Methods for the assessment of human airway ciliary function

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Airway ciliary function analysis underpins PCD diagnostics and *ex vivo*/*in vitro* mucociliary clearance studies. It is an important measure of airway culture model integrity in health and after microbial/viral infections or airway drug therapies. https://bit.ly/3EXsG5J

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Introduction

Motile cilia (figure 1a) are organelles that extend from the apical membranes of differentiated epithelial cells [1]. Cilia waveform is coordinated by their ultrastructure and microtubule arrangement; *e.g.* "9+2" cilia (figure 1b) perform metachronal "whip-like" movement, "9+0" embryonic nodal cilia have rotational movement. Axonemal dyneins are adenosine triphosphate (ATP) driven, mechano-chemically regulated, motor proteins responsible for cilia motility. Over 200 "9+2" cilia per cell line mucosal surfaces of several body sites (*e.g.* airway, reproductive oviducts, brain ependyma) where mucociliary clearance (MCC) and fluid flow is required. Airway MCC is critical for host defence, removing inhaled pathogens, particulates and mucus (figure 1c).

Primary ciliary dyskinesia (PCD) is mostly an autosomal recessive condition (except rare autosomal dominant and X-linked cases) affecting approximately 1:7554 individuals [2]. MCC impairment causes recurrent airway infection, chronic wet cough, progressive irreversible lung damage, bronchiectasis and mucus obstruction [3]. Mutations in over 50 different genes cause different cilia abnormalities, some causing a worse prognosis than others. Genetic testing (sequencing a panel, whole exome or whole genome) can identify up to 70% of PCD [4, 5]. Variants of unknown pathogenicity require functional and structural diagnostic tests to elevate the status of these variants or discount them. Secondary ciliary dyskinesia (SCD) is not inherited or caused by structurally defective cilia. Airway diseases such as idiopathic bronchiectasis [6], COPD [7] and asthma [8] incur SCD due to cellular damage. Thick mucus in cystic fibrosis can prevent normal ciliary waveform [9].

Airway MCC *in vivo* has been studied with clearance time for dye [10], saccharin and/or charcoal [11, 12] or radioactive isotopes [13]. Recently, *in vivo* MCC has been advanced through optical coherence tomography [14, 15]. Ciliary function can be studied using ciliated single cell organelles, *e.g.* *Chlamydomonas* [16], small organisms, *e.g.* planaria [17], nasal/tracheal brushing biopsies, or surgical explants from larger animals, such as mouse [18, 19], rat, guinea-pig, rabbit, dog, pig and cow [20], as well as human *ex vivo* and *in vitro* cell culture samples.

State-of-the-art ciliary function testing

Airway cilia sampling

Thin layers/clusters of epithelial cells are preferable for live cilia imaging. Nasal epithelial cell samples may be acquired by curette [21] or brushing biopsy [22, 23]. Tracheal or bronchial epithelial cell samples can be obtaining by brushing biopsy during bronchoscopy [24]. Excessive nasal mucus that impedes access to ciliated epithelium can be reduced by nasal douching with saline prior to sampling [22]. Excess mucus may be reduced post-sampling by adding additional medium, agitating and centrifuging to re-pellet ciliated cells. Air–liquid interface (ALI) cultures may be washed in processing to reduce mucus build-up before use [22]. Inhaled anaesthetics depress ciliary function [25, 26], therefore anaesthetic or topical nasal

