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REVIEW

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Contemporary techniques in human otopathology and promise for the future

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Abstract

Contemporary histopathology of the ear is based on an evolution of equipment and histological techniques over the last 500 years, including the invention of the light microscope and protocols for fixation, embedment, sectioning, and staining of tissue samples, and visual documentation of findings. Several recent techniques which can be utilized in otopathology hold promise for significant improvement in methods and a better understanding of pathologic processes in diseases of the ear.

KEYWORDS

balance disorders, deafness, ear, histopathology, human, temporal bone

1 | INTRODUCTION

Contemporary histopathology of the ear is based on an evolution of equipment and histological techniques over the last 500 years, including the invention of the light microscope and protocols for fixation, embedment, sectioning, and staining of tissue samples, and visual documentation of findings. Several recent techniques which can be utilized in otopathology hold promise for significant improvement in methods and a better understanding of pathologic processes in diseases of the ear.

2 | THE FIRST 500 YEARS

2.1 | Light microscope (invention and evolution of applied uses)

The origin of the contemporary light microscope can be traced to sometime between 1590 and 1618 when Zacharias Janssen (1585-1638), a Dutch spectacle-maker, created an early microscope with multiple lenses held in a single tube. The derivative instrument, the compound microscope, can be traced to Galileo Galilei (1564-1642), an Italian astronomer, in 1609. Many decades passed before microscopic study was applied to the study of biology. Thus in 1665, Robert Hooke (1635-1703) described what he called "cells," which were the plant cell walls within a piece of cork. The first description of the nucleus of a biological cell was made by Antonie van Leeuwenhoek in 1700.

Several more decades passed before microscopic anatomy and pathology were applied to medical subjects. In the 17th century, Marcello Malpighi, in Bologna, used the light microscope to study normal animal and plant anatomy. Rudolf Virchow (1821-1902), a German pathologist, demonstrated that diseases have a cellular basis, and he is considered by many to be the father of cellular pathology. The first published otopathologic observations were made by Giovanni Morgagni of Padua (1682-1771), who has been called the father of pathologic anatomy.[1] In letter XIV of *De Sedibus et Causis Morborum* (1769), the pathologies of various ear diseases, including stapes fixation, syphilis of the ear, and labyrinthitis were described based on anatomic dissections of autopsy specimens. Although camera lucida renderings of the normal labyrinth were available in the first half of the 19th century based on studies by Antonio Scarpa (1752-1832) and Alfonso Corti (1822-1876), it was not until much later that microscopic techniques were applied to the anatomy and pathology of the ear. This fact led Joseph Toynbee in his text entitled "Diseases of the Ear"[2] (1860) to lament:

> To quote from Mr. Wilde's introduction to his valuable treatise on Aural Surgery, medical men are too ready to

> affirm that "they know nothing about the diseases of the organ of hearing" and many looking upon the difficulties that surround the investigation as insurmountable, have tacitly abandoned its pursuit. Yet if we carefully survey the history of the rise and progress of aural surgery, as a distinct branch of scientific surgery, one main cause of the disrepute into which it had fallen, may be traced to the neglect of the pathology of the organ of hearing - a neglect that doubtless also led to the ignorance which has prevailed as to the structure and functions of some of the most important of its parts.

The first otopathologic studies using temporal bone sections were described by Moos and Steinbrugge in 1884.[3] A flurry of descriptions of otologic disease as studied with the light microscope was forthcoming, including otosclerosis,[4,5] Meniere's disease,[6,7] and presbycusis.[8]

Protocols for preparing tissue samples for light microscopic study likewise evolved in the 19th and 20th centuries.

2.2 | Fixation methods

The early use of heat, alcohol, acetic acid, and compound fixatives including Muller's fluid (c. 1860), Zenker's fixative (c. 1894), and Carnoy's fixative (c. 1887) has been largely replaced by the use of formalin, first described as a fixative by Blum in 1893.[9,10] Heidenhain's Susa solution, popular as an otopathologic fixative in the 19th and early 20th centuries, has been largely abandoned because of its content of toxic mercuric chloride.

