APS Renewal: Macromolecular Crystallography Subgroup-

Executive Summary-

Structural biology has made a fundamental impact on the understanding of biological organization, function and mechanism at the molecular level. Although techniques such as NMR and microscopy have contributed significantly in transforming biology from a descriptive science into a molecular-based one, it is X-ray crystallography that has made by far the largest contributions. Its impact on biology can be traced directly to a set of technical advances made over the last decade. Notable among these are MAD phasing techniques, cryocrystallography, enhanced computing capabilities and a broad range of new structure refinement techniques. Additionally, advances in molecular biology and biochemical techniques have been instrumental in the structure determination process by providing an ample supply of highly pure protein samples.

Nevertheless, the impact on structural biology of all these advances in aggregate pales in comparison to the impact of synchrotron radiation. Put bluntly, structural biology would still be a cottage industry without synchrotron X-ray sources. Approximately 50% of all users of US synchrotron facilities are life science researchers and the majority of these scientists are macromolecular crystallographers. More than 80% of all macromolecular crystal structures deposited to the PDB during 2007 were determined from diffraction data collected at a synchrotron source, a total of more than 5500 structures during that year alone (http://biosync.rcsb.org/BiosyncStat.html).

In some sense, the experimental infrastructure to perform high-level macromolecular X-ray crystallography at synchrotron sources (and at the APS in particular) is mature. However, our subgroup identified two areas of R&D that would have a transformative effect on studies of complex structures and elucidating relationships between structure-dynamics and function. The first area is micro X-ray crystallography where the objective is to develop methodologies that enable the study of microcrystalline samples. Since large, well-ordered crystals remain hard to obtain, this would enormously increase both the number and types of systems that can be studied. Thus, we concur with conclusion reached by a subcommittee of the Biological and Environmental Research Advisory Committee of the Department of Energy that addressed questions relating to synchrotron structural biology resources (http://www.sc.doe.gov/ober/berac/SMB_Report.pdf). A conclusion of the subcommittee was:

> *"One area of special note for macromolecular crystallography is the need for beamlines/instrumentation optimized for microcrystal and microbeam studies."*

A second area of significant opportunity is high-resolution time-resolved crystallography, which involves proteins that can perform chemical reactions in crystals. This is a very specialized field and requiring sophisticated instrumentation and software that is tailored for the time-resolved experiments. Nevertheless, it is uniquely powerful in its capability to produce high-resolution time slices of structural transitions along reaction pathways. These data provide a vital connection between structure/dynamics and their effects on function. This represents a major unmet need that has greatly limited our understanding of structure-function linkages in biological processes in general. Additionally, it can provide an experimental framework to guide computational efforts to

model conformational changes occurring during biological processes. We note that the ability to use microcrystals for these time-resolved experiments is also important. Since these experiments are initiated by introducing a stimulus like light, temperature, pH, etc. to commence the reaction cascade, it is important that as many molecules as possible undergo reaction in the crystal. The efficiency of reaction initiation is highly dependent on crystal shape and volume; typically, the smaller the crystal, the more rapidly and the more uniformly can the reaction be initiated. Thus, the two areas of microcrystals and time-resolved studies are in part linked.

To realize these objectives it will be essential to develop technologies on two fronts. First, micro-beams on the order of a few micrometers will be needed. However, this is technically challenging because of present limitations in X-ray beam stability at the APS. Beam stability is not an issue confined to micro X-ray crystallography; solving this problem has important ramifications for a broad range of experiments and proposals have been put forth to deal with it. Second, a new generation of pixel array detectors will need to be developed with much faster readout times. Overlaid on these two major objectives is a set of complementary requirements. In order for micro X-ray crystallography to be practical, significant improvements in automation for crystal mounting and positioning have to be achieved. For time-resolved crystallography, ultra-fast X-ray pulses would have an impact by enhancing the time resolution that can be achieved.

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Micro X-ray crystallography: Opportunities and Issues

A major misconception is that the principal bottleneck to solving high profile structural biology problems is that crystals cannot be obtained. While this is certainly an on-going issue, the fact is that for many problems microcrystals can be obtained, but they are not of a size adequate to acquire excellent data using current technology. The problem of small crystals is especially pronounced for cutting edge problems in biology: large proteins; complexes of multiple proteins; complexes of proteins with nucleic acids and lipids; and partially-ordered proteins. There is no hard data on the percentage of projects that are abandoned due to crystal size limitations but based on anecdotal data, it is not an insignificant percentage. Thus, it is widely recognized that developing new methodologies to deal with microcrystals would be transformative for structural biology. Importantly, the facility that provides researchers with effective capability to work in the microcrystal size domain will be the next Mecca for structural biologists and their research. Further, facilities for micro X-ray crystallography should be useful in all areas of chemical and geochemical crystallography in which only small specimens are available.

