

THE REGISTRY

Newsletter of the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry



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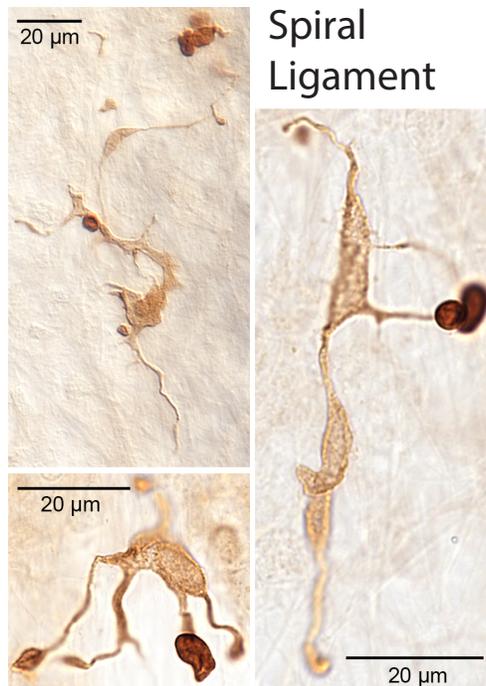
MISSION STATEMENT

The NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry was established in 1992 by the National Institute on Deafness and Other Communication Disorders (NIDCD) of the National Institutes of Health to continue and expand upon the former National Temporal Bone Banks (NTBB) Program. The Registry promotes research on hearing and balance disorders and serves as a resource for the public and the scientific community about research on the pathology of the human auditory and vestibular systems.

Microglia in the ear in health and disease

Jennifer O'Malley, Felipe Santos, M.D., and Michael McKenna, M.D.
Department of Otolaryngology, Harvard Medical School,
Massachusetts Eye and Ear, Boston, MA

Microglia are a class of cells present in the central nervous system. They represent 10 percent of the total cells in the central nervous system (CNS) but vary in density by region^{1,2,3}. They are important in the development and maintenance of organ function and are the sensor and effector cells of the innate immune system. They have also been shown to play a role in the development of several neurodegenerative diseases including multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS) and Parkinson's disease⁴.



We have demonstrated that these cells are present in abundance in the human inner ear in a variety of locations, including the spiral ligament, (figure 1), stria vascularis, (figure 2), osseous spiral lamina, (figure 3), spiral (figure 4) and Scarpa's ganglions, and within the auditory (figure 5) and vestibular nerves⁵. Almost certainly, they play a role in the maintenance of normal function of the inner ear. Based on a growing body of data from other organ systems, including the CNS, we hypothesize a fundamental role in the maintenance of cochlear homeostasis and in the development of otologic disease, including several disorders that have been considered heretofore idiopathic, including sudden hearing loss, auto-immune inner ear disease, Ménière's

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Figure 1. Three ramified cells staining positive for Iba1, a marker known to be specific for microglia, are from the area of the spiral ligament. The cells were from the cochleae of two different male individuals ages 70 and 88 with no known otologic issues other than those associated with age.



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In the Summer 2015 issue of *The Registry* on page 5, Dr. Kumiko Hosokawa's name is misspelled. The editors of *The Registry* regret this error.

Stria Vascularis and Otic Capsule

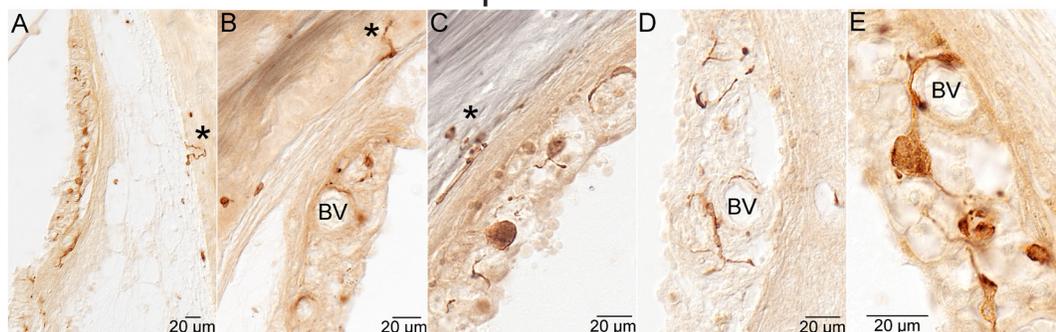


Figure 2. Iba1 positive cells in the stria were present around blood vessels (BV) and within the intermediate cell layer. There were microglia/macrophages that extended from the spiral ligament into the bone of the otic capsule (*). A, B, and E are from a 58 year old male individual with normal hearing for age. C and D are from the 70 year old individual in Figure 1.

