Contemporary techniques in human otopathology and promise for the future

Joseph B. Nadol Jr. MD

Otopathology Laboratory, Department of Otolaryngology Head and Neck Surgery, Massachusetts Eye and Ear, Harvard Medical School, Boston, Massachusetts

Correspondence
Joseph B. Nadol Jr, Otopathology Laboratory, Department of Otolaryngology Head and Neck Surgery, Massachusetts Eye and Ear & Harvard Medical School, 243 Charles Street, Boston, MA 02114. Email: joseph_nadol@meei.harvard.edu

Funding information
NIDCD (NIH), Grant/Award Number: U24DC013983

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, embedding, 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, embedding, 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 some time 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. A flurry of descriptions of otologic disease as studied with the light microscope was forthcoming, including otosclerosis, Meniere's disease, and presbycusis.

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. 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. 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. The 19th and 20th centuries saw the introduction of the Cambridge Rocker microtome (c. 1885), the Cuthcart microtome (c. 1880s), the rotary microtome (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.

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 new techniques that promise to revolutionize otopathologic study in the future.

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.

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
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.\(^\text{24,25}\) 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).

3.5  |  Mass spectrometry

It is well known that chemotherapy using cisplatin may cause a permanent sensorineural hearing loss.\(^\text{26}\) Inductively coupled plasma mass spectrometry (ICP-MS) has been employed\(^\text{27}\) 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.\(^\text{28,29}\) Using these retrieval techniques, Burgess et al\(^\text{30}\) demonstrated the presence of Herpes simplex type 1 (HSV-1) genomic DNA in the geniculate ganglion of a patient who

---

**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\(^\text{25}\) and reprinted with permission of Wolters Kluwer Health, Inc, publisher

**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\(^\text{27}\) and reprinted with permission of Springer Nature, publisher
had Bell’s palsy during life. In a subsequent study, 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 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.

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. 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.L114P COCH mutation (DFNA9) was demonstrated (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 (Figure 7). This fact dramatically enhances the scientific value of archival formalin-fixed temporal bone specimens.

**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 and reprinted with permission of Karger Publishers.

**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 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 signifi-
cantly enhanced via the use of immunohistochemistry. Kamakura
et al 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 Vla immunostaining. Inner and outer
pillar cells and Deiter cells are reliably stained with antitubulin anti-
bodies, 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 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 preser-
vation of inner and outer hair cells and synapses on these cells using
confocal microscopy and immunostaining using anti-neurofilament,
anti-myosin VI or VlA, 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 tis-
sues. A thin sheet of light is used to optically section tissue rendered
transparent by chemical means following routine fixation, decalcifica-
tion, and dehydration (Figure 8). Thin-sheet microscopy may be
considered a nondestructive imaging methodology which may comple-
ment 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
(Figure 9). The application of this technique may greatly reduce the prepa-
ration time needed for conventional light microscopy and holds
the potential for possible in vivo imaging.

In summary, the current “gold standard” for clinicopathological cor-
relation 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 tech-
niques including the use of SEM and TEM, confocal microscopy, DIC
microscopy (Nomarski microscopy), synchrotron radiation phase con-
trast 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 immunohis-
tochemistry is also possible using these specimens and dramatically
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.

