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# Correcting for the hemoglobin absorption artifact in fiber photometry data

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**Fiber photometry is a widely used fluorescence approach for measuring neuronal activity in freely behaving animals; however, these signals can be contaminated by hemoglobin absorption artifacts. Zhang et al. propose a computational method to quantify and correct hemoglobin photon absorption effects using spectral fiber photometry, resulting in more accurate neuronal activity measurements.**

Over the past few years, there has been a significant increase in the use of fiber photometry (Adelsberger et al., 2005; Gunaydin et al., 2014) to monitor brain dynamics *in vivo*. Fiber photometry is performed by delivering bulk excitation light into the brain through an implanted optical fiber. Neurons below the fiber expressing an activity indicator, e.g. GCaMP6 (Chen et al., 2013), emit activity-dependent fluorescence that is captured by the same optical fiber and relayed through a patch cord back to either a photodetector (Gunaydin et al., 2014), camera sensor (Kim et al., 2016), or spectrometer (Meng et al., 2018). These activity-dependent fluorescence signals can be corrected for motion artifacts by regressing out changes measured from a simultaneously collected static fluorophore or from the isosbestic (activity-independent) wavelength of the sensor (Lerner et al., 2015). Fiber photometry's ease of use, affordability, and compatibility with other methodologies such as optogenetics make it highly amenable to studying neuronal circuits underlying complex behaviors in rodents. Furthermore, with the advent of new fluorescent activity sensors across different wavelengths, fiber photometry is increasingly becoming the method of choice to enable fast, multiplexed read-outs of cellular physiology in the brain.

However, despite its many advantages, in contrast to cellular-resolution imaging techniques, fiber photometry can only collect fluorescence signals emitted from cells and cellular compartments averaged across a small volume of brain tissue. As a result, fiber photometry data cannot spatially resolve individual neuron activity, and furthermore it cannot circumvent unwanted spatial artifacts such as blood vessels. Thus, the fluorescence signals can be affected by endogenous absorbers like hemoglobin (Hb). Hb in the brain volume absorbs both excitation and emission photons. The Hb absorption effect is wavelength dependent and varies in accordance with the dynamic changes of oxyhemoglobin (HbO) and deoxyhemoglobin (HbR) concentration *in vivo*. Previously, studies have shown that the absorption spectra of HbO and HbR overlap significantly with the excitation and emission spectra of GCaMP and other fluorescent sensors (Prahl, 1999). Even with brighter GCaMPs or other fluorophores, this Hb absorption artifact still cannot be overcome due to the multiplicative effect of absorption on fluorescence. Hence, Hb absorption artifacts can have an impact on the accuracy of measured fluorescence data and may lead to misleading results and erroneous data interpretation (Ma et al., 2016; Prahl, 1999).

Methods have previously been developed to estimate Hb concentration and correct the Hb absorption artifact in the context of wide-field fluorescence imaging (Ma et al., 2016; Valley et al., 2020). In these cases, the proportion of backscattered or reflective light that returns to the detector after multiple scattering events within the tissue can be used to estimate the Hb absorption effect. The concentrations of Hb are then calculated by relating the Hb absorption to the mean scattering pathlength in the tissue using a modified Beer-Lambert relationship. Backscatter measurements enable continuous recording of hemodynamics without interfering with the fluorescence recording (Ma et al., 2016). However, the accuracy of the backscatter method largely relies on estimation of parameters like mean scattering pathlengths. More recently, Valley et al., (2020) described a spatially detailed regression-based method that fits every pixel in the image and does not rely on estimation of scattering pathlength. This regression method can distinguish differences in optical properties with single-pixel resolution and provides good Hb absorption correction near blood vessels.

Despite these advances in correcting Hb absorption in wide-field imaging data, how the Hb-absorption affects fiber photometry fluorescence readouts is still largely unknown. Therefore, there is a need for methods that quantify Hb concentration changes and correct Hb absorption artifacts in fiber photometry data. In this issue of *Cell Reports Methods*, Zhang et al. (2022) report on a computational model that enabled them to estimate the change in HbO and HbR and then remove the total Hb concentration changes from the activity-dependent fluorescence readings (Figure 1). Their model requires the well-documented molar extinction coefficients of HbO and HbR, simulated photon-traveling pathlengths through brain tissue (which they collected here using a Monte Carlo simulation), and spectral fiber photometry data points

