Published in final edited form as:

J Neurosci Methods. 2009 May 15; 179(2): 201–207. doi:10.1016/j.jneumeth.2009.01.034.

Visualization of spiral ganglion neurites within the scala tympani with a cochlear implant *in situ*

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Abstract

Current cochlear histology methods do not allow *in situ* processing of cochlear implants. The metal components of the implant preclude standard embedding and mid-modiolar sectioning, and whole mounts do not have the spatial resolution needed to view the implant within the scala tympani. One focus of recent auditory research is the regeneration of structures within the cochlea, particularly the ganglion cells and their processes, and there are multiple potential benefits to cochlear implant users from this work. To facilitate experimental investigations of auditory nerve regeneration performed in conjunction with cochlear implantation, it is critical to visualize the cochlear tissue and the implant together to determine if the nerve has made contact with the implant. This paper presents a novel histological technique that enables simultaneous visualization of the *in situ* cochlear implant and neurofilament – labeled nerve processes within the scala tympani, and the spatial relationship between them.

Keywords

auditory nerve; cochlear implant; immunohistochemistry; peripheral processes; regeneration; scala tympani; stereoscope

Introduction

In mammals, cochlear hair cell loss is a permanent and irreversible event, which often initiates auditory nerve deterioration. This nerve degeneration begins at the level of the afferent and efferent processes that innervate the hair cells, and may continue with the primary ganglion cell bodies (Spoendlin, 1975). In such cases of severe hearing loss, cochlear implants (CIs) offer an effective treatment option. CIs function by electrically stimulating the remaining auditory nerve and bypassing the function of lost hair cells. The status of the nerve, and its ability to receive and process electrical signals, is therefore a critical factor in the success of cochlear implantation. While mammalian hair cells cannot currently be regenerated to a functional level, there is well-established evidence that the introduction of growth factors can promote auditory nerve ganglion cell survival following hair cell loss (Marzella and Clark,

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1999; Roehm and Hansen, 2005). In addition, it has been shown that an increase in ganglion cell survival is correlated with improved electrophysiological thresholds for CI stimulation (Yamagata et al., 2004; Shepherd et al., 2005).

Once distal processes are regressed, the primary auditory neurons remain relatively far from the CI stimulation source. It may be beneficial for CI users if the peripheral processes of the auditory nerve could be preserved or directed to regrow towards and make contact with a CI. Decreasing the distance between the auditory nerve and the CI has been shown to lower thresholds and decrease the spatial spread of excitation in human CI patients (Cohen et al., 2005), animal subjects (Shepherd et al., 1993), and model systems (Briaire and Frijns, 2006). By stimulating nerve fibers instead of the more distant cell bodies, a lower level of current may be required to elicit a neural response, which may increase the battery life of the implant and reduce cellular damage. Battery power is a limiting factor in the parameters of electrical stimulation available from a CI; increasing the battery life could allow for more complex stimulation strategies. In addition, there is the potential for increased specificity of electrical stimulation and reduced channel interaction when neural impulses are initiated at nerve fibers instead of the cell body. Any of these improvements in electrical signal processing could improve the hearing experience for CI recipients.

Spontaneous regeneration of auditory nerve peripheral processes has been reported in mammals following both acoustic trauma (Bohne and Harding, 1992; Lawner et al., 1997) and aminoglycoside ototoxic deafening (Webster and Webster, 1982). Growth factor-induced regeneration of nerve fibers has also been seen both *in vivo* and *in vitro* (Malgrange et al., 1996; Staecker et al., 1996; Cho et al., 1998; Wise et al., 2005; Glueckert et al., 2008). *In vivo* studies have shown fiber growth both within the habenula perforata, where processes are normally located (Wise et al., 2005; Glueckert et al., 2008), as well as within the scala tympani (ST), the fluid-filled space within the cochlea where CIs are placed (Leake and Hradek, 1988; Staecker et al., 1996). While several lines of research are aimed at promoting peripheral processes regrowth into the ST to contact a CI, current histological assays are not well suited to visualizing the results. If future work on auditory nerve regeneration proceeds to the level of directing growth to make contact with a CI, it will become necessary to visualize the implant and the regenerated fibers simultaneously while minimizing the perturbation of the regrown nerve fibers.

