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Development and application of cryo-EM tools to study the ultrastructure of microbes in changing environments

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CHAPTER 1

General Introduction

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Bacteria are ubiquitous in nature and certain species have adapted to thrive in even the most extreme environments. Moreover, cells from individual species can adapt to changing conditions, such as in soil, in fresh, brackish, or salt water as well as inside a host organism. Yet, much is still unknown about how bacteria can adapt to these changing conditions and thrive as individual cells, as part of microbial communities, or in close interaction with cells of a eukaryotic host.

The structural makeup of bacterial cells contributes to their ability to navigate and persist in environmental niches: they can produce a variety of molecular machines which enable the cells to sense and interact with their environment. They can even adjust their overall cell morphology. Gaining insight into bacterial ultrastructure in different growth conditions is essential to understand how bacteria adjust and change in complex environments. This is especially relevant for pathogens that cause hard-to-treat infections. Detailed insight into their specific morphological and structural characteristics during infection may open new treatment avenues.

However, the tools required to examine the ultrastructure that enables environmental interactions have only recently become available to the larger scientific community. The aim of this thesis is to develop and use tools and workflows for electron microscopy (EM) to better understand these interactions.

Cryogenic EM (cryo-EM) has become an especially powerful technique to peer inside of cells and to study specific protein complexes. Two types of cryo-EM are primarily used for these types of studies. To study whole cells and their associated molecular machines *in vivo*, cryo-electron tomography (cryo-ET) is used (outlined in Fig. 1). With this technique, cells are flash-frozen on an EM support grid using a cryogen cooled to liquid nitrogen temperatures (-194˚C). These samples are preserved in a near-native state because the water molecules do not have time to crystalize, instead forming an amorphous state that does not impact the sample (1). The frozen samples are then transferred to an electron microscope, where a series of images are collected while the sample is tilted. The resulting images can then be computational combined to reconstruct a three-dimensional (3D) volume of the sample. These data can then be used to analyze structures of interest and can be further processed to obtain high resolution information about the structures at the macromolecular scale (2-4 nm, Fig. 2).

Figure 1, Overview of cryo-ET sample preparation and imaging workflow. Samples with a thickness of less than 5-10µm are typically frozen by plunge freezing and then directly imaged using cryo-ET. However, if the area of interest falls within the thicker region, then thinning of the sample may be required. New freezing techniques allow for freezing directly on the cryo-stage (microfluidic cryo-fixation), though this technique is not assessed in this work. For samples greater than 10µm, high pressure freezing is the typical process for vitrification. Once frozen, the sample requires thinning using focused ion beam (FIB) milling, FIB lift-out, and or CEMOVIS. Sample thicknesses of approximately 200nm provide a good balance between sample stability, image quality and sampling of the cell. The thinned sample is transferred to the cryo-EM for imaging and further analysis. Adapted from (2).

Figure 2. Examples of structures visible by cryo-ET. A. A single slice of a tomogram showing a whole *Vibrio cholerae* cell grown in LB overnight at 30˚C. Different structural aspects denoted include the inner and outer membrane, (IM, OM), the ribosomes (selected examples are circled with white), areas of ribosome exclusion where the DNA is located, and two types of chemotaxis arrays (F6 & F7). B-D. Other structures that are identifiable using cryo-ET include the MSHA pili and its basal body in B, storage granules (SG) in C, and the flagella apparatus (flagellar motor (FM), the flagellum (FL), and the flagellar sheath (FS) in D. Scale bar = 200nm in A, and 50nm in B-D.

Alternatively, cryo-EM can be used to study individual proteins and protein complexes in solution, which typically allows for higher resolution information. As with cryo-ET, the sample is applied to an EM support grid and plunge frozen to embed the sample in an amorphous buffer solution. Using the electron microscope, individual images are taken from numerous positions on the EM grid. Each image contains a large number of individual particles, each randomly orientated with respect to the electron beam. These particles are then selected from each image, and computationally combined to create a 3D structure. With this technique, resolutions similar to crystallography can be obtained (sub nm, in ideal cases 2 Å or better have been achieved) which can provide significant insight into the structure of interest.

