Three-dimensional confocal microscopy of the mammalian inner ear

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This protocol is an updated version of our original work published in MacDonald and Rubel, Hearing Research, 2008; 243(1-2):1-10, and subsequently published in a proceeding issue of Audiological Medicine. We found some very important nuances of methodology greatly affected the results of the immuno-labeling.

Previous work in our laboratory obtained three-dimensional images by confocal microscopy from bisected adult mouse cochlea embedded in epoxy resin (4). We have now obtained three-dimensional images of the mature mammalian inner ear with confocal microscopy by creating a homogeneous refractive index throughout this complex structure (5). The clearing agent is composed of 5 parts methyl salicylate: 3 parts benzyl benzoate (MSBB). It was initially applied to the mammalian cochlea for orthogonal plane fluorescent optical sectioning (OPFOS) (7,8), a sheet-illumination imaging method. It was subsequently used with high-resolution OPFOS (9,10) and more recently employed for thin-sheet laser illumination microscopy (TSLIM) (11). It is possible to obtain confocal volumes up to 900 mm in thickness through an intact, fluorescently labeled cochlea utilizing a low NA objective. Minimal dissection allows high NA objective lenses to record optical volumes limited by the working distance of the objective lens, rather than by spherical aberration created by the specimen.

Materials and methods

Inner ears were collected from mice raised in the University of Washington vivarium in accordance with IACUC regulations. The mice, 129/CBA, were killed by cervical dislocation at ages ranging from post-natal day 7 (P7) to adult. Each temporal bone was dissected to expose the cochlea, the stapes was removed and a small hole made in the apex of the cochlea, over the helicotrema. A slow perfusion of 0.1 ml 4% paraformaldehyde buffered with 0.1 *M* sodium-potassium phosphate, pH 7.4, was followed by an overnight post-fixation in 4% paraformaldehyde at 4°C, on a nutator. The specimens were washed with 0.01 *M* sodium potassium phosphate buffer, pH 7.4, containing 0.9% sodium chloride (PBS), three times for 10 min each, with gentle rotation at room temperature. Each specimen was placed in a polystyrene Petri dish, covered with PBS, and gently dissected free of extraneous soft tissues while viewed under a dissecting microscope (MZ-8, Leica Microsystems, Wetzlar, DE) with oblique illumination from a 150-W fiberoptic illuminator (Techniquip, Livermore, CA). Enough bone was removed from the cochlea to allow it to lie on its lateral or ventral side. Circulation of the immunolabeling reagents through the inner ear was encouraged by opening the round window, dissecting a hole in the bone overlying the lower turn and enlarging the hole in the apical turn. Our previously published protocol (5), decalcificied by submerging specimens for three days in 10% ethylenediamine tetra-acetic aciddisodium salt (EDTA, Sigma, St. Louis, MO) in PBS, pH 7.4, at 4°C, with rocking. The EDTA was removed by rinsing with three changes of PBS, 10 min each, at room temperature with rotation. Recently, specimens were prepared with a rapid decalcification by means of 15-min immersion in RDO Rapid Decalcifier (Apex Engineering Products Corp., Aurora, IL) at

