

**DESY Summer School 2012**

**Sample preparation, characterization and  
delivery for Free Electron Laser protein  
serial crystallography experiments**

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## I. FEL crystallography

X-ray crystallography provides the vast majority of macromolecular structures, but the success of the method relies on growing crystals of sufficient size. In conventional measurements, the necessary increase in X-ray dose to record data from crystals that are too small leads to extensive damage before a diffraction signal can be recorded [1][2][3]. It is particularly challenging to obtain large, well-diffracting crystals of membrane proteins, for which fewer than 300 unique structures have been determined despite their importance in all living cells. I learnt a method for structure determination where single-crystal X-ray diffraction 'snapshots' are collected from a fully hydrated stream of nanocrystals using femtosecond pulses from an X-ray Free Electron Laser [4]. The problem of radiation damage is mitigated in crystallography by using pulses briefer than the timescale of most damage processes [5]. I used an approach to structure determination of macromolecules that do not yield crystals of sufficient size for studies using conventional radiation sources I prepared protein crystal material, with a lysozyme used as a model protein. I learnt how different conditions affect size distribution and shapes of growing protein crystals. I built injection system, that can bring the samples in a stream into XFEL focusing area [6][7].

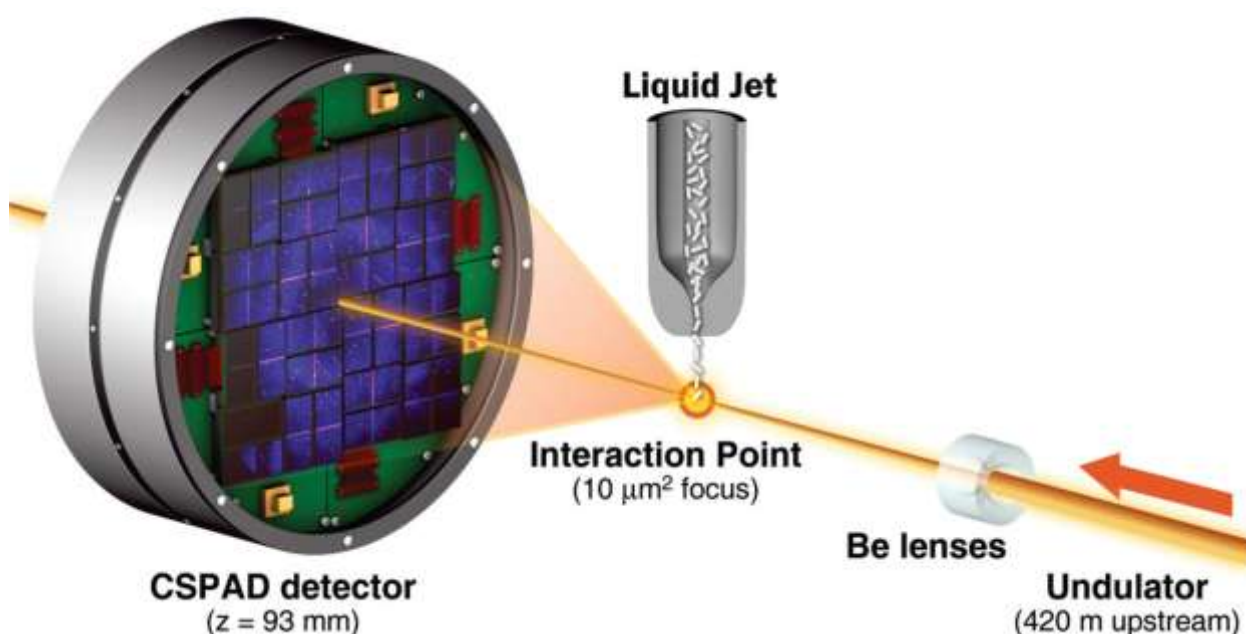


Fig. 1 Experimental geometry for serial femtosecond crystallography at the Coherent X-ray Imaging instrument. Single pulse diffraction patterns from single crystals flowing in a liquid jet are recorded on a CSPAD at the 120 Hz repetition rate of LCLS. Each pulse was focused at the interaction point using 9.4 keV x-rays. The sample-to-detector distance ( $z$ ) was 93 mm.

## II. Sample Preparation

Since the serial crystallography approach requires hundreds of thousands of crystals, it is necessary to develop protocols to get showers of small crystals. My work was aiming at optimizing protocols to get them, taking into account how different pHs, temperatures, salt concentrations and other conditions affect size distribution and shapes of protein crystals.

In order to develop protocols to prepare, characterize and inject protein microcrystal, lysozyme was used as a test sample. Lysozyme is indeed one of the first crystallized proteins [8](Fig. 2), and in particular

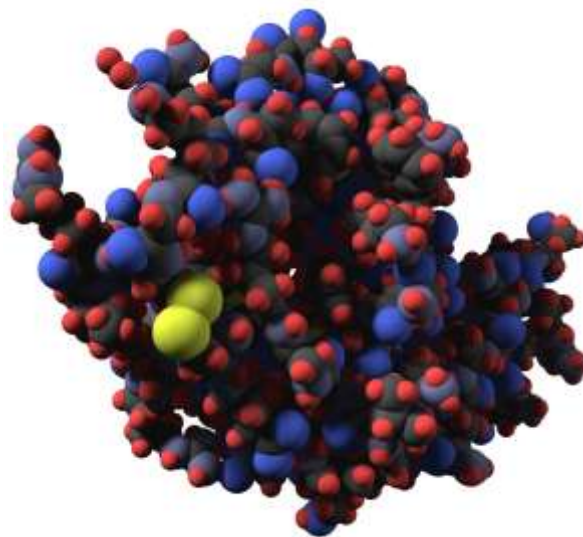


Fig. 2 Lysozyme molecule, reconstructed with X-ray crystallography

the first protein whose structure was solved using X-ray diffraction [9][10]. It is a protein easy to crystallize and to get in large amounts. For this reasons, it has been widely used as a test system for many structural biology experiments. It can be considered the 'hydrogen atom' of protein crystallography.

