Spatiotemporal precision and hemodynamic mechanism of optical point spreads in alert primates

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In functional brain imaging there is controversy over which hemodynamic signal best represents neural activity. Intrinsic signal optical imaging (ISOI) suggests that the best signal is the early darkening observed at wavelengths absorbed preferentially by deoxyhemoglobin (HbR). It is assumed that this darkening or “initial dip” reports local conversion of oxyhemoglobin (HbO) to HbR, i.e., oxygen consumption caused by local neural activity, thus giving the most specific measure of such activity. The blood volume signal, by contrast, is believed to be more delayed and less specific. Here, we used multiwavelength ISOI to simultaneously map oxygenation and blood volume [i.e., total hemoglobin (HbT)] in primary visual cortex (V1) of the alert macaque. We found that the hemodynamic “point spread,” i.e., impulse response to a minimal visual stimulus, was as rapid and retinotopically specific when imaged by using blood volume as when using the initial dip. Quantitative separation of the imaged signal into HbO, HbR, and HbT showed, moreover, that the initial dip was dominated by a fast local increase in HbT, with no increase in HbR. We found only a delayed HbR decrease that was broader in retinotopic spread than HbO or HbT. Further, we show that the multiphasic time course of typical ISOI signals and the strength of the initial dip may reflect the temporal interplay of monophasic HbO, HbR, and HbT signals. Characterizing the hemodynamic response is important for understanding neurovascular coupling and elucidating the physiological basis of imaging techniques such as fMRI.

fMRI | imaging | macaque | visual | neurovascular coupling

Cerebral hemodynamics respond quickly and specifically to local neural activity (1, 2). Hemodynamic signals are thus used extensively as proxies for such activity in functional neuroimaging techniques like fMRI and intrinsic signal optical imaging (ISOI). There has been considerable debate, however, as to which of the possible hemodynamic signals, e.g., changes in local blood oxygenation, volume, or flow, with their distinct response properties, constitutes the “best” signal for inferring neural activity (1, 3–5).

The debate regarding the best signal sharpened with reports of initial dips in both ISOI and fMRI signals. ISOI studies consistently found a brief stimulus-evoked darkening followed by a strong brightening at imaging wavelengths preferentially absorbed in deoxyhemoglobin (HbR) [e.g., at 605 nm (6)]. The darkening, later termed the initial dip (7), was interpreted as a local conversion of oxyhemoglobin (HbO) to HbR caused by increased oxygen consumption by local neurons before any active vascular response (1, 7). The subsequent brightening was taken to measure the “rebound” in [HbO] caused by a delayed, stimulus-triggered increase in cerebral blood flow (7).

In parallel, some fMRI studies reported finding an initial dip before the rise in the blood oxygen level-dependent (BOLD) signal (8). Because the BOLD signal measures changes in [HbR] alone, the fMRI initial dip was seen as clear evidence for an initial increase in [HbR]. It was equated to the ISOI dip (8) and was argued to be the best marker of neural activity, free of the venous artifacts seen in positive BOLD (8). By extension, positive BOLD was equated to the ISOI rebound (9). A key implication of these studies was the presence of a significant delay between the start of neural activity-triggered oxygen consumption, believed to underlie the initial dip, and the subsequent active vascular response generating the rebound in fMRI or ISOI (4, 7, 9, 10). The ISOI studies also suggested that the rebound was more spatially diffuse and less stimulus-specific than the initial dip (4, 7, 10). The initial dip thus became the signal of choice in a number of functional imaging studies, not only in ISOI (11–15) but also in fMRI (16).

There are a number of unresolved issues, however, questioning the interpretation of the ISOI initial dip as local deoxygenation preceding the active vascular response. First, the initial dip, while robust and reliable in ISOI, has proved to be elusive in fMRI (3, 9). Next, oxygen consumption preceding increased blood flow should reduce [HbO] in addition to increasing [HbR]. However, even studies proposing an initial deoxygenation failed to see the expected [HbO] decrease (7). Finally, a number of ISOI studies in the rodent that quantitatively separated the imaging signal into [HbR] and [HbO] either failed to see any transient [HbR] increase (17–20) or found a simultaneous rise in [HbO] consistent with a stimulus-evoked increase in total hemoglobin [HbT] (21, 22).

