Cryoprotection of delicate crystals, in 2 easy steps (and 16 hard steps).

Artem G. Evdokimov (artem@xtals.org)

Introduction

The procedure below summarizes one of the approaches to cryoprotection commonly employed in our lab. This approach worked for me on many occasions, but obviously I cannot promise that it works with every crystal, all the time.
I would like to mention that this is not the easiest approach, and certainly not the first that we try on a new crystal (I usually start with 20-25% ethylene glycol in crystal mother liquor; this seems to be satisfactory for at least 50% of crystals I worked with). However, if you have a few crystals to spare, and the simpler things did not pan out, this method is worth trying.

This approach has been hashed out during the late-night hours at various synchrotron beam lines, and has not been put to a rigorous statistical test, so it's not likely that this kind of semi-anecdotal stuff will see the light of day as a real paper. Many colleagues and friends have contributed through discussion, for which I am very grateful ☺. It is likely that someone has described similar, if not the same considerations before. If this is the case, I would appreciate the reference so that I could give proper credit for earlier work.

I am always most grateful for comments, suggestions, or anecdotes of success/failure.

Crystal handling

I do most of this kind of work on the cover slip (or NeXtal device, etc.) – often on the very slip that's been used to grow the crystals. I like to work directly under the microscope because I can immediately see what is happening. Others may be more comfortable with larger volumes of solutions loaded in depression plates, 24-well plates, *etc.* – there is no limit to what can be done as long as one works rapidly and consistently, in order to minimize liquid-air exchange, crystallization of small molecules out of solutions, and so forth.

There are many ways to soak, but I personally like two: (I) a direct soak in the final solution or (II) a two-step transfer, the first step being into 50/50 mixture of final solution and crystal mother liquor. By default I use the two-step transfer first; it is more successful in my hands. These methods are briefly summarized at the end of the document.

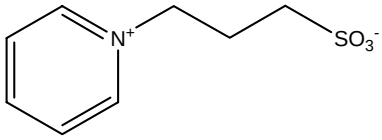
Looking for the right cryosolution

The procedure is generally iterative, for as long as you have crystals and patience to continue. For every attempt, if the crystal looks OK in the cryo I would normally obtain a diffractogram before trying more solutions – and if diffraction is acceptable I'd collect data, because as the proverb goes '*the best* is the worst enemy of *good*'. There are plentiful scary anecdotes out there about how decent diffraction was observed with one

attempt, but all further attempts to improve it were not successful and at the end, the decent diffraction wasn't reproduced.[♣]

We use the following stock solutions:

- 100% ethylene glycol (**EG**)
- 500 mg/ml solution of NDSB-201 in distilled water (**NDSB**)
- 50% sucrose solution in distilled water (**S**)



NDSB-201

We also use the well solution (**W**) and/or the mother liquor (**ML**). If crystals were grown by methods other than vapor diffusion, of if well solution quantity is unacceptable; I try to make up artificial equivalent of the well solution. Likewise, it is sometimes necessary to prepare artificial mother liquor.

It is useful to have the following 8 solutions prepared in a convenient container - a 96-well plate with a silicon resin lid, for example.

	100% Ethylene Glycol (µl)	**500 mg/ml NDSB-201 (µl)**
"1"	0	100
"2"	10	90
"3"	20	80
"4"	40	60
"5"	60	40
"6"	80	20
"7"	90	10
"8"	100	0