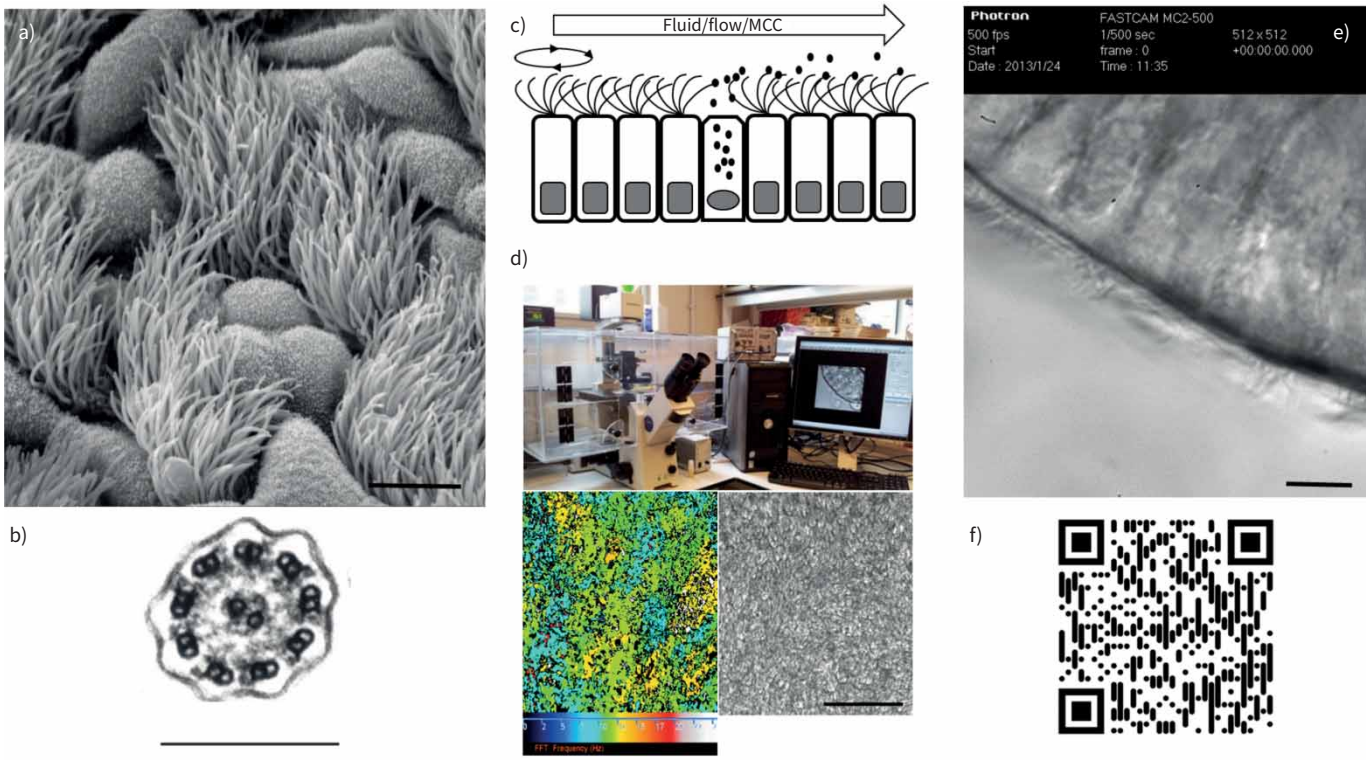

FIGURE 1 Microscopic cilia assessment by various methods. a) Scanning electron microscopy of ciliated airway epithelium (scale 10 µm). b) Transmission electron microscopy of airway cilia in cross-section (scale 200 nm). c) Diagram demonstrating ciliated airway epithelium and goblet cell secreting mucus into the peri-ciliary layer, with direction of ciliary movement and mucociliary clearance (MCC). d) High-speed video microscopy analysis (HSVA) (at 37°C) of air–liquid interface (ALI) cultured airway epithelium on Transwell insert (20× objective), with fast Fourier transform "heat-map" analysis of ciliary beat frequency in Fiji ImageJ (scale 100 µm). e) Image of ciliated nasal brushing biopsy taken by HSVA (100× objective; scale 10 µm). f) QR code for representative HSVA video data before and after ALI culture to remove secondary dyskinesia and verify primary ciliary dyskinesia.

agents that potentially modify ciliary function should be avoided or washed out before baseline measurements. Sampling and infection damage increase SCD [27, 28]. Donors should be 4–6 weeks free of infection, sampling methods need to be practised, and cell culture considered to maximise sample quality and ciliary function interpretation [29, 30].

