Functional Anatomic Imaging of the Airway Surface

Ren-Jay Shei^{1,2}, Jacelyn E. Peabody^{1,2,3}, and Steven M. Rowe^{1,2,4,5}

¹Department of Medicine, ²Gregory Fleming James Cystic Fibrosis Research Center, ³Medical Scientist Training Program, ⁴Department of Pediatrics, and ⁵Department of Cell Developmental and Integrative Biology, the University of Alabama at Birmingham, Birmingham, Alabama

ORCID IDs: 0000-0002-7733-643X (R.-J.S.); 0000-0003-3490-8474 (J.E.P.); 0000-0001-9045-0133 (S.M.R.).

Abstract

The airway surface functional microanatomy, including the ciliated airway epithelium and overlying mucus layer, is a critical component of the mucociliary escalator apparatus, an innate immune defense that helps to maintain a clean environment in the respiratory tract. Many genetic and acquired respiratory diseases have underlying pathophysiological mechanisms in which constituents of the airway surface functional microanatomy are defective. For example, in cystic fibrosis, mutations in the cystic fibrosis transmembrane conductance regulator gene, which normally produces a secretory anion channel protein, result in defective anion secretion and consequent dehydrated and acidic mucosal layer overlying the

airway epithelium. This thick, viscous mucus results in depressed ciliary beating and delayed mucociliary transport, trapping bacteria and other pathogens, compromising host defenses and ultimately propagating disease progression. Thus, developing tools capable of studying the airway surface microanatomy has been critical to better understanding key pathophysiological mechanisms, and may become useful tools to monitor treatment outcomes. Here, we discuss functional imaging tools to study the airway surface functional microanatomy, and how their application has contributed to an improved understanding of airway disease pathophysiology.

Keywords: mucociliary clearance; mucus; cilia; airway surface liquid

(Received in original form June 18, 2018; accepted in final form July 5, 2018)

Supported by National Institutes of Health grants T32HL105346, PI Victor J. Thannickal (R.-J.S.), T32HL134640, PI William E. Swords (J.E.P.), and R35HL135816 and P30DK072482 (S.M.R.).

Correspondence and requests for reprints should be addressed to Steven M. Rowe, M.D., M.S.P.H., MCLM 706, 1918 University Boulevard, Birmingham, AL 35294-0006. E-mail: smrowe@uab.edu.

Ann Am Thorac Soc Vol 15, Supplement 3, pp S177–S183, Nov 2018

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DOI: 10.1513/AnnalsATS.201806-407AW

Internet address: www.atsjournals.org

The respiratory tract is continuously exposed to exogenous environmental and pathogenic challenges, such as microbes, toxins, and harmful inhaled particles (1). Innate airway defense is crucial to maintaining a clean environment throughout the lungs and respiratory tract. A key component of innate airway defense is the mucociliary escalator apparatus, which functions to trap microbes and particles and transport them out of the respiratory tract by a combination of ciliary beating and coughing (2). The ciliated epithelium of the airways, which spans the cephalic end of the trachea to the terminal bronchioles, and associated mucus layer overlying the epithelial surface comprise the primary

structures of the mucociliary escalator apparatus (3). The mucociliary escalator apparatus is depicted in Figure 1. The epithelial surface is a mosaic of secretory and ciliated cells, which function in conjunction to secrete the mucus gel layer and transport secreted mucus from the distal airway to the proximal (cephalic) end of the respiratory tract, where the mucus is either swallowed or expectorated. Secretory cells are responsible for releasing a host of compounds, which contribute to airway defense, including mucins and liquid, antimicrobial molecules, immunomodulatory molecules, and protective molecules (2). In particular, the secretion of mucins and fluid to form the

airway surface liquid (ASL), or mucus gel layer, provides a physical barrier that protects the epithelial surface of the airway lumen. This heterogeneous mucus gel overlies a presumably more homogenous periciliary liquid (PCL) layer, which is approximately 7 μm in depth and is composed primarily of membrane-bound mucins (including MUC4, MUC1, and MUC16) and liquid to form a thick gel layer that surrounds the cilia to facilitate their beating. The morphology and components of both the ASL and PCL have been identified as critical determinants of the mucociliary transport (MCT) rate, and have been implicated in the pathogenesis of several respiratory diseases (4–6).

