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Sample delivery effects on the viability of microorganisms for X-ray free electron laser imaging

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Title (English)

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Abstract

This report describes the first X-ray Free Electron Laser (FEL) imaging experiments at the Linac Coherent Light Source, at Stanford Linear Accelerator Centre, where a number of diffraction patterns with the highest quality as of today were collected from aerosolized cells, virus particles and protein microcrystals. Also described are a number of methods to evaluate the effects from the sample delivery process on organism integrity and viability. It is shown that *Roseobacter* and *Escherichia coli* cells maintain the ability to reproduce, and that *Synechococcus elongatus* strain 22344 has retained membrane integrity after injection into high vacuum. The possible infectivity loss for the marine virus Φ40:2 was investigated, but must be further evaluated. Methods to study the transmission efficiency of the sample injector were successfully investigated. The results presented in this report show that FEL imaging has the possibility to image alive and viable cells.

Keywords

X-ray Free Electron Laser Imaging, Viability, Vacuum, Aerodynamic lens stack, Aerosol , *Roseobacter, Escherichia coli, Synechococcus elongatus,* phage *Φ40:2,* Stain

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Sample Delivery Effects on the Viability of Microorganisms for X-ray Free Electron Laser Imaging

Olof Jönsson

Sammanfattning

Det finns ett starkt samband mellan struktur och funktion inom biologin. Det här arbetet är en del av utvecklingen av en ny teknik för högupplöst avbildning av biologiska objekt, röntgendiffraktionsavbildning med frielektronlaser. Bakom det långa namnet döljer sig en metod som använder sig av extremt korta röntgenpulser med hög energi för att avbilda enskilda viruspartiklar, celler och mikrokristaller av proteiner. I denna rapport beskrivs det första avbildningsexperimentet vid Linac Coherent Light Source, Stanford Linear Accelerator Centre. En förutsättning för relevanta resultat är att de prov som man ska avbilda inte har skadats i processen. Detta är inte självklart eftersom proven skjuts in i vakuum med hög hastighet i form av en aerosol. Samtidigt ger den höga hastigheten hopp om att bakterier och viruspartiklar inte hinner påverkas innan man får en bild. Återväxtförsök av bakterierna *Escherichia coli* och *Roseobacter* visar att de fortfarande kan dela sig efter vakuumexponeringen. Med infärgning visas att cyanobakterien *Synechococcus elongatus* har ett oskadat cellmembran. Effekten på marina fager har undersökts men är svårtydd. Metoder för att utvärdera förluster i antal partiklar har framgångsrikt använts. Sammantaget visar detta arbete att röntgendiffraktionsavbildning med frielektronlaser har ett gott hopp om att avbilda levande och välmående celler.

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Contents

Abbreviations used

AmAc	Ammonium Acetate
BG-11-media	Growth media for marine photosynthetic bacteria
CFU	Colony forming units
DESY	Deutsches Elektronen Synchrotron
FEL	Free Electron Laser
FLASH	Free Electron Laser in Hamburg
LB-media	Lysogeny broth media, or Luria Bertani media
LCLS	Linac Coherent Light Source
MLBss	Growth media for *Cellulophaga baltica*
MSM	A buffer suitable for *Cellulophaga baltica* phages
PFU	Plaque forming units
SLAC	Stanford Linear accelerator centre
TBA	Solid media plates for *Escherichia coli*, optimal for CFU-counts
TBS-buffer	Tris Buffered Saline, a phosphate free buffer with physiological salt concentration.
VNC	Viable but non-culturable

1 Introduction

1.1 Aim and scope of the project

Imaging with X-ray free electron lasers is a new field, where many components in the process must be further developed. The second facility where this imaging technique is available was first opened for experiments late 2009, and the first part of this project describes the work preparing samples for the first beam-time there. The quality of the images is dependent on the physical capabilities of the X-ray radiation and the data collection and processing. This will only be discussed briefly. A requirement for relevant images is that the sample is unharmed when imaged. This report describes for probably the first time methods to study the viability of samples when delivered to the X-ray beam.

1.2 Imaging of small biological objects with X-ray free electron lasers

Humans have always been interested in understanding nature, and the methods to image nature's structures have been under constant development ever since the very first cave-paintings. There is a close relationship between structure and function in biology, which stretches from the large to the small, from the shape of whale fins down to the shape of neural cells in our brain.

Development of new imaging techniques has given us access to ever increasing resolution, and a possibility to study many biological processes at the scale in which they happen. The light microscope has made it possible to see things smaller than what the naked human eye can see. Electron microscopy, and especially its variant cryo-electron tomography, has given us three-dimensional pictures of virus particles [1]. X-ray crystallography has given us the ability to image proteins to atomic resolution using diffraction from crystallized proteins.

Most of the imaging techniques use radiation in some way (AFM microscopy is a notable exception). Some limiting factors are common to all radiation-based imaging techniques. The wavelength of the radiation seriously limits the possible resolution, under normal conditions the imaging is diffraction-limited meaning that only features up to half the wavelength used can be detected. Another limiting factor is the damage that inevitably results from the interaction between the sample and the radiation. X-ray crystallography successfully overcomes this limit by spreading the damage between all proteins in the crystal [2]. Finally, the sample handling and sample delivery process might interfere with the object or process studied. For electron microscopy, the sample is imaged in vacuum and often needs a coating of conductive material. A dehydrated cell coated in gold is obviously not alive anymore, and any processes depending on live organisms are thus impossible to study with this technique. Another notable example where sample preparation might affect the relevance is X-ray crystallography. All the proteins must have the same shape to allow crystallization, thus effectively freezing them in one of the many possible conformations.

All imaging techniques used today have their advantages and disadvantages, but there is obviously room for new fields in imaging of biological objects overcoming some of the limitations in resolution and sample disturbance inherent in current imaging techniques.

One emerging field in imaging is called free electron laser imaging, or FEL-imaging. In this project I have worked with some aspects of this technique. A combination of a very short wavelength (soft to hard X-ray regime) and femtosecond short pulses with a very high intensity makes it possible to image single biological particles without crystallizing them. The theory for FEL imaging was first presented in a paper from 2000 [3]. The publication of this paper was the starting point for a whole field, which eventually lead to the construction of the first laser sources of this kind. Using these

sources, high-resolution images of single virus particles or even single protein molecules will be possible. The author predicts that an extremely short X-ray pulse, with very high photon brilliance, would be diffracted by the sample and produce a detectable diffraction pattern before the radiation damage destroys the sample. A two-dimensional structure of the sample can be calculated from the diffraction pattern, and theory predicts that even the three-dimensional structure can be calculated [4]. The first successful imaging experiment was done in 2005 and showed that the predicted methods worked in reality. [5]. Since then the resolution of acquired images has greatly increased, and progress in the field is an ongoing endeavor.

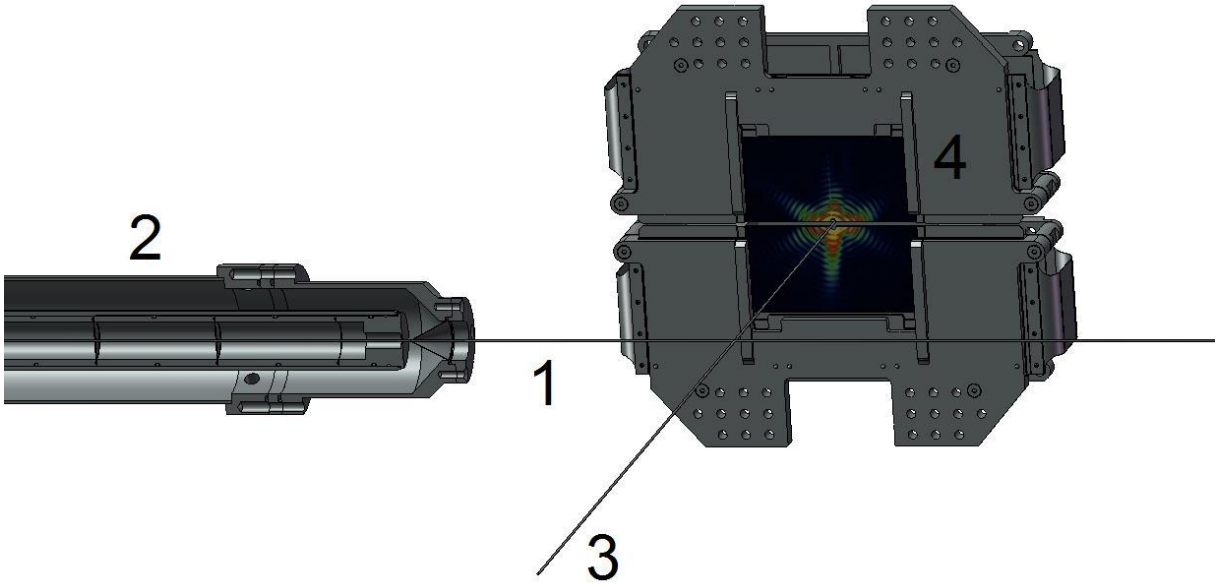

Figure 1: Interaction between sample and the X-ray pulses. The particle beam (1) sprayed from the particle injector (2) must intersect the X-ray pulses (3) and diffract on the detector (4). CAD rendering done by Daniel Westphal, used with permission.

The X-ray pulses are shot at the sample (see Figure 1). When an X-ray pulse hit a particle it will interact with it and give rise to a unique diffraction pattern that is dependent on the characteristics of the sample (see Figure 2). The radiation is so intense that it will turn the sample into plasma, but the damage barrier is overcome by the short pulses. The damage process takes longer time than the time the X-ray pulses require to diffract from the sample, and a pattern from an undamaged object can be detected. Mathematical algorithms are used to recalculate the structure of the imaged object.

The interaction between the sample and the X-ray pulses has to take place in vacuum, to reduce the background from the otherwise diffracting gas molecules present between the X-ray source and the detector. The whole beam line and the interaction chamber are pumped down to high vacuum using turbomolecular pumps. This seriously complicates sample delivery to the X-ray pulses. One way to introduce samples into the X-ray beam is to use a solid support with the sample attached to the surface, but the silicon nitride used so far has proven to give a severe background that limits the resolution. To reduce background, container-less introduction of samples has been developed. A liquid jet injector has been tested that creates a very thin liquid stream[6], but the

background diffraction from the water in the jet still interferes with the diffraction from the sample. The technique that to this day provides the least background focuses an aerosolized sample into the interaction region using an aerodynamic lens stack (see section 3.2).

The resulting diffraction patterns will have different characteristics depending on whether the sample is periodic or not. A sample with orderly repeating patterns, such as a crystal, will give a pattern similar to those obtained by X-ray crystallography with the characteristic circles of Bragg peaks. The reconstruction algorithms for these patterns are well known. For particles without repeating units such as cells, virus particles and even non-crystallized proteins, the diffraction pattern will be continuous. (See Figure 2) Reconstruction algorithms for non-continuous diffraction images acquired by FEL imaging is a new field under constant development.

Three-dimensional structure of reproducible biological objects such as virus capsids and single proteins will be possible to image down to atomic resolution in three dimensions. This will require several diffraction patterns covering many angles from copies of the same sample. The knowledge that all of these patterns should come from the same original protein can be used in the reconstruction algorithm.

Several FEL imaging experiments have been done since the first proof-of-principle experiment [5]. These experiments were done at the Free-Electron Laser in Hamburg or FLASH, at the Deutsches Elektronen-Synchrotron (DESY) facility in Hamburg. The second FEL source, Linac Coherent Light Source or LCLS, was completed 2009 at Stanford Linear Accelerator centre, USA. Experiments as a part of this project at LCLS proved that the wavelengths and properties available today at these sources are enough to create low-resolution images of cells and virus particles as continuous diffraction patterns, and protein crystals to probably atomic resolution as non-continuous diffraction patterns. Preliminary results show that these diffraction patterns are possible to reconstruct (see section 2). However, with the new development of X-ray free electron lasers, no crystallization will be needed to image proteins. It is here that this imaging technique really excels, as it might be possible to determine the structure of many proteins that are very difficult to crystallize. This includes most of the important membrane proteins that are vital in the energy metabolism and signaling in the cell. This group of proteins is very important as drug targets, and given that the structure is known more efficient cures can be developed against many diseases.

There is no theoretical limit that will stop FEL imaging from imaging individual cells and virus particles to subnanometer resolution when the technology is fully developed [4]. This would allow positioning of individual macromolecules within the cell, and whole system approaches to studying the foundation for all life. A requirement is that the samples are undamaged when imaged, and the following section discusses why this is questioned.

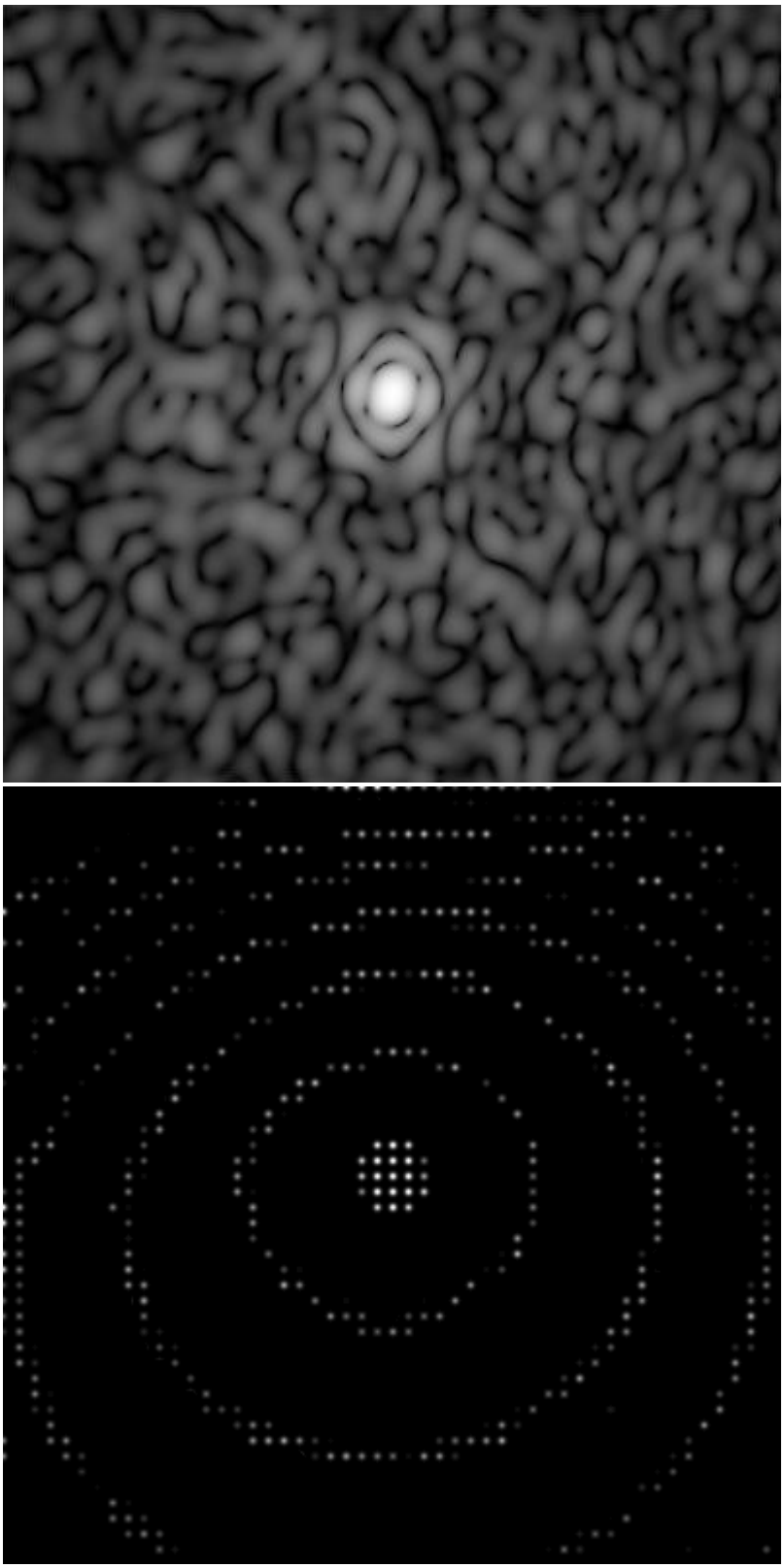

Figure 2: Simulated diffraction patterns from lysozyme. Both patterns are based on theoretical and not experimental data. Top: Continuous diffraction pattern from single protein. Bottom: Non-continuous diffraction pattern of crystallized protein, with the characteristic Bragg-peaks. Based on PDB structure 132L [7]. Simulation done by Tomas Ekeberg, used with permission.

