Ice formation in cell freezing media

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September 4, 2019

Abstract

X-ray diffraction methods give the opportunity to find the difference in the number of crystals and their sizes in solutions characterized by the addition of separate non-penetrating components.

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INTRODUCTION

Nowadays different methods of working with cells associated with low temperatures such as cryosurgery, cryofixation and cryopreservation are widely used in modern biology and medicine. Cryopreservation – freezing and storage of living biological objects with the possibility of warming them without losing their functions is actively used to create gamete cryobanks for assisted reproductive technologies, to preserve stem cells and etc. [1] Despite the high rates of low temperature methods development 30-70% of cells die during the freezing process. [2] The main reason of the cell damage is ice formation for the appearance of which crystallization nuclei are required. These nuclei can be both inside and outside the cell. Ice formation inside the cell irreversibly leads to damage to the cell membrane and intracellular structures because during the formation of ice crystals their volume will be bigger than that of the original liquid. Ice outside the cell is also dangerous because crystallization leads to a decrease in the amount of water that can interact with electrolytes in the solution. This causes pH changes and increase in osmotic pressure. There are special compounds which are used to reduce the negative effects of freezing. They are called cryoprotectors and are divided into two groups: penetrating cellsn(form hydrogen bonds with water inside the cell) and non-penetrating (prevent osmotic changes outside the cell). In addition there are different velocities of freezing which also help to prevent ice formation: slow freezing and vitrification. Slow freezing means cooling the cryoprotectant solution with cells in liquid nitrogen vapors and gradually putting the falcon tube with the sample into a foam with liquid nitrogen. Vitrification is type of ultrafast freezing by putting the falcon tube with the sample directly to the liquid nitrogen. It allows to achieve amorphous state of the solution with the cells but this type of freezing is not suitable for all cell lines and it depends on the concentration of cryoprotectant. That is why it is important to understand how concentration of different penetrating and non-penetrating cryoprotectants influence the process of freezing.

MATERIALS AND METHODS

1. Samples

In this project water-glycerol solutions were used as cryoprotectant samples because glycerol is one of the most common penetrating cryoprotectant. The concentration of glycerol was 12% vol., it was chosen due to the physiological characteristics. All samples contained water with HEPES and salts in concentrations commonly used in cell media. In addition some samples contained penetrating cryoprotectants: albumin and sucrose.

№	Components
1	water
2	glycerol
3	water, glycerol (50% vol.)
4	water, glycerol (50% vol.), HEPES
5	water, glycerol (50% vol.), sucrose (0.5 M)
6	water, glycerol (12% vol.)
7	water, glycerol (12% vol.), salts
8	water, glycerol (12% vol.), HEPES
9	water, glycerol (12% vol.), albumin
10	water, glycerol (50% vol.), sucrose (0.25 M)
11	water, glycerol (50% vol.), sucrose (0.5 M)
12	water, glycerol (12% vol.), salts, HEPES
13	water, glycerol (12% vol.), salts, HEPES, albumin
14	water, glycerol (12% vol.), salts, HEPES, sucrose (0.5 M)
15	water, glycerol (12% vol.), salts, HEPES, sucrose (0.5 M), albumin
16	water, glycerol (12% vol.), salts, HEPES, sucrose (0.25 M), albumin
17	water, glycerol (15% vol.), salts, HEPES, sucrose (0.25 M), albumin
18	water, glycerol (15% vol.), salts, HEPES, sucrose (0.25 M), albumin

Table 1. Measured samples

In this table samples which are the closest to the real cryoprotectant solutions used in clinics nowadays are selected by yellow colour.

2. Measurements

2.1. Experimental setup

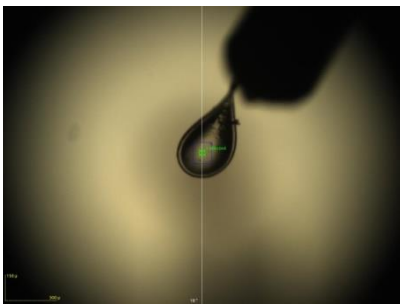
The X-ray diffraction experiments were performed at Beamlines P14 and P11 of PETRA III synchrotron source at DESY. Table 2 presents the experimental setup. Most of the parameters were equal to each other. The main difference is the type of detector and pixel size. Also during these beamtimes different ways of freezing were used, they will be described later.

