment can be saved in compressed form (use *Dynamic Fl > Save as*, resolution will be lost), or regenerated to full resolution before saving.

Montages of Compressed Images

Experiments often contain a large number of images, with only a few demonstrating effects of interest. **MCID Elite** allows selection of representative images from a larger set. For example, we may have told **MCID Elite** to acquire 48 ratios. Before or after the acquisition, use *Dynamic Fl > Channel management* to select only those timed images that are to be shown in the montage.

Camera Setup

MCID Elite can be connected to a number of cameras. In fluorescence applications, we might have a video camera connected to an intensifier, and an integrating digital camera. For static fluorescence, the selection among these cameras is controlled via *Settings > Input select*, accessed from the main menu bar. *Settings > Input select* is used to adjust the **MCID Elite** gain and offset for use with intensified CCD cameras (ICCDs).

Setting Up an ICCD Camera

ICCD cameras usually offer gain and offset controls on the video camera, and a gain control for the intensifier. Only video cameras with adjustable gain and offset should be used with intensifiers, to allow optimal adjustment under difficult conditions.

There are also digital gain and offset controls offered in **MCID Elite**, via the *Settings > Input select > Adjust* function. Balancing all of these camera and **MCID Elite** adjustments for optimal response is a bit of an art. The critical settings are those on the ICCD camera itself, and we will give some guidelines for adjusting them for typical dynamic fluorescence studies. For the Dage 72, the camera control unit should be set to gain of about 300 and offset of about 900 with well-lit specimens. These settings may vary when an intensifier is used.

Gain

Gain is the amount of light amplification. Both the camera and the intensifier contain amplification circuits. Your goal is to achieve the best signal-to-noise ratio (SNR) by achieving an optimal gain setting in each device. The higher the gain, the more light amplification. With high gain, even dim specimens can be visualized. However, higher gain also generates greater amounts of noise.

We usually start with the video camera set for optimal response with well-lit specimens. We then amplify light using the intensifier gain. Theoretically, this yields a better SNR than adjusting the camera gain upward.

Offset

The offset control (referred to as the “pedestal” or “black level” on some cameras) adjusts the response of the camera under black conditions. We want a small level of signal, even while the camera is viewing a completely black field. This small level of black response ensures that the camera is not cutting off any of the low level signal and will respond to very dim specimens. Please remember that if you adjust the camera to yield no response with a black image, there is a good possibility that relevant specimen details will be lost. For dynamic fluorescence, we usually set the offset to yield 5-10 gray levels on a black image. This is a bit higher than the offset that we would use with bright light imaging (about 1 gray level).

With the Dage 72, for low light imaging, set the offset switch to the “Manual” condition, and adjust the offset potentiometer until you have 5-10 gray levels with a blank image.

Using Gain and Offset to Adjust Dynamic Range

Gain and offset settings affect the dynamic range of the camera. That is, we can affect the contrast of our specimens by adjusting gain and offset. Increasing dynamic range decreases contrast. The general rule for gain and offset adjustment is:

Action	Effect on black image	Effect on real image	Contrast	Dynamic range
Increase offset	Brighter	Brighter	Decrease	Larger
Increase gain	Small increase	Brighter, noisier	Increase	Smaller

Optimal adjustment of gain and offset yields the best image contrast. For example, you find that your cells span only the range from 100 -200 gray levels. Therefore, you are not using the full dynamic range of the camera. Decrease the offset on the camera control unit, slightly, until the darkest parts of the cells are closer to black (e.g., 20 gray levels). Then, increase gain to once again yield saturation with the brightest details. Now, a narrower range of incident light intensities drives the system from black to saturation. This allows your specimens to fill more of the system response range, and can increase sensitivity to subtle changes in image density. Be careful with this, as enhancing contrast decreases the dynamic range of the system. That is, you will be more likely to saturate on bright details and lose dim details in the noise floor.

MCID Elite Gain and Offset Settings

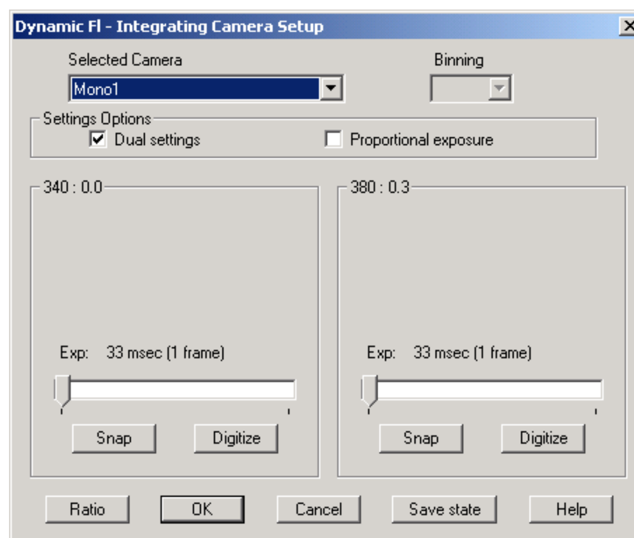
It is always best to adjust gain and offset in the camera, so that the signal is optimal before reaching **MCID Elite**, if your camera allows this. **MCID Elite** gain can usually be set to 50 and offset to 100. These **MCID Elite** settings are a good starting point for adjusting the analog controls on any camera. The goal is to have the imaging system just yield pure white at maximum camera input, and near-black at minimum camera input. Optimal settings for specific specimen and video camera characteristics may be arrived at by experimentation.

Setting Up an Integrating Camera

Digital integrating cameras (e.g., Photometrics® Cascade®) require more flexible controls than ICCDs. For example, we need to adjust the exposure time independently for each of two

excitation wavelengths. This type of control is achieved by selecting D*ynamic Fl > Integrating camera setup*.

Figure 10: *The Dynamic Fl Integrating Camera Setup dialog box allows adjustment of the way in which digital cameras perform dynamic fluorescence imaging.*



When the **Integrating camera setup** is first selected **Current system camera** will be selected. This means that the system is using its default camera. The default camera and its settings will have been previously selected from the main menu bar, using *Settings > Input select*. Deselecting **Current system camera** allows cameras to be selected from the **Selected Camera** drop down menu in the *Integrating Camera Setup* dialog box.

Selecting **Dual Settings** will allow discrete exposure settings to be selected for wavelength 1 and for wavelength 2.

Selecting **Proportional exposure** will allow you to adjust the exposure for wavelength 1 and at the same time the exposure for wavelength 2 will be adjusted proportionally. For example, if the wavelength 1 exposure is set to 100 msec and the wavelength 2 exposure set to 50 msec, changing the wavelength 1 exposure to 200 msec will also change the wavelength 2 exposure to 100 msec.

Adjusting the Integration Time

With a digital camera selected in the **Selected Camera** drop down menu, we are ready to set the integration time. To adjust the exposure time (in milliseconds) slide the pointer along the horizontal bar. Each time the exposure is adjusted, press **[Snap]** to see the effects. Pressing the **[Digitize]** button will put the camera into a continuous digitization mode.

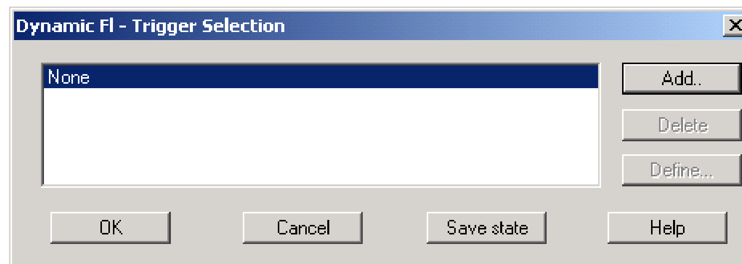
Once you have made all the adjustments to camera exposure, press the **[Ratio]** button to get an idea of what the ratio image will look like with the chosen exposure settings.

