User's Guide

FluoroPlex

ION OPTIX

FluoroPlex is a trademark of IonOptix.

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Introduction

Overview

For many years, a vast number of tissue and whole organ experiments have been done in water-jacketed tissue chambers. These experiments can be as simple as the measurement of force generated by smooth or striated muscle tissue or as elaborate as monitoring the EKG and chamber pressures from a beating heart. Multiple preparations are often examined simultaneously and it is not uncommon to arrange arrays of 4 or even 8 tissue chambers to increase data throughput. With the introduction of the IonOptix FluoroPlex, it is now possible to add ***fluorescence ion recording*** to tissue bath experiments in parallel with force, pressure, or other measurements. The ability to monitor regulatory intracellular ion levels promises to advance the insights revealed by this workhorse methodology.

The FluoroPlex epifluorescence illumination and detection assembly lies at the heart of the complete FluoroPlex Tissue Bath Fluorometry System. The system includes not only the FluoroPlex but also a suite of force transducer/ amplifier combinations and specially-designed tissue baths built by Radnoti Glass Technologies Inc. to accommodate the solution-resistant tip of our liquid light guides. The complete system also includes an optional light-tight enclosure to house the Radnoti baths and stand, as well as an optional Radnoti thermo circulator. Data is handled by the optional ADInstruments PowerLab interface and LabChart and Scope software. The FluoroPlex System allows fast fluorescence recordings from multiple tissue baths with simultaneous force measurements. Sampling occurs via two modes: *burst* and *continuous*. Burst mode sampling occurs at 1000 Hz with single wavelength excitation and 250 Hz with dual excitation. Continuous mode ratiometric sampling occurs at up to 14 Hz for four baths. Sampling modes, frequencies, duration and associated tasks such as signal averaging and background collection are programmed within a simple user interface.

FluoroPlex Components

FluoroPlex base unit

The FluoroPlex base unit consists of an LED based light source, a light-multiplexing assembly, the MultiPlexer, and a photodiode based light detector. Excitation light emerges from the MultiPlexer through one of up to eight quartz liquid light guides (LLGs). Fluorescence emission light is collected through the LLGs and subsequently steered and filtered before collection by the photodiode sub-system.

FluoroPlex Controller

The control unit coordinates the excitation light, tissue bath path selection, and data collection. The stand-alone controller features a digital interface for programming collection. Outputs include a digital TTL sync, two raw analog channels and eight analog data channels.

Optics

Emission filters and dichroic mirrors are included with each system. The specification of these is dependent on the fluorophore to be interrogated.

Liquid Light Guides

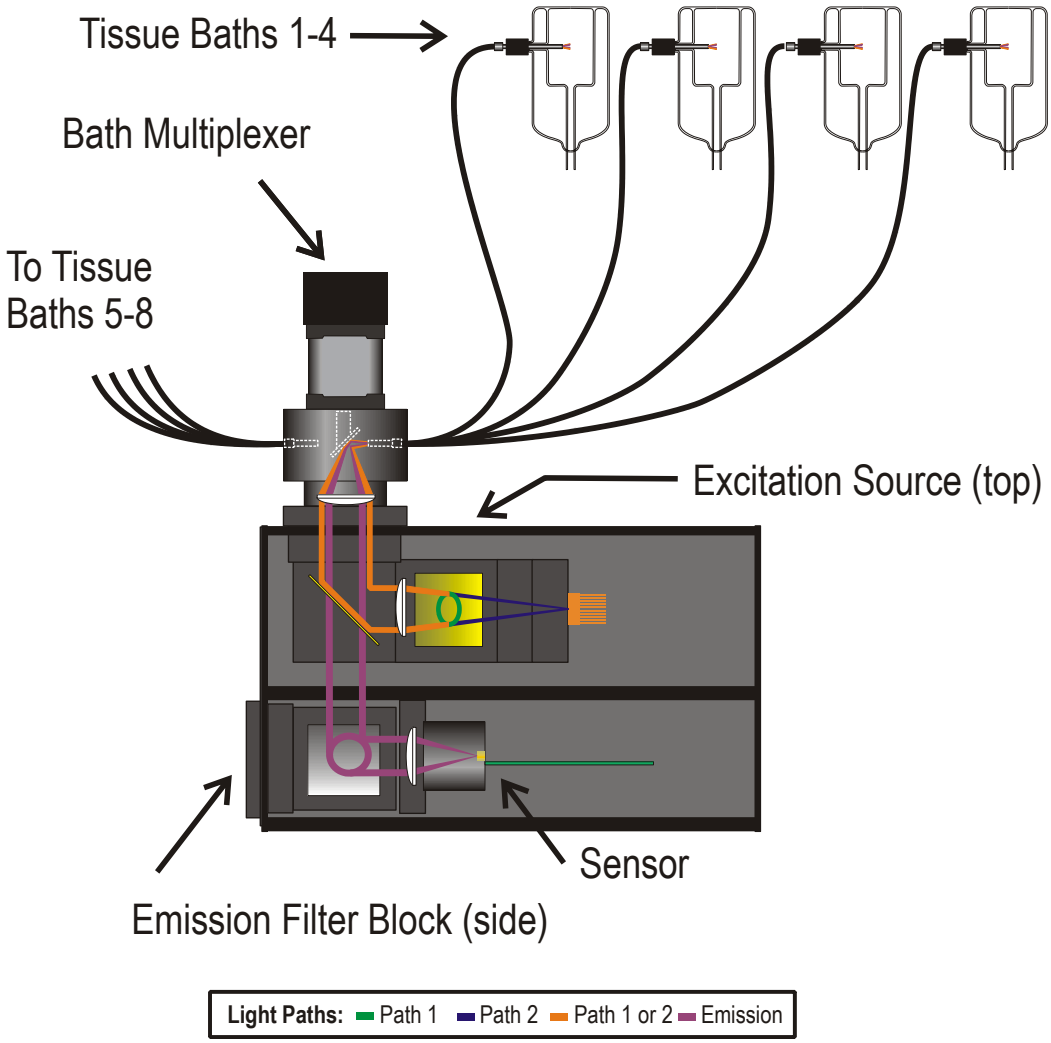
The LLG fits into the Radnoti Quick Disconnect fittings to insure a convenient, leak-resistant connection.

