An improved loopless mounting method for cryocrystallography*

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Based on a recent loopless mounting method, a simplified loopless and bufferless crystal mounting method is developed for macromolecular crystallography. This simplified crystal mounting system is composed of the following components: a home-made glass capillary, a brass seat for holding the glass capillary, a flow regulator, and a vacuum pump for evacuation. Compared with the currently prevalent loop mounting method, this simplified method has almost the same mounting procedure and thus is compatible with the current automated crystal mounting system. The advantages of this method include higher signal-to-noise ratio, more accurate measurement, more rapid flash cooling, less x-ray absorption and thus less radiation damage to the crystal. This method can be extended to the flash-freeing of a crystal without or with soaking it in a lower concentration of cryoprotectant, thus it may be the best option for data collection in the absence of suitable cryoprotectant. Therefore, it is suggested that this mounting method should be further improved and extensively applied to cryocrystallographic experiments.

Keywords: cryoprotectant, crystal mounting, data collection, flash cooling

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1. Introduction

Crystal mounting is an important step in the data collection of protein crystallography. The ability to mount protein crystals effectively increases the diffraction dataset both quantitatively and qualitatively. In particular, better crystal mounting methods will conduce to the improvement of experiment in some respects, such as crystal screening,[1] high-throughput data collection,[2] and structure-based drug design.[3]

A series of developments in crystal mounting devices have historically made important contributions to protein crystallography. Protein crystals were first mounted in a sealed glass or quartz capillary, together with some mother liquor, in order to retain the internal crystalline order.[4] However, this capillary mounting device has restrictions on the crystal shape and shortcomings in non-uniform x-ray absorption and poor heat-transfer. The loop method developed by Teng[5] and modified later by using low x-ray absorption materials[6] is simple, fast and reliable, in which the surface tension of the mother liquor is utilized to hold a crystal. While it is the most widely used mounting device in protein crystallography, the loop mounting method has drawbacks: the loop and the excess liquid surrounding the crystal will not only cause background x-ray scattering and thus lower the signal-to-noise ratio, but also decrease the cooling rate and thus may increase the cooling-induced crystal damage.[7] A loopless and bufferless crystal mounting technique was recently developed by Kitago *et al.*[8] for the sulphur single wavelength anomalous diffraction (S-SAD) method. In their novel mounting method, the cryo-buffer as well as the loop around the protein crystal was removed prior to flash cooling in order to eliminate radiation absorption. It is shown that this loopless method increases the quality of the overall dataset and the accuracy of the measured anomalous difference between the Bijvoet pairs. However, the complicated operating procedure may restrict its practical application, especially in high-throughput protein crystallography.

This work aims to develop a more practical and more reliable procedure for mounting a crystal without the help of a loop and buffer in order to improve the overall quality of the diffraction dataset. Inspired by the work of Kitago *et al.*[8] the specific task involved is to refine and simplify the loopless and bufferless crystal mounting procedure. Furthermore, high-throughput data collection for macromolecular

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crystallography requires an automated sample mounting system.[9] So by simplifying the existing method, the possibility exists that the loopless and bufferless crystal mounting procedure could be performed by a robot, instead of only by a skilled experimenter.

In this paper, the design and implementation of a simplified loopless and bufferless crystal mounting method is described. The simplified method is tested for its applicability to flash cooling of tetregonal lysozyme crystals. It is shown that the simplified method retains most of the advantages of the method of Kitago *et al.*[8] It is demonstrated in experiment on lysozyme crystals that our simplified method can also flash cool crystals by soaking in a lower concentration of cryoprotectant.

2. Materials and methods

2.1. Preparation of crystals

Tetragonal crystals of hen egg-white lysozyme were obtained by the hanging-drop vapour diffusion method using a precipitant solution of 4% NaCl in 2×10^4mol/dm^3 sodium acetate at pH 4.4 and a protein concentration of 14 mg·ml^{-1}. The drop size was 4 microlitre. They crystallized into a space group $P4_32_12$ with the unit cell parameters shown in Table 1. It took about 5–8 days to yield isomorphous lysozyme crystals. In the diffraction experiments, only crystals of similar size, about 0.3 mm×0.3 mm×0.2 mm, were selected. Diffraction datasets were collected on a Rigaku rotating anode with a radiation wavelength of 1.5418 Å (1 Å=0.1 nm), equipped with an R-AXIS IV^{++} imaging-plate detector. All datasets were processed with the CrystalClear v1.3.5 program.

Table 1. Dataset statistics for tetragonal lysozyme. Data were collected by using different mounting methods. The first two datasets, labelled CryoNoLoop, were collected by soaking in 15% glycerol using our mounting method, while the next two datasets, labelled CryoLoop, were collected by using the standard loop method. The last two datasets, labelled NoLoop, were collected by using our method without soaking in any cryoprotectant. Values in parentheses are for the highest resolution shell.

