A protocol for preparing GFP-labeled neurons previously imaged *in vivo* and in slice preparations for light and electron microscopic analysis

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Published online 16 July 2009; doi:10.1038/nprot.2009.114

***In vivo* imaging of green fluorescent protein (GFP)-labeled neurons in the intact brain is being used increasingly to study neuronal plasticity. However, interpreting the observed changes as modifications in neuronal connectivity needs information about synapses. We show here that axons and dendrites of GFP-labeled neurons imaged previously in the live mouse or in slice preparations using 2-photon laser microscopy can be analyzed using light and electron microscopy, allowing morphological reconstruction of the synapses both on the imaged neurons, as well as those in the surrounding neuropil. We describe how, over a 2-day period, the imaged tissue is fixed, sliced and immuno-labeled to localize the neurons of interest. Once embedded in epoxy resin, the entire neuron can then be drawn in three dimensions (3D) for detailed morphological analysis using light microscopy. Specific dendrites and axons can be further serially thin sectioned, imaged in the electron microscope (EM) and then the ultrastructure analyzed on the serial images.**

INTRODUCTION

This protocol describes the procedure for using light and transmission electron microscopy to locate and image axons and dendrites of neurons expressing green fluorescent protein (GFP) that had previously been imaged live, either in slice preparations[1,2] or anaesthetized animals[3–7] using 2-photon laser scanning microscope. The method uses an immunocytochemistry approach to show the imaged cells, and then a preparation protocol to embed them in resin ready for serial sectioning and imaging in the transmission electron microscope (TEM).

Imaging live cells containing GFP, or its derivatives, is a widely used method in many biological fields, and recent studies have extended this approach to enable the visualization of fluorescent cells in the brains of live animals. The technique uses 2-photon microscopy to detect the fluorescence contained within cells inside the live tissue. This permits real-time imaging with temporal resolution that allows for imaging over minutes, hours or days to see changes in structure (see accompanying paper by Holtmaat *et al.*[8]). These experiments have given the first insights into how the structure of neurons in the adult brain change on a day-to-day basis.

In vivo imaging, however, needs that only a small fraction of neurons is labeled. The limits of resolution make it impossible to separate, or even classify, apposed elements or visualize small subcellular structures like synapses. When observing the appearance of spines along the dendritic shaft or boutons along axons, it is impossible to interpret changes in fluorescence as alterations in synaptic connectivity with light microscopy alone. Combining these analyzes with electron microscopy of the same structure extends the understanding of the modifications seen. Imaging the same fluorescent structure in the electron microscope (EM) allows researchers to analyze not only the imaged cells, but also all the surrounding elements. GFP-labeled neurons, imaged on a daily basis, show a certain degree of structural change at the level of the dendritic spines[3,4,7,9] and axons[6]. Electron microscopy can localize these structures, identify synapses and the connected axonal boutons, as well as surrounding elements, e.g., astrocytes and unconnected neurites. The nanometer resolution also allows for the analysis of even the very small organelles, such as vesicles and ribosomes, essential additions for the interpretation of the changes seen *in vivo*.

The protocol described here can be used when electron microscopy is needed to analyse the ultrastructure of GFP, containing axons and dendrites that have been previously imaged in either the living brain or live slice preparations. It has previously been used in a number of studies[3–6,9]. However, in the case of *in vivo* labeling, only mice of the GFP-M line were used in which GFP is expressed under the Thy1 promoter and show fluorescence in a subset of layer V pyramidal neurons, and some in layer II/III[10]. The GFP in these mice is distributed in the cytosol and at a level that gives a strong immuno reaction and clear labeling. In the cultured slices, it has also been used on cells that have been transfected to produce GFP, and again the labeling was adequate[1]. However, clearly the levels of GFP can vary and weak fluorescence may not be sufficient to give enough signal after the immunocytochemistry that can be seen the thin serial EM sections.

The protocol includes details of how the same labeled axons and dendrites, as imaged *in vivo* can be identified after they have been embedded in resin ready for EM. We outline how the imaged regions are mounted onto a resin block and trimmed until only the required part remains, ready for serial sectioning. We explain our approach to serial sectioning of the resin block to obtain long series of thin sections, in which it is possible to find and image the needed axon or dendrite. Finally, we show how the serial images can then be processed so that morphometric measurements can be made of the neuronal structures that were imaged, or those surrounding them, in the live tissue.

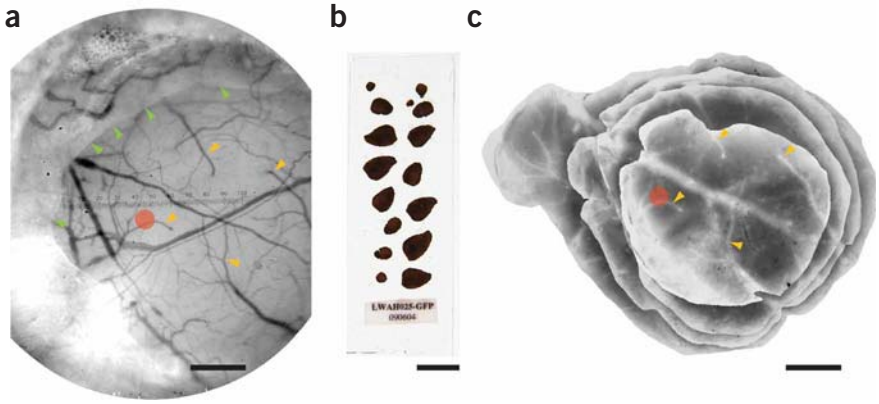

Figure 1 | Finding the location of the imaged cell in the resin embedded sections. (**a**) Photograph through the cranial window with the edge of the skull indicated by the green arrowheads. The blood vessels (some indicated with orange arrowheads) lying across the top of the brain are clearly visible where the skull is missing. After fixation, cutting and labeling sections are embedded on glass slides. The red spot indicates the site where the investigated neuron is positioned. (**b**) Serial vibratome-cut sections in resin. The sections were cut tangential to the brain surface, over the region where the cranial window was positioned. Once the sections are photographed with the light microscope (LM) and aligned (**c**), the same vessel pattern can be seen in these sections and also in the image of the live brain surface (**a**). Orange arrowheads indicate the blood vessels visible in both images, (**a**) and (**c**). The position of the imaged cell is known relative to the blood vessels (indicated with a red spot), which will, therefore, indicate where the cell can be found in the resin sections. Scale bars in **a** and **c** are 0.5 mm, and scale bar in **b** is 1 cm. All experiments involving animals were carried out according to institutional and national guidelines.

