Quantitative light-microscopic evaluation of the human temporal bone classically begins with hair cell counts and ganglion cell counts extracted from serial celloidin sections, stained with hematoxylin and eosin. The “cytocochleogram,” as it has been called, is sometimes supplemented by a more qualitative analysis of the stria vascularis, the spiral ligament, the limbus and/or the peripheral axons of auditory nerve fibers in the osseous spiral lamina. More quantitative assessments of the afferent and efferent synapses and terminals within the organ of Corti has, historically, been restricted to electron microscopic studies, which typically require labor-intensive serial section analysis, and thus are always focused on very small samples of hair cells and nerve fibers.

Our laboratory has long studied the afferent and efferent neurons connecting cochlear hair cells with the brain. Over the years, we have developed numerous techniques at both the light- and electron-microscopic levels for quantifying this innervation in normal ears, and in ears with acquired sensorineural hearing loss.\(^4\,5\,6\,7\,8\,9\) Recently, we have shown in animal models that the synapses between auditory nerve fibers and inner hair cells are the most vulnerable elements in the inner ear. In both noise-damage\(^9\) and aging\(^10\) and perhaps also in aminoglycoside ototoxicity,\(^11\) auditory-nerve synapses disappear before the hair cells die. Thus, many compromised ears have a full complement of hair cells, despite significant (as much as 50%) synaptic loss. Depending on the etiology and species, the delay between synaptic loss and spiral ganglion cell death can be months, years, or even decades. However, once disconnected from its hair cell, the auditory nerve fiber is unresponsive, without a cochlear implant. Since the synapses are hard to see in normal
light-microscopic preparations, since ganglion cell death is slow, and since this primary neural degeneration degrades auditory performance on difficult tasks (like hearing in noise) without changing performance on simple tasks (like detecting pure-tone thresholds), the phenomenon has been called “hidden hearing loss.”

To better study the phenomenon of hidden hearing loss in both animal and human ears, we have developed immunostaining approaches that allow us, at the light-microscopic level, to count hair cells, cochlear nerve synapses, efferent terminals and the peripheral axons of both afferent and efferent neurons in the osseous spiral lamina throughout the entire cochlea. We use antibodies to neurofilament protein to image axons and terminals of both afferent and efferent neurons, including both myelinated and unmyelinated portions (Fig. 1). We use antibodies to an enzyme in the biosynthetic pathway for acetylcholine to highlight the cholinergic olivocochlear pathway and thus to distinguish afferent from efferent axons in the osseous spiral lamina. Note, in the low power view of the double-stained osseous spiral lamina, that the efferent (ChAT-positive) axons take a spiral course (Fig. 1), as expected based on numerous prior anatomical studies. This provides compelling evidence that the antibody is working properly and that it provides a robust method for distinguishing afferent from efferent neurons. Further evidence is provided in the organ of Corti, where the ChAT antibody labels large endings underneath the outer hair cells and smaller endings in the inner hair cell area (Fig. 2A,B), exactly as expected from numerous ultrastructural studies.

To label hair cells, we use antibodies to a myosin variant (myosin VIIa) that is highly expressed throughout the cytoplasm of both inner and outer hair cells. As seen in Figure 3, the myosin staining is most robust in the cuticular plate. This makes it particularly easy to count present and missing hair cells for construction of a cytocochleogram. To label afferent synapses, we use antibodies to a protein called C-terminal binding protein (CtBP2, Fig. 4), which is a major component of the pre-synaptic ribbon present at each synapse between an inner hair cell and an auditory nerve terminal. Since most auditory-nerve fibers contact a single inner hair cell, via a single unmyelinated terminal, and since most hair cell synapses contain a single pre-synaptic ribbon, counts of inner hair cell ribbons should closely match counts of peripheral axons in the osseous spiral lamina (once efferents are accounted for): indeed, recent data from immunostained human temporal bones show that this is the case. As can also be seen in Figure 4, the CtBP2-positive puncta are only found within the hair cell cytoplasm, and are only present around the basolateral region of the hair cells. Since this is exactly the pattern expected from the known ultrastructural

