FP3002 Manual

Spring 2021

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NEUROPHOTOMETRICS

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Important Safety Warnings

1. Ensure that your microB (the cable from your PC, USB) is plugged into the front stub wall of your system before booting FP3002.
2. Ensure that any BNC connections are made before booting FP3002.
3. Prior to turning on your system, decide if you will be using the 450nm or 635nm 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). Also before turning on the system - make sure that your ribbon wire is connected to the appropriate laser.
4. Do not ever look directly into a patch cord.
5. Wear the appropriate laser safety goggles when using the lasers.
6. Please prepare your inputs and outputs with care. Input signals should be no larger than 5V. Do NOT check Out0 and Out1 if you do not intend to record the state of each LED.

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FP3002 Quick Start Guide

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 front stub wall, plug your USB-3 adaptor into the top-left port and connect to a USB-3 port on your computer. Please do NOT use a USB extension cable, we cannot guarantee that your system will work properly.
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. While this is happening, open your Bonsai program and make sure your parameters are as desired.
5. 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.

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. The system was shipped to you with the focus locked. Open the lid, and use the L-hex-key (1.5 mm) to unlock the translator nob, and then the straight hex-key (2 mm) 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.
8. You are ready to record!

IMPORTANT: Read the manual and associated safety instructions prior to use.

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FP3002 Technical Specifications

Acquisition Rate: 1-200 Hz; 80 Hz standard
Absolute Sensitivity Measure: 4.51 (photons needed to equal noise)

OptoStim Lasers:
Red Laser Power Max: ≥10mW from a single 200 µm fiber
Wavelength: 635 nm +/- 5 nm
Blue Laser Power Max: ≥30mW from a single 200 µm fiber
Wavelength: 450 nm +/- 5 nm
Stimulation Frequency: 1-100Hz

Simultaneous Recordings from multiple fibers:
8 branching patch cord (200 µm each)
4 branching patch cord (400 µm each)
**please note, we always recommend*
200 µm over 400 µm, and it is critical to ensure that the outer diameter of the bundle does not exceed 1mm.

LEDs for recording:
*415 (isosbestic)**: 0.1 - ≥400 µW
*470 (e.g. GCaMP, dLight)**: 0.1 - ≥560 µW
*560 (e.g. RCaMP)**: 0.1 - ≥130 µW
Step Size: 25 nW
*min and max through 200 µm fiber

Numerical Aperture: 0.37 - 0.4

Digital Inputs: 2
Digital Outputs: 1 + laser

Timing Synchronization: sub-millisecond synchronization with external equipment

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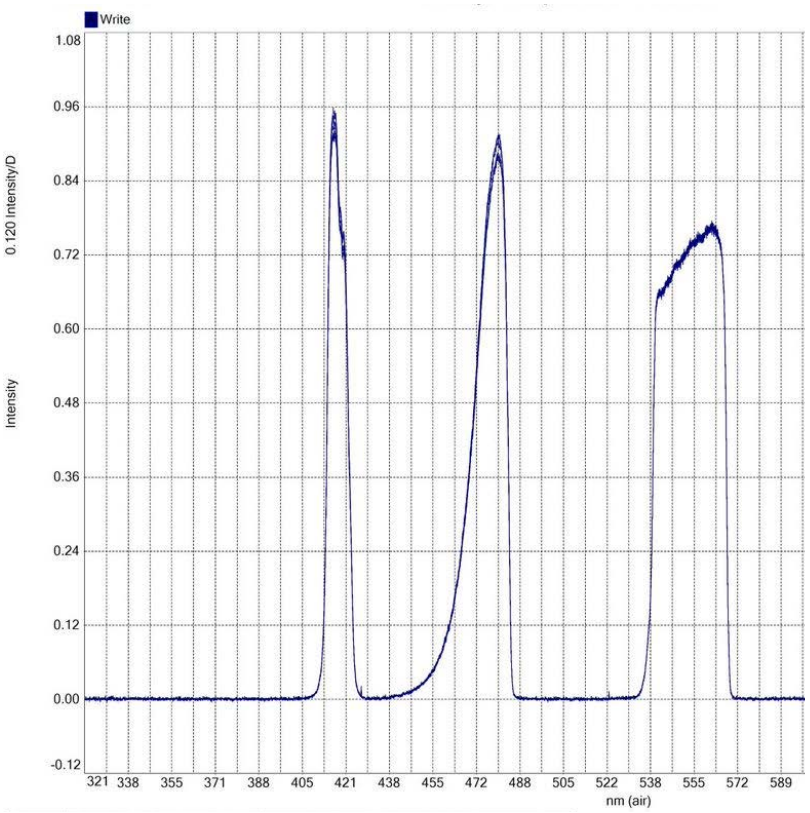
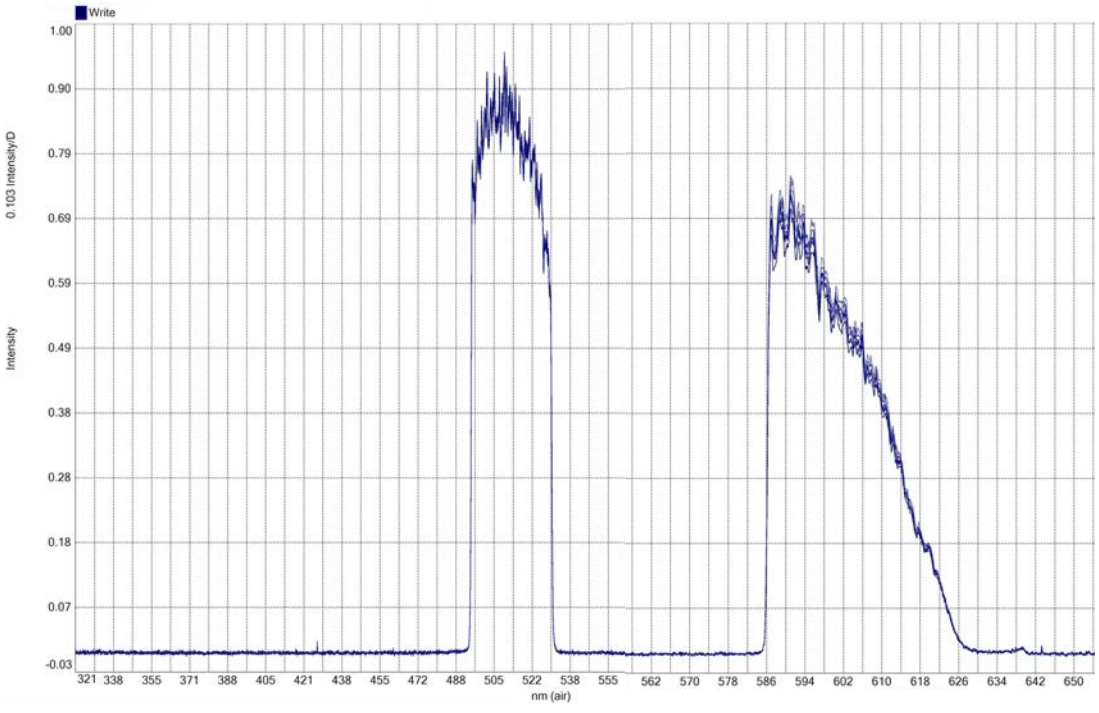
FP3002 Excitation and Emission Spectra

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. The channels are named these wavelengths because each LED emits the highest intensity at that wavelength. 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 - 531nm

Red Emission Channel : 586 - 627nm.

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What is Included in the FP3002 System

Anatomy of the System

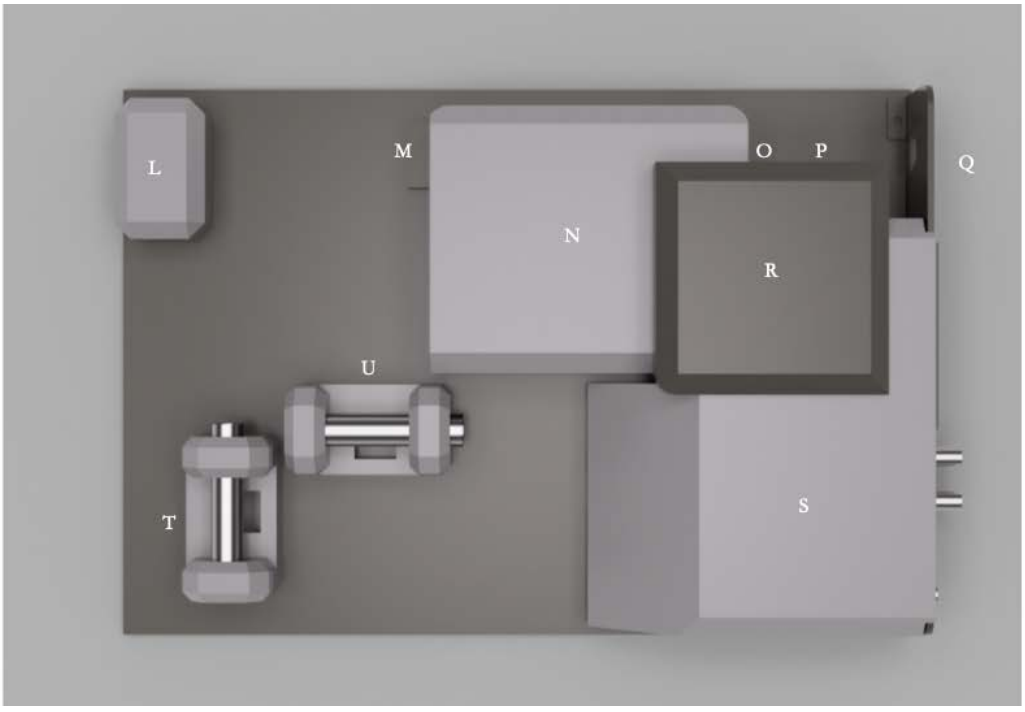
Stub Wall

A. USB 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. Patch Cord In
K. One Axis Translator Knob

Internal Components

L. Power in and on/off switch
M. X/Y Translator for 635 nm Laser
N. Optical Housing
O. Objective (20x, NA = .4)
P. One Axis Translator with Magnetic Connector
Q. Stub Wall
R. LED Housing
S. Electronics Housing
T. 635 nm Fiber Coupled Laser
U. 450 nm Fiber Coupled Laser



Accessories

- ❏ Micro - B to USB 3 Cable (3 meters)
  - ❏ Connect the USB plug to a USB 3 port on your computer and connect the Micro - B plug to port A specified in the diagram above. **Please do NOT use a USB extension cable, we cannot guarantee that your system will work properly.**
- ❏ ⅛ inch Audio Cable (1 meter)
  - ❏ 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
  - ❏ Connect the male grounded plug to a three prong outlet and the female plug to the Power Supply Brick.
- ❏ Power Supply Brick
  - ❏ Connect the DC plug to port L.
- ❏ 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 the handle.
- ❏ 2mm Straight Hex Key
  - ❏ Thread through hole K to attach to the 1 axis translator knob. 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 connector
  - ❏ Connect a patch cord to one of the connectors. Thread through hole J and connect to the translator carriage.

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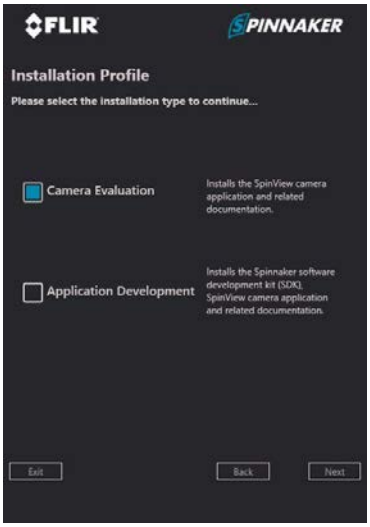
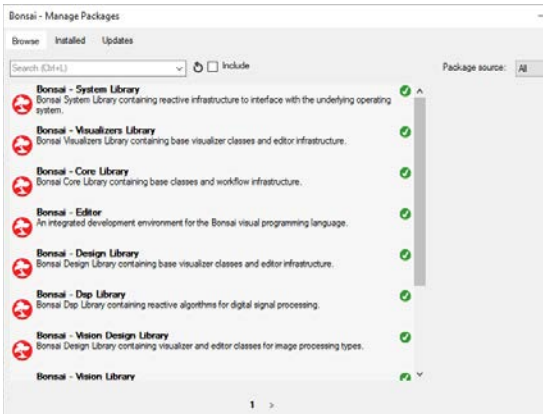
Download Software

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 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 all packages until the 3rd page. These packages include: Bonsai Starter Pack (This will automatically install Arduino, Audio, DSP, DSP Design, Osc, Scripting, Vision, Vision Design and Window Input), 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. Ensure that all dependencies are installed, too.

Please read the documentation on the FP3001 Bonsai Nodes linked here.

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How to Update Bonsai

Tools needed to update: Your laptop with Bonsai.
Time requirement: Maximum 5 mins.

An update to any installed package is available when this icon is present in your toolbar. See the image ***(Image 2)*** below for a reference as to where the update icon will be displayed. Please save any open workflows and close out of them. Please left click once on the icon to open the update manager.

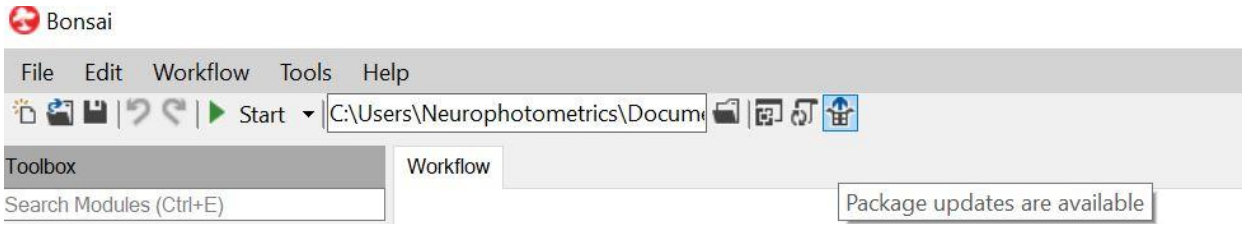


Image 1*: Zoomed in image of the Bonsai toolbar. The update icon will be displayed as the rightmost icon in your toolbar. Hovering over the icon will highlight the icon blue and display the message "Package updates are available".*

The update manager will open automatically, showing what packages are available. Note that if you are using a version of Bonsai older than 2.6.0, please ensure that you are looking at "**Stable Only**" packages, not "Include Prerelease". This option can be found in a drop down menu located to the top left of the package selector, see the image below ***(Image 2a)*** for location reference. You can left click and select "**Update**" on individual packages to update packages selectively; however, we recommend left clicking the "**Update All**" button. If you are using Bonsai 2.6.0 or newer, please click on the "**Update"** tab and left click the "**Update All**" button (***see Image 2b***).

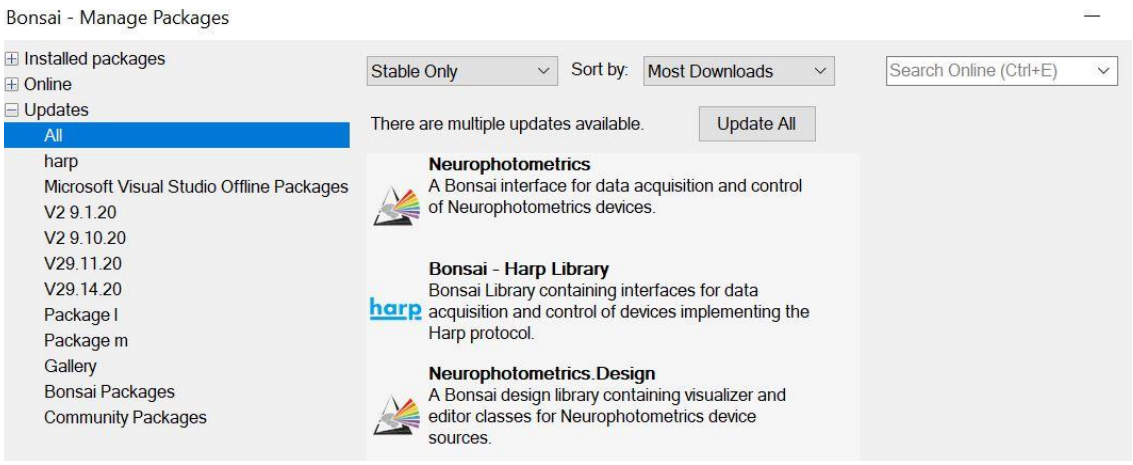


Image 2a: *Cropped image of the Update Manager for Bonsai versions older than version 2.6.0. Note that "Stable Only" updates are displayed.*

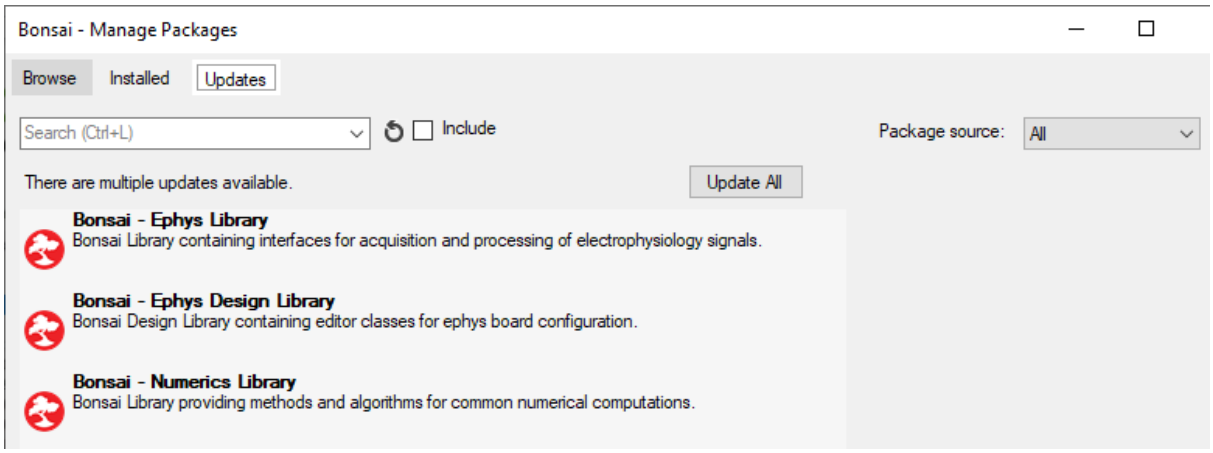


Image 2b: *Cropped image of the Update Manager for Bonsai version 2.6.0. All updates will be "Stable Only" updates.*

A window will pop up to ensure dependencies are also up to date. If you see a message uninstalling an old package, do not worry as the updated package will be installed in its place. The window will automatically close and you can exit out of the update manager to return to the main Bonsai window. You can open a previous workflow and continue experiments as normal!

Please note that we, the Neurophotometrics team, will send an update email whenever an update is released. If you do not see the update icon in your tool bar, please manually access the Update Manager by clicking on Tools > Manage Packages. Click

on the word Updates to open the Update Manager. If the Update Manager displays the error “The remote server returned the error. (404) Not Found.” this means that there is a network connection issue between Bonsai and the Internet. First, please attempt to resolve the connection issue by checking your connection to the internet. Please disconnect and reconnect to the Internet if possible.

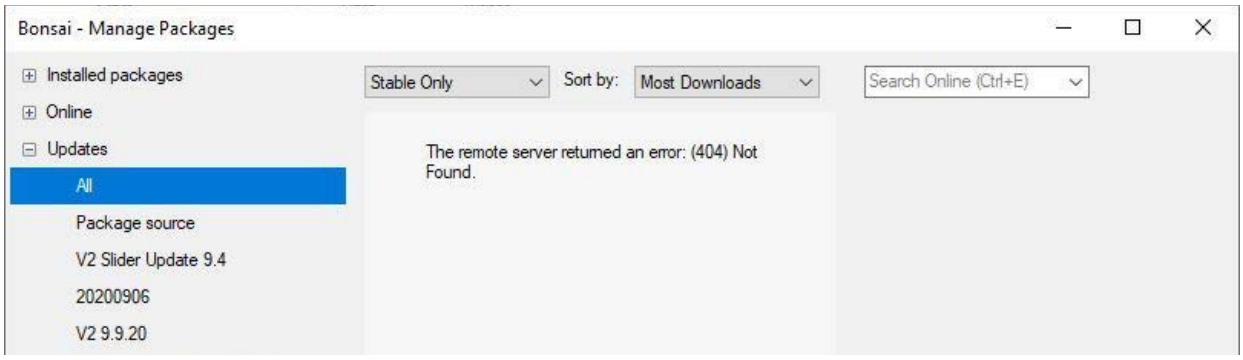
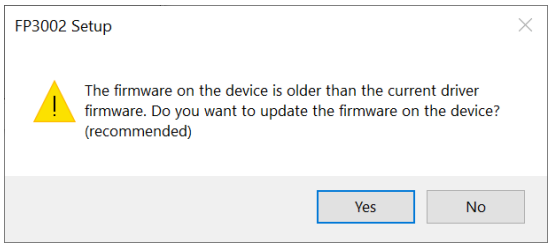


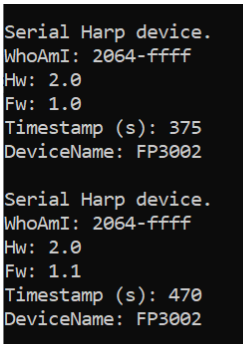
***Image 3**: Example of a network connection error in the Update Manager.*

If that does not work then access the Package Updater by clicking on Online/Browse. If this page is able to connect to the Internet, please search for the Neurophotometrics packages by clicking on Community packages and installing the Neurophotometrics and Neurophotometrics.Design packages. This will download the most up to date version.

Update 1.1 — Released January 12th, 2021

Update 1.1 will require you to update your firmware as well. The firmware will be uploaded through the FP3002 node. You can use a previously saved workflow or you can access your Toolbox and insert a new FP3002(Neurophotometrics) node. Please double left click on the FP3002 node to open up the FP3002 Configuration window. A pop-up window will appear requesting you update the firmware. Left click yes. Bonsai will automatically upload firmware and restart your system. When the system has booted up, you can continue with your normal experiments. If you are unsure if your system has





successfully installed this firmware, close out of your FP3002 node and double left click to open the node again. Hover over the Bonsai logo located on your Windows toolbar. Select the black screen. Your firmware (Fw) should now say 1.1 instead of 1.0.

Update Functionality: Updates the system firmware. Cross-talk between Digital In ports is resolved. 470 nm LED power is automatically 0 when opening up the ROI calibration window.

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Using the New FP3002 Bonsai Nodes

This document will cover all new nodes unique to the FP3002 system, new nodes include **FP3002, Command, DigitalInput, and DigitalOutput**. **GroupRegions, PhotometryData, PhotometryWriter and WithLatestTimestamp** are compatible with the FP3002 node and their functionality is detailed in FP3001 Bonsai Documentation.

Differences between FP3001 and FP3002

Unlike FP3001, the first version of the Neurophotometrics multi-fiber photometry system, FP3002 does not have an external driver box. Instead, your system is driven by a software interface in Bonsai. The only hardware interface on the FP3002 system is the ON/OFF switch along with both translators.

Before Starting an Experiment

Plug the micro-B to USB cord into the plug on the stub wall of the system. Then turn the system on. If you turn the system on before plugging in the USB cord, please wait for the system to fully boot up before plugging in the USB cord. Open up Bonsai and either use this template or search for FP3002 in the toolbox and enter it into the workflow.

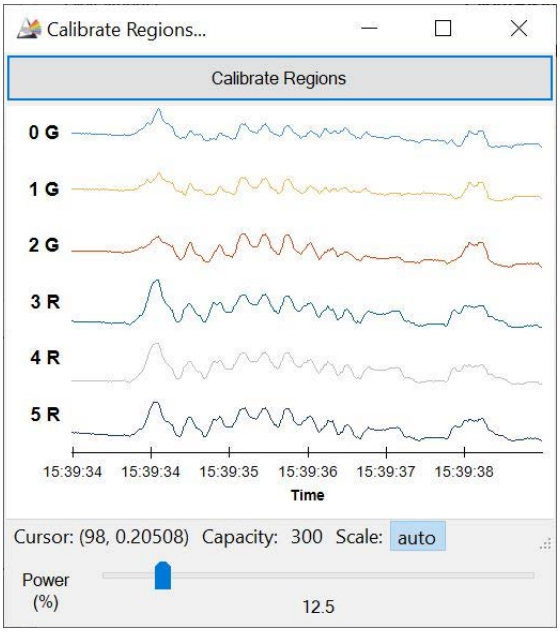
To begin, simply click on the FP3002 node once, under properties on the right hand side of the screen, select the port name of your FP3002 system (COMx). Double click the FP3002 node to configure your settings.

Load/Save

The top left corner of the FP3002 Setup Window allows one to save and load settings as an .xlm file. The top button "**Load Device Settings…**" will load a previously saved .xlm file. The middle button "**Save Device Settings…**" will save the current configuration. When clicked, use the directory window to select the save location of the .xlm file.

Another window will pop up prompting: "Do you want to save the register values on persistent device memory?" Clicking **Yes** will save the configuration to the board

indefinitely. Every time the system is turned on the saved configuration will automatically populate. Clicking **No** will save the configuration until the system is turned off. The next time the system is turned on, the configuration saved to the board will be loaded; if nothing has been saved to the board, factory settings will be populated. Please save configurations before starting each recording. Closing out of the window will not guarantee that your configuration is saved. The bottom button "**Reset Device Settings…**" will reset the configuration to factory settings. Please note that "**Save Device Settings…**" will save the configurations of the electronics. This button will not save Regions of Interest (ROI's). ROIs are saved within the Bonsai file. Saving a Bonsai file can be done by clicking "**File**" on the topmost Bonsai toolbar, and clicking "**Save As**".

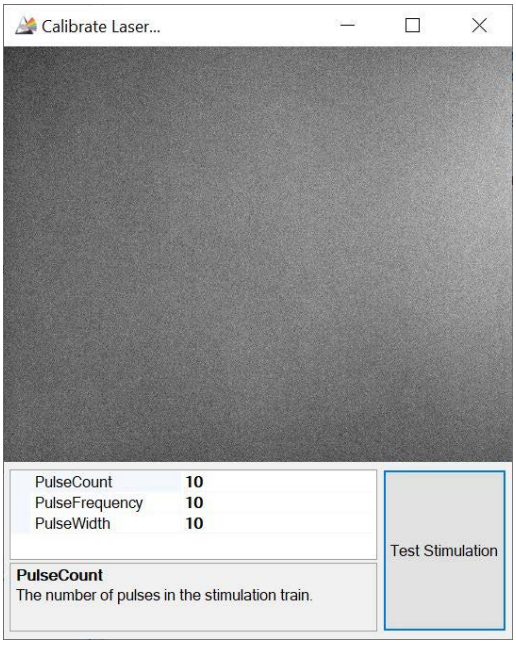


Setup

The bottom left corner of the FP3002 Setup Window contains two calibration windows. The top button "Calibrate Regions…" will open a window to visualize and define ROI activity. A graph displaying trace 0 representing the average pixel value of the entire window will display. The graph is normalized to the maximum pixel value, that maximum pixel value is dependent if the camera's pixel format is in Mono 8 or Mono 16. Note that this is how data are formatted; each data point is the normalized average pixel value of each ROI. The x-axis represents time of day (hour, minute, second) [starting time,∞] and the y-axis represents a normalized average pixel value [0,1]. Cursor will display the coordinates your cursor arrow is hovering over (frame number, normalized average pixel value). Note that this will not "snap" to the coordinates on the graph. Capacity alters the amount of points displayed. Increase Capacity to display more data points, decrease Capacity to display less. The bottom slider bar represents power of the 470nm LED (%). This power will not save when exiting out of the calibration window and should be used to aid patch cord alignment.

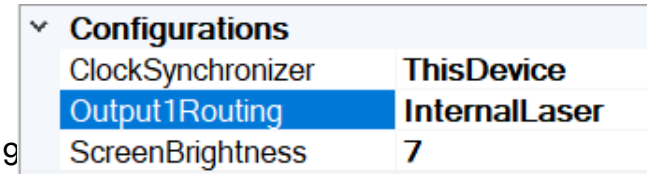
The button at the top called Calibrate Regions, will open the ROI Picker. Use this window to define ROIs. Left click and drag to draw and create new ROIs. To modify an existing ROI, left click to select an ROI and right click and drag to redefine. To delete an ROI, left click to select and hit delete on your keyboard. Each ROI should be defined by a number and a letter, either G or R. Bonsai will automatically detect which ROIs collect data from the green (G) or red (R) channel based upon a 50/50 split of the field of view. Exit out of the window and you will now see a trace for all ROIs. Exit out of the window to save your ROIs.

The middle button, “Calibrate Power…” will open a window with 3 slider bars. Each bar modulates the amount of amperage sent to an individual LED. This amount is represented as a percent. To calibrate power, slide one slider to a percent and use a power meter to configure power. Out of the tip of a fiber, the power should be ~50µW across all LEDs for a 200um patch cord. This corresponds to ~4% for both the 470nm and 415nm LED and ~30% for the 560nm LED. Please return the slider back to 0 when calibrating a different LED. Not doing so will inflate your power readings. When you close out of the Calibrate Power window, the number the slider is positioned over, will automatically write to the Power Configuration table.



The bottom button, “Calibrate Laser…” will open a window to align the 635nm laser. This button will be blocked unless ‘635’ is typed into the Laser Wavelength selector. Clicking on the “Calibrate Laser…” button will open a window displaying the field of view of the camera. The bottom left corner configures pulse settings. PulseCount sets the number of pulses to occur. Pulse Frequency how often a pulse occurs (in Hz). Pulse Width is the laser exposure time (ms). The bottom right corner will allow you to pulse the laser at the specified pulse settings by clicking on “**Test Stimulation**”. Power is automatically set at 10% power. Use the XY Translator to align your laser to a fiber core. You will configure power before running the Bonsai workflow.

Configuration



The configuration panel alters where specified information is routed. The **Clock Synchronizer** specifies if data is time locked to "**This Device**" or to an "**External Device**". This setting will also need to match with the clock in and clock out ports on the stub wall. If plugged into the clock in, please select "**External Device**". If plugged into the clock out or not plugged in at all, please select "**ThisDevice**".

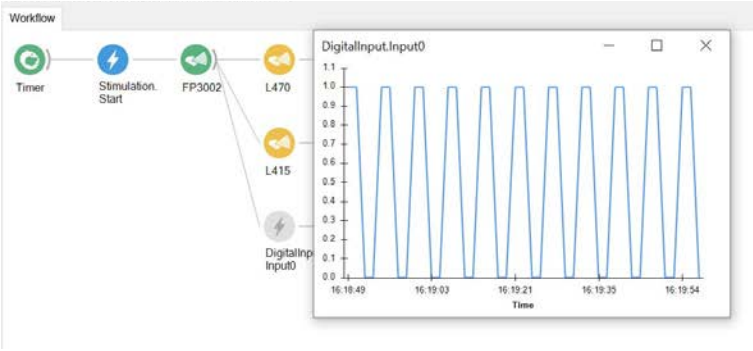
Output1Routing communicates the state of the output port 1 to the specified selection. This option is implemented as another safety key to the laser. The laser will not turn on if the state is routed to only **BNC**. If the state is routed to **InternalLaser**, then the BNC will not output data. If the state is routed to **Both** then both the Internal Laser and BNC port will function as specified.

Brightness alters the brightness of the screen [0,15]. 0 turns the screen off completely while 15 is the brightest the screen can go. Please note that if either the 450nm or the 635nm laser is selected the screen will not dim lower than 7.