Acquisition Trigger

In dynamic fluorescence **MCID Elite** has the ability to utilize an acquisition trigger to carry out a number of timed events. To set up an acquisition trigger, select *Dynamic Fl > Trigger*

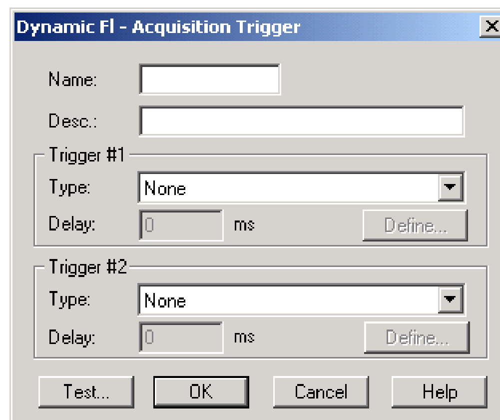
from the main menu. This will display the *Dynamic Fl - Trigger Selection* dialog box, (Figure 11).

Figure 11: *The Dynamic Fl Trigger Selection dialog box.*



The *Dynamic Fl - Trigger Selection* dialog box allows you to select predefined acquisition trigger setups or define your own. Press the **[Add]** button to add and define a new acquisition trigger function, (Figure 12).

Figure 12: *The Dynamic Fl Acquisition Trigger dialog box.*



Two different triggers can be defined within the *Acquisition Trigger* dialog box. These are labeled **Trigger #1** and **Trigger #2.**

There are four different types of trigger functions to choose from:

- **None** – No trigger function has been selected.
- **Parallel Port (in)** – The acquisition trigger expects an incoming TTL signal on the parallel port.
- **Parallel Port (Out)** – The acquisition trigger will send out a TTL signal on the parallel port.
- **Beep** – The acquisition trigger will sound a beep.

The **Delay** refers to the delay in milliseconds that occurs from the start of the trigger to the start of the experimental image acquisitions by **MCID Elite**.

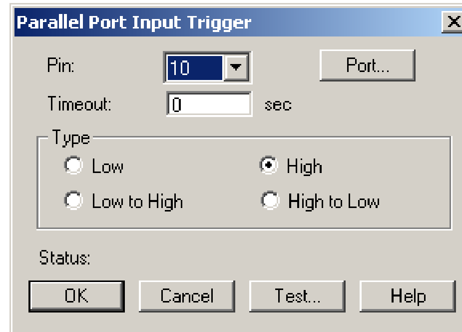
Using the Trigger Functions

Each of the three acquisition trigger functions has its own setup which can be reached by pressing the **[Define]** button after selecting the appropriate trigger.

Parallel Port (In)

Pressing the **[Define]** button when **Parallel Port (In)** has been selected will display the *Parallel Port Input Trigger* dialog box, (Figure 13).

Figure 13: *The Parallel Port Input Trigger dialog box.*

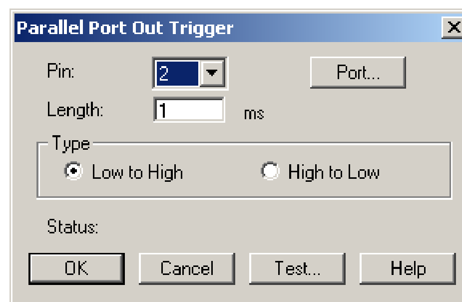


Pressing the **[Port]** button will bring up a dialog box from which you can define the parallel port that is to be used for the trigger signal. The input pin number is selectable and defines the pin for the TTL signal. The **Timeout** defines the length of time **MCID Elite** will wait for an incoming signal before returning an error. The **Type** section of the dialog box is used to define the TTL signal characteristics of the input trigger. Selecting either **Low** or **High** instructs **MCID Elite** to look for either a 0 volt or a +5 volt TTL signal to start the acquisition trigger. Selecting either **Low to High** or **High to Low** instructs **MCID Elite** to look for a TTL signal that changes from 0 to +5 volts or +5 to 0 volts.

Parallel Port (Out)

Pressing the **[Define]** button when **Parallel Port (Out)** has been selected will display the *Parallel Port Out Trigger* dialog box, (Figure 14).

Figure 14: *The Parallel Port Out Trigger dialog box.*

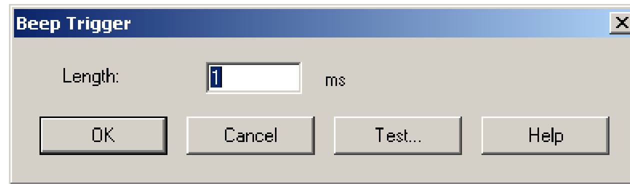


Pressing the **[Port]** button will bring up a dialog box from which you can define the parallel port that is to be used for the trigger signal. The output pin number is selectable and defines the pin for the TTL signal. The **Length** refers to the length of the TTL pulse that **MCID Elite** will send out and is defined in milliseconds. The **Type** section of the dialog box is used to define the TTL signal characteristics of the output trigger. Selecting either **Low to High** or **High to Low** instructs **MCID Elite** to send a TTL signal that changes from 0 to +5 volts or +5 to 0 volts in order to trigger an external event.

Beep

Pressing the **[Define]** button when **Beep** has been selected will display the *Beep Trigger* dialog box, (Figure 18).

Figure 15: *The Beep Trigger dialog box.*

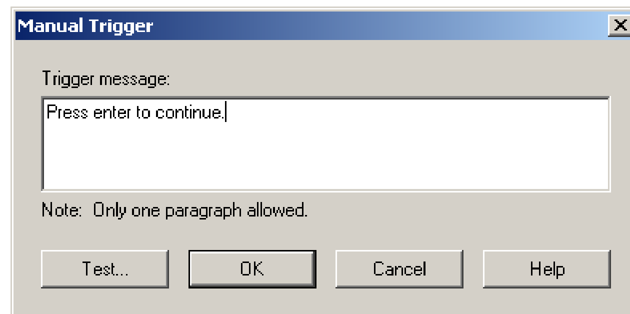


The Beep trigger is used to sound a beep for the length of time determined by the **Length** in milliseconds.

Manual Trigger

Pressing the **[Define]** button when **Manual** has been selected from the drop down menu will display the *Manual Trigger* dialog box, (Figure 16).

Figure 16: *The Manual Trigger dialog box.*



The entered trigger message will appear when the manual trigger is defined.

Background Definition

Each ratio value is created from two components. One component is the background fluorescence at each of the two excitation wavelengths. The other component is due to the binding of fura-2 to Ca^{++} in the specimen. The background component must be removed before a ratio is constructed, so that the ratio values reflect only Ca^{++}. **MCID Elite** offers three modes for handling background fluorescence.

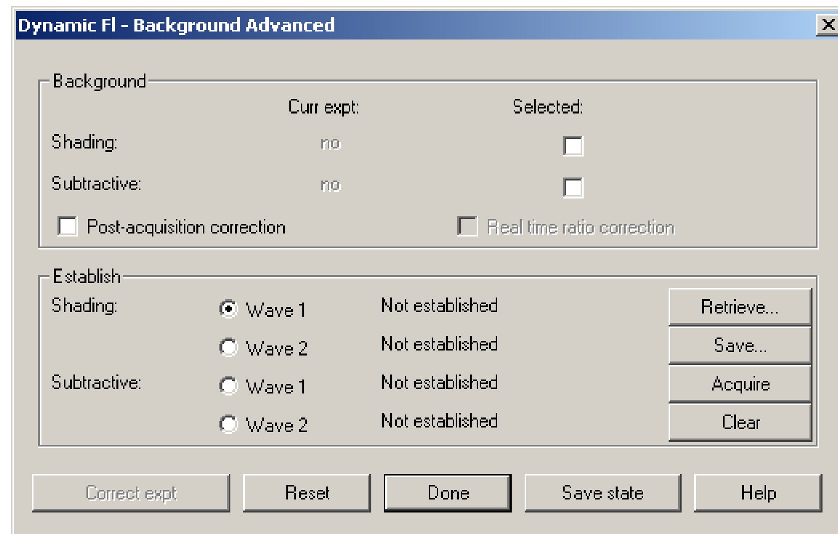
- **None** – No background correction.
- **Single value** – Set everything up so as to optimally illuminate your specimens. Then, *Dynamic Fl* > *Acquire* a pair of images. Select *Background* > *Single value*. Place the sampling window near a cell on a 340 nm image and click. **MCID Elite** will automatically fill in the 340 nm background value from that sample. Repeat from the 380 nm image, preferably from the same position as in the 340 nm image. The background values may also be typed directly into the entry field in the dialog box. All parts of the image will receive the same background correction.

