Visualizing collagen fibers in the basilar and tectorial membranes using CNA35-OG, a fluorescently labelled bacterial-adhesion protein

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Abstract

Background: The tectorial membrane (TM) is a complex acellular structure that extends from the spiral limbus and covers a part of the organ of Corti, running along the entire cochlear spiral. It is required for normal hearing, interacting with the stereocilia of the hair cells that are essential for mechanoelectrical transduction. The TM is comprised of 97% water, collagen fibrils (II, V, IX and XI), and various non-collagenous glycoproteins such as α-tectorin, β-tectorin, ceacam16, otogelin, otogelin-like, and otoancorin. As an acellular matrix, the TM is not easily stained with vital dyes and is therefore difficult to visualize. We produced a fluorescently labeled collagen-specific probe using a technique that takes advantage of the inherent specificity of collagen-binding protein domains present in a bacterial adhesion protein (CNA35), obtained from *E. coli*, which allows for a detailed view of collagen fibers. In this study we present confocal images of regions of gerbil cochleae stained with CNA35-OG.

Methods: Recombinant poly-histidine-tagged CNA35 (from the Maarten Merkx lab in the Netherlands) was expressed in E. coli and purified by cobalt-affinity chromatography before labeling with OregonGreen488-succinimidyl ester. OregonGreen488-labeled CNA35 was further purified by gel filtration chromatography. Mongolian gerbils of both sexes were used. Freshly harvested bullae were harvested at the end of experiments. The bullae were opened, middle ear ossicles were removed, and the cochleae were irrigated with 1-2 μM CNA35-OG through the oval window. The samples were immersed in approximately 1.5 ml of CNA35-OG and stored at room temperature for times ranging from 2 hrs - overnight. The cochleae were then placed in 4% formaldehyde and fixed at 7 °C overnight (or up to 4 days), and then decalcified in EDTA (120 mM) for 3 days. Dissection was done under phosphate buffer saline (PBS), and samples from the basal and middle turn were obtained. The dissected turns were imaged using the 488 nm laser on a Nikon A1R laser scanning confocal microscope.

Results: CNA35-OG dye stains both the basilar membrane (BM) and tectorial membrane. In addition, volumetric images allowed us to visualize the shape of these structures and the organization of the collagen fibrils within them.

Conclusion: The confocal images illustrated in this report represent a unique view of the tectorial membrane in the fixed and decalcified gerbil cochlea. The use of CNA35-OG dye allowed a detailed visualization of the TM collagenous fibers and the attachment of the TM to the spiral limbus. Future studies will analyze the effect of injecting collagenase into scala media, and its impact on TM structure, cochlear mechanics and cochlear amplification.

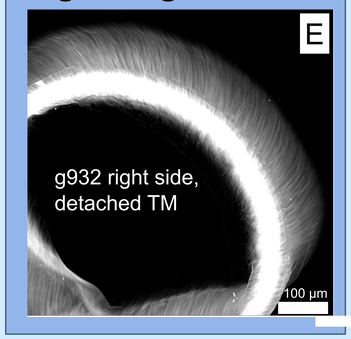
Dissection approach

Using a silicone-coated petri dish filled with PBS, the basal turn was separated from the middle and apical turns. One blade of spring scissors was inserted into the oval window and cuts were made along the lateral wall of the basal turn. The scissors were repositioned by inserting the other blade into the region just cut and the first blade medial to the oval window to isolate the basal turn. Decalcified bony tissue basal to the basal organ of Corti was removed, and a series of small cuts were made to remove the spiral ligament/lateral wall from the organ of Corti. This is a crucial step, since the TM can be accidentally detached from the spiral limbus. To avoid this, the basal turn should be held away from the bottom of the petri dish to prevent deforming, while gentle and precise cuts are made with the scissors, with the plane of the blades at ~ a 45° angle with respect to the plane of the organ of Corti.

Results

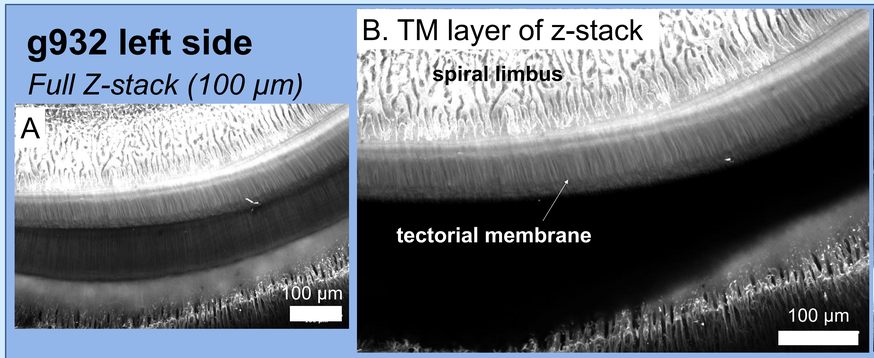
To date, retention of cochlear structure has been variable due to the challenging dissection. In gerbil 932 the left cochlea retained anatomical structure, with the TM attached to the limbus and the BM anchored between the lamina and limbus. In the right cochlea the TM became detached. The off-radial, apical tilt of the TM fibers was more extreme in the detached TM, likely because the imaged portion was from a more apical cochlear region, and possibly also due to the detached state. The left cochlea's scala media had been injected in-vivo with a solution of polyethylene glycol (8 kDa PEG) in artificial endolymph, which is expected to penetrate the TM (Sellon et al.,2014). Either PEG treatment or dissection variability may have led to the different right/left retention of the TM.

