

# FP3002 Manual- Hardware

Summer 2023

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## Table of Contents

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<b>Important Safety Warnings</b>	4
<b>FP3002 Quick Start Guide</b>	5
<b>FP3002 Technical Specifications</b>	6
FP3002 Excitation and Emission Spectra	7
<b>Anatomy of the FP3002 System: <u>Micro B connection</u></b>	8
<b>Anatomy of the FP3002 System: <u>USB-B connection</u></b>	9
Accessories	10
<b>Required Materials for Fiber Photometry Experiments</b>	12
Patch Cord Guidelines	14
Fiber Optic Cannula Guide	18
Ceramic Split Sleeve Guide	19
System Requirements	20
<b>First FP Experiment Setup</b>	21
Download Software	21
Connect FP3002 System and Open Bonsai	23
Connect and Align Patch Cord	24
Set LED power	26
Configure Settings in the FP3002 Node	27
Build a Bonsai Workflow for Recording	28
Signal Check	30
Shut Down System	31
<b>Recommended Recording Parameters</b>	32
<b>Using Integrated Lasers for Optogenetics</b>	33
635 nm Laser Alignment	34
Using Light Blocks with 635 nm Stimulation	36
450 nm Laser Stimulation	38
<b>Determining LED Duty Cycle</b>	39
<b>Tips for Successful FP Recordings</b>	40
How to Cleave Fibers	40
Independence Test	42

Photobleaching Patch Cords	43
Example Surgical Protocol	44
Photometry-Guided Stereotaxic Surgery	46
Considerations for Behavioral Experiments	49
Setting up a Camera for Behavior Tracking	50
Patch Cord Management	51
<b>Additional Information and Scientific Background</b>	54
Isosbestic Points	54
Choosing GCaMP Variants	61
Best Practices for Novel Neurotransmitter Biosensors	63
<b>Troubleshooting</b>	66
Software and Hardware	67
Experiments and Photometry Signal	70

## Important Safety Warnings

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1. Ensure that your USB-B or microB (the cable from your PC, USB) is plugged into the back power housing unit of your system *before* booting FP3002.
2. Ensure that any BNC connections are made *before* booting FP3002. Please prepare your inputs and outputs with care. Input signals should be **no larger than 5V**.
3. Prior to turning on your system, decide if you will be using the 450nm or 635nm laser - make sure that your ribbon wire is connected to the appropriate laser. If you intend to use the 450nm laser, ensure that you have connected your secondary patch cord to the FC-FC connector on the front stub wall ([C] in diagrams below). Do not ever look directly into a patch cord.
4. Wear the appropriate laser safety goggles when using the lasers.
5. Do not unscrew any of the internal lids. Opening any internal lid will yield to a voided warranty. As of June 29th, 2021, an anti-tamper sticker has been added to the system to show signs of tamper, do not remove this sticker.

## FP3002 Quick Start Guide

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Inside your pelican case, you will find your FP3002. To get started:

1. Remove FP3002 from the Pelican case, and place right-side-up on the table.
2. On the back of the system, carefully plug in your USB-B cable and connect to a USB-3 port on your computer. Do NOT use a USB extension cable, or USB-hub, this will decrease data acquisition potential.
  - a. If your system has a Micro-B connection, your USB cable will connect to the front stub wall of the system.
3. Plug the power in - and toggle the “ON/OFF” switch in the back to “ON”.
4. The front facing LCD screen will show the system booting up.
5. The system was shipped to you with the focus locked. To unlock, lift the safety lid, and use the L-hex-key (1.5 mm) to unlock the translator knob set-screw, and then the straight hex-key (2 mm - through the front stub wall) to advance the carriage closer to the objective (i.e. further away from you).
6. Screw the appropriate (SMA or FC) connector onto your photometry patch cord. Thread this through the front face of the stub wall, and connect to the carriage with magnets.
7. After the system has booted up, open your Bonsai program (set the COMx port accordingly ) and make sure your parameters are as desired. Align your patch cord. Note that the focal point should be approximately 0.5mm from the objective.
8. If you intend to use the laser (for opto-stim) during this experiment, be sure to flip the laser key to “ON”. This key is in the box of accessories inside the Pelican case.

**CAUTION:** If you are using a 450nm laser, make sure your second patch cord is attached to the FC-connector on the front stub wall of FP3002.

9. You are ready to record!

## FP3002 Technical Specifications

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**Acquisition Rate:** 16-200 Hz;

**Absolute Sensitivity Measure:** 4.51 (photons needed to equal noise)

### **OptoStim Lasers:**

*Red Laser Power Max:*  $\geq 10\text{mW}$  from a single 200  $\mu\text{m}$  fiber

Wavelength: 635 nm +/- 5 nm

*Blue Laser Power Max:*  $\geq 30\text{mW}$  from a single 200  $\mu\text{m}$  fiber;  $\geq 5\text{mW}$  from bifurcating 200 $\mu\text{m}$  patch cord

Wavelength: 450 nm +/- 5 nm

Stimulation Frequency: 1-100Hz

### **Simultaneous Recordings from multiple fibers:**

8 branching patch cord (200  $\mu\text{m}$  each)

4 branching patch cord (400  $\mu\text{m}$  each)

*\*Please note, we always recommend 200  $\mu\text{m}$  over 400  $\mu\text{m}$ , and it is critical to ensure that the outer diameter of the bundle does not exceed 1mm.*

### **LEDs for recording:**

415 (*isosbestic*)\*: 0.1 -  $\geq 300 \mu\text{W}$

470 (e.g. *GCaMP, dLight*)\*: 0.1 -  $\geq 300 \mu\text{W}$

560 (e.g. *RCaMP*)\*: 0.1 -  $\geq 130 \mu\text{W}$

Step Size: 25 nW

\*min and max through 200  $\mu\text{m}$  fiber

**Numerical Aperture:** 0.37 - 0.4

**Digital Inputs:** 2

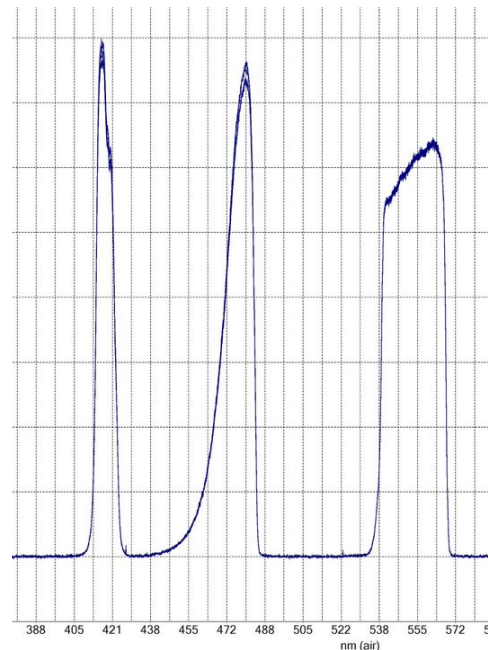
**Digital Outputs:** 1 + laser routing

**Timing Synchronization:** sub-millisecond synchronization with external equipment

## FP3002 Excitation and Emission Spectra

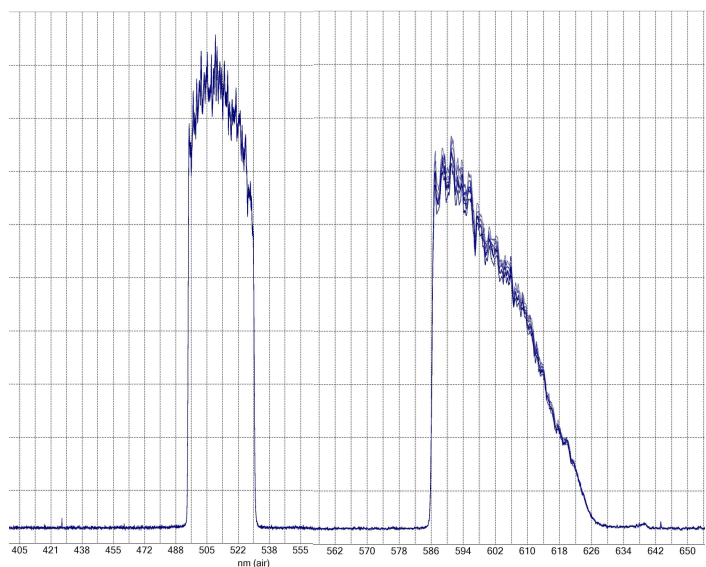
The FP3002 system comes with three excitation channels and is capable of recording two emission channels. Throughout this manual the three excitation channels will be referred to as the 415nm, 470nm and 560nm channels. These are the central wavelength but because the system is LED based, excitation spectra is a band within the following range for each LED:

- 415nm LED** : 400 - 425nm
- 470nm LED** : 445 - 486nm
- 560nm LED** : 535 - 569nm.



The two emission channels will be referred to as the red and green channels. The emission channels have been optimized to record from green fluorescent proteins and red fluorescent proteins. The following is the spectral range for the green and red emission channels:

- Green Emission Channel:**  
494 - 531 nm
- Red Emission Channel:**  
586 - 627 nm



## Anatomy of the FP3002 System: Micro B connection

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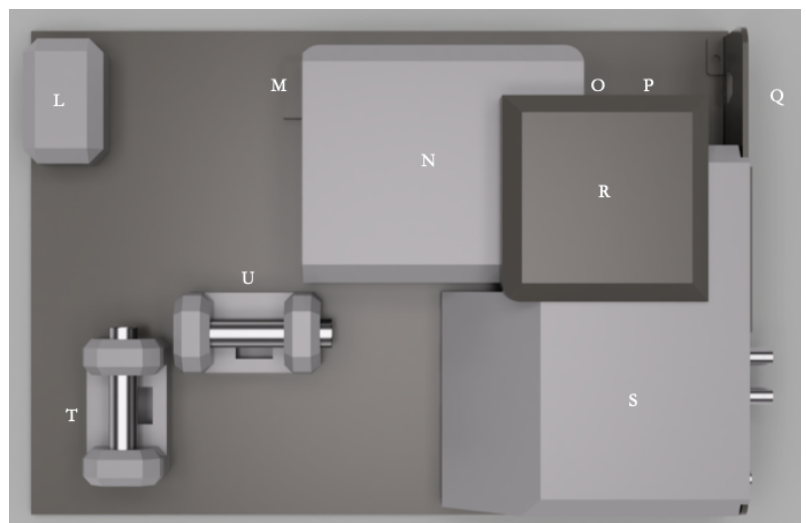
### Stub Wall

- A. MicroB Out (USB-3 ONLY)
- B. Laser Enable/Disable Key Switch
- C. 450nm Laser Out, FC Connector
- D. General Purpose Input/Output (GPIO - Hirose connector)
- E. Timing Synchronization In (1/8 inch Audio connector)
- F. Timing Synchronization Out (1/8 inch Audio connector)
- G. Digital Inputs (BNC connector)
- H. Digital Output (BNC connector)
- I. LCD Screen
- J. Recording Patch Cord In
- K. One Axis Translator Knob



### Internal Components

- L. Power in and on/off switch
- M. X/Y Translator for 635nm
- N. Optical Housing
- O. Objective (20x, 0.40NA)
- P. One Axis Translator with Magnetic Connector
- Q. Stub Wall
- R,S. LED & Electronics Housing
- T. 635nm Fiber Coupled Laser
- U. 450nm Fiber Coupled Laser





## Anatomy of the FP3002 System: USB-B connection

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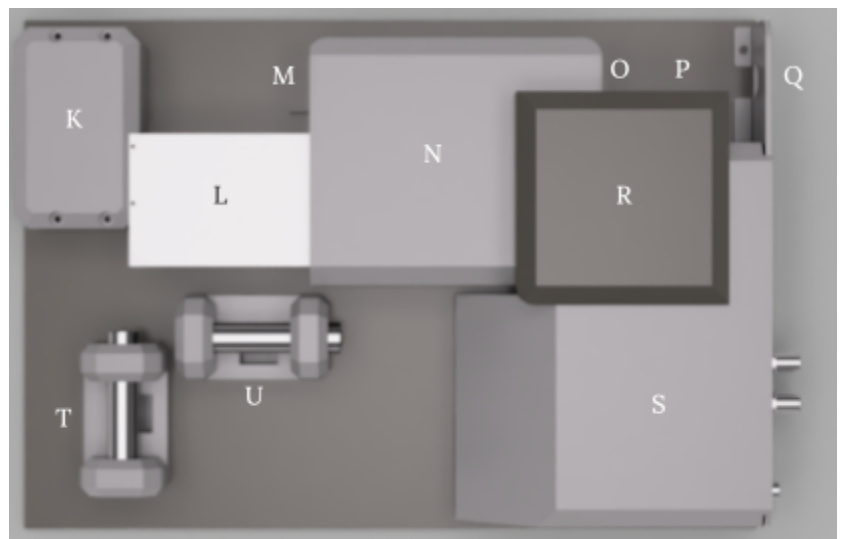
### Stub Wall

- A. Laser Enable/Disable Key Switch
- B. 450nm Laser Out, FC Connector
- C. General Purpose Input/Output (GPIO - Hirose connector)
- D. Timing Synchronization In (1/8 inch Audio connector)
- E. Timing Synchronization Out (1/8 inch Audio connector)
- F. Digital Inputs (BNC connector)
- G. Digital Output (BNC connector)
- H. LCD Screen
- I. Recording Patch Cord In
- J. One Axis Translator Knob



### Internal Components

- K. Power in, on/off switch, USB B
- L. Camera Cover
- M. X/Y Translator for 635nm
- N. Optical Housing
- O. Objective (20x, 0.40NA)
- P. One Axis Translator with Magnetic Connector
- Q. Stub Wall
- R,S. LED & Electronics Housing
- T. 635nm Fiber Coupled Laser
- U. 450nm Fiber Coupled Laser



## Accessories

- ❑ USB - B or MicroB to USB 3 Cable- Connect the USB plug to a USB 3 port on your computer. For MicroB systems: connect the Micro B plug to port A specified in the diagram above. For USB-B systems: connect the USB-B to the port on the back of the power unit. **Do NOT use a USB extension cable, or USB hub.**
- ❑ 1/8 inch Audio Cable (1 meter)- Optional: connect one side of the cable to either port E or F. The other side of the audio cable should be connected to another Harp controlled system.
- ❑ Power Supply Cable
- ❑ Power Supply Brick- Connect the male grounded plug to a three prong outlet and the female plug to the Power Supply Brick. Connect the DC plug to port L (Micro B) or port K (USB-B).
- ❑ 1.5mm L-shaped Hex Key - Use to lock or unlock the position of the 1 axis translator. The set screw is accessible from a top-down view of the system, near (but not on) the silver handle.
- ❑ 2mm Straight Hex Key - Use for focusing your patch cord without opening the lid. Turning clockwise will translate the patch cord carriage towards the objective. Turning counterclockwise will translate the patch cord carriage away from the objective.
- ❑ Magnetized FC and SMA connectors - Connect a patch cord to one of the connectors. Thread through hole J and connect to the translator carriage.
- ❑ Fiber Cleaving Jig- Insert your fiber into the hole corresponding to your target length such that the ferrule is as far down as it will go and the end of the fiber core is sticking out. Score fiber with a scribe and gently flick to cleave. Make sure to test light transmission of cleaved fiber prior to implant
- ❑ Replacement Carriage- Unscrew and replace carriage installed on translator if magnets come out.
- ❑ Replacement Carriage Screws

- ❑ Replacement Washers- If your patch cord wiggles inside of the FC or SMA connector, place a washer on the bundle of the patch cord and reattach connector.

## Required Materials for Fiber Photometry Experiments

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Your FP3002 system will arrive with all equipment necessary for the system to run, but there are additional materials required for fiber photometry experiments.