	CryoNoLoop1	CryoNoLoop2	CryoLoop1	CryoLoop2	NoLoop1	NoLoop2
crystal dimensions/mm	0.3×0.3×0.2	0.3×0.3×0.2	0.3×0.3×0.2	0.3×0.3×0.2	0.3×0.3×0.2	0.3×0.3×0.2
crystal-to-detector distance/mm	100	100	100	100	100	100
oscillation range/(°)	1	1	1	1	1	1
rotation range/(°)	360	360	360	360	360	360
exposure time/min	1	1	1	1	1	1
unit-cell parameters/Å						
a	78.53	78.74	78.79	78.81	78.27	78.75
b	78.53	78.74	78.79	78.81	78.27	78.75
c	37.02	36.97	36.97	36.90	37.27	37.18
space group	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$
resolution limit	35.12–1.70 (1.76–1.70)	35.21–1.70 (1.76–1.70)	39.39–1.70 (1.76–1.70)	39.40–1.70 (1.76–1.70)	33.65–1.70 (1.76–1.70)	39.38–1.70 (1.76–1.70)
reflections measured	352143	354529	345209	345696	348539	341103
unique reflections	13211	13244	13266	12680	13199	13258
completeness/%	99.7 (99.5)	99.6 (100.0)	99.6 (99.8)	95.4 (91.1)	99.8 (100.0)	99.1 (100.0)
$R_{\rm sym}$/%	0.055 (0.187)	0.053 (0.156)	0.057 (0.216)	0.060 (0.259)	0.079 (0.243)	0.053 (0.197)
redundancy	26.66 (25.16)	26.77 (26.43)	26.02 (25.56)	27.26 (26.98)	26.41 (26.46)	25.73 (25.23)
average $I/\sigma(I)$	38.4 (15.7)	34.8 (16.8)	31.1 (11.0)	32.7 (11.0)	28.4 (12.9)	34.0 (11.9)
mosaicity	0.777	0.637	0.860	0.646	1.123	0.912

2.2. Crystal mounting device

Our simplified crystal mounting device is composed of the following components: a glass capillary, a brass seat for mounting the glass capillary, plastic tubing, a flow regulator, and a vacuum pump for evacuation. The glass capillary used in our experiments was home-made by pulling a glass tube. The wall of the glass capillary should be thin enough to allow rapid cooling, but thick enough to allow normal manipulation without breaking. According to our approximate measurement, the inner diameters of our home-made glass capillaries should range from 100 μm to 250 μm. They were at least 12 mm long. In addition, two small notches were poked on the tip of the capillary as shown in Fig. 1. These notches could prevent the complete blocking of the air flow by a large crystal. Continuous air flow is necessary for sucking away all the mother liquor and holding the crystal on the tip.

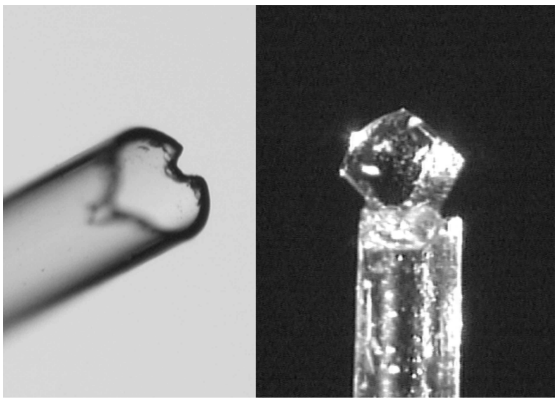


Fig. 1. Home-made glass capillary and a lysozyme crystal mounted on its tip.

In the experiments on lysozyme crystals, a $\sim$ 30 μm thick glass capillary with an inner diameter of 160 μm was used to fit the size of the lysozyme crystals mentioned above. The home-made brass seat was mounted on a goniometer head. On the top of the brass seat, there was a small hole for holding the thin glass capillary, which was fastened and sealed with clay for an airtight connection. On the side face of the brass seat, there was an outlet for connecting to the vacuum pump with plastic tubing. In order to maintain a stable suction pressure, a Porter flowmeter with an adjustable controller was connected between the vacuum pump and the brass seat. Finally, vacuum grease was used for sealing the plastic tubing connections.

