



Novel Imaging Methodologies for Improved Study and Diagnosis of Sensorineural Hearing Loss

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Novel Imaging Methodologies for Improved Study and Diagnosis of Sensorineural Hearing Loss

A dissertation presented

by

Janani S. Iyer

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Speech and Hearing Bioscience and Technology

Harvard University

Cambridge, Massachusetts

February 2021

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Novel Imaging Methodologies for Improved Study and Diagnosis of Sensorineural Hearing Loss

Abstract

Sensorineural hearing loss is the most common sensory deficit in the world. Despite its widespread prevalence, diagnostics and therapeutics for hearing loss remain limited in part due to the lack of an effective clinical imaging technology for visualizing biomarkers of sensorineural hearing loss in humans. Results from experiments conducted in animal models of hearing loss and human cadaveric inner ear specimens suggest that this type of hearing loss is characterized by damage to the cochlea – the small, snail-shaped sensory organ that facilitates hearing – and in particular to the cochlea’s sensory epithelium, which comprises a mosaic of sensory cells and nerve fibers that relay acoustic information from our environments to our brains. These findings are yet to be confirmed in living humans, however, due to the human cochlea’s small size, complex three-dimensional configuration, fragility, and deep encasement in dense bone, which render it inaccessible via conventional clinical imaging techniques such as computed tomography and magnetic resonance imaging. Our inability to visualize these sensory structures, and importantly, the specific morphological and physiological transformations that induce hearing impairment, has precluded personalized and meaningful diagnosis of sensorineural hearing loss in patients, and has slowed therapy development. The present dissertation introduces two novel imaging technologies that are promising for clinical translation and inner ear applications, and demonstrates their efficacy for visualizing the micron-scale structures that are implicated in sensorineural hearing loss in humans. The first, synchrotron radiation phase contrast imaging, leverages the penetration power and resolving capability of synchrotron-facilitated X-ray imaging to enable cellular-level imaging of the cochlea’s interior through its dense bony shell *in situ*, without contrast enhancement or bone decalcification. The second, micro-optical coherence tomography, is a high-resolution, minimally-invasive interferometric imaging technique that can be interfaced with sub-millimeter-diameter endoscopes to access the cochlea’s internal lumina for micron-scale imaging. Together, the results presented herein motivate further investigation into the utility of high-resolution X-ray- and optical coherence tomography-

based imaging techniques for improving both the study and clinical diagnosis of sensorineural hearing loss in humans.

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CHAPTER 1. INTRODUCTION

The peripheral auditory system is extraordinary in its ability to collect, transform, and relay acoustic information from our environments to our brains. It accomplishes these feats by converting sound pressure waves, through a series of transformations, into electrical signals that are decoded by the auditory nerve and interpreted by the brain. When a sound pressure wave enters the external auditory canal (**Figure 1.1**), it is amplified and funneled toward the tympanic membrane. The tympanic membrane vibrates in response, and its intricate pattern of vibrations is then transmitted through the small middle ear ossicles – first the malleus and the incus, and finally the stapes, which thumps in accordance with this pattern of vibrations on the oval window of the fluid-filled *cochlea* – the bone-encased, snail-shaped auditory sensory organ (**Figure 1.2**). The cochlea is only a centimeter across its base, 5-7 centimeters from base to apex, and 35 millimeters in length¹, but what it lacks in stature it makes up for in significance. When the stapes vibrates against the cochlea's oval window, its movement displaces the cochlea's internal fluids, sending a traveling wave through the basilar membrane, which separates two of the cochlea's three fluid-filled chambers, and that is maximally deflected at different locations along its length in correspondence with the incoming sound's frequency. Sitting atop the basilar membrane is the *organ of Corti* – a tessellation of auditory nerve fibers and sensory hair cells, the latter of which, when displaced with the basilar membrane in response to an incoming sound, exchange ions with the fluids that bathe them, facilitating mechanoelectrical transduction. The resulting electrical signals are relayed via nerve fibers to the auditory nerve, and ultimately up the ascending auditory pathway to the brain. The organ of Corti's constituent cells and nerve fibers are arranged tonotopically, much like the keys on a piano, and respond uniquely to specific frequency inputs. Structures at the cochlea's base respond to higher frequencies, and structures at its apex responding to lower frequencies.²

A malfunction in this series of precisely orchestrated events results in either or both of two categories of hearing loss. *Conductive* hearing losses are characterized by damage to the outer and/or middle ear portions of the peripheral auditory system (**Figure 1.1**). These types of hearing losses can result from a condition as simple as a build-up of cerumen in the auditory canal, to as complex as tympanic membrane rupture or ossicular fracture or fusion. Conductive hearing losses are typically easily treated in the clinic, via, for example, cleaning, fluid drainage, or surgery. The patient's hearing is typically mostly or entirely restored post-procedure.

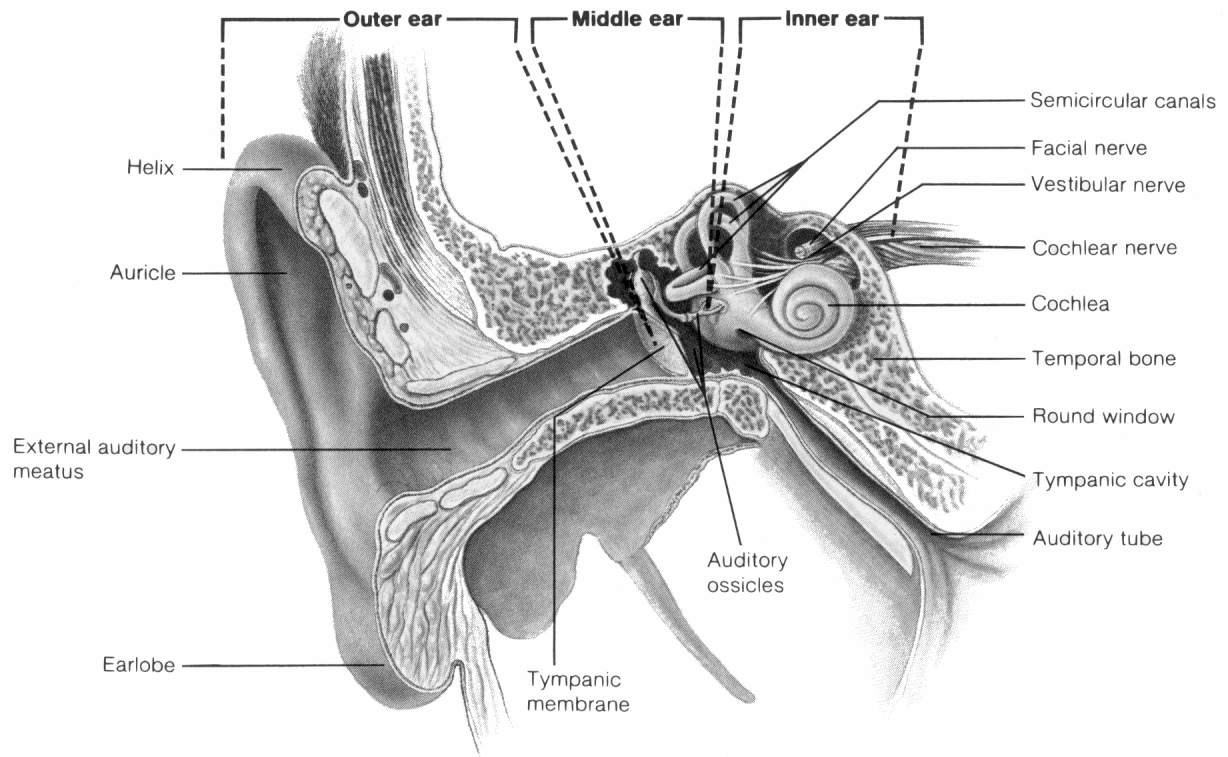


Figure 1.1. Schematic of the peripheral auditory system, showing the outer, middle, and inner ear structural components.³

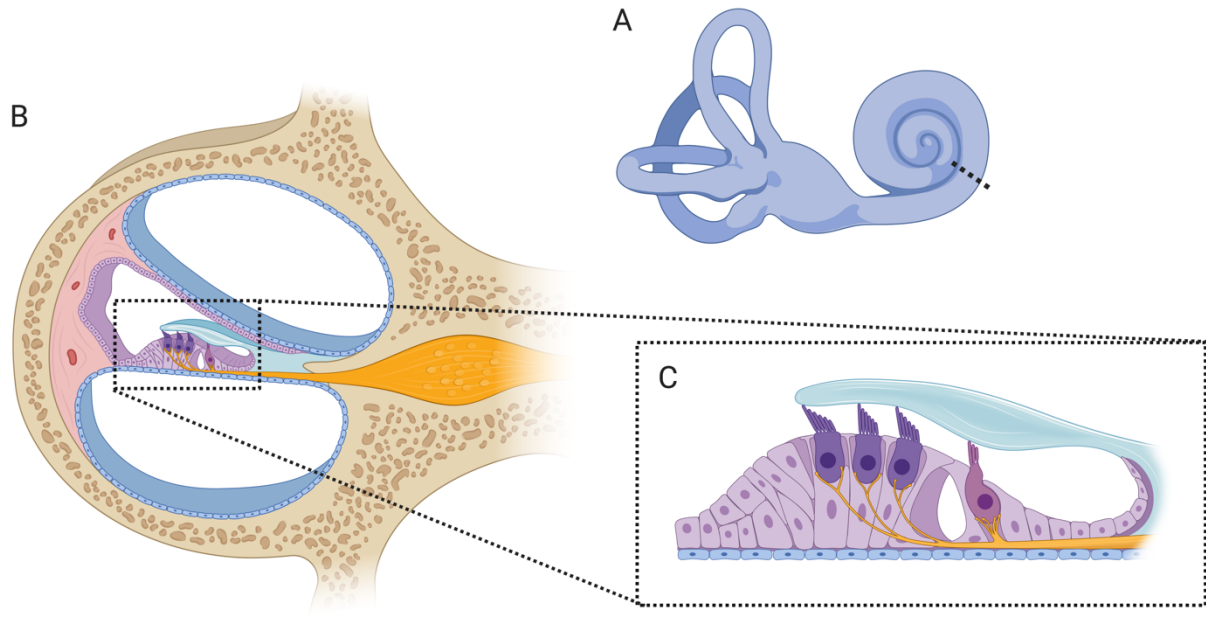


Figure 1.2. (A) Schematic of the human cochlea. Dashed line indicates cross-section along which the drawings in panels B and C are depicted. (B) Schematic of a mid-modiolar cross-section through the cochlea, showing the location and orientation of the organ of Corti. (C) Zoomed-in schematic of the boxed region in panel C, showing the sensory and supporting cells and auditory nerve fibers that comprise the organ of Corti.⁴

By contrast, *sensorineural* hearing losses (SNHLs) result from damage to the cochlea. SNHL is typically caused by aging, overexposure to noise or ototoxic drugs, or genetic mutation. Studies conducted in animal models and cadaveric human temporal bone specimens suggest that SNHL can be characterized by damage to sensory and supporting cells and auditory nerve fibers comprising the organ of Corti. Despite over a century of auditory research and groundbreaking advances in biotechnology, SNHL remains incurable, irreversible, and extremely challenging to treat. Indeed, treatments for hearing loss today are limited to hearing aids and cochlear implants, neither of which comes close to replicating the cochlea's dynamic range or resolving capability.

Treatments for SNHL remain limited in part due to the fact that diagnostic tests for hearing loss are incapable of elucidating the pathology underlying hearing loss in humans. The gold standard test for evaluating human hearing ability remains pure tone audiometry – a metric that reports the level at which a tone of a particular frequency needs to be played in order for the patient to hear it, for a relevant range of frequencies. Pure tone audiometry remains the standard for assessing hearing ability despite compelling evidence that this metric is subjective, inconsistent within and across patients, unreliable in certain patient populations, and insensitive to certain critical underlying pathologies that are now known to disrupt hearing long before they are detectable via audiometry.^{5,6,7} There is thus a clear clinical need for an objective diagnostic tool that enables an audiologist or otologist to identify the specific source of a patient's hearing loss, and ultimately relate a patient's experience of hearing loss to the corresponding underlying pathology. Such a tool would allow us to establish biomarkers of human SNHL, which would not only enable personalized and accurate diagnosis and therapy recommendation for patients, but would also dramatically increase the rate at which novel therapeutics for SNHL are developed and tested in humans.

Historically, the advent of novel imaging technologies that are safe for use in humans has revolutionized the way that diseases are diagnosed and treated. The discovery of X-rays in the late 1800s revolutionized the screening, diagnosis, and monitoring of disease, (especially those involving skeletal fracture or breakage), and its predecessor, computed tomography, is now routinely used in the clinic to render entire organs in high resolution and in three dimensions. Ultrasound, first used clinically in the mid-1900s, continues to be the gold standard for monitoring pregnancies, and magnetic resonance imaging,

first conducted in a live human in 1977, is the current standard of care for diagnosing and monitoring soft tissue lesions such as those that occur in the brain. More recently, optical coherence tomography, invented in the early 1990s, has dramatically improved diagnostics in ophthalmology and dermatology, and in its endoscopic iterations is becoming more common in gastroenterological and cardiovascular applications. These imaging techniques rely on inherently different mechanisms for image acquisition, but share their ability to facilitate meaningful visualization of remote regions of the body's interior.

Such a clinical imaging tool, if capable of accessing the cochlea's interior, would similarly revolutionize diagnostics in otolaryngology. It would allow physicians and researchers to not only directly relate perceived hearing impairment to its specific biological origin in humans for the first time ever, but it would also allow direct visualization of the effects of newly developed drugs and gene therapies for hearing loss on intracochlear morphology in patients. However, the human cochlea has historically evaded attempts at clinical imaging due to its small size, fragility, complex 3D configuration, and deep encasement within the dense petrous temporal bone. Indeed, even high-resolution computed tomography (CT) and 11.5T magnetic resonance imaging (MRI) are incapable of penetrating through the cochlea's dense otic capsule and providing the micron-scale resolution that is required for visualizing the organ of Corti's cytoarchitecture, limiting their diagnostic utility to identification of gross anatomical defects in the cochlea's exterior.^{8,9} Importantly, the vast majority of SNHL is caused by damage to the cochlea's delicate internal structures, rather than its bony exterior. CT and MRI also often require contrast enhancement via iodine or gadolinium injection respectively to achieve high contrast in soft tissue^{9,10}, adding a layer of potential complication to these diagnostic procedures.

Thus, alternative and/or improved classes of clinical imaging technologies are necessary for providing safe, meaningful, and objective diagnosis of SNHL in patients. To this end, the present dissertation demonstrates the utility of two novel imaging techniques that are promising for applications in clinical otolaryngology. **Chapter 2** introduces synchrotron radiation phase contrast imaging, an X-ray-based imaging method that leverages the capability of synchrotron particle accelerators to generate high energy X-rays for penetration through dense bone and resolution of delicate soft tissue structures. **Chapter 3** introduces micro-optical coherence tomography, a high-resolution predecessor to standard optical coherence tomography that offers not only improved resolution but also increased depth of focus.

Chapter 4 describes a technique for interfacing flexible, miniature endoscopes with a micro-optical coherence tomography imaging system for future intracochlear investigation in human patients. The dissertation concludes with implications of and proposed future directions of the enclosed work (**Chapter 5**).

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**CHAPTER 2: Visualizing the 3D cytoarchitecture of the human cochlea in an intact temporal bone
using synchrotron radiation phase contrast imaging**

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Visualizing the 3D Cytoarchitecture of the Human Cochlea in an Intact Temporal Bone Using Synchrotron Radiation Phase Contrast Imaging

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Abstract

The gold standard method for visualizing the pathologies underlying human sensorineural hearing loss has remained post-mortem histology for over 125 years, despite awareness that histological preparation induces severe artifacts in biological tissue. Historically, the transition from post-mortem assessment to clinical evaluation in living humans through non-invasive biomedical imaging has revolutionized diagnosis and treatment of disease; however, innovation in non-invasive techniques for

cellular-level intracochlear imaging in humans has been difficult due to the cochlea's micron-scale small size, complex 3D configuration, fragility, and deep encasement within bone. Here we investigate the ability of synchrotron radiation-facilitated X-ray absorption and phase contrast imaging to enable visualization of sensory cells and nerve fibers in the cochlea's sensory epithelium *in situ* in 3D intact, non-decalcified, unstained human temporal bones. Our findings show that this imaging technique resolves the bone-encased sensory epithelium's cytoarchitecture with unprecedented levels of cellular detail, and is capable of distinguishing between healthy and damaged epithelium at the cellular level. These data suggest that synchrotron radiation phase contrast imaging has the potential to replace histology as a gold standard for evaluating intracochlear structural integrity, and motivate further optimization for translation to the clinic.

Introduction

Sensorineural hearing loss is the most common sensory deficit in the world¹, but the mechanisms underlying the progression of SNHL in the human inner ear remain unknown and there is no cure. This void in our understanding of the human peripheral auditory system exists in part due to the lack of a method enabling *in vivo* or even *in situ* visualization of the delicate mosaic of micron-scale cells and auditory nerve fibers that facilitate hearing from within the cochlea: the tiny, snail-shaped organ that lies deeply nestled within the densest bone in the human body, the petrous temporal bone's otic capsule (**Figure 2.1**). Indeed, the cochlea's small size, fragility, complex 3D configuration, and deep encasement within bone have precluded visualization of its interior at cellular-level resolution in the intact human temporal for the entirety of the auditory research field's existence.

