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Discovery and functional evaluation of ciliary proteins in *Tetrahymena thermophila*

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Abstract

The ciliate *Tetrahymena thermophila* is an excellent model system for the discovery and functional studies of ciliary proteins. The power of the model is based on the ease with which cilia can be purified in large quantities for fractionation and proteomic identification, and the ability to knock out any gene by homologous DNA recombination. Here, we include methods used by our laboratories for isolation and fractionation of cilia, *in vivo* tagging and localization of ciliary proteins and the evaluation of ciliary mutants.

Keywords

Cilium; axoneme; ciliary cap; *Tetrahymena*; ciliate

1. Introduction

For studies on cilia, *Tetrahymena thermophila* has attractive features, including the high number of cilia, ability to deciliate and regenerate cilia, ease of culturing in axenic media, short generation time (3 hr at 30°C), high maximal cell concentration (10⁶ cells/ml), and well developed classical and molecular genetic approaches. One of the most useful features of *Tetrahymena* is that its genes can be modified by homologous DNA recombination allowing for routine gene knockouts (reviewed in (Chalker, 2012).

Tetrahymena has locomotory and oral cilia (for a recent review of the *Tetrahymena* cell organization see (Wloga and Frankel, 2012). Locomotory cilia are organized in approximately 20 longitudinal rows and beat metachronally. Oral cilia are organized into membranelles that sweep food particles into the oral cavity. *Tetrahymena* swim in a complex pattern that involves rotations of the cells and switches between forward and backward motility. Also, *Tetrahymena* cells chemotax in response to chemical gradients. This abundance of cilia-based behaviors provides a basis for simple assays that can be used as a rapid readout of functionality of cilia (Hennessey and Lampert, 2012).

Tetrahymena cells can be deciliated to produce a population of regenerating cells with assembling cilia (Rosenbaum and Carlson, 1969). Cells arrested in G1 by starvation maintain non-assembling cilia (Mowat et al., 1974; Vonderfecht et al., 2011). When cultured vegetatively, *Tetrahymena* assembles new basal bodies near existing basal bodies without resorbing the old cilia (Allen, 1969). The time at which a newly assembled basal body grows a cilium depends on its position in the cell and may be delayed until shortly before cell division (Frankel et al., 1981). Therefore, a single *Tetrahymena* cell carries cilia that were assembled during multiple generations and differ vastly in age (Thazhath et al., 2004), which provides a unique opportunity to study ciliary maintenance. The length of locomotory cilia is non-uniform, as cilia located in the anterior region are shorter than those in the mid and posterior region (Wloga et al., 2006). Most models of ciliary length control are based on *Chlamydomonas*, that contain two equal-length flagella that grow at the same time, or epithelial cells that have a single primary cilium (reviewed in (Avasthi and Marshall, 2012)). Because *Tetrahymena* contains both growing and nongrowing cilia and maintains unequal length cilia in the same cell, studies of *Tetrahymena* may provide valuable insight about the subcellular location-specific mechanisms that regulate ciliary assembly and maintenance.

Based on proteomic, phylogenomic, and gene expression analyses, cilia appear to contain more than 1000 different polypeptides (recently reviewed in (Arnaiz et al., 2009)). The location and function of many of these proteins remains to be discovered. Great progress has been made in our (still incomplete) understanding of the structure of the axonemal microtubules and associated motility-related protein complexes including the radial spokes, dynein arms and IFT particles. Recent studies also have made significant advances in our understanding of proteins localized to the ciliary base and transition zone, including proteins that regulate the entry and exit of components into the ciliary compartment (recently reviewed in (Czarnecki and Shah, 2012; Qin, 2012)). By contrast, the composition and function of the distal ends of cilia remain relatively unexplored. In particular, the ciliary caps that link the lumens of the central and A-tubules of each doublet microtubule to the plasma membrane (Dentler, 1980; Fisch and Dupuis-Williams, 2011) (Fig. 1) remain to be characterized. With its robust biochemical and genetic approaches *Tetrahymena* remains a model of choice for identification of cap proteins.

Here we describe a set of protocols that our laboratories use to study cilia in *Tetrahymena*, with the focus on purification and fractionation of cilia for proteomic studies and protein localization by *in vivo* tagging. Methods that we routinely use to study cilia-related phenotypes in *Tetrahymena* mutants are included in sections 4.4. and 5.