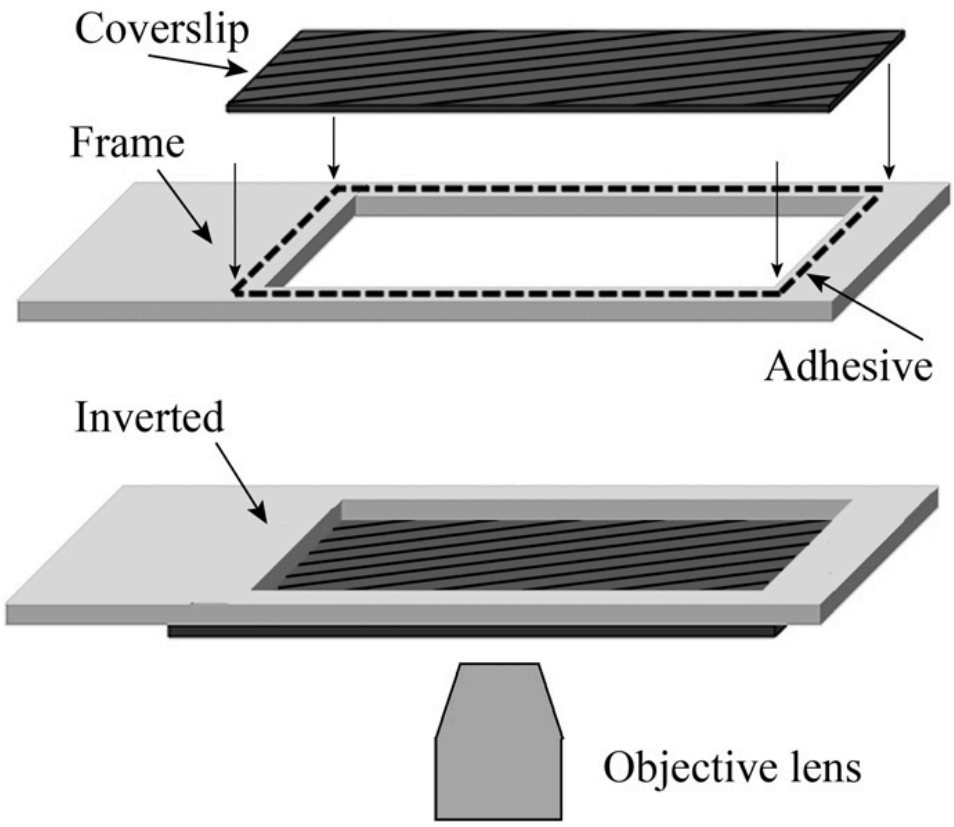
room temperature, with gentle rotation. The RDO was removed with three rapid exchanges of PBS followed by three changes of PBS for 5 min each, at room temperature, with rotation.

Immunocytochemistry
This labeling protocol employed a rocking platform ('nutator') to circulate reagents. However, the quality and consistency of labeling are controlled by the interplay of agitation force, vessel dimensions, reagent volume and degree of dissection. The incubation vessel required sufficient inner diameter to allow a noticeable wave action on the reagent surface. A reagent volume of 1 ml in a 4-ml glass vial with a polyethylene snap cap (Cat. No. 225532, Wheaton, Millville, NJ) incubated on a nutator (Model 260100F, Fisher Scientific, Pittsburgh, PA) provided acceptable labeling without allowing the specimens to tumble in a damaging manner. The plastic snap-on caps were resistant to the clearing agent. A volume of 1 ml was sufficient to keep the specimens submerged to avoid entrapment of air bubbles that may block the flow of reagents within the cochlear duct. Note that the MSBB mixture will dissolve polystyrene dishes (as well as computer keyboards and microscope focus knobs). Decalcified specimens were transferred into the 4-ml glass vials with up to four inner ears per vial. All labeling incubations were carried out at 4°C, with agitation on the nutator, and rinses were carried out at room temperature, on a platform rotator, unless otherwise noted. The inner ears were incubated in a minimal volume of Image-iT FX (Molecular Probes, Eugene, OR) for 30 min at room temperature, on a rotator, to reduce interactions between fluorophores and tissue. This was replaced with an immuno-block solution of PBS containing 10% normal serum (Vector Laboratories, Burlingame, CA), 0.5% bovine serum albumin (A-7030, Sigma, St. Louis, MO) and 0.1% Triton X-100 (Sigma). The immuno-block was removed after an incubation of 4 h carried out at room temperature, with rotation. The specimens were covered with 1 ml of immuno-block containing the primary antibodies and incubated for three days. The primary antibodies employed in this report were mouse monoclonal anti-parvalbumin (MAB1572, Chemicon, Temecula, CA) and chicken anti-200 kD neurofilament (AB5539, Chemicon) diluted 1/500 in immuno-block solution as a 'cocktail' mixture. After incubation, the primary antibodies were removed and the specimens were washed three times in PBS for 2 h each. The final PBS wash was replaced with a 1-ml volume of immuno-block solution containing the secondary antibodies, each diluted 1/500, and the nucleic acid label DAPI (Sigma) at a concentration of 0.2 μg/ml, and then the specimens were incubated for three days. Secondary antibodies employed were goat anti-mouse IgG conjugated to Alexafluor 488 and goat anti-chicken IgY conjugated to Alexafluor568 (Molecular Probes). The inner ears were washed three times with PBS, 2 h each, at the end of incubation. *Clearing and mounting* The labeled inner ears were dehydrated with a graded series of ethanol from 70% through absolute ethanol, as previously described. They were transferred into a clearing medium made of 5 parts methyl salicylate and 3 parts benzyl benzoate (MSBB). The RI of this clearing mixture was calculated to be 1.556 by multiplying the proportion of each reagent in the final mixture by its RI and summing the results. Each cleared specimen was mounted for imaging by placement in an imaging chamber and covering by several drops of MSBB. An imaging chamber was created by attaching a 24-mm X 50-mm coverslip to a 25-mm X 75-mm X 1-mm aluminum frame slide containing a large

