TITLE:

Dextran Labeling and Uptake in Live and Functional Murine Cochlear Hair Cells

AUTHORS AND AFFILIATIONS:

Angela Ballesteros[1] and Kenton J. Swartz[1].

[1]Molecular Physiology and Biophysics Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

Email addresses of co-authors:

Angela Ballesteros (angela.ballesteros@nih.gov)

Kenton J. Swartz (Kenton.Swartz@nih.gov)

Corresponding author:

Angela Ballesteros (angela.ballesteros@nih.gov)

KEYWORDS:

Dextran, inner ear hair cells, mechanotransduction channel, dye uptake, endocytosis, intracellular vesicles, confocal microscopy.

SUMMARY:

Here, we present a method for visualizing the uptake of 3 kDa Texas Red-labeled dextran in auditory hair cells with functional mechanotransduction channels. In addition, dextrans of 3 - 10 kDa can be used to study endocytosis in hair and supporting cells of the organ of Corti.

ABSTRACT:

The hair cell mechanotransduction (MET) channel plays an important role in hearing. ~~, h~~ However, the molecular identity and structural information of MET ~~still~~ remain unknown. Electrophysiological studies of hair cells revealed that the MET channel has a large conductance and is permeable to relatively large fluorescent cationic molecules, including some styryl dyes and Texas Red-labeled aminoglycoside antibiotics. In this protocol, we describe a method to visualize and evaluate the uptake of fluorescent dextrans in hair cells of the organ of Corti explants that can be used to assay for functional MET channels. We found that 3 kDa Texas Red-labeled dextran specifically labels functional auditory hair cells after 1-2 h incubation. In particular, 3 kDa dextran labels the two shorter stereocilia rows and accumulates in the cell body in a diffuse pattern when functional MET channels are present. An additional vesicle-like pattern of labeling was observed in the cell body of hair cells and surrounding supporting cells. Our data suggest that 3 kDa Texas-Red dextran can be used to visualize and study two pathways for cellular dye uptake; a hair cell-specific entry route through functional MET channels and endocytosis, a pattern also available to larger dextran.

INTRODUCTION:

The hair cells of the inner ear are the sensory cells that detect sound and covert the mechanically stimuli in electrical signals, which are ultimately interpreted by our brain. These cells have a staircase-shaped bundle of three rows of actin-based filaments, known as stereocilia, which

protrude from their apical region[1,2]. The mechanical stimuli deflect the stereocilia filaments toward the longest row and trigger the opening of the mechanotransduction (MET) channels[3]. The opening of the MET channels leads to an influx of cations that depolarizes the cell and consequently signals the release of synapse vesicles at the basal region of the hair cell.

The biophysical properties of the MET channel essential for hearing have been extensively characterized. Among other properties, these channels are cationic selective and have a relatively large conductance (150-300 pS in low Ca^{2+})[4-10]. Remarkably, large fluorescent molecules such as FM1-43 and Texas Red-labeled aminoglycosides are permeant blockers of the MET channel, resulting in their accumulation in the hair cell body that can be visualized using fluorescence microscopy[11-14]. Conversely, the molecular identity and the structure of the MET channel and its permeation pathway have remained elusive. Increasing experimental evidence indicates that the transmembrane-like channel protein 1 (TMC1) is a component of the MET channel in mature hair cells[15-19]. Mutations in the transmembrane-like channel 1 (TMC1) alter the MET channel properties[19-22] and cause deafness. In addition, TMC1 localizes to the site of the MET channel[18,23] and interacts with the tip-link responsible for transmitting the mechanical force to the MET channel[24,25]. Furthermore, recent bioinformatics analysis has identified the TMC proteins as evolutionary related to the mechanosensitive channels TMEM63/OSCA proteins and the TMEM16 proteins, a family of calcium-activated chloride channels and lipid scramblases[26-28]. A structural model of TMC1 based on the relationship between these proteins revealed the presence of a large cavity at the protein-lipid interface[27]. This cavity harbors the two TMC1 mutations that cause autosomal dominant hearing loss (DFNA36)[27,29-32], and selective modification of cysteine mutants for residues in the cavity alter MET channel properties[28], indicating that it could function as the permeation pathway of the MET channel. The large size of this predicted cavity in TMC proteins could explain the ability of large molecules to permeate the MET channel. To test the prediction that the MET channel contains an unusually large permeation pathway and to push the limits of the size of the cavity observed in TMC1, we developed a protocol to perform uptake experiments in the organ of Corti explants with a larger molecule, 3 kDa dextran fluorescently labeled with Texas Red.

Dextran is a complex branched polysaccharide composed of many D- glucose molecules bound by alpha-1,6 glycosidic linkages. Its high solubility in water, low cell toxicity, and bioinertity make it a versatile tool to study several cellular processes. In addition, dextran is available in a wide range of sizes and fluorescently labeled with fluorophores of several colors. Fluorescently labeled dextrans are commonly used in cell and tissue permeability research[33,34], to study endocytosis in multiple cellular systems[35,36], and for neural tracing[37,38]. In the auditory field, dextran molecules have also been used to assess the disruption of the cell-cell junction and loss of the auditory sensory epithelium integrity after exposure to intense noise in the chinchilla organ of Corti[39,40].

