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Review Article

Frontiers in optical imaging of cerebral blood flow and metabolism

Anna Devor[1,2,3], Sava Sakadžić[3], Vivek J Srinivasan[3], Mohammad A Yaseen[3], Krystal Nizar[1], Payam A Saisan[1], Peifang Tian[1,4], Anders M Dale[1,2], Sergei A Vinogradov[5], Maria Angela Franceschini[3] and David A Boas[3]

[1]*Department of Neurosciences, UCSD, La Jolla, California, USA;* [2]*Department of Radiology, UCSD, La Jolla, California, USA;* [3]*Martinos Center for Biomedical Imaging, MGH, Harvard Medical School, Charlestown, Massachusetts, USA;* [4]*Department of Physics, John Carroll University, University Heights, Ohio, USA;* [5]*Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania, USA*

In vivo **optical imaging of cerebral blood flow (CBF) and metabolism did not exist 50 years ago. While point optical fluorescence and absorption measurements of cellular metabolism and hemoglobin concentrations had already been introduced by then, point blood flow measurements appeared only 40 years ago. The advent of digital cameras has significantly advanced two-dimensional optical imaging of neuronal, metabolic, vascular, and hemodynamic signals. More recently, advanced laser sources have enabled a variety of novel three-dimensional high-spatial-resolution imaging approaches. Combined, as we discuss here, these methods are permitting a multifaceted investigation of the local regulation of CBF and metabolism with unprecedented spatial and temporal resolution. Through multimodal combination of these optical techniques with genetic methods of encoding optical reporter and actuator proteins, the future is bright for solving the mysteries of neurometabolic and neurovascular coupling and translating them to clinical utility.**
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Introduction

With this *Special Issue* we celebrate 50 years of dedicated symposia on *Cerebral Blood Flow and Metabolism*. During the past half century we, as a research community, have accumulated a considerable body of experimental and theoretical knowledge on cellular metabolic pathways in health and disease, identified a variety of vasoactive substances, established correlations between vascular, metabolic, and neuronal parameters, developed computational models and took aboard a broad suite of methodologies. Yet, a central piece of the cerebrovascular puzzle is missing: Despite a number of hypotheses (for recent reviews see Attwell *et al*, 2010; Cauli and Hamel, 2010; Hamilton *et al*, 2010; Iadecola and Nedergaard, 2007; Kleinfeld *et al*, 2011; Paulson *et al*, 2010), we still do not have a clear mechanistic understanding of local regulation of cerebral blood flow (CBF) and metabolism by neuronal activity. By 'mechanistic' we mean determining causal relationships and identifying molecular messengers, which communicate a change in neuronal activity to the vasculature causing dilation or constriction. What makes the neurovascular signaling so difficult to grasp and what is required for a breakthrough? In this essay, we argue that further advancement in a mechanistic understanding of neurovascular communication and dynamic regulation of blood flow critically depends on the advent of new imaging technologies with microscopic resolution applicable to *in vivo* studies.

Correspondence: Dr A Devor, Departments of Neurosciences and Radiology, University of California San Diego, San Diego, La Jolla, CA, USA.
E-mail: adevor@ucsd.edu or
Dr DA Boas, Martinos Center for Biomedical Imaging, MGH, Harvard Medical School, Charlestown, MA 02129, USA.
E-mail: dboas@nmr.mgh.harvard.edu
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The most intuitive scenario for neurovascular coupling might be that in which consumption of energy by neuronal tissue provides a feedback signal to the feeding vasculature: Changes in neuronal activity drive changes in energy metabolism, which then drive vasodilation/constriction and the associated changes in blood flow. This idea, usually referred to as the 'metabolic hypothesis,' comes in different flavors with relation to the putative molecular mediators, including lactate, NAD^+/NADH (nicotinamide adenine dinucleotide) ratio, ATP/ADP ratio, adenosine, and an (unidentified) O_2 sensor (Paulson *et al*, 2010; Raichle and Mintun, 2006). As an alternative hypothesis, changes in neuronal activity can drive vasodilation and vasoconstriction by feed-forward mechanisms releasing neurotransmitter and neuropeptide molecules related to neuronal signaling (Attwell *et al*, 2010; Cauli and Hamel, 2010). In this 'neurogenic hypothesis,' blood flow and energy metabolism are driven in parallel by neuronal activity. Astrocytes, 'more than a glue' of the central nervous system (Allaman *et al*, 2011; Fiacco *et al*, 2009; Giaume *et al*, 2010; Iadecola and Nedergaard, 2007; Koehler *et al*, 2009), can potentially have a role in both scenarios: via release of vasoactive metabolic biproducts (metabolic) or synthesis and release of vasoactive gliotransmitters in response to neurotransmitters and neuropeptides (neurogenic). Supportive evidence for both hypotheses has been derived from experiments in isolated tissue: brain slices, excised vessels, and even cell cultures. Of these, brain slice preparation produced a wealth of data in experiments with controlled perfusion, pharmacological manipulations, and excitation of single neurons with identified phenotypes (Cauli *et al*, 2004; Gordon *et al*, 2008; Zonta *et al*, 2003). However, homeostasis of brain slices departs from that *in vivo* in many ways (Huchzermeyer *et al*, 2008; Turner *et al*, 2007). Significantly, many of these departures are unknown or difficult to quantify, sometimes making extrapolation of the observed phenomena to the *in vivo* situation uncertain.

The ability to descend to the single-cell and single-capillary levels *in vivo* and observe firing of individual neurons, vasodilation, glucose uptake, and infusion of O_2 into the tissue—all while directly controlling neuronal activity—has long been a dream of scientists interested in understanding the complex regulation of blood flow and metabolism as related to neuronal activity. However, in contrast to the detailed and elegant mechanistic studies in isolated tissue, *in vivo* reports have, in the main, focused simply on correlations between the 'observables,' limited by the available methods. This 'too hard to do' *status quo* for mechanistic studies *in vivo* is starting to change, due to rapid developments in optical microscopy. In fact, already today, a versatile suite of optical tools is available for high-resolution, high-sensitivity measurements of vascular, metabolic, and neuronal parameters in deep tissue and local, cell-type specific manipulations of neuronal activity. Below, we consider the current state of the art of a number of key optical microscopy technologies that will be critical in the effort of graduating from correlation driven to mechanistic approaches for studies *in vivo*. The technological requirements necessary for this endeavor include

- Resolving single cells and single blood vessels; distinguishing local effects from global effects—'*spatial resolution*'
- Sampling fast enough to reconstruct the time course of dynamic processes—'*temporal resolution*'
- Directly measuring variables of interest taking advantage of natural changes in the optical properties of tissue—'*intrinsic optical contrasts*'
- Developing '*optical reporters*' or '*probes*' with high sensitivity and specificity
- Directly manipulating neuronal, vascular, and metabolic activity—'*optical actuators*'
- Imaging deep under the cortical surface—'*depth penetration*'

The tools suitable for unraveling the mechanics of neurovascular and neurometabolic coupling will be complemented by other noninvasive optical technologies that will enable translation of the physiological findings from animal to human studies and clinical application. Importantly, these noninvasive optical technologies can be used in both animals and humans and thus can facilitate the connection of microscopic to macroscopic observables from animals to humans.

The arsenal of optical tools for neurovascular and neurometabolic studies

The use of novel optical technologies has been instrumental for a number of central discoveries in both basic and clinical neuroscience. Examples from basic neuroscience include the fine mapping of cortical functional organization (Grinvald *et al*, 1986) and the discovery of glial calcium excitability (Cornell-Bell *et al*, 1990; Nedergaard, 1994). Among the clinical applications, optical tools played an important role in the study of neurovascular and neurometabolic disregulation in animal models of stroke (Zhang and Murphy, 2007), epilepsy (Schwartz and Bonhoeffer, 2001), migraine (Bolay *et al*, 2002), and cancer (Barretto *et al*, 2011). Likewise, noninvasive optical technologies have started making inroads into bedside imaging of blood flow and oxygen consumption in human patients (Grant *et al*, 2009; Mesquita *et al*, 2011).