### II. 1. Lysozyme batch crystallization

In order to grow the crystals I followed the protocol described in [11]. It is a protocol for batch crystallization, so it is naturally well suited for preparation of large amount of crystals.

More in details, I followed the following steps:

1. Buffer preparation: lysozyme can be crystallized in batch by using salt solutions at different pHs. I used NaCl solution at different concentrations. I prepared solutions either with or without 01 mM ammonium acetate and I changed the pH by adding acetic acid or sodium hydroxide.
2. 50 mg/ml of protein are dissolved in the crystallization buffer. The sample is then carefully mixed to avoid aggregation of protein molecules. I also pre-filtered the solution using 2  $\mu$ m and 0.5  $\mu$ m peek

filters in order to remove big protein aggregates. Since this step might also affect the presence of small impurities that can act as crystallization centers, I also prepared the same samples without pre-filtering.

3. The samples are placed in an environment with a constant temperature, either at 20°C or at 4°C.

To crystallize lysozyme I used two methods [11][12].

1. BUFFER 1: 2 M NaCl (110 g / l), 0.1 M ammonium acetate (7.7 g / l)

Sample	LYSOZYME_A	LYSOZYME_B	LYSOZYME_C	LYSOZYME_D	LYSOZYME_E
Buffer	1.88 M NaCl 0.1 M AcONH <sub>4</sub>	1.87 M NaCl 0.1 M AcONH <sub>4</sub> 0.09 M AcOH	1.86 M NaCl 0.1 M AcONH <sub>4</sub> 0.17 M AcOH	1.84 M NaCl 0.1 M AcONH <sub>4</sub> 0.34 M AcOH	1.81 M NaCl 0.1 M AcONH <sub>4</sub> 0.67 M AcOH
pH	7	6	6>pH>5	6>pH>5	5

In all cases 50 mg / ml of lysozyme were added to the buffer solution.

Samples LYSOZYME\_A, LYSOZYME\_B, LYSOZYME\_C, LYSOZYME\_D, LYSOZYME\_E were placed in a constant temperature of 20 ° C.

2. BUFFER 2: 0.86 M NaCl (50 g / l)

To adjust the pH of the buffer I used acetic acid (to reduce pH) and sodium hydroxide (to increase pH).

Sample	LYSOZYME_F	LYSOZYME_G	LYSOZYME_H	LYSOZYME_I
Buffer composition	0.86 M NaCl 0.34 M AcOH	0.86 M NaCl	0.43 M NaCl 0.08 M NaOH	0.86 M NaCl 0.15 M NaOH
pH	4	8	12	13

Lysozyme protein was placed in each buffer in concentration of 50 mg / ml.

The second set of experiments is also aimed at studying the effect of temperature on lysozyme. To this purpose, in addition to samples LYSOZYME\_F, LYSOZYME\_G, LYSOZYME\_H, LYSOZYME\_I, stored at 20 ° C, I prepared samples LYSOZYME\_F\_4, LYSOZYME\_G\_4, LYSOZYME\_H\_4, LYSOZYME\_I\_4, stored at 4 ° C .

### III. Sample characterization

Crystal suspensions can be analyzed using several complementary techniques to reveal the size distribution, shape and concentration of particles in the samples.

#### III. 1. Optical microscopy

Optical microscopy is the easiest way to check if there are any crystals in the

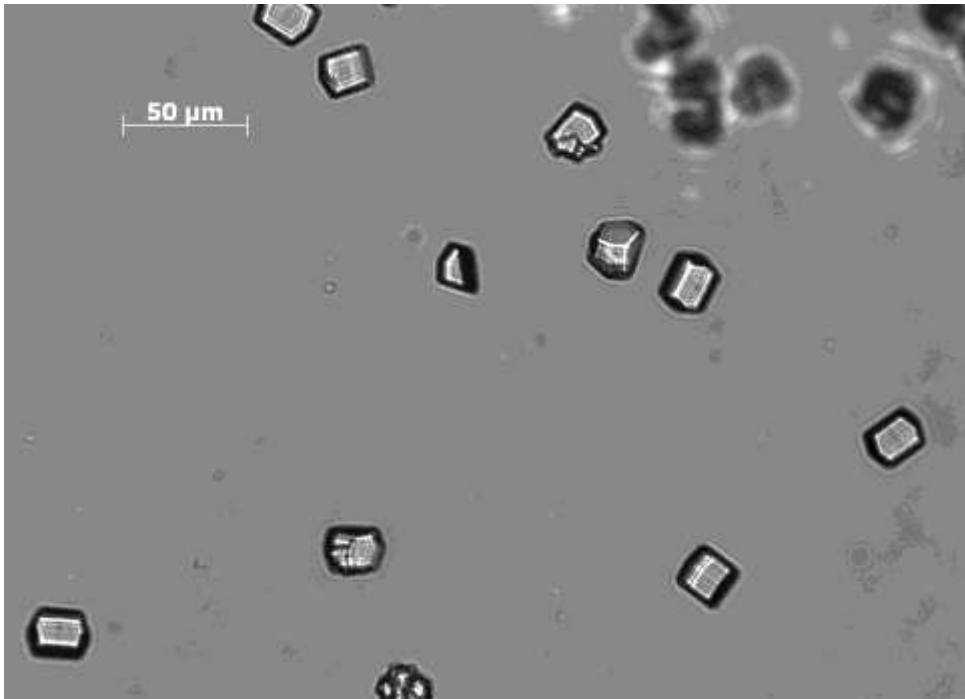


Fig. 3 Optical picture of the sample LYSOZYME\_H\_F\_4: 0.43 M NaCl, 0.08 M NaOH, 50 g/l HEWL, 0.5 μm pre-filtered, pH 12 temperature 4°C.

sample. Picture on Fig. 3 was made with optical microscopy:

Crystals of tens of microns can be clearly observed in the solution. This sample was crystallized in high (12) pH and at 4°C.

