Spectral fiber photometry derives hemoglobin concentration changes for accurate measurement of fluorescent sensor activity

**Highlights**

- Hemoglobin absorbs light and creates artifacts in fiber photometry recordings *in vivo*
- A method to derive hemoglobin concentration changes from spectral photometry
- Correction of this artifact provides accurate measurements of fluorescent sensor signals
- Quantification of regional differences in neurovascular transfer function

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**In brief**

Changes in hemoglobin concentration affect the accuracy of fiber photometry measurements *in vivo*, even flipping the measured signal polarity under certain conditions. Zhang et al. develop an approach to derive oxy- and deoxy-hemoglobin concentration changes from spectral fiber photometry data and a pipeline to correct for these artifacts in fluorescent sensor measurements.
Spectral fiber photometry derives hemoglobin concentration changes for accurate measurement of fluorescent sensor activity

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SUMMARY
Fiber photometry is an emerging technique for recording fluorescent sensor activity in the brain. However, significant hemoglobin absorption artifacts in fiber photometry data may be misinterpreted as sensor activity changes. Because hemoglobin exists widely in the brain, and its concentration varies temporally, such artifacts could impede the accuracy of photometry recordings. Here we present use of spectral photometry and computational methods to quantify hemoglobin concentration changes and correction of hemoglobin absorption artifacts in fiber photometry data. We also demonstrate the added benefit of our methods to compute hemodynamic response function using the derived hemoglobin concentration information.

INTRODUCTION
Fiber photometry relies on an implanted optical fiber to excite and detect fluorescence signals and is best known for its ability to measure ensemble neuronal or neurochemical activity in small volumes of brain tissue in animal models. The simplicity and cost effectiveness of the optical system make the technique highly accessible (Meng et al., 2018), and lightweight, miniature fiber optics make photometry compatible with a wide variety of experimental settings, such as freely moving behavioral assessment.
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Kim et al., 2016; Meng et al., 2018) or head-fixed brain-wide imaging (Chen et al., 2019; Pais-Roldán et al., 2020; Figure 1A). Several genetically encoded fluorescent sensors of cellular activity and neurotransmitters have become widely available (Chen et al., 2013; Dana et al., 2016; Marvin et al., 2013, 2019; Patriarchi et al., 2018; Sun et al., 2018; Tian et al., 2009; Wang et al., 2018a, 2018b), expanding the range of applications for fiber photometry. The tunable spectral specificity has also enabled new parallel sensing capabilities with wavelength multiplexing. Given its rapidly increasing usage in neuroscience (Cui et al., 2018a, 2018b), expanding the range of applications for fiber photometry is crucial for interpreting hemodynamics-based neuroimaging recordings and demonstrate the added benefit to compute the transfer function between neuronal and vascular activity, which is crucial for interpreting hemodynamics-based neuroimaging data such as fMRI, functional ultrasound, and intrinsic optics.

RESULTS

Theoretical Hb absorption effect on photometry recordings

We solved the following equation using the generalized method of moments (GMM) (Hansen, 1982) with spectrometer outputs, which provides quantifiable photon counts across spectra:

\[
\frac{F(t, \lambda_{Em})}{C(t)} = e^{-\Delta\mu_{a}(t, \lambda_{Ex})X/\lambda_{Ex}} \frac{C(t)}{C(t_0)} \quad \text{(Equation 1)}
\]

where \(\Delta\mu_{a}(t, \lambda_{Ex})\) and \(\Delta\mu_{a}(t, \lambda_{Em})\) represent the absorption coefficient changes at excitation wavelength \(\lambda_{Ex}\) and emission wavelength \(\lambda_{Em}\), respectively, at time point \(t\) compared with a reference time point \(t_0\). \(\Delta\mu_{a}(t, \lambda_{Ex})\) and \(\Delta\mu_{a}(t, \lambda_{Em})\) are affected by \(\Delta\text{C}_{\text{BO}}\) and \(\Delta\text{C}_{\text{BR}}\) (STAR Methods, Equation 16). \(X(\lambda_{Ex})\) and \(X(\lambda_{Em})\) represent the photon traveling path lengths (see STAR Methods for details) at the excitation and emission wavelengths, respectively. To solve Equation 1, we first performed a Monte Carlo simulation using the pipeline shown in Figure S2A to obtain \(X(\lambda_{Ex})\) and \(X(\lambda_{Em})\). We determined the average photon traveling path lengths for emission wavelengths from 488–700 nm in the simulated brain tissue from a total of 29 simulations (2–2.5 × 10^6 photons each; Figure 1C, detailed numbers tabulated in Table S1). These results are expected to be generalizable and can be used across labs. As an example, we illustrated spatial probability and luminance-distance profiles for the commonly used 488-nm excitation, 515-nm emission and 580-nm emission in Figures S2B–S2D (see also Video S1). These results allowed calculating the simulated effects of Hb absorption on fluorescence signal measurements, for which we observed significant nonlinear, wavelength-dependent effects (Figure 1D).

Ex vivo quantification of Hb absorption effects on photometry recordings

We first performed ex vivo measurements and showed that adding 1 mL of fresh venous mouse blood into a 10-mL mixture of Alexa 488 and 568 fluorescent dye solution significantly reduced both fluorescence signals measured by fiber photometry. Compared with adding the blood, adding 1 mL vehicle (0.9% saline) induced significantly less reduction in Alexa 488 and 568 signals (Figures S3A–S3C). Subsequently, we saturated the mixture with 100% oxygen to increase the HbO-HbR ratio and observed marked increases in the Alexa 568 signals but decreases in Alexa 488 signals. We did not observe significant signal changes when infusing oxygen into the solution with 1 mL saline added (Figures S3B and S3D).

In vivo quantification of Hb absorption effects on photometry recordings

We performed an in vivo experiment to measure the Hb absorption of activity-independent fluorescence signals in fiber photometry. We selected a commonly used fluorophore, enhanced yellow fluorescent protein (EYFP), whose fluorescence signal is

A step-by-step protocol of this method could be found in Zhang et al. (2022).
Figure 1. Spectrally resolved fiber photometry system and Hb absorption effects
(A) Schematic of a spectrally resolved fiber photometry system used for recording fluorescence signals.
(B) Hb in red blood cells absorbs light in two phases: excitation light from the optical fiber (left) and emission light to be recorded by the optical fiber (right). The color density of arrowheads indicates light intensity. An increase in Hb is expected to decrease the number of photons in both phases.
(C) Simulated average excitation and emission photon traveling path lengths across 488–700-nm emission wavelengths from our database of paired excitation/emission light paths.
(D) Effect of HbO and HbR concentration on ΔF/F. Shown are simulated effects of HbO and HbR concentration changes on measurement of fluorescence signals. ΔC_{HbO} and ΔC_{HbR} ranging from −90 to +90 μM were used to simulate fluorescence signal changes (ΔF/F) between 500 and 700 nm using the molar extinction coefficients of HbO and HbR (Figure S1) and simulated light paths (Figure 1C). Graphs shaded in blue indicate an overall decrease in total Hb (HbT), and graphs shaded in red indicate an overall increase in HbT. See also Figures S1, S2, Video S1, and Table S1.
activity independent and has an emission spectrum peaking at 526 nm. We virally expressed EYFP using an adeno-associated virus (AAV) in the primary somatosensory cortex of the forelimb region (S1FL) under the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII\textalpha) promoter and then implanted an optical fiber into this target area (Figures 2A and 2B). On the day of photometry recording, we intravenously administered Rhodamine B, a long-circulating red fluorescent dye, Rhodamine B. (D–J) Peri-stimulus heatmaps showing repeated trials and average response time courses of the changes in measured rhoCBV, measured EYFP, derived HbO, derived HbR, corrected rhoCBV, corrected EYFP, and derived HbT, respectively. The gray-shaded segment indicates the forepaw stimulation period. Hb absorption induces pseudo-negative changes in measured EYFP signals during stimulation. (K and L) Correction of the contaminated EYFP and rhoCBV signals, respectively, in 5 hemispheres and 30 trials (**p < 0.001, paired t test). Measured and corrected EYFP signals are compared. Measured rhoCBV was normalized to 0 to show the difference before and after correction. All color bars use the same unit as the y axis of the time course. Error bars represent standard deviation. See also Figures S3–S5.

Figure 2. In vivo quantification of HbO and HbR changes using EYFP emission in fiber photometry and correction of EYFP signals for Hb absorption
(A) Preparation for fiber photometry measurement of EYFP signals in S1FL.
(B) Confocal image showing EYFP expression.
(C) Simultaneous dual spectral photometry recording of EYFP signals and hemodynamic responses (CBV) was achieved by intravenously injecting a long-circulating red fluorescent dye, Rhodamine B.

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every single time point and therefore could use numerous empirical data to solve two unknowns (ΔCHbR and ΔCHbT; see also Figures S4A and S4B for the effect of input spectral datapoints). The derived concentration changes (Figures 2F and 2G) were within physiological ranges and comparable with those reported in the literature (Ma et al., 2016), and the summation of the two (i.e., total Hb concentration changes, termed ΔCHb(t)) informs CBV changes (Ma et al., 2016; Figure 2J). The calculated ΔCHbT(t) highly resembled the rhoCBV changes measured from a distinct and less affected red spectrum (Figures S4C–S4E). With ΔCHb(t) and ΔCHbR(t) obtained, we then calculated Δμ(t, λEx) and Δμ(t, λEm) and entered these parameters into Equation 1 to correct the rhoCBV changes (Figure 2H) and EYFP signals (Figure 2I). Figure 2I shows a flattened EYFP trace after the proposed correction, demonstrating the validity of our approach. Although the influence of Hb absorption on EYFP signals varied by trials, our proposed method accurately corrected EYFP signals in all trials (Figure 2K). Despite the smaller influence on the rhoCBV signal measured from the red spectrum, the absorption effect remained statistically significant (Figure 2L). In addition to the fluorescence signal changes induced by forepaw sensory stimulations, we also replicated these findings using a 5% CO2 hypercapnic challenge paradigm and successfully restored the contaminated EYFP signal changes (Figure S5).