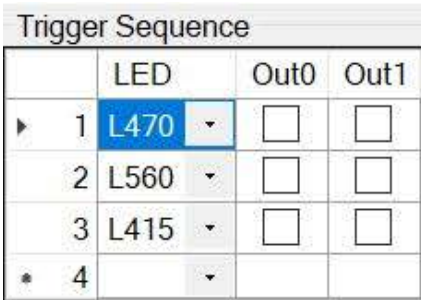
Digital IO

The Digital IO panel can be used to timelock behavioural equipment/data to the fiber photometry system. The system comes with two digital input and two digital output ports.

Digital0Input and **Digital1Input** take TTLs coming in from an external system. Please do not send more than 5Vs into the system. **EventRising** will only acquire the time point at which the signal changes from LOW to HIGH — active high. **EventRising** produces a 1 when the signal changes. **EventFalling** will only acquire the time point at which the signal changes from HIGH to LOW — active low. **EventFalling** produces a 0 when the signal changes. **EventChange** will acquire the time point the signal changes, producing a square wave consisting of 0's representing a change from HIGH to LOW and 1's representing a change from LOW to HIGH. StartStimulation will enable the laser to activate when the incoming signal is HIGH. You will not need to configure the laser settings besides the Laser Wavelength and Pulse Amplitude.

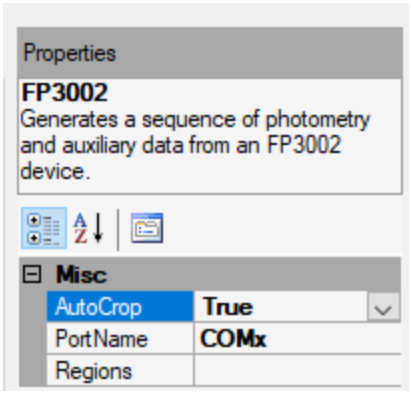


Neither will you need the Command node to start Stimulation. StopTrigger, Stop ExternalCamera, StartTriggerExternalCamera, ControlTrigger, ControlExternalCamera, ControlTriggerExternalCamera, ControlStimulation are works in progress to be available in a future update.

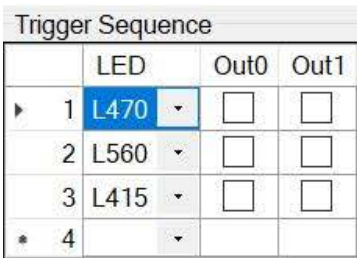


Digital0Output and **Digital1Output** specifies the output TTLs source. This signal can range from 0V to 5V, either positive or negative polarity. This signal can be sourced from the **Software** where Bonsai can act as the function generator via line(s) of code in the workflow. A **Strobe** signal can be output from the ports, which sends out a binary signal whenever the camera is acquiring data. **TriggerState** will output a binary signal whenever a specified LED is on. Please select the LEDs to examine on the right hand window labelled **TriggerSequence**. Note that Out1 or Out0 and **TriggerState** for the respective port need to be checked and selected in order to output a signal for LED activity. **<u>Do NOT check these boxes if you are not recording the LED trigger state.</u>**

Photometry



FrameRate can be set within a range of 15-200 using the dropdown menu slider. The **FrameRate** establishes the acquisition rate of the camera in frames per second (fps). The required frame rate for the fluorophore you are using depends on the kinetics of the fluorophore itself. <u>If the camera's field of view is not cropped, the maximum frame rate is 80fps.</u> Cropping the field of view will increase the camera's acquisition rate. To do this, select the FP3002 node and on the right hand side under the 'Properties' menu, 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. If AutoCrop is set to False and an attempt to increase the FrameRate past the camera's capability is made, an error will pop up notifying you to either turn down the FrameRate or set AutoCrop to True.



Trigger State

Trigger state is represented by an array configured on the right hand side of the FP3002 window called **Trigger**

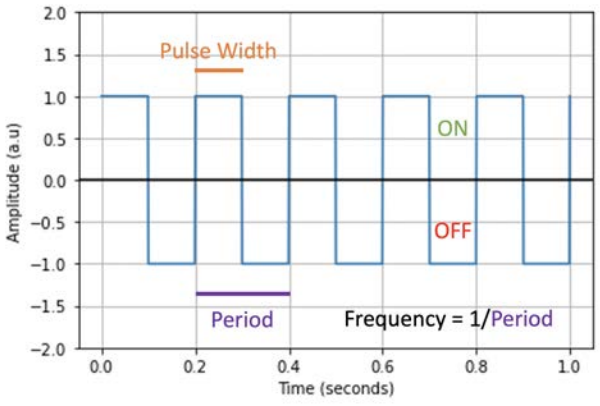
Sequence. The order of the LEDs can be chosen within this window. The order of the LEDs does not matter, the **PhotometryData** node will deinterleave the data based upon the trigger sequence. The LED sequence is sequential rather than simultaneous. This means that no LED will be active at the same time as another LED, thus your effective frame rate is the frame rate specified in the Photometry panel divided by the number of rows in your Trigger Sequence. Selecting only 1 LED will result in that LED activating constantly, not pulsed. This action is only advised for calibration purposes, not during recording. Note an LED will not be included in the sequence until a blank row appears below it, this is achieved by clicking the wavelength from the drop down menu. To delete an LED in the sequence, select the row and hit delete on the keyboard. Click on the Out0 or/and Out1 check boxes if you wish to have a record of when the LEDs are turned on. Please note that for this to be recorded, the DigitalOut selection will need to be set to TriggerState. Do NOT check these boxes if you are not recording the LED trigger state.

Power

The **Power** panel controls the power of the LEDs, respective to their wavelength. The power is represented in percent of the maximum amperage [0.00%, 100%]. To calibrate power, you will need to set the percentage and check the power output with a power meter. Out of the tip of a fiber, the power should be ~50µW across all LEDs. This corresponds to ~4% for both the 470nm and 415nm LED and ~30% for the 560nm LED. Power can be set lower or higher, this is dependent on implantation and viral expression, power should be balanced between good SNR and bleaching rate.

Stimulation

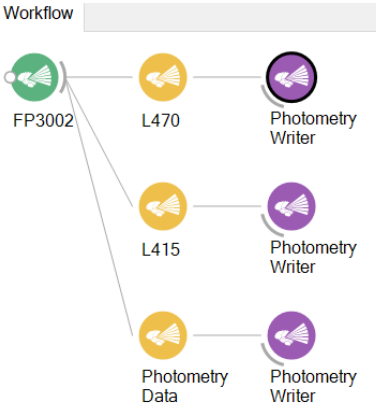
The **Stimulation** panel should be used to configure the laser settings. Please note that the key switch will need to be connected in order for the laser to be used. The wavelength will need to be typed into the cell for **LaserWavelength**. The wavelength needs to be exactly either 450 or 635 in order for the laser to turn on. Additionally every time the system is booted, you will need to re-enter the laser wavelength. This setting will not save to



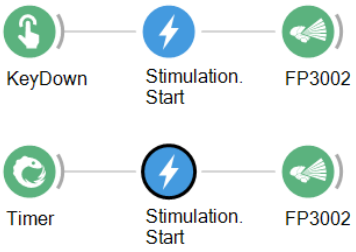
the system's memory. Entering 635 will make the "**Calibrate Laser…**" available. Entering 450 will pop up a window reminding you to attach the secondary patch cord. **Pulse Amplitude** sets the power of the laser in % power [0.00%, 100%]. To calibrate power, you will need to set the percent and run the workflow. 1-3mW should be sufficient for opsin stimulation. **PulseCount** sets the number of pulses to occur. **Pulse Frequency** is the time one pulse begins and ends (Hz). **Pulse Width** is the laser exposure time (ms).

Bonsai Workflow

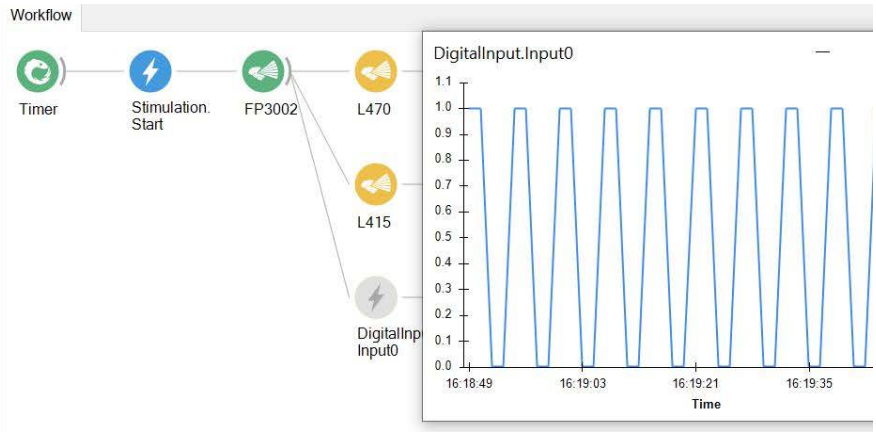
In order to start LED activity and data acquisition, configuring the **FP3002** node and playing the workflow is sufficient. In order to record data, you will need to attach **PhotometryData** nodes to specify which deinterleaved channel to examine and attach a **PhotometryWriter** at the end. If no filter is selected in **PhotometryData**, then the interleaved signal will be displayed. Note that unlike the **FP3001** node, a **PhotometryWriter** node cannot be connected directly to the **FP3002** node.



To turn on the laser, a **Command** node will need to be connected to the **FP3002** node — the laser will not turn on with just the **FP3002** node. Please have the laser key connected to the system and laser wavelength selected in the **FP3002** node. There are many ways to command the laser to turn on, a few easy ways is to use either a **KeyDown** node or the **Timer** node. If using a **KeyDown** node, select a filter under properties to define a key that activates the laser. Using a **KeyDown** node will require you to press the key every time the **PulseCount** completes. If using a **Timer (Reactive)**, the laser will start at the same time as the start of the workflow. If using the **Timer** node, laser activity can be delayed by setting the **DueTime** (Hour, Minutes, Seconds). Similar to the **KeyDown** node, the laser will turn off after the pulse count is completed. However, if the **Period** (Hour, Minutes, Seconds) is set to a certain time frame the pulse count will replay until the recording is stopped or a command to stop stimulation is executed. Within properties, the **Command** node allows the user to select the **Mode**, this defines if the Command **Start**s or **Stop**s the **Type**. The **Type** defines what is being controlled: **Stimulation**, **External Camera** or **Photodiode**.



Connect the **DigitalInput** node after the **FP3002** in order to visualize and record an input signal. To record the input signal, please use a **CsvWriter** to save your data. Please ensure that the **FP3002** DigitalIO panel reflects the same information on the **DigitalInput** node. A timestamp can be included within the properties by selecting **True** or **False**, the timestamp is represented in seconds. The **Type** can be specified to define where information is routed from, either **State**, **Input 1** or **Input 0**. The **DigitalOutput** node will function similarly, but is still a work in progress to synchronize and perform closed loop paradigms.



NEUROPHOTOMETRICS
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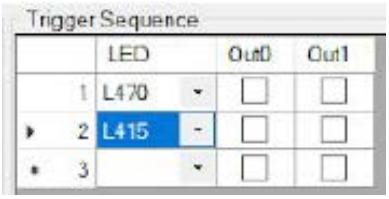
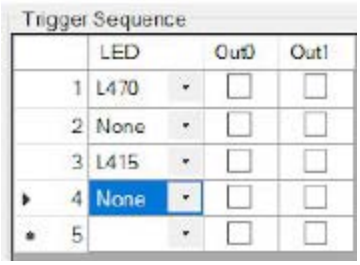
Determining LED Duty Cycle

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 duty cycle as the percent time the device is on. Within this section, we will discuss how to control the duty cycle of your LEDs which excite fluorescent indicators such as GCaMP. A decreased duty cycle is particularly advantageous for overnight recordings. The less time the LED is on, the slower photobleaching will occur. You should not have to decrease the duty cycle for recordings shorter than 1-2 hours. To simplify this process, the Neurophotometrics system modulates the amount of time an LED is on in a given frame by inserting "blank" frames.

For this, 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.

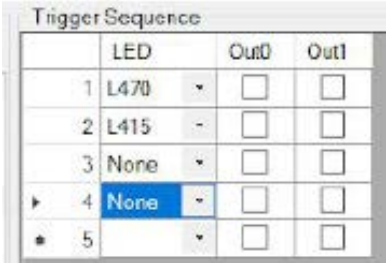


You want an effective frame rate of 25Hz per wavelength, you need to set your system to 50Hz. Keep in mind that the effective frame rate is determined by the interval of time it takes for a specific LED to complete a frame and turn on at a new frame. At 50Hz your LEDs will be on for just under 20msec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



Now let's say that you want the same effective frequency but a decreased duty cycle of 50% (10msec). 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 25Hz per wavelength, but now the LED is only on for 10msec.

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.



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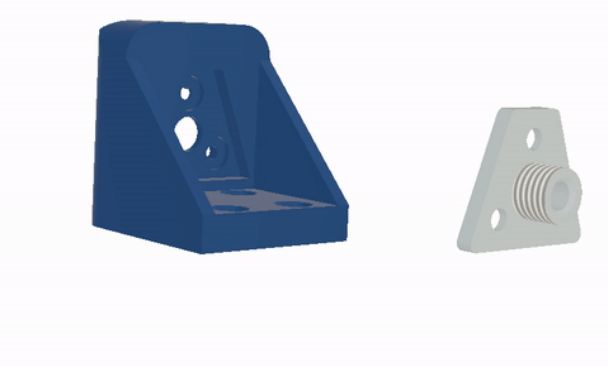
Connecting and Aligning My Patch Cord

The NPM 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 be proud of 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.
7. Insert the straight hex-key (2 mm) into hole [K] (please reference page 6 of this manual) 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. You can illuminate your fibers by tapping or shining light down the tip(s) of the patch cord. An example of a two branching 200um core FC patch cord is below.

Image 1: Image of an aligned two branched 200um core FC patch cord, illuminated by tapping on the ends of a patch cord with a gloved finger.

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Laser Alignment

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 a fellow researcher's or your own face.
- 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.

Laser Configuration

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 one to do simultaneous green channel fiber photometry recordings and red opsin stimulation. The system comes with a two axis translator (XY translator) to focus your laser beam onto only one branch. Note that you may be able to split the beam into two branches, but this is not recommended due to the drastic decrease in power efficiency. This translator can be found at location M referenced on page 7 of this manual —it is the translator located nearest to the ON/OFF switch.

To begin, please make sure to first align your patch cord to the system using the one axis translator. You will not get adequate power output if your patch cord is not aligned. Next, type in the laser wavelength under the laser settings in the FP3002 configuration window. You will need to type in '635' exactly. When you press 'Enter', the Calibrate Laser button will activate. Press on the button to align your laser. Within the Calibrate Laser window, you are only able to configure the Pulse Count, Pulse Frequency and Pulse Width. For your safety the laser power has been automatically configured to 10%. Configure the parameters per your experiment, the pulse variables will copy over to the FP3002 configuration window when you exit out of the Calibrate Laser window.

Laser Alignment

Click ‘Test Stimulation’ to begin pulsing the laser. You should see the laser show up on the left side of your screen. Use the knobs on the XY translator to center the laser beam on a core. To aid alignment, shine a light (i.e. at the ceiling lights or a flashlight) down the tip of your patch cord. The external light is a means to locate your patch cord in the image and visualize each branch. Ensure that the laser is still running by checking the LCD screen on the system. The screen should like the image to the right.

Once you have verified that the laser is on, use the XY translator to point the laser beam onto an illuminated fiber. Remove the external light when you have selected your branch. From this point, the laser beam should illuminate the whole fiber. You can fine tune the laser alignment without the external light.

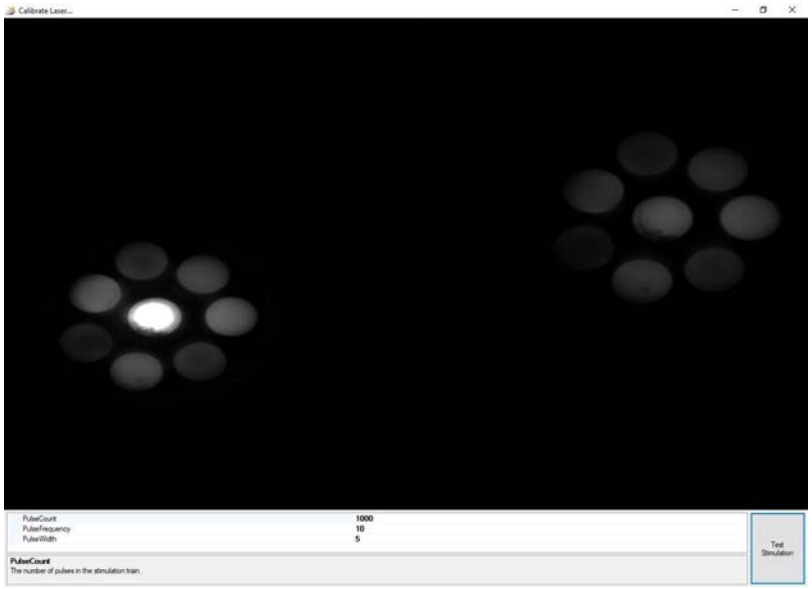


Image 2: The tips of an 8 branching patch cord were pointed at an external light source. The laser beam was translated to the middle core.

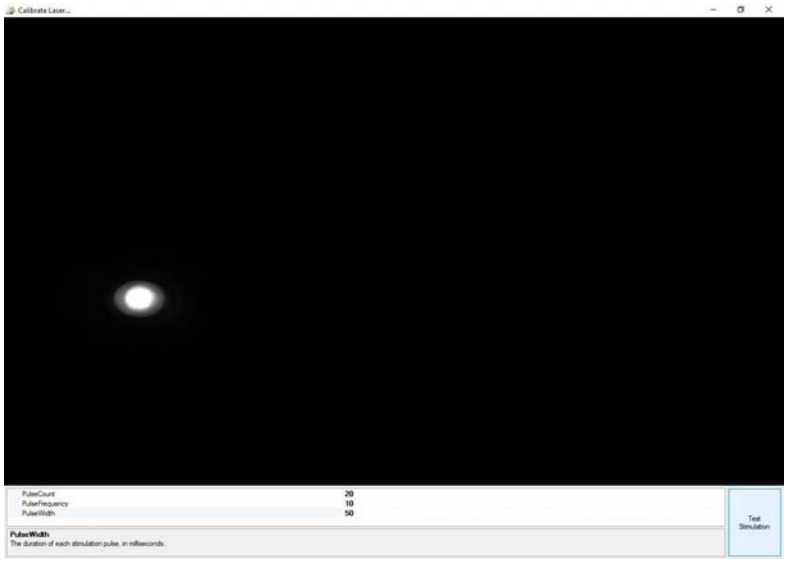
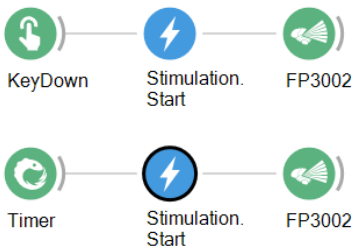


Image 3: With the external light source removed, the beam was translated to the middle of the fiber.

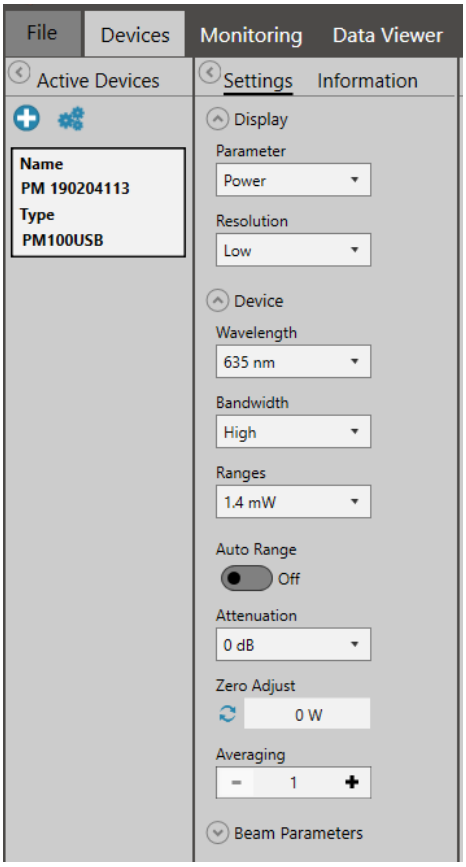
How to Test Laser Power

The integrated lasers on the FP3002 are locked to function when pulsed. Please inquire if your experiments necessitate CW (continuous wave). Taking power of a pulsed laser is different from taking power of an LED that is constantly on. Many top tier power meters will automatically spit out this reading by switching to a different mode; however, these power meters can cost at least $2,000. As we recommend the Thorlabs PM100USB power meter (coupled with the S120C sensor), this guide will detail how to achieve an accurate reading with that power meter. Therefore you will need the PM100USB power meter, S120C or similar sensor, the Thorlabs Optical Power Meter software and the FP3002 system.



There is no calibrate laser power button, this will need to be done by running Bonsai code. Use either of the Bonsai lines shown on the right to run the laser. We recommend setting the Pulse Amplitude to 30. This will give you a power of approximately 1mW out of a 200um fiber. This process may take a few minutes as you start, stop and alter the pulse amplitude to meet your desired power output. Note that you will need to turn LED power to 0 to avoid inaccurate readings. If you have a PhotometryWriter in your workflow, you can right click and select **Disable** to avoid saving unnecessary data.

Access the power meter settings under the Thorlabs Optical Power Meter application. Since the power meter will need to integrate data from a slow frequency, you will need to set your **Bandwidth** to **High** (100 kHz). This will increase the rate of data transfer thereby allowing you to integrate peak to peak more efficiently. Please use a Low (20Hz) bandwidth when measuring LED power as the higher data transfer rate will increase noise levels. Set **Averaging** to **1**. Any value higher may result in a value that is averaged with the time that the laser is off. You can set this to any value, recommended 100, when measuring LED power. Lastly, set **Resolution** to **Low**. Setting the resolution to **Low** will decrease the noise level. All other parameters are no different than the parameters set when taking LED power measurements. Remember to wear laser safety glasses when taking power measurements!



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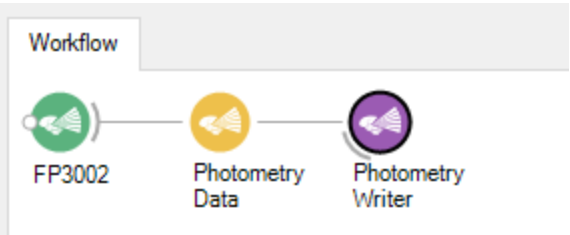
Independence Tests

An independence test examines how much signal of one fiber bleeds into another fiber. This can vary based upon the polishing of your patch cord therefore independence tests are important to do every time you align a new patch cord. The test can be done qualitatively or quantitatively. Tapping one fiber should result in no noticeable signal change in any other fiber. Accurate independence tests will need to be done *post hoc* instead of online. 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. This section will cover how to quantitatively check independence on Excel. This process can be automated with analysis software, once you understand how an independence test is conducted. You will need the FP3002 system aligned with a branching patch cord, an item to cover the tips of your patch cord and an item that is green and red. We recommend autofluorescent slides, but a smart phone application displaying coloured light works similarly.

First configure your settings. All settings are negligible except for **LED Powers**, **Trigger Sequence** and **Frame Rate**. We recommend setting your LED powers to the same number that you will record with or setting them between 10-20%. We recommend setting Frame Rate to the lowest setting 16. This is to decrease your file size. Please select only one LED, either the 470 or 560, in the Trigger Sequence. For this guide we will examine the green channel so select the 470 LED. You will need to repeat the following steps for the red channel if you are using a red fluorescent protein. First align your system with your branching patch cord using the Calibrate Regions window. Within that same window, draw ROIs over each core for the green channel only. We highly suggest that you label each branch with a piece of tape or nail polish. Please note what #ROI corresponds to what branch. Exit out of your ROI picker window and save your settings.



Return to the Bonsai workflow. You will only need three nodes for this test: FP3002, PhotometryData and PhotometryWriter, connected in that order. Define the save patch in your PhotometryWriter

node by clicking the ellipsis button. Next prepare for the recording. Grab one branch of your patch cord and hide all other branches under a cover such that no light will interfere with them. Play the Bonsai workflow and open the PhotometryData node by double left clicking. Take your green object and wave it over the tip of the selected branch. You will want to replicate a square wave. You will not want to saturate the sensor, so the object should be no less than 6mm from the tip of the patch cord. You will want to wave at least 5 times. At this point you can stop the workflow, grab another branch and repeat the process above for all branches.

Open the csv file in Excel. The goal of an independence test is to compare the signal change of each branch. For this example, a 4 branching patch cord was used with 0G representing the branch receiving signal. <u>First, calculate the change between each frame.</u> In cell I2 type in '=D3-D2'.

=D3-D2

	C	D	E	F	G	H	I	J	K	L
	Flags	Region0G	Region1G	Region2G	Region3G					
	16	0.003922	0.003922	0.003922	0.003922		0.033352	0.022288	0.056528	0.032054
	18	0.037274	0.02621	0.060449	0.035975		-0.00024	-4.9E-05	-7.9E-05	-7.8E-05
	18	0.037037	0.026161	0.060371	0.035898		-1.2E-05	-5.2E-05	3.98E-05	-1.9E-05
	18	0.037025	0.02611	0.06041	0.035879		5.22E-05	-3.8E-06	-4.8E-05	5.58E-05
	18	0.037077	0.026106	0.060362	0.035935		-0.00014	2.05E-05	-8.1E-05	-2.5E-05
	18	0.036736	0.026119	0.060183	0.035828		6.59E-05	-2.6E-05	-1.1E-05	-2.1E-05
	18	0.036719	0.026034	0.060087	0.035795		0.054459	0.000163	2.85E-05	0.000109
	18	0.091178	0.026197	0.060115	0.035904		0.191422	0.000255	0.000255	0.000376
	18	0.2826	0.026452	0.060371	0.03628		-0.00661	-5.9E-05	-4.4E-05	8.63E-06
	18	0.275992	0.026392	0.060326	0.036289		-0.21693	-0.00028	-0.00032	-0.00048
	18	0.059065	0.026114	0.060007	0.03581		-0.0223	-4.9E-05	6.1E-05	2.59E-05
	18	0.036767	0.026065	0.060068	0.035836		-5.2E-05	3.8E-05	-2.7E-05	-4.8E-05
	18	0.036715	0.026103	0.060041	0.035787		0.004245	-9.9E-06	2.78E-05	4.65E-05
	18	0.04096	0.026093	0.060068	0.035834		0.113911	0.000129	9.14E-05	0.000187
	18	0.15487	0.026222	0.06016	0.036021		0.004422	-1.7E-05	-1.5E-05	4.32E-05

I	J	K	L	M	N	O	P
0.033352	0.022288	0.056528	0.032054		1.496375	0.590006	1.040494
-0.00024	-4.9E-05	-7.9E-05	-7.8E-05		4.876452	3.01015	3.054882
-1.2E-05	-5.2E-05	3.98E-05	-1.9E-05		0.230578	-0.29993	0.641306
5.22E-05	-3.8E-06	-4.8E-05	5.58E-05		-13.7381	-1.08	0.93651
-0.00014	2.05E-05	-8.1E-05	-2.5E-05		-6.91328	1.741782	5.777531
6.59E-05	-2.6E-05	-1.1E-05	-2.1E-05		-2.54734	-5.84747	-3.19963
0.054459	0.000163	2.85E-05	0.000109		334.649	1911.552	500.0987
0.191422	0.000255	0.000255	0.000376		751.4235	750.447	508.4436
-0.00661	-5.9E-05	-4.4E-05	8.63E-06		111.4112	148.8675	-765.555
-0.21693	-0.00028	-0.00032	-0.00048		779.4164	679.2893	453.1184
-0.0223	-4.9E-05	6.1E-05	2.59E-05		451.1162	-365.818	-861.06
-5.2E-05	3.8E-05	-2.7E-05	-4.8E-05		-1.37381	1.922927	1.077628
0.004245	-9.9E-06	2.78E-05	4.65E-05		-429.429	152.5587	91.33418
0.113911	0.000129	9.14E-05	0.000187		881.1553	1245.872	610.508
0.004422	-1.7E-05	-1.5E-05	4.32E-05		-252.817	-303.363	102.4511
-0.07537	-2.3E-05	-8.3E-05	-0.00016		3303.929	910.1086	457.7166
-0.04723	-0.00015	-1.6E-05	-1.5E-05		323.5024	2970.426	3233.348
4.2E-05	5.93E-05	1.52E-05	-3.4E-05		0.708345	2.757165	-1.2407

You can now hover over to the bottom right corner of the cell until a '+' appears. Left click and drag to apply the formula to the right three cells. Now highlight the four cells that you have made and using the '+' to drag them down to the end of the recording, applying the formula for all rows. <u>Second, identify the spots when the jump in signal occurred.</u> We recommend using conditional formatting to highlight cells with a value above 0.1within column I. <u>Third, you want to</u>

compare the change in each fiber to the change of fiber 0G. You will want a change 200 times larger in fiber 0G than the change in any other fiber. You can calculate this ratio by entering '=I2/J2' in cell N2, '=I2/K2' in cell O2, and '=I2/L2' in cell P2. You can use the '+' to drag and apply this formula to all rows or just apply the formula to the highlighted rows. **This number should be larger than 200.** You can reverse this ratio to determine the percent bleed of one fiber into others. Repeat for all remaining fibers and the red channel. Note that you will select the 560 LED under Trigger Sequence and wave the red object in front of the patch cord branch.

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Walkthrough

This section will walk you through a typical GCaMP recording with 635nm laser stimulation. For details on some of the methods mentioned in this section please look at the details on the specified pages of this manual. We advise reading the manual before proceeding with a recording.

1. Plug in the USB to micro B cable. The micro B end will attach to the system. The USB end will attach to a USB 3 port on your computer. **Please do not use a USB extension cable, we cannot guarantee that your system will work properly.**
2. Plug in the power supply and turn on the system. I represents ON and O represents OFF.
3. Patiently wait for your system to boot up. Boot up is complete when you see the image on the right.
4. Open Bonsai and start a new workflow (Reference page 10 for software details).
5. Access your toolbox and type in 'KeyDown'.
6. Double left click on **KeyDown(Windows.Input)**.
7. Access your toolbox and type 'Neurophotometrics'.
8. Left click on the **KeyDown** node once.
9. Double left click on **Command** to insert the node into your workflow. Without clicking on anything else, double left click on **FP3002**, then double left click on **PhotometryData**, then double left click on **PhotometryWriter** to connect all five in that order.

10. Attach your patch cord to the one axis translator (Reference page 21 for details).

11. Access the Calibrate Regions window. Align your patch cord and define ROI's (Reference page 14-15).
12. Align your laser and configure laser power (Reference page 23-24).
13. Conduct an independence test if using a new patch cord (Reference page 27-29).
14. Configure settings and LED power within the FP3002 node (Reference page 14-18).
15. Connect your animal with a ceramic sleeve, open Calibrate Power and turn off all of the room lights. Please ensure that you do not see any light escaping from the ceramic sleeve. If you see light, fix the connection between the patch cord and fiber optic cannula.
16. Save your settings and return to the workflow.
17. Press play on Bonsai to begin recording.
18. Press a key on the keyboard to begin pulsing the laser.
19. When the recording is complete, click stop on Bonsai to stop recording.
20. 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 main window.
21. Turn 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.

NEUROPHOTOMETRICS
The fiber photometry company

Troubleshooting

Q: I do not see any of the Neurophotometric 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.