1.3 What happens to small living cells when injected into vacuum?

Many factors in the sample delivery process (described in detail in section 3) may interfere with the samples in a negative way. If the sample is damaged in any way during the sample delivery process, the relevance of the structural data will be decreased. If the structure is altered in a predictable and known way, this information can be used to still get useful data. Ironically, the best way to measure the structure is with the FEL itself. A high-resolution image obtained with the FEL would be the ultimate proof that the structure is unchanged, as this structure could be compared to low resolution images of the same sample achieved with other imaging techniques. The first results from FEL imaging suggest that the samples have the same shape as before, but the resolution is unfortunately yet too limited to give any definite answers.

No method has been developed to monitor damage on the samples in real time as they are injected into the vacuum chamber. This would require a very fast technique, as the sample is travelling at a speed of 100 m/s. Any method to study sample damage must inevitably take longer time to perform than the vacuum exposure time during an actual imaging experiment (a few ms). In this project, methods to capture the sample and indirectly measure the damage were developed.

The samples are aerosolized before they are injected into vacuum. The properties of cells in aerosol have been studied before. Microbes are present in atmospheric droplets [8], and have been studied as a possible risk factor for human health [9]. An aerosol made with bio-electrospray containing zebrafish embryos showed that the embryos developed normally after the treatment [10]. These results show that aerosolization of sample should be possible without damaging the cells.

Vacuum effects on microbes have also been studied before. The food industry uses freeze drying and vacuum packaging techniques as means to sterilize and preserve food. These studies have focused on how to kill organisms as efficiently as possible, and the general message is that long time vacuum exposure can be very harmful to most microorganisms [11].

Another area that investigates vacuum effects is space biology, describing how microbes can survive space conditions. Outer space is a very hostile environment to living organisms, with high radiation, thermal extremes, high-velocity debris and high vacuum.

These studies have identified the main vacuum effects that can be expected. In vacuum, all liquids will evaporate. This will cause a very harmful dehydration of the living cell. Transport of energy, protein synthesis, signaling and all other vital processes in the cellular metabolism rely on water to function. When a cell is dehydrated, the lipid membrane might irreversibly change conformation and form cylinders instead of sheets. Without water, Maillard-reactions (amino-carbonyl reactions) might cross-link and polymerize proteins with each other, nucleic acids and carbohydrates. When the nucleic acids are damaged it will cause mutations, and coupled with the other damage done to the cell it will not be able to recover. Non-metabolizing cells have no means of repairing induced damage, which might be easy to repair if the cell was active. [12]

Many of the results from space biology experiments are interesting in their own right, but are not always applicable to this project. All studies expose the organisms to vacuum for far longer time than what is relevant to FEL-imaging. In space the most lethal factor is the combination of vacuum dehydration and radiation damage, and only a few studies have isolated these effects from each other. It can be noted that the “survival” of these organisms often means survival of spores and other long-term survival forms that are not metabolically active. Some extremophiles might survive for a longer time in an active form, but a sample delivery technique for FEL imaging must be able to deliver a broad range of samples. The interest for FEL imaging is in living fully metabolizing cells of any type.

1.4 Modes of sample damage

There are several requirements that must be fulfilled if an organism is to be claimed healthy and undamaged. The question whether a cell is alive or not touches on the philosophical question of what life actually is, and as with most philosophical questions, no clear answers exist. With viruses, the question is even more complicated as they are not defined as alive to begin with. The exact terminology describing the different states between on the one hand fully active and dividing cells, and dead cells on the other is complex and under discussion [13]. A number of factors can be identified as being important when a cell is to be declared severely damaged:

1.4.1 Loss of membrane integrity

The cell must be well defined and distinct from its environment. If the cell membrane is damaged enough, it will lose the membrane potential that is crucial to energy metabolism, and the cell content can freely diffuse out in the surroundings.

1.4.2 Loss of reproducibility

This includes both the ability to divide, and the ability to pass on genetical information. To many this criterion is the very definition of life, as the ability to create copies of oneself under the influence of evolutionary forces clearly distinguishes life from other processes. There is still some controversy that complicates this as a standalone criterion. The discussion about cells that are “viable but non-culturable” (VNC) is growing, and many researchers agree that dormant states and metabolically active but non-reproducing cells should be classified as alive.[14]

1.4.3 Loss of metabolism

The ability to reproduce and maintain membrane integrity is strictly dependant on a constant intake of energy and material from the surroundings. A cell that has lost its ability to respond to its environment is nothing more than a gathering of molecules waiting for entropy to do its work.

2 Beam-time at Stanford

One of the major milestones in FEL imaging was made during this project; the first series of experiments done at the new Linac Coherent Light Source (LCLS) facility (see Figure 3). This is the second full scale X-ray free electron laser in the world, and the first that will have the hard X-ray capabilities needed for single protein imaging. The unique properties of free electron lasers with a short pulse length attract scientists from many fields, and proposals of possible experiments have to be prioritized by a committee. Each experiment that goes through this needle's eye gets a number of beam-time shifts. Around 8 out of 100 proposed experiments were performed during the first round. One of these experiments was the imaging experiment described here.

The experimental time was divided into two parts; the first studied protein structure using diffraction from microcrystals (section 2.2), and the second studied single virus particles and cells (section 2.3). The experiment was an international collaboration with around 50 scientists from twelve universities, including nine scientists from the Molecular Biophysics group in Uppsala.

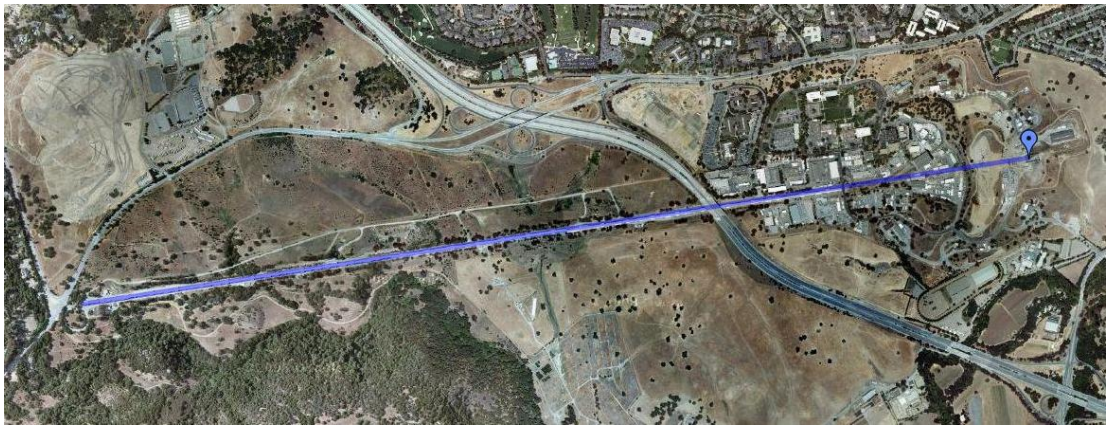

Figure 3: SLAC, US. This satellite picture shows the over 3 km long linear accelerator building (marked in blue) from above. It houses the LCLS X-ray free electron laser. The small blue flag is the location of the experimental hall where the X-ray pulses will hit their targets. © 2010 Google – Imagery © 2010 DigitalGlobe, USDA Farm Service Agency, GeoEye, U.S Geological Survey. Used under fair use licence.

2.1 LCLS

The Stanford Linear Accelerator Centre, SLAC, has been home to some of the most spectacular experiments in physics since it was built in the 1960s. It was here that the quark was discovered, and several other exotic particles were investigated at this institution for the first time. One of the many instruments available to researchers is the circular synchrotron Stanford Synchrotron Radiation Laboratory (SSRL). It was used to determine the structures of important components of the transcription in eukaryotic organisms. This work gave one of the six Nobel prizes awarded to scientists working at the facilities.

Today SLAC has the most advanced free electron laser source in the world, LCLS (Figure 3). This 4th generation laser source, that was first available for experiments in 2009, can produce the extremely short and brilliant pulses needed for FEL imaging. In [15], the author describes much of the instrumentation at LCLS.

2.2 Structure of protein microcrystals

The beam-time at LCLS was mainly intended for structural studies of protein microcrystals. Theory predicts that the structure of the protein can be determined even if the crystal is much smaller than what is required in a normal continuous X-ray synchrotron. The collaborating groups focused on photosystem I microcrystals crystallized by a group from Arizona State University [16] .

These crystals were injected into the experimental chamber using a liquid jet sample delivery system [17]. Many diffraction patterns were collected, and the data is still under processing.

2.2.1 Lysozyme microcrystals

As a part of this project work, an attempt was made to create microcrystals of lyzozyme. Even though the imaging experiment was focused on photosystem I microcrystals, back-up samples were needed. The following sections describe the attempt to create the needed sample (2.2.1.1-2.2.1.5).

2.2.1.1 Principle

A liquid containing as high concentration of stable lysozyme crystals as possible is required for imaging. The crystals should also have a dimension smaller than 2µm. Different conditions and techniques for manufacturing these were examined. The size distribution was evaluated with microscopy (see section 3.5). The crystals must be stable for a long time if they are to be shipped to LCLS, or easy to manufacture on site.

2.2.1.2 Initial screening

Lysozyme (MERCK 50 000u/mg) dissolved in destilled water was mixed with NaCl dissolved in NaAc buffer with pH between 4.7 and 5.0. Conditions examined were all combinations of 10, 20, 40, 80, 100 mg/ml lysozyme and 6, 12 and 20 weight percent NaCl. The solutions were mixed in clear plastic test tubes and were left for 3 days at 25°C. The results are summarized in Figure 4.

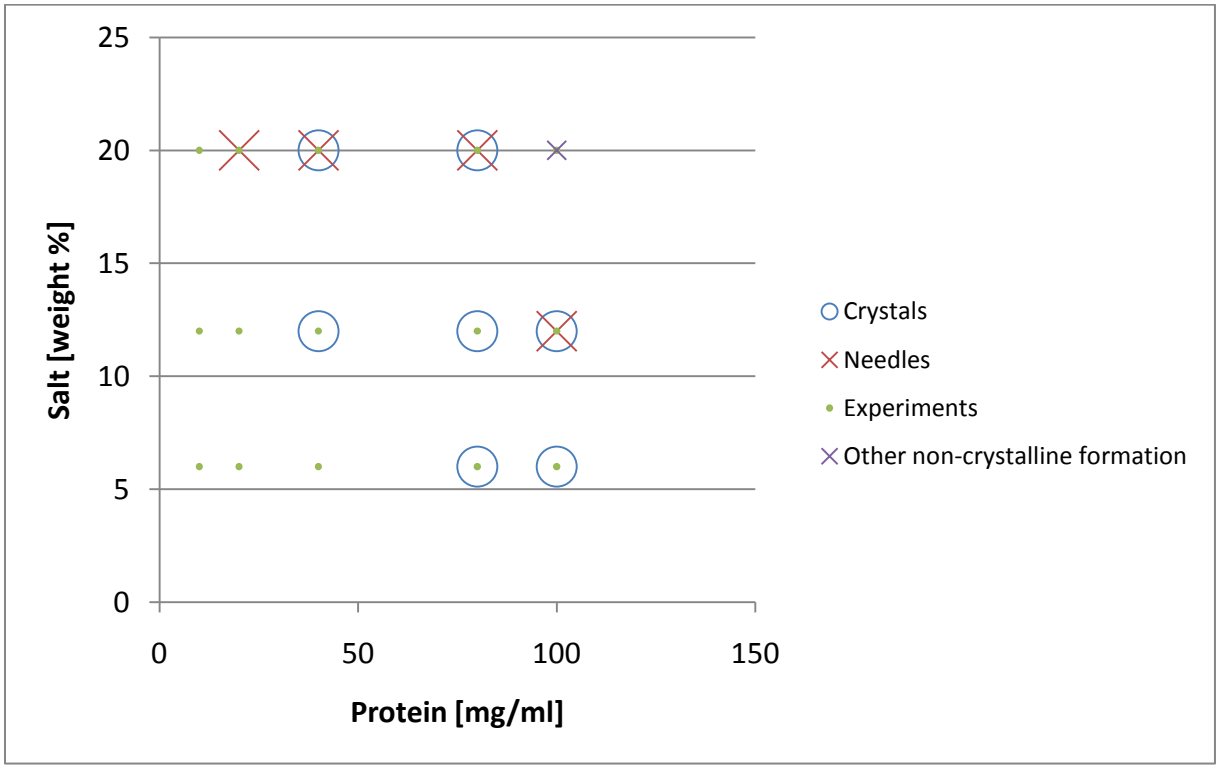

Figure 4: Results from initial screening experiment for exploring crystallization conditions. Crystals refer to visible crystals of any shape and size, needles refer to long needle-shaped formations.

2.2.1.3 Examining crystallization conditions

The most promising experimental region was between 40 and 100 mg/ml lysozyme, and between 6 and 12 weight percent NaCl. Further experiments were performed in this region. Some crystals stuck to the walls of the test tube, and some of these crystals had the desired size. Several different crystallization tubes were examined (1.5 ml VWR Microtube Superspin, Costar 24-well Cell

Culture Cluster Tissue Culture Treated 3424, Linbro Tissue Culture 76-033-05, 15 ml Falcon tube model VWR 21008-216, Untreated glass test tubes). The choice of wall material had a significant effect on the size of the crystals, which ranged in size from several mm down to a few µm for the same conditions in different vessels. The smallest crystals stuck to the walls, and most of them were destroyed when scraped off. Replicates with the same conditions were very unreliable. Even if small crystals were formed, they tended to grow with time, and after a week most of the tubes either had their walls covered with lysozyme crystals, or a few big crystals (>100µm).

