Fiber Photometry

USER GUIDE FOR RZ5P PROCESSOR

TUCKER-DAVIS TECHNOLOGIES

Updated: 3/12/2020

Notices

The information contained in this document is provided "as is," and is subject to being changed, without notice. TDT shall not be liable for errors or damages in connection with the furnishing, use, or performance of this document or of any information contained herein.

Contents

[This page is intentionally left blank]

Welcome

Hello and welcome to the Fiber Photometry User Guide. We appreciate you taking the time to view this document. First, if you are a TDT customer, then thank you – we greatly appreciate your business and we hope to help you meet your research goals. If you are considering purchasing a fiber photometry system from us, thank you as well – TDT is the industry leader in fiber photometry systems, and we have many successful and happy customers who use our products. We would enjoy nothing more than having you join the TDT family.

The objective of this document is to be a *hardware and software* instructional reference for all levels of fiber photometry users. This guide will not go into any meaningful details about the biological underpinnings for fiber photometry, calcium (Ca++) imaging, optogenetics, or other related fields. The successful use of your fiber photometry equipment is predicated on you knowing how to get fluorophores to express in cells and perform surgeries for *in vivo* monitoring of neural targets.

Getting Started

This section will cover initial hardware and software setup. Please carefully unbox your equipment and install the PO5e card or UZ3 interface according to your System 3 manual. Briefly – power down your computer* and place the PO5e card into an available PCIe slot in your computer. Next, install your TDT drivers and Synapse software from the USB Storage Drive that was provided with your shipment.

* TDT drivers only function on Windows machines. Synapse will not run on Mac or Linux.

Below is a table of helpful online TDT resources with which users should be familiar before starting.

**Table of TDT Resources**

Synapse Training Videos Narrated walk-throughs of the Synapse software. These are very helpful for beginner users first learning the Synapse environment	https://www.tdt.com/training-videos/
Lightning Videos ⚡ Short, unnarrated videos that demonstrate specific actions in TDT software. These are referenced several times throughout this document, so look out for the blue icon	https://www.tdt.com/lightning/
Knowledgebase Contains documentation for all TDT hardware and software. This is a great first resource for troubleshooting	https://www.tdt.com/knowledgebase/
Tech Notes Contain information about known hardware or software issues and asssociated solutions or workarounds	https://www.tdt.com/technotes/
Support Help TDT Tech Support offers phone and remote screen sharing support via GoToAssist to customers M – F, 8 AM – 5 PM Eastern Time. For remote screen sharing assistance, please email support@tdt.com to schedule an appointment.	https://www.tdt.com/support/

Setup

Establishing RZ processor and PC communication

Once the PO5e card is seated and TDT drivers and software are installed, you are ready to connect the RZ processor (designated as RZ5P henceforth but could be any RZ processor) and PC together. The orange fiber optic cables will be used for PC-RZ communication (see Sys III manual for more details). Please connect the fiber optics to the correct ports on the RZ5P and PO5e card, as shown in the diagram below (red optical connector to 'Out' or Red-labelled ports on RZ and PC). Note* that your fiber optic cable may be a different length.

PC-RZ communication
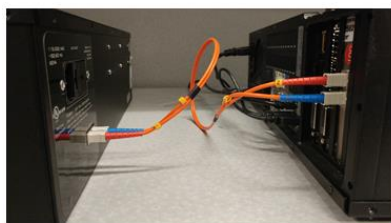
Full fiber optic connection   RZ5P   PC PO5e

Next, turn the RZ5P on. The display screen on the processor should illuminate with information about the unit's DSP cards (Run! u1 u2…). To check whether there is communication between the RZ5P and the PC, open zBusMon (shown to the right). The RZ processor should appear with information about the driver version and number of DSP cards. Click *Transfer Test* to test communication.

If there is an error in zBusMon, or you do not see your RZ appear, please contact TDT for assistance.
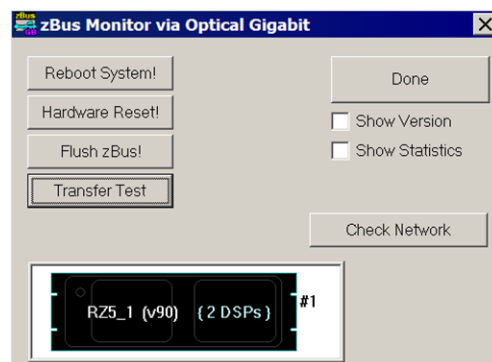
zBusMon

Launching Synapse

With your RZ5P on and connected, launch Synapse. The Rig Editor will appear, but it will be blank. Click *Detect* for Synapse to recognize your RZ5P. A PC, RZ5P, two DSPs, and a PZ5 will show up in the tree. If you have a PZ5, this is where you would enable it by checking the PZ5(1) box. Finally, click *Ok* to exit the Rig Editor. The Rig Editor may be accessed later for modification through the Synapse Menu.
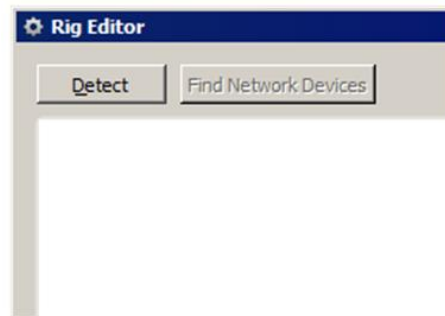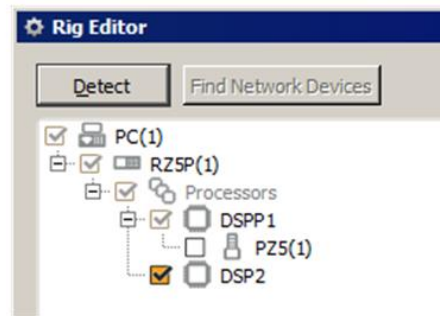

Detect →   Rig Detected

Your processor and any peripheral equipment declared in the Rig Editor will appear in the Processing Tree. For basic fiber photometry recordings, the experimental setup is simple. With the RZ5P selected, find the Fiber Photometry gizmo*‡. Drag and drop, or double-click, the gizmo onto the RZ5P to form a connection.

 * Version 90 and earlier of Synapse has the Fiber Photometry gizmo in the Neural category (red). In later versions, Fiber Photometry is in the Specialized category (blue). There is no functional difference between the two versions.

‡ You can learn more about gizmos and experimental connections in the Synapse Manual.
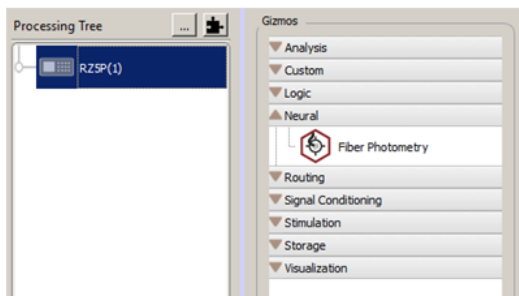
Drag Fiber Photometry Gizmo →   Connected
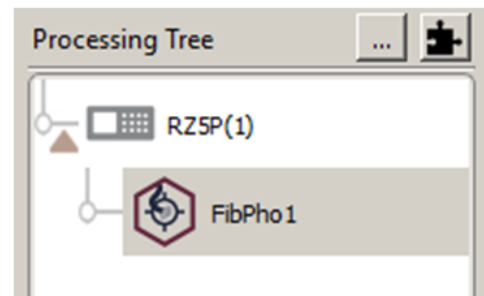
Connecting your fiber photometry equipment

Generic connection diagram for fiber photometry equipment.

