Video Article

A Rapid Approach to High-Resolution Fluorescence Imaging in Semi-Thick Brain Slices

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Abstract

A fundamental goal to both basic and clinical neuroscience is to better understand the identities, molecular makeup, and patterns of connectivity that are characteristic to neurons in both normal and diseased brain. Towards this, a great deal of effort has been placed on building high-resolution neuroanatomical maps[1-3]. With the expansion of molecular genetics and advances in light microscopy has come the ability to query not only neuronal morphologies, but also the molecular and cellular makeup of individual neurons and their associated networks[4]. Major advances in the ability to mark and manipulate neurons through transgenic and gene targeting technologies in the rodent now allow investigators to 'program' neuronal subsets at will[5-6]. Arguably, one of the most influential contributions to contemporary neuroscience has been the discovery and cloning of genes encoding fluorescent proteins (FPs) in marine invertebrates[7-8], alongside their subsequent engineering to yield an ever-expanding toolbox of vital reporters[9]. Exploiting cell type-specific promoter activity to drive targeted FP expression in discrete neuronal populations now affords neuroanatomical investigation with genetic precision.

Engineering FP expression in neurons has vastly improved our understanding of brain structure and function. However, imaging individual neurons and their associated networks in deep brain tissues, or in three dimensions, has remained a challenge. Due to high lipid content, nervous tissue is rather opaque and exhibits auto fluorescence. These inherent biophysical properties make it difficult to visualize and image fluorescently labelled neurons at high resolution using standard epifluorescent or confocal microscopy beyond depths of tens of microns. To circumvent this challenge investigators often employ serial thin-section imaging and reconstruction methods[10], or 2-photon laser scanning microscopy[11]. Current drawbacks to these approaches are the associated labor-intensive tissue preparation, or cost-prohibitive instrumentation respectively.

Here, we present a relatively rapid and simple method to visualize fluorescently labelled cells in fixed semi-thick mouse brain slices by optical clearing and imaging. In the attached protocol we describe the methods of: 1) fixing brain tissue *in situ* via intracardial perfusion, 2) dissection and removal of whole brain, 3) stationary brain embedding in agarose, 4) precision semi-thick slice preparation using new vibratome instrumentation, 5) clearing brain tissue through a glycerol gradient, and 6) mounting on glass slides for light microscopy and z-stack reconstruction (**Figure 1**).

For preparing brain slices we implemented a relatively new piece of instrumentation called the 'Compresstome' VF-200 (http://www.precisionary.com/products_vf200.html). This instrument is a semi-automated microtome equipped with a motorized advance and blade vibration system with features similar in function to other vibratomes. Unlike other vibratomes, the tissue to be sliced is mounted in an agarose plug within a stainless steel cylinder. The tissue is extruded at desired thicknesses from the cylinder, and cut by the forward advancing vibrating blade. The agarose plug/cylinder system allows for reproducible tissue mounting, alignment, and precision cutting. In our hands, the 'Compresstome' yields high quality tissue slices for electrophysiology, immunohistochemistry, and direct fixed-tissue mounting and imaging. Combined with optical clearing, here we demonstrate the preparation of semi-thick fixed brain slices for high-resolution fluorescent imaging.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=2807

Protocol

1. *In situ* brain fixation

*Prepare a 10 ml syringe (28 gauge needle) filled with phosphate buffered saline (PBS).
*Prepare a 10 ml syringe (28 gauge needle) filled with 4% paraformaldehyde (PFA) in PBS. Reserve an additional 5-10 ml of PFA/PBS for post fixation.

1. Inject experimental mouse intraperitoneally with a lethal dose of Avertin or Nembutal.
2. Once the mouse is sedated, wet the abdomen with ethanol and secure the mouse to the bottom of a tray layered with cork, wax, or silicone elastomer using dissection pins. With abdomen facing up, secure the four paws to the surface - spreading them as wide as possible.

3. Grab the skin with forceps at the level of the sternum, and cut crosswise to expose the liver. Cut laterally and then up through the ribs and diaphragm. Remove the rib tissue flap and continue cutting until the heart is accessible.
4. Pierce or snip the right atrium to drain circulated blood.
5. Flush residual blood from circulatory system by perfusion of PBS through left ventricle.
6. Accessing the same needle hole, fix whole mouse by subsequent perfusion of PBS/PFA through left ventricle.