**Effect of Hb absorption on acute GCaMP signal measurements using fiber photometry**

We virally expressed GCaMP6f (hereafter called GCaMP), one of the most widely used genetically encoded calcium indicators (Chen et al., 2013), using an AAV under the CaMKIIz promoter in the S1FL. We also expressed tandem dimer tomato (tdTomato), an activity-independent red fluorescent protein, under the CAG promoter (Figures 3A and 3B) and then implanted an optical fiber into this target area. Our rationale for choosing tdTomato (Chen et al., 2013), using an AAV under the CaMKIIz promoter (Figures S4C–S4E). With ΔCHb(t) and ΔCHbR(t) obtained, we then calculated Δμ(t, λEx) and Δμ(t, λEm) and entered these parameters into Equation 1 to correct the rhoCBV changes (Figure 2H) and EYFP signals (Figure 2I). Figure 2I shows a flattened EYFP trace after the proposed correction, demonstrating the validity of our approach. Although the influence of Hb absorption on EYFP signals varied by trials, our proposed method accurately corrected EYFP signals in all trials (Figure 2K). Despite the smaller influence on the rhoCBV signal measured from the red spectrum, the absorption effect remained statistically significant (Figure 2L). In addition to the fluorescence signal changes induced by forepaw sensory stimulations, we also replicated these findings using a 5% CO2 hypercapnic challenge paradigm and successfully restored the contaminated EYFP signal changes (Figure S5).

To demonstrate the effect of Hb absorption on GCaMP signals over an extended period of time, we leveraged an acute ethanol challenge protocol (Broadwater et al., 2018; Vaagenes et al., 2015) that has been shown to decrease neuronal activity (Lotfullina and Khazipov, 2018) as well as cerebral perfusion (Broadwater et al., 2018; Kelly et al., 1997; Sano et al., 1993). We virally expressed GCaMP using an AAV under the CaMKIIz promoter and tdTomato under the CAG promoter in the prelimbic cortex (PrL) (Figure 4A) and then implanted an optical fiber in this target area. On the day of the experiment, ethanol (2 g/kg, 20% [v/v], intraperitoneally [i.p.]) was given 5 min after onset of fiber photometry recording. After ethanol administration, we observed robust increases in the raw, measured tdTomato (Figure 4B) and GCaMP (Figure 4C) signals. These changes were ethanol specific because we did not observe any changes in a separate session where the same group of subjects received a vehicle injection (an equal volume of 0.9% saline, i.p.) (Figures 4B and 4C). Next, we derived ΔCHbO(t), ΔCHbR(t), and ΔCHbT(t) (Figure 4D) from the ethanol data using correction methods identical to that described in Figure 3 and found robust decreases in ΔCHbO(t) and ΔCHbR(t), in agreement with studies showing reduced Hb affinity for oxygen (Van De Borne et al., 1997) and vasoconstriction (Kudo et al., 2015) following acute alcohol administration. Following our proposed correction, the tdTomato trace flattened as expected (Figures 4B and 4E).
Importantly, the trend of the GCaMP signal shifted from unexpected positive changes to the expected negative changes (Lotfullina and Khazipov, 2018; Figures 4C and 4F). In another two proof-of-concept experiments, we also noted the influence of Hb absorption on anesthetics- and pharmacology-induced fluorescence signal changes (Figure S7). These data highlight the importance of correcting for Hb absorption in fiber photometry studies because misinterpretation may otherwise occur.

Correction of Hb absorption artifacts in other fiber photometry settings
To expand the utility of our method, we modeled and compared the Hb absorption correction techniques among three settings. The first method involves using a spectrometer with a sequence that interleaves between 405 and 488 nm excitation. The GCaMP isosbestic point is around 405 nm (Tian et al., 2009; Dana et al., 2019), where excitation light delivered at this range would...
we observed a rapid increase in GCaMP488 signal, followed by a pseudo-undershoot (Chen et al., 2013; Mittmann et al., 2011). We then compared the three settings using a single set of in vivo data, thus avoiding subject and trial-by-trial variability confounds. With respect to the three settings, we present (1) corrected GCaMP488 using $\Delta C_{\text{HbO}}(t)$ and $\Delta C_{\text{HbR}}(t)$ derived from the full GCaMP405 spectrum in Figure 5D, (2) corrected GCaMP488 using $\Delta C_{\text{HbO}}(t)$ and $\Delta C_{\text{HbR}}(t)$ derived from three spectral datapoints from the GCaMP405 spectrum in Figure 5E, and (3) corrected GCaMP488 by regressing out the GCaMP405 signal intensity in Figure 5F. Comparisons of the post-stimulus pseudo-undershoot artifacts suggested successful restoration of the GCaMP488 signals (i.e., signals rapidly return to zero) via both Hb absorption-based correction methods but not via the regression-based method (Figure 5G). Because tdTomato signals are available from 488-nm excitation (Figure 5B), we performed a similar set of analysis using tdTomato instead of GCaMP405 emission signals (Figures 5H–5K) and observed successful restoration of GCaMP488 signals using both Hb absorption-based correction methods but sustained elevated signals using the regression-based method (Figures 5H–5L). No significant difference was observed between the full spectrum versus the three-point correction methods (Figures 5G and 5L). As expected, the calculated HbT from the GCaMP405 spectrum exhibited a lower CNR when using only three spectral datapoints compared with the full spectrum (Figure 5M).

**Brain-regional differences in hemodynamic response function**

Because our proposed technique allows simultaneous measurements of neuronal activity (e.g., GCaMP) and vascular responses evoking GCaMP emission photons irrelevant to the changes in calcium concentration. The GCaMP emission signals from the 488-nm excitation is abbreviated as GCaMP405, in contrast to GCaMP488 excited by the 488 nm laser. The fact that apparent Hb absorption exists at the GCaMP405 emission spectrum (Figure S1A) makes this an appealing alternative approach when another activity-independent fluorescent protein is not expressed. In essence, this provides reference fluorescence signals similar to that of EYFP (Figure 2) or tdTomato (Figure 3) and allows $\Delta C_{\text{HbO}}(t)$ and $\Delta C_{\text{HbR}}(t)$ to be calculated for Hb absorption correction. The second method involves using GCaMP405 data from a spectrometer but only selecting three spectral datapoints to mimic the settings using band-pass filters with a limited number of photodetectors (i.e., without using the full GCaMP405 spectrum for correction). The third method involves regressing out the activity-independent GCaMP405 fluorescence signal intensity as in Kim et al. (2016). To provide data for these evaluations, we virally expressed GCaMP6f using an AAV under the CaMKIIα promoter and tdTomato under the CAG promoter in the primary motor cortex (M1) of the left hemisphere and implanted a microelectrode into the M1 of the right hemisphere, allowing electrical stimulation to be delivered (60 Hz, 300 μA, 0.5-ms pulse width for 2 s); thus, time-locked GCaMP signal changes can be induced in the left M1 (Figure 5A). The interleaved excitation and acquisition sequences are shown in Figure 5B. The raw responses of GCaMP405 and GCaMP488 to stimulation are shown in Figure 5C. Similar to Figures 3Q and 3R, we observed a rapid increase in GCaMP488 signal, followed by a pseudo-undershoot (Chen et al., 2013; Mittmann et al., 2011).
Figure 5. Correction of Hb absorption artifacts in other fiber photometry settings

(A) Preparation for fiber photometry measurement of GCaMP and tdTomato signals in the left M1, with the right M1 receiving local micro-electrical stimulation.

(B) Interleaved acquisition paradigm with 405- and 488-nm excitation lasers. Ca²⁺-independent GCaMP signals (GCaMP₄₀₅) were excited by a 405-nm laser, whereas Ca²⁺-dependent GCaMP signals (GCaMP₄₈₈) and tdTomato signals were excited by a 488-nm laser. This interleaved paradigm was used because GCaMP₄₀₅ and GCaMP₄₈₈ share nearly identical emission spectra and cannot be spectrally unmixed. Data were acquired at 50% duty cycle every 50 ms using a box paradigm to avoid spectrometer frame loss during trigger mode. The final sampling rate is 10 Hz for all signals.

(C) Measured GCaMP₄₈₈ and GCaMP₄₀₅ signals without any correction.

(D) Corrected GCaMP₄₈₈ using HbO and HbR dynamics derived from the full GCaMP₄₀₅ spectrum.

(E) Corrected GCaMP₄₈₈ using HbO and HbR dynamics from 3 datapoints in the GCaMP₄₀₅ spectrum (at 502, 523, and 544 nm).

(F) Corrected GCaMP₄₈₈ by regressing out the GCaMP₄₀₅ signal dynamics.

