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Observing planar cell polarity in multiciliated mouse airway epithelial cells

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Abstract

The concerted movement of cilia propels inhaled contaminants out of the lungs, safeguarding the respiratory system from toxins, pathogens, pollutants, and allergens. Motile cilia on the multiciliated cells (MCCs) of the airway epithelium are physically oriented along the tissue axis for directional motility, which depends on the planar cell polarity (PCP) signaling pathway. The MCCs of the mouse respiratory epithelium have emerged as an important model for the study of motile ciliogenesis and the PCP signaling mechanism. Unlike other motile ciliated or planar polarized tissues, airway epithelial cells are relatively easily accessible and primary cultures faithfully model many of the essential features of the *in vivo* tissue. There is growing interest in understanding how cells acquire and polarize motile cilia due to the impact of mucociliary clearance on respiratory health. Here, we present methods for observing and quantifying the planar polarized orientation of motile cilia both *in vivo* and in primary culture airway epithelial cells. We describe how to acquire and evaluate electron and light microscopy images of ciliary ultrastructural features that reveal planar polarized orientation. Furthermore, we describe the immunofluorescence localization of PCP pathway components as a simple readout for airway epithelial planar polarization and ciliary orientation. These methods can be adapted to observe ciliary orientation in other multi- and monociliated cells and to detect PCP pathway activity in any tissue or cell type.

Introduction

Motile multicilia on the epithelial cells lining the respiratory tract beat in a concerted, directional manner. Inhaled contaminants trapped by the mucus layer covering the airway surface are thereby propelled from the lungs. Directional ciliary motility is essential for a healthy respiratory system, and the breakdown of this process is associated with asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis.

Multiciliated cells (MCCs) of the airway epithelium each contain 200—300 motile cilia. For concerted, directional movement, each cilium must be physically aligned with respect to the proximal—distal (P-D, oral-lung) axis of the airways (Figure 1). This can be observed at the ultrastructural level: the basal foot appendage on individual cilia points toward the beat direction and a line passing through each microtubule of the axonemal central doublet is perpendicular to it (Boisvieux-Ulrich, Laine, & Sandoz, 1985; Gibbons, 1961) (Figure 1). This type of alignment along the plane of the epithelium is termed planar cell polarity (PCP). In order to beat in the correct direction, an individual cilium has to be aligned with respect to all the other cilia in the same MCC, with respect to cilia in neighboring MCCs and with respect to the P-D tissue axis.

The PCP signaling mechanism, which was first shown to control the tissue-level polarity of body hairs and eye facets in *Drosophila* (Vladar, Antic, & Axelrod, 2009), underlies this orientation. Although the mammalian pathway is still poorly understood, conservation with flies suggests that a multitier pathway controls motile ciliary orientation (Boutin et al., 2014; Guirao et al., 2010; Mitchell et al., 2009; Vladar, Bayly, Sangoram, Scott, & Axelrod, 2012). Motile cilia can be found on the mammalian airway, ependymal, and oviduct epithelium and the embryonic frog skin, and appear to align in a similar manner. At the heart of this mechanism is the alignment of neighboring cells with each other by two groups of core PCP proteins that are distributed at the proximal and distal side apical cell junctions, respectively (Figure 2). Within MCCs, individual cilia are linked into a common orientation by microtubules and actin filaments (Werner et al., 2011), then as an intracellularly polarized layer, appear to be linked by additional cytoskeletal elements to the proximal side core PCP domain, aligning them with the axis of P-D polarity (Vladar et al., 2012). How the directionality of the P-D core PCP domains, also termed “crescents,” is established remains unknown. In flies, the position of the crescents is determined, at least in part, by another set of PCP proteins, which are conserved in vertebrates and may also act in this capacity. In addition to PCP signaling, directional hydrodynamic forces generated by the propulsion of extracellular fluid by the cilia themselves also contribute to P-D alignment (Guirao et al., 2010; Mitchell, Jacobs, Li, Chien, & Kintner, 2007).

