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Microscale imaging of cilia-driven fluid flow

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Abstract

Cilia-driven fluid flow is important for multiple processes in the body, including respiratory mucus clearance, gamete transport in the oviduct, right-left patterning in the embryonic node, and cerebrospinal fluid circulation. Multiple imaging techniques have been applied towards quantifying ciliary flow. Here we review common velocimetry methods of quantifying fluid flow. We then discuss four important optical modalities, including light microscopy, epifluorescence, confocal microscopy, and optical coherence tomography, that have been used to investigate cilia-driven flow.

Keywords

Microfluidic flow; mucus; velocimetry; particle tracking; digital particle image velocimetry; confocal microscopy; fluorescence microscopy; optical coherence tomography

1 An Overview of Ciliary Physiology

1.1 Motile Cilia Drive Fluid Flow in Multiple Organ Systems

Cilia are hair-like organelles projecting from specialized epithelial surfaces in the body. *Motile cilia* are responsible for generating microfluidic flow in a variety of organ systems (Fig. 1), and defects in cilia-driven fluid flow can have severe disease manifestations. In the lung, bacteria and particulate matter are removed by *mucociliary clearance*, the process whereby mucus, a biochemical barrier to pathogens, is transported by motile cilia out of the respiratory tract [1]. Defects in mucociliary clearance can result in recurrent pulmonary infections in diseases such as cystic fibrosis [2] and primary ciliary dyskinesia (PCD) [3]. In the oviduct, cilia drive the transport of ova [4], and defects in cilia such as in PCD can lead to infertility. A transient organ during embryonic development known as the *embryonic node* also contains cilia and is responsible for determining right-left asymmetry [5]. Patients with PCD and hence impaired nodal flow can exhibit *situs inversus*, a condition in which organs are located in the mirror position in the body. Finally, the ventricles of the brain are lined with cilia that contribute to the circulation of cerebrospinal fluid (CSF) [6].

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Intriguingly, an accumulation of CSF known as *hydrocephalus* also is associated with PCD [7].

Given the numerous sites where cilia play a critical role in the body as well as the severe disease manifestations that occur when cilia are defective, there is much interest from both clinicians and basic scientists in imaging cilia-driven fluid flow. Although cilia were described as early as 1671 by Leeuwenhoek [8], because of the complexity of studying ciliary physiology, there continues to be active research in techniques for ciliary imaging. One reason why studying cilia can be challenging arises from the fact that ciliary action takes place over a large range of length scales, and any single imaging technique can only probe a portion of those length scales. Thus, no single modality tells a complete story. Another reason is that current-generation technologies are not sufficiently fast to capture two important dynamic processes: the three-dimensional motion of individual cilia, and the three-dimensional, three-vector component cilia-driven flow field.

In this review we will first give an overview of the multiple scales of ciliary physiology. We will then discuss velocimetry as a general matter and describe common physical methods of interrogating fluid flow, including image and point-based techniques. We will lastly review the main optical modalities for imaging ciliary flow, including historically important methods (light microscopy), the current standards, (epifluorescence and confocal fluorescence microscopy), and promising emerging methods (optical coherence tomography).

1.2 Cilia Exert Their Effects Over Multiple Length Scales

On the smallest, nanoscopic scale, motile cilia are made up of microtubules, each about 25 nm wide [9]. These microtubules are arranged either in a “9+2” pattern (Fig 2, *top*) in most sites [10] or a “9+0” pattern at the node [11]. ATP-powered molecular motor proteins known as *dyneins* are attached to the microtubules [12,13]. According to the sliding filament model of ciliary motion, dynein motors walk along microtubular filaments while pulling on adjacent filaments in order to cause the cilium to beat [14]. In order to study ciliary structure at this nanoscopic scale, transmission electron microscopy (TEM) has the requisite resolution to see features such as dynein arms and microtubular structure. Sample fixation, however, is necessary for TEM preparation and thus precludes study of the dynamics of functioning cilia [15].

On a slightly larger size scale, an individual cilium extends on the order of ~10 μm in length [16]. Each cilium beats at a given tempo, known as the *ciliary beat frequency* (CBF) [17]. Because measuring CBF does not require resolving ultrastructure, but rather probing movement of the ciliary stalk, CBF can be measured by techniques with less stringent resolution requirements. The most common modalities for measuring CBF include (1) *light scattering*, focusing light on a small area of a ciliated surface and analyzing the frequency content of a backscattered signal on a photodiode [17], and (2) using *white light* or *differential interference contrast* (DIC) microscopy combined with high speed video imaging to resolve actual cilia themselves [18,19]. Other forms of microscopy such as confocal fluorescence microscopy to directly visualize cilia have also been applied [20,21].

On yet a larger length scale of approximately 10–100 μm , many cilia line a surface (Fig 2, *middle*). There may be 200 cilia on a given epithelial cell, and many cells adjacent to each other [14]. These adjacent cilia need to beat in the same direction in order to drive flow [22], and they may also beat in coordinated, *metachronal waves* to properly propel fluid [16]. Additionally, in the respiratory tract specifically, the cilia themselves are situated in the *airway surface liquid (ASL)*, the fluid that covers the epithelial lining of conductive airways (Fig 1, *left*). The ASL in turn is composed of the *periciliary layer (PCL)*, an approximately 7 μm thick fluid layer where the cilia themselves reside [23], and the *mucus layer (ML)*, the viscoelastic mucus material that sits directly above the periciliary layer [24]. The mucus layer can vary considerably in thickness and has been measured to be anywhere from 8 – 200 μm [25]. At these length scales, flow is characterized by a low Reynolds number and is often denoted as microfluidic flow [26,27]. For the purposes of this article, we denote the intermediate, microscopic scale over which cilia generate flow as *microfluidic cilia-driven fluid flow*, and the investigation of fluid flow at this scale will be the subject of this review article.

The largest length scale of ciliary action is the anatomic scale of multiple centimeters. Microfluidic flows must coalesce to create bulk transport of mucus through the conductive airways of the lungs, which range in size from sub 1 mm diameter bronchioles to the ~2 cm diameter trachea [1,28], or through the oviduct, for example, which can be ~10 cm long [29]. Although flow is still low Reynolds number, given the larger size scale of movement, we refer to this anatomic clearance of fluid (Fig. 2, *bottom*) as *macroscopic flow*. Clearance on this macroscopic scale is most often investigated through radioactive methods, e.g., by having a patient inhale radioactively labeled colloids and imaging movement with a gamma camera [15]. Other methods, such as timing transit time of saccharin in the nasal sinuses [30], fluoroscopic methods [31], or using real time CT to track tantalum discs [32,33] can also be used to assay anatomic clearance.