Radnoti and ADI Components

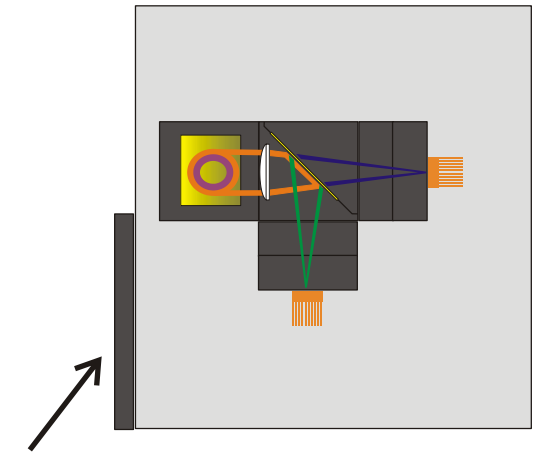
The FluoroPlex System includes Radnoti force transducer and amplifier combinations as well as tissue baths that have been specially adapted for use with this system. Radnoti also produces the optional light tight enclosure and thermocirculator. The complete system with data recording also includes ADInstruments PowerLab USB interface and LabChart and Scope software. Please see the appropriate manufacturer's documentation accompanying the system for proper use of this equipment.

Hardware

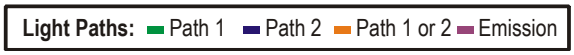
Hardware Overview



Excitation Source Overview

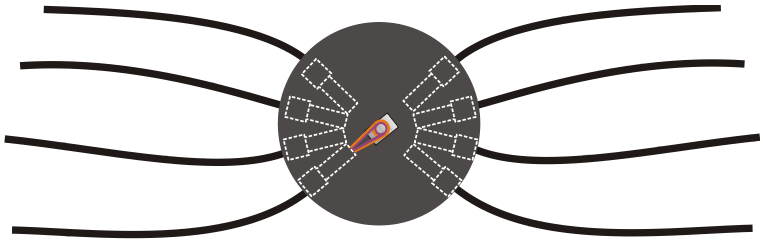


Emission Filter Block (side)



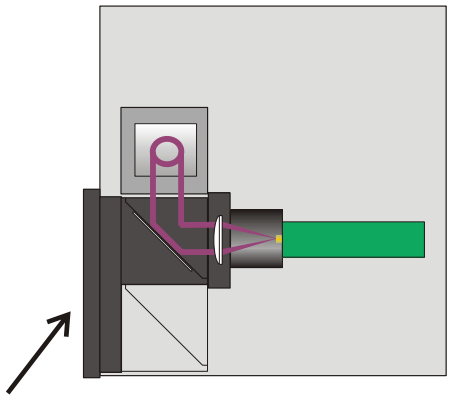
Two LEDs selected for their wavelengths are mounted underneath the FluoroPlex lid. A dichroic mirror steers the light from both LEDs into the MultiPlexer by a vertically reflecting dichroic.

MultiPlexer Overview

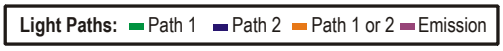


The MultiPlexer consists of a mirror mounted on a micro-stepping motor. The mirror steers focused light into one of up to eight available light guides. Excitation light emerging from the light guide directly illuminates the sample. Light is then collected through the same light guide and reflected by the MultiPlexer mirror back into the FluoroPlex.