Experimental design

Experimental approach. When the regions of interest within the live brain have been imaged, a low-resolution image of the cortex below the cranial window is made using the 2-photon microscope objective, taking a series of images that are later stitched together to provide a view of most of the exposed region of the brain. A further image is then taken using a low power objective (×5) with transmitted white light, of the vessels below the cranial window, which will serve as landmarks to help locate the imaged cells (**Fig. 1**, panel a; and see accompanying paper by Holtmaat *et al.*[8]). The same vessels can be seen later on in the resin-embedded sections (**Fig. 1c**), and are essential for indicating where the cell of interest can be found.

With the animal maintained under deep anesthesia, it is perfused, the brain removed, vibratome sectioned and processed for immunocytochemistry. The sections are then stained for EM and embedded in resin. At this point they are imaged under the light microscope (LM) to locate the neuron of interest. This region is then serially sectioned for EM and the sections imaged at high resolution.

Methods to increase permeability of reagents in the tissue. To increase the density of staining, many immunocytochemistry protocols use detergents. These dissolve the membranes and improve the penetration of reagents into the tissue. This, however, will reduce the quality of the membranes, making them less defined with lower contrast. They should not be used in this protocol. Although very low concentrations of detergents are sometimes used in EM-immunocytochemistry protocols, they will greatly decrease the ability to follow labeled structures across serial sections, and should be avoided, if possible.

The process of freeze/thaw is an essential step for improving the permeability of the fixed tissue to the reagents, allowing the GFP containing axons and dendrites to be labeled through the entire thickness of the slice (**Fig. 2**, panel b). The process fractures the section giving the solutions greater access to structures deeper in the tissue. In the tissue containing GFP-expressing neurons, as used here, the labeling of axons and dendrites occurs through the diffusion of reagents along the inside of these neurites. This is evident by the weak labeling that occurs in neurites that lie deep in the section and parallel to its surface. However, where a crack in the

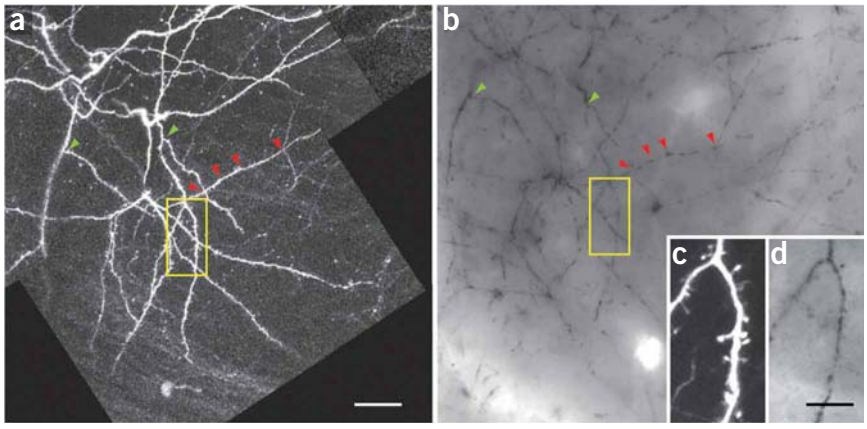

Figure 2 | Locating the imaged dendrite in the resin embedded sections. (**a**) A2-photon microscope image of dendrites of a layer V pyramidal cell shown through the cranial window. This image was made by tiling together high-resolution images of the cell to show the extent of the dendritic arborization in upper cortical layers, and helps to recognize the cell after the immuno-labeling. Each image was a projection of 60 images collected at 3-μm intervals in the *z* axis. (**b**) The same cell, imaged with light microscope (LM), but now vibratome-sectioned and embedded in resin at the point indicated by the red spot in **Figure 1**. The image is a projection of ten images collected at intervals of 3 μm. The same dendrite in **a** and **b** is indicated with a series of red arrowheads and the green arrowheads indicate two other dendritic branch points. The yellow box in **a** and **b** highlights the same dendritic fork that is shown in higher magnification in **c**. Imaging this region at a higher magnification (**d**) in the resin section, shows that the dendrite and some of its spines are clearly labeled with DAB along its length. Scale bar in **a** is 20 μm, and in **d** is 10 μm.

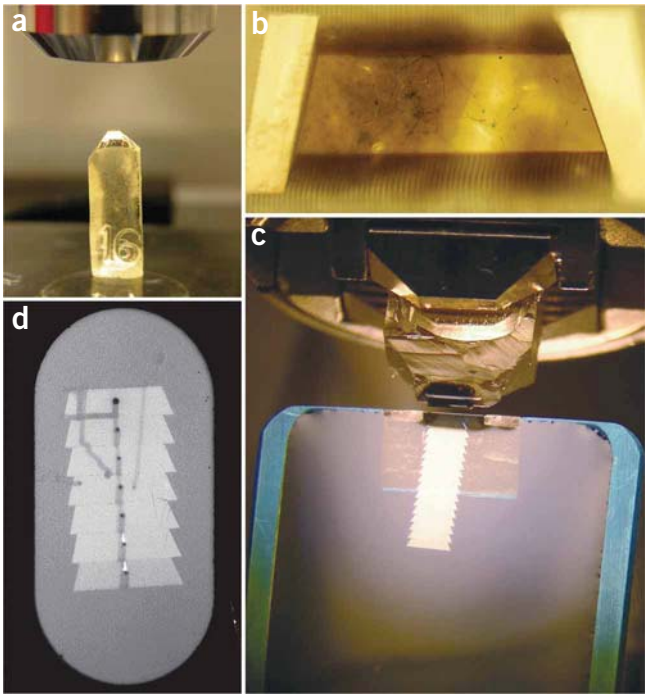

Figure 3 | Trimming and serially sectioning the region of interest. Trimmed resin blocks can be placed under a low power objective of a light microscope (LM) (**a**) to visualize the labeled cell in the resin block. This block is trimmed under a dissecting microscope and microtome, using glass knives, so that only the dendrites of interest are remaining in the center. (**b**) The block face imaged with the LM showing the trapezium shape of the block with the labeled neurites within (maximum width of this block is 600 μm). By focusing through the block, the distance between the block surface and the neurites of interest can be measured. This provides an estimate of how many sections must be cut to section the entire region of interest. (**c**) The diamond knife sectioning the block and the ribbon of sections aligned perpendicular to the knife-edge on the surface of the water. These sections are then collected onto single slot grids. (**d**) The aperture of a single slot grid illuminated with darkfield illumination. The oval shaped slot (width of 1 mm) has a formvar support film stretched across it on which the ribbon of sections were placed. These sections have been imaged with an electron beam that leaves a trace of its path. Dark spots within the trace indicate where the beam dwelt, and burnt the section, for a few minutes while an image was taken.

section breaks this structure it is more clearly labeled, especially close to the crack, decreasing further away from the open ends. Very thin axons will not be easily labeled, and many dendritic spines, with thin necks, will also show limited labeling, or none at all, because of the difficulty of reagents to diffuse along these thin tubes. However, this also means that the labeling of dendrites, inside a tissue slice (e.g., a cultured slice containing a GFP-expressing neuron), can be improved by simply cutting through part of the cell with a scalpel blade, taking care not to cut too close to an important imaged region. This will allow the reagents access to the dendrite through the cut ends, significantly enhancing the labeling.