In the developing mouse trachea, PCP signaling precedes motile ciliogenesis (Vladar et al., 2012). The core PCP proteins Van Gogh-like 1 (Vangl1) and Frizzled 6 (Fzd6) localize to P-D aligned crescents starting at embryonic day 14.5 (E14.5). Thus, motile cilia emerge at E16.5 in an already molecularly polarized cell. Cilia are initially immotile and poorly aligned, but gradually refine their orientation to produce robust, directional fluid flow starting at postnatal day 9 (P9). PCP crescents persist for the life of the organism (Vladar et al., 2012) and are restored following injury to the airway epithelium (EKV, unpublished observation).

The sequential acquisition of PCP signaling, motile ciliogenesis and directional ciliary motility can be observed in vivo as well as in de novo differentiated primary mouse tracheal epithelial cell (MTEC) cultures (Vladar & Brody, 2013; You, Richer, Huang, & Brody, 2002). It is important to note that in the absence of a developmentally defined P-D tissue axis, MTECs will show local, but no global cilium and PCP crescent alignment. This is likely mediated by the core PCP cassette in the absence of global signals. Human and

porcine TECs will occasionally spontaneously globally align in a circular pattern (Oldenburg, Chhetri, Hill, & Button, 2012). However, this does not tend to occur in MTECs.

Ciliary planar polarity can be detected and assessed in multiple ways. Here, we describe the electron and super-resolution microscopic observation of the basal foot and microtubule doublet of cilia, which reveal beat direction, and the localization of core PCP proteins, which correlate with both the orientation of ciliary beat direction and the P-D axis of polarity. Beat direction can also be directly observed by time lapse microscopy of cilia or the transport of fluorescent particles, methods for which have been recently presented elsewhere (Francis & Lo, 2013).

1. Acquisition or Generation of Multiciliated Airway Epithelial Cells

MCCs can be found in the lining of the mouse nasal cavity, sinuses, trachea and bronchi, but they are most easily observed in the trachea. Mature MCCs with maximally P-D aligned motile cilia are ideally observed starting at three weeks after birth (Vladar et al., 2012). MCCs can also be generated by in vitro differentiation of epithelial progenitor cells isolated from trachea.

1.1 Obtaining in Vivo Multiciliated Airway Epithelium

The polarity of ciliary ultrastructure can be observed in isolated mouse trachea by electron microscopy (EM, Section 2.1) or super-resolution microscopy (Section 2.2). The directionality of asymmetric core PCP protein localization can be observed by immunofluorescence labeling (Sections 3.1 and 3.2). Planar polarity is a tissue-level phenomenon, therefore these assays require intact multiciliated epithelium where the P-D tissue axis is tracked throughout the experiment. Tracheal P-D orientation can be easily tracked by the position of the larynx (proximal side) and/or the bifurcation (distal side).

1.1.1 Materials

1. Mice at desired age
2. Sterile surgical tools
3. Ham's F12 cell culture medium (cat. no. 11765-054, Life Technologies) +1% penicillin—streptomycin (cat. no. 15140-148, Life Technologies).

1.1.2 Method

1. Euthanize mouse by CO₂ asphyxiation following guidelines set by the Institutional Animal Care and Use Committee. Do not perform cervical dislocation as this ruptures the tracheal rings.
2. Pin mouse in the supine position to dissecting board, douse with 70% alcohol and isolate trachea as follows:
 - a. Cut through the skin using dissecting scissors to expose the region from abdomen to mandible.
 - b. Separate and remove the salivary glands to reveal the tracheal area.

- c. Cut open the rib cage offset from the midline, then resect the neck muscles to expose the trachea.
 - d. Remove trachea, including the larynx and the bifurcation, by first transecting it above the larynx (the trachea will recede slightly into the rib cage). Then grasp the larynx with forceps and gently pull the trachea up and out of the body and carefully trim away the muscles that hold it in place. Once exposed, transect the trachea below the bifurcation.
 - e. Place trachea in Ham's F12 +1% pen-strep on ice.
3. Dissect the esophagus, thyroid, thymus, blood vessels and muscle away from the trachea in a Petri dish with ice cold Ham's F12 + 1% pen-strep.
 4. While grasping the larynx with forceps, expose the lumen of the trachea by cutting it open longitudinally along the dorsal surface with small dissecting scissors or spring scissors.
 5. Proceed with preparation for immunofluorescence, electron or time-lapse microscopy or with epithelial cell isolation for primary culture.

