Two-photon imaging of cerebral vasodynamics in awake mice during health and disease

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Abbreviations:
ACSF = artificial cerebral spinal fluid
BOLD fMRI = blood oxygen level dependent functional magnetic resonance imaging
PoRTS = polished and reinforced thinned skull
RBC = red blood cell
TPLSM = two-photon laser scanning microscopy

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Abstract

The energy demands of the brain are supplied by a dynamic and topologically organized vascular system. In vivo two-photon laser-scanning microscopy has become an essential tool for studying several aspects of cerebrovascular function, including neurovascular coupling, collateral arterial flow, and vasculopathies associated with stroke and neurodegeneration. Recently there has been a strong effort to perform imaging studies on mice in the awake state. This is to avoid the dampening effect that anesthetics have on neurovascular dynamics, and also to enable studies on trained behaviors. Studying awake mice has revealed a rich dynamism of cerebral blood flow control, but also raised a number of challenges in the collection of rigorous and meaningful datasets. In this chapter, we describe techniques routinely used for two-photon blood flow imaging in awake mice. This includes chronic window implantations and head fixation techniques to reduce movement during imaging, as well as blood flow data processing techniques that are robust to movement artifacts. Finally, we describe a non-invasive photothrombotic stroke model that can be performed on awake mice for imaging of ischemic pathology.

The constant energy demands of brain function are supplied by a reliable and dynamic cerebrovascular system (Attwell and Laughlin 2001). In the cerebral cortex, this system begins with the great cerebral arteries that emanate from the Circle of Willis and source a planar network of highly interconnected pial arterioles on the brain surface (Mchedlishvili 1963). Blood flow in this network is relatively insensitive to blockages (Schaffer et al. 2006; Blinder et al. 2010), but also allows the dynamic redistribution of blood toward regions of heightened electrical activity (Devor et al. 2007; Derdikman et al. 2003)(Fig. 1). Pial vessels are in turn connected to an underlying, three-dimensional network of microvessels by radially directed penetrating arterioles, which form bottlenecks in flow to columns of cortical microvasculature (Blinder et al. 2013). Blood is then drained from the microvasculature back to the cortical surface through penetrating venules and emptied into the central sinus to complete the supply chain. While the topology of the cortical vasculature is well established, the cellular and molecular mechanisms underlying moment-to-moment regulation of flow are a topic of considerable debate (Attwell et al. 2010). As we begin to unravel these mechanisms in vivo using animal models, it is important to consider not only how the use of anesthetics impacts
coupling of neuronal activity to blood flow in the normal brain, but also its impact on the progression of pathology in disease models.

**Figure 1 goes about here.**

*In vivo* two-photon laser-scanning microscopy (TPLSM) is an established method to visualize cerebrovascular topology and quantify blood flow at the level of single vessels in the rodent cortex (Kleinfeld et al. 1998; Shih et al. 2012b). In this respect, TPLSM has become a key tool to understand the basis of neurovascular coupling in health and disease (Helmchen and Kleinfeld 2008; Misgeld and Kerschensteiner 2006). While a rich body of data has resulted from studies on anesthetized animals, there remains a striking paucity of studies on vasodynamics in the awake state. Anesthetics greatly dampen the activity of neurons (Franks 2008) and astrocytes (Thrane et al. 2012), and alter vascular dynamics in basal and stimulated states (Drew et al. 2011). Anesthetics further preclude the study of vascular function during voluntary sensorimotor function. To image brain vasculature in the awake state, only minor enhancements upon routine *in vivo* imaging techniques are necessary. First, chronically implanted cranial windows are required to allow imaging well after recovery from initial surgeries. Second, a rigid head mount and gradual habituation to head restraint is needed to minimize movement during imaging. Here, we describe methods to visualize and quantify blood flow at the level of individual vessels in the cerebral cortex of awake, head-restrained mice. We further provide an example of how this technique can be used to study the effects of sensory-evoked neuronal activity on vascular responses. Finally we consider how non-invasive stroke models might be useful to study the trajectory of stroke injury on vascular function in the awake mouse.