Together, these techniques provide a tremendous opportunity to better understand the bacteria at the nanoscale. However, there are still some challenges associated with these techniques. Sample thickness is especially challenging due to the limited ability of the electrons to penetrate the sample of interest. This becomes most clear when using cryo-ET to image intact bacterial cells. For instance, *Vibrio cholerae*, a common bacterial species used for studying a diverse set of molecular machines, can exceed 0.5 µm in diameter. This thickness is approaching the penetration limit of the electron beam when operating at 300 keV, which is a typical voltage for cryo-EM studies (3). In addition, we must consider that as the sample is tilted, the thickness further increases, meaning the less information that can be recovered. Thus, other techniques are necessary to prepare EM grids from samples of greater thicknesses.

Sample thickness is not just a challenge during cryo-EM imaging. Only relatively thin samples can be frozen by directly plunging them in a cryogen (ethane or ethane-propane mixture)(1, 4). Using thicker samples, such as larger cells, biofilms, or tissues that exceed 5 µm, the freezing rate during plunge-freezing is not fast enough to prevent ice crystal formation. Therefore, other methods must be used to vitrify thick samples. The most common technique for freezing volumes up to 250 µm is high pressure freezing. This technique combines the application of high pressure with rapid cooling to freeze the sample in amorphous water, buffer or growth media (5). Samples prepared by high-pressure freezing need additional thinning steps in order for the sample to be imaged with cryo-EM.

Samples less than 20 µm can be thinned using an ion beam, a technique referred to as cryogenic focused ion beam scanning electron microscopy (cryo-FIB SEM). The ion beam is used to ablate the material in a targeted way. The SEM beam is used to monitor the milling progress, ultimately producing so-called lamella with a thickness of approximately 200 nm (which is considered a good balance between thickness and the ability for electrons to pass through the sample).

Samples with a greater thickness must be thinned by other methods prior to imaging, either by cryogenic ultramicrotomy or 'cryogenic lift out'. This is due to the limited depth of focus for the ion beam. In cryo-ultramicrotomy, the sample is thinned at cryogenic temperatures using a diamond knife, which removes 75-100 nm sections with each stroke (6, 7). These sections can then be transferred to an EM support grid for imaging. For the cryo-lift out method, a specialized cryo-FIB SEM is used. In this case, the focused ion beam is used to create trenches on either side of the area of interest and then the remaining tissue is lifted out with a mechanical arm, transferred to a specialty grid, and then milled to create lamella

for imaging (8, 9). However, both techniques are challenging and time consuming, and require someone specifically trained in the techniques.

An alternative method for preparing large volume samples lies in classical electron microscopy. Serial block face scanning electron microscopy (SBF SEM) continues to improve, allowing the automated trimming and image collection of larger volumes of samples (10). With this technique, the sample is prepared using chemical fixatives rather than physical fixation (such as vitrification), followed by subsequent steps to exchange the water molecules with non-ionic solvents and finally embedded in resin. The sample is roughly trimmed, and then inserted into a SEM that is equipped to trim the sample during imaging (Fig. 3). Imaging using this instrument allows the visualization of large volumes (in our case, up to 150 µm) because of its ability to repeat the process of section removal, imaging, removal, imaging, etc. unsupervised. The individual images of the sections can then be combined to reconstruct the volume in 3D. An alternative of this technique combines high pressure freezing with chemical fixation, which has been shown to greatly improve sample preservation (11). However resolution is still limited to larger features such as cell shape, storage granules, and flagella. Smaller structures below this resolution (i.e. < 2 nm) require methods that preserve samples in near native states, as demonstrated by cryo-ET.

Figure 3. Overview of serial block face scanning electron microscopy. The sample is fixed, stained, and embedded in resin. Once polymerized, the block containing sample is roughly trimmed and then inserted into the SBF SEM instrument. During data collection, the sample is trimmed using a diamond knife with a determined thickness, followed by imaging using the SEM beam. This process is repeated through the volume, and the resulting images are then computationally combined into a 3D volume (Image alignment and computational reconstruction). Areas of interest can then be segmented for further analysis (3D volume analysis and segmentation). Adapted from (10).

Together, these techniques allow an unprecedented view into the microbial world and provide opportunities to examine the multicellular environment. Cryo-ET methods also allow resolutions that approach the atomic scales, providing key insights into the structural biology of the molecular machines that are important to a bacterium's ability to adjust to changing environments. The goal of this thesis is to employ these techniques to better understand how bacteria interact with their environment, and to develop and employ the tools necessary to engage these techniques in answering this question.