1.3 Optical Techniques Are Well-Suited for Microfluidic Imaging

In order to image microfluidic cilia-driven flow, then, we require techniques with 1–10 μm spatial resolution but that can also image over a relatively large field of view of at least 1 mm. Additionally, the techniques should be non-destructive to cells and tissue as not to perturb functional dynamics. Optical microscopy satisfies these criteria and is well suited towards studying cilia-driven flow at the microfluidic scale.

As shown in Fig. 3, when imaging cilia-driven fluid flow, there are three main choices to make. First, one must choose which ciliary system to investigate. Second, one must decide on the optimal optical imaging modality. And third, one must apply a specific velocimetry technique to that imaging modality. Each of these choices can be made relatively independently, and many combinations of flow system, modality, and velocimetry technique have been demonstrated in the literature. Given the many ways of imaging cilia-driven flow, familiarity with both the optical modalities, discussed in Section 4, and the various velocimetry techniques, discussed in Section 2, is crucial to selecting the proper approach for studying the ciliary physiology in question.

2. Microfluidic Velocimetry

Velocimetry refers to the measurement of the velocity of objects. Because fluids may vary in their properties over space, one way of describing fluid velocity is by describing the *fluid flow velocity field* [34]. A fluid flow velocity field, often simply called a flow field, is a type of vector field that specifies the three-component velocity $\mathbf{v}=(v_x, v_y, v_z)$ of a fluid at every point in space $\mathbf{r}=(x, y, z)$ and time t [35]. An example of a flow field, generated by nodal cilia, is shown in Fig. 4. The velocity is a vector quantity specified by the magnitude of velocity or speed defined as $s=(v_x^2+v_y^2+v_z^2)^{1/2}$, as well as the direction of flow at a specific location $\mathbf{r}=(x, y, z)$. The velocity can often be approximated as stationary or *steady-state*, in which case it does not vary in time [35]. In the context of this review, we assume that cilia-driven fluid velocities are steady-state over the measurement period.

In measuring steady-state flow, then, one must measure six parameters to specify the flow velocity at a given point: three components of velocity, and three spatial coordinates. In addition to specifying velocity as a function of spatial location, the translational diffusivity of the particles, $D(x, y, z)$, or the rotational diffusivity $D_r(x, y, z)$ [26] can also be measured as a function of spatial location. Commonly, however, it may only be possible to measure the two-dimensional flow field, in which case the velocity $\mathbf{v}=(v_x, v_y)$ is specified at $\mathbf{r}=(x, y)$.

The goal of any implementation of microfluidic velocimetry is to measure velocity throughout a fluid. Velocimetry can be performed in many ways. Although these techniques are not necessarily neatly divisible into individual categories, we have found it useful to divide ciliary flow techniques based on two criteria. Experimenters will typically choose between (1) dye versus particle-based methods, and between using (2) image information (2D or 3D) to track features versus point-wise (0D), time-varying information to deduce velocity.

2.1 Dye-Based Approaches

2.1.1 Dye Visualization—One straightforward method for visualizing flow is to inject dye into fluid and image its subsequent transport [36]. Visualization of dye movement is often enough to qualitatively differentiate the presence versus the absence of flow. In a slightly more sophisticated implementation, by recording a movie of the moving dye and calculating the displacement of the dye over some period of time, one can also estimate a linear flow speed. Similarly, in order to estimate linear flow speed, instead of imaging the dye, one can also use other chemical detection methods such as microdialysis probes located downstream of fluid flow to measure dye transport time over some distance. Grubb et al. used this method to quantitatively estimate linear flow speed in the respiratory tract [37].

2.1.2 Molecular Tagging Velocimetry—Injecting dye has limitations, as it is not always possible to evenly seed the dye, and the needle itself may interfere with flow. One alternative strategy is to pre-mix a dye throughout the fluid and then use an optical pulse to activate, bleach, or otherwise modify the dye in a specific spatial region. This process is often called molecular tagging velocimetry [38,39], and it has the advantage of being able to non-invasively generate a line of dye at a given instant. For instance, caged fluorophores can be released at a given location with a UV pulse, and the movement of the subsequent

fluorescent dye can be traced in order to provide an estimate of velocity [38]. Although not as common, such approaches have been used to image mucociliary flow [23].

2.1.3 Experimental Considerations of Dye-Based Imaging—The main criterion for a dye is that it provides imaging contrast with the rest of the fluid. That is, based on the physical property being measured by the imaging modality, the dye must have some discernible signal that differs from the surrounding fluid [36]. In the case of simple color video microscopy, this contrast can be as simple as colored dye in a clear fluid. In the case of molecular tagging velocimetry, contrast can be given by other physical parameters such as fluorescence, bleaching, or absorptivity.

Another important consideration is that dye will simultaneously diffuse as it is transported. Diffusion leads to a root mean-squared (RMS) displacement of $r^{\text{RMS}} = \langle r(t)^2 \rangle^{1/2} = (2nD \Delta t)^{1/2}$, where n is the dimensionality of the measurement, D is the diffusivity, and Δt is the interframe time [40]. This RMS displacement can manifest itself as a source of noise in quantifying flow. Thus, the average displacement due to flow should be significantly larger than RMS movement due to dye diffusion [41].

2.2 Particle-Based Approaches

Another common approach to measure cilia-driven flow is to seed and track particles in the fluid. Many freely available programs exist to automatically implement particle tracking [42]. Particle-based approaches can be divided into three related techniques based upon the density of particle seeding [43]. In the least dense case, known as *particle tracking velocimetry* (PTV), individual particles can be identified, localized, and matched from frame to frame. Increasing the density of particles to an intermediate regime makes particle matching less feasible, so instead subregions of an image are tracked in *digital particle image velocimetry* (DPIV). Finally, in the densest seeding where multiple particles occupy each focal volume in a laser-illuminated imaging system, *speckle tracking* is used to also to track subregions of an image. In the two-dimensional implementations of these techniques, particles can undergo three types of motion: (1) in-plane translation, (2) out-of-plane translation orthogonal to the imaging plane, and (3) diffusion. As we will discuss, each of these types of motion will affect the particle-based techniques.