1.2 In Vitro Differentiation of Multiciliated Airway Epithelial Cells

The primary MTEC culture protocol has been recently described in detail (Vladar & Brody, 2013; You & Brody, 2013). In short, epithelial cells are isolated from adult mouse trachea using protease digestion and seeded onto a porous Transwell filter (Corning). Initially, the filter is submerged in culture medium, which supports the proliferation of the progenitor basal cells (You et al., 2002). Upon confluency, an air—liquid interface (ALI) is created by supplying medium only below the filter, which promotes the de novo differentiation of multiciliated and secretory cell types. ALI day 14 MTEC cultures typically model the adult airway epithelium with many locally aligned MCCs (Vladar et al., 2012).

2. Assessment of Ultrastructural Planar Polarity of Motile Cilia

The planar polarity of individual cilia is revealed by the orientation of its ultrastructural features: the basal foot appendage of the basal body points in the direction of the beat and a line passing through both microtubules of the central axonemal doublet is perpendicular to it (Boisvieux-Ulrich et al., 1985; Gibbons, 1961) (Figure 1). Due to the small size of these structures and the high density of motile cilia at the apical cell surface, EM remains the gold standard for detailed observation of ciliary planar polarization. However, immunofluorescence microscopy of cilium proteins can also be used to assess orientation.

2.1 Transmission Electron Microscopy of Polarized Ciliary Structures

2.1.1 Materials

1. Trachea or MTEC cultures at desired age or stage
2. EM-grade reagents (Electron Microscopy Sciences), handle hazardous materials and collect waste appropriately

2.1.2 Method

1. Prepare samples for fixation:
 - a. If processing mouse trachea: obtain trachea by dissection in Ham's F12 medium on ice (see Section 1.1). Take care to include the larynx and/or the bifurcation to serve as tissue polarity markers during the sample preparation. Grasp the trachea by the larynx using forceps and cut open longitudinally along the dorsal side using dissecting scissors to expose the lumen. Fix and process in 2 mL screw cap tubes.
 - b. If processing MTEC cultures: obtain Transwell filter cultures by differentiating basal cells isolated from mouse trachea (see Section 1.2). Cut filter out of plastic support by placing it cell-side down on a clean Petri dish and carefully cutting around the inside edge with a fresh razor blade. The filter may be cut into multiple segments. Fix and process in 2 mL screw cap tubes.
2. Fix in 2% glutaraldehyde, 4% paraformaldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, for 15 min at room temperature, then change to fresh fixative and fix overnight at 4 °C.
3. Stain with 1% aqueous osmium tetroxide for 2 h at 4 °C, rotating.
4. Rinse three times with dH₂O.
5. Stain in 1% uranyl acetate in dH₂O overnight at 4 °C, rotating.
6. Dehydrate by incubating in:
 - a. 50% ice cold ethanol, 5 min on ice
 - b. 70% ice cold ethanol, 5 min on ice
 - c. 95% ice cold ethanol, 10 min at room temperature
 - d. 100% room temperature ethanol, twice for 10 min at room temperature.

Tannic acid may be added to the last ethanol wash at 1 % to improve the visualization of ciliary structures and cytoskeletal elements.
7. Incubate in acetonitrile, 5 min at room temperature, rotating.
8. Gradually infiltrate with EMbed 812 embedding resin:
 - a. 1:1 EMbed 812:acetonitrile, 1 h at room temperature, rotating
 - b. 2:1 EMbed 812:acetonitrile, overnight at room temperature, rotating
 - c. 100% EMbed 812, with tubes uncapped, 2—4 h at room temperature, rotating
9. Fill embedding capsule with 100% EMbed 812 resin.
10. Embed samples as follows:

- a. If processing mouse trachea: using a razor blade, trim off the larynx and the bifurcation to liberate a relatively flat, rectangular area of tissue. The trachea may curve back slightly into a cylinder. Take care to track orientation and place the trachea into the bottom of the capsule with the luminal side facing up and proximal side facing north. Be sure to note the orientation of the trachea on the capsule label.
- b. If processing MTEC cultures: place filter cell side (identified by gently scraping the edge of filter under a dissecting microscope) facing down into the bottom of capsule.

