

# Two-photon microscopy- sequential imaging studies *in vivo*

Mario Valentino, Christian Zammit, Giuseppe Di Giovanni, Massimo Pierucci, R. Muscat

## Abstract

Microscopists have always desired to look inside various organ tissues to study structure, function and dysfunction of their cellular constituents. In the past, this has frequently required tissue extraction and histological preparation to gain access. Traditional optical microscopy techniques, which use linear (one-photon) absorption processes for contrast generation, are limited to use near the tissue surface (< 80  $\mu\text{m}$ ) because at greater depths strong and multiple light scattering blurs the images. Scattering particularly strongly affects signal strength in confocal microscopy, which achieves three-dimensional resolution and optical sectioning with a detection pinhole that rejects all light that appears not to originate from the focus. New optical microscopy techniques have been developed that use nonlinear light-matter interactions to generate signal contrast only within a thin raster-scanned plane. Since its first demonstration over a decade ago, two-photon microscopy has been applied to a variety of imaging tasks and has now become the technique of choice for fluorescence microscopy in thick tissue preparations and in live animals. The gain in resolution over conventional *in vivo* imaging techniques has been several orders of magnitude. Neuroscientists have used it to measure calcium dynamics deep in brain slices and in live animals, blood flow measurement, neuronal plasticity and to monitor neurodegenerative disease models in brain slices and in live rodents. These types of applications define the most important niche for two-photon microscopy - high-resolution imaging of physiology, morphology and cell-cell interactions in intact tissue. Clearly the biggest advantage of two-photon microscopy is in longitudinal monitoring of rodent models of disease or plasticity over days to weeks. The aim of this article is to discuss some methodological principles, and show some applications of this technique obtained from our laboratory in the area of acute experimental stroke research.

## Keywords

Stroke, two-photon microscopy, brain slice, *in vivo* imaging, cranial windows vasculature, blood flow

### **Mario Valentino MSc, PhD**

Department of Pathology, Faculty of Medicine & Surgery  
University of Malta, Msida, MSD 2080, MALTA.  
Email: mario.valentino@um.edu.mt  
Tel.: (00356) 23402775 Fax: (00356) 21 310577

### **Christian Zammit MD MSc**

Department of Anatomy, Faculty of Medicine and Surgery,  
University of Malta. Msida MSD 2080, Malta.  
Email: christian.m.zammit@um.edu.mt

### **Giuseppe Di Giovanni, MSc, PhD**

Department of Physiology and Biochemistry,  
Faculty of Medicine and Surgery, University of Malta,  
Msida MSD 2080, Malta.  
Email: giuseppe.digiovanni@um.edu.mt

### **Massimo Pierucci, MSc, BSc**

Department of Physiology and Biochemistry,  
University of Malta, Msida MSD 2080, Malta.  
Email: massimo.pierucci@um.edu.mt

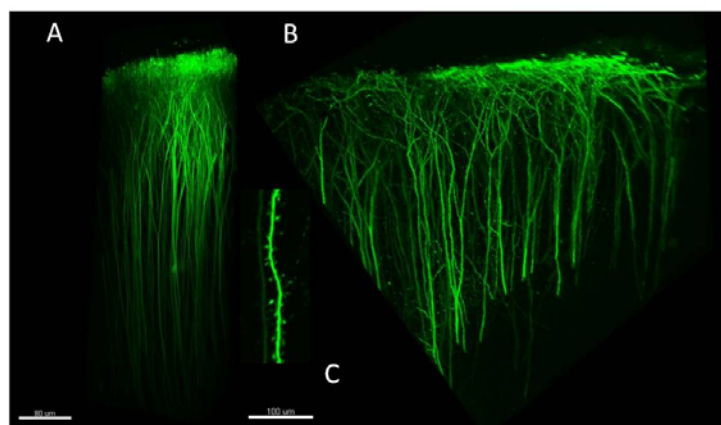
### **Richard Muscat, MSc, PhD**

Department of Physiology and Biochemistry,  
Faculty of Medicine and Surgery, University of Malta,  
Msida MSD 2080, Malta.  
Email: richard.muscat@um.edu.mt

