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# Isolation of Primary Cilia by Shear Force

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**ABSTRACT**

The cell's primary cilium is both a mechanical and chemical sensor involved in many signaling pathways. In order to ascertain protein enrichment in the primary cilium or study sub-ciliary localization of various proteins, it is advantageous to remove the primary cilium from the cell body. The protocol described here gives detailed instructions on purifying primary cilia by separating them from the cell body using shear force. This simple technique avoids using harsh purification conditions that may affect signaling proteins in the cilium or cause the ciliary membrane to disintegrate. In addition, as the cell body remains mostly intact, contamination of the isolated cilia by proteins from the cell body is minimized. This protocol is ideally suited for isolating cilia from renal cell lines, as primary cilia in these cells grow to greater lengths than in other cell types (up to 50 μm long in *Xenopus* A6 toad kidney cells as opposed to 1-5 μm in NIH3T3 fibroblast cells).

**Keywords: Primary cilia, isolation, purification, shear force**

## INTRODUCTION

Primary cilia are hair-like sensory organelles that project from the surface of most vertebrate cells and play an essential role as detectors and transducers of both extracellular and intracellular signals (for reviews see Berbari et al., 2009; D'Angelo and Franco, 2011; Deane and Ricardo, 2012; Farnum and Wilsman, 2011; Gerdes et al., 2009; Goetz and Anderson, 2010; Hoey et al., 2011; Jones and Nauli, 2012; Lancaster and Gleeson, 2009; Lienkamp et al., 2012; Satir et al., 2010; Sloboda, 2009; Veland et al., 2009; Zaghloul and Brugmann, 2011). Although primary cilia were discovered in 1898 (Zimmerman, 1898), their ubiquity and significance were not revealed for almost 100 years. In 1980, Gallagher demonstrated that primary cilia were not an infrequent cell trait, as previously thought. Rather, a single cilium was detectable in the majority of corneal endothelial cells, if care was taken not to expose the tissue to turbulent solution flow during mounting and fixing procedures. Flushing of the cell surface with liquid led to rupture of the fragile cilium stem, resulting in partial or total deciliation of cells.

The ease with which primary cilia detach from cells exposed to mechanical shear was exploited by Mitchell et al. (2004), leading to the development of the first procedure for the isolation of this organelle in amounts sufficient for biochemical and structural analysis. This protocol is described here and relies on the use of cell lines that express long cilia. Renal cells such as

*Xenopus* A6 kidney cells are ideal. The cilia of A6 cells reach lengths of ~24 μm (Mitchell et al., 2004) after 7-10 days post-confluence in culture (Figure 1). Other renal cell lines grow cilia with average lengths ranging from 8-24 μm (Roth et al., 1988; Wheatley and Bowser, 2000; Wheatley et al., 1996). In contrast, most cells have much shorter (average 1-4 μm) primary cilia.

The technique is simple, rapid, easily scaled up, and does not require special laboratory equipment or chemicals. Four 150 mm dishes of A6 cells yield 6-20 μg of isolated primary cilia, an amount that compares well with the maximal yield of 12 μg of protein for a totally pure preparation of full length (24 μm) cilia obtained by Mitchell et al. (2004). The conditions during isolation are mild and cells remain intact and adherent, minimizing contamination of the primary cilium fraction with proteins from the cell body. Effectiveness of purification can be assessed using both immunocytochemistry and biochemical analysis (Figures 2, 3), as protein and membrane compositions differ between primary cilium and the cell body/plasma membrane (Hu and Nelson, 2011). To analyze purity via biochemical analysis, SDS-PAGE should be performed, loading equal amounts of protein from the purified primary cilia preparation and from a whole cell lysate. The gel can either be stained with a protein stain such as Coomassie Brilliant Blue, or transferred in preparation for Western blotting. After transfer, the blot should be probed with an antibody against acetylated α-tubulin (Figure 2). The ciliary fraction should show an enrichment in acetylated α-tubulin (Piperno et al., 1987). In the author's experience, the ciliary fraction had an average of a 17-fold enrichment in acetylated α-tubulin compared to the *Xenopus* A6 whole cell lysate (Mitchell et al., 2009). Although thorough discussion of the protein composition of the primary cilium is beyond the scope of this paper, hundreds of proteins have been identified in other studies as primary cilium components (Davenport and Yoder, 2005; Ishikawa et al., 2012; Narita et al., 2012). An additional protocol using electron microscopy (EM) to visualize the isolated fraction is included (Basic Protocol 2).