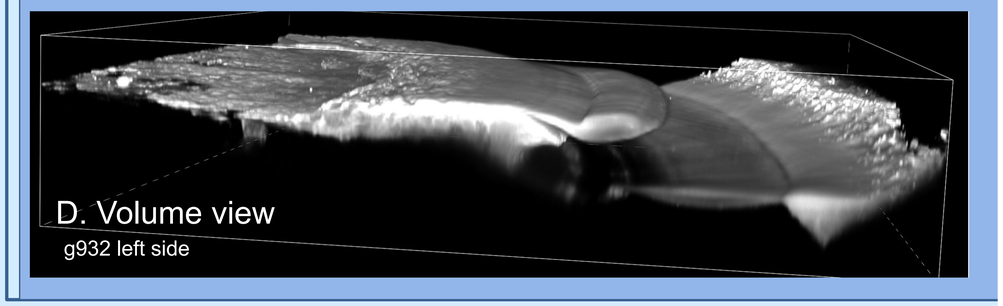
g932 right side



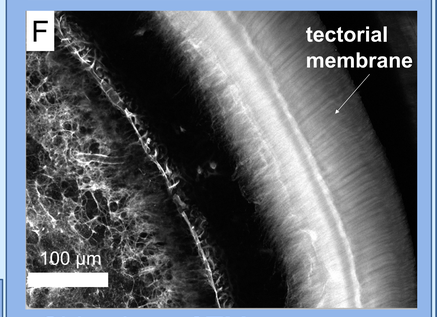
g932 left side

Full Z-stack (100 μm)

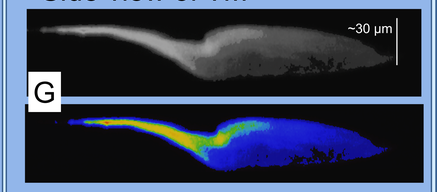




Gerbil 901: Another example of good retention of TM structure (In this case, PEG was not applied).



Side view of TM

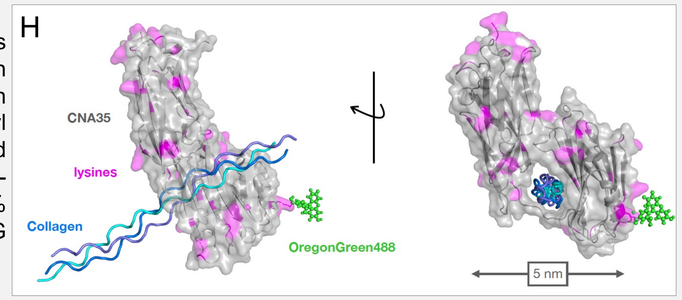


Heat map of fluorescence intensity highlights concentration of collagen in the limbal attachment zone of the TM.

Background on CNA35

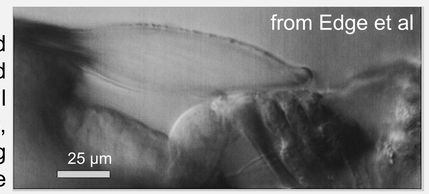
CNA35 is a collagen adhesion protein from *Staphylococcus aureus* bacteria, the A-region of which (amino acids 30-344) has been adapted for fluorescent detection of collagen in cells and tissues (Aper et al., 2014). CNA35 binds with collagen through hydrophobic interactions between residues on the CNA35 protein and Glycine-Proline-4-Hydroxyproline repeats of collagen. The two subdomains in the A-region (N1 and N2) wrap around the collagen's triple helical structure allowing for tight binding (Zong et al., 2005). CNA35 is used in unfixed tissue because CNA does not bind to denatured collagen (Krahn et al., 2006; Zong et al., 2005).

The CNA35 protein was fluorescently labeled with Oregon Green 488 (an amine-reactive succinimidyl ester dye). We produced two batches of CNA35-OG488 with 60% and 135% labeling (around one OG per CNA35).



Discussion

- Our successful preparations showed a plump TM similar to that observed in the base of the unfixed gerbil hemicochlea (Edge et al, 1998), suggesting that our processing allowed for the shape of the TM to be retained.



- CNA35 staining revealed the collagen fiber density and orientation in the TM and BM.
- CNA35 stained the spiral limbus and ligament, and outlined the habenula perforata of the spiral lamina.
- The stiff and firmly anchored BM was more readily maintained than the TM, which was often not retained in good condition through the dissection process.

Future Directions: This work is part of a study in which the TM's physical properties are altered with an endolymphatic injection of collagenase (to disrupt the TM fibers), and PEG (to permeate the TM and increase viscosity). These anatomical studies will document changes and relate them to changes in the cochlea's physiological responses to sound stimulation.

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