In Vivo Microendoscopy of the Hippocampus

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Protocol

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Conventional intravital microscopy has generally been limited to superficial brain areas such as the olfactory bulb, the neocortex, or the cerebellar cortex. In vivo optical microendoscopy uses gradient refractive index (GRIN) microlenses that can be inserted into tissue to image cells in deeper areas. This protocol describes in vivo microendoscopy of the mouse hippocampus. The general methodology can be applied to many deep brain regions and other areas of the body.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Agarose, Type III-A (Sigma-Aldrich)
Analgesic (e.g., buprenorphine)
Anti-inflammatory (e.g., carprofen, dexamethasone) (optional; see Step 10)
Artificial cerebrospinal fluid (ACSF; e.g., from Harvard Apparatus)
Dental acrylic (e.g., Ortho-Jet, Lang Dental Manufacturing Co., Inc.)
Ethanol (70%)
Local anesthetic (e.g., 1% lidocaine) as needed (see Step 13)
Ophthalmic ointment (e.g., Puralube Vet Ointment, PharmaDerm/Nycomed US)
Physiologic saline or lactated Ringer's solution (e.g., from Electron Microscopy Sciences)
Skin disinfectant (e.g., betadine, Baxter)
Tissue adhesive (e.g., Vetbond, 3 M)

Equipment

Balance (for weighing animals; e.g., Mettler Toledo International, Inc.)
Capillary tubing (e.g., thin-walled glass 1.0–2.5-mm inner diameter, Vitrocom, Inc.)
Cold light source (e.g., KL 1500, SCHOTT North America, Inc.)
Connection bar (metal) and adaptors

Adapted from *Imaging: A Laboratory Manual* (ed. Yuste). CSHL Press, Cold Spring Harbor, NY, USA, 2010.

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Containers to store guide tubes (e.g., sterile culture dishes)

Cotton swabs

Coverslips (#0 thickness; e.g., from Electron Microscopy Sciences)

Curing light (e.g., COLTOLUX 75, Coltène Whaledent)

DC temperature regulation system (e.g., FHC Inc. 40-90-8; 40-90-5; 40-90-2-07)

Diamond-scribing tool (e.g., from Electron Microscopy Sciences)

Gel foam (optional; see Step 18)

Glass bead sterilizer (e.g., model BS-500, Dent-EQ)

Gloves

Heating blanket

Imaging setup:

- Microscope that has infinity optics and that has been adapted for in vivo imaging (e.g., Ultima IV, Prairie Technologies, Inc.)
- Microendoscope probe
- Microscope objectives for optical coupling to the microendoscope probe

*For a detailed description of the components, see **In Vivo Optical Microendoscopy for Imaging Cells Lying Deep within Live Tissue** (Barretto and Schnitzer 2012). Parameters for one- and two-photon imaging are outlined in Step 30.*

Instruments/surgical tools, aseptic (e.g., from Fine Science Tools)

Laboratory animal anesthesia system (e.g., VetEquip Inc. 901806) with the following:

- Anesthetic gas (e.g., isoflurane, Southmedic, Inc.)
- Carrier gas tank (e.g., medigrade oxygen from Praxair)
- Waste anesthetic gas system (recommended; e.g., VetEquip Inc. item 933101)

Alternatively, use an interperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg) to anesthetize the animal.

Lens paper

Microdrill (e.g., Osada, Inc. EXL-M40)

Microwave, standard (for agarose gel preparation)

Mounting post (custom-made; aluminum 15 × 3 × 2-mm bar with 2.7-mm through hole on end)

Mounting-post holder (custom-made; aluminum bar with M2 tapped hole)

Needles (30-, 29-, 27-gauge)

Optical adhesive (e.g., NOA 81; Norland Products, Inc.)

Sandpaper, fine-500 grit (e.g., 3 M) or glass polisher (e.g., ULTRAPOL, ULTRA TEC Manufacturing, Inc.)

Shaver

Sonicator (e.g., Model 1510, Branson Ultrasonics Corp.)

