Original Research

In vivo and *in vitro* observation of nasal ciliary motion in a guinea pig model

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Impact statement

Cilia play an important role in the airway defense mechanism. So far, studies on ciliary function have mainly been based on *in vitro* methods. Images of *in vivo* ciliary motion are very difficult to capture. In this study, we describe a novel approach to observe and analyze nasal ciliary motion in living animals with comparison to *in vitro* observation. Such images of ciliary motion from living animals have not been reported to date. The result of the study indicates that *in vivo* ciliary physiological function differs from *ex vivo* and *in vitro* conditions in many ways, such as the stability over time and response to temperature variation. This is a good foundation for further *in vivo* analysis of airway ciliary physiological function in animals as well as humans.

Abstract

In vitro airway specimens are widely used to evaluate airway ciliary function. However, the function of *in vitro* ciliated cells may be far different from their actual *in vivo* physiological conditions. Due to the lack of a valid technique, direct images of *in vivo* airway ciliary motion have never been captured and analyzed before. This study aims to examine nasal ciliary motion in living guinea pigs with comparison to *in vitro* observation. Nasal septum mucosa was exposed in anaesthetized guinea pigs and directly examined using a digital microscopy system. The study included three parts: (1) measurement of ciliary beat frequency (CBF) of nasal mucosa at room temperature in living guinea pigs and immediately after death, and in dissected mucosa specimens/cells for comparison; (2) monitoring of nasal ciliary motion, CBF, and ciliary beat distance (CBD) over 12 h in both living guinea pigs and dissected mucosa specimens/cells; and (3) measurement of ciliary motion changes in responses to temperature variations. Compared with when the animal was alive, the CBF after death and in dissected mucosa specimens/cells was lower by about 20% ($P < 0.05$). CBF and CBD variation in living guinea pigs was within 10% over time. The slope of CBF/temperature profile was 0.18 ± 0.01 Hz/°C in living guinea pigs, 0.51 ± 0.02 Hz/°C for dissected mucosa specimens, and 0.48 ± 0.03 Hz/°C for isolated ciliary cells. The technique described in this study makes it feasible to study ciliary motion in living animals using the digital microscope system. Ciliary function changes immediately after death. Ciliary motion in a living animal is more stable over time and has a different response to temperature change as compared with *in vitro* observation results.

Keywords: Cilia, ciliary beat frequency, *in vivo*, ciliary beat amplitude, nasal mucosa, temperature

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Introduction

Cilia, a special organelle, are widely distributed in mammals. For upper airway cilia, the primary function is to eliminate pathogenic particles by a fast and synchronous beat pattern, as a principal component of mucociliary clearance (MCC).[1] It is clear that the mucociliary clearance rate is positively correlated with the ciliary motion.[2] However, due to the lack of a valid *in vivo* observation method for ciliary motion, factors that influence the ciliary motion in living subjects remain unclear and subject to debate.

Currently, observation and studies of ciliary motion rely mainly on *in vitro* methods, using biopsy specimens[3] and cell culture,[4] and ciliary motion can be recorded with a high-speed camera to measure ciliary beat frequency (CBF)[5] and ciliary beat amplitude.[6] These techniques have been widely used in recent decades as a result of the even observation surface and improved control of experimental conditions. However, *in vitro* tissue conditions are far different from those *in vivo*. Therefore, firstly, it is difficult for *in vitro* experimental findings to reflect the actual status in a

living animal. For instance, while *in vitro* observations suggest that nasal spray inhibits CBF,[7] no significant difference in MCC has been found in living subjects.[8] Actual CBF change in a living subject remains unknown. Secondly, most likely due to factors such as the consumption of nutrients and toxic waste accumulation, *in vitro* ciliated cells have a limited lifespan, with their function changing over time,[9,10] potentially jeopardizing the stability and accuracy of *in vitro* ciliary motion measurement, especially as such experiments can be time-consuming. Thirdly, during the *in vitro* specimen preparation process, the function of ciliated cells may be mechanically and chemically disturbed.

Since the 1980s, many researchers have ever made efforts to analyze *in vivo* ciliary motions. A heterodyne mode correlation analysis light-scattering system was used to measure the *in vivo* ciliary motion of canine trachea.[11] Another light reflection technique was used to measure the *in vivo* CBF of the rabbit maxillary sinus,[12] and it was later further applied in human noses.[13] These pioneering techniques basically managed to measure *in vivo* CBF, but no direct images were obtained. Due to the technique limitations at that time, the data reliability and accuracy were difficult to evaluate, and so they have rarely been used recently. Meanwhile, optical coherence tomography (OCT) is now a rapidly maturing, interferometry-based, and noninvasive technique to image micro-structures in living tissues.[14] An extension of the technique, Doppler OCT, was used to quantify CBF.[15,16] It detects the phase shift of backscattered light caused by ciliary motion to measure CBF, but no *in vivo* application has been reported yet. Another extension of OCT, micro-OCT (μOCT), has been used by Chu *et al.*[17] to image ciliary motion of primary human bronchial epithelial (HBE) cells, and then to measure CBF of the tracheal epithelium in a living porcine model.[18] This is a very promising method to image *in vivo* ciliary motion. It has an ultra-high resolution of 1–2 μm and can also noninvasively measure airway surface liquid (ASL) and periciliary liquid (PCL) layer depth. However, it is still a proprietary technology in development and has many technical challenges to overcome, including short working distance, small field of view, low capture frequency, and image instability.

