

Electron Tomography for the Study of Synaptic Ultrastructure in Fixed Brain Sections

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Abstract

Synaptic function depends upon interactions among sets of proteins that assemble into complex machines. Molecular biology, electrophysiology, and live-cell imaging studies have provided glimpses into the inner workings of the synapse, but the functional organization of these supramolecular nano-assemblies remains obscure. Electron tomography reveals the internal structure of synapses in three dimensions with exceptional spatial resolution. We here describe an approach to the study of ultra-structure that relies on plastic-embedded aldehyde-fixed material stabilized with tannic acid instead of osmium tetroxide. This approach offers a new window into the structural basis of synaptic processing in the mammalian brain.

Key words Dendritic spine, Synapse, Actin, 3D visualization, Software, Electron tomography, Fixation, Embedding

1 Introduction

Structure provides the basis for physiological function, motivating continuing study of synaptic morphology. Notwithstanding dramatic recent advances in super-resolution light microscopy that permit visualization of features well below the classical Abbe diffraction limit of ~250 nm [1–3], transmission electron microscopy (TEM) still provides superior resolving power. High resolution is especially important for studying excitatory synapses in the mammalian forebrain, in view of their minuscule dimensions and the dense packing of supramolecular complexes crucial for their function.

TEM, which relies on exposure of thin sections to a focused electron beam inside a high vacuum chamber, requires specialized sample preparation procedures. Preparation of biological specimens involves inevitable compromises in an effort to maintain *in vivo* morphology while preserving structural detail; ideally, one would also like to maintain antigenicity, to permit the use of

immunocytochemical probes. There are important practical advantages to methods that are reasonably simple and robust; furthermore, one would prefer methods that minimize hazard to the histologist and the environment.

Physical cryo-fixation can stabilize cells within milliseconds, but limited thermal conductivity leads to unacceptable damage from ice crystals beyond a few micrometers from the site of freezing. Moreover, cryo-electron microscopy is inherently low contrast, and vulnerability to beam damage severely limits sample exposure. High-pressure freezing (which minimizes disruption of tissue by ice crystals) followed by low-temperature dehydration and freeze-substitution into acrylic plastic offers a more feasible approach to TEM study of neuronal structure, but current equipment limits samples to small ($\sim 1 \text{ mm}^3$) tissue blocks. Thus, high-pressure freezing is unsuitable for mammalian brain unless tissue is first dissected out, with inevitable surgical and hypoxic insult; for this reason, the approach has been limited to small invertebrates, peripheral tissue, and culture systems. TEM analysis of mammalian brain continues to rely on initial chemical fixation with mixed aldehydes, followed by postfixation with heavy metals, dehydration, and embedding into a suitable plastic resin. After polymerization of the plastic, thin sections may be cut with an ultramicrotome from a highly stable block.

Ultrastructural studies routinely use osmium tetroxide to stabilize and contrast lipid-rich membranes. However, OsO_4 is a hazardous volatile chemical, requiring special handling and disposal precautions. Moreover, osmium can lead to loss of protein from tissue [4, 5], and its oxidizing effect denatures antigens, making postembedding immunolabeling unfeasible for most proteins. Finally, intense staining of the osmiophilic lipid bilayer can hide subtle details of membrane substructure while obscuring trans-membrane proteins. Analysis of protein-rich compartments like the postsynaptic density (PSD) requires techniques that better preserve protein.

While TEM is physically capable of sub-nanometer resolution, technical constraints make it impractical to cut tissue sections thinner than $\sim 40 \text{ nm}$, much larger than most proteins. By generating virtual ultrathin sections, electron tomography (which relies on a computational strategy formally identical to that used for CAT scans) can greatly reduce the image degradation arising from finite section thickness [6–8]. This chapter describes our approach to electron tomographic analysis of synapses in the mammalian fore-brain, using an osmium-free protocol that provides excellent preservation, while emphasizing proteins over lipids to provide a “proteocentric” view of synaptic structure.

2 Materials

2.1 Fixative

Put 10 g paraformaldehyde (Sigma #P6148) into a flask with 500 ml of phosphate buffer (0.1 M pH 6.8). Heat with mixing (caution: use fume hood to exhaust toxic fumes); solution should begin to clear before boiling; continue until solution is almost entirely clear. Remove from hot plate and put in bucket with crushed ice until mixture reaches room temperature. Vacuum-filter to remove undissolved paraformaldehyde. Add 20 ml 50 % glutaraldehyde (EMS #16310, EM grade). Fixative should be used within 2 h.

2.2 Reagent Preparation

1. Sodium acetate, 0.1 M (store in refrigerator): sodium acetate trihydrate, 1.36 g; distilled water, 100 ml.
2. 1 % tannic acid in sodium acetate. The solution should be made fresh and kept in the dark.
3. 0.1 % calcium chloride. Make a 10 % calcium chloride dihydrate solution in water; for use, dilute at 1:100 in acetate buffer.
4. 1 % uranyl acetate + 0.1 % platinum tetrachloride in acetate buffer. May require prolonged stirring to dissolve; stable for several weeks in refrigerator.
5. Buffered 50 % ethanol: equal parts 100 % ethanol and 0.1 M sodium acetate. Note: buffer is necessary because tissue not treated with osmium is sensitive to osmotic pressure.
6. 100 % ethanol (freshly opened or stored over molecular sieves) (do not shake or insert pipet into the sieve layer).
7. Embedding resins: ERL 4221, 5 g; DER 736, 3 g; nonenylsuccinic anhydride, 9 g; dimethylaminoethanol, 0.17 g (available as "Spurr low-viscosity embedding kit" from Electron Microscopy Sciences). Weigh sequentially into plastic disposable beaker, cover tightly with foil, and stir gently on magnetic stir plate for 20 min. Transfer to plastic 20 ml syringes with tip caps. Wrap in foil and store in freezer; let come to room temperature before opening. Dedicated magnetic stir bar is cleaned by wiping off as much resin as possible with Kimwipe, then coating with liquid soap, working up a lather, and only then rinsing with water. Used beaker, pipets, paper, vials, etc., can be placed in oven to polymerize the resin and then disposed of in common trash, but unpolymerized resin is toxic.
8. Uranyl acetate solution: uranyl acetate, 0.5 g; distilled water, 50 ml; glacial acetic acid, 45 μ l. Make at least 2 h in advance. Store in refrigerator in foil-wrapped plastic syringes.

