FLIPR™

Fluorometric Imaging Plate Reader

User Manual

Version 3.4
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1. System Overview

1.1. Introduction

This section gives a general and brief overview of what comprises the FLIPR optical screening system. Section 1.2 gives a brief description of the basic functionality found in FLIPR. Section 1.3 lists the various system operating requirements for FLIPR and Section 1.4 gives start-up and shutdown operating procedures. Section 1.5 lists recommended maintenance procedures.

Caution
It is recommended (and perhaps a necessity) that the user familiarize themselves with the entire manual "before" running FLIPR.

1.2. System Description

1.2.1. Overview

FLIPR was initially designed to perform as a large-volume screening tool for measurements of membrane potential of adherent cells in 96 well microtiter plates. FLIPR has since been applied to other fluorescent assays, such as in the measurement of intra-cellular calcium and intracellular pH. More recently, FLIPR has also been used in non-fluorescent assays, for example in measuring luminescence for reporter assays such as luciferase.

In fluorescent mode, FLIPR works by illuminating the bottom of a 96 well microplate and measuring the fluorescence from all 96 wells simultaneously using a cooled CCD camera. The excitation optics in FLIPR illuminate the plate from the bottom and the emission optics read the plate from the bottom. This requires that the microplate that is optically measured in the machine has a clear bottom.

The typical measurement assay consists of using one 96 well plate with cells and one (or two) 96 well plate with the compounds to be screened. Details dealing with cell culturing and protocol for the membrane potential assay are described in Section 4. Specifics of setting up FLIPR for measurements of intracellular calcium can also be found in that section.

FLIPR can be programmed via a general Experiment Setup Window to take multiple time point exposures of the cell plate, as well as aspirate and subsequently dispense the compounds from the addition plate (or plates) into the cell plate at a specified time. FLIPR continues to take camera exposures of all 96 wells recording the changes in fluorescence, which occur upon addition of the experimental compounds to the cells. Each of these fluorescent time points is actually a picture frame of the bottom of the plate, thereby containing data from all 96 wells.

The FLIPR control software provides a display of fluorescence versus time for all 96 wells of the microplate on the system monitor. These screen updates occur in real-time if the computer has enough time to read back and plot the data before the time point is to be acquired. If the time points are gathered quickly, the data will be plotted at the end of the run. Once the run is completed, the user can then export the data for post-run analysis. The data can be converted to fluorescence vs. time using various scaling options, or it can be converted to one number (statistic) per well. These

statistics can be defined to be the peak signal after the stimulus, the summed area under the curve etc.. This data is then stored in an ASCII text file format for input into a spreadsheet program, for example Microsoft Excel.

Because of the multitude of fluorescent probes now available, we have tried to build into FLIPR the functional tools necessary to perform screens on a large number of optical-based assays. These functional tools are as follows:

a.) FLIPR provides very precise temperature control for assays which are temperature sensitive. This is accomplished by actively heating and humidifying the cell incubation chamber.

b.) A computer controlled 96 well pipettor is built into the instrument in order to perform simultaneous sample additions to the cell test plate. Additions can be made from either of two 96 well holding plates. The pipettor uses disposable pipette tips thereby eliminating any possibility of compound and/or reagent carry-over. The ability to "mix" the fluid additions is also available in multiple formats. The pipettor can dispense volumes in a range from 5 to 250 µl. The pipettor height and dispense speed are also configurable within the Experiment Setup Window. Section 4.4 of this manual lists recommended vendos and ordering information for disposables to be used with FLIPR

c.) All 96 wells of a microtiter plate are analyzed in parallel. Fluorescent measurement updates for each of the 96 wells can be made as short 0.5 seconds apart, depending on the available fluorescent signal. Typical updates are on the order of 1.0 second.

d.) The optical detection scheme used in FLIPR allows for fluorescent signal isolation on cell-monolayers. This helps in reducing the background fluorescence that may be present in certain assays. Both adherent and non-adherent assay protocols have been established.

e.) The optical scheme can potentially work with any optical probe. However, there are practical limitations based on the spectral transmission of the microplates, which are typically comprised of polystyrene. FLIPR has proven more useful as a screening tool with dyes which excite in the visible, i.e. 450 nm and longer.

f.) The system is supplied an Argon laser source, providing discreet spectral lines between 350nm and 520 nm.

g.) Once collected, the fluorescent data is "exported" via various processing options into a data file format compatible with spreadsheet programs such as Microsoft Excel.

1.2.2. Calibration

In order to establish signal calibration within a single 96 well plate (denoted as a run) as well as plate to plate (run to run), it has proven useful to provide the user with some very simple data scaling features built into the FLIPR Control Software. This will also be discussed in more detail in Section 4.

FLIPR measures changes in fluorescence versus time very accurately. Calibrating these optical changes to actual physiological changes, such as membrane potential or intra-cellular calcium usually requires a second measurement of the same physiological event. Usually this is not of great importance to those interested in using FLIPR as a screening tool, as relative fluorescent changes between wells will usually suffice.

However in order to insure that the optical signals are relatively consistent within the wells of a single test plate, or even between different plates, FLIPR provides control scaling options. When graphing

or exporting data, the user has the ability to use some wells as negative controls (for temporal baseline correction) and other wells as positive controls (for comparative scaling) of the optical signals.

The negative controls are typically additions of a non-stimulating fluid balanced salt buffer, e.g. in the case of the membrane potential assay, EBSS, DiBAC and perhaps any solvent being used. The negative controls are used to make sure the instrument as well as the assay is working properly, yielding flat baselines and no response where there should not be any. The negative control wells can be used to temporally correct the data in cases where there is an artifact, for example a temperature drift, dye leakage effect or photobleach effect, which is consistent in all the data. In a way, this can be thought of as a differential type measurement, where a few of the wells are used as part of the ratio pair.

The positive control wells are used to monitor changes in signal gain. The gain calibration is useful in scaling all of the data on a given plate with respect to a known agonist on the same plate. This corrects for system gain discrepancies such as a change in camera exposure time, laser power , cell density or response, all of which can effect the absolute fluorescent change measured. By scaling the data on a given plate to a known agonist of a given concentration on the same plate, one can in effect correct for subtle gain differences between data runs.

1.3. FLIPR System Requirements

The following is a list of room requirements, electrical, cooling, space and air, required for operation of the FLIPR system. All of this is covered in the FLIPR Pre-Installation Manual.

1.3.1. Electrical

One room outlet (110V, 15 Amp) is required to power everything but the light source.
A 220 V, 3-phase, 50 AMP power source and main disconnect is required for the laser. In Europe a transformer will be supplied converting the room outlet to 110V. A separate transformer must be purchased from Coherent for proper conditioning of the 3-phase laser power.

1.3.2. Light Source Cooling

A water supply and drain capable of 2.5 gal/minute for cooling the laser. MDC can provide a calibrated flow meter to insure the flow rate (which depends supply pressure and restriction on the water line) is adequate. Most users flow the water to a drain, however Coherent offers other cooling options including a water to water chiller (LaserPure20) and an air/water heat exchanger closed loop system (PolarPure 18). Please contact MDC for more information, or consult the FLIPR pre-installation manual.

1.3.3. Space

The optical table provided with the FLIPR is system is 96 inches by 30 inches. The operator will need at least 24 inches to the side for loading tips and 24 inches behind the machine for maintenance. Therefore the room must be at least 10 ft in the long dimension of the table, and preferable 8 feet in the short dimension. The table can be supplied with wheels (an option) for those users who wish to converve the 24 inches of space required behind the system by wheeling the system out from the wall during maintenance.

1.3.4. Air

FLIPR requires two air sources. For loading pipette tips as well as for providing proper air pressure for many of the pneumatic controls within the instrument, a steady, clean air supply of 80-90 PSI is necessary. House air can be used as long as it is regulated and reliable. Some users prefer using a nitrogen tank and two-stage regulator if they are concerned about the stability of the house air. Compressed air can also be used.

The second air source is for temperature sensitive assays only. This provides the air for the humidification system in FLIPR. This source should be regular air (as opposed to nitrogen) since the gas will be used to humidify the cell incubation chamber. The pressure required for this air line is minimal (5PSI) and MDC provides a low pressure regulator with FLIPR for this requirement. This source is only required for temperature controlled assays. This should not be run off an air tank however. Although the flow rate is small, it will drain a typical air tank in a few days. Normally this is run off a house air system.

*Please consult MDC or refer to the FLIPR pre-installation manual for specific room layout requirements and recommendations.

1.4. FLIPR Start-Up and Shutdown Procedures

Because of the computer-controlled nature of FLIPR, as well as for proper system operation, certain components need to be turned on before others. These are described as follows:

Start-Up Procedure:
1.) Turn on the laser water flow and main electrical power disconnect.

2.) Turn on the laser. Typically, the laser will require about 15 to 30 minutes to stabilize. Coherent recommends a 30-minute warm-up stabilization period for the laser. They also recommend (due to tube lifetime) turning the laser off only if you plan on being down for more than 3 hours, otherwise let it run.

3.) Turn on the outlet strip which provides power for the PC, camera controller etc..
Make sure that the CCD camera is at temperature before taking data with the camera. Proper temperature is indicated by a lit green "status" light on the camera controller. Normally camera cool-down takes about 5 minutes.

4.) Turn on FLIPR.
Make sure PC is booted up before turning on FLIPR.

5.) Turn on the regulator for 80 psi air

6.) Clear any interlocks by making sure the cell drawer is in and the pipette tips are up.

7.) Start the airflow for the humidity chamber (temperature dependent assays only)

8.) Start the FLIPR code by selecting the FLIPR control software from the programs menu.
The pipettor will go through the normal homing cycle immediately. If temperature control is being used, this will also start the temperature control warm-up cycle for FLIPR that takes about 30 minutes. If no temperature control is being used you can ignore this 30 minute delay.

9.) Wait for the temperature warning indicators to clear on the display window if temperature control is being used. You are now ready to take data.

The Shutdown-Procedure is as follows:

1.) Stop any running experiments from the "Stop" pull-down menu. Wait for any pipettor operations to complete.

2.) Exit the FLIPR code by choosing "Exit" from the FILE pull-down menu.

3.) Turn of the laser at the remote controller.

4.) With the laser off for at least 5 minutes, turn off the laser water flow lines. Turn off water supply line first and then the drain line. *Make sure the laser has had a chance to cool for at least 5 minutes before turning off the water.*

5.) Turn off the 80 psi air line.

6.) Turn off the 5 psi air line.

7.) Turn off the FLIPR power switch on the back of the enclosure

8.) Turn off the power strip, or selectively turn off the camera controller and PC.

1.5. System Maintenance

In general FLIPR is a very low maintenance machine. Certain areas, which require at least a regular check, are:

1.) Laser Alignment: (Check daily)
Each day the laser should be checked for system alignment. In general, turn on the laser, and let it run 20-30 minutes before doing any adjustment. Then using the calibrated yellow plate and the signal test tool, check the uniformity of the beam across the plate. Depending on the specific laser, the horizontal and vertical adjustment knobs may need to be slightly adjusted on the back of the laser head. MDC personnel should explain this procedure in person. Also it is a good idea to keep track of the typical laser tube current required for a certain optical power output for your particular laser (for example 15.0 Amps for an output of 300 mW). If the laser is running suboptimally, for example due to mis-alignment or dirty optics, the result will be a larger current required to maintain a given light output. Monitoring this parameter daily (after warm-up and tuning) can aid in the diagnosis of a system problem.

2.) Water Fill: (Check daily, *only used with temperature control*)
The water bottle on the back of FLIPR, which is used to fill the humidity chamber, should be kept with some visible water level. FLIPR automatically fills the humidity tank from the water bottle via a water level switch and solenoid valve. We would recommend filling the bottle with distilled water when the level falls below 1/3 of full. Under normal use, using temperature control, this bottle will need filling every couple of weeks. A fungicide should be used to keep the tank free of contamination. However, avoid using too much, as most fungicides are soapy and will produce

bubbles that will inhibit the proper function of the humidifier system. We generally would recommend no more that 300 µl of a fungicide like Roccal II (10%) to every liter of water added.

3.) Air Flow: (Check daily, *only used with temperature control*)
Check that proper airflow is maintained through the humidity chamber. After start-up is complete pull out the cell drawer, and check for airflow at the intake located in the rear of the instrument. If condensation has built up the air line can fill with water restricting air flow. If this happens the line should be drained of water.

4.) Every six months it is a good idea to lube the lead screws on the pipettor. There are three to lube: the horizontal positioning lead screw (17" long) as well as the up-down and aspirate-dispense lead screws (3-4") on the pipettor head itself. A lightweight lithium –based grease should be used.

2. Hardware Overview

2.1. Cell Drawer

In the front of the FLIPR enclosure is a manually opened drawer which holds up to three 96 well microtiter plates. This is referred to as the cell incubation chamber. The middle position (with the 96 holes) holds the plate that is illuminated by the light source and read by the camera. The other two plate positions (one to the right and one to the left) hold the fluid addition plates. The entire drawer is temperature controlled and filled with high humidity air so as to retard evaporation and provide better temperature uniformity across the microplates.

The 96 well plates are gently placed into position and held in place by small ball-plungers in the sidewalls of the plate manifold. Since the two ball plungers push from the bottom and from the right, it is easiest to load the microplate by sliding it in from the top left corner. This avoids the "snap" that can occur from pushing straight down. This "snap" should be avoided so as not to intermix fluid in the microplate wells. Please note the plate orientation when placing microplates into FLIPR. *To be consistent with the software, well* A1 *should be to the left and towards the user when loading plates into FLIPR.*

Caution:
Make sure when loading plates that they are lying flat on the machined surfaces on which they are to sit. This is important for proper temperature conduction from the plate manifold to the microtiter plates as well as for proper optical alignment of the wells to the camera.

2.2. Temperature and Relative Humidity Control

Depending on the assay, temperature and relative humidity controls are an integral part of the FLIPR detection scheme. This is especially true of the membrane potential assay using the voltage sensitive (and very temperature sensitive) dye DiBAC. Temperature control is maintained by conduction of heat from the cell drawer to the microtiter plates, as well as by eliminating fluid evaporation.

As shown on the FLIPR Control Main Window, there are 4 primary temperature control feedback systems. The operator has individual control of all of these systems and can set different set points and temperature warning limits on each individually by choosing Heaters under the SetUp pull-down menu. These heaters can also be disabled from within this menu. Depending on whether the user intends use temperature control, either heaters on or heaters off can be made the default value.

Once enabled, the individual temperature control set points are set at the factory for operation at 35 deg C. It is recommended that the user consult MDC before altering these defaults settings as there is a unique relationship between the individual subsystem temperature settings and the desired set point temperature of the microplates inside FLIPR.

The four temperature control systems are
1.) Drawer
2.) Cell drawer lid
3.) Humidifier water

4.) Air heater

The cell drawer and cell drawer lid are normally kept at the desired temperature of cells and addition plates. This is maintained by PID control loops, which read probes on the plate and the lid, and via electronic feedback apply voltage to the resistive heater elements strategically located in FLIPR.

To prevent evaporation of the fluid in the microtiter plates, humidified air is generated by bubbling air through a heated water chamber. During this process the air is nearly 100% humidified, as well as being brought to the temperature of the water. The input air line for this chamber is the "5 PSI air" input on the back control panel of the FLIPR enclosure. This line is regulated via a low-pressure regulator supplied with the system and set to about 5 PSI. This insures a constant steady flow of air through the chamber. In order to avoid condensation, the humidified air leaving the water chamber is then re-heated to a temperature slightly above that of the drawer and lid. The water heater set point is normally kept 1^{o}C below the drawer and lid "set point" temperatures and the air heater set point kept 0.1^{o}C above these set points. This insures that no condensation builds up in the cell chamber, and at the same time minimizes the amount of evaporation which occurs in the test plates.

Caution:
The temperature probes used in FLIPR are meant for high precision to allow for temporal control of temperature to less than 0.1 deg C peak to peak. As such, they are only calibrated between 33 and 37 deg C. Outside of this temperature range the probes will not read accurately. If temperature settings outside this range are desired please consult MDC for proper set points. The heaters can also be disabled for operation at ambient temperature.

2.3. Integrated 96 Well Pipettor

Within FLIPR is an integrated, computer-controlled 96 well pipettor. The purpose of the pipettor is to aspirate, dispense and mix fluid additions, from one of three compound addition positions into the optically tested microplate. All operations of the pipettor are controlled via the various Experiment Setup Windows within the Setup pull-down menu (see Section 3).

FLIPR software allows for full control of all pipettor functionality. Features include, when the aspirate and dispense cycles occur during an experiment, fluid volume, fluid height, dispense speed, mixing speed etc.

The pipettor is controlled via 3 stepping motors inside FLIPR. The stepping motors perform the following functions: a.) Motion of the pipette head up or down b.) Aspirate and dispense motions of the pipette cylinder needles c.) Motion of the pipettor horizontally between the addition trays and the cell plate. The communication between the computer and the motion control board inside FLIPR is via an RS232 line which connects up to a D-type connector on the back of FLIPR.

One of the key requirements of the pipettor for drug screening is the ability to perform accurate and precise fluid additions. This is to insure well to well data fidelity. The pipettor inside FLIPR will hold 1% accuracy and 1% rms precision for an aspirate/dispense cycle of a nominal 20 μL fluid volume. At 200 μL volumes the error and precision are closer to 0.2 % rms.

A typical data collection will involve a multiple point temporal baseline, followed by a compound addition and then an additional multiple time point observation sequence. It should be noted that in FLIPR, optical readings are made during the fluid stimulus addition. In membrane potential experiments using the DiBAC, it is necessary to pre-soak the pipettor tips in dye in order to minimize absorption of DiBAC into the plastic tips during the experiment. The software can perform a pre-

soak by aspirating fluid from the designated “pre-soak” addition plate and holding it for a 1 minute period before the start of an experiment.

2.4. Loading Pipette Tips

One of the key features of FLIPR is the elimination of potential reagent carry-over by the use of disposable pipette tips. The current pipettor in FLIPR is made to be used with tips provided by Robbins Scientific Cat 1043-24-0. These tips come in 96 well racks, are non-sterile and black in color and are 250 μL in capacity (Consult Section 4.4 for more information). The FLIPR software limits the dispense volumes to the full usable range of the tip. Blue tips can also be purchased to allow the user to “see” fluid levels in the tips for a visual inspection of the pipettor function.