Emission Overview



Emission Filter Block (side)



Emission light passes through the vertically reflecting dichroic and is steered through an emission filter before encountering the photodiode. The photodiode's output is sent to and processed by the FluoroPlex Controller.

Processing and Analog Output Overview

The FluoroPlex controller collects and integrates the photodiode output for a millisecond, performs desired calculations, and outputs analog voltages to be recorded by any analog data collection system.

CE and ROHS

This device has been designed and manufactured to be ROHS compliant and meet CE requirements.

Hardware Operation

Most aspects of the FluoroPlex hardware operation are controlled through the software that has been loaded onto the FluoroPlex Controller. Please refer to the Software Operation section for proper operation of the FluoroPlex hardware.

FluoroPlex Controller

The controller box has a simple LCD display and knob which turns to scroll and clicks to select. It has one digital TTL output, 2 raw analog outputs, and 8 analog data outputs. It also has a 25 pin connector to be used with the custom FPX cable for bringing power and communication to the Optics Box.

FluoroPlex Control Box

Digital Output

A TTL pulse output allows synchronization with external hardware. The TTL rising edge is hardware-locked in both Continuous and Burst modes. A TTL output can be coordinated with one of four hardware events: (1) at the beginning of an experiment protocol, (2) at the beginning of an acquisition cycle when the MultiPlexer is positioned at the first bath, (3) accompanying each move to a new bank, or (4) accompanying the output of each new data point. The nature of the investigation and capability of available external hardware to receive TTL pulses will dictate the user's choice of the above options.

Control Box Back Panel

Analog Outputs

There are 10 analog output BNCs on the back of the FluoroPlex Control Box. The 12-bit analog outputs are single ended with a range of 0 to +5V. Eight are the Bath Outputs. The numbers correlate to the MultiPlexer positions the baths are connected to. (Bath 1 correlates with the white triangle on the MultiPlexer and the bath number increases by adjacent positions of the MultiPlexer). Excitation type, background subtraction, output configuration and averaging all affect the calculated data emerging from these 8 BNCs. This voltage range is mapped to a default ratio range of 0-10 in the case of the data outputs for dual excitation. There are two Raw outputs. Num/Sing is the output correlating with the Path 1 LED, which is the signal pathway for single excitation experiments and the numerator value for dual excitation experiments. Den correlates with the Path 2 LED, which is inactive for single excitation experiments and the denominator value for dual excitation experiments. Background subtraction, output configuration and averaging do NOT affect the raw data emerging from these 2 BNCs. The bank these signals correlate to is set by the Lock Raw line on the Run Menu (See Software).

Optics Box Connection

A custom 25 pin D-Sub cable carries power and communication signals between the Control Box and Optics Box.

Power Entry

The Control Box is compatible with both 120V/60Hz and 240V/50Hz AC standards. Fuses are accessible with the use of a small flat head screwdriver in the power entry unit. Fuses should be replaced with 5X20mm, 3A, slow blow fuses if necessary.

FluoroPlex Optics Box

There are four lockable dials on the FluoroPlex Optics Box that are available for the adjustment of the LEDs' brightness and the photodiode's gain and offset. All other hardware control is handled through the FluoroPlex Controller interface. Please see the section on the Hardware Control Menu to learn about gaining direct access to LED and bank control and the photodiode readout. See the Edit Protocol Menu section to learn about setting up the run time protocol.

Power should be applied to the Optics box at least ten minutes before data collection begins to allow the output of the sensor electronics to warm up and stabilize.

Optics Box

To FluoroPlex Controller

Only the IonOptix custom cable that is shipped with the system should be used to connect the FluoroPlex Optics Box to the Controller.

To Optical MultiPlexer

The MultiPlexer 9-pin cable should be plugged into this connector.

Excitation Intensity Control

Excitation Intensity Control Dials

The dial labeled "Path 1" controls the LED used to create the numerator in ratiometric measurements and the output signal in single excitation measurements. "Path 2" is unused in single excitation experiments and creates the denominator value in dual excitation experiments. See the Hardware Adjustments App Note for further discussion.