Stereomicroscope (e.g., MZ12.5, Leica)

Stereotaxic apparatus (custom-made)

Surgical eye spears (e.g., 1556455, Henry Schein Medical)

Tape (flexible) or adhesive dressing

Waste liquid suction line (custom-made)

METHOD

Glass Guide Tube Construction (~25 min)

An optically transparent guide tube (Fig. 1B) is often used to assist in delivering the microendoscope to the tissue of interest. Because the tube is sealed at the tip with a small cover glass that permits optical but not physical access to the tissue, microendoscopes can be quickly delivered and can be interchanged with minimal mechanical disturbance to the field of view under inspection.

1. Choose a thin-walled capillary glass of appropriate diameter. Typical inner diameters safely exceed the microendoscope diameter by 10%–15%.
2. Prepare the guide tube as follows:
   i. Cut the thin-walled capillary glass to the desired length.
   ii. Use a microdrill to uniformly thin the circumference of the glass at the location of the cut.
   iii. Snap the glass at the thinned portion, and coarsely smooth with the microdrill or sandpaper.
3. Polish one end of the guide tube with a fiber-optic polisher or a fine grit sandpaper. Inspect the guide tube end under a stereomicroscope, and ensure flatness. Repolish as necessary.

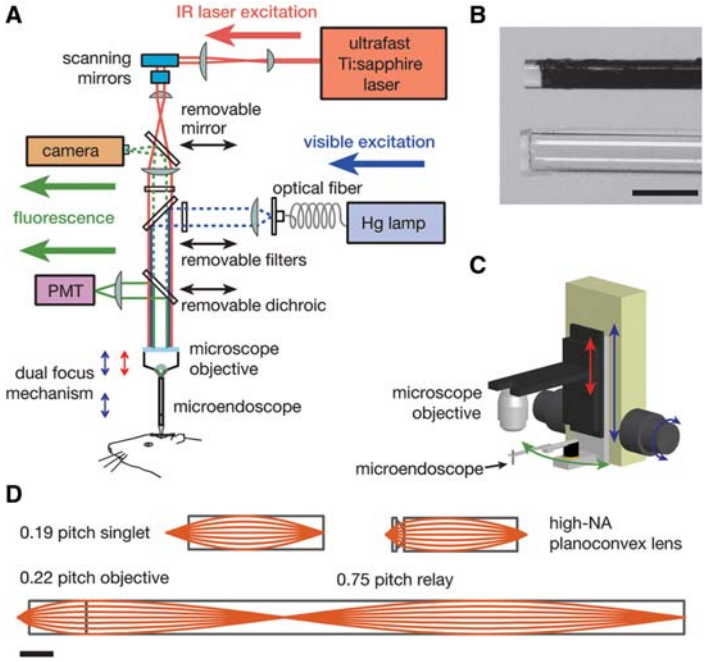

FIGURE 1. Methodologies for in vivo optical microendoscopy. (*A*) Optical schematic of an upright microscope modified to permit both one- and two-photon fluorescence microendoscopy. For two-photon imaging, the beam from an ultrashort-pulsed infrared (IR) Ti:sapphire laser is scanned within the focal plane of the microscope objective. By adjusting the axial separation between the objective and the microendoscope (red arrow of the dual-focus mechanism; see also *C*), this focal plane of the microscope objective is also set to the microendoscope's back focal plane. Another focal adjustment (blue arrows of the dual mechanism) is used to lower the objective and the microendoscope in tandem toward the animal. For one-photon imaging, a mercury (Hg) arc lamp provides illumination. In both imaging modes, fluorescence emissions route back through the microendoscope and to either a camera or a photomultiplier tube (PMT) for one- or two-photon imaging, respectively. (*B*) Photographs of the tips of a 0.5-mm-diameter microendoscope of doublet design (*top*) and a 0.8-mm-outer-diameter glass capillary guide tube (*bottom*) into which this microendoscope can be inserted. The relay of the microendoscope is coated black. A glass coverslip is attached to the tip of the guide tube. The guide tube facilitates the rapid exchange of microendoscopes without perturbation to the underlying tissue. Scale bar, 1 mm. (*C*) The microscope objective and the microendoscope probe are mounted on a pair of cascaded focusing actuators that provide dual-focus capability. This allows the objective to be moved either alone (red arrow) or together with the microendoscope (blue arrow). The microendoscope can also be swung out of the optical axis (green arrow) to permit conventional microscopy. (*D*) Optical ray diagrams for sample microendoscopes of the singlet GRIN (*top left*), compound plano-convex and GRIN (*top right*), and GRIN doublet (*bottom*) types. Scale bar, 1 mm.