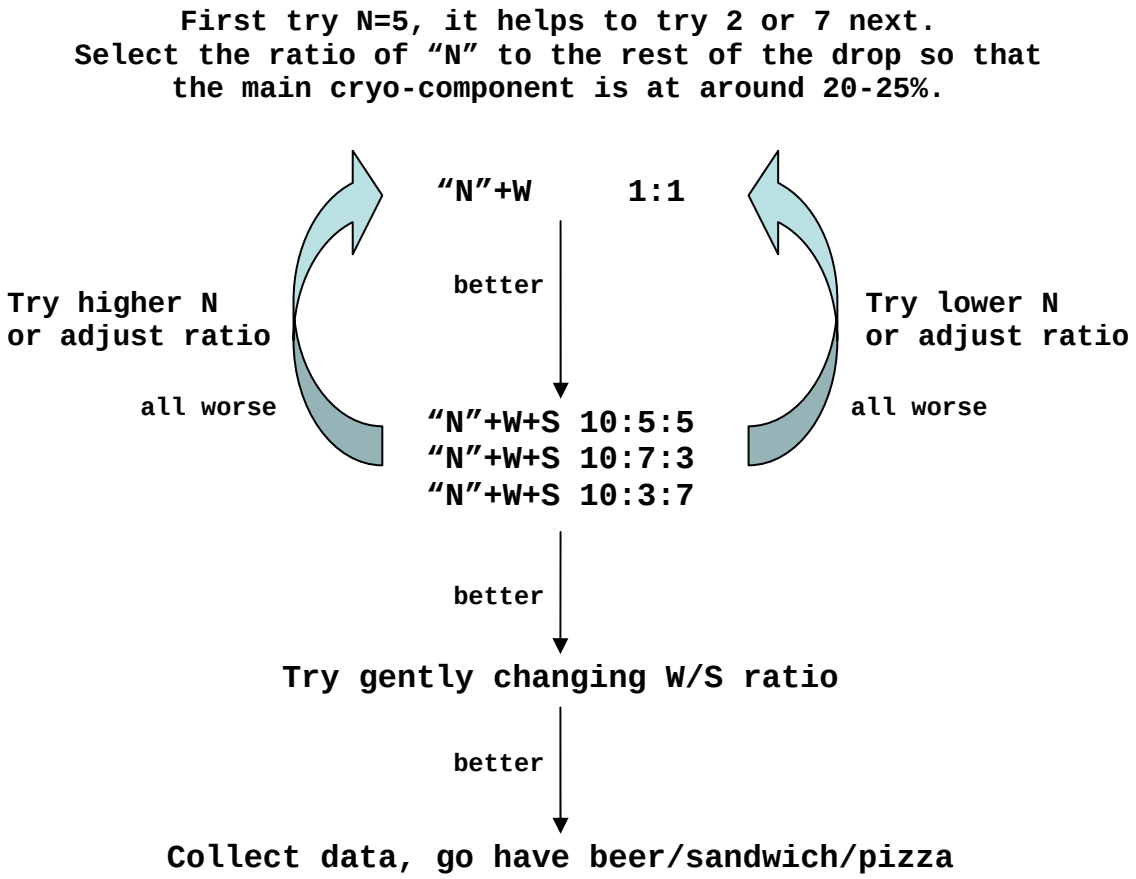
It is also very helpful to have ~100µl of well solution from the crystallization experiment available - again, a separate well of a 96-well plate works for me.

The principle of this test scheme is simple – we hope that there is a combination of **EG** and **NDSB** which allows the crystals to maintain internal order while also allowing the mother liquor to vitrify upon flash-freezing. Oftentimes, however, protein crystals crack and/or deform in such cryo-solutions. In my experience this can be alleviated by adjusting the density of the solution by adding sucrose, while maintaining the concentrations of the other two components. Sucrose itself can be used for vitrification however so far I've had bad luck with sucrose alone.

[♣] If you can take frozen crystals off the beam and store them in LN_2, then life becomes less unpredictable, but to me a collected dataset on my hard drive beats a frozen crystal in the Dewar every time.

A typical diagram of testing is shown below. This is by no means the only way to try this – and I would love to hear what people have succeeded and failed with. For me personally, solutions "4" and "5" diluted 1:1 with mother liquor or well solution, and adjusted to the right density with sucrose worked in at least 5-6 very unpleasant cases. Of course, there is no way to know if this will work at all for anyone else ☺.

