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TITLE:

Preparation of Newborn Rat Brain Tissue for Ultrastructural Morphometric Analysis of Synaptic Vesicle Distribution at Nerve Terminals

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SHORT ABSTRACT:

We describe procedures for processing newborn rat brain tissue to obtain high-resolution electron micrographs for morphometric analysis of synaptic vesicle distribution at nerve terminals. The micrographs obtained with these methods can also be used to study the morphology of a number of other cellular components and their dimensional structural relationships.

LONG ABSTRACT:

Our laboratory and many others have exploited the high resolving power of transmission electron microscopy to study the morphology and spatial organization of synaptic vesicles. In order to obtain high-quality electron micrographs that can yield the degree of morphological detail necessary for quantitative analysis of pre-synaptic vesicle distribution, optimal specimen preparation is critical. Chemical fixation is the first step in the process of specimen preparation, and of utmost importance to preserve fine ultrastructure. Vascular fixation with a glutaraldehyde-formaldehyde solution, followed by treatment of vibratome-sectioned specimens with osmium tetroxide, stabilizes the maximum number of molecules, especially proteins and lipids, and results in superior conservation of ultrastructure. Tissue is then processed with counterstaining, sequential dehydration and resin-embedding. En bloc staining with uranyl acetate (i.e., staining of vibratome-sectioned tissue before resin embedding) enhances endogenous contrast and stabilizes cell components against extraction during specimen processing. Contrast can be further increased by applying uranyl acetate as a post-stain on ultrathin sections. Double-staining of ultrathin sections with lead citrate after uranyl acetate treatment also improves image resolution, by intensifying electron-opacity of nucleic acid-containing structures through selective binding of lead to uranyl acetate. Transmission electron microscopy is a powerful tool for characterization of the morphological details of synaptic

vesicles and quantification of their size and spatial organization in the terminal bouton. However, because it uses fixed tissue, transmission electron microscopy can only provide indirect information regarding living or evolving processes. Therefore, other techniques should be considered when the main objective is to study dynamic or functional aspects of synaptic vesicle trafficking and exocytosis.

INTRODUCTION:

We describe methods for the preparation of newborn rat brain tissue to obtain high-quality electron micrographs for in-depth morphometric analysis of synaptic vesicle spatial distribution at nerve terminals[1,2]. The high-contrast micrographs that can be obtained by processing specimens following these methods can also be used to study the detailed morphology of a number of cellular components and their dimensional structural relations[3,4].

The transmission electron microscope (TEM) is a powerful tool to study the morphology of organelles and other cellular structures quantitatively. As of this decade, there are no other methods of investigation that can provide the same degree of resolution of lipid membranes and organelles without immuno-tagging, with the exception of cryofixation by high pressure freezing. However, freeze substitution techniques are not widely used, and normally require expensive equipment and long preparation times.

In order to take advantage of TEM's high resolving power, optimal specimen preparation is of paramount importance. The main goals of specimen preparation are to preserve tissue structure with minimum alteration from the living state, enhance specimen contrast, and stabilize the tissue against extraction of cellular components during processing and exposure to the electron beam. Numerous protocols for TEM tissue preparation have been introduced and perfected by several laboratories over the years. Many of them have focused on methods for optimal visualization of synaptic vesicles[5-11]. Among a number of well-established, gold standard methods currently in use, we chose procedures for chemical fixation, post fixation, en bloc staining, sequential dehydration, resin embedding and post staining that aim to preserve optimal tissue structure and achieve excellent image contrast. Of note, preservation of fine ultrastructure can be particularly challenging when working with newborn rat brain tissue. In fact, the central nervous system of very young animals is characterized by a higher water content than the adult brain, more prominent enlargement of extracellular spaces, and looser connections between cells[12]. This makes newborn rat brain tissue profoundly sensitive to changes in osmolarity, and exquisitely prone to artifactual shrinkage and/or swelling when processed through sequential solutions of different tonicity[12]. Therefore, our methods employed solutions for specimen processing that are of osmolarity as close as possible to that of rat newborn brain. Our goal was to obtain high-quality, high-resolution electron microscopy images for quantitative assessment of synaptic vesicle spatial distribution at nerve terminals. Specifically, we sought to measure the number of vesicles within the nerve terminal, the distance of synaptic vesicles from the pre-synaptic plasma membrane, the number of vesicles docked at the pre-synaptic membrane, the size of synaptic vesicles and the inter-vesicle distances[1].

Satisfactory chemical fixation is a prerequisite for obtaining high-quality electron micrographs that can provide the morphological detail necessary to study synaptic vesicle morphology and spatial organization. Although several modes of fixation exist, fixation of brain tissue by vascular perfusion is decidedly superior to other methods. Since fixation via vascular perfusion begins immediately after the arrest of systemic circulation, it shortens the interval between deoxygenation of the brain tissue and cross-linking of proteins with fixatives, resulting in minimum alterations in cell structure. Furthermore, it accomplishes fast and uniform penetration, because of the rapid flow of the fixative from the vascular bed to the extracellular and cellular compartments[12-14]. Primary fixation with glutaraldehyde, followed by secondary fixation (post-fixation) with osmium tetroxide, yields excellent preservation of the fine structure[15-17]. A mixture of glutaraldehyde and paraformaldehyde has the additional advantage of more rapid penetration into the tissue[12].

Since biological tissues are not sufficiently rigid to be cut into thin sections without the support of a resin matrix, they need to be embedded in a medium before thin sectioning. Water-immiscible epoxy resins are commonly used as an embedding medium in TEM. When this type of matrix is used, all specimen's free water must be replaced with an organic solvent before resin infiltration. Water is removed by passing the specimen through a series of solutions of ascending concentrations of ethanol and/or acetone[12]. In this protocol, specimens are first flat embedded between flexible aclar sheets, then embedded in a capsule. The final result is tissue situated at the tip of a cylindrical resin block, which has the ideal geometry to be least affected by vibrations arising during microtome sectioning.

Staining with heavy metals to enhance endogenous tissue contrast is another important aspect of specimen preparation. Image contrast in TEM is due to electron scattering by the atoms in the tissue. However, biological materials consist largely of low atomic weight molecules (i.e., carbon, hydrogen, oxygen and nitrogen). Therefore, the generation of sufficient scattering contrast requires the incorporation of high atomic weight atoms into the cellular components of the tissue. This is achieved through staining of the specimen with heavy metals[12,18,19]. Osmium tetroxide, uranium and lead, which bind strongly to lipids, are the most common heavy metals used as electron stains.

Osmium (atomic number 76) is one of the densest metals in existence. It is both a fixative and a stain, although its primary role in TEM is as a reliable fixative[12]. Among various fixation protocols in use, the method of double fixation with glutaraldehyde followed by osmium is the most effective in reducing the extraction of cell constituents during specimen preparation. These two fixatives are used to stabilize the maximum number of different types of molecules, especially proteins and lipids, and result in superior preservation of tissue ultrastructure[12,14-17].

Uranium (atomic number 92) is the heaviest metal used as electron stain, most typically in the form of uranyl acetate. Similarly to osmium, it acts as a stain and fixative, although its primary role in TEM is as a stain[20,21]. Nucleic acid-containing and membranous structures are strongly and preferentially stained with uranyl salts in aldehyde-fixed tissues[22,23]. Treatment of tissues with uranyl acetate after osmication and before dehydration results in stabilization of membranous

and nucleic acid-containing structures, as well as enhanced contrast, and permits identification of some structural details that would not be easily detected in specimens stained with osmium alone[12,24,25]. It is thought that uranyl acetate may stabilize the fine structure by combining with reduced osmium that has been deposited on lipid membranes during osmication[24]. Maximum contrast is achieved when uranyl acetate is applied before embedding and as a post-stain in thin sections[12].

Lead (atomic number 82) is the most common stain used for TEM and is mainly employed for post-staining of thin sections. Lead salts have high electron opacity and show affinity for a wide range of cellular structures, including membranes, nuclear and cytoplasmic proteins, nucleic acids and glycogen[26,27]. When the double staining method is employed (i.e., staining with uranyl acetate is followed by treatment with lead), the latter acts as a developer of uranyl acetate staining. For instance, lead post-staining of chromatin fixed with glutaraldehyde increases uranyl acetate uptake by a factor of three[28-32]. Lead also enhances the staining imparted by other metals such as osmium. It is thought that lead salts stain the membranes of osmium-fixed tissues by attaching to the polar group of phosphatides in the presence of reduced osmium[33]. A potential disadvantage of staining with both uranyl acetate and lead, especially for prolonged durations, is that many different structural elements are stained equally and non-specifically, and thus may not be easily distinguished from one another[12].