Methods for imaging the cochlea in living humans today are limited to computed tomography (CT) and magnetic resonance imaging (MRI). Standard clinical CT and MRI systems typically afford resolution capabilities in the 0.5mm range^{2,3} – over two orders of magnitude lower than what is necessary for resolving diagnostically-relevant detail in the cochlea's sensory epithelium (the organ of Corti). Thus, CT and MRI are used primarily to detect gross anatomical anomalies in the cochlea (e.g. abnormally small or deformed cochlear bone or missing auditory nerve)^{4,5}. More advanced clinical CT (e.g. cone beam, multislice, flat-panel volume) and MRI (histology, 3D fluid attenuation inversion recovery, and constructive

interference in steady state) systems resolve down to 70⁶ and 300 microns⁷, respectively; these systems afford better visualization of the radius of curvature and inner diameter of the cochlear lumen as it spirals toward its apex, and are thus useful for surgical planning and assessing depth and quality of cochlear implant electrode array insertion. However, the resolving power and penetration depth afforded are still insufficient for visualizing the single-micron scale cells and auditory nerve fibers that are known from studies in animal models to be heavily implicated in SNHL. Importantly, achieving even the aforementioned extent of visualization often requires injecting the patient intravenously with contrast-enhancing agents (e.g. iodine for CT, gadolinium for MRI).



Figure 2.1. Micro-CT image of the human head, virtually sectioned through the temporal bone to reveal the outer, middle, and inner ear portions of the peripheral auditory system. The cochlea (•)'s spiral shape and deep encasement within bone are appreciated. * = auricle (outer ear); ■ = malleus, first of the three middle ear ossicles. Scale = 2.5cm.

Due to the ironically snail-like pace at which advances in imaging techniques for noninvasive human intracochlear evaluation have occurred, the gold standard method for assessing the structural integrity of the human organ of Corti and its association with hearing ability has remained *post-mortem* temporal bone histology for over 125 years⁸ (**Fig. 2.2A**). This technique involves harvesting the temporal bone at patient autopsy, and subsequently fixing, decalcifying, dehydrating, manually sectioning, staining, and mounting the specimens on glass slides for light microscopic evaluation⁹. This method is crude for several reasons: **1)** it introduces artifacts to the tissue at every stage in processing, limiting the evaluator's ability to distinguish between tissue damaged due to histological preparation versus pathology¹⁰⁻¹²; **2)** it is extremely laborious and time-consuming, requiring months to years of preparation before analysis is possible; and **3)** it reduces the level of study possible to what is visible along a single orientation plane (3D reconstruction of micro-dissected, stained temporal bone sections has been attempted in the past, but is also extremely labor intensive, and requires expensive equipment^{13,14}). These issues are compounded by profound recent evidence from re-analyses of hematoxylin and eosin (H&E)-stained cochlear sections from several patients suggesting that this method of cochlear analysis, which has dominated the field since its nascence, has resulted in significant underestimation of the amount of cellular damage and has thus led to inaccurate conclusions regarding associations between extent and location of intracochlear pathology and hearing ability in the corresponding patient¹⁵. Thus, there is an enormous unmet need for a non-destructive technique that allows clinicians and researchers to visualize the structural integrity of the organ of Corti at the cellular level and establish accurate correlations between location and degree of damage and cochlear function; such a method would revolutionize the way we diagnose, treat, and study SNHL.

Recent innovations in X-ray imaging promise to yield new methods for intracochlear diagnostic evaluation that would address the aforementioned need. The cochlea has historically resisted X-ray-based techniques such as CT (**Fig. 2.2B**) and its higher resolution successor, micro-computed tomography (μ CT; **Fig. 2.2C**), because of the highly X-ray-absorbent petrous bone encasing its interior. However, recent harnessing of synchrotron radiation (SR), which provides coherent, collimated X-rays with high photon flux and submicron source stability, has enabled significantly deeper penetration through bone and thus ultra-high resolution imaging of the organ of Corti¹⁶⁻²¹. The recent integration of phase

contrast imaging (PCI) with SR-facilitated X-ray imaging enables enhanced soft tissue contrast in tandem with strong absorption contrast in bone by **a)** transforming the sample-induced phase shift into detectable variations in X-ray intensity, and **b)** enhancing contrast at boundaries between individual structures within the field of view. Studies implementing synchrotron radiation phase contrast imaging (SR-PCI) have demonstrated this technique's ability to reveal the organ of Corti's cytoarchitecture *in situ* in unprecedented levels of detail; structures such as the basilar membrane, spiral ligament and stria vascularis, spiral ganglion cell bodies and nerve fibers, and Reissner's membrane have been resolved despite being imaged *in situ* in the temporal bone (**Fig. 2.2D**)¹⁶⁻²¹.

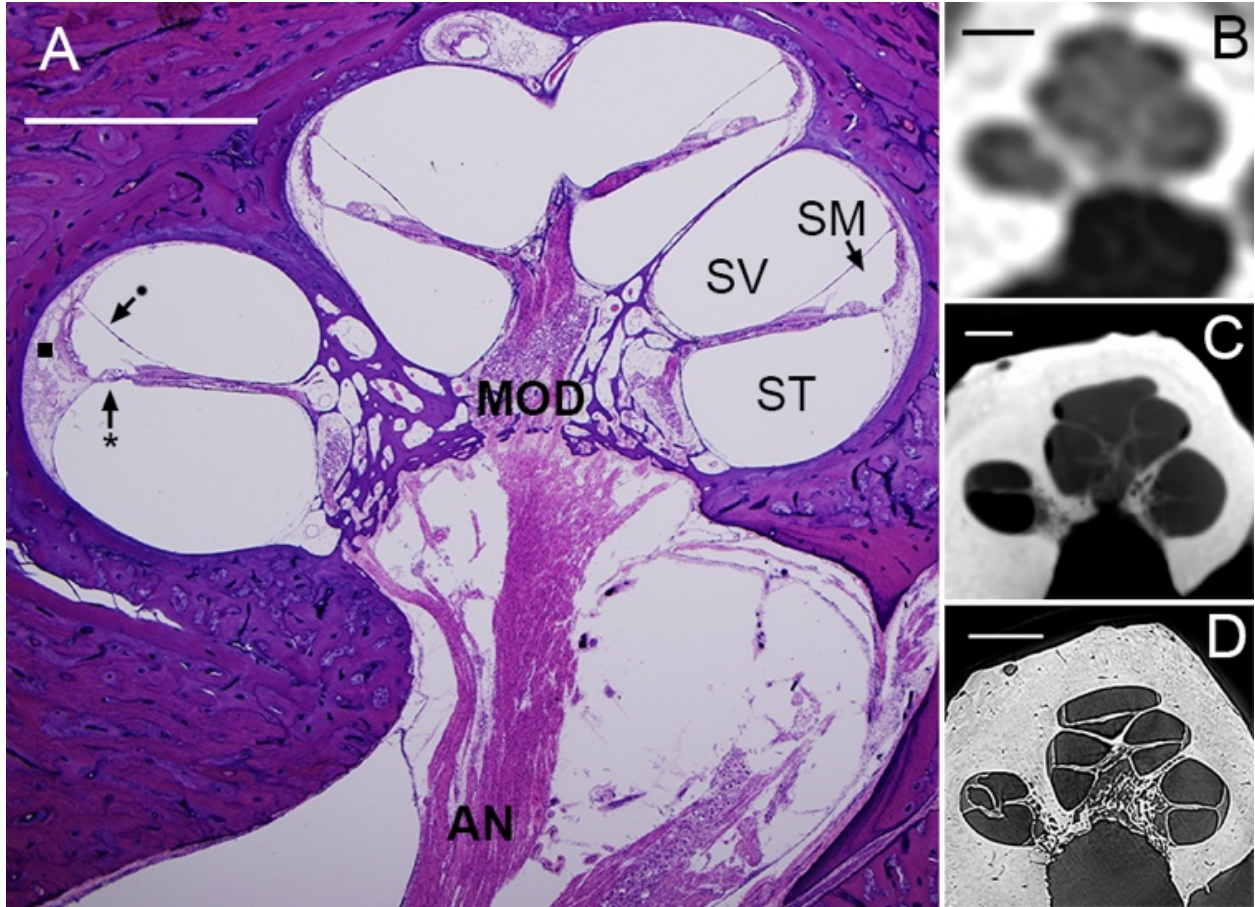


Fig. 2.2. (A) Mid-modiolar section of an H&E-stained human cochlea. Five cochlear half-turns, corresponding to the $2\frac{3}{4}$ cochlear turns in the human cochlea, are seen spiraling around the modiolus (MOD), the bony trunk that contains spiral ganglion neuronal cell bodies and axons traveling to and from the auditory nerve (AN). The basilar membrane (*) and Reissner's membrane (•) define the boundaries between the cochlea's three fluid-filled lumina: scala tympani (ST), scala media (SM), and scala vestibuli (SV). The lateral wall (■) attaches the basilar membrane to the cochlea's osseous shell, the otic capsule. Standard CT (B), μ CT (C), and SR-PCI (D) images of virtual mid-modiolar section through a human cochlea in situ are also shown. The differences in visualizability of intracochlear structures across the three X-ray-based imaging modalities are dramatic. All scales = 2mm.

While these studies are promising, most have relied on sample decalcification and staining to significantly enhance contrast¹⁸⁻²¹, and many of the experiments were performed in animal cochleae^{20,21}. An impressive amount of structural detail has been previously resolved in samples that were fixed and dehydrated only^{16,17}, but these data were not prepared or presented in ways that facilitate easy detection of intracochlear sensorineural pathologies or comparison with histology^{16-18, 20, 21}.

Here, we demonstrate the power of SR-PCI combined with a novel image analysis technique to provide profoundly detailed 3D images of the non-decalcified, unstained human cochlea's interior *in situ*. Our objectives were to demonstrate **a)** SR-PCI's ability to distinguish between damaged and healthy organ of Corti tissue, and **b)** the minimal effect of sample dehydration on image quality. These data demonstrate SR-PCI's potential to accelerate the discovery of the microstructural basis of human deafness by enabling rapid, thorough assessment of intact, non-decalcified, unstained human temporal bone specimens.

Materials and methods

Specimen preparation

Human temporal bones were harvested at autopsy from patient donors. Post-extraction, specimens were processed for imaging in one of two ways:

- 1) A fresh specimen was fixed in 10% formalin for 2 weeks and the surrounding petrous bone was drilled to expose the round and oval windows. Post-fixation, a soft tube of roughly the same flexibility as a cochlear implant electrode array (outer diameter = 580µm) was repeatedly inserted into scala tympani to a depth of roughly 10mm to mimic insertion trauma.
- 2) Three fresh-frozen specimens were cylindrically trimmed to a diameter of 40mm and length of 60mm around the cochlea, and were fixed in a 4F1G (3.7% formaldehyde + 1% glutaraldehyde in phosphate buffer) bath for 5 days. The samples were rinsed twice in double distilled water, and subsequently dehydrated according to an eight-stage ethanol series (50%, 60%, 70%, 80%, 90%, 95%, 100%, and 100%), where in each stage the samples were submerged in 300mL of solution for 30 minutes.

No additional processing (e.g. decalcification, staining, sectioning) was performed on any specimens. The Massachusetts Eye and Ear Human Studies Committee and Western University permitted use of these specimens for research purposes.

SR-PCI image acquisition

The SR-PCI technique implemented in these experiments (SR combined with propagation-based PCI) has been previously described^{16,17}.

In brief, specimens were scanned using the Biomedical Imaging and Therapy (BMIT) 05ID-2 beamline at the Canadian Light Source Inc. in Saskatoon, SK, Canada. The SR beam is produced by a superconducting wiggler source²² and is filtered using a monochromator, yielding an energy bandwidth of $\Delta E/E \approx 10^{-3}$ over a 30-150 keV range. Samples were imaged at 40 keV; the photon flux delivered to the imaging station at this energy is about 5×10^{10} phot/sec/mm². The imaging station, located 58 meters from the source, comprises a motorized stage and an X-ray detector that consists of a 10 μ m-thick Gadox scintillator screen and a tandem objective system that relays a luminescence image to a charge-coupled device camera (C9300-124, Hamamatsu Photonics, Shizuoka, Japan) without magnification. The imaging field of view is 36×8.6 mm² and effective pixel size is 9×9 μ m². The distance between the sample and detector is 2 meters.

For high-resolution tomography, 3000 angular projections were acquired over 180° of sample rotation. Acquisition time per “view” was roughly 30 minutes; multiple views were acquired if the region of interest exceeded the vertical size of the X-ray beam (8.6 mm).

Experimental setup and image analysis

During SR-PCI, specimens were firmly fixed to the motorized sample stage in the imaging station. The acquisition field of view was optimized for imaging the cochlea within the temporal bone through fine adjustment of the sample’s position in X, Y, and Z directions.

SR-PCI images were reconstructed in NRecon (Bruker MicroCT, Kontich, Belgium) and the resulting 2D grayscale images were converted into tagged image file format (.tif). When multiple views were acquired for a single specimen, 2D images were stitched together along the z-axis using Fiji²³. 2D

image stacks were then imported into OsiriX (Pixmeo SARL, Bernex, Switzerland), a software program routinely used by medical practitioners to visualize clinical CT and MRI images. Multiplanar reconstruction and volumetric rendering were performed in OsiriX to visualize the SR-PCI images in 3D, generate maximum intensity projections, rotate, enlarge, and crop the images, set image opacity, and generate scale bar measurements. Image brightness and contrast were adjusted and schematics were drawn in Adobe Photoshop (Adobe, Inc., San Jose, CA). 3D volumetric SR-PCI “fly-through” videos (10 frames per second) were rendered using an endoscopy perspective in OsiriX.

Results and Discussion

Virtual sectioning of the 3D intact human temporal bone reveals micron-scale cytoarchitecture within the cochlea in unprecedented levels of detail (**Fig. 2.3**). Mid-modiolar virtual sections through dehydrated (**Fig. 2.3A**) and non-dehydrated (**Fig. 2.3B**) specimens are tilted in 3D toward the viewer to reveal both surface and cross-sectional views of the organ of Corti in a single image. The three rows of outer hair cells and single row of inner hair cells are clearly visualized in their fan-shaped pattern as they spiral toward the cochlear apex, as are the region of supporting cells and the spiral limbus. Scala tympani, the intracochlear lumen through which electrode arrays are inserted during cochlear implantation, is well-defined. The three trunks of the vestibulocochlear nerve, individual spiral ganglion nerve fiber bundles, and branches of the common cochlear blood vessels and labyrinthine artery are striking in the basal region of the modiolus (**Fig. 2.3A**, **Fig. 2.4**, [Visualization 1](#)); spiral ganglion nerve fibers are also seen traveling through the osseous spiral lamina to where they synapse at inner and outer hair cells (**Fig. 2.3A**). Rosenthal’s canal, the tunnel that contains spiral ganglion neuron cell bodies, is also visualized.

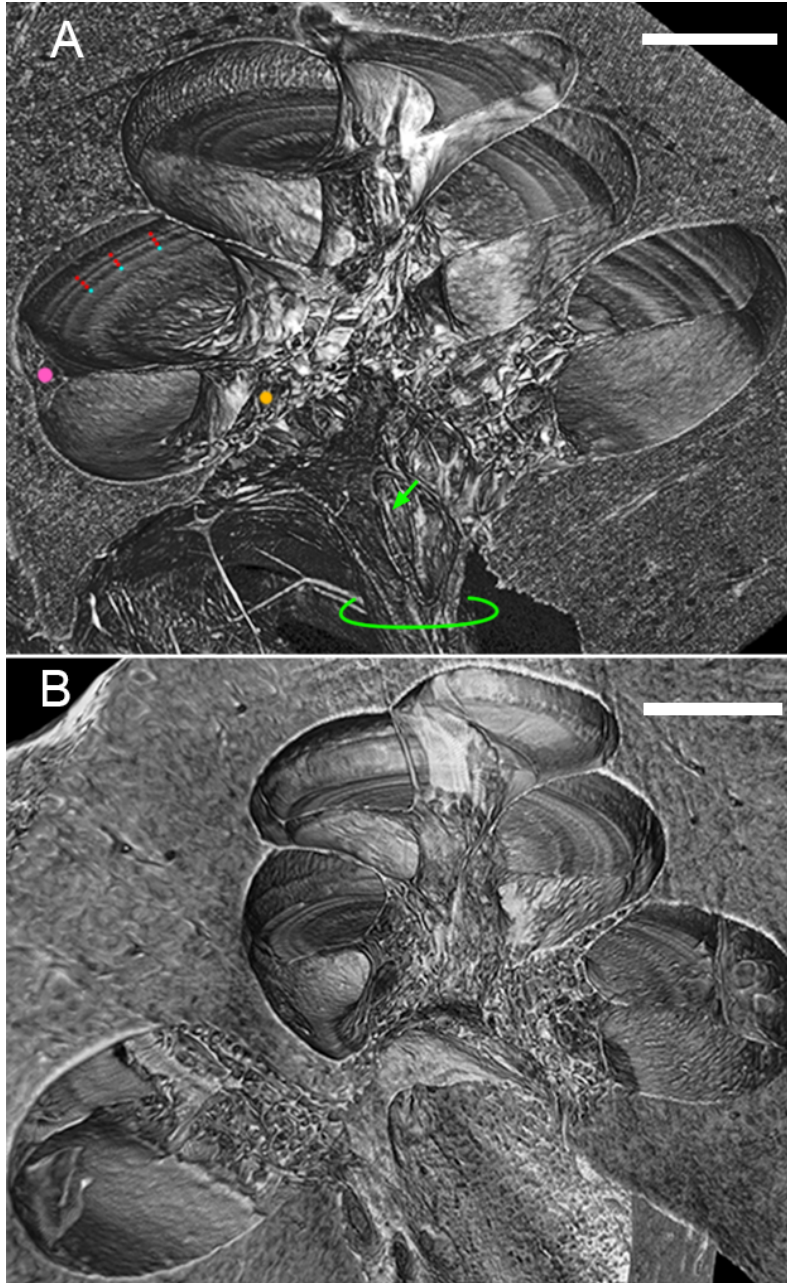


Fig. 2.3. (A) Mid-modiolar 3D virtual cross-section through the dehydrated human cochlea. Three rows of outer hair cells (red dots), row of inner hair cells (blue dots), region of the spiral ligament and stria vascularis (pink dot), and Rosenthal's canal (orange dot) are clearly visualized. Individual bundles of spiral ganglion neuronal fibers (green arrow) and the auditory nerve trunk (green outline) are also

visualized. **(B)** Mid-modiolar 3D virtual cross-section through the non-dehydrated human cochlea, illustrating that similar visualization is achieved through dehydration and fixation without dehydration. Both scales = 1mm.

