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ISBN

9781510647411

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Publication Date

2022-03-09

DOI

10.1117/12.2607390

PROCEEDINGS OF SPIE

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Asher C. Park, Zhikai Zhu, Lidek Chou, Katelyn Dilley, Akarsh Lal, Edward C. Kuan, Zhongping Chen, Brian J. F. Wong, "Validation of spectrally encoded interferometric microscopy (SEIM) in finding ciliary beat frequency of human ex vivo upper airway tissue," Proc. SPIE 11935, Imaging, Therapeutics, and Advanced Technology in Head and Neck Surgery and Otolaryngology 2022, 1193505 (9 March 2022); doi: 10.1117/12.2607390

SPIE.

Event: SPIE BiOS, 2022, San Francisco, California, United States

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Keywords
Spectrally encoded interferometric microscopy, ciliary beat frequency, phase contrast microscopy, interferometry, microscopy, doppler effect, chronic rhinosinusitis, allergic rhinitis

Imaging, Therapeutics, and Advanced Technology in Head and Neck Surgery and Otolaryngology 2022, edited by Brian J. F. Wong, Justus F. Ilgner, Proc. of SPIE Vol. 11935, 1193505
 · 1605-7422 · doi: 10.1117/12.2607390

Abstract
Mucociliary clearance facilitated by healthy cilia beating is crucial to normal upper airway function. Phase-contrast microscopy (PCM) is the current golden standard for measuring ciliary beat frequency (CBF) and has limitations. With PCM, one cannot appreciate how CBF varies across the complex landscape of the nasal vault and sinus tissues. With Spectrally encoded interferometric microscopy (SEIM), *en face* imaging of cilia can be achieved, providing insight into the changes in CBF across tissue surfaces. This study aims to validate the use of SEIM to quantify ciliary beat frequency across *ex vivo* upper airway tissue.

Introduction
Motile cilia play a major role in a number of physiologic processes. In the upper airway, cilia largely contribute to immunologic defense.[1] Mucus in the upper airway traps dust, debris and pathogens and is cleared by the constant rhythmic beating of cilia. This mucociliary clearance that cilia provide is critical for normal airway function. Ciliary dysfunction has predominantly been studied in association with conditions such as cystic fibrosis and ciliary dyskinesia. However, sinonasal diseases such as chronic rhinosinusitis (CRS) and allergic rhinitis (AR) are relatively overlooked and have substantial economic impact. In 2012, 17.6M adults and 6.6M children were diagnoses with AR in the United States, costing approximately $11B annually.[2-4] Additionally, CRS affected 10-15% (~50M) of Americans and had an economic cost of $24B.[5-7] Currently, there are qualitative measures used to evaluate these CRS and AR patients which include visual inspection with a nasal endoscope as well as the Sinonasal Oucome Test (SNOT-22) to help clinicians describe the severity of disease and subsequent measurements in response to therapeutic interventions. Both of these methods are neither standardizable nor quantifiable and fail to provide adequate information regarding mucosal pathophysiology.[8-10] At this point in time, a quantitative means of measuring the mucosal physiologic responses to pharmacologic and surgical treatments of AR and CRS are needed.

Although, ciliary beat frequency (CBF) is a direct, quantifiable measurement of cilia function, this metric currently has limited clinical practicality. The current golden standard for finding CBF is taking a nasal swab brushing of mucosa and imaging cilia under a phase-contrast microscope.[11] This method is limited to *ex vivo* use and has a limited profile view of cilia, failing to appreciate other ciliary parameters such as ciliary beat pattern (CBP) and metachronal waves (MW) which contribute to the functional characteristics of cilia. Recent developments have led to optical coherence tomography (OCT) use to acquire cross-sectional one-dimensional (1-D) images of the microstructure of mucosa overtime, allowing for imaging of cilia.[12] Although this imaging modality is attractive as it allows for endoscopic OCT probes to be used to measure ciliary beat frequency *in vivo*, 1-D scanning again does not allow for measurement of the functional parameters, CBP and MW. This highlights the need for a cilia imaging modality that can not only find CBF *in vivo*, but that can also provide data on ciliary beat pattern and metachronal waves.