The ISOI dip has been successfully used to map cortical columns (e.g., orientation or ocular dominance) where activated columns darken more than inactive ones. However, despite only a transient initial dip in the global signal, the mapping signal (i.e., the additional darkening in active columns) remains throughout the entire duration of the hemodynamic response despite the rebound observed in the global signal. The mapping signal is typically interpreted as a maintained increase in [HbR] in the activated columns, persisting through the presumed spatially diffuse rebound of oxygenated blood (7, 23). Such a maintained [HbR] increase demands a sustained elevation of local oxygen consumption for many seconds (>10 s) after cessation of neural activity (23). This interpretation is not supported by fMRI. The BOLD mapping signal shows a tuned increase in activated columns, indicating a spatially specific decrease of [HbR], during the rebound (24). The observed orientation-tuned decrease in [HbR] suggests that the prolonged mapping signal is not caused by deoxygenation and, by extension, also raises doubts about the standard interpretation of the initial dip.

These conundrums might be resolved by considering alternate explanations, other than deoxygenation, for the darkening seen at typical imaging wavelengths (e.g., 605 nm). Darkening at these wavelengths may result from a rise in local blood volume, increasing...
absorption by both HbO and HbR, or even an increase in [HbO] alone. Most hemoglobin in cerebral blood is highly oxygenated [saturation between 95% and 60% (25)] and HbR is a significant absorber at all imaging wavelengths. Even small fractional increases in [HbO] could yield increased absorption. The current study was designed to test these distinct interpretations of the ISOI signals with a focus on the initial dip.

Results
We analyzed stimulus-evoked hemodynamic signals in the brain by using dual-wavelength ISOI in primary visual cortex (V1) of the alert behaving macaque (ref. 26; two animals V and S). With this technique we imaged the hemodynamic point spread, i.e., the impulse response to a minimal visual stimulus (a ¼ deg line) flashed (1 s) while the animal held its gaze steady in a fixation task (4-s fixation periods, trial length 10 s). Stimulated trials alternated with nonstimulated trials (giving 20 s between stimuli for hemodynamic response to return to baseline). The imaging wavelengths were chosen pairwise from three light-emitting diode (LED) sources (Fig. L4): a “volumetric” LED (green, centered at 530 nm) that was absorbed equally in HbO and HbR and giving a measure of [HbT] or blood volume; and “oximetric” LEDs (orange: center 605 nm; red, center 630 nm), absorbed more strongly in HbR than HbO, and thus being sensitive to blood oxygenation changes. Consistent with earlier findings, the signals at oximetric wavelengths showed a robust initial dip followed by a rebound and then a delayed undershoot (secondary darkening). Notably, however, volumetric signals started at the same time as the initial dip and showed similar spatial specificity.

To get a quantitative measure of the responses as blood oxygenation and volume we used the absorption spectra of HbR and HbO to spectrally decompose the measured imaging signals into [HbO], [HbR], and [HbT]. Notably, we used the Beer-Lambert Law modified to account for the wavelength-dependent optical path lengths in cortical tissue at different imaging wavelengths (see Methods and refs. 17 and 27). Path length correction, although missing from prior work in monkeys and cats, is necessary to avoid errors in the spectral decomposition and interpretation of ISOI signals (17, 27). This quantitative analysis showed that the earliest stimulus-evoked response was driven by a rapid increase in [HbT] with no change in [HbR]. The full multiphasic time course of typical imaging signals, including the initial dip, rebound, and undershoot, could be well explained by a fast increase in [HbT] preceding a more transient decrease in [HbR].

Spatial and Temporal Properties of Absorption Point Spreads Measured at Different Wavelengths. The point spread at oximetric wavelengths (605 and 630 nm) had a triphasic time course with an
initial darkening [i.e., increased absorption; Figs. 1B and 2A: initial dip; 605 nm: peak at 1.5 s, dR/R = 0.31 (0.05)%; 630 nm: peak at 1.3 s, dR/R = 0.05 (0.03)%]⁴, a subsequent larger decrease in absorption [rebound; 605 nm: peak at 3.7 s, dR/R = 0.69 (0.09)%; 630 nm: peak at 3.6 s, dR/R = 0.49 (0.05)%], and finally a small secondary increase in absorption [undershoot; 605 nm: peak at 8.0 s, dR/R = 0.15 (0.04)%; 630 nm: peak at 8.8 s, dR/R = 0.04 (0.02)%]. Volumetric responses at 530 nm, by contrast, were large and monophasic [peak at 2.9 s, dR/R = 3.3 (0.2 %)]. The spatial properties of these signals are summarized in Table 1. For comparison, the 75% half-width of the point spread measured in V1 using voltage-sensitive dyes was ~0.6 mm (estimated from ref. 28).

