Available online at www.sciencedirect.com
SCIENCE DIRECT®
Methods 34 (2004) 415–423
METHODS
www.elsevier.com/locate/ymeth

Macromolecular cryocrystallography—methods for cooling and mounting protein crystals at cryogenic temperatures

J.W. Pflugrath*

Rigaku/MSC, Inc., 9009 New Trails Drive, The Woodlands, TX 77381, USA

Accepted 24 March 2004

Abstract

Cryocrystallography is routinely used in macromolecular crystallography laboratories. The main advantage of X-ray diffraction data collection near 100 K is that crystals display much less radiation damage than seen at room temperature. Techniques and tools are described to facilitate cryoprotecting and flash-cooling crystals for data collection.

Keywords: Cryocrystallography; Cryoprotectants; Flash-cooling; Crystal mounting; Annealing

1. Introduction

Diffraction data collection at cryogenic temperatures from flashed-cooled protein crystals has become routine in macromolecular crystallography laboratories and at synchrotron beamlines. Indeed, cryocrystallography is virtually required nowadays to get any kind of reasonable diffraction data from protein crystals. The theory and many of the techniques used in cryocrystallography have been reviewed extensively elsewhere [1–6], so only experimental details will be covered here. The reader is strongly encouraged to read all of these references. The rewards of low-temperature data collection over room-temperature data collection are so extensive that the latter is often not even attempted. The advantages of diminished observed radiation damage to crystals (at least while the crystal is kept at low temperature), the need for fewer crystals to collect complete datasets and solve the crystal structure, the ability to transport crystals at near liquid nitrogen temperatures, and the sometimes perceived higher diffraction resolution limit have made flash-cooling of crystals an extremely important technique to master in macromolecular crystallography. Furthermore, one can mount and store crystals at convenient times for use days and even weeks later. The disadvantages of flash-cooling crystals have diminished over the years as equipment and techniques have improved. There is often an increase in crystal mosaicity and lack of isomorphism among different crystals, but modern data processing and analysis software can overcome these drawbacks. Sometimes ice forms on the crystal during data collection and becomes a problem, but this can be obviated by proper technique. Other disadvantages are the cost of the hardware and the time and practice required to master good technique.

Safety is a primary concern when working with fluids and items at cryogenic temperatures. Safety glasses should be worn at all times. Loose-fitting thermal gloves that can be 'thrown-off' hands if accidentally soaked in liquid nitrogen should be worn when filling and carrying dewars. Hoses should be jacketed of flexible metal (not rubber). For filling open vessels such as the desktop dewars, a phase separator attachment of sintered-bronze allows liquid nitrogen to dribble out the end of a transfer hose into a dewar and avoids splashing when filling dewars. See http://www.shef.ac.uk/safety/guidance/cryogenics.html for more guidelines.

* Fax: 1-281-364-3628.
E-mail address: jwp@RigakuMSC.com.

1046-2023/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2004.03.032

2. Hardware

The assembly of a proper and extensive kit for cryocrystallography is a must in order to have any chance of successfully flash-cooling crystals. Not only are major items like the cryosystem itself and dewars for storage and shipping important, but also the smaller items such as pins, vials, tongs, racks, desktop dewars, covers for dewars, magnets, wands, canes, pucks, and other odds and ends. Generally, a laboratory will standardize on a set of matched tools to create a 'system' and one such system will be described next.

Cryogenic nitrogen gas is blown across the crystal position by a cryosystem and its nozzle [5]. The cooled gas is created by boil-off from liquid nitrogen in a dewar or by helium refrigeration of gaseous nitrogen extracted from room air and maintained near 100 K and at least below 130 K. Such a cold gas stream would freeze out any moisture that it came in contact with, so to prevent such contact and ice formation, a concentric shrouding gas stream of very dry air or nitrogen sheaths the inner cold stream for a short distance from the end of the nozzle. If the inner cold stream and outer dry warm stream move at similar velocities, then gaseous water molecules will not come in contact with a crystal positioned within 7–10 mm from the end of the nozzle (Fig. 1).

The angle of attack of the gas stream upon the crystal is important [5]. The cold gas stream should impinge upon the crystal first. An angle that is nearly co-linear with the mounting pin can be helpful in that the velocity of the gas keeps water molecules (and ice) off the crystal.

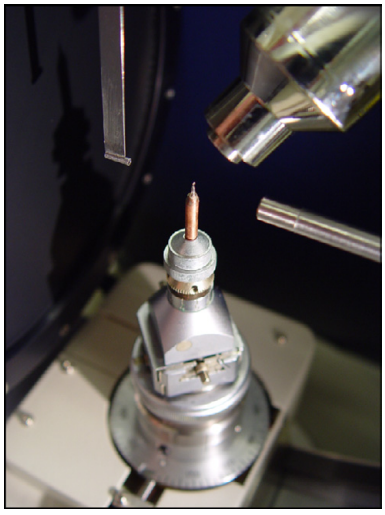

Fig. 1. Cryocrystallography data collection setup. The cryonozzle (upper right) blows cryogenic nitrogen gas upon a crystal (center) mounted in a loop at the end of a copper pin. The copper pin is fastened to a magnetic steel base that is held onto a magnet mounted in a goniometer head (center bottom). The cryonozzle is positioned 8 mm from the crystal and angled such that the cold gas stream does not impinge on the pin base. The collimator is on the right and the beamstop on the left. Note that the cold gas stream hits only the copper pin and neither its steel base nor the goniometer head below it.