2.2.1 Particle Tracking Velocimetry—In particle tracking velocimetry (PTV) particles are first identified and then matched from frame to frame [43]. By following a particle from image to image and knowing the frame rate of the imaging device, one can estimate the velocity $\mathbf{v}$ of each particle as $v_x(x,y,z) = \Delta x / \Delta t$, $v_y(x,y,z) = \Delta y / \Delta t$, and $v_z(x,y,z) = \Delta z / \Delta t$, where (x,y,z) is the vectorial position where the particle is tracked, $(\Delta x, \Delta y, \Delta z)$ is the displacement vector between two frames, and Δt is the interframe time. By tracking particles over an entire two-dimensional image, one can obtain a two-component velocity estimate as a function of two spatial locations, i.e. a two-dimensional flow field.

Two-dimensional PTV is the most common implementation of PTV, and two velocity components can be recovered. In order to gain three components of velocity, there are several strategies that can be employed [44], including using two perspectives (stereoscopic PIV) [45], using two planes of imaging (dual plane PIV) [46], or using holographic imaging

to digitally refocus the image [47]. These implementations, which yield three-component velocity fields in two spatial dimensions, also require more complicated optical setups. Another way of recovering three-components, now in three spatial dimensions, is to image sequential volumes. Many confocal microscopes, for example, are already equipped with volumetric imaging capabilities. Volumetric imaging, however, is typically much slower, and the limited imaging speed may limit the particle speed that can be tracked [48].

2.2.2 Experimental Considerations of PTV—Similar to dyes selected for imaging, particles should provide sufficient imaging contrast with the fluid. Contrast in particles is typically achieved through color, light scattering, or fluorescence [49]. As with dye, beads also undergo diffusion, which is manifest as an RMS displacement r^{RMS} in between each image frame. Diffusion, if unaccounted for in experimental design or data modeling, may limit velocimetry measurements but can also be measured separately (discussed below).

Another consideration of PTV is imaging speed. In two-dimensional PTV, in-plane motion is estimated as a particle is tracked. If a particle also has motion orthogonal to the imaging plane, it may transit through the imaging plane. The particle must be captured in at least two sequential frames in order to be tracked. Thus, the degree of out-of-plane motion should be estimated to determine the minimum imaging frame rate. Additionally, if a particle is accelerating, either changing in speed or directionality as it moves, it can still be tracked. Between frames, however, the particle is assumed to undergo negligible acceleration.

Finally, one must ensure that particle movement is actually reflective of the velocity of the surrounding fluid. The difference in velocity of a particle with respect to the surrounding fluid is known as *slip velocity* [49]. For spherical particles, the slip velocity $\mathbf{u}_s$ is given by $\mathbf{u}_s = d^2(\rho_p - \rho)\mathbf{a}_p / (18\mu)$. That is, the velocity difference between the fluid and particles is increased with larger particle radius d , particle acceleration $\mathbf{a}_p$, and differences in density between particle and fluid ($\rho_p - \rho$), while it is decreased with greater viscosity μ . Thus, particles with low slip velocity should be chosen. Notably, the formula for slip velocity assumes that the molecules of the fluid are much smaller than the particle itself. This assumption may be violated in the case of mucus, which consists of large polymers that may approach the size of particles and may also have a complex nanotopology [50]. Moreover, mucus may be viscoelastic, having a component of frictional and restoring force, in which case more sophisticated models are required to interpret particle tracking data [51].

2.2.3 Measuring Diffusivity with PTV—Once particles have been successfully, tracked, PTV can additionally yield estimates of viscosity of the fluid. By tracking the Brownian motion of particles and specifically calculating the mean-squared displacement of particles, one can estimate the diffusivity $D = \langle \mathbf{r}(t)^2 \rangle / (2nT)$, where $\langle \mathbf{r}(t)^2 \rangle$ is the mean-squared displacement over some time interval T , and n is the dimensionality of measurement [51]. To convert these particle measurements into estimate of fluid parameters, the diffusivity D can be related to fluid viscosity by the Stokes-Einstein relationship [52]. Typically one must have an *a priori* measurement of particle diameter to estimate viscosity from Stokes-Einstein. Consequently, these measurements are usually performed with exogenously seeded particles, the properties of which are known in advance.

The diffusivity and velocity of a particle can be simultaneously estimated from particle tracking. In this case, the particle trajectory has an average drift, which gives its velocity, in addition to random displacements, which gives its diffusivity. The mean square displacement is then no longer linear with time, as it is in pure diffusion, but now also contains an additional quadratic term from velocity [51]. As mentioned previously, the particle is assumed not to undergo significant acceleration during the interrogation period. In estimating diffusivity and velocity simultaneously, however, the interrogation period is no longer the interframe time t , but a longer period T that lasts multiple frames.

2.2.4 Digital Particle Image Velocimetry—In PTV particles must be seeded sparsely in order to accurately match individual particles in adjacent frames. By definition, then, one recovers only a sparse number of velocity measurements in each frame. Increasing the particle density becomes problematic because particles cannot always be tracked with high fidelity. In order to increase the density of sampling, subregions of the image rather than individual particles are tracked in *digital particle image velocimetry* (DPIV) [53]. In such methods, the images are divided into subregions large enough to encompass features of the image, and these subregions from consecutive frames are compared against each other using *spatial cross-correlation*. When the maximum interframe cross-correlation is found, a feature is “tracked,” and a velocity $\mathbf{v}$ can be assigned at a specific location (x,y,z) just as in particle tracking. This cross-correlation procedure then allows for mapping of flow fields even when exogenous particles cannot be applied or when the fluid has densely scattering particulate matter.

DPIV has many of the same characteristics as particle tracking. Like PTV, the number of velocity components is the same as the number of dimensions tracked unless specialized techniques are used [44]. In two-dimensional DPIV, in-plane translation of particles causes the same imaging feature to be translated in the image at a later time. Significant out-of-plane motion, as in PTV, may cause features to move through the imaging plane before they can be tracked. Additionally, if particles are undergoing significant diffusive motion, the random motion with respect to one other may cause imaging features themselves to change, limiting the ability to track features accurately [54].

