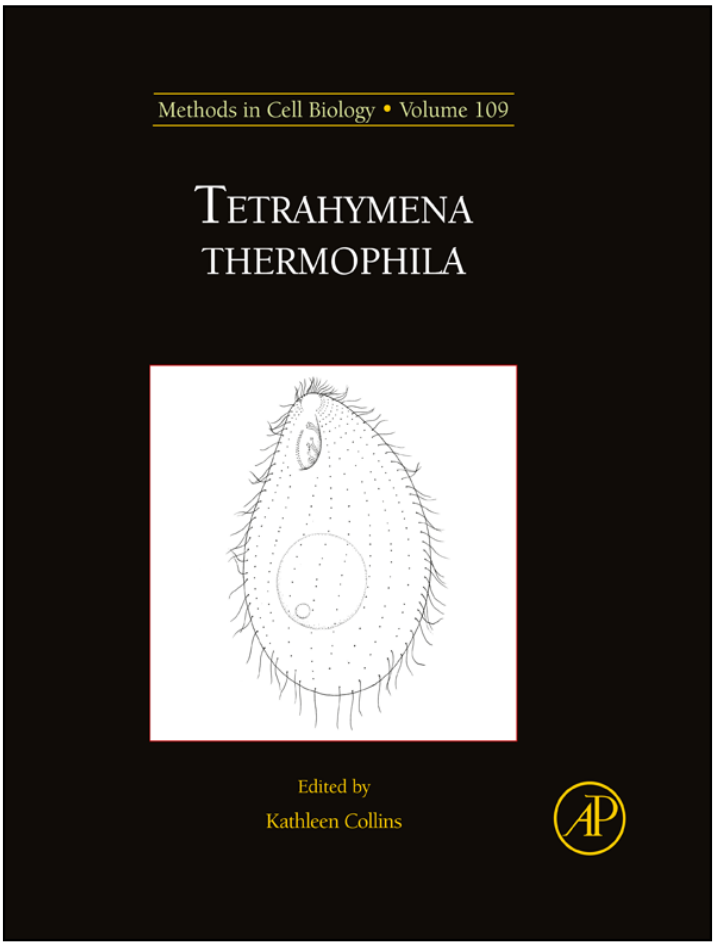

From Mark Winey, Cytological Analysis of Tetrahymena thermophila. In: Kathleen Collins, editors: Tetrahymena Thermophila, Vol 109, USA: Academic Press; 2012, p. 357-378.
ISBN:978-0-12-385967-9

CHAPTER 13

Cytological Analysis of *Tetrahymena thermophila*

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Abstract

Since their first detection in pond water, large ciliates such as *Tetrahymena thermophila,* have captivated school children and scientists alike with the elegance of their swimming and the beauty of their cortical organization. Indeed, cytology – simply looking at cells – is an important component of most areas of study in cell biology and is particularly intriguing in the large, complex *Tetrahymena* cell. Cytological analysis of *Tetrahymena* is critical for the study of the microtubule cytoskeleton, membrane trafficking, complex nuclear movements and interactions,

METHODS IN CELL BIOLOGY, VOL 109

0091-679X/10 $35.00
DOI 10.1016/B978-0-12-385967-9.00013-X

and the cellular remodeling during conjugation, to name a few topics. We briefly review previously reported cytological techniques for both light and electron microscopy, and point the reader to resources to learn about those protocols. We go on to present new and emerging technologies for the study of these marvelous cells. These include the use of fluorescent-protein tagging to localize cellular components in live cells, as well as for tracking the dynamic behavior of proteins using pulse labeling and fluorescence recovery after photobleaching. For electron microscopy, cellular and antigenic preservation has been improved with the use of cryofixation and freeze-substitution. The technologies described here advance *Tetrahymena* cell biology to the cutting-edge of cytological analysis.

I. Introduction

Tetrahymena cells and their behavior during their life cycle offer a wealth of interesting cytology (Wloga and Frankel, *this volume*). Simply examining the cells in the light or electron microscope can be informative about a variety of cellular processes. In fact, we routinely cite classic *Tetrahymena* cytological studies, such as Dick Allen's description of basal body assembly based on electron microscopy (Allen, 1969); see also http://www5.pbrc.hawaii.edu/allen/ch18/ and also the ASCB cell image library http://www.cellimagelibrary.org/). As detailed below, the previous 2000 edition of this volume contains still relevant chapters describing protocols for light or electron microscopy. However, since that time gene discovery and analysis in *Tetrahymena* has been greatly enhanced by the availability of the genome sequence and facilitated by improved fluorescent protein tagging constructs coupled with efficient means to alter gene function. Given such advances, robust methods for protein localization and structural analyses are necessary to accomplish the outstanding cell biology that can be done in these cells. It has been demonstrated that large-scale proteomic or genomic screens followed by localization of proteins can effectively reveal genes of interest to the investigator (e.g., Bright *et al*., 2010; Cole *et al*., 2008; Jacobs *et al*., 2006; Kilburn *et al*., 2007). Furthermore, sensitive live-cell and high-resolution electron microscopy techniques are critical for phenotypic analysis of mutant strains. We present methods and reagents for important microscopy techniques that have been implemented over the last 10 years, and we suggest emerging cytological techniques that should be valuable when applied to *Tetrahymena thermophila*.

II. Rationale

As noted above, cytology is an important component of cell biological investigations in *Tetrahymena*, as in other organisms. The major applications for cytological protocols are protein localization and phenotypic analysis. Both of these applications can be pursued in the light microscope or the electron microscope, and some of the protocols for these applications are presented below. Furthermore, the combined

use of both types of instruments to determine protein localization or to reveal a mutant phenotype can provide the most robust and revealing description. *Tetrahymena* is a wonderful subject for microscopy, but it does offer some challenges – its large size can make it difficult to effectively fix for EM, and its motility can be an issue for live-cell imaging. Nonetheless, many of the liabilities of doing cytology in *Tetrahymena* can be mitigated with newer reagents and techniques described here.

III. Light Microscopy

Specific model systems in cell biology are used because of the advantages that they possess. *Tetrahymena* is a good cytological model system because cells are large, highly organized, and several cellular structures are amplified, allowing the researcher to more easily identify structures and events of interest. Protein tagging strategies for fixed cell protein localization using light microscopy has generated a wealth of novel information that contributes to our understanding of the cortical cytoskeleton, nuclear architecture, and cilia function. While still in its infancy, the utility of *Tetrahymena* and specific live-cell imaging strategies are now being developed for a real time view of these biological events. Such strategies allow us to view cellular events as they occur, the dynamics of proteins and DNA, and the short-lived, transient structural events that lead to a final product. The ability to visualize transient events that may not be captured by fixed time-point studies, because they are short-lived, will help reveal key functional events that happen during the cell cycle.

A. Previous Methods and Resources

In the previous edition, Stuart and Cole (2000) presented protocols for the preparation and imaging of immuno-fluorescently labeled fixed cells. In a separate chapter, these authors (Cole and Stuart, 2000) described classical staining techniques for bright-field microscopy. In a later publication, Cole *et al*. (2002) and others described additional techniques for *in situ* hybridization and for live-cell imaging using devices, such as the rotocompressor, that immobilize cells (e.g., Aufderheide, 2008; Cole *et al*., 2002; Loidl and Scherthan, 2004; Wolfe and Colby, 1981). All of these techniques are still relevant and applicable to *Tetrahymena*. We present further "low-tech" solutions for live-cell imaging, along with new applications based on the ability to tag genes with various fluorescent proteins.