Cochlea

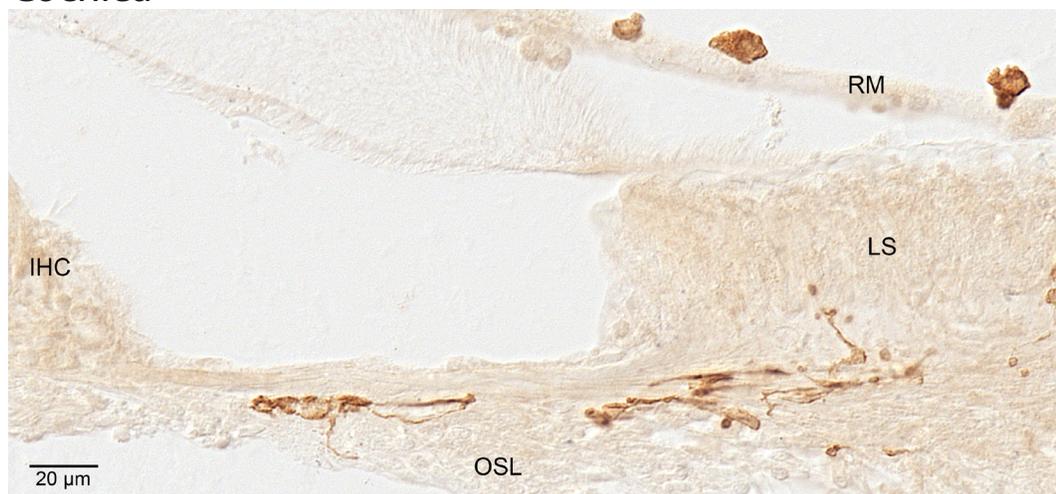


Figure 3. Microglia/macrophages were present in the osseous spiral lamina (OSL) and limbus spiralis (LS). Those demonstrated here have a ramified morphology. There were often amoeboid cells present along Reissner's membrane (RM) on the scala vestibuli side. One inner hair cell (IHC) can also be seen here. This specimen is from an 82 year old female with no known otologic disease other than changes due to age.

disease and cochlear otosclerosis. As such, otic microglia represent a new potential target for treatment and possible prevention of a host of otologic disorders with drugs that have been developed for the treatment of other disorders of similar etiology.

Until recently, microglia within the inner ears of humans could not be appreciated or evaluated. Improvements in the immunostaining of archival human temporal bones^{6,7} have made possible the identification of abundant microglia throughout the inner ear. These cells are morphologically similar to microglia in the CNS and to date express some of the same specific markers.

What are microglia?

Microglia are derived from erythromyeloid progenitor cells that migrate into the CNS during embryologic development. These progenitors develop lineage-specific gene expression and mature into microglia^{8,9}. Microglia play multiple roles in homeostasis. Residing in close proximity to synapses and dendrites, microglia are fundamental in axonal development and pruning. They can respond to changes in neuronal activity and can even modulate the firing pattern of neurons^{10, 11, 12}. Microglia are phagocytic and in

development they can clear neurons that have undergone apoptosis¹³ and they can also directly instruct neurons to undergo apoptosis^{14, 15, 16}. With maturation in the CNS, microglia become relatively inconspicuous by standard histology. The cell bodies contract and they develop long highly motile processes that participate in autocrine and paracrine signaling with cells in their immediate environment. These motile processes are constantly moving and sampling the surroundings¹⁷. They possess specific receptors³ for cellular injury and invading pathologic organisms. These stimuli result in a rapid response during which microglia enlarge with lysosomal cytoplasmic components, retraction of the microprocesses, and the assumption of a motile tissue macrophage morphology and function. This process of microglial activation can be associated with the recruitment of circulating monocytes and other inflammatory cells.