Silanization is a method that has been reported to minimize wall attachment. VWR 15 ml 21008-216, glass test tubes and 1.5 ml Treff 96-7246-9-01 were used. After a few seconds of flushing with dichlordimethyl-silan, the test tubes were rinsed with distilled water. 40 to 90 mg/ml lyzozyme, 1 to 2 M NaCl was mixed in the vessels. Crystals were still formed on the walls, but mild shaking was enough for them to leave the surface. 90 mg/ml, 1 M NaCl produced crystals sized 1-10 µl. Filtering and mild centrifugation were tried to size differentiate the crystals, but the crystals dissolved or aggregated.

Big crystals were shattered in an ultrasonic bath to small fragments with a size ranging from less than a µm up to 20 µm, but the resulting crystals quickly grew large if in the mother liquor. If the buffer was exchanged, the crystals dissolved within minutes.

2.2.1.4 Centrifugal evaporator

In an article from 2003, the authors describe a method for the creation of small microcrystals. They successfully created crystals ranging in size from 100 nm to a few µm using a centrifugal evaporator [18]. A centrifugal evaporator centrifuges the sample under vacuum, making the evaporation of water or other volatiles very quick, concentrating the remaining material. Their protocol was ambiguous, so different conditions were examined. Equal amount of 12.5 weight percent PEG 2000, 75 mM NaCl, 50, 100 and 200mM NaAc pH 4.5 were mixed with 25 mg/ml lysozyme dissolved in destilled water. A Savant Speed Vac SVC100H was used. According to the protocol, the solution should be evaporated until half of the remaining liquid remained. This proved to be difficult to estimate in time, as the evaporation speed accelerated when a sample froze. The timing of this freezing event varies irregularly even between tubes with the same solution. Some tubes contained microcrystals after centrifugal evaporation (see Figure 5). Many replicates were done with varying conditions (protein concentration, cooling the solution before concentrating, evaporating in steps, different total volume), but no reproducible conditions could be found.

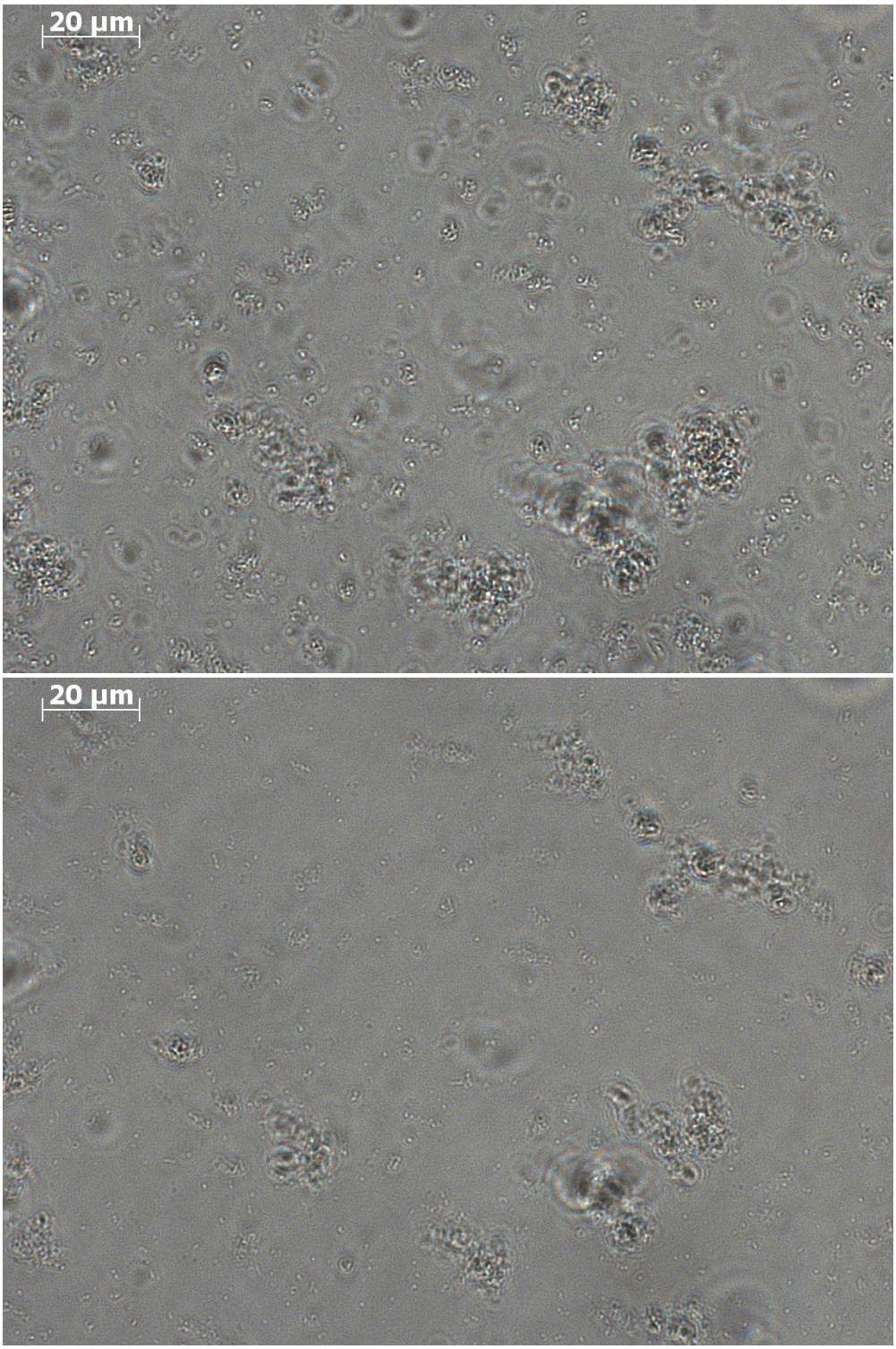

Figure 5: Lysozyme microcrystals sized around 1 µm. These crystals were created with the centrifugal evaporator method described in section 2.2.1.4. The bottom picture was taken 10 minutes after the top picture in the same region. The pictures illustrate some of the problems with this method. The crystals created may dissolve after only a short time. Aggregates of particles could make transfer in thin capillaries impossible, and it is difficult to verify that the particles really are uniform crystals without using some other method.

2.2.1.5 Conclusions

Even though some microcrystals were formed during the experiments, no method provided microcrystals that were stable long enough for use in real imaging experiments. No reproducible method was found that created high enough concentration of crystals in the desired size region. It would also have been necessary to confirm that the smallest crystals really were ordered crystals and not a precipitate with random composition. Small crystals are often unstable, as they tend to either grow or dissolve. Growing crystals were unacceptable as the liquid jet used relies upon thin capillaries for sample delivery.

It can be noted that cross-linking was used to create some of the other back-up microcrystals made by other groups. In the future, when shorter wavelengths are available at free electron laser sources, it may be possible to skip the crystallization step entirely.

2.3 Structure of cells and virus particles

The second part of the beam-time was dedicated to imaging cells and virus particles. Similar experiments have previously been performed at FLASH in Hamburg, creating images with a quality up to the limit of that facility. LCLS is a second generation FEL-source, with much higher potential. This was the first attempt to image cells and virus there, with a newly constructed beam-line and interaction chamber, and a new setup of sample delivery methods and detectors. The three sample delivery methods used were a liquid jet (briefly described in section 1.2 and [6]), wafers with sample deposited on thin silicon nitride windows, and the aerodynamic lens stack (described in section 3.2) The samples studied during the beam-time were the giant Mimivirus, *Cellulophaga baltica* phages Φ40:2 and Φ4:1, various strains of the cyanobacteria *Synechococcus* and Prochlorococcus and the parasitic bacteria Bdellovibrio.

Many samples were cultivated on site in a mobile laboratory shipped to the site (Figure 7, Figure 18), as the facility is so recently constructed to lack fully functional labs suitable for work with biological objects. To minimize any background in the diffraction patterns from salt crystals in the solution, a buffer exchange into volatile Ammonium Acetate was done on all samples. A flowchart for the imaging experiments can be seen in Figure 6.

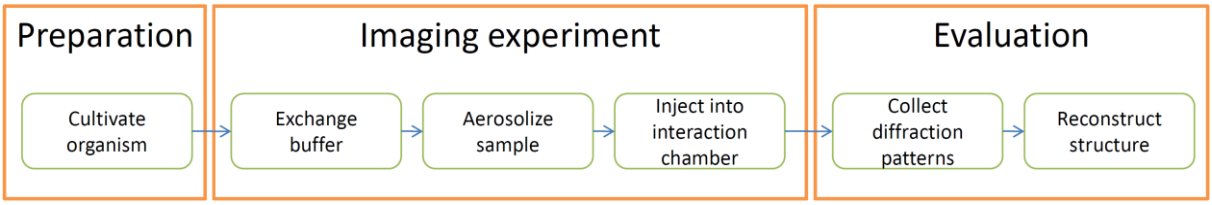

Figure 6: Flowchart for imaging experiments.

Figure 7: The interior of the mobile laboratory where all samples were prepared before imaging.

2.3.1 Outcome

Both the liquid jet and the wafers with deposited sample created background that will severely limit the resolution of the reconstructed structure of the samples. The aerodynamic lens successfully delivered particles as an aerosol, and many interactions with following diffraction were recorded with only a small amount of background noise. The diffraction patterns that were collected had the highest resolution ever achieved in FEL imaging. The original data and the reconstructed images are still unpublished and under processing. It is still unclear how high the resolution will be, but the preliminary results show that the structure of the samples injected is possible to recalculate, and that they are imaged before any radiation damage has taken place (personal communication with Janos Hajdu, Filipe Maia and Tomas Ekeberg).

3 Experimental setup for viability studies in Uppsala

This section describes the experimental setup used for measuring the viability in the molecular biophysics lab in Uppsala. To verify the relevance of the FEL imaging experiment (2.3), a method is needed to evaluate the potential damage caused by the sample delivery process. First a method must be developed that can catch the samples in a state as close as possible to the state when they are imaged. Then methods to study the viability must be developed. As the organisms studied are diverse, many different strategies may be needed.

The experimental procedure for viability studies should be considered as a toolbox with many available methods. First a model organism must be selected (see section 3.6) and a method to measure its viability has to be developed (see section 3.7). The sample is turned into an aerosol (see section 3.3) and sprayed into the vacuum chamber (see section 3.1) using the injector (see section 3.2) with the desired experimental configuration, and finally the sample is to be captured (see section 3.4) and evaluated (see sections 3.5 and 3.7). See Figure 8 for a flowchart of the experiments.

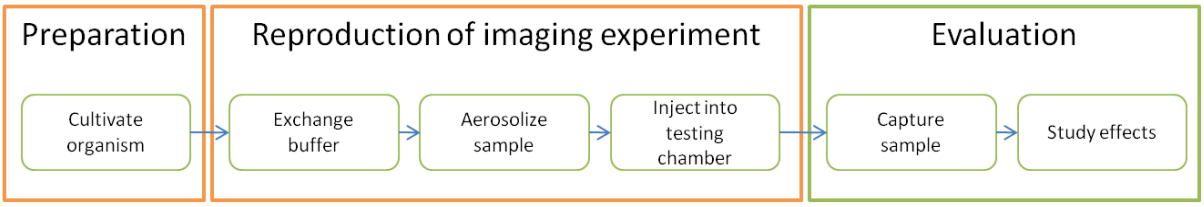

Figure 8: Flowchart for viability experiments. Compare to Figure 6

3.1 Vacuum chamber and pumping

As mentioned in section 1.2, the imaging experiments have to be performed in vacuum in order to reduce background noise. The testing chamber available in the Molecular biophysics lab in Uppsala can be used to emulate the conditions at a real imaging experiment (Figure 9).

One of the biggest limitations with characterization of the injector has been the long time it takes to pump and vent the vacuum chamber. The pumping must be done in several steps in order to reach the desired pressure. First, normal vacuum pumps are used to reach the low-vacuum regime (10^{-1} mBar). Turbomolecular pumps are then used to reach into the high-vacuum regime (final chamber pressure is down to $2*10^{-6}$mBar). These pumps have jet-engine-like propellers that throw the molecules out by hitting them extremely fast with a turbine blade rotating up to 1000 Hz. The turbine blades are very sensitive and must operate in a pumped environment because of the high friction heat that would develop when interacting with gas molecules at atmospheric pressure. It takes up to an hour for the turbomolecular pumps to slow down after they are turned off, and the vacuum must be maintained during this time. It is very difficult to perform controlled vacuum exposure experiments under these conditions.

The exposure to vacuum during sample delivery for imaging is very short in relation to the time it takes to vent the chamber. This waiting time may seriously interfere with the viability studies, as long time vacuum exposure is known to be harmful for most organisms (see section 1.3). The interlock system (see section 3.4) solves this problem.

