Video Article

Multiphoton Microscopy of Cleared Mouse Brain Expressing YFP

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Abstract

Multiphoton microscopy of intrinsic fluorescence and second harmonic generation (SHG) of whole mouse organs is made possible by optically clearing the organ before imaging.[1,2] However, for organs that contain fluorescent proteins such as GFP and YFP, optical clearing protocols that use methanol dehydration and clear using benzyl alcohol:benzyl benzoate (BABB) while unprotected from light[3] do not preserve the fluorescent signal. The protocol presented here is a novel way in which to perform whole organ optical clearing on mouse brain while preserving the fluorescence signal of YFP expressed in neurons. Altering the optical clearing protocol such that the organ is dehydrated using an ethanol graded series has been found to reduce the damage to the fluorescent proteins and preserve their fluorescent signal for multiphoton imaging.[4] Using an optimized method of optical clearing with ethanol-based dehydration and clearing by BABB while shielded from light, we show high-resolution multiphoton images of yellow fluorescent protein (YFP) expression in the neurons of a mouse brain more than 2 mm beneath the tissue surface.

Video Link

The video component of this article can be found at http://www.jove.com/video/3848/

Protocol

1. Animal Perfusion[5] and Whole Mouse Brain Clearing

1. The entire length of the procedure can vary depending on the length of time used per dehydration step, but in total the whole process can be conducted in two days.
2. Weigh YFP mice and then deeply anesthetize with an intraperitoneal injection of ketamine/xylazine (100 mg/kg:10 mg/kg).
3. Confirm a surgical plane of deep anesthesia before proceeding to surgery. Check the animal every 5 min to see if it reacts to a firm toe or tail pinch. If the animal reacts, a supplemental dose (1/3 of original dose) of ketamine/xylazine is required.
4. Once deeply anesthetized, restrain the mouse by adhering each limb to a surgical bed using lab tape so that the mouse is in a supine position (dorsal recumbancy), exposing its chest for surgery. The surgical bed is usually made of a metal or plastic mesh and placed into a sink or on top of a lipped-pan so that blood and fixative waste can easily be collected.
5. To begin, make an incision below the xyphoid process. Cut along the base of the rib cage using scissors and tweezers and pull back skin as the cut is made. Make two cuts along either side of the mouse sternum (through the ribs) to create a flap of tissue that is held away from the chest cavity using a hemostat to leave the heart exposed.
6. Insert a 23 g needle into the left ventricle of the heart and make a small incision in the muscle wall of the right atrium to allow blood to escape. Please refer to JoVE article 2497 for a video of this procedure.[5]
7. Immediately after the right atrium is cut, begin a perfusion with 4 °C phosphate buffered saline, PBS, (pH 7.2) until blood is no longer observed draining from the right atrium of the heart (30 - 40 ml at a rate of about 5 ml/min).
8. Once all the blood has been drained (fluid exiting the right atrium is clear), switch the perfusion medium to a chilled 4% PFA solution. Perfuse until the mouse's body becomes noticeably rigid and cold to the touch (approx. 30 - 40 ml at a rate of about 5 ml/min). (Concentrated 16% PFA solution is diluted according to manufacturer's instructions and NaOH is added until pH 7.2 is reached. Wear gloves and a lab coat and handle and mix chemicals inside a fume hood.)
9. After perfusion, remove the mouse from the surgical bed and decapitate to begin the excision of the brain.
10. Using forceps and iris scissors, remove the skull in small sections starting from the back of the skull and moving forward. Make small cuts every 2 - 4 mm with the scissors across the skull while using the forceps to carefully pull the bone away from the brain in small sections. Do this until the entire top surface of the brain is exposed.
11. Excise the brain from the skull using a 5 mm wide, flat spatula and place into a glass vial. Submerge in 4% PFA for 6 hr at 4 °C for post fixation.