2.3. Crystal mounting procedure

Our crystal mounting procedure is as follows. First, fix the glass capillary into the brass seat and bring the tip of the glass capillary into the focus of the x-ray beam. Second, begin to evacuate by turning on the vacuum pump and adjust the flowmeter control valve so that the air flow rate stabilizes at an appropriate value. Third, block the nitrogen gas cooling stream with a small piece of paper card, pick up a protein crystal from the mother liquor by using a standard loop, and then quickly move the crystal to the tip of the glass capillary. This step is a little bit hard because one needs to aim and position the crystal well. Under the suction pressure, the crystal together with the buffer around it will flow to the tip of the glass capillary, and the buffer will be removed immediately after the crystal has been mounted. Finally, flash cool the crystal on the tip of the glass capillary by quickly taking away the blocking paper card. A comparison between our procedure and the standard loop mounting procedure shows that the main difference is that in our procedure the crystal is sucked and stuck onto the tip of the glass capillary. The whole mounting process from picking up the crystal to flash cooling it and to mounting it onto the tip of the glass capillary takes approximately less than 10 s. In most cases, it is not difficult for a skilled crystallographer to do so.

2.4. Soaking in cryoprotective solvent

Most diffraction data are collected at cryogenic temperature in order to reduce radiation damage to the protein crystals.[6,10–13] The common practice is to first soak the protein crystals in a suitable cryosolution (mother liquor plus a cryoprotectant such as glycerol, polyethylene glycol (PEG), or 2-methyl-2, 4-pentadiol (MPD)) to prevent ice formation during flash cooling. In our experiment, the lysozyme crystals were first soaked in glycerol with increasing concentration from 5% to 15% and then mounted according to the procedure described above.

2.5. Flash cooling without soaking in cryoprotective solvent

The process of finding a suitable cryoprotectant and a corresponding optimal concentration for cooling a protein crystal is sometimes difficult and time-consuming. A common problem of soaking a protein

crystal in a cryo-solution is that any change in the property of the mother liquor may adversely affect or even ruin the crystal. In practice, the optimal cryoprotectant and its concentration must be determined by experiment. Sometimes no appropriate cryoprotectant is available even after extensive searching.

By using the simplified loopless crystal mounting method, flash cooling a protein crystal without soaking in any cryoprotectant may be an alternative. As shown by Hope,[14] the probability P that a volume of water V is frozen in a time length t is given by

$$P = J(T)Vt, \tag{1}$$

where $J(T)$ is the nucleation rate at temperature T. Since the mother liquor and the loop around the crystal have been removed before flash cooling, the volume and thus the heat capacity of the system can be greatly reduced. Therefore, it takes less time to reach the glass transition temperature. The corresponding probability of ice formation also decreases.

Cooling crystal without any cryoprotectant was originally shown to be possible by using a glass fibre or micro-spatula.[10,11] In that mounting procedure, the protein crystal was first soaked in hydrophobic oil to remove the solvent around the crystal, and then it was flash cooled in the cold stream. This technique can be applied to the loopless and bufferless crystal mounting method. The mounting procedure is the same except omitting the soaking step. A crystal is simply picked up from the crystallization mother liquor and directly mounted on the tip of the glass capillary for flash cooling. It should be especially noted that the speed of the flash cooling is vital to the success of this procedure because in the absence of cryoprotectant the protein crystal is more susceptible to ice formation. In our experiments, mounting a lysozyme crystal using our device without soaking it in any cryoprotectant was tested.

3. Results

3.1. Tests with the simplified method of mounting a crystal without using a loop and mother liquor

Since the suction pressure exerts a mechanical force on the mounted crystal, there is a range of optimal values for the air flow rate. The force must be not too big, otherwise mechanical damage to the crystal takes place, but still big enough, so that the crystal can be held. A Porter flowmeter with a flow rate ranging from 4 ml/min to 55 ml/min was used for controlling the air flow rate. For example, when a plate-shape crystal was mounted at a flow rate of 40 ml/min, it was immediately broken into two pieces by the high suction pressure. By trial and error, 12 ml/min was found to be an optimal flow rate for the glass capillary with ~ 160 μm inner diametre, which resulted in a high successful mounting rate and a high diffraction data quality as well. It should be mentioned that the bore of the glass capillary should fit the size of protein crystals, that is, a large diametre capillary and a high air flow rate should fit a large size crystal. Our 160 μm sized glass capillary with a flow rate around 12 ml/min was sufficient for most of the chunky lysozyme crystals used in the tests.

Tests were carried out to verify the performance and the reliability of our simplified crystal mounting method. Datasets were collected on lysozyme crystals of similar size by using different mounting methods. As shown in Table 1, the datasets labelled CryoNoLoop were collected by using our simplified method, while those labelled CryoLoop datasets were collected by using the standard loop. It can be seen from the data statistics that the total number of measured reflections and completeness are almost the same for the four datasets. However, the $I/\sigma(I)$s are higher and the $R_{\rm sym}$s and mosaicities are lower for the datasets labelled CryoNoLoop. An exception is noted for datasets. It is shown that for CryoNoLoop2 and CryoLoop2, they have much small improvement on $I/\sigma(I)$, whose values are 34.8 and 32.7 respectively. This exception is not surprising because mounting the crystal to the tip of the glass capillary is more susceptible to errors in manipulation than to the standard loop method itself.