	P14 beamline, PETRA III	P11 beamline, PETRA III
X-ray beam		
Energy	12.7 keV	12 keV
Size	200x200 um	100 um
Beam	1e12	1e12
Detector		
Name	Eiger 16M	Pilatus 6M
Pixel size	75 um	178 um
Sample-detector distance	198.6 mm	200 mm
Edge resolution		
Type of freezing	in test tube	fast/slow

Table 2. Experimental setup

2.2. Sample delivery

Samples during the first experiment (beamline P14) were put in the loops (Pic.1 (a)). This method has a disadvantage: volume of the solution is not fixed and measured data cannot be compared. That is why for the second experiment (beamline P11) new single used sample holders were invented and made on 3D-printer (Pic.2(b,c)). They allow to fix the measured volume.

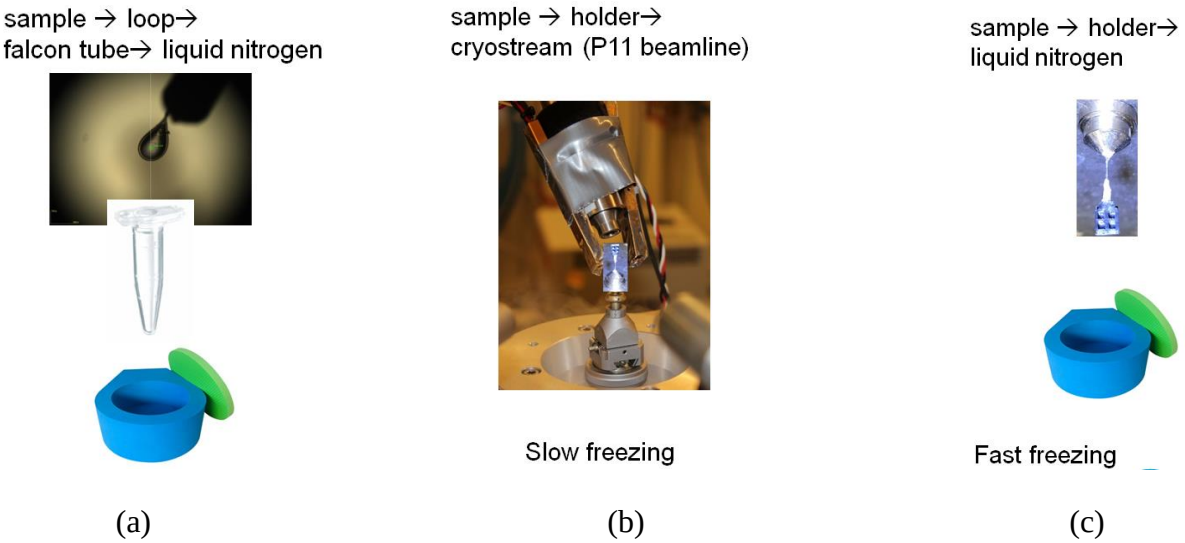


(a) (b) (c)

Pic.1 Ways of sample delivery (a- P14 beamline, b/c –P11 beamline)

2.3. Ways of freezing

In this project three different ways of freezing were used. Samples from the experiment provided on the beamline P14 were frozen in the loops which were put in the falcon tubes situated in the liquid nitrogen foam (Pic.2 (a)). During the second experiment (beamline P11) freezing was made in two ways: slow and fast one. During the slow freezing solution in the special holder (Pic. 1(c)) was put under the cryostream of the beamline P11. This allows to reproduce the method of slow cell freezing the only difference is that the volume of solutions in cell biology is much bigger and time of freezing is shorter. Other samples were immersed in liquid nitrogen and then were put under the cryostream. This method is close to the vitrification process(Pic.2 (b,c)).



Pic.2 Ways of freezing (a –P14 beamline, b/c – P11 beamline)

3. Data processing

The first step of data analysis is center finding which requires measurement of known crystals with good resolution. In this experiment phycocyanin and lysozyme were used. Below the main commands and scripts are represented:

1. Find good crystal with the same detector distance
2. Change detector distance and beam center in the geometry file to that one which is in the cbf file
3. Run CrystFEL:

1) Make the list of all cbf files:

find path/ -name '.cbf' > center_finding.lst (write path to the files you need to analyze, see the script make_sum,)*

2) Examine the peaks stored in the HDF5 files (none indexing)

indexamajig -i center_finding.lst -g geometry_file .geom --peaks=peakfinder8 --min-snr=4 --max-res=1000 --threshold=0 --min-pix-count=1 --max-pix-count=50 --min-peaks=20 --local-bg-radius=2 --indexing=none -o center_finding.stream -j 30 (orange color indicates the parameters that can be changed depending on the parameters of the experiment)

3) Sum all the peaks

path/sum-peaks center_finding.stream

4) See the peaks with hdfsee and use simple rings for understanding the position of the center. Use Calibration Mode to change the center position and save new geometry file (press "S"):