2.3 | Embedding media

The routine use of paraffin wax was introduced in the late 19th century.[10] Celloidin was introduced as an embedding medium in 1877 and remains the current gold standard for otopathologic study. Celloidin does not cause the tissue shrinkage and therefore the resultant rupture of delicate inner ear membranes has been associated with the use of paraffin wax. Plastic embedding materials such as epon, araldite, or methyl methacrylate are used for special applications such as transmission electron microscopy.

Sectioning has evolved from free hand techniques to the use of microtomes, introduced by John Hill, MD, in 1770.[11] The 19th and 20th centuries saw the introduction of the Cambridge Rocker microtome[10] (c. 1885), the Cuthcart microtome[10] (c. 1880s), the rotary microtome[10] (Minot, c. 1885), and the sliding microtome, which remains popular in otopathology because of its capacity to section large blocks of tissue.

2.4 | Staining

The earliest staining techniques included the use of carmine, to stain glycogen, and mucicarmine to stain acid muco-polysaccharides. The use of hematoxylin was first reported in 1863. Basic fuchsin was manufactured first in 1865. Combined staining with hematoxylin and eosin was introduced by Wissowzky in 1875.[12]

2.5 | Imaging of pathological change

Visual documentation of the pathologic findings achieved by histopathologic study included camera lucida renderings of normal and pathologic material. Photomicroscopy was introduced circa 1876 by J.J. Woodward, an American Civil War surgeon.

Histopathology is still undergoing evolution with the application of new techniques that promise to revolutionize otopathologic study in the future.

3 | INTRODUCTION OF NEW TECHNIQUES IN OTOPATHOLOGY

3.1 | SEM and TEM

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) significantly increase the resolving power of microscopic examination of normal and diseased tissue in human disease.

SEM projects a beam of electrons onto the surface of a specimen, which creates an image based on the surface topography. Resolution of 1 nm is achievable. Development of SEM in the 1950s and early 1960s led to the first commercial instrument by 1965. SEM study of biologic materials, such as living cells, requires chemical fixation for stabilization using glutaraldehyde and/or formaldehyde followed by post-fixation treatment with osmium-tetroxide and dehydration in ethanol acetone.

Examples of the use of SEM in otopathology include the definition of the normal variation in the number of cilia per hair cell in the normal human cochlea[13,14] (Figure 1A), as well as the study of pathologic changes in the hair cell bundles of inner and outer hair cells of the mammalian inner ear induced by genetic mutations[16] and noise trauma.[17]

TEM was first developed in the 1930s, and Ernst Ruska, a German physicist, was awarded the Nobel Prize in physics in 1986 for the development of TEM.

In TEM, electrons pass through a thin section of tissue, thus creating an image of subcellular detail of the anatomy and pathology. Examples of the use of TEM in human otopathologic specimens include the morphology and numbers of synapses on inner and outer hair cells on the organ of Corti[15,18,19] (Figure 1B).

3.2 | Confocal microscopy

Confocal laser scanning microscopy (CLSM) was patented in 1957 by Marvin Minsky, an American mathematician and computer scientist. The technique captures multiple two-dimensional (2D) images at varying depths in a biologic sample, thus allowing reconstruction of a three-dimensional (3D) structure by "optical sectioning." Using confocal microscopy, it has been recently demonstrated that synaptic