Serial sectioning. Reconstructing imaged dendrites and axons, from electron micrographs is dependent on being able to serially section them in the resin block, and collecting them all on the slot grids. Cutting thin sections is more easily achieved with smaller block faces and these are typically a trapezoid shape with a maximum width of around 600 μm and a depth of 50 μm (**Fig. 3**). The final trimming is made with a glass knife to ensure that the sides are smooth to facilitate serial sectioning. For further details about serial-sectioning technique see also references 11 and 12.

Vibration and air currents will hamper the ability to cut long ribbons of sections, and their effects can be minimized with devices such as anti-vibration tables and enclosures around the microtome[7]. However, one very important, and often overlooked, factor is the level of static close to the knife and block. Static, and its effects on cutting, appear to be greatest when the humidity is at its lowest, often during very dry climatic conditions (it is always best to cut after it has rained, and the atmosphere is more humid). In this situation static can build up quickly, and only short series of sections are possible before they begin to separate from one another. One approach to reduce the static is to increase the humidity within the cutting room by using boiling water vessels in the immediate vicinity. A relative humidity measurement of 60% is often a good level for serial sectioning. Another method of reducing static is to use an antistatic device, consisting of a thin metal cathode, at high voltage, positioned close to the cutting knife and block. However, it is important that the device is switched off when the sections are collected due to the risk of electric shock, also the reduced static can dramatically change the behavior of the meniscus and affect the sticking of the sections to EM grids in the water bath. A reduction in static leads to the sections not adhering to the grid's support film, and sliding back into the water when the grid is removed from the bath.

Once the sections are collected onto the grid, the tweezers (reverse action) and grid are placed together on a hot plate maintained at 60 °C. It is important to leave the grid on this plate until it has dried as the center of the plate will always remain dust free. Once dry, the grids are placed quickly into a grid storage box ready for contrasting with uranyl acetate and lead citrate.

Electron microscopy. To avoid excessive distortions of the sections, caused by the heating effect of the electron beam, the support film is coated with a thin carbon layer. This makes the film more rigid; however, it is important that this layer is not too thick as it will also decrease the image contrast. The carbon layer is deposited before sectioning on the opposite to the one used for the sections. During carbon coating, the sections are placed on filter papers that will turn a very pale brown during this process. This is used to indicate a suitable thickness of the carbon coat, and is between 1 nm and 2 nm.

MATERIALS

REAGENTS

Fixation and immunocytochemistry-reagents

- 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma-Aldrich, cat. no. D5637-1G)
- Agarose (Chemie Brunschwig, cat. no. CEPAGA07-64)
- AntiGFP antibody (Chemicon, cat. no. AB3080)
- Avidin–biotin complex (Vectastain, Vectorlabs, cat. no. PK-6100)
- Biotin-SP-affinipure goat anti-rabbit IgG, F(ab′)2 (Jackson Immuno Research Laboratories, cat. no. 111-065-047)
- BSA-c (Aurion, cat. no. 900.022)

- DMSO (Sigma-Aldrich Chemie GmbH, cat. no. 41641) ! CAUTION Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Gelatin (Sigma-Aldrich Chemie GmbH, cat. no. G2500-100G)
- Glutaraldehyde (EMS, cat. no. 16222) ! CAUTION Very toxic if inhaled, comes in contact with skin and if swallowed. Wear suitable clothing, gloves and eye protection.
- Glycerin (Sigma-Aldrich Chemie GmbH, cat. no 49780)
- Hydrogen peroxide (Merck, cat. no. 107209) ! CAUTION Causes burns. Wear suitable clothing and gloves.
- Liquid nitrogen ! CAUTION Cryogen, causes severe burns. Use only in well-ventilated areas. Wear suitable clothing, gloves and eye protection.
- Osmium tetroxide (EMS, cat. no. 19110) ! CAUTION Very toxic if inhaled, comes in contact with skin and if swallowed. Wear suitable clothing, gloves and eye protection.
- Paraformaldehyde (EMS, cat. no. RT 19208) ! CAUTION Toxic if inhaled, comes in contact with skin and if swallowed. Wear suitable clothing, gloves and eye protection.
- Phosphate buffered saline (PBS)
- Phosphate salts for phosphate buffer (Sigma-Aldrich Chemie GmbH, cat. nos. 71642 and 71496)
- Propylene oxide (Sigma-Aldrich Chemie GmbH, cat. no. 82320) ! CAUTION Extremely flammable, toxic if inhalation, comes in contact with skin and eyes. Wear suitable clothing, gloves and eye protection.
- Tris base (TBS) (Sigma-Aldrich Chemie GmbH, cat. no. T1378) ! CAUTION Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Sodium cacodylate (Sigma-Aldrich Chemie GmbH, cat. no. 20840) ! CAUTION Irritant, toxic if swallowed. Wear suitable clothing and gloves.
- Sodium hydroxide (Sigma-Aldrich Chemie GmbH, cat. no. S8045) ! CAUTION Causes severe burns. Avoid contact with skin and eyes. Wear suitable protective clothing, gloves and eye protection.
- Sodium pentobarbitone (Sigma-Aldrich Chemie GmbH, cat. no. P3761) ! CAUTION Harmful if swallowed. Wear gloves.
- Uranyl acetate (EMS, cat. no. RT 22451) ! CAUTION Radioactive. When dealing with radioactive material, appropriate safety precaution must be followed.

EM embedding

- Ethanol ! CAUTION Flammable and toxic. Wear suitable clothing and gloves.
- Double-distilled water (dd H_2O)
- Durcupan resin: supplied in four separate parts (Sigma-Aldrich Chemie GmbH, Durcupan resin A/M, cat. no. 44611; B hardener, cat. no. 44612; D hardener, cat no. 44614; DMP 30 (EMS), cat. no. RT3600) ! CAUTION Toxic by inhalation, in contact with skin, and if swallowed. Wear suitable clothing, gloves and eye protection.

