



LCP injector for use in crystallography

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Abstract

7 weeks of research done at CFEL, DESY during the summer of 2015, with main weight on preparing LCP injection for a beamtime at PETRA 3. The LCP injector was found to be stable and straight for gas pressures of 200 to 300 psi, and water pressure of 40 to 80 psi, and have a flow rate from 90 nl min^{-1} . Inner capillaries of $50 \mu\text{m}$ has been found reliable, but the possibility of having $30 \mu\text{m}$ or $40 \mu\text{m}$ inner capillaries has been looked at to further decrease sample consumption.

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1 Introduction

This is a report from a 7 week long summer student internship at the photon science department at CFEL at DESY. The programme consisted of weekly lectures on science conducted at DESY as well as individual projects. Most of the work consisted of getting the LCP (Lipidic Cubic Phase) injector ready for use in a beamtime at PETRA P11 at the end of September 2015. Even though the LCP injector was developed for femtosecond crystallography at XFEL's, it has proved to be useable (Nogly et al. 2014 [3]) for millisecond crystallography at synchrotron rings, which are more easily accessible for beamtimes. This report is mainly a description for how one can use the LCP injector, how it was tested and what was found.

2 LCP-injector

2.1 Background

The LCP (Lipidic Cubic Phase) injector has been developed by the State University of Arizona (James 2015 [2]) in order to conduct serial femtosecond crystallography on viscous proteins like membrane proteins that can be grown in LCP. When doing serial X-ray scattering experiments, a jet system that can deliver crystals with the required frequency is feasible. For this purpose, the already well developed GDVN (Gas Dynamic Virtual Nozzle) is normally used to create a flow focused microjet. The GDVN consists of an inner sharpened capillary tube with inner diameter of typically $50\text{ }\mu\text{m}$ inside a capillary tube with an opening of approximately the same size. Through the outer capillary runs a gas flow that focuses the flowing liquid sample jet from the inner capillary by a factor of about 10. However, the GDVN is not well adapted for use with viscous mediums and for the purpose of imaging proteins with higher viscosities, the LCP injector was made.

LCP is a growth medium for membrane protein crystals and was chosen as a delivery material to fill the high-viscosity injector because of its ability to grow well ordered but not extremeley big crystals. One of the reasons to conduct serial crystallography instead of the most established way of using a goniometer and rotate a big crystal was that not all proteins can grow big crystals. Proteins in LCP have also been shown to remain active (Rayan et al. 2014 [4]) which is necessary for time resolved x-ray imaging.

Worth mentioning: the GDVN has a way higher sample consumption than LCP. A complete dataset for GDVN might consume 10 mg to 100 mg while the LCP reduces this 50-100 fold (Nogly et al., 2015 [3])

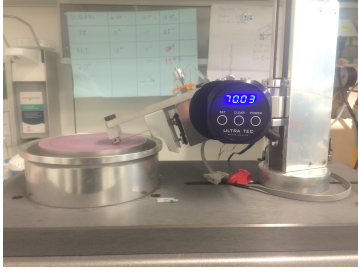
2.2 Assembly and parts

The LCP injector consist of several parts to be assembled, most of them obtained from the State University of California. Before starting injection, a nozzle must be made. Meanwhile, the other parts should be clean and ready for use.

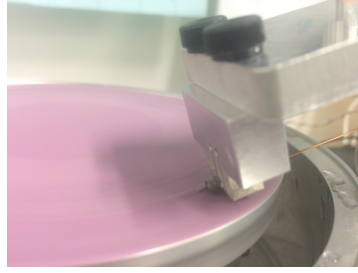
To obtain the special parts for the LCP injector, see Table 4.1 and 4.2 in James 2015 [2]. PEEK fittings and sleeves (Upchurch, IDEX Health & Science) has been used for connections and kapton coated glass capillaries of varying inner diameter but with outer diameter 360 μm (TSP07537, Polymicro Technologies).

2.2.1 Nozzle

As the GDVN, the LCP injector has an inner capillary and an outer square glass capillary. The nozzle is also assembled with a steel 1 cm rod which must be cut in workshop/machineshop to obtain a straight cut. The inner capillaries used for this project



(a) Set the grinder detector to 70.00° and lower until the tip touches the grinder and the detector shows 70.04°.



(b) The tip should be protruding a few mm from the holding position. 661X lapping disk was used.



(c) The rotating motor is set to 3000 rpm. Accessory power is turned on and the lap direction set to (-).

Figure 1: Correct grinding of the innermost capillary.

had an inner diameter of 30 and 50 μm and outer diameter 360 μm . The inner capillary is made by sharpening to a 15° to 20°, which corresponds to setting the grinder at the nozzle lab at CFEL to 70.00° as in Figure 1a and the weight not too far from perpendicular position (the glass capillary is fragile and may break under too much pressure). Cut the capillary with a diamond cutter to ensure a clean cut and lower until the grinder shows 70.04°. Some water should be sprayed on the grinding surface to transport away debris and the capillary should be sticking out by a few mm as seen in Figure 1b. In this project the 661X diamond lapping disk was used. The rotating motor should be set to 3000 rpm. Accessory power should be on and the lap in negative direction (-) as seen in Figure 1c so that debris is not transported into the nozzle tip. When the detector angle is back at 70.00°, the capillary can be removed and the tip examined in the microscope, it should be symmetric and unbroken. Some reengineering by dipping the tip straight down on the grinder plate might be required (can be protected by a sleeve) if it turns out that the cut was not straight. Flush clean with water and air.

The outer glass capillary is melted in a flame as in Figure 2 so that it stops the capillary from shooting through and directs the gas flow. The cut should be as straight as possible, the grinder can be used to grind down uneven edges. Use a long piece of glass capillary and a bunsen burner flame and hold the glass capillary in the flame



Figure 2: Melt the outer glass capillary in a bunsen burner flame while rotating it. Have a look in the microscope and try to fit a grinded inner capillary. Iterate until satisfactory protrusion length ($200\text{ }\mu\text{m}$ to $350\text{ }\mu\text{m}$).