Below, we highlight many of the optical methods used for vascular, hemodynamic, metabolic, and neuronal imaging at different resolution scales—from cellular to macroscopic—with an emphasis on *in vivo* methodology (Figure 1). We apologize in

advance for the less-than-comprehensive coverage of this exceedingly broad topic. We have had to cite the literature sparsely, but have strived to include sufficient citations to lead the reader to more detailed information. Subcellular optical imaging methods (e.g., fluorescence resonance energy transfer (FRET)-based fluorescent methods to track protein–protein interactions) are beyond the scope of the current review.

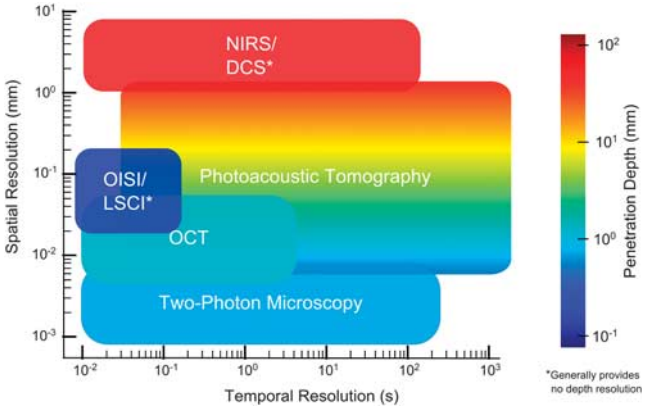

Figure 1 Comparison of spatial resolution, temporal resolution, and penetration depth of neurovascular and neurometabolic optical imaging techniques. Plot of the spatial and temporal resolutions of different optical techniques, with color-coded penetration depth. These are guidelines, intended to relate the currently reported capabilities of different optical methods. Technological advances continue to improve the resolution and penetration depth of each technique. Consequently, this figure does not constitute a definitive comparison of these techniques.

Vascular and hemodynamic imaging

Optical imaging can utilize several endogenous contrast mechanisms for vascular imaging, including hemoglobin absorption, red blood cell (RBC) motion-induced Doppler shifts, and many exogenous fluorescent contrast agents for labeling the blood plasma or RBC. These different contrast mechanisms are used to image hemoglobin concentration and oxygenation changes, to image blood flow, and to obtain angiograms of the microvascular network (Table 1).

Hemoglobin Concentration and Oxygenation

Optical intrinsic signal imaging: Since hemoglobin is a dominant absorber in the brain tissue in the visible and near-infrared spectrum, changes in hemoglobin concentration and oxygenation associated with neuronal activity can be monitored and imaged via optical intrinsic signal imaging (OISI) (Figure 2A). Imaging of cerebral function using these intrinsic absorption changes *in vivo* was demonstrated over 25 years ago (Grinvald *et al*, 1986).

Optical intrinsic signal imaging is limited to imaging of the cortical surface, generally requiring a cranial window or thinned skull to illuminate the cortex with light and image the reflected light with a camera. The amount of light reflected from the cortical surface is modulated by changes in the absorption coefficient of the tissue. These changes are related to changes in the concentrations of HbO (oxy-hemoglobin) and Hb (deoxy-hemoglobin). Estimating the hemoglobin concentration changes,

Table 1 Overview of measurable parameters of cerebral metabolism obtainable using optical techniques, and their associated contrast mechanisms

	Measured parameter						
	Hb/SO_2	*Flow/velocity*	*Angiograms*	*Energy metabolism*			*Electrical activity, ionic concentrations, and synaptic release*
				pO_2, NADH, FAD, glucose	*Cytochromes*	*$CMRO_2$*[a]	
Contrast mechanism							
Absorption	NIRS OISI PAT		PAT		NIRS OISI	NIRS OISI PAT	Widefield (CCD) (VSD)
Fluorescence/ phosphorescence		TPM	TPM	TPM Widefield (CCD)		TPM Widefield (CCD)	TPM Widefield (CCD) (VSD, ionic indicators, pH dyes)
Scattering/ Doppler	OCT	LSCI Doppler OCT DCS	OCT				OISI (fast signals)

CCD, charge-coupled device; $CMRO_2$, cerebral metabolic rate of oxygen; DCS, diffuse correlation spectroscopy; FAD, flavin adenine dinucleotide; Hb, deoxy-hemoglobin; LSCI, laser speckle contrast imaging; NADH, nicotinamide adenine dinucleotide; NIRS, near-infrared spectroscopy; OCT, optical coherence tomography; OISI, optical intrinsic signal imaging; PAT, photoacoustic tomography; pO_2, partial pressure of oxygen; SO_2, hemoglobin oxygen saturation; TPM, two-photon microscopy; VSD, voltage-sensitive probes or dyes.
Widefield imaging (CCD) represents a technique nearly identical to OISI, with the distinction that an exogenous contrast agent is observed.
[a]Obtainable when oxygenation measurements are coupled with flow measurements.

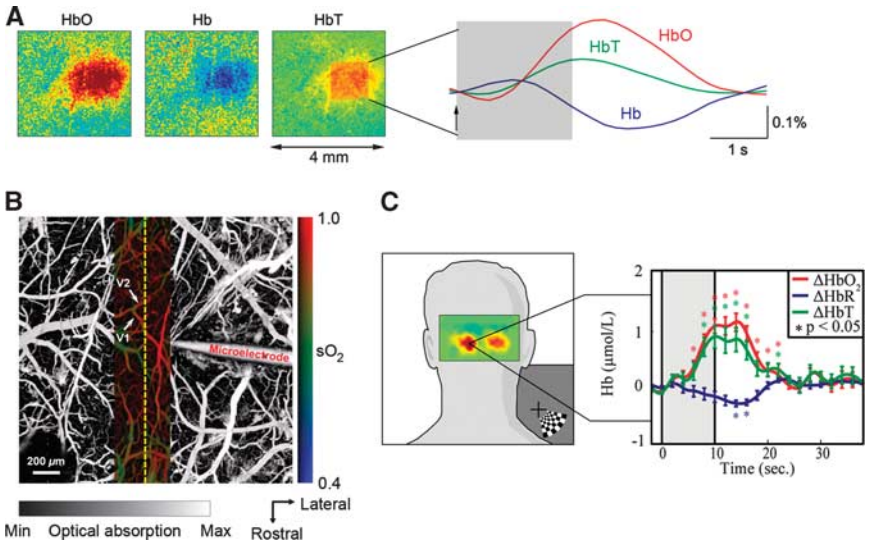

Figure 2 Measurements of hemoglobin concentration and oxygenation. (**A**) Optical intrinsic signal imaging (OISI) of 2-second forepaw stimulation in rodent somatosensory cortex results in a transient increase in oxy-hemoglobin (HbO) (red) and total hemoglobin concentration (HbT = HbO + Hb) (green) and a decrease in Hb. (**B**) Photoacoustic tomography (PAT) can provide high-resolution angiograms of microvessels and estimate the hemoglobin oxygen saturation (SO_2) (Tsytsarev *et al*, 2011). (**C**) Near-infrared spectroscopy (NIRS) can be used to image hemodynamic changes from visual stimulation noninvasively in humans (Gregg *et al*, 2010).

therefore, requires measurements at two or more wavelengths and depends on knowledge of the path length of light through the tissue (Kohl *et al*, 2000). The tissue scattering of light degrades image contrast and spatial resolution, with lateral resolutions ranging from 1 to 100 μm, increasing with depth. The depth sensitivity is limited to the top 500 μm with exponential weighting toward the surface (Tian *et al*, 2010*a*).

The use of OISI was in the center of the discovery of fine details of functional mapping and plasticity in the cerebral cortex (Grinvald *et al*, 1991; Kalatsky *et al*, 2005; Shtoyerman *et al*, 2000; Vnek *et al*, 1999) and played a key role in temporal parsing of the hemodynamic response, with implications for the order of neuronal, metabolic, and vascular events underlying functional hyperemia (Mayhew *et al*, 2000; Sheth *et al*, 2005; Vanzetta and Grinvald, 1999). Optical intrinsic signal imaging also played a significant role in studying pathological departures in neurovascular coupling in disease, such as an increase in Hb associated with focal epileptic seizures (indicating inadequate increase in CBF) (reviewed in Schwartz *et al*, 2011).