### Required Materials

1. **Fiber Optic Cannulae (FOC)** - These can be purchased through Neurophotometrics. We carry a variety of sizes and can custom order if special specs are needed.
2. **Patch Cord** - 4-branching patch cords can be purchased through Neurophotometrics. If you prefer different specs, we recommend ordering from Doric. For more information, see Patch Cord Guidelines section.
3. **Ferrule Sleeves** - These connect the patch cord to implanted FOC. We carry sleeves for both 1.25 and 2.5mm ferrules.
4. **Computer Running Windows**- A computer (we recommend a laptop) is needed to record data. See System Requirements section for technical specifications.

### Recommended Materials

1. **Fiber Optic Scribe** - This is used to cleave FOC to the appropriate length. We recommend getting them from Specialized Products. The ruby and carbide versions both work well and can be ordered [here](#).
2. **Power Meter** - This allows you to measure the LED and optogenetic stimulation laser output from the system for each wavelength. It can also be used to test light transmission through a fiber prior to implantation. We test all fibers ordered through Neurophotometrics, but we recommend that you retest after cleaving fibers, as this can impact light transmission. We recommend this [photodiode power sensor](#) and [power meter interface](#) from ThorLabs.
3. **Fiber Jig** - We make jigs to assist in cleaving fibers. You received one with your FP3002 system, but if you would like additional jigs, they can be purchased.
4. **Polishing Paper** - Lightly polishing FOC after cleaving to the desired length can improve light transmission. We recommend [polishing paper from Thor labs](#), 0.3-3um should be fine.
5. **Stereotaxic Cannulae Holders(s)** - These allow you to implant FOC with excellent precision. If your stereotax has two arms and you plan to implant more

than one FOC, having two fiber adaptors will speed up surgery. We recommend [this one from Doric Instruments](#).

6. **Cement to attach fibers to the skull** - You will want specialized adhesive to secure FOC and prevent them from detaching from the skull. We recommend OptiBond Bonding Reagent or Parkell Metabond.
7. **A small cart** - The FP3002 system is entirely contained within a 1 x 2' box and can be easily moved between rooms for different experiments.

## Patch Cord Guidelines

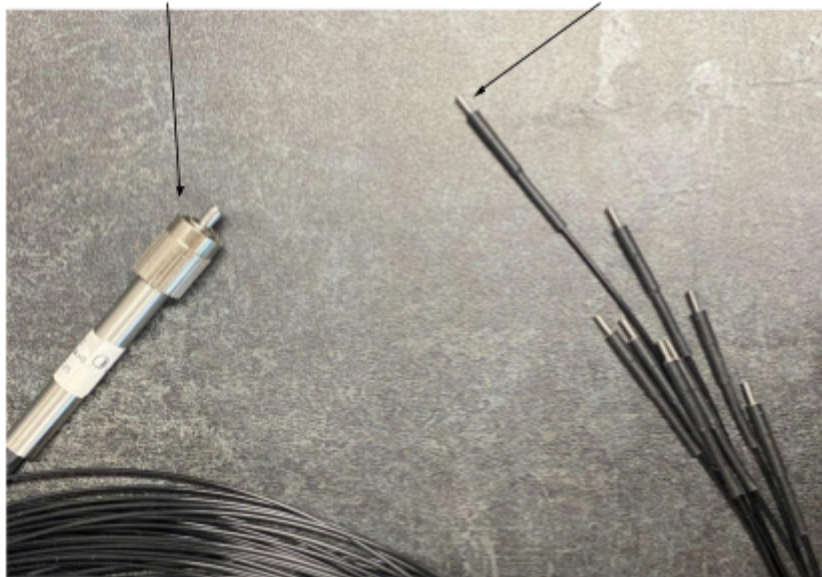
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Prior to conducting your first fiber photometry experiment, you will need a patch cord. You can purchase a 4x-branching (200um) cord from us. If you would like more or less branches, the details below will help you order one elsewhere (we recommend Doric).

The most important thing when choosing a patch cord is ensuring you have the correct numerical aperture (NA). Neurophotometrics systems come with an objective that has a 0.4 NA. To maximize your signal, the patch cord (and fiber optic cannulae) should match this NA, **0.39-0.37 NA will work appropriately**. Matching the NA will ensure that there is no light loss from the excitation or emission side. Having mismatched NAs at any of these points will cause significant light loss, giving you less than optimal signal - or none at all!

Connector (FC)

Ferrule (1.25 mm metal)



Next, choose the ferrule and core diameters of the patch cord. These can vary between experiments, but must match specifications of the fiber optic cannula(e) that you will implant into the animal. **Common ferrule diameters are 1.25mm and 2.5mm.** **Common core diameters are 200um and 400um.** The most popular combination is

1.25mm ferrule diameter with a 200um core diameter. You can pick any combination to fit your experiment — just be sure that the patch cord matches the fibers.

Another consideration for your patch cord is the connection to the system. The Neurophotometrics FP3002 fiber photometry system comes with **both an FC and SMA connector**. We recommend using an FC connector because the connector contains an “alignment” pin that ensures that your fibers do not spin while you secure the connector. It is important to choose FCM — where the M stands for metal — this is important because metal is less autofluorescent than zirconia/white ceramic. It is also important to note in the special instruction box, that you need the back of the SMA or FC connection, to have an **inner diameter of 0.9mm max**.

The patch cord also must connect to the animal subject, so you will need to specify this type of connection as well. **This connection type should be MF, followed by the diameter size of your ferrule**. Note that MF stands for Metal Ferrule. For example, if you are using fiber optic cannulae with 1.25mm ferrules, the connection would be “MF1.25.”

Patch cords come in a variety of lengths. **We recommend a length of 3 meters (or more)**. This will make multiplexing the system and managing the patch cord easier. The longer the patch cord, the more resistant it will be to twisting (i.e. it can absorb a higher number of twists before the signal starts to attenuate).

Patch cords can also have several branches. The number of branches you choose is completely up to you and dependent on your experimental needs. How many animals and/or brain regions you wish to record from at once will influence this choice. **We recommend starting with at least two branches, even if you are only recording from one brain region to start**. You can simply cover up the one unused core with aluminum foil and tape it up during the experiment. Be sure to order a “bundle branching” cord, and not a “splitter branching” cord.

Once you’ve determined what kind of patch cord you need, we recommend buying that patch cord from Doric Lenses. Please note, however, that they can have quite long lead times; you’ll want to place your order at least 6 weeks before you plan to do any experiments.



Low-Autofluorescence Bundle Branching Fiber-optic Patch Cord

**Number of Output Fibers:** 4  
**Fiber-optic Type:** 200  $\mu\text{m}$  - NA0.37  
**Branches1:** Type A  
**Protection Jacket:** 900  
**Cable Length (m):** CUSTOM  
**Cable Length (m) - Custom:** 3  
**Connector A:** FCM  
**Connector B:** MF1.25  
**product\_sku:** BBP(4)\_200/220/900-0.37\_3m\_FCM-4xMF1.25\_LAF

The screenshot to the left is an example order for a [branching patch cord](#), 3m long, 1.25mm ferrule diameter, 200um core, 0.37NA.

Note that if you have a system with 450nm laser, you will also want a second cable for stimulation to a certain brain region. You can use a [mono-branch patch cord](#) or if you are interested in bilateral

stimulation, we recommend using a [bifurcated patch cord](#). **You must use a patch cord with a FC connector at one end.** For optimal power output, please use a 200 um core, 0.39-0.37 NA patch cord.

Some labs that are just beginning to do fiber photometry might already have patch cords. A common question we receive is whether or not these pre-existing patch cords will work for fiber photometry experiments, even if the NA, ferrule size, and/or core diameter don't perfectly match their new system or fiber optic implants. We highly recommend that you purchase a patch cord that will perfectly match up with the system.

There will be a significant amount of emission loss when these qualities are mis-matched, resulting in a diminished signal. It is always a good idea to double check the specs of your patch cord before recording, especially if you have multiple patch cords.

If you are performing experiments that require

Pigtailed 1x1 Fiber-optic Rotary Joint



**Optical Fiber Core** - 200 \* ?

**Optical Fiber NA** - 0.37 \*

**Input fiber length (m)** - 1.0 ?

**Input Receptacle** - FCM ?

**Output Receptacle** - FCM ?

**Ordering Code:**  
 FRJ\_1x1\_PT\_200-0.37\_1m\_FCM\_0.15m\_FCM



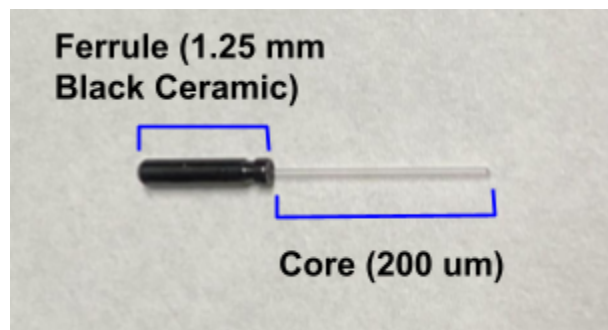
long recording periods, you may want to purchase a [rotary joint or commutator](#) as well. However, rotary joints can cause a loss of light at the connection point of the rotary joint and patch cord. We advise that you start recording without one, and add one only if necessary. If you decide that you need a rotary joint, Doric makes a pigtail commutator that is compatible with fiber photometry.

## Fiber Optic Cannula Guide

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You will also need fiber optic cannulae (FOC) to implant before starting your first experiment. Like with patch cords, you will need to match the NA of the optical components in the system and patch cord. FOC with an NA of **0.39-0.37 NA will work appropriately.**

Next, choose the ferrule and core diameters of the FOC. **It is critical that the specs of your FOC match your patch cord.** If you have a patch cord with a 200 um diameter core, you will want fibers with a 200 um diameter core. You will also want the ferrule diameter to match the ferrules on your patch cord (typically either 1.25mm or 2.5mm). You can pick any combination to fit your experiment — just be sure that the patch cord matches the fibers.



We recommend starting with 200 um FOC as they cause significantly less damage to the brain when implanted. You can also fit up to 8 ROIs within the camera's field of view. Using 400 um fibers *may* help capture more light when recording from a sparse population, but they will also increase the background light collected and can negatively impact signal to noise ratio. With 400 um fibers, you can fit up to 4 ROIs in the field of view.

Another consideration when choosing FOC is the ferrule material and color. We recommend black ceramic because it has low autofluorescence. We suggest avoiding white ceramic as it can allow light from the room to leak in and contaminate FP signal. Stainless steel ferrules work fine as well.

You can purchase black ceramic FOC from us with the following options:  
200 or 400 um core diameter  
1.25 mm or 2.5 mm ferrule diameter  
10 mm length (in stock) or custom length (allow 3 weeks for production)

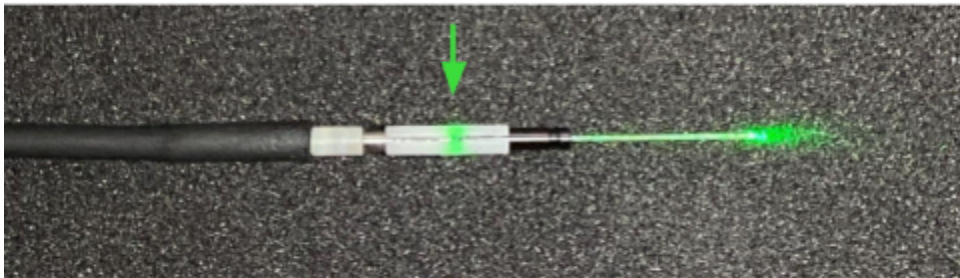
## Ceramic Split Sleeve Guide

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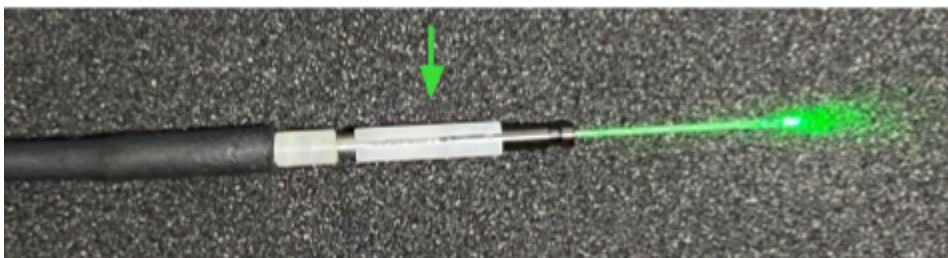
You will also need ceramic split sleeves in order to attach a patch cord to FOC implants. These are tightly-fitting ceramic tubes that fit around the patch cord and implant ferrules to hold them together while you are recording. They have an open slit to allow you to see if you have made a full connection. You will want to match the diameter of the sleeve to the diameter of your implant and patch cord ferrules. Both 1.25 and 2.5mm split sleeves can be ordered from Neurophotometrics.

When attaching a patch cord, we recommend putting the sleeve 50% on the patch cord ferrule, and 50% on the implant ferrule. To tether an animal, first put the sleeve onto the patch cord. Gently wiggle the sleeve onto the implant by twisting back and forth between your thumb and forefinger. **It is critical that the ferrules are touching with no gaps in between.**

**Poor connection with visible light leak**



**Good connection with no light leak**



## System Requirements

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### **Operating System**

Bonsai is only compatible with Windows. Mac and Linux are not supported. Windows 10 is recommended.

### **Processor**

Any Intel or AMD processor with a minimum of four logical cores and clock speed of 1.6GHz.

### **Graphics**

No graphics cards are required, Bonsai's visualizers run off of your CPU.

### **RAM**

Recommended 8GB or more.

### **Disk Space**

0.9GB of space is required for Bonsai and packages, and Spinnaker.

### **Ports**

At least one USB A 3.0. Converters or extension cables are not recommended.

### **Example computers for fiber photometry**

- ASUS VivoBook K571 Laptop
- Lenovo Thinkpad T480 Laptop
- Lenovo Thinkpad T14 Laptop
- Dell Inspiron 3501

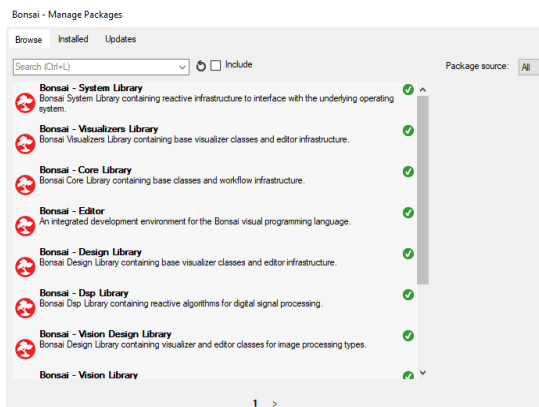
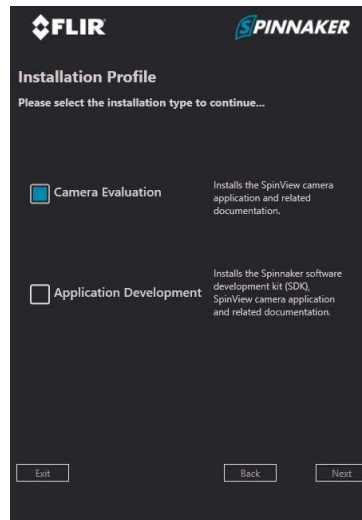
## First FP Experiment Setup

This section will walk you through setting up the system and software for a basic recording with 635nm laser stimulation. Please see our [Bonsai manual](#) for instructions on all nodes and workflows. We advise reading the manual before proceeding with a recording.

## Download Software

### Spinnaker

Follow this [link](#) to download Spinnaker 1.29.05. Version 1.29.05 needs to be installed for Bonsai to recognize the camera sensor. (Please use exactly this version). Download the .exe file and open it to run installation. Follow the installation instructions, when choosing between Camera Evaluation or Application Development choose **Camera Evaluation**. Uncheck "I will use GigE Cameras" and install.