12. After post fixation, wash the brain twice in room temperature PBS by pouring the PFA out of the glass vial and replacing it with PBS. Swirl the brain in PBS solution before pouring out and replacing with PBS for a second washing.
13. Dehydrate the brain at room temperature by a graded series of ethanol incubations (once in 50%, 70%, 95%, and twice in 100%) at 2 hr per incubation, and then 12 hr for the second 100% ethanol incubation, to extract the water from the fixed tissue. For each incubation, pour out the previous ethanol solution from the glass vial and replace it with the subsequent solution until the brain is completely submerged.
14. After the second incubation in 100% ethanol, pour out the solution and replace with equal parts ethanol and the clearing solution containing benzyl alcohol and benzyl benzoate (1:2 vol:vol ratio). After 2 hr of incubation, decant this solution and replace with a 100% solution of benzyl alcohol and benzyl benzoate (1:2 vol:vol ratio). The refractive index of the clearing solution is n = 1.54.
15. Once in BABB, the brain will become noticeably transparent, within 4 - 5 hr. For best clearing results, leave the brain to clear for 6 days at room temperature while shielded from bright light.

2. Microscope Setup

1. Once the brain is cleared and ready for imaging, affix it to the bottom of a Petri dish using cyanoacrylate. Allow the adhesive to dry before proceeding.
2. After drying, submerge the brain in BABB and place the Petri dish under the microscope objective for imaging.
3. We use a multiphoton microscope that incorporates a Mai Tai titanium sapphire laser adjustable between a 710 nm to 990 nm excitation wavelength. The excitation wavelength we use to generate YFP signals is 886 nm. Laser power varies from 30 - 100 mW depending on imaging depth.
4. Capture the reflected fluorescent signal using a Nikon 5X objective (NA, 0.5) that allows for large field-of-view imaging (2 x 2 mm).
5. Filter the reflected fluorescent signal using a 535/50 bandpass filter and collect it using a GaAsP PMT (H7422PA-40, Hamamatsu, Bridgewater, New Jersey).
6. Process images using ScanImage software[6] at a resolution of 2048 x 2048 pixels using a scan rate of 2 ms per line to generate high-resolution, YFP images.
7. Once imaging is complete, remove the brain from the Petri dish using forceps and store in BABB shielded from light for future imaging. For glued samples, remove the tissue by running a razor blade between the glue and the Petri dish at an angle no greater than 30 degrees. We have stored samples in BABB for up to 1 year with no visible degradation.

3. Representative Results

The representative images and videos shown here demonstrate the high-resolution multiphoton imaging capability made possible by optical clearing. Whole brain imaging allows for YFP-labeled neurons in the different layers of the hippocampus and cortex to be clearly visible as deep as 2 mm below the tissue surface. **Figure 1** shows a representative image of a coronal view corresponding to the anatomical features of the hippocampus at 2.92 mm caudal to bregma[7]. The depth of imaging in the sample was 1.94 mm. Some of this difference was due to removal of the cerebellum at the caudal end of the brain and the balance was due to shrinkage from the dehydration process. Another image from the stack shows layer V/VI pyramidal neurons of the neocortex expressing YFP 2 mm beneath the tissue surface (**Figure 2**). All images were acquired using the Nikon 5X objective and magnification was done using the digital zoom feature for image acquisition in ScanImage software.

By zooming in on the neocortex, the individual axons and neuron cell bodies of pyramidal neurons in Layer V of the neocortex are clearly distinguishable up to 1.02 mm beneath the tissue surface (**Figure 3**). Using the image stack from **Figure 3**, a 3D reconstruction of the neuron region was made using ImageJ software[8] (**Figure 4**).

The high-resolution capability of MPM also allows us to present images of whole, individual neurons. Here we show a reconstruction of a Layer V pyramidal neuron of the neocortex in which individual dendritic processes are clearly visible (**Figure 5**).

