Quantifying ciliary dynamics during assembly reveals step-wise waveform maturation in airway cells

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Abstract

Motile cilia are essential for clearance of particulates and pathogens from airways. For effective transport, ciliary motor proteins and axonemal structures interact to generate the rhythmic, propulsive bending, but the mechanisms that produce a dynamic waveform remain incompletely understood. Biomechanical measures of human cilia motion and their relationships to cilia assembly are needed to illuminate the biophysics of normal cilia function, and to quantify dysfunction in ciliopathies. To these ends, we analyzed cilia motion from high-speed video microscopy of ciliated cells sampled from human lung airways compared to primary-culture cells that undergo ciliogenesis *in vitro*. Quantitative assessment of waveform parameters showed variations in waveform shape between individual cilia; however, general trends in waveform parameters emerged, associated with progression of cilia length and stage of differentiation. When cilia emerged from cultured cells, beat frequency was initially elevated, then fell and remained stable as cilia lengthened. In contrast, the average bending amplitude and the ability to generate force gradually increased and eventually approached values observed in *ex vivo* samples. Dynein arm motor proteins DNAH5, DNAH9, DNAH11, and DNAH6 were localized within specific regions of the axoneme in the *ex vivo* cells; however distinct stages of *in vitro* waveform development identified by biomechanical features were associated with the progressive movement of dyneins to the appropriate proximal or distal sections of the cilium. These observations suggest that the step-wise variation in waveform development during ciliogenesis is dependent on cilia length and potentially outer dynein arm assembly.

Keywords: airway epithelial cell, cilia beat frequency, dynein, primary ciliary dyskinesia

Introduction

Motile cilia extend from the apical surface of cells and beat rhythmically to move fluid in the brain, airways, and oviduct (1-3). Similarly, the motile flagella which propel sperm and swimming cells (such as the biflagellate alga *Chlamydomonas reinhardtii*) oscillate with propulsive waveforms to perform their function. In the airway, hundreds of cilia on each cell must beat in coordination to achieve effective mucociliary clearance (4). Ciliary motion is the product of ATP-dependent activation of dynein motor proteins between pairs of microtubule doublets that are the major structural component of the ciliary axoneme (4). Dynein motor complex activity and structural proteins link and stabilize neighboring pairs of microtubule doublets to limit their sliding and produce bending. Mutant *Chlamydomonas* and humans with the genetic motile cilia disease, primary ciliary dyskinesia (PCD), demonstrate that failure of production or proper localization of an axonemal motor protein can result in abnormalities in beat frequency or cilia waveform (5, 6).

How ciliary proteins direct the waveform remains unresolved. Studies of model organisms have provided insight into the importance of dynein motor proteins for cilia beat and waveform by manipulating the major motor complexes called the inner and outer dynein arms, in reference to their position inside the axoneme. In 1973, Gibbons et al. showed that chemical removal of the outer dynein arms of flagella in sea urchin sperm halved the beat frequency but qualitatively appeared to have little effect on the waveform shape (7). Similarly, *Chlamydomonas* mutants lacking outer dynein arm components exhibit decreased beat frequency, while mutants lacking inner dynein arm components have altered waveform shape (8). Flagellar motion and waveform have been quantified in wild type and dynein mutants of *Chlamydomonas* to determine parameters such as shear angle, stroke width or amplitude, velocities, and forces exerted on surrounding fluid during swimming (8-10). There are few studies of human ciliary dynamics (11-13). Most of these are detailed studies of a small number of cells and have not explored potential differences between cilia motion *in vivo* and *in vitro*. Importantly, the normal human ciliary waveform and its development have not been described statistically. Moreover, changes in waveform relative to motor protein incorporation is unknown.

Ciliary waveform analysis is one approach proposed for the diagnosis of primary ciliary dyskinesia (PCD) (14). Cilia motion is typically assessed by high-speed video microscopy

(HSVM) of cells obtained by fresh biopsy, while cells cultured from patient samples have been claimed to be a more accurate tool for diagnosis of ciliopathies (14, 15). The interpretation of HSVM is limited by the fairly subjective description of waveforms, as opposed to rigorous quantitative and statistical analysis, leading to inconsistency between observers and trials (14). For example, analysis of cilia motion often employs qualitative measures such as dyskinesia scores of "abnormal", "stiff," or "asynchronous beating" (16, 17). On the other hand, studies such as those of Papon and colleagues, describe human ciliary characteristics in more quantitative terms, such as cilia length, beat angle, the distance traveled by the cilium tip, and duration of the power (or effective) and recovery strokes (12). Sears and co-authors developed tools to fit curves to videos of beating cilia to describe waveform positions, curvature, and velocity, and to then form composite waveforms (11). However, isolation and precise quantification of the motion of individual cilia remains challenging, due to the large number of cilia on each airway cell and the difficulty in resolving structures in videos.

