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Flash-cooling of macromolecular crystals in a capillary to overcome increased mosaicity

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This paper describes the usefulness of flash-cooling in a capillary (FCC) for X-ray diffraction data collection from macromolecular crystals. FCC may be applied when conventional cooling using a cryoloop fails. This technique cools crystals in a capillary instead of in a cryoloop and thus cools crystals more slowly than conventional cooling. Measurements of cooling rates have shown that the time taken to reach the final temperature is two to eight times longer using FCC than using a cryoloop, depending on the volume of cryoprotectant solution around the crystal. Using this cooling technique, the crystal structures of isocitrate dehydrogenase and protein L-isoaspartate *O*-methyltransferase have been solved at 1.95 and at 2.50 Å resolution, respectively. Both crystals could not be cooled by the conventional method using a cryoloop. Moreover, diffraction data from crystals of the hypothetical proteins PH-A and PH-B were also collected successfully using the FCC method. These results show that some crystals, especially larger ones, need to be cooled slowly.

1. Introduction

In the field of macromolecular crystallography, X-ray diffraction data collection under cryogenic temperatures using a loop (cryoloop) has become a standard technique with both in-house and synchrotron sources (Teng, 1990). This cryocrystallographic technique reduces radiation damage and increases the crystal lifetime, enabling the collection of higher quality diffraction data or complete multiwavelength anomalous diffraction (MAD) data using a single crystal. Using the cryocrystallographic technique, sample handling during data-collection experiments is also simplified and the crystal can be stored for later reuse after diffraction experiments. However, the cooling step nearly always increases the crystal mosaicity, resulting in a reduction in diffraction quality. Many different trials have been employed in attempts to overcome this problem by experimenting with cryoprotectant solutions, cryogenic temperature and annealing methods (Mitchell & Garman, 1994; Garman & Mitchell, 1996; Garman & Schneider, 1997; Garman, 1999; Rubinson *et al.*, 2000; Harp *et al.*, 1998, 1999; Yeh & Hol, 1998). However, there remain some crystals that cannot be cooled to cryogenic temperature because the level of mosaic spread increases to such an extent that integrating diffraction intensities becomes impossible.

In general, protein crystals are imperfect crystals that can be considered to be constructed of small mosaic blocks. These mosaic blocks are generally spread at an angle of $<0.02^{\circ}$. Moreover, lattice spacing in such imperfect crystals exhibits a typical distribution of <0.05% (Kriminski *et al.*, 2002; Dobrianov *et al.*, 1998). When these crystals are flash-cooled to

cryogenic temperature large lattice stresses are introduced into the crystals; the unit-cell parameters vary with temperature and their average shrinks by 5–7% during cooling from 300 to 80 K (Frauenfelder *et al.*, 1987; Kurinov & Harrison, 1995). Moreover, during the cooling process the unit-cell parameters are generally not constant throughout the whole volume of the crystals. These stresses placed on the crystals by the temperature change increase the distribution of both mosaic blocks and lattice spacing. The former makes diffraction spots broad and the latter strongly affects the resolution limit (Kriminski *et al.*, 2002). Rapid cooling is generally considered to minimize the increase in mosacity of crystals. However, the cooling cannot be extremely rapid because of the size of the crystals and the subsequent amount of heat transfer. It usually takes 1–2 s to reach the final cryogenic temperature under liquid-nitrogen gas using a cryoloop (Teng & Moffat, 1998; Walker *et al.*, 1998). Therefore, in order to minimize the increase of mosaic spread, the cooling rate has to be carefully optimized.

Table 1
Crystals that have been successfully cooled by the FCC method.

	Precipitant	Cryoprotectant	Crystal dimensions (mm)	Solvent fraction	Space group
IDH	PEG 6000, 20% glycerol	20% glycerol	0.3 × 0.4 × 0.5	0.52	$P2_1$ or $P2_12_12_1$
PIMT	PEG 4000, 10% glycerol	20% glycerol	0.3 × 0.5 × 0.6	0.44	$C2$
PH-A	46–50% MPD	46–50% MPD	0.15 × 0.15 × 0.6	0.62	$P4_32_12$
PH-B	PEG 6000, 10% sucrose	35% sucrose	0.2 × 0.2 × 0.5	0.52	$P4_12_12$ or $P4_32_12$

Table 2
Data-collection statistics.

Values in parentheses refer to data in the highest resolution shell.

