Sample Delivery Method Optimisation for X-ray Serial Crystallography Experiments

Agathe Depraz Depland, University Claude Bernard Lyon 1, France

Jana Wittmann, University of Heidelberg, Germany

Supervisor: Dr. Dominik Oberthür, CFEL, Germany

September 4, 2019

Abstract

In this report several steps involved in a typical X-ray serial crystallography experiment are described. The main focus is on the optimisation of current techniques used for sample delivery at beamline experiments, such as EuXFEL, PETRA III or LCLS. First, with an introduction of a mobile set-up for nozzle sample delivery and then with an optical "catch and release" set up. We will also share our common experiences from beamline experiments in which we participated, assisting international collaborators during their shifts.
This report has been written equally by both authors as we collaborated on the same projects, and shared the same tasks during this program.

Contents

1 Introduction

Structure determination of macromolecules is crucial for understanding chemical mechanisms underlying biological functions. Thus, solving a protein's structure plays a central role in many fields, including cell and molecular biology, chemistry and drug development in medical research for example.
Different approaches such as electron microscopy (EM), nuclear magnetic resonance (NMR) or X-ray crystallography have been developed to determine molecular structures experimentally[1], each having different advantages and disadvantages. For the high resolution necessary to gain insight in the structure of macro-molecules small wavelengths corresponding to high energies are used in X-ray crystallography and electron microscopy. These high energies lead to radiation damage which is the fundamental limit of these techniques. Nowadays, X-ray crystallography is by far the most widely used method - with approximately 90 % of the structures added to the protein database in 2018 determined using X-rays diffraction methods[2].
In general, structure determination is a two phases process consisting of, an experimental part and a computational data analysis and refinement part[3]. In X-ray crystallography the first step of an experiment is the purification and crystallisation of the sample, where molecules of interest self-assemble into periodic single crystal lattices under specific conditions. Afterwards, the sample has to be delivered to an X-ray beam, which nowadays mainly are monochromatised and focused synchrotron or free-electron laser (FEL) beams. As X-rays are not much bend by matter, building focusing optics, and thus X-ray microscopes with sufficient resolution to determine molecular structures of big molecules, has not been achieved so far. For this reason, the X-ray's intensity of the scattered beam is detected. The reciprocal space images obtained in that way are transformed back into real space using a several step computational procedure to obtain the molecular structure.
The main focus of the work presented here is the delivery phase in such an experiment, but also the process and setup of X-ray crystallography experiment and the computational stage will be explained in more detail in the background section (section 2).
Then, different setups to improve the sample delivery pipeline and a beamline experiment will be introduced: the mobile nozzle setup which has been designed and constructed to support the sample delivery at X-ray experiments and an optical catch and release setup. This can be found in the part on 'sample delivery optimisation' (section 3). The second half of the main part (section 4 'beamline experiments') contains a report of our experiences at the Petra III and XFEL beamtime. Lastly, a conclusion will be drawn.

2 Background: Steps in X-ray diffraction experiments

Performing X-ray diffraction experiments includes multiple challenging steps of which an outline will be given in this section. A schematic overview of a typical X-ray diffraction experiment to determine the structure of macro-molecules can be seen in figure 1.

