

Semiautomated correlative 3D electron microscopy of *in vivo*-imaged axons and dendrites

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This protocol describes how *in vivo*-imaged dendrites and axons in adult mouse brains can subsequently be prepared and imaged with focused ion beam scanning electron microscopy (FIBSEM). The procedure starts after *in vivo* imaging with chemical fixation, followed by the identification of the fluorescent structures of interest. Their position is then highlighted in the fixed tissue by burning fiducial marks with the two-photon laser. Once the section has been stained and resin-embedded, a small block is trimmed close to these marks. Serially aligned EM images are acquired through this region, using FIBSEM, and the neurites of interest are then reconstructed semiautomatically by using the ilastik software (<http://ilastik.org/>). This reliable imaging and reconstruction technique avoids the use of specific labels to identify the structures of interest in the electron microscope, enabling optimal chemical fixation techniques to be applied and providing the best possible structural preservation for 3D analysis. The entire protocol takes ~4 d.

INTRODUCTION

Visualizing the fine morphological details of cells can only be achieved with EM. With its nanometer resolution, EM gives greater structural context by revealing all the membranes and large stained macromolecules. When combining EM with *in vivo* light microscopy, the structural dynamics captured across various timescales¹ can be correlated with the underlying ultrastructural morphology. The synaptic connections between axons and dendrites, for example, can only be seen with EM. The growth of dendritic spines in the adult mammalian brain, seen with two-photon *in vivo* microscopy, has been verified as new synaptic connections by using *post hoc* serial section transmission EM^{2,3}.

Locating the same structure in images obtained using the two imaging methods has typically relied on transforming the label seen with light microscopy into an electron-dense stain that can be identified in the electron microscope^{2,4}. In the case of correlative *in vivo* light microscopy and EM of neurons⁵, the GFP present in the cytosol is stained using immunocytochemistry to produce the electron-dense precipitate of 3, 3'-diaminobenzidine tetrahydrochloride. After resin-embedding, the sample is then serially thin-sectioned, and imaged with transmission EM. This technique is labor-intensive, requiring high levels of skill to manually collect hundreds of sections followed by many hours of microscopy to locate and image the region of interest. These images are then aligned, again manually, and the analysis of the ultrastructure can then begin. As well as being time-consuming, this targeted ultrastructural analysis relies on having sufficient labeling in the thin sections to recognize the previously imaged structures. This is achieved by using weaker fixatives, and tissue permeabilization is used to improve the antibody penetration and strengthen the labeling. However, this results in suboptimal ultrastructural preservation.

FIBSEM produces large series of aligned images through tissue samples with limited human intervention^{6,7}. In addition, the sequential imaging and milling with the electron and ion beam results in isotropic image stacks from which structures such as axons, dendrites and synapses can be segmented automatically by using computer software.

This protocol describes how FIBSEM can be used to image volumes of brain tissue containing axons and dendrites previously studied *in vivo* in the adult mouse brain. With the microscope's ability to accurately target specific regions, no labeling of the fluorescent neurites is used. Instead, the neurites of interest are identified after the tissue has been chemically fixed and fiducial marks have been burned around this region with the two-photon laser by using the near-infrared branding (NIRB) technique⁸. The use of these NIRB marks to indicate the region of interest allows optimal fixation and staining methods to be used, giving well-preserved and contrasted cell structures.

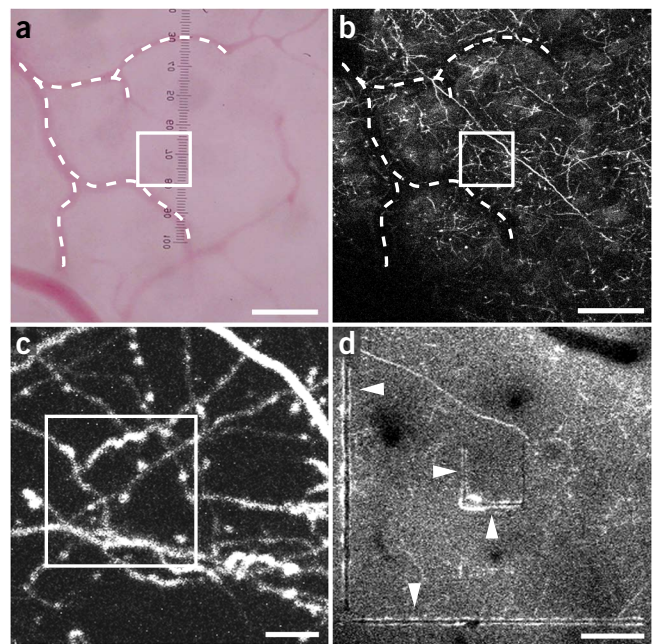
In comparison to the previous correlative method using transmission EM for analyzing *in vivo*-imaged neurites³, this approach with FIBSEM is more reliable as there is no handling of fragile ultrathin serial sections that may break or become contaminated during the staining process. The imaging occurs entirely in the microscope, and the entire series, acquired automatically, has near-isotropic voxels with which segmentation software can help to reconstruct the features of interest^{9,10}.

FIBSEM imaging does, however, have a limited field of view with a maximum imaging area of ~20 μm × 20 μm achievable when very precise milling depths across the entire face are required. The alternative serial block-face scanning method that uses a diamond knife to remove layers¹¹ can be used to image far larger areas (114 μm × 132 μm), although only to a minimum section thickness of ~25 nm (ref. 12). Both of these methods destroy the imaged region and thus give only a single opportunity to obtain the best image series. TEM, in contrast, is non-destructive, and the same region can be imaged repeatedly at different magnifications.

Experimental design

The procedure starts after the completion of *in vivo* imaging¹ of axons and dendrites. It describes how the tissue is prepared, and the same neurites that had been identified are then imaged with EM. The procedure can be applied to any of the fluorescent

Figure 1 | Two-photon *in vivo* imaging and fiducial laser-branding marks. (a) Blood vasculature of the cortical surface as seen through the cranial imaging window. (b) The pattern of the blood vessels (white dashed lines) is used to locate the region of interest in the two-photon image. (c) High-magnification image of tdTomato-labeled GABAergic axons and dendrites shown in the white square in b. White square in c indicates the region of interest. (d) After fixation and vibratome sectioning, the section containing the region of interest was identified, and a two-photon laser was used to burn fiducial laser marks (white arrowheads) around the structure of interest. Note that the TdTomato-positive neurites are not clearly visible at the end of the procedure owing to photobleaching caused by previous imaging. Scale bars are 50 μm (a,b), 5 μm (c) and 25 μm (d). Experiments using animals were carried out under institutional and national ethics guidelines.



structures that were imaged with light microscopy as long as they can be seen in the tissue after the fixation step.

Localizing the previously imaged neurites in the fixed tissue. For EM analysis of *in vivo*-imaged structures, the mouse should be imaged immediately before perfusion fixation and a low-magnification image of the brain's surface vasculature is acquired through the cranial window⁵ (Fig. 1). The image of the vasculature is an important map for locating the region where the neurons of interest can be found. This pattern of vessels is seen in the first tangential vibratome sections that are cut from the fixed brain at the position of the cranial window. These sections are photographed, the images are aligned and, by comparing these with the live image of the surface vasculature, the exact location of the imaged neuron is found. The identified section, containing the neurites of interest, is then imaged with the two-photon microscope, and a laser is used to burn fiducial NIRB marks, which will be visible with transmitted light microscopy once the tissue has been stained and embedded in resin. These laser-branding marks cause autofluorescence and need to be imaged, as soon as they are made, through the depth of the section so that their precise location is known relative to the required axons and dendrites. As the embedding process of this section will remove any remaining

fluorescence, these fiducial marks are crucial for correctly positioning the block for FIBSEM.

