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Bharti Katbamna
Cleveland State University

Atha Ralston
Ohio State University

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GLYCOL METHACRYLATE EMBEDDING AND MICROWAVE STAINING FOR LIGHT MICROSCOPY OF THE MOUSE COCHLEA

Bharti Katbamna[1*] and Atha Ralston[2]

[1]Cleveland State University, Department of Speech and Hearing, Cleveland, Ohio, and
[2]Otological Research Laboratories, Ohio State University, Columbus, Ohio 43210

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Abstract

This study examined the utility of a methacrylate-based embedding medium and microwave staining for light microscopic quantification of hair cells and spiral ganglion cells in the mouse cochlea. The most important phase of the preparation process involved slowing down the polymerization process. The tissue molecules so locked within the plastic matrix produced excellent preservation of the organ of Corti and adjacent structures including the spiral ganglion, as well as tissue ionic charges. Excitable by microwaves, these ionic charges accelerated the movement of the basic dye (hematoxylin) into the tissue, reducing the time for this segment of the staining process from approximately 45 minutes to 1-2 minutes. When embedded in glycol methacrylate (GMA), acidic dyes show less stain-cell affinity so that staining intensity and time cannot be improved significantly. However, addition of color extenders to the counterstain eosin produced distinguished staining of all tissue constituents. Thus, a combination of GMA embedding medium, use of the microwave for staining and addition of color extenders to the counterstain generated excellent structural resolution and contrast. This made both hair cell and spiral ganglion cell counts possible from within the same specimen and provided an opportunity for qualitative evaluation as well.

Key Words: GMA, microwave staining, light microscopy, mouse cochlea, organ of Corti, spiral ganglion cells.

*Address for correspondence:
Bharti Katbamna
Department of Speech and Hearing,
Cleveland State University,
1983 East 24th Street,
Cleveland, OH 44115
Telephone number: (216) 687-6988
FAX number: (216) 687-9366

Introduction

Although the range of hearing of the house mouse extends from approximately 0.5 kHz to 100 kHz, the basilar membrane spans only 6.8 mm (Ehret and Frankenreiter, 1977). While this structural dimension makes the tissue size very small, it also poses technical problems for routine examination of both cochlear hair cells and spiral ganglion cells within the same specimen. A combination of appropriate embedding medium and staining procedure can, however, alleviate time constraints and enhance structural details. The properties of glycol methacrylate (GMA), such as its miscibility with water and ethanol, its ability to infiltrate soft and hard tissues which are not completely dehydrated, polymerization at low temperatures (0-4°C) and thin sectioning (0.5 to 3.5 μm), makes it an ideal embedding medium for light microscopic examinations (Cole and Sykes, 1974). Microwave processing of tissue, likewise, can accelerate tissue preparation time, produce excellent preservation of tissue and in many instances improve the stainability of the tissue (Hellstrom and Nilsson, 1992; Kayser and Bubanzer, 1990; Kok and Boon, 1990). Although the microwave oven can be employed for fixation, decalcification, dehydration, and embedding purposes, it was utilized in this study for staining purposes only. Since the mouse otic capsule was dissected prior to tissue processing, the specimen size was reduced substantially, thereby, reducing processing time. The purpose of this study was to determine if a methacrylate-based embedding medium, in coordination with microwave staining, provides sufficient structural resolution for routine quantification of hair cells and spiral ganglion cells in the mouse cochlea.

Materials and Methods

Fixation and Dissection

Four-month-old CBA/Ca and C57BL/6 strains of mice were utilized for this study. The mice were overdosed with pentobarbital and perfused intracardially with physiological saline followed by buffered neutral 10% formalin (pH 7.0). The otic capsules were retrieved and

stored in neutral buffered 10% formalin for 12 hours. The fixed specimens were partially dissected in 10% formalin. The bulla and the ossicles were removed with the help of fine jeweler's forceps, thus isolating the cochlea and the semicircular canals. Semicircular canals were also detached from some specimens with fine scissors or drills before and after decalcification.