\* corresponding author

## Introduction

Incremental advances in neuroscience have often been characterized by advances in visualization methods. The ability to observe fine brain structure using routine light microscopy enabled Cajal to map synaptic pathways and even deduce functional relationships between brain regions. Approximately 100 years after Cajal's work using the Golgi silver staining technique, Denk, Webb, and others<sup>1</sup> developed the two-photon microscope, which allows visualizing small structures within the functioning brain of a living organism. By using a pulsed infrared laser that excites fluorophores by the combined power of two long-wavelength photons, it was possible to achieve optical sectioning based on the use of infrared light necessary for the 2-photon effect and the deep tissue penetration advantage.



**Figure 1.** Three-dimensionally constructed images of neurons expressing YFP-H in the cerebral neocortex of a mouse under anesthesia. Images captured using the Olympus Fluoview FV1000-MPE multiphoton system with a 25x objective.

(A) Live imaging of anaesthetized mouse through a cranial window up to 614 $\mu$ m deep below the pial surface showing distinct pyramidal neurons with axons extending down to layer IV and (B) at a of 450 $\mu$ m. In the cortex, most studies of dendritic plasticity have taken advantage of apical tuft dendrites of layer 5 neurons and layer 2 and layer 3 neurons that reach near the surface of the cortex. Near the cortical surface, the tuft dendrites curve and almost run parallel with the pial surface. These parallel running dendrites have spines (C) projecting laterally taking full advantage of two-photon microscopy's good resolution with depth.

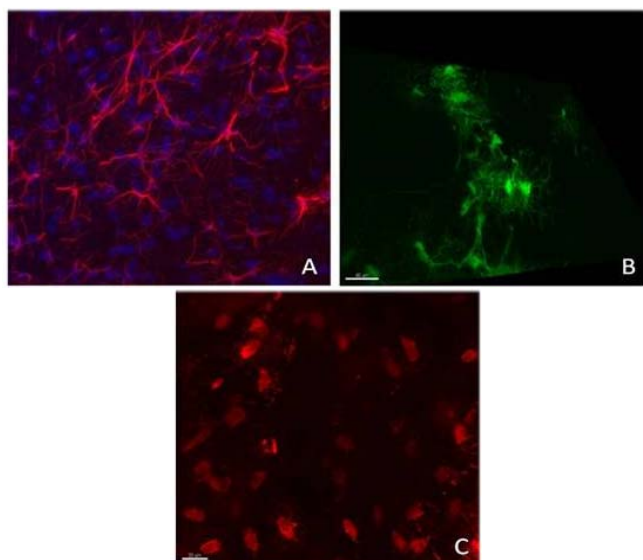
The greatest advances in two-photon microscopy arose from the ability to perform high-resolution imaging in highly light-scattering tissues such as the brain of live vertebrates over several hundred micrometers of depth.<sup>2,4</sup> Over the years, greatly improved staining approaches made it possible to label specific neurons and pathways with genetic tools. Among the optical techniques, multiphoton microscopy is best suited for *in vivo* studies

due to the (a) quadratic dependence of optical absorption, such that under most circumstances optical sectioning is performed by the incident light, (b) use of IR light which scatters less than visible light and hence increases the depth of focal penetration, (c) pulsing and pre-chirp of the IR light to optimize its penetration into the brain yet moderate its average power, and (d) use of a point-scanning system to optimize spatial resolution.<sup>5</sup>

From our experience, routine use of our custom-modified Olympus BX50W1 upright multiphoton microscope achieves imaging depths in brain of typically  $\sim$ 800  $\mu$ m (Fig.1). However the ultimate imaging depth is dependent on many parameters, including the age of the animal, tissue dispersion and the optical geometry.<sup>6</sup> Recently, the appropriate use of a low-magnification, high numerical aperture objectives in combination with the regenerative amplification of 200-kHz pulses to achieve high pulse peak powers have extended the depth of imaging close to 1 mm and offer the enhanced opportunity to reach even deeper lying structures. In this respect, the upper layers (I-IV) of neocortex and thick live slice preparations (400 $\mu$ m) are ideally suited for high-resolution two-photon imaging.

