In vivo imaging of airway cilia and mucus clearance with micro-optical coherence tomography

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Abstract: We have designed and fabricated a 4 mm diameter rigid endoscopic probe to obtain high resolution micro-optical coherence tomography (µOCT) images from the tracheal epithelium of living swine. Our common-path fiber-optic probe used gradient-index focusing optics, a selectively coated prism reflector to implement a circular-obscuration apodization for depth-of-focus enhancement, and a common-path reference arm and an ultra-broadband supercontinuum laser to achieve high axial resolution. Benchtop characterization demonstrated lateral and axial resolutions of 3.4 µm and 1.7 µm, respectively (in tissue). Mechanical standoff rails flanking the imaging window allowed the epithelial surface to be maintained in focus without disrupting mucus flow. During in vivo imaging, relative motion was mitigated by inflating an airway balloon to hold the standoff rails on the epithelium. Software implemented image stabilization was also implemented during post-processing. The resulting image sequences yielded co-registered quantitative outputs of airway surface liquid and periciliary liquid layer thicknesses, ciliary beat frequency, and mucociliary transport rate, metrics that directly indicate airway epithelial function that have dominated in vitro research in diseases such as cystic fibrosis, but have not been available in vivo.

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1. Introduction

Optical coherence tomography (OCT) [1] has become a mainstay biomedical imaging technology due to its ability to non-invasively acquire cross-sectional images with high resolution compared to ultrasound. Endoscopic devices that deliver OCT imaging to a variety of organ systems have been developed [2–5], particularly since the advent of second generation Fourier domain OCT [6–8], which enabled speedier acquisition and improved practicality for in vivo applications.

However, the optical resolution conferred by OCT has been typically lower than other forms of microscopy that operate on similar wavelengths. High numerical aperture (NA) improves OCT lateral resolution, as in conventional optical microscopy. Depth of focus scales inversely with NA in both modalities, but in OCT, a small depth of focus is undesirable, unlike in conventional microscopy, in which depth of focus defines axial resolution. OCT
depth of focus determines instead the axial field of view, while axial resolution is derived independently from the bandwidth of the light source. This creates conflicting pressures for the choice of NA: high NA yields superior resolution, while low NA allows cross-sectional OCT imaging over greater depths.

Our laboratory has previously developed a spectral-domain implementation of OCT that combines a new generation of ultra-broadband supercontinuum light sources with an engineered beam shape to produce 2-µm lateral resolution, 1-µm axial resolution, and 300-µm depth of focus performance. We have termed this technique micro-optical coherence tomography (µOCT). We first demonstrated this technology in coronary artery plaques ex vivo [9].

More recently, we have utilized µOCT for in vitro imaging of the airway surface, including the cilia [10–14]. Cilia are microscopic organelles that line respiratory epithelia and beat repeatedly in a sweeping motion to clear mucus from the airways. Mucociliary clearance (MCC) is an essential airway defense mechanism, as foreign particles and microbes are trapped in mucus and swept out of the lungs and airways. In diseases characterized by impaired mucociliary clearance, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), the failure of effective MCC is believed to contribute to significant morbidity and mortality [15, 16]. Others have also utilized conventional OCT to interrogate ciliary beating, but without the microscopic resolution to directly visualize the cilia [17].

We now present a fiber-optic endoscopic instrument for imaging of airway cilia, and demonstrate its use in swine trachea in vivo. Our probe utilized an apodizing beam splitter that created the depth-of-focus-enhancing annular beam shape and also enabled a common-path design that fully enclosed the OCT reference path in the endoscope itself. This first-generation design employed a rigid design for reasons of simplicity; a flexible probe would likely be subject to much greater scanning hysteresis as the driveshaft transitions from compression to tension.