The recent introduction of alternative light sources, such as in optical super-resolution photo-activated localization microscopy, has significantly improved light microscopy resolution[34]. However, because light microscopy relies on histochemical and immune-cytochemical methods to visualize individually-labelled proteins or enzymes, the power of TEM to display all structural elements at once remains unsurpassed for in-depth study of the morphology and dimensional relationships of tissue structures. In particular, no other technique can provide the morphological detail necessary to perform morphometric analysis of synaptic vesicle distribution at pre-synaptic nerve boutons. Nevertheless, it is important to note that electron micrographs capture the structure of the tissue after the organism dies, and therefore they cannot provide information regarding the dynamics of pre-synaptic vesicle trafficking and exocytosis. Hence, other tools, such as FM dye-live imaging and patch-clamp electrophysiology, should be considered when the main objective is to study dynamic and/or functional aspects of synaptic vesicle trafficking and exocytosis.

PROTOCOL:

All studies were approved by the Institutional Animal Care and Use Committee at the University of Virginia (Charlottesville, VA) and conducted in accordance with the National Institutes of Health guidelines.

1. Fixation by vascular perfusion

NOTE: A general description of the method for rat brain vascular perfusion has been already detailed in this journal[13] and is beyond the scope of this protocol. However, the following steps

are specific for the preparation of newborn rat brain tissue to obtain high-quality electron micrographs for quantitative analysis of synaptic vesicles distribution at pre-synaptic terminals.

1.1. **Prepare 4% paraformaldehyde**

1.1.1. Place 800 mL of 0.1 M Phosphate Buffer (PB) in a 2 L glass beaker on a stirring plate under a fume-hood. Heat to approximately 60 °C without boiling the buffer.

1.1.2. Add 40 g of paraformaldehyde powder. Stir continuously

1.1.3. While measuring pH, add small drops of 10 N NaOH until the solution clears. Final pH should be 7.2-7.4.

1.1.4. Add remaining 0.1 M PB to a final volume of 1 L. Let cool, filter with a 0.45 µm membrane and store at 4 °C overnight.

NOTE: Prepare fresh paraformaldehyde the day before perfusion.

CAUTION: Inhalation of aldehyde vapors can cause nasal symptoms and airway irritation. Contact with skin causes dermatitis. Aldehydes should be handled in a fume-hood while wearing gloves, a protective gown and safety goggles

1.2. **Prepare Tyrode solution**

1.2.1. Place 1 L of distilled water in a glass beaker on a stirring plate. Add NaCl (8 g), KCl (0.15 g), $CaCl_2$ (0.1 g), $MgCl_2$ (0.006 g), NaH_2PO_4 (0.055 g), $NaHCO_3$ (1 g) and dextrose (1 g) in the beaker.

1.2.2. Stir continuously and measure pH. Keep pH between 7.2 and 7.4.

NOTE: Tyrode solution is also commercially available.

1.3. Mix 4% paraformaldehyde with glutaraldehyde at a final concentration of 2%. Add 40 mL of electron microscopy-grade 50% glutaraldehyde to 1 L of 4% paraformaldehyde. Mix well in a stirring plate

NOTE: Approximately 100 mL of the paraformaldehyde-glutaraldehyde fixative solution is needed to perfuse the body of a newborn rat. Therefore, at least 10 newborn rats can be perfused with 1 L of fixative. Do not mix paraformaldehyde and glutaraldehyde until immediately before use. Bring all solutions to room temperature before perfusion.

1.4. Once access to the left ventricle has been gained (see whole animal perfusion protocol[13]), flush the vascular system with Tyrode solution for 30 s at a perfusion pressure of 120 mmHg.

NOTE: The success of the perfusion depends in part on the complete exclusion of blood from the vascular bed

1.5. Flush with paraformaldehyde-glutaraldehyde solution at the same pressure for 10 min.

NOTE: Maintenance of constant perfusion pressure is essential to avoid introduction of artifacts. In addition, the temperature of the perfusate should not be below the rat's body temperature to avoid vasoconstriction

1.6. Remove the fixed brain from the skull and place in fresh paraformaldehyde-glutaraldehyde fixative at 4 °C overnight.

NOTE: The protocol can be paused here.

2. Brain slicing

2.1. Embed the fixed brain in 4% agarose and glue the brain-agarose block on a vibratome stage

NOTE: Other laboratories have obtained good quality sections by achieving a stable block on the vibratome without agar support

2.2. Section slices of 50 µm thickness. Set the microtome to low frequency and speed.

NOTE: Sections can be stored in 0.1 M PB with 0.05% sodium azide at 4 °C for several years.

2.3. Place the sections in 0.1 M PB in a Petri dish, examine the sections under a dissection microscope and select specimens for embedding

NOTE: The protocol can be paused here.

3. Rinsing

NOTE: It is important to rinse the specimen after fixation with aldehydes and before post-fixation with osmium, since residual fixatives may produce osmium precipitates

3.1. Pipette 0.1 M PB into a short, wide-mouth glass vial with cap. Place one specimen per each vial. Fully cover the specimen so that it does not dry.

NOTE: Keep specimens in the same vial from this step through all the solution changes of fixation, dehydration and infiltration, until they are ready for flat embedding.

3.2. Rinse the specimen in 0.1 M PB for 3 min x 2, then remove PB with a micropipette.

NOTE: Since the brain of the newborn rat is profoundly sensitive to changes in osmolarity[12,35-37], carry out the washings in the same vehicle as that used in the fixative mixture

4. Post-fixation with osmium

4.1. Preparation of osmium tetroxide (OsO_4)

NOTE: This Author's lab utilizes OsO_4 4% supplied in an aqueous solution in glass ampoules (5 mL of OsO_4 4% in H_2O). Other labs have used OsO_4 crystals successfully. However, several hours are necessary to dissolve OsO_4 crystals in a vehicle.

4.1.1. Take 5 mL (one ampoule) of 4% OsO_4/H_2O, open it and place it in a brown glass bottle

NOTE: OsO_4 is a strong oxidizing agent and readily reduced by exposure to light. Reduction can be avoided during preparation by placing OsO_4 in a brown glass bottle

4.1.2. Add 5 mL of 0.2 M PB. This will yield 10 mL of 2% OsO4/0.1 M PB.

NOTE: Since the brain of the newborn rat is profoundly sensitive to changes in osmolarity, use the same buffer to prepare aldehyde fixatives and OsO_4 solution[12,35-37]

4.1.3. Add additional 10 mL of 0.1 M PB. This will yield a final solution of 20 mL of 1% OsO_4 in 0.1 m PB.

4.1.4. Use a 20 mL syringe fitted with a long needle to draw 1% OsO_4 and place it in a brown glass bottle or a scint vial covered with aluminum foil.

CAUTION: OsO_4 is extremely volatile and its fumes are toxic to nose, eyes and throat. All work should be performed in a fume-hood using gloves and protective clothing, and no body part should be exposed to OsO_4. Handling and waste disposal should be done according to your institution's guidelines. Store the unused OsO_4 in a tightly stoppered brown glass bottle with Teflon liner on the glass stopper, wrap the bottle in double aluminum foil and store in a dessicator. In the presence of leaking fumes, OsO_4 can discolor internal surfaces and contents of the refrigerator. Under the above mentioned storage conditions the OsO_4 solution is stable for several months. When solutions get oxidized during storage, they turn gray, in which case they need to be disposed of.

4.2. Add 1% OsO_4 in 0.1 M PB in the specimen vial and let sit for 1 h. Extract OsO_4 after 1 h with a micropipette.

NOTE: Before applying OsO_4 to the section, it is critical to unfold and flatten the specimen. Avoid application directly on top of the specimen, instead use the vial's walls to gently drip OsO_4 to the bottom of the vial. The specimen becomes brown and rigid shortly after OsO_4 application. Handle gently from now on to avoid tissue damage

5. Rinsing

NOTE: It is important to rinse the specimen after post-fixation with OsO_4 and before dehydration, since residual fixatives may react with dehydration agents[37].

5.1. Rinse with 0.1 M PB for 3 min x 3.

NOTE: Since the brain of the newborn rat is profoundly sensitive to changes in osmolarity, carry out the washings in the same vehicle as that used in the fixative mixture[12,35-37].

5.2. Place the osmium solution and first two PB rinses into OsO_4 waste and dispose of it according to your institution's guidelines.

6. Sequential dehydration and staining with uranyl acetate

NOTE: This author's laboratory uses water-immiscible epoxy resins for embedding. When epoxy resins are used, all specimen's free water must be replaced with an organic solvent before infiltration by the embedding medium. Water is removed by passing the specimen through a series of solutions of ascending concentrations of ethanol and acetone[12].

6.1. Preparation of uranyl acetate (UA)

6.1.1. Place a 200 mL volumetric glass flask containing 100 mL of EtOH 70% on a stirring plate.

6.1.2. Add 4 g of UA to the flask. Wrap in aluminum foil (UA precipitates when exposed to light) and stir continuously.

NOTE: UA dissolves slowly and incompletely in 70% EtOH. Allow undissolved crystals to settle down before using the solution. UA solution should be filtered with a 0.45 µm filter before use. UA can be prepared ahead of time and kept in a brown bottle wrapped in aluminum foil at 4 °C for months.

CAUTION: UA is mildly radioactive and highly toxic. The inhalation of UA powder can cause upper respiratory tract disorders and disease of the lungs, liver and kidneys. UA is dangerous when ingested or when it comes in direct contact with skin and mucous membranes. All work should be performed in a fume-hood using gloves and protective clothing. Handling and waste disposal should be done according to your institution's guidelines.