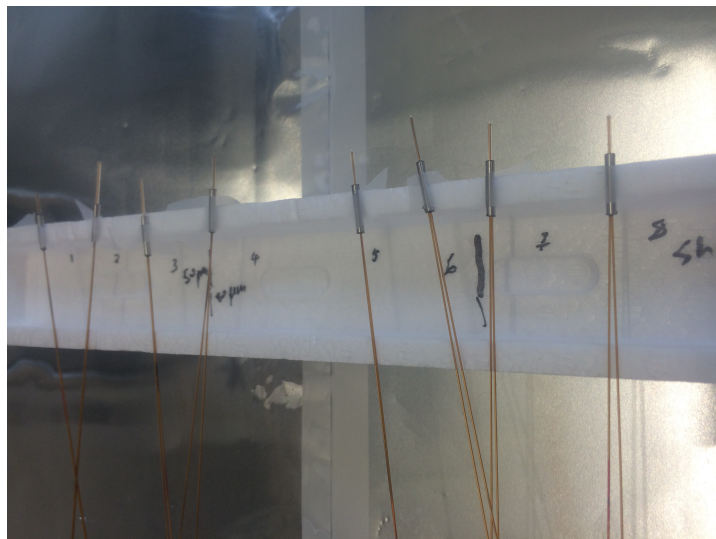


Figure 3: The outer glass capillary should be inserted a few *mm* into the steel tube with the inner glass capillary inside. Apply blue proxy glue and push the outer glass capillary another millimeter into the steel tube. After the first part has dried, fix the inner capillary with another capillary of the same size while making sure the inner capillary protrudes and glue the back of the steel tube shut.

for a few seconds while rotating. Have a look in the microscope and check if an inner sharpened capillary goes through. Repeat until satisfactory protrusion, which according to James 2015 [2] is about $200\text{ }\mu\text{m}$ to $300\text{ }\mu\text{m}$. Note that a too tight tip still has potential by grinding it down. Cut the glass capillary tip to about 2 cm and clean together with

the steel rods in an ethanol bath in the ultrasonic cleaner for 20 minutes.

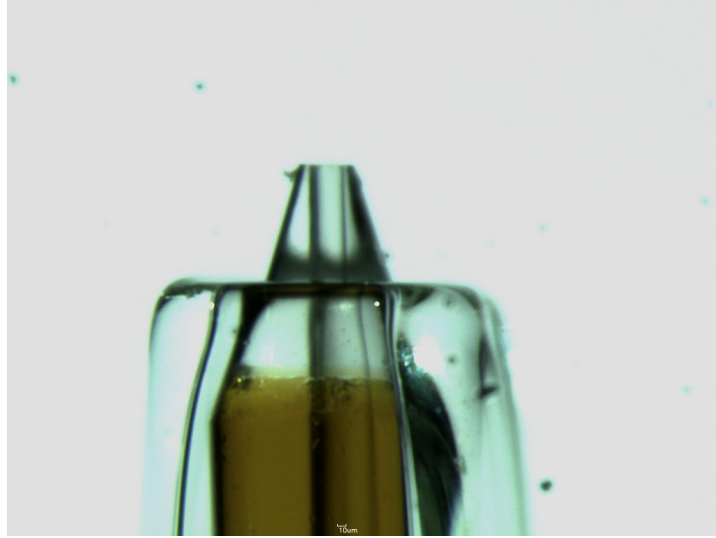


Figure 4: The tip of a fully assembled nozzle with inner diameter $50\text{ }\mu\text{m}$ and protrusion $218\text{ }\mu\text{m}$. The protrusion length should be between 200 and $300\text{ }\mu\text{m}$ but the nozzle has been found to be quite forgiving and both somewhat longer and shorter protrusion is acceptable.

With all parts clean and dry, assembling of the nozzle can start. Line up the steel rods with tape on any movable stable surface. Place the square glass capillaries into the steel tube a few millimeters with the inner capillary inside. The inner capillary need not be put in place, but this way one does not risk breaking the inner capillary tip when inserting it into the steel rod. Use the blue proxy glue and mix 50:50. Use another unsharpened capillary (or any other small tool at hand) to apply a small amount of glue around the glass capillary and push it just an extra millimeter into the steel rod. Let it dry in the clean room. Next, fix the inner capillary in place with another capillary which will be used for gas flow and make sure that the inner capillary is fixed in the right position with your microscope. The gas flow fixing capillary was cut a little shorter than the protruding one so as to tell them apart easily. The inner capillary should be protruding about $200\text{ }\mu\text{m}$ to $300\text{ }\mu\text{m}$ as in Figure 4, but the exact protrusion length was found not to be a crucial factor and nozzles with protrusion up to $350\text{ }\mu\text{m}$ has yielded good testing results as well. As opposed to the GDVN, the LCP injector does not need gas to focus the jet, but rather to stabilize, straighten and lengthen the jet. Glue the back of the steel tube.

2.2.2 Assembly

Main parts to ready for assembly consist of A) nozzle body, B) reservoir, C) hydraulic stage body and D) piston as showed in Figure 5. Notice that the gas line feedthrough (part E) of the original setup (James 2015 [2]) is not used here, as the running is done

