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Visualization of live primary cilia dynamics using fluorescence microscopy

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Abstract

Here we describe methods that are useful for exploring the formation and functions of primary cilia in living cells. First we describe multiple protocols for visualizing solitary cilia that extend away from the cell body. Primary cilia collect, synthesize, and transmit information about the extracellular space into the cell body to promote critical cellular responses. Problems with cilia formation or function can lead to dramatic changes in cell physiology. These methods can be used to assess cilia formation and length, the location of the cilium relative to other cellular structures, and localization of specific proteins to the cilium. The second protocol describes how to quantify movement of fluorescent molecules within the cilium. The microtubules that form the structural scaffold of the cilium are also critical avenues for kinesin and dynein-mediated movement of proteins within the cilium. Assessing intraflagellar dynamics can provide insight into mechanisms of ciliary-mediated signal perception and transmission.

Keywords

Primary cilium; cilia; intraflagellar transport; kymograph

Introduction

There are many ways to visualize primary cilia in cultured cells. Primary cilia can be distinguished nicely by differential interference contrast (DIC) microscopy when a microscope is properly aligned (see protocol described in (Salmon and Canman, 2001)). Wide-field and confocal imaging of immunostained cilia are commonly used to assess cilia formation and protein localization. However, upon fixation, many cilia deform, curl and shrink. Cilia in living cells maintain their structural integrity. In addition, live cell imaging avoids artifacts caused by fixation and permeabilization. The first two basic protocols described here employ fluorescent-tagged proteins that localize to primary cilia. These methods can be used to assess the cilium as a whole, and are also valuable for assaying the localization of specific proteins. For some investigations, staining of live cilia is preferable to expression of tagged proteins because researchers have concerns about effects of overexpression or functions of fluorescent-tagged proteins. In these situations the alternate protocols described here which utilize fluorescently tagged lipids, cholesterol and lectins can be good alternatives. These methods may also be useful for research questions related to the ciliary membrane and cilia glycoproteins.

Internet Resources

Image J <http://rsb.info.nih.gov/ij/Image> J kymograph pluggins http://www.embl.de/eamnet/html/body_kymograph.html

Another important benefit of imaging live cilia is that they are dynamic structures. Proteins are constantly moving into and out of the cilium. Sheathed by the ciliary membrane, 9 microtubule doublets extend from the centriole toward the tip of the cilium. Motor proteins drive along the microtubules toward the tip (kinesin - anterograde) or toward the base (dynein - retrograde). Intraflagellar transport (IFT) complex components link soluble and membrane proteins to the motors and facilitate directed transport (Pedersen and Rosenbaum, 2008). The third basic protocol details how to use time-lapse imaging to quantify movement of proteins within the cilium. The alternate protocols employ photobleaching and photoconversion of fluorescent molecules to highlight a subpopulation of the fluorescent proteins in the cilium. This can be especially useful when the movement of molecules is difficult to distinguish using time-lapse imaging alone. Characterizing and quantifying the movement of proteins within the cilium can provide important insights when trying to discern the mechanisms of cilia localized cellular functions.

The methods described are for visualization of primary cilia of cultured canine kidney epithelial cells (MDCK cells) and mouse fibroblasts (NIH3T3 cells), which each present different advantages. When grown on a filter that promotes cell polarization, MDCK cells pack closely, and form long cilia (8–15µm) on almost every cell. These long cilia signal in response to bending by fluid flow (Praetorius et al., 2003; Praetorius and Spring, 2001; Praetorius and Spring, 2003). Importantly, filter-grown MDCK cell cilia can be positioned right along the coverslip, which is useful for many applications. NIH3T3 cell cilia appear much more quickly (one day after plating instead of the 3 to 5 days needed for MDCK cells) and can be positioned on the dorsal or ventral side of the cell (either near the cover glass, or projecting into the medium). The cilia of NIH3T3 cells have been shown to mediate responses to sonic hedgehog and platelet derived growth factor alpha (Schneider et al., 2005; Taipale et al., 2000). Basic Protocols 1 & 2 describe how to culture these cell types for cilia visualization. However, in principle the protocols can be adapted to visualize cilia in other cultured cells, in multi-ciliated cells and potentially *in vivo*.

Basic Protocol 1

Imaging fluorescent proteins in live, filter-grown, MDCK cell primary cilia

The baso-lateral and apical membranes of polarized epithelial cells perform different functions and have different membrane protein compliments. When MDCK cells grow directly on plastic or glass, the basal surface lacks direct access to fluids so the membrane specialization is not as complete. In contrast, epithelial cells grown on filter supports (such as the commercially available transwell filters) form columnar cells that are taller and more uniform. In addition, we have found that the percentage of ciliated cells is higher, and the cilia are longer, on filter grown MDCK cells. One challenge of conventional transwell filters is that they are opaque, so it is difficult to image cells grown inside the transwell. Here we describe a method of culturing the MDCK cells on the underside of the transwell filter. Based on the work of Wakabayashi et al. (Wakabayashi et al., 2007), this method enables live imaging of filter-grown MDCK cell cilia. There are new filter systems available that are transparent (for example chambers manufactured by SIMPore), and these may also be good for some applications. Figure 1 shows MDCK cells stably expressing chimeras of Tubulin and green fluorescent protein (tubulinGFP) or Smoothened and yellow fluorescent protein (SmoYFP) that were cultured in this way and imaged on inverted confocal microscopes.

MDCK cells have been used extensively for studies of polarized cells. Additional information for culturing these cells are provided in (Lipschutz et al., 2001). Primary cilia also form on MDCK cells that form cysts in 3D cultures. Refer to (Elia and Lippincott-Schwartz, 2009) for protocols for culturing and imaging cells in that system.

All solutions and **Materials** coming in contact with live cells should be sterile.