NIRB technique. Two sets of NIRB marks are made in the section. The first are burned at a depth of 4–6 μm above the neurites of interest (Fig. 1d), forming a square shape of 15–20 μm in length. The second set of laser marks are burned on the section surface as an asymmetric shape, 100 μm in length and around the first small laser marks. These second marks are then easily seen with a low-power ($\times 5$) objective in the transmitted light microscope. They serve to indicate the approximate location of the small marks, highlighting the position of the neurites of interest. The shape of these fiducial marks ensures that the orientation of the neurites is easily recognized during the block-making process, and their height in the section helps the block to be trimmed close to these features without removing them.

The original description of this laser-branding method used photoconversion of 3, 3-diaminobenzidine tetrahydrochloride to form a dense precipitate of the NIRB marks to help locate them in

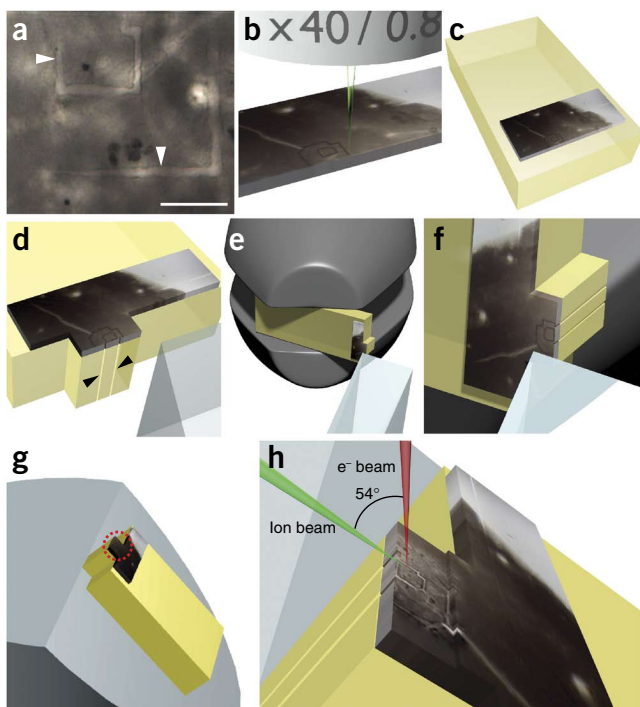
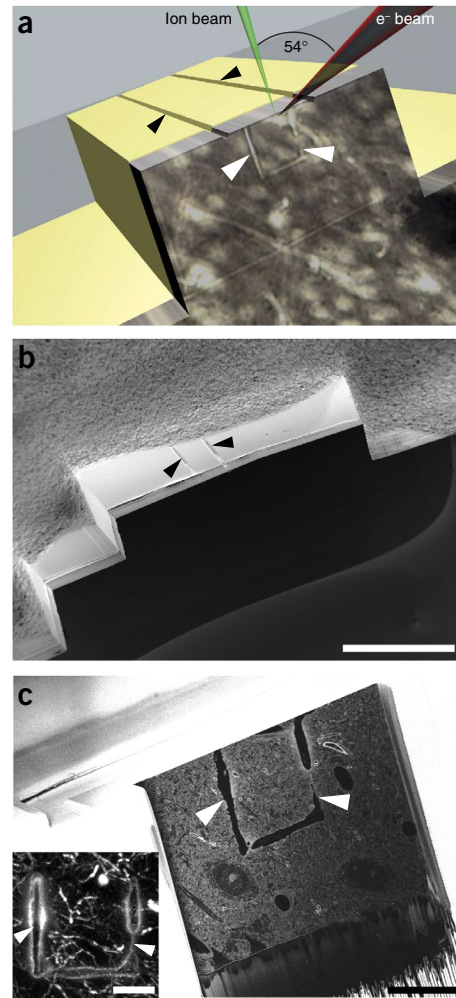


Figure 2 | Block preparation for the FIBSEM. (a) Fixed-vibratome section processed for EM and resin-embedded with clearly visible fiducial laser marks inside the tissue (white arrowheads). (b) A laser dissection microscope is used to replicate the original NIRB marks on the top surface of the resin-embedded sample to facilitate the trimming process. (c) The piece of resin containing the NIRB marks is cut from the section and glued onto a flat slab of empty resin. (d) One side of this slab is trimmed away until the NIRB marks lie at the edge. Steps are cut on either side of this region to expose the region of interest, and two score marks are made down this front edge (arrowheads) as additional fiducial marks to locate region of interest inside FIBSEM. (e,f) The block is positioned in the jaws of a ultramicrotome holder to allow excess resin, above the NIRB marks, to be removed with the glass knife. (g) The block is then glued on an inclined aluminum SEM stub and coated with a thin protective layer of gold (~30 nm). The red dotted circle indicates the position of the final trimmed block face (enlarged in h). (h) The stub is placed inside the FIBSEM, and the focused ion beam is oriented parallel to the block face, which is parallel to the plane of the NIRB marks. The electron beam is 54° relative to the milling ion beam. Scale bar in a is 25 μm . Experiments using animals were carried out under institutional and national ethics guidelines.



PROTOCOL

Figure 3 | Imaging the region of interest with FIBSEM. (a) A model showing the orientation of the block inside the FIBSEM with the electron and ion beams. (b) Secondary electron image of the block before milling (in the same orientation as the model in a). The score marks at the edge of the block are clearly visible (black arrowheads) (also in a) and indicate the position along the face at which the NIRB marks will be found (white arrowheads in a and c). (c) Back-scattered electron image of the milled face exposing the NIRB marks inside the resin (white arrowheads). The shape of these marks is also seen in the two-photon image of the same region in the fixed-vibratome section (inset). Scale bar in b is 500 μm , in c it is 20 μm and in c inset it is 10 μm . Experiments using animals were carried out under institutional and national ethics guidelines.



the resin⁸. This step is omitted here, as the marks are clearly visible in resin without further enhancement (Fig. 2a). However, the sections are cut no thicker than 60 μm . Thicker sections cannot be adequately illuminated in the light microscope once they have been stained and resin-embedded. If the sections are too thick, the densely labeled tissue will obscure the NIRB marks.

The chemical fixation of the fluorescent tissue also renders any fluorescent structures susceptible to bleaching. Therefore, it is important that all imaging be performed as rapidly as possible to acquire information about the positions of these structures in relation to the NIRB marks as well as in relation to other structures such as cell bodies and blood vessels. As the smaller NIRB marks are the most crucial, these need to be made first to decrease the chances of fluorescence bleaching. As fluorescence is also attenuated by the fixed tissue, the image quality may be poor. However, it is only necessary to acquire images that show the position of the neurites in relation to the marks. The neurites themselves do not need to be identifiable at this stage.

Preparation for FIBSEM. The EM embedding part of the protocol produces highly contrasted membranes. However, although there are many variations of stains that can be used, this particular recipe will still allow the NIRB marks to be seen easily in the transmitted light microscope. If more contrast were needed, then it would be necessary to cut thinner vibratome sections and increase the membrane contrast by increasing the staining times. However, these sections will be more fragile and break more easily during resin-embedding.