### Bonsai

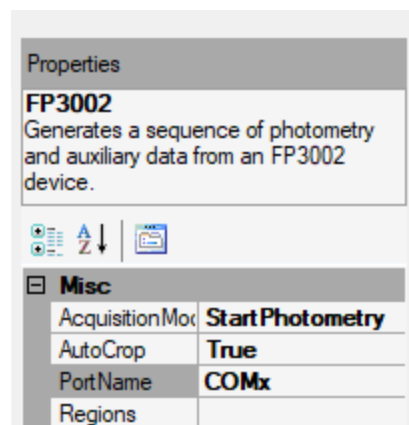
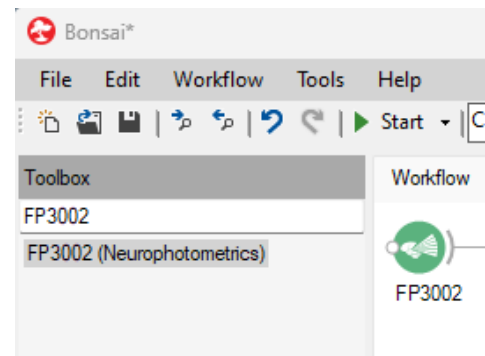
Follow this [link](#) to download the newest version of Bonsai. Follow the installation instructions. When installation is complete, please click **Launch** instead of Close to install all dependencies. At the start up window, click on **Manage Packages**. Under Browse → Package Source: All. Download: Bonsai Starter Pack, Bonsai – System, Bonsai – Visualizers, Bonsai – Editor, Bonsai – Core, Bonsai – Design,

Bonsai – Video, Bonsai – System Design, Bonsai – Video Design, Bonsai - Player, and

Bonsai - Spinnaker Library. The FP3002 nodes are found in the Community Packages. Please search and install the Neurophotometrics, Neurophotometrics Design and Harp Libraries which can be found under Community Packages. **Do not check the box next to Include Prerelease**. This will download unstable packages still in development stages.

## Connect FP3002 System and Open Bonsai

1. Plug the USB-B to USB cord into the plug on the back of the system. If your system has a Micro-B, it will connect to the front stub wall of the system instead. The USB end will attach to a USB 3 port on your computer. **Please do not use a USB extension cable or USB hub as we cannot guarantee that your system will work properly.**
2. Plug in the power supply and turn on the system.
3. Wait for your system to boot up. Boot up is complete when you see the image on the right.
4. Open Bonsai and create a new workflow
5. Under toolbox on the left hand side of the Bonsai window, search 'FP3002' and double click to add the FP3002 node to your workflow. This node is where you will interface with the system and configure all of your settings for recording.
6. Click on the FP3002 node once, then under properties on the right hand side of the screen, select the correct port name of your FP3002 system (COMx). **Also select 'True' from the dropdown menu next to 'AutoCrop'.** This will automatically crop the camera field of view around your selected ROI(s), increasing the capacity by which the camera can now collect data at the increased frame rate.



## Connect and Align Patch Cord

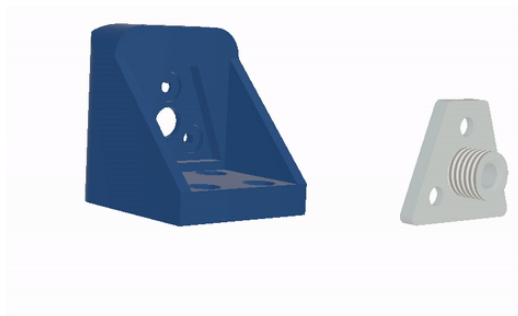
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The FP 3002 system is compatible with both FC and SMA connectors and the procedure for connecting both patch cords to the system is identical. The system is not pre-focused, this guide will walk you through patch cord alignment. Note that the one axis translator is locked when shipped. You will need to unlock the translator with a 1.5mm L-hex-key.

1. Screw the patch cord into either FC or SMA-NPM magnetic connector. The end of the patch cord should stick out slightly from the flat face (opposite the threaded side) of the connector. Note that the FC connector has an alignment pin and the SMA connector does not. Please make sure that the pin fits into the pin sized slot **before** screwing.



2. Open the lid of the system.
3. Insert the FC or SMA-NPM connector through the “Patch Cord In” [J] port.
4. Fit FC or SMA-NPM connector into the carriage. It is held in place by magnets and should click into place.

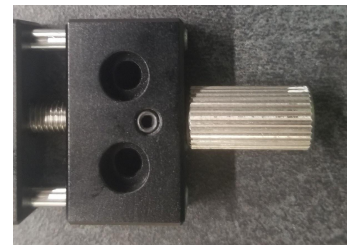






5. Unlock the translator with the 1.5mm L-hex-key. The set screw location can be found in the image below.

6. Visualize camera feed in Bonsai by opening the FP 3002 node and opening Calibrate regions and clicking the Calibrate Regions button at the top of the window. Using the slider at the bottom of the window, turn on the 470 LED to illuminate your patch cord.



7. Insert the straight hex-key (2 mm) into the small hole under the patch cord opening on the stub wall of the system and turn counterclockwise to advance the carriage closer to the objective (i.e. further away from you). Your focal point should be about 0.5 mm from the objective. You should see an image of the tip(s) of your patch cord. Continue to move the carriage until the image looks crisp. Below is an example of a correctly aligned PC with two branches.



8. You will see two images of the end of your patch cord. The image on the right is the green emission, and the image on the left is the red. Fluorescent emission coming into the system is split by wavelength before being reflected onto the camera sensor, producing two separate images. The number of circles in each image will depend on the number of branches on your patch cord.

## Set LED power

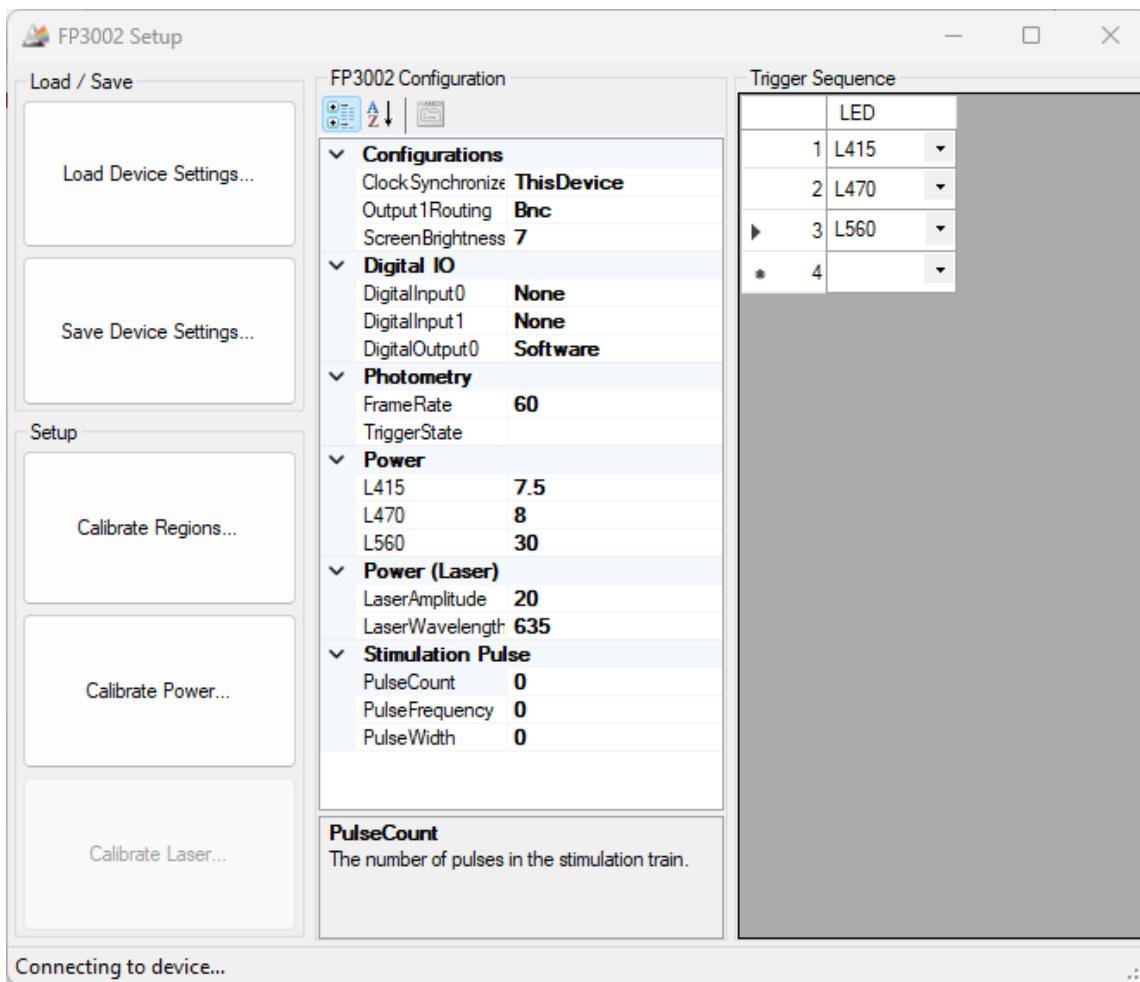
---

Light power for each wavelength can be set using the 'Calibrate Power' button on the left side of the FP3002 window. You will need an external power meter for this step. Use the sliders to turn on LEDs and adjust the power level while measuring the light coming out of each patch cord branch using a power meter. These values correspond to the percentage of maximum power being sent to each LED. **These values are not light intensity** and should not be used in place of external light power measurements.

You will want to measure light power out of each patch cord branch **without any connectors or split sleeves attached**. We recommend aiming for 50uW of light in each wavelength (for a 200um PC core diameter). Write down the power settings for each wavelength, as these values are not saved once the window is closed and will need to be entered elsewhere. Be sure that only one wavelength is on at a time, and that you change the wavelength on your power meter accordingly.

## Configure Settings in the FP3002 Node

All system settings can be configured in the FP3002 node. Please refer to the [Bonsai Manual](#) for information on each parameter. Details begin on page 19. Settings can be saved and loaded for each experiment using the ‘Save Device Settings’ and ‘Load Device Settings’ buttons on the left hand side of the FP3002 window.



The screenshot shows the 'FP3002 Setup' window with the following sections:

- Load / Save:** Buttons for 'Load Device Settings...' and 'Save Device Settings...'.
- Setup:** Buttons for 'Calibrate Regions...', 'Calibrate Power...', and 'Calibrate Laser...'.
- FP3002 Configuration:**
  - Configurations:** Clock Synchronize: **ThisDevice**, Output 1 Routing: **Bnc**, Screen Brightness: **7**.
  - Digital IO:** Digital Input 0: **None**, Digital Input 1: **None**, Digital Output 0: **Software**.
  - Photometry:** Frame Rate: **60**, Trigger State: (empty).
  - Power:** L415: **7.5**, L470: **8**, L560: **30**.
  - Power (Laser):** Laser Amplitude: **20**, Laser Wavelength: **635**.
  - Stimulation Pulse:** Pulse Count: **0**, Pulse Frequency: **0**, Pulse Width: **0**.
- PulseCount:** The number of pulses in the stimulation train.
- Trigger Sequence:** A table with columns for LED and a dropdown menu.

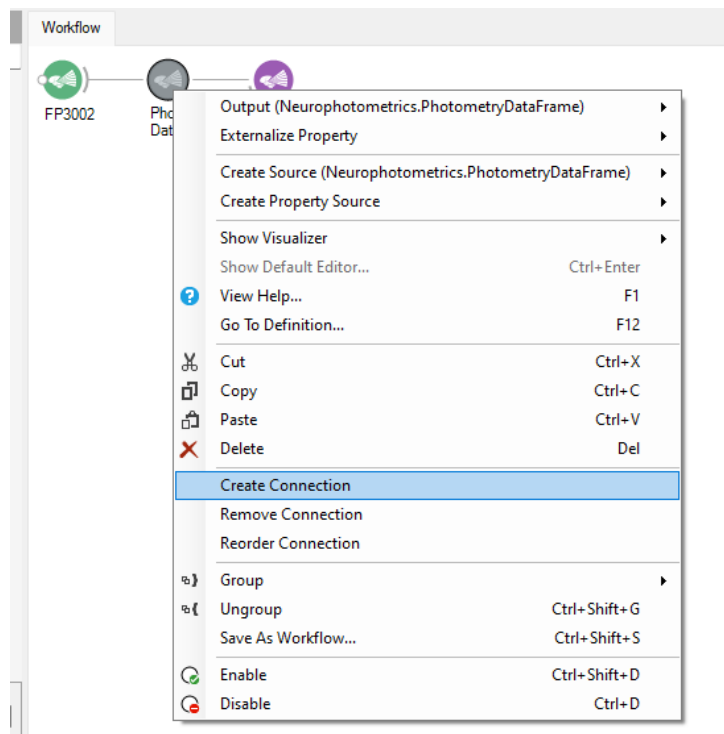
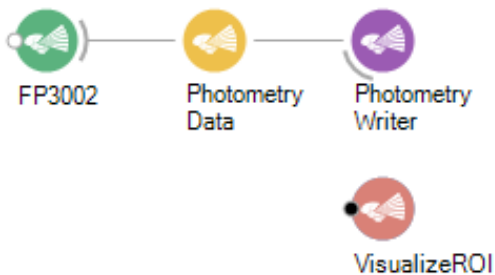
	LED
1	L415
2	L470
▶ 3	L560
* 4	

Connecting to device...

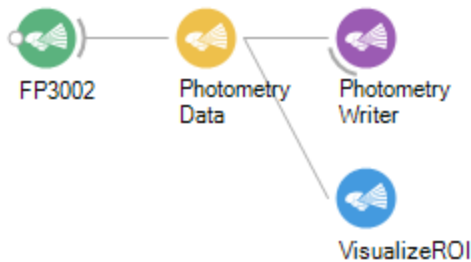
## Build a Bonsai Workflow for Recording

Below are details on how to configure a simple Bonsai workflow for recordings without hardware synchronization, timestamping, video capture, etc. Bonsai is a highly flexible platform that allows for synchronization with multiple data streams. For details on how to interface with other data streams, please refer to the [Bonsai Manual](#). There are also premade workflows that you can download from our website that correspond to each example in the manual. The workflow detailed below will help you get started with recordings.

1. Open up Bonsai and search for FP3002 in the toolbox and double click to enter it into the workflow, if you have not already.
2. Search for the Photometry Data node and double click to add, then Photometry Writer. The nodes should connect in a single straight line
3. Then click into the white space in the Bonsai window and search for Visualize ROI, double click to add. It should not be connected to any other nodes



- Right click on the Photometry Data node and select 'Create Connection' then click on the Visualize ROI node. The finished workflow should look like the example below.



You will need to configure a few settings in order to use this workflow.

- Click on the FP3002 node once, then under properties on the right hand side of the screen, select the correct port name of your FP3002 system from the dropdown menu that appears when you click 'COMx'. **Also select 'True' from the dropdown menu next to 'AutoCrop'.**
- Click the Photometry Writer node and define your filename by clicking the ellipsis next to 'Filename'. You will need to add .csv at the end in order for it to save correctly. 'Buffered' should be set to 'True'. All other settings are optional.