## In Vivo Imaging With Endogenous Fluorophores

The greatest asset for high-resolution brain imaging has been the development of transgenic animals with neurons or glia labeled with fluorescence proteins (XFPs). The first successful animals were developed by Sanes and Litchman.<sup>7</sup> This approach has yielded high resolution imagery into the structure of the cortex during normal development, into its plasticity, and into disorders of the brain.<sup>8-9</sup> There are 2 major advantages to using XFP labeling. The first is simply that fluorescent probes are already resident within neurons or glia of interest and preparation time and complexity is reduced. The second major advantage is that XFP labels tend to be bright and relatively resistant to photobleaching.<sup>10</sup> XFPs can be delivered to neurons in brain slices and *in vivo* using a variety of noninvasive techniques, including transfection, viral transduction, and transgenesis methods. We routinely use GFP (line M) to image the structure and dynamics of dendritic spines in brain slices and *in vivo* rodent preparations. Interestingly, cellular expression from transgenic mice with specific cell promoter expression reveals the true cellular cytoarchitecture as opposed to immunocytochemical labelling with specific antibodies (Fig 2).

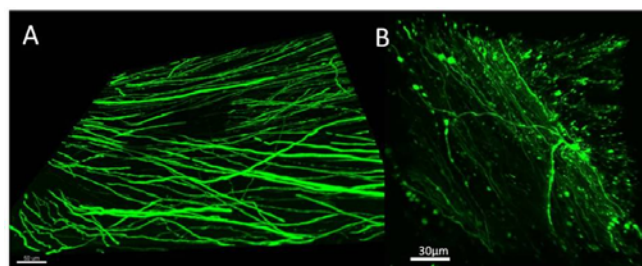


**Figure 2**

- A) GFAP-labelled fibrous astrocytes (red) along with interspersed nuclei labelled with hoechst (blue) obtained from a thin (16 $\mu$ m) slice of the corpus callosum and imaged under epifluorescence.
- B) GFP-GFAP (line C) labelled fibrous astrocytes imaged by two-photon microscopy in thick live brain slice (400 $\mu$ m) from corpus callosum. Transgenic mice with expression of GFP-GFAP show the true morphology and fine structure of astrocytes over other conventional staining techniques.
- C) Two-photon micrograph of SR-101-labelled protoplasmic cortical astrocytes imaged at 150 $\mu$ m below the pial surface. Notice the well defined processes and large cell bodies.

Another very useful spectral variant which is routinely used in our laboratory is the THY1-YFP line H (kind gift from Prof Jochen Hermes, Munich), which express intense expression of YFP (a variant which is yellow-shifted and substantially brighter than GFP) in layer 5 cortical neurons which project axons in the corpus callosum. These mice express green fluorescent protein (GFP) and spectral variants under control of the neuron-specific promoter, *thy1*, and are thus ideal candidates for deep *in vivo* imaging. However, we had difficulty in visualization of the axonal bundles in the subcortical white matter which is at a critical depth of  $\sim$ 1mm. YFP demonstrates axon structural disruption without reliance on cytoskeletal labeling. The method represents a substantial improvement over existing techniques because it shows the entire axoplasm, does not require disruption in axonal transport, and is not sensitive to potential changes in specific epitopes such as cytoskeletal proteins. This method also allows for the possibility of imaging axons and their progression to injury in living slices. With conventional confocal imaging in white matter we had difficulty visualizing fluorescence deeper than 50  $\mu$ m in the slice, which is near the border of spontaneous injury. In preliminary experiments using two-photon imaging, we demonstrate excellent axon

visualization  $>$ 100  $\mu$ m in living slices. Brain slice preparations are widely used to study neuronal injury and offer important advantages for investigation of white matter. Slices preserve anatomical and structural integrity of the mature nervous system. In contrast, dissociated cell cultures exclude many cell-cell interactions, and isolated white matter preparations may lack continuity of the axon with the cell body. Unlike dissociated or slice cultures, acute slices can be prepared from adult animals, allowing assessment of myelinated axons and fully differentiated oligodendrocytes so that sequential injury can be followed over time. (Fig.3)



**Figure 3.** Sequential time lapse imaging in live brain slice  
Sequential time lapse imaging of YFP-H labeled callosal axons from 400 $\mu$ m thick live brain slice under two-photon microscopy.

