

Chapter 14

Functional Imaging of Cerebral Oxygenation with Intrinsic Optical Contrast and Phosphorescent Probes

**Anna Devor, Sava Sakadžić, Mohammad A. Yaseen,
Emmanuel Roussakis, Peifang Tian, Hamutal Slovin, Ivo Vanzetta,
Ivan Teng, Payam A. Saisan, Louise E. Sinks, Anders M. Dale,
Sergei A. Vinogradov, and David A. Boas**

Abstract

Microscopic *in vivo* measurements of cerebral oxygenation are of key importance for understanding normal cerebral energy metabolism and its dysregulation in a wide range of clinical conditions. Relevant cerebral pathologies include compromised blood perfusion following stroke and a decrease in efficiency of single-cell respiratory processes that occurs in neurodegenerative diseases such as Alzheimer's and Parkinson's disease. In this chapter we review a number of quantitative optical approaches to measuring oxygenation of blood and cerebral tissue. These methods can be applied to map the hemodynamic response and study neurovascular and neurometabolic coupling, and can provide microscopic imaging of biomarkers in animal models of human disease, which would be useful for screening potential therapeutic approaches.

Key words O₂ sensing, Phosphorescence quenching, Intrinsic optical signals, Energy metabolism, *In vivo* imaging, Hemoglobin, Two-photon microscopy, CCD

1 Introduction

Cerebral oxygenation and O₂ metabolism are key parameters defining healthy brain function. Until recently, though, our knowledge of the microscopic dynamics of cerebral oxygenation has been limited by a lack of methods with the spatial and temporal resolution needed to follow oxygenation changes induced by varying neuronal activity. Most of what we know about the behavior of cerebral oxygenation during the hemodynamic response comes from measurements of light absorption by hemoglobin in the blood. This method, commonly referred to as “optical imaging of intrinsic signals (OIS)”, is based on differential absorption spectra of oxy- and deoxy-hemoglobin (HbO and Hb) in the visible range and does not require

loading of extrinsic O₂-sensitive probes. Following the original demonstration [1], the intrinsic imaging method was widely used for investigation of neuro-hemodynamic coupling [2–11] and mapping of cortical neuronal responses [12–15] (Box 1).

The hemoglobin molecule is composed of four monomers, each containing an O₂-binding heme group. Hemoglobin tetramer

Box 1 Optical Imaging of Intrinsic Signals—A Historical Perspective

One hundred and twenty years ago, Roy and Sherrington [16] argued that neuronal activation causes the local vasculature to respond. While unequivocal confirmation of this claim had to wait nearly a century, until radioactive methods became available [17–20], large reflectance changes of brain tissue during localized seizure activity could be visualized already in the late 1930s [21]. A few decades later advances made it possible to detect and analyze the much smaller optical signals during activity of the normal cortex. These were accounted for by activity-associated changes in cerebral blood flow (CBF) and volume (CBV) [18, 22]; in addition, Chance [23] and Jöbsis [22] observed that neuronal activity is often accompanied by oximetric signals that can be detected optically by monitoring the absorption (and/or fluorescence) of hemoglobin and other intrinsic chromophores.

In the late 1980s, Grinvald et al. [1] showed that the small light absorption changes induced by these activity-evoked hemodynamic responses can be used to explore cortical functional architecture *in vivo*, by using a CCD camera to image the cortex upon illumination at specific wavelengths during the presentation of sensory stimuli. The resulting cortical images can then be used to produce functional maps at the spatial resolution of a few tens of microns, more than enough to image the columnar structure of the mammalian neocortex.

Since then, the interpretation of intrinsic signals in terms of neuronal activity—and thus their utility for functional brain mapping—has been tightly linked to our understanding of the mechanisms underlying neurovascular coupling. In fact, such knowledge is necessary to distinguish between optical signals resulting from changes in the activity of local neuronal populations and those resulting from the vascular effects of remote neuronal events (e.g., venous drain). To address this issue, Frostig et al. [4] imaged the sensory-evoked optical responses in the cortex at several wavelengths and tried to decompose them spectroscopically into CBV and O₂ saturation. A few years later, Malonek and Grinvald [6] used a continuous wavelength spectroscopic approach, optical imaging spectroscopy, to investigate the hemodynamic response in further detail. Both studies concluded that some hemodynamic events colocalize better with neuronal activity than others. In particular, an early increase in deoxy-hemoglobin concentration (Hb)—the so-called initial dip—was detected, and interpreted as resulting from local O₂ consumption induced by neuronal activity while the vasculature is still at rest. Thus, the initial dip is expected to colocalize more accurately with neuronal activity than the subsequent hemodynamic events, i.e., increased CBV and CBF, which are mediated by the complex spatiotemporal transfer function of the active vascular response [11, 24]. Which hemodynamic response component is best for functional mapping and how to optimally choose the imaging parameters (wavelength, timing of data acquisition, etc.) are therefore still the object of some debate [8, 25–31].

Whatever its conclusion, *de facto*, imaging the activity of visual stimuli in early visual cortex using oximetric wavelengths (600–630 nm, emphasizing changes in [Hb] over those in [HbO]) has allowed investigators to obtain high-quality single-condition functional maps, i.e., maps obtained by comparing stimulated conditions to rest. This has not been possible using isosbestic wavelengths, where Hb and HbO absorb with equal strength and which thus emphasize changes in CBV. At those wavelengths, differential approaches (the comparison of several stimulated conditions one to another) are needed to visualize functional architecture at the columnar level [4, 24], at least upon stimulation with large visual stimuli. Interestingly, in other cortices such as auditory [32–34] and rat somatosensory cortex [26], CBV-based optical signals have turned out to warrant at least as good mapping as oximetric ones. Moreover, in primate visual cortex [30] it has been shown that, upon stimulation with small

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Box 1 (continued)

retinotopic stimuli, the resolution of CBV-dominated signals is as precise as that obtained using oximetric ones.

Taken together, these accumulating results indicate that the optimal choice of the signal to be used for functional mapping might depend on the relation between the topology of the local vascular network and that of the functional architecture to be mapped, that is, on the specific cortical area. However, kind and duration of the stimulus, as well as the signals' etiology, must also be taken into account (e.g., the CBV response appears to have an early capillary component and a delayed macrovascular one), in rodents [27, 35, 36] and primates [30]. Finally, it has been shown that, under some circumstances, imaging at long wavelengths (>700 nm) is advantageous in spite of the small signal sizes, because it reduces the contribution of surface vasculature [37]. Yet, the precise composition of the optical signal at those wavelengths remains to be determined [38].

Irrespective of the above issues, detailed maps of the cortical functional architecture have been obtained in a large variety of preparations—from rodent through cat and ferret up to the primate—in several cortical areas, including both sensory (visual, auditory, somatosensory) and motor. In early visual cortex, intrinsic imaging of functional domains was first reported by Grinvald et al. [1] and later on by many others [39–42]. These studies have laid the groundwork for optical imaging of higher visual areas such as MT [43], V4 [44], and inferotemporal cortex [45]. Fine functional organization of auditory cortex revealed by Fourier optical imaging was reported by Kalatsky et al. [46], after earlier work by Frostig and co-workers [47] and other groups. In the somatosensory cortex, the visualization of whisker barrels in the rat has been a standard protocol for nearly 20 years now [48]. Moreover, the representation of functional domains for digits [49–51] and recently the representation of mechanical nociceptive stimuli within SI have been reported [52].

The elimination of anesthesia has been another critical step. High-resolution optical imaging in alert monkeys was first reported by Grinvald et al. [53], and later in trained monkeys by Roe's group [54]. However, the challenge of long-term imaging in the awake behaving primate could be met only with the development of an artificial, transparent dura substitute [14, 55, 56]. This has allowed investigation not only of the functional architecture but also of its stability over time [14], as well as its behavioral correlation with neurophysiology [15]. Optical imaging has also enabled long-term developmental studies such as imaging the development of orientation preference maps [57, 58] and the layout of functional domains in strabismic animals [59, 60].

Due to its high resolution and non-invasiveness, optical imaging of intrinsic signals has also been used to explore pathological situations, such as the exploration of epilepsy, in animal models as well as in humans [61, 62] and in intraoperative imaging in humans [63, 64], with the goal of finely localizing the site of specific cortical functions.

exists in equilibrium between two states (T and R), characterized by different affinity to O₂. The O₂ binding to hemoglobin is allosterically regulated in the sense that the binding itself shifts the equilibrium towards the higher affinity R state (for review see [65]). Conformationally the T and R states are different, but their optical absorption spectra are very similar. In contrast, binding of O₂ to a heme group induces significant spectroscopic changes. Therefore, each monomer in the hemoglobin molecule, whether it exists in the T or in the R state, can be considered an independent chromophoric unit.