Handling of cells and solutions should occur in a tissue culture cabinet.

Materials

MDCK II Cells

MDCK cell growth media: Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 3mM glutamine

0.25%trypsin/2.21mM EDTA in Hank's Buffered Salt Solution (HBSS) 1xPBS

Hemocytometer or cell counter

Transwells (Corning # 3460 or 3470)

2-well LabTek chambers

Imaging media such as Liebovitz-15 or CO₂-Independednt media with 4mM Glutamine

Serum free media or OptiMEM

Lipofectamine 2000

DNA

Inverted confocal microscope

Culturing polarized MDCK cells for imaging

- 1 MDCK cells will pack tightly and begin to polarize when grown in plastic culture dishes. Before passaging, culture cells until they pack tightly to ensure the cells are healthy and competent to polarize when plated on a filter. Typically this is one day after they are confluent.
- 2 Rinse MDCK cells briefly with 1xPBS, then trypsonize cells in 0.25%trypsin/ 2.21mM EDTA in HBSS for 5 to 15 minutes at 37 °C. Because they have formed a tightly packed monolayer, high concentrations and temperature help speed cell-cell separation.
- 3 After the cells have separated from the substrate they often remain in clumps. To separate clumps of cells into individuals, add media to neutralize the Trypsin/ EDTA (4 volumes), suck the cells into a pipette, press the pipette tip firmly against the bottom of the dish, and expulse the cells. This generates sheering forces that will separate the clumps.
- 4 Pellet the cells (1,000g for 5 min), remove supernatant, and resuspend cells in growth medium to the same volume used for trypsonization. Count the cells and resuspend cells to a final concentration of 4×10^5 cells per mL (see (Phelan, 2001) for cell counting procedure). These cells settle quickly. Be sure to pipette up and down immediately before removing a sample to count or dilute.
- 5 Transwell filters come in a multi-well plate. **Invert the plate containing the transwell filters** and remove the bottom of the plate (which is now on top). Now the underside of the transwell filter will be facing up.
- 6 Add diluted cells directly to the transwell. For a 6mm transwell, add 38 μ L. To 12mm transwells, add 100 μ L.
- 7 Replace the bottom of the multi-well dish. The liquid should touch the dish and surface tension will keep the cells in place.

- 8 Place the cells (still inverted) into the incubator for 2–6 hours. **Then flip the entire dish.** Look under the microscope. The cells are ready when you see that many of the cells have adhered to the filter and have spread out.
- 9 Add 0.75 mL (6mm) or 1.5 mL (12mm) media to the outside of the transwell (i.e. the bottom of the multi-well dish). To the inside of each transwell add 0.35 mL (6mm) or 0.75 mL (12mm).
- 10 Return the cells to the incubator. Once the cells become confluent (approximately the third day) move the transwell to a fresh multiwell plate (so cells on the bottom of the dish don't compete for resources). After the cells become confluent replace the media every day.
- 11 By day 5 most cells should have cilia. By day 7 the cilia should be longer (8–15 μ m). Although cilia are still present on some cells, general health of the culture deteriorates beyond the ninth day.

Transient transfection of MDCK cells—Transfect the MDCK cells two days before you want to image them. We get reasonable transfection using the Lipofectamine 2000 reagent (Invitrogen).

- 12 Set up two tubes for each reaction (see Table 1). Add serum-free medium or Opti-MEM to both of the tubes. Then add either DNA (Tube A) or lipofectamine2000 (Tube B). If multiple DNAs are being transfected, combine these first, then dilute them in the transfection medium to maximize the number of cells that express all desired proteins.
- 13 Incubate the dilutions for 5 minutes at room temperature.
- 14 Add the solution in tube A to tube B and mix gently.
- 15 Incubate solution for 20 minutes at room temperature.
- 16 Remove media from inside and outside transwell.
- 17 Invert plate (similar to what was done when the cells were plated). Remove the bottom of the dish (which is now on the top). The cells are now exposed, facing up, on the outer side of the transwell.
- 18 Add the transfection reaction drop-wise to the cells.
- 19 Replace the bottom of the dish so the transfection solution contacts the inside of the multi-well dish. The surface tension should hold the solution in place over the cells. If all of the media was not removed from the bottom of the dish, the transfection solution can be diluted and the solution may not spread evenly across the transwell.
- 20 Place inverted dish in the incubator and leave for 4 hours.
- 21 Flip the dish over and add culture media to the inside and outside of the transwell filter.

Live imaging of MDCK cilia expressing fluorescent proteins—In general the idea is to place the transwell on top of the coverslip of an imaging chamber so that the cilia will be close to the objective. Lab-Tec chambered coverglass (Nunc) is a good option. MatTek dishes, which have a coverglass bottom, or coverslip holders (such as the ChamSlide from Quorum Technologies) also work.

- 22 Replace culture medium both inside and outside the well with medium for imaging. CO₂ independent medium (Invitrogen) or Liebovitz media are two good options.
- 23 If you want to fit a lid onto the imaging chamber, it is possible to clip the plastic ring off the top of the transwell using a pair of wire cutters while you hold the main body of the filter mount with tweezers.
- 24 The volume of media between the cells and the coverslip impacts the orientation of the cilia. If cells are in a large volume of media, then the cilia will be perpendicular to the coverslip. However, if you gently wick most of the media from the underside of the transwell and place them in 0.1 mL of imaging media, the space will be reduced and the cilia will bend and flatten a little. To completely flatten the cilia against the coverslip, wick most of the media off the cells side of the transwell and place the cells on an empty imaging chamber. Many cilia will lay flat against the coverslip. Because there is media nourishing the cells from the basal side, the cells can stay alive in these conditions for many hours.
- 25 Image cells on an inverted microscope using an objective with high magnification and a high numerical aperture.

