Microprisms for In Vivo Multilayer Cortical Imaging

Thomas H. Chia and Michael J. Levene

Department of Biomedical Engineering, Yale University, New Haven, Connecticut

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Chia TH, Levene MJ. Microprisms for in vivo multilayer cortical imaging. J Neurophysiol 102: 1310–1314, 2009. First published June 3, 2009; doi:10.1152/jn.91208.2008. Cortical slices allow for simultaneous imaging of multiple cortical layers. However, slices lack native physiological inputs and outputs. Although in vivo, two-photon imaging preserves the native context, it is typically limited to a depth of <500 µm. In addition, simultaneous imaging of multiple cortical layers is difficult due to the stratified organization of the cortex. We demonstrate the use of 1-mm microprisms for in vivo, two-photon neocortical imaging. These prisms enable simultaneous imaging of multiple cortical layers, including layer V, at an angle typical of slice preparations. Images were collected from the mouse motor and somatosensory cortex and show a nearly 900-µm-wide field of view. At high-magnification imaging using an objective with 1-mm of coverglass correction, resolution is sufficient to resolve dendritic spines on layer V neurons. Images collected using the microprism are comparable to images collected from a traditional slice preparation. Functional imaging of blood flow at various neocortical depths is also presented, allowing for quantification of red blood cell flux and velocity. H&E staining shows the surrounding tissue remains in its native, stratified organization. Estimation of neuronal damage using propidium iodide and a fluorescent Nissl stain reveals cell damage is limited to <100 µm from the tissue–glass interface. Microprisms are a straightforward tool offering numerous advantages for research into neocortical tissue.

INTRODUCTION

Cortical slices provide a powerful platform for neurophysiology, with easy access to multiple cortical layers for chemical, electrical, and optical measurement and manipulation. However, slices lack important aspects of physiological context, including inputs from intra- and extracortical regions and an active circulatory system. Although in vivo experiments provide this context, optical access to deeper layers is exceedingly difficult and almost always provides image planes confined to a single cortical layer (Helmchen and Denk 2005). Here, we demonstrate that microprisms inserted into mouse cortex enable simultaneous imaging of multiple cortical layers with a 1-mm field of view (FOV), submicron spatial resolution, and an imaging perspective typical of slice preparations.

Attempts to image deep layers of neocortex are made difficult by the highly light scattering nature of brain tissue. Although multiphoton microscopy is well suited for fluorescence imaging in the brain, imaging depths typically reach <500 µm. Generally, this depth is superficial of cortical layers IV–VI, where many pyramidal cell bodies are located. Given the importance of deep cortical layers, there is strong demand to clearly see the unique structures and functions of these layers in their native context with high resolution and signal-to-noise ratio (SNR).

METHODS

Microprism optics and imaging parameters

Our microprisms consist of 1-mm, BK7 glass right-angle prisms with an enhanced silver reflective coating (>97.5% reflectivity from 400 to 2,000 nm) on the hypotenuse for internal reflection (Optosigma, Santa Ana, CA) (Fig. 1A). This allows the raster-scanning pattern of the excitation laser to be translated from an x–y-plane to an x–z-plane (Fig. 1B). This gives the advantage of imaging the tissue adjacent to the prism leg.

The face of the prism flush with the cortical surface was positioned normal to the laser excitation beam to maximize light transmission and minimize optical aberrations. A 40×, 0.60 numerical aperture (NA) objective set for 1 mm of glass was used in some experiments to correct aberrations caused by the microprism or coverglass.