- **Advanced** – The advanced background modes attempt to correct for spatial inhomogeneities. Thus, different parts of the image will receive different corrections.

Using Advanced Corrections

MCID Elite offers two ways to apply spatial background correction. **Shading** is a proportional correction. It is best suited to the correction of optical or illumination-induced shading. **Subtractive** is a simple linear correction, and may be best suited for removing autofluorescence.

Figure 17: *Setting advanced background corrections. Both shading and subtractive corrections are available.*

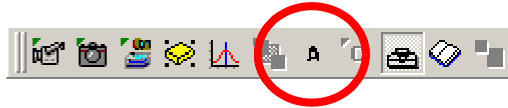


To establish a shading correction, first arrange illumination so as to have a clearly visible amount of fluorescence from a blank field. A rhodamine-coated slide or a solution of fura-2 containing some calcium can be useful in this. Then, acquire a background image at each excitation wavelength. From within each background image, a grand mean is calculated and the proportional deviation of each pixel from that mean is stored. Subsequent images are corrected for the proportional deviation appropriate to each pixel. The two shading correction images can be saved to disk and retrieved.

Subtractive correction also requires that you obtain an image of autofluorescence at each excitation wavelength. The autofluorescence images are subtracted from the subsequent excitation images. If your experimental condition is less bright than your autofluorescence image, the result is a 0 image value. This is not an unlikely occurrence given the variability of fluorescence illumination. You may wish to save a few autofluorescence images to accommodate various levels of brightness that might occur during an experiment.

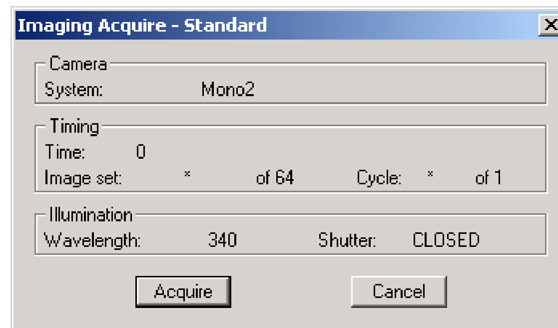
Acquiring Images

The image acquisition process is initiated by clicking the **Experiment Acquisition** icon (“**A**”) located in the Application toolbar:



MCID Elite takes a moment to set up its memory and internal procedures and then presents a dialog box containing **[Acquire]** and **[Cancel]** buttons (Figure 18). Pressing **[Acquire]** sets the process in motion.

Figure 18: *Starting the acquisition process.*



If a timed series of ratio images with controls has been specified, acquisition is a two-stage process. **MCID Elite** acquires the specified number of control images. Then, it halts, and waits for a signal to continue acquisition. Perform an experimental manipulation at this time, and then hit any key. **MCID Elite** will wait for the specified inter-ratio interval and then continue with automatic acquisition until the full timed series is complete.

Channel Management

MCID Elite uses the first four channels for brightfield, 340 nm, 380 nm, and ratio in that order. That is, first digitize a phase or Nomarski image (we will refer to this as the phase image) into channel 1 (this is optional). Then select *Dynamic Fl > Acquire*. The phase image will remain in place during the subsequent acquisition, and may be used as a reference for sampling from the ratio image. We could then view any of the four displays (phase, 340 nm, 380 nm, ratio). **MCID Elite** displays four images simultaneously, each at full 640 x 480 pixel resolution.

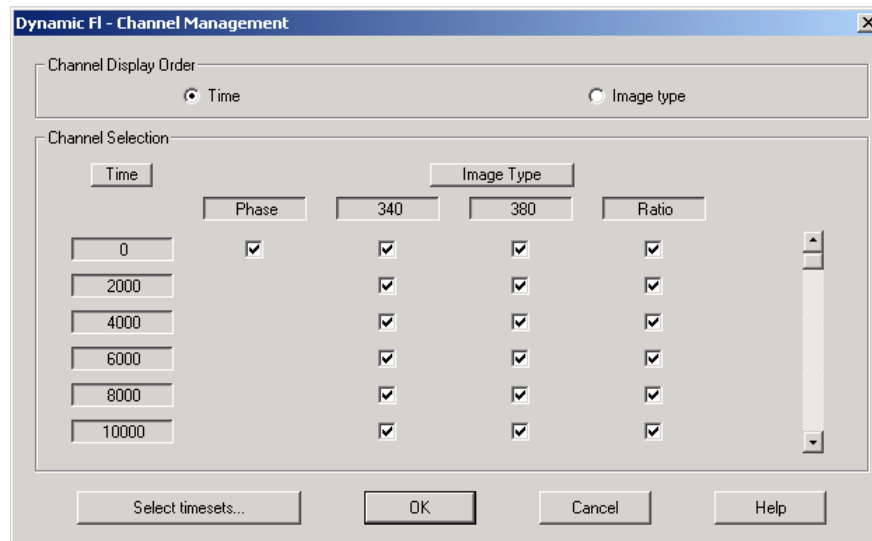
Display of the phase, 340 nm, 380 nm, and ratio images is controlled by selecting *Dynamic Fl > Channel management*. Click on column headings in the *Channel Management* dialog box (Figure 19) to select or deselect a particular type of image display. For example, **MCID Elite** may be directed to display only ratio images. This makes paging through a series of timed ratios much more convenient, as we do not have to see each of the wavelength components as we page.

Channel Display Order controls the order in which images are displayed. Usually, we display 340 nm, 380 nm, and ratio in that order. We then repeat for the next time set. This

display mode gives priority to **Image type**. We could also elect to give priority to **Time**, displaying all 340 nm images across time, then all 380 nm images, and then all the ratios.

To control which images are displayed from within a series, use the **[Select timesets]** button to specify a spacing interval. For example, you could display every fourth image. You may also want to turn on or off some other images at critical points. For example, we often use **[Select timesets]** to specify a spacing interval, and then use a closer spacing for those times when an agent is initially affecting the tissues. If any individual images need to be turned on or off, click on that button in the **Time** column.

