Fluctuation of cilia-generated flow on the surface of the tracheal lumen

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When we inhale air to obtain oxygen, we sometimes also inhale small particles, such as viruses, bacteria, pollen, and dust. These particles are trapped on the surface of the tracheal lumen and transported towards the larynx by cilia-generated mucous flow, known as the clearance function. If the clearance function does not work properly, harmful substances may remain in the body and may cause various diseases and infections, such as pneumonia and influenza (1, 7, 10, 16–19).

The clearance function has been investigated from many points of view. In 1934, Lucas and Douglas (14) reported the presence of two fluid layers on the epithelium of the trachea. A periciliary layer (PCL) exists on the epithelial cells and has low viscosity, while a mucus layer (ML) exists on top of the PCL. and has a much higher viscosity than that of the PCL. The classical picture of the two-layer structure was recently modified by BUTTON et al. (4), so that the PCL is not just a watery fluid but contains membrane-spanning mucins and mucopolysaccharides. It is also known that cilia of the tracheal lumen show two kinds of strokes, an effective stroke and a recovery stroke, with a frequency of about 10–20 Hz (6, 20, 22, 27, 28). Due to the asymmetry of the strokes, the cilia generate a net flow towards the larynx (23, 27).

Velocity analyses of cilia-generated flow have been performed both experimentally and numerically (8, 12, 15, 24–26). Hussong et al. (8) clarified the time-averaged flow structure around epithelial cells and discussed the effect of ATP stimulation on particle transport. These former studies are important in understanding the time- and space-average flow field; however, the time and space fluctuations of the flow have received little attention to date. Flow fluctuation is one of the key quantities in particle transport, given that transport phenomena are governed by advection, due to the bulk flow, and diffusion, due to flow fluctuations and Brownian motion.

In this study, we investigated the time and space fluctuations of cilia-generated flow experimentally in mice. To understand the origin of flow fluctuation, we first measured the distribution of ciliated cells in the trachea and their ciliary motion. Next, we measured the in-plane flow field at different heights of observation using a confocal micro-PTV system. This system enabled us to accurately measure the flow with high resolution in time and space. Finally, we quantified the effect of flow fluctuation on bulk flow by evaluating the Peclet number (cf. Eqs. 7 and 2) of the system.

MATERIALS AND METHODS

Preparation of samples. Experiments were conducted with approval by the Animal Ethics Review Board of Tohoku University. Figure 1 illustrates the procedure for sample preparation. Tracheas were obtained exclusively from wild-type mice (Crlj:CD1), 4–16 wk old. The mice were killed by cervical dislocation. The trachea was then dissected from the larynx to the bronchial main branches and doused with L-15 medium (GIBCO) + 10% fetal bovine serum (FBS; ThermoScientific). Muscle and vascular tissues were removed from the trachea in cold medium. The trachea was opened longitudinally, and 3-mm square specimens were excised. The exposed tracheal lumens were soaked in L-15 medium including 5 mM dithiothreitol (DTT; Fluka) to gently remove the original mucus and contaminating...
impurities during the preparation process. After incubating in L-15 medium with DTT, the specimens were washed with L-15 + 10% FBS. Then, we added a drop of working solution including fluorescent particles. When we measured the flow field, tracers of 1-μm diameter were used to minimize the effect of Brownian motion while maintaining high resolution. When we measured ciliary motion, on the other hand, tracers of 0.5-μm diameter were used to reduce the viscous drag caused by the tracer. The specimen was placed on a slide glass and covered by a coverslip (Matsunami), in which two spacers prevented the specimen from being pressed directly. The distance between the coverslip and the slide glass was ~100 μm, and the gap at the edge was sealed. Curved air-liquid interfaces may generate inhomogeneity in surface tension and Marangoni stress at the interface, leading to complex flow. To avoid such uncertainty and to measure the flow field precisely, we used a coverslip.

**Experimental setup.** When we measured the flow field, a confocal micro-PTV system was used, similar to that in our former studies (13, 21) but with an upright instead of an inverted microscope. Figure 2 shows a schematic of the system used, consisting of a fluorescence upright microscope (BX51WI; Olympus), a Nipkow lens-type confocal unit (CSU-X1; Yokogawa), a high-speed camera (SA3; Photron), and a ×100 objective lens (Olympus). The observation height was controlled by a piezo actuator (E-665; PI). The temperature of the sample was regulated at 37°C using a thermo plate (MATS-55RAF20; Tokai Hit). When we measured ciliary motion, we also used another setup, consisting of a fluorescent microscope (DM4000B; Leica), a
mercury lamp (ebq 100; LEj), and a high-speed camera (HAS-220; Ditect).