However, the gas may then hit the pin base causing turbulence and ice on the goniometer head. In such a case, a less co-linear angle is better. An angle that is perpendicular to the pin may not be ideal either. Re-positioning of the nozzle may be required to determine conditions where ice does not form on the setup, where there are no shadows on the detector and where one can mount and dismount crystals without bumping into the nozzle, beamstop or collimator (Fig. 1).

If ice forms on the crystal, then several details should be investigated: (1) Are the gas velocities mismatched? (2) Are the gas streams dry? (3) Is excess turbulence avoided by keeping beamstops and pin bases out of the gas stream? (4) Is the end of the pin with the crystal the very first item in the gas stream? (5) Is the path of the gas stream unobstructed so that no turbulence is created by hitting the pin base, detector, beamstop, goniometer head or anything else? (6) Are random drafts near the setup avoided, perhaps by creating a constant draft with a fan that sucks air away from the crystal?

A magnet securely mounted on a goniometer head is another piece of the system. The magnet should not wobble because then any pin gripped by it will wobble. The magnet is selected based on the pin base used. Some laboratories have weak magnets, some have strong. Some magnets have a hole through which a small centering nib protrudes. A medium strength magnet sized to fit the pin base should suffice.

There are many different kinds of pin bases and more are introduced every year. A pin base should be attracted by a magnet, should not rust or oxidize, should conduct heat well, and should fit in a cryovial without allowing the crystal to bump against the inner walls. Plastic screw-threaded pin bases do not conduct heat well and do not fit squarely into a cryovial without aligning the threads. The Hampton Research (Laguna Nigel, CA) CrystalCap Copper Magnetic base with a copper pin works well in our laboratory (Fig. 1).

The crystal will be scooped up in a loop or other micro-device [7,8]. The loop should be reasonably transparent to X-rays, give a minimal increase in X-ray background and not produce a diffraction pattern on the detector. While loops can be hand-made from a variety of threads and come in many sizes, it is easier to purchase pre-made loops from Hampton Research that have been pre-glued into steel pins. The 10 μm thread loops have been found to be insufficiently stiff enough for the gas stream, so 20 μm thread loops should be used. The length of the stalk of the loop should be long enough to prevent the metal pin from intersecting the X-ray beam, but short and stiff enough to prevent the loop from flapping in the gas stream. Other crystal-holding devices are under development and appear from time to time [8].

The loop in a steel pin should be attached by glue or set screw into another pin that is fixed in a magnetic

stainless steel or nickel base. Copper is a good material for this pin. The copper pin is long enough so that the cold stream gas does not impact the base of the pin which would cause excess turbulence. The base of steel conducts heat from the goniometer head up through the copper. This prevents ice build-up at the intersection of the copper pin and the interface between the inner cold gas stream and the outer warm dry gas stream. There are many variations of this same theme.

When not on the goniometer head, the crystal and pin are stored in a cryovial or a magnetic puck for use with a robotic sample changer which in turn are stored in liquid nitrogen in a dewar. Plastic *cryovials* can be either threaded or have an embedded ring magnet to hold the metal pin in place. Threaded pins and vials are problematic in that it may be difficult to screw them together while they are in liquid nitrogen. The vials can be placed in a cane and then in a canister in the dewar. A protective plastic sleeve around the cane helps prevent the vials from being knocked off the cane during shipment. The Rigaku/MSC (The Woodlands, TX) ACTOR magazines and rack hold vials even more securely for shipment. This system can be used with or without a robotic sample changer.

A variety of *tongs* and *wands* are required when flash-cooling crystals (Fig. 2). Locking clamps for vials are used to manipulate vials in liquid N_2 and the pins they contain. Pin bases can be attached to magnetic wands so that crystals can be fished out of crystallization drops and plunged into liquid nitrogen. A magnetic wand with a plunger facilitates detaching a pin from the wand. Another variety of tong is described for transfer of pins from liquid nitrogen to the goniometer head magnet [3]. Transfer tongs have a metal cylinder divided into two halves with a hollowed out volume in which a crystal on a pin and base can fit snugly. When pre-chilled in liquid N_2 and closed around a crystal + pin, the cryogenic temperature of the crystal is maintained for several seconds and the crystal is shielded from water in the atmosphere, so that no ice forms on the crystal. Such tongs can be used to transfer crystals in liquid nitrogen to the goniometer head magnet and gaseous cold nitrogen stream. The reverse operation can be performed, too: the crystal + pin can be removed from the diffractometer and replaced in liquid nitrogen.