During an increase in neuronal activity, the associated increase in flow and volume of blood results in a decrease of O₂ extraction fraction (from blood to tissue) producing a decrease in deoxy-hemoglobin concentration ([Hb]) and an increase in oxy-hemoglobin concentration ([HbO]). This effect can be detected,

for example, by a decrease in light absorption at 580 nm and the corresponding changes in the absorption-based image (for a recent example from rat somatosensory cortex see [66]). Thus, mapping of neuronal activity can be achieved using a simple epi-illumination/reflectance setup and a CCD camera for detection. Multi-wavelength illumination [6] can be used to extend this method to quantify HbO and Hb changes (as % change from the baseline) [38, 67, 68]. Such absorption-based measurements are linearly related to the fraction of O₂ bound to hemoglobin, while their relationship to the blood pO₂ (dissolved O₂) is defined by the non-linear hemoglobin O₂ binding curve.

An alternative approach to quantifying intravascular O₂ changes in the brain is based on introducing extrinsic phosphorescent probes into the blood stream [25, 69–71,] and measuring phosphorescence lifetime. The method—namely the use of phosphorescence quenching to quantify O₂ tension—was originally developed by Wilson and colleagues [72, 73] and relies on the fact that molecular oxygen (O₂)—a triplet molecule in the ground state—is able to quench emission from excited triplet states of other molecules, such as phosphorescent metalloporphyrin-based probes. Thus, while the intrinsic imaging method measures hemoglobin oxygenation, the phosphorescence quenching technique detects O₂ by measuring kinetics of quenching. Therefore, it can be applied for both intravascular and tissue measurements, given an adequate strategy for delivery of the probe. The phosphorescence lifetime of a probe depends on the partial pressure of O₂ (pO₂) in the immediate vicinity of the probe, providing a spatially localized measurement. O₂ is the only effective dynamic quencher of phosphorescence present in biological systems in sufficient concentration, so measurements of phosphorescence report O₂ levels with high specificity. Importantly, the decay lifetime (rather than the phosphorescence intensity) is measured, which makes the technique insensitive to local variations in the probe concentration and, very importantly, to changes in tissue optical properties. As such, lifetime measurements are insensitive to the absorption and scattering changes associated with the hemodynamic response *in vivo*, as long as a sufficient number of photons reach the detector for the accurate fitting of the phosphorescent decay. A brief discussion of phosphorescent probes can be found in Box 2 below as well as in recent papers (see, for example, [74, 75]).

Imaging with intrinsic hemoglobin-based contrast or with phosphorescence in wide-field mode using a CCD camera has no true depth resolution: the signal at every pixel represents a weighted sum of the response through the whole depth of light penetration with the highest sensitivity to the cortical surface. This limitation might be acceptable for certain types of intravascular studies, in particular, studies of the “columnar” organization of circulation in cerebral cortex: the vertical orientation of diving arterioles and, more importantly, surfacing venules, transporting

Box 2 Phosphorescent O₂ Sensors

The ideal phosphorescent O₂ probe for in vivo applications consists of a phosphorescent chromophore of the maximal attainable brightness, encapsulated inside a protective jacket, the role of which is to regulate O₂ diffusion to the chromophore (and thereby constant k_q , Eq. 2) in order to maintain high O₂ sensitivity throughout the physiological range. Unrestricted O₂ access (too high k_q) leads to complete quenching of phosphorescence already at low O₂ concentrations, whereas overly restricted access (low k_q) results in a narrow dynamic range, i.e., the difference between the phosphorescence of the quenched and unquenched probes is too small. Similarly, too short triplet lifetimes result in low sensitivity because of low probability of collisions with O₂.

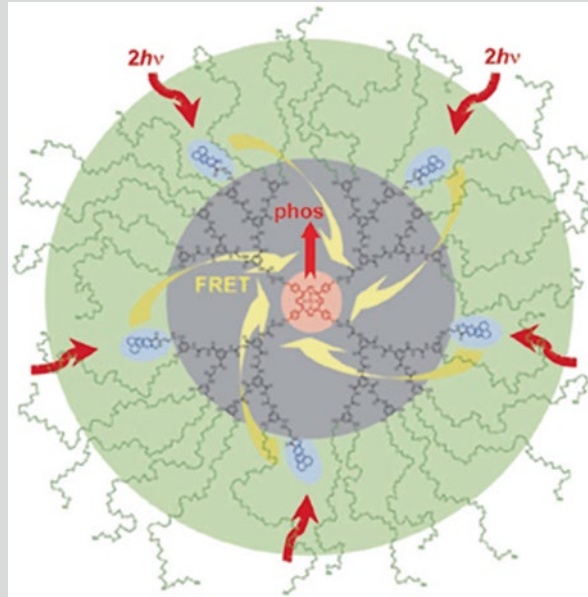


Fig. B1 Molecular structure of PtP-C343. PtP-C343 is a dendritic O₂ probe, in which phosphorescence of metalloporphyrins upon two-photon excitation is enhanced by intramolecular Förster resonance energy transfer (FRET) from two-photon coumarin-343 antennae chromophores (*blue*). The O₂ sensitivity of the probes is regulated by dendritic encapsulation of the core metalloporphyrin (*gray*), while peripheral polyethyleneglycol residue groups on the dendrimer isolate the probes from contact with biological macromolecules in the environment (*green*).

Only a few chromophores exhibit bright phosphorescence in solutions at ambient temperatures. Based on the optical spectra, emission quantum yields, and triplet lifetimes, porphyrins of Pt and Pd are by far the best suited for O₂ measurements in the physiological pO₂ range. The original probes were based on simple derivatives of Pd porphyrins [73, 76]. Such porphyrins are relatively hydrophobic and need to be prebound to serum albumin (usually bovine albumin) to improve their solubility and prevent aggregation in aqueous media. As a result, exogenous protein becomes a part of the injected material, which may lead to unwanted immunogenic reactions and possible toxicity.

Introduction of dendritic polyglutamic porphyrins [77, 78], known as Oxyphors R2 and G2, alleviated the necessity of pre-binding probes to albumin, thanks to polyglutamic branches which render the compounds highly water-soluble. Nevertheless, Oxyphors R2 and G2 still require binding to endogenous albumin, because only albumin complexes of these probes are suitable for measurements in the physiological O₂ range [79]. As a result, O₂ measurements with R2 and G2 can be performed only in albumin-rich environments (e.g., blood plasma). Even in the blood, though, incomplete binding, easily

(continued)

Box 2 (continued)

encountered at high probe concentrations (above $\sim 10^{-5}$ M), can lead to heterogeneous decays and compromise measurement accuracy.

Keeping the calibration constants of probes unaltered in biological systems is a significant challenge, but also a stringent requirement for quantitative imaging. Indeed, it is not sufficient to have a luminescent probe whose signal simply changes in response to O_2 . It is important that both k_q and τ_0 (Eq. 2) remain exactly the same as they were determined during the calibration. These probes' parameters are defined by the probes' immediate environment; to insure their stability the probe molecule must remain in aqueous phase, forming no complexes with bio-macromolecules, cellular membranes, or other biological objects. These requirements have been fulfilled in the so-called fully protected third-generation probes [74, 80]. Pt or Pd porphyrins in these probes are encapsulated inside hydrophobic poly-arylglycine (AG) dendrimers, whose exteriors are modified with hydrophilic polyethyleneglycol (PEG) residues. PEGylated jackets regulate the sensitivity and dynamic range of the measurement and insure the probes' high aqueous solubility and inertness with respect to the biological system. This general design is inherent in both one- and two-photon (vide infra) O_2 probes.

