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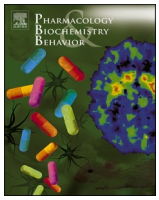

Opinion paper

Practical opinions for new fiber photometry users to obtain rigorous recordings and avoid pitfalls

Jennifer Mejaes [a], Dhruvi Desai [a], Cody A. Siciliano [c,d,e], David J. Barker [a,b,*]

[a] Department of Psychology, Rutgers, The State University of New Jersey, Piscataway, NJ, USA
[b] Brain Health Institute, Rutgers University, Piscataway, NJ, USA
[c] Department of Pharmacology, Vanderbilt University, Nashville, TN, USA
[d] Vanderbilt Brain Institute, Vanderbilt University, Nashville, TN, USA
[e] Vanderbilt Center for Addiction Research, Vanderbilt University, Nashville, TN, USA

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1. Introduction

Fiber photometry allows for quantification of neuronal activity in freely behaving animals through an implanted fiber optic alongside a genetically encoded fluorescent biosensor (Adelsberger et al., 2005). One reason for the growth of this technique is the ongoing development of biosensors that measure general cellular activity, or the activity of specific neurotransmitters such as glutamate, GABA, acetylcholine, serotonin, dopamine, norepinephrine, and orexin. Nonetheless, fiber photometry is not without its shortcomings, and a proper understanding of the technique is critical for recording reproducible results. Obtaining rigorous fiber photometry recordings requires understanding the contribution of extraneous signals such as hemodynamic changes or movement artifacts, understanding the recording system and signal sources, learning what constitutes high quality data, and developing an effective histological and data processing pipeline. To help with this, we have developed a list of our top 5 tips for new users:

1. Start simple

Experimental designs for fiber photometry are becoming increasingly complex and often involve the combination of fiber photometry with cre-driver lines, pharmacology, or optogenetics. While complex experimental designs can yield exciting results when they work, when they don't, the user is often left trying to disentangle problems by testing each moving part. For this reason, the best *first* (pilot) experiment is a simple one. Our suggestion would be to design an experiment where the biosensor and fiber are targeted to the same location, with the choice of location, viral promoter, and targeting strategy allowing for the robust activation of the biosensor.

2. Understand your system and how to troubleshoot it

A strong technical understanding of fiber photometry systems is just as important as any other neuroscience technology. At their core, fiber photometry systems function in the same manner as any fluorescence microscope – a typical system is, in essence, a widefield microscope with a fiber optic held in the focal plane of the objective. Learning about fluorescence microscopy is therefore advantageous for understanding any problems that might arise within fiber photometry. Articles like such as the review from Sanderson et al. (2014) are good starting points. While developing an understanding of these systems, we suggest generating a stepwise process for checking the system prior to use and during troubleshooting. A common approach for this is to progress stepwise through the system by verifying the activation and modulation of LEDs for excitation, confirming the passage of light through each relevant dichroic mirror or photocube, measuring the power of excitation at the subject connection via a working fiber, and confirming that

* Corresponding author at: Department of Psychology, Rutgers, the State University of New Jersey, 152 Frelinghuysen Road, Piscataway, NJ 08854, USA.
E-mail address: David.Barker@rutgers.edu (D.J. Barker).

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cameras or photodetectors have sufficient power and can record emitted signals using fluorescence test slides or other positive controls without clipping.

3. Incorporate positive and negative controls

Perhaps the most important advice for new users is to incorporate positive controls, negative controls, or both, into their experimental protocols. These controls can help to validate that signals being measured are an accurate representation of neuronal activity. Indeed, biosensors can capture a broad range of activity including somatic and nonsomatic activity depending on the experimental design or choice of sensor (Legaria et al., 2022). Common controls include pharmacological manipulations, electrical stimulation, optogenetic or chemogenetic excitation and inhibition, and behavioral controls.

Pharmacological controls are easily implemented and allow for the bi-directional modulation of signals through appropriate agonists and antagonists. While fewer papers have implemented pharmacological controls (e.g., Jones-Tabah et al., 2020), their utility is likely to increase with the expanding toolkit of neurotransmitter-specific biosensors. Electrical stimulation has also been effectively used as a positive control, including during the original ex vivo validation of isosbestic control channels (Lerner et al., 2015) and during our initial validation when recording GCaMP signals at axon terminals (Barker et al., 2017, unpublished data).