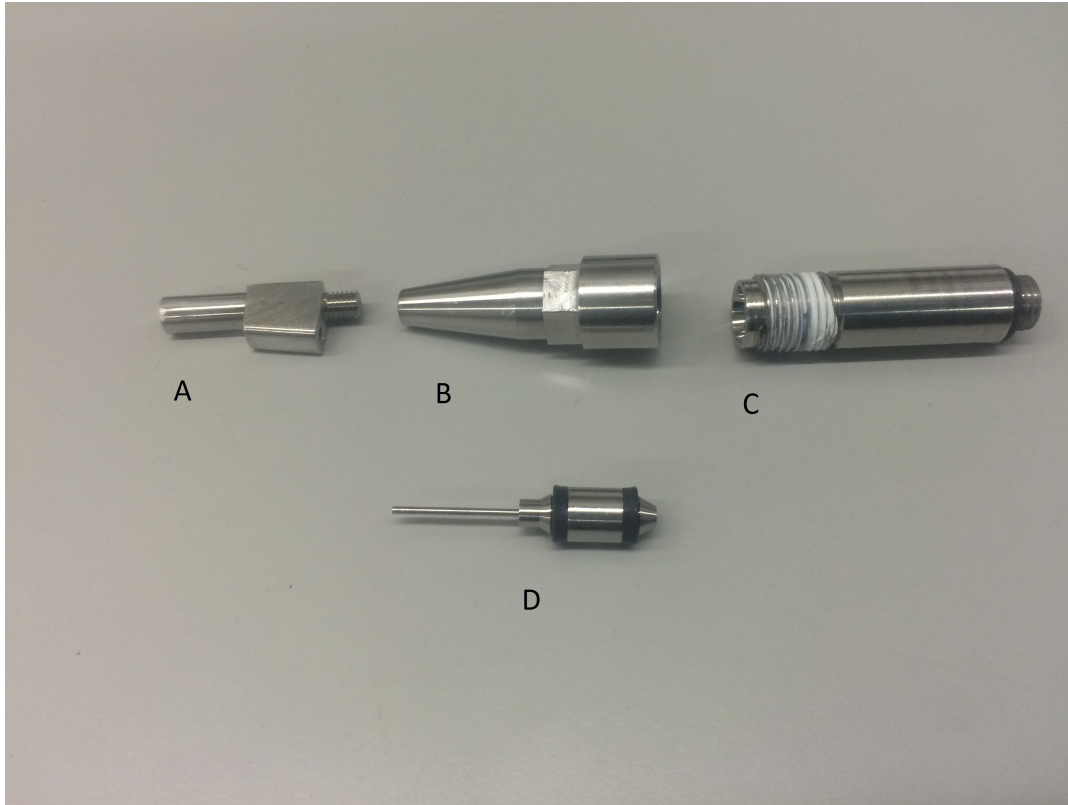


Figure 5: Main parts of the LCP injector used in this project in a disassembled state are (as in James 2015 [2]) A) nozzle body, B) reservoir, C) hydraulic stage body and D) piston. Note that the gas line feedthrough (part E) of the original injector is not used in this project, as vacuum is not needed.

in free air and not vacuum. These parts should be cleaned with ethanol in an ultrasonic bath before use.

First, the nozzle should be fastened in the nozzle body. Parts needed are A.1) nozzle body, A.2) specially fitted screw, A.3) metal ferrule with plastic top ferrule with green sleeve, A.4) plastic ferrule and A.5) nozzle as seen in Figure 6.

The shortened capillary meant for gas flow should be cut close to the steel tube and fit A.4) plastic ferrule sleeve over the front of A.5) nozzle carefully as showed in figure 7a. Place A.5) nozzle into A.1) nozzle body and screw A.2) screw in with a specially made screw driver fitted to this particular screw as seen in Figure 7b. Fit A.3) metal ferrule with green sleeve on from the back of the capillary as in Figure 7c and cut the capillary close to the end of the sleeve.

A gas line capillary with PEEK fittings and sleeves before connection with the nozzle body is shown in Figure 8. The gas line should be tightened with a special pair of pliers with rounded edges so that it is sealed. The fully assembled nozzle body can be seen in Figure 9 (The capillary has not yet been cut close to the body).

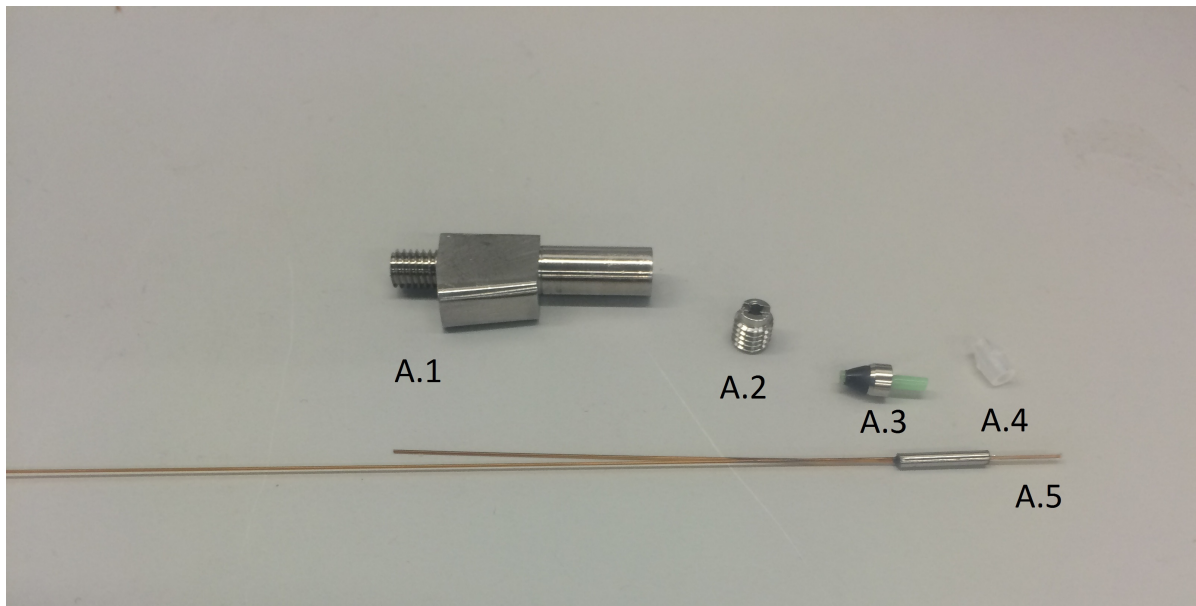
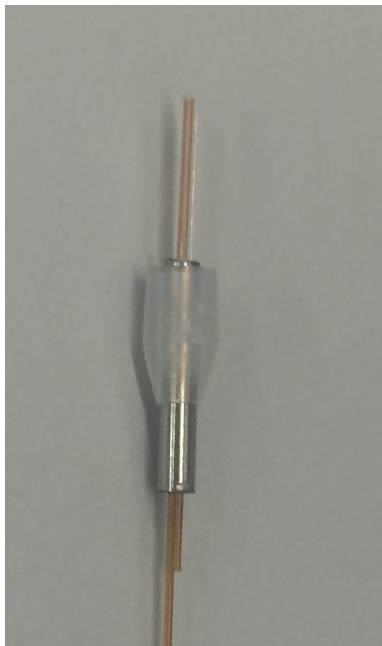
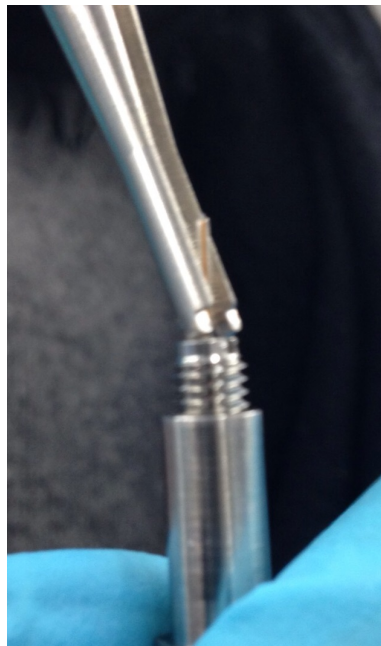