Spectrally encoded interferometric microscopy (SEIM) can detect displacement on the nanometer scale at a kilohertz frame rate and when coupled with a wavelength-swept laser and a spectral disperser, SEIM can image tissue *en face*.[13] Our group developed a SEIM system based on a swept source OCT system using a vertical-cavity surface emitting laser (VCSEL) with the

intention of eventually measuring functional parameters of cilia across the surface of upper airway tissues. In this study, we aim to validate this SEIM system as a means of measuring ciliary beat frequency in *ex vivo* human upper airway tissue with high-speed video of phase-contrast microscopy.

Materials and Methods

Ex vivo Mucosal Sample Acquisition and Preparation

The Institutional Review Board of the University of California, Irvine (UCI) reviewed and approved of all procedures. Tissue was collected from a patient undergoing primary functional endoscopic sinus surgery. Our patient was perioperatively administered topical epinephrine at 1:100,000 concentration via intranasal cotton pledgets for hemostasis. Tissue samples were collected from the inferior turbinate. Following resection, tissue samples were immediately placed in Hank's Balanced Salt Solution (HBSS) without Calcium and Magnesium (Fisher Scientific International, Inc., Hampton, NH) maintained at approximately physiologic temperature during tissue transport for imaging. To maintain this temperature, a specimen cup was filled with HBSS at physiologic temperature and was placed in a vacuum insulated container (Stainless King Vacuum Insulated Food Jar, THERMOS, Schaumburg, IL) along with three individual air-activated hand warmers (Hot Hands, Dalton, Georgia). We found that this method adequately maintained the sample at physiologic temperature during tissue transport to our imaging facilities. After incubation, tissue samples were placed in in culture dishes; a thin layer of HBSS was poured into these dishes to lightly cover the tissue. These culture dishes were then placed on heated stages (Microscope Temperature Control Stage Slide Warmer, Amscope, Irvine, CA) at 37 degrees Celsius for imaging. Imaging was performed within 90 minutes after tissue removal.

Image Data Acquisition

Cilia imaging was conducted with a SEIM system driven by a swept-source laser (Thorlabs, Newton, NJ). The A-line rate for this system is 100 kHz while the center wavelength is 1.3 um. The lateral resolution and axial displacement sensitivity are 1.2 um and 0.3 nm, respectively. This system can image *en face* displacement up to 100 fps with 1000 A-lines per image. During imaging with this device, the tissue was placed on a heated stage that was fastened to a manual translation stage (Thorlabs, Newton, NJ). This stage was manually adjusted in the x, y and z-axes in order to clearly resolve the surface of the tissue mucosa. Once a patch of ciliated cells was resolved within the field of view, data was collected.

Our SEIM system was validated with phase-contrast imaging of the cilia. We collected our high-speed videography according to methods described in a previous study.[14] By attaching a smartphone adaptor (Amscope, Irvine, CA) to a phase-contrast microscope (32 x 16, Axiovert 10; Zeiss, Jena, Germany), we were able to take high speed video of ciliary beat frequency at 60 fps with a smartphone (iPhone 11 Pro, Apple, Cupertino, CA). With this method, we traced the outer edges of the tissue mucosa until we encountered ciliated cells. The least disrupted ciliated cells were then recorded and were saved for further analysis.

Data Analysis

In this study, our SEIM data was processed similarly to previous studies.[13] We used the Hilbert Transform to create the analytical form of the raw SEIM data. From this, the phase term can be extracted. Then, the phase value is converted to the displacement information by the PRD algorithm using Eq. 1. These two steps are conducted at every spatial position and the algorithm is able to map out the *en face* displacement images over time. Using a Cooley-Tukey and Bluestein fast Fourier Transform (FFT) to incorporate the Hilbert transform, the total time complexity to get an *en face* displacement map is O(MNlog2N). Here, M is the number of A-lines per image and N is the number of points per A-line. With the *en face* displacement images, we were able to perform analysis on the pattern and frequency of ciliary beats. By performing a Fourier domain over the entire patch of cilia, the CBF over the surface of the tissue can be visualized as shown in the representative figure (Figure 1).