Cell staining. We performed phalloidin staining for visualization of actin filaments and immunofluorescent staining of respiratory cilia using anti-acetylated tubulin antibody, according to Francis et al. (6). For immunostaining, tracheas were fixed in 4% paraformaldehyde and then permeabilized with PBST (0.2% Triton in PBS), blocked with buffer (5% goat serum + 0.1% Triton in PBS), and incubated overnight at 4°C with a monoclonal anti-acetylated tubulin antibody (Sigma). Then, fluorescent labeling was achieved with an Alexa Fluor 532-conjugated anti-mouse IgG as the secondary antibody, diluted in PBST2 (×500, 0.1% Triton in PBS), and Alexa Fluor 488-conjugated phalloidin, diluted in PBST2 (×40). The tissues were imaged using the Leica microscope with a ×100 oil immersion objective lens. In total, 26 locations were selected randomly, and an area of 12 × 96 μm was recorded at each location. Images of epithelial cells and cilia were taken, colored separately, and merged using Adobe Photoshop CS4 (Adobe Systems).

Analysis of ciliary beat. Cilia were labeled individually with a polystyrene tracer particle of 0.5-μm diameter (wavelength 505 nm; Molecular Probes) by nonspecific binding to facilitate visualization of ciliary motion. The claw-like structures called ciliary crown is elongated from the surface of the ciliary tip (5), to which we intend to bond the tracer particles. The sample was washed before the experiment to remove nonbinding tracer particles. The motions of tracer particles were tracked at 200 frames/s in bright-field images. Oscillation of each tracer particle was analyzed along the beat axis e (see Fig. 3B and Supplemental Movie S1; Supplemental Material for this article is available online at the Am J Physiol Lung Cell Mol Physiol website), and the time history of the tracer oscillation along the beat axis was plotted (Fig. 3C) using the ImageJ software (National Institutes of Health). After a time history of tracer oscillation had been obtained, the beat frequency (BF) could be calculated readily by counting the peaks during a 1-s period. The velocity of effective and recovery strokes were defined as the slopes at the crossing points of the line of average e value (Fig. 3).

Analysis of cilia-generated flow. For flow visualization, fluorescent polystyrene beads of 1-μm diameter (wavelength: 505 nm; Molecular Probes) were coated with bovine serum albumin (BSA; Sigma) to prevent nonspecific binding to tissue surfaces and then added to the working solution. The trajectories of particles were recorded using the confocal micro-PTV system at a frame rate of 60 frames/s. The observation plane was controlled by the piezo actuator. The observation height H was measured from the plane of the tips of the cilia, and particle motions in the planes with H = 0, 5, 10, 15, and 20 μm were measured. We note that the focal plane thickness is ~0.7 μm in the present setup. In each movie, motions of five particles during an at least 1-s period were analyzed using the ImageJ software.

Let the velocity of particle i at time t be \( v_i(t) \), and the time-average velocity of particle i be \( \overline{v}_i \). Flow fluctuation may be quantified by calculating the standard deviation of the velocity (SDV). We, thus, calculated the SDV of each bead in the parallel and the normal directions to \( \overline{v}_i \), i.e., SDV<sub>bead,para</sub> and SDV<sub>bead,norm</sub>, given by:

\[
SDV_{\text{bead,para}} = \sqrt{\frac{1}{N_p N_f \sum_{i=1}^{N_f} \sum_{t=1}^{N_i} [v_{i,\text{para}}(k\Delta t) - \overline{v}_i]^2},
\]

\[
SDV_{\text{bead,norm}} = \sqrt{\frac{1}{N_p N_f \sum_{i=1}^{N_f} \sum_{t=1}^{N_i} [v_{i,norm}(k\Delta t)]^2},
\]

where \( N_p \) is the total number of particles, \( N_f \) is the total number of frames, \( v_{i,\text{para}} \) and \( v_{i,norm} \) are the parallel and the normal components of \( v_i \), and \( \Delta t \) is the time interval between two successive images. SDV<sub>bead</sub> indicates how an individual bead fluctuates relative to its time-average motion.

