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TITLE:

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

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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, 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 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 available to perform inner ear mouse hair cell uptake experiments and explore the size of the permeation pathway of the inner ear hair cell MET channel. In addition, we used Airyscan super-resolution confocal microscopy 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 guide for the Care and Use of Laboratory Animals and 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 semicircular canals, vestibular organs, and surrounding cochleae tissue with surgical forceps (**Figure 2F**).

2.11. Perform two incisions on the dissected cochleae with the surgical forceps; one on the round window and other at the apical cochlear region to allow the dextran and media to enter the cochlear duct.

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 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 phosphate-buffered saline (PBS).

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 finally 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 using 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 cover slip, 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 confocal laser scanning microscope 880 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 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 suggest 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 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 Texas Red molecule (magenta) is linked by a succinimidyl ester reaction 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 previous publication[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 previous publication[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 previous publication[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 previous publication[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 calcify[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 more

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 that is coupled to the dextran and the presence of 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 Airyscan super resolution confocal microscope 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 Airyscan could be reach using a conventional confocal microscope with oversampling and deconvolution, although the SNR would be lower than the one reached with the Airyscan.

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.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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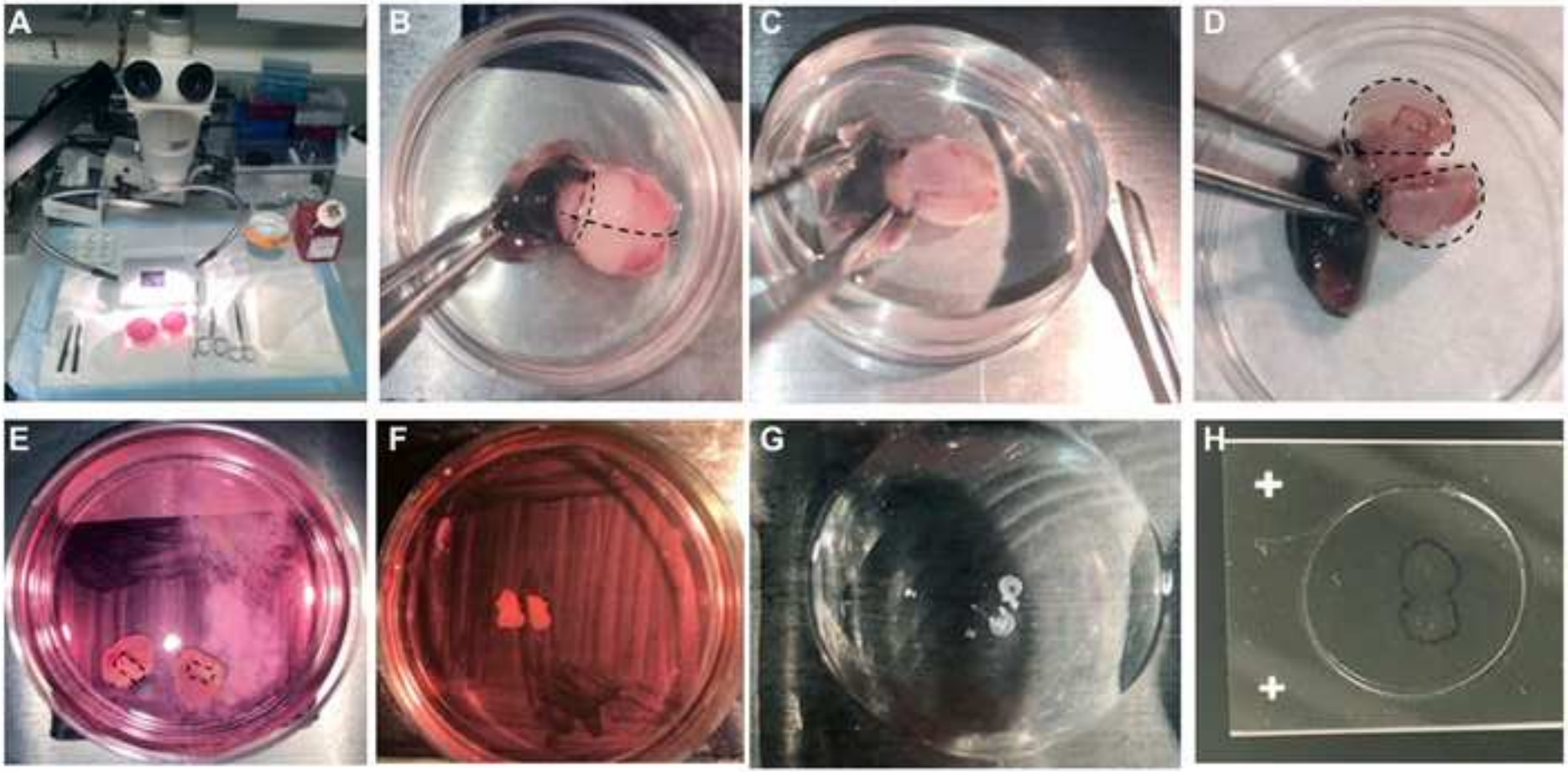