2. Cell culture

Tetrahymena strains (available from the *Tetrahymena* Stock Center) are routinely cultured in SPPA (Gorovsky, 1973) medium containing 1% proteose-peptone (Difco 211684), 0.1% yeast extract, 0.2% glucose, 0.003% EDTA ferric sodium salt (Sigma-Aldrich E6760) and 1% antibiotic-antimycotic mix (VWR 12001-712). Most often, cells are grown in 50 ml of SPPA with shaking at 70–125 rpm at 30°C. For large scale purifications, cells are grown in 1 liter of SPP (without antibiotics) in 2800 ml Fernbach flasks with shaking at 70 rpm. Strains that completely lack cilia are not viable on SPPA but can be grown on MEPPA

medium that supports viability in the absence of phagocytosis (Orias and Rasmussen, 1976). To prepare MEPPA, make the following solutions:

- A. 2% proteose-peptone (1 liter, autoclave)
- B. 2.5 mg/ml folinic acid (25 mg in 10 ml, filter sterilize)
- C. 0.2 M sodium citrate (3 g in 50 ml, filter sterilize)
- D. 0.1 M FeCl₃, 3 mM CuSO₄ (1.35 g of FeCl₃ and 22 mg of CuSO₄ in 50 ml, filter sterilize).

Measure 50 ml of A and add, with mixing, (the order of addition is important): 20 µl of B, 0.5 ml of C, 0.5 ml of D, and 1 ml of the antibiotic/antimycotic mix. To study non-assembling cilia, cells are arrested in macronuclear G1 by replacing the growth medium with 10 mM Tris pH 7.5 and incubation for 6 hr at 30°C. Note that starved cells have morphostatic locomotory cilia but periodically resorb and reassemble the oral apparatus (Williams and Frankel, 1973).

3. Deciliation, purification, and fractionation of cilia

Tetrahymena can be easily deciliated by pH shock (protocol 3.1), calcium/pH shock (Calzone and Gorovsky, 1982; Rosenbaum and Carlson, 1969) or dibucaine (Thompson et al., 1974). The pH shock method (protocol 3.1) is useful for isolation of cilia and axonemes from small volumes (100 ml). The dibucaine method (protocol 3.2) produces large yields of cilia that are suitable for fractionation. A modified calcium/pH shock method is used for deciliation of 5–10 ml of cells and is particularly suitable for observations of cilia regeneration (protocol 5.3.).

3.1. Purification of cilia and axonemes using deciliation by pH shock

The method (modified from (Lefebvre, 1995)) produces cilia with low amounts of mucus contamination, that are suitable for a variety of purposes including isolation of axonemes for *in vitro* reactivation of microtubule sliding (Suryavanshi et al., 2010) and for *in vitro* post-translational modification of axonemal tubulin (Akella et al., 2010).

1. Grow cells in 100 ml SPPA in 500 ml Erlenmeyer flask to 2×10^5 cells/ml at 30°C with shaking at 120 rpm.
2. Collect cells by centrifugation ($1700 \times g$ 3 min; swinging bucket rotor, 50 ml conical tubes), wash once with 10 mM Tris-HCl pH 7.5 and gently suspend in 20 ml of the deciliation medium (10 mM Tris-HCl pH 7.4, 50 mM sucrose, 10 mM CaCl₂, protease inhibitors (Complete, Roche)) in a 250 ml flask.
3. Add 420 µl of 0.5 M acetic acid while swirling gently for 1 min (some strains may require longer exposure to acetic acid), and then add 360 µl of 0.6 M KOH and mix briefly. Verify that cells have stopped moving by inspecting a drop under a microscope.
4. Collect the deciliated cell bodies by centrifugation (5 min at $1700 \times g$ at 4°C). Using a pipette, transfer the supernatant to a new tube but leave about 1 cm of the

supernatant above the pellet to reduce the amount of contaminating cell bodies. Verify the purity of cilia under a microscope (phase contrast, 40× lense).

5. Repeat step 4 to collect any remaining cell bodies. Collect the supernatant.
6. Centrifuge the supernatant for 30 min at $21000 \times g$ in a fixed angle rotor at 4°C to collect cilia. Suspend the ciliary pellet in 500 μl of ice-cold axoneme buffer (20 mM potassium acetate, 5 mM MgSO_4 , 0.5 mM EDTA, 20 mM HEPES, pH 7.6).
7. To obtain axonemes, suspend cilia in 500 μl of ice-cold motility buffer (5 mM MgSO_4 , 1 mM EGTA, 30 mM HEPES, 1% PEG, pH 7.6). Add 100 μl of 1% NP-40, incubate for 10 min on ice, spin down at $10000 \times g$ for 10 min and suspend the axoneme pellet in the motility buffer. The axonemes can be stored frozen at -20°C .