Properties

**FP3002**  
Generates a sequence of photometry and auxiliary data from an FP3002 device.

**Misc**

AcquisitionMode	Start Photometry
AutoCrop	True
PortName	COMx
Regions	

Properties

**PhotometryWriter**  
Writes photometry data frames into a CSV text file.

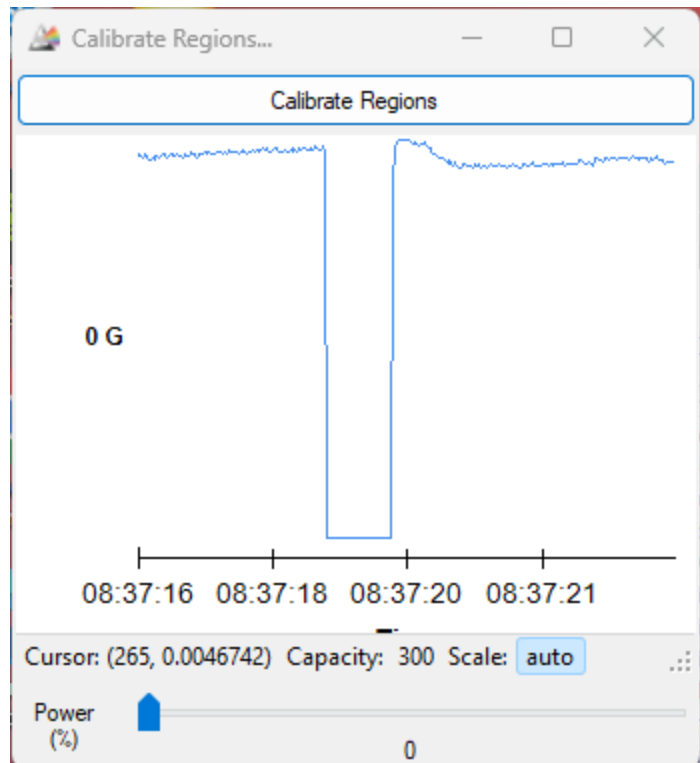
**Misc**

Buffered	True
FileName	...
IncludeChart	False
IncludeRegion	False
Overwrite	False
Suffix	None

## Signal Check

Once your settings have been configured and your Bonsai workflow created, it is time to plug in an animal and look at the FP signal.

1. Place a ceramic split sleeve on the tip of a patch cord branch such that 50% of the sleeve is covering the tip. The other 50% will cover the tip of the implanted fiber.
2. Connect your animal by gently wiggling the ceramic sleeve onto the fiber implant. Ensure that there are no gaps between the implant and the patch cord.
3. Open the Calibrate Regions window (your ROIs should have been drawn before connecting the animal). Use the power slider at the bottom of the window to send a small amount of light into your fiber (<7% power).
4. While looking at the traces for ROIs that are connected to your animal, turn the room lights off and on. If there is a step function drop/increase in your signal then the FOC:Patch Cord connection should be optimized further. If the room light change is *not* visible in these graphs, then you are ready to continue with your experiment.



## Shut Down System

---

1. If this is your last recording, exit out of all FP3002 windows first, do not close the Bonsai black screen. This will automatically close when exiting out of the Bonsai workflow main window.
2. Power off the system, unplug the USB cable, remove the laser key and exit out of Bonsai. **It is very important to unplug the USB cable at the end of each day.** This will avoid overheating of the camera. \*If you are running overnight recordings, this is okay as the camera has an active heatsink to cool it off while the system is running.

## Recommended Recording Parameters

---

Recording parameters needs will depend on the sensors and experiment, but we recommend the following as a starting point.

### **Trigger Sequence:**

The order of your LEDs does not matter. If you are just recording a green sensor, we recommend using the 415 and 470 channels.

### **LED power**

50 uW +/- 25uW per wavelength for a 200 um diameter fiber

100-200 uW per wavelength for a 400 um diameter fiber

Note: this is the output from the patch cord, measured with an external power meter. You will need to adjust the LED power in Bonsai while measuring output through the patch cord in order to set your parameters. Be sure to measure each branch without a ferrule sleeve attached, and to change the wavelength on the power meter to match.

### **Frame rate**

30 hz / wavelength

This means if you are using 2 LEDs (or have 2 trigger states), you will want to record at 60 hz.



## Using Integrated Lasers for Optogenetics

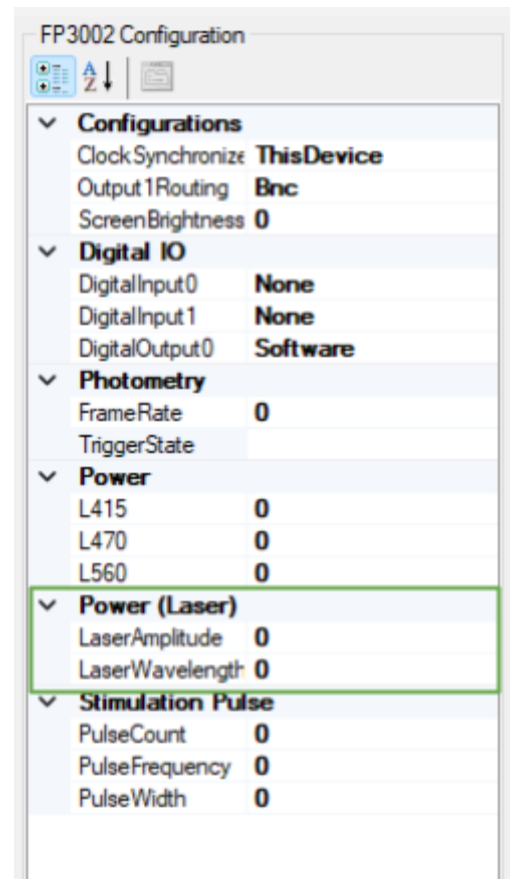
### Safety Warnings

- Wear laser safety glasses whenever the laser is in use.
- **Please do not look or use optical instruments to look directly at the laser.** This especially applies to your patch cord. Handle the patch cord with care when the laser is on, please do not shine the tip of the patch cord in your eyes.
- Remove the laser key after every use. Although there is still a software governor, please remove the laser key to avoid any accidents.
- To switch between the 635nm and 450nm laser, please turn off the system to swap cables. Do not unplug and plug in the laser cable while the system is running.

FP3002 systems come with either a 635 nm laser, 450 nm laser, or both. The two lasers are different in terms of how they are integrated into the light path of the system. The 635 (red) laser is routed into the same light path as the excitation LEDs, and can be used to stimulate in the same fiber you are recording in. The 450 laser is not integrated into the same light path, and must be used with a separate patch cord. The lasers cannot be used at the same time.

Settings for both lasers are configured in the FP3002 node. The 'Calibrate Laser' button is grayed out as a safety feature until the exact wavelength (635 or 450) is typed into the 'LaserWavelength' Box under the Power (Laser) settings. Hit enter to enable Calibrate Laser. The settings in this window differ between lasers.

To enable either laser, insert the laser key included with your system into the key switch on the stub wall of the system and turn to the right.



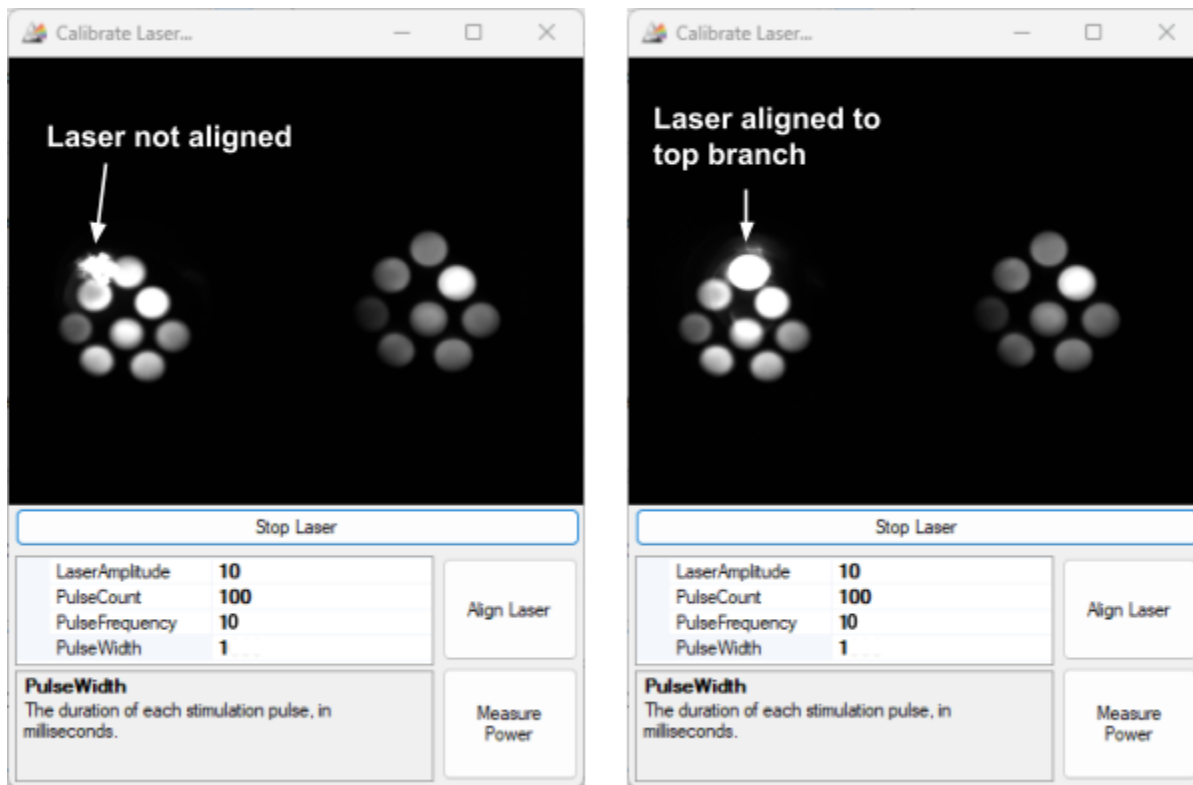
## 635 nm Laser Alignment

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With the FP3002 system, you are able to align the 635nm laser to a single branch on your multi-branching patch cord. This method allows for simultaneous green channel fiber photometry recordings and red opsin stimulation. The system comes with a two axis translator (XY translator) to align your laser beam into only one branch. This translator can be found at location M referenced in the ‘Anatomy of the FP3002’ section of this manual –it is the translator located nearest to the camera sensor.

Before aligning the laser, focus your recording patch cord to the objective using the one axis translator. You will not get adequate power output if your patch cord is not aligned. You will also need to ensure that the laser cable is plugged into the 635 laser. If you have both lasers and are switching between them, please turn off the FP3002 system before moving the white ribbon cable between lasers.

To align the laser, open the ‘Calibrate Laser’ window. This will open an image of your patch cord, similar to the ‘Calibrate Regions’ node. You may need to shine light into your patch cord in order to see the branches. Point the patch cord branches away from your eyes. Click ‘Trigger Laser’ to start the laser. You will see an irregularly-shaped flashing circle appear over the red emission image. Turn the knobs on the XY translator to align the circle with the branch you would like to use for stimulation. If the circle is very faint, adjust the laser power. You will likely see light coming out of all branches, though the light power coming out of branches the laser is not aligned to will be <1% of the aligned branch.



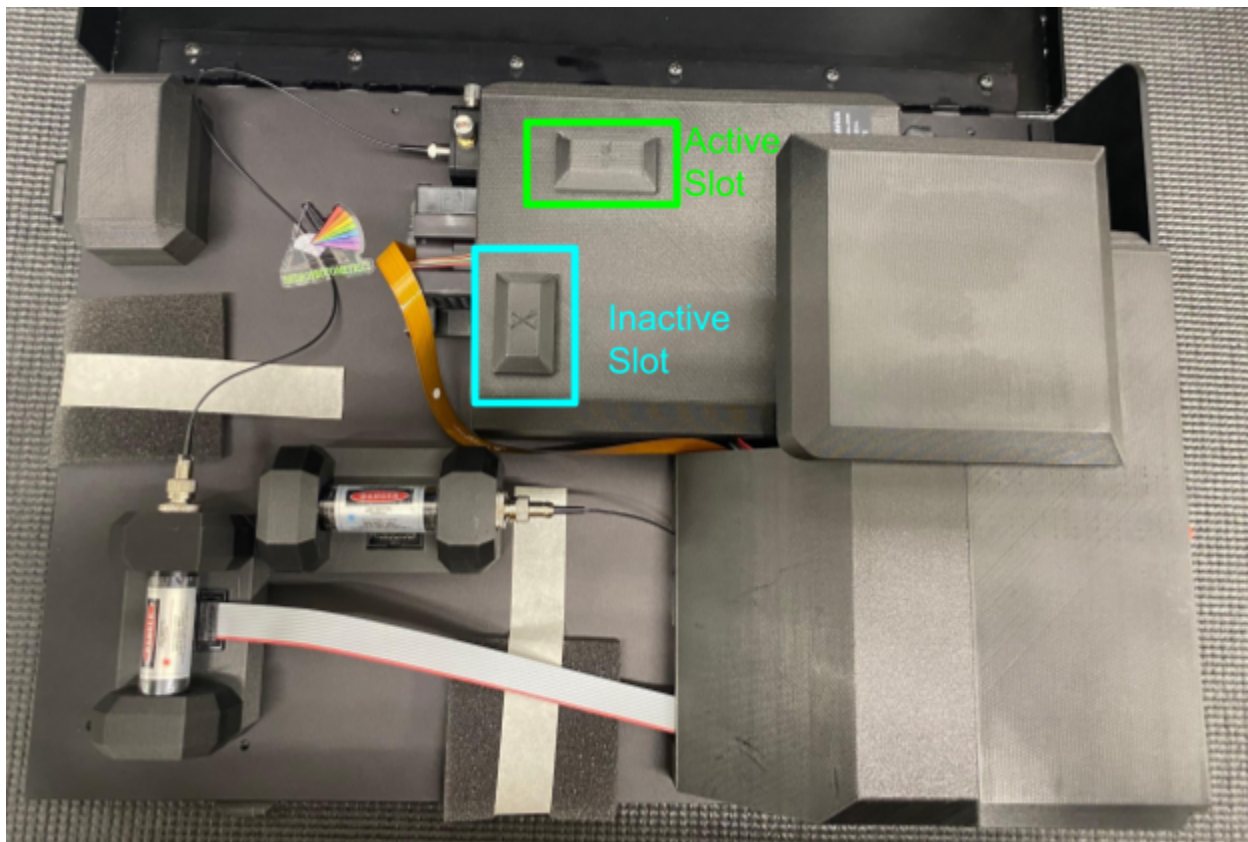
Like with the LEDs, the power setting refers to the percentage of maximum power delivered to the laser, not the light output. Light output will need to be measured with an external power meter. If you need to turn the laser on without pulsing to measure power output, set the frequency to 1 and the pulse width to 1000. Write down the desired power settings, as parameters in this window are for calibration purposes only and will not be saved once the window is closed.

Once you have determined what laser power to use, enter your laser parameters in the 'Power (Laser)' and 'Stimulation Pulse' sections of the FP3002 node. For details on how to configure the timing of stimulation delivery, please see the Stimulation section of the [Bonsai Manual](#), starting on page 118.

## Using Light Blocks with 635 nm Stimulation

---

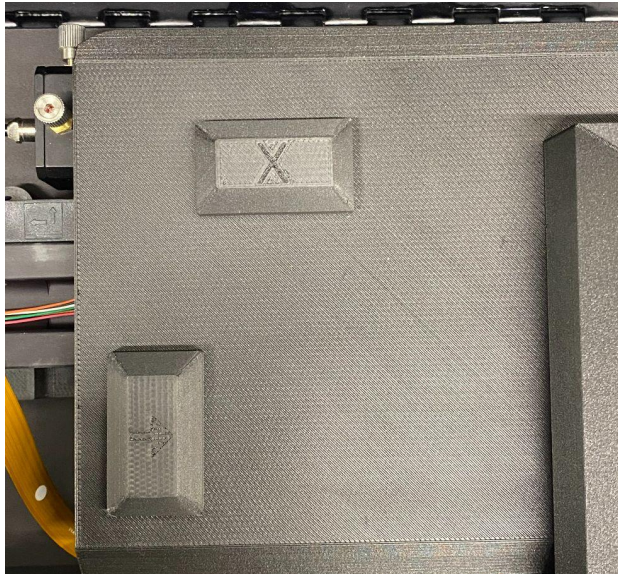
To prevent 635 nm laser light from reaching the camera sensor and creating artifacts in FP data, the FP3002 system uses physical light blocks. You will need to move the light blocks when switching between optogenetic stimulation and dual color experiments. They are not necessary when using 450 nm stim, as the 450 laser does not go into the same light path. Light blocks are located inside the system, highlighted below. There are 2 different light blocks, one long and one short. There are also 2 slots for the light blocks, the one closest to the hinge of the box that we will refer to as the “active slot”, and the other, the “inactive slot”.



