# In Vivo 2-Photon Imaging of Fine Structure in the Rodent Brain

## Before, During, and After Stroke

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***Abstract*—The recent application of 2-photon microscopy to biological specimens has allowed investigators to examine individual synapses within live animals. The gain in resolution over conventional in vivo imaging techniques has been several orders of magnitude. We outline steps for the preparation and maintenance of animals for 2-photon microscopy of fine brain structure. We discuss the in vivo resolution of the method and the ability to image blood flow and synaptic structure in vivo. Applications of in vivo 2-photon microscopy include the study of synapse turnover in adult animals under normal conditions and during pathology such as stroke. In the case of stroke, 2-photon imaging has revealed marked swelling of dendrites and loss of spines within minutes of ischemic onset. Surprisingly, restoration of blood flow during reperfusion was associated with a return of relatively normal structure. Over longer time scales, 2-photon imaging revealed elevated rates of synaptogenesis within peri-infarct tissues recovering from stroke. These results provide an example of how high-resolution in vivo microscopy can be used to provide insight into both the acute pathology and recovery from stroke damage. (*Stroke*. 2010;41[suppl 1]:S117-S123.)**

**Key Words:** 2-photon microscopy ■ anatomy ■ blood flow ■ brain recovery ■ dendritic spine morphology ■ excitotoxicity ■ fluorescent proteins ■ focal ischemia ■ imaging ■ microscopy ■ mouse model ■ neuropathology



For over the last 100 years, incremental advances in neuroscience have often been characterized by advances in visualization methods. Around the turn of the 20th century, Cajal's prolific work using the Golgi staining method largely founded the field of modern neuroanatomy.[1] Specifically 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 later, Denk, Webb, and others[2] developed the 2-photon microscopy technique, 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 2 long-wavelength photons, it was possible to achieve optical sectioning based on physical principles alone without difficulty in aligning optics or light losses due to confocal pinholes.[3] The use of infrared light was not only necessary for the 2-photon effect, but it had the added advantage of good tissue depth penetration. Optical sectioning results since the probability of 2-photon excitation is relatively low and is restricted to a narrow focal plane where excitation power is highest.[3]

The greatest advances in 2-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.[4–6] Later extension of the method from anesthetized animals to freely moving animals using fiber-based endoscopic systems heightened imaging potential even more.[7,8] One major limitation of this method is the need of a fluorescent specimen. Greatly improved staining approaches, however, made it possible to label specific neurons and pathways with genetic tools.[9] The purpose of this article is to first introduce readers to the preparation of an animal for high-resolution 2-photon imaging and, second, to articulate various approaches and preparations for imaging of fine structure within the central nervous system of live rodents and their relation to stroke (Figures 1 and 2).

## Results and Procedures

### Preparation of Animals for In Vivo Imaging

For reference material on basic in vivo imaging techniques, including basic surgical techniques, anesthesia, monitoring of physiological parameters, and imaging applications such as imaging of blood flow or synaptic structure, we direct readers to articles by Svoboda and Kleinfeld and colleagues in "*Imaging Neurons* and *Imaging in Neuroscience and Development.*"[10,11]

### Brain Windows

To obtain the highest resolution images and the deepest imaging, we suggest a craniotomy in which a section of skull

