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Kimberly A. P. Mitchell
Liberty University, kamitchell@liberty.edu

Gabor Szabo

Angela de S. Otero

METHODS FOR THE ISOLATION OF SENSORY AND PRIMARY CILIA-AN OVERVIEW

Kimberly A. P. Mitchell[2], Gabor Szabo[1] and Angela de S. Otero[1]

[1]Department of Molecular Physiology and Biological Physics
University of Virginia School of Medicine
Charlottesville, VA 22903

[2]Department of Biology and Chemistry
Liberty University
Lynchburg, VA 24502

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Corresponding author:
Gabor Szabo
Dept. of Molecular Physiology and Biological Physics
University of Virginia School of Medicine
P.O. Box 800886
480 Ray C. Hunt Dr. - Snyder Bldg.
Charlottesville, VA 22903
email: gs3z@virginia.edu

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ABSTRACT

Detailed proteomic analyses of mammalian olfactory and rod photoreceptor sensory cilia are now available, providing an inventory of resident ciliary proteins and laying the foundation for future studies of developmental and spatio-temporal changes in the composition of sensory cilia. Cilia purification methods that were elaborated and perfected over several decades were essential for these advances. In contrast, the proteome of primary cilia is yet to be established, because purification procedures for this organelle have been developed only recently. In this chapter we review current techniques for the purification of olfactory and photoreceptor cilia, and evaluate methods designed for the selective isolation of primary cilia.

1. INTRODUCTION

Primary and sensory cilia of higher organisms play an essential role as detectors and transducers of extracellular and intercellular signals (for a recent review, see Berbari *et al.*, 2009). The primary cilium present in the majority of vertebrate cells responds to chemical and mechanical stimuli (reviewed in Praetorius and Spring, 2005; Wheatley, 2008; Gerdes *et al.*, 2009), while modified cilia of specialized cells in the retina and olfactory epithelium sense odorants and light. Multiple olfactory cilia arise from a knob at the extremity of apically oriented dendrites of olfactory receptor neurons (ORN) and project into the mucous layer on the epithelial surface (reviewed in Menco, 1997 and McEwan *et al.*, 2009). The photoreceptor outer segment is the enlarged tip of the connecting cilium, which is anchored to a basal body in the inner segment (Besharse and Horst, 1990; Fig. 1). Disfunctions of non-motile cilia are associated with a number of human diseases, referred to as ciliopathies (reviewed in Tobin and Beales, 2009).

Outer segments from retinal photoreceptor cells have been isolated by mechanical agitation followed by fractionation in density gradients for more than 70 years (Saito, 1938), and the techniques for isolation of olfactory and primary cilia rely on classical protocols developed over several decades for the purification of flagella and traditional “9+2” cilia from invertebrates and protozoa. The invaluable contribution of these methods to recent and remarkable developments in the understanding of the structure and function of sensory and primary cilia cannot be understated (Ostrowski *et al.*, 2002; Pazour *et al.*, 2005; Keller *et al.*, 2005; Gherman *et al.*, 2006; Liu *et al.*, 2007; Mayer *et al.*, 2009). As pointed out by Pazour *et al.* (2005), comparative genomics and *in silico* approaches provide important information on the identity of ciliary proteins, but cannot substitute for direct proteomic analysis of purified cilia, which is a

critical step toward establishing a definitive inventory of the proteins involved in the structure and function of any organelle. Here we recapitulate techniques developed for the isolation of olfactory and photoreceptor cilia of a purity suitable for proteomics studies, and assess existing methods for isolation of primary cilia for their potential value in future structural and functional studies.

2. ISOLATION OF OLFACTORY CILIA

Early methods for removing cilia from *Tetrahymena* utilized ethanol treatment followed by exposure to digitonin, high concentrations of KCl or glycerol (Child and Mazia, 1956; Child, 1959). In 1962 Watson and Hopkins published a method for isolating *Tetrahymena* cilia that combined ethanol treatment with high calcium, a procedure that became the basis for deciliation and deflagellation protocols in subsequent years (Gibbons, 1963; Winicur, 1967; Rosenbaum and Child, 1967; Witman *et al.*, 1972; Adoutte, 1980). Eventually the use of ethanol was discontinued (Adoutte, 1980), and deciliation through incubation in high Ca^{2+}, often combined with mechanical agitation, became known as the Ca^{2+}-shock method, a classic procedure that is still the method of choice for detaching olfactory cilia from ORNs (Anholt *et al.*, 1986; Chen *et al.*, 1986; Schandar *et al.*, 1998; Delgado, *et al.*, 2003; Mayer *et al.*, 2008, 2009). The common event triggered by all these treatments is the entry of calcium into cells, which activates the molecular machinery that results in the severance of cilia or flagella from the cell body (reviewed in Quarmby, 2004).

