Chapter 1: Introduction

Minimal Cells

Cell biology, genomics, and proteomics have flooded us with a "parts list" for cells. Genetic, structural, and biochemical studies may tell us what each part does, but rarely do these parts act in isolation. Their distribution in a cell is often non-random. Many organize into large complexes or form into complex organelles. Fully understanding and modeling the final product, a living cell, requires knowledge of how all the parts fit together. While EM is not a new technique, recent advances have given new life to the field. Improved instrumentation, automation, and sample preservation allow for the three-dimensional (3D) reconstruction of whole cells trapped in frozen-hydrated life-like states.

Understanding multi-component systems is difficult and this drives the search for model systems where the complexity is reduced while maintaining the features of interest. In this light "minimal cells," with a shrunken cell size and genome, offer obvious appeal. The parasitic bacteria *Mycoplasma pneumoniae* has one of the smallest known genomes, but preserves features of importance, including cell polarity and motility. *Ostreococcus tauri* is the smallest free-living eukaryotic known, but still contains a single copy of each major organelle, including the chloroplast. These two minimal cells were used as models to explore prokaryotic and eukaryotic ultrastructure.

Electron Microscopy and Sample Preservation

An intuitive understanding of spatial systems is best developed visually. New means to visualize biology have lead to great advances in cell biology. Four hundred years ago Anton van Leeuwenhoek invented the light microscope, and so began cell biology. Over three hundred years later, in 1931, Max Knott and Ernst Ruska invented the electron microscope. By the 1940s, EM was being used to visualize cells (Porter and Yegian, 1945). Today most of our cell ultrastructural knowledge comes from EM.

The study of cell ultrastructure and macromolecular protein complexes is heavily indebted to transmission EM, one of the few techniques that can visualize details at the 3-7 nm range. Despite its crucial importance, electrons are in many ways non-ideal for imaging biological samples. Even modest doses rapidly destroy biological material. Using a limited dose results in loss of contrast between the biological molecules and the surrounding water. In addition, for adequate electron beam penetration of the sample, without excessive inelastic scattering of the electrons, samples must be kept as thin as possible.

To overcome the dose and contrast limitations, traditional EM does not directly image the biological sample, but relies on heavy metal staining to surround molecules of interest. Samples are chemically fixed and dehydrated, allowing for the water to be replaced with a plastic resin. Samples are then sectioned and stained with heavy metals (osmium, lead, and uranium). These preparation techniques lead to alterations of the native cell ultrastructure. Sample preservation can be improved by carrying them out at low temperatures, a technique known as high-pressure freezing and freeze substitution (McDonald and Auer, 2006). By rapidly freezing the sample and not warming the sample until after it has been fixed and

embedded in resin, the cell ultrastructure has less ability to change. This leads to substantially improved membrane and protein structure.

For the investigation of biological specimens, the electron dose is the main limiting factor. Improvements in imaging, computers, and robot technology in the last two decades resulted in electron microscopes that can be automated and controlled by computers. These systems help minimize unnecessary electron beam exposure to the sample. By practically eliminating the overhead dose the sample receives, the entire dose can be used for image capture. A second major discovery was that biological samples tolerate the radiation damage from the electron beam up to an order of magnitude better at liquid nitrogen temperatures compared to room temperature (Fujiyoshi, 1998; Glaeser, 1971). Thus significantly higher doses can be used. Imaging of frozen samples can now produce a high enough signal-to-noise ratio that useful data can be recorded from the native biological structure. Today, cells can be rapidly immobilized in a frozen-hydrated, life-like state with minimal preservation artifact. Automated, climate-controlled environments, such as the Vitrobot have greatly facilitated this process (Appendix A).