**Visualizing the cerebral vasculature**

To gain optical access to the mouse cortex, a portion of the overlying skull must be thinned to translucency or removed entirely. This window is contained within a larger head-piece that is constructed, typically from dental acrylic, directly on the cleaned skull surface (**Fig. 2A**). Detailed methods have been described for chronic bone-removed cranial windows (Holtmaat et al. 2009; Mostany and Portera-Cailliau 2008) and semi-chronic thinned skull windows, which can provide excellent imaging quality for months (Yang et al. 2010)(See Chapter X, by Mostany *et al.*). Recent work, however, indicates that complete bone removal can lead to cortical inflammation and gliosis that might affect the phenomenon under study (Xu
et al. 2007). For this reason, chronically-implanted polished and reinforced thinned-skull windows (PoRTs), which avoid exposure of the cortex to air and compression of the brain with an overlying coverslip, may be preferred for awake imaging experiments (Fig. 2B) (Drew et al. 2010b; Shih et al. 2012a). PoRTs windows are stable for several weeks of imaging and provide optical access to the upper 250 µm of cortex with conventional TPLSM imaging systems. To study deeper cortical layers, however, one must use bone-removed cranial window, which can provide access to cortical structures as deep as 600 to 800 µm, or even the full 1 mm depth of cortex with special techniques (Kobat et al. 2009). Thus, the type of window used depends largely upon the goal of the experiment, and the factors to consider are imaging depth and potential affects of surgically-induced inflammation (Shih et al. 2012b). Both chronic bone-removed and PoRTs window types are suitable to awake two-photon imaging.

**Figure 2 goes about here.**

**Habituation to head restraint for imaging**<1° heading>

Artifacts caused by animal movement account for the majority of the noise during imaging of animals in the awake state. Movements caused by struggling can be greatly reduced through a gradual process of habituation to the head restraint apparatus designed to hold the head of the animal steady during imaging sessions. One to two days following surgery, animals can begin the process of habituation to head restraint. The animal’s head-piece must include a metal flange to hold the head steady during imaging (Fig. 2C). The apparatus for holding the flange typically consists of an optical breadboard with miniature optomechanical components commercially available from Newport or ThorLabs, and a custom-machined component designed to couple to the flange (Shih et al. 2012b). While the design of the head restraint apparatus differs widely between labs, stability is key. Thus, having at least two anchoring points for the custom flange holder, and as few degrees of freedom as possible will help minimize movement artifacts.

A new animal can be gradually accustomed to head restraint over a period of 3 to 7 days, starting with 15 min sessions without imaging and working up to several hours (Drew et al. 2011). Head-restrained mice tend to struggle less when their bodies are enclosed within a loose-fitting plastic or cardboard tube. The first two-photon imaging session should be at least 3 days after the initiation of habituation when the animal has become more accustomed to the apparatus. For each imaging session, restraining the animal for two hours at a time is
reasonable, but times can differ for each animal. Discomfort should be gauged by the extent of struggling and vocalizations emitted, and the restraint time adjusted accordingly. For imaging sessions lasting hours, drinking water should be supplied with a pipette. In our experience, approximately 10% of mice never become habituated to head-fixation and cannot be used for awake imaging.

In practice, a properly habituated animal will provide movement-free data for several minutes at a time even during sensory stimulation. While, intermittent periods of animal movement are unavoidable, it is important to identify significant movement artifacts such that the data can be omitted from analysis. Typically, several trials are collected for each stimulus paradigm and trials with excessive movement will be discarded. As a quality control measure, data acquisition can be tested on capillaries, which are only 3 to 5 μm in diameter and thus most sensitive to motion. Similarly, a surface arteriole, which may move due to dilations/constriction, can be scanned simultaneously with a neighboring venule that should exhibit little or no change in lumen diameter. A movement in both vessels would indicate movement of the animal. Finally, low-cost piezoelectric sensors and accelerometers can be used to identify movement alongside vasodynamic measurements, allowing data sets with excessive movement to be removed (Drew et al. 2011).