In this work, we exploited the properties of some of the smallest (3 and 10 kDa) fluorescent dextrans to perform uptake experiments in murine inner ear hair cells and explore the size of the permeation pathway of the inner ear hair cell MET channel. In addition, we used a laser-scanning confocal microscope (LSM) 880 equipped with an Airyscan detector to visualize and localize

fluorescent dextran at the stereocilia and the cell body of auditory hair cells.

PROTOCOL:

The animal care and experimental procedures were performed following the guidelines for the Care and Use of Laboratory Animals, which were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke (Animal protocol #1336 to KJS).

1. Mice

1.1. Set a couple of breeding pairs of C57BL/6J wild-type to breed in the animal facility to control the date of birth of the litters and keep track of the age of the pups.

2. Cochleae dissection

2.1. Set a clean space close to a stereomicroscope to perform the dissections (**Figure 1A**). Use 70% ethanol to clean the space and surroundings and place a clean bench pad. A Medical Pathological Waste (MPW) plastic bag would be required to discard the animal carcasses.

2.2. Prepare several 35 mm dishes with some Leibovitz's L15 media.

NOTE: Leibovitz's L-15 cell media contains 1-2 mM Ca^{2+}, which is required to maintain the integrity of the tip-links, and contains essential amino acids, vitamins, and sodium pyruvate to improve cell health and survival. Serum was excluded to avoid experimental variability due to its poorly defined composition and potential interference with the dextran.

2.3. Euthanize postnatal-day-6 (P6) mice by decapitation.

NOTE: Six-day old mice are somewhat resistant to inhalant anesthetics. Although isoflurane or prolonged CO_2 exposures (up to 50 min) may be used for euthanasia, a secondary physical method is recommended to ensure death.

2.4. Use surgical scissors to remove the skin of the skull by making a superficial cut from the anterior to the posterior end and across the external auditory canals.

2.5. Fold the skin towards the nose to expose the cranium (**Figure 2B**).

2.6. Make an incision from the back to the front of the skull and across the eye line (**Figure 2B-C**).

2.7. Separate the skull in two halves and remove the brain with the use of a small spatula to expose the temporal bones (**Figure 2C-D**).

2.8. With small scissors, cut around the temporal bones, and excise the tissue.

2.9. Place both temporal bones in a 35 mm dish and make sure they are covered with L15 media (**Figure 2E**).

NOTE: The following steps are performed under the stereomicroscope. A black background usually helps to visualize the tissue during the fine dissection steps.

2.10. Under a stereomicroscope equipped with a widefield eyepiece (a 10X magnification power (WF10X) and an external alternating current (AC) halogen light source), remove the surrounding cochleae tissue, semicircular canals, and vestibular organs with surgical forceps (**Figure 2F**).

2.11. To allow the dextran and L15 media to enter the cochlear duct, perform two puncture bounds on the dissected cochleae , one on the round window and other at the apical cochlear region.

2.12. Add 300 µL of Leibovitz's L15 media in each well from a 9-well glass depression plate.

2.13. Place at least three dissected cochleae on each well.

3. Dextran labeling

3.1. Reconstitute the dextran in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-CFM) at a final concentration of 10 mg/mL. This stock solution must be aliquoted in opaque black tubes (protected from light) and stored at -30 °C until use.

NOTE: The use of lysine-fixable dextran is critical for a successful outcome of this protocol.

3.2. Prepare each dextran at a final concentration of 2 mg/mL in 500 µL of Leibovitz's L15 media.

3.3. Remove the media from the cochlea and add Leibovitz's L15 media containing the dextran of interest at a final concentration of 2 mg/mL.

NOTE: Although a proportion of the MET channels are open at rest[41,42], the dextran incubation was performed with a gentle shaking of the explants to increase the open probability of the MET channel.

3.4. Incubate at room temperature for 2 h with gentle shaking (25 rpm) by using a 3-dimensional shaker with a tilted angle of 25°.

NOTE: Fluorescently labeled dextran must be protected from light when possible. To protect the dextran during the 2h incubation, place the 9-well glass plate inside a cell culture P150 dish wrapped in aluminum foil.

4. Sample preparation for imaging

4.1. After incubation with the dextran, wash the tissue for 2 min twice with media and once with HBSS.

4.2. Incubate the tissue at room temperature for 30 min with 4% paraformaldehyde in Hanks' balanced salt solution (HBSS).

CAUTION: Exposure to formaldehyde can be irritating to the eyes, nose, and upper respiratory tract. In certain individuals, repeated skin exposure to formaldehyde can cause sensitization that may result in allergic dermatitis. Formaldehyde is a known human carcinogen and a suspected reproductive hazard.

4.3. Quickly and gently wash the fixed tissue twice with HBSS to remove the paraformaldehyde.

NOTE: Decalcification of the temporal bones is not needed at this developmental stage of the cochlea.

4.4. Remove the spiral ligament and the tectorial membrane with fine tip forceps to dissect the organ of Corti (**Figure 2G**).

4.5. Remove all the small pieces of tissue and wash the tissue with HBSS.

4.6. Permeabilize the tissue in 0.5% Triton X-100 in PBS containing fluorescently-labeled phalloidin (conjugated to green or red when testing the uptake of TR- or FITC-labeled dextran, respectively) at a 1:200 dilution for 30 min to label F-actin and visualize the actin-based stereocilia.