9. Sato's lead [9]: lead nitrate, 1 g; lead acetate, 1 g; lead citrate, 1 g; sodium citrate, 2 g; distilled water, 82 ml; 1 M sodium hydroxide, 18 ml (make fresh, limit air exposure). Rinse beaker with dilute sodium hydroxide. Boil distilled water for 10 min, cool slightly, add lead salts, and stir 3 min. Add sodium citrate and stir for another minute. Add the 18 ml fresh 4 % sodium hydroxide; the cloudy solution should start to clear. Limit air exposure while solution cools. Store in capped syringes in refrigerator. When staining, dispense drops through a fresh 0.2 μm syringe filter into a CO_2 -depleted staining dish (put a small container of sodium hydroxide pellets in the staining dish and keep the lid closed except when moving a grid). Note: uranyl and lead salts are persistent toxins and must be handled appropriately.
10. 12 nm colloid Gold-APure donkey anti-goat IgG (Jackson ImmunoResearch Laboratories).

2.3 Image Acquisition and Processing

1. FEI Tecnai G² Twin equipped with tomography package and a tilt range of $\pm 70^\circ$.
2. Apple Mac Pro (2 2.8 GHz Quad-Core Intel Xeon, 14 GB memory, NVIDIA GeForce 8800 GT).

3 Methods

3.1 Fixation

Satisfactory chemical fixation is a prerequisite for structural analysis. The concept is simple, but in practice the procedure can be technically challenging. Results are not entirely predictable even in experienced hands; consequently, unless the material is precious, it may be wise to recognize that only a fraction of the perfusions will yield satisfactory material. Our underlying principles are to minimize surgical hypoxia and then fix quickly with a minimum of physiological disruption. We use constant pressure (~ 1.2 m gravity feed) instead of a perfusion pump, since physiological perfusion is closer to a constant pressure than a constant flow system. We use isotonic saline (0.9 % NaCl in water) instead of buffer for the initial flush.

1. *Aldehyde reagents.* For optimal structure we normally use a mixture of 2 % glutaraldehyde and 2 % depolymerized paraformaldehyde in 0.1 M phosphate buffer, pH 6.8, at room temperature. Glutaraldehyde is a highly effective cross-linking agent almost indispensable for good structural preservation, but its exact concentration is of little consequence. Though a less effective fixative, formaldehyde penetrates tissue more quickly than glutaraldehyde, providing a rationale for the use of mixtures of glutaraldehyde and formaldehyde.
2. *Fixative solution.* The solution should be in buffer, since the fixatives can be quite far from neutral, and reactions with tissue

during the fixation process can further disturb the pH balance. Isotonic fixative would seem preferable, but tonicity is almost impossible to calculate properly, since the fixatives are somewhat membrane permeable. We use phosphate buffer for its stability and buffer efficacy; it is cheaper and far less toxic than cacodylate (a buffer formerly in wide use). There are theoretical advantages to some of the Good buffers (e.g., MOPS and PIPES [10]), but aside from their expense, direct evidence for their superiority is meager.

3. *Surgical procedure.* Our work uses rats and mice. We deliver an overdose of anesthetic (typically IP pentobarbital, 80 mg/kg); besides providing anesthesia, this attenuates possible vascular reflexes while lowering brain metabolism. When the animal becomes areflexic to noxious stimuli (e.g., paw pinch), it is placed on its back on a small platform inside a sink with all four paws held extended by adhesive tape. Biological fixatives are toxic; take appropriate precautions, including gloves and safety glasses; suitable arrangements must be made to protect the surgeon from toxic fumes. We run cold water to insure a clear operative field.

The chest is opened; the right atrium is cut open, and a small cut is made in the left ventricle. Saline is then allowed to flow through a small cannula (to ensure that no bubbles remain), which is carefully inserted into the ventricle. For adult rats, the cannula is gently pushed into the proximal aorta. Tear in the aorta would ruin the procedure, so for mice and baby rats (whose aorta is small and fragile), the cannula tip is simply left in the ventricle.

Once proper placement is assured (by visual inspection, effective flushing of blood, and initiation of rigor), the cannula should be clamped in place. After a brief saline flush (30 s–1 min), the saline is replaced by fixative. Assuming technical success, rigor should begin within ~30 s after onset of fixative flow, and the body should be hard and rigid within 10 min. After at least 10 min of fixation, the flow is stopped; we then immediately remove the brain, carefully dissecting and exposing it with scalpel and bone rongeurs, and then remove the brain, which is put into a vial of fixative at 4 °C for 12–24 h prior to cutting.

3.2 Preparation and Embedment of Fixed Tissue

1. A suitable block of fixed brain containing the area of interest must be prepared. We use a standard double-edged razor blade, broken in half, to cut a block of brain ~5 mm thick; for best cutting the block should have a pyramidal shape. The block is immersed in buffer in a glass petri dish. Large pieces of connective tissue (arteries, pia) are peeled off the block with jeweler's forceps under a dissecting microscope. The block is blotted quickly to remove excess liquid without allowing it to dry,

glued to the top of a rectangular holder with a thin coating of quick-drying cyanoacrylate glue (Krazy Glue), and put back in buffer. The holder is mounted in a Vibratome, and 50 μm sections are cut, collected, and stored in phosphate buffer in tissue culture trays. These sections may safely be stored for up to ~1 week at 4 °C with minimal structural degradation.