Figure 6: Parts needed to fasten the nozzle in the nozzle body consist of A.1) nozzle body, A.2) specially fitted screw, A.3) metal ferrule with plastic top ferrule with green sleeve, A.4) plastic ferrule and A.5) nozzle.



(a) A.4) plastic ferrule sleeve fitted from the front onto A.5) nozzle.



(b) A.5) nozzle placed in A.1) nozzle body with A.2) screw tightened.



(c) A.3) metal ferrule is slipped on from the back of the capillary.

Figure 7: Steps of nozzle fitting into nozzle body.

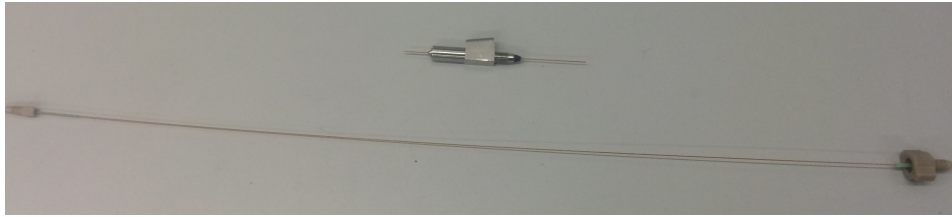


Figure 8: A gas line capillary with PEEK fittings and sleeves before connection with the nozzle body.

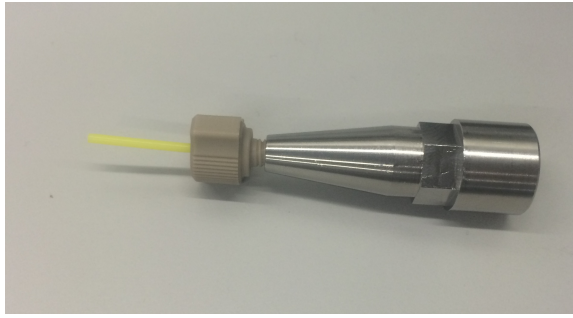


Figure 9: A fully assembled nozzle body shown with the special pliers for tightening the gas line.

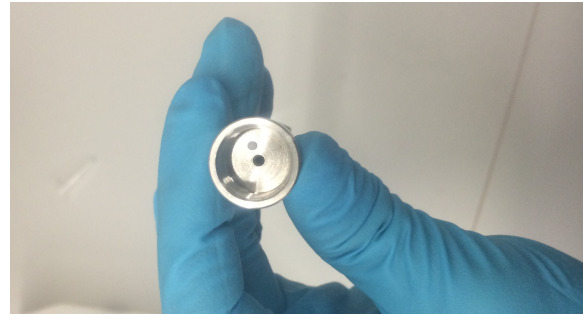
Troubleshooting:

- Part A.4) plastic ferrule must either be new or the hole widened some if it has been used and compressed.
- It is easy to break the outer glass capillary of the nozzle when fastening it into the nozzle body with the screw, a possible solution is to use longer steel tubes to protect the nozzle.
- To check that the assembly has been successful, one should try to flow air through the air channel with a pressure of 100-300 psi, one should be able to feel the air flow with a moistened finger.

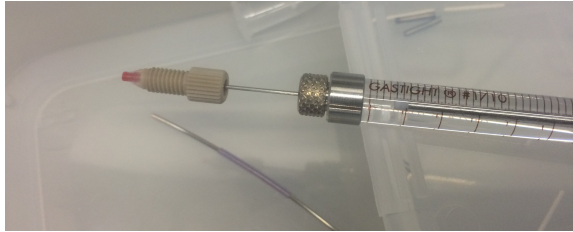
Next is the filling of B) reservoir. First, a preprepared PEEK fitting with sleeve is inserted into the front of the reservoir in Figure 10a and the teflon ball pushed in through the back as in Figure 10b. For this purpose, a steel thread that fitted into the



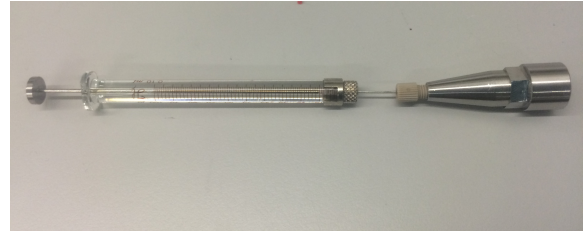
(a) Distance measure for teflon ball.



(b) Teflon ball in reservoir.



(c) Syringe needle with PEEK fitting.