6.2. Dehydrate in 50% EtOH for 1 min. Remove 50% EtOH before adding UA.

6.3. Add 4% UA in 70% EtOH. Let sit for 1 h or overnight. If overnight, place in the refrigerator at 4 °C.

NOTE: Cap vial to avoid EtOH evaporation and cover with aluminum foil to avoid exposure to light. The protocol can be paused here.

6.3.1. Dispose of UA waste and the two following rinses according to your institution's guidelines

6.4. Dehydrate in 70% EtOH for 1 min.

6.5. Dehydrate in 90% EtOH for 5 min.

6.6. Dehydrate in 100% EtOH for 5 min x 2.

6.7. Rinse the specimen in acetone for 2 min x 3.

7. Infiltration and embedding

NOTE: Tissues are not sufficiently rigid to be cut into thin sections without the additional support of a resin matrix. Therefore, infiltration and embedding must precede sectioning[12].

7.1. Preparation of EPON resin

7.1.1. Use a 60 mL gavage syringe. Cap the syringe with a safety needle and remove the syringe plunger.

7.1.2. Place the syringe with the open side up and add the volume of each ingredient of the resin mix incrementally. Add 22 mL of Embed-812 (resin). Add DDSA (hardener) to a total volume of 37 mL. Add NMA (hardener) to a total volume of 50.5 mL. Add 525 µL of DMP-30 (accelerator) with a pipette. Move the plunger of the pipette very slowly as DMP is very viscous.

NOTE: it is important to add the embedding reagents in the order listed. The accelerator (DMP-30) must be added last. To obtain a cured block that has the desired characteristics, it is critical to use the exact amount of the hardener and the accelerator. Freshly prepared embedding mixtures are preferred.

7.1.3. Put the plunger back and place the syringe on a rocker with continuous shaking for at least 30 min. Color will change from yellow to amber.

NOTE: All ingredients must be mixed very thoroughly. Failure to do so results in uneven impregnation of the tissue specimen and a block of uneven hardness

7.2. Mix 1 volume of EPON resin with 1 volume of acetone in a scint vial and shake to mix. Apply the 1:1 EPON:acetone mixture on the tissue after removing the last acetone rinse. Keep covered to avoid acetone evaporation. Remove the 1:1 mixture of resin and acetone after 2-4 h or keep overnight.

NOTE: the protocol can be paused here.

7.3. Replace the mixture of resin and acetone with full resin. Let sit for 4 h or overnight. Put all EPON waste in a collection container under the hood to be polymerized and disposed of later.

NOTE: the protocol can be paused here.

8. Flat-embedding

NOTE: Specimens are flat-embedded between two aclar films in a sandwich-like fashion.

8.1. Cut two rectangular pieces of clear aclar sheet. Wipe the films clean with EtOH 70%. Trim the sheets so that there is at least 1.5 cm of tissue-free plastic on every side of the section. The aclar sheet on top should have the same width as the bottom, and its height should be approximately two thirds of the bottom sheet.

8.2. Slowly tilt the vial with the specimen and gently lift the tissue from the bottom of the vial.

8.3. Use a micro flexible spatula and a fine brush to carefully move the section along the vial walls and transfer onto the aclar sheet.

NOTE: Handle sections with caution to avoid specimen damage.

8.4. Gently place the aclar sheet on top of the specimen.

NOTE: Make sure there is enough resin between the two sheets to seal the sandwich

8.5. Gently push out any trapped air bubbles without exerting direct pressure onto the section. Wipe out the excess EPON.

NOTE: The elimination of air bubbles is important, as their presence makes visualization of the specimen difficult and weakens the stability of the resin bonds.

8.6. Label the sheets with a solvent resistant pen and place in oven at 60 ˚C for 2-3 days to polymerize.

NOTE: the protocol can be paused here.

9. Capsule embedding

NOTE: Ultramicrotomes are supplied with chucks to hold cylindrical blocks obtained from embedding specimens in capsules. Cylindrical blocks have the ideal geometry to be least affected by vibrations arising during sectioning.

9.1. Gently open the sandwiched aclar films. The specimen will adhere to one of the two aclar sheets.

9.2. Use a solvent resistant pen to mark the side of the aclar sheet that contains the section. Mark near the tissue.

NOTE: It is critical to perform this step before punching out the tissue of interest.

9.3. Use a disc punch to obtain a circle sample of the section. The pen mark should be part of the punched out tissue.

9.4. Prepare the cap of an embedding capsule side up on a capsule holder.

9.5. Place the punched disc inside the cap with the tissue section facing up. The pen mark will gleam when light is shined on the specimen.

9.6. Insert a capsule into the cap and use fine tweezers to insert labeling. Roll and lower a printed 2 cm-long piece of paper into the capsule. Make the label to fit to the curvature of the side walls of the capsule. Wait for polymerization to be complete, so that the label becomes permanently embedded in the resin.

9.7. Pour the embedding resin inside the capsule and fill until the edge of the capsule.

9.8. Push the tissue specimen down to the bottom of the capsule with the aid of a pointed wooden stick and place in the oven at 60˚C for 2-3 days.

NOTE: Take care not to press the tissue too hard, instead let it lie loosely so that a thin layer of the embedding medium is present between the tissue and the capsule surface. The protocol can be paused here.

10. Trimming of block face

NOTE: Small size and appropriate shape of the block face are prerequisites for satisfactory sectioning. Therefore, trimming of the specimen block is a necessity.

10.1. Use a sharp one-edge razor blade. Clean with acetone immediately before use.

10.2. Mount the capsule block in a block holder and place the block holder on the stage of a stereomicroscope.

NOTE: The trimming procedure should be carried out under microscope binoculars and oblique illumination.

10.3. Remove the aclar sheet from the tip of the capsule using a razor blade. The aclar sheet appears as a shiny layer under the light of the dissecting scope.

10.4. Hold the razor blade at an angle of 45 degrees and make cuts down four sides of the capsule block.

NOTE: Better control of the trimming is achieved if the blade is held with both hands.

10.5. Make short cuts so that the block takes the form of a short pyramid with wall angles of approximately 45 degrees.

NOTE: The specimen face is supported much better if the sides leading to it are kept short. If the block tip is too thin and long, it will vibrate during thin sectioning. Ideally, the area of interest should be centered in the block face.

10.6. Trim the tip of the capsule to discard superfluous tissue and only preserve the area of interest.

NOTE: No part of the section should be without tissue, as variations in the density of the block face are a major cause of difficulty in sectioning. Ideally, the surface area of the capsule face should be no more than 1 mm^2.

10.7. Trim the capsule face in the shape of a scalene trapezoid. Because in a scalene trapezoid no side is equal, this shape is best to orient the area of interest relative to the rest of the specimen.

NOTE: During sectioning, the trapezoid-shaped face is supported much better than that of any other shape.

11. Microtome sectioning

NOTE: The majority of biological specimens are too thick in their natural state to be penetrated by an electron beam. Therefore, the material must be cut in thin sections that can be penetrated by the electron beam.

11.1. Cut thin sections (silver interference color, 600-900 Å) on a ultramicrotome at planes parallel to the surface.

11.2. Place the sections on grids. This author’s lab uses copper grids of 3.05 mm in outer diameter.

NOTE: The protocol can be paused here.

12. Post-staining with uranyl acetate

12.1. Prepare 2% UA in distilled water. Place a 200 mL volumetric glass flask containing 100 mL of distilled water on a stirring plate. Add 2 g of UA to the flask. Wrap in aluminum foil (UA precipitates when exposed to light) and stir continuously.

NOTE: For details of UA preparation, see section 6 of this protocol.

12.2. Place several individual drops of 2% UA on a clean sheet of dental wax in a Petri dish.

12.3. Float the grid with the specimen (section side down) on a drop of UA for 25 min.

NOTE: Float each grid on a separate drop of UA.

12.4. Hold the grid at its edge with forceps and rinse twice under a gentle jet of boiled distilled water at room temperature from a plastic wash bottle.

NOTE: Do not allow the grid to dry before staining with lead.

13. Post-staining with lead

13.1. Prepare a CO_2-free chamber (CO_2 in the air is the primary source of lead precipitation). Place a piece of filter paper soaked with 1 N NaOH in a Petri dish. Place a small sheet of clean dental wax on top of the filter paper and place several NaOH pellets on one side of the dish. Cover the dish.

NOTE: Prepare this setup before the grids are stained with UA, so that the atmosphere in the chamber is free of CO_2 and ready for lead staining by the time UA staining is complete.

13.2. Make 4% NaOH. Add 0.2 g of NaOH to 5 mL of distilled water.

13.3. Prepare Sato's triple lead solution. To prepare 10 mL, add 0.1 g of lead nitrate, 0.1 g of lead citrate, 0.1 g of lead acetate and 0.2 g of sodium citrate into a glass bottle. Add 8.2 mL of distilled water and shake vigorously for 5 min. Sonicate for 30 s, then add 1.8 mL of freshly made 4% NaOH.