Ciliary beat frequency analysis

Ciliary beat frequency (CBF) is a quantitative measure of cilia speed. CBF is environmentally dependent (*e.g.* temperature, pH, medium type, chemical additives, mechanical vibration and time from sampling) and varies by sample, donor or organism. CBF is reduced on single cells, therefore measurements from intact cell clusters are representative [31]. Chilvers and O'Callaghan[32] demonstrated that different methods of CBF measurement are not interchangeable. The photomultiplier [33] and photodiode [34] methods both significantly under-recorded mean CBF compared to digital high-speed video with manual analysis, under standardised 37°C and pH 7.4 conditions (n=200 measurements per method across 20 donors) [32]. The relationship between CBF and temperature is sigmoidal (linear between 7°C and 32°C) [35]. Testing at unregulated ambient temperature or below 32°C risks increased CBF variability and reduced reproducibility. Local normal/reference CBF ranges need to be established with specific methods and equipment, and are not transferable across centres.

Advances in ciliary beat pattern analysis

High-speed video microscopy analysis (HSVA) facilitates CBF and ciliary beat pattern (CBP) and waveform analysis, in real-time and slow motion, adding invaluable evidence. A light microscope (inverted or upright) requires a long working distance, high numerical aperture (plan apochromatic) and magnification objective lens (*e.g.* 63× and above). Lower lens magnifications or lower camera digital resolutions (Southampton uses a Photron FASTCAM MC2 with 512×512 pixel resolution) risk poor

resolution image data that is more challenging and often impossible to interpret, *e.g.* for subtle reductions in ciliary beat amplitude and flexibility. A high-speed video camera should be able to image upwards of 120 frames per second (fps), ideally 500 fps, to acquire enough frame-by-frame CBP detail. For example, if cilia moving at 20 Hz [22, 23, 36] were recorded, 25 frames per ciliary beat would be taken at 500 fps, opposed to only six frames if recorded at 120 fps.

For PCD diagnostics, CBP analysis is conducted using software that facilitates slow-motion playback (30–60 fps recommended [30]). CBP analysis is mostly subjective with arbitrary measures of side and top views [36] (see [22] with supplementary videos at https://zenodo.org/record/4115168; figure 1e and f). It is imperative that investigators develop ciliary function analysis expertise to conduct reproducible data. The European Respiratory Society Clinical Research Collaboration BEAT-PCD (http://beat-pcd.squarespace.com), the European Reference Center for Rare Lung Diseases (ERN-LUNG) (https://ern-lung.eu/) and the UK Cilia Network (https://www.cilianetwork.org.uk) provide training and access to researchers and clinicians with expertise in cilia structure and function. The UK PCD diagnostic centres have shared standard protocols and analyse ciliary function (after sample equilibration at the microscope, heated to 37°C) within hours of sampling to maintain sample integrity [37], also enabling same day results for PCD-likely cases [36]. We [38] and others [39] have reported that cooling cilia from 37°C to ambient temperature caused the abnormal ciliary waveform in several PCD samples to become less evident, which could risk PCD misdiagnosis if transmission electron microscopy is normal, or testing resources/expertise are limited.

As well as maintaining a stable sample pH 7.4 (*e.g.* Hanks' balanced salts, HEPES buffering or 5% CO_2 equilibration), addition of a broad-spectrum antibiotic (*e.g.* penicillin-streptomycin) is advisable to inhibit bacterial growth. It is also important to avoid mechanical–vibrational cilia stimulus and consider how sample additives such as ATP, calcium, anaesthetics or mucolytics may affect sample health and ciliary function. If the effect of drug treatment on ciliary function is being assessed, it is important to consider pre-treatment baseline and temporal variability of ciliary function with drug action and half-life. Time-lapse coupled HSVA can facilitate continuous temporal cilia analysis of multiple experimental conditions in different wells, from specific x, y, z locations offering data repeatability [40, 41]. The caveat of this method is that it relies on the ciliated cells remaining *in situ*, *e.g.* nasal brushing samples grown on plastic or ALI cultures on membranes, rather than free floating spheroids able to move out of position.