The interpretation of the initial dip as a focal deoxygenation preceding a delayed and spatially diffuse active vascular response leads to specific predictions when comparing oximetric and volumetric signals. (i) The oximetric point spread should appear first. Any volumetric response should appear considerably later, well after the onset of the initial dip. (ii) This volumetric response should be distributed more diffusely over cortex than the initial dip. (iii) During the oximetric rebound (which is presumably dominated by the blood volume response), the oximetric and volumetric responses should have comparable spatial extents. To test these predictions, we compared the volumetric and oximetric point spreads at the three phases of the oximetric signal. Our results failed to bear out any of the above predictions.

### Table 1. Half-widths of the hemodynamic response point-spreads over V1

<table>
<thead>
<tr>
<th>Time</th>
<th>Oximetric</th>
<th>Volumetric</th>
<th>HbR</th>
<th>HbO</th>
<th>HbT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 s (dip)</td>
<td>0.48 (0.08)</td>
<td>0.5 (0.2)</td>
<td>–</td>
<td>0.5 (0.2)</td>
<td>0.5 (0.1)</td>
</tr>
<tr>
<td>3.0 s (rebound)</td>
<td>1.15 (0.08)</td>
<td>0.60 (0.05)</td>
<td>0.80 (0.03)</td>
<td>0.66 (0.04)</td>
<td>0.61 (0.05)</td>
</tr>
<tr>
<td>9.0 s (undershoot)</td>
<td>0.6 (0.2)</td>
<td>0.57 (0.05)</td>
<td>0.59 (0.03)</td>
<td>0.58 (0.04)</td>
<td>0.58 (0.04)</td>
</tr>
</tbody>
</table>

①All data cited are averaged across 11 experiments in two animals.

②Half-widths measured at the 75% assess the focal spatial resolution of the point spread.

### Delayed Oximetric Rebound.
Volumetric Signal Remains Spatially Focused, Unlike the Diffuse and Delayed Oximetric Rebound. Both the volumetric and oximetric responses broadened during the rebound. However, the spatial spread at the center of the volumetric response remained largely unchanged, maintaining a sharp profile. At its peak (3 s after stimulus onset), the 75% half-width of the volumetric response grew by only 13% [half-width: from 0.5 (0.2) to 0.60 (0.05) mm] despite a 13-fold increase in amplitude [from 0.26 (0.09) % to 3.36 (0.16) %] (Table 1 and Fig. 2A, C, and E). By contrast, the spatial spread at the center of the oximetric response broadened significantly during the rebound, taking on a distinctly rounded profile [75% half-width grew from 0.48 (0.08) to 1.15 (0.08) mm, an increase of a factor of 2.4; P = 9e-6, n = 11]. It is noteworthy that the cross-sectional shape and spatial extent of the volumetric point spread closely matches those observed with voltage sensitive dyes (28, 29). This finding suggests that the volumetric response, is not only distinct from the oximetric rebound, but is likely a better neuroimaging signal tightly coupled to underlying neural activity.

### Spectral Decomposition.
The spectrally decomposed [HbO], [HbR], and [HbT] signals showed similar spatially localized responses, each monophasic in time (Table 1 and Fig. 3). To relate absorptive changes at oximetric wavelengths during the initial dip, rebound, and undershoot to these analyzed hemodynamic components we examined the point-spreads in HbR and HbT separately during these phases.

### Initial Dip Is Dominated by HbT.
Surprisingly, we saw no significant change in [HbR] during the initial dip (0.8 s after stimulus, P = 0.9, n = 11; Fig. 3 A and B and Fig. S2). Unlike the darkening at oximetric wavelengths, [HbR] at this time point had no reliable spatial structure. Instead, the response was entirely dominated by [HbO] (P = 0.02, Fig. 3 B–D), whereas [HbR] showed only a delayed monophasic decrease. The [HbT] point spread was identical to the volumetric point spread (Figs. 1 and 2 and Table 1). Our data suggest that the initial dip at oximetric wavelengths is indeed a result of an early and spatially localized increase in [HbT] and not an increase in [HbR]. These results were independent of the wavelength pairs used for imaging (Figs. S2 and S3) or the model parameters used for decomposition (see Methods and Fig. S4).

