Video Article

Ex vivo Method for High Resolution Imaging of Cilia Motility in Rodent Airway Epithelia

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URL: https://www.jove.com/video/50343
DOI: doi:10.3791/50343

Keywords: Biomedical Engineering, Issue 78, Developmental Biology, Cellular Biology, Molecular Biology, Anatomy, Physiology, Respiratory Mucosa, Trachea, Ciliary Motility Disorders, Animal Experimentation, Microscopy, Fluorescence, Interference, Polarization, Video, Airway, mucociliary clearance, microscopy, animal model

Date Published: 8/8/2013

Citation: Francis, R., Lo, C. *Ex vivo* Method for High Resolution Imaging of Cilia Motility in Rodent Airway Epithelia. *J. Vis. Exp.* (78), e50343, doi:10.3791/50343 (2013).

Abstract

An *ex vivo* technique for imaging mouse airway epithelia for quantitative analysis of motile cilia function important for insight into mucociliary clearance function has been established. Freshly harvested mouse trachea is cut longitudinally through the trachealis muscle and mounted in a shallow walled chamber on a glass-bottomed dish. The trachea sample is positioned along its long axis to take advantage of the trachealis muscle to curl longitudinally. This allows imaging of ciliary motion in the profile view along the entire tracheal length. Videos at 200 frames/sec are obtained using differential interference contrast microscopy and a high speed digital camera to allow quantitative analysis of cilia beat frequency and ciliary waveform. With the addition of fluorescent beads during imaging, cilia generated fluid flow also can be determined. The protocol time spans approximately 30 min, with 5 min for chamber preparation, 5-10 min for sample mounting, and 10-15 min for videomicroscopy.

Video Link

The video component of this article can be found at https://www.jove.com/video/50343/

Introduction

Analysis of motile cilia function in the airway epithelia is experimentally important for elucidating the genetic and environmental factors that can affect mucociliary clearance and pulmonary health [1]. The simple protocol developed for imaging the mouse airway epithelia provides an efficient method to interrogate airway cilia motility in mutant and knockout mouse models and require only basic skills in mouse tracheal tissue dissection and *ex vivo* imaging of airway cilia motility with high resolution videomicroscopy. This protocol was established and refined during a large-scale mouse mutagenesis screen to allow rapid evaluation of motile cilia function (cilia beat frequency, cilia beat shape, cilia generated flow) in mutants with congenital heart disease associated with heterotaxy [2-5].

Current techniques used to study airway cilia motility can be grouped into either the acute *ex vivo* type or longer term *in vitro* experimental approaches. Acute experiments include *ex vivo* visualization of human nasal/airway brush biopsies [6,7] and analysis of simple transverse airway sections [8]. The *in vitro* approaches utilize various cell culture techniques to generate sheets of differentiated ciliated epithelia such as in air liquid interface cultures or airway suspension cultures[9-11]. However, these airway epithelia reciliation techniques require very significant investment in time and training before any useable ciliated epithelial cells are produced for experimentation (4-6 weeks [9,10]). While acute *ex vivo* analysis of airway epithelial brush biopsies are commonly used for human clinical studies, this method is not usable in mouse studies due to exacerbated mechanical tissue injury [12].

The technique outlined in this protocol for analysis of mouse tracheal airway epithelia is not only simple to execute, but it requires no special dissection skills nor any specialized equipment besides those standard for imaging by videomicroscopy. There are many advantages to this simple protocol. First, as the mouse trachea tissue harvest is quick and easy to perform, it allows for rapid assessment of airway cilia function in a large number of mice. This can include acute analysis of the short term effects of different *in vitro* treatments. Second, being an *ex vivo* technique, the ciliated airway epithelium remains attached to it underlying supporting tissues and thus retain associated cell signaling pathways. Therefore in comparison to *in vitro* reciliated airway epithelia, this preparation is a better representation of the natural *in vivo* tissue environment. Third, this protocol allows the acquisition of a number of different quantitative parameters that can provide the objective assessment of motile cilia function. Finally, in contrast to other current methods for airway cilia visualization, this protocol allows for visualization of the cilia at right angles to the cilia beat direction, allowing profile view of the cilia that is optimal for high resolution imaging of cilia beat and metachronal wave generation.