2. Stabilization and dehydration of the tissue sections is performed on a shaker, using the following steps. Low temperature helps to maintain structure; therefore, steps a–h are performed over crushed ice, and the subsequent steps over salted ice (~–15 °C).
 - (a) 2 $\times$ 5' in 0.1 M Na acetate
 - (b) 40' in 1 % tannic acid in 0.1 M Na acetate (tannic acid helps to stabilize membrane and cytoskeletal components and acts as mordant for subsequent heavy metals)
 - (c) 2 $\times$ 5' in 0.1 M Na acetate
 - (d) 20' in 0.1 % $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 0.1 M Na acetate (calcium ion also helps to stabilize membranes)
 - (e) 2 $\times$ 5' in 0.1 M Na acetate
 - (f) 40' in 1 % uranyl acetate + 0.1 % PtCl_4 in 0.1 M Na acetate (these heavy metal salts further stabilize ultrastructure while providing electron contrast)
 - (g) 2 $\times$ 5' in 0.1 M Na acetate
 - (h) 5' in 50 % buffered ethanol (equal parts of 100 % ethanol and 0.1 M Na acetate)
 - (i) 5' in 70 % ethanol (3 parts of 50 % buffered ethanol and 2 parts of 100 % ethanol)
 - (j) 5' in 80 % ethanol
 - (k) 5' in 95 % ethanol
 - (l) 2 $\times$ 5' in 100 % ethanol
3. Sections are then embedded in resin.
 - (a) 30' in 1:1 ethanol/Spurr resin (on a rotator at room temp.)
 - (b) 30' in 1:3 ethanol/Spurr (rotator/room temp.)
 - (c) 2 $\times$ 1h or overnight in Spurr (rotator/room temp.)
 - (d) Place a Kimwipe on a foil-backed folded paper towel. Place a slide on the Kimwipe, covered with a piece of Aclar plastic (7.8 mil thickness, EMS), cut slightly larger than the slide. Using a wooden applicator with a tapered tip, transfer a small amount of resin to the Aclar on the slide and spread it a little down the length (avoid making bubbles). Then use the applicator to transfer a section to the Aclar. It may be necessary to manually rotate the vial to get the section(s) to float up in the resin, so you can get the applicator under it

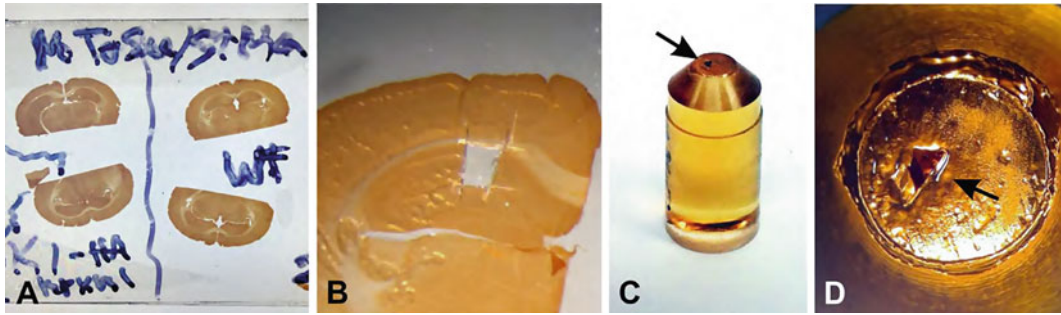


Fig. 1 Preparation of tissue block: (a) plastic-infiltrated brain sections on Aclar wafer, after polymerization. The color arises from deposition of heavy metals during processing. (b) Close-up shows a wafer-embedded section after a block of hippocampus has been cut out for sectioning. (c) A blank plastic block. Tissue block (arrow) has been glued to its upper surface. (d) Close-up showing block glued to the plastic blank

to make the transfer. Keep the sections away from the edges of the Aclar and do not overcrowd, because sections will migrate when the second piece of Aclar and the capping slide are placed on top of them. When applying the second Aclar, ease it on from one end and gently tap the sections to chase out air bubbles and to stop the migration. Once the second slide is in place, being careful not to lose orientation, wrap the Kimwipe tightly around the wafer and apply gentle pressure. The Kimwipe should absorb most of the excess resin while preventing the sections from oozing beyond the edges of the Aclar/slide. Remove the Kimwipe, wipe off any excess resin from the slide surfaces, and label as needed. Place in oven on foil, with a ~20 g weight on top.

(e) Cure 36–48 h at 60 °C

3.3 Grid Preparation

1. The wafer should be opened as soon as it is removed from the oven. Take a single edge razor blade and carefully work it between one slide and the Aclar, sliding it down the edges until the glass slide separates and can be removed. Then set the remaining wafer slide side down on a flat surface (making sure any labels or critical orientations are noted), and start separating the two pieces of Aclar at one end with a fingernail or blade. When you can get both index fingers between the Aclars, slide them down the length of the slide to separate the Aclars without excessive bending. The sections will stick to one or the other sheet of Aclar (or some on each). Transfer any labels, and note which side of the Aclar the tissue is on.
2. Under a dissecting microscope, use a scalpel (blades #11, 15, or 10 work well) to cut out the area of interest and then glue it to the surface of a blank block with Krazy Glue (Fig. 1). If the tissue cannot be removed without taking the Aclar as well, make sure to glue the tissue side to the block, not the Aclar side.

3. Colloidal gold particles are deposited on the section to serve as fiducial marks. Grids are incubated for 5 min in secondary antibodies conjugated to 12 nm gold particles (1:50, concentration should be adjusted to achieve a density of 10–20 gold particles per field of interest), and air-dry. Once dry, place the grid in storage box.
4. Cut 120-nm sections onto 75 mesh gold grids coated with Coat-Quick E-pen. 300 mesh copper grids can be used for preliminary screening, to select samples suitable for tomography.

Grids are counterstained on dedicated silicone grid mats in closed petri dishes: 5 min submerged in drops (filtered with 0.2 μm syringe filter) 1 % uranyl acetate + acetic acid, quickly transferred to drops of rinse water (boiled 10 min, cooled with tightly wrapped foil cover), dipped five times in each of three 10 ml beakers of rinse water, and then quickly submerged in drops of Sato's lead for 5 min, followed by a similar rinse in fresh beakers of boiled water.

