

C0080 Correlative *In Vivo* 2-Photon Imaging and Focused Ion Beam Scanning Electron Microscopy: 3D Analysis of Neuronal Ultrastructure **16**

Bohumil Maco^{*,†}, Anthony Holtmaat[‡], Anne Jorstad[†], Pascal Fua[†]
Graham W. Knott^{*}

^{*}BioEM Facility, Centre of Electron Microscopy, EPFL, Lausanne, Switzerland

[†]Computer Vision Laboratory, EPFL, Lausanne, Switzerland

[‡]Department of Neuroscience, University Medical Center (CMU), University of Geneva, Geneva, Switzerland

CHAPTER OUTLINE

Introduction	340
16.1 Method	342
16.1.1 Fixation and Vibratome Sectioning	342
16.1.2 Laser Fiducial Marks Around Region of Interest (NIRB Technique)	344
16.1.2.1 Method	344
16.1.3 Staining and Resin Embedding	345
16.1.3.1 Method	345
16.1.4 Preparation of the Block Face and Mounting the Block for FIBSEM	346
16.1.4.1 Method	346
16.1.5 FIBSEM Imaging	349
16.1.5.1 Method	350
16.1.6 3D Reconstruction and Analysis	350
16.1.6.1 Method	353
Conclusion	359
Acknowledgments	359
References	360

Abstract

This protocol describes how dendrites and axons, imaged *in vivo*, can subsequently be analyzed in 3D using focused ion beam scanning electron microscopy (FIBSEM). The fluorescent structures are identified after chemical fixation and their position highlighted using the 2-photon laser to burn fiducial marks around the region. 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 then reconstructed semi-automatically using the Ilastik software (ilastik.org). This fast and reliable imaging and reconstruction technique avoids the use of specific labels to identify the features of interest in the electron microscope and optimizes their preservation for high-quality imaging and 3D analysis.

INTRODUCTION

s0005

p0005

Visualizing the morphology and connectivity of neurons is crucial to understanding how they function. The increasing use of live-imaging methods has enabled neuroscientists to study neurons in their native environment. However, this has also demanded greater use of electron microscopy to give further interpretation to the changes seen with the light microscope. With its higher resolution and ability to give more structural details, electron microscopy remains the only technique that is able to see fine cellular architecture and synaptic connectivity of neurons. In recent years, the development of new EM methods has enabled large volumes of tissue to be imaged with only limited human interaction. These have opened up many different opportunities for exploring the brain.

p0010

This chapter gives a detailed explanation of one technique that has recently been used in a number of high-profile studies combining the imaging of neurons in the live brain, using 2-photon microscopy, with electron microscopy using the focused ion beam scanning electron microscope (Allegra Mascaro et al., 2013; Cane, Maco, Knott, & Holtmaat, 2014; Canty et al., 2013; Grillo et al., 2013; Mostany et al., 2013). These studies carried out extensive series of live-imaging experiments showing how the morphology of axons and dendrites of fluorescently labeled neurons changed during aging and in response to injury.

p0015

Previous CLEM experiments on *in vivo*-imaged neurons had used serial-section transmission electron microscopy (Holtmaat, Wilbrecht, Knott, Welker, & Svoboda, 2006; Knott, Holtmaat, Trachtenberg, Svoboda, & Welker, 2009; Knott, Holtmaat, Wilbrecht, Welker, & Svoboda, 2006; Trachtenberg et al., 2002; Zito, Parnas, Fetter, Isacoff, & Goodman, 1999), however, these studies were rare, requiring considerable amounts of time and skill to manually section large volumes of tissue that is then imaged one section at a time to locate the region of interest. Maintaining a complete series of sections, in perfect condition, is critical for this method to work. The first study of this kind appeared soon after green fluorescent protein (GFP) started to be used in live-cell imaging, and was undertaken in *Drosophila* (Zito et al., 1999) to understand how the neuromuscular junction developed and synapses inserted into

Au4

the growing boutons. This pioneering work required the axons of interest to be found in the large series of sections without any label being used to highlight its whereabouts. Later studies of neuronal plasticity in the mammalian adult brain used antibodies against the GFP in a preembedding protocol that revealed the location of the structure of interest in the resin-embedded tissue. This also allows specific neurites to be found in the bewilderingly complex networks of the central nervous system.

p0020 Labeling fluorescent cells, so that they can be found later with electron microscopy, however, risks being counterproductive as the label itself may obscure the ultrastructural details. For this reason, the method outlined here has been developed using the near-infrared branding (NIRB) method (Bishop et al., 2011; Maco et al., 2013) to make fiducial marks inside the tissue close to the structures of interest, but not associated with them. Using a 2-photon laser, the fixed tissue, with the fluorescent structures still visible, is branded with easily identifiable marks. These are seen both in the resin-embedded blocks and in the electron microscope and are used to locate the position of the features of interest. This region is then imaged with FIB-SEM, making sure that the entire volume indicated by the NIRB marks is captured.

p0025 This approach has several advantages to previous correlative methods. Without using any immunolabeling converting the fluorescence into an electron-dense signal ensures that optimal fixation protocols can be used without compromising the quality of the ultrastructure. Using FIBSEM provides an automated imaging method requiring only limited human intervention to obtain large series of uninterrupted images. This block-face scanning approach not only gives aligned images, but as the ion beam is able to remove a few nanometers from the block surface, image stacks with isotropic voxels of only a few nanometers in each dimension are possible (see, e.g., Fig. 16.5). As well as giving a more accurate view of 3D structures, it also provides better opportunities to use computer vision algorithms for automatically segmenting and reconstruction of certain features (Kreshuk et al., 2011; Straehle, Köthe, Knott, & Hamprecht, 2011).

p0030 With the continued development of block-face scanning electron microscopy, detailed analysis using manual methods on ever-larger series of images is becoming unfeasible. The advances in computer vision algorithms have made significant strides toward automated methods for recognizing and reconstructing different features, particularly in serial FIBSEM images (Becker, Ali, Knott, & Fua, 2013; Lucchi, Smith, Achanta, Knott, & Fua, 2012; Lucchi, Smith, Achanta, Lepetit, & Fua, 2010). This includes structures such as mitochondria, synapses, and neurites. This chapter describes how, once the correct region has been imaged, axons and dendrites can be reconstructed automatically from the serial images using the Ilastik software (www.ilastik.org). This task would take a few days of work if attempted by hand. Instead, it is now possible in a few hours to generate a computer model of the structures of interest. This is then ready for further analysis. The final part of this chapter describes how measurements can be made of these models using a set of software tools that run on the open-source 3D imaging platform Blender (www.blender.org). Measuring the sizes of structures like synaptic contacts, dendritic spines, and axonal boutons is an important means of comparing different

342 CHAPTER 16 2-Photon Imaging and FIBSEM

neurites, to understand how they function. These tools offer an accurate and rapid means to make morphometric measurements in a 3D workspace, with rapid access to the serial images from which the models were derived.

p0035 This complete protocol represents a significant advance in the field of correlative light and electron microscopy. As well as using optimal fixation and staining conditions for this type of material, the imaging is automated, as well as the reconstruction process. This is a method that is therefore not only more reliable but faster and more accurate.

s0010 **16.1 METHOD**

s0015 **16.1.1 FIXATION AND VIBRATOME SECTIONING**

p0040 The fixation of the living brain is the most crucial part of the entire procedure to ensure high-quality EM images. The fixative is perfused through the circulatory system as soon after opening the chest cavity as possible, with the animal deeply anesthetized throughout. The brain is therefore exposed to fixatives without it experiencing hypoxic conditions for more than 30 s. Any delay in this part will result in alterations to the ultrastructure (Tao-Cheng, Gallant, Brightman, Dosemeci, & Reese, 2007). The fixation conditions are considered very standard and have changed little from the original EM fixation studies in the 1960s (Schultz & Karlsson, 1965). The process is complete after 2 h, and therefore, the brain can be removed and vibratome sectioned shortly after fixation. AUS

p0045 Vibratome sections need to be cut parallel to the imaging plane of the 2-photon microscope to ensure that the orientation of the neurites in the fixed material is similar to those seen in the live brain. If the orientation is too dissimilar, recognizing the same neurites in the final EM image stack is too difficult.

p0050 The protocol begins once the live 2-photon imaging has been completed with the animal deeply anesthetized, nonreflexive, and ready to be perfusion fixed.