Each experiment is denoted as component mixture followed by a ratio. "**N**" corresponds to one of the eight solutions above. Whenever possible I prefer to use mother liquor rather than the well solution, but one tends to run out of that pretty quickly (unless multiple wells with good crystals are available). Therefore, when the diagram calls for '**W**' it actually means '***ML is preferred, but if you're out of it then use W***'.



Assumptions and observations

a) For the purposes of this document we assume that the crystals that are being frozen are at least 'decently diffractive' at room temperature, or become 'decently diffractive' upon careful freezing. Personally (when I bother to check diffraction at r.t.) this means at least 3.0-4.0 Å diffraction in a capillary mount. Having said that, I'd like to add that capillary mounting skills differ greatly from person to

person, and capillary mount is not always the gentlest treatment of protein crystals. Freezing is known to improve diffraction, sometimes dramatically beyond the improvement arising from thermal motion considerations. Therefore, I usually try cryoprotection anyway even if at r.t. the crystals are not promising, and, in fact, I often try cryoprotection first.

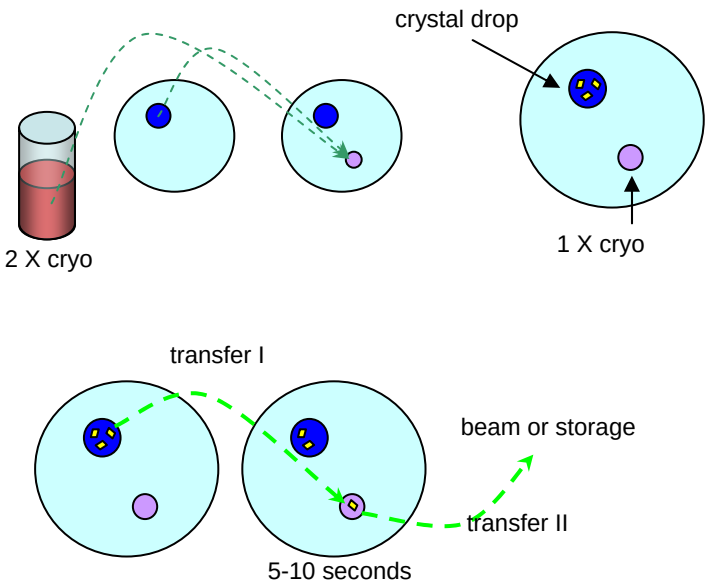
b) I suggest (and it may be entirely wrong) that ethylene glycol and NDSB are 'orthogonal' cryoprotectants. I think that there are two relevant considerations: vitrification of the drop, and interactions between the cryo-solute and the protein inside the crystal. Ethylene glycol is a small, polar, hydroxyl-rich substance, whereas NDSB-201 is a larger, highly polar, zwitterionic substance, with no hydroxyls, which also has a lot of hydrophobic character. An interesting summary of the complex physics of vitrification can be found in *e.g.* the paper of Rao *et al.* [http://eprints.iisc.ernet.in/archive/00000257/01/kjrao.pdf]. As my understanding of the subject matter is not very extensive, I can only suggest that the *ionicity* and *characteristic distance* parameters of concentrated solutions of the two reagents are different, resulting in different vitrification behavior - likewise, the interactions between EG or NDSB and protein molecules are probably quite different. In practice, the two cryo-agents display interesting (and different) peculiarities with respect to individual proteins and well conditions. I have had less success with freezing in NDSB alone, than with freezing in EG alone – however I've had at least one case of NDSB-alone cryoprotection where nothing else worked. Other types of NDSB's are definitely worth exploring, as well as glycerol, propylene glycol, MPD, and so forth.

c) Crystals dipped in various cryoprotectants often develop cracks, bends, dents, rips, etc. indicative of stress forces. Even the crystals dipped into pure well solution can be destroyed, which suggests that even small variations in composition of the medium can be very detrimental to crystal health. While obviously there is a score of complex effects responsible for these unwanted stress forces, a lot of them seem to be related to the density/osmolarity differential between the protein crystal and the solution in which it is placed. I have observed time and time again that sucrose is very useful for adjusting the density of the solution to a value that the crystal 'likes' (i.e. survives in). By itself, however, sucrose seems to be a much worse cryo-agent than ethylene glycol or glycerol.

