Hydrostatic measurement and finite element simulation of the compliance of the organ of Corti complex

Daniel Marnell,¹ Talat Jabeen,¹ and Jong-Hoon Nam^{2,a)}

¹Department of Biomedical Engineering, University of Rochester, 204 Goergen Hall, Rochester, New York 14627, USA

²Department of Mechanical Engineering, University of Rochester, 212 Hopeman Engineering Building, Rochester, New York 14627, USA

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In the mammalian cochlea, the geometrical and mechanical properties of the organ of Corti complex (OCC, consisting of the tectorial membrane, the organ of Corti, and the basilar membrane) have fundamental consequences for understanding the physics of hearing. Despite efforts to correlate the mechanical properties of the OCC with cochlear function, experimental data of OCC stiffness are limited due to difficulties in measurement. Modern measurements of the OCC stiffness use microprobes exclusively, but suffer ambiguity when defining the physiologically relevant stiffness due to the high nonlinearity in the force-displacement relationship. The nonlinearity stems from two sources. First, microprobes apply local force instead of fluid pressure across the OCC. Second, to obtain the functionally relevant stiffness, the OCC is deformed well beyond *in vivo* levels ($>10\text{ }\mu\text{m}$). The objective of this study was to develop an alternative technique to overcome challenges intrinsic to the microprobe method. Using a custom-designed microfluidic chamber system, hydrostatic pressures were applied to the excised gerbil cochlea. Deformations of the OCC due to hydrostatic pressures were analyzed through optical-axis image correlation. The pressure-displacement relationship was linear within nanoscale displacement ranges ($<1\text{ }\mu\text{m}$). To compare the results in this paper with existing measurements, a three-dimensional finite element model was used. © 2018 Acoustical Society of America. <https://doi.org/10.1121/1.5023206>

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I. INTRODUCTION

In the mammalian cochlea, passive mechanics have fundamental consequences in encoding sound amplitude and frequency into neural signals (Lighthill, 1981; Olson *et al.*, 2012). Structurally, the cochlea consists of fluid-filled cavities whose compartments are divided by an elastic cochlear partition. Acoustic pressures mediated by the cochlear fluid vibrate the partition between the scala media and the scala tympani called the organ of Corti complex (OCC; the tectorial membrane, the organ of Corti, and the basilar membrane). The hair cells embedded in the organ of Corti are deflected and activated by the relative vibrations between the tectorial membrane and the basilar membrane (Fettiplace and Kim, 2014).

The organ of Corti mechanics are drawing increasing attention with the introduction of new experimental (Karavitaki and Mountain, 2007b; Karavitaki and Mountain, 2007a; Chen *et al.*, 2011; Ghaffari *et al.*, 2013; Xia *et al.*, 2013; Ramamoorthy *et al.*, 2014) and theoretical approaches (Andoh and Wada, 2004; Steele *et al.*, 2009; Meaud and Grosh, 2010; Nam and Fettiplace, 2010; Nam and Fettiplace, 2012; Ó Maoiléidigh and Hudspeth, 2013; Meaud and Grosh, 2014; Nam, 2014; Zagadou *et al.*, 2014). The mechanical properties of the OCC are fundamental for understanding cochlear function. Despite increasing data

regarding the mechanics of the OCC, there exists a significant gap between the information that theoretical models require and available measurement data. Arguably, the most fundamental mechanical property that still lacks agreement is the stiffness of the OCC, especially in the middle of apical turns of the cochlea. The stiffness of the OCC is considered the primary factor to determine the frequency range of hearing (Olson *et al.*, 2012; Liu *et al.*, 2015).

As the basilar membrane is the most prominent mechanical structure in the OCC, the OCC stiffness has been measured at and represented by the basilar membrane. Compliant microprobes have been used to measure the mechanical properties of the basilar membrane in various species (Gummer *et al.*, 1981; Miller, 1985; Olson and Mountain, 1991, 1994; Naidu and Mountain, 1998; Emadi *et al.*, 2004; Emadi and Richter, 2008). Although the microprobe technique is intuitive and efficient, it has acknowledged complications. For example, the applied stimulation is a localized contact force unlike natural fluid pressure across the OCC, and the OCC is deformed more than $10\text{ }\mu\text{m}$ to obtain the functionally relevant stiffness. As a result, the measured force-displacement relationship is highly nonlinear.

The OCC deformation due to *static* fluid pressure is believed to better represent the intrinsic compliance of the OCC than the deformation due to contact deformation (von Békésy, 1960). Under static stimulation, the elastic (stiffness) term of OCC vibrations can be separated from the viscous and inertial components and thus can be measured

^{a)}Electronic mail: jong-hoon.nam@rochester.edu

explicitly. However, it is non-trivial to apply a hydrostatic pressure to the cochlear partition. Hydrostatic pressures cannot be applied across the cochlear partition in the intact cochlea because the top and bottom fluid spaces across the cochlear partition are interconnected through the helicotrema, an opening at the apex of the cochlea. In a study by von Békésy (von Békésy, 1960), the helicotrema was closed, hydrostatic pressure was applied through a water column attached to the round window, and the deformation of the Reissner's membrane was measured through an opening made in the apical turn of the cochlea. Since then, to our knowledge, no attempts have been made to measure the OCC deformation under hydrostatic pressure.