In order to insure proper "seating" of the tips to the cylinder heads, each tip requires about 2 lbs. of force. For 96 tips this equates to about 200 lbs. of force. This is achieved in FLIPR via an air cylinder located at the top of the pipettor, inside the FLIPR enclosure. The air feed for this cylinder comes form the air input line marked (80 psi) on the back of the enclosure. Using less air pressure than 80 psi can cause certain tips not to seat properly, eliminating proper vacuum and subsequently causing erratic volumes. In addition, much higher air pressures (e.g. 100 PSI) can make "unseating" of the tips difficult. The pressure should be kept above 80 psi (nominal) and *not greater than 90 psi*. If the pressure falls below 80 PSI, a software interlock will warn the user.

Loading tips involves transferring of the 96 tips from the rack that the tips are shipped in to the removable tip loading tray in FLIPR. This procedure is best described in person, but briefly the tips are unseated by throwing the air switch (located on the upper rear back panel of FLIPR) to the down position. Note: FLIPR must be powered up to load and unload tips due to a safety interlock on the tip tray. Once the tips have been unseated, the user removes the tip loading tray which pulls out of FLIPR and disposes any used tips. The new tips are then transferred from the 96 well rack to the FLIPR tip tray via the tip transfer plate (plate with 96 pins in it) provided with the system.

A safety interlock is provided on the air switch which disables the air cylinder when the tip tray is pulled out. This insures that the cylinder cannot be engaged with the tip tray partially pulled out, thereby preventing potential damage to the pipettor. Proper operating procedure would be to unseat tips by throwing the switch "down", removing the tip tray, loading new tips, replacing the tip tray and then seating the tips by throwing the switch to the "up" position.

Caution:
Avoid throwing the manual tip load switch when the tip tray is not in place; although nothing will happen because of the interlock, leaving the switch in the “up” position will cause the air cylinder to immediately engage as soon as the interlock is defeated (i.e. by replacing the tip drawer). While this won’t necessarily harm anything, it may be a big surprise to the operator.

1.) It is not recommended that tips other than those recommended by MDC be used in FLIPR. This insures proper seating of the tip on the tip cylinder heads as well as prevents potential damage to the pipettor.

2.) In order to insure proper tip seating there must be at least 80 psi at air input to FLIPR. It is also recommended that the tip air not exceed 90 psi.

3.) Always make sure that the tip load switch is in the "down" position when inserting the removable tip tray into FLIPR.

2.5. Illumination Optics

The purpose of the illumination optics is to simultaneously illuminate all 96 wells of the cell plate in the most efficient manner as possible. In the current version of FLIPR, a series of turn mirrors, a 24 faceted polygon scanner and cylindrical optics do this. The laser light also runs through a shutter which maintains the light exposure on the cells just long enough to include the exposure interval for the camera. This helps to reduce any dye bleaching effect, which could be potentially present. However, for most assays, the power density on the cells does not produce any noticeable photo-bleach effect. If fast camera updates are desired (e.g. less than 2 seconds), the laser shutter may stay "on" during the entire experiment. The software decides if it has enough time to shutter the laser illumination between camera exposures.

It is important to note that with the laser source no excitation filters are required. This would not be the case if a broadband light source were used. Coarse tuning of the vertical tune knob on the back of the laser head chooses different spectral output lines. A dielectric filter in front of the camera provides spectral discrimination of the fluorescent signal from the excitation light. This filter is held by a filter slider which is accessible through a side access door on the right side of the FLIPR enclosure. The filter slider has positions for two optical emission filters. The filters used are specially designed, 2" optics with special blocking characteristics. Consult MDC for the proper filters for any given application. Special filters can be ordered, however if non-standard may have lead times of 6-8 weeks.

In the current implementation, the laser illumination beam profile at the bottom of the cell plate is not perfectly uniform. Variations on the order of 6 –7 % (standard deviation) are typical from well to well. The wide range of illumination geometries required to illuminate the entire microplate surface causes this variation. The processing software takes this non-uniformity into account and spatially corrects the data to remove this gain factor if so desired. This correction is referred to as the "Spatial Uniformity Correction" and is discussed in more detail in Section 3.

2.6. CCD camera

The FLIPR detection scheme is founded on the use of a thermo-electrically cooled CCD camera. The camera is an integrating type detector, in that it must temporally integrate to build up signal to noise ratio. Depending on the light level available (which in turn depends on dye efficiency and laser power) it may be necessary to utilize longer camera exposures such that the measured fluorescent signal is not dominated by detector noise. Usually the user will perform a signal test to determine if the system (exposure time, light source power) is in a useful part of the total optical dynamic range. Usually this will be achieved in a range from 10,000 to 30,000 measured video units as measured by running a signal test (refer to Section 3.7.2) Saturation occurs at 65,536 video units.

The camera controller, located on the bottom shelf of the FLIPR table, houses most of the electronics that run the camera. On the controller are two switches, one for power and the other to turn on the cooler. Both these switches will normally be turned on. The thermostat control dial on the camera controller should not be changed. The camera requires about 5 to 10 minutes to reach a cooled operating temperature, around - 40 degrees C.

Warning: Using the camera before it has reached its cooled temperature will result in noisy data. The camera is at temperature when the green status light indicator is lit. When not cooled the yellow status light indicator is lit.

The camera images the bottom of the plate. The camera format is 512 x 512 pixels which are spread out over a 96 well microtiter plate which equates to about 190 μm resolution at the cell plane. The

FLIPR processing algorithm goes into each individual camera frame and extracts/averages the proper camera pixels to obtain a single fluorescent measurement for each well. Being comprised of silicon, the useful detection range for the camera is primarily between 400 and 900 nm, although the response of the camera at or below 400 nm can be enhanced using a UV coating on the CCD chip.

A 2.0" diameter interference filter is used before the camera to separate out the emission band of the dye being used. This filter is chosen not only to isolate out the emission band but also to reject unwanted excitation light. The most common configuration in FLIPR is use of the 488 nm Argon laser line in conjuction with a 510 to 560 nm bandpass interference filter.

2.7. System Laser

This section is intended only to fill out some specifics with regards to using the laser with FLIPR. For a general tutorial on the operation and/or maintenance of the laser refer to the Coherent I 90-6 laser manual supplied with your system. If there are any questions on the proper operation of the laser, please consult MDC.

The user must determine the optimum light level to run the experiment at. As a general rule of thumb it is better to use no more laser light than is required to reach viable signal levels. Using "Signal Test" and keeping in mind that the camera will saturate at 65,000 counts, the user should choose an optimum light level for his or her assay (usually around 15,000 to 20,000 counts at basal conditions). The light level can be adjusted in three ways: 1.) Adjustment of the laser output power via the laser remote 2.) Adjustment of the camera exposure (integration) time (see experimental setup windows) or 3.) Adjustment of the camera lens F-stop.

We have found that a warm-up time of 30 minutes is plenty for proper laser stability. Since FLIPR requires more time than this for warm-up the operator should be safe by following the "start-up " procedures listed previously. Spatial beam quality is not a big factor for FLIPR, we therefore operate with the laser aperture set to "0" which is full open. Details of these settings are on page 4-21 of the I-90 manual.

After the warm-up period it quite often is advantageous to tune the laser to minimize tube current. This is done by adjusting the vertical and horizontal tilt knobs on the back of the laser to minimize tube current while maintaining optical output. An MDC representative can instruct you on this operation. We would recommend that this be checked daily. Care should be taken not to make huge adjustments while tuning the laser. This is because one of the adjustments is also the wavelength adjustment for the laser. By turning this adjustment far enough, it is possible to detune off onto another optical line (e.g. 456 nm) which has different power output, and may not be optimized for the particular filters being used.

Caution:
When turning off the laser always let the water run a few minutes to allow the tube to cool after the laser has been shut off.

2.8. System Computer

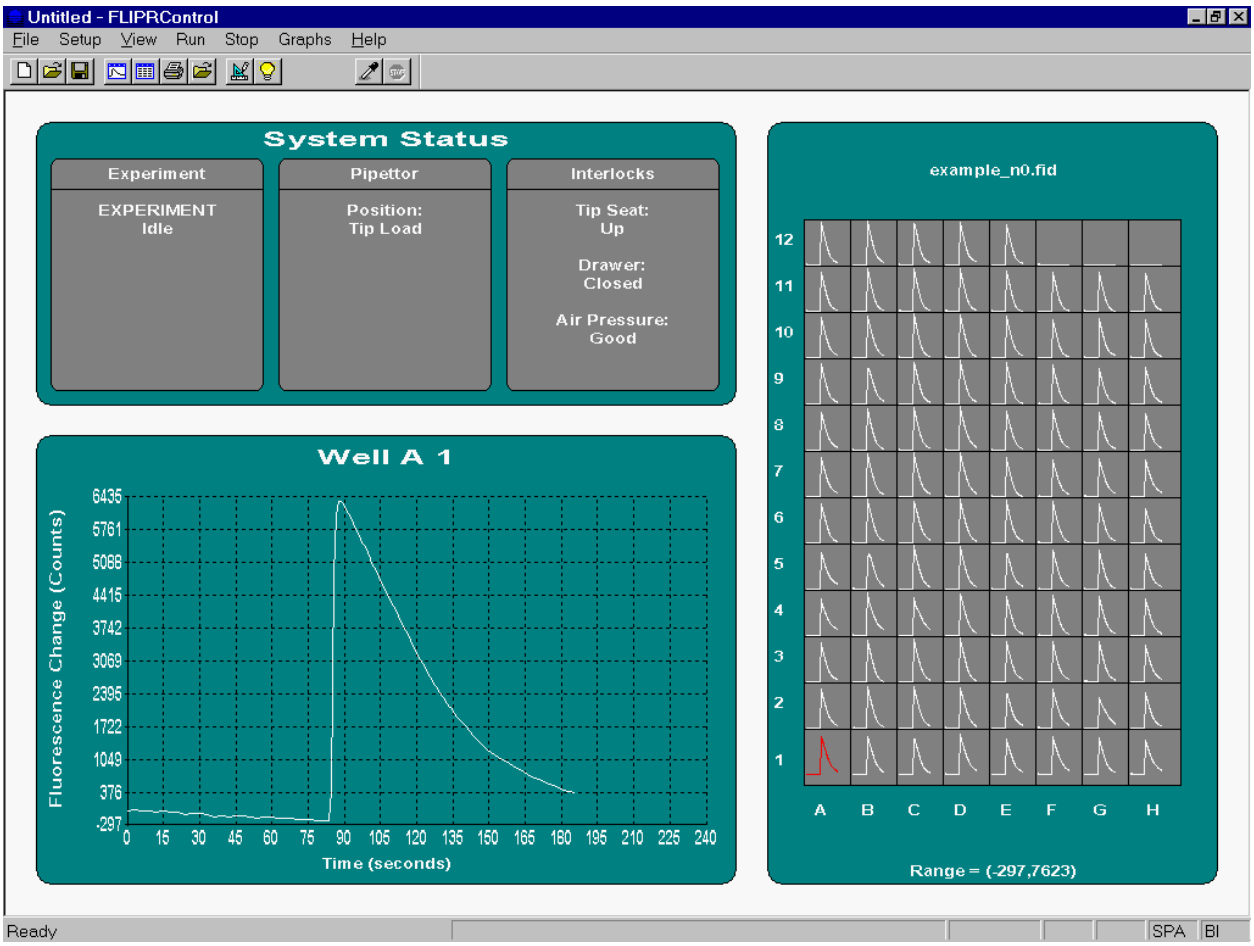
The FLIPR control software runs in the Windows NT 4.0 operating system on Intel processor based computers. The minimum hardware configuration recommended includes a 166 MHz Pentium processor, 64 Mbytes of RAM, a 2 Gbyte hard drive, a graphics card with 2 MB of video memory capable of 1024X768 resolution at 65K colors and a 17 inch color monitor.

Users often require a network connection or data backup device, which require the addition of adapter cards to the FLIPR computer. Several different brands of network and SCSI adapters have been installed and there are currently no known incompatibilities.

3. Software Overview

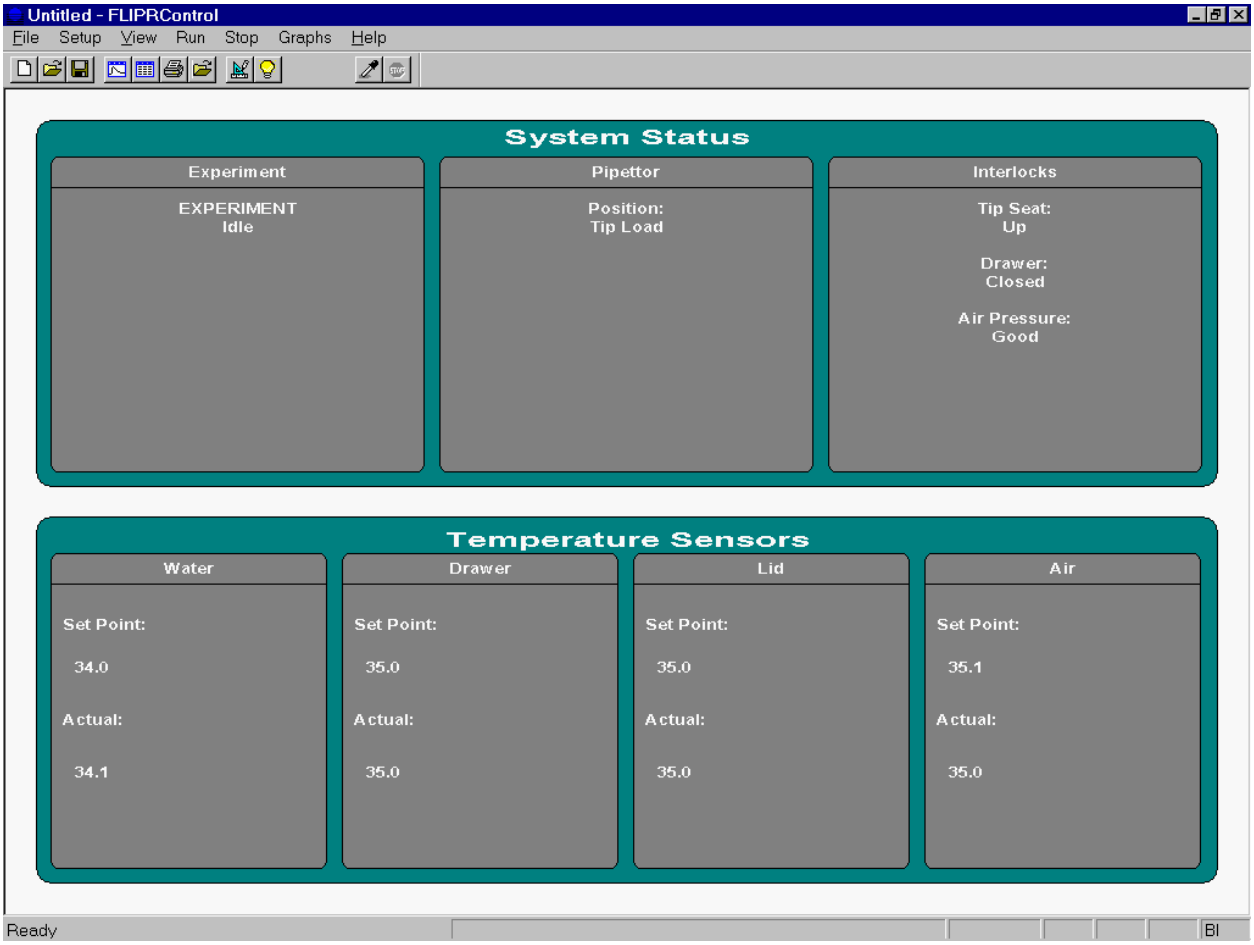
3.1. FLIPR Control Software Start-Up

Before starting the FLIPR control software, the hardware should be powered on by following the procedure described in section 1.4. The control software can then be started by selecting the FLIPR control shortcut in the start menu on the taskbar. Once started, the program window will look as shown below. It is recommended that the program window be maximized during operation so that all indicators are clearly visible.



All FLIPR setup and operations are controlled by making selections from either the menu bar, or the tool bar across the top of the window. In this view, the main program window is divided into three sections. The upper left section, labeled system status provides information describing the current operating parameters. The lower left section plots the signal from user specified wells of the current image data file. The right section shows the signal from all 96 wells of the microplate. Clicking on

any of these small signal plots will select that well into the larger plot in the lower left section of the screen. By choosing the Graphs command from the View menu the user can deselect the chart view and change the main window to the view shown below.



In this view, the screen is divided into two sections with system status at the top and temperature sensor information at the bottom. The following is a brief description of the information that appears in each of the areas on the screen.

System Status Section	Description
Experiment	This area shows the current status of a running experiment, including the type of experiment, and the number of measurements made. On system start-up, no experiment is active so this area indicates that the system is idle.
Pipettor	This area shows the current position of the pipettor. When an experiment is running, it will indicate when fluid additions will be made.
Interlocks	There are three system interlocks that can keep the system from operating. These are on the cell drawer, on the pipettor tip loading mechanism, and on the 80 psi air supply. The cell drawer must be completely in, the pipette tips must be in the loaded position, and the 80 psi air must be on for the system to operate. This area on the screen indicates the status of these interlocks and displays a red error light if any one of the interlocks locks out system operation.

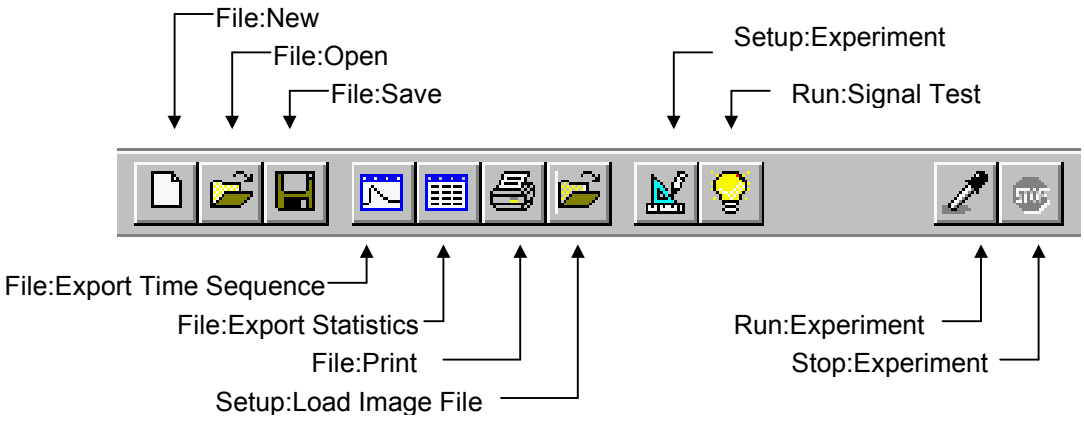
Temperature Sensor Sections	Description
Drawer	This area shows the current temperature and setpoint of the cell drawer. It will also display a red error light if the current temperature is out of limits, which are user settable.
Air	This area shows the current temperature and setpoint of the humidified air entering the cell drawer. It will also display a red error light if the current temperature is out of limits, which are user settable.
Lid	This area shows the current temperature and setpoint of the cell drawer lid. It will also display a red error light if the current temperature is out of limits, which are user settable.
Water	This area shows the current temperature and setpoint of the humidifier water. It will also display a red error light if the current temperature is out of limits, which are user settable. The temperature of the humidifier water is used to control the relative humidity in the cell drawer.