Photoacoustic tomography: Greater depth penetration and depth resolution than is possible with OISI can be achieved with photoacoustic tomography (PAT) (Figure 2B), up to several centimeters deep, with spatial resolution of 5/15/500 μm at depths of 0.7/3/50 mm, respectively (Wang, 2009). Photoacoustic tomography utilizes the photoacoustic effect (Bell, 1880), in which a pulse of light scattering through the tissue is absorbed by hemoglobin (or any other absorber), producing local heating and thermal expansion, resulting in an acoustic wave. The position of the optical absorption is then recovered based on the time-of-flight of detected ultrasound waves, yielding a three-dimensional (3D) image based on optical contrast and ultrasound resolution. Exploiting hemoglobin absorption, PAT has been used to image vascular structure and oxygenation in small rodents at various spatial scales, frequently through the intact skull (Hu *et al*, 2009; Laufer *et al*, 2009; Wang *et al*, 2003).

Since diffuse photons contribute to the signal as much as ballistic photons, generation of ultrasound waves can be achieved deep inside the sample: the low scattering of ultrasound in soft tissue further facilitates detection from increased depths. Two main PAT system types have been introduced (Wang, 2009): reconstruction-based PAT (Wang *et al*, 2003) and raster scan-based photoacoustic microscopy (Hu *et al*, 2009; Zhang *et al*, 2006). Application of ultrasound arrays and pulsed lasers that can rapidly change excitation wavelength will increase acquisition speeds. Photoacoustic tomography systems are now becoming commercially available.

Although PAT is a new optical imaging method and its potential for neurovascular research being explored, it is likely to have a broad impact in the next 5 to 10 years due to improved resolution and a greater depth penetration in comparison with OISI.

Near-infrared spectroscopy: Noninvasive human measurements of hemoglobin concentrations are routinely performed with near-infrared spectroscopy (NIRS) (Figure 2C). The principle of NIRS is

equivalent to that of OISI, described above, in that near-infrared light is used to monitor the changes in hemoglobin concentration via absorption spectroscopy. While OISI is generally performed through a cranial window or thinned skull to provide direct viewing of the cortical surface, NIRS measurements are usually obtained through the intact skull and scalp. Near-infrared spectroscopy measurements of hemoglobin concentration changes in the human brain through the intact scalp and skull are feasible because of the weak absorption of near-infrared light by hemoglobin and sufficiently small tissue scattering, as first demonstrated by Jobsis (1977). By 1993, NIRS was being used to measure hemoglobin responses to brain activation in humans (Hoshi and Tamura, 1993; Villringer *et al*, 1993) similar to blood oxygen level-dependent functional magnetic resonance imaging (fMRI). While fMRI has the advantage of spatial resolution and whole brain sensitivity, NIRS has the physiological advantage of sensitivity to HbO and Hb as well as the advantage of being a portable instrument suitable for continuous bedside measurements. While continuous-wave NIRS is used to measure changes in hemoglobin, frequency-domain and time-domain NIRS devices enable estimation of the absolute hemoglobin concentrations (Wolf *et al*, 2007).

Because of the ease in applying NIRS to infants relative to fMRI, it is enabling the identification of neural regions associated with and responsible for the emergence of various behavioral traits in developing human infants (Lloyd-Fox *et al*, 2010). Clinically, it has revealed a hypermetabolic response to acute brain injury in newborn infants, potentially providing a tool to guide therapies aimed at reducing metabolic burden (Grant *et al*, 2009). Further, NIRS is broadly important in studying language function in healthy and psychiatric populations, as it permits natural language production and conversation (Dieler *et al*, 2011).

Blood Flow

Laser speckle contrast imaging: Laser speckle contrast imaging utilizes RBC-induced Doppler shifts of laser light to measure blood flow (Figure 3A). A temporally coherent light source is required to have sensitivity to the Doppler shifts. Laser speckle contrast imaging is analogous to laser Doppler methods (Briers, 2001), but instead of resolving the CBF-induced temporal fluctuations of the light at a single point, it images the blurring of the speckle contrast that occurs over the integration time of the camera, thus enabling near-real-time imaging of blood flow. The relationship between speckle contrast and blood flow and the underlying assumptions were recently reviewed in Boas and Dunn (2010).

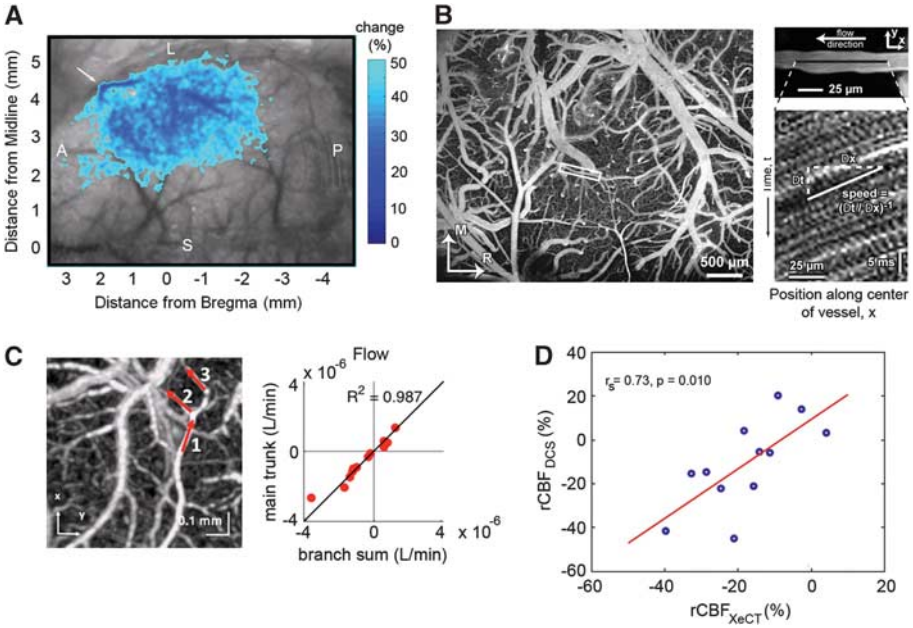

Figure 3 Different measurements of velocity and flow. (**A**) Laser speckle contrast imaging (LSCI) measures changes in blood flow as exemplified here for a middle cerebral artery occlusion in a rodent (Ayata *et al*, 2004). (**B**) Two-photon microscopy (TPM) is used to obtain a depth-resolved angiogram of the microvessels and red blood cell (RBC) velocity within individual vessels (Schaffer *et al*, 2006). (**C**) Doppler optical coherence tomography (OCT) can obtain angiograms of the microvessels as well as quantify volumetric flow within individual vessels, as illustrated by flow conservation at branching vessels (Srinivasan *et al*, 2011). (**D**) Diffuse correlation spectroscopy (DCS) can obtain noninvasively an index of flow changes in humans that correlates with Xenon computed tomography (Kim *et al*, 2010).

A recent advance of note is the ability to measure the temporal speckle contrast instead of spatial contrast. Temporal contrast affords better spatial resolution at the expense of temporal resolution and is less sensitive to the contaminating effects of static scattering from, for example, the skull. The spatial resolution and depth sensitivity of laser speckle contrast imaging (LSCI) are comparable to those of OISI.

Our own work with LSCI has revealed that cortical spreading depression during the aura preceding a migraine headache, activates trigeminal afferents, which results in inflammation of the pain sensitive meninges generating the migraine (Bolay *et al*, 2002), and that cortical spreading depression results in a prolonged state of hypoperfusion in mouse stroke models that results in further growth of ischemic cortex (Shin *et al*, 2006). Others have used LSCI to show local retinal vasodilation in response to focal light stimulation, revealing a neurovascular coupling similar to that observed in cortex (Srienc *et al*, 2010).