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4. Cut circular pieces of #0-thickness cover glass with diameters matching the outer diameter of the guide tube. Using a diamond scribe, score circular patterns onto the cover glass, and break with the forceps.

   Tolerances for the cover-glass dimensions are set by the inner and the outer diameters of the guide tube.

5. Clean all glass pieces by sonication while they are immersed in the cleanser, and store in ethanol until assembly. In subsequent steps, use gloves, and work in a dust-free area.
6. Apply a thin layer of ultraviolet-curing optical adhesive to the polished end of the guide tube. Using a high-magnification stereomicroscope, orient the guide tube toward the objective, and use a fine 30-gauge needle to apply the adhesive onto the guide tube.
7. Attach the circular coverslips to the guide tube. Use forceps to hold the cover glass, and gently drop the coverslip onto the guide tube. Ensure that glue does not enter the central area of the guide tube and that an epoxy seal is formed around the entire circumference of the guide tube. Set the epoxy using an ultraviolet light source.
8. Store guide tubes in clean containers until use (e.g., sterile culture dishes). If possible, allow at least 12 h for the optical epoxy to cure before use. Rinse with saline solution before implantation.

Surgery (~1 h)

The following animal procedures are outlined for the examination of the dorsal hippocampus in adult mice but are applicable to other regions. All procedures were approved by the Stanford Administrative Panel on Laboratory Animal Care (APLAC). Consultation with those overseeing institutional guidelines for animal surgery care and anesthesia is recommended.

9. Deeply anesthetize mice with isoflurane gas (2.0%–2.5%; mixed with 2-L/min oxygen) or interperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). Assess depth of anesthesia by monitoring toe pinch withdrawal, eyelid reflex, and respiration rate.
10. (Optional) Administer dexamethasone (2-mg/kg intramuscular) and carprofen (5-mg/kg subcutaneous) to minimize tissue swelling and inflammation.
11. Secure the animal in a stereotaxic frame. Maintain body temperature at 37°C with a heating blanket. Apply ophthalmic ointment to the eyes.
12. Trim or shave the fur from the top of the head, and disinfect the exposed skin with alternating washes of 70% ethanol and betadine.

    The use of a bead sterilizer to disinfect surgical instruments is recommended.

13. Expose the cranium in the vicinity dorsal to the brain structure of interest. Remove the periosteum using a probe or a scalpel, and rinse with 0.9% saline solution. After rinsing, use a cotton swab to dry the exposed skull.

    Subcutaneous lidocaine or other local anesthetic may be administered before exposing the cranium, as necessary.

14. Apply a thin layer of cyanoacrylate tissue adhesive (e.g., Vetbond) to the regions of exposed skull outside of the expected craniotomy site. Use a fine applicator (e.g., hypodermic needle) to spread the cyanoacrylate over the boundaries of the exposed cranium to seal the skin cut sites. Allow the cyanoacrylate to dry for 5 min.
15. Drill a round craniotomy centered over the stereotaxic coordinates of interest (e.g., 2.0-mm posterior and 2.0-mm lateral to the bregma in the hippocampus). Remove the dura with forceps.

    A trephine is helpful in marking craniotomy dimensions matched to the microendoscope diameter.

