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Immuno-TEM/STEM in retinal research

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Abstract

Immuno-EM is a method that can determine the localization of a protein in a tissue at the ultrastructural level. Generally, membrane structures in immuno-EM specimens have very low contrast because fixation is performed without osmium. Here, by using high angle annular dark field (HAADF)-scanning transmission electron microscopy (STEM) instead of transmission electron microscopy (TEM) for observation of immuno-EM samples, we demonstrate that photoreceptor disk membranes are clearly visible, and that the procedures described in this article can be extended to visualize other membrane structures.

Keywords

Immuno-EM; HAADF-STEM; TEM; photoreceptor; disk (disc) membrane; LR white resin and rhodopsin

1 Introduction

Photoreceptor cells are highly polarized retinal neurons characterized by their outer segment structures which are optimized for photon absorbance. These membrane rich structures consist of thousands of stacked and regularly aligned disk membranes. Different degenerative diseases of the retina are caused by abnormalities associated with disk membrane proteins which then disturb the structure of the disks as well as the outer segment as a whole. To unravel the etiologies of these blinding disorders, it often is crucial to determine the localization of abnormal proteins in relationship to disrupted membrane structures. Transmission electron microscopy (TEM) is a powerful tool to analyze the relationship between membrane structure and protein localization at the ultrastructural level. For conventional TEM imaging of plastic thin sections of tissue, membrane contrast is achieved by osmium staining of membrane components. Unfortunately, osmium fixation is not compatible with immuno-EM because tissue fixation with osmium can result in masking of the antibody epitope. However, if EM samples are prepared without osmium, it is possible to label sections with antibodies. However, membrane contrast is then low for these specimens. The confounding properties of osmium fixation and immunolabeling make it difficult to study the membrane structure while accurately determining the localization of proteins. In this manuscript, we describe the application of high angle annular dark field (HAADF)-scanning transmission electron microscopy (STEM) to visualize immunolabeled photoreceptor disks without osmium fixation. Membrane contrast was enhanced by staining

retinal sections with uranyl acetate and lead after immunolabeling. With HAADF-STEM, uranyl acetate and lead staining provide clear membrane contrast. HAADF-STEM mainly relies on amplitudes of transmitted scattered electrons for contrast formation. Therefore, sensitivity towards heavy metals in the specimen can be higher with HAADF-STEM than with conventional TEM. By taking advantage of this difference, HAADF-STEM has been successfully applied to image ultra-small gold particles (< 3 nm) which are difficult to image with high-contrast by conventional TEM [1, 2]. Compared to other widely-used immuno-EM techniques, the method described here also does not to require incubation of retina with antibodies prior to resin embedding. This advantage allows labeling of serial thin sections (~80 nm thickness) with different antibodies.

2 Materials

All glassware and magnetic stir bars should be carefully washed and then rinsed with Milli-Q water. Use 0.22 μm filtered Milli-Q water for both the final rinse and preparing solutions for EM. Solutions that need to be stored at 4 °C are: 1% $CaCl_2$ stock solution, 1% $MgCl_2$ stock solution, 0.187 M, 0.195 M, 0.202 M and 0.312 M, Millonig's buffer, 0.1 M phosphate buffer (pH 7.4), 1X PBS, antibody reaction/washing solution, 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), uranyl acetate staining solution, modified Sato's lead-staining solution, 20% paraformaldehyde (formaldehyde) aqueous solution, and 30% ethanol solution. Liquids that need to be stored at −20 °C are: 50%, 70%, and 95% ethanol solutions and blocking solutions for antibody reactions. All other solutions can be stored at room temperature (RT). Use only the highest quality chemicals or EM grade chemicals. Solutions that should be made with EM grade chemicals are noted in the Methods section. Handle toxic chemicals following local safety guidelines.

2.1 Preparing immuno-EM blocks

1. Millonig's buffer (0.312 M): A) Dissolve 58.76 g of $NaH_2PO_4{\cdot}2H_2O$ (Sigma, 71505) into 1 L of filtered Milli-Q water. B) Dissolve 10.8 g of NaOH into 200 ml of filtered Milli-Q water. Mix 415 ml of solution A and 85 ml of solution B using a magnetic stirring bar. The pH should be 7.3 at 4 °C [3]. Leave magnetic stirring bar in the bottle and store at 4 °C.

2. Fixative solution: 4% formaldehyde, 0.05% glutaraldehyde, 0.1% DMSO, 0.00025% $CaCl_2$ and 0.00025% $MgCl_2$ in 0.156 M Millonig's buffer. Prepare fixative solution on the same day as tissue fixation. Mix ~19 ml of filtered Milli-Q water, 25 ml of 0.312 M Millonig's buffer, with 50 μl of DMSO (*e.g.*, EMS, 13390), 12.5 μl of 1% $CaCl_2$ and 12.5 μl of $MgCl_2$. Then, add 10 ml of 20% EM grade paraformaldehyde (formaldehyde) aqueous solution (EMS, 15713) and 0.1 ml of 25% EM grade glutaraldehyde aqueous solution (EMS, 16220) in a chemical fume hood. Make up to 50 ml of solution with filtered Milli-Q water and keep at room temperature. Both the 20% paraformaldehyde and 25% glutaraldehyde aqueous solutions from EMS come in glass vials. Prepare fixatives from newly opened vials. Filter with a 0.22 μm filter. Use undiluted DMSO to make fixative solution. Keep DMSO stock with molecular sieves in it and wrap lid with parafilm (*see* Note 1).

3. Quenching solution: 50 mM glycine (Sigma, G7403) in 0.187 M Millonig's buffer. Prepare quenching solution just before use. Mix 20 ml of filtered Milli-Q water and 30 ml of

0.312 M Millonig's buffer to make 50 ml of 0.187 M Millonig's buffer. Dissolve 0.187 g of glycine into 50 ml of 0.195 M Millonig's buffer. Filter with a 0.22 μm filter. Store at 4 °C.

4. Washing solution: 0.195 M Millonig's buffer. Mix 18.75 ml of filtered Milli-Q water and 31.25 ml of 0.312 M Millonig's buffer to make 50 ml of 0.195 M Millonig's buffer. Store at 4 °C.