To enable two-photon phosphorescence lifetime microscopy (2PLM), special phosphorescent probes were recently developed with enhanced two-photon absorption cross sections. One such probe is PtP-C343 [81], which has been successfully utilized in vivo in brain imaging studies [82–84]. The functional elements of PtP-C343 are Pt tetraarylporphyrin (PtP), which acts as a phosphorescent core, and several C343 units, which play the role of the two-photon antenna (Fig. B1). Near-infrared energy (e.g., at 840 nm) is absorbed by the C343-antenna and within fractions of a nanosecond channeled to PtP via intramolecular FRET, where it is utilized to populate the emissive triplet excited state. The redox potentials of PtP and C343 and the distances between them within the probe molecule are optimized to minimize the unwanted triplet quenching by electron transfer [80, 81]. The C343-antenna enhances phosphorescence in PtP-C343 approximately 25-fold compared to that of a regular one-photon probe. Because of the effect of the dendritic layer and peripheral PEG groups, PtP-C343 is pH-insensitive in the physiological range and its signal is independent of interactions with proteins in the blood.

the deoxygenated blood along the imaging axis. Sensing tissue oxygenation, however, has a stronger requirement for the resolution in depth. Indeed, metabolic differences between cortical layers have been demonstrated with other methods, e.g., cytochrome oxidase staining and incorporation of 2-deoxyglucose [85–87]. In recognition of the importance of depth-resolved tissue pO_2 measurements, numerous studies have employed point polarographic O_2 sensors, also called “ O_2 electrodes” [88]. While measurements with O_2 electrodes have provided invaluable insight into tissue O_2 dynamics in cerebral [89, 90] and cerebellar cortex [91], the point nature of the measurement, blind positioning, and invasiveness of the method—the need for repetitive penetrations throughout the cortical tissue for mapping the signal at different locations—put significant constraints on throughput and data interpretations. Indeed, O_2 electrode measurements have demonstrated a significant degree of unaccounted for variability between nearby locations, which could result from differences in the electrode position relative to the blood vessels and/or tissue damage associated with electrode insertion [92, 93]. O_2 electrodes have been successfully used to map oxygenation on the cortical surface [94–96], circumventing the uncertainty of blind positioning. Other methods of measuring tissue O_2 include binding of nitroimidazole-based

drugs [97] and electron paramagnetic resonance (EPR) oximetry [98, 99]. These methods differ in spatial and temporal resolution, but generally cannot be applied to real-time tissue O_2 microscopy.

Several new approaches to tissue oximetry have recently been disclosed. First, imaging modalities based on photo-acoustic effect (photo-acoustic tomography [PAT], photo-acoustic microscopy [PAM], optical resolution photo-acoustic microscopy [OR-PAM]) are capable of volumetric measurements of the Hb/HbO ratio [100] with an optical contrast and, except in the case of OR-PAM, ultrasound resolution. Due to the low scattering of sound waves, the imaging resolution of PAT is much higher than that of Diffuse Optical Tomography (DOT), e.g., sub-millimeter resolution at depths as high as 50 mm. However, direct comparisons of O_2 images obtained by the two methods have yet to be performed. Also, penetration depth of PAM is higher than that of current optical microscopy technologies (e.g., two-photon microscopy and optical coherence tomography). Second, a microscopic O_2 imaging method has been described by Warren and collaborators [101] who quantified the Hb/HbO ratio by measuring the excited state absorption of the two components using multiphoton absorption and excitation by shaped femtosecond infrared pulses. This technique is subject to the same depth limitations as all methods relying on ballistic photons and, like DOT, suffers from low sensitivity. A variant of luminescence quenching technique has recently been described by Ashkenazi et al. [102] who measured triplet kinetics using transient triplet-triplet absorption and acoustic detection. However, generation of adequate acoustic signals requires very high probe concentrations and/or near-saturation excitation regime in order to produce adequate amounts of the triplet state. Finally, a method is being developed, which is based on the dependence between the time-averaged fluorescence intensity of regular fluorescent dyes and population of dark triplet states of these dyes in O_2 -dependent fashion [103, 104]. This approach also requires extremely high excitation intensities, unlikely to be sustainable by biological tissues.

Seeking to overcome the problem of spatial resolution in CCD-based wide-field imaging of pO_2 based on O_2 -dependent phosphorescence quenching, some studies have attempted to combine the phosphorescent quenching method with two-photon excitation [71, 105]. Unfortunately, regular metalloporphyrin-based phosphorescent probes have very low two-photon absorption cross sections, and therefore require extremely high probe concentrations and high laser powers to observe adequate signals. Probes such as Oxyphor R2 [77, 79] and simple hydrophilic Pd porphyrins [73], which can operate only as complexes with albumin (vide infra), partially exist in unbound form at such high concentrations. As a result, the phosphorescence decays are characterized by complex lifetime distributions and cannot be interpreted quantitatively. To enable 2PLM of O_2 , special two-photon-enhanced phosphorescent probes have recently been

designed [80, 81, 106]. Here, two-photon-“antenna” chromophores are included in the probe molecules for the purpose of capturing excitation energy and transmitting the excitation to phosphorescent cores via intramolecular Förster resonance energy transfer (FRET).

The other major obstacle precluding widespread use of two-photon excitation of phosphorescent probes is a temporal mismatch between high repetition rates of femtosecond Ti:sapphire lasers, typically used for multiphoton excitation (~80 MHz), and long triplet lifetimes of porphyrin-based probes (tens to hundreds of microseconds). To circumvent this problem, the excitation must be gated to allow for acquisition of the phosphorescence decay. Commercial systems suitable for simultaneous measurement/imaging of phosphorescent lifetimes during the gate off-time and fluorescent lifetimes during the gate on-time are just starting to appear (Becker & Hickl data acquisition boards SPC-830 and SPC-150, <http://www.becker-hickl.de>). However, it is possible to modify or build a 2PLM system in a laboratory with optical expertise [71, 81–83]. 2PLM allows measurement of both intravascular and tissue pO_2 with unprecedented spatial resolution and is well suited to imaging of pO_2 changes during functional activation [84]. Furthermore, it has been recently demonstrated that 2PLM enables estimation of blood flow in individual capillaries simultaneously with intravascular pO_2 [83].

Despite its advantages in spatial resolution and applicability to measurements of tissue oxygenation, 2PLM does not replace measurements of intrinsic signals. Rather, it complements the suite of tools available for metabolic/hemodynamic measurements. Multiple measurement techniques need to be employed to obtain the full picture of the underlying physiological process and to cross-validate one another. For example, one can combine measurements of oxygenation with extrinsic measurements of blood flow to estimate the cerebral rate of O_2 metabolism: 2PLM with optical coherence tomography [107, 108] for microscopic resolution, or intrinsic imaging with either laser speckle [66] or wide-field red blood cell velocity measurements [109] for a larger “mesoscopic” view. Below we discuss the technical aspects of the multi-wavelength CCD-based method for imaging of intrinsic signals and the application of phosphorescence quenching for imaging of intravascular and tissue pO_2 , and provide specific examples and references for further reading.

2 Optical Imaging of Hemoglobin Oxygenation

Researchers have long recognized intrinsic optical changes in fluorescence, absorption, and scattering associated with neuronal and metabolic activity. The sources of fluorescence changes are small, intrinsically fluorescent metabolites such as NADH and FAD, whose signals change during a metabolic response to increased

neuronal activity or changes in O₂ availability (e.g., during hypoxia) [110]. These molecules have characteristic chromophore-specific excitation bands. The hemoglobin in blood absorbs visible light and is the main source of intrinsic contrast in brain tissue in the visible spectrum [4, 6, 25, 111–114]. At longer wavelengths, where Hb and HbO have negligible absorbance, light-scattering effects dominate. Near-infrared scattering changes, for example, underlie the contrast in optical coherence tomography [115, 116].

Since hemoglobin-based OIS was introduced to measure the functional architecture of the cortex in vivo [1], the method has been widely applied (Box 1). The popularity of OIS for in vivo studies is due largely to the robustness of the hemodynamic response, utilization of endogenous contrast agents, and moderate complexity of the optical instrumentation setup. In addition, intrinsic signals have a relatively large percentage change from the baseline ($\Delta I/I$) in response to neuronal activation, enabling data collection with minimally invasive procedures, such as imaging through the thinned skull.

Whereas OIS relies on functional hemoglobin changes to create contrast, increases in blood volume and the amount of hemoglobin during the hemodynamic response produce unwanted “artifacts” for fluorescence imaging techniques in vivo, such as calcium or NADH imaging. Absorption and scattering changes typically affect both the illuminating light on the way to the fluorophore and the emitted fluorescence. To correct the signal, one can employ algorithms based on the reflectance changes at an additional wavelength [117–119] or fluorescence signals from a physiologically inert fluorophore such as SR101 or GFP [120, 121].

OIS typically uses a CCD camera for signal detection and provides spatial resolution in the XY plane ranging from $\sim 10\ \mu\text{m}$ on the cortical surface to hundreds of micrometer in cortical layers 2–3, and no true depth resolution (see Chapter 2 in this volume). By measuring the absorption changes at multiple wavelengths of light, and given knowledge of the path length of light through the tissue, it is possible to quantify absolute hemoglobin concentration changes [38, 68]. Below we consider the quantitative estimation of Hb and HbO from multi-wavelength OIS data and the spatial resolution of common OIS instrumentation setups.

