COMMENTARY

Expanding the femtosecond crystallography toolkit

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The "21st century of biology" is built on advances in protein structure determination developed over the last five decades. Exponential growth in the number of determined protein structures has largely been driven by new technologies in protein X-ray crystallography, including synchrotron X-ray sources, X-ray detectors, computational tools, and methods of handling protein crystals. In consequence, the time to acquire a routine, complete crystallographic dataset has gone from years to minutes. However, many important macromolecular structures are still undetermined and many challenges remain, especially in cases where crystals are either hard to obtain, particularly sensitive to X-ray damage, or exhibit transient states that are difficult to capture by structural analysis. The latest tool in the crystallographic arsenal, the X-ray free electron laser (XFEL) (1), promises to help surmount these challenges (2), provided efficient ways are found to handle crystals in the demanding XFEL environment. In PNAS, Cohen et al. (3) show that crystal handling technology developed primarily for use at synchrotron storage ring X-ray sources can also be effectively adapted for use at XFELs.

Short-wavelength XFELs suitable for crystallography have only become available since the Linac Coherent Light Source turned on at the Department of Energy's California SLAC laboratory in 2009. The relevant difference between storage ring and XFEL sources is that storage ring sources deliver many low-intensity X-ray pulses per second, whereas XFELs do the same with a much smaller number of ultra-short pulses that are each millions of times as intense as storage ring pulses. The result is that the XFEL can deliver roughly as many X-rays in a single tens-of-femtoseconds duration pulse as a storage ring source delivers over the course of a second. At the LCLS this occurs 120 times per second.

A femtosecond is truly a short period. If all of the energy of, for example, a 10-keV X-ray were magically and instantaneously imparted into kinetic energy of a protein carbon atom, the atom would only travel a few angstroms in a femtosecond. In fact, the energy transfer mechanism is slightly slower. If the X-ray beam is focused to micrometer sizes to hit a protein microcrystal, so many electrons are photo-ejected quickly enough that the remaining positively charged crystal blows itself apart via Coulomb repulsion; this takes some tens of femtoseconds. However, the X-rays, moving at the speed of light, can outrace the Coulomb explosion. The result is that the X-rays in the first few tens of femtoseconds of the X-ray pulse have already been diffracted and left the crystal before the atoms in the crystal have had a chance to move appreciably. This is the basis for the term "femtosecond crystallography" (FSC), also known as "diffract before destroy" (4). Thus, although each illuminated microcrystalline region in a highly focused XFEL beam is destroyed by the first X-ray pulse it sees, the diffraction pattern is of the state before the explosion event.

The problem is that it isn't easy to hit a crystal the size of a bacterium with an equally small X-ray bullet moving at the speed of light, especially if crystals have to be positioned within 1/120th of a second. Pioneering FSC experiments (5) used stochastic positioning: microcrystals were streamed in a straight line that intersected the path of the X-ray beam with the hope that a crystal would occasionally be in the right place at the right time. The probability of hits increases with the number density of crystals, but if this number is too high then two crystals might be next to one another and the combined diffraction is very confusing.

Clever stochastic microinjectors were developed at Arizona State University, in which crystals dispersed in a water solution were ejected as a liquid jet or microdroplet stream a few microns in size moving at a few meters per second (Fig. 1) (6, 7). Most of the protein was wasted because only a few percent of the crystals were actually hit. Another pioneering method involved extrusion of a column of lipidic phase gel studded with membrane protein microcrystals. The gel column was a few microns wide and was extuded so as to intersect the X-ray beam (8). This