Two-photon microscopy: The technical aspects of two-photon microscopy (TPM) (Denk *et al*, 1990) and the spectrum of its applications in neuroscience have been extensively covered elsewhere (Svoboda and Yasuda, 2006). In the context of blood flow, the technique has enabled depth-resolved measurements of RBC velocities (Kleinfeld *et al*, 1998) and vascular diameters (Devor *et al*, 2007; Tian *et al*, 2010*b*) routinely at depths of up to 500 μm deep in the cortex and as deep as 1 mm when utilizing advanced laser systems (Kobat *et al*, 2009). In contrast with LSCI, which utilizes Doppler contrast from RBC motion, TPM velocity measurements typically utilize a fluorescent dye to image the blood plasma and track the RBC 'shadows' to estimate velocity in individual capillaries (Figure 3B). While LSCI is generally performed through a thin skull, TPM typically requires a cranial window, although thin skull measurements are now being conducted at the expense of depth penetration (Drew *et al*, 2010). Velocity and diameter measurements are performed on no more than a few vessel segments at a time. As a result, studies that require measurements throughout the vascular geometry to ascertain the collective behavior are not practical with TPM.

Two-photon microscopy vascular measurements were instrumental in studying the reorganization of blood flow following experimental disruption of the vascular network (targeted 'microstrokes') (Nishimura *et al*, 2006, 2007, 2010; Schaffer *et al*, 2006) and of dilation of cortical microvasculature below the confocal reach (Stefanovic *et al*, 2008; Tian *et al*, 2010*b*).

Optical coherence tomography: Doppler optical coherence tomography (OCT) enables depth-resolved imaging of blood flow in individual diving arterioles and ascending venules (Figure 3C). The penetration depth of OCT in highly scattering media can exceed 1 mm (Izatt *et al*, 1994). Full volumetric imaging of blood flow over a cortical surface area of 1 mm^2 is possible in $\sim$1 minute. Commercial systems are now available, facilitating widespread adoption of OCT. Doppler OCT promises to be an important tool for studying cerebrovascular pathology.

Optical coherence tomography is in many ways analogous to ultrasound, though instead of measuring the scattering of sound waves by tissue, it measures the scattering of light waves. Optical coherence tomography uses the principle of low-coherence interferometry to resolve the delay between different light scattering 'echoes.' A low-temporal-coherence light source provides a coherence gate that rejects multiply scattered light (Izatt *et al*, 1994) to improve contrast of tissue structure at depths greater than can be achieved with confocal microscopy, with a typical depth resolution of $\sim$5 μm and diffraction-limited lateral resolution typically ranging from 1 to $\sim$20 μm. In common practice, OCT is implemented as a scanning method like confocal or TPM and forms images by moving the light beam over the surface of the tissue.

All scanning methods result in a tradeoff between field of view and image acquisition rate. While confocal and TPM form an image at a single depth, OCT and PAT form images over a range of depths simultaneously. Simultaneous measurements over this range of depths is achieved in OCT by implementing recently developed Fourier domain detection techniques (Choma *et al*, 2003; Leitgeb *et al*, 2003), which offer tremendous improvements in the volumetric image acquisition rate relative to time-domain OCT (Huang *et al*, 1991). Importantly, OCT is sensitive to Doppler shifts in the scattered light that arise from moving RBCs, enabling high-resolution measurements of RBC velocities (Chen *et al*, 1997) and blood flow (Srinivasan *et al*, 2011; Wang *et al*, 2007*b*).

While the spectrum of brain OCT applications is still being explored, its utility for minimally invasive quantitative measurements of blood flow *in vivo* (Srinivasan *et al*, 2011) will become an important tool because of its improved spatial and temporal resolution over conventional gold-standard methods and because it can be applied in longitudinal studies.

Diffuse correlation spectroscopy: Diffuse correlation spectroscopy (DCS) offers the ability to noninvasively measure CBF in humans through the intact scalp and skull (Kim *et al*, 2010), albeit with only superficial cortical sensitivity, lateral resolution of 1 to 3 cm, and no depth resolution. It has been extensively crossvalidated against other blood flow measures (Kim *et al*, 2010) (Figure 3D). Diffuse correlation spectroscopy measurements are generally obtained using a long-coherence-length laser at around 800 nm to exploit the weak absorption of tissue and enable light to propagate through thick tissues. A long-coherence-length laser is used so the light paths that travel long distances through the

tissue still interfere with the short light paths. The tissue spatial sensitivity profile is identical to that of NIRS measurements; therefore, the partial volume effects of the overlying scalp and skull must be considered when estimating CBF. Diffuse correlation spectroscopy is similar to arterial spin labeling fMRI in that it provides a measure of blood flow. Near-infrared spectroscopy and DCS both hold the same advantage over fMRI: that of being portable and suitable for continuous bedside measurements.

Diffuse correlation spectroscopy has been cross-validated extensively against other blood flow measures (Mesquita *et al*, 2011). It has been shown in acute stroke patients that cerebral perfusion varies with the elevation of the patient's head in the bed and that while optimal perfusion is usually achieved with the patient lying down, that some patients are optimized in a more elevated position. Diffuse correlation spectroscopy has also demonstrated a flow—volume uncoupling in newborn infants during the first 8 weeks of life that is a result of dramatic reductions in hematocrit as fetal hemoglobin is replaced with adult hemoglobin (Roche-Labarbe *et al*, 2010).

Angiogram

Two-photon microscopy and OCT, in addition to providing measures of RBC velocity and blood flow, are able to provide high-resolution angiograms of the microvasculature. Two-photon microscopy angiograms are commonly performed (Kleinfeld *et al*, 1998) (Figure 3B); OCT angiograms have recently appeared in the literature (Wang *et al*, 2007*a*) (Figure 3C). In contrast with TPM, OCT angiography does not require the administration of dyes or extrinsic contrast agents. Also, OCT angiography performs 3D imaging on time scales of minutes, whereas TPM requires time scales of hours to achieve comparable fields of view (Vakoc *et al*, 2009). While OCT is able to penetrate deeper than TPM can, the limit is slightly >1 mm. Photoacoustic tomography (Figure 2B) offers the ability to obtain angiograms at depths of several millimeters (Hu and Wang, 2010). In all cases, greater depth penetration requires a craniotomy.

Using TPM angiograms, Nishimura *et al* (2007) have demonstrated that while pial arteries provide a mesh network of redundant blood flow, penetrating arterioles are bottlenecks of flow to deeper levels, such that occlusion of a penetrating arteriole will result in downstream ischemic damage. Optical coherence tomography is playing an important role in longitudinally quantifying angiogenesis following brain injury and investigating the effect of different agents on promoting angiogenesis (Jia *et al*, 2011).

Metabolic imaging

Optical methods are well suited to measure oxygen delivery by blood, and through multimodal approaches, estimating oxygen consumption by tissue. Because of the autofluorescence of the coenzymes NADH and flavin adenine dinucleotide (FAD), it is possible to monitor cellular energetics directly. Fluorescent analogs of glucose have recently become available and these will likely enable more direct measures of glycolysis with optical resolution.

Phosphorescence Lifetime Imaging of Oxygen

Phosphorescence lifetime imaging of oxygen ($PLIO_2$) measures oxygen-dependent phosphorescence lifetimes of an exogenous contrast agent (Rumsey *et al*, 1988; Vanderkooi *et al*, 1987). It can be used to image both cerebral intravascular and tissue oxygenation using widespread optical imaging systems such as widefield charge-coupled device (CCD) imaging (Sakadzic *et al*, 2009) or different microscopy modalities (Sakadzic *et al*, 2010; Yaseen *et al*, 2009) (Table 1). Combining $PLIO_2$ with TPM (Finikova *et al*, 2008; Sakadzic *et al*, 2010) enables measurement of cortical oxygen delivery with subcapillary resolution in tissue and deep microvasculature (Devor *et al*, 2011; Lecoq *et al*, 2011; Sakadzic *et al*, 2010) (Figures 4A and 4B).