B. New Methods

1. Live-Cell Imaging

A key technological advance in recent years with *Tetrahymena thermophila* studies is the application of green fluorescent protein (GFP) and its spectral variants,

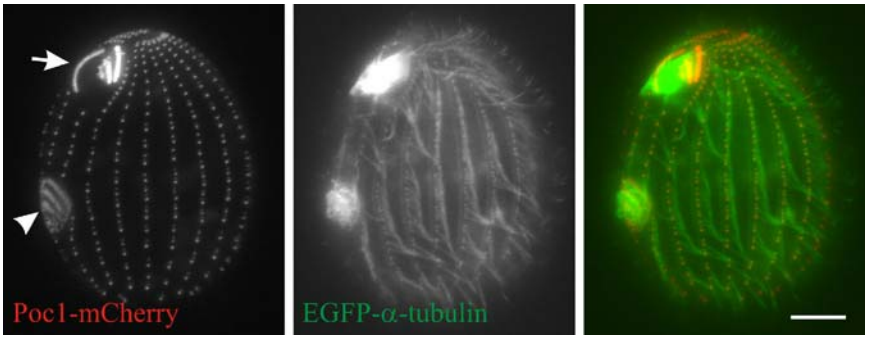

Fig. 1 Live cell, two color colocalization using EGFP and mCherry fusion proteins. Co-localization of Poc1-mCherry (left panel, red) and EGFP-α-tubulin (center panel, green) in a live *Tetrahymena thermophila* cell that is in mitosis. Immobilized cells were imaged for red and green fluorescence using a through volume Z-series. A maximum fluorescence intensity projection was generated using Metamorph Imaging Software (Molecular Devices). Arrow denotes the old oral apparatus and the arrowhead denotes the nascent oral apparatus or oral primordium. For visualization of individual cilia, the image brightness was increased leading to saturation of the oral apparatus GFP-α-tubulin fluorescence. Scale bar, 10 μm. (See color plate.)

which have been used to create fusion proteins labeled with different colors in the same cell (Fig. 1). With this advance comes the ability to visualize the assembly and structural localization of proteins and chromosomes.

Cell Immobilization

Because *Tetrahymena* are vigorous swimmers, live cells must be adhered to a substrate before cellular structures can be followed for imaging. A number of historical studies have identified methods for limiting cell motility during live-cell imaging (Cole *et al*., 2002). This is still a significant challenge given the hundreds of undulating cilia that propel cells to almost 0.5 mm/s. A range of tactics has been used to slow or stop cell motility (Aufderheide, 2008; Bright *et al*., 2010). We find that a combination of compression of cells between the glass coverslip and slide in the presence of a high viscosity medium works well for short imaging times of up to 30 min.

While the below methods have worked well for many of our experiments, we expect to develop robust strategies for long time period imaging in the future. We have, so far, been unsuccessful in using optical lithography with microfabricated chambers, and this technology may also provide a means to immobilize cells in the future. Furthermore, an exciting new possibility is the use of temperature-regulated polymers that have already shown promise in the Turkewitz lab (Bright *et al*., 2010). The techniques described below provide a short-term imaging tactic to follow localization in live cells (Pearson *et al*., 2009a, 2009b; Fig. 1).

Methods

1. Grow cells to mid-log phase.
2. Spin down ~1 mL of cells at 3000 × g for 30 s.
3. Aspirate supernatant.
4. Gently resuspend cells in ~0.2 mL of *Imaging Media*. Allow the cells to equilibrate for at least 20 min in the viscous *Imaging Media*.
5. Prepare a microscope slide by placing four small (~1 mm^2) spots of silicone vacuum grease on each corner of an 18 mm^2 coverslip.
6. Apply 50 μL of fresh *Imaging Media* on the center of a microscope slide.
7. Apply 20 μL of the resuspended cells on top of the *Imaging Media* pad.
8. Cover with the above prepared coverslip so that media is dispersed and cells become trapped in the viscous solution between the coverslip and slide. The silicone creates a buffer distance between the slide and coverslip so that the cells are not overly compressed or lysed. Use a pipette tip to apply pressure to all four corners of the coverslip. To minimize tilting of the coverslip, it is best to apply pressure to all four corners at the same time.
9. Aspirate or wick away any residual media that is dispersed from the coverslip.
10. Monitor cell motility using a transmitted light microscope at low magnification.
11. If required, apply additional pressure to the corners to inhibit cell motility.
12. Once cell motility is abated, evaporation of the media from the chamber can be minimized by sealing the coverslip to the microscope slide using a thin film of melted *VALAP* around the edges. This allows for air exchange but reduces evaporation.
13. Transfer prepared sample to an appropriate light microscope to visualize cells.

Materials

Imaging Media (modified 1% SPP Cole and Stuart, 2008)

Proteose peptone	1%
Yeast extract	0.1%
Glucose	0.2%
Poly(ethylene oxide) MW 900,000 (PEO, Sigma)	3%

VALAP

Vasolin	30%
Lanolin	30%
Paraffin	30%

Notes

It is important to minimize background autofluorescence to obtain a high signal-to-noise ratio when imaging fluorescent proteins. Media is an unfortunate source of background autofluorescence. We minimize this source of background signal by

keeping the proteose peptone at 1% as opposed to the 2% that we use to grow cells in culture. Also, we do not autoclave our media with glucose but rather add sterile glucose at the appropriate concentration (0.2% final) after autoclaving to minimize caramelization of the sugar. Reducing the total media concentration also improves the efficiency with which cells are immobilized on the glass surface.

2. Fluorescent Protein Pulse Experiments

Meselson and Stahl (1958) famously used pulse labeling of DNA to show that DNA is semiconservatively replicated. Additionally, George Palade used strategies to pulse label proteins and define the mechanisms of the cellular secretory pathway (Caro and Palade, 1964; Jamieson and Palade, 1967a, 1967b). These pioneering techniques elucidated the mechanisms of fundamental cellular processes with radioactive markers.

Pulse-chase experiments can now be used in live cells to follow the fate of newly expressed proteins fused to EGFP to assess the assembly and turnover dynamics of both nucleic acids and proteins. Reagents exist for the expression and repression of genes fused to EGFP in *Tetrahymena*. Transient regulation of gene expression can be controlled by promoters that are sensitive to metals in the media (e.g., cadmium and copper, Boldrin *et al*., 2008; Shang *et al*., 2002). GFP-tagged proteins are then pulsed on with the addition of metals. Within minutes, EGFP is visualized and the incorporation and dynamics of proteins at their site of activity can be monitored with high temporal resolution. Examples of these experiments are provided in Fig. 2. Furthermore, the ultrastructural localization of the dynamics can be visualized using immuno-EM that is described in the EM section of this chapter and as shown in Pearson *et al.* (2009a).

Methods

1. Grow cells containing MTT-EGFP-Your Favorite Gene (YFG) to mid-log phase or to a cell cycle arrest in SPP media. The constructs for generating these strains are described below.
2. Image live cells to ensure that the EGFP signal is not detectable (this indicates that the promoter is not leaky; see *Notes*).
3. Induce the expression of EGFP-YFG by adding 0.1–1.0 μg/mL $CdCl_2$ to the culture. Concentration will depend both on YFG and the media that you are growing your cells in (less $CdCl_2$ is required in Starvation Media (10 mM Tris pH 7.4)). We use low concentrations of cadmium to minimize deleterious effects from protein overexpression. In addition, high concentrations of $CdCl_2$ (>1.0 μg/mL) affect cell growth rates (Larsen, 1989).
4. After EGFP-YFG is synthesized, cellular expression can be stopped by washing the cells three times with fresh SPP media. Alternatively, EGFP-YFG expression can be constitutively expressed by maintaining the $CdCl_2$ in the media.