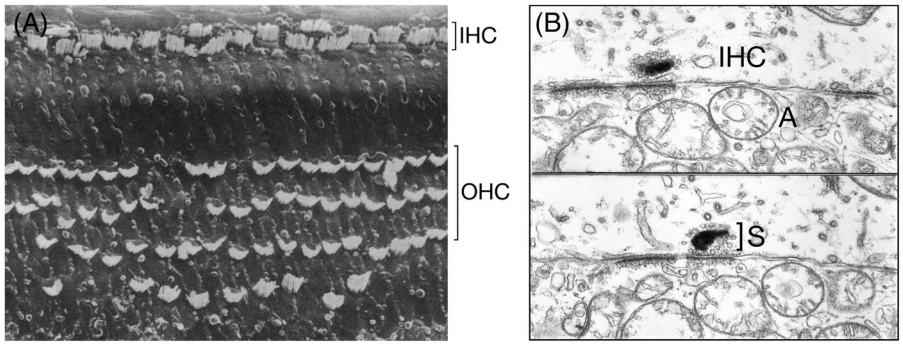

FIGURE 1 A, Surface view of the human organ of Corti by scanning electron microscopy (SEM). Both inner (IHC) and outer (OHC) hair cells and their stereocilia were visible (picture with 20 μm). *Source*: Previously published[14] and reprinted with permission of Sage Publications Ltd. B, Two representative micrographs by transmission electron microscopy (TEM) from serial sections of the synaptic area between an IHC and a large afferent dendrite "A" in the basal turn of the human cochlea. Synaptic contacts "S" consisted of an asymmetrical thickening of the apposed membranes, a layer of granular material subjacent to the thicker post-synaptic membrane, and a presynaptic body surrounded by vesicles. *Source*: Previously published[15] and reprinted with permission of John Wiley and Sons, Inc, publisher

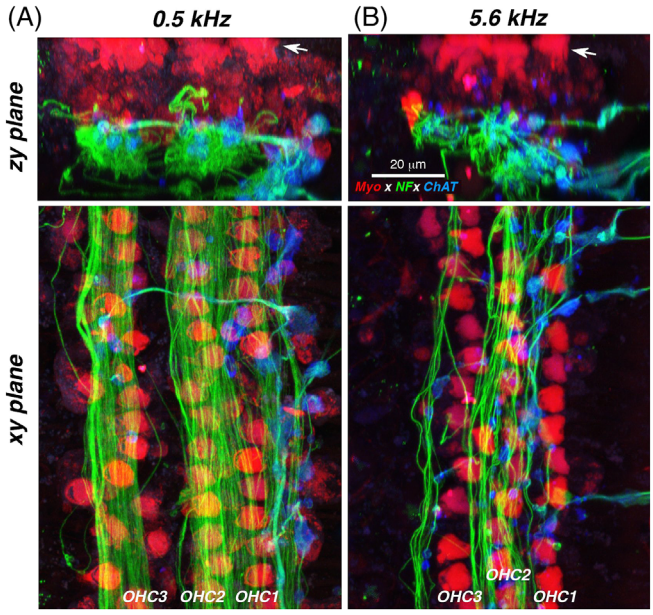

FIGURE 2 Confocal projections from two whole mounts of the organ of Corti in the human in the outer hair cell region at two cochlear locations in a 39-year-old female. First, second, and third rows of outer hair cells were seen (OHC 1, 2, 3). White arrows in (A) and (B) showed brightly stained cuticular plates of the hair cells. Scale bar, staining key in (B) applies to all panels. *Source*: Previously published[21] and reprinted with permission of Elsevier, publisher