Figure 1. (A) Microoptical coherence tomography (μ OCT) image of excised swine trachea. The epithelium (ep) and lamina propria (lp) are visible inferior to the airway surface liquid (ASL), as indicated by the yellow bar, and the periciliary liquid (PCL), as indicated by the red bar. (B) Hematoxylin and eosin histology of excised swine trachea illustrating cilia (c), epithelium (ep), and lamina propria (lp). Scale bars = 10 μ m. A and B are adapted by permission from Reference 12. (C) Cartoon schematic demonstrates the four main parameters measurable by μ OCT. ASL depth is indicated by the yellow bar. Periciliary liquid (PCL) is shown by the red bar. Mucociliary transport (MCT) rate is shown as a dark green particle moving along the MCT apparatus over time. Ciliary beat frequency (CBF), as defined by the cycles of power and recovery strokes, is demonstrated in the orange box (adapted by permission from Reference 22). (D) Cartoon schematic compares microsurface anatomy with normal cystic fibrosis transmembrane conductance regulator (CFTR) with that of defective CFTR (4, 18, 19, 23, 28); note the dehydrated ASL and PCL and the decreased MCT.

Abnormally delayed mucociliary clearance (MCC) is a characteristic and critical component of a variety of both rare and prevalent airway diseases, leading to accumulation of thick, sticky mucus that obstructs the airways and precipitates recurrent and chronic infection with pathogens (7–9). For example, pulmonary disease is the primary cause of morbidity and mortality in patients with cystic fibrosis (CF) (10) of which aberrant MCC is thought to play a crucial role; delayed MCC is prominent in many other respiratory diseases as well, including primary

ciliary dyskinesia (PCD) and non-CF bronchiectasis, and its importance in chronic obstructive pulmonary disease (COPD) is beginning to be realized. Progress in understanding the airway surface functional microanatomy, including components, such as ion transport, glandular fluid secretion, ASL/PCL morphology, mucus rheology, and ciliary motion, has begun to advance our understanding of the underlying pathophysiology for a variety of pulmonary diseases. The importance of mucus and cilia to the MCC apparatus is becoming

appreciated with these advances; however, the biology of how abnormal mucus interacts with cilia and subsequently affects MCC is in the early stages of investigation (11). Therefore, the study of mucus clearance pathophysiology has been of great interest, and recent advances in imaging technology have enabled quantitative, real-time imaging of the functional epithelial surface of living airways, including the advent of 1- μ m-resolution optical coherence tomography (12), particularly when coupled with other complementary techniques, such as particle tracking

microrheology (13) and traditional molecular biology.

Challenges of Studying Airway Surface Functional Microanatomy

The airway surface functional microanatomy is challenging to study because of the resolution necessary to fully resolve its components ($\sim 1 \mu\text{m}$), the need to quantitatively characterize its components in living cells and tissues without disturbing the MCT apparatus, and the need to simultaneously image its elements to study dynamic interrelationships (12). Characterization of ASL, PCL, MCT, and ciliary beat frequency (CBF) has been achieved *ex vivo* and *in vitro* using a variety of techniques, including X-Z scanning confocal microscopy (ASL), osmium tetroxide fixation with perfluorocarbon preservation of the ASL (for PCL measurement), particle and radiolabeling for MCT, and high frame rate phase-contrast microscopy for quantifying CBF (12). However, many of these techniques require the addition of exogenous dyes, destruction of tissue, and specialized equipment that does not readily characterize these metrics simultaneously. Furthermore, these techniques have limited potential for *in vivo* imaging of the airway surface microanatomy. This need inspired the development of a microoptical coherence tomography (μOCT) imaging, which uses reflected light from a study sample to construct cross-sectional images to study tissue structure and quantitative metrics of the airway surface microanatomy in living cells and tissues without the need for exogenous dyes or other manipulation (14). μOCT is an interferometric imaging modality that can be considered the optics analog to ultrasound imaging performed at an approximately $1\text{-}\mu\text{m}$ resolution and scanned at rapid rates to generate cross-sectional, functional imaging over a two-dimensional plane.