It is also important to discuss how each bead fluctuates relative to the particle-average motion. We, thus, calculated the particle-average velocity in each movie and define SDV<sub>mov</sub> as:

\[
SDV_{\text{mov}} = \sqrt{\frac{1}{5N_m \sum_{m=1}^{N_m} \sum_{i=1}^{N_i} [v_{i,m} - \overline{v}_m]^2},
\]

where \( N_m \) is the number of movies, \( v_{i,m} \) is the time-average velocity of particle i in movie m, and \( \overline{v}_m \) is the average \( v_{i,m} \) of five particles in movie m. SDV<sub>mov</sub> indicates the fluctuation in time-average motion of an individual bead relative to the bulk motion of beads in the movie. Furthermore, we analyzed the deviation angle of the \( \overline{v}_{i,m} \) vector from the \( V_m \) vector, defined as:

![Fig. 3. Analysis of ciliary beat. A: observation of tracer particles attached to the tips of cilia (bright field). B: magnified image of A, where e is the beat axis (see also Supplemental Movie S1). C: time history of the tracer motion during 0.5 s. D: slope indicates the velocity of the ciliary beat. The velocity of the effective stroke is shown as the slope of dashed lines, whereas that of the recovery stroke is shown as the slope of chain lines.](http://ajplung.physiology.org/)
The deviation angle is defined in the range 0 to 180°. When all particles move in the same direction it is 0°, while when particle motion is almost random, it becomes 90°. Thus the deviation angle indicates the fluctuation in the flow direction.

Analysis of particle spreading. To quantify the spreading of particles relative to the bulk motion, we calculated the dispersion coefficient, defined as:

$$D' = \frac{\langle R^2 \rangle}{4k\Delta t},$$

(5)

where $k\Delta t$ is the time interval, $R_i$ is the position vector of bead $i$ relative to the bulk motion, defined as:

$$R_i(k\Delta t) = \int_0^{k\Delta t} [v_{i,m}(t) - V_m(t)] dt.$$  

(6)

$\langle R^2 \rangle$ is the ensemble average of the square displacements relative to the bulk motion. By making the time interval sufficiently large, $D'$ may converge to the well-known diffusion coefficient if the spreading process can be described as a diffusion process.

Once the dispersion coefficient is obtained, we can define the Peclet number ($Pe$) as (2):

$$Pe = \frac{VL}{D'},$$

(7)

where $V$ is the characteristic velocity, and $L$ is the characteristic length. $Pe$ indicates the ratio of advection to diffusion. When $Pe >> 1$, mass transport is dominated by advection, and particles move with the bulk fluid motion. When $Pe << 1$, on the other hand, mass transport is dominated by diffusion. The particles diffuse similar to Brownian diffusion and background flow has little effect. Thus $Pe$ is one of the most important dimensionless parameters in discussing mass transport.

RESULTS AND DISCUSSION

Distribution of ciliated cells. Epithelial cells and cilia were stained separately (Fig. 4). We see that ciliated cells were not distributed homogeneously in the tracheal lumen but were distributed discretely at the cellular scale. Such inhomogeneity in the ciliated cell distribution is likely to induce flow fluctuations at the cellular scale.

The ratio of ciliated cells to all cells was also calculated from the stained images. It was found that the ratio of ciliated cells was about 36.7 ± 9.6% (n = 26, where n is the number of samples). This value is similar to that reported by Francis et al. (6). We note that the ratio changes not only with individual mice but also with the observation region of a single specimen, such as the annular ligament region, tracheal cartilage region, larynx side, or lung side. Such inhomogeneity may have lead to the ratio standard deviation of 9.6%. The ciliated cell distribution is thus inhomogeneous, even at a millimeter scale, and flow in the tracheal lumen could become inhomogeneous, not only on the cellular scale, but also on the millimeter scale.

Ciliary beat. We first show typical motions of particles that are attached to the tips of cilia (Fig. 5A and Supplemental Movie S2). The major axis $e$ is calculated by image analysis (cf. Fig. 5B), and the motion of individual tracers in each $e$ direction is shown in Fig. 5C. We see that all particles oscillate with similar amplitude, which indicates that the particles are attached at similar positions, i.e., at the tips of cilia. Supplemental Movie S2 also shows that particles other than the oscillating particles flow away from the frame. This fact illustrates that particles are not attached on the cell surface or

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Fig. 4. Sample cell staining image. Cell morphologies were visualized by phalloidin staining of actin (green), while cilia were visualized by immunofluorescence analysis of acetylated-tubulin (red).

Fig. 5. Motion of tracers attached to the tips of cilia based on a same ciliary cell. A: number of tracked tracers attached to the tips of cilia (see also Supplemental Movie S2). B: trajectory of a tracer and its major axis $e$. C: motion of 4 tracers in each $e$ direction.
particles attached on the cell surface are invisible due to the thin focal plane of our confocal system.