2.1 Multi-wavelength Imaging Method for Quantification of Oxy- and Deoxy-hemoglobin

Performing OIS with a number of different illumination wavelengths (referred to hereafter as “spectral” imaging) enables estimation of changes in oxy-hemoglobin (ΔHbO), deoxy-hemoglobin (ΔHb), and total hemoglobin (ΔHbT) [66–68, 122, 123]; for a recent review see [124].

For spectral imaging, one can employ a white illumination source filtered with a rotating filter wheel [9, 26, 66–68, 123, 125–127], a set of electronically controlled flashing LEDs optically chopped with appropriate filters [128], or a grating monochromator

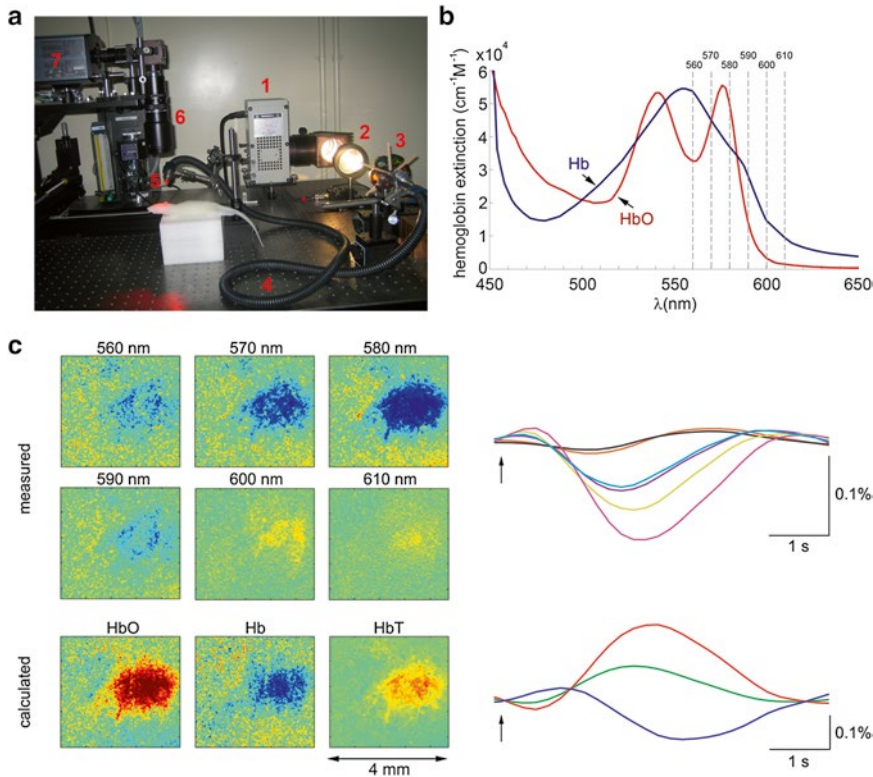


Fig. 1 Conversion of multi-wavelength signals to HbO, Hb, and HbT. **(a)** Optical instrumentation setup with a light source (1), focusing lens (2), filter wheel (3), fiber bundle (4) that brings illumination to the specimen (5), objective lens (6), and CCD camera (7). **(b)** Absorption spectra of hemoglobin in the visible range. **(c)** Single-filter maps of absorption changes (*left*) and the corresponding signal time-courses extracted from the active region (*right*). Signal time-courses for each of the six wavelengths are superimposed. **(d)** Estimated HbO, Hb, and HbT maps (*left*) and time-courses (*right*).

[129]. Figure 1a shows an example of a simple instrumentation setup used in our laboratory. Illuminating light (labeled “1” in the figure) from a tungsten–halogen light source (Oriol, Spectra-Physics) is directed by a lens (“2”) through a 6-slot filter wheel (“3”), coupled to a 12-mm fiber bundle (“4”) that brings the illuminating light to the specimen (“5”). In this filter wheel, the center wavelengths of the filters range between 560 and 610 nm at 10-nm intervals. The filter wheel is mounted on a DC motor and rotates at ~18 Hz, resulting in a ~3 Hz acquisition rate per wavelength. The light is collected by an objective lens (“6”) and images are acquired by a cooled 16-bit CCD camera (Cascade 512B, Photometrics) (“7”). Image acquisition is triggered by individual filters in the filter wheel passing through an optic sensor. The image set at each wavelength is averaged across trials and the averaged data are converted to ΔHbO and ΔHb at each pixel using the modified Beer–Lambert relationship:

$$\Delta A(\lambda, t) = (\varepsilon_{\text{HbO}}(\lambda) \Delta C_{\text{HbO}}(t) + \varepsilon_{\text{Hb}}(\lambda) \Delta C_{\text{Hb}}(t)) D(\lambda) \quad (1)$$

Table 1
The pathlength correction factor and extinction coefficients for commonly used illumination wavelengths.

λ (nm)	560	570	580	590	600	610
$D(\lambda)$	0.0537	0.0500	0.0503	0.0909	0.1587	0.2044
$\epsilon_{\text{HbO}}(\lambda), \times 1.0\text{e} + 05$	0.7511	1.0247	1.1539	0.3316	0.0737	0.0346
$\epsilon_{\text{Hb}}(\lambda), \times 1.0\text{e} + 05$	1.2387	1.0380	0.8526	0.6523	0.3380	0.2174

The values for extinction coefficients are corrected for the concentration of hemoglobin in tissue.

where $\Delta A(\lambda, t) = \log(R_o/R(t))$ is the attenuation at each wavelength, R_o and $R(t)$ are the measured reflectance intensities at baseline and time t , respectively, ΔC_{HbO} and ΔC_{Hb} are the changes in concentrations of HbO and Hb, respectively, and ϵ_{HbO} and ϵ_{Hb} are the wavelength-dependent molar extinction coefficients (Fig. 1b). This equation is solved for ΔC_{HbO} and ΔC_{Hb} using a least-squares approach. The differential pathlength factor, $D(\lambda)$, accounts for the fact that each wavelength travels slightly different pathlengths through the tissue due to the wavelength dependence of scattering and absorption in the tissue. It is estimated using the approach described by Kohl et al. [38] through Monte Carlo simulations of light propagation in tissue (Table 1). Baseline concentrations of 60 and 40 μM are assumed for HbO and Hb, respectively [3, 8]. Results for relative hemoglobin changes during functional activation are only weakly sensitive to these assumed baseline values [66]. Figure 1c–d shows an example of $\Delta\text{HbO}/\Delta\text{Hb}$ estimation from the six-wavelength data in rat barrel cortex in response to a single deflection of one whisker. Larger hemodynamic changes induced by various pathological manipulations (e.g., middle cerebral artery occlusion, cortical spreading depression) require application of nonlinear fitting algorithms to estimate hemoglobin changes [130].

2.2 Optical Resolution and Depth Sensitivity Considerations

In two-dimensional (2D) camera-based OIS, a collimated beam in the visible spectrum incident onto the brain surface propagates inside the tissue. The reflected light from the brain is collected by the objective lens and imaged onto the CCD chip, forming a 2D image. Tissues at different depths of the cortex contribute to the image on the camera, such that the measured signal intensity of each pixel represents a weighted sum of the response through the whole depth of light penetration (with the highest sensitivity to the cortical surface) [131, 132]. Because of light scattering in the cortex, even a point source in the brain tissue will not result in an image corresponding to a diffraction-limited spot but to a much larger region. The size of this region is determined by the configuration of the optical imaging system (numerical aperture (NA) and the depth at which the image is in focus, referred to as “focal plane