2. Dissection and brain extraction

1. Remove the skin from head, neck, and cranium by cutting around ear canal and eye orbits, followed by pulling skin anteriorly to expose the cranium.
   *See Figure 2 for an illustration of steps 2.2) to 2.5)
2. Using a bone scissors, cut crosswise from eye the socket through the nasal turbinate bones. Do this for each side. The crosscut should be anterior to the eyes and olfactory bulbs.
3. Cut lengthwise from each ear canal to eye socket.
4. Using a small dissection scissors, cut the occipital bone plate overlying the cerebellum from one ear canal to the other.
5. With the same dissection scissors, cut anteriorly along the midline to the level of the olfactory bulbs.
6. Using forceps, remove each bone plate from the brain, taking care to remove any connective tissue so that the brain remains intact when lifting off the bone.
7. Snip optic and cranial nerves, cut the top cervical vertebrae, and remove the brain into fixative.
8. Post-fix for 1-2 hrs at 4° C. Wash 3 times 15 min each in PBS.

3. Agarose embedding

1. Melt 2% high-strength, low-gel point agarose type I-B (Sigma catalog number: A0576) in PBS saline with microwave oven.
2. Transfer molten agarose to a test tube. Place test tube in a warm incubator or water bath (40°-41° C) until temperature equilibration.
3. Glue fixed brain tissue onto the plunger of the specimen syringe with cyanoacrylate glue (**Figure 3A**). Brush a thin layer of saturated sucrose-PBS solution on the surface of the brain tissue to facilitate the later release of the agarose ring from the brain slices.
4. Draw down the plunger with brain tissue into the plunger housing (**Figure 3B**).
5. Pipette or pour warm agarose into the plunger housing, covering the brain tissue. Avoid trapping air bubbles into the agarose. Air bubbles in the agarose will compromise the slice quality.
6. Clamp the specimen syringe with a cold (0° to -25° C) chilling block for 1 minute to set the agarose (**Figure 3C**).

4. Sectioning brain slice with the Compresstome

1. Assemble the specimen syringe containing the brain tissue into the Compresstome (**Figure 3D**).
2. Align the edge of the razor blade closely with the outlet of the specimen syringe (**Figure 3E**).
3. Fill up the buffer tank with PBS solution.
4. Rotate the micrometer one slice thickness forward to extrude the agarose-brain tissue block out of the specimen syringe.
5. Press the “start” button on the control unit of the Compresstome to initiate the sectioning process.
6. Repeat steps 4.4) to 4.5) until desired slices are obtained at determine thickness.
7. Harvest brain slices with brush or pipette to vials or staining wells filled with PBS (**Figure 3F**).

5. Optical Clearing

1. Prepare a 75% vol:vol solution of glycerol:PBS.
2. Pour (or pipette) off half the volume of PBS wash from the collected brain slices.
3. Add back the volume removed with glycerol/PBS.
4. Allow to equilibrate with gentle mixing at 4° C until the slices sink.
5. Repeat steps 6.2) to 6.4) until a final glycerol concentration reaches 75%.
6. Replace solution with 90% glycerol in PBS and allow to equilibrate.
   Note: Take care not to introduce air bubbles while adding glycerol solutions. Pour solutions slowly and mix gently, since air bubbles will interfere with uniform equilibration and will be carried over to slide mounting.

6. Slice mounting for imaging

1. Cut double-sided seal adhesive (SA-S-1L, Grace Bio-Labs) into an open rectangle to fit the viewable area of a micro slide (Superfrost Plus, 25 X 75 X 1 mm, cat. # 48311-703, VWR) with sides 2-3 mm wide.
2. Place brain slices inside the center of the adhesive tape rectangle using a pipette or paintbrush. Remove excess glycerol with gentle aspiration.
3. Arrange brain slices on slide in desired order with a paintbrush.
4. Gently lower cover glass (24 X 60 mm, cat. # 48383-139, VWR) onto the adhesive tape, taking care not to introduce air bubbles. Gently apply pressure onto cover glass to ensure contact with adhesive tape.
5. Aspirate excess glycerol from edges between slide and coverglass.
6. Seal slide and coverslip using clear nail polish.
7. Allow to dry before imaging, or store long-term in slide boxes at 4° C.

