A microfluidic device for *in situ* fixation and super-resolved mechanosensation studies of primary cilia

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ABSTRACT

The primary cilium plays an important role in mechanosensation in mammalian cells. To understand mechanosensation in the primary cilium, we combined a microfluidic device with super-resolution microscopy to study the primary cilium phenotypes. The microfluidic system enabled the precise control of the flow shear within a well-confined cell-culture environment. In addition, *in situ* cilia fixation was possible by switching from the culture medium to the fixation buffer instantaneously, which preserved the real-time cilium phenotype under the flow shear. After fixation, multiple cilium-specific proteins were immunostained to quantify the cilia bending behavior. We found that >50% of the primary cilia of mouse inner medullary collecting duct cells were highly aligned with the direction of flow under 11 Pa shear stress. Finally, we used super-resolution microscopy to observe the redistribution of two major cilium-specific proteins under flow shear, acetylated alpha-tubulin, and intraflagellar transport protein 88. To the best of our knowledge, this is the first platform to combine a microfluidic device with super-resolution microscopy to enable flow stimulation and *in situ* fixation for the observation of ciliary protein. This system can potentially be applied to the future development of a stimulation-enabled organ-on-a-chip to observe the intercellular signaling of primary cilia or for the analysis of disease mechanisms associated with ciliary mutations at the organ level.

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INTRODUCTION

The primary cilium is a rod-shaped organelle enclosed by the ciliary membrane. It has nine microtubule doublets at the center that act as the structural backbone and can be submerged within a cell or protrude out of the cell membrane. The primary cilium plays a central role in chemical and mechanical signal transduction, e.g., during vertebrate development.¹ It is known that primary cilia can sense the concentration gradients of specific molecules such as those in the sonic hedgehog and Wnt families of morphogens.² For example, cilium size is affected when sensing various chemical molecules such as interleukin-1³ and SIRT2⁴ and by death

inducer-obliterators-dependent targeting of histone deacetylase 6.⁵ Primary cilia also act as mechanosensors in the human body, deflecting according to the surrounding fluid flow, pressure, or vibration. A review paper has provided a complete summary of the different roles of primary cilia such as acting as a Ca²⁺ signal amplifier or as a flow sensor.⁶ Other researchers have studied the equilibrium shape and dynamics of primary cilia under a hydrodynamic load.⁷ Due to the complicated composition of the primary cilium, it cannot simply be modeled as a homogenous cantilever.⁸ Researchers have also shown that cilia stiffen under flow stimulation, and oscillatory fluid flow can influence primary cilia and microtubule mechanics.⁹

To enable a comprehensive understanding of the mechanosensation of primary cilia, it is important to provide a well-controlled microenvironment. Microfluidics provide an ideal platform to precisely control different flow conditions, including velocity and duration, for epithelial cell-layer culture and characterization.¹⁰ In addition, cell culture conditions such as seeding density and nutrient concentration can be well maintained or adjusted inside the microchamber, which facilitates dense and uniform ciliary growth. Different researchers have fabricated various biomimetic artificial cilia (e.g., magnetic-activated¹¹ and photo-activated polymeric artificial cilia¹²) inside microfluidic devices to produce sequential fluidic manipulations such as mixing and propulsion at the micro-scale.^{13,14} To study natural primary cilia in microfluidics, Rydholm *et al.*¹⁵ developed a microfluidic channel consisting of multiple inputs and outputs to control the flow direction and monitored the corresponding ciliary deflection angle inside the microchamber. Rahimzadeh *et al.*¹⁶ developed a parallel-plate microfluidic chip with fluorescence resonance energy transfer microscopy to study actinin's response under flow-induced stress. Tkachenko *et al.*¹⁷ found that under strong flow shears, endothelial cells disassemble their primary cilia and sense the strength and direction of the flow through the hydrodynamic drag on their nuclei.