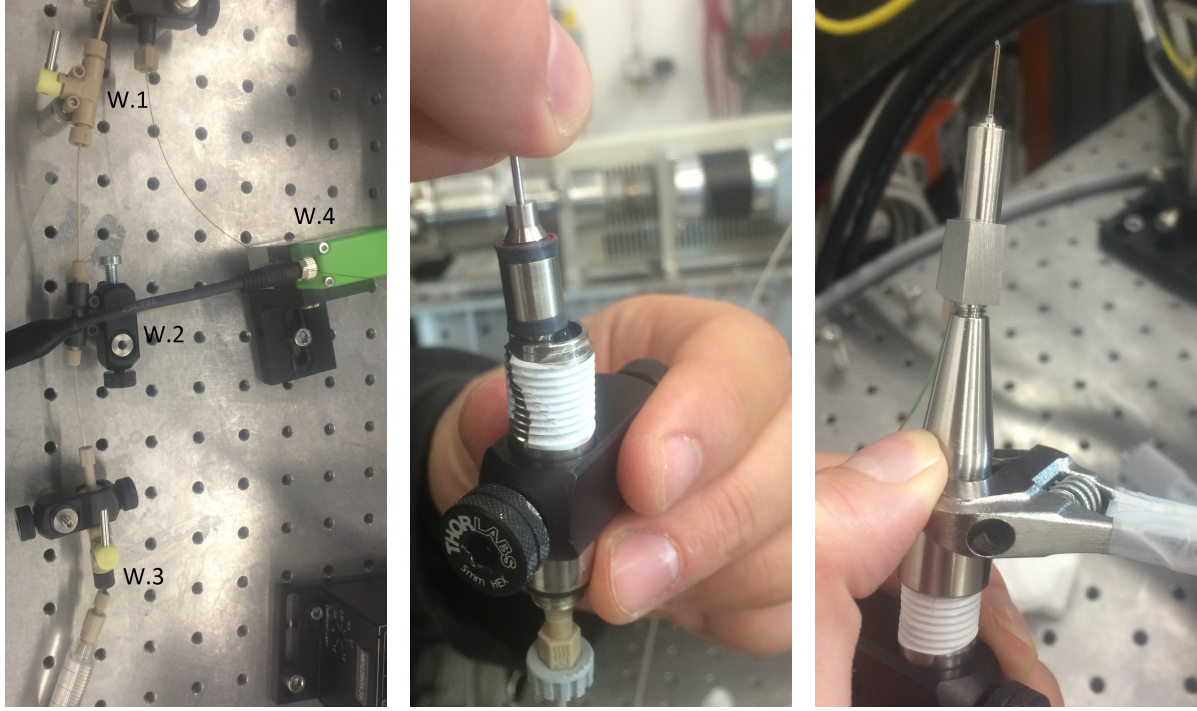


(d) Filling the reservoir

Figure 10: Steps of filling the reservoir. A preprepared PEEK fitting with sleeve is inserted into the front of the reservoir in Figure 10a and the teflon ball pushed in through the back in Figure 10b. For this purpose, a steel thread that fitted into the hole was used. In Figure 10c the steel thread can be seen with a sleeve indicating the position of the teflon ball from the back. In Figure 10c the flat syringe needle is also seen, with a PEEK fitting and sleeve. The needle is then screwed into the reservoir as seen in Figure 10d and the plunger pressed in. Note that the needle consumes around 5 μl of sample.

hole was used. In Figure 10c the steel thread can be seen with a sleeve indicating the position of the teflon ball from the back. In Figure 10c the flat syringe needle is also seen, with a PEEK fitting and sleeve. The needle is then screwed into the reservoir as seen in Figure 10d and the plunger pressed in. Note that the needle consumes around 5 μl of sample, it is a temporary solution until shorter needles arrive.

When the reservoir is ready, A) nozzle body as seen in Figure 9 can be screwed into B) reservoir with an adjustable spanner wrench. Before final assembly, D) plunger must be inserted into C) hydraulic stage body in a way that ensures no air bubbles. To obtain this, a syringe is placed in the waterline as seen in Figure 11a. The waterline then consist of a water pump that can be controlled by valve W.1), a T-junction W.2) and a syringe with a controlling valve W.3). W.4) is a flowmeter in the LCP injector line. When inserting the plunger, W.1) is shut and pressure is applied to the syringe by hand so a meniscus form in C) the hydraulic stage body. D) plunger is inserted into the mensicus and pushed in. In order to avoid cold welding of the stainless steel, a teflon tape is wrapped around C) the hydraulic stage body. The nozzle body with reservoir can be fastened to the hydraulic stage body by tightening with the adjustable spanner wrench.



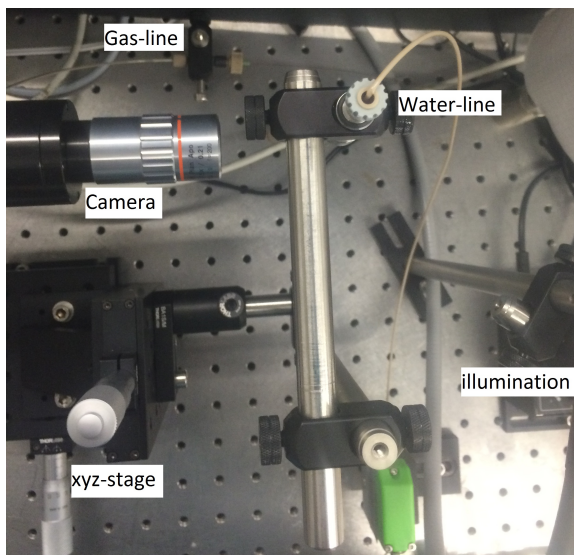
(a) Pump system with flow meter. (b) Plunger inserted into water meniscus. (c) Final tightening with adjustable spanner wrench.