The method above describes how to plate cells in the inverted culture system and how to transiently transfect cells on the filter. However, stably transfected cell lines also work very well in this system. To transiently transfect an additional marker into a stable cell line, culture the cells in the absence of any antibiotics or selection reagents for at least 1 hour prior to transfection.

A similar protocol can be used to culture cells inside the transwells and the cells can then be fixed and imaged (for example for immunostaining). Follow the procedure above but instead of inverting the transwells in step 5, omit steps 5, 7, and 8. Add media to the wells as described in step 9, then add the volume of cells indicated in step 6 directly to the media inside of the transwell.

Basic Protocol 2

Imaging fluorescent proteins in primary cilia of live NIH3T3 cells

NIH3T3 cells are mouse fibroblasts. Under typical growth conditions a small percentage of the cells will form a cilium. However, upon serum starvation, which causes the cells to stop cycling and to enter the G₀ phase, we observe as many as 70% of the cells will ciliate. Fibroblast cilia are generally much shorter than the cilia of epithelial cells and NIH3T3 cell cilia range from 1 to 5 microns in most cases. Although the cilia are shorter, there are many advantages to working with these cells including: 1) the cells are responsive to several signaling pathways such as sonic hedgehog and platelet derived growth factor (Schneider et al., 2005; Taipale et al., 2000); 2) if serum is removed shortly after cells are plated, serum starvation can begin and cilia can be seen on the next day (Schneider et al., 2005) instead of waiting 5 to 7 days; 3) higher transient transfection efficiencies can be obtained with the NIH3T3 cells than with filter-grown MDCK cells. Figure 1 shows the cilium localization of SmoYFP in NIH3T3 cells.

ATCC recommends culturing NIH3T3 cells in the presence of calf serum, not FBS. Presumably, the FBS has more nutrients and causes cells to divide more quickly. We pay special attention to passage numbers - the cells we received from ATCC were passage 125 and we typically use them for no more than 30 additional passages. Never let NIH3T3 cells become confluent. Over time, cells that have been confluent can lose their contact inhibition.

Generally, cells that are approximately 80% confluent split 1:10 will be 80 % confluent after 3 days. When plated twice as densely, the cells will be ready one day earlier.

For imaging, cells can be plated onto coverglass bottom imaging dishes, such as LabTek or MatTek chambers, or onto coverslips that are mounted into imaging chambers (see descriptions above). The method below provides details for plating NIH3T3 cells onto a 10 mm coverslip in a 24 well dish, or a single well of a 4-well LabTek chamber (both have an area of approx 1.8 cm²), but the protocol can be scaled for dishes with other surface areas. To visualize fluorescent proteins, stable cell lines can be generated, or cells can be transiently transfected. Using Lipofectamine2000 (Invitrogen) we observe the optimal transient transfection efficiency when the cells are plated much less densely than the manufacturer suggests. An optimized protocol is provided below.

Materials

NIH3T3 cells

NIH3T3 growth media: Dulbecco's Modified Eagle Medium (DMEM) + 110 mg/mL sodium pyruvate + 2mM Glutamine + 10% calf serum

NIH3T3 starvation media: DMEM + 110 mg/mL sodium pyruvate + 2mM Glutamine 1xPBS

0.05% Trypsin/0.53 mM EDTA in HBSS

Serum Free medium or OptiMEM

Lipofectamine2000

DNA

Imaging media such as Liebovitz-15 or CO₂-Independednt media with 4mM Glutamine

Culturing NIH3T3 cells for live imaging

- 1 Trypsinize NIH3T3 cells when they are approximately 80% confluent (i.e. there are few large spaces, but cells are not overlapping or close together), using 0.05% Trypsin/0.53mM EDTA in HBSS at room temperature for <5 minutes. Gently resuspend the cells in culture medium and, if needed, put a fraction of the cells into a fresh dish to maintain the culture.
- 2 Pellet the remaining cells by centrifugation for 5 minutes at 1,000g. Resuspend in 1/5 to 1/10th the original culture volume and count the cells.
- 3 Dilute NIH3T3 cells to 6×10^4 cells/mL and plate 0.5 mL per well (final concentration of 1.6×10^4 cells per cm²). Return cells to the incubator overnight.
- 4 If cells are stably expressing a fluorescent protein, growth medium can be replaced by starvation media the next day, and then imaged on the third day. If you plate the cells at a slightly higher density, you can start the starvation treatment after 6–8 hours and image the cells the following day. If cells are not stably expressing a fluorescent protein, proceed with the transient transfection.

Transfection of NIH3T3 Cells

- 5 For each sample, label two initial reaction tubes. Tube A is for dilution of DNA and Tube B is for the Lipofectamine 2000 reagent dilution.

- 6 Dispense 50 μ L of Opti-Mem (Invitrogen) or serum free medium into each tube (tube A and tube B).
- 7 Pre-mix DNAs to maximize co-transfection efficiency, if multiple plasmids are being used. Add a total of 1 μ g of DNA to tube A.
- 8 Dispense 2 μ L of Lipofectamine 2000 reagent to tube B.
- 9 Let the solutions sit at room temperature for 5 minutes.
- 10 Transfer the contents of Tube A into Tube B.
- 11 Incubate the reaction at room temperature for 20 minutes.
- 12 Toward the end of the incubation replace the 500 μ L of growth media on the target cells with 300 μ L fresh growth medium (includes serum, but never antibiotics).
- 13 Add the transfection reaction drop wise to the cells. Gently rock the dish to mix, and then place the cells in the incubator.
- 14 After 3–5 hours, replace the growth media with starvation media to induce cilia formation.