BEFORE installing those, please install the FTDI drivers:
https://bitbucket.org/fchampalimaud/downloads/downloads/UsbDriver-2.12.26.zip

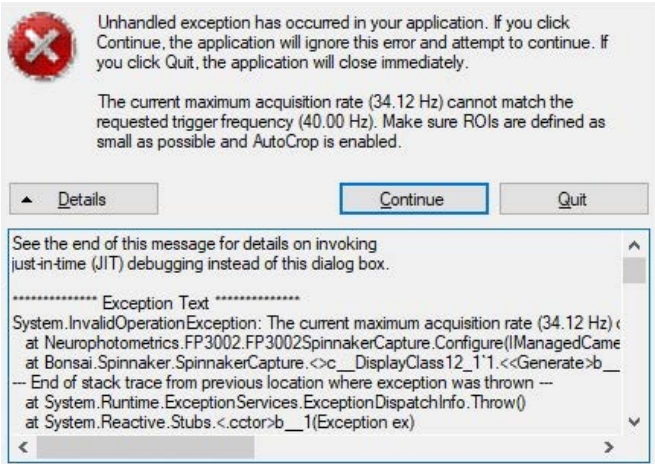
When you reinstall the others, you would want to make sure that the correct version of Spinview is installed. Bonsai accesses the camera's data via the Spinnaker app. Follow the steps for installation, when choosing between Camera Evaluation or Application Development choose Camera Evaluation. Uncheck "I will use GigE Cameras" and install. https://flir.app.boxcn.net/v/SpinnakerSDK/file/622481657674

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.

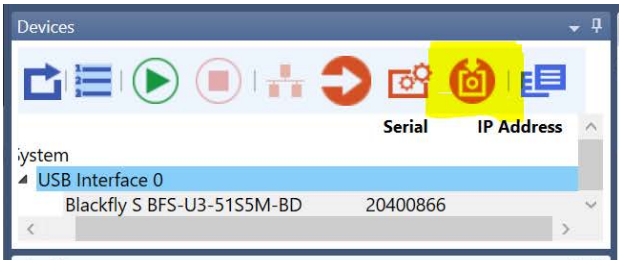
Q: My system’s connection to Bonsai keeps on dropping.
A: Most likely your USB connection is faulty - if you have used an alternate USB cable from that which was provided, this is the cause. Similarly, this can occur if an extension cord is used.

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.

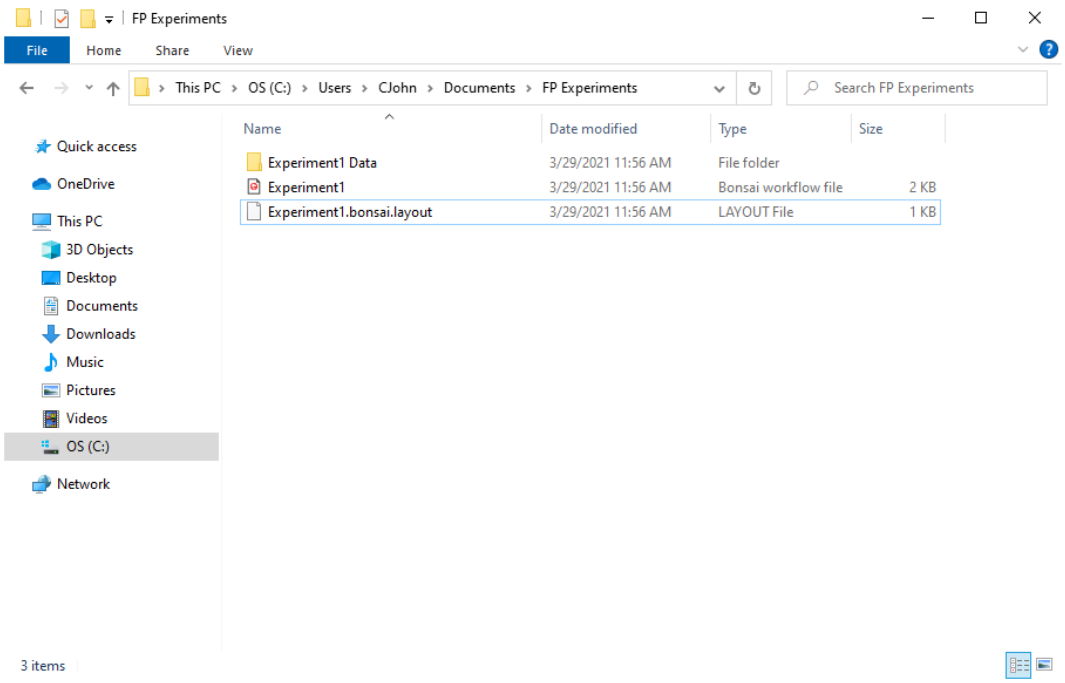
Q: I have received a similar error message located to the right when I click “Calibrate Regions” or when I run the workflow.



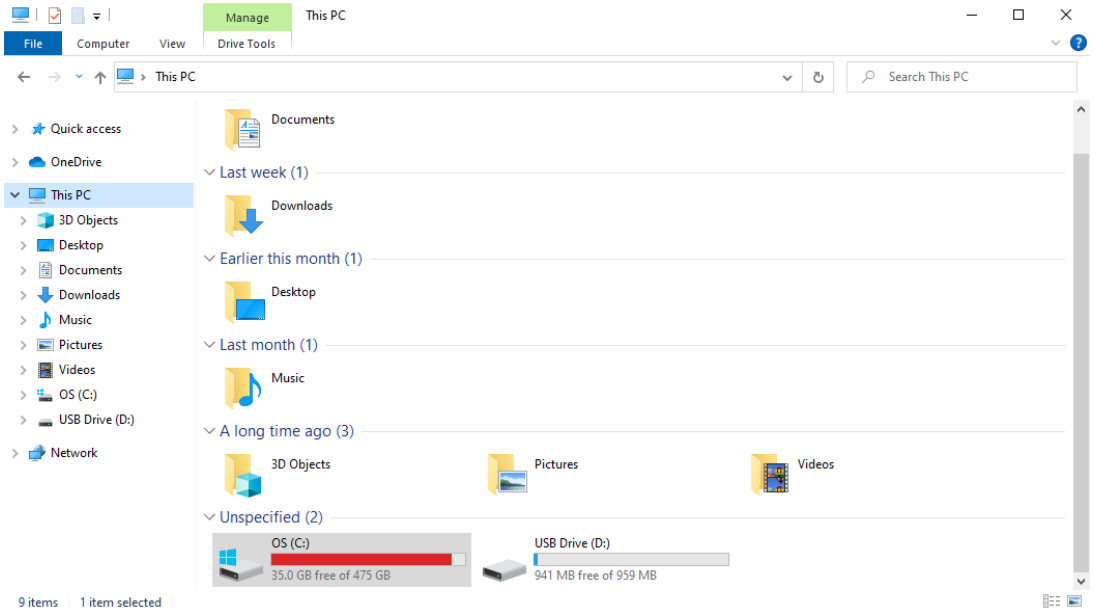
A: If this message pops up when you run the workflow, please set “AutoCrop” to True. AutoCrop can be found by clicking on the FP3002 once and accessing the Properties on the right hand side of your Bonsai window. If this message pops up when you click “Calibrate Regions” this means that the system has 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, you will need to access the SpinView application. Click once on the camera name "Blackfly S BFS-U3-51S5M-BD". Under the "Settings" tab, scroll down to the bottom and on the slider bar next to "Device Link Throughput Limit" slide it all the way to the right. Then click on the red circle with an arrow pointing downwards at a camera on the toolbar. Below is a picture highlighting the red circle to click. Close out of SpinView and return to 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. One basic organizational scheme is to separate your data by experiment, as shown below.



In the search bar for the file explorer window, one can see that the path for The “Experiement1 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.



NEUROPHOTOMETRICS
The fiber photometry company

Additional Information: Isosbestic Points

In brief:

When you excite many GFP-based indicators with 415-nm light the emission level is proportional to the number of molecules underneath your implant making it a useful indicator of photobleaching and some movement artifacts.

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 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 ($F_{bound} - F_{unbound}$) is what drives the high signal-to-noise ratio (SNR) of this indicator.[1]

If you UV-shift the excitation wavelength (i.e. reduce the wavelength), this delta decreases. At a certain point, known as the "isosbestic point", the two values are equal ($F_{bound} == F_{unbound}$). 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.

[1] Barnett, L. M., Hughes, T. E., & Drobizhev, M. (2017). Deciphering the molecular mechanism responsible for GCaMP6ms Ca2 -dependent change in fluorescence. Plos One, 12(2). doi:10.1371/journal.pone.0170934

Why do we care?

In short: 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 (Fig. 1). [2]

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-relative 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 comes in handy! As the isosbestic or 415nm-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-470nm-relative 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).

Even more details:

The biophysically inclined will note that the true isosbestic point of GCaMP is closer to 420-425nm. They'll also know that it is pH dependent and can be a bit of a moving target across preparations. Any way you slice it, though, 415nm is indeed shorter than the isosbestic wavelength. So, why did we choose to excite at this wavelength?

[2] Motion artifacts of this kind, the brain moving relative to the implant, are relatively rare – accounting for ~5% of those observed in photometry recordings. Their effect size is also fairly small as a photometry recording is spatially averaging a signal from a volume of tissue (many orders of magnitude larger than a pixel in a 2-photon recording).

The delta (F_{bound} - $F_{unbound}$) changes asymmetrically about the isosbestic point. If you red-shift the excitation wavelength, you will quickly see a signal where F_{bound} >>> $F_{unbound}$ 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, $F_{bound} < F_{unbound}$. 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.[3]

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. So, we express GCaMP in this region, implant a fiber, hook it up to our photometry setup, and we're off to the races. We record a 10-minute baseline, then administer the drug. For the purposes of this discussion, we also happen to be omnipotent and we know 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.

[3] 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.

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.[4] 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 a jRGECO and RCaMPs, 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[5]). Our experience, generally, corroborates these results as the predominant response one sees in the 415nm-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.

---

[4] [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.

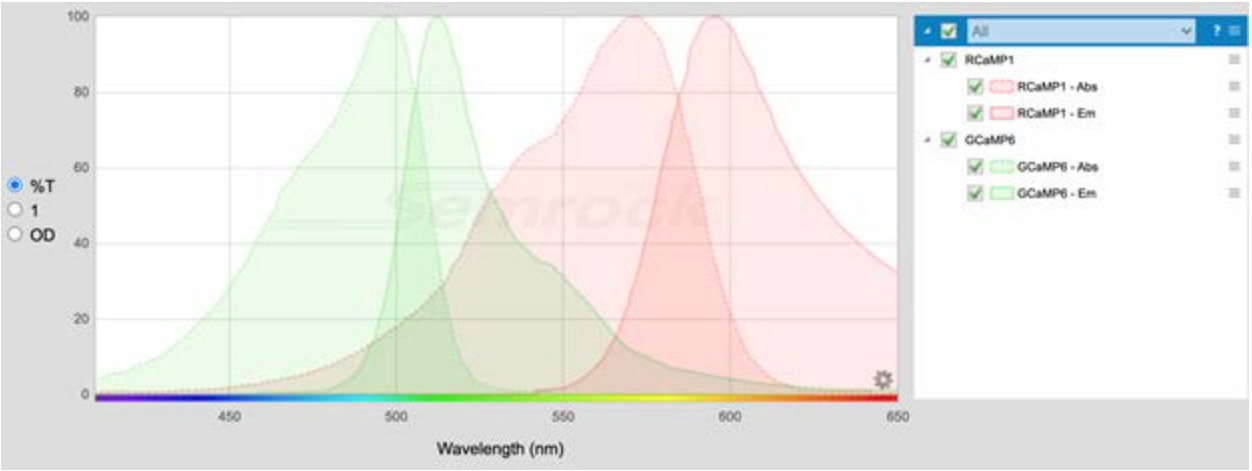
[5] Kim, C. K., Yang, S. J., Pichamoorthy, N., Young, N. P., Kauvar, I., Jennings, J. H., . . . Deisseroth, K. (2016). Simultaneous fast measurement of circuit dynamics at multiple sites across the mammalian brain. Nature Methods, 13(4), 325-328. doi:10.1038/nmeth.3770

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

This type of photometry setup operates using “time division multiplexing (TDM)” which is fancy-speak for “alternating.” In a case where we are recording from green and red indicators, we record one frame with the 415nm LED on, then one frame with the 470nm LED on, then another with the 560nm 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 560nm 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.[6] [7] So, in practice, we are getting four usable signals from each fiber.



Looking at the above absorption/emission spectra for GCaMP6 and RCaMP1[8], we see that there is a good portion of the green emission (~500-530nm) where there is no red

[6] Some caveats to this in Molina et al., 2019.
[7] Molina, R., Qian, Y., Wu, J., Shen, Y., Campbell, R., Hughes, T., & Drobizhev, M. (2019). Understanding the Ca2 -dependent Fluorescence Change in Red
[8] Genetically Encoded Ca2 Indicators. Biophysical Journal, 116(10), 1873-1886. doi:10.1101/435891

emission; and we chose a bandpass filter to ensure this! GCaMP's emission, though, has a super 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 560nm 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.

Calcium-Independent Red = 415nm Signal in Red + X*415nm 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.

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

Additional Information: Surgery Tips

First, modify your ferrules by cutting / scoring the base so the glue has something to grip to. This reduces the risk of a ferrule coming out later in your experiments. This occurs more with the 2.5 mm ferrules -- but can be a worry if you need to use an extra tight sleeve with the 1.25s.

Once you have exposed the skull, clean it thoroughly and dry it. We recommend compressed air (not from a can!) for this. Then use OptiBond Bonding reagent. This does 2 things: 1) It chemically etches the skull (i.e. is super corrosive), 2) Once the solvent evaporates, there is a light-curable compound that creates a porous layer on top of the skull. This is super useful as you will no longer need screws and your caps will be much tighter!

This is how it works:

Next, implant your fibers normally. Use Tetric N-Flow (and little syringe applicator) to put a small dab at the base of the ferrule. Use blue light for ~10 seconds to cure. You can buy one of these things for about $20 on Amazon. It is very viscous and allows you to apply the glue focally which is necessary when implanting multiple fibers. You can then use this to build up the cap properly. Don't make a huge cap and we recommend putting a little antibiotic ointment at the edges and under the skin. Finally, use surgical glue to coat the outside of the cap so the skin adheres.

Implanting 2 fibers takes 30-40 minutes tops. All of these things are available on eBay. Otherwise, you can get them through a Dentist -- but they might be much more expensive there.

NEUROPHOTOMETRICS
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How to Cleave Fibers

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.

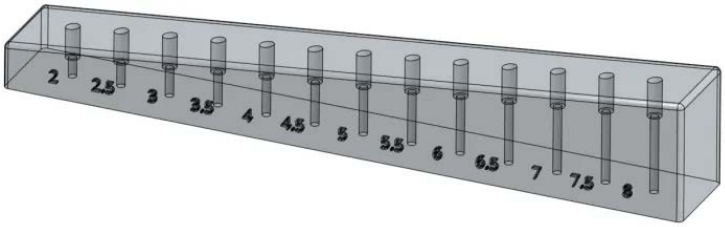
Fibers purchased from Neurophotometrics are sold at 10 mm in length, so they will need to be cleaved to size dictated by your experimental needs. Consult a brain atlas to choose the proper coordinates for your implants. For fiber photometry experiments, it is very important that the fiber is implanted 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). 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.

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 page.

NEUROPHOTOMETRICS
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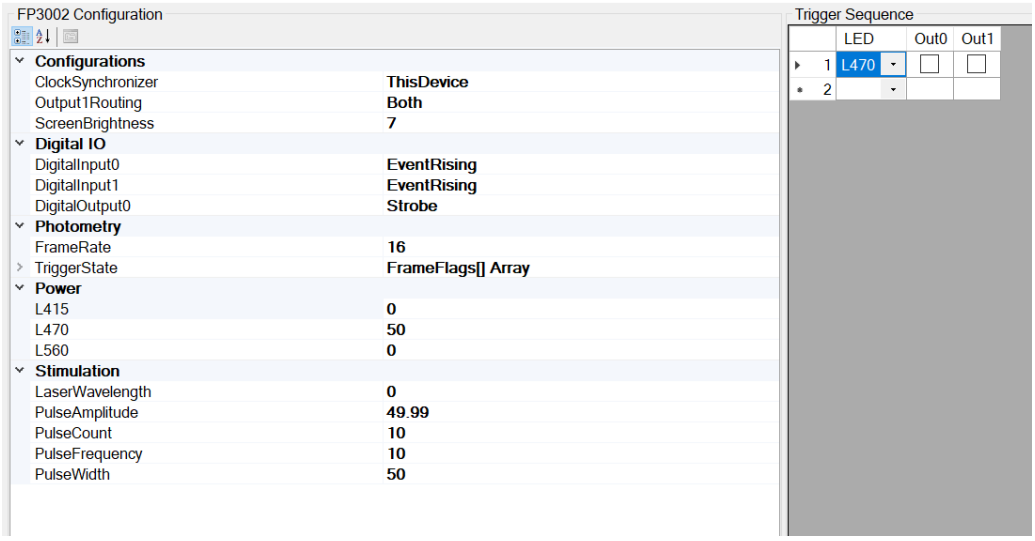
How to Photobleach Patch Cords

Before recording an experiment, we recommend to 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 “Connecting and Aligning my Patch Cord on page 23 of this manual. In the main FP3002 Setup window, under Trigger Sequence, delete the 560 and 415 LED so that only the 470 LED remains. Then under power, slide the L470 power to 50. Save the configuration and play the workflow. Please repeat this protocol once every month.



Patch cord bleaching configurations are shown in the image above.

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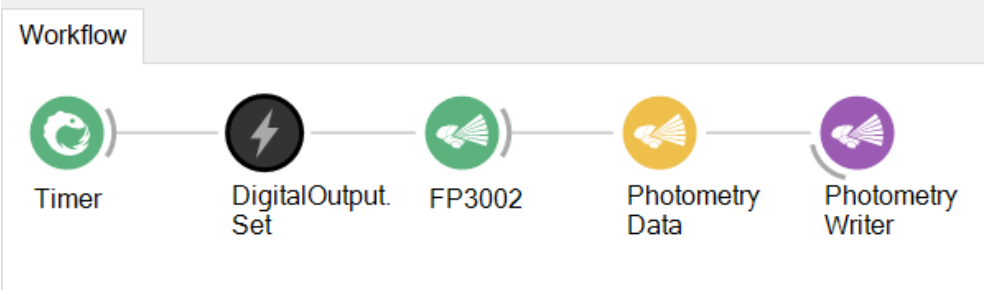
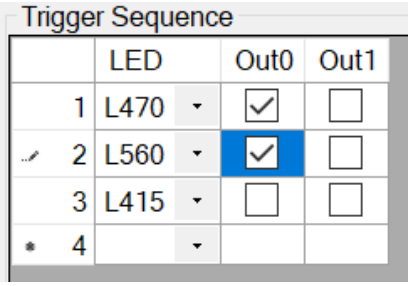
Additional Information: Using the Digital Output Port 0

Digital Output Port 0 is a BNC port, enabling the user to send a signal from the Neurophotometrics fiber photometry system to an external device. The signal can range from 0V to 5V, either as a HIGH or LOW signal. In order to configure the signal, you will need to access the Calibration window by double clicking the **FP3002** node. Under the section labelled **DigitalIO**, click on the drop down menu for **DigitialOutput0**. There are 3 preset configurations on this drop down menu, **Software**, **TriggerState** and **Strobe**.

The **Software** configuration utilizes the **DigitalOutput(Neurophotometrics)** node. This configuration allows Bonsai to act as a function generator via line(s) of code in the workflow. The **DigitialOutput(Neurophotometrics)** node will receive input from most data types and output to the FP3002 node.



In the example above, a signal will output from digital output port 0 every time the timer completes a cycle. Within the **DigitalOutput** node, you can select a **Command**. This Command will determine how the incoming signal will function. For users sending a simple HIGH/LOW signal to the **DigitialOutput** node, we highly suggest using the **Set** command. **Set** will set the signal to HIGH when a sequence is sent to the node. **Clear** will set the signal to LOW when a sequence is sent to the node. **Toggle** will switch the signal from LOW to HIGH and vice versa when a sequence is sent to the node. **Write** will set the signal to the state that is input to the DigitalOutput node. Note that the **Mask** should always be set to **Output0**. A signal dictated by the software can be beneficial for timelocking external behavioural rigs or controlling an external device such as an external laser or camera.



The **Strobe** configuration does not utilize any external nodes. Instead the configuration sends a HIGH signal whenever the camera is acquiring a frame. Selecting **Strobe** can be beneficial for timelocking external devices. The **TriggerState** configuration will output a HIGH signal whenever a specified LED is on. Please select the LEDs to examine on the right hand window labelled **TriggerSequence**. In the example on the left, a HIGH signal is produced every time the 470nm and 560nm LEDs are on. Note that Out1 or Out0 and **TriggerState** for the respective port need to be checked and selected in order to output a signal for LED activity. **Do NOT check these boxes if you are not recording the LED trigger state.**

NEUROPHOTOMETRICS
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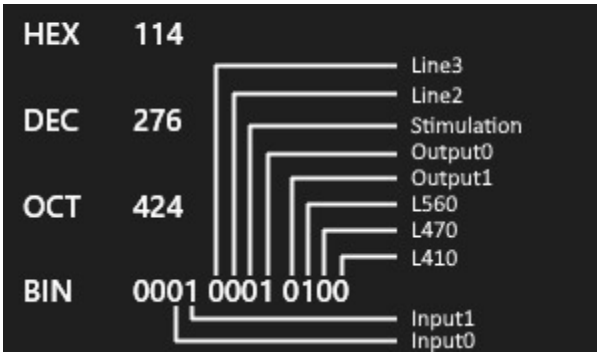
Additional Information: Understanding Frame Flags



Frame Flags are a way to understand what events occurred during a time period in a recording. Frame Flags can be found in the 3rd column of a csv file that is saved from the PhotometryWriter node. The frame flags are determined by a bitwise shift. The starting value 1 is shifted by x bits to the left depending on the state of certain components of the system. The shift is depicted in the image to the right. Using frame flags can aid *post hoc* data processing by noting if a component was simultaneously on while data was recorded. By using frame flags, you can more efficiently compare fiber photometry data when the laser was ON versus OFF.

	Byte Position	Value/No LED ON	L415	L470	L560
No additional signal	0	0	1	2	4
Output 1 signal HIGH	3	8	9	10	12
Output 0 signal HIGH	4	16	17	18	20
Stimulation ON	5	32	33	34	36
GPIO Line 2 HIGH	6	64	65	66	68
GPIO Line 3 HIGH	7	128	129	130	132
Input 1 HIGH	8	256	257	258	260
Input 0 HIGH	9	512	513	514	516

Table 1: Frame flags representing when a LED is on simultaneously with an additional signal.

Above is a table laying out what a frame flag would be if an individual component was on. As a base the 415nm LED is flagged with value 1, the 470nm LED is flagged with value 2 and the 560nm LED is flagged with value 4. These values will only change if

another component on the system was operational simultaneously with an LED. For example, if the Output0 port was in a HIGH state only while the 415nm LED was ON, the flag for the 415nm LED would write as 17 while the 470nm LED flag would be 2 and the 560nm LED flag would be 4. Please note that these values compound. If the laser(Stimulation) was ON simultaneously with the Output0 port and the 415nm LED, the flag would write as 49— 1(415nm base value) + 16(bit shift of 4) + 32(bit shift of 5).

More Example Frame Flags

	No LED ON	L415	L470	L560
No additional signal	0	1	2	4
Output 0 signal HIGH + Stimulation	48	49	50	52
Output 0 signal HIGH + Input 0 signal HIGH	528	529	530	532
Input 0 signal HIGH + Stimulation	544	545	546	548
Output 0 HIGH + Input 0 HIGH + Stimulation	560	561	562	564

Table 2 : Example frame flags when multiple signals are on simultaneously.

The table above shows some example frame flags if many components are on simultaneously. These values have been calculated by adding the Value of each specific signal in **Table 1** with the LED base value (found in the row labelled “No additional signal”).

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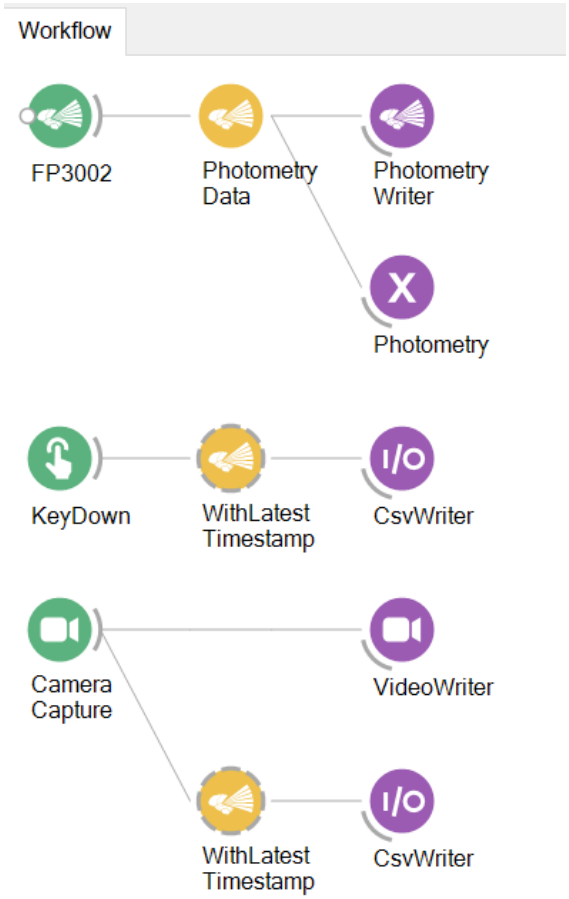
Bonsai: Basic Bonsai Workflow



This basic Bonsai workflow will allow one to record interleaved fiber photometry data and record an external web camera and keystrokes. The first part of this workflow aims to record your fiber photometry data and save it into a csv file. The **FP3002** node configures all of your settings such as LED power levels and Region of Interest (ROI) definition. Configuration occurs before the workflow is played. The **PhotometryData** node not only passes your data to the **PhotometryWriter**, but also populates a graph of your photometry data in real time. To view this graph, double left click on the **PhotomeryData** node while the workflow is playing. The automatic Capacity that is displayed is 300 points. You can change this by right clicking once on the bottom of the pop-up window. **PhotometryWriter** saves all data into a csv file. The **PhotometryWriter** will save the frame number, timestamp, frame flag, and a normalized average pixel value for each ROI. Define the save path by left clicking on the node and selecting FileName under Properties on the right hand side of the window. **PhotometryWriter** can save a jpeg of a graph of the entire recording and an image of your ROIs. The Photometry node is actually a PublishSubject node renamed as Photometry. PublishSubject casts the information from one node to be used by another node. This node is crucial if using the **WithLatestTimestamp**.

The second part of the workflow saves keyboard strokes and the time at which a key was pressed into a csv file. The KeyDown records when any key on the keyboard was pressed down. WithLatestTimestamp selectively filters out the timestamp from the Photometry node. WithLatestTimestamp records the nearest photometry timestamp

with the input keystroke. The information is passed to the CsvWriter where the key and timestamp is saved in a csv file. Similarly with PhotometryWriter, save path definition is found under the properties section of the node. This part of the workflow is great for note taking, i.e. if an unexpected door slams or to mark behaviour during signal checks.

The last part of the workflow saves the video from an external web camera and saves the timestamp of each frame. CameraCapture can pass data from most USB web cameras. A specific webcam can be specified by inputting a number in Index found under the Properties section. Note that 0 will most likely be an integrated web camera, from there each camera will be specified based upon the order that it was plugged in. VideoWriter will save the video from the CameraCapture as an avi file. Define the save path under FileName found in the Properties section of the node. WithLatestTimeStamp and CsvWriter behave similarly to the ones found in the second section of the workflow. However, the WithLatestTimestamp node will record the nearest photometry timestamp to each external camera frame. The CsvWriter will only record the timestamp as the index is indicative of frame number.

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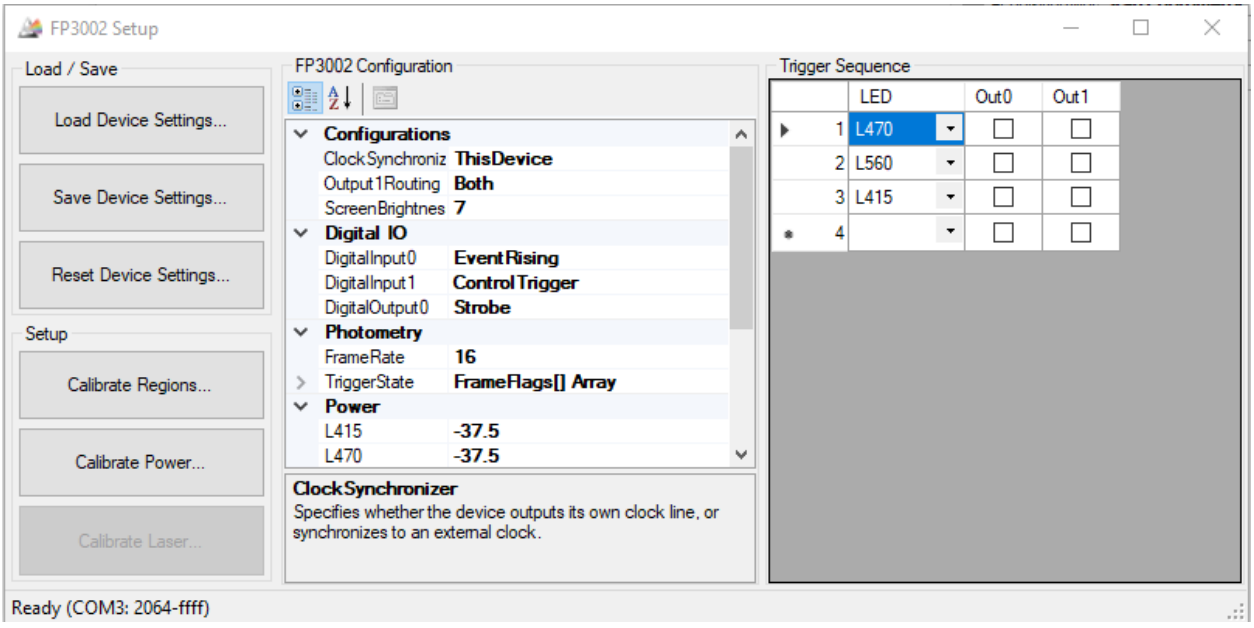
Bonsai: Control LED Activity on TTL

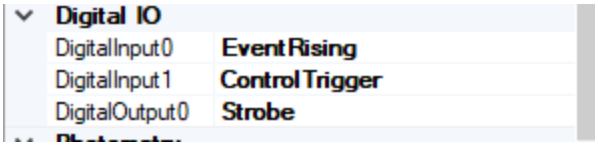
This document discusses a Bonsai Example Code that allows for an external TTL signal to control the LED activity of the FP system. Using this method, the FP system will always collect data, but the LED sequence will turn on when a HIGH digital signal is present on the BNC Digital Input port.

First, double check that your Bonsai Packages are up to date by clicking “Tools -> Manage Packages…”. This will close your current Bonsai window and open up the “Bonsai - Manage Packages” window. Check under the updates sections for any available Neurophotometrics packages that need to be updated.

Once, all your packages are up to date, click the “close” button for the “Bonsai - Manage Packages” window, and your usually Bonsai window will appear. Now bring a “FP3002” node into your workflow, specifying the “PortName” value. Double click the “FP3002” node to open the “FP3002 Setup” window.



In the “FP3002 Configuration” section, find the “Digital IO” menu. Change the value for “DigitalInput1” to “ControlTrigger”.



Then connect your TLL signal to the BNC Digital Input 1 port of the system. Once you press “Start” in the Bonsai code, the LED sequence will turn on while there is a HIGH signal on the TTL. However, the system will continuously acquire data from the moment the workflow is started until it is stopped.