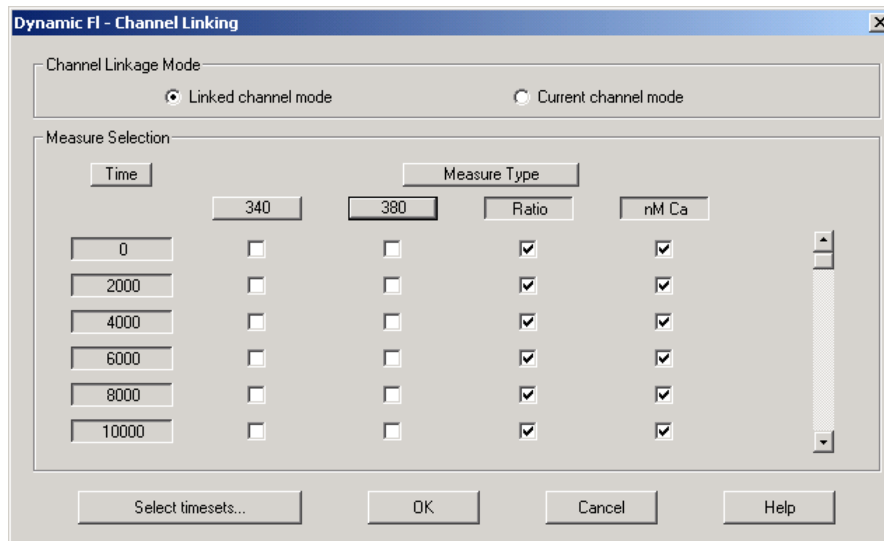
Figure 19: *Controlling the display of phase, 340 nm, 380 nm and ratio images from within the Dynamic Fl Channel Management dialog box.*



Multiple Ratios and Channel Linking

A ratio experiment generates component 1 (e.g., 340 nm), component 2 (e.g., 380 nm), ratio, and nM calcium concentration data. Each of these data types can appear as a column on the host monitor. The *Dynamic Fl > Channel linking* selection (see Figure 20) specifies which columns are present (340 nm, 380 nm, ratio, concentration). It also allows any number of timed acquisitions to be linked together, so that they are all read from with a single click of a sample tool.

Figure 20: *Controlling the data reported for each sample using the Dynamic Fl Channel Linking dialog box.*



Specifying Component, Ratio, and Concentration Data

When you place a sample tool on an image and click, **MCID Elite** reports the time, 340 nm, 380 nm, ratio, and concentration values for that sample point. However, we may not want all this detail. For example, we may wish to see only the data for the ratios and data for the concentrations. To do this, turn off the **340** and **380** columns by clicking on the column head under the **Measure Type** button.

The **Time** column can be used to turn on or off the report of data from specific acquisitions within a series. Click on the **[Time]** button to turn all of the times on/off. Click on specific time buttons to turn only that acquisition on or off.

Simultaneous Reading of Data From Multiple Times

Dynamic Fl >Channel linking allows 340 nm, 380 nm, ratio, and nM calcium values to be read simultaneously, across as many timed sets as desired. For example, we have just acquired ten ratio images and wish to define regions of interest on the phase image, while **MCID Elite** reports 340 nm, 380 nm, ratio, and nM calcium values from all ten acquisitions. From the *Channel Linking* dialog box, press the **[Time]** button then the **[Measure Type]** button. This will turn on all of the 340 nm, 380 nm, ratio, and nM Ca entries. View the phase image (or any of the other images) and place sample windows on it. As each window is placed, **MCID Elite** reports the data values across all the ten time sets.

You can turn on or off the report of any time set by clicking on that time button. You may also turn on or off any discrete data type by clicking in its check box.

Maximizing and Minimizing the Data Display

A lot of data points may be reported on each linked sample. It is possible to have the entire array of data points represented as a single line of data showing the concentration at time 0 only. At the top of the target number column is a button containing a “+” or a “-”. Clicking

this button will minimize or maximize the data display. If the button shows “-”, clicking it will minimize the display. The button then shows “+”, and clicking it will maximize the display. The maximized display shows data for each individual timeset.

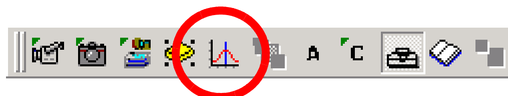
Figure 21: *Portion of a minimized and maximized data display. The minimized display (left) shows data at time 0 only. Data from each sample (target) is displayed on a separate line. The maximized display (right) shows data for each time point.*

+	Time	Ratio	Ratio
Target	ms	Ratio	nM Ca
1	0	0.97	94.94
2	0	0.59	43.53
3	0	0.95	92.16

-	Time	Ratio	Ratio
Target	ms	Ratio	nM Ca
1	0	0.97	94.94
	784	0.97	94.77
	1567	0.97	95.50

Graphing Ion Kinetics

The multiple data values gathered during a linked sample may be graphed. Enter a Region label and sample any region from a linked set of ratios. Do this for as many regions as you wish displayed on the graph. It is easiest to do this with the target data display minimized. Then, highlight the data values to be graphed on the host monitor by clicking the target number. Then, click the **Profile** icon:



A *Dynamic Fl Graph Mode* dialog box appears, prompting you to choose a **Fluorescence timing** graph or a **Transept line** (Figure 22). Select the **Fluorescence timing**, option and click **[OK]**. **MCID Elite** then displays a graph of changes over time (Figure 23). See the chapter describing Profiles and Histograms in the online *Reference Manual* for more detailed instructions.

Figure 22: *The Dynamic Fl Graph Mode dialog box.*

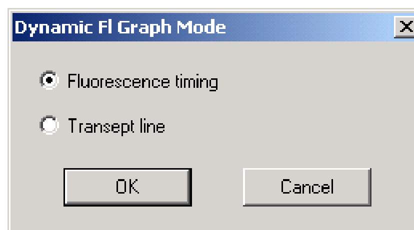
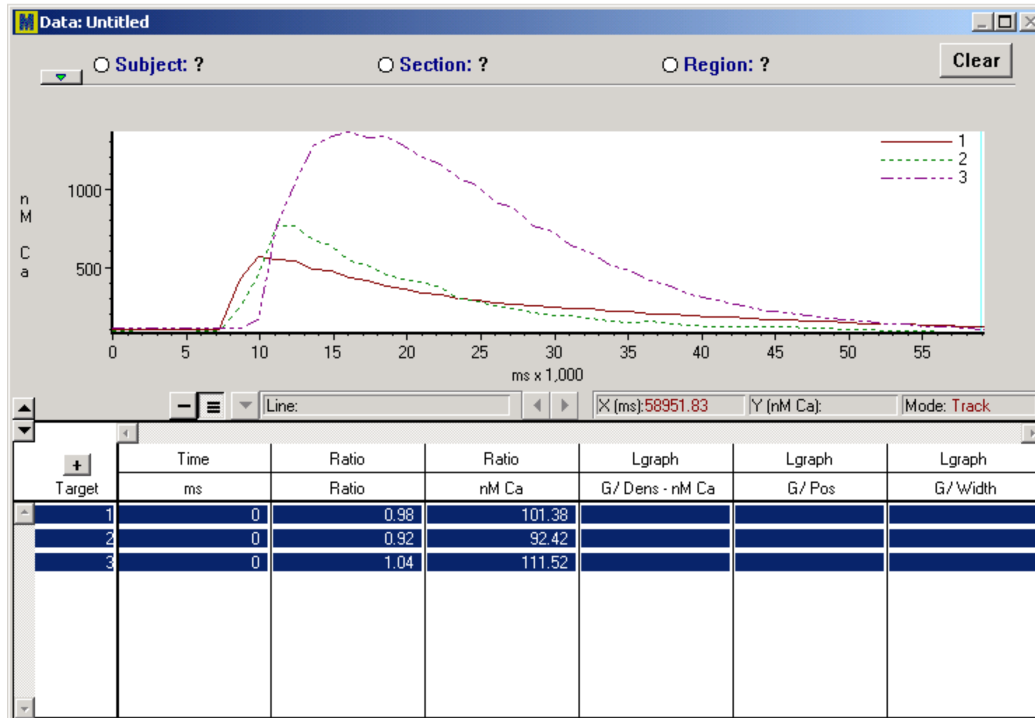


Figure 23: *Graphical display of intracellular Ca^{++} concentrations during the course of a 58 sec experiment. The inter-ratio interval was approximately 1.2 sec. Samples were taken from three individual cells, depicted as Targets 1, 2 and 3. Numerical data for the time 0 sample are shown under the graph.*