Sensor Control

Sensor Gain and Offset Control Dials

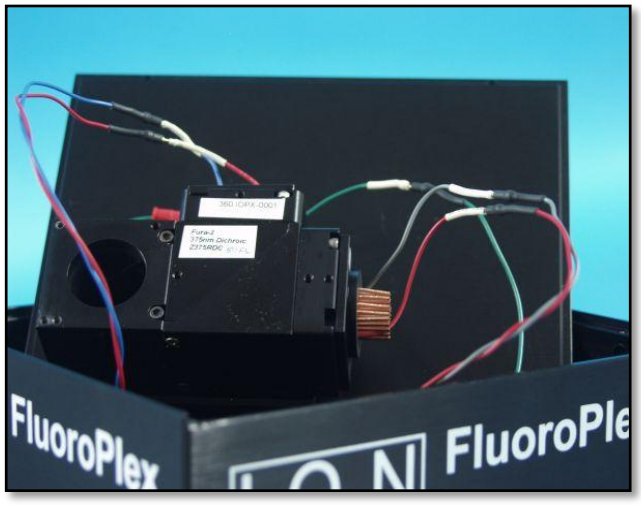
The photodiode "Gain" knob controls the amplification of the signal produced by the photodiode. There are some cases when the background signal from auto fluorescent tissue components is expected to be high relative to the signal. There is also a small dark signal produced by the hardware itself. The "Offset" adjustment is intended to allow the user to subtract off this baseline in order to focus in on the region of interest. See the Hardware Adjustments App Note for further discussion.

Emissions Optics

Emissions Optics

The emissions optics come assembLED onto a 2X4 inch plate that is labeled with the intended dye and mounted onto the side of the Optics Box.

Excitation Source



Excitation Source and Optics

The LEDs and excitation optics are mounted onto the lid, which is labeled with the intended dye. The 5 pins on the lid's wiring harnesses should be plugged into Optics Box wires of the matching color (Red = 12V, Green = GND, Blue = Path 1 signal, Gray = Path 2 signal) that are thread into the upper compartment of the Optics Box.

MultiPlexer

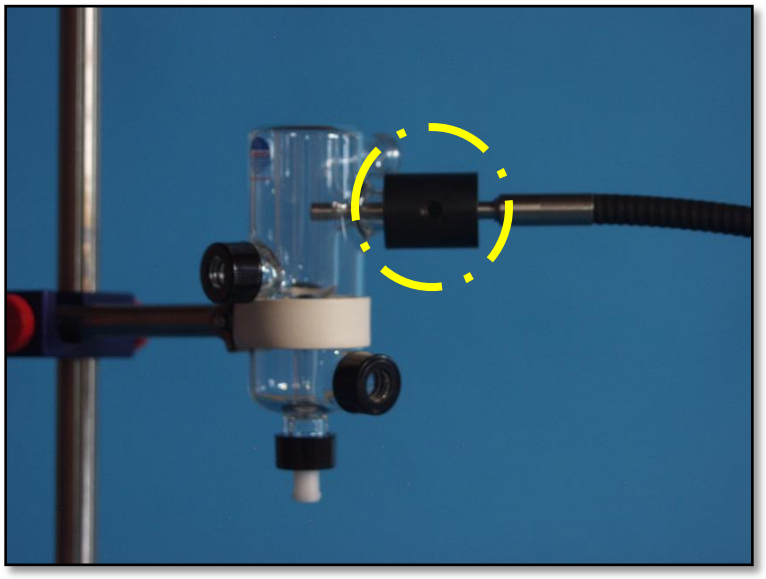
The set screws securing the light guides are only accessible when the MultiPlexer is removed from the lid. A white triangle marks bank position #1. Insert the light guides with their spacers into the available spots, starting with position 1 and secure them with set screws using a .050 hex driver. Secure blockers into any remaining positions to minimize unwanted background noise.

The MultiPlexer should then be inserted into the MultiPlexer mounting block on the lid, plugged into the Optics Box connector labeled “To Optical MultiPlexer”, and then secured by tightening the set screw on the side of the mounting block using a 5/64 hex driver.

It will zero automatically when the Control Box is powered on.

Liquid Light Guides

The light guide tip should be positioned such that it abuts the specimen under investigation. A black threaded collar screws onto the bath's FluoroPort to affix and seal the septum. A set screw fastens the collar and locks the position of the light guide.



Radnoti bath with FluoroPort and LLG (The collar and fastening screw are highlighted.)

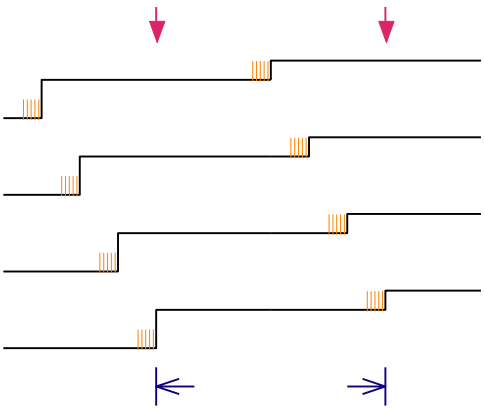
The LLG's accompanying the FluoroPlex system are designed to withstand saline solution. Care should be taken to clean the tip to remove salt and protein at the end of data collection. Please rinse the tip with DI water several times, followed by 70% ethanol. Dry the tip carefully with objective lens paper to prevent scratching the quartz.