The phosphorescence lifetime of a probe depends on the partial pressure of oxygen (pO_2) in the immediate vicinity of the probe, providing a spatially localized measurement of dissolved oxygen. Probe molecules were specially designed for either linear or two-photon excitation regimes, with a high degree of encapsulation that ensures stability of lifetime calibration in a complex biological environment (Finikova *et al*, 2008; Lebedev *et al*, 2009). Unlike spectroscopy-based hemoglobin saturation measurements, $PLIO_2$ lifetime imaging is insensitive to changes in tissue optical properties during imaging. The acquisition speed is currently limited to 0.2 to 1 second per measurement point by relatively long phosphorescence lifetimes and the number of decay averages required at each point.

Through combination of $PLIO_2$ with TPM, we recently demonstrated that the increase in blood oxygenation during the hemodynamic response, which has been perceived as a paradox, may serve to prevent a sustained oxygenation drop at tissue locations remote from the vascular feeding sources (Devor *et al*, 2011). We have also observed that a significant amount of oxygen is delivered to the tissue from the arteries, and that venous intravascular pO_2, surprisingly, is higher than that in the capillary bed on average (Sakadzic *et al*, 2010).

Oxygen Consumption

As noted above, OISI, PAT, and NIRS are all able to measure hemoglobin oxygenation through absorption spectroscopy, while $PLIO_2$ can measure pO_2 in blood and tissue. Tissue oxygen consumption is commonly estimated based on the difference

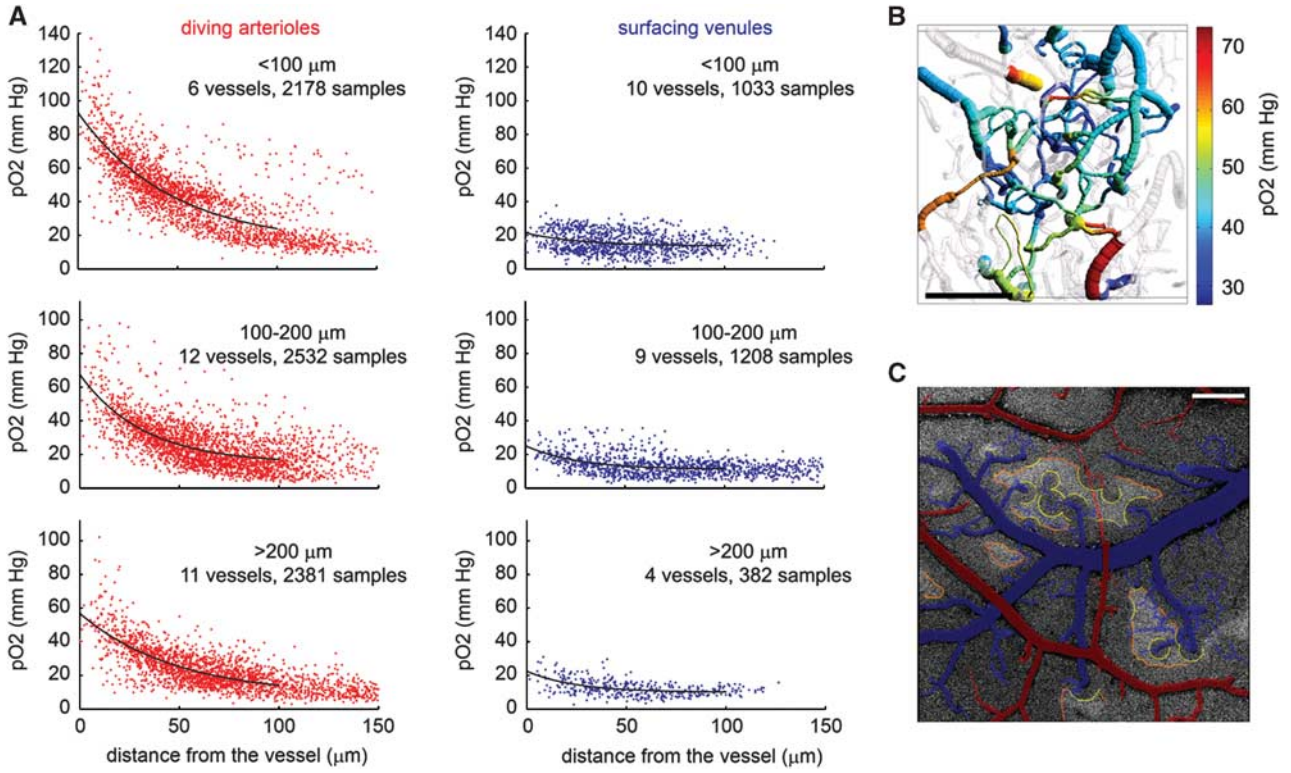

Figure 4 Optical imaging of oxygen availability and metabolism. (**A**) Two-photon partial pressure of oxygen (pO_2) imaging in cerebral tissue. Each plot shows baseline pO_2 as a function of the radial distance from the center of the blood vessel—diving arteriole (left) or surfacing venule (right)—for a specific cortical depth range. Data from multiple vessels are overlaid on each plot (Devor *et al*, 2011). (**B**) Two-photon measurement of pO_2 in cortical microvasculature. Measured pO_2 values are overlaid on a vasculature graph (grayscale) (Sakadzic *et al*, 2010). Scale bar: 200 μm. (**C**) Spatial variation in baseline nicotinamide adenine dinucleotide (NADH) fluorescence, imaged with two-photon microscopy (TPM), can be explained by distance from the vasculature. NADH fluorescence is superimposed with pseudocolored arterioles (red) and venules (blue). The yellow sketches highlight circular borders enclosing perpendicularly oriented vessels; the orange sketches highlight linear borders parallel to horizontally oriented vessels (Kasischke *et al*, 2010). Scale bar: 200 μm.

between oxygen flowing into a region through arteries and out through veins. Thus, combining optical measures of oxygen with a measure of flow, it is possible to estimate oxygen consumption. This has been performed by combining OISI with LSCI (Dunn *et al*, 2005), by combining $PLIO_2$ with LSCI (Sakadzic *et al*, 2009) and Doppler OCT (Yaseen *et al*, 2011), by using PAT (Yao *et al*, 2011), and by using NIRS in combination with DCS (Durduran *et al*, 2010).

Metabolic Coenzymes Nicotinamide Adenine Dinucleotide and Flavin Adenine Dinucleotide

Nicotinamide adenine dinucleotide and FAD are important coenzymes for energy metabolism and both are intrinsically fluorescent (for recent reviews see Heikal, 2011; Shuttleworth, 2010; Turner *et al*, 2007). *In vivo* studies generally focus on measuring changes in NADH or FAD fluorescence (Table 1). Nicotinamide adenine dinucleotide fluorescent changes can serve as an indicator of the balance between glycolysis and oxidative phosphorylation rate changes. Flavin adenine dinucleotide increases indicate an increase in oxidative phosphorylation. Fluorescent changes can be imaged using single- and two-photon excitation (Harbig *et al*, 1976; Huang *et al*, 2002; Kasischke *et al*, 2010; Weber *et al*, 2004) (Figure 4C). However, measuring FAD in the brain can be challenging, because of its low two-photon absorption cross-section, possible low concentration, and emission overlap with lipofuscin fluorescence and other flavin proteins (Heikal, 2011).

Single-photon NADH and FAD imaging was used to map cortical activity (Husson *et al*, 2007; Reinert *et al*, 2007; Sirotin and Das, 2010) and demonstrate that an increase in metabolism occurs faster than an increase in CBF (Weber *et al*, 2004). Two-photon imaging of NADH in brain slices indicated that the ratio and time course of oxidative and nonoxidative metabolism can differ in neurons and astrocytes (Kasischke *et al*, 2004). Two-photon imaging of NADH also has been applied *in vivo* in healthy cerebral cortex (Kasischke *et al*, 2010) and during experimentally induced cortical spreading depression (Takano *et al*, 2007). These studies showed a

close spatial association between NADH fluorescence and arteriolar geometry, implying that arterioles serve as oxygen sources, and demonstrated vulnerability of tissue midway between capillaries for hypoxia caused by large-scale pathological increases in neuronal activity.