Thesis Outline

In this thesis, I describe the use of the various cryo-EM techniques to gain insight into the structural changes of the human pathogen, *Vibrio cholerae*, and its transitions between different environments. A combination of techniques is used that describe how to prepare the individual cells for cryo-ET using a locally designed manual plunging apparatus and how to visualize changes to the cells' morphology and structure when transitioning from the environment and back into the host. I also show how environmental conditions, such as exposure to ultraviolet-C radiation, affects the ultrastructure of *V. cholerae* and the associated ICP1 bacteriophage. Together with biochemical techniques and microbiology assays, cryo-EM proves to be a powerful technique for elucidating how bacteria adapt to changing environments.

In **chapter two**, I begin with an overview of bacterial and archaeal ultrastructure, highlighting molecular machines that have been investigated by methods including classical and cryo-EM. Many of these machines have been shown to be important for interacting with the microbe's environment, and a significant amount of the current knowledge that is known is because of methods in cryo-EM. For instance, I describe the F6 chemotaxis array, which is responsible for sensing chemical signals in the environment, and the flagellar apparatus, which is the then used to moves toward or away depending on that signal. This chapter lays the foundation for several of the following chapters.

Chapter three describes the development of a portable manual plunge freezing device. The inspiration for this device came when the sample preparation for a collaborative project could not be carried out within our own group, and the collaborating lab did not have a cryo-EM setup. The portable manual plunge freezer developed here is an inexpensive alternative to commercially available options and allows the device to be easily moved from lab to lab, locally, nationally, or internationally. Subsequently, I used this device for the analysis of the

ultrastructure of *V. cholerae* that had been excreted by the natural host, the zebrafish (*Danio rerio)*. In this case, I was able to travel to the lab with the expertise in the infection model and use the device to prepare the cells.

Using the manual freezing device, in **chapter four** I examine *V. cholerae* for changes in its morphology and the molecular machines as this bacterium transitions from the environment, into a zebrafish host, and then back into the environment. This important piece of work demonstrates the journey through a natural host's digestive track results in a more resilient bacterium that is better able to adjust to environmental changes. This resilience likely primes the bacterium to move from host to host more efficiently, and analysis of the molecular machines showed that cells that maintained their vibrioid or near-viroid shape also retained the molecular machines that are important to sensing and attachment during colonization. To gain these insights, I used cryo-EM, confocal imaging, SBF-SEM, and microbiological techniques to characterize the bacterium at different stages in the infection cycle.

Working with a pathogenic species requires the adherence to strict biosafety guidelines to ensure the safety of staff, researchers, and the surrounding environment. In **chapter five**, in another effort to increase access to cryo-EM, I focused on the effect of ultraviolet C (UVC) irradiation on sample integrity and the ability to access high resolution structural information after irradiation. In this study, I targeted two types of samples, the bacterium *V. cholerae* for subtomogram averaging, and the ICP1 bacteriophage for single particle analysis. The importance of this study became even more apparent with the outbreak of the COVID-19 pandemic as UVC irradiation is a well-established protocol for disinfection. Exposure to UVC causes changes to the DNA which in turn prevents replication of the bacterium or virus (12). Our study demonstrated that UVC exposure at cryogenic temperatures is a suitable method for inactivation of pathogens. Furthermore, the structural information gained from the inactivated pathogen is indistinguishable to untreated organisms. For the bacterial sample, I achieved a resolution of 22.6Å after inactivation with 30 seconds of UVC using our home-built device. For the bacterial virus sample, I achieved a resolution of 5.1Å for the capsid after 60s UVC treatment on the carbon and 30s of treatment on the copper side of the support grid. In addition to the economical device used for UVC treatment, this chapter also demonstrates the possibility of using UVC irradiation as a way to lower the biosafety level of pathogens which can then be imaged in most cryo-EM facilities, even those lacking biosafety certifications.

In **chapter six**, I describe the current state of large volume sample preparation using microfabricated biopsy needles. The challenges associated with these sample types

and the promising workflows that I am developing in collaboration with members of the group and our local fine mechanical department are given. I first tested glass needles for the biopsy of various tissue types. I then utilize 3D printed needles to retrieve samples from a bacterial colony of *Streptomyces coelicolor*, which were subsequently frozen by high pressure freezing in preparation for sample thinning by cryo-ultramicrotomy and cryo-FIB SEM. In addition, we began the steps of optimal sample preparation by freezing of sample directly on EM support grids. Together, I demonstrate that our biopsy needles are suitable for the cryo-EM workflow.

Overall, as summarized and discussed in **chapter seven**, this thesis provides unique insight into the infection life cycle of *V. cholerae* while also developing devices that invite more users into the field of cryo-EM.