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Crystallization and cryocrystallography inside X-ray capillaries

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This paper presents a modification of the gel acupuncture method to grow isolated crystals inside X-ray capillaries. Protein crystals are grown from 2–12 µl of gelled agarose–protein solution, cryoprotected and immobilized by the gel matrix. The same X-ray capillary that acts as a crystallization reactor is used to transport the crystals to the X-ray source and to collect data at both room temperature and 100 K, without any post-crystallization manipulation. To enhance the flash-cooling stage, two additional elements are proposed for inclusion in the cryosystems currently in use: a laser pointer to illuminate the crystal to be flash-cooled and a trap to divert the N_2 flow and switch from room temperature to 100 K without misalignment of the crystal. With the proposed implementation, data can be collected at different temperatures from the same crystal in exactly the same orientation. This permits the study, at lattice level, of changes in unit-cell parameters, mosaic spread and crystal quality induced by cryogenic temperatures and annealing techniques.

1. Introduction

Nowadays, the Human Genome project is making available thousands of sequences of potential biotechnological interest. But in most cases, the function of these genes remains unknown. One clue, however, may be provided by the three-dimensional structure of the proteins that they encode. So a new effort, known as structural genomics, is ongoing to crystallize and determine the three-dimensional structure of thousands of proteins, much as the genome project determined gene sequences *en masse*. This structural information will lead to a better understanding of the molecular basis of life and diseases, and to drugs that specifically bind to the target protein. That outlook obviously depends on improvements in crystallization techniques and minimization of the post-crystallization manipulation of crystals.

Hanging-drop, sitting-drop and dialysis-button techniques are among the classical approaches currently used to crystallize proteins (McPherson, 1999; Ducruix & Giegé, 1999). All these traditional methods share a common feature: once protein crystals are obtained they must be either placed in a capillary that is transparent to X-rays or transferred to a cryoloop prior to collecting diffraction data. In both cases, this manipulation is problematic because it is time consuming, affects the integrity of the crystal (*i.e.* crystal quality and, hence, resolution limit) and provokes inevitable losses of some crystals.

Our group has developed a new crystallization method based on the counter-diffusion of the precipitant agent and the protein solution under conditions starting far from equilibrium. A practical implementation is the gel acupuncture method (GAME), in which the protein solution is confined in a capillary punched in a gel layer and the precipitating agent is poured on the gel layer (García-Ruiz, 1991; García-Ruiz & Moreno, 1994; García-Ruiz & Moreno, 1997). The capillary acts as a long protein chamber and removes convection from the system. When the precipitating system contacts the protein solution, a wave of supersaturation is triggered (García-Ruiz, Otálora *et al.*, 2001). This wave travels along the protein chamber and screens different crystallization conditions of progressively lower supersaturation values as it moves further from the lower end of the capillary.

Unlike traditional methods, one of the main advantages of GAME is its ability to yield crystals obtained under different nucleation and growth conditions in every experiment. This implies that a single GAME experiment is equivalent to many experiments with traditional methods. As well as crystals of higher quality, GAME is the only technique that produces crystals that fill the capillary diameter and do not slip, allowing data collection at room temperature from the same capillary in which the crystals were grown (Otálora *et al.*, 1996; Gavira, 2000). However, when the crystals do not fill the capillary or do not attach to the capillary walls, they sediment and have to be mounted in a new capillary in order to collect data.

Furthermore, if one is seeking to perform cryocrystallography, crystal manipulation is considered to be mandatory, since, until now, it has been presumed that crystals in capillaries are not suitable candidates for data collection under cryogenic conditions.

We present in this paper a modification of the experimental setup to implement counter-diffusion experiments. The new setup allows the collection of X-ray data from crystals in the same X-ray capillary where they were grown. The proposed experimental setup avoids post-crystallization handling of crystals and makes possible cryocrystallography inside capillaries. The practical implications of this improvement are also discussed.

2. Materials and methods

2.1. Chemicals

Lysozyme, thaumatin and ferritin were purchased from Sigma (Lot. 65H7025, Lot. 108F0299 and Lot. 125H70402); recombinant human liver fructose-1,6-bisphosphatase (HLFBPase) was produced in our laboratory as described by El-Maghrabi *et al.* (1993). The precipitating agent and starting conditions used to crystallize each of these proteins are summarized in Table 1. Agarose powder was supplied by Hispanagar. The gelling and melting temperatures of the agarose used in this study are 309.7 and 362.4 K, respectively.