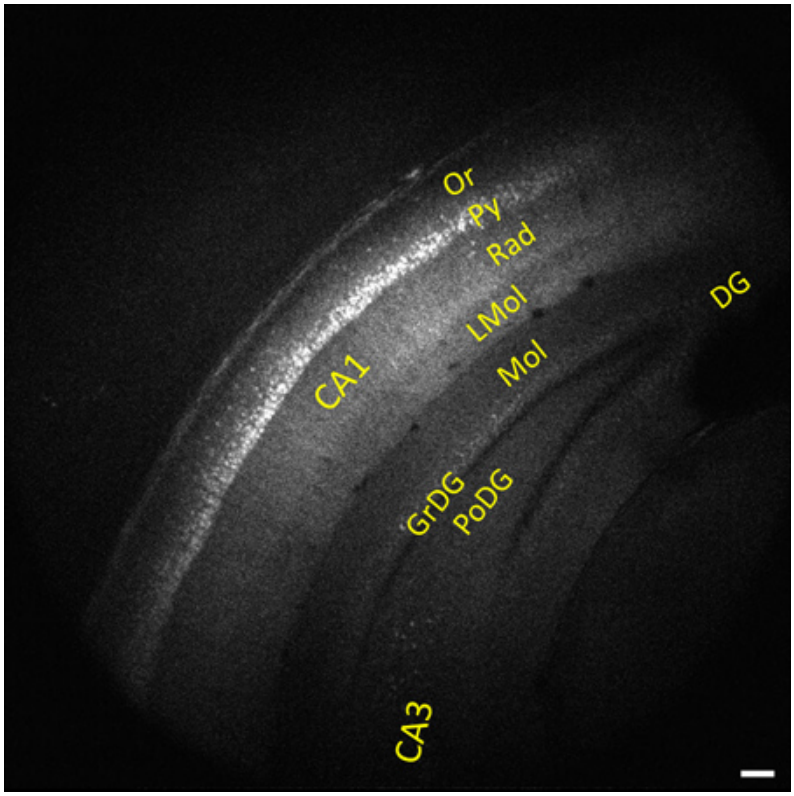

Figure 1. Representative image from a 1.2 mm image stack (0.8 - 2 mm beneath the tissue surface) of whole mouse brain showing neocortex and the different layers of the hippocampus. Features of the hippocampus are visible 1.1 mm below the tissue surface and have been labeled using the following code: DG = dentate gyrus, GrDG = granular layer of DG, Lmol = lacunosum moleculare layer, Mol = molecular layer DG, Or = oriens layer, PoDG = polymorph layer DG, Py = pyramidal cell layer, Rad = stratum radiatum.[7] Image is 1.8 x 1.8 mm in size, scale bar = 200 µm. The rostral end of the brain was affixed to a Petri dish with the caudal end facing up toward the objective. This facilitated imaging in the coronal plane.