5. Sample storage buffer: 0.202 M Millonig's buffer. Mix 17.5 ml of filtered Milli-Q water and 32.5 ml of 0.312 M Millonig's buffer to make 50 ml of 0.202 M Millonig's buffer. Store at 4 °C.

6. Dehydration solutions: 30%, 50%, 70%, and 95% ethanol. Mix ethanol and filtered Milli-Q water to make a series of solutions with increasing concentrations of ethanol. Store the 30% ethanol solution at 4 °C, the 50%, 70% and 95% ethanol solutions at −20 °C. (*see* Note 2).

7. Resin: Pour fresh LR white resin (EMS, 14381) into a clean brand new disposable plastic beaker (*e.g.*, EMS, 60974) in the chemical fume hood. Wrap the sides of the beaker and then cover it with aluminum foil. Then open ~2/3 of the aluminum foil cover for effective degassing. Vacuum ~ 2 h in a vacuum desiccator. Use unexpired resin.

8. Capsules: Gelatin size 3 (EMS, 70104).

9. Holder for capsule cap: Make a holder with a metal wire and a small foot such as a cork stopper for flask (Fig.1).

10. Rack for capsule body (*see* Note 3).

11. Rack for polymerization of resin in the capsule (*see* Note 4)

12. Microcentrifuge tubes: 0.6 mL.

13. Minus 20 °C freezer for sample processing: a chest type freezer is preferred.

14. Incubator: equipped with shaker is preferred.

15. Glass vials with polyethylene caps for sample processing (*e.g.*, 2 ml vials, EMS, 72630–05). (*see* Note 5).

16. Ice cooler to keep the sample at −20 °C during processing in the chemical fume hood.

17. Microdissecting spring scissors with a curved tip (*e.g.*, Roboz, RS-5671).

18. Microdissecting spring scissors with a fine straight tip (*e.g.*, FST, 15003–08).

19. Plastic petri dishes.

20. Rotator.

21. Wash bottles (*e.g.*, Fisher, 03-409-10AA).

22. Butyl gloves (*e.g.*, Honeywell, B074GI).

23. Surgical blades (*e.g.*, Bard-Parker™ Surgical Blades, size 11, BD-371211).

24. Transfer pipets (*e.g.*, EMS, 70960–1S).

25. Glass Pasteur pipets (*e.g.*, Fisher scientific, 22-230-482).

26. Microplates: 24 well.

27. Conical tubes: 50ml.

28. Water resistant thick paper

2.2 Preparing sections

1. Ultramicrotome (*e.g.*, RMC, MT6000-XL, PTX or PTXL or Leica, EM UC6 or EM UC7)

2. Specimen holder (*see* Note 6)

3 a. Diamond knife for thin sectioning, 70–80 nm (*e.g.*, Diatome, ultra 45°).

3 b. Diamond knife for thick sectioning, ~400 nm (*e.g.*, Diatome, Histo).

4. Glass knife for trimming: make glass knives from ultramicrotome grade glass knife strips (*e.g.*, EMS, 71012) using a glass knife maker (*e.g.*, Ted Pella, LKB 7800 KnifeMaker).

5. Double edged stainless steel razor blades (*e.g.*, EMS, 72000).

6. Two-part epoxy (*e.g.*, Loctite, Quick Set Epoxy).

7. Small glass beaker to hold removed gelatin capsule.

8. Hammer.

9. Razor blade.

10. Non-drying, non-hardening modeling clay.

11. Plain glass microscope slides.

12. Staining solution: 0.5% Toluidine Blue O in 0.1 M phosphate buffer (pH 7.4). Dissolve 0.5 g of Toluidine Blue O (*e.g.*, Polyscience, 01234) into 100 ml of 0.1 M phosphate buffer (pH 7.4). Mix well with a magnetic stir bar. Store at room temperature. For use: fill a disposable plastic syringe with staining solution, place a 0.22 μm filter at the end of the syringe to prevent contamination of sections with undissolved Toluidine Blue O.

13. 0.2 M NaH_2PO_4. Dissolve 15.6 g of $NaH_2PO_4 \cdot 2H_2O$ into Milli-Q water. Adjust volume to 500 ml with Milli-Q water. Autoclave and store at RT.

14. 0.2 M Na_2HPO_4. Dissolve 35.6 g of $Na_2HPO_4 \cdot 2H_2O$ in Milli-Q water. Adjust volume to 1 L ml with Milli-Q water. Autoclave and store at RT.

15. 0.1 M Phosphate buffer (pH 7.4). Mix 38 ml of 0.2 M NaH_2PO_4, 162 ml of 0.2 M Na_2HPO_4 and 200 ml of Milli-Q water to make 400 ml of 0.1 M phosphate buffer (pH 7.4). Store at 4 °C.

16. Upright microscope equipped with 63X oil-immersion objective lens.

17. Lens oil.

18. Lens paper.

19. Xylene.

20. Black ultra-fine point marker.

21. Compressed air dusters.

22. Whatman™ Qualitative Filter Paper. Circles, Ø 90 mm: Grade 5 or Grade 1 (GE Healthcare Life Sciences, 1005–090 or 1001–090).

23. Fine tip non-magnetic tweezers, #5 with a clamping ring.

24. Filtered Milli-Q water: For filling diamond knife boat, use a syringe with a 0.22 μm filter and needle. For washing the diamond knife and sections, use filtered Milli-Q water in a wash bottle.

25. EM grids (*e.g.*, EMS, T400-Ni): Place grids onto a clean lens paper or a filter paper. Hold tab of grid with fine tip tweezers as shiny side up. Dip grid into freshly prepared formvar solution in the chemical fume hood for less than 1 s (see Note 7). Then, quickly absorb excess formvar solution on the grid by attaching the matt side of the grid edge to filter paper at less than a 90° angle. Place grid as shiny side up on a clean filter paper placed in a petri dish. Repeat this process to make sufficient dipped grids. Once enough girds are placed on a filter paper, let them completely dry, then transfer grids one by one with fine tip tweezers onto a new filter paper placed in a glass petri dish. Make sure the shiny side is facing to up (*see* Note 8). Store grids at RT. Dispose chemicals following local rules (*see* Note 9).