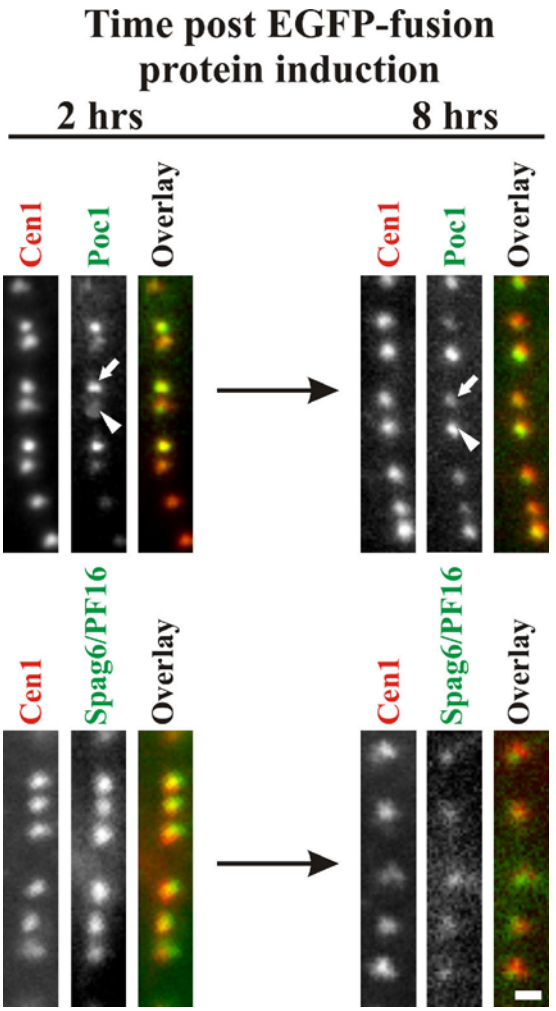

Fig. 2 Pulse-labeling using EGFP fusions. Pulsed expression of EGFP fusion proteins can be used to visualize protein assembly at their binding sites. Expression of basal body proteins Poc1 (top panels) and Spag6/PF16 (bottom panels) controlled by the metallothionein (MTT1) promoter was induced by addition of $CdCl_2$ to the media. All basal bodies were uniformly labeled using α-centrin staining. Fluorescence signal of the EGFP is not observed prior to induction (data not shown). By two hours post-induction basal bodies are labeled in a manner that represents the turnover dynamics of each component. Poc1 labels existing basal bodies with a low level of fluorescence (arrowhead) that represents dynamic protein turnover. Basal bodies that assemble in the presence of EGFP-Poc1 are brightly labeled (arrow) indicating that new basal body assembly is required for complete incorporation of signal. These assembly dynamics correspond to the turnover dynamics measured by FRAP (Fig. 3). In contrast, EGFP-Spag6/PF16 exhibits rapid assembly at all basal bodies shortly after EGFP-Spag6/PF16 induction indicating that it exhibits rapid exchange with its basal body binding sites, independent of new basal body assembly. By eight hours, most basal bodies have assembled in the presence of EGFP tagged proteins and these levels represent steady state incorporation dynamics. (Figure adapted from Pearson *et al*. (2009a, 2009b) Scale bar, 1 μm. (See color plate.)

5. At defined time points after expression, EGFP-YFG localization to the site of activity can be followed by EGFP fluorescence.

Materials

$CdCl_2$Stock
$CdCl_2$ 1 mg/mL
ddH_2O
Filter sterilize

Starvation Media
Tris base 10 mM
ddH_20
pH to 7.4 and filter sterilize.

Notes

It is important to monitor the level of EGFP fluorescence signal that is expressed in the absence of $CdCl_2$. The metallothionine (MTT) promoter can exhibit a low level of expression without induction. This is likely due to small amounts of metals in the media that activate the promoter. This can be limited by growing cells in limited media defined by the Gaertig lab (J. Gaertig, personal communication). Finally, new promoter systems are available for use with copper (MTT2; Boldrin *et al*., 2008). This advancement limits the use of toxic heavy metals.

3. Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP) is a powerful strategy to measure protein dynamics and complement pulse-chase studies. The quantitative analysis of protein interactions and dynamics has historically been studied *in vitro*. However, GFP tagged proteins allow for the study of interactions and dynamics in live cells. This technique can be used to measure diffusion rates, dynamics of protein binding, and to measure interactions with other components. Several complementary studies and reviews describe FRAP technology and analysis in more detail (Salmon *et al*., 1984a, 1984b; Sprague and McNally, 2005; Walczak *et al*., 2010). We provide a brief introduction to the methods required for photobleaching and live-cell imaging of fluorescence recovery in *Tetrahymena* (Fig. 3).

Methods

1. Grow cells expressing EGFP-YFG to mid-log phase.
2. Prepare immobilized cells as described above.
3. Several methods exist for photobleaching. The most common are to either use a laser scanning confocal to scan and bleach a region of interest (ROI) or to use a

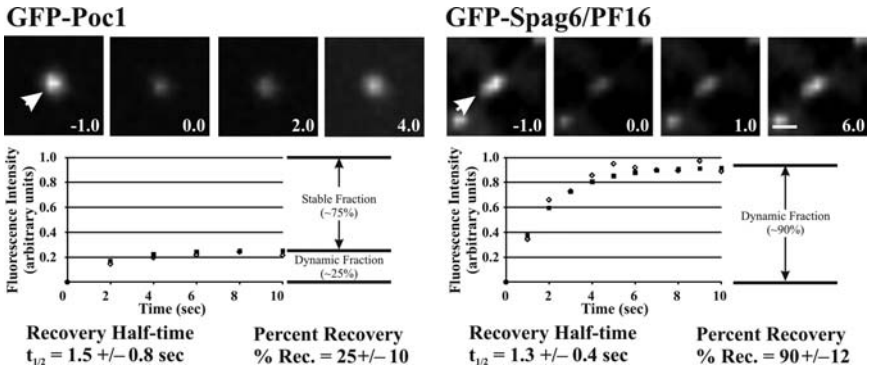

Fig. 3 Fluorescence recovery after photobleaching (FRAP) to visualize protein dynamics. Protein exchange at basal bodies is visualized by photobleaching bound EGFP-tagged protein at basal bodies and visualizing fluorescence recovery over time. Fluorescence recovery (FRAP) represents unbleached molecules in the cytoplasmic pool that replace the bleached GFP molecules. Basal body components exhibit divergent protein exchange. Poc1's binding to basal bodies is bimodal. ~25% of the basal body localized Poc1 protein is dynamic and ~75% is stable and no fluorescence recovery is observed. In contrast, Spag6/PF16 is almost completely dynamic with ~90% of the protein exchanging with rapid kinetics. (Figure adapted from Pearson et al. (2009a, 2009b)) Scale bar, 1 μm.

focused laser beam to bleach a specific region of interest. We will describe the later technique, as we believe this is an effective technique for FRAP studies. We use a Nikon TiE stand with a motorized X–Y stage. Laser light (either 488 nm (for GFP) or 564 nm (for mCherry)) is fiber-optically coupled to the back aperture of the objective, and the collimated laser beam is focused on to the specimen plane as a point. The beam spot size can be expanded by defocusing the laser beam. The site of the beam is then identified as an ROI.

4. Once the cellular target is chosen, the specimen is centered at the laser site (ROI) using the motorized stage driven by Nikon – NIS-Elements Software.
5. A prebleach image is acquired to determine the sample fluorescence intensity prior to photobleaching.
6. The samples are exposed to a short laser pulse (~50 ms) using a shuttered laser light. The laser exposure time, spot size, and intensity is altered depending on the experiment.
7. Immediately following photobleaching, a post-bleach image is then acquired ($t = 0$ s).
8. A time course is then acquired to follow the fluorescence recovery. The time interval between acquisition time points is critical for obtaining appropriate recovery curves to determine the protein turnover kinetics. Often this is achieved by trial and error.
9. Following FRAP image acquisition, the data are analyzed (Salmon *et al*., 1984a, 1984b; Sprague and McNally, 2005; Walczak *et al*., 2010).