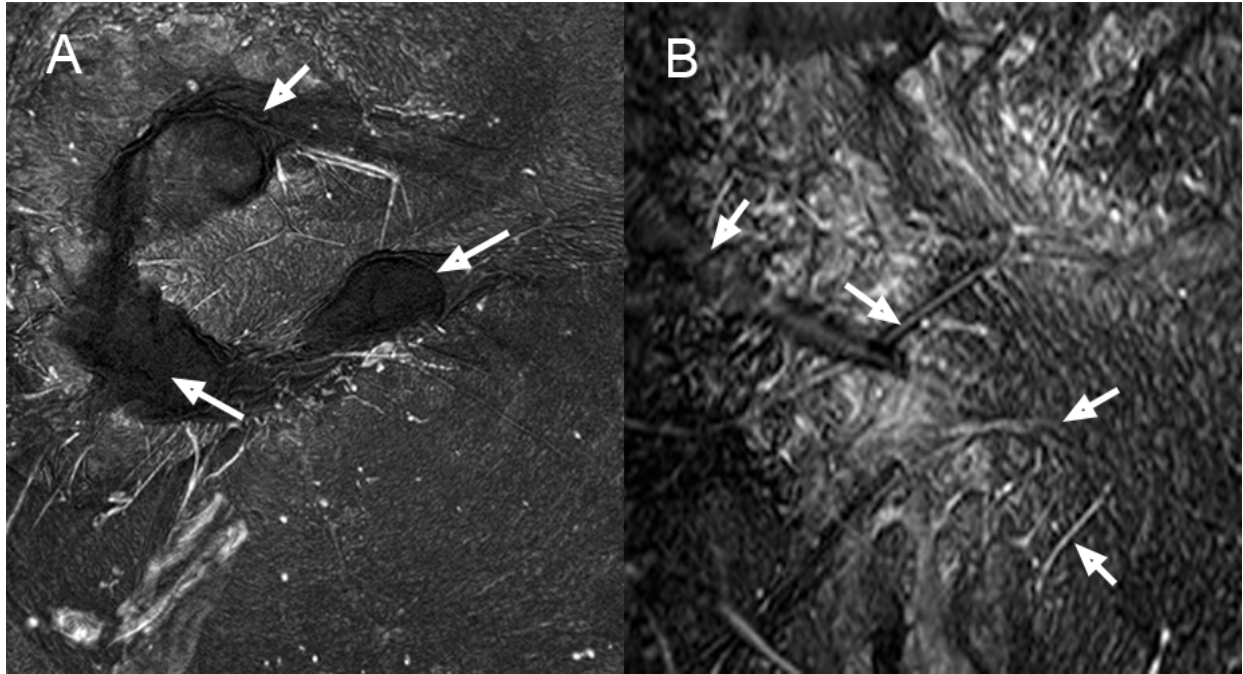


Fig. 2.4. Still images corresponding to two sequential time points in a virtual endoscopic video of the internal auditory meatus ([Visualization 1](#)), highlighting the three trunks of the vestibulocochlear nerve (**A**; white arrows), and bundles of nerve fiber axons and branches of the common cochlear vessels and labyrinthine artery (**B**; white arrows).

When comparing visualization quality in dehydrated (**Fig. 2.3A**) versus non-dehydrated (**Fig. 2.3B**) temporal bone specimens, it is evident that dehydration yields slightly better results; this is consistent with previous work demonstrating that dehydration improves soft-tissue contrast in absorption-based μ CT imaging¹⁸. However, regions, and in some cases individual structures, of relevance to SNHL are still visualized in the non-dehydrated specimen, motivating further optimization of SR-PCI imaging parameters to allow for maintenance of as much specimen purity as possible.

Virtual whole mounts providing a clear surface view of the organ of Corti were prepared by sectioning along a plane parallel to the organ of Corti (**Fig. 2.5**). The full cytoarchitecture of the organ of Corti is visualized in this virtual cross-section, from the modiolar core to the region of outer supporting cells and the lateral wall. This viewing orientation in combination with the amount of visible cellular-level detail is impossible to achieve even through 3D reconstruction of histological micro-sections of the human temporal bone, as slice thickness limits resolution in the z-dimension¹³. These data support our method as the first, to our knowledge, to facilitate cellular-level visualization of the cochlea along an infinite number of cross-sectional planes in the unstained, non-decalcified, 3D intact human temporal bone.

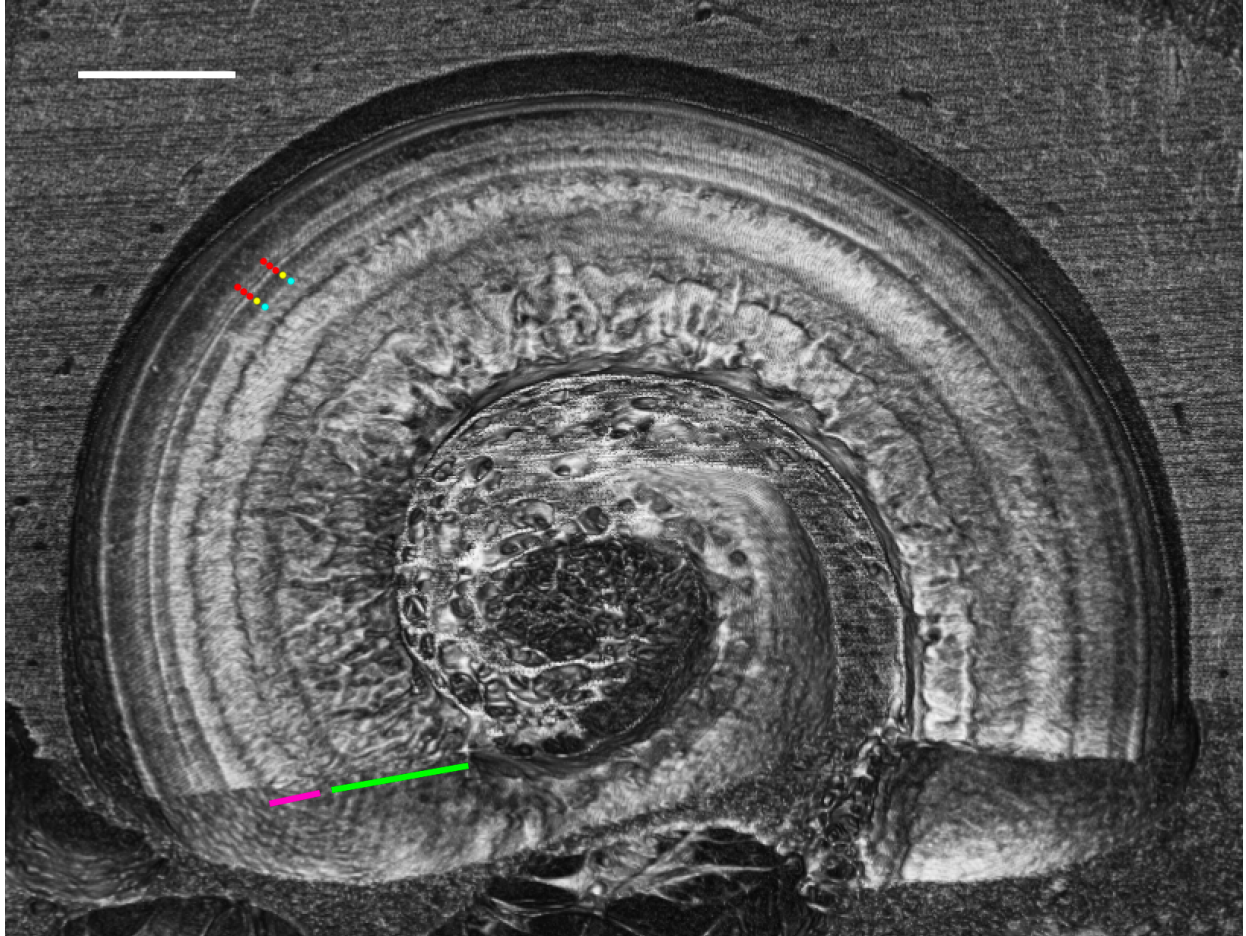


Fig. 2.5. 3D virtual whole mount section revealing a surface view of the organ of Corti in situ in the temporal bone using SR-PCI. The complex cytoarchitecture of the organ of Corti, including the three rows of outer hair cells (red dots), single row of inner pillar cells (yellow dot), single row of inner hair cells (blue dot), spiral limbus (pink underline), and fan of auditory nerve fiber bundles (green underline) is clearly visualized. Scale = 1mm.

Different organ of Corti regions were virtually sectioned to illustrate the degrees and types of pathology that SR-PCI is capable of detecting (**Fig. 2.6**). **Figure 2.6A** shows healthy organ of Corti tissue, virtually sectioned from the upper basal-to-middle turn; as in previous figures, rows of hair cells and supporting cells and nerve fiber bundles are clearly visualized.

Figure 2.6B shows the hook region of one cochlear specimen, where damage manifests as a dark region covering what was measured to be a length of roughly 3.5mm. The entire length of the organ of Corti was measured in this specimen and a modified Greenwood function^{24,25} was applied to estimate the frequency range corresponding to the region of damage. The damaged band spans frequencies to roughly the 13kHz region of the cochlea. Given the natural progression of cellular damage visible in the tissue morphology, the elderly age of the temporal bone donor, and the typical clinical presentation of age-related hearing loss, or presbycusis, we hypothesize that this damage is characteristic of an early stage of presbycusis. **Figure 2.6C** shows the hook region from the specimen that underwent simulated insertion trauma. Complete perforation and severe tissue damage in the basilar membrane and lateral wall were seen in the SR-PCI scan of this specimen (**Fig. 2.6C**). **Figure 2.6** demonstrates SR-PCI's ability to distinguish across varying degrees of tissue health/damage at the cellular level in the 3D intact human temporal bone.

An endoscopic "fly-through" video was created to enable visualization of the cochlea's interior from within the cochlea and in 3D (**Fig. 2.7, Visualization 2**). This method of visualization motivates the development of instructional tools that help the viewer fully understand and appreciate the cochlea's complex 3D geometry and internal cellular structure.

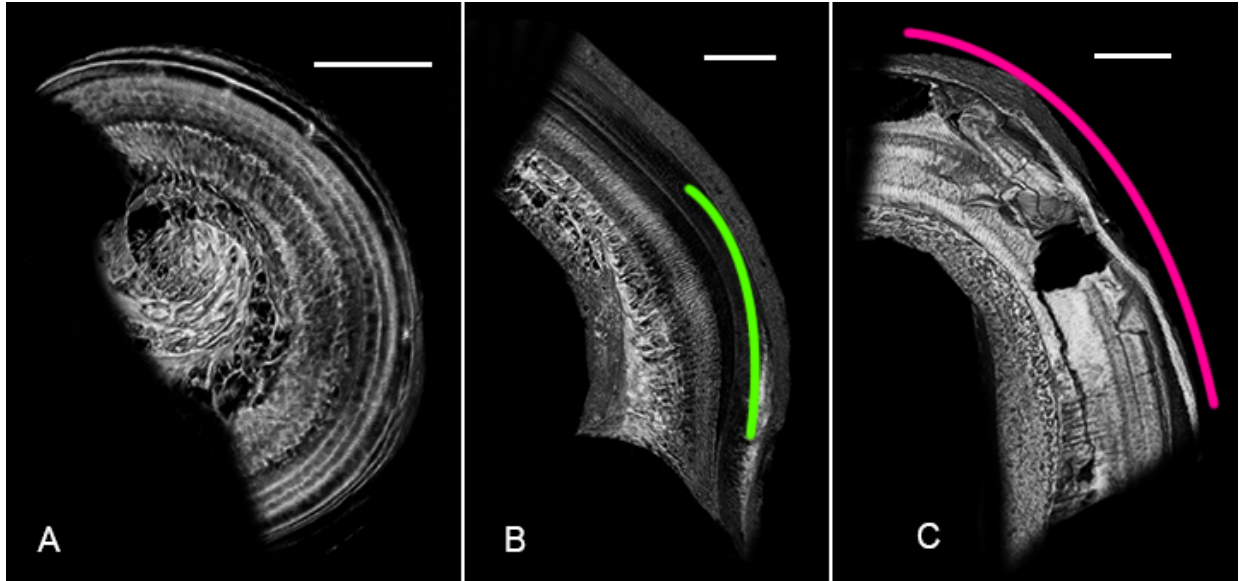


Fig. 2.6. Virtual whole mounts of three regions of the human organ of Corti, illustrating SR-PCI's ability to distinguish between healthy (**A**) and damaged (**B, C**) tissue. (**A**) SR-PCI virtual whole mount sectioned from the upper basal-to-middle turn; individual rows of hair cells and the fan of auditory nerve fibers are clearly visible. (**B**) SR-PCI virtual whole mount sectioned from the base; hypothesized region of presbycusis is outlined in green. (**C**) SR-PCI virtual whole mount sectioned from the base of a cochlear specimen which had undergone simulated insertion trauma (pink outline). All scales = 1mm.

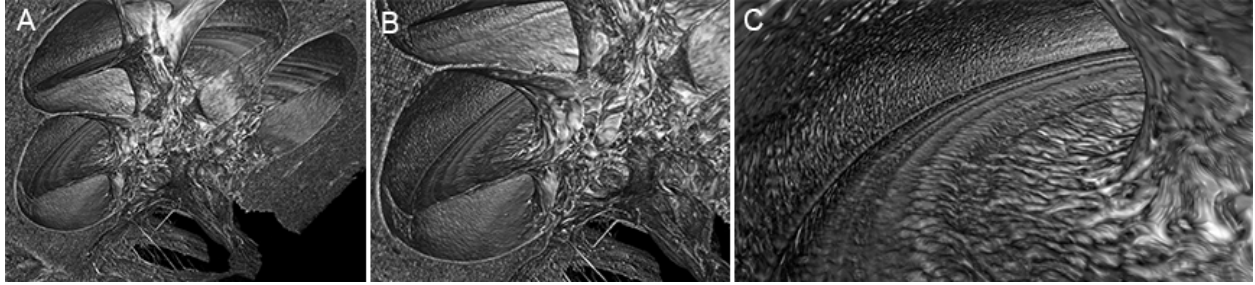


Fig. 2.7. Still images acquired at three different time points (A, B, C) in an endoscopic “fly-through” video ([Visualization 2](#)) allowing the viewer to fly into scala vestibuli to view the cochlea’s interior in 3D from within.

In one specimen, the entire inner ear including vestibular (balance) organs and the cochlea was imaged across three different views in order to assess SR-PCI's ability to resolve the vestibular system's sensory epithelia. **Figure 2.8** shows a 3D volumetric reconstruction of the region of the temporal bone containing the vestibular organs; the stack was virtually sectioned along several planes to show cross-sections through multiple structures at once, including the ampullae of the superior and posterior semicircular canals and their internal cristae ampullaris, and the utricular macula. The superior semicircular canal (SSC) is also striking in this orientation (**Fig. 2.8C**), and a corresponding fly-through video ([Visualization 3](#)) highlights the location and orientation of the SSC and three proximal sensory epithelia. Cellular-level structural detail was not visible in the vestibular epithelia; we hypothesize that this is because **a)** the cells here are surrounded by a gelatinous cupula that may have affected X-ray penetration to these cells, **b)** the sensory epithelium itself is significantly more uniform in its cellular organization than the cochlea's organ of Corti, and thus phase contrast-mediated edge enhancement effects were not as strong for these structures, or **c)** the epithelium had simply deteriorated over time or through fixation. The discovery that SR-PCI resolves the human vestibular system's sensory epithelia *in situ* and without any specimen manipulation aside from fixation unlocks doors to entire fields of study that are not possible to explore with today's histological methods. Falls are the leading cause of death in the elderly and are often caused by vestibular dysfunction, yet extremely little is known about the changes in vestibular organ morphology that are associated with symptoms of the variety of vestibular disorders that people commonly experience.