##### III. 1. 1. Counting crystals

Using optical microscopy one can not only check if there are any crystals in the sample, but measure average concentration of crystals in the sample, too. To do it one can follow these steps:

1. put a uniform droplet of known volume;
2. measure the surface of the droplet;
3. count the number of crystals in different parts of the droplet;
4. calculate the total number of crystals.

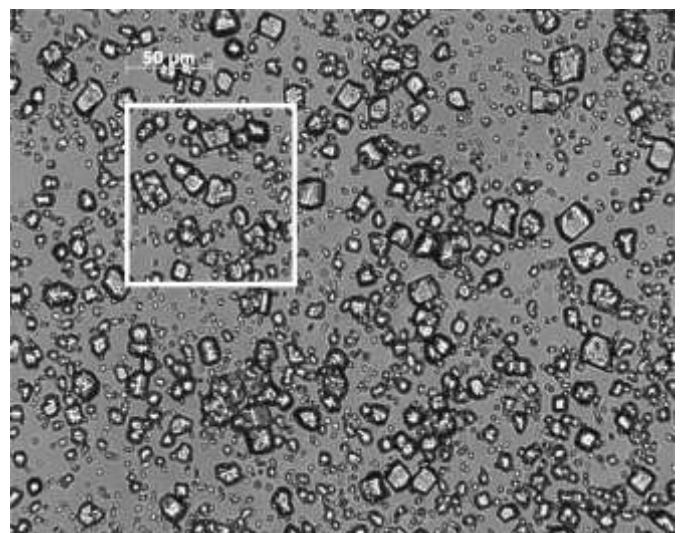


Fig. 4 Optical picture of the sample LYSOZYME\_H\_F\_4: 0.43 M NaCl, 0.08 M NaOH, 50 g/l HEWL, 0.5 μm pre-filtered, pH 12 temperature 4°C.



As an example, on Fig. 4 we can see the sample LYSOZYME\_H\_F\_4:

$$V_{\text{droplet}} = 2 \mu\text{l}$$

$$S_{\text{droplet surface}} = 6 \text{ mm}^2$$

$$\# / 10\,000 \mu\text{m}^2 = 160$$

$$(6 \text{ mm}^2 / 0.01 \text{ mm}^2) * 160 \# / 2 \mu\text{l} = 50\,000 \# / \mu\text{l} = 5 * 10^7 \# / \text{ml}$$

The average concentration of protein crystals in LYSOZYME\_H\_F\_4 is about  $10^7$  crystals/ml.

### III. 1. 2. Crystal shape and size distribution

Crystals of different sizes and shapes are observed according to the different crystallization conditions:

Needles –length: from few  $\mu\text{m}$  to few hundreds of  $\mu\text{m}$ . Width: from about  $1 \mu\text{m}$  to about  $30 \mu\text{m}$ . Needles were observed in sample crystallized with 90 mM NaCl salt concentration, high (11) pH and  $4^\circ\text{C}$ . Relatively high concentration of protein crystals (about  $10^6 \# / \mu\text{l}$ ) is observed.

On Fig. 5 Most part of crystals are needles with a length of tens of microns.

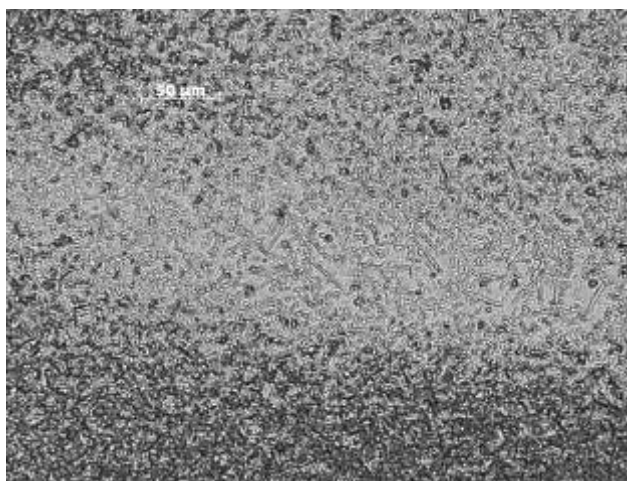


Fig. 5 Optical picture of the sample LYSOZYME\_M\_F\_4: 0.09 M NaCl, 0.01 M NaOH 50 g/l HEWL, 2  $\mu\text{m}$  prefiltered, pH 11, temperature  $4^\circ\text{C}$ . Most part of crystals are needles with a length of tens of microns.

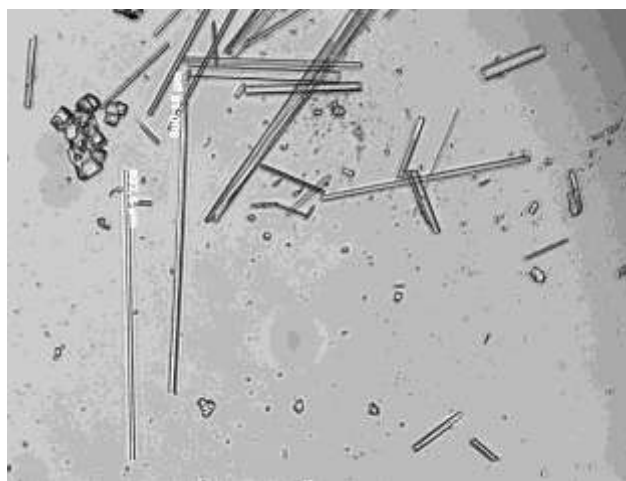


Fig. 6 Optical picture of the sample LYSOZYME\_G\_F: 0.9 M NaCl, 50 g/l HEWL, 0.5  $\mu\text{m}$  prefiltered, pH 8, temperature  $20^\circ\text{C}$ .

Needles can be even bigger. In the sample LYSOZYME\_G\_F (Fig. 6) with higher salt concentration, lower pH and holding in higher temperature needles of hundreds of microns are observed.

Tetrahedrons – is another crystal shape observed in the samples. Clear tetrahedron of  $40 \mu\text{m}$  can be seen in Fig. 7. Tetrahedrons usually have size of tens of  $\mu\text{m}$ .