11. Bake capsules 24 h at 60 °C to cure EMbed 812.

12. If processing trachea, keep track of tissue orientation by trimming the block face into a trapezoidal shape. Generate a “map” of the tissue based on toluidine blue stained thick sections, which will be used to orient the thin sections under the transmission electron microscope (TEM).

13. Cut 80—100 nm thin sections and mount onto copper grids.

14. Stain with uranyl acetate and lead citrate and analyze with TEM.

2.1.3 Analysis—In wildtype trachea, the directionality of the ciliary basal foot and the central axonemal microtubule doublet will reveal the direction of motility (Figure 1). PCP defects can lead to the misalignment of cilia within cells, with respect to neighboring MCCs or with respect to the P-D tissue axis (Vladar et al., 2012).

To assess cilium directionality based on central axonemal microtubule doublet orientation, draw a line passing through both microtubules of the doublet and calculate the angle formed by the line and the proximal direction as marked by the position of the larynx. For maximally P-D cilia, the angle will be close to 90°. Note that this assay can only detect a deviation $\pm 90^\circ$ of orientation.

To assess cilium directionality based on basal foot orientation, draw a line passing through the center of the basal body and the tip of the basal foot and calculate the angle made by the line and the proximal direction as marked by the position of the larynx. For maximally P-D cilia, the angle will be close to 0°. This assay can detect a deviation $\pm 180^\circ$ of orientation.

The ImageJ (National Institutes of Health) software (Schneider, Rasband, & Eliceiri, 2012) is ideal for analyzing images and calculating the angles of deviation between the position of the ciliary structures and the proximal direction. Assessing and quantifying parameters of ciliary orientation requires the use of circular statistics software such as Oriana (Kovach Computing Services), the Circular package for R (R Foundation for Statistical Computing), or the CircStat toolbox for Mat-lab (The Mathworks, Inc.). Data can be obtained for intracellular or tissue-wide ciliary orientation and can be depicted on circular graphs or “rose plots.”

2.2 Super-Resolution Light Microscopy of Polarized Ciliary Structures

EM provides extremely high resolution, and with immunoelectron microscopy, can be used to determine the nanoscale localization of specific proteins at ciliary structures. However, it is also a cumbersome technique, and polarized ciliary structures such as the basal foot can be observed with light microscopy. By using appropriate immunofluorescence markers, the basal body (~ 250 nm in diameter) can be distinguished from the basal foot (~ 100 nm in length) with conventional diffraction-limited light microscopy, which has ~ 200 nm resolution.

For light imaging of closely apposed ciliary structures, super-resolution microscopy techniques (Schermelleh, Heintzmann, & Leonhardt, 2010), which have effective resolutions of ~ 20—100 nm, are more appropriate. In addition, super-resolution immunofluorescence microscopy can be used to distinguish the relative localizations of proteins within polarized ciliary structures such as the basal foot. This would not be possible with conventional light microscopy and cumbersome to do routinely with immunoelectron microscopy. Either antibodies against known markers of the basal body and the basal foot or lentivirally expressed epitope-tagged transgenes can be used with the various super-resolution microscopy techniques. Here, we will focus on three-dimensional structured illumination microscopy (3D-SIM) immunofluorescence imaging of the transition fiber component Cep164 (Graser et al., 2007; Lau, Lee, Sahl, Stearns, & Moerner, 2012) and the basal foot component γ -tubulin (Hagiwara, Kano, Aoki, Ohwada, & Takata, 2000) (Figure 3). 3D-SIM requires minimal adjustment to established immunofluorescence protocols, while permitting super-resolution imaging in up to four channels. We and others have successfully used the Applied Precision Delta Vision OMX instrument to image basal bodies in MTECs (Lee et al., 2014; Zhao et al., 2013), but the methods described here should be broadly applicable to other 3D-SIM platforms. In addition, stimulated emission depletion microscopy has also been used to image Cep164 (Lau et al., 2012) and several distal centriole end and transition zone proteins (Lee et al., 2014) in MTECs.