	IDH	PH-A	PIMP
Beamline	BL41XU, SPring-8	BL41XU, SPring-8	BL38B1, SPring-8
Space group	$P2_1$	$P4_32_12$	$C2$
Unit-cell parameters (Å, °)	a = 110.4	a = 127.7	a = 164.8
	b = 119.0	b = 127.7	b = 94.7
	c = 128.2	c = 105.9	c = 198.7
	β = 99.0		β = 99.2
Wavelength (Å)	0.9000	0.9794	0.9700
Resolution range (Å)	40–1.95 (2.06–1.95)	40–3.1 (3.27–3.1)	40–2.5 (2.59–2.5)
No. of unique reflections	235314	16425	93248
Completeness (%)	98.9 (96.7)	99.8 (99.7)	89.6 (86.4)
Multiplicity	3.6 (3.2)	12.9 (13.3)	1.8 (1.6)
Average $I/\sigma(I)$	8.6 (2.0)	6.1 (2.5)	17.5
$R_{\rm meas}$†	0.083 (0.388)	0.104 (0.298)	0.114 (0.339)

† $R_{\rm meas} = \sum_{hkl}[m/(m-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i |I_i(hkl)|$, where $\langle I\rangle$ is the mean intensity of a set of equivalent reflections and m is the multiplicity of the data set.

Here, we introduce a useful flash-cooling in a capillary technique. With this technique, the crystal is mounted in a glass capillary, which is then placed in a nitrogen-gas stream to cool the crystal. The cooling rate of flash-cooling in a capillary (FCC) is slower than that using a cryoloop. Applications of this technique to crystals of monomeric $NADP^+$-dependent isocitrate dehydrogenase (IDH) from *Azotobacter vinelandii*, protein L-isoaspartate *O*-methyltransferase (PIMT) from *Sulfolobus tokodaii* as well as the hypothetical proteins PH-A and PH-B from *Pyrococcus horikoshii* are described here. These results show that cooling rates are optimized through the use of a capillary.

2. Materials and methods

2.1. Crystals

Crystals of IDH–isocitrate–Mn^{2+} and IDH–$NADP^+$ were obtained as described previously (Yasutake *et al.*, 2001, 2003). The IDH–isocitrate–Mn^{2+} crystals grew in two forms; one grew to 0.3 × 0.3 × 0.2 mm in six months and belongs to space group $P2_12_12_1$, with unit-cell parameters a = 108.4, b = 121.7, c = 129.7 Å (two molecules in the asymmetric unit), and the other grew to 0.5 × 0.5 × 0.4 mm in one month and belongs to space group $P2_1$, with unit-cell parameters a = 110.4, b = 119.0, c = 128.2 Å, β = 99.0° (four molecules in the asymmetric unit). The crystal of IDH–$NADP^+$ complex belonged to space group $P2_1$, with unit-cell parameters a = 113.5, b = 110.4, c = 133.7 Å, β = 90.2°. The three crystal forms have nearly the same $V_{\rm M}$ value of 2.6 Å^3 Da^{-1}, corresponding to a solvent content of 52%.

The crystals of PIMT were grown from a reservoir solution of 0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 4000, 10%(*v*/*v*) glycerol in 3–5 d. This crystal belongs to the monoclinic system and there are 12 molecules in an asymmetric unit with a $V_{\rm M}$ value of 2.8 Å^3 Da^{-1}.

The best crystals of PH-A grew to dimensions of 0.15 × 0.15 × 0.6 mm from 0.1 *M* acetate pH 4.5–4.7, 47–50%(*v*/*v*) MPD in a week, while crystals of PH-B grew to 0.2 × 0.2 × 0.6 mm from 0.1 *M* citric acid pH 4.6–5.0, 10–12.5%(*w*/*v*) PEG 6000, 10%(*v*/*v*) sucrose in 3–5 d. Crystals of both PH-A and PH-B belong to the tetragonal system (Tables 1 and 2).

2.2. Cooling crystals in a capillary

Since the IDH and PH-A crystals were grown in cryoprotectant solutions containing 20.0% glycerol and 44–50% MPD, respectively, it was possible to cool them directly under cryogenic conditions without any soaking step. The IDH and PH-A crystals were first mounted in a glass capillary (0.7 mm diameter) and the mother solution around the crystal was removed. After the capillary was mounted on the goniometer head and the crystal was centred on the X-ray beam position, with the nitrogen-gas stream shielded, the crystal was flash-cooled in the capillary under the nitrogen-gas stream as shown in Fig. 1(*a*). Prior to data collection, the nitrogen-gas stream

was turned around to a forward position along the capillary axis (Fig. 1*b*).