Typical cochlear preparations involve decalcification, embedding, and sectioning to produce mid – modiolar images of the interior of the cochlea. These mid – modiolar sections do not allow visualization of both the implant and the scala tympani because highly specialized sectioning tools are required to cut through the metal wire used in the construction of CIs, and the implant must therefore be removed prior to analysis. Surface preparations of various cochlear structures are also commonly used, but this technique typically exposes only one surface. Any microscopic analysis would require plating and coverslipping the specimen, eliminating the ability to examine the space within the ST. Inverted confocal microscopes are also unsuitable because the magnification levels are typically too high for proper visualization of the entire implant and its spatial relationship with the cochlea. We have therefore developed a technique that utilizes a stereoscope and preserves the placement of the CI, yet allows visualization of fluorescently – labeled neuronal fibers and preserves the spatial relationship between the implant and the fibers. The method detailed in this paper includes both a specialized cochlear dissection and the subsequent tissue processing. This method represents a novel visualization technique that provides a means for characterizing the presence and location of nerve fibers within the scala tympani with respect to a CI in a way that preserves the fidelity of the *in vivo* relationship.

Methods and materials

Subjects

Eleven male, pigmented guinea pigs (Elm Hill, Chelmsford, MA) were used in this study. Guinea pigs have cochleae that are mature at birth, similar to humans, which makes them a better animal model for cochlear implant research than other rodents. Animal weights ranged from 400 – 600 g at the time of implantation. Following systemic deafening, animals were implanted with two – electrode CIs through a cochleostomy near the round window. Animals retained their implants for four weeks, after which they were sacrificed for temporal bone removal and cochlear dissections. This study was performed in accordance with National Institutes of Health Guidelines (Guide for the Care and Use of Laboratory Animals, 1996). The University Committee on the Use and Care of Animals at the University of Michigan approved the experimental protocols. The Unit for Laboratory Animal Medicine at the University of Michigan provided veterinary care and animal husbandry, in facilities certified by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl).

Deafening procedure

Animals were systemically deafened using a combination of kanamycin (400 mg/kg, SQ) and ethacrynic acid (40 mg/kg, IV, two hours following kanamycin). This procedure is a standard means of mimicking human hearing loss, and effectively eliminates cochlear hair cells bilaterally (West et al., 1973; Xu et al., 1993). Hearing loss was confirmed with auditory brainstem responses (ABRs). ABRs were measured prior to deafening and four weeks post – deafening and hearing loss was defined as a shift of 40 dB SPL between the two measurements. Hair cell loss was confirmed with post – mortem histological analysis. Animals were deafened prior to implantation to initiate a regression of peripheral processes and increase the likelihood of fiber growth into the ST.

Implant construction

Implants consisted of two stimulating electrodes and were constructed in – house, as previously described (Miller et al., 1995). Briefly, a wire electrode was wrapped in a helix (300µm length and diameter) around an insulated wire leading to ball electrode (450µm diameter). All wires were platinum/iridium (A & M Systems, Sequim, WA). The lead wires from the electrodes were threaded through a piece of silicone tubing 35 mm long and connected to a base pedestal. The implant was inserted approximately 2 mm into the ST (Fig. 1).