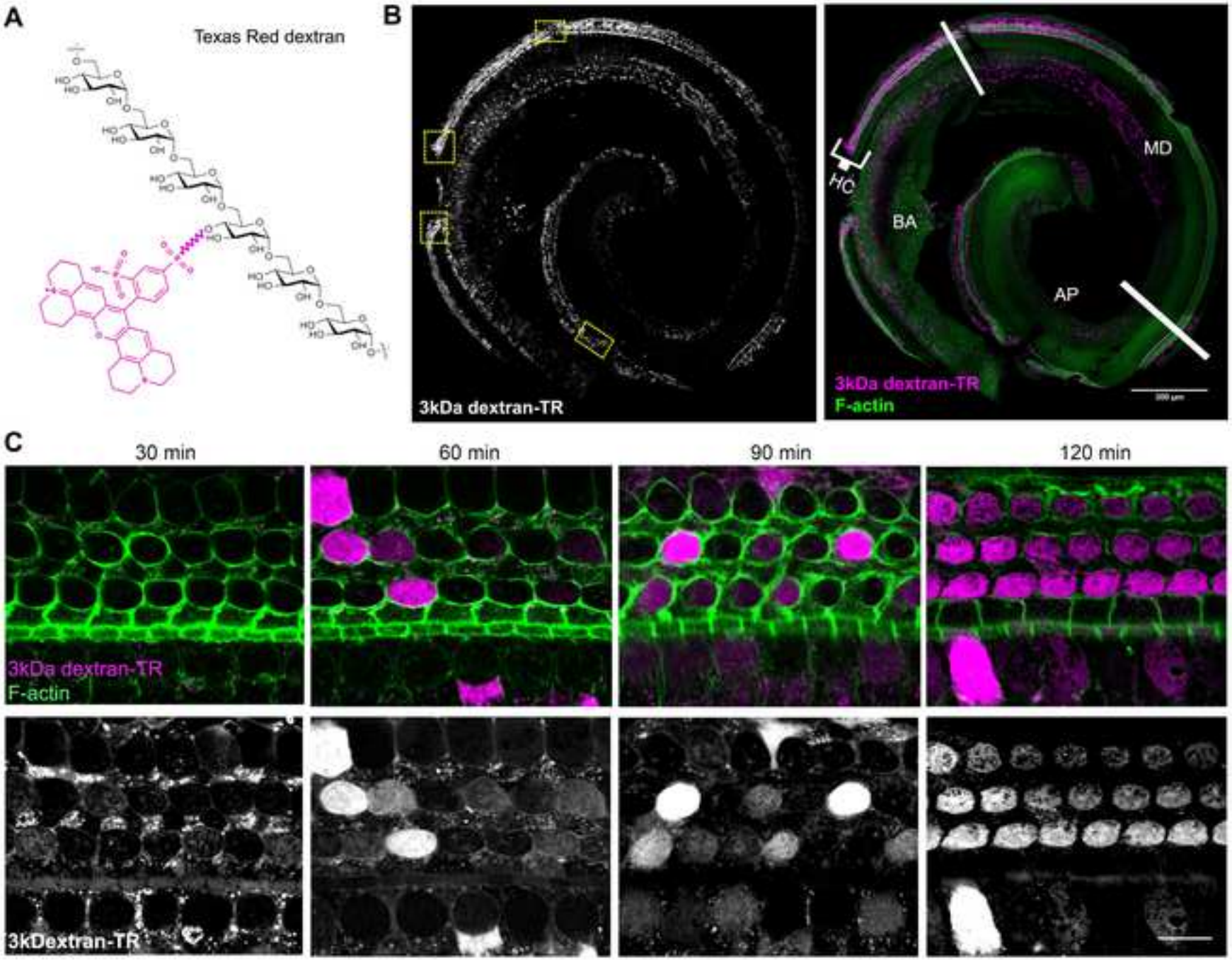

Figure3

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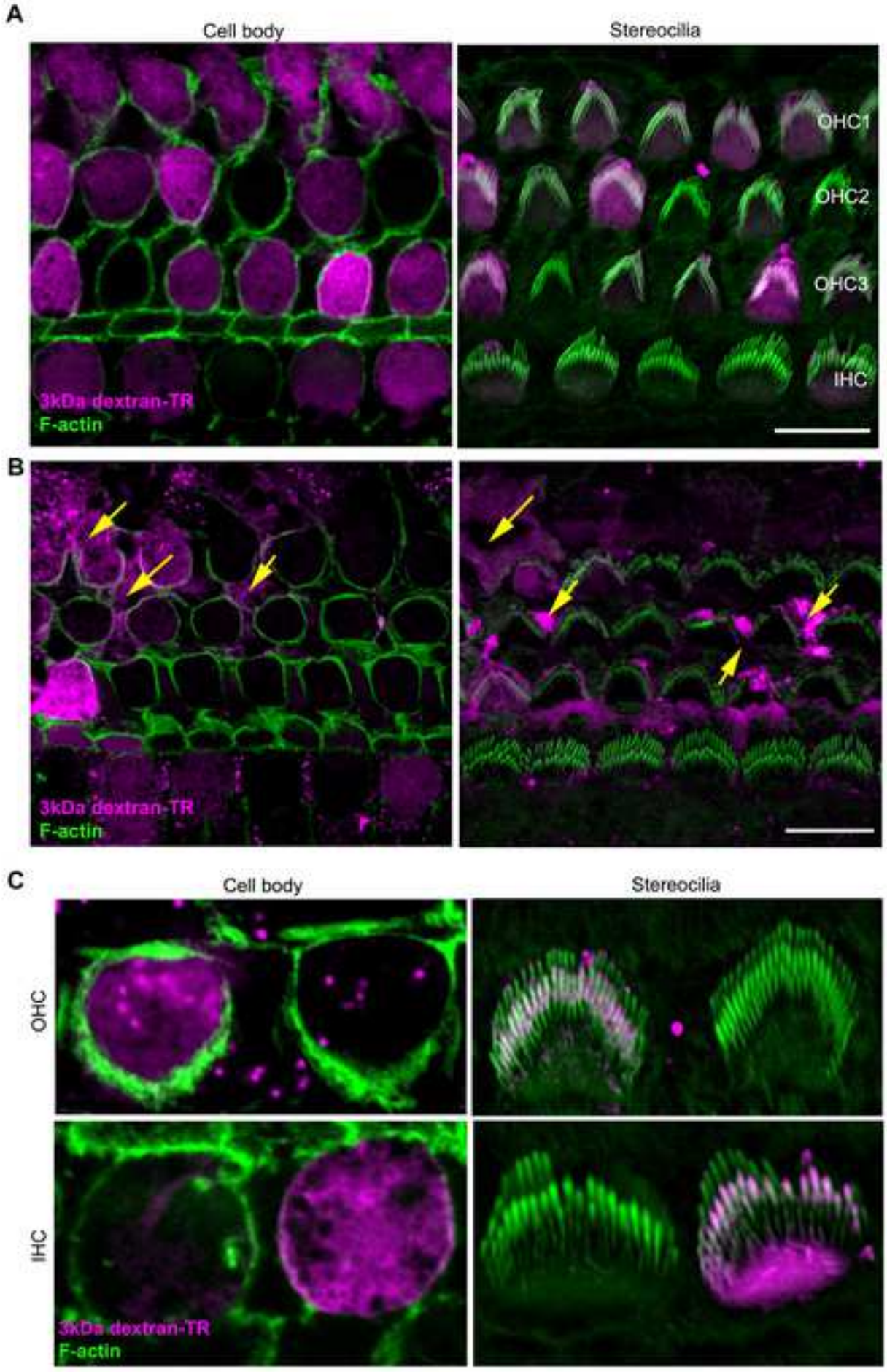