2.2. Experimental setup

The crystallization reactors consisted of glass X-ray capillaries with 0.2, 0.3, 0.5 and 1 mm internal diameters. The protein solution was gelled inside the capillary (Fig. 1) with a small amount of agarose by the following procedure. An agarose sol was prepared by dissolving agarose powder in the buffer solution and heating at 368 K (above the melting point of the agarose used) until a clear solution was obtained. Then it was cooled to 313 K (a few degrees above the gelling point). The appropriate volumes of agarose sol and protein solution, pre-warmed at 313 K, were mixed to yield a 0.075% (*w*/*v*) agarose protein–agarose solution, which was kept at 313 K to prevent gelling. The capillary was loaded by immersing its lower end in the protein–agarose solution, which rose and filled the capillary by capillary forces. Then the lower end of the capillary was sealed with nail varnish and the capillary stored at room temperature for a few minutes until the protein–agarose solution formed the gel. Afterwards, the precipitating agent and the cryobuffer solution (Table 1) were poured into the upper part of the capillary, which was then sealed with vacuum grease.

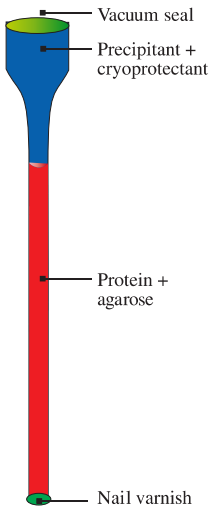


Figure 1
Experimental setup to implement the crystallization technique. The protein solution is gelled, the precipitant with/without cryobuffer is placed on top and the upper and lower ends are sealed. Note that the capillary itself becomes the crystallization reactor.

Isolated crystals grew along the capillaries and were available for diffraction within 10 days.

2.3. Data collection

In experiments carried out at Hamburg EMBL Synchrotron outstation (beamline BW7B), fourteen 0.5° oscillation images were collected at 100 K from a lysozyme crystal, of age one month, in the same 0.2 mm diameter capillary in which it was grown with 20% (*v*/*v*) glycerol in the precipitating system. The exposure time was 30 s in dose mode. Images were recorded on a Mar345 image-plate detector placed at 200 mm and were processed with the *XDS* package (Kabsch, 1993).

In an experiment carried out using an Enraf–Nonius rotating anode at 40 kV × 90 mA, a single 0.15° oscillation image was collected at room temperature from a thaumatin crystal in the same capillary in which it was grown; it was then flash-cooled and moved back to the starting φ position, and the image collected again. The exposure time was 180 s in each case. These two images were collected on a Mar345 image-plate detector placed at 130 mm and were analysed with *MOSFLM* 6.01 (Leslie, 1999).

3. Results and discussion

The goal of this work was to collect X-ray diffraction data from the same capillary in which the crystal was grown. This

Table 1
Concentrations of protein and precipitant solutions that yield isolated crystals along the capillary.

Protein solutions gelled in 0.07% agarose	Precipitant solutions plus cryoprotectant
80 mg ml^{-1} lysozyme in 50 m*M* sodium acetate pH 4.5	20 NaCl in 50 m*M* sodium acetate pH 4.5, 20% glycerol
80 mg ml^{-1} thaumatin in 100 m*M* phosphate buffer pH 6.5	30% Na/K tartrate in 100 m*M* phosphate pH 7, 20% glycerol
16 mg ml^{-1} ferritin in 200 m*M* sodium acetate pH 5.03	6% $CdSO_4$ in 200 m*M* sodium acetate pH 5.03, 20% glycerol
15.3 mg ml^{-1} HLFBPase in 50 m*M* malonate buffer pH 7.2, 1 m*M* DTT, 0.5 m*M* PMSF, 0.1 m*M* edta	40% PEG 4000 in 50 m*M* Tris-HCl pH 9, 0.1 m*M* Li_2SO_4, 0.1 m*M* citrate, 1 m*M* 2-mercaptoethanol, 1 mM AMP, 20% glycerol

had already been achieved for the particular case in which the crystal fills the capillary diameter (*i.e.* rod-like crystals) and cannot slip during the data collection (Otálora *et al.*, 1996), but some problems arose with smaller crystals floating in the solution. To overcome these difficulties, two modifications were introduced (Fig. 1), as follows.

(*a*) The protein solution was gelled into the capillary with 0.075% (*w*/*v*) agarose. The agarose gel holds crystals that are unattached to the capillary walls and also removes convective flow from the system.