13.4. Place several drops of Sato's lead on the wax in the Petri dish.

13.5. Place the grid stained with UA (section side down) on the drop of lead.

NOTE: Each grid should be floated on a separate drop of lead. Each drop should be small enough to allow the grid to float on top of the dome of the drop instead of sliding down on the sides.

13.6. Cover the dish and let stain for 5 min.

13.7. Hold the grid at its edge with forceps and wash thoroughly under a gentle jet of boiled distilled water at room temperature from a plastic wash bottle.

13.8. Blot dry the grid on filter paper and store the grid.

NOTE: Make sure the section does not come in direct contact with the filter paper.

REPRESENTATIVE RESULTS:

General criteria that are mostly accepted as indicative of satisfactory or defective preservation of specimen for TEM have been established. These criteria are exemplified in four selected electron micrographs (two examples of optimal preparation, two examples of defective preparation) that were obtained by treating young rat brain tissue following the methods described in this protocol.

In general, a good-quality electron micrograph appears as an orderly, distinct and overall grayish image. In a satisfactorily prepared specimen, spaces between membranes should be filled with granular material, and should not be empty. Similarly, no empty spaces should be found in the cytoplasmic ground substance or within organelles (compare **Figure 1** and **Figure 2A** with **Figure 3** and **Figure 4**). Nevertheless, it is important to note that the central nervous system of very young animals shows a certain degree of enlargement of extracellular spaces when compared to the adult brain, with looser connections between cells and an overall whiter, less electron-dense appearance. Membranes should be continuous, without distortion or breakage (**Figure 1** and **Figure 2A**). The stroma of mitochondria should appear uniform and dense, with no empty spaces. Cristae should be intact and not swollen, and the mitochondrial outer double membrane should be unbroken (compare **Figure 1A** with **Figure 3**). To facilitate morphometric analysis of pre-synaptic vesicle organization, pre- and post- synaptic membranes need to be intact and essentially parallel to each other (see **Figure 1**). Synaptic vesicles should be traceable and bound by a continuous single membrane (see **Figure 2**).

Importantly, even when best practices are followed, treatment of the specimen with fixatives, stains and resins introduces artifacts. Since artifacts cannot be eliminated, it is critical to understand what process originates them, so that the appearance of the specimen can be interpreted with respect to the treatment that it underwent[12]. One example of an artifact - among several that can be generated during specimen preparation for TEM - is a myelin figure, a membranous lamellar inclusion that resembles myelin sheaths. Although myelin figures can be seen in pathologic conditions[12], they most often result from extraction of membrane lipids during fixation with aldehydes (see **Figure 4**).

FIGURE LEGENDS:

Figure 1. Electron micrograph representative of satisfactory preservation of cell structure (example 1). (A) Neuronal cell membranes are layered and without breaks. The cytoplasm is finely granular and without empty spaces. Mitochondria are neither swollen nor shrunk. Their outer double membrane is conserved, and internal cristae are intact. **(B)** Detail of panel A,

exemplifying one method for measuring the distance of synaptic vesicles from the pre-synaptic membrane.

Figure 2. Electron micrograph representative of satisfactory preservation of cell structure (example 2). (A) Synaptic vesicles are distinct and lined by an unbroken single membrane. To allow morphometric analysis of synaptic vesicle distribution, pre-synaptic and post-synaptic membranes need to be parallel and their continuity preserved. **(B)** Detail of panel A, exemplifying the counting of synaptic vesicles within the pre-synaptic terminal.

Figure 3. Electron micrograph representative of defective preservation of tissue structure (example 1). Note the distortion and breakage of neuronal cell membranes and the presence of markedly enlarged extracellular spaces (marked with *). Mitochondria appear distended and have swollen cristae (marked with arrow).

Figure 4. Electron micrograph representative of defective preservation of tissue structure (example 2). Note the presence of large white empty spaces within the cytoplasm (marked with ‡), in place of finely granular cytoplasmic substance. Extracellular spaces appear enlarged. An artifactual membranous whorl (myelin figure), likely resulting from mobilization of lipids during fixation with glutaraldehyde, is marked with the acronym MF.

DISCUSSION:

Handling of tissue sections during specimen preparation for TEM requires a considerable degree of finesse, concentration and patience. When using a micropipette to add and remove solutions, specimens can be sucked into the pipette tip by surface tension, so great care should be taken to avoid tissue damage by the pipette. Also, certain steps of the dehydration sequence can be as quick as 1 min, hence the operator needs to work swiftly to ensure that the next dehydration step is started on time and the specimen does not dry or wrinkle. One procedure that requires special attention is post-fixation with osmium. Sections become rigid after treatment with osmium and can be easily damaged. Before adding osmium, it is imperative that the sections are flattened at the bottom of the vial, else any fold will result in tissue fracture. Handling of osmicated tissue is particularly challenging when transferring sections onto aclar films for flat embedding. Special care is needed when lifting the specimen from the bottom of the vial to avoid fragmentation, and when pushing air bubbles out of the film sandwich. While it is important to push out any trapped air, as it makes visualization of the specimen difficult and weakens the stability of the resin bonds, direct pressure onto osmicated tissue can easily inflict damage. Another step that necessitates extra care is preparation of the resin mixture for specimen infiltration and embedding. EPON, the most widely used embedding resin, can be hardened with the addition of a hardener and an accelerator. It is imperative to use the exact amount of hardener and accelerator in order to obtain a cured block with the desired characteristics. Both gravimetric and volumetric methods have been described for measuring viscous resins. Although gravimetric methods have been traditionally considered more precise[12], this Author's laboratory has had good success with volumetric modes (i.e., adding the volume of each ingredient of the resin mix incrementally by means of a gavage syringe). After the resin components have been carefully measured, they must be mixed very thoroughly to accomplish uniform impregnation,

since they possess different viscosities and rates of polymerization. Failure to achieve complete mixing will result in a block of uneven hardness that is unsuitable for thin sectioning.

Marked changes in the tonicity of the solutions used to process sections of young rat brain can cause shrinkage and/or swelling of the extracellular space and cellular components[12,35-37]. During fixation, the best results are obtained when the osmotic pressure of the perfusate is kept as similar as possible to that of the tissue under study. Rat brain osmolality is approximately 330 mOsm. A 2% glutaraldehyde in 0.1 M PB solution is only slightly hypertonic (400-450 mOsm) and minimizes expansion of the extravascular space[12]. Notably, membranes remain sensitive to changes in the osmotic pressure of the rinsing and dehydration solutions after fixation with aldehydes. Therefore, it is also important to minimize the differences between the osmolarity of the fixative and the solutions used subsequently[12,35-37]. For this reason, the same vehicle (0.1 M PB, approximate osmolarity 440 mOsM) is used as a solvent for all solutions in this protocol. However, it should be noted that several different buffers have been used successfully during specimen preparation for TEM and no single buffer can claim universal superiority over the others. When buffers of lower tonicity are preferred, other laboratories have chosen to increase the osmolarity with electrolytes or non-electrolytes[12].

Several steps in this protocol require the use of chemicals that can be toxic when not handled properly. The importance of working under a fume-hood and wearing personal protective equipment while handling aldehydes, osmium, uranium and lead compounds cannot be overstated. While automated contrasting systems can help attenuate some of the risk and are commercially available, they can be quite expensive and may not be affordable by laboratories that do not make routine use of TEM. Although the electron microscope laboratory can potentially be a hazardous place, specimen processing for TEM is overall safe when performed rigorously.

Recently, the introduction of super-resolution light microscopy has increased optical imaging resolving power to about 15 nm[34]. However, when the goal is to perform morphometric analysis of synaptic vesicle spatial organization at pre-synaptic terminals, no technique provides the same degree of morphological detail. Importantly, TEM is limited by the need for the specimen to be dead before it can be processed and visualized. Therefore, when the study objective is to investigate dynamic or functional aspects of synaptic vesicle trafficking and exocytosis, tools other than TEM should be considered.

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DISCLOSURES:

The Authors have nothing to disclose.

REFERENCES:

1. Lunardi, N., Oklopcic, A., Prillaman, M., Erisir, A., Jevtovic-Todorovic, V. Early exposure to general anesthesia disrupts spatial organization of presynaptic vesicles in nerve terminals of the developing subiculum. *Molecular Neurobiol*ogy. **52** (2), 942-51 (2015).

2. Lunardi, N., Ori, C., Erisir, A., Jevtovic-Todorovic, V. General anesthesia causes long-lasting disturbances in the ultrastructural properties of developing synapses in young rats. *Neurotoxicity Research.* **17** (2), 179-188 (2010).

3. Sanchez, V. et al. General anesthesia causes long-term impairment of mitochondrial morphogenesis and synaptic transmission in developing rat brain. *Anesthesiology.* **115** (5), 992-1002 (2011).

4. Boscolo, A. et al. The abolishment of anesthesia-induced cognitive impairment by timely protection of mitochondria in the developing rat brain: the importance of free oxygen radicals and mitochondria integrity. *Neurobiology of Disease.* **45** (3), 1031-41 (2012).