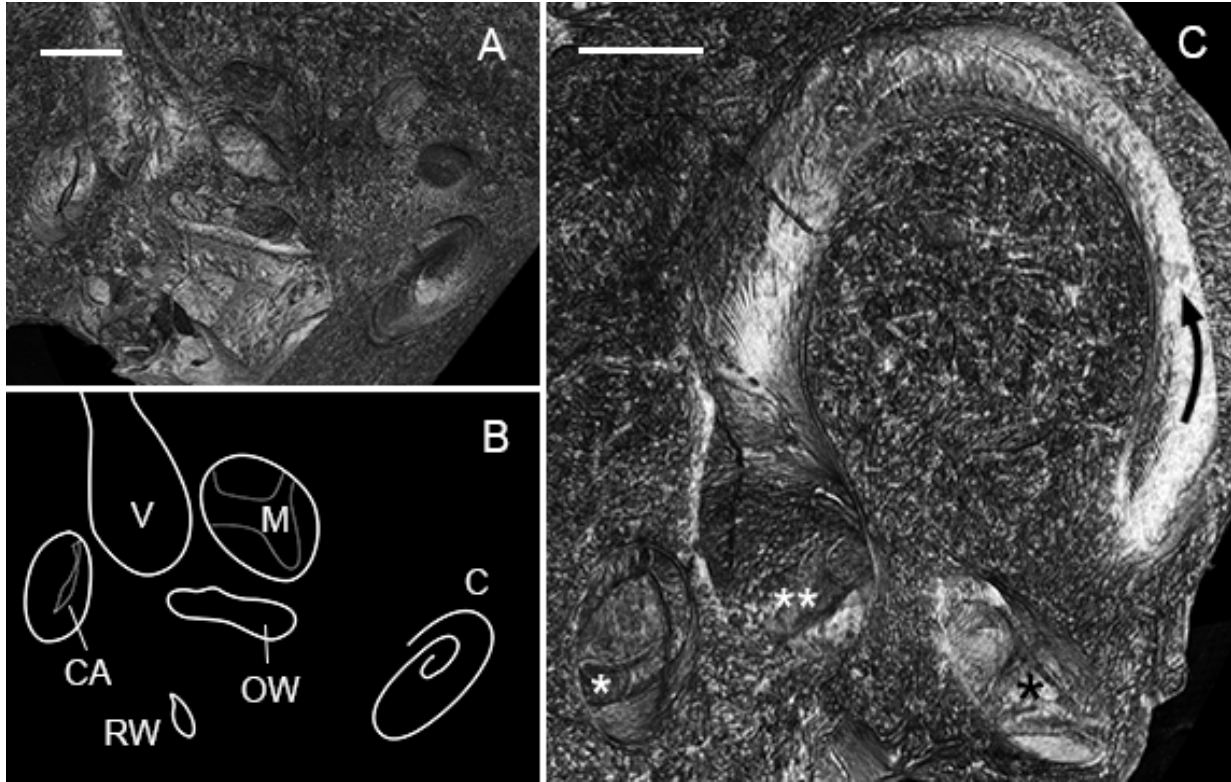


Fig. 2.8. 3D volumetric analysis of the vestibular organs in human temporal bone. **(A)** Bone was virtually sectioned along several planes to reveal two of the vestibular system's sensory epithelia. A schematic orienting the reader to these structures is in panel **(B)**. V = vestibule; M = utricular macula; C = cochlea; OW = oval window; RW = round window; CA = crista ampullaris (posterior ampulla). **(C)** Still image highlighting the vestibular structures seen in [Visualization 3](#); the black arrow indicates the position and orientation of the superior semicircular canal; double white asterisks mark the utricular macula; single white asterisk marks the crista ampullaris of the posterior ampulla; single black asterisk marks the crista ampullaris of the superior ampulla. Scales = 0.5mm.

Conclusions

Our data demonstrate that synchrotron radiation X-ray imaging in combination with phase detection algorithms for edge enhancement enables visualization of the cochlea's organ of Corti at cellular level resolution in the 3D intact, unstained, non-decalcified human temporal bone. The method of imaging and analysis described here has the potential to revolutionize and accelerate the study and diagnosis of human inner pathologies, as it promises to replace traditional histologic sectioning and staining as the gold standard method for human organ of Corti evaluation and disease diagnosis. Future work should aim to **a)** further optimize SR-PCI parameters for intracochlear evaluation and vestibular organ assessment, and **b)** identify ways to minimize radiation energy so that this technology can be translated to the clinic.

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Disclosures

The authors declare that there are no conflicts of interest associated with this work.

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CHAPTER 3: Micro-optical coherence tomography of the mammalian cochlea

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Micro-optical coherence tomography of the mammalian cochlea

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Abstract

The mammalian cochlea has historically resisted attempts at high-resolution, non-invasive imaging due to its small size, complex three-dimensional structure, and embedded location within the temporal bone. As a result, little is known about the relationship between an individual's cochlear pathology and hearing function, and otologists must rely on physiological testing and imaging methods that offer limited resolution to obtain information about the inner ear prior to performing surgery. Micro-optical coherence tomography (μ OCT) is a non-invasive, low-coherence interferometric imaging

technique capable of resolving cellular-level anatomic structures. To determine whether μ OCT is capable of resolving mammalian intracochlear anatomy, fixed guinea pig inner ears were imaged as whole temporal bones with cochlea *in situ*. Anatomical structures such as the tunnel of Corti, space of Nuel, modiolus, scalae, and cell groupings were visualized, in addition to individual cell types such as neuronal fibers, hair cells, and supporting cells. Visualization of these structures, via volumetrically-reconstructed image stacks and endoscopic perspective videos, represents an improvement over previous efforts using conventional OCT. These are the first μ OCT images of mammalian cochlear anatomy, and they demonstrate μ OCT's potential utility as an imaging tool in otology research.

Introduction

Few treatments for human hearing loss exist, largely because the relationship between an individual patient's cochlear pathology and their degree of hearing loss is poorly understood. A large obstacle to achieving this understanding is the inability to perform noninvasive imaging on patients' inner ears at a resolution sufficient to assess potential physiological contributions to hearing impairment. Hearing loss can result from physiological damage to the sensory hair cells and spiral ganglion, malformation of or damage to areas necessary for sound conduction through bone, or a mixture of these pathologies¹. However, conventional clinical imaging methods, such as magnetic resonance imaging (MRI) and computed tomography (CT), are limited in spatial resolution to approximately 1 mm and 0.5-1 mm, respectively^{2,3}. Consequently, these modalities can only detect gross abnormalities, such as profound malformations in the bony anatomy of the cochlea. MRI and CT are largely insensitive to intracochlear defects that fall beneath this detection range, such as missing and damaged cells within the cochlea's sensory epithelium, the organ of Corti⁴.

The organ of Corti is a heterogeneous matrix of sensory and non-sensory epithelial cells that contribute to both the perception and fine-tuning of frequencies within the range of mammalian hearing⁵. Supporting cells, such as pillar, Deiters, and Hensen's cells, provide structural and molecular support to the sensory hair cells. Hair cells are organized as a single row of inner hair cells (IHC), which receive 90% afferent innervation, and three rows of outer hair cells (OHC), which receive 90% efferent innervation. The inner hair cells transduce sound via mechanical shearing forces imparted by the basilar membrane's

vibration and the cells' protruding actin stereocilia. Stereocilia deflection prompts neurotransmitter release into the post-synaptic space near spiral ganglion neurites, generating electrical signals in response to frequency-specific stimulation that is sent to brainstem and cortical processing regions. The cochlea contains other soft tissue microstructures that are critical for hearing: Reissner's membrane, which serves as a diffusion barrier separating the contents of two of the cochlea's fluid-filled cavities; the tectorial membrane, which contacts hair cells' stereocilia during sound transduction; the stria vascularis in the spiral ligament, which maintains ionic gradients of endocochlear fluids and provides a blood barrier; and the neurites of the spiral ganglion, which form one branch of the auditory nerve, and extend both radially and diagonally along the sensory epithelium⁶. These structures fall beneath the detection limits of both MRI and CT.

Optical coherence tomography (OCT) is a non-contact, cross-sectional imaging technique that applies low coherence interferometry to image opaque subsurface structures with a resolution typically from 10-15 μm in axial and 30-40 μm in transverse planes^{7,8}. During OCT imaging, infrared laser light is backscattered by microstructural features within a structure or organ of interest. The dimensions of these features can be determined by applying low coherence interferometry, which enables the backscattered sample light to be resolved in depth. OCT is characterized by high detection sensitivity, as small as 10^{-10} of the incident optical power⁸, and a penetration depth of 1-3 mm, depending on tissue type⁷. OCT is routinely used in clinical ophthalmology to image the retina and cornea^{9,10} and in dermatology^{11,12}, and has previously been used for cellular and submicrometer imaging¹³⁻¹⁵. Intracochlear morphology and mechanics have also been observed with OCT in rodent models, with axial and lateral resolution ranging from 10-20 μm ¹⁶⁻²⁰, *ex vivo*^{16,17,19} and *in vivo*^{13,18,20}. OCT has enabled the identification of larger structures including Reissner's membrane¹⁶⁻²⁰, the basilar¹⁶⁻²⁰ and tectorial membranes¹⁸⁻²⁰, the spiral ligament¹⁷, the three scalae of the cochlea^{16,18,20}, and the region of the sensory epithelium¹⁸⁻²⁰, in addition to spaces between various structures such as the demarcation between the osseous and membranous labyrinths¹⁷, the tunnel of Corti¹⁹, and spaces between regions of inner and outer hair cells¹⁹, *ex vivo*^{16,17,19} and *in vivo*^{18,20}. Cochlear mechanics and measurements of the motion and displacement of intracochlear structures in response to frequency-specific auditory stimulation have also been studied with OCT *ex vivo*^{21,22} and *in vivo*²⁰. However, similar to traditional imaging methodology, these studies have been

limited by OCT's resolution threshold, and have not been capable of resolving smaller anatomical features including the major therapeutic targets in hearing loss such as inner and outer hair cells, supporting cells, and nerve fibers.

To improve the spatial resolution of conventional OCT, we introduced a successor called micro-optical coherence tomography (μ OCT) and demonstrated its ability to resolve individual endothelial cells, leukocytes, lymphocytes, and monocytes in human cadaver coronary arteries, at a resolution of $2\ \mu\text{m} \times 2\ \mu\text{m} \times 1\ \mu\text{m}$ (x, y, z)²³. μ OCT has more recently been utilized to detect cholesterol crystals within macrophages in atherosclerosis²⁴, to visualize functional anatomy, including individual beating cilia involved in mucociliary clearance and transport in airway epithelium^{25,26}, and to resolve cellular details in zebrafish larvae *in vivo*²⁷. μ OCT technology may also be suitable to resolve cochlear microanatomy at a cellular level. Thus, this study's objective was to determine whether μ OCT was capable of resolving major and micro-anatomical structures within the mammalian inner ear, and to generate the first μ OCT images of fixed guinea pig intracochlear anatomy *in situ*.

Results

Figure 3.1A-C depicts cross-sections of a guinea pig cochlea cut along its longitudinal axis, and stained with hematoxylin and eosin (H&E) to highlight cellular structures. **Figure 3.1A** shows 8 cochlear half-turn cross sections, corresponding to the four cochlear turns, spiraling around the bony, neuron-filled core, the modiolus (M). The basilar membrane (BM) and Reissner's membrane (RM) delineate the cochlea's three fluid-filled chambers: scala tympani (ST), scala media (SM) and scala vestibuli (SV). The bony otic capsule surrounding the cochlear tissue is stained purple. **Figure 3.1B** zooms in on the single half-turn (boxed in blue in **Figure 3.1**), and **Figure 3.1C** zooms in on the region boxed in red in **Figure 3.1B**, depicting the sensory cells (inner and outer hair cells) and non-sensory cells (including inner and outer pillar cells) of the organ of Corti, as well as other supporting cells and the tectorial membrane.

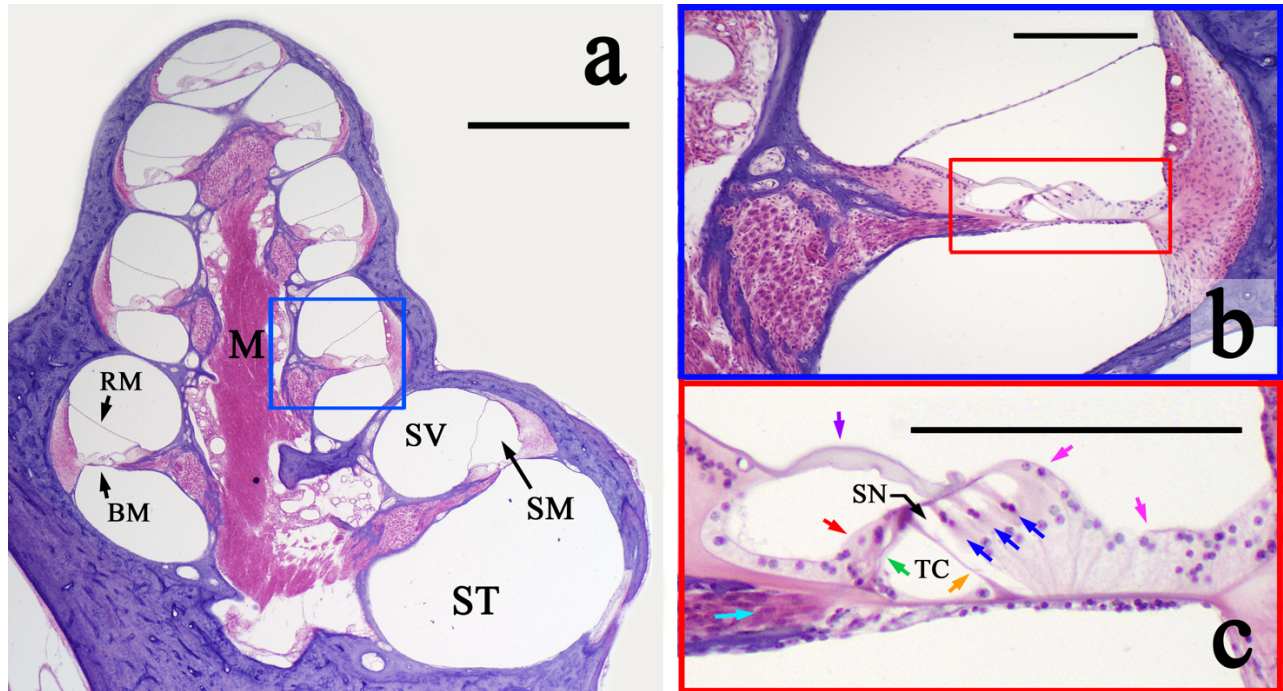


Figure 3.1. Micrographs of a sectioned guinea pig cochlea. (A) Cross-section of a guinea pig cochlea, stained with H&E, and cut along its longitudinal axis. M: modiolus. BM: basilar membrane; RM: Reissner's membrane, ST: scala tympani; SM: scala media; SV: scala vestibuli. Magnification = 2X; Scale = 1 mm. (B) A single half-turn of the guinea pig cochlea, representing the region boxed in blue in **Figure 1A**. Magnification = 10X; Scale = 200 μ m. (C) Zoomed-in view of the organ of Corti (boxed in red in **Figure 1B**). Colored arrows point to specific cell types: inner (red) and outer (blue) hair cells, inner (green) and outer (orange) pillar cells, and neuronal fibers (turquoise), which travel through the tunnel of Corti (TC) and space of Nuel (SN). Pink arrows: supporting cells; purple arrow: tectorial membrane. Magnification = 10X; Scale = 200 μ m.

Excised guinea pig temporal bones were imaged with μ OCT via a 0.5-1 mm diameter cochleostomy in the otic capsule, corresponding to either a) the region of the second of the four cochlear turns, exposing the area from the top of the third turn to the top of the second turn, laterally, or b) the apex. μ OCT permitted imaging in 1 mm x 1 mm and 500 μ m x 500 μ m fields of view. Raw images of the cochlea's apical turn are shown in **Figure 3.2**. **Figure 3.2A** reveals the region of inner hair cells and inner pillar cells, a row of outer pillar cells (OPCs), and three rows of OHCs. The dark space between the OPCs and OHCs is the space of Nuel; the dark space between the OPCs and the inner pillar cells is the tunnel of Corti. **Figure 3.2B** shows a single plane image of the region where outer hair cells reside – individual outer hair cells are identifiable. For reference and orientation, **Figures 3.2C** and **3.2D** show immunohistochemically labeled cells and neuronal processes in the guinea pig organ of Corti.

After performing a volumetric reconstruction of the raw 2D scans, neuronal fiber bundles became visible at several levels within the tunnel of Corti and space of Nuel along the length of the imaged tissue (**Figure 3.3**). Due to their location and radial trajectory across the tunnel of Corti and space of Nuel (the endolymph-filled epithelial lumens situated between the inner and outer pillar cells and the outer pillar and hair cells, respectively), these nerve fiber bundles are hypothesized to be synaptically connected to outer hair cells.