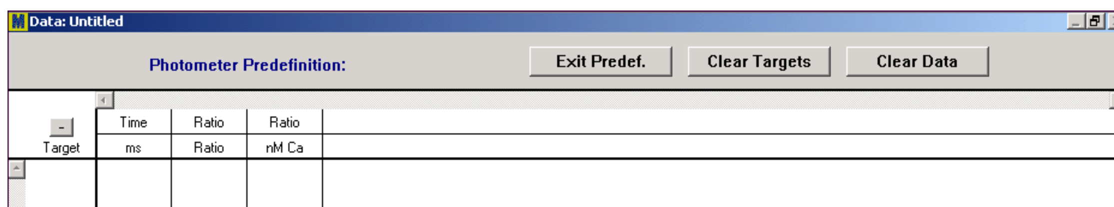
Target	Time	Ratio	Ratio	Lgraph	Lgraph	Lgraph
	ms	Ratio	nM Ca	G/ Dens - nM Ca	G/ Pos	G/ Width
1	0	0.98	101.38			
2	0	0.92	92.42			
3	0	1.04	111.52			

Using Photometer Mode

We may be willing to give up image formation, if we only need to see numerical values from identified regions of the specimen. In this case, one or more “photometer windows” are placed on the image. **MCID Elite** then reads the density values of these windows to construct the ratio. Frame averaging is not usually required. The removal of frame averaging permits somewhat more rapid operations, though each additional photometer window must be read and will slow the inter-ratio interval somewhat.

To enter photometer mode, select *Dynamic Fl* > *Settings*. Under the **Timing** section select **Photometer** and deselect **Imaging**. Set the various timing parameters appropriately. The total experiment time is expressed in msec. When you leave the *Settings* dialog box, **MCID Elite** presents a *Dynamic Fluorescence Photometer Predefinition Sample* screen (Figure 24).

Figure 24: *The Dynamic Fluorescence Photometer Predefinition Sample screen allows the placement of "photometer windows" on an image from which the photometer readings are taken.*



Digitize an image. This can be a brightfield or fluorescent image. Then, place any **Sample** tool onto regions of interest. As each tool is placed, its target number appears on the host monitor. Any number of tools may be placed, and each will result in its own set of photometer values. However, each region of interest must be read during acquisition. Therefore, the more the number of regions, the longer the inter-ratio interval will be.

Once a suitable number of targets have been defined and highlighted, select the **Profile** icon and specify **Fluorescence timing** (Figure 22).

MCID Elite presents a blank graph, ready for acquisition. Select *Dynamic Fl > Acquire*, and **MCID Elite** will perform the photometer readings, displaying the values of the selected targets on the graph as it goes. Once the graph is complete, all the usual graph data reading functions are available. To leave photometer mode, deselect the **Profile** icon, and select *Dynamic Fl > Settings* to return to the *Settings* dialog box to deselect **Photometer**.

Photometer mode generates a set of Ca^{++} concentration values across time. Any period of time may be used, as there are no memory storage requirements for the photometer data. The numerical values may be graphed, either during the acquisition process or following it.

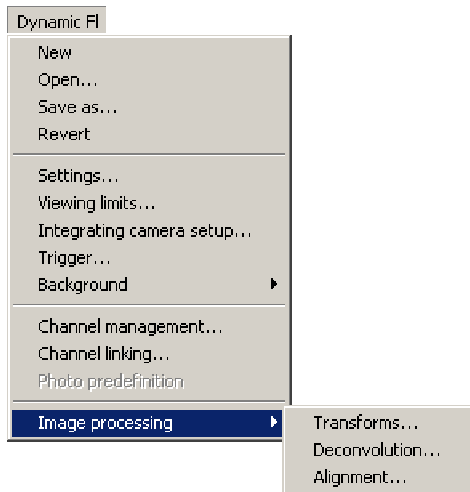
Mixing Photometer and Imaging Mode

It is possible to view a graph of ratios from a set of photometer targets, while conducting an imaging experiment. Enable both the **Photometer** and **Imaging** boxes in the **Timing** section of the *Settings* dialog box. Predefine the photometer targets in the normal way. Then, select the **Profile** icon and **Acquire** your ratios. As each ratio is acquired, photometer readings are taken from the defined targets and shown on the profile graph. This mode works best with longer IRIs, as the photometer readings increase the time devoted to each ratio acquisition and storage.

Using Image Processing Functions

Selecting *Image processing* option from the *Dynamic Fl* menu brings up a further sub-menu consisting of *Transforms, Deconvolution*, and *Alignment*. All these functions can only be accessed once an experiment has been acquired or opened from a saved disk file.

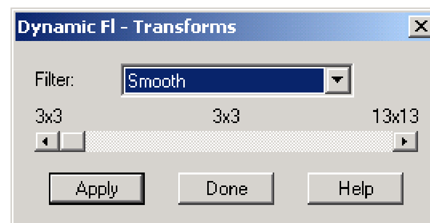
Figure 25: *Selecting Image processing will display a sub-menu giving access to a number of image processing functions.*



Using the Transforms Functions

In the **Dynamic Fluorescence** study type, **MCID Elite** is set up in a very specific way. Therefore, our standard Transform operational window is not available. Instead three image processing operations are available within the *Dynamic Fl - Transforms* dialog box (Figure 27). Select these from the **Filter** drop down menu. These filters are all convolutions. That is, they operate by passing a matrix of values across the image. This matrix is called the convolution kernel. The size of the convolution kernel can be adjusted by moving the slider. The larger the kernel, the larger the features that the filter will work on.

Figure 26: *Image transformation functions are available in the Dynamic Fl - Transforms dialog box.*



Press **[Apply]** to apply the filter to all the images for wavelength 1 and wavelength 2. The ratio images will also change, because the ratio is recalculated following the transformation process.

- **Smooth** – This is a low pass filter. High spatial frequency components are attenuated, while low spatial frequencies are unaffected. Larger kernel sizes smooth more coarsely.
- **Median** – This filter replaces each pixel value with a median value of the neighboring pixel brightness values. It is useful in decreasing noise spikes, such as shot noise in low light images.

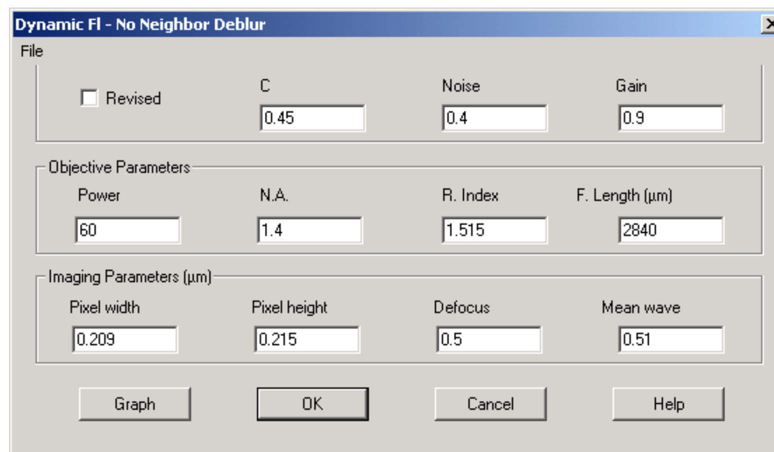
- **Enhancement** – This is a high pass filter which accentuates high frequency components. The resulting image will appear sharper and more detailed, but will also contain more noise and some filter artifacts.

Please remember that filtering images will change ratio data values. Use caution in applying filters.

Deconvolution

Deconvolution is a process by which, out of focus blur is removed from your images. It is often referred to as "deblurring" or "digital confocal". Various forms of deconvolution are available, most of which require that you take images at multiple focal planes. These "nearest neighbor" procedures are not very suitable for dynamic fluorescence imaging. Therefore, **MCID Elite** offers a "no neighbor" deconvolution.