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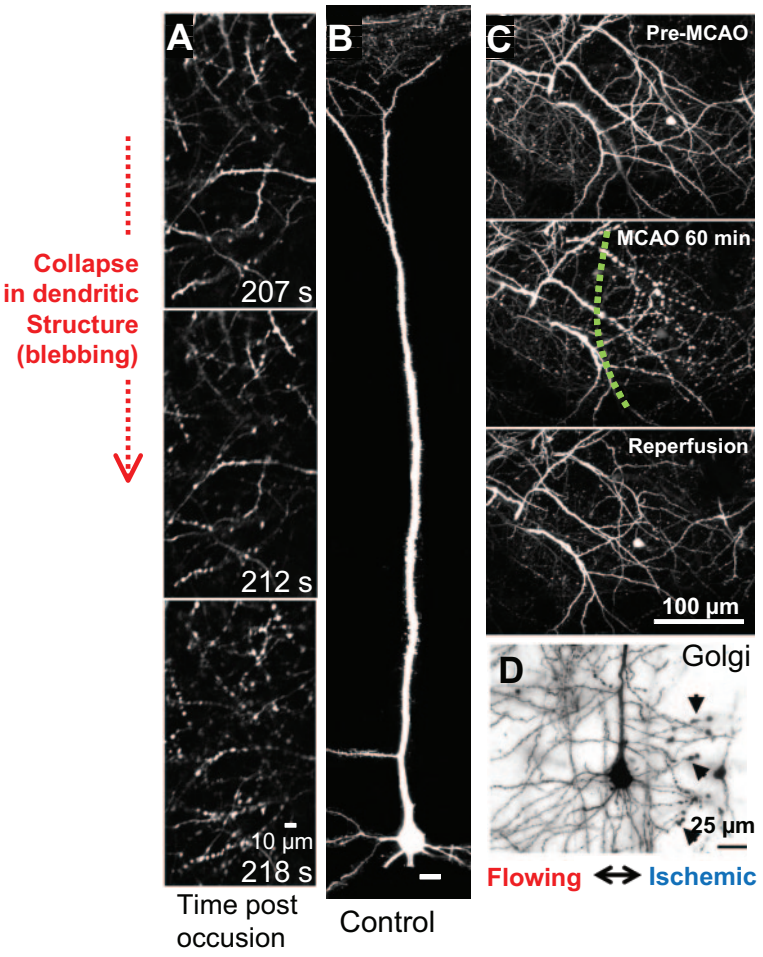


**Figure 1.** Stroke induces rapid and selective damage to brain circuitry. A, Two-photon in vivo images taken from layer I of a live adult GFP-M mouse somatosensory cortex at time points after induction of global ischemia. Between 207 and 212 seconds after ischemic induction, a collapse in dendritic structure and beaded dendrites is apparent (data similar to Murphy et al[42]). B, Stroke-induced changes in dendritic structure are observed in focal stroke models, including middle cerebral artery occlusion (adapted from Li and Murphy[41]). Shown is a confocal image from a fixed and sectioned layer 5 cortical neuron with its apical dendrite and tuft in a region not subjected to ischemia (contrasted with C). Scale bar, 20 $\mu$m. C, Two-photon in vivo imaging performed from the surface scanning into the brain of an adult live GFP-M transgenic mouse indicates selective regional vulnerability of dendrites to middle cerebral artery occlusion. In the second panel down, taken during occlusion, dendrites on the right are further within the middle cerebral artery territory and more extensively beaded. The damage is partly reversible on reperfusion after 60 minutes of focal ischemia. D, Ischemic changes in dendritic structure can be visualized using Golgi histology techniques on fixed and sectioned tissue (adapted from Brown et al[53]). In this case, the cortical neuron shown was close to the infarct border with the left side of the neuron projecting toward areas with increased blood flow, whereas the right side (as shown) projects toward the center of the infarct. An increase in dendritic beading is apparent toward the center of the infarct (see arrowheads marking blebs).

is removed. Besides the protocols mentioned previously, a video tutorial of the procedure is available.[12] The craniotomy may take up to 4 hours for a high-quality preparation. 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,[13] prestaining of the dura with a fast green solution (1%) permits staining of the dura before teasing it away from the underlying brain. In rats, the dura is thicker and generally needs to be removed for 2-photon experiments.[10,11]

For stable image acquisition, the head of the anesthetized animal must be immobilized. In acute imaging, we use a stainless steel plate and stabilize the brain with a glass coverslip placed over a layer of agarose as outlined by Kleinfeld.[14] If animals are to be used chronically, we glue a coverslip to the skull[10,11] and immobilize the head with ear- and toothbars (or a small holding post). Under these condi-