2.2.1 Materials

1. ALI day 14 MTEC cultures.
2. Fixative: 20 °C methanol; 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, may be used depending on antibodies.
3. Bovine serum albumin (cat. no. 15561020, Life Technologies).
4. High precision #1.5 glass coverslips (size 0.170 ± 0.005 mm, Marienfeld).
5. Label transition fibers and distal appendages with Cep164 rabbit polyclonal antibody (Graser et al., 2007).
6. Label basal feet with γ -tubulin mouse monoclonal antibody (clone GTU-88, cat. no. T6557, Sigma-Aldrich).
7. Appropriate secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 or other stable fluorophores that match the microscope laser lines and filters. 3D-SIM requires the collection of multiple images to reconstruct a

single image, thus stable fluorophores are required to prevent photobleaching that could result in reconstruction artifacts.

8. Slowfade Gold antifade mountant (cat. no. S36937 or S36936, Life Technologies) or other appropriate nonhardening antifade mountant. Self-hardening mountants may have inconsistent refractive indices in different areas of the sample or the refractive index could vary over time.
9. Microscope oils with refractive indices varying by 0.002 to ensure proper matching of refractive index between sample and objective. A set of microscope oils is supplied with the Delta Vision OMX instrument.
10. Humid chamber: cover the outside surface of a 150 mm Petri dish with aluminum foil and line edges with moistened strips of thick filter paper or folded laboratory wipes. The filters are immobilized on a sheet of Parafilm taped to the bottom.

2.2.2 Method

1. Fix cultures by adding -20°C methanol to apical and basal chambers of the Transwell filter, incubate at -20°C for 10 min.
2. Rinse twice with PBS, pH 7.4.
3. Cut filters out of plastic support (Section 2.1.2). Place filter cell side facing up into humid chamber by grasping edge with fine forceps and immediately add PBS.
4. Block filters in 3% bovine serum albumin in PBS +0.1% Triton X-100 (PBSBT) for 30 min at room temperature. Other antibodies may benefit from different blocking reagents, such as normal serum matched to secondary antibody. Add liquids by gently pipetting over the filter; remove liquids by carefully approaching filter edge with a pipet tip connected to a vacuum aspirator. Avoid contacting the cells on the filter or scraping the Parafilm.
5. Incubate with primary antibodies diluted in PBSBT for 1 h at room temperature. Titration of primary antibody concentration is critical to ensure appropriate labeling for super-resolution microscopy; the appropriate concentration may differ between conventional and super-resolution imaging (Lau et al., 2012).
6. Rinse three times with PBST, then incubate for 5 min in final rinse.
7. Incubate in fluorescent dye conjugated secondary antibodies diluted in PBSBT for 30 min at room temperature.
8. Rinse three times with PBST, then incubate for 5 min in final rinse.
9. Place filter cell side down on glass slide, place a few microliters of mountant on top of filter and quickly invert coverslip onto glass slide. This helps ensure that the apical surface of the cells is as close to the coverslip as possible.
10. Remove excess mountant by aspiration.
11. Seal coverslip to slide with clear nail polish. Sample can be imaged immediately or up to one to two weeks later.

12. Samples can be imaged in conventional wide-field or confocal fluorescence microscopes.
13. For super-resolution experiments, samples should be imaged in a 3D-SIM microscope and image reconstruction and alignment carried out according to manufacturer's instructions. An important step is proper matching of microscope oil refractive index to the refractive index of the sample. This can be done by imaging 0.1 μm multispectral fluorescent beads (Tetraspeck beads, Life Technologies) using the same mountant as in experimental samples. The appropriate microscope oil will minimize distortions to the point spread function of a single fluorescent bead, which approximates a point source.

2.2.3 Analysis—Deltavision OMX 3D-SIM images can be manipulated and analyzed in the proprietary softWoRx software or in the Fiji distribution of the ImageJ software (Schindelin et al., 2012), where necessary plugins are preinstalled.

