Microprisms for in vivo multi-layer cortical imaging

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Abstract:
Cortical slices allow for simultaneous imaging of multiple cortical layers. However, slices lack native physiological inputs and outputs. While 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 an ~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 of the tissue-glass interface. Microprisms are a straightforward tool offering numerous advantages for research into neocortical tissue.
Main Text

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 extra-cortical 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), sub-micron 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).

Two-photon imaging depths of 1000 μm in cortical tissue have been obtained using a regenerative amplifier to increase excitation power (Theer et al. 2003). However, images at depths greater than 600 μm suffer from low SNR and poor contrast. Gradient-index (GRIN) lenses, inserted perpendicular to the neocortex, have also imaged deep layers of the brain (Levene et al. 2004; Jung et al. 2004). However, the FOV was limited to <130 μm, and apical dendrites belonging to the neurons imaged directly below the GRIN lens were destroyed. Half-millimeter prisms have been used with 1-photon excitation to measure the net fluorescence emission from layer V apical dendrites in rats (Murayama et al. 2007; Murayama et al. 2009). However, fluorescence was collected from
large regions-of-interests in superficial cortical layers and did not produce high-resolution fluorescence images.

**Materials and 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 nm - 2000 nm) on the hypotenuse for internal reflection (Optosigma Corporation, Santa Ana, CA) (**Fig. 1, A**). This allows the raster-scanning pattern of the excitation laser to be translated from an x-, y-plane to an x-, z-plane (**Fig. 1, B**). 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 40x, 0.60 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 nm - 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 4x, 0.28 NA air objective (XLFLUOR 4x/340, Olympus) or a 40x, 0.60 NA coverglass-corrected air objective (LUCPLFLN, Olympus) set for 1-mm of glass. Anesthetized animals were placed on a motorized 3-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 Hamamastu (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 (Pologruto et al. 2003). Images were collected at a resolution of 512 x 512 pixels or 1024 x 1024 pixels with 2 ms or 4 ms scan time per line.

**Animal Surgeries**

Mice were anesthetized with an I.P. 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, USA) 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 burr was used to create a craniotomy hole approximately 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 ~2 minutes 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 neocortical surface were severed. The animal's body temperature was maintained at ~36°C Celsius using a water-filled heating pad. The sides of the microprism were held by Dumont forceps away from the vertical imaging face to minimize damage upon insertion. In addition, the long-side of the forceps handle was parallel with the vertical imaging face to help guide the prism perpendicular to the cortical surface. The prism was completely inserted into the cortical tissue with a single, steady motion until the top surface of the microprism was placed flush with the surface of the cortex. No tissue was excised from the region 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 out 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 approximately 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-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 at least 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 H&E staining, a mouse underwent the typical surgery to insert a microprism into the cortex.
The mouse was properly anesthetized for three hours 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 435/455, Molecular Probes) and a DNA intercalating dye,
propidium iodide (10% wt/vol in dH2O, Molecular Probes). The Nissl stain labels neurons, while the
propidium iodide (PI) will enter any cells with compromised cell membranes. This procedure was
adapted from Blanche et al. in which neurons with damaged membranes are co-labeled with both
dyes (Blanche et al. 2005). 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 one hour, 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; KCl, 2.5; CaCl₂, 0.5; MgSO₄, 7.0; NaHPO₄, 1.25; and glucose, 7 for 1-2
minutes. 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 per the
manufacturer’s instructions. Upon completion of staining, slices were imaged with a two-photon
microscope using an excitation wavelength of 770 nm for the PI and 830 nm for the NeuroTrace. A
total of 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 minutes 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
hours prior to imaging. Following recovery, slices were placed into an imaging chamber (Warner
Instruments, Hamden, CT) and perfused with oxygenated artificial cerebral spinal fluid (ACSF) at a
rate of 0.8 mL / min using a two-channel peristaltic pump (Cole-Parmer, Vernon Hills, Illinois). The
ACSF contained (in mM) NaCl, 130; KCl, 3.0; CaCl₂, 2.0; MgSO₄, 1.25; NaHCO₃, 28; NaH₂PO₄, 1.25
and glucose, 10. For slice imaging with coverglass, a standard 1-mm thick microscope slide (12-422-
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 approximately 900 μm below the cortical surface (Fig. 2, 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 and F). We could resolve dendritic spines on layer V pyramidal cells (Fig. 2, H).

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 numerical aperture (NA), the microprism and 1-mm coverglass have minimal impact on image quality when compared to the direct slice image (Fig. 2, A, C, E and G). At high numerical apertures, the 1-mm microprism and coverglass still permit imaging of dendritic spines. However, the ability to resolve smaller spines appears limited when compared to 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 10-50 μm diameter vessels extending from deeper layers towards the pial surface (Fig. 3, D and I). This method clearly reveals the delicate network of small caliber vessels spanning the entire field-of-view. 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, F, G, and H). Flux and velocity measurements were also obtained at the pia mater, before a
microprism was inserted (Fig. 3, A, B, and C). RBC velocity at the pia mater was $0.59 \pm 0.06$ mm/second with a flux of $44.7 \pm 2.5$ RBCs/second. For comparison, blood flow parameters in the superficial layers following microprism insertions showed a velocity of $0.65 \pm 0.04$ mm/second with a flux of $54.7 \pm 10$ RBCs/second. Blood flow in deep cortical blood vessels measured a velocity of $0.56 \pm 0.03$ mm/second with a flux of $49 \pm 4.5$ RBCs/second. Measurements taken pre- and post-insertion compare well with each other and do not indicate major damage to the vasculature by the microprism. All measured values are consistent with measurements obtained by other groups (Kleinfeld et al. 1998). However, imaging less than 100 μm from the microprism’s vertical face reveals dye leakage from severed vessels (Fig. 3, J).