The longer light block is labeled with an X, and the shorter is labeled with an arrow. The blocks are secured in place by magnets. When you are not using 635 nm stimulation, you should keep the X block in the inactive slot, and the arrow block in the active slot. This will allow red emission to reach the camera. When you are using stimulation, put



the X block in the active slot, and the arrow block in the inactive slot. Below are pictures of these configurations.



Using 635 nm stim  
(and no red-emission recording)



**NOT** using 635 nm stim

## 450 nm Laser Stimulation

---

The 450 nm laser is not integrated into the same light path as the LEDs and requires a separate patch cord. We recommend using either a single branch or bifurcating patch cord; these can be ordered from Doric or other manufacturers. The patch cord for the 450 laser attaches to the FC connector on the stub wall of the system.

If you have both lasers and are switching between them, please turn off the FP3002 system before moving the white ribbon cable between lasers.

Type '450' into the Laser Wavelength box to enable the 'Calibrate Laser' button. This will allow you to open the window and turn on the laser to measure power output. Like with the LEDs, the power setting refers to the percentage of maximum power delivered to the laser, not the light output. Light output will need to be measured with an external power meter. Write down the desired power settings, as parameters in this window are for calibration purposes only and will not be saved once the window is closed.

Once you have determined what laser power to use, enter your laser parameters in the 'Power (Laser)' and 'Stimulation Pulse' sections of the FP3002 node. For details on how to configure the timing of stimulation delivery, please see the Stimulation section of the [Bonsai Manual](#), starting on page 118.

## Determining LED Duty Cycle

Duty Cycle refers to the ratio of time that a device is on versus the time that it is off within one period. It is easiest to think of the duty cycle as the percentage of time the device is on. Within this section, we will discuss how to control the duty cycle of your LEDs.

A decreased duty cycle is particularly advantageous for long (>6 hour) recordings. The less time the LED is on, the slower photobleaching will occur. To simplify this process, the Neurophotometrics system pseudo modulates the amount of time an LED is on in a given frame by inserting “blank” frames, leading to a virtual “snapshot” of activity. It is not necessary to do this for shorter recordings.

It is easiest to think of the desired frequency/duty cycle per wavelength. So if for example you are only interested in imaging in Green with a full duty cycle, you will set up your LEDs as represented in the image on the right.

Trigger Sequence	
	LED
1	L470
2	L415
3	

For an effective frame rate of 25 Hz per wavelength, you need to set your system to 50 Hz. Keep in mind that the effective frame rate is determined by the interval of time it takes for a specific LED to complete a frame and turn on at a new frame. At 50 Hz your LEDs will be on for just under 20 ms per frame.

Trigger Sequence	
	LED
1	L470
2	None
3	L415
4	None
5	

Now let's say that you want the same effective frequency but a decreased duty cycle of 50% (10 ms). You will set up your LEDs to have blank frames as represented in the image on the left. Set the

frequency to 100Hz. This will still give you an effective frequency of 25 Hz per wavelength, but now the LED is only on for 10 ms.

Because it would be most advantageous to have the isosbestic control as close to the calcium dependent recording as possible, we recommend you actually place the 470 and 415 LEDs consecutively, leaving the two blank frames at the end, as pictured in the image on the right.

Trigger Sequence	
	LED
1	L470
2	L415
3	None
4	None
5	

## Tips for Successful FP Recordings

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### How to Cleave Fibers

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After deciding on an experimental paradigm, you will need to cleave your fibers to reach your brain region(s) of interest. It is vital that the fiber is cleaved properly, as fiber photometry employs very low levels of light. Proper fiber cleaving ensures the maximum light power can be emitted.

In order to cleave your fibers, you will need a scribe. We recommend getting them from Specialized Products. The ruby and carbide versions both work well. We also recommend using a ferrule jig when cleaving fibers. We 3D print the ferrule jigs in-house to help standardize the fiber cleaving process; if you have ordered a system from us, you likely received one with your system. They allow you to cut fibers in 0.5mm increments from 2mm to 10mm. The jigs eliminate the need for rulers and leave you with a perfectly cleaved fiber, ready for implantation.

Consult a brain atlas to choose the proper coordinates for your implants. For fiber photometry experiments, it is very important that the fiber is **implanted at the viral injection site**. This differs from optogenetics, where the fiber is usually implanted ~200um above the injection site. We are working with considerably lower levels of light in fiber photometry than in optogenetics, so it is important that the fiber is directly implanted in the brain area you are wanting to record from. It is important to note that many other factors beyond target brain regions go into choosing a fiber length as well. These include surgical method, type of animal subject, and where your target brain region is located relative to the varying thickness of the skull.

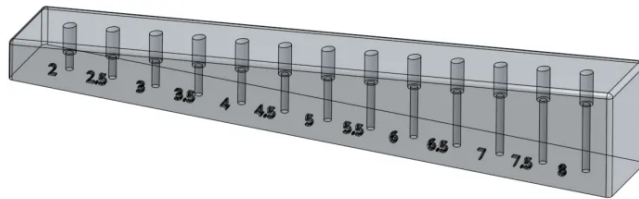
It is imperative that fiber optics meant for brain implantation are flat and polished at the tip. Jagged, irregular, or slanted tips will diminish the power efficiency and limit the cell populations that you can reach. Using scissors, or chopping all the way through the fiber, will result in a fractured core. A clean and symmetrical cut allows for fluorescent traces in the nW range to be picked up by your fiber photometry system. Polishing the tip of your fiber will ensure that the tip is flat and clean. Polishing the tip of the fiber is not



necessary, but highly recommended. Polishing the tip of the fiber will not only increase power efficiency but also maximize the collection field below your fiber. A fiber with a low efficiency will effectively lower your SNR.

## How To Cleave with a Neurophotometrics Ferrule Jig

1. Place the fiber in the well of the ferrule jig that corresponds to your desired fiber length (the fiber goes into the side of the ferrule with the measurements).



2. Holding the bottom of the ferrule in place with one finger, lightly score ONE side of the fiber using the scribe. The pressure applied should be light enough that the fiber does not snap off, but firm enough to push it up against the side of the well.
3. Flick the excess fiber off gently with your finger.
4. Check the efficiency of the fiber using a power meter to ensure it is about 80%. This can be accomplished by taking a power measurement out of the patch cord with the fiber attached and dividing it by the power coming out of just the patch cord (sans fiber **AND split sleeve**). If it is slightly below this threshold (3-4%), you may lightly polish the fiber by rubbing it gently on polishing paper placed on a rubber pad. We recommend [polishing paper from Thor labs](#), 0.3-3um should be fine.

While the instructions above include the use of a ferrule jig, you can still cleave a fiber without one. Using the same scribe, tape the ferrule to a ruler and carefully score one side of the fiber and flick off the excess. All rules still apply — make sure to use gentle pressure and try not to chop all the way through the fiber.

To mitigate scratches on the ferrule while the animal is in the home cage we recommend changing the caged lid to a raised caged lid. You can also install dust caps on the top of the ferrule. However the caps can easily get wedged into the caged lid or your animal can pull it off. If you have the raised cage lid and no dust cap, you can simply clean the ferrule with ethanol and a kim wipe and proceed with your experiment. For a cleaving instructional video, please visit our [website](#).

## Independence Test

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An independence test examines how much signal in one patch cord branch bleeds into another branch. Independence is tested on all FP3002 systems with an 8-branch patch cord (maximum number of branches that will fit in the field of view) prior to shipping. In most cases, you will not need to re-test independence, but it is a useful way to identify if a patch cord is damaged or is improperly aligned. Independence tests are only needed if you are using a branching patch cord. If your patch cord only has one fiber, an independence test is not needed.

Independence can be roughly assessed by following the steps below.

1. Connect and align your patch cord
2. Draw ROIs over each branch. It is best to draw your circles slightly smaller than the branches.
3. Cover all branches except for one so that no light is reaching them. Aluminum foil works great for this.
4. Create a basic Bonsai workflow as described in 'Build a Bonsai Workflow' section
5. Configure Bonsai settings
  - a. Set your **LED powers** to the same values you will use for recordings
  - b. **Frame rate** = 16
  - c. **Trigger sequence** = 470 only
6. Start your Bonsai workflow
7. Open the Visualize ROI node
8. Gently tap the end of the exposed branch with your finger
9. Look at the size of the peak in the branch you are tapping
10. Compare this change to the peaks in other branches. You want the change in signal in the branch you are tapping to be 200x larger than the change in other channels.
11. Repeat test with the 560 LED on if you are recording in the red channel

## Photobleaching Patch Cords

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Before recording an experiment, we recommend that you photobleach your patch cord overnight, or at least 12 hours, every month. Photobleaching your patch cord before a recording is important as to minimize the effects of photobleaching on your signal. Photobleaching your patch cord for an extended period of time will inhibit fluorescence of any fluorescent molecule within the fiber core and cladding. As a result, the rate of decay is less compared to the rate of decay from an unbleached patch cord.

To photobleach your patch cord, please attach your patch cord to a Neurophotometrics system. Open Bonsai and insert an FP3002 node into the workflow. Align your patch cord using the 1-axis translator, for more information please see section 'Connect and Align Patch Cord'. In the main FP3002 Setup window, under Trigger Sequence, select 470 and 415 nm LEDs. Then under power, slide the LED powers to 50%. Save the configuration and play the workflow. Please repeat this protocol once every month.

## Example Surgical Protocol

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Below is a sample fiber implant surgery protocol. These types of surgeries follow the same basic steps as a virus injection surgery, with a few added steps.

1. Put the animal under and move to stereotax.
2. Prep and clean the head according to your protocol.
3. Make an incision, no need to make it larger than you would for a virus injection
4. Dry the skull, we recommend using compressed air (not from a can!) for this
5. Level the skull.
6. Optional but highly recommended: chemically etch the skull to improve cement adherence. We recommend OptiBond Bonding reagent or Metabond dentin activator (green). Be careful to avoid touching any part of the skin with etching reagents. In general, if you etch the skull, you shouldn't need skull screws to anchor your headcap.
7. Drill holes for your injections and fibers. No need to drill a large hole, as only the core of the fiber will need to pass through the skull.
8. Infuse virus if you are using virally-delivered indicators.
9. Optional if you are having issues with losing headcaps: drill a shallow divot and attach skull screws.
10. Attach your fiber holder accessory of choice, then attach the fiber. Wipe down the fiber with 70% alcohol to sterilize. Bregma your fiber. Make sure the alcohol has evaporated, then lower it into the brain. You will want to implant your fiber at the same depth as your virus injection (not above).
11. Cement the fiber in place using your cement of choice. We like Tetric N-Flow which cures with ~10 seconds of blue light. It is very viscous and allows you to apply the glue focally which is necessary when implanting multiple fibers. We also like opaque Parkell Metabond (green jar). Be sure that whatever cement you use is opaque, as light contamination leaking through the headcap can impact your photometry signal.
  - a. If you are implanting more fibers than you have stereotax arms for, always cement the fiber furthest from bregma first, and be sure that you do not cover bregma.
  - b. Take care not to get cement on the sides of the fibers where the patch cord will attach. If you do get some on there, it is best to wait until your

cement is hardened to attempt clean-up so that you are not moving the fiber around inside of the brain.

12. Build up a headcap to cover any exposed skull. It is not necessary to make a large headcap. Make sure not to let the cement spread too close to the animals eyes.
  - a. Optional: use surgical glue to coat the outside of the cap so the skin adheres.
13. Once the cement is dry, gently remove fiber holders.
  - a. If any cement has gotten on the sides of fibers, gently scrape it off with a scalpel
14. Optional: dab antibiotic ointment around the edge of the headcap

If you are implanting multiple fibers that are close together (2 mm or so for 1.25 mm ferrule diameter), consider angling one or both. The bulkiness of the patch cord connection may make it difficult to connect both FOC at the same time.

For those who do not have a stereotaxic apparatus, we recommend using those from Harvard Apparatus or David Kopf Instruments. We recommend using a Stereotaxic Cannula holder, such as one from Doric Instruments linked [here](#), in order to implant your fiber optic ferrules. [These electrode holders](#) are also great for implanting 1.25 mm fibers that are close together (item no. 68216).

A note on virus expression time- for cell body expression, we recommend waiting at least 2 weeks before recording. For terminal expression, it can take up to 6 weeks. If using a flip-dependent virus, you may need to wait 8 weeks for cell body expression.

## Photometry-Guided Stereotaxic Surgery

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Manipulating or recording from the brain of a freely moving animal requires the precise implantation of an optical element (in most cases, a fiber optic cannula). Improper implantation will yield poor to no results during an experiment. The most common surgical implantation approach approximates coordinates of the brain from sutures of the skull (ie Bregma and Lambda). Unfortunately, the relationship between skull sutures and brain structures can vary animal-to-animal. This results in lower “hit rates” or the percentage of time your region of interest is accurately targeted.

There are a few factors that can reduce one’s hit rate:

- Implanting a fiber optic cannula over projection fibers.
- Use of steep angles and/or targeting deeper structures; this can compound errors resulting from improper leveling or abnormal sutures.
- Tiny brain regions and/or sparse expression.
- Targeting multiple brain regions; This compounds any implantation error. Even with a high hit rate of 90%, if you try to implant 4 brain regions your overall hit rate drops to 66%.

If you are experiencing a low hit rate that is affecting your efficiency or muddying your results, you may want to consider Photometry-Guided Stereotaxic Surgery.

Express your indicator (whether it be GCaMP, ChR2 with a fluorescent tag, or anything with a compatible absorption/emission spectrum) 2-4 weeks prior to your implantation surgery. If you are using a virus to express your indicator, this requires a total of two surgeries instead of one. The second surgery involves implanting a fiber optic cannula while it is connected to the photometry system.

Procedure:

1. After allowing for proper expression of your fluorophore, prepare the animal for surgery. Note that there will be considerably more scar tissue and bleeding compared to the initial surgery. Spend the time to remove all scar tissue and ensure the skull is completely dry prior to the next step.

# NEUROPHOTOMETRICS

The fiber photometry company



2. Setup your photometry system as with any other experiment. But set the frame rate to 16 Hz. Ensure you can visualize the de-interleaved signal in Bonsai (both 415 and 470-related emissions).
3. Connect your fiber optic cannula to a patch cord connected to the photometry system using a ceramic split sleeve. Unlike a traditional photometry experiment, have 2/3 of the split sleeve on the patch cord to make it easier to detach later.
4. Use the stereotax to locate bregma/lambda, mark your implantation site (AP and ML coordinates should be determined by a brain atlas, see notes at the bottom of this section for more information) and drill a fiber optic sized hole in the skull.
5. Connect the patch cord to the stereotax such that:
  - a. Fiber optic cannula is coaxially with the stereotax arm.
  - b. Fiber optic cannula is ONLY connected to the stereotax arm by the ceramic split sleeve.
  - c. There is sufficient slack in the patch cord to allow for movement of the stereotax arm.
6. Zero your z-axis to the tip of the fiber optic cannula as you would with a normal implantation.
7. Lower the fiber optic cannula into the previously drilled hole until it contacts the brain.
8. **WARNING:** If you are visualizing the surgery under a dissection scope, the photometry light may appear incredibly bright. Use of light attenuating goggles is recommended. Turn on the photometry system, interleaving 415/470 (for a GFP-based fluorophore).
9. Slowly lower the probe into the brain at an approximate rate of 1mm/minute. This rate is important for reasons discussed below.
10. You can use previous histological measurements from animals where you did not hit your target to determine the uncertainty associated with your implantations. We recommend starting to focus within 500um of the expected DV value.
11. ~500um above your expected DV value: You do NOT want to overshoot your ROI, as this could cause unnecessary damage. Slowly lower your probe, keeping a careful eye on the live traces from Bonsai. As you approach cells expressing GCaMP, you will see a rapid rise in the 470-related trace and a more modest rise in the 415-related trace. Continue lowering until you reach a peak or the rate of rise begins to decrease.
  - i. Note: The 470-related signal is most likely a real calcium signal caused by physical perturbation of expressing cells. Thus, this

signal will decay as the cells either return to homeostatic levels of intracellular calcium or die. This is why the speed with which you lower your probe can affect your accuracy.