CO_2 contamination during the lead staining can result in lead carbonate contamination on the grids. To avoid this, we place fresh sodium hydroxide pellets in a small flat plastic cap into the petri dish before the lead drops (filtered with a 0.2 μm syringe filter) are introduced. The lid to the petri dish is only lifted enough to insert or remove a grid to or from a drop, and then closed while the next grid is being picked up or rinsed. When removing a grid from the lead, a drop of boiled water from a syringe is dropped onto the grid as it emerges from the lead, and that grid is quickly immersed in the first of 3 beaker rinses. As soon as the grid is below the surface of the water, replace the petri dish cover. Do not exhale immediately before opening the petri dish, nor while it is open. You can breathe once the grid is under water. Limit talking and traffic by other personnel, and be aware of airflow patterns. Once rinsed, wick excess water from the grid and forceps and let it in a dust-free place.

3.4 Tilt Series Acquisition

Grids are observed under the electron microscope, and synapses of interest selected (Fig. 2). Data collection requires the acquisition of a “tilt series,” systematically collecting micrographs as the specimen is tilted in the electron beam.

1. The quality of the reconstruction is dependent on the total range of tilt angle and the spacing of angular increments, so in principle one should collect as many tilt images over as wide an angular range as possible. However, this must be balanced by practical considerations of acquisition time, cumulative beam damage to the specimen, and processing capacity. Computed tomography assumes projections from the entire range of tilt angles ($\pm 90^\circ$), but for biological electron microscopy there is an inherent limit to the range of tilt angles, resulting in a missing wedge of information in Fourier space. This reduces the

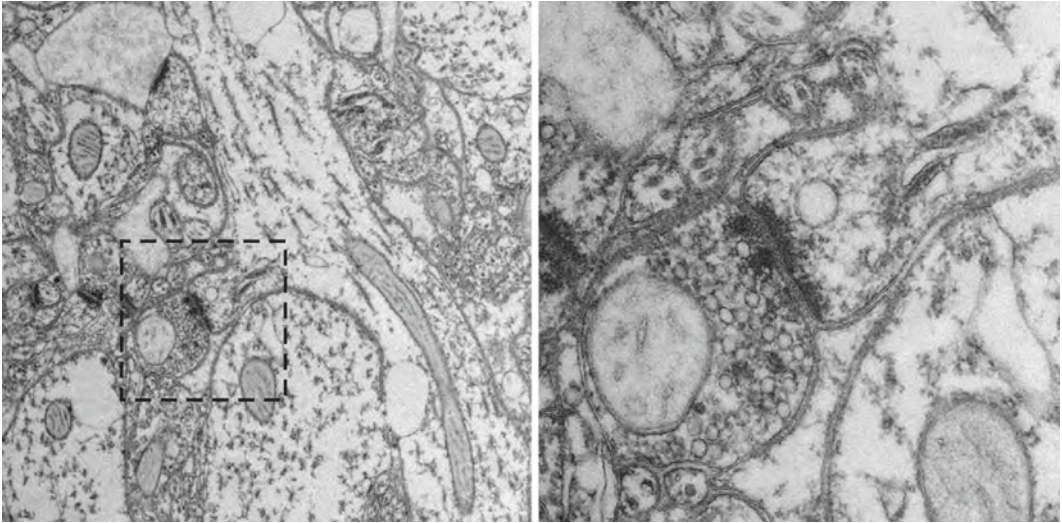


Fig. 2 Axospinous synapse (stratum radiatum of CA1 hippocampus) from non-osmicated material, prepared according to techniques described in this chapter. Protein-rich compartments (e.g., the PSD and the presynaptic active zone) are highly contrasted, and cytoskeletal features within the spine are prominent

resolution in the dimension perpendicular to the plane of the section and introduces reconstruction artifacts, the most visible being an elongation of the reconstruction perpendicular to the tilt axis. This problem can be ameliorated by acquiring an additional tilt axis perpendicular to the original axis (“dual tilt”) [11]. The missing volume in Fourier space becomes a pyramid significantly smaller than the wedge, resulting in significantly improved tomogram. High-angle triple-axis specimen holders are now in development [12].

2. The accelerating voltage is also an important factor for data collection. At a 70° tilt, the path length of the electron beam through a 100 nm section is 292 nm, resulting in severe image deterioration at the conventional accelerating voltage (80 kV), though energy filtering can compensate to some degree (Fig. 3). Moreover, at higher voltages inelastic scatter is reduced, leading to less damage to the specimen. In principle, best results would be with a specialized high-voltage (>1 MV) microscope. However, besides their scarcity and expense, image contrast is so severely impaired at these voltages that high-voltage microscopy is impractical except for special purposes. Thus, for sections of thickness <400 nm, intermediate-voltage (200–400 kV) microscopes are preferred.
3. Projections are taken at a series of orientations, typically 2° increments over an angular range of $\pm 60^\circ$ to 70° about a single axis (“single tilt”) or two orthogonal axes (“double tilt”). A rapid thinning of plastic sections occurs when first exposed to the electron beam. This phenomenon is important for tomography,

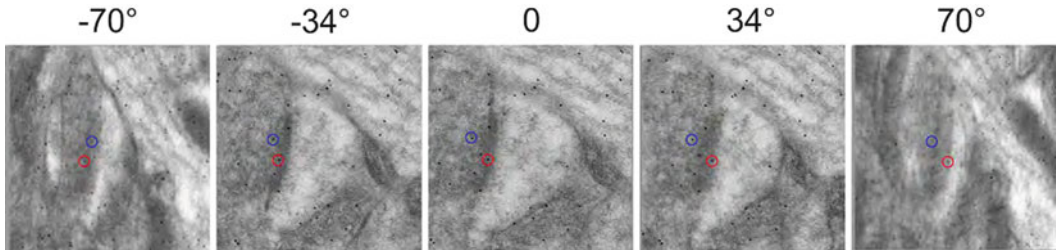


Fig. 3 Images of a single axospinous synapse acquired at multiple tilts, as noted above. Two gold particles (marked by *circles*) are on the two surfaces of the section. Contrast has been enhanced for reproduction

since changes in section thickness during data collection complicate computation of the 3D reconstruction. To minimize such problems, the specimen should be preexposed to the beam before collecting the tilt series. Further improvement in specimen stability can be achieved by coating the grid with a thin (~10 nm) layer of carbon.