Figure 27: *The Dynamic Fl - No Neighbor Deblur dialog box is used to control the deconvolution process.*



The **MCID Elite** deconvolution function uses a variant of the no-neighbor algorithm which was first described by Agard, (1984) and revised by Monck et al. (1992). A nearest neighbor algorithm would be more efficient but would require multiple exposures which is not practical during dynamic fluorescence imaging. The no-neighbor algorithm accepts input from the user regarding the point spread function of the lens/camera system, and then models the nearest neighbors to improve the image quality obtained from a single exposure.

Three sets of parameters are required to be input by the user: **algorithm** parameters, **objective** parameters and **imaging** parameters.

Algorithm Parameters

The default settings use the revised algorithm of Monck et al. (1992). By deselecting **Revised**, the original algorithm of Agard (1984) can be applied.

- **C** – The empirical algorithm constant. This should be set to between 0.45 and 0.49 for optimum results. Increasing this value will cause an overall increase in gain, but more so at the lower spatial frequencies.
- **Noise** – The square of the standard deviation of noise contained in the image, expressed in gray levels. Typically, noise lies in the range between 0.1 and 0.5.

- **DC Gain** – A multiplier which is applied to the average gray level of the image at a spatial frequency = 0. The deconvolution process is not particularly efficient. It loses some of the light in your specimens and will tend to darken them in the processed image. Use the DC gain setting to adjust brightness in the transformed image. Values greater than 1 will cause brightening.

Objective Parameters

These describe the optical properties of the objective used in creating the image.

- **Power** – The magnifying power of the objective lens.
- **N.A.** – The numerical aperture of the objective lens.
- **R. Index** – The refractive index of the medium surrounding the objective, such as air, oil or water.
- **Focal length (um)** – The focal length of the objective lens expressed in microns.

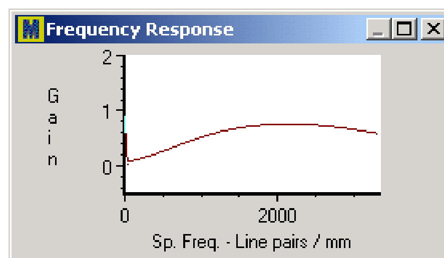
Imaging Parameters

These describe the spatial and wavelength characteristics of the images.

- **Pixel width & height** – These values can be obtained by calibrating the objective to linear distance before using it in dynamic fluorescence imaging. Pixel width and height will be available from the calibration screen. Make a note of these values, so that you can enter them (in microns) when required.
- **Defocus** – The estimated distance in microns to the nearest neighbor image. This is not a real image. Rather, the algorithm reconstructs this image during the calculation procedure.
- **Mean wave** – The wavelength of light (microns) under which the image was viewed. For fura-2 this would be 510 nm, entered as 0.51 microns.

Once all the parameters have been entered, you have defined an operation that will differentially affect the image spatial frequencies. Some spatial frequencies will be accentuated. Others (particularly those which constitute blur) will be minimized. Click **[Graph]** to display a frequency response graph that displays the effects of the current deconvolution parameters (Figure 28).

Figure 28: *The Frequency Response graph displays the effects of the various deconvolution parameters.*



The graph is useful, because the deconvolution process is rather slow. Examining the graph can give you some idea as to how the parameters you have set will affect the image. If the effect looks suitable, select **[OK]**. This will apply the deconvolution to all the wavelength 1

and wavelength 2 images. A new set of ratios are then recalculated from the deconvolved images.

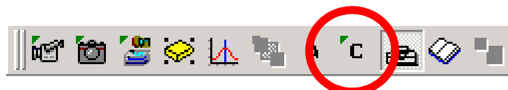
“Confidence” Mapping

Confidence mapping (Tsien and Harootunian, 1990) is a way of making ratio images more visually interesting. Without confidence mapping, we use hue to create a lookup table showing color mapped onto ion concentration. For example, we might construct a color map in which a concentration of 100 nM Ca^{++} is given a blue color. If we have a cell which exhibits a uniform concentration all across its surface, it appears a homogeneous blue. In fact, this type of mapping can lead to rather uninteresting images, particularly if flat cells form a sheet on a culture plate. At best such cells appear as homogeneous blobs. At worst, they might merge into a sheet of blue with little discrimination between individual cells.

When we enable confidence mapping, we create a lookup table, which uses two dimensions of information, instead of one. We still use hue to map color onto the calibrated concentration of ion in the ratio image, but we also use intensity to reflect the brightness of the specimen in the component images. Confidence mapping can help to diminish the problem of uninteresting calcium images. It makes the assumption that brightness in the component images (340 nm and 380 nm for fura-2) is related to the quality of the ratio image calculated from those components. Brighter image components are assigned more confidence than dim image components. This is not an unreasonable assumption, if care is taken to avoid overdriving the intensifier. Very dim parts of the image are more likely to fall into the nonlinear area of intensifier response.

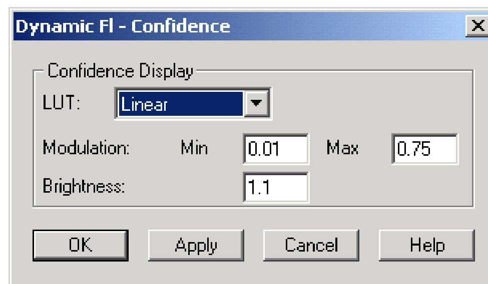
Lets summarize this. Now we have two dimensions upon which a lookup table map can be built. Concentration is mapped to color and the brightness of the component images modulates the brightness of colors in the ratio image. For example, we map blue onto a concentration of 100 nM Ca. In examining the component images used in creating the ratio, we find that the edges of the cell envelope are dimmer than the central portions. We use confidence mapping to modulate the blue color. The ratio image takes on various intensities of the same blue hue, from dim to bright, reflecting the spatial distribution of brightness in the component images. Now, the cell edges are a dim blue, and the central portions are a brighter blue. We have a more visually interesting ratio image that might also be more meaningful.

Confidence is mapped as the lightness or brightness component in the Hue, Lightness, and Saturation paradigm, using a full range of 256 lightness levels. Similarly, hue is also mapped using a full 8 bit range. The resultant 24 bit color image is visually pleasing and does not appear as grainy or blotchy. To apply confidence mapping to your experiment click on the **Color Confidence** icon (“**C**”) in the Application toolbar:



To access the various confidence display options, hold down the <Ctrl> key when clicking on the **C** icon. This will bring up the *Dynamic Fl - Confidence* dialog box (Figure 29).

Figure 29: *Controls available for confidence mapping.*



Confidence Display Options

Entry fields in the *Dynamic Fl - Confidence* dialog box allow adjustment of the appearance of the ratio image. These options sound rather complex, but a brief period of experimentation with real data will give you a feel for their use. Like confidence mapping in general, none of these options affects your actual ratio values. Only the image appearance is affected.

LUT

A linear lookup table (LUT) will give equal emphasis to all confidence intervals. The three exponential LUTs assign more of the available confidence levels to the higher confidence parts of the lookup table. We usually use an EXP2.

Modulation

"Modulation" adjusts the saturation of the colors used for concentration mapping. Values range between 0 and 1, where:

0	black
0.1	dim color
0.75	bright color
1.0	white

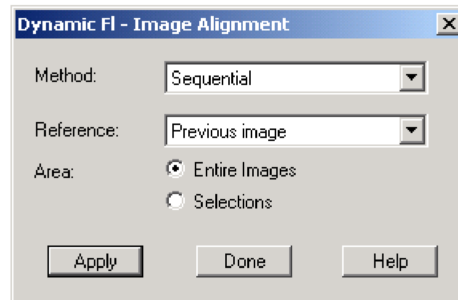
Brightness

Confidence calculation can have the effects of making a ratio image dim. The "Brightness" entry field accepts a factor that will increase overall brightness of the ratio image. Higher values will increase brightness.

Alignment

Alignment is an option that allows you to align a series of images that have been acquired as part of a dynamic fluorescence experiment. It is most useful in instances where the image has moved during the acquisition period (i.e., cells moving across the field of view with time).