The isolation of olfactory cilia is not a straightforward process, given that the cilia and the dendritic knob are surrounded by numerous microvilli from supporting cells which can contaminate the final product. For instance, the standard Ca^{2+}-shock method produces a fraction

containing significant amounts of non-ciliary material (Mayer *et al.*, 2008), a finding that led to modifications of the procedure that yield highly purified olfactory cilia appropriate for proteomic analysis. This method is summarized below.

2a. Ca^{2+}/K^{+}-shock and NaBr treatment; Mayer *et al.*, 2009

In this modified Ca^{2+}-shock procedure Mayer *et al.* (2009) reduce contaminants of the cilia purified by the traditional Ca^{2+}-shock method (Mayer *et al.*, 2008) through the inclusion of K^{+} in the deciliation buffer, an approach previously adopted for the isolation of *Paramecium* cilia (Adoutte, 1980).

Briefly, the rat olfactory epithelium is washed for 5 min in a neutral isotonic buffer containing EGTA to remove mucus. Subsequently, the cilia are severed by gentle agitation in the same buffer, this time containing 20 mM $CaCl_2$ and 30 mM KCl -but no EGTA- for 20 min at 4°C. Cells and debris are pelleted by low speed centrifugation. The detached cilia are collected by ultracentrifugation on a sucrose cushion (45% sucrose in deciliation buffer), and the interface band is diluted and pelleted. The ciliary fraction is washed twice with NaBr (2M, followed by 1M) by resuspension and ultracentrifugation. The resulting pellet is washed by resuspension in a hypotonic buffer containing EGTA, and the olfactory cilia are recovered through a final ultracentrifugation step.

The yields in olfactory cilia protein using this procedure are one fifth of those observed with the traditional Ca^{2+}-shock method, presumably due to the decrease in contaminating cellular proteins and microvilli. However, these modifications increase the specificity of the deciliation process, so that larger amounts of olfactory cilia are detached from the tissue while contaminating cellular proteins from microvilli and other sources remain behind, leading to an

estimated enrichment of ~100-fold in ciliary proteins as assessed by immunoblotting of marker proteins. Examination of the deciliated tissue shows stubs of the ciliary proximal segments, suggesting that the fraction obtained through this method consists chiefly of the distal portions of olfactory cilia. Analysis of the purified cilia by mass spectrometry led to the identification of 377 proteins, the majority of those being linked to the specialized function of olfactory neurons. Clearly, this is not a complete inventory of the proteins present in olfactory cilia, but these results give new insights and perspectives regarding the components of the complex molecular machinery involved in olfactory signaling.

Note that isolation of olfactory cilia by a Ca^{2+}-shock may inflict damage to cellular processes sensitive to high calcium levels, such as signaling mechanisms, and may promote proteolytic activity. With this caveat in mind, Washburn *et al.* (2002) devised a method for the purification of olfactory cilia membranes in which cilia are detached by mechanical agitation in a neutral, isotonic buffer containing EDTA. The ciliary yields in the supernatant are slightly higher than those obtained with a standard Ca^{2+}-shock method. In addition, the presence of ciliary markers (evidenced by immunoblotting) and the adenylyl cyclase activities of the two preparations are comparable. However promising, this protocol has yet to be utilized specifically for the isolation of intact cilia, and has not been characterized in terms of purity and integrity. Until this is done, the Ca^{2+}/K^{+}-shock method described above will likely remain the standard means of isolating olfactory cilia.

3. ISOLATION OF ROD PHOTORECEPTOR CILIA

Intact, sealed photoreceptor sensory complexes (PSC) which comprise the outer segments and their ciliary axoneme, as well as the basal bodies and ciliary rootlets from the inner segment