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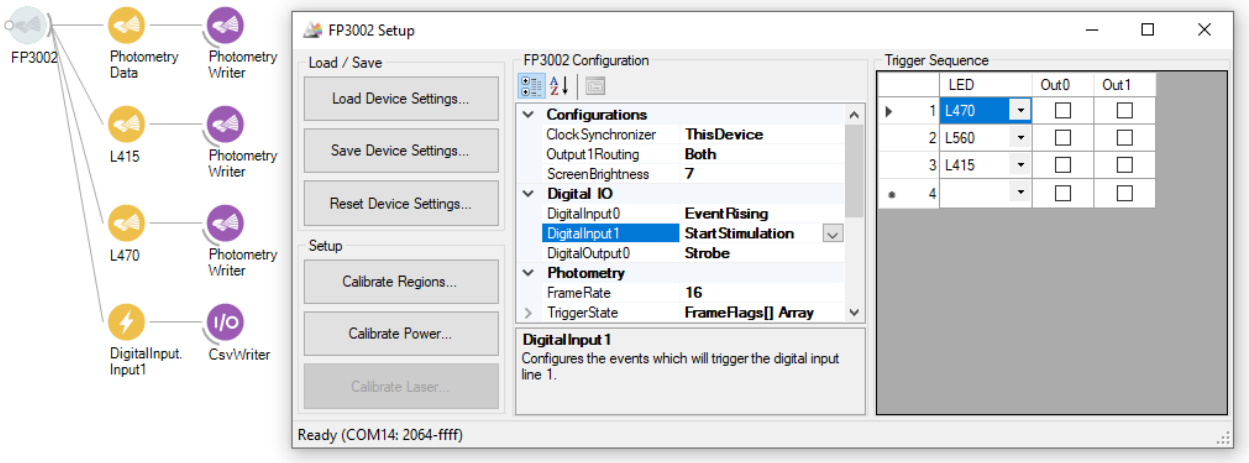
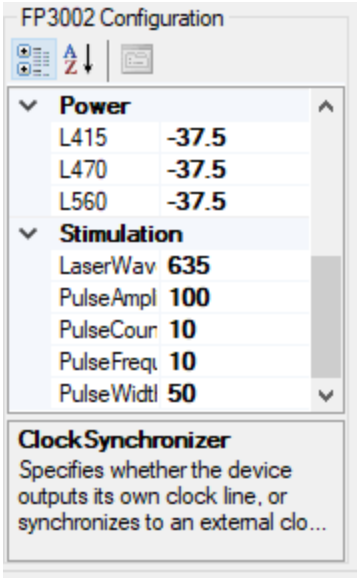
Bonsai:
Triggering the Internal Laser with an External TTL

This document discusses how to trigger the internal laser using an external TTL signal. The “FP3002” node is actually already set up to handle this so there is no need for a complicated workflow. First, connect the BNC cable carrying the TTL signal to the “In 1” BNC port of the V2 system. Then, in Bonsai double click the “FP3002” node to open the “FP3002 Setup” window. Find the “Digital IO” section of the “FP3002 Configuration”. Find the “DigitalInput1” and change its value to “StartStimulation”.

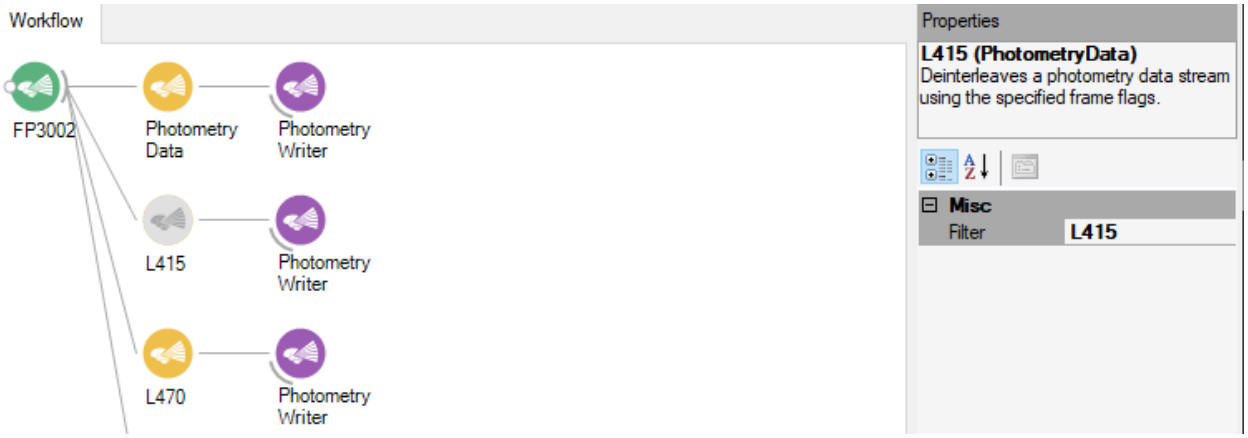


Next scroll down to the “Stimulation” section and specify the “Laser Wavelength” and the “Pulse Amplitude”. This will let the system know how bright to shine the laser.

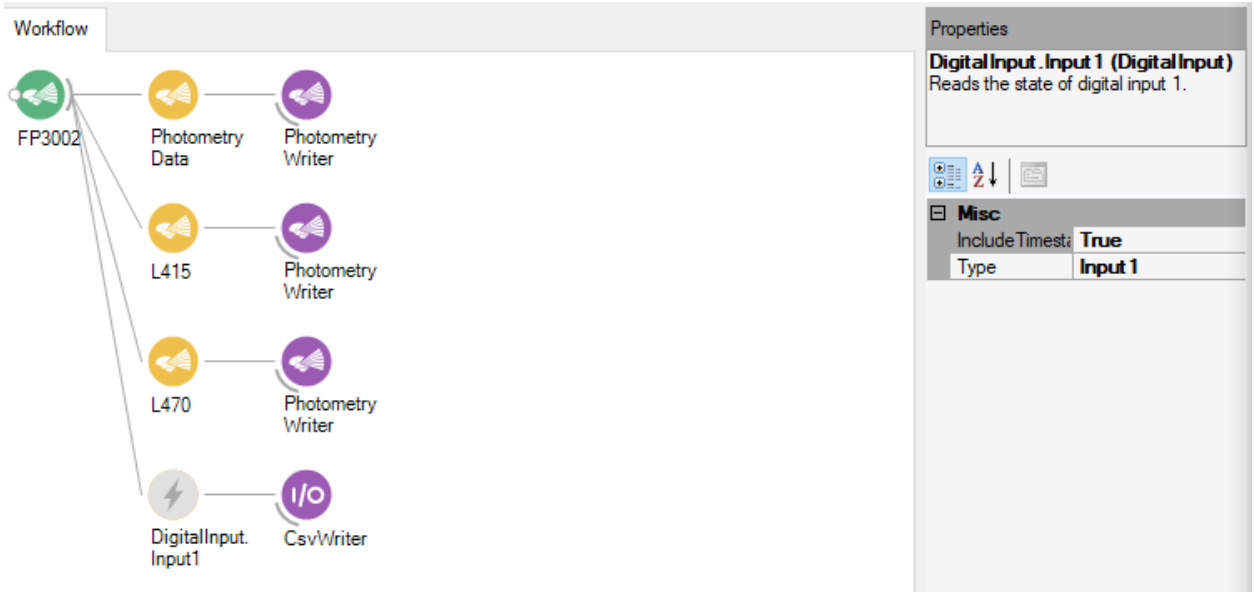


This Bonsai Example Code will collect the interleaved and deinterleaved FP data as well as the TTL signal/timestamps. The laser will be ON whenever the TTL signal is HIGH, and OFF when the TTL signal is LOW.

This Bonsai Example Code use the “Photometry Data” node three time, once with no filter, once with the L415 filter, and once with the L470 filter. This way we can use the “Photometry Writer” nodes to save three files, one with the full data set, one with the deinterleaved 415 data, and one with the deinterleaved 470 data.



We also use the “Digital Input (Neurophotometrics)” node to save the timestamps of the TTL input. To do this we simply set the “Include Timestamp” value to “True” and the “Type” value to “Input 1”.



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Bonsai:
External Sensors - Using Arduino

This document discusses how to implement external sensors in an experiment using Arduino. There are a variety of basic sensors that may be able to provide much needed data, some examples include: temperature sensors, proximity sensors, accelerometers, IR sensors, pressure sensors (microphones), voltage/capacitive sensors (lickometers), etc. Most of the time, these basic sensors output an analog voltage signal. With some basic wiring to an Arduino microcontroller, we can bring the values measured by these sensors into Bonsai for data collection purposes.

Arduino microcontrollers are powerful devices, and even the most basic one of the series, the Arduino Uno, should be more than capable of sending all sensor data to Bonsai in FP experiments without performance issues. Below is a workflow for setting up the Circuitry/Hardware:

Circuitry/Hardware:

1. Find out what materials are needed for your setup. You will always need an Arduino microcontroller (usually an Arduino Uno) and a USB 2.0 Cable Type A/B. Then depending on what sensor you are using, and your experimental setup you may need a soldering iron, solder, wiring, BNC Connectors, and/or BNC Cables.
2. Wire the sensor to the Arduino by connecting the sensor's output pin to the A0 pin of the Arduino and connecting the rest of the pins as the datasheet recommends. Often this involves connecting the sensor's ground pin to the GND pin of the Arduino and connecting the sensor's positive voltage pin to the +5V pin of the Arduino. NOTE: Always double check the datasheet of the sensor you are using to see what you need to connect to what pin. Sometimes, when your sensor is far away from your computer, it is best to send it through a BNC cable. For this, you wire the output of the sensor to the positive pin of a BNC connector and you connect the ground pin of the sensor to the ground pin of the BNC Connector. Then wire another BNC Connector to the Arduino, with the positive pin of the BNC Connector to the A0 pin of the Arduino, and the negative pin of the BNC Connector to the GND pin of the Arduino. Once these BNC Connectors

are wired to the sensor and the Arduino, you can connect a BNC Cable between them.
3. After we have set up the circuitry, we must connect the Arduino to the computer using the USB 2.0 Cable Type A/B.

Once we are confident in our Circuitry/Hardware setup, we can set up the code for our Arduino. We will use a simple analog firmata code which sends the voltage value on the A0 pin of the Arduino to the computer. Below are the steps to follow to upload the code:

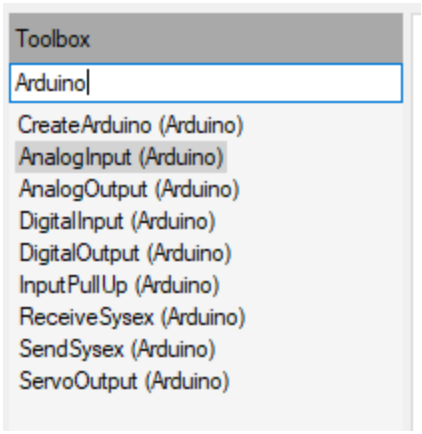
Arduino Software:

1. Visit the GitHub link: https://github.com/firmata/arduino
2. Download Zip (in the "Code" drop down menu in the upper right).
3. Open up the Arduino IDE.
4. Click Sketch -> Include Library -> Add .ZIP Library
5. Click on the .ZIP file you just downloaded (should be in your downloads folder)
6. Open the "SimpleAnalogFirmata.ino" (Click File -> Examples -> Firmata -> SimpleAnalogFirmata)
7. Check that the Arduino is connected to the Computer via a USB.
8. Click Tools and double check that the "Board:" is filled with the correct board type (usually Arduino Uno) and that "Port:" is the correct port (should be COMX, where X is a number).
9. Finally, click the Upload button in the Arduino IDE to upload the code onto your Arduino.

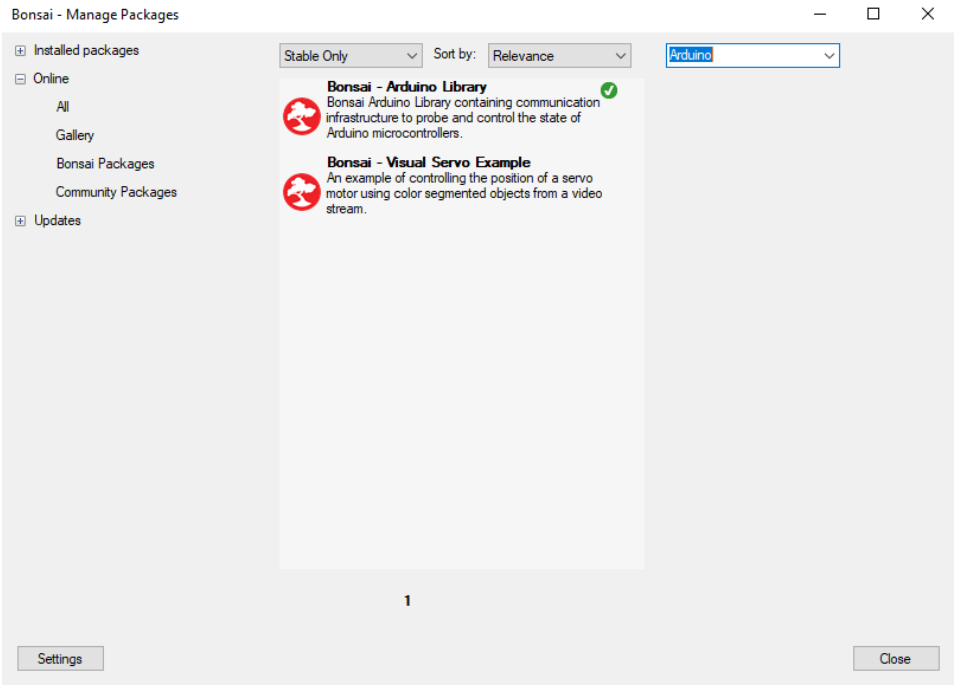
Once our Arduino is encoded, we can set up our Bonsai code to accept information from the Arduino. Below are the instructions for setting up Bonsai:

Bonsai Code:

1. Double check you have the Arduino pack downloaded by searching for "Arduino" in the toolbox search bar.

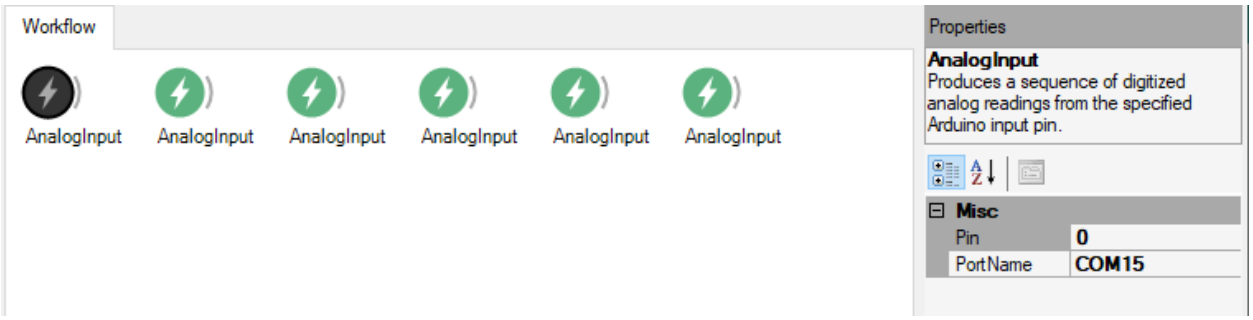


2. If no "Arduino" nodes pop up in the toolbox, Click Tools -> Manage Packages. The Bonsai Workflow will close out and the "Bonsai - Manage Packages" window will open. Click the "Online" tab on the left and search for "Bonsai - Arduino Library" in the search bar on the top right. Click it and click install. Once finished you can close the Manage Packages window and the Bonsai Workflow window should open back up.

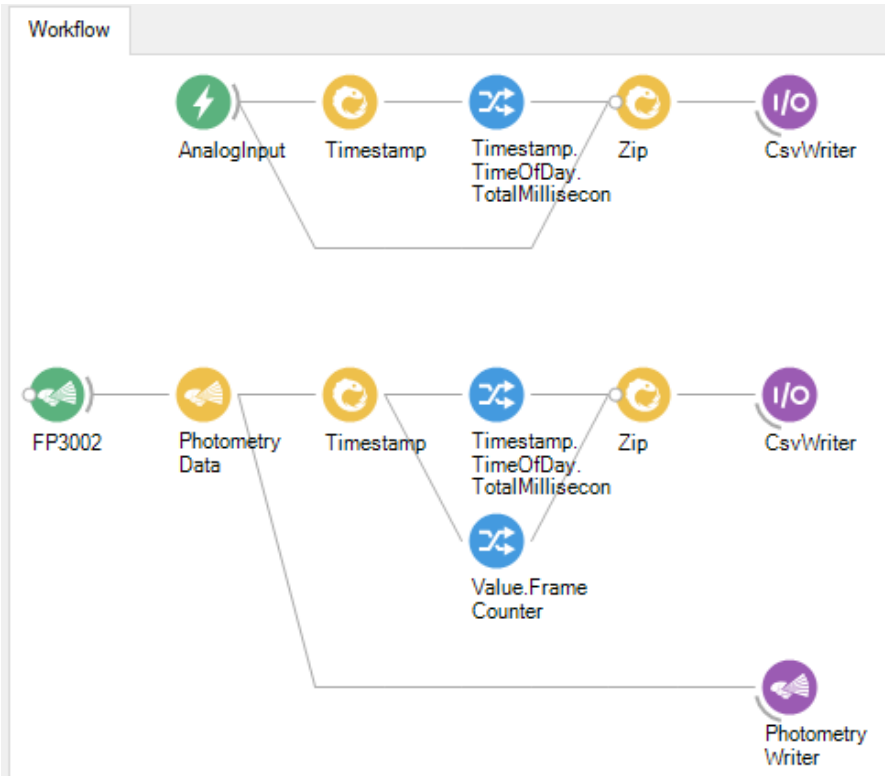


3. Search for "Arduino" in the toolbox search bar and add one "AnalogInput (Arduino)" node for each analog pin on the Arduino you wish to sample.
4. For each "AnalogInput (Arduino)" node select the pin number you wish to sample and the port name (Should be the same as the port name you set in the

Arduino IDE, and should be called something like "COMX" where "X" is a number). NOTE: Each Arduino Uno setup in this way can read values from six different sensors at the same time.



5. Once you click start on Bonsai, the ports on the Arduino should be sampled, all at the same frequency.
6. You can synch this data with the FP data by adding a "Timestamp" node to the "AnalogInput (Arduino)", then right click the "Timestamp" node and navigate to Output -> Timestamp -> TimeOfDay -> TotalMilliseconds.
7. Combine the "TotalMilliseconds" and "Analog Input" nodes in a "ZIP" node and attach a "CsvWriter".



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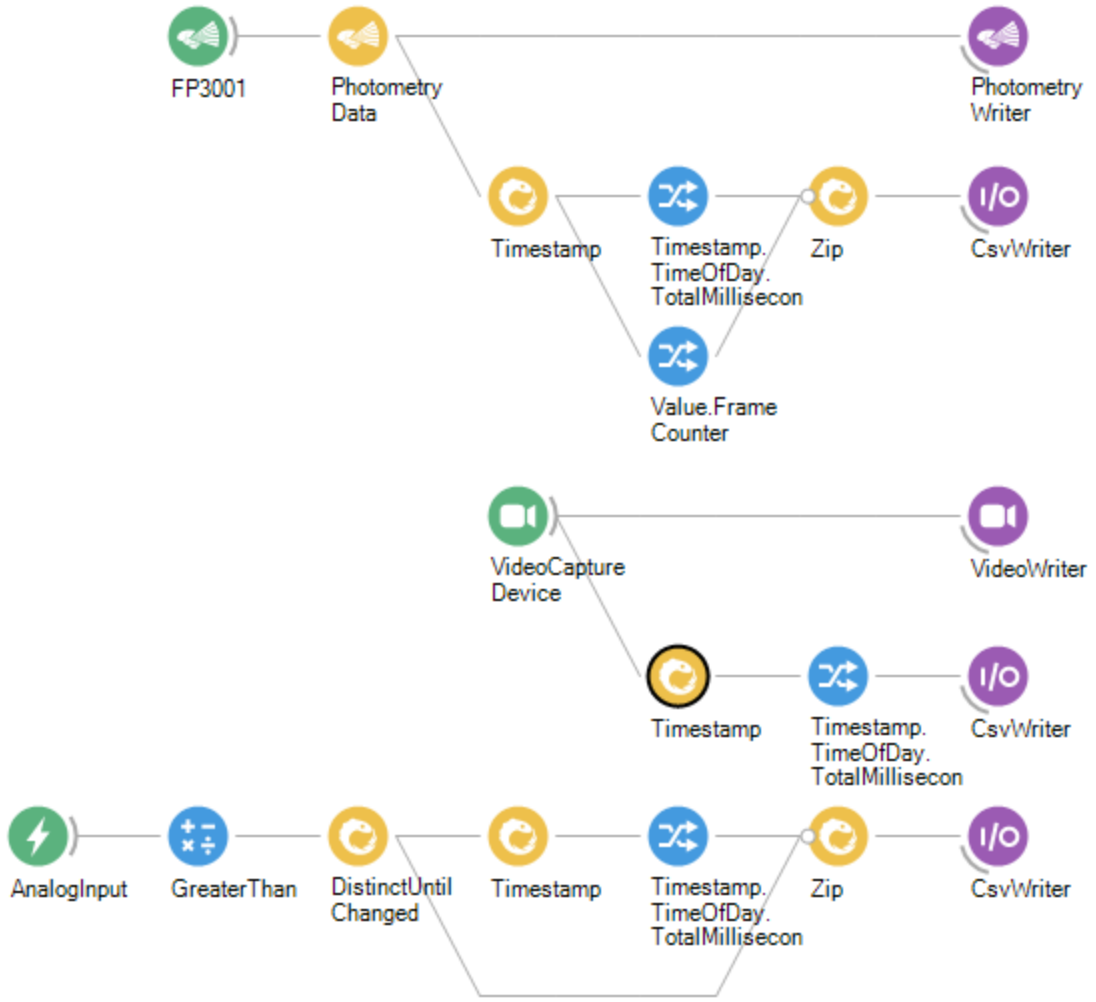
Bonsai: Synchronizing Datasets

This document discusses two methods for synchronizing the timestamps of various datastreams. These methods are used when an experiment requires use of external cameras, sensors, and/or DAQ boards that must have their data synchronized with the fiber photometry data. The main difference between the below methods is that one uses the system's internal clock to timestamp, while the other uses the computer's clock to timestamp.

Computer's Clock:

Since the "FP3001" and "FP3002" nodes utilize the internal clock of the system, the timestamp of the "PhotometryWriter" node is always in seconds since the system turned on. An external camera or a DAQ board does not have access to this information, so to synchronize the data, we save the Time of Day, Total Milliseconds, of every frame of the FP data. This way the FP data can be timestamped using a clock that all other datastreams have access to. So, if we also timestamp all of the datastreams with Time of Day, Total Milliseconds, then all the data will be synchronized in time.



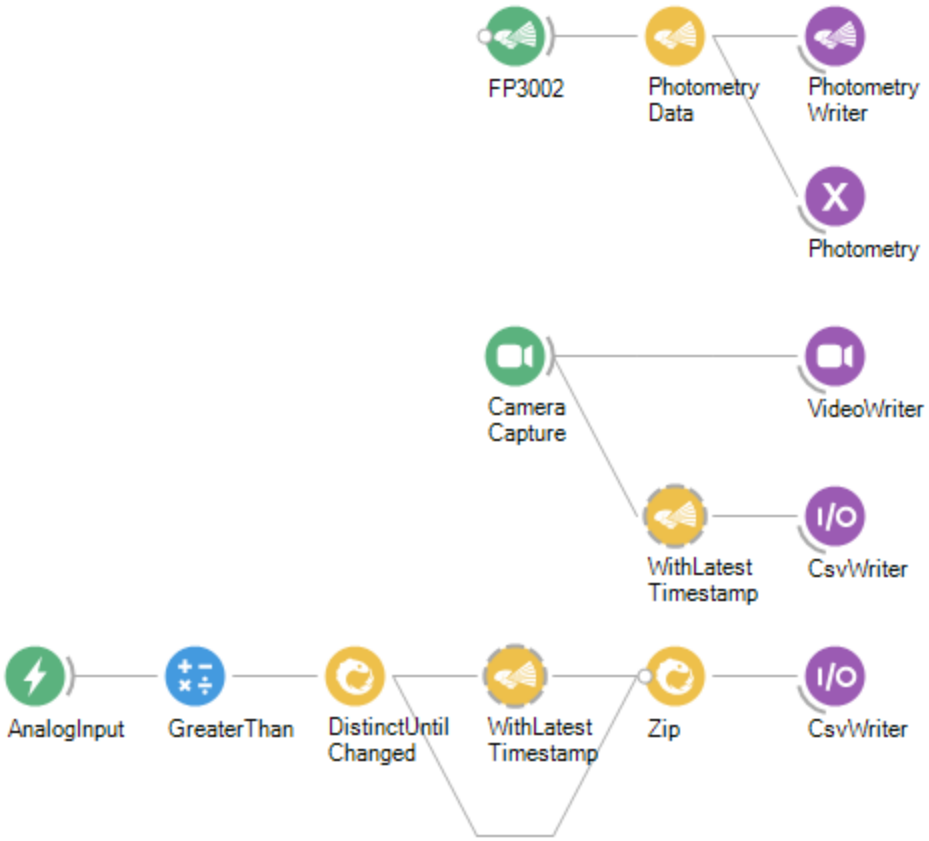
In the example code above, there are three data streams. First is the FP datastream, which saves the “PhotometryData” using the “PhotometryWriter” node. This is the standard way of saving the FP data. However, in a parallel stream, we also “Timestamp” every frame and “Zip” the frame number along with the Time of Day, Total Milliseconds value, to save to a “.csv”. This way we can have the Computer’s Clock timestamp of each FP frame to align to the other datasets. The second datastream records video from an external camera and timestamps every frame. Here you can also employ video processing techniques (see the “Animal Tracking” documents for more details). Finally, the third datastream, deals with timestamping a TTL signal using our DAQ board. Here we timestamp every time the TTL signal changes from HIGH to LOW or from LOW to HIGH. Here you could also save the raw data from any sensor value sent from an Arduino. NOTE: Saving the raw data of a sensor can create large file sizes

because sensors tend to send a lot of data. Since the example is sampling a TTL signal we first convert the signal to digital using the “GreaterThan” node, and use the “DistinctUntilChange” so data is only saved when the value changes. For example, an Arduino Uno can send values of a sensor at ~1250 samples per seconds, so without the “GreaterThan” and “DistinctUntilChange” nodes, you would be saving 4500000 rows of data per hour of recording in this datastream alone.

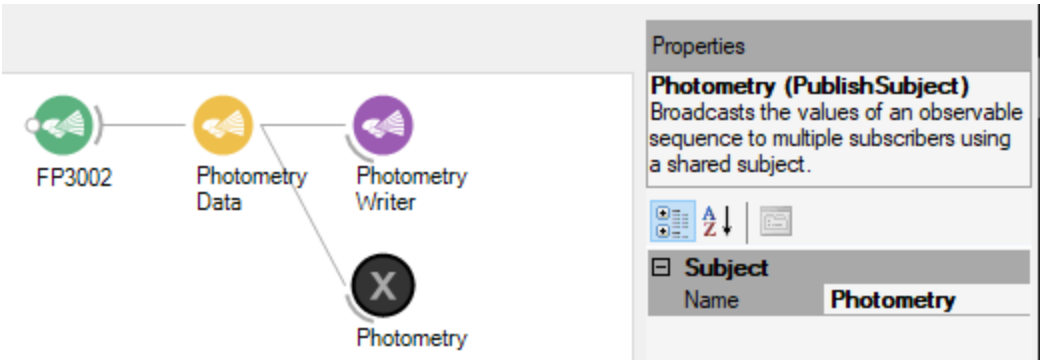
The key similarity between each datastream in this code is the use of the “Timestamp” and “Timestamp. Time Of Day. Total Milliseconds” nodes. This can use the computer’s clock to timestamp any datastream in Bonsai, making this method quite a robust data synchronization method.

System’s Clock

Since external cameras, sensors, etc, do not have access to the system’s internal clock, this method utilizes the “WithLatestTimestamp (Neurophotometrics)” node. This node will use the latest FP data timestamp as the timestamp for every other datastream. For example, when a datastream, such as an external camera feed, pushes an element into the “With Latest Timestamp” node, it will use the latest timestamp in the FP datastream as the current timestamp for the external camera feed.



Implementation of this method is quite similar to the computer's clock method. First, we must connect the "Photometry Data" node to a "Publish Subject" node and in the properties for the "Publish Subject", we must specify the "name" value as "Photometry". This "Publish Subject" node is needed for the "With Latest Timestamp" nodes to have access to the FP timestamps.



Ultimately, this method synchronizes the datasets much the same as the computer clock method. However, there are some differences between the two methods. First, using the system's clock method, the timestamps of the datasets other than the FP dataset, will be less accurate. This is because the other datastreams will use the latest FP data timestamp, which could be several milliseconds before the actual time of recording for that frame. The accuracy of the FP dataset will not be affected, only the datasets of the external camera, sensors, etc. Since this accuracy decrease might be negligible for some experiments, this method is sometimes used to make data analysis more efficient. This is because every timestamp of every dataset will have their timestamps line up exactly with a timestamp from the FP dataset.

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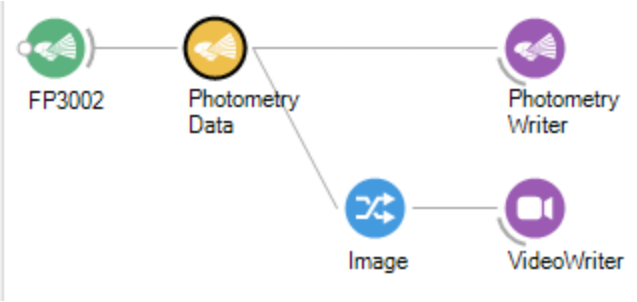
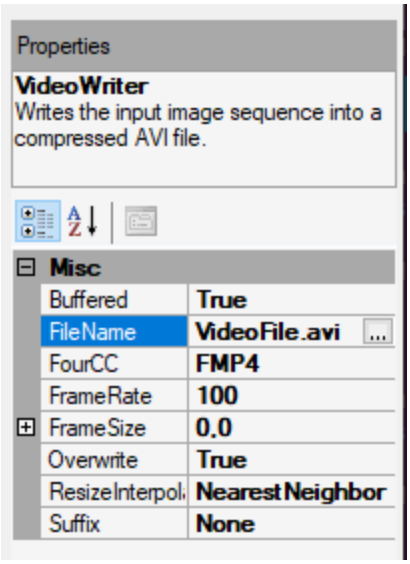
Bonsai: Recording Raw Data from the Internal Camera

This document discusses how to record the raw data from the internal camera of the system as a ".avi" file. This can be used for post hoc analysis of the images coming from the system's camera. First, we start with the standard FP data workflow. Then, right click the "Photometry Data" node and navigate to "Output -> Image" so that the "Photometry Data" node has an "Image" node coming from it. Connect the "Image" node to a "VideoWriter" node to save each frame's image into a ".avi" file.



Under the "VideoWriter" setting, be sure that the "FrameRate" value is greater than or equal to the "FPS" of the FP system. This way you do not lose frames in your saved ".avi" file.