3.1.1. Pull-down Menu Descriptions

This section describes each of the pull down menu options. The entire menu structure for the FLIPR control code is shown below.

- File
  - New Control File
  - Open Control File
  - Save Control File
  - Export
    - Time Sequence
    - Statistics
  - Print
  - Print Preview
  - Print Setup
  - Print Selections
  - Exit
- Setup
  - Load Image File
  - Experiment
  - File Names
  - Control Well Layout
  - Heaters
  - Alignment
  - Open Shutter
- View
  - Toolbar
  - Status Bar
  - Graphs
  - Data Cursor
  - Notes Editor
- Run
  - Experiment
  - Signal Test
  - FID Viewer
  - Plate Viewer
- Stop
  - Experiment
- Graphs
  - Single
  - Overlay
  - Average
  - Setup
  - Increment Group
  - Decrement Group
  - Group
  - Reset Groups
- Help
  - About FLIPR

Several of these functions can also be selected using the toolbar. In this version of the FLIPR program, the toolbar is completely dockable. The user can drag the toolbar to the center of the main window, which will make it a floating toolbar or it can be docked at any edge of the main window. The correspondence between the menu and the toolbar is shown below.



The following tables give a brief explanation of each menu selection.

File Menu

Selection	Description
New Control File	This selection is used to create a new FLIPR control file. When selected, all system parameters will be reset to their default values.
Open Control File	This selection is used to open an existing FLIPR control file. The operator will be presented with the standard windows file open box which can be used to move through the disk directory structure to locate the desired file.
Save Control File	Save the current control file using its current name.
Save Control File As	Save the current control file using a user supplied file name.
Export Time Sequence	This menu item is selected to process FLIPR data and export the resulting time sequence for each well. The output of the processing will be saved in a text file which can be easily read into a spreadsheet program.. For a complete description of data processing, see section 3.4
Export Statistics	This menu item is selected to process FLIPR data and export the resulting single value for each well. The output of the processing will be saved in a text file, which can be easily read into a spreadsheet program. For a complete description of data

	processing, see section 3.5
Print	Selecting this option will print all items chosen in the Print Selections dialog box. These can include, the mini graphs, the main graph, experiment setup parameters and the notes page.
Print Preview	This selection will present the user with a standard print preview window showing the selected pages.
Print Setup	Allows the user to select a printer and options associated with that printer.
Print Selections	Allows the user to select which items will be printed each time a print command is received.
Exit	This option exits the FLIPR code.

Setup Menu

Selection	Description
Load Image File	Allows the user to reload an existing image file for display in the graphs or for data export.
Experiment	This menu selection opens the experiment setup dialog box which is used for entering all parameters which define an experiment. For a complete description of the parameters used to define an experiment see section 3.2.
File Names	The FLIPR code automatically names the .fid files generated when an experiment is run. This menu selection allows the user to configure how the name is created. The name always has the form XXXXXX_n0.fid, where XXXXXX is a base name which can be the current date or a user supplied character string and 0 is a counter which increments each time an experiment is run.
Control Well Layout	Displays a dialog box, which allows the user to input the configuration of positive and negative control wells. These wells will be used in the data processing for displaying signals in the graphs and for exporting data.
Heaters	This menu selection is used to setup the FLIPR temperature control system. The default values for each control section have been factory set to minimize the temperature variation across the sample plate. These values should only be changed if a significantly different operating temperature is required. If temperature control is used, it should be turned on at least 40 minutes before collecting data.
Alignment	This menu selection is used to change the system alignment parameters. Under normal usage, these parameters will only occasionally need to be checked (see section 1.4 for recommended schedule). The proper operation of FLIPR is

	dependent on these values being correctly entered. It is recommended that users trained by MDC only do this procedure. See section 3.7 for a complete description of the alignment process.
Open Shutter	Selecting this option opens the laser shutter illuminating the microplate in the cell chamber. This option is useful when aligning optical components in FLIPR, which should only be done by trained personnel.

View Menu

Selection	Description
Toolbar	Display the FLIPR toolbar.
Status Bar	Display the status bar.
Graphs	This selection will display or hide the data graphs.
Data Cursor	This selection will display a cursor on the main chart and information about the corresponding data point in the status bar at the bottom of the main FLIPR window. The cursor can be moved between data points by clicking the left mouse button on the main chart or by using the left and right arrow keys on the keyboard. The data cursor only works in the single or average graphing modes.
Notes Editor	Display a dialog that allows the user to input notes, which will be appended, to the FID file.

Run Menu

Selection	Description
Experiment	This selection starts an experiment as defined in the experiment setup dialog box.
Signal Test	This selection is used to check the exposure time to ensure that the signal strength measured by FLIPR is reasonable, and should be done before each experiment. For a detailed description of using the signal test, see section 3.7.
Fid Viewer	This selection starts the plate viewing tool. This tool is useful in diagnosing problems such as cell layer non-uniformity, dust, etc. For a complete description on using this tool see section 3.7.
Plate Viewer	This selection starts the plate viewing tool. This tool is useful in diagnosing problems such as cell layer non-uniformity, dust,

	etc. For a complete description on using this tool see section 3.7.

Stop Menu

Selection	Description
Experiment	This selection is used to stop an experiment that is currently running. Data collection will stop and the pipettor will return to the tip loading position.

Graphs Menu

Selection	Description
Single	In this mode, the main graph shows a data curve corresponding to a single well. The displayed well can be changed by clicking with the left mouse button on the desired well in the 96 well mini graphs.
Overlay	In this mode, the main graph shows multiple traces corresponding to individual wells in the microplate. The wells can be defined in up to twelve groups. Clicking or dragging with the left mouse button does selection. Deselecting is done using the right mouse button.
Average	In this mode, the main graph shows multiple traces corresponding to averages of groups of wells in the microplate. Up to twelve groups can be defined. Clicking or dragging with the left mouse button does selection. Deselecting is done using the right mouse button.
Setup	This selection opens the dialog box shown below, which is used for configuring the graphs.
Increment Group	Increment the active group number by one.
Decrement Group	Decrement the active group number by one.
Group	Opens a drop down menu for direct entry of the active group number.
Reset Groups	Deselects all wells from all groups and sets the active group to group 1.

Help Menu

Selection	Description
About FLIPR	This selection displays the current version of code and the most current address and phone number that can be used for technical support.

3.1.2. Status Bar Indicators

The status bar at the bottom of the main FLIPR window is composed of 7 panes as shown below.

From left to right these panes display the following information:

1) Brief description of menu selections
2) Data cursor output. This includes the sample number and time, the bias value that has been subtracted from the plot and the signal delta (change) at the specified point.
3) Indicates the active group number for the graphs.
4) Indicates with "POS" when positive control scaling is enabled on the graphs.
5) Indicates with "NEG" when negative control correction is enabled on the graphs.
6) Indicates with "SPA" when spatial uniformity correction is enabled on the graphs.
7) Indicates with "BI" when bias subtraction is enabled on the graphs.

3.2. Defining an Experiment

To define an experiment, you must choose Experiment from the Setup menu, to display the experiment setup dialog. A typical FLIPR experiment consists of three basic functions, controlling the strength of the fluorescent signal, periodically measuring (sampling) the fluorescence from the microplate and pipetting fluid into the microplate.

The signal strength is controlled within the software by varying the length of each camera exposure, which constitutes a fluorescence sample. The signal strength, or equivalently system sensativity, can also be varied external to the FLIPR software by changing the LASER output power or the f-stop setting of the camera lens. Section 3.7.2 describes the process of selecting an appropriate exposure length.

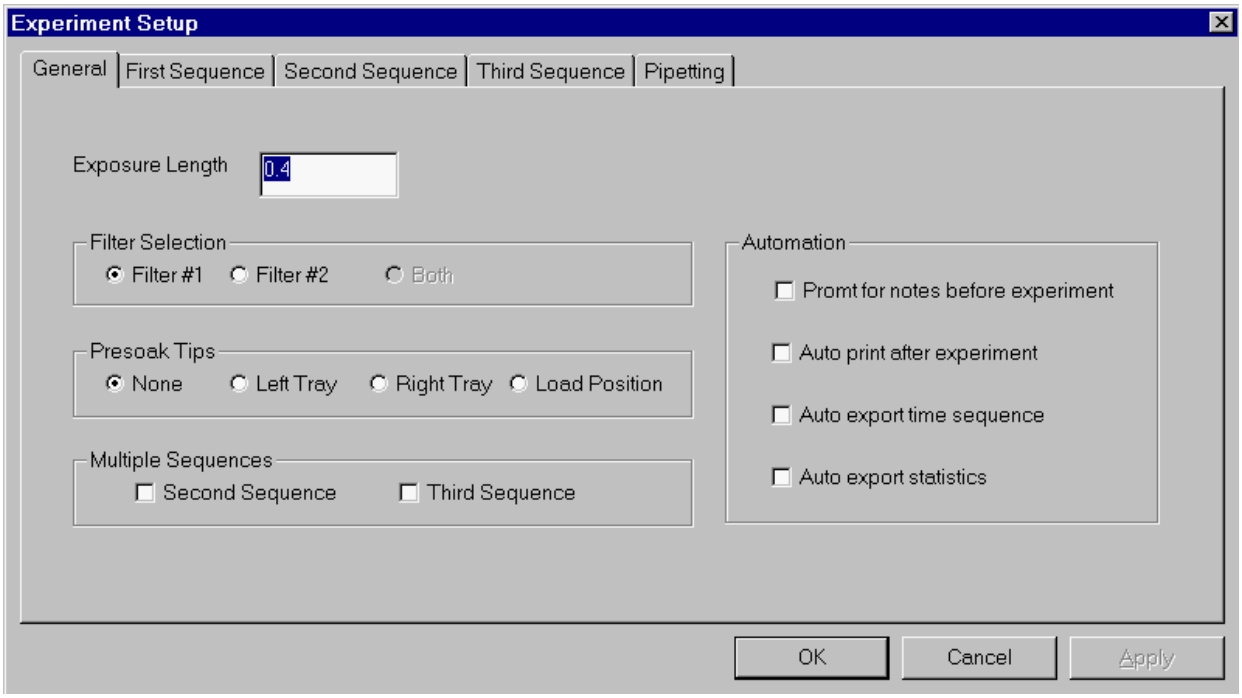
The sampling of the fluorescence is controlled using two basic parameters, a sample interval and a sample count. The interval is the time in seconds between samples and the count is the number of samples taken at the specified interval. A FLIPR experiment can be broken into three sequences. A sequence can be thought of as a sub-experiment, which can contain two different sampling intervals, and one fluid addition. Many, simple experiments may consist of a single sequence with only one sampling interval. In this case, multiple sequences are not selected, and the sample count for the second interval of the first sequence is set to zero. In experiments with multiple sequences, there will be a delay between sequences as the pipettor moves to aspirate fluid and prepare for the following sequence. The FLIPR software keeps track of this time, and appends all of the data from the multiple sequences into a single file with time recorded starting from the beginning of the experiment.

All fluid additions during an experiment are done after a specified sample in a sequence (sub-experiment). Also, parameters in the experiment setup allow the user to specify the fluid volume added, the height of the pipettor, fluid mixing and dispense speed.

The following sections describe the entries in the experiment setup dialog box. Once an experiment has been defined, the parameters can be saved in a FLIPR control file (*.fcf) using the "Save Control File As" and "Save Control File" commands under the File menu.

3.2.1. Experiment Setup Dialog

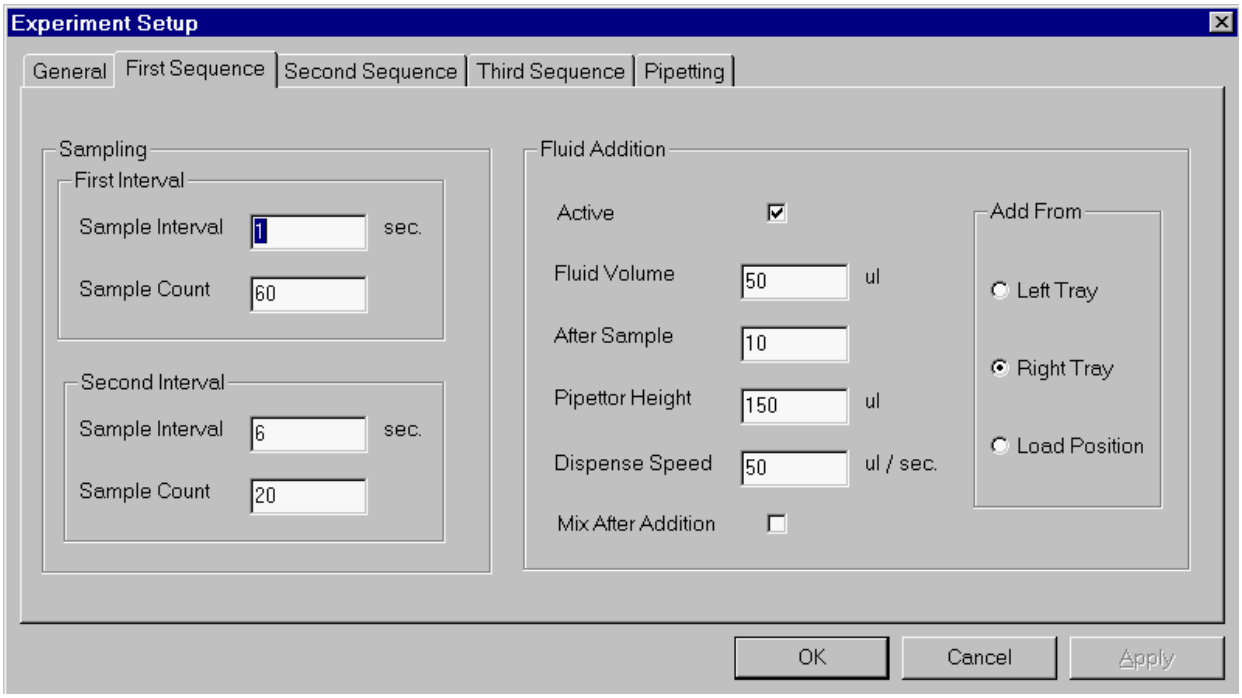
This dialog is separated into five pages labeled General, First Sequence, Second Sequence, Third Sequence and Pipetting. The following tables describe each parameter that can be set in this dialog.



Experiment Setup Dialog General Page

Parameter	Description
Exposure Length	This parameter is the time in seconds that the camera shutter opens to expose the detector. This parameter is chosen using the Run:Signal Test menu selection as described in section 3.7.
Filter#1-Filter#2 Radio Buttons	Selects the filter to be used for the experiment
Presoak Tips Radio Buttons	Selects the type of presoaking done for this experiment. Presoaking will be done by drawing the volume of fluid specified in the first addition from the specified position. The fluid will be held in the tips for one minute and then replaced prior to the start of the experiment.
Multiple Sequence Check Boxes	Checking these boxes makes the specified sequences active. The first sequence is always active.
Prompt for Notes before experiment	If selected, the user will be prompted for experiment notes before the experiment begins.
Auto print after experiement	Pages specified in the Print Selections dialog box will be printed immediately at the completion of the experiment.

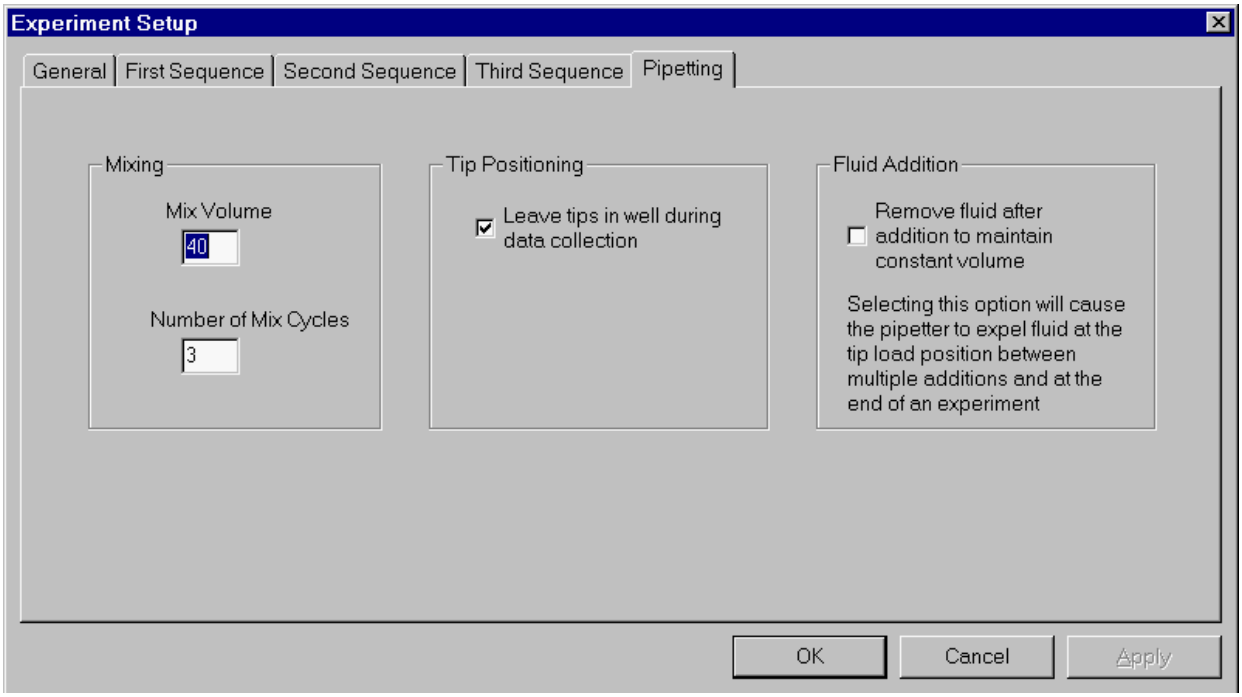
Auto export time sequence	A time sequence will be exported upon completion of the experiment.
Auto export statistics	Statistics will be exported upon completion of the experiment



Experiment Setup Dialog Sequence #1, #2 and #3 Pages

Parameter	Description
Sample Interval	The time in seconds between optical measurements. This parameter must be greater than the exposure length by 0.575 seconds.
Sample Count	The number of samples collected at the corresponding interval. The first interval must contain at least one sample, but the second can be zero.
Fluid Addition Active	This check box specifies whether an addition will be done. If it is not checked, the addition will not be done, and the other fluid addition parameters are irrelevant.
Add From Radio Buttons	This radio button selection specifies which tray the fluid addition will be made from. The fluid addition is always made to the center position.
Fluid Volume	This parameter specifies the amount of fluid in ul moved from the addition tray to the center.
After Sample	This parameter specifies when the addition will be performed.
Pipettor Height	This is the approximate height of the tips in the cell plate

	measured in ul of fluid. This height only applies to pipetting done in the cell plate. All pipetting in the addition plates is done at a height of approximately 50 ul.
Dispense Speed	This is the speed in ul/s at which all dispenses and aspirations are done in the cell plate. Any pipetting done in the addition plates is done at a fixed rate.
Mix After Addition	If selected, the mixes defined on the pipetting page will be done.