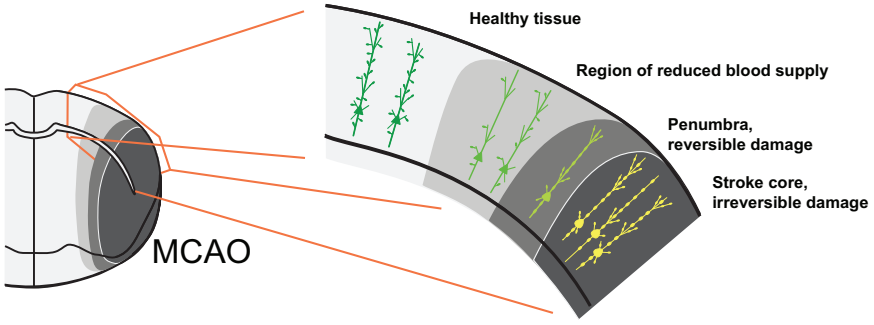


**Figure 2.** Relationship between synaptic circuit damage and local blood flow. Cartoon of a cross-section through the rodent cortex showing the stroke core (black) and penumbra (lighter shades of gray)[54] after occlusion of the middle cerebral artery, a common experimental stroke model. The core has <20% of baseline blood flow and fails to regain its fine dendritic structure after reperfusion.[41] In the penumbra, blood flow increases moving toward the midline as tissues in this region are supplied by other artery systems that were not blocked during the stroke. Within the penumbra, some loss of dendrite structure will reverse when reperfusion occurs and this is where rewiring over longer time scales will occur to replace connectivity lost due to ischemia.[23,41,50]

tions, images with dendritic spine-level resolution (1 $\mu$m) can be acquired with motion artifacts small enough that synchronizing image acquisition with the heartbeat or breathing rate is not required.

## Thin Skull Preparations

In addition to the cranial window, in the past, investigators for intrinsic signal imaging have developed thin skull preparations in mice. In these preparations, it was sufficient to thin the overlying bone to the point where it becomes partially transparent. For intrinsic signal imaging, the degree of thinning to get sensory-evoked responses is relatively modest.[15,16] To image dendritic spines, investigators such as Gan have thinned the skull above the cortex down to 10s of micrometers and have obtained excellent images with less potential for trauma, which could occur during bone removal.[17] Excellent images of blood vessels filled with fluorescent dextrans have also been obtained through thinned bone.[18] Recently the laboratory of Gan has challenged the integrity of chronic craniotomy preparations in mice and published an article comparing the 2 preparations.[19] They concluded that the chronic craniotomy results in significant changes in synapse turnover due to possible trauma. Although these results were reported, it is clear that success or failure of any of these preparations lies in the skill of the operator. Therefore, it is unclear whether problems with the craniotomy preparation would necessarily be experienced by all laboratories and efforts are now underway to standardize and refine such protocols.[20] Nonetheless, these findings suggest that it is important for future studies to have controls to determine whether the means of brain window preparation is affecting the experimental outcome. Clearly the biggest advantage of 2-photon microscopy is in longitudinal monitoring of rodent models of disease or plasticity over days to weeks.[17,20–23] Although this is a goal of many, it is the most difficult experiment to perform. Experiments should be designed so that one can assess whether the imaging preparation and procedures are impacting results.

## Fluorescent Dextran Imaging of Vascular Structure and Function: An Informative Test Specimen

After making a craniotomy using 1 of the methods described previously, it is advantageous to have a robust test specimen for 2-photon microscopy. We find fluorescent dextran imaging of the vasculature[24] 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 of the technique using an in vivo preparation. 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.[25,26] Two-photon microscopy has been used to monitor blood flow in vivo by labeling blood plasma using (largely) membrane-impermeable fluorescent dextrans. Injection of fluorescent dextran into the tail vein of a rat or mouse results in robust labeling of all vasculature within seconds.[14,24] In this case, the dextran is excluded from blood cells and they are resolved as dark structures on a well-stained plasma background. Using modifications of procedures developed for video microscopy,[27] Kleinfeld and colleagues pioneered the approach of using fluorescent dextrans to monitor blood flow in live rats and mice using 2-photon microscopy.[14,24] These techniques can be used to assess the efficacy of blood vessel blockade during stroke models.[26] Advantages of 2-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. By using 2-photon microscopy, blood flow values can be expressed in units of red blood cell velocity or supply rate (cells/second).[14,24] The advantages of 2-photon imaging of blood flow include the ability to directly measure velocity of individual cells within identified vessels rather than inferring it from the behavior 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.[28]

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, producing relatively selectively clotted vessels.[25,29] This approach allows one to make very precise regions of ischemia. We have recently used it to examine the relationship between changes in blood flow and changes in neuronal structure and function with a modified setup.[30] The work by Zhang and Murphy[26] combined structural assessment of neurons using yellow fluorescent protein fluorescence with 2-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, discussed in more detail subsequently.