The objective of this study was to develop a novel experimental approach that enables us to apply a known hydrostatic pressure to the OCC and measure its deformation. We tested the hypothesis that, the pressure-displacement relationship is linear within the nanoscale physiological displacement range ($<1\ \mu\text{m}$). To compare our results with existing microprobe measurements, a computational model of the OCC was used.

II. METHODS

A. Micro-chamber design

Micro-chambers were designed using computer aided drawing software (Pro/ENGINEER, PTC, Needham, MA) and fabricated using stereolithography (ProtoLabs, Maple Plain, MN). This study focuses on measuring OCC deformations due to hydrostatic pressures. However, the chamber

was designed for general physiological experiments such as acoustical and electrical stimulations and measurements. Each micro-chamber has two compartments that are connected through a small slit opening where a tissue section is placed (Fig. 1). The slit opening is rectangular, with dimensions of 0.5 mm by 1 mm after considering the scala tympani width of the gerbil cochlea (Kim *et al.*, 2008). The top compartment is open, allowing a clear view of samples at the slit by an immersion objective as well as the access of micro-manipulators and electrodes. The bottom compartment is a circular channel ($d = 0.5\ \text{mm}$) that connects four openings: a stimulation port, a release port, and two perfusion ports. The stimulation port is at one end of the micro-channel 12 mm away from the slit, and the channel continues from the slit to a pressure release port at the other end. During tissue placement, the open release port serves as a pressure release, ensuring the tissue is not subjected to excessive pressures that could cause damage during the placement procedure. Before the experiment begins, the release port is also closed with a stiff cap. Polytetrafluoroethylene (PTFE) tubing with 1 mm inner diameter inserted into the perfusion ports is connected to a perfusion system that circulates fresh fluid into the microchannel and perfusion channel. Before hydrostatic measurement, the perfusion ports are used to refresh the solution. During hydrostatic experiments, all ports are closed except the inlet perfusion port. The inlet port remains connected to a fluid column that sets the pressure level [Fig. 1(C)]. As a result, the micro-chamber has the capability to apply hydrostatic pressure to the slit. To provide a clear

FIG. 1. (Color online) Micro-chamber and experimental design. (A) Top-down view of the micro-chamber. The slit opening is at the center. The stimulation port (SP) and release port (RP) are connected by a micro-channel that runs down the length of the chamber and connects to the slit opening. Fluid is perfused through the micro-channel through perfusion ports (PP) on the side of the chamber. (B) Magnified view of the excised turn preparation placed across the micro-chamber slit. The apical and basal scalae openings, the modiolar regions, and the periphery around the lateral walls are sealed with glue (opaque red color). At the targeted region of the OCC, the inter-scalae bone is removed along with the Reissner's membrane to expose the tissue. (C) Overall experimental setup, viewed along the cross-section of the perfusion channel. Perilymph is perfused to fill the micro-channel and the top well. The fluid efflux tube is connected to a three-way valve, which controls the connections between the micro-chamber, the leak rate column, and the pressure vessel. Adjustment of the depth of the cylinder into the vessel applies hydrostatic pressure to the slit sample. The black dashed line indicates the fluid equilibrium level (P_0).

optical pathway, the base of the micro-chamber directly underneath the slit was covered by a transparent glass cover-slip attached by optical glue [Fig. 1(C)].

B. Tissue preparation

Fresh cochleae were harvested from deeply anesthetized Mongolian gerbils according to methods approved by the University Committee on Animal Resources at University of Rochester. Young gerbils of either sex aged 16–21 days old were used for experiments. At this age, the cochlea at the middle and apical turns has been shown to be fully developed in terms of its mechanical (Emadi and Richter, 2008) and anatomical (Schweitzer *et al.*, 1996) properties.

The dissection and preparation of tissues for experiments were performed in a chilled bath solution containing HBSS (+CaCl₂, +MgCl₂), 10 mM HEPES, and 20 mM glucose. Following animal decapitation, the cochlea was isolated [Fig. 2(A)]. The basal turn and apical turn of the cochlea were removed using forceps. An entire turn spanning between 6.0 and 9.5 mm from the base was obtained including the modiolus and the lateral bony shell. A 1 mm long region, centered at 8.5 mm from the basal end was our target location for measurements. The target location for measurement was exposed by picking away the inter-scalae bone on both the top [separating the middle and apical turns; red outlined region, Fig. 2(B)] and bottom (separating the middle and basal turns) sides. About a half-turn away from the target location, there are the apical and basal scalae that are left open after removal of the apical turn and the basal turn. To separate the top and the bottom fluid spaces, those scalae openings were sealed [apical seal and basal seal, Fig. 1(B)] using cyanoacrylate glue (Loctite, Rocky Hill, CT).

The micro-chamber was filled with artificial perilymph (in mM: 154 NaCl, 6 KCl, 1.5 CaCl₂, 10 HEPES, 8 glucose, and 2

Na-pyruvate; pH 7.4; 320 mOsm). The excised turn was placed in the micro-chamber, with the apical aspect of the preparation facing up and the exposed target region overlying the slit opening. The perimeter of the excised cochlear turn was sealed by applying glue between the remaining bony lateral wall of the preparation and the surface of the micro-chamber [peripheral seal, Fig. 1(B)]. After completion of the sealing tasks, the Reissner's membrane was removed at the target location. When the peripheral and scalae openings were sealed properly and the OCC was not damaged, the fluid spaces above and below the OCC were fully separated [Fig. 1(B)]. This preparation allows hydrostatic pressure application through the bottom compartment to the entire OCC of the excised turn.