2. Materials and methods

2.1 µOCT platform and probe

The general layout of the optical system is shown in Fig. 1. The light source was a supercontinuum laser (SuperK Extreme OCT, NKT Photonics, Birkeroed, Denmark) with broadband output nominally spanning 600 nm to 1800 nm with 4 W total laser power. Though OCT designs frequently prescribe optical circulators to direct illumination light towards the interferometer while shunting the returning interference signal to a spectrometer, no optical circulator was available with sufficient performance over our desired wavelength range of 600 – 1000 nm. We chose instead to utilize a 90/10 beam-splitter (BS029, Thorlabs Inc., Newton, NJ) to both limit the excessive illumination power sent to the probe, as well as to maximally preserve the light returning from the probe.
An achromatic lens (AC050-010-B, Thorlabs) focused the laser light into the single-mode fiber (SM600, Thorlabs) of the endoscopic probe. The returning interference signal from the probe was 90% reflected by the BS029 splitter and focused by an achromatic lens (AC050-010-B, Thorlabs) to a 10 µm pinhole acting as a spatial filter to remove contributions from stray reflections in the light delivery optics. The output of the pinhole was collimated (AC254-060-B, Thorlabs) and directed towards a volume phase holographic grating with 940 line pairs/mm (Wasatch Photonics, Logan, UT). A photographic prime lens with f = 85 mm (Nikon, Tokyo, Japan), focused the dispersed light onto a linear CCD array camera (Sprint spL4096-140k, Basler AG, Ahrensburg, Germany). Data was streamed from the camera to an acquisition computer through a Camera Link interface card (KBN-PCE-CL4-F, Bitflow Inc., Woburn, MA). Analog and trigger signals were generated by a data acquisition card (PCI-6221, National Instruments, Austin, TX). Custom written acquisition software provided system control and real-time image rendering. All spectrometer and computer components were enclosed in a wheeled steel-frame cart (Cabbage Cases, Columbus, OH) for portability.

The optical and mechanical components of the µOCT endoscope are shown in Fig. 2. The endoscope was a fiber-optic probe comprising a gradient-index focusing optic, common-path interferometric geometry, and side-viewing beam output. Figure 2(A) and 2(B) illustrate the probe optical design and housing assembly, respectively. Light emanating from the SM600 fiber diverged within the N-BK7 glass spacer, which was joined to the fiber with an 8° angle-polished interface to prevent back-reflection. A gradient-index lens (Grintech, Jena, Germany) acted as a single-element objective. The converging light then passed through a custom beam-splitter composed of two N-BK7 right-angle prisms, one of which was gold-coated with a circular uncoated center, causing the reflected beam to be annularly apodized. All optical joints were cemented with UV-curing epoxy (NOA65, Norland Products, Cranbury, NJ).
The apodizing coating of the prism was performed by placing an approximately 200 µm sized bead of epoxy on the center of the prism’s hypotenuse. The prism was then gold-coated by a commercial metal deposition facility (Evaporated Coatings, Willow Grove, PA), covering the hypotenuse, including the raised epoxy bead. The epoxy was then manually removed, leaving an circularly apodizing reflective surface. The light traveling forward through the center of the apodizing prism served as the reference beam.

The glass ferrule was cemented within a rigid stainless steel tube (Microgroup, Medway, MA) of 2 mm outer diameter and 1 mm inner diameter using fiber connector epoxy (Thorlabs) and polished to a common plane for joining with the glass spacer. The remaining optics were encased in a machined brass shuttle tube. The tip of a polycarbonate 4-40 screw was polished to a flat surface, and the screw was inserted into a tapped hole at the end of the shuttle. The polished surface was placed in the path of the reference beam, which provided an approximately 4% reflection to serve as the reference mirror. The reference position was adjustable by turning the screw. A 4 mm outer tube of stainless steel (Microgroup) acted as the outer housing of the probe. The length of the tube was 50 cm, with 7 cm held within the probe scanner housing, resulting in an insertable length of 43 cm. A slot was machined to provide a window for the imaging beam.

To provide mechanical stability, a custom polymer cap was 3D printed (PicoPlus33, Asiga, Anaheim Hills, CA) and fitted over the probe end with a pair of parallel rails that flanked the imaging window and protruded at a distance corresponding to the focal plane of the µOCT beam. The rails permitted the probe to be brought into contact with the airway lumen surface without disrupting the flow of mucus at the site of imaging. Figure 2(C)
illusrates the placement of the standoff rails against the trachea surface before and after balloon inflation (described in the section 2.3), and Fig. 2(D) shows a 3D rendering of the standoff rails integrated into the probe cap. The cap was attached to the probe steel housing by epoxy, and a polyolefin heat-shrink overwrap (Vention Medical, Marlborough, MA) was used to seal the imaging window.

Scanning was transduced longitudinally, with the probe’s inner and outer steel tubes connected to differential ends of a piezoelectric stack linear motor (P-602.5SL, Physik Instrumente, Karlsruhe, Germany) driven by a matching piezoelectric amplifier/controller (E-625, Physik Instrumente). The scan pattern was a sinusoid with amplitude of 400 µm and a frequency of 40 Hz.