Notes

The quantification of FRAP is an important aspect to define the turnover dynamics. For simplicity, we refer the reader to prior publications (Salmon *et al.*, 1984a, 1984b; Sprague and McNally, 2005; Walczak *et al.*, 2010). The key parameters to correct for are background fluorescence and the photobleaching that occurs from excitation light exposure during image acquisition. It is also important to ensure that the photobleaching laser irradiation is not causing cellular damage and affecting the biological event of interest.

C. Fluorescent Protein Tagging Strategies

Several technical advances have made creating fluorescent fusion proteins in *Tetrahymena* increasingly efficient. These include the availability of the *Tetrahymena* macronuclear genome sequence, use of metal-sensitive promoters, an increasing number of fluorescent proteins, a choice of drug resistance markers, and development of new vector systems designed to target gene fusions either to exogenous or endogenous loci. These strategies are equally applicable to other kinds of tags, but the focus here is on fluorescent tags for live-cell imaging.

Initially *Tetrahymena* GFP constructs were based on rDNA processing vectors, which, when introduced into the *Tetrahymena* macronucleus following mating, provide resistance to paromomycin. As rDNA sequences are processed and amplified in the maturing macronucleus, the transforming sequence is greatly amplified (Tondravi and Yao, 1986). Two vectors designed to create N-terminal GFP fusions were built into this system, pVGF-1 and pIGF-1 (Table I, Malone *et al.*, 2005; Wiley *et al.*, 2000; Yao *et al.*, 2007), the primary difference being in the promoters used to drive the constructs. pVGF-1 utilizes the rpL29 promoter, which is constitutive during vegetative growth, whereas pIGF employs the MTT1 promoter, which is responsive to the addition of $CdCl_2$ to the media. These constructs accept the gene in sites engineered just 3′ of the GFP-coding region. rDNA processing vectors have been modified further to employ YFP and CFP (Yellow and Cyan Fluorescent Protein, respectively), and the Gateway recombinase cloning system (Invitrogen), as well as to create C-terminal fusions (Cole *et al.*, 2008; Malone *et al.*, 2005; Yao *et al.*, 2007). Because these constructs are highly amplified, one must be cautious in interpreting data due to overexpression of the fusion protein (Stemm-Wolf *et al.*, 2005).

GFP-fusions have also been targeted to the rpL29 locus by using an rpL29 allele that confers resistance to cycloheximide (Matsuda *et al.*, 2010; Yao and Yao, 1991). These constructs take advantage of the Gateway cloning system and are controlled by the MTT1 promoter. Additionally, variants have been made replacing GFP with monomeric Cherry (C. G. Pearson, unpublished). These constructs have several advantageous properties: because they are not reliant on rDNA processing, the gene copy number is considerably lower, and vegetative cells can be transformed by biolistic bombardment. Furthermore, the use of cycloheximide as a selectable marker leaves paromomycin resistance, encoded by any

Table I
Vectors for fluorescent tagging of proteins in *Tetrahymena*.

Vector type	Vector name	*Tetrahymena* selection	Description	Reference
rDNA processing vectors	pVGF-1	Paromomycin	N-terminal EGFP, rpL29 promoter	Wiley *et al*., 2000 Yao *et al*., 2007
	pIGF-1	Paromomycin	N-terminal EGFP, MTT1 promoter	Malone *et al*., 2005
	pIGF-gtw	Paromomycin	N-terminal EGFP, MTT1 promoter	Yao *et al*., 2007
	pICC-gtw	Paromomycin	C-terminal CyanFP, MTT1 promoter	
	pICY-gtw	Paromomycin	C-terminal YellowFP, MTT1 promoter	Cole *et al*., 2008 Malone *et al*., 2005
rpL29 exogenous	pBS-MTT-GFP-gtw	Cycloheximide	N-terminal EGFP, MTT1 promoter	Matsuda *et al*., 2010
	pBS-MTT-mCherry-gtw	Cycloheximide	N-terminal mCherry, MTT1 promoter	Pearson unpublished
Endogenous	pEGFP-NEO4	Paromomycin	C-terminal EGFP, codon optimized	Kataoka *et al*., 2010
	pmCherry-NEO4	Paromomycin	C-terminal mCherry, codon optimized	
	ploxP-NEO4-loxP	Paromomycin	N-terminal EGFP, codon optimized. Requires abortive mating to CRE556 or some CRE expressing strain.	Busch *et al*., 2010
	pmCherryLAP-NEO2	Paromomycin	C-terminal S-peptide-PreScission protease site-mCherry, codon optimized	Stemm-Wolf unpublished
	pNEO2-MTT1pr-mCherryLAP	Paromomycin	N-terminal mCherry-PreScission protease site-RGS6HIS, codon optimized, MTT1 promoter	
	pNEO2-MTT1pr-GFP	Paromomycin	N-terminal EGFP, MTT1 promoter	

number of NEO cassettes engineered for *Tetrahymena*, available for additional vegetative transformations.

Because *Tetrahymena* executes high-fidelity homologous recombination (Dave *et al*., 2009; Yao and Yao, 1991), fluorescent tags can be targeted directly to the endogenous locus, and systems have been devised to allow expression from the endogenous promoter for both C- and N-terminal fusions. Furthermore, the entire gene does

not have to be cloned into these vectors, as sequence is required only to promote homologous recombination at the target locus. This can be a tremendous advantage when studying proteins encoded by large genes. PCR strategies have been employed that entirely bypass the need for cloning new gene specific vectors for transformation (Kataoka *et al.*, 2010). Furthermore, cassettes have been developed that optimize codon usage for expression in *Tetrahymena* (Kataoka *et al.*, 2010). Endogenous C-terminal tagging is straightforward as a drug resistance marker can be inserted downstream of the fluorescent tag, but tagging the N-terminus requires either the addition of an exogenous promoter (such as the MTT1 promoter) or the subsequent removal of the selectable marker after transformation in order for the fusion protein to be expressed. This has been accomplished by introducing the Cre recombinase into a transformed strain that has the selectable marker flanked by loxP sites (Busch *et al.*, 2010).

Once a strain has been constructed, it is sometimes necessary to observe the fluorescent tag following cell fixation for antibody staining of a different protein. In such cases, it is important to minimize the extent of the fixation in order to preserve the fluorescent protein signal. We have effectively used the "Double Fix" (Cole and Stuart, 2000), which employs a short formaldehyde fix followed by an ethanol fix, and a 30 min 2% formaldehyde fix has been reported to be effective as well (Matsuda *et al.*, 2010).

Now that a variety of vector systems are well established, new developments in fluorescence microscopy can be easily incorporated into *Tetrahymena* research. Amongst these are Localization and Purification (LAP) tags which pair a fluorescent protein with a second tag well suited for protein purification and function similarly to TAP tags (Cheeseman and Desai, 2005; Puig *et al.*, 2001). Newer fluorescent proteins, such as Dendra, which can be converted from green fluorescence to red fluorescence by blue or UV light, have the potential to distinguish between unactivated (green) and activated (red) populations of the same protein within the cell (Gurskaya *et al.*, 2006). SNAP and CLIP-tags are flexible tags that can bind a number of fluorescent substrates whose use in *Tetrahymena* is just now being explored (New England Biolabs).

IV. Electron Microscopy

Electron microscopy (EM) is an important tool that allows the cell biologist to peer into the cell and directly image the structures of interest at a resolution of 2 nm or better. Indeed, EM investigation of ciliates has been invaluable in the understanding of basal body assembly, the identification of structures associated with cortical patterning, and the description of cellular membrane systems. Despite outstanding advances in live-cell imaging using light microscopy electron microscopy remains the only means of discerning the ultrastructure of the cell at the macromolecular level. The combination of modern electron and light microscopy techniques provides a powerful approach to the study of cellular processes, protein localization, and correlation of structure and function through the investigation of mutant phenotypes.