In addition to their ability to respond to local insults, their function is highly modulated by circulating cytokines including those liberated by distant inflammation. Microglial processes are also intimately associated with the microvasculature and control vascular permeability, extracellular fluid volume and composition. In the CNS, aberration in the activation of microglia leads to an amplified inflammatory state that can result in neurotoxicity implicating their role in virtually every neurodegenerative disorder. It seems likely that given the abundance of microglia in the ear that similar biological processes are occurring within the ear in some individuals with auditory and vestibular dysfunction.

The challenge lies in defining the role of microglia in human otopathology. Animal models, although essential, have significant and fundamental differences from human disease processes. These differences have been an impediment to the development of effective treatment as successful approaches in mice have often been unsuccessful in humans with disorders of the CNS.¹⁸

Approach to human otopathology

To understand the role of microglia in human otopathology, it is necessary to characterize what aspects of processing archival specimens impact our ability to immunostain these cells. Next, it will be necessary to characterize microglial density and staining patterns in normal individuals over all age ranges. Any assessment of pathological cases will require this normative data. Cases of Meniere's disease, sudden hearing loss, autoimmune inner ear disease, and spiral ligament degeneration in cochlear otosclerosis will be examined. We know from studies in the CNS as well as other organ systems that characterization of the molecular and functional phenotype is important in understanding the role of these cells in the pathogenesis of disease. To accomplish this, it may be necessary to employ other techniques including proteomics and RT-PCR. This will require technique development and refinement.

Spiral Ganglion

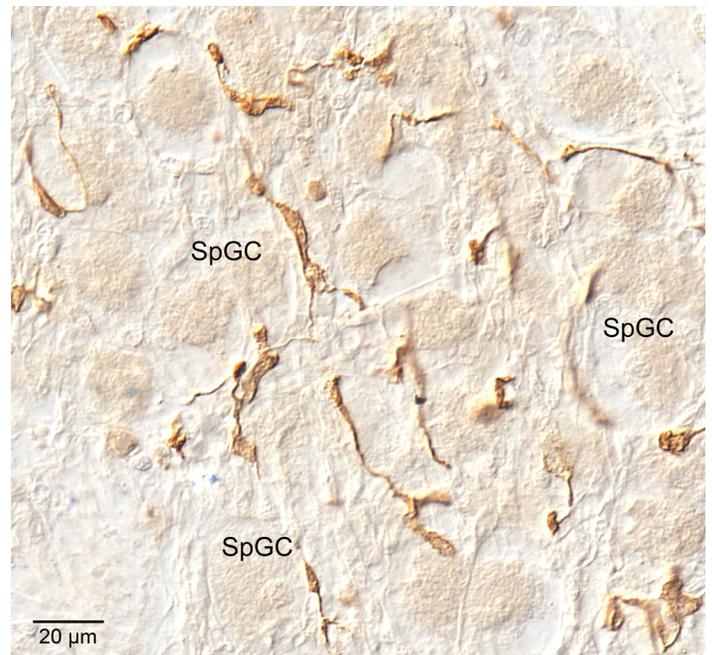


Figure 4. Microglial cells were present around spiral ganglion cells (SpGC) in a 70 year old male (same ear as Figure 1). Most of those shown here have a ramified morphology.

Animal models and what they offer

Perhaps the most fundamental question concerns the role of microglia in cochlear and vestibular physiology. Do they solely serve as sentries of the innate immune system or do they play other regulatory roles in normal cochlear function? To answer these questions, investigators have focused efforts on depleting microglia and determining organ specific effects. Until recently these studies were confounded by side effects of the depletion methods. The recent development of a drug that inhibits the receptor of Colony Stimulating Factor 1 (CSF R1) has been shown to deplete 99% of microglia in the CNS and ear. When the drug is discontinued, the microglial population rapidly regenerates from progenitor stem cells which reside within the CNS and presumably within the ear¹⁹. Remarkably, these mice remain healthy despite microglial depletions for extended periods of time. With such models, it is possible to determine the role of microglia in modulating the response to trauma, including noise. Such models will allow us to understand whether microglia are important in the synaptic loss and neuronal pathology that occurs in response to insults to the inner ear such as noise.