2.2 Benchtop characterization

Optical resolution was characterized separately in lateral and axial directions. Lateral resolution was determined by placing the probe sample arm beam at the focus of a 20x conventional brightfield microscope (BX61, Olympus, Tokyo, Japan), which served as a high-resolution beam profiler. Input laser power was substantially reduced by intentional misfocus at the fiber coupling to prevent detector saturation. A short-pass filter at 950 nm was used to truncate the spectrum to match the range detected by the μOCT spectrometer. The full-width half maximum (FWHM) of the in-focus spot was considered to be the lateral resolution limit. Axial resolution was measured by determining the axial FWHM of the image of a glass surface reflector.

Ex vivo imaging was also performed as a validation. A swine trachea was explanted and immediately imaged. The probe was inserted through the proximal end of the intact trachea and manipulated by hand.

2.3 Swine imaging

All animal procedures were reviewed by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC) as described in protocol 2013N000124. An adult female Yorkshire swine was procured and anesthetized by injected telazol and xylazine for transport to the procedure room, whereupon the animal was intubated and anesthesia was continued by inhaled isoflurane. An 8.5 mm inner diameter endotracheal (ET) tube was placed during intubation. The μOCT probe was inserted into the trachea through the ET tube, followed by a small-diameter bronchoscope (EB-1170K, Pentax Medical, Montvale, NJ) for visualization. During probe insertion, mechanical scanning and laser illumination were disabled. Once the μOCT probe reached the trachea by extending beyond the end of the ET tube, as confirmed by direct visualization, scanning and illumination were activated. The imaging region of the probe was placed on regions of the trachea that were accessible by the μOCT probe, which was limited by the rigidity of the device. A bronchial balloon (Cook Medical, Bloomington, IN) was used to stabilize the μOCT probe against the lumen momentarily during imaging to alleviate motion artifacts.

2.4 Post-processing and quantitative analysis

Though real-time previews of μOCT images were shown during the procedure, only raw image data were stored to disk. μOCT images were then produced in post-processing using standard Fourier-domain image reconstruction techniques [18]. The μOCT images were interpolated laterally to remap the sinusoidal scan pattern to a linear scan.

Additional processing was performed to further mitigate the effect of motion artifacts. First, a series of images was selected that included a consistent field of view, except for minor motion-induced shifts. A region of the image corresponding to the epithelial surface was selected by the user. The corresponding region in each frame was cross-correlated in two dimensions with the first frame in the sequence to determine the shift error between the frames, and each frame was translated in the opposite direction of the shift error to negate the effect of motion.
Previously described methods were used to quantify respiratory epithelial functional parameters [13] which are briefly summarized here. Geometric measurement tools in ImageJ (NIH, Bethesda, MD) were used to measure the depth of the airway surface liquid (ASL) and periciliary liquid (PCL). The stabilized image sequence was also analyzed for pixels exhibiting periodic oscillations in image intensity, the frequency of which represented the ciliary beat frequency (CBF). To quantify mucociliary transport (MCT) rate, a line was drawn through the mucus layer and parallel to its direction of transport, and the intensity along this line as a function of time was projected as a 2D image (Fig. 8). In the resulting space vs. time image, moving particles were visualized as slanted streaks, the slopes of which represented the component of mucus velocity aligned with the probe, similar to other particle streak velocimetry methods [19, 20].

3. Results and discussion

The probe optical design centered around the apodizing beam splitter, which was the cornerstone of the common-path annular beam design. The manual epoxy mask method we employed was effective at creating an annular apodization, but imperfect in several ways. The repeatability of the manual application of the epoxy dot and its subsequent removal after coating was limited, even under microscope guidance. Additionally, a circular mask on a 45 degree prism surface resulted in an elliptical apodization, which introduced an anisotropy to the probe’s lateral performance. Critically, however, the reference and sample arms were separated for only several millimeters, minimizing the effect of chromatic dispersion. The common path design also obviated polarization diverse detection, which is ordinarily needed to avoid contrast-depleting unmatched polarization shifts in the sample and reference fibers [21]. Polarization diversity is difficult to achieve in SD-OCT due to the need for two detectors, which in the case of spectrometers, requires extremely precise alignment [22], particularly given the wide bandwidth utilized in µOCT.

Figure 3(A) shows the lateral beam profile generated by the µOCT probe in the plane of best focus, located at 1 mm outside of the probe’s outer housing, which is the working distance. The beam spot is largely circular, with FWHM along the major and minor axes respectively measured at 3.41 µm and 3.32 µm. Figure 3(B) also shows the lateral beam profile in a 3D mesh plot, which demonstrates that sidelobe energy was minimal but slightly anisotropic. The axial response of a glass slide surface is shown in Fig. 4. The FWHM of the axial profile was 2.29 µm in air, which corresponds to 1.72 µm in aqueous environments such as cells and tissue (n = 1.33).
Fig. 3. Normalized lateral beam profile of μOCT beam measured at plane of best focus. A) 2D image of beam profile. B) 3D plot of beam profile, with portion of beam exceeding half-maximum colored in red, and the remainder of the profile colored in blue.