H&E histology shows the imaged tissue in a native state and retaining its stratified organization (Fig. 4, A and B). Deeper structures such as the hippocampus also appear unaffected as evident by the smooth curvature of the cell bodies in the CA fields (Fig. 4, C). Some accumulation of blood is seen at the deepest point where the microprism was placed. However, the vast majority of this blood is found on the non-imaging side (backside) of the microprism (Fig. 4, A and C). Using the combination of a fluorescent Nissl stain and propidium iodide, results show the majority of damaged neurons occur within the first 50 μm from the imaging face of the microprism (Fig. 5, A and B). Only ~7% of neurons are damaged at a distance of 75 – 100 μm away. No evidence of neuronal damage is present beyond 100 μm from the imaging face (Fig. 5, C). Labeled cells within the microprism region are due to cells pulled into the volume during the prism removal process. It is important to note these estimations of neuronal damage are only approximate. Blanche et al. claim this method will overestimate damage since some PI labeled neurons will not actually be dead, but rather only have severed processes that allow the uptake of PI into the cell. In addition, the tissue-glass boundary point cannot be exactly pinpointed and the surrounding brain tissue decompresses following the removal of the microprism leading to its distortion relative to the cortical tissue.
**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 slice, but in an in vivo preparation. Furthermore, this technique allows for deep cortical imaging with uniform image intensity, a wide field-of-view, and spatial resolutions sufficient to resolve dendritic spines.

The optimal spatial resolution and maximum numerical aperture is located in center of the microprism’s imaging plane. The resolution gradually decreases towards the edges of the field-of-view due to clipping of the excitation beam (Fig. 6). However, overall image quality is comparable to 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 affect on the spatial resolution compared to 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 non-normal 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 in slice imaging.
Our experiments have allowed imaging up to 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, optimization of light collection in the microscope, to name a few. The cortical tissue being imaged remains steady over the course of the experiment (~1 hour). Although some tissue expansion following the prism insertion might be expected, our experiments have not revealed any significant changes in the field-of-view 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. post-insertion).

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. 6, C). All images collected in these experiments are from at least 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., using 0.5-mm microprisms, found normal resting potentials of layer V neurons surrounding the microprism (Murayama et al. 2007). 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:


**Figure Legends:**

**FIG. 1.** Micro prism size and placement in neocortex for two-photon imaging. *A:* One-millimeter micro prism next to Lincoln’s nose on a U.S. penny. *B:* Illustration depicting the micro prism’s placement into the neocortex and the resulting translation of the excitation laser for side-on imaging. Brain image in *B* used with permission from http://brainmuseum.org.

**FIG. 2.** Neocortical images of layer V YFP neurons taken from a traditional slice preparation, a slice with 1-mm of coverglass, and in vivo preparations using the micro prism. Low- *A, C, E, F, G* and high- *B, D, H* magnification images of layer V YFP pyramidal neurons with apical dendrites extending into layer I. Yellow arrows point to dendritic spines. *A, B:* Slice images without coverglass. *C, D:* Slice images with 1-mm coverglass to simulate optical aberrations. *E, F, G, H:* In vivo images collected with 1-mm micro prism (*G* from a different animal). *F:* Detailed image of a single layer V neuron. *H:* High-magnification image from yellow box in *F*. Scale bars: 200 μm (*A, C, E, G*), 100 μm (*F*), and 15 μm (*B, D, H*). Slice images taken ~200 μm from surface. Micro prism images taken ~150 μm from the vertical imaging face in *E, F, H* and ~200 μm away in *G*.

**FIG. 3.** Imaging cortical blood vessels and blood flow. *A:* Fluorescently labeled blood vessels at the pia mater prior to micro prism insertion. Box indicates location of capillary for line-scanning in *B*.
**B**: Close-up image of a capillary. Black streaks correspond to RBCs flowing through the capillary.

**C**: Line-scan of blood vessel along dotted line shown in (B). Measurements show a velocity of $0.59 \pm 0.06$ mm/second and an average flux of $44.7 \pm 2.5$ RBCs/second.

**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 $0.65 \pm 0.04$ mm/second and an average flux of $54.7 \pm 10$ RBCs/second.

**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 $0.56 \pm 0.03$ mm/second and an average flux of $49 \pm 4.5$ RBCs/second.

**I**: Image of fluorescently labeled blood vessels visualized through the microprism from a different animal preparation.

**J**: Cortical blood vessels imaged approximately 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 ms (C, y-axis), 100 μm (D), 10 μm (E), 5 μm (F, x-axis) and 100 ms (F, y-axis), 10 μm (G), 5 μm (H, x-axis) and 100 ms (H, y-axis), 200 μm (I), 200 μm (J). Microprism images taken ~100 μm from the vertical imaging face in (D), ~150 μm in (I), and ~50 μm in (J).

**FIG. 4.** H&E staining of tissue surrounding microprism.  

**A**: Histology reveals imaged tissue remains in native context and the stratified organization of different cell types are preserved.

**B**: Large layer V pyramidal neurons are easily seen at a higher magnification (region inside the box from A).

**C**: Histological images of the hippocampus immediately below the site of microprism insertion reveal the smooth curvature of the cell bodies in the CA fields indicating little to no compression damage. Some blood accumulation is located on the non-imaging side of the microprism. Scale bars: 200 μm (A, C), 100 μm (B).
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 co-labeled 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 (non-imaging 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 greater than 100 μm from the imaging face. Scale bars: 25 μm (**A**), 10 μm (**B**). Error bars in (**C**) are standard error, 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 decreasing due to the partial clipping of the excitation light towards 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.