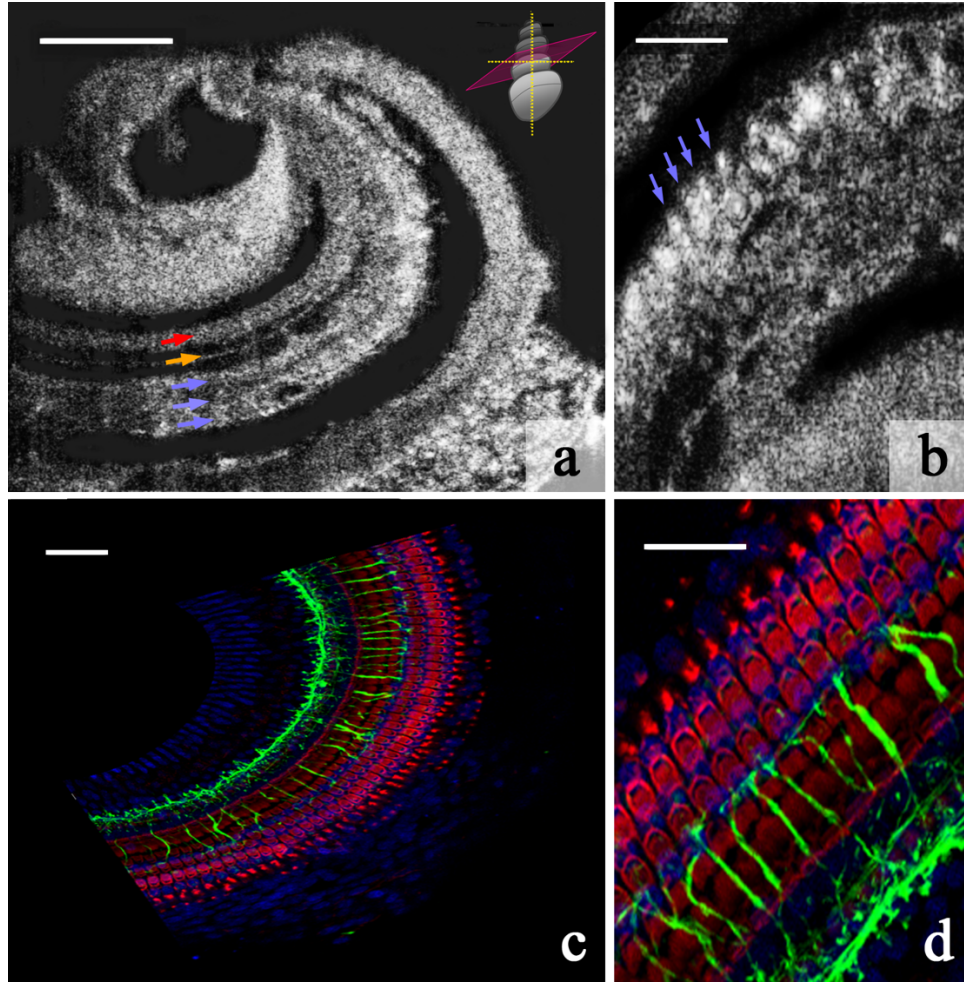


Figure 3.2. μ OCT images and immunohistochemically-stained regions of sensory and supporting cell rows within the guinea pig organ of Corti (A) Single 2D image from a μ OCT imaging stack, depicting the regions of the inner pillar cells and inner hair cells (red arrow), outer pillar cells (orange arrow), and 3 rows of outer hair cells (blue arrows). The schematic in the top right-hand corner shows the orientation of the plane (pink) along which the image was sectioned relative to the orientation of the cochlea. Scale = 100 μ m. (B) Single 2D image depicting individual outer hair cells (examples indicated with blue arrows). Scale = 50 μ m. (C) Immunohistochemically-stained guinea pig organ of Corti whole mount, corresponding to the orientation presented in **Figure 3.2A**. Cytoskeletal actin within hair cells and supporting cells is labeled with rhodamine phalloidin (red), neuronal processes are labeled with neurofilament-H (green), and cell nuclei are labeled with Hoechst stain (blue). Scale = 50 μ m. (D) Zoomed-in view of immunohistochemically-stained guinea pig organ of Corti depicting the orientation presented in **Figure 3.2B**. Color convention as in **Figure 3.2C**. Scale = 25 μ m.

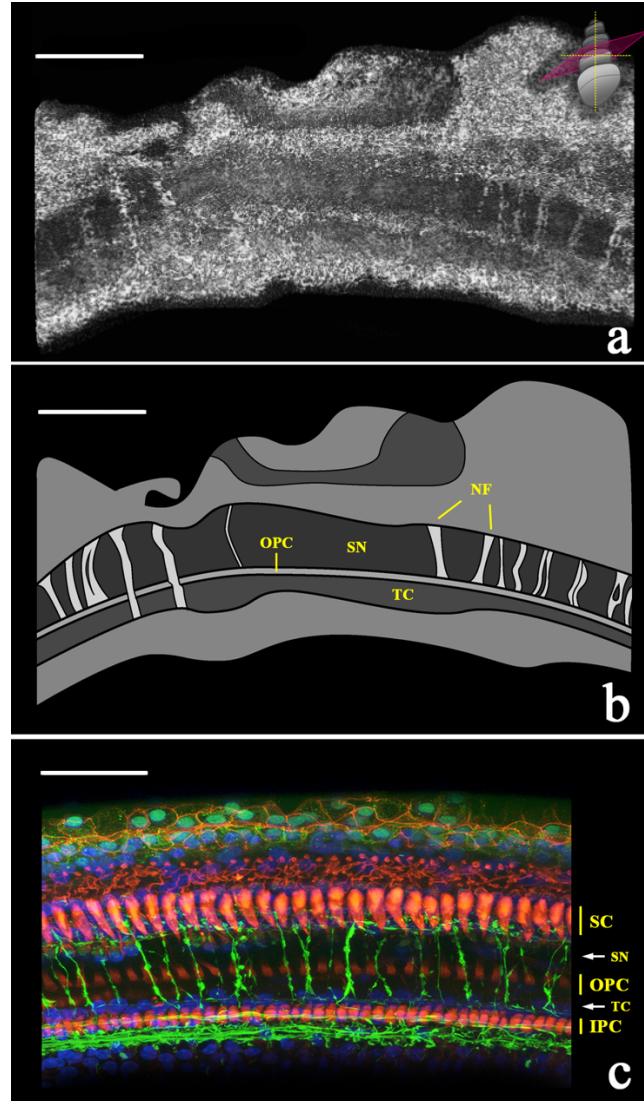


Figure 3.3. μ OCT image of nerve fiber bundles traversing the tunnel of Corti and space of Nuel to innervate outer hair cells ($500\ \mu\text{m} \times 500\ \mu\text{m}$). (A) Volumetric reconstruction of maximum-projected μ OCT image stack, depicting bundles of nerve fibers traversing the organ of Corti towards the outer hair cell region. The schematic in the top right-hand corner shows the orientation of the virtual sectioning plane. Scale = $150\ \mu\text{m}$. (B) Schematic representation of the microanatomy in the top panel, with bundles of nerve fibers (NF) crossing the tunnel of Corti (TC) and/or the space of Nuel (SN). OPC = outer pillar cells. Scale = $150\ \mu\text{m}$. (C) For reference, a confocal laser scanning microscopy image of the guinea pig organ of Corti. Rhodamine phalloidin (red) marks supporting cells (SC), and outer and inner pillar cells (OPC and IPC, respectively), Hoechst stain (blue) marks cell nuclei, and neurofilament-H (green) marks neuronal fibers. Scale = $50\ \mu\text{m}$.

Visualizations 1a and **1b** allow the viewer to virtually “fly through” the space of Nuel and tunnel of Corti, respectively, in volumetric reconstructions of two μ OCT imaging stacks of the guinea pig organ of Corti. The orientation visualized here is the same as those depicted in **Figures 3.1A-C**. In **Visualization 1a**, bundles of neuronal fibers are observed traversing the basal region of the space of Nuel (labeled still image shown in **Figure 3.4A**). In **Visualization 1b**, a single bundle of neuronal fibers is observed crossing the central region of the tunnel of Corti (labeled still image shown in **Figure 3.4B**). We hypothesize that this is a fascicle of medial olivocochlear efferent nerve fibers, based on its radial trajectory and location within the tunnel.²⁸ A different nerve fiber bundle is observed traveling longitudinally along the medial wall of the tunnel of Corti for the length of the reconstructed tissue section. The longitudinal trajectory and specific location of this bundle are characteristic of the tunnel spiral bundle (TSB), a mass of primarily lateral olivocochlear nerve fibers. The TSB is also visualized in cross-section in **Figures 3.5A-C**, which display its precise location in three 2D orientations. Our interpretation regarding the tunnel spiral bundle and tunnel-crossing bundle of medial efferent fibers are consistent with previously reported arborization patterns of efferent fibers in the cat cochlea.^{29,30}

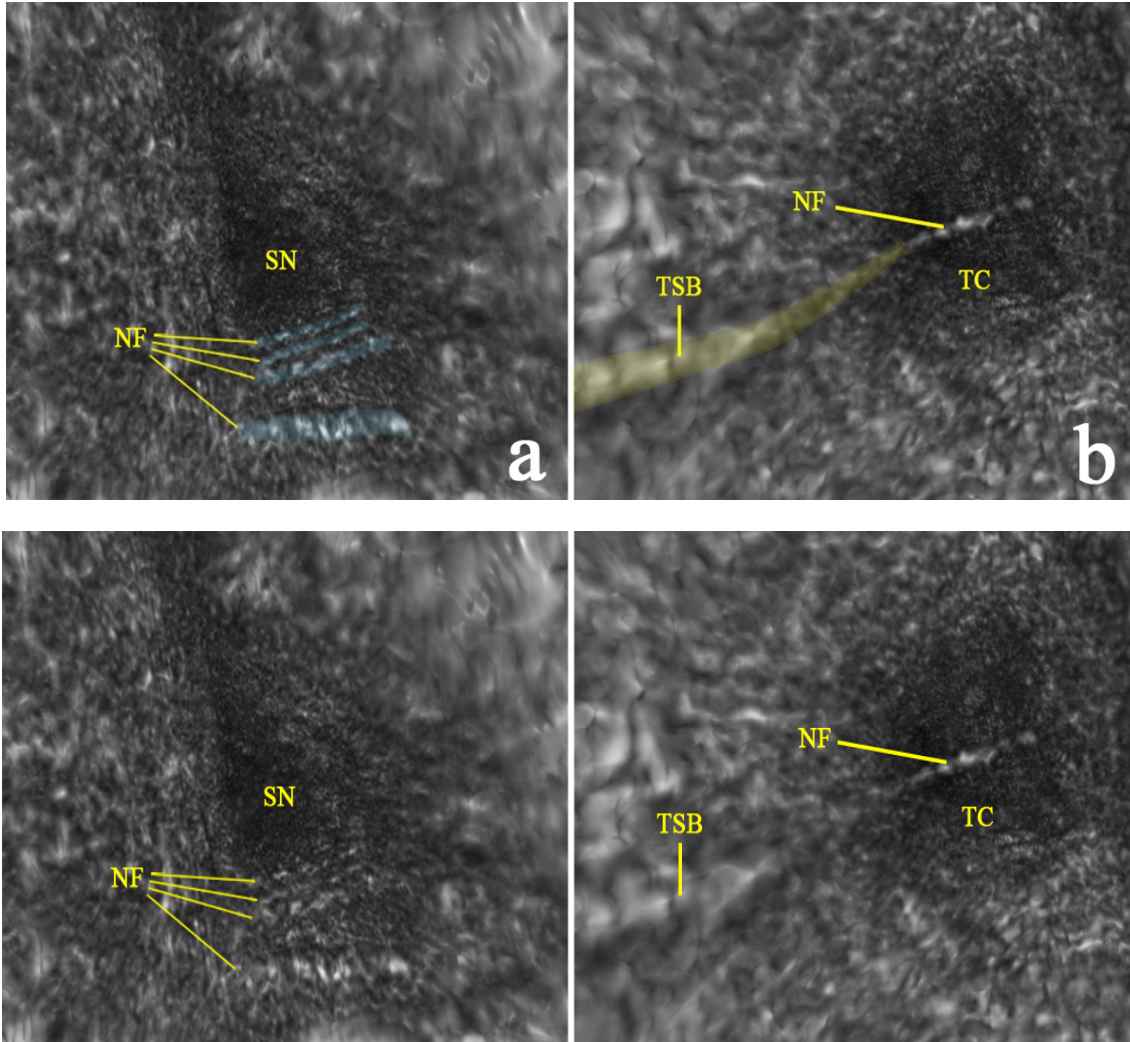


Figure 3.4 (top) and Visualization 1 (video stills of Visualization 1a [left] and Visualization 1b [right] shown in bottom as video placeholders). (A) A labeled, colorized still from a 3D volumetric reconstruction of a μ OCT image stack (**Visualization 1a**), “flying through” the space of Nuel (SN), showing bundles of nerve fibers (NF, blue) crossing the basal region of the SN. (B) A labeled, colorized still from a 3D volumetric reconstruction of a μ OCT image stack (**Visualization 1b**), “flying through” the tunnel of Corti (TC), showing a single bundle of medial efferent nerve fibers crossing the central region of the TC, and the tunnel spiral bundle (TSB, yellow) running along the tunnel’s medial wall. **Visualization 1:** Endoscopy perspective videos of reconstructed μ OCT images of the space of Nuel (left; **Visualization 1a**) and tunnel of Corti (right; **Visualization 1b**), which reveal several bundles of nerve fibers traversing radially towards the outer hair cell region. Composed of 200 frames, captured at 15 fps; 500 μ m x 500 μ m field of view.

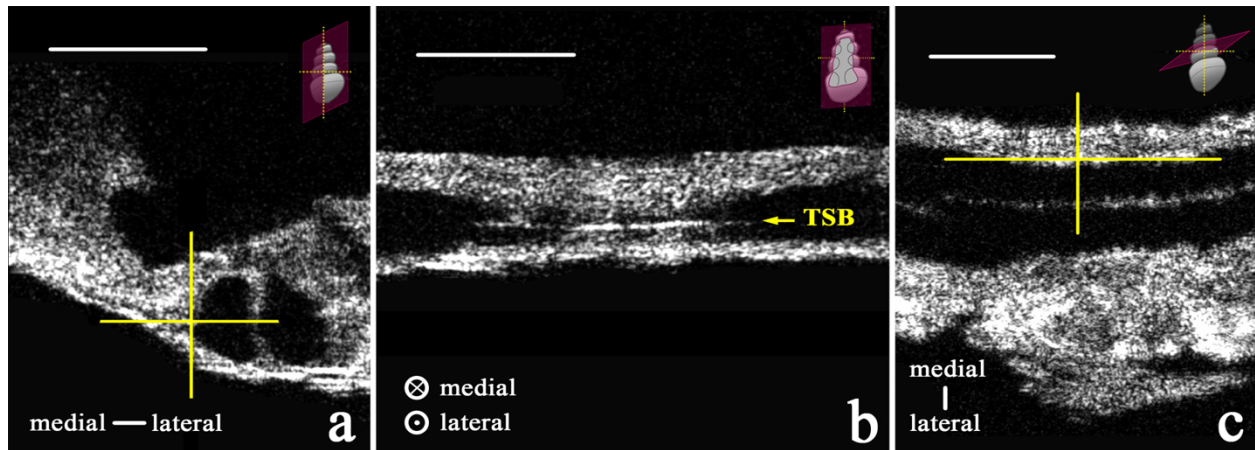


Figure 3.5. Two-dimensional μ OCT images of the tunnel spiral bundle (TSB) ($500\ \mu\text{m} \times 500\ \mu\text{m}$) within the guinea pig organ of Corti, in three perspectives. Yellow cross hairs in (A) (cross-section of the organ of Corti and two fluid lumens, separated by a pillar cell) and (B) (looking down from above the organ of Corti) indicate the TSB's (medial section, shown in (C)) specific position along the medial wall of the tunnel of Corti. The schematics in the top right-hand corner of each panel show the orientation of the 2D plane depicted, respectively. Scale = $100\ \mu\text{m}$.

A volumetric reconstruction of the imaged section of the guinea pig organ of Corti viewed in cross-section revealed discernable individual cell types *in situ* (**Figure 3.6**). In this image, the scalae tympani and media are clearly visualized, separated by the basilar membrane and the organ of Corti atop it. Other identifiable structures include the bony modiolus (MOD) and the spiral limbus (SL; medial and medio-apical to the basilar membrane, respectively), inner and outer pillar cells (IPC and OPC, respectively), outer hair cells (OHC), the tunnel of Corti (TC), and space of Nuel (SN; inferior and medial to the outer hair cells). The inner hair cells are medial to the tunnel of Corti; their embedded location did not permit visualization here. Bundles of nerve fibers (NF) were observed traveling from within the spiral lamina across the space of Nuel (SN) to the region of the outer hair cells.

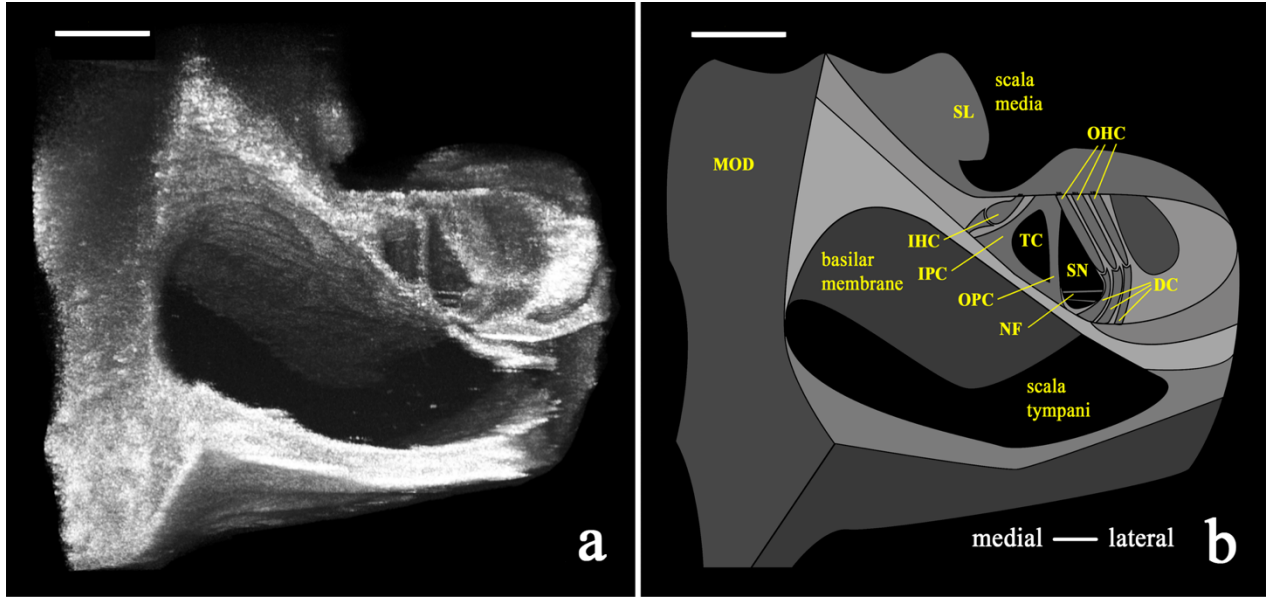


Figure 3.6. Volumetrically reconstructed μ OCT image ($500\ \mu\text{m} \times 500\ \mu\text{m}$) and schematic of the guinea pig organ of Corti in situ. (A) Volumetric reconstruction of μ OCT-visualized sensory and non-sensory cells of the organ of Corti from the 2nd-3rd turn of the cochlea. **(B)** Schematic labeling structures visualized in the left panel, such as outer hair cells (OHC), bundles of nerve fibers (NF), and inner and outer pillar cells (IPC and OPC, respectively). MOD = modiolus; SL = spiral limbus; IHC = inner hair cell; TC = tunnel of Corti; SN = space of Nuel. Both scales = $100\ \mu\text{m}$.

