

10.2. CRYOCRYSTALLOGRAPHY TECHNIQUES AND DEVICES

sizes as low as 5 μl and allow a piece of dialysis membrane to be stretched and held securely over the opening. Dialysis times range from 1 to 24 h depending on the size of the cryoprotectant and the viscosity of the solution.

Another technique, growth of the crystals directly in cryoprotectant solutions, is particularly convenient and effective. In some cases, the primary precipitant, MPD for example, may provide cryoprotection if the concentration used in crystallization is sufficiently high. More commonly, however, additives such as glycerol are included in the crystallization buffer. An advantage of this technique is that crystals can be mounted directly from the crystallization drop, eliminating potential damage in transferring to a harvest or cryoprotective solution.

When it is not possible to identify a cryosolvent compatible with the crystals, a brief exposure to the cryoprotective solution may allow successful flash cooling. Apparently, the water in crystal solvent channels is constrained sufficiently to prevent nucleation, and simply exchanging the external aqueous solution with cryosolvent provides protection. The 'quick dunk' in the cryosolvent may be as short as a few seconds, and for some crystals it is possible to combine this technique with prior equilibration in lower, non-damaging concentrations of cryoprotectant. The same principle of preventing ice formation in the external solution forms the basis of an alternative technique developed by Hope (1988). Here, the external solution is replaced by a hydrocarbon oil before flash cooling.

Finding suitable cryoprotection conditions is a trial-and-error process. Two problems must be overcome: the cryoprotectant must be introduced without significant damage to the crystal, and damage during the flash-cooling process must be minimized. A scheme for systematically determining conditions for flash cooling is given in Fig. 10.2.2.1. In order to assess the effect of subsequent manipulations, it is important first to establish the resolution and rocking curve of the crystals under normal harvest conditions. Then

one or a few cryoprotectants can be added to the harvest solution under conditions that allow equilibration with the crystal. The minimum concentration of cryoprotectant necessary to prevent ice formation can be determined by flash cooling candidate solutions using the loop-mounting technique described in Section 10.2.3. A sufficient concentration of cryoprotectant will result in a transparent glass upon cooling, while too low a concentration will produce opaque microcrystalline ice. A solution of cryoprotectant 2–3% above this minimum value should be used to allow for the added volume and therefore slower cooling when the crystal is present. If the crystals crack or dissolve in a cryosolvent, then the cryoprotectant should be introduced more slowly, the solution conditions (precipitant concentration, ionic strength, pH) altered, or the cryoprotectant eliminated from consideration.

The diffraction quality of crystals that show no visible sign of damage should be assessed at the crystal-growth temperature, and solution conditions should be altered if there has been a significant loss in resolution or an increase in rocking-curve width. For crystals that are incompatible with a wide range of cryosolvent conditions, quick-dunk and oil-coating techniques should be considered. Limited cross-linking (with glutaraldehyde, for example) can sometimes stabilize crystals for the introduction of cryoprotectant or improve stability during flash cooling.

When conditions that result in little or no damage have been identified, the crystals should be flash cooled and the diffraction assessed again. The formation of even small amounts of microcrystalline ice can be detected after flash cooling as characteristic powder rings at low-order spacings of 3.90, 3.67, 3.44 Å. If ice forms, a greater concentration of cryoprotectant must be used. An increase in the rocking-curve width of the crystal at this stage is common, probably due to the thermal stress on the lattice or changes in solution properties on cooling. If this increase is more than 50%, or if any loss of resolution occurs, solution conditions should be altered and the process repeated. The concentration of the cryoprotectant can be increased and different cryoprotectants tested. Other solution parameters, as noted above, can also be adjusted in an attempt to decrease the damage from flash cooling. In addition, different flash-cooling techniques (discussed below) can be tested to determine whether they produce less damage. Suitable cryosolvent conditions are usually established after a few trials, and even in difficult cases it has generally proven possible to find acceptable conditions by continuing to refine solution parameters.

10.2.3. Crystal mounting

A mounting technique suitable for flash cooling should allow for rapid heat exchange by providing a large surface area and a minimum of extraneous material that must be cooled. The technique should also subject the crystals to little mechanical stress and should result in a relatively compact sample that can be immersed in the narrow gas stream used to maintain the temperature during data collection. The glass capillary tubes conventionally used to mount macromolecular crystals are not well suited to flash-cooling procedures since they insulate the sample, reducing cooling rates, and their bulk interferes with cryogenic equipment. A number of alternative mounting methods used for flash cooling are shown in Fig. 10.2.3.1. Crystals can be affixed directly to thin glass fibres with cement or grease (Haas & Rossmann, 1970; Dewan & Tilton, 1987), or they can be scooped up on thin glass spatulas, a procedure first used in conjunction with the oil-coating method described in Section 10.2.2 (Hope, 1988). A loop-mounting technique introduced by Teng (1990) has proven the most generally applicable, however, and has become the method of choice. Here, the crystal is held suspended in a thin film of cryosolvent formed in a small loop.