Animal perfusion fixation

- o0005 1. At the end of the 2-photon *in vivo* imaging session, take a low-magnification image of the blood vessel vasculature through the cranial window (Fig. 16.1A) to ensure that the cell of interest can be located relative the blood vessel pattern.
- o0010 2. Pin the mouse to the dissection board, exposing its underside, and then, without delay, cut into the chest cavity to reveal the heart and lungs using surgical scissors.
- o0015 3. Push the blunt perfusion needle (1" Straight Cannula Blunt End Tip, 15 gauge; Fisinin) into the left ventricle, taking care not to damage the walls of the heart. Fix the needle in place with a heart clamp.
- o0020 4. Perfuse the animal with 10 ml of isotonic PBS, with a perfusion pump, at rate of 12 ml/min followed immediately with fixative solution. To avoid stopping and restarting the pump to change solutions, backfill the tubing with PBS, after the entire tubing is initially filled with fixative.
- o0025 5. Perfuse the animal with 300 ml of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The perfusion takes

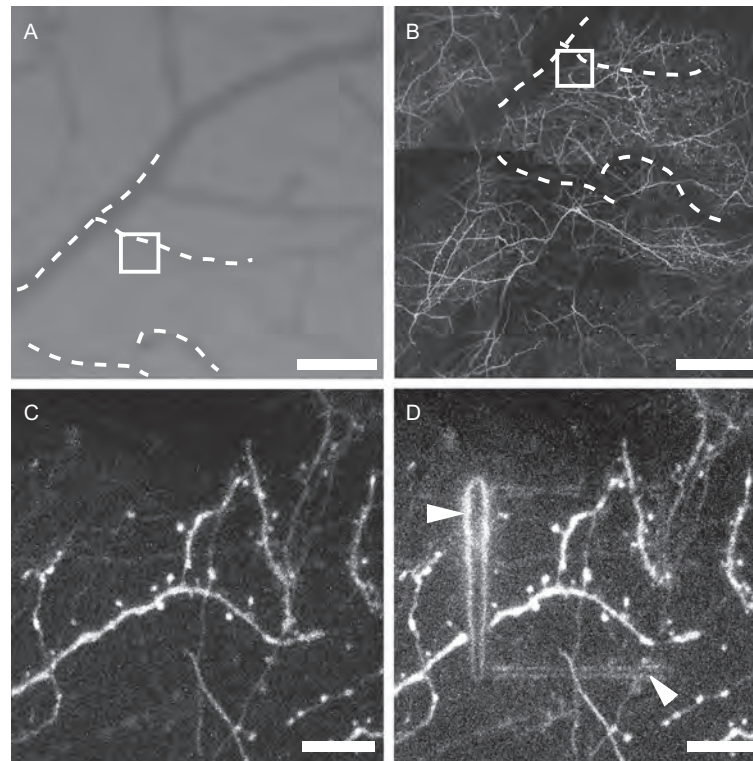


FIGURE 16.1
In vivo imaging and near-infrared branding (NIRB) of the fiducial laser marks. (A) Blood vasculature on the surface of the brain, seen through the cranial window. The pattern of the blood vessels (white dotted lines) is seen in 2-photon microscope (B) and is used to locate the region of interest (white square). (C) High-magnification image of the region of interest with GFP-labeled neurites indicated by white square in (A) and (B). (D) 2-Photon laser was used to burn the fiducial laser marks (white arrowheads) around the structure of interest on the fixed vibratome section containing the region of interest previously imaged *in vivo*. Scale bar in (A) and (B) is 100 μm , and in (C) and (D) is 10 μm .

Modified from Maco et al. (2013); reproduced with permission.

approximately 25 min, and once completed, the animal is then left for a further 90 min at room temperature, and sealed inside a plastic bag.

6. Dissect the brain from the skull using thin, blunt forceps, taking care not to damage the brain tissue. Once the brain is removed immerse in PBS.

Vibratome sectioning

7. Sink the brain in molten agarose (5%, in PBS) that has been cooled to 30 °C, and manipulate its position until the region of the cranial window is approximately

344 CHAPTER 16 2-Photon Imaging and FIBSEM

positioned uppermost. Then place in the fridge for 10 min or until the agarose sets.

- o0040 8. Trim the agarose block, containing the brain, so that the area of cortex imaged through the cranial window is parallel to the upper surface.
- o0045 9. Glue the agarose block to the vibratome support using cyanoacrylate glue.
- o0050 10. Vibratome section 60- μ m thick slices (Leica VT1200S, Leica Microsystems) from the region of the cranial window making sure that the sections are cut in the same plane as the focal plane as the 2-photon microscope. Cut sections picked up with a paint brush and place sequentially in a 24-well culture plate.

s0020 **16.1.2 LASER FIDUCIAL MARKS AROUND REGION OF INTEREST (NIRB TECHNIQUE)**

p0105 As the protocol relies on making fiducial marks in the fixed tissue, it is crucial that there is enough visible fluorescent signal after using this type of fixative with a high concentration of glutaraldehyde (2.5%). Without any label to see the presence of the fluorescent signal in the electron microscope, the neurites of interest need to be pinpointed together with fiducial marks. This gives their relative position allowing them to be found in the serial images.

p0110 The number of NIRB marks made depends on how many features are already present in the slice. If there is a clear pattern of blood vessels, for example, it would only be necessary to make a single square around the neurites. However, if natural landmarks are less obvious, it may be necessary to include larger burn marks around the first. These help only in finding the small set of marks in the resin-embedding section. It may also be useful to make NIRBs that form an asymmetric shape which will indicate from which side the section should be imaged and attached to the block resin slab.

s0025 **16.1.2.1 Method**

Localization of neurites of interest on the fixed vibratome section

- o0055 1. Using a dissecting microscope (Leica M205C), capture images at approximately 12 \times zoom to visualize the blood vessels. These are most prominent in the first sections cut from the surface of the brain.
- o0060 2. Overlay each image, into a stack, and align them (e.g., Photoshop, ImageJ, or GIMP). This stack will reveal the complete pattern of blood vessels on the brain's surface.
- o0065 3. Match the image of the blood vessels from the fixed sections with the image of the blood vessel pattern taken of the live brain through the cranial window. This will reveal in which section to find the fluorescent structures of interest.
- o0070 4. Place the identified section on a glass microscope slide, with adequate PBS to ensure that it does not dry. Place a coverslip on top and seal with nail varnish.

Making the NIRB marks

- o0075 5. Identify the neurites of interest (Fig. 16.1B and C) using the 2-photon microscope. Then set the laser to a wavelength of 850 nm, with a power of

300 mW at the back focal plane of the objective. Here, a 2-photon laser scanning microscope equipped with a pulsed titanium sapphire laser (Chameleon Ultra II, Coherent) was used.