Figure 1: Surface view of the osseous spiral lamina (OSL) and organ of Corti from the 1 kHz region of a 54 yr old male, immunostained with anti-NF (neurofilament, green) and anti-ChAT (choline acetyltransferase, red) to reveal the afferent and efferent innervation respectively. Red arrows point to two of the many bundles of efferent axons spiraling through the OSL.
Because we tag the antibodies with fluorescent markers, the tissue can be imaged in the confocal microscope. Confocal microscopes can be outfitted with multiple lasers to excite fluorophores at multiple wavelengths from 400 to 700 nm. We typically use four fluorophores (with optimal excitation at 405, 488, 568 and 647 nm). This allows us to use four different primary antibodies (each visualized with a different fluorophore-coupled secondary antibody). Thus, we are able to simultaneously image four elements in each piece of tissue, e.g. hair cells, nerve terminals, efferent fibers, and afferent synapses. The only limitation is finding the primary antibodies that work well in human post-mortem tissue. If a particular primary antibody works well in isolation, we find that combining it with others in double-, triple- and quadruple-stained samples is never a problem, providing that each primary antibody was raised in a different species and thus can be coupled to a different type of fluorinely labeled secondary antibody. We have also been pleasantly surprised at how many of the primary antibodies that we have developed for use in well-fixed animal ears have also worked well in human tissue, even with post-mortem times of as much as 12 hours.

Confocal microscopy also provides the best resolution available at the light-microscopic level, and allows 3-D image stacks to be acquired by scanning the tissue (in x and y), at each of many finely spaced (e.g. 0.25 micron) focal levels (z). Once such a “z-stack” is acquired, it can be digitally analyzed, quantified and computationally “re-sliced” to view the tissue from any desired angle. Thus, as shown in Figure 2C,D, an image stack of peripheral axons acquired by scanning a surface-mounted piece of the cochlear spiral, can be digitally “re-sliced” to view as if the axons had been cut in cross-section, allowing them to counted just as easily as if the tissue had been laboriously embedded and re-sectioned in a plane perpendicular to the osseous spiral lamina, as temporal bone studies have done in the past. Image stacks can also be digitally processed in 3-D, using one of many powerful image-analysis software packages. We find Amira (http://www.fei.com/software/amira-3d-for-life-sciences/) to be particularly useful. We use it, for example, to automate the counting of synaptic ribbons in our confocal z-stacks of the inner hair cell area.

Most of our immunostaining work to date on human temporal bones has been on “surface preparations” of the inner ear, i.e. manual dissection of drilled and decalcified cochleas, in which the entire spiral is dissected into roughly 6-8 pieces, each containing roughly ½ cochlear turn with the osseous spiral lamina and the organ of Corti. Before imaging at the confocal, we derive a cochlear map for each case, by tracing an arc along the pillar cells in low-power digital images of each piece. Armed with the map, we can then choose to capture data from precisely specified cochlear frequency locations. The cochlear mapping is done automatically with the aid of a free-ware “plug-in” to Image J, the image-analysis software. Image J is free and available here: http://rsbweb.nih.gov/ij/download.html. Our plug-in is free and

Figure 2: Confocal image stacks of the organ of Corti (A,B) and the osseous spiral lamina (C,D), immunostained with anti-NF (green), anti-ChAT (red) as in Figure 1, plus anti-MyosinVIIa (blue) to show the hair cells. A,B - These two panels are different views of one confocal z-stack from the 5.6 kHz region of a 56 yr old male: A is the surface view, and B is re-imaged to show the “side view”. C,D – These two panels are different views from one confocal z-stack from the 0.5 kHz region of the same individual: C is the surface view, and D is a digital “section” through the image stack taken at the position of the dotted line in C. Red-filled arrowheads in all panels point to efferent terminals (A) or axons (C and D); green-filled arrows in C point to the auditory nerve terminals in the inner hair cell area.

Figure 3: Confocal image from the 0.35 kHz region of an 89 yr-old male, showing how easy it is to spot missing hair cells (white arrowheads) when myosin VIIa is used to immunostain the inner and outer hair cells. This cochlea was also immunostained with anti-CtBP2 (red) to show the pre-synaptic ribbons inside the hair cells (red-filled arrowheads).
Thus, though limited in scope, the data cells in the human organ of Corti. II. outer hair cells. Laryngoscope 93(6): 780-791.


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For the surface preparations, the tissue is immunostained after the dissection. Double-, triple- and quadruple-staining with fluorescent markers has also been successfully applied to archival human tissue, after using techniques for removing the celloidin described in previous immunohistochemical work using individual primary antibodies followed by generation of brown/black “reaction product” that is visible in routine brightfield microscopy.22

Our analysis of the innervation status of human ears with minimal hair cell loss is only in the early stages, but we have already demonstrated, in aging humans with no explicit history of otologic disease, that there are many fewer synapses on the remaining hair cells than would be predicted based on spiral ganglion cell counts.16 Thus, though limited in scope, the data thus far available are consistent with the idea that spiral ganglion counts greatly underestimate the degree of cochlear neuropathy and that primary neural degeneration in the form of loss of synaptic connections between ganglion cell and hair cell is a major contributor to the problems understanding complex stimuli like speech in a noisy environment, the classic complaint of those with idiopathic presbycusis. ●

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Figure 4: High-power confocal views of the 4 kHz region of a 67 yr-old female, showing the pre-synaptic ribbons, immunostained with anti-CtBP2 (red), at the basolateral poles of the inner hair cells. Hair cells are immunostained with anti-mysosinVIIa (blue) and nerve terminals are immunostained with anti-neurofilament (green). A and B are views of the same image stack: A is the surface view and B is rotated to view the stack from the side.