- ii. The 415-related signal should be invariant of calcium concentrations. However, neural tissue (especially white matter) is quite auto-fluorescent when excited by the near-UV light. For example: You can clearly tell which layer of the hippocampus you are in as you lower your probe.
12. When you have reached your target, affix the probe to the skull as you would with any other surgery. Carefully detach probe from patch cord.
  13. Follow through postoperative care and wait ~7 days before recording an experiment to allow for recovery.

#### Concluding Notes:

While you can remove the probe to try a new XY coordinate, doing this can cause excessive damage to the brain. If you do wish to implant in a different coordinate, use a small probe (~50um or smaller) to find your ROI, before implanting a larger probe. Almost all brain regions are quiet under most anesthetics. It is quite rare to see any “real” calcium dynamics while performing this experiment. The increase in 470-related signal is specific to somatic GCaMP.



## Considerations for Behavioral Experiments

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Fiber photometry allows for recordings in awake and freely-moving animals and can be used during behavior. Below we discuss implications and considerations for behavioral experiments.

### **Habituation to Tethering**

We recommend habituating animals to patch cord tethering prior to the start of experiments to minimize disruption to behavior. Old or broken patch cords are perfect for this, but functional ones work too.

### **Labeling Patch Cord Branches**

If you are using a branching patch cord, it is important to label your branches so that you know which data column corresponds to each implanted fiber. Pieces of different colored tape or nail polish work great for this. Be sure to record in your notes which branch was used for each fiber.

### **Cleaning FOC Before Recording**

FOC can get quite dirty, especially in single-housed animals. Dust or lint on the FOC can interfere with light transmission and make it difficult to connect patch cords. We recommend wiping down FOC with an alcohol swab or alcohol-soaked Kimwipe before each recording.

### **Properly Connecting Patch Cords**

Any gaps between the patch cord ferrule and implanted ferrule can result in a significant loss of light transmission that can severely impact recordings. Always be sure the ferrules are touching, and that no light escapes. Split ferrule sleeves should allow you to see any gaps, but if you are recording in the low light, briefly turning on the LEDs can help find gaps, too. If no light escapes when you turn on the LEDs, your connection is good. You can also turn the room lights on and off, and if it shows up in your signal, you can assume there is some light leak. If there is cement or glue on the side of the FOC preventing full connection, we recommend putting the animal under and using a scalpel or dremel to scrape away excess cement.

## Setting up a Camera for Behavior Tracking

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Many labs set up cameras to record behavior to later align with FP signal. If you don't already have cameras set up, we highlight best practices for getting a clear and stable video. A fancy camera is not necessary for most experiments, a basic webcam like [this one](#) works great! For information on how to record videos through Bonsai alongside your photometry data and track subjects, see the [Bonsai Textbook](#) on our website.

1. **Pick a camera angle**- for most behavior, a birds-eye view is ideal. This view makes tracking in Bonsai or other software easier. However, for some experiments like feeding, observing motor control, etc. you may want to record from the side to get a better view of what the animal is doing.
2. **Secure your camera** - There are many ways to do this. If your behavior chamber has a top, taping (or screwing, if possible) the camera works well. If your chamber is open, we recommend securing the camera to a ring stand or flexible tripod, and positioning that above the chamber.
3. **Adjust your Field of View** - Adjust the camera position so that it captures the entire behavior arena and is centered as much as possible. Make sure that nothing is obstructing the camera view, i.e. whatever the patch cord is secured with. If you are recording in the home cage, you may want to remove any huts or items that may obstruct the camera.
4. **Mark an outline for your behavior chamber on the bench**- lab tape works great for this. This makes it easy to realign your behavior chamber if you have to move it to clean between trials. This will help to keep your videos consistent for tracking purposes.

## Patch Cord Management

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Patch cords are made of specialized glass that transmits light with high efficiency. However, they are still glass and must be handled carefully to avoid damage. Damage to your patch cord can greatly impact your photometry signal.

### **Anchoring Patch Cords**

We always recommend securing the patch cord above the behavior chamber to prevent excess bending. Our favorite way to do this is just to tape the cord to the top of the chamber or to a ring stand if the chamber is open. If you are having trouble with the cord twisting, you can also bend open a paperclip so that the two loops are perpendicular to each other, then tape one side to the top of the chamber. Feed the patch cord through the other loop so that it can still move and absorb any twists. Be sure not to close the chamber on the cord itself.

We do not recommend just letting the extra patch cord flop over the side of an open behavioral chamber or drag behind an animal. At best, this can introduce motion artifacts, and at worst, can cause your cord to bend too far and crack. **Always secure your patch cord above your animal when you are recording.**

### **Bending and Looping**

Bending the patch cord past the point that it can maintain total internal reflection will result in attenuation of signal. Generally, these look like dips or downward spikes across all channels. Artifacts like this can happen if the animal is sniffing/grooming/eating too close to the edge of the behavioral chamber, causing the patch cord to push against the side of the chamber. Sometimes, these types of artifacts are unavoidable, but good patch cord management can help reduce them.

If a patch cord gets very twisted, it can cause it to loop on itself. You want to avoid this. Generally, this leads to a tight bend in the patch cord, and significant attenuation of signal. If the animal continues to twist or pull on the looped patch cord, this can damage the cord. Having a long patch cord can help prevent this by absorbing more twists (we recommend 3m). Helping the slack of the patch cord to twist as needed can also reduce the risk of looping.

### **Leaving Proper Slack**

Leaving enough slack in patch cords is also essential to allow animals to behave normally and to protect the patch cord. There should be just enough slack in your patch cords to allow animals to reach all corners of the behavior chamber without having to pull against the cord. However, there should not be enough slack for animals to chew the cord. It doesn't take much for a mouse to chew completely through a patch cord!

You also want to minimize excess swinging of the patch cord, as this can introduce motion artifacts into your signal. Some movement is inevitable when you are recording from freely moving animals, but taping some of the slack can help to reduce motion.

### **Avoiding Cracks**

If the patch cord bends too far, it can cause the glass to crack and will severely impact signal. Sometimes this happens without any apparent damage to the outside of the cord. Wrap your cord loosely when storing, and do not allow it to twist tightly during experiments.

### **Extra Branches**

Some twisting and wear on the patch cord are inevitable. You will likely need to replace your patch cord periodically. We recommend always having a backup on hand in case you need to switch cords mid experiment. Having extra branches can also help. If one branch gets damaged, you can quickly swap to another without needing to change and re-align the whole patch cord.

If you are using fewer branches than your patch cord has, we strongly recommend covering and securing unused branches. Our favorite way to do this is to leave them loosely coiled on top of the FP system, secured with a single piece of tape. Be sure to also cover the ferrules. The white caps that come with the patch cord work great for this, but a piece of foil or tape works fine too. Just be sure not to get tape residue on the ferrule itself.

### **Labeling Branches**

It is also important to label patch cord branches, especially if you are recording from multiple regions. Numbering them and attaching a small, folded over piece of tape or

putting a small stripe of different colored nail polish works great. **It is critical that you note which branch corresponds to which ROI, as there is not a way to do this post-hoc.** For example- branch 1 in PAG and branch 2 in VTA.

### **Commutators**

We strongly discourage the use of commutators or rotary joints with fiber photometry, as they can lead to light loss of up to 50%. While generally this is not an issue with excitation power, it can make the difference between a great and an unusable emission. Robust and bright signals from GCaMP might still be usable with a commutator, but we would not recommend using one with novel biosensors that have a lower SNR. We always suggest starting without a commutator, and only adding one in if it becomes necessary.

## Additional Information and Scientific Background

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### Isosbestic Points

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GCaMP and other indicators fluoresce somewhat in their unbound state, and fluorescence increases sharply upon  $\text{Ca}^{2+}$  binding. The excitation/emission curves for bound and unbound GCaMP differ slightly. The point at which these two curves intersect is called the isosbestic point. At the isosbestic point (~420-425 nm for GCaMP), the strength of fluorescent emission is the same for both unbound and bound GCaMP. This means that at this wavelength, the fluorescent signal will be the same whether or not there is  $\text{Ca}^{2+}$  present. Accordingly, any changes in fluorescence at this wavelength can be ascribed to other factors like motion, photobleaching, changes in pH, and absorption of light by hemoglobin. Recordings using the 415 channel can be used as a control for GCaMP signals.

#### **More information:**

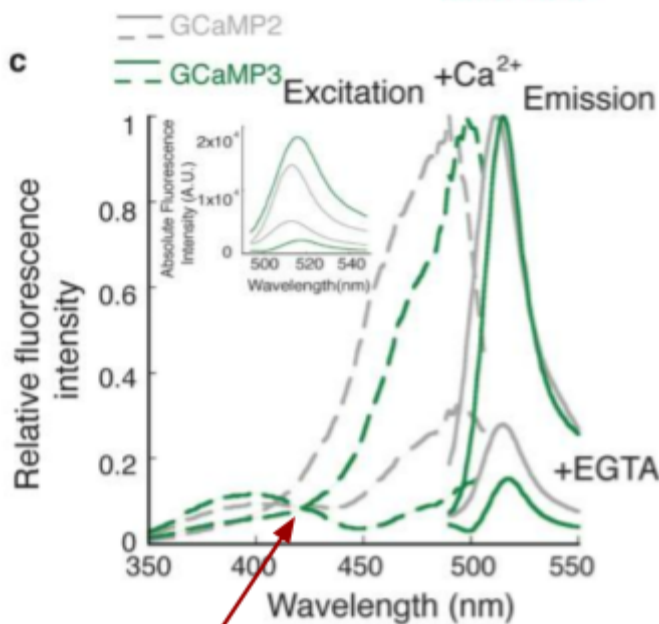
Green fluorescent protein (GFP) can be transiently excited by photons in the blue range (~470nm). When it relaxes from this unstable state, energy is released in the form of a photon. Due to conservation of energy, it must have less energy than the photon that was absorbed – so, it releases a photon in the green range (~520nm). This difference is known as a Stokes shift.

GFP-based indicators (GCaMP, dLight, GRAB-NE, etc) link the ability to fluoresce (absorb one photon and release another lower energy photon) to a binding event. Conceptually, in the case of GCaMP, when it is bound to calcium, it behaves similarly to standard GFP. However, when it is unbound, it is much less fluorescent. This delta ( $F_{\text{bound}} - F_{\text{unbound}}$ ) is what drives the high signal-to-noise ratio (SNR) of this indicator.<sup>1</sup>

If you UV-shift the excitation wavelength (i.e. reduce the wavelength), this delta decreases. Note: the emission *wavelength* is unchanged - only the amplitude/SNR are

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<sup>1</sup> Barnett, L. M., Hughes, T. E., & Drobizhev, M. (2017). Deciphering the molecular mechanism responsible for GCaMP6ms  $\text{Ca}^{2+}$ -dependent change in fluorescence. Plos One, 12(2). doi:10.1371/journal.pone.0170934



Isosbestic point

Modified from Tian et al.  
2009 *Nature Methods* (2)

changing. At a certain point, known as the “isosbestic point”, the two values are equal ( $F_{\text{bound}} = F_{\text{unbound}}$ ). An important caveat that we exploit with photometry is that, though diminished, we still observe fluorescence at this point. So, if we excite GCaMP with a higher energy photon, we can get an emission signal that does not depend on whether it is bound to calcium or not. In essence, it behaves like a lower SNR GFP molecule.

## How can we use this information?

The brain is squishy and optics/the skull are not. This mechanical mismatch leads to movement of

neural tissue relative to your implant. Emission levels when excited at the isosbestic point are only sensitive to the number of GCaMP molecules beneath your implant – so movement will result in a change in that signal. If we were only recording the highly calcium-dependent 470 nm-related signal, we would not be able to determine whether this change was due to movement or a change in the fraction of molecules that are bound to calcium.

Another important function of this isosbestic control is to quantify the impact of photobleaching on your recording. Photobleaching is a phenomenon where a fluorescent molecule loses its ability to fluoresce in a light-dependent manner. More light leads to faster rates of photobleaching. This is HUGELY important as the same underlying biological event will produce a larger 470-related photometry event early on in a recording compared to later. This can skew your interpretation of the results and can produce both false positives and false negatives. That’s where the isosbestic-control

<sup>2</sup> Tian L.; et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* 2009, 6, 875–881. 10.1038/nmeth.1398.

comes in handy! As the isosbestic, or 415 nm-related emission, is proportional to the *number* of fluorescent molecules beneath your implant, the emission will decrease as a function of time as more and more molecules lose their ability to fluoresce. This allows you to correct your raw 470 nm-related emission so as to make it ONLY dependent on the biochemical event of interest (in the case of GCaMP, this would be the fraction of molecules that are bound to calcium).

The true isosbestic point of GCaMP is closer to 420-425 nm, however, it is also pH dependent and can be a bit of a moving target across preparations. Regardless, 415nm is indeed shorter than the isosbestic wavelength. So, why did we choose to excite at this wavelength?

The delta ( $F_{\text{bound}} - F_{\text{unbound}}$ ) changes asymmetrically about the isosbestic point. If you red-shift the excitation wavelength, you will quickly see a signal where  $F_{\text{bound}} \gg F_{\text{unbound}}$  that will approximate your 470-related signal, just with lower SNR. Experimentally, this would not be that useful as a control. If you UV-shift the excitation wavelength from the isosbestic point, as we did,  $F_{\text{bound}} < F_{\text{unbound}}$ . This delta is quite small, and for most recordings, the resulting emission is explained by the number of fluorescent molecules under your fiber more-so than its state (bound or unbound).

Experimentally, this signal serves two purposes.

- 1) Correcting for photobleaching
- 2) Validating slow changes in signal

*Correcting for photobleaching:* For most recordings that are > 10 minutes in length, you will observe a decay in your 415-related signal that can be fit with a biexponential decay. If you divide your 470-related data by a linearly scaled version of this fit, you will, largely, correct for photobleaching.<sup>3</sup>

*Validating slow changes in signal:* Photometry is great at detecting relatively fast (seconds-long) changes in signal related to a discrete event. Things get trickier when the biochemical event has kinetics that begin to approach those of photobleaching (10s of minutes). For the purposes of discussion, let's say we wanted to see a drug decrease activity in a particular brain region. We express GCaMP in this region, implant a fiber,

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<sup>3</sup> This is an estimate. The length of the recording required to observe this decay, and to effectively correct for bleaching, is proportional to your SNR. Higher SNRs require longer recordings to observe a downward trend in your 470-related signal.



hook it up to our photometry setup, record a 10-minute baseline, and then administer the drug. For the purposes of this discussion, we assume that this drug drives the mean firing rate of neurons in this brain region to decrease, linearly, for 20 minutes and then plateau.

If we look at the slower kinetics of the 470-related signal, it will always be trending downward, as photobleaching and the effect of the drug are working in **concert** to decrease emission. Without the 415nm control, we would be at risk of the photobleaching occluding our biological effect resulting in a false-negative result.