Microscope apparatus

We used a custom-built multiphoton microscope based on an Olympus BX51 WI upright fluorescence microscope (Olympus America, Center Valley, PA). The excitation source was an 80-MHz pulsed Ti:sapphire laser (Mai Tai, Spectra-Physics, Mountain View, CA), tunable between 710 and 990 nm with a 100-fs pulsewidth. The excitation wavelength for the yellow fluorescent protein (YFP) was 886 nm. The excitation wavelength for the fluorescein-dextran was 830 nm. Images were collected using a 4×, 0.28 NA air objective (XLFLUOR 4x/340, Olympus) or a 40×, 0.60 NA coverglass-corrected air objective (LUCPLFLN, Olympus) set for 1 mm of glass. Anesthetized animals were placed on a motorized three-axis microscope stage (ASI Imaging, Eugene, OR).

The emitted fluorescence was reflected into a custom-built, two-channel photomultiplier tube (PMT) housing; both PMTs were built by Hamamatsu (HC-125-02, Bridgewater, NJ). A 525/50-nm emission filter (Chroma, Rockingham, VT) was used for the YFP and fluorescein-dextran fluorescence collection. Images were acquired using ScanImage software (Polguruto et al. 2003). Images were collected at a resolution of 512 × 512 or 1,024 × 1,024 pixels with 2- or 4-ms scan time per line.
Animal surgeries

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) to a level suitable for surgical procedures. Mice were immobilized using a stereotactic device (#51625; Stoelting, Wood Dale, IL) to minimize motion artifact during imaging. The skull was properly cleaned and cleared of skin and hair. The ear bars on the stereotactic device were adjusted to tilt the animal’s head until the exposed skull was level. This provided a suitable surface for surgical procedures. A dental bur was used to create a craniotomy hole about 2–3 mm in diameter without damaging the brain tissue. Forceps were used to remove the bone flap. A Von Graefe knife carefully pulled across the side of the exposed brain helped remove the dura without damaging the cortical tissue. Bleeding was minimal and primarily occurred only during the initial surgical procedure to excise the dura following bone flap removal. Any bleeding that occurred was allowed to continue for about 2 min to allow for coagulation. Once the blood had coagulated, the excess blood could be removed with a dry, triangular surgical sponge without promoting additional bleeding. Subsequent bleeding following prism insertion was minimal, provided no large blood vessels at the neocortex and flow imaging, the microprism was first properly inserted from the surrounding tissue shortly after insertion, resulting in imaging of only superficial layers. The animal was ready for imaging once the region was clear of any active bleeding. For all experiments, the location of the microprism was about 1.5 mm caudal and 1 mm lateral to the bregma. At this location the region imaged is the primary and secondary motor cortex along with parts of the primary somatosensory cortex (hindlimb region) and retrosplenial agranular cortex. Mice used for microprism imaging were between the ages of P30 and P60. The genetic background of the mice used for YFP layer V imaging experiments were YFP-H (Feng et al. 2000). For blood vessel visualization and flow imaging, the microprism was first properly inserted into the cortex prior to tail vein injection. Then the blood serum was fluorescently labeled with 5% (wt/vol) fluorescein-dextran (70 kDa, Sigma, FD70) in physiological saline solution. Approximately 70 μl of dye was administered through a tail vein injection. Acquired images are ≥100 μm away from the imaging face of the prism to avoid a region of damaged tissue surrounding the microprism. All animal procedures were approved by Yale University Institutional Animal Care and Use Committee.

Histology

For hematoxylin-eosin (H&E) staining, a mouse underwent the typical surgery to insert a microprism into the cortex. The mouse was where the prism was placed. Any resulting blood was absorbed from the region surrounding the prism insertion site using a surgical sponge. Care was taken not to disturb the prism. The prism remained in its original position without any additional intervention. In rare instances (3 of 25 experiments) the microprism partially displaced itself from the surrounding tissue shortly after insertion, resulting in imaging of only superficial layers. The animal was ready for imaging once the region was clear of any active bleeding. For all experiments, the location of the microprism was about 1.5 mm caudal and 1 mm lateral to the bregma. At this location the region imaged is the primary and secondary motor cortex along with parts of the primary somatosensory cortex (hindlimb region) and retrosplenial agranular cortex. Mice used for microprism imaging were between the ages of P30 and P60. The genetic background of the mice used for YFP layer V imaging experiments were YFP-H (Feng et al. 2000). For blood vessel visualization and flow imaging, the microprism was first properly inserted into the cortex prior to tail vein injection. Then the blood serum was fluorescently labeled with 5% (wt/vol) fluorescein-dextran (70 kDa, Sigma, FD70) in physiological saline solution. Approximately 70 μl of dye was administered through a tail vein injection. Acquired images are ≥100 μm away from the imaging face of the prism to avoid a region of damaged tissue surrounding the microprism. All animal procedures were approved by Yale University Institutional Animal Care and Use Committee.