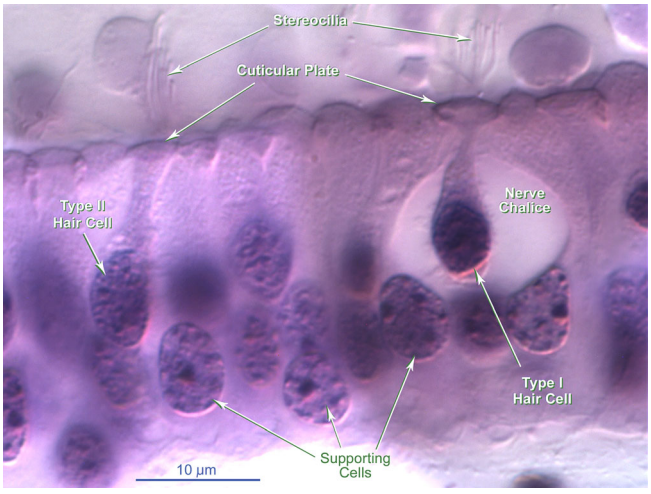

FIGURE 3 Nomarski microscopy image of the human utricular macula in an archival temporal bone section of a male, age 54 years. Specimen removed 3 days after death. The cytologic feature of hair cells and supporting cells from the nucleus to the cuticular plate and stereocilia bundle are shown. Type 1 hair cells are recognized by their flask shape, spherical nucleus, and nerve chalice surrounding the cell body. Type 2 hair cells are identifiable by a relatively cylindrical shape, oval nucleus, and absence of the nerve chalice. Supporting cells have lightly stained nuclei with chromotin material distributed in clumps, and lack stereocilia and cuticular plates. *Source*: Previously published[22] and reprinted with permission of Sage Publications Ltd

connections between sensory hair cells and first order cochlear neurons may be interrupted by exposure to loud noise, ototoxic drugs, and even the aging process[20,21] (Figure 2).

3.3 | Phase contrast and differential interference contrast microscopy (DIC)

The phase contrast microscope is a derivative of the optical microscope. A phase shift in light passing through a transparent specimen results in changes in brightness in the image, making the phase shift visible. Invention of the phase contrast microscope earned Frits Zernike, a Dutch physicist, the Nobel Prize in physics in 1953.

A closely related microscopic technique has been termed "differential interference contrast microscopy (DIC)" or "Nomarski microscopy," which results in the improvement of the image contrast. Merchant et al[22] used Nomarski microscopy to better differentiate type I and type II vestibular hair cells in the human (Figure 3). Liberman et al[23] used DIC microscopy to achieve more

accurate counts of inner and outer hair cells that can be achieved using bright field microscopy in fixed specimens of the cochlea in the human.

3.4 | Energy-dispersive X-ray spectroscopy

Energy-dispersive X-ray spectroscopy by scanning electron microscopy (EDS-SEM) is an analytical technique that is based on the induction of an emission of X-rays by electron irradiation of a specimen, producing characteristic patterns, thus allowing identification of component elements or chemical characterization of the specimen. For example, the presence of platinum and silicon particles along the electrode track in human temporal bone specimens following cochlear implantation has been demonstrated.[24,25] It has been suggested that these elements were derived from the cochlear implant electrode and served as foreign bodies, inducing a cellular immunologic response to the presence of the electrode array (Figure 4).

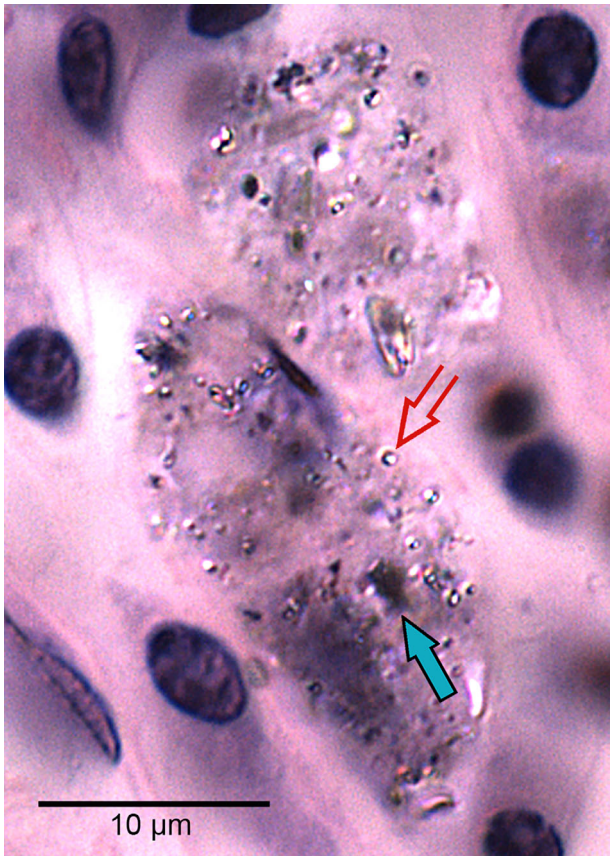

FIGURE 4 Macrophages containing both black particulate material (solid arrow) and birefringent material (hollow arrow) are consistent with phagocytized platinum and silicone, respectively, confirmed by energy-dispersive X-ray spectroscopy, and were commonly found in the fibrous tissue sheath surrounding a cochlear implant electrode in the human (H&E stained). *Source*: Previously published[25] and reprinted with permission of Wolters Kluwer Health, Inc, publisher