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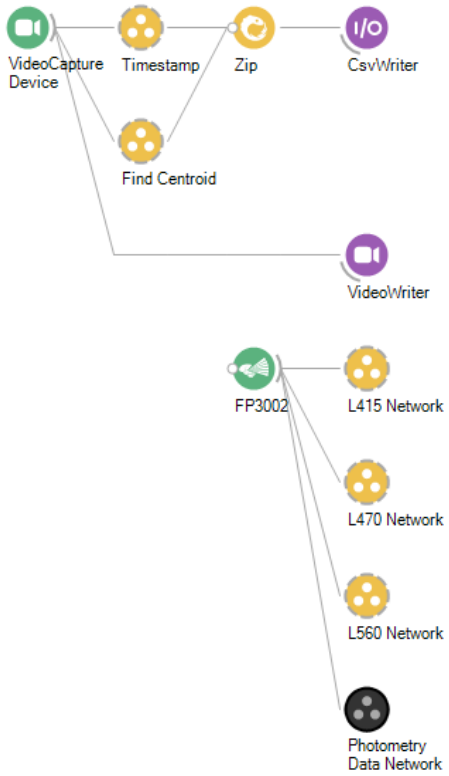
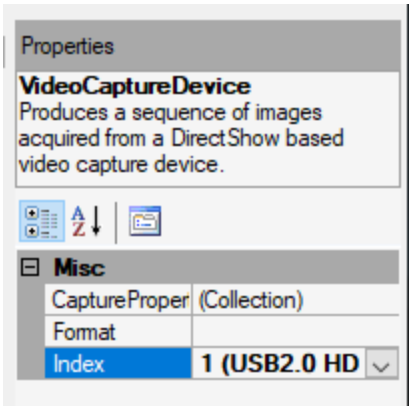
Bonsai: Basic Animal Tracking

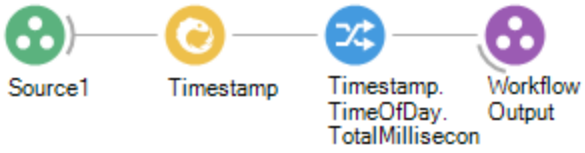
This document discusses a setup for basic animal tracking while collecting FP data. The Bonsai Example Code shown below has two datastreams, the animal tracking datastream and the FP datastream. The FP datastream collects and saves the raw FP data and deinterleaved FP data, both with timestamps in Time of Day, Total Milliseconds, so it can be synchronized with the data collected from the animal tracking datastream. The animal tracking datastream uses the video stream from an external camera to find the position of an animal in an arena. It saves the centroid of the animal (in pixels) and the timestamp (in Time of Day, Total Milliseconds) every frame. It also records the raw video stream and saves it as a ".avi" file for you to reanalyze at a later time if required.



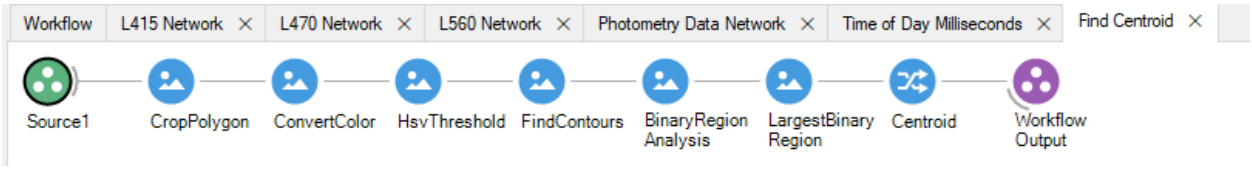
Starting with the animal tracking datastream, first we set up the "Video Capture Device" node, making sure the correct camera is connected. When you have a camera plugged into your computer via USB, you should see options in the "Index" value of that node, one will be the camera you want to use the other might be the webcam built into your computer.



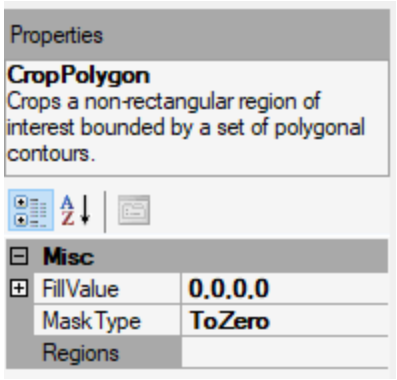
Second, we set up the “Timestamp” nested workflow, this is reused multiple times throughout the file and is used to timestamp in Time of Day, Total Milliseconds. Create a “Timestamp” node, right click it and navigate to “Output -> Timestamp -> TimeOfDay -> TotalMilliseconds”. To put these two nodes in a nested workflow, highlight them and click “Group”. Below is what the inside of the nested workflow should look like.



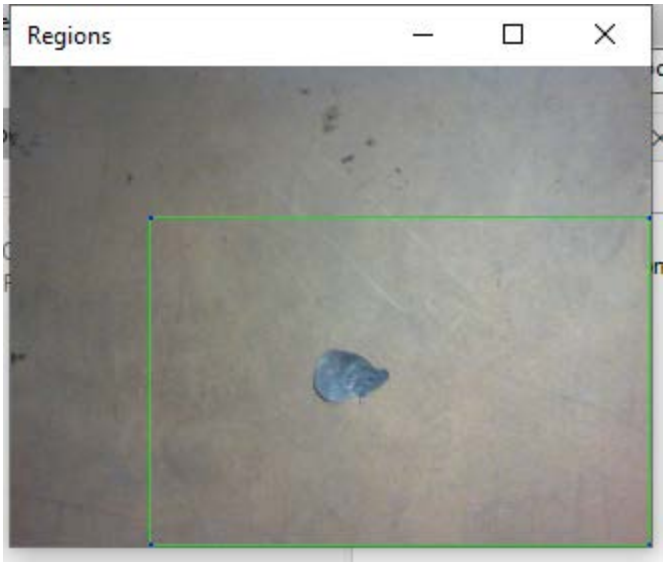
Next, create the “Find Centroid” nested workflow. Create the nested workflow shown below, then we will have to change the values of some of the nodes.



Take a look at the "Crop Polygon" node, this is what crops the video footage to your arena. While the Bonsai file is running, Click the "Crop Polygon" node, and then click the "..." at the right of the "Regions" value.

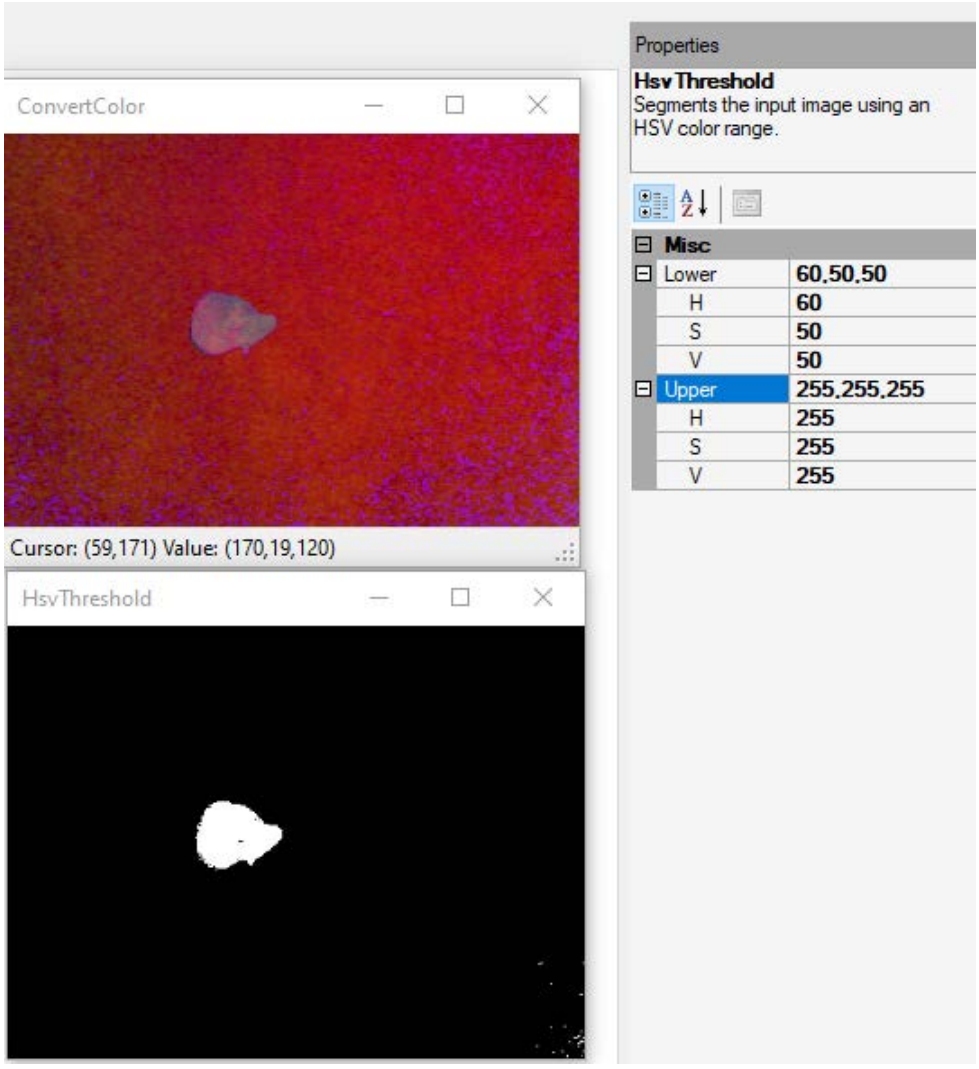


This will open the video feed from the camera. Now you can crop to your arena by left clicking and dragging a rectangle in the "Regions" window. If you mess up simply press delete and try again. Make sure you only have one rectangle, to check this, close the "Regions" window and click the "+" to the left of the "Regions" value of the "Crop Polygon" node. Check that there is only one "Point[] Array" value called "[0]". If there is more than one, (called "[1]", "[2]", ... "[N]"), simply delete the "Crop Polygon" node, add it again, and retry drawing the regions.

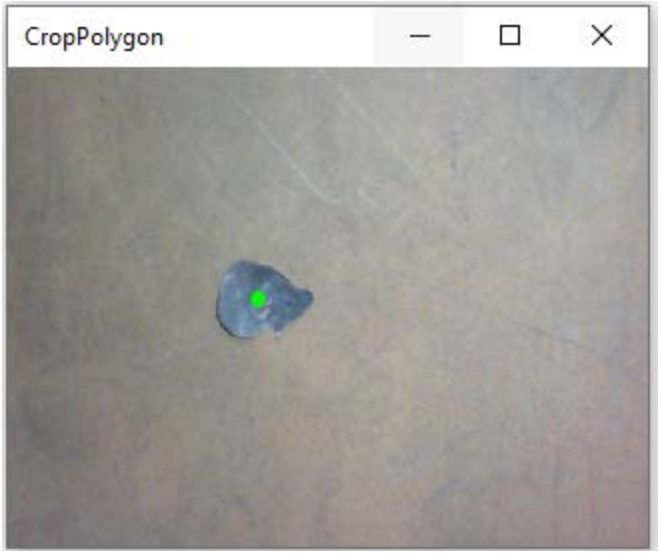


Next, probably one of the most important nodes, is the "HsvThreshold" node, this is what is used to find the animal in your arena. While Bonsai is running and the animal is

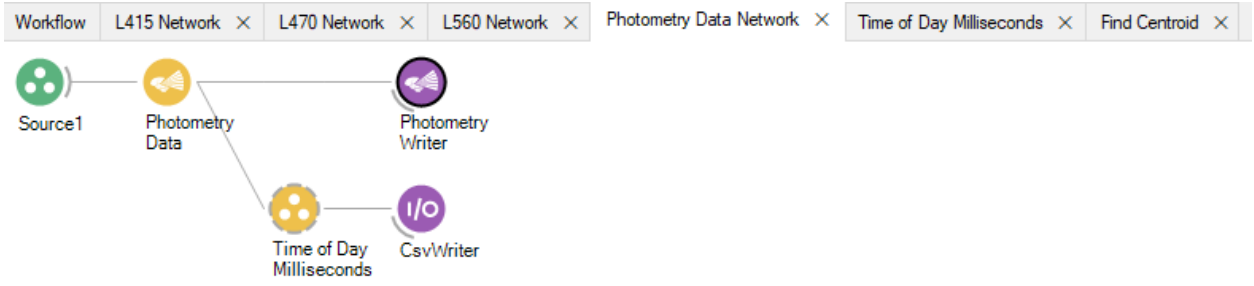
in the arena, double click this "HsvThreshold" node, you will see a black and white image of your animal in your arena. You want to adjust the "Lower" and "Upper" values of this node so that you only see an all-white figure of the mouse. To help with this, you can double click the "ConvertColor" node to open its visualizer (while the Bonsai file is still running). If you right click the window, you can see the HSV values of what your cursor is hovering over in the window. You want only the HSV values of the animal to be in the "LOWER" to "UPPER" range of the "HsvThreshold" node.



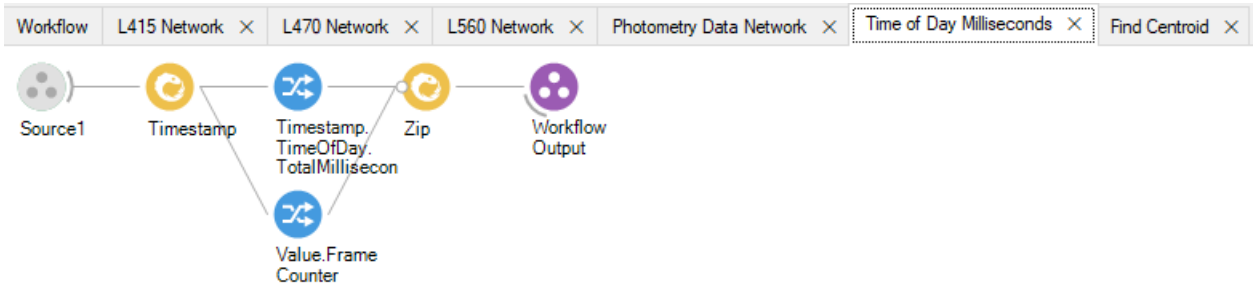
To double check this is all working properly, while the Bonsai file is running double click the "CropPolygon" node to open the video feed window. Then click and drag the "Centroid" node onto the "CropPolygon" window. A green dot will appear in the window, and it should always be in the center of the animal.



The seconds datastream, which begins with the "FP3002" node, is what collects your FP Data. In each network node (i.e. "L415 Network'', ... , "Photometry Data Network") you have your standard "Photometry Data" node (with or without a filter for deinterleaving) and your standard "Photometry Writer" node.



This will collect your FP data, however, the "Photometry Writer'' node alone will not have synchronized timestamps with the animal position data. This is why we have included a "Time of Day, Milliseconds" node to write the synched timestamp data for every frame of the FP data. During analysis, you will simply combine the columns of the "Time of Day, Milliseconds'' data to a matrix containing the "Photometry Writer" data.



NEUROPHOTOMETRICS
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Bonsai:
Animal Tracking, With Multiple Chambers and Laser Stimulation

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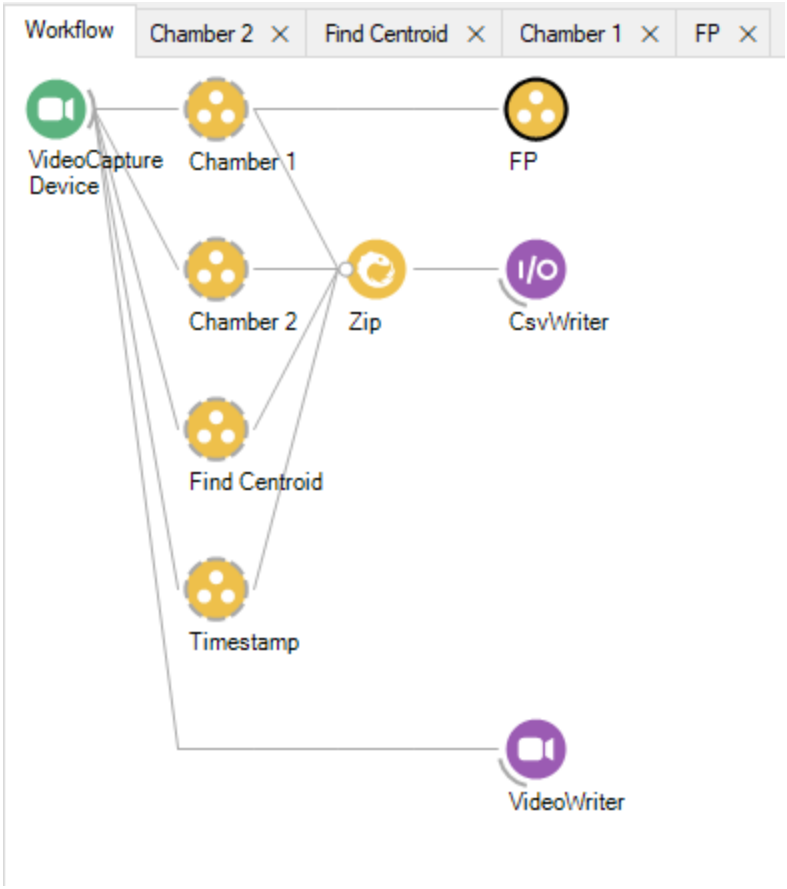
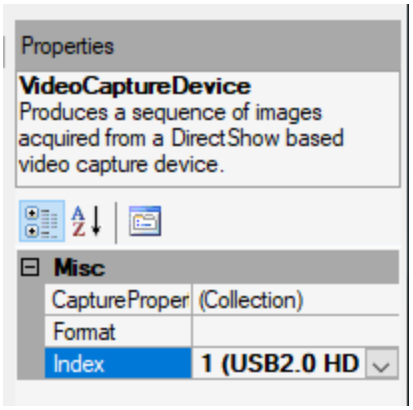
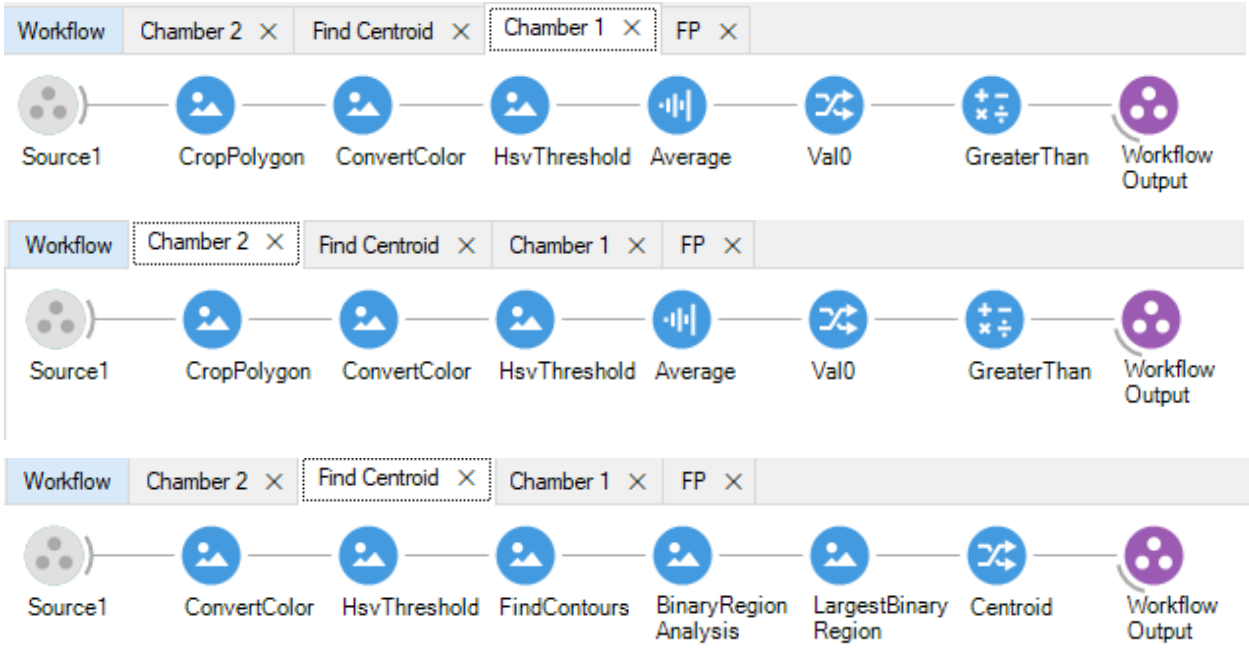
This document discusses a Bonsai Example Code that collects FP data while tracking an animal's position in a multi-chamber arena and stimulating when the animal enters a specified chamber.

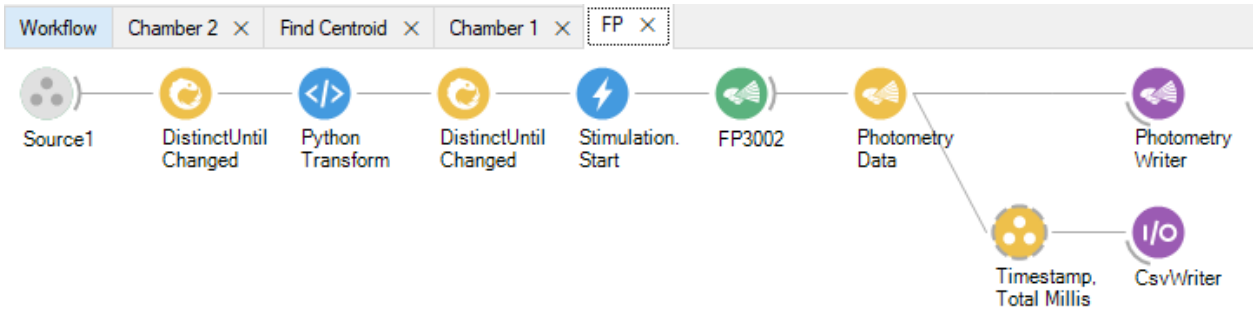


We begin with the "VideoCaptureDevice" node, and specify the "Index" value of your external camera.



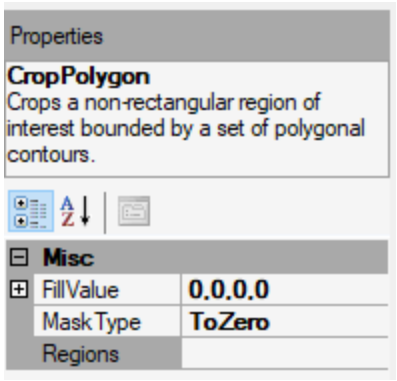
Then you must make changes to some nodes inside the "Chamber1", "Chamber2", "FindCentroid", and "FP" nested workflow nodes. Start by creating the nested workflows shown below. Connect them as you see in the “Workflow” screenshot above.



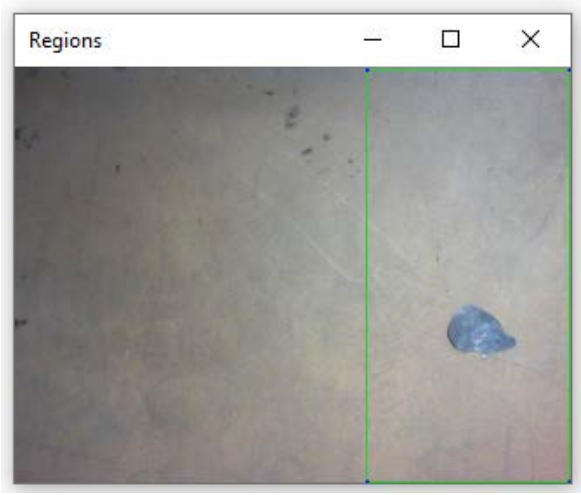


We will need to change the values of some of these nodes while the Bonsai file is running. **So, for now, disable all of the writer nodes as well as the "FP" nested workflow. This way you do not save a ton of .csv and .avi files nor flash the laser while you adjust the parameters of the Bonsai code for your particular setup.**

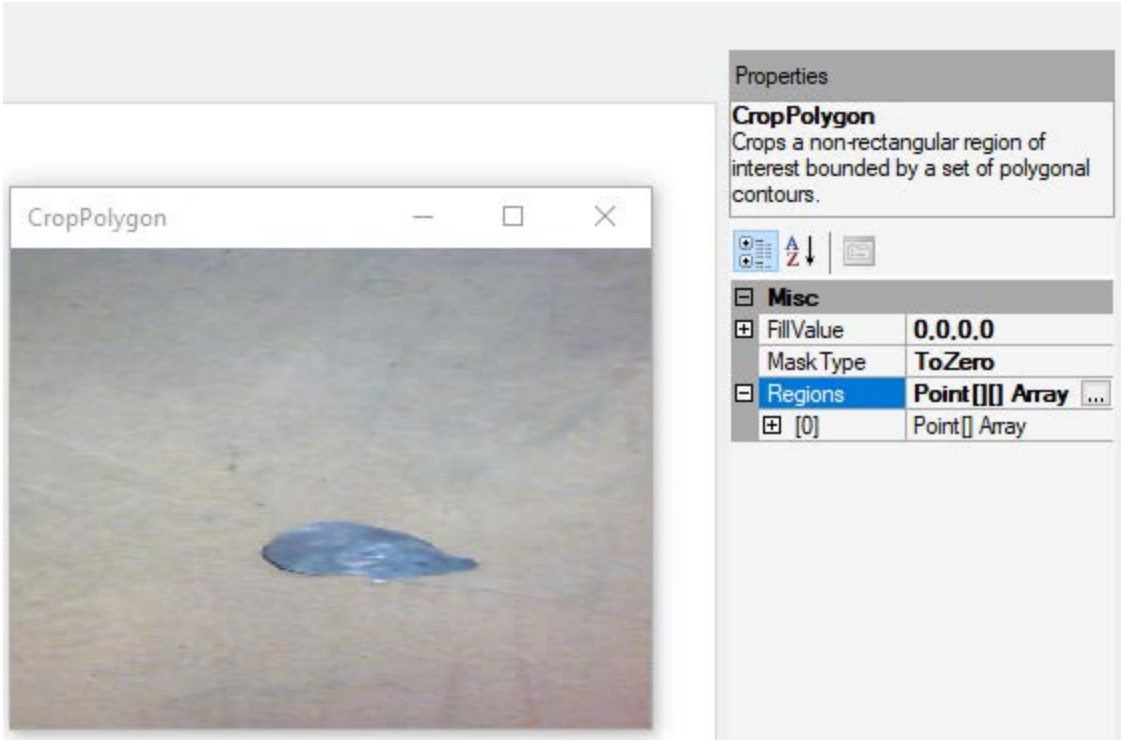
Now navigate inside the "Chamber1" node, this is the chamber that will trigger the laser when the animal enters it. Here, we first need to specify the "Regions" value of the "CropPolygon" node.



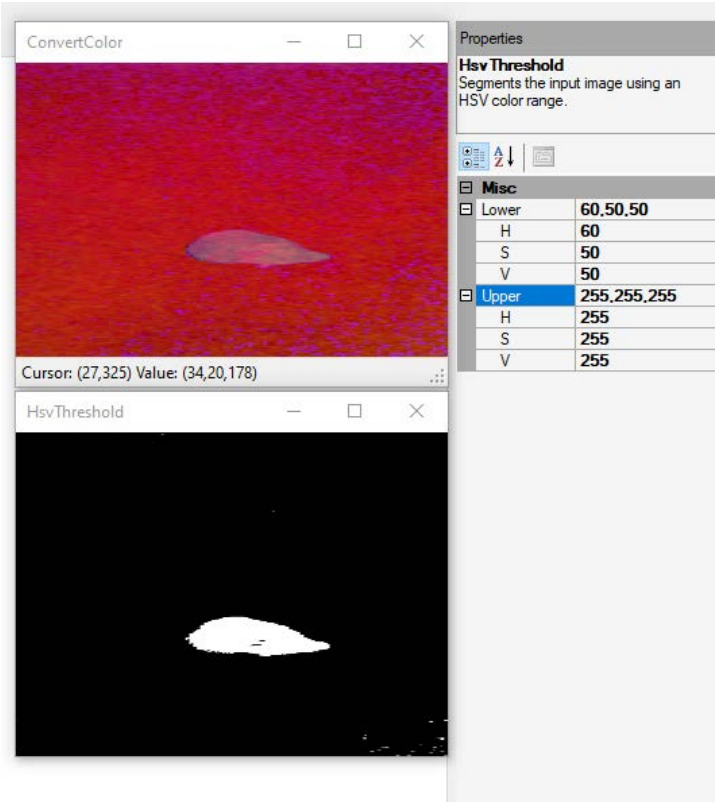
While the Bonsai code is running, click the "..." in the "Regions" value of the "CropPoly" node inside the "Chamber1" nested workflow. This will pop open a window for you to crop the video to only analyze the chamber that will trigger the laser. Left click and drag a rectangle around the region of interest, making sure not to have any extra clicks.



When completed, check the "Regions" value, press the "+" to extend to see your ROI values. Double check that there is only one labeled "[0]". Also double click the "CropPolygon" node to make sure that you see only the chamber that will trigger the laser.



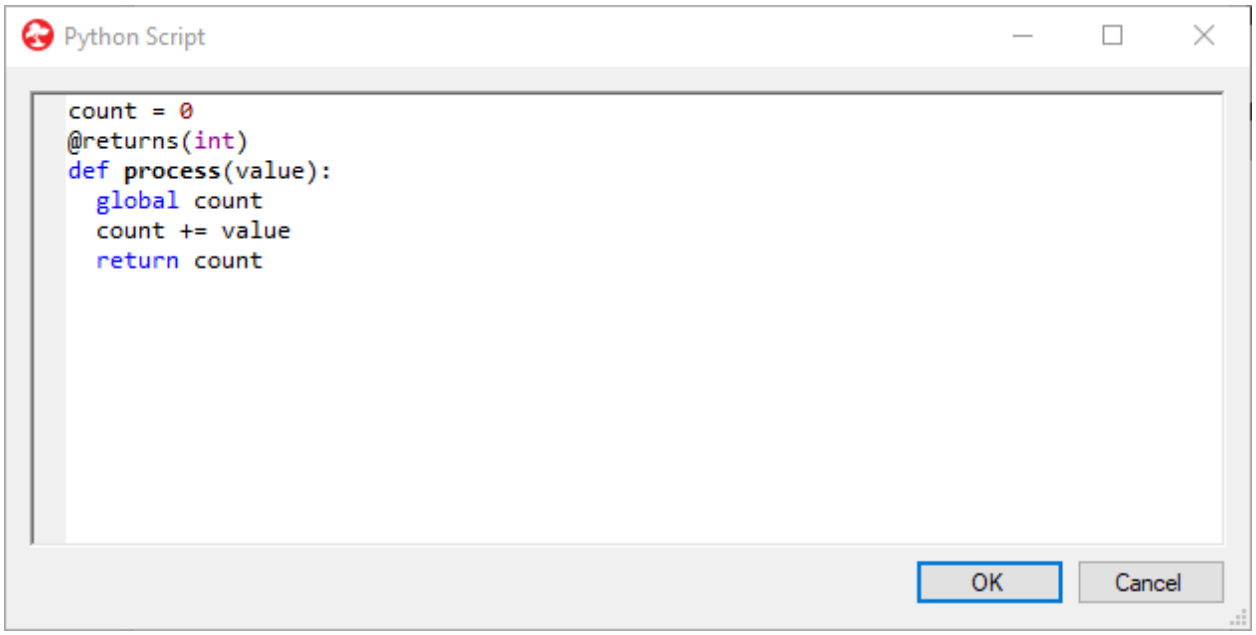
Now you must adjust the values of the "HsvThreshold" node in the "Chamber1" nested workflow. This is the node that is used to find the animal in your arena. It turns every pixel of the video feed inside the specified HSV range to white, and every pixel outside the HSV range to black. You want this to output an image that is all black except for the animal which is all white. While the Bonsai file is running, double click the "HsvThreshold" node to see what it is outputting, and change its values until you only see the animal in all white, with an all black background.
Note: if you are having issues finding the correct HSV values, double click the "ConvertColor" node while the Bonsai is running to see the video feed in HSV color domain. Then right click that "ConvertColor" popup window, and in the bottom right it will tell you the current HSV value of the pixel that your cursor is hovering over. This will help you know what HSV values correspond to the color of your animal and what HSV values correspond to the color of the arena/chamber.



Make these same changes to the "Chamber2" nested workflow. Then for the "FindCentroid" all you need to change is the "HsvThreshold" values, since there is no "CropPolygon" node. Note: The values of ALL of the "HsvThreshold" nodes will probably be quite similar, so you might be able to get away with simply copy and

pasting the values from the "HsvThreshold" node in the "Chamber1" nested workflow, to the other two "HsvThreshold" nodes.