The prepared micro-chamber was moved to an upright microscope (Olympus BX51, 40× water immersion objective with numerical aperture of 0.8 and working distance of 3.3 mm). The outlet tube of the perfusion system was connected to the three-way valve and accompanying fluid columns [Fig. 1(C)] used to set the fluid (pressure) level. At this stage, the fluid levels of the micro-chamber release port and the fluid columns are in equilibrium. The release port was gently closed with a stiff cap. Measurements typically began within 30 min post-mortem, and lasted 1 h.

The structural integrity of prepared tissues was inspected visually. During surgical processes such as bone removal, tissue samples often exhibited visible damage. Because this study relies on stable tissue structures for image analysis, any disrupted structures led to failure in image analysis. A tissue preparation was considered viable for our purpose of passive mechanical measurement when: (1) hair cells and their stereocilia bundles exhibited no signs of morphological damage and retained their orderly arrangement; (2) the tectorial membrane fully extended over the three rows of outer hair cells, and showed no sign of detachment [Figs. 2(D) and 2(E)].

FIG. 2. Tissue preparation in the micro-chamber. (A) The isolated gerbil cochlea, viewed from the apical side. The red markers and broken line denote the target region, 8–9 mm from the base. (B) Apical side view of excised turn preparation, showing glue seal at apical opening. Red outline indicates the exposed target region for study. (C) View of tissue section after placement onto the micro-chamber over the slit. The three arrows mark the three rows of outer hair cells. White arrows indicate the radial (y) and longitudinal (x) axes. Tectorial membrane is not seen at this focus level. (D) High-magnification at the level of the tectorial membrane. The edge of the tectorial membrane is marked by the dashed red line. (E) High-magnification image of the same tissue preparation shown in panel (D), but with the focus level adjusted to the level of the outer hair cells. The image is also taken from the same location. The three rows of outer hair cells (white arrowheads) are clearly visible, and the location of the tectorial membrane edge from panel (D) is marked by the red dotted line.

C. Automated image acquisition and apparatus

The focal plane was set at the middle of the second-row outer hair cell body (the origin of the optical axis). The microscope was motorized so that a stepper motor (4118L, Lin Engineering) adjusts its focus level. With a gear ratio of 4:1 coupling the motor to the focus adjustment knob of the microscope, the focal plane level was adjustable with a step size of $0.125\ \mu\text{m}$. As the focus level was adjusted, reference images were captured every $0.25\ \mu\text{m}$ along the optical axis. The images were captured by a CCD camera (ORCA 05-G, Hamamatsu) through the image acquisition toolbox in Matlab. After a set of reference images were acquired, the focus level returned to the origin level. Then, a series of hydrostatic pressures were applied to the tissue by adjusting the fluid level within a vessel connected to the perfusion outlet port of the micro-chamber [Fig. 1(C)]. A linear rail stepper motor (S17 RGS04, Haydon Kerk) controlled the depth of the cylinder in the vessel. With an area ratio of 3 between the fluid surface and the cylinder cross-section ($6300\text{--}2100\text{ mm}^2$), each displacement step of the cylinder ($13\ \mu\text{m}$) resulted in a $4.2\ \mu\text{m}$ fluid level change (equivalent to a 41 mPa pressure change). Twelve successive steps of the cylinder provided the fluid level increment of 0.05 mm, at which deformed (measurement) image was captured. The rate of pressure change was 3 Pa/min. The two stepper motors were driven by a data acquisition device (NI USB-6008, National Instruments) through Matlab. A 1 s delay separated the motor steps and image acquisition to ensure images were not blurred by vibrations transmitted to the sample from the stepping of the motors.

D. Leak rate measurement

A practical concern was the pressure loss originating from fluid leakage due to an incomplete seal of the tissue preparation. In order to confirm the quality of the placement and scalae opening seals, the leak rate was measured prior to and following each experiment. The leak rate was quantified by closing the connection between the outlet tube of the chamber and the fluid vessel, and opening the connection between the chamber and a 2-mm diameter fluid column using a three-way valve [Fig. 1(C)]. A 2-mm thick tube was used for leak rate measurements because it provided greater sensitivity (fluid level change) for the same volumetric flow rate compared to the wide vessel. After allowing the fluid spaces between the chamber's top compartment and the leakage test column to equilibrate, the connection was closed at the valve, and the fluid level in the leakage test column was set to be 2 mm higher (+20 Pa) than the equilibrium level. The connection at the valve was re-opened, and the fluid level within the column was observed over a period of 2 min. Any preparations that showed greater than 0.1 mm change in the column's fluid level over the 2 min time period ($0.16\ \mu\text{L}/\text{min}$ of leak rate at 20 Pa) either before or after the experiment were not considered for further analysis. Given the large fluid surface area of the vessel compared to the leakage test column, this leak rate corresponds to a rate of fluid level change of 25 nm/min in the large vessel. Since the rate of fluid level change during the measurement (pressure)

sequence is 0.3 mm/min (equivalent to the 3 Pa/min), the rate of pressure loss due to leakage is at least four orders of magnitude slower than the rate of the pressure change applied by the cylinder. Therefore, for well-sealed preparations, our pressure application can safely be considered static.