Translation to the human condition

The above research can only bring us so far. Ultimately, drugs that prove successful in the management of parallel disorders will need to be evaluated in clinical trials in humans with otologic disorders. The above research will however make

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Cochlear Nerve

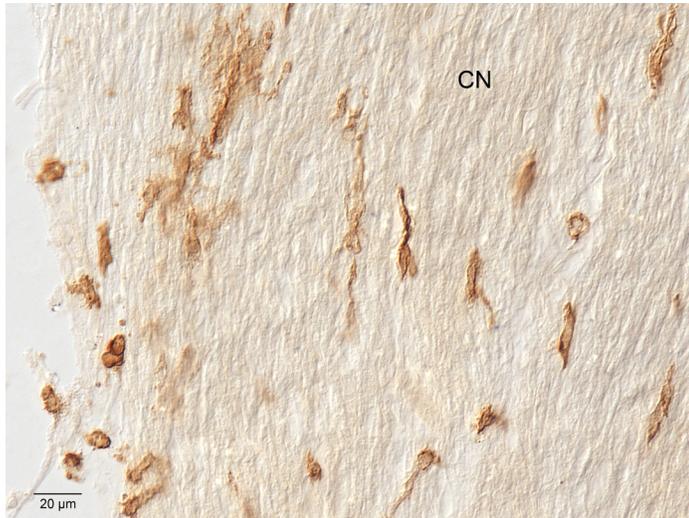


Figure 5. Both ramified and amoeboid Iba1+ microglia/macrophages were seen all along the cochlear nerve (CN) in the internal auditory canal of an 82 year old female with no known hearing disorder other than changes related to age.

possible the identification of parallel pathologies. Already, there is emerging evidence that microglia participate in the inflammatory state associated with aging suggesting the potential for a therapeutic window in delaying the progression of presbycusis. ●

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UPCOMING EVENT

ARO 39th
MidWinter Meeting
FEBRUARY 20-24, 2016
Manchester Grand Hyatt
San Diego, California, USA



Light-Sheet Microscopy for Imaging Whole Cochleas

Peter A. Santi, Ph.D.
University of Minnesota

We have developed a light-sheet microscope that is capable of imaging whole cochleas from the size of a mouse to that of a human. This microscope/microtome is called a scanning Thin Sheet Laser Imaging Microscope (sTSLIM).¹⁻² Figure 1 shows a CAD diagram of sTSLIM with the exception of the Olympus MVX 10 microscope for recording the image. sTSLIM has x,y,z motorized controllers, different size specimen chambers, a blue and green laser, the capability for structured illumination, and the whole system is automated by a custom LabView program. The light-sheet is generated not by a cylindrical lens but by a scanning galvanometer which produces a wide, thin light sheet. sTSLIM can be constructed for the cost of a one-year maintenance contract for a confocal microscope.

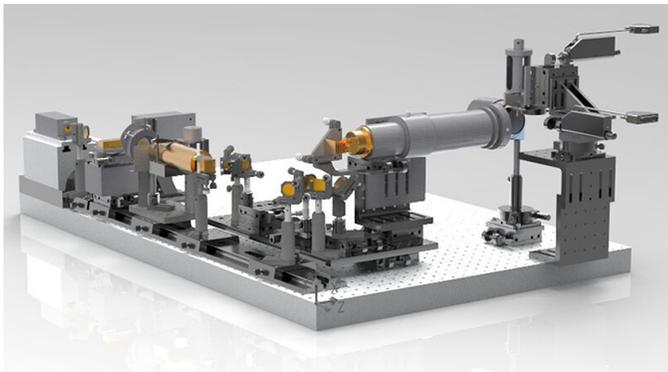


Figure 1. CAD diagram of sTSLIM.

A light-sheet microscope uses a thin sheet of light to optically section transparent tissue. The light-sheet induces a 2D fluorescent image plane within a tissue that is recorded orthogonal to the axis of illumination. Only the illumination plane is exposed to the light and therefore photobleaching is minimized. Light-sheet microscopes are able to image deep within a tissue at high resolution. By stepping through the z-axis, well-aligned, serial sections can be obtained through the complete length, width and thickness of a cochlea. These image stacks are excellent for preparing 3D reconstructions of cochlear structures. We use 1X and 2X objective lenses so that the full width of a cochlea can be imaged in a single section. Higher magnifications can be taken within the cochlea due to the low photobleaching of the imaging process. In addition, sTSLIM imaging is non-destructive and the tissue can be used for subsequent processing by celloidin sectioning⁴, TEM or SEM after imaging. Other investigators have developed different types of light-sheet microscopes and I have reviewed their microscopes in a recent publication.³