26. Formvar solution: 0.3% formvar [15/95 (poly[vinyl formal]) in 1,2-dichloroethane. Just before use, freshly mix 15/95 polyvinyl formvar (Polyscience) and 1,2-dichloroethane (Sigma, 34872) to make 5 ml of 0.3% Formvar 15/95 solution in a clean glass vial with a polyethylene cap.

27. Glass petri dishes (Ø 100 mm for storage of a large number of grids and Ø 60 mm for storage of a small number of grids, *e.g.*, Fisher, 08–747D and 08–747A) (*see* Note 10).

28. Toothpick with eyelash: Clean eyelash by soaking it in xylene. Glue one piece of eyelash at the end of a long toothpick with crazy glue or an alternative glue. A straight eyelash is suitable for handling sections.

29. Tooth pick for picking up sections: Trim a high quality of bamboo tooth pick on one side to flatten its shape and the other side to obtain a pencil shape.

30. Chemical waste container for staining solution.

31. Hot plate that can heat to ~120 °C.

32. Mechanical or carbon pencils.

33. Cover glass.

34. Cure type mounting media or resin used for making plastic blocks.

Optional: Zerostat Anti-Static Gun (EMS, 60610).

Optional: Light microscope equipped with a 63X oil-immersion objective lens and a color camera.

2.3 Antibody reaction

1. Sections (for 80 nm thickness section on a grid).

2. Silicon pads with slits (*e.g.*, Ted Pella, 10523) (*see* Note 11).

3. Primary antibody (*see* Note 12).

4. Secondary antibody conjugated with gold particles (*e.g.*, British Biocell International (BBI), 5 nm gold particles conjugated with secondary antibodies).

5. 10X PBS stock; 1.36 M NaCl, 20 mM KCl, 80 mM Na_2HPO_4,10 mM KH_2PO_4, pH7.4. Dissolve 80 g of NaCl, 2 g of KCl, 14.4g of $Na_2HPO_4{\cdot}2H_2O$ and 2 g of KH_2PO4 in Milli-Q water. Adjust volume to 1 L and autoclave. Store at RT.

6. Autoclaved Milli-Q water.

7. Blocking solution: 5% dry milk, 0.01% Tween 20 in PBS. Dissolve 2.5 g of dry milk (Nestle, Carnation instant nonfat dry milk) into a mixture of ~40 ml of autoclaved Milli-Q water and 5 ml of 10X PBS. Add 500 μl of autoclaved Milli-Q water into a 0.6 ml microcentrifuge tube. Then, add Tween 20 up to 0.6 ml indicated on the tube (100 μl of Tween 20 and 500 μl of Milli-Q water). Mix well by vortexing. Transfer 30 μl of diluted Tween 20 into ~45 ml of PBS containing dry milk. Adjust volume to 50 ml with autoclaved Milli-Q water. Mix well and then filter with a 0.22 μm filter and store at −20 °C.

8. Antibody reaction/washing solution: 1% BSA, 0.01% Tween 20 in PBS. Dissolve 0.5 g of BSA into a mixture of ~40 ml of autoclaved Milli-Q water and 5 ml of 10X PBS. Add 500 μl of autoclaved Milli-Q water into a 0.6 ml microcentrifuge tube. Then, add Tween 20 up to the 0.6 ml line on tube (100 μl of Tween 20 and 500 μl of Milli-Q water). Mix well by vortexing. Transfer 30 μl of diluted Tween 20 into ~45 ml of PBS containing BSA. Adjust volume to 50 ml with autoclaved Milli-Q water. Mix well and then filter with a 0.22 μm filter. Store at 4 °C. Make this solution just before use.

9. 0.1 M Phosphate buffer (pH 7.4). Mix 38 ml of 0.2 M NaH_2PO_4 solution, 162 ml of 0.2 M Na_2HPO_4 solution, and 200 ml of Milli-Q water to make 400 ml of 0.1 M phosphate buffer (pH 7.4). Store at 4 °C.

10. 2.5% Glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Mix 0.95 ml of 0.2 M NaH_2PO_4 solution, 4.05 ml of 0.2 M Na_2HPO_4 solution and 4 ml of autoclaved Milli-Q water. Add 1 ml of 25% EM grade glutaraldehyde (EMS, 16220) in the chemical fume hood. Store at 4 °C (*see* Note 13).

11. Filtered Milli-Q water. Filter Milli-Q water with 0.22 μm filter (*e.g.*, Millipore Sigma, SCGPT01RE).

2.4 Staining and carbon coating

1. Six 100–150 ml glass beakers with mouths wide enough for silicon pad washing.

2. Flat-tip tweezers (*e.g.*, EMS, 78334–34A).

3. Silicon pad with slits (*e.g.*, Ted Pella, 10523).

4. Uranyl acetate staining solution: 2% uranyl acetate in 50% methanol. Dissolve 0.5 g of uranyl acetate (*e.g.*, EMS, 22400) into 12.5 ml methanol and 12.5 ml of filtered Milli-Q water. Wrap in aluminum foil and mix with a magnetic stir bar for more than 2 h at RT. Add 7 μl of glacial acetic acid. Filter with a 0.22 μm filter and store at 4 °C in a light-sealed container.

5. Modified Sato's lead-staining solution [4]: 68 mM sodium citrate, 4.6 mM lead(II) citrate, 11 mM lead nitrate, 9.6 mM lead(II) acetate, 0.18 M NaOH in filtered Milli-Q water. Prepare this solution at least one week before use. Dissolve 0.5 g of $HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$, 0.1 g of $(C_6H_5O_7)_2Pb_3 \cdot 3H_2O$, 0.075 g of $Pb(NO_3)_2$ and 0.075 g of $Pb(CH_3CO_2)_2 \cdot 3H_2O$ in 20.5 ml of filtered Milli-Q water in a glass flask. Seal mouth with parafilm and mix with a magnetic stir bar for more than 2 h at RT. Add 4.5 ml of 1 N NaOH and stir for about 10 min. Filter solution with a 0.22 μm filter. Draw solution into a 20 cc syringe with an 18 G needle. Place a 0.22 μm filter between the syringe and needle. Wrap with paraffin to minimize exposure solution to air. Store at 4 °C. Wait 1 week before use (*see* Note 14).