Fig. 7 Optical picture of LYSOZYME\_G\_F: 0.9 M NaCl, 50 g/l HEWL, 0.5 μm prefiltered, pH 8, temperature 20°C. Clear tetrahedron lysozyme crystal is observed.

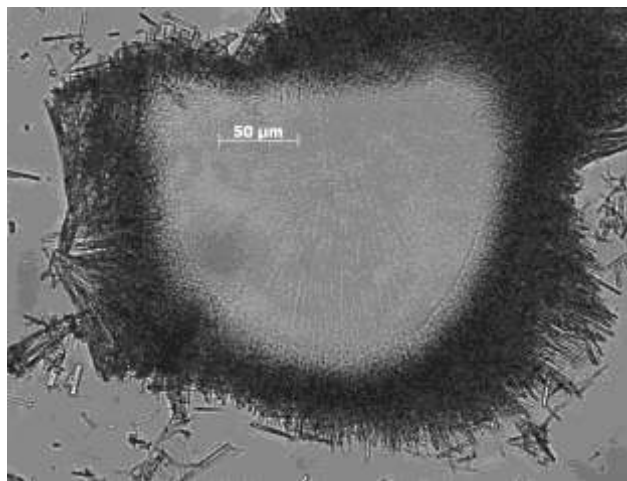


Fig. 8 Optical picture of the sample LYSOZYME\_D: 1.84 M NaCl, 0.1 M amm. ac, 0.34 M ac. acid, 50 g/l HEWL, pH 5.5, temperature 20°C.

Huge aggregates – their size is about hundreds microns. Aggregates seem to contain amorphous parts, but sometimes an aggregate clearly consist of little needles or tetrahedrons, as on Fig 8.

### III. 1. 3. Temperature effects

In original article [12], performing the second protocol used, based on BUFFER\_2, it was mentioned, that there is a dependence between crystal growth during batch crystallization and temperature in what samples are hold.

To study this dependence samples prepared in BUFFER\_2 were hold either at 20°C (LYSOZYME\_F, LYSOZYME\_G, LYSOZYME\_H, LYSOZYME\_I) or at 4°C.

Crystals obtained at 4°C are on average are smaller. For example, LYSOZYME\_H\_F was hold in 20°C (Fig. 9) and LYSOZYME\_H\_F\_4 was hold in

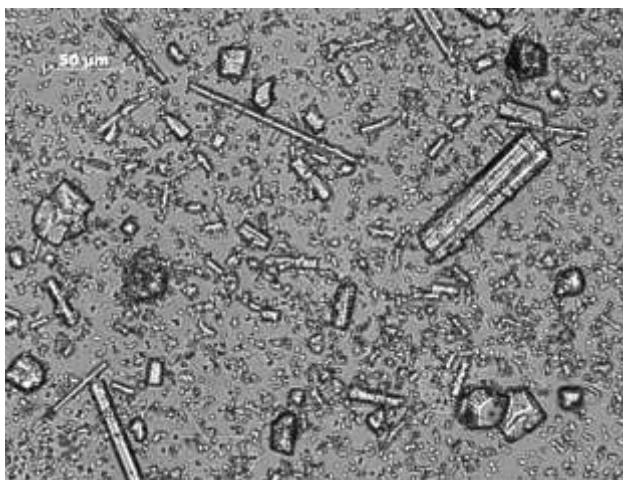


Fig. 9 Optical picture of the sample LYSOZYME\_H\_F: 0.43 M NaCl, 0.08 M NaOH, pH 12, 0.5 μm prefiltered, temperature 20°C – after one day of crystallizing.

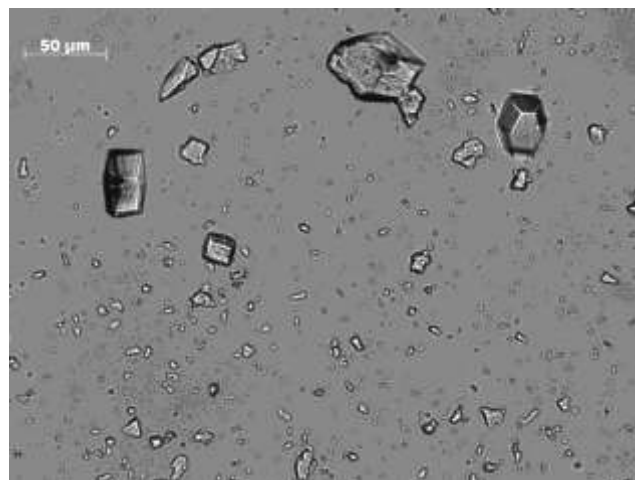


Fig. 10 Optical picture of the sample LYSOZYME\_H\_F: 0.43 M NaCl, 0.08 M NaOH, pH 12, 0.5 μm prefiltered, temperature 4°C – after one day of crystallizing. Crystal concentration is clearly lower.

## 4°C (Fig. 10).II. 2 Crystal grinding protocols

In some conditions lysozyme successfully crystallizes , but it produce crystals with bigger sizes than the ones needed for protein serial crystallography experiments.

It is therefore important to apply procedures to crush the big crystals into a lot of little ones. There are two main crushing protocols:

1. Manual grinding: the crystal containing solution is manually grinded using an agate mortar and pestle (Fig. 11);

2. Vortexing with glass beads: the sample is vortexed for a few minutes with 2 glass beads. On Fig. 12-13 the sample

LYSOZYME\_G (0.9 M NaCl, 50 g/l HEWL, pH 8, holding temperature 20°C) can be observed before and after vortexing with beads for 10 minutes. It is clearly seen that all the big crystal structures are gone. After vortexing with beads only objects less than 5 microns are observed. As shown in the dedicated paragraph, x-ray diffraction is used to confirm the crystallinity of the vortexed samples.



Fig. 11 Agate mortar and pestle used for crushing huge crystals and crystal aggregations.