- (A) Intact morphology of axons under control conditions
- (B) Injury as revealed after 60 minutes of glucose deprivation.

### Why use mice in our experimental setup ?

The mouse is the most commonly used mammalian animal model in biomedical research because its anatomy and physiology has been extensively studied. Mice reach sexual maturity by 4-6 weeks with a gestation period of usually 19-21 days and breed very fast. Apart from this, there are several technical reasons for using mice. First, the cortical thickness in mice is thin as compared to in rats ( $\sim$ 1.6 mm vs. 2.2 mm), and thus more of the cortical anatomy may be imaged in mice. Second, since the skull and dura mater are substantially thinner in mice as compared to rats, it is possible to use multiphoton microscopy to image directly through intact, thinned skull in mice. Third, the thin dura mater of the mouse does not impede an intracranial injection via a beveled glass pipette. Fourth, the bulk of mammalian transgenic animals are mice. Fifth, the thin dura mater of mice may also allow easier access of drugs into a brain via a cannula over a cortical window than would the thicker dura mater of rats. Lastly, genetically engineered transgenic mammals are in the great part, mice.

### Hardware for two-photon imaging *in vivo*

In all our experiments, high-resolution *in vivo* two-photon imaging was performed with a custom-modified Olympus BX50W1 upright microscope (Olympus, Tokyo, Japan) designed for low dispersion. The system includes Keplerian beam expanders with IR introduction light paths to achieve perfect excitation efficiency and highly resolved multiphoton images. A mode-locked MaiTai HP DeepSee laser system (Spectra-Physics) with a tuneable Ti: sapphire oscillator (690-1040 nm) used as the excitation light source (pulse width < 100fs; pulse repetition rate 80MHz) and controlled through an acousto-optical-modulator to allow for precise changes in laser intensity. The Group Velocity Dispersion was electronically compensated by a prism-coupled pre-chirper and the beam diameter adjusted by a Kepler telescope. Images were acquired with a water-based 25X IR-corrected Olympus XLPLN25xWMP multiphoton objective, NA 1.05, WD 2.0. and FluoView imaging software.

### Combining High- and Low-Resolution Imaging

Two-photon imaging provides a high-resolution look at brain structure and function. The ability to perform such studies *in vivo* also allows one to relate microscopic brain structure to macroscopic brain function. To do this, one needs to be able to navigate the brain on both levels. We perform two-photon imaging on an upright microscope allowing us to readily use charged coupled device cameras to generate video images of the cortical surface (by using a mirror to switch between imaging modes) without affecting the ability to perform parallel two-photon imaging.<sup>11</sup> On a rough scale, one can use video images of surface arteries and veins to identify particular regions in chronic imaging preparations. In addition to examining the vascular structure of animals and navigating the brain by this mechanism, one can also perform functional imaging *in vivo* at a macroscopic level and subsequently define the microscopic properties of such functional maps using two-photon imaging. We anticipate a bright future in which multiple modes of imaging will enable both microscopic and regional assessments of brain structure and function to help unravel the mechanisms of stroke damage and recovery.

### Cranial Cortical Windows

To obtain the highest resolution images and the deepest imaging, we use a craniotomy in which a section of skull is removed. The craniotomy may take up to 3 hours for a high-quality preparation. A 1-2 mm<sup>2</sup> window of skull is removed over the imaging region with a surgical drill and 1/4 bur (Fine Science Tools). The dura mater – which, at least in our mice strain, is of negligible thickness – is left intact and does not interfere with imaging. The most critical step of the procedure is cutting and removing