(Fig. 1) are readily detached from the retina by mechanical agitation, and can be separated from the remainder of the photoreceptor cell by centrifugation in density gradients. Versions of this procedure have been used for many years to obtain large amounts of virtually pure PSCs from bovine retinas, and were instrumental in building our present understanding of signal transduction in the visual system (Saito, 1938; Papermaster and Dryer, 1974; Zimmerman and Godchaux, 1982; Schnetkamp and Daemen, 1982). Published methods differ in the technique adopted to detach PSCs from retinas (e.g., vortexing or swirling), the composition and osmolarity of buffer solutions, the gradient-forming material (sucrose, Percoll, Ficoll, metrizamide), and the type of gradient utilized (continuous vs. discontinuous) and will not be reviewed here. The ciliary cytoskeleton of PSCs, comprising the axonemes, basal bodies and rootlets, can be isolated by detergent treatment, which solubilizes the plasma membrane and the outer segment discs (Horst, 1987; Fleischman and Denisevich, 1979; Pagh-Roehl and Burnside, 1993; Schmitt and Wolfrum, 2001). Note that the separation of connecting cilia from the remainder of the PSC has not been achieved, since this bridge-like structure is quite small (1 μm long and 0.3 μm in diameter; Schmitt and Wolfrum, 2001) and is effectively buried between the inner and outer segments.

We summarize below a procedure that was chosen for the purification of ROS for proteomic analysis (Liu *et al.*, 2007) providing a wealth of qualitative and quantitative information on the composition of the photoreceptor cilium.

3a. Mechanical shear; Liu *et al.*, 2007

This procedure is a modification of those of Papermaster and Dryer (1974) and Liu *et al.* (2004). Mouse retinas are gently vortexed for 1 min in a hypertonic buffer containing 50% (w/v) sucrose. The suspension is centrifuged at high speed to pellet debris and tissue. The PSC band at the top is diluted to reduce the sucrose concentration and layered on a 50% sucrose shelf and re-centrifuged. Purified PSCs are collected at the interface, diluted and pelleted. This fraction contains highly purified photoreceptor cilia. The same purification procedure was followed to isolate PSCs from retinas from rootletin KO mice, which do not form ciliary rootlets and therefore are essentially free of inner segment components (Fig. 1). Additionally, wild-type PSCs were treated with detergent to allow detection of low abundance cytoskeletal proteins that otherwise would be obscured by the copious amounts of signaling proteins such as rhodopsin.

Analysis of these three preparations by mass spectrometry led to the identification of ~2,000 proteins, along with an estimate of their individual copy numbers. The assignment of the proteins to outer vs. inner segments was accomplished through the assessment of overlaps and divergences between the three datasets. Comparison with the proteomes of cilia from lower organisms shows that numerous proteins are conserved and expressed in the mammalian photoreceptor cilium, in particular those involved in ciliogenesis and maintenance of mature cilia. A significant number of the proteins identified in this work are linked to diseases that impair vision, e.g., retinal degeneration, and others are the product of genes linked to ciliary anomalies associated with human ciliopathies such as Bardet-Biedl syndrome (Tobin and Beales, 2009).

4. ISOLATION OF PRIMARY CILIA

Anyone attempting to isolate primary cilia in amounts compatible with biochemical or functional analysis faces a formidable task. Most vertebrate cells display a single cilium of diminutive proportions, with a diameter of approximately 0.2 μm and lengths that vary from 1-4 μm. These forbidding characteristics stack up against a single encouraging feature: in the majority of cells the primary cilium protrudes from the cell surface, its accessibility being a trait that in principle should simplify the purification process. The situation is somewhat improved in confluent cultures of renal epithelial cells which express long primary cilia, with average lengths of 8-25 μm depending on the specific cell line (Roth *et al.*, 1988; Wheatley *et al.*, 1996; Wheatley and Bowser, 2000; Mitchell *et al.*, 2004). Still, the ratio of the volume of a primary cilium to that of a typical renal epithelial cell in culture is very low, e.g., about 1:3,000 in Madin-Darby canine kidney (MDCK) cells for a cilium with a length of 8 μm (Praetorius and Spring, 2002). Because cell volume is proportional to protein content (Erlinger and Saier, 1982), a confluent 10 cm dish of MDCK cells with 3 mg protein can yield at best 1 μg of pure primary cilium protein. This is a highly optimistic estimate, which assumes 100% of full-length cilia are harvested by breakage at the ciliary necklace. In reality, primary cilia are very fragile structures prone to fragmentation (Gallagher, 1980), and are easily severed along the axoneme. Indeed, EM and immunofluorescence microscopy analysis of isolated primary cilia fractions (Mitchell *et al.*, 2004; Raychowdhury *et al.*, 2005) show cilia with shortened shafts and numerous detached tips, indicating that fracture of the ciliary stem often takes place at its distal portion and not at the base. Thus, it is essential to start with generous amounts of cells in order to obtain sufficient material for further analysis. Equally important is to use the gentlest possible deciliation process

in order to preserve the integrity of the cilium membrane, retain components of the ciliary matrix, and reduce the odds of contamination by other organelles or cell components.