- o0080 **6.** In the line scan mode, with a speed of approximately 2 ms/line, burn stripes in the fixed tissue around the region of interest. Between 500 and 2000 sweeps were used until the marks became visible from the autofluorescence generated from the line edges (Fig. 16.1D). The more sweeps used, the wider the burnt stripe, and the further apart the two fluorescent edges. Make sure that the photomultipliers are switched off for this marking phase.
- o0085 **7.** NIRB marks are made around the neurites of interest in the form of a square or rectangle approximately 15–24 μm along one side (Fig. 16.1D). These are also placed approximately 3 μm above the neurites of interest in the Z-axis.
- o0090 **8.** Image this region, with the NIRB marks, using the 2-photon laser, and then again using transmitted light, to capture its position in relation to other features, such as blood vessels and cell bodies.

s0030 16.1.3 STAINING AND RESIN EMBEDDING

p0160 The staining and resin embedding part of this protocol uses a standard method with conventional reagents. This approach has been used for years by many laboratories. The most important consideration is to stain the membranes as fully as possible to give good contrast with FIBSEM, while being careful not to overstain the tissue that would make it difficult to view the laser marks in the embedded section using transmitted light microscopy. Therefore, the timings are important. Although thinner sections could be considered as a solution, these often do not remain flat during the dehydration process and end up with an undulating appearance in the final hardened resin. This gives regions of interest that are difficult to mount horizontally on the blank resin slab.

s0035 16.1.3.1 Method

o0095 Staining, dehydration, and resin embedding of the fixed sections

- o0095 **1.** Wash the section 3 \times for 5 min each change in 0.1 M sodium cacodylate buffer (pH 7.4), in a 20 ml glass scintillation vial mixing continuously on a rotating mixer.
- o0100 **2.** Stain in 1% osmium tetroxide and 1.5% ferrocyanide in 0.1 M sodium cacodylate buffer (pH 7.4) for 40 min.
- o0105 **3.** Stain with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 40 min.
- o0110 **4.** Rinse the sections in ddH₂O, 2 \times for 5 min each rinse.
- o0115 **5.** Stain section with 1% uranyl acetate in ddH₂O for 40 min.
- o0120 **6.** Rinse section in ddH₂O, 5 min.
- o0125 **7.** Dehydrate in increasing concentrations of ethanol: 2 \times in 50%, 1 \times in 70%, 1 \times in 90%, 1 \times in 95%, and 2 \times in 100% ethanol, 3 min each change.

Au6

346 CHAPTER 16 2-Photon Imaging and FIBSEM

- o0130 8. Infiltrate the sections with 1:1 mixture of Durcupan resin and 100% ethanol for 30 min.
- o0135 9. Infiltrate the section for 2×30 min with fresh resin each change.
- o0140 10. Exchange for fresh resin and agitate for 4 h.
- o0145 11. Arrange the section onto treated glass slide (coated with mold separating agent), add more resin and overlay a second glass slide to sandwich the section between.
- o0150 12. Polymerize the resin and section in 65 °C oven for 24 h and then separate the glass slides from the resin using a razor blade.

s0040 **16.1.4 PREPARATION OF THE BLOCK FACE AND MOUNTING THE BLOCK FOR FIBSEM**

p0230 The goal of the block preparation process is to have a block face in the microscope on which the observer can pinpoint the exact position of the NIRB marks and without having to undertake long periods of milling before the final image series is collected. This requires trimming with glass knives mounted in the ultramicrotome so that the NIRB marks lie close to the edge of the resin block and within $\sim 4 \mu\text{m}$ of its surface (Fig. 16.3F).

p0235 To help guide the trimming process, a laser-dissecting microscope can also be used to etch the block surface and mark the position of the NIRB marks within the block (Fig. 16.2C). These surface marks are visible with the stereomicroscope and also in the ultramicrotome. This means that the block does not need to be removed from its mounting and imaged under a transmitted light microscope to check each time the position of the required region.

p0240 To locate the exact region to be imaged with FIBSEM, a small indentation is also made each side of the final imaging face (Fig. 16.3C), as well as score marks running across the top of the block (Fig. 16.3D). The position of these marks are also carefully measured, in relation to the NIRB marks, as they will be the only indicators with which to place the final imaging window for the collection of the FIBSEM image stack.

s0045 **16.1.4.1 Method**

- o0155 1. Image the resin-embedded section in the light microscope to pinpoint the position of the NIRB marks (Fig. 16.2B).
- o0160 2. Etch the shape of the NIRB onto the surface of the resin section, using the pulsed laser of the laser dissection microscope (Fig. 16.2C). These marks serve to indicate the position of the NIRB marks inside the resin block.
- o0165 3. Cut a 4×20 mm strip of blank resin, with thickness of 1 mm, using a jeweler's saw, and rinse with water and then dry, to remove all the dust particles.
- o0170 4. Glue the section of interest, containing the NIRB mark, to the resin slab with a small drop of Durcupan resin and polymerize in the oven. The drop of resin should be small so that it does not raise the section too much from the surface of the slab (Figs. 16.2D and 16.3A).

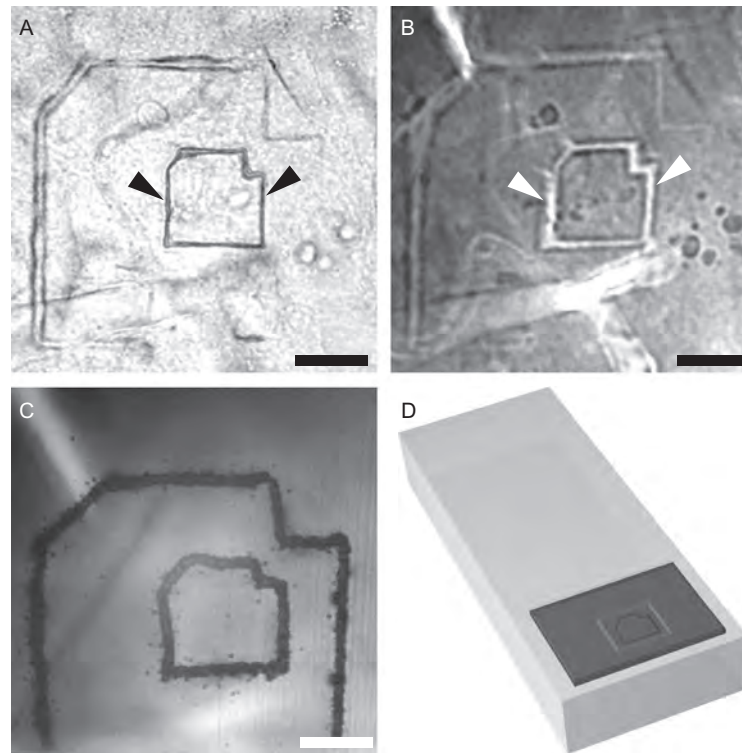


FIGURE 16.2

f0010

Tissue preparation for the FIBSEM. (A) Fiducial laser marks can be seen inside the fixed tissue section (black arrowheads) as well as after staining and embedded in resin (B, white arrowheads). (C) Using a laser-dissecting microscope, the original NIRB marks were replicated on to the top surface of resin-embedded sample to help indicate the region of interest during the trimming process. (D) The piece of resin, with the NIRB marked region of interest, was cut from the section and mounted onto a flat slab of empty resin providing a thicker support for easy manipulation during the trimming process. Note the asymmetric shape of the 2-photon laser marks used to distinguish the correct orientation, as well as the larger laser marks, used to facilitate the search and identification of the region of interest on the resin-embedded section. Scale bar: 20 μm .

