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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 7

General Discussion & Summary

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To understand the biological world around us, we need to investigate it across multiple scales. While some of it can be directly observed with our own eyes, such as large organisms interacting with their environments, most of the fundamental processes enabling life occur inside individual cells and tissues and thus are hidden from plain sight. To gain insight into how cells work at the (macro)molecular scale, we rely on specialized tools including model systems and a wide range of light and electron microscopes.

Laboratory animal models, such as the *z*ebrafish (*Danio rerio*) system, have allowed us to gain a better understanding of a variety of fundamental processes. This has been possible due to the ability to manipulate the organism genetically and to directly observe the resulting effects on the larvae using fluorescence microscopy (140). However, many cellular processes and interactions between cells and invading pathogens occur on an even smaller scale and cannot be understood by using light microscopy techniques alone.

To better understand cells and how they interact with their environment at the micro- and nanoscale level, we must zoom in using other, more powerful microscopy methods such as cryogenic electron microscopy (cryo-EM)(75). For questions at the cellular level and involving heterogeneous samples, cryogenic electron tomography (cryo-ET) has become a tool of choice because it can peer directly into samples at near-native preservation. For example, we can examine whole bacteria (***C*hapter 4**) and analyze different macromolecular machines such as their chemotaxis arrays, flagella, or pili (**Chapters 2, 4, 5**). For looking at isolated protein complexes in purified, homogenous samples, we can apply another cryo-EM method called single particle analysis (SPA). This technique enables us to visualize individual proteins or protein structures with nanometer resolution. In this thesis, I used this method to examine a bacterial virus, the ICP1 bacteriophage, and a human protein, apoferritin (**Chapters 3, 5**).

The investigation of cells, viruses, or protein complexes with cryo-EM requires specialized sample preparation techniques. For samples less than 5-10 µm in thickness, we can use plunge freezing to embed the sample in vitreous ice (**Chapters 3, 4, 5**). Samples with greater thicknesses, such as larger cells, tissues or microbial biofilms, require high pressure freezing to avoid artefacts caused by ice crystal formation and subsequent sample thinning (**Chapter 6**)(5, 129). In this thesis, I developed new tools and workflows based on these methods to gain new

insight into the ultrastructure of the pathogen *Vibrio cholerae* and how it adapts to changing environments during the infection of a natural host, the zebrafish.

In the past two decades we have gained much insight into the ultrastructure of microbes. Much of these insights into bacterial ultrastructure have been gained using cryo-ET (75, 141). In **chapter 2**, I review our current knowledge on the cellular ultrastructure and how specific macromolecular machines enable the cells to interact productively with their environments. First, I outline how the different structures of the cell envelope act as boundaries between the cell's inner contents and its external environment. I then link these to cell shape and discuss the process of cell division. Finally, I discuss structures that directly interact with the external environment, such as the various appendages and secretion systems.

Advances in cryo-EM specimen preparation

While we have gained a lot of insight into structure and function of bacterial cells in laboratory monocultures, less is known about bacterial ultrastructure in natural environments, such as when bacteria are interacting with a host during infection. However, this poses a major practical challenge: the biological experiments for host infection must be performed in specialized laboratories that often lack the necessary equipment for cryo-EM specimen preparation. Commercially available machines are cost prohibitive for a non-EM focused lab and require dedicated space and expertise for their operation. Therefore, I created a device that could simply and affordably prepare samples for cryo-EM that could be easily used in any laboratory. I designed the device to be suitable for travel (lightweight and compact design for easy to transport), as well as capable of producing high quality samples for both cryo-ET and SPA. Only some generally available accessory components need to be acquired onsite, such as liquid nitrogen and a cryogen for the vitrification (generally pure ethane or ethane propane mixture). After testing and optimizing the device on a variety of samples in our own laboratory, I used it to prepare samples in our collaborators' laboratory at Wayne State University (Detroit, MI USA). This device was pivotal in collecting data that contributed to **chapter 4**. In addition to my own experiments that relied on the device, it will be directly applicable to labs that have very specialized animal housing or other set-ups for generating biological samples. For example, the device would be useful to study bacteria that rely on specialized anaerobic environments or pathogenic organisms requiring stringent safety measures.