Implantation

All animals were unilaterally implanted in the left ear. For general anesthesia, animals were given a mix of ketamine (40 mg/kg) and xylazine (10 mg/kg) (IM). Animals were also given atropine (0.05 mg/kg, SQ) to aid in respiration, and kept warm using a heating blanket. Lidocaine was used as a local anesthetic, and an incision was made in an arc caudal to the pinna of the ear to be implanted. The underlying muscle and tissue were pushed back to reveal the bulla and a small hole was made in the bulla with the tip of a scalpel blade, exposing the middle ear space, the basal turn of the cochlea, and the round window. A bone drill and a 0.7 mm diamond burr was used to make a cochleostomy in the wall of the ST apical to the round window, and the two – electrode CI was placed into the base of the cochlea. Implants were inserted approximately one – third to one – half of the way into the basal turn of the cochlea. A secondary hole was made in the bulla, and a silk suture was threaded through the two bulla holes and used to tie the implant to the temporal bone and prohibit movement of the implant within the cochlea. Carboxylate cement covered both holes in the bulla and secured the position of the implant. The silicone tubing containing the lead wires of the implant was tunneled under

the skin from the incision behind the ear to the midline of the skull where the implant base pedestal was secured to the skull of the animal using screws and dental acrylic. The skin incision was sutured in two layers and the animal was given warm subcutaneous saline and yohimbe (1 mg/kg) to reverse the effects of xylazine and aid in recovery.

Sacrifice and initial dissection

Four weeks post – implantation, animals were given an overdose of anesthesia (as above) and decapitated. Excess tissue and muscle from the neck and head were carefully removed until the silicone tubing of the implant leading into the left temporal bone was visible. The implant was cut with scissors outside the temporal bone and the remainder of the connective tissue and muscle attached to the temporal bone was removed so that the entire left (implanted) temporal bone could be dissected out. The right temporal bone was also removed. Each temporal bone was immediately submerged in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH = 7.3). While in 4 % PFA, the temporal bone was carefully trimmed to reveal the cochlea. The region of the temporal bone that contained the carboxylate cement and silk suture, which held the implant in place, was left undisturbed. A small portion of the bone covering the apex of the cochlea was removed and one to two mL of PFA were locally perfused into the fluid spaces of the cochlea via this apical hole. The entire temporal bone, with the implant held in place by the suture tie in the bulla, was left in 4 % PFA for 24 hours to allow thorough fixation.

Basal cochlear bone removal

Following fixation, temporal bones were rinsed in PBS and placed in a 35 mm Petri dish in PBS. The temporal bone was trimmed such that the cochlea and the portion of the temporal bone that held the implant in place remained intact and the implant was immobile. The bone where the basal turn of the cochlea meets the temporal bone wall (black arrow, Fig. 1) was carefully thinned using a bone drill and a 3.0 mm carbide cutting burr (Osteon™). Once the bone was thin and translucent, a 1.0 mm diamond burr (Osteon™) was used to carefully create a cochleostomy in the basal cochlear wall, apical to the original implant cochleostomy. This hole was carefully expanded (dashed line, Fig. 1), using either the 1.0 mm diamond burr or fine-tipped forceps, to approximately 3 mm long, until the entire CI within the ST could be visualized.

Immunohistochemistry and fluorescent stereoscopy

Following removal of the basal turn outer wall, the entire temporal bone specimen was rinsed by submerging it twice in PBS for 5 minutes each. Specimens were permeabilized in 0.3 % Triton X – 100 (Sigma) for 10 minutes and rinsed with PBS for 5 minutes. Specimens were then exposed to a mouse monoclonal anti – neurofilament antibody (160kD, Millipore) diluted 1:100 in PBS/0.1 % bovine serum albumin (BSA, Sigma)/1 % goat serum, for one hour at room temperature. The 160kD neurofilament subunit was chosen for this study because it labels both type I and type II ganglion cells and their processes and labeling is not dependent on phosphorylation (Berglund and Ryugo, 1991). For this primary antibody step, which would have required a large amount of antibody to fully submerge the entire temporal bone, specimens were placed cochlear side down in a glass vial and enough solution was added to fully cover the cochlea but not all of the temporal bone (approximately 1 mL). During the soaking time, several local perfusions of the primary antibody solution were rinsed directly into the cochlea. Specimens were rinsed twice in PBS/0.1 % BSA/1 % goat serum, for 5 minutes each, and then placed into a solution containing the secondary antibody: goat anti-mouse FITC (Jackson), diluted 1:200 in PBS/0.1 % BSA/1 % goat serum. Specimens were left in the secondary antibody solution overnight at 4°C. As with the primary antibody solution, temporal bones were not completely submerged in the secondary antibody solution, but instead only the