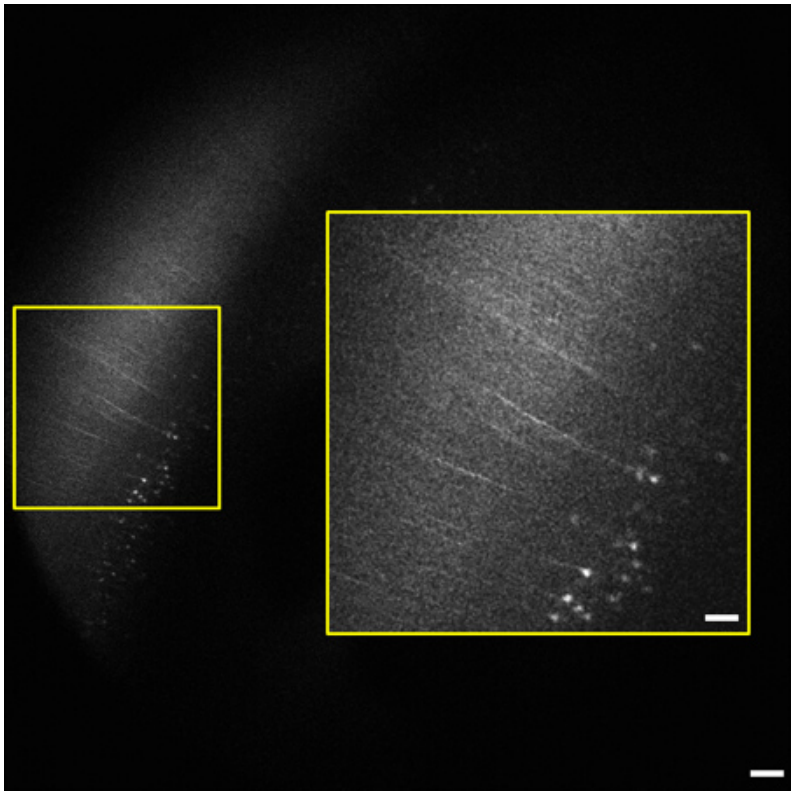

Figure 2. Representative frame from the 1.2 mm image stack (0.8 - 2 mm beneath the tissue surface) of whole mouse brain highlighting the neocortex. Pyramidal cells and processes expressing YFP in layer V/VI of cortex are visible 2 mm below the tissue surface. The degree of labeling is consistent with previous reports of the sparse labeling in the neocortex.[9] Inset (right) is enlarged from boxed area on left. Image is 1.8 x 1.8 mm in size, scale bar = 200 μm (50 μm inset).

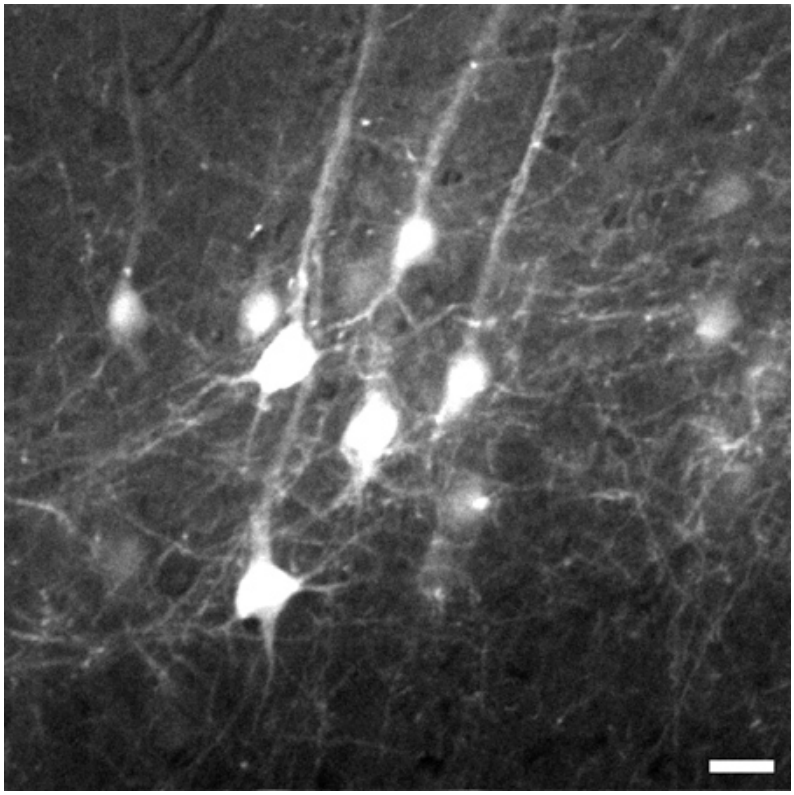

Figure 3. Representative frame taken 774 µm beneath the tissue surface from the image stack of layer V pyramidal neurons in the neocortex of the brain. Stack goes from 700 µm to 1,020 µm beneath the tissue surface. An 8X digital zoom was used to capture details such as fine processes. Image is 225 x 225 µm in size, scale bar = 20 µm.

