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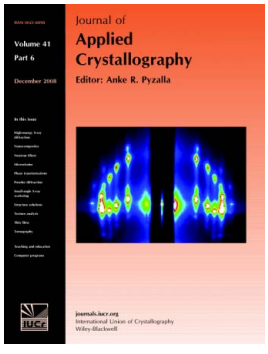

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Is your cold-stream working for you or against you? An in-depth look at temperature and sample motion

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It is normally assumed that a commercial gaseous nitrogen cold-stream provides a sample environment near 100 K and that the force of the cold-stream does not induce movement in the sample. As might be expected, the reality is much more complex. Here, an investigation of one cold-stream, starting with the temperature profile, is presented. Using silicon single crystals and flexible mounting loops, an approximate force/vibration profile of the cold-stream is obtained. Results indicate that the center of the temperature profile is offset from the position suggested by the manufacturer-supplied alignment tool and coincides with the area within the cold-stream that has the most consistent force profile. Tests indicate that this region is only about one-third of the width of the cold-stream nozzle opening. To verify that the results were relevant to protein crystallographic data collection, the impact of cold-stream position on the final data quality for lysozyme crystals was analyzed. On the basis of the observations it is recommended that users perform a temperature profile of their cold-streams to ensure proper alignment instead of relying only on the alignment tool for setup. In addition, suggestions are made on what users can look for in data processing to identify problems with loop movement and what users can do to minimize the impact of these problems on their experiments.

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1. Introduction

Macromolecular crystallography at third-generation synchrotrons has only been able to achieve its full potential thanks to the development of low-temperature devices and the subsequent apparatus used with them. The main motivation for these low-temperature techniques as far as protein crystallography is concerned has been the reduction in radiation damage of the protein sample and reduced atomic motion, leading to high-resolution structures. Garman & Schneider (1997) give an excellent review of the subject, and Garman & Owen (2006) discuss the impact of cryocooling on radiation damage.

With the maturing of protein crystallography at the synchrotron, most users have come to expect the X-ray source to be stable and that beamlines will provide good optics, accurate goniometer-shutter timing and high-quality detectors. Since most nitrogen cold-streams in use are commercial devices, user confidence in their reliability and quality is high. Most users only think about the impact of the cold-stream when they are confronted with the rare occasion when it fails to operate properly. Given that the beamline equipment is performing correctly, users assume that if their processed data has high $R_{\rm merge}$ values it must be as a result of sample quality.

At the Structural Biology Center bending magnet (19BM) and insertion device (19ID) beamlines at the Advanced Photon Source, routine testing is performed after each maintenance period to verify system performance before releasing the beamlines to user operation. The final test is to collect and process data from a reasonably low mosaic lysozyme crystal. From a sample that had displayed normally low $R_{\rm merge}$ values during a previous verification, unusually high $R_{\rm merge}$ values were obtained. After some investigation it was found that the nitrogen cold-stream was misaligned. When the cold-stream alignment was restored, data quality returned to normal.

Beamline 19BM uses either a Cryostream 600 or 700 (Oxford Cryosystems Ltd, Oxford, UK) as its source of cold gaseous nitrogen. These devices are in routine use at the Structural Biology Center and have performed well over many years. To ensure peak performance, these units are rotated in and out of service depending on maintenance requirements. For this study the Cryostream 600 was employed. However, subsequent measurements indicate that these results are equally applicable to the Cryostream 700.

The Cryostream 600 is designed to provide a constant flow of gaseous nitrogen regulated to a constant temperature, typically 100 K. To prevent icing around the cold-stream nozzle and in the area downstream of the sample, the cold gas stream is surrounded by an ultra-dry (dew point less than 213 K) co-axial warm air stream. Flow regulation of the device is set by a needle valve and the bulk flow rate is monitored *via* a standard gas flow meter. Although these devices are designed to provide laminar flow over a diameter of several millimetres, disturbances inside the flow can change

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depending on how the gas travels through all the components of the cold-head, the design of the exit nozzle, how the cold-stream gas mixes with the warm air stream of the shield gas near the edge of the laminar region and the angle of the cold-stream head. Getting a measure of these characteristics is no easy task since bulk flow meters are too coarse to provide any detailed information and optical methods based on density are likely to average over the entire cross section of the gas stream rather than probing specific parts. The ideal probe would act very much like a protein sample under real experimental conditions and, if sufficiently sensitive, would clearly show areas of constant force within the cold-stream. Changes in force would point to areas of changing gas density.

While there are few detailed studies to suggest how the cold-stream can contribute to overall data quality, the need to align the cold-stream accurately so that the sample resides in the coldest part of the gas stream has been emphasized by Mitchell & Garman (1994). One widely held belief by users has always been that it is better to push the cold-stream in close to the sample for the highest quality data. This would appear to be confirmed by one study (Flot *et al.*, 2006), but another recommendation (Garman & Owen, 2006) suggests that a range of 5–8 mm is acceptable.

It is impossible to separate the impact of the cold-stream from the loops used to mount protein samples. Several authors have emphasized the importance of using rigid loops (Thorne *et al.*, 2003; Alkire *et al.*, 2005; Garman & Owen, 2006), and it is clear that if the loops are not able to withstand the force of the cold-stream, data quality will suffer. Although testing loops of differing strength is not directly part of this study, the characteristics of different loops were utilized to help characterize the cold-stream. Based on the results of this study some suggestions are made on how users might benefit from specific loops and how to prepare loops to get the most rigidity out of them.

In order to quantify the impact of the cold-stream, both a temperature profile and a force/vibration profile were measured for our existing system. The validity of the force/vibration profile depends on the sensitivity of the probe, and a few attempts were made to achieve adequate sensitivity using different size mounting loops. Once the probe had been decided, the vertical, horizontal and on-axis profiles of the cold-stream were mapped. To assess the impact on real protein crystallographic data, numerous lysozyme data sets were measured and the processing statistics analyzed for any correlation with the measurement profiles.

2. Cold-stream measurements: temperature

The cold-stream was mounted to a set of X–Y–Z translation stages with dove-tail construction, each capable of movement in 0.0254 mm increments. The X (horizontal) and Y (vertical) stages are quite massive, capable of moving 68 kg each, and the Z (on-axis, parallel to the cold-stream head) stage has a weight rating of 45 kg. These heavy-duty stages are critical to keeping the cold-stream positioned accurately, particularly during sample-mounting robotic operation. The cold-stream head was mounted at 90° to the incident beam, directly across from the sample position, and inclined at an angle of 42° to the horizontal direction. This was well within the manufacturer's suggested range of 20–90°. The nitrogen gas sample flow rate was set to 5 l min^{-1}, the warm air stream to 7–8 l min^{-1} and the controller temperature to 100 K; these parameters are indicative of typical cold-stream operation at the Structural Biology Center beamlines.

To measure the temperature at the sample a type K thermocouple was used. This thermocouple is a fully exposed beaded wire (Omega Engineering Inc., Stamford, CT, USA, part No. 5SC-TT-K-40-36) with the soldered tip roughly 0.2 mm in diameter. Individual wires are 0.076 mm in diameter and Teflon coated away from the solder tip. The readout display is a benchtop digital thermometer with type K connector (Omega Engineering Inc., Stamford, CT, USA, Monogram series model DP41-TC). Fig. 1 shows the cold-stream and thermocouple measurement setup.