Histology

For hematoxylin-eosin (H&E) staining, a mouse underwent the typical surgery to insert a microprism into the cortex. The mouse was...
properly anesthetized for 3 h with the prism inserted. At the end of the experiment, mice were perfused with PBS followed by 4% paraformaldehyde. The microprism was taken out of the brain following fixation. The brain was then carefully removed from the cranium and embedded in paraffin for slicing and staining. Slices were cut 5 μm thick.

Quantification of neurons damaged by the microprism was accomplished using a combination of a fluorescent Nissl stain (NeuroTrace 435/445, Molecular Probes) and a DNA intercalating dye, propidium iodide (PI; 10% wt/vol in dH2O, Molecular Probes). The Nissl stain labels neurons, whereas the PI will enter any cells with compromised cell membranes. This procedure was adapted from Blanche et al. (2005), in which neurons with damaged membranes are colabeled with both dyes. PI-coated prisms were inserted into the neocortex of wild-type mice using the previously described procedure. After the microprism had been in place for 1 h, the microprism was carefully removed from the neocortex and the animals were immediately decapitated. Their brains were rapidly removed and placed in an ice-cold cutting solution containing (in mM): sucrose, 219; NaHCO3, 28; NaH2PO4, 1.25; and glucose, 10. For slice imaging with 1-mm coverglass to simulate the optical aberration, comparison between traditional cortical slice images, slice with 1-mm coverglass to simulate the optical aberration, and in vivo “slice” images collected using a Vibratome. Slices were allowed to recover in oxygenated ACSF at a rate of 0.8 ml/min using a two-channel peristaltic pump (Cole-Parmer, Vernon Hills, IL). The ACSF contained (in mM) NaCl, 130; KCl, 3.0; CaCl2, 2.0; MgSO4, 1.25; NaHPO4, 1.25; and glucose, 7 for 1–2 min. Brains were then blocked into a section including the neocortex where the microprism was inserted and mounted on the stage of a Vibratome (St. Louis, MO). Sagittal neocortical slices were cut 300 μm in thickness. Slices were transferred to PBS and stained with NeuroTrace according to the manufacturer’s instructions. On completion of staining, slices were imaged using a two-photon microscope using an excitation wavelength of 770 nm for the PI and 830 nm for the NeuroTrace. In all, 167 neurons were analyzed from three different tissue slices from the same animal. The percentage of damaged neurons was calculated within each 25-μm region located away from the imaging face of the microprism.

**Neocortical slice imaging**

Mice were anesthetized with a pentobarbital sodium solution (50 mg/kg) and then decapitated. Their brains were rapidly removed and placed in an ice-cold cutting solution for 1–2 min and then blocked into a section including the neocortex and cut 400 μm in thickness using a Vibratome. Slices were allowed to recover in oxygenated (95% O2-5% CO2) artificial cerebral spinal fluid (ACSF) for ≥2 h prior to imaging. Following recovery, slices were placed into an imaging chamber (Warner Instruments, Hamden, CT) and perfused with oxygenated ACSF at a rate of 0.8 ml/min using a two-channel peristaltic pump. The ACSF contained (in mM) NaCl, 130; KCl, 2.5; CaCl2, 0.5; MgSO4, 7.0; NaHPO4, 1.25; and glucose, 7 for 1–2 min. Brains were then blocked into a section including the neocortex where the microprism was inserted and mounted on the stage of a Vibratome (St. Louis, MO). Sagittal neocortical slices were cut 300 μm in thickness. Slices were transferred to PBS and stained with NeuroTrace according to the manufacturer’s instructions. On completion of staining, slices were imaged using a two-photon microscope using an excitation wavelength of 770 nm for the PI and 830 nm for the NeuroTrace. In all, 167 neurons were analyzed from three different tissue slices from the same animal. The percentage of damaged neurons was calculated within each 25-μm region located away from the imaging face of the microprism.