2.3. Cooling crystals in a capillary with cryoprotectant solution

To cool crystals of PIMT and PH-B, cryoprotectant solutions were screened by varying their contents, including PEGs, MPD, glycerol and sucrose. The final cryoprotectant solutions of PIMT and PH-B were reservoir solutions containing 20%(*v*/*v*) glycerol and 32%(*w*/*v*) sucrose, respectively. Crystals of both PIMT and PH-B were stable after being soaked in cryoprotectant for 5–10 min.

Both crystals were flash-cooled in four steps. Firstly, 1.2–1.6 µl cryoprotectant solution (3–4 mm in height in a 0.7 mm diameter capillary) was injected into a short glass capillary at the top and the capillary was closed at the other end using capillary wax in order to fix the position of the cryoprotectant solution. In the second step, the crystal was transferred from the crystallization drop to cryoprotectant in the capillary using a cryoloop, as shown in Fig. 2(*a*). After shielding the nitrogen-gas stream, the capillary was mounted on a standard goniometer head and the crystal was centred on the X-ray beam position along the rotation axis. In the final step, the nitrogen-gas stream was moved back to the beam position and the crystal was flash-cooled in the capillary with cryoprotectant solution under the nitrogen-gas stream, as in Fig. 2(*b*); the cooled crystal was then centred at the beam position again.

2.4. Data collection and structure determination

Diffraction data from IDH, PH-A, PH-B and PIMT crystals were successfully collected under cryogenic conditions (100 K) using FCC techniques with a CCD detector on the BL41XU and BL38B1 stations, SPring-8, Japan. The data from IDH, PH-A and PH-B crystals were processed using the program *MOSFLM* (Leslie, 1993) for integration and the program *SCALA* (Evans, 1997) for scaling and merging, whereas the PIMT data were processed using *HKL*2000 (Otwinowski & Minor, 1997).

The structure determination of the IDH–isocitrate–Mn^{2+} complex was carried out using Mn-MAD phasing (Yasutake *et*

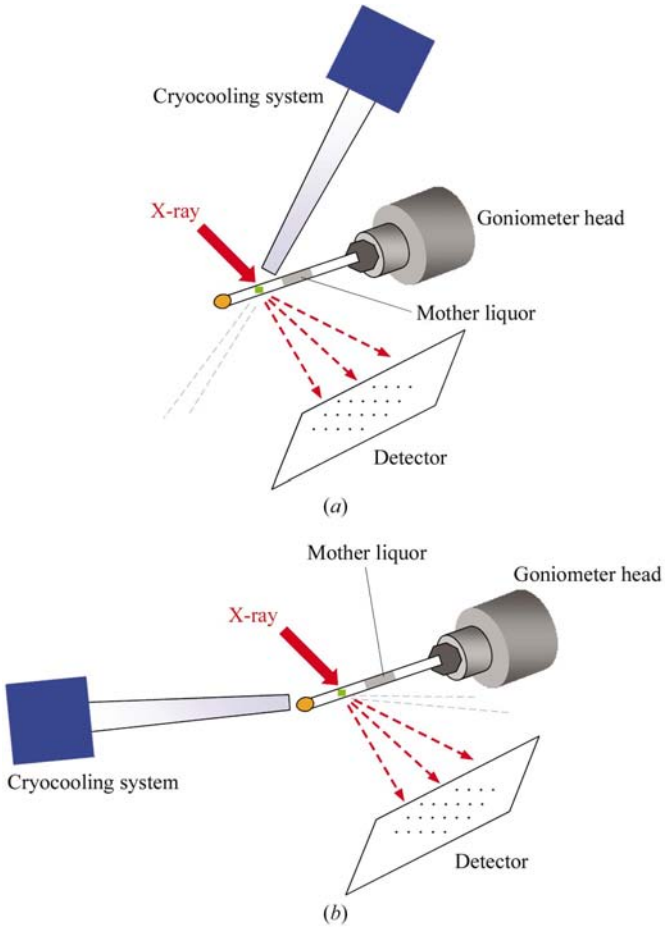


Figure 1
Experiment setup for flash-cooling a crystal in a glass capillary for data collection. (*a*) Mounting the capillary onto a diffractometer. (*b*) The nitrogen-gas stream is turned to a forward position along the rotation axis.