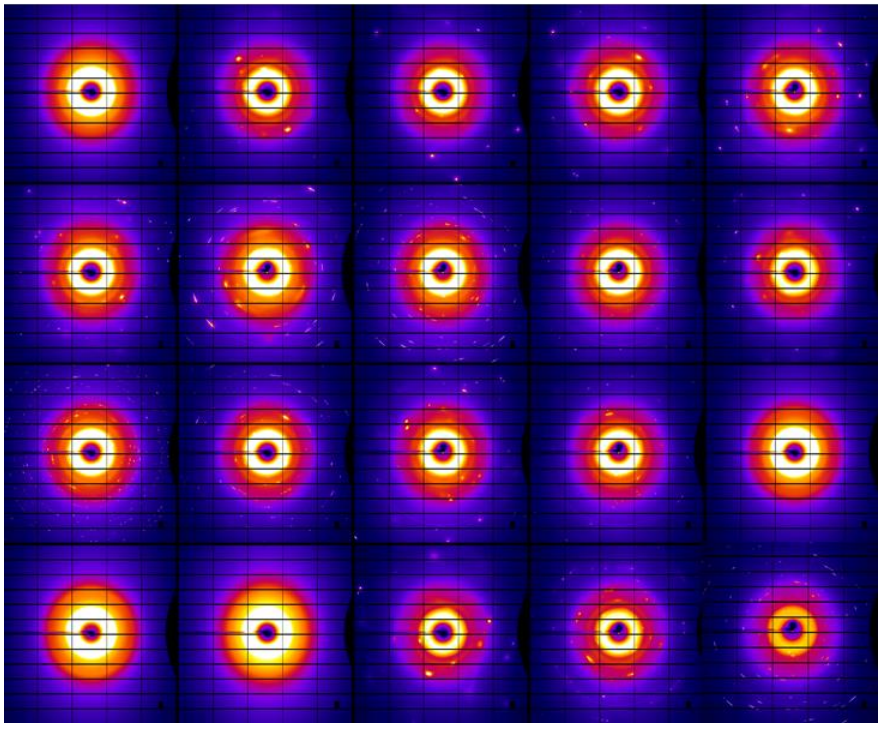
hdfsee center_finding-powder.h5 -g geometry_file.geom --simple-rings=50,100,200,300

5) Mosflm indexing

indexamajig -i center_finding.lst -g geometry_file.geom --peaks=peakfinder8 --min-snr=4 --max-res=1000 --threshold=0 --min-pix-count=1 --max-pix-count=50 --min-peaks=20 --local-bg-radius=2 --indexing=mosflm -o center_finding.stream -j 30

5) Use script detector-shift to correct the position after indexing

ANALYSIS AND RESULTS

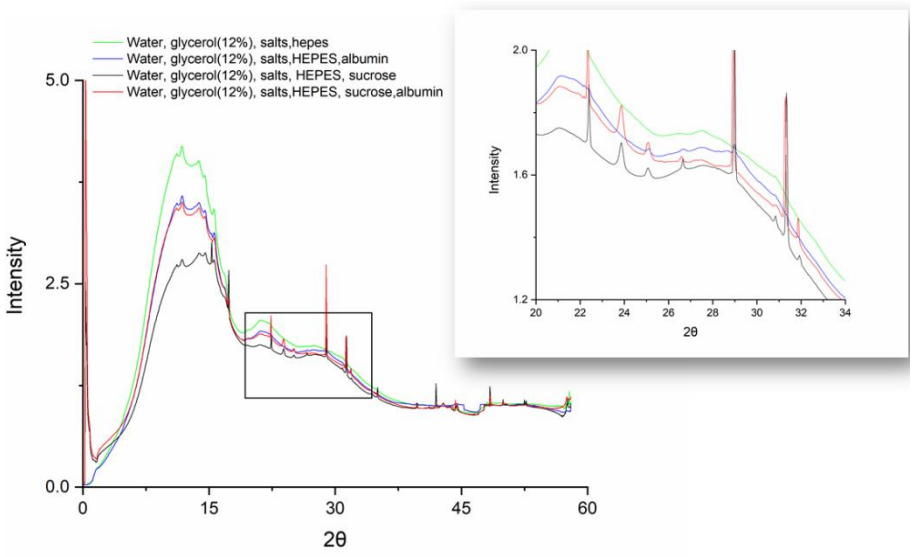


Pic.3 Patterns for measured water-glycerol solutions

Picture 3 presents the main patterns from data sets which were collected on beamlines P14 and P11.