**RESULTS**

Using mice expressing YFP in layer V cortical neurons, we present images from several experiments showing a clear band of layer V pyramidal neurons about 900 μm below the cortical surface (Fig. 2, A, C, E, and G). The apical dendrites extend from the cell soma and eventually undergo bifurcations in the upper layers before branching to tufts in layer I (Fig. 2, E). We could resolve dendritic spines on layer V pyramidal cells (Fig. 2H).

Imaging through 1-mm of glass is expected to induce significant amounts of spherical aberration. Therefore we show a comparison between traditional cortical slice images, slice images with 1-mm coverglass to simulate the optical aberrations of the microprism, and in vivo “slice” images collected through the microprism. Under a low NA, the microprism and 1-mm coverglass have minimal impact on image quality when compared with the direct slice image (Fig. 2, A, C, E, and G). At high NA values, the 1-mm microprism and coverglass still permit imaging of dendritic spines. However, the ability to resolve smaller spines appears limited when compared with direct slice imaging (Fig. 2, B, D, and H).

A 70-μl tail vein injection of fluorescein-dextran (5% wt/vol in physiological saline) in conjunction with the microprism allowed for the visualization of neocortical blood vessels from a new perspective. Images produced using this technique show 10- to 50-μm-diameter vessels extending from deeper layers toward the pial surface (Fig. 3, D and I). This method clearly

**FIG. 3.** Imaging cortical blood vessels and blood flow. A: fluorescently labeled blood vessels at the pia mater prior to microprism insertion. Box indicates location of capillary for line-scanning in B. B: close-up image of a capillary. Black streaks correspond to red blood cells (RBCs) flowing through the capillary. C: line scan of blood vessel along dotted line shown in B. Measurements show a velocity of 0.59 ± 0.06 mm/s and an average flux of 44.7 ± 2.5 RBCs/s. D: fluorescently labeled blood vessels visualized through the microprism. Horizontal line indicates a depth of 500 μm from the brain surface. E: image of a capillary from the superficial part of the neocortex (<500 μm deep). Box indicates line-scan location. F: line scan of blood vessel in E measures a velocity of 0.65 ± 0.04 mm/s and an average flux of 54.7 ± 10 RBCs/s. G: image of a capillary from the deep neocortex (>500 μm deep). Box indicates line-scan location. H: line scan of blood vessel in G measures a velocity of 0.56 ± 0.03 mm/s and an average flux of 49 ± 4.5 RBCs/s. I: image of fluorescently labeled blood vessels visualized through the microprism from a different animal preparation. J: cortical blood vessels imaged about 50 μm away from the vertical imaging face. Boxes indicate severed vessels leaking fluorescent dye into the extracellular matrix. Scale bars: 100 μm (A), 10 μm (B), 10 μm (C, x-axis) and 200 μs (C, y-axis), 100 μm (D), 10 μm (E), 5 μm (F, x-axis) and 100 μs (F, y-axis), 10 μm (G), 5 μm (H, x-axis) and 100 μs (H, y-axis), 200 μm (I), and 200 μm (J).
reveals the delicate network of small-caliber vessels spanning the entire FOV. Line scans of a capillary in the direction of blood flow created an image containing information on red blood cell (RBC) flux and velocity. Measurements through the microprism taken in three different animals were divided into two groups to compare values at the superficial layers (<500 μm deep) and the deep layers (>500 μm deep) (Fig. 3, E–H). Flux and velocity measurements were also obtained at the pia mater, before a microprism was inserted (Fig. 3, A–C). RBC velocity at the pia mater was 0.59 ± 0.06 mm/s with a flux of 44.7 ± 2.5 RBCs/s. For comparison, blood flow parameters in the superficial layers following microprism insertions showed a velocity of 0.65 ± 0.04 mm/s with a flux of 54.7 ± 10 RBCs/s. Blood flow in deep cortical blood vessels measured a velocity of 0.56 ± 0.03 mm/s with a flux of 49 ± 4.5 RBCs/s. Measurements taken pre- and post-insertion compare well with the removal of the microprism, leading to its distortion relative to the surrounding brain tissue decompresses following the removal of the microprism, leading to its distortion relative to the cortical tissue.