HSVA recordings can be *post hoc* analysed to determine mean CBF across a whole field of view or within a region of interest. Manually calculated, CBF (Hz) is equal to the recording frame rate (fps) divided by the number of frames for one ciliary beat (averaged from 6–10 separate areas) [36]. CBF and percentage area of ciliary movement can also be measured computationally, *e.g.* Sisson-Ammons Video Analysis (SAVA), ciliaFA [42], Fiji ImageJ with fast Fourier transform (FFT) custom plugin [22] (figure 1d) or CiliarMove [43], to name common software platforms. When there are mixed beat pattern phenotypes (*e.g.* static and hyperfrequent twitching [4, 18, 36] with high variation in CBF in PCD, a mean CBF is not representative. When subtle beat pattern PCD abnormalities occur in PCD (*e.g. HYDIN* mutation cases) often with normal CBF [5, 36, 44], then only cilia waveform assessment is diagnostically informative. ALI culture can be employed to regrow cilia *in vitro* to help identify PCD and reduce patient recall, by removing confounding secondary health/infection issues [22, 23, 45, 46]. Marthin *et al.* [47] described how three-dimensional organoids (spheroids) can be cultured from nasal brush samples by preventing cell attachment with repeated agitation during the initial 4 h of incubation. Single spheroids can be immobilised by flattening between glass slide and cover slip, permitting HSVA on side views of the spheroids. HSVA is a staple validation tool for cilia culture models, *e.g.* employed to determine CBF of the advanced "airway-on-a-chip" ALI cultures amongst other tests [48]; airway epithelial cells are differentiated at an ALI under continuous perfusion *via* a basolateral microchannel.

HSVA is an important diagnostic and research tool in the field of PCD [4, 49] and when conducted by experts has good accuracy to identify PCD patients [36]. Whilst HSVA has good diagnostic accuracy it is not available at every diagnostic centre due to limited resources [50]. A major challenge for HSVA remains the lack of unified language or quantitative measures to describe CBPs for PCD or SCD [51, 52].

Quantitative ciliary beat pattern analysis

Novel quantitative parameters can track the position of a single cilium over an entire cycle of beating. The position of the cilium base as well as the positions of the cilium tip at the start and the end of the active stroke are measured in a series of frames, but require repeating on at least 10 spatially distant individual cilia (per sample) to be representative. The distance travelled by the cilium tip or the angle described by the cilium may be calculated through trigonometry [53, 54]. The entire cilium position from base to tip can

be "curve-fitted" providing data on waveform in space and time. Waveform shape, curvature and bend amplitude can be mathematically described, and kinematics can be applied to measurements of flow velocity [55–59]. Lack of commercial software prevents widespread application of these quantitative mathematical descriptors of CBP.

MCC analysis

The mucociliary interface consists distinctive gel-like layers, a watery periciliary ciliary layer (PCL) and a soluble transporting mucus layer. Cilia move asymmetrically within the PCL to create flow (at low Reynolds number, where viscous forces overcome inertial effects). The transporting mucus layer contains two major heavily glycosylated mucins, MUC5AC and MUC5B, and many other globular proteins, produced by mucus-secreting goblet cells [60]. Mucins enable dynamic mucus attachment to cilia to facilitate MCC to protect the airway [61]. MCC, or cilia driven flow, can be quantified by dynamically imaging the transport of cellular debris, synthetic microbeads (1 to 3 µm, with or without fluorescence) or fluorescent dyes across the surface of tissue explants or ALI cultures when added to the sample's media. The benefit of using uniformly shaped microbeads opposed to tracking debris, particularly with added fluorescence, is the ease of particle identification by microscopy and for velocimetry analysis. It is important to measure the distance (in x, y, z plane) between cilia and microbead or debris item when tracking the velocity, as mucociliary flow rate decreases with increased distance from the cilia [62]. Differentiated epithelial cell ALI cultures develop mucus vortices as an artefact of their environment [63]. Microfluidic devices to direct fluid flow [64] or culture membrane modifications, such as collagen substrate patterning [65], help polarise epithelial cell growth which promotes unidirectional cell–cilia alignment. No specific studies have assessed the quality of ciliary function in these instances.