1. Radial average analysis

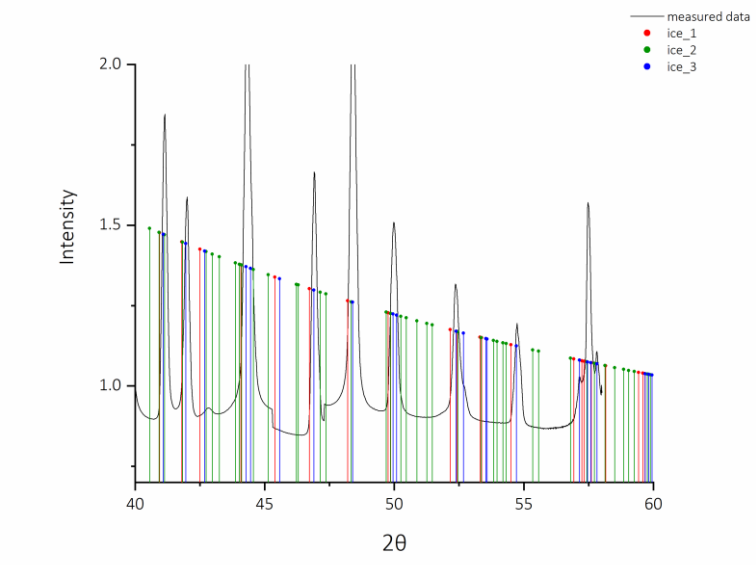
One of the most important part of data processing is radial average analysis. In aim to get the txt files with the radial average parameters for each solutions script intor was used. Processed data then can be used to see the difference between ice formation in exploring solutions. For the main solutions (closest to the clinical ones) we can see (Pic.4) that for the solutions without non-penetrating components: sucrose and albumin the intensity dependence from angle is smooth without peaks (green). Addition of sucrose and albumin leads to the appearance of sharp peaks.



Pic.4 Radial average analysis

2. Powder plot analysis

Also powder plot analysis was provided manually. It requires the plot of intensity from angle and allows to find out the type of ice formed in the solution. For this aim ices with different space groups and unit cell parameters were simulated (Pic.5).



Pic. 5 Powder plot analysis for different types of ice

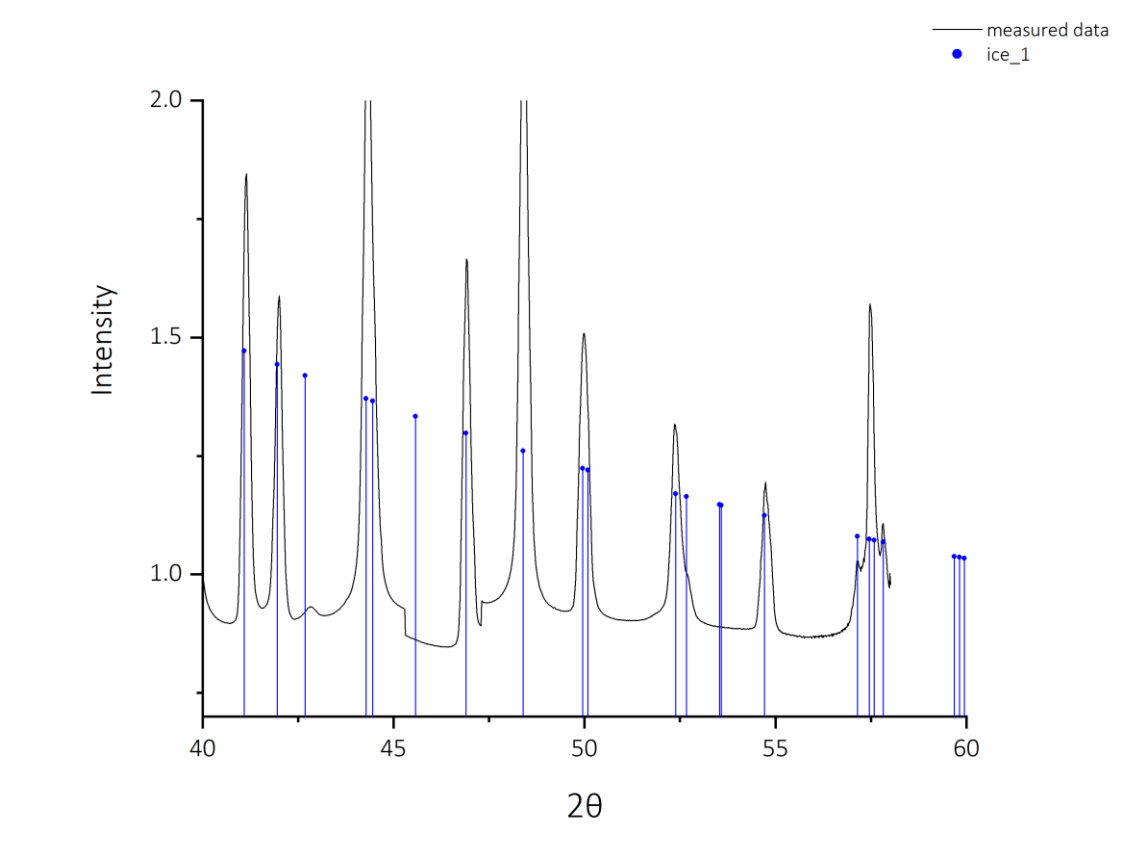
Then one of them which approximate the measured data better than others was chosen (Pic.6). It was ice with