Serial section preparation, cutting and staining

- Sodium citrate dihydrate (Calbiochem, cat. no. 567446)
- Lead nitrate (Sigma-Aldrich Chemie GmbH, cat. no. 15334) ! CAUTION Do not breathe the dust, avoid contact with skin or eyes.

EQUIPMENT

- 24-well culture dishes (Nunc, cat. no. 142475)
- Forceps (Marcel Blanc, cat. no. BD220R)
- Glass scintillation vials (20 ml) (EMS, cat. no. 72634)
- Glass histology slides (Menzel-Gläser, cat. no. AA00008032E)
- Paint brush, size 6, artificial sable
- Perfusion pump (Witec AG, cat. no. PK100)
- Rodent heart clamp (Marcel Blanc et Cie, cat. no. BH020R)
- Surgical scissors (Aichele Medico AG, cat. no. 14110-15, 14088-10)
- Shaker table: (Rotamax 120, Hiedolph, cat. no. 544-41200-00)
- Vibratome (Leica VT1000S, Leica Microsystems)
- Wooden cocktail sticks
- Syringe with 23 gauge needle (Polymed Médical Center, cat. no. 03.341.01)

EM embedding

- Acetate sheets (clear plastic sheet used in overhead projectors).
- Glass scintillation vials 20 ml (EMS, cat. no. 72634)
- Glass histology slides
- Mold separating agent (Glorex, cat. no. 62407445)
- Tri pour beaker, 100 ml (EMS, cat. no. 60970)

Block preparation

- Cyanoacrylate glue
- Dissecting microscope (Leica MZ8, Leica Microsystems)
- Glass knife maker (Leica EM KMR2, Leica Microsystems)
- LM with digital camera (microscope: AxioPlan 2, Zeiss; camera: AxioCam HRc, Zeiss)
- Ultramicrotome (Leica UCT, Leica Microsystems)

Serial section preparation, cutting and staining

- Diamond knife: 45° ultra (Diatome)
- Deionizer (One Point Ionizer, Haug)
- Formvar 15/95 powder for making support film (EMS, cat. no. 15800)
- Grid support plate (EMS, cat. no. 71560-10)
- Grid storage box (EMS, cat. no. 71155)
- Hot plate (maintained at 60 °C)
- Reverse action tweezers (Biologie N5, Dumont)
- Single slot grids with formvar support film and carbon coated; slot dimensions, 2 mm × 1 mm (EMS, cat. no. GG2010-Ni)
- Ultramicrotome (UCT, Leica Microsystems)

Imaging and 3D reconstruction–LM

- Computer
- LM with a digital camera and a motorized stage interfaced with a Neurolucida control-unit.
- Software from Microbrightfield, Neurolucida program and Neuroexplorer

Imaging and 3D reconstruction–EM

- TEM with digital camera (microscope: CM12, Philips (now FEI); camera: Megaview III, Olympus)
- Computer
- Photoshop software (Adobe)
- Neurolucida software (Microbrightfield)

REAGENT SETUP

Fixative (4% wt/vol paraformaldehyde and 0.2% vol/vol glutaraldehyde in 0.1 M PB, pH 7.4) The fixative is to be prepared the same day and should not be stored for longer than 3 h. Heat 100 ml of dd H_2O to 65 °C and add 16 g of paraformaldehyde. Add two drops of 5 M sodium hydroxide and place on a heated stirrer. Add a magnetic bar and stir until the solution clears (~2 min). Add the solution to 200 ml of cold 0.2 M PB and then add 3.2 ml of freshly opened glutaraldehyde (25% vol/vol aqueous solution). Correct pH to 7.4, and then make volume up to 400 ml with dd H_2O. Leave to cool to 20 °C.

Osmium tetroxide 1% wt/vol in cacodylate buffer Add 10 ml of 2% aqueous osmium tetroxide (made by adding 1 g of osmium tetroxide crystals in 50 ml of dd H_2O) to 10 ml of 0.2 M cacodylate buffer. Can be stored for weeks at 4 °C.

Cacodylate buffer (0.2 M stock solution) Prepare 100 ml of 0.4 M solution of sodium cacodylate (8.56 g to 100 ml of dd H_2O) and add ~10.8 ml of 0.2 M HCl. Adjust the pH to 7.4 and make up the final volume to 200 ml. Can be stored at 20 °C.

Uranyl acetate (1% wt/vol in dd H_2O) Add 0.2 g of uranyl acetate powder to 20 ml of dd H_2O, and agitate gently until completely dissolved (~2 h). Can be stored at room temperature for more than 1 week.

Tris buffered saline (TBS; pH 7.4, 0.1 M) From a 1 M stock solution of Tris-HCl (121.1 g of Tris base in 800 ml of dd H_2O and adjusted to pH 7.4 with concentrated HCl, made up to 1,000 ml) take 100 ml and add 9 g of NaCl and 900 ml of dd H_2O. Can be stored at 4 °C for more than 1 week.

Tris buffered saline (TBS; pH 8.0, 0.05 M) From a 1 M stock solution of Tris-HCl (121.1 g of Tris base in 800 ml of dd H_2O and adjusted to pH 8.0 with concentrated HCl, made up to 1,000 ml) take 50 ml and add 9.0 g of NaCl and 950 ml of dd H_2O. Can be stored at 4 °C for more than 1 week.

Phosphate buffer (PB, 0.2 M stock solution) Prepare two stock solutions, A and B. A = 0.2 M sodium dihydrogen phosphate (NaH_2PO_4); B = disodium hydrogen phosphate (Na_2HPO_4). Take 19 ml of A and add 81 ml of B, and check that the pH is 7.4. Can be stored at 4 °C for more than 1 week.

Phosphate buffered saline (PBS, 0.01 M, 0.9% NaCl) To 50 ml of 0.2 M PB stock add 950 ml of dd H_2O and 9 g of NaCl. Can be stored at 4 °C for more than 1 week.

PBS/BSA-c Dilute 50 ml of 0.2 M PB with 50 ml of dd H_2O, and add 0.9 g of NaCl and 0.5 ml of BSA-c. Agitate gently for 1 h to ensure complete mixing. Can be stored at 4 °C for ~1 week.

15% wt/vol gelatin solution Mix 15 g of gelatin powder and add to 100 ml of 0.1 M PB and warm gently to 50 °C. Can be stored at 4 °C for ~3 d.