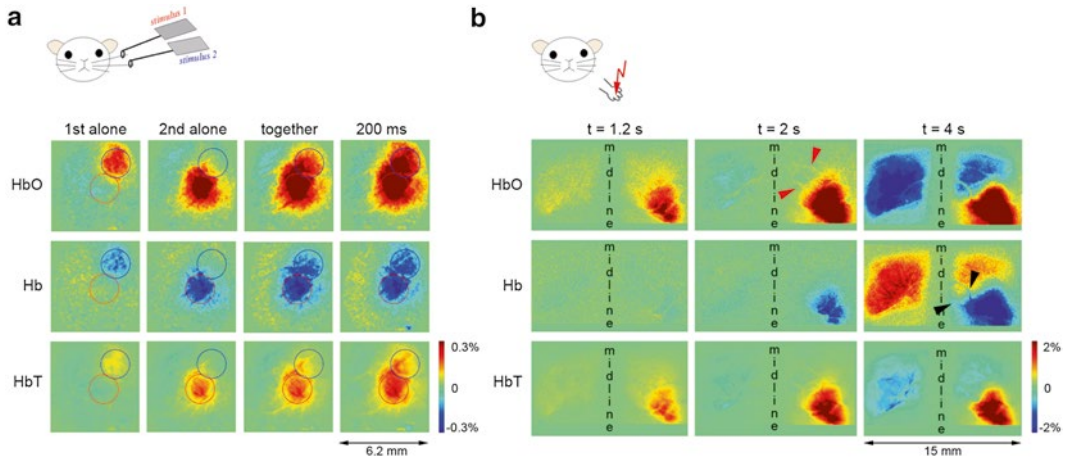


Fig. 2 Imaging of hemoglobin oxygenation in the rat primary somatosensory cortex. **(a)** Example HbO, Hb, and HbT maps in response to a tactile stimulus of single whiskers. The maps correspond to 2.5 s after the stimulus onset for four stimulus conditions: one deflection of a single whisker (“1st alone” and “2nd alone”), simultaneous deflection of both whiskers (“together”), and a sequential deflection with a 200-ms interval (“200 ms”). The signal for Hb and HbO is expressed in percent change relative to its own baseline concentration (40 and 60 μ M, respectively). HbT was calculated as a sum of Hb and HbO. **(b)** Example HbO, Hb, and HbT maps in response to an electrical forepaw stimulus. *Red* and *black arrows* point to the surface arteries and veins, visible on HbO and Hb images, respectively.

depth”) and the optical properties of the tissue (such as the scattering and absorption coefficients).

Propagation of light in a highly scattering medium such as cortical tissue is complicated and cannot be modeled analytically with adequate accuracy. Instead, numerical methods, such as Monte Carlo methods, are widely used to simulate light transport in tissues [133–135]. A systematic Monte Carlo study of spatial resolution and depth sensitivity for 2D optical imaging methods with configurations typically encountered in functional brain imaging demonstrated that more than 97 % of the signal comes from the top 500 μ m of the tissue [134] (see Chapter 3 in this volume). Therefore, no significant contribution is expected to originate in cortical layers 4 or 5. The spatial resolution using the common NA and focal depth settings is less than or comparable to the size of cortical columns in rodent barrel cortex.

2.3 Example Application: Mapping of Rat Barrel Cortex Using Multi-wavelength Optical Imaging

OIS provides a sensitive tool for mapping the location of neuronal responses to sensory stimulation and for studying neurovascular coupling while varying the evoked hemodynamic response in the temporal and spatial domain [4, 11, 14, 48, 136]. Figure 2a shows an example from the rat barrel cortex. The skull over the barrel cortex contralateral to the stimulated whisker pad was thinned until transparent, and tactile stimuli were delivered to single whiskers at different time delays [137]. The hemodynamic response to

tactile stimulation of individual whiskers is clearly localized (red and blue circles in Fig. 2a). Moreover, one can study the additive properties of the response by stimulating multiple whiskers, either simultaneously or sequentially.

Figure 2b shows a different example of $\Delta\text{Hb}/\Delta\text{HbO}/\Delta\text{HbT}$ maps in response to a much stronger stimulus: an electrical stimulus delivered to a forepaw in the rat. In this case, the skull was thinned bilaterally over the forepaw region of the primary somatosensory cortex. Note that the amplitude of the signal change is almost an order of magnitude larger than in the previous case. The maps in both cases have a spatial structure, which can be exploited to study the transformation of neuronal circuit activity to hemodynamic signal changes [126, 127].

3 Direct Methods for O₂ Imaging Using Phosphorescent Probes

The phosphorescence quenching method exploits exogenous molecular probes, which emit from their triplet spin states (as opposed to fluorescent probes, which emit from singlet spin states), to quantify O₂ content in living tissue [73]. O₂ can react with excited state molecules in the environment, quenching their luminescence. Collisional quenching is much less probable on the time scale of excited singlet states (nanoseconds) than of triplet states (microseconds to milliseconds), making phosphorescence much more sensitive than fluorescence to dynamic O₂ quenching. The phosphorescence decay time (τ) of a probe depends on the partial pressure of O₂ in the physiological pO₂ range according to the Stern–Volmer relationship:

$$\frac{\tau_o}{\tau} = 1 + k_q \tau_o pO_2 \quad (2)$$

where τ_o and τ are the phosphorescence lifetimes in the absence of O₂ and at O₂ pressure pO₂, respectively, and k_q is the O₂ quenching constant. Parameters k_q and τ_o are characteristic of the probe molecule and the environment (solvent, pH, temperature, etc.) and do not depend on the measurement system. Phosphorescence quenching is arguably the most direct method of O₂ quantification, since the physics of quenching is well understood. In biological systems O₂ is the only small-molecule quencher present in sufficiently high concentrations: hence the specificity of the measurement.

Phosphorescence quenching as a form of biological oximetry has been developed specifically for *in vivo* applications. Phosphorescent probes (Box 2) are introduced directly into the medium of interest, where they report on the oxygenation in their local environments. External excitation and detection are used to retrieve the signal. Using phosphorescence, O₂ can be imaged in large

areas/volumes [69, 72, 138, 139] potentially in 3D [140]; no mechanical damage is done to the tissue, and the measurement can be performed with high temporal and spatial resolution. If phosphorescence is excited by ballistic photons, as in microscopy applications [141–143], the spatial resolution is limited only by the optical diffraction. Provided that the volume contains a sufficient number of probe molecules, temporal resolution is limited only by the triplet lifetime (sub-millisecond range). However, in practice, collection of multiple decays is required to obtain a reliable measurement. Thus, phosphorescent probes with high emissivity are the key to high temporal and spatial resolution.

4 Single-Photon Excitation of O₂-Sensitive Probe

Regular phosphorescence-based pO₂ measurements by linear (one-photon) excitation can be performed with relatively simple instrumentation. For example, using a time-gated CCD camera synchronized with a pulsed- or frequency-modulated excitation source [69, 70, 138, 139, 144–147], spatially resolved oxygenation maps can be collected in tissue or vasculature with temporal resolution on the order of seconds.

Frequency-modulated illumination can be provided by an optically chopped halogen source light or an LED [144, 145]. Alternatively, in the time domain, one can use flash lamps [69, 70, 138] or a pulsed laser such as a q-switched, frequency-doubled Nd:YAG laser that delivers ~6-ns pulses at a fixed frequency (10 Hz) [139, 147]. As with OIS, the excitation light is typically coupled into a multimode fiber positioned at an angle relatively oblique to the cortical surface. The sample can also be excited with a 0° incidence angle if a dichroic mirror is used to couple the excitation and detection beams. In a wide-field microscopy system operating in one of our laboratories [139], the phosphorescence light is collected by a low-magnification infinity-corrected objective (e.g., Olympus XL Fluor 4x/340, 0.28 NA), and an image is formed on the CCD sensor by a 100-mm focal length tube lens. A long-pass filter is positioned between the objective and the tube lens to suppress the excitation light, and the signal is detected from the entire field of view at once. Higher signal-to-noise ratio (SNR) can be achieved by binning pixels on the CCD chip (at a price of lower spatial resolution). A single lifetime image (reconstruction of the phosphorescent decay) requires multiple camera exposures temporally jittered relative to the illumination. Using a pulsed laser source, each camera exposure starts at a specific programmed delay with respect to its corresponding excitation pulse (Fig. 3a). For each pixel, intensity values are plotted against the delay between the excitation pulse and CCD exposure to generate the phosphorescence decay. If frequency-modulated

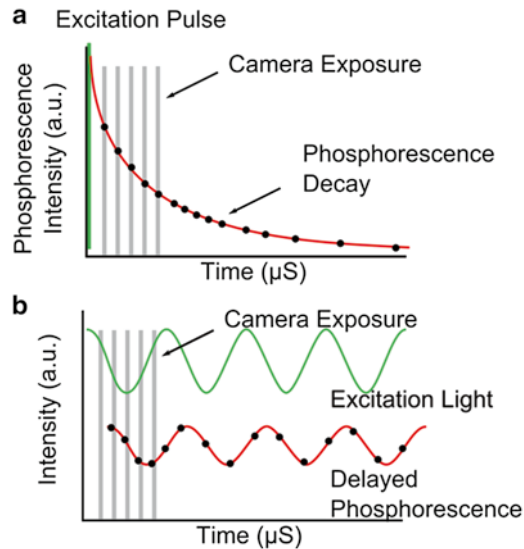


Fig. 3 Phosphorescence lifetime measurements—time-domain and frequency-domain methods. **(a)** Timing relationship for time-domain measurement of phosphorescence decay. A pulsed laser delivers light at a fixed repetition rate, with each pulse generating a phosphorescent decay. Each laser pulse triggers a corresponding brief camera exposure, delayed by a programmed period after the laser pulse. Each camera exposure captures a different portion of the phosphorescence decay. After several laser pulses, the phosphorescence decay can be measured and the lifetime is estimated by an exponential fit. **(b)** Timing relationship for frequency-domain lifetime measurement. A sinusoidally modulated excitation beam runs continuously, and a synchronized camera collects multiple brief exposures at programmed phase delays. After several exposures, the delayed sinusoidal phosphorescence waveform can be estimated, and the lifetime is estimated by $\tan(\theta)/2\pi \times \text{modulation frequency}$, where theta is the phase delay of the phosphorescence waveform [98].

excitation is used rather than pulsed excitation, the lifetime can be computed from the phosphorescence waveform's phase delay with respect to the excitation waveform (Fig. 3b) [145]. Maps of pO_2 are computed per pixel using the Stern–Volmer relationship (Eq. 2).