space group: P63

unit cell parameters:

a	**b**	**c**	**α**	**β**	**γ**
4.5	4.5	7.3	90	90	120

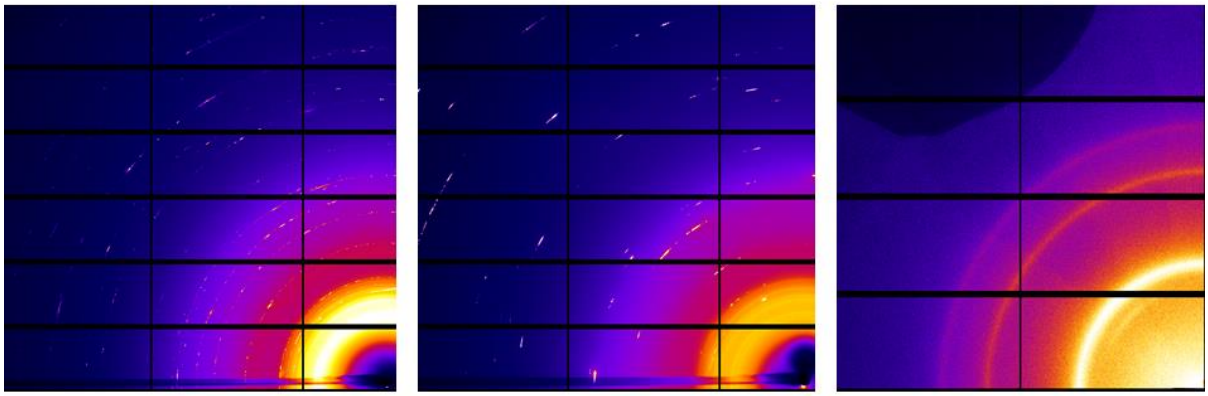
But there are still some ice reflections which cannot be found on the plot for measured data. One of the assumptions is that there is additional symmetry due to stress in the crystal.



Pic.6 Results for the best approximating ice

3. Analysis for different ways of freezing

In the Pic.7 we can see the difference in the results for three kinds of freezing process. The most interesting question was how non-penetrating component for example sucrose influences the ice formation.