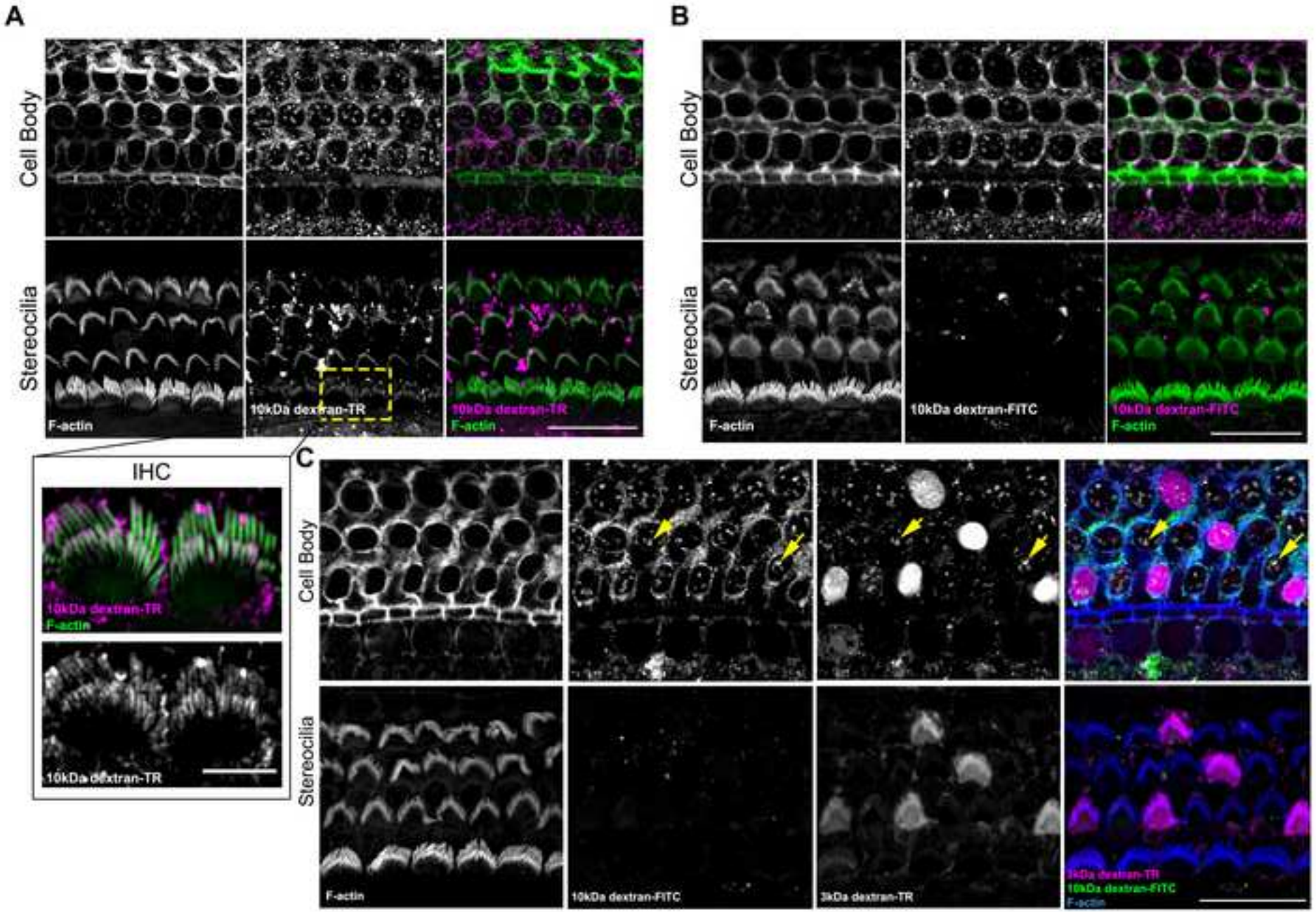