E. Finite element model

A finite element model of the OCC was modified from a previous work (Nam and Fettiplace, 2010). The major structural components of the organ of Corti, such as the pillar cells, outer hair cells, and Deiters' cells in addition to the anisotropic tectorial and basilar membranes are represented by beam elements that allow axial and bending deformations [Fig. 3(A)]. In the previous study, the supporting cells in the organ of Corti such as the Hensen's, Claudius and inner sulcus cells were not incorporated considering that their structural contribution can be neglected (i.e., they do not have organized cytoskeletal structures such as microtubules or crystallized actin bundles). These supporting cells were newly incorporated in this study to facilitate comparison with experimental measurements by adding more nodes to represent the entire top surface of the organ of Corti. They are represented by three-dimensional (3-D) tetrahedral elements that take up volume (blue shaded regions, Fig. 3), but add negligible stiffness ($<2\%$ of total stiffness). Radial sections repeat every $10\ \mu\text{m}$ along the longitudinal direction, corresponding to the spacing of the outer hair cells [Fig. 3(B)]. Longitudinal sections are coupled through beam elements along the tectorial membrane, reticular lamina, and

FIG. 3. (Color online) Finite element model of the organ of Corti complex. (A) Cross-sectional view of model geometry, including all nodes. Filled black markers (●) indicate fixed nodes where there is no displacement. The major structural elements are composed of beam elements, while the regions of supporting cells (such as Hensen's and Claudius cells) are composed of solid elements (blue shaded regions) containing mass but little stiffness. The cross marker (+) indicates the focal origin of imaging. (B) Three-dimensional view of model. The cross-section from panel (A) is repeated every $10\ \mu\text{m}$ along the longitudinal axis (x axis).

basilar membrane in a continuous manner, with discrete coupling through the Deiters' cell process.

Detailed geometrical information of the gerbil OCC was obtained from the literature (Edge *et al.*, 1998). The mechanical properties of fine structures were determined based on available data such as the stiffness of the basilar membrane (Gummer *et al.*, 1981; Olson and Mountain, 1991; Olson and Mountain, 1994; Naidu and Mountain, 1998; Emadi *et al.*, 2004), outer hair cells (Iwasa and Adachi, 1997), hair cell's stereocilia (Nam *et al.*, 2015), tectorial membrane (Richter *et al.*, 2007; Gu *et al.*, 2008), Deiters' cells (Laffon and Angelini, 1996; Zetes *et al.*, 2012), and pillar cells (Tolomeo and Holley, 1997). After incorporating the mechanical properties of individual components, the overall cochlear responses agree to other measured cochlear responses, such as elastic longitudinal space constant, the wavelength of the traveling waves, and frequency-location relationship (Liu *et al.*, 2015). These properties were not adjusted from the values in the previous study (Liu *et al.*, 2015).

A 1-mm-long cochlear section from a location 8.5 mm from the base was simulated. Considering the attachment to the spiral lamina and spiral ligament, the medial and lateral edges of the basilar membrane, and the medial edge of the tectorial membrane were clamped [filled circles, Fig. 3(A)]. The two longitudinal ends (nodes at $x = 8$ and 9 mm) were also clamped. Although these boundary conditions at the longitudinal ends are artificial, the results are minimally affected because the longitudinal space constant of the basilar membrane is < 0.1 mm (Naidu and Mountain, 2001). To simulate the experimental conditions, the basilar membrane of the model was subjected to a uniform and unilateral pressure.

III. RESULTS

Hydrostatic pressures were applied to nine gerbil tissue preparations and deformations of the OCC were measured and analyzed. Among the nine measured preparations, five were rejected due to tissue damage and/or incomplete seal between the two fluid spaces. The experimental and analysis schemes were validated using artificial membranes before being extended to tissue measurements. A finite element

model was used to analyze experimental data in further detail.

A. Static displacement measurement by image correlation

The compliance of the OCC was calculated by measuring the tissue deformation under hydrostatic pressure by an image cross-correlation method [similar to Taute *et al.* (2015)]. With the fluid level in the vessel set to zero ($H = 0$, no pressure applied), a stack of images at different heights along the optical axis [z -axis, Fig. 4(A)] were obtained. The origin ($z = 0$) was set to the level of the midpoint of the outer hair cell bodies [the cross marker in Fig. 3(A)]. At this level, the image contrast was superior due to the characteristic structural patterns such as the pillar cells and three rows of the outer hair cells. Considering the tissue displacement is expected to be less than $1 \mu\text{m}$ at a maximum applied pressure of 5 Pa , reference images were taken from $z = -4$ to $4 \mu\text{m}$ with a $0.25 \mu\text{m}$ increment to form a library of 33 reference images. The hydrostatic pressure was applied through a fluid-filled vessel connected to one of the perfusion (fluid-in) ports of the micro-chamber [Figs. 1(A) and 1(C)]. The fluid level in the vessel was changed from $H = -0.5 \text{ mm}$ to $+0.5 \text{ mm}$ with a 0.05 mm increment and measurement images were taken at each height, corresponding to a pressure range between -5 and 5 Pa , in increments of 0.5 Pa ($\Delta p = \rho g \Delta h$, where ρ is the mass density of the fluid, g is the gravitational acceleration, and Δh is the height difference of water column).