4.7. Wash the tissue 2-3 times for 2 min each time with HBSS buffer to remove the excess of triton and phalloidin, and once with PBS to remove the salts.

4.8. Mount the organ of Corti tissues on a microscope slide and cover it with mounting media.

NOTE: When mounting the tissue, make sure that the side of the tissue containing the hair cell stereocilia is facing the coverslip.

4.9. Remove any potential bubbles generated during the addition of the mounting media and prevent the generation of new bubbles during the placement of the coverslip.

NOTE: Aspirate with a pipette any bubble generated during the addition of the mounting media. To prevent air bubbles from being trapped under the coverslip, place an edge of the coverslip close to the sample and carefully and slowly lower the coverslip over the tissue using

forceps or a pipette tip.

4.10. Cover the tissue in mounting media with a glass coverslip (**Figure 2H**).

NOTE: Objectives with a numerical aperture above 0.4 are designed to use #1.5 coverslips (0.17 mm thickness). Using the wrong coverslip may have severe implications for the intensity and quality of the images.

4.11. Incubate the mounted tissue overnight at room temperature to let the mounting media dry and store the slides at 4 °C until imaging.

5. Image acquisition and image processing

NOTE: The confocal images were taken with a LSM 880 confocal microscope equipped with a 32 channel Airyscan detector in the super-resolution (SR) mode[43] and a 63X objective.

5.1. Add a small drop of immersion oil on the objective.

5.2. Place the microscope slide containing the mounted tissue sample in the microscope stage with the glass coverslip facing the immersion oil.

5.3. Focus on the sample and set the imaging parameters using the image acquisition software.

5.4. Use identical image acquisition settings and optimal parameters for x, y, and z resolution for each independent experiment. A piezo-driven focus system is required to quickly and precisely move the objective when acquiring the z-stack of images.

NOTE: To image the entire apical region of the hair cells, collect a z-stack of images from the stereocilia to the apical half of the hair cell body using the optimal settings. It is crucial to collect a large z-stack along the hair cell to assure the imaging of the vesicle-like particles.

5.5. Use the image acquisition software to process the raw confocal images using the Airyscan 3D reconstruction algorithm with the automatic default deconvolution filter settings.

5.6. Open the confocal images in an image processing software to adjust the brightness and contrast, add the scale bar, and export the images for the final figures.

REPRESENTATIVE RESULTS:

We observed robust and specific labeling of hair cells after 2h incubation of organ of Corti explants from wild-type postnatal-day-6 (P6) mice with 3 kDa dextran fluorescently labeled with Texas Red (dextran-TR) (**Figure 2A-B**). Dextran labeling was observed in both inner and outer hair cells (IHC and OHC) at the basal, middle, and apical regions of the organ of Corti (**Figure 2B**).

Fluorescently labeled phalloidin was used to counterstain filamentous actin (F-actin) and visualize the actin-based hair cell stereocilia. We also performed similar experiments at shorter incubation times, and although we observed dextran-TR accumulation in the hair cell body, the signals were weaker and more variable than those at 2 h incubation (**Figure 2C**).

We next imaged both stereocilia and cell bodies of the hair cells and observed that only those cells that incorporated 3 kDa dextran-TR in their cell body showed fluorescent labeling of their stereocilia (**Figure 3A**). This relationship between stereocilia and cell body labeling was absent in hair cells from damaged tissue, which also presented unspecific labeling of dextran in several cell types of the sensory epithelium (yellow squares in **Figure 2B**, and **Figure 3B**). Importantly, we observed a uniform fluorescence signal along the stereocilia with enrichment at the tips of the shorter stereocilia rows where the MET channel is located[18,23] (**Figure 3C**). Also, we noticed vesicle-like structures in the cell body of the hair cells and the neighboring supporting cells. The vesicle-like pattern of uptake in these cells suggests that dextran-TR can also be taken up by endocytosis (**Figure 3C**).

The protocol described here also allows for the examination of the uptake of larger dextrans and combinations of different dextrans. Larger dextran of 10 kDa labeled with Texas Red (dextran-TR) or fluorescein (dextran-FITC) also produced a vesicle-like pattern in the cell body of hair cells and supporting cells, in addition to accumulating around the hair cell membrane in a patchy pattern (**Figure 4A, B**). The 10 kDa dextran-TR also superficially labeled the three stereocilia rows of all the hair cells (**Figure 4A**, inset), probably due to the negatively charged surface of the hair cell plasma membrane[44-46]. We next examined the uptake of 3 kDa dextran-TR and 10 kDa dextran-FITC simultaneously in the organ of Corti explants. For the 3 kDa dextran-TR, we observed both a diffuse and a vesicle-like pattern. However, the 10 kDa dextran-FITC only displayed a vesicle-like pattern (**Figure 4C**). These data suggest that dextran uptake occurs by at least two distinct mechanisms that are dependent on the size of the dextran.