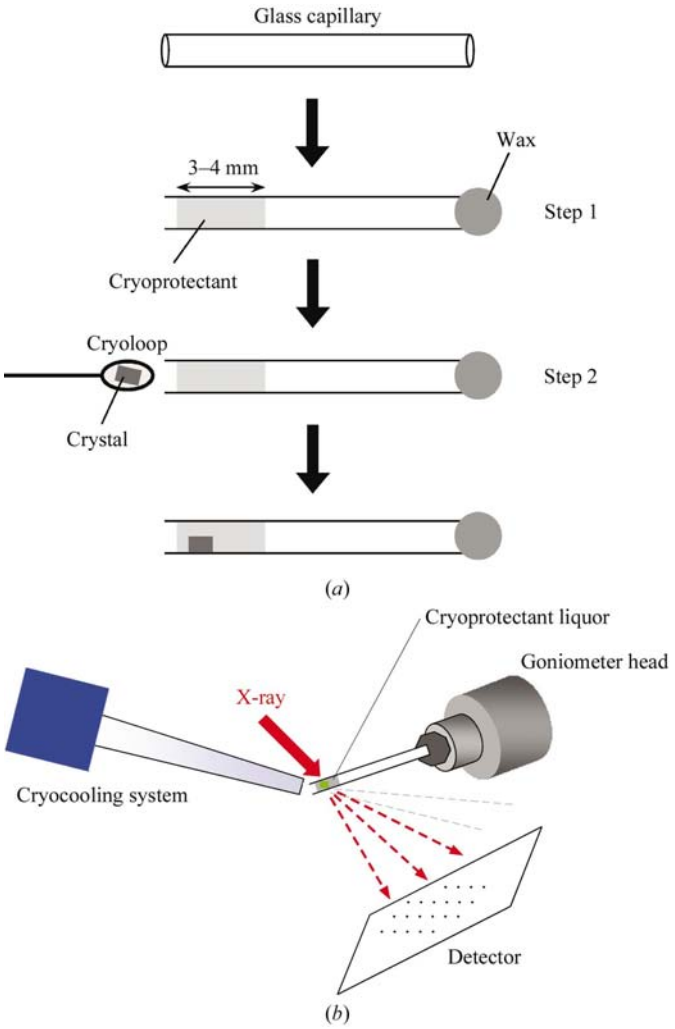


Figure 2
Data-collection setup for flash-cooling crystals in a capillary with cryoprotectant solution. (*a*) Placing the crystal into a shortened glass capillary with a cryoprotectant-solution volume of 1.2–1.6 µl. (*b*) Mounting the capillary onto a diffractometer and flash-cooling it under a nitrogen-gas stream.

al., 2002). The MR (molecular-replacement) method was used in the structure analysis of the IDH–NADP$^+$ complex (Yasutake *et al.*, 2003) and PIMT.

2.5. Measurements of the cooling rate

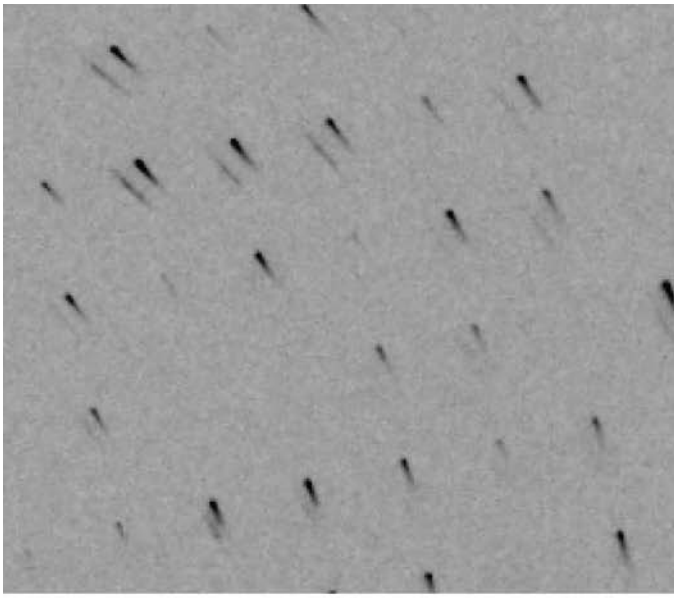
For measurement of the cooling rate, thermocouples were mounted on a standard goniometer head under a nitrogen-gas stream. The time-course of the temperature decrease during flash-cooling was measured using different sizes of thermocouple in three cooling methods: flash-cooling in air, flash-cooling in a capillary and flash-cooling in a capillary with cryoprotectant solution. The thermocouples were Chromel-Alumel fine wires with an exposed junction. The temperatures were measured with a WAVE THERMO 1000 data-acquisition system (Keyence Inc.) and readings were taken at 0.1 s intervals. The measured plots differed slightly depending on the cryosystem and nitrogen-gas conditions. In this study, measurement was carried out using nitrogen-gas cryogenic conditions (100 K) produced using a 600 series Cryostream cooler (Oxford Cryosystems Inc.). For measurement in the capillary, the thermocouples were in contact with the capillary wall as much as possible. The measurements were repeated five times for each case. The final plots of the cooling rate from room to cryogenic temperature were determined by averaging five measurements.