Fig. 4. Normalized axial profile of reflecting plane located at z = 0. Measured axial resolution (FWHM) = 2.29 μm in air, corresponding to 1.72μm in tissue (n = 1.33).

Compared to benchtop μOCT, both the lateral and axial resolution performance of the μOCT probe were similar, but slightly decreased. The lateral resolution loss of approximately 1 μm may have been caused by the beam apodization irregularities described above. The axial resolution loss likely originated from the placement of the reference reflector in a converging, non-collimated beam. To avoid the complex conjugate ambiguity common to Fourier-domain OCT systems, the reference path length must be slightly shorter than the in-focus sample path length. Because the beam was converging where it was split and apodized in our design, the reference beam was also converging, and the reflector had to be placed slightly nearer than its focal plane. This defocus is wavelength-dependent, causing some wavelengths to be more defocused than others which effectively narrows the bandwidth available for interference.

Probe scanning transduced by the linear motor also presents significant differences when compared to results obtained by the galvanometer mirror scanning benchtop system. The closed-loop piezoelectric stack was limited in bandwidth to 40 Hz, necessitating a sinusoidal scan pattern to eliminate higher harmonics of the scan pattern that were outside the motor bandwidth. During post-processing, the sinusoidal pattern was remapped to a linear scan.

Figure 5 (movie online) shows an image sequence acquired from freshly excised intact swine trachea. The still image shown for Fig. 5 is an average of 16 frames taken from the video sequence, with a green channel overlay added to accentuate pixels exhibiting interframe variance exceeding an arbitrary threshold, allowing the ciliated areas to be accentuated in a static image. In the video itself, the ciliated epithelium can be clearly visualized, including the...
beating of the cilia. The image quality was comparable to those obtained by the benchtop system from swine trachea. The airway surface liquid can also be seen. The image motion in this \textit{ex vivo} case was caused solely by the motion of the manually-held probe. The sinusoidal scan pattern has been remapped to a linear scale, resulting in an undistorted image throughout the field of view.

![Image](image.png)

Fig. 5. (See Visualization 1 for video) \textit{Ex vivo} swine trachea epithelium imaged by handheld µOCT probe. Still frame displays 16x average (video content is not averaged) with a green channel overlay indicating pixels exhibiting high interframe variance, which accentuates the beating cilia.

Figure 6 (movie available in Visualization 2) shows an image sequence acquired from a swine trachea \textit{in vivo}. In this experiment, a blocker balloon situated behind the µOCT probe (on the opposite side of the imaging window) was briefly inflated to compress the probe into the epithelial surface. As in the \textit{ex vivo} case, the beating cilia and mucus layer were visualized. However, a rhythmic motion artifact was still observed, due to a combination of cardiac and tidal breathing motion. An additional artifact that could be seen in Fig. 6 was the reflection from the transparent polyolefin covering that serves as the imaging window, seen as two bright lines that appear to be just below the epithelium. This artifact was the phase conjugate of the polyolefin window, which was nearer to the probe than the reference position.

Computational image stabilization resulted in a much more stable image (Fig. 7 and Visualization 3). The individual frames in this image sequence have been shifted to maximize the 2D cross-correlation with the initial frame, causing the epithelium surface to remain in the same position while the edges of the frame appear to shift.

The stabilized image sequence was sufficiently free of motion to enable quantitative measurements of the depths of the airway surface liquid and periciliary liquid for benchtop imaging [13]. By sampling 5 equally spaced positions along the epithelium, we found ASL and PCL thickness to be 19.5 ± 1.7 µm and 6.0 ± 0.2 µm respectively, as shown in Fig. 8 (mean ± standard deviation). The cilia towards the left of the frame were determined to be beating at 12.0 Hz based on its Fourier-domain spectral peak. The velocity of mucus transport in this sequence was also measured to be 71 ± 28 µm/s. These values were in accordance with previous results derived from \textit{ex vivo} swine trachea [10, 11, 13].

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Fig. 6. µOCT image sequence acquired in vivo by probe. Images were acquired while airway balloon was inflated to depress probe tip towards trachea wall. No software stabilization applied. A) 25 frame average shows blurring due to remaining motion artifacts. Scale bar: 50 μm. B) A depth-time profile of one A-line from the approximate location indicated by the red dashed line in (A). Oscillatory tissue surface motion is evident (blue arrow). See Visualization 2 for full image sequence.