**Measurement of blood flow dynamics in single cortical vessels**

Prior to imaging, the mouse must be briefly anesthetized with isoflurane for an intravenous injection of fluorescent dextran dye. Typically, 25 to 50 μL of 5% (w/v) fluorescent-dextran dye, fluorescein-dextran (FD2000S; Sigma) or Texas red-dextran (D1830; Invitrogen), is dissolved in saline and injected either through the tail vein or infraorbital vein to label the blood serum. The dye will remain in circulation for approximately three to four hours, and supplements can be given as necessary if the animal is re-anesthetized.

When the vasculature is labeled with an intravenous bolus of fluorescent-conjugated dextran, red blood cells (RBCs) exclude the high molecular weight dextran dye and will appear as dark shadows moving against a bright fluorescent background of serum within the vascular lumen. This differential staining is the basis for measuring RBC velocity using laser-scanning microscopy (Villringer et al. 1989; Kleinfeld et al. 1998). Conventionally, single line-scans have been used to sample the RBC velocity and diameter of a vessel separately (Schaffer et al. 2006; Shih et al. 2009). However, blood flow is dynamic and could change within the time...
between each scan. In order to collect more samples simultaneously and with varying trajectories within the imaging plane, we use custom software to direct the imaging laser beam in a user-defined path (Fig. 3A, 4B and 5C) (Valmianski et al. 2010; Driscoll et al. 2011), following earlier work (Göbel et al. 2007; Göbel and Helmchen 2007; Lillis et al. 2008). Linear segments of constant scan speed traverse along the length of the center of the vessel and across the width of the vessel to measure RBC speed and lumen diameter, respectively (Fig. 3A). These linear scan segments are connected by polynomial splines, where connecting portions of the scan are accelerated to allow for rapid data collection across multiple vessels (Driscoll et al. 2011). The resulting line-scan is a space-time plot, typically displayed with the individual scan lines stacked on top of each other (Fig. 3B, 4C and 5D).

**Figure 3 goes about here.**

*Calculating red blood cell velocity.* <2° heading>

Portions of the scan path along the centerline of the vessel lumen reveal angled streaks within the space-time plot (Fig. 3B, bottom right, and 4D). Moving RBCs in flowing vessels sampled at a sufficient rate will appear as diagonal streaks. Stalls in flow will result in vertical streaks with distance as the abscissa and time as the ordinate. This is a common occurrence when measuring from capillaries, and may also occur in pial arteriolar and venous anastomoses (Shih et al. 2009; Nguyen et al. 2007). In the limiting case of extremely fast flowing vessels, the streaks will become horizontal and velocity data cannot be extracted unless faster scanning methods are used (see Future Directions). The centerline velocity is proportional to the slope of the RBC streaks. This slope can be efficiently determined with a Radon transform of windowed portions of the data (Drew et al. 2010a), which is available in the MATLAB™ Image Processing Toolbox (Mathworks). The direction of flow can be ascertained from the sign of the slope and the direction of the line-scan sweep.

A velocity time series is calculated by taking successive time-windowed portions of the line-scan (Fig. 3C center, 4E and 5D). The size of the window must be sufficiently short to resolve the highest velocity modulation frequency, the heart rate, which is 8 to 10 Hz for awake mice (Drew et al. 2011). In addition, the window size must be large enough to capture a sufficient portion of the streak lines for the Radon transform to calculate an accurate slope, but small enough to prevent smoothing out the higher frequencies of the velocity data. When sampling with line-scan rates of ~1 kHz, we find that a window size of 40 ms is a good
compromise, which yields a Nyquist frequency of 12.5 Hz. We further use a window spacing of 10 ms. Oscillating physiological rhythms within the RBC velocity are a first indicator of good data quality. In addition to heart rate (Fig. 4E and 5E), other physiological signals detected in the RBC velocity may include breathing at ~1 to 2 Hz, and vasomotion at ~0.1 to 1 Hz (Kleinfeld and Mitra 2011; Mayhew et al. 1996)(Fig, 5E). Breathing rate, however, is not always detectable in the flow data collected from awake mice (Drew et al. 2011).