3.5 | Mass spectrometry

It is well known that chemotherapy using cisplatin may cause a permanent sensorineural hearing loss.[26] Inductively coupled plasma mass spectrometry (ICP-MS) has been employed[27] to investigate the retention by the cochlea of cisplatin introduced by chemotherapeutic regimens during life using archival human temporal bone specimens (Figure 5). It was demonstrated that the cochlea retains cisplatin for months to years following treatment in both mice and humans. Furthermore, using laser ablation coupled to the ICP-MS, the distribution of cisplatin within the human cochlea was determined to be largely in the stria vascularis.

3.6 | Molecular genetics and histochemical localization in otopathology

It has been demonstrated that it is possible to isolate DNA from archivally collected formalin-fixed and celloidin-embedded human temporal bone specimens.[28,29] Using these retrieval techniques, Burgess et al[30] demonstrated the presence of Herpes simplex type 1 (HSV-1) genomic DNA in the geniculate ganglion of a patient who

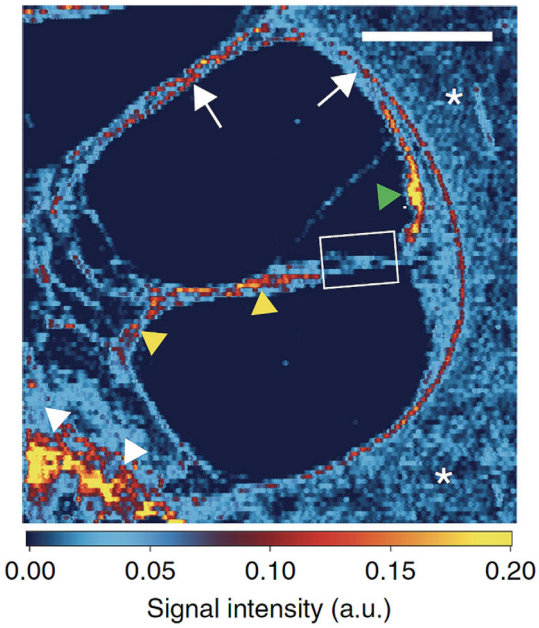

FIGURE 5 Mass spectrometry. Laser ablation ICP-MS (inductively coupled plasma mass spectrometry) image of platinum distribution in the organ of Corti from a patient who was treated with cisplatin. This patient died 25 days after the last cisplatin infusion. Green arrowhead marks the stria vascularis; yellow arrowheads mark cochlear nerve fibers; white arrowheads mark the boundary between the cochlear nerve and the bone of the cochlear modiolus; white arrows mark the endosteum which lines the cochlear canal; asterisks mark surrounding cochlear bone, and white box frames the organ of Corti. Scale bar = 500 µm. Platinum signal intensity values are in arbitrary units. *Source*: Previously published[27] and reprinted with permission of Springer Nature, publisher

had Bell's palsy during life. In a subsequent study,[31] the presence of Herpes varicella-Zoster viral (VZV) DNA in celloidin embedded human temporal bone sections was demonstrated in two patients who suffered from Ramsay Hunt syndrome during life. McKenna et al[29] demonstrated the presence of a 115-base pair sequence of the measles nucleocapsid gene in human temporal bone specimens with histologic evidence of otosclerosis, but not in control specimens without the evidence of otosclerosis, suggesting a causative association between the presence of measles nucleocapsid and histologic otosclerosis in the human.