It is interesting to note, but not well appreciated, that the cochlea was the inspiration for development of light-sheet microscopy. Arne Voie and David Burns in Francis Spelman's laboratory at the University of Washington developed the first, modern light-sheet microscope with the goal of improving the cochlear implant. Their microscope was called the orthogonal-plane fluorescence optical sectioning microscopy (OPFOS). Voie and colleagues published several papers in *Journal of Microscopy and Hearing Research* on OPFOS beginning in 1993.⁵⁻⁸ However, since auditory research is a niche field, the general body of science did not recognize the potential of light-sheet microscopy until 2004 with the publication of a Science article describing a light-sheet microscope called the Selective Plane Illumination Microscope (SPIM).⁹ SPIM is very different from OPFOS in that it uses high NA objectives immersed in the specimen chamber to image small, live specimens for developmental biology. To date, most of the commercial light-sheet microscopes are SPIM-like and designed for imaging small specimens and not large structures such as the cochlea, brain and organs of the body. We were fortunate that Arne Voie imaged a few mouse cochleas for us using OPFOS. However, since he did not develop OPFOS for commercial use, we had to develop our own light-sheet microscope. Since 2008 we have continued to develop this microscope and improve its ability to image larger specimens, including the human cochlea. Development of this microscope has been successful because of a collaboration between the Technical University in Illmenau, Germany and the University of Minnesota. Six engineering masters degree students have worked in my laboratory for 6-month stays improving the microscope. The students are: Matthias Hillenbrand, Peter Schacht, Tobias Schroeter, Michel Layher, Meike Lawin, and Oliver Dannberg, all mentored from Germany by Kerstin John, Stefan Sinzinger and Rene Theska.

In order to image tissue by a light-sheet microscope it must be transparent and fluorescent. However, the cochlea is opaque and it must be made transparent using chemical treatments. Figure 2 shows a human cochlea during the process to make it transparent and fluorescently labeled for imaging.

Cochleas are fixed, decalcified, dehydrated and then stained and cleared for imaging. Voie and colleagues used methyl salicylate for clearing to transparency and benzoyl benzoate to make the refractive index of the tissue and solution close to that of the glass specimen chamber¹⁰. Voie and colleagues also made the whole cochlea fluorescent by immersion in Rhodamine-b isothiocyanate. Figure 3A shows an OPFOS image of the scala media of mouse cochlea and figure 3B shows an sTSLIM image. Image quality of sTSLIM has improved due to optical improvements and the use of a high resolution digital camera.

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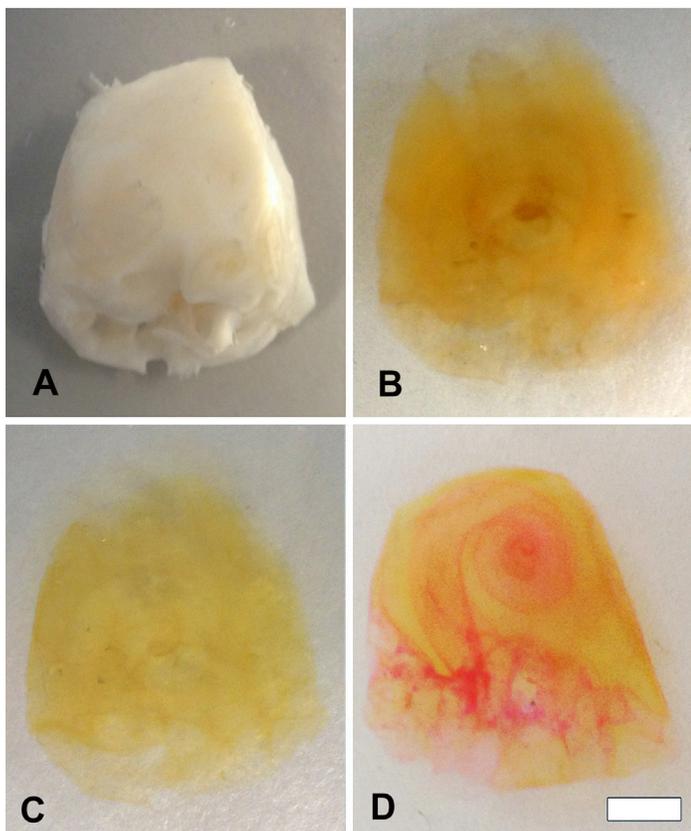


Figure 2. (A) Decalcified cochlea, (B) Dehydrated and cleared, (C) After bleaching, (D) Scala media fluorescently labeled by perfusion. Bar=3mm.