In order to image and analyze *in vivo* ciliary motion, we developed a method for direct observation of ciliary motion in a living guinea pig model using a digital microscopy system. In this study, the feasibility of this method was verified, and ciliary motion in living guinea pigs was compared to that in dissected specimens/cells, which we believe is the first such study reported. Furthermore, *in vivo* ciliary motion stability over time and its response to temperature variation were also studied and compared to the *in vitro* specimens.

Materials and methods

Experimental animals and preparation

Healthy adult guinea pigs (250–350 g) were used for all studies reported in the present article and were provided by the Medical Animal Centre of Chinese PLA General Hospital (Beijing, China). Experimental procedures were approved by the local ethics committee in accordance with institutional animal protection regulations.

Guinea pigs were anaesthetized by intraperitoneal administration of sodium pentobarbital (60 mg/kg; Merck Company, Kenilworth, New Jersey, USA) and maintained on continuous intravenous infusion of pentobarbital (6 mg/h) through the jugular vein. Heart rates were monitored by a stethoscope and all remained within a normal range from 243 to 308 beat/min.

In vivo observation and ciliary motion recording

An incision was made from the anterior naris to 0.5 cm in front of the eye on one side and the lateral wall of nasal cavity was removed to expose the nasal septum mucosa. Slight incision bleeding could be controlled by gentle gauze pressing. A custom-made platform with a heating pad was used to hold the animal, whose head and body were properly secured on the platform to reduce image instability caused by respiratory and cardiac motions. The animal on the platform was then placed on the digital microscopy system (VHX6000, Keyence Corporation, Osaka, Japan) with the observation lens (VH-Z100R, Keyence Corporation, Osaka, Japan) set perpendicular to the mucosal surface. An illumination adapter (OP-88164, Keyence Corporation, Osaka, Japan) was used to satisfy the variable observation conditions and uneven mucosal surfaces (Figure 1). To obtain a surface environment identical to that of *in vitro* specimens, the mucosal surface was wetted with Leibovitz's L-15 medium (L15; supplemented with 10% FBS and 100 units/mL penicillin G sodium and 100 ug/mL of streptomycin sulfate). When not recording, the observation area was covered with a saline gauze to reduce evaporation. Each region of ciliary beat motion was recorded with a high-speed digital microscopy camera system (VW-9000, Keyence Corporation, Osaka, Japan) for 5 s at 150 frames per second.

In vitro observation and ciliary motion recording

Upon finishing *in vivo* observation, the nasal septum mucosa was immediately removed and placed in a Petri dish, submerged in the same L15 medium mentioned above. Part of the mucosa specimen was cut into about 2×2 mm square segments. The rest of the mucosa was scraped using fine forceps to obtain isolated cells from epithelial debris, mimicking biopsy brushing. Both *ex vivo* mucosa specimens and *in vitro* isolated ciliated cells (hereinafter referred to as '*in vitro* specimens') were placed in a capped Petri dish and examined under an inverted phase-contrast microscope (DMi 8, Leica Microsystems GmbH, Wetzlar, Germany). Only beating cilia with normal pattern and morphology[5] were recorded using a high-speed digital camera (EoSens-4CXP, Mikrotron GmbH, Unterschleissheim, Germany) and a movie acquisition software at 150 frames per second. Abnormal beat patterns were defined as obviously reduced beat amplitude, stiff beat pattern, failure to bend along the

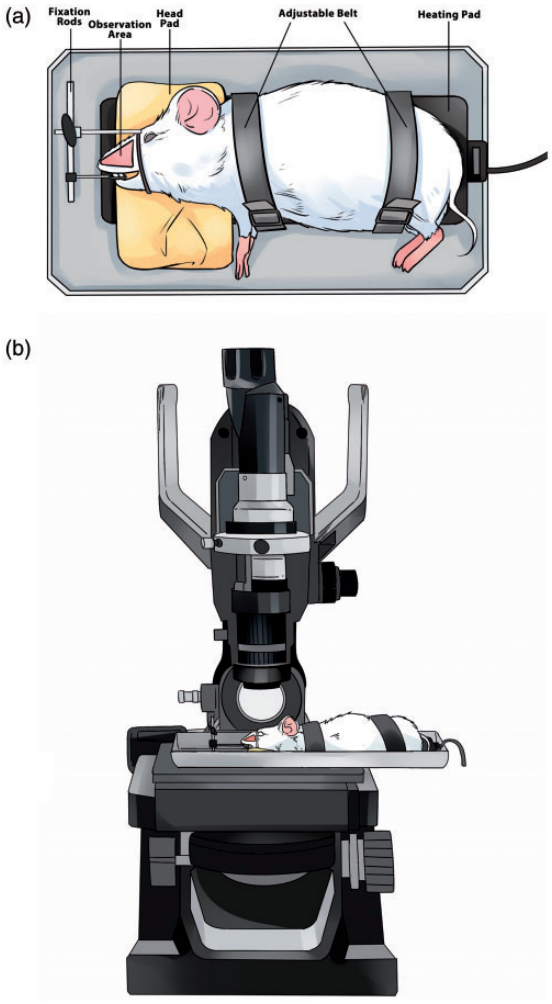

Figure 1. (a) The custom-made platform with proper fixation of the animal's body and the observation area. The head pad propped the head and the observation area up a little bit. (b) The animal on the platform placed on the digital microscopy system. The observation lens was set perpendicular to the septum mucosal surface. (A color version of this figure is available in the online journal.)