cochlear portion was covered. Following the overnight incubation, the tissue was rinsed three times with PBS for 5 minutes each. Specimens were observed and photographed under a Leica MXFL III stereo fluorescence microscope (Leica, Eaton, PA) equipped with a 10x eyepiece, a 1.6x lens, and 0.8 – 10x focus knob. This allowed for a magnification range of 12.8x – 160x. Adobe Photoshop™ software was used to adjust contrast levels in all images.

Results

Our neurofilament immunohistochemistry protocol was successful in labeling the auditory nerve and the spiral ganglion neurites in the scala tympani with a cochlear implant *in situ*. These processes could be visualized in the same field of view as the implanted electrode with a low-magnification stereoscope, following our cochlear dissection which left the implant intact within the cochlea.

Immunohistochemistry of the central auditory nerve

The auditory nerve central processes in each temporal bone were examined under fluorescent light to verify successful labeling of neurons with neurofilament. Each temporal bone was turned cochlear side down (apex down) to reveal the auditory nerve as it exited the temporal bone at the internal auditory meatus. This was the region of the auditory pathway where the nerve would pass from the internal auditory meatus to the cochlear nucleus in an intact system. However, because only the temporal bone itself was preserved for dissection and immunohistochemistry, the auditory nerve was severed from the rest of the auditory pathway and could be visualized. Images of the nerve were taken in both bright light and fluorescent light, and Fig. 2 shows an example of fluorescing auditory nerve fibers. This image contains two control specimens that verify the specificity of our immunohistochemistry protocol. The specimen in Fig. 2A & B received the full immunohistochemical protocol, the specimen in Fig. 2C & D did not receive the primary antibody step of the protocol, and the specimen in Fig. 2E & F did not receive any staining. There were no fluorescing fibers seen in the auditory nerve of any specimen that did not receive the full immunohistochemical protocol. The images under fluorescent light (right panel, Fig. 2B, D, F) show the auto – fluorescence of the temporal bone. There was a noticeable lack of illumination in the region where the auditory nerve is (black dashed lines) without the full immunohistochemical protocol (Fig. 2D, F) but a significant increase above background levels in the auditory nerve that received the full protocol (Fig. 2B). Fluorescing central auditory nerve fibers were verified in all specimens prior to further analyses.

Immunohistochemical visualization of neurofilament – labeled processes within the scala tympani

Fig. 3 shows both bright light and fluorescent light images of the exposed ST from one animal at two magnification levels. At lower magnification levels (Fig. 3A & B), neurofilament – labeled processes are visible where the central auditory nerve exits the temporal bone (black arrow), as well as within the scala tympani (white arrow). As the magnification level is increased (Fig. 3C & D), neurofilament – labeled processes can be seen more clearly (white arrows). The scala tympani from three additional animals are shown in Fig. 4. Fig. 4A and 4B demonstrate that it is possible to view single fibers and the implant in the same view. At the highest level of magnification possible with the Leica stereoscope, 160x, neurofilament – labeled fibers are visible in the direct vicinity of the implant (white arrows, Fig. 4B). Fig. 4C shows a normal non – deafened, non – implanted cochlea following our immunohistochemical protocol, where no neurofilament – labeled fibers were visible within the scala tympani.