(*b*) The precipitant was placed at the top of the capillary and both the upper end and the lower end of the capillary were sealed to turn the X-ray capillary into a crystallization reactor.

The use of agarose gels to avoid convection is a well known technique (Robert *et al.*, 1999), but it is also known that agarose modifies the crystallization behaviour of proteins (Vidal *et al.*, 1998*a*,*b*). In a separate study, we have demonstrated that agarose at concentrations below the percolation threshold can ensure mass transport dominated by diffusion (García-Ruiz, Novella *et al.*, 2001). Thus, as seen from the transport control, the 0.075% (*w*/*v*) agarose used in this study is as effective as higher concentrations typically utilized in gel crystallization, but minimizes the side effects on crystal nucleation and growth.

The gelling point of the agarose is an important parameter to take into account since the protein has to be pre-warmed some degrees above such a temperature before preparing the protein–agarose solution. In our experimental conditions, we have used an agarose with a gelling temperature of 309.7 K and the protein solution was pre-warmed to 313 K. Most proteins are stable at 313 K, but for particular cases where the pre-warming temperature is a problem, low-melting-point agaroses that gel at temperatures as low as 281–290 K (ultra-low-gelling-temperature agarose from Amersham Pharmacia Biotech) can be used.

The proposed approach was successfully tested with four different proteins and four precipitating agents, as presented in Table 2. By tuning the starting conditions, isolated crystals grew along the capillary in the same location where they nucleated and they remained fixed in the agarose matrix. The capillaries were transported to the X-ray source for diffraction at room temperature without any further manipulation.

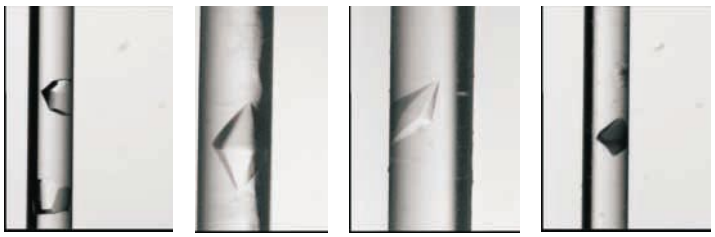
Next, we faced the problem of performing cryocrystallography with the above crystallization setup, *i.e.* of freezing the crystals in the same capillary in which they were grown without any post-crystallization manipulation. This implies two problems to be overcome: (i) crystals have to be grown with cryoprotectant in the crystallization buffer and (ii) crystals have to be flash-cooled inside the capillary without ice formation.

The feasibility of growing crystals in the cryobuffer was demonstrated by Kim & Lipscomb (1993). Since glycerol is considered to be a 'universal cryoprotectant' (Garman & Schneider, 1997) in the sense of being the most widely used, our effort was focused on growing crystals with glycerol as an additive in the crystallization buffer. Different glycerol concentrations were tested with lysozyme and we found that 20% (*v*/*v*) was high enough to cryoprotect and does not affect the crystal growth process. Lysozyme, thaumatin, ferritin and recombinant HLFBPase were successfully crystallized in capillaries of different diameter from gelled protein solutions and 20% (*w*/*v*) glycerol in the precipitating agent.

The first attempt to flash-cool crystals inside capillaries was carried out on lysozyme crystals, of age one month, grown from a gelled protein solution in a capillary of 0.2 mm diameter, with 20% (*v*/*v*) glycerol in the precipitating system. The diffraction pattern showed a high mosaic spread and six weak rings at 1.91, 2.06, 2.24, 3.43, 3.65 and 3.88 Å, which correspond to hexagonal ice (Garman & Schneider, 1997) (Fig. 2). A closer analysis of these ice rings revealed that they are very thin when compared with the spots at the same position and slightly more intense than the background (Fig. 2, right-hand side). These two facts made possible the processing, with the *XDS* package, of data from fourteen images without masking the ice rings (Fig. 2, left-hand side).