Decalcification

The specimen was washed in distilled water for removal of the fixative and left overnight in 0.1 M Tris buffer (pH 6.95) placed on a shaker. The buffer was replaced with a decalcifying solution of 10% ethylenediaminetetraacetic acid (EDTA; disodium dihydrate salt) in 0.1 M Tris buffer (pH 6.95). The EDTA solution was changed twice the first day and every 48 hours thereafter until the used solution tested negative for calcium salts (Seilly, 1982). The specimen capsules/vials during this and subsequent procedures involving rinsing, dehydration, clearing, and infiltration were placed on a shaker at 4°C. A gentle shaking cycle was used to ensure bubble-free agitation.

Dehydration and infiltration

After decalcification the specimens were washed in distilled water and placed in 70% ethyl alcohol for 24 hours. Further dehydration was accomplished through graded solutions of ethyl alcohol of 80%, 95% and 100% (two changes of the latter two concentrations) for one hour each. The specimens were then cleared in 1:1 solution of 100% ethyl alcohol and 0.9% catalyzed GMA solution (Polysciences, Inc.) followed by the original strength 0.9% GMA solution for two hours each. The GMA solution was changed every 24 hours thereafter until the specimens turned translucent and submerged to the bottom of the container indicating completion of infiltration; infiltration was complete within 3-4 days.

Embedding and sectioning

The infiltrated specimen along with a few drops of 0.9% GMA solution were placed in a plastic tray (histomolds) with one or more 6 x 8 x 5 mm wells. The specimen was oriented with the oval and round windows of the cochlea facing down and the activated GMA solution was pipetted slowly along the edge of the well. The activated GMA solution was freshly prepared by adding 1 part of the activator to 20 or 25 parts of cooled 0.9% catalyzed GMA solution. The flooded wells were tightly capped with aluminium block holders (chucks), placed in a dark-colored cardboard box and transferred to a refrigerator or cold room to further slow down the speed of polymerization and retard the formation of gas bubbles. The blocks in this experiment polymerized within 24 hours. The polymerized blocks were stored in plastic bags placed in a vacuum jar at room temperature.

An automated retracting microtome equipped with a Ralph knife holder (LKB Historange) was used to section the specimens. After rough cutting (to ensure sectioning in the desired portion of the specimen), 3.0-4.0 μm serial sections were cut with a rapid cutting stroke, followed by a long enough pause to enable section collection and floatation in a warm water bath. The floating section was picked on a positively charged slide (Fisher superfrost plus) to facilitate section adhesion and allowed to dry on a hot plate (60°C) for an hour.

Staining

The slides were cleaned, hydrated and placed in a jar containing filtered Richard Allan's hematoxylin (no. 1 or no. 3). The microwave oven process controller (Bio-Rad, Polaron Div.) was programmed to a power level of 100%, and minimum and maximum temperatures of 28°C and 37°C, respectively. The staining jar along with a water load were placed in the microwave oven, the temperature probe and bubbler being inserted in the staining jar. The microwave oven was operated for 50-60 seconds and the stain checked under the microscope for appropriate darkness. Up to 80 seconds of time were required depending on the age of the stain and reuse of the solution. After staining, the slides were washed in warm running tap water for 10 minutes and placed in Richard Allan's clarifier (no. 1) for 15 minutes or until no more stain remained in the plastic. The slides were washed again in warm running tap water for 10 minutes and placed in Richard Allan's bluing reagent (no. 1) for 4-6 minutes. The slides were washed once again in tap water for 10-15 minutes, rinsed in distilled water and dried at room temperature. The slides were dehydrated in 80% and 95% ethyl alcohol and counter-stained with eosin-phloxine.

Eosin was prepared with eosin Y (2.0 g), orange G (0.5 g), acid fuschin (0.25 g) and glacial acetic acid (0.1 ml) dissolved in 95% ethyl alcohol (200 ml). Working solution of eosin-phloxine was prepared by diluting the eosin solution (125 ml) with 95% ethyl alcohol (675 ml) and adding 2.5% alcoholic phloxine B (0.5 ml) and glacial acetic acid (1.5 ml). Filtered eosin-phloxine was warmed in the microwave oven for approximately 30-40 seconds using the same settings as with the hematoxylin microwave procedure. The slides were placed in warm eosin-phloxine for 2-4 minutes, dehydrated and air dried. The dried slides were placed in xylene and mounted with Richard Allan's mounting medium. Mounted sections were examined with a Zeiss 405M Axiovert microscope.