In our research, we combined a microfluidic platform with super-resolution microscopy to systematically observe primary cilium phenotypes under various flow conditions. Our microfluidic device consisted of three cell culture regions with different cross-sections, which allowed for the direct observation of primary cilia under different flow shears precisely controlled by a syringe pump. By connecting the device to a three-way valve, the cell-culture medium and fixation buffer could be seamlessly switched and loaded into the device. This seamless reagent switching was essential to enable *in situ* ciliary fixation during flow stimulation, which preserved the real-time cilium phenotype while under the flow shear. As a proof-of-concept, we first cultured mouse inner medullary collecting duct (IMCD) cells in the microfluidic device and recorded the real-time cilium dynamics at different flow shear levels. After the bending of primary cilia could be clearly observed, we applied a flow stimulation to them and then fixed them *in situ*. We systematically analyzed the projection lengths and alignment angles of the primary cilia relative to the flow direction under three different flow shears. Finally, we used super-resolution microscopy to observe the redistribution of two cilium-specific proteins, acetylated alpha-tubulin (ac- α -tubulin) and intraflagellar transport protein 88 (IFT88), within primary cilia exposed to a flow shear generated by the microfluidic device. Overall, combining the microfluidic device with super-resolution microscopy would be an ideal platform for studying ciliary protein responses under flow stimulation.

MATERIALS AND METHODS

Fabrication of the microfluidic device

The microfluidic device was made of polydimethylsiloxane (PDMS) prepolymer composed of precursor A and precursor B at a ratio of 10:1. PDMS prepolymer was first poured

onto a poly(methyl methacrylate) (PMMA) mold fabricated with a CNC engraving machine (EGX-400, Roland). Detailed chip fabrication parameters can be found in our previous study.¹⁸ After heating in a 60 °C oven for 4 h, the fully cured PDMS structure was peeled off the mold. The inlet and outlet of the microchannel were then punched with 0.5 mm and 1.0 mm biopsy punches, respectively. For easier immunostaining, the design included the detachable microfluidic portion composed of the 5 mm-thick PDMS microfluidic device and a poly-L-lysine coated #1.5 coverslip. The two structures were clamped together by the combination of a steel holder, a PMMA board, and four screws and nuts. After the flow stimulation and *in situ* fixation were complete, the cell adhesion slide was detached for immunostaining.

Flow protocol in the microfluidic device

To produce the flow stimulation, a steady fluid flow was introduced into the microfluidic device by a syringe pump (Fusion 200, Chemyx Inc., TX). Two 10 ml syringes filled with culture medium [DMEM F-12 minus fetal bovine serum (FBS)] and fixation buffer [4% paraformaldehyde in phosphate buffered saline (PBS)] were connected to a three-way valve, and the liquids were drawn into the microfluidic device at 0–500 μ l/min for 5 and 10 min, respectively. The channel height of the cell culture region was 100 μ m. To create different flow shear regions at one flow rate, three channel widths of 270, 420, and 640 μ m were used. Shear stress is proportional to the flow rate as per the below equation:

$$\tau = \mu \left. \frac{\partial u}{\partial z} \right|_{z=0}, \quad (1)$$

where τ and u are the shear stress and flow velocity, respectively, which depend on the width and height of the microchannel, μ is the viscosity of the fluid, and z represents the height above the channel wall. We used a commercial finite-element-method (FEM) software (COMSOL 5.3a, Multiphysics) to confirm that the shear stress in the channel was in a range similar to that in the human vascular system or epithelial tissue.⁷

Cell culture conditions

We used mouse IMCD cells transfected with the cilium-targeted proximity labeling enzyme APEX fused to GFP (a gift from Maxence Nachury, UCSF). The use of such a construct may facilitate proteomic studies under mechanical stimuli in the future. Before being placed in the microfluidic device, IMCD cells adhered on poly-L-lysine coated coverslips were cultured in medium (DMEM F-12 with 10% FBS and 1% penicillin/streptomycin) to 90% confluence in a cell incubator at 37 °C with 5% CO₂. To make the cilia more recognizable, we added 200 nM cytochalasin D to the medium and incubated for another 18 h, which increases the cilium length.¹⁹ The fluorescence signal from the cilia was excited by the 488 nm laser line during epifluorescence microscopy.