The general connection scheme for fiber photometry equipment is shown in the above diagram. The RZ5P is equipped with four analog-to-digital (ADC) and four digital-to-analog (DAC) outputs. These connect to the optical components via BNC (coaxial) cables. The RZ5P DAC outputs connect to one or more LED Drivers for external modulation of Light Sources. The Light Sources then output light through a series of filters and dichromic mirrors, which send excitation light to the subject and receive fluorescence back.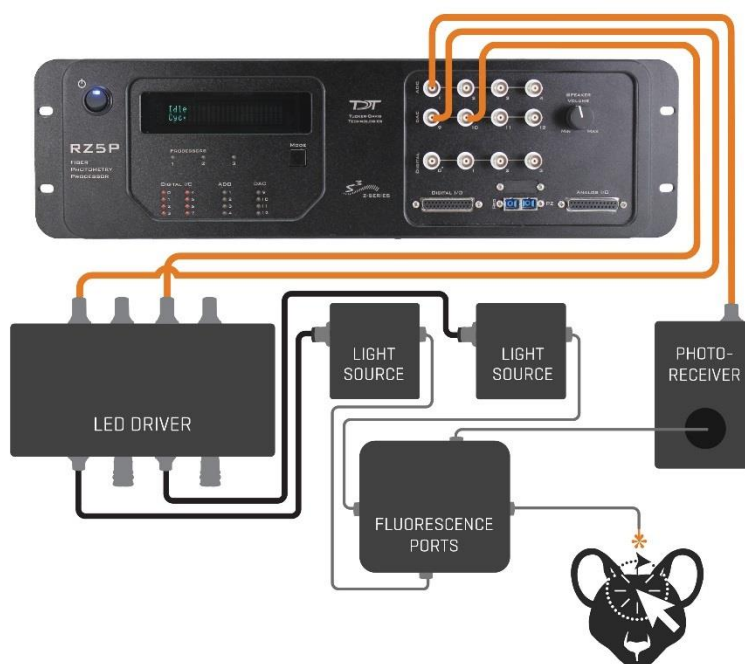 The fluorescence signals are then sent to a photo receiver, which sends its signal back to the RZ5P through an ADC input.

The most common LED Drivers are from Doric Lenses and Thor Labs. The same is true for light sources. Many labs will use Doric Mini Cubes as their light filters instead of creating their own optical benchtop, but both options are feasible. Finally, the most commonly used photodetector to date is the Newport Femtowatt photoreceiver with the appropriate fiber optic connector.

LED Driver settings – LED drivers must be set to external modulation mode (EXT or MOD). On the LED driver, the current for each LED channel will set the max output range. However, you will adjust the actual current level in Synapse. Fiber photometry is a low light power application, so the max output does not need to be very high. Setting the output ranges to 200 mA or greater should work well for most modern setups.

Common light sources – 405 nm (autofluorescence detection, isosbestic control), 465 nm (GCaMP), 560 nm (TDtomato, mCherry, RCaMP).

Fiber optic patch cords – We suggest a low auto-fluorescent specification if the manufacturer offers it. Some labs recommend getting silica clad fibers for this purpose. At least for Doric equipment, it is

common to use patch cords with attenuation filters (1%, 5%, or 10%) to reduce the power output of the excitation light sources before light reaches the fluorescent ports. Fiber photometry is a low light power application, and the Thor and Doric LED light sources can output very high power. Be careful NOT use an attenuating filter when sending light to the photoreceiver; this will severely diminish fluorescent output.

Mini Cube or Optical Benchtop – these need to be configured specifically for the wavelengths of light sources and fluorescent signals that are expected. Be sure to route the appropriate light wavelengths to the correct bandpass filter ports.

For example: with a 465 nm GCaMP + 405 nm isosbestic setup that uses a four-port Doric Minicube, the 465 light will route to E1, the 405 light to AE, the subject will be connected to Sample, and the output to the photoreceiver will be the F1 port.

Photoreceiver – for the Newport photoreceiver, the gain should always be set to DC Low. This provides the widest bandwidth of light detection and will enable the user to more easily detect signal clipping. Here is a link to the photoreceiver frequency response plots.

BNC (coaxial) cables are used to connect the to and from the ADC and DAC outputs of the RZ5P.

Adding a USB Camera

Configuration of low frame rate (20 fps) subject monitoring via USB cameras is simple in Synapse. Cameras can be added in the Rig Editor. Please follow this *Lightning Video* for specific instructions *https://www.tdt.com/lightning/#AddCameraToRig*

The Fiber Photometry Gizmo

The fiber photometry gizmo is the main interface for setting up and controlling your fiber photometry equipment. There are three tabs to configure your light sensors, light drivers, and demodulated data streams. Any single fiber photometry gizmo can support up to two photodetectors and four light sources. This is applicable for up to two subjects with independent control of two lights each. Additional gizmos can be added to the experiment easily to increase subject or sensor count.

Sensor(s)

This tab is used to configure settings for conditioning photodetector signals.

Name

This is the name assigned to the photodetector. While there is no standard convention, it is often easiest and least confusing to use A and B. The first letter of the sensor name will be appended to the store name of the demodulated data.

Source

The source of your sensor is the ADC input channel to which the output of the photodetector is physically connected. Thus, if the BNC for your photodetector is going to ADC Channel 1 on the front face of the RZ5P, you will select ADC-1 as the Source.

Calibration Factor

This value scales the data from the photodetector, but in general should need to be changed.

Clip Threshold

This value will be set once you know the maximum voltage the photodetector can receive. The clip threshold sets a voltage level above which a red clipping indicator light will turn on in the fiber photometry Run-Time window. The clipping threshold is a dummy light, so it cannot tell when the photodetector is clipping. It must be set correctly, by the user, to be calibrated. More on this in the Adjusting Parameters section.

A good practice is to add in a factor of safety to the threshold value of 0.5 V. For example, if my photodetector clips at 7.5 V (a common value for most Newport Femtowatt Photodetectors), I will set the Clip Threshold at 7.0 V. If the clipping indicator turns red once my Clip Threshold is calibrated, then I know too much light is going to my photodetector.

Demodulator

These settings affect the smoothness of the demodulated data stream. They are applied in real-time, so set these according to how you want the data to be saved.
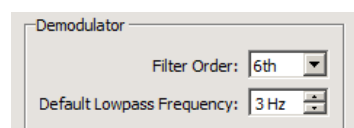
> **Filter Order -** This setting determines how sharp the low pass filter is that smooths the data. The default 6th order is most commonly used.

> **Default Low Pass Frequency -** This setting will determine the extent of the frequency content in the demodulated data stream. The minimum value is 1 Hz and the maximum is 20 Hz. Increasing the low pass corner frequency will add higher frequency content into your demodulated waveform. I think the default of 3 Hz is too low, as Ca++ signals can have a rise time of 100 ms – 300 ms, so some of the response characteristics may be attenuated. The value I prefer is 6 Hz, since this provides a nice visualization of Ca++ transients (fast rise and slow decay) during runtime. However, saving the full bandwidth at 20 Hz could be advantageous if later scientific reports show meaningful response dynamics above 6 Hz.

Light driver(s)

This tab is used to configure settings for modulating light sources.

Name

This is the name assigned to each light source. The typical convention is to name them after the wavelength of light each source is generating. For example, if Output 1 is your GCaMP signal, then you might provide a name of 465. The first three characters of this name will appear on the demodulated data stream store, with the last letter being the first of the sensor name.

Output

This option selects which DAC channel output drives a specific channel on the LED driver. The RZ5P has DAC channels numbers 9 – 12. When a light driver is enabled, and a DAC channel has been selected for the output, that DAC channel cannot be used again elsewhere in Synapse as an output.
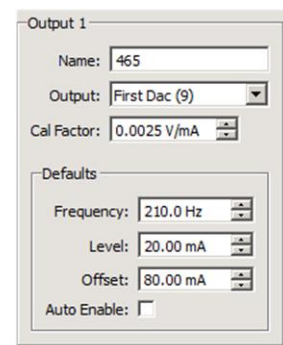
Cal Factor

The calibration factor is based on the mA/V conversion that the LED Drivers use for external modulation mode. As of January 2019, based on the LED Driver manuals, the standard Doric conversion is 400 mA/V

in normal power mode, 40 mA/V in low power mode, and Thor is 100 mA/V. The calibration factor is the inverse of these numbers. Thus, **the Cal Factor for a Doric LED Driver is 0.0025 V/mA or 0.025 V/mA and Thor is 0.01 V/mA.**

Defaults

These are adjustable parameters for modulating the light sources. The default values set here in design time will appear the first time the user goes into Preview or Record mode, or if the user chooses a Fresh persistence. At runtime, if any of these values are changed, and the user has Best persistence selected, then these values will not be used upon the next Preview or Record. Instead, the last value set in run time will be used. The defaults will, however, not be updated in the design time gizmo settings unless changed by the user.