Vibrio cholerae interaction with a natural host

In my thesis, I focused on the ultrastructural changes of the bacterium *Vibrio cholerae*, which must rapidly adjust to changing environments during its infection

cycle. The pandemic-related strains are the cause of cholera, a severe diarrheal disease that affects humans and animal hosts (51, 52, 57, 66, 142, 143). The bacterium primarily lives in environmental water bodies including fresh-, brackish- and salt-water, where it exists as free floating bacteria, or in multicellular biofilms attached various surfaces such as copepods (57). When a human ingests contaminated water, the bacterium quickly transitions through the hostile environment of the stomach to the small intestine where colonization occurs (Fig. 1.2; Peterson & Gellings, 2018; Singh & Barnard, 2016). Within hours of ingestion, dissemination back into the environment occurs in the form of diarrhea, which typically restarts the infection cycle (58).

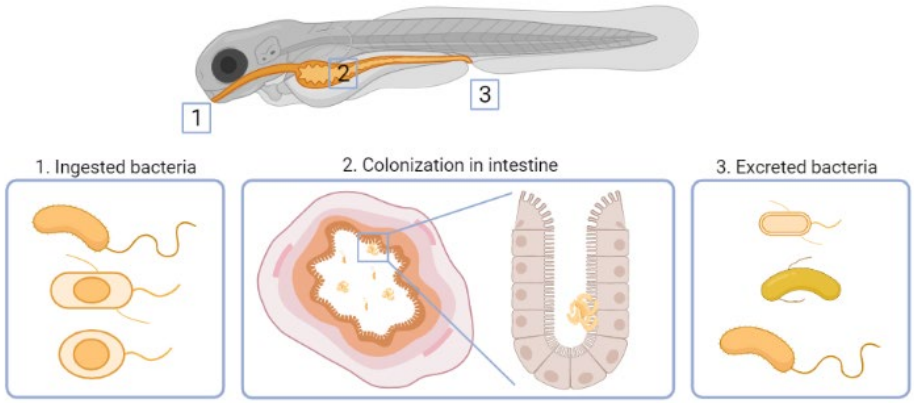

Figure 1. Overview of *V. cholerae* infection cycle in the zebrafish larvae characterized by cryo-EM and SBF SEM (Chapter 4). Bacteria in the environment (1) are ingested naturally by the zebrafish larvae leading to the colonization of the intestine (2) and eventual dissemination back into the environment (3). Created in Biorender.com.

Here, I wanted to gain a better understanding of how the bacterial ultrastructure changes during the infection cycle. To do this, I used a recently described natural host of *V. cholerae*, the laboratory model organism of the zebrafish (66). Our study in **chapter four** first describes the morphology and molecular machines of cells that are transitioned from the laboratory environment of a nutrient rich culture media (LB) into a controlled nutrient poor freshwater environment (AFW). I paid specific attention to cell shape, and the molecular machines that had previously been shown to be involved in colonization: the F6 chemotaxis array, the flagella, and the pili (53, 57, 58, 63). I found that while there are significant changes in cell shape, including a significant increase in the fraction of cells with a large periplasmic space (dehiscence), the expression of the molecular machines changes only slightly (Fig. 1.1). Next, I was interested in understanding the colonization dynamics within the