This protocol can be modified in a number of ways to address a wide range of experimental needs such as the role of pharmacological agents, genetic factors, environmental exposures, and/or mechanical factors such as mucus load on airway cilia function and generation/maintenance of airway cilia beat and metachronal wave propagation.

Protocol

1. Reagents Setup

1.1 Dissection and Imaging Medium

Leibovitz's L-15 medium (L15) is supplemented with FBS (10%) and Penicillin-Streptomycin (100 units/ml of penicillin G sodium and 100 µg/ml of streptomycin sulfate) is used during both harvest and imaging of trachea samples.

2. Shallow Walled Culture Chamber Assembly

The chamber used to hold trachea tissue is shown in **Figure 1**. The floor of the chamber is the glass bottomed 35 mm culture dish. The top and side of the chamber is generated by placing a small piece of 0.3 mm thick silicone sheeting over the glass dish. The edge of the sheet is cut to fit the round bottom dish and the center is further trimmed to form a shallow central chamber (see steps 2.1-2.3). On top of this assembly, a 18 mm round glass cover slip is placed to generate an enclosed chamber suitable for imaging.

1. Completely cover a 18 mm round glass cover slip with a square of 0.3 mm thick silicone sheeting.
2. With standard sharp dissection scissors trim off the excess silicone sheeting from around the edge of the cover slip (resulting in a circular layer of silicone sheeting).
3. With a sharp scalpel cut out and remove a central square of silicone sheeting (approximately 10 mm square) from the middle of the covered cover slip (thus forming the top and sides of the imaging chamber).

3. Trachea Harvest and Preparation

1. Euthanize mice in accordance with local institutional animal care and use committee and/or governmental guidelines and remove trachea into a standard plastic 35 mm culture dish with enough L15 media to submerge tissue (authors have used mice just before birth at gestation day 16.5, through to newborn, neonatal, and adult animals [2]).
2. Remove non-trachea associated tissue attached to the outside of the trachea using blunt dissection.
3. Remove the larynx with a transverse cut just below the cricoid cartilage using micro dissection scissors (**Figure 2A**, line 1).
4. Remove the left and right primary bronchial branches with a transverse cut just above the carina using micro dissection scissors (**Figure 2A**, line 2).
5. Gently hold the trachea with fine forceps and flush the lumen 2-3 times with ~1 ml of the bathing L15 medium with a P1000 Pipetman and P1000 tip to clear any mucus and blood.
6. Cut out a 3-4 ring segment of trachea using a transverse cut with micro dissection scissors (**Figure 2B**, line 1).
7. Cut longitudinally through the middle of the trachealis muscle of this short trachea segment using micro dissection scissors (**Figure 2B**, line 2).
8. Cut longitudinally through the middle of the tracheal cartilage rings using micro dissection scissors (**Figure 2C**, line), leaving two sections of trachea tissue suitable for imaging (single section shown in **Figure 2D**).