From the standpoint of micro X-ray crystallography "microcrystal" samples fall into two general categories: 1) very small but highly ordered, and 2) large and nonuniform, but with small areas of low mosacicity. These situations require different strategies that overcome particular sets of technical challenges to yield high quality data. Nevertheless, if the technical framework can be put into place to routinely facilitate collection of microcrystal data from both cases, the impact would be enormous. Below are described the issues and opportunities that each of these categories of microcrystals presents.

1) Highly ordered microcrystals Clearly, small crystals with typical dimensions in the 1-5 μm range with low mosaic spread represent the major class of microcrystal. If practical methods can be developed for manipulation, mounting, freezing, and taking data from these microcrystals, there are several potential attributes that can be taken advantage of. Small crystals have the advantage that they are sometimes better ordered than larger crystals. This means that structures can, in some cases, be determined to higher resolution using microcrystalline samples. Additionally, microcrystals may suffer less from radiation damage since the mean free path of outgoing photoelectrons exceeds the crystal dimensions[1, 2]; the photoelectrons escape from the crystal before causing the full extent of radiation damage. Reduced radiation damage will – if confirmed - be extremely important. Otherwise, each microcrystal would yield only 1 or 2 diffraction patterns.

A pertinent example of the power of microcrystallograpy is the high resolution analysis of amyloid producing peptides by the Eisenberg group[3, 4]. This class of peptides has been the subject of intense investigation because of their relevance to major classes of amyloid-related disease states such as Huntingtons, Parkinsons, Alzheimers and ALS among others. It had been known that these peptides formed β-sheet-like structures, but the atomic detail displayed by these structures was controversial.

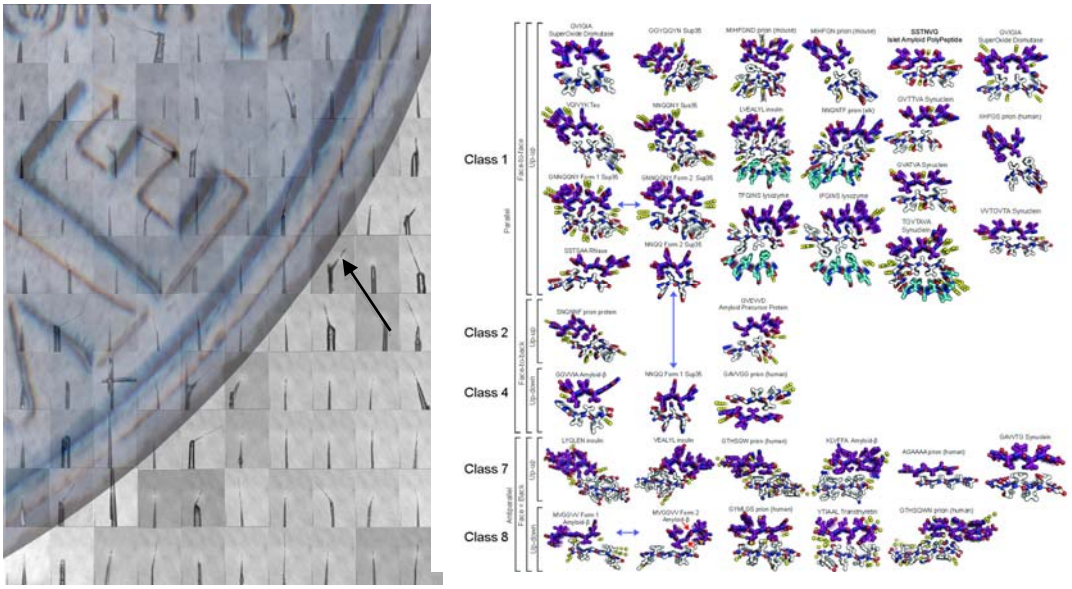

Figure 1 Left- Comparison of size of mounted microcrystals to the size of a dime. Arrow designates crystal attached to mounting pin. Right- Examples of different β-sheet arrangements among the 45 crystal structures analyzed.