16. Perform blunt dissection and aspiration to gradually remove a cylindrical column of neocortical brain tissue with a 27-gauge blunt needle. Continuously irrigate the applied area with sterile ACSF or Ringer's solution.

Bleeding from disrupted vasculature is normal; increase irrigation rates to maintain visibility within the column.

17. As the desired imaging area is approached, expose the imaging area by aspiration with a fine 29-gauge needle.

    Under optimal conditions, a thin layer of tissue remains overlying the cells of interest, to minimize direct mechanical tissue damage from aspiration. In the hippocampal preparation, the overlying corpus callosum can be readily identified by its stereotyped white matter tract patterns.

18. Minimize bleeding from the sides of the aspirated column by following applications of saline irrigation and aspiration with 5-sec pause intervals to allow clot formation.

    Gel foam may be applied to control bleeding. Take care not to allow a clot to form over the imaging area.

19. (Optional) Examine animal for fluorescence labeling, using a low-magnification long working distance objective. (See Imaging Session below.)

20. Gradually insert a closed-end glass guide tube into the aspirated column. Lower the guide tube until it is in contact with the distal tissue regions. Check that neither air pockets nor bleeding regions are present under the guide tube. If necessary, irrigate with buffer, and repeat guide tube insertion.

    The tissue should be visible on inspection through the guide tube with a stereomicroscope.

21. Suction any liquids that are present on the cyanoacrylate layer.

22. Apply melted agarose (~1.5%) to the sides of the guide tube, filling gaps between skull and the guide tube. Allow agarose to harden. Remove excess agarose by dicing with a scalpel blade.

23. Apply a layer of dental acrylic over all of the exposed skull and sides of the guide tube. Affix a metal connection bar approximately parallel to the plane of the guide tube surface. The distal end of the bar must be at least 1 cm away from the guide tube to prevent obstruction during imaging. Wait 10 min for the acrylic to harden.

24. Affix a piece of flexible tape or adhesive dressing over the guide tube. This will prevent dirt from entering the tube.

25. Allow the animal to recover from anesthesia. Return mouse to a clean home cage, and maintain heating until righting reflex is shown. Administer analgesics (e.g., buprenorphine or carprofen) as necessary.

Imaging Session (>30 min)

26. Anesthetize mice with isoflurane gas (2.0%–2.5%; mixed with 2-L/min oxygen) or interperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). Assess depth of anesthesia by monitoring toe pinch withdrawal, eyelid reflex, and respiration rate.

27. Secure animal into a position suitable for imaging. Use appropriate adaptors to clamp the metal connection bar. Maintain body temperature at 37°C with a heating blanket. Apply ophthalmic ointment to the eyes as necessary.

28. Insert the microendoscope probe into the guide tube. Remove protective tape to expose the guide tube. Examine the guide tube for any dirt particles. If necessary, deliver H_2O into the guide tube, and rinse. Using air suction through a 25–29-gauge blunt needle, remove all fluid from the guide tube.

    Take care not to damage the bottom face of the guide tube with excess pressure.

29. Using an eyepiece and bright-field illumination, focus the microscope objective onto the proximal microendoscope surface. Align the microendoscope to the optical axis of the microscope by adjusting the clamp orientation.

    Under bright-field illumination, a well-aligned microendoscope will appear circular, not elliptical (which would indicate tilt relative to the optical axis).

30. If available, use one-photon fluorescence imaging to locate the desired tissue region. Use the minimal intensity of light necessary to illuminate the tissue. Typically, one gradually adjusts the focal plane of the microscope objective upward (i.e., away from the specimen), assuming that the tissue plane of interest is located closer to the face of the micro-optical objective than to the microendoscope probe's design working distance. Optionally, switch to the two-photon fluorescence mode.