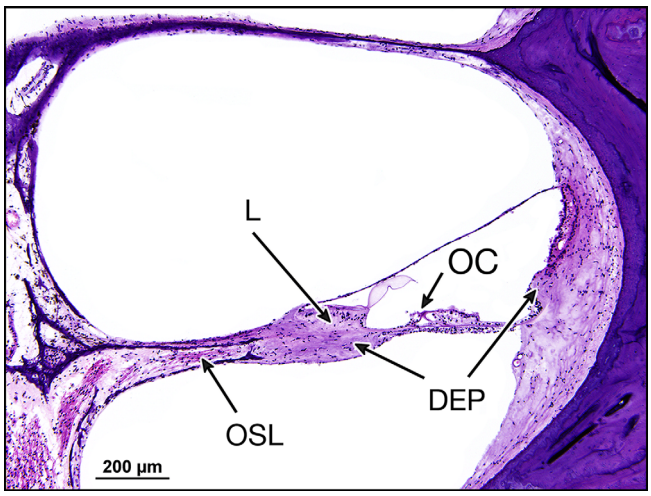

FIGURE 6 A mid-modiolar section from the left cochlea in a patient with a p.L114P COCH mutation (DFNA9). There was a deposit of extracellular amorphous material (DEP) in the spiral ligament (SL), distal end of the osseous spiral lamina (OSL), and at the base of the limbus (L). The organ of Corti (OC) was seen. *Source*: Previously published[33] and reprinted with permission of Karger Publishers

In addition, it has been demonstrated that Sanger sequencing of DNA can be accomplished with DNA obtained from formalin-fixed temporal bone sections in the human. Using archivally collected and formalin-fixed human temporal bone tissue from a patient with sensorineural hearing loss, Sanger sequencing of DNA demonstrated a pathogenic variant in the DFNA5 gene, thus allowing the otopathologic findings to be correlated with a known genetic mutation.[32] Sanger sequencing of DNA can also be accomplished using frozen muscle obtained at autopsy of a temporal bone donor. Using this technique, the histopathology of the human inner ear caused by the p.L11p Coch mutation (DFNA9) was demonstrated[33] (Figure 6).

Immunohistochemistry provides a histologic technique that may identify and localize antigens (proteins) within tissue sections based on antibody binding. It has been recently demonstrated that immunostaining can be successfully accomplished using archival human temporal bone specimens[34,35] (Figure 7). This fact dramatically enhances the scientific value of archival formalin-fixed temporal bone specimens