Sample thickness is a second critical problem for transmission EM. Each individual image is a 2-D projection of the sample making it impossible to determine its 3-D. Tomography solves this problem. Multiple 2D projections of the same sample are taken as the specimen is incrementally rotated. A computer then recombines the images using a weighted back projection algorithm to construct a 3D model of the sample (Lucic et al., 2005a; Radermacher et al., 1992). 200–700 nm thick samples are commonly used in tomography, but for samples greater than 500 nm the resolution begins to worsen rapidly. Sample thickness is still narrow, but the new limiting factor is the electrons penetrating the

sample. Most modern tomography EMs have 300 keV accelerating voltages. This voltage will accelerate the electrons up to approximately 80% the speed of light. At lower voltages, electrons cannot adequately penetrate the sample. Using higher voltages, electrons have limited interaction with the sample and detectors have difficulty localizing the electrons. New detectors are being developed to decrease noise and allow for thicker samples to be imaged (Milazzo et al., 2005; Yonekura et al., 2006).

Cellular electron cryotomography (ECT) has combined the advantages of tomography with frozen-hydrated samples to create 3D life-like snapshots of the cells. Perhaps the most profound proof of concept for the advantages of ECT compared to conventional EM has been its ability to prove the existence of prokaryotic cytoskeletons *in vivo* (Briegel et al., 2006; Henderson and Jensen, 2006; Komeili et al., 2006; Kurner et al., 2005; Scheffel et al., 2006), something previous EM techniques have not generally been able to do with confidence. These earlier limitations had lead to the mistaken belief that these filaments were absent in bacteria. ECT has also been successfully used to reveal startling new details of macromolecular machines, including two motors used for cell motility: the bacterial flagella motor (Murphy et al., 2006), and the motor complex of *M. pneumoniae*'s attachment organelle (Chapter 2) (Henderson and Jensen, 2006). ECT is more challenging in eukaryotes. Cell size, typically far greater than one micrometer, precludes most cells from being imaged whole. Thin cellular extensions such as the leading edge of a Dictyostelium cell (Medalia et al., 2002b), the eukaryotic flagella (Nicastro et al., 2006; Sui and Downing, 2006), and nerve processes (Garvalov et al., 2006) are thin enough to be imaged. Progress is still limited on sectioning frozen-hydrated samples for ECT (Hsieh et al., 2006). To date, there are no publications on whole cell tomography of frozen-hydrated eukaryotic cells

(Chapter 3). Most tomography in eukaryotes has focused on samples that have been high-pressure frozen, embedded, and sectioned.

Mycoplasmas and the Attachment Organelle

Despite decades of ultrastructural studies on bacteria, many fundamental questions remain unanswered: How do cells maintain their shape and polarity? What roles do the cytoskeletons play? How is the genome organized and segregated? And how do different types of bacteria move? With only 700 genes, *M. pneumoniae* has but one-sixth the genes of *Escherichia coli* and is nearly 25 times smaller in volume. Nonetheless, it can be used to try to answer each of the questions above.

M. pneumoniae is the leading cause of pneumonia in older children and young adults. Mycoplasmas are characteristic in their lack of a peptidoglycan cell wall; thus *M. pneumoniae* can be pleomorphic. When moving over a surface, it will orient itself along a line with a leading head, an ellipsoid body, and a long trailing tail. The cell's most remarkable structure is its leading head, the attachment organelle. The attachment organelle mediates adhesion of the cell to a surface, and has now been shown to also mediate motility (Hasselbring and Krause, 2007), something suspected but not proven at the time of my publication. Prior to the observations by ECT (Chapter 2) the attachment organelle was known to be a membrane-bound cell extension coated by surface adhesion proteins. Internally, there is a large protein core known as the electron-dense core. The core was identified by the first EM studies, but traditional EM could do no more than resolve the core as two rods that were assumed to be identical (Hegermann et al., 2002b). Models assumed

the core was homogenous, perhaps made of one or two proteins. The core's role was assumed to be to organize the surface proteins that would mediate cell adhesion and motility. Such models were reasonable because another well-studied mobile mycoplasma, *M. mobile*, appeared to rely on its surface proteins for adhesion and motility, though these proteins lacked homologs in *M. pneumoniae*. Further research has now shown that most of the proteins involved in motility in *M. pneumoniae* are also not found in *M. mobile* (Hasselbring and Krause, 2007).