o0175

5. Clamp the block in the ultramicrotome so that the embedded section with NIRB mark is held vertically.

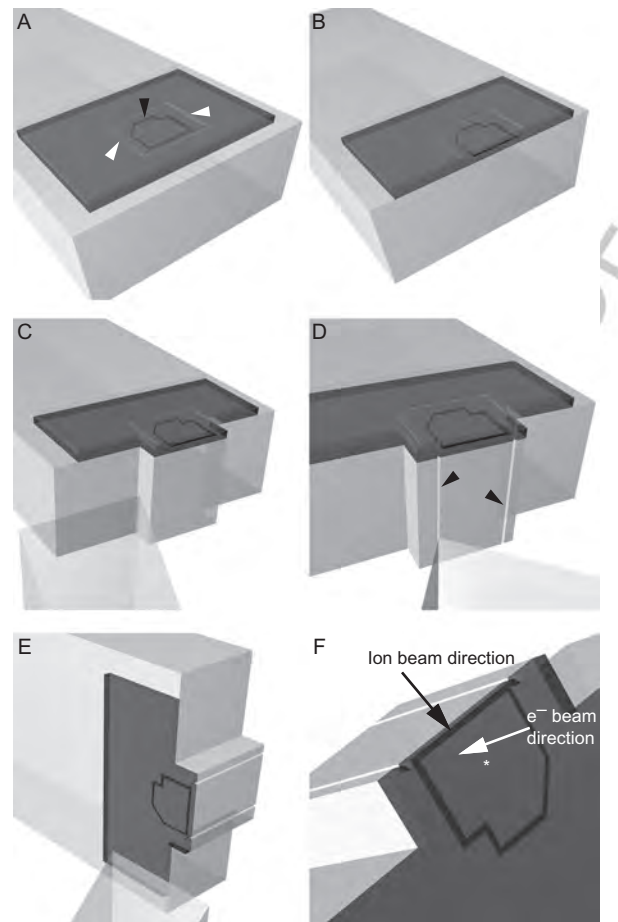
o0180

6. Trim away the resin, using a glass knife, so that the region of interest marked by the NIRB marks lies less than 10 μm from this front face (Fig. 16.3B). Use the etched surface of the slab to guide the trimming process, and avoid too much resin being removed.

o0185

7. Rotate the block so that the NIRB region is lying horizontally (Fig. 16.3C) and trim an indentation of <50 μm to the right and left of the region of interest,

348 CHAPTER 16 2-Photon Imaging and FIBSEM



f0015 **FIGURE 16.3** Trimming process and preparation of the block face for the FIBSEM. (A) Piece of resin-embedded section with 2-photon laser marks (black arrowhead) replicated on the top surface (white arrowheads) by the laser dissection microscope and mounted on the slab of empty resin. (B) The slab is then mounted on the ultramicrotome, and using the glass knife, one side is trimmed away until the NIRB marks are exposed at the edge. (C) To locate the region of interest inside the FIBSEM, indentations are made either side of the laser marks and (D) two score marks are made down the front edge (black arrowheads) serving as additional fiducial marks. These are easily seen in the electron microscope. (E) The block is then orientated to allow any excess resin above the NIRB marks to be removed. (F) The block is then mounted on a 45° inclined aluminum SEM stub, and coated with a protective layer of gold (approximately 30 nm thick) inside the FIBSEM. The focused ion beam is oriented parallel to the block face (black arrow) and about 54° relative to the electron beam (white arrow).

Au2

about 50 μm from the NIRB marks (Fig. 16.3C). Additionally, score marks can be made down the face of the block marking the position of the NIRB mark in the block face. This is achieved using the edge of the glass knife held at an angle of 45° (Fig. 16.3D).

- o0190 **8.** Hold the resin block in the jaws of the ultramicrotome holder so that the surface of the block, parallel to the NIRB mark, is also parallel to the edge of the glass knife. Trim away the resin from above the NIRB marks (Fig. 16.3E). Ensure that only approximately 5 μm of resin remains above the NIRB marks.

Mounting the block to SEM stub and gold coating

- o0195 **9.** Photograph this final block, using the light microscope, to show the position of the NIRB mark inside the resin block relative to the block's edges, the indentation made on the right-hand side of the block, and the score marks on the front edge.
- o0200 **10.** Match this final block image with the 2-photon image of the NIRB marks that also includes the structures of interest. These two images are used to precisely position the imaging face in the FIBSEM.
- o0205 **11.** Glue the final trimmed block to the scanning electron microscopy (SEM) stub, on the included surface, using carbon conductive cement.
- o0210 **12.** Place the stub in the sputter coater and deposit a 30 nm layer of gold over the surface.

s0050 16.1.5 FIBSEM IMAGING

p0310 FIBSEM images are taken from the resin-embedded tissue, at the same angle as the 2-photon images were collected in the live brain. This means that the block face is milled with the ion beam at the same plane as the focus plane of the 2-photon microscope (Fig. 16.3F). This results in imaging of the neurites of interest at the same orientation as they were imaged live.

p0315 The initial work in the FIB microscope is to roughly mill the block face according to the position indicated by the score marks, described above. Once the underlying tissue is exposed milling continues until other fiducial marks are seen, such as the NIRB marks. Once this has been achieved the block face can then be finally polished a protective layer deposited on the top surface of the block. This layer of platinum or carbon ensures a consistent milling without streaking and the curtaining effect.

p0320 Unfortunately, at this point it is not advised to immediately start capturing the final image series. During these milling steps, to prepare the final imaging window, the block has been heated and will drift while a thermal equilibrium is reached. For this reason, it is advisable to switch off the electron beam, close the valve of the ion beam, and leave the microscope for at least 2 h. During this time, a thermal and mechanical stability will have been reached and less drift of the block will occur.

p0325 During the imaging phase, accurate focus and consistent milling thickness is paramount. Although the thickness is given by the initial settings of the ion beam, in reality this can be different and needs to be calculated *post hoc*, using the cylindrical diameters method (Fiala & Harris, 2001).

350 CHAPTER 16 2-Photon Imaging and FIBSEM

s0055 **16.1.5.1 Method**

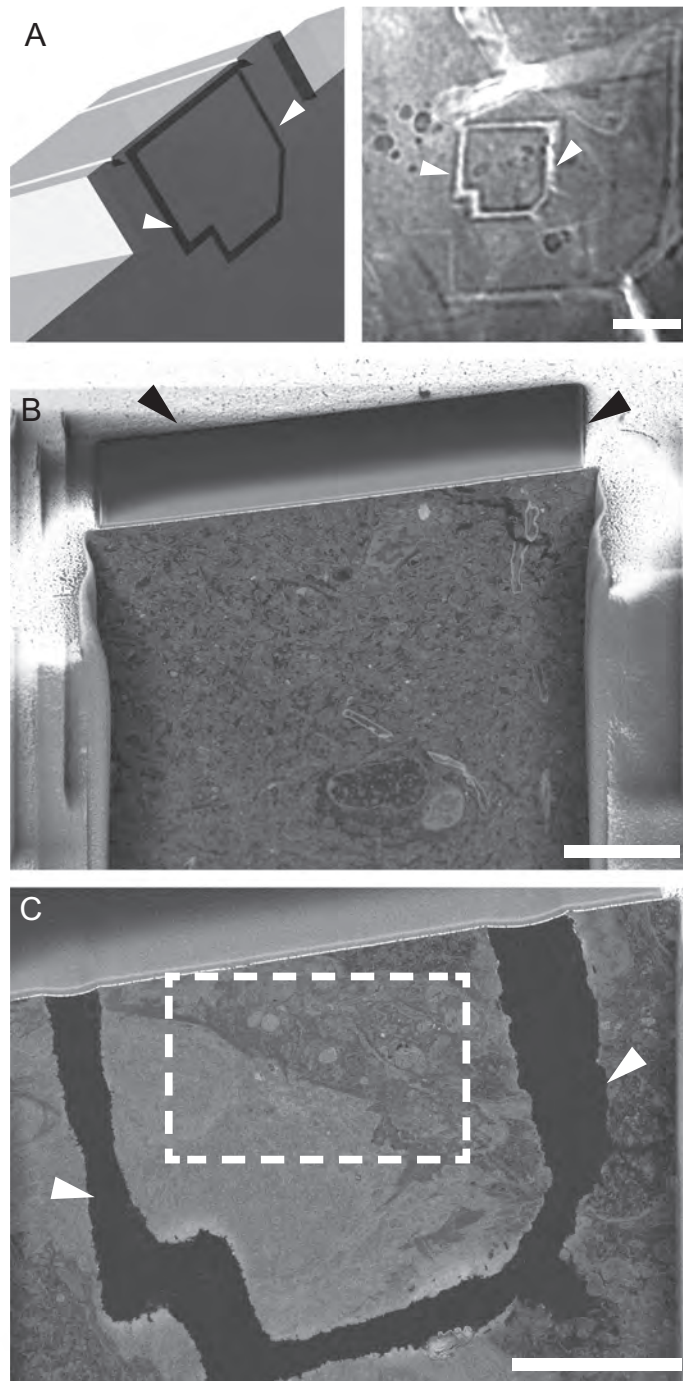
- o0215 1. Load the sample into the FIBSEM microscope.
- o0220 2. Position the ion beam so that it is parallel to the block face. As the face is already inclined at 45°, the SEM stub needs only to be tilted by around 10°, using the microscope stage to ensure the ion beam is parallel to this face. This imaging face is scanned with the electron beam during image acquisition.
- o0225 3. Locate the position of the NIRB marks along the imaging face using the images acquired during the block preparation.
- o0230 4. Mill this region of the face, about 60 μm wide, with an ion beam current of approximately 6 nA. The region of interest should be positioned in the center. Continue milling into the block until some underlying features in the tissue appear when imaging the face in the back-scattered imaging mode. These features, like blood vessels, or cells bodies, or even the NIRB marks themselves are used to calculate the exact position of serial milling and image acquisition.
- o0235 5. Deposit a protective layer of platinum (or carbon, 1-μm thick) onto the top surface of the block, above the region of interest. This patch of deposited material is approximately 30 μm wide and 10 μm deep (Fig. 16.4C).
- o0240 6. Finely mill the imaging face with a weaker ion beam of current 0.7–1.5 nA. This creates a more vertical and smooth face.
- o0245 7. Choose the exact region for sequential milling and imaging (approximately 15 × 10 μm) to capture the neurites of interest (Fig. 16.4).
- o0250 8. Set the ion beam current to 700 pA with a voltage of 30 kV and set the width of the milling window to three times wider than the imaged face. This ensures that no redeposition occurs on the face to be imaged.
- o0255 9. With an electron beam current of 800 pA, voltage of 1.5 kV, and grid tension on the ESB detector (Zeiss microscopes only) of 1.3 kV start imaging the block face.
- o0260 10. Acquire images with pixel size of 4–5 nm per pixel and dwell times of around 10 μs. Mill the face with a thickness of around 5–10 nm each slice.
- o0265 11. Use microscope dynamic focus, image tracking, and tilt correct functions to keep the images focused while the block face retreats during sequential milling, and in focus down the entire face of the tilted sample.