Software

Software Overview

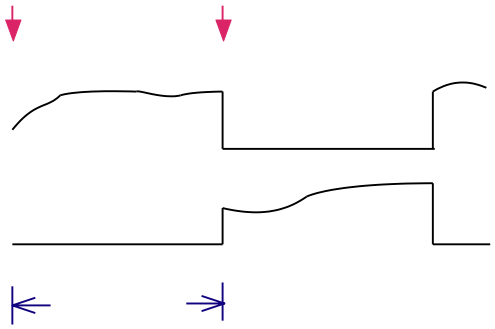
The FluoroPlex's intelligent control provides a simple means to execute experimental programs as well as background collection and signal averaging tasks.

Continuous Mode



Continuous Mode will constantly monitor fluorescence from multiple baths, ideal for high-throughput calcium recordings of multiple smooth muscle baths. In the Continuous Mode, data will be collected at a certain bank for a user-specified time (milliseconds). This data is averaged and output as a single data point before the FluoroPlex switches to the next bank. Signal averaging is an effective means of reducing signal noise when fluorescence changes are slow enough to allow it. It takes approximately 70 ms to complete ratiometric data collection from 4 banks if only 1ms of data is collected per bank; i.e., no averaging. A 10Hz data rate allows 4 averaged points per bank for a 4-bank system. It is also possible to select slower data rates if minimizing bleaching is necessary. Analog data will be updated immediately at the end of the data collection at that bank, and the value will be held until the new data for that particular bank is ready.

Burst Mode



Burst Mode is ideally suited for monitoring fast changes in fluorescence, such as calcium transients in striated muscle. In Burst Mode, data is collected at each bank for a specified number of seconds. Each data point taken is immediately available on the analog output for that bank allowing data collection at 250 Hz for dual excitation or 1000 Hz for single excitation. Banks that are not currently collecting data will have their outputs set to the lowest value to make differentiating the active bank from inactive banks easier.

Background Collection

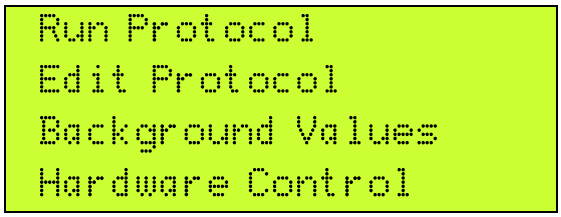
Prior to data collection, background signals should be collected for each tissue bank. 64 data points will be collected and averaged to calculate Numerator and Denominator values for each bank. These values will be stored and subtracted from the signal values prior to ratio calculation (Dual excitation mode) or conversion to analog representation (Single excitation mode). See the Background Collection App Note for further discussion.

Software Operation

There are several options/controls available to the user through the interface. Turning the knob performs scrolling and adjustment functions and pushing in the knob ("clicking") performs a select/confirm function. There is the Main Menu and four submenus: the Run Menu, the Edit Menu, the Background Menu and the Hardware Menu.

Main Menu

The Main Menu initiates data collection and provide access to the submenus.



Run Protocol The selection of Run Protocol immediately initiates data collection and pulls up the Run Menu.

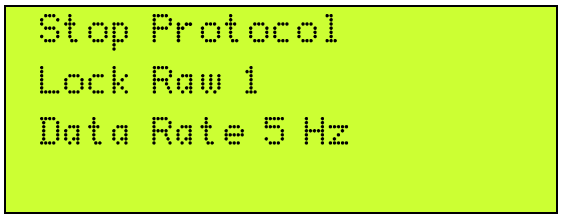
Edit Protocol The selection of Edit Protocol pulls up the Edit Menu where all the protocol setup is done.

Background Values The selection of Background Values pulls up the Background Menu where values can be collected, zeroed, and viewed.

Hardware Control The selection of Hardware Control pulls up the Hardware Menu where the outputs can be configured and the hardware manually controlled for testing purposes.

Run Menu

While data is being collected, the Run Menu will be displayed.



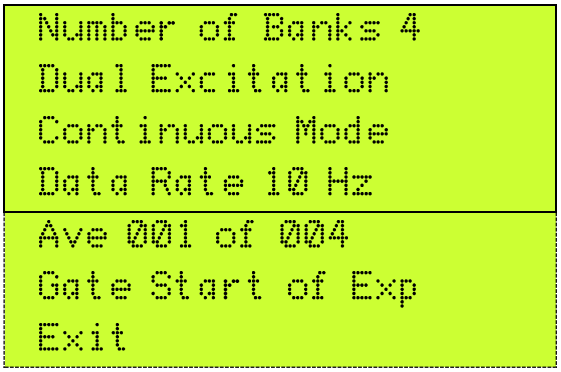
Stop Protocol — The selection of Stop Protocol immediately stops data collection and returns the user to the Main Menu.