Summary

State-of-the-art ciliary function analysis of airway epithelium underpins PCD diagnostics and also enables understanding of how cilia move in health or when temporarily damaged. Ciliary function analysis can underpin investigations of epithelial cell differentiation, integrity, disease, infection and drug therapy evaluation in airway culture models [41, 66–68].

Ciliary function assessment through HSVA is predominantly carried out manually, and requires expertise to meaningfully assess CBP. Quantitative cilia analysis could replace non-standardised, subjective assessment to better study subtle CBP changes; the lack of commercially available software hinders this. Artificial intelligence, used for the first time in the transmission electron microscopy assessment of cilia for PCD diagnostics [69], could potentially quantify cilia waveforms and model ciliary function. If developed, such platforms will enable future standardisation of testing and time-saving.

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References

1 Mitchison HM, Valente EM. Motile and non-motile cilia in human pathology: from function to phenotypes. *J Pathol* 2017; 241: 294–309.
2 Hannah WB, Seifert, BA, Truty, R, *et al.* The global prevalence and ethnic heterogeneity of primary ciliary dyskinesia gene variants: a genetic database analysis. *Lancet Respir Med* 2022; 10: 459–468.
3 Munkholm M, Mortensen J. Mucociliary clearance: pathophysiological aspects. *Clin Physiol Funct Imaging* 2014; 34: 171–177.
4 Lucas JS, Davis SD, Omran H, *et al.* Primary ciliary dyskinesia in the genomics age. *Lancet Respir Med* 2020; 8: 202–216.
5 Shoemark A, Rubbo B, Legendre M, *et al.* Topological data analysis reveals genotype-phenotype relationships in primary ciliary dyskinesia. *Eur Respir J* 2021; 58: 2002359.
6 De Iongh RU, Rutland J. Ciliary defects in healthy subjects, bronchiectasis, and primary ciliary dyskinesia. *Am J Respir Crit Care Med* 1995; 151: 1559–1567.
7 Thomas B, Koh MS, O'Callaghan C, *et al.* Dysfunctional bronchial cilia are a feature of chronic obstructive pulmonary disease (COPD). *COPD* 2021; 18: 657–663.