Otosclerosis is a bone disease that occurs only in the human otic capsule as single or multiple foci, where there has been repeated resorption and remodeling of bone. Otosclerosis has been used to describe both spongiotic and sclerotic lesions. Active otospongiosis, which involves the endosteum over the spiral ligament, can result in hyalinization of the spiral ligament and sensorineural hearing loss. In contrast, if the lesion is sclerotic, the spiral ligament may be normal, and the bone conduction hearing thresholds may be normal.

Histopathological studies of otosclerosis have been well documented by many anatomists and pathologists beginning with Valsava in 1704. Cochlear and vestibular lesions have been identified in microdissected temporal bones, and light microscopic descriptions have been made by several investigators, including Politzer (1893-1894). Transmission electron microscopy and cytochemistry have provided additional insight into the molecular pathology of otosclerosis, including the involvement of lysosomes and the role of calcium and the enzyme alkaline phosphatase bone in metabolism.

Recent molecular genetic studies, proteomics, and immunohistochemistry have revealed several specific proteins and pathways involved in the pathophysiology of otosclerosis. Studies of differential gene expression between the otic capsule and other bones have provided clues to the roles of specific genes in otosclerosis. Using proteomics and immunohistochemistry in temporal bones affected with otosclerosis, we have identified several proteins present in the otosclerotic foci, including TGFB-1, HSP90, sialyltransferase, ribophorin II, and superoxide dismutase (SOD). Several other proteins remain to be identified.

We continue our immunohistochemical studies on the localization of proteins present in the otic capsule of temporal bones affected with otosclerosis. Among these proteins, ubiquitin (UBA52), collagen IX, nidogen and bone sialoprotein (BSP) are likely to be expressed. It was surprising to find (in some bones) that these proteins, suggesting bone remodeling activity, were immunolocalized mainly in sclerotic lesions and, to a lesser degree, in spongiotic lesions, suggesting that active remodeling of bone may be occurring in the “inactive” sclerotic lesions.

It has been generally accepted that otospongiosis is the primary active lesion and otosclerosis is the inactive healed phase. However, based on the effect of the various types of lesion on the spiral ligament, we suggested that there may be two types of sclerotic dyscrasias. This concept was based on the findings that destruction of bone may signal the surrounding bone to remodel and increase in density, perhaps to prevent further incursion of the advancing spongiosis.

Hyalinization of the spiral ligament is found in human cochleae where the endosteum is breached by an otosclerotic or otospongiotic process (Fig. 1). Hyalinization of the spiral ligament may be the end result of necrosis of cells of the spiral ligament nearest the cochlear endosteum. An uncommon finding is hyalinization adjacent to an otosclerotic lesion (Fig. 2); whereas, in other specimens, there is no hyalinization adjacent to the spiral ligament, possibly suggesting two variants of the otosclerotic lesion.

Figure 1. Hyalinized spiral ligament (h) adjacent to an active otospongiotic lesion (sp). (hematoxylin and eosin [H&E] X 200).
In large otosclerotic lesions surrounding the cochlea, the perimeter of the process is sclerotic and the part closer to the cochlea is spongiotic (Fig. 3). This seems inconsistent with the hypothesis that otosclerosis is the healing phase of otospongiosis. Labeling large lesions containing both sclerosis and spongiosis with ubiquitin (a protein that mediates intracellular selective protein degradation)\(^1\) indicates that some sclerotic lesions are active while others are quiescent, and the same is true for spongiotic lesions (Fig. 4).

We suggest that the active sclerotic lesion represents a reactive hyperplasia in the cochlear capsule attempting to impede the destructive spongiotic process as it progresses through the cochlear capsule (Fig. 5). The areas of sclerosis that do not label with ubiquitin, indicating quiescence, are the resolving inactive, formerly spongiotic, lesions. The application of immunolabeling, and more recently, proteomics, to archive celloidin-embedded temporal bone sections are enabling us to unlock more of the mysteries of otosclerosis.\(^1\)

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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:
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- 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:

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