### Late Undershoot at the Oximetric Wavelengths Matches the Spatial Extent of the Volumetric Signal.
At oximetric wavelengths we often detected a small increase in absorption after the rebound that was spatially localized [half-width at 75% = 0.6 (0.2) mm; Table 1 and Fig. 2F]. Other investigators have shown this “late undershoot” emerging many seconds after the cessation of neural activity and have suggested that it may indicate a secondary increase in [HbR] (30). This late oximetric signal matched spatially the long-lived volumetric signal [volumetric half-width = 0.57 (0.05) mm], suggesting that both could reflect lingering blood volume.

### Observed Oximetric Rebound Is Likely Shaped by an Interaction Between HbT and HbR.
We found that the [HbT] signal during the rebound had a spatial spread that was consistently, albeit slightly narrower than the concomitant decrease in [HbR] (Table 1 and Fig. 3E arrowhead: [HbR] width = 0.80 (0.03); [HbT]: 0.61 (0.05); P = 0.0017) (31, 32). Notably, both the [HbR] and [HbT] profiles were...
sharply peaked at the center and much narrower than the raw oximetric rebound [half-width $/H_{11015}/1.15$ (0.08) mm; $P/H_{11015}/0.0007$; Table 1 and Fig. 2]. Thus, neither the $[HbT]$ nor the $[HbR]$ signal by itself can account for the width and the diffuse peak of the oximetric rebound.

We hypothesize that the oximetric rebound reflects the difference between the absorption increase from increasing $[HbT]$ and the somewhat broader absorption decrease from decreasing $[HbR]$. A simple model of such a difference image, using computed profiles for $[HbR]$ and $[HbT]$, plausibly reproduces the shape of the rebound (Fig. S5).

Late Undershoot Is a Result of Residual HbT. Our analysis also confirmed that the late darkening is dominated by slowly decaying $[HbT]$, persisting for 15 s, rather than a late increase in $[HbR]$.

Predicting the Imaging Signal Time Course Across Wavelengths. Our calculated hemodynamic time courses, although obtained with three imaging wavelengths, correctly predict ISOI time courses observed across the full spectrum (Fig. 4). These predictions were made by using the calculated early increase in $[HbT]$ (increased darkening) and delayed decrease in $[HbR]$ (decreased darkening). Signals at isosbestic wavelengths result from a rapid, monophasic darkening matching the $[HbT]$. For wavelengths away from the isosbestic point, with progressively stronger absorption by $HbR$ vs. $HbO$, the delayed $[HbR]$ decrease (Fig. 3B) becomes progressively more prominent. This delayed $[HbR]$ decrease leads to a delayed reduction (e.g., at 592 nm) or reversal of the initial $HbT$-linked darkening (e.g., at $/H_{600-720}/$ nm where the predicted signal displays the familiar initial dip and rebound). These predicted time courses match published results (e.g., figure 2 of ref. 19).
Fig. 4. Changes in blood volume predict size of initial dip. (A) Molar extinction coefficient for HbR and HbO. Vertical dashed lines indicate isosbestic points. Shaded regions indicate: ranges where HbR absorbs more strongly than HbO. (B) Predicted response time courses at wavelengths moving from isosbestic to oxymetric marked in A, showing progression from a monophasic signal at the isosbestic 584 nm to a biphasic signal at 600 nm. (Note: The predicted signal time courses here are for the pure spectral wavelengths indicated; while qualitatively similar to our measured imaging signals they are quantitatively different because our LED sources had finite bandwidths giving the corresponding admixture of responses. All quantitative calculations using our LED sources accounted for this finite bandwidth; see Methods). (C) The predicted ratio of the size of the initial dip relative to the rebound across wavelength. Note an explosive increase in this ratio toward isosbestic points. (D) Predicted spatial profiles at 3 s poststimulus onset normalized to maximum response amplitude. Wavelengths same as in B. Note broader spreads at wavelengths showing a rebound. (Inset) Not normalized for amplitude.

particular, wavelengths with stronger absorption in HbR vs. HbO show stronger and more prolonged rebounds after weaker and more transient initial dips (Fig. 4 B and C) matching our own (Fig. 2B) and previous observations (30) of stronger dips closer to the isosbestic point. By contrast, a model of the initial dip as deoxygenation failed to account for these observations, yielding similarly sized dips across wavelengths, and delayed darkening near isosbestic points (Fig. S6).

Our findings suggest that the multiphasic temporal profile of the ISOI signals at typical oximetric wavelengths, including the magnitude and duration of the initial dip, rebound, and undershoot, reflect the interplay of absorptions by HbT and HbR and are problematic as estimates of neural oxygen metabolism.