In this study, we used HSVM to obtain quantitative, statistical measures of the human cilia waveform by (i) using ciliated cells freshly isolated from human airways and (ii) tracking waveform dynamics in primary culture human airway epithelial cells during ciliogenesis. We uncovered trends in cilia beat frequency, length, curvature, and forces between samples in the statistical characterization of the waveform over time. To investigate possible relationships between ciliary dynamics and structural changes during cilia biogenesis, we assessed dynein localization. These studies show that the cilia waveform develops slowly during ciliogenesis as dynein axonemal proteins are integrated in a multistep process to achieve features of the mature cilia present in human airways.

Materials and Methods

Details are in Supplemental Methods.

Human airway epithelial cells

The Institutional Review Board at Washington University reviewed the use of human tissues. Human tracheobronchial epithelial cells (hTECs) were isolated from surgical excess of tracheae and proximal bronchi of lungs donated for transplantation. For *in vitro* studies, hTECs were expanded on collagen-coated dishes (18, 19), then cultured on collagen-coated membranes (Transwell, Corning, Corning, NY) using air-liquid interface (ALI) conditions at first passage.

High-speed video microscopy

Ciliated airway epithelial cells were placed onto a microscope slide with medium, then covered with a No. 1.5 square coverslip (20). Clusters of ciliated cells were imaged on an inverted microscope (Eclipse Ti-U, Nikon, Tokyo, Japan) with a high-speed video camera (Ace acA1300-200um, Basler AG, Ahrensburg, Germany) at 37 °C with 240 or 360 frames per second. Cultured hTECs were imaged live on Transwell membranes to capture an *en face* view of distal tips of beating cilia. To view the cilia waveform from the side, cells were imaged on a microscope slide. Mucociliary transport was measured from recordings of microspheres applied to the apical surface of ALI cultures.

Cilia beat frequency and waveform analysis

Cilia beat frequency (CBF) was calculated across each video of *ex vivo* or *in vitro* cells using the fast Fourier transform (FFT) in Matlab (R2015a, Mathworks, Natick, MA). A parameter called "synchrony" was defined using the phase data from the FFT analysis.

Cells were selected for cilia length measurements if the full length of cilia was visible and in focus. For waveforms, cilia were manually traced from video recordings of regions typically along the edge of a raft of cells. For each cilium, one beat cycle was traced and fitted using custom Matlab code to identify the Cartesian coordinates of points and estimate the local angle and curvature (21). Statistics for local angle (θ) and curvature were obtained at each point along the arc length and throughout the beat cycle (21).

Parameters of cilia dynamics were determined using equations in Table 1. The accumulated bend was calculated as the average of the absolute value of curvature multiplied by length. To describe the amplitude of bending at each time point, the standard deviation of local angles was calculated for each cilium analyzed. This value was averaged to generate the average bend amplitude. Stroke width was defined as the maximum horizontal distance spanned by the waveform. The average force over the entire beat and the maximum force during the power stroke were also estimated (9).

Immunofluorescent staining and imaging

Cells on Transwell membranes and from tissue samples (*ex vivo* cells) were immunostained with the following primary antibodies: rabbit anti-DNAH5 (HPA037470, Sigma-Aldrich; 1:400), rabbit anti-DNAH9 (PA5-45744, ThermoFisher, Waltham, MA; 1:50), rabbit anti-DNAH11 (HPA045880, Sigma-Aldrich; 1:50), rabbit anti-DNAH6 (HPA036391, Sigma-Aldrich; 1:500), and mouse anti–acetylated α-tubulin (clone 6-11B-1, Sigma-Aldrich; 1:5,000).

Statistical analysis

Analysis for statistical significance between groups was assessed with Sigmaplot 11.0 software (Systat Software, San Jose, CA). Linear regression analyses and K-means clustering was performed in Matlab.

Results

Variation in cilia beat frequency, synchrony, and cilia length of cilia from large airways

To begin to characterize the human motile ciliary waveform, we first studied motile cilia in *ex vivo* samples obtained directly from excess tracheobronchial tissues of lungs donated for transplantation (Supplemental Table E1). Cells scraped from the airways were placed on glass slides under cover slips, and examined with brightfield HSVM (Fig. 1A, B, Supplemental Video 1). The CBF varied between samples, ranging from 3.1 Hz to 19.7 Hz with an average CBF across all *ex vivo* samples of 9.3 Hz (Fig. 1C, E); however, only minor differences in standard error of the means were observed among samples from 13 donors (Fig. 1F).

To quantify synchronization of cilia within cell clusters, the phase and overall synchrony, representing the relative time at which each cilium begins to move in a new direction, were calculated for each video (Fig. 1D, G). Selected samples of high and low synchrony were confirmed by kymography (Fig. E1, Supplemental Videos 1, 2). Synchrony averaged 0.67 across all *ex vivo* samples signifying that cilia were not beating in perfect unison (i.e., a value of 1.0).

Changes in ciliary length have been previously associated with disease and smoking history (22), suggesting length as an important factor in cilia function. In our study, the average cilia length across all *ex vivo* samples was 7.0 μm (Fig. 1H). While cilia length appeared mostly uniform within a single cell (e.g., Fig. E1A, D), length varied considerably between cells within each sample and between samples ($P<0.001$ for ANOVA on Ranks). In the tissues analyzed, differences in length were not related to donor smoking history ($P=0.28$ by *t*-test).