As basal bodies in MTECs are likely to be in slightly different focal planes, Z-projections of image stacks obtained from 3D-SIM may be performed. Relative localizations of Cep164 and γ -Tubulin (Figure 3) in the lateral axis (XY plane) in the 3D-SIM images can be assessed by eye, or by using plot profiles.

To determine the relative localization of proteins in the axial dimension, XZ or YZ projections of stacks can be performed and analyzed by eye or using plot profiles. Quantitative measurement of biophysical features such as the diameters of rings formed by centriolar proteins are described elsewhere (Lau et al., 2012; Lee et al., 2014; Mennella et al., 2012).

3. Localization of PCP Signaling Pathway Proteins in Multiciliated Airway Epithelial Cells

The asymmetric localization of core PCP proteins in crescents at the proximal and distal side apical junctions of airway epithelial cells is at the heart of the cell-to-cell alignment mechanism mediated by the PCP pathway (Vladar et al., 2009), and it is also a convenient readout for motile cilium orientation. The core PCP protein Vang-like 1 (Vangl1) localizes to the distal side cell cortex in airway epithelial cells, while Frizzled 6 (Fzd6) localizes to the proximal side, or the direction of the ciliary active stroke (Vladar et al., 2012). Immunofluorescence labeling with PCP protein antibodies or lentiviral expression of epitope tagged PCP cDNAs in MTEC cultures will reveal these membrane domains and ciliary beat direction.

3.1 Observing Vang-Uke 1 and Frizzled 6 Localization in the in Vivo Multiciliated Airway Epithelium

Asymmetrically localized Vangl1 and Fzd6 can be observed in the mouse trachea after day E14.5 of embryonic development (Vladar et al., 2012). To visualize the apical epithelium *en face*, the luminal side of the tissue is exposed and whole-mount labeled while immobilized on a silicone surface.

3.1.1 Materials

1. Label distal airway cortex with Vangl1 rabbit polyclonal antibody (cat. no. HPA025235, Sigma-Aldrich), use at 1:800.
2. Label proximal airway cortex with Fzd6 goat polyclonal antibody (cat. no. AF1526, R&D Systems), use at 1:350.
3. Label adherens junctions at cell boundaries with E-cadherin rat polyclonal antibody (cat. no. 13-1900, Life Technologies), use at 1:350.
4. Label cilia with acetylated α -tubulin mouse monoclonal antibody (cat. no. ab24610, Abcam), use at 1:2000.
5. Minutien insect pins (cat. no. 6002-10, Fine Science Tools).
6. Sylgard 184 silicone (cat. no. DC4019862, Krayden Inc.) surface prepared in a 60 mm Petri dish following the manufacturer's instructions. Activated charcoal powder (Sigma—Aldrich) can be added to the silicone slurry before hardening to create a dark surface for contrast.
7. Humid chamber.
8. Glass slide adapted for mounting tracheas: cover slide surface with a layer of electrical tape and use a razor blade to cut out a window to accommodate the size of the trachea. This will create a dam for the mounting medium and a raised edge around the tissue for placing the coverslip.
9. Vectashield (cat. no. H-1400, Vector Laboratories) or similar self-hardening mounting medium.

3.1.2 Method

1. Obtain mouse trachea by dissection in Ham's F12 medium on ice (Section 1.1). Take care to include the larynx and/or the bifurcation to serve as tissue polarity markers during the sample preparation.
2. To prepare trachea for labeling:
 - a. Grasp the trachea by the larynx using forceps and cut open longitudinally along the dorsal side using dissecting scissors.
 - b. Place it onto the silicone surface with luminal side facing up and proximal side (larynx) facing north, and insert a Minutien pin into the left edge of the larynx.
 - c. Using forceps, gently pull on the right edge of the larynx to flatten it against the surface and secure in place with a second pin. Similarly pin the left and right edges of the tissue at the distal end, gently stretching the trachea to expose the luminal surface.
3. To fix, add -20°C methanol over the tissue, incubate at -20°C for 10 min.