6. Petri dish: to hold silicon pad with grids inside.

7. Light blocking container: to hold two petri dishes inside.

8. Filtered Milli-Q water.

9. A wash bottle (*e.g.*, Fisher, 03-409-10AA).

10. Chemical waste bottles for uranyl acetate and lead solutions.

11. Whatman™ Qualitative Filter Paper, Circles, Ø 90 mm: Grade 5 or Grade 1 (GE Healthcare Life Sciences, 1005–090 or 1001–090).

12. Fine tip non-magnetic tweezers, #5.

13. Carbon coater (*e.g.*, Denton Vacuum, DV-401).

14. Carbon yarn and rod.

2.5 Imaging of immuno-EM samples

1. TEM (*eg.* a JEOL Ex1200, 80 kV).

2. STEM (*eg.* a Zeiss Libra 200FE, 200 kV).

3. EM grids for imaging.

4. Fine tip non-magnetic tweezers (*see* Note 15).

3 Methods

3.1 Sample preparation, day 1

1. Prepare all dissection tools and solutions listed in 2.1.

2. Start to de-gas fresh LR white resin.

3. Turn on the - 20 °C freezer.

4. Euthanize a mouse following local laws and regulations.

5. Remove eye balls using a micro dissecting spring scissors with a curved tip (*see* Note 16*).*

6. Soak eye balls in fixative solution in a 24 well plate. Then, transfer eye balls into ice cold 1X PBS solution in a petri dish. Make slits on cornea using a surgical blade in solution, and then transfer eye balls into fixative solution again. A few minuts later, transfer eye balls into 1 X PBS. Remove cornea with scissors, and then transfer eye balls into fixative solution. About 10 min later, transfer eye balls into 1X PBS. Remove lens in 1X PBS, and then transfer resulting eye cups into fixative solution (*see* Note 17).

7. Transfer eye cups onto a water resistant thick paper and cut eye cups into small pieces by sliding two double edge blades across the eye cup. With 2-week old mice, cut eye cups into 4 pieces but with adult mice, cut into 6 pieces (*see* Note 18).

8. Transfer small pieces of eye cups with a transfer pipette into fixative solution. Then, transfer them into fresh fixative solution. Incubate at 37 °C for 30 min with gentle agitation (*see* Note 19).

9. Transfer de-gassed fresh LR white resin into 50 ml tubes and keep at −20 °C.

10. Prepare quenching solution.

11. Transfer small pieces of eye cups into a 24 well plate filled with freshly prepared quenching solution. Incubate at 4 °C for 5 −10 min on a rotator set to gentle speed.

12. Transfer small pieces of eye cups into a 24 well plate filled with 0.187 M Millonig's buffer. Incubate at 4 °C for 5 min on a rotator set to a gentle speed.

13. Briefly remove solution from each well and then add 0.195 M Millonig's buffer from a wash bottle. Incubate at 4 °C for 5 min. Repeat two more times.

14. Transfer small pieces of eye cups into a glass vial filled with 0.202 M Millonig's buffer (*see* Note 20).

15. Mix one part of −20 °C cold white resin and one part of −20 °C cold 95% ethanol in a 50 ml conical tube. Store at −20 °C.

16. Mix two parts of −20 °C cold white resin and one part of −20 °C cold 95% ethanol in a 50 ml conical tube. Store at −20 °C.

17. Remove 0.202 M Millonig's buffer from the sample vial. Add ice cold 30% ethanol. Incubate at 4 °C for 10–15 min (*see* Note 21).

18. In a −20 °C freezer, remove 30% ethanol, and then add −20 °C cold 50% ethanol. Incubate at −20 °C for 10–15 min (*see* Note 22).

19. In a −20 °C freezer, remove 50% ethanol, and then add −20 °C cold 70% ethanol. Incubate at −20 °C for 10–15 min.

20. In a −20 °C freezer, remove 70% ethanol, and then add a −20 °C cold mixture of white resin and 95% ethanol at a 1:1 ratio (prepared at step 15) with a glass Pasteur pipette. Incubate at −20 °C for 10 min.

21. Move the vial into a chemical fume hood with an ice cooler. Remove solution, and then add a −20 °C cold mixture of white resin and 95% ethanol at a 2:1 ratio (prepared at step16). Bring the vial back to −20 °C. Incubate 15 min.

22. Move vial back into a chemical fume hood with an ice cooler. Remove the solution, and then add −20 °C cold white resin. Return the vial back to −20 °C. Incubate 10 min.

23. Repeat step 22 three more times.

24. Return the sample in white resin from −20 °C back to 4 °C. Keep at 4 °C overnight.

3.2 Sample preparation, day 2

1. De-gas fresh LR white resin for over 2 h (*see* Note 23).

2. Remove white resin from sample vials and then add freshly de-gassed white resin (prepared at step1). Incubate at RT for 15 min.

3. Remove white resin from sample vials and then add freshly de-gassed white resin again (prepared at step1). Incubate at RT for 1.5 h.

4. Remove white resin and add freshly de-gassed white resin (prepared in step 1). Place vial without cap in a desiccator and cover the sample and remaining unused resin with aluminum foil. Incubate at RT for 1 h under vacuum.

5. Repeat step 4. At the same time, start to de-gas fresh resin aliquoted from the original bottle stored at 4 °C.

6. Open gelatin capsules and place the bodies of capsules into a PCR tube rack.

7. In a chemical fume hood, transfer the sample and white resin from the glass sample vial into a capsule.

8. Remove white resin and then add freshly de-gassed white resin (prepared in step 5). Cover sample and remaining unused resin with aluminum foil. Incubate at RT for 1 h under vacuum. At the same time, prepare extra capsules with white resin in it.

9. Prepare labels for the immuno-EM samples. Place one sheet of filter paper on a plastic petri dish lid. Tape one side of double sided tape on the filter paper. Write name of sample on a white copy paper with a mechanical or carbon pencil. Alternatively, print the sample name with laser jet printer and press the ink with a tacking iron (*e.g.*, Sealector II Tacking Iron). Cut paper with scissors to make a label about 2.5 mm x15 mm. This will fit into a size of 3 gelatin capsule. Using fine tweezers, stick one long side of the label on double-sided tape to stand it up. Close the lid and keep labels in a plastic petri dish until use.