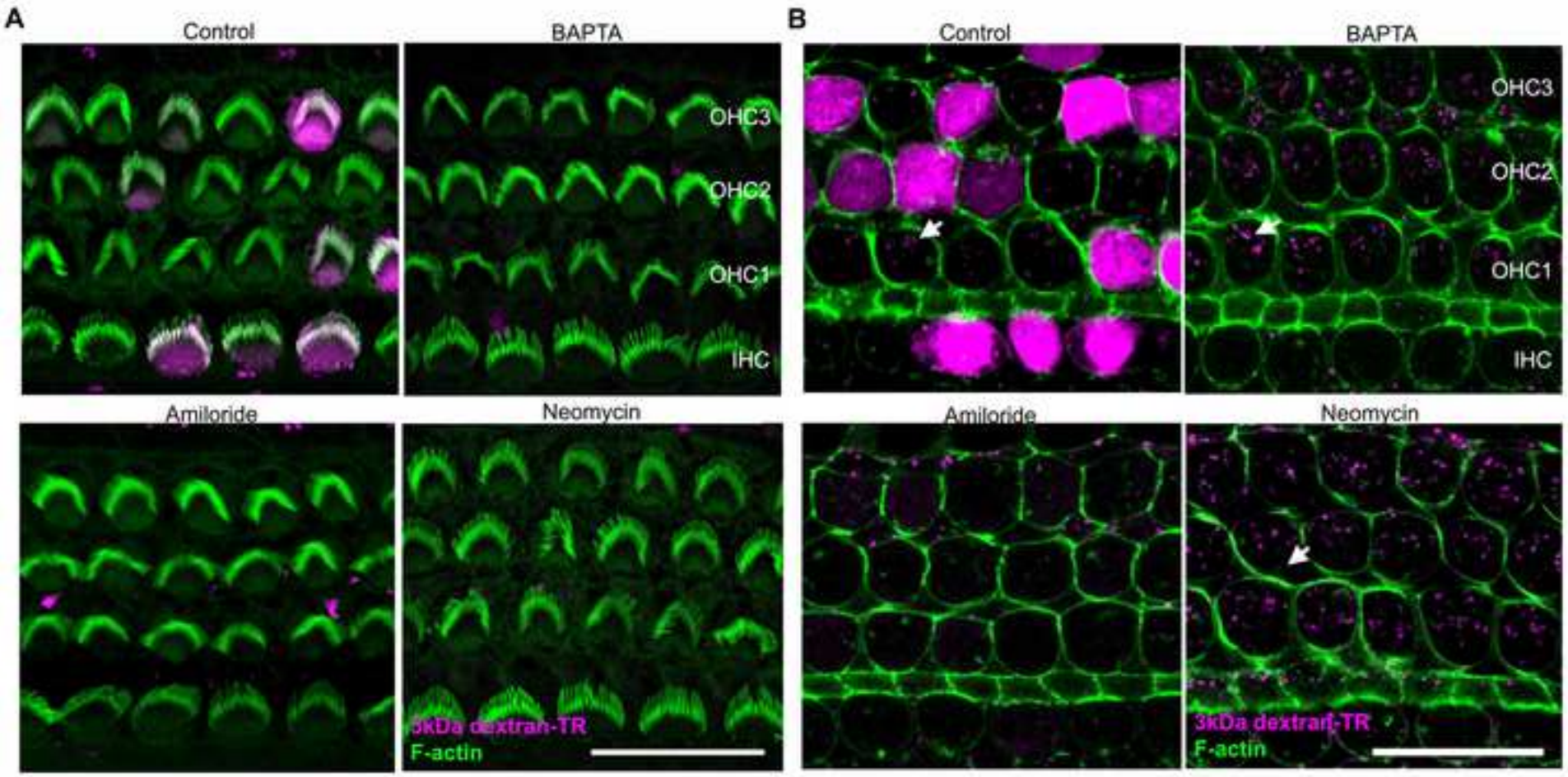