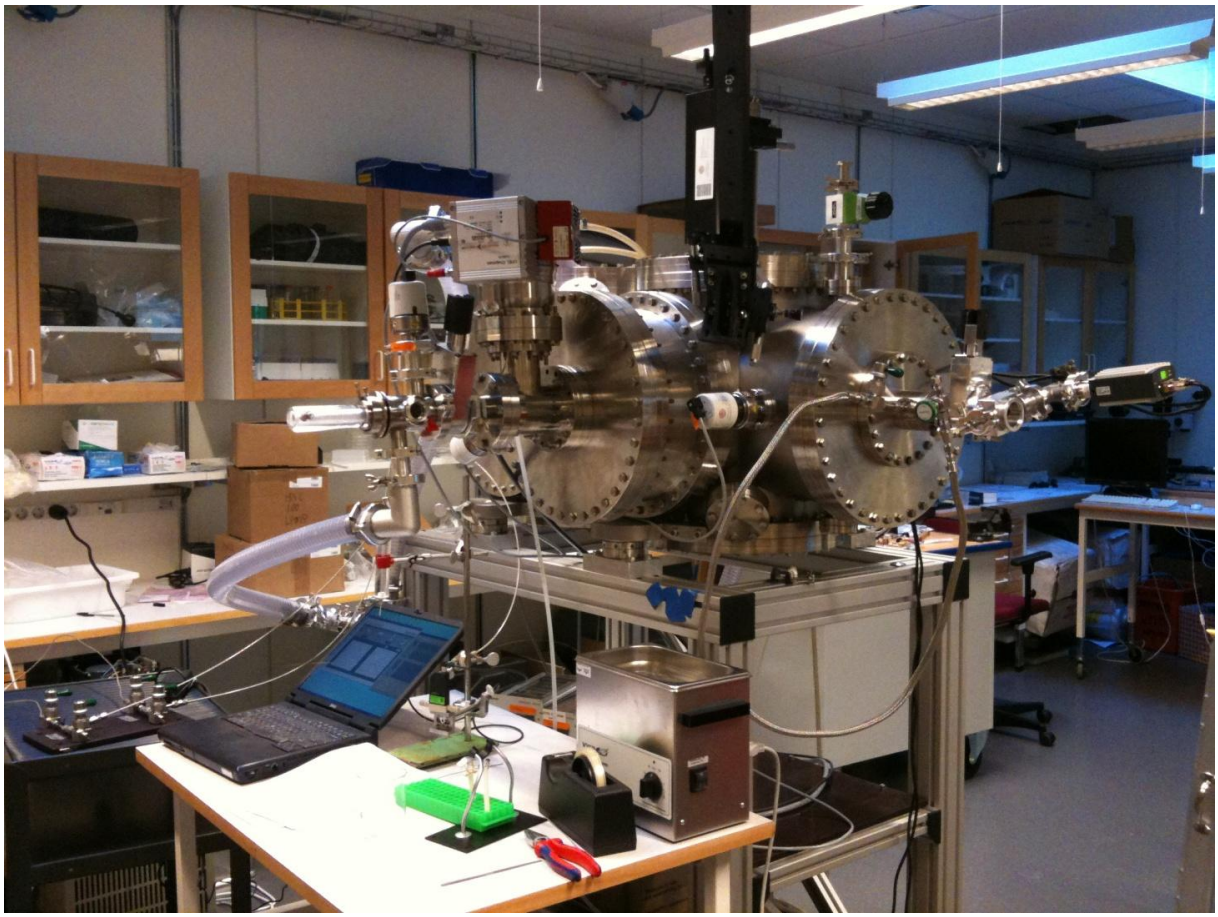

Figure 9: The vacuum chamber used for all vacuum experiments in Uppsala.

3.2 Aerodynamic lens sample injector

The optimal X-ray pulse beam is very thin, with a diameter down to the size of the sample. This is a way to optimize the number of photons that hit the sample without increasing the photon flux. The width of the beam is a parameter that is continuously improved at the FEL-facilities (The current pulse width at LCLS is around 10 µm). The distance between the diffraction event and the detector must be well defined in order to recalculate the structure of the sample. This creates a very well defined interaction region into which the sample must be delivered. It is possible to shoot a very high number of x-ray pulses per second. The pulse repetition rate used at the LCLS experiment was 30 Hz, but the FEL sources under construction might have a rate of up to 30 000 Hz. This leads to a situation where the aim of sample delivery is to make a concentrated straight train of particles, which flies through the interaction region.

The injector previously used at FLASH and LCLS has been developed in Uppsala according to the principles first suggested in [19, 20] and it is similar to the aerodynamic lense stack described in [21]. Using this sample injector, an aerosol can be focused into the thin particle train required for interacting with the X-ray pulses. The apparatus will also transition the samples through the pressure gradient between the atmospheric pressure outside the chamber and the inside of the interaction chamber.

The underlying principle is quite simple. Because of the higher inertia, particles in a gas will to some extent retain their path even if the gas is diverging. The light gas molecules can change their path much more easily than the bigger and heavier particles. By leading the aerosol through a series of openings and holes over a pressure gradient, the bigger particles are forced to be focused by the

aerodynamic properties of the system. At the end of the aerodynamic lens stack sits an accelerating nozzle, where the gas rapidly diverges but the particles continue as the desired particle train. The details of the aerodynamical theory are outside the scope of this work.

Because of the high speed of the sprayed particles (around 100m/s), the time the sample spends in vacuum is very short, around 10 milliseconds, before reaching the interaction region. The short exposure time gives hope that the samples are virtually unaffected.

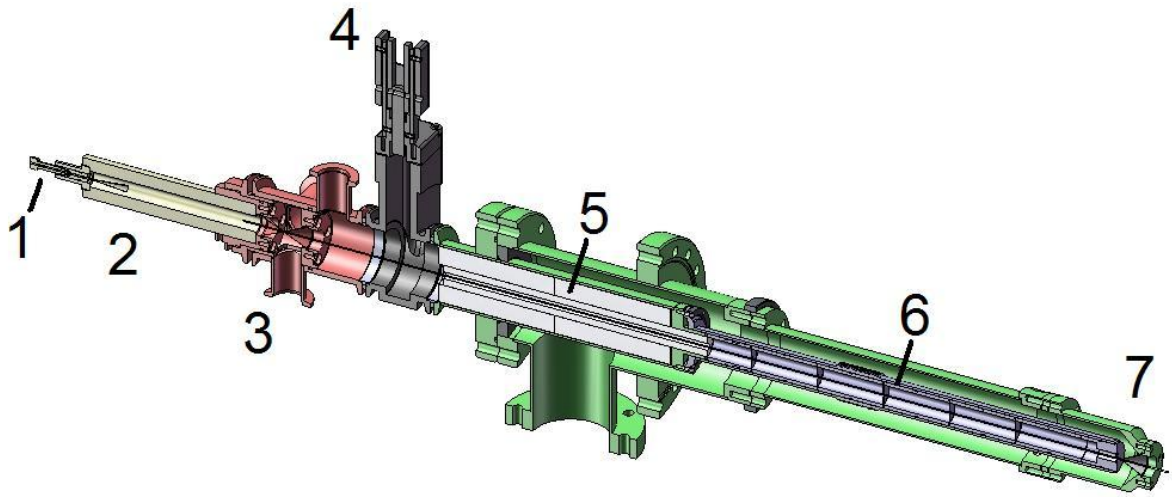

Figure 10: The particle injector. Legend: Aerosolization nozzle(1) Aerosol chamber (2) Adjustable nozzle box (3) Valve to interrupt the particle beam (4) Relaxation chamber (5) Lens stack (6) Exit nozzle (7) CAD rendering by Daniel Westphal, used with permission.

3.3 Aerosol creation

Almost all interesting biological samples exist as particles in a liquid. This is true for all cell cultures, virus suspensions and macromolecular complexes. The liquid surrounding the sample would create a severe background for the imaging process, so the optimal solution is to aerosolize the sample. This requires a device that will aerosolize liquids into a mist. In [17], the authors describe the droplet source used in all aerosol experiments described in this report (see Figure 11).

The liquid is placed inside a sample holder (Figure 12). The sample is pressed through a 5 µm filter into a capillary using inert gas (N_2). The flow in this capillary is measured with a very precise liquid flow meter (CMOSens® SLG1430-480). The capillary ends in the aerosolization nozzle situated inside the aerosol chamber of the injector. The exit of this capillary is situated inside the exit of another tube. From this tube a fast flow of helium sheaths the liquid, and breaks up the exiting liquid stream into small droplets. The capillary with sample has an inner diameter of only 20-50 µm, and the droplets formed are only 20 µm in diameter. These droplets will shrink in size as the liquid evaporates during travel trough the aerodynamic lens. This can be compared to the typical sample size of some µm.

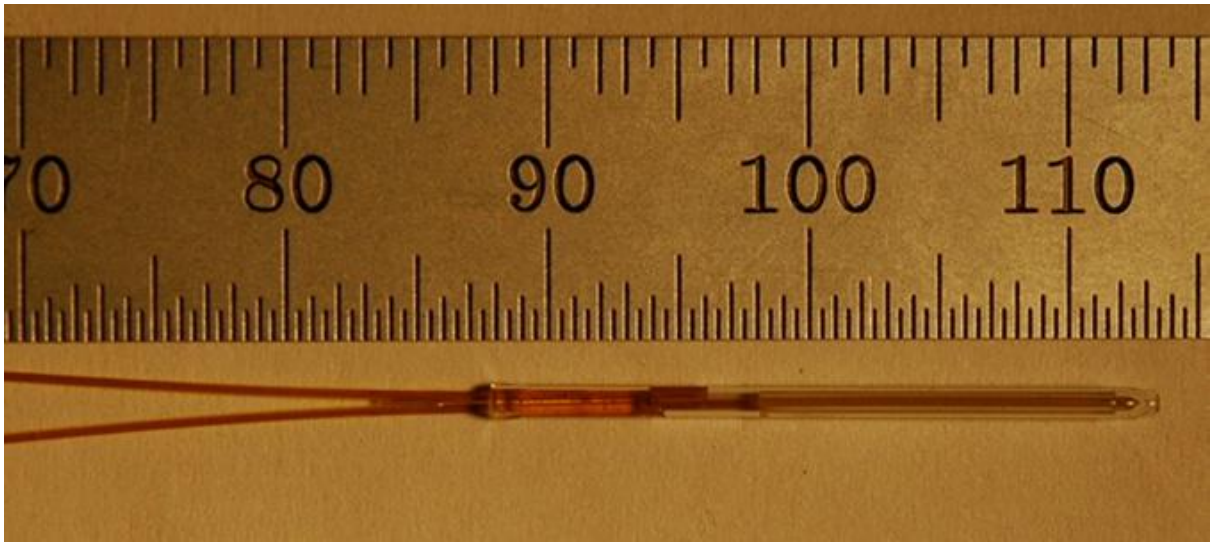

Figure 11: The aerosolization nozzle. The ruler is scaled in mm. A thin stream of aerosol will exit to the right. The outer translucent tube will sheath the emerging liquid flow from the inner brown capillary with helium, causing it to break up into small droplets.

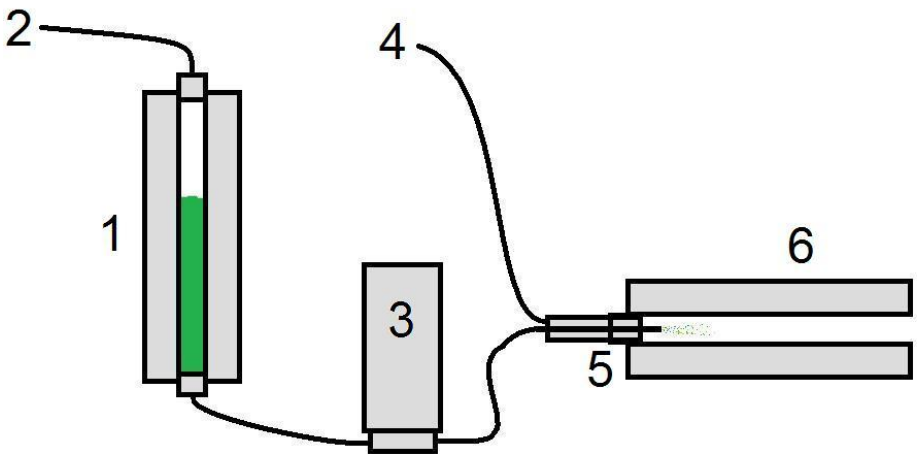

Figure 12: Aerosol creation system. The sample liquid is transferred to the sample holder(1). Nitrogen gas (2) is pressing on the surface of the liquid. This will press the liquid through a 5µm filter to the capillary at the bottom of the holder. The flow is measured with a very precise flow meter (3). A fast flow of helium gas(4) is sheathing the liquid at the exit of the aerosolization nozzle(5). The gas flow will disrupt the liquid stream and produce a fine aerosol of sample liquid in the aerosol chamber (6).

3.4 Capturing sprayed particles

3.4.1 Capturing device

In order to investigate the injected particles, a capturing device for capturing the injected particles when they enter the chamber is needed. The material that the cells are sprayed upon may influence the recovery of cells. This might be due to both mechanical reasons (collision dampening, stickiness to the cells) and chemical (toxicity, degradability by the organism). The optimal device should have the following properties:

- **Adhesiveness to samples**
  The sample must stick to the surface.
- **Easy to clean or available in many copies**
  Several replicates of each experiment must be made within a short time interval.
- **Vacuum resistant**
  Many materials change their properties in vacuum. Agar and agarose gels quickly lose their water content in high vacuum, even though gels of these material could be used for low vacuum (data not shown).
- **Non-interfering**
  The material must be non-toxic, and non-interfering with the sample.
- **Translucent**
  The optimal material should be translucent so that captured particles can be seen or illuminated from the back side

Several candidate materials were discussed (agar or agarose gels, glass microscopy slides uncoated or covered in poly- trimethylene-carbonate [22] or vacuum grease) . The capturing device which was chosen was gel from Gel-Box AD-22T-00-X0 from Gel-Pak. Gel-Box is a plastic box with a snap lid and a bottom covered in sticky gel, made for carrying e.g. sensitive equipment. The composition of the gel is, according to the manufacturer, a highly cross-linked polymer. The main reason for choosing this material was because of its good capturing abilities and its translucency. The material is also easy to cut into pieces, it is autoclavable and was readily available in the lab.

A square of Gel-box gel is cut out of the box and placed on a glass microscopy slide. This slide can be held by a movable arm reaching into the interlock.

3.4.2 Shutter

A translation stage is added to the top of the chamber. A microscopy slide attached to a stick is connected to the z-axis of the stage, that is controlled by an electric drill. This setup makes it possible to quickly change the position of the covering slide either blocking or allowing the particle beam onto the capturing slide (see Figure 13) Together with the flow meter a very precise volume can be sprayed.

3.4.3 Interlock

The long venting and pumping times for the chamber is partly overcome by using an interlock system, which makes it possible to quickly get samples in and out of the chamber while it is being pumped. With some practice, the time to get samples from high vacuum to atmospheric pressure can be reduced to a few seconds.