Experiment Setup Dialog Pipetting

Parameter	Description
Mix Volume	Volume in ul apirated and dispensed by the pipettor for each mix cycle.
Number of Mix Cycles	Specifies how many aspirate and dispense cycles will be done each time a mix is conducted.
Leave tips in well during data collection	During experiments, which have short sampling intervals (less than 5 sec.), artifacts may be seen due to the pipettor motions during sampling. This can be minimized by leaving the tips stationary in the well during a run. If this option is selected, the height of the tips should be set such that they are above the initial volume in the cell plate but below the final (after experiment addition) volume. This ensures that fluid will not leach out of the tips before the time specified for the addition,

	and that a drop of fluid will not remain on the tips.
Remove fluid after addition to maintain constant volume	In experiments which use a dye in solution with the cell samples (such as DiBac), artifacts can be seen in experiments due to changing the fluid level in the cell plate. Adding solutions, mixing and then removing fluid to maintain a constant fluid volume can minimize this. Note the warning in the dialog box. When this option is selected, there will be fluid in the tips after each addition. The pipettor is programmed to dump this fluid at the tip load position before another addition or at the end of an experiment.

3.2.2. Saving Your Experiment Setup

All of the parameters in your experiment setup can be saved in a FLIPR control file (*.fcf), by using the options under the File menu. In addition to the experiment setup, almost any parameter entered into FLIPR dialogs is saved. This includes graphing parameters, data processing and export parameters. The name of the currently active fcf file is visible in upper left corner of the main FLIPR window. At startup, default parameters are loaded and the name is "Untitled". The standard Windows Save and Save As (in this case Save Control File, and Save Control File As) functions can be used to save parameters with the current fcf file name or with a user specified fcf file name.

3.3. Running an Experiment

To run the current experiment, simply select experiment from the run menu. The software will generate a new fid file name, run the experiment and save the acquired data in the fid file. At any time during an experiment, the user can select stop experiment. Data collection will be terminated, and if the pipettor has fluid in its tips, it will return the fluid and then move to the tip load position. Any automation options (see the following section), which were to be executed upon experiment completion are cancelled, but data already collected is saved to disk.

3.3.1. Experiment Automation

To help streamline the process of running many plates, the experiment setup includes several "automation" options, these are: prompt for notes before experiment, auto print after experiment, auto export time sequence, and auto export statistics. To maximize the usefulness of these automation features, many other parameters must be wisely selected.

For example, if the auto print after experiment option is selected, data will be sent to the default Windows printer upon experiment completion. The print selections menu should be used to define which pages to print, graph scales should be set appropriately (or auto-scaling can be used), and wells of interest should be selected into the main graph (assuming the main graph is to be printed), all before the experiment is run.

When using the auto export options, the appropriate exporting parameters should be set in the export dialogs. This must be done by manually exporting a file (by choosing the export time sequence or statistic menu option). Remember, all experiment setup, exporting and graphing parameters are saved in fcf files, so once all of the parameters are properly defined, choose "save control file as" to save the configuration.

3.3.2. The Notes Editor

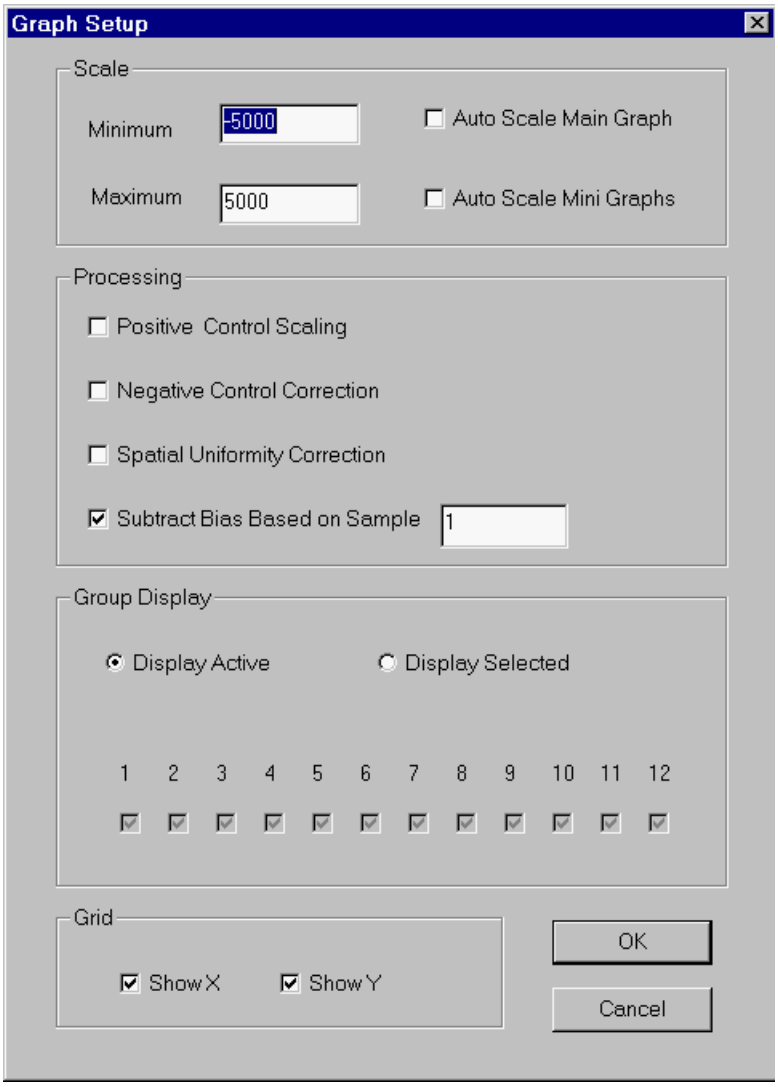
The software allows the user to enter notes that will be saved with an FID file. Select Notes Editor from the view menu to view and/or edit the notes associated with the current FID file. Selecting the "prompt for notes" automation option in the experiment setup dialog box will present the operator with this dialog each time start experiment is selected. The notes will be printed each time the print option is selected if the appropriate box is checked in the print selections dialog.

3.4. Data Graphs

There are two areas of the FLIPR main window that show graphical information about the fluorescence of each well. These are the main graph in the lower left portion of the screen and the mini graphs in the right portion of the screen. Many parameters can be set to configure these graphs and all are accessible through the graph menu selection.

There are three different types of graphs that can be displayed in the main graph area. These are single, overlay and average. When the single graph type is selected, a single trace corresponding to the fluorescence signal from a single well is displayed. The displayed well can be changed by left clicking on the desired well in the mini graphs. In overlay mode, groups of wells are displayed simultaneously in the main graph, were each trace corresponds to the fluorescence signal from a single well. In average mode, multiple traces can be shown, but each trace will correspond to an average of a group of wells.

There are many processing and scaling options that can be specified for any of the three graph types. Selecting Setup from the Graphs menu, which displays the dialog box shown below, accesses these.



Graph Setup Dialog

Parameter	Description
Minimum	Sets the minimum value used for plotting in both the main plot and the mini graphs when auto-scaling is not activated.
Maximum	Sets the maximum value used for plotting in both the main plot and the mini graphs when auto-scaling is not activated.
Auto Scale Main Graph	If selected, the main plot will automatically scale to the range required to view all of the signal. If it is not selected, the graph will be clipped at the minimum and maximum values as described above.
Auto Scale Mini Graphs	If selected, the mini graphs will automatically scale to the range required to view all of the signals. If it is not selected, the graphs will be clipped at the minimum and maximum values as described above.

Positive Control Scaling	If selected, data is scaled such that the average of the selected positive control wells is 100%. See section 3.5 for more information on data processing.
Negative Control Scaling	If selected, the average change of the selected negative control wells is removed as a percentage from all wells. See section 3.5 for more information on data processing.
Spatial Uniformity Correction	If selected, the signal from each well is scaled by a constant that makes the starting value equal to the mean starting value of all wells on the plate. See section 3.5 for more information on data processing.
Subtract bias based on sample	The value at the specified sample is subtracted from the time sequence for each well. See section 3.5 for more information on data processing.
Display Active/ Selected Radio Buttons	If display active is chosen, only the currently active group of wells is display on the main graph. When display selected is chosen, any group that is checked will be displayed.
Show X	Display a grid for the time axis of the large graph.
Show Y	Display a grid for the fluorescence axis of the large graph.

When displaying data in overlay and average graphs it is often useful to arrange wells into groups. The FLIPR software currently allows the definition of twelve groups. When the user first changes a graph to an overlay or average, group 1 will automatically become active as can be seen in the status bar. Wells can be selected into the active group by clicking or dragging with the left mouse button. Wells are deselected from the group by clicking or dragging with the right mouse button. The active group number can be changed in a variety of ways. These include, up/down arrow keys, choosing increment or decrement group in the graphs menu or direct selection in the graphs group menu. After the group number has been changed, wells can be selected or deselected into the new group as before. By default only the active group is displayed in the main graph. To display multiple groups, the user must choose the Display Selected Radio button in the graph setup dialog. This will enable the twelve check boxes to select which groups should be displayed. In this mode, the default is to display all groups, however no trace will be shown for a group which contains no selected wells.

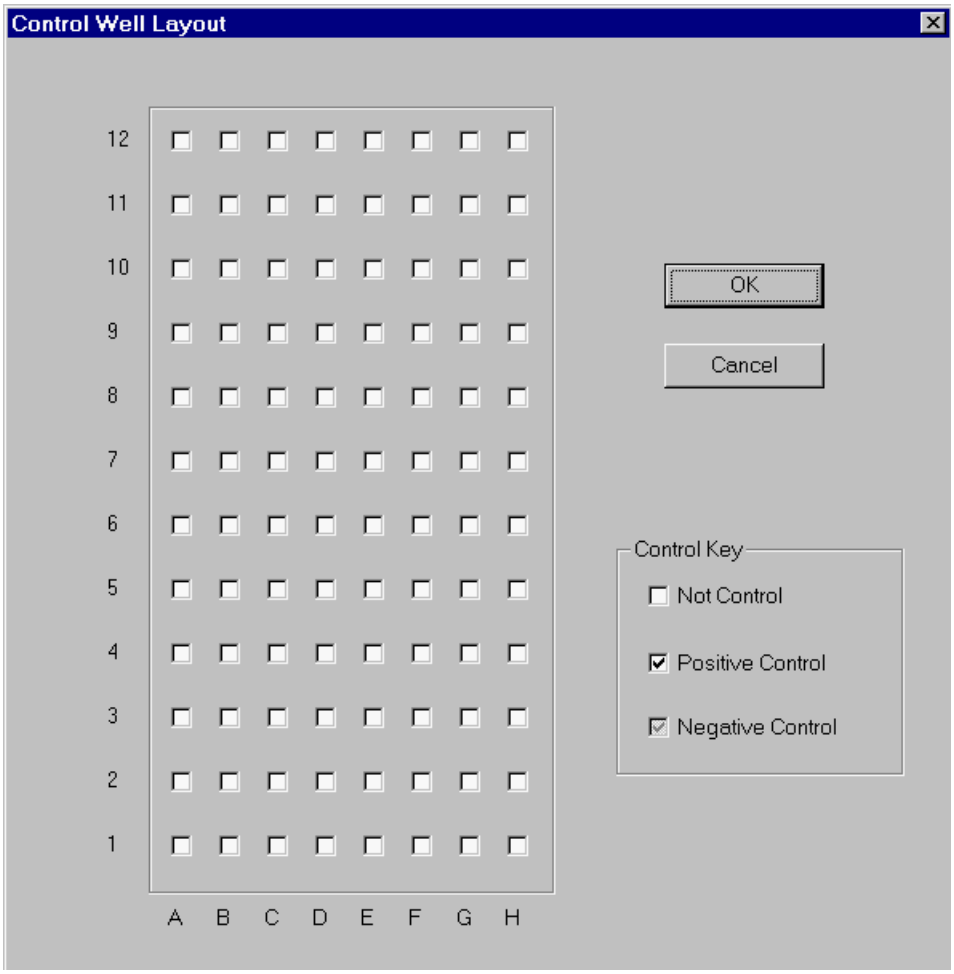
Another useful tool when analyzing data displayed in the graphs is the data cursor. By selecting Data Cursor from the View menu, a cross-hair cursor will be shown on the main chart. This cursor can be moved between data points by clicking the left mouse button with the cursor in the main plot window, or by using the left and right arrow keys on the keyboard. When the cursor is visible, data corresponding to the selected point will be displayed in the status bar at the bottom of the main window. This data includes the time since the start of data collection, the bias fluorescence value subtracted from the sequence, and the signal change (delta) from the bias value. Any processing options selected in the graph setup dialog will be reflected in the displayed data. To avoid confusion about which well is being analyzed, the data cursor is only available when in the graph is a single or average.

3.5. Processing Data

After a data set has been gathered using Run:Experiment, it must be processed and saved in a spreadsheet readable form. This is done with the File:Export menu selection, which has sub-menu choices for outputting a time sequence or statistics.

3.5.1. Defining Control Wells

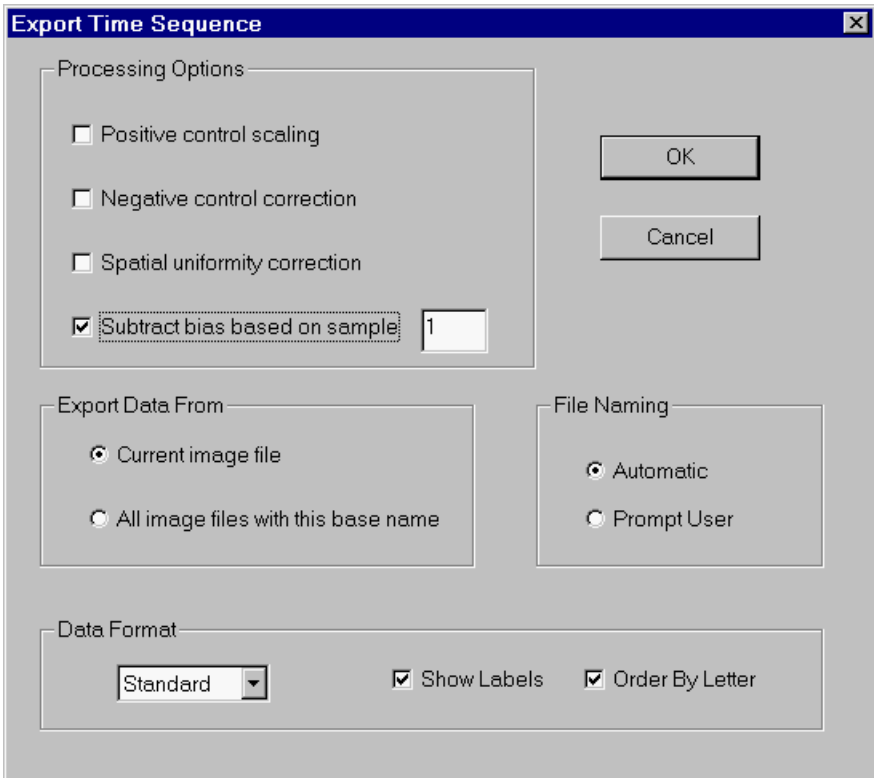
Wells to be used during processing for positive and negative controls should be selected before exporting data by selecting Setup:Control Well Layout. This will display the dialog box shown below.



The box for each well is a tri-state check box. Clicking once will change the state to a check, indicating that the well is to be treated as a positive control. A second click will change the state to a grayed check indicating a negative control. The controls defined in this dialog, will be used in any subsequent data export or data graphing if the positive and negative control options are selected.

3.5.2. Time Sequence Output

Selecting File:Export Time Sequence presents the user with the following dialog box. Several processing options can be selected using the check boxes on the left side of the dialog box. These options are described below.