zebrafish intestine. I infected zebrafish larvae with *V. cholerae*, and then used serial block face scanning electron microscopy (SBF SEM) to characterize sites of colonization and the cell shape. I found that the bacterium typically exists in small microcolonies that can be found free floating in the lumen and associated with the microvilli (Fig. 1.2). Segmentation of individual cells revealed a typical vibrioid morphology, as expected, and measurements of cell lengths were comparable to cells found in a nutrient rich environment. Lastly, I was interested in the cells that were excreted back into the environment from *V. cholerae*-infected zebrafish, both from the adult fish as well as the larvae. To do this, I travelled to Wayne State University with the manual plunger device produced in **chapter three**, and working with the local experts, infected adult fish and larvae with *V. cholerae* and collected the bacteria that were excreted over a 24 h period. Using cryo-ET, I examined the same features as the cells in LB and AFW and found significant differences when comparing their morphology. The cell shape was either typical vibrioid or ovoid in structure, and much less occurrences of dehiscence were seen in these cells (Fig. 1.3). From this research, I conclude that the transition through the zebrafish gut primes the bacteria for release back into a nutrient poor environment, likely aiding the bacteria in colonizing another host and continuing its infection cycle. In addition, I also noted that storage granules were present in the cells that colonized the zebrafish larvae intestine and the excreted bacteria, which has also been shown to support a successful transition from the host back into the environment (82). Lastly, these finding support the use of the zebrafish model for understanding colonization and dissemination factors.

Taken together, this piece of work is the first time the detailed, subcellular, ultrastructural changes of a pathogen have been investigated throughout the entire infection cycle. It provides further insight into why cells found in stool samples or cells in biofilms as used in other studies, are hyperinfectious, thus requiring a less infectious dose than cells that might be found in freshwater alone (80, 81).

Ultraviolet light inactivation of pathogens for cryo-EM

Working with a pathogen such as *V. cholerae* (biosafety level II, BSL-II) can be quite challenging as it requires strict adherence to biosafety procedures and containment measures. At the onset of this thesis, the local microscopy cryo-EM center, the Netherlands Centre for Electron Nanoscopy (NeCEN), was not certified to image BSL-II pathogens. While the BSL-II certification was obtained for NeCEN in the course of this thesis, it demonstrated that access to cryo-EM can be limited for BSL-II or higher samples. Therefore, I was interested in developing methods for reducing the activity of a microorganism to facilitate easy access to cryo-EM facilities that are not certified for higher biosafety level samples. In this case, I used a well-known

disinfectant, ultraviolet-C (UVC, wavelengths 185-280nm) irradiation, to inactivate cryo-EM samples of two types of microorganisms, *V. cholerae* and its associated bacteriophage, ICP1 (a bacterial virus). While much is known about how UVC affects the activity of microorganisms at ambient temperatures, what the impact of UVC light on ultrastructure and viability at cryogenic temperatures was still unknown (12, 96, 122, 145). Here, I took a step-by-step approach by using cryo-ET and SPA methods to determine both viability after UVC treatment and to investigate if resolution was adversely affected using the bacterial and viral samples (**Chapter 5**). A vitrified grid containing *V. cholerae* was inactivated completely (as quantified by colony forming units) in just 30 s of UVC irradiation. Importantly, there was no visible damage to the cells, and subtomogram averaging of its F6 chemotaxis array achieved resolutions ranging from 22-26 Å, which is comparable to data collected from cells that were not UVC irradiated. For the ICP1 bacteriophage, treatment of the vitrified sample with UVC for 60 s on the front of the grid and 30 s on the back resulted in a 99.9999% reduction in phage activity. Using SPA, I achieved cryo-EM structures of 5.1 Å and 6 Å for the UVC-treated and untreated DNA-containing capsid, respectively. As with the bacterial sample, visual inspection of the phage did not show any obvious damage, and the achieved resolution was well within expectations for the number or phage particles used in the analysis. The resolution was actually slightly higher for the UVC treated sample. This can be partially explained by having more data for the averaging and is consistent with the UVC treatment not causing any detectable damage despite rendering the samples harmless.