We next assessed whether functional MET channels were required for the uptake of 3 kDa dextran-TR. To do this, we tested dextran incorporation in hair cells from the organ of Corti explants in the presence of MET channel blockers (neomycin and amiloride)[13,14,47] or the Ca^{2+} chelator BAPTA, which abolishes MET currents by breaking the tip-links and preventing the gating of the channel[25,48,49]. In these experiments, the tissue explants were previously incubated for 30 min with neomycin (500 μM), amiloride (150 μM), or with BAPTA (5mM) before the addition of 3 kDa dextran-TR in the presence of the corresponding MET blocker. The presence of BAPTA or the MET channel blockers prevented the stereocilia labeling (**Figure 5A**) and the uptake of 3 kDa dextran-TR in hair cells (**Figure 5B**). However, blockade of the MET channel preserved the vesicle-like pattern (**Figure 5B**), indicating that this pattern of uptake is independent of functional MET channels. Similar results have been observed in hair cells from TMC1/TMC2 double knock-out mice, which lack MET[27]. Intriguingly, these vesicle-like structures were not observable in the presence of amiloride, which is known to inhibit the Na^{+}-H^{+} exchanger and thereby inhibit endocytosis[50,51]. These results indicate that 3kDa dextran-TR enters the hair cells through two different pathways, one common to larger dextrans involving vesicle-like structures and another that depends on functional MET channels.

FIGURE LEGENDS:

Figure 1: Steps of the dissection of a murine organ of Corti. (**A**) Clean area and materials required for the dissection. (**B**) Exposed mouse cranium. Forceps are holding the skin, which has folded towards the nose. Black lines indicate the two incisions that are required for the removal of the brain. (**C**) Incised and opened cranium to allow for removal of the brain with a spatula (on the right). (**D**) Cranium and temporal bones after brain removal. (**E**) Excised skull, including the temporal bones (dashed black squares) in a P35 plate covered with media. (**F**) Excised cochleae. (**G**) Two dissected organs of Corti on the well of a glass depression plate. (**H**) Mounted sample on a glass coverslip ready for imaging.

Figure 2: Hair cells uptake 3 kDa dextran-TR. (**A**) Schematic representation of Texas Red-labeled dextran (dextran-TR) containing six molecules of glucose corresponding to a molecular weight of 1.08 kDa. A succinimidyl ester reaction links a Texas Red molecule (magenta) to a glucose monomer.
(**B**) Representative confocal image showing specific labeling of sensory hair cells (HC) with 3 kDa dextran-TR across the whole organ of Corti from a 6-day-old mouse. The basal (BA), middle (MD), and apical (AP) regions of the organ are indicated. Yellow squares indicate tissue damaged regions. (**C**) 3 kDa dextran-TR fluorescence after 30, 60, 90, or 120 min incubation with the organ of Corti explants is shown in magenta merged with F-actin in green (top images) and independently in grayscale (bottom images). The image display range was linearly adjusted in each one of the gray images independently for visualization of the 3 kDa dextran-TR fluorescence. Scale bar represents 20 μm. This figure has been modified from[27].

Figure 3: Hair cell body and stereocilia labeling with 3 kDa dextran-TR. (**A**) Confocal images displaying 3 kDa dextran-TR fluorescence (magenta) at the cell body and stereocilia counterstained with phalloidin to label F-actin (green) to visualize hair cell boundaries and stereocilia. The three rows of outer hair cells (OHC) and one row of inner hair cells (IHC) are indicated. Scale bar represents 20 μm. (**B**) Confocal images of a damaged tissue area displaying 3 kDa dextran-TR fluorescence (magenta) at the cell body and stereocilia. Yellow arrows indicate the labeling of non-sensory supporting cells. Scale bar represents 20 μm. (**C**) Closer view of 3 kDa dextran-TR fluorescence at the cell body and stereocilia of outer hair cells (OHC, top) and inner hair cells (IHC, bottom). Scale bar represents 5 μm. This figure has been modified from [27].

Figure 4: Labeling of larger 10 kDa dextran and a combination of 3 and 10 kDa dextran. (**A**) Confocal image of hair cells at the hair cell body (top) and stereocilia (bottom) levels incubated with 10 kDa dextran-TR. The dextran fluorescence signal is shown in magenta merged with F-actin in green and independently in grayscale. A couple of IHC are shown at higher magnification in the inset to appreciate the superficial labeling of stereocilia observed with the 10 kDa dextran-TR. The scale bar corresponds to 5 μm. (**B**) Confocal image of hair cells incubated with 10 kDa dextran-FITC represented as in A. (**C**) Confocal image of hair cells at the hair cell body (top) and stereocilia (bottom) levels incubated simultaneously with 3 kDa dextran-TR and 10 kDa dextran-FITC and imaged using independent channels and depicted separately in gray. In the right panel,

F-actin (blue), 10 kDa dextran-FITC (green), and 3 kDa dextran-TR (magenta) are shown together along with phalloidin (blue). The scale bar represents 20 μm, and the vesicle-like pattern of uptake is indicated with yellow arrows. This figure has been modified from[27].

Figure 5: Functional MET channels are required for 3 kDa dextran-TR uptake. Representative confocal images of hair cells at the stereocilia (**A**) or cell body (**B**) level after incubation with 3 kDa dextran-TR in the absence (control) or presence of BAPTA or the MET channel blockers amiloride and neomycin. 3 kDa dextran-TR fluorescence is shown in magenta. Tissue was counterstained with phalloidin to label F-actin for visualization of the hair cell stereocilia and boundaries (green). White arrows point to vesicle-like structures. The three rows of outer hair cells (OHC) and one row of inner hair cells (IHC) are indicated, and the scale bar represents 20 μm. This figure has been modified from[27].