A. Previous Methods and Resources

Traditional chemical fixation of *Tetrahymena* cells with aqueous glutaraldehyde and osmium tetroxide generated a wealth of structural information about these cells. In the previous edition, Dentler (2000) and Gavin *et al.* (2000) presented techniques for the chemical fixation of *Tetrahymena* for morphology and for protein localization by antibody staining, respectively. In addition, we have found that simultaneous fixation with a mixture of glutaraldehyde and osmium tetroxide (modified from the method of Orias *et al*., 1983) yielded particularly well-preserved and stained cellular ultrastructure (described in Giddings *et al*., 2010).

We describe here methods for cryofixation of *Tetrahymena* by high-pressure freezing and freeze-substitution (HPF/FS) for morphological analysis in thin sections and for the immuno-EM (IEM) localization of proteins. These techniques have been introduced into the analysis of *Tetrahymena* since the previous edition of this manual and have been reviewed elsewhere (Giddings *et al*., 2010; Meehl *et al*., 2009). High-pressure freezing, as the name implies, involves rapidly freezing the cells under conditions of high pressure such that the formation of damaging ice crystals is greatly reduced or prevented (Glkey and Staehelin, 1986). Freeze-substitution is the process during which the water in the samples is replaced with an organic solvent, stains, and fixatives at low temperatures in preparation for embedding the cells in plastics. The use of HPF/FS on *Tetrahymena* specimens has resulted in excellent preservation of overall cell structure with very little extraction of material (Meehl *et al*., 2009; Fig. 4). Immuno-labeling of thin sections (50–70 nm) from similarly prepared HPF/FS samples has been used to localize proteins in cells and to specific domains of cellular structures (Kilburn *et al*., 2007; Fig. 4). Finally, cells prepared by HPF/FS can be used for electron tomography (ET). ET produces three-dimensional models based on a tilt-series of electron micrographs of semithick sections (~300 nm) to reveal intricate details of cellular structures previously unobserved in traditional thin-section EM. ET is an advanced EM application that generally requires higher voltage TEMs, specialized software and significant expertise. The number of laboratories equipped to perform ET is increasing steadily. We have discussed the application of ET to the study of *Tetrahymena* ultrastructure elsewhere and presented models of basal bodies and other structures (Giddings *et al*., 2010).

B. High-Pressure Freezing and Freeze-Substitution of *Tetrahymena* Cells

1. High-Pressure Freezing

Small cell pellets are prepared from 8–10 mL of *Tetrahymena* culture by centrifugation in a 15 mL conical centrifuge tube at 500 × g for 2 min. Quick removal of the supernatant from the pellets prevents cells from swimming out of the pellet. Each pellet is gently resuspended in a cryoprotectant solution (500 μL of SPP media supplemented with 15% dextran (Sigma) and 5% Bovine Serum Albumin (BSA) (Sigma)). This cell slurry is centrifuged at 800 – 1000 × g for 4 min, and the

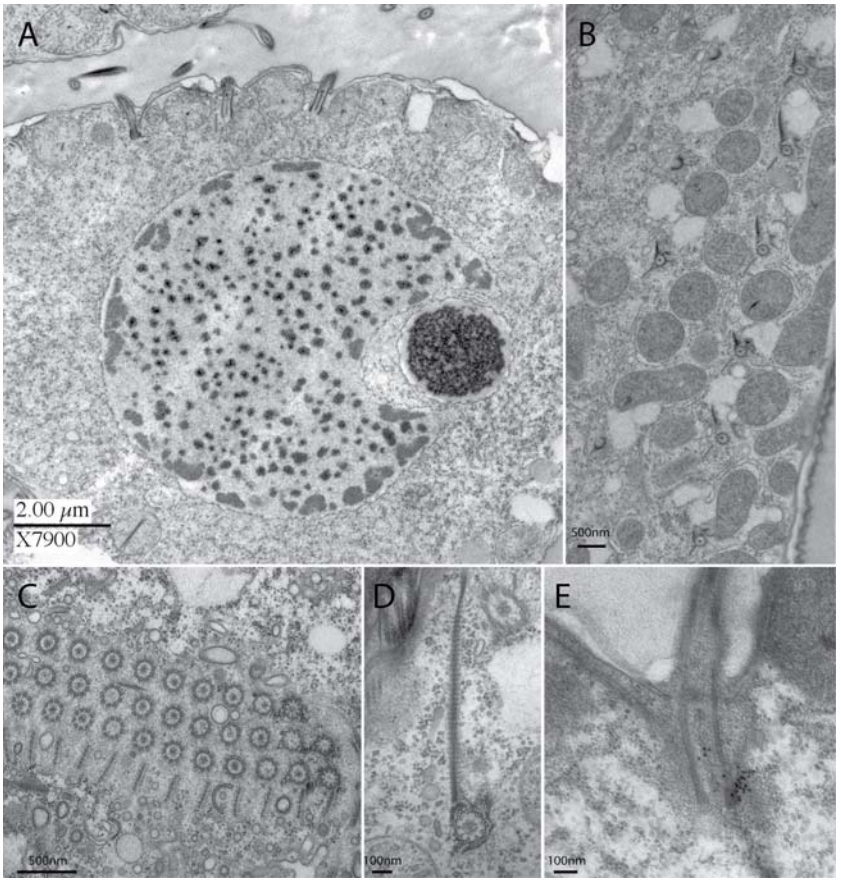

Fig. 4 Thin section transmission electron microscopy and immuno-EM of *Tetrahymena thermophila* prepared by high pressure freezing and freeze substitution. (A) A lower magnification image showing preservation of the two nuclei as well as organelles in the cytoplasm and basal bodies anchored at the cell surface. (B) A lower magnification image of a section near the cortical surface in which two cortical rows of basal bodies are visible. Basal bodies and some of their associated microtubules and structures, as well as mitochondria are visible. (C) Basal bodies and associated microtubule structures in one of the oral apparatus membranelles. Connectors between basal bodies on the right side of the image are visible. (D) A basal body in cross-section along with its associated kinetodesmal fiber. E. A longitudinal section of a basal body that has been stained with anti-Cen1 antibodies followed by a secondary antibody conjugated with 15 nm gold particles. Cen1 is asymetrically localized at the proximal end of the basal body, and is visible at the basal body midzone. The cells in panels A–D were freeze-substituted with osmium/uranyl acetate and embedded in Epon. The cell in panel E were freeze-substituted with glutaraldehyde/uranyl acetate and embedded in Lowicryl HM20.

supernatant is removed leaving a minimal residue of cryoprotectant media with the pellet. The small residue of the cryoprotectant allows for the cells to be somewhat resuspended and separated. Loosely packed *Tetrahymena* cells retain their normal shape, freeze better, and retain more of their cortical cilia. Two to three microliters of the cell preparation are pipetted into the 100 μm deep well (shallow side) of an aluminum Type B specimen carrier (Technotrade International). The samples are then capped with the flat side of a Type A specimen carrier coated with hexadecene (Sigma). The tip of the specimen holder is clamped around the specimen carriers and tightened gently before insertion into the HPF instrument. With the Bal-Tec HPM 010, the freezing process is initiated by simply pressing a button. Immediately after the freezing event and cessation of the audible venting of the freezing chamber, the sample holder is rapidly moved to a tray of liquid nitrogen for unloading the sample. Under liquid nitrogen, the aluminum carrier hats containing the sample are pried apart and transferred to cryovials that contain 1 mL of *FS medium*. The samples will lie on top of the frozen *FS medium*, but sink into it once the vial is warmed sufficiently to initiate freeze-substitution.