The electron-dense core is part of what has been called *M. pneumoniae*'s "cytoskeleton." Washing eukaryotic cells in a non-ionic detergent, such as Triton X-100, leaves behind the cells' cytoskeletons. By the same criteria, *M. pneumoniae* has a cytoskeleton that roughly adopts the shape of the cell body. More specifically, 5-nm filaments have been demonstrated within the cell body, sometimes associated with the proximal end of the attachment organelle (Hegermann et al., 2002b; Meng and Pfister, 1980). These filaments were demonstrated long before the more recent evidence of filaments in many other bacteria. Importantly, traditional candidate genes for cytoskeletal proteins have not been found in *M. pneumoniae* (Himmelreich et al., 1996; Regula et al., 2001). Along with the cytoskeleton, the attachment organelle itself may play a role in maintaining cell shape. Knocking out the proteins that localize to the attachment organelle leads to abnormally shaped cells (Krause and Balish, 2004).

The role of the attachment organelle in cell division is a rapidly evolving story. By 2001 it was believed that the replication of the attachment organelle preceded cell division (Seto et al., 2001). This coordination occurs despite a lack of common transcriptional regulators in M. pneumoniae (Himmelreich et al., 1996). Based upon fluorescently labeled

attachment organelle proteins and cellular DNA from fixed cells, a model was proposed. New attachment organelles were thought to form near the original one. The newly formed attachment organelle then migrated to the opposite end of the cell as the genome replicated. Once the attachment organelle reached the other side of the cell, the cell divided (Seto et al., 2001). Many structural questions can be asked about these steps: (1) Did the attachment organelle replicate by a semi-conserved splitting of the electron-dense core, or was the attachment organelle synthesized *de novo*? (2) Was there a cytoskeletal path that the new attachment organelle migrated along to reach the opposite side of the cell? (3) How were these processes coordinated with genome replication and division? Was the genome somehow physically connected to the attachment organelle?

With my publication and other subsequent publications (a combination of ECT (Chapter 2) (Henderson and Jensen, 2006; Seybert et al., 2006) and time-lapse light microscopy (Hasselbring et al., 2006a)), an answer has started to evolve. (1) New evidence now suggests that the attachment organelle forms *de novo*. First, attachment organelles form *de novo* in knockout mutants that lack an attachment organelle but subsequently form them when the missing genes are reintroduced (Krause and Balish, 2004). Second, ECT shows the two halves of the electron-dense core are non-identical. Finally, observations of fluorescently labeled proteins being added to new attachment organelles suggest *de novo* synthesis (Hasselbring *et al*., 2006a). (2) Once two attachment organelles form, they separate. ECT fails to find any structural connection between adjacent attachment organelles. Time lapse microscopy shows the original attachment organelle begins to move, pulling the cell body away from the newly formed attachment organelle, which remains non-motile and attached to the surface during the process (Hasselbring et al., 2006a). (3) No

physical connection is seen by ECT between the attachment organelle and the genome. However, the genome is notoriously difficult to visualize by EM in bacteria. That said, light microscopic data shows that most cells actually produce multiple (> 2) attachment organelles before cytokinesis, which questions a direct link between attachment organelle replication and genome replication.

Many bacteria move, but the mechanism of *M. pneumoniae*'s motility appears to be exclusive to just a small number of closely related species (*M. pneumoniae*, *M. genitalium*, and *M. gallisepticum*) (Clyde and Hu, 1986). No homologs for other bacterial motility genes have been found in *M. pneumoniae* (Himmelreich *et al*., 1996). Therefore to identify the involved proteins, screens have been set up to find mutants with cytadherence defects, and, more recently, motility defects. At this point, the role of most of the discovered proteins remains unknown.