4. Remove fixative using a pipettor or vacuum aspirator, then rinse tracheas twice with PBS, and proceed to labeling immediately. During the labeling, the silicone surface should be kept in a humid chamber.
5. Block tissue with 10% normal serum matched to the secondary antibody in PBS, pH 7.4 + 10% Triton X-100 (PBST) for 1 h at room temperature.
6. Incubate with primary antibodies diluted in blocking solution for 1 h at room temperature.
7. Rinse three times with PBST, then incubate for 5 min in final rinse.
8. Incubate in fluorescent dye conjugated secondary antibodies diluted in blocking solution for 30 min at room temperature.
9. Rinse three times with PBST, then incubate for 5 min in final rinse.
10. Prepare slide for mounting the trachea.
11. Using spring or other fine scissors, cut laterally across the pinned trachea below the top and above the bottom set of pins to liberate a relatively flat, rectangular area of tissue. The trachea may curve back slightly into a cylinder. Take care to track orientation.
12. To mount trachea onto microscope slide:
 - a. Using forceps, place the trachea into the electrical tape window on the slide with the luminal side facing up and proximal side facing north.
 - b. Flood with mountant and gently place a 22 × 30 mm or longer coverslip on top. This will flatten out the trachea and expose the luminal surface. If all excess tissue has been properly dissected from the exterior of the trachea, the coverslip will sit flat over the top of the tissue. Be sure to note the orientation of the trachea on the slide.
 - c. Place the slide with coverslip side down on a flat surface at 4 °C overnight to allow the mountant to dry before imaging.
13. Image slides with confocal microscope.

3.1.3 Analysis—In mature trachea, the asymmetric distribution of Vangl1 or Fzd6 can be assessed by a partial overlap with E-cadherin at the apical junctions (Figure 4). In wildtype tissues, the position of the Vangl1 or Fzd6 crescents will reveal the direction of ciliary motility and the tissue axis of polarity. PCP defects can lead to loss of Vangl1 or Fzd6 asymmetry in cells or a deviation between the P-D tissue axis and the crescents (Vladar et al., 2012).

A simple method to measure the extent of Vangl1 or Fzd6 asymmetry in cells involves determining the overlap between the PCP protein and E-cadherin signals. The Just Another Colocalisation Plugin (Bolte & Cordelieres, 2006) in the ImageJ (National Institutes of Health) software (Schneider et al., 2012) is ideal for analyzing images and calculating the fraction of overlap and correlation between the two signals.

More advanced methods have been developed to assess and quantify both the extent of PCP protein asymmetry and the directionality of PCP protein crescents (Aigouy et al., 2010; Matis, Axelrod, & Galic, 2012).

3.2 Observing Vang-Like 1 and Frizzled 6 Localization in in Vitro Differentiated Multiciliated Airway Epithelial Cells

Asymmetrically localized Vangl1 and Fzd6 can be assessed in MTEC cultures starting at the time of ALI creation. MTEC cultures are also ideal for studying the establishment of asymmetric PCP domains by observing Vangl1 and Fzd6 distribution in proliferating cells during the submerged phase (Vladar et al., 2012). MTECs can also be transduced by lentivirus carrying epitope tagged cDNAs (ex. *Vangl1::GFP*) to observe localization (Vladar & Stearns, 2007). Note that MTEC cultures will show only locally, not globally aligned cells.

3.2.1 Materials—See Section 3.1.1 for antibodies and humid chamber.

3.2.2 Method

1. Fix cultures by adding -20°C methanol to apical and basal chambers, incubate at -20°C for 10 min.
2. Rinse twice with PBS, pH 7.4 (fixed filters can be stored at 4°C in PBS).
3. Cut filter out of plastic support (Section 2.1.2) and place cell side up onto a sheet of Parafilm taped into the bottom of the humid chamber.
4. Block tissue in 10% normal serum matched to the secondary antibody in PBST for 30 min at room temperature. Add liquids by gently pipetting over the filter; remove liquids by carefully approaching filter edge with a pipet tip connected to a vacuum aspirator.
5. Incubate in primary antibodies diluted in blocking solution for 1 h at room temperature.
6. Rinse three times with PBST, then incubate for 5 min in final rinse.
7. Incubate in fluorescent dye conjugated secondary antibodies diluted in blocking solution for 30 min at room temperature.
8. Rinse three times with PBST, then incubate for 5 min in final rinse.
9. Place filter cell side up on microscope slide, place a few microliters of self-hardening mounting medium on top of filter and cover with coverslip.
10. Place the slide at 4°C overnight to allow the mounting medium to dry before imaging.
11. Image slides with confocal microscope.