5. Aghajanian, G.K., Bloom, F.E. The formation of synaptic junctions in developing rat brain: a quantitative electron microscopy study. *Brain Research.* **6** (4),716-727 (1967).

6. Akert, K., Sandri, C. Significance of the Maillet method for cytochemical studies of synapses. In: *Golgi centennial symposium; Perspectives in Neurobiology.* (Santini M editor). Raven Press, NY. 387-399 (1975).

7. Bloom, F.E., Aghajanian, G.K. Cytochemistry of synapses: selective staining for electron microscopy. *Science.* **154** (3756), 1575-1577 (1966).

8. Bloom, F.E., Aghajanian, G.K. Fine structural and cytochemical analysis of the staining of synaptic junctions with phosphotungstic acid. *Journal of Ultrastructure Research.* **22** (5-6), 361-375 (1968).

9. Burry, R.W., Lasher, R.S. A quantitative electron microscopy study of synapse formation in dispersed cell cultures of rat cerebellum stained either by O-UL or by E-PTA. *Brain Research.* **147**(1), 1-15 (1978).

10. Pellegrino de Iraldi, A., Suburo, A.M. Electron staining of synaptic vesicles using the Champy-Maillet technique. *Journal of Microscopy.* **91** (2), 99-103 (1970).

11. Pfenninger, K.H. The cytochemistry of synaptic densities. I. An analysis of the bismuth iodide impregnation method. *Journal of Ultrastructural Research.* **34** (1), 103-122 (1971).

12. Hayat, M.A. Chemical fixation. In: *Principles and techniques of electron microscopy. Biological applications*. (Hayat MA editor). Cambridge University Press. 4-84 (2000).

13. Gage, G.J., Kipke, D.R, Shain, W. Whole Animal Perfusion Fixation for Rodents. *Journal of Visual Experiments.* (65), e3564, doi:10.3791/3564 (2012).

14. Hayat, M.A. *Fixation for electron microscopy*. (Hayat MA editor) Academic Press, San Diego (1981).

15. Behrman, E.J. The chemistry of osmium tetroxide fixation. In: *The science of biological specimen preparation for microscopy and microanalysis.* (Revel JP et al editors), Scanning Electron microscopy Inc, AMF O'Hare, Chicago. 1-5 (1983).

16. Nielson, A.J., Griffith, W.P. Reactions of osmium tetroxide with protein side chains and unsaturated lipids. *Journal of the Chemical Society, Dalton Transactions.* (6)1084-1088 (1979).

17. Nielson, A.J., Griffith, W.P. Tissue fixation by osmium tetroxide. A possible role for proteins. *Journal of Hystochemistry & Cytochemistry.* **27** (5), 997-999 (1979).

18. Hanker, J.S., Deb, C., Wasserkrug, H.L., Seligman, A.M. Staining tissue for light and electron microscopy by bridging metals with multidentate ligands. *Science.* **152** (3729), 1631-1634 (1966).

19. Zobel, C.R., Beer, M. The use of heavy metal salts as electron stains. *International Review of Cytology.* **18**, 363-400 (1965).

20. Silva, M.T., Guerra, F.C., Magalhaes, M.M. The fixative action of uranyl acetate in electron microscopy. *Experientia.* **24** (10),1074 (1968).

21. Terzakis, J.A. Uranyl acetate, a stain and a fixative. *Journal of Ultrastructural Research.* **22** (1-2), 168 184 (1968).

22. Feldman, I., Jones, J., Cross, R. Chelation of uranyl ions by adenine nucleotides. *Journal of the American Chemical Society*. **89** (1),49-53 (1967).

23. Shah, D.O. Interaction of uranyl ions with phospholipid and cholesterol monolayer. *Journal of Colloid and Interface Science.* **29** (2), 210-215 (1969).

24. Daems, W.T., Persijn, J.P. Section staining with heavy metals of osmium-fixed and formol-fixed mouse liver tissue. *Journal. Royal Microscopical Society.* **81** (pts 3&4), 199-201 (1963).

25. Lombardi, L., Prenna, G., Okolicsanyi, L., Gautier, A. Electron staining with uranyl acetate: possible role of free amino groups. *Journal of Histochemistry & Cytochemistry.* **19** (3), 161-168 (1971).

26. Cattini, P.A., Davies, H.G. Kinetics of lead citrate staining of thin sections for electron microscopy. *Stain Technology*. **58 (1)**, 29-40 (1983).

27. Reynolds, E.S. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology.* **17** (1), 208-212 (1963).

28. Cattini, P.A., Davies, H.G. Observations on the kinetics of uranyl acetate and phosphotungstic acid staining of chromatin in thin sections for electron microscopy. *Stain Technology.* **59** (5), 291-304 (1984).

29. Derksen, J.W., Meekes, H. Selective staining of nucleic acid containing structures by uranyl acetate-lead citrate. *Micron and Microscopica Acta.* **15** (1), 55-58 (1984).

30. Frasca, J.M., Parks, V.R. A routine technique for double-staining ultrathin sections using uranyl and lead salts. *Journal of Cell Biology.* **25** (1), 157-161 (1965).

31. Hanaichi, T., Sato, T., Iwamoto, T., Malavasi-Yamashiro, J., Hoshino, M., Mizuno, N. A stable lead by modification of Sato's method. *Journal of Electron Microscopy.* **35** (3), 304-306 (1986).

32. Sato, T. A modified method for lead staining of thin sections. *Journal of Electron Microscopy.* **17** (2), 158-159 (1968).

33. Lever, J.D. A method of staining tissues with lead for electron microscopy. *Nature.* **186**,810-811. (1960).

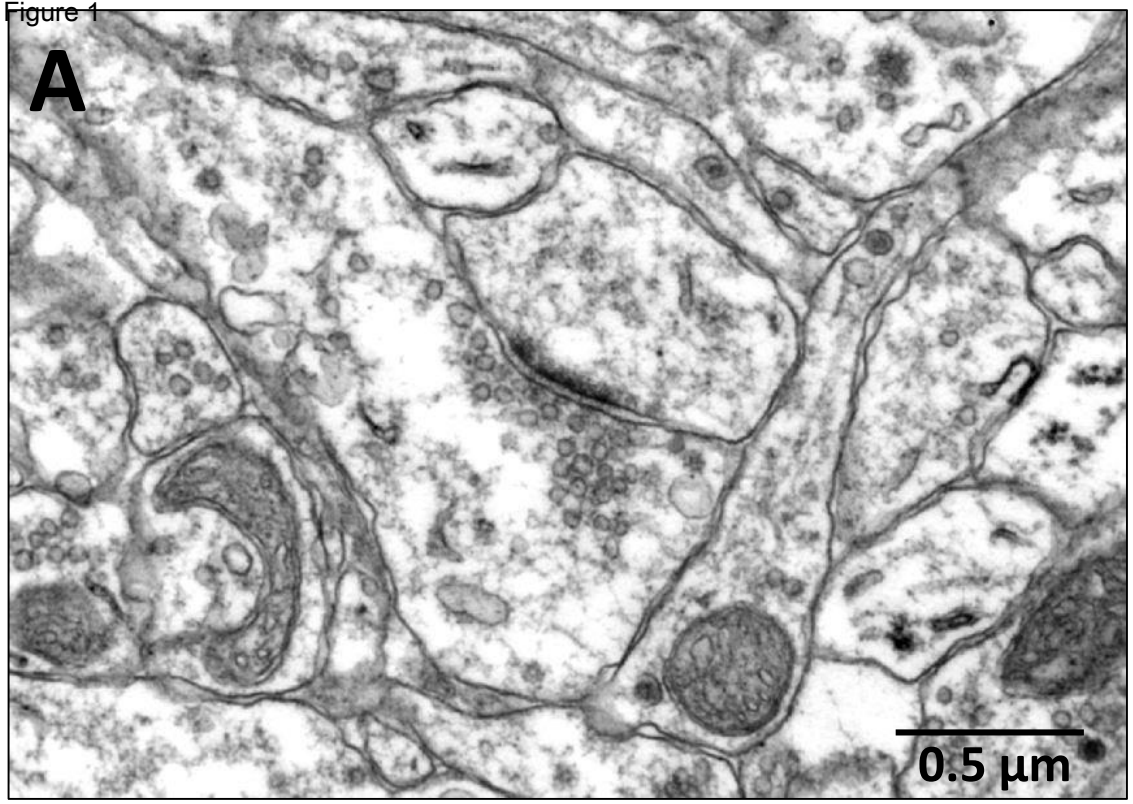
34. Derek, G. et al. Self organization of the Escherichia Coli chemotaxis network imaged with super-resolution light microscopy. *Public Library of Science Biology.* **7**(6):e1000137. doi: 10.1371/journal.pbio.10000137 (2009).