> *Frequency –* This is the frequency at which the light source will be modulated. Each light source on each subject should be modulated at a different frequency for lock-in amplification to work effectively. Frequency has no effect on the power output. For more on choosing the frequency values, see the Run-Time section.

> *Level –* This is the peak-to-peak amplitude of the light source modulation. This will be the main parameter to adjust when changing power levels. This setting will be adjusted based on the desired light power output or level of response signal observed.

> *Offset –* This is the DC current offset to bias the light source. We will set this to the minimum current that turns the light on through a full modulation cycle. For a Doric-based setup, this is typically 1 – 50 mA. For a Thor-based setup, this is typically 0 – 5 mA. This setting will be adjusted during run time based on the characteristics of the raw photodetector signal.

> *Auto Enable –* This setting automatically turns on the LED drivers at the start of run-time if enabled.

Outputs and data saving

Gizmo Outputs

These options are used to output select demodulated data streams to other gizmos. By default, these are not enabled, and are not needed to save data. An example use case would be to send a demodulated GCaMP stream to another gizmo for online ΔF/F approximation.

Demodulator Save Options

This cross table (picture, right) is used to configure demodulated data streams. Users have the option to select a specific light driver demodulated from a specific sensor. The appropriate configuration will depend on how many lights and sensors are being used and on which subjects. The example picture is setup for a subject with 405 nm and 465 nm light sources, and fluorescent responses going to the same photodetector. This configuration will result in two demodulated data streams 405A and 465A that save during runtime. If a second sensor and additional drivers (a 560 light, or maybe more 465 and 405 for a second site or animal) were added, the 'B' column would be active, and the user would select the Driver x

Sensor combinations for each driver-sensor pair. Typically, one Driver is only ever crossed with one sensor, so having both A and B active for any one driver would not be desired.

"Demodulated" simply means that the relevant fluorescence data has been extracted from the raw photodetector signal and low pass filtered. You should think of this as your un-normalized ΔF/F or z-score*. These data are created using the principles of lock-in amplification. The basic concept is shown in the diagram below.

* ΔF/F and z-score are mathematical paradigms used to normalize and quantify relative change of a continuous time series. These are commonly used metrics in the calcium imaging field and, while methods for how to implement ΔF/F or z-score many vary, almost every report will have some version of them when showing demodulated data traces.
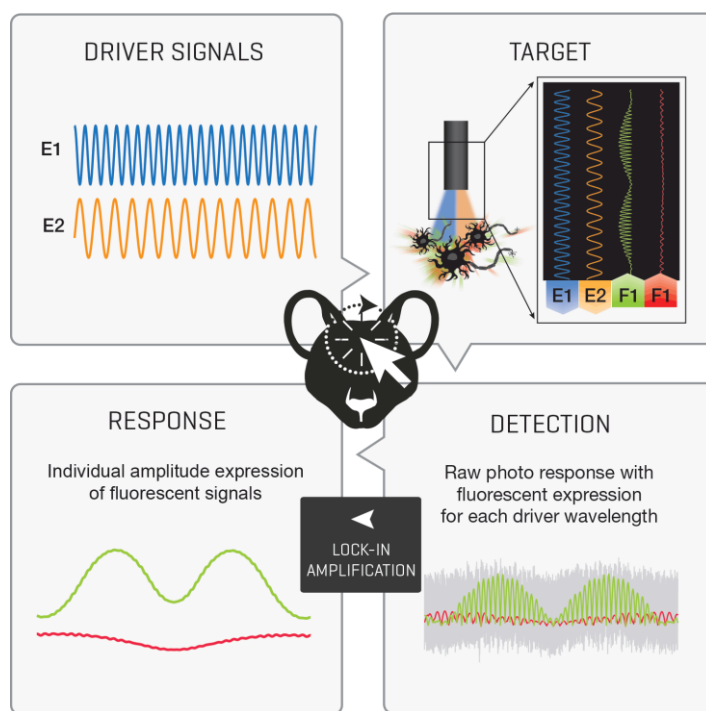
Lock-in Amplification

Misc Saves

Store Broadband Raw Signals – These data are saved as Fi1r. They are the sine waves used to modulate the LED Driver channels and the analog signal from the photodetector. For n Light Drivers, the first n channels of the Fi1r group are the driver waveforms. The remaining channels are the raw photodetector signal(s).

Store Driver Parameters – These data contain information about each light driver's parameters. A new timestamp containing these parameters is saved when the Light Drivers are enabled and whenever a setting is changed during runtime.

Run-Time

The run-time layout

The default layout – To the right is the default run-time setup for a fiber photometry gizmo configured to save two drivers, one sensor, broadband raw signals, and driver parameters. Continuous data streams are displayed in the Flow Plot tab. Order of data streams, or creation of multiple Flow Plots, can be achieved by adjusting RT or FP Setup at design or run-time. *Note, on first run, you need to autoscale the data stream by clicking the icon highlighted by the blue arrow.
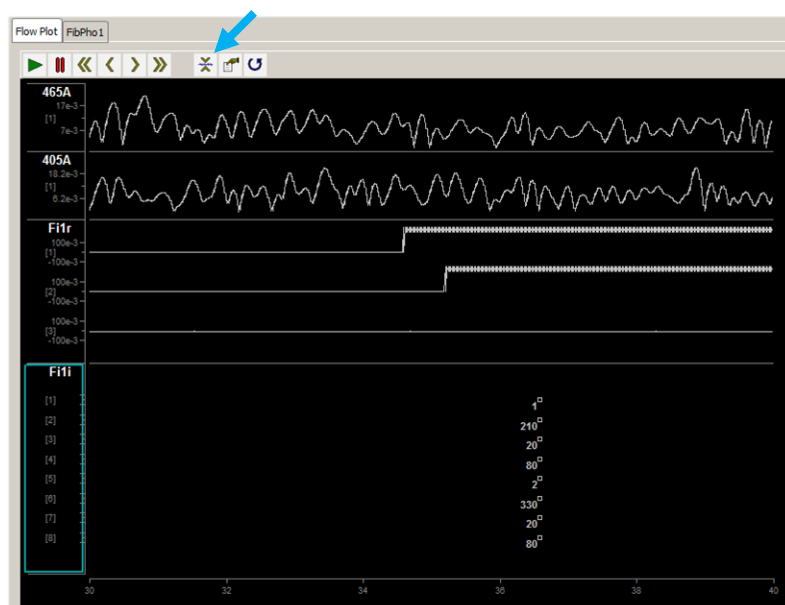
From top to bottom: 465A is the 465 nm driver data demodulated from sensor A; 405A is that for the 405 nm driver; Fi1r is the broadband raw signals (two driver waveforms in channels 1 and 2, one raw photodetector signal in channel 3); Fi1i are the driver parameters for each LED driver (driver number, driver frequency, driver level, driver DC offset, driver number…).

My preferred layout – My preferred setup is to view both the Flow Plot and the fiber photometry controls (and camera data, if applicable) in the same view. To do this, select the tab you want to move, right-click "FibPho1" → split → right.

I also want to easily recognize which demodulated data stream I am observing, so I color-code them (green is chosen for 465 nm instead of blue because blue and purple (of the 405 nm) look too similar, and 465 nm is for GCaMP). You can change the color of data streams by double-clicking the y-axis of that stream → Color Mode → Set Color. I also do not plot the Fi1i parameters because they take up a lot of space (FP Setup → move Fi1r to "don't plot). In most cases, I will also only view a single channel of the Fi1r group - the raw photodetector signal (double-click → Channels in View → 1, then autoscale just the Fi1r by right-clicking that data stream and selecting autoscale).
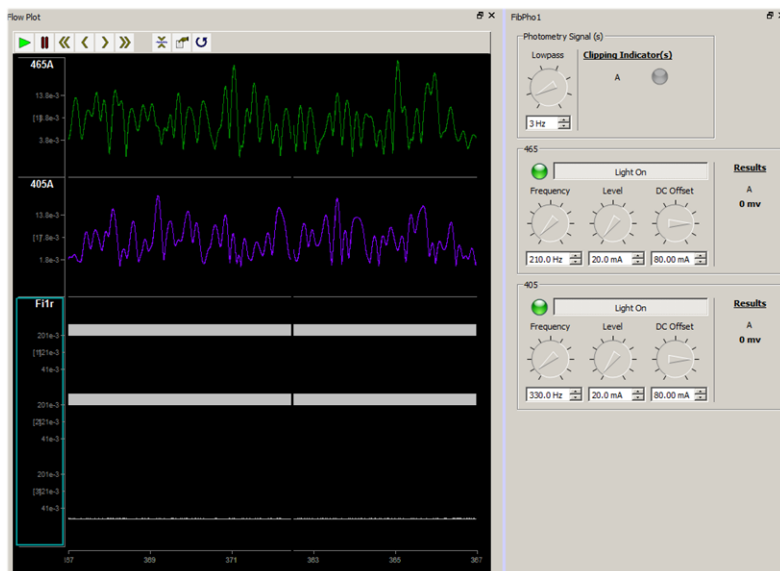
Fiber Photometry Controls – The FibPho1 tab contains all the parameter controls for that gizmo (there would be multiple control tabs for each separate Fiber Photometry gizmo). These are the same controls that were discussed in the Sensor(s) and Light Driver(s) tabs in The Fiber Photometry Gizmo chapter.