Changes in cilia beat frequency and length during *in vitro* ciliogenesis

To investigate changes in ciliary dynamics during cilia growth, first passage, cultured cells were imaged *en face* for up to 150 days using HSVM for CBF and phase measurements (Fig. 2A-H, Supplemental Video 3). The onset of beating cilia was observed in most preparations between ALI days 7 to 14 (Fig. 2E). The initial detected CBF exhibited a mean value of 8.3 Hz (range 3.8 Hz to 19.4 Hz) at ALI days 7 through 14, but after ALI day 14, CBF decreased and approached a fairly constant mean value of 6.0 Hz (range 2.8 Hz to 10.8 Hz), which was significantly lower than the average CBF in *ex vivo* samples (9.3 Hz, $P<0.05$ by one-way ANOVA and Dunn's method). The standard error of CBF within each video was highest in the

first week of beating and then was consistently decreased across all preparations (Fig. 2F). The average CBF measured *en face* at all days, compared to measurements in cells removed from the cultures and imaged from the side on slides (6.8 Hz) was slightly different (Fig. E2, P=0.047, Mann-Whitney rank sum test).

Higher cilia beat frequency in *ex vivo* compared to *in vitro* cells suggested that culture conditions might impair CBF. To examine the capability of cultured cells to increase CBF, the apical surface of culture membranes was stimulated by shear stress using a wash with PBS or medium (23). The CBF was transiently increased 3 to 5 hours later, regardless of the number of days in culture at ALI (Fig. 2G). This increase was significantly above baseline, but returned to baseline levels the following day. We also considered that treatment with the gamma-secretase inhibitor DAPT (used to inhibit Notch signaling and increase the number of motile ciliated cells) may affect CBF. Cells were treated for only two days between ALI days 2 and 6, which was prior to the emergence of cilia. Cilia appeared earlier in preparations that were pre-treated with DAPT compared to non-treated cultures. Cilia in the DAPT-treated preparations had a higher CBF than non-treated preparations (Fig. E3A, 9.0 Hz vs 5.9 Hz non-zero averages, P<0.001 for days 7-14 by Mann-Whitney rank sum test). DAPT added to the medium of a well differentiated preparation of cells that had attained a stable CBF (ALI day 33) did not increase CBF during two days of treatment or in the two weeks following (Fig. E3B).

Cilia length increased in the first month of *in vitro* culture and remained fairly steady but varied within and across samples (Fig. 2I). We considered that the CBF was related to cilia length, since mean length of *ex vivo* cilia was greater than *in vitro* cells (6.9 μm vs. 5.8 μm, P<0.001 by Mann-Whitney rank sum test). There was no direct correlation between CBF and cilia length from *ex vivo* or *in vitro* cells analyzed under coverslips (Fig. E2). Pre-treatment with DAPT did not affect final cilia length of well-differentiated cells (ALI day>30; P=0.08, Mann-Whitney rank sum test).

The synchrony calculated from the phase of frequency analysis varied considerably across samples (Fig. 2H) with a few preparations found to be more synchronized earlier in ciliogenesis. The mean synchrony across all cultured preparations was only 7.5% higher than observed in *ex vivo* samples (0.72 vs 0.67, P<0.001, Mann-Whitney rank sum test).

Mucociliary transport was assessed by measuring the velocity of microspheres applied to the apical surface of ALI preparations. Application of beads increased the CBF (Fig. E4),

similar to stimulation of the apical surface by washing. Regardless, bead velocity was zero when the cilia first appeared, then gradually increased over the time in culture, with a plateau approximately after ALI day 40 (Fig. 2J).

Comparison of waveform analysis of *ex vivo* and *in vitro* cilia

Cilia generate characteristic waveforms, similar to those that have been comprehensively evaluated in wild-type and mutant *Chlamydomonas* using statistical analysis of angle, curvature, and velocity of the flagellum (8, 9, 21). To quantify human cilia waveform parameters, we analyzed images obtained from HSVM of *ex vivo* and *in vitro* ciliated cells under coverslips. The images from each video were examined across multiple beat cycles to identify, then manually trace, a cilium that was visible for a full beat cycle and visually consistent with other cycles in the video (Fig. 3A, Fig. E5). Each time point of the traced waveform was fit to a curve (Fig. 3B) with the power stroke oriented in the positive X-direction. The overall waveform (Fig. 3C), the angle of each point along the curve (Fig. 3D), and the curvature of each point over time (Fig. 3E) were determined. The power stroke direction was determined by identifying the time with greatest velocity and positive curvature (see example curvature and force vectors in Fig. 3E, F) (11). The distribution of length and CBF of the cilia analyzed were representative of those observed in *ex vivo* and *in vitro* samples (Fig. 3G, H).