A variety of *dewars* are needed (see Table 1). Small desktop dewars can hold racks of cryovials or magazines. A shallow wide-mouth dewar (160 mm diameter × 100 mm tall) is suitable for manipulating pins on wands into transfer tongs. Smaller dewars (100 mm diameter × 135 mm tall) are better for ACTOR magazines. Tall ones are suitable for holding canes of crystals. Shipping dewars that hold a single canister are suitable for filling with liquid nitrogen and pouring off the

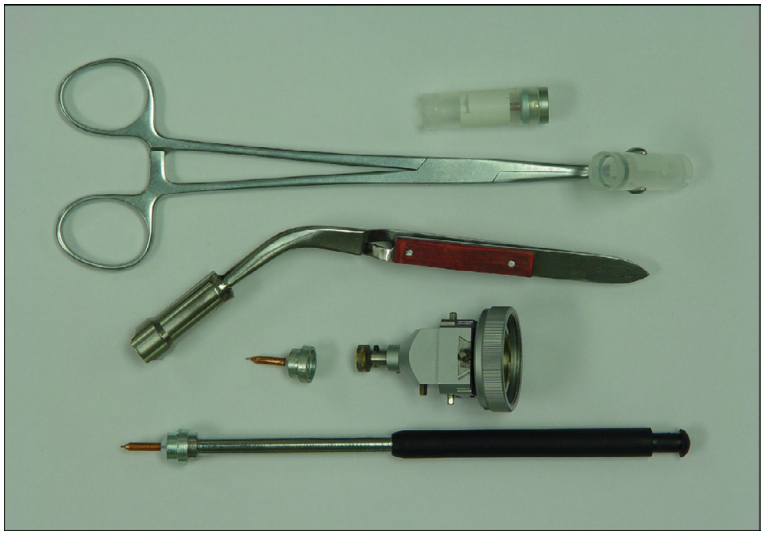

Fig. 2. Small tools for cryocrystallography. From top to bottom: cryovial with magnetic ring holding a pin; locking clamp holding a cryovial; transfer tongs; copper pin + base, goniometer head with magnet; magnetic wand holding a pin.

Table 1
Desktop dewars

Dewar designation	Outer dimensions	Purpose
ACTOR magazine	100 mm diameter × 135 mm tall	Plunging pin into LN_2. Holds cryovials in a single ACTOR magazine
Dog dish	160 mm diameter × 100 mm tall	Plunging pin into LN_2. Grip pin in transfer tongs
Short desktop	120 mm diameter × 180 mm tall	Plunging pin into LN_2. Holds rack of cryovials
Tall desktop	120 mm diameter × 310 mm tall	Holds entire cane of cryovials

LN_2[1] before shipping. Storage dewars usually hold six canisters. Stainless steel dewars are more durable than glass dewars and less likely to break. Sometimes only a glass dewar is available. Dewars are available from a variety of vendors including major laboratory suppliers, Cole-Parmer and TaylorWharton.

The bane of dewars is *ice*. To keep ice off dewars and out of the liquid nitrogen keep them topped up with liquid nitrogen. Also do not pull anything out of the dewar unless it is full of liquid nitrogen otherwise the change in internal gas volume will suck moist air into the dewar and frost will form inside. For desktop dewars, covers that extend over the *outside* of the dewar and down the sides at least 2–3 cm are better than cork lids or aluminum foil. Suitable covers are the clear covers of CDROM or DVD packs or the clear round plastic boxes that cryopins are shipped in. In a pinch, even a plastic bag is better than a cork lid. With lids that extend down the outside of the dewar boil-off nitrogen gas fills the volume above the liquid and displaces any moisture-laden air that might cause frosting and ice in and on the dewar.

Now that you have all your dewars, tongs, wands, pins, loops, racks, and your cryosystem setup, you are almost ready to begin freezing crystals.

3. Cryoprotectants

In general, the solutions that crystals are grown in and that cover them are unsuitable for flash-cooling and vitrification because ice can form in these solutions during the process. The trick is to replace the solution around the crystal with a *cryoprotectant*. There are two required characteristics of a cryoprotectant: (1) the cryoprotectant must vitrify without ice forming and (2) it must not degrade or damage a crystal that is placed in it before the crystal is flash-cooled. If the cryoprotectant in a loop freezes to yield a glass-clear solid, then there is a high probability that it meets the first condition. The freezing characteristics of cryoprotectant solutions should be tested first without a crystal. A loop with frozen cryoprotectant can be subjected to X-rays and a diffraction image can be collected. The image should show no powder rings from ice diffraction or buffer precipitation.

The second characteristic is more troublesome to establish, but there are several rules of thumb published that are helpful [1–4,6,9]. The first rule is that anything that works is good. The second rule is that something that is similar to what the crystal is already in is a good choice. For example, if the crystal was grown in PEG 400, then PEG 400 would be something to try. If the crystal was grown in high salt, then high salt is something to try, but high concentrations of sugars also work well in high salt solutions. When adding a cryoprotectant to the crystal growth solution, it is important to not reduce the concentration of the precipitant and other compounds in the solution except for water. That is, replace water with cryoprotectant and not the salt or PEG. Simply adding a liquid like glycerol or ethylene glycol is considered a bad idea because it reduces the concentration of the other chemicals in the crystal growth solution. On the other hand, adding solid sucrose does not seem to have the same deleterious effect. Reasonable cryoprotectant concentrations that work are in the 25–50% range for glycerol, ethylene glycol (v/v), for PEGs (v/v or w/v), for saturated sugars (% saturation), and alcohols (v/v). For cryosalts such as lithium salts or the carboxylic acids formate, acetate, and malonate concentrations up to the 8 M range are reported to have worked [10]. One can even combine cryoprotectants in the same solution. For example, sucrose and ethylene glycol work well for lysozyme and thaumatin crystals.