Discussion

In presenting the first, to our knowledge, μ OCT images of the mammalian cochlea, this report provides evidence of μ OCT's utility as a high-resolution intracochlear imaging tool. Our μ OCT imaging system resolved cellular anatomy in the guinea pig organ of Corti, including nerve fiber bundles, which have eluded conventional clinical imaging methods thus far. Importantly, results of the present study also reveal that high resolution is not the sole criterion for achieving informative images; indeed, our data suggest that μ OCT resolves some of the organ of Corti's anatomical and cellular features, such as lumens and bundles of neurites, more readily than other structures that are more deeply embedded in the sensory epithelium, such as inner hair cells. Thus, it is evident that factors such as sample contrast and speckle noise, in addition to resolution, are important consideration for future improvements on this technology³¹.

Hearing loss is the most common sensory deficit in the world³² and the most common disability in the United States³³. Because mammalian cochlear hair cells and neurons do not spontaneously regenerate, hearing loss is permanent and irreversible in the vast majority of cases. A significant barrier to developing otologic therapies is a limited understanding of how cochlear pathology relates to the degree and type of hearing loss³⁴. The cochlea remains a "black box" in living subjects, closed to direct or conventional imaging due to its embedded location, fragility, and complex structure. Our knowledge of cochlear physiology and morphology today thus comes primarily from post-mortem analyses of human temporal bones and experiments using animal models, which have revealed many physiological sources of hearing impairment: sensory hair cell loss or damage^{35,36}; damage to stereocilia due to noise over-exposure³⁷; malformations of the tectorial membrane³⁸; loss of auditory nerve fibers and spiral ganglion neurons³⁹; and atrophy of the stria vascularis⁴⁰.

Numerous studies have noted the value of traditional OCT imaging for the cochlea^{16-21,41-43}; however, the improvements that μ OCT affords over OCT in resolution and depth-of-focus (DOF) make it better-suited for imaging the cochlea. The anatomical undulations in the cochlea require high DOF to simultaneously capture peaks and valleys; OCT's shorter DOF may be responsible for limiting earlier studies to 5-10 μ m resolution. We have previously reported concurrent gains in DOF and resolution in μ OCT and established the utility of this enhanced performance in cardiovascular²³ and airway imaging

applications⁴⁴. Compared to other conventional imaging, the advantages of μ OCT include that it 1) can be conducted on the benchtop, 2) requires no contrast agent, 3) can image whole structures within its detection field near-instantaneously, and 4) employs primarily infrared and near-infrared light sources, theoretically safer than higher-energy (lower wavelength) laser exposure⁴⁵. While significant development is required before this technology can be employed to assess cochlear integrity in living humans, the present study demonstrates μ OCT's potential to image this organ and motivates further miniaturization of μ OCT technology. We have recently reported progress in the development of miniaturized μ OCT instrumentation for *in vivo* applications^{46,47} and in human cochlear endoscopy via the external auditory canal⁴⁸. However, the adaptation of our imaging configuration to the constraints of the small size and embedded location of the human cochlea remains a significant technical challenge.

Clinical realization of μ OCT cochlear imaging faces significant surgical and engineering hurdles; however, applications for otolaryngology research in animals may be more immediately realized. In contemporary studies employing animal models of hearing impairment, the techniques most commonly used to detect and visualize changes in cochlear morphology include confocal and two-photon microscopy in tandem with histology and immunohistochemistry. These techniques are applied post-mortem, and both specimen preparation and imaging itself may be extremely time-consuming. An imaging technique such as μ OCT, capable of resolving cochlear microanatomy in experimental animals as genetic models of human hearing loss, could potentially be adapted for *in vivo* longitudinal analysis of cochlear anatomy in healthy and pathological states. Potential *in vivo* applications in animals include monitoring physiological changes resulting from genetic mutations associated with progressive hearing loss, facilitating surgical guidance when advancing therapy-delivering probes, targeting distinct intracochlear spaces or frequency-specific locations through narrow cavities in the ear⁴⁹, assessing and measuring vibration in the organ of Corti prior to and post exposure to noise or regenerative therapies⁵⁰, and acquiring an improved understanding of the natural progression of age-related hearing loss. Future directions of this work include conducting *in vivo* μ OCT experiments in guinea pigs, to determine optimal surgical approaches and the instrumentation's performance in a living subject.

The present work is subject to several limitations, namely that the described experiments were conducted in an animal model and within healthy, normal cochleae; the specimens used were excised,

partially dissected, and fixed for ease of manipulation; and a small number of cochleae (7) were examined. Nonetheless, these findings represent an important incremental advance regarding μ OCT's ability to perform cellular-level imaging of the mammalian cochlea, which we hope will accelerate the technology's improvement and customization for broader applications.

Conclusions

Innovation of imaging systems capable of resolving mammalian middle and inner ear anatomy is essential for understanding the link between otopathology and hearing function. μ OCT, shown here to be capable of resolving microanatomy in the mammalian cochlea, affords the resolution and speed necessary to be considered a promising candidate for otologic imaging development; however, significant progress remains to be made. Future efforts should aim to improve μ OCT's resolution and penetration depth, determine whether similar imaging results can be reproduced in living animal subjects, and define the limits of the instrumentation and surgical approaches to permit optimal imaging access.

Method

Animals

The guinea pig is a well-studied animal model that is commonly used in translational research studies on hearing and hearing loss because its frequency sensitivity and susceptibility to ototoxic medications are similar to that in humans⁵¹⁻⁵⁴, and its entire cochlea is surgically accessible. Albino adult male guinea pigs of approximately 400 grams were used in this study (7 cochleae were imaged). The Institutional Animal Care and Use Committee (IACUC) of Massachusetts Eye and Ear Infirmary (MEEI) approved all experimental protocols for this study, and all procedures were carried out in accordance with approved institutional guidelines of the IACUC. Guinea pigs were maintained at MEEI's animal care facility in Boston, MA.

Instrumentation and image collection

The system was a customized, spectral-domain OCT (SD-OCT) instrument with improvements to standard OCT that yield higher resolution in lateral and axial directions. The instrumentation layout has been previously described²³ and is illustrated in **Figure 3.1**.

In brief, a circular obscuration was created in the sample beam path to enhance axial depth-of-focus (DOF) and field-of-view, maintaining an extended DOF of approximately 300 μm with a numerical aperture of 0.12. The resulting lateral resolution was 2 μm .²³ The high axial resolution of 1 μm was derived from a high bandwidth custom OCT spectrometer, spanning 650-950 nm combined with an ultra-broadband supercontinuum laser source (NKT Photonics, Birkerød, Denmark).

The optical power at the sample was less than 15 mW. Transverse (x, y) scanning across the sample was performed using software-controlled galvanometer scanning motors (Thorlabs, Newton, NJ); the same software was used to simultaneously acquire spectral data through the spectrometer camera. Images comprising 512 A-lines were acquired at 40 frames per second (fps), with each frame spanning either 500 μm or 1 mm in lateral space. Three-dimensional (3D) volumes were acquired by scanning over a square region spanning either 500 x 500 μm or 1 x 1 mm. A large working distance (25 mm), defined as the distance between the objective lens and the focal plane, was chosen to allow flexible positioning of specimens on the sample stage. Cross-sectional and 3D images are displayed using logarithmic, logarithmic inverse, or linear grayscale lookup tables, which depict liquid-filled regions (e.g. the scalae) as dark, and highly-scattering regions (e.g. bone and tissue) as light.

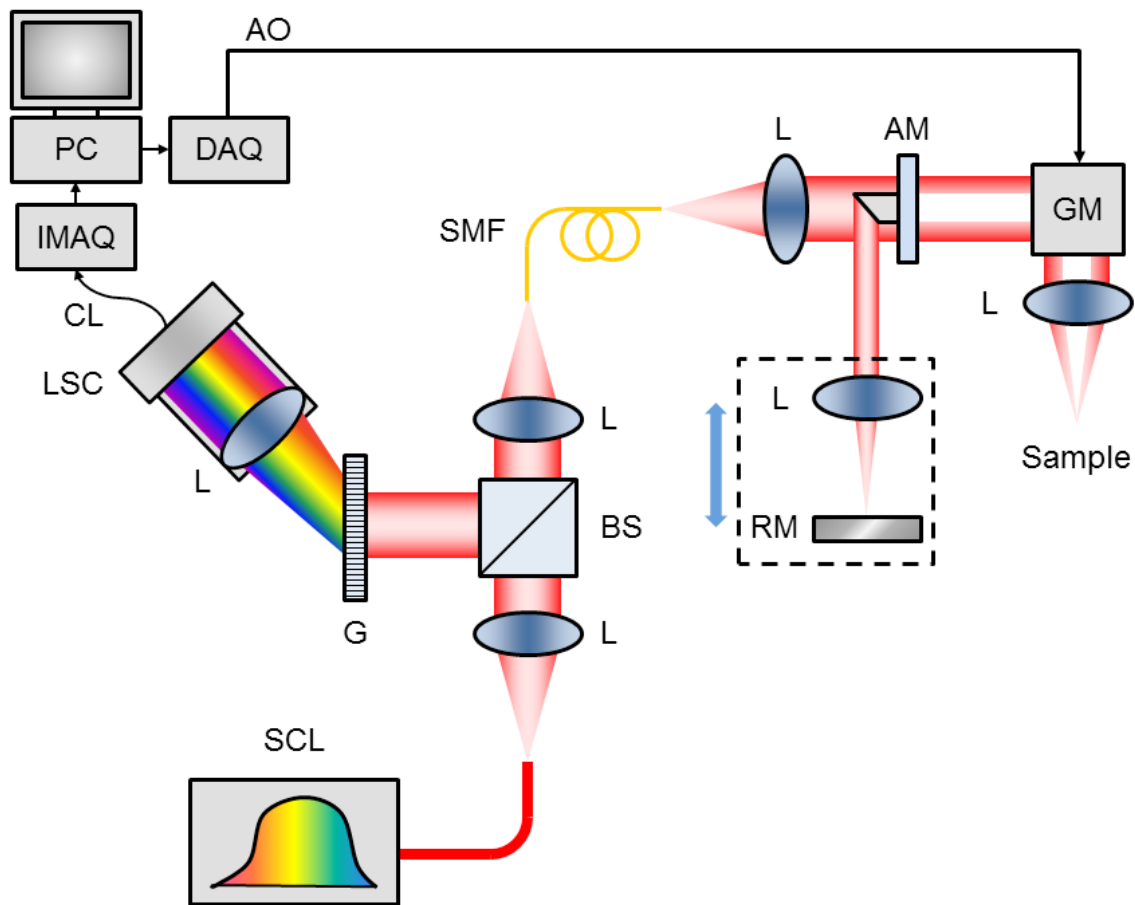


Figure 3.1. μ OCT instrumentation. Schematic diagram of μ OCT system. Supercontinuum laser (SCL) power is directed by collimating and focusing lenses (L) through single mode fiber (SMF). Output light from SMF is collimated and passed through an apodizing mirror (AM), resulting in a circular obscuration of the transmitted light, which is steered by a galvanometer mirror (GM) through an objective lens onto the sample. Light reflected by the AM is focused onto a reference mirror (RM), and the reference lens and mirror assembly can be translated in unison to adjust the reference path length. Back-scattered light from the sample is re-integrated at the SMF with light reflected by the RM. The return light is collimated and directed by the beam-splitter (BS) towards a diffraction grating (G). The spectrally dispersed light is then focused onto a line scan camera (LSC), which outputs raw spectrograms through a CameraLink (CL) interface to an image acquisition board (IMAQ) installed in a PC. The PC also controls scanning through a data acquisition card (DAQ), which produces an analog output (AO) voltage signal that controls the GM.

Specimen processing and μ OCT imaging

Guinea pig temporal bones were extracted following euthanasia (intraperitoneal injection of Fatal-Plus Solution [0.1 mL/kg; Vortech Pharmaceuticals, Dearborn, MI]) and decapitation. Temporal bones were dissected in cold 4% paraformaldehyde (PFA) (diluted in Phosphate Buffer Saline [PBS]) to expose the cochlea's interior to fixative by opening the apical otic capsule and puncturing the round and oval window membranes with forceps. Approximately 1 ml of cold 4% PFA was slowly infused into the apical hole until it flowed out of the oval and round windows. The temporal bones were immersed in cold 4% PFA in PBS for 4-8 hours on a shaker. Seven specimens were further dissected in PBS to remove some of the otic capsule, exposing a section or the entire cochlea. Specimens were stored at 4° C in PBS prior to μ OCT imaging. Two additional specimens were placed in 0.12M EDTA for two weeks to decalcify the otic capsule for whole mount preparation and immunostaining (see section 'Immunohistochemistry' below).

μ OCT imaging and image analysis

During μ OCT imaging, specimens were removed from the PBS and positioned beneath the μ OCT laser aperture in a dry plastic culture dish. The imaging apparatus was adjusted in X, Y, and Z directions to achieve optimal focus.

Raw data were converted into tagged image file format (.tif) stacks using standard SD-OCT processing routines⁵⁵ and then imported into OsiriX (Pixmeo SARL, Bernex, Switzerland), a software program routinely used to analyze clinical CT and MRI images. OsiriX was used to reconstruct (multiplanar reconstruction and volumetric rendering) the μ OCT images in 3D, generate maximum intensity projections, rotate, enlarge, and crop the images, set image opacity, and generate scale bar measurements. Images were labeled, structures were colorized with a partially transparent brush (for ease of visualization), and scale bars were traced in Photoshop (Adobe, Inc., San Jose, CA). 3D volumetric μ OCT images were constructed using an endoscopy perspective in OsiriX to create “fly-through” videos (15 fps) revealing intracochlear structures.

Haematoxylin/eosin (H&E) histology

Animal euthanasia and specimen extraction and fixation followed the protocol described above. For H&E staining, guinea pig cochleae were perfused with 10% formalin and decalcified in 0.27M EDTA for 25 days. Specimens were then dehydrated in ethanol and embedded in celloidin (1.5% celloidin for 1 week, 3% for 2 weeks, 6% for 3 weeks, 12% for 3 weeks). Hardened ears were mounted on a fiber block for sectioning with a sliding microtome. The sections were mounted on glass slides, stained with H&E, and preserved in 80% alcohol. Imaging of these sectioned specimens was conducted with an Olympus BH2 microscope (Olympus, Tokyo, Japan) at 2X and 10X magnifications for **Figures 3.2A** and **3.2B**, respectively. **Figure 3.2B** was cropped using Adobe Photoshop CS5.1 (Adobe, Inc., San Jose, CA) to highlight cellular detail (**Figure 3.2C**).

Immunohistochemistry and confocal imaging

Guinea pig cochleae were extracted, fixed, and decalcified as described above. Under a stereomicroscope, the decalcified otic capsule was peeled away from the cochlea, and the 4 turns of the cochlea were sectioned into 8 pieces. Each piece was further microdissected to reveal the organ of Corti. The spiral ligament, stria vascularis, and tectorial and Reissner's membranes were removed.

After being rinsed in PBS for 15 minutes, cochlear sections were blocked with 5% Normal Horse Serum (NHS; Sigma-Aldrich, St. Louis, MO) in 1% Triton X-100 (Integra Chemical, Kent, WA), and were placed on a shaker for 30 minutes at room temperature. Sections were incubated with a primary antibody against neurofilament-H (polyclonal chicken; AB5539, Lot #2701573; EMD Millipore, Temecula, CA) overnight. After rinsing for 15 minutes in PBS, the tissue was incubated in a secondary antibody (AlexaFluor 488 goat anti-chicken IgG, A-11039, Lot #898239; ThermoFisher Scientific, Inc., Waltham, MA) diluted in 1% NHS with 0.4% Triton X-100 for 90 minutes in combination with 1:200 rhodamine phalloidin (ThermoFisher Scientific, Waltham, MA). Finally, the tissue was placed in Hoechst stain 33342 (Life Technologies, NY; 1 nM in PBS), and washed for 15 minutes in PBS and briefly in distilled water. Stained tissue was mounted under coverslips on glass slides with Vectashield mounting media (Vector Laboratories, CA, #H-1000).

Cochlear whole mounts were imaged with 20X (PlanApochromat, oil immersion, NA=0.7; #H1LG/02; Leica, Wetzlar, Germany) and 63X (PlanApochromat, oil immersion, NA=1.3, #506194; Leica, Wetzlar, Germany) Leica objectives, using a Leica TCS SP5 laser-scanning confocal microscope. Images (1024 x 1024 pixels) were collected in z-stacks and masked as maximum intensity projection images in the Leica software. The final images were cropped and scale bars were retraced in Photoshop CS5.1.

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Author disclosures

The authors have no competing interests to declare.

Author contributions

K.M.S and G.J.T. conceived of and supervised the work. K.M.S, G.J.T., S.A.B., K.K.C and J.S.I. designed the experiments. J.S.I., S.A.B., K.K.C., M.I.S, and H.M.L. performed the experiments. J.S.I., S.A.B., K.K.C., M.I.S, K.M.S., and G.J.T. analyzed data. S.A.B., J.S.I., and K.M.S. wrote the manuscript. All authors critically edited the manuscript and approved the final version.