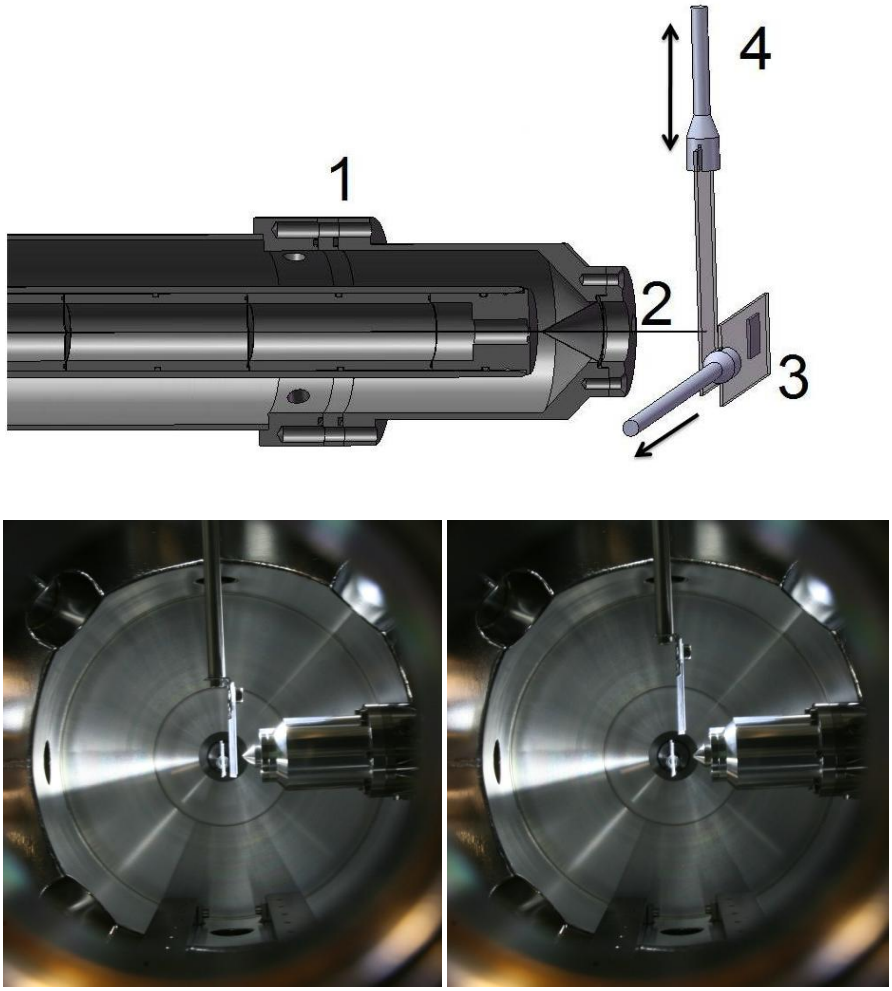

Figure 13: The shutter and capturing mechanism. Top: The injector(1) sprays particles(2) that can be interrupted from reaching the capturing slide (3) using the shutter(4). When the particles hit the capturing slide, a spot will form in the impact area. The slide can be moved out of the vacuum chamber very quickly using the interlock (see section 3.4). The injector setup and the distance is exactly the same as the one used for real imaging experiments (Compare to Figure 1). Bottom: The shutter mechanism seen from the side. Left is closed position, right is opened. Top: CAD rendering by Daniel Westphal, used with permission.

3.5 Microscopy

A fully automated Zeiss Axio Imager.M1 with a COLIBRI LED-module for fluorescence illumination was used for all microscopy. It is connected to a computer with Axiovision 4.6.3 software. Several microscopy techniques require deeper explanations:

3.5.1 Measuring cell concentration with a counting chamber

To determine the cell concentration in a liquid, a counting chamber was used. It is a special kind of multiple-use microscopy slide with a very well defined depth and a printed grid. The volume of every square in the grid is precisely known, and the number of cells/square can with an easy change of unit be expressed as cells/ml. The chamber used had a Neubauer Improved pattern, a depth of 0,01 mm and a square area of 0,0025 mm^2. According to [23], a minimum of 100 cells are required to get a statistical error of less than 20%. This usually means that the cell number in many squares must be counted. A standard pattern was used to select squares to remove any bias.

3.5.2 Merging multiple pictures

The stage that holds the microscopy slides is fully automated and monitored by the software. The exact stage position of every picture is known, and this makes it possible to merge several images into a single picture. This is important for imaging the whole impact area (see section 4.1.4). A setting with Overlap=0%, ScanMode=Meander, and several manually selected focus correction points were found to be optimal. For imaging spots sprayed on a Gel Box gel , 5*7 tiles of each 550µm*330µm was used. The exposure time must carefully be adjusted to the required application to avoid photo bleaching of the sample, but still capture enough light to actually see the cells. An exposure time of 500 ms was an optimal compromise for visualizing strain 22344 (data not shown).

3.5.3 Fluorescence

The COLIBRI unit together with the right filter provides easy access to light with known and well-defined wavelength. Filters can be used to limit the incoming light, thus allowing well-defined excitation. If the sample is fluorescent, it will emit light in a longer wavelength. With the correct filter, very specific excitation/emission properties can be examined. The Zeiss filters used in this project were the following:

- Filter set 20, excitation 546 +/-6 nm, emission 575-640 nm (filter useful for visualizing red fluorescence from Synechococcus cells and propidium iodide stain)
- Filter set 62 HE, excitation 370 +/- 20 nm, emission 395 +495+610 nm (multipurpose filter for visualizing fluorescence in green and red at the same time, optimal for SYTOX® Green staining of Synechococcus)
- Filter Set 38, excitation 470+/-20 nm, emission 525 +/-25 nm (filter useful for visualizing green fluorescence from SYTO®9)

3.6 Choice of model organism

Not all organisms studied in imaging experiments are suitable for development of methods to study viability. The organism must be easy to cultivate and study to allow fast method improvements. Almost any organism studied will be interesting in its own right, as long as the effects can be measured, in this early stage of sample delivery effect studies. Still, priority should be given to samples already studied with FEL imaging. Before publishing the calculated structure, it must be made likely that the organism was still in its native state and not altered or damaged in any way.

There will not be a single optimal model organism. This is not only because of the relevance to previous imaging experiments, but also because different organisms might be optimal for different investigation methods. The optimal cell model organism should have several attributes that will be discussed in sections 3.6.1 to 3.6.4. The organisms chosen and the reasons are presented in the “Experiments” chapter (section 4).

3.6.1 Cultivation and growth

All cells investigated must be cultivatable in liquid media. The growth must be reasonably fast, so that experiments can be planned and evaluated. This is most important for re-growth experiments. The easiest way to study the concentration of cells, if the number is too low for direct counting in a counting chamber, is to make a Colony Forming Unit-count (CFU-count, see section 3.7.1). For this study an organism that can easily switch between solid and liquid media is desired. All media with yeast extract or similar cell-debris matter will disturb nucleic acid staining techniques. The organism must be able to survive the transfer to a new non-interfering media.

3.6.2 Size and shape

The size is a major factor for injection behavior. The injector can be optimized for particles ranging in size from 20 nm to 3 µm. Some organisms are obviously unsuitable for this sample delivery method; these include filamentous bacteria and sensitive mammalian cells. A roughly homogenous population is also desired if effects are to be quantified. If the organisms in the same population have different properties to begin with it can be difficult to characterize the effects from sample delivery.

It is also important that the cells do not attach to each other. This is especially important when cell numbers are estimated by CFU-counts. If the cells under some conditions stick to each other, the number of cells that grow on a spread plate will be seriously underestimated as many cells will appear as one colony forming unit.

3.6.3 Knowledge/reliability

The cell must be must be stable in order to minimize the need for replicates. If cells are treated and compared to non-treated cells, it is very important that the comparison shows a real effect and is not diminished by normal variations in the population. It must also be known that the organism is non-pathogenic.

3.6.4 Detectablility

It is sometimes difficult to see non-treated cells in a light microscope. Many of the interesting model organisms are fluorescent by nature, as they are photosynthetic. Staining techniques may help visualizing otherwise invisible or hard-to-detect cells or virus particles.

3.7 Measuring viability

To just look at the organisms retrieved from vacuum with the microscope is not enough to say that they are unharmed by the sample delivery process. A number of methods can be used to determine the effects.

3.7.1 Regrowth on plates

One of the standard methods in microbiology is to transfer cells from liquid to solid media. By streaking the liquid evenly on the plate, the cells in the liquid will be randomly distributed. After incubation all cells that divide will eventually become a visible colony consisting of hundreds of thousands of cells. If the concentration is low enough, it will be possible to count the number of colony forming units per volume in the starting liquid. This number has often been taken as a direct measurement of the number of cells in the starting liquid. This assumption is not as obvious as often thought. Environmental genomics has proven that most organisms that appear in nature are actually uncultivable. Many organisms can only be cultured under very specific conditions, and even if they can grow with different media types, the switch between different culturing conditions in itself might be harmful. Research regarding this sensitivity has met an increased interest. [24]

Cells with metabolic properties like live cells that still lack the ability to reproduce unless very specific conditions have been fulfilled (viable but non-culturable, VNC-cells) have been discussed since 1984. These cells are obviously not countable with CFU-counting [25].

In an article from 2006 [24], the authors describe what harmful effects plating might have on bacteria that until that moment were viable. Three key factors have been identified: The increased oxygen pressure, hydrogen peroxide that has formed on the plate and the increased distance between bacteria. Even exponentially growing cells showed a lag-phase when plated, and

heat shock and oxidative-stress regulons were induced. This first lag phase was around 180 min long for the studied E coli, and independent of the initial cell concentration, the physiological state of the cells and the composition of the solid media. The authors perceive that the stress response gene activation show that this is a recovery time, and not a harmless adaption time. CFU counts are still useful as an estimate of the minimum number of cells in the plated liquid, even though not all of them will form colonies. If a CFU-count is performed before and after a treatment, the lost fraction will be an estimate of the treatments effect.

3.7.2 Plaque assay

Virus particles are most of the time too small to be visualized with a microscope. Even with staining techniques or with electron microscopy, the structure is difficult to see. Infectivity will rely on an intact structure, and this ability is the only thing that distinguishes a viable virus particle from a random collection of proteins and nucleic acids. One method that can be used for evaluating infectivity is called plaque assay. It measures the ability of the investigated solution to infect a carpet of host cells on a solid media plate. A single virus particle can start a chain of infection, reproduction, lysis and release of more virus particles that will be visible as a clearing where no cells grow. The starting suspension can have any concentration of virus particles, but it must be diluted before being streaked onto a plate. The discrete number of clearings on the plate is directly correlated to the number of infectious particles in the starting solution, the Number of Plaque Forming Units or the PFU-count.

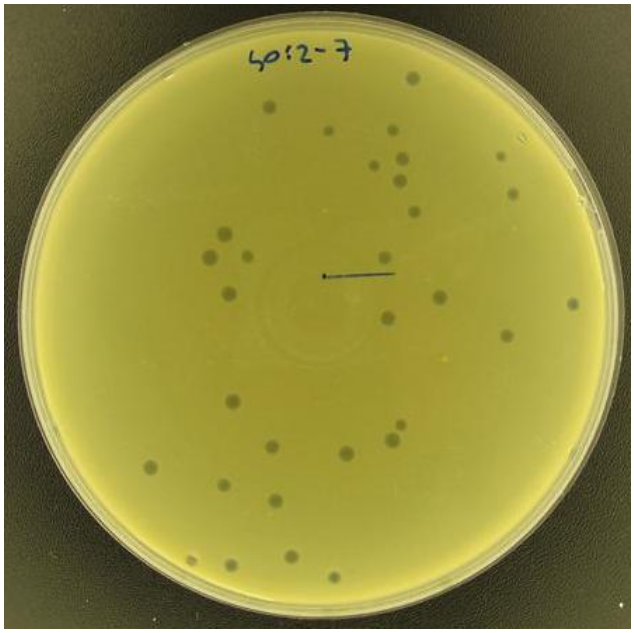

Figure 14: Plaque assay. Presence of infectious virus particles can be detected as darker clearings (plaques) in the yellow carpet of bacteria. The virus suspension must often be diluted to avoid interference between the plaques. This plate has 31 plaques. Photo: Dusko Odic, used with permission

3.7.3 Viability /membrane integrity measurement with stains

Fluorescent probes have been under increased research interest as a method to measure the viability of cells. A number of different stains are commercially available, with different target processes and properties [26]. Two different kits have been used in this study; LIVE/DEAD® BacLight™ and SYTOX® Green, both from Invitrogen™. Both contain nucleic acid binding stains that have

different membrane permeability properties depending on the membrane integrity of the target cells, which results in different colors for viable and non-viable cells.

3.7.3.1 LIVE/DEAD® BacLight™

LIVE/DEAD® BacLight™ from Invitrogen is a composite stain, with propidium iodide (PI) and SYTO®9. The two components in this kit will be excited at 485 nm and emit 530 nm (green) in live cells, and 630 nm (red) in dead, see Figure 15. The manufacturer has tested the stain with E coli.

It has been used in other studies with Synechococcus [27], and comparisons have been made to other stains. The authors report that the stain will be unreliable if the stain concentration is lower than the standard recommendation in the protocol [28]. Initial studies were initiated with this stain and *E coli* as a part of this project, but as no damage experiments with this organism was done during the project the full data will not be published here. It can be said that the stain works well with *E coli.*

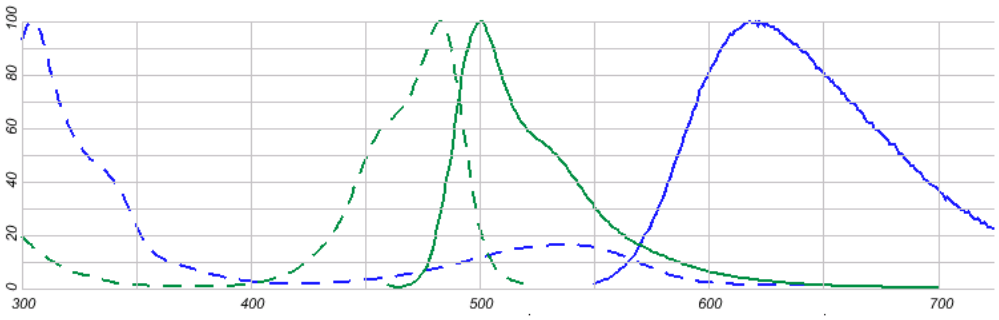

Figure 15: Excitation spectra for propidium iodide (blue) and SYTO®9 (green). The x-axis is wavelength in nm. The y-axis is normalized light intensity. The dashed lines represent the excitation energy absorbed by the stain, the filled lines represent the emitted light.

3.7.3.1.1 Staining protocol

The powder in the prefilled Pasteur pipettes containing the stain was dissolved in a suitable liquid according to the manufacturer's instructions. Filtered water from Antarctica was used for staining *Roseobacter*, distilled water was used for the other bacteria. Excitation with 555nm, filter set 20, was used to visualize propidium iodiode that stained the cells with damaged membrane integrity red. 470 nm and filter set 38 was used for SYTO 9, which stain living cells green. The sample was incubated at room temperature for at least 15 minutes. The final concentration was 6µM SYTO 9 and 30 µM PI.

3.7.3.2 SYTOX® Green

SYTOX® Green is a nucleic acid stain that will only penetrate damaged cell membranes. It is manufactured by Invitrogen and will be excited at 488 nm. It will emit light in 504 to 523 nm (blue-green), see Figure 16. The manufacturer has tested the stain with *E coli*. This stain has also been successfully tested with *Synechococcus*. [29, 30].

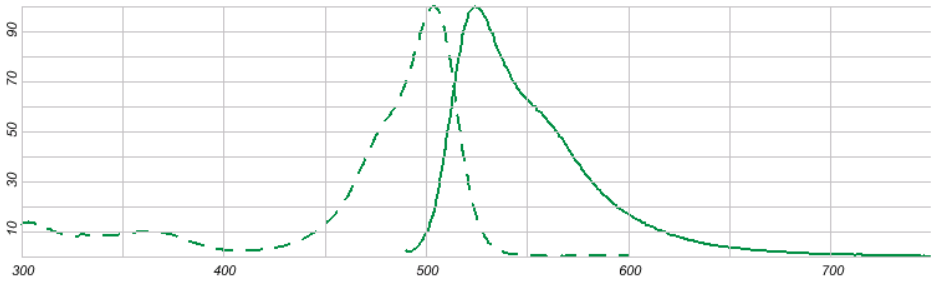

Figure 16: Excitation spectrum for SYTOX® Green. The x-axis is wavelength in nm. The y-axis is normalized light intensity. The dashed line represents the excitation energy absorbed by the stain, the filled line represents the emitted light.