Fluorescent Glucose

Glucose metabolism is routinely assessed with 2DG autoradiography postmortem. FDG positron emission tomography enables *in vivo* measurements. Both of these require radioactive glucose accumulation over tens of minutes. Fluorescent glucose analogs offer the exciting ability to estimate glucose transport into individual cells *in vivo* (Chuquet *et al*, 2010), but still require accumulation of the glucose analog over minutes (Table 1). In addition, the interpretation of the data may be complicated due to the difference in kinetic parameters of individual glucose transporters with respect to glucose and fluorescent glucose analogs (Barros *et al*, 2009), as well as the existence of different glucose transporters in neuronal and glial cells (Simpson *et al*, 2007). Further advances in the design of novel glucose probes (Lee *et al*, 2011) or glucose fluorescent sensors (Pickup *et al*, 2005) are needed to address these challenges.

Neuronal imaging and photoactivation

The ability to both observe and experimentally manipulate neuronal activity is a prerequisite for conducting successful mechanistic neurovascular/neurometabolic studies with unambiguously interpretable results. A change in neuronal activity is associated with multiple processes that can be measured optically, including changes in transmembrane voltage (depolarization or hyperpolarization), intracellular changes in ionic concentration (e.g., increases in $[Ca^{2+}]$), release of neurotransmitters, and changes in pH (Table 1). None of these processes alone can be considered an absolute measure of neuronal activity. Rather, each reflects a particular aspect of neuronal activity and various of these aspects (e.g., voltage changes and the amount of released neurotransmitter) can be nonlinearly related.

Well-controlled experimental manipulation of neuronal activity on a cellular scale provides a powerful tool for understanding the associated metabolism and testing the role of specific cell types in control of vasodilation/vasoconstriction.

Voltage Imaging

Voltage-sensitive probes or 'dyes' (VSD) (Cohen and Salzberg, 1978; Grinvald and Hildesheim, 2004) reside in the plasma membrane of neurons and act as molecular transducers that transform changes in membrane potential into optical signals: absorption, emitted fluorescence, a shift in the spectrum of the dye, or a change in its second-harmonic generation properties (for a recent review see Peterka *et al*, 2011). Since dendritic arborizations constitute a large percentage of the total membrane area, VSD signals are sensitive to subthreshold neuronal activity.

Synthetic VSD bind to all plasma membranes; targeting to particular neuronal cell types is not feasible. To overcome this problem, approaches for genetically encoded and 'hybrid' voltage sensors have been explored (Akemann *et al*, 2010; Siegel and Isacoff, 1997; Wang *et al*, 2010). Although cell-type specific expression of genetically encoded voltage sensors has not yet been demonstrated, it is an active area of research (Homma *et al*, 2009). Another challenge is development of voltage probes suitable for two-photon excitation (Kuhn *et al*, 2008).

Neuronal changes in membrane voltage occur on a millisecond timescale, introducing a strict requirement for the temporal response properties of the sensor. In common practice, voltage sensors are excited in the single-photon regime, and images are generally acquired using a camera detector with no depth resolution (but see Kuhn *et al*, 2008).

Due to sensitivity of VSD signals to subthreshold changes in neuronal polarization, VSD imaging is very useful for detection of neuronal inhibition. By implementing VSD imaging, we previously demonstrated the occurrence of CBF decrease and vasoconstriction in inhibited cortical regions (Devor *et al*, 2008). Within the realm of pathophysiology, VSD imaging provided a significant insight in stroke-induced neuronal reorganization, including short- and long-term sensory remapping (Brown *et al*, 2009; Sigler *et al*, 2009) and the recent finding of the immediate neuronal disinhibition in the unaffected hemisphere (Mohajerani *et al*, 2011).

Ionic Indicators

Fluorescent ionic-sensitive indicators are widely employed as measurements of neuroglial activity. Among them, calcium indicators have become an important tool in brain research due to the importance of calcium in neuronal and astrocytic physiology and the availability of bright and sensitive acetoxymethyl ester derivatives (Tsien, 1981) that can be delivered *in vivo* and excited in the two-photon regime (for recent reviews see Garaschuk *et al*, 2006; Grewe and Helmchen, 2009; Kerr and Denk, 2008).

In contrast to voltage sensors, where usable genetically encoded variants are just starting to arrive, genetic calcium probes are widely used (Hires *et al*, 2008; Mank and Griesbeck, 2008). Intracellular calcium concentration changes on a slower time scale than transmembrane voltage. Therefore, relatively slow kinetics of genetically encoded sensors—ultimately limited by the rate of conformational

change of proteins—is less of an issue in the design of calcium probes.

When imaged with TPM, calcium increases within individual neuronal cell bodies can be used to reconstruct spike trains (Vogelstein *et al*, 2010). Different types of neurons vary in their expression level of particular calcium channels, and multiple types of calcium channels exist (Tsien *et al*, 1995). Although cytosolic calcium can fluctuate within the subthreshold range of membrane potentials (Ross *et al*, 2005), calcium imaging is believed to reflect spiking and not subthreshold (e.g., synaptic) activity (Cossart *et al*, 2005).

The use of calcium indicators enabled the discovery of astrocytic excitability (Cornell-Bell *et al*, 1990; Nedergaard, 1994), and, in combination with TPM, has truly revolutionized both basic and applied neuroscience allowing visualization of microscopic cortical functional organization (Ohki *et al*, 2005), abnormal waves of astrocytic activity in Alzheimer's disease (Kuchibhotla *et al*, 2009), and functional rewiring after a stroke (Winship and Murphy, 2008), just to name a few.

Synaptic Release

Synaptic release of (potentially vasoactive) neurotransmitters and neuropeptides involves depolarization of the presynaptic terminal, calcium entry through voltage-gated channels located on the plasma membrane in the vicinity of docked and ready-to-release synaptic vesicles, fusion of the vesicles with the plasma membrane, and loss of the acidic intravesicular environment. The change of pH experienced by the luminal side of a vesicle upon fusion provides the foundation for pHluorin-based reporters of synaptic release (Miesenbock *et al*, 1998). These reporters are genetically encoded and have been used extensively in the olfactory bulb. Another approach is to target genetically encoded calcium indicators to the cytoplasmic side of vesicular membranes (Dreosti *et al*, 2009). Imaging the release of specific transmitters requires specific optical probes. One such probe, suitable for TPM, has recently been developed for imaging of extracellular glutamate (Okubo *et al*, 2010). Genetically encoded probes have been applied in the olfactory bulb to demonstrate that functional hyperemia was highly correlated with glutamate release but not with postsynaptic activity (Petzold *et al*, 2008).

Optogenetic Actuators

Genetically encoded optical actuators for manipulation of neuronal activity *in vivo* have recently become available due to the development of methods based on expression of light-activated ionic channels and pumps of bacterial origin (Miesenbock, 2011) (Figure 5A). These methods, together with genetic methods for reporting of neuronal activity—such as the genetically encoded voltage and calcium probes discussed above—comprise a new field called 'optogenetics.' Current optogenetic tools for optical control of neuronal activity offer both excitation and inhibition (Miesenbock, 2011; Yizhar *et al*, 2011).