ORCID
Joseph B. Nadol Jr. https://orcid.org/0000-0002-4033-3104

REFERENCES
1. Ghosh SK. Giovanni Battista Morgagni (1682-1771): father of patho-
logic anatomy and pioneer of modern medicine. Anat Sci Int. 2017;92
(3):305-312. https://doi.org/10.1007/s12565-016-0373-7.
2. Toynbee J. The Diseases of the Ear: Their Nature, Diagnosis, and Treat-
ment. Philadelphia, PA: Hathi Trust Digital Library, Blanchard and Lea;
1860.
3. Hawkins JE. Early histopathologic studies of ear disease and deafness.
Ann Otol Rhinol Laryngol. 1989;98(12 suppl):6-10.
4. Hartmann E. Zwei neue Fälle von doppelseitiger Knochen Stape-
sankyllose. Z Ohrenheilkd. 1898;33:103-151.
5. Mudry A. Adam Politzer (1835-1920) and the description of otoscle-
rosis. Otol Neurotol. 2006;27(2):276-281.
6. Hallpike CS, Cairns H. Observations on the pathology of Meniere's
syndrome. J Laryngol Otol. 1938;53:625-655.
7. Yamakawa K. Über die pathologische veränderung bei einem
Meniere-Kranken. J Otorhinolaryngol Soc Jpn. 1938;44:2310-2312.
8. Crowe SJ, Guild SR, Polvogt LM. Observations on the pathology of
high-tone deafness. Bull Johns Hopkins Hosp. 1934;54:315-379.
9. Puchter H, Meloan SN. On the chemistry of formaldehyde fixation
and its effects on immunohistochemical reactions. Histochemistry.
1985;82(3):201-204.
10. Tifford M. A short history of histopathology technique. J Histotechnol.
2006;29(2):99-110.
11. Hill J. The Construction of Timber from Its Early Growth Explained by
the Microscope, and Proved from Experiments. London; 1770.
12. Wissowsky A. Ueber das eosin als reagenz auf hemoglobin und die
Bildung von Blutgefässen und Blutkörperchen bei Säugtier und Hühnerei-
bryonen. Arch für Mikroskopesch. 1876;13:479-496.
13. Wright A. Scanning electron microscopy of the human cochlea—the
organ of Corti. Arch Otorhinolaryngol. 1981;230(1):11-19.
14. Wright A. Scanning electron microscopy of the human organ of Corti.
J Royal Soc Med. 1983;76:269-278.
15. Nadol JB Jr. Serial section reconstruction of the neural poles of hair
cells in the human organ of Corti. I. Inner hair cells. Laryngoscope.
1983;93(5):599-614.
16. Friedman LM, Avraham KB. MicroRNAs and epigenetic regulation in
the mammalian inner ear: implications for deafness. Mamm Genome.
2009;20(9-10):581-603.
17. Hultcrantz M, Li HS. Inner ear morphology in CBA/ca and C57BL/6J
mice in relationship to noise, age and phenotype. Eur Arch Otorhinolar-
yngol. 1993;250(5):257-264.
18. Nadol JB Jr. Serial section reconstruction of the neural poles of hair
cells in the human organ or Corti. II. Outer hair cells. Laryngoscope.
1983;93(6):780-791.
19. Nadol JB Jr, Thornton AR. Ultrastructural findings in a case of
Meniere's disease. Ann Otol Rhinol Laryngol. 1987;96(4):449-454.
20. Liberman MC, Kujawa SG. Cochlear synaptopathy in acquired senso-
rineural hearing loss: manifestations and mechanisms. Hear Res. 2017;
349:138-147.
21. Wu PZ, Liberman LD, Bennett K, deGruttola V, O'Malley JT, Liberman
MC. Primary neural degeneration in the human cochlea: evidence for
hidden hearing loss in the aging ear. Neuroscience. 2019; 407:8-20. https://doi.org/10.1016/j.neuroscience.2018.07.053.
22. Merchant SM, Tsuji K, Wall C, Velasquez-Vila Senor L, Glynn RJ,
Rauch SD. Temporal bone studies of the human peripheral vestibular
system. 1. Normative vestibular hair cell data. Ann Otol Rhinol Laryngol.
2000;109:3-13.
23. Liberman et al (in press).
24. Nadol JR Jr, O'Malley JT, Burgess BJ, Galler D. Cellular immunologic
responses to cochlear implantation in the human. Hear Res. 2014;
318:11-17.
25. O'Malley JT, Burgess BJ, Galler D, Nadol JB Jr. Foreign body response
to silicone in cochlear implant electrodes in the human. Otol Neurotol.
2017;38(7):970-977.
26. Frisina RD, Wheeler HE, Fossa SD, et al. Comprehensive audiometric
analysis of hearing impairment and tinnitus after Cicplatin-based che-
motherapy in survivors of adult-onset cancer. J Clin Oncol. 2016;34
(23):2712-2720.
27. Breglio AM, Rusheen AE, Shide ED, et al. Cisplatin is retained in the
cochlea indefinitely following chemotherapy. Nat Commun. 2017;8:1654.
28. Wackym PA, Simpson TA, Gantz BJ, Smith RJ. Polymerase chain reac-
tion amplification of DNA from archival celloidin-embedded human
temporal bone sections. Laryngoscope. Jun;103(6):583-588;1993.
29. McKenna MJ, Kristiansen AG, Haines J. Polymerase chain reaction
amplification of a measles virus sequence from human temporal bone
sections with active otosclerosis. Am J Otol. 1996;17(6):827-830.
30. Burgess RC, Michaels L, Bale JF Jr, Smith RJ. Polymerase chain reac-
tion amplification of herpes simplex viral DNA from the geniculate
ganglion of a patient with Bell's palsy. Ann Otol Rhinol Laryngol.
1994; 103(10):775-779.
31. Wackym PA. Molecular temporal bone pathology: II. Ramsay hunt syn-
drome (herpes zoster oticus). Laryngoscope. 1997;107(9):1165-1175.
32. Nadol JB Jr, Handzel O, Amr S. Histopathology of the human inner
ear in a patient with sensorineural hearing loss caused by a variant in
DFNA5. Otol Neurotol. 2015;26:1616-1621.
33. Burgess BJ, O'Malley JT, Kamakura T, et al. Histopathology of the
human inner ear in the p1144P COCH mutation (DFNA9). Audiol Neu-
rotool. 2016;21(2):88-97.
34. O'Malley JT, Merchant SN, Burgess BJ, Jones DD, Adams JC. Effects
of fixative and embedding medium on morphology and immuno-
staining of the cochlea. Audiol Neurotol. 2009;14(2):78-87.
35. O'Malley JT, Burgess BJ, Jones DD, Adams JC, Merchant SN. Techni-
quines of celloidin removal from temporal bone sections. Ann Otol
Rhinol Laryngol June;118(6):435-441;2009.
36. Kamakura T, O'Malley JT, Nadol JB Jr. Preservation of cells of the
organ of Corti and innervating dendritic processes following cochlear
implantation in the human: an immunohistochemical study. Otol Neu-
rotool. 2018;39(3):284-293.
37. Santi PA, Johnson SB, Hillenbrand M, Grandpre PZ, Glass TG,
Leger JR. Thin sheet laser imaging microscopy for optical sectioning
of thick tissues. Biotechniques. 2009;46:287-294.
38. Iyer JS, Zhu N, Gasilov S, Ladak HM, Agrawal SK, Stankovic KM. Visu-
alizing the 3D cytoarchitecture of the human cochlea in an intact
temporal bone using synchrotron radiation phase contrast imaging.
Biomed Opt Express. 2018;9(8):3757-3767.

How to cite this article: Nadol Jr. JB. Contemporary
techniques in human otopathology and promise for the future.
Laryngoscope Investigative Otolaryngology. 2020;5:145–151.
https://doi.org/10.1002/lio2.341