DISCUSSION:

This protocol describes how to perform uptake experiments in murine organ of Corti explants with 3 kDa dextran Texas Red. The goal of this method is to test whether molecules larger than others previously tested were also able to specifically label auditory hair cells and permeate through the MET channel. Similar experimental protocols have been previously used to evaluate the permeability of hair cells to other fluorescent dyes such as FM1-43 (0.56 kDa)[12,19,20] and Texas Red-labeled aminoglycosides (1.29-1.43 kDa)[13,52]. The time needed for labeling hair cells using these fluorescent molecules varies with their size. The maximal labeling requires a few minutes for FM1-43[13,53], 30-60 min for Texas Red-labeled aminoglycosides[13], and 1.5 h for Texas Red-labeled 3 kDa dextran. Thus, long incubation times are crucial for this experiment as we saw only minimal labeling of the hair cell body at short incubation times (**Figure 2C**). We chose to study P6 mice for these experiments because, at this age, most apical and basal hair cells carry transduction currents and express both TMC1 and TMC2 proteins[12,18,54,55]. It should be possible to study younger animals as MET currents are present earlier than P6, in particular near the base of the cochlea[12]. Studying older animals may be more challenging because dissection of the bony cochlea becomes more difficult as this structure continues to ossify[56].

There are several critical steps in this protocol. The most crucial one being the successful dissection and preparation of the organ of Corti tissue (**Figure 1**). The delicate structure of the organ of Corti and encasement in the boney cochlea presents a challenge for histological and biochemical analysis. Several detailed protocols for the successful dissection of this tissue have been previously published in this journal[56-58] and others[59]. Disruption of the cell-cell junction and loss of the auditory sensory epithelium integrity may lead to the uptake of dextran of up to 40 kDa and the labeling of the affected cells[39,40]. In agreement with this, we observed unspecific labeling of hair cells and the surrounding supporting cells when the tissue was unintentionally damaged with the forceps during dissection before dextran incubation (**Figure 2B** and **Figure 3B**). There are no tricks or guidelines to reach proficiency at dissecting an intact organ of Corti other than practice and patience. However, the presence of 1-2 mM Ca^{2+} during tissue dissection and uptake experiments is crucial to maintain the integrity of the tip-link that transmits the mechanical force to the MET channel and thereby preserve MET channel function. Once the tissue is fixed with 4% PFA, the presence of calcium in the buffer or media becomes trivial.

One of the limitations of this study resides in the heterogeneous nature of the dextran molecules. The dextran elaborated by the *Leuconostoc* bacteria is a polymer of anhydroglucose composed of approximately 95% alpha-(1-6) D-linkages (**Figure 2A**). The remaining linkages account for the branching of dextran, which correlates with its length, so dextran of lower molecular weight are more rod-like and have a narrower size distribution while larger dextrans are polydisperse[60]. Therefore, the actual molecular weight of the molecules in each preparation of the 3 kDa dextran ranges from 1.5 to 3.0 kDa, including the fluorescent molecule[61]. Additionally, the fluorescently labeled dextran differ in the number of Texas Red molecules attached to the dextran (degree of labeling). It is advisable to use dextran with a degree of labeling of at least 0.4 since a lower degree of labeling will hinder the ability to detect the fluorescently labeled dextran by microscopy. The fluorescent dye coupled to the dextran and the lysine residues in the fixable versions, will determine the overall charge of the dextran. This is an important consideration since the cationic selectivity of the MET channel would preferably uptake cationic dextran[27]. Lastly, a lysine-fixable dextran is necessary to reach the resolution displayed in these experiments. A non-fixable dextran will continue to diffuse into the tissue hampering its accurate localization and visualization.

The LSM 880 confocal microscope equipped with an Airyscan detector used to image the organ of Corti samples provided us with twice the resolution, and better signal-to-noise ratio (SNR) compared to a conventional confocal microscope[62]. Therefore, this microscope allowed us not only to observe the robust dextran accumulation at the cell body but also to pinpoint the accumulation of the dextran at the stereocilia tips and the vesicle-like pattern at the cell body. A conventional confocal microscope would allow for the visualization and quantification of the accumulation of fluorescent dextran at the cell body, but it would difficult to distinguish the three rows of stereocilia and the precise localization of the dextran at the tips of the shorter stereocilia rows. A similar resolution to the one achieved with the LSM 880 Airyscan confocal could be reached using a conventional confocal microscope with oversampling and deconvolution, although the SNR would be lower than the one reached with the Airyscan detector.

Formatted: Font: (Default) +Body (Calibri)

Formatted: Font: (Default) +Body (Calibri), 12 pt

Formatted: Font: (Default) +Body (Calibri), 12 pt

In addition to the use of 3 kDa dextran-TR to assay for functional MET channels, this protocol could be further used to study endocytic processes in hair cells since all the dextrans tested reveal an intracellular vesicle-like pattern resembling endocytosis. This process was not the focus of our work, and additional studies would be required to fully characterize this phenomenon.