Measurement of RBC velocity is the limiting factor for the measurement of flow in larger arterioles on the cortical surface. In practice, conventional galvanometric mirrors are sufficiently fast to simultaneous capture RBC velocity and diameter from one penetrating arteriole at a time in the anesthetized state (Shih et al. 2013). However, in the awake state RBC velocity tends to increase, and thus atypical fast scanning and/or special analysis techniques (Kim et al. 2012) may be necessary or smaller pial vessels should be sampled. Venules and deep microvessels exhibit slower RBC velocity and thus 3 to 4 vessels can be measured simultaneously with spatially-optimized line-scans (Fig. 4 and 5C-E). Greater distances traversed by the laser will reduce the sampling frequency and a 1 to 2 kHz line-scan rate is recommended for accurate sampling of the movement of RBCs in pial arterioles (Driscoll et al. 2011).

**Calculating lumen diameter.** <2° heading>

As with RBC velocity, the diameter calculations are also taken from time-windowed portions of the line-scan data. The same window size and spacing used for velocity is typically used for diameter so that both parameters can be calculated on the same time scale. Vessel diameter is calculated as full-width at half-max of the vessel profile for each window (Fig. 3B, bottom left, and 3C, left). Note the intensity profile tends to increase near the edges due to the exclusion of RBCs from the glycocalyx, which generates two peaks. The two outermost half-max points of these peaks are used to calculate the vessel boundary. Linear interpolation adds sub-pixel accuracy to the diameter measurement.

**Estimating the flux of red blood cells.** <2° heading>

There are two limiting cases for the flux. When the diameter of the vessel allows the passage of RBCs only in single file, as in the case of capillaries, the flux of RBCs is just the number of RBCs that pass per second (Kleinfeld et al. 1998; Chuquet et al. 2007). In this limit, the blood plasma has an essentially constant velocity profile as a function of distance from the center of
the vessel, then the velocity falls rapidly to zero near the walls. The thin layer of plasma near the walls acts as a lubricating layer that, together with the glycocalyx, minimizes friction (Secomb et al. 1998).

When the diameter of the vessel is much greater than that of the RBC, as is the case for all pial surface vessels, the flow is laminar and nearly parabolic as RBCs flow in parallel streams (Schaffer et al. 2006; Rovainen et al. 1993). The two vascular parameters, RBC velocity and lumen diameter, are combined to calculate the volume flux, i.e., RBCs and plasma, for each vessel (Fig. 3C, right panel). Flux is a more complete description of blood flow in single vessels, as RBC velocity and lumen diameter can change independently of each other (Shih et al. 2009; Kontos 1989). The volume flux through the vessel is given by

\[ \bar{F} = \langle \ddot{v} \rangle A = \frac{\pi}{8} \ddot{v}(0) d^2 \]

where \( \langle \dot{v} \rangle \) is the average RBC velocity over \( A \), the cross-sectional area of the vessel lumen, \( v(0) \), is the time-averaged RBC velocity at the center line of the vessel, and \( d \) is the lumen diameter. Note that this formula underestimates the flux as the nonzero spatial extent of the RBC flattens the parabola of Poiseuille flow; empirical corrections have been discussed (Nishimura et al. 2010).

**Measurement of vessel diameter from 2-D planar images.**

If the goal is to examine only changes in vascular diameter, the collection of 2-dimensional planar images may be more desirable than line-scans. Arteriolar dilations occur on the order of seconds and do not require fast acquisition rates. Thus, movies collected at 5 Hz, for example, would be adequate to capture diameter changes associated with sensory-evoked hemodynamic responses and vasomotion (Drew et al. 2011). Heart-beat and breathing rates do not typically result in appreciable diameter changes. A further advantage of planar scans is that conduction of dilatory or constrictive changes may be easily observed along a length of vasculature. As with line-scan data, the full-width of half maximum of a segment of vasculature is calculated from each image using custom-written software to generate a diameter time-series from the movie.