An illustration of CCD-based intravascular pO_2 imaging is presented in Fig. 4. In this example, a simple laser speckle imaging system was used simultaneously with the pO_2 measurement to record relative blood flow changes (rCBF) for estimation of the cerebral metabolic rate of O_2 ($CMRO_2$) [139]. See Chapter 15 in this volume for review of laser speckle imaging.

As discussed in Sect. 2.2, CCD-based imaging (in combination with wide-field excitation of the phosphor) suffers from poor spatial resolution due to the scattering of light in tissue. Confocal microscopy can be employed to improve the spatial resolution in a single-photon excitation mode [143]. In the confocal regime, the scanning galvanometer mirrors can be programmed to direct the

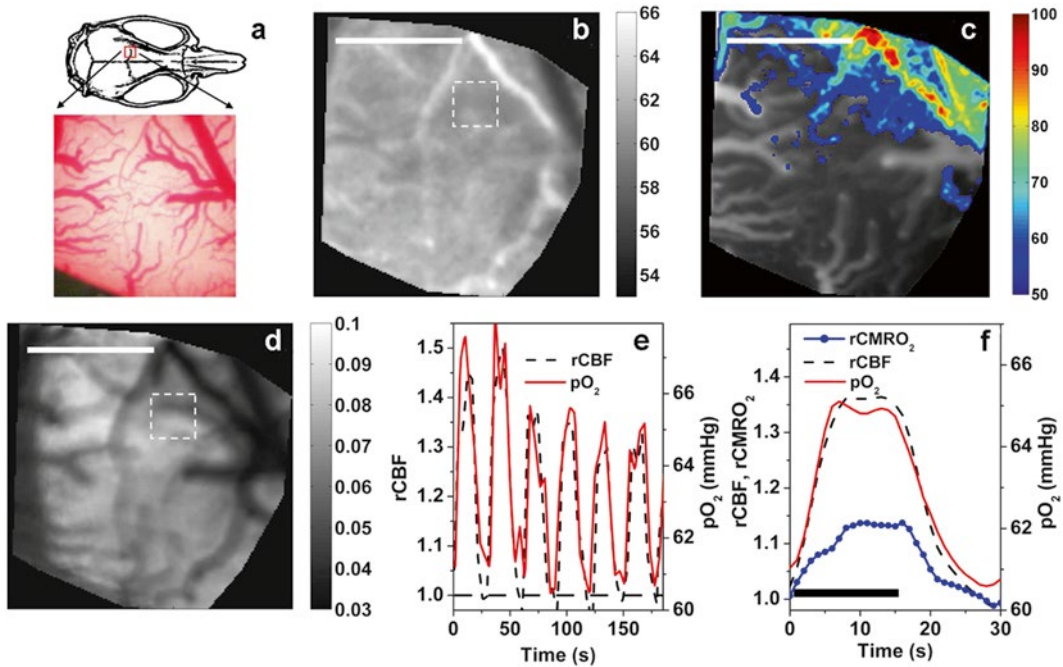


Fig. 4 CCD measurement of intravascular pO_2 with simultaneous laser speckle imaging during forepaw stimulation. **(a)** Position of the cranial window over the forepaw area of the somatosensory cortex and a photograph of the cortical vasculature. **(b)** Baseline pO_2 map. **(c)** Composite image consisting of phosphorescence intensity (*gray*) and activation area (*color*). **(d)** Baseline speckle contrast image. **(e)** Time-courses of pO_2 (*red solid curve*) and $rCBF$ (*black dashed curve*) during several stimulation sequences extracted from the ROI marked by the *white rectangles* in **(b)** and **(d)**. *Black horizontal bars* in **(e)** and **(f)** denote stimulus duration. **(f)** Trial-averaged pO_2 , $rCBF$, and $rCMRO_2$ responses. Scale bar is 1 mm.

excitation beam to distinct locations from which pO_2 measurements are made. The detected light must be de-scanned by placing the detector after the galvanometer scanners and spatially filtered using a pinhole in front of the detector. Thus, spatial resolution is improved by (1) confining the excitation to a small volume and (2) rejecting out-of-focus phosphorescence by implementing the pinhole.

5 Two-Photon Phosphorescence Lifetime Imaging (2PLM) of O_2

In addition to single-photon excitation of O_2 -sensitive probes, two-photon excitation is now being employed to achieve better depth resolution and higher spatial localization [71, 81–83, 148]. In two-photon microscopy, ultrashort laser pulses and a high NA objective are required to induce nonlinear excitation of the probe, confining the generation of the excited state to the immediate vicinity of the focal volume. In two-photon microscopes constructed in our laboratories [81, 82], phosphorescence is excited by trains of femtosecond pulses from a Ti:sapphire oscillator, gated