μOCT as a Tool to Study the Airway Surface Microanatomy

μOCT is an interferometry-based technique that collects reflected light from subcellular structures while simultaneously disregarding diffusely scattered photons, which minimizes the background signal (12, 15–17). Because

contrast is derived from the natural reflectance of back-scattered light, the ASL and PCL can be visualized noninvasively in native airways, and the $1\text{-}\mu\text{m}$ resolution of the μOCT allows for visualization of the ciliary stroke pattern, CBF, and MCT (14). Consequently, μOCT has been successfully applied to quantitatively and accurately characterize the airway surface microanatomy *in vitro* (4, 18, 19), *ex vivo* (4, 20–22), and *in vivo* (23, 24). Further efforts to miniaturize the μOCT system to enable *in vivo* imaging of the respiratory tract are ongoing (20, 23, 24). Preliminary results demonstrate that a miniaturized, flexible bronchial probe performs comparably to a benchtop μOCT system (20), although enhancing the stability of *in vivo* μOCT probes is still necessary to refine imaging of the lung where respiratory motion represents a challenge. Future efforts should aim to develop three-dimensional imaging by μOCT , which has not been done to date, and could potentially be implemented by altering the scanning pattern and reveal additional insight into complex systems. As native or exogenous particles as small as 500 nm diameter can be reliably captured, particle tracking microrheology can also be implemented via μOCT imaging, providing an estimate of mucus viscosity *in situ*, as long as movement of the cilia is halted (18, 23). Coupling fluorescent imaging probes to capture ion transport readouts colocalized on the time-domain and XY plane has been successfully implemented for Ca^{2+} , and theoretically could be implemented for pH, chloride, or sodium to help provide a more composite view of the airway surface as it relates to ion transport (25).

Functional Characterization of Mucus Transport in CF and Other Airway Diseases

Mucus transport regulation is multifaceted, which has made its study *in situ* challenging. New insights garnered from functional imaging of the airway surface microanatomy using μOCT , and other imaging technologies, have made essential contributions to our understanding of the physiology and pathophysiology of mucus transport and diseases of abnormal mucus clearance (Figure 2). In particular, using CF as a model, recent data have demonstrated aspects of the functional anatomic defects present in the airway surface microanatomy in CF (4). In CF, inherited mutations in

the CF transmembrane conductance regulator (*CFTR*) gene cause multiorgan pathologies, with morbidity and mortality primarily resulting from pulmonary disease (8). Abnormalities in *CFTR* protein resulting from *CFTR* mutations produces dysregulated epithelial anion secretion uncoupled with epithelial sodium channel-mediated sodium transport (7), thus affecting the airway mucosa. Consequently, airway surface dehydration (4), together with other defects in host defense, including deficient bacterial killing and acidification of the airways due to the deficiency of bicarbonate transport (26), makes patients with CF susceptible to inflammation, chronic infection, and progressive obstructive lung disease. Using functional imaging of the airway surface microanatomy, depletion of the ASL and PCL layers and blunting of the MCT rate in excised tracheas of *Cftr* knockout porcine and rat models is evident (4, 27, 28), and CBF was similarly shown to be depressed in porcine tracheas (4). These data are not without controversy, however, given conflicting evidence in the excised trachea of CF swine (29), which independently demonstrated no alteration of PCL depth in *Cftr* knockout pigs. The latter work studied excised tracheal sections of 1-day-old piglets, which were fixed with OsO_4 and subsequently imaged by light microscopy and electron microscopy to quantify PCL depth. In contrast, Birket and colleagues (4) measured PCL depth using μOCT , which does not require fixation, and thus could be more sensitive to differences in PCL depth than OsO_4 fixation. Furthermore, μOCT also incorporates the mucus layer, which is more variable across anatomic regions. Experiments in which shear stress was applied to excised trachea further demonstrated a persistent difference in PCL between wild-type and CF piglet trachea, thus suggesting that physiologic conditions produce relative PCL depletion in CF. On the other hand, samples subjected to μOCT analysis are manipulated after excision, sometimes including 24-hour transport to the imaging facility, which could also have important effects. These conflicting data ultimately highlight the need for *in vivo* validation in human subjects with CF while breathing.