Using a thermocouple to measure the temperature is not recommended by Oxford Cryosystems owing to the possibility of incorrectly applying the technique. In order for this measurement to be accurate it is necessary to have the thermocouple unshielded so that there is no material in front of or around the thermocouple to either block the cold-stream or act as a heat source near the thermocouple tip. Standard metal-jacketed thermocouple probes typically used for measuring bake-out temperatures at synchrotron beamlines are not suitable for measuring the gas temperature because of the large heat shield surrounding the probe. For these measurements the thermocouple wire was wrapped around the stainless steel pin of a mounting base (for support) and the Teflon-coated wires were secured to the pin near its base with epoxy. The beaded tip of the thermocouple extended at least 2 mm beyond the tip of the stainless steel pin and the ther-

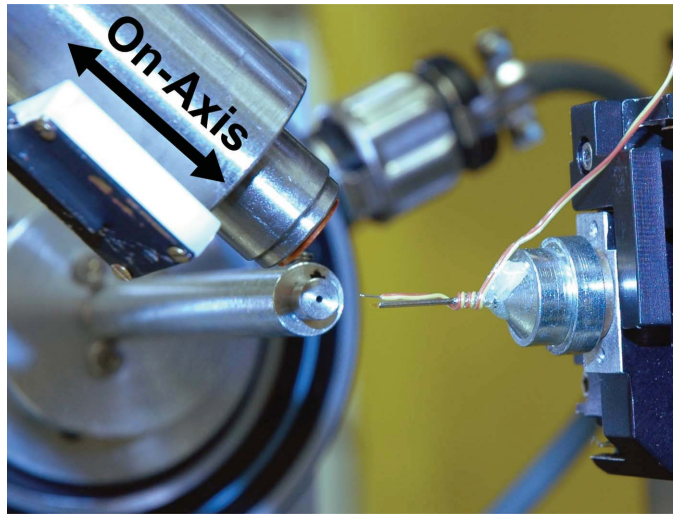

Figure 1
Cold-stream and thermocouple setup used for making the temperature measurements. The arrow on the cold-stream head illustrates the direction the cold-stream moves when changing the (on-axis) nozzle-to-sample distance. The white box near the cold-stream nozzle forms part of a moveable illumination paddle used in sample alignment and does not block the warm air stream.

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mocouple wires were positioned above the pin, so that any turbulence created by the pin would occur well beyond the thermocouple. After using this design for a while it was realized that the stainless steel pin was unnecessary as long as the wires were first secured to the base. Without the stainless steel pin the thermocouple assembly can be positioned in any orientation for use.

At the beginning of each temperature measurement the Cryostream alignment tool, which is nearly identical to the one developed by Mitchell & Garman (1994), was placed on the end of the cold-stream nozzle. The tip of the alignment tool can be reproducibly positioned with an accuracy of about 0.1 mm if no attention is paid to the orientation of the tool. If the device is installed in the same orientation each time, for example, with the locking screw always pointing up, the positioning precision can be improved to about 0.05 mm.

Using the translation slides the tip of the alignment tool was centered on the crosshairs of the images from the sample visualization cameras, *i.e.* the κ goniometer rotation axis center. 19BM has cameras aligned in both the vertical and the horizontal planes, which allows the alignment tool to be positioned in three dimensions with the same precision as a protein sample. It is convenient to refer to the cold-stream in space with three coordinates, one for each direction. With the alignment tool positioned at the rotation axis center, these coordinates become vertical (V) 0.0, horizontal (H) 0.0 and the on-axis nozzle-to-sample distance (NSD) 7.0, or in combined form (V0.0, H0.0, NSD7.0), with all dimensions in millimetres. During these measurements the thermocouple was kept stationary at the rotation axis center, while the cold-stream was moved using the translation slides. Cold-stream movement was isolated from the sample position and camera optics. This made it possible for the sample alignment cameras to monitor the thermocouple location continuously as the cold-stream changed position, ensuring the thermocouple remained stationary to within 0.050 mm of the crosshair center at all times.

Fig. 2(*a*) shows the vertical temperature profile measured using the type K thermocouple at a starting position NSD7.0. Unless otherwise stated, the horizontal starting position was always H0.0. The center of the vertical temperature profile has been chosen as the midpoint between 110 K readings taken at the top and bottom of the cold-stream profile. With the cold-stream temperature set to 100 K, this definition makes it straightforward to establish the temperature endpoints. If the coldest temperature was used to establish the temperature center, any calibration errors or asymmetry in the temperature profile could lead to inconsistent or, in the worst case, incorrect placement of the cold-stream head. Knowing where to position the cold-stream is very important, and the coldest temperature is not necessarily a sufficient indicator.

If the cold-stream gas were aligned with the alignment tool, the center of the temperature profile would coincide with the rotation axis center, *i.e.* V0.0. It is evident from Fig. 2(*a*) that the two centers are displaced, the difference being 1.27 mm. Since the alignment tool was used to center the cold-stream before starting the measurement, this result is quite unexpected. Assuming that the nitrogen gas stream leaving the nozzle is roughly circular in cross section, the horizontal temperature profile should show an increased width if the raised vertical setting is correct.

Fig. 2(*b*) shows the horizontal profile at two different vertical settings: one at V0.0 and the other at V1.27. Assuming that the two points at 110 K define the width of the horizontal profile, the raised position is wider by 0.8 mm. This confirms that the positioning of the cold-stream head using the align-

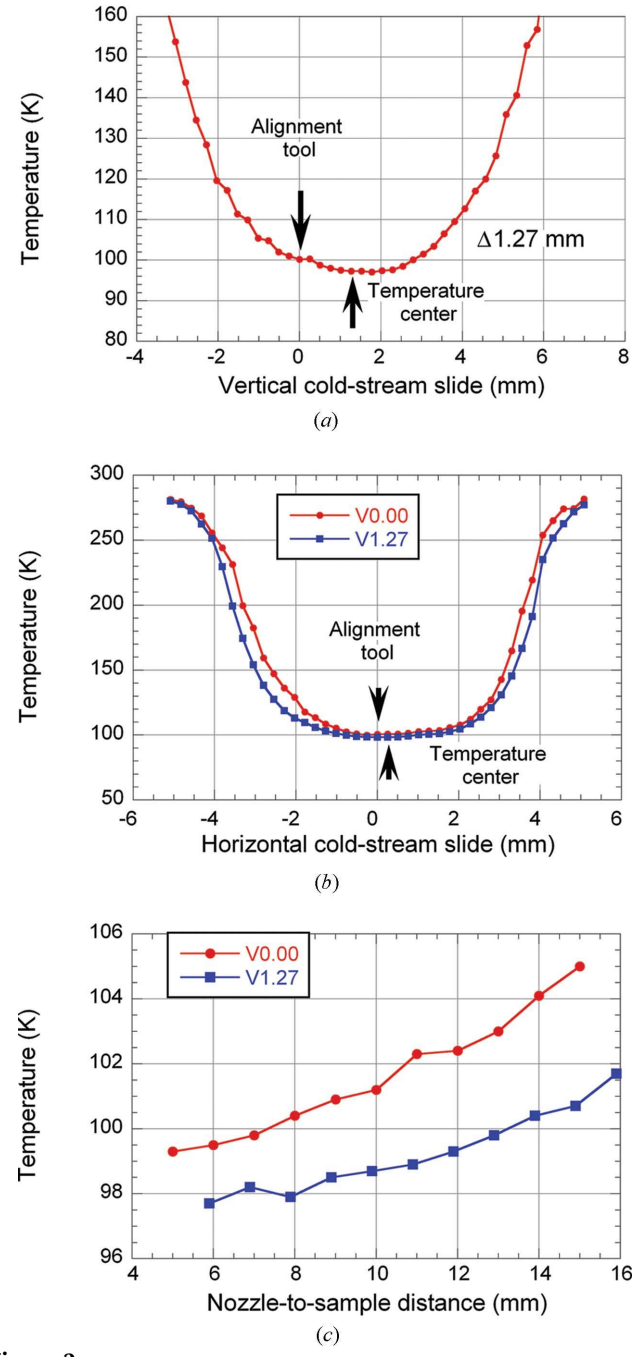

Figure 2
Temperature profile measured with a type K thermocouple as the cold-stream slide is moved (*a*) vertically, (*b*) horizontally and (*c*) along the cold-stream axis. The nozzle alignment tool is centered on the rotation axis at (V0.0, H0.0, NSD7.0).