Figure 30: *The Dynamic Fl - Image Alignment dialog box.*



Alignment Options

Four different methods of performing an alignment are available. These can be selected from the **Method** drop down menu.

Method

- **Sequential** – With the sequential method each image is aligned in sequence with its preceding neighbor image, e.g. starting with the first image at 340 nm at time 0, then the 380 nm image at time 0 and so on.
- **Wave 1 / Wave 2** – With this method first the Wave 1 and Wave 2 images are aligned for a given time point. The alignment is averaged before being applied to the corresponding Wave 1 and Wave 2 images.
- **Wave 1** – The alignment algorithm is only calculated for the Wave 1 images. The resulting correction is then applied to both Wave 1 and Wave 2 images.
- **Wave 2** – The alignment algorithm is only calculated for the Wave 2 images. The resulting correction is then applied to both Wave 1 and Wave 2 images.

Reference

- **Previous image** – Selecting this option from the **Reference** drop down menu will ensure that as each image is aligned to the previous one, the newly aligned image becomes a template for the next alignment in the image series.
- **340 nm: 0 ms** – This and all subsequent **Reference** options make the chosen image in the series the reference image to which all the subsequent images are aligned to. The labels in the **Reference** drop down menu will change depending on the type of **Method** chosen.

Area

This option refers to whether entire images are aligned or just user defined selections within an image.

Saving and Retrieving Experiments

On-Line

To save a ratio experiment, select *Dynamic Fl > Save as*. Experiments are saved as a single large file (*.fre) containing all the discrete wavelength images. By default, images are saved in the **FRE** subdirectory of **MCID Elite**. Please remember that a series of ratios occupies a lot of disk space, and it is best to save experiments to a secondary backup medium (e.g., CD-R disk) to leave enough disk free for current operations.

Note: Ratio experiment files (*.fre) do not contain sampled data. See Saving Data from Ratio Experiments, below.

Experiments are retrieved with *Dynamic Fl > Open*. As each experiment is saved as a single file, it only requires specification of that file name to bring back all the component images, calibrations, and settings. You can also quickly save and retrieve experiment settings by creating experiments without images. For example, we use the *Settings* dialog box to define a set of parameters for a typical experiment. We then save the experiment without generating any images. This set of parameters can be retrieved by opening the experiment.

Saving Data from a Ratio Experiment

Ratio experiment files (*.fre) contain images, calibrations and experiment settings. They do not, however, contain any data gathered from the component images (e.g., 340 nm, 380 nm, ratio, and nM calcium values). To save such data to disk, you must save the data as a discrete data file (*.lg2 file). Select *File > Save as* and specify a filename for the data file. Data files are stored in the **MCID Elite 7.0/LOG** folder by default.

Exporting Images

Images from dynamic fluorescence experiments can be exported in TIFF format (*.tif) for use with other applications. You can save multiple images in a single TIFF file or create separate files from individual images.

PROCEDURE:

1. Select *Dynamic Fl > Save as*.
2. Select **Export image (*.tif)** as the image type (Figure 31).
3. Specify **Archive** or **Presentation** format (see below).
4. Specify the **Components** to include in the file (Presentation format only).
5. Press **[Select images]** to specify which experiment image(s) to include in the output file.
6. Enter a filename for the output file and press **[Save]** to store the file to disk.

Archive or Presentation?

You can export image files for **Archive** or **Presentation** purposes. Select **Archive** if you wish to use the images with other image analysis programs. The images are formatted as grayscale TIFF files, without any color information (i.e., no pseudocolor, no colored overlays). Original bit densities and gray level data are preserved.

If you want to create a TIFF file for use with graphics, desktop publishing, or word processing programs, use the **Presentation** option. This option retains the visual information

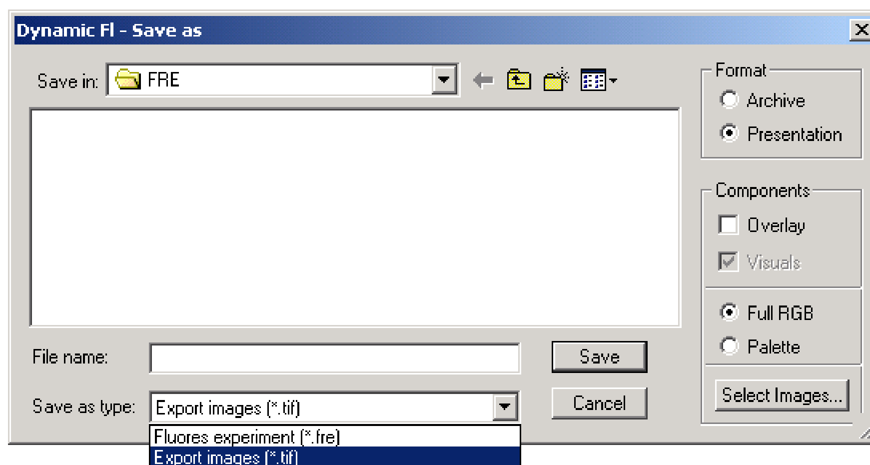
contained in the original image(s), but not the gray level values. Select this option if you want the output images to look like the images you see on your monitor.

Components

Presentation files can be saved in **Palette** or **Full RGB** format. The **Palette** option produces an 8-bit color TIFF file with 256 colors. The **Full RGB** option produces a high-quality, 24-bit color TIFF file.

Check the **Overlay** box if you wish to include image overlays (e.g., sample outlines, annotations) with the file.

Figure 31: *Exporting images from a ratio experiment.*



Saving a Ratio Experiment as a Single Image

The usual way to display ratio images is by opening a ratio experiment. Then, all the data from the experiment are available, including calibrations. However, you may wish to save the results as an uncalibrated image, for demonstration or photographic purposes. To do this, use *Dynamic Fl > Channel management* to create a single image screen showing the ratios and or 340/380 images that are required. Then, use the *Image > Save as* selection from the main menu line. From within the *Save Image As* dialog box select **Presentation** as the **Format** and select **Entire screen** from the **Area and Components** drop down menu. This will save the screen as an uncalibrated image. If you wish, save a calibration bar with the image to show the mapping of grays/colors onto ion concentration values.

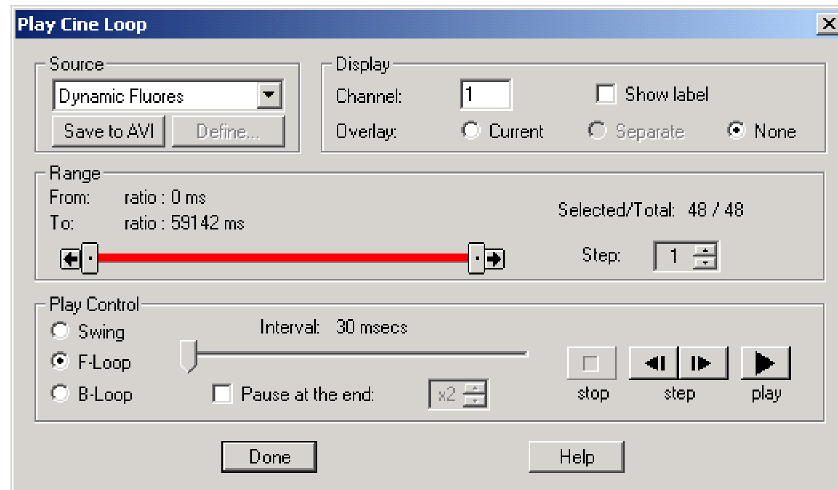
Cine Loops

Cine loops are like movies, which display a series of images very rapidly to create the impression of movement. Apparent motion is achieved by copying multiple channels into a single display channel in rapid succession, or by rapidly loading image files into a single display channel. Ratio images from dynamic fluorescence experiments are particularly striking when viewed in a cine loop, as the influx of Ca into the cells becomes visually apparent.