Changes in waveform dynamics during *in vitro* ciliogenesis

We hypothesized that the waveform would exhibit changes during cilia growth and assembly, as a function of length and ALI day. To characterize these changes, we investigated waveform parameters previously found to be sensitive to changes in flagellar waveform in *Chlamydomonas*. Such parameters consolidate the spatiotemporal position, angle, curvature, and force data from each waveform into concise, meaningful metrics of cilia motion, (21) (Figs. 3, 4). For example, the stroke width, previously measured in *Chlamydomonas* (10) and human cilia studies (12), describes the distance from start-to-finish that the tip travels in one beat. The stroke width increased linearly with cilia length (Fig. 4A) in almost a 1:1 proportion (slope of linear best-fit is 1.1).

Dysfunctional cilia are frequently described by the qualitative term “stiff” (versus “normal”) in PCD (24). To capture this qualitative difference in waveform shape, a simple,

quantitative metric was sought. First, we averaged the total curvature within each waveform (Fig. 4B), which led to values close to 0 due to the presence of positive and negative curvature in the cilia. Subsequently, we calculated the accumulated bend angle, which emphasizes the magnitude of the waveform curvature (10); this metric exhibited a linear relationship with cilia length (Fig. 4C). Finally, we quantified cilium shape by analyzing the variation of angle (θ) data without explicitly incorporating curvature. The standard deviation of θ at each time point produces large values for 'S'-shaped or 'C'-shaped cilia and low values for straight cilia (Fig. 3D, E). Averaging this standard deviation within each waveform produced the average bend amplitude (Fig. 4D), which exhibited a strong, linear relationship with cilia length (r^2=0.51).

The propulsive force generated by the cilium is one of the key functional parameters of the waveform. Force estimates, obtained using methods previously established in *Chlamydomonas,* showed that average force generated throughout the beat (Fig. 4E) and the maximum force in the power stroke (Fig. 4F) both increased as a function of cilia length. The highest values of maximum force typically occurred in *ex vivo* samples, while average force was not significantly different between *in vitro* and *ex vivo* samples.

We also sought to determine whether beat frequency was related to ciliary waveform. Unlike cilia length, CBF was not well correlated to waveform metrics (r^2 was less than 0.05 for all waveform parameters except maximum force with r^2=0.17).

This analysis of cilia dynamics during growth indicates that: (i) at each time point a longer cilium exhibits greater total bending, (ii) over a complete cycle, a longer flagellum sweeps across a longer distance, (iii) the maximum force of cilia increases as a function of length, but (iv) cilia beat frequency does not correlate with changes in ciliary dynamics.

Stages of waveform development

To compare the maturation of the ciliary waveform to cilia structure, four stages of waveform development relative to ALI day in culture were identified based on our previous observations (19) and milestones of function noted by others (11, 25). The separation into these stages was supported by similar results via K-means clustering of CBF (calculated boundaries at ALI 18, ALI 35, and ALI 67). The *Initial* stage encompasses the period when most cilia began to beat (ALI days 7-14). The *Early* stage includes the period of culture when cilia are still growing (ALI 15-30). The *Mid-stage* includes the following four weeks of culture at ALI (ALI days 31-

59), and the *Mature* stage includes periods after 2 months of culture (ALI days 60+). An example of a characteristic waveform from each stage was identified and plotted relative to dynamics of waveforms in each stage (Fig. 4G, numbers 1-5 in Figs. 4A-F). These observations suggest that waveforms analyzed at different stages have distinct quantitative features.

Spatial distribution of axonemal dynein during ciliogenesis

Having identified changes in waveform parameters during cilia maturation, we sought to determine a relationship to the localization of molecular motors within the ciliary axoneme. Large protein complexes containing dynein motors power the cilia beat, and genetic mutations affecting individual dynein proteins can alter the waveform in mature cilia leading to PCD (5). Dynein motor proteins are found in large complexes called the inner and outer dynein arms, and some distribute within specific regions of the axoneme (26, 27). Their temporal and spatial localization during ciliogenesis is not known. Immunostaining of *ex vivo* cilia for outer arm dyneins showed that DNAH5 was present along the length of the cilia, DNAH9 was typically within the distal portion, and DNAH11 restricted to the proximal region; inner arm DNAH6 was present along the entire length. These regional patterns are consistent with prior reports (Fig. 5A) (26, 28). However, the dyneins did not immediately assume these positions during cilia growth, but instead, slowly localized to these regions over time, corresponding to the identified stages of maturation (Fig. 5B, Supplemental Table E2).

DNAH5 was typically absent, or occasionally variably positioned within the cilia, during the initial stages of *in vitro* ciliogenesis (Fig. 5B). In several cells, DNAH5 was visible along basal bodies or within the cytoplasm before entering the short cilia (Initial stage). By the Early stage, and during the Mature stage, DNAH5 was present throughout the entire cilia. The location of DNAH9 was highly variable, often present throughout the full length of cilia or absent in the cilia of Initial, Early, and Mid-staged cells, before assuming a distal position in the Mature stage. In contrast, DNAH11 was absent only in the short cilia of the Early-stage, but then consistently present with the proximal region at Early, Mid, and Mature stages. DNAH6 was absent in the cilia at the initial stage, present at low levels throughout the cilium length until increasing levels at the Mature stage. DNAH6, DNAH9, and DNAH11 were also present within the cytoplasm, often in the Early-stage. These findings may imply that the slow incorporation of dynein proteins contributes to the delayed maturation of an effective waveform *in vitro*.