One last node to setup is the “Python Transform” node within the “FP” nested workflow. This node works as a count and has a simple script shown below. Simply double click the “Python Transform” and enter the script below.



Once the code is all set up to track the animal position and which chamber it is in, go ahead and enable the "FP" nested workflow and double click the "FP3002" node to set the parameters for the V2 system (especially the “stimulation” parameters). Then go to all of the writers and specify the file names. When this is all set up you will save 4 files. The first contains the normal FP data from the "PhotometryWriter". The seconds contains the timestamp of every FP frame in Time of Day, Total Milliseconds as well as the frame count (this way you can synchronize the FP frames with the external camera frames). The third has the first column saying if the animal is in chamber 1, the second column saying if the animal is in chamber 2, the third column saying the x position of the animal, the fourth column saying the y position of the animal, and the fifth column saying the timestamp in Time of Day, Total Milliseconds. Finally, the fourth file is the raw .avi (video file) from the external camera so you can reanalyze it at a later date, should the need arise.

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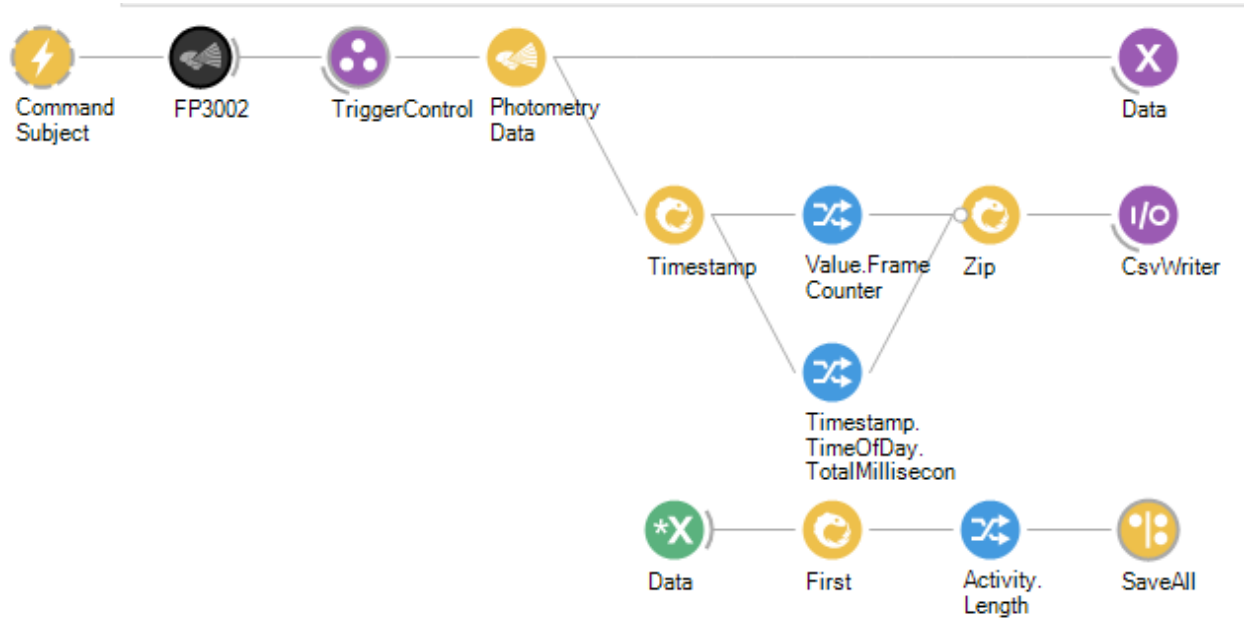
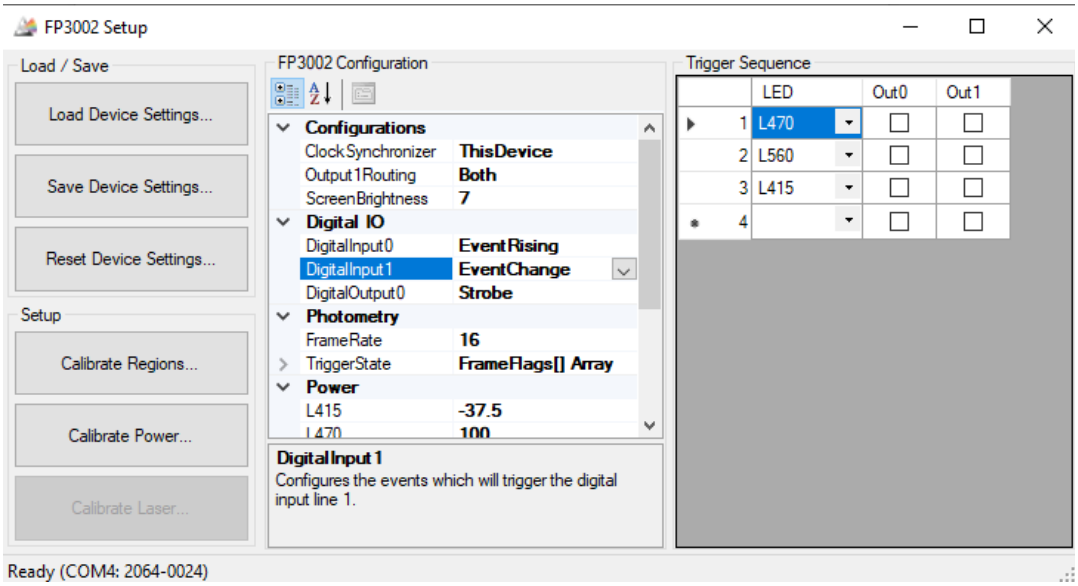
Bonsai: Start Data Acquisition On TTL Input

This document discusses a Bonsai Example Code that allows for an external TTL signal to control the data acquisition of the FP system. Using this method, the FP system will only collect data when a HIGH digital signal is present on the BNC Digital Input port.

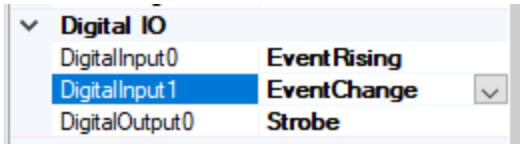


First, double check that your Bonsai Packages are up to date by clicking “Tools -> Manage Packages…”. This will close your current Bonsai window and open up the “Bonsai - Manage Packages” window. Check under the updates sections for any available Neurophotometrics packages that need to be updated.

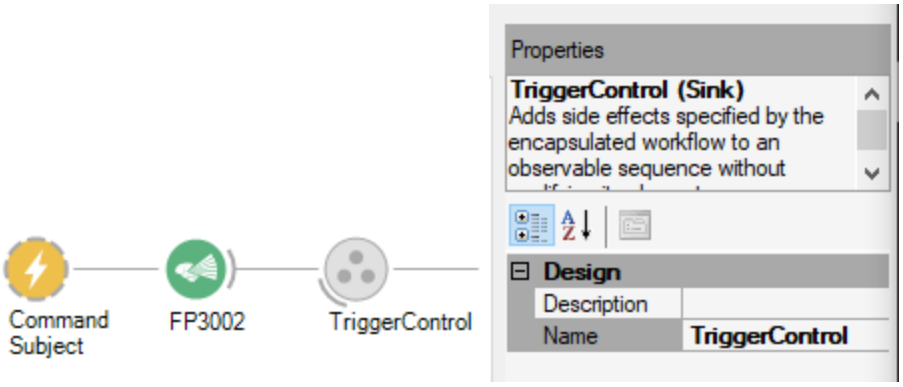
Once, all your packages are up to date, click the “close” button for the “Bonsai - Manage Packages” window, and your usually Bonsai window will appear. Starting with the “FP3002” node, specify the “PortName” value and double click the node to open the “FP3002 Setup” window.



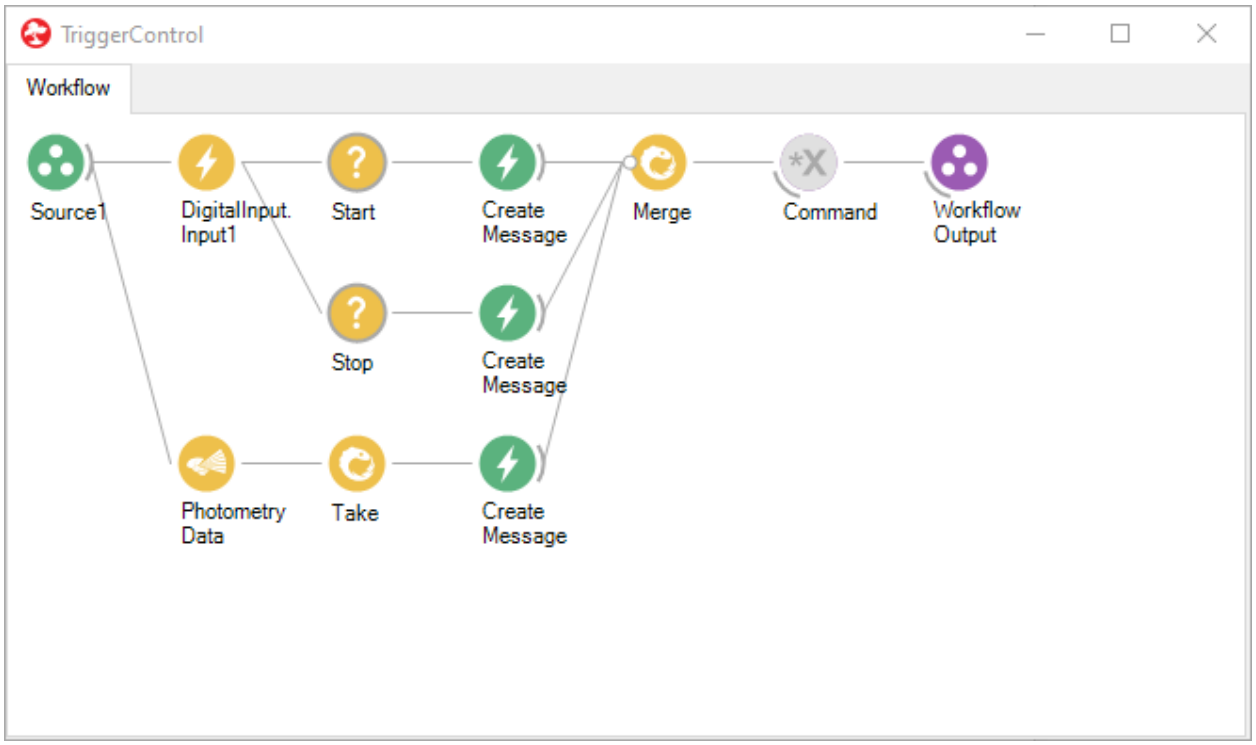
In the “FP3002 Configuration” section, find the “Digital IO” menu. Change the value for “DigitalInput1” to “EventChange”.



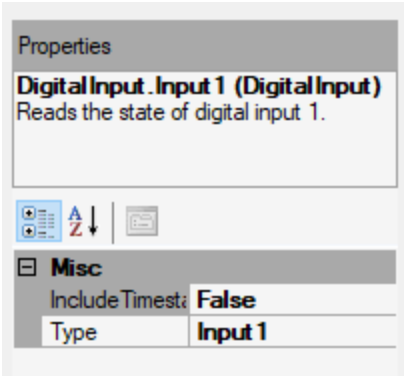
Then we put a “Command Subject” node before the “FP3002” node, and a “Sink” node after, which we name “TriggerControl”



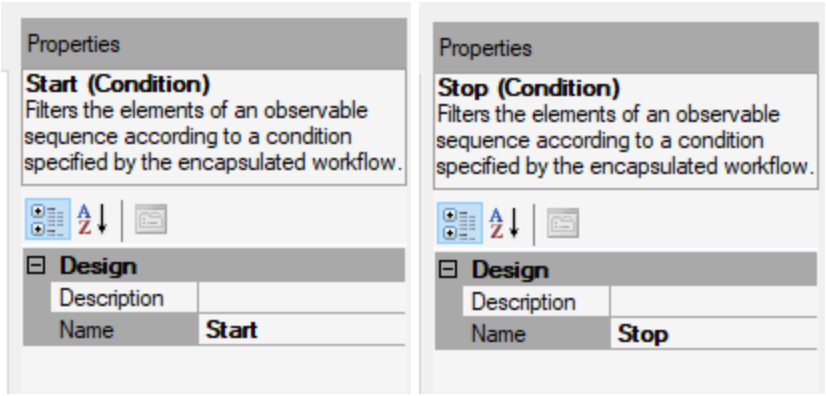
Now we must configure the “Trigger Control” node as shown below.



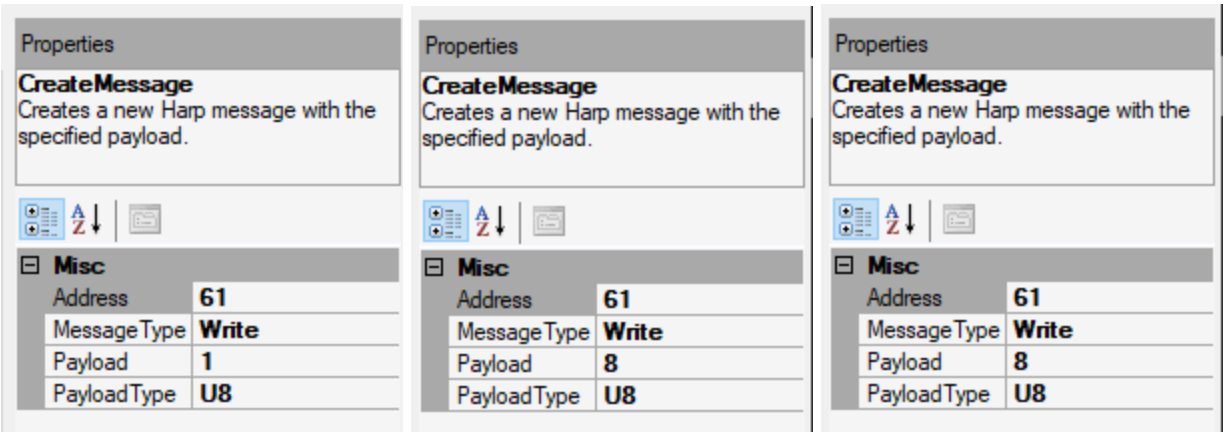
The “DigitalInput.Input1” node is the “Digital Input (Neurophotometrics)” node with the “Type” value as “Input 1”.



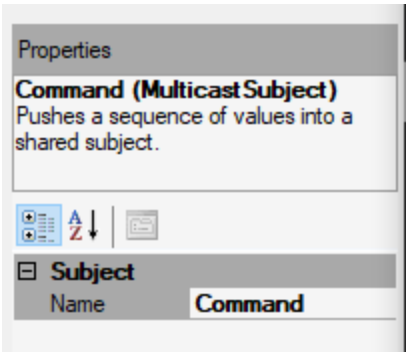
The “Start” and “Stop” nodes are “Condition” nodes with “Start” and “Stop” values for the “Name”.



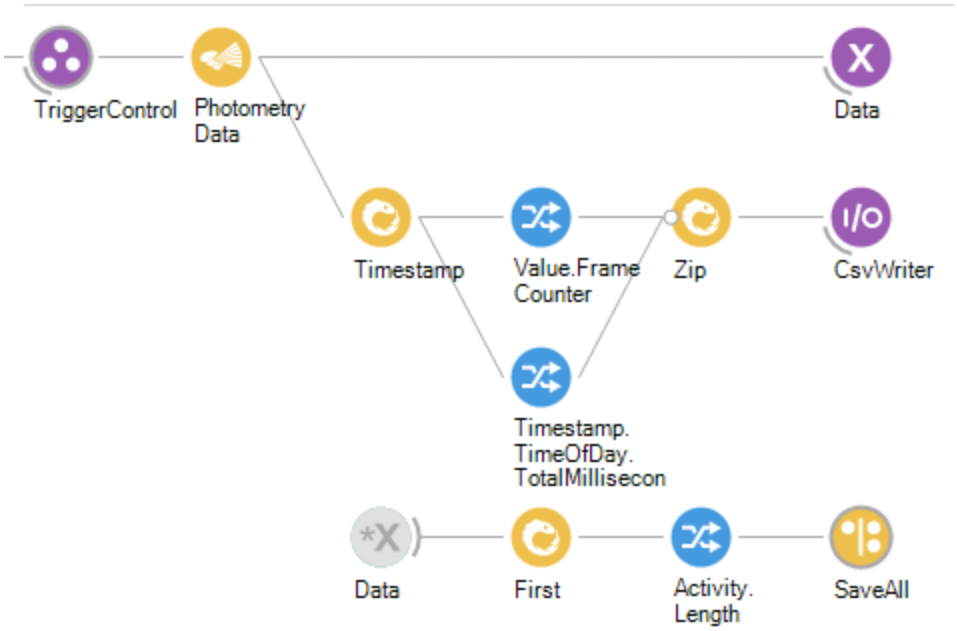
Below are the values you must specify for each “Create Message” node after the “Start”, “Stop” and “Take” nodes (in that order).



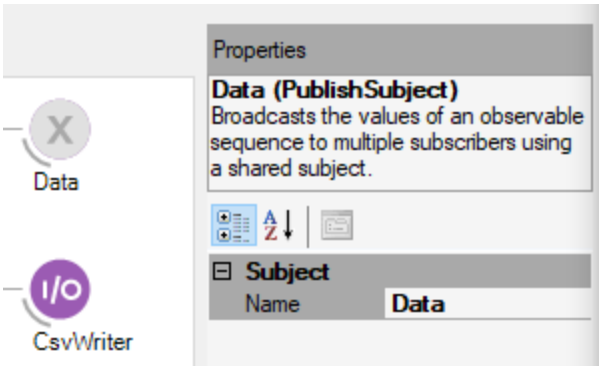
After the “Merge” node is a “MulticastSubject” node with the “Name” value as “Command”.



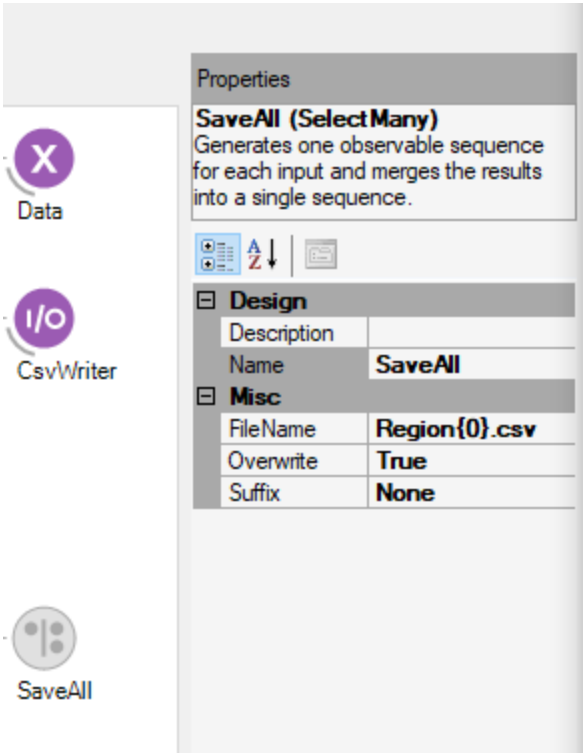
Now that the “TriggerControl” node is configured, we can move on to the second half of the program shown below.



After the “Photometry Data” we create a “Publish Subject” node and name it “Data”.

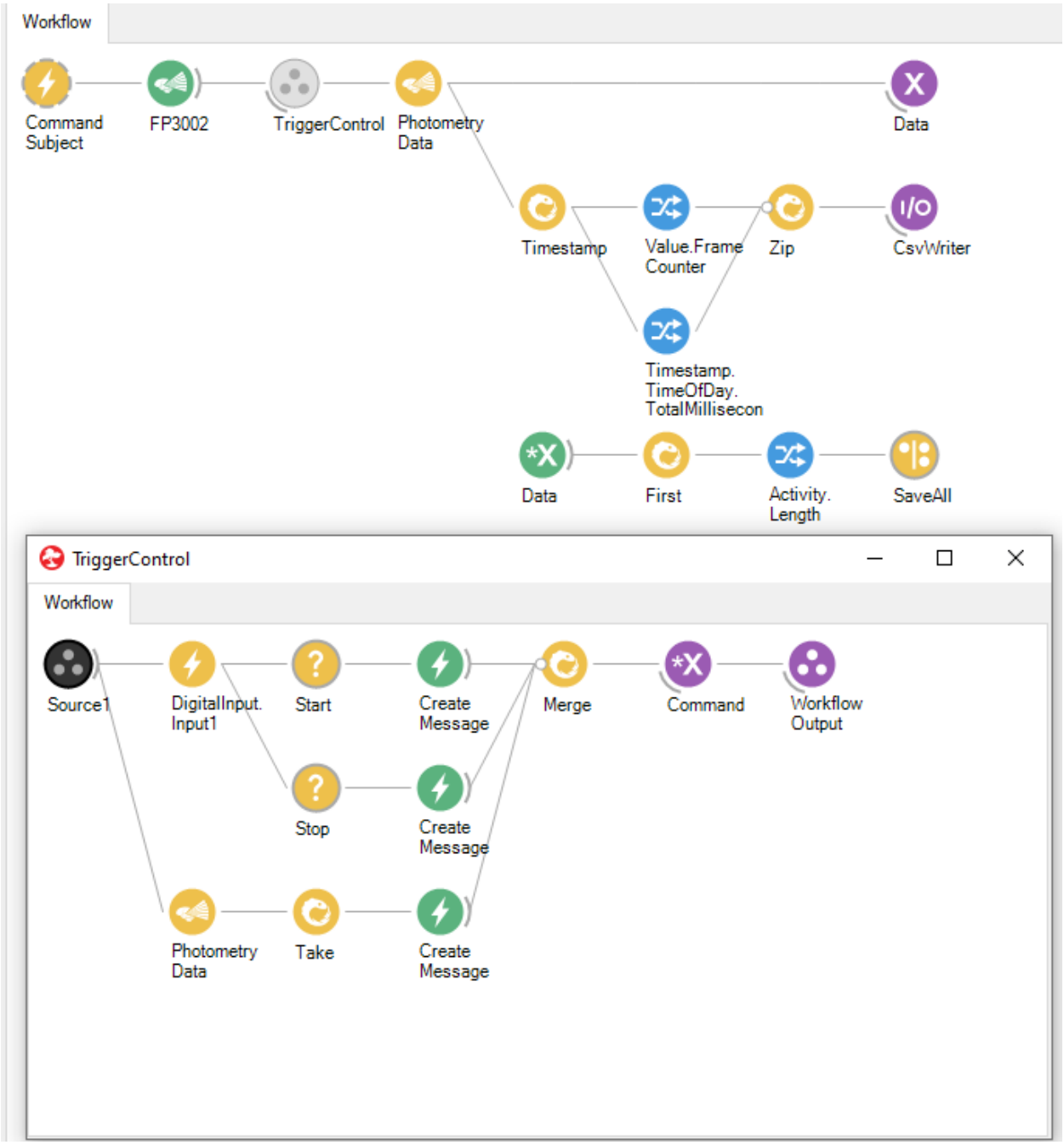


Then in a separate data stream we make a “Subscribe Subject” with the exact same name of “Data”. After the “Subscribe Subject” is the “First” node, then right click the “First” node and navigate to “Output -> Activity -> Length”. End the datastream with a “Select Many” node with the values below.



All that is left is the “Timestamp” datastream coming from the “Photometry Data” node. Where we “Zip” the frame count and Time of Day, Total Milliseconds outputs from the “Timestamp” node and save to .csv.

Once the Bonsai code is configured then connect your TLL signal to the BNC Digital Input 1 port of the system. Once you press “Start” in the Bonsai code, the system will only acquire data while there is a HIGH signal on the TTL.



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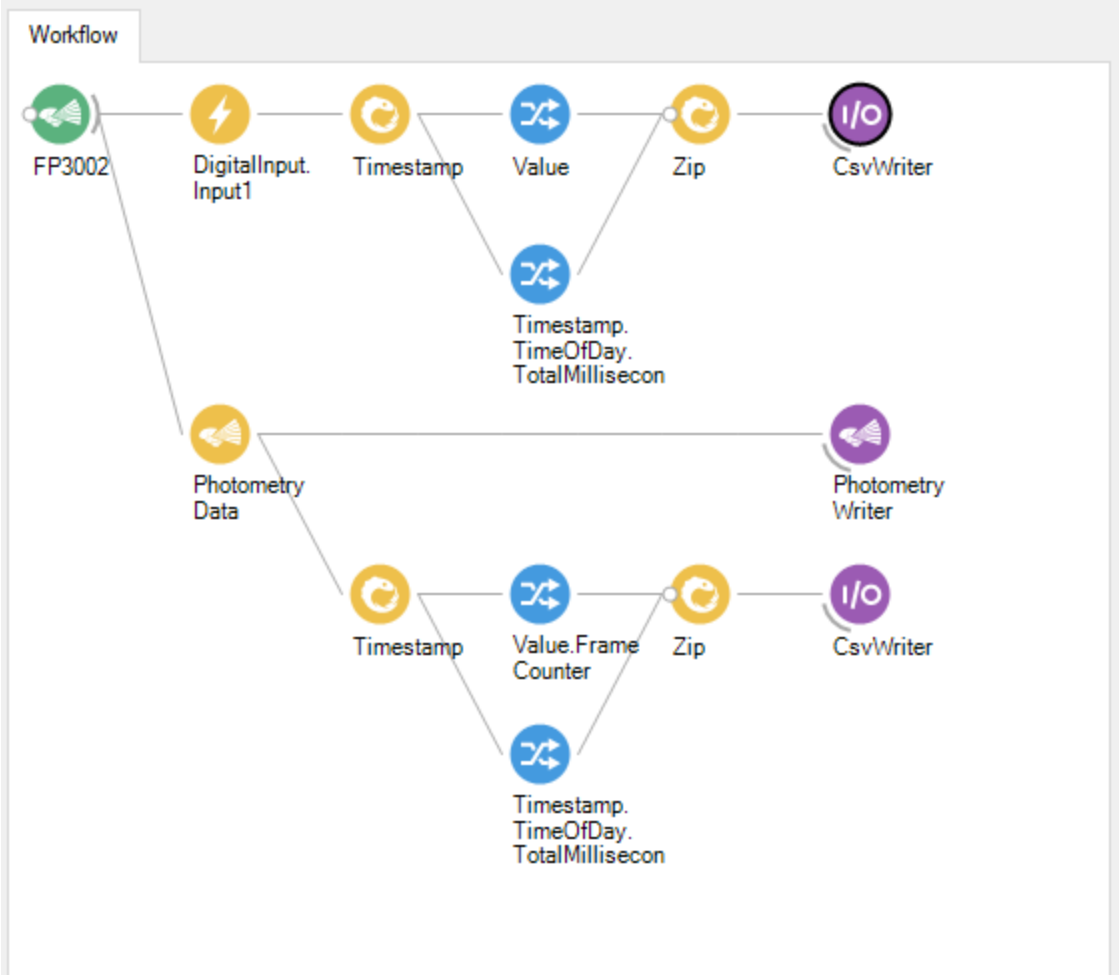
Bonsai: TTL Synchronization

This document discusses a Bonsai Example Code that allows for the timestamps of the FP system to synchronize with external devices via a TTL signal. We attach a BNC cable carrying the TTL signal from another device to the Digital Input 1 of the FP system. Then we can timestamp that TTL signal and the FP data frames using the same clock.



The Bonsai code above has two data streams coming from the “FP3002” node. The top data stream timestamps the TTL signal coming into the Digital Input 1 of the FP system using the Time of Day, Total Milliseconds method.

The bottom data stream uses the “Photometry Writer” node to save the FP data. However, this node timestamps using the FP system’s internal clock, while the Time of Day, Total Milliseconds method timestamps using the computer’s internal clock. So, we also use a “CsvWriter” node to save the timestamps of each FP data frame using the computer’s clock. This way the timestamps of the TTL signal and FP data are aligned. You can take this method of timestamping further by timestamping any datastream using the Time of Day, Total Milliseconds (i.e. “Video Device Capture”, “Analog Input (Arduino)”, etc.). Bringing in all of the experimental data to Bonsai and using this timestamping method allows for all the data sets to be timestamped using the same clock, causing the data to be aligned.

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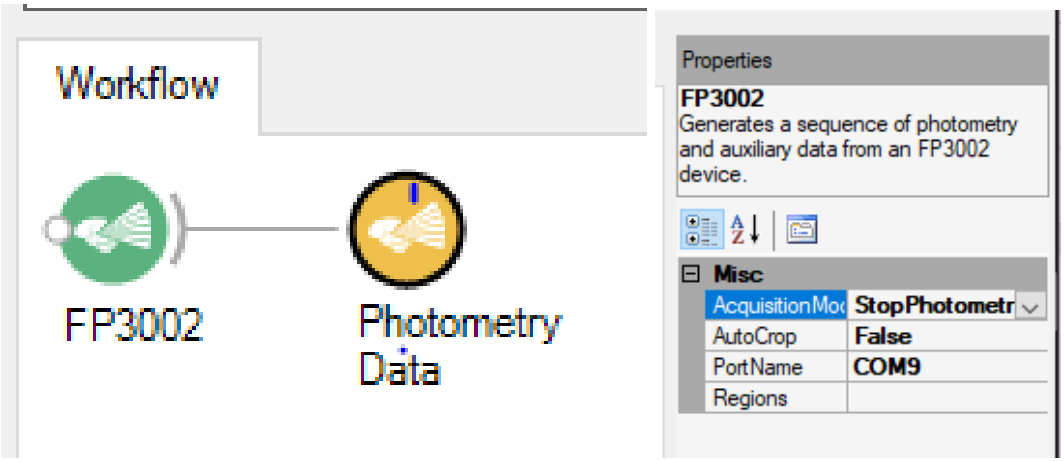
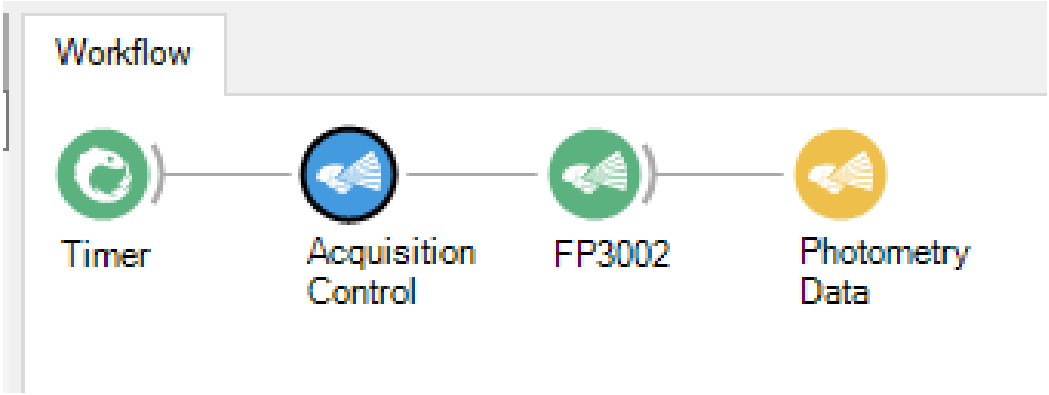
Bonsai: Timer Delayed Data Acquisition

This document discusses a Bonsai Example Code that allows for photometry data acquisition to be delayed by a specified amount of time. Starting with a basic setup of using a “FP3002” node connected to a “Photometry Data”node, specify the “PortName” and set the “AcquisitionMode” to “StopPhotometry”. This current setup will not acquire photometry data until it is commanded to do so.