Figure 2 shows the distribution of $R_{\rm sym}$s and $I/\sigma(I)$s against frame (rotation degree) in the process of data collection. It is apparent that after eliminating x-ray absorption and scattering by the cryo-buffer and the loop, the overall quality of diffraction datasets has been improved. Figure 3 shows the changes of the scale factors. Typically, using the loopless and bufferless crystal mounting method, the crystal is stabler and shows few signs of radiation damage in the process of data collection. In comparison, for the crystal mounted using the loop, radiation damage is shown clearly by gradually increasing the scale factor for higher (or later) frames.

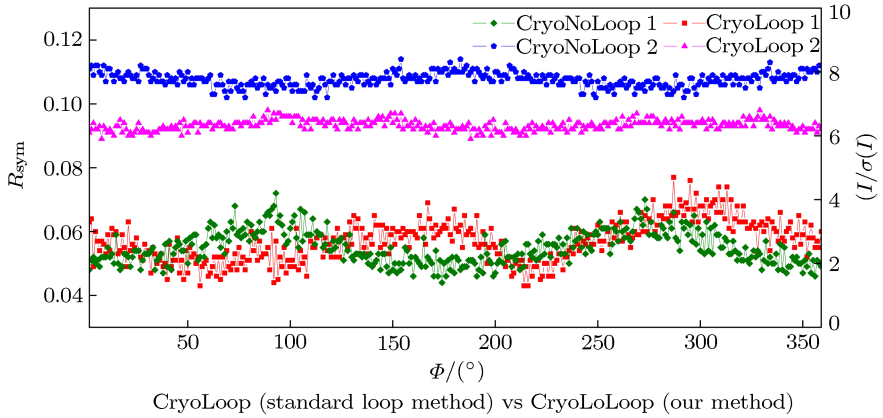


Fig. 2. Comparison between $R_{\rm sym}$s and $I/\sigma(I)$s obtained from different crystal mounting methods. $R_{\rm sym}$s and $I/\sigma(I)$s are plotted against frames (rotation degree) during the data collection. The diamonds and circles (green and blue), representing $R_{\rm sym}$ and $I/\sigma(I)$ respectively, are for our method (CryoNoLoop). The squares and triangles (red and purple) are for the standard loop method (CryoLoop).

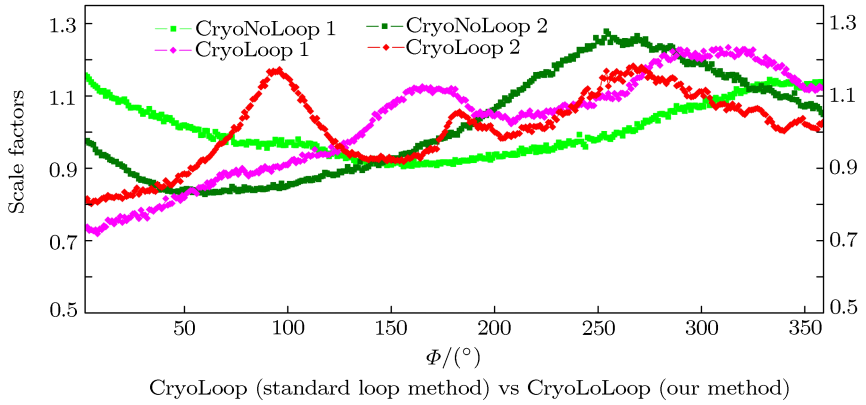


Fig. 3. Comparison between distributions of scale factors with soaking in cryo-solution for both the standard and our methods. The scale factors are plotted against frame (rotation degree) during data collection. The squares (light and dark green) are for our method (CryoNoLoop) and the diamonds (purple and red) are for the standard loop method (CryoLoop).

3.2. Flash cooling crystals without using cryoprotectant

Tests on the feasibility of flash cooling crystals without using any cryoprotectant were carried out by our new crystal mounting method. In total, 18 lysozyme crystals were sampled in one batch, with each crystal mounted and checked by subsequent data collection of four equally-spaced frames. The results are summarized in Table 2.