Discussion

With ISOI in alert macaque we observed the expected, robust initial dip. Our interpretation of this dip is very different, however, from earlier interpretations in macaque or cat. The likely reason for this difference is that we separated our imaging signal into [HbR] and [HbO] by using a model that incorporates changes in optical path length caused by the large changes in absorptivity across wavelengths (27). The earlier results (7, 23) were obtained with fixed path lengths, which, as now widely acknowledged (4, 10), can lead to significant errors in spectroscopic analysis (17, 27). Our results are robust over a broad range of model parameters when using wavelength-dependent path lengths (Fig. S4). However, control analyses of our imaging data assuming fixed path lengths resulted in transient initial increases in [HbR] similar to the earlier published results.

Although we applied these analysis techniques to ISOI in the macaque, our results are consistent with a large body of work pioneering similar analysis in rodents [including alert rodents (22)] and alert humans (33). Those studies arrived at conclusions similar to ours: that the primary response after stimulus onset is a rapid and robust increase in [HbT]. Those groups either failed to see any initial increase in [HbR] (e.g., refs. 18–20) or found only a modest increase accompanied by a large rise in [HbO], consistent with a net increase in [HbT] (e.g., refs. 21, 22, and 34). Discrepancies between rodent and earlier monkey results had been attributed to species differences (35, 36). By contrast, our findings add to a growing body of evidence suggesting that neurovascular coupling mechanisms may be conserved across species.

Independent of modeling, we see that the volumetric (530 nm) signal is as fast as the oximetric (605 or 630 nm) initial dip, indicating no delay in the rise of [HbT]. We identified signal onset times by using a quantitative statistical measure. Our findings are consistent with those from fMRI (35) and rat ISOI (18, 19). Prior work in cats and monkeys suggested that changes in HbT are delayed, but that delay could be an artifact of signal normalization and appears to be contradicted by other data from the same group (e.g., figure 3 a and b in ref. 7). Our results argue against any significant delays between the start of oxygen extraction from blood and the initiation of the active vascular response (4, 7).

It is important to note that the lack of increased [HbR] does not preclude increased oxygen consumption or decreased tissue pO2 caused by neural activity (19). Stimulation leads to a rapid (<400 ms) increase in local cerebral blood flow (18, 19, 37), increasing [HbO] in local blood vessels (38). This increase in flow could support increased oxygen consumption and a decrease of tissue pO2 even as the local blood supply gets enriched in [HbO] and depleted of [HbR] (19). Our findings highlight the complexity of the relationship between measures of blood volume, flow, and oxygenation with local metabolic demand or neural activity.

Our observations could shed light on the elusive nature of the fMRI initial dip compared with the ISOI. Unlike in ISOI, a dip in BOLD equals increased [HbR]. However, a large number of careful fMRI investigations have either failed to see any initial dip (e.g., refs. 24 and 35), found it unreliable (39), or obtained it only after unnaturally intense stimulation (40). Our work together with the large body of work in rodents suggests an explanation: the hemodynamic response during the ISOI dip (unlike in fMRI) is driven by a rapid active vascular response, dominated by increases in [HbT], and only occasionally accompanied by small increases in [HbR].

Our results suggest that blood volume ([HbT]) is a more reliable signal to use for neuroimaging than the initial dip. The initial dip likely carries no privileged information about neural activity or metabolic demand. The [HbT] signal is as rapid and spatially focused and more than an order of magnitude stronger and
alert-monkey optical imaging techniques (23) were used to record the intrinsic cortical signal, continuously, through a clear artificial dura and glass-fronted recording chamber mounted over the animals’ V1. The brain surface was illuminated by using two LED arrays chosen from three sets with wavelengths centered at 530, 605, and 630 nm. The LED arrays were switched on and off alternately in synchrony with the camera (15 Hz) giving, in effect, simultaneous optical imaging at 7.5 Hz at the separate LED wavelengths.

Importantly, the imaging signals were spectrally decomposed by using modified Beer-Lambert equations (3, 4) to estimate wavelength-dependent optical path lengths; the cortical tissue was modeled as a scattering medium with embedded absorbers (HbO, HbR). The path lengths of light traveling into and out of cortex were estimated separately for each LED wavelength by using Monte Carlo simulations. These wavelength-dependent path lengths were then used to decompose the measured image signal into the underlying [HbR] and [HbO]

Details of animal training, visual stimuli, optical imaging, and data analysis are in SI Appendix. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of Columbia University and the New York State Psychiatric Institute.