The work prior to my publication was based upon cytadhesion mutants. Two general defects were recognized: 1) loss of adhesion surface proteins, and 2) failure to localize the adhesion proteins to the attachment organelle. The two major surface adhesion proteins are P1 and P30. Antibodies to either will block cytadherence. Similarly, loss of either protein leads to non-adherent cells, but does not affect the localization of the other adhesion molecule. P1 is believed to localize over the entire attachment organelle, while P30 is believed to localize more to the distal end (Seto and Miyata, 2003).

Three other surface proteins are B, C, and P65. P1 co-localized with proteins B and C, which are cleaved from a common 130 kDa precursor protein that is cotranscribed with P1. In *M. genitalium* and *M. gallisepticum*, the homologous precursor protein is not cleaved.

There is evidence that this precursor protein shares low-level sequence similarity to P1 and may adopt a similar conformation (Papazisi et al., 2000). Loss of protein B and C results in a failure to localize P1 and loss of cytadherence (Baseman et al., 1982). P30 co-localized with P65 but the role of P65 is unknown (Jordan et al., 2001; Seto et al., 2001; Seto and Miyata, 2003).

Failure to localize the adhesion proteins often results from abnormalities in the electron-dense core. Cells lacking either of two large proteins, HMW1 or HMW2, do not construct an electron-dense core. A third protein characterized by core defects is HMW3. Earlier EM findings showed that cells lacking HMW3 occasionally have attachment organelles where the core looks split like a "V," with the vertex pointing away from the cell body. These earlier observations of the split core and the mistaken belief that the two halves of the core were identical lead to a hypothesis that HMW3 held the two halves of the core together and loss of HMW3 lead to splitting of the core. These observations also generated a hypothesis about how the attachment organelle could replicate by dividing its core into two equal parts. More recent observations with ECT suggest that in cells lacking HMW3 the two halves of a single core do not in fact split, but instead two cores occupy the same attachment organelle (personal observation).

Work published since my paper has brought forth a new set of at least 30 proteins that affect motility (Hasselbring et al., 2006b). Many of these have no known function. Some are involved in processes like metabolism and may have only secondary effects on motility. The most startling finding was for the protein P41. Cells lacking P41 still developed the attachment organelle and could move and adhere to surfaces. Amazingly, the attachment organelle would sometimes separate from the cell body and continue moving on its own.

This conclusively demonstrated that the attachment organelle contained the motor for cell motility (Hasselbring and Krause, 2007).

While ECT of wild type *M. pneumoniae* was an important and necessary first step, a much fuller picture of the attachment organelle's structure and function will be achieved by future ECT studies of the attachment organelle and the cytadherence and motility mutants. These studies promise to localize precisely some of the proteins of the attachment organelle and to facilitate the understanding of their functions in this unique form of motility.

Eukaryote Ultrastructure

ECT has helped to show that bacteria are not "bags" of free-floating enzymes; even the simplest cells such as *M. pneumoniae* use multiple means to organize themselves. This truth is all the more evident in eukaryotic cells, where people have long appreciated the fact that the cells contain multiple organelles and a complex cytoskeleton. Knowing this is different from understanding its implications. Current techniques have seriously hampered our ability to effectively map eukaryote ultrastructure in whole cells. Classical light microscopy, for all its advantages of being able to image live cells in 3-D, is limited to ca. 200 nm resolution and 40 nm resolution for fluorescently labeled molecules (Willig et al., 2006). Shorter wavelength X-ray tomography has the promise of sub-15 nm resolution (Chao et al., 2005) but published results are of 40 nm or higher (Larabell and Le Gros, 2004; Le Gros et al., 2005). Traditional thin-sectioned EM has sufficient resolution but effectively gives only two-dimensional information. Electron tomography alone combines high resolution with 3D data. Unfortunately, it is restricted to samples less than 1000 nm thick.

To overcome this disadvantage, either thin samples must be used or thicker samples must be sectioned.