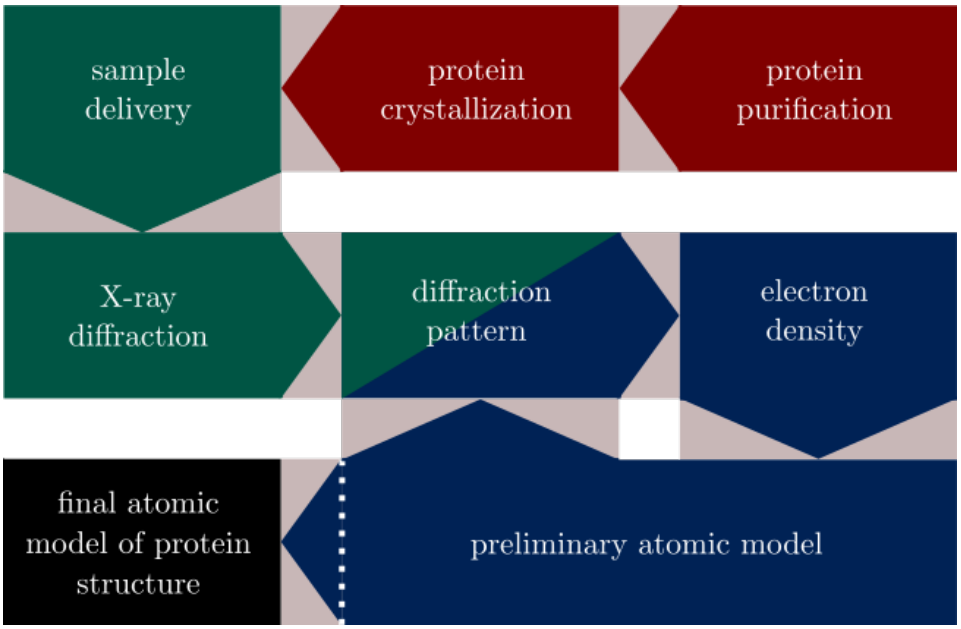

Figure 1: Overview of steps in an X-ray crystallography experiment: The sample preparation steps are marked in red, steps performed at the beamline experiment in green and the data analysis part is coloured blue.

As a first step, the sample has to be prepared, meaning protein crystals must be grown. To achieve crystallisation, a high protein concentration is necessary and optimal conditions that yield protein growth of well-diffracting crystals have to be found.
Afterwards, the diffraction experiment is performed: The protein crystals are delivered to the X-ray beam which is diffracted by the crystals. The intensity of the diffracted signal is measured by a detector. In modern crystallography experiments the beam brightness is so high, that the sample is vaporised. Thus many diffraction patterns of different crystals of the same sample are collected in a serial fashion, giving rise to the name 'serial crystallography'.
These patterns have to be merged and, to obtain real space images, the diffracted wave has to be Fourier-transformed. The computational procedure for this is difficult, as only the intensity is measured by the detector and the phases of the incident beam are unknown. The result is the famous 'phase problem'. Hence, simply performing a Fourier-transformation is not possible and the phases have to be reconstructed. Several approaches to overcome this problem exist, but to obtain a high resolution, an iterative approach can not be avoided. The iterations include performing a transformation with

preliminary phases, obtaining an electron density map, constructing an atomic model, then performing a Fourier transformation back to reciprocal space and calculating the difference between the detected intensity and the intensity resulting from the atomic model to update the phases for the next iteration.

2.1 Sample preparation

A proteins ability to form crystals depends on its intrinsic properties, like surface charge distribution or flexibility [4]. In order to obtain a single crystal, the proteins must separate from the solution and self-assemble in a periodic crystal lattice. In general, protein crystals are fragile, meaning they are stabilised by relatively few weak bonds and hence sensitive to environmental changes such as temperature, mechanical stress and pH-value. To achieve crystallisation, usually one starts from a high protein concentration in the order of mg per ml and adds precipitants, reagents that reduce the proteins solubility. Precipitants that lead to conditions for well-diffracting crystals are hard to predict. Therefore, for growing protein crystals of regular shape that are in the size of 20-50 μm needed for synchrotron experiments or 10 μm needed for micro-focus synchrotron experiments, crystallisation under many different conditions is tested until suitable crystals are forming. Parameters that can be varied include pH, temperature, reagent concentrations and most importantly reagents.