Cryoprotectant solution Add 2 ml of glycerin and 20 ml DMSO to 78 ml of PBS and agitate gently for 2 min to mix completely. Must be freshly prepared.

Lead citrate (Reynolds stain) Dissolve 1.33 g of lead (II) nitrate in 30 ml of dd H_2O and then add 1.76 g of sodium citrate dihydrate. Mix the milky solution well and then add 0.8 ml of 10 M NaOH and mix well until the solution clears. Make the volume up to 50 ml and store in a glass, light-tight container, under paraffin oil, at 4 °C, for as long as 3 months.

Durcupan resin Weigh 33.3 g of resin A/M, 33.3 g of hardener B and 1 g of hardener D into a plastic pouring flask. Stir continuously with magnetic stirrer for at least 30 min. Add 16 drops of DMP 30, and stir for a further 10 min. This can be stored in sealed 50 ml syringes at −20°C for ~2 weeks.

! CAUTION Resin should be prepared and used in a fume hood at all times.

Coated slides Dilute the mold separating agent 1:1 with dd H_2O and dip each slide into the solution until completely covered. Withdraw from the solution and place in a slide drying rack in a 60 °C oven for 24 h, until the varnish completely dries.

PROCEDURE

Fixation and preparation

1| For fixation of the whole brain, cultured brain slices or acute brain slices, follow options A, B or C, respectively.

(A) Fixing and preparing the whole brain ● TIMING **2.5–3 h**

(i) With the animal maintained under deep anesthesia, pin the forelimbs to the dissection board.

! CAUTION All experiments using animals should be carried out under institutional and national guidelines.

(ii) Using scissors and forceps open the chest cavity, exposing the heart and lungs.

(iii) Quickly insert the perfusion needle into the left ventricle gently pushing upwards and towards the aorta, then clamp in place using the heart clamp. Cut a hole in the right auricle.

▲ CRITICAL STEP Completing Steps 1A (I–III) must take less than 1 min.

(iv) Switch on the perfusion pump and perfuse the whole animal through the left ventricle, at 12 ml min^{-1}, with 300 ml minimum of 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 0.2% glutaraldehyde. The perfusion should take about 30 min to complete.

▲ CRITICAL STEP The rate of perfusion must not be too great as some of the weaker vessels in the circulatory system may burst, creating a low resistance circuit and decreasing the delivery of fixative to the brain. A useful check of perfusion quality is to check that the liver quickly turns light brown within a few seconds after the perfusion pump is started.

? TROUBLESHOOTING

(v) Leave the perfused animal for further 90 min, and then remove the cranial window assembly from the head of the animal and make three holes (1 rostral and 2 caudal) at the edges of the craniotomy with a 23-gauge needle that has been dipped in 2% wt/vol toluidine blue solution.

(vi) Remove the brain from the skull using the forcep and place in PBS solution. The needle holes should appear blue in the region where the cranial window was in place. Do not touch the region of the brain that was imaged.

(vii) Immerse the brain in 5% wt/vol agarose solution at 50 °C (in PBS and fully dissolved), so that the somatosensory cortex is uppermost and parallel to the top surface.

? TROUBLESHOOTING

(viii) Allow the agarose to solidify by cooling to room temperature.

(ix) Trim the block so that the region delimited by the blue needle holes is parallel to the upper surface of the block.

(x) Cut 60-µm thick vibratome sections in PBS, taking care to remove every section and place in the multi-well culture dish with PBS.

? TROUBLESHOOTING

(B) Fixing and preparing cultured slices ● TIMING **2.5–3 h**

(i) Immerse the slice in cold (4 °C), fresh fixative for 2 h, and then transfer them to PBS.

■ PAUSE POINT Slices can be stored in PBS at 4 °C for ~24 h.

(ii) To ensure that the labeled structures have an adequate exposure to the immunocytochemistry reagents, cut part of the labeled cell by dragging a fine scalpel blade across the slice, at a position that will sever a dendrite of the cell in question.

▲ CRITICAL STEP Take care not to cut too close to the region of interest.

? TROUBLESHOOTING

(C) Fixing and preparing acute slices ● TIMING **2.5–3 h**

(i) Immerse the slice in cold (4 °C), fresh fixative for 2 h, and then transfer them to PBS.

■ PAUSE POINT Slices can be stored in PBS at 4 °C for ~ 24 h, and during this time they can be re-sectioned.

(ii) Acute slices that are greater than 150 µm in thickness need to be resectioned. Place the slice on a flat copper block at 4 °C, and place two-glass histology slides either side to form two sides of a mold.

(iii) Pour warm 15% gelatin solution into the space between the slides to cover the slice and then place a small acetate sheet across the slides bridging the gap between them, ensuring that the liquid gelatin forms a flat surface over the slice.

(iv) Once cooled, the glass slides and acetate sheet can be taken away and a new razor blade is used to remove the slice, embedded in gelatin, from the copper block.

(v) Leave the block in 4% wt/vol paraformaldehyde in PB (0.1 M) for 30 min to further harden the gelatin block.

(vi) Glue the block, using cyanoacrylate glue, section uppermost, to the vibratome stage and cut sections cut at 60–80 µm thickness.

Immunocytochemistry ● TIMING 24 h

2| Wash sections in PBS for three changes of 5 min each change, agitating continuously, making sure that the sections are completely covered in solution at all times.

3| Change the PBS for the cryoprotectant of 2% vol/vol glycerin and 20% vol/vol DMSO in PBS and agitate continuously for 10 min.

4| Plunge each section separately in the liquid nitrogen, using the wooden cocktail sticks to pick them up. Hold them in the liquid until they freeze. Then, remove and plunge the sections back into the cryoprotectant until they completely defrost.
? TROUBLESHOOTING
▲ CRITICAL STEP This process causes a certain degree of fracturing in the tissue and considerably improves the labeling of structures deep in the slices.

5| Repeat Step 4.

6| Transfer sections to a solution of 0.3% peroxide in PBS and agitate for 10 min, ensuring the sections are covered in solution at all times.

7| Wash sections in PBS/BSA-c for three changes of 5 min each change and agitate continuously.

8| Incubate sections in primary antibody (1:500) with PBS/BSA-c overnight at 4 °C, agitating continuously, by placing them on a rotating plate.
? TROUBLESHOOTING

9| Wash in PBS/BSA-c for three changes of 5 min each, agitating continuously.

10| Incubate sections in secondary antibody (1:300) in PBS/BSA-c for 90 min, agitating continuously.

