Methods for the assessment of human airway ciliary function

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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 defense, 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. Airways diseases such as idiopathic bronchiectasis [6], chronic

obstructive pulmonary disease [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 R.* [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. 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 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 practiced, 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 7.3 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 own methods and equipment, and are not transferable across centres.

Advances in ciliary beat pattern analysis

High-speed video microscopy analysis (HSVA) facilitates both 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., 63x and above). Lower lens magnifications or lower camera digital resolutions (Southampton uses a Photron FASTCAM MC2 with 512x512 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 ciliary beat pattern 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 6 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 https://zenodo.org/record/4115168, Figure 1e, f). It is imperative that investigators develop ciliary function analysis expertise to conduct reproducible data. The European Respiratory Society (ERS) Clinical Research Collaboration (CRC) 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 centers 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 mis-diagnosis if TEM is normal, or testing resources/expertise are limited.

As well as maintaining a stable sample pH 7.3 (e.g., 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 (FOV) or within a region of interest (ROI). Manually calculated, CBF (Hz) is equal to the recording frame rate (fps) divided by the number of frames for 1 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 3D organoids (spheroids) can be cultured from nasal brush samples by preventing cell attachment with repeated agitation during the initial 4 hours 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 polarize 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 but 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 manually done 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 (AI), used for the first time in the transmission electron microscopy assessment of cilia for PCD diagnostics [69], could potentially quantify cilia waveforms and to model the ciliary function. If developed, such platforms will enable future standardisation of testing and time-saving.

1984 words

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.

c) Diagram demonstrating ciliated airway epithelium and goblet cell secreting mucus into the peri-ciliary layer, with direction of ciliary movement and MCC (MCC).

d) High-speed video microscopy analysis (HSVA) (at 37°C) of ALI-cultured airway epithelium on Transwell insert (20x objective), with Fast Fourier Transform (FFT) 'heat-map' analysis of CBF in Fiji ImageJ (scale 100 μm).

e) Image of ciliated nasal brushing biopsy taken by HSVA (100x objective; scale 10 μm).

f) QR code for representative HSVA video data before and after ALI-culture to remove secondary dyskinesia and verify PCD.

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