Immunostaining

To perform immunostaining, the cell seeded glass slide was first detached from the microfluidic channel. The cells were then permeabilized in 0.5% Triton X-100 for 10 min and blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature, incubated with primary antibodies in 3% BSA overnight at 4 °C, and rinsed at least 3 times in PBS with 0.5% Tween 20 (PBST). The cells were then incubated with secondary antibodies for 60 min at room temperature and washed 3 times in PBST. The primary antibodies used in this study visualized (1) ac- α -tubulin (mouse anti-acetylated α -tubulin IgG; Sigma 6-11B-1; 1:1000 dilution), used as a ciliary marker during epifluorescence and direct stochastic optical reconstruction microscopy (dSTORM) imaging; (2) IFT88 (rabbit anti-IFT88 IgG; Proteintech Group 13967-1-AP; 1:200 dilution), used as a ciliary marker during dSTORM imaging; and (3) CEP164 (goat anti-CEP164, N-14; Santa Cruz Biotechnology sc-240226; 1:200 dilution), used as a marker for the centriole distal end during epifluorescence imaging. Alexa Fluor 568 secondary antibodies (donkey anti-rabbit or donkey anti-mouse; Jackson ImmunoResearch Laboratories, Inc., 1:500 dilution) were used for labeling the anti-ac- α -tubulin and anti-IFT88. Alexa Fluor 647 (donkey anti-goat, Jackson ImmunoResearch Laboratories, Inc., 1:500 dilution) was used for labeling the anti-CEP164.

Epifluorescence and super-resolution microscopy

The fluorescent IMCD cells and their primary cilia were imaged on an inverted fluorescent microscope (Olympus IX-81) with a 100 $\times$ oil-immersion objective (UPlan FL, NA 1.30, Olympus) and a CCD camera (C8484-03G01, Hamamatsu). The 488 nm (Coherent) laser line was used to excite GFP signal inside cilium. For super-resolution microscopy, we used a dSTORM imaging system based on a Nikon Eclipse Ti-E microscope with a 100 $\times$ 1.49 NA oil-immersion objective. This system was based on previous dSTORM studies.^{20–22} Briefly, the 637 nm (Coherent) and 561 nm (Cobolt) laser lines were used to excite Alexa 647 and Cy3B, respectively, while the 405 nm laser was used for the photoconversion of Cy3B. During the acquisition, the laser power was set to ~ 4 kW/cm² excitation intensity, with <5 W/cm² used for the activation light. The emissions from the samples were spectrally cleaned with single-band filters (Chroma 593/40 nm and 700/75 nm) and imaged on an EMCCD camera (Evolve 512 Delta, Photometrics) with a pixel size of 93 nm. During acquisition, the samples were incubated in a standard switching medium containing TN buffer (50 mM Tris, pH 8.0, and 10 mM NaCl), an oxygen scavenging system [0.5 mg/ml glucose oxidase (Sigma), 40 μ g/ml catalase (Sigma), and 10% (w/v) glucose], and 80–100 mM mercaptoethylamine (Sigma). The samples were placed at a consistent focus using a Perfect Focus System (PFS; Nikon), and a stack of 10,000–20,000 frames were recorded at an acquisition rate of 33 frames s^{−1}. For dual-color imaging, Alexa 647 and Cy3B were imaged sequentially. The localization analysis of individual molecules was performed using the MetaMorph Super-Resolution Software module (Molecular Devices).

Data analysis

The projection lengths and alignment angles of the cilia were analyzed using ImageJ. We first found the CEP164 marker to identify the distal end of the centriole (the origin of the cilium). The projection length of the cilium was then quantified by the GFP or ac- α -tubulin signal. The alignment angle was defined as the angle between the axis of the flow direction and the projection line of the cilium [Fig. 2(b)].

RESULTS AND DISCUSSION

Device design and system setup

A schematic of our system is shown in Fig. 1(a). It consists of (1) the microfluidic device, (2) the flow control system, and (3) the epifluorescence and super-resolution microscopes. The microfluidic device consists of three cell culture regions with areas of various cross-sections. This design generates three flow shears inside the microchannel with only one flow rate requirement. FEM simulation was used to calculate the shear stress distribution of the device. As shown in Fig. 1(b), if the injection flow rate was 500 μ l/min, the shear stresses in the narrow and middle regions were 11 and 7.14 Pa, which are 2.39 $\times$ and 1.54 $\times$ higher than the shear stress in the wide region (4.60 Pa), respectively. Each culture region contained more than 5 mm² of cell-seeding area with uniform flow shear, which enabled the simultaneous mechanical stimulation of large numbers of cilia. The outlet of the device was connected to a syringe pump that could draw the reagents from the inlet at adjustable flow rates. The three-way valve connected to the inlet allowed for seamless switching between the culture medium and fixation buffer. After the primary cilia were deformed under the flow and fixed in their deformed geometry, the epifluorescence or super-resolution microscope was used to observe the ciliary morphology or protein distribution within the primary cilia.