## In Vivo Imaging With XFP

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.[9] This approach has yielded a high-resolution look into the structure of the cortex during normal development, into its plasticity, and into disorders of the brain.[31,32] 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.[33]

## In Vivo Resolution and Parameters for Spine Imaging

Potentially the resolution of in vivo 2-photon microscopy should be similar to in vitro 2-photon microscopy. However, in vivo, a number of issues conspire to degrade resolution. Presumably if one is imaging the surface of the brain in vivo within the first few hundred micrometers, the images should not appear to be too different from those obtained in a brain slice. 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.[34] Furthermore, regular pulsation of the brain caused by the heart rate and vasomotion results in slight movements of labeled structures limiting resolution. In our laboratory, we have estimated the in vivo resolution of our 2-photon microscope system to be 0.65 $\mu$m laterally using injected fluorescent beads.[22] Although lateral resolution is typically adequate to resolve small structures such as dendritic spines, the Z-resolution of in vivo 2-photon microscopy was significantly lower.[22] Depth penetration, which has been covered extensively by others,[35] is limited by a number of variables in vivo. 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 projecting laterally taking full advantage of 2-photon microscopy's good lateral resolution in vivo.

## Imaging Brain Structure During Plasticity

It is assumed that changes in brain function come about through altering the hard wiring of neurons. Therefore, the degree to which hard wiring changes during development and maturation is a sought-after statistic. Using in vivo brain imaging of layer 5 neurons expressing yellow fluorescent protein (YFP) or green fluorescent protein (GFP), 2 major laboratories have examined the long-term turnover of dendritic spines, the sites of excitatory synapses in the mouse somatosensory cortex in vivo.[17,21,36,37] Although results differ somewhat between laboratories and brain regions/target neuronal populations, an overall theme is that in adult animals, the brain is relatively hard-wired and changes relatively little over time. Repeated in vivo imaging also offers investigators the ability to alter sensory experience of an animal by performing manipulations such as whisker trimming in mice or rats to understand how sensory experience affects structural hard wiring. Recent data also indicate a clear role for changes in spine turnover being associated with enduring motor memories.[38,39]

## Imaging of Brain Structure and Function During Acute Stroke

We have used 2-photon imaging to provide insight into the behavior of synapses during stroke in vivo. We furthermore combined imaging of fine structure using neurons labeled with fluorescent proteins (Figure 1) and imaging of blood flow using fluorescent dextrans. Our goal has been to determine the relationship between microcirculation and dendritic spine structure in mouse somatosensory neurons during acute stroke. Normally dendritic spines were on average 13 $\mu$m from a capillary and were supplied by approximately 100 red blood cells/second.[26,40] Moderate ischemia produced by local injection of the vasoconstrictor endothelin (approximately 50% supply) did not significantly affect spines within 5 hours. However, severe ischemia ($<$10% supply) caused a rapid loss of spine and dendrite structure within as little as 10 minutes.[26,40] Surprisingly, if reperfusion occurred between 20 and 50 minutes, dendrite and spine structure was largely restored.[40] Similar findings were observed in global ischemia models and in a middle cerebral artery occlusion model[41,42] (see Figures 1 and 2).