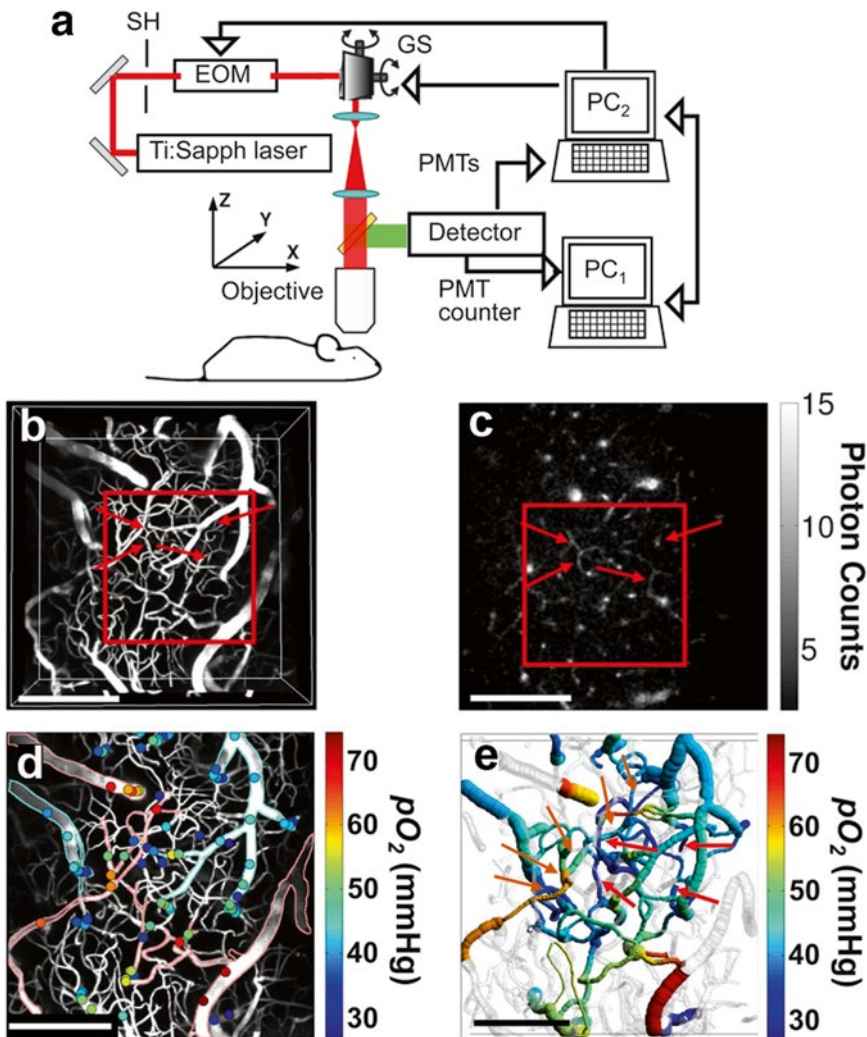


Fig. 5 Two-photon detection of phosphorescence. (a) Schematic of the experimental setup. *EOM* electro-optic modulator, *GS* galvanometer scanner, *PMTs* analog-mode photomultiplier tubes, *PMT counter* a Geiger-mode photomultiplier tube. (b) Maximum intensity projection along the *z* direction of a 250- μm -thick stack in the mouse cortex. The vasculature was labeled with dextran-conjugated fluorescein isothiocyanate (FITC). (c) Phosphorescence intensity image of microvasculature obtained at a depth of 240 μm below the cortical surface. The *color bar* shows the average number of photon counts in each pixel collected during a single phosphorescence decay. Four *arrows* mark the locations of the same capillary vessels in panels (b), (c), and (e). (d) Measured $p\text{O}_2$ values in microvasculature at various depths (*colored dots*), overlaid on the maximum intensity projection image of vasculature structure (grayscale). Digital processing was performed to remove images of the dura vessels. Edges of the major pial arterioles and venules are outlined in *red* and *blue*, respectively. (e) Composite image showing a projection of the imaged vasculature stack. *Red arrows* mark $p\text{O}_2$ measurement locations in the capillary vessels at a depth of 240 μm . *Orange arrows* point to the consecutive branches of the vascular tree, from pial arteriole (*bottom left arrow*) to the capillary and then to the connection with ascending venule (*top right arrow*). Scale bar, 200 μm .

by an electro-optic modulator. The phosphorescent signals are detected by photon-counting modules (Fig. 5a). For example, the probe can be excited by a 10- μ s excitation gate, followed by a 300- μ s data acquisition period. The counts are binned and subsequently used to reconstruct the decay, which is analyzed by the least-squares method to obtain the phosphorescence lifetime. The SNR of the measurement is determined solely by the number of the counted photons. Only very few photons can be collected from a near diffraction-limited volume per excitation gate at a reasonable probe concentration [81]. Therefore, decays are typically analyzed after averaging multiple excitation cycles. The size of the excitation volume is dependent on the laser power and the duration of the excitation gate [148]. These parameters can be adjusted to select the optimal combination of the excitation volume (spatial resolution) and pixel dwell time (temporal resolution). For example, to measure baseline pO₂ in the mouse cortex we usually acquire 500–2,000 excitation cycles per point using PtP-C343 (for description of this and other probes see [74, 75, 77, 80–81]) at intravascular concentration of <15 μ M and with 10- μ s excitation gates. The average laser power during the excitation gate is <10 mW at the focal plane. Using 2,000 cycles, a grid of 600 points can be acquired in ~6 min. We estimate that under this excitation regime the emitting volume has ~2 μ m lateral ($X\gamma$) and ~5 μ m axial (Z) dimensions.

Using 2PLM in an upright setup [82], we can image the cortex of anesthetized mice and rats through sealed cranial windows, either injecting the phosphorescence probe into the vasculature through the femoral artery or pressure-injecting it directly into the brain tissue. Structural images of the cortical vasculature can be obtained either by imaging fluorescence emission from coumarin-343 (C343) chromophores constituting the two-photon antenna of the PtP-C343 probe or, to increase contrast, by using intravenously administered fluorescein isothiocyanate (FITC) conjugated with dextran (Fig. 5b).

O₂ imaging in the cerebral cortex is typically performed in two steps. First, we raster-scan the excitation beam over the field of view, rendering 2D survey maps of the integrated emission intensity (150 \times 150 or 250 \times 250 pixels, acquired in ~9 or ~25 s, respectively). A single-plane scan performed 240 μ m below the brain surface in Fig. 5c demonstrates the ability of the system to resolve the structure of the microvasculature down to the capillary level. After mapping the tissue, we average multiple excitation cycles in selected locations for accurate pO₂ determination. Given the average of 500–2,000 cycles, the acquisition time corresponds to a temporal resolution of 0.16–0.76 s per single-point pO₂ measurement. Finally, using Stern–Volmer calibration plots (Eq. 2), we convert phosphorescence lifetimes, obtained by fitting, into pO₂ values (Fig. 5d–e).

5.1 Example
Application: Two-Photon Imaging of Tissue pO_2 Dynamics in Rat Somatosensory Cortex with Phosphorescent Nanoprobe PtP-C343

Measurement of pO_2 based on 2PLM is uniquely capable of assessing pO_2 directly in the cortical tissue. In the example shown in Fig. 6, PtP-C343 was microinjected into the interstitial space (tissue) of the rat primary somatosensory cortex through a glass pipette attached to a pressure injector, as is usually done with calcium indicators [149]. In contrast to calcium indicators, PtP-C343 is easily dissolved in artificial cerebrospinal fluid and does not require additional solvents (such as pluronic acid or DMSO). Following microinjection, PtP-C343 quickly diffuses within ~ 1 mm from the injection site. Using our protocol, there is no significant decrease in the signal intensity up to 4 h after the injection. After that, the signal slowly reduces. Thus, a single injection per subject is sufficient. FITC is administered intravenously to delineate the vasculature (Fig. 6a) and allows coregistration of pO_2 measurements relative to the vascular O_2 sources. To investigate the spatial distribution of tissue pO_2 at the baseline (resting) level of neuronal activity, one can acquire grids of points (Fig. 6b). Plotted as a function of the radial distance from the arteriole (Fig. 6c), these data demonstrate a gradient indicating that O_2 diffuses into the tissue through the walls of diving arterioles [84].

When the probe is also injected intravenously, one can perform simultaneous intravascular and tissue pO_2 measurements. However, given the resolution of $\sim 2 \times 2 \times 5$ μm , capillary signals cannot be resolved if the probe is present in both intra- and extravascular compartments. Nevertheless, one can resolve intravascular pO_2 in diving arterioles, although the ontogeny of the signal at the vessel border would be uncertain (Fig. 6d). Alternatively, capillary pO_2 can be readily imaged if the probe is present only in the vasculature (but not in the tissue around it) as demonstrated in Fig. 5.

When the beam is moved from point to point, a short time period is required to stabilize the galvanometer mirrors. Therefore, the most time-efficient way to accomplish a point pO_2 measurement is to stay at the same point and collect multiple decays before moving to the next point. For functional measurements, however, the sampling rate per point must match the kinetics of the dynamic process to be imaged. Figure 7 shows an example of a dynamic pO_2 measurement performed at ~ 1 Hz. In this example, 50 decays were collected at each point and the number of points was adjusted to allow us to revisit the point within ~ 1 s (1 Hz sampling rate). Therefore, after determining the target acquisition speed, one has to compromise between the overall number of measurement points and the number of excitation cycles collected per point. Multiple stimulus trials can be averaged before fitting for the decay to increase the SNR of the measurement. For example, 20 trials were averaged in Fig. 7.