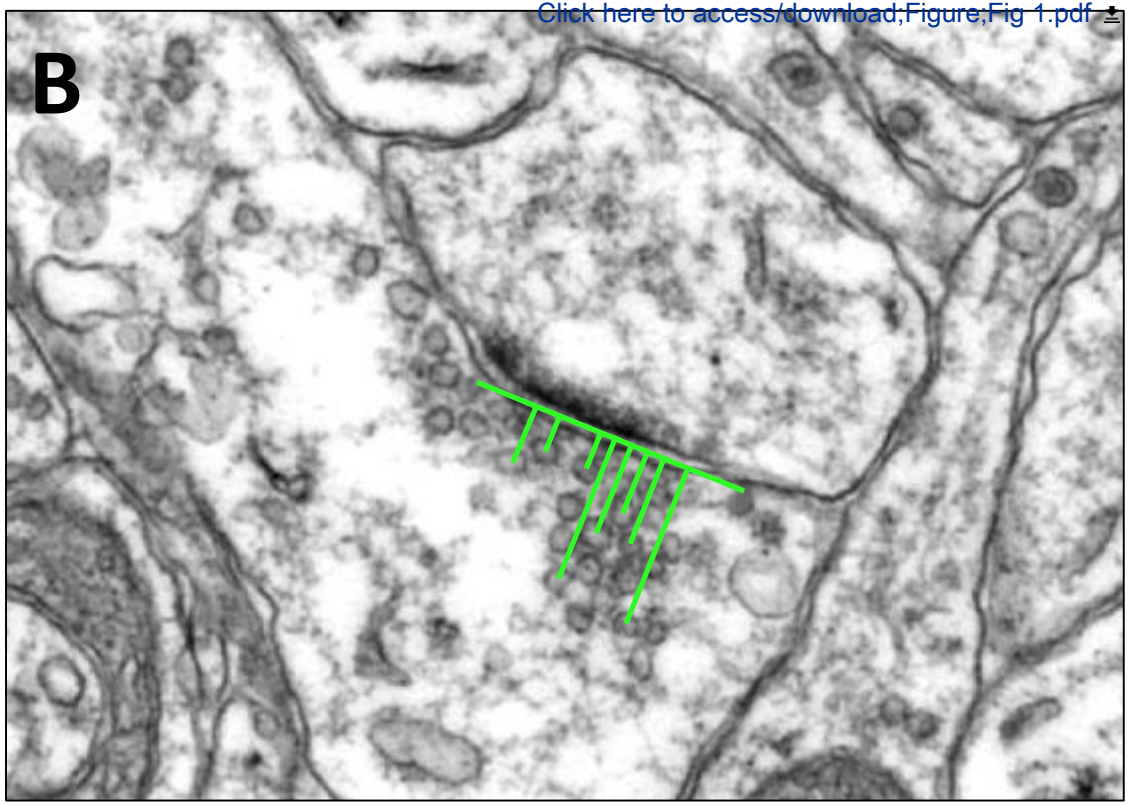
35. Richards, J.G. Ultrastructural histochemistry of nervous tissue. In: *Techniques in Neuroanatomical Research.* (Heyn C and Forssmann WG editors), Springer, Berlin. 277-292 (1981).

36. Wood, R.L, Luft, J.H. The influence of the buffer system on fixation with osmium tetroxide. *Journal of Ultrastructural Res*earch. **12** (1-2), 22-45 (1965).

37. Louw, J., Williams, K., Harper, I.S., Walfe-Coote, S.A. Electron dense artifactual deposits in tissue sections: the role of ethanol, uranyl acetate and phosphate buffer. *Stain Technology*. **65** (5), 243-250 (1990).

Figure 1





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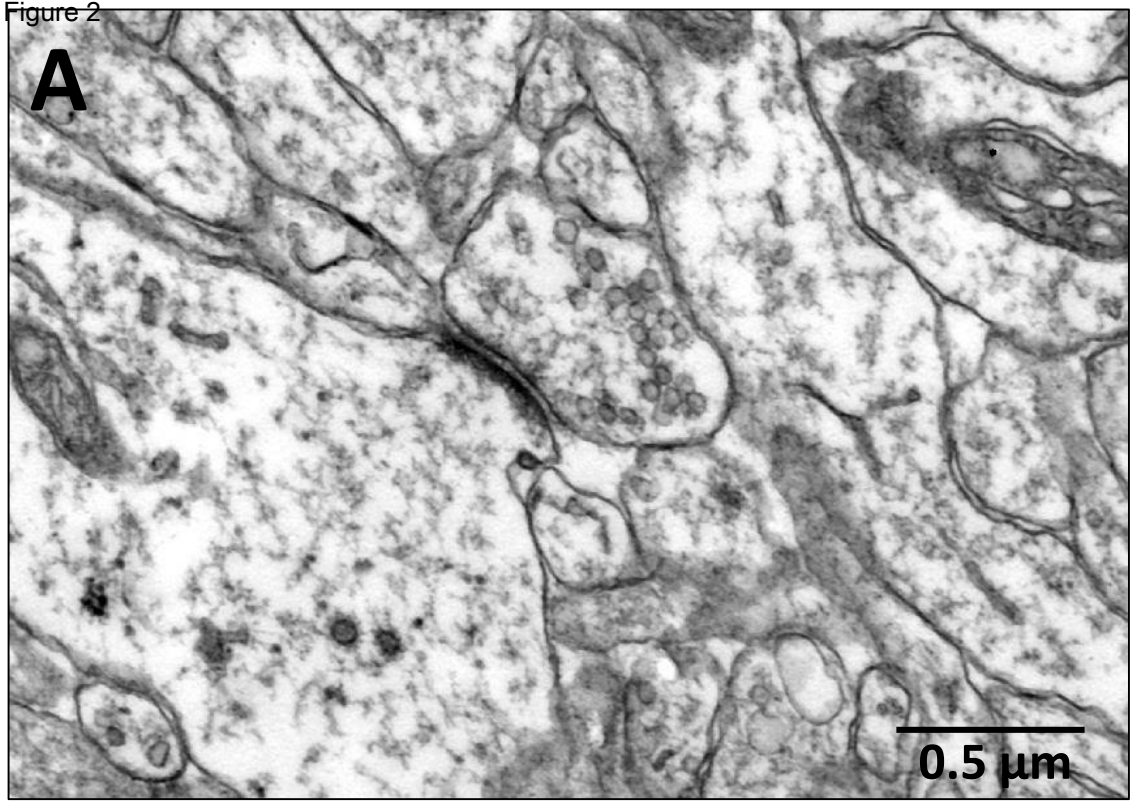