Imaging NIH3T3 cilia

- 15 Unless using a microscope outfitted with 5% CO₂, the samples cannot go directly onto the stage. For short-term experiments, mineral oil can be added gently to the media to form a permeability layer, which prevents oxygen exchange. Alternatively, CO₂ Independent media (Invitrogen) or Liebovitz (L15) media can be used. Add glutamine, but *no* serum.
- 16 Image the cilia on an inverted microscope while keeping the cells at 37°C.

Alternate Protocol 1

In addition to using genetically encoded markers to visualize primary cilia, it is also possible to stain ciliary proteins or the ciliary membrane with exogenously added dyes. Here we describe three such methods: 1) Incorporation of a fluorescently tagged lipid into the ciliary membrane; 2) Incorporation of fluorescently labeled cholesterol into the ciliary membrane; and 3) Binding of fluorescent lectins to the glycoproteins of the cilium. The advantages of these methods are that they do not require transfection or overexpression of proteins. Also, the reagents are available with multiple fluorescent or non-fluorescent conjugates. Generally, however, non-cilia membranes may also take up the stains, and the enrichment at the ciliary membrane can be short lived as the lipids and proteins exchange with unlabeled populations in the cell and decrease the background fluorescence.

As with the methods for visualizing fluorescent proteins described above, cells must be grown to promote cilia growth. This means looking at MDCK cells at least 5 days after plating on a transwell, or at NIH3T3 cells after starvation. Alternate protocols 1 and 3 describe the use of rhodamine-conjugated probes, however, other fluorescent conjugates also work. Similarly, we describe methods for the use of specific phospholipids and lectins. We have found that alternate lipids or lectins can also be used. In polarized MDCK cells, it is possible to add the stain to just the apical surface, which reduces the amount of dye taken up into the cell.

Staining primary cilia with fluorescently labeled lipids

Fluorescently tagged phospholipids can incorporate into the lipid bilayer of cells. Initially they localize just to the outer cellular membranes, but in time they can be taken up into vesicles and distribute across the many membrane containing cellular compartments. DOPE rhodamine incorporates into the apical plasma membrane and the ciliary membrane, which makes it difficult to discriminate in widefield images, but as seen in the side projection of cells that have been labeled with DOPE rhodamine (Figure 2) the ciliary signal is visible. In our experience the cilia were discernable for at least 30 minutes (Ott et al., 2012).

Materials

Ciliated MDCK or NIH3T3 cells cultured as described in protocol 1 and 2

1 mg/mL DOPE rhodamine (Avanti polar lipids) in ethanol

Imaging Media such as Liebovitz-15 or CO₂-Independednt media with 4mM Glutamine

Protocol Steps

1. Dilute a stock solution of 1 mg/mL DOPE rhodamine (Avanti polar lipids) in ethanol to a final concentration of 50 µg/mL in imaging media.
2. Incubate the cells for 5 minutes in the staining solution. Then replace the staining solution with fresh staining solution and incubate for an additional 5 minutes.
3. Quickly rinse the cells twice in imaging media. Then leave the cells in a third rinse solution for five minutes.
4. Image the cells. Look for the distinctive cilia morphology, location, and co-localization, if appropriate.

Alternate Protocol 2

Staining primary cilia with BODIPY cholesterol

BODIPY cholesterol has been used to study the trafficking and transport of cholesterol in live cells. It has been well established that ciliary membranes are cholesterol-rich (Cuevas and Gutierrez Diaz, 1985). When complexed with methyl-β-cyclodextran, BODIPY cholesterol can be loaded into cells (Holtta-Vuori et al., 2008; Leppimäki et al., 2000). We have found that the conditions described below can be used to highlight the membrane of the primary cilium, as well as the rest of the apical plasma membrane (figure 2).

Materials

Ciliated MDCK or NIH3T3 cells cultured as described in protocol 1 and 2

BODIPY cholesterol stock solution: 4mM BODIPY cholesterol in a 1:10 molar ratio with methyl-β-cyclodextran (MβCD) in 1xPBS. Sonicated 20 min.

Imaging Media such as Liebovitz-15 or CO₂-Independednt media with 4mM Glutamine

Protocol Steps

1. Pre-warm imaging media.
2. Dilute 4mM BODIPY cholesterol stock solution 1:200, to a final concentration of 20 µM in imaging media.
3. Incubate cells for 2–5 minutes at 37 °C.
4. Rinse one time in imaging media, then image.

Alternate Protocol 3

Lectin staining of primary cilia

Cilia contain a host of glycosylated membrane proteins, some of which are important for mediating cilia-cilia adhesion (Ott et al., 2012), and lectin staining has been used as a tool to visualize primary cilia (Praetorius et al., 2004). Here we describe a staining protocol for use with wheat germ agglutinin, which binds to N-acetylglucosamine and sialic acid. Figure 2C shows the co-localization of the WGA-rhodamine and tubulinGFP. We observed that succinyl WGA does not bind sialic acid and does not label primary cilia; this suggests that WGA is recognizing sialylated proteins on the cilium.

Materials

Ciliated MDCK or NIH3T3 cells cultured as described in protocol 1 and 2

1mg/mL stock solution of WGA rhodamine

Imaging Media such as Liebovitz-15 or CO₂-Independednt media with 4mM Glutamine

Protocol Steps

1. Dilute a 1mg/mL stock solution of WGA rhodamine 1:200 to 5 µg/mL in pre-warmed imaging media.
2. Rinse the cell briefly in imaging media and then apply the 5 µg/mL WGA solution.
3. Place the cells at 37 °C for 30 minutes. (Note: if using CO₂ Independent media place the cells in an incubator that has ambient CO₂ levels.)
4. Rinse the cells once in pre-warmed imaging media and examine them on the microscope.