2. Freeze-Substitution

We use two different freeze-substitution protocols for fixation and embedding of HPF-prepared *Tetrahymena* cells depending on the experiment (Meehl *et al*., 2009). To achieve a thorough fixation with strong staining of both membranous and cytoskeletal organelles, we freeze-substitute in 2% osmium tetroxide (OsO_4; Ted Pella) and 0.1% uranyl acetate (UA; Electron Microscopy Sciences, Hatfield, PA) in acetone followed by embedding in Epon-Araldite (Electron Microscopy Sciences, Hatfield, PA). To retain antigenicity for immuno-labeling of plastic-embedded sections, we use a milder fixation with 0.25% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 0.1% UA in acetone followed by embedding in Lowicryl HM20 (Electron Microscopy Sciences, Hatfield, PA). The Lowicryl low-temperature embedding method has also proven to yield excellent preservation of cellular ultrastructure for high-resolution EM analysis including tomography. Our general practice is to high-pressure freeze enough samples to carry out both FS and embedding protocols on the same batch of cells.

Freeze-Substitution with Osmium Tetroxide for Embedding in Epon-Araldite Epoxy Resin

A metal block cooled to –80 °C is used to hold the cryovials containing the samples in *FS media*. This block with the samples is nestled in a chest of dry ice and placed in a standard –20 °C freezer for 3–4 days. Gradual overnight warming of the samples to –20 °C is achieved by removing the lid from the chest allowing a small residue of the dry ice to evaporate. After remaining at −20 °C for several hours, the metal block containing the samples is moved to 4 °C for 4–6 h and finally to room temperature for 1 h. An alternative method is to use an automated freeze-substitution device. Once the samples have reached room temperature, the *FS media* is removed

and the samples are rinsed twice with acetone. The samples are now removed from the aluminum carrier hats prior to embedding. The freeze-substituted cells and cryoprotectant solution typically form a cohesive disk that either falls off or can be removed gently from the aluminum carrier hats by means of dissecting needles or similar tools. It can be worthwhile to work under a dissecting microscope to retrieve any small fragments. Samples are rinsed again in fresh acetone, and then infiltrated with increasing concentrations of Epon-Araldite resin (without DMP30 accelerator, Electron Microscopy Sciences, Hatfield, PA) diluted in acetone. The embedding regimen is 25% Epon in acetone overnight; 50% Epon 8–10 h; 75% Epon overnight; and two changes of 100% Epon during the next day. The samples are then left in Epon with accelerator overnight, transferred to BEEM capsules with fresh embedding resin the next day and placed in a 60 °C oven to polymerize for at least 48 h.

Freeze-Substitution in Glutaraldehyde for Embedding in Lowicryl HM20

As described above, samples of frozen cells were freeze-substituted at −80 °C for 3–4 days followed by gradual warming to –20 °C overnight. The samples are then held at −20 °C for acetone rinses and infiltration with increasing concentrations of Lowicryl HM20 in acetone. After rinsing with acetone chilled to –20 °C, the FS samples were separated from the specimen carriers. The procedure for separating the specimens from the specimen carriers is the same as above except for the use of chilled acetone. Working quickly is best to minimize sample warming that can cause extraction and cause morphological changes. As soon as samples are returned to the cryovial they are immediately rinsed in fresh –20 °C acetone and then infiltrated with increasing concentrations of Lowicryl HM20 diluted in acetone. The infiltration schedule is 25% HM20 in acetone overnight; 50% HM20 for 6–8 h; 75% HM20 overnight. The final incubation of the samples is in 100% HM20 for approximately 1.5 days. During this incubation, four changes with fresh resin are made to ensure the removal of any residual acetone. The samples are transferred to embedding capsules that are half filled with fresh HM20, and then the capsule is filled to the top and capped. Polymerization under UV illumination is carried out at –45 °C in a home-made device (see below).

We have also freeze-substituted high-pressure frozen *Tetrahymena* cells using only 0.1% UA in acetone. The rest of the procedure is identical to the above-described glutaraldehyde/UA FS and HM20-embedding protocol and result in nearly indistinguishable morphology. In other cell types, this has allowed us to obtain significant labeling of aldehyde-sensitive antigens (e.g., Pearson *et al*., 2009b).

3. Ultramicrotomy and Staining of Sections

Epon or Lowicryl HM20 plastic resin block faces are trimmed to short, wide trapezoids to optimize the number of cells per section while allowing a large number of serial sections per grid. Cells can then be easily tracked from one section to the next in a ribbon. Copper slot grids are used to pick up serial thin sections (50–70 nm).

The sections are stained in 2% UA in 70% methanol for 6 min; rinsed in the same solvent and dried; and then stained in Reynolds lead citrate for 4 min and thoroughly rinsed with water. For immuno-electron microscopy (IEM), staining times for both UA and lead can be reduced to better visualize colloidal gold particles over electron dense structures.

C. Immuno-Labeling Thin Sections

Sectioned *Tetrahymena* cells prepared by HPF/FS and embedded in Lowicryl HM20 as described above are used for immuno-electron microscopy (IEM). We have been successful with either primary antibodies to selected proteins or with antibodies to tags such as GFP fused to your favorite gene (YFP) (Kilburn *et al*., 2007; Stemm-Wolf *et al*., 2005). Sections of cells fixed by light chemical fixation and embedded in LR White (Electron Microscopy Sciences, Hatfield, PA) have also been used for immuno-localization studies in *Tetrahymena* (Ueno *et al*., 2003).

We have previously published IEM methods for the localization of *Tetrahymena* proteins (Meehl *et al*., 2009). Serial thin sections (50–70 nm) of Lowicryl-embedded cells are collected on Formvar-coated nickel slot grids. The grids are placed, sections side down, onto 15 μL drops of blocking solution for 30 min, followed by 2 h on primary antibody diluted in blocking solution. The grids are then rinsed with a steady stream of Phosphate-Buffered Saline with Tween (PBST) for 20 s before labeling with an appropriate secondary antibody (conjugated to 10 or 15 nm gold) for 1 h. Grids are then rinsed with PBST followed by distilled water, which is removed by careful blotting and air-drying. The visibility of colloidal gold secondary antibody can be improved by using thinner sections and reduced staining times. Expression of GFP-fusion proteins is a valuable technique for the study of *Tetrahymena,* as described earlier. GFP has proven to be robust tag for IEM. To date, we have used two GFP rabbit polyclonal antibodies on *Tetrahymena* cells harboring GFP-tagged proteins (see *Materials* section). Both of these antibodies yield strong signal with low background following this IEM protocol. Of course, the abundance of the given GFP-tagged protein and its concentration at a cellular location contribute to the success of localizing the protein by IEM.

D. Instrumentation and Materials

1. High-Pressure Freezing and Freeze-Substitution Instrumetation

The technology underlying high-pressure freezing and general techniques for its use have been described (e.g., Glkey and Staehelin, 1986; McDonald, 1999). Our instrument is a Bal-Tec HPM 010 (currently available from RMC, Tucson, AZ). Other available models include the Wohlwend HPM 01 (available in the United States through Technotrade International, Manchester, NH) and two models from Leica, the Leica EM PACT2 (McDonald *et al*., 2007) and the Leica EM HPM100.

We commonly grow, HPF/FS, and embed *Tetrahymena* cells for investigators whose local EM facility lacks a freezer. Once embedded in plastic, the bullets can be easily shipped to the investigator for sectioning, staining, and imaging at their home EM facility. High-pressure freezers and associated technological expertise can be found in numerous electron microscopy core facilities at universities, medical schools, or research institutes. Our freeze-substitution system employs a simple Styrofoam box filled with dry ice that is used to maintain the samples at –80 °C for freeze-substitution. The box is placed in a standard refrigerator-freezer unit for gradual warming to −20 °C and embedding in Lowicryl. We use a metal block with holes drilled in it to hold the cryovials of *FS media* upright and to provide a slower rate of temperature change during warming from −80 °C to –20 °C.