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ment tool offsets the actual gas temperature center from the rotation axis. Horizontally, the temperature center was displaced only slightly (0.25 mm) from the alignment tool position.

The effective width of the gas stream in the vertical direction depends on the angle of the cold-head relative to the translation slide. In the horizontal direction there is no angular dependence, so the horizontal temperature profile is a good representation of the true cold-stream width. Any changes resulting from a horizontal misalignment are likely to be felt much sooner, owing to the smaller width, than the same relative offset in the vertical direction. These measurements showed the horizontal width to be 1 mm narrower than the corresponding vertical measurement.

Mapping the on-axis temperature profile, *i.e.* the profile of the temperature as the nozzle-to-sample distance is changed, becomes more informative when the vertical offset is considered. Fig. 2(*c*) illustrates the on-axis profile for both the V0.0 and the V1.27 positions. At V1.27 the center of the nozzle is 0.9 mm farther away from the sample than the V0.0 position for the same *Z*-slide setting. At a cold-stream position of (V1.27, NSD7.9), the temperature is lower than the much closer (V0.0, NSD5.0) position. In fact, it is necessary to move the nozzle beyond 11.9 mm before the temperature at V1.27 gets above the (V0.0, NSD5.0) value. This provides additional evidence that the raised position is a better optimized position than that suggested by the alignment tool.

Instead of centering the cold-stream by moving up vertically, the same adjustment could be achieved by moving closer to the sample, parallel to the spindle. Because of the way the cold-stream is mounted and the potential for collisions with the κ stage, moving the cold-stream in this direction at 19BM is not an option. Moving the cold-stream up vertically moves the nozzle 0.9 mm farther from the sample. Since the longer nozzle-to-sample distance had little temperature impact, the raised position was subsequently used as the new standard setting.

Given the geometry of the cold-stream relative to the sample at 19BM, not correcting the vertical temperature offset can have a major impact on the temperature of the sample if the overall length of the sample pin is significantly shorter than the length of the pin currently in use on the instrument. Some of the commonly used nylon loops come attached to pins that vary in 3 mm length increments. If the cold-stream is positioned at the alignment tool position, mounting a pin 3 mm shorter than what the instrument is set up for can result in the sample being placed into an area of the cold-stream that is close to 140 K. This would lead to ice formation inside the crystal (Garman & Schneider, 1997) before the sample could be optically aligned. It is important, therefore, to get the cold-stream centered as closely as possible on the rotation axis, *i.e.* correct the cold-stream vertical offset, and to take the time to check pin lengths before starting an experiment.

3. Cold-stream measurements: force and vibration

Mapping out the temperature profile is informative, but to achieve a detailed understanding of how the cold-stream might affect data quality it is important to obtain knowledge of the force that exists inside the cold-stream. To perform this task, silicon single crystals were chosen as the sensing tool. Silicon is ideal because it will not undergo appreciable radiation damage, can be cycled to room temperature, has a mosaicity that will likely be lower than the divergence of the incident beam and is a very strongly diffracting crystal. For this study cut silicon single-crystal cubes, 0.2 × 0.2 × 0.2 mm, and silicon single-crystal rods, 0.2 × 0.2 × 10.0 mm, were obtained from the Advanced Photon Source Optics Fabrication Laboratory. These crystals were cut from a 0.20 mm-thick single-crystal wafer using a diamond saw.

The method of choice was to mount a silicon crystal in place of the protein sample and use the CCD detector to determine the crystal orientation. Once the position and angle of a single X-ray reflection of interest were known, a photodiode detector was installed and positioned to intercept only one reflection [Si(220)] from the crystal. As the crystal goes through the reflecting position, the photodiode output was measured with an oscilloscope (Tektronix Inc., Richardson, TX, USA, model TDS 2024). The oscilloscope was triggered at a known starting angle using a constant rotation speed of $1^\circ\ s^{-1}$, and the oscilloscope timing correlated with the reflection angle. To ensure an accurate statistical measure, each measurement was repeated 5–6 times at each cold-stream position. The cold-stream was moved in a single pass over the entire scan range of interest (vertical, horizontal or on-axis) to complete the measurement. From the data a center-of-mass (centroid) for each reflection was computed. Deviations in the centroid positions across the span of the cold-stream give a relative measure of the force that the cold-stream exerts on the sample. When combined with the statistical variation in each measurement, a map of this force emerges.

In order to establish an instrumental baseline it is necessary to measure what happens when no loop is present. For this measurement a silicon rod was mounted with epoxy inside a

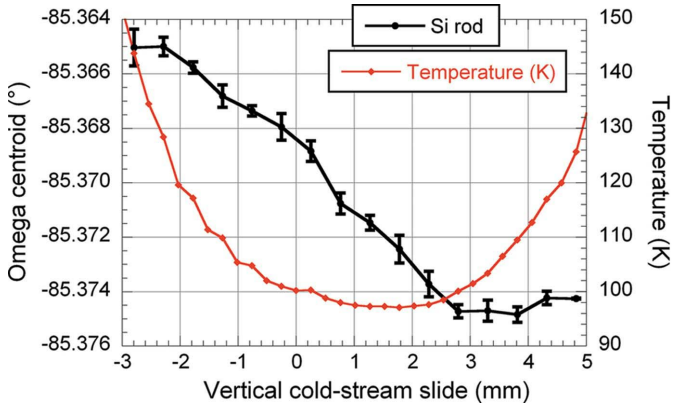

Figure 3
Reflection centroid positions are shown for the silicon rod as the cold-stream is scanned vertically, along with the corresponding vertical temperature profile. These high-precision baseline measurements allow very small changes in the centroid positions to be easily tracked as the cold-stream is moved vertically. The positional shift of 0.01° over the course of the measurement is likely due to rotation induced by thermal contraction of the mount used to hold the silicon rod.

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copper pin with stainless steel insert. About 2 mm of silicon rod protruded beyond the stainless steel, and the X-ray beam hit the silicon rod 0.5 mm from the end. Fig. 3 shows the reflection centroid positions for the silicon rod as the cold-stream was moved in the vertical direction along with the corresponding temperature profile. The intensity on the diode was always less than 0.3 V, well below the photodiode saturation level of 0.34 V. The reflection full width at half-maximum (FWHM) from a typical point in the curve was 0.0368 (3)°, the standard deviation of the reflection centroid was ±0.00035° and the standard deviation of the measured reflection intensity, without compensating for beam decay in top-up mode operation of the storage ring, was ±0.7%.

Starting at a sample temperature of 145 K, where the cold-stream was mostly below the silicon rod, the first two points show the reflection centroid angle unchanged. As the vertical cold-stream slide is moved up and the temperature falls at the sample position, the reflection centroids start an angular shift that is linear with cold-stream position. This linearity stops and the angle becomes constant again about 1.5 mm after the temperature center of the cold-stream passes above the rotation axis center. Given that the positions of the reflection centroids are not symmetric with temperature and the change in the thermal expansion coefficient for silicon is quite small over this temperature range, the likely cause of the 0.01° shift is rotation induced by thermal contraction of the mounting assembly. In fact, thermal contraction of the pin base will have little impact on the overall profile determined by the cold-stream probe used to make the force measurements. Nevertheless, it is important to establish how moving the cold-stream around may influence the measurement and with what precision these measurements can be made.