To analyze the data collected with phase-contrast microscopy, we utilized a code written in MATLAB (MathWorks Inc, Natick, Massachusetts). A three-second video was cropped from each recording and a 3 x 3-pixel area was selected, creating a region of interest (ROI) for analysis (Figure 2). Within each ROI, a FFT was sued to extract frequency information from the signal and a power spectrum and time domain graph was created to find the CBF range for the mucosal sample (Figure 3). A median filter was used to the FFT to reduce noise. This was repeated five times yielding five measurements of CBF.

Results

The frequency found with SEIM was 2.66 ± 0.001 Hz (average ± standard error) while the frequency found with phase-contrast microscopy was 2.61 ± 0.119 Hz. Although the sample size is limited, trends can be inferred between the two cilia imaging modalities.

Discussion

Cilia contribute to the proper function of the immune system in the respiratory tract. Ciliary beat frequency can provide a quantifiable and standardizable means of evaluating mucosal pathophysiology which would have application across a number of clinical settings including the treatment of chronic rhinosinusitis and allergic rhinitis. However, current methods of imaging ciliary beat frequency are limited to high-speed imaging with phase-contrast microscopy of nasal brushings. This method is limited to *ex vivo* imaging and is also labor and time intensive. The *ex vivo* nature of this method does not allow for observation of cilia in their native environment and leaves the tissue subject to damage of ciliated cells, preventing accurate representation of ciliary functional parameters. Previous studies using optical coherence tomography as a means of measuring ciliary beat frequency affords a step towards *in vivo* imaging of cilia which could help progress drug and device development for respiratory disorders by providing better measures of cilia pathophysiology at different time points in response to treatment. However, there still exists a need for a comprehensive *in vivo* imaging modality that can observe more functional measures of ciliary health.

In this study, the ciliary beat frequency acquired with spectrally encoded interferometric microscopy was similar to that acquired with phase-contrast microscopy. Although our sample size is limited to a single patch of cilia from tissue from a single patient subject, this trend

indicates that SEIM can be used as a means of measuring ciliary beat frequency *ex vivo.* One limitation of this study stemmed from the *ex vivo* nature of this study. Not only is the tissue being sampled out of the native environment of the upper airway, but removal of the tissue can lead to changes in ciliary beat frequency due to damage of ciliated cells. Previous studies have found that removal of cilia using the nasal brushings method led to disrupted ciliated edges which significantly decreased CBF of samples.[15] This suggests that tissue damage may also occur with removal of our mucosal samples in this study. However, with SEIM imaging the entire surface of the tissue can be imaged rather than just the edges which is not possible with phase-contrast microscopy. This provides a more native means of imaging cilia than phase-contrast microscopy which images cilia along the edge of mucosa that are more likely to be damaged during dissection.

Although this study was conducted *ex vivo* and was limited to characterizing CBF, future studies aimed to look at CBF, ciliary beat pattern and metachronal waves *in vivo* will be conducted to progress towards the goal of a quantifiable and standardized measure of mucosal disease conditions clinically. Additionally, our sample size in this study is limited; expanding this study in a systematic process can result in more comprehensive results on the effect of different parameters on ciliary beat frequency. Furthermore, *in vivo* imaging in future studies will afford a more thorough understanding of ciliary parameters in a native environment.

Conclusion

Using the gold standard method for finding ciliary beat frequency, high speed videography with phase contrast microscopy, spectrally encoded interferometric microscopy was validated as a means of finding ciliary beat frequency in *ex vivo* human upper airway mucosa. This technology has the potential to better serve as a method of collecting metrics on ciliary health. The benefits of SEIM include the ability to image tissue *in vivo*, image ciliary beat frequency *en face* across tissue surfaces, allowing for the data collection of the metachronal wave and ciliary beat pattern. Future studies are needed to further develop and validate this technology to optimize this device for clinical use.