Real-time ciliary bending behavior and simulation

Mechanical stimulation was applied to primary cilia by flowing the culture medium into the microfluidic device, where the IMCD cells were seeded on the glass bottom surface. An initial fluorescent image of the primary cilia was acquired at flow rates of 0, 100, 300, and 500 μ l/min, corresponding to 0–11 Pa of shear stress. This is the range that cells experience in the human body at/within normal physiological conditions. Under each flow rate condition, the flow was maintained for 30 s. A final image was acquired after the flow was turned off. Using the images, we calculated the projection length and alignment angle corresponding to the flow-induced shear stress in polar coordinates for each primary cilium. Figure 2(a) shows a plot derived from real-time fluorescent images of 10 cilia. We found that all cilia became bent within this shear-stress range. However, the bending behavior could be categorized into two types (shown in red and blue). Two cilia [red and blue boxes in Fig. 2(a)] are shown as representative cases. Figures 2(b) and 2(c) show two series of fluorescent images of cilia under flows of 0–500 μ l/min (0–11 Pa

FIG. 1. (a) Schematic of the microfluidic device combining epifluorescence and super-resolution microscopy. The fixation buffer and culture medium were switched by a three-way valve and seamlessly drawn into the microfluidic devices using the syringe pump. The inset image is of the microfluidic channel, consisting of three observation zones with different shear stress levels. (b) The velocity field and shear stress simulation inside the microfluidic channel using finite element method (FEM) software.

FIG. 2. (a) The projection length and alignment angle distribution of 10 bent cilia. The solid dots represent the distribution under flow rates of 0, 100, 300, and 500 $\mu\text{L}/\text{min}$ (0, 2.2, 6.6, and 11 Pa). Hollow dots represent the distribution when the flow was turned off. The bending behavior can be divided into red and blue types. (b) and (c) Fluorescent images of cilia under the various flow rates (shear stress) in the inset red and blue boxes in (a). Yellow arrows indicate the basal body of the primary cilia. Scale bar: 2 μm . Two time-lapse videos were shown in the [Supplementary material](#) [Fig. S1A (red box) and Fig. S1B (blue box)]. (d) and (e) Cilium simulation model of the inset red and blue boxes in (a). (f) and (g) The simulated cilium bending behavior corresponding to the images in (b) and (c).

shear stress). The yellow arrows point to the origins of the cilia. We suspect the different bending behaviors were due to different cilia growth orientations, as illustrated in Figs. 2(d) and 2(e). If the cilia growth direction was perpendicular to the flow direction [Fig. 2(d)], the cilia would have a distinct projection length increase along the direction of flow (red case). In contrast, if the cilia growth direction was in the same plane as the flow direction [Fig. 2(e)], the cilia had a distinct angle change along the direction of flow with a minimum change in projection length (blue case).

To model the cilia bending behavior, we used the finite element method (FEM) to simulate the two cilia growth cases under flow stimulation. To simplify the simulation complexity, the cilium was modeled as a homogenous beam-shaped structure with a fixed Young modulus anchored to a cell. Based on the experimental observations, the cilium diameter was set to $0.3\ \mu\text{m}$ and the length to $6\ \mu\text{m}$. To simulate the cilia bending under different flow rates, we employed the fluid-structure interaction model using the following equations:

$$\begin{aligned} \rho \frac{\partial \mathbf{u}_{\text{fluid}}}{\partial t} + \rho (\mathbf{u}_{\text{fluid}} \cdot \nabla) \mathbf{u}_{\text{fluid}} &= \nabla \cdot \{-p\mathbf{I} + \mu[\nabla \mathbf{u}_{\text{fluid}} + (\mathbf{u}_{\text{fluid}})^T]\} \\ + F_v \rho \nabla \cdot \mathbf{u}_{\text{fluid}} &= 0, \quad \rho \frac{\partial^2 \mathbf{u}_{\text{solid}}}{\partial t^2} - \nabla \cdot \boldsymbol{\sigma} = \mathbf{F}_v \end{aligned} \quad (2)$$

where μ and ρ is the dynamic viscosity [$\text{kg}/(\text{m s})$] and the density of the fluid (kg/m^3), respectively; p is the pressure (Pa); $\mathbf{I}$ is the identity matrix; $\mathbf{F}_v$ is the volume force of the fluid; $\mathbf{u}_{\text{fluid}}$ is the flow velocity given by the Navier-Stokes equations; and $\mathbf{u}_{\text{solid}}$ is the structural velocity, which acts as a moving wall for the fluid domain.