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Supporting Methods.

Behavioral Task: We trained two macaque monkeys to perform a simple fixation task for liquid reward (N=9 experiments in monkey S; N=2 experiments in monkey V). We tracked the monkeys’ eye position using an infra-red eye tracker and custom software (1). Each trial began with the onset of a small red fixation point (0.05 deg) at the center of the monitor (20” Sony Trinitron; 100 Hz refresh). When the monkey attained fixation, the fixation point would turn green and the monkey would be required to maintain fixation within 0.5 deg for 4 sec. Stimuli were presented for 1 sec during the fixation period. If the monkey maintained proper fixation throughout the fixation interval it received a drop of fruit juice as reward. Any premature eye movements aborted the trial and it was subsequently excluded from analysis. To ensure that there was no crosstalk between the long-lived responses to each stimulus presentation, we put additional, non analyzed, blank trials between each stimulus (and blank) trial making the total inter-stimulus-interval 20 sec. The experiment continued until the monkey received enough juice and stopped working.

Visual Stimuli: Stimuli were short 0.25 deg bars of light generated as bitmaps in MATLAB (Mathworks, Natick, MA) presented for 1 sec on a dark background at maximum contrast using the VSG2/5 graphics card (Cambridge Research Systems, UK). To measure any trial-related imaging signals, some of the trials carried no stimulus, but were otherwise identical (‘blank trials’).
Optical imaging: Surgery, recording chambers, artificial dura: After the monkeys were trained on visual fixation tasks, craniotomies were performed over the animals’ V1 and glass-windowed stainless steel recording chambers were implanted, under surgical anesthesia, using standard sterile procedures. The exposed dura was resected and replaced with a soft, clear silicone artificial dura. After the animals had recovered from the surgery, cortical activity from their V1 was optically imaged, routinely, while the animals engaged in relevant behavioral tasks. Recording chambers and artificial dura were fabricated in our lab using published methods (2).

Optical Imaging: Data acquisition: Images were acquired using a CCD camera (Dalsa 1M30, resolution 256x256 pixels, 15 frames / sec) through an Optical PCI Bus Digital Frame Grabber (Coreco Imaging, Boston, MA). Imaging software was developed in our lab based on a system by V. Kalatsky (3). The camera was coupled to a back-to-back lens combination macroscope (two 55mm Nikon lenses; 1:1 magnification, Numerical Aperture = 0.4) and focused on the cortical surface. Illumination was provided by a custom made LED ring illuminator powered by a DC power supply (Hewlett Packard). For each experiment, we loaded the illuminator with garlands of LEDs (Avago Technologies) at two wavelengths with known emission spectra (Fig 1). One of the wavelengths was typically at the hemoglobin isosbestic point of 530 nm. The other garland was loaded with LEDs at one of two oximetric wavelengths (605 nm, N=4; 630 nm, N=7). The two garlands were synched to the frame acquisition of the imaging camera so that illumination switched on alternate frames (frame frequency = 15 Hz; effective led frequency = 7.5 Hz). The entire imaging assembly was covered by light proof baffles that blocked any stray illumination.
**Optical Imaging: Image Processing:** All images were first ‘shift-corrected’ to correct for any residual movement of the cortical surface that remained despite the specially designed camera mount, animal head post and overall structural framework (NOTE: most of the residual movements were due to the brain moving relative to the animal’s head, when the animal shifted body position etc). This ‘shift-correction’ consisted of aligning each image frame to the first frame of a given experimental session, using the imaged blood vessels as references. Each image frame was cross-correlated with the reference frame, and a gradient descent method used to maximize this correlation value as a function of lateral shifts in the image position, frame by frame. These ‘shift-corrected’ images were then used for all subsequent processing.

We calculated responses at each wavelength as the ratio between 105 raw signal frames acquired post stimulus onset and 15 frames immediately preceding the stimulus (dR/R). To correct for any trial-related responses, we subtracted the time-course of activity obtained on ‘blank trials’ (2, 4).

**Spatio-Temporal Profiles:** To calculate the spatiotemporal pattern of the optical signals, we first determined the center of the focal response evoked by the visual stimulus. The image of the response was low-pass filtered (Gaussian σ=0.4 mm) and the pixel with the peak response was taken as the center position. We found that the response center was the same whether localized using the initial dip or blood volume signals and therefore we relied on blood volume to localize as it offered a more reliable signal. We excluded any response
contribution from large vessels by generating vascular masks using the cortical image at the isosbestic wavelength (Fig SM1 Below).