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$$\Delta d(x,\ t) = \frac{\lambda}{4\pi n} * \Delta\varphi(x,\ t)$$

Equation 1

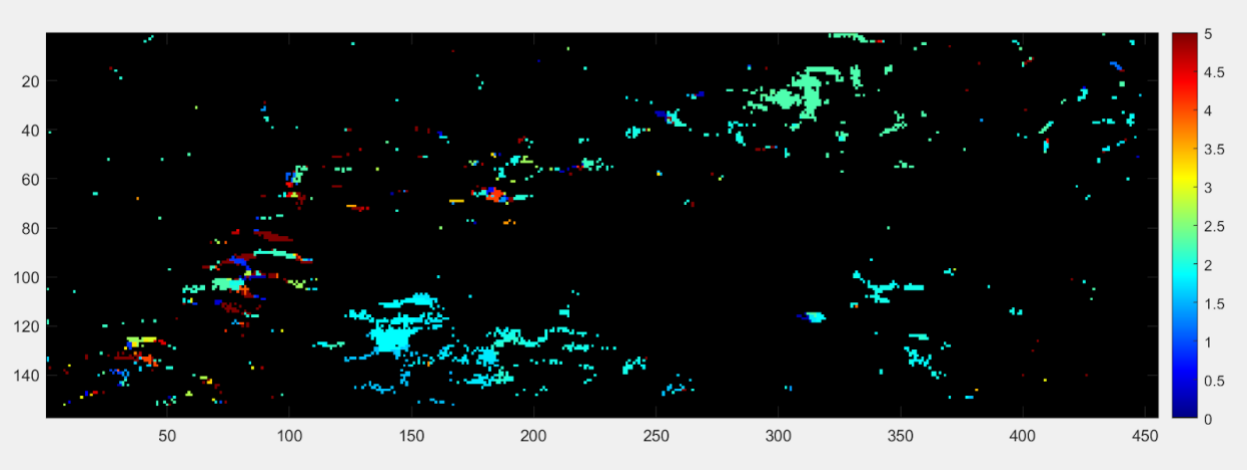

Figure 1: Representative image of a heat map looking at mucosa tissue *en face* acquired with SEIM. A color legend with corresponding frequencies is on the right-hand size. The x and y-axes represent arbitrary coordinate values.