3.5 Tomographic Reconstruction

For tomography the tilt series must be reconstructed into a 3D volume. The reconstruction can be performed using a variety of software packages (Table 1). To illustrate the general strategy required, we will outline the main steps in reconstruction using the IMOD software suite [24, 25].

1. *Convert files and edit slice info.* eTomo provides the graphical user interface to the IMOD reconstruction tools and can be started from the command line with the command “*etomo*.” eTomo opens image files in the “MRC” format. Raw tilt image stacks must have the extension .st, and dual axis stacks must have a common name ending with a.st and b.st. IMOD provides several tools to convert image file format. To convert the .mrc file generated by the FEI software, you need to use IMOD’s “*newstack*” command by typing the following command line: “*newstack file_name.mrc file_name.st*”.
2. *Prealignment.* As goniometers in electron microscopes are not perfect, it is necessary to perform a certain amount of specimen tracking and refocusing during collection of the tilt series. The tilt images must then be properly aligned before the 3D reconstruction, using the simultaneous iterative reconstruction technique (SIRT). The first step is to prealign the stack using a coarse alignment by cross-correlation. This step will allow automatic tracking of fiducial gold particles.
3. *Fine alignment.* The positions of fiducial gold on each tilt image are then used to bring the images into full register (Fig. 4). IMOD offers two ways to make a fiducial gold model that can be used to align the tilt series. In one method, gold beads are marked on the zero-degree image and then “*Beadtrack*” is used

Table 1
Software packages for electron tomography

	OS	Notes	References
<i>Amira</i> (commercial)	Linux, Windows, OSX	Powerful 3D visualization for image stack	[13]
<i>CoAn</i> (free)	Linux, OSX	State-of-the-art software package for correlation-based docking of atomic models into lower-resolution densities generated by electron microscopy and image reconstruction	[14, 15]
<i>Cytoseg</i> (free)	Linux, Windows	Promising tool for automatic segmentation of 3D biological datasets, with emphasis on 3D electron microscopy	[16]
<i>EM3D</i> (free)	Linux, Windows, OSX	Alignment and reconstruction of the tilt-series data, segmentations, and analysis tools	[17–19]
<i>Fiji</i> (free)	Linux, Windows, OSX	An easy to install version of ImageJ with a large number of preinstalled plugins	[20]
<i>JUST</i> (free)	Linux	Automatic segmentation with 3D watershed algorithms	[21, 22]
<i>ImageJ</i> (free)	Linux, Windows, OSX	Outstanding Java-based image processing program designed for analysis of various types of microscope data; ImageJ has numerous plugins for electron tomography, filtering, and 3D visualization	[23]
<i>IMOD</i> (free)	Linux, Windows, OSX	Outstanding software package for alignment and reconstruction of the tilt-series data and segmentations	[24, 25]
<i>RAPTOR</i> (free)	Linux	Automatic alignment of the tilt-series data. Also part of IMOD software package	[26, 27]
<i>Reconstruct</i> (free)	Windows	Montage, alignment, analysis, and visualization of serial sections	[28, 29]
<i>TOM Toolbox</i> (free)	Linux, Windows	A collection of functions that extend MATLAB and its Image Processing Toolbox. The toolbox supports a wide range of functions for tomography, including collection of tilt series	[30, 31]
<i>TOMOBLOW</i> (free)	Linux, OSX	Feature-preserving noise filtering in electron tomography	[32, 33]
<i>TomotJ</i> (free)	Linux, Windows, OSX	ImageJ plugin for alignment and reconstruction of the tilt-series data	[34]
<i>TxBR</i> (free)	Linux	State-of-the-art software package for alignment and reconstruction of the tilt-series data from various schemes of data acquisition takes into account the underlying electron trajectories	[35, 36]
<i>SerialEM</i> (free)	Windows	SerialEM is a program to acquire tilt series for electron tomography on Tecnai and JEOL microscopes	[24, 37]
<i>UCSF Tomography Software</i> (free)	Windows	To collect single-axis tilt series on electron transmission microscopes. Implemented based upon a novel approach in which the stage tilting is modeled as geometric rotation	[38]

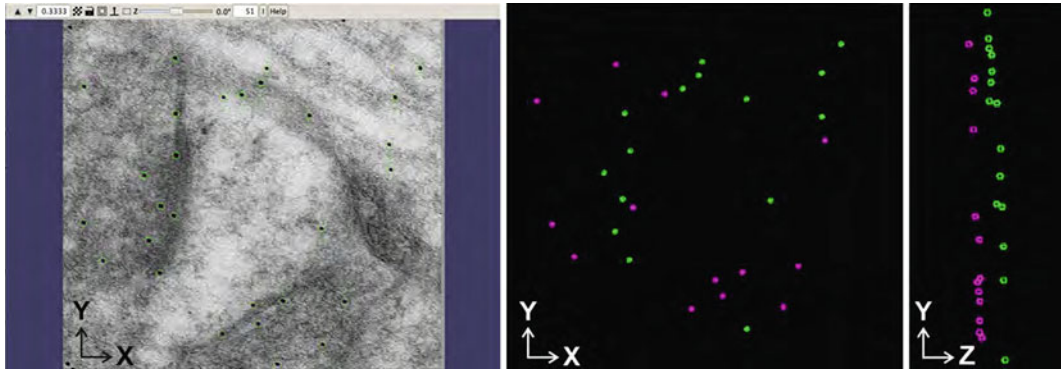


Fig. 4 The axospinous synapse shown in Fig. 3 illustrates how gold fiducial markers are used in IMOD. (a) Marked gold beads on the zero-degree view. (b, c) 3D view of the fiducial model; beads on the two sides of the ~120 nm thick section are shown in two colors

to find their locations in the rest of the tilt series. An alternate method uses RAPTOR [26, 27] to find beads automatically. Tilt series without fiducial marker can be aligned by tracking small patches of the image through the series by cross-correlating each patch from one view to the next with “*Tiltxcorr*.”

