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Comparison of traditional histology and TSLIM optical sectioning of human temporal bones

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Abstract

Hypothesis—Thin-sheet laser imaging microscopy (TSLIM) optical sectioning can be used to assess temporal bone soft tissue morphology before celloidin sectioning.

Background—Traditional human temporal bone (TB) celloidin embedding and sectioning is a lengthy and involved process. Although bone morphology can be assessed with microCT prior to traditional histology, soft tissue structures are difficult to resolve until after celloidin sectioning. A potential solution is TSLIM, a high resolution, non-destructive optical sectioning technique first developed to image bone and soft tissue in animal cochleae.

Methods—Two temporal bones from one individual were used to evaluate TSLIM's capacity to image human temporal bones (bone and soft tissue) before traditional histology. The right TB was trimmed to the cochlea, prepared for and imaged with TSLIM, then processed for celloidin sectioning. The left TB, serving as a control, was directly prepared for traditional celloidin sectioning.

Results—TSLIM imaging of the right TB showed adequate resolution of all major tissue structures, but barely resolved cells. Celloidin sections produced from the TSLIM-imaged right TB were equivalent in cytological detail to those from the traditionally prepared left TB. TSLIM three-dimensional (3D) reconstructions were superior to those obtained from celloidin sections because TSLIM produced many more sections that were without mechanical sectioning artifacts or alignment issues.

Conclusion—TSLIM processing disturbs neither gross nor detailed morphology and integrates well with celloidin histology making it an ideal method to image soft tissue before celloidin sectioning.

1. Introduction

Histological preparation of human temporal bones (TBs) is a well-established, yet time-consuming process. Excised TBs are relatively large ($2 \times 2 \times 2$ cm) and dense, requiring

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months of decalcification followed by prolonged and labor-intensive embedding, sectioning and staining. However, when processed correctly the resulting sections show excellent histology if postmortem artifacts are reduced. When a study requires such detail, the time and labor are worthwhile, but in many cases it would be beneficial to check for anatomical abnormalities before undertaking the lengthy process of celloidin sectioning.

Micro computed tomography (μ CT) is a nondestructive imaging method often used for preliminary TB imaging because it does not require lengthy decalcification or sectioning. Unfortunately it can be difficult to resolve soft tissue, and produces only relatively low-resolution ($5.9 \times 5.9 \times 5.9 \mu\text{m}^3$) images of bony structures¹. A variant of μ CT called synchrotron radiation-based micro computed tomography (SR μ CT) at HASYLAB in Hamburg, Germany has been used to image human temporal bones stained with osmium tetroxide². This method, with results similar to our light-sheet microscope, produces high resolution ($4.3 \times 4.3 \times 4.3 \mu\text{m}^3$) images of bone and soft tissue but is limited to a $3.6 \text{ mm} \times 3.6 \text{ mm}$ field of view and requires that tissues be fixed in aldehyde and osmium tetroxide. However, the storage ring DORIS III that produces the x-rays was turned off in 2012. Other agents such as phosphotungstic acid can reveal soft tissue in microCT datasets but can also obscure the borders between bone and soft tissue structures³.

Optical sectioning is another category of non-destructive imaging methods; one that is already common in animal auditory models^{4,5,6,7,8}. Two vital preparation steps make optical sectioning of such tissues possible. Unlike μ CT x-rays, visible light waves cannot pass through calcified bone. This necessitates immersion in a calcium chelator such as disodium ethylenediamine tetraacetate (EDTA), typically for 4-7 months⁹. Next, the decalcified bone is dehydrated and optically cleared in a solution that matches the refractive index of the tissue, such as Spalteholz solution¹⁰. The transparent TB can then be imaged by confocal or light-sheet fluorescence microscopy.