The lowpass filter on the demodulated data can be changed in real time from 1-20 Hz by manually entering a value or adjusting the knob.

The Clipping Indicator will illuminate red if the voltage levels of the analog photodetector signal exceed the clipping threshold set in the Sensor(s) tab in design time.

Each light driver can be toggled On or Off by pressing the Light On switch button. The light drivers are on when the green LED next to the button are illuminated. There is an option to Auto Enable lights in design time. Note* the physical light might not turn on until the appropriate Level and DC Offset are configured for that LED.

Frequency, Level, and DC Offset can be manually entered or adjusted using the knob. Valid Frequency values range from 1 Hz – 5 kHz.*

*Lock-in amplification works best when the driver frequency is high; the default values of 210 Hz, 330 Hz, etc. are good choices. Higher frequencies** (1 kHz and above, Synapse v90 or greater) can be used for specialized applications such as TEMPO (voltage sensor photometry). Valid Level values range from 0 mA – 1000 mA. Valid DC Offset values range from 0 mA – 100 mA.

** When running drivers at higher frequencies, make sure the acquisition processor rate (in the RZ gizmo) or the Required Sample Rate in the Fiber Photometry Gizmo (Synapse v91 or greater) is set high enough to avoid aliasing (at least double the driver frequency, e.g if you want to run a driver at 5 kHz you must set the acquisition processor rate to 12 kHz or preferably higher in Synapse).

The Results indicator displays the amplitude of the demodulated waveform for that Driver x Sensor. At low amplitudes (< 1 mV) the value rounds down to 0 mV.

Adjusting parameters

The best way to setup your LED driver parameters to start is to examine the raw analog photodetector signal in the Fi1r data stream on the benchtop. This signal will reveal characteristics of the LEDs with respect to Level and DC Offset. This section will start by setting up the driver parameters to get a desirable response in the Fi1r signal outside of an animal. The later portions will discuss how this setup translates to demonstrating correct signal demodulation with multiple LED colors and *in vivo* recordings.
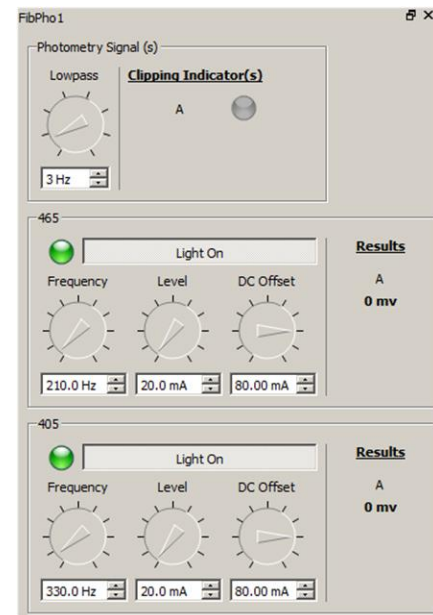
Summary Steps

1. Turn LEDs On/Off through Synapse control
2. Identify minimum DC Offset needed for full LED modulation
3. Identify the maximum voltage the photoreceiver can detect, then set the clipping threshold
4. Adjust the Level parameter and measure the light power output for protocol

Fi1r

With all your equipment turned on **(this guide is using an *older** Doric LEDRVP_2ch_1000, Analog mode, 1000 mA** per channel, 5% attenuating patchcords, 4-port (AE, E1, F1, Sample) minicube, Newport Femtowatt Photodetector on Gain=DC Low)**, start by isolating just the photodetector signal in the Fi1r stream in the flow plot (double-click Fi1r → Multi-Channel Viewing → Channels in View → 1 → Okay → drag up or down on the Fi1r plot with your cursor to change channels → autoscale as needed). We will focus on one light source at a time, so turn off all LED Drivers except one.

*Older LED Driver models + ConnectoriLEDs require more current for activation and do not have a low-power mode.

** For both Doric and Thor LED drivers, setting a value of 200 mA – 500 mA should be enough for these tasks. This setting will be the maximum current output for the LEDs, but the runtime value will be set in Synapse (valid up to the LED Driver current setting).

Our immediate goal is to make the light turn on/off at the end of the cannula when the Light On switch button is toggled. Find a DC offset value that enables this functionality. I recommend you start with 20 mA and increase the DC offset by 10 mA steps until the light turns on/off with the toggling of the Light On button.

Once you can turn the light on/off, double click the x-axis on the bottom of the flow plots and change the time span to 1 second. Next, change the frequency of the light driver in use to a low value, such as 17 Hz. Always autoscale and shift + drag as needed to better resolve the waveform. Changing the time resolution and lowering the frequency of the LED modulation will help us better view that is happening in the Fi1r signal. Recall that frequency has no effect on light power.

With the default light driver values, you may see waveforms like the image shown, where the Fi1r is at some DC voltage level, but no clear signal is resolvable. Likewise, you may see a sinusoid signal. Regardless, toggle the Light On switch button and check at the cannula whether your light (blue light, in the case of 465 nm) is turning on or off. With most LED drivers, a DC Offset = 80 mA should be enough to power on the LED. If your LED

is not turning on, try increasing the DC offset until it does or checking the LED Driver channel settings (these should be > 200 mA, MOD or External Modulation mode). Note that the Fi1r is on channel 3, which is the photodetector signal (drag up or down on Fi1r plot to change channels).