3. Results and discussion

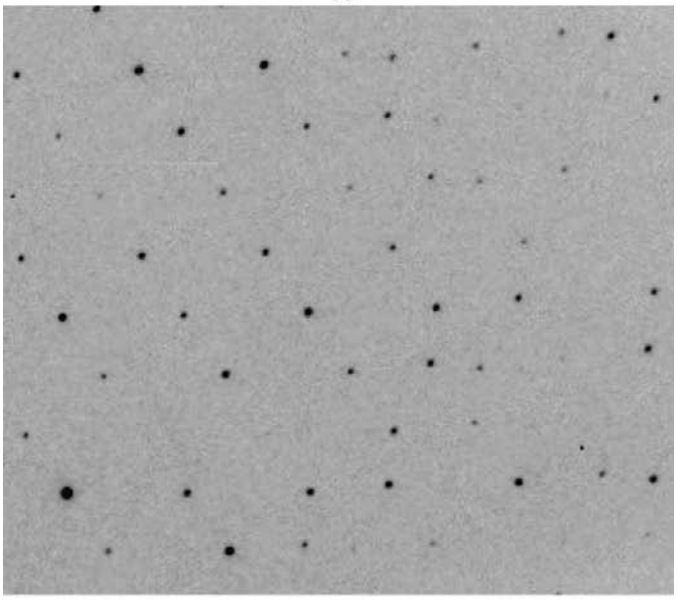
3.1. Cooling crystals of IDH and PH-A

The IDH crystals were nearly cube-shaped with 20% glycerol and the quality of the crystals was good enough for diffraction experiments at synchrotron beamlines at room temperature. However, the potential for serious radiation damage at room temperature made it necessary to collect data under cryogenic conditions. The crystals were not stable when transferred to a higher concentration of glycerol than 20% or to other cryoprotectant solutions such as PEG 400, MPD and Paratone-N by quick dipping, long soaking, stepwise transfer or dialysis. Plunge-cooling crystals into liquid nitrogen and flash-cooling using cryoloops were not successful for data collection, since the mosaicity was greatly increased and the diffraction patterns subsequently obtained had long tails (Fig. 3*a*). Annealing treatments were also tried, but degradation occurred. Flash-cooling crystals in a capillary as described in §2 was first applied to orthorhombic crystals (Fig. 3*b*) and a 2.9 Å Mn-MAD data set was successfully collected at the Mn *K* edge (peak, 1.8923 Å; edge, 1.8934 Å; remote, 1.8783 Å) over 6 h. Subsequently, a 1.95 Å resolution data set from IDH–isocitrate–Mn^{2+} was measured at 0.9 Å wavelength from a monoclinic crystal using the same FCC method. The structure of the IDH–isocitrate–Mn^{2+} complex was solved by Mn-MAD phasing followed by multi-crystal-form averaging. The high-quality data obtained using the FCC method enabled Mn-MAD phasing using only one Mn atom per novel 80 kDa protein (Yasutake *et al.*, 2002; PDB code 1itw). We were also able to collect diffraction data from the IDH–NADP$^+$ complex using the FCC method. This complex structure was determined by the MR method at 3.2 Å resolution. After a few cycles of refinement, both $2F_o - F_c$ and $F_o - F_c$ density maps showed the NADP molecule clearly (Yasutake *et al.*, 2003; PDB code 1j1w).

Protein PH-A is another example of successful use of the FCC method. Similar to the process for obtaining IDH crystals, the PH-A crystals were first obtained from a reservoir solution containing 44–46%(*v*/*v*) MPD as cryoprotectant. Flash-cooling using a cryoloop increased the mosaic spread significantly. Several annealing treatments have been tried. Flash-annealing in the loop by shielding the cold gas stream



(*a*)



(*b*)

Figure 3
Diffraction images taken from the crystals of isocitrate dehydrogenase. Image (*a*) is a typical diffraction image obtained using a cryoloop as a general method. The diffraction in (*b*) was obtained using the FCC method.

for 2–10 s followed by re-flash-cooling (Yeh & Hol, 1998) and annealing by returning crystals to the original cryoprotectant solution for 1–3 min followed by re-flash-cooling (Harp *et al.*, 1998, 1999) produced no improvement. Using the FCC technique enabled us to measure a complete 3.1 Å resolution data set. The data-processing statistics are shown in Table 2. Subsequently, the crystals of PH-A were improved by optimizing the crystallization conditions by increasing the concentration of MPD up to 50% and good-quality crystals were obtained for standard flash-cooling. Thus, the Se-MAD data and higher resolution (2.5 Å) native data were obtained by normal flash-cooling and the diffraction data obtained using the FCC technique were not used for structure determination.