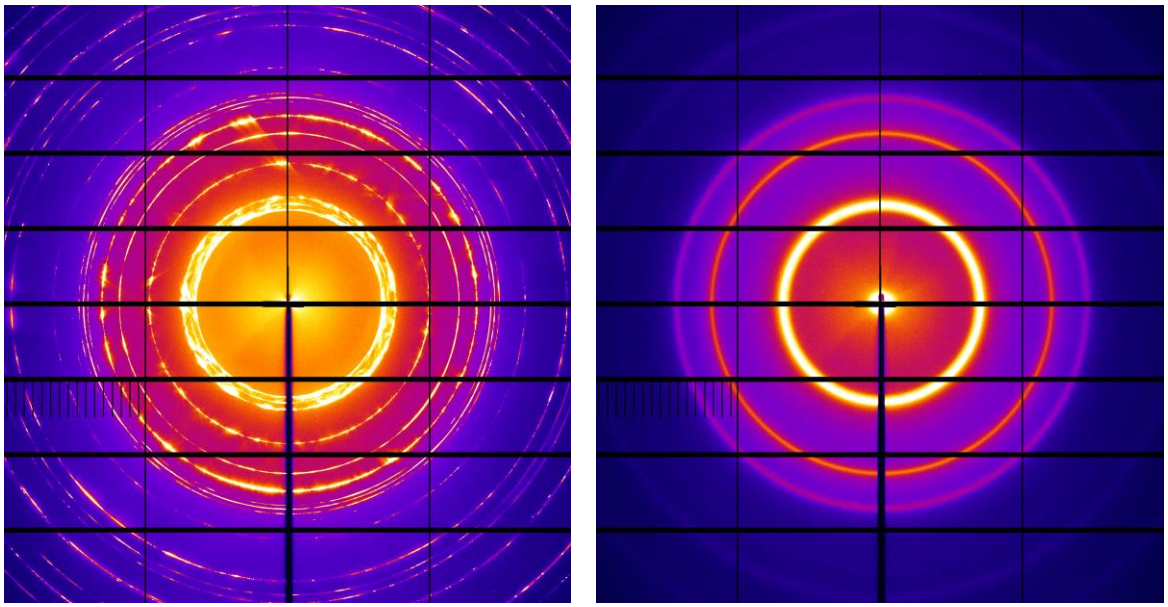
Beamline: P11
Freezing: fast in liquid

Beamline: P11
Freezing: slow in cryostream

Beamline: P14
Freezing: in the falcon tube

Pic.7 Patterns for different ways of freezing

At these images we can see the difference between the number of crystals and their sizes for solutions with and without sucrose. The rings which are presented in the Pic.8 (b) can appear only if there are lots of small crystals inside the frozen solution and there sizes should be smaller than that ones in the basic solution.



Water, glycerol (12%vol.), HEPES, salts

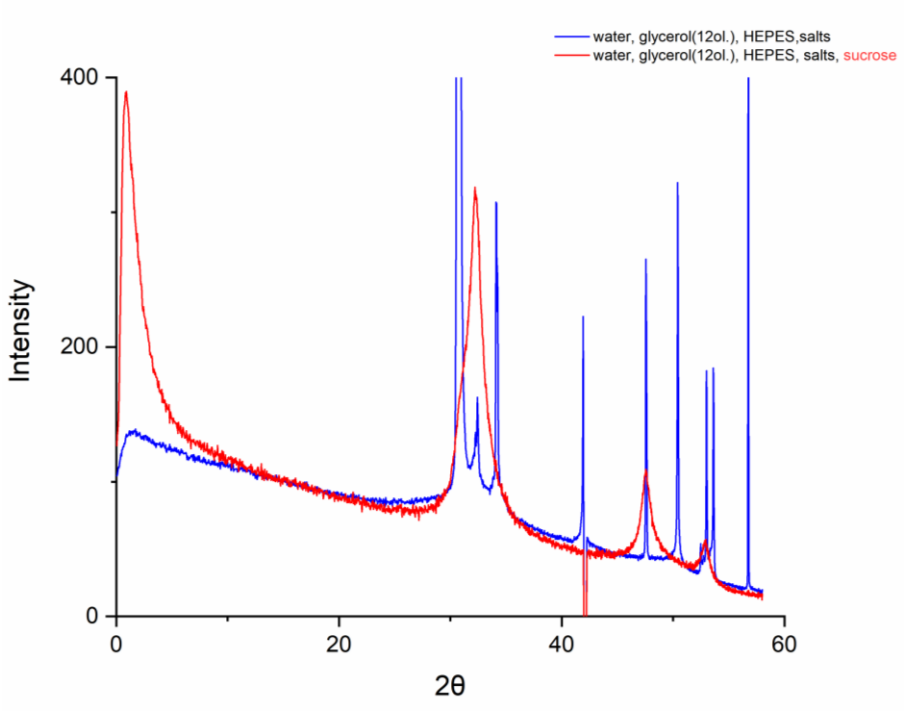
(a)

Water, glycerol (12%vol.), HEPES, salts, sucrose

(b)

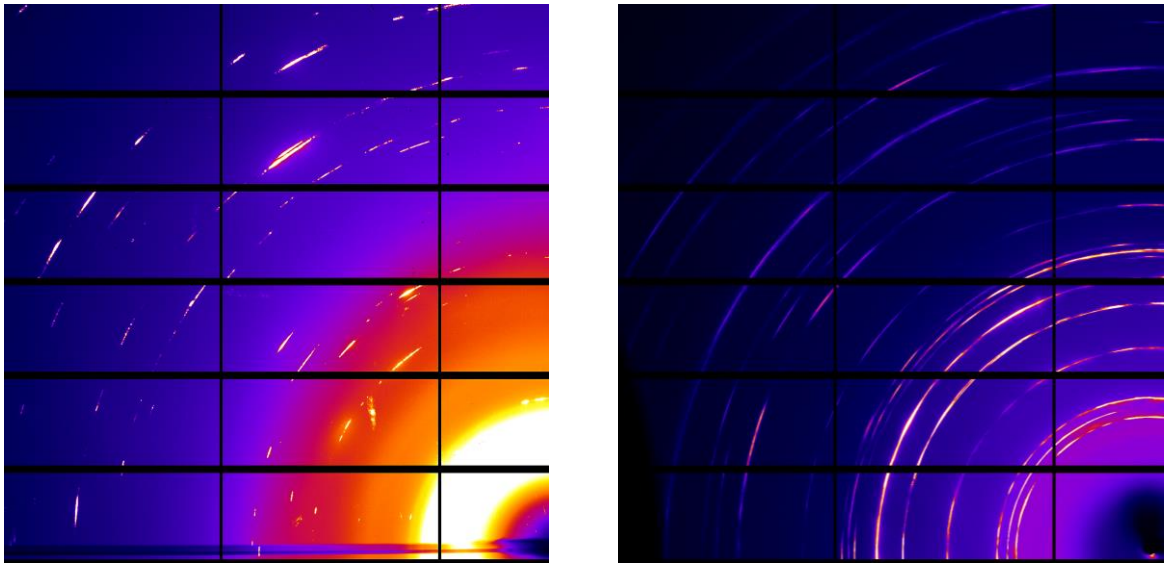
Pic.8 Patterns for basic solution with addition of sucrose measured on the beamline P14