8 Thomas B, Rutman A, Hirst RA, *et al.* Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma. *J Allergy Clin Immunol* 2010; 126: 722–729.e2.
9 Haq IJ, Gray MA, Garnett JP, *et al.* Airway surface liquid homeostasis in cystic fibrosis: pathophysiology and therapeutic targets. *Thorax* 2016; 71: 284–287.
10 O'Neil LM, Jefferson ND. Direct visualization of laryngeal mucociliary clearance in adults. *Ann Otol Rhinol Laryngol* 2019; 128: 1048–1053.
11 Andersen I, Camner P, Jensen PL, *et al.* A comparison of nasal and tracheobronchial clearance. *Arch Environ Health* 1974; 29: 290–293.
12 Rutland J, Cole PJ. Nasal mucociliary clearance and ciliary beat frequency in cystic fibrosis compared with sinusitis and bronchiectasis. *Thorax* 1981; 36: 654–658.
13 Walker WT, Young A, Bennett M, *et al.* Pulmonary radioaerosol mucociliary clearance in primary ciliary dyskinesia. *Eur Respir J* 2014; 44: 533–535.
14 Jonas S, Bhattacharya D, Khokha MK, *et al.* Microfluidic characterization of cilia-driven fluid flow using optical coherence tomography-based particle tracking velocimetry. *Biomed Opt Express* 2011; 2: 2022–2034.
15 Gil DA, Sharick JT, Mancha S, *et al.* Redox imaging and optical coherence tomography of the respiratory ciliated epithelium. *J Biomed Opt* 2019; 24: 1–4.
16 Wan KY, Goldstein RE. Coordinated beating of algal flagella is mediated by basal coupling. *Proc Natl Acad Sci USA* 2016; 113: E2784–E2793.
17 King SM, Patel-King RS. Planaria as a model system for the analysis of ciliary assembly and motility. *Methods Mol Biol* 2016; 1454: 245–254.
18 Lucas JS, Adam EC, Goggin PM, *et al.* 11/DNAH11: a mouse model of PCD. *Hum Mutat* 2012; 33: 495–503.
19 Gui M, Farley H, Anujan P, *et al. De novo* identification of mammalian ciliary motility proteins using cryo-EM. *Cell* 2021; 184: 5791–5806.e19.
20 Joki S, Saano V. Ciliary beat frequency at six levels of the respiratory tract in cow, dog, guinea-pig, pig, rabbit and rat. *Clin Exp Pharmacol Physiol* 1994; 21: 427–434.
21 Olin JT, Burns K, Carson JL, *et al.* Diagnostic yield of nasal scrape biopsies in primary ciliary dyskinesia: a multicenter experience. *Pediatr Pulmonol* 2011; 46: 483–488.
22 Coles JL, Thompson J, Horton KL, *et al.* A revised protocol for culture of airway epithelial cells as a diagnostic tool for primary ciliary dyskinesia. *J Clin Med* 2020; 9: 3753.
23 Hirst RA, Jackson CL, Coles JL, *et al.* Culture of primary ciliary dyskinesia epithelial cells at air-liquid interface can alter ciliary phenotype but remains a robust and informative diagnostic aid. *PLoS One* 2014; 9: e89675.
24 Low PM, Luk CK, Dulfano MJ, *et al.* Ciliary beat frequency of human respiratory tract by different sampling techniques. *Am Rev Respir Dis* 1984; 130: 497–498.
25 Raphael JH, Strupish J, Selwyn DA, *et al.* Recovery of respiratory ciliary function after depression by inhalation anaesthetic agents: an *in vitro* study using nasal turbinate explants. *Br J Anaesth* 1996; 76: 854–859.
26 Feldman KS, Kim E, Czachowski MJ, *et al.* Differential effect of anesthetics on mucociliary clearance *in vivo* in mice. *Sci Rep* 2021; 11: 4896.
27 Dixon M, Shoemark A. Secondary defects detected by transmission electron microscopy in primary ciliary dyskinesia diagnostics. *Ultrastruct Pathol* 2017; 41: 390–398.
28 Shoemark A, Boon M, Brochhausen C, *et al.* International consensus guideline for reporting transmission electron microscopy results in the diagnosis of primary ciliary dyskinesia (BEAT PCD TEM Criteria). *Eur Respir J* 2020; 55: 1900725.
29 Barbato A, Frischer T, Kuehni CE, *et al.* Primary ciliary dyskinesia: a consensus statement on diagnostic and treatment approaches in children. *Eur Respir J* 2009; 34: 1264–1276.
30 Lucas JS, Barbato A, Collins SA, *et al.* European Respiratory Society guidelines for the diagnosis of primary ciliary dyskinesia. *Eur Respir* 2017; 49: 1601090.
31 Thomas B, Rutman A, O'Callaghan C. Disrupted ciliated epithelium shows slower ciliary beat frequency and increased dyskinesia. *Eur Respir J* 2009; 34: 401–404.
32 Chilvers MA, O'Callaghan C. Analysis of ciliary beat pattern and beat frequency using digital high speed imaging: comparison with the photomultiplier and photodiode methods. *Thorax* 2000; 55: 314–317.
33 Dalhamn T, Rylander R. Frequency of ciliary beat measured with a photo-sensitive cell. *Nature* 1962; 196: 592–593.
34 O'Callaghan C, Smith K, Wilkinson M, *et al.* Ciliary beat frequency in newborn infants. *Arch Dis Child* 1991; 66: 443–444.
35 Smith CM, Hirst RA, Bankart MJ, *et al.* Cooling of cilia allows functional analysis of the beat pattern for diagnostic testing. *Chest* 2011; 140: 186–190.
36 Rubbo B, Shoemark A, Jackson CL, *et al.* Accuracy of high-speed video analysis to diagnose primary ciliary dyskinesia. *Chest* 2019; 155: 1008–1017.
37 Reula A, Pitarch-Fabregat J, Milara J, *et al.* High-speed video microscopy for primary ciliary dyskinesia diagnosis: a study of ciliary motility variations with time and temperature. *Diagnostics* 2021; 11: 1301.