## The Spatial Relationship Between Blood Flow and Synaptic Structure and Function

Using 2-photon microscopy of XFP-labeled dendrites, we found remarkably sharp borders for focal stroke damage in vivo.[26] These sharp borders allowed us to determine the distance scale over which interruptions in blood flow[26] affect synaptic hard wiring and brain function. In this work, high-resolution microscopy of live mice was used to image cerebral cortex synapses in real time during targeted interruptions of cortical blood flow that model small survivable strokes. A mouse photothrombotic stroke model was used to reduce somatosensory cortex blood flow in discrete regions of cortical maps. This approach allowed us to define relationships among blood flow, cortical structure, and function on scales not previously achieved with macroscopic imaging techniques. Acute ischemic damage to dendrites was triggered within 30 minutes when blood flow to $>$0.2 mm$^2$ of cortical surface was blocked.[26] Rapid damage was not attributed to a subset of clotted or even leaking vessels (extravasation) alone. Assessment of stroke borders revealed a remarkably sharp transition between intact and damaged synaptic circuitry that occurred over 10s of micrometers and was defined by a transition between flowing and blocked vessels. Although dendritic spines were normally approximately 13 $\mu$m from small flowing vessels, we found that an intact dendritic structure can be maintained (in areas without flowing vessels) by blood flow from vessels that are on average 80 $\mu$m away.[26] Functional imaging of intrinsic optical signals associated with activity-evoked hemodynamic responses in the somatosensory cortex indicated that sensory-induced changes in signal were blocked in areas with damaged dendrites but were present 200 to 600 $\mu$m away from the border of dendritic damage.[26] Complementary results were also obtained using voltage-sensitive dye imaging to assess sensory-stimulus evoked depolarization directly[43] (Figure 3). These results define the range of influence that blood flow can have on local cortical fine structure and function and also demonstrate that peri-infarct tissues can be functional within the first few hours after stroke and are well positioned to aid in poststroke recovery. By being able to monitor blood flow and fine dendrite structure in vivo, we could establish a relationship between perfusion and structure within a single animal (Figure 2). In contrast, similar previous work using histology required extremely labor-intensive brain sectioning and a complete electron microscope reconstruction.[44] Using a histological approach, it is possible to view the location of clotted vessels (that were imaged using 2-photon microscopy) if they are filled with a fluorescent dextran because it will be retained within them after fixation of the animal by cardiac perfusion.[45] Three-dimensional reconstruction of the cortical vascular network can also be performed (in nonstroke animals) by perfusion during histological preparation with liquid fluorescent dextran–agarose.[46]