Aside from changes in hydration of the ASL and PCL layers, *in vitro* data in human bronchial epithelial cell cultures grown on an air-liquid interface and *ex vivo* data from

Figure 2. Microoptical coherence tomography (μ OCT) video stills showing intact, (A) full-thickness cystic fibrosis transmembrane conductance regulator (CFTR) (+/+) piglet tracheal explant and (B) a CFTR (-/-) piglet tracheal explant. Scale bars = 10 μ m. Airway surface liquid (ASL) shown by the red bar; periciliary liquid (PCL) layer shown in the yellow bar. Epithelium (ep), lamina propria (lp), a gland (gl), and gland duct (gd), which transect the image, are also visualized. (C–F) Quantitative analysis of μ OCT images ($n = 6$ trachea/condition) for ASL (C), PCL (D), ciliary beat frequency (CBF) (E), and mucociliary transport (MCT) (F); * $P < 0.05$, ** $P < 0.005$. (G) Relationship between viscosity of mucus and MCT rate. Adapted by permission from References 4 and 28.

excised porcine and rat trachea demonstrate that changes in mucus rheology also have substantial effects on MCT rate, and can, in fact, be the dominant parameter in some anatomic locations (4, 28). The intragranular mucin matrices in CF and non-CF cells are not different with regard to their maturation or swelling states, but

do have distinct viscosity distributions (30). In contrast, in the absence of mature glandular secretion, as in young CF rats, PCL depletion, even when accompanied by increased solid content and acidification, is not sufficient to depress MCT rate completely. *Cftr*^{-/-} rats exhibit PCL depletion, reduced airway pH, and increased

solid content at a young age without slowed MCT (28). Instead, MCT defects emerge with age in *Cftr*^{-/-} rats, suggesting that mature CF secretions are required to recapitulate MCT defects fully in the rat. Subsequent regression analysis confirmed that lower mucus viscosity is associated with faster MCT rate, and there appears to be a

critical viscosity threshold near 50 cP, above which MCT decreases sharply (28); similar thresholds are emerging in other model systems. The relative contributions of airway fluid homeostasis, pH-induced (31) and, potentially, Ca^{2+} -dependent changes in mucus viscosity (32, 33), and their interactions will ultimately require *in vivo* studies in humans to fully unravel.

Application in Other Diseases of Mucus

Functional anatomic defects of the MCT apparatus extend beyond CF, and could help improve our understanding of the pathophysiology of these disorders. Because cigarette smoke exposure has been shown to induce dysfunction in CFTR (34–37), functional studies have confirmed ASL dehydration and blunted CBF after cigarette smoke exposure in airway monolayers (without the contribution of glands) (21, 35). Separately, comprehensive studies on the ciliary motion in PCD demonstrated that different murine models of PCD did not possess the same defects in ciliary movement (22, 38), with consequent effects on MCT (22). In *Ccdc39*^{-/-} mice, which display a mutation in the axonemal organization of the cilia, the regions of motile cilia were reduced, which nearly abolished MCT completely. Conversely, *Dnah5*^{-/-} mice, which display a mutation in the outer dynein arm of the cilia, had preserved motile cilia area, no changes in ciliary stroke, but a substantial reduction in CBF. The *Dnah5*^{-/-} mutation resulted in reduced, but partially preserved, MCT. Finally, *Wdr69*^{-/-} mice, possessing a mutation predicted to cause PCD, but which has not been formally characterized, maintained normal motile cilia area as well, with preserved CBF. The ciliary arc was abnormal in these mice, however, leading to partially delayed MCT as well. Together, these data demonstrate that multiple aspects of ciliary motion contribute to MCT rate, and that μOCT is a useful tool for interrogating the functional airway surface microanatomy.