Note, the gain on the photoreceiver should be **DC Low** for all fiber photometry applications using Newport or Doric's integrated photodetectors. DC low provides the widest bandwidth of signal and will make low- and high-end clipping detection easier. If poor signal characteristics exist on DC, and "disappear" when the gain is set to AC low or AC high, then the problems are just being masked.
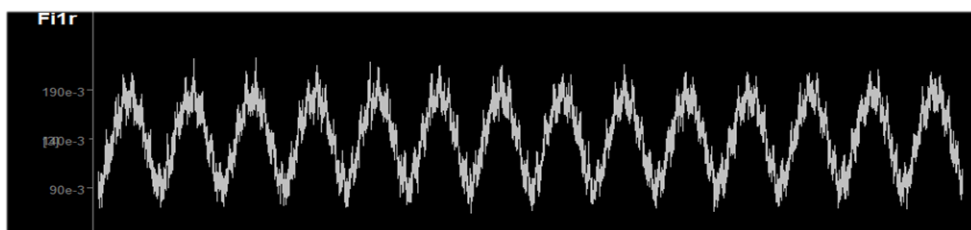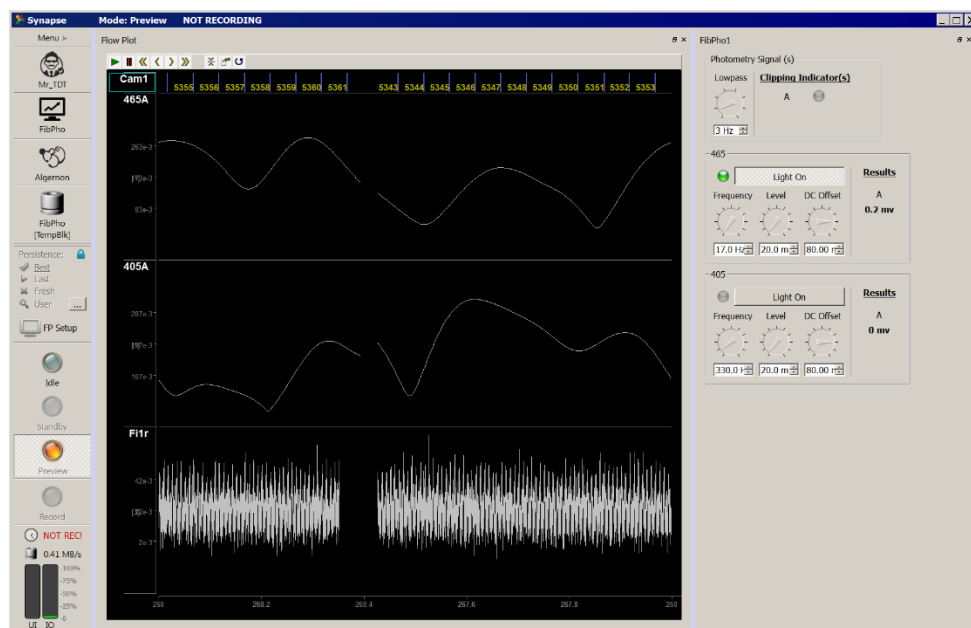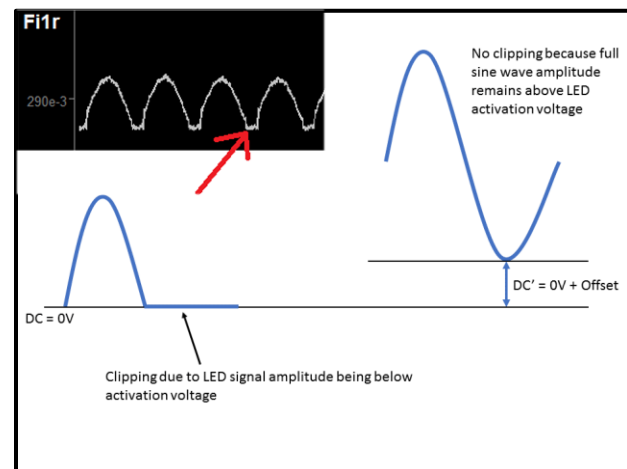
Setting the DC Offset

Once you are able to successfully turn the LEDs On/Off through Synapse, the next step is to set the DC Offset properly. It is best if you can see a sinusoid in the Fi1r signal, such as the one shown in the figure to the right. To accomplish this, point the cannula at a surface that will fluoresce, such as a yellow sticky note, a white piece of paper with yellow highlighter, or a fluorescent slide.

For my setup, I had to use a large Level to see a strong enough response. For most setups, this will not be the case and a Level < 100 mA should be more than enough.
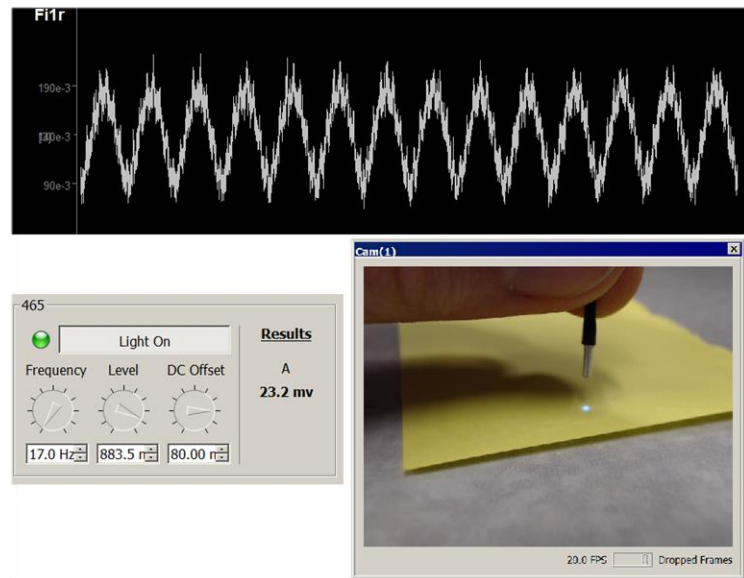
Our goal is to set the DC Offset to be as low as needed whilst still providing a full LED modulation signal. Shown in the diagram is the effect of DC offset on the LED waveform. LEDs require a bias current to work in a full linear range. The DC offset shifts the mean current going to the LED in order to fully activate the light. If the DC Offset is too low, the light will turn off during part of its period, as indicated by the red arrow in the Fi1r signal. The sine wave is flat at the bottom, and the LED is turning *off* for this part of its period. In this case, I had the DC Offset = 0 mA. Depending on your setup, this low-end clipping may be more pronounced or less.

Once you determine what the lowest DC offset is to prevent low-end clipping, double that value – this is the DC offset at which you should run your system. Doubling the value helps prevent artifacts in your demodulated signals caused by noisy environments. For Doric in normal power mode (cal factor 0.0025 V/mA) this typically yields a DC Offset = 40 mA. In Doric low power mode (cal factor 0.025 V/mA) the DC Offset is typically 10 mA. For Thor (cal factor 0.01 V/mA), a DC offset of 10 mA is common. These values can be higher than what is common, just make sure you are not saturating your light detector. Note that increasing the DC offset does increase the power intensity of your light, so be sure to measure the power output with a meter.

Setting the Clip Threshold

The clipping indicator in the fiber photometry run-time control tab can be useful for quickly identifying whether your raw photodetector signal is clipping. By default, the value is set to 3.5 V. However, the value must be calibrated for each photodetector. The Fi1r signal will help with this.

With the cannula still pointing at a fluorescent surface, increase the level of your light significantly, and get closer to the surface. The amplitude of the Fi1r signal should increase by a lot. Ideally, you will either see a sinusoid with a flat top, or a completely flat line (indicated by the blue arrow). Whatever the value of the flat line is the maximum voltage output of the photodetector (typically 6.5 V – 7.5 V). Use the shift + drag and ctrl + drag to narrow in on the exact value. Subtract some factor of safety, say 0.5V, from this value and input it into the clip threshold.
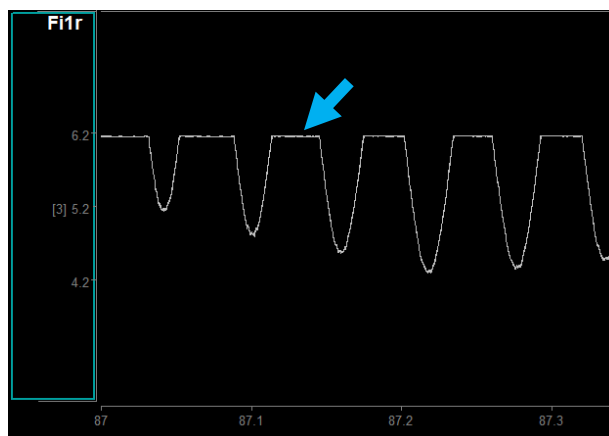
Setting the Level

For benchtop testing, you can use a Level that provides a clear sinusoidal waveform on your LEDs. In general, you should aim for using low light power, which means using as low a level as possible while still seeing a meaningful signal response. Ideally, your protocol would use a specific light power for all trials and subjects. Overall, the best way to set the level *in vivo* is to find the LED Driver current* (on LED driver, not the DC Offset) + Level setting that provides a desired power output at the cannula tip when measured with an external light power meter.

* The LED Driver current should be set at the maximum of your desired current range. The Level setting in Synapse will output the set current if it is within the Driver Current range.

Below is a table of approximate expected power output with a given Doric hardware configuration. This is meant to provide a general idea of current vs light power through a standard patchcord.

Doric Light Power Table: 400 µm/ 0.48 NA cannula system

| Configuration	Driver Current (mA)	Power at Cannula Tip (µW)
Standard	40 – 1000	400 – 10000
Low-Power Mode	4 – 200	20 – 2000
Low-Power with 5% attenuating patchcord filter	4 – 200	2 – 100

Benchtop Testing

Please change the LED driver frequencies back to the defaults of 210 Hz and 330 Hz. Lock-in amplification of the low-frequency fluorescent signals works best at high frequencies with a wide frequency separation between driver frequencies. Make sure your driver frequencies are not multiples of one another and not a multiple of mains power (50 Hz or 60 Hz).