38 Jackson CL, Goggin PM, Lucas JS. Ciliary beat pattern analysis below 37°C may increase risk of primary ciliary dyskinesia misdiagnosis. *Chest* 2012; 142: 543–544.
39 Bricmont N, Bonhiver R, Benchimol L, *et al.* Ciliary videomicroscopy at room temperature might lack sensitivity for primary ciliary dyskinesia diagnosis. *Eur Respir J* 2021; 58: Suppl. 65, PA2362.
40 Jackson C, Rassmann S, Stüven B *et al.* Automated time-lapse analysis of ciliary function. *Eur Respir J* 2016; 48: Suppl. 60, PA4003.
41 Dabrowski M, Bukowy-Bieryllo Z, Jackson CL, *et al.* Properties of non-aminoglycoside compounds used to stimulate translational readthrough of PTC mutations in primary ciliary dyskinesia. *Int J Mol Sci* 2021; 22: 4923.
42 Smith CM, Djakow J, Free RC, *et al.* ciliaFA: a research tool for automated, high-throughput measurement of ciliary beat frequency using freely available software. *Cilia* 2012; 1: 14.
43 Sampaio P, Da Silva MF, Vale I, *et al.* CiliarMove: new software for evaluating ciliary beat frequency helps find novel mutations by a Portuguese multidisciplinary team on primary ciliary dyskinesia. *ERJ Open Research* 2021; 7: 00792-2020.
44 Raidt J, Wallmeier J, Hjeij R, *et al.* Ciliary beat pattern and frequency in genetic variants of primary ciliary dyskinesia. *Eur Respir J* 2014; 44: 1579–1588.
45 Hirst RA, Rutman A, Williams G, *et al.* Ciliated air-liquid cultures as an aid to diagnostic testing of primary ciliary dyskinesia. *Chest* 2010; 138: 1441–1447.
46 Lee DDH, Petris A, Hynds RE, *et al.* Ciliated epithelial cell differentiation at air-liquid interface using commercially available culture media. *Methods Mol Biol* 2020; 2109: 275–291.
47 Marthin JK, Stevens EM, Larsen LA, *et al.* Patient-specific three-dimensional explant spheroids derived from human nasal airway epithelium: a simple methodological approach for *ex vivo* studies of primary ciliary dyskinesia. *Cilia* 2017; 6: 3.
48 Benam KH, Villenave R, Lucchesi C, *et al.* Small airway-on-a-chip enables analysis of human lung inflammation and drug responses *in vitro*. *Nat Methods* 2016; 13: 151–157.
49 Lucas JS, Evans HJ, Haarman EG, *et al.* Exploring the art of ciliary beating: the benefits of high-speed video analysis. *Chest* 2017; 152: 1348–1349.
50 Rumman N, Jackson C, Collins S, *et al.* Diagnosis of primary ciliary dyskinesia: potential options for resource-limited countries. *Eur Respir Rev* 2017; 26: 160058.
51 Kempeneers C, Seaton C, Chilvers MA. Variation of ciliary beat pattern in three different beating planes in healthy subjects. *Chest* 2017; 151: 993–1001.
52 Kempeneers C, Seaton C, Garcia Espinosa B, *et al.* Ciliary functional analysis: beating a path towards standardization. *Pediatr Pulmonol* 2019; 54: 1627–1638.
53 Papon J-F, Bassinet L, Cariou-Patron G, *et al.* Quantitative analysis of ciliary beating in primary ciliary dyskinesia; a pilot study. *Orphanet J Rare Dis* 2012; 7: 78.