Serial sections must be done on fixed samples using current techniques. Fixed EM samples, though, have the serious disadvantage in that they undergo major shrinkage when they are under the electron beam. Samples will typically shrink 40 to 50% in the direction of the beam and 5 to 10% in the perpendicular direction (Lucic et al., 2005a); it is not clear if this shrinkage occurs uniformly or not. ECT using frozen-hydrated samples avoids this shrinkage as well as staining artifacts. But again it is severely restricted by sample thickness; serial sectioning for frozen-hydrated samples remains unavailable. Because *O. tauri* is so small, it is the only eukaryotic cell known that can be imaged whole by ECT throughout its entire life cycle. *O. tauri* is thus a powerful model for the study of whole eukaryote cell ultrastructure.

Other examples of whole cell tomography are very limited. X-ray tomography has been used to generate 60 nm resolution tomograms of frozen-hydrated *Saccharomyces cerevisiae* (Larabell and Le Gros, 2004). The cells were 5 μm in diameter, far thicker than can be imaged by electron tomography. In addition, multiple cells could be imaged rapidly to provide better statistics. However at 60 nm resolution, structures like the endoplasmic reticulum and the mitochondria could not be differentiated. Higher resolution EM tomograms of whole cells using serial sections is a feat that has rarely been done. In March of this year, a paper appeared on serially sectioned embedded fission yeast, *Schizosaccharomyces pombe* (Hoog et al., 2007). Though the paper focused on interphase microtubules, they reported that one of the cells had been completely reconstructed by combining multiple 250 nm sections. Gathering data from serial sections is time intensive.

Having only one or two reconstructions of cells significantly limits our knowledge of whole cell ultrastructure. Not only do individual cells vary, but also the ultrastructure varies in response to the environment (Mannella, 2006a, b) and cell cycle (discussed in Chapter 3).

Generally, tomography of eukaryotes has been limited to individual organelles or sections through cells. These works have provided important functional insight into organelles and sub-organellar structure such as the mitochondrion (Mannella, 2006a; Nicastro et al., 2000), chloroplast (Shimoni et al., 2005), nucleus-embedded nuclear pore complex (Beck et al., 2004), and the cytoskeleton (Hoog et al., 2007; Medalia et al., 2002a). In contrast, organelles such as the endoplasmic reticulum and Golgi body (Ladinsky et al., 1999; Storrie and Nilsson, 2002) have only been studied piecemeal by EM because they cannot be readily purified and are extremely large.

For a detailed look at the role of EM in the study of sub-cellular structure, the example of the mitochondria is illustrative. The earliest EM images of mitochondria were thin-sectioned. These erroneously gave rise to a "baffle model" in which the inner membrane of the mitochondria was assumed to fold inward like the baffles of an accordion into wide-open compartments. As technology improved, electron tomography was used on thick sections cut from high-pressure frozen and embedded sections. These showed the cristae to be connected to the inner membrane through narrow junctions, not wide-open enfoldings. The cristae shapes were pleomorphic, varying from tubular to lamellar depending on the environment the mitochondria were in (Frey and Mannella, 2000). This led to new models of the cristae as being local environments connected to the inter-membrane space through junctions that limited diffusion. Still, the use of chemical fixation resulted in the inner and outer membrane looking rough and uneven. It was not until ECT was used on

isolated mitochondria that the inner and outer membranes were shown to be smooth and parallel. Additional features were found including wider crista junctions and new interconnections between the cristae (Nicastro et al., 2000). Since effects like widening of the crista junctions are thought to occur in apoptosis (Scorrano et al., 2002), it remains unclear whether the ECT observations were physiological or an artifact of isolating the mitochondria from the cells.

An *in vivo* model of frozen-hydrated mitochondria would eliminate any artifacts from organelle isolation. It would also open the door to ask new questions through tomography. One could probe whether the mitochondria had any set positional relations to other organelles and what were the underlying structural bases for these relations. One could also observe how these relations changed during the cell cycle. The cell cycle is often discussed in terms of the changes the nucleus and the genome undergo, but every organelle must replicate and partition itself during cell division. This process cannot easily be observed at the ultrastructural level with current techniques.