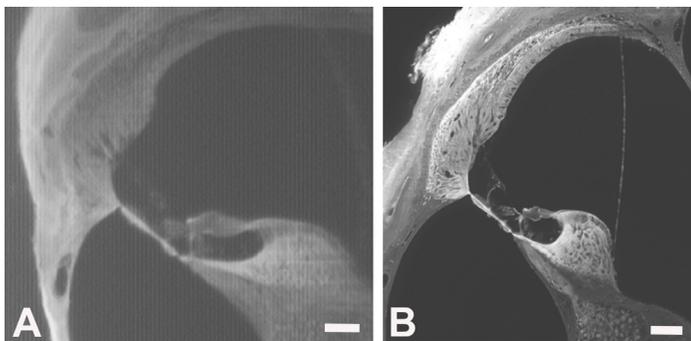


Figure 3. (A) OPFOS image of the scala media of the mouse cochlea, (B) sTSLIM image of the mouse cochlea. Bar=50µm.

The smaller the cochlea the better the image quality presumably due to less light scatter and absorption. Figure 4 shows sTSLIM imaging of several different types of cochleas from the mouse, guinea pig, cat, monkey, porpoise and human. For small cochleas fluorescent labeling can be accomplished by immersion of the whole temporal bone in the dye. However, for larger cochleas we have had better results if the scala media is perfused with the dye rather than having the dye in the bone which causes increased light scatter. Panel F in figure 4 is a cross section of the human cochlea shown in figure 2D. In addition to fluorescently labeling all structures in the cochlea we have also been able to label certain specific structures within the cochlea by whole cochlea immunohistochemistry or ligand labeling (figure 5).

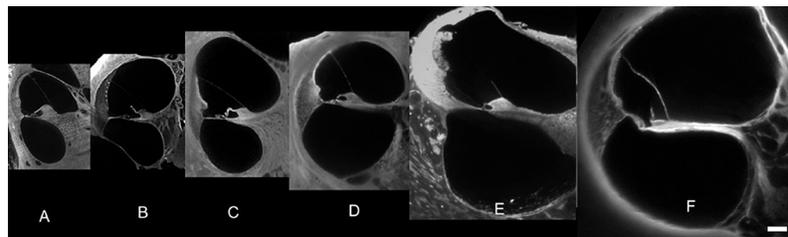


Figure 4. sTSLIM cross sections of the scala media in (A) mouse, (B) guinea pig, (C) cat, (D) monkey, (E) porpoise and (F) human. Notice image quality decreases with the increasing size of the cochlea. Bar=100µm

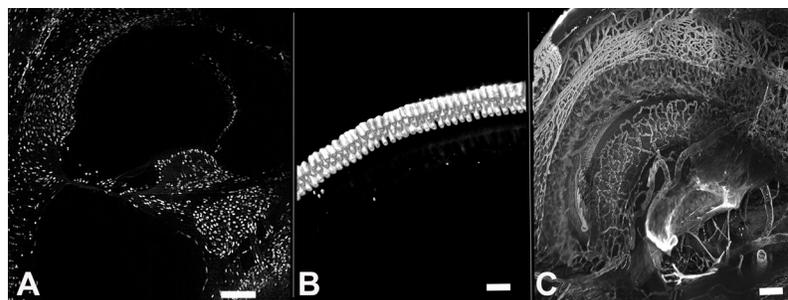


Figure 5. (A) Sytox labeling of DNA in nuclei, (B) Prestin labeling of outer hair cells, (C) Type IV collagen labeling of vessel basement membranes in decellularized tissue. Bars=40µm.