4. *Positioning of the tomogram.* This step shifts and rotates the reconstruction so that it is as flat as possible, allowing it to fit into the smallest volume possible. Two rotations are adjusted: the rotation about the tilt axis (to make the section level when viewed in the X - Z plane) and a rotation about the X axis (to make the section come out at the same Z height throughout the length of the tomogram). This is done through the creation of a boundary model by sampling three regions of the tomogram, computed from the top, middle, and bottom of the tilt images. Based on the model contours, *tomopitch* determines parameters to flatten the reconstruction and to fit in the smallest volume possible.
5. *Final alignment.* The alignment transformations are then applied to the full tilt series to produce the final aligned stack. Subsequently, several optional steps for contrast transfer function correction, erasing fiducial particles, and filtering the aligned stack can be applied.
6. *Tomogram generation.* Once the tilt images are aligned, the 3D reconstruction is computed using the simultaneous iterative reconstruction algorithm by pressing “*Generate Tomogram*.”
7. *Post-processing.* The tomogram is scaled, trimmed, and reoriented. When working with a very large dataset, the tomogram can be downsampled.
8. *Resolution.* Several methods have been proposed to calculate the resolution of tomographic volumes, but none of these is

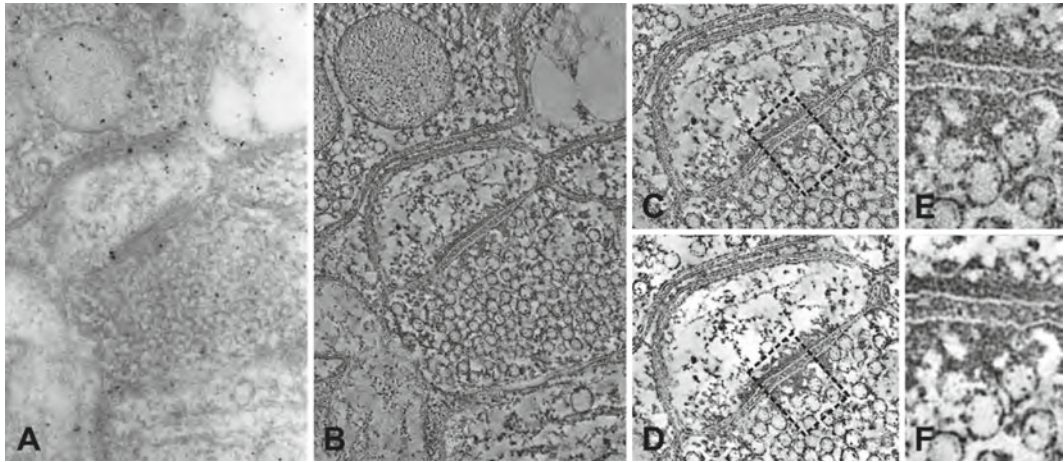


Fig. 5 Electron tomography of an axospinous synapse in cerebral cortex (layers II–III of S1). **(a)** A raw TEM image of the field used to generate the tomographic stack. The general impression of fuzziness reflects the relatively thick (~120 nm) section and high (400 kV) accelerating voltage used for image acquisition. **(b)** A representative ultrathin virtual tomographic section. **(c, d)** Enlargement of the spine head before **(c)** and after noise filtering **(d)**. **(e, f)** Enlargement from *boxes* in **(c)** and **(d)**, respectively. Filtering process removes extraneous density that may confuse automated segmentation algorithms, though it may also remove biologically relevant details (Reprinted from [39]. The original dataset can be viewed and downloaded from the Cell Centered Data Base (<http://ccdb.ucsd.edu>), under project P1194)

entirely satisfactory; the most suitable approach to measuring the quality of the 3D reconstructions remains controversial.

9. *Noise reduction.* Tomograms are inherently noisy. Post-processing noise reduction techniques can facilitate visualization and analysis. Standard linear filtering techniques based on local averages or Gaussian kernels should be avoided, as they blur edges and features. Anisotropic nonlinear diffusion techniques are better, but are intensive in terms of computation time and memory consumption (Fig. 5).

3.6 Visualization and Analysis

The specific tools and methods used to view and analyze a tomogram will depend on the biological question at hand. Several software packages are available for this (Table 1) [40].

1. Tomograms can be viewed with ImageJ [23, 41], as a series of images in two dimensions, as movies of image stacks, or in interactive 3D using volume rendering technique, using one of the several available 3D viewer plugins (Fig. 6).
2. Tomograms typically contain massive amounts of information; their interpretation often requires segmentation. By grouping sets of voxels into meaningful objects and regions, segmentation increases the interpretability of the tomogram and allows for quantification. The most common approach is manual segmentation, which requires the user to manually trace the features