Also radial average analysis shows that for the basic solution: water, glycerol(12%), salts and HEPES the plot has sharp peaks and solution with sucrose has parts with amorphous state and the whole number of peaks is smaller, they are smoothed.



Pic.9 Radial average analysis for the basic solution with and without sucrose

These results are reproducible for different ways of freezing. Patterns of fast frozen samples measured on the beamline P11 also have more smaller crystals in the solution with non-penetrating component- sucrose (Pic.10)



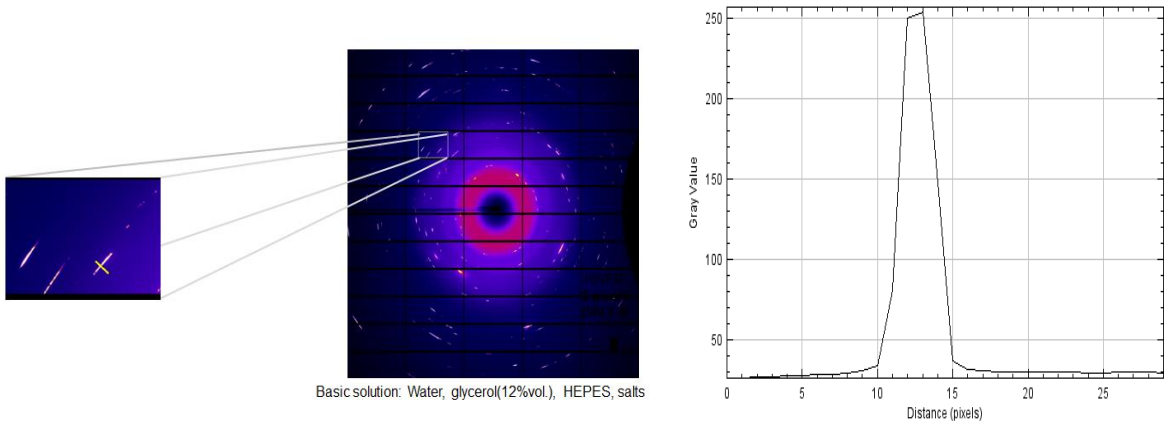
Water, glycerol (12%vol.), HEPES, salts **Water, glycerol (12%vol.), HEPES, salts, sucrose**

Pic.10 Patterns for basic solution with addition of sucrose measured on the beamline P11

4. Single pattern analysis

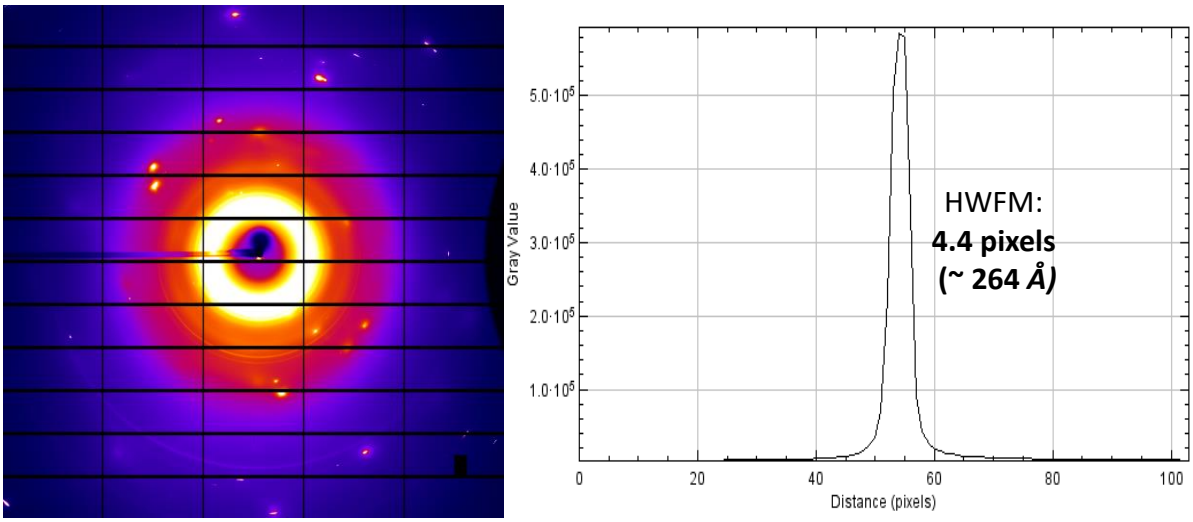
Another method which can be used is single pattern analysis. It can show the difference in size of crystals between different water-glycerol solutions and the sizes themself by measuring the half width (HWFM). Pic.11 shows the main idea of this method. HWFM