**Vasodynamics in awake mice.**

We show an example study of pial surface and capillary vasodynamics measured under
normal conditions (Fig. 5). A mouse was habituated to head fixation and blood flow was measured using planar movies through a chronically implanted PoRTS window (Fig. 5A). Consistent with hemodynamic coupling of blood flow to neural activity, robust arteriole dilations could be evoked by prolonged contra-lateral whisker stimulation with puffs of air (Fig. 5B). Dilatory events consisted of an early peak followed by a secondary slowly-rising plateau, in averaged data, during sustained stimulation. The dilatory affect returned to baseline over ten seconds following cessation of stimulation. Pial venules, typically thought to be static in terms of diameter, show a weak dilation in the awake state (Fig. 5B). These data suggest that functional hyperemia detected by blood oxygen level dependent functional magnetic resonance imaging (BOLD fMRI) may be dominated by large changes in arteriole volume, in agreement with recent studies (Kim and Kim 2011), rather than in venules as predicted by the “balloon” model (Buxton et al. 1998).

The speed of RBCs within arterioles is high during the awake state and thus fast line-scan frequencies are required to capture this data in a smaller pial arteriole. However, RBC velocity can be routinely measured within the full range of capillaries for awake mice at 1 kHz scanning frequency (Drew et al. 2011; Drew et al. 2010b). While the basal vasomotor activity manifest as spontaneous arteriole dilations on the cortical surface, this translates into changes in microvascular RBC velocity as the blood flows into the brain tissue. These oscillations range from 0.1 to 1 Hz in frequency and are commonly observed in the awake state but may be dampened or non-existent during anesthesia. Vasomotor blood flow changes are more globally spread over the cortical mantle, compared to relatively local sensory-driven hemodynamic responses. Consistent with this idea, spontaneous oscillations in capillaries are strongly correlated across distant sites within the cranial window (Fig. 5C-E).

Figure 4 goes about here.

Both basal and stimulated responses of arterioles and venules are strongly suppressed by the use of urethane, a common anesthetic (Drew et al. 2011), that can attenuate the magnitude of the overall dilatory response by up to 75 % in the anesthetized state. Other commonly used anesthetics, including isoflurane (Takuwaa et al. 2012), α-chloralose (Dudley et al. 1982), and propofol (Alkire et al. 1995), similarly suppress the neurovascular response. In addition to the direct neural effects, anesthetics exert strong depressing effects on cardiovascular function (Janssen et al. 2004). Consistent with the depressive effects of
anesthetics on neural and cardiovascular system, optical imaging studies of neurovascular coupling have found differences between the awake and anesthetized animal (Nakao et al. 2001; Jones et al. 2008; Martin et al. 2006).

**Awake imaging and disease models** <1° heading>

The awake imaging methods we describe are also amenable to the study of cerebrovascular pathology in any mouse disease model. Here we focus here on pathology associated with ischemia. Anesthesia alters key mechanisms of stroke injury and repair and will therefore affect the trajectory of the disease. In the acute stage of ischemic injury, this can occur by suppression of neuronal activity or by engaging collateral blood flow sources. Common anesthetics, such as isoflurane, reduce the cerebral metabolic rate for oxygen consumption and likely the extent of excitotoxic neuronal toxicity (Scheller et al. 1988). Ketamine, in particular, is an antagonist of the NMDA receptor and attenuates production of the potent vasomediator, nitric oxide, during stroke (Olney et al. 1991; Lin et al. 1996). Cortical spreading depression (CSD), a wave of hyperactivity followed by a wave of depression (Siesjo and Bengtsson 1989), contributes to the expansion of tissue infarction (Lauritzen et al. 2011). The frequency of CSD events has been shown to be attenuated or modulated by numerous anesthetics, including ketamine (Hernández-Cáceres et al. 1987; Verhaegen et al. 1992), volatile anesthetics halothane, isoflurane and sevoflurane (Kudo et al. 2008; Kitahara et al. 2001), and urethane-chloralose (Guedes and Barreto 1992). Thus, many anesthetics mediate an overall protective effect in stroke by attenuating excitotoxic injury (Kawaguchi et al. 2005).