***BASIC PROTOCOL 1***

## ISOLATING PRIMARY CILIA USING SHEAR FORCE

This section gives step-by-step instructions on isolating primary cilia from cells using shear force. In order to carry out this protocol, cells must be confluent and quiescent, as primary cilia are formed during interphase and reabsorbed during mitosis. In addition, primary cilia should be mature and allowed to grow to their maximal length, which varies among cell types. Renal cells develop the longest cilia and are therefore ideal for this procedure. Several renal cell lines grow long primary cilia, including *Xenopus* A6, BSC-40, LLC-PK, MDCK, PtK1 and PtK2 with average lengths of 24, 11, 13, 8, 12 and 13 μm, respectively. (Mitchell et al., 2004; Pazour and Witman, 2003; Praetorius and Spring, 2001; Roth et al., 1988). Once cilia are mature, the cell culture dishes will be shaken in PBS to remove the primary cilia from the cell bodies, followed by centrifugation to remove any cell bodies/large organelles, and then an additional centrifugation step to pellet the primary cilia. The isolated primary cilia will then be resuspended in Resuspension Buffer (Hastie et al., 1986) and either studied directly or frozen

and stored for later use. To test efficacy of purification, cells should be grown on 2 coverslips. Primary cilia should be removed from one coverslip according to the protocol below. After cilia are removed, immunofluorescent staining should be performed on both coverslips using antibodies against acetylated $\alpha$-tubulin. Additionally, an antibody against any other cellular/cytoplasmic protein or structure should be used as a control to identify individual cells. Comparison of the two coverslips will reveal effectiveness of primary cilia removal (Figure 3).

***Materials***

Cells such as *Xenopus* A6 cells
150 mm tissue culture dishes
A6 tissue culture medium (see recipe)
PBS (see recipe)
Resuspension Buffer (see recipe)
~50 ml centrifuge tubes appropriate for chosen rotor
~24 ml ultracentrifuge tubes appropriate for chosen rotor

Humidified incubator with $CO_2$ supply
Rotary shaker
Refrigerated centrifuge (Beckman J6 or equivalent) with either swinging-bucket or fixed-angle rotor capable of volumes of 45 ml or more
Refrigerated ultracentrifuge with fixed-angle rotor (Beckman 60Ti, or equivalent)

***Grow cells to produce long primary cilia.***

1. In 150 mm tissue culture dishes, plate cells in appropriate medium, such as A6 culture medium (see recipe).
   *Kidney cells such as A6 Xenopus cells yield long primary cilia. These can be grown in a humidified incubator at 28 °C in 1% $CO_2$.*
   *Primary cilia form during interphase, so cells must be confluent and quiescent. Plating cells at higher density will allow them to reach confluence more quickly.*
2. Continue to grow for 7-10 days beyond confluence, changing medium as necessary to keep cells supplied with nutrients.

***Remove primary cilia from cells.***

3. Remove tissue culture dishes from incubator and remove medium from dishes using vacuum aspiration.

4. Rinse cells by adding 5 ml PBS (see recipe) against the inside edge of the dish to avoid lifting cells with fluid flow, and gently rocking back and forth; remove PBS by vacuum aspiration.

5. Gently add 10 ml PBS to the inside edge of the dish; replace dish covers.

6. Place dishes on a rotary shaker and shake at 360 rpm [*Au: x g] for 4 min at room temperature.

7. Remove dishes from shaker and, with a micropipetter, transfer the PBS solution containing primary cilia into a 50 ml centrifuge tube or other appropriate tube.

***Remove any contaminating cells and debris from suspension.***

8. Centrifuge the solution containing primary cilia at 1,000 x g for 10 min at 4 °C in a Beckman J6 centrifuge or equivalent.

9. Mark position of pellet (P1) with a felt tip pen. Using a micropipetter, transfer supernatant (S1) to an ultracentrifuge tube, being careful not to disturb the pellet. Discard the pellet (P1) containing cells and debris.

***Pellet primary cilia out of suspension.***

10. Centrifuge the supernatant (S1) from Step 9 at 40,000 x g for 30 min at 4 *°C* in an ultracentrifuge.

11. Note position of pellet (P2). Taking care not to disturb pellet, remove supernatant (S2) with a micropipetter and discard.

12. Resuspend pellet (P2) containing primary cilia in 20 µl Resuspension Buffer (see recipe) by pipetting up and down with a micropipetter.

    *The typical concentration of final preparation can range from 0.2 to 0.8 µg/µl.*

13. Resuspended primary cilia can be frozen.

    *Storage at -80 °C or in liquid nitrogen is recommended.*

**Basic Protocol 2: VISUALIZATION OF ISOLATED PRIMARY CILIA FRACTION USING ELECTRON MICROSCOPY (EM)**

This protocol will allow visualization of the primary cilia isolated in Basic Protocol 1. The primary cilia preparation is applied to Formvar-coated grids by floating the grids upside-down on the sample of isolated primary cilia. The grids are allowed to dry; the sample is stained and, finally, examined with an electron microscope.