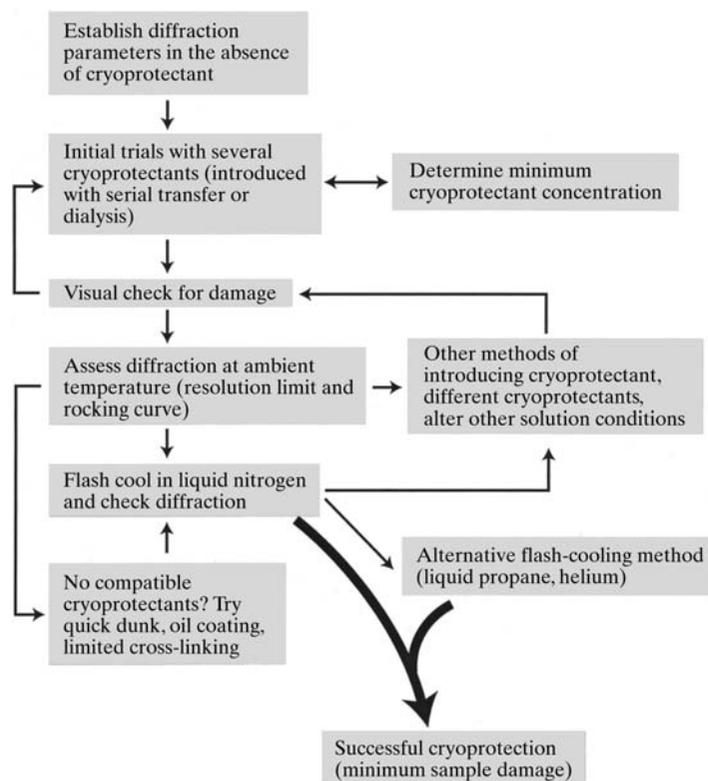


Fig. 10.2.2.1. Recommended pathway for optimizing cryoprotectant conditions and flash cooling.

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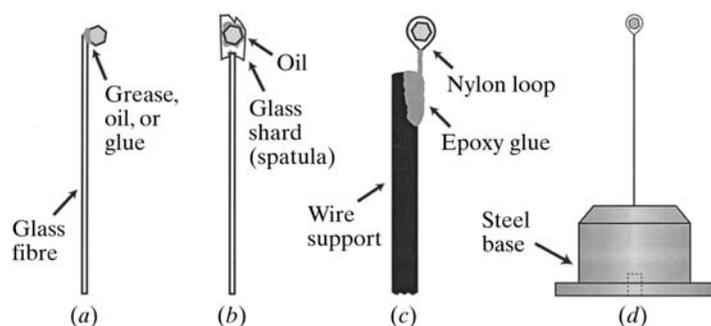


Fig. 10.2.3.1. Different crystal mounts for flash cooling and cryogenic data collection. (a) Crystal mounted on a thin glass fibre with adhesive, grease, oil, or glue. (b) Crystal placed in a hydrocarbon oil and then scooped onto a thin glass shard. (c) Crystal suspended in a film of aqueous solution within a nylon loop. The loop is attached to a thin (~ 0.25 mm diameter) wire support. (d) A diagram of the entire loop-mount assembly. The base is made of plain steel or a magnetic alloy and has two holes, one for the wire post and one for a locating pin, which reproducibly positions the assembly on the goniometer.

The technique is quick and straightforward, remarkably gentle to the crystal, and provides a large surface area for cooling.

The loops are generally formed from nylon fibre, although glass wool is useful for larger versions because its rigidity keeps them from collapsing under the surface tension of the suspended film. Both types of fibres should have a diameter of approximately $10\ \mu\text{m}$. This small cross section reduces absorption and scattering from the material itself and also minimizes the thickness of the film in the loop. Several methods of making the loops have been described in detail (Rodgers, 1997; Garman & Schneider, 1997), and nylon loops of different sizes are available commercially. The loop is usually glued to a thin metal wire or other heat-conductive post. The ability to conduct heat rapidly is required to minimize ice formation at the point where the wire or post exits the cold gas stream of the cryostat, which occurs in some orientations of the loop assembly. This post is in turn attached to a steel base, which is used with the magnetic transfer system described below.