3.2. Purification and fractionation of dibucaine-released cilia

This procedure first was originally developed by Thompson and colleagues (Thompson et al., 1974) and has been modified to produce high quantities of pure cilia with intact ciliary membranes (Dentler, 1995a, b; Suprenant and Dentler, 1988). When the membrane is solubilized by a nonionic detergent, the axonemes retain dynein arms, spokes, central microtubules and ciliary capping structures. Pure ciliary membrane vesicles and fractions containing ciliary caps can be isolated from these cilia as described below. Note that the HEPES buffer used originally is now replaced with Tris-HCl to make the method more compatible with trichloroacetic acid (TCA) or perchloric acid (PCA) precipitation required for concentration of proteins in diluted fractions prior to proteomic studies. Examination of cilia by transmission electron microscopy (TEM) and SDS-PAGE revealed no differences in cilia or axonemes isolated with HEPES or Tris-HCl. To monitor the fractionation process, samples should be negatively stained with uranyl acetate and examined by TEM (Fig. 1). The ciliary fractions produced by this protocol are suitable for fractionation and identification of proteins by MuDPIT mass spectrometry (Wolters et al., 2001) (WD unpublished data).

1. Culture *Tetrahymena* cells in 1–2 liters of SPP.
2. Harvest cells by centrifugation ($700 \times g$, 5 min, 500 ml Nalgene centrifuge bottles, room temperature).
3. Suspend cells in 1 liter of fresh SPP and centrifuge at $700 \times g$, 5 min, to concentrate cells.
4. Suspend cells in fresh SPP to a final volume of 50–80 ml in a 125–250 ml flask.
5. Deciliate cells by adding dibucaine (Sigma-Aldrich D-0638) to a final concentration of 1 mg/ml. Dissolve dibucaine in approximately 1 ml of SPP, add to the cells, and stir the flask by hand for no longer than 4 min. Periodically examine a drop of cells with a microscope to verify that cells are becoming immotile.
6. Dilute cells by adding 3 volumes of ice-cold SPP. Transfer deciliated cells into centrifuge bottles and keep on ice. All subsequent steps are done at 4°C or on ice.

7. Pellet deciliated cells by centrifugation ($4420 \times g$, 7 min, 4°C). Recover the cilia supernatant.
8. Pellet cilia ($17,000 \times g$, 30 min, 4°C). Cilia form a tight white pellet covered with a fluffy layer of mucus. If the pellet is not pure white, it is likely that the cilia are contaminated with cell debris. This generally is due to cell disruption during dibucaine treatment and it is better to start a new preparation than continue with a contaminated one. Decant the supernatant and the mucus layer and place inverted centrifuge bottles on paper towels to drain as much of the mucus as possible. The tight cilia pellet should not dislodge from the centrifuge bottle. Remove any remaining mucus by gently rinsing the pellet with the cilia wash buffer (CWB: 50 mM Tris-HCl pH 7.4, 3 mM MgSO_4 , 0.1 mM EGTA, 250 mM sucrose, 1 mM DTT) using a Pasteur pipette. Avoid dislodging the pellet during rinsing.
9. Gently suspend cilia in 100 ml of ice-cold CWB. To avoid shearing cilia, use a large bore pipette (25 ml glass or plastic pipette). Examine the suspension by phase contrast microscopy to be certain that cilia are not contaminated with cell bodies. If cell bodies are present, try to pellet them by centrifuging for 5 min at $484 \times g$ at 4°C . Pellet cilia from the supernatant by centrifugation at $7740 \times g$ for 5–10 min 4°C . If there is a small mucus layer above the cilia pellet, gently remove it with a Pasteur pipette before suspending the pellet. Keep cilia concentrated if you plan to recover the membrane+matrix (M+M) fraction for further studies (see below). Negatively stain a sample with 1% uranyl acetate and use TEM to determine if the membranes remain intact on the purified cilia.
10. Add 10% Nonidet P-40 (NP-40) or Triton X-100 to a final concentration of 1%. Swirl gently and leave on ice for 10 min.
11. Centrifuge the suspension at $5930 \times g$, 10 min, 4°C . Centrifuging in a 15 ml round bottom tube will prevent the cilia from packing into a tight pellet that cannot be resuspended without damaging the demembrated axonemes. Remove and save the supernatant (M+M fraction). Suspend the pellet, containing axonemes, in 1–2 ml of cold CWB. Negatively stain a sample with 1% uranyl acetate and examine by TEM to confirm that the axonemes are intact. Caps should be visible at the tips of at least 75% of axonemes.
12. To separate ciliary caps from the distal tips of axonemes, add MgCl_2 to a final concentration of 75 mM to the suspended axonemes. Gently mix by swirling and incubate on ice for 10 min. Negatively stain a sample and examine by TEM to ensure that caps are released.
13. Centrifuge at $12000 \times g$, 10 min, 4°C , to pellet Mg-extracted axonemes (MgP).
14. Remove the supernatant (MgS) and centrifuge at $48400 \times g$, 30 min, 4°C . Separate the high speed supernatant (MgHSS) and pellet (MgHSP). Ciliary caps rapidly disassemble, so their proteins will be present in the MgCl_2 -solubilized fractions. The high speed centrifugation removes remaining pieces of microtubules and membrane vesicles from the solubilized fractions. Store at -20°C or concentrate MgHSS proteins with 10% TCA or PCA (below).