11| Wash sections in TBS (pH 8, 0.1 M, 0.9% NaCl) for three changes of 5 min each change.

12| Incubate sections in ABC solution (100 µl solution A + 100 µl solution B in 10 ml TBS, pH 8.0, 0.1 M) for 90 min agitating continuously.

13| Wash sections in TBS (pH 7.6, 0.05 M, 0.9% NaCl) for three changes of 5 min each change.

14| Incubate sections in TBS (0.05 M, pH 7.6, 0.9% wt/vol NaCl) with 0.04% wt/vol DAB and 0.015% vol/vol H_2O_2 for 20 min, at an ambient temperature of 21 °C. For higher or lower temperatures the incubation times will need to be adjusted.
? TROUBLESHOOTING

15| Wash sections in TBS (pH 7.6, 0.05 M, 0.9% wt/vol NaCl) for three changes of 5 min each change.

Embedding sections for electron microscopy ● TIMING 20 h

16| Using the wooden cocktail sticks, transfer sections to glass scintillation vials. About 20 sections can be placed in a single vial. For the remaining steps these vials are placed into a rotating mixer set to ~4 rev min^{-1}.

17| Wash sections in cacodylate buffer (0.1 M, pH 7.4) for three changes of 5 min each change.

18| Carry out secondary fixation and staining with 1.0% osmium tetroxide in cacodylate buffer (0.1 M, pH 7.4) for 40 min.
? TROUBLESHOOTING

19| Wash sections for 5 min in dd H_2O.

20| Stain sections for 15 min in 1% wt/vol uranyl acetate in dd H_2O.
? TROUBLESHOOTING

21| Wash sections for 5 min in dd H_2O.

22| Dehydrate sections in graded alcohol series 2 min each change (2 × 50%, 2 × 70%, 1 × 90%, 1 × 95% and 2 × 100%).
▲ CRITICAL STEP Do not allow the sections to air dry at any time during this step.

23| Replace the final alcohol with 100% propylene oxide for 5 min.

24| Replace half the propylene oxide with Durcupan resin and agitate gently by hand, taking care not to damage the now brittle sections, until the liquid has mixed completely. Rotate the vial continuously for 30 min.

25| Replace half the Durcupan/propylene oxide mix with Durcupan and rotate for 30 min.

26| Replace all the Durcupan/propylene oxide mix with Durcupan and rotate for a minimum of 6 h.

27| Replace with fresh Durcupan and rotate for 1 h.

28| Carefully remove each section from the resin, using the wooden cocktail sticks, and place into a glass microscope slide that has been coated with the mold separating agent. Once all sections are arranged on the slide, cover with another identical slide. Make sure there is enough resin to fill the gap between the slides, but not too much that may cause sections to drift out from between the slides.

29| Place section in 65 °C oven for a minimum of 24 h. When the resin has hardened the glass slides can be separated from the resin and sections between them with the use of a razor blade. The mold-separating agent prevents the epoxy resin from bonding to the glass.

■ **PAUSE POINT** Once the sections are resin embedded and the resin is hardened, they can be left at room temperature for any length of time until needed.

Localizing imaged dendrites in the resin sections using light microscopy ● TIMING 2–3 h

30| Photograph every resin-embedded section (**Fig. 1b**), using a low power objective lens (×1–×2.5) so that most of the entire section is seen in the single field of view, without having to stitch images together.

31| Using the Photoshop software, place the image of each section into a different layer in a single file (**Fig. 1c**).

32| Arrange each image so that the smallest section from the surface of the brain is situated at the top of the stack and the subsequent sections lie below (**Fig. 1c**).

▲ **CRITICAL STEP** Image alignment is helped by using the holes in the sections from the blood vessels as fudicial markers.

33| Place the bright-field image of the surface of the brain (**Fig. 1a**, taken with the low power objective using reflected white light, while the mouse is still immobilized in the 2-photon microscope setup) on top of the stack of images, aligning the blood vessels with those seen in the resin embedded sections.

34| Mark the position of the cell in the bright field image. This will also mark the position of the cell in the resin sections.

35| Draw the labeled structures in 3D using a computer controlled microscope and digital drawing system (Neurolucida, Microbrightfield, Williston, Vermont). Despite their staining with osmium tetroxide and uranyl acetate, the sections are transparent enough to see the detail of the labeled structures, e.g. axons and dendrites. It is also possible to use immersion oil on the sections for imaging at higher magnifications without affecting the resin. The oil can be removed afterwards with 70% alcohol.

Serial sectioning and imaging in the EM ● TIMING >2 d

36| Cut out the region of the section (2 mm × 2 mm), using a razor blade, containing the imaged cell, and stick to a resin block with cyanoacrylate glue.

? TROUBLESHOOTING

37| Trim the resin block with glass knives, in the ultramicrotome (**Fig. 3**).

▲ **CRITICAL STEP** To ensure that serial sections can be cut from the block face, it must be trimmed with new glass knives, and it must be 600 µm wide and 150 µm high. The imaged neurites must not be more than 10 µm below the surface of the block to avoid the need to cut many hundreds of sections before cutting the neurites of interest. A smaller block face is easier to serially section.

38| Draw the block with the labeled axons and dendrites using a LM with a drawing tube attached .

▲ **CRITICAL STEP** This drawing is used as a map to find the position of the labeled structure in the thin sections in the EM.

39| Serially section the block with a well-serviced, clean diamond knife, collecting sections onto carbon-coated single slot grids. Ensure that the block is well fixed in the block holder and does not protrude too far. Sections are cut at 60 nm (indicated on the ultramicrotome, and these have a silver-grey appearance when floating on the surface of the water bath, **Fig. 3**). Use the antistatic device to prevent sections folding into one another, or separating, and not forming a ribbon.

▲ CRITICAL STEP A thin carbon layer of less than 1 nm helps to stabilize the section under the electron beam and prevents the grids from breaking during the staining process. Single-slot grids are given a formvar support film that is first cast in water, where it has a silver-grey color.
? TROUBLESHOOTING

40| Load grids into the plastic grid-supporting plate, and then place into a petri dish.

41| Flood all the grids with dd H_2O using a Pasteur pipette and leave for 5 min. Then remove the dd H_2O ensuring that the grids are not exposed to the air, and flood the plate and grids with 1% uranyl acetate. Leave for 15 min.
? TROUBLESHOOTING

42| Remove the uranyl acetate making sure that not all the solution is removed so that the grids are not exposed to the air. Flood the grids with dd H_2O and then replace with lead citrate solution. At the same time place some sodium hydroxide pellets in the petri dish (to remove any carbon dioxide) and cover for 5 min.