Figure5

Click here to access/download;Figure;Figure5.jpg



Name of Material/ Equipment	Company	Catalog Number
#1.5 glass coverslips 18mm	Warner Instruments	64-0714
Alexa Fluor 488 Phalloidin	ThermoFisher	A12379
Alexa Fluor 594 Phalloidin	ThermoFisher	A12381
alpha Plan-Apochromat 63X/1.4 Oil Corr M27 objective	Carl Zeiss	420780-9970-000
Amiloride hydrochloride	EMD MILLIPORE	129876
Benchwaver 3-dimensional Rocker	Benchmarks scientific	B3D5000
C57BL/6J wild-type mice	strain 000664	The Jackson Laboratory
Cell impermeant BAPTA tetrapotassium salt	ThermoFisher	B1204
Dextran, Fluorescein, 10,000 MW, Anionic, Lysine Fixable	ThermoFisher	D1820
Dextran, Texas Red, 10,000 MW, Lysine Fixable	ThermoFisher	D1863
Dextran, Texas Red, 3000 MW, Lysine Fixable	ThermoFisher	D3328
Formaldehyde Aqueous Solution EM Grade	Electron microscopy science	15710
HBSS, calcium, magnesium, no phenol red	ThermoFisher	14025
HBSS, no calcium, no magnesium, no phenol red	ThermoFisher	14170
Image J or FIJI	NIH	http://fiji.sc/
Immersol 518F oil immersion media	Carl Zeiss	444970-9000-000
Leibovitz's L-15 Medium, GlutaMAX Supplement	ThermoFisher	31415029
neomycin trisulfate salt hydrate	Sigma	N6386
PBS (10X), pH 7.4	ThermoFisher	70011069
Phalloidin-CF405M	Biotium	00034
ProLong Diamond antifade mounting media	ThermoFisher	P36970
superfrost plus microscope slide	Fisherbrand	22-037-246
Triton X-100	Sigma	T8787
Zen Black 2.3 SP1 software	Carl Zeiss	https://www.zeiss.com/micros

Comments/Description

scopy/us/products/microscope-software/zen.html

Manuscript: JoVE_60769

Title: Dextran labeling and uptake in live and functional murine cochlear hair cells.

Authors: Angela Ballesteros and Kenton J. Swartz.

We are very grateful to the reviewers and editorial board for their careful evaluation and constructive suggestions that have significantly improved our manuscript. We have answered all their questions (in blue) and revised the manuscript accordingly. As requested by both reviewers, we have modified the title and abstract to emphasize the applications of this protocol. In addition, we have revised the text in our manuscript to reduce overlap with our eLife paper, corrected several grammatical errors and typos, and removed the commercial language.

Editorial comments:

The manuscript has been modified and the updated manuscript, 60769_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully read our manuscript and corrected several grammatical errors and typos.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Our paper was published with a Creative Commons CCO public domain license and permissions are not needed to reproduce figures. We have uploaded the information on the copyright permission from eLife into our Editorial Manager account. The citation on the figure legends has been modified to “This figure has been modified from[27].”, being reference 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).

3. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.

We have thoughtfully revised our manuscript to reduce the sections that presented some overlap with our previously published work.

4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We have removed all the commercial language from our manuscript.

5. Please do not use more than 1 note for each step.

Our protocol has been modified to include a maximum of 1 note per step.

6. Please avoid long steps/notes (more than 4 lines).

The steps and notes of our protocol have been modified to be no longer than 4 lines.

7. Step 1.1: Please write this step in the imperative tense.

We have written this step in the imperative tense.

8. Step 1.2: Please write this step in the imperative tense.

As suggested by reviewer number 2 (comment 5), we have removed this step due to its limited relevance for a successful outcome of this protocol.

9. Please do not abbreviate journal titles for references.

We have revised our references and removed any abbreviations.

10. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Commercial symbols have been removed from the Table of Equipment and Materials. We have also included information on the shaker that was used in our experiments.

11. Please sort the items in alphabetical order according to the name of material/equipment.

We have alphabetically ordered the items listed in the Table of Equipment and Materials.

Reviewer #1:

Manuscript Summary:

This paper provides an important new methodology for assaying the presence of functional mechanotransduction (MET) channels in cochlear hair cells. Ballesteros and Swartz describe the use of fluorescently-tagged 3 kDa Dextrans molecules to assay cellular uptake in mouse cochleas. The protocol is well described and images convincingly show specific uptake of fluorescent Dextrans by stereocilia, where sensory mechanotransduction channels reside, in addition to endocytotic entry in the cell body. Furthermore, when tip links are broken or mechanotransduction channels are pharmacologically blocked, dextrans uptake is abolished, implicating that the route of entry into stereocilia is through functional MET channels. This is an important methodological paper that could be useful for many labs assaying mechanotransduction function in cochlear hair cells.

Major Concerns:

None

Minor Concerns:

I have only minor comments that would improve the impact and utility of the paper.

1) Perhaps amend the title and abstract to emphasize that the dextran uptake experiments can be used to assay functional mechanotransduction channels. That is the major use of this method as presented.

We are trying to cover both pathways of uptake in the title, and the presumed endocytic mechanism doesn't require functional MET channels. However, to partially address this issue and the related one by reviewer #2, we have added '..live and functional..' to the title before "..hair cells.." and we have mentioned the specific goal of using the assay to assay functional MET channels in the abstract.

2) Have the authors attempted this method in vestibular hair cells? If so, it would be noteworthy to include that information and any similarities/differences in the protocol for cochlea/vestibule. It is not necessary to include a detailed description of dissection of the vestibule, however.

This is an excellent point, but we haven't tried vestibular hair cells yet.

3) The authors note that prolonged exposure to dextrans, even dextrans larger than 3 kDA, can lead to superficial labeling of stereocilia. This is distinct from the dextrans that are presumably entering through the functional mechanotransduction channels. It would be important to include details about how to differentiate between this nonspecific labeling and the desired uptake.

We observed that the 10kDa dextran-TR labels the surface of the stereocilia of all the hair cells (Figure 4A) while the 3kDa dextran accumulates at the tips of the shorter stereocilia rows and labels the stereocilia core (Figure 3C). We have now included an enlarged image of a couple of IHCs displaying the superficial labeling of the stereocilia observed with the 10kDa dextran (Figure 4A, inset). We hope that this would help the reader to differentiate between the labeling of the 3 and 10 kDa dextran-TR. However, because this difference would be hard to appreciate using conventional confocal microscopes (see new paragraph in the discussion), evaluating the dextran accumulation at the cell body would be a better way to differentiate between the uptake of these two dextrans.

4) It would also be important to include a bit that explains that a proportion of mechanotransduction channels are open at rest due to tension on the channels from tip links (i.e. in the absence of stimulation), and it is because of this that the dextrans are able to enter stereocilia in the absence of stimulation.

This is an excellent point. We now state that some MET channels will be open at rest due to tension on the channel by the tip links (Beurg, M, Nam J, Chen Q, Fettiplace R. J Neurophysiology, 2010. and Johnson SL, Beurg M, Marcotti W, Fettiplace R. Neuron. 2011). We added gentle shaking of the explants to increase the open probability of the MET channel. We also included more detailed information about our shaking conditions in the protocol and information on the shaker in the Table of Equipment and Materials.

5) The authors describe some control experiments that suggest that the dextrans is entering through the MET pore (Figure 5). It would be helpful for them to suggest even more precise controls—is Dextrans uptake abolished in mice in which MET is genetically abolished (i.e. Tmc1/2 dKO mice)?

We now mention in the result section these control experiments that were recently published in eLife (Ballesteros, A, Fenollar-Ferrer, C. and Swartz, K. J. eLIfe. 2018).

6) Have the authors tried this experiment at other early postnatal ages (P0-P8). They report use at P8, but previous work by the Ricci/Holt/Geleoc labs show that cochlear mechanotransduction currents are measurable between P0-P2 in the base of the cochlea.

This is an excellent point, but we have not looked at ages other than P6.

7) Are there any indications for why the uptake is so slow relative to FM1-43? FM1-43 takes seconds, while dextrans take 1.5 hours. If the bundles are stimulated (to increase the open probability), does the rate of uptake increase?

In the discussion, we mention that the lower permeation rate of the dextran is likely due to its larger size. The styryl dye FM1-43 (0.56 kDa) labels the hair cell body in seconds while the Texas Red-labeled aminoglycosides (1.29-1.43 kDa) need longer times of 30-60 min to label the hair cell body (Alharazneh, A., et al. Plos One. 2011). These two molecules permeate through the MET channel, so if the 3kDa dextran-TR permeates using the same permeation pathway, we would expect it to take a longer time due to its larger size. We have not attempted experiments to determine whether the stimulation of hair cells increases the update of dextran.