Since the capillary was tube-shaped and the nitrogen-gas stream could not cover its whole surface, the capillary became frosted on the opposite side to the nitrogen stream after about 2 h of cooling. To overcome this problem, we tried orienting and turning the nitrogen-gas stream so it would blow along the capillary axis and directly flash-cool the crystal, but this was not successful because we could not mount crystals close to the edges of the capillary. The best way was to turn the nitrogen-gas stream to a forward position along the capillary axis after flash-cooling (Fig. 1*b*).

3.2. Cooling crystals of PIMT and PH-B

The reflections from the crystals of both PIMT and PH-B had sharp diffraction spots during our X-ray diffraction experiments at room temperature. However, they were sensitive to radiation damage at room temperature, making cryogenic data collection essential. We tried to flash-cool more than 20 crystals after soaking them in cryoprotectant solutions, but flash-cooling using a cryoloop increased the mosaicity dramatically and the crystals were damaged. The annealing and FCC techniques were not effective for crystals of either PIMT or PH-B.

In an attempt to stabilize the crystal, we maintained a large volume (about 1.2–1.6 µl) of PIMT crystal cryoprotectant solution [0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 4000, 20%(*v*/*v*) glycerol] around the crystal in the capillary and then flash-cooled the crystal in the capillary as described in §2. Using this method, sharp diffraction spots were obtained with a mosaicity of about 0.35° (as estimated by the diffraction data-processing program). The diffraction images of the PIMT crystal using a cryoloop and the capillary method are compared in Fig. 4. It is very clear that the mosaic spread and spot sharpness were improved by the FCC method with a large volume of cryoprotectant solution. The structure of PIMT was solved by molecular replacement at 2.5 Å resolution.

A similar effect was seen in crystals of PH-B. Application of FCC with a large volume (about 1.2–1.6 µl) of PH-B crystal cryoprotectant solution [0.1 *M* citric acid pH 4.6–5.0, 10–12.5%(*w*/*v*) PEG 6000, 35%(*v*/*v*) sucrose] to protein PH-B is a unique method for collecting a complete Se-MAD data set at 4.0 Å resolution (the same resolution as at room temperature).

In this case of FCC with cryoprotectant solution, using an in-house X-ray beam may result in high background because of the large volume of cryoprotectant solution. Therefore, this method may be applicable to data collection at synchrotron beamlines, where a well focused intense beam is available.