43| Remove the lead citrate ensuring once again that the grids do not get exposed and flood with dd H_2O.
▲ CRITICAL STEP Throughout the staining procedure the grids must not be exposed to the air and plenty of dd H_2O must used to rinse the grids when the staining is complete.

44| Blot away all H_2O around the grids and allow them to dry for at least 1 h in a dry, dust-free chamber.

45| Remove the grids from the grid holder and place them back into the grid box.

46| Place the first grid into the EM and using a low power ($\sim\times$ 3,000) magnification, and with the camera lucida drawing of the block made immediately before cutting (Step 35), determine the position on the section where the labeled dendrite should be found.
▲ CRITICAL STEP It may take many hours to find the correct neurite, requiring many grids to be loaded in and out of the TEM. It is important that each time a grid is placed into the microscope it is first viewed at low magnification (less than $\times$3,000). This will bake the sections and render them more resistant to beam damage and distortion during subsequent imaging.

47| Continue to observe the same position on each section on this grid and each subsequent grid until the labeled structure is seen.

48| Photograph the labeled structure on each section using an approximate resolution of 4 nm per pixel.

Reconstructing the imaged neurites ● TIMING **> 2 d**
49| Using the Photoshop software arrange each image as a new layer in a single file. Using the transparency and product function on one layer at a time, align the superimposed images so that images from the same section can be stitched correctly together and the images from subsequent sections can be aligned one on top of the other.

50| Separate out the individual layers in the file into a series of image files, numbered in sequence.

51| Open image series in the Neurolucida software using the confocal module. Draw contours around each of the labeled structures that appear on each image in the series.
▲ CRITICAL STEP Make sure that before drawing any structures, the correct magnification (lens) is setup and selected.

● TIMING
Step 1, Fixation and brain sectioning: 2.5–3 h
Steps 2–15, Immunocytochemistry: 24 h
Steps 16–29, Embedding sections for electron microscopy: 20 h
Steps 30–35, Localizing imaged dendrites in the resin sections: 2–3 h
Steps 36–48, Serial sectioning and imaging in the EM: > 2 d (depending on the size of the imaged neurite)
Steps 49–51, Reconstructing the imaged neurites: > 2 d, depending on the number of images

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible Reason	Solution
4	Labeled axons and dendrites seen only within the first few microns below the surface of the sections	Insufficient membrane disruption in the freeze/thaw process	Increase the number of freeze/thaw cycles
8		GFP labeling too weak	Increase the amount of GFP expression
			Increase the concentration of the primary antibody and the incubation time with DAB
1Aiv, 1Bi		Tissue too well fixed	Decrease the glutaraldehyde concentration to 0.05%
1Aiv	Sections are cracked when resin hardens	Tissue poorly fixed before osmium staining	Ensure that perfusion setup is delivering fixative at a sufficient rate to the animal
1Ax		Vibratome sections too thin	Ensure the vibratome sections are not thinner than 60 μm
18		Sections not flat when osmium stain added to glass vials	As soon as the osmium tetroxide is added to the sections, swirl them around by hand for a few seconds ensuring that no sections stick to the side of the vial
1Avii	Impossible to identify the imaged dendrite in the resin-embedded sections	Vibratome sections were not cut parallel to the focal plane of the 2-photon microscope, and directly under the imaging window	Identify the three holes made in the cortex around the imaging window before the removal of the brain from the skull
18		Alignment of the sections does not clearly identify the vessels overlying the brain	Ensure that images of the resin sections show the entire sections, including the vessels at the very edges
1Ax		Impossible to align the *in vivo* image of the overlying blood vessels with the vessel pattern seen in the resin sections	Ensure that the sections at the very surface are collected and embedded in resin. Often the imaged dendrites are in the very first few sections cut from the brain, therefore, the smallest sections
		Labeled dendrites in the resin sections have been separated between two sections, and therefore the pattern of dendritic arborization is unclear	Ensure there are only a sparse number of labeled cells so their arborization patterns do not overlap. Select animals which only show a very sparse population of GFP-labeled neurons
		Too many labeled dendrites in the region that was imaged	See above comment
1Aiv	Ultrastructure of the membranes is too disrupted to allow dendrites and axons to be followed through the neuropil	Poor fixation because of poor perfusion	Ensure a sufficient flow of fixative to the animal, and check after fixation through the imaging window that no red blood vessels are present on the surface of the cortex
1		Glutaraldehyde not fresh enough when used for the fixative	Make sure that only glutaraldehyde that is stored in glass vials is used for the fixative. Do not use glutaraldehyde that is kept in re-sealable bottles
1		Fixative not freshly made immediately before fixation	Only use fixative that is made immediately before perfusion. Do not store the fixative in the fridge
		Detergent present in solutions before osmium fixation	Ensure that clean glassware is used at all times and no detergent residue is present
4		Incorrect concentrations of DMSO and glycerin in the cryoprotectant	When the sections are returned to the cryoprotectant, after being frozen, check they return to their normal shape. If not, the cryoprotectant has an incorrect concentration

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible Reason	Solution
39	Thin sections do not stay in a ribbon but float apart	Too much static in the vicinity of the diamond knife	Increase the humidity in the room to at least 65%. Increase the setting on the antistatic device to increase its ability to reduce static
		Block not held firmly enough	Ensure that only a very small part of the block protrudes from the holder. Remove the block from the holder reposition and reclamp firmly
		Diamond knife edge dirty or not sharp	Have the diamond knife checked for sharpness and cleanliness
36		Block edges are not smooth enough, or face too large	Reduce the size of the block face and ensure that a new sharp glass knife is used for the final trim
39	Support film holding the sections breaks in the electron microscope	Support film too thin	Ensure that the formvar support film is strong enough by testing section-less grids in the microscope prior to collecting sections
41		Sections contaminated with precipitate that creates hotspots	Ensure that sections do not come into contact with the air during the staining phase with uranyl acetate and lead citrate
39			Carbon coat the support film before collecting the sections.
39	Sections do not stick easily to the grids	Detergent present in the knife bath	Ensure that the diamond knife bath has been thoroughly rinsed with dd H_2O before sectioning
		Too much static in the vicinity of the bath	Increase the humidity in the cutting room to at least 60% and increase the antistatic setting on the antistatic device
18, 20	Contrast of the membranes is weak, and therefore difficult to identify axons and dendrites	Not enough staining of the tissue sections with osmium and uranyl acetate	Increase the staining time of the tissue sections with the osmium tetroxide and uranyl acetate
39		Support membrane is too thick	Ensure that the support membranes are tested in the electron microscope before using for collecting sections. Check that they do not restrict the illumination too much
		Too much carbon coating on the support film	Ensure that when carbon coating there is only the faintest shadow on the grids
41		Insufficient staining of the thin sections with uranyl acetate and lead citrate	Increase the grid staining times with the uranyl acetate
14	Difficult to see the DAB labeling within the imaged dendrite	DAB staining too weak	Increase the incubation time for the DAB reaction. Be careful to ensure that there is no increase in the non-specific background staining
8		Too much membrane contrast in all the neuropil, which decreases the contrast between stained and unstained elements	Increase the antibody concentrations. Be careful not to increase the background staining
4			Increase the number of times that the freeze thaw cycles are carried out
41	Difficult to identify the labeled dendrite in the serial sections		Reduce the on-grid staining with uranyl acetate and lead citrate to diminish the general membrane staining