By recording diffraction data from microcrystals on microfocus beamline ID13 at the European Synchrotron Research Facility (ESRF), Nelson et. al. were able to determine structures for two polymorphs of the heptapeptide GNNQQNY (refined at 1.8 Å resolution) and of NNQQNY (refined at 1.3 Å resolution) [5]. These peptides are extended in conformation and are hydrogen-bonded to each other in standard Pauling-Corey parallel β-sheets. Because the strands are perpendicular to the long axis of the microcrystals, hydrogen-bonded addition of GNNQQNY molecules to the growing β-sheet accounts for the elongated shape of the crystals as well as the fibrils. As previously suggested from X-ray powder diffraction of the microcrystals, the GNNQQNY β-strands within each sheet are parallel and exactly in register. A parallel, in register arrangement is also seen for A β molecules in their fibrils. Each member of a pair of sheets is related to the other by a 2_1 screw axis: the strands in one sheet are antiparallel to those in the neighboring sheet, and each sheet is shifted along the screw axis relative to its neighbor by one half the strand-strand separation of 4.87 Å. Thus, side-chains extending from a strand in one sheet nestle between side-chains extending from two strands of the neighboring sheet.

More recently, Sawaya et al (2007) have identified some 70 segments of fibril-forming proteins, which themselves form both fibrils and microcrystals[4]. Virtually all of the crystals are microcrystals, some of which are shown in Fig. 1, where they are compared in scale to a dime. To date, structures of 45 of these microcrystals have been determined, at resolutions between 0.8Å and 2.0Å. One result of these studies is that the fibrils associated with numerous disease states have similar structures at the atomic level.

2) Large, non-uniform crystals Although it has been shown that the application of small X-ray beams (1-10 μm diameter) to very small, but uniformly highly ordered crystals can bring excellent results as illustrated above, the application of small X-ray beams to larger, less-ordered crystals is equally important. It has been generally observed that complex systems like large protein assemblies, membrane proteins and multidomain proteins are inherently susceptible to producing crystals with surface heterogeneities that impede crystal growth. A major frustration is that many times crystals grow preferentially along a single axis to produce long thin needles, or along two axes to produce very thin plates. In both cases the crystals are fragile and prone to mechanical deformation; and their volume is far too small to generate diffraction of sufficient signal-to-background with a conventional beam. In other cases, larger 3-D crystals can be grown, but they have significant non-uniformities. When probed with an X-ray beam that is matched to the crystal size, such crystals produce split, smeared, broad diffraction maxima that extend only to limited resolution, which in many cases precludes measurement of useful data. However, within some of this these crystals there exist diffraction "sweet spots", small regions of lower mosaicity that can be the source of high resolution data. But the sweet spots have to be identifiable and separable from the rest of the crystal by using an X-ray micro-beam. Improved strategies for automatically scanning crystals to locate sweet spots are very important.

One striking example of a successful diffraction experiment is the structure determination of human beta-2-adrenergic receptor from needle crystals using a beam of 7 μm FWHM at Sector 23 of the APS[6, 7]. The structure determination of this 7-transmembrane helix GPCR membrane receptor was one of the most anticipated

structures in the last decade. It could not have been solved without using a very small beam and a search strategy to target the regions of the crystal that had maximal order.

Another common practice is to systematically move the beam along the long axis of the crystal to access fresh regions for collecting partial data that can be merged. This practice is made much more efficient using micro-beams.

Present status of micro X-ray facilities.

Today, methods of structure determination from very small crystals (approaching 1 μm in typical dimension) are being developed and implemented at 3rd generation synchrotron sources throughout the world, including beamlines at APS [8];Fischetti et al. J. Synch. Rad. (in press). Since Fall 2007 mini-beams of 5 μm and 10 μm diameter have been in routine use on both undulator beamlines at GM/CA CAT (23-ID-B and 23-ID-D). The mini-beam capability was so successful in initial user tests that it was offered in automated form in February 2008. Users quickly learned the benefits of a 5 μm or 10μm beam when it was made rapidly selectable at the click of a button without need for re-alignment. In the first two months of availability, 75% of users took advantage of a mini-beam for at least some of their crystals. Several reported that data were collected from crystals that would have been discarded if examined only with the large beam also available at these beamlines (~30 μm x 100 μm). While 10 μm micro-beams have made an impact, even smaller beams will be needed to attack the types of cutting edge problems that we envision in the future.