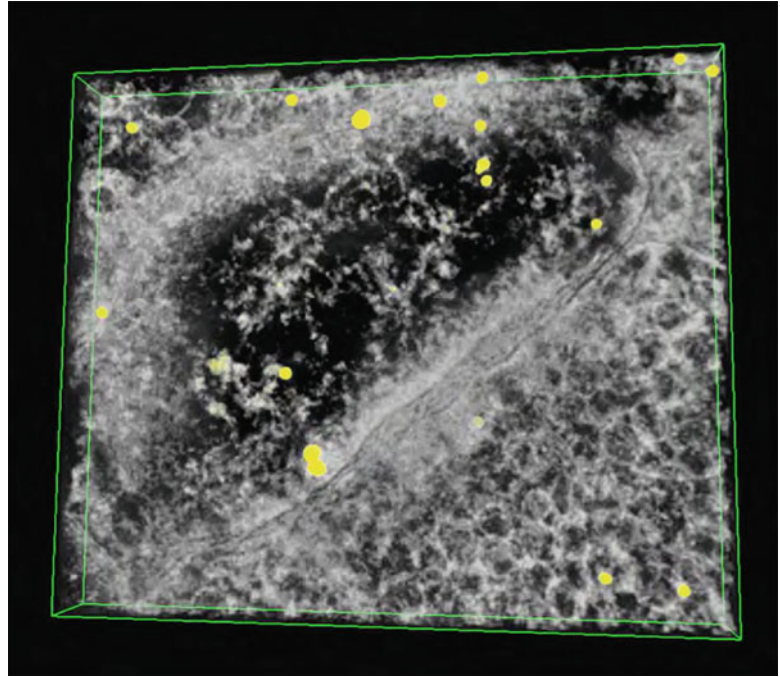


Fig. 6 A volume rendering projection (using ImageJ) of 20 tomographic slices from the tomogram shown in Fig. 5. The *yellow spheres* are gold particles. Overlap from the multiple slices obscures some details; this difficulty is less serious when working on-screen, because the projection can be rotated interactively

of interest. Though simple and robust, the method is very time consuming and inherently subjective. Contours are traced in 2D on successive tomographic slices. The contours are then meshed and displayed as solid surfaces. Color-coding is used to distinguish individual objects. IMOD provides many tools for manual segmentation and for visualizing and analyzing the 3D model generated. Figure 7 illustrates manual segmentation as applied to a small piece of neuropil.

3. Because of these limitations in manual segmentation, considerable effort has been put into the development of automated segmentation tools. While these can be very useful, the reader must recognize that tomographs represent a formidable challenge for current segmentation algorithms, and in practice most segmentations are somewhat arbitrary, relying as much on human vision as on computer algorithms. General purpose software applications such as Amira from Visage Imaging [13] offer a number of alternative segmentation, visualization, and analysis methods, including brush (to select the volume of interest, VOI, by painting the pixels), lasso (to draw the VOI), magic wand (which performs region

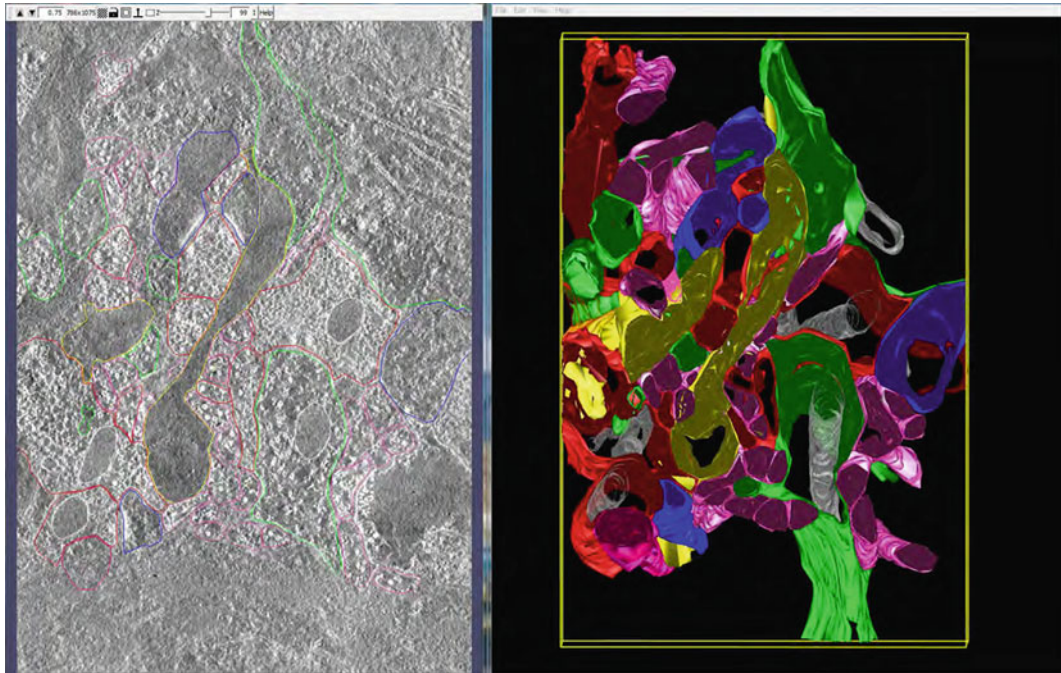


Fig. 7 Manual segmentation of a small piece of neuropil from stratum radiatum of CA1 hippocampus, using IMOD. **(a)** Is a tomographic slice (from a 200 slice tomogram) with modeling contours overlaid. **(b)** View of the completed model. The original dataset can be viewed and downloaded from the Cell Centered Data Base (<http://ccdb.ucsd.edu>), under project P1194. Movie of the 3D model can be viewed at <http://www.youtube.com/watch?v=Ux6JL8pUdk>

growing either in 2D or 3D), and filament tracing. Figure 8 illustrates the use of Amira to segment the landscape of the presynaptic active zone and to semiautomatically reconstruct the network of postsynaptic filaments.

4. Electron tomography has the potential to provide information with a resolution sufficient to depict individual molecules within their native cellular environments. The computational tools to extract objective information at the molecular level from tomograms are still in their infancy and, so far, have been mostly applied to the detection of isolated macromolecular assemblies. Template matching approaches are the primary tools for extracting molecular level information from tomograms. This approach uses a known crystallographic or high-resolution cryo-electron microscopy signature to search for a macromolecule or individual protein molecules in a reconstructed volume. Figure 9 illustrates the use of the correlative analysis software system “pyCoAn” [14, 15] to identify F-actin within a tomogram using an atomic model for F-actin, derived from high-resolution cryo-electron microscopy.