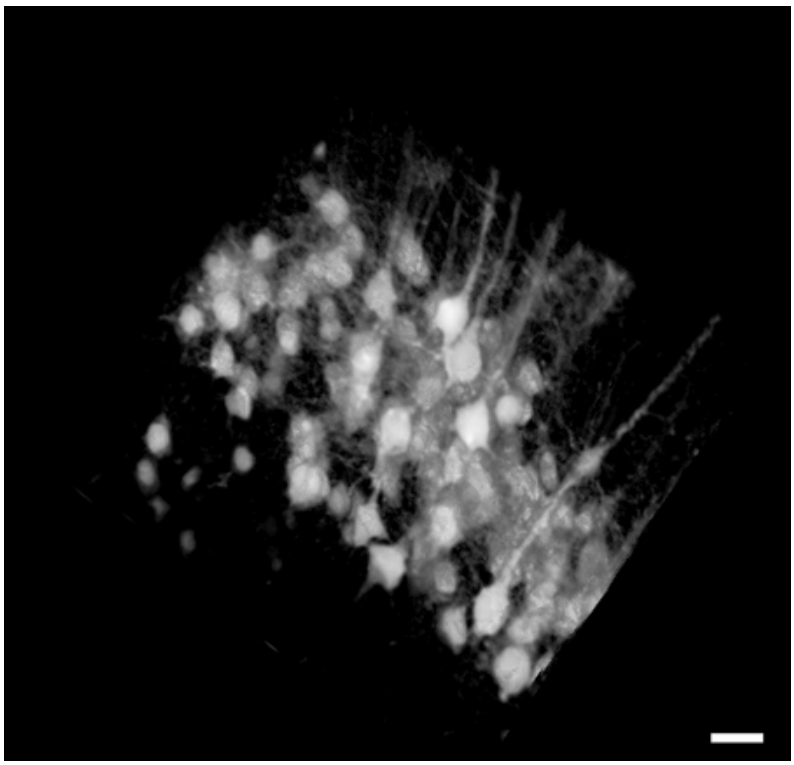

Figure 4. Representative image of 3D reconstruction (225 x 225 x 320 µm) of the image stack in **Figure 3**. Image is 225 x 225 µm in size, scale bar = 28 µm.

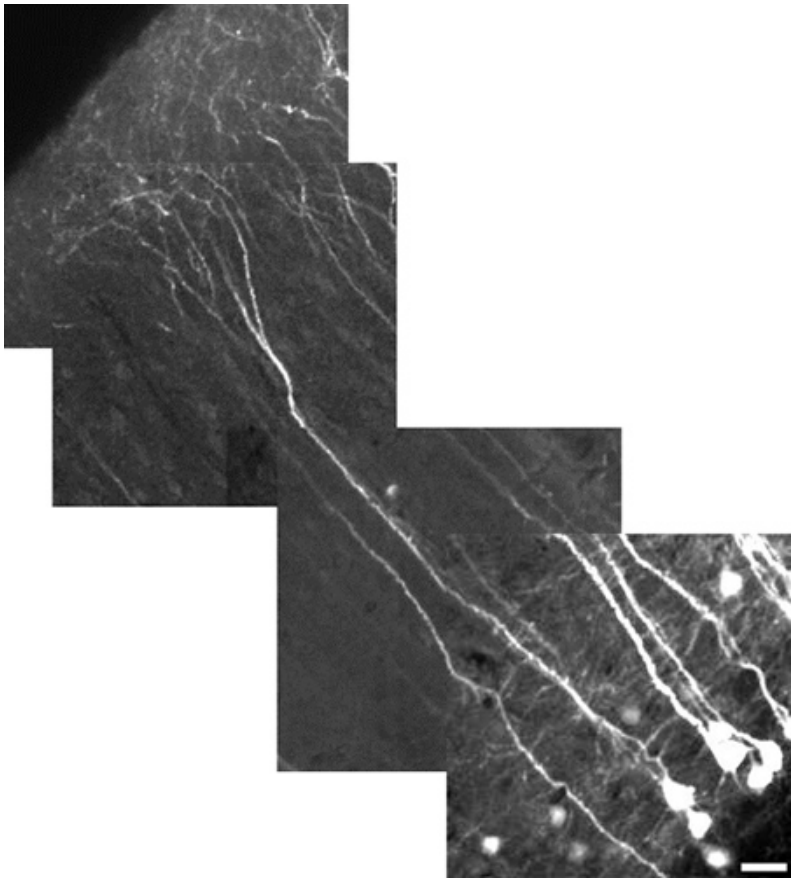

Figure 5. High-resolution reconstructed image of a layer V pyramidal neuron of the neocortex using a 10X digital zoom. Neuron measures 485 µm in length, scale bar = 25 µm. The field of view for each image tile is 180 µm x 180 µm. Each tile is at a different depth (z-level). The cortical apical dendrites do not, in general, express YFP as well as the soma. To have both in the same field of view, as shown here, the power was lowered to minimize saturation of soma; this makes the fine process look dimmer. Our emphasis here was on demonstrating field-of-view rather than fine detail. To reveal finer details, use a digital zoom, focus on a region without soma, and increase the laser power.