3.2.3 Analysis—To assess the extent and directionality of Vangl1 or Fzd6 asymmetry in MTEC samples, see Section 3.1.3.

Conclusions

The methods presented here for the observation and quantitation of the planar polarized orientation of motile cilia and the activity of the PCP signaling pathway in the airway epithelium will enable deeper understanding of ciliary polarity mechanisms and facilitate the identification of novel components and regulatory processes. The observation of motile ciliary polarity is challenging because MCCs can only differentiate in vivo or in complex primary culture systems, and imaging approaches are time-consuming and cumbersome. However, we anticipate that rapid advances and new methodologies, especially in super-resolution and correlative light and EM will make it easier to visualize the polarity of cilia in MCCs and tissues. Polarized ciliary motility is fundamental to directional mucociliary airway clearance, which is essential for respiratory function. Planar polarized monociliated cells exist also in the inner ear, kidneys, and the neural tube, and ciliary or PCP defects in these tissues lead to human disease. Improved understanding of developmental and disease mechanisms that impact PCP signaling and ciliary orientation and function will certainly aid in the development of new therapeutic approaches. We also hope that robust techniques will soon exist to instill tissue-wide polarity in vitro, which will greatly facilitate tissue engineering of functional planar polarized epithelia, a major unmet goal in regenerative medicine.

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Figure 1. Motile cilia are planar polarized at the ultrastructural level

(A) Schematic and EM images of a motile cilium in longitudinal (left) and cross-section (right) with its planar polarized structures: the basal foot appendage of the basal body and the central microtubule doublet of the axoneme. (B) Schematic of the trachea shows the proximal (oral) to distal (lung) tissue axis of polarity. An electron micrograph of an oblique section through a MCC demonstrates that the basal feet of cilia point to the direction of the beat (white arrow) and a line passing through the central axonemal microtubule doublet (white lines) is perpendicular to it. (See the color plate.)

Figure 2. The PCP pathway orients motile cilia

Schematic shows the three tiers of the PCP signaling mechanism. The core PCP cassette is responsible for the coordination and propagation of cell polarities via intercellular communication across adjacent cell boundaries by two PCP complexes. In the airway epithelium, the proximal complex contains Fzd6 and the distal complex contains Vangl1. Cytoskeletal elements link the basal feet of cilia to the proximal cortex for directional motility. Thus Vangl1 and Fzd6 asymmetric cortical localization serves as a readout for both PCP pathway activity and ciliary orientation. (See color plate)

Figure 3. Transmission and super-resolution microscopy of ciliary planar polarization
 (A) TEM images showing P-D aligned motile cilia in wildtype MCC with basal feet pointing in the proximal direction (left), PCP mutant MCC with intracellularly aligned, but globally misaligned basal feet (middle) and PCP mutant MCC with intracellularly misaligned basal feet (right). (B) EM image of a cilium (left) in longitudinal section showing the approximate localization of γ -tubulin at the basal foot and Cep164 at transition fibers, which form a ring at the top of the basal body. Super-resolution (center) and electron (right) microscopy images show the direction of ciliary motility based on basal foot directionality. For super-resolution imaging, MTECs were labeled with γ -tubulin (green (white in print versions)) and Cep164 (red (white in print versions)) antibodies; the γ -tubulin puncta correspond to the basal feet and the Cep164 rings to the transition fibers viewed in cross-section.

Figure 4. Observation of core PCP protein localization

Confocal microscopy images of trachea (left) and MTECs (right) labeled with Vangl1 (green), acetylated α -tubulin (red) and E-cadherin (blue) antibodies. The directionality of the Vangl1 signal indicates P-D aligned cells in vivo and locally, but not globally aligned cells in vitro. Compare Vangl1 signal position to schematic in Figure 2. (See color plate)