length of the ciliary shaft, flickering or twitching motion, and static cilia.[19]

Ciliary beat frequency and amplitude measurement

Both *in vivo* and *in vitro* images can be analyzed following the technique described by Richard Francis *et al.*[20] Briefly, movies of ciliary motion were processed using the Fiji/ImageJ software package. A line was drawn across the *in vivo* ciliary wave/*in vitro* cilium of interest along their moving direction. The "Reslice" feature was used to generate a kymograph type image with a wave pattern. By measuring the length of each wave to calculate how many waves in each second. CBF(Hz) = Ps/Pw (one pixel = one frame, Ps = pixels per second, Pw = pixels of each wave) (Figure 2). Ciliary beat distance (CBD) was measured to represent ciliary beat amplitude. The ImageJ software enabled us to visualize the *in vitro* cilia tips frame by frame. The CBD of *in vitro* cilia was determined by the distance between maximum power stroke and recovery stroke of the cilia tips.[21] *In vivo* CBD was determined by the distance travelled by the front edge of each ciliary wave also from its maximum recovery stroke to power stroke (Figure 3). At room temperature, 24°C, the baseline CBD was recorded at the start of each experiment and was defined as 100%. The normalized CBD (CBD ratio) was calculated to compare the ciliary beat amplitude among experiments. For both *in vivo* and *in vitro* observation, the average CBF or CBD of five randomly chosen points in a region of interest was calculated to represent the CBF/CBD of this region. The recordings were analyzed twice by two separate observers (Pang Chuan and An Fengwei) blinded to each other's results.

Temperature control

For *in vivo* observation, the room temperature was maintained at 24°C with relative humidity at 40–50% by an air conditioner. The mucosal surface temperature was monitored by an infrared thermometer (Fluke 561, Fluke Corporation, Everett, USA) and maintained at 24–26.5°C. During *in vitro* observation, a temperature control platform (MATS, Leica Microsystems GmbH, Wetzlar, Germany) was used to maintain the specimen at the same temperature as for *in vivo* observation. Variation of both room and mucosal surface temperature was controlled within 0.5°C for each guinea pig (more details below).

Comparison between in vivo and in vitro results

Upon completion of *in vivo* observation, the animal was sacrificed by decollation and the same mucosa was examined and recorded immediately after the animal's death. After this 5–10 min procedure, mucosa specimens and isolated cells were obtained as described above and examined once the desired surface temperature was reached for 5 min. The overall experiment duration after the animal sacrifice was kept to within 30 min.

Recording of ciliary motion changes over time

To document the temporal stability of ciliary beat, changes of ciliary motion over time were recorded. Ciliary beat in living animals was recorded every 15 min for the first hour and every 30 min for another 11 h (total = 12 h). In our preliminary experiments, ciliary motion in living animals had no significant change for 12 h and maintained morphology and function comparable to those collected from a freshly sacrificed animal. So, *in vitro* specimens were still collected from the same mucosa immediately after *in vivo* observation. Then they were recorded for another 12 h *in vitro*.

Recording of ciliary motion changes in response to temperature variation

To confirm *in vivo* ciliary beat response to environmental conditions, the temperature was chosen as a typical influencing factor.[2] Before the animal preparation procedure, a contact probe attached to the infrared thermometer was used to record the temperature in the animal's nasal cavity (the unexperimented side, 3 cm to anterior nares). To manipulate temperature during i*n vivo* observation, a cylindrical polystyrene shell was used to cover the animal's