Functional Microanatomy as a Tool for Preclinical Assessment and Prediction

Physicochemical properties of mucus such as specific mucin composition,

viscoelasticity, pH, pore size, ionic strength, and charge can impact transmucosal drug delivery and impact efficacy of the MCT apparatus (39–43). Using imaging modalities like μOCT to monitor response to treatment should aid in rational drug design and development and provide a better understanding of the functional outcomes of these treatments, especially those targeting ion transports or mucus structure. Together with clinical outcome measures, and basic measures of mechanistic interest, functional imaging of the airway surface microanatomy can give a more comprehensive overview of individual drug treatments, aiding in translation from benchtop to clinic. For example, in human bronchial epithelial cells exposed to cigarette smoke extract, which exhibit a reduced ASL and CBF as a consequence of acquired CFTR dysfunction, treatment with the CFTR potentiator, ivacaftor, restores ASL and improves CBF (21). Furthermore, in a pilot trial in patients with COPD and chronic bronchitis, treatment with ivacaftor produced nonsignificant improvements in CFTR function, detected by sweat chloride concentrations and nasal potential differences (44). Patients also had nonsignificant enhancements in Breathlessness, Cough, and Sputum Scale, which exceeded the minimally clinically relevant difference of 1 unit. Patients who experienced the most substantial improvement in sweat chloride and Breathlessness, Cough, and Sputum Scale were those who possessed the highest sweat chloride concentration at baseline, suggesting that more severe CFTR dysfunction was present in these patients, and this dysfunction was partially reversible with ivacaftor treatment. Confirmatory studies are presently underway. Similarly, the phosphodiesterase-4 inhibitor roflumilast has been shown to restore CFTR function after cigarette smoke exposure *in vitro* and *in vivo*, which may explain why it is most effective in those with chronic bronchitis and frequent exacerbations, as opposed to those with a pure emphysema phenotype (45–48). These data demonstrate that airway diseases that share common pathophysiological mechanisms may benefit from the translation of therapies targeting these mechanisms from one disease to others, such as from CF to COPD (49). Furthermore, in addition to clinical measures, such as sweat chloride concentration and nasal potential

difference, the application of functional airway surface microanatomy imaging can provide additional insights into both the pathophysiological mechanisms and the response to treatments.

Future Applications of Functional Imaging of the Airway Surface Functional Microanatomy

Translating of functional imaging tools, including μOCT , to more widespread use will help answer critical questions that remain difficult to study. In particular, the miniaturization of a human μOCT probe capable of *in vivo* imaging of nasal and endobronchial imaging will significantly aid in validating mechanistic animal studies examining specific aspects of disease pathophysiology and progression. Specifically, elucidating the relationship between CFTR function and ASL homeostasis and mucus viscosity, and how mucus interacts with the cilia and how ciliary motion defects confer delayed MCT, should be achievable using an *in vivo* μOCT system. Characterization of these features may also aid in understanding the pathophysiological mechanisms in other airway diseases, particularly those that feature aberrant mucus clearance. Knowledge garnered from these studies should assist in the development of next-generation, rationally designed drugs to ameliorate defects in MCT and MCC. For example, exploring whether new pathways can be used to normalize mucus and restore MCC will be of great interest in a variety of airway diseases, such as CF, COPD, and PCD, but there first needs to be the translation of basic benchtop imaging techniques to the clinic. It remains to be seen whether *in vivo* findings using μOCT will recapitulate results observed in *ex vivo* tissue samples and cell culture models. Furthermore, presently, *in vivo* μOCT is an investigational technology, and therefore unavailable for widespread use as a research and clinical tool. Further development is necessary to expand and optimize the capabilities of *in vivo* μOCT , such as the development of three-dimensional imaging and efficient stabilization of imaging probes. Advancing these capabilities, among others, should help drive the commercialization and adoption beyond current uses.

Conclusions

In summary, the use of μ OCT in conjunction with other imaging tools and clinical outcome measures has dramatically enhanced our understanding of the airway surface microanatomy, particularly the disorder that occurs in the airway surface microanatomy

in specific airway diseases, such as CF, COPD, and PCD. Even in this early stage of development, its application has helped elucidate new insights into pathophysiological mechanisms of disease, and to monitor responses to treatments targeting fundamental molecular and physiologic defects in disease. Further development and translation of such

functional imaging tools to more widespread use should prove useful in enhancing our understanding of disease mechanisms *in vivo*, and to track treatment responses over time. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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