Once the resin has hardened, the block containing the section is then trimmed and is ready for FIBSEM. To help with this trimming process, a dissecting laser is used to etch the surface of the resin to indicate the position of the NIRB marks in the embedded tissue below (Fig. 2b). These surface marks are easily visible under the stereomicroscope and act as guides when this region is cut away from the rest of the section to be mounted on a 1-mm-thick resin slab (Fig. 2c). This provides a solid support and allows the sample to be easily manipulated when it is mounted on the ultramicrotome. The etched marks on the surface can be seen at all times during the trimming process and help to avoid accidental removal of the required region.

The goal of the trimming process is to expose the smaller NIRB marks to the point where they lie at the edge of the block and are within 3–4 μm of its surface (Fig. 2d–f). This is achieved by completely removing all the resin from one side of the sample up to and including the edge of the smaller NIRB marks. This creates one side of the block at 90° to the plane of the section (Fig. 2d). Next, the top surface of the sample is trimmed away, parallel to the section, a few micrometers at a time, until the NIRB marks are 3–4 μm

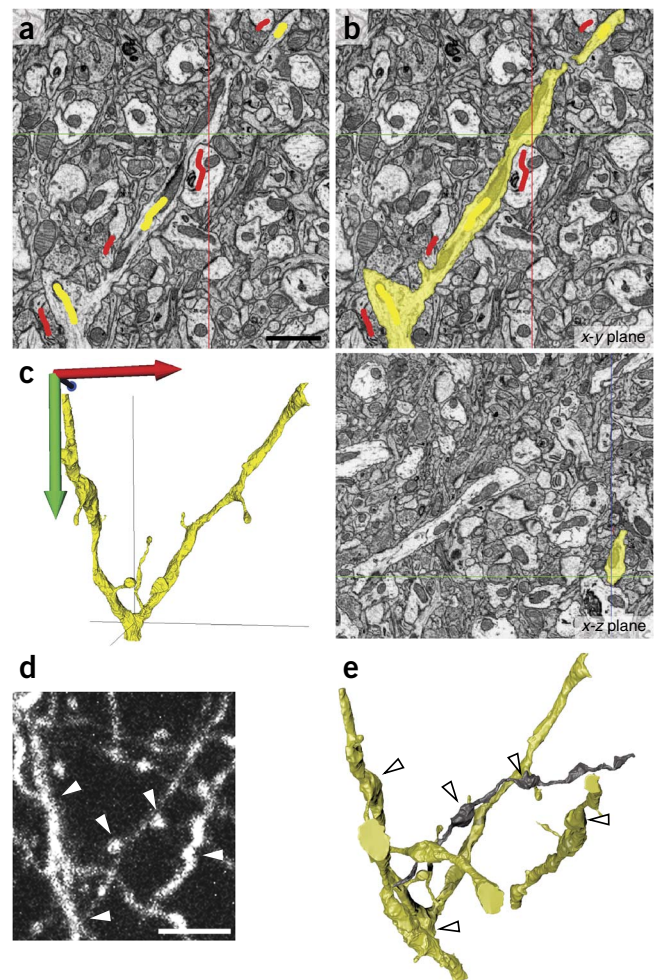
from the surface (Fig. 2e,f). The precision of this trimming process helps to minimize the time for which the focused ion beam is used to mill away material before any imaging is performed.

Throughout the trimming, a transmitted light microscope is used to repeatedly check the position of the NIRB marks inside the block. At this point, any other features in the tissue, such as blood vessels, lying close to the region of interest are noted. These can be useful fiducial marks that may appear during the initial preparation with FIBSEM and are used as indicators of where the marks lie.

The final block is then mounted onto a 45° inclined aluminum SEM stub (Fig. 2g). This angle means that the sample is pre-tilted in the microscope, with the microscope stage being used to attain the final exact position for imaging.

FIBSEM approach. The block is imaged inside the focused ion beam (FIB) scanning electron microscope (for example, Zeiss NVision 40) with the FIB milling the face parallel to the NIRB marks and, therefore, the plane of the section (Figs. 2h and 3a,b). The initial imaging face made by the ion beam, with a milling current of 1.5–13 nA, is much larger (60–80 μm) than the region of interest. This quickly reveals the underlying tissue. At this point, the coordinates measured during the block-preparation phase, which pinpoint the exact position of the underlying NIRB marks, are used to determine where imaging will occur. As the marks lie close to the edge of the block, the protective 1- μm -thick layer of platinum (or carbon) can immediately be deposited on the

Figure 4 | Segmentation and 3D reconstruction. **(a)** The ilastik program was used for interactive seeded watershed segmentation of the structures of interest. An image from the stack shows yellow marks made by the user to indicate the structure of interest (object) and red marks for the background. **(b)** On the basis of these ‘seeds’, the segmentation process proceeds automatically through the entire image stack. *x-y* (top) and *x-z* (bottom) views of the segmented piece of dendrite (in yellow). **(c,d)** The 3D model is then generated automatically **(c)** and can then be compared with the *in vivo* image **(d)**. **(e)** The final 3D model of the tdTomato-labeled GABAergic axons (gray) and dendrites (yellow) can then be rendered and visualized with Blender rendering software. Scale bar in **a** is 1 μm , and in **d** it is 5 μm . Images in panel **b** are at the same scale as panel **a**. Experiments using animals were carried out under institutional and national ethics guidelines.



top face by using a gas-deposition system. Here, a precursor gas is ionized with the FIB to leave a metal deposit on the surface below. This layer serves as a hard, even crust along the top edge of the imaged face that ensures even milling. This reduces milling artifacts such as the curtaining effect, which is seen as vertical streaks running down the imaging face. Once the exact position of the imaging window is selected (Fig. 3c), the FIB is then used to make the final polish at a low milling current of ~ 700 pA. The machine is then left unattended for ~ 2 h to allow any thermal or mechanical drift to dissipate.

A series of images is collected automatically from the FIB scanning electron microscope during a single run. Any interruption to this imaging procedure will cause irregular thicknesses to be removed by the milling beam. It is crucial, therefore, that the microscope be left undisturbed as much as possible, with only brief checks of the focus and astigmatism. The imaging parameters used are magnifications of ~ 5 – 6 nm per pixel, at an acceleration voltage of 1.3–1.5 kV and with a current of between 800 pA and 1,000 pA. The dwell time is optimized to maintain the milling/imaging cycle to below 2 min. This allows consistent milling depths as the block face reaches a steady-state thermal equilibrium. The FIB is set to mill 5–10 nm from the surface of the block after each image. However, because of a certain amount of thermal drift of the sample forward or backward, which can alter the section thickness, the actual distance between each image is calculated after imaging is finished by using the cylindrical diameters method¹³. Briefly, the section thickness is calculated by dividing the number of images across which a longitudinally milled mitochondrion is seen by its maximum diameter. This is repeated through the entire stack on >40 mitochondria.

Image segmentation and 3D reconstruction

Although FIBSEM can be used to remove thicker layers from the imaging face during each cycle, and therefore capture volumes faster, it is important to acquire images with small *Z* steps. This optimizes the segmentation performance of the algorithms

used to reconstruct the neurites, speeding up the reconstruction process with fewer errors. By using the ilastik program^{9,10} (<http://ilastik.org/>; Fig. 4), the different axons and dendrites are selected by scribbling their inner regions on a single image (intercellular space and organelles), and also by indicating the background or the regions that are not part of the reconstruction. The program grows these seeds until they fill the entire volume within less than a second. The resulting segmentation can then be refined or corrected by placing additional seeds at any time. To indicate how quickly reconstruction can be completed, the total time to reconstruct the volume of $15.00 \mu\text{m} \times 15.00 \mu\text{m} \times 13.10 \mu\text{m}$ and dendrites and axons in a stack containing 1,505 images was 2 h. The final 3D model can then be exported to 3D modeling software (Blender, <http://www.blender.org/>) for measurements and rendering (Fig. 4e).