Lock Raw — Adjust this value to select the bank or banks reflected in the analog signals on the Raw Output Num and Den BNCs.

Data Rate — This read only line displays the rate at which new data is available. The data rate can be adjusted in the Edit Menu.

Edit Menu

The Edit Menu allows access to all user programmable features. All values are saved immediately upon adjustment.



Number of Banks — Adjust this value to set the number of banks to collect from. Eight banks are available unless Dual Excitation and Output Raw are selected, in which case only 4 are available.

Excitation Type — This option selects for use with a dual excitation or single excitation dye. If single excitation is selected, the fluorescence light source will stay in the numerator path (Path 1).

Protocol Type — Continuous Mode and Burst Mode protocols are available. In Burst Mode, the option has the form "Burst Mode ### sec". The number of seconds selects the length of time collection occurs at a bank before switching to the next bank.

Data Rate — The frequency on this line reflects the rate at which new data is available at the outputs. In continuous mode, the rate reflects the rate at which new data is available

for all banks. In burst mode, the rate reflects that of the stream of data being output by the currently active bank. Decreasing the data rate creates a delay during which either the LEDs will be turned off to reduce bleaching or additional data points can be collected and averaged together. The maximum rate is affected by excitation method (single or dual), protocol type (Burst mode or Continuous mode), acquisition time and number of banks.

Averaging

"Ave Pts ### of ###" describes the number data points being averaged out of the maximum available. The maximum number is affected by the number of banks, excitation type, protocol type and data rate. Decreasing the data rate will make more data points available for averaging. Data will be collected at each wavelength for the specified number of data points. This data will then be averaged to create one output data point. While averaging data points together provides an effective means of diminishing signal noise, care should be taken not to reduce the data rate and subsequent temporal resolution too severely. Care should also be taken to minimize photobleaching and phototoxicity. Averaging data points will increase the amount of time that tissue and fluorophore are exposed to illumination.

Gate

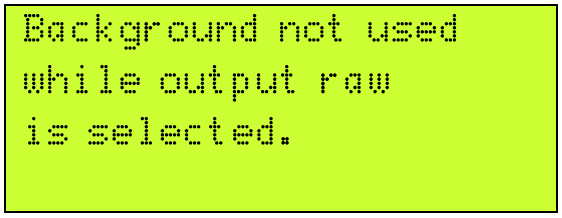
These options coordinate a TTL output pulse on the BNC labeled "Gate" on the front panel with one of four hardware events: (1) at the beginning of an experiment protocol, (2) at the beginning of an acquisition cycle when the MultiPlexer is positioned at the first bath, (3) accompanying each move to a new bank, or (4) accompanying the output of each new data point.

Exit

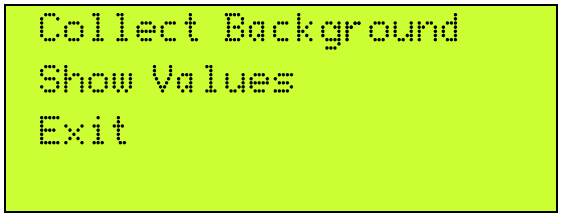
Selecting "Exit" returns to the Main Menu.

Background Menu

One of two possible menus will be displayed depending on the "Output" setting in the Hardware Control Menu.



If "Output Raw" is selected, no background subtraction will occur and so the display will temporarily show the above message.



If "Output Ratio" is selected, background subtraction will occur and the following menu options will be displayed.

Set Background Values

Three options are available on this line.

- Use Current Values does nothing.
- Collect Background initiates the collection of new values. Collect Background should be selected when it is appropriate to do so; most often, when the tissue to be investigated is mounted in the chamber but not yet loaded with fluorophore. The FluoroPlex will initiate interrogation of the tissue at all banks. Background values reflect the endogenous fluorescence of the tissue as well as other sources of stray light. These values will be automatically subtracted from the recorded photodiode values, reflecting only the values originating from the fluorophore. No adjustement of LED intensity or sensor gain should be made after background collection.
- Zero Background sets all background values to zero.

Show Values

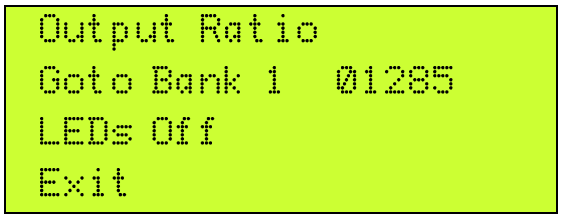
Selecting "Show Values" pulls up a screen that will cycle through the current values and return the user to the background menu. The values screen is not manually editable and the user will be locked out of the controls until all banks have been displayed.

