Article

Mechanical Properties of a Primary Cilium As Measured by Resonant Oscillation

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ABSTRACT Primary cilia are ubiquitous mammalian cellular substructures implicated in an ever-increasing number of regulatory pathways. The well-established ciliary hypothesis states that physical bending of the cilium (for example, due to fluid flow) initiates signaling cascades, yet the mechanical properties of the cilium remain incompletely measured, resulting in confusion regarding the biological significance of flow-induced ciliary mechanotransduction. In this work we measure the mechanical properties of a primary cilium by using an optical trap to induce resonant oscillation of the structure. Our data indicate 1) the primary cilium is not a simple cantilevered beam; 2) the base of the cilium may be modeled as a nonlinear rotatory spring, with the linear spring constant k of the cilium base calculated to be $(4.6 \pm 0.62) \times 10^{-12}$ N/rad and nonlinear spring constant α to be $(-1 \pm 0.34) \times 10^{-10}$ N/rad^2; and 3) the ciliary base may be an essential regulator of mechanotransduction signaling. Our method is also particularly suited to measure mechanical properties of nodal cilia, stereocilia, and motile cilia—anatomically similar structures with very different physiological functions.

INTRODUCTION

The primary cilium is a microtubule bundle that extends from the mother centrosome into the extracellular space and is hypothesized to be a mechanotransducing structure (1–4). A decade of experimental results has demonstrated that bending the primary cilium is correlated with initiation of a variety of signaling cascades (1,2,5–26). Measurements of the essential mechanical properties of this mechanical sensor are surprisingly few, and have relied on static deformations induced either by steady fluid flow (9,16,27), by glass pipette (7), or by optical trapping of a bead attached to the cilium tip (3). Experiments that infer the mechanical properties through relaxation methods have been performed on microtubules and cilia (3,28) as well. It must be emphasized that while models of the mechanical properties of motile cilia and flagella are plentiful (for example, see the literature (29–42)), they are not relevant here due to the significant physiological and structural differences between motile and nonmotile cilia. Nodal cilia (43) are structurally identical to a primary cilium, but again, while the mechanical properties of nodal cilia have been inferred from models of induced flow (43–45), they have not yet been directly measured.

In contrast to the previous methods, we have directly excited a resonant oscillation of a primary cilium with a single beam three-dimensional optical tweezer (46,47). Measuring dynamic responses of the cilium provides information that cannot be measured using static methods such as, for example, recording mechanical properties of the cilium base. In addition, use of dynamics rather than statics potentially obviates the need for detailed shape fitting, which requires either side-on views of the cilium (48) or insertion of fluorescent transmembrane proteins (16). We will demonstrate that a single dynamic measurement provides a wealth of reliable information about not only the mechanical properties of the primary cilium, but of the cilium-fluid interaction as well. The method described here provides results equivalent to multiple independent experiments, and our method could be applied to the study of motile cilia (49), nodal cilia, and more complex structures such as hair-cell stereocilia (50). Our experimental approach compares favorably with similar experiments performed using magnetic tweezers on motile cilia (49). One advantage of optical trapping over magnetic tweezers is the noncontact generation of force; magnetic tweezers require a paramagnetic bead to be affixed to the cilia, significantly altering the fluid flow in the neighborhood of the cilium.

MATERIALS AND METHODS

Cell culture

Experiments were carried out with a mouse cell line derived (51) from the cortical collecting duct (mCCD 1296 (d)) of a heterozygous offspring of the Immortomouse (Charles River Laboratories, Wilmington, MA). The Immortomouse carries as transgene a temperature-sensitive SV40 large T antigen under the control of an interferon-γ response element. Cells were maintained on collagen-coated Millicell-CM inserts (inner diameter 30 mm, permeable support area 7 cm^2; Millipore, Billerica, MA) to promote a polarized epithelial phenotype. Cells were grown to confluence at 33°C, 5% CO_2 and then maintained at 39°C, 5% CO_2 to enhance differentiation. The growth medium consisted of the following (final concentrations): Dulbecco's Modified Eagle's Medium w/o glucose and Ham's F12

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at a 1:1 ratio, 5 mM glucose, 5 μg/mL transferrin, 1 nM T3 (triiodothyronine), 5 μg/mL insulin, 10 ng/mL EGF (epithelial growth factor), 4 μg/mL dexamethasone, 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.06% $NaHCO_3$, 2 mM L-glutamine, 10 ng/mL interferon-γ, 50 μM ascorbic acid 2-phosphate, 20 nM selenium, and 5% FBS (fetal bovine serum). For differentiation, FBS, EGF, insulin, and interferon-γ were omitted from the apical medium and insulin, EGF, and interferon-γ from the basal medium.

Optical tweezers

The source for the single-beam three-dimensional trap was a Crystalaser IRCL-0.5W-1064 (Reno, NV), a diode-pumped Nd:YAG continuous-wave single mode laser providing 0.5-W optical power from a 10-W electrical power supply. The optical tweezer breadboard layout was constructed using optomechanical mounts (Qioptiq, Munich, Germany; Excelitas Technologies, Fremont, CA). Achromatic doublets were used for the beam expansion. The first lens has a 10-mm focal length, while the second has a 200-mm focal length. Both lenses were anti-reflection-coated for 1064 nm. The focal lengths were chosen simply for convenience: the distance between the entrance port of the microscope and the objective lens is 140 mm, and the laser beam was expanded a factor of ~6× to fill the aperture. The objective lens used was a 63× NA 0.9 U-V-I HCX long-working-distance Plan Apochromat dipping objective (Leica Microsystems, Buffalo Grove, IL) with a 2.2-mm working distance. The tweezer couples into the microscope through an existing lateral port. A side-looking dichroic mirror (Chroma Technology, Bellows Falls, VT) mounted within the fluorescence turret provides the ability to perform normal microscope viewing while the tweezers are operating. The fixed-position optical trap has a beam waist of 0.3 μm and Rayleigh length of 0.4 μm.