As shown in Figs. 2(d)–2(g), the simulated bending behavior was quite similar to the real bending result. However, when the flow was off, the real cilium [Figs. 2(d) and 2(e)] did not return to their original position as did those in the simulated cases [Figs. 2(f) and 2(g)]. We suspect this was due to a deformation of the basal anchor caused by the large axonemal stress. Still, both the simulated and real cilium bending

behaviors imply that when the flow was turned off, the cilia would not maintain their original shape during stimulation. Therefore, an *in situ* ciliary fixation process during the flow stimulation was necessary to preserve the geometry of the bent cilia under flow conditions.

In situ fixation

To confirm that the *in situ* fixation could be realized, we designed three experiments with various flow stimulation and fixation conditions. The first experiment served as the control. In this experiment, once the cilia grew during cultivation, we did not apply any flow to stimulate the IMCD cells. The glass slide was directly detached from the microfluidic device, and the primary cilia were fixed by immersing the slide in fixation buffer. In this case, most cilia maintained their original position after fixation [Fig. 3(a)]. For the second experiment, the cilia were stimulated by 11 Pa shear stress for 5 min. The flow was then stopped, and the fixation was again performed by immersion in fixation buffer. As shown in Fig. 3(b), the cilia bending behavior was similar to that in the control. Although a few cilia were bent, the directions of the bends were fairly random, and the projection lengths were limited. Lastly, we flowed the culture medium into the device under the same flow conditions (11 Pa shear stress for 5 min) and, maintaining the flow, used the three-way valve to immediately switch to the fixation buffer for another 10 min. As shown in Fig. 3(c), most cilia were clearly bent along the direction of flow. An increase in their projection lengths was also obvious. Overall, the results demonstrated that compared to the traditional immersion-based fixation process, *in situ* fixation could preserve the bent geometry of the cilia.

Observing the mechanosensation of primary cilia

Once the *in situ* fixation protocol was confirmed, we detached the cell seeding glass slide from the microfluidic device for immunostaining. The α -tubulin was stained orange (Alexa Fluor 568), the CEP 164 of the distal appendages

FIG. 3. Images of cilia after the flow stimulation and fixation processes. (a) Both processes were performed in static solutions. (b) Stimulation under 11 Pa shear stress and fixation under static conditions. (c) Both processes performed under 11 Pa shear stress. Scale bar: $1\ \mu\text{m}$.

was stained red (Alexa Fluor 647), and the nuclei were stained blue (DAPI). Three individual experiments were performed for each shear stress level (0.92, 4.6, and 11 Pa), and over 20 fluorescent images were acquired. Each image consisted of ~ 5 –16 cilia. **Figures 4(a)–4(c)** show a representative image at each shear stress level. Clearly, more cilia were aligned toward the direction of flow when under the highest level of shear stress [Fig. 4(c)]. To further quantify the actual projection lengths and

alignment angles of the cilia, we eliminated the cilium if it was out of focus or the projection length was less than $1\mu\text{m}$ or more than $6\mu\text{m}$. Based on these criteria, the total numbers of cilia for each shear stress level from the three independent experiments were 281, 356, and 256, respectively. For those cilia counted, we quantified the cilia projection length and alignment angle along the flow direction, and then plotted them in polar coordinates [Figs. 4(d)–4(f)]. On the plots, all cilia

FIG. 4. (a)–(c) Images of immunostained cilia showing their bending under 3 shear stress levels. (d)–(f) The distribution profile of individual primary cilia in polar coordinates. Three individual experiments were performed under each condition. Cilia from an individual experiment are shown in the same color. The total numbers of cilia under the different shear stress levels were 281, 356, and 256, respectively. (g)–(i) Angular distribution of the cilia in polar coordinates. The proportion of each interval between $\pm 45^\circ$ is also shown next to the bar. The orange and yellow colors represent those highly ($\pm 15^\circ$) and moderately ($\pm 45^\circ$) correlated to the direction of the flow stimulation, respectively.

in the same experiment are labeled in the same color. By comparing the three plots, we found that the higher the shear stress, the more the cilia aligned toward the direction of flow. To quantify this trend, we created polar coordinate histograms [Figs. 4(g)–4(i)]. If the cilium was within a $\pm 15^\circ$ (orange) or $\pm 45^\circ$ (yellow) region on the plot, it was defined as highly or moderately correlated with the flow shear, respectively. Based on the data in Figs. 4(d)–4(f), we calculated the cumulative percentage of the cilia within these polar coordinates. As shown in Figs. 4(g)–4(i), the proportion of highly ($\pm 15^\circ$) and moderately ($\pm 45^\circ$) correlated cilia increased as the flow shear (0.92, 4.6, and 11 Pa) increased ($\pm 15^\circ$, $11.8 \pm 1\%$, $16.0 \pm 3\%$, and $22.4 \pm 2.5\%$, respectively; $\pm 45^\circ$, $33.6 \pm 1\%$, $40.7 \pm 1\%$, and $54.1 \pm 1\%$, respectively).