3.3 Purification of ciliary membrane vesicles

Most (not all, see (Dentler, 1995a)) ciliary membranes are solubilized by 1% nonionic detergent and will be found in the membrane+matrix fractions (M+M). However, ciliary membranes can be recovered more completely from the dibucaine-isolated cilia and purified as described below.

1. To purify ciliary membrane vesicles (CMV), first purify cilia as described (steps 1–9 above) from 2 liters of culture. Suspend cilia in 5 ml of CWB and add NP-40 to a final concentration of 0.05–0.2%.
2. Incubate the suspension on ice for 10 min in a 14 ml glass or plastic centrifuge tube. Remove the tube every minute and vortex rapidly for 5–10 sec.
3. Layer the suspension over 2 ml of 50% sucrose in CWB and 2 ml of 30% sucrose in CWB in a glass (Corex) centrifuge tube.
4. Centrifuge in for 1 hr at $26900 \times g$, 4°C in a swinging bucket rotor. Ciliary membrane vesicles will form a white band at the interface between the 30% and 50% sucrose layers.
5. Use a pipette to remove the layer containing membranes and dilute with CWB. Examine the suspension by light microscopy or by negative staining and transmission electron microscopy to determine the purity of the vesicle fraction. It should not contain axoneme or cell fragments.
6. Transfer the suspension to a 15 ml plastic centrifuge tube and pellet the vesicles by centrifugation for 60 min, $48400 \times g$.

3.4 Acid precipitation

For proteomic analyses, pellets (axonemes, MgP, and CMV) can be stored frozen and used directly. Proteins in soluble fractions (M+M, MgHSS) are precipitated with 10% TCA or PCA. For acid precipitation, add TCA or PCA to a final concentration of 10%, incubate 10–20 minutes on ice, and centrifuge in plastic microcentrifuge tubes ($23645 \times g$). Wash the pellets 3–4 times with cold acetone, dry briefly in a 100°C temperature block and store at -20°C . When examined by SDS-PAGE, no differences could be detected between freshly prepared ciliary fractions and suspended TCA precipitates (WD, unpublished data).

4. Localization of proteins in *Tetrahymena*

4.1. Expression of epitope-tagged proteins by targeting to the macronuclear *BTU1* locus

We express epitope-tagged protein genes under the cadmium-inducible promoter MTT1 (Shang et al., 2002b) by inserting transgenes into the non-essential *BTU1* locus (Gaertig et al., 1999). The structure of a required targeting fragment is shown in Fig. 2 (top). The advantage of the method is that the targeting fragment does not require a positively selectable marker. Cloning of the extremely AT-rich DNA of *Tetrahymena* in *E. coli* is challenging. We believe that the relatively small size of the *BTU1* targeting plasmids facilitates cloning of *Tetrahymena* ORFs. We have cloned and obtained *Tetrahymena* transformants for ORFs up to 6 kb long. The principle of the transgene targeting into *BTU1*

is based on negative selection. *Tetrahymena* has two genes that encode exactly the same β -tubulin protein: *BTU1* and *BTU2*, that are partially functionally redundant (Xia et al., 2000). The CU522 transformation host strain carries a *BTU1* allele encoding a K350M substitution, that confers sensitivity to paclitaxel and resistance to oryzalin (Gaertig et al., 1994). Replacement of the *BTU1-K350M* coding region by a transgene ORF confers paclitaxel resistance characteristic of wildtype cells (Gaertig et al., 1999). While the transgene targeting requires a specific genetic background (*BTU1-K350M*), this background can be introduced into other strains by crosses (see (Wloga et al., 2006)). The following protocol describes introduction of an MTT1-driven transgene into *BTU1*.