Of the 18 lysozyme crystals, 15 were successfully mounted, while the other 3 lysozyme crystals were mismounted due to error in manipulation. Of the 15 successfully mounted crystals, 12 lysozyme crystals diffracted beyond 1.7 Å. In addition, two large datasets (360 frames with $\Delta\varphi = 1^\circ$ each frame), labelled NoLoop1 and NoLoop 2 in Table 1, were collected to evaluate the quality of the crystals and their diffraction without soaking them in any cryoprotectant. The dataset statistics are also given in Table 1. Comparing $I/\sigma(I)$ of the dataset labelled NoLoop1 with the other two datasets labelled CryoLoop shows that the NoLoop1 $I/\sigma(I)$ is about 3% lower and $R_{\rm sym}$ is about 2% higher than the CryoLoop $I/\sigma(I)$. However, for the dataset NoLoop 2, the $I/\sigma(I)$ is high and the $R_{\rm sym}$ is low compared with CryoLoop $I/\sigma(I)$. This is because our crystal mounting procedure has been optimized by ensuring a faster rate of flash cooling when taking away the paper card. Figure 4 shows the distributions of the scale factors, obtained by different methods. Even without using any cryoprotectant, the changes of scale factors, obtained by our simplified loopless method, indicate that the crystals are also stable in the process of data collection.

Table 2. Summary of continuously mounted crystals without using cryoprotectant. Crystals were mounted by using the simplified loopless method without soaking them in any cryoprotectant. Crystals Nos. 2, 6 and 14 were mis-mounted due to error in manipulation. NA stands for not available. Diffraction resolution was judged by visible diffraction spots on displayed frames.

crystal number	dataset statistics			
	resolution/Å	unit cell dimensions/Å	mosaicity	explanation
1	2.1	$a = b$=78.21, c=37.02; $\alpha = \beta = \gamma$=90°	1.25	
2	NA	NA	NA	mismounted
3	1.7	$a = b$=78.67, c=37.11; $\alpha = \beta = \gamma$=90°	0.86	
4	1.7	$a = b$=78.69, c=37.17; $\alpha = \beta = \gamma$=90^o	1.06	
5	1.9	$a = b$=78.32, c=37.01; $\alpha = \beta = \gamma$=90°	1.17	
6	NA	NA	NA	mismounted
7	1.7	$a = b$=78.52, c=37.04; $\alpha = \beta = \gamma$=90°	0.95	
8	1.7	$a = b$=78.75, c=37.20; $\alpha = \beta = \gamma$=90°	0.93	
9	1.7	$a = b$=78.49, c=37.14; $\alpha = \beta = \gamma$=90°	0.72	
10	1.7	$a = b$=78.61, c=37.21; $\alpha = \beta = \gamma$=90°	0.67	
11	1.8	$a = b$=78.45, c=37.22; $\alpha = \beta = \gamma$=90°	0.98	
12	1.7	$a = b$=78.58, c=37.17; $\alpha = \beta = \gamma$=90°	0.93	
13	1.7	$a = b$=78.66, c=37.11; $\alpha = \beta = \gamma$=90°	0.85	
14	NA	NA	NA	mismounted
15	1.7	$a = b$=78.72, c=37.17; $\alpha = \beta = \gamma$=90°	0.75	
16	1.7	$a = b$=78.51, c=37.16; $\alpha = \beta = \gamma$=90°	0.98	
17	1.7	$a = b$=78.59, c=37.14; $\alpha = \beta = \gamma$=90°	0.75	
18	1.7	$a = b$=78.75, c=37.18; $\alpha = \beta = \gamma$=90°	0.91	

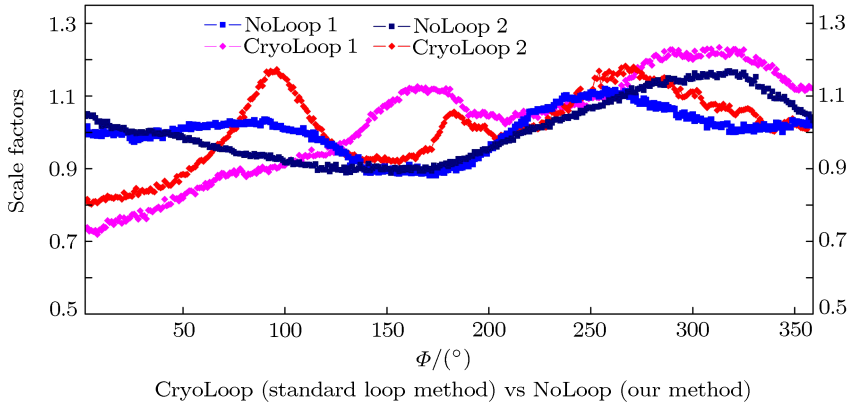


Fig. 4. Comparison between distributions of scale factors without soaking in cryo-solution. The scale factors are plotted against frame (rotation degree) during the data collection. The squares (light and deep blue) are for our method (NoLoop) and the diamonds (purple and red) are for the standard loop method (CryoLoop).