The tissue displacements were calculated from 2-D cross-correlation of images. Each image was discretized into square sections. The size of a section was $16 \times 16 \mu\text{m}^2$ for the measurement images and $18 \times 18 \mu\text{m}^2$ for the reference images [Fig. 4(B)]. Considering the width of the basilar membrane ($250 \mu\text{m}$, Fig. 2), this section size is small enough to resolve the peak displacement along the radial (y) axis. Each measurement image was cross-correlated with the 33 reference images. The cross-correlation coefficient, $r(u, v)$, was obtained for each reference-measurement image pair by normalizing 2-D cross-correlation between a reference image **C** and a measurement image **D** as the latter is shifted by (u, v) ,

$$r(u, v) = \frac{\sum_{x,y} (\mathbf{C}(x, y) - \bar{\mathbf{C}}_{u,v})(\mathbf{D}(x - u, y - v) - \bar{\mathbf{D}})}{\left\{ \sum_{x,y} (\mathbf{C}(x, y) - \bar{\mathbf{C}}_{u,v})^2 \sum_{x,y} (\mathbf{D}(x - u, y - v) - \bar{\mathbf{D}})^2 \right\}^{0.5}}, \quad (1)$$

where, $\bar{\mathbf{C}}_{u,v}$ represents the mean value of $\mathbf{C}(x, y)$ corresponding to the section $\mathbf{D}(x - u, y - v)$,

$$\bar{\mathbf{C}}_{u,v} = \frac{1}{n^2} \sum_{x=u}^{u+n-1} \sum_{y=v}^{v+n-1} \mathbf{C}(x, y). \quad (2)$$

The maximum correlation coefficient was computed for each of the reference images to plot maximum coefficients vs elevation (z) curve corresponding to each measurement height [Fig. 4(C)]. The data points near the peak of the r - z curve were fitted with a parabolic curve to find the elevation of maximum r value. The shift between two correlation

FIG. 4. (Color online) Compliance measurement using image correlation. (A) Displacement measurement using image correlations. Left: hydrostatic pressure was applied to the tissue through the micro-chamber. Right: a library of reference images are obtained at different focal depth (z). (B) Deformed images were taken at different pressure levels (h_i and h_j) and their section of images correlated with (indicated by the star symbol) the reference image sections. (C) The point of maximum correlation indicates the position of the measurement image section in the z -axis. As the fluid column height changes from h_i to h_j , the shift of the correlation-position (r - z) curve indicates the displacement in the z -direction. (D) An artificial membrane was subjected to hydrostatic pressure and its 3-D deformation was measured using image correlations. (E) Radial deformation pattern (circles) agrees with plate theory (solid curve). (F) Displacement-pressure relation of the artificial membrane.

curves (Δz) is the displacement of the tissue in the z -direction due to the corresponding pressure change, $\rho g(h_i - h_j)$.

It was difficult to obtain reliable data over the entire field of view—in some locations, the image analysis failed. There were two criteria to determine the success/failure of image analysis at a specific point. First, when the image correlation coefficient curve showed no obvious peak [unlike the two curves in Fig. 4(C)], the analysis result was not used. Second, when the displacement-pressure relation was non-monotonic, the data point was rejected. The failure of image analysis was ascribed to different causes. For example, it is difficult to analyze the images when the analyzed section is optically homogeneous (e.g., fluid spaces), when local tissue damage results in structural instability, or when there is leakage between the two fluid spaces. It took about 15 min to run one set of measurements at a location (acquiring reference images, and applying a sequence of pressures).

B. Hydrostatic stimulation of artificial membranes

Since we propose a new stimulation and measurement technique, we wanted to verify that: (1) hydrostatic pressures are successfully delivered to the slit sample with a known pressure step, and (2) the image correlation method can resolve deformations on the scale of tens of nanometers. Our measurement and analysis schemes were verified using an artificial membrane with known mechanical properties (Teflon 50 A, DuPont Young's modulus $E = 0.4$ – 0.552 GPa, Poisson's ratio $\nu = 0.46$, thickness $t = 12.5$ μm) on a circular glass pipette [Fig. 4(D)]. To provide image contrast and optical variation, 10 μm diameter microbeads were spread across the membrane surface. The focus level was set to the center of the microbeads. As the artificial membrane is stiffer than biological tissue, greater pressure levels were applied. The deformed shape along the radial axis x [circular markers,

Fig. 4(E)] conformed to the solution from plate theory [solid curve, (Reddy, 2007)],

$$\Delta z = \Delta p(a^2 - x^2)^2 / 64D, \quad (3)$$

where the bending rigidity $D = Et^3 / 12(1 - \nu^2)$, and a is the radius. The fitting curve was obtained with $E = 0.5$ GPa and $a = 0.38$ mm.

The pressure-displacement relation was highly linear ($R^2 > 0.95$) as was expected for an elastic plate subjected to small deformation [that is, the maximum displacement is at least one order of magnitude smaller than its thickness, Fig. 4(F)]. The measured displacement per unit pressure at the center of the membrane [3.2 nm/Pa, Fig. 4(F)] agrees to the value measured with an acoustic method, in which a calibrated low-frequency pressure stimulus from a speaker is applied to the artificial membrane, and its displacement is measured by laser interferometry. The elastic modulus of the membrane could be estimated from the theory of plate deformation. The measured value of 3.2 nm/Pa is equivalent to an elastic modulus of 0.5 GPa, which agrees with known value of the material [0.4 – 0.552 GPa at room temperature, (Ashby, 2005)].