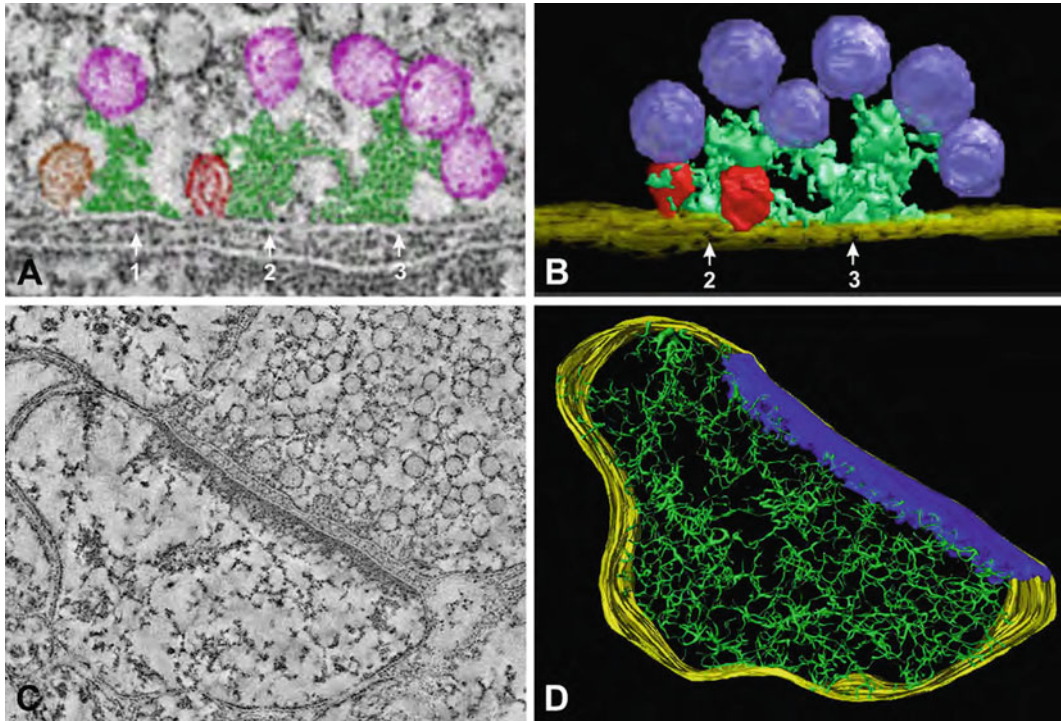


Fig. 8 (a) Is colorized tomographic slice of the presynaptic active zone. Three dense projections are shown in *green*. Vesicles in the cytoplasm that contact dense projections are shown in *purple*. A docked vesicle directly contacting the plasma membrane (*left, brown*) is also associated with a dense projection. A vesicle that has fused with the plasma membrane is seen in the center of the field (*red*). (b) Is a 3D reconstruction of the area shown in (a), using semiautomatic segmentation tools from Amira. Dense projections are *green*, presynaptic vesicles are *indigo*, and vesicles fused with the plasma membrane are *red*. (c) Is a tomographic slice from a large axospinous synapse in stratum radiatum of CA1 hippocampus. (d) Shows semiautomated reconstruction of filaments in the spine shown in (c), using the “autoskeleton” tool in Amira (Reprinted from [39])

3.7 Publication and Sharing

The size and the 3D nature of tomograms and associated segmented models make it difficult to adequately convey results with standard publication formats. Moreover, tomograms may contain more information than could ever be analyzed by a single researcher and may also address issues unrelated to the original purpose for which they were acquired. Online databases can be used to help manage and share high-resolution electron microscopic imaging data and 3D reconstruction models. For example, the Cell Centered Database (CCDB) [43, 44] at the National Center for Microscopy and Imaging Research aims to make unique and valuable datasets (2D, 3D, and 4D data from light and electron microscopy) available to the scientific community for visualization, reuse, and reanalysis [40].

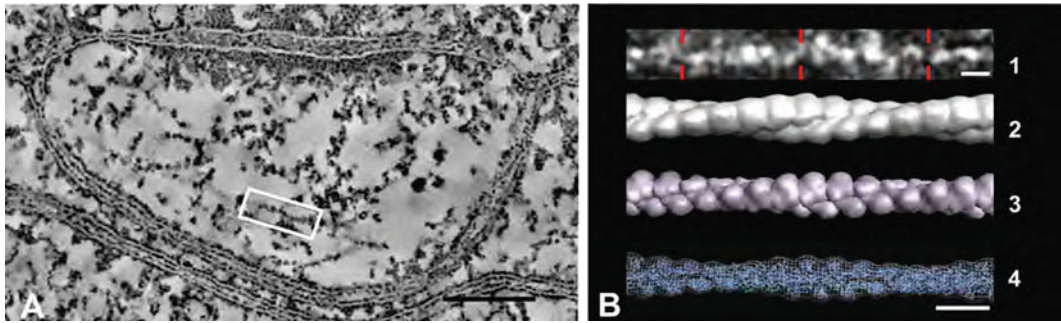


Fig. 9 (a) Shows a denoised virtual slice through the postsynaptic spine shown in Fig. 5. A volume containing a long, straight filament is highlighted by the *white box*. (b₁) Shows a projection of the filament density extracted from the boxed volume. The helical symmetry of the filament can be seen at a qualitative level; several putative actin-binding proteins are attached to the filament. To facilitate comparison with the known structure of F-actin, intensities were inverted so that electron density corresponds to bright intensity values. The extracted filament volume was subjected to a search for helical symmetry parameters, yielding values of 2.8 nm for helical rise and -167.4° for angular increment around the axes, very close to the canonical symmetry observed for in vitro actin filaments (2.7 nm and -166.7°). (b₂) Shows a surface representation of the extracted density after application of the deduced helical symmetry. The fact that individual subunits are discernible with sharp edges indicates that the helical symmetry is quite accurate; otherwise the subunits would blur into each other along the filament axis. (b₃) Shows a low-resolution representation of an atomic model of a canonical actin filament obtained from high-resolution electron microscopy analysis [42], adjusted for the difference in symmetry parameters. (b₄) Shows the fit of this atomic model (*blue* cartoon representation) into the symmetrized, extracted filament density (*white mesh* representation of the surface shown in b₂) (Reprinted from [39])

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