Six out of the eleven implanted cochleae (55%) that were examined exhibited neurofilament – labeled processes within the scala tympani. Our visualization technique allowed us to see

neurofilament – labeled processes in various locations throughout the basal turn of the cochlea, including near the lateral cochlear wall (Fig. 4A), near the cochlear implant (Fig. 4B), near the original cochleostomy, and near the basilar membrane. This diversity was expected, as peripheral processes growth can be random and disorganized (Bohne and Harding, 1992;Staecker et al., 1996), and there is currently no method to consistently attract nerve growth into the scala tympani. In addition, some cochleae exhibited fibrotic tissue growth within the scala tympani, which is common with long-term cochlear implantation (Ni et al., 1992;Shepherd et al., 1994). This tissue growth did not label with the neurofilament antibody, indicating that our labeling process was specific to neuronal processes only.

Discussion

In this study, we show that it is possible to visualize neuronal fibers at stereoscopic levels in relation to a CI *in situ*. Our technique leaves the CI in place within the cochlea, allows for very little movement of the implant during processing, allows visualization of the space within the ST, and reduces damage to cochlear tissue by performing minimal dissection. This represents a new technique to aid experiments that need to characterize the physical relationship between neurites and a CI *in vivo*.

The highest magnification level available with the stereoscope used in this study was 160x, a relatively low level compared to other histological methods. However, this level was sufficient to show individual nerve processes within the scala tympani (Fig. 4B). Lower magnification levels also showed fibers in both in the central auditory nerve (Fig. 2) and the scala tympani (Fig. 3). These figures demonstrate that the method described here can simultaneously provide images of the small nerve fibers and the larger implant, as well as the three-dimensional space around them.

The method described here may be best employed in conjunction with other cochlear histology methods. Due to the size of the implant, a stereoscope is necessary to visualize the entire implant within the cochlea, but the coarse nature of stereoscopic focus may make it difficult to determine the exact location of fluorescently – labeled fibers within the ST. Finer resolution could be found using mid-modiolar sectioning, whole mount preparations of the basal turn, or confocal microscopy, any of which could be implemented following the method described in this paper. For example, a series of z – stacks using a confocal microscope could provide images that are more accurate and include more detail of the location of fluorescently – labeled fibers. However, the stereoscope must first be used to determine the general location of fibers because the high magnification of the confocal microscope does not permit visualization of the entire implant and the fibers in the same view. The technique described in this paper could therefore be an important preliminary histological step in auditory nerve regeneration studies.

This technique should be used as qualitative assessment of neurites within the cochlea and not a quantitative metric, for several reasons. First, the focal adjustments on the stereoscope are not fine enough to ensure accurate imaging of neurites. Second, there is limit to the time spent capturing images of fluorescing fibers, and anti – fade medium is cannot be applied to these specimens because of air exposure. Individual variability in the strength of labeling is quite possible with images taken without anti – fade medium due to the length of light exposure prior to image capture. This can be seen in the difference in signal strength between the central nerve fibers seen in Fig. 2 and the peripheral fibers seen in Fig. 3. The images of the central nerve fibers were taken prior to the images of the peripheral nerve fibers, to verify successful immunohistochemical processing. The processes in the scala tympani images therefore received longer light exposure and did not fluoresce as brightly as the central nerve fibers. Finally, there is the possibility that fibers grew medial to the cochlear implant and were blocked from view by the implant. These neurites could be seen in cross sections of the cochlea, and

potentially counted in these images, but these sections are taken after implant removal, which will most likely disrupt or shear any fibers that have grown within the scala tympani. Any of these complications would likely make any neurite count an underestimate.

While we focused on neurofilament labeling, this protocol could be easily adapted for use with a host of neuronal and synaptic markers. Neurofilament labeling does not tell us whether the nerve growth seen within the scala tympani was afferent or efferent, regenerated fibers, or whether the nerve is functional. However, other markers, such as ChAT, synapsin, or GAP43, that could further define the fiber growth. Additional immunohistochemistry could be performed following this initial characterization, allowing gross visualization of nerve processes while not precluding the use of sectioning if microanalysis is also desired. Modifications to the immunohistochemical protocol, such as the choice of blocking agent and antibody hosts, should be considered if further labeling will occur.