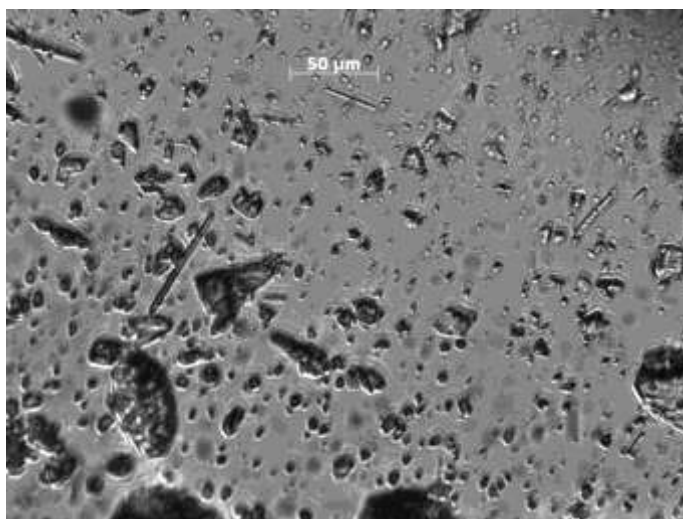


Fig. 12 Optical picture of the sample LYSOZYME\_G: 0.9 M NaCl, 50 g/l HEWL, pH 8, temperature 20°C, before vortexing with beads.

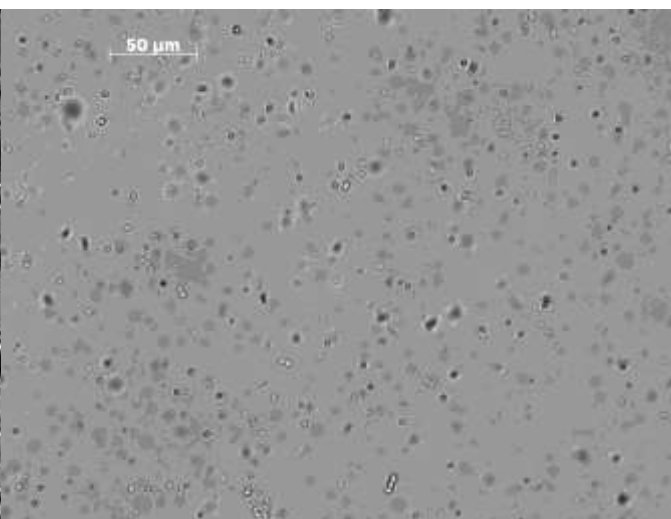


Fig. 13 Optical picture of the sample LYSOZYME\_G: 0.9 M NaCl, 50 g/l HEWL, pH 8, temperature 20°C, after vortexing with beads for 10 minutes.



### III. 3. Electron Microscopy

Electron microscope uses a beam of electrons to illuminate a specimen and produce a magnified image. Electron microscope has greater resolving power than a light-powered optical microscope because electrons have wavelengths about 100,000 times shorter than visible light photons . It uses electrostatic and electromagnetic "lenses" to control the electron beam and focus it to form an image. These lenses are analogous to but different from the glass lenses of an optical microscope that form a magnified image by focusing light on or through the specimen.

I also used electron microscopy to study shape and size of lysozyme microcrystals. Fig. 14 was obtained by using an electron microscope operating at 2 kV.

Since samples for electron microscopy have to be dry, salt forms crystal that are usually bigger than the protein ones. Using electron microscopy protein crystals or their fragments can be found between or on these salt surfaces.

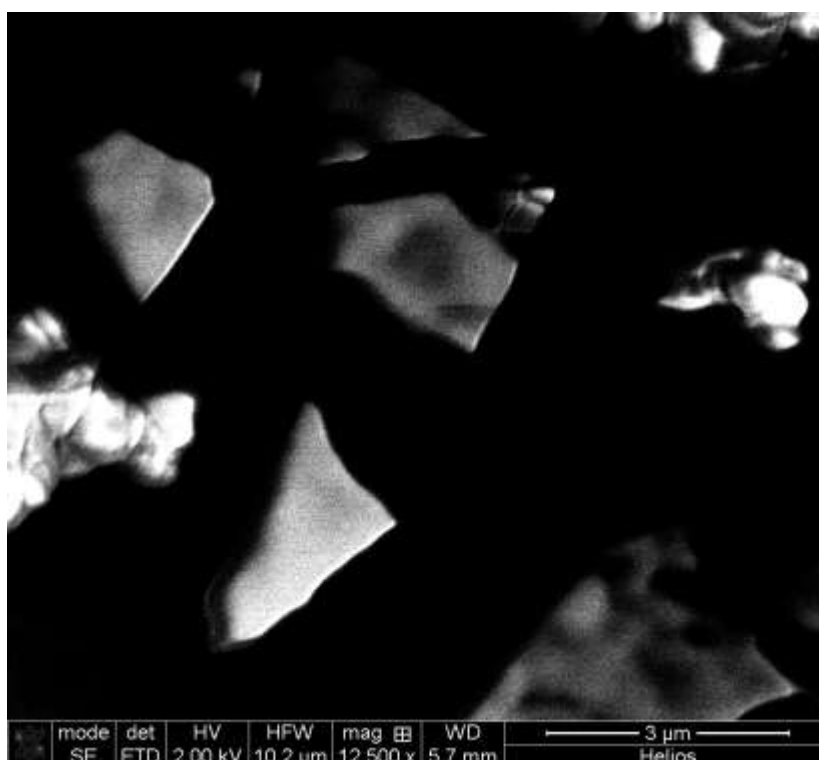


Fig. 14 Electron microscopical picture of the sample LYSOZYME\_P\_F\_4: 0,85 M NaCl, 0,0005 M NaOH, 50 g/l HEWL, 2 μm prefiltered, pH 11, temperature 4°C.