Relationship of cilia waveform to assembly

While dynein arm localization cannot be directly compared in the same cells that were live-imaged for waveform analysis, we grouped the localization of dynein motors and cilia motion parameters by stage of cilia maturation to observe trends and possible correlations (Fig. 6A, B). DNAH5, DNAH11, and DNAH6 localization of *in vitro* samples resembled *ex vivo* samples in the Early stage of ciliogenesis. However, *in vitro*, DNAH9 localization was not distal until the Mature stage. Relationships to waveform parameters revealed that Initial-stage cilia had higher CBF than all other stages of *in vitro* culture, but Mature-stage cilia did not reach the level of CBF of the *ex vivo* observed in samples, despite proper localization of the dynein heavy chain proteins at this time (Fig. 6C). Cilia length was significantly different between most stages of ciliogenesis (Fig. 6D). Mature, *in vitro* cilia achieve a similar length as *ex vivo* cilia associated with similar localization of all three dynein motor proteins only at this stage.

Four waveform parameters of maturing cilia exhibited a trend toward levels found in *ex vivo* samples. Stroke width generally increased during ciliogenesis but varied substantially during most cilia stages (Fig. 6E); therefore, *ex vivo* stroke width was not significantly different than the stroke width of *in vitro* samples. Average bend amplitude also increased during ciliogenesis (accumulated bend, not shown, exhibited similar trends). Initial, Early, and Mid-stage cilia had significantly lower average bend amplitude than *ex vivo* cilia while Mature cilia were nearly identical to *ex vivo* cilia (Fig. 6F). Even the earliest cilia had levels of average force that were statistically similar to Mature stage and *ex vivo* cilia, suggesting either that cilia are functional even at primitive stages of cilia assembly or that the sample size was too small to detect subtle changes in force. In contrast, the maximum force in the power stroke increased across stages of ciliogenesis with a significant difference between Initial stage cilia and *ex vivo* cilia (Fig. 6G, H). These findings indicate that there is a distinct pattern of biomechanical maturation of the human cilia waveform and suggest an association with the assembly of ciliary motor components.

Discussion

The elegant strokes of flagella and cilia have long intrigued scientists, from the first microscopic view of beating cilia in 1675 (29), to the imaging of 9+2 axoneme structure in 1949 (30). More recently, knowledge of the components of the cilium machinery and discovery of diverse genetic causes of motile cilia disease have motivated the investigation of structure-function relationships, including waveform (31). Assessing ciliary waveform dynamics remains challenging in airway epithelial cells with huge clusters of cilia. Moreover, little is known about how the waveform develops relative to cilia assembly. As a result, the variation of normal ciliary activity has been primarily addressed almost solely in terms of beat frequency, and clinical diagnosis still relies heavily on subjective terminology (32). Thus, we addressed two goals: (i) to characterize biomechanical parameters for the statistical description of the normal waveform, and (ii) to determine how these parameters change as cilia assemble. To identify quantitative descriptors of the human ciliary waveform, we analyzed metrics of shape and force during the complete cilium beat cycle. Statistical measures, in particular the average bend amplitude, captured the maturation of the waveform as cilia grew in culture and became similar to *ex vivo* cilia. The step-wise development of the waveform correlated to key time points in ciliogenesis and the incorporation of dynein arms.

We initially evaluated CBF, since it is the most commonly used metric of cilia function, in experimental conditions and for clinical assessment of disease in human samples (15, 24). CBF of *ex vivo* preparations was variable, ranging from 3.1 Hz to 19.7 Hz with statistically significant differences between several donors, suggesting that selecting a representative single value would be difficult. Variations in CBF were noted by others (24, 33, 34). It is possible that CBF varies with the anatomical locations of airway cilia; however, insufficient information is available for this conclusion. Although we made every effort to be consistent in handling samples, CBF may be sensitive to handling and environmental factors such as temperature (35) but does not appear to be affected by passage number (Fig. E6). Overall, large numbers of samples from different individuals and sites must be evaluated to make robust comparisons of CBF.

After examining hundreds of recordings from cultured cells of over 20 different donors, we found that in culture, nascent cilia had a higher beat frequency than at the later stages. After

stimulating the surface of *in vitro* preparations with microspheres or medium rinses, the CBF temporarily increased to match CBF of *ex vivo* samples; however, young cilia still maintained the highest CBF. This early elevation of CBF was consistent with measurements of elevated CBF in the trachea of newborn mice (36), which indicates that the finding is not unique to our culture system. After the early stage high CBF, the average CBF fell and remained stable during ciliogenesis, despite changes in length, waveform shape, and force generation. This finding emphasizes the central role of waveform mechanics, rather than CBF, for assessing cilia function.