4a. Mechanical shear; Mitchell *et al.*, 2004

The rationale behind this procedure is two-fold: to harvest large amounts of primary cilia by using a cell line which has long cilia, and to prevent damage to the cilium by avoiding the use of anesthetics or extremes of pH, osmolarity or high calcium. The cells chosen, from the A6 line, derive from the distal nephron of *Xenopus* and have a high incidence of primary cilia that extend to an average length of 23 μm after 7-10 days of culture in complete medium (Mitchell *et al.*, 2004). Therefore, in principle one could obtain 3 μg of pure primary cilia from a confluent, 150 mm dish of A6 cells. Additionally, when A6 cells are plated at high density they express sizeable primary cilia within 24h, a feature that can be exploited to study changes in the composition (Mitchell *et al.*, 2004) and function of cilia during the elongation process. The technique selected for deciliation is based on the observation that shear forces can easily lead to deciliation of specimens (Gallagher, 1980). The procedure consists of brief mechanical agitation in phosphate-buffered saline followed by differential centrifugation and can be completed in less than 1 hour. With this gentle approach the cell monolayer and its apical surface suffer a minimum of mechanical damage, reducing contamination of the primary cilium fraction with proteins from the cell body. Examination of cells by immunofluorescence microscopy after agitation shows that the apical microvilli, which are stained by an antibody to NDP kinase, remain intact after deciliation. Note that not all cilia are removed, since the surface of the confluent monolayer is uneven due to dome formation (Moberly and Fanestil, 1988), so the drag force on stems and tips of cilia is not uniform across the culture dish.

Electron microscopy of negatively stained specimens (Fig. 2a) reveals structures with the characteristic lollipop shape of primary cilia (Roth *et al.*, 1988; Wheatley and Bowser, 2000). The bulb-like tips often appear as discrete structures. Immunofluorescence analysis of preparations fixed and then stained for both acetylated α-tubulin and NDP kinase, which is present in primary cilia as well as in microvilli (Mitchell *et al.*, 2004; Donowitz *et al.*, 2007), confirms that the preparation contains not only typical cilia, but also round structures of roughly the size of ciliary tips observed in cells stained for acetylated α-tubulin. Structures labeled solely by antibodies to NDP kinase, presumably non-ciliary contaminants, constitute an estimated 40–60% of the isolated primary cilia fraction.

Note that the ciliary shafts are significantly longer in samples visualized by immunofluorescence (Fig. 2b) when compared with those seen with negative stain microscopy (Fig. 2a), suggesting that fixation of the specimen helps to preserve ciliary structure

Yields from four 150 mm dishes are 6-20 μg of isolated primary cilia, falling within the range of the theoretical number of 12 μg of protein for an ideal, 100% pure preparation of full length, 24 μm cilia, and the procedure is easily scaled up. Analysis of immunoblots (Fig. 2c) shows a considerable enrichment in acetylated α-tubulin in comparison with whole A6 cell extracts, on average 17-fold (n=4). Note that in some preparations a purification factor could not be estimated, because of the absence of a signal for acetylated α-tubulin in the cell extract (e.g., Fig. 2c), as expected from an antigen present at low levels in cells (Piperno *et al.*, 1987).

In brief, this protocol suffers from the usual trade-off: a high degree of enrichment is achieved, but yields are relatively low. The same approach can be utilized to isolate primary cilia from MDCK cells (Otero and Mitchell, unpublished results), although one should keep in mind

that the process of ciliogenesis in this line is slower than in A6 cells (Praetorius and Spring, 2005).

4b. Ca^{2+}-shock; Raychowdhury *et al.*, 2005

This procedure uses a Ca^{2+}-shock protocol (see section 2a) to purify cilia from the LLC-PK1 cells, a renal cell line that has well developed primary cilia when grown to confluence for 2-3 weeks (17 μm; Roth *et al.*, 1988). The major modification to the classic Ca^{2+}-shock is a preliminary step in which cells are scraped from the culture dish in Ca^{2+}-free saline. The cells are then pelleted, resuspended in a high Ca^{2+} solution (112 mM NaCl, 3.4 mM KCl, 10 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 2 mM HEPES, pH 7.0) and shaken for 10 min at 4°C. After low speed centrifugation (7,700x*g* for 5 min) to remove debris, the suspension is placed on a 45% sucrose shelf in high Ca^{2+} solution and centrifuged at 100,000xg for 1h. The interface band is removed, and cilia are pelleted by centrifugation for 1h at 100,000xg after a 10-fold dilution of the band at the saline/sucrose interface. The final pellet is resuspended in normal PBS containing 2.0 mM EGTA and 0.5 mM sucrose. Yields were not reported. This method was successfully adapted by Wang and Brautigan (2008) to purify primary cilia from human retinal epithelial cells.