The sensitivity of the force probe can be adjusted by mounting a silicon single-crystal cube either inside or on the edge of a loop and by varying the total length of the loop assembly. Attempts to attach silicon to loops using epoxy led to significant strain in the crystals upon freezing, causing the reflection profiles to broaden and thereby reducing the precision of the measurement. As a replacement for epoxy a small amount of Apiezon-T grease was used to lightly coat the loop before touching it to the silicon crystal. Apiezon-T (Apiezon Products, Manchester, UK; http://www.apiezon.com) is a silicone-free, medium-temperature hydrocarbon vacuum grease with very high sticking power. Results from this technique were very impressive. Reflection profiles remained narrow, typically 0.035–0.06° (FWHM), and in some cases the standard deviations in reflection centroids were as low as when measured with a silicon rod.

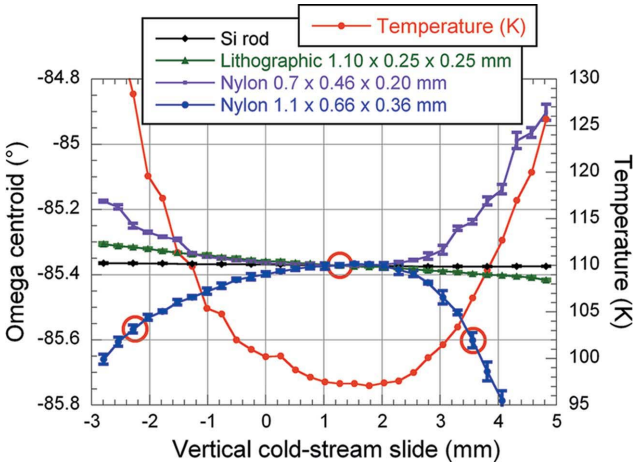

Figure 4
Reflection centroid profiles from silicon crystal cubes mounted in three different types of loops are shown as a function of vertical slide position. Baseline measurements from the silicon rod have been included in the plot for reference. Changes in centroid positions reflect changes in the force applied by the cold-stream. Individual scans from the three points circled on the 1.1 mm-long nylon loop curve will be displayed in Fig. 6 to illustrate more clearly the effects of force changes exerted by different parts of the cold-stream.

Fig. 4 shows the silicon rod baseline measurements (for reference) along with three different measurements of the vertical cold-stream force profile. Data from each loop have been normalized so their reflection centroids coincide at a vertical slide position of 1.27 mm. The dimensions of each loop are given in the plot legend as the combined length of the loop and loop stem, followed by the approximate dimensions of just the open area of the loop.

The lithographic loop is a 0.25 mm-diameter LithoLoop (product code MD7-137) made by Molecular Dimensions Inc. (Apopka, FL, USA; http://www.moleculardimensions.com) from 0.025 mm-thick Mylar. It is composed of three distinctive regions: a long stem ~0.4 mm wide, a short neck 0.08 mm wide by ~0.15 mm long, followed by the actual loop. The total length of the loop assembly from the top of the loop to the point on its base where it meets the stainless steel insert is 1.10 mm. Since the open loop portion of a LithoLoop is circular the dimensions of 0.25 × 0.25 mm represent the length and width of the circular opening. The actual width of the loop, not just the open area, is at least 0.050 mm wider owing to the thickness of the material used to construct the loop. Nylon loops from Hampton Research (Aliso Viejo, CA, USA; http://www.hamptonresearch.com) are made from 0.020 mm fibers. Because of the wrap-around nature of the nylon loop the open area approximates an oval instead of a circle. In all cases the loops chosen were purchased commercially, pre-mounted in stainless steel pins.

As can be seen from Fig. 4, the Si(220) reflection centroids from the lithograhic mount change gradually over the vertical range but give little indication of changes in cold-stream force. This lack of movement is due in part to the width of the stem, which is ten times greater than a twisted fiber nylon loop. The silicon crystal was sitting completely inside the loop and this added to the overall stability. Of course, this is good news for limiting sample movement in the cold-stream but not appropriate for creating a sensitive probe.

To increase sensitivity new silicon crystals were mounted on the edges of nylon loops. The 0.7 mm-long nylon loop data show a measure of sensitivity to the cold-stream force, with a constant reflection centroid position, *i.e.* constant force, from −0.3 to 2 mm. Outside this range the reflection centroids no longer maintain a constant position, indicating that the force of the cold-stream is changing. Note that the top of the cold-

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stream corresponds to the negative vertical slide region because the thermocouple is stationary during the measurements. These results indicate that the change in slope near the top of the cold-stream is much less severe than that at the bottom of the cold-stream. To increase the sensitivity further, the nylon loop assembly was lengthened to 1.1 mm, with an opening width of 0.36 mm. Reflection centroids change faster at both ends of the cold-stream with this loop, as do the error bars, indicating even more sensitivity to gas flow. The change in direction between the two nylon loops as a function of position (convex *versus* concave) is simply due to the direction in which the loop moved because of its orientation in the pin base. For our purposes it is the shape that matters.

Looking at the temperature profile in Fig. 4, it is important to remember that the nozzle alignment tool puts the cold-stream vertical height at V0.0. On the basis of the measurements using the 1.1 mm-long nylon probe, the temperature center at V1.27 is also the area where the reflection centroids are most constant. Neither nylon probe suggests vertical V0.0 as the best region. Furthermore, if a sample loop is sensitive to changes in cold-stream force, the most uniform region of force is probably from V0.7 to V2.0. For the shorter nylon loop assembly, the region extends about 1 mm further in the negative direction (to V−0.3) but only 0.25 mm further in the positive direction. Beyond the gas cold-stream temperature center at V1.27 the positive vertical slide positions represent an increasingly lower region of the cold-stream hitting the rotation axis center. It is this lower region that induces the greatest instability for both nylon loops. At V2.80 the force variation would be considered unacceptable, even though the temperature is 100 K. On the negative side of the plot, V0.0 is just on the edge of acceptability for the stiffer nylon probe and too far for the more flexible nylon. Although far from ideal, it is fortunate that the forces in the top of the cold-stream induce less disruption to the reflection centroid than the bottom of the cold-stream, since this is the default direction for cold-stream positioning when relying on the alignment tool.

Using the 1.1 mm-long nylon loop assembly as the force probe, force profiles in the horizontal and on-axis directions were then measured. Fig. 5 combines the measurements from these two directions with the vertical so that a direct comparison can be made. The 'optimal position' axis has been constructed so that each curve on the plot demonstrates what happens when the cold-stream is moved along each individual axis. The reference position of 0.0 mm is defined as the cold-stream position *after* making all the necessary offset corrections. This corresponds to individual slide settings of V1.27, H0.5 and NSD7.9.

Unlike the vertical profile, the horizontal direction is reasonably symmetric, with the uniform force region centered at a horizontal slide position of H0.5, slightly more positive than our H0.25 temperature center. The slope changes exhibited on both ends are steeper than was exhibited in the vertical direction. These force changes are accelerated because of the narrower width of the gas stream in the horizontal direction. On average, the corresponding horizontal standard deviations are twice as large as those measured over an equivalent vertical distance. Combined, these two directions form an area of constant force about 2.0–2.5 mm in diameter, or roughly one-third of the diameter of the 7 mm nozzle opening. The size of this area makes it essential that the cold-stream be aligned accurately in both the vertical and the horizontal directions.

With the vertical cold-stream offset corrected, there is relatively little change in force on going from a nozzle-to-sample distance of 5.9 to 7.9 mm. Beyond this point the probe registers a change in force that is essentially linear with distance and is significantly less than the changes measured in either the vertical or the horizontal direction.