We identified useful quantitative descriptors of the human ciliary waveform by comparing changes in ciliary dynamics during assembly to those observed in *ex vivo* samples. We adapted parameters developed to describe waveforms of flagella of *Chlamydomonas*, sea urchin sperm, and cilia of human airway cells (7, 21, 37). Among several metrics estimated over the period of waveform maturation, we found the parameter average bend amplitude the most useful and intuitive metric of the shape of the human ciliary waveform. This parameter was not described in prior studies but has specific value as changes in average bend amplitude correlate with increases in cilia length and the assembly of dynein motor proteins. The average bend amplitude captures both forward and backward bending, in contrast to average curvature in which positive and negative values cancel arithmetically. This parameter is independent of cilia beat frequency and appears to reflect changes in maturation, consistent with the step-wise development of bending along the length of the cilium. Clinical use of this measure will depend on improving image acquisition of airway cell waveforms.

An essential component of this work was the acquisition of cilia waveforms by manual tracing, which is much more difficult in the multiciliated human airway cell than *Chlamydomonas*. Other researchers have reported both the difficulty of manually tracing human cilia waveforms (11) and the variability of cilia motion in human donors (16). In the latter study of 13 nasal samples from healthy subjects, there was significant variation of qualitative measures of motility and significant inter-observer differences (16). We suggest replacing qualitative measures of cilia waveform shape with quantitative parameters, with the goal of improving the precision of diagnosing PCD with HSVM. Papon et al. measured 12 quantitative parameters of cilia motion (without waveform tracing) in 34 patients being evaluated for PCD (12). Their results include an overall bend angle (correlated to stroke width) and a weighted distance

traveled per second at the tip. They report high sensitivity and specificity of some metrics with respect to clinical diagnosis of PCD. However, the shape of the cilium was not described by values of curvature or bending angles at each time step. These parameters could possibly be quantified and compared at a few, key positions during the beat cycle as proposed by Sears et al. (11) to more efficiently quantify shape analysis. To speed the overall process, efforts to automate waveform acquisition are ongoing. Quinn et al. developed a computational method to automatically assess and classify cilia motion as healthy or diseased; however, this strategy uses broad classifications of movement (termed by the authors as a "black box" approach) with relatively less focus on waveform (38). Upcoming systems should endeavor to combine the benefit of automated algorithms with biophysically-relevant descriptors.

Our findings further suggest that comparing human waveforms of airway cilia with the classical waveform models derived from *Chlamydomonas* has limitations as well as implications for understanding cilia function. For example, our waveform analysis revealed that cilia beat more symmetrically than expected. Some studies have observed small differences in height between the power and recovery strokes (11, 13) that is often exaggerated in diagrams of cilia beating. More dramatic differences between the power stroke and recovery stroke (as clearly observed in *Chlamydomonas*, where the recovery stroke has a consistent, large bend) would have produced greater average force values during the beat cycle. This suggests that each cilium is less effective than a *Chlamydomonas* flagellum that uses two flagella to swim. It is consequently reasonable that coordinated motion of multiple cilia is necessary to develop directed flow in the airway. In a sense, this lack of asymmetry "gears down" the force on each cilium and means that each cilium does less work per cycle. Mathematical models of cilia beating have shown the mechanical efficiency of beating in a metachronal wave (39, 40), which is difficult to measure by mucociliary transport in cultures due to the "patches" of ciliated cells whose cilia are not oriented in the same direction.

Surveying ciliogenesis *in vitro*, we observed the birth and maturation of the waveform by tracking cilia growth over time until their structure and function approached those of *ex vivo* cells. It was unexpected that the maturation of the human waveform biomechanics was relatively slow *in vitro*, requiring two months in our culture system to achieve most of the biomechanical and structural features of the cilia studied *ex vivo* from human large airways. Distal, proximal, and full length dynein proteins in *Chlamydomonas* have also been shown to incorporate at

different times in growing flagella, but the effect on waveform not determined (41). Dynein arm assembly was associated with waveform maturation, most notably tied to a failure of DNAH9 to achieve a distal position, but likely also linked to the assembly of other axonemal proteins. It is possible that the inclusion of distal DNAH9 was particularly important since our findings, and those of others show that force is generated in the distal tip, where even a small bend has a large impact.

Our results have two important implications: First, regarding basic cilia biology, the sequential assembly of the dynein motors over time is contrary to the theory that inner and outer dynein arms are moving in large aggregates into the axoneme (42, 43). Second, as related to PCD, the failure of cilia to generate appropriate curves in the absence of complete dynein incorporation may explain the so-called "stiff" cilia observed in some PCD mutations (12, 32). One limitation of these findings is that we have not examined the impact of single, specific dynein protein deficiencies on cilia waveform; however, this is complex, as multiple dynein proteins are often absent in PCD mutations (27, 44).