Results

Specimen preparation

Fixation of specimens with 10% neutral buffered formalin not only minimized tissue shrinkage, but also enhanced stainability of tissue. Dissection of the specimens with the help of drilling burrs and sandpaper left deposits

of fine bone dust on the specimen and even sand from the sandpaper which could not be washed away during processing. This resulted in frequent breakdown of Ralph glass knives used for specimen sectioning. Finer dissections were better accomplished after partial or complete decalcification of the specimen. In fact once the bulla was opened, unwanted tissue, like the semicircular canals in this case, could be simply trimmed away with a fine scissors after decalcification. Keeping the semicircular canals intact not only increases the specimen size and processing time, but requires considerable specimen maneuvering for appropriate orientation during embedding.

Infiltration was considered to be complete when the specimen became uniformally translucent, took on a pinkish tinge and sank to the bottom of the container. In infiltrated specimens, the pigmented stria vascularis along with the cochlear turns could be clearly delineated with the naked eye. Incomplete infiltration caused tears in the sections, in addition to damaging the edge of the Ralph glass knife. Polymerization was regulated appropriately by using precooled catalyzed GMA solution and by refrigerating the blocks. Change in the ratio of the activator to catalyzed solutions from 1:20 to 1:25 further slowed down the rate of polymerization without increasing the polymerization time substantially.

Approximately 1-2 minutes were required for hematoxylin staining, one hour for differentiating, bluing and drying, and 2-4 minutes for counterstaining. Staining with Richard Allan's hematoxylin was enhanced with the triple strength solution (no. 3) compared to the original strength solution (no. 1). Orange G, acid fuschin and phloxine B added to the eosin solution served as color extenders providing overwhelming structural details not visible otherwise.

Cochlear structures

Approximately 200 serial sections were obtained for each cochlea and the status of the organ of Corti along the entire length of the cochlea could be delineated. Figures 1A and 1B show mid-apical and mid-basal turns of the cochlea, respectively, obtained from the CBA/Ca mouse. Higher magnifications of the organ of Corti from these portions of the cochlea, shown in Figures 1C and 1D, respectively, clearly show structural integrity of the inner and outer hair cells, the adjacent pillar cells, and the overlying tectorial membrane. In addition to the changes in the dimensions of the tectorial membrane from apex to the base seen in these figures, the sites of attachment of the tectorial membrane on either end, the inner sulcus cells and the supporting cells in the periphery of outer hair cells, are also evident. The basilar membrane, likewise shows changing characteristics from the apex to the base of the cochlea. While both the figures show the attachment of the basilar membrane to the osseous spiral lamina, the basal turn (Fig. 1D) shows both pars tecta and pars pectinata portions of the membrane.

Spiral ganglion cells

Figure 1D also shows spiral ganglion cells in the Rosenthal's canal, some of the nerve fibers traveling through the habenula perforata, and some crossing the tunnel of Corti. A mid-modiolar section obtained from the same CBA/Ca mouse is shown in Figure 2A. It displays the entire spiral ganglion housed within the modiolus, the section magnified in Figure 2B depicting cytoplasmic and nuclear details of normal spiral ganglion cells. Abnormalities associated with hearing loss can be seen in the mid-modiolar sections obtained from a C57BL/6 mouse shown in Figures 2C and 2D. Figure 2C displays an early phase of disintegrating ganglion cells in the center of the spiral ganglion. Several clumps of degenerating cells with old and new recruits can be seen in Figure 2D. The contours of the adjacent bone appear readjusted due to the loss of neighboring neurons.