We use a homemade UV polymerization chamber for polymerizing blocks of Lowicryl HM20. It consists of an insulated box mounted with two 7-W UV lights. BEEM capsules with samples in liquid resin are held in a wire rack immersed in a temperature-controlled bowl of isopropyl or methyl alcohol. Dry ice is placed in the bottom of the box, and the temperature is maintained at –45 °C by means of a thermocouple-based controller and a heating element wrapped around the bowl.

Commercially available alternatives combine freeze-substitution and UV polymerization capabilities in a single instrument. Such devices include the Leica EM AFS (Leica Microsystems). These instruments offer a versatile and convenient means of achieving controlled, reproducible freeze-substitution and UV polymerization of low-temperature embedding resins. These units have the advantage of offering a wide range of temperatures for initial FS, low temperature fixation, resin infiltration and polymerization, and controlled rates of temperature change throughout the protocol.

2. High-Pressure Freezing and Freeze-Substitution Reagents

The cryoprotectant solution for HPF is 15% dextran (average molecular weight 9.5 kDa, Sigma), 5% BSA in SPP (growth media; see above). We have evaluated a variety of cryoprotectants with respect to the freezing of *Tetrahymena* cells. Consistent and high-quality results are obtained with a mixture of 15% dextran (average molecular weight 9.5 kDa; Sigma) and 5% BSA in culture media. Low MW dextran (9.5–11 kDa) is less viscous at the same concentration than the more commonly used 40 kDa dextran and allows for easier handling of the *Tetrahymena* cells.

The aluminum specimen carriers (hats), Type A and Type B, can be purchased from Technotrade International and are available from several sources. These and many other styles of specimen carriers have been reviewed (McDonald *et al.*, 2007). The freeze-substitution media (*FS media*) for Epon embedding is 2% OsO_4 and 0.1% UA in acetone. It is prepared by placing 12.25 mL of anhydrous acetone in a vial and using 1 mL of the acetone to dissolve the 0.25 g OsO_4 in a glass ampoule (EMS: Electron Microscopy Sciences, Hatfield, PA). The dissolved

osmium tetroxide was returned to the vial and placed on dry ice. Repeating the process quickly dissolved all of the OsO_4. Then 0.25 mL of 5% UA (EMS) in methanol (stored at −20 °C) is added to the solution. This *FS media* is kept on dry ice until aliquoted (1 mL/vial) into 1.8 mL cryovials (Nunc), which are stored under liquid nitrogen until needed.

For embedding in Lowicryl HM20, the freeze-substitution media (*FS media*) is 0.25% glutaraldehyde and 0.1% UA in acetone. It is prepared by adding 0.25 mL 10% glutaraldehyde in acetone (EMS) and 0.2 mL of a 5% UA/methanol stock solution to 9.55 mL acetone. The *FS media* is then aliquoted to cryovials and stored as described above.

3. Immuno-Labeling of Thin Sections Instrumentation

Immuno-labeling is done in a covered glass Petri dish lined with moist filter paper and Parafilm. The droplets of blocking solution and antibodies are placed on the Parafilm, and the dish is set on a magnetic stir plate. Adjust the speed of the stirrer to cause very slow rotation of the nickel grids on the droplets. Be sure to use nonmagnetic-self-closing tweezers when handling nickel grids.

4. Immuno-Labeling of Thin Sections Reagents

PBST is 10 mM sodium phosphate, 150 mM sodium chloride and 0.1% Tween-20. The blocking solution is 1% nonfat dry milk powder (w/v) in PBST. The solution is centrifuged at 1500 × g prior to use to remove undissolved solids.

We have had success with various rabbit polyclonal antibodies to GFP prepared by individual investigators. Unfortunately, we have yet to identify a commercially made α-GFP antibody that works reliably for IEM. Goat-anti-rabbit-15 nm gold or 10 nm gold (Ted Pella, Redding, CA) secondary antibodies were diluted 1:20 in blocking solution.

V. Discussion

We have presented imaging techniques for *Tetrahymena* cells focused on the use of fluorescent proteins to tag genes for live-cell light microscopy, and on the preparation of cells for electron microscopy by high-pressure freezing and freeze-substitution. These techniques along with previously published techniques for either light or electron microscopy make for a strong suite of technologies that enable high quality cytology in *Tetrahymena*. We look forward to seeing additional tools and techniques deployed in these cells, such as photoconversion of Dendra tags, the use of SNAP tags, the application of super resolution imaging, and the incorporation of computational modeling to develop predictive models of cellular processes, as future advances to achieve the cutting-edge cell biology research that can be accomplished in *Tetrahymena*.

Acknowledgments

We thank Aaron Turkewitz and Eric Cole for helpful discussions in using *Tetrahymena* cell immobilization, Doug Chalker for discussions of fluorecent protein tagging strategies, and Jacek Gaertig for discussions of fluorescent protein tagging strategies and for information on limiting leakiness from MTT promoters. We thank Eileen O'Toole (Boulder Laboratory for 3D EM of Cells), Janet Meehl, and Christina Clarissa for helpful advice and assistance with electron microscopy and tomography. Our work on *Tetrahymena* has been supported by the NIH (GM74746 to MW), the March of Dimes Foundation (#1-FY07-422 to MW), as well as the Damon Runyan Cancer Research Foundation, Pew Charitable Trust Scholars Program in Biomedical Sciences, and American Cancer Society (ASC-IRG#57-001-50) to CGP.

The authors declare no conflicts of interest.

References

Allen, R. D. (1969). The morphogenesis of basal bodies and accessory structures of the cortex of the ciliated protozoan *Tetrahymena pyriformis*. *J. Cell Biol.* **40**, 716–733.

Aufderheide, K. J. (2008). An overview of techniques for immobilizing and viewing living cells. *Micron* **39**, 71–76.

Boldrin, F., Santovito, G., Formigari, A., Bisharyan, Y., Cassidy-Hanley, D., Clark, T. G., and Piccinni, E. (2008). MTT2, a copper-inducible metallothionein gene from *Tetrahymena thermophila*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **147**, 232–240.

Bright, L. J., Kambesis, N., Nelson, S. B., Jeong, B., and Turkewitz, A. P. (2010). Comprehensive analysis reveals dynamic and evolutionary plasticity of Rab GTPases and membrane traffic in *Tetrahymena thermophila*. *PLoS Genet.* **6**, e1001155.

Busch, C. J., Vogt, A., and Mochizuki, K. (2010). Establishment of a Cre/loxP recombination system for N-terminal epitope tagging of genes in *Tetrahymena*. *BMC Microbiol.* **10**, 191.

Caro, L. G., and Palade, G. E. (1964). Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study. *J. Cell Biol.* **20**, 473–495.

Cheeseman, I. M., and Desai, A. (2005). A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. *Sci. STKE.* **2005**, l1.

Cole, E. S., Anderson, P. C., Fulton, R. B., Majerus, M. E., Rooney, M. G., Savage, J. M., Chalker, D., Honts, J., Welch, M. E., Wentland, A. L., Zweifel, E., and Beussman, D. J. (2008). A proteomics approach to cloning fenestrin from the nuclear exchange junction of *Tetrahymena*. *J. Eukaryot. Microbiol.* **55**, 245–256.

Cole, E. S., and Stuart, K. R. (2000). Nuclear and cortical histology for brightfield microscopy. *Methods Cell Biol.* **62**, 313–322.

Cole, E. S., Stuart, K. R., Marsh, T. C., Aufderheide, K., and Ringlien, W. (2002). Confocal fluorescence microscopy for *Tetrahymena thermophila*. *Methods Cell Biol.* **70**, 337–359.