Electron tomography of eukaryotes is a challenging field, but modeling cellular physiology and pathophysiology relies on ultrastructural details. Improvements in techniques will drive this area. Cryosectioning (Hsieh et al., 2006) and focused ion beam milling (Marko et al., 2006) may one day make routine the imaging of frozen-hydrated sections of cells and tissues. *O. tauri* will continue to play a key role though because of its simple cellular organization and its ability to be imaged whole. *O. tauri* is becoming a well-developed system for the analysis of the cell cycle and will hopefully be amenable for future genetic manipulation. These two properties of *O. tauri* will enable systematic high-

resolution ultrastructural analyses of a *bona fide* eukaryote in fundamentally important cellular states.

Personal Contributions

I joined Dr. Jensen's laboratory at its very beginning and began working on mycoplasmas and *O. tauri*. Chapter 2 summarizes my work with ECT on *M. pneumoniae*. I was the sole student responsible for this work. The project evolved out of an effort to characterize *M. genitalium*, at the time the smallest and simplest known cell. I decided to focus on the attachment organelle and proposed to switch to *M. pneumoniae* where more work had been done. I arranged to attend a conference in Athens, Georgia, where I was able to establish collaborations with Dr. Krause and arranged to obtain samples of *M. pneumoniae*. Since then I have secured other *M. pneumoniae* mutants to work with. I learned how to culture them and prepare samples. I set up the necessary bio-safety level two precautions with the help of Dr. Dias. I prepared all samples. Data was taken by either myself or with the assistance of Dr. Tivol. I did all the reconstruction and segmentation. I drafted the paper and all figures. Dr. Jensen revised the paper. I submitted the paper and helped craft replies to the reviewers' comments. I also created the cover figure for that issue of *Molecular Microbiology*. I have traveled to three meetings to present this work, including giving an oral presentation at the 16th International Congress of the International Organization for Mycoplasmology in England. Under Dr. Jensen's supervision, I peer-reviewed a paper from another laboratory on *M. pneumoniae*'s attachment organelle and reviewed a grant proposal on mycoplasmas for the NIH.

Chapter 3 summarizes my work on whole cell tomography of *O. tauri* throughout its cell cycle. I began working on this project at the onset of joining the lab. I learned how to optimally grow and work with the cells and visited the laboratories of Dr. Moreau (France) and Dr. Palenik (San Diego) to work with *O. tauri* and *O. lucimarinus* respectively. I worked with flow cytometry to try to select the smallest cells possible. I learned conventional EM fixation and sectioning at the Caltech transmission electron microscopy facility and I traveled to Berkeley with Dr. Jensen to perform high-pressure freezing with Dr. McDonald. Undergraduate Bingni Wen worked under my guidance in a project to attempt to isolate organelles from *O. tauri*. Most of the ECT data was collected one year ago, at which time I began working with a new post-doc, Dr. Lu Gan. Dr. Gan helped culture the cells and imaged the cells by light microscopy. I prepared most of the ECT samples and collected most of the data on the EM. Dr. Gan helped with the reconstruction of the data. I prepared data for Dr. Jane Ding to run the necessary programs on the Caltech supercomputer to search *O. tauri* for ribosomes by template matching. I wrote the majority of the paper, though Dr. Gan wrote the section on the nuclear pore complex and a portion of the section on the chloroplast. He created figures two and seven while I prepared the other figures and the movie. Dr. Gan also revised the paper multiple times. The final paper was revised by Dr. Jensen, and will be submitted to *PLoS Biology*.

Appendix A is a protocol paper written by Dr. Iancu. I along with many other laboratory members contributed our experiences in using the Vitrobot (FEI) for frozen-hydrated sample preparation. I also helped revise the paper.

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