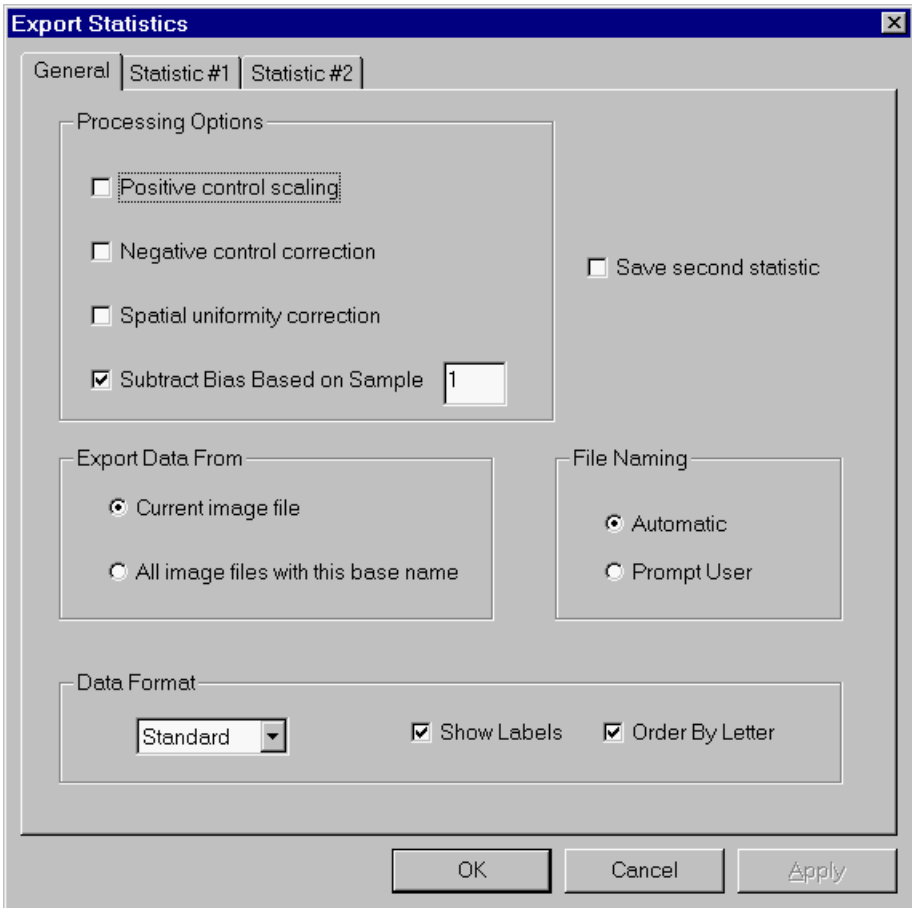
Export Time Sequence Dialog

Option	Description
Scale By Positive Controls	If this option is selected, the algorithm scales all signal changes so that the positive control changes average to 100. This makes plate to plate comparisons less dependent on factors such as cell density.
Time Correct with Negative Controls	If selected, any time fluctuation which is present in the averaged control wells is removed from all wells. This minimizes any global effects such as laser power or temperature drifts.
Spatial Uniformity Correction	This processing algorithm normalizes the signal in each well using the wells initial value to remove variations due to non-uniform laser illumination and well to well variations in cell density or dye loading.
Subtract Bias Value	When selected, the value at the specified sample is subtracted from all time points in the sequence from each well. This has the effect of forcing all graphs to zero at the specified sample,

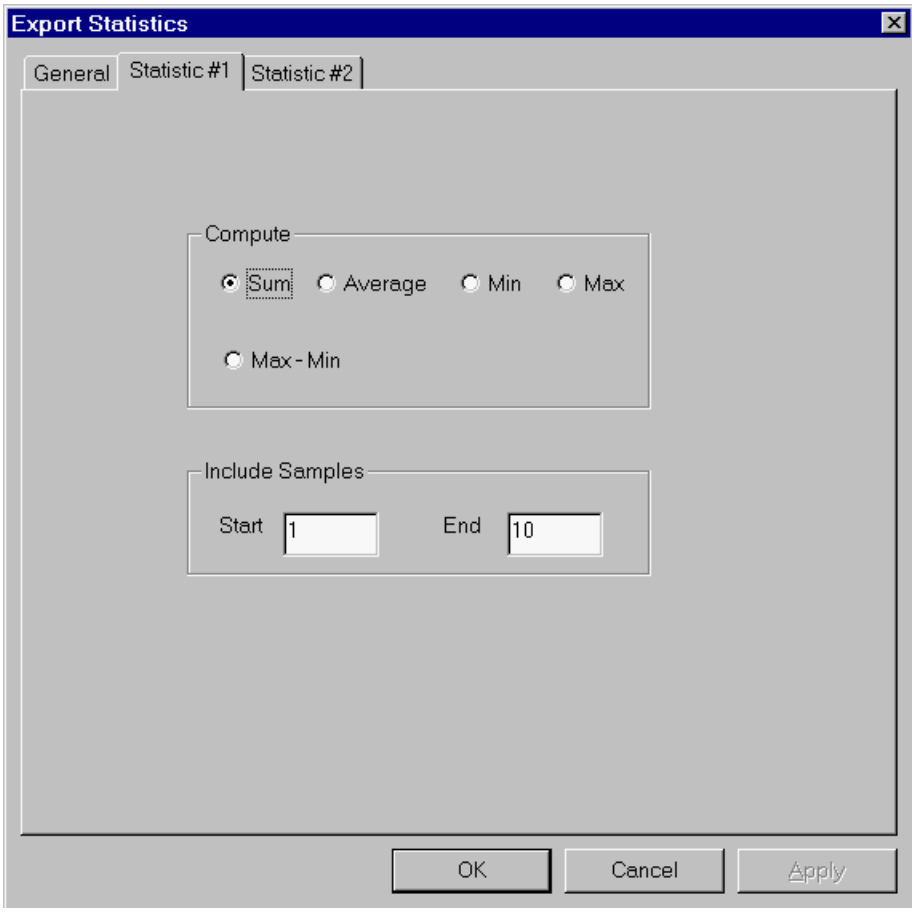
	which can make well to well comparisons easier.
Current Image / All Image Files Radio Buttons	When All Image files are selected, the FLIPR software will search for files (starting at number zero) with the same base name as the current file. All files will be processed into a single output file. If the automatic naming feature is used (as described below) the name of the resulting file will be basename_mult.squ.
Automatic / Prompt User Radio Buttons	If automatic naming is selected, a file will be generated with a name in the form of basename_nX.squ, where X is the number of the FID file. The basename used will be the same as the FID file regardless of the settings in the file names dialog.
Data Format	Currently only the Standard format is available.
Show Labels	When selected, the output file will contain information about the processing options selected, the name of the FID file and well labels.
Order By Letter	If checked, the columns in the output file will be ordered A1,A2,A3…, otherwise, the order will be A1,B1,C1…

3.5.3. Statistics Output

Selecting File:Export Statistics presents the user with the following dialog box.



The processing options are the same as described above for exporting a time sequence. Exporting statistics differs in that a single value is written for each well (actually two values are written if save second statistic is checked). As seen in the dialog page below, this value can be a sum, average, minimum or maximum of a series of samples from the corresponding time sequence. When automatic naming is used, the output files will have names of the form basename_nX.stat and basename_nX_stat2.stat. When all files with the same basename are processed, the output files will be basename_mult.stat and basname_mult_stat2.stat.



3.5.4. Understanding FLIPR Data Processing Algorithms

There are four basic processing options incorporated in the FLIPR software. Listed in the order they are performed, these are:

1) Spatial Uniformity Correction

2) Negative Control Correction

3) Positive Control Scaling

4) Subtract Bias Value

The following paragraphs explain each of these options in detail. In the descriptions, the term "Δf" is used to denote changes in fluorescence which are produced by a stimulus which is part of a FLIPR experiment. The term "initial fluorescence" implies the absolute value of fluorescence measured by FLIPR at the first sample in an experiment (before the application of a stimulus).

3.5.4.1.Spatial Uniformity Correction

The spatial uniformity correction is designed to minimize well to well variations in measured Δf. These variations may be due to non-uniform laser illumination, cell density variations, or any other

effect which produces a change in the initial fluorescence of a well which is proportional to Δf. A spatial correction factor is computed for each well according to the formula:

Well X Correction Factor = (96 well average of initial fluorescence) / (Well X initial fluorescence)

Each time point in the data sequence for a well is multiplied by the correction factor to produce the output sequence.

Output[n] = (Well X Correction Factor) * Input[n]

where n is an integer index corresponding to the sample number and Input[n] is the fluorescence signal sequence for well X.

This spatial correction algorithm can be thought of as separate automatic gain functions for each well, which force the initial fluorescence of all wells to equal the average. This correction works best when all samples in the microplate are prepared the same. For example, if several wells in a plate are empty, the low fluorescence in these wells will bring down the 96 well average. Also, the correction factor will be very large for these wells. Think of it as turning up the auto gain on these wells until the noise is equivalent in strength to the plate average initial fluorescence. Obviously, the signal in these wells will look very poor after this correction is applied.

The correction also works best when the initial fluorescence of the samples are much larger than the background fluorescence from the plate and buffer. This is because a component of the initial fluorescence from the plate and buffer will not produce a corresponding Δf signal when the experiment is conducted.

3.5.4.2.Negative Control Correction

This option was designed to remove fluctuations in fluorescence which are shared by all wells and can cause errors in interpreting Δf signals. Examples of such fluctuations include, cells which leak dye producing a steady drift of signal levels throughout an experiment, or fluid addition artifacts such as dilution of dye in solution with the cells.

A single negative correction time sequence which will be applied to all wells in the plate is calculated using the following steps. First, the fluorescence time sequences from the wells which the user has identified as negative controls are averaged into a single sequence. Next, each value in the resulting sequence (AverageNegative[n]) is converted to a correction factor using the formula:

NegativeCorr[n] = AverageNegative[1] / AverageNegative[n]

This sequence and the fluorescence signal sequence from each well are then multiplied element by element to produce the output time sequence.

Output[n] = NegativeCorr[n] * Input[n]

3.5.4.3.Positive Control Scaling

The purpose of positive control scaling is to increase the consistency of Δf measurements which are made on different plates with possibly different instrument settings. The measured value of Δf is a function of many variables including laser power, exposure length, cell density, dye type, loading

parameters etc. The value is only truly meaningful when it is in some sense calibrated. This calibration could be a full conversion from fluorescence change to the absolute quantity which the fluorescence change is reporting such as intracellular calcium, membrane potential etc. However, in many instances this full calibration requires collection of additional data which complicates experiment protocols. When the instrument is used in screening, this full calibration is almost always unnecessary. Positive control scaling serves as a simple calibration scheme by reporting each Δf as a percentage of the Δf measured in the positive control wells. A typical positive control would be a response to a fixed concentration of a known agonist.

As in the negative control processing an average time sequence is computed by averaging the signals from the user specified positive control wells. The maximum deviation from the initial value is found in this average time sequence and a scaling parameter is computed using the formula

Positive Scale Factor = 100/(Maximum Positive Control Deviation)

The fluorescence sequence of each well is multiplied by this factor to produce the output sequence.

Output[n] = (Positive Scale Factor) * Input[n]

3.5.4.4.Subtract Bias Value

A constant equal to the value of the fluorescence sequence at the user specified sample is subtracted from each well. This forces all wells to zero at the specified sample and makes well to well comparisons much simpler. In practice, the bias value subtracted is usually based on the first sample of an experiment, which is before any fluid addition has been made. However, in experiments which have signal artifacts due to the fluid addition, it is often useful to subtract a bias based on a sample taken shortly after the addition.

3.5.4.5.Example Computation

To aid in the understanding of the FLIPR data processing, it is helpful to follow several fluorescence sequences through each step. Assume that A1[n], B1[n], and C1[n] are the fluorescence time sequences collected from three wells in a microplate during a FLIPR experiment. In this example, n is the index of each sample, and will run from 1 to 10. We will also assume that well A1 is a positive control and well B1 is a negative control, and that the user has chosen all of the processing options. The data simulates an increase in fluorescence which is initiated by a fluid addition after the third sample.

Assume that the unprocessed sequences are,

A1[n] = (20100, 20050, 20000, 22300, 26500, 27200, 26000, 25000, 24000, 22000)

B1[n] = (23000, 22950, 22900, 22850, 22800, 22750, 22700, 22650, 22600, 22550)

C1[n] = (19500, 19450, 19400, 20300, 22700, 23100, 22500,
22000, 21100, 20000)

For simplicity, all values in this example will be rounded to integers. However, you should be aware that in the FLIPR software each is treated as a floating point value. We will assume that the 96 well average of the initial values is equivalent to the wells shown which is 20867. The results of the spatial uniformity correction are:

Correction factor for A1 = 1.03816
Correction factor for B1 = 0.90726

Correction factor for C1 = 1.07010

A1[n] = (20867, 20815, 20763, 23151, 27511, 28238, 26992, 25954, 24916, 22840)

B1[n] = (20867, 20822, 20776, 20731, 20686, 20640, 20595, 20549, 20504, 20459)

C1[n] = (20867, 20814, 20760, 21723, 24291, 24720, 24077, 23542, 22579, 21402)

The next step in the processing is negative control correction. The results of the computations are:

NegativeCorr[n] = (1.00000, 1.00218, 1.00436, 1.00656, 1.00877, 1.01099, 1.01321, 1.01545, 1.01770, 1.01995)

A1[n] = (20867, 20860, 20854, 23303, 27753, 28548, 27349, 26355,25357, 23295)

B1[n] = (20867, 20867, 20867, 20867, 20867, 20867, 20867, 20867, 20867, 20867)

C1[n] = (20867, 20859, 20851, 21866, 24504, 24991, 24395, 23906, 22979, 21829)

The results of the positive control scale are as follows:

Positive Scale Factor = 0.013019

A1[n] = (272, 272, 271, 303, 361, 372, 356, 343, 330, 303)

B1[n] = (272, 272, 272, 272, 272, 272, 272, 272, 272, 272)

C1[n] = (272, 272, 271, 285, 319, 325, 318, 311, 299, 284)

And finally the results of subtracting the bias value (assuming the user has selected sample 1 as the basis) is:

A1[n] = (0, 0 , 0, 32, 90, 100, 84, 71, 58, 32)

B1[n] = (0, 0, 0, 0, 0, 0, 0, 0, 0, 0)

C1[n] = (0, 0, 0, 13, 47, 54, 46, 40, 27, 13)

The processing has produced three time sequences that are much easier to interpret than the originals. Note that the positive control well maximum Δf is forced to 100. This will only be true when a single positive control well is used in the processing. If multiple wells are used, the average Δf of these wells will be 100 but no individual Δf is guaranteed to be 100. Second, the negative control is forced to 0 at all times. Again, this is only true if a single negative control well is used. An finally, well C1 is readily seen to produce a Δf which is 54% of the Δf produced by the positive control.

3.6. Analyzing Data

The FLIPR software does not include any functions specifically for data analysis. The built in data charting in FLIPR is meant primarily as a real time feedback to insure proper system operation and was not intended to be used for serious data analysis. Most data analysis should be done on data which is processed using the File:Export menu selection. Since each assay may require slightly

different analyses, it is recommend that users develop spreadsheet tools for plotting, computing statistics and flagging data of interest from the FLIPR collected and exported data.

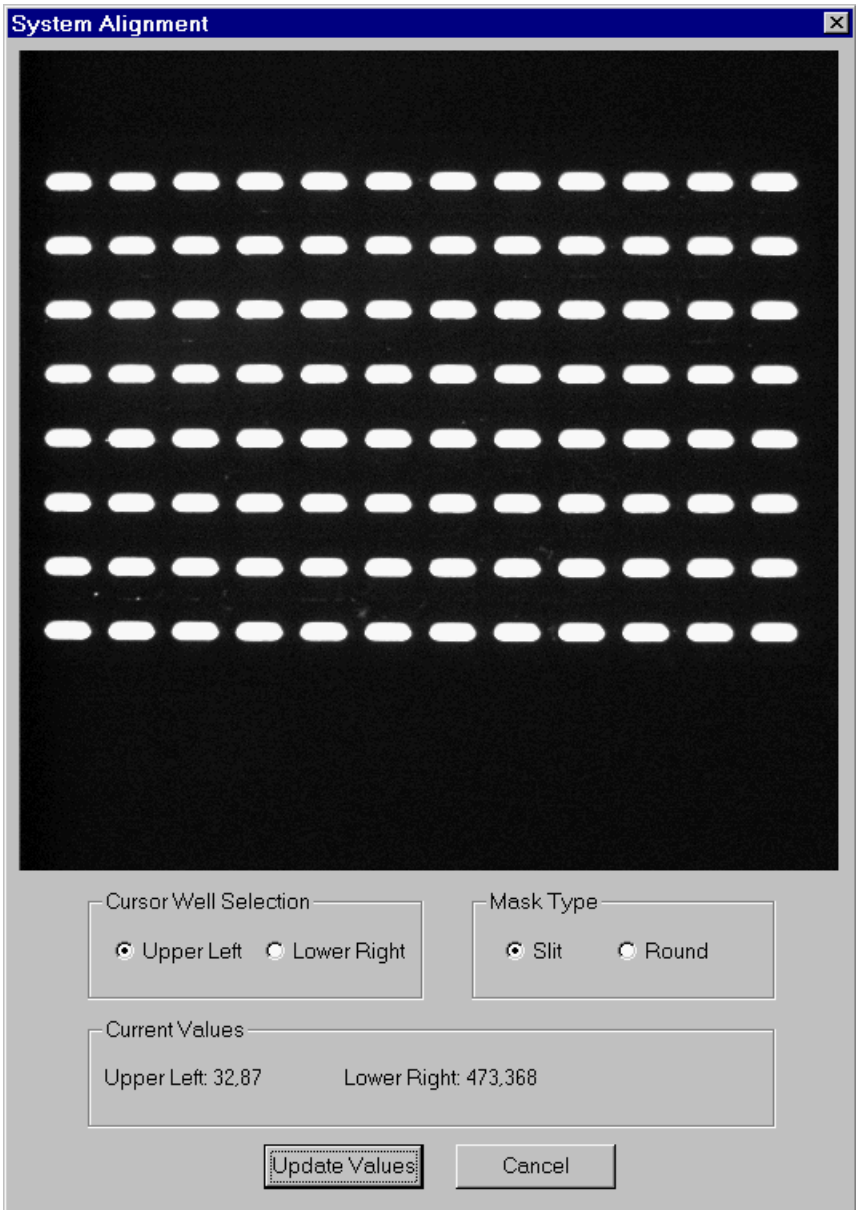
3.7. Diagnostic Tools

Several tools available from the FLIPR menus are used when setting up and conducting an experiment to insure that FLIPR is operating properly. These include Setup:Alignment, Run:Signal Test, Run:Plate Viewer and Run:FID Viewer.

3.7.1. Checking System Alignment

For proper operation of the FLIPR system, accurate information regarding the alignment of the imaging sensor is required. This alignment data is input using the tools available under the Setup:Alignment menu selection which displays the following dialog.

For all fluorescence-based measurements, the slit mask should be selected. Before choosing Setup:Alignment, a test plate should be placed in the sample plate position. After selecting Setup:Alignment, the alignment setup dialog should appear with an image of the bottom of the plate as shown below. The system alignment is specified by two points, one on the upper-left well and one on the lower-right well. To specify a point, select upper left or lower right with the radio button selection. Then move the cursor to the center of the bottom edge of the appropriate well and click the left mouse button. The new coordinates for that well will be displayed in the dialog box. Repeat this procedure for the other well and press update values to save these new values or cancel to keep the old values.