ACKNOWLEDGMENTS:

We thank Vincent Schram from the NICHD microscopy and imaging core for assisting in the confocal image acquisition, and Tsg-Hui Chang for invaluable help with colony management and mice care. This research was supported by the Intramural Research Program of the NINDS, NIH, Bethesda, MD, to K.J.S. A.B. was supported by the Intramural Research Program of the NINDS, NIH, and by a Robert Wenthold Postdoctoral Fellowship from the intramural research program of the NIDCD.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Furness, D. N. & Hackney, C. M. *The Structure and Composition of the Stereociliary Bundle of Vertebrate Hair Cells*. (Springer New York, 2006).

2 Barr-Gillespie, P. G. Assembly of hair bundles, an amazing problem for cell biology. *Mol Biol Cell.* **26** (15), 2727-2732, doi:10.1091/mbc.E14-04-0940, (2015).

3 Shotwell, S. L., Jacobs, R. & Hudspeth, A. J. Directional sensitivity of individual vertebrate hair cells to controlled deflection of their hair bundles. *Ann N Y Acad Sci.* **374** 1-10, doi:10.1111/j.1749-6632.1981.tb30854.x, (1981).

4 Fettiplace, R. & Kim, K. X. The physiology of mechanoelectrical transduction channels in hearing. *Physiol Rev.* **94** (3), 951-986, doi:10.1152/physrev.00038.2013, (2014).

5 Fettiplace, R. Hair Cell Transduction, Tuning, and Synaptic Transmission in the Mammalian Cochlea. *Compr Physiol.* **7** (4), 1197-1227, doi:10.1002/cphy.c160049, (2017).

6 Corey, D. P. & Hudspeth, A. J. Ionic basis of the receptor potential in a vertebrate hair cell. *Nature.* **281** (5733), 675-677 (1979).

7 Beurg, M., Evans, M. G., Hackney, C. M. & Fettiplace, R. A large-conductance calcium-selective mechanotransducer channel in mammalian cochlear hair cells. *J Neurosci.* **26** (43), 10992-11000, doi:10.1523/JNEUROSCI.2188-06.2006, (2006).

8 Geleoc, G. S., Lennan, G. W., Richardson, G. P. & Kros, C. J. A quantitative comparison of mechanoelectrical transduction in vestibular and auditory hair cells of neonatal mice. *Proc Biol Sci.* **264** (1381), 611-621, doi:10.1098/rspb.1997.0087, (1997).

9 Kros, C. J., Rusch, A. & Richardson, G. P. Mechano-electrical transducer currents in hair cells of the cultured neonatal mouse cochlea. *Proc Biol Sci.* **249** (1325), 185-193, doi:10.1098/rspb.1992.0102, (1992).

10 Ohmori, H. Mechano-electrical transduction currents in isolated vestibular hair cells of the chick. *J Physiol.* **359** 189-217 (1985).

11 Gale, J. E., Marcotti, W., Kennedy, H. J., Kros, C. J. & Richardson, G. P. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *J Neurosci.* **21** (18), 7013-7025 (2001).

12 Lelli, A., Asai, Y., Forge, A., Holt, J. R. & Geleoc, G. S. Tonotopic gradient in the developmental acquisition of sensory transduction in outer hair cells of the mouse cochlea. *J Neurophysiol.* **101** (6), 2961-2973, doi:10.1152/jn.00136.2009, (2009).

13 Alharazneh, A. *et al.* Functional hair cell mechanotransducer channels are required for aminoglycoside ototoxicity. *PLoS One.* **6** (7), e22347, doi:10.1371/journal.pone.0022347, (2011).

14 Marcotti, W., van Netten, S. M. & Kros, C. J. The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the mechano-electrical transducer channels. *J Physiol.* **567** (Pt 2), 505-521, doi:10.1113/jphysiol.2005.085951, (2005).

15 Corns, L. F., Jeng, J. Y., Richardson, G. P., Kros, C. J. & Marcotti, W. TMC2 Modifies Permeation Properties of the Mechanoelectrical Transducer Channel in Early Postnatal Mouse Cochlear Outer Hair Cells. *Front Mol Neurosci.* **10** 326,