When lysozyme, thaumatin and ferritin crystals of two weeks of age were flash-cooled, a massive ice formation occurred along the capillary; this led us to hypothesize that although crystals grow within ten days and are suitable for data collection at room temperature, glycerol needs one month to diffuse along the capillary and reach a concentration high enough to act as cryoprotectant. This hypothesis was verified later, when capillaries from the same batch were flash-cooled under the same conditions. We conclude that crystal growth and cryoprotection are two different events that take place at different times as a consequence of the

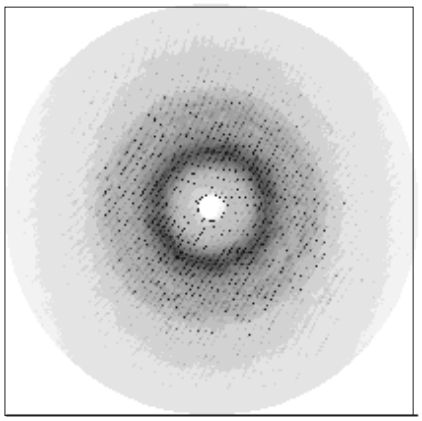
Table 2
Main features of the four crystallization systems assayed to collect data from isolated crystals in the same capillary where they were grown.



Protein	Lysozyme	Thaumatin	HLFBPase	Ferritin
Molecular weight†	14.3 kDa monomer	22 kDa monomer	147 kDa tetramer	456 kDa 24-mer
Predicted isoelectric point†	9.3	8.5	6.6	5.4
Precipitant	NaCl	Na/K tartrate	PEG 4000	$CdSO_4$
pH of crystallization	4.5	7	9	5.03

† Estimated from the amino acid sequence.

Lysozyme	
Wavelength (Å)	0.91
Oscillation angle (°)	0.5
Detector distance (mm)	200
Number of crystals	1
Space group	$P4_32_12$
Unit cell ($a = b$, c in Å)	79.33, 37.88
Resolution range (Å)	10.00–1.2
Unique reflections	12057
Completeness	32.3%
R(symmetry)	5.4%
$I/\sigma(I)$	8.27
Reflections with $I/\sigma(I) > 2$	30.6%



Resolution	I/B
1.91 Å	1.14
2.06 Å	1.14
2.24 Å	1.24
3.43 Å	1.22
3.65 Å	1.31
3.88 Å	1.15

Figure 2
Diffraction pattern obtained at Hamburg EMBL synchrotron outstation (beamline BW7B) from a lysozyme crystal, of age one month, grown inside a 0.2 mm diameter capillary from a gelled protein solution and with 20% glycerol in the precipitating system. Six ice rings at 1.91, 2.06, 2.24, 3.43, 3.65 and 3.88 Å appeared. They are thinner than the spots and slightly more intense than the background ($I/B > 1$ tabulated on the right-hand side), but data from 14 images were processed with *XDS* without masking them out (tabulated on the left-hand side).

different diffusion coefficients of glycerol and the precipitating agent: the precipitating agent diffuses faster than glycerol, yielding crystals grown at very low concentrations of glycerol, and later on, when the glycerol reaches the suitable concentration, crystals become cryoprotected.

Although we have flash-cooled crystals in capillaries of up to 1 mm in diameter, the importance of the surface-to-volume ratio (S/V) in the flash-cooling technique is well known and needs consideration (Garman & Schneider, 1997): low values of S/V lead to slow cooling rates and a temperature gradient will occur, causing thermal stress and an increment in the mosaicity (Garman, 1999). Thus, to enhance the flash-cooling stage, we propose the use of capillaries of up to 0.3 mm in diameter and two modifications to the currently available cryosystems: a laser pointer on the cryonozzle to illuminate and single out the crystal to be flash-cooled, and a trap to divert the N_2 stream (Fig. 3). Both the laser pointer and the trap are intended to facilitate the steps of placing the crystal under the cryonozzle precisely and switching the data collection from room temperature to 100 K without any misalignment of the sample. This has an important implication since it allows the study of changes induced by cryo- and annealing techniques (Harp *et al.*, 1999) in the mosaic spread, the unit-cell volume and the crystal quality at the lattice level, in the same crystal at exactly the same orientation. To confirm the feasibility of this approach, a 0.15° oscillation image from a thaumatin crystal grown in a capillary of 0.3 mm diameter as described above was collected for 180 s on a rotating anode at room temperature. The crystal was flash-cooled inside the capillary without ice formation and rotated 0.15° back to the starting φ position. Then the corresponding image was collected at 100 K for exactly the same exposure time. Both images were integrated using *MOSFLM* 6.01 (Leslie, 1999). The comparison of the two images showed an increase by up to 0.4 in the estimated mosaic spread, a shrinkage of the unit-cell dimensions and an improvement of $I/\sigma(I)$ at 100 K (Fig. 4).