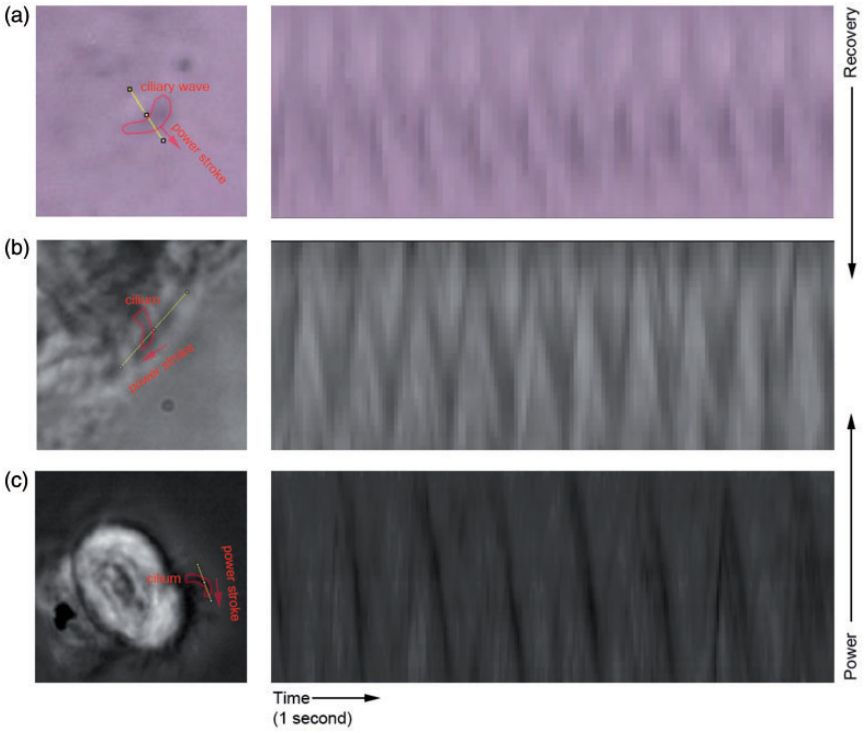

Figure 2. Use ImageJ to draw a line (yellow line) across the ciliary wave/cilium of interest to "reslice" a kymograph of ciliary motion. The *in vivo* ciliary wave and *in vitro* cilia are outlined in red. (a) Measurement of *in vivo* ciliary motion. (b) Measurement of ciliary motion of an *ex vivo* mucosa segment. (c) Measurement of ciliary motion of an *in vitro* isolated cell. (A color version of this figure is available in the online journal.)

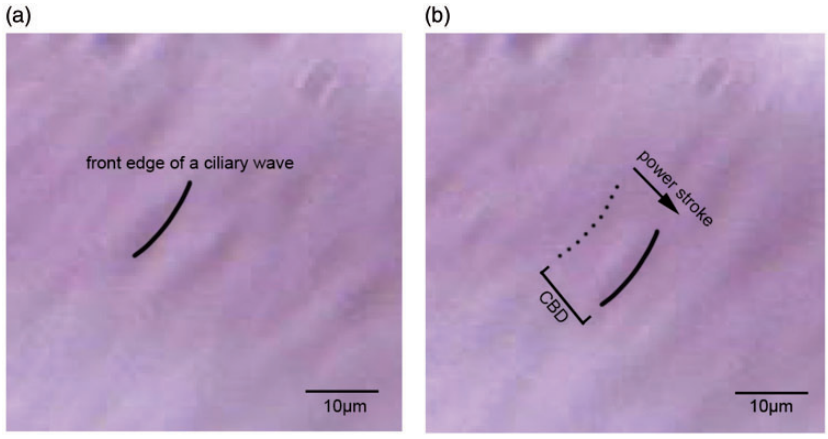

Figure 3. *In vivo* ciliary beat distance (CBD) is defined as the distance travelled by the front edge of a ciliary wave between maximum recovery stroke (Figure a, solid line; Figure b, dotted line) and maximum power stroke. (Figure b, solid line). (A color version of this figure is available in the online journal.)

nasal region in living animals, which was wrapped with crushed ice bags, avoiding any direct physical contact with the exposed mucosa. After cooling, the ciliary motion was immediately recorded, and the infrared thermometer was used to monitor the mucosal surface temperature. The mucosal surface temperature was allowed to recover from 0°C to room temperature and then gradually heated up to 40°C by a heating pad under the nose while the ciliary motion was recorded continually. The rewarming is usually completed within 30 min.

For *in vitro* observation, the capped Petri dish containing specimens was placed in a polystyrene chamber and cooled down by wrapping ice bags for 30 min. The Petri dish was then immediately placed on a temperature-controlled platform under the microscope. The temperature was controlled by the temperature controller and varied from 0 to 40°C within 30 min, while ciliary beats of mucosa specimens and isolated cells were recorded.

Statistical analysis. The GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. Friedman's test was used to compare *in vivo* and *in vitro* data. Regression analysis was used to correlate CBF with temperature. P values less than 0.05 were considered statistically significant. Agreement between the two

observers (interclass correlation) and within each observer was also determined.

Results

Ciliary beat in living animals

Ciliary motion waves were visible at 100× magnification but were much clearer at 500×–1000× magnification. The motion area did not cover the whole mucosal surface but was regional, consistent with the existence of non-ciliated cells.[22] Captured images *in vivo* reflected an overall result of regional large-scale ciliary beats, synchronously propelling surface liquid and forming regular, synchronous, and metachronal waves. We believe the observed waves were reflections on the liquid surface, propelled by beating cilia beneath (Figure 4(a)). Video 1 (supplementary file) showed a region of ciliary motion on a guinea pig's nasal septum magnified from 100× to 1000×.