Fig. 7. µOCT image sequence as shown in Fig. 6, with cross-correlation software stabilization applied. A) 25 frame average shows improved resolution due to removal of motion artifacts. Visible features include air, mucus, cilia, epithelium (epi), and the complex conjugate artifact of a reflection from the imaging window (IW). Scale bar: 50 μm. B) A depth-time profile of one A-line from the approximate location indicated by the red dashed line in (A). As a result of software stabilization, tissue surface motion is stationary (blue arrow). See Visualization 3 for full image sequence.

Fig. 8. Quantitative analysis from in vivo µOCT sequence shown in Fig. 7. Image shown is average of 40 frames (1 second) and lookup table inverted for contrast enhancement. Airway surface liquid (ASL) and periciliary liquid (PCL) depths were measured geometrically as shown. Ciliary beat frequency (CBF) was computed by Fourier analysis of the periodic motion of a single pixel (orange dash and inset * illustrate one cross-sectional line from the surface projected through time. Periodic motion of the cilia is visible in the region enclosed by the orange bars. Mucociliary transport (MCT) was measured by projecting a cross-sectional line through the mucus through time (blue dashed line and inset **). The slope of the particle trajectories indicated velocity.
The addition of the dual rail standoffs around the imaging window allowed the probe to be braced against the tissue surface. The rails served both to place the focal position of the probe at the epithelial surface and to mitigate motion artifacts by providing a static point of contact. The rail geometry avoided disruption of the mucus directly in front of the window, though epithelial contact in the proximity of the imaged cilia is still undesirable. However, the use of rails proved essential to suppressing excessive motion.

The appearance of cilia on respiratory epithelium is typically somewhat patchy due to the presence of non-ciliated cell types on the surface [23], but challenges unique to in vivo imaging may have contributed to the relative sparseness of cilia shown in Figs. 6 and 7. The requirement that cilia be imaged over several periods of motion in order to capture beat frequency, combined with their small size, creates a narrow constraint on the amount of tolerable motion artifacts. Even with our motion-suppressing solutions, some motion remained, and visualization of some regions of cilia was likely compromised as a result.

The most limiting aspect of the rigid \( \mu \)OCT probe’s performance was tissue access. Though the trachea is a relatively straight lumen that permits entry of the rigid probe, the short working distance of the imaging optics necessitated near-contact with the imaging window, which constrained the accessible regions of the trachea to those regions that protruded slightly towards the center. A flexible probe design that is navigable through a bronchoscope imaging channel will be able to be placed onto more regions of the airway surface, as well as navigated into smaller airways, representing a substantial advantage for biological applications. A flexible probe may also better able to move along with the tissue following the cardiac and respiratory rhythms, which would result in less relative motion to the cilia and enable a higher proportion of beating cilia to be captured by \( \mu \)OCT. However, the rigid probe did have the advantage of being operable with a single hand, since it did not require a separate hand to control flexure.

The primary challenge for the flexible probe will likely be the prevention of hysteresis during scanning. A flexible driveshaft undergoing push-pull transitions within a curved outer tube will alternately seek the outside and inside portions of the curve, resulting in lost motion at the distal end.

4. Conclusion

We have designed and fabricated an endoscopic \( \mu \)OCT probe with a 4 mm outer diameter. The probe was utilized in a living adult swine airway to acquire live high-resolution OCT images of the airway epithelium, showing individual cilia, ciliary beat, and mucociliary clearance, which were quantified from the image sequence.

The images of beating cilia in the living swine airway obtained by the \( \mu \)OCT probe are, to our knowledge, the first demonstration of in vivo optical imaging of cilia with sufficient spatial and temporal resolution to quantify beat frequency and mucociliary transport. Both measurements were obtained using only native reflectance contrast, with no exogenous tracers or dyes. The structural context of the cross-sectional image sequence also enabled quantitative geometric measurements of airway surface liquid and periciliary liquid layers.

The ability to acquire the quantitative metrics of functional airway microanatomy has the potential to enhance clinical research and diagnostics involving cilia and mucociliary clearance by providing coordinated biomarkers of airway function. In cystic fibrosis, for example, the failure of mucociliary clearance is considered to be one of the hallmarks of CF lung disease, and restoration of mucus transport is one of the primary goals of CF treatments. Yet CF disease monitoring relies largely on spirometry and subjective reporting by patients, in part due to the lack of direct monitoring tools. Continued development of this technique, including implementation in a flexible sheath and further miniaturization, should expand clinical and biological utility further.
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Disclosures
Several authors are named co-inventors on a pending patent application (US 20150253240), which discloses methods utilized in this manuscript.