Sugars such as sucrose and trehalose can be ideal cryoprotectants. They are also reported to help stabilize proteins in solution [11]. Make a saturated solution of sugar by mixing solid sugar in 10 μl or less of the crystal mother liquor or reservoir solution and test whether it freezes glass clear. If not, reduce the sugar concentration by adding drop or reservoir liquid to the 100% saturated solution. This entire experiment can be done on a coverslip at the viewing microscope and requires only a micropipettor and not a weighing balance.

An alternative to mixing in a cryoprotectant would be to simply coat the crystal with an oil. Excess water is removed from the crystal surface while in the oil. Then the oil-coated crystal is ready to plunge into liquid nitrogen. Many different oils and oil mixtures have been reported to work [12]. In our laboratory, we have had positive results with Paratone-N, perfluoropolyether, turbomolecular pump oil, olive oil, and cod liver oil. Mineral oil and silicone oil have not worked for us, but mixtures of mineral oil and Paratone-N have worked. There is anecdotal evidence that old oil solutions do not work perhaps because they have absorbed moisture from the atmosphere.

4. Techniques

4.1. Transfer crystal to cryoprotectant

Once a cryoprotectant is chosen, the crystal must be introduced to it. Macromolecule crystals are fragile entities and do not tolerate well a change in their environment. Thus, a second criterion of a good cryoprotectant

[1] *Abbreviations used:* LN_2, liquid nitrogen; mp, melting point; bp, boiling point; PEG, polyethylene glycol; MPD, 2-methyl-2,4-pentanediol; MW, molecular weight.

is whether the crystal remains intact and well-ordered when transferred into cryoprotectant. A cryoprotectant is of no use if the crystal immediately dissolves when the two come in contact. There are many transfer techniques to introduce a crystal to a cryoprotectant.

For the first attempt do not use the best crystal to conduct the initial test. Instead, select a crystal unsuitable for data collection and test it in the cryoprotectant. While there are many techniques for getting the crystal and cryoprotectant together, usually the simplest works best. Try the simple techniques first, but use the more difficult techniques if the simple ones fail.

One of the easiest transfer techniques is not a transfer at all, but does require some planning. Simply grow the crystal in a cryoprotectant. We routinely test glycerol, ethylene glycol, sugars, MPD, and low MW PEGs in our crystal growth experiments. Often crystal growth tolerates or even improves with these additives. Empirical evidence suggests that glycerol and ethylene glycol make proteins more soluble, so if they are present, then a higher protein or precipitant concentration may be required to achieve crystal growth. Even if crystals cannot be grown in a cryoprotectant concentration high enough to prevent ice formation, lower concentrations of cryoprotectant in the growth conditions can be helpful. It is possible that the cryoprotectant compound binds to the protein in a specific way (e.g., sucrose to lysozyme), so introducing it during growth may be better than soaking it in. That is, a conformational change in the protein may occur when a new chemical is introduced to the crystal and cause cracking or other degradation. If the chemical was already present during growth, the different conformation already exists and higher amounts of the chemical will be unlikely to degrade the crystal.

Another easy transfer technique is to simply swish the crystal through the cryoprotectant solution. The crystal spends very little time in the cryoprotectant before it is plunged into liquid nitrogen or placed in the cryogenic gas stream or otherwise flash-cooled. A crystal is simply picked out of the crystal growth solution with a loop and immediately the loop is stroked into a drop of the cryoprotectant. In the process, the crystal often falls out of the loop and the 'swish' time is the time it takes to recapture the crystal in the loop and flash-cool it. Since this technique is fast, try to make sure the cryoprotectant solution is as similar to the environment of the crystal as possible. Pay special attention to pH and osmolarity. The crystal in its growth solution may be vapor equilibrated with the cryoprotectant to help minimize differences.

Other transfer techniques that one might use are:

Shock transfer—drop the crystal in the cryoprotectant. This is the same as swish but a longer time is spent in the cryoprotectant. Sometimes if cracks appear in the crystal, they will re-anneal if given time.

Step transfer—drop the crystal in a lower concentration of cryoprotectant, then in increasing concentrations until the final concentration is achieved. One can also place cryoprotectant solutions of increasing concentrations on the crystals.

Dialysis—this is slow and gradual and used when all else fails. There are many variations of the theme. One can try to make the cryoprotectant iso-osmolar with a drop of containing crystals by equilibrating them through the vapor phase much like a sitting or hanging drop crystallization experiment. However it is done does not really matter.