1. Amplify a predicted ciliary protein ORF from total genomic DNA with a high fidelity DNA polymerase and clone into a *BTU1* targeting plasmid (Fig. 2). Digest 15 μ g of plasmid DNA with restriction enzymes to separate the targeting fragment from the plasmid backbone (for most of our *BTU1* targeting plasmids these are *ApaI* and *SacII* restriction enzymes). Clean the digested DNA using the Qiaquick PCR product purification kit (Qiagen, 28104). Elute the digested DNA in 50 μ l of water. Store at -20°C .
2. Grow a 50 ml culture of CU522 cells in SPPA to $2\text{--}2.5 \times 10^5$ cells/ml.
3. Spin down cells in a 50 ml conical tube for 3 min at $1700 \times g$, wash once and suspend in 50 ml of 10 mM Tris-HCl pH 7.5 with 1% antibiotic-antimycotic mix in a 250 ml flask. Incubate cells at 30°C for 18–22 hrs with shaking at 80 rpm.
4. On the next day, adjust the cell concentration to 2×10^5 cells/ml.
5. Use 15 μ g of digested plasmid DNA to coat 3 mg of gold particles (S550d, Seashell Technology) using the manufacturer protocol and reagents as follows. Mix 60 μ l of 50 mg/ml gold with 40 μ l of the binding buffer. Add the digested DNA (50 μ l). Vortex briefly. Add 150 μ l of the precipitation buffer. Vortex for 2 min and let stand for 3 min. Spin down the sample at $9600 \times g$ (10000 rpm in a microcentrifuge) for 10 sec. Remove the supernatant. Add 500 μ l of cold 100% ethanol. Briefly sonicate the tube inside a water bath sonicator for ~ 20 sec, until no gold aggregates are visible on the microcentrifuge tube surface. Spin down at $9600 \times g$ for 10 sec. Remove as much of supernatant as possible. Add 15 μ l of cold ethanol. Briefly sonicate as above. Immediately spread gold onto the center of the macrocarrier.
6. Proceed to perform biolistic bombardment of starved CU522 cells with gold particles coated with the digested DNA using the PDS1000/He biolistic gun (Biorad). For details see (Dave et al., 2009). The only modification is that we now use a 1100 psi rupture disc and the helium pressure is set at 1300 psi.
7. After biolistic bombardment, transfer cells into a 250 ml flask with 50 ml of SPPA medium and incubate at 30°C for 2 hr without shaking.
8. Add paclitaxel to 20 μM . We prepare paclitaxel (LC Laboratories P-9600) as a 10 mM stock solution in DMSO, store at -20°C in 100 μ l aliquots.

9. Plate cells on 96-well microtiter plates (flat bottom) using a multichannel pipette at 100 μ l per well and incubate plates at 30°C in a moist box. Non-transformed cells fail to grow, become larger, have irregular shape and are completely paralyzed within 2–3 days of selection. Transformant cells are motile and grow (with reduced rate as compared to wildtype unselected cells). Note that false positives occasionally appear that contain unrelated loss of function mutations in *BTU1-K350M*. The background of false positives can be reduced by passing CU522 cells on SPPA with 10 μ M oryzalin a few times prior to step 2 (personal communication, Donna Cassidy-Hanley, Cornell University, NY).
10. Propagate a few positive wells by transferring 1 μ l into 200 μ l of fresh SPPA with 20 μ M paclitaxel on a 96-well plate. To induce transgene expression, grow transformant clones in drug-free SPPA and suspend at 1×10^5 cells/ml of SPPA with 2.5 μ g/ml CdCl₂. Incubate for 2–4 hrs. As appropriate, either observe directly live cells or use for immunofluorescence with anti-tag antibodies (see protocol 4.4). In the case of fluorescent protein imaging in live cells, if the signal is weak, fix the cells in the presence of a detergent to reduce autofluorescence (see protocol 4.3).
11. *Tetrahymena* has ~45 copies of each gene in the macronucleus. Using the above approach, initially only some of the 45 endogenous *BTU1-K350M* alleles are replaced by the transgene. The allele replacement can be completed using phenotypic assortment. The macronucleus divides by amitosis and alleles are segregated randomly (reviewed in (Orias, 2012)). Cells that grow with paclitaxel selection accumulate the transgene copies in expense of the endogenous copies of *BTU1-K350M*. Eventually in some selected cells all endogenous *BTU1-K350M* copies are lost. To select assorted transgenic cells, propagate transformant clones in SPPA with 20 μ M paclitaxel by transferring every 1–2 days. Usually this is done at least 12 times. Make 48 single cell isolations in SPPA (without the drug) on a 10 cm Petri dish. Let the drop cultures grow and replicate onto fresh SPPA 5–7 times. Replicate isolates onto SPPA with 2.5 μ g/ml CdCl₂. Check a few clones for transgene expression. If all clones show a consistent uniform epitope signal, most likely the transgene had been completely assorted at the time when cells were isolated into a drug free SPPA. Pick up 1–2 of assorted clones and grow without paclitaxel.