Anesthesia can also affect cerebral blood flow, which may have opposing effects on stroke outcome. Pentobarbital and isoflurane, in particular, cause global cerebral vasodilation that lead to a hypoperfusion, which can further decrease perfusion in the stroke penumbra and exacerbate injury (Hendrich et al. 2001). Conversely, the dilation of cerebral arterioles can conceivably promote tissue survival by engaging collateral blood supply through leptomeningeal anastamoses. Another important consideration is that hemodynamic responses measured by BOLD fMRI are widely used to infer tissue recovery after stroke (Cramer et al. 1997). Given the suppressive effects of anesthetics on arterial and neuronal reactivity, it is unclear when models using anesthesia fail to mimic the human condition (Austin et al. 2005; Antognini et al. 1997). Studies on awake preparations will be important to establish whether changes in arterial reactivity during stroke are masked or altered by the anesthetics.
The effects of anesthetics can extend beyond the acute phase of stroke when the infarct stabilizes and the peri-infarct zone begins to recover. Longitudinal in vivo imaging studies typically involve repeated periods of anesthesia that could affect repair/recovery processes. Anesthetics impact the activity of microglia directly by altering the expression of inflammatory mediators and also indirectly by reducing CSD, which promotes microglial activation (Gehrmann et al. 1993). The effect varies with the type of anesthetic used, as isoflurane appears to promote cytokine expression (Ye et al. 2013; Wu et al. 2012), while ketamine has an inhibitory effect (Chang Y 2009). Further, the activity of microglia is tightly linked to post-stroke repair process through release of matrix metalloproteinases (MMPs). MMPs are associated with the breakdown of tight junction proteins in acute stroke (Patel et al. 2013), but also appear to be involved in remodeling of neuronal connectivity (Wake et al. 2009) and possibly nascent blood vessel formation, which involves MMP activity (Arai et al. 2009). In summary, awake imaging preparations are expected to reveal the natural evolution of stroke injury, and will therefore provide more clinically relevant data on i) the spatiotemporal features of tissue injury and repair processes, ii) the timing of interventional therapies, and iii) the metrics used to gauge brain recovery, such as hemodynamic responses.

One limitation in current preclinical stroke studies is that the models are often invasive, necessitating deep and prolonged anesthesia. This precludes the visualization of early changes in stroke, which can in turn dictate the extent and long-term trajectory of the injury. We suggest that photothrombosis, a technique to non-invasively occlude blood vessels by light-mediated focal activation of the intravenous photosensitizer Rose Bengal, can be easily applied to awake preparations (Shih et al. 2013; Shih et al. 2011; Nishimura et al. 2007). An animal previously habituated to awake imaging would only need to be briefly anesthetized, (< 1 minute) for intravenous injection of a photosensitizer, minimizing the exposure time and allowing the stroke to develop more naturally during the period of the study. Photothrombotic stroke also provides the advantage of targeting the location of ischemia within the imaging window. Collimated laser beams, ranging in areas between 0.16 to 2 mm², have been used to ablate specific areas of cortex in sophisticated studies of functional remapping after stroke (Winship and Murphy 2008; Brown et al. 2009). While such lesions are spatially specific, they possess a limited ischemic penumbra (Zhang et al. 2005). A second approach using focused laser beams to irradiate single cortical penetrating vessels leads to similar areas of damage,
depending upon the RBC flux of the vessel, but generates a more gradual degradation of tissue due to a substantial penumbra (Shih et al. 2013; Shih et al. 2011). This allows for imaging of mechanisms involved in tissue death at the evolving core-penumbra interface.

**Figure 5 goes about here.**

**Future directions**

We show that methods to transition from anesthetized to awake imaging preparations are straight-forward and will be an important step to truly understand mechanisms of vascular control in the normal brain state. Since anesthesia has clear effects on the trajectory of stroke injury, it is also critical to understand how vascular degradation during acute stroke and repair in chronic stroke differ between anesthetized and awake preparations.

Two-photon imaging fills an important gap in the existing range of tools for blood flow quantification and enables the study of vasodynamics in awake mice. While intrinsic optical imaging, laser speckle, and BOLD fMRI enable measurement of flow over broader areas of brain, two-photon microscopy complements these techniques and provides a view of dynamics at the resolution of individual small cortical vessels. Further, TPLSM can be concurrently used to examine activity of surrounding cells that could be driving vascular activity (Takano et al. 2006; Nizar et al. 2013). Recent advances in hardware and optics now allow TPLSM images to be collected over entire cranial windows several millimeters in size, potentially enabling the spatiotemporal features of broader hemodynamic responses to be studied at capillary resolution (Tsai et al. 2013).