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Chapter 4. Endoscopic Micro-optical Coherence Tomography of the Human Cochlea

Iyer, JS, Yin, B, Stankovic, KM, Tearney, GJ. *Endomicroscopy of the human cochlea using a micro-optical coherence tomography catheter. Light: Science and Applications Letters. In prep.*

Endomicroscopy of the Human Cochlea using a Micro-Optical Coherence Tomography Catheter

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Sensorineural hearing loss (SNHL) is one of the most profound public health concerns of the modern era, affecting 466 million people today, and projected to affect 900 million by the year 2050¹. Despite its widespread prevalence and debilitating effects on quality of life and cognition², SNHL remains incurable in humans, due in part to the nonspecific and in some cases unreliable diagnostic tools that remain the gold standard methods for assessing a patient's ability to hear³. Advances in both diagnostics and therapeutics for SNHL have been impeded by the human cochlea's inaccessibility for *in vivo* imaging, resulting from its extremely small size, convoluted coiled configuration, fragility, and deep encasement in dense bone. Here, we develop and demonstrate the ability of a sub-millimeter-diameter, flexible endoscopic probe interfaced with a micro-optical coherence tomography (μ OCT) imaging system to enable micron-scale imaging of the inner ear's sensory epithelium in cadaveric human inner ears.

When a sound pressure wave enters the ear canal and interacts with the middle ear's ossicular chain of bones, it transforms into a complex and highly sophisticated pattern of mechanical vibrations that is ultimately detected, transduced, and delivered to the auditory nerve by the cochlea, the sensory organ that facilitates hearing (**Figure 4.1**). The cochlea's sensory epithelium, the organ of Corti, comprises a mosaic of micron-sized cells and nerve fibers that selectively respond to different sound frequencies based on their location along the length of the spiraling epithelium. Studies conducted in animal models and histologically-prepared post-mortem human cochlear specimens suggest that age-, drug-, and noise-induced SNHL are caused by damage to the organ of Corti's composite structures^{4,5}; these structures are thus the targets for developing drug and gene therapies for SNHL⁴.

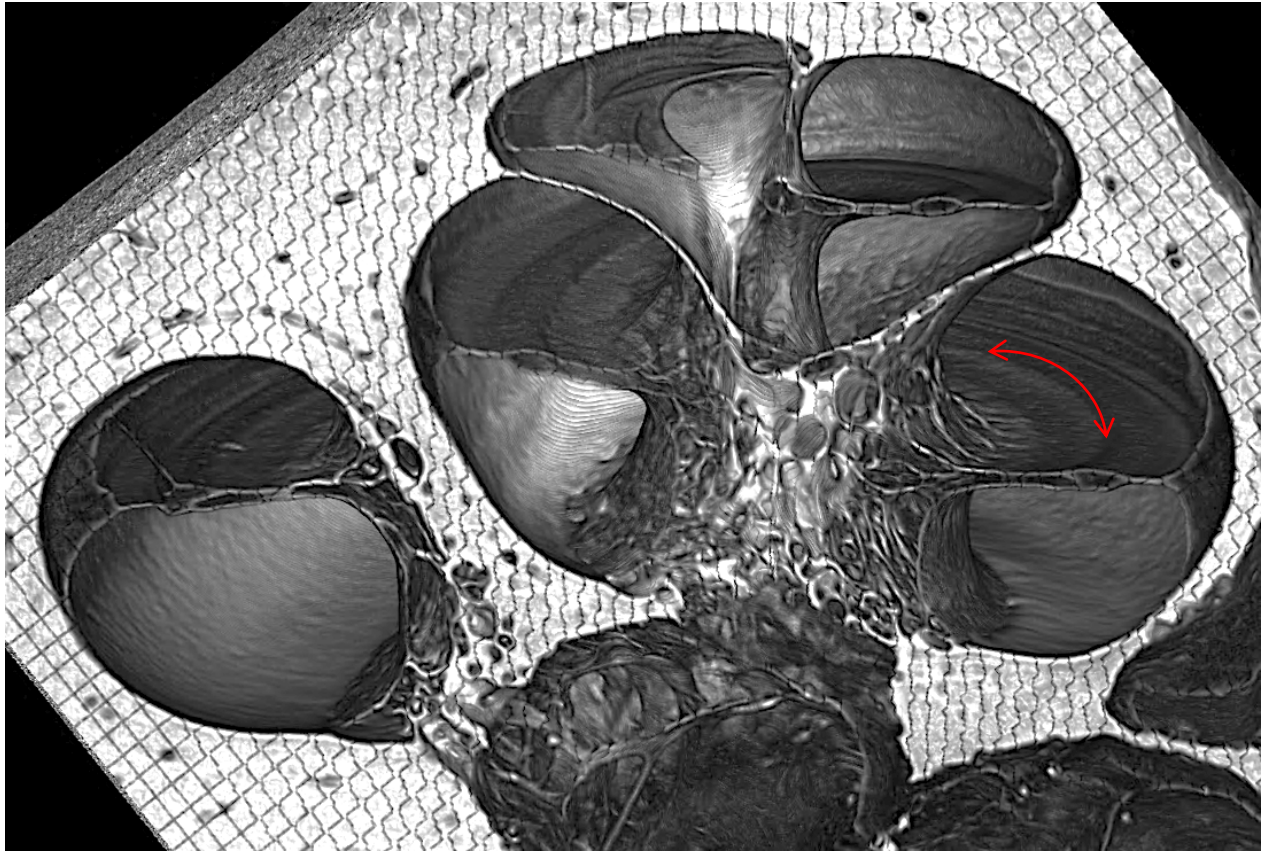


Figure 4.1. Virtual mid-modiolar cross-section through a human cochlea imaged using synchrotron radiation phase contrast imaging (SR-PCI). The location and orientation of the organ of Corti are indicated with the red arrow.

Clinical imaging techniques for human cochlear evaluation have historically failed at resolving the aforementioned cells and nerve fibers that are implicated in SNHL. Specifically, high-resolution computed tomography (CT) and magnetic resonance imaging (MRI) are incapable of providing cellular-level information about the organ of Corti's integrity in hearing-impaired patients, precluding objective diagnosis of the pathologic causes of hearing loss. In addition, the lack of a clinical intracochlear imaging technique prevents otologists from positioning electrode arrays during cochlear implantation with specificity and consistency; indeed, blind electrode array insertion may in part explain the high variability in hearing outcomes for cochlear implant patients^{6,7}.

To address these clinical needs, we developed a sub-millimeter diameter, flexible endomicroscopic probe, interfaced it with a system that performs μ OCT imaging with micron-scale resolution, and tested its ability to image the organ of Corti from within the cochlea in intact cadaveric human temporal bone specimens. μ OCT is a high-resolution descendant of optical coherence tomography (OCT), a cross-sectional imaging technique that generates two- and three-dimensional images by measuring properties of the back-scattered or back-reflected light from different depths within a biological tissue. OCT has achieved recent attention and popularity for its diverse range of clinical applications^{8,9,10}, and it is particularly well-suited for intracochlear imaging because of its minimally invasive nature, its ability to afford micron-scale resolution, and the ease with which it can be interfaced with endoscopes to access the body's interior¹⁰. The present effort was inspired by our group's demonstrations of μ OCT's ability to **a)** enable visualization of individual cells and nerve fibers in the guinea pig organ of Corti *in situ*¹¹, **b)** distinguish between healthy and noise-damaged mouse organ of Corti *in situ* (**Figure 4.2**), and **c)** allow extended depth-of-focus, micron-scale imaging when interfaced with submillimeter-diameter, flexible endoscopes¹².

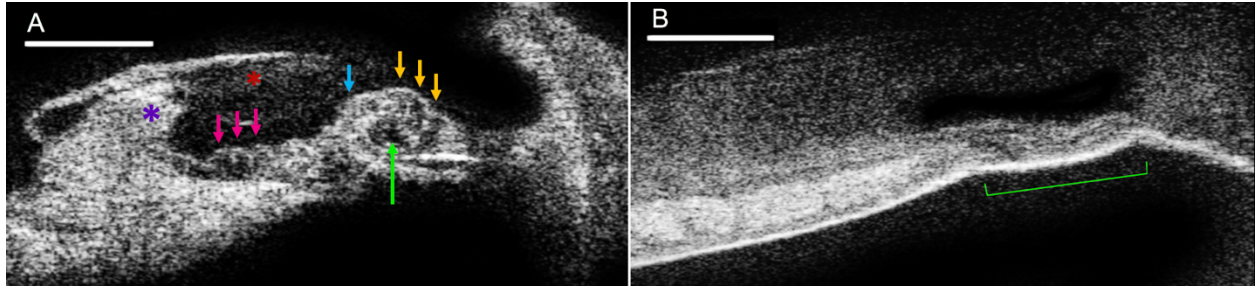


Figure 4.2. Mouse organs of Corti imaged using benchtop μ OCT. (A) Organ of Corti dissected from the basal cochlear turn of a healthy mouse. Purple = spiral limbus; red = tectorial membrane; Pink = supporting cells; blue = inner hair cell; orange = outer hair cells; green = tunnel of Corti. (B) Organ of Corti dissected from the basal cochlear turn of a mouse that was exposed 8-16kHz octave band noise at 116dB SPL for 2 hours. Flat epithelium morphology is marked with a green bracket. Scales = 100 μ m.

Our spectral domain μ OCT system achieves an axial resolution of approximately $1\mu\text{m}$ ^{12,13}. A supercontinuum laser source directs near-infrared light to a beamsplitter, which reflects 10% of the incident light through a common-path fiber optic probe (described below) and ultimately to the tissue; light reflected from the tissue is interfered with reference light to generate an interferogram and is coupled into a custom-made spectrometer (**Figure 4.3A**). A depth-resolved image is reconstructed using standard OCT image reconstruction techniques. This system is interfaced with a self-imaging wavefront division optical probe¹³ that **a)** achieves tight, perpendicular focusing via a GRIN lens-mirror complex (**Figure 4.3B**), and **b)** extends the imaging depth-of-focus via the addition of a cylindrical waveguide (in this case a multimode fiber element) positioned before the focusing lens (**Figure 4.3B**). The addition of the cylindrical waveguide results in the propagation of multiple co-axially focused pseudo-Bessel fields (**Figure 4.3C**). Light back-reflected from the distal surface of the GRIN lens was leveraged for reference in realizing a common-path interferometry system configuration. Resolution was characterized by calculating the full widths at half maxima (FWHM) of the point spread functions of a nanoparticle phantom at different distances from the probe's outer sheath (**Figures 4.3D**).

Circumferential cross-sectional images are acquired via a helical scanning mechanism. The probe is spun inside a transparent sheath such that the light, emitted perpendicular to the probe's long axis, is directed in a circular pattern. An optical rotary junction couples light from the fixed optical fiber emerging from the μ OCT system to the probe's rotating optical fiber, and the probe's optical fiber is fixed inside a driveshaft that delivers the torque to the distal end of the probe. The rotary junction was mounted onto a pullback stage that provides axial translation. During experiments, a micromanipulator was used to insert the probe through the cochlea's round window and into scala tympani, following the insertion procedure that is routine for electrode array insertion during cochlear implantation; real-time imaging was used to locate the basilar membrane and organ of Corti during insertion.

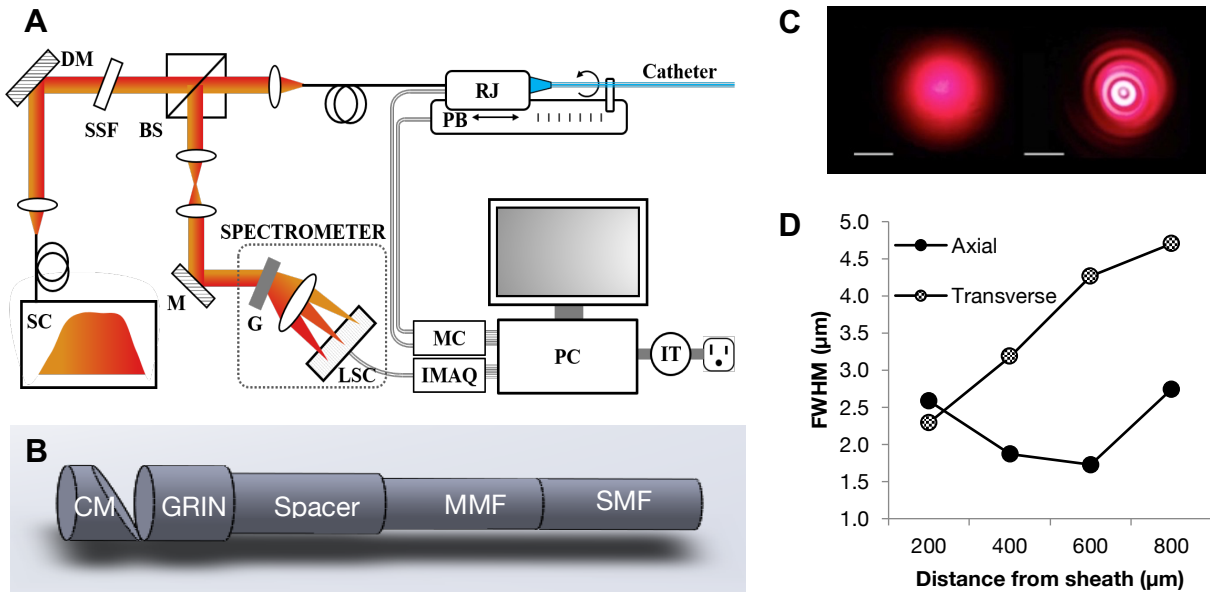


Figure 4.3. (A) Schematic depicting the elements comprising the μ OCT system that is interfaced with an endomicroscope for intracochlear imaging. DM = dichroic mirror; SSF = spectral shaping filter; BS = beamsplitter; PB = pullback stage; RJ = rotary junction; SC = supercontinuum laser; M = mirror; G = diffraction grating; LSC = line scan camera; MC = motor controller; IMAQ = image acquisition board; PC = personal computer; IT = isolation transformer.¹³ **(B)** Schematic depiction of the μ OCT probe. CM = cylindrical mirror; GRIN = graded refractive index lens; MMF = multimode fiber; SMF = single mode fiber. **(C)** Plot showing system and probe's axial and transverse resolution, measured as the full width at half maximum (FWHM) of point spread functions as a function of distance from the probe's outer sheath. **(D)** Far-field beam output profiles in the transverse plane for Gaussian (left) vs. pseudo-Bessel (right) beams.¹² Scales = 1 cm.

The current probe design facilitated insertion depths up to 8mm via the round window, corresponding to roughly the 13kHz frequency region¹⁴, in three intact human temporal bone specimens that were drilled to expose the round window membrane. Cross-sectional images acquired from within scala tympani reveal this lumen's boundaries in detail (**Figure 4.4**). The organ of Corti was clearly visualized, and in some cases, clear distinctions between damaged and intact organ of Corti were detected (**Figure 4.5**). In hypothesized healthy organ of Corti regions, mechanosensory hair cells and the tunnel of Corti were clearly identifiable, in addition to the spiral limbus and the tectorial and Reissner's membranes (**Figure 4.5A**); by contrast, flat epithelium morphology was visualized in the hook region of some specimens, suggesting organ of Corti deterioration and corresponding high frequency hearing loss in these individuals (**Figure 4.5B**). As expected, highest levels of OCT signal were visualized in the regions of the osseous spiral lamina and bone-encased lateral wall.

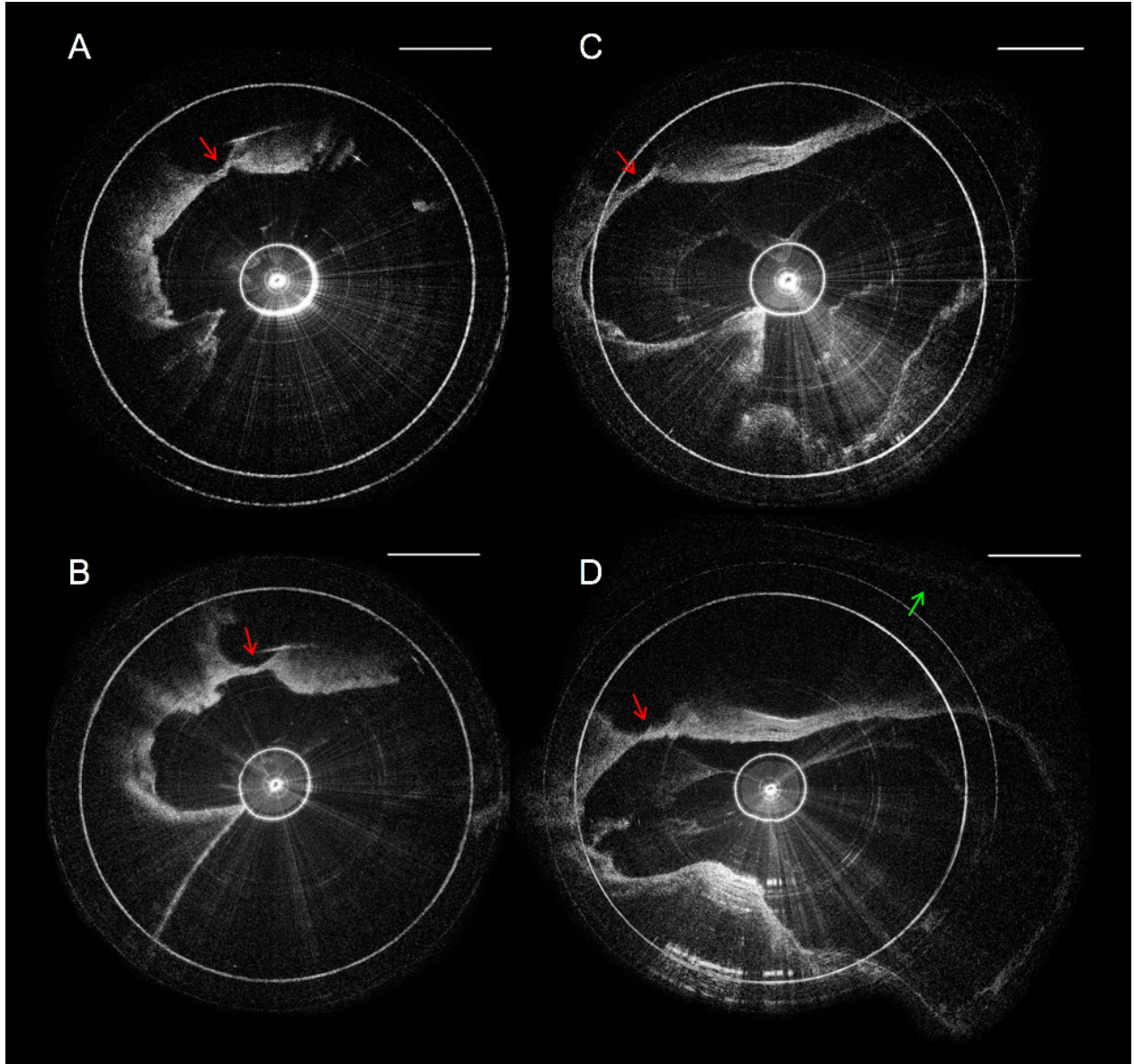


Figure 4.4. Four circularized μ OCT images of the human cochlea's interior, acquired using an intracochlear μ OCT endoscope. The 2D images depicted in panels A-D were acquired at sequentially increasing distances from the round window. The red arrow in each panel indicates the location of the organ of Corti. The green arrow in panel D indicates the upper boundary of scala vestibuli. All scales = $50\mu\text{m}$.