Differences between in vivo and in vitro

From the 20 guinea pigs, ciliary motions were recorded from 60 regions on the mucosa while the animal was alive and immediately after death, and from 60 mucosa specimens as well as 60 isolated cells (Figure 4(b) and (c), Videos 2 and 3). The median CBF (interquartile range, IQR) in living animals [9.95 (8.14–11.31) Hz] was higher than after animal death [7.74 (6.53–10.31) Hz] ($P = 0.022$), in mucosa specimens [8.24 (6.59–9.46) Hz] ($P = 0.045$) and in isolated cells [7.91 (5.77–9.69) Hz] ($P = 0.022$). Compared with when the animal was alive, CBFs decreased by 22.2% after death, 17.2% in resected mucosa specimens, and 20.5% in isolated cells, respectively. The IQR was the highest (3.92 Hz) in isolated cells (Figure 5).

Temporal changes of ciliary motion

In the 20 guinea pigs, CBF variation over 12 h was recorded in 40 regions of the mucosa while the animal was alive and in 40 *ex vivo* mucosa specimens and 40 *in vitro* isolated cells. Figure 6(a) to (d) shows changes of mean CBF over 12 h. CBF was essentially stable in living animals, fluctuating within 10% of the initial frequency. In contrast, CBF in mucosa specimens and isolated cells appeared to increase in the first few hours and decrease in later hours (Table 1). Although recording did not continue after 12 h, it is believed that *in vitro* CBF would decrease continually until zero as reported.[10] CBD was also stable in living animals, while it gradually decreased over time in resected specimens (Figure 6(e)).

Ciliary motion changes in response to temperature manipulation

At room temperature of 24°C, the temperature in guinea pigs' nasal cavity ranged from 31.6 to 35.4°C, 34.3°C on average. Ciliary motion data were collected from 60 regions on mucosa in living animals, 60 mucosa specimens, and 60 isolated cells. The CBF/Temperature profiles were apparently different between living animals and *in vitro* specimens (Figure 7(a)). With temperature rising from 0°C, ciliary motion was initially in a few mucosa specimens at 7°C and in a few isolated cells at 4 °C, and in majority of both types of *in vitro* specimens at 10°C, from where CBFs increased in a linear fashion along with temperature up to

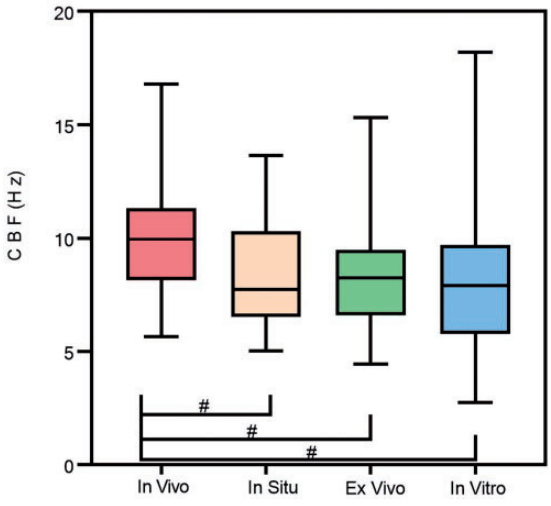

Figure 5. Ciliary beat frequency (CBF) of living animals (*in vivo*, red), immediately after death (*in situ*, yellow), *ex vivo* mucosa specimens (*ex vivo*, green) and in isolated cells (*in vitro*, blue). Boxes represent the IQR, and the median is marked with a solid line. # $P<0.05$. (A color version of this figure is available in the online journal.)

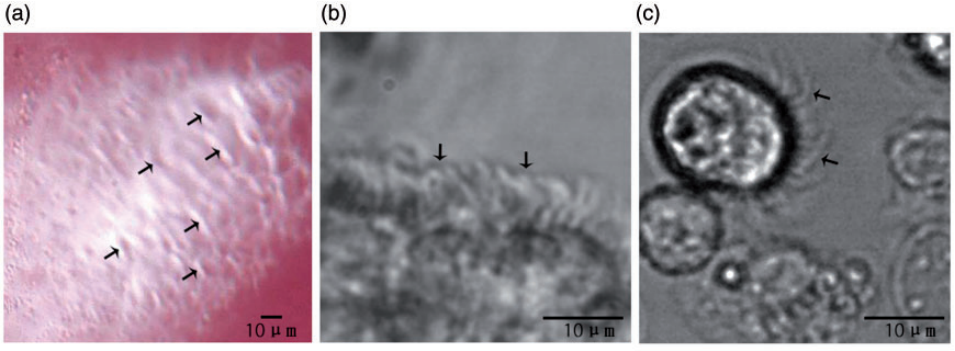

Figure 4. (a) Ciliary waves (black arrows) directly observed by the digital microscope system. (b) Intact *ex vivo* mucosa specimen edge. Beating cilia (black arrows) can be observed on the mucosa edge. (c) *In vitro* isolated single ciliated cell. Black arrows indicate cilia. (A color version of this figure is available in the online journal.)