Confocal microscopy (CM) has been used by MacDonald and Rubel⁴ and Kopecky et al.¹¹ to image mouse cochleae, but CM is generally limited by specimen size ($\sim 500 \mu\text{m}$) and sectioning depth ($300 \sim \mu\text{m}$) within a tissue. These dimensions fall well below the minimum dimensions of the human TB in which the cochlea is $\sim 6.4 \text{ mm}$ and the vestibular apparatus is 6.2 mm ¹². Light-sheet fluorescence microscopy (LSFM) is potentially better suited to this task. LSFMs generate a laser light-sheet orthogonal to, rather than coaxial with, the optical axis. Separating the illumination and detection axes leads to greater flexibility to image larger specimens, permits superior imaging depth, and reduces photobleaching¹³. The first LSFM, orthogonal-plane fluorescence optical sectioning (OPFOS), took advantage of this principle to image guinea pig cochleae, and later, human temporal bones^{4,14}. TSLIM is an improvement on OPFOS and other LSFMs, but operates on the same principles: a light-sheet is formed by passing a laser through a beam expander, then cylindrical lens⁷. The focused light-sheet then enters an open top glass chamber containing clearing solution and a specimen mounted to a plastic rod and a micropositioner translation system. The specimen is translated in the X dimension to collect each image, then in the Z dimension to collect a new image plane. These translation steps are repeated until the entire 3D volume (up to $1 \times 1 \times 1 \text{ cm}^3$) is collected as a z-stack of high-resolution images.

Even though the two procedures share decalcification and dehydration steps, it is unclear how compatible TSLIM histological preparation is with the standard celloidin embedding and sectioning method for human TBs. A comparison between OPFOS imaging and physical sectioning was performed with guinea pigs, but the procedures were not performed on the same cochleae and 2-hydroxypropyl methacrylate was used instead of celloidin¹⁵. Therefore this study proposes to assess the compatibility of histological sectioning after the same specimen has been imaged with TSLIM.

2. Methods

2.1 Histology

This study was approved by the Institutional Review Board of University of Minnesota (# 0206M26181). Two temporal bones were removed from one normal, female patient [age 59] at 8 hrs post-mortem. The left TB was fixed in 10% formalin with 0.1% glutaraldehyde and 1% acetic acid and was decalcified for 458 days in 0.27M EDTA without formalin. After decalcification, it was rinsed in distilled water (2 changes in a day) and dehydrated in increasing ethanol concentrations from 50% up to 100% (over a period of 10 days), exchanged with ether alcohol 1:1 (3 days), and then transferred to increasing concentrations of celloidin (from 1.5% to 12% over a period of 3-4 months). The block was hardened from 12% celloidin and sectioned.

The right TB was fixed in 10% formalin with 0.1% glutaraldehyde. It was decalcified for 298 days in 0.27M EDTA without formalin and transferred to 10% formalin for three days, then shipped in formalin soaked gauze for TSLIM analysis. At Minnesota the right TB was rinsed in phosphate buffered saline, trimmed down to the cochlea and dehydrated in an ascending ethanol concentration series: 50% day 1, 75% day 2, 100% days 3-4 (two solution changes per day). Afterward the right TB was immersed in Spalteholz solution for 2 days (two solution changes per day) then stained with Rhodamine B isothiocyanate in Spalteholz solution according to Voie et al. (1993) then rinsed twice with Spalteholz solution.

2.2 TSLIM imaging

The cleared right temporal bone was mounted to a plastic rod with epoxy and submerged in a Spalteholz solution-filled, optical glass specimen chamber. The TB was translated through a 5 μm thick (z-dimension), 1 cm wide (y-dimension) light-sheet produced by a 532 nm laser with a beam waist thickness of 4.2 μm , beam expander, cylindrical lens and focusing objective. The low magnification image stack had a voxel size (XYZ) of $6 \times 6 \times 20 \mu\text{m}^3$ and consisted of 336 images of the cochlea. The high magnification stack's voxel size was $1.5 \times 1.5 \times 5 \mu\text{m}^3$ and consisted of 288 images.