Basic Protocol 3

Visualizing and quantifying movement within primary cilia

Small molecular complexes moving in the cilium can be difficult to detect and quantify. To be successful, one must optimize two key factors: the signal to noise ratio and the time sampling interval. The signal of the moving particle must be brighter than the background signal of non-moving molecules. In addition, if the rate of image collection is not fast enough, movements could be missed or movements could be perceived that are actually just an artifact of the collection method. The three methods described here present methods to optimally collect and analyze time series of molecules moving within primary cilia. The alternate methods use photobleaching and photoconversion to improve the signal to noise to make the moving particle stand out.

Kymographs have been used in the cilia field and in many other areas to visualize and quantify movements within cells (see Figure 3). To generate a kymograph, a linear array (track) of pixels are extracted from a time series and straightened into a horizontal line. For example, a line can be drawn along the length of a cilium. Then, the values of the pixels at the same locations are extracted from each frame of a time series and the straightened pixels are placed below the original horizontal line to create a new image. In this image, distance is represented by the change in position along the x dimension, and change in time correlates to the y -axis. Movement of a particle along the track in the raw data will appear as a diagonal line in the kymograph. The slope of the line describes the change in distance over a set time interval and thus can be used to determine particle velocity. Often researchers hypothesize that their molecule of interest is moving through intraflagellar transport. To

support this hypothesis the rates of particle movement are measured to determine if they are consistent with the speeds that have been reported for IFT in a particular system.

Resolution in x, y, and t: Nyquist sampling—A kymograph makes patterns in data more obvious and facilitates velocity measurements. However, if data is not properly collected, patterns of movement could be missed, or artifactual patterns could appear (aliasing). As with many aspects of imaging, trade-offs must be made when optimizing. Optimal spatial and temporal sampling may not be possible simultaneously. Below we discuss the ideal conditions, and then give some practical advice for finding a compromise.

Every pixel in an image corresponds to a physical distance in real space. The pixel size is determined by the detector and the imaging conditions used. When assaying movement within cilia, it is critical to know the pixel size in order to determine the optimal time sampling. The Nyquist sampling theorem indicates that the sampling frequency must be 2.3 times the frequency of the event that is being assessed (explained in (Smith, 2001)). For optimal x,y resolution, this frequency is dependent on the optimal resolution of the objective. A spinning disk confocal microscope has a set pixel size for each objective, but a line scanning confocal microscope can alter the pixel size by zooming in on the scanning area. For conventional light microscopy, the Abbe limit restricts resolution to approximately 200nm. Imaging optimally, you would want a pixel size that is $200\text{nm} / 2.3 = 87\text{nm}$.

How frequently do you need to sample in order to avoid aliasing (artifact patterns in your data) or missing information? Again the Nyquist criterion can be used as a guide. In *Chlamydomonas* IFT rates are approximately $2.5\text{ }\mu\text{m/sec}$ (anterograde) and $4\text{ }\mu\text{m/sec}$ (retrograde) (Kozminski et al., 1993). In *C. elegans* and mammalian cells, rates from 0.5 to $1.2\text{ }\mu\text{m/sec}$ have been reported (Follit et al., 2006; Snow et al., 2004). This means that if a pixel is 87 nm , and a particle is moving at $1\text{ }\mu\text{m/sec}$, we would need to collect 11.5 images per second to see the particle in every pixel in the track. Our rate needs to be approximately twice as fast to optimally discern the movement (Nyquist sampling), which means collecting an image every 38 msec. But if the particle were moving $2.5\text{ }\mu\text{m/sec}$ one would need to halve the sampling interval.

Practically speaking, it may not be possible to image a live sample at these optimal resolutions. Some samples are not bright enough to acquire enough photons in such a short period, without turning up the lasers and photobleaching the fluorophores. Some detectors are limited in how fast they can go. For example, on a laser scanning confocal the frame size can be reduced to improve speed, but the frame must be large enough to get the entire cilium. To optimize, adjust the balance of these two parameters. Increase the pixel size and calculate what temporal resolution would be optimal. When using a spinning disk microscope, which has a fixed pixel size for each objective, calculate the optimal interval then try it. To increase pixel size change to an objective with higher magnification or set the camera to bin pixels.

Materials

Ciliated MDCK cells expressing a fluorescent protein cultured as described in protocol 1

Inverted Confocal Microscope

Image J with kymograph plugins

Time-lapse imaging

- 1 Prepare cells expressing a fluorescent protein that localizes to the cilium. Because it is necessary to optimize resolution in x, y, and t, it is generally best to analyze movement in cilia that are as flat as possible (i.e. in a single focal plane). MDCK cells placed onto the coverslip without imaging media (as described in Basic Protocol 1) work well for this type of analysis.
- 2 Optimize the imaging conditions for resolution in x, y, and t. As mentioned above, it is important to know the exact physical distance represented by each pixel. This can be used for computing the optimal time resolution, and will be needed during data analysis to calculate the speed of molecules observed.
- 3 Collect a time series to record movement within the cilium. The duration of the time series may depend on the abundance and intensity of the fluorescent protein and how quickly it bleaches. 200–300 frames often provide several tracks that can be analyzed.
- 4 Review the data once it has been collected. Hopefully some particle movements can be observed by eye. Quantification of the speed of movement will be calculated after generating a kymograph (see below).
- 5 Image several cilia using similar imaging conditions. Be sure to note the exact time interval for each time series. This information is needed to calculate the speed of particles.

Once a time series has been collected, it may be possible to generate a kymograph using the proprietary software of the microscope. Alternatively, one can use the kymograph pluggins for Image J [written by J. Rietdorf (FMI Basel) and A. Seitz (EMBL Heidelberg)]. Below is a description of the workflow for analysis using Image J based on the detailed plugin description available online. Filtering the raw data can reduce noise in an image set. Median filtering and walking average filtering are two such tools. Generally, we generate kymographs with the raw data and the filtered data (see figure 4A for an example). Tracks should be discernable under all conditions, but may be more obvious in one versus another. It doesn't matter which is used for the velocity calculations, but one should always be consistent within an experiment and clearly describe their procedure in any publication.