MATERIALS

REAGENTS

Fixation reagents

- Anesthetized mouse containing a cranial window (see ref. 1 for further details) **! CAUTION** Adhere to all institutional and national ethics guidelines for animal experiments.
- Agarose (Eurobio, cat. no. GEPAGA 07-64)
- Glutaraldehyde (25% (vol/vol) EM grade; EMS, cat. no. 16220) **! CAUTION** It is toxic on inhalation and skin contact. Wear gloves and eye protection, and always work in a fume hood.

- Paraformaldehyde (EMS, cat. no. RT 19208) **! CAUTION** It is an irritant on inhalation and skin contact. Wear gloves and eye protection, and work in a fume hood.
- PBS
- Disodium hydrogen phosphate (Na_2HPO_4 ; Sigma-Aldrich, cat. no. 71642)
- Sodium dihydrogen phosphate (NaH_2PO_4 ; Sigma-Aldrich, cat. no. 71496)
- Sodium cacodylate (Sigma-Aldrich, cat. no. 20840) **! CAUTION** It acts as an irritant on inhalation and skin contact. Wear gloves and work in a fume hood.

PROTOCOL

- Osmium tetroxide (OsO_4 ; EMS, cat. no. RT 19110) **! CAUTION** It is a highly toxic, volatile chemical that is strongly oxidizing, is fatal if swallowed or inhaled, and causes severe irritation to eyes, skin and respiratory tract. Avoid all direct skin contact and inhalation; wear gloves and eye protection, and work only in a fume hood. To handle any solution containing OsO_4 , only use glassware (glass Pasteur pipettes, glass scintillation vials and glass measuring cylinders).
- Potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$; Sigma-Aldrich, cat. no. 455989) **! CAUTION** It is a harmful reagent and is an irritant on inhalation and skin contact. Wear gloves and eye protection, and work in a fume hood while handling it.
- Uranyl acetate (Sigma-Aldrich, cat. no. 73943 FLUKA) **! CAUTION** This is a radioactive and light-sensitive reagent. It is toxic on inhalation. Wear gloves and work in a fume hood while handling it. Appropriate institutional safety guidelines and regulations must be met.

EM dehydration and embedding

- Ethanol (Merck, cat. no. 1.00983.2500) **! CAUTION** It is flammable. Wear gloves.
- Double-distilled water (ddH_2O)
- Durcupan resin: four components supplied separately: single component A, M epoxy resin (Sigma-Aldrich, cat. no. 44611), single component B, hardener (Sigma-Aldrich, cat. no. 44612), single component D, plasticizer (Sigma-Aldrich, cat. no. 44614), and DMP-30, accelerator (EMS, cat. no. RT 13600) **! CAUTION** This resin is toxic on inhalation and skin contact. Wear gloves and eye protection, and work in a fume hood while handling it.

EQUIPMENT

In vivo imaging and generation of laser fiducial marks

- Microscope slides (Thermo Scientific, cat. no. 10143562CE)
- Coverslips
- Syringe with 23-gauge needle (Polymed Medical Center, cat. no. 03.341.01)
- Two-photon laser scanning microscope equipped with a pulsed laser (we used a custom-built microscope and a femtosecond-pulsed titanium-sapphire laser (Chameleon Ultra II, Coherent))
- Nail polish
- Parafilm M (Fischer Scientific, cat. no. 13-374-10)
- Imaging chamber (made of three layers of Parafilm spacer, size of coverslip, placed on glass microscope slide and sealed from outside with nail polish)

Perfusion fixation

- Surgical scissors
- Forceps
- Dissecting board (polystyrene lid)
- Ampoule cracker (EMS, cat. no. 60605-10)
- Perfusion needle (1-inch Straight Cannula Blunt End Tip, 15 gauge; Fisnar, cat. no. 5601137)
- Perfusion peristaltic pump (Witec, cat. no. PK100)

Vibratome sectioning

- Histology plastic embedding molds, 22×22 mm (EMS; cat. no. 70182)
- 24-well culture dish (Nunc, cat. no. 142475)
- Glass scintillation vials, 20 ml (EMS, cat. no. 72634)
- Surgical scissors
- Forceps
- Paint brush, size 6
- Vibratome (Leica VT1200S, Leica Microsystems)
- Wooden cocktail sticks
- Cyanoacrylate glue
- Single-edged razor blades (EMS, cat. no. 71950) **! CAUTION** Sharp blade, avoid injury.

EM embedding

- Wooden cocktail sticks
- Glass scintillation vials, 20 ml (EMS, cat. no. 72634)
- Rotating mixing wheel
- Ampoule cracker (EMS, cat. no. 60605-10)
- Microscope slides (Thermo Scientific, cat. no. 10143562CE)
- Mold-separating agent (Glorex, cat. no. 62407445)
- Ultrasonic cleaner (VWR International, cat. no. 142-6044)
- Tri-corn beaker, 100 ml (EMS, cat. no. 60970)
- Glass Pasteur pipettes, 150 mm (VWR International, cat. no. 612-1701)
- Plastic Pasteur pipettes, disposable, 3 ml (Brand, cat. no. 747760)
- Single-edged razor blades (EMS, cat. no. 71950) **! CAUTION** Extremely sharp blade; avoid injury.

Block preparation

- Stereomicroscope (Leica M205 C, Leica Microsystems)
- Laser dissecting microscope (Leica LMD, Leica Microsystems)
- Single-edged razor blades (EMS, cat. no. 71950) **! CAUTION** Extremely sharp blade, avoid injury.
- Jeweler's saw (EMS, cat. no. 72009)
- Fine-toothed rasp
- Dust-off can (CRC Industries, cat. no. 207936041201)
- Flat slab of blank resin, 1 mm thick. (This is prepared by pouring fresh Durcupan resin between two glass slides held 1 mm apart. The exact spacing is achieved by using other glass slides sandwiched in this space. All slides are coated with mold-separating agent. Avoid introducing any air bubbles and polymerize in a 65°C oven. Remove the polymerized slab from between the glass slides with a razor blade.) **! CAUTION** Extremely sharp blade and high risk of breaking the glass slides when disassembling the casting frame. Wear eye protection and avoid injury.
- Tweezers
- Glass knife maker (Leica EM KMR3, Leica Microsystems)
- Glass strips (Leica, $400 \times 25 \times 8$ mm, cat. no. 840032)
- Ultramicrotome (Leica Ultracut UCT, Leica Microsystems)
- Transmitted light microscope (AxioCam 2 Plus, Zeiss) equipped with digital camera (AxioCam HR, Zeiss)
- Computer

FIBSEM stub preparation

- 12.5-mm aluminum SEM stub for mounting resin block, with 45° chamber (Agar Scientific, cat. no. G3020)
- Carbon conductive cement (CCC carbon adhesive, EMS, cat. no. RT12664) **! CAUTION** It is highly flammable, is harmful by inhalation and in contact with skin, and is an irritant to eye and skin. Wear gloves, eye protection and mask, or use a fume hood while handling it.
- Wooden cocktail sticks
- Tweezers
- Gold coater (Cressington vacuum evaporation system)

FIBSEM imaging and 3D reconstruction

- FIBSEM microscope (Zeiss NVision 40, Carl Zeiss SMT)
- Fiji software package (<http://fiji.sc/wiki/index.php/Fiji>)
- Image manipulation software, e.g., Photoshop (<http://www.adobe.com/>) or ImageJ (<http://rsbweb.nih.gov/ij/>) or GIMP (<http://www.gimp.org/>)
- ilastik software (version 0.5.12; <http://ilastik.org/>)
- Blender software (version 2.66; Blender Foundation; <http://www.blender.org/>).
- Powerful computer with a good graphics card and sufficient RAM memory (>8 GB) by using either Linux or MacOSX operating system

REAGENT SETUP

Phosphate buffer (0.2 M stock solution) Prepare aqueous stock solution A (0.2 M NaH_2PO_4) and stock solution B (0.2 M Na_2HPO_4). For 100 ml phosphate buffer (PB), mix 19 ml of solution A and 81 ml of solution B, check and adjust the pH to 7.4 if necessary. Store the buffer at 4°C , and use it within a week.