Our goal for this section is to demonstrate detection of fluorescent responses for each of our LED signals. To do this, we will need surfaces of different colors to serve as controls. The figure to the right depicts how LEDs of different colors would respond to Black, White, Yellow, and Pink surfaces. For our 405 nm and 465 nm setup, we will be using Black as a neutral control, white paper as a control for 405, and yellow highlighter as a control for both lights. Highlighter is a cheap solution for accomplishing this task. If more specific responses are desired, you can purchase fluorescent slides from Ted Pella.
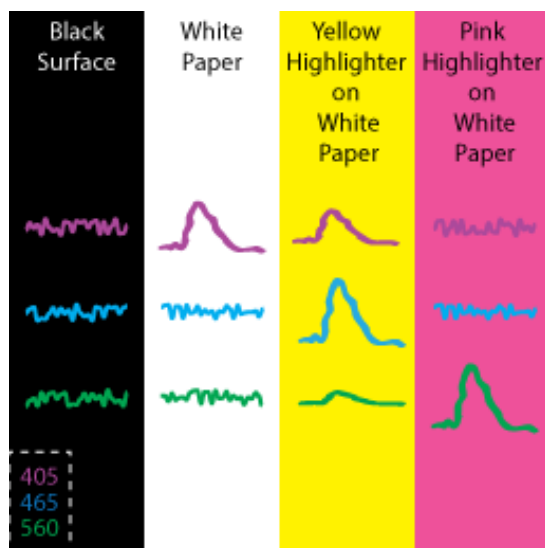
Shown in the Cam(1) images below is my *in vitro* setup for testing fluorescent demodulation. Both LEDs are on. To the right is a time-series of my response to each surface. Using a time span of 30 or 60 seconds is helpful for viewing (double click the x-axis on the bottom of the flow plots to change the time span). The black surface should have no significant fluorescence. As the cannula moves over the yellow highlighter surface, the amplitude of all signals increases because the fluorescence is non-specific but strong. When the cannula is over the white paper, only the 405 signal increases significantly.

Monitor your Fi1r signal and clipping indicator while doing this task. The demodulated signal will drop out if the light clips. This is because a clipped light is a DC signal, and thus there is no sinusoidal characteristics to demodulate. If you are clipping, try increasing the distance from your surfaces or decreasing the Level. If issues persist, see the Troubleshooting FAQ for more information.
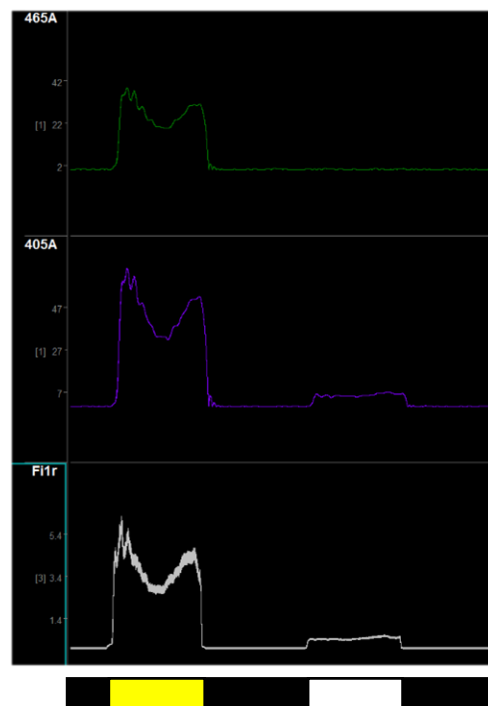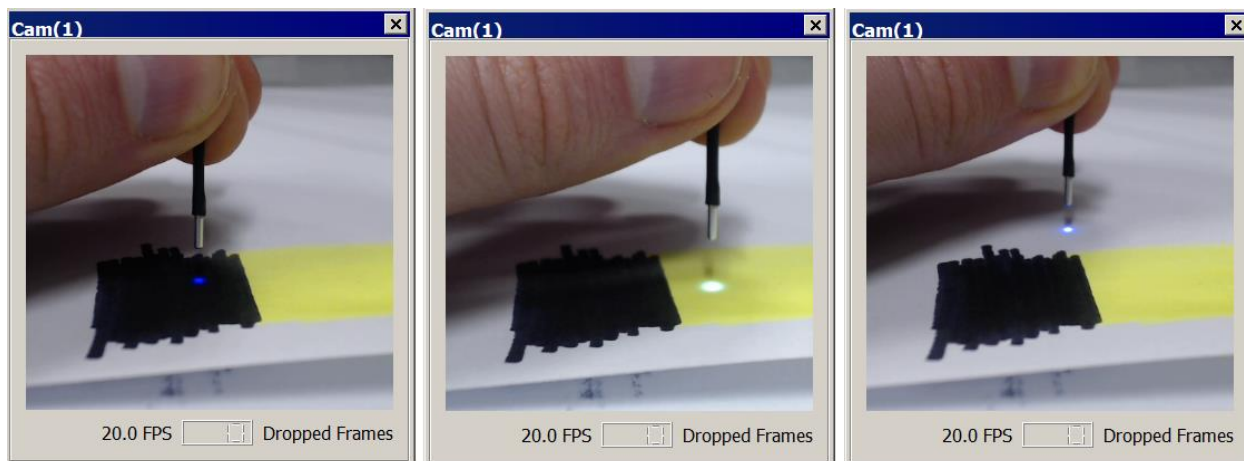
In-vivo Testing

Once you have confirmed system functionality, you are ready to test on a prepped subject. The procedure for checking the Fi1r signal and adjusting parameters is very similar to the benchtop method. The DC Offset you set *in vitro* should work *in vivo*. The Level you choose will depend on the desired light power and whether you see a response. If your light was very bright during *in vitro* testing, you will likely want to turn down the Levels as to prolong the risk of photobleaching*.

* Photobleaching is the overexposure of GFP to a light source that involves an irreversible change in the structure of the GFP protein. Long-term low-level light exposure and high-intensity light exposure will cause photobleaching. With photobleaching, users will see a decrease in response from the GFP and the response will be at a constant lower level.

With the cannula inserted into the implant sleeve, turn your LEDs on. Temporarily adjust the frequencies of your lights to low, but separate values (such as 17 Hz and 28 Hz) and look at the Fi1r signal. You should see the sinusoids like in the benchtop testing. Make sure you are not clipping on the high-end or low-end and adjust the Level if your waveform is too large. You should expect to see some noise on top of the Fi1r signal – if the Level is too high, the noise fuzz will not be present.

If the Fi1r characteristics are okay, adjust the frequency back to their original values (defaults or high frequencies with a wide frequency separation between driver frequencies that are not multiples of each other or mains power). Also adjust the time span to 30 seconds so you can observe fluorophore activity.

Allow the signal and the subject to settle for a couple minutes. Ideally, there will not be downward drift in your demodulated data streams. If there is, then consider turning down the Level. Once settled, perform either a startle (air puff, startle stimulus) or tail/foot pinch test, or another action that will invoke an expected response, and observe the demodulated data streams.