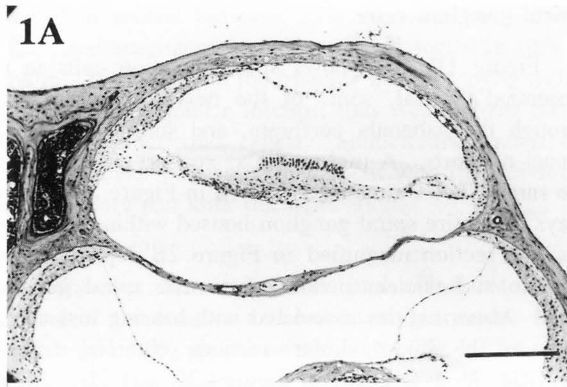
Vestibular structures

Although the semicircular canals were detached and no attempts were made to preserve any aspect of the sensory organs, most specimens retained the crista and macula. The hair cells along with some nerve fibers, and the cupula can be seen in the sensory organs of both the semicircular canal (Figures 3A and 3B) and the utricle (Figure 3C). Even the otoliths of the macula were well preserved in most sections (Figure 3C).

Discussion

The rationale for using GMA resins as embedding media is that the small mobile monomer molecules have the ability to infiltrate the tissue. When the plasticizer (activator) is added the monomer molecules link with each other to form tangled chains in which the tissue molecules become enclosed. This polymerized network is porous enough to allow solution contact with the tissue constituents and yet strong enough to provide both intra- and extracellular support. It is an ideal medium for preserving the organ of Corti and surrounding structures including the spiral ganglion cells, and hence for studying the effects of drugs or noise on the organ of Corti. Unlike paraffin embedding GMA provides preservation of fine structures like microvilli, cilia, goblet cells and fat cells. Unlike celloidin, embedding GMA blocks lend themselves to thin sectioning, so that finer details can be visualized. The most crucial phase of the preparation involves slowing down the polymerization process for the heat produced during rapid polymerization can generate many bubbles and fry the tissue.

Microwave irradiation not only reduces the staining time, but provides intense staining with crisp details. Due to the hydrophilic properties of GMA, tissue ionic charges are well preserved in the sections. These charged particles are excited by microwaves, causing the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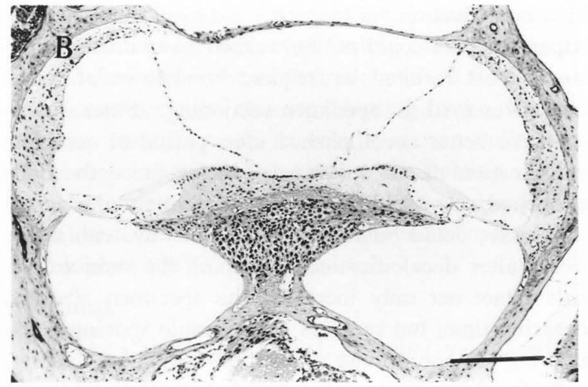
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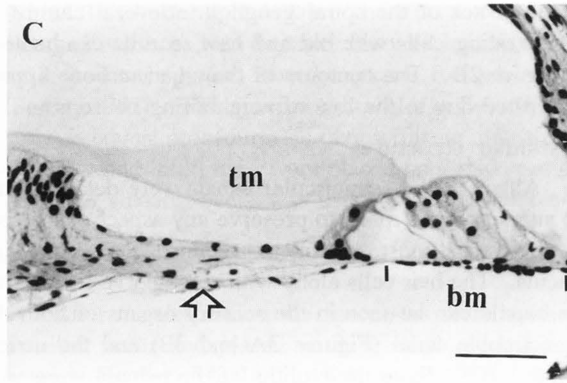
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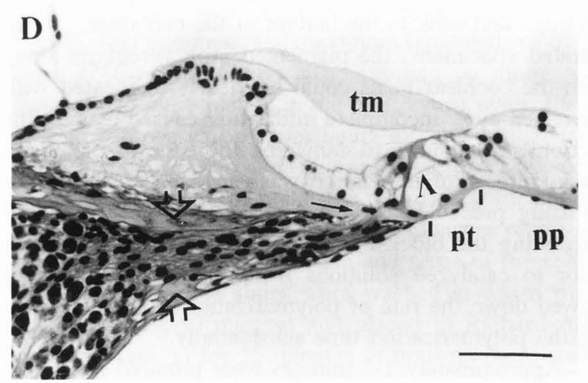
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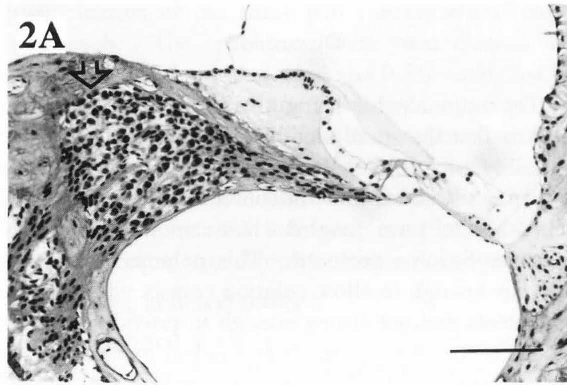
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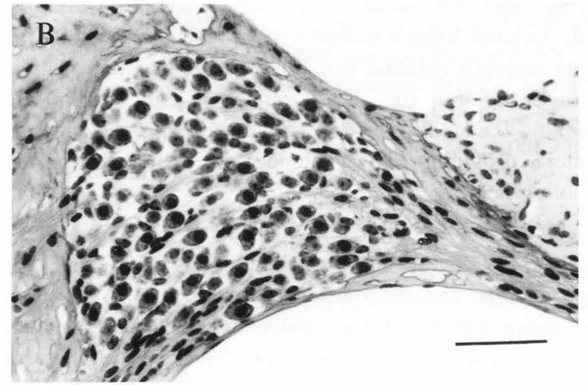
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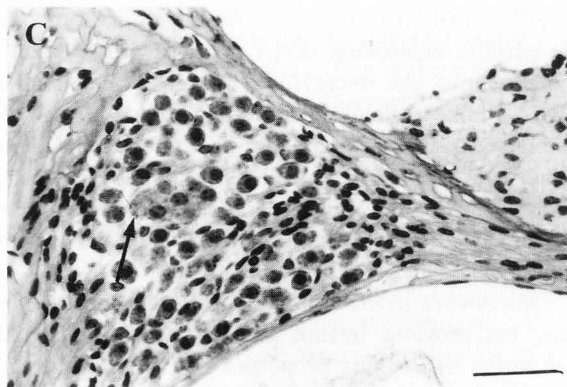
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D