2.3 Celloidin sectioning and imaging

After TSLIM imaging, the right TB was rinsed 3x in 100% ethanol to remove Spalteholz solution and Rhodamine. Processing continued for celloidin embedding in the same manner as stated previously for the left TB. Each temporal bone was serially sectioned in the horizontal plane at a thickness of 20 μm . Every 10th section was stained with H-E and mounted on a glass slide for light microscopic study. The digitized celloidin stack consisted

of 40 sections that were imaged with a digital camera for a final voxel size of $6.1 \times 6.1 \times 200 \mu\text{m}^3$.

2.4 Image segmentation and 3D reconstruction

TSLIM and celloidin image stacks were loaded in Amira 5 (VSG; Burlington, MA) for segmentation and 3D reconstruction. While the TSLIM stack was aligned as it was collected, the celloidin stack had to be aligned with Amira's "AlignSlices" module. To isolate individual structures within a stack of images we traced the borders of all three scalae using a Wacom tablet in every optical section of each stack in a process called segmentation. Once each structure was fully segmented, the labelfields were resampled in the XY plane by a factor of 5 to produce smoother reconstructions. Finally, Amira's "SurfaceGen" module was used to produce 3D isosurfaces from the resampled segmentation data. These 3D surface reconstructions were comprised of triangles that were fit to each structure's boundaries to produce a better representation of its true 3D shape.

3. Results

3.1 2D Low Magnification Sections

Low magnification TSLIM sections had adequate resolution, but inconsistent illumination (Fig. 1A). Celloidin sections exhibited fine detail consistently across each image that was only limited by the resolution bottleneck imposed by the camera (Figs. 1BC). TSLIM sections had enough detail to distinguish scalae in the cochlea, but the borders of certain structures such as Rosenthal's canal were less distinct. TSLIM's left-to-right orthogonal beam propagation can lead to uneven illumination (absorption stripes) in highly absorptive specimens, which was the case with our specimen. In contrast, bright field celloidin images have more even illumination because they are composed of transmitted light that passes through relatively thin sections.

Physical sectioning artifact was observed as a detached Reissner's membrane in the basal turn of the treated TB, which was not seen in right TB TSLIM sections or untreated left TB. The TSLIM treated, right TB celloidin sections exhibited much greater hematoxylin (blue-purple) staining relative to eosin (pink). Both issues are common to celloidin histology and were not attributed to TSLIM processing.

3.2 2D High Magnification Sections

High magnification TSLIM and celloidin sections showed detail of tissue structures (Fig 2ABC). Structures such as stria vascularis, spiral ligament, organ of Corti and Reissner's membrane were visible in each image. While individual cells and nuclei were barely resolved in TSLIM images (Fig. 2A), they were clearly visible in celloidin sections (Figs. 2BC). Celloidin sections were largely free of imaging artifacts (but did contain mechanical sectioning and alignment artifacts), but tissue inhomogeneities introduced diagonal absorption lines that obscured some cellular details in TSLIM optical sections.

3.3 Z Dimension - 3D Reconstructions

TSLIM produced an image stack with a z-resolution of 20 μm and was superior to that of traditional histological sectioning (Fig. 3A). In addition, since TSLIM stack images were obtained nondestructively, they are well aligned and contain none of the mechanical or alignment artifacts typical in celloidin sectioning. Also, the coarse z-resolution (here, 200 μm) typically obtained in celloidin sectioning produced rough 3D reconstructions at the tangential ends of the image stack (Fig. 3B).