We analyzed the BF by tracking a tracer particle attached to a cilium tip. The BF was about \(11.8 \pm 6.4\) Hz \((n = 24)\), showing good agreement with previous reports \((20, 22, 27, 28)\). The deviation of beat direction \(e\) (cf. Fig. 3B) among four cilia in each movie was about \(8.4 \pm 6.8^\circ\). We then analyzed the velocities of the effective and recovery strokes. The velocity of the effective stroke was about \(47.4 \pm 10.6\) \(\mu\)m/s \((n = 4)\), while the velocity of the recovery stroke was about \(28.6 \pm 5.8\) \(\mu\)m/s \((n = 4)\). The effective stroke was \(~1.7\) times faster than the recovery stroke. Such reciprocal strokes generate oscillation of the flow on a time scale of \(~100\) ms. Combining the flow oscillation and the spatial inhomogeneity discussed above, cilia-generated flow in the tracheal lumen fluctuates strongly relative to the bulk steady flow.

Cilia-generated flow. We first tracked tracer particle motion at \(H = 0\) \(\mu\)m in the fluorescent field, and then identified the positions of epithelial cells and cilia in the bright field at the same location. The results are shown in Fig. 6 (see also Supplemental Movie S3). We see that particles moved from the lungs side (Fig. 6, bottom) to the larynx side (Fig. 6, top), on average. The trajectories were not a straight line but erratic and unsteady. We consider that the fluctuations in the trajectories were caused by the reciprocal motion of the ciliary beat and the spatial inhomogeneity of ciliated cells.

The average velocity of all particles at each height of observation plane is shown in Fig. 7. The velocity was largest at the tip of cilia (i.e., \(H = 0\) \(\mu\)m) at \(13.8 \pm 8.6\) \(\mu\)m/s \((n = 20)\). The velocity decays rapidly as the height increases, which may be explained in terms of fluid mechanics. The cilia-generated flow can be classified as Stokes flow, in which the viscous force is dominant and the inertial force is negligible. Stokes flow often appears in cellular-scale phenomena, because the system size is small enough to neglect body forces, such as the inertial force, compared with surface forces, such as the viscous force. In a Stokes flow regime, the velocity disturbance induced by a point force near a wall decays rapidly as the distance from the wall increases \((3)\). Because the flow observed in the present study satisfies the Stokes flow condition, the velocity induced by the force of the ciliary beat also decays rapidly with height from the ciliated cells. We note that the velocity distribution can be modified considerably by the boundary conditions. If a stress-free surface exists just above the cilia, we may not observe such decay in the velocity.

The velocity field may also be affected by rheological properties of the working solution. Lai et al. \((11)\) reported that the viscosity of human mucus is about \(0.1–1\) Pa.s when the shear rate is about \(100\) s\(^{-1}\). To clarify the effect of such high viscosity, we increased the viscosity of our working solution by adding methyl cellulose 400 (Wako Pure Chemical). The effect of viscosity on the flow velocity \((H = 0\) \(\mu\)m) and the BF of cilia is shown in Fig. 8. The horizontal axis is normalized by the viscosity of the original working solution, i.e., \(1.2 \times 10^{-3}\) Pa.s, whereas the vertical axis is normalized by the velocity or the BF with the working solution. We see that both velocity and BF decrease as the viscosity is increased. However, the velocity is still \(>20\%\) even when the viscosity is increased up to 100 times. Similar tendency was also reported by Johnson et al. \((9)\). These results illustrate that the effect of viscosity on the velocity is not significant, because the cilia...
tend to generate larger force when the viscosity is increased. We should note that real mucus is not only highly viscous but also viscoelastic. This effect was not investigated here due to experimental difficulties, which is a shortcoming of this study. We hope to address it in our future studies.

To discuss the flow disturbance, the standard deviation of the velocity (SDV) of each bead was calculated. SDV_{bead,para} and SDV_{bead,norm}, defined by Eqs. 1 and 2, are plotted in Fig. 9 (n = 20 at each height). The dashed lines in the figure indicate the SDV of beads in the absence of ciliary flow (n = 19; i.e., pure Brownian motion). We see that SDV_{bead,para} was significantly larger than the average velocity (cf. Fig. 7), meaning that the tracer particles do not move constantly in one direction but move erratically, back and forth, as seen in Fig. 6. SDV_{bead,para} is considerably larger than SDV_{bead,norm}. This is because particles near the ciliated cells oscillate more in the flow direction, due to the reciprocal effective and recovery strokes. At H = 0 μm, the SDV was much larger than the dashed line of pure Brownian motion, indicating that the particles fluctuate mainly by the reciprocal motion of the ciliary beat and the spatial inhomogeneity of ciliated cells, and any effect of Brownian motion was small. At H = 20 μm, on the other hand, the SDV was only slightly larger than the dashed line, indicating that particles fluctuate strongly, also by Brownian motion. Thus the mechanism of the particle fluctuation changes with height.