central opening. These slide frames had been used to support plastic films used for laser microdissection. The film was scraped off with a razor blade and residual glue removed with absolute ethanol. The coverslips were measured to be of 170–173 mm thickness by means of a micrometer with an accuracy of 1 um (No. 293-765-30, Mitutoyo America, Aurora, IL) and attached with silicone aquarium sealant (All-Glass Aquarium Co., Franklin, WI). If desired, a second frame could be attached to the side opposite the coverslip in order to create a chamber 2 mm deep to allow greater volume of clearing agent or to provide clearance for a cover over the chamber to reduce evaporation. The silicone sealant was allowed to dry for 24 h under a weight, then the frame was inverted so that the coverslip became the floor of a specimen chamber (Figure 1). Each specimen was placed in the imaging chamber, covered with a few drops of clearing agent, and positioned while observing under a dissecting microscope. Fragments of coverglass were sometimes employed as shims to maintain the position of the cochlea. Microscopy of the organ of Corti using high-NA objectives with short working distances required that the side of the cochlea be sliced off in a plane roughly parallel to the modiolus to remove the lateral portion of the cochlear duct. This was accomplished by placing the specimen in a Petri dish containing a layer of clear silicone rubber (Sylgard 184, K.R. Anderson Co., Morgan Hill, CA) and slicing the cochlear shell with a scalpel blade.

Confocal microscopy

Mounted specimens were viewed on a Fluoview-1000 laser scanning confocal microscope with an IX-81 inverted microscope (Olympus America, Center Valley, PA). The fluorescent labels DAPI, AlexaFluor 488, AlexaFluor 568 and AlexaFluor 660 (not shown) were observed using laser lines of 405 nm, 488 nm, 561 nm and 635 nm, respectively, for excitation. Dichroic mirrors and bandpass emission filters used for the four fluorescence detectors on this instrument were 490 nm longpass dichroic and 425–475 nm bandpass interference filter, 560 nm longpass dichroic and 500–560 nm bandpass interference filter, 640 nm longpass filter and 575–640 bandpass interference filter, a front surface mirror and 655– 755 nm bandpass interference filter, respectively. The specimens were viewed with the following dry objectives: a 4x/NA 0.16 of 13 mm working distance, a 10x/NA 0.4 with 3.1 mm working distance and a 20x/NA 0.75 UPLSAPO with 600 mm working distance. A 40x/NA 1.3 UPFL oil-immersion objective lens with 200 mm working distance was also employed. Confocal images were collected with 12-bit digitization and then saved in a 16-bit OIF format. The oil-immersion objective was used with an immersion oil of RI 1.554 (Type A, Cargille Laboratories, Cedar Grove, NJ). Confocal image volumes were deconvolved by Huygens software (Scientific Volume Imaging, Hilversum, NL) using a constrained maximum likelihood estimate algorithm. Current work utilized Huygen Professional 3.5.5 on a Macintosh Pro computer, OS 10.6.2. Maximum intensity projections (MIP) for two-dimensional (2D) images and three-dimensional (3D) rotations were created using Huygens and ImageJ, version 1.42 (Rasband, National Institutes of Health, Bethesda, MD, http:// rsb.info.nih.gov/ij/). ImageJ was used on Macintosh Intel-based computers, OS 10.6.2 (Apple, Inc., Cupertino, CA). Final figures were created with Photoshop (Adobe, San Jose, CA). All figures were subjected to histogram stretch and gamma adjustment to increase contrast.



A specimen holder was fabricated from an aluminum frame originally used to support laser microdissection specimen films. The completed holder is flipped over after attaching the coverslip to create a sealed depression in which the specimen is placed. distance.

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