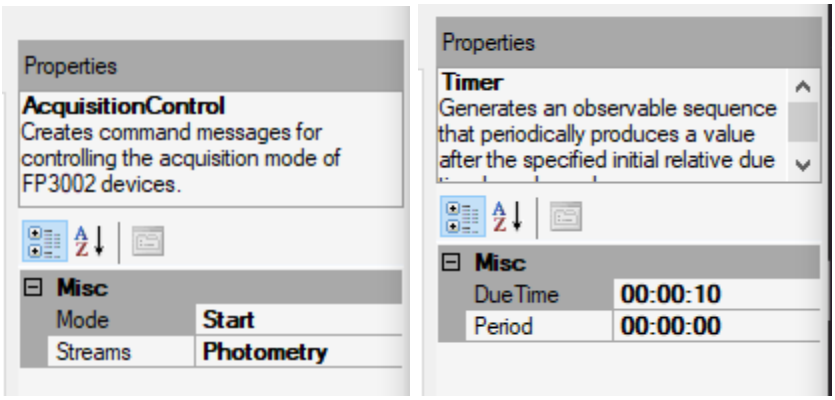


To command the “FP3002” node to “StartPhotometry” after a specified amount of time, we must use a “Timer” node connected to an “AcquisitionControl” node.



Now, we want the “Acquisition Control” node to command the “FP3002” node to “Start Photometry” after a specified amount of time. So in the “Acquisition Control” properties menu, set the “Mode” to “Start” and the “Streams” to “Photometry”. This means that

once the “Acquisition Control” node is passed a value (almost any value), then it will command the “FP3002” node to start collecting photometry data. To have this occur after a specified amount of time, we simply set the “Due Time” value of the “Timer” node to the amount of time we want. In the case below, we start acquiring photometry data 10 seconds after we click start on the Bonsai program.



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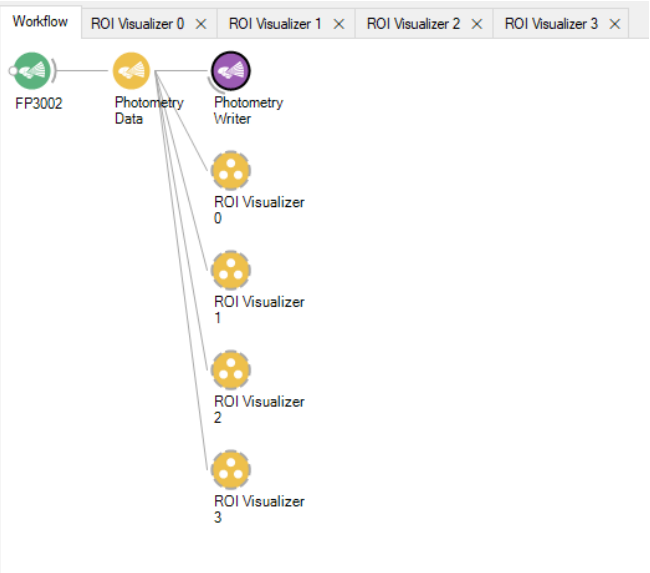
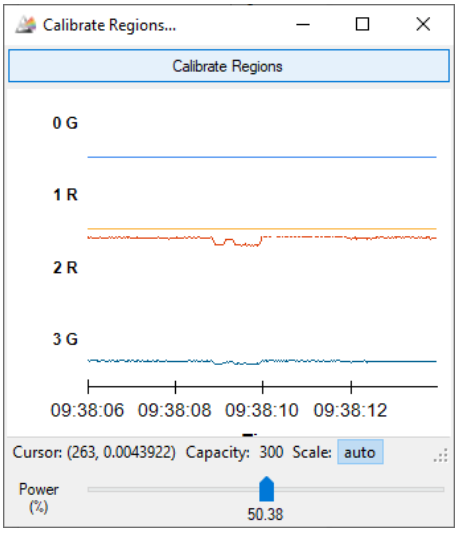
Bonsai: ROI Visualizers

This document discusses a Bonsai Example Code allows the user to separate the ROI data such that the data for each ROI is on a separate visualizer graph.

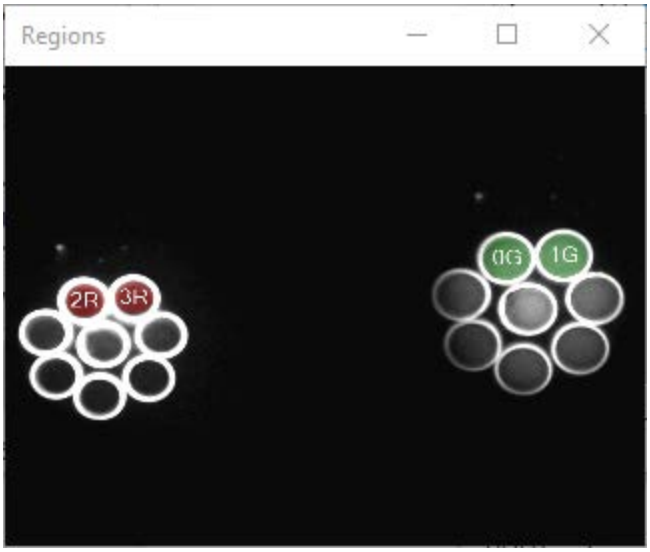


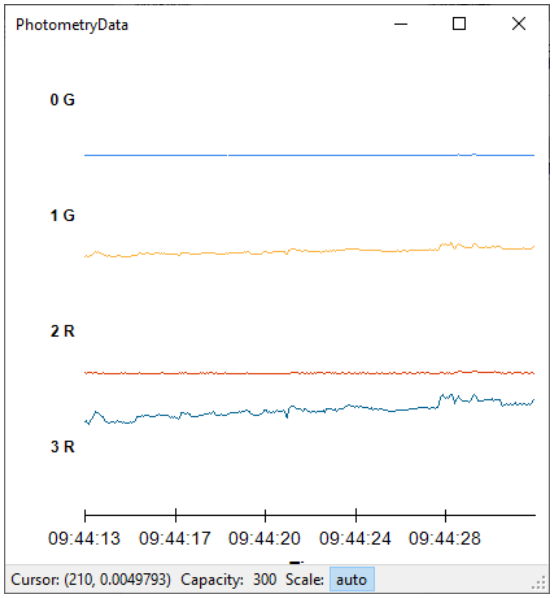
Setting up our ROIs, double click the “FP3002” node to open up the “FP3002 Setup” window. Then click the “Calibrate Regions”, to open the “Calibrate Regions…” window, slide the “Power (%)” bar up.

Now we can click the “Calibrate Regions” button in this window to open the “Regions” window. Here you want to click and drag to create oval ROIs in the cores of your patch cord.

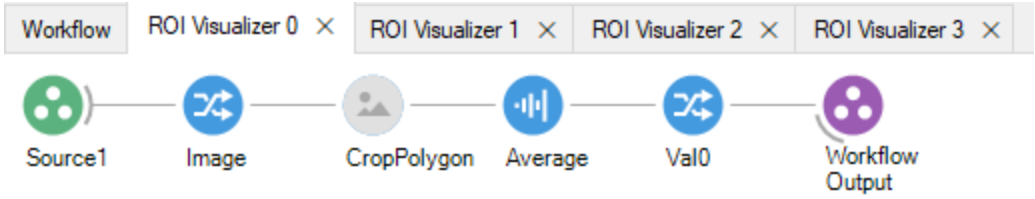


Now, when we click “Start” on the Bonsai file, and double click the “Photometry Data” node to open its visualizer window, we see that all of the ROIs are on one plot. This is normal, but below is a method for how to separate each of these ROI plots into their own windows.

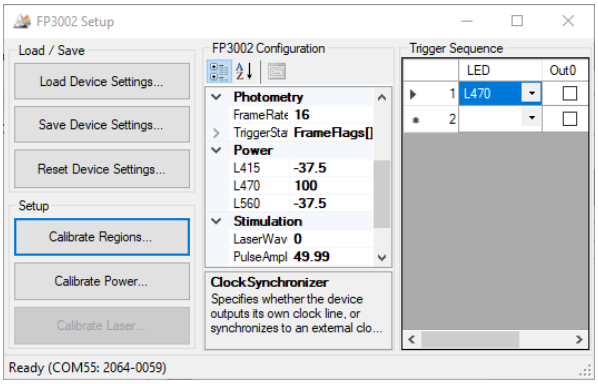




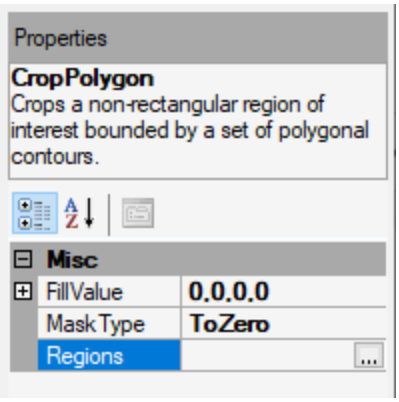
Using the “ROI Visualizer” nested workflows, we can approximately recreate what the “FP3002” and “Photometry Data” nodes do to acquire data from the ROIs. Enter the “ROI Visualizer” nested workflows by double clicking each of them. The only thing that needs edited in these nested workflows are the “Crop Polygon” nodes.

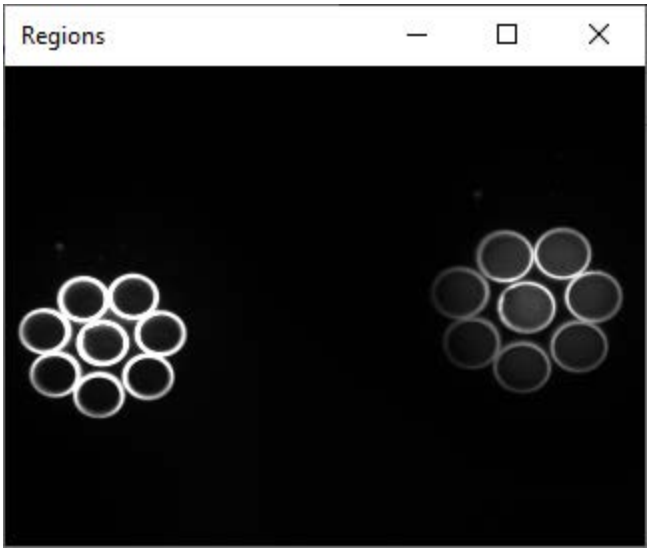


The “Crop Polygon” nodes are going to crop the incoming images to your ROIs, however, we will need light for this to be doable. So open up the “FP3002 Setup window, and under “Power”, change the L470 value to 100. In the “Trigger Sequence” menu, delete the “L560” and “L415” rows as shown below.

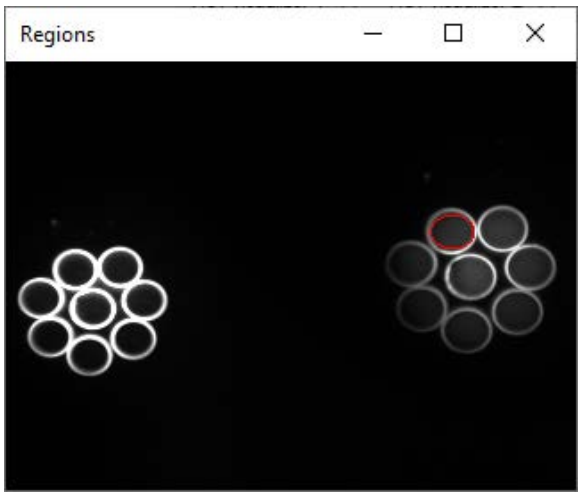


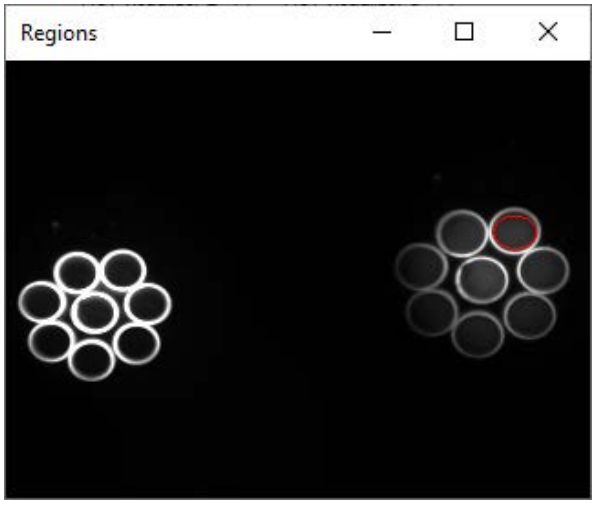
Now, click “Start” to run the Bonsai file, navigate inside an “ROI Visualizer” nested workflow, and click on the “Crop Polygon” node. Click on the regions value under “Properties” and click the “...”
symbol to open up the “Regions” window.

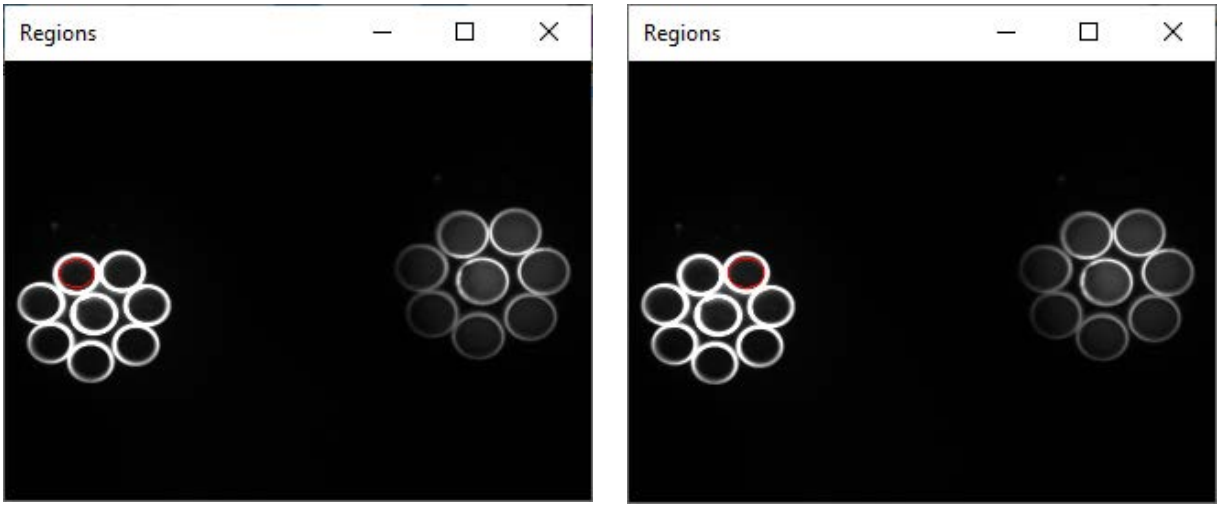




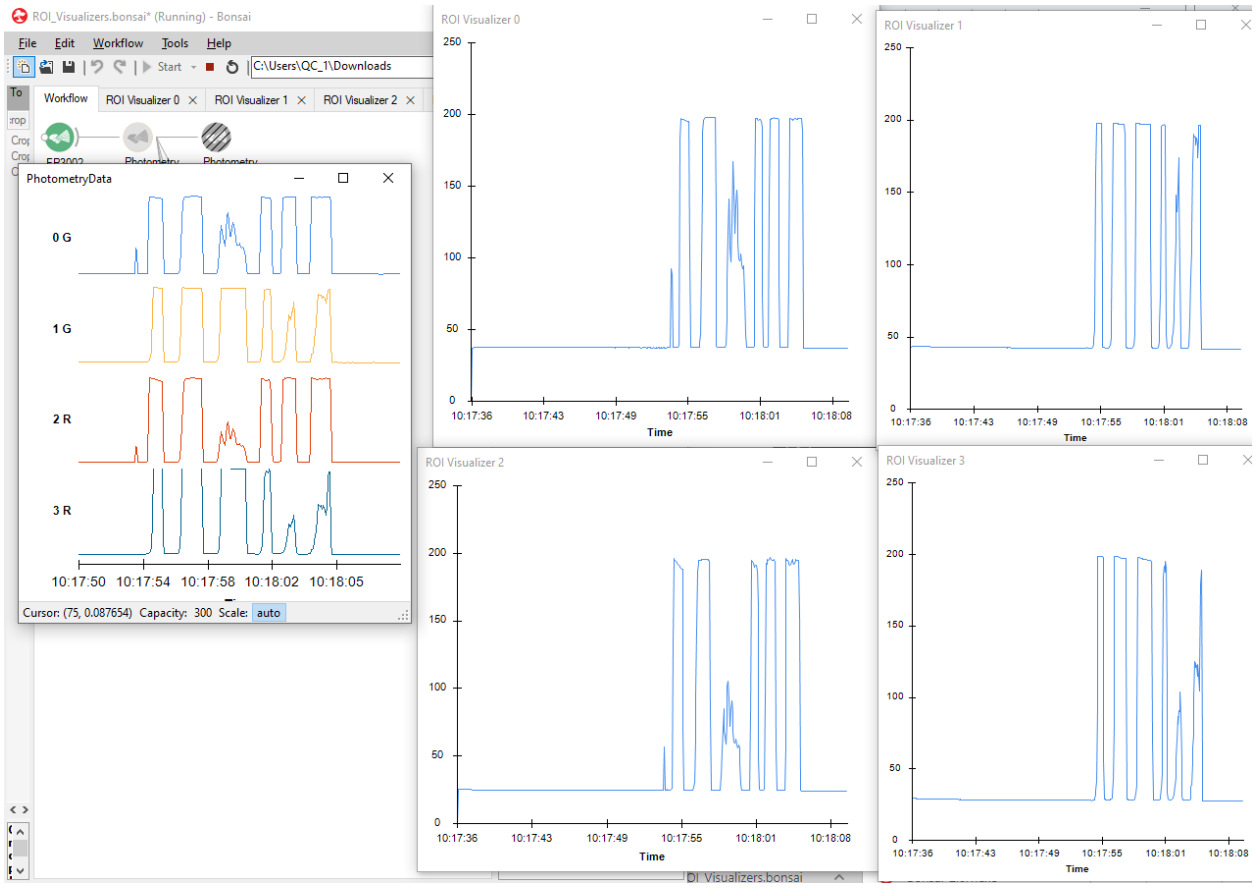
We see the same “Regions” windows from before when we were setting up our ROIs for the “FP3002” node. Now we need to recreate each ROI, one ROI per “ROI Visualizer” node. To draw these as ovals, you must Shift Click and drag to size.







Once all of the “Crop Polygons” are setup properly we can see the effect of this by double clicking each “ROI Visualizer” while the Bonsai file is running:



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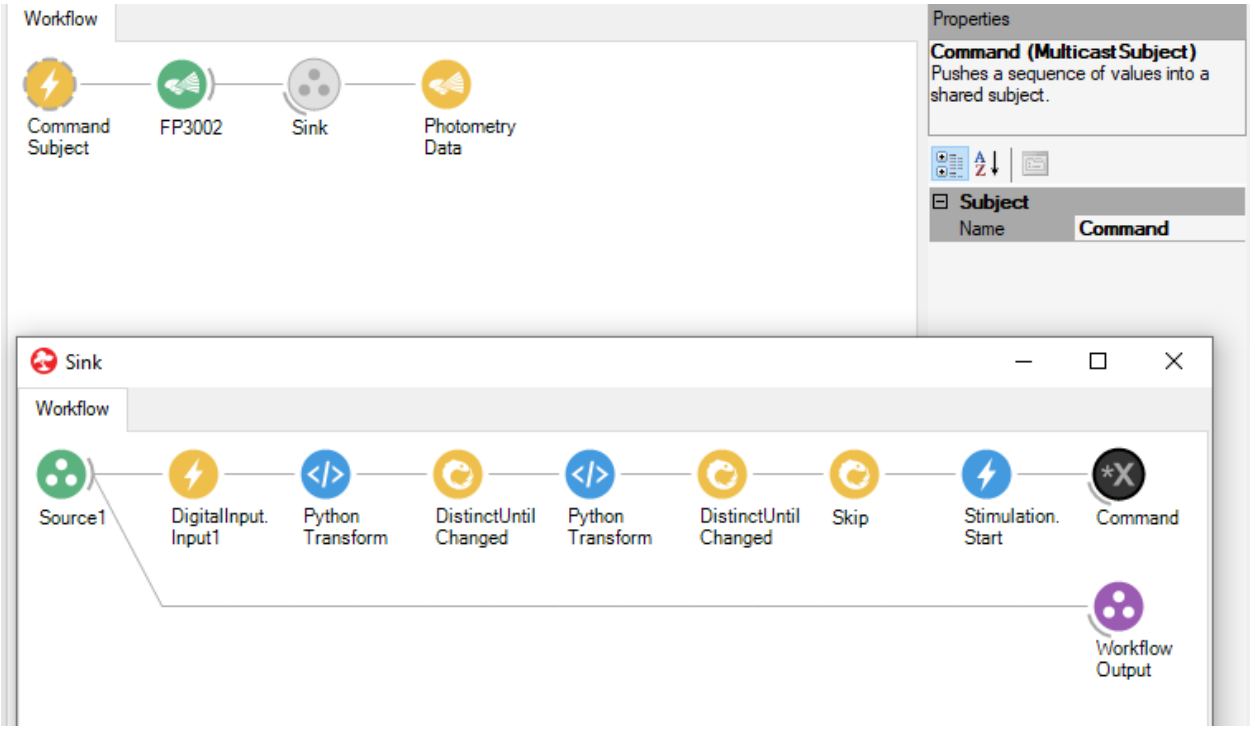
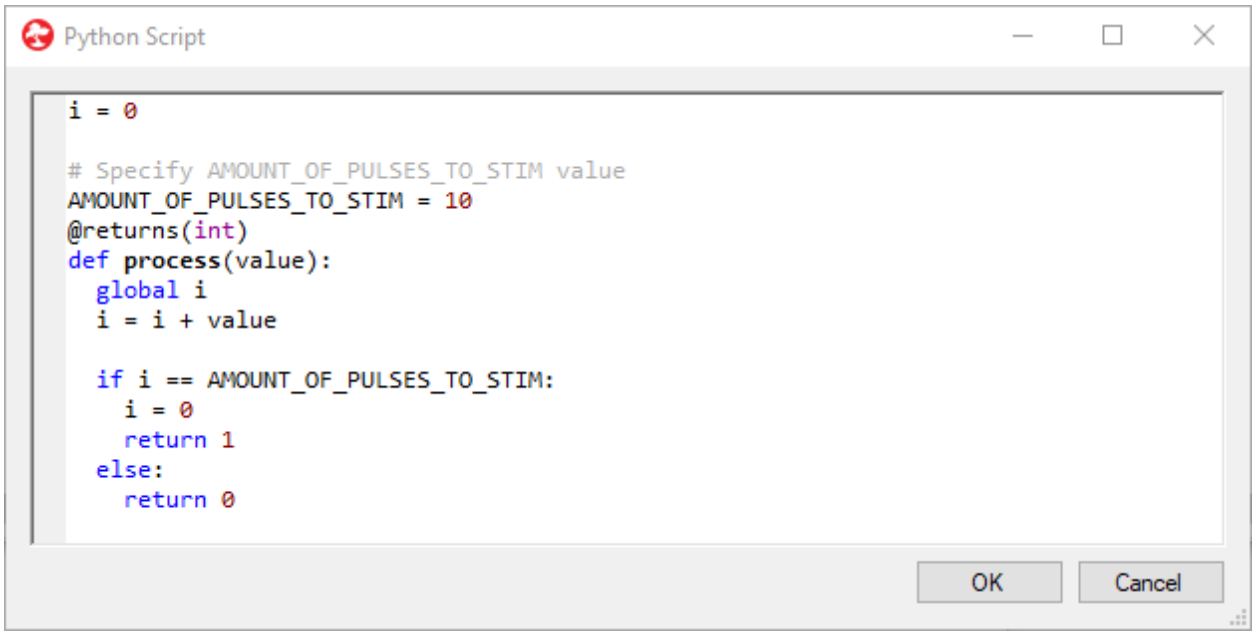
Bonsai: Start Stimulation After "X" TTL Pulses

This document discusses a method for activating the internal laser after X number of pulses from a TTL input.

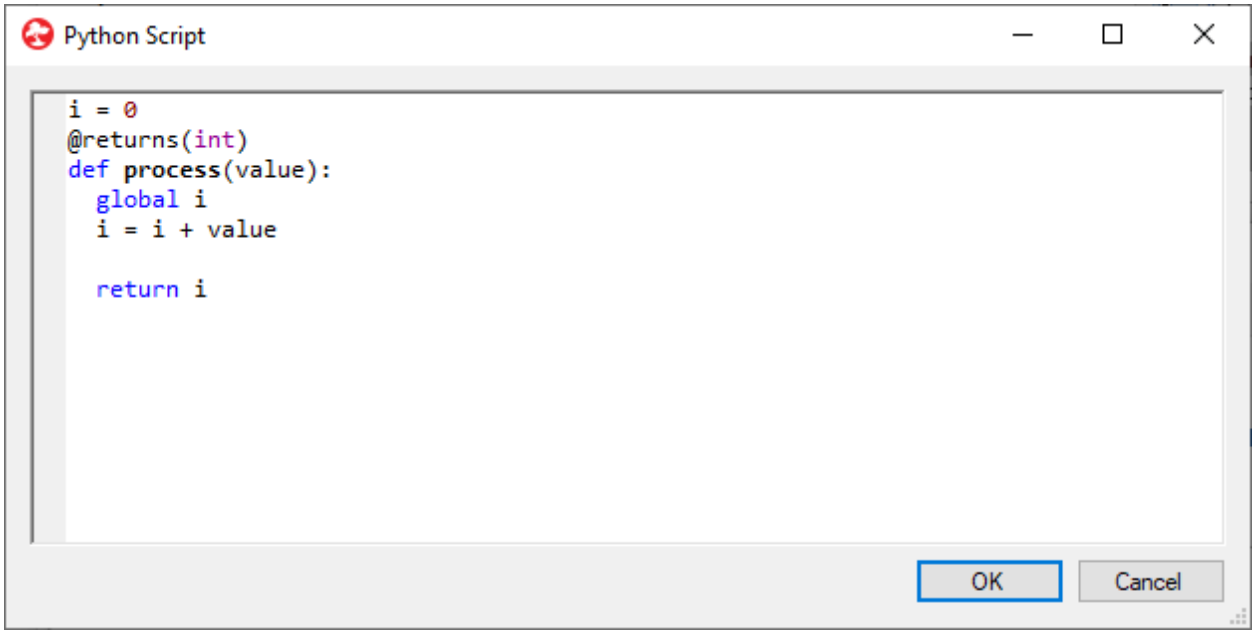


We start our workflow with a "Command Subject" node connected to a "FP3002" node and a "Sink" node. This is the basic setup for any feedback related Bonsai code. We will use the "Sink" to send a command back to the "Command Subject" node and thus back to the "FP3002" node. Within the "Sink" node we take in the "Digital Input. Input 1" value which reads the TTL signal on the Digital Input 1 port of the System. Then alternate between "Python Transform" and "Distinct Until Changed" nodes to format the data stream so that the "Stimulation. Start" node sends commands to start stimulation at the appropriate times.
The first "Python Transform" node contains the script below which counts how many TTL pulses have been read, returning a "1" when it has counted the desired number of pulses and "0" otherwise.



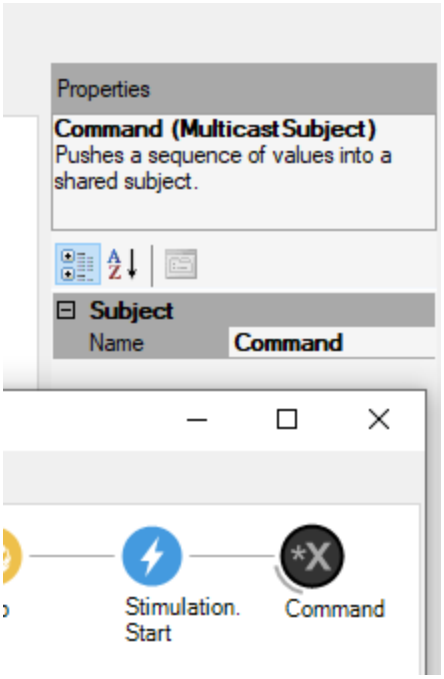
The first “Distinct Until Changed” will then only output a value when it changes, giving you a datastream of 0, 1, 0, 1, …, 0, 1, instead of “X” amount of zeros between each one.

Keep in mind that the “Command” node, in this case, the “Stimulation. Start” node, sends its command every time a value is passed to it. So we want to only have the “1”s being passed to it. So we chain another “Python Transform” counter node in combination with another “Distinct Until Changed” node so the value only changes when a “1” is passed, meaning that a value is only passed from the “Distinct Until Changed” node to the “Stimulation. Start” node when “X” amount of TTL pulses have been read. Below is the script used for the second “Python Transform” node.



We include a “Skip” node with a “Count” value of “1” so that the first value passed from the second “Distinct Until Changed” node is skipped, otherwise the laser will trigger its first laser pulse train immediately on start. With the “Skip” node, the first laser pulse train does not occur until the first “X” number of TTL pulses. Then the laser stimulation re-triggers after another “X” number of TTL pulses, repeating.

After the “Stimulation.Start” node, we use a “Multicast Subject” node with a “Name” value of “Command” to send the stimulation command to the “Command Subject” node.



Finally, double click the “FP3002” node to open the “FP3002 Setup” window. Double check that the “Digital Input 1” value is set to “Event Rising”, otherwise the logic in our “Sink” node will need to be changed. Then specify the “Stimulation” settings.

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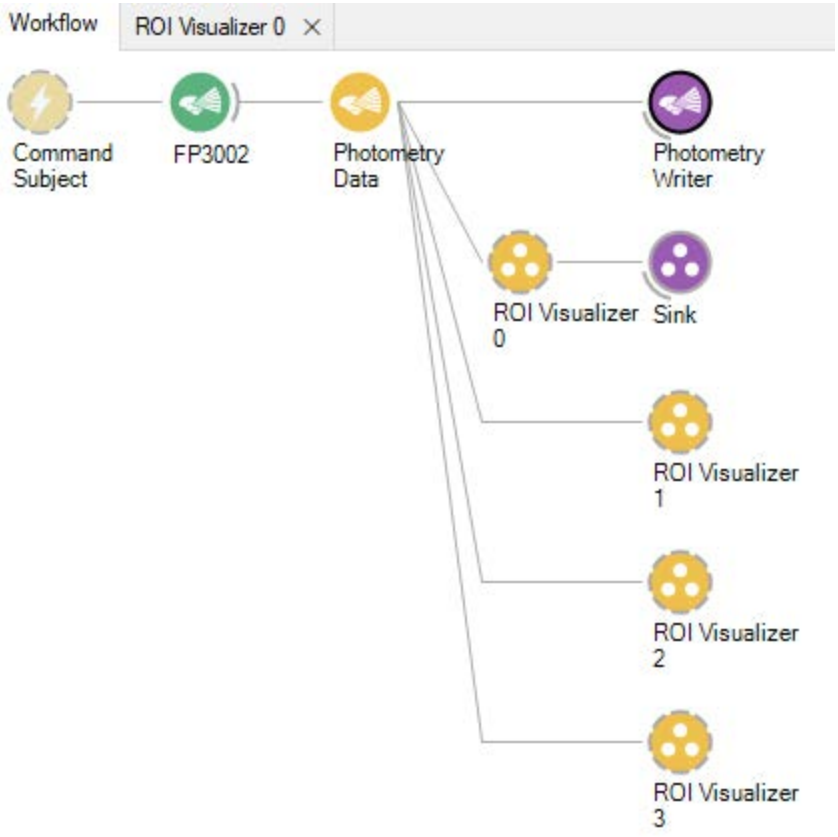
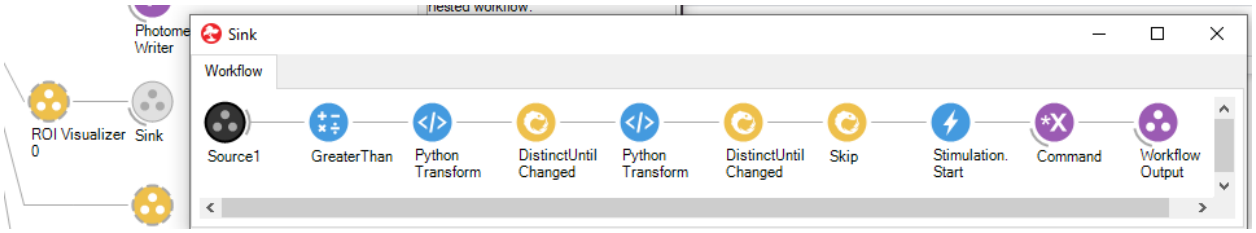
Bonsai: Using Real-Time FP Data to Trigger Laser Stimulation

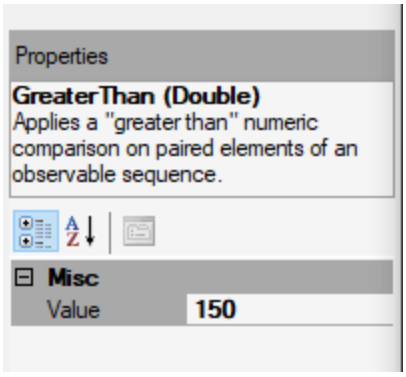
This document discusses how to use spikes in the FP data to trigger laser stimulation. We create a feedback loop in Bonsai that can send a “Start Stimulation” command to the “FP3002” node when FP data from one or more of the ROIs rise above a certain threshold.