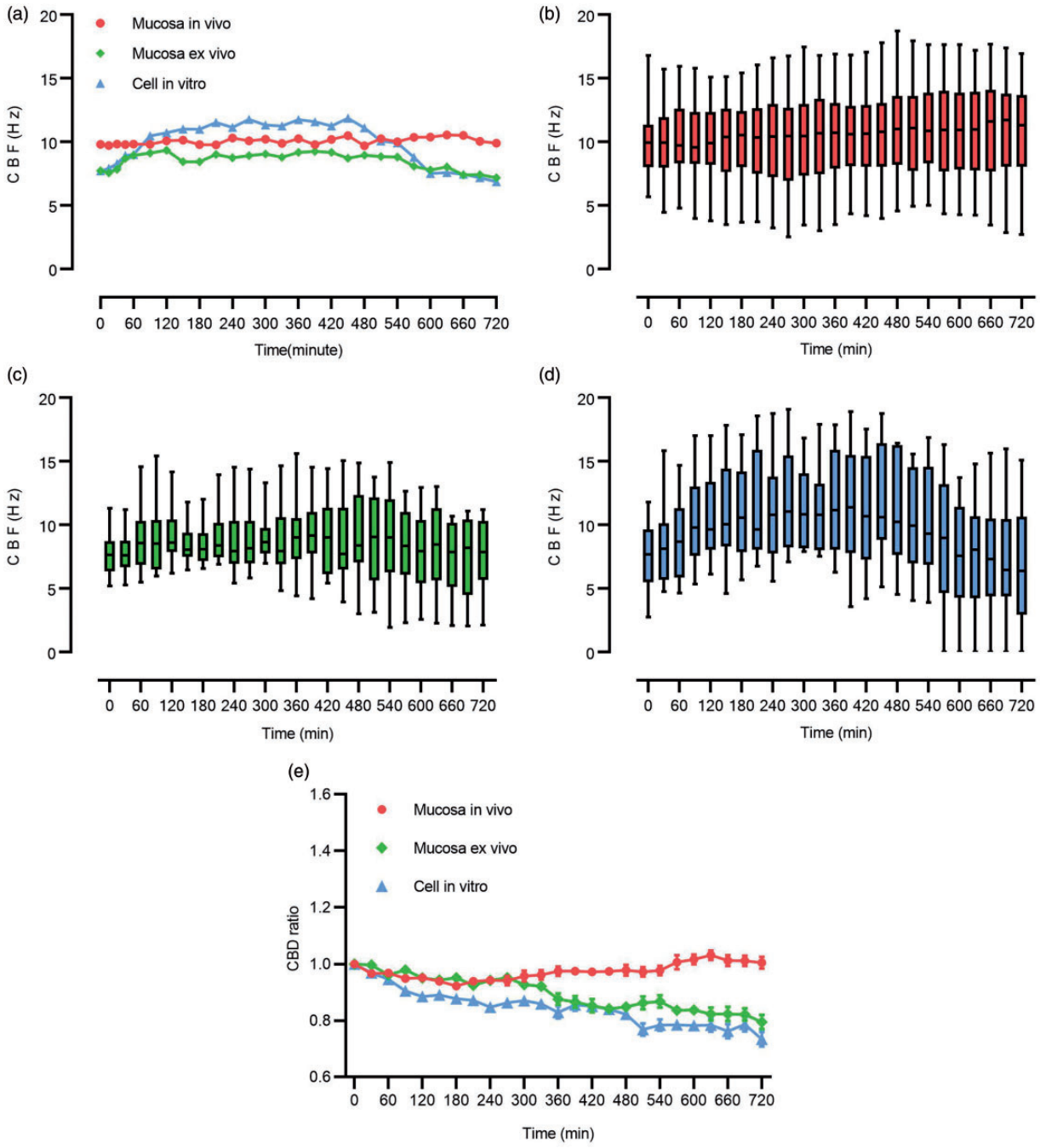

Figure 6. (a) Variation of mean CBF over 12 h. (b–d) The box-whisker plot of CBFs in living guinea pigs (b), mucosa specimens (c) and isolated cells (d), respectively. The box represents the IQR. The median is marked with a solid line. CBF IQR in living animals increased slightly in the first 3 h only, whereas it continues to gradually increase over time in mucosa specimens and isolated cells. (e) Ciliary beat distance (CBD) ratio profiles of three models over time. (A color version of this figure is available in the online journal.)

Table 1. CBF characteristics in living guinea pigs, mucosal specimens, and isolated cells over 12 h.

	Sample type (*n* = number of measurement)		
	Living animal (*n* = 40)	**Mucosa specimen (*n* = 40)**	**Isolated cell (*n* = 40)**
Increasing	None	0–120 min	0–150 min
Stable	0–720 min	150–540 min	150–450 min
Decreasing	None	>540 min	>450 min
Initial mean CBF	9.81 Hz	7.73 Hz	7.71 Hz
Peak mean CBF	10.49 Hz	9.34 Hz	11.85 Hz
(Time point; increase over initial CBF)	(450 min; 6.9%)	(120 min; 20.8%)	(150 min; 53.7%)

35°C. Thereafter, a plateau was reached after 36°C with a maximum mean CBF of 15.53 Hz for mucosa specimens and 13.98 Hz for isolated cells. The slope of the linear increase was 0.51 ± 0.02 Hz/°C (mean ± S.E.M.) for mucosa specimens and 0.48 ± 0.03 Hz/°C for isolated cells, with increasing standard deviations as the temperature rose, particularly for isolated cells. In contrast, some mucosal regions in living animals were found beating as soon as the temperature was above 0°C. CBF soon increased rapidly followed by a more gradual linear increase from 9°C to 34°C at 0.18 ± 0.01 Hz/°C, significantly shallower than that with *in vitro* specimens ($P < 0.001$) (Figure 7, Table 2). Thereafter, the profile reached a plateau after 34°C with a maximum mean CBF of 11.95 Hz. CBD ratios of *in vitro* specimens have no significant correlation with the rising temperature. *In vivo* CBD ratio has a linear increase until 27°C, with a slope of $0.65 \pm 0.005\%$/°C (Figure 7(b)).