The possibility of contamination of the preparation by basolateral membranes was explored in a subsequent publication (Raychowdhury *et al.*, 2009) through immunostaining with antibodies to tubulin, type 2 vasopressin receptor (V2R) and Na^+-K^+-ATPase. The results are not shown, but the authors report a negligible contribution of basolateral membranes to the isolated cilia fraction.

The cilia preparation obtained through this approach was characterized by immunofluorescence and electron microscopy, as well as electrophysiological methods. Staining

with antibodies to acetylated α-tubulin shows rounded structures and cilia that are short and appear much thicker than the native structures seen in cells, although comparison between native and isolated primary cilia is hampered by the absence of magnification bars (Raychowdhury *et al.*, 2005; Li *et al.*, 2006). Additional immunolabeling experiments show the presence of epithelial sodium channels in cilia isolated from wild-type cells, and V2R-*gfp* and adenylyl cyclase in primary cilia isolated from V2R-*gfp* expressing LLC-PK1 cells.

Negative stain electron microscopy shows numerous cilia tips and relatively long shafts of irregular diameter (Raychowdhury *et al.*, 2005). As stated in Raychowdhury *et al.* (2005), this procedure swells the cilia to the extent that electrophysiological recordings can be obtained using a very small, high resistance (140 mΩ, or approximately 0.1 μm) patch pipette. However, a true gigaseal (that is, a pipette-to-membrane electrical seal with a resistance greater than 5 GΩ) could not be established. The poor quality of the membrane-pipette seal may result from membrane damage, the presence of debris on the membrane surface or, similarly to what happens in spermatozoa (Kirichok *et al.*, 2006), the existence of a tight connection between the plasma membrane and the axoneme preventing seal formation. This leaky seal, in combination with the high resistance of very small patch pipettes, precludes proper voltage clamping of the preparation and accurate measurement of channel properties, since the leak current (~1 pA) is far from negligible when compared to the size of the single channel currents observed. Within these constraints, two levels of ion currents attributed to non-selective ion channels can be detected in cilium-attached patches, as well as a small Na^+-permeable channel induced by vasopressin. The activity of the latter is increased by bath application of cAMP-dependent protein kinase (PKA) and ATP, and is inhibited by amiloride. The mechanism by which PKA gains access to the inner surface of the membrane is not known; it is speculated that the enzyme can cross the leaky

membrane, and/or diffuse through the open end of the isolated cilia (Raychowdhury *et al.*, 2005). The latter hypothesis is unlikely to be correct, because the ciliary lumen is a congested compartment, mostly filled with the axoneme and attached proteins, and the cilia isolated by calcium shock retain not only axonemal proteins but also cytosolic and membrane proteins such as soluble and membrane-associated adenylyl cyclase, cyclic nucleotide phosphodiesterase (Raychowdhury *et al.*, 2009), the catalytic subunit of protein phosphatase 1 (PP1C) and phosphatase inhibitor-2 (Wang and Brautigan, 2008). Thus, the tears in the ciliary membrane are likely to be larger than 50 Å, since the hydrodynamic radius of PKA complexed with ATP is 24 Å (Yang *et al.*, 2005).

Reconstitution of the cilia preparation onto lipid bilayers indicates the presence of several types of ion channels, including a cation-selective channel inhibited by an antibody to polycystin-2 and an anion-selective channel (Raychowdhury *et al.*, 2005; Li *et al.*, 2006; Raychowdhury *et al.*, 2009). Nevertheless, in the absence of a rigorous assessment of the degree of contamination by subcellular components released by the initial scraping step, the origin of these channels remains undefined.

In summary, this approach produces a fraction enriched in primary cilia, but may lead to significant contamination of the final material by proteins from other cell compartments (Mayer *et al.*, 2009). The physical damage to the membrane is also a disadvantage, interfering with the direct electrophysiological recording of ion channel currents *in situ*. Note that scrape-loading experiments have demonstrated that the tears in the plasma membranes of cells are unable to reseal quickly at low calcium levels (McNeil and Steinhardt, 1997), so both problems may be reduced if scraping in a Ca^{2+} free solution is avoided. An alternative and milder approach would

be to apply this Ca^{2+}-shock protocol to cells lifted with normal saline containing 1 mM EDTA, a technique that preserves primary cilia (Mitchell *et al.*, 2004).