Kymograph analysis

- 6 Open the desired image.
- 7 Create a maximum intensity projection of the time series.
- 8 Use the segmented line tool to draw a segmented line across the cilium in the projection.
- 9 Click the image opened in step one and use the “restore selection” command to copy the segmented line from the projection to the raw data.
- 10 Generate the kymograph of the raw data. Typically, we use a line width of 3 pixels, which averages the pixel values perpendicular to the line at every position.
- 11 Click the image opened in step one and execute the walking average filter.
- 12 Use the “restore selection” command to copy the segmented line from the projection to the walking average filtered data. Typically we filter across 4 frames.
- 13 Generate the kymograph of the walking average filtered data.

- 14 Click the image opened in step one and execute a two pixel median filter.
- 15 Generate the kymograph of the median filtered data.
- 16 Save kymograph files.
- 17 Open the Multi Measure tool.
- 18 Use the line tool to draw a line over a track in a kymograph. (It is helpful to use the color picker to change the color of the line that is applied to the image.)
- 19 Click “Add and Draw” in the Multi Measure tool.
- 20 Select and add as many lines as can be discriminated.
- 21 Select all lines in the Multi Measure tool window and click “Measure”.
- 22 Save the kymographs with the lines traced and the values generated upon measuring.
- 23 Use the angle (given in radians) to calculate the velocity. Because the units of the cotangent of the angle equals the velocity in pixels per time interval, the pixel size and time interval are needed in the calculation:

$$speed(\mu\text{m}/\text{sec}) = \frac{\cot\theta \times \text{pixelsize}(\mu\text{m}/\text{pixel})}{\text{timeinterval}(\text{sec})}$$

The sign of the speed will indicate the direction of movement. If the base is oriented to the left in the kymograph, a negative value will represent anterograde movement and a positive value indicates movement toward the base.

- 24 Collect and analyze tracks from many cilia to gain statistical confidence in the velocities observed.

It is helpful to always orient the cilia the same direction for analysis. This will ensure that you can discriminate anterograde and retrograde movements in the data set. Then, when drawing lines, start with the earliest time point and go toward the latest. This will ensure that values of the data will correspond to the direction.

Cilia and cells can shift during data collection. Using the maximum intensity projection to select the pixels for the kymograph enables the researcher to draw a line that covers the data most of the time. If there is one major shift in cilia location, it is possible to draw two lines and generate a kymograph for each.

When tracing the tracks on the kymograph, it is not essential to select the entire length of the track because the calculation uses the angle to determine change in distance over time.

Alternate Protocol 4

Improved discrimination of protein movement using cilia photobleaching

If many molecules localize to the cilium, it can be difficult to discriminate movement. To get around this, it can be helpful to decrease the number of excited molecules. This can be done by decreasing the laser power or by using an excitation wavelength that is suboptimal (for example, exciting YFP with 488nm instead of 514nm). Another helpful alternative is to photobleach a fraction of the molecules in the cilium. As the fluorescent molecules travel into the bleached region, they stand out. In time, the bleached and fluorescent molecules may redistribute, but because the overall signal has decreased, it may still be easier to discriminate individual tracks than it was before bleaching. Figure 4A shows kymographs of

polarisGFP movement before and after photobleaching a small portion of the cilium. The exact procedure for setting up the photobleaching experiment will depend on the type of confocal microscope being used. Below is a general guide.

Materials

Ciliated MDCK cells expressing a fluorescent protein cultured as described in protocol 1

Inverted Confocal Microscope capable of photobleaching a specific region of interest

Image J with kymograph plugins

Protocol Steps

1. Prior to executing a photobleaching experiment, optimize the photobleaching conditions. Empirically determine the minimum laser power and duration required to bleach a region approximately half the size of the cilium. To determine the minimum laser power required, set up a time series in a mode that will alternate collecting an image and bleaching multiple times. Set the bleaching laser to a high power and few iterations. Execute the time series and monitor the fluorescence in the bleach region over time. Determine how many iterations are needed to bleach, the fluorophores. Repeat the bleach series using a different laser power and/or iterations to determine the optimal settings.
2. Set up the imaging conditions as described for the basic time series in Basic Protocol 3. Optimize resolution in x, y, and t.
3. Draw a region of interest around half of the cilium. Configure the software to bleach this region using the optimized parameters established in step 1.
4. Program the software to collect 5–10 images prior to bleaching, to bleach, and then to acquire post-bleach images for several minutes.
5. Use this data to generate a kymograph and measure particle speeds as described above.

Photobleaching can also be used to analyze protein dynamics based on the rate of exchange between bleached and unbleached molecules (Snapp et al., 2003).

Alternate Protocol 5

Assessing movement of select pools of cilia proteins using photoconversion

An alternative for highlighting and tracking a subset of molecules in the cilium is to employ photoconvertible fluorescent molecules. As shown in Figure 4B, this process can make it easier to discriminate IFT tracks. The excitation and emission spectra of these fluorophores change after activation with high-energy light. Photoconvertible proteins are better suited for this application than photoactivatable proteins (which fluoresce very little before activation) because it is necessary to identify the cilium to select the region of interest. Eos and Dendra are examples of photoconvertible proteins. Upon activation with 405nm light, Eos shifts from a peak excitation at 506nm and peak emission at 516nm to a peak excitation at 571nm and peak emission at 581. For Dendra the excitation/emission peaks shift from 490/507 to 553/573. For most microscopes, this means switching between the configuration used for imaging GFP to that used for red fluorescent protein (RFP). Here we describe a protocol for seeing movement of proteins within the cilium. A similar protocol can be used to visualize movement of proteins from the cilium to other cellular destinations.