4.2. Epitope tagging of proteins in the native gene locus

While the *BTU1* targeting is a straightforward, it has the disadvantage in that the transgene is expressed using a non-native promoter. Epitope tagging of an ORF in the native locus is expected to produce a more natural pattern of expression. We routinely add an epitope tag to the 3' region of an ORF using a linked *neo2* marker. The targeting plasmid is composed of the following elements in the exact order (see Fig. 2 bottom): 1) 3' fragment of an ORF with a removed stop codon (1.5–2 kb), 2) in frame GFP (or another epitope tag) coding region with a stop codon TGA, 3) transcription terminator region of *BTU2*, 4) *neo2* gene cassette for positive selection with paromomycin, and 5) 3'-UTR fragment of the targeted locus (1–1.5 kb). The use of a heterologous transcription terminator (*BTU2*) prevents undesired homologous DNA recombination events that lead to incorporation of the *neo2* marker alone.

The targeting fragment is used to transform vegetatively growing wildtype CU428 cells using biolistic bombardment (Dave et al., 2009) and transformants are selected with paromomycin. The native gene copies are replaced completely with epitope tag-expressing genes by phenotypic assortment, by growing cells in increasing concentrations of paromomycin. Plasmids for tagging the C-terminal end of the protein at the native locus with multiple epitope tags have recently been constructed by the Mochizuki group (Kataoka et al., 2010). The same group has developed a method for tagging proteins at the N-terminus in the native gene locus using a marker inserted into the 5'UTR region that is subsequently deleted by a Cre recombinase to restore the native promoter (Busch et al., 2010).

4.3. Detection of a GFP transgene protein in fixed cells by fluorescence microscopy

When GFP is used as an epitope tag, in some cases, the transgene signal is below the level of detection for live imaging using standard epifluorescence microscopy. One solution is to use immunofluorescence with polyclonal anti-GFP antibodies to enhance the signal. Another simpler way is to reduce the autofluorescence level to unmask a potential weak GFP signal by very brief permeabilization followed by immediate fixation. This method is particularly useful for detection of GFP signals in cells with genes tagged at the native locus, whose expression level is often low.

1. Grow a GFP transgene strain to 1×10^5 cells/ml
2. Place 15 μ l of cells on a cover slip (22×22 mm).
3. Add 15 μ l of 0.5% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, pH 6.9). Wait for 20 sec and add 10 μ l of 2% paraformaldehyde in PHEM (earlier, to prepare this solution add 0.2 g of paraformaldehyde to 10 ml of PHEM in a small glass flask, and warm up on a hot plate inside a chemical hood until the solution is clear. Avoid boiling the solution. Cool down to room temperature and store at 4°C).
4. Spread the fixed cells evenly on the cover slip and let dry at room temperature or 30°C.
5. Rehydrate cells by adding of PBS-T-BSA (PBS with 3% bovine serum albumin fraction V and 0.01% Tween-20; PBS alone contains 130 mM NaCl, 2 mM KCl, 8 mM $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 2 mM KH_2PO_4 , 10 mM EGTA, 2 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$) solution. Wait 17 min.
6. Wash the cover slip with PBS (by immersing the cover slip into a small Coplin staining jar or by placing the solution directly onto the cover slip).
7. Remove the excess of PBS by draining the cover slip on a piece of filter paper, and mount the cover slip onto a 10 μ l drop of the DABCO mounting medium (100 mg/ml of 1,4-diazobicyclo-[2,2,2]-octane (Sigma-Aldrich), dissolved at in 90% glycerol, 10% PBS solution). Seal the cover slip with nail polish.

4.4. Comparative (mutant versus wildtype) immunofluorescence

Many protocols are available for immunofluorescence in *Tetrahymena* that produce excellent results (recently reviewed by Winey et al., 2012). Here we present a relatively

low-tech approach that has a few advantages. While cells are dried on cover slips, surprisingly, their morphology and the integrity of cilia are well preserved. The protocol can be used to label a single population or a mixed population (mutant and wildtype). By mixing two strains, it is possible to image two genetically distinct strains side by side and detect even subtle phenotypic differences (see Fig. 3).