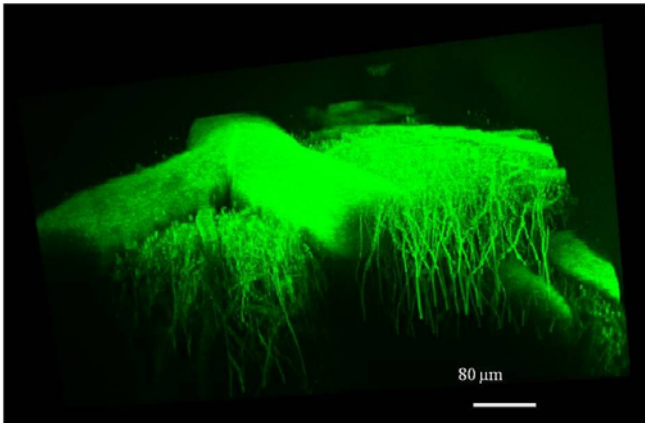
the bone window without disturbing the underlying vasculature. In the case of mouse imaging, it is not necessary to remove overlying dura membranes for green fluorescent protein or calcium indicators. For fluorophores to which the dura is impermeable such as voltage-sensitive dyes,<sup>12</sup> prestaining of the dura with a fast green solution (1%) permits staining of the dura before teasing it away from the underlying brain. Combined with topical loading of the astrocyte specific indicator SR101 (0.04mmol/L) on the intact dura, neurons and astrocytes can be easily distinguished because SR101 and neuronal (YFP) labeling show no overlap (see images).

For stable image acquisition, the head of the anesthetized animal must be immobilized. In acute imaging, we use a stainless steel plate cemented to the surrounding skull with fast curing dental cement. The brain is then stabilized with a glass coverslip placed over a layer of 1% low melting-point agar as outlined by Kleinfeld.<sup>13</sup> If animals are to be used chronically, we glue a coverslip to the skull and immobilize the head with ear- and toothbars (or a small holding post). Moisture of exposed brain regions is maintained by a surgical gelatin sponge (Upjohn) saturated with artificial cerebral spinal fluid (ACSF) throughout and in between the surgical procedures. This sponge is then removed just prior to sealing the exposed brain surface. Under these conditions, images with dendritic spine-level resolution (1µm) can be acquired with motion artifacts small enough that synchronizing image acquisition with the heartbeat or breathing rate is not required. This method allows for the maximum focal penetration into neocortex that may presently be attained with optical techniques. It does not suffer from high light scattering by the skull, or unmatched refractive indices between the skull and aqueous solutions (e.g. ACSF, agarose, or ddH<sub>2</sub>O). A caveat of this method is that the removal of skull renders the brain susceptible to pressure and temperature changes, which hastens the decline of the animals and thus reduces recording time.

### Fluorescent Dextran Imaging of Vascular Structure and Function

The ability to image the cerebral vasculature (from large vessels to capillaries) and record blood flow dynamics in the intact brain of living rodents is a powerful technique. Using *in vivo* two-photon microscopy through a cranial window it is possible to image fluorescent dyes injected intravenously (Fig 4). This permits one to image the cortical vasculature and also to obtain measurements of blood flow. This technique was originally developed by David Kleinfeld and Winfried Denk.<sup>13</sup>





**Figure 4.** FITC-dextran labelled pial vasculature merged with the upper cortical dendritic layers.

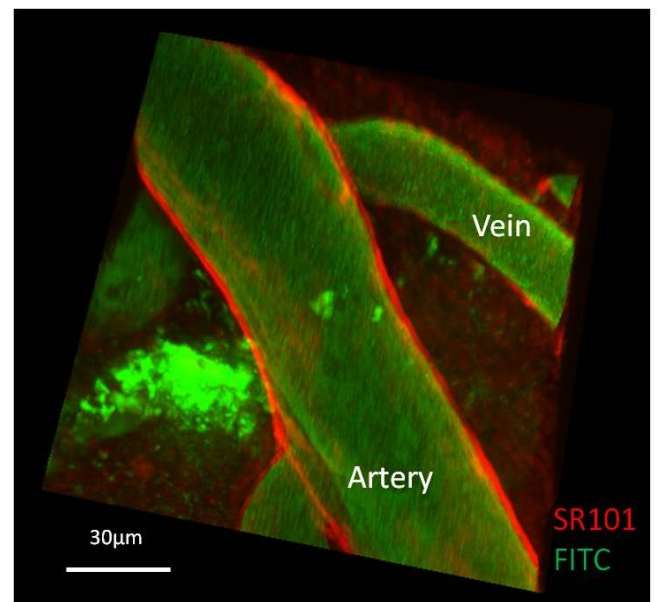
This image represents a 3-D projected Z-stack of 200 slices separated by 1 $\mu$ m spacing in an anesthetized YFP-H transgenic mouse with a cranial window of 1mm<sup>2</sup> area. The image was taken under two-photon microscopy at 800nm.