Detectors for microcrystallography

The size of diffraction spot on a detector depends on several factors such as the beam size and divergence, crystal quality and detector spatial resolution. For a microcrystallography experiment on a high quality crystal one might expect the width of the diffracted beam at the detector to be on the order of 30 to 50 μm. The resolution element of current CDD detectors is ~100 μm; and therefore, the diffracted intensity is spread over several pixels in two-dimensions. This reduces the signal-to-noise ratio of the measured peaks and limits the maximum resolution to which data can be observed. To address this issue, vendors are offering new thinner X-ray sensitive phosphors to reduce the point-spread-function and hence resolution element of their CCD detectors. However, there is a trade off between phosphor efficiency and point-spread-function so the true benefits have to be evaluated.

Another class of detectors has recently come to market. These pixel-array-detectors (PAD) have very fast read-out times allowing a data set to be collected 10-15 times faster than with a CCD detector. However, the only available large-format detector suitable for macromolecular crystallography, the Pilatus 6M, has a resolution element of 172 μm. This considered too large for microcrystallography experiments. It is anticipated that in the near future, large-format PAD detectors with smaller resolution elements will come to market. These will provide both increased data throughput and improved signal-to-noise.

Beam stability issues present a barrier to developing micro-beams at the APS-

To serve the community best, small beams on the order of 1 μm must be routinely, rapidly and robustly available. The APS is uniquely positioned among sources

in the US to develop micro-beams if its capability of producing beams with low divergence and high brilliance can be exploited. However the reality is that it will be impossible to achieve this goal without major improvements in beam stability. Experience with the GM/CA-CAT micro-beam indicates that beams of 5-μm to 10-μm diameter are at today's limit of stability of the X-ray beam, the beamline optics, the sample goniometry and the sample visualization. The proven success of a small beam for both micro-crystals and "ordinary" crystals argues that improved beam stability be given high priority in the APS renewal.

Currently, the beam stability at all the macromolecular crystallography stations is suboptimum. The problem is in stabilizing the angular position of the beam as an error as small as 0.1 microradian leads to a positional error on the sample of over 5 microns, depending on the beamline design. This misalignment must be constantly corrected for, and even during a single experiment this can lead to significant degradation of data quality. Misalignment will become an even more serious problem with smaller crystals and beam sizes. Positional stability of the X-ray beam at the experiment, as much as 70 meters downstream from the source, must be within less than 1 μm on very short (sub-second) time scale. The long term positional stability of the beam on a scale ranging from minutes to days – its drift - must also be ensured. It is also important to recognize the importance of the stability of the beam intensity for experiments that utilize monochromatic X-rays (though not for polychromatic, Laue experiments). An issue for effective utilization of small beams is the minimization of vibration arising from mechanical systems on the experimental floor and from other events external to the experimental hutch.

Automation for sample changing and alignment- Sample manipulation presents another major technical challenge for micro X-ray crystallography. Dr. Christian Riekel of ESRF has pioneered the development of several important aspects for micro-beams for X-ray diffraction[9]. He and his collaborators have produced highly-focused X-ray beams (currently under 1 μm in diameter); built a goniostat with a small sphere of confusion, suitable for orienting microcrystals; and have begun to develop methods for mounting and positioning small crystals. This set-up needs to be investigated to establish its strengths and weaknesses and its portability to the APS.

The importance of mounting and alignment cannot be over-emphasized. Methods for manipulation and mounting are still rather crude and need improvement to increase throughput. An essential future development will be the built-in capability for massive automatic sample changing. With current methods, a trained crystallographer can mount and center a microcrystal in 10-20 minutes. Because the small crystals are delicate, mounting and freezing them will also present numerous challenges. The experience and expectation is that generally many crystals must be screened prior to data collection to identify the best, from which suitable diffraction data can be collected. In the Eisenberg lab, experience suggests that about 20-40 crystals must be screened to find one that yields high resolution data. For this reason, the lab routinely mounts 500 or more crystals before going to the synchrotron. At the beam, most of the time goes into crystal screening. The whole process could be vastly speeded up by an automatic sample changer that would permit each crystal to be screened in seconds, rather than in tens of minutes. This is essential, because without a highly automated and efficient freezing and

positioning capability, micro X-ray crystallography will never reach its full potential. Even if the unlikely circumstance holds, all crystals are excellent and no crystal screening is necessary, automation is essential since no microcrystals will yield more than a few diffraction patterns before radiation damage becomes too severe and the crystal must be discarded and replaced by a fresh crystal.