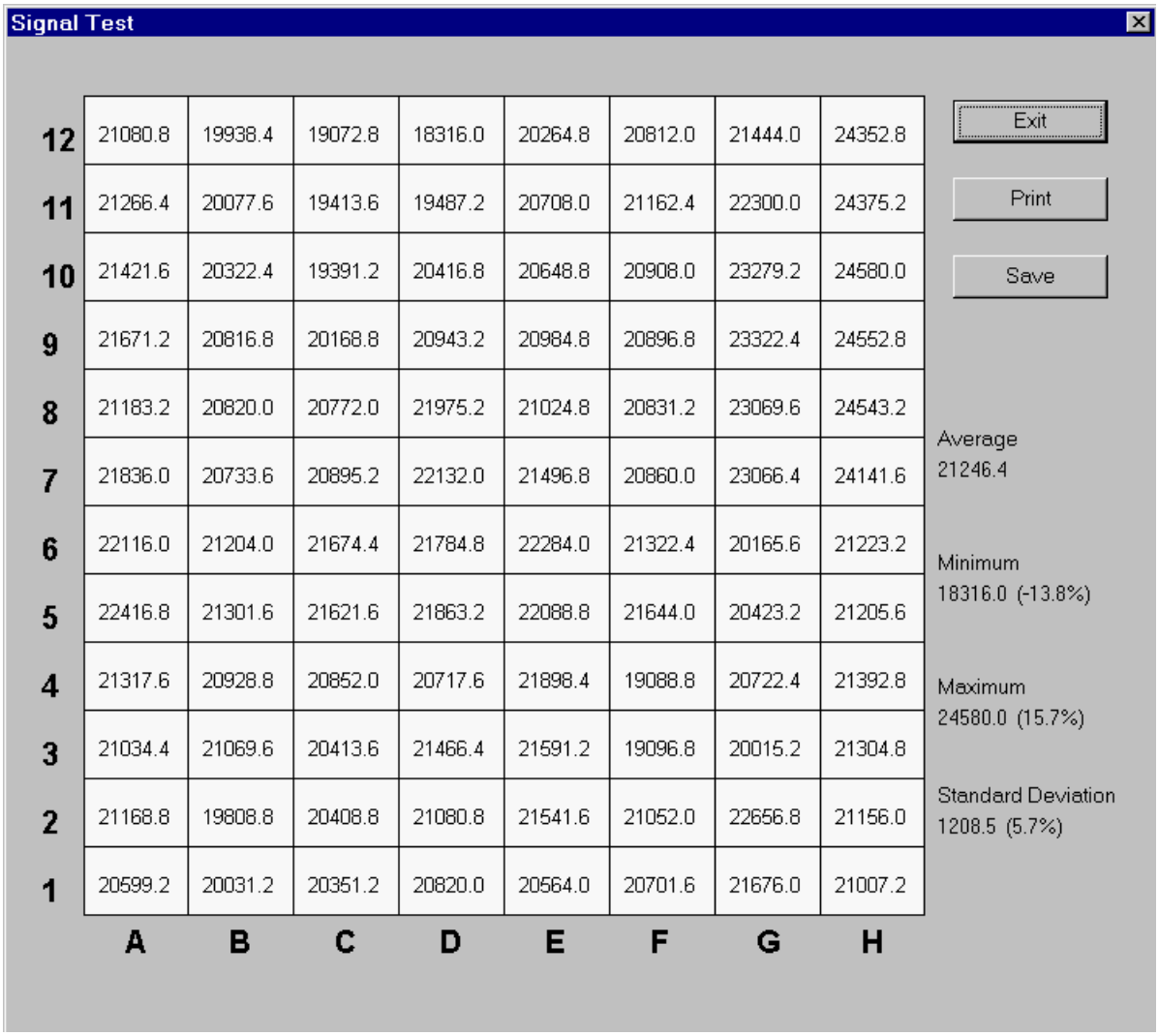
The proper operation of FLIPR is dependent on these values being correctly entered. It is recommended that this procedure only be done by users trained to do so.

When using FLIPR for making luminescence measurements with a round holed mask, the alignment procedure varies only slightly from what is described above. The round mask type should be selected, and when entering upper left and lower right wells, the center of the circular well should be clicked.

3.7.2. Checking Signal Levels

The Run:Signal Test menu selection is used to check the exposure time to ensure that the signal strength measured by FLIPR is reasonable. This check should be done before each experiment, after loading the sample and addition plates into FLIPR, and setting up the experiment (most importantly exposure length). After making an exposure, the statistics dialog will be displayed as shown below.

The dialog shows the signal level for each well and the minimum, maximum, average and standard deviation of all wells. The minimum, maximum and standard deviation are also given as a percentage of the average. If possible, it is desirable to have the maximum between 10,000 and 30,000 counts, and the minimum no lower than 5,000 counts before starting an experiment. If the values displayed in the dialog are not close to these ranges, exit the dialog and change the exposure (Setup:Experiment) using the knowledge that the number of counts is very nearly proportional to the exposure time and laser power. After resetting the exposure time or laser power select Run:Signal Test again to verify that values are within range.



	A	B	C	D	E	F	G	H
12	21080.8	19938.4	19072.8	18316.0	20264.8	20812.0	21444.0	24352.8
11	21266.4	20077.6	19413.6	19487.2	20708.0	21162.4	22300.0	24375.2
10	21421.6	20322.4	19391.2	20416.8	20648.8	20908.0	23279.2	24580.0
9	21671.2	20816.8	20168.8	20943.2	20984.8	20896.8	23322.4	24552.8
8	21183.2	20820.0	20772.0	21975.2	21024.8	20831.2	23069.6	24543.2
7	21836.0	20733.6	20895.2	22132.0	21496.8	20860.0	23066.4	24141.6
6	22116.0	21204.0	21674.4	21784.8	22284.0	21322.4	20165.6	21223.2
5	22416.8	21301.6	21621.6	21863.2	22088.8	21644.0	20423.2	21205.6
4	21317.6	20928.8	20852.0	20717.6	21898.4	19088.8	20722.4	21392.8
3	21034.4	21069.6	20413.6	21466.4	21591.2	19096.8	20015.2	21304.8
2	21168.8	19808.8	20408.8	21080.8	21541.6	21052.0	22656.8	21156.0
1	20599.2	20031.2	20351.2	20820.0	20564.0	20701.6	21676.0	21007.2

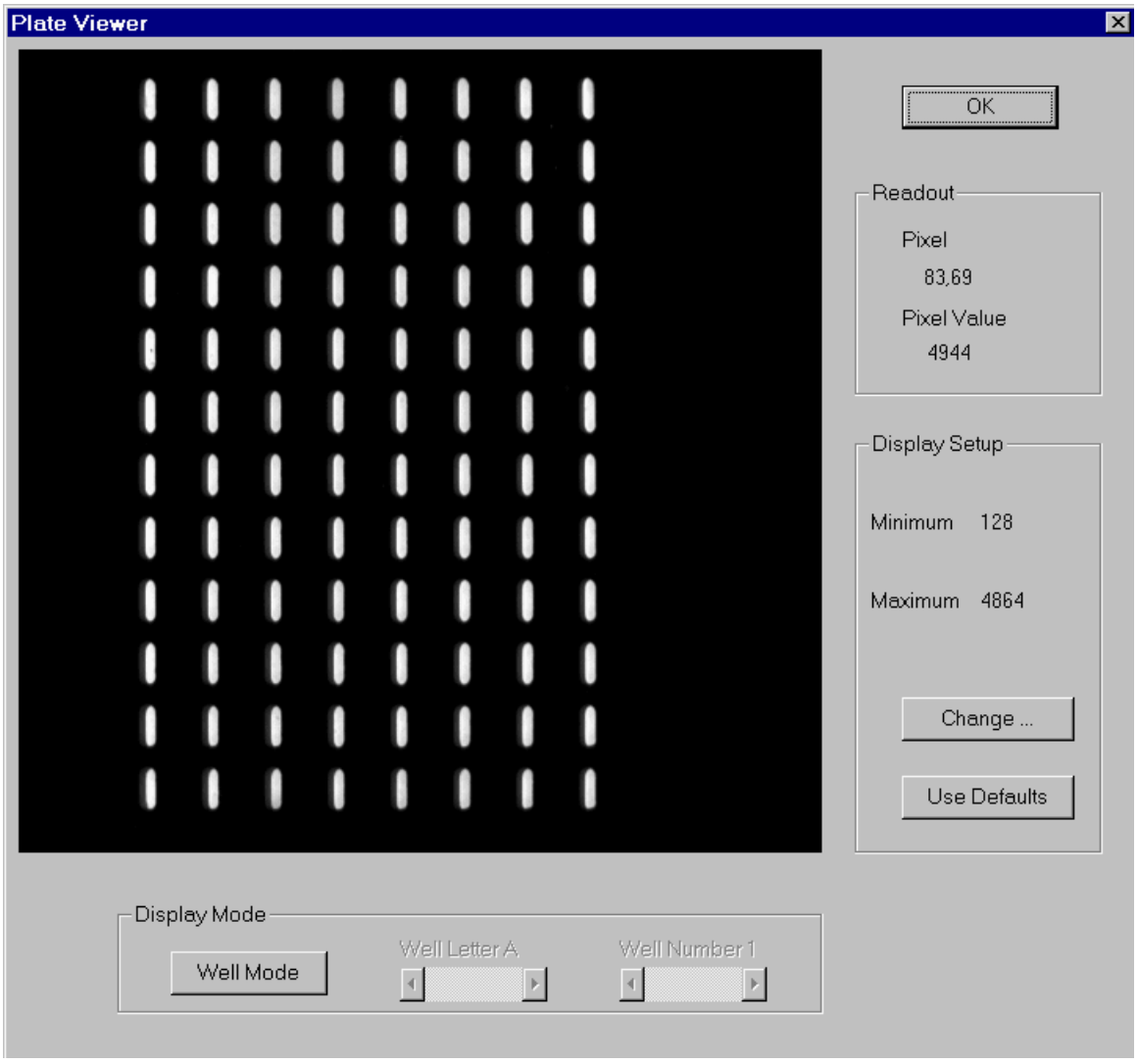
An error message will appear at the bottom of the signal test dialog if sections of the camera have been saturated. Saturation can result in erroneous value in the dialog, so the signal test should be immediately rerun with a lower exposure. To aid in locating bad wells, signal levels less than 75% of the mean level are displayed with green text and those greater than 125% of the mean are blue. Selecting the print button will make a print of the data from this dialog. Selecting save will prompt the user for a file name that will be used for saving the data in an ASCII text file.

When running an assay for the first time, it is advisable to try a very low exposure and work upward to avoid confusion from saturation.

3.7.3. Diagnosing Problems with Cell Samples

When fluid additions are made to a plate of cells, there are several ways that erroneous signals can be generated. If the adherence of the cells to the well bottom is not good, cells may be blown around the well. This usually results in a loss of signal since FLIPR reads the center of the well and cells tend to settle near the edges. Erroneous signal increases or decreases can be seen if dust particles are present in the cell plate or the fluid being added.

To help diagnose such problems, it is often useful to see an image of the fluorescent light emitted from the plate. Choosing Plate Viewer from the Run menu will cause FLIPR to acquire an image using the current exposure length and display the dialog box shown below.

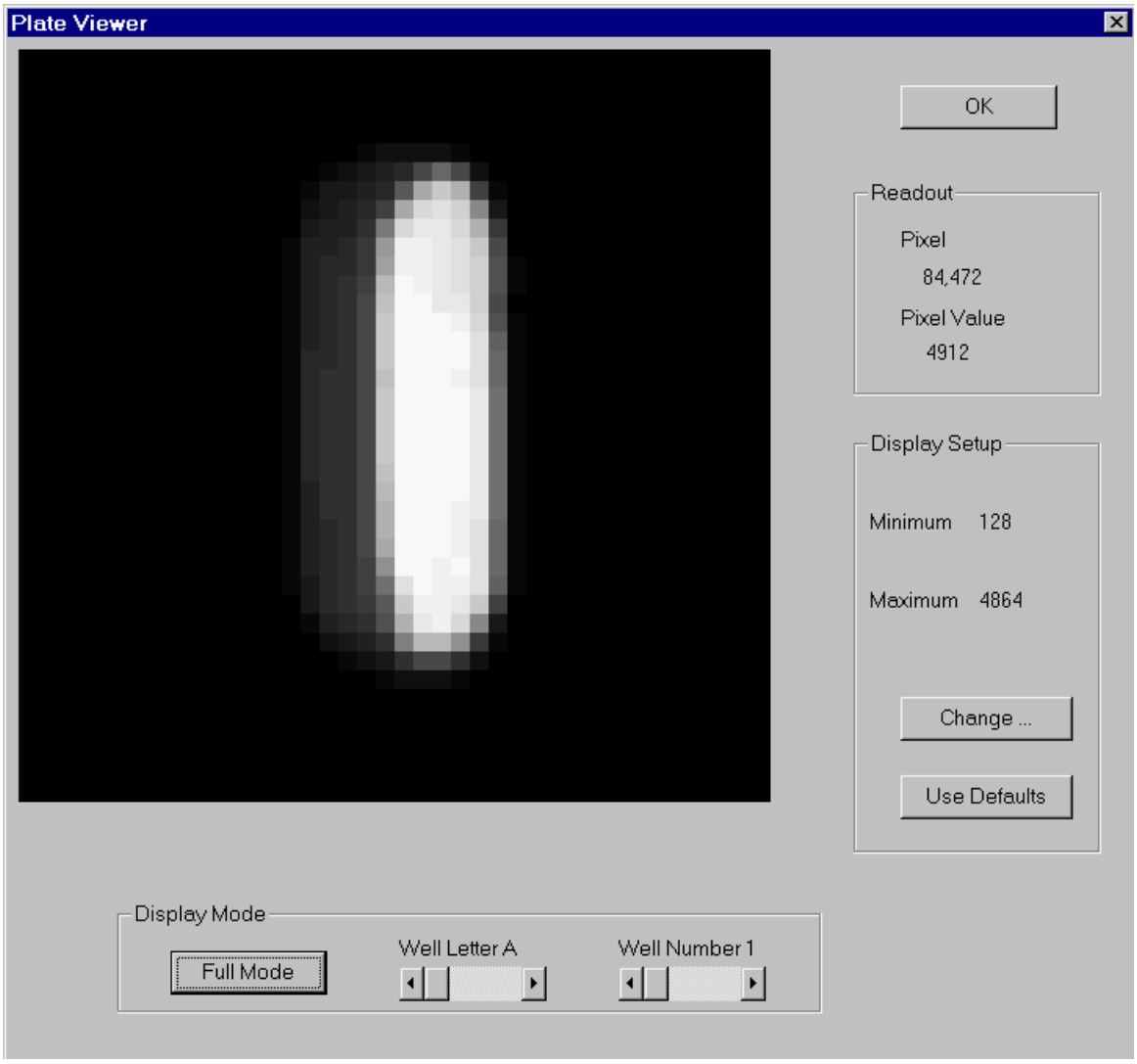


In this view all 96 wells are visible through the slits in the mask, with well A1 in the lower left. The display automatically scales to show the full range of light intensities in the image. At times this may not produce a desirable image, so the user can select a display range by clicking the Change button in the display setup box. Clicking the Use Defaults button will reset the parameter to the full range.

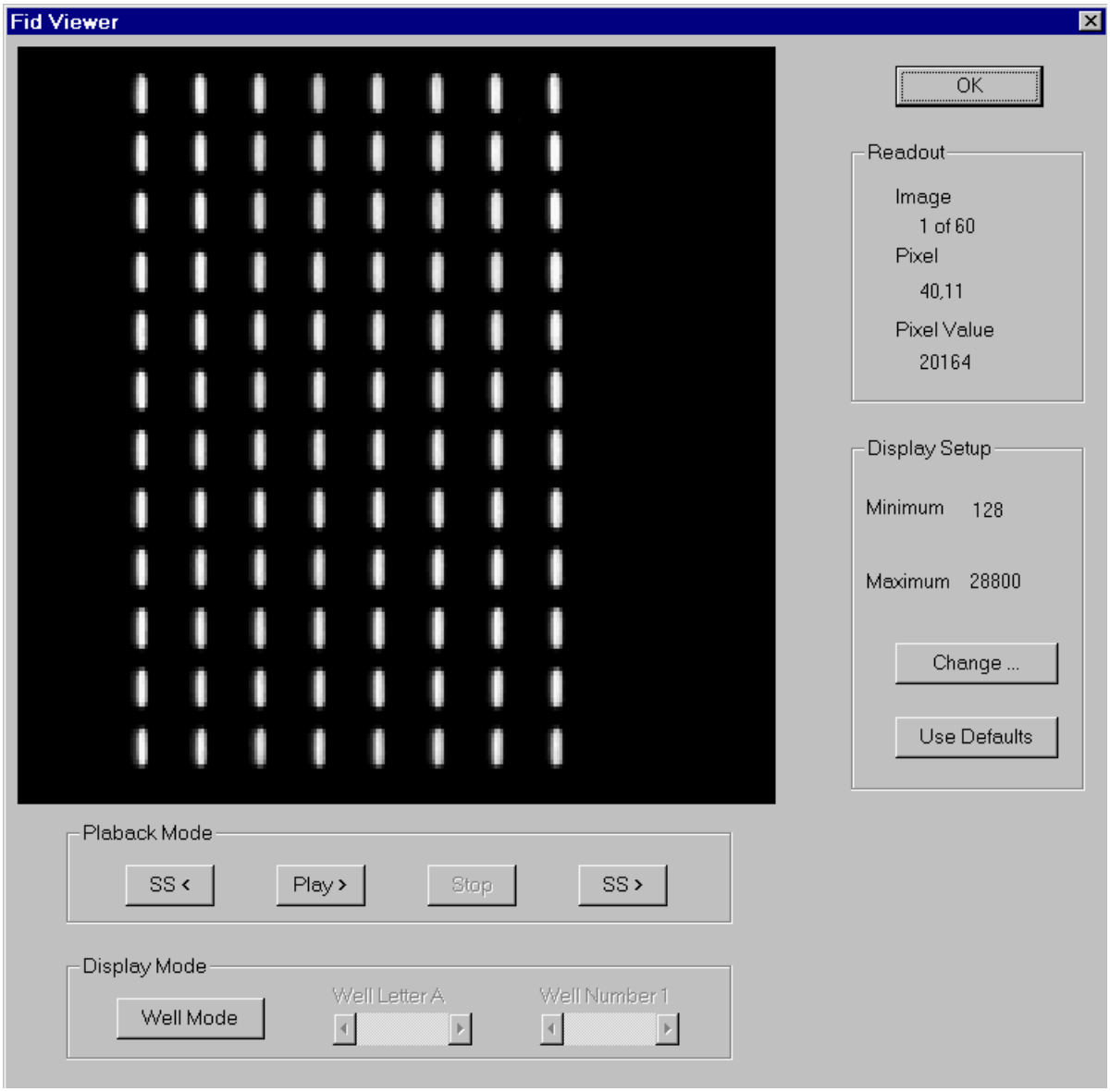
Moving the cursor over any pixel in the image displays the pixel coordinates and intensity value in the readout box. This is useful in making well to well comparisons of fluorescence or examining the relative signal differences within a well. However, the relationship between this intensity value and the signal value computed for a well during a FLIPR run is complicated. Because the signal values computed during a FLIPR run are sums of several pixels, (the number and location of pixels summed varies with experiment type) they will usually run 3 to 6 times larger than the values seen in this dialog.