The method can be used to study blood flow dynamics during or after cerebral ischemia, in neurodegenerative disorders, in brain tumors, or in normal brain physiology. For example, it has been used to study how stroke causes shifts in blood flow direction and changes in red blood cell velocity or flux in and around the infarct.<sup>14</sup>

After making a craniotomy, the easiest method of introducing fluorescent contrast into the brain is to use cerebral angiography, via intravenous injection of a fluorophore. We find fluorescent dextran imaging of the vasculature to be an ideal test specimen because it can be performed on any animal (after a routine intravenous injection) and permits one to image vessels of varied size and depth to test the resolution and depth penetration *in vivo*. The fluorophore should be conjugated to dextran (minimum MW 70,000) in order to prevent leakage of dye from the vasculature. Importantly, vascular imaging also provides a means of assessing damage to the preparation because leaking or stalled blood vessels indicative of trauma will be readily apparent.<sup>14,15</sup> Injection of fluorescent dextran (75-100  $\mu$ l of a 5% v/v solution of rhodamine dextran or FITC-isothiocyanate dextran dissolved in saline) into the tail vein of a mouse results in robust labeling of all vasculature within seconds. In this case, the dextran is excluded from blood cells and they are resolved as dark structures on a well-stained plasma background. The speed of the red blood cells (RBCs) is captured by repeated line-scans along the axis of the vessel lumen that form a space-time image when stacked sequentially and leads to the generation of streaks caused by the motion of RBCs. The speed of RBCs is given by the inverse of the slope of these streaks and the direction of flow is discerned from the sign of the slope.<sup>16</sup>

In larger vessels, such as surface pial arterioles, these techniques can be used to assess the efficacy of blood vessel blockade during stroke models. Advantages of two-photon microscopy for blood flow imaging are its greater penetration depth and ability to resolve individual small vessels such as capillaries and red blood cells within them. The advantages of two-photon imaging of blood flow include the ability to directly measure velocity of individual cells within identified vessels rather than inferring it from the behaviour of a large population. In addition, it is possible that methods such as laser Doppler are more sensitive to changes in velocity rather than supply rate (number of cells per second) and may not accurately assess flow in pial vessels.<sup>17</sup>

Vessels are typically identified as arteries, veins, or capillaries based on morphology, flow speed, and the direction of blood flow (Fig 5). Arteries typically have fewer branches, more linear morphology, and higher flow speeds relative to veins. Serial stacks of 250 x 250  $\mu$ m<sup>2</sup> images (400 x 400 pixels at 400,000 Hz pixel rate) separated by 1 to 2  $\mu$ m increments and running from the cortical surface to depths of 500 $\mu$ m were acquired using a water based 25X 1.05 N.A objective. These image stacks recorded the interactions between astrocytes and cerebral blood vessels at multiple cortical depths through layer IV, which terminates at around 450  $\mu$ m below the surface of the mouse somatosensory cortex (see figures).



**Figure 5.** Perivascular sheathing.

Pial arteries exhibit bright perivascular SR101 staining, whereas pial veins show no evidence of such staining. SR101, sulforhodamine 101. Image taken through a 1mm<sup>2</sup> cranial window in an anesthetised YFP-H mouse. The dendritic arbors are seen in green in the background.