Observation repeatability

Agreement between the two observers (interclass correlation) was 0.98 and agreement within each observer (interclass correlation) was 0.99.

Discussion

Direct images of ciliary motion in living animals or humans have been very difficult to capture before. In this study, we describe a novel approach to observe and analyze nasal ciliary motion in living animals with comparison to *in vitro* observation. Such forms of ciliary motion images have not been reported to date. The technique is simple and poses minimal disturbance to the normal physiological state. Ciliary motion on living animals' nasal mucosa can be directly observed and analyzed using this technique, without either sample acquisition or preparation process. It allows high-quality, stable, wide-field, and direct *in vivo* imaging of ciliary motion. Additionally, this technique also makes it possible to observe *in vivo* ciliary motion on nasopharyngeal region, trachea, biliary duct, and oviduct. One limitation of this technique is the anesthesia of animals, potentially influencing normal physiological conditions. The other one is the destruction of the nasal lateral wall, making it invasive and limiting its further application on humans. Compared with the recent μOCT system, our system has its advantages such as the images being more stable, wider-field, and easier to read. Nevertheless, the μOCT system has the advantages of being completely noninvasive and can simultaneously measure airway surface liquid and periciliary liquid layer depth. Our system does have the potential for completely noninvasive and direct observation of ciliary motion airway epithelium and is a promising approach for application in humans, provided the observation lens can be modified and attached to an endoscope. It can be transformed into a clinical examination that can facilitate the assessment of airway diseases such as primary ciliary dyskinesia (PCD).[23]

Compared with cell culture, ciliated cells directly collected by biopsy are considered to have the most similar function with those in a living animal, and isolated cells may be

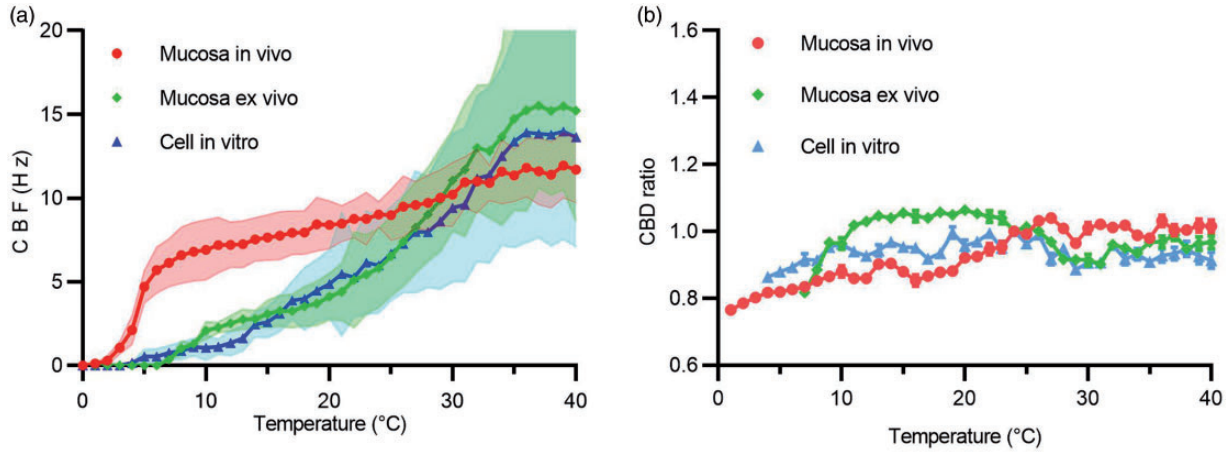

Figure 7. (a) Mean CBF/temperature profiles of mucosa in living guinea pigs (red), mucosal specimens (green), and isolated cells (blue). Colored area flanking the curve indicates corresponding standard deviation. (b) CBD ratio/temperature profile of three different models.

Table 2. CBF/temperature profiles in living guinea pigs, mucosa specimens, and isolated cells.