2.2.5 Speckle Tracking—In the highest density of seeding, multiple particles occupy a single focal volume on average. If a laser or other spatially coherent light source (e.g. superluminescent diode) is used to illuminate the sample, then the image will exhibit a property known as *speckle*. Speckle is a high-contrast intensity noise manifest as random-appearing patterns [55]. Like in DPIV, as particles undergo translational motion in the imaging plane, speckle patterns will move in the imaging plane. In this case, spatial cross-correlation analysis, similar to that used in DPIV, can be applied to track speckle features and infer in-plane, translational motion. The application of feature tracking to an imaging system characterized by speckle is known as *speckle tracking* [43,56,57].

Like in DPIV, if the particles that generate the speckle pattern move orthogonal to the imaging plane, then the speckle patterns will change over time. As particles diffuse relative to one another, the speckle patterns will also change, making feature tracking more difficult.

Thus imaging frame rates should be fast enough to track features before they change due to diffusion or out-of-plane motion.

2.3 Point-Based (0D) Approaches

While imaging-based approaches exploit spatial information to identify particles or features and track them, flow information can also be inferred by analyzing the time-varying signal at a single (0D) point.

2.3.1 Decorrelation-Based Approaches—Another method that exploits speckle for flow quantification is *speckle decorrelation* analysis. Diffusive and translational motion through an image pixel causes the signal from that (0D) point to decorrelate over time. The rate at which decorrelation occurs depends on how fast particles translate through the pixel, as well as how quickly particles diffuse. By fitting the rate of decorrelation to a mathematical model of decorrelation, the translational speed s and diffusivity D at the pixel location (x,y,z) can be estimated. If the signal originates from the backscattered light of particles in optical coherence tomography (OCT), then the analysis is known as *dynamic light scattering (DLS)-OCT* [58,59]. Decorrelation-based OCT approaches have been applied towards imaging fluid speed and diffusion [60–62].

In contrast to speckle tracking, which uses spatial information for cross-correlation, 0D techniques rely on the *temporal decorrelation* of a signal at a single pixel. While only out-of-plane motion causes speckle patterns to change in speckle tracking, any type of translational motion will cause a signal at a single point to decorrelate. Diffusion causes the signal to decorrelate over time in both cases.

2.3.2 Fluorescence Correlation Spectroscopy and Fluorescence Recovery After Photobleaching—In fluorescent imaging systems, a temporal analysis also can be applied to a 0D time signal. For example, if the signal arises from a fluorescent molecule as imaged by a confocal microscope, then the technique is known as *fluorescence correlation spectroscopy* (FCS) [63]. In the case of FCS, decorrelation occurs due to translational or diffusive motion of dye molecules through a focus. The decorrelation of the signal can be fit to yield the translational speed s and diffusivity D [64]. In a related technique known as *fluorescence recovery after photobleaching*, the time varying signal is fit to a model function predicted by the diffusion of fluorophores into a previously photobleached confocal volume, and diffusivity and flow speed can be recovered [65].

2.3.3 Doppler Velocimetry—In *Doppler velocimetry*, the frequency shift of light due to the Doppler effect is measured to give an estimate of the axial component of flow (the component directed along the propagation path of light), denoted here as v_z , at some location (x,y,z) . As with the other 0D approaches, the frequency shift is calculated from a time-varying signal at a single point. Doppler velocimetry is commonly used in ultrasound to measure blood flow and has also found use for blood flow imaging in OCT [66–68]. Because Doppler only captures a single velocity component v_z , however, and because cilia-driven fluid flow is typically parallel to the surface (that is containing primarily v_x and v_y components of flow), using Doppler alone has limitations for ciliary imaging. Recently,

though, there has been work in developing more complex models of the OCT signal and incorporating Doppler into a framework that also includes methods for qualifying *x-y* (*en face*) flow [59,69]. These methods hold promise for quantifying cilia-driven fluid flow.

3. Model Systems of Cilia-Driven Fluid Flow

There are numerous model systems for studying ciliary flow ranging from human, to cell culture, to animal models, and each of these models has their own advantages and disadvantages [15,24,70–77]. Although a complete discussion of model systems of ciliary-flow is outside the scope of this review, we would like to highlight two model systems we believe to have been important for imaging cilia-driven fluid flow.

The first model system is the frog embryo from the *Xenopus* genus (*Xenopus laevis* and *Xenopus tropicalis*). *Xenopus* species have traditionally been used to study embryonic development [78,79]. They also, however, express cilia in multiple organ systems, and in particular a ciliated surface epithelium that is accessible with no dissection. Cilia-driven flow has been investigated in *Xenopus* epithelium, CSF circulation [80], and embryonic nodal flow [81]. Simultaneously, the many genetic manipulation techniques available for *Xenopus* are significantly faster to implement as compared to mammalian systems [82]. As such, *Xenopus* embryos are useful not only for readily imaging ciliary flow, but also for biologically manipulating ciliary flow in the context of respiratory disease, central nervous system disorders, and disorders of left-right patterning.

The second important model system is the airways liquid interface (ALI) *in vitro* models system [83,84]. ALI cultures can be derived from vertebrate cells, including mouse and human, and extracted cells differentiate into both mucus-secreting goblet cells and ciliated epithelial cells. Because ALI cultures recapitulate both the mucus and ciliary component of respiratory flow and because cells can be cultured from patients with specific mutations, ALI cultures have proven to be very important in the study of ciliary disease [85].

4. Microscale Imaging of Cilia-Driven Fluid Flow

As shown in Fig 3, there are many combinations of flow system, velocimetry technique, and imaging modality that have been used to image cilia-driven fluid flow. The choice of these categories, then, depends on several factors. One factor is the level of quantification required for the study. The various velocimetry techniques can achieve different degrees of measurement sophistication, ranging from differentiating flow versus no flow, to estimating linear flow speed, to fully quantifying the flow field. The level of quantification, in turn, depends on the physiological question to be investigated. Another consideration is that different optical imaging modalities have different advantages and disadvantages in terms of resolution, imaging speed, depth sectioning, and contrast.

As shown in Fig. 5, the bulk of microscopic ciliary flow imaging have been performed using four optical imaging techniques: traditional light microscopy, epifluorescence microscopy, confocal fluorescence microscopy, and optical coherence tomography (OCT). Historically, light microscopy was the most important. Since then, epifluorescence and confocal fluorescence have become the standard methods of measuring flow. More recently,

however, OCT has shown great promise as a new method. In this section, we review applications of these various microscopic techniques towards ciliary flow imaging and discuss the advantages and disadvantages of each.