After making the vertical offset correction it is anticipated that only a modest improvement in data quality will be achieved by moving the nozzle closer than would be suggested by the alignment tool. Using the average reflection centroid positions as a guide, the angular change measured between V0.0 and V1.27 was more than ten times greater than the shift determined between NSD5.9 and NSD7.9. While some improvement may result by moving the nozzle closer to the sample, a much larger gain was achieved by correcting the vertical offset.

Although error bars are presented on the plots to indicate changes in centroid positions, this representation cannot accurately convey the effect that different areas of force inside the cold-stream have on the sample. Reflection profiles from the silicon rod give an excellent representation of what the silicon cube reflection should look like if no sample motion is present. In reality, the cold-stream force can cause the crystal to twist, vibrate or do both at the same time. Using data from the 1.1 mm nylon loop assembly presented in Fig. 4, three (circled) positions have been selected to show actual data instead of the resultant error bars. The outer points were chosen because they provide a good visual representation of the difference in force at the top (V−2.29) and bottom (V3.55)

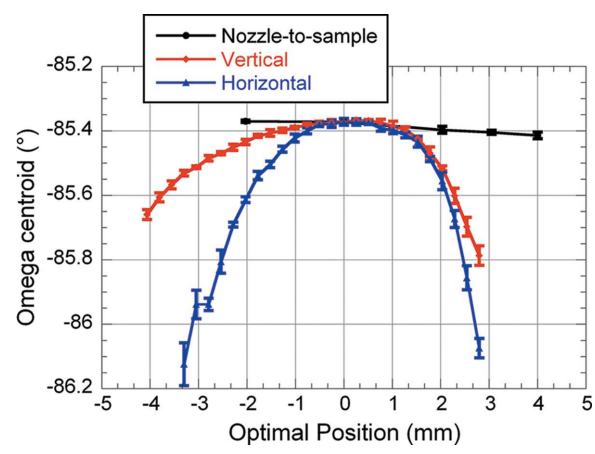

Figure 5
Combined force profile of the cold-stream in the vertical, horizontal and on-axis directions. Profiles from each direction were measured using the 1.1 mm nylon loop silicon probe. Changes along the plot *x* axis reflect how the force changes in each direction. The reference 0.0 mm point on the plot corresponds to (V1.27, H0.5, NSD7.9). On the basis on these measurements, the cold-stream produces an area of constant force 2.0–2.5 mm in diameter or about one-third of the width of the nozzle opening.

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of the cold-stream. Fig. 6 shows the overlapping five scans repeated for each measurement position; data from the silicon rod have been included for the purpose of comparison. Each region is isolated on the plot for clarity and labeled with the vertical slide position followed by the corresponding temperature at that position.

It is tempting to think of reflections from a crystal as having similar rocking curves or at least profiles that follow mosaicity as the crystal moves through the diffracting condition. As Fig. 6 shows, wildly different output can be measured for the same reflection depending on the flexibility of the loop and its position within the cold-stream. The profiles at V1.27, owing to the sensitivity of our probe, already have a standard deviation ten times that of the silicon rod. Even so, the shapes of the curves are reasonably consistent. At V−2.29 (125 K) the shape begins to break up, with small satellite peaks easily visible. At V3.55 (107 K) there is no longer any comparison to the shape of the original reflection. Instead, we get what appears to be a rapidly vibrating crystal moving in and out of the diffracting condition.

If temperature were the only variable to consider when aligning the cold-stream and the choices were between 125 and 107 K, it is obvious that the colder temperature would be advantageous. What Fig. 6 shows is that temperature is not the only factor to consider. This is particularly important if the cold-stream has an asymmetric temperature profile. When we have seen temperature asymmetry the bottom of the cold-stream has always been the area with the coldest temperature. As this study shows, the bottom of the cold-stream is capable of inducing large vibrations in the sample. To avoid this it is important to choose the temperature midpoint when aligning the cold-stream, not the coldest temperature.

4. Force and lysozyme data

To investigate how the cold-stream properties affect actual protein crystallographic data, tetragonal hen egg-white lysozyme (space group $P4_32_12$, $a = b = 78.87$, $c = 36.84$ Å) is the crystal of choice because it is reasonably radiation resistant and can be cryocooled with a relatively low mosaic spread. In the plots that follow all data sets were collected at 12.66 keV using a single pass scan and 1° frames exposed for 1 s each. To limit radiation decay, the incident photon flux was attenuated to $\sim 3 \times 10^{10}$ photons s^{-1}. Each point on the plot represents a complete data set of 90–100° processed to a resolution of 1.5 Å using *HKL-3000* (Minor *et al.*, 2006); the final linear $R_{\rm merge}$ is reported without rejecting any reflections. The mosaicities of all lysozyme crystals studied varied between 0.2 and 0.4°. To impose real world limits, the maximum temperature of any data set collected in the vertical and horizontal direction was 110 K; these temperature limits correspond to the endpoints of those scans. All lysozyme data sets were collected at a vertical setting of V1.27 unless otherwise stated.

Since each crystal was used for multiple measurements, radiation damage is inevitable. Before each series we always collected a data set at the (V1.27, H0.0, NSD7.9) position, *i.e.* the raised vertical setting and the alignment tool position in the horizontal and on-axis directions. Multiple data sets at the same position are plotted along with the data for the entire scanning direction. Data sets were collected from left to right on the plot, with the final data set re-collected at the starting reference position. In many cases the data sets overlap, leading to what looks like a single point on the plot. Although present in the data, radiation damage does not play a significant role in determining the outcome of the areas of force in these measurements. The change was simply not enough to alter the results. However, since it is present we caution the reader to take information from each crystal on each plot as a distinct series and not try to compare linear $R_{\rm merge}$ absolute values from the same crystal in different directions.

Fig. 7(*a*) shows the statistics from data sets collected at different vertical cold-stream positions using two crystals mounted in nylon loops plus two crystals mounted in loops reinforced on the stems with Apiezon-T grease. The non-greased loops will be discussed first. For the 1.67 mm-long nylon loop assembly, a measurable amount of radiation damage occurred during the measurement. Although difficult to see on the plot, there are actually three data sets collected at V1.27. The linear $R_{\rm merge}$ values for the three data sets at the reference position in order of their collection are 0.056, 0.058 and 0.063.

As is evident from the shape of the curves, the impact of the cold-stream force is strongest near the bottom of the cold-stream, in agreement with our silicon measurements. For the most sensitive (1.67 mm-long) nylon loop assembly, a decrease in data quality is apparent at the V0.0 (alignment tool) position and beyond in the negative direction. For the stiffer 0.85 mm-long loop assembly, the V0.0 position shows virtually no change from the V1.27 position and only a slight decrease in data quality at the negative extreme. At the positive extreme the 0.85 mm-long nylon loop assembly shows a distinct decrease in data quality compared with the V1.27 position. It is worth repeating that the temperature of the

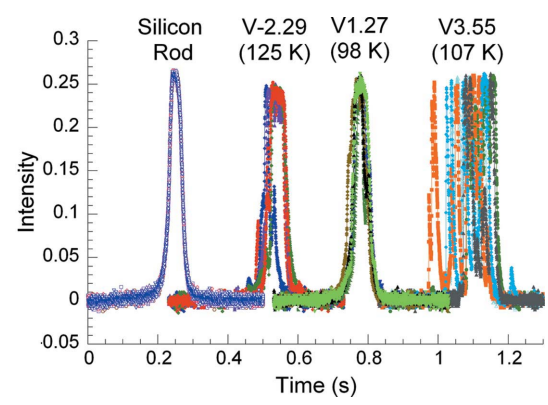

Figure 6
Oscilloscope scans (five each) from the silicon single-crystal force probe at V−2.29, V1.27 and V3.55 positions taken from the 1.1 mm nylon loop positions circled in Fig. 4, along with reference scans from the silicon rod. The temperatures corresponding to each vertical position are also listed. Evidence of changes in the force away from center is shown by the breakup of the reflection profiles. Changes in the cold-stream force at V3.55 are enough to completely eliminate the single peak profile, even though the temperature is below 110 K.