Genetically encoded photoactuators, expressed in cortical pyramidal cells, were used to drive the hemodynamic response in a recent fMRI study (Lee *et al*, 2010), providing a prove-of-principle for

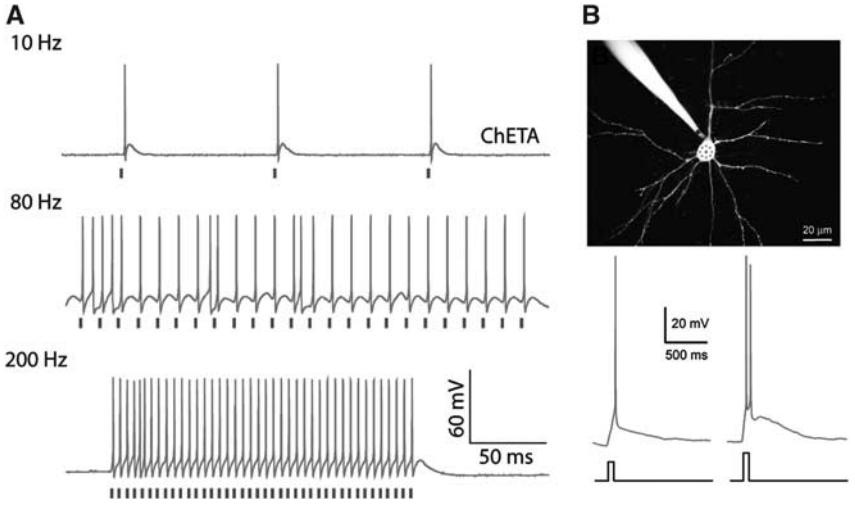

Figure 5 Optical manipulation of neuronal activity. (**A**) Intracellular electrophysiological recordings from an inhibitory cortical neuron that was genetically engineered to express a light-sensitive protein, *ChETA* (Gunaydin *et al*, 2010). Bars under the electrophysiological traces indicate light stimulation (472-nm, 2-milliseconds light pulse widths). (**B**) Photoactivation of a pyramidal cortical neuron by photolysis of a caged-glutamate compound (Fino *et al*, 2009). The upper panel shows the neuron loaded with a fluorescent dye through the recording pipette. Uncaging laser targets are indicated by dots within the soma. Scale bar: 20 μm. The lower panel shows electrophysiological recordings of neuronal spikes triggered by uncaging. The duration and relative intensity of the uncaging light are indicated by square-pulse traces below the electrophysiological recordings.

the utility of optogenetics in neurovascular and neurometabolic research.

Photolysis of 'Caged' Compounds

Another approach for photoactivation (with a longer history of applications) is based on the use of synthetic derivatives of transmitters and second messengers in which addition of a chemical bond makes them biologically inert (Adams and Tsien, 1993). Photolysis ('uncaging') breaks a bond, liberating active properties of the compound. Caged compounds designed for two-photon photolysis allow targeted manipulations on a cellular and even subcellular level (e.g., mimicking a synaptic input to a single spine) (Figure 5B). The large majority of uncaging studies have been performed in brain slices and cell cultures. This is because the need to deliver extrinsic caged molecules presents a challenge for *in vivo* studies (Noguchi *et al*, 2011). Nevertheless, future improved delivery strategies and technical advances in two-photon photostimulation (Nikolenko *et al*, 2007) are likely to promote two-photon uncaging to a valuable manipulation method in microscopic studies of the neurovascular unit physiology *in vivo*.

Future directions in development and application of optical tools: progress toward a mechanistic understanding of neurovascular and neurometabolic physiology

The spectrum of optical technologies outlined above offers a comprehensive set of tools for measurement of a wide range of physiological parameters. How can we use these optical tools to make progress toward a mechanistic understanding of the regulation of flow and metabolism? First, let us consider the following specific *Questions*, significant for achieving this understanding, which can be tackled with the use of optical imaging tools:

(1) What is the specificity, or biological relevance, of known vasoactive messengers (neurotransmitters, neuromodulators, gliotransmitters, and metabolic biproducts) with regards to normal or pathophysiological conditions and particular phases in the overall hyperemic response (e.g., initiation of dilation/constriction, maintenance of sustained dilation, or propagation of dilation along the arteriolar walls)?
(2) What is the primary vascular target of specific neurovascular messengers: capillaries, precapillary arterioles or larger diving arterioles? What is the origin of the depth dependence of hyperemic response? Is it related to differences in neurovascular coupling mechanisms or in response properties of the target vessels?
(3) What is the metabolic cost of neuronal activity across different modes of healthy brain function and in pathological conditions and how is it met? What is the ratio of oxidative phosphorylation to glycolysis, and to what degree are these metabolic processes compartment-specific (i.e., occur in neurons versus astrocytes)?
(4) What modulatory systems regulate the gain of hyperemic response and how? What happens with disregulation of this gain control in neurological disease?
(5) What are the fundamental principles that describe the healthy hyperemic response of the 3D vascular network: what is the location of the initial dilation, what are the summation rules of locally induced and propagated dilation/constriction, how diameter changes within the vascular network translate into changes in tissue oxygenation? How is this organization altered in neurological disease and stroke?
(6) What is the biological significance of astrocytic excitability, including calcium transients, calcium waves, and gliotransmission in healthy and diseased brain?
(7) How is microscopic neurovascular and neurometabolic function of the neurovascular unit reflected in noninvasive macroscopic signals relevant for human imaging? How can the insights from animal models be translated to human studies?

Below are a number of approaches, which, in our view, will be essential in addressing these questions.

Descending to a Single-Cell Level *In Vivo*

Availability of high-resolution optical microscopy tools will have a central role in addressing *Questions 1 to 6* in the list above. In the context of *Question 1*, recent reports in brain slices have demonstrated that stimulation of neurons of different types produces specific responses in the embedded vascular segments: dilation or constriction (Cauli *et al*, 2004; Rancillac *et al*, 2006). Can these experiments be translated *in vivo*? In other words, can we identify the microscopic *in vivo* vascular 'signature' of activation in neurons with known phenotype and neurotransmitter content? While one-photon excitation of photoactuators currently used in optogenetics affects a population of cells, eliciting spikes in a single cell can be achieved by two-photon uncaging as has been demonstrated *in vitro* (Fino *et al*, 2009) (Figure 5B). Two-photon photoactivation of channelrhodopsin-2 (Rickgauer and Tank, 2009) and delivery of a transgene to a single cell *in vivo* (Kitamura *et al*, 2008) have also been demonstrated.

Another potential strategy for evaluating vascular diameter changes induced by the firing of specific neurons *in vivo* is based on triggering the diameter measurements on spikes in a particular cell. Spike timing can be estimated from calcium imaging

(Vogelstein *et al*, 2010). Identification of cell types *in vivo* can also be achieved through the use of genetically encoded fluorescent markers (Tsien, 2005), postmortem immunolabeling (Kerlin *et al*, 2010), or single-cell PCR if activation is achieved by a targeted whole-cell patch recording (Margrie *et al*, 2003). This strategy requires that firing in the neuron of interest does not temporally coincide with firing in other neighboring neurons to avoid release of additional types of vasoactive substances. Therefore, the analysis would be limited to spontaneous firing, rather than firing in response to stimulation, and use of either awake animals or anesthesia that does not induce neuronal synchronization.

Merging Advanced Optical Imaging with Optical Manipulation

Combining novel and improved optical imaging technologies with the recent revolutionary advances in optogenetics (Miesenbock, 2011; Yizhar *et al*, 2011) will allow *in vivo* measurement of vascular and metabolic consequences of controlled experimental manipulation of neuronal activity, critical for addressing *Questions 2 and 3*. Already today, a number of transgenic mouse lines with cell-type specific expression of light-gated ionic channels have been developed; some are already commercially available. Photoactivation of these and other genetically encoded optical actuators results in activation (or inhibition) of cells expressing the transgene. Therefore, when expression is specific to a particular cell type, it might be well suited to addressing the neuronal cell-type specificity of vascular regulation: which cell types induce vasodilation/constriction upon depolarization (firing), and which vasoactive neurotransmitters are released. To ensure specificity in such an experiment, one has to prevent propagation of activation to other neuronal cell types. For example, unless synaptic communication is inhibited, photoactivation of excitatory pyramidal neurons is instructive in elucidating connectivity between the brain regions (Lee *et al*, 2010) but might not provide any advantage for the study of neurovascular coupling over a sensory stimulation: in both cases firing of many neuronal cell types results in release of a mixture of neurotransmitters and peptides.