4.1 Light Microscopy

Light microscopes were the first type of microscope to be invented, and they are still the most common microscope available today. Here we denote any standard microscope that uses white light in either a transmissive or epi-illumination mode as *light microscopy*. Light microscopy uses intrinsic scattering and absorption of light by samples to provide contrast. Light microscopy is typically one of the simplest optical setups to implement, and, perhaps due to this simplicity, was historically an important modality for imaging cilia-driven fluid flow.

4.1.1 Epithelial Imaging with Light Microscopy Imaging—The first studies of cilia-driven fluid flow were performed using standard white light microscopes to visualize movement of a dye or track moving particles in order to estimate linear flow speed. As far back as the 1870's, in experiments involving amphibian epithelial models of ciliary flow, researchers visually tracked weights and timed movement with a stopwatch to determine linear flow speed [86,87].

Even using this simple technique, early researchers made sophisticated measurements determining the amount of work that cilia could output as a function of ciliary load. It was shown, for example, that cilia increased work and oxygen consumption in response to increased loading, but only up to a certain load [88,89]. Simple dyes such as carmine and India ink were used to qualitatively assess flow speed and path in amphibian models [90,91] as well as in human (nasal cavity) and monkey, either *in vivo* or *ex vivo* [92,93]. More standardization of protocol in particle tracking occurred in the 1930's, when carbon particles or sterilized spores were used as seed particles [94–96]. It was at this time that ciliary beat frequency was also studied using light microscopy, including the use of 200 frames-per-second cinematography [91,97,98]. Fig. 6 shows examples of such ciliary studies from the 1930's, demonstrating a high level of sophistication even 80 years ago.

4.1.2 Respiratory Imaging with Light Microscopy—Since its initial implementation in the early 20th century, light microscopy has continued to be important for ciliary imaging in the respiratory tract. Light microscopy-based particle tracking and ciliary beat frequency measurements were refined in the 1950's and 60's [18,99] and were later used to study the effects of pharmacological agents [100], air pollution [101], and smoking [102] on mucociliary flow. With the increasing use of bronchoscopy, other particles such as Teflon discs or charcoal could be seeded in the tracheas of large animal model and even humans in order to image *in vivo* mucociliary clearance [103] and CBF [104], allowing further study of the effects of drugs [105] and cigarette smoking [106,107] on respiratory mucus clearance.

Even as more technically complex imaging modalities such as fluorescence microscopy and optical coherence tomography (discussed below) have come into more common use, light microscopy continues to play an important role in quantifying mucociliary flow speed. Researchers have used light microscopy to investigate physiological principles of flow and

the role of mucus [75,108–110], pharmacological and chemical signaling pathways [111–113], and the effects of infection on mucociliary clearance [114]. Moreover, there have been continued technical developments to increase contrast in light microscope-type setups [115] in order to obtain higher quality images, as well as to use of new types of metrics such as entropy to quantify mixing efficiency, as shown in Fig. 7 [116].

4.1.3 Oviduct Imaging with Light Microscopy—Light microscopy has also played an important role in investigating other types of cilia-driven fluid flow. Beginning in the 1960's and 70's, light microscopy particle tracking approaches were initially implemented in imaging cilia-driven fluid flow in the oviduct [70,117–120]. Oviduct flow results from the combination of ciliary motion, muscular contraction, and fluid production. Follow-up studies used light microscopy to elucidate the role of ciliary beating versus muscular contraction [121,122], as well as the effects of sex hormones [123] and pharmacological agents [124] on flow. Notably, unlike the lungs and sinuses, the oviducts are not optically accessible without surgery, and thus studies were typically performed on *ex vivo* human or mammal tissue. Nonetheless, light microscopy continues to be important today for studying the role of cilia in the oviduct [4], including recent work on gamete transport [125–127].

4.1.4 Nodal Imaging with Light Microscopy—The node is a transient structure that is present during a portion of embryonic life. Cilia in the node are thought to rotate in a clockwise fashion to generate a leftward flow (Fig 1 *right*), thereby creating asymmetric patterning signals [5]. Light microscopy has been used to study nodal flow [128], but it is used less commonly than epifluorescence, which will be discussed in the following section.

4.1.5 Limitations of Light Microscopy—Historically, the most common uses of light microscopy were to (1) qualitatively judge the presence or absence of flow or (2) manually track particles in order to estimate a linear flow speed, without directionality. For many experiments, this level of information sufficed to answer any relevant physiological questions. As researchers continue to investigate ciliary physiology in more detail, however, sometimes more complete descriptions of flow such as the vectorial flow field are required.

Although light microscopy can be used to measure a two-dimensional flow field, there are several limitations. First, even if two-component velocimetry can be implemented, it may not be sufficient to fully characterize flow. In applications such as the lung and the oviduct, flow is confined geometrically by the tube-like structures of the trachea or oviduct, and knowledge of two-components of flow in combination with the orientation of the tube often allows full characterization of flow. In less constrained geometries such as the CSF, node, or epithelium of amphibian models, however, flow may not be directed exactly parallel to the ciliated surface. In these cases, full characterization of flow requires estimation of three-component flow fields.

More practically speaking, two-dimensional particle tracking velocimetry can be challenging to reliably implement with light microscopy. Because light microscopy does not have optical sectioning capabilities [129], tracked particles cannot always be localized to a specific depth. Additionally, particle tracking velocimetry requires the ability to identify individual particles, which in turn requires imaging contrast between particles and the

surrounding fluid. Although standard reflective light microscopy was sufficient for early experiments, it became apparent that the mucus in the lungs itself refracted and scattered light, making it difficult to resolve tracer particles from the surrounding environment [130]. Thus, in certain cases, light microscopy may not provide sufficient imaging contrast to reliably identify particles from background.

These limitations of light microscopy were addressed by subsequent developments in optical techniques. Fluorescent-based methods overcame the imaging contrast limitation by eliminating non-specific background. Methods such as confocal microscopy and optical coherence tomography addressed the limitation in optical sectioning by (1) employing mechanisms for rejecting out-of-focus light and (2) quantitatively localizing every pixel in three-dimensional space.

4.2 Fluorescence Microscopy

Fluorescence microscopy refers to any microscopic implementation that involves excitation of fluorophores with light and formation of an image by detection of the emitted light. In the context of ciliary flow imaging, fluorescence tends to take two forms: epifluorescence and confocal fluorescence. *Epifluorescence* refers to the fluorescent excitation of a large area of a sample and the imaging of the emitted fluorescence with a camera. Like standard light microscopy, epifluorescence does not offer any depth sectioning.