Figure 1 (facing page, top). A section through (**A**) the mid-apical and (**B**) mid-basal turns of the cochlea obtained from the CBA/ Ca mouse. (**C**) and (**D**) Higher magnifications of the organ of Corti from these portions of the cochlea clearly show structural integrity of the sensory and supporting cells, respectively. In addition to the changing dimensions of the tectorial membrane (tm) from apex to the base seen in these figures, the sites of attachment of the tectorial membrane on either end is also evident. The basilar membrane (bm), likewise, shows changing characteristics from the apex to the base of the cochlea. While both the figures show the attachment of the basilar membrane (bm) to the osseous spiral lamina (bold arrowhead), the basal turn (Fig. 1D) shows both pars tecta (pt) and pars pectinata (pp) portions of the membrane. Spiral ganglion cells (nuclei) can also be seen coursing through the Rosenthal's canal [canal between the bony plates of the spiral lamina (bold arrowhead)], some of the nerve fibers traveling through the habenula perforata (small arrow) and some crossing the tunnel of Corti (open arrowhead) in the basal turn (Fig. 1D). Bars = 200 μm (in A, B), and 50 μm (in C, D).

Figure 2 (facing page bottom). A mid-modiolar section obtained from the CBA/Ca mouse showing (**A**) the entire spiral ganglion (bold arrowhead) housed within the bony modiolus; and (**B**) a higher magnification of this section depicting cytoplasmic and nuclear details of normal spiral ganglion cells. (**C**) An early phase of disintegrating ganglion cells (arrow) associated with hearing loss can be seen in the mid-modiolar section obtained from the basal cochlear turn of a C57BL/6 mouse. (**D**) In the apical turn of the same cochlea several clumps of degenerating cells (arrows) can be identified indicating more severe and advanced degeneration. The contours of the adjacent bone (filled arrowhead) appear readjusted due to the loss of neighboring neurons. Bars = 100 μm (in A) and 50 μm (in B, C, D).