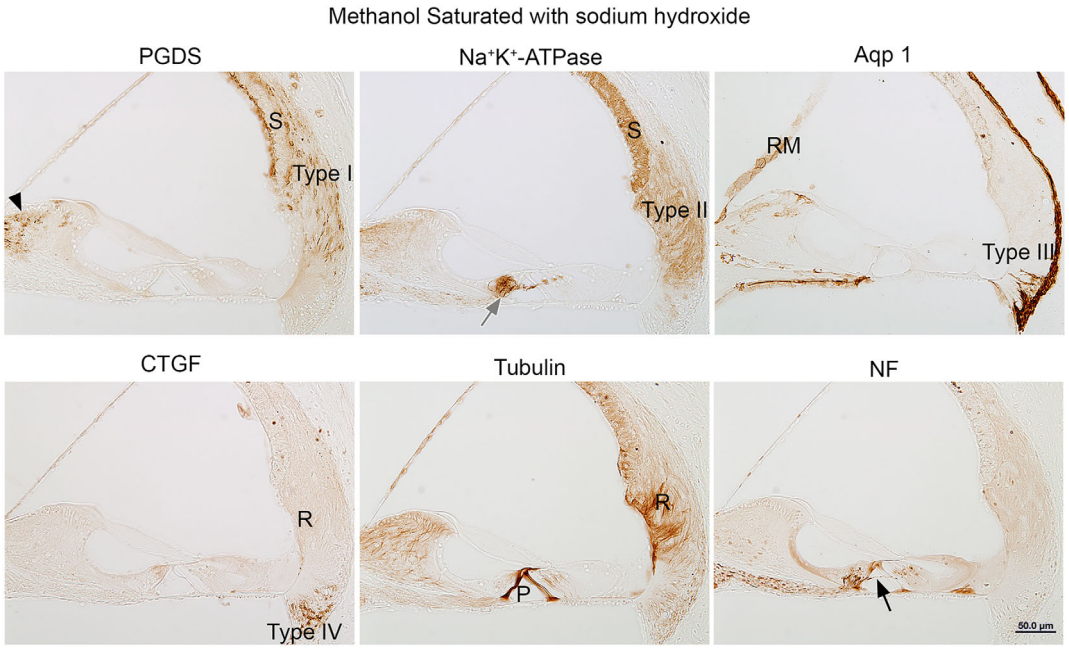

FIGURE 7 Immunostaining following celloidin removal with methanol, saturated with sodium hydroxide. Immunostaining for six antibodies was accomplished. Each antibody showed selectivity for appropriate cells, and there was very little background. PGDS (prostaglandin D synthase) staining was evident in marginal and basal cells of the stria vascularis (S), type 1 fibrocytes of the spiral ligament (type I), and fibrocytes of the spiral limbus. Aquaporin 1 antibody stained selectively for type 3 fibrocytes of the spiral ligament (type 3), as well as the medial portion of Reissner's membrane (RM), cells lining the bone of the scala tympani and some cells in the spiral limbus. The CTGF (connective tissue growth factor) antibody stained type 4 fibrocytes of the spiral ligament (type 4). Antibody against tubulin stained pillar cells (P), root cells (R), and spiral limbus. Neurofilament antibodies (NF) stained nerve fibers in the osseous spiral lamina, nerve fibers below the inner hair cell, tunnel crossing fibers (black arrow), and nerve fibers below the outer hair cells (calibration bar 15 µm). *Source*: Previously published[35] and reprinted with permission of Sage Publications, Ltd

which can be re-examined using immunohistochemical techniques for antigen localization.

For example, the capacity to accurately identify the remaining cells in a pathologic specimen of the organ of Corti has been significantly enhanced via the use of immunohistochemistry. Kamakura et al[36] demonstrated that the identification of hair cells in the organ of Corti in archival temporal bone human sections can be significantly improved by the use of myosin VIIa immunostaining. Inner and outer pillar cells and Deiter cells are reliably stained with antitubulin antibodies, and dendritic processes in the osseous spiral lamina or spiral bundles below inner and outer hair cells can be accurately identified by the use of anti-neurofilament antibody in archival formalin-fixed and celloidin embedded human specimens.

Wu et al[21] demonstrated that human temporal bones prepared by microdissection of formalin-fixed material as well as de-celloidinized archival temporal bone sections can be used to help quantify the preservation of inner and outer hair cells and synapses on these cells using confocal microscopy and immunostaining using anti-neurofilament, anti-myosin VI or VIIA, and ChAT (choline acetyltransferase) antibodies.

3.7 | Other imaging techniques

3.7.1 | Scanning thin sheet microscopy

Santi and his colleagues have developed a technique called scanning thin-sheet laser imaging microscopy for optical sectioning of thick tissues. A thin sheet of light is used to optically section tissue rendered transparent by chemical means following routine fixation, decalcification, and dehydration[37] (Figure 8). Thin-sheet microscopy may be considered a nondestructive imaging methodology which may complement more traditional examination by light, scanning, and transmission electron microscopy.

3.7.2 | Synchrotron radiation phase contrast imaging

Synchrotron radiation phase-contrast imaging and its application to visualization of the 3D cytoarchitecture of the human cochlea within the intact temporal bone has been developed by Iyer and colleagues[38] (Figure 9). The application of this technique may greatly reduce the preparation time needed for conventional light microscopy and holds the potential for possible in vivo imaging.