s0060 **16.1.6 3D RECONSTRUCTION AND ANALYSIS**

p0390 The analysis part begins with finding the neurites in the serial images. This can be achieved simply by overlaying the first EM image onto the fluorescent NIRBs image that also includes the neurites of interest. This will show the location, in x and y , at which the different neurites will be seen in the EM image stack.

p0395 The 3D models of the neurites of interest are generated from the image stack with the Ilastik software (Ilastik.org). This uses advanced watershed algorithms and allows the user to carry out segmentations of selected features, and importantly, to rapidly make corrections to the generated 3D models when mistakes are made (Ilastik.org; Fig. 16.5A and B). The small spacing in the Z direction optimizes the accuracy of the reconstruction and reduces the number of errors. Here, the protocol explains

B978-0-12-801075-4.00016-1, 00016



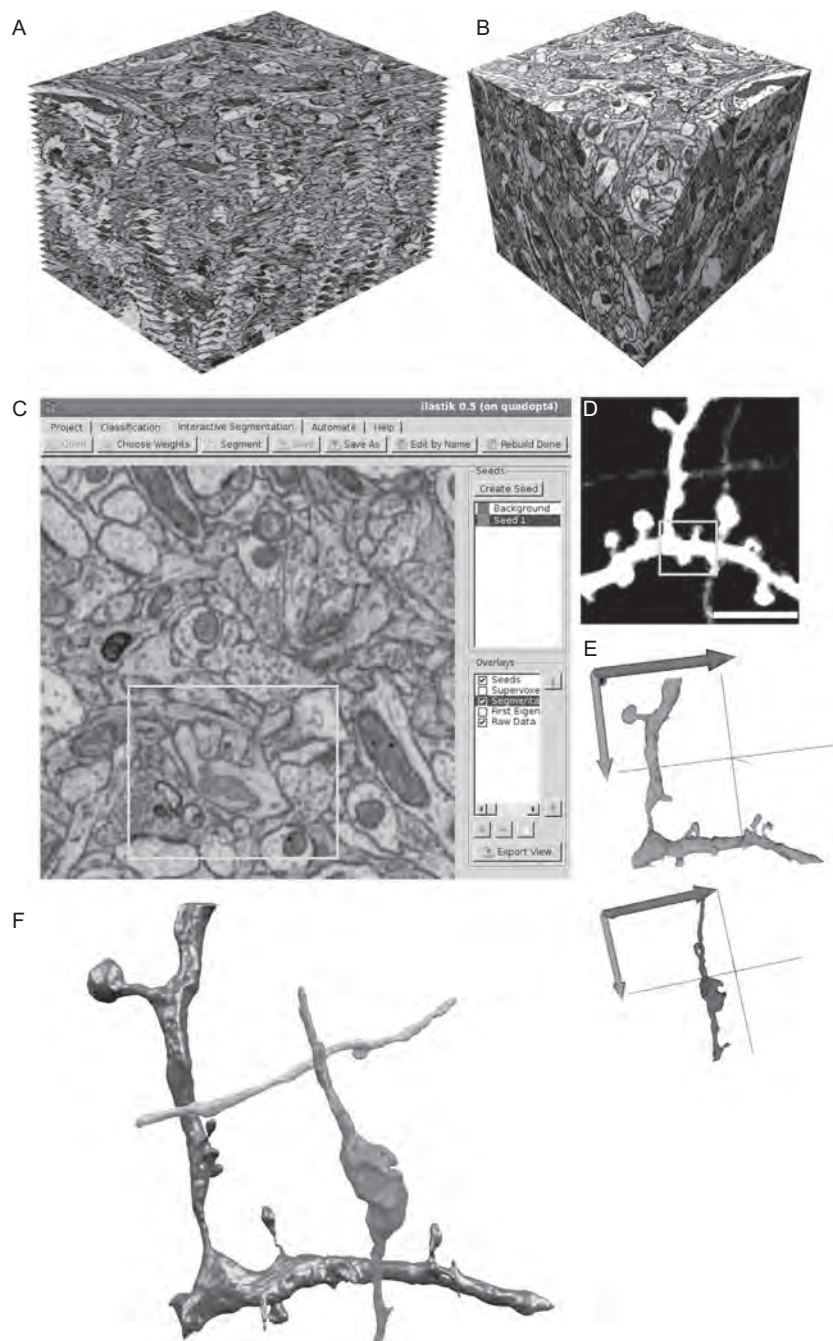
f0020

FIGURE 16.4

FIBSEM imaging. (A) A model representing the orientation of the block inside the FIBSEM (left) and resin-embedded tissue (right) with visible NIRB laser marks (white arrowheads). (B) The block face showing the edge of the block and the trench milled out by the ion beam imaged by back-scattered electrons showing the embedded tissue in the block. The top face of the block is coated with a layer of carbon (arrowheads). (C) The milled face covers the entire region of the NIRB marks (arrowheads) and the images were collected from a smaller area contained within (indicated with the dotted square). Scale bars: in (A) is 20 μm , and in (B) and (C) is 10 μm .