Confocal fluorescence, on the other hand, refers to microscope setups in which fluorescent light is only collected from a very small focal volume. Most confocal fluorescent setups scan a microscope focus over a sample and reconstruct an image in a point-wise fashion. Because fluorescent emission is localized to a specific location, determined by the pinhole of the microscope, confocal fluorescent images are by their nature cross-sectional and provide optical sectioning.

4.3 Epifluorescence Microscopy

Epifluorescence offers extremely low background and thus high specificity imaging contrast [131]. Indeed, although mucus can be auto-fluorescent, the use of fluorescently labeled microspheres offers increased contrast between mucus and tracer when compared to standard light microscopy [21,130], and is thus attractive for implementing particle tracking and related techniques. Additionally, fluorescent techniques in general allow multiplexing of chemical labeling based on different fluorophores. Although epifluorescence does not offer depth sectioning, if particles are seeded sparsely, particle tracking velocimetry can be implemented successfully.

4.3.1 Epithelial Imaging with Epifluorescence—Epifluorescent particle tracking velocimetry was first implemented by Winet et al., to study flow in the ciliated epithelium in frog [130]. They were able to quantify vectorial flow in a mucus preparation seeded with fluorescent beads and later use depth-dependent changes in flow to calculate shear stress [132]. After the initial demonstrations, epifluorescent techniques continued to be important in studying the ciliated epithelium of amphibian models [21]. For example, one important ongoing research question in ciliary physiology is how ciliated surfaces develop directional

flow. Quantifying directional flow development requires by its very nature requires making directional, vectorial measurements of velocity. Epifluorescent imaging was used for a series of studies in *Xenopus laevis* that investigated the interplay between tissue patterning, already known to be important in embryonic life [133], and subsequent development of flow directionality [20,22,134].

4.3.2 Respiratory Imaging with Epifluorescence—Epifluorescent-based particle tracking has been very important in studying mucus flow in the respiratory tract [73]. It has been used to study the effects of mucus removal on flow [108,109], the effects of physiological stressors [135], and development of directional flow in the trachea [136]. It also became the primary method of estimating mucus velocity in bronchoepithelial cell culture [23,137–139]. Although the most common implementation of epifluorescence is particle tracking of beads, other methods such as tracing out streaks in time lapse [23] or digital particle image velocimetry in more densely seeded media [140] have also been implemented in respiratory imaging.

4.3.3 Nodal Flow Imaging with Epifluorescence—Most studies of cilia-driven fluid flow in the node have used epifluorescence in order to quantify flow [141,142]. Important studies have been performed to study the effects of knocking out specific genes such as *kif3a* [143], *kif3b* [144], and *dnah9* [145]. Epifluorescent imaging was also used to study the fluidic dynamics of the node in mammalian [41,146], zebrafish [147,148], amphibian [81], and artificial cilia systems [149].

4.3.4 Cerebrospinal Fluid (CSF) Imaging with Epifluorescence—CSF flow is thought to be driven by three factors: the production and drainage of CSF, vascular pulsations, and ciliary action [150–152]. Although it is known that patients with ciliary defects developed hydrocephalus [7,153,154], the exact degree to which cilia contribute to CSF flow remains an open question. Imaging of CSF flow with fluorescence, either by injection of beads *in vivo* [80] or by dissection of the ventricles is helping to elucidate the role that cilia play in CSF flow. Epifluorescent particle and dye tracking have since been used to investigate defects in genes such as *mdnah5* [155] and *tg737* [156], to characterize normal development of CSF flow [147,157], and to study the role of tissue patterning in the development of flow directionality [158].

4.3.5 Oviduct Imaging with Epifluorescence—Epifluorescence has also been used to image oviduct flow [125], but its use in the oviduct appears to be more limited than light microscopy. This may be due to the fact that ova themselves are often tracked in the oviduct, which minimizes the need for seeding fluorescent tracers.

4.3.6 Limitations of Epifluorescence—In epifluorescence, as in light microscopy, light emanating from out-of-focus point objects is blurred but not rejected, and light from out-of-focus particles can enter the image. Careful experiments have been able to identify in plane particles to reconstruct flow profiles [130,132], but the techniques are limited by (1) the fact that there is no genuine localization and (2) there is potentially significant amounts of defocused, out-of-plane signal preventing particle identification unless particles are very sparse. For these reasons, if vectorial flow information is desired, then confocal and OCT

imaging systems, which are cross-sectional in nature, may offer more robust depth localization for flow field estimation.

4.4 Confocal Fluorescent Microscopy

Confocal fluorescent microscopy uses a pinhole to reject out-of-plane light and offers true depth sectioning [159] with spatial resolution of less than 1 μm [129]. Because it combines the low background and molecular specificity of fluorescence with the cross-sectional nature of confocal microscopy, confocal fluorescence became a very attractive technique especially for imaging the layered airway surface liquid (ASL).

4.4.1 Respiratory Imaging with Confocal—The first use of confocal fluorescent microscopy in cilia-driven fluid flow that we could locate comes from Matsui et al. They labeled the airway surface liquid with fluorescently-tagged dextrans and used confocal microscopy to image the clearance of dye from a small area where caged-fluorophores had been released [23], a technique similar to molecular tagging velocimetry. In this study, the high resolution of confocal microscopy was leveraged in order to demonstrate a novel physiological finding with regard to the airway surface liquid. While the periciliary layer was previously thought to be non-flowing, the authors found that indeed both the mucus layer and the periciliary underwent the same rate of fluid transport.

Building on this work, researchers continued to use confocal fluorescence to image the airway surface liquid and showed, for example, the importance of defects in regulating airway surface liquid depth in cystic fibrosis [160–164]. Fluorescence microscopy also offers the major advantage that the layers of the airway surface liquid can be labeled with either antibody [85,165,166] or chemical probes [139], thus allowing differentiation of mucus layer from periciliary layer. Because of the ability to differentiate the layers, confocal microscopy was also used to study compartmental transport between the mucus and periciliary layer [138,166].

Because confocal microscopy requires point scanning, imaging frame rates are generally slower than epifluorescence. PTV requires sufficiently high frame rates needed to accurately track particles. This speed limitation of scanning confocal may explain why although particle tracking velocimetry has been implemented in mucociliary flow imaging [167], the more common implementation of confocal fluorescence appears to be in static imaging of airway surface liquid structure [137].