Exit

"Exit" returns the user to the Main Menu.

Hardware Control Menu

The Hardware Control Menu allows the configuration of the outputs and provides some direct access to hardware.



Output Configuration

- In the "Output Ratio" configuration, the bath output connectors labeled 1-8 on the back panel always represent the outputs of banks 1-8 respectively. In the case of a single excitation experiment, they represent background subtracted data. In the case of a dual excitation experiment, they represent the ratio of background subtracted numerator to background subtracted denominator.
- In the "Output Raw" configuration, no background subtraction or ratio computation is done. In the case of a single excitation experiment, the bath outputs connectors labeled 1-8 on the back panel represent the outputs of banks 1-8 respectively. In the case of a dual excitation experiment, both the numerator and denominator values are output and only banks 1-4 are available. The mapping of bath output connector to signal is shown in the following table.

	Bank 1	Bank 2	Bank 3	Bank 4
Numerator	**1**	**2**	**3**	**4**
Denominator	**5**	**6**	**7**	**8**

MultiPlexer and Photodiode

Line 2 of the Hardware control menu allows the user to move the MultiPlexer between banks while watching the output of the photodiode either on the display or by way of Bath Output 1. The output of the photodiode is displayed as a number between 0-4096 (or slightly higher). A display reading of 0 correlates with an analog output on the Bath Output connector of 0 V and a display reading of 4096 correlates with 5V viewed.

LED and Photodiode

Line 3 of the Hardware control menu allows the user to turn the LEDs on and off while watching the output of the photodiode either on the display or by way of Bath Output 1. Only one LED is turned on at a time, so the options are LEDs Off, Denominator, and Numerator. The output of the photodiode is displayed as a number between 0-4096 (or slightly higher). A display reading of 0 correlates with an analog output on the Bath Output connector of 0 V and a display reading of 4096 correlates with 5 V. An unchanging reading of over 4000 means that the signal from the photodiode circuit is saturated. Turning down the LED brightness, the photodiode gain or the photodiode offset are all effective at lowering the value. An unchanging reading of 00001 means that the photodiode circuit is in negative range. The photodiode offset should be turned down until the signal is in range.

Exit

The MultiPlexer will move back to bank 1 and both LEDs will turn off. The user is then returned to the Main Menu.

Appendix

Application Notes

Hardware Adjustments

Excitation Intensity and Sensor Control

There are four knobs on the Optics Box that the user can use to control the signal. It will probably be a bit of an iterative process at the beginning to find the desired settings. It is the intent that once these values are found, that the dials will be locked and not need further adjustment. The value of the dials should be recorded by the user so they can be returned to if inadvertently changed. These values must not be changed after background values have been collected (see Background Subtraction App Note). Changing these values will have an effect on the ratios, making it difficult to compare two experiments.

The brightness of the LEDs are independently controlled by the Excitation Intensity Path 1 and Path 2 control knobs. The signal emitted by the photodiode can be offset using the Sensor Offset knob and amplified by the Gain knob.

Hardware Control

The easiest way to see the effects of your adjustments is by going into the Hardware Control Menu by selecting "Hardware Control" from the main menu. The third line will default to LEDs off. Scrolling to that line will allow you to select "Path 1 ON" or "Path 2 ON". That selection will turn that LED on (the other will be turned off) and will display the readout from the Sensor. The full range of numbers is zero to slightly over 4000. Concurrently, a voltage representation will be sent on the Raw Num output with its range of 0-5V.

The Goal and Factors to Consider

The goal is to record the complete signal with as good signal to noise and as little bleaching as possible. There are several factors.

Signal Amplitude

Turning the LED intensity up and turning the Sensor gain up will both result in signal increase. There are some differences to understand.

- The sensor gain will amplify the signal from both Path 1 and Path 2 equally. Adjusting the sensor gain will have some effect on ratio, but not much. The LED intensity adjustment is independent. Adjusting one LED without the other will have a huge effect on the ratio.
- The sensor gain amplifies noise along with the signal. Turning the gain down and turning the excitation intensity up will increase your signal to noise.
- Light bleaches the dye and creates phototoxins. The less light, the better for your tissue preparation.

The denominator (Path 2) value will go down with the influx of Calcium. The nominator value (Path 1) will either stay the about the same (if the isosbestic wavelength of the dye is being used) or will increase. At rest, a ratio of about 1 is usually chosen. Adjusting the hardware so that resting tissue gives values about halfway into the range of FluoroPlex (about 2000 on the Hardware Control Menu display or 2.5V on the output BNC) will start you with a ratio of one and give both signals space to move.