PBS (0.01 M, 0.9% (wt/vol) NaCl) For a final volume of 1 liter of PBS, mix 50 ml of 0.2 PB with 950 ml of ddH_2O and add 9 g of NaCl to it. Store the saline at 4°C , and use within a week.

Cacodylate buffer (0.2 M stock solution) Add 10.8 ml of 0.2 M HCl to 100 ml of 0.4 M solution of sodium cacodylate (8.56 g in 100 ml of ddH_2O). Adjust the pH to 7.4 and make up to 200 ml of final volume with ddH_2O . Store the buffer at 4°C indefinitely.

Fixative solution (2.5% (vol/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in 0.1 M PB, pH 7.4) Prepare solution immediately before use. Dissolve 6 g of paraformaldehyde in 100 ml of ddH_2O heated to 65°C . Add 1–2 drops of 5 M sodium hydroxide and stir with magnetic stirrer on a heated stirrer until all deposits have dissolved and the solution is clear. Add dissolved paraformaldehyde to 100 ml of cold 0.2 M PB, followed by 30 ml of freshly opened 25% (vol/vol) aqueous glutaraldehyde, by using an ampoule cracker. Adjust the pH to 7.4 and add ddH_2O up to a final volume of 300 ml. Leave it to cool to 20°C before use.

OsO_4 stock solution (2% (wt/vol) in ddH_2O) Open sealed ampoule of 1 g of OsO_4 crystals by using an ampoule cracker and dissolve them in 50 ml of ddH_2O . The solution must be stored under a fume hood in a double-sealed container at 4°C and can be used within a few months.

OsO₄ (1% (vol/vol)) and potassium ferrocyanide (1.5% (wt/vol)) in 0.1 M cacodylate buffer Mix 5 ml of 2% aqueous OsO₄ with 5 ml of 0.2 M cacodylate buffer and dissolve 0.15 g of potassium ferrocyanide. Always prepare this solution fresh.

OsO₄ (1% (vol/vol)) in 0.1 M cacodylate buffer Add 5 ml of 2% aqueous OsO₄ to 5 ml of 0.2 M cacodylate buffer. Always prepare this solution fresh.

Uranyl acetate (1% (wt/vol) in ddH₂O) To 10 ml of ddH₂O, add 0.1 g of uranyl acetate and agitate until dissolved or use water bath sonicator at maximum speed for ~5–10 min. Always prepare this solution fresh.

Durcupan resin Weigh 33.3 g of single component A (M epoxy resin), 33.3 g of single component B (hardener) and 1 g of single component D

(plasticizer) into a 100-ml Tri-corn beaker and stir with magnetic stirrer for 30 min. Add 1 g of DMP-30 (accelerator) and stir for additional 20–30 min. In any stage of mixing, avoid air bubble formation. Unused resin can be stored in a sealed 20-ml syringe at –20 °C for 2 weeks. **! CAUTION** Handle resins and their components only in a fume hood.

Mold-separating agent-coated slides Dip each cleaned glass microscope slide completely into mold-separating agent solution. Take the slide out of the solution and place in a slide-drying rack in a 60 °C oven for 2 h. Repeat the coating process a second time and let the slides completely dry in a 60 °C oven for 24 h. Store coated slides in a dust-free container until needed.

PROCEDURE

Fixation and vibratome sectioning ● TIMING 3–4 h

▲ CRITICAL This procedure starts after the acquisition of *in vivo* images of axons and dendrites through a mouse cranial window, as described by Holtmaat *et al.*¹. The animal is maintained under heavy anesthesia throughout. The handling of mice used in these experiments adhered to all institutional and governmental animal ethics guidelines for experimentation.

- 1| Take a low-magnification image of the blood vessel pattern through the cranial window by using transmitted light (**Fig. 1a**). Ensure that the position of the fluorescent structures is known relative to these blood vessels.
- 2| Fix the forelimbs of the deeply anesthetized animal to the dissection board.
- 3| Open the chest cavity with scissors and forceps to expose the heart and lungs.
- 4| Insert the perfusion needle into the left ventricle toward the aorta and fix the needle in place with the heart clamp.
- 5| Switch on the peristaltic pump and flush the animal with 10 ml of isotonic PBS at a speed of 12 ml min⁻¹. Filling the tubing first with fixative, then backfilling with PBS from the needle end ensures a smooth transition between the two perfusates, rather than using a three-way tap and switching between different sources.
- 6| Immediately perfuse the whole animal with 300 ml of 2.5% (vol/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in 0.1 M PB (pH 7.4). The perfusion fixation should be complete within 25–30 min.
- ▲ CRITICAL STEP** As a quality check of perfusion, monitor color change of the liver. It should change to light brown within 30 s.
- ? TROUBLESHOOTING**
- 7| Leave the animal for a further 2 h to complete the fixation process.
- 8| Remove the fixed brain from the skull by carefully peeling away the skull with forceps, and place it in 0.01 M PBS.
- 9| Fill the histology plastic mold with 5% (wt/vol) of agarose solution at 50 °C in PBS, allowing it to cool to ~30°C but ensuring that it remains liquid.
- 10| Immerse the brain in the molten agarose and orient it so that the region imaged via the imaging window is parallel to the surface.
- 11| Place the mold containing the agarose-embedded brain in a refrigerator (at 4 °C) for 10 min.
- 12| Remove the solidified agarose block from the mold, and trim with a razor blade to leave a minimal amount of agarose around the brain.
- 13| Mount the trimmed block onto the vibratome support table with cyanoacrylate glue, ensuring that the imaged region is uppermost and parallel to the upper surface.
- 14| Cut 60-μm-thick vibratome sections in PBS, and transfer them into a 24-well culture dish by using a paint brush.

Locating the axons and dendrites of interest in the fixed sections ● TIMING 30 min

- 15| Image each vibratomed section at low magnification (12–16 time magnification) by using the dissecting microscope. Blood vessels are best seen by using oblique illumination directed from below.

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16| Superimpose and align the images of the sections. These will reveal the blood vessel pattern on the brain's surface. This is the same pattern that is seen *in vivo* (captured at Step 1). The alignment of these images is carried out by using image manipulation software, e.g., Photoshop, ImageJ or GIMP to identify the section containing the region previously imaged *in vivo*. The position of the neurites of interest is located according to blood vessel pattern. This indicates in which section the neurites can be found.

17| Mount the identified section inside an imaging chamber made from a sheet of Parafilm, with a rectangle cut out of its center, placed under a coverslip. Flood the chamber with PBS.

▲ **CRITICAL STEP** Avoid any air-bubble formation, minimize light exposure and do not allow the section to dry.