(legend continued on next page)
(e.g., $\Delta\text{CHbT}$) with minimized Hb absorption artifacts, it offers a significant added benefit for studying the relationship between the two. This relationship, often termed the hemodynamic response function (HRF), governs data interpretation of many noninvasive neuroimaging technologies such as fMRI, intrinsic optics, and functional ultrasound (Taylor et al., 2018). Although use of a canonical HRF to model activity throughout the brain remains the most common practice in the field, accumulating evidence shows that HRF is region dependent (Devonshire et al., 2012; Taylor et al., 2018) and that a thorough understanding of HRF affected by regional cell types, neurochemicals, and subject conditions is crucial to accurately interpret most neuroimaging data (Lecrux and Hamel, 2016). To demonstrate the utility of our proposed technique for computing region- and sensor-specific HRFs (Chao et al., 2022), we first described the method to compute HRF (Figure S8A), similar to that in Chao et al. (2022), and then validated the results on a dataset not used in HRF computation (Figure S8B). Next, we virally expressed GCaMP6f using an AAV under the CaMKII promoter and tdTomato under the CAG promoter in the M1 of the left hemisphere and the caudate putamen (CPu; also known as the dorsal striatum) of the right hemisphere (Figures 6A and 6B). We implanted a microelectrode into the M1 of the right hemisphere (Figure 6A) so that transcallosal and corticostriatal stimulation could be achieved simultaneously. Upon electrical stimulation (60 Hz, 300 μA, 0.5-ms pulse width for 2 s), we observed robust GCaMP changes in the left M1 and right CPu (Figures 6C and 6D), which are both monosynaptically connected to the stimulation target. After calculation of Hb concentration changes, Hb absorption correction, and computation of HRFs, we observed that the M1 and CPu exhibited distinct HRF profiles (Figures 6C and 6D). Interestingly, we also observed distinct HbR polarities between these two regions.

**DISCUSSION**

Techniques measuring fluorescence signals in vivo, such as fiber photometry (Liang et al., 2017; Schlegel et al., 2018; Chao et al., 2022), one-photon microscopy (Bollimunta et al., 2021), two-photon microscopy (Baraghis et al., 2011), or macroscopic imaging through a thinned skull or implanted window (Cramer et al., 2012), one-photon microscopy (Bollimunta et al., 2021), and conventional two-photon microscopy (Bollimunta et al., 2021; Prahl, 1999; Figures S1A and S1B). Our simulation suggested that the accuracy of the fluorescence signal could be compromised even without a net change in CBV (see Figure 1D for plots without a shaded background). This makes it crucial to quantify $\Delta\text{CHbO}$ and $\Delta\text{CHbR}$ so that the true changes in the fluorescence signal can be restored.

At present, two major fluorescence-measurement strategies are used in fiber photometry. One is non-spectrally resolved...
using a narrow band-pass filter paired with a photodetector (Gunaydin et al., 2014; Kim et al., 2016; Lazarjan et al., 2021). The other is spectrally resolved using a photomultiplier tube array (Cui et al., 2013, 2014) or a spectrometer (Meng et al., 2018; Sun et al., 2018; Chao et al., 2022). The non-spectrally resolved method has the advantage of recording fluorescence signals at a faster speed and can be used with modulated excitation lights and a lock-in amplifier (Gunaydin et al., 2014). In contrast, the spectrally resolved method provides wavelength-dependent information for the fluorescence, where the detected photons can be matched with the specific spectral profile of the sensor to improve specificity or perform spectral unmixing for multiplexing (Meng et al., 2018). If a single non-spectral photodetector is used and \( \Delta \text{GCaMP} \) and \( \Delta \text{Hb} \) cannot be obtained, the Hb absorption spectra (Figures S1A and S1B) and our simulation results (Figure 1D) indicated that far-red sensors are less prone to Hb absorption artifacts. This highlights the importance of continually developing fluorescent sensors in the red-shifted and far-red range (Patriarchi et al., 2020; Shcherbakova, 2021).

Although we did not obtain photodetector-based data in this work, we mimicked the photodetector-based setting by sampling data only from three specific spectral datapoints to enter our Hb absorption correction pipeline. These modeling data support the utility of our proposed technique in the photodetector setting (Figures 5E and 5J). The detector in the spectrometer used in this study is a high-end back-thinned charge-coupled device (CCD) array (90% quantum efficiency) with TE cooling (40°C–60°C below ambient) to reduce the dark current (Ocean Insight, 2022). For a rough comparison of the reported detector sensitivity, the most common commercial photodiode detectors used in fiber photometry have a sensitivity (reported as responsivity) of 0.38 A/W at 550 nm (Doric, 2021), which is similar to 0.40 A/W at 550 nm of the system used in this study (calculated based on 90% quantum efficiency). We do not have access to ground-truth GCaMP signals (i.e., the “real” GCaMP signal) using fiber photometry to make an unequivocal comparison. However, GCaMP measurements from cultured neurons without Hb or using two-photon imaging to circumvent blood vessels have collectively demonstrated the absence of post-stimulus undershoot (Chen et al., 2013; Mittmann et al., 2011). Leveraging these observations in the literature, together with the results we obtained in Figures 2E and 3F, we believe in the utility of our proposed Hb absorption correction method. We recognize that the post-stimulus undershoot artifacts may not significantly compromise all fiber photometry studies, especially for those studying brief time-locked stimulations/tasks. We consider the post-stimulus undershoot artifact to be a useful marker to benchmark methods dealing with Hb absorption. We suggest caution when studying spontaneous ongoing neuronal/neurochemical activity dynamics or slower brain-state changes using fiber photometry because the HbO and HbR concentration changes may cause erroneous readings of the desired sensor activity (Figures 4 and S7). Our results also suggest that regression-based models may be suboptimal to restore the GCaMP
signal changes in fiber photometry (Figures 5F–5K) and highlighted the benefits of CNR when using a spectrometer system to derive HbO and HbR changes (Figure 5M).

Fluorescence signals generated from GCaMP isosbestic excitation can be used as the activity-independent fluorescent reference signal in the analytical method proposed in this study and derive ΔCHbO and ΔCHbR to restore the functional GCaMP signals (Figure 5). Potential limitations of this approach are (1) trade-off in temporal resolution because interleaved excitation is required, (2) relatively low CNR (Figure 5M; approximately one-tenth of the emission signal was obtained compared with 488-nm excitation), (3) higher susceptibility to pH-related confounding factors (Shaner et al., 2005; Barnett et al., 2017), and (4) although GCaMP has an isosbestic point at an amenable wavelength for this method, other functional fluorescent sensors may perform optimally at a different wavelength (Akerboom et al., 2012; Sun et al., 2020).

The opportunity to examine the interactions between cell types and/or neurotransmitters has become increasingly popular in fiber photometry. When the green and the red spectrum are used to encode fluorescent sensor activities, it may be possible to use the interleaved sequence, as shown in Figure 5, or spectral signals from the unmixing residuals (i.e., the remaining autofluorescence) to achieve hemodynamic correction (Figure S10). In our hands, the residual method suffers significantly from CNR issues because of very low residual photon counts. However, this approach may demonstrate its utility with the advent of a more sensitive spectrometer system or an improved unmixing algorithm. Considering the increasing need for multiplexing, a valuable next step should consider establishing an experimental pipeline to achieve motion and Hb absorption artifact-free measurement of green and a red fluorescent sensor (e.g., GCaMP and jRGECO) in freely moving animals. This may be achieved by incorporating a far-red activity-independent fluorescent sensor for motion correction first (because Hb absorption is negligible in this range; Figure S1), followed by Hb absorption correction using data from isosbestic GCaMP excitation, as shown in Figure 5.

Given the irreplaceable roles of noninvasive hemodynamics-based neuroimaging techniques (such as fMRI, near-infrared, intrinsic optics, functional ultrasound, positron emission tomography, etc.) for studying brain function, identifying brain region-dependent HRF is crucial for accurate interpretation of hemodynamics-based neuroimaging data. Current standard fMRI analysis uses one HRF shape throughout the brain. Increasingly, literature has pointed to potential problems with this analysis pipeline because HRF is brain region dependent (Devonshire et al., 2012; Ekstrom, 2021; Jin et al., 2020; Sloan et al., 2010). The current study and other pioneering studies (Albers et al., 2017; He et al., 2018; Liang et al., 2017; Schlegel et al., 2018; Schmid et al., 2016; Schulz et al., 2012; Schwalm et al., 2017; Wang et al., 2018a, 2018b; Zhang et al., 2022; Chao et al., 2022) have demonstrated that fiber photometry could be seamlessly coupled with fMRI and provide ground-truth cellular activity to help unravel the relationship between neuronal and hemodynamic responses. These studies have bridged a crucial knowledge gap in interpretation of fMRI data. The method proposed in this study expands the scope of this research area because it generates hemodynamic signals “free of charge” with better sensitivity than fMRI (Figure 3N), and the results can be readily compared with the concurrently recorded fluorescent sensor activity. With spectral photometry recording becoming increasingly popular, the proposed method opens up a unique opportunity to compute HRF in a cell-type-dependent, stimulation-circuit-dependent, and neurotransmission-dependent manner. We expect that this information can be obtained by performing a meta-analysis on fiber photometry data shared through an open-science platform and contribute significantly to a more accurate interpretation of hemodynamics-based neuroimaging results.

We demonstrated that significant Hb absorption artifacts in fiber photometry data may cause inaccurate measurements and, therefore, erroneous interpretation. We proposed a method that addresses this issue by quantifying dynamic HbO and HbR absorption changes using activity-independent fluorescence spectra and demonstrated that these additional quantitative measurements were efficient in correcting the artifact and retrieve the desired, unperturbed fluorescence signals in fiber photometry. This approach simultaneously measures HbO, HbR, and the activity of a selected fluorescent sensor, providing an additional experimental capability to investigate the influence of neuronal or neurochemical activity on cerebral hemodynamics. Through a proof-of-principle study, we provide direct evidence that HRF is brain region dependent, suggesting that analysis of hemodynamics-based neuroimaging data should not apply a single HRF across all brain regions.