	Sample type (n = number of measurements)		
	Living animal ($n = 60$)	**Mucosa specimen ($n = 60$)**	**Isolated cell ($n = 60$)**
Temperature at start of ciliary motion	>0°C	7°C	4°C
Temperature at start of linear phase	9°C	10°C	10°C
Slope of linear phase + S.E.M. (Hz/°C)	0.18 ± 0.01	0.51 ± 0.02	0.48 ± 0.03

subject to mechanical and chemical influences in comparison with mucosa specimens.[24] Therefore, we chose both mucosal specimens and isolated cells obtained by biopsy for comparison with the mucosa in living animals. In addition, *in situ* mucosa recording was continued after animal death to control potential impacts on ciliated cells by the specimen collection process, which showed immediate but similar CBF decrease as seen with *in vitro* specimens. The *in vivo* CBF measured in our study (9.95 Hz) is also higher than those previously reported in some *in vitro* guinea pigs studies at room temperature (7.49–8.6 Hz), although test conditions are not exactly the same.[25,26] The factor 'death' here plays a role in the fall of ciliary motions, which involves complex physiological mechanisms calling for further studies. One of our hypotheses is that the loss of blood supply after death resulted in a declined supply of oxygen and nutrients. Hypoxia may further reduce adenosine triphosphate (ATP). It is clear that ciliary motion is sustained by ATP-mediated motor activity of axonemal dynein,[27] and ATP can increase *in vitro* CBF.[28] Hypoxia also causes intracellular acidification, which can attenuate CBF as well.[29] In the second part of this study, temporal changes of CBF, specimens recovered from the impact of 'death' within the first 150 min after being set into a medium. This can partly support our hypothesis because the specimens may regain nutrients and oxygen from the medium. Furthermore, since the *in situ* ciliated cells have a similar CBF with those after collection, the mechanical damage seems relatively slight in our study. Although mechanical stimulation does have an impact on ciliary motion,[30] mechanical damage during a collection process may directly lead to cell death or severe impairment of cellular morphology and function, which is not difficult to identify and exclude for researchers.

In vitro CBF is time-dependent due to the limited lifespan of ciliated cells.[9,10] CBF change in living animals over time has not been studied before. In the present study, as hypothesized, *in vivo* CBF and CBD were relatively steady over time during the 12-h observation period under our experimental conditions. The CBF increase in the first few hours and its decrease during the later stage of the 12 h seen with *in vitro* specimens are consistent with previous research.[10] This not only indicates that time is less of an issue for *in vivo* experiments, but also suggests that in the *ex vivo* tissues, CBF starts at a lower level, and then recovers from the effect of "death." The mechanism needs further study. Moreover, compared with isolated cells, the ciliary motion of mucosa specimens appears to be more stable. We believe that this results from the main distinction between isolated cells and mucosal segments, i.e. isolated cells are completely surrounded by the medium, whereas ciliated cells on a mucosa specimen are still closely connected with adjacent cells and the basement membrane. For one thing, the preserved connection between epithelial cells enables intercellular communication to regulate ciliary motion and coordination.[31] For another, more contact with surrounding medium means more chemical impact, which may explain why isolated cells have greater variations during the study.[10] We observed a slowly decreasing ciliary beat amplitude *in vitro*, without correlation with CBF. The dissociation of ciliary beat amplitude from beat frequency was reported by previous studies,[21] probably due to the different regulatory mechanism.[6]

It has been clearly shown by *in vitro* studies that CBF rises with temperature in a sigmoid pattern.[32] Consistent with our study, cilia are reported to stop beating at 4°C and beating motion increases with warming at a rate of 0.3–0.7 Hz/°C by *in vitro* observations,[32–35] but CBF response to temperature change in living animals has not been reported until this study. The present study indicates that the CBF/temperature profile in a living animal also follows a sigmoid pattern with an earlier onset at just above 0°C, a more rapid early-stage rising CBF and a significantly shallower linear phase compared to resected specimens. In our study, cilia in a guinea pig's nose usually beat in an environment of 32.6 to 36.4°C. The *in vivo* CBF/temperature profile reached a constant plateau exactly in this temperature range, indicating that there is an ideal physiological temperature range for the ciliary motion. The finding is similar to that of human nasal samples where the temperature ranges between 30 and 35°C, and the CBF/temperature plateau is between 32 and 40°C.[36] We believe that these *in vivo* responses are what happen in actual physiological conditions. It can be speculated that the distinct *in vivo* result mainly originates from the complex regulation on the ciliary motion under physiological conditions in a living animal. One possible regulatory factor is the neural innervation. Both sympathetic and parasympathetic nervous systems were confirmed to regulate ciliary motion.[37–39] Multiple neuropeptides were found to have regulatory effects on cilia such as substance P,[40] neurotransmitter neuropeptide Y,[41] etc. Moreover, several signaling molecules are already known to regulate ciliary motion, including cAMP, Ca^{2+}, nitric oxide, and progesterone.[42] It has been reported that protein kinase C and Ca^{2+}/calmodulin-dependent kinase II regulate the profile of human nasal CBF in its response to temperature variation by altering the onset temperature for the ciliary motion and linear response slope.[32] The physiological ciliary regulation must be the interaction of complex factors including all of the above, in order to sustain a relatively stable ciliary motion to adapt to a wide range of temperature encountered in the environment. Extensive research remains to be done to further our understanding in this regard.

Conclusion

In summary, the present experimental system is a feasible, effective, and easy-to-implement method for direct observation and analysis of ciliary motion in living subjects. Compared with *in vitro* cilia observation, ciliary motion in living animals is different in frequency, amplitude, stability over time, and response to temperature variations. Results from *in vitro* studies of ciliated cells cannot accurately represent their status in a living subject.

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SUPPLEMENTAL MATERIAL

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