Materials

Ciliated MDCK cells expressing a fluorescent protein cultured as described in protocol 1

Inverted Confocal Microscope capable of photoconverting a specific region of interest

Image J with kymograph plugins

Protocol Steps

1. Begin by empirically determining the power and duration of the 405-laser pulse required to photoconvert the fluorescent protein. Like other fluorophores, the photoconvertible protein can be bleached, so you must expose the fluorophores enough to convert, but not so much that the red shifted fluorescence gets bleached. Optimize the photoconversion parameters on a region of interest that is similar to the size of the region you intend to use in your experiments. To determine the minimum laser power required set up a time series in a mode that will alternate collecting an image and bleaching multiple times. Set the bleaching laser to a low power and few iterations. Execute the time series and monitor the fluorescence in the bleach region over time. Determine how many iterations are needed to convert, but not bleach, the fluorophores. Repeat the bleach series using a different laser power and/or iterations to determine the optimal settings.
2. Optimize the imaging parameters as described for the basic time-lapse protocol to be sure you can resolve structures of interest in x, y, and t. Photoconvert a test location and use that to optimize the imaging parameters for the red channel.
3. Focus on a cilium in the green channel. Draw the region of interest for photoconversion. Often a region covering a quarter of the cilium or less is sufficient.
4. Program the imaging software to: image both the red and the green channels for at least 10 frames prior to photoconversion; photoconvert according to the optimized parameters established; and image (again, both channels) at optimal x, y, and t parameters for two to three hundred frames, or as long as fluorescent molecules are visible.
5. Generate kymographs from each channel as described in Basic Protocol 3. Analyze the tracks to determine particle speed.

COMMENTARY

Background Information

Primary cilia are solitary organelles that extend from the mother centriole and project into the extracellular space. Microtubules provide structural support for the cilium and as act as a central freeway for the movement of proteins along the length of the cilium. The plasma membrane of the cilium is continuous with, but somewhat distinct from, the plasma membrane of the cell body. Many cell types generate a cilium *in vivo*. Investigation of processes happening in and through primary cilia was re-vitalized by studies published between 2000 and 2005 that revealed that signaling through cilia is essential for both development and homeostasis (reviewed in (Bloodgood, 2009)). Signaling pathways, such as the sonic hedgehog pathway, use the cilium as an essential antenna for ligand detection and signal transduction (Goetz and Anderson, 2010). Primary cilia are mechanosensitive and trigger increases in intracellular calcium in response to changes in fluid flow rates (Praetorius and Spring, 2001). A class of diseases called ciliopathies is caused by improper

cilia formation or function. Many of these diseases impact function of multiple organs including kidney, liver, heart, and eyes (Sharma et al., 2008).

Studies pioneered by Keith Kozminski in the laboratory of Joel Rosenbaum demonstrated movement within the flagellum of *Chlamydomonas reinhardtii*, which they named intraflagellar transport (IFT) (Kozminski et al., 1993). Subsequent research by this group and others characterized this kinesin and dynein mediated movement and identified many proteins that make up the IFT complexes that associate with the motors during transport (Taschner et al., 2011). Flagella and cilia are structurally very similar and share common pathways for biosynthesis. Similar to flagella, proteins within cilia also move by IFT. This directed movement is essential for building primary cilia because microtubules grow from the plus end, which is located at the tip of the cilium. In addition, components of signaling pathways such as the Gli transcription factors in the Hedgehog pathway also require IFT protein movement (Haycraft et al., 2005). The protocols provided here provide the framework for measuring movement of proteins within cilia.

Three strategies can be very useful when determining whether a protein of interest localizes to the primary cilium:

1. **Morphology** The cilium is a very distinct structure. Localization to a solitary elongated protrusion suggests that a protein may be in the cilium
2. **Geography** Generally, cilia form in a characteristic location in a given cell type. In NIH3T3 cells, the cilium is usually perinuclear. In MDCK cells, cilia form at the center of the apical surface. Localization of a fluorescent marker to a structure that fits the expected geographic location of the cilium is also suggestive.
3. **Co-localization with a known cilia marker** This is considered the gold standard. For imaging fixed cells, antibodies to modified forms of tubulin are often used. In live cells, fluorescent tubulin goes to the cilium, but the signal can be somewhat obscured by the fluorescence of tubulin in the cell body. Many other fluorescent proteins that also localize to the cilium and can be used to assay for co-localization are listed in table 2.

Critical Parameters

It is important to maintain cultured cells optimally at all times. This will ensure cell behavior and cilia formation is consistent between experiments. For NIH3T3 cells, this means making sure that the cells never become confluent. Before passaging MDCK cells, however, it is good to let them become confluent and to make sure that the cells in the dish pack together. MDCK cells should be compact, not spread out or “S” shaped. We have found that decreasing the amount or quality of L-Glutamine can have a negative impact on MDCK cell cultures.

Select fluorophores that will be optimal for your imaging configuration. For imaging experiments it is generally best to set the imaging parameters so that the signal of the fluorophores is as bright as possible, but not saturating the detector. Be sure that the imaging chamber is at 37 °C for optimal cell viability.

Troubleshooting

Cannot see cilium very well signal too faint—Try another fluorophore, as some fluorescent proteins are brighter than others. A monomeric fluorescent protein might help - multimers of some protein do not target to the cilium as well. Alternatively, it can be useful to know where the cilium is in order to determine what signal is actually in the cilium. There are two methods that can be useful: 1) co-localization with a protein that is known to

localize to primary cilia, and 2) visualize the cilium using transmitted light by DIC microscopy.

Cannot see cilium very well background (non-cilia fluorescence) too high—If the background fluorescence is very high, it might be helpful to use (total internal reflection fluorescence) TIRF microscopy. MDCK cell cilia can be placed against the coverslip and are visible by TIRF. A fraction of the cilia on NIH3T3 cells grow between the cell and the coverslip. These can also be seen by TIRF.