7. Representative Results:

Processing, imaging, and analyzing fluorescently labeled brain tissue have become indispensible to the study of neurobiology. Many of these investigations require sophisticated genetic manipulations to obtain reporter expression in targeted neuronal subsets, followed by both low- and high-resolution image analyses. Often experimental and technical limitations make it impossible to obtain these types of data from the same animal, or in a rapid manner. Preparation of optically-cleared, semi-thick brain sections for fluorescent imaging aids in this challenge. An example

of an intact thick brain slice labeled with a genetically modified rabies virus engineered to express EGFP, and imaged using epifluorescent and confocal microscopy is shown in **Figure 4** and **Figure 5** respectively. For epifluorescent imaging we used a Leica M205 FA, and for confocal image acquisition we used a Zeiss LSM 510. Due to the relative simplicity of the attached protocol, this method is capable of yielding useful image data from tissue expressing fluorescent reporters with a turnaround time of less than one day, and is compatible with both low- and high-resolution light microscopy.

If the investigator chooses to incorporate additional postmortem labeling methods, immunohistochemical staining, or make thinner sections, the protocol lengthens accordingly. However, the method described above represents a simple and relatively high-throughput method for screening patterns of vital reporter expression in intact brain.

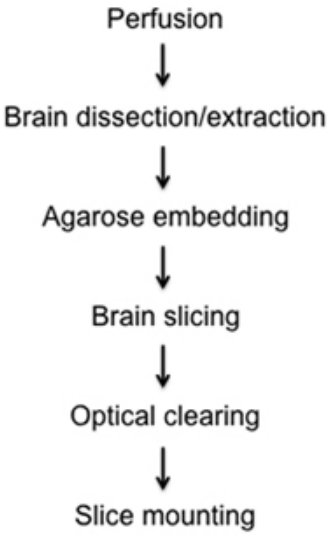

Figure 1. Flow chart diagraming the fixation, dissection, slicing, clearing, and mounting procedure of semi-thick brain slices.

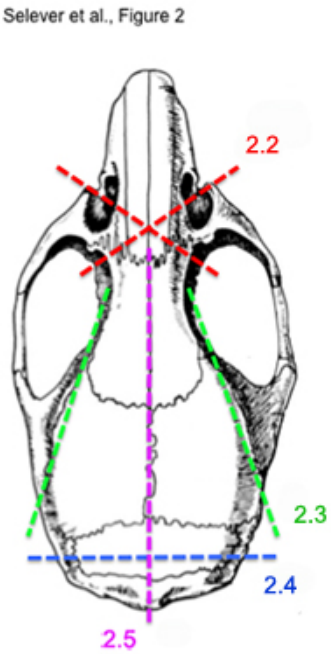

Figure 2. Diagram of sequential cutting steps to extract the intact mouse brain.

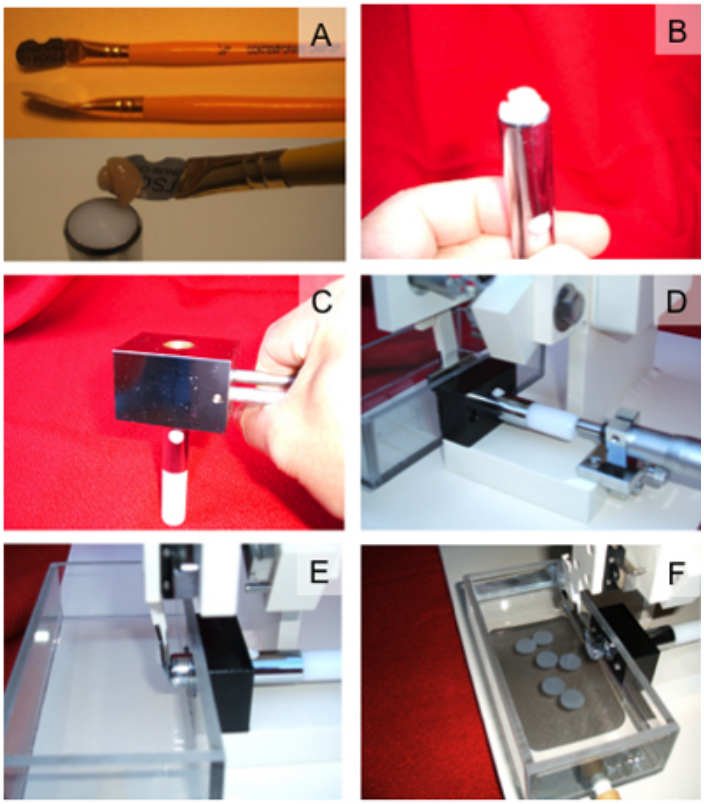

Figure 3. Steps to mount and slice brain tissue using the Compresstome. A) Placement of brain tissue onto cutting plunger using superglue. B) Drawing down the mounted brain tissue into the plunger for agarose embedding. C) Solidifying an agarose brain plug using a chilled compression block. D) Insertion of agarose brain plug and plunger into Compresstome cutting chamber. E) Alignment of razorblade to the plunger device. F) Cutting and collection of brain slices into Compresstome buffer chamber.