The current paper does not attempt to regrow auditory nerve peripheral processes, but rather develop a method for visualizing nerve fibers and their spatial relationship to a cochlear implant. Animals were deafened prior to implantation and tissue processing to disconnect peripheral processes from hair cells and encourage growth into the scala tympani. Indeed, neurofilament-labeled processes were only seen within the deafened cochleae, not within the non-deafened cochleae (Fig. 4). Growth of these processes into the scala tympani following hair cell loss has been seen previously (Leake and Hradek, 1988; Staecker et al., 1996). The fibers seen in these previous studies and in the images of this paper (Fig. 3 and Fig. 4) exhibited uncontrolled and indistinct growth patterns. The technique detailed in this study could provide a valuable tool for viewing nerve growth and help to refine future experiments directed at controlling and promoting the regrowth of auditory nerve fibers. This method provides an easily adaptable means to view fibers within the three-dimensional space of the scala tympani in conjunction with a cochlear implant.

Acknowledgements

We thank Lisa Beyer, Mark Crumling, Debbie Colesa, and Gina Su for technical support and assistance in completion of this study. This work was supported by the R. Jamison and Betty Williams Professorship, the Alfred A. Taubman Institute, and NIH/NIDCD Grants R01-DC007634, F31-DC009134, T32-DC00011, P30-DC05188.

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Figure 1. Basal cochlear dissection with implant in place

The left cochlea of a guinea pig is shown with the in – house constructed cochlear implant within the scala tympani four weeks post – implantation. The basal cochlear bone around the implant was carefully removed to reveal the location of the implant within the scala tympani without disturbing the implant or tissue within the cochlea. The dashed line indicates the perimeter of the area of bone removal. The black arrow shows the dissection starting point, where the temporal bone meets the cochlea and the bone is very thick relative to the rest of the cochlear bone. The white arrow indicates the original cochleostomy where the implant was inserted into the cochlea. Labels: b = ball electrode, h = helix electrode, ST = scala tympani, SV = scala vestibuli. Scale bar = 500µm.

Figure 2. Neurofilament staining of the central auditory nerve

The auditory nerve as it exits the internal auditory meatus is shown under bright light (left panel) and fluorescent light (right panel). Dashed lines indicate the auditory nerve. Neurofilament – labeled fibers are visible within the auditory nerve under fluorescent light at mid – level magnification (51.2x) only in tissue samples that underwent the entire immunohistochemical protocol (A, B) and not in samples that did not receive the primary antibody (C, D) or did not receive any staining (E, F). Scale bar = 200 μ m.

Figure 3. Neurofilament staining within the cochlea

The interior of the cochlea and the cochlear implant (b = ball electrode) following our dissection from the same specimen is shown at two magnification levels, under bright light and fluorescent light. (A) and (B) are low magnification images that show the entire cochlea, and the central auditory nerve exiting the temporal bone shows neurofilament – positive fibers (black arrow). (A') and (B') are middle magnification level images and neurofilament – labeled processes are visible within the scala tympani under fluorescent light (white arrows). Scale bar = 500µm.

Figure 4. Examples of neurofilament – labeling within the cochlea

Images from three different animals are shown. In (A), the ball electrode of the cochlear implant (b) and neurofilament – positive processes (white arrow) can be seen in the same view, although the implant is at a lower focal plane than the processes. In (B), at the highest magnification level possible with the stereoscope used here (160x), the implant electrode is clearly recognizable and individual processes can be seen (white arrows). In (C), a normal, non – deafened, non – implanted cochlea does not show any neurofilament – positive fibers within the scala tympani, ST = scala tympani, SV = scala vestibuli. Scale bar = 200µm in all images, dashed lines indicate the area where the lateral wall of the cochlea was removed to view the implant.