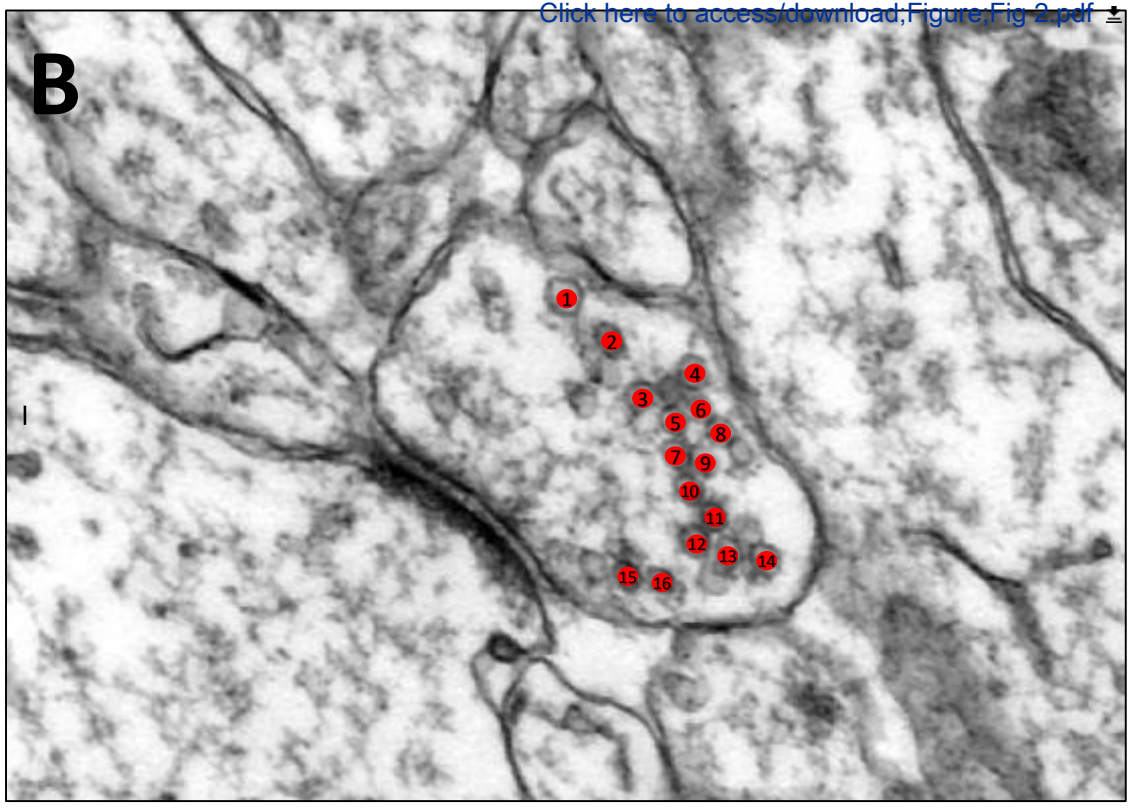

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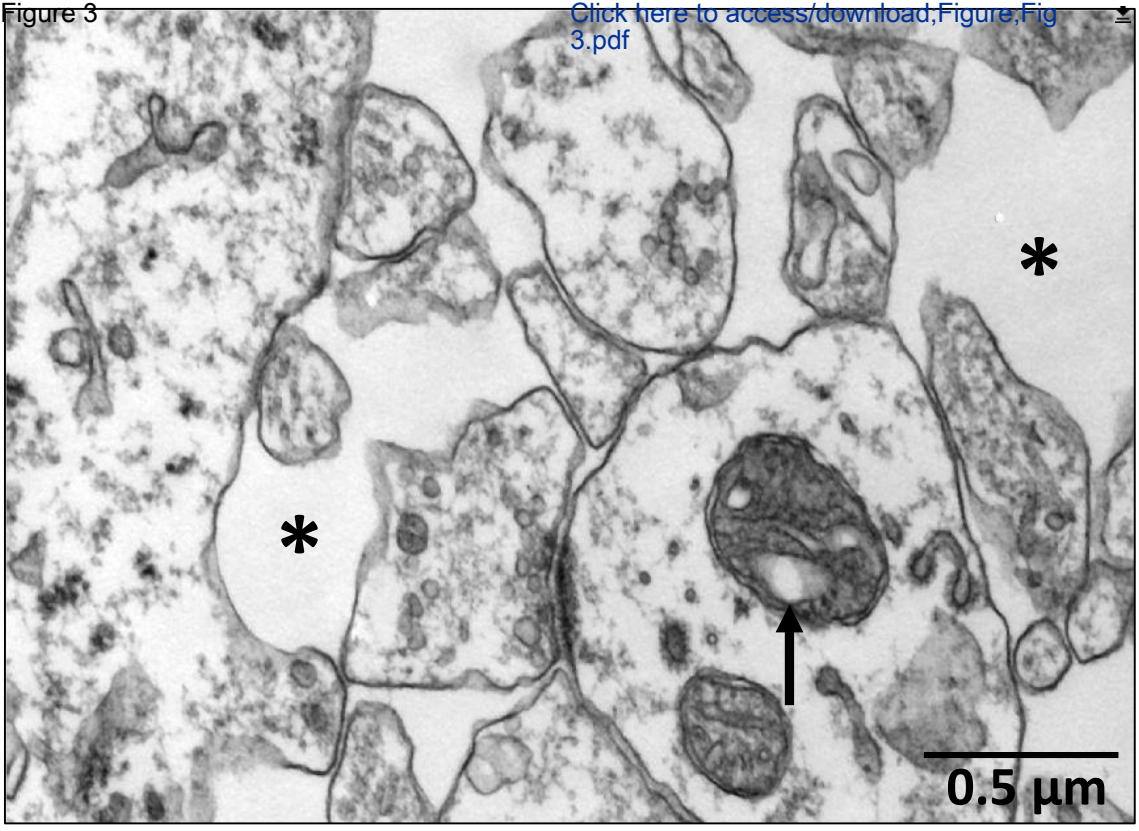

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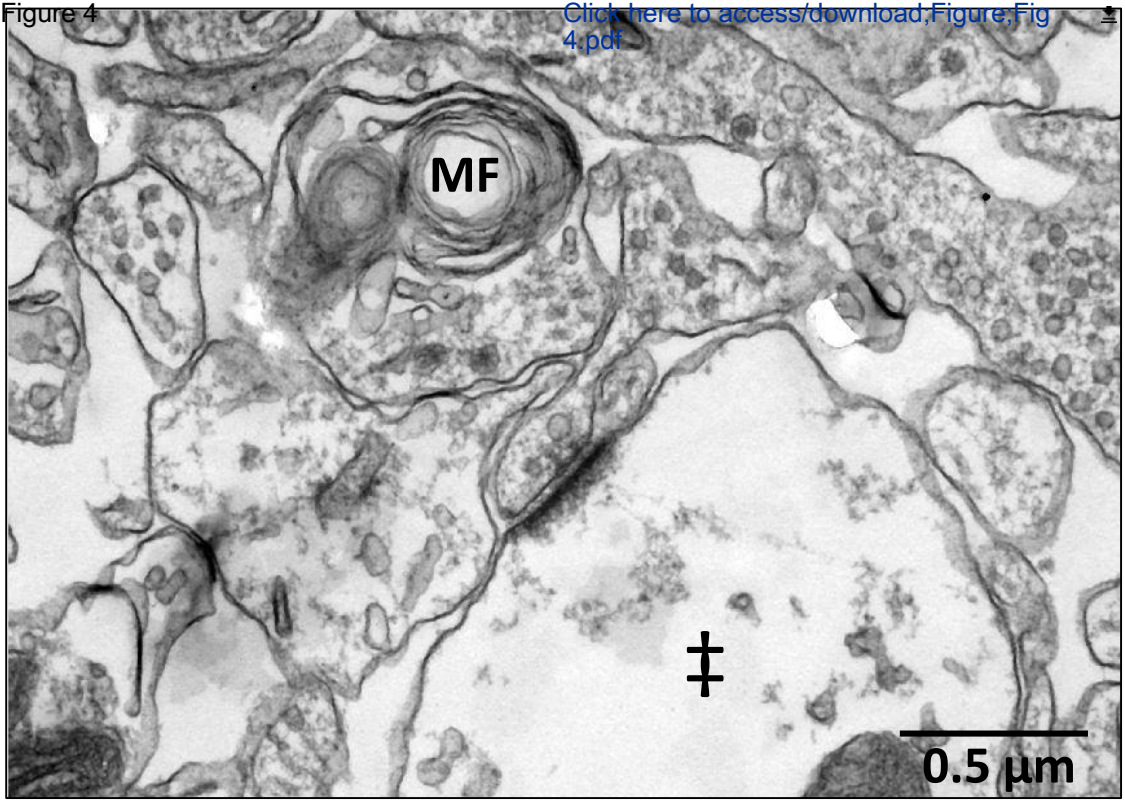

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
4% Osmium tetroxide	Electron Microscopy Sciences	19170	acqueous
50% Glutaraldehyde	Electron Microscopy Sciences	16310	EM grade, acqueous
Aclar 33 C embedding film	Electron Microscopy Sciences	50425-25	7.8 mil thickness, size 8"x10"
BEEM capsule holder	Electron Microscopy Sciences	69916	holds size "00" capsules
BEEM embedding capsules	Electron Microscopy Sciences	70021	size "00", flat
Butler block trimmer	Electron Microscopy Sciences	69945-01	
Camel hair paint brush	Electron Microscopy Sciences	65576-01	
Disc punch	Electron Microscopy Sciences	77850-09	
Embed 812 kit	Electron Microscopy Sciences	14120	
Lead acetate	Electron Microscopy Sciences	17600	
Lead citrate	Electron Microscopy Sciences	17800	
Lead nitrate	Electron microscopy Sciences	17900	
Leica UC7 ultracut microtome	Leica		
Micro scale	Electron Microscopy Sciences	62091-23	
Paraformaldehyde	Electron Microscopy Sciences	19208	EM grade, granular
Precision Thelco laboratory oven	Thelco	51221159	
Sodium azide	Sigma-Aldricht	S2002	

StatMark pen	Electron Microscopy Sciences	72109-01	
Tyrode solution	Electron Microscopy Sciences	11760-05	
Uranyl acetate	Electron Microscopy Sciences	22400	powder

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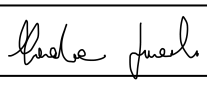
Signature:	
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EDITOR:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has now been checked carefully and edited for spelling and grammar errors.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

None of the figures in the manuscript have been previously published.

3. Please use single-spaced text throughout the manuscript.

The manuscript has now been edited to adopt single-spaced text throughout.

4. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

The manuscript text has now been modified to adopt SI unit symbols throughout.

5. Please use h, min, s for time units.

The manuscript text has been edited to adopt the abbreviations h, min and s for time units.

6. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns have now been removed from the manuscript text.

7. Please highlight complete sentences (not parts of sentences).

Only complete sentences are now highlighted in yellow for video production purposes.

8. Please use greek characters for SI unit prefixed, e.g. use 'µm' instead of 'um', use 'µL' instead of 'uL'.

The greek characters µm and µL are now used in the manuscript, in place of um and uL.

9. Step 1.1.1: What's the size of the beaker?

We apologize for not clarifying this in the original submission. The size of the beaker (2 L) is now specified on Page 6, Protocol, line 220.

10. 1.1.4: What's the concentration of NaOH solution?

The NaOH concentration is 10 normal (10 N). This is now specified on Page 6, Protocol, line 225.

11. 4.3: How to extract? What is used to extract?

A micropipette is used to extract osmium. This is now clarified on Page 9, Protocol, lines 340-341.

12. 9.7.1: Please write this step in the imperative tense.