Parameters for one-photon imaging:

- *Excitation filter: Approximately 470/40 nm for fluorescein-conjugated dextrans (for blood-flow imaging), green fluorescent protein (GFP) and yellow fluorescent protein (YFP)*
- *Emission filters: Approximately 525/50 nm for fluorescein-conjugated dextrans, GFP, and YFP*
- *Images/frame rate: 512 × 512 pixels at 100 Hz with a high-speed electron-multiplying charge-coupled device (CCD) camera (e.g., iXon DU-897E, Andor Technology), or 1392 × 1040 pixels with a cooled CCD camera (e.g., Coolsnap HQ, Roper Scientific)*
- *Recording duration: Typically 30–40 sec for a given field of view*

Parameters for two-photon imaging:

- *Excitation wavelength: Approximately 800 nm for fluorescein-conjugated dextrans in vascular imaging, ~920 nm for GFP and YFP*
- *Excitation power at sample surface: Always <25 mW for tissues proximal to the microendoscope, more distal tissues require greater power*
- *Images/dwell times: 512 × 512 pixels (typically ~0.8–4 µsec per pixel or as permitted by tissue motion). For high-resolution imaging, multiple images can be acquired and can be averaged, after motion correction, to produce an improved image.*
- *Section/stack (3D imaging): Approximately 5–10-µm axial spacing for GRIN singlets and doublets that have ~10-µm axial resolution, 1–2.5-µm axial spacing for high-resolution microlenses*
- *Recording duration: Typically 5–10 min for a given field of view*

See Troubleshooting.

31. Interchange microendoscope probes as needed without displacing the animal by using suction to remove the microendoscope from the glass guide tube.

See Troubleshooting.

32. At the end of the experiment, clean microendoscopes by rinsing and gentle scrubbing with H_2O and lens paper. Sacrifice animal as appropriate for post hoc histological examination.

TROUBLESHOOTING

Problem (Step 30): There is excessive tissue motion during imaging.
Solution: Consider the following:

1. Most commonly observed tissue motions are caused by breathing rhythms. First, check the depth of anesthesia during imaging. Second, adjust the head position relative to the animal's trunk to facilitate unconstrained breathing while providing modest mechanical decoupling of the head from motions of the trunk.
2. Another common cause of tissue motion is an excess gap between the tissue and the end of the guide tube; this is the fault of either an improper guide tube placement during the surgery or any swelling that occurred then and later subsided. As the brain tissue stabilizes, the guide tube may no longer be optimally positioned for the desired imaging experiment.
3. Reducing the overall duration of surgery, adjusting the dosage of anti-inflammatory agents, and decreasing the potential heating of the tissue during skull drilling all generally improve experimental quality.

Problem (Step 31): Image quality degrades during image acquisition.
Solution: Consider the following:

1. Clean and inspect the microendoscope, and replace it as necessary. Excessive laser power focused to surfaces of the microendoscope can result in damage to the glass. When this occurs, background photon levels in the image typically increase. Inspection of the microendoscope with an epifluorescence microscope will reveal autofluorescent patterns in which laser scanning occurred on the glass surface.
2. Image degradation may be an indication of cellular damage. During imaging of subcellular structures such as dendrites or axons, blebbing may appear as well as general fading of fluorescence in the scanned regions across the imaging sessions. In such cases, use lower intensity illumination.
3. As an alternative to acquiring a single image at a higher illumination power, averaging of multiple images each taken at a faster acquisition speed and lower power may also improve image quality.

DISCUSSION

Optical microendoscopy is suited for cellular level imaging deep within tissue in live animals or humans. Researchers can choose among a wide variety of microendoscope probe designs to select those best matched to their needs. For the combined acquisition of high-speed videos and 3D image stacks from the same specimen, it is useful to have a microscope that allows online toggling between one-photon fluorescence and laser-scanning imaging (Jung et al. 2004) (Fig. 1A). Laser-scanning second-harmonic generation microendoscopy can generally be performed on any microscope intended for intravital two-photon imaging by an appropriate choice of emission filter (Llewellyn et al. 2008). Overall, microendoscopy is a flexible technique that can be used with multiple modes of contrast generation, at different tissue depths, and with a wide variety of imaging parameters. In the brain, this flexibility has enabled the examination of intracellular calcium dynamics, microcirculatory flow, and neuronal morphology. Because the microendoscope is conceptually, at core, an optical relay, any fluorescent marker that performs well under conventional one- or two-photon fluorescence microscopy will generally perform comparably well under microendoscopy in similar optical conditions. Figure 2 shows examples of images acquired by fluorescence microendoscopy in live mice.