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cold-stream at V2.8 is 100 K. This position, based on our knowledge of the cold-stream force, would be completely unacceptable for routine operation but the distinction might be lost if temperature alone were used as a criterion for setting the cold-stream position.

Fig. 7(*b*) shows two crystals mounted in nylon loops as the horizontal cold-stream position is changed. Data quality degrades quickly as the cold-stream moves away from the horizontal center, with the changes roughly symmetric about H0.0. Careful examination of Fig. 7(*b*) shows that the data are not exactly centered at H0.0 but at a slightly more positive value. The symmetry and slightly positive center position are in good agreement with the silicon results.

Fig. 7(*c*) shows data collected while varying the nozzle-to-sample distance. It is clear that there is a force change upon moving farther from the sample. The magnitude of the change in the recommended 5–8 mm range is generally small, as seen with the silicon results. Beyond this distance the amount of change depends largely upon the stiffness of the loop.

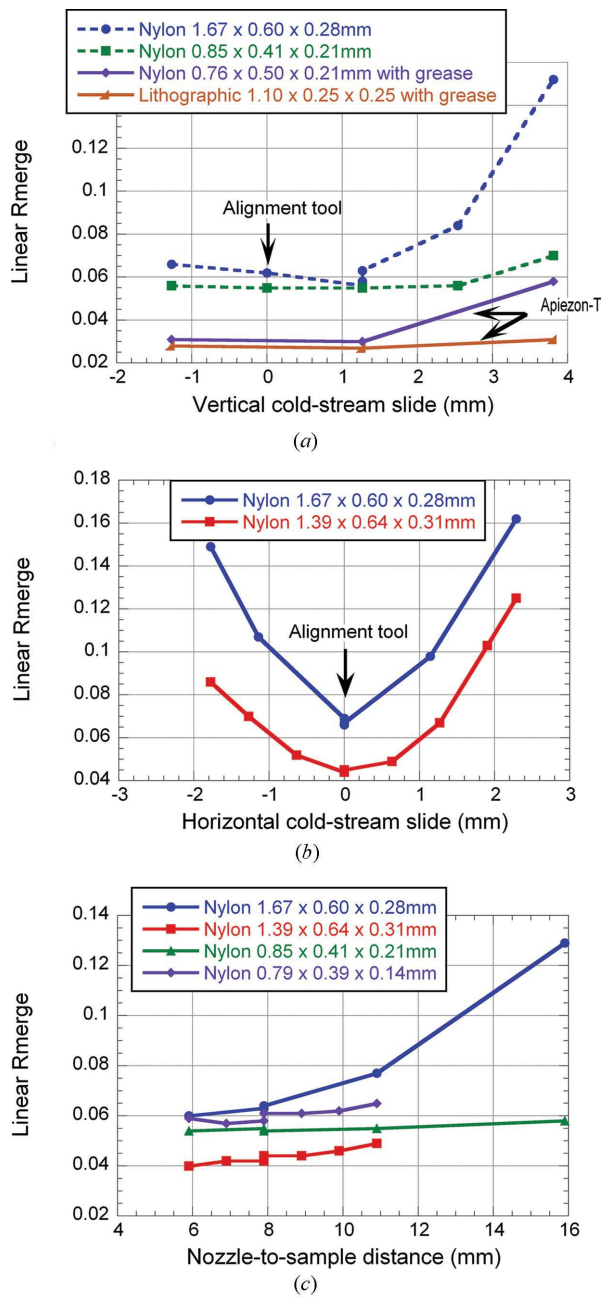

Figure 7
Linear R_{merge} for lysozyme data sets taken as the cold-stream position is changed in the (*a*) vertical, (*b*) horizontal and (*c*) on-axis directions. As shown in (*a*), adding grease to the stems of two loops before mounting the crystals (solid curves) helped reduce loop motion and improved data quality even in the areas of high force.

5. Loop motion

The cold-stream would have little direct impact on data quality if the sample loops were completely rigid. Obviously they may not always be motion free so the question becomes 'Can I tell if motion is present from the data?' In fact, the answer is no, you cannot tell explicitly if loop motion is causing a problem with the data. If, however, you make some reasonable assumptions, such as the beam is stable, the instrumentation is functioning properly and the crystal is of good quality, *e.g.* there is no twinning and the crystal has not suffered excessive radiation damage during the experiment, then you can use the data as a reasonable guide to detect loop motion.

The 1.39 mm-long nylon loop assembly curve in Fig. 7(*b*) shows a somewhat symmetric profile of linear R_{merge} away from center. *HKL-3000* output from scaling produces a normalized χ^2 *versus* integrated intensity plot. Results from the χ^2 plots for all data sets in the curve were collected and overlaid in Fig. 8. The linear R_{merge} value for each data set along with the respective horizontal position is provided next to the corresponding plot symbol in the legend.

The lowest R_{merge} corresponds to the flattest profile, *i.e.* the smallest χ^2 variation over the full range of intensities. As the linear R_{merge} increases, the normalized χ^2 increases. Because the data are from the same crystal there is a direct link of R_{merge} with the shape of the profile. Deviation from a flat profile indicates some intensity-related problem in the data,

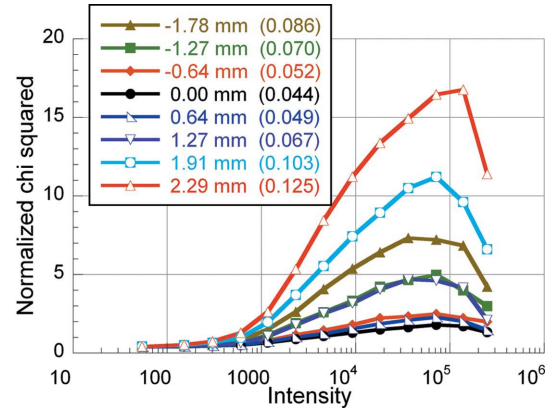

Figure 8
Normalized χ^2 values from the 1.39 mm-long nylon loop data sets collected in Fig. 7(*b*). The horizontal cold-stream positions and corresponding linear R_{merge} values for the data are listed in the legend. Because these data are all from the same crystal, larger deviations from linearity correlate with an increasingly higher R_{merge}.

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and for loop motion this deviation is usually most pronounced in the high-intensity region. Assuming all instrumental factors are under control and the data are processed with the correct space group, loop motion is the likely reason for the increased (nonlinear) χ^2.

Using data in Fig. 8 as a guide, the impact of cold-stream misalignment can be described more quantitatively by looking at the number of reflections marked for rejection between the data sets at the two extremes. R_{merge} for data taken at H0.0 is 0.044; the highest R_{merge} is 0.125 produced at H2.29. The number of reflections marked for rejection is, respectively, 146 and 6282. For lysozyme data at 1.5 Å resolution, this may not produce a significant impact on structure solution. However, increasing the number of rejections by more than a factor of 40 is a clear indication that cold-stream placement should be attended to carefully.