allows to determine the size of the crystals but it requires future research because one peak is not enough to make conclusions about average size of the crystals in the solution.



Pic.11 Main steps of single pattern analysis

Other problem of this method is presented in the Pic. 12. When small crystals are in the correct orientation they can give wide HWFM. Ewald's sphere does not always cross the entire Bragg peak. Therefore, this analysis without rotation is not entirely complete.



Pic.12 Single pattern analysis for the solution: water, glycerol (12%vol.), sucrose

CONCLUSION

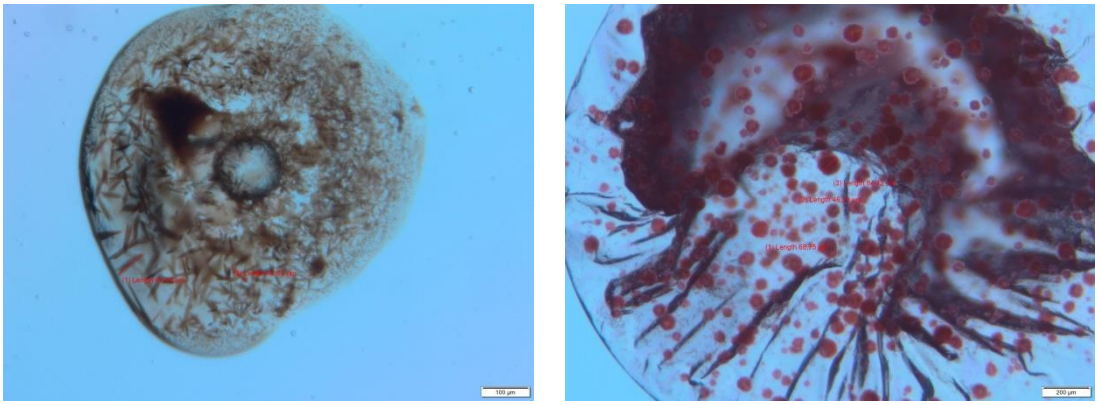
During this project:

- 20 water-glycerol solutions with different additional components were measured and analyzed
- Different ways of freezing were explored
- Powder analysis was used to determine type of formed ice
- Analysis of individual Bragg peaks was used to determine ice crystals' sizes

- It was found out that addition of non-penetrating cryoprotectant (sucrose, albumin) make more crystals with smaller size which correlates with observation that sucrose helps to protect cells from damage during the freezing

OTHER PROJECTS

During my summer school program I was also working in the laboratory where I was making crystals from proteins: Phycocyanin, Cytochrome, Hemmoglobin (Pic.12) for this I used different crystallization methods: SONICC, running SDS-gels, hanging drop way of crystallization.



(a) (b)

Pic.12 Hemmoglobin and Cytochrome crystals

Also I was processing some data sets from different experiments. The main points are presented below:

- XDS on different data sets:

Beamtime P11 in March → Phycocyanin, Aquaporin, Cytochrome, Green protein

Beamtime P14 in August → Green protein in different cryo conditions

- CrystFEL on cryo data to find detector geometry
- Cheetah and CrystFEL on LCLS data:

Experiment S.Boutet 2011: Cathepsin B, Lysozyme, PS1

These points are described in details in another report by Natalia Lybaykina because we were working on these projects together.

References:

1. Bruder S. P., Jaiswal N., and Haynesworth S. E., 1997, Cell. Biochem. 64 , 278
2. Libo S. P., Picton H. M., and Godson R. G., 2001, In Current Practices and Controversis in Assisted Reproduction, World Health Organization, Geneva, p.152–165