Optogenetic or chemogenetic approaches also provide the opportunity for bi-directional control of neuronal activity and have previously been combined with fiber photometry (e.g., Calipari et al., 2016). The major advantage of these approaches over pharmacological or electrical stimulation is the ability to combine them with cre-driver lines to confer genetic specificity. One major caveat with optogenetic stimulation is that there is a strong potential for optical crosstalk, which can result in artifactual signals and/or continuous activation of the opsin throughout the recording. Newer sensors with greater spectral separation are being developed that may alleviate this problem, but anatomical segregation is also a wise approach. It should also be noted that while DREADDs can both enhance or decrease neuronal firing, the longevity of their effects makes them better suited as a negative control. Exposure to fast-acting anesthetics, such as isoflurane, can also serve as a straightforward negative control in many cases.

Finally, behavioral controls are always an option for new users and often the simplest first option. This can include behavioral group expressing a control fluorophore (e.g., GFP) as a negative control. Such viral controls lack the dynamic responses of biosensors, meaning their responses during behavioral testing aid in revealing any extraneous influences on the recording (see below). Alternatively, strong acute stimuli such as a brief footshock (Barker et al., 2017) or airpuff induce broad and robust neuronal activation, making them a good behavioral option.

4. Correct for movement, photobleaching, and hemodynamics

Besides implementing positive and negative controls, it is also important to use controls for extraneous changes in recorded signals caused by photobleaching, movement, and hemodynamics. Two primary methods used for making these corrections involve exponential fit equations or references to isosbestic control channels. The isosbestic point is the wavelength at which the total absorbance of a sensor is the same in the presence and absence of the relevant ligand or ion, allowing an isosbestic control to capture and subtract changes not associated with the dynamics of the sensor of interest. Corrections using exponential modeling or isosbestic channels both have notable advantages, so choosing the best approach may be situation specific.

Regarding photobleaching, the signal in fiber photometry systems decreases over time across repeated exposure to light. Multiple sources contribute to this observed decrease, including bleaching of the optics, fluorescent reporters, or tissue autofluorescence. In accounting for all of these changes, corrections using exponential or biexponential fits may provide an advantage over isosbestic controls when adjusting for photobleaching, especially across longer timescales.

Other common noise sources include movement and changes in hemodynamics at the recording site. Movement artifacts involve mechanical changes to the patch cable connecting the animal to the remainder of the photometry system. Additionally, hemodynamic activity can also result in changes in signal. While these signals reflect true biological activity, they occur independent from any biosensor expression and can lead to spurious observations. These signals arise from the fact that the absorption spectrum of hemoglobin shifts depending on whether it is in an oxygenated versus deoxygenated state, making them difficult to disentangle from biosensor-driven transients (Zhang et al., 2022). Several other approaches, such as intrinsic optical imaging, rely on this phenomenon for label-free activity measures. As such, the optical signals related to hemodynamics have been extensively characterized, and this literature can provide a useful resource when interpreting fiber photometry data. Both movement and hemodynamic related changes are arguably best handled through the use of corrections via an isosbestic control channel (Zhang et al., 2022), especially when data analysis is handled over shorter timescales (Bruno et al., 2021).

5. Use histology and data analysis for feedback

Our last tip is to develop your data processing pipeline early to gain critical feedback about the quality of your targeting and recordings. Beyond the more general need to ensure correct viral expression and targeting of a region of interest, there is a strong correspondence between recording quality and histology. Fiber optics record from a limited volume of tissue (Pisanello et al., 2018), making it critical to ensure that optic fibers are proximal to viral expression (Generally <200 μm). Giving attention to these details will help improve recording quality over the long-term.

Finally, it is best to begin learning and applying basic analytical approaches early on. An examination of recordings throughout the data collection process will help you identify problems as they occur, develop an understanding of signal quality, and will overall increase the rigor of your experiments. Several well validated examples of analysis scripts exist across the prevailing photometry literature (e.g., London et al., 2018). Additionally, the *pMAT* or the *GuPPy* analysis packages offer simple point and click interfaces that are simple to use for users with no coding experience (Sherathiya et al., 2021; Bruno et al., 2021).

Data availability

No data was used for the research described in the article.

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