Because we found no obvious damage of the structures at resolutions up to 5 Å, I finally aimed to determine the structural effects of the UVC treatment using apoferritin (ApoF), a sample that is often used for high-resolution structural testing. I exposed the purified ApoF to UVC using the same conditions as the ICP1 phage sample and used SPA to determine the resolution. Similar to other published apoferritin structures, I achieved a resolution of 2.1 Å. A detailed comparison with other untreated structures did not show any noticeable changes. Ultimately this study demonstrated that UVC is a viable method for inactivating potentially dangerous cryogenic samples, and that there is no detectable impact on the structural information of proteins and protein complexes that can be achieved from this method up to resolutions of 2 Å. This is an important contribution to the field because it expands the toolbox for sample preparation and could increase access to the microscope infrastructure by reducing the pathogen's safety level to one that is acceptable by the facility.

However, questions remain about what the actual impact of the UVC is having on the sample, and additional experiments would need to be done to show how UVC is inactivating the vitrified samples. At room temperature, UVC is known to damage DNA by causing the formation pyrimidine dimers, but the extent of this damage is not known at cryogenic temperatures (12, 96). Presumably the damage caused by UVC irradiation would be randomly dispersed within the sample. However, this randomness would pose a challenge with the averaging required to improve the signal-to-noise ratio in cryo-EM, as any small defects are averaged out, making them effectively invisible within the dataset. While this method is useful for reducing the biosafety level without negatively impacting the final result, characterizing the precise damage would be a challenge.

Pushing the boundaries of sample thickness for cryo-EM

As noted in **chapter four**, I was interested in understanding the colonization dynamics of the *V. cholerae* within the zebrafish host. To do this, we used SBF SEM, which, while capable of providing important information about colonization, does not achieve the resolution required to see most of the molecular machines that are involved in colonization. In contrast, cryo-ET is capable of seeing these complexes at a near-native state. However, this method is typically applied to individual or small groups of cells because the technology is currently limited by sample thickness. For cryo-ET, ideal sample thicknesses are approximately 200 nm, though depending on the wavelength of the electron beam, can be greater than 400 nm (3). Ultimately, what this means is that any samples that are larger than 400 nm in thickness require an additional processing step for sample thinning to ensure the highest possible resolution. The potential for gaining new insights has been demonstrated by large volume samples, including new insights into septal junctions in cyanobacteria, pore structures important to RNA replication in a coronavirus infected cell line, and a structural understanding of cytosolic 80S ribosomes in a *Caenorhabditis elegans* worm (8, 88, 146). Thus, the development of new methods for gaining insight into even thicker samples would enable the application of this method to other biological disciplines, such as cancer research where studying the intact tumor tissue is critical, or intracellular pathogens.

The current methods for cryo-EM of thin samples utilizes plunge freezing, and thus can go directly into the cryo-FIB SEM to create lamella for imaging using cryo-ET. Alternatively, thicker samples that are high pressure frozen use the cryo-FIB SEM to create trenches at specific sites in the large volume, and then a cooled micro gripper to remove the remaining tissue (less than 20 µm in width and 5 µm in depth), which can then be thinned using the ion beam to create lamella. However, both techniques require extensive training and time and offer relatively low

throughput. Thus, I set out to improve this process, especially when very thick samples are involved.

In **chapter 6**, I describe an updated workflow for the processing of extremely thick samples such as tissues with a thickness greater than 50 µm and without the use of a micro-gripper. These samples require high pressure freezing, which can vitrify a sample up to 250 µm (5, 129). After vitrification, additional thinning steps must be developed to make these samples amendable to cryo-EM analysis. Here, I applied available tools and techniques such as cryo-ultramicrotomy and cryo-FIB SEM (7, 9, 147) on samples extracted with a novel type of 3D printed biopsy needle. The needle is critical to obviating the need for a micro-gripper. In our case, we succeeded in obtaining an initial sample from a *Streptomyces coelicolor* colony. In the future, we aim to optimize the current design to be suitable for tissue extraction from animal and plant systems. This will be done by further iterations of the needle design that inherently allows rapid changes, printing, and testing.