2.2 Beamline experiment

In modern X-ray experiments performed at synchrotron or free-electron laser beamlines, the very intense photon beam leads to extreme radiation damage.
At a photon energy of 12 keV, corresponding to a wavelength of 1 Å, only roughly 10 % of X-rays interacting with the sample are scattered elastically (Thomson scattering). The remaining approximately 90 % cause radiation damage by inelastic scattering with particles (Compton scattering) or photo-ionisation of atoms or molecules. The latter also causes secondary damage through Auger electrons which lead to the formation of radicals in water which then chemically 'attack' the sample. Additionally, fluorescent X-rays irradiated by atoms that were ionised causes further destruction of the sample. These effects not only cause local damage, such as decarboxylations, S-S bond breakage and photo-reduction of redox systems, but also lead to global damage. Global damage, meaning deformation of the crystal, causes a loss of resolution, broadening of diffraction spots and changes in unit cell constants.
To minimise the effect of radiation damage at modern beamlines with extreme brightness, the exposure time to the X-ray beam is decreased. A single 'shot' of one protein crystal's diffraction pattern is taken, afterwards the crystal has to be replaced due to the severe radiation damage. Sample delivery is the process of exchanging the sample continuously. As protein crystals are sensitive to the parameters of their environment, sample delivery methods have to be in accordance with these[5]. As many macromolecular protein crystals contain a lot of solvent, injecting the sample in the solvent is desirable, but scattering in the liquid also increases the background signal. Furthermore, purifying the

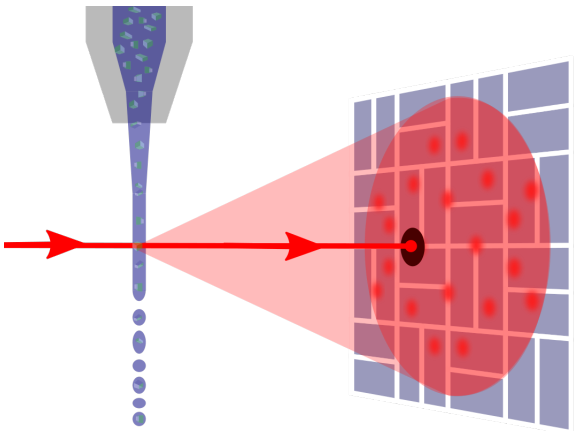

Figure 2: Scheme of typical setup at a beamline experiment: The sample protein crystals are injected with the precipitant using a special nozzle. The X-ray beam is diffracted at the sample and the intensity of the resulting beam is collected at a detector. To protect the detector, the direct X-ray beam is stopped.

protein and crystallising it can be an expensive, time-consuming process, thus economical sample use is beneficial. An additional challenge is injection *in vacuo* to reduce the background or detector needs.
For this purpose special gas dynamic virtual nozzles (GDVN) have been developed and currently are further optimised[6]. These devices consist of a relatively large capillary for sample delivery and a surrounding gas capillary. The sheath gas stream focuses the liquid jet, in this manner reduces the background and sample consumption, and prevents ice formation *in vacuo*.
At the beamline the beam is scattered at the protein crystals in the jet that is formed by a nozzle. The resulting diffraction patterns are collected at a detector and stored. A typical setup is shown in figure 2.

2.3 Computational data analysis

The diffraction patterns collected during a beamline experiment show the intensities of the reciprocal lattice of a crystal. This reciprocal lattice is the Fourier transform of the so-called 'direct lattice', the periodic spatial function of the electrons in real space [3].
A reciprocal vector is the momentum difference between the incoming and the diffracted beam. The goal of the data analysis is to find this direct lattice and thus solve the protein by obtaining its real space structure.
This is done by computing the Fourier transformation and finding the 'reciprocal of the reciprocal', the direct lattice. However, only the intensities of diffraction spots are detected, but for a correct reconstruction also the phases are needed. The issue that the phases are not detected is called 'phase-problem'. There are several experimental and computational methods to work around it.
In general, these methods can be divided into two groups:

1. *De novo* phase determination methods, where the initial phases are found by educated guessing or from additional information using reporters placed in the lattice. For example in 'isomorphous replacement' heavy atoms are added to the crystal to make measurable changes. Phase information can be obtained by calculating the change in the overall diffraction pattern.