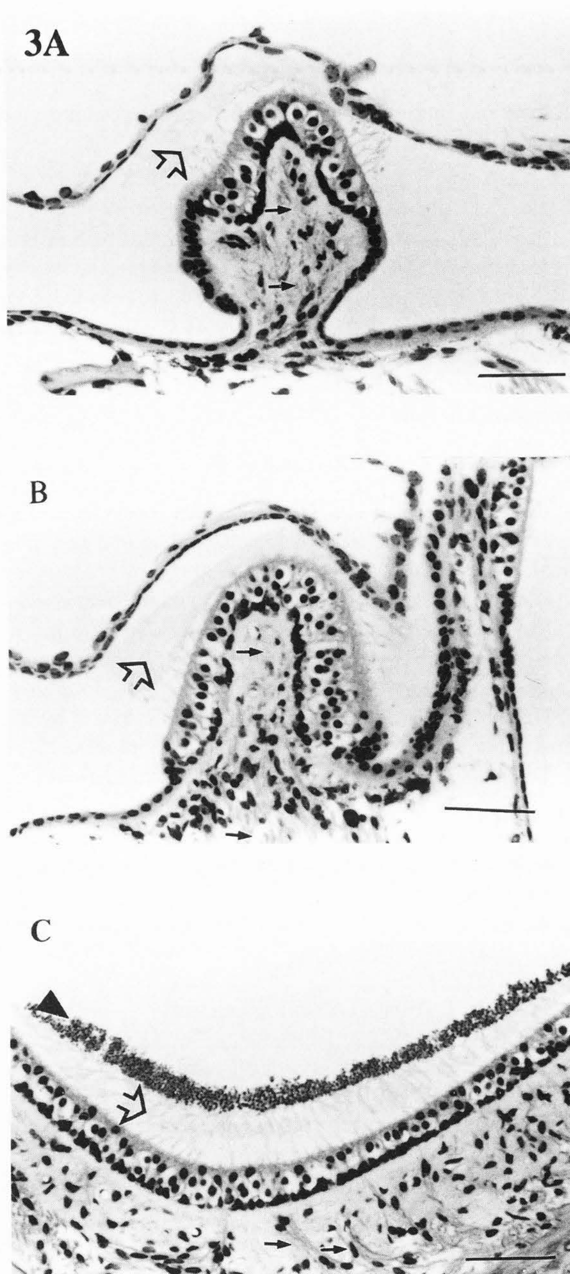

Figure 3 (at right). Longitudinal sections from (**A**) superficial and (**B**) deep levels of the semicircular canal showing hair cells along with some nerve fibers (arrows), and the cupula (open arrowhead) of the vestibular sensory organ. These structures can also be identified in (**C**) the utricular macula along with the otoliths (filled arrowhead). Bar = 50 μm.

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stain to move rapidly from the solution to the cells and to anchor on to the dye binding sites. Basic dyes like hematoxylin have a greater stain-cell affinity when embedded in GMA compared to acidic dyes like eosin. Thus, addition of color extenders like orange G, acid fuschin and phloxine B to eosin serve to greatly improve cell morphology. Crisp and contrasting pictures of all the components within the cochlea are related in part to less non-specific binding and color enhancement. This allowed qualitative analysis of cell status like those of the spiral ganglion cells. Glycol methacrylate sections stained with conventional hematoxylin and eosin method can produce somewhat comparable results, since it is the hydrophilic properties of GMA that promotes brilliant staining. However, the time required for hematoxylin staining ranges from 45 minutes to an hour, compared to 1-2 minutes when a microwave oven is used. The extended time has a tendency to produce both specific and non-specific binding, thereby reducing contrast and sharpness of the images. Although there are no substantial differences in counterstaining times between the two methods, eosin stain still needs to be prepared with color extenders to observe all the constituents of the tissue. Thus, microwave irradiation reduces the staining time by an hour (one-third of the total time required conventionally), but more importantly, this procedure produces greater contrast.