With the bacterial sample we were able to high pressure freeze the sample, retrieve it from the carrier, and store the sample for further processing. This became quite routine for agar-based samples, and testing is ongoing with a variety of different samples (bacterial colonies, plant roots, zebrafish larvae, etc.). So far, we have tested eleven different versions of the needle design. In conjunction with the needle development, we were also successful in freezing a plant root sample directly on a grid, trimming the sample with a cryo-ultramicrotome, and subsequently imaging within the cryo-FIB SEM. By freezing the root directly on a grid, we remove one step in the processing pipeline, which suggests that some additional focus should be given to freezing a needle directly on a grid. This project is still in progress, and each successful step in the process brings us closer to being able to use cryo-ET on multicellular samples. Current methods do exist for examining large volume samples, but most rely on chemical fixation and dehydration or techniques that require specialized training (9, 128, 148, 149). These all introduce unavoidable artifacts that preclude examination of complexes at the molecular level.

Concluding remarks and outlook

Taken together, this dissertation uses a variety of sample preparation techniques for cryo-EM to better understand how *V. cholerae* is impacted by changes in the environment. I was able to demonstrate morphological changes in *V. cholerae* bacteria when they pass through the zebrafish gut, which likely aid the released bacteria in colonizing a new host (outlined in Fig. 1, **Chapter 4**). This information is important for understanding how bacteria adjust to a new host, and how this

interaction prepares the bacterium for life unassociated with the host. Improvements in the preparation of large volume samples for cryo-EM would greatly benefit this research area.

The technical developments in sample preparation and new knowledge could be applied to a number of up-and-coming biological systems including a better understanding of the microbiome and its role in disease, an example of microbiome disruption by *V. cholerae*, and the role of various molecular machines such as type 4 pili in the gut (150–153). Combined with other techniques, such as correlative light and electron microscopy for targeting of trimming efforts or *in vivo* click chemistry, it is possible to hone in on specific areas that answer biological questions. However, without addressing the challenges regarding the processing of large volume samples for cryo-EM, many of these systems can only be explored to a limited extent. Currently, cryo-EM of large volume samples lags behind compared to other techniques such as light microscopy that offers much higher throughput and better statistics for comparable resource investment. Instead, my hope is that once our workflow is optimized, it will be possible to automate it and quickly adapt it to new biological questions, especially without the need to have a very specialized skill set.

With the continued development of new tools for sample processing, it will also be important to re-examine how we use our current tools and innovate in new methods. For instance, our current method of high pressure freezing was established in the 1980s and little has changed in its design and use. Other groups are focusing on different ways of vitrifying samples, such as constant volume freezing or microfluidic vitrification (154, 155), though neither technique has become routine. Besides the freezing step, advances in the cryo-FIB SEM instrumentation could also benefit the large volume workflow. Efforts are being made to replace the current gallium-based FIB with an oxygen plasma source, which would improve the beams milling ability to larger volume samples without a significant loss in precision (156). Additional efforts are also being made in integrating a fluorescent light microscope directly into the cryo-FIB SEM. In addition to reducing transfer steps between instruments, this advance would also allow better targeting during the sample thinning process (157, 158). Taken together, these types of advances would simplify the workflow by removing transfer and thinning steps, thereby increasing access to these highly specialized sample preparation techniques. Once the tools are developed I can next focus on simplifying the techniques for the non-expert, thereby answering more biological questions that require high resolution information to better understand the biological world.

In summary, this thesis contributes to important developments in the field as cryo-EM, providing access to techniques that have traditionally been restricted to a limited number of well-equipped labs. With the creation of regional and national facilities that house the microscopes, new users are invited to prepare samples locally before engaging these facilities. In addition, for users who work with organisms that are considered BSL II or higher, I introduce a new tool for sample preparation that could allow these higher-level organisms to be brought down to levels acceptable for such facilities. Combining the UVC technique with the manual plunger significantly reduces the investment for preparing these samples and will yield tremendous insight into the *in vivo* organism. These techniques together with other emerging techniques in large volume sample preparation and new models of host pathogen interactions, offer an entirely new set of tools for exploring microbes and how they interact with their environments.