4. Cilia Imaging

1. Transfer trachea tissue segments, lumen side down, onto the middle of a 35 mm glass bottom culture dish with 100-200 µl of L-15 medium.
2. To quantify cilia generated flow add a small amount of fluorescent 0.20 µm microspheres to the L-15 medium bathing the trachea tissue sample (~1 x 10^{11} particles/ml or 20 µl/ml of the Fluoresbrite 2.5% aqueous microsphere suspension).
3. Place the imaging chamber prepared previously (See Procedure 1-3 above) on top of the trachea sample silicone sheet side down, with the trachea sample located in the middle of the shallow walled chamber formed by the window cut into the silicone sheeting (**Figure 1**).
4. Gently push down on the coverslip to secure in place and remove any excess L-15 media from the edges using a P1000 tip and P1000 Pipetman.
5. Mount the 35 mm glass bottom culture dish and trachea sample on a inverted microscope with 100X objective and DIC optics.
6. Using a high-speed camera and movie acquisition software collect movies of cilia motility at 200+ frames per second along the ciliated tracheal epithelia of the curled up edge of the trachealis muscle (**Figure 2D** Outlined box, **E:** example still image from recorded movie) (**Supplementary Movie 1**).
7. Using FITC epifluorescent imaging and a low light CCD camera (See equipment list above), collect movies of fluorescent microsphere movement across the surface of the ciliated tracheal epithelia to allow later quantification of cilia generated flow (**Supplementary Movie 2**).

5. Quantitation of Cilia Motility

1. Load DIC imaged movies into the ImageJ software package and following the technique described by Iain Drummond [13] use the line tool to draw a raster line crossing the beating cilia. A "reslice" of this line generates a kymograph type image where cilia movement across the line generates a wave pattern. The number of pixels between each wave peak is them measured (one pixel = one movie frame) from which the number of beats per minute (*i.e.* Hz) can then be calculated.

2. To measure cilia generated flow load fluorescent bead movies into the ImageJ software package, and use the MTrackJ plugin [14] to manually track fluorescent beads across the surface of the trachea epithelia and let the software generate bead velocity and directionality for each bead tracked.

Care should be taken when using fluorescent beads to quantify flow as the distance from the beating cilia can strongly influence measured flow magnitude. The bead movement in Supplemental **Movie 2** is a good example of this. In this example bead movement is fast at the surface of the beating cilia and significantly drops off for beads further away in the bathing media. To control for this, bead tracings should only be collected at a fixed distance above the ciliated epithelia in all samples to allow correct comparisons to be made. Finally, to obtain representative data on fluid flow, beads should be tracked for as long as possible; we prefer to use bead tracks that cover 10+ ciliated cells for calculating fluid flow.

Representative Results

Control airway cilia should be clearly visible and seen to beat in a coordinated manner (**Supplemental Movie 1**; movie playback is slowed to 15% real time), with noticeable flow in the direction of cilia beat (**Supplemental Movie 2**; movie playback is 100% real time). Quantitation of cilia movies should yield results similar to that seen in **Figure 3**. Collection of high-speed DIC movies makes possible the quantification of cilia beat frequency (**Figure 3C**) and gives good images for qualitative assessment of cilia beat shape, while the tracking of fluorescent beads allows for the measurement of cilia generated flow velocity (**Figure 3D**) and directionality (**Figure 3E**).

We use directionality to give an index of linear flow. Directionality is calculated by dividing the total displacement traveled by a fluorescent bead (*i.e.* the shortest distance between the start and end points of the tracing) by the distance a fluorescent bead moves over the whole trace. Thus, a fluorescent bead moving in a straight line would have a directionality of one while a fluorescent bead meandering extensively from a straight path will have a directionality much less than 1.

We have imaged samples for up to an hour with out any noticeable alteration in cilia beat function.