doi:10.3389/fnmol.2017.00326, (2017).
16 Kawashima, Y., Kurima, K., Pan, B., Griffith, A. J. & Holt, J. R. Transmembrane channel-like (TMC) genes are required for auditory and vestibular mechanosensation. *Pflugers Arch.* **467** (1), 85-94, doi:10.1007/s00424-014-1582-3, (2015).
17 Kim, K. X. & Fettiplace, R. Developmental changes in the cochlear hair cell mechanotransducer channel and their regulation by transmembrane channel-like proteins. *J Gen Physiol.* **141** (1), 141-148, doi:10.1085/jgp.201210913, (2013).
18 Kurima, K. *et al.* TMC1 and TMC2 Localize at the Site of Mechanotransduction in Mammalian Inner Ear Hair Cell Stereocilia. *Cell Rep.* **12** (10), 1606-1617, doi:10.1016/j.celrep.2015.07.058, (2015).
19 Pan, B. *et al.* TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. *Neuron.* **79** (3), 504-515, doi:10.1016/j.neuron.2013.06.019, (2013).
20 Kawashima, Y. *et al.* Mechanotransduction in mouse inner ear hair cells requires transmembrane channel-like genes. *J Clin Invest.* **121** (12), 4796-4809, doi:10.1172/JCI60405, (2011).
21 Beurg, M., Goldring, A. C. & Fettiplace, R. The effects of Tmc1 Beethoven mutation on mechanotransducer channel function in cochlear hair cells. *J Gen Physiol.* **146** (3), 233-243, doi:10.1085/jgp.201511458, (2015).
22 Corns, L. F., Johnson, S. L., Kros, C. J. & Marcotti, W. Tmc1 Point Mutation Affects Ca2+ Sensitivity and Block by Dihydrostreptomycin of the Mechanoelectrical Transducer Current of Mouse Outer Hair Cells. *J Neurosci.* **36** (2), 336-349, doi:10.1523/JNEUROSCI.2439-15.2016, (2016).
23 Beurg, M., Fettiplace, R., Nam, J. H. & Ricci, A. J. Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. *Nat Neurosci.* **12** (5), 553-558, doi:10.1038/nn.2295, (2009).
24 Maeda, R. *et al.* Tip-link protein protocadherin 15 interacts with transmembrane channel-like proteins TMC1 and TMC2. *Proc Natl Acad Sci U S A.* **111** (35), 12907-12912, doi:10.1073/pnas.1402152111, (2014).
25 Assad, J. A., Shepherd, G. M. & Corey, D. P. Tip-link integrity and mechanical transduction in vertebrate hair cells. *Neuron.* **7** (6), 985-994, doi:10.1016/0896-6273(91)90343-x, (1991).
26 Medrano-Soto, A. *et al.* Bioinformatic characterization of the Anoctamin Superfamily of Ca2+-activated ion channels and lipid scramblases. *PLoS One.* **13** (3), e0192851, doi:10.1371/journal.pone.0192851, (2018).
27 Ballesteros, A., Fenollar-Ferrer, C. & Swartz, K. J. Structural relationship between the putative hair cell mechanotransduction channel TMC1 and TMEM16 proteins. *Elife.* **7**, doi:10.7554/eLife.38433, (2018).
28 Pan, B. *et al.* TMC1 Forms the Pore of Mechanosensory Transduction Channels in Vertebrate Inner Ear Hair Cells. *Neuron.* **99** (4), 736-753 e736, doi:10.1016/j.neuron.2018.07.033, (2018).
29 Kitajiri, S., Makishima, T., Friedman, T. B. & Griffith, A. J. A novel mutation at the DFNA36 hearing loss locus reveals a critical function and potential genotype-phenotype correlation for amino acid-572 of TMC1. *Clin Genet.* **71** (2), 148-152, doi:10.1111/j.1399-

0004.2007.00739.x, (2007).
30 Makishima, T., Kurima, K., Brewer, C. C. & Griffith, A. J. Early onset and rapid progression of dominant nonsyndromic DFNA36 hearing loss. *Otol Neurotol.* **25** (5), 714-719 (2004).
31 Noguchi, Y. *et al.* Multiple quantitative trait loci modify cochlear hair cell degeneration in the Beethoven (Tmc1Bth) mouse model of progressive hearing loss DFNA36. *Genetics.* **173** (4), 2111-2119, doi:10.1534/genetics.106.057372, (2006).
32 Vreugde, S. *et al.* Beethoven, a mouse model for dominant, progressive hearing loss DFNA36. *Nat Genet.* **30** (3), 257-258, doi:10.1038/ng848, (2002).
33 Hulstrom, D. & Svensjo, E. Intravital and electron microscopic study of bradykinin-induced vascular permeability changes using FITC-dextran as a tracer. *J Pathol.* **129** (3), 125-133, doi:10.1002/path.1711290304, (1979).
34 Mayhan, W. G. & Heistad, D. D. Permeability of blood-brain barrier to various sized molecules. *Am J Physiol.* **248** (5 Pt 2), H712-718, doi:10.1152/ajpheart.1985.248.5.H712, (1985).
35 Makarow, M. Endocytosis in Saccharomyces cerevisiae: internalization of alpha-amylase and fluorescent dextran into cells. *EMBO J.* **4** (7), 1861-1866 (1985).
36 Clayton, E. L. & Cousin, M. A. Quantitative monitoring of activity-dependent bulk endocytosis of synaptic vesicle membrane by fluorescent dextran imaging. *J Neurosci Methods.* **185** (1), 76-81, doi:10.1016/j.jneumeth.2009.09.016, (2009).
37 Allman, B. L., Keniston, L. P. & Meredith, M. A. Adult deafness induces somatosensory conversion of ferret auditory cortex. *Proc Natl Acad Sci U S A.* **106** (14), 5925-5930, doi:10.1073/pnas.0809483106, (2009).
38 Warr, W. B., Boche, J. B. & Neely, S. T. Efferent innervation of the inner hair cell region: origins and terminations of two lateral olivocochlear systems. *Hear Res.* **108** (1-2), 89-111, doi:10.1016/s0378-5955(97)00044-0, (1997).
39 Hu, B. H. & Zheng, G. L. Membrane disruption: an early event of hair cell apoptosis induced by exposure to intense noise. *Brain Res.* **1239** 107-118, doi:10.1016/j.brainres.2008.08.043, (2008).
40 Zheng, G. & Hu, B. H. Cell-cell junctions: a target of acoustic overstimulation in the sensory epithelium of the cochlea. *BMC Neurosci.* **13** 71, doi:10.1186/1471-2202-13-71, (2012).
41 Beurg, M., Nam, J. H., Chen, Q. & Fettiplace, R. Calcium balance and mechanotransduction in rat cochlear hair cells. *J Neurophysiol.* **104** (1), 18-34, doi:10.1152/jn.00019.2010, (2010).
42 Johnson, S. L., Beurg, M., Marcotti, W. & Fettiplace, R. Prestin-driven cochlear amplification is not limited by the outer hair cell membrane time constant. *Neuron.* **70** (6), 1143-1154, doi:10.1016/j.neuron.2011.04.024, (2011).
43 Korobchevskaya, K., Lagerholm, B. C., Colin-York, H. & Fritzsche, M. Exploring the Potential of Airyscan Microscopy for Live Cell Imaging. *Photonics.* **4** (3), doi:ARTN 41
10.3390/photonics4030041, (2017).
44 Gil-Loyzaga, P. & Brownell, W. E. Wheat germ agglutinin and Helix pomatia agglutinin lectin binding on cochlear hair cells. *Hear Res.* **34** (2), 149-155, doi:10.1016/0378-5955(88)90102-5, (1988).
45 Santi, P. A. & Anderson, C. B. Alcian blue staining of cochlear hair cell stereocilia and other cochlear tissues. *Hear Res.* **23** (2), 153-160, doi:10.1016/0378-5955(86)90012-2, (1986).