2. The second type are methods based on solved structures using these as a basis to generate phases for similar structures. An example would be difference Fourier, where the phases are obtained assuming that if two proteins have exactly the same lattice their structure must be similar. Thus the unknown protein crystal's amplitudes are combined with the known protein crystal's phases. The differences between the Fourier amplitudes calculated and the measured diffraction pattern is computed. This difference and the known protein's phases are used to perform a reverse Fourier transform. This 'difference' Fourier shows 'new' features as positive peaks and 'missing' features as wells and is used to update the phases and find the correct ones in an iterative process.

Once the electron density map has been found, an interpretation is necessary to find the molecular structure. For this, many known constraints, such as bond lengths and angles are applied to find a molecular model.

3 Sample delivery optimisation

When integrating laser beam workflows at third generation synchrotron and XFEL experiments [7], proteins crystals can be delivered as a liquid micro-jet into the vacuum chamber. For that purpose, micro sized nozzles have been developed. However, this type of delivery might observe difficulties such as clogging of the line due to viscosity or other factors, instability of the jetting, ice formation around the nozzle due to the high vacuum of its jetting environment and others. To prevent those phenomenons from happening and check the quality of the jet prior beam line experiments, we have tested methods that we will detail in the following sections.

3.1 Mobile sample delivery set up

In order to optimise the sample delivery of a nozzle, one can observe the behaviour of the liquid jet depending on the applied parameters chosen by the user. This set up being designed for conference demonstration, it monitors the jet optically through a camera with magnification up to 250x. It is mobile, compact and easy to disassemble. The nozzle used for experimentation was a double flow-focusing nozzle [8], flowing sample of various compositions and viscosity. The flows of ethanol, nitrogen and sample, were controlled using an Elveflow device and flow meters. The set up has been designed using ThorLabs parts and 3D printed objects as one can see in Fig.3.
Tests using different sample viscosity and jet illuminations have been performed to obtain the best possible image of the jet.

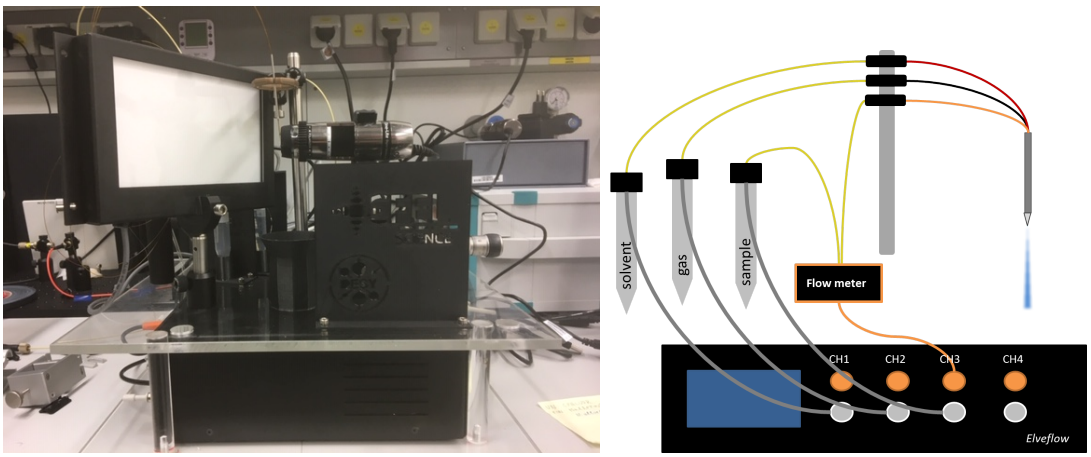

Figure 3: On the left: Front image of the mobile set up for nozzle sample delivery observation. On the right: Figure of the connections between the Elveflow, the sample tubes and the nozzle capillaries.

Figure 4: Image of a jet capture with Dinocam at magnification 222.8x, illumination from the camera alone.