In conclusion, human motile airway cilia exhibit a wide variety of waveforms and beat frequencies, reflected by substantial variation in quantitative waveform parameters. Analysis during ciliogenesis *in vitro* reveals a step-wise evolution of the maturing waveform. These parameters could be used for the quantitative characterization of cilia in the diagnosis of PCD. Additionally, these cilia waveform parameters can be compared rigorously to predictions from computational models of cilia and flagella, to assess underlying hypotheses of dynein activation and illuminate the biophysics of ciliary motility.

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Table 1. Summary of waveform parameters and equations to characterize each waveform over one beat cycle.

Waveform Parameter Name (Reference)	Description	Equation
Stroke width (10, 12)	Maximum distance spanned by the waveform in the X-direction	$\max(x) - \min(x)$
Curvature (21)	Derivative of the angle with respect to arc length	$C = \frac{\partial \theta}{\partial s}$
Avg curvature (10)	Average curvature	$\text{avg}(C)$
Accumulated bend (10)	Average, absolute value of curvature multiplied by length (L)	$\text{avg}(\lvert C \rvert) * \text{L}$
Avg bending amplitude (This report)	Standard deviation of angles at each time, averaged over a cycle	$\text{avg}(\text{SD}(\theta))$
Avg force in X-direction (9)	Average of the cilium force in X-direction	$\text{avg}(F_X)$
Max force in X-direction (9)	Maximum value of the force in X-direction	$\max(F_X)$

Abbreviations: Avg, average; Max, maximum; Min, minimum.

Figure Legends

Figure 1. Variability of cilia beat frequency and parameters of *ex vivo* cilia motion. (A) Airway epithelial cells from tracheobronchial segments of human lungs donated for lung transplantation were placed under a cover slip for high-speed video microscopy (HSVM). (B) Representative video frame of ciliated cells imaged by HSVM (see Supplemental Video 1). (C) Cilia beat frequency (CBF) and (D) phase calculated using the fast Fourier transform (FFT) of the video in B. Color bar in C indicates CBF (average value of 10.8 Hz in this video); color wheel in D indicates the normalized phase (~time delay) of local cilia motion (synchrony parameter value for this video is 0.74). (E) Variation of average CBF in samples (S1-S13, n=13 donors, n=95 total videos). Each point represents the mean of all cilia motion in each video. Average across all samples is 9.3 Hz. (P<0.001, one-way ANOVA. Significant differences exist between seven pairs by Holm-Sidak method) (F) Standard error of CBF within each video for a sample. (G) Ciliary beat synchrony calculated as 1 minus the circular standard deviation of the normalized oscillation phase within each video. Mean synchrony of all values is 0.67. (P=0.15 by one-way ANOVA.) Samples excluded due to short video file length (*). (n=73 total videos). (H) Variation in cilia length in samples. Length was measured in multiple cells for each video. Each color indicates raw data for a single video from a sample (n=1349 measurements, 62 videos). Dunn's post-hoc test indicates significant differences between 16 pairs, (P<0.001, ANOVA on ranks.) Black bars indicate mean values in E, G, and H. Scale bar in B is 10 μm.

Figure 2. Progression of cilia beat frequency and length during *in vitro* ciliogenesis. (A) Airway epithelial cells isolated from human tracheobronchial segments were expanded on tissue culture plates, then differentiated on supported membranes using air-liquid interface (ALI) conditions. (B) Representative frame of cilia on cultured cells imaged *en face* with high-speed video microscopy (HSVM) at ALI day 100 (see Supplemental Video 3). (C) Cilia beat frequency (CBF) and (D) phase were calculated with the fast Fourier transform (FFT) of the video in B. Color bar in C indicates CBF (average of 6.1 Hz in this video); color wheel in D indicates the normalized phase (~time delay) of the cilia motion (average synchrony value of 0.68 in this video). (E) Variation in average CBF for each video during the indicated day of differentiation. Each point represents the average CBF of cells from the entire field. Average of all beating videos is 6.5 Hz. (n=20 donors, n=505 total videos with 362 average values greater than 0 Hz) (F) Standard error of each video from E (n=20 donors, n=362 total videos). (G) The effect of apical medium wash on CBF prior to and post rinsing. Average CBF values for pre-wash, 3-5 h post-wash, and the next day are 7.5 Hz, 12.8 Hz, and 6.6 Hz respectively. (n=9 unique donors tested 3 times with 1 week between each trial; $P<0.001$ across wash times, P=0.008 across ALI days, two-way ANOVA. Holm-Sidak method indicates significant differences between pre-wash and 3-5 h post-wash but no significant difference between pre-wash and 1-day post-wash. n=428 total videos) (H) Variation in synchrony throughout ciliogenesis ($r^2=0.07$, n=276 total videos, n=15 donors). (I) Cilia length in cultured cells sampled from the membrane and imaged under a coverslip as in Figure 1. Average of all values is 5.8 μm (n=17 donors, n=125 videos). (J) Mucociliary transport. Mean velocity of microspheres on cell surface for each video (n=10 donors, n=126 total videos). Scale bar in B is 10 μm. Each color represents the same unique donor in E, F, H, and I. A separate cohort of donors was studied in J.