***Materials***

Parafilm
Formvar-coated carbon grids (200-mesh; Ted Pella Inc., Redding, CA)
Resuspension Buffer (see recipe)
2% uranyl acetate solution
Filter paper

Electron microscope such as Phillips CM12 or equivalent

***Place sample on EM grid and stain.***

1. Place 10µl of a primary cilia preparation on a small piece of Parafilm.

2. Apply primary cilia preparation to an EM grid by placing Formvar-coated carbon grid on the 10µl drop on the Parafilm using fine forceps. Float for 2 min.

3. Pick up grid with forceps. Wash grid by floating on drop of Resuspension Buffer on Parafilm for 2 min.

4. Dry grid using a small sliver of filter paper touched to grid to absorb Resuspension Buffer.

5. Stain preparation for 1 min by floating the grid on 10 µl of aqueous 2% uranyl acetate.

6. Remove excess stain with filter paper (as in Step 4) and allow grid to air-dry.

***Examine sample using EM.***

7. Examine grids on an EM such as a Phillips CM12 at 80kV. See Figure 4 as an example.

**REAGENTS AND SOLUTIONS**

*Use deionized distilled water in all recipes.*

***Xenopus* A6 Cell Culture Medium**

*All ingredients must be sterile.*
46.9 ml Leibowitz L-15 medium (40% final)
20.1 ml F-12K Nutrient Mixture (Kaighn's modification) (17% final)
40 ml $H_2O$ (33% final)
10 ml fetal bovine serum (Hyclone; filtered) (8% final)
1 ml L-glutamine-200 mM (100X) (1.68 mM final)
1 ml penicillin - streptomycin 100X Solution (10000 units) (84 U/ml penicillin and 84 µg/ml streptomycin final)
Follow shelf-life guidelines from media supplier.

**PBS**

*For 5x stock:*

to 800 mL $H_2O$ add:

40g NaCl (684 mM final)

1g KCl (13mM final)

7.2g $Na_2KHPO_4$ (51 mM final)

1.2g $KH_2PO_4$ (0.9 mM final)

Bring volume up to 1 L with water.

Before using, add 200 ml 5X PBS to 700 ml water. Adjust pH of 1X to 7.2 with 1N NaOH. Bring volume up to 1L with water.

Store 5X up to 24 months and 1X up to 2 months at 4 °C.

**Resuspension Buffer (Hastie et al., 1986)**

200 μl of 1 M Tris-HCl solution, pH 8 (final 20 mM)

37.2 mg KCl (final 50mM)

4.8 mg $MgSO_4$ (final 4mM)

1.5 mg DTT (final 1 mM)

10 μl of 0.5 M EDTA solution, pH 8 (final 0.5 mM)

Bring volume up to 10 ml with water.

Store up to 2 months in freezer.

**COMMENTARY**

**Background Information**

The primary cilium, often called the cell's antenna (Pazour and Witman, 2003), initiates responses to stimuli that control cellular processes such as development, differentiation and proliferation. While investigating the role of nucleoside diphosphate kinase in various subcellular locations, Mitchell et al. (2004) desired to isolate the primary cilia from the cell body to perform biochemical analyses.

Traditional deciliation methods (used to isolate motile cilia and flagella) involve either vigorous stirring with the addition of acetic acid to rapidly lower the pH, or high levels (~10 mM) of calcium (Anderson, 1974; Hastie et al., 1986; Linck, 1973; Rosenbaum and Child, 1967; Watson and Hopkins, 1962; Zhang et al., 1991). Extremes of pH may damage the ciliary membrane or the cell's plasma membrane, leading to contamination of the primary cilia fraction by components of the cell body. Due to the primary cilium's involvement in many signaling pathways, it is preferable to avoid high levels of calcium that could alter the sub-organellular

localization of various proteins and affect signaling mechanisms. Therefore this protocol uses more physiological conditions that are less likely to disrupt potential signaling components. In addition, other primary cilia purification methods such as the "peel off" and "slide pull" (Huang et al., 2006) also avoid harsh conditions, but lead to less pure fractions of primary cilia. The "peel off" technique involves pressing down on primary cilia with a coverslip, removing the culture medium and lifting the coverslip from the cells (ideally with primary cilia attached). The primary cilia are then either stained for microscopy or scraped from the coverslip for biochemical analysis. This method results in contamination of the preparation by cell body components (Huang et al., 2006), which is not surprising, as it is based on a procedure originally used to remove portions of the plasma membrane (Perez et al., 2006; Sanan and Anderson, 1991) and endocytic vesicles (Doctor et al., 2002). In addition, scraping primary cilia from the coverslip most likely causes ciliary damage.