4.4.2 Oviduct and Nodal Imaging with Confocal—Confocal fluorescence-based particle tracking has been applied towards imaging flow in the oviduct [168,169]. With regards to nodal imaging, Supatto et al. used femtosecond laser ablation to generate fluorescent debris within Kupfer's vesicle, the analogous structure of the node in zebrafish embryo [170]. They used three-dimensional confocal imaging to generate three-dimensional, three-component velocity vector fields (Fig. 4) and showed that flow can be either directional or vortical, depending on depth. Because vorticity cannot be calculated without directional velocity components, their work underscores the importance of three-component velocity imaging.

4.4.3 Other Confocal-Based Measurements: Ciliary Beat Frequency, Fluorescence Recovery After Photobleaching, and Fluorescence Correlation Spectroscopy—Due to its high resolution and well-defined focal volume, confocal fluorescent microscopy offers the ability to measure additional quantities related to ciliary beat frequency and diffusivity. Because cilia themselves can be fluorescently labeled, using a rapid enough confocal microscope allows quantification of ciliary beat frequency by direct visualization of the cilia themselves [21,167,171]. Moreover, methods of measuring diffusivity such as fluorescent recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) require well-defined, reasonably Gaussian focal volumes to properly extract diffusion [65,172]. Indeed, FRAP has been used to interrogate the diffusivity of mucus layer versus periciliary layer in cystic fibrosis versus non-cystic fibrosis patients [173], and FCS has been used to quantify diffusivity of viruses in cervical mucus [174].

4.4.4 Limitations of Confocal—Confocal fluorescence has allowed for the elucidation of important mucociliary physiology and will likely continue to be essential to microfluidic ciliary flow imaging. Nonetheless, it suffers from a limitation arising from scanning. In most confocal setups, the signal from just a single focus is recorded at any given time. That focus is then scanned in the *xy*-plane (the *en face* plane), and the process is repeated in the axial direction (the *z*-axis, along which light propagates) to acquire an entire volume [175].

Scanning can be performed in the *xy*-plane through the use of galvanometric mirrors with no physical attachment to the sample or objective. For scanning in the *z*-dimension, however, most setups require physical scanning of either the objective or the sample, usually with a piezoelectric element. That mechanical scanning can itself perturb the sample [176]. Due to experimental considerations, the airway surface liquid must be imaged from above [130], meaning that in order to resolve the layered structure, scanning must occur in the *z*-dimension. Additionally, for volumetric particle tracking, scanning must necessarily occur in the *z*-dimension. Overall, then, axial scanning is important for ciliary flow imaging but is currently a limitation of confocal microscopy. The ability to image axially without mechanically scanning is one important advantage of optical coherence tomography.

4.5 Optical Coherence Tomography

Optical coherence tomography (OCT) [177–179] is a microscopic imaging technique that uses the low temporal coherence light to perform depth ranging. It is often considered the light-based analogue of ultrasound. Axial resolution in OCT is typically in the ~3–10 μm regime. The axial resolution is determined by the center wavelength and bandwidth of the light source, two parameters that determine the temporal coherence length of the light source. Lateral resolution in OCT is typically in ~5–20 μm regime. In comparison, conventional confocal microscopy typically has spatial resolution in the micron to sub-micron regime. The major advantage of OCT over confocal, however, is that OCT inherently parallelizes acquisition in the *z*-dimension, thus eliminating the need for mechanical *z*-scanning.

OCT has additional advantages over confocal microscopy, as well. Because objective lenses with longer focal lengths are used, typical OCT setups have a long working distance objective of ~2–3 cm, eliminating any possibility that the ciliary flow system is affected by the physical presence of the microscope objective. OCT also has the ability to interferometrically reject multiply scattered photons, allowing for relatively deep tissue imaging (~1–2 mm). Similar to non-fluorescence, reflectance confocal microscopy, OCT uses differences in backscattered light as contrast, thus allowing for the possibility of label-free mucus imaging. Finally, OCT is a coherent detection method, allowing implementation of Doppler flow imaging. Because of these advantages of OCT over confocal fluorescent microscopy, there has been a great deal of recent interest in applying OCT towards imaging cilia-driven fluid flow.

4.5.1 Optical Coherence Tomography-Based Feature Tracking—In 2011, Jonas et al., showed the first implementation of OCT in cilia driven fluid flow. In this work, they implemented particle tracking velocimetry on polystyrene microspheres driven by the ciliated skin of *Xenopus* embryos [180]. Because acquisition was inherently depth resolved, they were able to quantify the flow vector field in two dimensions (xz -plane) and show that the flow speed and directionality varied significantly as a function of the distance from the ciliated surface. These findings again reinforced the importance of both directional and depth-resolved velocity measurements.

Mucus inherently backscatters light and is composed of many particles that generate a speckle pattern. A second application of OCT towards mucociliary flow imaging involved the use of speckle tracking to calculate the two-dimensional flow field of mucus in air-liquid interface cultures [181]. Oldenburg et al. were able to show label-free, directional measurement of mucociliary flow in a depth-resolved manner. Additionally, they were able to use time-dependent signals, similar to analysis in light scattering-based approaches, in order to quantify relative changes in ciliary beat frequency (CBF) due to gaseous anesthetic. Notably, however, they were unable to measure absolute CBF, likely because spatial resolution was not sufficiently high.

4.5.2 μ OCT for Mucociliary Imaging—Most OCT systems have tradeoff between lateral resolution and axial field of view. That is, one can increase lateral resolution by increasing the numerical aperture of the objective lens to increase light focusing, but the axial field of view (FOV) decreases simultaneously. For a typical OCT system, lateral resolution may be ~10 μ m, while axial FOV is ~2 mm. Liu et al. were able to circumvent this tradeoff by using extended depth of focus imaging sources [182] in order to extend high lateral resolution (~1 μ m) imaging over a ~1 mm FOV [183]. Consequently, they were able to use their μ OCT system to image the layered airway surface liquid structure with confocal-level resolution, as shown in Fig. 8 [184]. They were able to achieve high enough resolution to image individual cilia in order to make absolute CBF measurements, while simultaneously using manual feature tracking on the mucus layer to estimate mucociliary flow. In a follow up study, they used μ OCT methods to investigate the effects of mucus loading on CBF and periciliary layer height [185]. Overall, their methods demonstrated that

OCT is capable of simultaneously imaging the layered structure of the airway surface liquid and cilia themselves simultaneously.