Although Fig. 9 clarifies how an individual bead fluctuates relative to its time-average motion, it is also important to discuss how each bead fluctuates relative to the particle-average motion. We thus calculated SDV_{mov}, defined by Eq. 3, and plotted it in Fig. 10A (n = 20 at each height). We see that SDV_{mov} is about half of the average velocity shown in Fig. 7, indicating that each bead fluctuates strongly relative to the particle-average motion. Because the high-frequency oscillation...
tion due to the ciliary beat has little effect in $SDV_{\text{mov}}$, we think the fluctuation is caused primarily by the spatial inhomogeneity of ciliated cells. We note that the difference of animal species may affect the results in some aspects, which should be clarified in future studies.

The direction of flow was not uniform in the observed area. We thus calculated the deviation angle, defined by Eq. 4, and plotted the results in Fig. 10B ($n = 20$ at each height). The deviation angle did not differ much with height, and the height-average value was $\sim 21.1^\circ$. The fluctuation in the flow direction could be caused by inhomogeneity in the ciliary beat direction, so the ciliated cells are inhomogeneous, not only in physical space, but also in orientation space. Because the directional fluctuation was not too large, directional bulk flow could be developed, on average, from the lung side to the larynx side.

**Advection vs. diffusion.** Next, we quantified the spreading of particles relative to the bulk motion. The ensemble average of square displacements of beads relative to the bulk motion was calculated, and the dispersion coefficient $D'$, defined by Eq. 5, was obtained. The results are plotted in Fig. 11 at each height ($n = 20$ at each height). The results of pure Brownian motion (i.e., in the absence of cilia-generated flow) were also plotted in the figure for comparison. We see that the values of $D'$ converged well when the time interval was larger than $\sim 0.5$ s. In this time scale, the beads could experience several directional changes, because the BF of cilia was $\sim 12$ Hz. The beads lost directional memory and the spreading became diffusive. We can conclude that the spreading of particles, relative to bulk motion, becomes diffusive if the time scale is sufficiently larger than the beat period.

In Fig. 11, the values of $D'$ are significantly larger than Brownian diffusion. At $H = 0$ $\mu$m, for example, $D'$ is about two orders of magnitude larger than Brownian diffusion. These results indicate that the particles spread much more intensively than by Brownian motion. Because the tracer particles move with the surrounding fluid, the mechanism of the enhanced diffusion can be explained by the flow fluctuations discussed above.

Once the dispersion coefficient is obtained, we can evaluate the Peclet number ($Pe$) of the system, which is the ratio of advection to diffusion, defined by Eq. 7. Here, we take the characteristic velocity $V$ as the average velocity at $H = 0$ $\mu$m (i.e., $V = 13.8$ $\mu$m/s) and the characteristic dispersion constant at $H = 0$ $\mu$m of $D' = 1.85 \times 10^{-11}$ m$^2$/s. The characteristic length, $L$, is taken as the typical thickness of the mucous layer on the surface of tracheal lumen, and $L = 10$ $\mu$m is assumed. This is because the film thickness is usually taken as the characteristic length scale for film flow in the field of fluid mechanics. With the use of these values, the $Pe$ of the system can be derived as 7.5. Because $Pe$ is larger than unity, particles are transported more by advection than diffusion. This explains why tracer particles in the present study could be transported as a bulk even in the presence of such strong flow fluctuations.

**Conclusions.** In this study, we investigated the fluctuation of cilia-generated flow on the surface of the tracheal lumen. To understand the origin of flow fluctuation, we first measured the distribution of ciliated cells in the trachea and individual ciliary motions. The results indicated that the spatial distribution as well as the beat direction of ciliated cells were inhomogeneous.

Next, we measured the in-plane flow field at different heights using a confocal micro-PTV system. The results showed that the mean velocity and the velocity fluctuation decayed rapidly with increasing distance from the epithelial cells. Strong flow fluctuations were observed, caused by the reciprocal motion of the ciliary beat and the spatial inhomogeneity of ciliated cells. The spreading of particles relative to the bulk motion became diffusive if the time scale was sufficiently larger than the beat period. Finally, we quantified the effect of flow fluctuation on bulk flow by evaluating the Peclet number of the system. The results illustrated that ciliated cells could generate directional transport despite the large fluctuations caused by the reciprocal motion of the ciliary beat and the spatial inhomogeneity of ciliated cells. $Pe = 7.5$, indicating that the directional transport is one order of magnitude larger than the isotropic diffusion. These results are important for understanding the transport phenomena of airways on the cellular scale.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