The "slide pull" technique (Doctor et al., 2002; Huang et al., 2006) uses a smaller dish placed on top of the culture dish containing primary cilia in a shaker. The mechanical agitation of the smaller dish against the cells pulls off portions or all of the primary cilia and most likely leads to cell damage, resulting in the high protein yield of the preparation (150-270 mg) due to contamination by portions of the cell body.

The shear force protocol presented in this unit seems to be the best choice for good yields of a relatively pure preparation of intact cilia that can be used for biochemical and immunofluorescent applications, provided the cilia are long enough to be removed by shear force.

**Critical Parameters / Troubleshooting**

In order to obtain a sufficient yield of primary cilia for biochemical analysis, it may be necessary to grow multiple large dishes of cells. It is advantageous to grow kidney cells, in particular A6 *Xenopus* cells, as the primary cilia in these cells reach great lengths (up to 50 μm with an average of 24 μm). This protocol has been successfully applied to cells that have smaller cilia, namely MDCK with an average length of 8 μm (Praetorius and Spring, 2001). To do so, larger numbers of cells were kept at confluence for longer periods; as expected, yields were inferior to those obtained with A6 cells. Several factors have been shown to affect cilia length, and becoming familiar with these may help increase the length of cilia in desired tissue, or at least avoid decreasing length by exposure to less-than-favorable conditions. (Armour et al., 2012; Chakravarthy et al., 2012; Marley and von Zastrow, 2012; Massinen et al., 2011; McGlashan et al., 2010; Miyoshi et al., 2011; Rich and Clark, 2012; Rondanino et al., 2011; Sharma et al., 2011; Wann and Knight, 2012). Various methods have been developed to visualize cilia and measure their length, which may be useful when determining cilia length of desired cells to ensure

suitability of this protocol. (Ott and Lippincott-Schwartz, 2012; Roth et al., 1988; Saggese et al., 2012; Sharma et al., 2011; Wheatley and Bowser, 2000)

**Anticipated Results**

A 15 cm dish of A6 *Xenopus* kidney cells can yield 1.5-5 μg primary cilia. Cells with shorter cilia would be expected to have a lower yield.

**Time Considerations**

It takes considerable time to grow cells to confluence and then allow primary cilia to grow to great lengths. Plating cells at a high density will allow them to reach confluence more rapidly. This protocol was developed using cells that were grown 7-10 days post-confluence. Once the actual purification procedure is started, the entire Basic Protocol 1 isolation process can be completed in an hour. Basic Protocol 2, visualization of the isolated primary cilia fraction using EM, can also be completed within an hour's time.

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**Figure Legends**

**Figure 1. Long primary cilia are found in A6 cells after 9 days in culture.** Xenopus A6 cells were plated at high density and grown for 9 days to allow for maximal cilia length. Fixation was performed under flow to align cilia and cells were stained with an antibody to acetylated $\alpha$-tubulin, a protein enriched in primary cilia. (Mitchell et al., 2004)

**Figure 2. Effectiveness of purification can be assessed using SDS-PAGE**. Primary cilia were isolated as described in Basic Protocol 1. Equal protein amounts of whole cell extract (WC) and isolated primary cilia fraction (PC) were resolved by SDS-PAGE, followed by staining with Coomassie Blue (A) or immunoblotting with an antibody to acetylated $\alpha$-tubulin (B). The size in kDa and the migration distance of molecular mass markers is shown between the two panels. The most prominent bands in the primary cilium fraction are indicated by asterisks. (Mitchell et al., 2004)

**Figure 3. Effectiveness of purification can be assessed using immunocytochemistry.** A6 cells before and after deciliation were fixed and stained with antibodies against acetylated α-tubulin, a protein marker for primary cilia, and nucleoside diphosphate kinase, a cytoplasmic marker. Many cilia (bright lines such as indicated by arrowheads) are seen in the “Before” panel, but very few in the “After” panel, indicating successful deciliation.

**Figure 4. Isolated primary cilia fraction visualized with an electron microscope.** Primary cilia were isolated as described in Basic Protocol 1. Negatively stained samples were examined by electron microscopy as described in Basic Protocol 2. *Arrows* indicate primary cilia, and *arrowheads* point to ciliary tip-like structures. (Mitchell et al., 2004)