It should be pointed out that the mosaicities labelled NoLoop are generally about 0.3 higher than those of the other four datasets. This deterioration of the data quality is because during the flash cooling process in the absence of any cryoprotectant, a small fraction of the solvent in the lysozyme crystals turned into ice, which adversely perturbed the crystal lattices. It should be mentioned that almost all the diffraction images of the 15 successfully mounted crystals showed some weak ice rings. This is also indicated by slightly reduced diffraction resolution for some of the crystals with respect to that of the soaked crystals. Nevertheless, as shown in Table 2, many of the successfully mounted crystals have retained the high resolution diffraction close to that with using cryoprotectants such as 15% glycerol for lysozyme crystals. Thus, the loopless and bufferless crystal mounting method when no cryoprotectant is used is still able to freeze crystals for data collection. Therefore, this is a potential useful alternative in cases where no suitable cryoprotectant can be found.

3.3. Flash cooling crystal soaked in lower concentration of cryoprotectant

It is obvious from Table 1 and Table 2 that the mosaicities of flash cooled lysozyme crystals without their soaking in any cryoprotectant tend to be higher than those of the same crystals pre-soaked in cryoprotectant

solutions. The increase of mosaicity will not only increase the number of overlaps, but decrease the signal-to-noise ratio of a reflection.[15] Generally, lower concentration of cryoprotectant should be less harmful to the crystal stability. This is because less cryoprotectant will diffuse into and change the solution content of the crystal channels. This in turn will reduce both the amount of lattice change and conformational re-equilibration, and thus may reduce the mosaic spread of the crystal due to repacking. Flash cooling the lysozyme crystals soaked in a lower concentration of cryoprotectant was tested by using our simplified loopless method to see the effect on the mosaicity.

As shown in Table 3, three kinds of commonly used cryoprotectants, namely, glycerol, MPD and isopropanol, were used in the tests, and all of them were prepared as 5% solutions. The protein crystals were first soaked into a certain cryo-solution for a specific time length, and then flash cooled by using our simplified loopless method. Four equally-spaced frames were collected to evaluate the quality of the diffraction. For 5% glycerol, lysozyme crystals diffracted better than 1.7 Å resolution. The averaged mosaicities are 0.77, 0.72, 0.68 corresponding to soaking times of 20 s, 1 min and 3 min, respectively. The results are similar to those for 5% MPD. Their resolutions were beyond 1.7 Å, and the averaged mosaicities reduced from 0.81 to 0.72 when the soaking time extended from 1 min to 3 min. However, it is totally different for soaking in a 5% isopropanol solution. For lsozyme crystals soaked in 5% isoproponal for 3 min, they diffracted only about 1.8 Å resolution with an averaged mosaicity 0.98. When the soaking time was reduced to 1 min, the resolutions again returned to 1.7 Å, and the averaged mosaicities reduced to 0.80. This shows that for suitable cryoprotectants for lysozyme crystals such as glycerol and MPD and using our mounting method, lower concentrations of cryoprotectants can still work. It also suggests that in a suitable cryoprotectant condition, a longer soaking time, thereby allowing the crystals to reach re-equilibration in a longer time, leads to a lower mosaicity. For unsuitable cryoprotectants such as isopropanol, shorter soaking time gives better results because it will reduce the damage to the crystals due to the bad cryoprotectants. In this case, the cryoprotection in effect is probably due to a quick equilibration of the cryoprotectant in the surrounding mother liquor and the crystal channels without extensive diffusion and repacking in the crystals.

Table 3. Dataset statistics for lysozyme crystal soaked in a lower concentration of cryoprotectant. The cryoprotectants used were glycerol, MPD and isopropanol and they were prepared in 5% solutions. For each cryoprotectant, lysozyme crystals were first soaked for various time lengths, and then mounted by our simplified loopless method.