Some further advances will be necessary to fully capitalize on awake imaging experiments. First, faster laser-scanning is necessary to capture RBC velocity within larger cortical arterioles. Acousto-optic modulators (Lechleiter et al. 2002) can in principle scan vessels with faster flow as well as sample from larger populations of vessels simultaneously. Recent advances in processing of line-scan data using particle image velocimetry will also be useful to assess RBC velocity in larger vessels (Kim and Kim 2011). Additionally, application of filtering techniques to line-scan data may provide more robust measurements in the face of small movement artifacts caused by animal movement (Chhatbar and Kara 2013). Second,
physiological parameters most relevant to blood flow including blood pressure and blood gas remain difficult to measure in awake mice. In particular, even when these parameters are measured invasively from catheters in peripheral vessels, they may not reflect conditions in the cerebral vessel being examined. Novel imaging probes need to be developed to assess real-time changes in blood pressure, gas, and pH at the vessel being examined. Finally, new tools to manipulate cell-specific vasoactive signaling cascades will be an important step in dissecting the chemical basis of neurovascular coupling (Kleinfeld et al. 2011).

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Figure captions

Figure 1. The vasculature of the mouse cortex. (A) Dorsal surface of the whole mouse brain. The vasculature was filled with a fluorescein-gelatin cast and the brain was removed from the cranium to obtain a whole brain image. (B) Three-tiered topology of vasculature in mouse cortex, showing highly interconnected surface pial networks (tier 1) and subsurface microvascular networks (tier 3) bridged by bottleneck penetrating vessels (tier 2). Arterioles are colored red and venules in blue. This piece of tissue was removed from the barrel field of the primary somatosensory cortex (demarcated by a yellow square, inset of panel A). A golden band denotes the border of each vibrissa barrel column. Adapted from Blinder et al. (Blinder et al. 2013). (C) A schematic diagram illustrating penetrating vessels as they project radially from the pial surface into deeper layers of cortex. The density of microvasculature across different layers of cortex is generally uniform until the white matter where the density decreases (Tsai et al. 2009). Adapted from Shih et al. (Shih et al. 2012b)

Figure 2. Polished and reinforced thinned-skull transcranial window for imaging mouse cortex. (A) Schematic diagram showing dorsal view of the head mount and position of various components. A cross bar mount is added for stability when imaging awake preparations. $\beta$ = bregma and $\lambda$ = lambda. (B) Schematic diagram showing cross section of a PoRTS window and the components involved in its construction. (C) Magnified view of PoRTs window generated over the somatosensory cortex. The cross bar is affixed to a custom mounting apparatus. Two attachment points greatly reduces degrees of freedom for repeated imaging of the same location within the window. In this example, a connector was also implanted to organize electrodes for repeated electrocorticogram recordings. Adapted from Shih et al. (Shih et al. 2012b).

Figure 3. Single vessel vasodynamics measured with spatially optimized line-scans in somatosensory cortex of an anesthetized Sprague Dawley rat. (A) Planar image of pial arteriole and venule labeled by intravenous injection of 2 MDa fluorescein-dextran. This animal was anesthetized with continuous intravenous delivery of alpha-chloralose. These vessels were located in the forelimb region of somatosensory cortex. The line-scan path is overlaid on the image, with the colored segments corresponding to scan regions when the mirrors moved
at constant velocity and interconnecting white lines as regions when the mirrors were accelerated to reduce total scan time. (B) The line-scans generated from the path are stacked sequentially as a function of time to produce a space-time plot. Bottom left, the diameter is calculated as the full-width half-maximum of a time-average of several scans across the width of a vessel. Bottom right, red blood cell velocity is calculated from the angle of the RBC streaks from portions of the scan that traverse the centerline of the vessel. (C) Data traces of diameter, RBC velocity, and flux for the arteriole before, during, and after electrical stimulation of the forelimb, processed to remove heart rate and smoothed with a running window. The arteriole shows an increase in flux in response to stimulation attributed to an increase in both lumen diameter and RBC speed. Adapted from Driscoll et al. (Driscoll et al. 2011).