To create a cine loop from a dynamic fluorescence experiment, select *Image > Cine loop* (Figure 32). This option allows you to display images from a fluorescence experiment as a

short movie on the image monitor. Please note, the cine loop will only play the channels which have been selected for display through the *Channel Management* dialog box (Figure 19). Make sure you have the correct images selected for display before you create the cine loop (e.g., ratio images).

Figure 32: *The Cine Loop dialog box.*



Source

The drop-down menu in the **Source** area of the *Play Cine Loop* dialog box is normally used to indicate whether to create the loop from a series of image files or from a series of channel displays. When you are in the Dynamic Fluorescence study type, however, cine loops can only be created from channel displays. **Dynamic Fluores** is consequently the only option listed in the menu.

If you wish to view the cine loop in other programs, press **[Save to AVI]** to export the loop in *.avi format.

Display

From the **Display** section, select the channel number that you would like the cine loop to be displayed in. If you wish to have the label displayed during the cine loop then select the **Show label** checkbox.

Range

This section of the *Play Cine Loop* dialog box deals with the display of the individual images that will make up the movie. The **From** and **To** labels tell you the starting and end points of the movie. By sliding the two slider bars you can adjust the starting and end points respectively. Adjusting the **Step** size will determine how many frames are to be skipped between successive images (e.g,. a **Step** size of 2 will cause the cine loop to skip alternate images in the series selected by the **Range** slider bars).

Play Control

This section of the *Play Cine Loop* dialog box deals with the way the movie will be played in the image monitor. Selecting **Swing** will display the images in a series from first to last and

then from last to first. This sequence is then repeated. Selecting **F-Loop** will display the images in a sequence from first to last and then repeating the sequence. Selecting **B-Loop** will display the images in sequence from last to first and then repeating the sequence. The slider bar below the **Interval** selects the time interval between each image as it is being displayed. Enabling the **Pause at the end** checkbox will cause a delay before the sequence if images is replayed. The **stop, step** and **play** buttons are there to stop, start and step through the cine loop.

Fura-2 External Calibration

This section is provided as an informal guide to the ways in which we perform fura-2 calibration in our own labs. We take no responsibility for these procedures, and offer them purely as a starting point from which you may develop your own calibration procedures.

Calibrating to a Series of Calcium Standards

Before using external standards for calcium measurements, run some fura-loaded cells on **MCID Elite**. Adjust the intensifier gain or camera integration time so as to yield good images. Balance the 340 nm and 380 nm illumination, so that the images at the two wavelengths are of about the same brightness. Remember that 340 nm will rise and 380 nm will drop as Ca^{++} binds to fura-2, so leave some latitude for these effects as part of the balancing procedure. Your system is now at the settings that will be used for ratiometric measurement.

PROCEDURE:

1. Make up a range of standards with known calcium concentrations that range from 10 nM to 10 μM. An example of a typical series is given in Table 2.
2. Add equal amounts of fura-2 (free acid form, not the AM ester), between 1 μM to 10 μM, to each of the standards. The actual amount of fura-2 to be added will depend upon the loading and response characteristics of your cells. Some brighter preparations will require larger amounts of fura-2 for calibration. Whichever concentration of fura-2 you select for addition to the standards, it must be the same across the entire range of calcium concentration standards.
3. Place a drop of standard solution on a glass slide and cover it with a coverglass. We have also seen special calibration slides containing a series of coverslipped chambers on the same slide. However you arrange the solutions on the slides, the object is to ensure an even and flat distribution.
4. Have **MCID Elite** acquire a single ratio from one of the calibration standards. Place a sample window over the ratio image and read the ratio value.
5. Repeat step 4 for each of the calcium concentration standards.
6. Plot the calcium concentrations against the ratio values to obtain a standard curve which should begin to demonstrate saturation at the higher calcium concentrations. The calibration to a series of calcium concentrations has two purposes:
   a) it demonstrates the linear portion of the system's response to calcium concentration;

b) it should, in theory, give an accurate value for the point at which changes in thefura-2 emission spectrum are no longer visible with increasing concentrations of calcium (the Rmax value).

7. Enter the minimum Ratio (Rmin, obtained at the lowest calcium concentration) and the maximum Ratio (Rmax, obtained from the standard curve when it begins to saturate) into the *Dynamic Fl > Settings* screen.
8. The Fo and Fs values are obtained from the gray level intensity readings, measured at the 380 nm wavelength, at minimum and saturating calcium concentrations. Enter the Fo/Fs ratio in the *Dynamic Fl > Settings* screen.

Points to Note

Regarding step 1, it is important to make up the standards in a solution containing a calcium buffer such as EGTA or BAPTA. This allows for an accurate determination of the “free” calcium concentration (reagent contaminants can contain calcium in the µM range). The free calcium concentration can be calculated from the dissociation constant of the buffer, it’s concentration, and the total amount of calcium added. See Table 2 for an example.

Table 2: *An example of external calcium standards. Solution composition: 10mM EGTA, 150mM K, 5mM Na, 20mM HEPES, pH 7.4*

Free [Ca] (nM)	Total [Ca] (mM)	Free [Mg] (mM)	Total [Mg] (mM)
10	0.963	1.0	1.483
50	3.477	1.0	1.349
100	5.159	1.0	1.259
500	8.421	1.0	1.084
1000	9.143	1.0	1.046
5000	9.821	1.0	1.010
1000	9.917	1.0	1.005

To make this whole procedure of calculating free calcium more convenient, you could purchase a calibration kit from Molecular Probes.

Make sure that the microscope optics, and the gain and offset settings of the intensifier and camera controls are not adjusted during calibration. Ideally, you should then run your cells at the same intensifier and camera settings as are used during calibration.

In steps 4-5, above, we describe the calculation of the ratio value for each standard concentration. It is important to ensure that shading error is not affecting the ratio values. If spatial variation in the ratio is observed, we suggest:

1. taking the ratio of the entire screen, to minimize the contribution of shading in any one part;
2. trying the *Dynamic Fl > Background > Advanced* proportional and/or subtractive shading correction options when acquiring calibration ratios.

An Alternative: Calibrating Within the Cells

There can be many differences between the conditions of calibration to the external calcium standards, and the conditions under which cells are actually run. Therefore, some researchers prefer to calibrate **MCID Elite** to alterations in calcium concentration within the cells themselves. This type of calibration is a bit more complex than the external calibration, but may be more suitable for some specimens. Steps in calibrating **MCID Elite** to the cells are given below.

1. First, place the cells in a solution of calcium-free buffer containing EGTA or BAPTA.
2. Introduce a calcium ionophore (e.g., 10 μM Ionomycin), which will make the cell membrane permeable to calcium.
3. There will be an initial increase in intracellular free calcium as the ionophore penetrates and liberates internal calcium stores.
4. The buffer will then soak up any free calcium, and over time, you should see the ratio drop to a stable Rmin level. Find the minimum ratio value, and use this as Rmin in the *Dynamic Fl > Settings* screen.
5. Allow the cells to recover in calcium-containing medium (usually between 1 and 10 mM).
6. Use a number of compounds that are known to cause an elevation of intracellular calcium levels in cells of your particular type. Obtain Rmax from this exercise. Typical compounds include CCCP (a mitochondrial inhibitor), and 100 mM KCl (for cells that depolarize). You could also use Ionomycin, having allowed a sufficient time for recovery (e.g., 30 minutes) from any initial exposure to the agent (as in steps 2-4). Enter the observed Rmax value in the *Dynamic Fl > Settings* screen.
7. Obtain the gray level density values from the Rmin 380 nm image, and the Rmax 380 nm image. Using these values, calculate Fo/Fs and enter the value in the *Dynamic Fl > Settings* screen.

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