MCB, 978-0-12-801075-4

352 CHAPTER 16 2-Photon Imaging and FIBSEM



f0025

FIGURE 16.5

Segmentation and 3D reconstruction. FIBSEM imaging results in aligned serial images (A) producing the image volume with near-isotropic voxel properties (B) through which the

how seeds are scribbled into the neurites of interest on a single image from any orthogonal plane, with background seeds added to indicate the regions for exclusion. The program grows these seeds until they fill the entire volume of the selected neurite and a reconstruction is then produced within a few seconds. This can be refined and corrected by adding additional seeds.

p0400 The final 3D model is exported in a wavefront .obj file format that can be imported into the Blender modeling software (www.blender.org). A set of tools have recently been constructed for this software, named NeuroMorph, that enable the measurement and analysis on the models in the 3D space (<http://cvlab.epfl.ch/NeuroMorph>; http://wiki.blender.org/index.php/Extensions:2.6/Py/Scripts/Neuro_tool). The toolset is composed of three tools or add-ons: Import, Measurement tools, and Image Stack Interactions. The Import tool opens the models in the Blender software with the correct size, calibrated to known units (microns). The Measurement tool allows the models to be analyzed and such parameters as surface area, volume, and length to be made of any part. The measurements are stored together with each object, and when exported appear in a single-character separated values (CSV) text file (.txt), hierarchically organized. Each value appears as a child of the original object that can be easy ordered in a spreadsheet program.

p0405 Finally, the Image Stack Interactions tool allows the models to be viewed in 3D, but also with the original EM images. These are placed at the correct position relative to the model. The user can scroll through the images and all images are shown at their current position in the z depth of the volume. Additionally, the image plane can also be used to select other objects that may have been reconstructed, but are not visible. This allows the user to search for other objects, using the EM images are maps.

s0065 **16.1.6.1 Method**

Alignment of the serial images

- o0270 1. Using open-source free FIJI software package (<http://fiji.sc/wiki/index.php/Fiji>), combine individual sequential images in tiff image format into a single stack by selecting “File”—“Import”—“Image Sequence” command.
- o0275 2. Select image slice in the middle of the stack as reference for stack alignment by selecting “Plugins”—“Registration”—“StackReg” command using the transformation option “translation only.”

sp0025 **FIGURE 16.5—Cont'd** features of interest can be visualized using arbitrary imaging planes. (C) The Ilastik program was used to interactively segment the structures of interest using seeded watershed segmentation. An image from the stack shows green marks made by the user to indicate the structures of interest (object) and red marks for the background. The yellow rectangle indicates the part of the segmented structure of GFP-labeled dendrite shown on 2-photon image (D). (E) After the segmentation process, the 3D models of the reconstructed dendrite (green) and axon (blue) are displayed inside the Ilastik program. (F) The final model of the GFP-labeled neurites is rendered using the Blender 3D modeling software (in gray is the dendrite; axons are in green and orange). Scale bar in (D) is 5 μm . (See the color plate.)

Modified from Maco et al. (2013); reproduced with permission

354 CHAPTER 16 2-Photon Imaging and FIBSEM

- o0280 3. If necessary, crop the aligned stack by using Rectangular selection tool and “Image”—“Crop” command.
- o0285 4. Invert image contrast by selecting “Edit”—“Invert” command and adjust image contrast by selecting “Image”—“Adjust”—“Brightness/Contrast” command.
- o0290 5. Save processed stack as sequence of individual 8 bit, gray-scale images by selecting “File”—“Save As”—“Image Sequence” command.
- o0295 6. Determine the exact section thickness to measure the cylindrical object (e.g., mitochondria) diameter by dividing the number of slices it spans using the cylindrical diameters method (Fiala & Harris, 2001).
- o0300 7. Identify the exact position of the neurites of interest within the image volume by overlaying and aligning their 2-photon image including the NIRB marks with the known position of the NIRB marks on the block face.

Semi-automated segmentation with Ilastik

- o0305 8. Segment the structure of interest and create a 3D model using the seeded watershed segmentation algorithm within the Ilastik software (version 0.5.12; <http://ilastik.org>). Create the new project by selecting “Project” tab—“New.” In “Project” window, select “Load Stack” and choose the folder containing the sequential images of the aligned stack. The entire stack is displayed in orthogonal view with the possibility to scroll through the stack in any of the x - y , x - z , or y - z planes.
- o0310 9. To specify a set of features required to identify the membrane boundaries select the “Classification” tab—“Select Features” button. In the opened “Spatial Features” window, select “Texture” (includes the Eigenvalues of the Hessian matrix as a membrane boundary indicator). Set the scale of the features to “Medium” (representing the sigma value of 1.0). The selected set of features for the entire image stack is calculated.
- o0315 10. Switch to “Seeded Watershed” tab and import previously calculated Eigenvalues of 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. The supervoxels graph used for watershed segmentation for the entire image stack is calculated.
- o0320 11. Select “Seed 1” (as default in green color) in “Seeds” preview window, adjust brush size (“M” key increase and “N” key decrease) and draw a line inside the structure of interest. Select the “Background” (as default in red color) and mark the outside of the selected structure or background seed (Fig. 16.5C). Note that the lines representing the seeds can be placed from any displayed planes of the orthogonal view. Additional seeds (in different colors) can be added using the “Create Seed” button or deleted by right-clicking on the selected seed in the list on the “Seeds” preview window.
- o0325 12. Select the “Segment” button to start the segmentation. The selected structure is colorized as “Segmentation” overlay in the “Overlays” preview window. The segmentation can be iteratively refined by placing more foreground and background seeds, or adding seeds for other objects. The “Bias” dialogue field

allows to tune the growth of the background seeds more (values around 0.8) or less (values around 0.97) aggressively than the default.

- o0330 **13.** Display the rendered 3D model of the segmented structure by right-clicking on the “Segmentation” overlay and select “Display in 3D” (Fig. 16.5E).
- o0335 **14.** Export the final model as a wavefront OBJ mesh file by selecting the “Export” button to render high-quality images, or movies, using the Blender 3D modeling software (version 2.69; Blender Foundation; <http://www.blender.org>) (Fig. 16.5F).

3D analysis using NeuroMorph tools in Blender

- o0340 **15.** Install the NeuroMorph tools into the Blender by selecting the “File”—“User Preferences.” Under the “Addons” tab, select “Install from File” button and locate where the addon files are stored. Select newly installed addon by checking the right-hand box. Click “Save User Preferences” button and close the “User Preferences” window. Repeat this procedure for all addon files.

Import tool

- o0345 **16.** Under the “Scene” context panel, calibrate the scene by checking left-hand box “Microns Per Pixel” and define the pixel size in microns (Fig. 16.6A).
- o0350 **17.** Check the left-hand box “Use Remesh” to apply the octree remesh modifier to imported object. This modifier reduces the number of vertices and memory allocation, but still preserves the object shape. This can be tuned in “Remesh resolution” field (higher number generates denser meshes and increases the memory allocation).
- o0355 **18.** To start import, click the “Import” button and locate the folder with stored OBJ files. User can select multiple files which are then loaded and displayed in Blender.
- o0360 **19.** Imported objects are listed in the “Outliner” context panel. Select the object and in the “Properties” context panel select “Modifier” tab. Further “Remesh” modifier optimization can be tuned. By pressing “Apply” button the modifier is applied.

Surface area/volume measurement

- o0365 **20.** Define the type of structure to be measured in the “Measurement Tools” under “Object Name” select the “Object type” pop-up menu with predefined categories: spine, bouton, synapse, dendrite, axon, and other to define any new category (Fig. 16.6B and C).
- o0370 **21.** To measure the surface area and/or volume of the chosen structure, with the right mouse button (RMB) select the object and switch to “Edit” mode using the “Tab” key.
- o0375 **22.** Activate “Vertex select” button and deactivate “Limit selection to visible” button (both located at the bottom panel of the 3D view window).