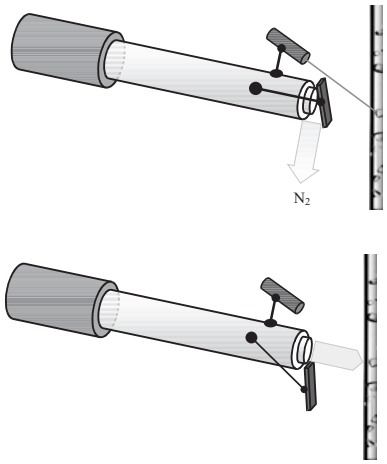


Figure 3
Proposed modification of the cryosystems currently in use: a trap and a laser pointer. During the crystal alignment, the laser pointer illuminates the crystal to be flash-cooled and the trap is switched on to divert the N_2 stream (top). For data collection at 100 K, the laser is turned off and the trap is switched off, avoiding crystal and/or cryonozzle realignment (bottom).

Since the temperature is the only difference between the two images, the flash-cooling is the only factor responsible for such variations, which, in any case, are of the order of those expected for flash-cooled crystals.

Moreover, the orientation matrix for the crystal at room temperature differs from that at 100 K by the order of one in one thousand. This was expected and can be explained on the basis of the increase in mosaicity and the shrinkage of the unit cell, but to rule out any slight movement of the crystal, sixteen strong spots, with Miller indices $h = 4$, $k = 2$ and $29 < l < 46$, were analysed. The plots of the x and y coordinates on the detector of these sixteen spots before flash-cooling *versus* their positions after flash-cooling fit to straight lines, with slopes of 0.989 and 0.973, respectively, and R equal to 1.000 (Fig. 5). This

	Room temperature	100 K
Wavelength	1.54179	1.54179
Detector distance (mm)	130.0	130.0
Oscillation angle (º)	0.15	0.15
Unit-cell parameters (Å, º)	58.796 58.796 151.595 90.000 90.000 90.000	57.977 57.977 150.574 90.000 90.000 90.000
Unit-cell volume (Å^3)	524059.31	506129.25
Estimated mosaicity	0.17	0.4
Resolution range (Å)	15.37–1.73	16.26–1.73
Number of reflections	2249	2800
$I/\sigma(I)$	18.67	18.70
Outer resolution shell		
Resolution range (Å)	1.93–1.73	1.93–1.73
Number of reflections	617	740
$I/\sigma(I)$	2.31	3.83

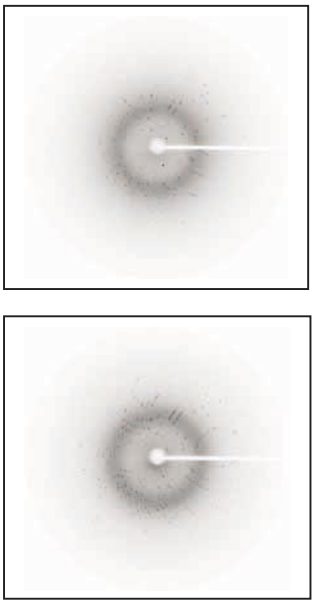


Figure 4
0.15° oscillation images of a thaumatin crystal before (top) and after (bottom) being flash-cooled inside the capillary. Both images were collected for 180 s, from the same crystal at exactly the same orientation. The tabulated data are some statistics and the changes in unit-cell size, mosaicity and $I/\sigma(I)$ at high resolution.

result proves that within the experimental error, the crystal did not slip during the transition from room temperature to 100 K. The analysis of the intensity of these sixteen strong spots also reveals that the flash-cooling enhances the quality of the data in terms of $I/\sigma(I)$ (Fig. 6) as a consequence of the reduction of the thermal vibration and thus the atomic B factor.

Finally, we would like to underline that the implementation of GAME does not require large amounts of protein. Table 3 shows the volume (V_{max}) of protein required to fill the capillary considering that the volume of a capillary is given by

$$V = \pi (\oslash/2)^2 L,$$

where $\oslash$ and L are the diameter and the length of the capillary, respectively, and that the optimum length of capillary to implement GAME is about 50–60 mm (Gavira, 2000). Note that for capillaries with diameters of 0.2, 0.3 and 0.5 mm, the maximum volume of protein is within 20 µl, the range described for hanging-drop experiments by Unge (1999). Hence, it can be stated that GAME can be implemented using as little protein as drop techniques do, but with two additional advantages: (i) a single capillary is equivalent to many single-drop experiments to search for the proper precipitant/protein ratio that yields the best crystals, and (ii) crystals obtained by GAME show higher quality (García-Ruiz, Novella *et al.*, 2001; García-Ruiz, Garvira *et al.*, 2001; García-Ruiz, Otálora *et al.*, 2001).