Figure 11: The waterline consist of water pump that can be controlled by valve W.1), a T-junction W.2) and a syringe with a controlling valve W.3). W.4) is a flowmeter in the LCP injector line. When inserting the plunger, W.1) is shut and pressure is applied to the syringe by hand. D) plunger is inserted into the mensicus and pushed in. In order to avoid cold welding of the stainless steel, a telfon tape is wrapped around C) the hydraulic stage body. A) nozzle body is screwed into B) reservoir which is fastened into D) hydraulic stage body. All is fastened with an adjustable spanner wrench.

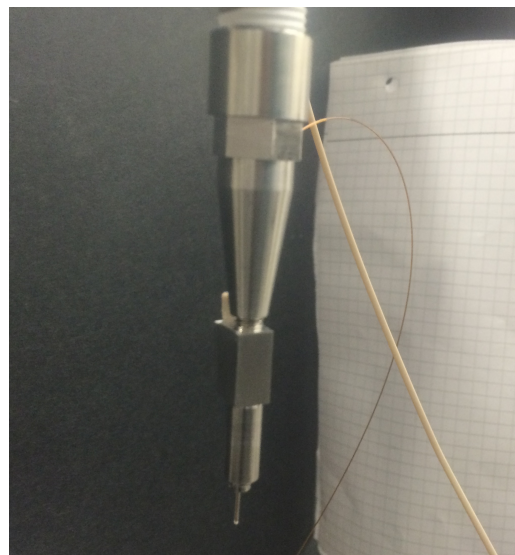
2.3 Testing

2.3.1 Without sample

The testing setup consist of a water pump (Prominence Liquid Chromotograph, 2×LC-20AD + Communication Bus Module CBM-20A) with water line seen in Figure 11a, illumination source (Xenon Nova 300, Storz Endoscope), moveable xyz-stage (Thorlabs assembly), camera (Teledyne DALSA with $f=200$ lens) and gas source (Aerojet Regulator). The nozzle was placed in a horizontal position for testing to see the effects of gravity, but will be mounted vertically as seen in Figure 12 at the beamtime.



(a) Test set-up.



(b) Vertical testing position.

Figure 12: The testing setup consist of a water pump (Prominence Liquid Chromatograph, 2×LC-20AD + Communication Bus Module CBM-20A) with water line seen in Figure 11a, illumination source (Xenon Nova 300, Storz Endoscope), moveable xyz-stage (Thorlabs assembly), camera (Teledyne DALSA with $f=200$ lens) and gas source (Aerojet Regulator).

2.3.2 Preparing LCP

Monoolein (MO) is warmed up until molten (45°C). Two syringes with a coupler should be clean and ready, one of the syringes should be somewhat warmed too, the wetlab at CFEL is cold and the MO solidifies quite fast. Weigh the warm syringe and pipette in $20\text{ }\mu\text{l}$ of MO either from the front. Use a high-tension tip for pipetting and pipette slowly. Make sure there is no air bubbles by pulling the plunger down and 'pump' the air out. Weigh the syringe again and put it somewhere warm (for example on top of the temperature controller) while you pipette $2/3$ times the weight of the MO (should be around $12\text{ }\mu\text{l}$) into the other syringe. Can be done both from front and back, main issue is that there should be no air bubbles. Connect the coupler and mix for a while, the solution should turn opaque. When done, the syringes should be sealed with parafilm around the coupler to make sure the LCP does not dry out. When sealed, the LCP can be stored for quite a while (at least 2 weeks in CFEL).

2.3.3 Testing without sample

The LCP injector was first tested with a $50\text{ }\mu\text{m}$ inner nozzle diameter and pure LCP in order to observe the jetting behavior. LCP is not cheap nor trivial to make, so obtaining a straight and stabile jet was prioritized above quantitative studies. We found that the nozzle in principle can not be reused because of dried LCP in the capillary that is hard to remove and because the nozzle often breaks on deassembly. It should be possible to



(a) Straight, pure LCP-jet.



(b) LCP solidifies over time.

Figure 13: Pure LCP tested with a 50 μm inner diameter nozzle. The jet was straight and stable at flow rates 3 $\mu\text{l}/\text{min}$ to 10 $\mu\text{l}/\text{min}$, water pressure 40-80 psi and air pressure 200-250 psi.

blow out the LCP with high enough pressure, but it has not been done successfully yet and the current strategy is to have many reservoir nozzles.

The jetting behavior seem to be predictable based on the symmetry of the nozzle, which means that a symmetric nozzle with the inner capillary protruding 200-350 μm and a flat tip is acceptable to jet with. The testing showed that the LCP can run for quite long (2 hours easily, depending on pumping speed) but that clogs form after a while and the LCP solidified and had to be wiped away or blown off by highly pressurized gas (up to 1000 psi). This might be improved by making sure that the LCP and all the equipment is completely clean. Dust and other small particles was also observed in the stream.

The jet was straight and stable at flow rates 3 $\mu\text{l min}^{-1}$ to 10 $\mu\text{l min}^{-1}$, water pressure 40-80 psi and air pressure 200-250 psi. This corresponds to a LCP flow of 90 nl min^{-1} to 300 nl min^{-1} .

2.3.4 With lysozyme sample

Small lysozyme crystals was made by the method described in Section 3.1.2. The crystals were about 5 μm as seen in Figure 15a. When making LCP, the water was replaced with crystal solution (elsewise same procedure) as in Section 2.3.2. The jet in a 50 μm nozzle can be seen in Figure 14. The crystals are clearly visible but not evenly distributed within the LCP. The buffers that the crystals were lying in did not mix well with the LCP and a straight and stable jet was not obtained. Different flows could be seen as the viscous LCP stuck to the inner edge of the nozzle and the crystals travelled closer to the middle. Clogs occurred and the pressure oscillated at around 10 psi.



Figure 14: Testing with lysozyme crystals did not give a stable jet. Flows with different speed was observed within nozzle.

To see that the crystals were actually flowing, two polarizing filters were set up, one in front of the camera and the other in front of the lightsource. Birefringence made the crystals light up as they moved.