10. Place the opened 0.6 ml microcentrifuge tubes in a rack.

11. Insert labels into capsules, take out label from a petri dish and make a ring with it. Hold the crossed part of label with tweezers and place it into the body of a capsule to release the label. Then, the curled label in the capsule should uncurl and relocate at the top of the capsule. The position of labels inside the capsules should be close to the top edge so the samples will be not hidden.

12. Bring Kimwipes and a chemical waste container into the chemical fume hood. Keep a box of disposable gloves and two pairs of butyl gloves nearby. Wear butyl gloves over a pair of disposable gloves.

13. Insert an empty capsule cap into a handmade capsule cap holder. A locking ring of the capsule cap should be fit to the holder.

14. Add freshly de-gassed white resin (prepared in step 5) into the cap until full. The white resin should make a dome with surface tension.

15. With one hand, hold the body of capsule with sample in it. Add resin until full.

16. Hold the cap in a handmade holder with other hand.

17. Invert the cap holder upside down. White resin should stay in the cap because of surface tension.

18. Tilt the cap and slowly close it from one side. Avoid formation of air bubbles on the inside of the capsule (*see* Notes 24 and 25).

19. Wipe the capped capsule carefully with a Kimwipe to remove spilled white resin.

20. Place the capsule in the open 0.6 ml tube prepared in step 10.

21. Repeat 13–20 for each sample and for a plain block (with no sample and no label in it). Plain capsules will be used later for re-embedding.

22. Polymerize resin at 40 °C for 5 days.

3.3 Sectioning

1. After complete polymerization of resin, soak blocks with gelatin capsules in Milli-Q water in a small beaker.

2. Once gelatin becomes soft, remove it completely.

3. Wipe block with a Kimwipe to remove water.

4. If required, change the orientation of samples by re-embedding. Usually, re-embedding is required for vertical sections of retina because the small pieces of eye cup usually sit on the bottom of the capsule in an orientation for horizontal sectioning. For re-embedding, record sample's name before cutting off label. Cut off bottom part of block containing sample with a razor blade and hammer. Trim block with a double edged blade. Do not trim tissue. Make a base by using a plain block without a sample and label. Cut off top part of the plain block to make a flat surface. Stand the plain block on modeling clay. Glue a piece of block containing a sample on this block at the desired angle. Wait until glue is completely hardened (*see* Note 26).

5. Set block into the specimen holder of ultramicrotome. Make sure block is tightly clasped by the specimen holder.

6. Set holder into the trimming block. Under the stereomicroscope of the ultramicrotome, trim block with a double edged blade (*see* Note 27).

7. Set holder into the ultramicrotome. Trim block surface with a glass knife (*see* Note 28).

8. After trimming block surface with glass knife, change to a diamond knife designed for thick sectioning. By using back light, which reflects light from knife onto block surface, adjust the knife parallel to block surface. Then, decrease the distance between the diamond knife and block surface.

9. Once diamond knife gets close enough to the block surface, but dones not touch it, transfer filtered Milli-Q water into the diamond knife boat until the water wets the entire surface of the knife. Then, remove excess water (*see* Note 29).

10. Set the cutting speed and slice thickness according to the maximum capability of the diamond knife and ultramicrotome. Run the ultramicrotome to make thick sections.

11. Once sections are generated, stop the ultramicrotome.

12. Drop a small amount of water on to a plain glass slide and mark the water's edge with fine black pen from the other side of the glass slide.

13. Pick up a floating section with the flat side of a bamboo tooth pick. Transfer section onto the water on the glass slide.

14. Let the section on the water dry.

15. Incubate the section on the hot plate (~120 °C) and then apply staining solution onto the section.

16. Incubate the section until the edge of the staining solution becomes dry.

17. Hold slide over the chemical waste container and wash out staining solution by applying a flow of Milli-Q water to the slide (*see* Note 30).

18. Absorb excess water with a paper towel. Place the slide back on the hot plate and let it dry completely.

19. (Optional) Repeat steps 15–18 if the staining is weak.

20. Directly apply lens oil to the section and observe section with an 63X oil-immersion objective lens. After observation, remove oil on the section by applying a drop of xylene and absorb them with lens paper. Gently slide off lens paper in one direction. Direct contact of a lens and oil on the section is not recommended if the oil-immersion objective lens is not dedicated for observation of stained sections without a coverslip. In that case, to prevent potential contamination of the lens with staining solution, first observe section with a low magnification air lens such as 20X. Then, mount the section with cure type mounting medium or epon resin used for a plastic block. Place cover-glass on the mounting medium and wait until it completely hardens. When you use epon resin is used as a mounting medium, you can polymerize it at RT in the chemical fume hood or at ~70 °C in the incubator. Do not apply too much resin to the section. Excess resin creates a thick plastic layer between the section and cover slip and prevents observation of the section with a 63X lens with short working distance (*see* Note 31).

21. If light microscopy shows the quality of sample is acceptable for immune-EM analysis, trim block with a double-edged blade to adjust block size to fit the EM grid.

22. Set block to ultramicrotome again and set diamond knife for thin sectioning. Align knife edge to block surface (*see* Note 32).

23. Decrease distance between knife edge and block surface.

24. Once the diamond knife is close to the block surface, but does not touch it, transfer filtered Milli-Q water into the diamond knife boat until the water wets the entire surface of the knife edge. Then, remove excess water.

25. Set the cutting speed and slice thickness according to the maximum capability of the diamond knife and the ultramicrotome. Then, run the ultramicrotome such that the diamond knife can cut sections with the largest dimensions. Stop ultramicrotome.

26. Decrease the cutting speed (~ 0.4 mm/s) and thickness to 80 nm and then run the ultramicrotome again (*see* Note 33).

27. Pick up a grid using tweezers with a clamping ring. Slide the clamping ring into a locked position. Prepare several tweezers with grids (*see* Note 34).

28. Once sufficient silver colored sections are made, stop ultramicrotome.

29. (Optional) Discharge sections with a Zerostat Anti-Static Gun.

30. Dissociate sections from ribbons by using a long tooth pick with an eyelash. Minimize direct contact of eyelash with sections to avoid damaging the latter.

31. With one hand, slowly insert a grid held with tweezers under the water with its shiny side up and scoop up a section floating on the water. With other hand, hold a long tooth pick with an eyelash to assure the section will stay at the middle of a grid. Again, minimize contact between the eyelash and sections.