![Image](image-url)

**FigSM1.** Example of a blood vessel mask used to avoid artifacts due to changes in major blood vessels. **a.** Image of the cortical vasculature illuminated with green light at 530nm. Both arteries and veins are clearly visible. **b.** The vascular mask generated by thresholding the image in a. **c.** The vascular mask overlaid on the radial map of the image. Each color represents the distance away from the center of the response. **d.** The pointspread imaged at 630nm during the rebound phase. Note that much of the signal appears localized to pial vessels. Arrows in c and d point to a large pial vessel whose contribution to the signal is removed by the vascular mask.

We then averaged the response image at each of the 120 frames along radial profiles extending from the center giving the spatial profile of the response at each timepoint. As the temporal profile of the response, we used the temporal profile of the response center.
To compare the spatial spreads of the responses at different wavelengths across signals of varying amplitude and sign, we normalized all radial response profiles to range from the signal intensity value at the center to the minimum value \( \left( \frac{r_{\text{norm}}}{r_{\text{norm}} - r_{\text{min}}} \right) \). We fitted the resultant profiles of response vs. radius with cubic spline functions (spline.m in MATLAB) and computed the value of the radius at 75 and 50 percent of the response at the center (radius = 0) which corresponds to the half-width. All statistical comparisons were done using t-test (MATLAB routines ttest.m and ttest2.m). Values are reported as mean (standard error of the mean).

**Spectral decomposition: Path length Correction:** We modeled cortical tissue as a scattering medium containing the absorbing chromophores HbO and HbR. Due to the 10-fold variation in light absorption in these chromophores over the spectral range used (5), different imaging wavelengths have very different effective path lengths in cortex while traveling into and then out of the cortex. We used a Monte Carlo simulation to estimate this differential path length. These path lengths were then used to decompose the signals measured simultaneously at pairs of imaging wavelengths into quantitative estimates of \( \Delta[HbO] \) and \( \Delta[HbR] \) and to predict expected imaging signals at other wavelengths across the spectrum (6-10).

**Spectral Decomposition: Parameter Selection:** Appropriate values for the mean angle of light scatter \( (g=0.95) \) and the scattering coefficient \( (\mu_s=60 \text{ mm}^{-1}) \) to use in the analysis of our data from monkey cortex were selected from (11) based on results from human tissue.
We used the value of 75% for baseline hemoglobin saturation and 6e-5 mM for baseline blood concentration. To assure ourselves of the generality of our solution, we ran simulations covering a range of plausible values in a grid ($g = 0.85, 0.90, 0.95; \mu_s = 20, 30, 40, 50, 60 \text{ mm}^{-1}$) as well as several different values for baseline tissue saturation and concentration (7, 10). The pattern of our results was reliable across this wide parameter space.

**Spectral decomposition: Effective Absorption for LEDs:** In order to compute the changes in HbR, HbO, and HbT from absorption changes observed under LED illumination, we first computed the effective absorption in HbR and HbO and path length through tissue for each LED used. Since the emitted LED light has a non-zero spectral width, the effective absorption can be calculated, to first order, from the modified Beer-Lambert law at a given wavelength $\lambda$ ($I_\lambda = I_0 \exp(-\mu_{\lambda} L C)$) by replacing the argument of the exponent ($\mu_{\lambda} L C$) by the mean of this quantity weighted by the intensity profile of the LED:

$$\mu_{\lambda}^{LED} L^{LED} = \frac{\int \mu_{\lambda} L C d\lambda}{I^{LED}},$$

where $I^{LED}$ is the incident light intensity from the LED, $I_0^{LED}$ is the reflected light intensity of the LED, $\mu_{\lambda}^{LED}$ is the effective absorption coefficient, $L^{LED}$ is the effective path-length for the LED, and $C$ is the concentration of the absorber. The term $I_0^{LED}$ represents the amount of total LED light reflected and is an integral of all the emitted wavelengths. We used the effective absorption and path length for the LED ($\mu_{\lambda}^{LED} L^{LED}$) to compute changes in HbR, HbO, and HbT as:
Where \( I_{LED}^t \) is the reflection signal from the LED at time \( t \), and \( I_{LED}^0 \) is the reflection signal from the LED at time 0.