3.7.3.2.1 Staining Protocol

The stain was prepared according to the manufacturer's instructions. The sample was incubated with the stain for at least 15 minutes. Excitation wavelength was 470 nm, filter set 38 was used and dead cells stained as green.

4 Experiments

The "Experiments" section of this report is split according to the organism used. Viability study of *Synechococcus elongatus* is described in section 4.1.2, transmission efficiency of the same species through the aerodynamic lens particle injector is described in 4.1.4. Re-growth studies of *Roseobacter* and *Escherichia coli* are described in sections 4.2.2 and 4.3.2, respectively. Finally, attempts to determine the potential loss of infectivity for *Cellulophaga baltica* Φ40:2 are described in section 4.4.2.

4.1 Experiments with *Synechococcus elongatus*

4.1.1 Background

Synechococcus elongatus and other planktonic cells have been used in real imaging experiments, and many diffraction images are waiting for reconstruction. This makes the evaluation of viability top priority, as viability data is needed to claim the relevance of the obtained structures.

The cyanobacterial genus *Synechococcus* contains many species that are abundant both in freshwater and in marine environments. The biggest advantage when working with photosynthetic cells is their autofluorescence. *Synechococcus* cells brilliantly fluoresce in red, which make them very easy to spot in a fluorescence microscope setup. This makes them especially well suited for direct count experiments, where the exact number of cells that are sprayed onto a surface is important. The cells are very easy to detect and count without tampering with them in any way. The autofluorescence might have a negative side-effect when fluorescent stains are used, as these may emit light in the same wavelength or even quench the signal from the probe. This was tested by a measurement in a FluoroLog® from Horiba Jobin Yvon, to get an understanding of the fluorescence spectrum at different wavelengths (see Figure 17). It was found that the autofluorescence would not interfere with SYTOX® Green stain but might interfere with LIVE/DEAD® BacLight™ stain. This was later confirmed by actual staining experiments (data not shown)

Compared with many other photosynthetic cells, 22344 is a strain of fast-growing, robust cells that withstand rough treatment. Compared to other bacteria used in laboratories, they stand out as very slow with their one-cell-division-per-day growth rate. Their extremely long adjustment time for changing between liquid to solid media (several weeks or more) also complicates experiments with this organism. The initial waiting time between streaking and the appearance of the first cultures is different for different cells, from a few days to several weeks. After a colony has started to grow, it might spread rather fast and possibly grow over smaller colonies. This makes it difficult, if not impossible, to determine the number of colonies (data not shown). It is possible to grow dense cell cultures in liquid media given a few weeks preparation time, and their fluorescence makes it easy to determine the cell numbers without bothering with plates and solid media.

Strain 22344 is grown in plastic bottles with bubbling of filtered air at 21°C in BG-11 media (see Figure 18)

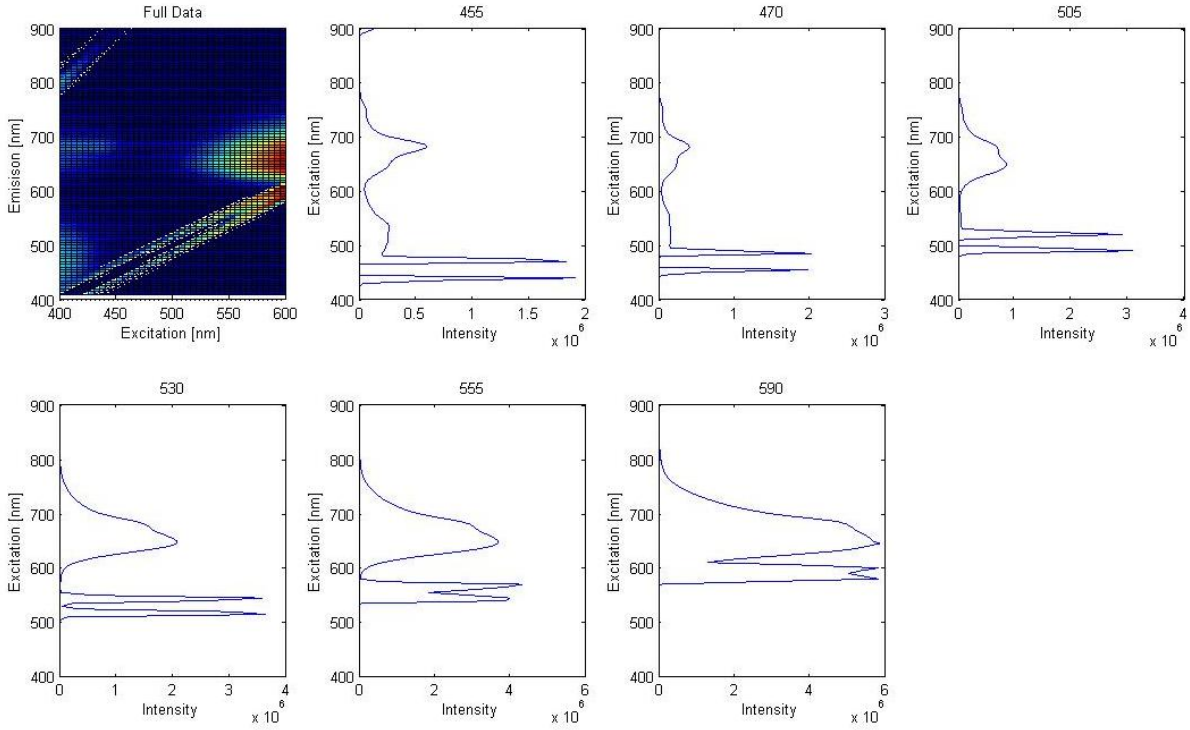

Figure 17: Autofluorescent spectrum for *Synechococcus elongatus* strain 22344, measured with a fluorescence spectrophotometer (see section 4.1.1). The top left is a full measurement of the whole spectrum (colormap: red is highest intensity, blue is lowest). The remaining charts describe the fluorescence at 455, 470, 505, 530, 555 and 590 nm, the available wavelengths for the microscope (see section 3.5.3). The incoming light is normally blocked to better measure the less intensive fluorescence. The slit size controlling the width of the incoming light was not calibrated at the time this measurement was done. This caused stray light that can be seen as unexplained peaks near the excitation wavelength.

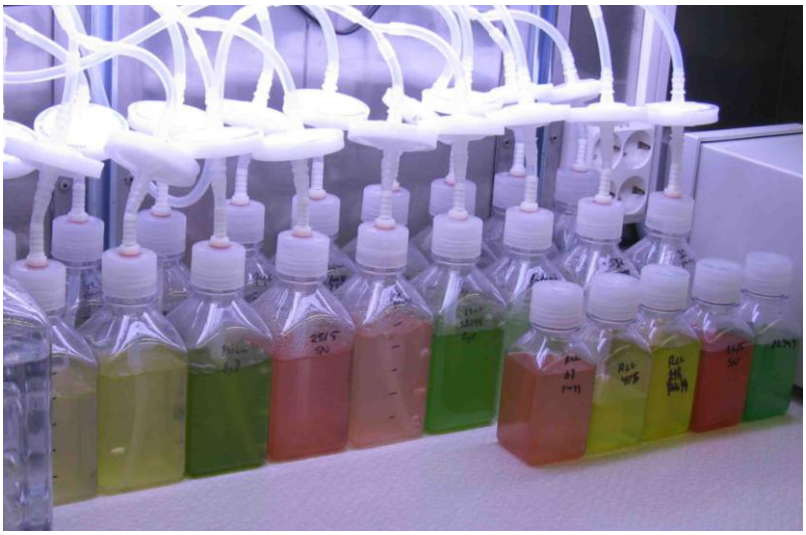

Figure 18: Cultivation of plankton. Different species of plankton may use different parts of the light spectrum for their fluorescence. This can be seen as different colors of the cultures. The tubes that go through the lids will pump filtered air into the cultures.

4.1.2 Viability studies

4.1.2.1 Principle

Synechococcus elongatus, strain 22344, is treated in different ways including spraying into vacuum. The cells were stained according to the description in section 3.7.3.2.

4.1.2.2 Protocol

1 ml *Synechococcus elongatus* growing exponentially with gas bubbling in BG-11 at 25°C was centrifuged at 6000 g for 10 min. The supernatant was removed and the pellet was resuspended in 25 mM 0.2 µm filtered Ammonium Acetate. The following treatments were compared:

- **Untreated**, starting culture
- **AmAc**, buffer exchanged into Ammonium Acetate (6000g 10 min)
- **Aerosolized dry under atmospheric pressure**, Sprayed 10 µl into empty test tube with the aerosolization nozzle, recovered with BG-11 media
- **Aerosolized into BG-11 under atmospheric pressure**, Sprayed 100 µl onto the liquid surface of a test tube containing BG-11 media with the aerosolization nozzle.
- **Short time vacuum exposure**, as low volume as possible sprayed into vacuum using the aerodynamic lens injector onto Gel Box gel with the parameters in Table 1
- **Long time vacuum exposure**, spraying for 30 min vacuum using the aerodynamic lens injector onto Gel Box gel with the parameters describe in Table 1

10 µl of each treatment was stained with 10 µl staining solution(500µl BG-11 +1µl Sytox Green stock). The samples were stained and evaluated according to the description in section 3.7.3.2. One microscopy slide was prepared for each sample. A number of photographs were taken of each slide. The total number of cells and the number of cells unaffected by the staining was counted from these pictures for each treatment.

Parameter	Value
He pressure	500 psi
N2 pressure	225 psi
Chamber pressure	4.0*10^-6 mbar
Injector pressure	1.0*10^-3 mbar
Relaxation chamber pressure	5.7 mbar
Nozzle box position	Unknown
Liquid flow rate	4.8 µl/min

Table 1: Experimental parameters for the sample delivery system in experiment 4.1.2

4.1.3 Results

There is a considerable fraction of damaged cells in all measurements, including the starting culture. The cells that have been aerosolized and left to dry under atmospheric pressure had the lowest number of unaffected cells. There is not a significant decline in affected cells due to vacuum exposure (Table 2).

Treatment	Total number of cells	Stained as damaged	Unaffected	Fraction unaffected
Untreated	393	248	145	36.90%
AmAc	287	178	109	37.98%
Aerosolized dry under atmospheric pressure	83	76	7	8.43%
Aerosolized into BG-11 under atmospheric pressure	324	218	106	32.72%
Short time vacuum exposure	82	49	33	40.24%
Long time vacuum exposure	23	11	12	52.17%

Table 2: Damage study of Synechococcus elongatus strain 22344, experiment 4.1.2 The cells were exposed to vacuum for a short time after aerosolization and injection onto a capturing Gel Box gel.

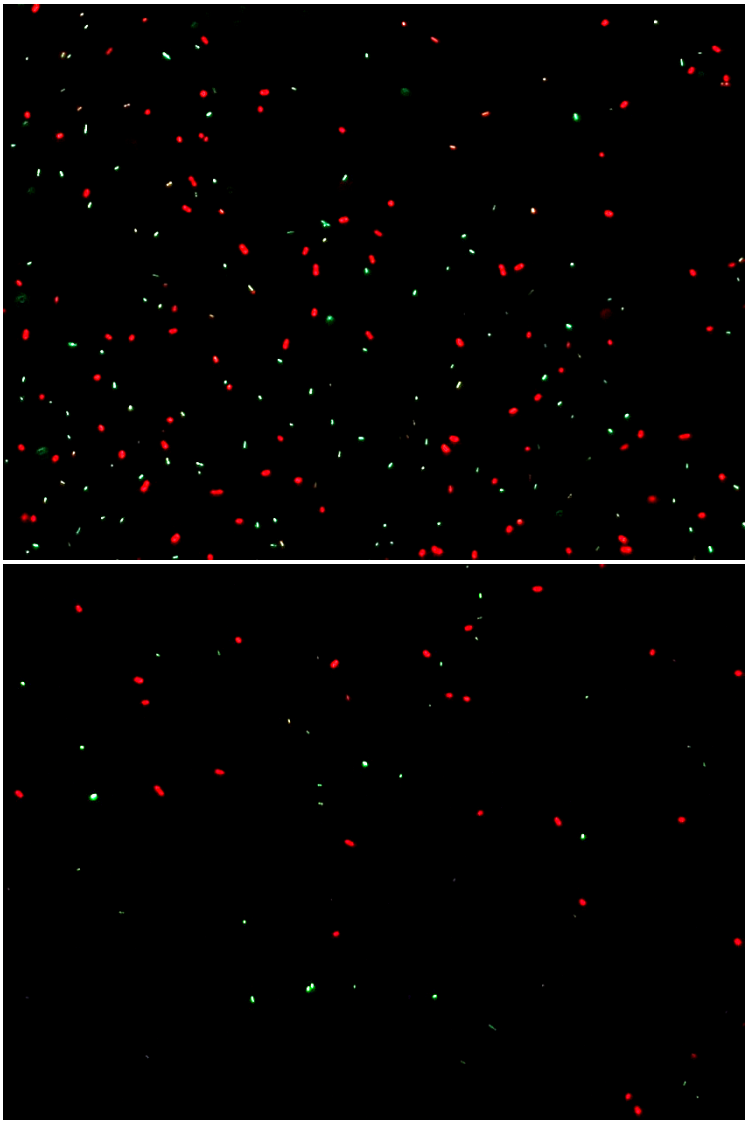

Figure 19: *Synechococcus elongatus* strain 22344 stained with SYTOX® Green stain, using excitation at 470 nm and 590 nm with filter set 62. Unaffected cells are red due to autofluorescence, stained cells appear green. Top: Starting culture. Bottom: After injection into vacuum.

4.1.3.1 Conclusions

The cells seem to be unaffected by the sample delivery method. The small rise in undamaged cell numbers is probably a statistical error, due to the low cell numbers counted. The reason for this was the dilution that was a result from the recovery from the capturing slide. It can be concluded that drying on a solid surface after aerosolization is harmful.

This experiment must be reproduced to better quantify the effect on membrane integrity.

4.1.4 Transmission efficiency of the injector

The number of diffraction patterns collected is dependent on the X-ray pulse rate and the rate at which the sample is injected into the interaction region. Two parameters can affect the number of samples in the interaction region, the concentration of sample injected and the ratio of lost particles in the injection process. Many parameters are expected to influence the transmission efficiency of the injector, and this experiment evaluates a method to investigate this. The method

must be as quick as possible to minimize the time required, as many experiments with varying conditions will be needed to make a full model.

4.1.4.1 Principle

A low and known concentration of *Synechococcus elongatus*, strain 22344, is injected onto a Gel-box gel in vacuum. A picture of the whole resulting spot is generated using the controlled stage and the picture merging capabilities of the automated microscope (see section 3.5.2). The experimental parameters are varied during the experiment to optimize the efficiency.