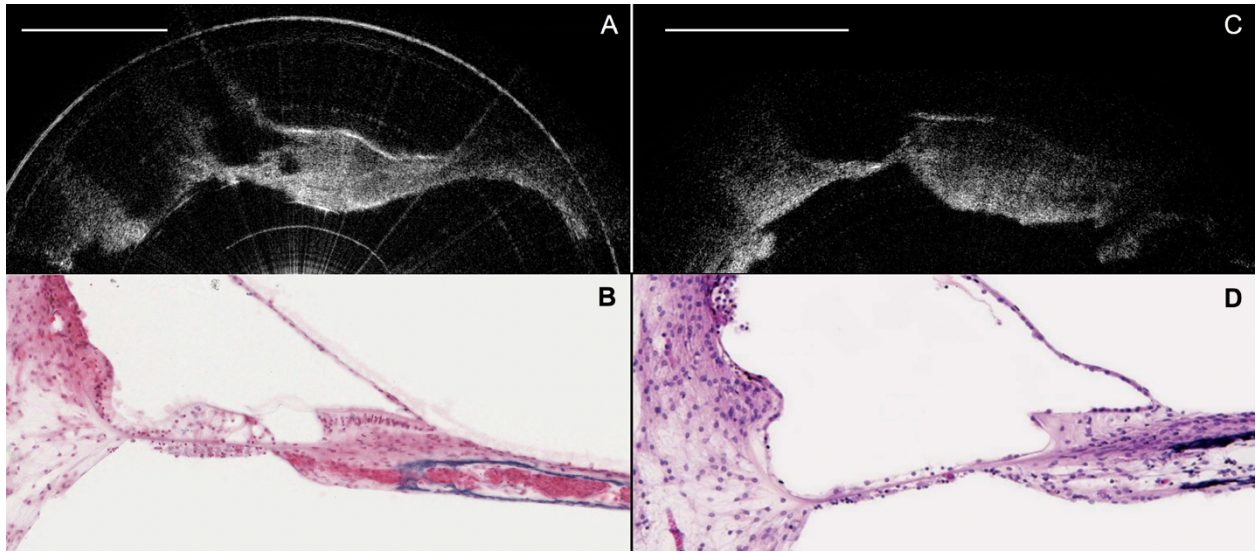


Figure 4.5. Circularized μ OCT images of the human cochlea's interior, acquired using a μ OCT endomicroscope inserted through the round window and positioned within scala tympani. (A) Region of intact organ of Corti, clearly showing the region of hair cells, the spiral limbus, and the tectorial and Reissner's membranes. Panel (B) shows a histological section acquired from a healthy region of human organ of Corti¹⁵. (C) Hypothesized region of damaged organ of Corti in the hook region of a human cochlea, showing flat epithelium morphology. Panel (D) shows a histological section acquired from a damaged region of human organ of Corti¹⁵. All scales = 50 μ m.

The present images are the first, to our knowledge, μ OCT-based images of the organ of Corti acquired with an imaging probe inserted into scala tympani via a round window approach. Previous attempts at intracochlear endoscopy have been limited predominantly to large, rigid endoscopes that are incapable of accessing structures at depths beyond a few millimeters from the round window¹⁶, or have required drilling cochleostomies for insertion. Several studies on cochlear implant electrode array insertion technique have shown that a round window approach is typically significantly less invasive than a cochleostomy approach. Lin et al. previously performed intracochlear OCT endoscopy in cadaveric human temporal bone specimens and demonstrated its ability to reveal scala tympani's boundaries, including the basilar membrane¹⁷; however, the authors had to perform an extended cochleostomy to acquire these images, and the image resolution was limited to 10-12 μ m. By contrast, we were able to demonstrate up to 2 μ m-scale resolution imaging via a minimally-invasive round window approach, and were able to see structural detail of diagnostic relevance for SNHL in the organ of Corti region.

The present results motivate further optimization of μ OCT endomicroscopy for inner ear applications. The primary limitations of the present design include the probe's rigid length (approximately 3mm), which limits insertion depth, and the helical scanning paradigm, which, due to the human cochlea's extremely tight radius of curvature, often causes nonuniform rotational distortion (NURD)-induced image artifacts¹⁸. Future iterations of this design should aim to address these limitations, in addition to further improving resolution and depth-of-focus to enable more sensitive detection of cellular-level intracochlear pathologies. If successful, the development of such an imaging tool would revolutionize the study and clinical treatment of SNHL, as it would allow, for the first time, **a)** direct association between inner ear pathology and hearing ability in a given patient, and thus more personalized and etiology-specific treatment recommendations, and **b)** real-time investigation of scala tympani for surgical guidance during cochlear implantation. In addition, it would allow researchers to accurately evaluate patient candidacy for gene therapy trials based on the integrity of their sensory epithelia¹⁹, and target drug-based SNHL therapies to specific regions in the inner ear and directly visualize the effects of administration. The present results should inspire hope for patients suffering from SNHL; this technological innovation has the potential to dramatically alter the course of therapy development for human SNHL, and will inevitably increase the speed and improve the quality of research on future cures.

Method

Specimen preparation

Four temporal bones were harvested from fresh human cadavers. A cylindrical saw was used to remove the portion of the skull containing the petrous temporal bone. Specimens were fixed in 10% formalin for two weeks, and were subsequently drilled to expose the round and oval windows. The bony round window niche was drilled away as necessary to allow full exposure of the round window region for probe insertion, and the round window membrane was punctured to provide a clear path for the probe.

Endomicroscope design and fabrication

The present self-imaging wavefront division μ OCT probe¹³ comprises a single mode fiber (630HP, Thorlabs, Newton, NJ, USA), a small segment of multimode fiber (FG050UGA, Thorlabs, Newton, NJ, USA), a spacer (FT400UMT, Thorlabs, Newton, NJ, USA), a GRIN lens (64-521, Edmund Optics, Barrington, NJ, USA), and a small custom-made mirror (**Figures 4.2B**) which directed the light in a direction nearly perpendicular to the probe's long axis. The single mode fiber, multimode fiber, and spacer components were directly spliced using a laser fusion splicer (LZM-100, AFL, Duncan, SC, USA). The small segment of multimode fiber serves as a waveguide which generates multiple propagation modes and thereby improves the system's depth of focus while maintaining high lateral resolution¹³. The light reflected from the distal surface of the GRIN lens was leveraged as reference in realizing a common-path interferometry system configuration.

Scanning

The μ OCT probe was housed within a driveshaft (Terumo Corporation, Tokyo, Japan). Helical scanning was achieved by coupling the probe's proximal end to a custom-made broad-bandwidth optical rotary junction which spun the driveshaft and the optics inside, thereby scanning the beam in a circular pattern. The rotary junction was mounted onto a pullback stage for axial translation. The rotary junction speed and system frame rate were matched at roughly 17Hz.

Imaging system and analysis

The present spectral domain μ OCT imaging system has been previously described in detail¹² (**Figure 4.2A**). In brief, light from a supercontinuum laser (SuperK Extreme EXR-15, NKT Photonics, Denmark) centered at 800nm is passed through a dichroic mirror and spectral shaping filter and is then directed toward a beamsplitter that sends 10% of the light to the sample. Light backscattered from the sample and reference is recombined and guided to a spectrometer detection module comprising a telescope system, grating, focusing lens set, and a line scan camera (Basler SPL8192, Basler AG, Ahrensburg Germany). Standard OCT image reconstruction methods²⁰ were used to generate depth-resolved images from the interferogram. The system has an axial point spread function with full width at half maximum of 2.5 μ m in water (**Figure 4.3D**), and a sensitivity of 90dB. The power output at the sample was roughly 18mW.

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Author contributions:

BY and GJT designed the imaging system and probe. JSI fabricated the probes and prepared the human temporal bone specimens. JSI and BY performed the experiments. JSI analyzed the results and wrote the manuscript. GJT and KMS conceived and led the project. All authors edited the manuscript.

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Chapter 5: Conclusions

The work presented herein demonstrates the potential of two novel imaging technologies for future clinical translation in otolaryngology: synchrotron radiation phase contrast imaging (SRPCI), and micro-optical coherence tomography (μ OCT). In the sections that follow, I summarize limitations and future directions for both of these technologies.

Synchrotron radiation phase contrast imaging for diagnosis of SNHL

The present data reveal that SRPCI is capable of resolving cellular-level detail of diagnostic relevance in the human cochlea and vestibular organs without requiring physical sectioning or staining of the respective sensory epithelia. These are compelling results because there is presently no other method that allows visualization of these sensory epithelia *in situ* – that is, in their natural 3D configurations, and without decalcifying or removing altogether the dense petrous temporal bone that encapsulates them. Indeed, even when the inner ear's bony shell is decalcified and its sensory epithelia are microdissected for research purposes, the tissue must subsequently be stained in order to achieve sufficient contrast for microscopic investigation. SRPCI, by contrast, offers cellular-level detail without any added contrast-enhancing agent. Finally, SRPCI at its core relies on an imaging method that has been used to diagnose human disease for over a century – namely, X-ray imaging. The ability to visualize the biological underpinnings of not only SNHL but also other debilitating maladies of the inner ear such as Menière's disease would inevitably lead to vast improvements in diagnostics and therapeutics for these conditions, and would allow us to establish specific targets for future treatments for these conditions.

Despite its promise, SRPCI poses several challenges that must be addressed prior to its clinical translation. The most apparent is that SRPCI presently requires a particle accelerator, typically the size of a football stadium or larger, and a large distance between the X-ray source and the sample (50 meters or more) in order to achieve the energy levels necessary to acquire the high resolution, high contrast images presented here. Clearly, requesting that all hospitals invest money and real estate in building such arenas is entirely infeasible. However, the advent of portable clinical X-ray and CT scanners inspires hope that further research efforts will eventually facilitate X-ray scanning capabilities that are similar to or better than what is achievable via SRPCI. In fact, Zeiss has already invented a microscopy system that fits comfortably inside standard laboratories and achieves the same resolution and contrast as that achieved

via synchrotron particle accelerators.¹ In addition, ongoing efforts to improve X-ray source and detection materials in addition to improved algorithms for phase contrast and k-edge detection in CT² suggest that CT technology may itself improve at a greater rate than SR technology, obviating the need to perform SRPCI specifically in more compact environments. Given X-ray imaging's widespread prevalence and demonstrated efficacy for medical imaging purposes, it is only a matter of time before single-micron resolution, high contrast imaging of the cochlea and its neighboring structures is capable via X-ray-based technology. The results presented here reveal that such advances in X-ray imaging may enable imaging of the organ of Corti at the cellular-level through the skull, revolutionizing diagnostics and therapeutics for SNHL.

Another significant limitation of SRPCI is the amount of X-ray radiation required to achieve the corresponding high resolution, high contrast images. Specifically, the amount of radiation exposure that the sample experienced in the experiments presented here is many times greater than what is currently acceptable for clinical use, precluding immediate translation. However, there has been an ongoing effort to reduce the amount of radiation and exposure time required to acquire X-ray images without sacrificing resolution since the technology's nascence. For example, a CT scan that 20 years ago took up to 20 minutes to acquire can be collected today in 1-2 seconds.³ In addition, advanced image reconstruction techniques and improved detector sensitivity have reduced the amount of radiation required for CT scanning by over 80% in the last 10 years alone.⁴ Labriet et al. demonstrated in 2018 that they are able to achieve similar resolution with lower radiation dose in the inner ear specifically in SR compared to CT images of the temporal bone in living humans, leveraging advanced acquisition and reconstruction methods to optimize resolution with minimal radiation exposure.⁵ They acquired their images using a slice thickness of 2mm (significantly larger than what is required for high resolution imaging of the cochlea), so it remains to be seen whether or not similar radiation doses can be used to achieve much higher resolution images of the cochlea specifically. Such advances and the rapid pace at which they have occurred inspire hope that this technology will eventually be capable of resolving intracochlear pathology through bone in a manner that is safe enough for use in living humans. With further optimization of controllable parameters such as energy level and sample-detector distance specifically for inner ear applications, in addition to exploration of whether or not there might be safe methods for introducing

contrast-enhancing agents into the organ of Corti (to minimize the amount of radiation required to resolve structures encapsulated in bone), SRPCI (and X-ray-based imaging techniques more broadly) promises to be an excellent candidate for a diagnostic tool for SNHL that allows us to establish biomarkers for the disease and thus therapeutic targets.

Micro-optical coherence tomography imaging for diagnosis of SNHL

The results presented herein demonstrate μ OCT's utility for enabling imaging of intracochlear structures relevant to SNHL *in situ*. Specifically, μ OCT facilitates clear visualization not only of sensory hair cells, supporting cells, and the broad cytoarchitecture of the organ of Corti, but also individual bundles of auditory nerve fibers that innervate the mechanosensory inner and outer hair cells that facilitate hearing. Given recent evidence that the auditory nerve fibers are more vulnerable to insult and demonstrate physical destruction prior to hair cells,⁶ and the facts that **a)** these nerve fibers are extremely small and notoriously difficult to isolate and track, and **b)** no contrast enhancer or stain was needed to resolve them, the latter finding is particularly remarkable and inspires hope for this technology to critically inform us about the progression of hearing loss and how to develop targeted therapies. The fact that μ OCT imaging systems are easily interfaced with miniature, flexible endoscopes, as demonstrated, makes the technique particularly well-suited not only for investigation of biomarkers of SNHL, but also for surgical guidance; indeed, our group is already developing a cochlear implant electrode array with an integrated μ OCT probe to allow specific placement of arrays during cochlear implantation. Providing neuro-otologists with visual guidance during cochlear implant surgeries would minimize the chances of poor electrode placement, ultimately improving outcomes, and allowing us to better understand the sources of variability in outcomes across patients.⁷ OCT is already used widely in clinical settings, its safety having been confirmed across several organ systems, and μ OCT imaging systems are easily transported from the benchtop to compact carts that can be wheeled safely through hospitals and that occupy minimal space in the surgical operating room. Such parameters and characteristics make μ OCT a strong candidate for clinical translation, and specifically for otologic applications.

However, intracochlear μ OCT endoscopy does pose a significant limitation: its requirement for invasive surgery. Specifically, μ OCT would likely require the same posterior tympanotomy surgical approach and insertion technique currently implemented for cochlear implantation. While this surgery is effective, its invasiveness restricts the population of hearing-impaired individuals who are encouraged to undergo this procedure to those who already have severe hearing loss, making a diagnostic evaluation at the respective stage minimally useful (especially when there is no cure). Thus, it would be unacceptable to perform this surgery purely for diagnostic purposes, i.e., in patients who have mild SNHL and who would not otherwise need to undergo invasive surgery for cochlear access, risking losing even more of their hearing or suffering from any of the other potential complications inherent to this surgery. To this end, our group is currently developing and fabricating μ OCT endoscopes that are 500-600 μ m in diameter, with rigid optics less than 500 μ m in length, and as flexible as the soft, silicone-encased cochlear implant electrode arrays that are routinely implanted today, leveraging novel fabrication techniques such as 3D-printing of optical components and lenses rather than fabricating them by hand. Such techniques may yield a device that can access the cochlea via the external auditory canal and the middle ear, eliminating the need for invasive surgery. The tympanic membrane is routinely lifted or gently punctured for treating middle ear conditions such as otitis media, and it is known to recover remarkably well from such disruption. Thus, a small, flexible endoscope that provides visual guidance as it traverses the external auditory canal, passes through or under the tympanic membrane, and ultimately reaches the cochlea's round window for subsequent insertion into scala tympani would ultimately be a much safer tool than one that requires a posterior tympanotomy surgical approach. While the first μ OCT intracochlear endomicroscopes will likely be integrated with cochlear implant electrode arrays for intracochlear investigation, our goal is to design a tool that can access the cochlea via the external auditory canal as described above, as such a tool would allow us to diagnose and establish causes of disease much earlier in the disease progression, enabling personalized intervention at appropriate times.

I hope that this work inspires hope for a future where a patient struggling with hearing loss visits their otologist, and, after a short and safe imaging session, is told what specifically is causing their impairment, how to stop it, and ideally, how to reverse it. I have learned over the course of engaging in the present work that one of the most serious impediments to scientific progress and technological

innovation is a lack of belief in the possibility of progress, especially in those in those who have the money and power to support relevant efforts. I hope that through my dissertation, I have contributed in a small way to an ongoing effort to communicate that, through creativity and interdisciplinary collaboration, *it is possible* to develop imaging methods that can image intracochlear structures in living humans and provide visual guidance during inner ear surgery, and that such developments are well under way and are promising.

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