8) Are these results unique to Texas-Red-3KDA dextrans, or can you use any fluorescently conjugated 3 kDa Dextrans?

We have included a paragraph in the discussion to address this question. We would expect hair cells to uptake other small cationic dextrans like the 3kDa dextran-TR but not anionic dextran based on the cationic-selectivity of the MET channel. In our recent eLife paper, we report that we do not see uptake with the negatively charged 3kDa dextran-FITC (Figure7-figure supplement 1. Ballesteros, A, Fenollar-Ferrer, C. and Swartz, K. J. eLIfe. 2018).

9) Summary (Line 20) should read "Uptake of fluorescently-labeled dextran….can be used to assess for functional mechanotransduction channels"

We have changed this sentence accordingly.

10) The text should be checked carefully for grammatical errors and typos.

The manuscript text has been thoroughly checked for grammatical errors and typos.

11) Some descriptions in the introduction should be slightly modified to allow for multiple interpretations of current data.

a. Line 55—"Increasing experimental evidences indicate that….TMC1 is the MET channel in mature hair cells" should be changed to "Increasing experimental evidence indicates that TMC1 is a component of the MET channel in mature hair cells."

We thank the reviewer for this suggestion and have changed this sentence accordingly.

b. Line 58—"the tip-link responsible for mechanical gating" should be changed to "the tip-link responsible for transmitting mechanical force to the channel". It has not been demonstrated that the tip-link gates the channel, although this is one hypothesis.

We have changed this sentence accordingly.

12) Change tense in 1.1 and 1.2 to present tense to make consistent with rest of protocol. As opposed to what you did, what do you suggest the reader do?

Step 1.1 has been edited accordingly. Step 1.2 has been removed following reviewer's 2 comments on its limited relevance for a successful outcome of this protocol.

13) Some portions of the protocol should be clarified to assist the reader/viewer

a. Section 2

i. For the dissection portion of the protocol (Section 2), it would be helpful to reference the figure images for each part of the dissection in Figure 1.

This is an excellent suggestion. We have now referred each dissection step of the protocol with its corresponding panel of figure 2.

ii. 2.9 should read, "Place both temporal bones in the 35 mm dish and make sure they are covered with L15 media"

We have modified this sentence accordingly.

iii. 2.10 please spell out acronyms (WF10x) and (ACE)

We have spelled WF10x and clarified the type of light source used.

iv. 2.12—the authors note to place media in a Pyrex 9 depression plate, but perhaps a note here could also indicate that any standard cell/tissue culture well plate could be used for this.

We are unfamiliar with the use and performance of cell/tissue culture wells for incubation of the organ of Corti explants and thus can't comment on it. We have always used a glass 9-wells depression plate for incubation of our tissue explants.

b. Section 3

i. Make a new 3.1 that includes how stock solutions of Dextrans are made and stored (i.e. move the note at the end of the section to the beginning as 3.1)

We have moved this note to the beginning of section 3 and named it as step 3.1.

ii. include a note to protect Fluorescently labeled dextrans from light.

We have included a note at the end of section 3 describing the procedure we follow to protect the fluorescently labeled dextran from light during the incubation with the tissue.

c. Section 4

i. General—how long are washes performed, and do they require shaking (applies to 4.1, 4.2, 4.3, 4.5, and 4.7)

We have now included more information about the washing steps of our protocol.

ii. 4.4—if possible, it would be helpful to show a diagram of the tectorial membrane and Reissners membrane, or a micrograph showing it being removed. Removal of the tectorial membrane is difficult but critical because it is a source of high background labeling that can cause misleading results. Would be very helpful for the reader/viewer.

We agree with the reviewer that it would be very useful to show this step, but unfortunately, we don't have a camera attached to our dissection microscope that would give us enough resolution to illustrate it. We hope to capture this step in the video protocol.

d. Section 5

i. Since many readers/viewers will not have access to a super-resolution microscope, but could still find the dextran uptake protocol useful, perhaps the authors could mention alternative imaging strategies (with conventional confocal microscopy or deconvolution microscopy).

We have added a paragraph in the discussion addressing the use of conventional confocal microscopy, including deconvolution, to visualize and evaluate the uptake of dextran in inner ear hair cells.

14) Discussion

a. can the authors include a Citation for molecular weights of the Dextrans?

The information on the molecular weight of the dextran was obtained from the product information provided by the company that commercializes the dextran. We have included a citation directing to this information.

Reviewer #2:

Manuscript Summary:

This paper describes a protocol to label hair cells with a functional mechanotransduction (MET) channel. The authors use a fluorescent dextran that is 3 kDa in size. Tissue is dissected from the cochlea and incubated in the fluorescent dextran for 2 hours. The dextran specifically labels hair cells with a functional MET channel and is excluded from hair cells with a non-functional channel. Hence the protocol would be of interest to researchers involved in hearing-related studies. I had no major concerns with the manuscript which was on the whole well written and had a clear description of the protocol.