4.2. Crystal and loop wafting

Picking up a crystal in a loop is not easily described, taught or learned. This is something that one has to do for oneself (see Fig. 3). Some prefer to hold a pin with a magnetic wand; some prefer to hold the pin in their fingers. Some prefer to use loop diameters smaller than the crystal; some prefer larger loops than the crystal. For plate-shaped crystals, a large loop may create a flat film of cryoprotectant that will help keep the plate flat and reduce observed crystal mosaicity. When wafting, pulling the loop out perpendicular to the drop may reduce the volume of liquid that accompanies the crystal in the loop. This in turn will reduce X-ray scatter from the excess solution. Another tip is to dab off the excess liquid by touching the edge of the loop to a coverslip (Fig. 3D). Care must be taken not to dislodge the crystal from the loop. If the crystal moves to the dabbed-off drop, simply pull liquid over the crystal and start anew.

4.3. Flash-cooling

The simplest method is to plunge the wand-held loop + pin base into liquid nitrogen held in a small desktop dewar (Fig. 4). The dewar holds a submerged cryovial that has been previously labeled with the proper identification. The liquid nitrogen level in the dewar should be topped up, so that the crystal does not freeze in the sub-zero gas above the cryogenic liquid. The distance and time traversed from plucking the crystal out of the cryoprotectant and plunging into the liquid nitrogen should be minimized. Some prefer to have an assistant present a liquid nitrogen-filled cryovial to the crystal manipulator, but this causes some delay as one hesitates to aim the crystal + pin into the cryovial. It is better to plunge into LN_2 first and save the aiming into a cryovial for later. Remember to keep the dewar covered to reduce ice build-up—an assistant can remove and replace the cover as needed. Some magazines or pucks now used with robotic crystal changers are especially suitable as racks to hold vials for this freezing process.

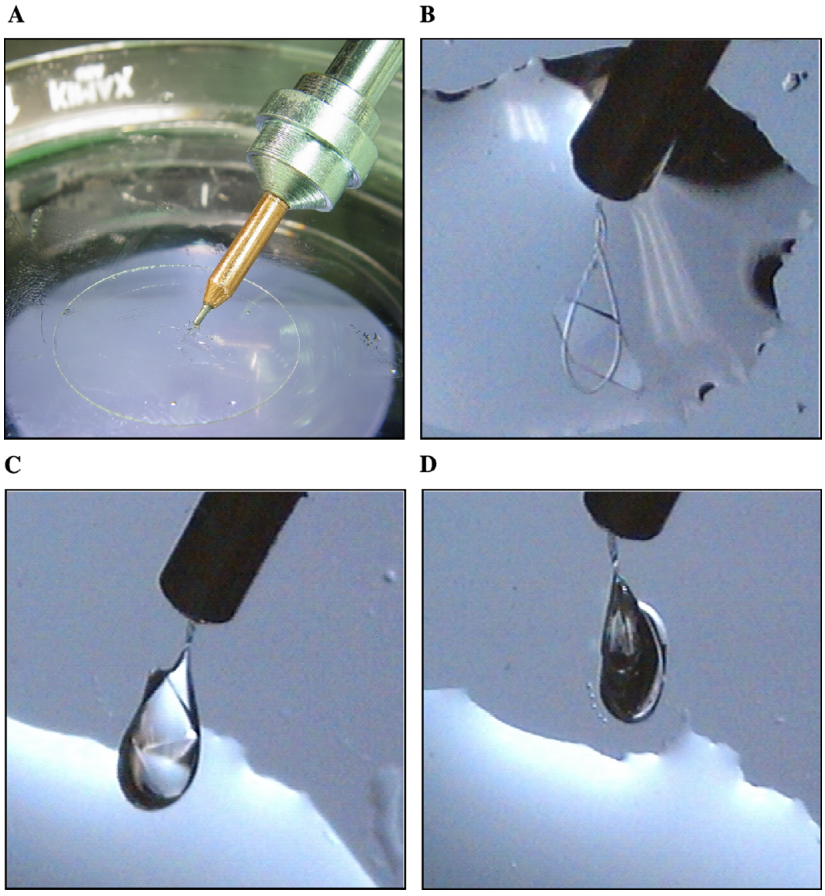

Fig. 3. (A) Loop in a pin held by a magnetic wand probing a drop of cryoprotectant on a silated glass coverslip on a microscope stage. (B) Loop wafting a crystal in the cryoprotectant. (C) Loop has captured the crystal which is being lifted out of the drop. (D) The loop is dabbed on a dry surface of the coverslip to remove excess cryoprotectant prior to flash-cooling by plunging into liquid nitrogen. This last step is optional, but may help reduce X-ray background due to scatter from the excess cryoprotectant.

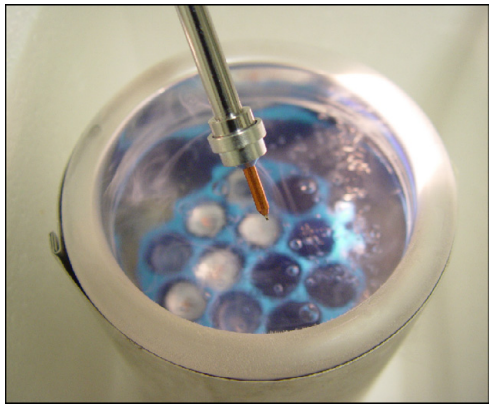

Fig. 4. A crystal in a loop on a pin is plunged directly into liquid nitrogen held in a small dewar. The dewar is filled to the top with LN_2 and contains a blue ACTOR magazine holding some cryovials both filled and empty. The crystal in a loop will be placed in an empty cryovial in the magazine. The cryovial will be inverted allowing the pin base to be held by the magnet at the base of the blue magazine. Transfer tongs (Figs. 2 and 5) can be used to remove the vial and then grip the base + pin for transfer to the goniometer head magnet.