Generating laser fiducial (NIRB) marks around the region containing the axons and dendrites of interest ● **TIMING 30 min**

18| Locate the previously imaged neurites (**Fig. 1a,b**) with the two-photon microscope. The image of the blood vessels from the section, and from the cranial window, will indicate exactly the position to find the fluorescent structures.

19| Tune the two-photon laser wavelength, λ , to 850 nm and laser power to ~300 mW at the back focal plane of the objective.

20| Use between 512 and 2,500 line scans at a speed of 2 ms per line to burn fiducial mark lines.

▲ **CRITICAL STEP** Control the size of the laser mark by using the auto-fluorescence generated from its edges as a guide. The wider the laser burns, the further apart the two fluorescent edges. If the two edges appear to be closer than 2 μm , then the mark will likely be invisible in the embedded section. During the line scans, turn the photomultipliers to a lower offset to prevent damage resulting from over-illumination. The relationship between the level of auto-fluorescence from line scans and the width of the laser marks should be determined empirically and can be tested on redundant sections.

21| Make a rectangle or square around the neurites of interest with a size of 15–24 μm along one edge. The marks should be placed 3–5 μm above the neurites of interest and not at the same Z height.

22| Image these smaller marks with the neurites enclosed through the depth of the section at a wavelength of $\lambda = 910$ nm and at high-magnification settings (e.g., image size of 0.2 μm per pixel and 2 μm steps in the Z direction).

23| Make an asymmetric shape (i.e., trapezium) around the first NIRB marks with a size of ~50–100 μm along any side (**Fig. 1d**).

24| Image these larger marks at a lower magnification with the two-photon microscope.

25| Image the laser marks by using transmitted light microscopy, including other features such as blood vessels and myelin bundles.

▲ **CRITICAL STEP** For future reference and correlation with FIBSEM, always collect two-photon image stacks before and after laser branding at both high and low resolution.

■ **PAUSE POINT** Fixed sections can be stored in 0.1 M sodium cacodylate buffer at 4 °C overnight.

Staining for EM and resin-embedding ● **TIMING 7–9 h (excluding resin polymerization)**

26| Transfer sections into a 20-ml glass scintillation vial with a paint brush.

27| Wash the sections in 0.1 M sodium cacodylate buffer (pH 7.4) for five washes of 5 min each wash, gently agitating closed vials on a rotating mixing wheel at a speed of ~4 rev min⁻¹. Agitate in this way for each of the following steps until the end of Step 36.

? **TROUBLESHOOTING**

28| Postfix and stain in 1% (vol/vol) OsO₄ and 1.5% (wt/vol) ferrocyanide in 0.1 M sodium cacodylate buffer (pH 7.4) for 40 min.

? **TROUBLESHOOTING**

29| Postfix and stain in 1% (vol/vol) OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4) alone for 40 min.

? **TROUBLESHOOTING**

30| Wash the sections in ddH₂O twice for 5 min each wash.

31| *En bloc* stain the section in 1% (wt/vol) uranyl acetate in ddH₂O for 40 min.

? **TROUBLESHOOTING**

32| Wash the sections in ddH₂O for 5 min.

■ **PAUSE POINT** Post-stained sections can be stored in ddH₂O at 4 °C overnight.

33| Dehydrate the sections in a graded alcohol series (vol/vol): 2 × 50%, 1 × 70%, 1 × 90%, 1 × 95% and 2 × 100% ethanol for 3 min each change.

▲ **CRITICAL STEP** Always prepare fresh solution immediately before use and prevent exposing sections to air at any time during the dehydration process. After dehydration, sections become brittle; therefore, handle them carefully.

34| Infiltrate the sections in 50% (vol/vol) Durcupan in ethanol for 30 min.

35| Infiltrate the section for 2 × 30 min with fresh Durcupan. Use fresh Durcupan for each change.

36| Replace with fresh Durcupan and leave for 2–4 h.

▲ **CRITICAL STEP** Extending the infiltration time will increase the viscosity of the resin.

37| Transfer and arrange section(s) onto glass slide coated with mold-separating agent by using the wooden cocktail stick. Fill the edges of the slide with a small amount of resin, place the label using a pencil and carefully cover with second coated glass slide to make a flat sandwich.

▲ **CRITICAL STEP** Do not try and readjust the position of the section once the sandwich has been made, as this risks breaking the section.

? **TROUBLESHOOTING**

38| Polymerize the flat-embedded sections in a 65 °C oven for 24 h. When the resin is completely cured, separate the glass slides from the resin by using the edges of the razor blade.

■ **PAUSE POINT** Cured resin-embedded sections can be stored at room temperature (~20–25 °C) for an unlimited period of time.

Preparation and trimming of the block face ● **TIMING** ~2–5 h

39| Locate the laser marks in the resin-embedded section by using a light microscope (**Fig. 2a**).

? **TROUBLESHOOTING**

40| Replicate the original NIRB marks on the section surface with the pulsed laser of the laser dissection microscope (**Fig. 2b**).

▲ **CRITICAL STEP** The etched marks on the surface of the resin are visible with the stereomicroscope fitted to the ultramicrotome during the subsequent trimming process. These marks also eliminate the need to remove the block from ultramicrotome to check the position of the region of interest inside the tissue.

? **TROUBLESHOOTING**

41| Wash the resin-embedded section with ddH₂O, and wipe it clean with soft tissue to remove any traces of mold-separating agent.

42| Make a 4 mm × 20 mm strip of 1-mm-thick slab of blank resin with a jeweler's saw, smooth the edges using the fine rasp and remove all dust with compressed air.

▲ **CRITICAL STEP** The slab of blank resin gives a solid support and ensures easy manipulation during the trimming process.

43| Wash the slab with ddH₂O, and wipe it with clean soft tissue to remove any traces of mold-separating agent.

44| Trim away the region marked by the laser dissection microscope from resin-embedded section with single-edged razor blade, and glue it to the slab of blank resin (Step 42) using a small droplet of Durcupan resin (**Fig. 2c**) under the dissecting stereomicroscope.

45| Place the slab with cut region into a 65 °C oven for at least 5 h, preferably overnight.

46| Mount the block in the ultramicrotome so that the embedded section (and the NIRB marks) is vertical. Set the edge of the glass knife parallel to the edge of this resin block and trim away the block, and embedded section, until the NIRB marks are close (<10 μm) to this cut edge.

▲ **CRITICAL STEP** Check the distance of the NIRB marks to the edge by removing the block from the holder and placing it in the light microscope.

47| Place the block back in the ultramicrotome and rotate until the section is horizontal (**Fig. 2d**). Trim a step into the right side of the face (<60 μm from the NIRB marks) by using the edge of a new glass knife close to within <50 μm of the edge of the NIRB marks (**Fig. 2d**).

? **TROUBLESHOOTING**



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48| Optionally, a second indentation can be made on the left side of the NIRB marks by rotating the sample holder 180°.

▲ **CRITICAL STEP** Indentations made on either side of the NIRB marks serve as fiducial marks from which measurements can be made to precisely position the FIBSEM imaging window. Additional score marks can also be made down the face of the block with the left corner of the glass knife (**Fig. 2d**) to serve as additional fiducial marks to precisely pinpoint the region of interest.

? TROUBLESHOOTING

49| Measure the depth of the NIRB marks inside the resin from the block's surface by using the transmitted light microscope equipped with a calibrated focusing stage.

▲ **CRITICAL STEP** After each trimming step, take calibrated images to record how far the NIRB marks are from the block edge and its surface.