In conclusion, quantitative and qualitative analyses were facilitated by utilizing GMA as an embedding medium and microwave irradiation for staining purposes. Both cytocochleograms and spiral ganglion cell counts can be made from within the same specimen.

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Discussion with Reviewers

Reviewer I: Other techniques that I have reviewed recently have shown that the initial embedding medium can be removed after the tissue block is sectioned and the sections prepared for SEM examination. Is this possible?

V.C. Barber: The methods described provide very good preservation at the light microscope level; have the authors observed their results at the electron microscope level either by thin sectioning or by de-embedding? Also, have the authors used other methods than formalin fixation with a view to improving tissue preservation at the ultrastructural level?

Authors: Unlike epon and paraffin that can be removed either from the block and/or the section, GMA cannot be removed or isolated from the tissue [Gerrits PO, Van Goor H (1988) J Histotechnol **11**: 245]. For special staining applications, such as immunostaining, GMA matrix is often expanded or softened through the process of etching; however, this process only facilitates penetration of antibodies into the tissue without removing GMA. Of course, an appropriate fixative for preservation of a specific antigen is assumed in this case. We had considered fixation with glutaraldehyde; however, this fixative produces very poor hematoxylin and eosin staining of GMA embedded material. We have not experimented with secondary or postfixation utilizing glutaraldehyde, nor have we found literature suggesting fixatives versatile for both light and electron microscopy of GMA embedded material. It appears that other embedding media like epoxy can better bridge the gap between light and electron microscopy.

Reviewer I: Some of the tissue used for this study was from mice which undergo progressive loss of hair cells and spiral ganglion cells. It should have been demonstrated that the loss of cells can be quantified.

Authors: Preliminary data on both hair cell and spiral ganglion cell densities were obtained; these data are consistent with those published in literature. The outcome of these data along with the electrophysiological results are addressed in a forthcoming article evaluating the effects of age and noise exposure on the mouse cochlea.

Reviewer I: None of the figures were taken at a sufficiently high magnification to be able to see hair cell stereocilia, unmyelinated nerve fiber bundles in the organ of Corti, or microtubules in the supporting cells. All of these details are visible with some other preparation techniques. There is an artifact in the shape/position of Reissner's membrane (Fig. 1A and 1B). In addition, the limbus and the hair cells in the cristae and maculae appear to be vacuolated. Higher power photomicrographs would demonstrate whether or not these structures are well preserved and prepared.

Authors: This technique was described primarily for studying the effects of noise or ototoxic drugs or aging on the cochlear hair cells and spiral ganglion cells. Typically, counts of surviving/damaged inner hair cells, outer hair cells, spiral ganglion cells and percentage of intact stria vascularis along the entire length of the cochlea are performed for such examinations. Evaluations of specific intracellular components are not generally intended; however, the fact that such details are visible is an added bonus. All photomicrographs are meant to demonstrate that such reconstructions are possible from examination of serial sections. The artifact associated with the Reissner's membrane (Figs. 1A and 1B) is not a true artifact: this specimen was slightly pinched by a pair of forceps after decalcification, when it was transferred from a vial to the embedding tray. Oddly enough, the organ of Corti along with the spiral ganglion cells were intact in this specimen. Also, since no attempts were made during dissection or processing to preserve the cristae or maculae (they were denuded of the supporting bone), it is hard to determine if the abnormalities seen in the photomicrographs are related to dissection, processing or true pathology. However, based on the structural preservation of the organ of Corti and surrounding tissue, we expect that vestibular sensory organs are also well preserved by this technique.

A. Forge: Concerning the "vacuolation" of the hair cells in the vestibular sensory epithelia, I suspect that this is most likely swelling of afferent nerve terminals, an artefact that results from sub-optimal primary fixation due to hypoxia. It is difficult to avoid, regardless of the fixative used, unless the inner ear tissue are directly perfused with fixative and the vestibule widely opened to allow fixative to enter. Fixation in cold may also help. Whole body perfusion does not seem to be satisfactory.

Authors: Thank you for your valuable comment.