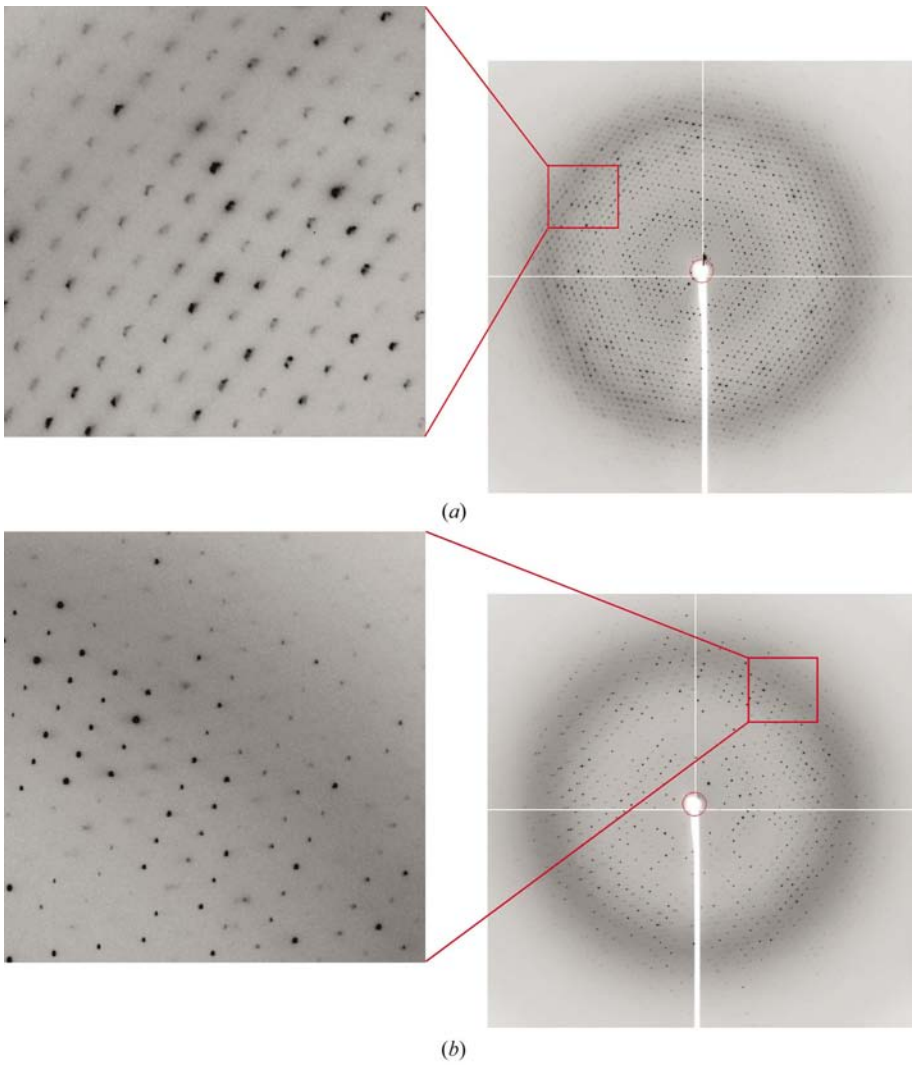


Figure 4
A comparison of diffraction patterns obtained using different flash-cooling methods from the crystals of protein L-isoaspartate *O*-methyltransferase to illustrate the effect of the FCC method. (*a*) Flash-cooling crystal using a cryoloop. (*b*) Flash-cooling a crystal using a capillary with cryoprotectant solution.

Additionally, a sensitive camera is necessary for centring the crystal, since it is difficult to find the crystal in cryoprotectant solution in a capillary under cryogenic temperature.

3.3. Comparison of cooling rates

To elucidate the characteristics of FCC methods of cooling crystals, we measured the flash-cooling rates in three cases: flash-cooling in air, flash-cooling in a capillary and flash-cooling in a capillary with cryoprotectant solution. Since a cryoloop is about 0.3–0.7 mm in diameter and the volume of cryoprotectant solution is normally very small when using a cryoloop to cool a crystal, we assumed that flash-cooling thermocouples in air can simulate flash-cooling in a cryoloop with a small volume of cryoprotectant solution. This was confirmed by the fact that our measured times for reaching the final cryogenic temperatures in air correspond to the results of Teng and Walker (Teng & Moffat, 1998; Walker *et al.*, 1998). To take the size of crystal into consideration, we used three thermocouples with different diameters (0.05, 0.07 and 0.1 mm) to measure temperatures, which produced evidence that the cooling rates are most sharply dependent on the size of the sample (thermocouple or crystal) in cryoloop cooling (Fig. 5*a*). The cooling rate is less dependent on the sample size in the FCC method (Fig. 5*b*). Furthermore, in the case of FCC with cryoprotectant solution, the cooling rates of the three thermocouples were very similar (Fig. 5*c*). Clearly, the cooling rates depended on the volume of cryoprotectant solution rather than the size of the sample.