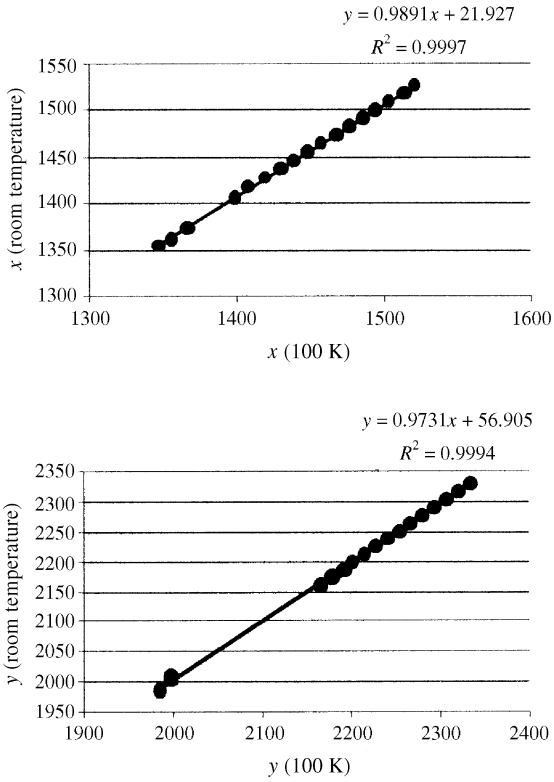


Figure 5
x and y coordinates of sixteen strong spots from a thaumatin crystal (Miller indices $h = 4$, $k = 2$ and $29 < l < 46$) plotted after collecting a single image from the same crystal at both room temperature and 100 K at exactly the same position. Both x and y fit a straight line with slope very close to 1, proving that the crystal from Fig. 4 was not misaligned during the flash-cooling stage.

4. Conclusions

The implementation of counter-diffusion described in this paper makes this crystallization technique more attractive than the previous gel acupuncture method. The crystallization is carried out in protein solutions gelled inside X-ray capillaries. It yields isolated crystals of very high quality, which are cryoprotected and suitable for collecting data at room

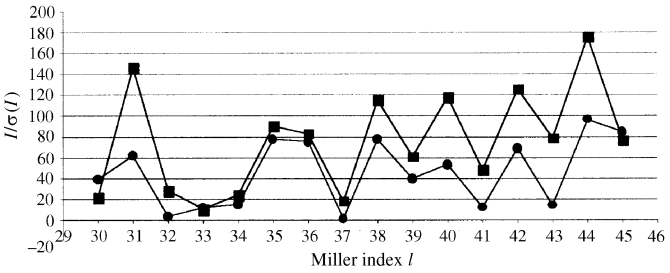


Figure 6
Analysis of the changes in intensity caused by flash cooling of the same thaumatin crystal as in Fig. 5. The analysis is performed in terms of $I/\sigma(I)$ of the sixteen strong spots with Miller indices $h = 4$, $k = 2$ and $29 < l < 46$. Squares: 100 K; circles: room-temperature data.

temperature or under cryogenic conditions from the same capillary where they were grown, and which are immobilized by a gel that avoids any slippage. These features have several interesting practical consequences, as follows.

(i) No post-crystallization manipulation is required. The only manipulation required is that to setup the experiment, saving time and minimizing the risk of losing or damaging the crystals.

(ii) Each capillary becomes a crystallization reactor that can be scanned at room temperature while crystals are still growing in order to identify those of potential interest without disrupting the growth process.

(iii) Crystals can undergo diffraction analysis at room temperature using 'home' X-ray sources and then those capillaries containing interesting crystals may be transported to a synchrotron facility to be flash-cooled for full data collection and structure solution.

(iv) There is no need for dewars of liquid N_2 to store or transport the crystals for diffraction analysis.

Since no manipulation that might provoke crystal misalignment is required to switch from room temperature to 100 K, the experimental setup proposed in this paper makes feasible the quantitative study at lattice level of the effects of cryo- and annealing techniques on the same crystal and with exactly the same orientation.

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Table 3
Volume (V_{max}) of protein required to fill the capillary.

	Capillary diameter (⊘)			
	0.2 mm	0.3 mm	0.5 mm	1 mm
V_{max} of protein (L = 60 mm)	1.9 µl	4.2 µl	11.8 µl	47.1 µl
V_{max} of protein (L = 50 mm)	1.6 µl	3.5 µl	9.8 µl	39.3 µl

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