Some advocate plunging into liquid propane or tetrafluoromethane because they possess a higher heat capacity than nitrogen. A simple trick to liquefy these gasses is to fill a balloon with 2 liters of gas and fix the filled ballon neck opening onto a 15 ml disposable centrifuge tube. The tube is placed in liquid nitrogen allowing the gas in the balloon to condense and freeze. The liquid propane (mp −190 °C, bp −42 °C) or CF_4 (mp −184 °C, bp −128 °C) can be thawed slightly and poured into pre-chilled cryovials held in a rack in a dewar of liquid nitrogen [6].

An alternative to plunging in a cryogenic liquid is to flash-cool in the cryogenic gas stream on the X-ray apparatus. While this may not seem ideal, some crystals seem to tolerate this method of freezing better than plunging in to LN_2 [13]. One may block the cold stream with a plastic card, place the crystal quickly at the sample position and unblock the cold stream. Variations of this include not blocking the cold gas stream and/or turning off the warm gas stream. Some crystals appear to flash-cool better in a gas stream than in a cryogenic liquid, but why this is so remains unclear.

All the above flash-cooling techniques should be performed as quickly as possible. When the loop and crystal are moved through the air there is a chance of drying and damaging the crystal. Thus, one should perform the actual vitrification step as quickly as possible and with the shortest distance from cryoprotectant to cryogenic temperature. If one has used oil-coating as the cryoprotectant method, then timing is not as critical.

4.4. Transfer from dewar to the gas stream on the X-ray diffractometer

Once the crystal is in liquid nitrogen—usually in a cryovial—it will have to be transferred to the X-ray diffractometer where a magnet in a goniometer head awaits. An empty pin + base of the same size should be used first to check the position and height of the goniometer head and nozzle in order to ensure that the crystal will be in the cold gas stream when mounted.

If the goniometer head magnet can be pointed downwards in a so-called inverse phi geometry, then a crystal + pin base in a cryovial can simply be lifted to be attached to the magnet and the vial lowered leaving the crystal + pin in the gas stream. Be careful because sometimes the pin will jump onto the magnet as it is brought close to the goniometer head. A way to avoid this is to come in at an angle and touch the pin base to the edge of the magnet, then 'roll' the base on until it seats properly on the magnet. One way to get the magnet to point downwards is to use an extended detachable arc for the goniometer head (Oxford Cryosystems, Oxford, UK).

If the goniometer head is mounted upright, then transfer tongs are an easy way to move the crystal from liquid nitrogen to the X-ray diffractometer. The use of an ACTOR magazine can facilitate this step since it can guide transfer tongs onto a crystal + pin without breaking the loop off the pin. How to use transfer tongs (Fig. 5) is described next.

1. While keeping a crystal + pin under liquid nitrogen in a "dog-dish" dewar (not shown), use a magnetic wand to help place the pin in the pre-chilled tongs and close the tongs around the pin. This step is simplified if an ACTOR magazine holds the pin and no special alignment of pin and tong is required (Fig. 6).
2. Place the captured pin (in the transfer tongs) securely onto the goniometer head magnet in such a way that when the tongs are opened, the cryogenic gas stream will blow directly on the crystal and will not be blocked by the tongs (Figs. 7A and B). The transfer tongs will hold the crystal for tens of seconds at low enough temperature, so one can be quick and deliberate.
3. Squeeze the tongs open, move the tongs *towards the goniometer head base* and expose the crystal to the gas stream (Fig. 7C). This is not a natural movement since one usually wishes to pull the tongs up and away from the goniometer head. By going the other way, the tongs will not accidentally knock the pin off the magnet nor block the cryogenic gas stream. The tongs can then be pulled away from the crystal + pin.

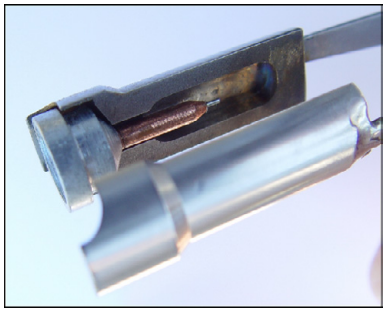

Fig. 5. Close-up of transfer tongs showing the chamber that shields and protects a crystal mounted in a loop on a pin when the tongs are clamped closed.