Discussion

While standard organic dyes are compatible with a range of organic solvents, and therefore do not pose a particular challenge for clearing protocols, fluorescent proteins are often less tolerant of changes in solvent.[4] The goal of the present work was to overcome a serious limitation of previous optical clearing protocols where fluorescence of XFPs was lost or severely degraded. The images that are presented here demonstrate that fluorescence from YFP was preserved. The optical clearing technique described here allows for high-resolution imaging of YFP-labeled neurons in whole mouse brain up to 2 mm beneath the tissue surface. Using ethanol dehydration, as demonstrated previously, will also preserve green and red fluorescent protein signals.[4] The images shown here demonstrate how this optical clearing technique can be applied to study whole organ regions, such as the hippocampus or neocortex that express fluorescent proteins. The high-resolution capability of multiphoton microscopy also allows for clear magnification of specific cells or areas of interest within the organ region. The key points for preserving protein fluorescence are to avoid over-fixation, to take care that the fixative and all solutions are maintained at close to neutral pH, to use ethanol rather than methanol for dehydration, and to store the samples in the dark.

Although we demonstrate here the use of optical clearing for imaging of mouse brain, the technique is generally applicable to most organs. In this study we showed high-resolution images of whole mouse brain regions (neocortex and hippocampus) and specific neurons within Layer V of the neocortex (The Thy1-YFPH mouse line labels pyramidal cells in the hippocampus and sparsely in the neocortex,[9] other regions appear dark.). Using the image stack of Layer V pyramidal neurons, a 3D reconstructed volume of the neurons was created. The ease with which these images were captured deep within the mouse brain at high-resolution after optical clearing makes this a powerful tool for studying neuroanatomy. However, while gray matter clears extremely well, imaging depth in brain was ultimately limited by incomplete clearing of dense white matter tracts near the center of the brain. This is not an issue in other organs.

One drawback of using BABB for clearing is that it is not compatible with conventional dipping objectives. The high refractive index of the solvent that enables clearing results in optical aberrations, and the solvent can dissolve the glue that holds the lenses in place in the objective. We therefore used a macro lens, the Nikon AZ PlanFluor 5X 0.5NA WD 15 mm, that combines an ~2-mm field-of-view with a lateral resolution of ~400 nm (1/e radius) and axial resolution of ~4.9 µm (1/e radius). The lens also has a correction collar to compensate for spherical aberrations. However, this lens has a 22-mm back aperture and therefore may not be easily adapted to standard commercial systems. One could machine a custom thread adaptor to enable mounting the lens onto a standard microscope, but care should be taken to ensure that the excitation beam

profile fills the back aperture as much as possible to make maximum use of the available numerical aperture. While this lens was able to focus through a relatively large depth (~2 mm) without immersion into the BABB solution, its 0.5 NA does not provide adequate resolution for imaging dendritic spines. A lens with a higher NA of at least 0.6 and ~40X magnification is capable of resolving dendritic spines. However, it must have sufficient working distance to avoid immersion in the BABB solution. Maintaining this distance will likely limit the depth of the z-stack image to well below the 2-mm depth available through clearing.

It should be noted that fixation and clearing can increase the signal from endogenous sources of fluorescence. This is best avoided by choosing longer-wavelength excitation (> 800 nm) and careful selection of emission filters to well match the fluorescence spectrum of the indicator dye.

Recently, a new urea-base clearing agent referred to as SCALE has been demonstrated to preserve the signal from fluorescent proteins[10], however, it takes 2 weeks - 6 months to clear a sample, compared with a few hours for BABB. SCALE also leaves the tissue very fragile, with large amounts of tissue expansion, and the use of urea, which denatures many proteins, is likely to be problematic for follow-up studies using immunohistochemistry. In contrast, although clearing process can result in some uniform tissue shrinkage (~20%), clearing with BABB has been shown to be compatible with standard histological processing[1,2].

Disclosures

Experiments on animals were performed in accordance with the guidelines and regulations set forth by Yale University's Institutional Animal Care & Use Committee.

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