3.2 Optical catch and release set up

Growing crystals is not always an easy exercise, and sometimes it is not possible to make enough of them to obtain a satisfying amount of data from beam line experiments. In fact, even if we can control the flow of our micro jet, the amount of dispensed crystals is still significant. To optimise the use of these crystals, an idea would be to catch

them optically with lasers[9], while they are flowing into the capillary and to release them synchronised with the beam frequency. Actually, the major part of the sample is wasted during an experiment, so only the smallest percentage of crystals is used for data collection and analyse. To realise this, we would split a laser beam into two and drive both beam with optical fibre on both sides of the square glass capillary, as one can see Fig.5 . In this way, at the point where the beams will meet, an intense field should allow the capture of particles, and maybe protein crystals.

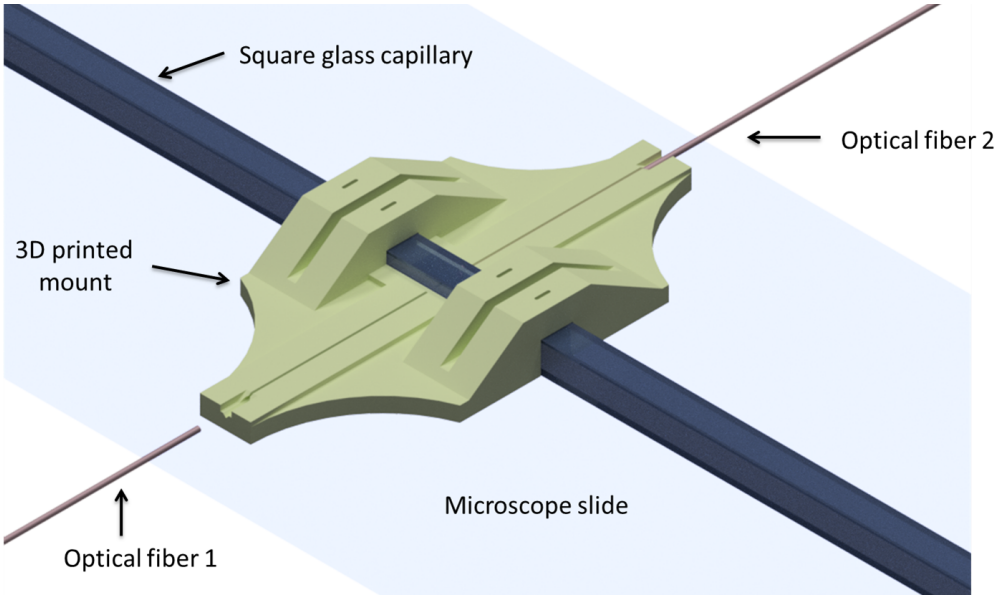

Figure 5: Image of the 3D printed design of the capillary and fibers holder for the optical catch and release set up.

4 Beamline experiments

We were able to engage in two beamline experiments during our stay. An experiment at the European XFEL, a free electron X-ray laser, and one at a synchrotron beamline at the Petra III.

4.1 Synchrotron experiment at Petra III

The P11 beamline at Petra III is built for diffraction experiments of biological samples. The X-ray energies can be tuned between 5.5 and 30 keV and a 'Pilatus 6M fast' detector is used to collect data [10]. The experiment performed was a follow-up experiment of a tape-drive experiment performed before [11]. In that experiment, the mixing and diffusion between lysozyme and the ligand N-N'-N"-triacetylchitotetraose and was studied, whereas in the experiment presented here the experiment is performed using lysozyme and N-N'-N"-N"'-tetraacetylchitotetraose is investigated.