4. Discussion

Human temporal bones with congenital middle and/or inner ear anomalies are among the most important specimens to evaluate histopathologically and histologic sectioning can be aided by 3D reference points. Currently, μCT scans reveal preliminary bony features of the middle and inner ear, but until recently it was difficult, if not impossible, to “preview” soft tissue morphology. TSLIM seems to fulfill this role well, providing an early look at areas of interest in the inner ear. Furthermore, its preparation steps do not disturb the morphology of structures prior to celloidin sectioning. Because TSLIM preparation shares both decalcification and dehydration steps with the celloidin procedure, the only time added is the few days needed to clear, image, then return to ethanol. In fact, TSLIM imaging could potentially save time if used to screen for pathology (i.e. hydrops), thus avoiding the need for up to five months of celloidin embedding, sectioning and H-E staining. As an automated, 3D technique TSLIM is able to collect enough Z-slices to represent the entire volume of a specimen. With these perfectly-aligned, digital slices an observer can easily scroll through a specimen and observe pervasive pathology or search for localized abnormalities that might have gone overlooked in coarsely Z-sampled celloidin sections.

For all its potential benefits, there are drawbacks to TSLIM imaging. At the moment, trimming is necessary to achieve the best possible image resolution because the clearing process needs to be improved to reduce light absorption and scatter. This imperfection prevents the beam from achieving its ideal focus, which in turn produces a poor quality image. This problem is only compounded by superfluous tissue, so the middle and inner ear are imaged separately and excess bone is removed from each. Another issue is inconsistent illumination caused by beam propagation across highly absorptive specimens. Absorption also leads to small-scale artifacts such as lines seen in higher magnification images. However, we have recently implemented *sTSLIM*, which produces a light-sheet by scanning a focused laser point through the specimen with a galvanomirror¹⁶. *sTSLIM* has virtually eliminated absorption artifacts. Addressing these issues will make TSLIM a better diagnostic tool, so we are currently looking into better clearing solutions to potentially reduce the amount of trimming needed. Also, we have begun perfusing Rhodamine B isothiocyanate through the scalae, rather than immersing the entire bone. This limits fluorescent dye to the areas of interest, allowing the light-sheet to pass unabsorbed through the transparent bone. Further developments, such as antibody staining of the whole TB, should expand on TSLIM’s usefulness in studying temporal bone pathology.

Acknowledgments

SBJ performed TSLIM processing and imaging, compiled the results, prepared the figures and wrote the manuscript. SC provided input on temporal bone anatomy and assisted with trimming the bone and writing the manuscript. JTO performed celloidin histology and imaging and helped write the manuscript. PAS helped compile the results, prepare the figures and write the manuscript.

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Figure 1.

A. Fluorescent, Rhodamine B stained, thin-sheet laser imaging microscopy (TSLIM) cross-section of the cochlea from the whole right TB. Reissner's membrane is preserved B. Hematoxylin and eosin (H-E) stained, serial celloidin section from the same TB and region as A. Note the preservation of Reissner's membrane in the basal turn of A (white asterisk) and the detached membrane in B (black asterisk). Most structure boundaries are visible in B, such as the border between scala tympani and the spiral ligament (black arrow), while this area has been obscured by the absorptive and refractive properties of the TSLIM-sectioned TB (A, white arrow). C. H-E stained, serial celloidin section from the left TB (flipped),

which was not TSLIM processed. Note the preservation of Reissner's membrane (asterisk) and typical appearance of H-E staining. Bar=500µm

Figure 2.

A. High magnification thin-sheet laser imaging microscopy (TSLIM) basal turn cross section of the right temporal bone (TB) showing absorption artifacts (dark lines at arrow). B. High magnification basal turn celloidin cross section of the TSLIM processed right TB. Reissner's membrane became detached from the spiral limbus in the right TB (panels A and B). C. High magnification basal turn celloidin cross section of the left TB (not TSLIM processed). Note the cell nuclei in the spiral ligament in panels C and D. Bar=100 μ m.

Figure 3. 3D reconstruction of scalae vestibuli (v), media (m) and tympani (t) from the right TB thin-sheet laser imaging microscopy (TSLIM) stack (A) and celloidin stack (B). Note the celloidin reconstruction is rougher in tangential regions of the Z-stack (black arrows) and an area of scala media is prematurely closed (white arrow). Bar=500μm.