### III. 4. DLS

Dynamic light scattering (also known as photon correlation spectroscopy or quasi-elastic light scattering) is a technique that can be used to determine the size distribution profile of small particles in suspension or polymers in solution. When light hits small particles, the light scatters in all directions (Rayleigh scattering) as long as the particles are small compared to the wavelength (below 250 nm). If the light source is a laser, and thus is monochromatic and coherent, then one observes a time-dependent fluctuation in the scattering intensity. This fluctuation is due to the fact that

the small molecules in solutions are undergoing Brownian motion, and so the distance between the scatterers in the solution is constantly changing with time. This scattered light then undergoes either constructive or destructive interference by the surrounding particles, and within this intensity fluctuation, information is contained about the time scale of movement of the scatterers. The dynamic information of the particles is derived from an autocorrelation of the intensity trace recorded during the experiment [13].

On the plot in Fig. 15 there is a clear peak in “1 $\mu$ m”, which shows that there are particle in solution having this size, although it doesn’t give information about their crystallinity.

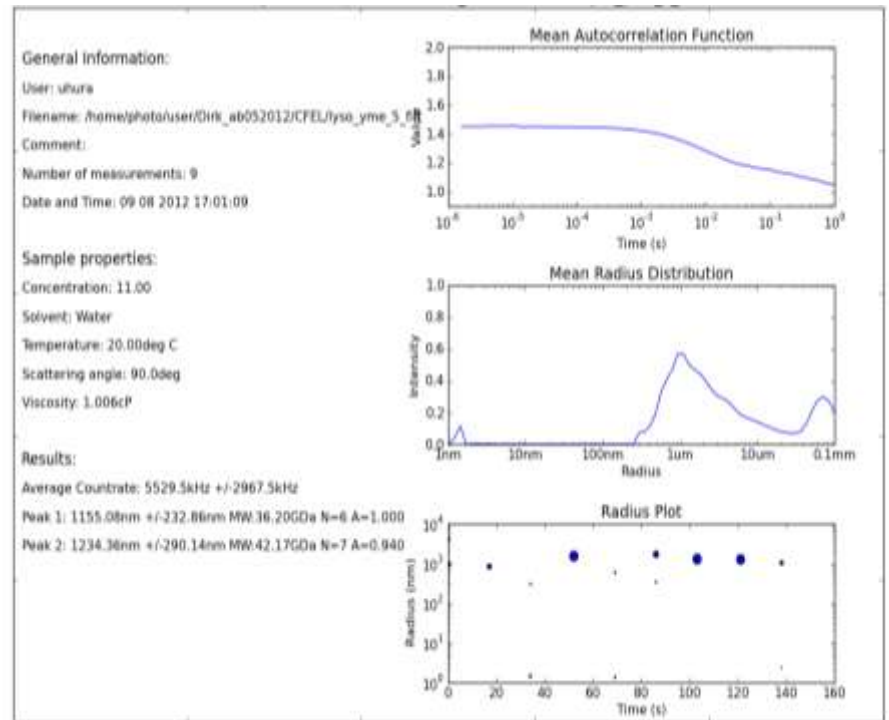


Fig. 15 DLS plot for the sample LYSOZYME\_E: 1.81 M NaCl, 0.1 M amm. ac., 0.67 M ac. acid, 50 g/l HEWL, pH 5, temperature 20°C.

### III. 2. X-ray diffraction

X-ray diffraction patterns of the samples described above are acquired at EMBL X11 beamline at DORIS. Measurements were made in the following conditions:

- Room temperature;
- MAR detector;
- 15 keV beam;
- Detector distance: 330 mm;
- Resolution at the corner: 1.15 Å.

Clear ring-structure even in low resolution in X-ray diffraction pattern of the sample LYSOZYME\_H\_F\_4 (0.43 M NaCl, 0.08 M NaOH, 50 g/l HEWL, 0.5 µm prefiltered, pH 12, temperature 4°C) (Fig. 16) proves that this is diffraction pattern of protein structure.

The second pattern (Fig. 17) was obtained in the same conditions as the same sample, but it was manually grinded for 10 minutes and then filtered using a 2 µm peek filter in order to remove all the big remaining crystals.

One can observe clear ring-structure up to the high resolution again, thus proving that our crushing protocols does not destroy protein crystal structure.

I have to mention that the intensity is lower in this second pattern, but the reason is that the concentration of the grinded sample is lower than the non-grinded one.

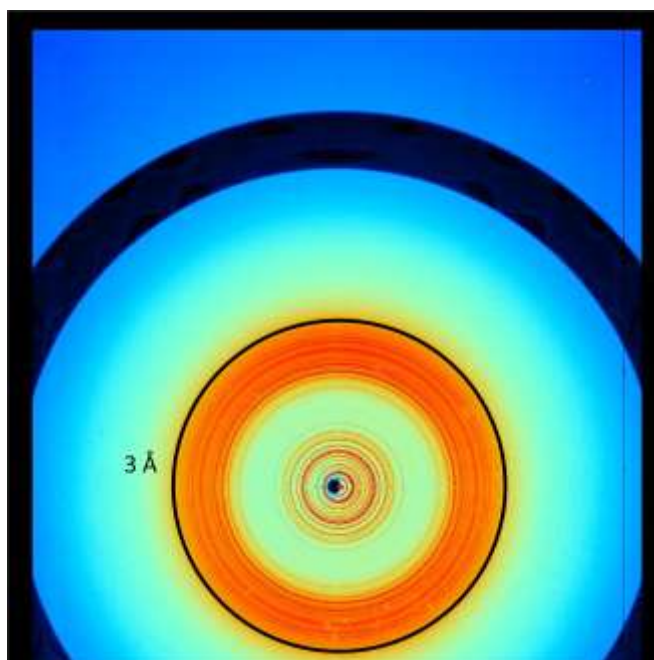


Fig. 16 X-ray diffraction pattern of the sample LYSOZYME\_H\_F\_4: 0.43 M NaCl, 0.08 M NaOH, 50 g/l HEWL, 0.5 µm prefiltered, pH 12, temperature 4°C.