4.1.1 Setup of a tape-drive experiment

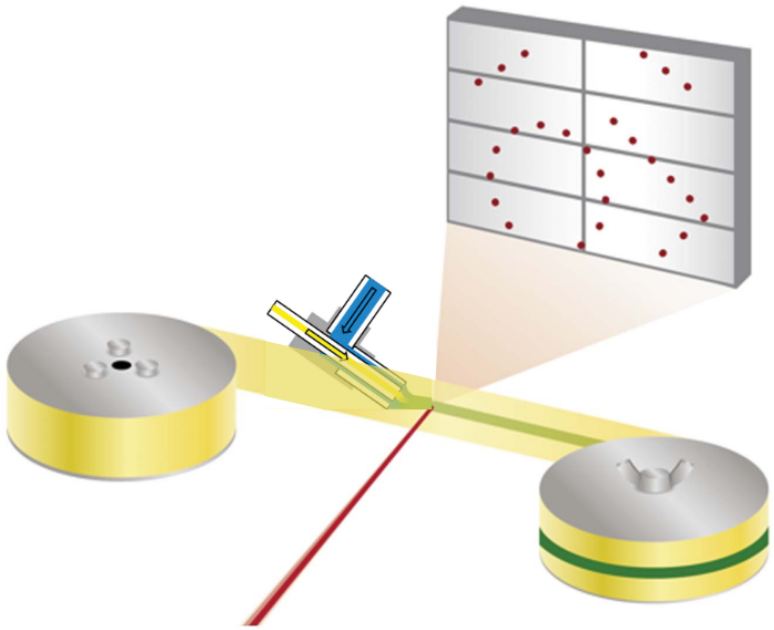

Figure 6: Schematic setup of a tape drive experiment, adapted from [11]:
A protein crystal sample in the precipitant (yellow) is mixed on a polyimide tape (yellow ribbon) with a ligand solution (blue). Using different setups for the mixing, different mixing times can be achieved. The X-ray beam is focused directly on the tape at a 90 ° angle and the diffraction pattern is collected at the detector.

The general setup at the beamline for the experiment is different from the setup shown in section 2. It is optimised to study mixing between two substances and schematically illustrated in figure 6. Instead of focusing the X-ray beam on a liquid jet from a nozzle, it is focused on a polyimide tape on which the protein crystals and a ligand are deposited. The tape is drawn from a roll at a constant speed. To study the mixing in a time-resolved fashion, the crystals and the substrate solutions are deposited at different tape speeds. In that manner, the mixing time delay, the time between the deposit of the sample and it being in the focus of the X-ray beam, differs.

4.1.2 Outline of the work flow

1. The samples, lysozyme crystals in precipitant and the sugar solutions are prepared. To achieve mixing at roughly the same time, a recipe that leads to smaller lysozyme crystals is used.

2. The beamline experiment as described in the previous section is performed with mixing time delays of 0.25 s, 0.5 s, 1.0 s, 2.0 s and 4.0 s.

3. The diffraction patterns collected by the detector are processed using the CrystFEL software [12]: The Bragg peaks are indexed, the intensities are integrated and the data collected during one scan is merged.

4. PHENIX, a computer program, is used with molecular replacement to find starting phases. Molecular replacement is one of the techniques used to overcome the phase problem discussed in section 2. The basic idea behind this method is that the proteins of two crystals are very similar, but the lattices are different [4]. The goal is to find the unknown protein in the lattice and then use its phases for the known protein. For this purpose, the auto-correlation functions of the diffraction pattern of the solved and the unknown protein are computed and different cross correlations are evaluated to find the position of the unknown protein.

5. The structure is refined in an iterative manner using the software COOT.

4.1.3 Results

The samples were prepared, checked under the microscope and the beamline experiment was conducted. During the beamtime there was a major power out at Petra and during this experiment there were power losses during the run where the beam current was down, then came back, but weakly and later came back to full power during a run.
When indexing the Bragg peaks in CrystFEL, only very few Bragg peaks could be found. Several attempts were made to overcome this issue, for example changing the signal-to-noise ratio, the distance to the detector or using different indexing methods. But none of them were successful. Thus the steps 3,4 and 5 described in the work flow could not be performed.

4.2 European XFEL experiment

During our stay at CFEL we had the opportunity to participate to an XFEL experiment by assisting collaborators from Australia.