1. Label a reference strain (e.g. CU428) by loading food vacuoles with India Ink via phagocytosis (see protocol 5.4). One ml of labeled cells is more than enough. Wash out the unused India ink with 10 mM Tris pH 7.5 by centrifugation (3 min 1700 × g). Combine equal number of cells of a studied strain (e.g. knockout or transgene-overproducing) and wildtype cells. Wash combined cells in a 10 mM Tris pH 7.5 by centrifugation and suspend in 10 mM Tris pH 7.5.
2. Transfer 10 µl of combined cells onto a cover slip. Typically we have 50–100 cells in a drop. Fewer cells can also be used – with this method most cells are recovered.
3. Add 10 µl of 0.5% Triton X-100 (or NP-40) in PHEM to the drop of cells and mix gently with a pipette tip.
4. After 40–60 sec add 15 µl of 2% paraformaldehyde in PHEM, gently mix and spread cells on the entire surface of cover slip. Let cover slips completely dry at 30°C. If the antigen is relatively soluble, at step 3 use 15 µl of 1:1 mixture of 0.5% Triton X-100 (or NP-40) in PHEM and 2% paraformaldehyde in PHEM solution and proceed to drying.
5. Cover dried cells with ~200 µl of PBS-T-BSA and incubate for 10–15 min at room temperature.
6. Prepare a 10 cm Petri dish with a piece of parafilm at the bottom (4–5 cover slips can be placed inside one 10 cm wide Petri dish). Place a 50 µl drop of a primary antibody in PBS-T-BSA on the parafilm. Using forceps, lift the cover slip, remove the excess of blocking solution by draining onto kimwipe and place on the top of the primary antibody drop with cells down. Incubate in the cold room overnight (or 2 or more hr at room temperature 30°C or, depending on the primary antibody type). To visualize cilia, most often we use the rabbit anti-polyglycylated tubulin (polyG) antibodies (use at 1:100 dilution) (Shang et al., 2002a). Note that these antibodies do not label the distal segment. To visualize the entire cilia, polyG can be combined with the mouse monoclonal anti- α -tubulin antibody 12G10 that label strongly the distal segment (Jerka-Dziadosz et al., 1995) available from DSHB, use at 1:25 dilution). A combination of both antibodies labels the entire axoneme (Fig. 4).
7. Wash the cover slips with PBS-T-BSA, 3 times for 5 min using a small Coplin staining jar.
8. Incubate the cover slip in the secondary antibodies as described in 6.
9. Wash the cover slip with PBS, 3 times for 5 min. To stain DNA with DAPI, add 1 µl of 0.1 mg/ml stock to 10 ml PBS during the first wash.

10. Drain the excess of liquid off the cover slip onto kimwipe, mount onto a 10 μ l drop of DABCO mounting medium on a microscope slide. Seal the edges with nail polish, dry and wash the top surface of cover slip with water (gently using a rinse bottle) to remove salt precipitates. Air dry.

5. Phenotypic studies on live ciliary mutants

Methods to generate strains with deletions of genes by homologous recombination were described elsewhere (Dave et al., 2009). We prefer to create heterokaryon strains in which genes are deleted in the germline micronucleus but not in the macronucleus (Hai et al., 2000). Heterokaryon strains can be maintained like wildtype strains. A cross of two heterokaryons produces progeny that has deleted genes in both the micronucleus and the macronucleus and expresses the mutant phenotype. The heterokaryon approach is useful for creating combinations of multiple gene knockouts by crosses in case of studies on paralogous groups or to study genetic interactions (mutants with severe ciliary phenotypes do not mate). Also, severe ciliary mutants are difficult if not impossible to preserve by freezing in liquid nitrogen. In some cases, the micronuclear gene could not be modified and thus heterokaryons could not be generated. In this case, we delete the macronuclear copies and complete gene replacement by phenotypic assortment (Dave et al., 2009).

5.1 Cell motility

Most strains are grown in SPPA but mutants with severe ciliary defects are grown in MEPPA (Section 2). The rate of cell motility is measured by capturing the paths of motile cells using video microscopy (Hennessey and Lampert, 2012). A variety of assays can be used to evaluate the ability of cells to change the frequency of ciliary beating, reverse the direction of motility and chemotax (Hennessey and Lampert, 2012; Rajagopalan et al., 2009).

5.2 Measuring the number and length of cilia

We label cilia by immunofluorescence as described in protocol 4.4. For consistency, we determine the number and average length of cilia on 10–20 cells using confocal optical sections that include the widest diameter of the macronucleus. The length measurements are done using NIH ImageJ (Schneider et al., 2012).

5.3. Cilia regeneration

To determine the rate of cilia regeneration we use the Calzone and Gorovsky method (Calzone and Gorovsky, 1982) that results in consistently complete deciliation. In our hands, wildtype cells regenerate full length cilia in 2 hr at 30°C.