To examine a well more closely, the user can click the Well Mode button to expand the view of a single well as shown below. In this mode, the scroll bars in the display mode box can be used to select the desired well.

If wells do not appear centered in the display when using well mode, system alignment should be checked as described earlier in this section.



To view data from an existing fid file, choose fid viewer from the run menu. This will display the dialog box shown below.



The tools available in this dialog are the same as those in the plate viewer described above, with the addition of playback tools, which allow the user to step through the sequence of frames collected. Images stored in fid files are compressed by reducing their spatial resolution. For this reason, if the plate being studied is still in existence, the plate viewer tool with its better spatial resolution may help the user diagnose problems.

3.8. Files generated by FLIPR

Several different files are created in a typical FLIPR session. These include a FLIPR control file that has an extension of .fcf. This file contains all the parameters entered into FLIPR dialogs when setting up an experiment. These files are saved and loaded using the Selections under File (i.e. File:Open Control File) menu selection.

When an experiment is run the FLIPR code also generates a compressed FLIPR image data file. This binary file contains the actual signal data that is processed using the File:Export menu selection. FLIPR image data files (*.fid) are named and stored automatically when an experiment is run. The name will have the form basename_n0.fid, where 0 is a counter that increments each time an experiment is run using the same base name. By default, the base name is the date, but choosing File Names from the Setup menu can enter a user supplied base name.

When data has been processed using File:Export, an ASCII text file is generated with a user supplied name and either a ".stat" or ".squ" extension. If a time sequence was selected, the file contains data in columns delimited by tab characters. The first column indicates the time in seconds since the start of data collection and each following column is labeled with the well number as shown on a standard 96 well plate. If statistics was selected, the data is written in a rectangular matrix with one value corresponding to each well. A check box in the export statistics dialog, allows the user to output labels for the matrix.

When exporting either a time sequence or statistics the user has the option of automatically naming the output files. The tables below show the automatically generated names.

Exporting Time Sequence Auto-Naming

Current Image File	All Image Files
Basename_nX.squ	Basename_mult.squ

Exporting Statistics Auto-Naming

Current Image File	All Image Files
Basename_nX.stat	Basename_mult.stat
Basename_nX_stat2.stat	Basename_mult_stat2.stat

An ASCII text file is also saved by FLIPR when the user presses the Save button in the Signal Test Dialog. This file is a tab-delimited matrix of values very much like the file produced by the file export statistics function.

3.9. Data Archival

A typical FLIPR run will generate an FID file of 2 to 6 Mbytes in size. This necessitates long term storage of data on media other than the system hard disk. Many different backup devices are compatible with the FLIPR computer. Many users choose to install a network adapter and use resources already available on their corporate networks for backing up FLIPR data. For a local backup many options exist, most of which use a SCSI interface adapter. Several different brands of network and SCSI adapters have been installed and there are currently no known incompatibilities.

4. Demonstrated Applications

This section discusses some standard assays for which FLIPR has been demonstrated; namely, membrane potential and intra-cellular calcium. The intention of this section is only to document some of the tissue culture and assay protocol issues that in our experience have proven useful. Any specific questions regarding specific cell types etc. should be forwarded to an MDC applications specialists.

Section 4.1 will describe a typical DiBAC (Molecular Probes) membrane potential assay with specific cell culture and loading protocol for A10 smooth muscle cells. Section 4.2 will then describe the actual experimental setup and operator steps in running a membrane potential experiment. Section 4.3 will summarize loading and protocol issues for measurements of intracellular calcium. Finally Section 4.4 lists some of the more relevant disposables and reagents used in FLIPR

4.1. Membrane Potential

The first assay for which FLIPR was developed was for the measurement of membrane potential using the voltage sensitive dye $DiBAC(4)_3$ (Molecular Probes B-438). The assay protocol was developed by Dr. Vince Groppi of Upjohn-Pharmacia in Kalamazoo, Michigan. Before discussing the assay loading protocol, it is probably informative to discuss some of the cell preparation techniques utilized.

4.1.1. Cell Culture

One criterion necessary for quality data from FLIPR is that the cells measured are at the bottom of the well. This does not necessary mean the cells are adherent. This necessity is due to the patented optical detection scheme in FLIPR which enhances sensitivity to fluorescence from the bottom of the microplate well. Non-adherent cells can also be used, however not in suspension. Generally non-adherent cells are spun down, forming a pseudo-monolayer at the bottom of the plate. There are then specific issues with regards to washing the cells and pipetting protocols for FLIPR so as not to disrupt the pseudo-monolayer.

As a general rule we have found it advantagous to treat cells that are normally adherent as adherent, whereas cells that are normally not adherent should be treated as non-adherent. Many times, for example with transfected HEK 293 cells, the cells do not like to adhere to the bottom of the microplate wells. Also some cell lines do not form nice monolayers. In these case we may follow a non-adherent cell protocol. This will be discussed in a later section. First, let's discuss the adherent cell issues.

Some cell types require a coating (e.g. Poly-D-Lysine) in order to insure adherence. Typical protocols would involve coating the microplates in a sterile environment before use. Please consult an MDC application specialist for protocols for microplate coatings.

Adherence of the cells is necessary in order to avoid artifacts generated by fluorescent cells which change position in the wells during a data collection cycle. This effect is amplified because the FLIPR optics, in order to have a limited depth of field, do not view the entire well bottom.

Therefore it is possible that cells could start in the field of view of the camera and due to pipetting subsequently be forced out of the field of view causing a large loss in fluorescent signal. The plating of most cell types into the polystyrene microplates used in FLIPR is pretty similar. Issues of adherence and cell growth are more specific. The following is an example of the tissue culture preparation and plating of A10 smooth muscle cells for use in FLIPR.

A10 smooth muscle cells are obtained from the American Type Culture Collection (Cat # CRL 1476) and are grown as previously described by Kimes and Brandt, Expt Cell Res 98:349 (1976). Briefly, stock cultures of A10 cells are grown in Dulbecco's modified Eagle's medium (DME) containing 20% fetal bovine serum. Stock cultures are passaged 1:15 using 1X trypsin/EDTA on a weekly basis and fed every second or third day. A10 cells are never allowed to remain at confluence for more than a day. A10 cells are never carried for more than 2 months in continuous culture. It is quite common for cell types to undergo a significant change in physiological response if carried too long in culture. This obviously varies cell line to cell line, but should always be considered in assay development.

The following procedure is used to prepare cells for FLIPR:

Day1: Stock cultures of A10's are detached from T75 flasks with 1X trypsin/EDTA. Typically 1 T75 flask will contain $2x10^6$ cells. A10 cells are diluted to approximately 0.25×10^5 cells/ml and 0.2 ml of cells are distributed to each well of a 96 well plate. In this way each well is plated with 0.5×10^4 A10 cells. In this manner 1 T75 flask will generate about four confluent 96 well plates after growing for 3 days.

Day 3: A10 cells are carefully fed with fresh medium. Care is taken not to disrupt the monolayer.

Day 4: Cells are ready for analysis in FLIPR.

Following this procedure, one should establish a uniform monolayer of A10 cells in each well of a 96 well plate. If the monolayer appears non-uniform in any way, then the experiment is terminated and fresh cultures are prepared.

Having followed the above procedure to Day 4 (see previous section) it is now time to prepare the plates for testing in FLIPR. Based on their reluctance to overgrow, the A10 cells can probably be used successfully on Days 4-6.

4.1.2. Calibration Issues

As described previously (Section 1.2.2), FLIPR can incorporate a calibration scheme using positive and negative control wells. This insures that the day to day results are as consistent as possible. The uniformity of the response of positive control wells, along with the “lack” of response to negative control wells is the ultimate check on instrument and biological quality control. These controls also allow for comparison of data plate to plate and day to day, effectively correcting for small changes in system gain which can occur. The data processing options provided in the FLIPR software are discussed in detail in Section 3.5.4.

The negative controls are typically additions of a non-stimulating fluid buffer, e.g. in the case of the membrane potential assay just EBSS, 5μM DiBAC and perhaps the proper concentration of any solvent (e.g. DMSO) being used. The negative controls are used to make sure the instrument as well as the assay is working properly, yielding flat baselines and no response where there should not be any. The negative control wells can be used to temporally correct the data in cases where there is

an artifact, for example a temperature drift, dye leakage effect or photobleach effect, which is consistent in all the data.

The positive control wells are used to monitor changes in signal gain. The gain calibration is useful to compare all of the data within a single run to known positives, thereby calibrating the plate. In addition, the use of similar positives for different data runs will correct for absolute gain discrepancies such as a change in camera exposure time, laser power, cell density or response which can occur day to day. The magnitude and uniformity of the positive control responses is the best check on both the instrument and the biology.

One processing option is to scale the data in percent based on the positive control well response being defined as 100%. In this mode, all of the positive control wells (as defined by the user) are measured and the average peak response is defined to be 100%. The rest of the wells are then normalized to the same scale. Other processing options allow the operator to look absolute FLIPR counts, thus allowing for absolute video level comparisons between plates. This may be useful in cases where the user may need to quantitatively compare different cell types, e.g. an endogenous cell line with a transfected cell line, where dye loading levels may be different.

4.1.3. Preparation of Solutions (Membrane Potential Assay)

The following protocol is intended to provide enough solutions to test approximately 2 microplates:

1.) Prepare a 20mM solution of HEPES Buffer in EBSS by adding 10 mL of 1M HEPES to 490 ml of EBSS. This solution is EBSS + H. Warm to 37 deg C by incubation.
2.) Prepare 100 ml of EBSS+H containing 5 μM DiBAC, by adding 50 μl of 10 mM DiBAC to 100 ml of EBSS+H. This solution is EBSS+H+D. Keep at 37 deg. C. This solution will be used in the negative control wells, to wash the cells and also as the media in solution with the cells during testing. Approximately 60 ml will be used per test plate. The 10 mM DiBAC stock is made up from the powder in DMSO. For the DiBAC 10 mM stock: DiBAC normally comes in 25 mg quantities and it is useful to make around ~20 300 μl aliquots in DMSO for freezing and later use.

3.) Prepare 50 ml of EBSS+H containing 10 μM DiBAC, by adding 50 μl of 10 mM DiBAC to 50 ml of EBSS+H. This solution is EBSS+H+D. Keep at 37 deg C. This 10μM dye (2X) solution will be used in FLIPR to presoak the pipette tips during a run. DiBAC will absorb into plastic, and it is normally a good idea to pre-soak virgin pipette tips when handling it. Note that in the DiBAC assay the stimulus compounds are prepared in DiBAC thereby maintaining the dye concentration in the optically measured microplate wells containing the cells.

4.) Prepare 100 ml of EBSS+H+D containing 300 mM KCl by adding 7.5 ml of 4M KCl to 92.5 ml of EBSS+H and 50 ml of 10 mM DiBAC. This solution is EBSS+H+D+300KCL. Keep at 37 deg C. This 10X KCl solution will be used for the positive control wells (20 ml addition to 180 ml : making the final concentration 30 mM KCL) in the addition plate.

4.1.4. Preparation of Cell Plate

1.) Remove growth media from cells with a multi-well pipettor. If using a 8 well pipettor do no more than 4 rows at a time to insure that the cells do not dry out. Add 250 μl of pre-warmed EBSS+H+D to the cells. Do this for the entire plate. This work should be carried out on a hot plate set to 37 deg C, to minimize temperature fluctuations. Do two washes with 250 μl of EBSS+H+D.

2.) Again doing 4 rows at a time, remove the 250 µl of EBSS+H+D and replace with 180 µl of fresh pre-warmed EBSS+H+D. Make sure that pipettor tips are pre-soaked and be consistent when handling dye washes to avoid variable dilution.

3.) After the cell plate is washed place it in the incubator to equilibrate to 37 deg C.

4.) The cells should be tested in FLIPR within 2 hours of the final wash.

4.1.5. Preparation of Drug Addition Plate

1.) Place 220-250 µl of EBSS+H+D in the negative control wells. The pipettor will go down to the 50 µl fluid level and will typically only aspirate 20 µl per run, therefore this plate can be used several times. Clear microplates can be used for the stimulus plates. Using the FLIPR 96 well pipettor with a flat bottom plate will result in a dead volume of about 50 µl whereas a v-bottomed plate can have dead volumes as low as 10-20 µl.

2.) Place 220-250 µl of EBSS+H+D+300KCl in the designated positive control wells.

3.) If the unknowns are in a solvent, be sure to include the same concentration of solvent in the negative control wells. In our experience try to stay below 0.1 % DMSO if possible.

4.) Place the drug addition plate in the incubator and equilibrate to 37 deg C.

4.2. Typical Measurement Sequence for Membrane Potential

This section is intended as a summary section to help walk the operator through an entire experiment. For completeness we will repeat the start-up procedure here.

4.2.1. Start-Up Procedure

1.) Turn on the laser water flow and electrical main disconnect.

2.) Turn on the laser. Typically, the laser will require about 15 to 30 minutes to stabilize. Coherent recommends a 30-minute warm-up stabilization period for the laser. They also recommend (due to tube lifetime) turning the laser off only if you plan on being down for more than 3 hours, otherwise leave the laser on.

3.) Turn on the computer, monitor and camera controller. Make sure that the CCD camera is at temperature before taking data with the camera. Proper temperature is indicated by a lit green "status" light on the camera controller. Normally camera cool-down takes about 5 minutes.

4.) Power up the FLIPR enclosure. Make sure the PC is booted up *before* powering up the FLIPR enclosure. The system may lock up otherwise.

5.) Turn on the regulator for 80 psi air source.

6.) Clear any interlocks by making sure the cell drawer is in and the pipette tips are up.

7.) Start the airflow for the humidity chamber (temperature dependent assays only).

8.) Start the FLIPR code by double clicking on the FLIPR control software icon.
The pipettor will also go through the normal homing cycle on start-up. If temperature control is desired, and it not set up in the setup.flp file as default on, turn it on via the Setup "heaters" pull down option. It will take approximately 30 minutes for the temperature sensors to stabilize. Recall that the temperature control option requires the low-pressure regulated air source be turned on also.

9.) Wait for the temperature warning indicators to clear on the display window if temperature control is being used. You are now ready to take data.

4.2.2. Data Collection

Assuming the cell plate and addition plate have been prepared via the previous section, you are now ready to take data with FLIPR. Because of the strong temperature dependence of DiBAC, it is a good idea to immediately place the cells in the FLIPR cell incubation chamber after removing them from the incubator.

Caution:
Once the cells are placed in solution with DiBAC, they should be tested within 2 hours.

To reduce the temperature stabilization time, the cell plate and the addition plate should be removed from the incubator and put into the cell incubation chamber in FLIPR as quickly as possible. The more careful the operator is in handling the plates, the quicker the DiBAC temperature stabilizes and the quicker the fluorescent baselines stabilizes. The cell plate of course goes in the middle drawer position and the addition plate typically goes in the right addition position. If a 2X (e.g. 10 μM) DiBAC presoak plate is being used it typically goes in the left addition plate position.

The operator then needs to set up the parameters to be used in the experiment. Choosing the Experiment option under the Setup pull-down menu does this. Typical parameters for DiBAC and A10's are as follows:

4.2.3. Typical System Setup for Measurements of Membrane Potential

System gain parameters:

Assuming the laser is set to around 300 mW
Since DiBAC is bright, the F/stop should be set around F/5.6, i.e. stopped down a little
Camera Exposure time around 0.4 seconds (how to set this is described below).

General Setup Parameters:
a.) Exposure Length: (camera integration time): usually around 0.4 seconds
b.) Filter #1 (standard filter)
c.) Presoak tips (usually checked, usually from left tray)
d.) Multiple additions (unchecked, as we will assume a single stimulus addition here)
e.) Automation: user's choice of options

Under the First Sequence Definition

a.) Sample Interval (typically 20 seconds)
b.) Sample count (depends on length of experiment desired), usually 15 minutes is enough for Dibac, therefore at 20 second updates, and 15 minute total run time, 45 samples would be taken.
c.) Second interval (usually not necessary with DiBAC, generally only used for fast updates)
d.) Fluid addition checked active
e.) Fluid volume to add (usually 20 µl, as DiBAC is temperature sensitive and smaller addition volumes are preferred.
f.) Pipettor height 150 µl (assuming there is 180 µl in the plate to start with)
g.) Dispense speed (depends on cell type, 20 µl/sec is slow, 80 µl is fast, better mixing the faster the mix speed, depends on what the cells will withstand without being relocated on the bottom of the plate.
h.) addition from the right try (default setting, assume presoak tray in left tray)

the Second Sequence is undefined (unchecked in the General setup window)
the Third Sequence is undefined (unchecked in the General setup window)

Under Pipetting
a.) Mix volume (generally 40 µl)
b.) Number of mixes (generally 3, we do mixing because of adding a small volume to a large volume, as well as the kinetics of DiBAC are slow enough to allow for mixing between updates.
c.) Tip position, uncheck, ie tips are out of well during data collection, since Dibac experiment is slow, the pipettor has time to go up and down into the fluid between sample points, by un-checking this box one can insure that compound does not leach out into the cell plate prior to the pre-programmed delivery
d.) Fluid Addition, generally for Dibac this will be checked, that is we will remove the same amount of fluid which was added, in this example 20 µl. This insures that there is no fluid height dependence associated with adding a very fluorescent background component, that being the Dibac in solution with the addition compounds. This may or may not be necessary. With this box unchecked, the fluid will be added, changing the total well volume to 200 µl (assuming we added 20 µl to 180 µl already in the cell plate).

Hit OK to save changes. Remember all the system parameters, once defined, can be saved in a FLIPR Control File (*.fcf file) by choosing Save under the File pull-down menu.