4c. Peel-off and slide-pull; Huang *et al.*, 2006

These two approaches were utilized to isolate primary cilia of medium size (7-10 μm) from cultures of normal mouse and rat cholangiocytes. Basically, a coverslip or small culture dish is coated with 0.1% poly-L-lysine (PLL) and placed atop the upper surface of the cells. After application of pressure (peel-off) or mechanical agitation (slide-pull), the coated surface with attached structures is removed and used for cilia purification. The composition of the solutions utilized during cilia isolation is not reported.

Both methods derive from the “rip-off" or "de-roofing" procedure originally designed for detaching sheets of plasma membrane from the cell surface (Sanan and Anderson, 1991, Perez *et al.*, 2006). This technique has been successfully utilized to isolate plasma membranes with attached endocytic vesicles and cytoskeletal components from the apical surface from normal rat cholangiocytes (Doctor *et al.*, 2002). Indeed, portions of the cholangiocyte apical membrane adhere to the PLL-coated surface and are detached along with the cilia (Huang *et al.*, 2006), contaminating the cilia preparation.

In the peel-off method, the coated coverslip is manually pressed onto the cells for 20s while the medium is removed, and then quickly lifted off with forceps. The preparation is either fixed and analyzed by microscopy, or scraped from the coverslip and purified in a sucrose cushion, as described by Raychowdhury *et al.* (2005). A deciliation efficiency of 70% was estimated by staining the coverslips with anti-acetylated α-tubulin.

In the slide-pull technique a 6 cm PLL-coated dish is placed on top of cells cultured in a 10 cm dish, and this assembly is placed on a sliding shaker at 70-100 rpm. After 5 min the supernatant is removed, vortexed for 1 min, and placed in an ice bath for 20-30 min to settle debris. The cilia are collected and purified as above.

To assess purity, ciliary pellets and extracts from the corresponding deciliated cell extracts were solubilized and analyzed by immunoblotting. Analysis of the isolated primary cilia shows the presence of acetylated α-tubulin, polycystins 1 and 2, and fibrocystin. However, amounts of the ciliary marker acetylated α-tubulin in the final fraction of the peel-off technique are only 50% higher than those measured in the whole cell lysate. The slide-pull approach leads to a slightly higher enrichment in acetylated α-tubulin: 3-fold. The yields of ciliary protein from three 10 cm dishes are 35-130 μg and 150-270 μg for the peel-off and slide-pull techniques, respectively. These excessively high yields (see section 4a), combined with the modest enrichment in acetylated α-tubulin, imply that both preparations are impure, consisting mostly of non-ciliary elements. The ciliary fraction obtained by the peel-off technique is likely to be heavily contaminated by plasma membranes, endocytic vesicles, caveolae, coated pits and the submembranous cytoskeleton (Huang *et al.*, 1997; Heuser, 2000; Parton and Hancock, 2001). Furthermore, the cilia recovered from the coated coverslip by scraping are likely to be irreparably damaged, and thus unsuitable for studies that require a minimum of structural integrity. As to the slide-pull method, movement of the PLL-coated dish may well wound the cells and even result in the detachment of membrane sheets, increasing the amounts of contaminants in the fraction.

4d. Perspectives

The emergence of methods to isolate primary cilia is a critical step toward understanding the normal functions of these complex structures and their role in human disease. The approaches described here use traditional techniques based on chemical or mechanical stress to sever primary cilia from cells. Methodological improvements will be required to reduce damage to this delicate organelle during purification. The discovery of selective deciliation agents that remove motile cilia reversibly, rapidly and at low concentrations (Semenova et al., 2008), raises hopes that analogous compounds may soon become available for the removal of immotile cilia, yielding preparations of intact and essentially pure primary cilia. Recent advances in patch clamp techniques for the study of ion channels in tiny structures such as the sperm head (Jiménez-González et al., 2007) imply that gigaseal recording of ion channel activity in isolated primary cilia is an attainable goal. Those are compelling reasons to believe that a comprehensive picture of the composition and function of primary cilia will be a reality in the near future.

FIGURE LEGENDS

Figure 1. **Purified PSC complexes and fractions** (From Liu *et al.*, 2007, reproduced with permission.)