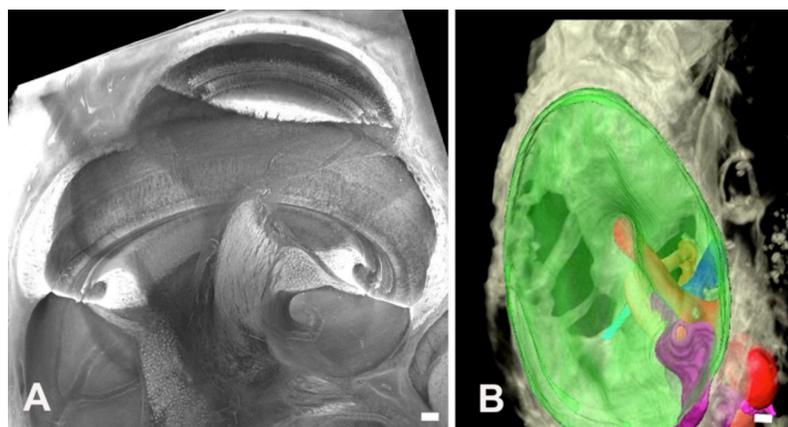


Figure 6. (A) Direct volume rendering of the apical portion of a hemisected mouse cochlea. (B) Three-dimensional volume rendering of the middle ear of the human with the ear drum (green) and segmentation of the ossicles (red, purple, orange). Bars=1mm.

For example, cell nuclei can be labeled by the DNA stain Sytox (figure 5A) and structures such as the outer hair cells that are accessible to anti-prestin antibodies (figure 5B) can be labeled. However, unlike mechanically sectioned tissues where the epitopes of structures are exposed, deep structures such as basement membrane proteins cannot be easily labeled in whole cochleas. However, creative tissue processing, such as decellularization, can be used to make deeper structures available for antibody labeling, such as type IV collagen (figure 5C).

Since light-sheet microscope produces a stack of well-aligned images, three-dimensional reconstructions of structures within tissues is possible (figure 6).

Volume renderings can be produced directly from the stacks of images (figure 6A) and are a convenient and rapid method for examining structures in three-dimension. However, for isolation of structures, segmentation is necessary and involves the labor intensive task of tracing the boundary of the structure in each section of the stack. Segmentation allows for isolation of the structure and its characterization by morphometry and by color (figure 6B).

Light-sheet microscopy is a complementary, nondestructive method to mechanical sectioning of the cochlea. Using air mounted objectives its resolution is subnuclear and not as good as sections examined by light microscopy after paraffin, celloidin or plastic sectioning. However, it allows for whole cochlea labeling of specific structures by fluorescent labels and their identification, tracking and quantification within the whole cochlea. It is a nondestructive method so that whole cochleas can be processed by other methods including: light, scanning and transmission electron microscopy. We are continuing to improve the image quality provided by light-sheet microscopy by using chemical methods to improve the clarity and fluorochrome labeling of the cochlea. We are also using different optical methods to probe deeper within large cochleas while reducing light scatter and absorption and by using different sectioning methods (e.g., translational vs. radial) in order to image around opaque structures, such as a cochlear implant. ●

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REFERENCES

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Otopathology Mini-Travel Fellowship Program

The NIDCD National Temporal Bone Registry is pleased to announce the availability of mini-travel fellowships. The fellowships provide travel funds for research technicians and young investigators to visit a temporal bone laboratory for a brief educational visit, lasting approximately one week. The emphasis is on the training of research assistants, technicians and junior faculty.

These fellowships are available to:

- U.S. hospital departments who aspire to start a new temporal bone laboratory.
- Inactive U.S. temporal bone laboratories that wish to reactivate their collections.
- Active U.S. temporal bone laboratories that wish to learn new research techniques.

Up to two fellowship awards will be made each year (\$1,000 per fellowship). The funds may be used to defray travel and lodging expenses. Applications will be decided on merit.

Interested applicants should submit the following:

- An outline of the educational or training aspect of the proposed fellowship (1-2 pages).
- Applicant's curriculum vitae.
- Letter of support from temporal bone laboratory director or department chairman.
- Letter from the host temporal bone laboratory, indicating willingness to receive the traveling fellow.

Applications should be submitted to:

Michael J. McKenna, M.D.
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Free Brochures for your Office or Clinic about Temporal Bone Research and Donation

The Gift of Hearing and Balance: Learning about Temporal Bone Donation is a 16-page, full-color booklet that describes in more detail the benefits of temporal bone research. It also answers commonly asked questions regarding the temporal bone donation process. *Dimensions: 7"x10"*

If you would like to display this brochure, please complete the form below and return it to the Registry by mail or fax. The brochures will be sent to you **free of charge**. Please circle the amount requested for each brochure or write in amount not listed.

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