Below is an example demodulated trace with GCaMP responses marked by black ticks. This has the iconic sharp rise at the onset of activity, then a slow decay back to baseline levels. GCaMP responses across experiment and observed cell group types may be different, and the amplitudes will vary light intensity, cell count, animal age, and GCaMP expression levels. Note, this is the demodulated response curve and not ΔF/F, although the waveform shapes would look nearly identical if it was ΔF/F.
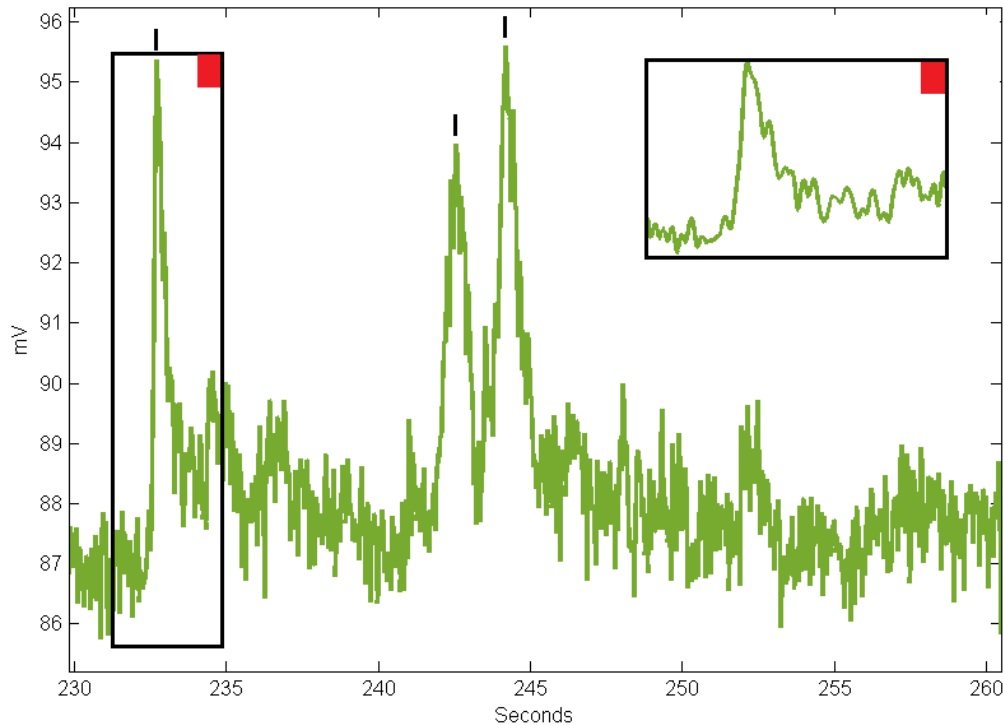
Data from Workbook Example

Motion Artifact

Motion artifact can occur during recordings. This shows up in the Fi1r and demodulated data streams as sudden changes in light and expression levels. The reason is because the cannula has shifted, so the cone of light, and thus the cone of fluorescent response, has changed. In order to detect motion artifacts, compare the isosbestic* 405 nm stream to the 465 nm stream. If you see similar sudden changes in the continuity of the streams (level is not important as each stream will be different) in both streams, then there was likely a motion artifact. An example is to the right, where you can see a sharp drop in the 405 nm signal, and an overall baseline shift in both signals after the event. It is important to recognize motion artifacts because they may sometimes appear as promising GCaMP responses in the demodulated streams.
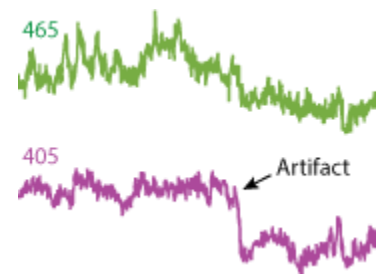
The ideal isosbestic control signal stays regular and flat during GCaMP activity, with only minor modulation that result from the demodulation process, as shown on the UV stream in the figure to the right. Data from Workbook Example
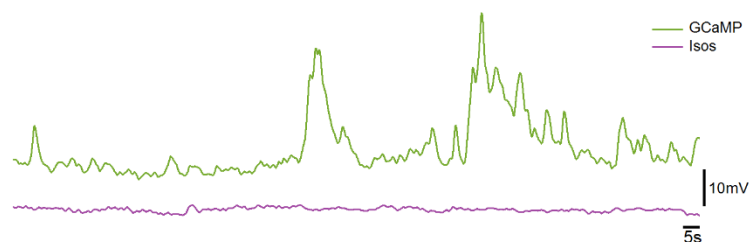
* From Wikipedia: *"In spectroscopy, an isosbestic point is a specific wavelength, wavenumber or frequency at which the total absorbance of a sample does not change during a chemical reaction or a physical change of the sample."*

* 405 nm is widely used as the isosbestic wavelength for GCaMP, as the total absorption of the UV light does not change during calcium activity changes (calcium independent measurement).

Easy First Targets and Controls

To verify system functionality in vivo, consider selecting easy areas that have GCaMP responses to simple stimuli (foot shock, tail pinch, reward), such as prefrontal cortex (PFC) or ventral tegmental area (VTA) or Barrel Cortex (stimuli is air puffs on whiskers), might be helpful for visualizing responses in subjects before approaching less characterized or harder to target populations.

Check with literature to see what standard controls are used to verify proper GCaMP activity. This often includes histochemical staining to confirm GCaMP expression within target cell types and sham recordings of animals without fluorophore expression during task trials.

If you are doing optogenetic stimulation, then performing controls is important to prove that the optogenetic light is not creating an artifact in the demodulated GCaMP data. This is because optogenetic stimulation wavelengths are close to those used in fiber photometry but are a much higher power, so there is a risk of light artifacts in the photodetector interfering with GCaMP data collection. A control could either be to stimulate in an animal without the opsin expression, but which has fluorophore expression, or to stimulate with the opsin expressed and record area without GCaMP expression. This is especially important if the opsin and fluorophore are in the same area and the light is being routed through the same fiber.

Run-time recording notes

Run-time recording notes are often very useful for marking when *in vivo* events, such as drug injections, occurred. These notes get saved as a text file in the data block. If Notes + Epocs is enabled, then a timestamp will also be added to the data. These epocs will be imported as a part of your data structure for later import. *Note, these should not take the place of programmatic timestamp markings of things like lever presses, foot shocks, lickometer events, etc. These more precisely-timed events are best implemented using the Digital I/O inputs (see Troubleshooting FAQ).

Lightning Video: https://www.tdt.com/lightning/#RunTimeNotes

Troubleshooting FAQ

My LEDs are not turning on.

Several factors affect whether the LEDs will turn on. First, check the LED Driver channels and make sure you are set to an external modulation mode and that the channel current is sufficiently high to drive an LED. Next, check the DC Offset setting in Synapse and make sure this is large enough to drive an LED. If this is still an issue, please check your BNC connections from the RZ5P to your LED driver and the connections from your LED Driver through to your cannula.

My Fi1r signal is always very high (6V or more) or a flat line at a high voltage.

If your signal is clipping on the high-end, try turning off the lights in your room. On the benchtop, ambient lighting gets picked up by the cannula and can add a lot of power to the photodetector signal. Ambient lighting will not be a problem *in vivo* because the brain is dark. If ambient lighting is not the cause of this issue, then adjust the power level of your LED driver down. If there is still a problem, then refer to the next FAQ point.

There is a very narrow range of LED Driver currents or Level settings that gives me a stable Fi1r signal. Outside of that, the LED is either off or I have high-end clipping.

The most likely issue is that too much power is going through your patchcords from the light source. Try lowering the current output on the LED driver, using Low-Power Mode (on Doric LED drivers), or putting an attenuating patch cord on the output of your LEDs.

My demodulated data stream has a steady downward slope in my subject.

You are likely experiencing bleaching or patchcord autofluorescence. One of the benefits of having an isosbestic control is that you can detrend signal bleaching in post processing using a 1st-order polyfit of the control to the GCaMP data (code in the Fiber Photometry Workbook Example) . However, it is best to reduce bleaching as much as possible online. Try reducing the power of your lights first and give it a few minutes to stabilize. If that doesn't help, there may be autofluorescence in your patchcords. To reduce this, connect each of your patchcords to the output of LED used to detect the fluorophore of interest (typically your 465 LED for GCaMP) and run the LED driver (don't need Synapse for this) on constant current (500 mA, CW) mode overnight (8 – 12 hours). This will photobleach the patch cord to remove the autofluorescence. You can read more about cable photobleaching from the Doric or Thorlabs manufacturer webpages.

I pick up 465 nm fluorescence on my 560 nm photodetector (crosstalk).

This is normal. If you are using two photodetectors (one for 405 + 465 or just one for 465, one for 560) and you are modulating the LEDs at different non-multiplicative frequencies (e.g frequency parameter set to 330 Hz, 530 Hz), then this is ok, because lock-in amplification will only extract the contributions of the relevant LED driver signal on each sensor. Just make sure that the 560 photodetector is not being saturated.

My demodulated signals have low-frequency or high-frequency sinusoidal artifacts in them.