A, schematic of a wild-type PSC complex showing that it is comprised of the outer segment and its cytoskeleton, including the rootlet, basal body, and axoneme. *B–D*, wild-type PSC complex preparation viewed with differential interference contrast and fluorescence microscopy. At higher magnification (*C* and *D*) it can be seen that the PSC complexes consist of outer segments with thin extensions at their bases. The extensions are the portions of the cytoplasmic cytoskeleton that were attached to the basal bodies as indicated by staining with antibodies to rootletin (*red*; *B* and *D*). The axoneme in the outer segment is demonstrated by staining with antibodies to Rp1 (*green*; *D*). At lower magnification (*B*), it is evident that the preparation consists of highly enriched PSC complexes with minimal contamination by the other structures noted. *E–H*, drawing of PSC complex-cytoskeleton (*E*) and isolated PSC complex-cytoskeleton preparation viewed with differential interference contrast (*G*) and fluorescence microscopy (*F* and *H*) using antibodies to rootletin (*red*) and Rp1 (*green*). The cytoskeletons consist of rootlets, basal bodies, and axonemes and are highly purified as illustrated in the images. *I–K*, drawing of a rootletin KO PSC complex (*I*) and rootletin KO PSC complex preparation (*J* and *K*) viewed with differential interference contrast and fluorescence microscopy and stained with antibodies to rootletin (*red*) and Rp1 (*green*). In these PSC complexes, *small dots* of rootletin staining are noted at the basal bodies, but no formed rootlets are present. *BB*, basal body;*RT*, ciliary rootlet; *TZ*, transition zone; *AX*, axoneme. *Bars* = 5 μm.

Figure 2: **Characterization of isolated primary cilia**

a. Electron micrograph of isolated primary cilia negatively stained with uranyl acetate. Arrows indicate primary cilia, and arrowheads point to ciliary tip-like structures. Bar: 2.5 μm. **b**. Immunofluorescence microscopy of isolated primary cilia doubly stained with antibodies to acetylated α-tubulin and NDP kinase. Red: acetylated α- tubulin; green, NDP kinase; yellow, overlap regions. Bars: 20 μm. **c**. Immunoblot analysis of isolated primary cilia: equal amounts of protein from whole cell extracts (WC) and isolated primary cilia (PC) were resolved by SDS-PAGE and probed with antibodies to acetylated α-tubulin (AcTub).(Modified from Mitchell *et al.*, 2004, with permission.)