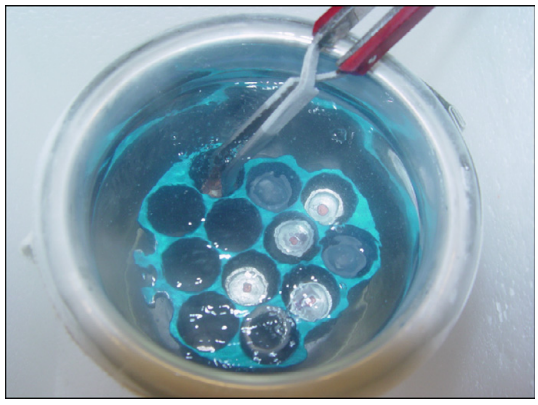

Fig. 6. Transfer tongs chilled in LN_2 and grasping a crystal + pin. The inner wall of the hole guides the tongs, so that no damage to the crystal or pin can occur. A magnet at the bottom of the hole holds pins in place. Other cryovials seen are both rightside up and upside down.

To dismount crystals with tongs:

1. Chill the tongs completely in LN_2, then bring them up to the crystal + pin from the downstream side of the cryogenic gas flow. *Do not open the tongs* until the tong cylinder touches the pin base. If you open the tongs beforehand, the inside of the cylinder has a greater chance of frosting up. Make sure that tongs are aligned so that when they open, the gas stream flows between the two halves of the cylinder. *Warning*: time is of the essence as the temperature of the tongs when not in LN_2 is rising steadily and quickly.
2. Now that the tongs are touching the pin base, squeeze them open and grasp the crystal + pin base by allowing the tongs to close completely around the base.
3. *Tilt* the tongs to break the magnetic attraction and return to liquid nitrogen. The tilt is important as the leverage helps break the magnetic force in a controlled manner.

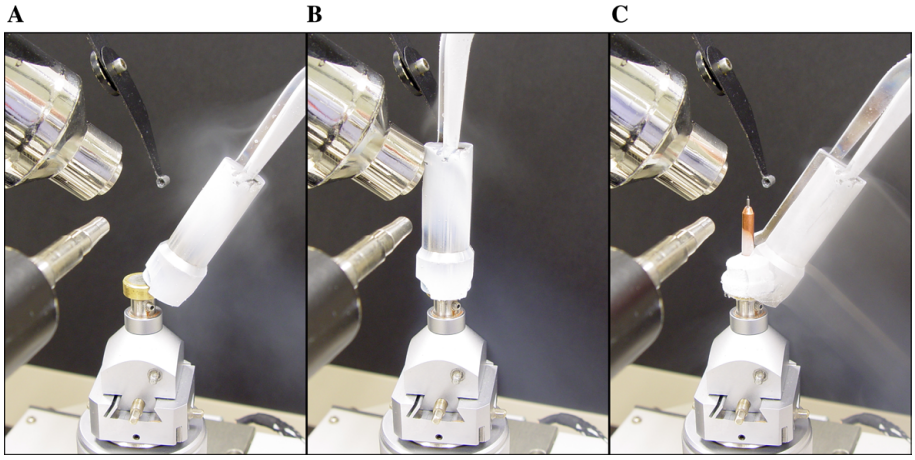

Fig. 7. (A) A pin and crystal held in transfer tongs approaching a magnet on a goniometer head. The cryogenic gas flows from the nozzle in the upper left. (B) The pin is securely seated on the magnet. (C) The transfer tongs are opened and moved *downwards* exposing the crystal to the cold gas stream. Reverse the steps to dismount the crystal.

4. Once the tongs are back in LN_2, they may be opened slightly to allow liquid to bathe the crystal. If held at a tilt, then gas boiled off from the chilling of the tongs will not hit the crystal.

One should practice until the operations are smooth and 100% successful.

4.5. Annealing or tempering

Despite one's best efforts, the diffraction experiment may show ice rings or not quite the best looking diffraction spots. Not to worry, the crystal may allow itself to be annealed—that is, the crystal is thawed momentarily and re-cooled. Two main ways of doing this are advocated [14,15]: in one method, the cryogenic gas stream is blocked with a plastic card from impinging on the crystal for a few moments (watch in the microscope to see the liquid thaw) and then unblocked to flash-cool the crystal again. Another method removes the crystal and places it back in the cryoprotectant for a specific amount of time (e.g., 3 min), then flash-cool again as before. It is not uncommon for such treatment to improve the quality of the diffraction images, so it should always be tried until proven that it does not work for your situation.

Another method is to warm the gas stream quickly to 250 K for a few minutes and then drop the temperature back to near 100 K [16]. However, this requires a special heater in the gas nozzle.

Annealing should probably always be tried on poorly frozen crystals that one would not collect diffraction images from. It is an inexpensive and quick method that just might improve the results.

4.6. Ice removal

If ice is on the surface of the crystal or loop, it can often be rinsed or rubbed off. Rinsing is more delicate and has less chance of damaging the crystal. Wear gloves and eye protection when doing this. Simply pour liquid nitrogen over the crystal. If LN_2 cannot be poured over perhaps partially filling a 10 ml plastic pipette by placing it in a tall dewar of LN_2 and quickly aiming it at the crystal will suffice (Fig. 8). Leave both ends of the pipette open when doing this. Make sure the LN_2 does not damage any microscope lenses or cameras. A small art brush or dental wick can be used to rub off the ice. Cool the brush or wick in the downstream side of the cryogenic gas stream before touching the ice otherwise one may melt the cryoprotectant if the brush or wick is too warm. The wick can be held in locking clamps during the operation. Annealing is also a way of removing ice, but does not work well with a marginal cryoprotectant since the condensed water dissolves in the thawed solution at the loop.