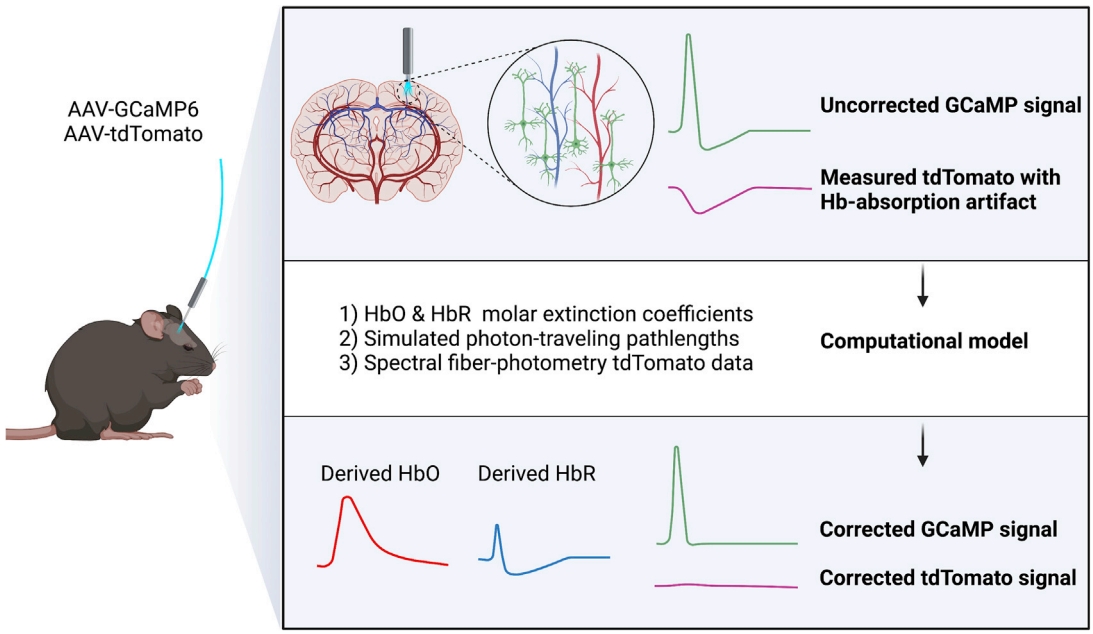


**Figure 1. Hb absorption artifact correction using spectral fiber photometry**

Hb absorption artifacts are measured in an activity-independent channel, such as tdTomato, along with the activity-related GCaMP signal. Their model derives the estimated hemodynamic changes and then removes the Hb absorption artifact from the GCaMP signal. Created with BioRender.com.

collected across a range of wavelengths over time from an activity-independent fluorophore.

They first validated their model *in vivo* while measuring fluorescence from the activity-independent fluorophore eYFP, along with simultaneous cerebral blood volume (CBV) measurements using intravenously injected Rhodamine B. They found that activity-induced CBV changes resulted in an unexpected decrease in eYFP spectral emission, which could be used to estimate the Hb changes with their model. Their derived total change in Hb was highly correlated with the directly measured CBV changes using either Rhodamine B or fMRI, highlighting the accuracy of their model. Then using simultaneous GCaMP and tdTomato fiber spectrometry recordings, they demonstrated how Hb changes can cause an aberrant "undershoot" in GCaMP fluorescence signal, seen as a non-activity-related dip in signal following a large transient (which might be incorrectly attributed to neural inhibition). More strikingly, large Hb changes induced by pharmacological agents can completely flip the polarity of expected GCaMP signals over the duration of a recording. Both types of unwanted artifacts could be corrected by estimating the change in HbO and HbR from the spectral tdTomato measurements. Their approach (which requires the collection of multiple spectrometry data points over time) is more accurate at correcting these Hb artifacts in GCaMP signals compared to regressing away the activity-independent 405 nm isosbestic channel—which is currently the standard for correcting motion-related artifacts in fiber photometry signals (Sherathiya et al., 2021).

In summary, Zhang et al. (2022) highlight the importance for recognizing and correcting Hb artifacts in fiber photometry data, beyond what the field currently does using isosbestic control wavelengths. It is particularly important to consider when performing fiber photometry recordings during pharmacological interventions *in vivo*, which can cause large changes in CBV. In these contexts, using their computational models along with a spectrometer can remove unwanted Hb absorption artifacts, leading to more accurate neuronal recording data with fiber photometry. Looking to the future, it may also be beneficial to design further red-shifted fluorescence sensors in wavelengths that are less affected by Hb absorption artifacts.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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