50| Clamp the block in the ultramicrotome so that the glass knife can trim the block's surface parallel to the NIRB marks. Trim away the resin until ~5 μm of resin remains about the NIRB marks (**Fig. 2e,f**).

▲ **CRITICAL STEP** Take care to trim only small amounts of resin at a time (<2 μm), checking the depth of the NIRB marks in the light microscope.

Mounting the block to SEM stub and gold coating ● **TIMING** ~1.5 h

51| Take an image of this final block in the light microscope (×20 objective). Ensure that it shows the position of the NIRB marks inside the tissue, relative to the block edge, steps cut on either side and the score marks on the block edge (**Fig. 3a**). The overlay and alignment of the final block image with both low-resolution two-photon image of the laser marks and high-resolution image of the structure of interest is later used to position the final imaging window for FIBSEM imaging.

52| Glue the block to the inclined (45°) surface of the 12.5 mm SEM aluminum stub by using carbon-conductive cement applied with a wooden cocktail stick. Position the block so that the edge to be imaged lies at the stub's center (**Fig. 2g**). Allow the cement to dry completely (<30 mins).

53| Coat the final block face with a 30-nm layer of gold by using a vacuum evaporation unit.

? TROUBLESHOOTING

■ **PAUSE POINT** The gold-coated block can be stored indefinitely in a desiccator until needed.

FIBSEM imaging face preparation and image acquisition ● **TIMING** ~2 h and 48–72 h

54| Insert the sample inside the FIBSEM microscope (e.g., Zeiss NVision 40).

55| Orient the block so that its upper face, parallel with the NIRB marks, is parallel to the ion beam, and therefore at 54° to the electron beam (for Zeiss microscopes). This upper face is the 'imaging face' (**Fig. 3a,b**).

56| Measure the position along the imaging face at which the NIRB marks are found.

57| Ion-beam mill the imaging face with a current of ~6 nA so that a 60-μm-wide portion of the underlying resin is revealed. This may reveal some underlying features that may help in positioning the imaging window more precisely.

58| Deposit a 1-μm-thick protective layer of platinum (or carbon) on the top surface of the block, above the region of interest. This is done by using the ion beam in conjunction with the gas injection system.

59| Polish the imaging face with a lower current of ~0.7–1.5 nA until all blemishes and imperfections have been removed, and the beam is milling the face consistently.

60| Stop milling and leave the microscope unattended for at least 2 h.

? TROUBLESHOOTING

61| Select a region to image, of ~15 μm × 10 μm in size and estimated to be situated in line with the NIRB marks, to capture the neurites of interest (**Fig. 3c**).

62| Select an ion beam current of 700 pA with a voltage of 30 kV and an imaging voltage of 1.5 kV, ~800 pA with a grid tension on the energy selective back-scattered (ESB) detector of 1.3 kV allowing for an energy loss of 200 eV.

63| Mill a window that is three times wider than the area to be imaged, i.e., 45 μm. Capture images with pixel sizes of 4–5 nm with pixel dwell times of ~10 μs, and a milling thickness between images of 5–10 nm. Use the microscope's focus and image-tracking functions (dynamic focus and TrackWD on the Zeiss NVision) so that the electron beam is properly focused on the tilted sample surface and follows the retreating face of the block. Tilt correction should be applied during the acquisition of the SEM

image so that the generated image will already be corrected for the foreshortening effect when imaging the tilted plane.

▲ **CRITICAL STEP** The milling and imaging cycle should be completed in 2 min. This comprises 90 s for the imaging and 30 s for the milling. Longer cycles should be avoided as the milling thickness becomes inconsistent.

? TROUBLESHOOTING

■ **PAUSE POINT** The rest of the procedure is image analysis, thus the experiment can be paused indefinitely here.

3D reconstruction and analysis ● TIMING ~3 h

64| Combine individual sequential images acquired by FIBSEM (in tiff image format) into a single stack by using an appropriate software package, for example Fiji (<http://fiji.sc/wiki/index.php/Fiji>). Combine images in Fiji by clicking on *File* → *Import* → *Image Sequence* command.

65| If the stack requires alignment, then align. Using Fiji, select the *Plugins* → *Registration* → *StackReg* command. Align from the middle of the stack by using the transformation option ‘translation only’.

66| Crop the aligned stack to the desired size. In Fiji, use the ‘Rectangular’ selection and *Image* → *Crop* command.

67| Invert the image contrast to easily interpret the FIBSEM-acquired images. In Fiji, use *Edit* → *Invert* command (alternatively, *Image* → *Lookup Tables* → *Invert LUT* command). Adjust the image contrast by using the *Image* → *Adjust* → *Brightness/Contrast* command. Select either the ‘Auto’ option or adjust contrast manually, and select *Apply*.

68| Save final processed stack as sequence of individual 8-bit, grayscale images. This is performed in Fiji by using the *File* → *Save As* → *Image Sequence* command.

69| Measure the section thickness by using the mitochondria and the cylindrical diameters method¹³. Briefly, the section thickness is calculated by dividing the number of images across which a longitudinally milled mitochondrion is seen by its maximum diameter. This is repeated through the entire stack on >40 mitochondria.

70| Locate the position of the neurites of interest within the image stack by overlaying their two-photon image, which also includes the NIRB marks (Steps 22–25), with the image of the same marks in the imaged block (Step 51). This step is carried out by using imaging software such as Photoshop, ImageJ or GIMP.

71| Segment the structure of interest and create a 3D model by using appropriate software. This is described here using an automated approach of seeded watershed segmentation using the ilastik software (version 0.5.12; <http://ilastik.org/>). This could also be achieved by manually segmenting the features on each image of the stack by using software such as TrakEM2 (ref. 14) in the FIJI program, although it would take considerably longer. On the splash screen of ilastik, choose ‘OpenGL’ for fastest 3D rendering or ‘Software’ in case OpenGL is not configured on the computer. Under the ‘Project’ tab, select ‘New’ button to load images. In the Project window, select ‘Load Stack’ and locate the folder where sequential images are stored. The entire stack is displayed in orthogonal views, giving the user the freedom to scroll through the stack in the *x-y*, *x-z* and *y-z* planes.

72| In the ‘Classification’ tab, select the ‘Select Features’ button to specify a set of features to be calculated to identify the membrane boundaries. In the ‘Spatial Features’ window, select ‘Texture’, which includes the Eigenvalues of the Hessian matrix as a membrane boundary indicator. Set the scale of the features to ‘Medium’ (representing sigma value of 1.0). The ilastik program will compute the selected set of features for the entire image stack.

73| Select ‘Seeded Watershed’ tab and import the previously calculated Eigenvalues of the Hessian matrix of Gaussian Sigma Channel 2 as chosen input weights. In the ‘Select Border Indicator’ window, specify whether the boundaries in ‘Calculated Weights’ are dark or bright. For Channel 2, select ‘Darkness’. Alternatively, if the selected Eigenvalue of Hessian matrix of Gaussian Sigma Channel is ‘0’, set the border indicator to ‘Brightness’. The ilastik program will then construct the supervoxel graph used for watershed segmentation.

74| Draw a line, or foreground seed, inside the structure of interest and in a different color mark the outside of the selected structure or background seed (Fig. 4a). As default, the background seed is in red and Seed 1 is in green. However, the user can create more seeds by using the button, or delete them by right-clicking on the list. The user can also change the color and width of these seeds.

75| Activate the ‘Segment’ button to start the segmentation process through the whole stack (Fig. 4b). The selected structure is colored as ‘Segmentation’ overlay in the ‘Overlays’ preview window. Note that the seeds can be placed from any displayed planes of the orthogonal view. The segmentation can be iteratively assessed and refined by placing more foreground and background seeds, or by adding seeds for other objects. The ‘Bias’ dialog field allows the background seeds to grow more (values ~0.8) or less (values ~0.97) aggressively than the default.