C. Assessment of fluid leakage

Preventing fluid leakage between the top and the bottom fluid spaces is a challenging but crucial task of our experiment. The leakage could occur due to incomplete sealing between the isolated cochlea and the chamber, incomplete sealing of the scalae openings, and/or tissue damage. When there is any leakage between the two fluid spaces, the levels of the two fluid surfaces (the top compartment of the micro-chamber and the pressure vessel) equilibrate over time, with the leakage rate proportional to the pressure applied. When

FIG. 5. (Color online) Deformation of the OCC due to hydrostatic pressure. (A) Tissue preparation on micro-chamber under 10 $\times$ objective. The solid and dashed arcs indicate the locations of inner hair cell bundles, and the second row of outer hair cells, respectively. The rectangular boxes indicate regions analyzed under 40 $\times$ objective. (B) Tissue deformation pattern for "L2" from (A). On the grid points along the longitudinal-radial axes, the displacement gains in nm/Pa are indicated. Points from which we could not produce reliable analysis results are indicated with "X" marks. (C) Cross-correlation curves at the pressure levels of -5, 0, and +5 Pa for a point located along the second row of outer hair cells [the fourth row from the top in panel (B)]. (D) Pressure vs displacement curves for all points ($n=8$) located along the second row of outer hair cells. The thick curve indicates the average of the individual curves.

the leakage is non-negligible, the pressure level can change within the measurement time scale (e.g., over a few minutes). Fluid leakage through or around the tissue sample can lead to an underestimation of the tissue compliance.

Two remedies were made to ensure the measured displacements were due to hydrostatic pressure, not affected by fluid leakage. First, leak rate was measured for every preparation twice—before and after tissue deformation measurements. Any preparation with a leakage rate exceeding 0.16 $\mu\text{L}/\text{min}$ was rejected. The distinction between leaking and non-leaking preparations was obvious. For leaky preparations, the fluid levels equilibrated quickly, typically within tens of seconds. The volumetric leak rate for poorly sealed preparations ($n=5$) was $>5 \mu\text{L}/\text{min}$, far exceeding the cutoff of 0.16 $\mu\text{L}/\text{min}$. For suitable preparations with good seals, the fluid level change in the leakage-test column was undetectable ($<0.1 \text{ mm}$) over 2 min. One set of the hydrostatic experiment took approximately 10 min. As a secondary remedy, we ensured that rate of pressure change during the measurement sequence far exceeded the rate of pressure loss due to small leakage by choosing a fluid vessel with a large fluid surface area.

In four out of nine preparations, there was no sign of leakage both before and after hydrostatic measurements (leakage rate $<0.16 \mu\text{L}/\text{min}$). As anticipated, those preparations with measurable leakage suffered from unreliable analysis outcomes such as non-monotonic pressure-displacement relationship or insufficient image correlations.

D. Hydrostatic deformation of the OCC

These hydrostatic measurement and analysis methods were applied to the excised OCCs (Figs. 5 and 6). The focus level was set to the midpoint of the cell bodies of the second row of outer hair cells. In principle, the tissue displacement could be obtained at any point of the field of view. However, some regions were not analyzable. Often, these regions were located in parts of the field of view that were of low image contrast or small optical variation [such as the most lateral sites in Fig. 5(B)].

Multiple regions of the exposed tissue section were measured under the 40 $\times$ water immersion objective [Fig. 5(A)]. The cross-correlation curves for successfully measured points all showed a similar trend, with positive

FIG. 6. Responses to hydrostatic pressure from multiple tissue preparations. (A) Pressure vs displacement curves for points located along the second row of outer hair cells for individual preparations. (B) Radial deforming patterns. The origin of the radial location is at the position of the inner hair cell. (C) Longitudinal deforming patterns. The origin is at 8.5 mm from the basal end of the cochlea. Note that, in one preparation ("1101"), three measurements were made at slightly different locations.

pressures showing peak cross-correlation at negative z (elevation) levels, consistent with the tissue moving upwards through the focal plane [Fig. 5(C)].

The pressure-displacement relationship for a given tissue section under hydrostatic pressure was highly linear within the pressure range between -5 and 5 Pa [Fig. 5(D)]. The R^2 value of linear regression was greater than 0.90 for over 90% of measured points. The measured displacement gain (i.e., the slope of pressure-displacement relationship, units in nm/Pa) was lower at points closer to the pillar cells, trending higher for more lateral positions [Fig. 5(B)]. The compliance of the OCC was represented by the displacement per pressure at the second-row outer hair cells. For example, for the sample shown in Fig. 5(B), the OCC compliance of the sample is represented by the mean of the displacement gain values along the fourth row from the top. The compliance of the OCC shown in Fig. 8(B) is 95 nm/Pa, with a linear fit $R^2 > 0.9$ [Fig. 5(D)].