Molecular localization of ciliary proteins upon flow stimulation

To gain a mechanistic insight into the molecular localization of the ciliary proteins upon flow stimulation, we employed dSTORM super-resolution microscopy to characterize the ciliary morphology as well as the protein distribution. Representative super-resolved images of immunostained IFT88 under 14 Pa shear stress are shown in Fig. 5(a). Interestingly, our results

showed that the IFT88 pattern under flow stimulation was significantly distinct from that without flow [Fig. 5(b)], revealing an enriched localization in the ciliary tip in most of the flow-stimulated cells. This finding of ciliary tip bulge is consistent with the results of the previous study by Mohieldin *et al.*, where they showed the flow induced ciliary tip swelling with molecules accumulated in this swollen tip, including functional proteins such as GM3 synthase (GM3S), bicaudal-c1 (Bicc1), and polycystin-2 (PC2).²³ To explore whether the bulge at the tip resulted from highly accumulated IFT88 proteins or an intrinsic change in axonemal morphology, we further conducted super-resolution imaging of ac- α -tubulin, a marker of the ciliary backbone. The ac- α -tubulin showed a pattern similar to that of IFT88, with a bulging localization at the tip, suggesting a physical enlargement within the compartment [Fig. 5(a)]. Moreover, the dual-color dSTORM images [Fig. 5(c)] revealed that the IFT88 and ac- α -tubulin distribution were spatially correlated, which indicates the localization of IFT88 is dependent on the axonemal alteration. It was also noted that the IFT88 was localized toward the periphery of the tip compartment bounded by the ciliary membrane [Fig. 5(c), enlarged]. Although the underlying mechanism is still not clear, this enlargement of the axonemal structure may form under the flow stimulation by reshaping the ac- α -tubulin

FIG. 5. (a) and (b) IFT88 and ac- α -tubulin (Actub) distribution along primary cilia using epifluorescence (WF) or dSTORM super-resolution (SR) microscopy imaging with (a) and without (b) flow stimulation. Yellow arrows indicate the basal bodies of the primary cilia. Green arrows indicate swelling at the tips. Scale bar: 500 nm. (c) IFT88 and ac- α -tubulin distribution along primary cilia using dual-color dSTORM super-resolution imaging. Scale bar: 200 nm.

pattern and facilitating the redistribution of IFT88 to the tip. It is possible that tubulin and IFT88 serve to create a structural framework to accommodate the abovementioned functional proteins at the swollen tip.

CONCLUSION

In summary, we combined a microfluidic device with super-resolution microscopy to perform flow stimulation followed by *in situ* fixation. The microfluidic device provided a well-confined cell culture environment with precise flow shear control. Importantly, the *in situ* fixation process preserved the ciliary phenotype and allowed for the observation of cilium-specific protein under different flow shears. We characterized individual cilium bending postures under different flow shears by measuring their projection length and alignment angle. In each flow shear case, ~250–350 cilia were individually imaged. Under high shear-stress stimulation, over 50% of the primary cilia were highly aligned with the flow direction, indicating uniform flow shear within the device. Finally, we used super-resolution microscopy to observe the cilium-related protein profile. To the best of our knowledge, this is the first platform combining a microfluidic device with super-resolution microscopy to enable precise flow stimulation and *in situ* cell fixation, allowing detailed observations of the proteins inside the cilia. These features could help us identify which proteins are involved in the mechanosensing of primary cilia. If we can also control the chemical concentration in the microfluidic device, we will also be able to determine gradient effects such as those on sonic hedgehog signaling in primary cilia. The device may also be useful if applied to the development of a stimulation-enabled organ-on-chip for studying the intercellular signaling of primary cilia or analyzing disease mechanisms associated with ciliary mutations at the organ level.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for two time-lapse videos of real-time cilia bending behavior under various flow shears.

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