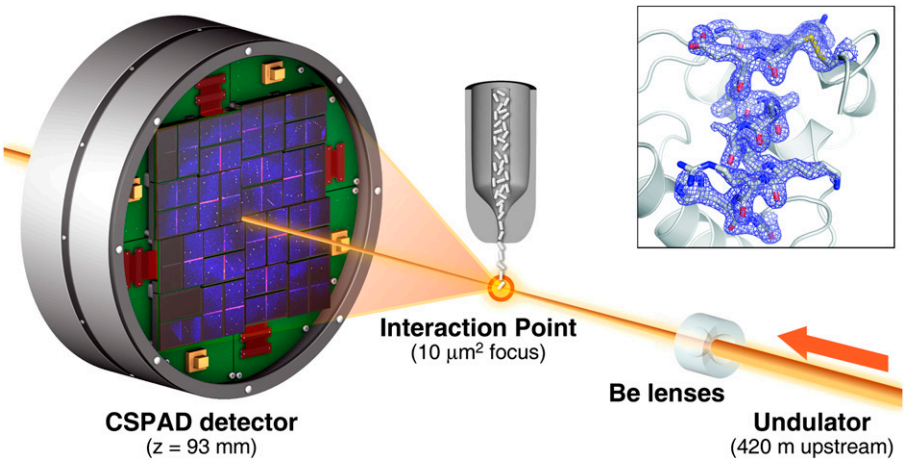

Fig. 1. Schematic of a pioneering femtosecond crystallography experiment performed at the Linac Coherent Light Source. XFEL pulses incident from the right are focused by beryllium lenses onto a stream of lysozyme microcrystals in a fast-flowing liquid jet (5). The diffraction pattern from a hit crystal is recorded on the Cornell-SLAC pixel array detector (CSPAD) at the left. The entire experiment proceeds in vacuum with pulses arriving at 120 Hz. The inset shows a refined electron density map at 1.9 Å resolution. Modified with permission from ref. 5.

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"toothpaste" extrusion method is more conservative of protein because the gel column can be extruded more slowly. In both cases, however, these methods rely on random position and orientation of the microcrystals, so they consume more protein than would be required if crystals could be systematically prepositioned for near unity hit probability. This can be a severe limitation if the supply of protein crystals is limited. Wasted hits also consume XFEL beam time, which is a problem because proposals for beam time greatly exceed availability. Given that XFELs are expensive and take many years to build, beam time oversubscription will be a problem for at least much of the remaining decade.

A second problem with FSC is that complete datasets require measurement of the Bragg peaks in a desired volume of reciprocal space. However, by Bragg's law, only those parts of peaks that intersect the Ewald sphere diffract for a given orientation of crystal. Typically, the widths of the Bragg reflections are wider than the thickness of the Ewald sphere, so the diffracted intensity is only partially representative of the full integrated Bragg spot (9). In standard crystallography this is solved by oscillating the crystal through a small angle (e.g., a few tenths of a degree) over the course of the measurement so the Bragg peak passes fully through the Ewald sphere at a uniform rate. However, in FSC, the crystal is effectively stationary over the time of diffraction; thus, Bragg peaks are only partially measured, greatly complicating the interpretation of the data.

Cohen et al. (3) address the obvious question: Can the highly refined goniometer-based procedures developed for crystal manipulation at storage rings be adapted for use at XFELs to help overcome the aforementioned problems? Over the years very sophisticated goniometer positioners and software tools have been developed to ease the task of positioning microcrystals in storage ring microbeams. Cohen et al. demonstrate that the storage ring toolkit can, indeed, be so adapted and used in a variety of ways to expand the data collection options for FSC. Examples include the goniometer-mounted microgrid, a thin strip of material with a regular array of small holes. The grid can be preloaded with one crystal per hole. Control software has been developed to allow the user to program the exact sequence of rapid positional and angular movements of the grid so that one crystal at a time is exactly in line for a given X-ray pulse. Another possibility that is enabled is raster data collection from crystals spread onto various kinds of mounting substrates, again using control software in use at the SLAC storage ring. One can also program step-wise helical and other types of data-collection strategies for larger crystals, wherein the illuminated part of the crystal is destroyed but a new part of the crystal is maneuvered into the beam with each pulse. Another useful strategy is to expand the X-ray beam focus to cover a large area, thereby decreasing the X-ray power density to nondestructive levels. This strategy can be combined with oscillations of cryo-frozen crystals to help resolve issues of partial reflections and indexing ambiguities. The end results of this toolkit are a reduction in the number of crystals and time required to do an experiment, as well as access to methods to resolve difficulties of data interpretation.

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The situations where these methods are likely to prove to be most useful are for problems that span the interface between what can be done at storage ring and XFEL sources. A good example is study of the structure of metalloproteins that are especially susceptible to radiation damage chemistry. These metalloproteins are difficult to fully analyze crystallographically at storage ring sources. FSC easily outraces the damage mechanism. One can envision experiments that combine low-resolution structure determination of small metalloproteins at storage ring sources with FSC determination of the details around the critical metal sites. Because XFELs are typically sited in proximity to storage ring sources, one might envision an experimental proposal that uses both types of sources with a common user interface. Another example is probing very fast time-resolved changes in photoproteins, where the basic static structure is determined at the storage ring sources and the time-resolved perturbations are performed at the XFEL.

These are exciting times for the biocrystallographic community. To be sure, XFELs have opened new possibilities in time-resolved structure determination and microcrystallography. However, XFEL developments have also catalyzed a surge of new ideas at storage ring sources. For example, a microcrystallography XFEL experiment on crystals grown in vivo in bacteria (10) was quickly and innovatively also performed at the PETRA III storage ring source (11). Both types of sources are still undergoing rapid technological advances. Seeded XFELs are coming (12), as are diffraction limited storage ring sources (13). It is unclear which will prove to be more capable for a given type of experiment. Combining the best features of both, as Cohen et al. (3) have shown, is the most desirable and likely outcome.

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