Across the four preparations, the compliance of the OCC was 85 ± 12 nm/Pa (mean $\pm$ standard error of the mean, Fig. 6). The radial deforming patterns were presented in Fig. 6(B). For each radial coordinate j , the values along the longitudinal grid points were averaged, or the displacement gain in nm/Pa at the radial location j , $g_j = (1/n_j) \sum_{i=1}^{n_j} g_{ij}$, where g_{ij} is the displacement gain at grid point (i, j) . Because of non-analyzable points [indicated with the cross symbols in Fig. 5(B)], the number of longitudinal data points (n_j) varies along the radial location [e.g., $n_1 = 4$, and $n_2 = 8$ in Fig. 5(B)]. Since the OCC is attached along the spiral limbus (medial edge) and the spiral ligament (lateral edge), the maximum deformation is expected in the middle of OCC, near the outer hair cells. The radial deforming pattern demonstrates that the measured deforming pattern conforms to these geometrical constraints. To observe the radial deforming pattern of the OCC in detail, there are other existing methods, such as hemi-cochlear preparation (Teudt and Richter, 2007), confocal microscopy (Fridberger et al., 2002), or optical coherence tomography (Chen et al., 2011).

In principle, we can measure the longitudinal gradient of OCC compliance even from a single set of images, considering that the viewable length is $220 \mu\text{m}$ (for the $40\times$ objective). In practice, within that cochlear length span, our method could not confidently capture the longitudinal stiffness variation. The longitudinal deformation patterns of the four tissue preparations are presented in Fig. 6(C). There was large variation not only within individual traces, but also between preparations (a linear fit R^2 value of 0.048). In order to obtain longitudinal trend of the OCC stiffness, more measurements over a longer span (from different longitudinal locations) is necessary.

E. Comparison between probe-measured stiffness and compliance due to pressure

To compare our measured compliance presented in displacement per pressure (nm/Pa) with previous point (local) stiffness values presented in force per displacement (N/m), we used the finite element model. Note that our finite element model cannot simulate the nonlinear force-displacement relationship because it does not have the details of composite

basilar membrane layers [see Kapuria et al. (2017)]. However, the model can simulate the difference between a locally concentrated force and evenly distributed force (pressure). The diameters of probe tips are typically between 10 and $30 \mu\text{m}$, and the probe tips are not flat. Therefore, there exists variation/uncertainty in the force distribution on the basilar membrane surface depending on the probe tip shape (Nam et al., 2015).

Previous studies applied the beam theory to consider the effect of probe tip size on the stiffness value [e.g., the probe force was distributed evenly over the span of probe tip thickness (Gummer et al., 1981; Olson and Mountain, 1991)]. In those studies, the longitudinal elastic coupling of the OCC was neglected, which is a fair assumption for the basal location measurements where the longitudinal space constant is $< 20 \mu\text{m}$ (Naidu and Mountain, 2001). A factor that is difficult to quantify is the tip shape (sharpness or curvature) as shown in Teudt and Richter (2014). In this study, to simulate the variation of probe tip shapes, the applied force was normally distributed to the basilar membrane. Or, the force at the distance r from the center of the basilar membrane is $f(r) = f_0 \exp(-r^2/2\sigma^2)$, where σ is the standard deviation. The probe-measured stiffness is defined as the net force divided by the displacement of the basilar membrane, $f_{\text{Probe}}/\delta_{\text{BM}}$, where $f_{\text{Probe}} = \sum f(r)$ [Fig. 7(A)]. The compliance due to fluid pressure is estimated by the displacement of the organ of Corti (δ_{OC}) divided by the pressure at the basilar membrane.

FIG. 7. (Color online) Nominal stiffness due to the distribution of applied force. (A) Deformation pattern when a local-distributed force was applied (red curves, the “Probe” case), and when evenly distributed force was applied to the basilar membrane (blue curves, the “Pressure” case). The light colored area indicates non-deformed configuration. δ_{OC} and δ_{BM} indicate where the displacements for the compliance are obtained when subjected to pressure, and for the probe force, respectively. (B) Longitudinal deforming pattern of the basilar membrane when it is deformed by probes with different sizes. To simulate different probe sizes, a 1 nN force was normally distributed with different spatial standard deviation ($\sigma = 0, 10$, and $20 \mu\text{m}$). The longitudinal space constant (λ) of the model was $35 \mu\text{m}$. (C) Nominal stiffness according to the force distribution (probe size).

The deforming pattern of the OCC is dependent on the applied force distribution. The blue and red lines in Fig. 7(A) are deformed shapes for evenly and locally distributed forces, respectively. The deforming pattern of the basilar membrane along the length is shown in Fig. 7(B). For the simulated location, the longitudinal space constant was $35\text{ }\mu\text{m}$ [Fig. 7(B)], which compares well with another experimental measurement (Naidu and Mountain, 2001). The force distribution representing the probe tip sharpness affects the *nominal* stiffness [Fig. 7(C)]. As the probe tip size increased from $\sigma = 0$ to $\sigma = 30\text{ }\mu\text{m}$, the stiffness increased from 55 to 140 mN/m.

The conversion factor between the two units may be considered as an effective area of the probe force A_{eff} , which can be obtained from

$$A_{\text{eff}} = k_{\text{Probe}} c_{\text{Press}}, \quad (4)$$

where k_{Probe} is the probe-measured stiffness in N/m and c_{Press} is the compliance due to fluid pressure in m/Pa. If a typical probe size is comparable to $\sigma = 10\text{ }\mu\text{m}$, the approximate stiffness of the probe-deformed case is 70 mN/m. For the evenly distributed force case, the compliance was 110 nm/Pa. For this case, $A_{\text{eff}} = (70\text{ mN/m})(110\text{ nm/Pa}) = 7700\text{ }\mu\text{m}^2$. Using this conversion relationship, for the apical model (8.5 mm from the basal end), our average measured value of 85 nm/Pa at the level of the outer hair cells corresponds to a probe-measured stiffness value of 91 mN/m ($k_{\text{Probe}} = A_{\text{eff}}/c_{\text{Press}}$).