Control protein doesn't localize to the cilium—It is possible cilia are not forming properly or that the protein isn't targeting. To test the former possibility, fix cells and stain them with an antibody to acetylated tubulin or another known cilia marker. It may be helpful to also immunodetect the fluorescent-tagged protein of interest using an antibody to the fluorophore because this will amplify the signal. If the protein is not targeting to the cilium, try tagging the protein with an alternate fluorophore. Sometimes fluorescent proteins can cause aggregation or mislocalization. Try a fluorophore that is designed to be monomeric.

Focus drifts during time-lapse acquisition—A kymograph can be generated for the portion of the time-lapse that is in focus and this data can be analyzed. During image acquisition, if focal plane drift occurs, slowly adjust the focus to bring the cilium back into view. You can then split the time series, or analyze as a whole. Tracks are unlikely to appear when the cilium is out of focus, so the unfocused time points will have little impact on the data analysis.

Cilium moves during time-lapse acquisition—If the cilium movement is slight, the data may be useable. If the movement is abrupt and the cilium stays in focus, the raw data can be used to generate two kymographs one for each position of the cilium. However, if the cilium is moving a lot, the data will not be useful. To minimize movement be sure that almost all media has been wicked away from the side of the transwell before it is placed in the imaging chamber. Also, verify that no vibration or other distortion is being created in your imaging system. If the problem persists, it may be helpful to coat the coverslip with polylysine prior to placing the transwell.

Cannot discern movement by eye in raw data—Some tracks will be more obvious than others, but movement should be visible by eye in the time-lapse series. If no movement is visible, then it is not likely that kymograph analysis will help. Instead, verify the imaging parameters to be sure that you have temporal resolution to see movement. Use a control protein, such as IFT88, to verify that configuration can detect IFT. If the signal is bright and you try the photobleaching and photoconversion methods described and still do not detect movement, this suggests that the protein of interest is not moving.

Anticipated Results

Proteins such as those listed in Table 2 should localize to the cilium. These can be used as markers for co-localization studies. There may be some cell type variability or mislocalization caused by improper folding of fluorophores (see troubleshooting).

When optimizing the alternate protocols to stain primary cilia it may be useful to use co-localization with a known, transfected, cilia marker to be sure the structure identified is the cilium.

IFT88 (polaris) GFP or another IFT protein can be used to verify that the imaging parameters will detect movement within the cilium.

Time Considerations

NIH3T3 cells will form primary cilia upon serum deprivation. Typically we serum starve cells overnight. MDCK cells form cilia in the presence of serum, but their appearance is dependent on polarization, and generally takes 4 to 5 days (Sfakianos et al., 2007). Cilia length continues to increase, so it is helpful to use cultures that have similar growth times for all experiments. We typically transfect NIH3T3 cells the day after plating and MDCK cells 2 days before imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Multiple fluorescent labels can be used to visualize primary cilia

A and B MDCK cells stably expressing Tubulin GFP. Without confocal imaging the signal in the cilium can be swamped out by the cellular pool of tubulin GFP. **A** Shows a side view of the cell (an xz slice). **B** shows a y-axis maximum intensity projection of a field of cells. **C** The enrichment of SmoYFP in MDCK cilia is clear in this z-axis maximum intensity projection. **D** An NIH3T3 cell expressing SmoYFP. In the top panel (this z-axis maximum intensity projection) the cilium appears to be a dot, but in the lower panel (an xz slice) it is clear that the cilium projects out of the cell. Levels were adjusted in this image to make the cilium more clear. Scale bars = 5 μ m.

Figure 2. Exogenously added fluorescent dyes can be used to visualize primary cilia
A x-axis maximum intensity projection of MDCK cells stained with DOPE rhodamine. **B** z-axis maximum intensity projection of NIH3T3 cells expressing inversinTdTomato (red) stained with BODIPY cholesterol (green). **C** MDCK cell expressing TubulinGFP labeled with WGA rhodamine (z-axis maximum intensity projection). Scale bars = 5 μ m.

1. Collect time series

2. Generate a line over the cilium using the projection of every time point

3. The pixel value at each point along the line is used to generate a new, straight line. This is done for each time point.

4. These lines are automatically combined to generate the kymograph

5. Trace the tracks and use the slope to calculate particle speed

Figure 3. Generation of a kymograph to quantify cilia movement

After collecting a time series, a kymograph can be used to calculate the velocity of particle movements. This figure provides an overview of the steps required to generate a kymograph. Scale bars = 2 μm .

Figure 4. Using photobleaching and photoconversion to highlight moving proteins with in the cilium

A Kymographs generated from a time series where a region of a polaris-GFP expressing cilium was photobleached during acquisition. The raw data was analyzed, and then processed with a walking average or median filter. Lines used for measurements are visible in the far right kymograph. **B** SmoEos in the cilium of an MDCK cell was imaged with both 488 and 561 nm light and then photoconverted. The movement of photoconverted particles is evident in the right panel (561nm).

Table 1

Transient transfection of MDCK cells

	Tube A		Tube B	
	Opti-MEM	DNA	Opti-MEM	Lipofectamine2000
6mm	30 μ L	200ng	30 μ L	0.8 μ L
12mm	80 μ L	800ng	80 μ L	3.2 μ L

Table 2

Select cilia-localized proteins that can be used as cilia markers

Protein	Reference
Adenylyl cyclase III	(Berbari et al., 2007)
Arl13b	(Casparly, 2007)
Inversin	(Morgan et al., 2002)
Polaris	(Taulman et al., 2001)
Rab8	(Nachury et al., 2007; Yoshimura et al., 2007)
Smoothened	(Corbit et al., 2005)
SSTR3	(Handel et al., 1999)