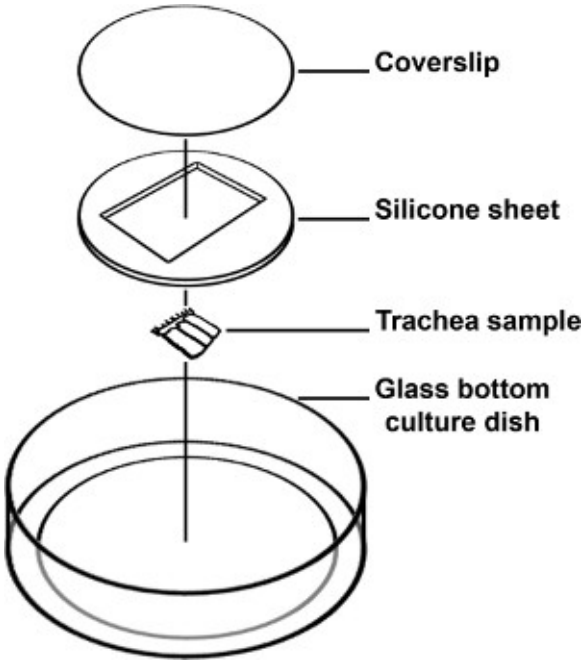

Figure 1. Enlarged isometric view of culture dish setup and trachea sample positioning. Note: the coverslip and silicone sheet should be assembled prior to putting the trachea sample into the culture dish.

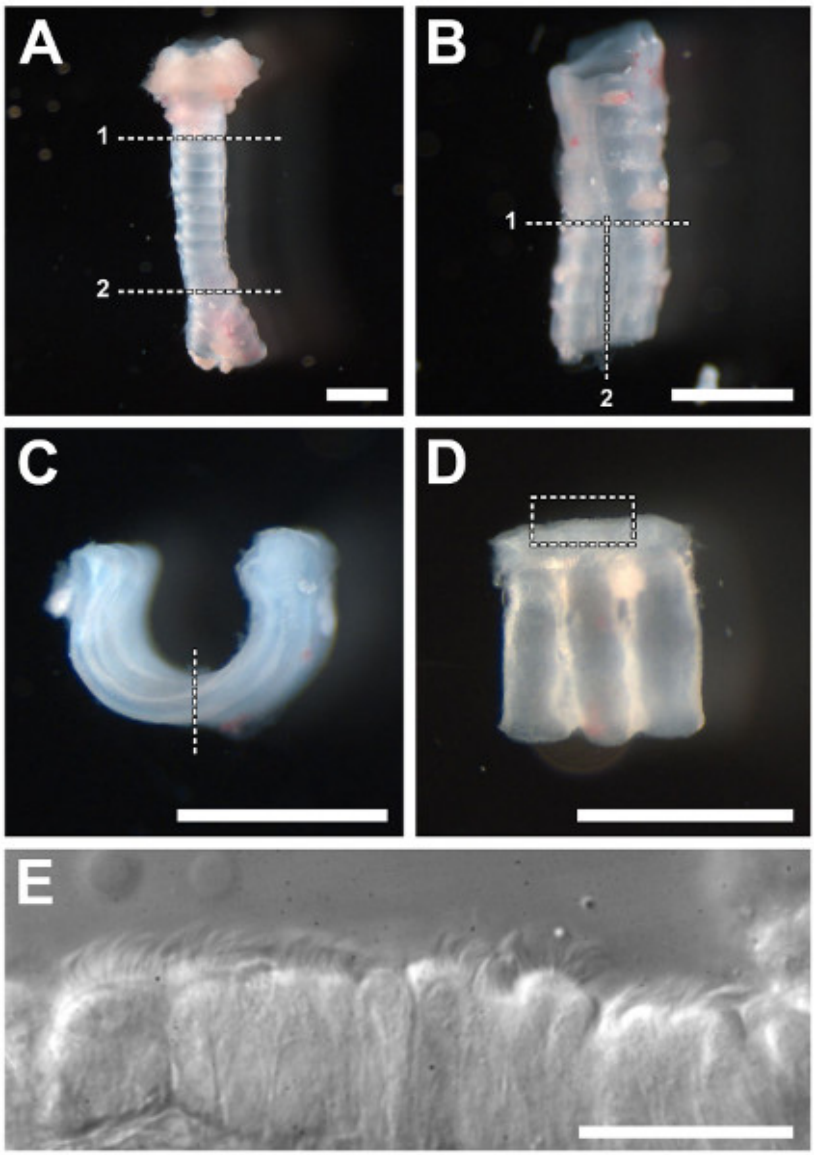

Figure 2. Example of a mouse trachea dissection using this protocol, starting from an intact trachea (A) to the final imaged result (E) (see procedure steps 3.2-3.8 for explanation of labels). Scale bars in A-D represent 500 µm. Scale bar in E represents 25 µm.