Assuming loop motion is a persistent problem, can anything be done to reduce it? To answer this question a silicon single crystal was mounted in a short nylon loop assembly and a separate silicon crystal was mounted in a lithograhic loop with a slightly larger opening. Data were collected starting at (V1.27, H0.0, NSD7.9) followed by an increase in vertical cold-stream position, thus placing the samples into the high-force region of the cold-stream. Results of these measurements are presented in Fig. 9. Both loops show a high degree of stability at the starting position, where the cold-stream should be positioned, indicating that *both* loops would be appropriate for routine data collection. As the cold-stream moves up, both loops show some variation in position with changes in cold-stream force. Owing to possible differences in response related to how these crystals were mounted in the loops, these measurements act as a baseline for the next step where grease is added to the loop stem in an attempt to reduce loop motion.

Silicon crystals attached using Apiezon-T grease showed remarkable stability during these measurements. For protein samples, the stability of a particular crystal on any given loop depends on the loop length and width combined with the rigidity of the loop stem. Loop stem reinforcement may occur as a natural process when the solvent surrounding the crystal covers the loop material and cools along with the crystal when picked up, or it may be the result of a conscious effort to reinforce the loop stem with something, such as epoxy, before scooping up a crystal. Given that Apiezon-T appears to freeze solid at low temperature but is relatively gel-like at room temperature, Apiezon-T was applied to the already tested loops used in Fig. 9. Once the non-grease measurements were made, each loop was removed from the cold-stream, the loop stem was coated with Apiezon-T while the crystal remained in place, and then the loop was returned to the cold-stream. In the case of the nylon loop, grease surrounded the stem all the way down to the attachment point of the pin and stopped just below the start of the loop. On the LithoLoop, the front and back sides of the 0.4 mm-wide stem base were coated, again down to the attachment point of the pin, but the short neck below the loop was left uncoated. These results are also shown in Fig. 9. While the coating process is not very precise, the results do show a marked decrease in loop movement for the nylon loop. The grease-coated LithoLoop also shows a flatter profile that extends much farther into the high-force areas of the cold-stream than before.

For crystallographic verification, two loop stems were coated with Apiezon-T grease in a similar manner before mounting lysozyme crystals in them to see what impact the added stability might have. Fig. 10(*a*) shows the as-coated nylon loop stem and Fig. 10(*b*) a side view of the lithographic loop. The uncoated region of the lithographic loop corresponds to the short neck portion of the loop stem.

Returning to the lysozyme crystal curves labeled Apiezon-T (Fig. 7*a*), both loops showed virtually no change on going from V1.27 to the negative limit of the range. In addition, the magnitude of the R_{merge} value at the V1.27 position is significantly lower than the respective values from the non-greased nylon loops. This represented an improvement for the nylon loop but was not totally unexpected for the LithoLoop on the basis of the previous silicon measurements. At the positive end of the range the nylon loop still shows significant motion, presumably because the nylon is still able to flex at the joint where there is no grease coating. LithoLoop data showed the smallest increase in R_{merge} of any loop at the end of the positive range, but there was an increase nevertheless. This

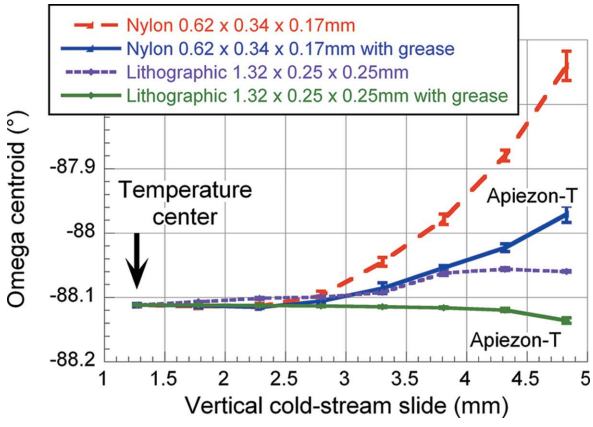

Figure 9
Silicon crystal cubes mounted in a nylon and lithographic loop before (dashed curves) and after (solid curves) adding Apiezon-T grease to the stems. Reinforcing the stems improved stem rigidity, leading to smaller deviations in reflection centroid positions, although both materials still exhibited flexibility in the area of highest force.

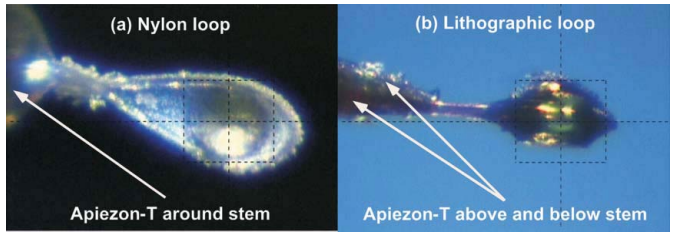

Figure 10
(*a*) Lysozyme crystal mounted in a nylon loop with Apiezon-T grease surrounding the length of the stem starting just below the loop. (*b*) Side view of a lysozyme crystal mounted in a lithographic loop coated with grease on the wide portion of the stem; the short stem neck was left uncoated. The dimensions of the crosshair boxes are 0.20 × 0.20 mm.

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says that the LithoLoop is also capable of flexing at low temperature.

Because both lysozyme crystals are mounted in a similar fashion, it appears that the LithoLoop design is more rigid in the high-force region of the cold-stream than a nylon loop assembly with even slightly smaller loop dimensions. However, if the cold-stream is aligned properly, *i.e.* positioned at V1.27, both greased reinforced loops appear rigid and produce excellent quality data.

6. Discussion

There is one more important piece of information obtainable from the lysozyme data and it goes to the heart of all protein crystallographic measurements. Because the lysozyme crystals are of good quality and the beamline is functioning properly, the only unknown is assumed to be whether or not crystal motion is present. Using the normalized χ^2 *versus* intensity plots, the lysozyme data sets can be checked for optimal data quality.

In this comparison only data collected at the position (V1.27, H0.0, NSD7.9) are considered. These data sets are the very first ones collected from each crystal so there will be minimal radiation damage. The magnitude of the linear $R_{\rm merge}$ from each data set, therefore, becomes directly comparable. If the normalized χ^2 curves are nonlinear, then it is likely that loop motion was the reason data quality was degraded.

Fig. 11 shows the statistics for data sets from five lysozyme crystals. In the normal *HKL-3000* plot the scale usually ranges from 0 to 10 instead of 0 to 3, so much smaller deviations are emphasized than would be the case for routinely processed data. The three dashed curves show significant nonlinear behavior, indicating that some amount of loop motion was present. The two solid curves, which represent Apiezon-T-greased loops, are quite flat, indicating that loop motion was probably not present in the data. As might be expected, data from the crystals that do not show evidence of loop motion produce the lowest linear $R_{\rm merge}$ values.

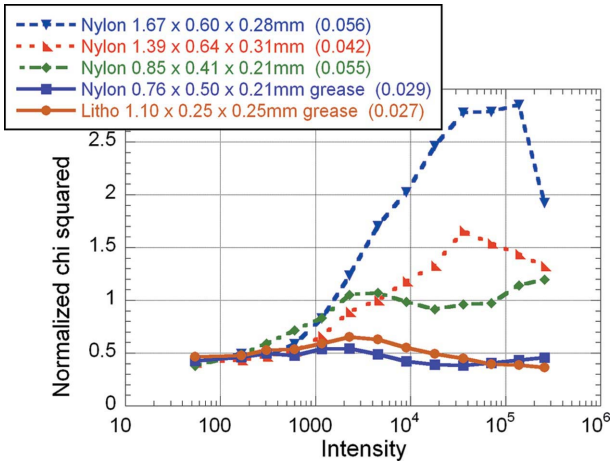

Figure 11
Normalized χ^2 values *versus* intensity for the first data set collected from each of five different lysozyme crystals. Linear $R_{\rm merge}$ values are listed beside the crystal identifiers in the legend. Nonlinear (dashed) curves indicate loop motion is present. Data from crystals with grease-reinforced stems (solid curves) are essentially flat, indicating loop motion is probably not present.