Optogenetic activation of specific modulatory (e.g., cholinergic) projections will have an important role in addressing *Question 4*. In this respect, transgenic mouse lines with targeted expression of photoactuator proteins in cholinergic and serotonergic neurons have been developed (Zhao *et al*, 2011) and are available from Jackson Laboratory (http://jaxmice.jax.org/).

Further Progress in the Design of Novel Optical Reporters

Our ability to grasp a physiological process and macroscopic 'observables,' relevant for noninvasive imaging in humans, critically depends on understanding the behavior of the underlying microscopic parameters. The most recent example illustrating the importance of specific, selective, and sensitive optical probes for high-resolution microscopic measurement is a new phosphorescent probe to measure pO_2 (Finikova *et al*, 2008), which will be instrumental is addressing *Question 5*. This probe, applicable for two-photon imaging, has already been utilized to study intravascular and extravascular oxygenation at baseline and during neuronal activation (Devor *et al*, 2011; Lecoq *et al*, 2011; Sakadzic *et al*, 2010). Other examples include a two-photon excitable probe that has also been recently developed for imaging of extracellular glutamate (Okubo *et al*, 2010) and novel cell-based fluorescent reporters for detection of acetylcholine release (Nguyen *et al*, 2010). Further progress in design of existing optical reporters (glucose) (Lee *et al*, 2011) and future development of new optical sensors—among them the ones for lactate and adenosine, important for addressing *Question 6*—will open unprecedented opportunities to visualize directly neurovascular and neurometabolic processes.

Combining Different Imaging Modalities

In the context of cortical function, even the most specific, sensitive and high-resolution measurement on its own provides only a descriptive view of a single aspect of the underlying multifaceted physiological processes. This being the case, integration of two or more imaging technologies, each sensitive to a different aspect of the physiological process under study (the 'multimodal' imaging approach) will allow simultaneous measurements of multiple relevant physiological and biophysical parameters and computational inference of processes that cannot be measured directly. For example, a combination of $PLIO_2$ (Sakadzic *et al*, 2010) with OCT (Srinivasan *et al*, 2011) may provide a microscopic measure of oxygen consumption. The multimodal principle is of course not limited to optical technologies—one can combine optical imaging with, for example, electrophysiological recordings (Berwick *et al*, 2008; Sheth *et al*, 2004) or MRI measurements (Kennerley *et al*, 2005).

Further Improvement in Resolution and Sampling Efficiency of the Available Methods

Optical technological innovation continually strives to improve spatial resolution, image acquisition rate, and depth penetration. We are now seeing tremendous advances on these fronts for scanning microscopies. The heterogeneous structure of the tissue distorts the optical wave front, degrading the spatial resolution from the diffraction limit. Adaptive optics is developing as a robust approach to counterbalance the tissue distortions and restore diffraction-limited resolution for TPM (Ji *et al*, 2010) and OCT.

The image acquisition rate is dictated by the raster scanning rate divided by the image volume. Scanning rate can be increased by using acousto-optic deflectors (Grewe *et al*, 2010). However, one must consider the implementation of acousto-optic deflectors so as not to degrade spatial resolution (Kirkby *et al*, 2010). Two-photon microscopy imaging in 3D is slow because of the need to physically move the objective, although piezo-electric resonators now enable rapid *z*-translation (Gobel *et al*, 2007). Faster *z*-scanning is being demonstrated by using adaptive optic strategies to dynamically adjust the focal depth of an objective with no moving parts (Grewe *et al*, 2011). Scanning rate is ultimately limited by the number of photons detected. Photon collection efficiency can be improved using novel approaches to increasing the effective numerical aperture of the microscope collection optics (Engelbrecht *et al*, 2009). More efficient excitation of fluorophores can be achieved by increasing the repetition of the pulsed laser sources used in TPM (Ji *et al*, 2008; Li *et al*, 2010). Larger image volumes can be scanned faster by essentially dividing the larger volume into smaller subvolumes that are imaged in parallel (Bewersdorf *et al*, 1998).

Tissue scattering is wavelength dependent and limits depth penetration. Reducing tissue scatter would therefore enable greater penetration. While tissue-clearing methods can be used *ex vivo*, we can use longer excitation wavelengths *in vivo* where the optical scattering is reduced. The penetration depth has been shown to increase from $\sim$600 to 1,000 μm when moving from 800 to 1,300 nm excitation with TPM (Kobat *et al*, 2009). Similar advantages have been shown for OCT. The challenge is to ensure that the fluorophore excitation extends out to these longer wavelengths. Even greater penetration depths are being achieved with TPM and OCT by using GRIN lenses and microprisms to invasively provide an optical window to deeper tissue structures (Barretto *et al*, 2011).

New Emerging Approaches for *In Vivo* Imaging

Several approaches not detailed here are worth mentioning because of their potential impact when applied *in vivo*. Intensity-based ionic imaging cannot be used to quantify absolute concentration of ions because the intensity depends on the ionic concentration and dye concentration. This limitation can be overcome by measuring fluorescent lifetime, as has been demonstrated for calcium (Wilms *et al*, 2006). Specifically, fluorescent lifetime provides an absolute measure when the ionic concentration alters the probe conformation, resulting in a change in lifetime that is not dependent on probe concentration. Fluorescent lifetime can also be used to distinguish different conformations of fluorescent molecules, such as bound and free NADH (Vishwasrao *et al*, 2005).

The fundamental diffraction limit of optical imaging has recently been shattered by a variety of clever solutions that achieved resolution of better than 20 nm (Hell, 2009; Huang, 2010). These new nanoscopic methods reveal fine-scale subcellular structures in culture, and have the potential to be applied *in vivo* with depth penetrations likely comparable to confocal microscopy of 50 to 100 μm.

Translation to human studies

We have described advances of optical imaging in obtaining microscopic measures of cellular and vascular functioning. For the foreseeable future, though, noninvasive human neuroimaging will only be able to access the collective behavior of large groups of cells and vessels. Thus, translation of our microscopic mechanistic understanding of neurovascular and neurometabolic coupling will depend on our understanding of how this microscopic behavior is reflected in macroscopic observables (*Question 7*). Because of their overlapping spatial and temporal resolutions, optical technologies enable us to perform experiments that step incrementally from the cell/capillary level to the few cell/capillary level and all the way to noninvasive imaging of cubic centimeters of tissue relevant to human studies. This transition can be made in an animal from super-resolution microscopy to NIRS/DCS to characterize the micro–macro relationship. Near-infrared spectroscopy/DCS, combined with noninvasive electrophysiological recordings, can then be used in humans to verify the physiological findings, and finally put into clinical utility. As an example, NIRS has been used in rodents to test whether neurovascular coupling is driven by cortico-cortical connections or thalamic inputs in the somatosensory cortex (Franceschini *et al*, 2010) and then translated to humans to confirm the findings (Ou *et al*, 2009). Confirming that the vascular response is driven by cortico-cortical processing rather than by thalamic inputs would have profound clinical importance because vascular-based neuroimaging methods (fMRI, NIRS, and DCS) could then be used to assess the integrity of the sensory system at the level of cortico-cortical circuits in injury (stroke and trauma) and disease, and when those systems become active in development or turn off due to aging.

Conclusions

The arsenal of available optical imaging technologies offers the ability to measure a spectrum of parameters related to vascular, metabolic, and neuronal activity at multiple scales. Merging these technologies with recent revolutionary methods in genetic labeling and remote control of neuronal activity, allowing targeted activation of identified neuronal cells and cellular populations, is going to be a hallmark of cerebrovascular research in the next decade, or maybe even the next '50 years of dedicated CBF and metabolism' research. In parallel, findings from animal imaging will be translated to human studies

through application of the noninvasive optical methods NIRS, DCS, and PAT. Although optical technologies are already broad and versatile, their performance is going to improve. In combination with continuous efforts in the development of novel optical sensors, and in expanding the array of transgenic animals with genetically encoded structural and functional fluorescent labels, the future of the brain imaging division of biomedical optics is as bright and exciting as ever before.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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