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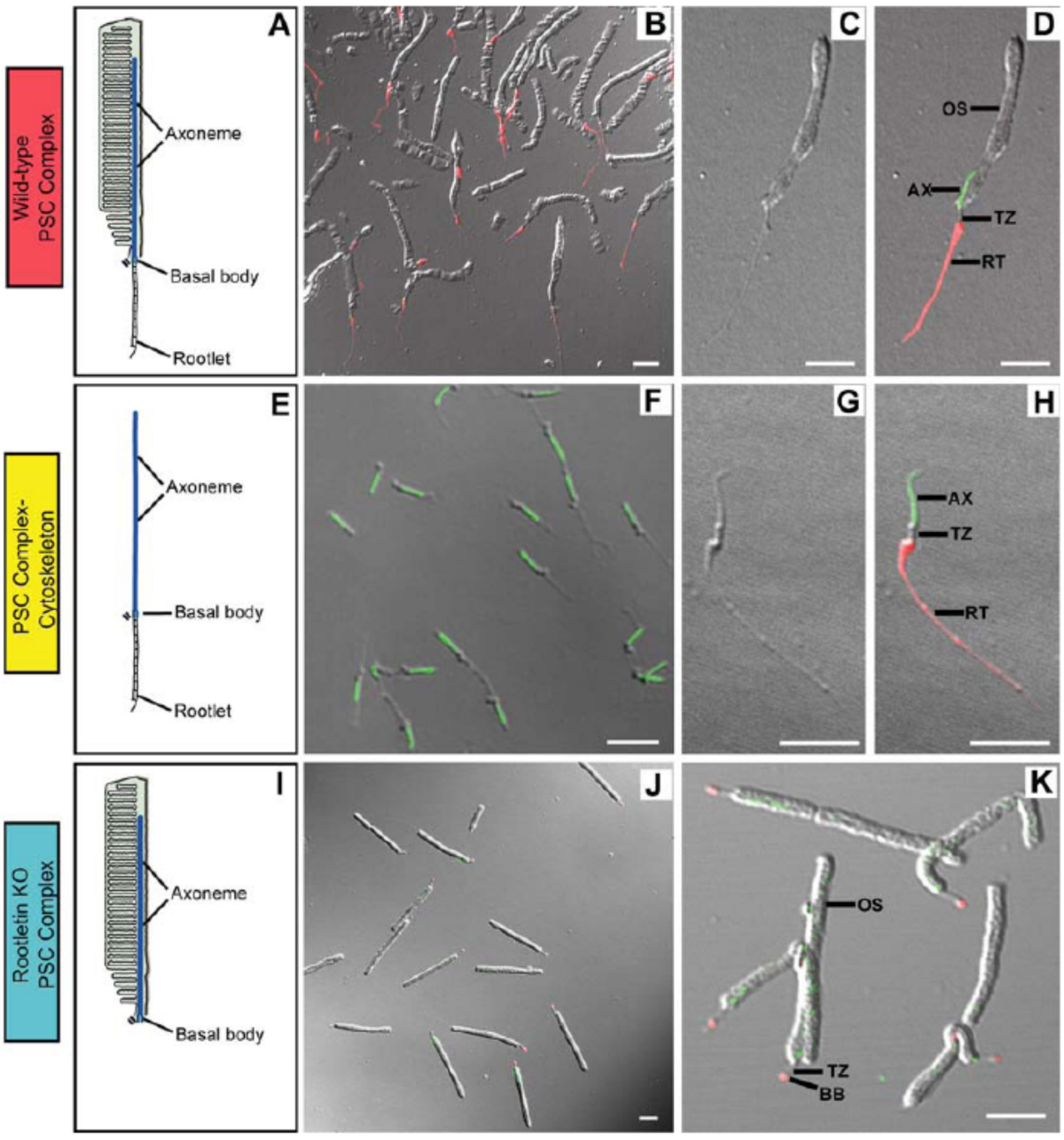
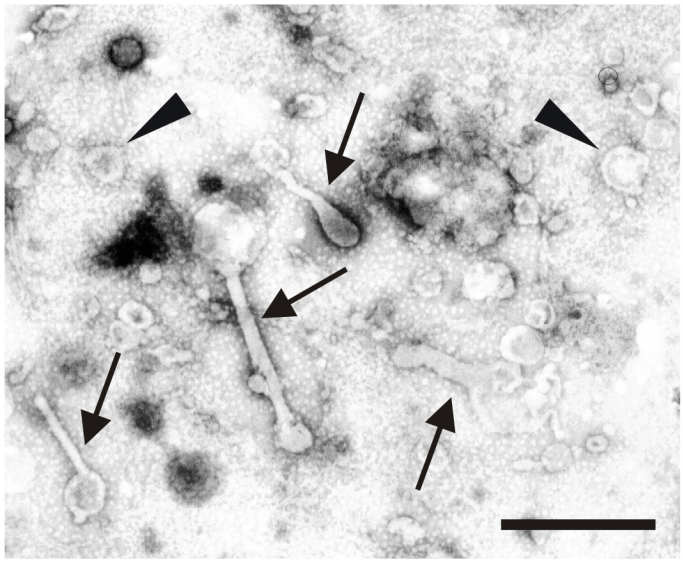


Fig. 1 Mitchell et al.

a



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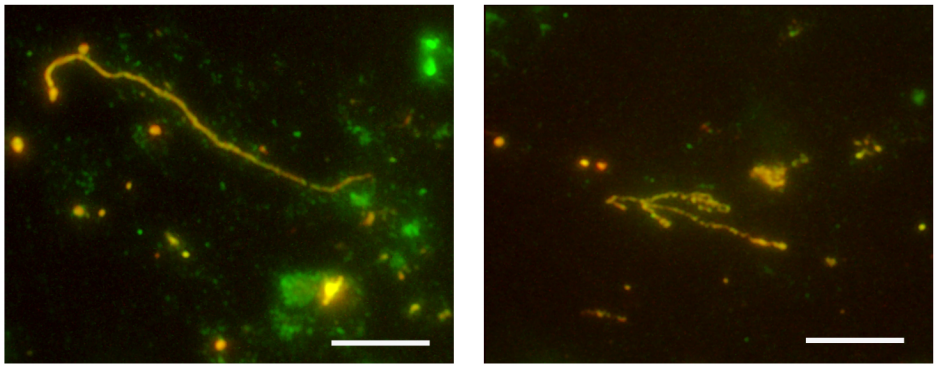

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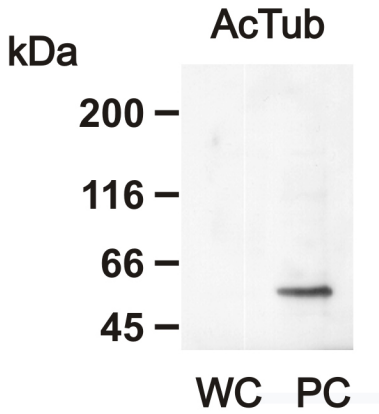

Fig. 2 Mitchell et al.