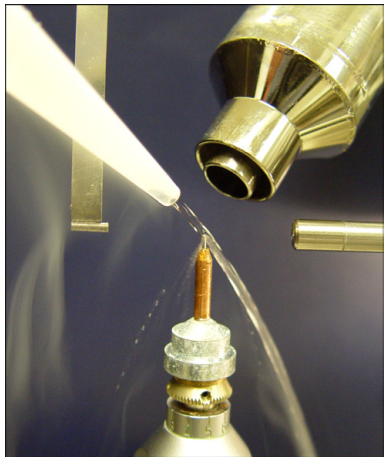

Fig. 8. Liquid nitrogen rinse of a crystal to help remove ice on the surface. A 10 ml plastic pipette was placed in a tall dewar of LN_2 to fill it, then quickly aimed at the crystal while *both* ends of the pipette are unobstructed. *Wear eye protection and gloves!*

4.7. Transport and storage

Crystals are easily stored in dewars of liquid nitrogen and easily shipped in so-called cryogenic shippers. These shippers are special foam-filled shipping dewars that hold one canister or a rack of ACTOR magazines. While not being shipped, the dewars are filled with LN_2 and kept that way. Just prior shipping, the liquid nitrogen is poured off. Upon receipt of a shipper, it should be filled with liquid nitrogen to prevent any frost build-up inside the dewar. These dewars need to be warmed and dried periodically because moisture can also fill the foam and degrade their performance.

The shipping dewars hold either a canister that can be filled with canes of cryovials or a special rack that holds ACTOR magazines or other robotic sample changer pucks. With the magazines there is no danger of a cryovial and crystal becoming separated. With canes, choose ones with tabs to help keep the pin bases in the vials. Furthermore, the vials should be held onto the canes with protective sleeves made for this purpose as shipping forces may dislodge the cryovials from the canes.

5. Conclusion

Cryocrystallography is performed routinely in macromolecular crystallography laboratories. Safety is of prime importance and cryogenic liquids are hazardous, so that gloves, face shields, a phase separator, and warning labels should always be used. The techniques described above are easy to master; nevertheless, attention to detail will make success a habit. A laboratory should standardize their techniques, tools, loops, pins, pin lengths, bases, and record keeping so that there is never any confusion about what to do as reproducibly as possible. Dewars should be kept covered to prevent any ice build-up.

As with many other endeavors in crystallography, new techniques in flash-cooling continue to be reported. For example, if flash-cooling in a loop fails, perhaps flash-cooling in a capillary will work [17]. Or perhaps the thermal contraction of the cryoprotectant solution needs to be optimized [18].

Acknowledgments

I thank my colleagues at Rigaku/MSC, Inc., for helpful discussions over the years as well as the instructors and students of the Cold Spring Harbor Laboratory X-ray Methods in Structural Biology course. I have learned many cryocrystallography techniques from them.

References

[1] D.W. Rodgers, Methods in Enzymology 276 (1997) 183–203.
[2] E. Garman, T.R. Schneider, J. Appl. Crystallogr. 30 (1997) 211–237.
[3] S. Parkin, H. Hope, J. Appl. Crystallogr. 31 (1998) 945–953.
[4] E. Garman, Acta Crystallogr. D 55 (1999) 1641–1653.
[5] H. Hope, Int. Tables Crystallogr. F (2001) 197–201.
[6] D.W. Rodgers, Int. Tables Crystallogr. F (2001) 202–207.
[7] T.Y. Teng, K. Moffat, J. Appl. Crystallogr. 31 (1998) 252–257.
[8] R.E. Thorne, Z. Stum, J. Kmetko, K. O'Neill, R. Gillilan, J. Appl. Crystallogr. 36 (2003) 1455–1460.
[9] E. Garman, E.P. Mitchell, J. Appl. Crystallogr. 29 (1996) 584–587.
[10] K.A. Rubinson, J.E. Ladner, M. Tordova, G.L. Gilliland, Acta Crystallogr. D 56 (2000) 996–1001.
[11] N.T. Saraswathi, R. Sankaranarayanan, M. Vijayan, Acta Crystallogr. D 58 (2002) 1162–1167.
[12] A. Riboldi-Tunnicliffe, R. Hilgenfeld, J. Appl. Crystallogr. 32 (1999) 1003–1005.
[13] S. Kriminski, M. Kazmierczak, R.E. Thorne, Acta Crystallogr. D 59 (2003) 697–708.
[14] J.I. Yeh, W.G.J. Hol, Acta Crystallogr. D 54 (1998) 479–480.
[15] J.M. Harp, D.E. Timm, G.J. Bunick, Acta Crystallogr. D 54 (1998) 622–628.
[16] S. Kriminski, C.L. Caylor, M.C. Nonato, K.K. Finkelstein, R.E. Thorne, Acta Crystallogr. D 58 (2002) 459–471.
[17] M. Yao, Y. Yasutake, I. Tanaka, Acta Crystallogr. D 60 (2004) 39–45.
[18] D.H. Juers, B.W. Matthews, Acta Crystallogr. D. 60 (2004) 412–421.