**Figure 4. Simultaneous measurement of red blood cell velocity from multiple subsurface capillaries in the cortex of an anesthetized mouse.** (A) Three dimensional reconstruction of image stack collected in vivo through a bone-removed cranial window of an isoflurane-anesthetized mouse. Note, that imaging depths of up to 700 µm below the pia can be achieved after complete removal of the overlying skull. (B) A single plane from the reconstruction shown in panel A, taken from the depth of the red box (325 µm below the pia). Three microvessels are scanned simultaneously with an optimized line-scan path. (C) The resulting space-time plot from the line-scan shows streaks generated by flowing RBCs in each microvessel. (D) The speed of RBC flow is extracted from the slope of the streaks generated by RBC movement. (E) Data traces of RBC speed collected over several minutes from microvessel 1. Heart-beat frequencies are present in the data, providing a check for data quality.

**Figure 5. Spontaneous and stimulus-induced vascular dynamics in the somatosensory cortex of awake mouse.** (A) Two-photon images of pial surface vessels taken from an awake mouse. Arterioles are shown in red, while venules are shown in blue. (B) Averaged time-series of the diameter of an arteriole and venule in response to a 30 s period of contralateral vibrissal stimulation using focused 8 Hz air puffs. The response of the arteriole during control air puffs to the tail provides a control for general arousal (black traces). Data are mean ± standard deviation. (C) Maximal z-dimension projection over 90 µm of images of fluorescein dextran–
labeled vasculature collected through a PoRTs window. The line-scan trajectory used to sample from two distantly separated capillaries is overlaid on the image; green and orange lines are constant velocity segments along capillaries and purple are minimum time segments between capillaries. (D) Space-time plots of one segment of line-scan data from each of two capillaries sampled. The calculated instantaneous RBC speed for the entire 300 s period of scanning is shown below each space-time plot. (E) Power spectra for the two RBC velocity traces in panel D (0.083 Hz bandwidth; top) and magnitude of the spectral coherence between the velocities of the two capillaries as a function of frequency (0.1 Hz bandwidth; bottom). Panels A and B are adapted from Drew et al. (Drew et al. 2011). Panels C to E are adapted from Drew et al. (Drew et al. 2010b).

**Figure 6. Generation of a localized ischemic stroke by targeted photothrombosis of single penetrating vessels in mouse cortex.** (A) Schematic showing focused green laser irradiation of a penetrating arteriole to generate a focal occlusion, following intravenous injection of Rose Bengal. Targeted photothrombosis can be performed through the thinned skull (Drew et al. 2010b). (B) Low magnification, maximally Z-dimension projected image of fluorescein dextran-labeled pial vasculature visualized through a thinned skull PoRTS window (Drew, 2010). A single penetrating arteriole within the window is identified for photothrombosis (yellow box). (C) Green laser irradiation of a single penetrating arteriole (left panel) immediately following intravenous administration of Rose Bengal leads to localized clotting (right panel). After successful photothrombosis, a dark clot is seen at the site of irradiation and the vessel becomes brighter upstream due to stagnation of RBC flow. (D) *Post-mortem* immunohistochemistry with the pan-neuronal marker NeuN demarcating the boundaries of the resulting infarct (yellow-dotted line), as observed 48 hours following occlusion.
Summers, Taylor and Shih. Figure 2
A

Scan path

Diameter Velocity

Diameter (µm)

0 5 10 15 20 25

Velocity (mm/s)

0 1 2 3 4

B

Scan path

Diameter Velocity Diameter Velocity

Scan across vessel (projection)

Scan along vessel

FWHM

50 µm

50 ms

C

Diameter (µm)

Time (s)

0 10 20 30 40 50

Velocity (mm/s)

Time (s)

0 10 20 30 40 50

Flux (pL/s)

Time (s)

0 10 20 30 40 50

Summers, Taylor and Shih. Figure 3
Blood flow dynamics of Microvessel 1

\[ v = \frac{\Delta d}{\Delta t} \]

RBC velocity, \( v \) (mm/s)

Summers, Taylor and Shih. Figure 4
Microinfarct

Histology

Depth below pial surface (µm)

In vivo two-photon imaging

B

C

D

Summers, Taylor and Shih. Figure 6