54 Blanchon S, Legendre M, Bottier M, *et al.* Deep phenotyping, including quantitative ciliary beating parameters, and extensive genotyping in primary ciliary dyskinesia. *J Med Genet* 2020; 57: 237–244.
55 Feriani L, Juenet M, Fowler CJ, *et al.* Assessing the collective dynamics of motile cilia in cultures of human airway cells by multiscale DDM. *Biophys J* 2017; 113: 109–119.
56 Oltean A, Schaffer AJ, Bayly PV, *et al.* Quantifying ciliary dynamics during assembly reveals stepwise waveform maturation in airway cells. *Am J Respir Cell Mol Biol* 2018; 59: 511–522.
57 Bottier M, Thomas KA, Dutcher SK, *et al.* How does cilium length affect beating? *Biophys J* 2019; 116: 1292–1304.
58 Chioccioli M, Feriani L, Kotar J, *et al.* Phenotyping ciliary dynamics and coordination in response to CFTR-modulators in cystic fibrosis respiratory epithelial cells. *Nat Commun* 2019; 10: 1763.
59 Chioccioli M, Feriani L, Nguyen Q, *et al.* Quantitative high-speed video profiling discriminates between *DNAH11* and *HYDIN* variants of primary ciliary dyskinesia. *Am J Respir Crit Care Med* 2019; 199: 1436–1438.
60 Kesimer M, Ehre C, Burns KA, *et al.* Molecular organization of the mucins and glycocalyx underlying mucus transport over mucosal surfaces of the airways. *Mucosal Immunology* 2013; 6: 379–392.
61 Sears PR, Davis CW, Chua M, *et al.* Mucociliary interactions and mucus dynamics in ciliated human bronchial epithelial cell cultures. *Am J Physiol Lung Cell Mol Physiol* 2011; 301: L181–L186.
62 Bottier M, Blanchon S, Pelle G, *et al.* A new index for characterizing micro-bead motion in a flow induced by ciliary beating: Part I, experimental analysis. *PLoS Comput Biol* 2017; 13: e1005605.
63 Khelloufi M-K, Loiseau E, Jaeger M, *et al.* Spatiotemporal organization of cilia drives multiscale mucus swirls in model human bronchial epithelium. *Sci Rep* 2018; 8: 2447.
64 Trieu D, Waddell TK, Mcguigan AP. A microfluidic device to apply shear stresses to polarizing ciliated airway epithelium using air flow. *Biomicrofluidics* 2014; 8: 064104.
65 Sears PR, Bustamante-Marin XM, Gong H, *et al.* Induction of ciliary orientation by matrix patterning and characterization of mucociliary transport. *Biophysical Journal* 2021; 120: 1387–1395.
66 Walker WT, Jackson CL, Allan RN, *et al.* Primary ciliary dyskinesia ciliated airway cells show increased susceptibility to *Haemophilus influenzae* biofilm formation. *Eur Respir J* 2017; 50: 1700612.

67 Collins SA, Kelso MJ, Rineh A, *et al.* 3'-Diazeniumdiolate NO donor prodrug PYRRO-C3D enhances azithromycin susceptibility of nontypeable *Haemophilus influenzae* biofilms. *Antimicrob Agents Chemother* 2017; 61: e02086-16.
68 Ong HX, Jackson CL, Cole JL, *et al.* Primary air-liquid interface culture of nasal epithelium for nasal drug delivery. *Mol Pharm* 2016; 13: 2242–2252.
69 Pinto AL, Rai RK, Hogg C, *et al.* Ciliary feature counter: a program for the quantitative assessment of cilia to diagnose primary ciliary dyskinesia. *Diagnostics* 2020; 10: 524.