32. Absorb excess water on the grid by attaching the matt side of the grid edge to clean filter paper. Absorb water between tips of tweezers with a triangular cut filter paper. Then, place the grip of the tweezers on a box or petri dish to hold the grid in the air with shiny side up. Repeat 31–32 to prepere a sufficient number of sections on grids.

33. Once a thin section on a grid becomes completely dry, place the grid in a grid storage box or on filter paper in a glass petri dish (*see* Note 35).

34. As a reference, stain thin sections by following steps 12–19 with the following modifications; a) pick up gold-colored thin sections with the pencil shaped side of a bamboo tooth pick. b) stain sections twice to enhance staining.

3.4 Antibody reactions

1. Thaw blocking solution. Vortex and centrifuge at 2,300 × g for 5 min.

2. Carefully clean silicon pads. To avoid cross reaction of antibodies, use one silicon pad for each antibody reaction.

3. Insert edge of grids in a slit of a silicon pad. Make sure that section on the grid is above the silicon pad.

4. Place filter paper in a petri dish and wet it with filtered Milli-Q water.

5. Place the silicon pad with grids on the wet filter paper in a petri dish.

6. Apply blocking solution to the both sides of a standing grid. Incubate at RT for 20 min.

7. Filter antibody reaction/washing solution with a 0.22 μm filter.

8. Dilute antibody with filtered antibody reaction/washing solution prepared at step 7 (*see* Note 36).

9. Remove blocking solution with pipetman (P200) and then apply antibodies with pipetman (P20). For negative control, apply antibody reaction/washing solution or immunoglobulins isolated from pre-immune or normal serum. Immunoglobulins for negative controls should be diluted to the same concentration as primary antibodies.

10. Close the lid of the petri dish and incubate at RT for 1–2 h.

11. Hold the silicon pad with grids with flat tip tweezers at the top of the beaker. Briefly wash out antibody by applying antibody reaction/washing solution from a wash bottle.

12. Apply antibody reaction/washing solution to grids. Close the lid of the petri dish and incubate at RT for 5 min.

13. Repeat 11–12 twice.

14. Briefly absorb excess solution on the silicon pad with a Kimwipe (*see* Note 37).

15. Apply gold particle conjugated secondary antibody diluted with antibody reaction/washing solution to both sides of a grid (*see* Note 38).

16. Incubate sections with secondary antibody at RT for 1–2 h.

17. Wash sections with antibody reaction/washing solution by repeating steps 11–12.

18. Wash sections twice with 0.1 M phosphate buffer (pH 7.4) by repeatingsteps 11–12, but using 0.1 M phosphate buffer (pH 7.4) instead of antibody reaction/washing solution.

19. Briefly absorb excess solution on the silicon pad with a Kimwipe (*see* Note 37).

20. Fix reacted antibodies on a section with glutaraldehyde. In chemical a fume hood, apply 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) to grids and incubate at RT for 10–30 min.

21. Rinse out glutaraldehyde with filtered Milli-Q water and then wash sections three times by following steps 11–12, but using filtered Milli-Q water instead of antibody reaction/washing solution (*see* Note 39).

22. Briefly absorb excess solution on the silicon pad with a Kimwipe.

23. Absorb water attached on grids and surface of silicon pad with small triangular cut pieces of filter paper. Insert triangular tip of filter paper between the edge of grid and surface of silicon pad. One can stop at this step. Close the lid of the petri dish and store grids at RT.

3.5 Staining and carbon coating

1. Place 0.22 μm filtered Milli-Q water into three 100 ml glass beakers and three 150 ml glass beakers. Cover beakers to avoid contamination.

2. Place filter paper in a petri dish and wet it with filtered Milli-Q water.

3. Place the silicon pad with grid on the wet filter paper in a petri dish.

4. Apply uranyl acetate staining solution to grids. Make sure the entire grids are soaked in solution.

5. Close the lid of the petri dish and incubate in a light-shielded box at RT for 30 min.

6. Bring modified Sato's lead-staining solution at 4 °C to bench.

7. Hold the silicon pad with flat tip tweezers and drop uranyl acetate staining solution onto grids held over a chemical waste container (*see* Note 40).

8. Rinse grids with filtered Milli-Q water from a wash bottle. Drop the washed solution into chemical waste container.

9. Keep holding the silicon pad with tweezers. Soak it in filtered Milli-Q water in a first beaker. Move the silicon pad up and down 50 times.

10. Transfer the silicon pad into a 2nd beaker filled with filtered Milli-Q water. Move the silicon pad up and down 50 times.

11. Hold the silicon pad with forceps and briefly wipe excess water with a Kimwipe placed on a paper towel.

12. Transfer the silicon pad into a 3nd beaker filled with filtered Milli-Q water. Move the silicon pad up and down 50 times.

13. Hold the silicon pad with tweezers and wash grid by applying filtered Milli-Q water from a wash bottle.

14. Hold the silicon pad with forceps and briefly wipe away water with a Kimwipe placed on a paper towel.

15. Lay the silicon pad on a Kimwipe.

16. Absorb water on the edge of silicon pad with a Kimwipe. Do not touch a grid with the Kimwipe.

17. Absorb water attached on grids and surface of silicon pad with filter papers cut in a triangular shape. Insert tip of filter paper between edge of grid and surface of a silicon pad to absorb water between them (*see* Note 41).

18. Apply modified Sato's lead-staining solution to grids from a syringe with a 0.22 μm filter and a needle. Make sure the entire grids are covered with solution.

19. Incubate at RT for 5 min.

20. Wash grids with filtered Milli-Q water and then absorb water on grids by repeating steps 7–17.

21. Change filter paper in the petri dish with clean dry filter paper. Place the silicon pads with grids on it. Close the lid and let sections dry completely.

22. Set carbon yarn and rod into the carbon coater.

23. Place a filter paper onto the lid of a petri dish. If carbon coating of more than one type of samples are performed at once, write border and name for each type of sample on filter paper with a mechanical or carbon pencil.

24. Transfer grids from the silicon pads onto labeled filter paper with their shiny side (with section on it) up (*see* Note 42).