4.1.4.2 Protocol

1 ml *Synechococcus elongatus* growing exponentially with gas bubbling in BG-11 at 25°C was centrifuged 6000 g for 10 min. The supernatant was removed and the pellet was resuspended in 25 mM 0.2 µm filtered Ammonium Acetate. The cell concentration was determined with a counting chamber (see section 3.5.1). A known volume was sprayed onto a microscopy slide with Gel box gel (see section 3.4). With a flow rate of around 5 µl/min the cells were in vacuum for at most around 2 minutes and 30 seconds until they were taken out to atmospheric pressure. The resulting spot was evaluated with the automated microscope (see section 3.5.2).

4.1.4.3 Results

The injection parameters have a strong influence on the fraction of successfully transmitted cells. The maximum transmission efficiency was 34 % (Table 3).

	I	II	III	IV	V
He pressure [psi]	350	700	700	700	500
N_2 pressure [psi]	225	225	225	225	225
Chamber pressure [mbar]	6.3*10^-6	7.4*10^-6	6.3*10^-6	3.4*10^-6	3.4*10^-6
Injector pressure [mbar]	8.6*10^-3	1.6*10^-2	1.6*10^-2	7.4*10^-3	6-3*10^-3
Relaxation chamber pressure [mbar]	4.5	130	110	4.6	3.5
Nozzle box position	Unknown, not tightest	Tightest possible	Tightest possible	¾ turn out	¾ turn out
Cell concentration in injected liquid	7*10^4/ml	7*10^4/ml	7.81*10^4/ml	7.81*10^4/ml	14.5*10^5/ml
Liquid volume injected	10 µl	10 µl	10 µl	10 µl	11µl
Theoretical number of cells injected	700	700	781	781	1600
Number of cells captured after injection	200	78	101	157	539
Fraction captured cells of theoretical number	29%	11%	13%	20%	34%

Table 3: Transmission efficiency of the injector for varying experimental parameters.

4.1.4.4 Discussion

The maximum transmission efficiency was around 30 %. This number can probably be improved by further systematic investigations, as even a small variation of parameters could almost triple the efficiency.

The method to investigate transmission efficiency is reasonably fast, but the sample preparation time and the manual count of cells are limiting factors. Automated count software could probably decrease the time required to do an experiment down to around 15 minutes.

A prerequisite for this investigation was that the cells investigated can be visualized in the microscope. Strain 22344 loses much fluorescence intensity when the cells are drying in open air, and this process takes different time in different cells. Photo bleaching by the fluorescent light will bleach some cells within minutes (data not shown). This effect is very strong for cells that have been exposed to vacuum for a long time. Automated microscopy and the quick retrieval of sprayed samples into atmospheric pressure by using the interlock made it possible to avoid this loss of intensity. The exact spray volume was achieved by the combined use of the exact flow meter and the quick shutter.

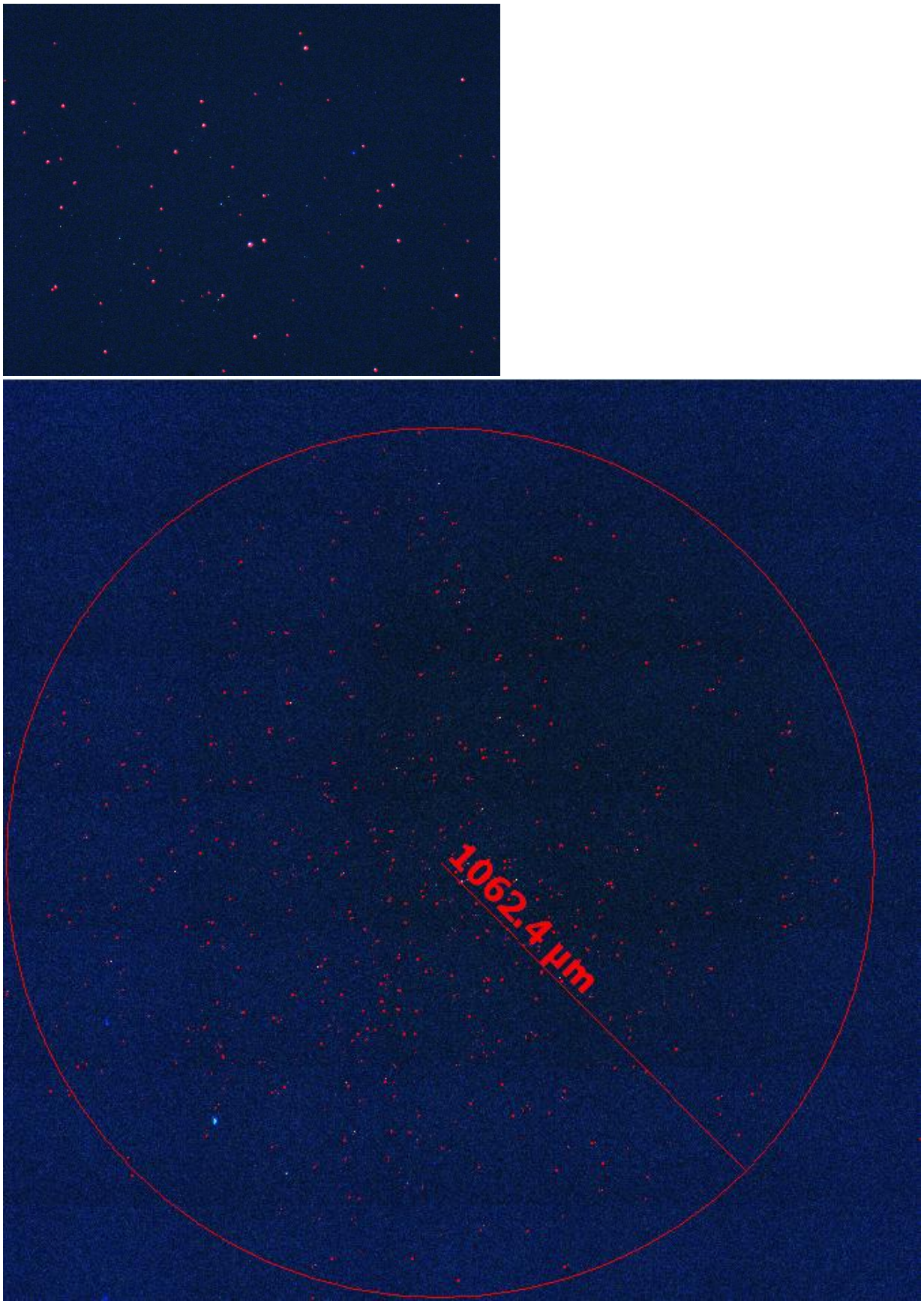

Figure 20: Transmission efficiency investigation using *Synechococcus elongatus* strain 22344. Top: Fluorescent cells on Gel-Box gel. Excitation 470 nm and 590 nm, filter set 62. Bottom: 35 pictures are merged into one collage of 6940x7280 pixels covering the whole spot. 539 red spots represent the counted particles.

4.2 Experiments with *Roseobacter*

4.2.1 Background

Roseobacter has not been used for FEL imaging experiments. *Roseobacter* is a group of gram-negative marine bacteria found in seas all over the world. Sequencing of 16s ribosomal DNA from seawater samples reveals that they are one of the most abundant bacteria in the marine waters (up to 30 % of all found sequences in one study). The metabolic versatility in the group has been the subject of many studies. The strain used in this project was isolated from southeastern United States coast-waters in a study that implied that *Roseobacter* have a major role in the sulfur cycling in the oceans. All strains isolated could degrade the sulfur compound dimethyl sulfoniopropionate into dimethyl sulfide, and many of the isolated strains could carry out other key processes [31]. The *Roseobacter* used here have an orange pigmentation, a size of around 1-2 µm and slightly elongated shape. The exact strain used is unknown, but it is of the GAI lineage (personal communication with Alexander Eiler).

Roseobacter are grown in Marine Broth, or MB-media, a yeast-extract and peptone based media with added salts to reach a marine salinity. For liquid media Difco™ Marine Broth 2216 was used, for solid plates Difco™ Marine Agar 2216. *Roseobacter* can grow on both media types and switch between them. The culturing time is a few days, both in liquid media and on plates. Different growth temperatures were examined, growth at 37°C was significantly faster than growth at 25°C. The ability to switch between liquid and solid media is very desirable, as cell concentration can only be quantified in liquids before injection (counting chamber) and with plating afterwards(diluting and streaking on solid media plates).

Many attempts were made to stain *Roseobacter* cells with the methods described in section 3.7.3, but the media interfered heavily with the staining, causing a severe background with stained particles the same size as the cells. When attempts were made to change buffer by centrifugation, the cells adhered to each other in large complexes, making quantification impossible. The cells grew significantly slower in filtered media (data not shown). The combination of the above led to the use of *E coli* as a model organism.

4.2.2 Regrowth of *Roseobacter*

4.2.2.1 Principle

A high concentration of *Roseobacter* is injected into vacuum with the sample injector. The injected cells are captured on a Gel-Box gel, recovered from vacuum and spread on solid MB plates. Any colonies formed on the plates will indicate survival.

4.2.2.2 Protocol

Roseobacter inoculated from a single colony growing on MB-plate was transferred into 100 ml liquid MB-media and grown for 2 days at 37 °C. 1 ml was centrifuged at 6000 g for 10 minutes. The supernatant was removed, and the pellet was resuspended in 25 mM 0.2 µm filtered Ammonium Acetate. This liquid was injected and the resulting spot was retrieved with Tris Buffered Saline (TBS) and spread on MB-plates. Four plates were prepared and incubated at 37°C.

Parameter	Value
He pressure	500 psi
N2 pressure	225 psi
Chamber pressure	2.5*10^-6 to 7.4*10^-6 mbar
Injector pressure	6.3*10^-3 mbar
Relaxation chamber pressure	3.4 to 3.5 mbar
Nozzle box position	¾ turn out

Table 4: Experimental parameters for the sample delivery system in experiment 4.2.2

4.2.2.3 Result

All plates were covered in colonies after 2 days.

4.2.2.4 Conclusion

Roseobacter are still viable after injection into vacuum and quick recovery to atmospheric pressure. This is a very important result for future free electron laser imaging experiments, as it can be claimed that the cells are still alive when they are imaged. The exact fraction of surviving cells is unknown. Further studies are needed to determine this number, with known starting cell number and dilution of the recovered cells. This would also require quantification of how the buffers used affect the viability, and an investigation of how many viable cells in liquid survive the transfer to plates. To determine the number of viable cells in liquid, a staining method must be developed, and this has proven to be difficult (data not shown).

4.3 Experiments with Escherichia coli

4.3.1 Background

Escherichia coli have not been used for any free electron laser imaging experiments, but the non-pathogenic wild-type strain K(12)λ F+ proved to be a good organism for this type of study. This strain was originally isolated from the intestines of a patient in the 1920s in Palo Alto, California, and has been the subject of many bacterial genomic studies (Personal communication with Karin Carlsson). *E coli* cells are rod shaped and around 2 µm long. Colonies growing on plates are white to yellow pigmented. It grows very fast, with an optimal doubling speed of 20 minutes.

The cell shape of this strain is very uniform during growth. This is a major advantage in relation to many other strains available in the laboratory, that often have a subpopulation of cells that continues to grow into extremely elongated cells (sometime up to tens of µm) instead of dividing. This would have been very unfavorable for this study (see section 3.6.2). (Data not shown for strain selection experiments).

E coli can be grown both in both liquid and on solid LB media (abbreviation for Lysogeny broth media or Luria Bertani media, a mixture of glucose, yeast extract and salts. Liquid LB can be made into a solid plate when mixed with agar). *E coli* can readily switch between growth on plates and in liquid. When growing *E coli* on plates, plates with solid TBA media give a higher and more reliable CFU count than LB-plates. The recovery rate from liquid LB media to TBA plates is very good but not fully 100% for optimal conditions (data not shown).

4.3.2 Regrowth of *E coli*

4.3.2.1 Principle

A low and known concentration of *E coli* is injected into vacuum with the sample injector. The conditions should be as similar to beam-time conditions as possible. The injected particles are collected on a gel, and plated on solid media plates. The number of growing colonies is compared to various control experiments to confirm the number of surviving bacteria.

4.3.2.2 Protocol

100 ml LB media was inoculated with *E coli* from a culture growing in liquid LB media in stationary phase. It was grown overnight at 24°C, 200 rpm in a Stuart Scientific Orbital Incubator SI50. Growth was monitored by OD at 600 nm. The culture was repeatedly diluted to half volume during a few hours to establish an absorbance region corresponding to exponential growth of the culture. 1 ml of an exponentially growing culture was centrifuged 5000g for 10 min. The supernatant was removed, and the pellet was resuspended in 25 mM 0.2 µm filtered Ammonium Acetate. The cell concentration was counted with a counting chamber (see section 3.5.1). The sample was diluted with a factor of 50 000 with Ammonium Acetate (same concentration). 10 µl of this liquid was spread on TBA-plates. 5 µl was sprayed directly onto TBA plates using the aerosolization nozzle (see section 3.2). 10 µl was sprayed on Gel Box gel under high vacuum using the injector (see section 3.2). The resulting spot was recovered with 100 µl TBS and was spread on TBA-plates. The remaining liquid was recovered from the sample holder and spread on TBA-plates. 10 µl of the starting culture was spread of TBA-plates, after 3 hours on the bench. 100 µl TBS from the same test tube was spread on a TBA-plate as a control. All plates were incubated at 37°C.

Parameter	Value
He pressure	500 psi
N2 pressure	225 psi
Chamber pressure	2.2*10^-6-3.4*10^-6 mbar
Injector pressure	3.4*10^-3 mbar
Relaxation chamber pressure	1.8 mbar
Nozzle box position	¾ turn out

Table 5: Experimental parameters for the sample delivery system in experiment 4.3.2

4.3.2.3 Results

No growth on the control plate with only TBS media without cells. A number of colonies were growing in sample retrieved at each step in the process, including after injection into vacuum (Table 6,Figure 21). The TBA plates that were sprayed directly upon could not be counted reliably, as the number of colonies was too high (>200) and to some extent grown together. It can be noted that the lag time before any cultures were seen on the samples injected into vacuum was over 2 days, whereas all other samples were visible after overnight incubation.