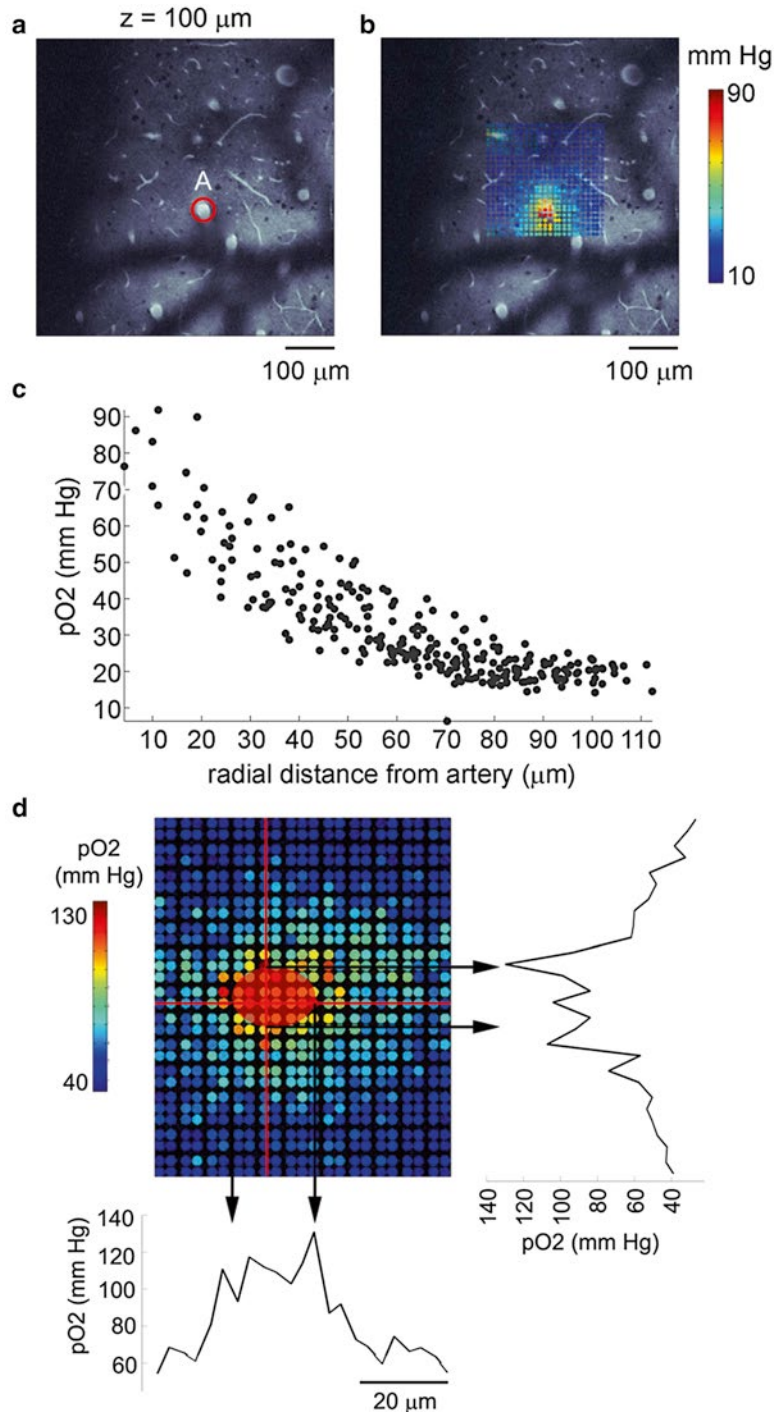


Fig. 6 Spatial distribution of baseline pO_2 values. (a) A reference vascular image with an arteriole ("A"). The fluorescent contrast is due to intravascular FITC. The background fluorescence is due to the excitation of coumarin molecules in PtP-C343. (b) The same image as in (a) with superimposed pO_2 values and segmented arteriolar territory. (c) pO_2 as a function of the radial distance from the arteriole. (d) The plots at the *bottom* and on the *right* show X and Y profiles cut through the *red lines* in the image. The *red circle* indicates the arteriolar boundary estimated from the FITC reference.

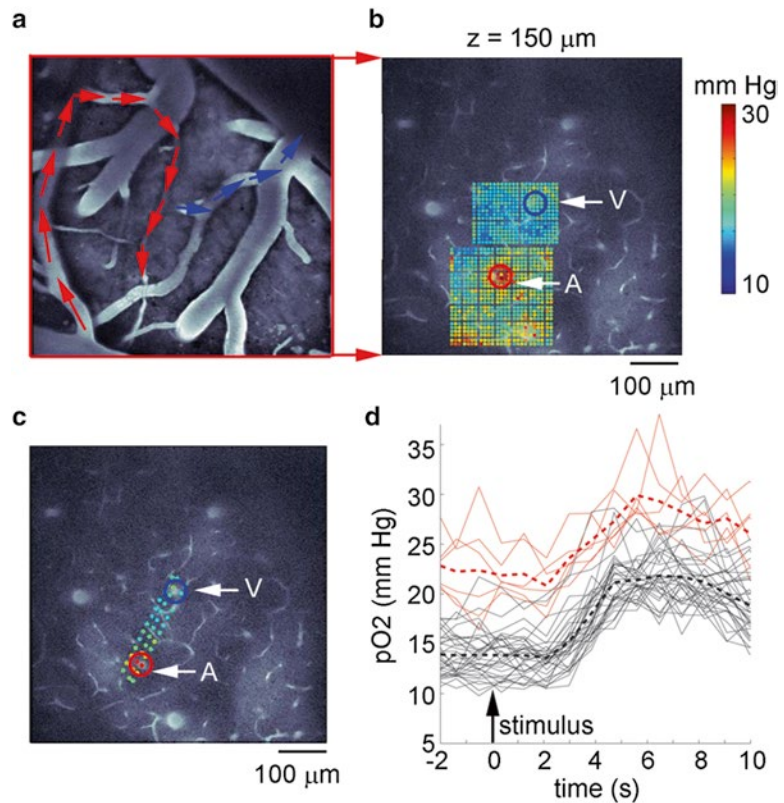


Fig. 7 Stimulus-induced tissue pO₂ changes in rat primary somatosensory cortex. (a) An image of the surface vasculature calculated as a maximum intensity projection (MIP) of an image stack 0–100 μm in depth using a 4× objective. Individual images were acquired every 10 μm. (b) The same region at the depth plane of pO₂ measurements, 150 μm below the surface. “A” and “V” indicate a diving arteriole and a surfacing venule, respectively. *Red* and *blue arrows* in (a) show the upstream and downstream surface arteriole and venule for “A” and “V,” respectively. A grid of measured baseline pO₂ values is superimposed on the vascular reference plane. (c) The same vascular reference plane as in (b) with superimposed pO₂ measurement points used for dynamic measurements. The points are color-coded according to the baseline pO₂. (d) Stimulus-evoked time-courses of pO₂ change, extracted from each of the measurement points in (c). The traces are divided into two categories: high baseline (*red*) and low baseline (*black*). The *dotted red* and *black lines* correspond to the average for each category. The *black arrow* denotes stimulus onset.

One additional limitation of 2PLM measurements is a decrease in SNR with increasing baseline pO₂. This limitation stems directly from the O₂ sensing mechanism of the probe: quenching of the triplet state by O₂ leads to a fewer emitted photons. At higher pO₂s, more of the probe molecules are quenched, resulting in fewer photons reaching the detector. The decrease in SNR at high pO₂ is usually not a problem for baseline measurements, where it can be compensated for by averaging more excitation cycles per point.

6 Conclusions and Outlook

In this chapter we have reviewed methods for measuring intravascular and tissue oxygenation, with a particular focus on applying OIS and phosphorescence quenching in the cerebral cortex. In the context of cortical function, these methods on their own provide a somewhat one-sided view of a single (but very important) aspect of the underlying physiological processes—oxygenation. A more complete picture can be obtained by combining these imaging modalities with other measurements of neuronal, glial, vascular, and metabolic activity. As such, OIS can be readily combined with simultaneous electrophysiological recordings [9, 27, 67, 150] or fMRI [105], and can serve as a bridge between the neuronal and fMRI signals, getting around the need to filter electrical potentials recorded in the environment of changing electromagnetic fields. Likewise, 2PLM can be combined with optical imaging of neuronal activity (e.g., two-photon calcium imaging [152], at least in alternating stimulus trials; see Chapter 9 in this volume) and optical coherence tomography measurements of blood flow [107, 108]. Measurements of capillary flow—the speed of red blood cells in individual capillaries—can be extracted directly from 2PLM data when a phosphorescent O_2 probe is applied intravascularly [83].

The choice of method for measuring oxygenation would depend on the requirement for spatial resolution, compartments of interest (intravascular or tissue), allocated budget, and availability of optical engineering expertise. OIS remains the method of choice for “mesoscopic” mapping of neuronal activity on the cortical surface: It does not require delivery of extrinsic probes and is inexpensive and easy to implement in a standard neuroscience laboratory setting. The most significant drawbacks of OIS are scattering and lack of depth resolution, which result in an inherent uncertainty in the spatial origin of the imaged signals. 2PLM is situated on the opposite side of the experimental/instrumentation complexity spectrum, but offers high-resolution, deep-penetration imaging of either intra- or extravascular pO_2 , depending on the location of the pO_2 probe. Confocal imaging might serve as a good compromise on the instrumentation side and does not require probes with high two-photon cross sections, offering more flexibility in the choice of probe molecules. In the two-photon regime, the most important parameter determining the temporal and spatial resolution of the measurement is the phosphorescence quantum yield of the probe. Probes based on metalloporphyrins and other chromophores exhibiting brighter phosphorescence are currently being developed. Likewise, strategies for intracellular delivery of phosphorescent O_2 probes are under investigation. In combination with transgenic methods for identification of specific neuronal cell types in the cerebral cortex, intracellular O_2 probes could be used for evaluation of cell-type specific pathology in neurodegenerative disease.

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