Dave, D., Wloga, D., and Gaertig, J. (2009). Manipulating ciliary protein-encoding genes in *Tetrahymena thermophila*. *Methods Cell Biol.* **93**, 1–20.

Dentler, W. (2000). Fixation of *Tetrahymena* cells for electron microscopy. *Methods Cell Biol.* **62**, 323–331.

Gavin, R. H., Hoey, J. G., and Garces, J. A. (2000). Immunoelectron microscopy of *Tetrahymena*. *Methods Cell Biol.* **62**, 333–343.

Giddings Jr., T. H., Meehl, J. B., Pearson, C. G., and Winey, M. (2010). Electron tomography and immunolabeling of *Tetrahymena thermophila* basal bodies. *Methods Cell Biol.* **96**, 117–141.

Glkey, J. C., and Staehelin, L. A. (1986). Advances in ultrarapid freezing for the preservation of cellular ultrastructure. *J. Electron Microsc. Tech.* **3**, 177–210.

Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S., and Lukyanov, K. A. (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat. Biotechnol.* **24**, 461–465.

Jacobs, M. E., DeSouza, L. V., Samaranayake, H., Pearlman, R. E., Siu, K. W., and Klobutcher, L. A. (2006). The *Tetrahymena thermophila* phagosome proteome. *Eukaryot. Cell* **5**, 1990–2000.

Jamieson, J. D., and Palade, G. E. (1967a). Intracellular transport of secretory proteins in the pancreatic exocrine cell: I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* **34**, 577–596.

Jamieson, J. D., and Palade, G. E. (1967b). Intracellular transport of secretory proteins in the pancreatic exocrine cell: II. Transport to condensing vacuoles and zymogen granules. *J. Cell Biol.* **34**, 597–615.

Kataoka, K., Schoeberl, U. E., and Mochizuki, K. (2010). Modules for C-terminal epitope tagging of *Tetrahymena* genes. *J. Microbiol. Methods* **82**, 342–346.

Kilburn, C. L., Pearson, C. G., Romijn, E. P., Meehl, J. B., Giddings Jr., T. H., Culver, B. P., Yates 3rd, J. R., and Winey, M. (2007). New *Tetrahymena* basal body protein components identify basal body domain structure. *J. Cell Biol.* **178**, 905–912.

Larsen, J. (1989). The influence of growth phase and culture conditions of *Tetrahymena* on effects of cadmium. *Toxicology* **58**, 211–223.

Loidl, J., and Scherthan, H. (2004). Organization and pairing of meiotic chromosomes in the ciliate *Tetrahymena thermophila*. *J. Cell Sci.* **117**, 5791–5801.

Malone, C. D., Anderson, A. M., Motl, J. A., Rexer, C. H., and Chalker, D. L. (2005). Germ line transcripts are processed by a Dicer-like protein that is essential for developmentally programmed genome rearrangements of *Tetrahymena thermophila*. *Mol. Cell Biol.* **25**, 9151–9164.

Matsuda, A., Shieh, A. W., Chalker, D. L., and Forney, J. D. (2010). The conjugation-specific Die5 protein is required for development of the somatic nucleus in both *Paramecium* and *Tetrahymena*. *Eukaryot. Cell* **9**, 1087–1099.

McDonald, K. (1999). High-pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling. *Methods Mol. Biol.* **117**, 77–97.

McDonald, K. L., Morphew, M., Verkade, P., and Muller-Reichert, T. (2007). Recent advances in high-pressure freezing: equipment- and specimen-loading methods. *Methods Mol. Biol.* **369**, 143–173.

Meehl, J. B., Giddings Jr., T. H., and Winey, M. (2009). High pressure freezing, electron microscopy, and immuno-electron microscopy of *Tetrahymena thermophila* basal bodies. *Methods Mol. Biol.* **586**, 227–241.

Meselson, M., and Stahl, F. W. (1958). The replication of DNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **44**, 671–682.

Orias, E., Flacks, M., and Satir, B. H. (1983). Isolation and ultrastructural characterization of secretory mutants of *Tetrahymena thermophila*. *J. Cell Sci.* **64**, 49–67.

Pearson, C. G., Giddings Jr., T. H., and Winey, M. (2009a). Basal body components exhibit differential protein dynamics during nascent basal body assembly. *Mol. Biol. Cell* **20**, 904–914.

Pearson, C. G., Osborn, D. P., Giddings Jr., T. H., Beales, P. L., and Winey, M. (2009b). Basal body stability and ciliogenesis requires the conserved component Poc1. *J. Cell Biol.* **187**, 905–920.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24**, 218–229.

Salmon, E. D., Leslie, R. J., Saxton, W. M., Karow, M. L., and McIntosh, J. R. (1984a). Spindle microtubule dynamics in sea urchin embryos: analysis using a fluorescein-labeled tubulin and measurements of fluorescence redistribution after laser photobleaching. *J. Cell Biol.* **99**, 2165–2174.

Salmon, E. D., Saxton, W. M., Leslie, R. J., Karow, M. L., and McIntosh, J. R. (1984b). Diffusion coefficient of fluorescein-labeled tubulin in the cytoplasm of embryonic cells of a sea urchin: video image analysis of fluorescence redistribution after photobleaching. *J. Cell Biol.* **99**, 2157–2164.

Shang, Y., Song, X., Bowen, J., Corstanje, R., Gao, Y., Gaertig, J., and Gorovsky, M. A. (2002). A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3734–3739.

Sprague, B. L., and McNally, J. G. (2005). FRAP analysis of binding: proper and fitting. *Trends Cell Biol.* **15**, 84–91.

Stemm-Wolf, A. J., Morgan, G., Giddings Jr., T. H., White, E. A., Marchione, R., McDonald, H. B., and Winey, M. (2005). Basal body duplication and maintenance require one member of the *Tetrahymena thermophila* centrin gene family. *Mol. Biol. Cell* **16**, 3606–3619.

Stuart, K. R., and Cole, E. S. (2000). Nuclear and cytoskeletal fluorescence microscopy techniques. *Methods Cell Biol.* **62**, 291–311.

Tondravi, M. M., and Yao, M. C. (1986). Transformation of *Tetrahymena thermophila* by microinjection of ribosomal RNA genes. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4369–4373.

Ueno, H., Gonda, K., Takeda, T., and Numata, O. (2003). Identification of elongation factor-1alpha as a Ca^{2+}/calmodulin-binding protein in *Tetrahymena* cilia. *Cell Motil. Cytoskeleton* **55**, 51–60.

Walczak, C. E., Rizk, R. S., and Shaw, S. L. (2010). The use of fluorescence redistribution after photobleaching for analysis of cellular microtubule dynamics. *Methods Cell Biol.* **97**, 35–52.

Wiley, E. A., Ohba, R., Yao, M. C., and Allis, C. D. (2000). Developmentally regulated rpd3p homolog specific to the transcriptionally active macronucleus of vegetative *Tetrahymena thermophila*. *Mol. Cell Biol.* **20**, 8319–8328.

Wolfe, J., and Colby, R. H. (1981). A method for the immobilization of living *Tetrahymena*. *Exp. Cell Res.* **134**, 313–317.

Yao, M. C., and Yao, C. H. (1991). Transformation of *Tetrahymena* to cycloheximide resistance with a ribosomal protein gene through sequence replacement. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9493–9497.

Yao, M. C., Yao, C. H., Halasz, L. M., Fuller, P., Rexer, C. H., Wang, S. H., Jain, R., Coyne, R. S., and Chalker, D. L. (2007). Identification of novel chromatin-associated proteins involved in programmed genome rearrangements in *Tetrahymena*. *J. Cell Sci.* **120**, 1978–1989.