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76 | Render a 3D model of the segmented structure by right-clicking on the ‘Segmentation’ overlay and selecting ‘Display in 3D’ (Fig. 4c).

77 | Export the final model as a wavefront OBJ mesh file by selecting the export button to render high-quality images or movies by using the Blender 3D modeling software (version 2.69; Blender Foundation; <http://www.blender.org/>) (Fig. 4e).

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
6	Brain is soft, or blood is not completely flushed from the vessels	Poor perfusion	Increase the amount PBS backfilled in the perfusion tubing Perfusion needle is not tightly clamped into heart
27	Dense precipitates seen in the EM images (‘black pepper effect’)	PBS is not properly rinsed out of the tissue	Increase number of washes with sodium cacodylate buffer to completely remove any traces of phosphate ions
28, 29, 31	NIRB marks are not visible in the section after resin embedding	Tissue is too heavily stained owing to excessive staining with reduced osmium tetroxide Tissue is too heavily stained with uranyl acetate Sections are thicker than 60 µm	Carefully monitor color of the section and reduce incubation time Ensure that brain is held firmly while it is sectioned in vibratome
37	Region of resin-embedded section, containing NIRB marks is not lying horizontally in the block	Insufficient laser power results in marks that are too thin Section is too thin and curled during dehydration, and embedding NIRB marks lie at the very edge of the section	Increase the laser power or number of line scans Check that section thickness is 60 µm when cut in the vibratome Ensure that regions of interest do not lie close to the section edges
39	Unable to find the laser marks in section after resin embedding	NIRB marks are too thin to be clearly visible with low-power objectives Not enough low-power images taken of section immediately after making NIRB marks	Increase laser power and number of line scans to increase the width of NIRB marks Image the section with transmitted light microscope at low and high power to capture NIRB marks and surrounding features, such as blood vessels and cell bodies
40	Laser-etched marks made with laser dissecting microscope are too large	Setting parameters for pulsed laser are too high	Reduce the power of pulsed laser. Take care to observe how the laser etches the surface more deeply when tissue is at the block surface.
47, 48	It is difficult to trim smooth surfaces while making the block	Glass knives are not sharp Block is not held firmly enough Knife is advancing too much each time	Use only freshly made glass knives Ensure that block is clamped firmly in the jaws of the holder Trim only 0.5 µm thickness each time
53	It is difficult to attain well-focused backscattered images in FIBSEM	Conductive gold layer on the block surface is too thin	Apply additional gold coat on the block face surface
60	Consecutive images are not from the same position on the block face	The block is drifting in relation to the electron beam	Ensure that microscope room temperature is not fluctuating Only visit the microscope briefly to check the image quality
63	Serial images do not show consistent Z spacing	No thermal steady-state has been reached, and block is expanding and contracting during the milling and imaging process	Imaging and milling should occur within ~2 min for each cycle

● **TIMING**

Steps 1–14, fixation and vibratome sectioning: 3–4 h

Steps 15–17, location of the neurites of interest in the fixed sections: 30 min

Steps 18–25, generation of laser fiducial marks around the region containing the axons and dendrites of interest: 30 min

Steps 26–38, staining for EM and resin-embedding: 7–9 h (excluding resin polymerization)

Steps 39–50, preparation and trimming of the block face: ~2–5 h

Steps 51–53, mounting the block to SEM stub and gold coating: ~1.5 h

Steps 54–63, FIBSEM imaging face preparation and image acquisition: ~2 h and 48–72 h

Steps 64–77, 3D reconstruction and analysis: ~3 h

ANTICIPATED RESULTS

Successful NIRB marking of sections is dependent on the experimenter being able to see the marks during all parts of the subsequent block preparation. As the burning effect of the laser decreases deeper in the tissue, it is important to check that the marks are clearly visible before embedding the sample in resin. This should be carried out by initially imaging with a two-photon microscope to ensure that all information is collected about the marks' location with respect to the neurites of interest. However, it should also be performed with a transmitted light microscope at medium magnification ($\times 20$). If the marks are still clearly visible, then they will be seen after the staining and resin-embedding steps.

NIRB marks are more easily made closer to the upper surface of the section; therefore, in this case, it may be better to turn a section over to have the NIRB marks closer to the upper face. This would mean that the block is then mounted upside-down on the resin slab. Therefore, the trimming and imaging need to account for the flipped orientation of neurites.

As the embedded section is densely stained, the NIRB marks may require intense illumination to be seen clearly during the block trimming. However, if they are located deep in the section, it is helpful to trim the excess material from above the region of interest to improve the illumination. The section in the resin can be imaged with a high-power objective ($\times 100$) with immersion oil in contact with its surface to produce the best images. The polymerized resin can be cleaned afterward with ethanol before it sticks to the resin slab. As the FIBSEM targets only the region of the NIRB marks, as the neurites are no longer fluorescent or specifically stained, it is important to gather as much information about this region before putting it in the electron microscope. It is also important to note that, although there is a certain degree of tissue shrinkage that occurs during the initial fixation, no discernible distortion occurs in the staining and embedding phase. Therefore, the orientation of the features seen in the resin clearly matches that seen in the two-photon images of the fixed section, i.e., distances between the features remain the same. However, when the sections are embedded they may not lie flat after polymerization, and slight undulations may appear. These can tilt the region of interest so that it does not lie parallel with the resin surface. This can be corrected in the trimming process by tilting the block so that a newly cut face is trimmed parallel to the NIRB marks.

The trimming phase of the protocol is the most crucial for ensuring that the least amount of time is spent using the FIB to expose the region of interest. The use of new glass knives is crucial for accurate trimming, as are the regular checks made with the light microscope to measure the distances between the NIRB marks and the edge of the block.

This protocol has been used in a number of studies in which dendrites and axons were imaged *in vivo*^{15,16}. These have included not only GFP-expressing neurites of excitatory neurons but also tdTomato-labeled inhibitory neurons⁷.

Most of the previous studies, which used immunocytochemistry to carry out this type of correlative microscopy, have focused on dendrites. Targeting axons with the labeling method proved difficult owing to problems with adequate staining of these structures that are often thin and not easily distinguished among the rest of the neuropil. This problem is removed in the current protocol as the neurites of interest are not labeled specifically, only the region in which they were seen is targeted, therefore optimal fixation and staining conditions can be used. The EM staining part of the protocol uses a conventional approach—staining recipe at ambient temperature; however, other protocols that maximize the preservation of the membranes while also allowing heavy contrasting of the membrane could also be envisaged¹⁷. Although this method only describes how this procedure can be carried out on dendrites and axons from live-animal experiments, it is equally applicable to acute or cultured slice preparations.

Concluding remarks

Block-face scanning EM techniques allow for automated collection of aligned serial images through substantial volumes of resin-embedded cells and tissues. Compared with the serial TEM approach, this is a fast and more reliable method and also allows EM images to be collected through volumes in which structures of interest, previously seen with light microscopy, are located. Therefore, if the required structures are recognizable in the EM images, or their exact position is pinpointed from the light microscope images, there is no need to use labels that can be seen in both imaging modes. This approach, without specific EM labels, means that any fluorophore seen in fixed material is compatible. This opens up many opportunities



to carry out correlative EM analysis on the vast array of different fluorophores to combine temporal information on many scales with high-resolution data in 3D.

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