But we're savvier than that! We've studied the exquisitely dense biophysics literature on calcium indicators and know that the 415-related signal is *slightly* more sensitive to unbound GCaMP, compared to bound. For the 10-minute baseline, our 415-related signal decreases as a function of photobleaching. However, as the drug begins to take effect, the fraction of GCaMP molecules that are calcium-bound decreases, and we observe an inflexion point as our 415-related signal begins to increase.<sup>4</sup> When the mean firing rate reaches its lowest point, the 415-related signal no longer increases, and the main effect is not photobleaching.

We will have to pivot our approach to correcting for photobleaching here depending on the relationship between the duration of the recording and the duration of the effect. Looking at the raw data, we can be confident that our effect is real based on the negative relationship between the 470 and 415nm signals.

### **How does this relate to red-shifted indicators?**

In photometry, red shifted indicators, such as jRGECO and RCaMP, can be used in conjunction with green indicators to record from multiple cell populations with a single fiber. Some research suggests one can use the same UV-shifted excitation wavelength to get a calcium-independent signal from these indicators (Supplementary Note and Fig. 2 in Kim et al., 2016<sup>5</sup>). Our experience, generally, corroborates these results as the

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<sup>4</sup> [1] Grain of salt: You would only observe an increase if the effect size was larger than the rate of photobleaching. Either way, the rate of decrease would decrease.

<sup>5</sup> Kim, C. K., Yang, S. J., Pichamoorthy, N., Young, N. P., Kauvar, I., Jennings, J. H., . . . Deisseroth, K. (2016). Simultaneous fast measurement of circuit dynamics at multiple sites across the mammalian brain. *Nature Methods*, 13(4), 325-328. doi:10.1038/nmeth.3770

Siciliano, C. A., & Tye, K. M. (2019). Leveraging calcium imaging to illuminate circuit dysfunction in addiction. *Alcohol*, 74, 47-63. doi:10.1016/j.alcohol.2018.05.013

predominant response one sees in the 415 nm-related red emission is a familiar biexponential decay and it is sensitive to motion artifacts.

Identifying motion artifacts with this signal works the same as with green emission and GCaMP. When thinking about photobleaching, though, things get a bit trickier. To do so, we have to think about how a green and red signal are recorded (near) simultaneously. This varies slightly if you are using a V1 (FP3001) or V2 (FP3002) system but, functionally, their behavior is the same.

This type of photometry setup operates using “time division multiplexing (TDM)”, i.e. “alternating.” In a case where we are recording from green and red indicators, we record one frame with the 415 nm LED on, then one frame with the 470nm LED on, then another with the 560 nm LED on. This is all happening very quickly (up to 200 times a second), much faster than any change in the relative emission of the indicators themselves (100s of ms at the fastest) so, effectively, we are able to record the resulting emission from three excitation frequencies simultaneously.

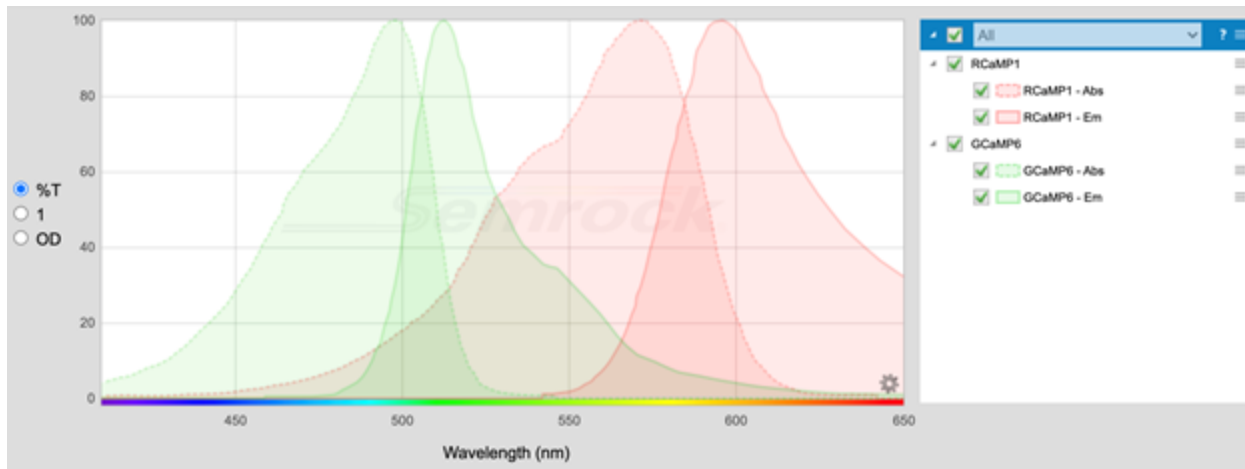
On the emission-side, the green and red signals are split by a dichroic mirror and filtered by respective bandpass filters, before being projected onto different locations on the camera sensor. This converts a *spectral* shift into a *spatial* shift – with one half of the sensor showing green emission and the other showing red. So, technically, from a single fiber, we are capturing six distinct readings.

Not all of these are useful. For instance, the green emission when the 560 nm LED is on does not give us information about our underlying biological construct. Same, for the most part, with the red signal with the 470nm LED on.<sup>6 7</sup> So, in practice, we are getting four usable signals from each fiber.

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<sup>6</sup> Some caveats to this in Molina et al., 2019.

<sup>7</sup> Molina, R., Qian, Y., Wu, J., Shen, Y., Campbell, R., Hughes, T., & Drobizhev, M. (2019). Understanding the Ca<sup>2+</sup>-dependent Fluorescence Change in Red



Looking at the above absorption/emission spectra for GCaMP6 and RCaMP1<sup>8</sup>, we see that there is a good portion of the green emission (~500-530 nm) where there is no red emission; and we chose a bandpass filter to ensure this! GCaMP's emission, though, has a long tail and there isn't a "clean" section in red where we don't get *some* green signal too.

Using TDM, we can skirt this issue for the calcium-dependent conditions by only looking at the red signal when the 560 nm LED is on. In this case, there isn't any GCaMP emission, and we don't have to worry about crosstalk. But in the calcium-independent case, 415nm excites *both* GCaMP and RCaMP. This isn't an issue for green, but our red emission does contain *some* signal from GCaMP. So, what to do? Luckily the amount of crosstalk is going to be constant and indicator specific.

$$\text{Calcium-Independent Red} = 415\text{nm Signal in Red} + X \cdot 415\text{nm Signal in Green}$$

(X = fraction of crosstalk)

Functionally, the rate of photobleaching across the two indicators is fairly similar (and they are being hit with the same power and wavelength light) so one could approximate the impact of photobleaching by generalizing the 415nm signal in green.

<sup>8</sup> Genetically Encoded Ca<sup>2+</sup> Indicators. *Biophysical Journal*, 116(10), 1873-1886. doi:10.1101/435891

To summarize: The isosbestic signal in the red channel can be used to detect motion artifacts and, with a few steps, to isolate the contribution of photobleaching to your signal.

### **Concluding Thoughts**

The 415nm-related emission is a powerful internal control that lets you:

1. Detect motion artifacts
2. Correct for photobleaching
3. Validate observations of slow changes in signal

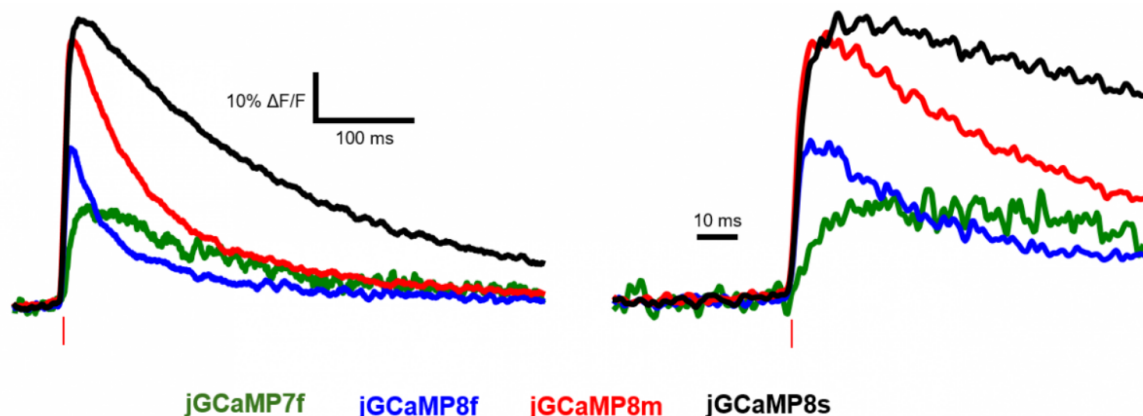
## Choosing GCaMP Variants

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There are many options available when it comes to calcium indicators. Below we discuss considerations for choosing the optimal sensor for your experiments.

### Choosing a GCaMP variant

GCaMP has gone through the most iterations of any sensor and has an excellent signal to noise ratio. There are numerous versions available. Generally, GCaMP variants are classified by their kinetics and maximum change in fluorescence in response to one or more action potentials. S (sensitive) variants have high sensitivity and a slow decay and are ideal for detecting sparse activity. F (fast) variants have a lower sensitivity and fast decay times, ideal for resolving individual events. M variants are somewhere in the middle. For further information on variants, see [here](#).



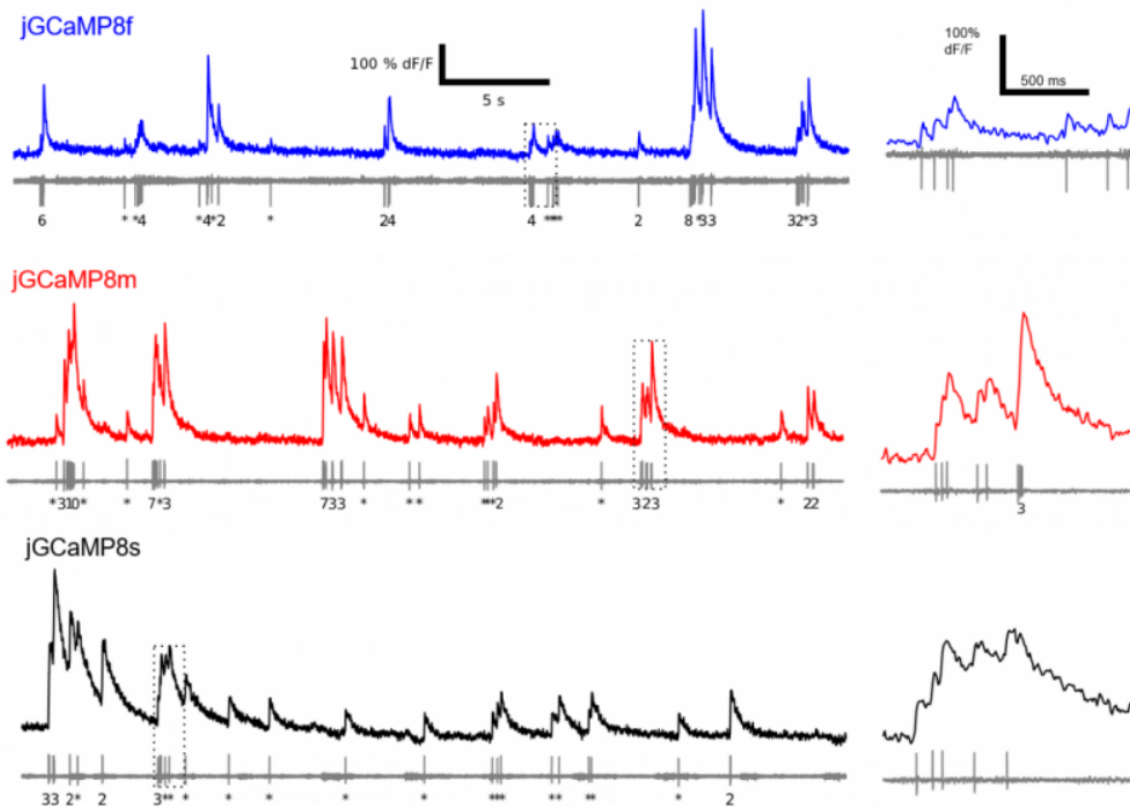
Zhang et al. 2020<sup>9</sup>

Choosing the best sensor for your experiment can sometimes require trial and error. If you are recording from a population of cells that are sparse or have low activity, we recommend starting with jGCaMP8s. If your population is dense, very active, or you want to look at discrete behavioral correlates that occur close in time (i.e. a series of lever presses), we recommend starting with jGCaMP8f. If you are unsure what your target population will look like, jGCaMP8m is a good place to start. Doing a pilot

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<sup>9</sup> Y. Zhang, M. Rózsa, D. Bushey, J. Zheng, D. Reep, G. J. Broussard, A. Tsang, G. Tsegaye, R. Patel, S. Narayan, J. X. Lim, R. Zhang, M. B. Ahrens, G. C. Turner, S. S.-H. Wang, K. Svoboda, W. Korff, E. R. Schreier, J. P. Hasseman, I. Kolb, L. L. Looger, *jGCaMP8 Fast Genetically Encoded Calcium Indicators* (2020). doi:10.25378/janelia.13148243.

experiment with multiple variants can be informative as well. Both cre-dependent and constitutive AAV vectors are available through [Addgene](#).



Zhang et al. 2020

### Virally-Delivered GCaMP v. Transgenic Crosses

There are options when it comes to how GCaMP will be delivered to the target population in mice, either via 1) injection of a viral vector to the region of interest or 2) crossing a transgenic cre line to a transgenic line expressing cre-dependent GCaMP (i.e. Jackson Laboratory strains [Ai148](#) or [Ai195](#)). Using a viral approach allows for greater flexibility in sensor variants, but adds an injection step to surgeries. The latter requires the maintenance of transgenic lines, but can speed up surgeries, especially if you are doing photometry-guided implantation. Both work for fiber photometry.

## Best Practices for Novel Neurotransmitter Biosensors

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Novel neurotransmitter-specific sensors (including dLight, iGABASnFR, GRAB 5HT, etc) allow for imaging of neurotransmitter activity with high specificity and temporal resolution. However, few standard practices have been established in the field when it comes to their use with fiber photometry, and more optimization is required for successful recordings. Below we highlight important considerations for experimental design when using these sensors.

### **Brightness and Signal to Noise Ratio**

In general, neurotransmitter biosensors (NT sensors) have not been optimized to the extent that GCaMP has and accordingly, are less bright and have a lower signal to noise ratio (SNR). This can make it difficult to detect changes in signal and to separate NT dynamics from noise. You may be unable to distinguish signal from noise if behaviors or stimuli of interest elicit only a small change in NT release, or in regions where signaling is not robust. Even excellent NT sensor signals are small compared to GCaMP. If you are used to looking at GCaMP data, do not be discouraged by a lack of clear spikes. You may not see spikes at all with certain neurotransmitters and neuromodulators depending on the characteristics of transmission and of the sensor itself. You also may not see changes in real time during recording sessions. We always recommend analyzing NT sensor traces before deciding whether or not to continue recording.

### **Choosing a Sensor**

Currently, there are sensors available for many neurotransmitters and neuromodulators, and for some, there are multiple options. Both GPCR and periplasmic binding protein-based sensors are suitable for use with the FP3002 system, as long as they are excited by 470 or 560 nm light.

One unique feature of GPCR-based sensors is that they are sensitive to drugs that bind to the receptors they are derived from. This is important to consider if you plan to combine pharmacology with fiber photometry- i.e. an antagonist will attenuate fluorescent signal. However, this can also be used as a control.

Sensor variants differ in their dynamic range, affinity, and kinetics, which can significantly impact recordings. For a comparison of various sensors, see [this review](#). Choosing the best sensor for your experiment based on these properties alone can be difficult. We recommend testing a few different sensors to see what works best with your applications. For more information on choosing sensors (particularly dopamine sensors) we recommend [this review](#).