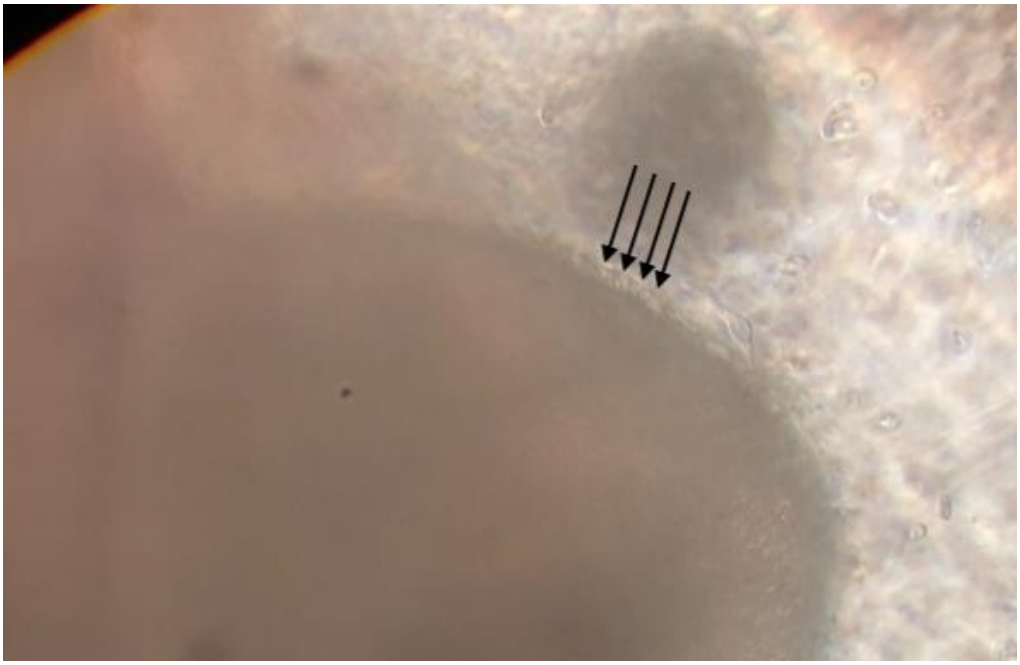

Figure 2: Representative image of a ciliated edge of mucosa under a phase contrast microscope at 20x. Arrows are pointing towards ciliated cells on the edge of the tissue.

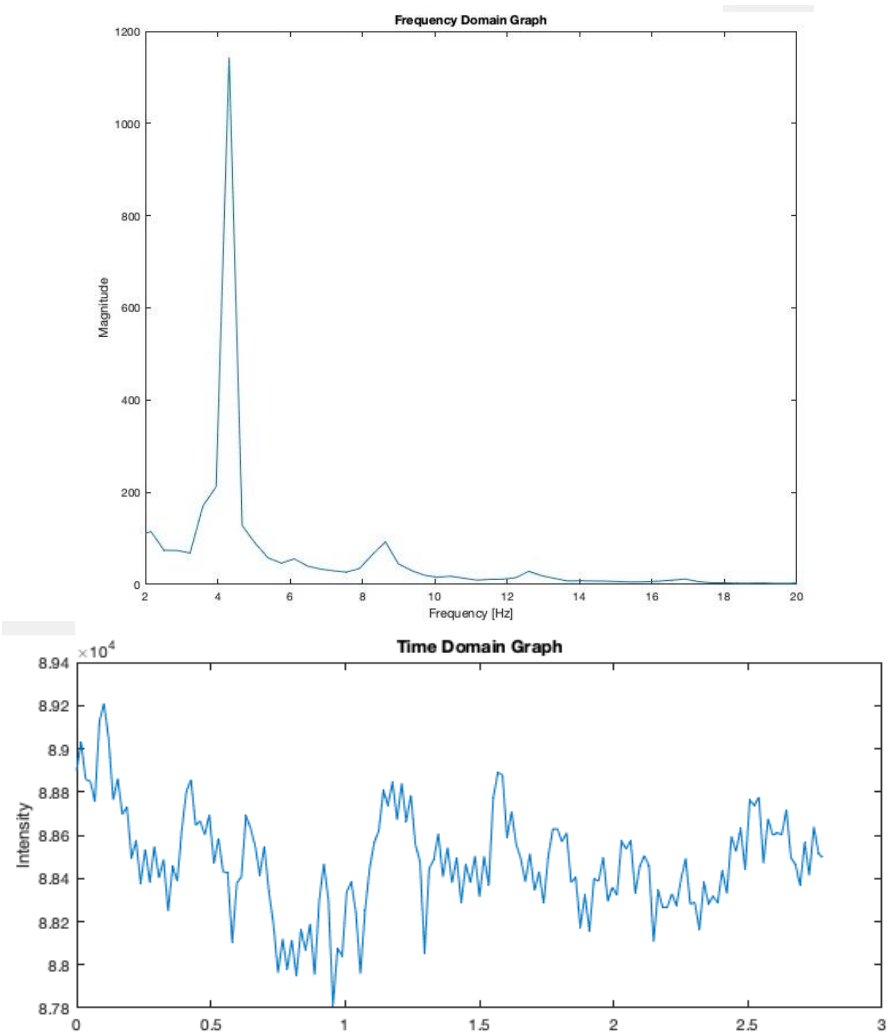

Figure 3: Representative frequency domain graph and time domain graph of ciliary beat frequency.