cryoprotectant	concentration/%	crystal number	soaking time	dataset statistics		
				resolution/Å	unit cell dimensions/Å	mosaicity
Glycerol	5%	1	20 s	1.70	a=b=78.58, c=37.06; $\alpha=\beta=\gamma$=90°	0.69
	5%	2	20 s	1.70	a=b=78.44, c=37.09; $\alpha=\beta=\gamma$=90°	0.75
	5%	3	20 s	1.70	a=b=78.67, c=37.02; $\alpha=\beta=\gamma$=90°	0.71
	5%	4	20 s	1.70	a=b=78.54, c=37.02; $\alpha=\beta=\gamma$=90°	0.70
	5%	5	20 s	1.70	a=b=78.31, c=37.13; $\alpha=\beta=\gamma$=90°	0.88
	5%	6	20 s	1.70	a=b=78.37, c=37.09; $\alpha=\beta=\gamma$=90°	0.87
	5%	7	1 min	1.70	a=b=78.52, c=37.05; $\alpha=\beta=\gamma$=90°	0.61
	5%	8	1 min	1.70	a=b=78.34, c=36.98; $\alpha=\beta=\gamma$=90°	0.73
	5%	9	1 min	1.70	a=b=78.50, c=37.03; $\alpha=\beta=\gamma$=90°	0.76
	5%	10	1 min	1.70	a=b=78.55, c=37.10; $\alpha=\beta=\gamma$=90°	0.85
	5%	11	1 min	1.70	a=b=78.54, c=37.10; $\alpha=\beta=\gamma$=90°	0.72
	5%	12	1 min	1.70	a=b=78.45, c=36.99; $\alpha=\beta=\gamma$=90°	0.75
	5%	13	3 min	1.70	a=b=78.64, c=37.09; $\alpha=\beta=\gamma$=90°	0.64
	5%	14	3 min	1.70	a=b=78.62, c=37.04; $\alpha=\beta=\gamma$=90°	0.64
	5%	15	3 min	1.70	a=b=78.70, c=37.10; $\alpha=\beta=\gamma$=90°	0.57
	5%	16	3 min	1.70	a=b=78.49, c=37.01; $\alpha=\beta=\gamma$=90°	0.74
	5%	17	3 min	1.70	a=b=78.51, c=37.03; $\alpha=\beta=\gamma$=90°	0.78
	5%	18	3 min	1.70	a=b=78.52, c=37.09; $\alpha=\beta=\gamma$=90°	0.72

Table 3. (*Continued*).

cryoprotectant	concentration/%	crystal number	soaking time	dataset statistics		
				resolution/Å	unit cell dimensions/Å	mosaicity
MPD	5%	19	1 min	1.70	a=b=78.68, c=37.06; $\alpha=\beta=\gamma$=90°	0.77
	5%	20	1 min	1.70	a=b=78.67, c=37.13; $\alpha=\beta=\gamma$=90°	0.87
	5%	21	1 min	1.70	a=b=78.81, c=37.10; $\alpha=\beta=\gamma$=90°	0.73
	5%	22	1 min	1.70	a=b=78.51, c=37.02; $\alpha=\beta=\gamma$=90°	0.80
	5%	23	1 min	1.75	a=b=78.41, c=37.01; $\alpha=\beta=\gamma$=90°	0.83
	5%	24	1 min	1.75	a=b=78.57, c=37.10; $\alpha=\beta=\gamma$=90°	0.85
	5%	25	3 min	1.70	a=b=78.72, c=37.28; $\alpha=\beta=\gamma$=90°	0.72
	5%	26	3 min	1.70	a=b=78.60, c=37.22; $\alpha=\beta=\gamma$=90°	0.72
	5%	27	3 min	1.70	a=b=78.64, c=37.20; $\alpha=\beta=\gamma$=90°	0.67
	5%	28	3 min	1.70	a=b=78.69, c=37.17; $\alpha=\beta=\gamma$=90°	0.81
	5%	29	3 min	1.70	a=b=78.62, c=37.26; $\alpha=\beta=\gamma$=90°	0.68
	5%	30	3 min	1.70	a=b=78.71, c=37.13; $\alpha=\beta=\gamma$=90°	0.73
Isopropanol	5%	31	1 min	1.70	a=b=78.66, c=37.05; $\alpha=\beta=\gamma$=90°	0.87
	5%	32	1 min	1.70	a=b=78.78, c=37.16; $\alpha=\beta=\gamma$=90°	0.79
	5%	33	1 min	1.70	a=b=78.77, c=37.11; $\alpha=\beta=\gamma$=90°	0.71
	5%	34	1 min	1.80	a=b=78.56, c=37.12; $\alpha=\beta=\gamma$=90°	0.87
	5%	35	1 min	1.70	a=b=78.65, c=37.13; $\alpha=\beta=\gamma$=90°	0.79
	5%	36	1 min	1.70	a=b=78.75, c=37.17; $\alpha=\beta=\gamma$=90°	0.77
	5%	37	3 min	1.70	a=b=78.66, c=37.14; $\alpha=\beta=\gamma$=90°	1.06
	5%	38	3 min	1.75	a=b=78.78, c=37.08; $\alpha=\beta=\gamma$=90°	1.06
	5%	39	3 min	1.70	a=b=78.74, c=37.05; $\alpha=\beta=\gamma$=90°	0.87
	5%	40	3 min	2.00	a=b=78.59, c=36.99; $\alpha=\beta=\gamma$=90°	0.98
	5%	41	3 min	1.75	a=b=78.83, c=37.18; $\alpha=\beta=\gamma$=90°	1.05
	5%	42	3 min	1.80	a=b=78.73, c=37.05; $\alpha=\beta=\gamma$=90°	0.83