Limitations of the study
Lack of access to ground-truth GCaMP signals is a limitation when making unequivocal comparisons between the “corrected” GCaMP and the “real” GCaMP signals. Second, post-stimulus undershoot may not be significant in all fiber photometry studies. Finally, we recognize that applying this method in a non-spectrally resolved fiber photometry system might be challenging because multiple band-pass filters, each combined with a photodetector, are required.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
- **EXPERIMENTAL MODELS AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - Monte Carlo simulation for photon transport in fiber-photometry recording
  - Correction of Hb-absorption
  - Stereotactic surgery
  - Experimental setup
  - Animal subject preparation and physiology management
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2022.100243.

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AUTHOR CONTRIBUTIONS

W.-T.Z., T.-H.H.C., G.C., and Y.-Y.I.S. conceived the project and designed the experiments. W.-T.Z., T.-H.H.C., T.-W.W., E.A.O., J.Z., and R.N. implemented the methods. W.-T.Z., T.-H.H.C., Y.Y., S.-H.L., and N.C.P. analyzed data. W.-T.Z., T.-H.H.C., G.C., and Y.-Y.I.S. wrote the manuscript with input from all authors. H.Z., G.C., and Y.-Y.I.S. supervised the study and provided funding support.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** |        |            |
| AAVS-CaMKIIα-EYFP   | UNC Vector Core | N/A        |
| AAV9-CaMKIIα-GCaMP6f-WPRE-SV40 | U.Penn Vector Core | AV-9-PV3435 |
| AAV9-CAG-tdTomato   | UNC Vector Core | N/A        |
| AAV9-CaMKIIα-ChR2-EYFP | UNC Vector Core | N/A        |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Rhodamine B, 70,000 MW, Neutral | Fisher scientific | D1841 |
| Phosphate Buffered Saline (10 x) | Sigma-Aldrich | P4417 |
| Lidocaine HCl jelly (2%, 5mL) | UNC SSC Pharmacy | N/A |
| Meloxicam oral suspension | Henry Schein | N/A |
| **Software and algorithms** |        |            |
| MATLAB               | Mathworks | R2019b      |
| RStudio              | RStudio Inc. | Version 1.0.136 |
| Oceanview            | Ocean Insight | N/A |
| Correction for Hb-absorption artifact in fiber-photometry | In this paper | https://github.com/CAMRlatUNC/HemoCorrection |
| Computation of hemodynamic response function | Chao et al., 2022 | https://github.com/CAMRlatUNC/Photometry-tools |
| **Other**            |        |            |
| 488 nm laser and remote | Coherent | OBIS 488 LS (60 mW) |
| 561 nm laser and remote | Coherent | OBIS 561 LS (50 mW) |
| Spectrometer         | Ocean Insight | OEProl-FL |
| Optic patch cable    | Thorlabs | FT200EMT (200 mm, 0.39 NA) |
| Uncleaved fiber optic cannula | Thorlabs | CFMC12U-20 (200 mm, 0.39 NA) |
| Bipolar Tungsten electrode | P1 Technologies | MS3037/2C-B/SP ELEC .908/.50MM TW TUNGSTEN (MRI) |
| Dual-edge dichroic mirror | Chroma | ZT488/561rpc |
| Dual-band emission filter | Chroma | ZET488/561m |
| Smart control panel  | Med Associate inc. | DIG-716P2 |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for data and resources should be directed to and will be fulfilled by the lead contact, Yen-Yu Ian Shih (shihy@unc.edu).

Materials availability
This study did not generate new materials or new reagents.

Data and code availability
All original code has been deposited at Github and is publicly available as of the date of publication. DOIs are listed in the Key Resources Table.
Data reported in this paper will be shared by the lead contact upon request.
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
This study employed a total of 26 male Sprague Dawley (SD) rats weighing between 300–600g. All procedures were carried out in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals) and approved by the University of North Carolina (UNC) Institutional Animal Care and Use Committee. These rats were separated into 6 cohorts. In the first cohort (n = 3), EYFP was expressed in the bilateral S1FL using AAV5-CaMKII-EYFP (titer $4.2 \times 10^{12}$ molecules/mL, UNC Vector Core) to demonstrate the impact of Hb concentration changes on activity-independent fluorescence signals and evaluate the proposed correction method. In the second cohort (n = 4), GCaMP and tdTomato were co-expressed in the bilateral S1FL using a mixture of AAV9-CaMKIIz-GCaMP6f-WPRE-SV40 (titer $\geq 1 \times 10^{13}$ vg/mL, Penn Vector Core) and AAV9-CAG-tdTomato (titer $3.8 \times 10^{12}$ molecules/mL, UNC Vector Core) in a 5:1 ratio to demonstrate the impact of Hb concentration on a commonly used fluorescent sensor and evaluate the proposed correction method. In the third cohort (n = 3), GCaMP and tdTomato were co-expressed in the left PrL using a viral mixture identical to the second cohort. The fourth cohort (n = 4) received a twisted tungsten electrode implanted into the M1, and GCaMP and tdTomato were co-expressed in the contralateral M1 using a viral mixture identical to the second cohort. The fifth cohort (n = 5) was used in a supplementary study of isoflurane challenge and only GCaMP was expressed in the left M1. The sixth cohort (n = 4) received a twisted tungsten electrode implanted into the M1, and GCaMP and tdTomato were co-expressed in the contralateral M1 and ipsilateral striatum using a viral mixture identical to the second cohort. The seventh cohort (n = 3) was used in a supplementary study and had channelrhodopsin2 (ChR2) expressed in the ventroposterior thalamus (VP) using AAV9-CaMKIIz-ChR2-EYFP (titer $3.6 \times 10^{12}$ molecules/mL, UNC Vector Core) and GCaMP expressed in the ipsilateral S1 using AAV9-CaMKII-GCaMP6f-WPRE-SV40 (titer $\geq 1 \times 10^{13}$ vg/mL, Penn Vector Core). All rats in this study were housed under environmentally controlled conditions (12 h normal light/dark cycles, lights on at 7a.m.; 20–23°C and 40–60% relative humidity), with ad libitum access to food and water.

**EXPERIMENTAL MODELS AND SUBJECT DETAILS**

**Monte Carlo simulation for photon transport in fiber-photometry recording**

The Monte Carlo simulation of photon transport during fiber-photometry recording was achieved in two steps: 1) excitation light path simulation and 2) emission light path simulation. The goal was to first build a database composed of a large number of possible excitation light paths. After which, an emission starting point can be randomly selected from the excitation light path database for emission light path simulation.

For the excitation light path simulation, we built the database by repeatedly launching excitation photon packets that started with a unity weight = 1. The photon packets deposited weight due to absorption at each step of traveling through the simulated brain, and the photon packet’s current locations and remaining weights were logged along the way. Each excitation photon packet repeatedly underwent the following steps (Figure S3, left panel), until it was either terminated or transmitted out of the brain.

**Step 1: Launching a photon packet (Figure S3, left panel a and b):**

In our model, we launched our photon packets from an optic fiber tip with a diameter of 200 μm; the range of possible launch directions, or so-called acceptance angles, would be limited by the fiber numerical aperture (NA) according to Snell’s law. For applications, the NA is most commonly expressed as:

$$NA_{fiber} = n_i \sin \theta_a$$

(Equation 2)

where $\theta_a$ is the maximum ½ acceptance angle of the fiber, and $n_i$ is the index of refraction of the material outside of the fiber.

With this in mind, we simply set the initial launch position randomly within the surface of fiber tip, along with a random initial launch direction that was within the range of acceptance angles. This can be easily done by using three Cartesian coordinates $(x, y, z)$ and three direction cosines $(v_x, v_y, v_z)$ to determine the initial position and direction, respectively.

For initial position:

$$x = r_{fiber} \sqrt{\frac{1}{\xi_1}} \cos(2\pi \xi_2)$$

$$y = r_{fiber} \sqrt{\frac{1}{\xi_1}} \sin(2\pi \xi_2)$$

$$z = 0$$

(Equation 3)

For initial direction:

$$v_x = \sin(\theta_3 \xi_3) \cos(2\pi \xi_4)$$

$$v_y = \sin(\theta_3 \xi_3) \sin(2\pi \xi_4)$$

$$v_z = \cos(\theta_3 \xi_3)$$

(Equation 4)

where $r_{fiber}$ is the radius of the optic fiber, and $\xi_{1-4}$ are the independent random numbers between 0 and 1.

**Step 2: Step size selection and photon packet movement (Figure S3, left panel 1-2 and right panel 5–6):**

The step size $s$ is the distance that the photon packet travels between interaction sites. In our study, we selected a step size using the following function derived from the inverse distribution method and the Beer–Lambert law:
where $\xi$ is a random number between 0 and 1, and $\mu_t$ is the total interaction coefficient (the sum of the absorption coefficient ($\mu_a$) and scattering coefficient ($\mu_s$)) of brain tissue. The $\mu_a$ and $\mu_s$ for distinct wavelengths of a traveling photon were derived from the optical absorption and scattering properties provided by an online database from Oregon Medical Laser Center, Oregon Health & Science University (https://omlc.org/software/mc/mcxyz/index.html). We assumed 3% blood with 75% average oxygen saturation in our simulated brain.