Once the experiment has been defined, the user will perform a signal level check using the “light bulb” or Run “Signal Test” option. This takes a camera exposure using the current exposure time defined in the Setup Experiment window and displays the signal counts for all 96 wells of the plate. It also displays some statistics of the average, minimum and maximum signal counts for the exposure. Since the total dynamic range of the camera is 65,000 counts is it a good idea to work with signal counts in the range of 25,000to 30,000 with DiBAC. At these gain levels, a good physiological DiBAC signal (e.g. a membrane depolarization) will be on the order of 5,000 counts. Thus starting with a basal fluorescent range of 25 to 30,000 counts helps to insure that the individual camera pixels will not saturate during the data collection.

If the signal counts are too high or low, we would recommend shortening or lengthening respectively, the camera exposure interval until the correct signal level is achieved. If exposure times less than 0.4 seconds are required to come down to the desired video levels, then we would turn down the laser source power or alternatively, stop down the camera aperture. Each F/stop increase of the camera cuts the detection sensitivity by a factor of two. General ranges for the system gain parameters are as follows:

Laser power: run between 150 mW and 1 Watt, generally we like to leave around 300 mW so laser warms up at a consistent place every day, increase power when more gain is needed.

Camera F/stop (run between F/2 and F/22), note that each stop is a factor of 2 in sensitivity with F/2 being the largest aperture, with the most sensitivity. This is the easiest thing to change to get rid of light, for example when running the DiBAC assay.

Camera Exposure time: Run above 0.2 seconds, for kinetic (multiple frame) data. This is because at very short exposure times the mechanical jitter in the opening and closing of the camera shutter can add noise to the temporal fluorescent traces. This is not an issue for a single (non-kinetic) exposure.

After the signal level is set, the operator is ready to start a baseline stability check. This is usually a good idea to insure that the fluorescent baselines are under thermal equilibrium resulting in flat baseline fluorescence. This can be done by starting an experiment, usually with 20 second time updates, with the pipetting deactivated. This is more of an issue with Dibac assays than with intracellular calcium assays. Depending on the temperature of the fluid when the cell plate was placed in FLIPR, stabilization can take a few minutes or up to 20 minutes. If after 20 minutes the baselines still have not stabilized, then generally it is not due to temperature instability and is usually associated with cells that are unhappy.

Assuming the baselines are flat, then it is time to start an experiment. Hitting the stop icon at any time can stop the baseline. Once the user is satisfied that the baselines are flat enough, the pipettor should be re-activated in the Experimental Setup window; “First Sequence” dialog page.

The user should make sure that a new set of tips has been loaded on the pipettor. Once new tips are loaded, the experiment is started by hitting Experiment under the Run pull-down menu or by hitting the "run experiment" tool. The pipettor will then immediately proceed to the presoak addition plate, and aspirate fluid for the tip pre-soak. This pre-soak volume will be held for one minute, at which time it will be re-dispensed back into the pre-soak plate and the pipettor will proceed to the stimulus addition plate for the programmed pick-up. This fluid will then be held in the tips until the pre-programmed delivery time, into the cell plate, is reached.

The experiment will continue, with the real-time display updating the measured fluorescent data values, until the total number of measurements has been completed. At this point the pipettor will return to the pipette load station on the right side of FLIPR.

Once the run is completed the user is then ready to export (or process) the data. This basically converts the raw data files, which have already been compressed and stored on the hard disk, into fluorescent signal counts per well. Exporting of data is covered in detail in Section 3.5.

4.3. Intracellular Calcium Measurements

As with the membrane potential assay description, the purpose of this section is to provide a starting point for the user. For specific protocol information for a particular cell type etc. please consult and MDC FLIPR applications specialist.

4.3.1. General Information

To date we have found FLIPR to work best with the visible wavelength calcium indicators Fluo-3 and Calcium Green-1. Both these dyes excite with the 488 nm excitation line of the Argon laser, and emit in the 500-560 nm range. Additionally, we have had some success with Oregon Green Bapta 1. This dye has a high affinity for calcium and can provide more optical signal for smaller calcium changes than calcium green in some cases.

Operation in the UV using the fluorescent probe Indo-1, for example, has yielded less fluorescent signal, partially due to the poorer UV transmission through the polystyrene microplates. The biggest drawback is that UV excitation causes the plates to fluoresce much more than when exposed to visible light (e.g. longer than 450 nm). The increased "background" signal in the UV, along with the higher probability of obtaining a signal artifact upon stimulus addition (due to a fluorescent compound) in the UV has made the visible wavelength dyes much more attractive.

Typically, we have found that 1 to 1.5 hours of loading with dye concentrations of 1 to 5 μM using 0.300 W of laser power, an F/2 lens setting and 0.4 second exposures have yielded plenty of signal to detect changes in calcium as small as 10 nM. This obviously depends on the cell type and density at which the cells have been plated. Cells have been loaded both in the presence and absence of serum. Usually loading in media and serum is preferable. We have had cases where loading in the presence of 0.1% BSA has helped with dye retention. We have also had cases, specifically with CHO cells, where loading in the presence of 2.5 mM Probenecid has helped with dye retention. The Probenecid helps shut down the MDR pump which in CHO cell inhibits dye loading. In general, the optimum conditions should be determined empirically for each cell type. Consult an MDC applications specialist for a head start in these areas.

After dye loading, several washes in a non-fluorescent salt buffer will be required to reduce signal artifacts associated with the presence of extra-cellular dye as well as background associated with fluorescent entities in media. The main artifact present with improper washing is a sharp signal decline upon addition of the stimulus due to the dilution of the strong background component. Recall that generally the compounds are mixed up in a non-fluorescent salt solution, e.g. Hanks, Earles etc. and that large volume additions are made (e.g. 50 μl to 100 μl) to insure rapid mixing.

The cell wash buffer should be the same solution used with the cells during testing. Likewise, this same buffer should be used to prepare the stimulus compounds to avoid unwanted pH, osmolarity, or morphology changes in the cells during a kinetic experiment. Typically balanced salt solutions like HBS, PBS or EBSS will be used. *It is very important that the compounds are prepared in "exactly" the same buffer as the cells are washed and sitting in prior to running the experiment.*

A typical protocol would be to load in dye for 1.5 hours, wash 3-4 times with buffer (200 μl per well), for example using a gentle washer such as the Denley CellWash. Exactly how many washes are necessary will be cell type dependent. Sometimes a wait period between washes (two before, two after) is good to allow the cells to re-equilibrate to a lower extra-cellular dye concentration, before doing the final wash. If the cells are only slightly adherent, there will be a trade-off between washing the cells thoroughly to remove extra-cellular dye, and washing cells off the plates.

Researchers who are interested in intra-cellular calcium measurements quite often want rapid temporal updates because they are trying to see the kinetics of fast calcium signals. To this end, it is necessary that the pipetting of a stimulus fluid addition be done in a fast manner. There is typically not enough time to do "mixing" with the pipettor as is done with the Dibac membrane potential protocol described above. We have found that adding fluid additions of 50 μl to a buffer solution of 150 μl already in the cell microplate wells (and using 4x concentrated stocks in the addition plate) provides nearly instantaneous mixing upon pipetting. Using less than 50 μl additions will probably slow down the kinetics of the response due to fluid mixing. It is also preferable to have at least 90μl of buffer on top of the cells prior to any stimulus addition in FLIPR. This volume provides a protective barrier to the cells, reducing the stress associated with pipetting volumes directly on the cell layer.

Through the Experimental Setup Window, the FLIPR software interface provides the user with the ability to alter the height of the tips in the wells, as well as the speed of the dispense cycle. This can be set for each of three pipette sequences independently. When the pipettor picks up fluid from the stimulus addition microplate, it is necessary to have a small air gap so that the fluid will not leak out

of the tips upon transfer. This air gap is the first to be dispensed when the pipettor starts its dispense motion. To avoid blowing air bubbles in the wells (which can cause random light reflections, and spurious signals) it is preferable to start the pipettor height above the resting fluid level of the wells. It is also preferable to have each pipettor tip submerged after the addition has been made to make sure that all the fluid is dispensed. If the pipette tips are in air at the end of the dispense cycle a drop can form on the end of the tip due to surface tension.

For example, suppose there is 100 µl in the wells that contain the cells. Also suppose we would like to make two fluid additions, each with 50 µl. The optimum pipettor height for the first addition would be about 125µl, insuring that the tips would be in air at the start of a dispense cycle (therefore would not blow bubbles, and not allowing for fluid to leach out of the tips), and would be submerged at the end (thereby not leaving any fluid drops on the tips). Correspondingly the second addition, now into a fluid volume of 150µl would be best done at a fluid height of around 175 µl.

The pipettor dispense speed should generally be about 50µl / second. However, if the cells are only slightly adherent, the user may need to reduce the dispense speed. A slow dispense speed is on the order of 20 µl/second, and a fast dispense speed is around 80 µl/sec. These values must be experimentally determined for each cell type, but generally it is preferable to dispense as fast as possible to enhance mixing. The trade-off is that the pipetting speed cannot be so forceful so as to dislodge the cells at the bottom of the well. This will in effect remove the fluorescent cells from the field of view of the camera pixels, thereby causing large decreases (artifacts) in the fluorescent traces upon the fluid addition.

At the beginning of a data run, it is useful to perform a "signal test" to check the dye loading level in the cells. When establishing loading level protocols, it is useful not to load dye into the entire microplate to get a reading on the background fluorescent counts. These unloaded wells should be treated just like the loaded wells in terms of wash steps etc. The dominant background component (assuming a salt solution is used for the buffer during the experiment) will be fluorescence from the plastic microplates. Most salt buffer solutions have negligible fluorescence at excitation wavelengths of 488 nm.

We would advise working with starting fluorescent levels (above background) of about 10,000 to 20,000 counts (recall saturation is 65,000 counts) for basal calcium levels. Again, depending on loading levels, this should be achievable with approximately 0.300 W of laser power, 0.4 sec of integration time and an F/stop setting of F/2. Assuming identical dye loading, Calcium Green-1 will have a larger fluorescent signal under basal calcium concentrations than FLUO-3. However, FLUO-3 will generally provide more change in fluorescence for a given change in intracellular calcium. We have gotten very good calcium signaling with only 5000 counts above background with FLUO-3. Calcium Green 1 may require loading levels of 10 to 15,000 counts above background for equivalent signal to noise ratio. The user should try all three (Calcium Green 1, Fluo-3 and Oregon Green Bapta 1) dyes when setting up a calcium new assay. We have had many cases where one dye will load well in a given cell type and the other not load at all.

The user should take advantage of the "dual" sampling interval feature available with FLIPR. This feature lets the user set up two different sampling intervals between time updates during a run. For example, at the start of an experiment and right after the pipettor applies the fluid stimulus to the cells, the user may want to sample every 1 second to fully resolve transient signals. However, the asymptotic response, which doesn't change very rapidly, can be sampled much slower without any loss in information (e.g. every 5 or 10 seconds). Making use of this feature can greatly reduce the amount of data having to be gathered and stored by FLIPR. This makes the system more efficient and faster. This dual sampling interval can be set up for each of the three separate programmable stimulus additions (defined as Sequences under the Experiment Setup Window) per experiment. The user is restricted to about 300 total samples per data run (time points) before the RAM of the computer is full.

4.3.2. Specific Dye Loading Protocol for A10 cells

As an example, here is the loading procedure and system parameters we have used on A10 smooth muscle cells using Calcium Green 1 (Molecular Probes C-3011). These procedures may differ with different cell types. Please consult Molecular Devices.

1.) Plate cells to confluence: need to be adherent

2.) Make ~2mM dye stock in anhydrous DMSO

3.) Make 1mM dye stock by diluting with 20%w/v pluronic acid (in our experience very necessary)

4.) Make 4 μM dye loading stock by adding 40 μl of 1mM stock to 10mls of loading buffer or media. Usually media without serum is used with A10 cells. This will do about 1 plate.

5.) Remove growth media and serum from plated cells. Alternatively, if loading in media and serum, just add dye/media/serum to existing media/serum. This removes an aspirate step, however dye concentration is reduced.

6.) Load cells with 4 μM dye (~100 μl per well) for 1.0 to 1.5 hours

7.) Wash cells with buffer solution twice. You want a good wash so use volumes of at least 200 μl per well.

8.) Re-incubate cells for 15 minutes.

9.) Wash twice more with buffer, leaving 100-150 μl in wells for single stimulus calcium experiment. The actual volume left depends on the amount of dilution desired in the preparation of the stimulus plate. It is important that the final volumes be consistent so as to produce good well to well consistency. It may also be appropriate to do 4 buffer washes consecutively, without the re-incubation. This is usually empirically determined based on whether or not a dye leakage artifact is present in the negative control wells. This artifact is represented by a loss in fluorescence upon addition of the non-fluorescent stimulus in FLIPR. If dye leakage is present, there is a strong non-cellular fluorescent background component, which is effectively diluted by the addition of the sample creating the artifact.

4.3.3. Typical System Setup for Measurements of Intracellular Calcium

Typical System gain parameters:

Generally the laser will be around 300 mW. More or less light may be needed depending on the cell type. This is determined by the number of basal fluorescent counts achieved above background in cell loading.

Assuming fast kinetics are to be measured, i.e. a 1 second kinetic time update, an exposure time of 0.4 seconds is often used.

The camera F/stop is set to F/2; this is the largest aperture recommended.

General Setup Parameters:

a.) Exposure Length: (camera integration time): usually around 0.4 seconds
b.) Filter #1 (standard filter)
c.) Presoak tips (none)
d.) Multiple additions (unchecked, as we will assume a single stimulus addition here)
e.) Automation: user's choice of options

Under the First Sequence Definition

a.) Sample Interval, typically 1 second for 60 seconds, to resolve fast signaling around stimulus addition
b.) Second sample interval, typically 5-10 second updates, for as long as required by the physiology of the calcium signaling. Up to 300 frames (samples) can be acquired for any one experiment, this includes those gathered with additional pipetting sequences.
c.) Fluid addition checked active
d.) Fluid volume to add (usually 20 μl, as DiBAC is temperature sensitive and smaller volume are preferred.
e.) Pipettor height 125 μl, assuming we are going to pipette 50 μl into 100 μl (already in the cell plate).
f.) Dispense speed (depends on cell type, 20 μl/sec is slow, 80 μl is fast, better mixing occurs with higher dispense speeds, depends on what the cells will withstand without being relocated on the bottom of the plate.
g.) addition from the right try . Typically for adherent cell lines 60-80 μl/sec is used; for non-adherent cell lines 20-40 μl/sec.

the Second Sequence is undefined (unchecked in the General setup window)
the Third Sequence is undefined (unchecked in the General setup window)

Under Pipetting
a.) Mix volume (not applicable)
b.) Number of mixes (usually 0 for fast experiments, kinetics rely on a significant fluid volume change, e.g. 50 μl to 100 μl to produce an instantaneous mix in the microplate well).
c.) Tip position, checked, tips are in the well during data collection to expedite fluid delivery
e.) Fluid Addition, unchecked, do not remove fluid after addition to maintain constant volume, this is only important for DiBAC assay

4.3.4. Protocol for Non-adherent Cell Lines

FLIPR has been extensively used for measurement of intracellular calcium and membrane potential with non-adherent cell lines. At first glance this may seem strange since the patented optical detection scheme in FLIPR was developed to isolate the fluorescent signal from an adherent cell monolayer. In order to circumvent this problem, we have found that non-adherent cells can be loaded and washed as non-adherents, i.e. via a centrifugation and re-suspension. The final loaded cells are then dispensed at a proper density into a microplate and the microplate is then centrifuged to sediment the cells at the bottom of the microplate wells. Quite often one additional wash step is required after the centrifugation step. This is generally believed to be necessary to remove extra-cellular dye present from the destruction of a few cells during centrifugation. This wash must be very gentle, as the cells are not physically adherent (or very weakly adherent) to the bottom of the microplate well.

The following is a generic protocol used for THP-1 cells in FLIPR.

For one 96 well plate:

1.) Spin down 35 x 10^6 cells from culture media
2.) Mix dye solution:
    8 ml of complete culture media with serum
    32 µl of 1 mM Fluo-3 stock with 10% pluronic acid in DMSO (see Sec. 4.3.2)
3.) Re-suspend cells into dye solution
4.) Transfer cell suspension to a 10 cm Petri dish
5.) Place into CO_2 incubator for one hour
6.) After one hour transfer cell suspension into test tube for wash
    1st wash: add equal volume culture media, spin
    2nd wash: re-suspend into 20 ml Hank's + 20 mM Hepes, spin
7.) Re-suspend into 16 ml of Hank's + Hepes, dispense into microplate, 150 µl per well
8.) Centrifuge plate 3 min/1200 rpm at RT (IEC Centra) with brake off
9.) Wash once in Denley CellWash using 300 µl fill volume at the slowest dispense speed, followed by an aspiration step to 90 µl final volume.

4.4. Recommended FLIPR Disposables and Reagents

Recommended microplates for optically measured cell plates:

Vendor: Corning Costar
Catalog number 3603.
Generally come TC treated and sterile, in individually wrapped packages.
Only order from lot numbers after Jan 1, 1997.
To order call: (508) 635 2200.

The compound addition plates can be any cheap, non-sterile microplate. Note that conical or v-shape bottoms generally have about a 10-20 µl dead volume, whereas flat-bottomed plates have about a 50 µl dead volume.

Recommended pipettor tips for FLIPR:

Vendor: Robbins Scientific
Part# 1043-24-0-96 (black, non-sterile)
96 tips/rack, 5 racks/case
To order call: 800 752 8585
In Europe: 011 44 (0) 1564 775525

Common Fluorescent Indicators used in FLIPR:

Vendor: Molecular Probes (www.probes.com)
In U.S. (541) 465 8300
In Europe: 31 (0) 71 5233378 Netherlands

Intracellular Calcium

Fluo-3 AM ester, cell permeant version, Cat# F-1241 (1mg pckg) also available in 50 µg
Calcium Green-1, AM ester, cell permeant version, Cat # C-3011
Pluronic acid, buy as 20% w/v solution (DMSO) or powder
Ionophore, 4-bromo A23187, Cat # B-1494

Membrane Potential

Dibac$_{(4)}$3, bis-oxonol, Cat# B-438

Intracellular pH

BCECF, B-1170
Nigericin, for calibration of pH measurements, N-1495

Recommended fungicide for humidity system
Vendor: Scientific Products: (800) 234 5227
Product: Roccal II (10%) fungicide: Cat# C6320-11
Use about 300 µl per liter of water added to humidity fill bottle. Do not use too much as the water tank, which is aerated, will foam over.