This step ("A printed 2 cm-long piece of paper is rolled and lowered into the capsule. The label is made to fit to the curvature of the side walls of the capsule") has now been changed to "Roll and lower a printed 2 cm-long piece of paper into the capsule. Make the label to fit to the curvature of the side walls of the capsule" (Page 13, Protocol, lines 512-513).

13. 9.7.2: Please write this step in the imperative tense.

The step ("After polymerization is complete, the label becomes permanently embedded in the resin") has now been changed to "Wait for polymerization to be complete, so that the label becomes permanently embedded in the resin" (Page 13, Protocol, lines 515-516).

14. 10.4: Please write this step in the imperative tense.

This step ("The razor blade is held at an angle of 45 degrees and cuts are made down four sides of the capsule block") has now been substituted with "Hold the razor blade at an angle of 45 degrees and make cuts down four sides of the capsule block" (Page 13, Protocol, lines 543-544).

15. 10.5: Please write this step in the imperative tense.

This step ("The cuts should be short so that the block takes the form of a short pyramid with wall angles of approximately 45°") has now been changed to "Make short cuts so that the block takes the form of a short pyramid with wall angles of approximately 45 degrees"(Page 13, Protocol, lines 548-549).

16. Please combine some short steps so that each step contains 2-3 actions.

Several short steps in the Protocol have now been combined in 2-3 actions steps.
In detail, step 1.1.1. (Place 800 mL of 0.1 M Phosphate Buffer (PB) in a 2 L glass beaker on a stirring plate under a fume-hood) and 1.1.2. (Heat to approximately 60°C while making sure not to boil) have now been combined into step 1.1.1. (Page 6, Protocol, lines 220-221). Step 1.2.1. (Place 1 L of distilled water in a glass beaker on a stirring plate) and step 1.2.2. (Add NaCl 8 g, KCl 0.15 g, $CaCl_2$ 0.1 g, $MgCl_2$ 0.006 g, NaH_2PO_4 0.055 g, $NaHCO_3$ 1 g and dextrose 1 g in the beaker) have been combined into step 1.2.1 (Page 6, Protocol, lines 238-239). Step 1.3.1 (Add

40 mL of electron microscopy-grade 50% glutaraldehyde to 1 L of 4% paraformaldehyde) and 1.3.2. (Mix well in a stirring plate) have been consolidated into one step (step 1.3.1, Page 7, Protocol, lines 247-248). Step 2.3. (Place sections in 0.1 M PB in a Petri dish) and 2.4 (Examine the sections under a dissection microscope and select specimens for embedding) have been combined into step 2.3 (Page 7, Protocol, lines 285-286). Step 3.2 (Rinse specimen in 0.1 M PB for 3 min x 2) and 3.3 (Remove PB with a micropipette) have been consolidated into step 3.2 (Page 8, Protocol, line 301). Step 4.2 (Add 1% OsO_4 in 0.1 M PB in the specimen vial and let sit for 1 h) and 4.3 (Extract OsO_4 after 1 h with a micropipette) have been combined into step 4.2 (Page 9, Protocol, lines 340-341). Step 7.3 (Replace the mixture of resin and acetone with full resin. Let sit for 4 h or overnight) has been combined with step 7.3.1 (Put all EPON waste in a collection container under the hood to be polymerized and disposed of later) into one step (step 7.3, Page 11, Protocol, lines 451-452). Steps 8.1 (Cut two rectangular pieces of clear aclar sheet) and 8.2 (Wipe the films clean with ETOH 70%) are now combined together in step 8.1 (Page 11, Protocol, line 460). Steps 8.7 (Label the sheets with a solvent resistant pen) and 8.8 (Place in oven at 60°C for 2-3 days to polymerize) are now consolidated in step 8.6 (Page 12, Protocol, lines 483-484). Steps 9.6 (Insert a capsule into the cap) and 9.7 (Use fine tweezers to insert labelling) are now one step (step 9.6, Page 13, Protocol, line 510). Step 9.9 (Push the tissue specimen down to the bottom of the capsule with the aid of a pointed wooden stick) and 9.10 (Place in the oven at 60°C for 2-3 days) are now together in step 9.8 (Page 13, Protocol, lines 519-520). Steps 13.1.2 (Place a small sheet of clean dental wax on top of the filter paper), 13.1.3. (Place several NaOH pellets on one side of the dish) and 13.1.4 (Cover the dish) are now one step (step 13.1.2, Page 15, Protocol, lines 611-612). Steps 13.3.2 (Add 8.2 mL of distilled water) and 13.3.3 (Shake vigorously for 5 min) are now in one step (step 13.3.2, Page 15, Protocol, line 626). Steps 13.3.4 (Sonicate for 30 s) and 13.3.5 (Add 1.8 ml of freshly made 4% NaOH) are now combined into step 13.3.3 (Page 15, Protocol, line 628). Steps 13.6 (Cover the dish) and 13.7 (Let stain for 5 min) are now one step (step 13.6, Page 15, Protocol, line 638).

17. Please do not abbreviate journal titles for references.

Journal titles in the “References” section are now spelled out.

REVIEWER#1:

1. It is unclear in the abstract, introduction, or discussion how this technique differs from other EM preparation techniques; and therefore why an investigator would choose this protocol over another. Please address why this particular technique may be beneficial. Have other techniques proved difficult in preparing newborn rat brains to look at synaptic vesicles? The title is very specific, so I am curious if this technique provides a particular advantage when investigating newborn brain vesicles.

We are grateful to the Reviewer for this comment, and we regret we did not sufficiently emphasize the added challenges of preserving fine ultrastructure when working with newborn rat brain tissue. The central nervous system of very young animals is characterized by a higher content in water than the adult brain, more prominent enlargement of extracellular spaces and looser connections between cells. Because of these unique characteristics, the neonatal rat

brain is very sensitive to changes in osmolarity, and artifactual shrinkage and/or swelling of extracellular spaces and cellular components can easily develop when using solutions of markedly different tonicity than that of newborn rat brain. We have now highlighted these challenges in the Introduction.

2. Protocol step 1.6: please clarify if the "fresh fixative" is the PFA-glutaraldehyde fixative.

Fresh fixative refers to paraformaldehyde-glutaraldehyde fixative. This is now clarified on Page 7, Protocol, lines 269-270.

3. Protocol step 6.3: The step between 6.2 and 6.3 is slightly vague. Is the 50% ETOH removed before adding 4% UA in 70% ETOH? I assume yes, but it would be helpful to the reader if it was more clear.

50% ETOH needs to be removed before UA can be added. This is now specified on Page 10, Protocol, line 387.

4. Protocol step 7.1.3: Although it's intuitive, authors should instruct readers to put plunger back on 60 mL syringe prior to rocker

A sentence has now been added to clarify that the syringe plunger needs to be put back in place before the syringe is placed on the rocker (Page 11, Protocol, line 435).

5*. Protocol step 7.2.1: How do you apply the EPON:acetone mixture? Add to the vial? Anything important in this step or simply add to vial?*

This step does not require any special consideration, i.e., the EPON:acetone mixture can simply be added to the vial containing the specimen. Therefore, no change has been made to the original manuscript.

6*. Protocol step 12.4: What is the temperature of this boiled water when used as a rinse? Room temperature?*

We apologize for this oversight in the original submission. The boiled water should be used at room temperature. This has now been clarified in the text (Page 15, Protocol, lines 600-601).

REVIEWER#2:

1. It would help the reader that all major parts of the procedure are briefly discussed in the introduction. Currently, some parts, such as the dehydration embedding and microtomy are not mentioned at all.

The Introduction has now been edited to include a brief discussion of sequential dehydration and embedding (Page 4, Introduction, lines 127-135). However, since in the protocol there are no microtomy steps that are specific to preparation of newborn rat brain tissue for ultrastructural analysis of synaptic vesicles, we elected to not mention microtomy.

2. (Line 51) It may be noted that imaging vitrified samples by cryo-electron microscopy or high

pressure frozen and freeze substituted samples yield better ultrastructural preservation and that they were used in the past to image brain slices. Certainly, these methods have their own limitations.

The method of cryofixation by high pressure freezing has now been acknowledged in the Introduction (Page 3, Introduction, lines 86-88).

3. (Line 118) It is not correct that carbon, oxygen and nitrogen are electron transparent, because they provide signal in cryo-electron microscopy.
This sentence has now been removed from the revised manuscript.

4. There is an inconsistency in numbering of some steps. In most cases where three number items describe specific steps, their corresponding two number headings serve as subtitles, while 7.2 , 7.3 and 8.1 are both sub-headings and specific steps.

We believe that three number items are used in the protocol to group together the explicit steps to prepare a certain solution or component. We agree with the Reviewer that three number items are mostly placed under a two number heading that is used as a subtitle. However, throughout the protocol two number headings are not exclusively used as a subtitle for three number items, and there are several two number headings that describe specific steps (in sections 1, 2, 3 and 5, for example). Steps 7.2, 7.3. and 8.1 do not contain specific steps to prepare a solution/component that we would group together under a three number item. Therefore, we believe that these steps should be assigned two numbers.