Once the step size was selected, the photon packet traveled for distance $s$ in the direction defined by direction cosines, and the coordinates were updated as follows:

\[
x \leftarrow x + v_x s, \quad y \leftarrow y + v_y s, \quad z \leftarrow z + v_z s
\]  
(Equation 6)

Step 3: Absorption and scattering (Figure S3, left panel 3–4 and right panel 7–8):

At each interaction site, photon weight was partially absorbed by brain tissue, and the decreased fraction of the weight $\Delta w$ can be defined as:

\[
\Delta w = w(1 - e^{-\mu_t s})
\]  
(Equation 7)

The weight of the photon packet was then updated as follows:

\[
w \leftarrow w - \Delta w
\]  
(Equation 8)

Following absorption, the photon packet was scattered. The scattering direction was defined by both scattering angle $\theta$ and polar angle $\varphi$. The scattering angle was determined using the Henyey-Greenstein phase function (Henyey and Greenstein, 1941):

\[
\cos \theta = \left\{ \begin{array}{ll}
\frac{1}{2g} \left[ 1 + g^2 - \left( \frac{1 - g^2}{1 + 2g\xi} \right)^2 \right] & \text{if } g \neq 0 \\
1 - 2\xi & \text{if } g = 0
\end{array} \right.
\]  
(Equation 9)

where $g$ refers to scattering anisotropy, which has a value between $-1$ and 1. When $g$ is 0, it generally indicates that the scattering is isotropic. As $g$ approaches 1 or -1, the scattering is confined to forwards and backwards, respectively.

The polar angle was assumed to be uniformly distributed between 0 and $2\pi$, as below:

\[
\varphi = 2\pi \xi
\]  
(Equation 10)

With these angles and the original direction cosines, we were able to calculate the new set of propagation direction cosines, which can be represented in the global coordinate system as follows:

\[
v_x' = \begin{cases}
\frac{\sin \theta (v_x v_z \cos \varphi - v_y \sin \varphi) + \mu_x \cos \theta}{\sqrt{1 - v_x^2}} & \text{if } |v_x| \neq 1 \land |v_z| \neq 1 \\
\sin \theta \cos \varphi & \text{if } |v_x| = 1 \cup |v_z| = 1
\end{cases}
\]  
(Equation 11)

\[
v_y' = \begin{cases}
\frac{\sin \theta (v_x v_z \cos \varphi - v_y \sin \varphi) + \mu_y \cos \theta}{\sqrt{1 - v_z^2}} & \text{if } |v_y| \neq 1 \land |v_z| \neq 1 \\
\sin \theta \sin \varphi & \text{if } |v_y| = 1
\end{cases}
\]

\[
v_z' = \begin{cases}
-\sqrt{1 - v_z^2} \sin \theta \cos \varphi + v_x \cos \theta & \text{if } |v_x| \neq 1 \land |v_z| \neq 1 \\
\cos \theta & \text{if } |v_y| = 1 \\
-\cos \theta & \text{if } |v_y| = -1
\end{cases}
\]

Step 4: Photon termination

Steps 2 and 3 were repeated until the weight of the photon packet reached $10^{-4}$ or less, then the simulation for this particular photon packet was terminated. The excitation light path and weight of each step were saved to the database, after which a new photon packet simulation was started from step 1.

The emission light path simulation was similar to the excitation light path simulation. Specifically, we need to first determine the initial position, the initial direction, and the initial weight before launching an emission photon packet (Figure S3, right panel C-D).
The initial coordinates of each emission photon packet were randomly selected from the excitation light path database. All coordinates in the database had an equal probability to be selected regardless of excitation light path. If the selected coordinates were within an area containing fluorophore, its initial coordinates were determined first, and then the corresponding excitation light path history (from the start to the point where the emission occurred) was recorded. In this study, we assumed the fluorophore existed equally within a 1 mm diameter sphere of viral expression area centered 0.3 mm below the fiber tip.

The initial direction of an emission photon was assumed to have isotropic probability in all directions; therefore, the direction cosines can be derived using the following:

\[
\begin{align*}
\nu_x &= \sin(\pi \xi_1) \cos(2\pi \xi_2) \\
\nu_y &= \sin(\pi \xi_1) \sin(2\pi \xi_2) \\
\nu_z &= \cos(\pi \xi_1)
\end{align*}
\]  

(Equation 12)

Lastly, the initial weights were inherited from the remaining weights of the excitation photon packet at the initial coordinates.

Steps 2 and 3 were repeated until the weight of the photon packet reached \(10^{-4}\) or less, and then the simulation of this photon packet was terminated (step 4). In emission step 4, only when the photon packet reached the fiber tip at smaller than \(\frac{1}{2}\) acceptance angle and a weight beyond \(10^{-4}\), the paired excitation/emission light path and the weight of each step were saved to the database.

In order to improve signal-to-noise ratio, we collected 2–2.5 \(\times 10^6\) pairs of excitation and emission light paths.

The final weight (\(W_{\text{final}}\)) when the emission photon reaching the optical fiber tip represents the survival rate of the current combined excitation/emission light path. Therefore, the total average pathlength of excitation and emission lights are then calculated by summing each survived photon’s \(W_{\text{final}}\) multiplied by its paired excitation and emission pathlength respectively:

\[
\begin{align*}
X_{\text{ex}}(\lambda_{\text{em}}) &= \sum_{n=1}^{N} X_{\text{ex}}(n) \cdot e^{-\mu_a(l_{\text{ex}}) \cdot X_{\text{ex}}(n)} \cdot e^{-\mu_a(l_{\text{em}}) \cdot X_{\text{em}}(n)} = \sum_{n=1}^{N} X_{\text{ex}}(n) \cdot W_{\text{final}} \\
X_{\text{em}}(\lambda_{\text{em}}) &= \sum_{n=1}^{N} X_{\text{em}}(n) \cdot e^{-\mu_a(l_{\text{ex}}) \cdot X_{\text{ex}}(n)} \cdot e^{-\mu_a(l_{\text{em}}) \cdot X_{\text{em}}(n)} = \sum_{n=1}^{N} X_{\text{em}}(n) \cdot W_{\text{final}}
\end{align*}
\]  

(Equation 13)

Since the \(W_{\text{final}}\) is affected during both excitation and emission phase, the emission measured at different wavelengths have not only distinct average emission pathlength (\(X_{\text{em}}(\lambda_{\text{em}})\)), but also distinct average excitation pathlength (\(X_{\text{ex}}(\lambda_{\text{em}})\)) even when these emission wavelengths are excited by the same excitation wavelength. In this study, only the 488 nm excitation wavelength was used and simulated.

To plot the 488 nm excitation light illuminating distribution (Figures S3A S3B), all excitation light paths’ coordinates generated from the excitation light path simulation, regardless to the emission light detection, were first compressed into 2D coordinates, and then used for the 2D histogram. To plot the 515 or 580 nm emission light receptive probability map (Figures S3B–S3C), the initial emission 3D coordinate of each successfully detected 515 or 580 emission photons were compressed into 2D coordinates and shown in a 2D histogram.

**Correction of Hb-absorption**

Given the different absorption of HbO and HbR (Figure S1), their dynamic concentration changes must be quantified in order to properly correct fluorescent sensor signals. To address this, we proposed the following methods to derive changes in the HbO and HbR concentration (\(\Delta C_{\text{HbO}}\) and \(\Delta C_{\text{HbR}}\)) using spectral datapoints.

In a simple, non-absorbing and non-scattering medium, the measured fluorescence signal \(F\) at any time \(t\) is linearly related to the fluorescent protein concentration \(C(t)\):

\[
\frac{F(t)}{F(t_0)} = \frac{C(t)}{C(t_0)}
\]  

(Equation 14)

where \(F(t_0)\) and \(C(t_0)\) represent the fluorescence signal and the fluorescent protein concentration at the reference time point 0, respectively. When the absorption of the medium is considered, and \(F\) is measured at multiple wavelengths \(\lambda_{\text{em}}\), Equation (14) can be further modified according to an established model (Ma et al., 2016):

\[
\frac{F(t, \lambda_{\text{em}})}{F(t_0, \lambda_{\text{em}})} = e^{-\Delta \mu_a(t, \lambda_{\text{ex}}) X(\lambda_{\text{ex}}) + \Delta \mu_a(t, \lambda_{\text{em}}) X(\lambda_{\text{em}})} \cdot \frac{C(t)}{C(t_0)}
\]

Then after natural logarithmic transformation:

\[
\ln \left( \frac{F(t, \lambda_{\text{em}})}{F(t_0, \lambda_{\text{em}})} \right) = - \left( \Delta \mu_a(t, \lambda_{\text{ex}}) X(\lambda_{\text{ex}}) + \Delta \mu_a(t, \lambda_{\text{em}}) X(\lambda_{\text{em}}) \right) + \ln \left( \frac{C(t)}{C(t_0)} \right)
\]  

(Equation 15)

where \(\Delta \mu_a(t, \lambda_{\text{ex}})\) represents the change of the absorption coefficient \(\mu_a\) at excitation wavelength \(\lambda_{\text{ex}}\) at the time point \(t\) compared to the time point 0; \(\Delta \mu_a(t, \lambda_{\text{em}})\) represents the change of the absorption coefficient \(\mu_a\) at emission wavelength \(\lambda_{\text{em}}\) at the time point \(t\) compared to the time point 0; \(X(\lambda_{\text{ex}})\) and \(X(\lambda_{\text{em}})\) represent the photon traveling pathlengths at the given wavelength, respectively. Next, HbO and HbR concentration changes (\(\Delta C_{\text{HbO}}\) and \(\Delta C_{\text{HbR}}\)) as a function of time need to be incorporated. When using
488 nm excitation, the absorption term in Equation (15) that contains $\Delta \mu_i$ could be expressed as:

$$
\Delta \mu_i(t, \lambda_{Em}) = [\xi_{HBO}(488 nm) \Delta C_{HBO}(t) + \xi_{HBR}(488 nm) \Delta C_{HBR}(t)]X(\lambda_{Em}) + \Delta \mu_i(t, 488 nm)X(488 nm) + \Delta \mu_i(t, \lambda_{Em})X(\lambda_{Em})
$$

where $\xi_{HBO}$ and $\xi_{HBR}$ are well-established molar extinction coefficients at different wavelengths of HbO and HbR, respectively (Prahl, 1999).