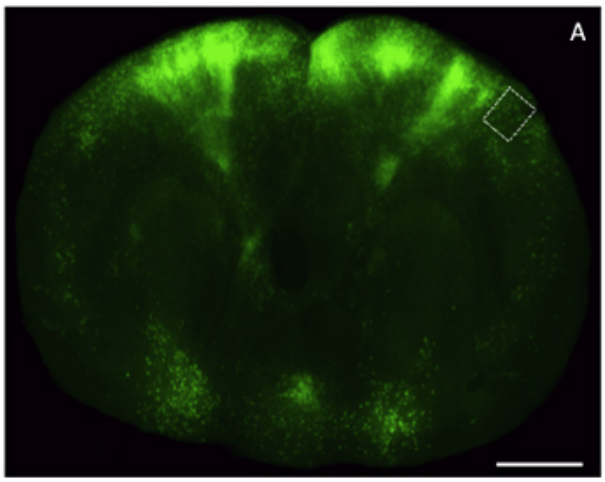

Figure 4. Light microscopy images of a thick slice from glycerol-cleared brain tissue through the frontal cortex expressing enhanced green fluorescent protein (EGFP). A) Coronal slice through a mouse brain (200 um thick) labeled with a viral vector expressing EGFP and imaged at low resolution using an epifluorescent stereoscope. Scale bar, 2 mm.

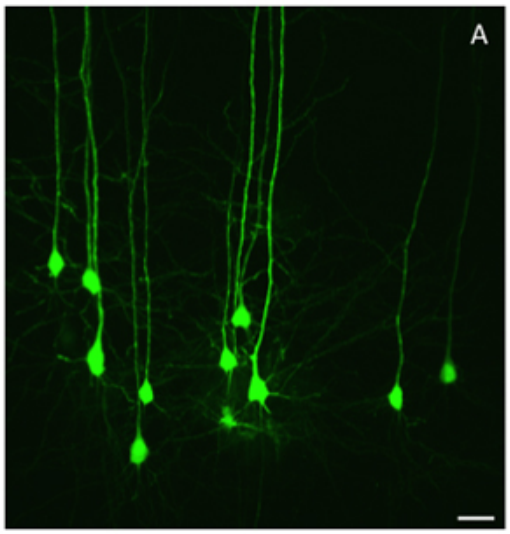

Figure 5. High magnification image of fluorescently labeled layer 5/6 cortical neurons in a cleared thick brain slice. A) High resolution confocal image of a maximum projection Z-stack (150 um thick) through the region highlighted in (Figure 4). Scale bar, 25 um.

Discussion

Given the widespread application of using fluorescent proteins to target neuronal subsets for investigation via light microscopy, the need to rapidly screen, image, and analyze neural networks within intact brain tissue has become invaluable.

Technical advances in the development of user-friendly viral vectors, *in vivo* electroporation techniques, and genetically modified mouse strains now provides a seemingly unlimited source of labeled cell types to investigate. However, image analysis of intact brain tissue still remains a bottleneck to experimentation. The method described here has proven to be extremely valuable for our routine analysis of brain tissue expressing fluorescent reporters. It significantly truncates the time needed to prepare and analyze tissue from brain sections, is compatible with both low-power and high-resolution fluorescence imaging, can be used in combination with immunocytochemistry, is translatable to other experimental animal models, and importantly, is broadly applicable to routine laboratory environments that may not have access to multiphoton imaging equipment. It is important to note however, that optical clearing with glycerol and other aqueous and organic solutions has practical limitations. Here we describe the use of glycerol to aid clearing of brain slices up to 200 um thick. Although this method works quite well to improve the imaging resolution in tissues up to 200 um, we do not see an improvement beyond this. Other organic solvents are capable of clearing tissue further, however there is always a trade off with the loss (or extraction) of fluorescent signal.

Possible variations of the method described above may include perfusion using perstaltic pumps, clearing with other aqueous or organic phase solvents that are compatible with FP fluorescence, automated slicing and serial imaging, or pairing with multi-photon micropscopy to allow imaging in even thicker brain slices.

Disclosures

Jian-Qiang Kong is an employee of Precisionary Instruments, Inc., that manufactures a product used in this article.

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