Luckily, the final crystals will be grown in LCP and the solution used at the beamtime will therefore probably behave much more like pure LCP than the lysozyme mix. A possible test that unfortunately is out of the question for this report due to time is to grow crystals directly in the LCP and see if clogging occurs.

2.3.5 The way ahead

Since there was some problems with the pressure fluctuating somewhat at low streams (low streams is what we want, 10 nl/min to 20 nl/min at the nozzle tip, 32 times more as flowrate for the water), a smaller inner diameter of the nozzle is considered. The crystals in question are $2\mu\text{m} \times 2\mu\text{m} \times 20\mu\text{m}$ and should fit in both $30\mu\text{m}$ and $40\mu\text{m}$. This would also save sample consumption. Both will be prepared before the beamtime. Every testrun with $30\mu\text{m}$ has had some problems, but in principle it should work. Remembering that a steady and stable flow has been achieved at 40 psi, the following table gives the increase in pressure one can expect to give about the same flowrate when exchanging the capillary. Assumed that the pressure is proportional to d^{-4} (Hagen-Poiseuille equation).

Capillary size [μm]	Amplification factor	P_{min} [psi]
50	1	40
40	7.71	308
30	2.44	98

Note that the LCP injector is not built for a water pressure of more than 300 psi and that the initial pressure needed to get the flow started might be somewhat more than what is used later to obtain a steady flow.

If there had been more time, a test with crystal-like material deposited in the LCP would have been made to better simulate the conditions and look at the behaviour if a clog occurs.

3 Crystallography

As a part of getting to know the process from protein to crystal in x-ray machine, and also because it would be useful to prove phasing, lysozyme and thaumatin was crystallized using different methods.

When the proteins were crystallized, x-ray imaging could be performed with Rigaku x-ray machine (wavelength 1.54 Å), up to 1.5 Å resolution. This was done overnight or over the weekend. The data was then processed in ccp4, xds, phenix and coot.

3.1 Crystallizing proteins

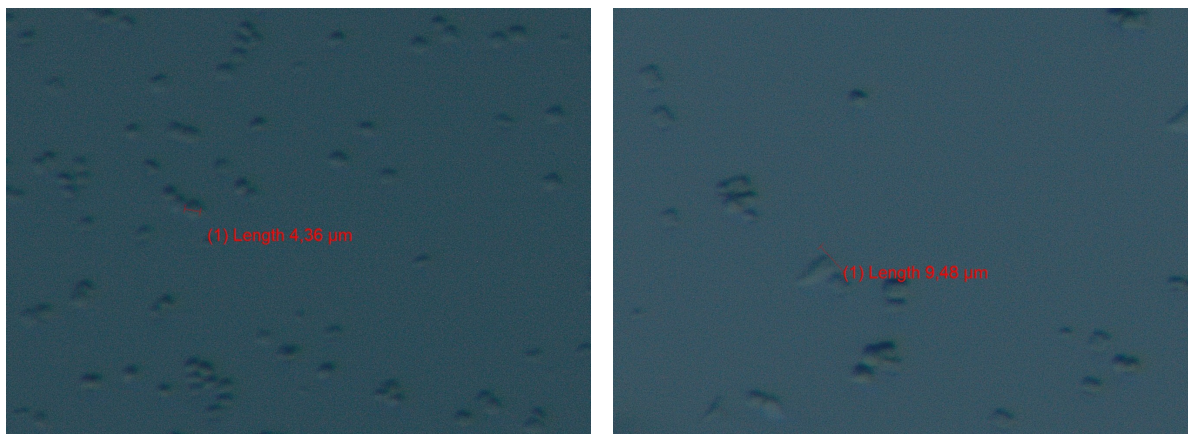
3.1.1 Making of lysozyme

Lysozyme was made by mixing 1.2 g of lysozyme powder with 10 ml acetate buffer at pH 3.5 until 50 ml. The solution was vortexed then centrifuged at cold temperatures in order to get rid of foam. Later the solution was concentrated by using a concentrator with a filter and centrifuge for 7 minutes. In the end a concentration of 127 mg ml⁻¹ was obtained.

3.1.2 Growing microcrystals

When performing serial crystallography, relatively small but homogenous crystals are feasible. Lysozyme was added to precipitant PREC AS at 1 C in 1:3 relation and then put straight on a vortex mixer. After a few minutes one can see that the solution contains crystals in that it is milky-white. In a microscope one can then see homogenous 5 μm crystals as in Figure 15a.

In order to change crystal size one can vary temperature, precipitant concentrations and the concentration of the protein that one wishes to crystallize. Low temperatures (preferably 0 °C, but not all of the available temperatur controllers could go below 1 °C) and higher concentrations of NaBr and the proteins accelerate crystallization and cause even smaller crystals. By increasing the temperature a little (or changing precipitant concentration), somewhat bigger crystals were obtained as seen in Figure 15b. They were



(a) 1 °C gave homogenous 5 μm crystals.

(b) 4 °C gave inhomogenous 10 μm crystals.

Figure 15: Lysozyme microcrystals crystallized with concentration 127 mg ml^{-1} at temperature 1 °C and 4 °C. The higher temperature gave bigger but more inhomogenous crystals. The unevenness of the crystals could be a result of uneven temperature control.

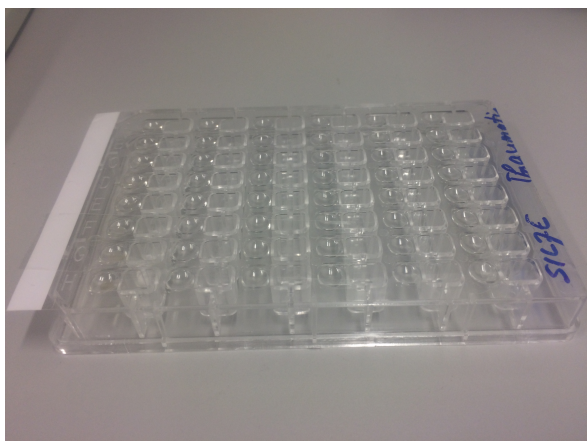
not as homogenous, which can be contributed to 1) bad temperature control - there were some problems with the eppendorf temperature controller or 2) too much NaBr. To fix 1), the temperature controller could have been placed in the cold room, the documentation mentions that temperature changes of more than 15 °C can be problematic. 2) could be easily remedied by making a new precipitant.