Major Concerns:

None

Minor Concerns:

1) I thought the title should specifically mention 'functional' hair cells as this is the crux of the story. This would help to distinguish it from the use of antibodies that could otherwise be used to simply label hair cells.

We have added 'live and functional' to the title as suggested. Now it reads "Dextran labeling and uptake in live and functional murine cochlear hair cells."

The steps listed would appear to lead to the desired outcome. I had only a few minor queries for the protocol:

2) Washing steps listed in lines 170, 180, 188, 193 and 194 (steps 4.1, 4.3, 4.7): How long were the washing steps? Is it simply wash buffer on and then remove or is there a short incubation?

We have included more information about the washing steps of our protocol.

3) On Line 145, the note regarding performing the steps under the stereomicroscope should go before step 2.10:

We have moved this note accordingly, and it is now before step 2.10 of our protocol.

4) I wonder whether the fact that lysine-fixable dextran is critical (mentioned in the discussion, line 335) should be included as a note in the protocol

We have now included an additional note after step 3.1. on the requirement for the use of lysine-fixable dextran.

5) Line 97 under Protocol - I thought the mouse breeding procedure was not necessary to the overall protocol. You could just state the strain of mouse that was used.

We have removed step 1.2 due to its limited relevance for a successful outcome of this protocol. However, we have maintained step 1.1 since keeping a good track of the age of the pups is relevant for these experiments. Step 1.1 has been edited to maintain the verb tense consistency through the protocol. Information on the wild type mouse strain used in these experiments is included in the Table of Equipment and Materials.

6) Line 154 2.13. Place at least three dissected cochleae on each well. Should the number of dissected cochleae per well be a recommendation rather than a requirement.

We believe that the use of replicates is as important as repeating the experiments three times, especially went working with live animal samples (Replicates and Repeats. Vaux, L.D., Fidler, F. and Cumming, G. EMBO Rep. 2012). We found some variability in our experiments, mainly due to damaged tissue or non-functional hair cells, and the use of replicas helped us to identify and interpret these differences.

7) Line 163 3.3. Incubate at room temperature for 2h with gentle shaking by using an orbital shaker. Is there a way to indicate how gentle is gentle. A setting?

We have now included in the protocol more detailed information on the shaking conditions used during the dextran incubation. The catalog number of the shaker used in these experiments has been included in the Table of Equipment and Materials.

8) Line 190 4.6. You mention a specific fluorophore for phalloidin but not for dextran. Readers would need to ensure that the 488 label is different to the fluorophore used on the dextran. You could suggest which specific fluorophore that you used for your experiment (you do this in the list but not in the protocol).

We have added a sentence clarifying the color of the conjugated phalloidin that was used in combination with the TR- or FITC-labeled dextran.

I think it might be worth mentioned or discussing whether the protocol would work on older mice? You mention younger mice in the discussion, but what about older tissue?

In the discussion, we mention why we choose P6 mice due to the expression of TMC proteins and the appearance of MET currents. We have added a brief comment on additional complications that may be encountered when studying older mice.

9) Figure 2 - What are the tissue damaged regions? Is this damage from forceps or handling. It would be worth mentioning why there are damaged regions and whether any damage was specifically inflicted in order to demonstrate cells with no dye uptake.

We have added more information about the damaged tissue shown in Figure 2 and 3. Now the sentence in the discussion reads: "……..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 3B)"

10) Line 209 Have you defined NA in the paper?

We have now defined NA in the manuscript.

11) Order of figures. Figure2A is mentioned first.

We have removed the citation to Fig. 2A in the introduction.

A few other minor points that I noticed along the way:

12) Line 43 "…protrude from their apical region. The mechanically stimuli deflects…" Do you mean Mechanical stimuli deflect…

We have changed this sentence accordingly and thoroughly revised the manuscript test for grammatical errors and typos.

13) Line 70 "…explants with a little bit of a larger molecule, 3 kDa dextran fluorescently…" A little bit larger is a rather vague term.

We have removed the words “a little bit of a” from this sentence. Now this sentence reads “...explants with a larger molecule, 3 kDa dextran fluorescently…..”

14) Line 73 "…composed by many D- glucose molecules bond by…" Do you mean bound?

We have changed this sentence accordingly.

15) Line 132 2.6. "Make and incision form the back to the frontal…" Do you mean Make an incision from…

We have changed this sentence accordingly.

16) 261 "…observed a uniform diffuse fluorescence signal along the stereocilia with enrichment at the tips of the shorter stereocilia rows where the MET channel 262 is located.." This sounds contradictory?

To clarify this sentence, we have removed the word “diffuse”.

17) 320 "…than loads of practice and patience" Just sounded a little colloquial.

We have removed the word “loads” from this sentence. Now it reads “…other than practice and patience.”.

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