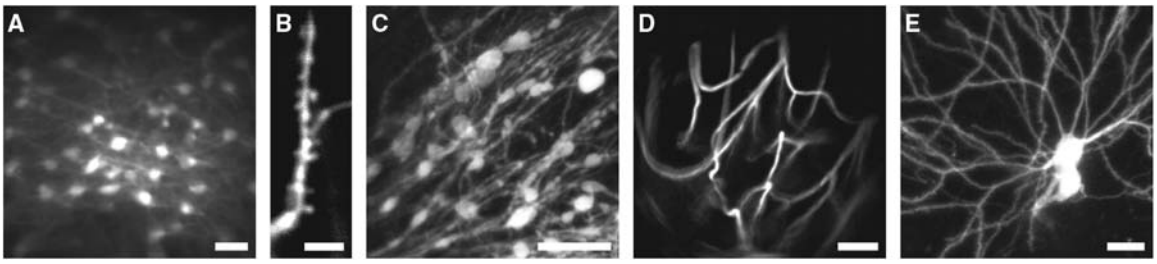

FIGURE 2. Images acquired by fluorescence microendoscopy in live mice. (*A*) GFP-labeled pyramidal neurons in CA1 hippocampus imaged with a 1-mm singlet probe. Scale bar, 50 µm. (*B*) High-resolution image of CA1 hippocampal dendritic spines acquired using an LaSFN9 high-resolution probe. Scale bar, 5 µm. (*C*) GFP-labeled neurons in the brainstem's external cuneate nucleus imaged with a 1-mm doublet probe of 20-mm length and a 0.75-pitch relay. Scale bar, 50 µm. (*D*) Fluorescein-labeled vasculature in CA1 hippocampus imaged with a 0.5-mm singlet probe. Scale bar, 50 µm. (*E*) GFP-labeled pyramidal neurons in CA1 hippocampus imaged with a 1-mm singlet probe. Scale bar, 50 µm. *A–C* and *E* are 2D projections of 3D stacks acquired by two-photon microendoscopy. These stacks were composed of 108 image slices acquired at 2-µm axial separation between adjacent slices for *A*; nine images with 1.6-µm axial separation for *B*; 50 images with 0.43-µm axial separation for *C*; four slices taken at 4.2-µm axial separation for *E*. *D* was obtained by one-photon microendoscopy and shows the standard deviation image of a high-speed video sequence of blood flow, which is a postprocessed image that highlights blood vessels.

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Some deep structures may be accessed by conventional microscope optics. In one strategy, more invasive aspiration of the tissue allows direct access to the tissue of interest (Mizrahi et al. 2004). A wide column of tissue must be removed to prevent blocking light to and from the specimen if imaging with a high NA is to be achieved. The applicability of this technique seems limited because deeper structures require surgery and aspiration that are substantially more invasive.

Another strategy for deep imaging extends the penetration depth of conventional two-photon microscopy to tissues as deep as 1 mm below the surface, as reviewed in Wilt et al. (2009). To achieve this, several methods exist to improve fluorescence generation, including the use of illumination sources with higher pulse energies and longer wavelengths and adaptive optics to improve the focusing of light in the tissue. In addition to providing a relatively noninvasive means of imaging structures at intermediate depths, such as the infragranular layers of the neocortex, these improvements are also compatible with microendoscopy. However, because of the exponential increase with depth of a photon's probability of being scattered, these methods for extending the reach of conventional light microscopy are unlikely to reach the tissue depths of several millimeters to ~1 cm that have already been demonstrated by microendoscopy.

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