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Comment on *Flash-cooling of macromolecular crystals to overcome increased mosaicity* by Yao, Yasutake & Tanaka (2004)

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In their article on the use of capillary *versus* loop-mounted crystals to obtain slower cooling rates to cryotemperatures, Yao *et al.* (2004) refer to our use of capillaries for cooling nucleosome core particle crystals (Luger *et al.*, 1997). They indicate that the cooling process we used is even slower than theirs. We note, however, that although our process takes 20 min to complete, the cooling rates achieved for vitrification are actually very fast. Considering the importance of flash-cooling in macromolecular crystallography, we describe our procedure in greater detail here.

Following transfer of nucleosome core particle crystals to a 24% solution of the cryoprotectant 2-methyl-2,4-pentanediol, our cooling procedure using capillary mounts involved four distinct steps: (1) transfer to 277 K, (2) cooling to 255 K over 5 min, (3) flash-cooling into liquid propane at 153 K, and (4) transfer into a cold gas stream at 103±5 K. While the first two steps in the liquid phase occurred relatively slowly, the cooling rate in the flash-cooling step was limited only by the heat-transfer physics of the system when using a mechanical device (*e.g.* Hope *et al.*, 1989) capable of plunging the mounted crystal rapidly into the cryobath. Rod-shaped crystals of about 0.2 mm in cross-section were mounted in thin-walled tapered capillaries, with the crystals lodged in the taper. Excess solution above and below the crystal was removed, leaving only the liquid held by capillary attraction between the crystal and the capillary wall. With this residual liquid ensuring optimal thermal contact with the bath, our calculations indicate that the expected initial cooling rates would be greater than 1000 K s^{-1} for the jump from 255 to 153 K. Experimental tests using a fine thermocouple indicated that the crystals reach 173 K within 50 ms and the final temperature within 120 ms (unpublished work).

Cooling rates with different flash-cooling strategies vary over ten to 100-fold, and as Yao *et al.* (2004) note, the optimal cooling rate will depend on the material in the crystal, crystal size and the cryoprotectant. Our observations indicate that the cryobath temperature can also be critical for optimal diffraction quality. For several protein/DNA complexes, including the nucleosome core particle, we have found that the optimal bath temperature for the vitrification step is always near 153 K. At higher temperatures, the intensity of the diffuse water ring increased significantly. At lower temperatures, mosaicity increased and the diffraction limit deteriorated. Once vitrified, the rate of transfer to 103 K had no apparent effect on diffraction quality.

With nucleosome core particle crystals prepared in capillaries as described in Luger *et al.* (1997), or later with loops (Davey *et al.*, 2002), no improvement in diffraction quality could be achieved with the annealing procedure reported by Harp *et al.* (1998), who flash-cooled crystals by direct transfer into a cold gas stream. Crystals that have suffered a substantial increase in mosaicity because of excessive thermal or osmotic shock may partially recover through a melting and revitrification cycle. We have seen that crystals that have developed cracks during transfer to cryoprotectant in many cases reanneal. However, use of smaller crystals that are less susceptible to cracking generally yields higher resolution single-crystal diffraction. We suggest that where cryo-annealing procedures result in improved diffraction quality, an optimized cooling protocol would be likely to yield better diffraction quality without a cryo-annealing step. In our experience, optimization of the cryocooling protocol used for different crystal types has generally resulted in improved data quality, and the capillary-mount method described by Yao *et al.* (2004) is a useful tool for this purpose.

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