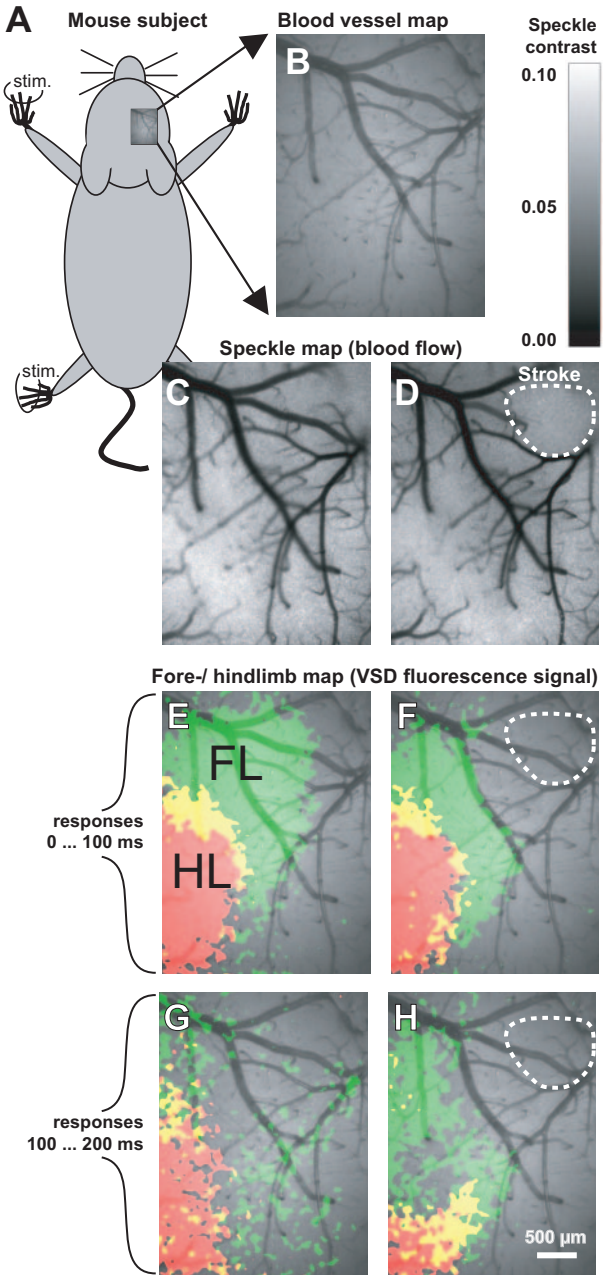


**Figure 3.** Redistribution of cortical activity after a ministroke. A, Schematic showing C57 Bl6 mouse after craniotomy. The fore- and hindlimb areas in the somatosensory cortex are stimulated by touching a contralateral paw (circled in scheme). B, Light reflection image of the cortex through the glass coverslip that was placed in the mouse scull after craniotomy. C–D, Mapping of blood flow by laser speckle imaging before (C) and within the first hour after induction of a focal stroke by photothrombosis in the area indicated by a dashed line (in D). Darker shade indicates a higher flow velocity. E–H**,** Mapping of evoked depolarization in response to tactile stimulation of forelimb (FL, green) and hindlimb (HL, red), before (E and G) and after photothrombotic stroke induction (F and H). The upper row (E–F) represents the immediate responses to stimulation with the first 100 ms after each stimulus. The lower row (G–H) represent the delayed component of the response recorded 100 to 200 ms after each stimulus to the fore- or hindpaw. Within the first hour after stoke, the area of responsiveness to forelimb stimulation relocates toward the hindlimb area, which indicates that cortical activity redistributes rapidly after an ischemic stroke using preexisting synaptic pathways.

## Plasticity of Dendritic Spines in the Peristroke Cortex After Weeks of Recovery

Evidence suggests that recovery from stroke damage results from the production of new synaptic pathways within surviving brain regions over weeks and that adjacent, surviving regions of the brain may be critically involved in this process.[47–50] The peri-infarct cortex is an exceptionally fertile area for plasticity, because there are robust changes in the expression of growth-promoting or -inhibiting proteins involved in neuronal rewiring.[49] However, these studies provide only end-point measures of reorganization without providing information on the process of how these changes came about.[32] We have used 2-photon microscopy to monitor real-time changes in dendritic structure in the living brain over a period of natural recovery occurring weeks after stroke.[22,23] In adult control mice, dendrites were relatively stable with very low levels of spine turnover. After stroke, however, the organization of dendritic arbors in the peri-infarct cortex was fundamentally altered with both apical dendrites and blood vessels radiating in parallel from the lesion.[22] On a finer scale, peri-infarct dendrites were exceptionally plastic, as manifested by a dramatic increase in the rate of spinogenesis that was maximal at 1 to 2 weeks (approximately 4-fold increase) and was still evident 6 weeks after stroke in measurements inferred from both acute and chronic imaging.[22,23] These changes were selective given that turnover rates were not significantly altered in ipsilateral cortical regions more distant to the lesion (>1.5 mm). These data provide a structural framework for understanding functional and behavioral changes that accompany brain injury[50] and suggest new targets that could be exploited by future therapies to rebuild and rewire neuronal circuits lost to stroke.[51]

## 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. Currently we perform 2-photon imaging on an upright commercial 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 2-photon imaging.[30] 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 2-photon imaging. Using a charged coupled device camera, we have captured intrinsic optical signal maps during forelimb or hindlimb stimulation for animals that were also subjected to 2-photon imaging at the level of individual dendritic spines.[26] By placing the camera equipment on the same stage as the 2-photon microscope, we greatly facilitated the image registration process. We anticipate a bright future in which multiple modes of imaging will enable both micro-

scopic and regional assessments of brain structure and function to help unravel the mechanisms of stroke damage and recovery.

## Conclusion

We have outlined how recent advances in 2-photon microscopy have enabled the study of micrometer-level structures such as synapses to be studied in situ (Figure 1) and to define the vulnerability of the synapse with respect to regional differences in blood flow during stroke and reperfusion (Figure 2). We anticipate that with advances in surgical techniques and further miniaturization of equipment,[7,52] such high-resolution measurements will be routinely performed in even awake animals to chart the effects of stroke and recovery. Recent data also suggest that these methods may be important for evaluating the transient effect of peri-infarct depolarizations.[55]

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## Disclosures

None.

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