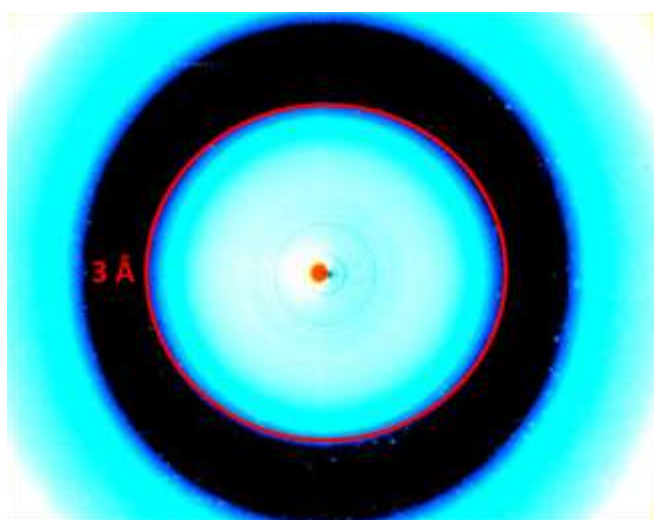


Fig. 17 X-ray diffraction pattern of the sample LYSOZYME\_H\_F\_4: 0.43 M NaCl, 0.08 M NaOH, 50 g/l HEWL, 0.5 µm prefiltered, pH 12, temperature 4°C. The sample was grinded for 10 minutes.

## V. Sample delivery

To deliver protein crystals into FEL (or synchrotron) x-ray beam gas focused virtual nozzles are used [14][15]. The injector design makes use of very well described in the literature Gas Dynamic Virtual Nozzle (GDVN) [16][17][18], which produces a liquid jet of micron to submicron diameter without the clogging problems associated with a Rayleigh jet of the same diameter. This nozzle uses a co-flowing sheath gas to reduce the diameter of the liquid jet. As the jet emerges from a tapered 50 micron capillary, it is focused down by the sheath gas to a few micron diameter (Fig. 18). [19]

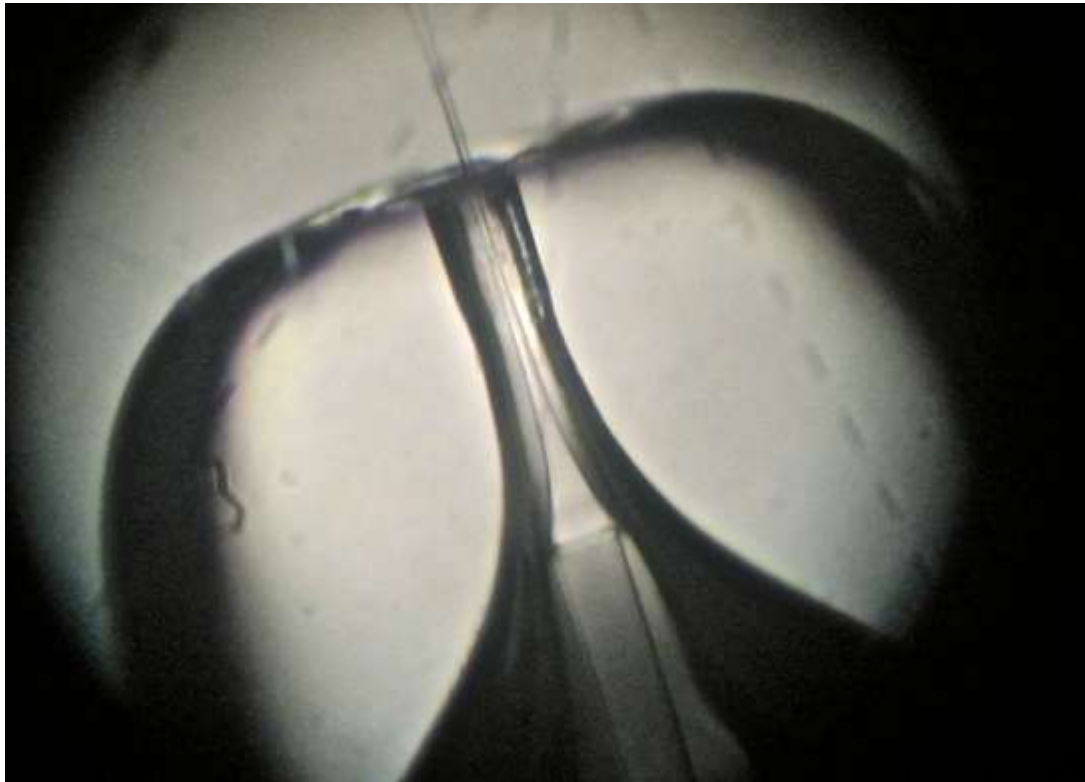


Fig. 18 Gas Dynamic virtual nozzle. A capillary with 50 micrometer ID is centered inside a glass tube, which is flame polished at the end to form a 100 micrometer diameter aperture. Gas flows parallel to the liquid jet and accelerates the liquid, thereby reducing the diameter of the liquid jet.



## V. 1. Nozzle making

To produce nozzles the one needs to follow this protocol:

1. A glass capillary is shrunk by fire to match the inner capillary shape;
2. Cleaning and sharpening of inner glass capillary;
3. Inner capillary should fit the exit hole of the tube very well. The end of the capillary should be close enough to the end of the tube to obtain a stable and small jet.

It is very important that the position of the inner capillary is as close as possible to the end of the external glass capillary. Only in this way the gas flow will be able to focus the liquid jet down to a few microns size.

## V. 2. Nozzle testing

I first tested the nozzle I made using pure water (Fig. 20), in order to avoid any clogging issue and to characterize them in terms of liquid and gas pressure necessary to obtain a stable jet.