25. Place grids on filter paper on the lid of a petri dish into a carbon coater and process the samples.

3.6 Imaging with EM

1. If a TEM is available which can provide high contrast images on a phosphor viewing screen at low magnifications (*e.g.*, Jeol Ex 1200, 80kV), view the sections with this instrument to make a map for STEM imaging. Once you define the regions for imaging with STEM, reduce the magnification and capture low magnification images (*e.g.*, 200 and 1.5K). Mark the regions for STEM imaging on these low magnification images. These maps dramatically reduce the time required for STEM imaging.

2. With TEM/STEM microcopy (*e.g.*, a Zeiss Libra 200FE, 200 kV), find a position for imaging with the TEM mode by referencing maps prepared at step 1. Pigmented granules in the RPE and grid bars can be useful markers for positioning. Once regions are found for STEM imaging, change to STEM mode and insert HAADF detector.

3. Take images with a HAADF detector. The 200 kV with 20 μm condenser aperture, spot size 9 (2.5 nm) works well for imaging of immune-EM samples described here. We generally use a magnification of ~17 K can be used for imaging photoreceptor outer segments and ~48 K-70 K for imaging photoreceptor disk membranes (Fig. 2).

4 Notes

1. The DMSO solution is easily diluted by exposure to air.

2. Store dehydration solutions in dispensing plastic bottles with caps (*e.g.*, Bel-Art Scienceware Dispensing/Drop Bottles/Bottle, Capacity: 125 mL, Fisher, 22–034751) for easy handling.

3. PCR tube rack with 96 holes (Ø 6 mm and depth ~8 mm) fit to a type 3 gelatin capsule body.

4. A tube rack which can hold 0.6 ml tubes that is resistant to 40 °C heating.

5. A 48 well microplate can be used for a rack of 2 ml vials.

6. If a collet-type specimen holder is available, use one that can hold a capsule shaped block more tightly than a universal holder.

7. Do not touch the precipitate in the formvar solution to the grid. Do not use grids dropped in the solution.

8. If it is hard to detach a grid from filter paper, discharge grid with a Zerostat Anti-Static Gun.

9. Recommend preparing one more glass vial filled with 1,2-dichloroethane to remove formvar from the tip of tweezers.

10. Ø 90 mm filter paper fits into the base of a Ø 100 mm glass petri dish.

11. Silicon pads with aligned shallow slits are more convenient for the staining of multiple grids at the same time (*e.g.*, HIRAOKA staining kit).

12. To find an antibody which works on immuno-EM samples, refer to past immuno-EM papers and test antibodies on cryo-sections that initially were fixed with fixative solution for immuno-EM. An atibody which has the potential to work on immuno-EM samples should operate at a significant dilution, such as ~1:10,000.

13. It is not necessary to make this solution freshly each time. A 3-week-old solution stored at 4 °C can be used.

14. If the color of solution is still white after adding NaOH, do not use. Make the solution again.

15. Tweezers has direct contact with a grid holder of EM. For selection of tweezers, follow instructions from the EM manager.

16. If tracking of the dorsal-ventral and nasal-temporal axis is required, mark the sclera with a blue fine point sharpie or an alternative marker prior to removing eye balls. Then, process the right and left eyes in individual wells.

17. When samples are collected from more than two mice, repeat 4–6 for each mouse to prepare fresh samples.

18. If tracking of the dorsal-ventral and nasal-temporal axis is required, transfer small pieces of eye cups from dorsal, ventral, nasal and temporal into individual wells.

19. When samples are collected from multiple mice, the fixation time at RT prior to fixation at 37 °C can range from 1 h to 3.5 h. Keep eye cups in fixative until all dissections is done. Then, cut all eye cups (step 7) into small pieces and then fix them at 37 °C for 30 min (step 8).

20. It is possible to stop sample processing at this step. In that case, fill the sample vial completely with buffer, so there is no space for air. Then, close the lid and store at 4 °C. However, if a high quality immune-EM sample is needed, continue sample processing to step 24.

21. To prevent drying of sample, retain a small amount of solution to keep the sample wet.

22. Glass vials tend to break after cooling down to −20°C. Take care when opening and closing lids.

23. Wrap with aluminum foil and use a vacuum desiccator. The desiccator should be connected to a vacuum line in the chemical fume hood. Do not turn on the light in the hood.

24. Resin near air bubbles will not polymerize. Once the cap is almost closed, ensure that it is closed completely without trapping an air bubble.

25. White resin is toxic. Also, it tends to degrade disposable laboratory exam gloves quickly. Thus, wear an undamaged butyl glove over a pair of disposable gloves. Wipe white resin spilled on the butyl glove with a Kimwipe to prevent damage. Contaminated Kimwipes and other dry waste should be disposed following local instructions.

26. Two-part epoxy can glue sample tightly on the base block.

27. Do not trim tissue with a blade. Trim only the part of the block which does not contain tissue.

28. Trimming also can be achieved with either a slightly damaged diamond knife not used for sectioning or a commercially available trimming knife. When sections are accumulated on knife, remove them by blowing away with dust off.

29. A syringe with a 0.22 μm filter and a needle, and a syringe with a needle are convenient for transferring and removing water, respectively.

30. Dispose of staining solution as dictated by local regulations.

31. To check color of section, a light microscopy equipped with an eye piece is recommended. For imaging, a color camera (e.g., Qimaging, Micropublisher 5.0 RTV) provides more information than a black and white camera.

32. Sections for immune-EM should be prepared from the appropriate depth of a tissue. Avoid dissected surface of tissue damaged by mechanical force. Avoid regions distant from the surface of a tissue and a dissected surface where fixation is weak.

33. Minimize water vibrations in the boat during sectioning. If breath shields are available, set them to minimize water vibrations from the air flow. Minimize trafficking around the ultramicrotome. Also, to minimize vibrations from the floor, install the microtome at a location free of vibrations and air flow. If the microtome need to be installed on a floor with vibrations, consider an anti-vibration table.

34. A handmade ring can be used as a clamping ring. Make a 2-turn spiral ring from an electronic wire with vinyl jacket. This ring works for tweezers without serrated grip.

35. Static causes grids to jump, so store and transport them on filter paper in a glass petri dish.

36. If this is the first time you've used a specific antibody on immune-EM samples, testing different dilutions is recommended. Usually, 25–30 μl of antibody solution can cover one grid. The amount required also depends on the hydrophobicity of the silicon pad.