4.2.1 The experiment

We were working with the Single Particles, Clusters, and Biomolecules and Serial Femtosecond Crystallography (SPB/SFX) instrument that is principally used for diffractive imaging, structure determination of biological samples, at atomic or near-atomic resolution and study of dynamic structures at femtosecond timescale. Our mission was to assist the sample preparation team mainly and the sample delivery team. The technique used for sample delivery was the nozzle one explained earlier in this report. After a long process of alignment of the beam onto the sample micro-jet, operated by instrument scientists, we filled reservoirs to provide sample that could be jet through the nozzle in the vacuum chamber in the experimental hutch. In order to get to the wanted position in the chamber, the nozzle tubing has to be long which then requires to apply a strong pressure over the sample to make it flow at a satisfying rate. For that we use HPLC

(High Performance Liquid Chromatography) delivery system, which required a special procedure for filing to avoid injecting air bubbles inside the vacuum chamber. The purpose behind this experimentation was to follow structural information during protein crystallisation over very short time scales.

4.2.2 Results

The beamtime wasn't successful because of a major power failure that shutdown the machine for the two last days of the experiment. Prior to that, we didn't collect any useful crystallisation data, as we spent the first two days dealing with technical issues related to sample injection. We did, however, collected buffers data and learnt precious information concerning sample preparation and delivery methods. We then realised that 2mJ pulses and 250 pulses per train can cause rapid clogging of the nozzle. Once diagnosed, it was easy to fix this by attenuating the beam. Another cause for non optimal sample delivery that we observed was a shearing in the filter used between the reservoirs and the nozzle line, for which solutions were found quite fast. Those information will be use to improve and optimise next beam time experiment.

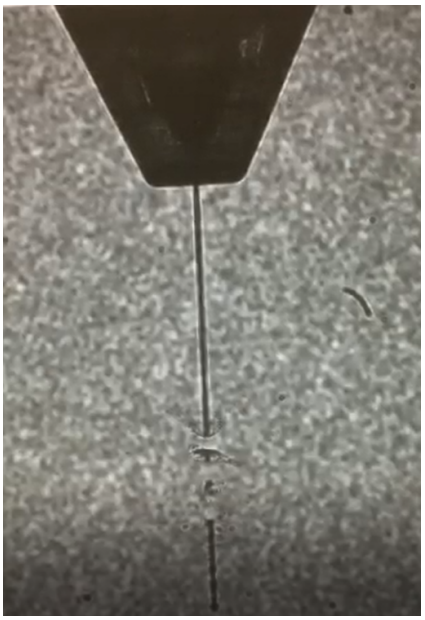

Figure 7: Image of the beam hitting the micro-jet of buffer inside the vacuum chamber from XFEL experiment.

5 Conclusion and Outlook

We had the opportunity to have a closer look at many different stages of X-ray crystallography experiments of biological samples, in particular sample delivery.
During the beamtime at the European XFEL, we experienced how central sample delivery can be for the successful performance of an experiment. The shearing in the filter at the XFEL experiment could have been detected prior to the beamtime, for example in a test using the mobile nozzle setup.
When preparing the lysozyme crystals for the tape drive experiment at the Petra III P11 beamline, we experienced some of the difficulties in the steps of preparing sample crystals. Undoubtedly, the preparation of lysozyme crystals is not the most difficult exercise. Still, this short digression raised our awareness for how challenging and delicate with respect to outer parameters crystallisation can be. With the development of more efficient sample delivery methods, for instance the optical catch and release setup, a lower amount of sample has to be prepared, saving resources and time. Thereupon, we hope this setup can successfully be proven to function and optimise sample delivery in the future.
The bottom line of our time at CFEL might be, that many gears have to work together precisely to make the clockwork of modern X-ray crystallography experiments possible. But these divers steps also allow for advances and thus lead to progress in the future of this engaging field.

6 Acknowledgements

First of all, we would like to thank Dominik for supervising us and introducing us to experimental techniques in the labs. Thank you for your patience, taking us to the beamtimes and answering all of our questions.
Thank you Salah for being so spontaneous and supervising us when the data processing got stuck. We would also like to thank the whole Chapman group as a whole, the overall atmosphere in your group is very pleasant and productive.
Last, but not least, a big thanks to Olaf and the organisation team for making everything go so smoothly for us.

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