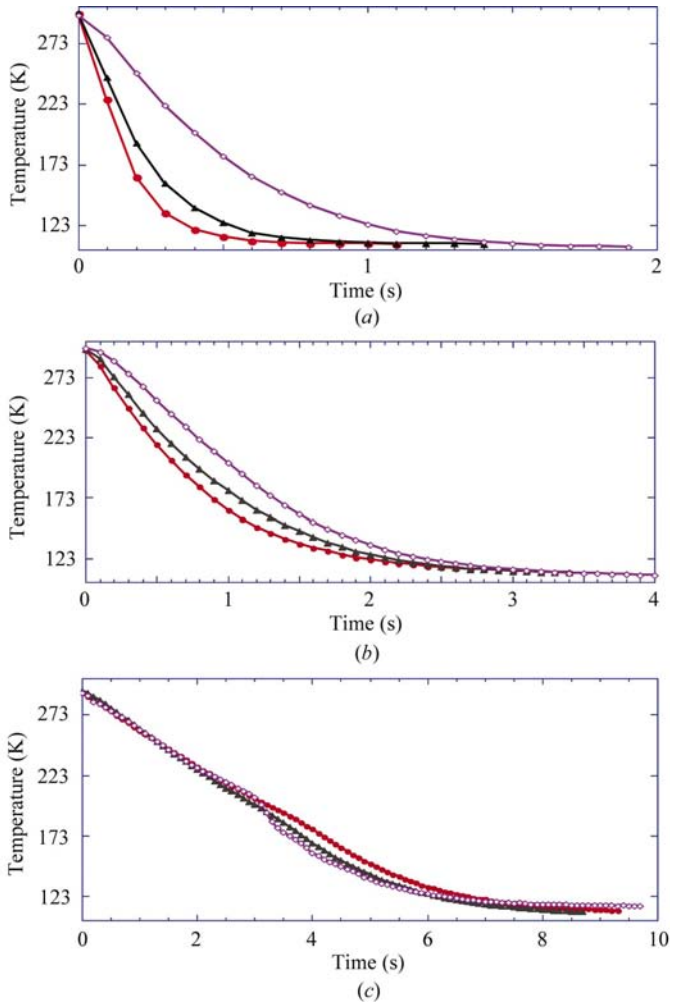


Figure 5
Representative cooling-rate plot for three sizes of thermocouple in three cooling cases. The red lines, black lines and purple lines represent 0.05, 0.07 and 0.1 mm diameters, respectively. (*a*) Flash-cooling in air, which simulates using a cryoloop, (*b*) flash-cooling using a capillary and (*c*) flash-cooling using a capillary with cryoprotectant solution.

Fig. 6 shows the temperature as a function of time for flash-cooling in a cryoloop, in a capillary and in a capillary with cryoprotectant solution, respectively, using a wire with a diameter of 0.05 mm. The time taken to reach cryogenic conditions is about 1 s or less than 1 s in the case of flash-cooling using a cryoloop, while it takes 2–3 s in a capillary. Moreover, the flash-cooling rate using a capillary with cryoprotectant solution was much slower, with an estimated time of about 8 s.

From these measurements, it is obvious that the cooling rates of the crystals of IDH, PIMT, PH-A and PH-B were reduced by the use of FCC methods. The cooling rates of FCC (in the cases of the IDH and PH-A crystals) were estimated to be about two times slower than that using a cryoloop and the cooling rates of FCC with cryoprotectant solution (in the cases of the PIMT and PH-B crystals) were even slower (in the range of 20–25 K s^{-1}). Obviously, in these cases the cooling speeds were optimized by reduction of the cooling speed through the capillary. All crystals used in the present study were relatively large (Table 1). These large crystals may need relatively long cooling periods to allow perfect variation of unit-cell parameters throughout the entire volume of the crystal and to complete relaxation of the crystal lattice before reaching the final temperature. Excessively fast cooling might add to the strain in the lattice, resulting in unbearably high mosaicity.

Previous studies of cooling techniques have concentrated mostly on the cooling environment to relax the lattice, such as using a larger loop (Garman, 1999), optimization of cryoprotectant (Garman, 1999; Rubinson *et al.*, 2000) and annealing (Harp *et al.*, 1998, 1999; Yeh & Hol, 1998); however, those

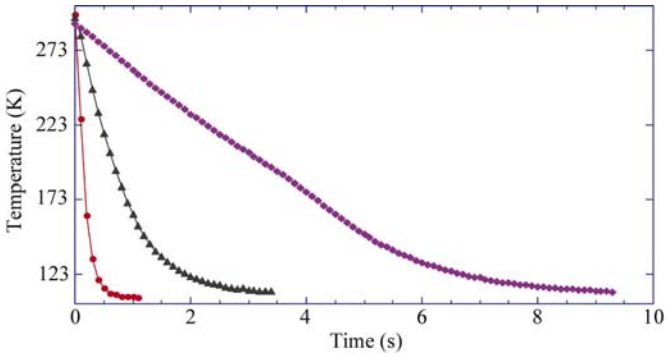


Figure 6
Cooling-rate plots of flash-cooling for a 0.05 mm diameter thermocouple using different techniques. The red plot shows flash-cooling using a cryoloop, the black plot shows flash-cooling in a capillary and the purple plot shows flash-cooling in a capillary with cryoprotectant solution.

techniques do not greatly affect the cooling rate. Our present results show that the cooling rate should be optimized over 2–8 s in order to minimize the increase in mosaicity during crystal cooling. Crystals of the nucleosome core have been successfully cooled with even slower cooling processes using a capillary (Luger *et al.*, 1997). The optimum speed depends on the size of the crystal, the crystallization solvent and the relaxation process and none of these parameters is easily controlled. The FCC method may be useful if nothing seems to work for cooling a crystal.

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