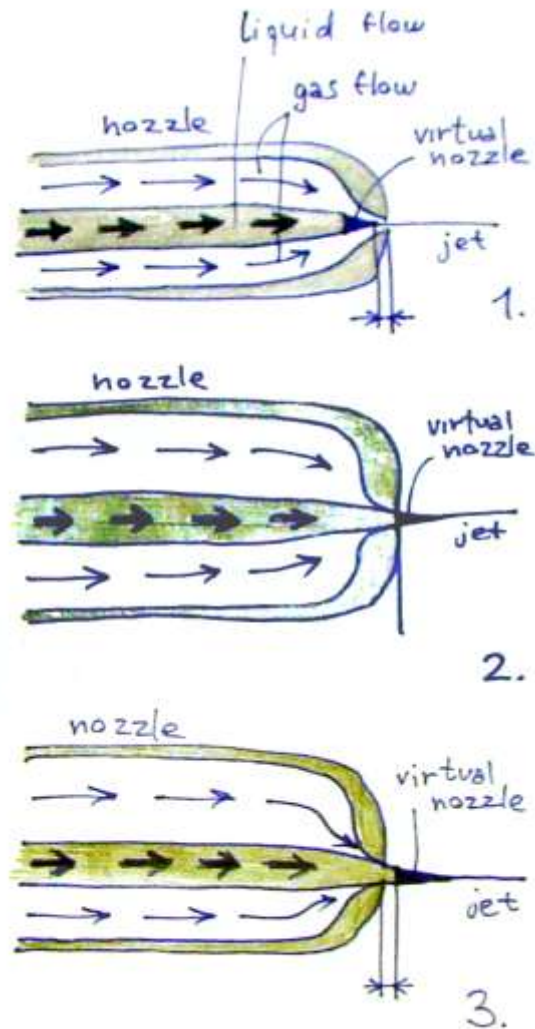


Fig. 19 There can be three configurations forming by differences between length of inner capillary and length of outer tube and taking to account that virtual nozzle always is formed at the end of the inner fiber: 1. Outer tube tips out; 2. Outer tube and inner capillary are of the same size; 3. Inner capillary tips out.

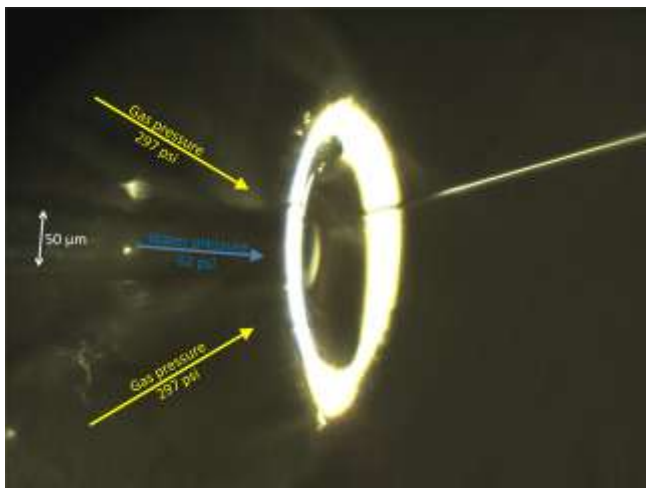


Fig. 20 NOZZLE\_3 (1st configuration) jets with pure water. Diameter of the jet is 5-6 microns. The gas pressure is 297 psi and the liquid pressure is 62 psi.



Fig. 21 NOZZLE\_3 (1st configuration) jets with the sample LYSOZYME\_A: 2 M NaCl, 0.1 M amm. ac., 50 g/l HEWL, pH 7, temperature 20°C, 2 μm filtered. Diameter of the jet is 5-6 microns. The gas pressure is 297 psi, the liquid pressure is 62 psi. NaCl crystal growth on the nozzle observed after 20 minutes jetting.

Fig. 20-21 perform jetting with water only (Fig. 20) and jetting with LYSOZYME\_A (2 M NaCl, 0.1 M amm. ac., 50 g/l HEWL, pH 7, temperature 20°C, 2 µm filtered) (Fig. 21).

Then I tried jetting the crystal containing solution. One of the problems is that solutions prepared for crystallization usually contain high concentration of salts. On the end of nozzle, where liquid starts dripping or jetting, salt crystals can quickly grow thus blocking the exit hole of the nozzle.

The right picture shows how salt crystals start blocking virtual nozzle after just half an hour after the jet started. This is an origin of an idea to decrease salt concentration as much as possible, what I tried to realize, choosing the second protocol with BUFFER\_2 (0.86 M NaCl) containing twice less salt than BUFFER\_1 (2 M NaCl), and preparing samples LYSOSYME\_H (0.43 M NaCl) and LYSOZYME\_M (0.09 M NaCl) with even less concentrations of salt.

## VI. Conclusions

1. Lysozyme was successfully crystallized. Temperature, pH and salt concentration effects are studied. Among the ones I prepared, the most suitable sample for FEL protein serial crystallography is one with high pH, low salt concentration, hold in low temperature after pre-filtering of the solution with a 0.5  $\mu\text{m}$  filter. The samples have then to be vortexed with glass beads to increase the concentration and get small crystals.

Sample	LYSOZYME_H
Protocol	0.45 M NaCl 0.08 M NaOH
pH	12
T	4°C

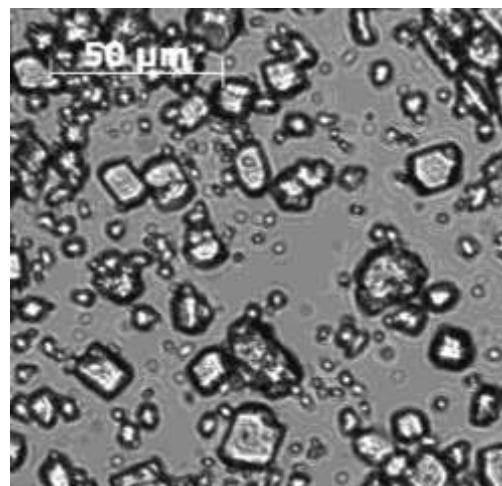


Fig. 22 Optical picture of the sample  
LYSOZYME\_H\_F\_4: 0.43 M NaCl, 0.08 M NaOH, 50  
g/l HEWL, 0.5  $\mu\text{m}$  pre-filtered, pH 12  
temperature 4°C.

2. X-ray diffraction measurements showed that the samples diffract up to high resolution, which proves, that structures looking like protein crystals in optical microscopy – are crystals in fact;
3. Nozzles to inject micro crystals for FEL protein serial crystallography experiments are produced and tested.

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