DMSO, dimethyl sulfoxide; GFP, green fluorescent protein.

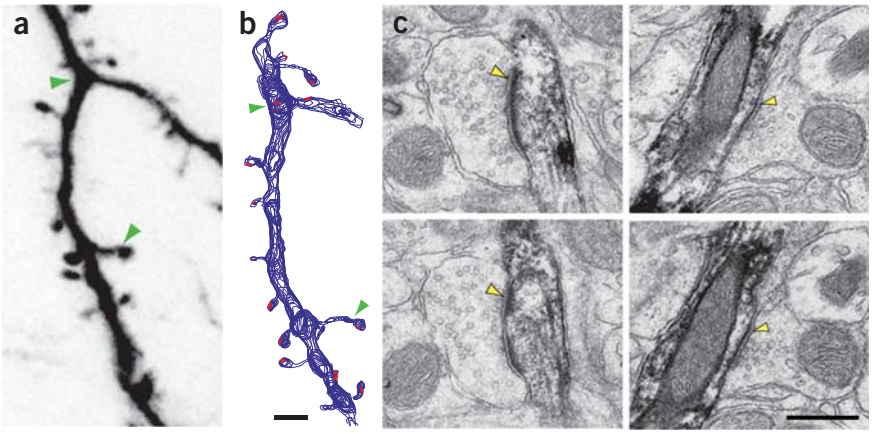

Figure 4 | Electron microscopy and reconstruction of imaged dendrite. The imaged dendrite (**a**) was serially sectioned and images were taken in the transmission electron microscope (TEM). The serial electron micrographs are first aligned using the Photoshop software and then the complete stack imported into the Neurolucida software so that the dendrite, its spines and synapses can be identified and reconstructed in three dimension (3D) (**b**, synaptic contacts are colored red). The reconstruction shows the complete dendritic structure, making it easy to identify the corresponding regions in the 2-photon image, e.g., the dendritic branch point and a dendritic spine indicated in both images with green arrow heads. This protocol will not heavily label the dendrites and obscure some of the features. This allows structures such as asymmetric synapses (**c**, left hand micrographs) and symmetric synapses (**c**, right hand micrographs) to be identified. Scale bar in **b** is 1 μm and in **c** is 0.5 μm.

ANTICIPATED RESULTS

The intensity of the staining after the immunocytochemistry can be assessed when the resin has polymerized. The embedded sections can be removed from between the glass slides with the use of a razor blade, and examined under a LM (**Fig. 2b**). The sections (**Fig. 1b**) have smooth upper and lower surfaces and the labeled cells within the sections can easily be seen. It can also be imaged at high magnification, with oil immersion objectives, using the immersion oil to stick the sections to a glass slide. At this point, the penetration of the labeling into the sections can be measured, as well as its density assessed in individual neurites. Axons and dendrites, seen clearly in these conditions, will normally have sufficient labeling to be easily detected in the EM (**Figs. 2,4**). Likewise, neurites that appear to be only weakly labeled, and are difficult to see in the LM, may be hard to identify in the EM. The level of staining throughout the axons and dendrites is never homogeneous and some regions will have more than others. Some part of the dendrites and some dendritic spines may even have no short stretches at all and these regions would, therefore, be invisible in the LM and impossible to locate in the EM. However, this can have its advantages for visualizing intracellular structures, as long as the correct neurite is identified.

It may take many hours to find the correct neurite, requiring many grids to be loaded in and out of the TEM. It is important that each time a grid is placed into the microscope it is first viewed at low magnification (less than ×3,000). This will bake the sections and render them more resistant to beam damage and distortion during subsequent imaging. It will also give the observer the chance to orientate the sections with the images that were made of the block face before sectioning (**Fig. 3**), to have a clear understanding as to where the labeled structures are in the sections. At this point, many labeled structures may be seen in the EM, so it is important to compare their position with the images of the block before it was serially sectioned. When the comparison between the EM images and those taken with the LM are made, the piece of axon or dendrite is usually identified. If not, then it will be necessary to measure the distance between the labeled structure, and the edge of the block in the LM images and make the same measurements in the EM images, to the edge of the sections.

When correctly identified, images are ready to be taken at a magnification of about 15,000 times (~3 nm per pixel). However, although the correct neurite is now identified, it is impossible in one EM thin section to say from which exact region of the *in vivo* image it corresponds. When a particular spine needs to be seen in the EM, it will often be necessary to reconstruct a large segment of dendrite before an uneqivocal identification can be made. Serial images must, therefore, be collected from as large a length of axon or dendrite as possible, and a reconstruction made, to avoid any ambiguity as to which region it corresponds to. Without characteristic landmarks, like dendritic branch points or groups of spines, it is not possible to compare the fluorescent images without the reconstruction.

During the initial phases of making the reconstruction it is common that some of the spines will not have been photographed entirely, or some may have been missed and are not included in the field of view. This happens often when spines do not contain the DAB reaction product. The reconstruction is then used as a map to find missed spines, and more images can be retaken and stitched together with the serial images to be used in constructing a complete 3D model.

ACKNOWLEDGMENTS This work was supported by the Swiss National Science Foundation (G.W.K. no. 3100A0-112335 and E.W. no. 310000-108245). The authors would like to thank Stéphanie Rosset for technical help in the development of this protocol.

AUTHOR CONTRIBUTIONS G.W.K., J.T.T., K.S. and E.W. conceived the strategy for carrying out the electron microscopy on the imaged neurites. J.T.T., A.H. and K.S. carried out all of the *in vivo* imaging. K.S. and E.W. provided equipment and reagents. G.W.K. carried out the electron microscopy and wrote the manuscript.

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