At the time point $t$, the measured fluorescence ratio after natural logarithmic transformation, ranged from $\lambda_{Em}$ to $\lambda_{Em}$, could be put together in a vector form $M(t)$:

$$
M(t) = \begin{bmatrix}
\ln\left(\frac{F(t, \lambda_{Em})}{F(0, \lambda_{Em})}\right) \\
\vdots \\
\ln\left(\frac{F(t, \lambda_{Em})}{F(0, \lambda_{Em})}\right)
\end{bmatrix}
\tag{Equation 17}
$$

Additionally, two vectors A and B could be used to represent the following:

$$
A = \xi_{HBO}(488 nm)X(488 nm) + \begin{bmatrix}
\xi_{HBO}(\lambda_{Em}) \cdot X(\lambda_{Em}) \\
\vdots \\
\xi_{HBO}(\lambda_{Em}) \cdot X(\lambda_{Em})
\end{bmatrix}
\tag{Equation 18}
$$

$$
B = \xi_{HBR}(488 nm)X(488 nm) + \begin{bmatrix}
\xi_{HBR}(\lambda_{Em}) \cdot X(\lambda_{Em}) \\
\vdots \\
\xi_{HBR}(\lambda_{Em}) \cdot X(\lambda_{Em})
\end{bmatrix}
\tag{Equation 19}
$$

With any activity-independent fluorescence signals, the last component in $C^{(n)}$ 0 should be 0. Practically, we designate an error value $\sigma$. Taken together, Equation (15) could then be simplified and reorganized as Equation (20). To solve $\Delta C_{HBO}(t)$ and $\Delta C_{HBR}(t)$ at any time point $t$, we used generalized method of moment (GMM) (Hansen, 1982) to minimize L in Equation (21). By performing the GMM computation for each time point, we can obtain time-courses of $\Delta C_{HBO}(t)$, $\Delta C_{HBR}(t)$ and $\sigma$.

$$
M(t) + \Delta C_{HBO}(t) \cdot A + \Delta C_{HBR}(t) \cdot B - \sigma = 0
\tag{Equation 20}
$$

$$
L = \sum_{i=1}^{n} (M(t)_i + \Delta C_{HBO}(t)_i \cdot A_i + \Delta C_{HBR}(t)_i \cdot B_i - \sigma)^2
\tag{Equation 21}
$$

In this case, we have numerous spectral datapoints that can be used to resolve two unknowns that are of interest — $\Delta C_{HBO}(t)$ and $\Delta C_{HBR}(t)$. After which, $\Delta \mu_i(t, \lambda_{Ex})$ and $\Delta \mu_i(t, \lambda_{Em})$ can be calculated using Equation (16) and $F(t, \lambda_{Em})$ can be subsequently calculated using Equation (15) to correct for HbO and HbR absorptions.

**Stereotactic surgery**

For all surgical procedures, rats were initially anesthetized with 5% isoflurane, and maintained with a constant flow of 2–3% isoflurane mixed with medical air. Rectal temperature was continuously monitored and maintained within 37 ± 0.5°C using a feedback-controlled heating pad (Harvard Apparatus, Model 557.020, Holliston, MA). Rats were head-fixed to a stereotactic frame (Kopf Instruments, Model 962, Tujunga, CA). The skin was opened to expose the skull surface, and burr holes were prepared according to experimental coordinates. All coordinates used for microinjection are listed as follows. S1: AP = +0.5 mm and ML = +3.7 mm, DV = 1.3 mm, M1: AP = +1.8 mm and ML = −2.5 mm, DV = 2.0 mm, Striatum: AP = 0.5 mm and ML = 4.0 mm, DV = 5.2 mm, VP: AP = −2.8 mm and ML = +2.5 mm, DV = 6.5 mm, PrL: AP = 2.2 mm and ML = 0.9 mm, DV = 4.1 mm. Microinjections were performed at a flow rate of 0.1 µL/min for 1 µL, and an additional 10 min was given for virus diffusion prior to slow retraction of the microsyringe needle. The burr holes were then sealed with bone wax (Fisher Scientific, Pittsburgh, PA), and the wound was sutured. A month after virus microinjection, the skin was reopened to expose the skull, then bone wax was removed and optical fibers (200 µm in diameter; NA: 0.39) were chronically implanted to the coordinates 0.2–0.5 mm above the virus injection sites. Four MR-compatible miniature brass screws (Item #94070A031, McMaster Carr, Atlanta, GA) were anchored to the skull, then the surface of the skull was covered with dental cement to seal implanted components and the wound was sutured to further protect the surgical site.
At the end of each surgical procedure, lidocaine jelly (#866096, Henry Schein Inc., Melville, NY) was applied around the surgical wound to relieve pain and prevent the rat from scratching the wound. Meloxicam (#6451720670, Henry Schein Inc., Melville, NY) was also given by oral administration for further pain relief. Rats were allowed at least 1 week to recover from surgical procedures before any further experiments.

**Experimental setup**

The spectrally resolved fiber-photometry system in this study replicates an established system described previously (Meng et al., 2018). Laser beams from a 488 nm 60 mW continuous wave (CW) laser (OBIS 488 LS-60, Coherent, Santa Clara, CA) and a 561 nm 50 mW CW laser (OBIS 561 LS-50, Coherent, Inc.) are aligned and combined by broadband dielectric mirrors (BB1-E02, Thorlabs, newton, NJ) and a long-pass dichroic mirror (ZT488rdc, Chroma Technology Corp), then launched into a fluorescence cube (DFM1, Thorlabs, newton, NJ). Extra neutral density filters (NEK01, Thorlabs, newton, NJ) are placed between the combined laser beam and the fluorescence cube to adjust the final laser power. The fluorescence cube contains a dichroic mirror (ZT488/561rpc, Chroma Technology Corp) to reflect and launch the combined laser beam through an achromatic fiber port (PAFA-X-4-A, Thorlabs, newton, NJ) into the core of a 105/125 mm core/cladding multi-mode optical fiber patch cable (FG105UCA, Thorlabs, newton, NJ). The distal end of the patch cable is connected to an implantable optical fiber probe for both excitation laser delivery and emission fluorescence collection. The emission fluorescence collected from the fiber travels back along the patch cable into the fluorescence cube, passes through the dichroic mirror and an emission filter (ZET488/561 m, Chroma Technology Corp, Bellows Falls, VT), then launches through an aspheric fiber port (PAF-SMA-11-A, Thorlabs, newton, NJ) into the core of an AR-coated 200/230 mm core/cladding multi-mode optical fiber patch cable (M200L025-A, Thorlabs, newton, NJ). The AR-coated multi-mode patch cable is connected to a spectrometer (QE Pro-FL, Ocean Optics, Largo, FL) for spectral data acquisition, which can be operated by a UI software OceanView (Ocean Optics, Largo, FL). In order to achieve concurrent recording during fMRI, trigger mode is used in OceanView, where the photometry system is synchronized with MRI using an Arduino micro-controller board.

The stimulation system uses a DAQ board (1208Hs-2AO, Measurement Computing Corp., Norton, MA) to send out stimulus triggers according to the stimulus paradigm set in a homemade software program. During fMRI experiments, the DAQ synchronizes stimulation pulses via triggers from the MRI system. The stimulation pulses were driven by a constant current stimulus isolator (A385RC, World Precision Instruments, Sarasota, FL) for forepaw and micro-electrical stimulation experiments; and by a 473 nm blue diode laser (Shang Laser and Optics Century, BL473T8-200FC + ADR-800A, Shanghai, China) for optogenetic experiments.

**Animal subject preparation and physiology management**

The rats were initially anesthetized with 4% isoflurane (Vaporizer #911103, VetEquip Inc., Livernmore, CA, USA) mixed with medical air and endotracheally intubated using a 14G x 2” (>400 g) or 16G x 2” (<400 g) i.v. catheter (Surflash Polyurethane Catheter, TERUMO, newton, MA) and a capnometer was used to monitor heart rate, peripheral blood oxygen saturation, and end-tidal CO2 between 2.8 and 3.2%. End-tidal CO2 values from this capnometer system were previously calibrated against arterial blood gas, reflecting a partial pressure of carbon dioxide (pCO2) level of 30–40 mm Hg (Shih et al., 2012, 2013). For studies using Rhodamine B for CBV measurements, a bolus dose of 40 mg/kg (Sigma–Aldrich, St. Louis, MO) was injected via tail vein.

**Concurrent fMRI scan with fiber-photometry recording**

All fMRI data in this study were collected on a Bruker BioSpec 9.4-Tesla, 30 cm bore system with 6.0.1 on an AVANCE II console (Bruker BioSpin Corp., Billerica, MA). An RRI BFG 150/90 gradient insert (Resonance Research, Inc, Billerica, MA) paired with a Cole-Parmer, Vernon Hills, IL, USA) and a capnometer was used to monitor heart rate, peripheral blood oxygen saturation, and end-tidal CO2 (SURGIVET V90041LF, Smith Medical, Dublin, OH, USA). Body temperature was maintained at 37 ± 0.5 °C using a circulating water blanket connected to a temperature adjustable water bath (Haake S13, Thermo Fisher Scientific, Waltham, MA, USA). Ventilation tidal volume was adjusted to keep the heart rate at 300 ± 50 beats per minute, peripheral blood oxygen saturation above 90%, and end-tidal CO2 between 2.8 and 3.2%. End-tidal CO2 values from this capnometer system were previously calibrated against invasive sampling of arterial blood gas, reflecting a partial pressure of carbon dioxide (pCO2) level of 30–40 mm Hg (Shih et al., 2012, 2013). For studies using Rhodamine B for CBV measurements, a bolus dose of 40 mg/kg (Sigma–Aldrich, St. Louis, MO) was injected via tail vein.