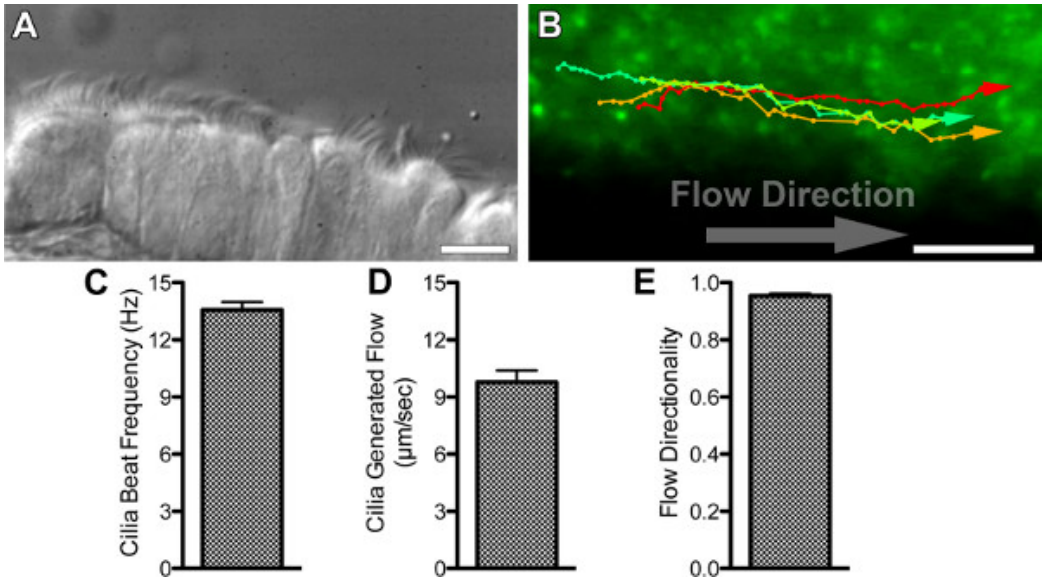

Figure 3. Anticipated results obtained using a wild type mouse trachea sample as prepared by the protocol described here. (A) Single frame from a high-speed DIC movie (See supplemental **movie 1** for representative DIC movie). **(B)** Movement tracks of four fluorescent beads across the surface of a tracheal epithelia sample (See supplemental **movie 2** for representative fluorescent movie). **(C)** Cilia beat frequency quantified from high-speed DIC cilia motility movies (n=41 random point measurements from 6 mouse tracheas). **(D)** Flow speed and **(E)** Flow directionality quantified from tracking fluorescent bead movement across the surface of tracheal epithelia samples (n=31 fluorescent bead tracings from 6 mouse tracheas). Data is displaced as mean ± SEM. Scale bars 10 μm.

Supplemental Movie 1. DIC movie showing cilia beat shape and cilia generated flow observed using a wildtype mouse trachea sample. The movie was collected at 200 fps, while playback is 30 fps. Click here to view movie.

Supplemental Movie 2. Movie of cilia generated flow across a wildtype mouse trachea sample using 0.20 μm fluorescent microspheres and epifluorescent microscopy. Movie collection and play back is 15 fps. Click here to view movie.