If you are experiencing low-frequency sinusoidal artifacts (< 2 Hz) in one or both of your demodulated data streams, then it could be because your DC Offset is too low. Tech

Note 0991 has more information about this issue. Please read Setting the DC Offset for more details about properly setting the DC Offset.

If you are experiencing high-frequency (> 2 Hz) sinusoidal artifacts in your demodulated data streams, try changing the driving frequency. For example, if your 210 Hz signal has fast periodic oscillations that look artificial, try increase the driving frequency by a few Hz to 217 Hz to see if it goes away. If that doesn't help, try picking a frequency further away that is not a multiple on main line noise (50 or 60 Hz) or a multiple of your other driver light. If that does not work, then refer to the DC Offset note above.

I have tried all sorts of stimuli and levels, but I cannot get a response.

This is not an uncommon result, especially when you are getting up and running. Many factors can attribute to this, not all of which include:

Fluorophore expression – this is dependent on injection accuracy and virus uptake. Histology should be done on all subjects after the completion of experiments to verify expression.

Targeting accuracy – If the cannulas are not within approximately 1 mm of the injection site, then the ability to detect a signal will be compromised. Cannula targeting can be verified during histology.

Time since infection – Levels of GCaMP expression will decrease over time. The longer the time post infections, the lower the overall expression will be.

Photobleaching – Long-term low-level or high intensity light exposure can cause photobleaching of the GCaMP proteins. With photobleaching, users will see a decrease in response from the GFP and the response will be at a constant lower level.

Low Light Power – Under driving the LEDs can make it difficult to pick up a noticeable response during runtime. Try slowly increasing the light levels and retest. Do not increase the level too much, or else you may photobleach any GCaMP that is in the area.

Try recording from different animals in the same cohort if you prepped multiple animals. If problems persist, consider trying an easier or more common target to demonstrate that the system and your methodology can work, then try targeting different areas.

What do I need to add a second animal or second site?

The general rule is one photodetector and one set of dichromatic mirrors or minicube per site/subject. The RZ5P can control up to 4 independent light sources. A two animal, fully-independent 405 nm + 465 nm setup would have: four LED Driver channels, two 405 nm and two 465 nm LEDs, two sets of proper dichromatic mirrors or minicubes, and two photodetectors. Each subject would use its own Fiber Photometry gizmo. Multi-site setups on the same animal could share LED sources using a bifurcating cable going from the LED to each minicube or set of mirrors.

I want to receive digital TTL communication from an external device, such as MedAssociates. How do I do this?

This is a common feature that customers add to their Synapse experiment when doing behavioral work. The RZ5P has 24 bits of digital I/O communication. Four BNC ports are accessible on the front panel of the unit that correspond to Bits C0 – C3. Adding epoc markers to timestamp digital communication in real time is easy in Synapse by enabling Bit Input , Word input, or using the User Input Gizmo (v90 or greater).

I want to add optogenetic or some other external TTL-triggered stimulation to my experiment.
The Pulse Gen or User Input gizmos may be used to accomplish this. Be sure to route the gizmo outputs to the desired Digital I/O port on your RZ. Pulse Gen can be set up to trigger pulse trains based on gizmo inputs or external TTL inputs.

Post Processing & Data Analysis

TDTbin2mat and the MATLAB SDK

Exporting data from Synapse into MATLAB is simple with the TDT MATLAB SDK. The main importing function of the MATLAB SDK is TDTbin2mat. The main argument for TDTbin2mat is the full file path to the data block that you want to import. Synapse makes copying this file path easy via the History dialog. See the *Lightning Video* to see this importing sequence. You can also copy the block file path via Windows Explorer.

Link to the *MATLAB SDK*: https://www.tdt.com/support/matlab-sdk/
https://www.tdt.com/lightning/#Import2Matlab
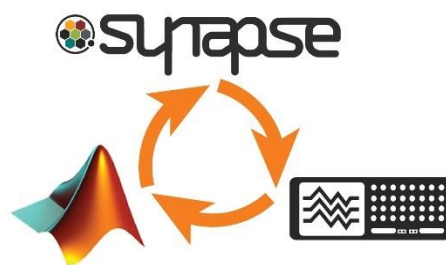
The TDT Python Package

Data can also be easily imported into Python 3 using the tdt package. If you already have Python 3 installed, you can add the tdt package in your cmd window: `pip install tdt`
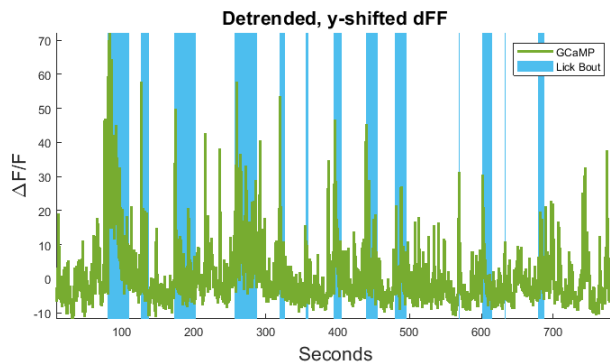
Link to Python Package and SDK: https://pypi.org/project/tdt/

https://www.tdt.com/support/python-sdk/

Release notes and select examples

MATLAB and Python Workbook Examples

TDT aims to help customers as much as possible with easy data import and analysis. We understand that not all customers have extensive MATLAB or Python experience, so we created fully-commented workbook examples that demonstrate how to do basic, but interesting operations with MATLAB or Python code. These examples are not intended to serve as a complete pipeline for your data analysis – please use wisely.

Link to Matlab Workbook examples: https://www.tdt.com/support/matlab-sdk/offline-analysis-examples/

Link to Python Workbook examples: https://www.tdt.com/support/python-sdk/offline-analysis-examples/

Fiber Photometry Epoch Averaging example (Matlab): https://www.tdt.com/support/matlab-sdk/offline-analysis-examples/fiber-photometry-epoch-averaging-example/

Fiber Photometry Epoch Averaging example (Python): https://www.tdt.com/support/python-sdk/offline-analysis-examples/fiber-photometry-epoch-averaging-example/

Lick Bout Epoc Filtering (Matlab):
https://www.tdt.com/support/matlab-sdk/offline-analysis-examples/licking-bout-epoc-filtering/

Lick Bout Epoc Filtering (Python):
https://www.tdt.com/support/python-sdk/offline-analysis-examples/licking-bout-epoc-filtering/

If you have other scripting needs, please reach out to TDT Tech Support.

View Data in OpenScope

For a first-pass replay of data, you can view any Synapse recording in OpenScope. This also takes advantage of the Synapse History dialog. OpenScope has extra features that make jumping around the data fast and intuitive. You can also use the Video Viewer feature to replay videos with the timestamp of each frame.

Using OpenScope: https://www.tdt.com/files/manuals/OpenEx_User_Guide.pdf#page=221

⚡ https://www.tdt.com/lightning/#ViewDataInScope

⚡ https://www.tdt.com/lightning/#VideoViewerScope

OpenBrowser – Exporting to Excel

If MATLAB is not your preferred data viewer, you can export data in an ASCII format into Microsoft Excel.

Using OpenBrowser: https://www.tdt.com/files/manuals/OpenEx_User_Guide.pdf#page=277

 https://www.tdt.com/lightning/#Export_to_EDF

Note* this video demonstrates exporting to an EDF file format, not ASCII.

More Resources

Here are some common resources that customers find helpful as they work to understand fiber photometry and conduct experiments.

TDT Fiber Photometry webpage: https://www.tdt.com/system/fiber-photometry-system/

Select fiber photometry papers:

Lerner et al. 2015 http://dx.doi.org/10.1016/j.cell.2015.07.014

Calipari et al. 2016 https://doi.org/10.1073/pnas.1521238113

Knight et al. 2015 http://dx.doi.org/10.1016/j.cell.2015.01.033

Barker et al. 2017 https://doi.org/10.1016/j.celrep.2017.10.066

Fiber photometry community forum: http://forum.fiberphotometry.org/

Tom Davidson's fiber photometry Google Drive:
https://drive.google.com/drive/folders/0B7FioEJAlB1afmRxa09oRlhmRzVCME9vSDVyZEQ0NkZh
RWFMbVh2MzItSkVmLUdQbTlkUUk

Lerner Lab Resources webpage: http://lernerlab.org/resources/