Starting culture	Starting culture after 2 h	Retrieved from sample holder	Injected into vacuum
81	118	67	120
37	89	177	42
31	99	220	351
	113	132	110

Table 6: CFU count on TBA plates for experiment 4.3.2

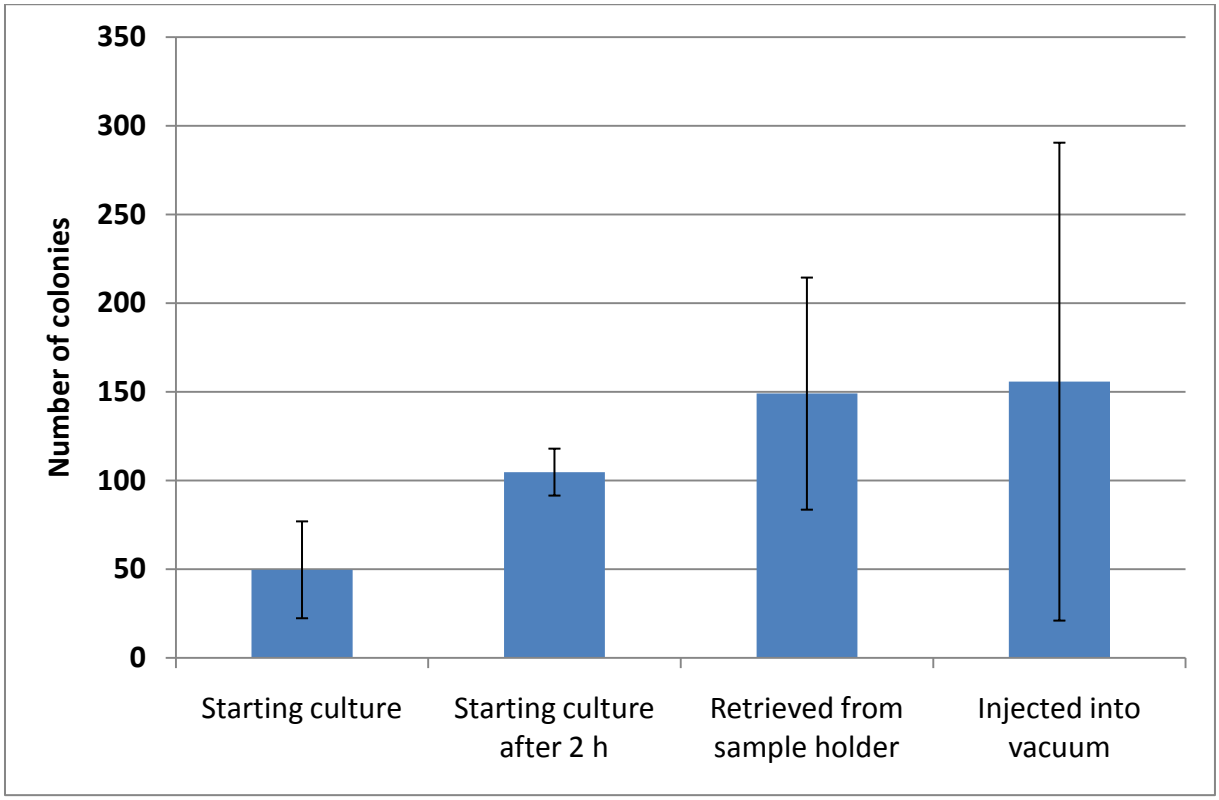

Figure 21: The number of growing *E coli* colonies after different treatments. The staples are the average number of colony forming units, the error bars represent the standard deviation. It can be noted that there is a very high uncertainty. (4.3.2)

4.3.2.4 Conclusions

E coli are still viable after injection into vacuum. There are some anomalies in the data, the most prominent being that the number of growing colonies after injection is larger than before from the same volume. Contaminating bacteria were present both in the liquid retrieved from the sample holder, the aerosolized liquid and the retrieved spots that has been injected into vacuum. The shape and color of the dominating colonies are like *E coli* colonies, and the bacteria have *E coli* shape when investigated with microscope. The larger number of colonies can be explained by a contaminated filter in the sample holder from previous experiments. The control plate shows that the TBS buffer was not contaminated.

The higher CFU in the liquid that has been standing on the bench can be explained with dividing bacteria during the experiment, even though the buffer is free from nutrients. The internal intracellular storage of energy was apparently enough for the bacteria to divide at least one time. This is important to keep in mind for future studies where the number of cells is counted in the starting culture.

As the transmission efficiency experiment with *Synechococcus elongatus* shows (see section 4.1.4), the transmission efficiency may be below 100%, this has to be evaluated with *E coli* as well.

The longer lag time is probably a result of some kind of stress (compare to section 3.7.1). This reaction must be further investigated before any definite answers can be given regarding the viability of *E coli* after short time vacuum exposure.

4.4 Experiments with *Cellulophaga baltica* phages

4.4.1 Background

Virus particles are good model samples for imaging experiments because of the symmetrical structure that simplifies reconstruction and the possibility to spray highly concentrated liquid containing identical particles. These phages have been studied by FEL imaging experiments, making evaluation of sample delivery effects a top priority project. The protocols for buffer exchange and injection are known, and needs to be evaluated to know the relevance of the structural data.

Recent studies have shown that the role of marine bacteriophages is very important. The interactions between bacteriophages and bacteria form a complex and ever changing ecosystem, where the turnover is very high. The lysed bacterial cells are a major source of dissolved organic matter that is used by other organisms to grow [32, 33]. Recent environmental genomic studies have shown that the ocean has an extreme abundance and diversity of viral genomes [34] and the number of phage particles is very high, making marine viruses the probably most common biological object in the oceans [35]. Two strains of marine phages are currently cultivated in the Molecular Biophysics laboratory in Uppsala, Φ40:2 and Φ4:1. They were isolated from the Baltic Sea together with their host cell, Cellulophaga baltica [36].

This study was made with pre-purified virus particles made by colleagues at the lab according to the following protocol: *Cellulophaga baltica* are grown in MLBss (a tryptone / yeast extract based medium with added salts). Virus inoculum is added to the host cells at a multiplicity factor of 0.1 and grown for 2 days. The virus particles are harvested and purified by centrifugation, filtering, ultracentrifugation, cesium chloride gradient ultracentrifugation and dialysis with MSM buffer. Buffer exchange to 25 mM ammonium acetate has been done with the ultrafiltration device Vivaspin 2 from Sigma-Aldrich®.

4.4.2 Infectivity studies

4.4.2.1 Principle

The key parameter for describing virus viability is the infectivity. The infectivity was measured at different steps in the sample delivery process. The first series of experiments tried to quantify the effects from the initial steps, and the final experiment tried to qualitatively determine the effects of the whole process.

4.4.2.2 Initial sample handling steps

Cellulophaga baltica phage Φ40:2 was treated according to conditions emulating those used during real imaging experiments: centrifuging, buffer exchange and exposure to Ammonium Acetate (AmAc). The infectivity is measured with plaque assays (see section 3.7.2). The virus particles are initially suspended in MSM buffer that must be exchanged into a volatile buffer for imaging experiments. This was done using centrifugal filter concentrator, Vivaspin 2 MWCO 10,000 from Sigma-Aldrich®, spinning speed 1000g. Different methods to retrieve sample and different numbers of buffer exchanges were investigated. AmAc concentrations tested were 2 mM, 250 mM and 2M. This was compared to retrieval into MSM buffer and buffer exchange by dialysis (1 ml of virus in original buffer into many washes of 200 ml 25 mM AmAc during 2 hours). Virus stored in bright light was compared to virus particles stored in the dark.

4.4.2.3 Full injection experiment

Initial experiments using plaque assay with diluted liquid (method explained in section 3.7.2) to evaluate possible loss of infectivity after injection into vacuum gave unreliable results. It was decided to do a qualitative study with a non-diluted sample.

Plates with a carpet of *Cellulophaga baltica* #3 host cells were prepared. These were divided into fields where differently treated virus samples were applied. A virus suspension, approximately 10^9 PFU, was diluted 1:100 in 25 mM AmAc. Methods are seen in Table 8. The experimental parameters used can be seen in Table 7. Because of the limit time available, an aerodynamic lense theoretically optimized for larger particles was used. The results are summarized in Table 8. Clearings were found on plates with starting solution, the plate sprayed by aerosolized virus particles, and the plate with virus suspension droplets covered by Gel Box Gel. No clearings were found where the virus particles had been exposed to vacuum, virus free AmAc solutions or Gel Box gel.

Parameter	Value
He pressure	600 psi
N2 pressure	225 psi
Chamber pressure	1.9*10^-6 mbar
Injector pressure	4.6*10^-3 mbar
Relaxation chamber pressure	2.4 mbar
Nozzle box position	¾ turn out

Table 7: Experimental parameters for the sample delivery system in experiment 4.4.2.3

Buffer exchange into AmAc	Virus	Volume	Method	Clearing
	x	10µl	Droplet directly onto plate	Yes
x		10µl	Droplet directly onto plate	No
x		5 µl	Sprayed onto plate, atmospheric pressure	No
	x	5 µl	Droplet directly onto plate	Yes
x	**x**	5 µl	Droplet directly onto plate	Yes
x	**x**	Several replicates, different volumes	Droplet directly onto plate, covered with Gel Box Gel	Yes
x	**x**	5 µl	Sprayed onto plate, atmospheric pressure	Yes
x	**x**	10 µl	Injected into chamber onto Gel Box Gel, upside down on plate	No
x	**x**	50 µl	Injected into chamber onto Gel Box Gel, upside down on plate	No
x	**x**	10 µl	Injected into chamber onto Gel Box Gel, flushed with MSM buffer, the buffer	No
x	**x**	10 µl	Injected into chamber onto Gel Box Gel, flushed with MSM buffer, the gel	No
no buffer			Gel Box Gel directly on plate	No

Table 8: Method and results for experiment 4.4.2.3

4.4.2.4 Conclusions

The data from the first experiments (4.4.2.2) are not complete and often contradictory. This makes interpretation very difficult. In order to get an understanding of the statistical reliability of the plaque assay method, sample diluted to different orders of magnitude are compared (see section 3.7.2). Often, these numbers varied even between replicates from the same test tube. A few results can be derived from this study. There is a strong correlation between sample retrieval and the number of infectious particles in the retrieved solution. The best method was to flush very gently and in several steps, to make sure that all virus particles left the filter. The AmAc concentration was a factor, lower concentrations were generally better. No significant difference could be seen for infectivity after a few hours exposed to bright light in 25 mM AmAc compared to sample that had been centrifuged in the same way but exchanged back into MSN buffer.

The phages are still infectious after aerosolization. No infectious phages were found after injection into vacuum, even when non-diluted sample was investigated qualitatively (4.4.2.3). These results might imply that infectivity is lost during injection, that the injector fails to focus the particles, or that the recovery process damages the particles. More experiments are needed to give a good answer. Staining experiments can show if the injector focuses the virus. Drying/recovery experiments in atmospheric pressure can show if the recovery process is harmful.

5 Discussion

5.1 Conclusions

The first imaging experiments at LCLS are yet to be fully evaluated, since the data is still under processing. It is not within the scope of this project to discuss the image reconstruction process and algorithms. The number of collected diffraction patterns was high, so the first part of the project must be considered to be successful even though the lysozyme microcrystals were not used.

The aim of the second part of the project was to develop methods to investigate the effects of the sample delivery systems on biological samples, and to use these methods to investigate and optimize the system. There are many parameters that can affect the viability of the samples prior to investigation with FEL imaging, and this work has established methodologies for answering many questions. The exact numbers are admittedly not statistically secure enough to be relied too much on, but some qualitative conclusions can be drawn.

A significant proportion of all injected cells shows no sign of membrane integrity damage or can be regrown after injection into and exposure to high vacuum for two minutes. This is much longer then the time spent in vacuum during sample injection for imaging.

Staining bacterial cells is a good way to measure damage on cells, this method works especially well for plankton that is growing in media without disturbing components. For bacteria that can grow both in liquid and solid media, regrowth experiments on plates is a good way to measure the fraction of cells that still have the ability to divide. Both of these methods are well established in literature (sections 3.7.1, 3.7.3), and are good indicators that the cells have maintained viability. Of the three criteria established in section 1.4 (structural integrity, ability to reproduce and maintained metabolism), the first two were shown for at least one organism. The long lag phase in the regrowth experiment of *E coli* cells might imply a disturbance that must be further investigated.

Transmission efficiency can be evaluated with a combination of a fluorescent diluted sample, interlock, shutter, gel box gel capturing and automated microscopy.

The virus experiments show that the particles are intact during the first steps of sample preparation, including aerosolization. It is unclear if or how long the particles are intact when injected into vacuum. A possible explanation to the inability to find infectious particles is that they might be lost in the injector. As described in 3.2, the aerodynamic lenses used in this experiment were theoretically optimized for far larger objects. Even if the viruses were focused and retrieved, they might just as well have lost their infectivity in the impact or during the far longer vacuum exposure than the millisecond exposure under real imaging experimental conditions.

The importance of studying effects from sample delivery must not be underestimated when it comes to the relevance of the obtained structures. This project has successfully shown that sample delivery of viable objects with an aerodynamic lens injector is possible.

5.2 Further studies

If the same culture is examined with CFU-counts, membrane integrity and metabolic capability staining and structural studies with microscope before and after injection into vacuum, a total picture of sample delivery effects for the same sample will be established . This experiment is within reach using the methods described here, but was not done during this project due to lack of time.

Many of the experiments presented here were done under non-sterile conditions, and a means to sterilize the equipment used will greatly improve the reliability of the obtained data. This is

also important for future imaging experiments where potentially pathogenic organisms may be studied. The data set is also too small to get an understanding of the statistical reliability of the results. Further experiments and replicates must be done to get an exact quantitative understanding of the spraying effects.

The quantitative reliability of the staining must be further investigated. A correlation study between membrane damage and re-growth of samples injected into vacuum has been started with E coli as the model organism, but it has not been finished yet. Studies investigating damage to the metabolism may give further insights into the nature of the sample delivery effects.

The methods used here are in many cases generic, and can be adapted to many organisms. A quick survey with staining can probably be used for most bacteria. All bacteria that can grow both in liquid media and on plates can be examined with regrowth methods. There are other injectors with similar configuration used by other groups, and the methods used here can without changes be used to compare these injectors to each other to better understand the underlying principles.

The methods for transmission efficiency can be used to further optimize the injector, but this will require much time. To evaluate the particle losses for all cell types would require reliable staining methods. A way to directly measure particle number and the shape of the particle beam in real-time is very high up on the wish list for the experimentalists in this field, especially if this could be used during actual imaging experiments.

Effects on virus particles have been studied in this project, and the results may indicate that the particles are damaged when sprayed with the injector. Still, the method to study this has many potentially harmful steps (the most prominent being the impact on the capturing slide) that must be eliminated.

This study shows that it may take time to develop a way to characterize the effects from sample delivery on the sample studied. Each organism may pose its own problems that must be overcome. The transmission efficiency experiment shows that the experimental procedure and parameters must be developed experimentally for each imaging experiment with a given organism.

According to the scientists in the field, the importance of X-ray Free-Electron Laser imaging will only increase within the near future. As only two facilities exist in the world today, research time is a very scarce resource. There are several FEL facilities under construction today, and within a few years there will be several sources with beam characteristics suitable for single-protein imaging. Soon, the structure of many virus capsids will be determined, and the results from these studies might help explain many diseases. Internal features of cells will be studied, and this project has shown that it is possible to deliver alive and viable cells into the beam.

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