Signal Offset

The goal of the offset is to eliminate signal that comes from anything besides your dye. The hardware itself produces some small signal and some tissue components are auto-fluorescent. Subtracting this signal off will make your ratio changes larger and truer. There are two methods to do this, the "Sensor Offset" knob and software background subtraction (see the Background Subtraction app note). The intent is that software background subtraction will be done for every single experiment because this offset will be slightly different for different pieces of tissue. The intent of the knob is to subtract off the offset that you can count on to be there every experiment so that the range of the device can be put to use focusing on the signal. If

you have a small signal on top of a large offset, you may saturate the signal before you can increase the gain of the sensor as much as you would like. By using the "Sensor Offset" knob to decrease the offset, you will be able to turn the gain up higher and stay in range. If you see a sensor readout of 00001, it means that the signal has become negative and you need to turn this knob down. If you are still seeing 00001 when the knob has been turned all the way down and the device has warmed up at least 10 minutes, call our technical support.

Suggested Pathway

- Block all MultiPlexer paths except bank 1. Set up your bank in a relatively light tight enclosure.
- Turn the device on and let warm up at least 10 minutes.
- Start with a non-loaded tissue sample in your bank.
- Turn the sensor gain all the way up.
- Turn the LEDs up to 4.
- Adjust the offset knob such that the signal is about 100 counts.
- Load your tissue with dye.
- Adjust the LEDs until the counts are about 2000.
- Exit from the Hardware Menu and try running an experiment. You will want to record both the output from bank 1 and the Numerator and Denominator outputs. Make adjustments such that all three signals stay in range.
- PRACTICE, PLAY AND REPEAT before you try to collect data. Dye loading will almost certainly present a very steep learning curve. It can be difficult to get dye into the cells at all. Dye can get trapped in areas of the cell that are not excitable. Too much dye can disrupt the normal Calcium flow. Timing, concentration of dye and temperature all have an effect. Feel free to adjust the hardware knobs all you want at this point to get the best looking data you can.
- TRY IT OUT. Once you have a loading procedure down, try locking down the knobs and seeing if they work for a number of tissue samples. Adjust as needed.
- RECORD SETTINGS AND LET IT BE. Ideally, you will want to leave the knobs alone so that you can most accurately compare the results of your experiments.

Background Subtraction

Background subtraction is a crucial part of ratio and ion calculation. Unfortunately it can be a very confusing subject. This section of the manual will attempt to clear up this issue.

What is background?

Background is the signal reported by the instrumentation in absence of indicator fluorescence. Background can be caused by stray light, internal reflections, electrical noise and auto-fluorescence. The amount of background is an indicator of how much noise there is in a system.

A fluorescence recording is the sum of the background emission and the emission of the indicator substance. Ratios calculated from non-background subtracted signals can be quite different from ratios calculated from corrected signals. This is because small changes in the denominator will have a greater change than small changes in the numerator. This will not be a baseline change from which the relative signal changes will still be informative. These are non-linear errors that will lead to false ratios from which no real information can be extracted. For this reason it is imperative to use background subtracted data to perform ratios.

Due to changes in auto-fluorescent components of tissues, electronics characteristics, power supply voltages, and filter performance it is necessary to get background values on a regular basis. Additionally, the background values are a loose indicator of overall system performance. Very large backgrounds may be indicative of failed filters, failed diachronic mirrors or stray light leaks.

Background Subtraction Options

- If the loading protocol allows it, it is easiest to acquire the background value first, before any fluorescent dye is loaded into the tissue. First, scroll to the FluoroPlex's "Hardware Control" option on the Main Menu. Make sure the first line is set to "Output Ratio". Exit to return to the Main Menu and select the "Background Values" option. Select "Collect Background" to automatically record, average and save a background value for each wavelenth from each bank. You can view these values by selecting "Show Values". These background values will be automatically subtracted before the ratio is calculated. The analog value on the "Bath Output" BNCs will reflect the true ratio stemming from the fluorescent dye.
- Loading protocols may make it impossible to collect background data before loading the fluorescent dye. In this case, manganese is used at the end of the experiment to quench the dye and background subtraction has to be done as a step in the analysis of the data. This means that the user must acquire both the numerator and denominator values. Scroll to the FluoroPlex's "Hardware Control" option on the Main Menu. Make sure the first line is set to "Output Raw". This will cause the FluoroPlex to send non-background subtracted, raw numerator and denominator signals to its Output Bath BNCs. (See the Hardware Control Menu section for more details.) The user should run the experiment, recording both numerator and denominator traces, and then record data after the dye has been quenched to get background traces. Average a background trace over a few seconds to get a single background value for that bank and wavelength. Now subtract that value from the data trace collected from that bank and wavelength. Repeat for all wavelengths and banks. Now divide the numerator trace by the denominator trace to obtain your ratio.