1. Grow cells in SPPA to $2\text{--}3 \times 10^5$ cells/ml and starve for 6–24 hr in 10 mM Tris-HCl pH 7.5.
2. Spin down 10 ml of starved cells by centrifugation at $1700 \times g$ for 3 min in a 15 ml conical tube. Remove the supernatant, add 10 ml of 10 mM Tris-HCl buffer pH. 7.5 and resuspend cells by gentle shaking.

3. Centrifuge cells again as above and concentrate cells in 1 ml of 10 mM Tris-HCl pH 7.5 in a 15 ml tube.
4. Add 10 ml the deciliation medium, (10 % Ficoll 400, 10 mM sodium acetate, 10 mM CaCl₂, 10 mM EDTA, pH 4.2 adjusted with acetic acid). Immediately transfer the solution to a 100 ml glass beaker and shear cilia by taking the cells into and forcing out of a 30 ml syringe with a 18 G 1 1/2 needle (twice).
5. Immediately add 55 ml of the regeneration buffer (15 mM Tris-HCl, pH 7.95, 2.0 mM CaCl₂). At this stage, cells can be concentrated by a brief centrifugation and suspended back to $2-3 \times 10^5$ cells/ml.
6. Monitor the extent of cilia regeneration as a percentage of motile cells using a microscope at a low magnification. Use a hemocytometer or a microscope slide without a cover slip to give cells enough of room for swimming.
7. To measure the rate of cilia elongation, at multiple time points fix and label cells with a combination of 12G10 and polyG antibodies as described above and measure the length of cilia using NIH ImageJ (Schneider et al., 2012).

5.4. Phagocytosis

To test the function of oral cilia, determine the rate of uptake of India ink by phagocytosis.

1. Grow cells in SPPA to 2×10^5 cells/ml.
2. Add 3 μ l of black India Ink to 1 ml of cells, incubate at 30°C.
3. Fix cells by combining 20 μ l of 2% paraformaldehyde in PHEM buffer with 20 μ l of cells in an Eppendorf tube at multiple time points between 10–30 minutes.
4. Examine 10 μ l of fixed cells on microscopic slide, using a brightfield microscope at low magnification. Determine the number of vacuoles with concentrated India ink per cell in a total of 50 cells.

6. Summary

A combination of the protocols presented here enables for discovery and functional analysis of individual proteins in multiple ciliary compartments. WD lab has used the fractionation protocol 3.2. to identify over 2000 proteins in ciliary fractions of *Tetrahymena* (unpublished data). We are now using the tagging protocols described here to search for proteins that localize to the distal parts of cilia, with the long-term goal of identifying components of ciliary caps.

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Figure 1.

A–C. Thin sections of the distal tips of *Tetrahymena* oral (A,B) and somatic (C) cilia. The central microtubule caps (c) link the distal tips of the central microtubules to the membrane (small arrowheads) and the distal filament caps (d) link the tips of the A-tubules of each doublet to the membrane (small arrowheads). The distal filaments (see F,H,I) at the tips of somatic cilia are thin and appear identical to those seen in *Chlamydomonas* flagella. The more bulbous distal filaments at the tips of oral cilia appear to be unique to *Tetrahymena*. D. *Tetrahymena* cilia purified after dibucaine deciliation. Cilia are intact and are completely enclosed by ciliary membranes. E. Purified ciliary membrane vesicles. F. Axoneme after demembranation with 1% NP-40. Distal filament caps at the tips of A tubules (d) and the central microtubule cap (c) crowns the tip of the central microtubules. G. Distal tip of an axoneme after extraction with MgCl_2 to release the capping structures. The tips of the A and central microtubules are intact but lack distal filaments and central microtubule caps (arrows). H,I. Negatively stained MgHSS containing central microtubule caps (c) and distal filaments (d) released from axonemes by MgCl_2 .

Figure 2.

Composition of plasmid fragments used for expression of epitope-tagged proteins either in the *BTU1* locus (top) or in the native locus (bottom). The locations of the translation initiation codon (ATG) and the translation termination codon (TGA) are shown. “X” shows intended location of a homologous DNA recombination event.

Figure 3.

A dark field (right) and a corresponding confocal immunofluorescence (left) image of *Tetrahymena* cells labeled with the polyG anti-polyglycylated tubulin antibodies using protocol 4.4. Two cells, each from a different strain, are imaged side by side. Note that the cell on the left has labeled food vacuoles.

Figure 4.
Confocal images of a single cell co-labeled by immunofluorescence (protocol 4.4) with the 12G10 anti- α -tubulin and poly-G anti-polyglycylated tubulin antibodies.