Cryo-soaking techniques

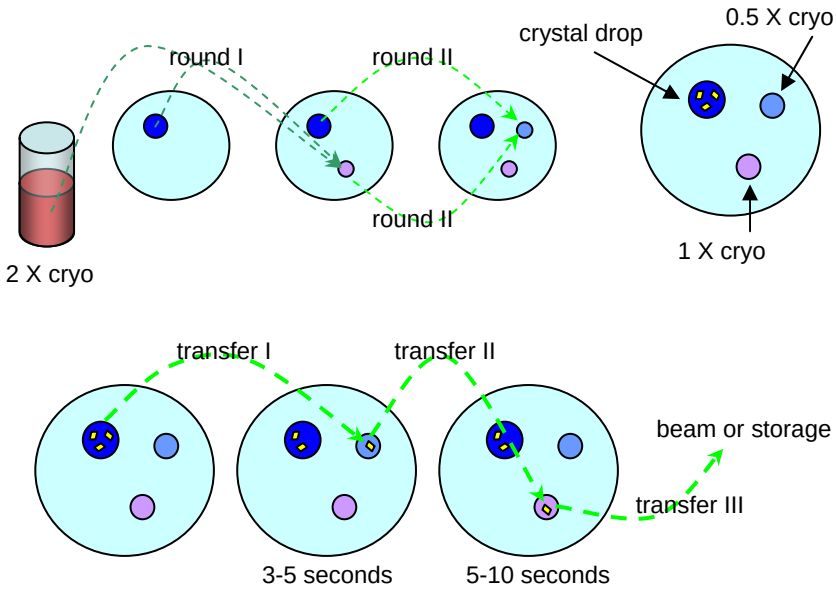
I (One-step): Quickly pipette 0.5µl of **2X cryosolution** on the cover slip. Carefully add 0.5µl of **mother liquor** (gently withdrawn from the crystal drop, or a surrogate). Mix well by pipetting or by stirring with a mounted loop. Pick up the crystal from the drop using a loop and quickly plunge it into the cryosolution. Gentle mixing of the drop with the loop (but don't touch the crystal if you don't have to) can help avoid persistent concentration gradients, especially if the solutions are viscous. Wait 5-10 seconds (observation is key here, sometimes crystals will tolerate 1-3 seconds but not 5, and

sometimes 10 seconds is also OK). Immediately snap-freeze the crystal in the stream of 100K nitrogen, or dip it into LN_2 for storage, etc.



II. (Two-step): Quickly pipette 0.5µl of **2X cryosolution** on the cover slip. Carefully add 0.5µl of **mother liquor** or its surrogate. Mix well. This is now the drop with **1X cryosolution**. Take 0.5µl of the resulting mixture and set a separate drop, to which again add 0.5µl of the **mother liquor** or its artificial substitute. Mix the second drop well – it contains the **0.5X cryosolution**. Pick up the crystal from the drop using a loop and very quickly plunge it into the **0.5X cryosolution** drop. It helps not to let the crystal move out of the loop – this way you can immediately transfer it into the next drop. Wait 3-5 seconds (and observe what happens, sometimes the prospects already are grim at this step). Transfer the crystal into the final cryosolution drop[♦] and wait 5-10 seconds. Immediately freeze the crystal.

[♦] If this causes the crystal to die, you could try merging the **0.5X** and the **1.0X** drops instead of transferring the crystal from one to another - sometimes it helps.


round I
round II
crystal drop
0.5 X cryo
round II
2 X cryo
1 X cryo
transfer I
transfer II
beam or storage
transfer III
3-5 seconds
5-10 seconds