In summary, the current "gold standard" for clinicopathological correlation of hearing loss and vestibular disturbance in the human depends on light microscopic study of human temporal bone specimens from patients who had hearing or balance disorders. Additional techniques including the use of SEM and TEM, confocal microscopy, DIC microscopy (Nomarski microscopy), synchrotron radiation phase contrast imaging, thin-sheet microscopy, energy dispersive X-ray spectroscopy, mass spectrometry, molecular genetics, and immunohistochemical localization are in their infancy in their application to human deafness and balance disorders. The recent demonstration that DNA may be retrieved from archival temporal bone specimens and that immunohistochemistry is also possible using these specimens and dramatically

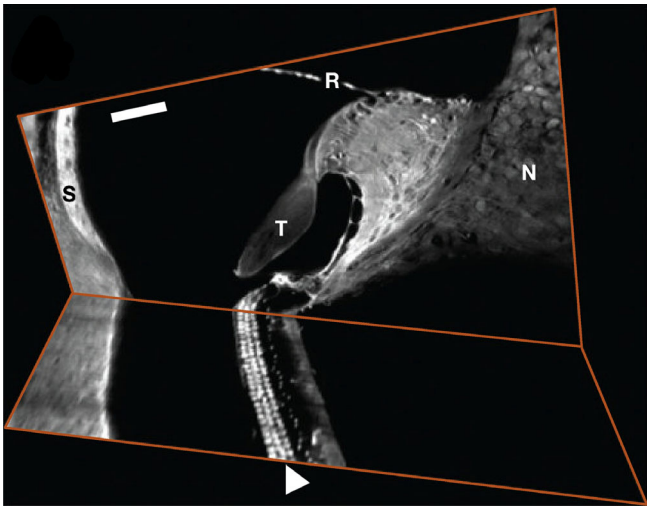

FIGURE 8 Thin-sheet laser imaging microscopy image of mouse scala media and high magnification from a stack that has been virtually resectioned using Amira software. The last 2D processing is shown in the back plane, and virtually resectioned images are shown in the front plane. The three rows of outer hair cells (arrowhead) of the organ of Corti and other tissues including the stria vascularis (S), Reissner's membrane (R), tectorial membrane (T), and neuron cell bodies (N) of the spiral ganglion were clearly seen. The light-sheet thickness was 7.5 μm for this stack of images. *Source*: Previously published[37] and reprinted with permission of Future Sciences, Ltd, publisher

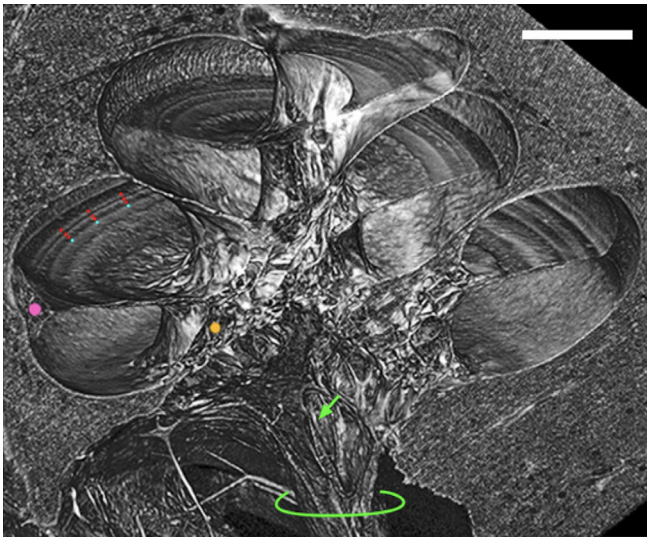

FIGURE 9 Synchrotron radiation phase contrast image of a human cochlea. A mid-modiolar 3D virtual cross-sectioned image is depicted. The three rows of outer hair cells (red dot), row of inner hair cells (blue dot) in the region of the spiral ligament and stria vascularis (pink dot), and Rosenthal's canal (orange dot) were clearly visualized. In addition, individual bundles of spiral ganglion neuronal fibers were shown (green arrow). The auditory nerve trunk (green outline) was also visualized. *Source*: Previously published[38] and reprinted with permission of The Optical Society, publisher

enhances the scientific value of archival formalin-fixed temporal bone tissue which can be re-examined using these techniques.

CONFLICT OF INTEREST

No conflicts of interest were identified.

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