The 0.76 mm greased nylon loop is very close to the dimensions of the ungreased 0.85 mm nylon loop, so the addition of grease to the stem significantly lowered the overall motion of the loop. There is no ungreased LithoLoop with which to compare, so it is unknown if the LithoLoop would have performed the same way in the ungreased state. LithoLoops have shown good stability, on the evidence of the silicon results, under normal conditions. However, it is impossible to say anything more about the greased LithoLoop other than that the data were of excellent quality and showed no obvious signs of loop motion.

On the basis of the silicon and lysozyme measurements, the cold-stream temperature center is a good indicator of the optimal position for the cold-stream. This location gives the most consistent force profile and, ultimately, the highest-quality data. Given that many beamlines using commercial cold-streams rely on the alignment tool to center the gas stream and that thousands of structures are solved all over the world, it is tempting to dismiss our vertical 1.27 mm offset as an anomaly.

The temperature profiles for five other cold-streams were also analyzed: two Cryostream 700s and three CryojetXLs (Oxford Diffraction Ltd, Oxfordshire, UK). The tilt angles for these devices ranged from 42 to 64°, with temperature settings all fixed at 100 K. The nitrogen gas sample flow rate was 5.0–7.4 l min^{-1} and the warm air stream flow ranged from 4 to 10 l min^{-1}. The width of the cold-stream was again taken as the distance between 110 K thermocouple readings. For the 19BM cold-stream the distance from the vertical alignment tool position to the temperature center was 1.27 mm and the total width of the cold-stream was 5.12 mm, yielding an offset of 25%. Expressing the offset as a percentage helps to normalize out the different angular settings of the cold-stream heads.

The Cryostream 600 and 700 operate in only two flow rate modes: in normal operation mode at 5 l min^{-1} and in turbo mode at 10 l min^{-1}. The flow rate was set at the normal operation mode for these units. The Oxford Diffraction CryojetXLs have controls that allow alterations in the flow rates. However, because each of the cold-streams was in use at a specific beamline at the time of measurement, flow rates were kept as they were during normal operation.

Using the alignment tool provided by each manufacturer to set the initial position, vertical offsets for this group of five cold-streams ranged from 20 to 37%. All cold-streams were offset in the same direction as described above. The range of flow rates encountered suggests that flow rate, as regards temperature, does not have a significant influence on the vertical offset. Measurements on most of these cold-streams showed reasonable alignment from a temperature perspective in the horizontal direction. However, in at least one case the horizontal profile was displaced by 0.75 mm from the alignment tool position. Even though the Cryostream 600 in this study was reasonably symmetric in the horizontal direction,

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the data were sensitive to a 0.5 mm misalignment. When possible, it is recommended that any misalignment, horizontal or vertical, should be corrected before starting operations.

It is tempting to speculate on why the offset occurs at all. These devices are designed to provide laminar flow and should be uniform across most of the nozzle opening. Gravity most likely plays an important role, but there may also be design issues common to both manufacturers. In the end, we cannot say why the offset occurs, only that it does and should be accounted for if the cold-stream is operated within the conditions described in this study.

Compared with cold-streams mounted at a relatively steep angle, such as those in this study, devices mounted horizontally, parallel to the spindle, may induce less sample motion because of the reduction in sample cross section afforded by the head-on flow direction, even though there may not be any significant change in the cold-stream force profile. Although a horizontal orientation was not examined in this study, we believe it would still be prudent to measure the temperature profile for a cold-stream in any orientation, including horizontal, before establishing the final cold-stream position.

One of the best ways to ensure an accurate cold-stream placement is to use the manufacturer's alignment tool for initial placement, then proceed with the required offset adjustments. These tools are well matched to the cold-stream nozzle and the positioning is reproducible to within 0.05 mm if care is used in orienting the locking screw the same way each time the tool is placed on the cold-stream nozzle.

For the cold-stream to perform its intended purpose it must be mounted with great precision and, equally important, good rigidity. The lever arm of aligning the cold-stream nozzle extends beyond the cold-head to include the support structure. It is, therefore, extremely important to use good quality, rigid components. Deviations in position can potentially have a large impact on the sample, particularly when these devices are subjected to rapid movement back and forth along the translation slides under robotic operations. Short translation slides and flexible mounts do not add to the stability of the cold-stream head and can lead to instantaneous as well as long term changes in cold-stream position. Because of the importance of the cold-stream in the experiment it should be considered a high-precision device and mounted with no less precision and rigidity than any goniometer table or slit system.

At the Structural Biology Center many data sets are collected using both nylon and lithographic loops. Many nylon loop data sets are of high quality and show exactly the type of normalized χ^2 profiles consistent with no loop motion. After all, to meet the needs of this study the nylon probe was tailored for increased sensitivity by placing the silicon crystal on the edge of the loop, a condition that would never be used in practice. It was also demonstrated that 0.25 mm-diameter LithoLoops are of excellent quality. However, there are a wide range of LithoLoop sizes and other types of lithographic loops, such as those made by MiTeGen (Ithaca, NY, USA; http://www.mitegen.com), that have not been analyzed in this way. For this reason it would be inappropriate to conclude that all nylon loops will exhibit motion or that all lithographic loops are completely rigid, even with the cold-stream positioned at its optimal setting. Some lithographic loops are certainly capable of significant motion and there are many styles of loops available with differing characteristics.

In making recommendations about loops a common sense approach should be followed. Loops should be kept as short as possible and as small in diameter as possible for the greatest amount of stability. If nylon loops are used, we recommend trying to add support to the loop base when practical, either using epoxy or Apiezon-T, since this grease appears to work well in stabilizing loops. Other types of Apiezon grease are available and these have very different characteristics; the correct type should be used.

Using data processing statistics as a guide, known crystals in different loops can be tried to see which loops work best for the particular application. With large diameter loops it is likely, but as yet untested, that the lithographic loops will have an advantage. At some point, however, the overall size of the loop may simply be too large to withstand the force of the cold-stream. When the broad face of the loop is perpendicular to the cold-stream the potential for motion is far greater than when the wide area is parallel to the cold-stream. Certainly in these cases a slower cold-stream flow rate will be less forceful and, therefore, less likely to induce sample motion. Ultimately, the decision on what loop to use depends on the end user. On the evidence of the results presented here, data quality will improve with any type of loop if the cold-stream is properly centered before data collection begins.

7. Conclusions

We have investigated the temperature and force profiles for an Oxford Cryosystems Cryostream 600. Our results indicate that the cold-stream alignment tool can only be relied upon to provide an accurate reference position for the nozzle opening. In order to center the cold-stream gas on the rotation axis it is necessary to map the temperature profile. The temperature center, based on force measurements, is a good place to position the cold-stream. Temperature profiles for different model cold-streams and those from one other manufacturer all showed offsets that would require corrections before use for optimal performance.

The alignment tool usually positions the cold-stream near the center of the horizontal temperature profile. However, measurements on other cold-streams indicate that it would be good practice to measure the temperature profile in this direction as well, particularly since the force of the cold-stream changes rapidly away from the center. The shortest distance between nozzle and sample gave the lowest linear $R_{\rm merge}$, but the force change within the recommended 5–8 mm range was small once the vertical offset had been corrected.

Assuming that the beamline optics and the X-ray source are performing within specifications and the sample is of good quality, processing statistics can be used to look for evidence of loop motion. Loops stems should always be kept as short as

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possible to reduce motion and, when practical, reinforced with epoxy or grease to increase rigidity.

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