Discussion

Measurement of cilia beat frequency (CBF) is relatively easy using high power microscope objectives and fast image acquisition hardware [13,15], and explains why CBF measurements form the basis of most studies investigating mucociliary clearance during health and disease. However, while CBF measurement is essential for understanding mucociliary clearance, measurement of CBF alone ignores the underlying importance of both ciliary generated flow and cilia beat waveform, both of which are more difficult to measure, are often ignored, and may not correlate with CBF. An example of how increased CBF may not correspond with increased ciliary generated flow is seen in some patients with DNAH11 mutations. DNAH11 is an axonemal dynein heavy chain protein, mutations in which have been reported to cause both hyperkinetic (faster) ciliary beat and abnormal cilia beat waveform that is predicted to cause problems with mucociliary clearance [16]. The significant advantage of our protocol is that it allows for easy visualization of the cilia at right angles to the cilia beat direction, allowing a profile view of cilia that is optimal for high resolution imaging of cilia beat shape and cilia generated fluid flow across the surface of the beating cilia, thus allowing direct comparison of CBF with cilia beat waveform and/or cilia generated flow within a single sample.

Another advantage of this technique is that cilia in freshly harvested trachea retain their ability to beat in a coordinated manner across the whole surface of the respiratory epithelium, thus allowing easy correlation between CBF and cilia generated flow. Such coordinated ciliary motion is not observed in reciliating mouse tracheal epithelial cultures, which often ciliate poorly and show cilia beat in random directions [9,10]. The coordinated ciliary beat observed using the tracheal epithelia technique described here can provide the means to interrogate the mechanisms regulating ciliary beat coordination and its potential role in mucociliary clearance.

The most challenging part of this protocol for researches will be trachea preparation, including clearing of fat and connective tissue from the trachea surface (**Figure 2a**), and longitudinally cutting through the middle of the trachealis muscle (**Figure 2b**, line 2). We find using good micro dissection scissors essential for both tissue clearing and subsequent trachealisotomy. Furthermore, we find it easier to use the lower portion of the trachea just anterior to the carina where the trachea bifurcates into the left and right primary bronchi as the trachealis muscle here is widest. However, with sufficient experience, any length of the trachea, or indeed the primary bronchi can be used successfully to generate high-quality movies of cilia motility and cilia generate flow.

Possible technical problems associated with this protocol include the trachea sample moving during imaging and/or the sample not being able to be brought into focus. Both of these problems can arise if too much L-15 was added with the sample and/or the imaging chamber cover slip was not pressed down securely; this causes the sample to float within the cover slip chamber resulting in sample movement or the sample floating above the focus plane making focus impossible. To avoid this it is important to try and minimize the amount of liquid used to mount each trachea sample.

A possible limitation of this technique is that measurement of cilia generated fluid flow may not completely match *in vivo* mucociliary transport. Our finding of cilia generated flow of ~10 μm/sec is significantly less than the 100 μm/sec mucociliary transport reported using *in vivo* mouse

trachea [17]. This difference is probably due to the cilia being submerged in media as opposed to simply moving a thin layer of mucus across the air-liquid interface. Nevertheless, this method has great utility for relative comparison of ciliary motility between different samples.

It should be noted that while the experimental technique and data presented here was conducted at room temperature, temperature itself plays a significant role in regulating cilia activity. CBF in human nasal and tracheal samples is seen to be linearly correlated with temperature between 5-20 °C, with a small increase in CBF seen between 20-45 °C [18]. We see a similar relationship between temperature and CBF in mouse airway samples (unpublished observations). Thus, while we record cilia activity at room temperature for quick assessment of possible defects in cilia motility in novel mutant mice, we also incubate some samples at 37 °C for determining cilia activity under physiological conditions. To accomplish this a standard microscope incubation chamber can be used to maintain samples at 37 °C.

Simple modifications to this protocol will make it a powerful tool for assessing the regulation of airway cilia activity by a wide range of factors such as the role of pharmacological agents, temperature, genetic factors, environmental exposures, and/or mechanical factors such as mucus load on airway cilia function and generation/maintenance of airway cilia beat.

Disclosures

Authors have nothing to disclose.

Acknowledgements

The project was supported by NIH grant U01HL098180 from the National Heart, Lung, and Blood Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

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