356 CHAPTER 16 2-Photon Imaging and FIBSEM

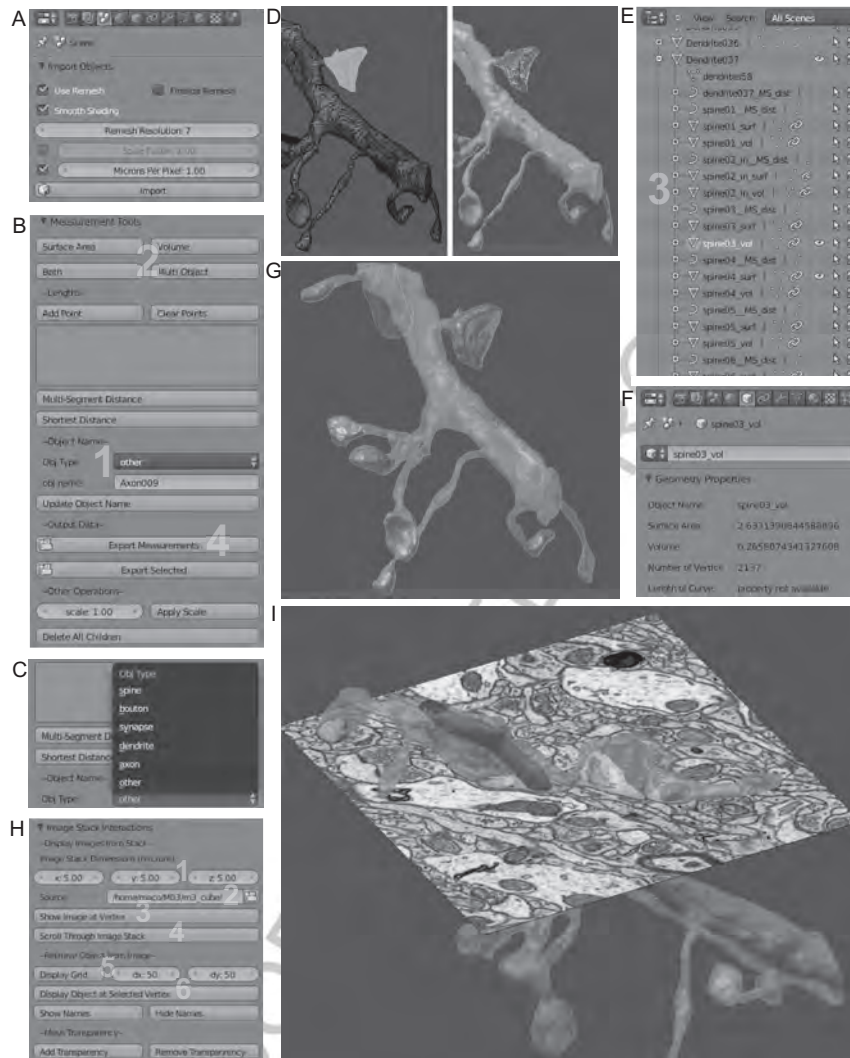


FIGURE 16.6

f0030

NeuroMorph import, Measurement and Image Stack Interaction toolset for Blender. (A) The mesh files created by Ilastik are imported into Blender using the Import tool that also calibrates the size of the models into a micron scale. During the import process, an algorithm (remesh modifier) is applied to reduce the number of vertices that comprises the mesh object while also preserving the objects shape. (B) The Measurement tool is able to name the objects being analyzed (here the names of neuronal structures are used: spine, bouton, synapse, dendrite, axon, or other; indicated with yellow 1 (light gray in the print version), and in C). After selecting the vertices of the chosen structure, the type of measurement is selected (yellow 2 (light gray in the print version)). The results can be exported into a text file for further analysis (yellow 4 (light gray in the print version)). (D) A model of dendrite with selected

- o0380 **23.** Using lasso selection, by simultaneously pressing “Ctrl” key and the left mouse button (LMB), select vertices of the chosen structure. Alternatively, “Border” selection can be used by pressing “B” key and with LMB select the region. Selected vertices are highlighted in orange (Fig. 16.6D).
- o0385 **24.** In the “Measurement Tools” the user can select the type of measurement by activating “Surface Area” or “Volume” buttons separately or the “Both” button to perform both measurements simultaneously (Fig. 16.6B). Note that for each measurement category a new child object is created and listed in the “Outliner” context panel (Fig. 16.6E). For the surface area, an open mesh named “surf” and for the volume a close mesh named “vol” are created. The measurement values are displayed in the “Properties” context panel under the “Object” tab and in the “Geometry Properties” section (Fig. 16.6F).

Length measurement

- o0390 **25.** To measure the length, select the object with RMB and switch to “Edit” mode “Tab” key.
- o0395 **26.** Activate both “Vertex select” and “Limit selection to visible” buttons (both located at the bottom panel of the 3D view window).
- o0400 **27.** Select points (vertices) along the object to be measured, using RMB, adding each point into the list by selecting “Add Point” button (or “Ctrl S” shortcut).
- o0405 **28.** Activate “Multi-Segment Distance” button to measure the length from multiple points. The new child object named “MS_dist” is created and listed in the “Outliner” context panel. The length values are displayed in the “Properties”

sp0030 **FIGURE 16.6—Cont’d** vertices that define a dendritic spine (highlighted in orange (light gray in the print version), left panel) is measured, creating a new mesh object (3) listed in the Outliner window (E). Other structures (colorized in magenta (gray in the print version)) on this dendrite model, and shown in the Object mode, can be measured (G), to form many mesh objects (shown in the Outliner in (E)). (F) The result of these measurements is displayed in the “Properties” context panel under the “Object” tab and in the “Geometry Properties” section. (H) The Image Stack Interaction tool is able to display the EM image (size is defined in microns; yellow 1 (light gray in the print version), and from the correct directory; yellow 2 (light gray in the print version)), from which the model is derived, at the correct position in the model (displayed in Edit mode after clicking on the button indicated with yellow 1 (light gray in the print version)). User can also scroll through images of the entire stack (yellow 4 (light gray in the print version)). The displayed image can also be used to retrieve the 3D model of any structure (object) by selecting the points density (in pixels) and displaying the grid over the image (5). Then by selecting any grid point(s), the corresponding object(s) is displayed at selected vertex (6). (I) Retrieved objects based on the selection the grid points are displayed: presynaptic bouton (in transparent green (light gray in the print version)), axonal mitochondrion (in green (dark gray in the print version)), synapse (in red (dark gray in the print version)), and dendritic mitochondrion (in magenta (dark gray in the print version)). The initial dendrite is shown in transparent magenta (light gray in the print version).

358 CHAPTER 16 2-Photon Imaging and FIBSEM

context panel under the “Object” tab and in the “Geometry Properties” section under Length of Curve. Alternatively, for shorter or relatively straight segments, adding two points (start–end) to the list is sufficient for length measurement by selecting “Shortest distance” button. The new child object named “2p_dist” is created and listed in the “Outliner” panel (Fig. 16.6E).

- o0410 29. When finished with length measurement, remove the points from the list with “Clear points” button.

Export measurement results

- o0415 30. Measurement results can be exported into a single CSV text file (.txt) for further analysis with a spreadsheet program (e.g., Excel, Open Office, Numbers). By activating the “Export Measurements” button in the “Measurement Tools” under “Output Data” section, user specifies the destination folder and the file name. All measurement results for all analyzed objects present in the blender file are exported and written to the text file.

- o0420 31. Alternatively, the measurement results for only selected object(s) can be exported by activating the Export Selected” button.

Image Stack Interaction tool

- o0425 32. This tool allows the interaction of the reconstructed model with the original EM images (Fig. 16.6H and I). In the “Display Images from Stack” section under “Image Stack Dimensions,” specify the reconstructed volume (in microns) size in x , y , and z dimensions in appropriate fields.

- o0430 33. In the “Source” section, locate and activate folder button (right-hand side) to select the storage folder with the sequential images of the volume stack used for the reconstruction.

- o0435 34. To overlay the model with the image, select the object with RMB and switch to edit mode pressing “Tab” key.

- o0440 35. Select any vertex from the mesh and activate “Show image at Vertex” button. The image from the volume stack at the position of selected vertex is placed to the scene (called “Empty” with EM image as a texture). Note: user can selected multiple vertices (RMB with “Shift” key) in which case for each selected point, the image plane is added.

- o0445 36. To see the EM image through the object, transparency can be changed by selecting the object with RMB and activating the “Add Transparency” button in the “Mesh Transparency” section. In the field “Alpha,” the opacity intensity can be adjusted as well as the color can be changed from the “Color Palette” button (next to “Alpha” button). To remove the transparency of the object, select “Remove Transparency” button (Fig. 16.6H).