Objects held within the trap diffract the trapping beam. The spatial dynamics of the diffracted beam were recorded using a quadrant photodiode (QPD) and the data analyzed as per Glaser et al. (52). Briefly, the QPD outputs the centroid location of the diffracted trapping beam, digitally sampled at 10 kS/s. The data was then analyzed to calculate the force applied by the trap to the trapped object. The relationship between the centroid position and the location of the trapped object is known, allowing calculation of the cilium tip position.

Applying the trap to a primary cilium proceeded as follows. First, the trap location was precisely determined by trapping a small piece of floating cell debris. Turning the trapping laser off and using bright-field illumination, a cilium was moved to the center of the trap and focus-adjusted to align the trapping plane to the cilium tip. The cilium was then laterally displaced slightly from the trap axis and recorded using the *XY* translation stage digital readout. The optical trap was turned on and QPD data acquired for several seconds. The trap was then turned off, another cilium moved into position, and the procedure repeated.

Microscopy

Imaging and manipulations of terminally differentiated epithelial monolayers were carried out using a DM 6000 upright microscope (Leica Microsystems) equipped with a heated and CO_2-controlled incubation chamber (Solent Scientific, Segensworth, UK). The microscope stage (Cat. No. H30XY2; Prior Scientific, Rockland, MA) was accurate to ± 0.04 μm. Bright-field image acquisition and optical trap monitoring were performed by a Flea digital video-rate camera (Point Grey, Richmond, British Columbia, Canada).

Cilium length measurement

After the trapping experiments, cells were fixed and stained for high-resolution imaging. Image stacks (0.1 μm *z*-step size) were obtained via the software MICRO-MANAGER (https://www.micro-manager.org/) (53) using a 100× 1.46 NA immersion lens (Leica Microsystems) and cilium lengths measured directly from the image stack. For the measurements reported here, the cilium length was measured to be $L = 2.1 \pm 0.05$ μm. It is important to note that we use the immunostained axoneme length as a proxy for the cilium length; the actual cilium length could be slightly different.

Immunocytochemistry

Fixation and immunocytochemistry were performed using standard techniques. The cells were fixed in 4% paraformaldehyde for 10 min. After rinsing, the monolayers were permeabilized for 10 min with a solution of 0.1% Triton-X and 0.5% saponin in a blocking buffer containing 5% donkey serum, 5% sheep serum, 1% BSA (bovine serum albumin), and 5% FBS (fetal bovine serum). The monolayers were then stained with a monoclonal mouse antibody against acetylated α-tubulin (Invitrogen, Carlsbad, CA) and a polyclonal goat antibody against Polycystin-1 (Abcam, Cambridge, UK) followed by an anti-mouse antibody labeled with AlexaFluor 488 (Invitrogen) and an anti-goat antibody labeled with AlexaFluor 594 (Invitrogen). The stained filter was cut out of the culture insert and transferred to a microscope slide, monolayer side up. The filter was mounted in a VectaShield (Vector Labs, Burlingame, CA) with DAPI. A No. 1.5 coverslip was placed on top of the monolayer, then sealed with nail polish and stored at 4°C for later imaging.

Culture media viscosity and density measurement

The dynamic viscosity of apical media was measured with a Cannon-Fenske Routine Viscometer (Induchem Lab Glass, Roselle, NJ) with the apparatus and media equilibrated to 37°C. The density of the media was measured with a pycnometer (Thermo Fisher Scientific, Waltham, MA).

RESULTS

An en face view of stained cells with primary cilia indicated is shown in Fig. 1. Most of the cells are ciliated, and the cilia are oriented vertically, appearing as a diffraction-limited point. The length of cilia were measured to be $L = 2.1 \pm 0.05$ μm. The viscosity of fluid was measured to be $\eta = 0.637 \pm 0.012$ cP, the density measured to be $\rho = 1.00 \pm 0.01$ g/cm^3. The density of the cilium is estimated to be 1.11 g/cm^3 (10) and the cilium diameter $a = 0.2$ μm.

The direct QPD output from a trapped cilium is shown in Fig. 2, showing the time-varying position of the cilium tip. This result was unexpected, because the cilium tip was not held steady within the trap as for trapped microspheres. The Fourier transform of the QPD output results in Fig. 3, clearly showing a resonant oscillation frequency and multiple harmonics.

We directly obtain the following: oscillation amplitude = 0.22 ± 0.047 μm and resonant oscillation frequency $f = 57$ Hz $= \omega/2\pi$. The Reynolds number (Re) associated with this motion is $Re = (\rho_{\text{fluid}} a^2 \omega_c^{\text{viscous}})/(4\eta_{\text{fluid}}) = 5.6 \times 10^{-6}$, indicating viscous effects dominate. We performed this measurement on ($N = 6$) cilia and obtained the oscillation amplitude and resonant frequency for each. Taken as a whole, the average resonant frequency is $f = 55.9 \pm 1.4$ Hz.