Upon stabilizing the animals, a pair of needle electrodes was inserted under the skin of forepaw for stimulation, or electrode/optical fiber patch cables were connected according to the experimental design. Before connecting the fiber-photometry patch cable, all light in the room was turned off, the final output power of the 488 nm laser and 561 nm laser were 51.3 ± 18.6 μW (mean ± SD, n = 26), which were adjusted to balance spectral amplitudes (Meng et al., 2018). The 100 μW power is the maximum we suggested when the virus expression is less robust. This power range is consistent with published fiber-photometry studies (Gunaydin et al., 2014; Jones-Tabah et al., 2021, p.; Liang et al., 2017; Zhang et al., 2021). A background spectrum was measured as a reference by pointing the fiber tip to a nonreflective background in the dark room. This background spectrum was then automatically subtracted by OceanView during photometry recording.
Following setup processes, the cradle was pushed into MRI bore, and a bolus of dexmedetomidine (0.025 mg/kg; Dexdormitor, Orion, Espoo, Finland) cocktail with paralytic agent rocuronium bromide (4.5 mg/kg; Sigma–Aldrich, St. Louis, MO) was injected into the tail vein (Chao et al., 2018). Fifteen minutes after bolus injection, a continuous intravenous infusion of dexmedetomidine (0.05 mg/kg/h) and rocuronium bromide (9 mg/kg/h) cocktail was initiated and the isoflurane concentration was adjusted to 0.5–1% for the entire scanning period.

Magnetic field homogeneity was optimized first by global shim and followed by local first- and second-order shims according to B0 map. Anatomical images for referencing were acquired using a rapid acquisition with relaxation enhancement (RARE) sequence (slice thickness = 1 mm, TR = 1000 ms, TE = 8.1 ms, matrix size = 345 × 345, in plane resolution 0.32 mm, bandwidth = 250 kHz), with the same image slice geometry imported from the previously acquired T2-weighted anatomical image. A session of GE-EPI scans with 300 repetitions was taken, and at about the 100th scan, Feraheme (30 mg Fe/kg, i.v.) was administered for CBV percentage change calculations.

CBV fMRI data processing and statistical analyses
All fMRI data were analyzed using the Analysis of Functional Neuroimages (AFNI) (Cox, 1996) general linear model (GLM) framework (Worsley and Friston, 1995). All EPI images were skull-stripped, and slice-timing was corrected. Then automatic co-registration was applied to realign time-courses within subjects to correct subtle drift of EPI images. Finally all EPI images were aligned to a T2-weighted rat brain template (Valdés-Hernández et al., 2011) to generate normalized fMRI images to allow for group-level comparisons. Gaussian smooth (FWHM = 0.5 mm) was performed before feeding into the GLM fitting. To test the significant consistency of the stimulus-evoked responses at the group-level, we employed a parametric one-sample t test implemented in AFNI. The false discovery rate correction was used to adjust for multiple comparisons of fMRI maps (p < 0.05). A 3x3 voxel region of interest (ROI) was placed at the fiber tip in the S1 to extract fMRI time-course data. To account for Feraheme kinetics during the course of the experiments, the following equations were used to calculate the \( \Delta R_2^*(\text{baseline}) \), \( \Delta R_2^*(\text{stim}) \), and \( \Delta \text{CBV} \) (Decot et al., 2017).

\[
\Delta R_2^*(\text{baseline}) = \frac{1}{TE} \ln \left( \frac{S_{\text{prestim}}}{S_0} \right) 
\]

\[
\Delta R_2^*(\text{stim}) = \frac{1}{TE} \ln \left( \frac{S_{\text{stim}}}{S_{\text{prestim}}} \right) 
\]

\[
\Delta \text{CBV} = \frac{\Delta R_2^*(\text{stim})}{\Delta R_2^*(\text{baseline})}
\]

where \( S_{\text{prestim}} \) and \( S_0 \) represents MR signal intensity after and before Feraheme injection, respectively, and \( S_{\text{stim}} \) are the MR signal intensities during the stimulation.

Fiber-photometry spectral unmixing
Mixed spectra acquired by fiber-photometry were analyzed using a spectral linear unmixing algorithm (Meng et al., 2018). Briefly, at any time point \( n \), the mixed spectrum \( Y(n) \) was modeled as:

\[
Y(n) = \text{Coeff}_1(n) \times S_1 + \text{Coeff}_2(n) \times S_2 + C + \epsilon(n)
\]

where \( S_1 \) and \( S_2 \) are the normalized reference emission spectra of the two fluorescence signal sources. \( \text{Coeff}_1 \) and \( \text{Coeff}_2 \) are the unknown regression coefficients corresponding to the \( S_1 \) and \( S_2 \) respectively. \( C \) is the unknown constant, and \( \epsilon(n) \) is random error. \( \text{Coeff}_1(n) \), \( \text{Coeff}_2(n) \), and \( \epsilon(n) \) at each time point were estimated using the lm() function in the RStudio package (RStudio Inc. V1.0.136, Boston, MA).

Modeling the hemodynamic response function
The relationship between neuronal activity and hemodynamic response can be expressed as:

\[
HbT(T) = N(T) \Delta HRF(t) + c + d(T)
\]

where the \( HbT(T) \) represents hemoglobin fluctuation time-course, the \( N(T) \) represents neuronal activity time-course, the \( HRF(t) \) represents an impulse hemodynamic response function with \( t \) sampling points, \( c \) is a constant for baseline offset and \( d(T) \) is for linear drift over time. Assuming \( T = \{0, 1, 2, ..., m\} \), \( t = \{0, 1, 2, ..., n\} \), this equation can be expressed as:
Therefore, the $\text{HRF}(t)$, $c$ and $D$ (slope of $d(T)$) can be solved using Ordinary Least Squares solution with known $\text{HbT}(T)$ and $N(T)$. To avoid physiological noise contamination, $\text{HbT}(T)$ were low-pass filtered with a cutoff frequency at 0.5 Hz before the HRF estimation.

**Histology**

At the end of the experiments, the rats were euthanized with a mixture of 1–2 mL of sodium pentobarbital and phenytoin sodium (Euthasol, Virbac AH, Inc., Westlake, TX), and transcardially perfused with saline followed by 10% formalin. The brains were removed and stored in 10% formalin overnight, then transferred to a 30% sucrose solution (in 0.1 M phosphate buffer) for 2 to 3 days, until the brains sunk to the bottom of storage bottles. These brains were cut into serial coronal sections (40 μm) using a cryotome (#HM450, Thermo Fisher Scientific, Waltham, MA) and mounted on glass slides. The Fluoro-Gel II Mounting Medium (#17985-50, Electron Microscopy Sciences, Hatfield, PA) was covered on the brain slides to provide DAPI stain and for fluorescence imaging. The slides were imaged using a Zeiss LSM780 confocal microscope.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The fiber-photometry data analysis was carried out using customized codes in MATLAB (Mathworks, R2019b). A customized program was written in R to fit Hb changes and called by MATLAB internally. Linear regression (in Figure 5), two-way ANOVA, and paired t test were carried out using MATLAB (Mathworks, R2019b). Statistical data including means, sample numbers $n$, what $n$ represents and significance values were indicated in the main body or figure legends. One hemisphere in cohort 1 and three hemispheres in cohort 2 were excluded due to the lack of response to forepaw stimulation. All error bars in the figures are SD.
Supplemental information

Spectral fiber photometry derives hemoglobin concentration changes for accurate measurement of fluorescent sensor activity

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Figure S1. Molar extinction coefficients of HbO and HbR and power spectral distribution of several commonly used fluorescent sensors, related to Figure 1

(A) Broad-band presentation of Molar extinction coefficients of HbO and HbR as a function of wavelength.

(B) Zoomed from (A) in the range commonly used to measure fluorescent sensor activity.

(C) Power spectral distribution. Note that CBV was measured by intravenously injecting a long-circulating red fluorescent dye, Rhodamine B (rhoCBV). Data were acquired continuously to generate time-courses and then converted to power spectra density using Fourier transform.
Figure S2. Monte Carlo simulation of the light path and results, related to Figure 1

(A) Flow diagram of the light path simulation.

(B) Illuminating distribution of the excitation 488 nm light. Left showing the spatial probability map, and middle and right showing normalized luminance-distance profiles in x and z directions.

(C) Receptive probability of the 515 nm emission light. Left showing the spatial probability map, and middle and right showing normalized luminance-distance profiles in x and z directions.

(D) Receptive probability of the 580 nm emission light. Left showing the spatial probability map, and middle and right showing normalized luminance-distance profiles in x and z directions.

Color bars indicate probability from 0 to 1.
Figure S3. Ex vivo quantification of Hb-absorption effects on photometry recordings, related to Figure 2

(A) ΔF/F changes before and after adding 1 mL of fresh mouse venous blood or 1 mL vehicle (0.9% saline) to a 10 mL saline solution containing 10 µL of Alexa 488 and 10 µL of Alexa 568 fluorescent dyes. Adding saline decreased the concentrations of Alexa 488 and 568, which caused the slight but significant reduction in signal intensity. Error bars represent standard deviation. *** p < 0.001, t-test (n = 100 time points). ### p < 0.001, two-way ANOVA (n = 100).