46 Slepecky, N. & Chamberlain, S. C. The cell coat of inner ear sensory and supporting cells as demonstrated by ruthenium red. *Hear Res.* **17** (3), 281-288, doi:10.1016/0378-5955(85)90072-3, (1985).
47 Rusch, A., Kros, C. J. & Richardson, G. P. Block by amiloride and its derivatives of mechano-electrical transduction in outer hair cells of mouse cochlear cultures. *J Physiol.* **474** (1), 75-86, doi:10.1113/jphysiol.1994.sp020004, (1994).
48 Zhao, Y., Yamoah, E. N. & Gillespie, P. G. Regeneration of broken tip links and restoration of mechanical transduction in hair cells. *Proc Natl Acad Sci U S A.* **93** (26), 15469-15474, doi:10.1073/pnas.93.26.15469, (1996).
49 Indzhykulian, A. A. *et al.* Molecular remodeling of tip links underlies mechanosensory regeneration in auditory hair cells. *PLoS Biol.* **11** (6), e1001583, doi:10.1371/journal.pbio.1001583, (2013).
50 Koivusalo, M. *et al.* Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling. *J Cell Biol.* **188** (4), 547-563, doi:10.1083/jcb.200908086, (2010).
51 West, M. A., Bretscher, M. S. & Watts, C. Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J Cell Biol.* **109** (6 Pt 1), 2731-2739, doi:10.1083/jcb.109.6.2731, (1989).
52 Park, S. *et al.* tmie Is required for gentamicin uptake by the hair cells of mice. *Comp Med.* **63** (2), 136-142 (2013).
53 Meyers, J. R. *et al.* Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. *J Neurosci.* **23** (10), 4054-4065 (2003).
54 Waguespack, J., Salles, F. T., Kachar, B. & Ricci, A. J. Stepwise morphological and functional maturation of mechanotransduction in rat outer hair cells. *J Neurosci.* **27** (50), 13890-13902, doi:10.1523/JNEUROSCI.2159-07.2007, (2007).
55 Beurg, M. *et al.* Variable number of TMC1-dependent mechanotransducer channels underlie tonotopic conductance gradients in the cochlea. *Nat Commun.* **9** (1), 2185, doi:10.1038/s41467-018-04589-8, (2018).
56 Montgomery, S. C. & Cox, B. C. Whole Mount Dissection and Immunofluorescence of the Adult Mouse Cochlea. *J Vis Exp.* (107), doi:10.3791/53561, (2016).
57 Landegger, L. D., Dilwali, S. & Stankovic, K. M. Neonatal Murine Cochlear Explant Technique as an In Vitro Screening Tool in Hearing Research. *J Vis Exp.* (124), doi:10.3791/55704, (2017).
58 May-Simera, H. Evaluation of Planar-Cell-Polarity Phenotypes in Ciliopathy Mouse Mutant Cochlea. *J Vis Exp.* (108), 53559, doi:10.3791/53559, (2016).
59 Ogier, J. M., Burt, R. A., Drury, H. R., Lim, R. & Nayagam, B. A. Organotypic Culture of Neonatal Murine Inner Ear Explants. *Front Cell Neurosci.* **13** 170, doi:10.3389/fncel.2019.00170, (2019).
60 Granath, K. A. Solution properties of branched dextrans. *Journal of Colloid Science.* **13** (4), 20, doi:https://doi.org/10.1016/0095-8522(58)90041-2, (1958).
61 Molecular Probes , I. D. T. *Product information on Dextran Conjugates*, 2006).
62 Korobchevskaya, K., Lagerholm, B. C., Colin-York, H. & Fritzsche, M. Exploring the Potential of Airyscan Microscopy for Live Cell Imaging. *Photonics.* **4** (3), 41 (2017).

617