Figure 3. Technique for analysis of waveforms in *ex vivo* and *in vitro* cilia samples. Cilia waveforms were analyzed from frames of HSVM of *ex vivo* and *in vitro* cells under coverslips. (A) Representative example of a manually-traced cilium at two time points (8 ms and 65 ms) from frames of a beating cilium in an *ex vivo* sample. (B) Traced cilia were fitted with a polynomial function describing angle, θ, in terms of the arc length, s. The tracing was oriented with the power stroke moving in the positive X-direction and the recovery stroke in the negative X-direction. (C) Example of a traced waveform of the *ex vivo* sample shown in (A) for one full beat cycle. Color indicates time in the beat cycle. (D) Angle, θ, and (E) curvature data, $\frac{\partial\theta}{\partial s}$, for the waveform in panel C. Curvature is shown on two example cilia with the bending amplitude (standard deviation of θ) indicated below. (F) Waveform with arrows indicating the local forces exerted by the cilium (arrows: purple=recovery stroke forces, green=power stroke forces, length is proportional to magnitude of force). Only one-half of the time points and one-tenth of the arrows are visualized. (G, H) Features of all traced waveforms from *in vitro* and *ex vivo* samples indicating the spectrum of (G) cilia lengths and (H) beat frequencies represented in the analyzed waveforms (n=28 waveforms).

Figure 4. Waveform analysis of cilia during ciliogenesis. Ciliary waveforms from *ex vivo* (filled black circles, n=7) and *in vitro* (filled colored circles, n=21) cell samples were quantified using the indicated waveform parameters; results were analyzed by linear regression (dashed line). (A) Stroke width (see inset) vs. cilia length (slope=1.1 μm, r^2=0.46). (B) Average curvature of each waveform vs. cilia length (slope=0.23 deg/μm, r^2=0.01). (C) Accumulated bend for each waveform (slope=10.1 deg, r^2=0.42). (D) The average bend amplitude (standard deviation of θ, see inset) of waveforms from *in vitro* and *ex vivo* samples as a function of cilia length (slope=2.2 deg, r^2=0.51). (E) The average force in the X-direction relative to cilia length (slope=0.0052 pN, r^2=0.25). (F) The maximum force in the power stroke direction relative to cilia length (slope=0.30 pN, r^2=0.38). (G) Summary of representative waveforms during four stages of ciliogenesis and an *ex vivo* example. Color bar indicates the relative stroke direction over time (t). Numbers 1-5 indicate the location of the representative waveforms in the above plots. Scale bar is 2 μm.

Figure 5. Spatial distribution of axonemal dynein during ciliogenesis. Ciliary location of dynein heavy chain of the outer arm (DNAH5, DNAH9, DNAH11) and inner arm (DNAH6) in (A) *ex vivo* and (B) *in vitro* cell samples. Representative images from cells immunostained using cilia marker acetylated a-tubulin (green) and the indicated dynein axonemal heavy chain (magenta). Region of co-localization is white. Nuclei were stained with DAPI (blue). Shown are two examples of *ex vivo* cells and from the *in vitro* preparations, two examples of Initial; singles examples of Early-stage, Mid-stage, and Mature stages. Orange brackets and arrows indicate the position of the dynein within the cilia. (n=18 *ex vivo* samples from 8 donors, n=67 *in vitro* samples from 12 donors). Scale bars are 10 μm.

Figure 6. Relationship between cilia waveform and assembly. (A) Cartoon of cells during stages of cilia growth observed *in vitro*, indicated by ALI day, and compared to *ex vivo* cells. (B) Trends of dynein localization from fluorescence imaging at each stage of cilia growth. (C, D) Changes in CBF and cilia length corresponding to stages in A. Box and whisker plot at each stage; box bounds indicate 25th and 75th percentiles. Black marker (+) indicates mean values; red marker (+) indicates outliers (points outside 1.5 times the box height). (C) CBF data from Fig. 1E and Fig. 2E were binned based on the cilia stage. (P<0.001, ANOVA on ranks. Dunn's test indicates 6 pairs with significant differences. 'a' indicates a significant difference to Initial cilia, and 'b' indicates a significant difference to e*x vivo* cilia.) (D) Cilia length data from Fig. 1H and Fig. 2I grouped by cilia stage. (P<0.001, one-way ANOVA. Holm-Sidak method indicates 8 pairs of significant differences. NS indicates pairs that are not significantly different.) (E-H) Waveform parameters from Fig. 4 grouped by stage including (E) Stroke width, P=0.042 for one-way ANOVA. Holm-Sidak test indicates a significant difference between Initial and Mature cilia stroke width (*). (F) Average bend amplitude, P<0.001 for one-way ANOVA. Holm-Sidak test indicates 5 differences ('c' indicates a significant difference to Mature cilia, and 'd' indicates a significant difference to *ex vivo* cilia). (G) Average force, ANOVA on ranks indicates no significant differences, P=0.187. (H) Maximum force, P=0.006 for ANOVA on ranks. Dunn's test indicates a significant difference in maximum horizontal force between Initial and *ex vivo* cells (*). Black bars indicate mean values.