## Recording Parameters

In general, NT sensors can be recorded using the same LED parameters as GCaMP. We recommend starting with ~50 uW light (for a 200 um fiber). Ideally, you should aim for the output from each ROI to be between 0.03 and 0.1 in the visualizer node. If your signal is below this, you can turn up LED power as needed.

Frame rate requirements vary greatly by sensor. Some have slower kinetics than GCaMP, and some are significantly faster. Be sure to read up on the kinetics of your specific sensor when designing experiments. Keep in mind that acquisition frequency is inversely proportional to the amount of time the camera shutter is open. The longer the shutter is open, the more light you will collect, boosting your signal, and vice versa.

## Controls

Little consensus has been reached when it comes to appropriate controls for imaging NT sensors with fiber photometry, but many options have been published.

1. **Co-infection with a static fluorophore of a different color** (i.e. tdTomato with dLight). With this approach, the control fluorophore provides a motion control in the same region as your recording<sup>10</sup>.
2. **Static Fluorophore in separate animals** (GFP). This approach does not provide a control for motion artifacts within the same region, but can control for other artifacts including light absorption by hemoglobin (more information [here](#)). Note- fluorescent proteins can be significantly brighter than NT sensors of the same color, making it nearly impossible to image both with the same parameters. We

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<sup>10</sup> Sun F, Zeng J, Jing M, Zhou J, Feng J, Owen SF, Luo Y, Li F, Wang H, Yamaguchi T, Yong Z, Gao Y, Peng W, Wang L, Zhang S, Du J, Lin D, Xu M, Kreitzer AC, Cui G, Li Y. A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell*. 2018 Jul 12;174(2):481-496.e19. doi: 10.1016/j.cell.2018.06.042. PMID: 30007419; PMCID: PMC6092020.



recommend diluting viruses to match the brightness of your sensor as much as possible<sup>11</sup>.

3. **Mutated Sensors**- These sensors are developed alongside functional sensors as controls, and are identical except that they do not bind the ligand of interest. Any changes in fluorescence should be independent of NT release. The concept of mutated sensor controls is similar to using a static fluorophore, except these sensors recapitulate the expression pattern, brightness, and optical properties of the functional sensors. These are only available for certain sensors<sup>11,12</sup>.
4. **Isosbestic channel**- The use of the isosbestic (415) channel for NT sensors has not been thoroughly validated. While some sensors have optical properties similar to GCaMP, not all green sensors have the same isosbestic point. Additionally, excitation/emission spectra are not published for all sensors<sup>12,13</sup>.

## Tips for Success

1. **Establish a robust positive control.** If possible, it is very helpful to come up with a temporally discrete stimulus that reliably elicits a response with your sensor of interest. A quick way to assess whether or not you have signal in an animal prior to starting experiments can save a lot of time. Often an airpuff or a tail pinch works.
2. **Pilot virus dilution.** Though it seems counterintuitive as the overall brightness of many NT sensors is low, you may want to dilute your viruses. Overexpression of your sensor will lead to smaller dynamic range or even saturation of your signal. The FP3002 system is extremely sensitive and will still detect changes in signal when the brightness is low.
3. **Consider restricting expression.** Restricting expression either via a sparse promoter, or using a cre-dependent sensor in a transgenic cre line can similarly prevent overexpression of your sensor. It can also improve the signal to noise ratio in regions where you might expect asynchronous NT release, for example, using a GABA sensor in a highly GABAergic structure. Keep in mind that a noisy signal does not necessarily mean a lack of activity, there may just be

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<sup>11</sup> Unger EK, Keller JP, Altermatt M, et al. Directed Evolution of a Selective and Sensitive Serotonin Sensor via Machine Learning. *Cell*. 2020 Dec 23;183(7):1986-2002.e26. doi: 10.1016/j.cell.2020.11.040. Epub 2020 Dec 16. PMID: 33333022; PMCID: PMC8025677.

<sup>12</sup> Wan J, Peng W, Li X, Qian T, et al. A genetically encoded sensor for measuring serotonin dynamics. *Nat Neurosci*. 2021 May;24(5):746-752. doi: 10.1038/s41593-021-00823-7. Epub 2021 Apr 5. PMID: 33821000; PMCID: PMC8544647.

<sup>13</sup> Sun, F., Zhou, J., Dai, B. *et al.* Next-generation GRAB sensors for monitoring dopaminergic activity in vivo. *Nat Methods* 17, 1156–1166 (2020). <https://doi.org/10.1038/s41592-020-00981-9>

asynchronous activity that when measured as a bulk signal, averages out. Restricting expression to a population of interest may help prevent this.

4. **Include many subjects.** NT sensor signals can vary widely from animal to animal, and often baseline signals can be unstable. Once you have your experimental design established, including more animals than you typically would for GCaMP experiments can help to average out these differences and prevent false negatives or positives in your data.
5. **Consider not using a commutator.** While using a commutator can help manage patch cord twisting, they can result in a 50% loss in signal. This can have a big impact on NT sensor signal. If you are using a commutator and having trouble getting a large enough signal, try recording without the commutator.

## Troubleshooting

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Below are common issues and solutions. Further troubleshooting questions can be sent to [support@neurophotometrics.com](mailto:support@neurophotometrics.com)

### Software and Hardware

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**Q: My system will not turn on**

**A:** Rotate the AC/DC connector plugged into the back of the system.

**Q: I do not see any of the Neurophotometrics nodes in my Bonsai toolbox.**

**A:** Download [Spinnaker 1.29.05](#). You will need this version in order to use any of the Neurophotometric nodes. See Downloading Software on page 9 of this manual.

If you have installed the correct Spinnaker and still have errors, you may be missing some drivers. The best thing to do is completely uninstall Bonsai from your computer, as well as SpinView/Spinnaker. Follow the steps for installation, when choosing between Camera Evaluation or Application Development choose Camera Evaluation. Uncheck "I will use GigE Cameras" and install.

BEFORE installing those, please install the FTDI drivers:

<https://bitbucket.org/fchampalimaud/downloads/downloads/UsbDriver-2.12.26.zip>

**Q: My Calibrate Laser button is disabled, or my laser will not turn on.**

**A:** You will need to ensure that both laser governors are enabled. First make sure that the key on your system stub wall is in the slot and turned 90 degrees clockwise. The second governor is found in the FP3002 calibration window. You need to manually type in your laser wavelength. This will be 450 or 635. Do not add in any spaces or symbols. There is not a range, the software will only enable the laser if you type in the exact wavelength.

**Q: My system’s connection to Bonsai keeps on dropping.**

**A:** Most likely your USB connection is faulty - if you have used an alternate USB cable from that which was provided, this is the cause. Similarly, this can occur if an extension cord is used.

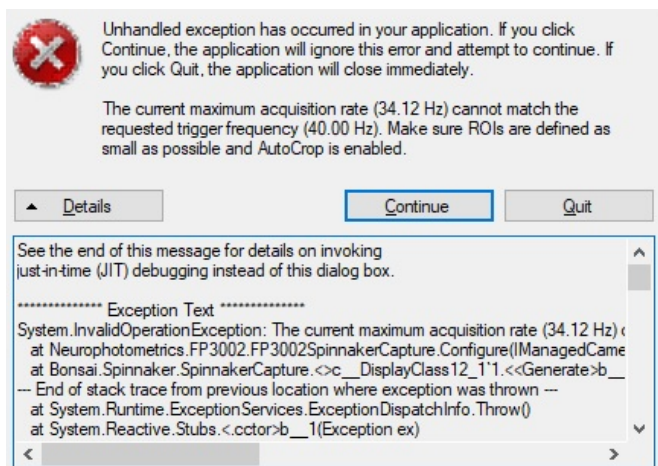
**Q: How do I save my ROIs so that they are the same each day?**

**A:** ROIs are defined and processed via Bonsai; therefore, your ROI configuration is saved in the Bonsai file. Even though the ROI configuration can be saved, we recommend checking ROIs before each experiment as good practice.

**Q: I have received a similar error message located to the right when I click “Calibrate Regions” or when I run the workflow.**

**A:** If this message pops up when you run the workflow, please set “AutoCrop” to True. AutoCrop can be found by clicking on the FP3002 once and accessing the Properties on the right hand side of your

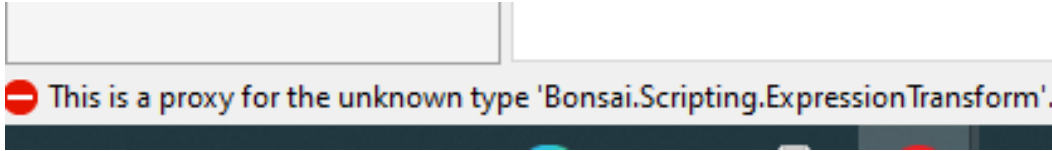
Bonsai window. If this message pops up when you click “Calibrate Regions” this means that the system has or currently is plugged into a USB 2.0 port - you have used an alternate USB3 cable - or the cable is not plugged directly into the computer (uses an extension cord or USB hub). To remedy this, please remove all hubs/extensions and ensure your USB3 is plugged directly into a USB3 port. Then power off the FP3002, unplug the USB cable, close Bonsai, replug the USB in the USB3 port, power on the FP3002, open Bonsai.



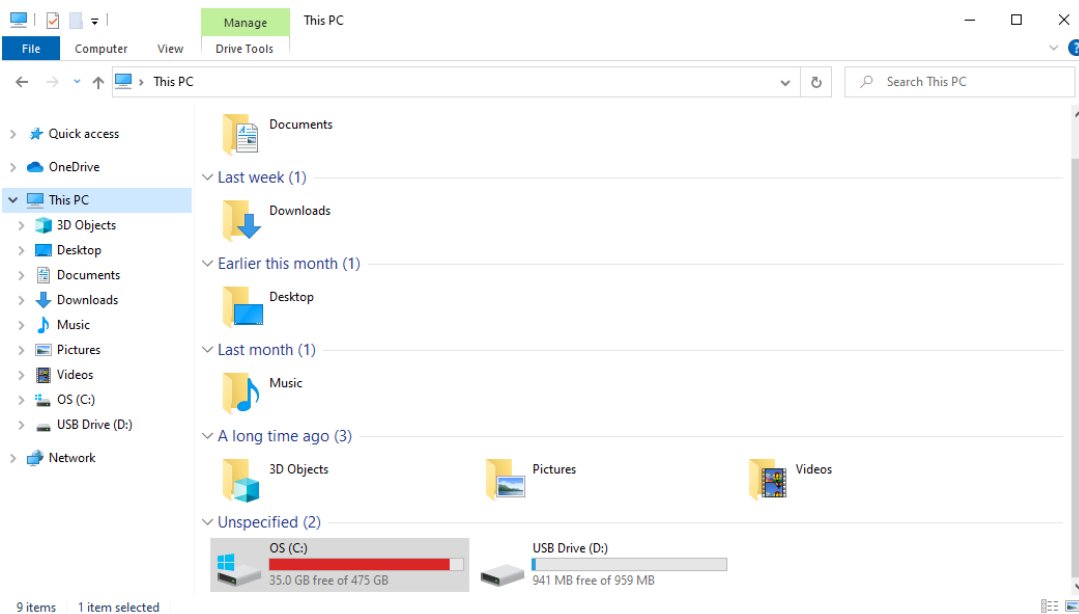
**Q: Where should I save my data and how do I navigate directories?**

**A:** Saving your data in organized directories is a key part of any experiment, else important experimental data can be lost. When setting up your Bonsai workflow, double click the writer node that you are using to save data (i.e. “Photometry Writer”, “CsvWriter”, “VideoWriter”, etc.). This should open your operating system’s “File Explorer” window. This is what you will use to navigate your directory to choose the location you want your file to be saved.

**Q: How do I fix the error “This is a proxy for the unknown type ‘Bonsai.Scripting.ExpressionTransform’”?**



**A:** Install the “Bonsai - Scripting Library” Package. In the search bar for the file explorer window, one can see that the path for The “Experiment1 Data” folder is “C:\Users\CJohn\Documents\FP\_Experiments”. Since this is currently saved on this PC’s SSD, it is inside of the “OS (C:)” drive. However, sometimes it is beneficial to save elsewhere. For example, if you wish to save data directly to an external drive, you can navigate all the way out to “This PC” and instead of selecting the “OS (C:)” drive, you can enter the “D:” drive and save there.



**Q: Why aren’t my ROIs starting at 0?**

**A:** They are! ROIs always start at 0 - so there must be some hidden “micro-clicks” that have made pixel sized ROIs. Draw/select a big ROI that you can click. Then hit “tab” on your keyboard to pass through the other hidden ROIs - with each one, hit “delete” on your keyboard to remove it. You can clear all the ROIs this way.

## Experiments and Photometry Signal

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**Q: I'm not seeing a clear signal.**

**A:** This is the most common question we get, and there are many reasons why you may not be getting a great signal. Below is a list of common issues that can impact signal quality.

- **Mismatch in optical parameters of patch cords and implanted FOC-** using a patch cord or FOC of incorrect numerical aperture or core diameter can significantly attenuate light transmission. See Patch Cord Guidelines for more information.
- **Not cleaving optical fibers with a proper scribe-** A properly cleaved optical fiber is key to efficient light transmission. Cutting fibers with scissors or a razor blade can result in a rough surface that will not transmit light well. See How to Cleave Fibers section for more information. We also recommend testing fibers for transmission efficiency prior to implant. If you prefer not to cleave fibers yourself, you can order specific lengths from us.
- **Patch cord is out of alignment-** Be sure to align your patch cord after attaching it to your system. A patch cord that is out of alignment will result in a blurry image and suboptimal signal. You will also want to double check alignment if you change the patch cord. See Connecting and Aligning My Patch Cord for more information.
- **Gap between patch cord and implanted ferrule-** Not connecting the patch cord such that the ferrule is touching the implanted ferrule can result in a significant loss of signal. You can check that your patch cord is fully connected by turning the room lights on and off. If you see a response in your photometry signal, you may have a gap. You can also turn on any of the LEDs and check for light leak.
- **Insufficient virus expression time-** If you are imaging cell bodies, we recommend waiting at least 2 weeks for virus expression. If you are recording projections, 6 weeks is ideal. Recording before you have full sensor expression will result in a weaker signal.

- **Implanting fiber above ROI-** For fiber photometry, you want to implant your fiber in, not above, the structure you are targeting (use the same coordinates as you would for virus infusions).
- **Use of a commutator-** While using a commutator may not have as dramatic an effect on your signal as other factors, they can lead to a 10-20% loss in signal. This can make the difference between a usable and noisy signal in some cases, particularly when recording from terminals or when using novel neurotransmitter sensors. We always recommend starting without a commutator, and adding one in only if necessary.
- **Tightly coiling excess patch cord-** Patch cords can only bend a certain amount until they can no longer maintain total internal reflection, meaning that beinding your cord too lightly can attenuate signal. If you coil excess patch cord, aim for a diameter of 6 inches or more.
- Population of interest is sparse-

**Q: Why is my signal a smooth flat line?**

**A:** Generally, this happens when your signal is maxed out. Lowering the LED output will help. If this is a persistent issue, diluting viruses prior to injection or restricting expression can help.

**Q: Can I use the same FOC that we use for optogenetics?**

**A:** Using the same FOC for optogenetics and fiber photometry is fine as long as the optical specs match those of the patch cord and FP3002 system (0.37 NA, 200 or 400 um core diameter). However, we do not recommend using white ceramic ferrules, as ambient light contamination can interfere with photometry signals. We recommend using metal or black ceramic ferrules.

**Q: An animal that had a good signal yesterday suddenly looks noisy or flat.**

**A:** This can happen if a piece of dust blocks light transmission. Unplugging the animal and using compressed air or an alcohol swab to clean both the FOC and the end of the patch cord will remove any debris. If the issue persists, the patch cord may need to be replaced. Patch cords do wear out, especially if they get chewed, kinked, or heavily twisted.