4.5.3 Dynamic Light Scattering OCT—As described in Section 2.3, point-based methods such as dynamic light scattering can be used to quantify the rate of decorrelation at a given location and estimate fluid flow speed [59]. Such approaches, in combination with Doppler velocimetry, have been proposed as an alternative technique for quantifying directional cilia-driven flow velocity with OCT [69]. Moreover, DLS-OCT has been used to quantify localized diffusion in the mucus of airway-liquid interface culture [50,186]. Thus, we believe that OCT holds promise in quantifying both flow and diffusion in ciliary systems.

4.5.3 Limitations of OCT—OCT has already shown excellent results in imaging cilia-driven fluid flow, but it does suffer from limitations. As previously mentioned, OCT has lower resolution than confocal microscopy. Additionally, much like light microscopy, image contrast in OCT arises from differences in light scattering, and thus it lacks the chemical specificity of fluorescence microscopy. Currently, however, there is development underway to combine OCT with more molecular specific imaging modalities such as Raman spectroscopy in order to achieve molecular specific signals [187]. As these dual modality techniques mature, they may also find a place in cilia-driven flow imaging.

5 Conclusions

Cilia-driven fluid flow is an important physiological process in multiple organ systems, including the respiratory tract, the oviduct, the ventricles of the brain, and the embryonic node. Defects in cilia-driven fluid flow have significant health ramifications, and thus quantifying fluid flow in these systems is of great importance. General methods of velocimetry can be divided into particle versus dye-based techniques, as well as techniques which rely on 2D or 3D imaging versus those which can be performed using the signal from a single point (0D).

These velocimetry techniques have been combined primarily with four optical modalities to study microfluidic flow: light microscopy, epifluorescence, confocal fluorescence, and optical coherence tomography. Light microscopy has historically been the most important modality for quantifying ciliary flow but has been partially replaced by epifluorescence and confocal fluorescence microscopy, which are the current standards. Optical coherence tomography, a high-speed, cross-sectional microscopic imaging modality, has recently shown great promise as an emerging method for microscale imaging of cilia-driven fluid flow. Advances in imaging speed and resolution will continue to drive advances in cilia flow physiology.

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Fig. 1.

Anatomic sites of ciliary action. Cilia are found in several organ systems, including (*left*) the respiratory and nasal tract, where the airway surface liquid is divided into the periciliary layer and the mucus layer, (*middle*) the ventricles of the brain and the oviduct, where no such layered structure exists, and (*right*) the transient embryonic node, in which ciliary-driven flow is thought to be generated by rotating cilia.

Length Scales of Ciliary Action

Fig. 2.

Ciliary physiology spans many length scales, requiring multiple imaging modalities. (*top*) Ciliary ultrastructure such as microtubules and motor proteins can be imaged by transmission electron microscopy (TEM) (image reproduced from [3]). (*middle*) Microfluidic flow can be imaged by optical modalities such as light microscopy, epifluorescence, confocal, and optical coherence tomography (OCT). The image here shows cilia in the ventricle as visualized by confocal fluorescence (image reproduced from [158]). (*bottom*) Anatomic clearance of mucus can be quantified by radiotracer methods (image reproduced from [188]) and computed x-ray tomography (CT).

Fig. 3. Workflow of ciliary imaging. For any given choice of flow system (*left column*) to be imaged, an optical imaging modality can be chosen (*middle column*) and combined with a choice of velocimetry technique (*right column*). Typically, the imaging modality will determine contrast and cross-sectional ability, while the velocimetry technique will determine the extent of velocimetry information recovered. CSF = cerebrospinal fluid. OCT = optical coherence tomography. PTV = particle tracking velocimetry. DPIV = digital particle image velocimetry.

Fig. 4.

Flow field generated by a single nodal cilium in a zebrafish embryo, reproduced from [170]. Here, the flow field specifies a magnitude (color) and direction of the velocity vector at every location in space, relative to epithelium (axes denoted as d - v dorsal ventral, a - p anterior posterior, and l - r left right). The flow field was estimated by employing three-dimensional, confocal fluorescent particle tracking velocimetry.

Light Microscopy		Easy Implementation High Speed
		No Depth Sectioning No Molecular Specificity
Epifluorescence		Low Background High Speed
		No Depth Sectioning
Confocal Fluorescence		Low Background Depth Sectioning
		Slow Acquisition Speed Small Field of View
Optical Coherence Tomography		Depth Sectioning Parallelized Depth Acquisition
		Speckle Artifact No Molecular Specificity

Optical Techniques for Microscale Cilia-Driven Fluid Flow

Fig. 5.

Commonly used optical techniques for investigating cilia-driven fluid flow, with advantages and disadvantages of each technique. (*top*) Light microscopy has historically been used (image showing tracheal mucus velocity measurement, reproduced from [109]). (*middle*) Epifluorescence (image showing epithelial ciliary flow, unpublished work from authors) and confocal fluorescence (image showing airway surface liquid, reproduced from [166]) are the current standard methods. (*bottom*) Optical coherence tomography (image showing epithelial ciliary flow, unpublished work from authors) is an emerging method showing promise for flow quantification.

Fig. 6.

Early works in the videomicroscopic study of ciliary physiology. Panels (a) and (b) are two consecutive frames from a 200 frames per second movie of abfrontal cilia motion in *Mytilus edulis* (blue mussel). Images reproduced from [97], published in 1930. Panels (c) and (d) are closely spaced frames from a movie that captured cilia-driven fluid flow in a rabbit trachea. Red blood cells were used as flow tracers. Images are reproduced from [189], originally produced in 1934. The scale bar in (b) is for frames (a) and (b) only.

Fig. 7. Quantification of ciliary performance using light microscopy and entropy of dye mixing, reproduced from [116]. (a) Microfluidic chip layout; (b, c) Time evolution of mixing process, false color insets show contrast-enhanced flow patterns; (d) Time-varying image entropy, a measure of flow performance; (e) Maps of local entropy, another measure of flow performance.

Fig. 8. Micro-optical coherence tomography (μ OCT) image showing airway surface liquid, reproduced from [184]. OCT can be used to differentiate the mucus layer (mu), periciliary layer (pcl), and individual cilia (green arrows) in relation to epithelial cell layer (ep).