Numerical Aperture / Spatial Resolution

Rolls Off Towards Microprism Edges

Fluorophore Distance from Face of Microprism

- 250 μm
- 50 μm

Deviation From Center of Imaging Plane (microns)

0.2 0.3 0.4 0.5 0.6 0.7

Effective Numerical Aperture

0 10 20 30 40 50 60

Distance From Imaging Plane (microns)

0 25 50 75 100 125 150

Percent of Neurons

0–25 25–50 50–75 75–100 100–125

Distance from Imaging Face of Microprism (μm)

A: neurons are labeled in green with a fluorescent Nissl stain. Damaged cells (neurons and glia) are labeled in red with propidium iodide. Yellow cells are colabeled with both dyes and indicate damaged neurons. The white lines denote the former location of the microprism in the cortex. The thick, solid line is the back side (nonimaging side) of the microprism; the thin, solid line is the front side (imaging side) of the microprism. The distance between each dotted line is 25 μm. B: a close-up image from A. Arrows point to damaged neurons in yellow. Healthy neurons (green) and damaged glia (red) are also visualized using this method. C: neuronal damage rolls off as a function of distance from the imaging face of the microprism. Approximately 7% of neurons are damaged at a distance of 75–100 μm. No indication of neuronal damage is present at distances >100 μm from the imaging face. Scale bars: 25 μm (A), 10 μm (B). Error bars in C are SE, n = 3.

FIG. 5. Estimating neuronal damage in tissue imaged through the microprism. A: neurons are labeled in green with a fluorescent Nissl stain. Damaged cells (neurons and glia) are labeled in red with propidium iodide. Yellow cells are colabeled with both dyes and indicate damaged neurons. The white lines denote the former location of the microprism in the cortex. The thick, solid line is the back side (nonimaging side) of the microprism; the thin, solid line is the front side (imaging side) of the microprism. The distance between each dotted line is 25 μm. B: a close-up image from A. Arrows point to damaged neurons in yellow. Healthy neurons (green) and damaged glia (red) are also visualized using this method. C: neuronal damage rolls off as a function of distance from the imaging face of the microprism. Approximately 7% of neurons are damaged at a distance of 75–100 μm. No indication of neuronal damage is present at distances >100 μm from the imaging face. Scale bars: 25 μm (A), 10 μm (B). Error bars in C are SE, n = 3.