4. Discussion

A new, simplified loopless and bufferless crystal mounting method has been described in detail. Compared with the method of Kitago *et al.*,[8] we have simplified their crystal mounting procedure, improved the design of the micro glass capillary, and optimized the aspiration system. It is shown that the removal of the loop and cryo-buffer around the crystal has resulted in higher $I/\sigma(I)$s and lower $R_{\rm sym}$s. Furthermore, the distribution of scale factors by using our method is stabler than that of the current loop method, indicating the radiation damage is reduced for this loopless and bufferless method. This may be because the removal of the buffer on the surface of a protein crystal reduces the amount of x-ray absorption and thus may mitigate the radiation damage to the crystal.[6,15,16] Therefore, crystals mounted by our simplified method should be stabler in the process of data collection. As also pointed out by Kitago *et al.*[8] the loopless and bufferless mounting method is especially useful for measuring anomalous signals at either normal (Cu) or longer (Cr) wavelengths.

In the last few years, automated crystal mounting systems have been developed to increase the capability of high-throughput protein crystallography.[9,17,18] The robotic sample mounting system will not only help a crystallographer systematically screen a large number of crystals, but also ensure much more reliable and accurate data collection. Since our simplified crystal mounting method has fewer steps than the method of Kitago *et al.*[8] it may be amenable to robotic automation. In particular, the most difficult step of requiring an operator to accurately and quickly position the crystal onto the tip of the glass capillary could be accomplished by the current robotic arm. It

has been shown that such a robotic arm has a positional repeatability of ±20 μm,[18] which should be sufficient for our purpose.

There are other interesting applications of our method in other types of data collection.[19,20] One of them that has been tested in this work is flash cooling a crystal without using cryoprotectant. The primary goal of flash cooling a protein crystal is to turn the bulk solvent water surrounding and inside the crystal into vitrified glass, instead of ice[6,21] when the temperature drops below −150 °C. Depending on the content of the bulk solvent in the crystal and the cooling speed, it has been shown that flash cooling a protein crystal without using any cryoprotectant by using our simplified loopless method may be possible to some extent. The cooling rate is a crucial factor and it depends on two processes: the heat transfer from the interior of the crystal to its surface and that across the surface to the cooling agent.[22] For flash cooling a protein crystal by the current loop method, the whole volume of the crystal together with the mother liquor is mainly determined by the mother liquor, while for our simplified loopless method, the mother liquor has been removed prior to flash cooling. Thus in the process of flash cooling, the equilibration process and the changes of the unit-cell dimension due to repacking between the molecules may be different for the two methods. Of the 18 unsoaked crystals shown in Table 2, 12 crystals give acceptable resolution better than 1.7 Å. This suggests that the success rate for achieving a fast cooling speed is repeatable.

It can be seen from Table 4 that by using our simplified loopless method, we could obtain good diffraction datasets for crystals soaked in an appropriate cryoprotectant even at a lower concentration, for example, 5% glycerol for lysozyme crystals. For those inappropriate cryoprotectants, we could still obtain stable datasets by shortening the soaking time or by further reducing the concentration. It must be pointed out that if the crystals are flash cooled by the current standard loop method at the same lower concentration of suitable cryoprotectants (5%), the frozen drops in the loop can turn opaque (in milky colour). Subsequent diffraction images also showed heavy ice rings. This suggests that lower concentration will not work for flash cooling by using the standard loop method but only for our mounting method. However, our simplified loopless mounting method should be further tested by using other protein crystals to see its applicability. Therefore, it is hoped that when an appropriate cryoprotectant is unavailable, this new method will provide another solution for obtaining useful cryodiffraction data.

Table 4. Summary of the averaged mosaicities for lysozyme crystals soaked in a lower concentration of cryoprotectants. Each averaged mosaicity was obtained by averaging over data of the six crystals for the corresponding condition as listed in Table 3.

cryoprotectant	concentration/%	soaking time	resolution/Å	mosaicity
Glycerol	5%	20 s	1.70	0.77
	5%	1 min	1.70	0.74
	5%	3 min	1.70	0.68
MPD	5%	1 min	1.72	0.81
	5%	3 min	1.70	0.72
Isopropanol	5%	1 min	1.72	0.80
	5%	3 min	1.78	0.98

5. Summary

We simplify and improve the loopless and bufferless crystal mounting method for protein crystallography. Compared with the standard mounting procedure using a loop, this method has almost the same operation steps and thus is amenable to robotic automation. Application of this method is extended to flash cooling of a protein crystal with a quick soaking in a lower concentration of cryoprotectant to reduce its damage to the crystal.

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