3.1.3 Vapor drop diffusion for macrocrystals

One can either perform hanging or sitting drop crystallization with well plates. For sitting drop crystallization, use a well crystallization plate as seen in Figure 16a and add 100 μl precipitant to the reservoir well and 2 μl protein solution to the protein well. Then add 2 μl from the reservoir to the well. The amount of protein solution and precipitant to be added can vary. Seal the plate and wait for crystals to appear while controlling temperature for optimal conditions. When one is not sure about the best conditions, one can perform a commercially available screen or make one oneself.

A sitting drop diffusion experiment that was done with Thaumatine (an artificial sweetener) is shown in Figure 16a and the result can be seen in Figure 17. The precipitant in use was 1.5 M NaTarTraTe. Tacsimate at pH 7.0 and 6.0 was also used as a precipitant and gave bigger crystals which could be used for X-ray diffraction.

Hanging drop crystallization can be performed with a pre-greased VDX plate as seen in Figure 16b where 400 μl precipitant is added to the reservoir. Use a silicone cover slip and add 3 μl of protein solution and 3 μl of precipitant from the well. Flip it carefully with a pair of tweezers and lay it over the well. Make sure that it seals properly. A disadvantage of the sitting drop is that the crystals stick easily to the well and can be easily destroyed when harvesting.



(a) Sitting drop diffusion.



(b) Hanging drop diffusion.

Figure 16: Common methods of vapor drop diffusion.

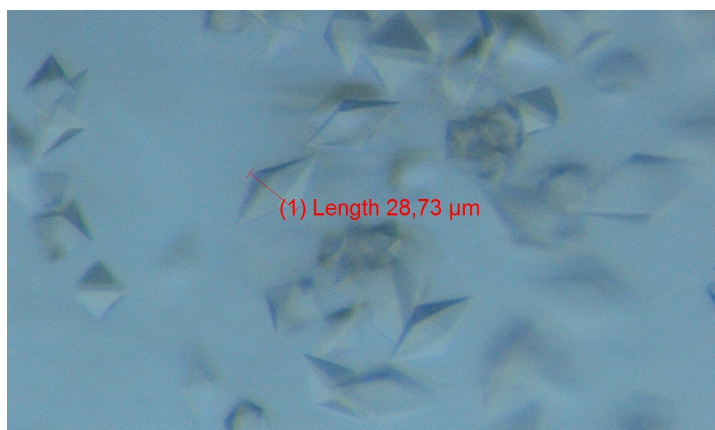


Figure 17: Thaumatin crystals of about 30 μm . Not big enough to do X-ray diffraction in the Rigaku, but other Thaumatin crystals were grown with a different screen that were big enough ($>100\ \mu\text{m}$) to perform standard X-ray diffraction as seen in Section 3.2.2.

3.2 X-ray diffraction

3.2.1 Powder diffraction

Powder diffraction is performed by centrifuging a crystal solution to form a pallet and mounting this in the X-ray beam. This was done with the small lysozyme crystals depicted in Figure 15a. The diffraction pattern can be seen in Figure 18a.

3.2.2 Macrocystal diffraction

To perform macrocrystal diffraction at the Rigaku, fish a suitable crystal (100 μm to 300 μm) with a loop and insert directly into cryo conditions or pull over a sleeve with

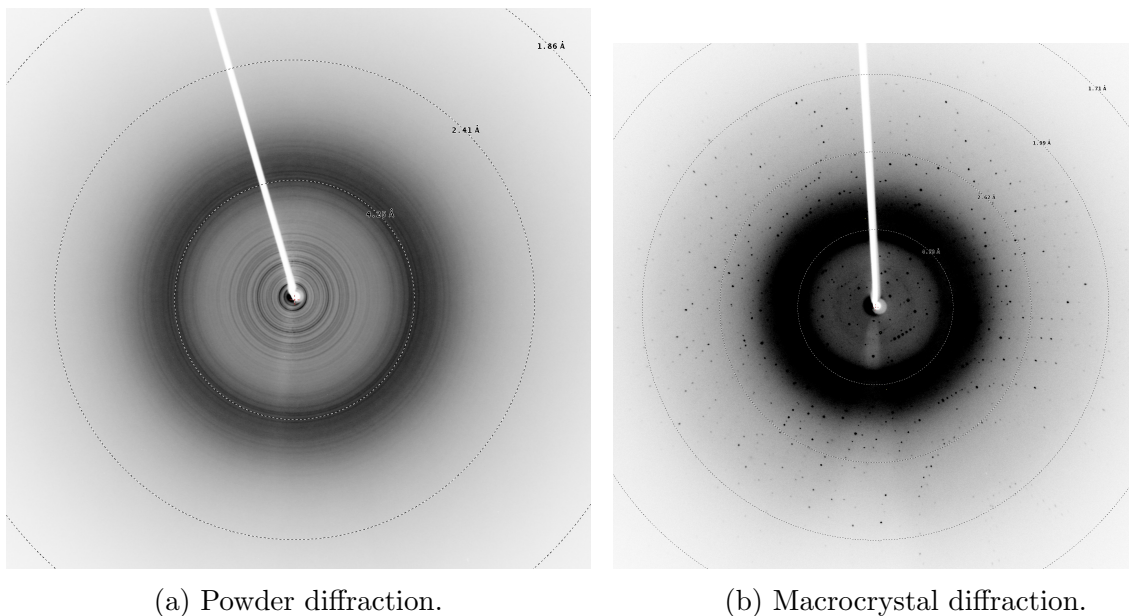


Figure 18: Diffraction with different methods on differently sized lysozyme crystals.

the well precipitant. The resulting diffraction of an approximately 150 μm lysozyme crystal can be seen in Figure 18b.

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