**Spectral decomposition: Predicting signals across wavelengths:** To predict imaging signals at different wavelengths, we placed our computed response time courses in [HbO] and [HbR] into the equations above. We used textbook hemoglobin absorptivity and our wavelength-dependent path lengths to generate predicted absorption time courses (Fig 4B). In comparing the size of the initial dip to the rebound, we computed the ratio of the maximum absorption increase observed during the dip and the maximum absorption decrease during the rebound (Fig 4C). Wavelengths with no distinct dip and rebound were excluded.
References


Fig. S1. Onset latencies of the raw and decomposed hemodynamic signals. (a) The slopes of the absorption changes for the different imaging wavelengths used. Asterisks denote slopes significantly different from the prestimulus baseline across sessions. Note that all signals reach significance by 400–533 ms after stimulus onset regardless of the imaging wavelength used. Error bars are SEM. (b) The $P$ value of the statistical comparisons in a Wilcoxon rank sum test. Dashed line denotes the 0.05 significance level. Note that the signal at 530 nm approaches significance quickly after stimulus onset. (c) Same as a, but for the decomposed signals. Note that HbO and HbT become significantly different from baseline by 400–533 ms after stimulus onset whereas HbR becomes significant only by 933 ms after stimulus. (d) Same as b, but for the decomposed values.
Fig. S2. [HbR], [HbO], and [HbT] time courses decomposed independently for each imaging wavelength pair or for each monkey consistently fail to show any increase in HbR during the initial dip. (a) (Left) Time courses of [HbR], [HbO], and [HbT] for experiments imaged by using the 605- and 530-nm LED pair. (Right) Time courses scaled to maximum. (b) Same as a, but for experiments using the 630- and 530-nm pair. (c) Based on data from monkey S alone (n = 9). (d) Same as c, but using data from monkey V alone (n = 2). Note no early increases in HbR, only delayed decreases in HbR relative to HbT in all of the time courses.
Fig. 53. The larger initial dip and undershoot observed at 605 nm relative to 630 nm is well predicted from spectrally decomposed hemodynamic signals. No early increase in HbR is observed from pairing 605 nm with 630 nm. (a) Normalized absorption changes at 605 and 630 nm. Arrows mark the initial dip and undershoot. (b) Responses at each imaging wavelength predicted by using the computed concentration changes averaged over all experiments and imaging wavelength pairs (as in Fig. 3B). (c) Predictions based solely on data from 605 nm paired with 530 nm. (d) Predictions based solely on data from 630 nm paired with 530 nm. Note that the basic pattern of larger dip and rebound for 605 nm is predicted in all conditions. (e) Results of an additional control experiment imaging simultaneously with 605- and 630-nm LEDs. (Left) Normalized raw absorption changes at 605 and 630 nm. Note the much larger initial dip at 605 than at 630 nm. (Center) Spectrally decomposed time courses in [HbR], [HbO], and [HbT]. Note the good agreement with results using the LED 605 + 530-nm combination; compare with Fig. 3B. (Right) Decomposed responses scaled to maximum. Note, as in other decomposed images, that the decrease in [HbR] is delayed and more transient than the increases in [HbT].
Fig. S4. Modeling results are robust against changes in parameters, giving the same patterns of computed signals. (a–c) Representative model responses across a range of parameters examined from $g = 0.85$, $\mu_s = 60 \text{ mm}^{-1}$ (a) to $g = 0.95$, $\mu_s = 20 \text{ mm}^{-1}$ (c); the plot in b shows the responses for parameter pair used throughout the text ($g = 0.95$, $\mu_s = 20 \text{ mm}^{-1}$), which is in the middle of the extremes in a and c. Note that although the amplitude of the signal changes from a to c (greatest amplitude for highest scatter), all parameters show an early increase in HbT with no increase in HbR.
Fig. S5. Spatial interactions of HbR and HbO can lead to a family of complex pointspread shapes depending on their relative contribution to responses at different wavelengths. (a) Normalized computed point-spreads for HbR and HbO (divided by maximum response) differ only slightly in their spatial profiles (arrow). (b–d) Family of normalized response shapes produced by simply subtracting these two shapes in different proportions. Note that pointspreads can be made narrower or wider depending on the exact proportions of HbR and HbO. This shows that wavelengths absorbed to different extents in HbR and HbO can, in principle, show substantially different pointspreads. Dashed line shows HbT profile for comparison.
Other Supporting Information Files

SI Appendix (PDF)