FIG. 6. Field-dependent spatial resolution. Imaging away from the central plane leads to a gradual roll-off of spatial resolution. Differences in resolution are attributed to the numerical aperture (NA) decreasing due to the partial clipping of the excitation light toward the edges of the prism. Therefore the effective NA is determined by calculating the fraction of the excitation light cone able to reach the sample. The effective NA (left y-axis) and spatial resolution (right y-axis) also depend on the distance of the focal plane from the face of the microprism. Calculations assume an 800-nm excitation wavelength and an upper NA limit of 0.60 due to the microscope objective. NA and spatial resolution are calculated using standard formulas.
DISCUSSION

Slice imaging provides an ideal imaging angle for neocortical tissue, but lacks the functional context of in vivo experiments. Traditional in vivo cortical imaging required large z-stacks to create an x-, z-projection. However, generating z-stacks can be highly sensitive to motion artifact, result in uneven image intensities, overlook important features if the sampling interval is too large, and is unable to rapidly measure fluorescent transients in both superficial and deep parts of a cortex during a single-image acquisition. Microprism imaging provides a viewing angle similar to that of slice, but in an in vivo preparation. Furthermore, this technique allows for deep cortical imaging with uniform image intensity, a wide FOV, and spatial resolutions sufficient to resolve dendritic spines.

The optimal spatial resolution and maximum numerical aperture are located in the center of the microprism’s imaging plane. The resolution gradually decreases toward the edges of the FOV due to clipping of the excitation beam (Fig. 6). However, overall image quality is comparable to that of images obtained in slice preparations using a 0.6 NA objective lens. Using a microprism made from a higher refractive index would allow improved light collection and increased imaging resolution. Resolution differences between direct slice imaging versus imaging though 1-mm coverglass or a 1-mm microprism may be due to imperfect spherical aberration correction by the microscope objective’s correction collar. There is no need to change the objective’s correction collar while collecting an image stack. The additional thickness of the cortical tissue has a relatively minimal effect on the spatial resolution compared with the aberrations induced by 1-mm of glass. With the correction collar optimized for imaging tissue 100–200 μm away from the imaging face of the prism, all the images in a three-dimensional image stack have comparable spatial resolutions. Further imaging quality differences seen in the microprism may be attributed to nonnormal incidence of excitation light onto the top prism surface. Although care is taken to properly align the microprism during in vivo experiments, it is difficult to achieve the same precision of alignment as that in slice imaging.

Our experiments have allowed imaging ≤300–350 μm away from the face of the prism. However, it is expected that this imaging “depth limit” depends less on the microprism itself and more on traditional two-photon imaging parameters that factor into imaging depth such as: age of the animal, numerical aperture of the objective, and optimization of light collection in the microscope, for example. The cortical tissue being imaged remains steady over the course of the experiment (~1 h). Although some tissue expansion following the prism insertion might be expected, our experiments have not revealed any significant changes in the FOV while imaging. It is likely the majority of the tissue rebound occurs immediately after the prism is inserted and has stabilized by the time the imaging experiment has commenced (~15 min postinsertion). A reasonable concern with any invasive probe is the extent of damage to structures of interest. The insertion of the microprism into the cortical surface creates a volume of tissue that is damaged and should be avoided in imaging experiments. This is similar to traditional sliced brain tissue that exhibits heavily damaged cells and structures at the incision surfaces. This region was found to be within 100 μm of the glass–brain interface (Fig. 6C). All images collected in these experiments are from ≥100 μm away from the imaging face of the prism to avoid this region of damage. In addition, blood flow data collected using the microprism indicated that the vasculature remains functional throughout the 1-mm depth of imaging. Murayama et al. (2007) using 0.5-mm microprisms, found normal resting potentials of layer V neurons surrounding the microprism. These data are not surprising given the imaging face of the microprism could cleanly shear the tissue. Evidence of a clean cut by the microprisms is seen in the H&E staining where the layered organization of cortical cells is preserved. The thickness of cortex varies depending on the brain region, specimen’s age, and animal model used. Therefore with consideration of these factors, it should be possible to simultaneously image all six cortical layers with the microprism.

Microprisms are an elegant approach to studying many facets of neocortical tissue. Our data have shown capabilities of imaging large FOVs, dendritic spines, and RBCs flowing through capillaries. This simple, easy-to-implement technique will open new doors to imaging studies of the mammalian neocortex.

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REFERENCES