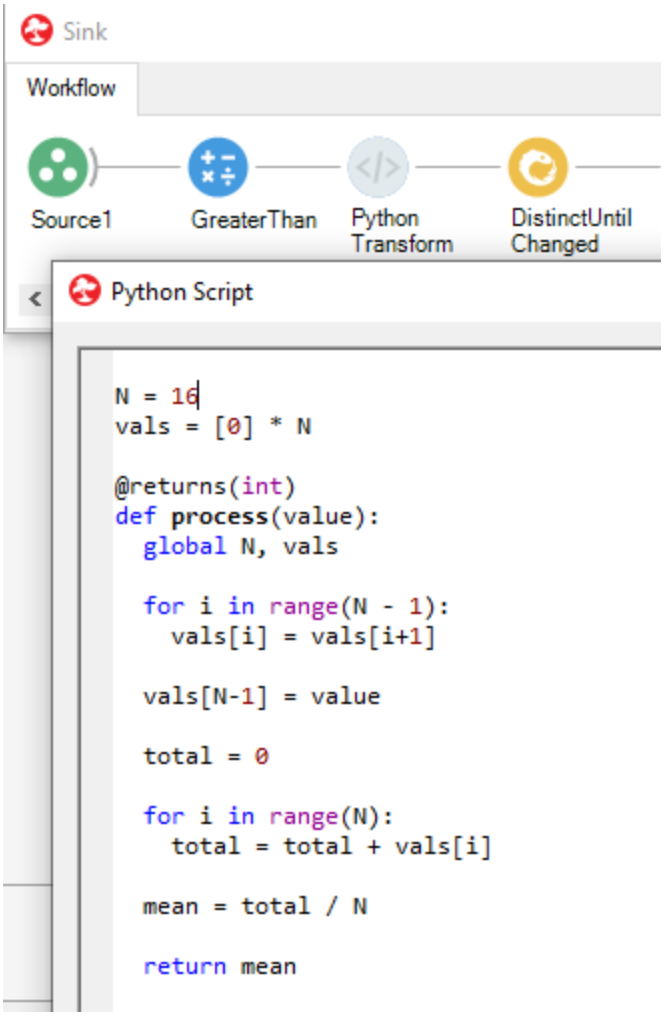
First, we must properly set up our ROIs in the “FP3002” node and within the “ROI Visualizer” nested workflows. Please read the “ROI Visualizers” documentation on how to set these up. Once the ROIs and ROI visualizers are properly implemented, we choose which ROI to use as a trigger for the laser stimulation. In the example case, we use ROI 0 to trigger the stimulation by connecting it to a “Sink” node.



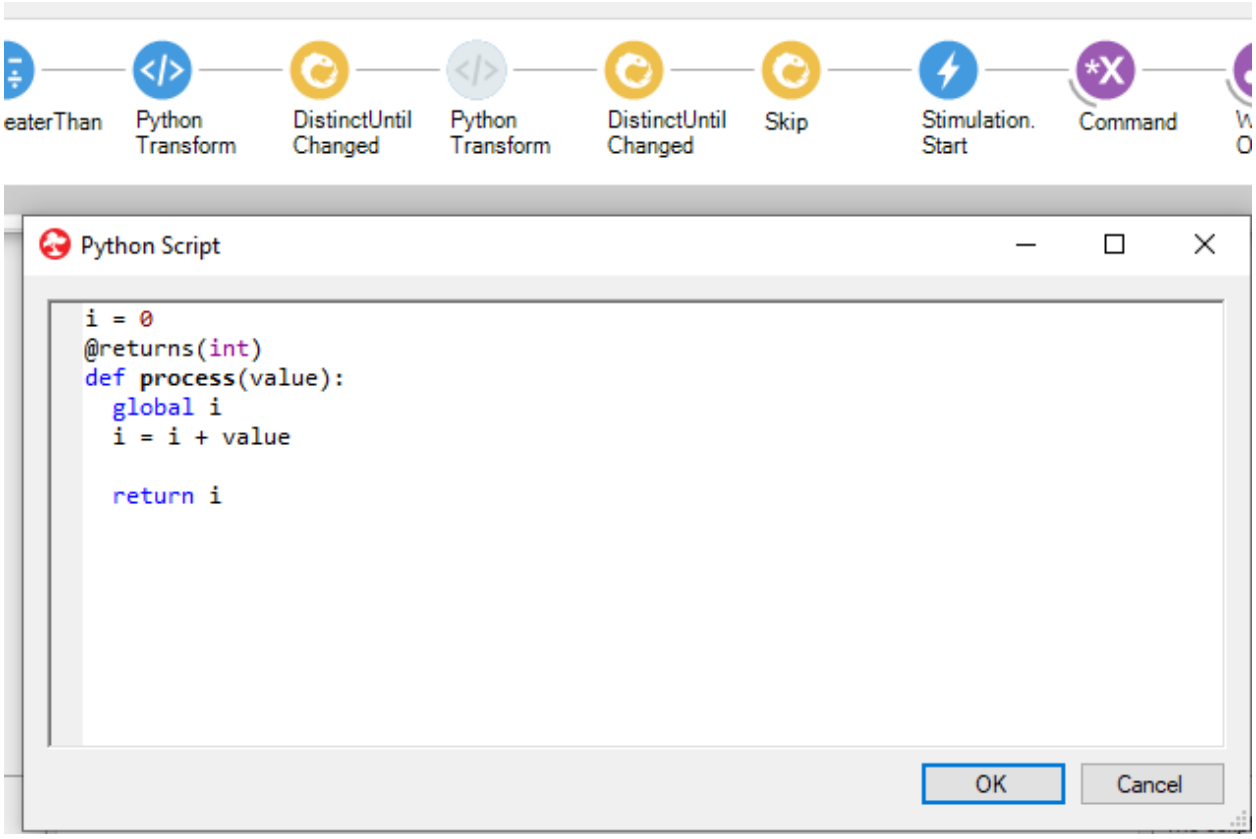
The “ROI Visualizer 0” node sends the raw FP data from ROI 0 into the “Sink” nested workflow shown above. The “Greater Than” node thresholds the raw value, outputting a “1” when the FP data is above the threshold, and a “0” when below the threshold. In the example case we want values above 150 to trigger the laser, change the “Value” value of the “Greater Than” node to your desired threshold.



Since brain activity is spontaneous and the noisy signal can occasionally jump above a threshold without a neural event, we use a “Python Transform” node to keep track of the last “N” FP data values and we only stimulate if all of the previous “N” values are above the threshold. In the example code, we only trigger laser stimulation after “N” successive values are above the specified threshold. To change this, double click the “Python Transform” node to open up the “Python Script” window and change the value of “N” to your desired amount of samples. This first “Python Transform” node will return a “1” when ALL of the previous “N” values are above the threshold and a “0” otherwise.

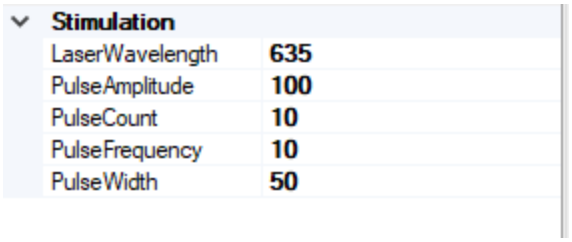


This allows us to use a similar counter method as in the “Starting Stimulation After X TTL Pulses” document, to format the data so that the “Stimulation. Start” node only sends a command when “1”s are passed through to it. So we follow the first “Python Transform” node with a “Distinct Until Changed” node so that we have a data stream in the form of 0, 1, 0, 1, ... , 0, 1, instead of having an arbitrary amount of zeros in between each 1. Next comes the seconds “Python Transform” node that acts as a counter adding each successive value being passed through it as shown below.



Since the value coming out of the “Python Transform” node only changes when a “1” is passed through it (i.e. when there is a spike in FP data) then we can use a “Distinct Until Changed” node so that a value is only passed to the “Stimulation. Start” node when there is a spike in FP data. We include a “Skip” node with a “Count” value of “1” so that the laser does not trigger immediately upon start. We use a “Multicast Subject” node with a “Name” value of “Command” to send the “Stimulation. Start” command back to the “Command Subject” node so that the “FP3002” node knows to begin stimulation. Ultimately, this code allows us to trigger the stimulation after a spike in FP data. One can expand upon this workflow by including more sophisticated event finding algorithms in the first “Python Transform” node.

Once the threshold and number of samples have been specified, then all that is left is setting up the “Stimulation” settings inside of the “FP3002” node.



Now, once the Bonsai code is started, spikes in the FP data will trigger the laser stimulation. To expand this technique, one can implement more sophisticated real-time event finding algorithms within the “Python Transform” node such as a calibrated running-average or curve-finding techniques.

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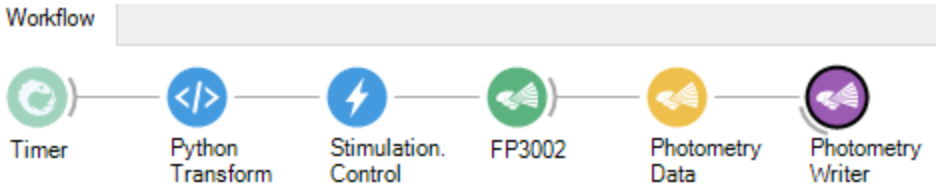
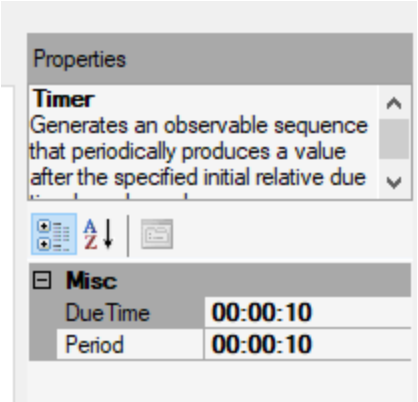
Bonsai: Timer Controlled Internal Laser Stimulation

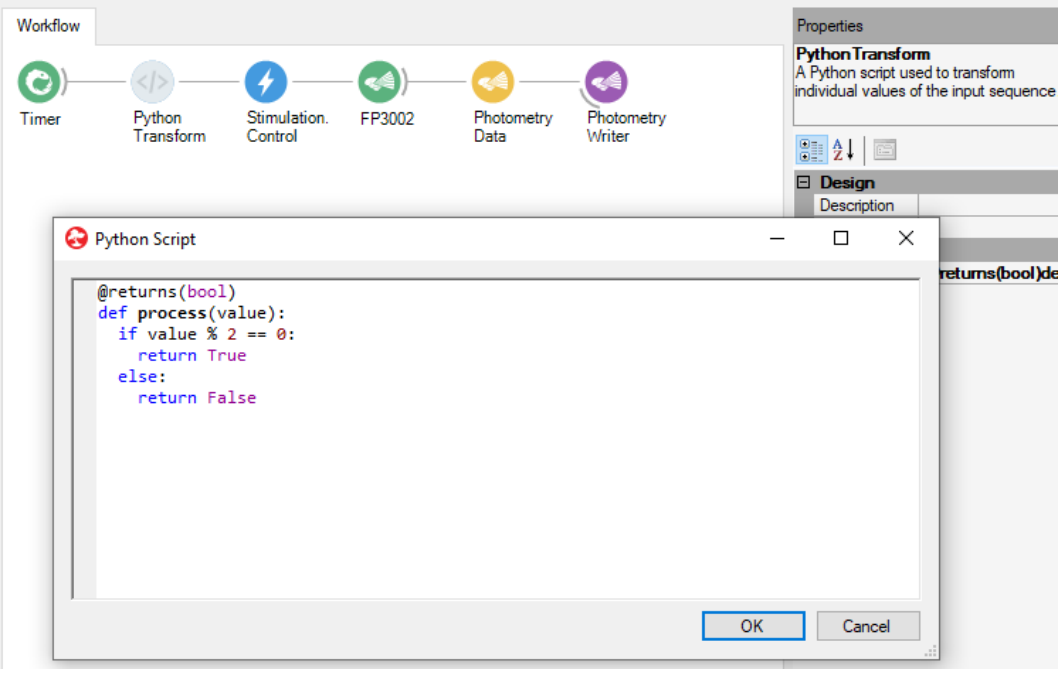
This document discusses how to use a timer to control the triggering of laser stimulation. For instance, when implementing this code, the laser will start OFF, wait “X” amount of seconds, pulse according to the “Stimulation” settings for “X” amount of seconds, turn OFF for “X” amount of seconds, and repeat in this manner until the code is stopped.



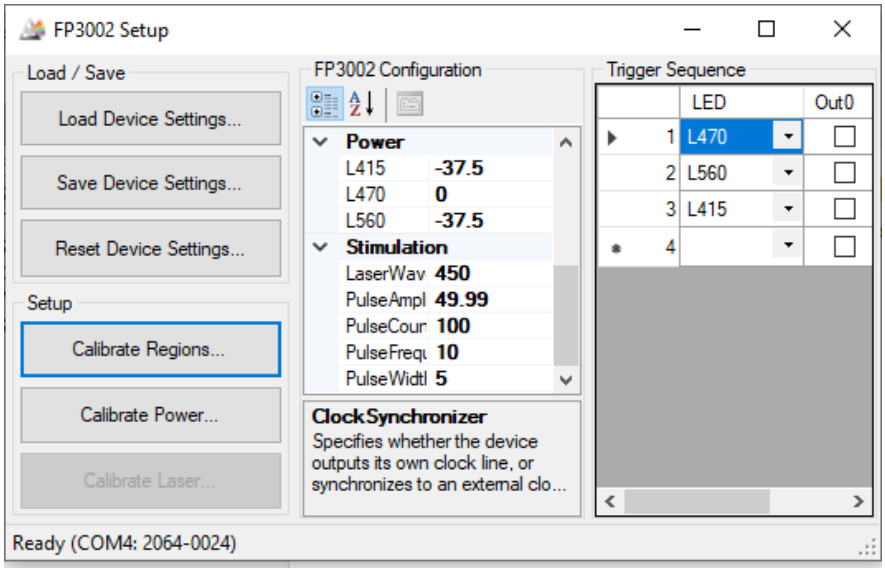
We start with a "Timer" node and specify values for the "Due Time" and the "Period". The "Due Time" will dictate how long it will take until the first laser pulse train. Then the "Period" will dictate how long to have the laser pulse train run and how long to have it stopped for. So in the example, we have set both value to 10 seconds meaning the laser will start OFF for 10 seconds, then ON for 10 seconds, then OFF for 10 seconds, etc.



The "Python Transform" node formats the data coming from the "Timer" node so that the "Stimulation. Control" node will command the "FP3002" node to trigger the laser in this way.



All that is left is to connect the "FP3002" node in the standard datastream for data acquisition ("FP3002" -> "Photometry Data" -> "Photometry Writer") and to set up the "FP3002" node configuration by specifying the "Stimulation" settings.



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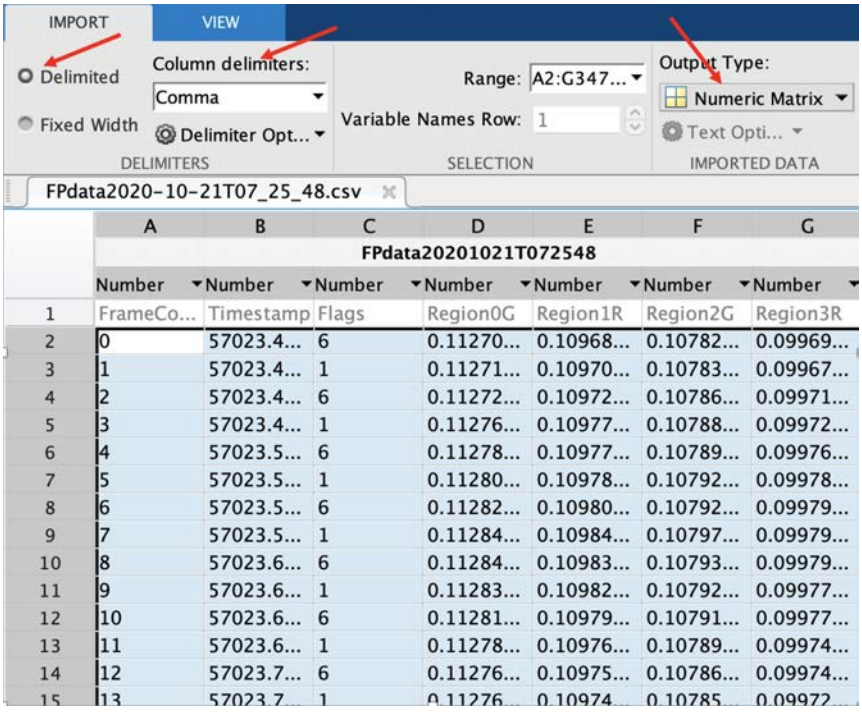
Introduction to Analyzing Your Data (MatLab)

Importing data into MATLAB

To import your data into MATLAB, click on “Import Data” in the toolbar.

The FP data are stored as a csv file (comma separated value) so make sure MATLAB knows this by selecting “Delimited” and “Comma.” Lastly, output the data as a “Numeric Matrix.”



	A	B	C	D	E	F	G
	Number	Number	Number	Number	Number	Number	Number
1	FrameCo...	Timestamp	Flags	Region0G	Region1R	Region2G	Region3R
2	0	57023.4...	6	0.11270...	0.10968...	0.10782...	0.09969...
3	1	57023.4...	1	0.11271...	0.10970...	0.10783...	0.09967...
4	2	57023.4...	6	0.11272...	0.10972...	0.10786...	0.09971...
5	3	57023.4...	1	0.11276...	0.10977...	0.10788...	0.09972...
6	4	57023.5...	6	0.11278...	0.10977...	0.10789...	0.09976...
7	5	57023.5...	1	0.11280...	0.10978...	0.10792...	0.09978...
8	6	57023.5...	6	0.11282...	0.10980...	0.10792...	0.09979...
9	7	57023.5...	1	0.11284...	0.10984...	0.10797...	0.09979...
10	8	57023.6...	6	0.11284...	0.10983...	0.10793...	0.09979...
11	9	57023.6...	1	0.11283...	0.10982...	0.10792...	0.09977...
12	10	57023.6...	6	0.11281...	0.10979...	0.10791...	0.09977...
13	11	57023.6...	1	0.11278...	0.10976...	0.10789...	0.09974...
14	12	57023.7...	6	0.11276...	0.10975...	0.10786...	0.09974...
15	13	57023.7...	1	0.11276...	0.10974...	0.10785...	0.09972...

Data formatting

There are a few ways to save your photometry data. While it is useful to deinterleave your data in Bonsai for visualization, we recommend saving the non-deinterleaved file. It makes analysis easier down the road.

The data themselves are pretty intuitive. For every frame, we save a packet of information letting you know when that frame was collected, which LEDs were on, and your signal.

FrameCounter: Sequentially counts the number of frames collected during an experiment.

Timestamp: Time when a frame was recorded using the internal clock in the FP3002 (<5us precision).

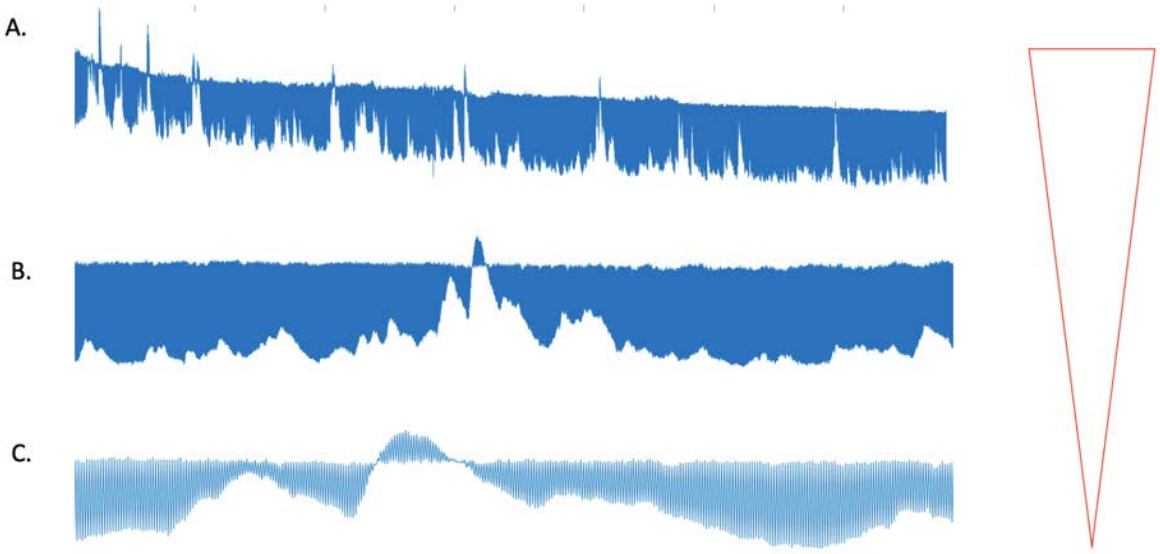
Flags: The flag indicates the internal state of the FP3002 when a frame was captured.

RegionXG: Mean pixel value within the confines of the user-selected region of interest (ROI) on the half of the image sensor that captures green emission. Note: These data are normalized as the fraction of the maximum value, so the range is 0-1. X is an integer starting at 0.

RegionYR: Mean pixel value within the confines of the user-selected region of interest (ROI) on the half of the image sensor that captures red emission. Note: These data are normalized as the fraction of the maximum value, so the range is 0-1. Y is an integer starting at 0.

It is important to familiarize yourself with what “raw” photometry data look like. To do this, plot the data from one of your ROIs. No need to worry about an X value.

```
plot(data(:,4))
```



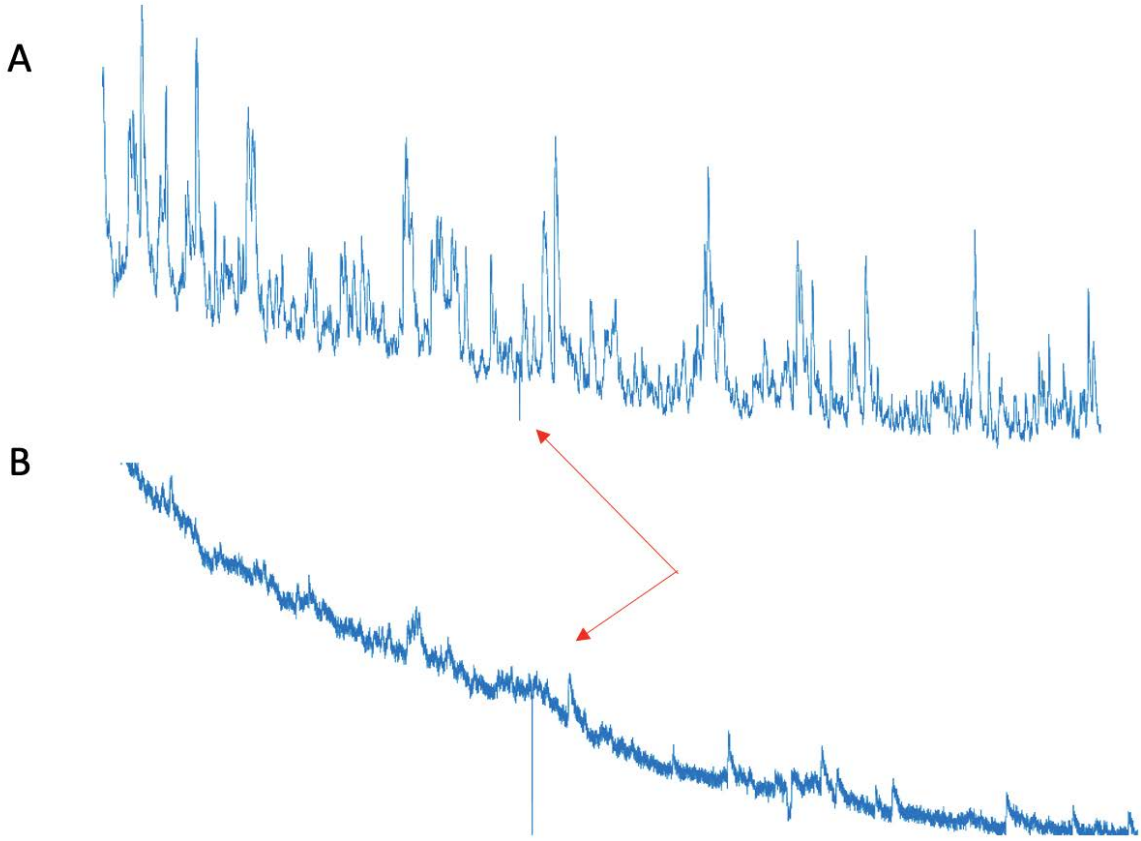
This is an example of a dataset with a high signal-to-noise ratio (SNR) in a brain region with quite a bit of spontaneous (and evoked) activity. Subplots A, B, and C are zoomed in versions of the same data.
Looking at A, we see there are two parts of the data -- the lower part where we see a lot of activity and the upper part that is relatively flat. Zooming in further, with B, it becomes even clearer and we can observe the telltale asymmetrical kinetics of GCaMP (in this case, 6s). Faster rise. Slower decay. Zooming in even further, with C, we can see that every-other-datapoint is either from the group with signal (lower group, in this case) or without (higher group, in this case).

With this dataset, researchers were oscillating between 415nm stimulation and 470nm stimulation resulting in a calcium-independent (415mm, upper group) and calcium-dependent (470nm, lower group) data streams.

Subsequent analyses will deinterleave these data using the “flags” variable. For example:

plot(data(data(:,3)==18,4)) %plots all data from column 4 (in our case, ROI0G, where the flag value was 18 indicating that the 470nm LED was on; data plotted in A below)

plot(data(data(:,3)==17,4)) %plots all data from column 4 (in our case, ROI0G, where the flag value was 18 indicating that the 415nm LED was on; data plotted in B below)



Just by looking through the deinterleaved data, without any fancy post-hoc analyses, we can glean a tremendous amount of useful information.
Note: You can have bonsai save a chart of your deinterleaved data. This is not only useful for all the reasons detailed below, but allows you to quickly scan through files (without opening MATLAB or any analysis software) so see what your signal looks like.

ALWAYS ALWAYS ALWAYS SCAN THROUGH DE-INTERLEAVED DATA FROM ANY PHOTOMETRY RECORDING.
For reference, here is a checklist of what to look for in raw, deinterleaved, data.

- Do we have good signal?
    - Look at the 470 data and see if there are clearly defined peaks.
        - Do they have asymmetrical kinetics?

- For calcium indicators, the rise time is always faster than the decay time and this is reflected in the photometry signal.
- Dopamine indicators, in our experience, have more symmetrical rise and decay kinetics.

        - Do they appear “smooth”?
            - This is an indication of a high SNR as the signal is so large that the data scale to wash out the noise. You can visualize this in Bonsai live too!
            - There are always exceptions. If you have a brain region with a very low mean firing rate (locus coeruleus and the dentate gyrus, for example) this can result in a fairly noisy flat signal with clearly defined peaks on top of it.
    - Find a large peak in the 470 and look at the corresponding 415 signal.
        - Do they look identical? If so, this is bad news and your signal is likely artifactual. Check additional peaks to confirm. Note: It is rare for a signal to have your expected kinetics and be artifactual -- but not impossible.
        - Is the 415 signal flat? Great! Your signal is likely real!
        - Does the 415 look like an inversion of your 470 signal? Double great! See section on the isosbestic point for more details.
- Was there a lot or a little spontaneous activity?
    - For the majority of brain regions, recording over 10 minutes or so, you’ll see some sort of spontaneous activity. This results in the 470 signal looking “busier” than the 415.
    - Are there periods with no activity? Or: Does there appear to be a signal floor?
        - This becomes important when correcting for photobleaching. With some regions, there is so much spontaneous activity that it becomes very hard to estimate baseline (most interneurons in cortex, for example). Take note of this. The more spontaneous activity you observe, the longer the recording needs to be to estimate baseline and correct for photobleaching.
- Was there substantial photobleaching during the recording?
    - Check out the decay of the 415 signal. Can it be explained by a biexponential decay?
    - Were there many motion artifacts?

        - This would appear as a transient deviation in your signal that is clearest in the 415nm signal. Most often, it presents as a decrease with symmetrical kinetics that exceed the kinetics you would expect with your indicator. See red arrows for an example.
    - Are there discontinuities in the 415 channel? Does it appear to jump (step-wise) from one value to another?
        - This is often an indication that the patch cord became disconnected from the animal during the recording or it was damaged. Mice can chew through patch cables if unsupervised.

Strategies

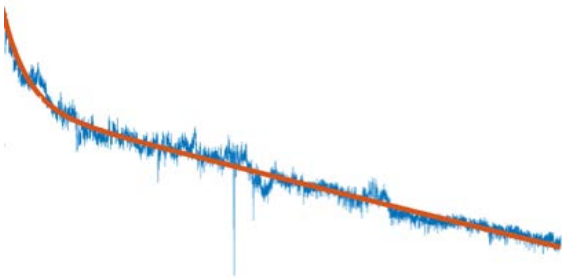
There are various strategies one can take once the data is scanned through, depending on the purpose of the experiment. We outline the most common one below.

1. Correct data for photobleaching
2. Align data to some event
3. Chop data up about said event
4. Profit

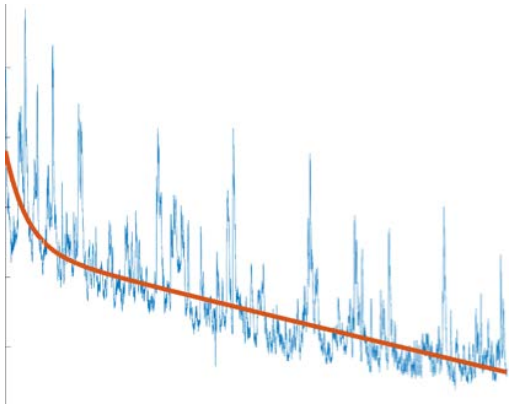
Correcting for photobleaching: The importance of this step is a function of the light power used during the experiment as well as the duration of the recording. For example, a five minute recording with 30uW of light will probably have negligible bleaching effects. Even if that is the case, ***always take bleaching into consideration!***

There are a couple of approaches out there. This is the one we recommend for 90% of datasets.

1) Fit isosbestic with biexponential decay



2) Linearly scale that fit to the calcium dependent data (using Robust Fit)



3) Divide your raw 470 data by the scaled fit