Perhaps the most widely used application for blood flow imaging is in defining areas of ischemia after induced local stroke-like lesions. Kleinfeld and his laboratory established a variant of a rose bengal photothrombotic stroke model that allows individual surface and penetrating arterioles to be targeted by a 532nm laser, producing relatively selectively clotted vessels.<sup>14,17-20</sup> This approach allows one to make very precise regions of ischemia. The work by Zhang and Murphy combined structural assessment of neurons using yellow fluorescent protein fluorescence with two-photon analysis of blood flow as well as functional measures, including intrinsic optical signals, that defined the range with which individual blood vessels maintain synaptic structure and function. We have recently used this technique to examine the relationship between changes in blood flow and changes in neuronal structure and function with a modified setup (unpublished).

### Problems and pitfalls during *in vivo* imaging

The *in vivo* situation is complicated by the presence of blood and hemoglobin that can limit the penetration of infrared light due to strong absorbance.<sup>18,20</sup> However several other critical issues can be involved either separately or together. One of the most common problems encountered during *in vivo* imaging is motion artifact. An obvious strategy for dealing with sources of motion external to the brain is to improve the stability of the interface between the head and the imaging apparatus, thereby removing or reducing the motion artifact during data acquisition. Failing this, corrections for motion during data analysis may be possible if a fiducial marker was present (be it from autofluorescence or experimentally-introduced contrast). In such a case, this might provide a means to track a region of interest (ROI) and reregister images. Generally, this requires that the range of motion for the ROI was fully captured within the dimensions (planar and 3D) recorded by the images. Motion internal to the brain is not readily alleviated, as it is often either part of a genuine physiological response or related to the “vasomotion” indicative of a living animal. Nevertheless, the impact of “physiological noise” may be minimized by collecting images at rates distinct from biological rhythms. Furthermore, signal fidelity may be improved by filtering the frequencies associated with respiratory and cardiac cycles.

Physiological monitoring of animals serves many purposes. It tracks the animal’s viability, to verify when the animal is healthy and to communicate in real time when it is not. Physiological monitoring can provide control data for systemic responses to experimental stimuli (such as the changes in breathing during hypercapnic stimulation). To the extent that respiration, heart rate, or

blood pressure generate “physiological noise”, the imaging SNR may be improved by gating image acquisition to a physiological signal or by using the physiological data to filter the imaging signals during post-processing. Further, as anesthetized animals do not properly thermoregulate, this function may be provided by feedback from a temperature monitoring system.

The use of anesthetized animals for *in vivo* imaging reduces motion artifact and allows for invasive procedures. However, its use introduces some confounding effects that are not fully understood. Some neuronal activity is resilient during anesthesia, in that electrical responses to stimuli are observed. However, glial activity may be directly antagonized by some anesthetics<sup>21</sup> and anesthetics exert wide-ranging effects on cerebral blood flow.<sup>22</sup> These matters should be considered when interpreting data collected on anesthetized animals.

### Conclusion

We have outlined how recent methodological advances in two-photon microscopy have enabled the study of micrometer-level structures *in situ* in the brain. This will clearly help to define the various aspects of the neurovascular unit with respect to regional differences in blood flow during stroke and reperfusion. Experimental models of stroke are also poised to gain from measurements of functional changes in neurons and glial cells concomitant to those of changes of blood flow in response to targeted occlusion of vessels. These methods may also be important for evaluating the transient effect of perinfarct depolarizations and regional assessments of blood flow patterns in the penumbra during stroke and recovery.

With the advances in surgical techniques and further miniaturization of equipment, such high-resolution measurements will be routinely performed in awake animals using fiber-based endoscopic systems to chart the effects of stroke and recovery. To date, all measurements on blood flow, their control by neuronal and glial activity, and changes in molecular markers of tissue viability, have involved anesthetized animals. While anesthesia does not necessarily block homeostasis, it does affect the extent of modulation of the pial and deep brain vasculature by small neuroactive molecules such as acetylcholine. It is thus important to move towards recording in awake animals. This is particularly important for the case of experimental stroke, where homeostasis may be compromised by anesthesia.

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