Crystals are placed in the loop as shown in Fig. 10.2.3.2. They can be mounted directly from the crystallization drop or after harvesting into any convenient container. Under a stereomicroscope, the crystal is teased to the surface of the solution, usually with the loop itself. Once at the surface, the crystal is carried through the interface by first resting it on the bottom of the loop and then moving the assembly vertically to pull it out of the solution. A practiced experimentalist can usually capture the crystal in the first few tries. The plane of the loop should be kept near the vertical to increase the chance of catching the crystal and to minimize the

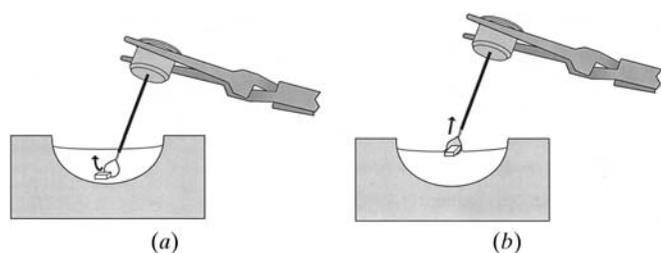


Fig. 10.2.3.2. Mounting a crystal in a loop. (a) While viewing with a stereomicroscope, the crystal is teased to the surface of the liquid using the loop. (b) It is then drawn through the interface and into the loop. The sizes of the loop and crystal have been exaggerated. Reproduced with permission from Rodgers (1997). Copyright (1997) Academic Press.

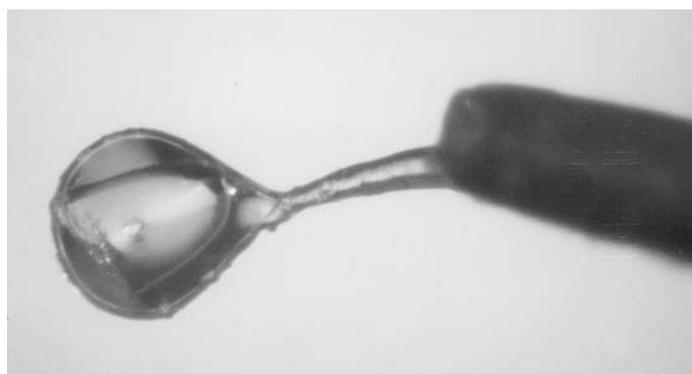


Fig. 10.2.3.3. Photograph of a flash-cooled crystal mounted in a nylon loop. The wire post holding the loop is visible on the right. Reprinted from Rodgers (1994) with permission from Elsevier Science.

amount of liquid drawn up with it. An alternative technique is to use a small pipette to place the crystal and a drop of cryosolvent into the loop and then draw off the excess solution with filter paper. In either case, it can be difficult to form a film in the loop with solutions high in organic solvent due to the lack of surface tension. For these solutions, adding PEG up to a few per cent usually allows a stable film to form. Fig. 10.2.3.3 is a photograph of a crystal mounted in a nylon loop. If the diameter of the loop is chosen so that it just accommodates the crystal, mounting is easier and the amount of extra scattering material in the X-ray beam is reduced. Also, asymmetric crystals can then be oriented relative to the assembly by preforming the loop into the appropriate shape.

The loop-mounting technique can also be used for data collection above cryogenic temperatures by sealing the loop and pin in a large diameter (3 mm) glass or quartz X-ray capillary (Fig. 10.2.3.4). A guard composed of stiff wax or a plastic plug cemented to the pin helps to guide the capillary over the sample before sealing it to the base with high vacuum grease or a cement low in volatile solvent. Loop mounting can be less damaging for many crystals than capillary mounting, and it results in a more uniform X-ray absorption surface.

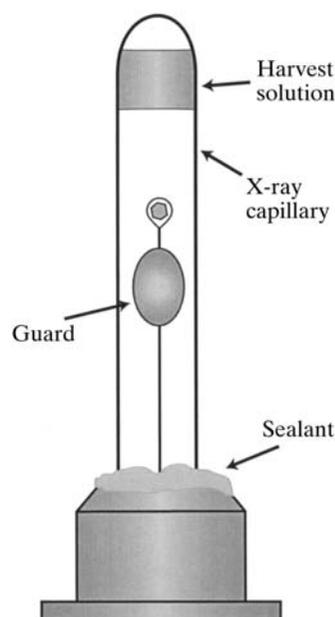


Fig. 10.2.3.4. Arrangement for using the loop-mounting technique at non-cryogenic temperatures.