(B) ΔF/F changes before and after 100% oxygen infusion into the solution containing blood or vehicle and fluorescent dye mixture. Error bars represent standard deviation. *** p < 0.001, t-test (n = 100 time points). ### p < 0.001, two-way ANOVA (n = 100).

(C) Normalized intensity spectra of the fluorescent solution mixture before and after adding blood or vehicle.

(D) Normalized intensity spectra of the blood-fluorescent solution mixture, or vehicle-fluorescent solution mixture, before and after oxygen infusion.
Figure S4. Number of continuous spectral datapoints needed to derive reliable changes in hemoglobin concentration and the comparison between rhoCBV and HbT, related to Figure 2.

(A) Representative $\Delta C_{HbO}$ time-courses derived from activity-independent EYFP data using different number of spectral datapoints ranging from 3 to 45 (centered at 523 nm).

(B) Reduction of white noise was observed with increased number of datapoints. The derived $\Delta C_{HbO}$ exhibited minimized white noise when the number of datapoints reaching 35.

(C) Power spectral distribution of the corrected rhoCBV and HbT derived from the EYFP signals.

(D) Two-dimensional histogram summarizing rhoCBV and HbT power. Each datapoint in the frequency series is assigned to a two-dimensional matrix to form the histogram. Correlation is significant at the 0.001 level (Pearson correlation, $r = 0.98$). The color bar indicates counts on the log scale.

(E) Two-dimensional histogram summarizing z-scores of rhoCBV and HbT. Each datapoint in the time-courses is assigned to a two-dimensional matrix to form the histogram. Correlation is significant at the 0.001 level (Pearson correlation, $r = 0.66$). The color bar indicates counts on the log scale.
Figure S5. Fiber-photometry in S1 with EYFP during hypercapnia (5% CO₂), related to Figure 2

(A-G) Peri-event heatmaps showing repeated trials and average response time-courses of the changes in measured rhoCBV, measured EYFP, derived HbO, derived HbR, derived HbT, corrected rhoCBV, and corrected EYFP, respectively. The gray-shaded segment indicates infusion of 5% CO₂. All color bars use the same unit as the Y axis of the time-course. Error bars represent standard deviation.

(H, I) Correction of the contaminated rhoCBV and EYFP signals, respectively, across all subjects and all trials (n=5, total trials =18; *** indicates $p < 0.001$, paired t-test).

(J) Power spectral distribution.

(K) Two-dimensional histogram summarizing rhoCBV and HbT power. Each datapoint in the frequency series is assigned to a two-dimensional matrix to form the histogram. Correlation is significant at the 0.001 level (Pearson correlation, $r = 0.98$). The color bar indicates counts on the log scale.

(L) rhoCBV exhibited a higher sensitivity than derived HbT. Error bars represent standard deviation. *** indicates $p < 0.001$ (paired t-test).

(M) Two-dimensional histogram summarizing rhoCBV and HbT z-scores. Each datapoint in the time-courses is assigned to a two-dimensional matrix to form the histogram. Correlation is significant at the 0.001 level (Pearson correlation, $r = 0.50$). The color bar indicates counts on the log scale.
Figure S6. Simultaneous optogenetic-evoked CBV-fMRI and fiber-photometry recording and comparison of the peak GCaMP responses before and after Hb-absorption correction, related to Figure 3

(A) GCaMP and channelrhodopsin-2 (ChR2) were virally expressed using AAV under the CaMKIIα promoter in the S1 and ventroposterior (VP) thalamus, respectively. Rhodamine B was injected intravenously to achieve CBV measurement.

(B) Confocal images showing GCaMP expression.

(C) MRI-CBV activation maps in response to optogenetic stimulation of VP.

(D) Uncorrected time-courses for GCaMP, rhoCBV, and MRI-CBV (n = 3, total trials = 33 trials). After a short optogenetic stimulation of VP (60Hz, 10mW power, 0.5 ms pulse width for 1 s), uncorrected GCaMP signal exhibited a strong undershoot with signal pattern mirrored the rhoCBV and MRI-CBV data, indicative of significant Hb absorption in vivo.

(E) The peak amplitude of the GCaMP responses is significantly higher in the corrected GCaMP compared to the uncorrected GCaMP. (***) indicates p<0.001, paired t-test, n=27)
Figure S7. Hb-absorption effects on fiber-photometry measurement during 5% isoflurane challenges and pharmacological studies, related to Figure 4

(A) Preparation for fiber-photometry measurement of GCaMP and Rhodamine B signals in left M1. Ca²⁺-independent GCaMP signal (GCaMP₄₀₅) was excited by a 405 nm laser and used for hemodynamic correction. rhoCBV signals were measured as described in Figure 2.

(B) Measured and corrected GCaMP₄₀₅ time-courses before, during, and after 2-min 5% isoflurane challenges (baseline = 0.5% isoflurane). The gray-shaded segment indicates the isoflurane period. Error bars represent standard deviation.

(C) Scatter plots of measured and corrected GCaMP₄₀₅ signals at 1, 2, 3, 4, 5, 6, and 7 min after the onset of isoflurane challenge (n = 5, p < 0.001, two-way ANOVA).

(D) Derived HbO, HbR, and HbT time-courses before, during, and after isoflurane challenge.

(E) Measured and corrected rhoCBV time-courses before and after the isoflurane challenge. Rhodamine B readout could be misinterpreted without hemodynamic correction.

(F) Scatter plots of measured and corrected rhoCBV signals at 1, 2, 3, 4, 5, 6, and 7 min after the onset of isoflurane challenge (n = 5, p < 0.001, two-way ANOVA).

(G) Fiber-photometry measurement of GCaMP and tdTomato signals in left M1.

(H) Measured and corrected tdTomato time-courses before and after administering an α₂ adrenergic antagonist Atipamezole (1 mg/kg, s.c., Zoetis, Parsippany, NJ).

(I) Scatter plots of measured and corrected tdTomato signals at 2, 4, 6, 8, and 10 min after atipamezole administration (n = 3, p < 0.001, two-way ANOVA).

(J) Derived HbO, HbR, and HbT time-courses before and after atipamezole administration. Increased CBV could be the result of redistribution of blood supply to active brain regions (Bekar et al., 2012).

(K) Measured and corrected GCaMP time-courses before and after atipamezole administration. The increased GCaMP activity in the cortex would have been otherwise unchanged without hemodynamic correction.
(L) Scatter plots of measured and corrected GCaMP signals at 2, 4, 6, 8, and 10 min after atipamezole administration (n = 3, p < 0.05, two-way ANOVA).
Figure S8. HRF calculation and validation, related to Figure 6

(A) Pipeline for estimating HRF from tdTomato-derived HbT and GCaMP signals. Yellow arrows indicate post-stimulus undershoot caused by Hb absorption. Note that using uncorrected GCaMP caused an HRF amplitude shift at t=0.

(B) Strategy to deconvolve HRF from preprocessed HbT and GCaMP signals.

(C) Validation of the HRF using a separate dataset naïve to the HRF calculation. HRF was convolved with GCaMP data to predict HbT changes (black trace). A high correlation was observed between the predicted and tdTomato-derived HbT (purple trace).
**Figure S9.** Simulated detection volume, correlation of signals drops with CBV changes, and using regression to correct GCaMP signals, related to Figure 3

**(A)** Simulated detection volume of fiber-photometry and its projected probability.

**(B)** Two-dimensional histogram summarizing % changes of corrected rhoCBV and measured EYFP signal. Each datapoint in the time-courses is assigned into a two-dimensional matrix to make the histogram. Correlation is significant at the 0.001 level (Pearson correlation, \( r = -0.62 \)). Color bars indicate datapoint counts. Linear fit (red line) and 95% confidence interval (pink lines) are also shown.

**(C)** Two-dimensional histogram summarizing % changes of MRI-CBV and measured tdTomato signal. Each datapoint in the time-courses is assigned into a two-dimensional matrix to make the histogram. Correlation is significant at the 0.001 level (Pearson correlation, \( r = -0.33 \)). Color bars indicate datapoint counts. Linear fit (red line) and 95% confidence interval (pink lines) are also shown.

**(D)** Correcting GCaMP signals by regressing out the HbT with short (1 s) stimulation

**(E)** Correcting GCaMP signals by regressing out the HbT with long (10 s) stimulation.
Figure S10. Quantification of Hb-absorption effects using residue signals of fiber-photometry, related to Figure 3

(A) Preparation for fiber-photometry measurement of the GCaMP signal in S1 of the forelimb region.

(B) Confocal images showing GCaMP expression.

(C) Simultaneous dual-spectral photometry recording of GCaMP and rhoCBV.

(D-I) Peri-stimulus heatmaps showing repeated trials and average response time-courses of the changes in uncorrected GCaMP, measured rhoCBV, derived HbO, derived HbR, corrected GCaMP, and derived HbT, respectively. The gray-shaded segment indicates forepaw-stimulation period. All color bars use the same unit as the Y axis of the time-course. Error bars represent standard deviation.

(J) Correction of the post-stimulus dip of the GCaMP signals (n = 2, total trials = 6; ** indicates p < 0.01, paired t-test).

(K) Two-dimensional histogram summarizing rhoCBV and HbT z-scores. Each datapoint in the time-courses is assigned into a two-dimensional matrix to make the histogram. Correlation is significant at the 0.001 level (Pearson correlation, r = 0.28). Colorbar indicates the number of datapoints. Linear fit (red line) and 95% confidence interval (pink lines) are also shown.