37. Never dry out sections on a grid.

38. Sensitivity of the secondary antibody depends on the size of the conjugated gold particle. Antibodies conjugated with smaller gold particles produce higher labeling densities. We used 5 nm gold particle conjugated goat anti-mouse IgG (H+L) from BBI, distributed by Ted Pella, cat No.15750. A 1:30 dilution worked well.

39. Solutions containing glutaraldehyde need to be disposed following local instructions.

40. Solutions containing uranyl acetate need to be disposed of following local instructions.

41. Remove remaining water which otherwise will dilute the lead-staining solution. However, do not dry a section completely.

42. Place grids slightly away from the edge of lid where the deposition of carbon is diminished.

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References

[1]. Sousa AA, Hassan SA, Knittel LL, Balbo A, Aronova MA, Brown PH, Schuck P, Leapman RD, Nanoscale, 8 (2016) 6577–6588. [PubMed: 26934984]

[2]. Stierhoff YD, Humbel BM, Homann R, Otten MT, Schwarz H, Scanning Microsc, 6, (1992) 1009–1022.

[3]. Millonig G, J. Appl. Physics, 32 (1961) 1637.

[4]. Hanaichi T, Sato T, Iwamoto T, Malavasi-Yamashiro J, Hoshino M, Mizuno N, J. Electron Microsc. (Tokyo), 35 (1986) 304–306. [PubMed: 2440973]

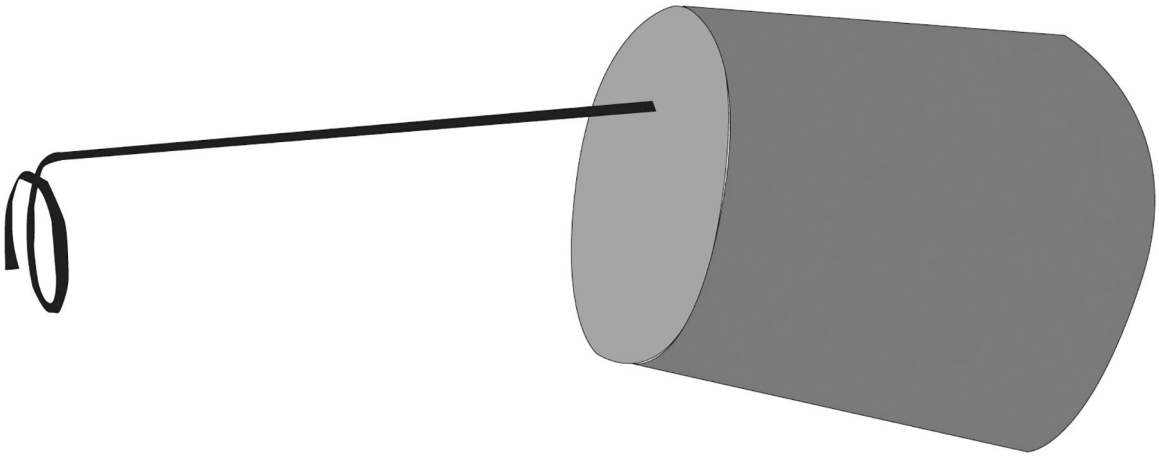

Fig. 1. Self-standing cap holder.

A ring-shaped wire is fit to an indentation in the cap of a gelatin capsule. The bottom part is large enough for manual handling.

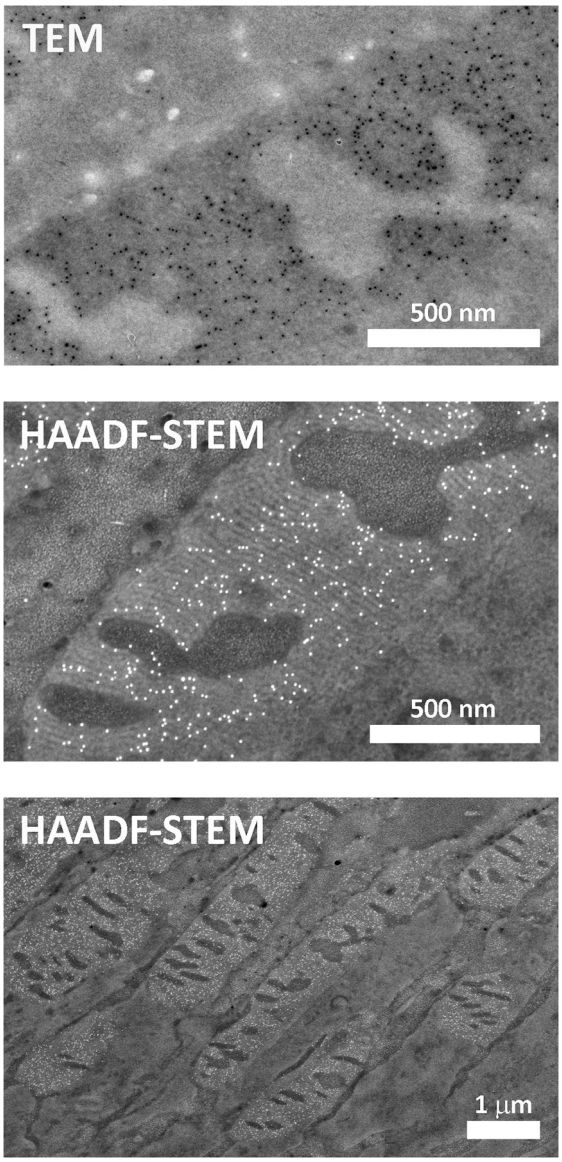

Fig. 2. Visualization of membrane structures in an immuno-EM sample by TEM and HAADF-STEM.

A thin section of an immuno-EM sample prepared from a PND14 wild type mouse retina was labeled with opsin antibody (4D2) and 5 nm gold particle-conjugated secondary antibodies. The same region of rod outer segment was imaged with a transmission electron microscope (TEM) and a high angle annular dark field scanning transmission electron microscope (HAADF-STEM) at 200 kV. In high magnification images (top and middle panels), a part of a photoreceptor cell outer segment is shown. Disk membranes are clearly visible in HAADF-STEM images in both high (69.90 k) and low (15.05 k) magnification images (middle and bottom panels).