IV. DISCUSSION

An alternative method for the measurement of OCC stiffness was developed and validated. This new approach overcame the ambiguity in the existing measurement technique using microprobes: nonlinear force-displacement relationships. In line with recent studies (Teudt and Richter, 2014; Kapuria *et al.*, 2017), we assumed that if the physiological form of mechanical stimulation (fluid pressure) is applied at physiological levels (tissue deformation $< 1\text{ }\mu\text{m}$), the cochlear mechanics will be linear. We obtained linear pressure-displacement relationship within physiological deformation ranges (Figs. 5 and 6). To our knowledge, since von Békésy's studies, this is the first measurement of OCC

compliance by applying hydrostatic pressures. In the following, we compare our measurement with existing measurements, and discuss how our method can be further improved.

Previous experiments have used compliant microprobes to measure the basilar membrane stiffness of various species (Gummer *et al.*, 1981; Miller, 1985; Olson and Mountain, 1991; Olson and Mountain, 1994; Naidu and Mountain, 1998; Emadi *et al.*, 2004; Emadi and Richter, 2008; Teudt and Richter, 2014). Most measurements were made at the basal turn of the cochlea where the microprobe can access the basilar membrane through the round window with minimal disturbance to the cochlear tissue. In a few exceptional studies, however, the longitudinal stiffness variation was measured. Mountain and his colleagues measured the basilar membrane stiffness at different longitudinal locations in the gerbil cochlea (Olson and Mountain, 1991; Olson and Mountain, 1994; Naidu and Mountain, 1998; Naidu and Mountain, 2001). Emadi *et al.* used the hemi-cochlea preparation to measure stiffness from different locations (Emadi *et al.*, 2004; Emadi and Richter, 2008). A similar approach was applied to measure the longitudinal stiffness variation in the mouse cochlea (Teudt and Richter, 2014). For measurements at basal locations (e.g., Miller, 1985), larger loads were often necessary to overcome the higher OCC stiffness compared to middle or apical locations (e.g., Emadi *et al.*, 2004). Despite using different tissue preparation methods, the force application method was consistent—compliant microprobes.

The force-displacement relationship obtained through the microprobe method is highly nonlinear [Fig. 8(A)]. There exists inevitable ambiguity in defining the stiffness from this nonlinear force-displacement relationship. Following the approach of Gummer *et al.* (1981), later studies considered that functionally relevant stiffness is the “plateau stiffness”—a linear region after the onset nonlinearity [the region iii in Fig. 8(A)]. The basilar membrane was deformed 5–25 μm to reach this plateau region. In contrast, our method applied hydrostatic pressure within physiological amplitudes ($< 5\text{ Pa}$), which result in OCC deformations less than 1 μm from its resting position. Within this physiological deformation range, the pressure-displacement relationship was highly linear [Figs. 5(D) and 6(A)]. In Fig. 8(B), after applying the conversion factor of

FIG. 8. (Color online) Comparison with probe measurements. (A) A sample data from Emadi *et al.* (2004). The original data presented in stiffness vs displacement were integrated over displacement to obtain this force-displacement curve. It was a measurement at 7.5 mm from the basal end of the gerbil cochlea. The slope of curve (stiffness) increases as the basilar membrane deforms further from (i) to (ii) and to (iii). (B) Comparison of three local slopes of the probe-measurements with our measurements. The three colored curves correspond to the curves in three squares in (A). The force was converted to pressure using the conversion factor in Eq. (4). (C) Comparison of the measured and modeled values of this study with existing measurements. The filled triangular symbol corresponds to the data point iii in (A) and (B).

Eq. (4), the local slopes of a probe measurement curve were compared with our measurements. Our result supports the argument that the plateau stiffness is physiologically relevant. Our measurement (converted to N/m) and model results were shown together with two sets of available data in Fig. 8(C).

This study can be enhanced further in several ways. First, the small number of measurements made it difficult to draw other meaningful scientific conclusions. For example, we cannot discuss if the stiffness gradient is sufficient to explain cochlear tonotopy. More measurements from different longitudinal locations will contribute to the discussion. Second, the solutions during experiment can be closer to physiological conditions. Unlike most existing experiments that use a single fluid chamber, we established a method (leakage test) to verify that the top and bottom fluid spaces across the OCC were separated. As a result, it is possible to provide physiological fluid conditions to excised cochlear turns—endolymph-like and perilymph-like solutions at the two fluid spaces. Although this study did not exploit the capability of fluid separation, a better physiological condition may further improve the quality of measurements. Third, different imaging modalities than the bright-field light microscopy of this study can provide more information. For example, both confocal microscopy and optical coherence tomography can capture the deforming pattern over the entire depth of the OCC. To measure the vibrations of OCC structures, those two imaging methods have been successfully exploited. For instance, Fridberger, Ulfendahl, and their colleagues (Fridberger *et al.*, 1997; Fridberger *et al.*, 2002) analyzed confocal microscopy images with a visual flow technique to obtain kinematic relationships between OCC fine structures. Recently, the optical coherence tomography was used to measure the OCC vibrations *in vivo* (Chen *et al.*, 2011; Lee *et al.*, 2015). These imaging techniques can readily be combined with our hydrostatic measurement system.

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