- o0450 37. By selecting the image plane (or Empty), the user is able to scroll the image plane up and down throughout the whole volume by activating the “Scroll Through Image Stack” and by using mouse middle button. This shows each

- image from the original stack at its correct position relative to the model. Pressing the “Esc” key deactivates this scrolling function.
- o0455 **38.** The image plane can be used to display any additional object in the scene based on the current image. Select the image plane (RMB) and define the grid density (distance between grid points in pixels) in x - and y dimensions in the “dx” and “dy” fields located under “Retrieve Object” from Image section.
- o0460 **39.** By activating the “Display Grid” button, the mesh grid is overlaid over the image plane. Select any point (RMB) or multiple points (RMB with “Shift” key).
- o0465 **40.** To display the object based on grid point selection, activate the “Display Object at Selected Vertex” button.

CONCLUSION

s0070

p0615 Recent years have seen significant advances in EM imaging methods for capturing volumetric data on a large scale. In particular, the rise of block-face scanning approaches now offers many opportunities for automated serial imaging. These are likely to improve, with more efficient detectors achieving better signal to noise ratios giving faster imaging rates. More automated procedures that ensure consistent image quality for longer periods will improve. All this will allow users to image more samples and allow these microscopes to be available to more users. Correlating light microscopy data with structural information from the electron microscope will, therefore, become increasingly more prevalent.

p0620 The protocol presented here circumvents a number of the steps that have made CLEM methods using transmission electron microscopy an exception rather than the norm. In many places, it reduces considerably the need of highly skilled hands to carry out such tasks as cutting, collecting, and imaging many hundreds of serial sections. It also describes how to use a freely available set of software tools, useable on any computer platform, to rebuild automatically, in the computer, the various targeted structures, as well as carry out detailed morphometric analysis.

p0625 The computer vision research providing the segmentation and reconstruction tools, described here is, however, dependent on large and varied images, as well as ground truth segmentations to develop and test their algorithms. As more laboratories use these imaging methods, more datasets becomes available and better software will lead to more opportunities to analyze larger image volumes, and reducing the time needed to proofread and correct the results. This will inevitably lead to greater insights into the structure and function of biological systems.

ACKNOWLEDGMENTS

- ac0005
- p0630 This work was supported by the Swiss National Foundation Synergia project grant CRF I1313470/1 (G. W. K.) and project grant 31003A_135631 (A. H.).

REFERENCES

- Allegra Mascaro, A. L., Cesare, P., Sacconi, L., Grasselli, G., Mandolesi, G., Maco, B., et al. (2013). In vivo single branch axotomy induces GAP-43-dependent sprouting and synaptic remodeling in cerebellar cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 10824–10829.
- Becker, C., Ali, K., Knott, G., & Fua, P. (2013). Learning context cues for synapse segmentation. *IEEE Transactions on Medical Imaging*, *32*, 1864–1877.
- Bishop, D., Nikic, I., Brinkoetter, M., Knecht, S., Potz, S., Kerschensteiner, M., et al. (2011). Near-infrared branding efficiently correlates light and electron microscopy. *Nature Methods*, *8*, 568–570.
- Cane, M., Maco, B., Knott, G., & Holtmaat, A. (2014). The relationship between PSD-95 clustering and spine stability in vivo. *The Journal of Neuroscience*, *34*, 2075–2086.
- Canty, A. J., Huang, L., Jackson, J. S., Little, G. E., Knott, G., Maco, B., et al. (2013). In-vivo single neuron axotomy triggers axon regeneration to restore synaptic density in specific cortical circuits. *Nature Communications*, *4*, 2038.
- Fiala, J. C., & Harris, K. M. (2001). Cylindrical diameters method for calibrating section thickness in serial electron microscopy. *Journal of Microscopy*, *202*, 468–472.
- Grillo, F. W., Song, S., Teles-Grilo Ruivo, L. M., Huang, L., Gao, G., Knott, G. W., et al. (2013). Increased axonal bouton dynamics in the aging mouse cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, E1514–E1523.
- Holtmaat, A., Wilbrecht, L., Knott, G. W., Welker, E., & Svoboda, K. (2006). Experience-dependent and cell-type-specific spine growth in the neocortex. *Nature*, *441*, 979–983.
- Knott, G. W., Holtmaat, A., Trachtenberg, J. T., Svoboda, K., & Welker, E. (2009). A protocol for preparing GFP-labeled neurons previously imaged *in vivo* and in slice preparations for light and electron microscopic analysis. *Nature Protocols*, *4*, 1145–1156.
- Knott, G. W., Holtmaat, A., Wilbrecht, L., Welker, E., & Svoboda, K. (2006). Spine growth precedes synapse formation in the adult neocortex *in vivo*. *Nature Neuroscience*, *9*, 1117–1124.
- Kreshuk, A., Straehle, C. N., Sommer, C., Koethe, U., Cantoni, M., Knott, G., et al. (2011). Automated detection and segmentation of synaptic contacts in nearly isotropic serial electron microscopy images. *PLoS One*, *6*, e24899.
- Lucchi, A., Smith, K., Achanta, R., Knott, G., & Fua, P. (2012). Supervoxel-based segmentation of mitochondria in em image stacks with learned shape features. *IEEE Transactions on Medical Imaging*, *31*, 474–486.
- Lucchi, A., Smith, K., Achanta, R., Lepetit, V., & Fua, P. (2010). A fully automated approach to segmentation of irregularly shaped cellular structures in EM images. *Medical Image Computing and Computer-Assisted Intervention*, *13*, 463–471, International Conference on Medical Image Computing and Computer-Assisted Intervention.
- Maco, B., Holtmaat, A., Cantoni, M., Kreshuk, A., Straehle, C. N., Hamprecht, F. A., et al. (2013). Correlative *in vivo* 2 photon and focused ion beam scanning electron microscopy of cortical neurons. *PLoS One*, *8*, e57405.
- Mostany, R., Anstey, J. E., Crump, K. L., Maco, B., Knott, G., & Portera-Cailliau, C. (2013). Altered synaptic dynamics during normal brain aging. *Journal of Neuroscience*, *33*, 4094–4104.
- Schultz, R. L., & Karlsson, U. (1965). Fixation of the central nervous system for electron microscopy by aldehyde perfusion. II. Effect of osmolarity, pH of perfusate, and fixative concentration. *Journal of Ultrastructure Research*, *12*, 187–206.

- Straehle, C. N., Köthe, U., Knott, G., & Hamprecht, F. A. (2011). Carving: Scalable interactive segmentation of neural volume electron microscopy images. *Medical Image Computing and Computer-Assisted Intervention*, 14, 653–660.
- Tao-Cheng, J. H., Gallant, P. E., Brightman, M. W., Dosemeci, A., & Reese, T. S. (2007). Structural changes at synapses after delayed perfusion fixation in different regions of the mouse brain. *The Journal of Comparative Neurology*, 501, 731–740.
- Trachtenberg, J. T., Chen, B. E., Knott, G. W., Feng, G., Sanes, J. R., Welker, E., et al. (2002). Long-term *in vivo* imaging of experience-dependent synaptic plasticity in adult cortex. *Nature*, 420, 788–794.
- Zito, K., Parnas, D., Fetter, R. D., Isacoff, E. Y., & Goodman, C. S. (1999). Watching a synapse grow: Noninvasive confocal imaging of synaptic growth in *Drosophila*. *Neuron*, 22, 719–729.

UNCORRECTED PROOF

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-801075-4.00016-1, 00016

Non-Print Items

Keywords: *In vivo* imaging, 2-Photon microscopy, CLEM, Focused ion beam SEM, FIB-SEM, Segmentation, 3D reconstruction, 3D model, Volumetric analysis, NeuroMorph tool, Laser branding, NIRB

UNCORRECTED PROOF

MCB, 978-0-12-801075-4