Probing reaction dynamics by time-resolved crystallography-

Structure determination by either traditional X-ray crystallography or NMR techniques provides a view of molecules that is time-averaged over the duration of data acquisition. While this averaged view alone has proved remarkably powerful and provides crucial information, in reality all reactions in chemistry, biochemistry and biology are highly dynamic processes involving structural transitions over a very wide time range - from femtoseconds to kiloseconds. Critical information about the interplay between structure, dynamics and function is consequently lost when all the structural transitions are superimposed. Thus, a major area of investigation has been to develop new crystallographic approaches that exploit the high resolution capabilities of diffraction, while also exploring the time scales that are relevant for biological phenomena.

Snap-shots defining reaction pathways- In time-resolved crystallographic experiments, sequential snapshots of structural changes are taken in real time after the reaction is trigger by an appropriate stimulus[10]. (Note, these experiments differ from and are complementary to more widely used trapping strategies such as chemical trapping or freeze-trapping, which seek to prolong the lifetime of short-lived intermediates by chemical or physical means[10, 11]).Time-resolved techniques could be described as "analytical trapping", in which the intermediate structures are isolated and "trapped", not during the data collection, but in the subsequent processes of data analysis. From the time-dependent electron density maps (or difference electron density maps relative to time zero) that are obtained, the goal is to identify and refine the time-independent structures of the transient intermediates that comprise the overall, chemical kinetic mechanism[10-15]. In practice, a stepwise reconstruction of the complete structural basis for the chemical process can be obtained. Unquestionably, this represents a major step in understanding the fundamental relationships between structure, function and dynamics.

Technical challenges-

1). Triggering the reaction- Initiating the reaction in an ultrafast and controlled way presents its own set of issues, in addition to the challenges of developing experimental setups for collecting diffraction data that faithfully represent time slices of a reaction pathway. In principle any extensive variable which influences protein structure can be used for reaction initiation, e.g. temperature jump, pressure jump, electric field jump, pH jump, or concentration jump via diffusion. The problem is that changes in almost all these variables can only take place very slowly, typically in the μs to hundreds of ms range, and such changes are therefore suitable only for exploring slow reactions. Even the more widely used photoactivation of caged compounds (for which caged ATP is the classical example) proceeds with modest quantum yield and is slow, only liberating the desired ATP in the tens to hundreds of μs range. Further, photoactivation by rupture of a covalent bond is irreversible and proceeds via highly reactive chemical intermediates that

often introduce unwanted side reactions. In contrast, in natural systems photoactivation by isomerization is ultrafast (on the fs to ps time scale), devoid of side reactions, usually reversible, and proceeds with high quantum yield. But chemistry R&D is needed to devise suitable photoisomerizable substrates, cofactors or inhibitors for this approach. For example, does a smoothly isomerizable, biochemically active analog of ATP or GTP exist?

Another approach offers promise: to confer light sensitivity on otherwise light-inert systems, by domain fusion. This approach takes note of the fact that natural photosensors are modular, in which an input, photosensor domain is coupled – usually covalently – to an output, effector domain whose biological activity is thereby made light-sensitive. In nature there are a very wide variety of such output domains e.g. histidine and serine/threonine kinases, phosphotransferases and DNA binding proteins. We have successfully conferred sensitivity to blue light on the Trp repressor[16]and on a normally oxygen-sensitive histidine kinase (Moeglich, Ayers and Moffat, submitted), and we know of a successful application to a small GTPase (Y. Wu, unpublished). In all three systems, a molecular biology-based approach was used to covalently link a blue light-sensitive, FMN-based LOV sensor domain to the N-terminus of the desired output domain. It was not necessary to prepare a large library of fusions and screen them. This observation and the wide diversity of the three successful output domains suggest that this approach may be quite widely applicable. More R&D is necessary to establish whether this is indeed so, and whether the design principles derived from these three systems apply generally. The obvious requirement for time-resolved crystallography is that good crystals be obtained of the newly light-sensitive proteins, and that they retain both photochemical and light-dependent biochemical activity in the crystal.

2). Detection of intermediates- Because of limitations in current detection technology, all sub-second time-resolved crystallographic experiments have been conducted in pump – probe mode, in which each pump or reaction initiation event is followed after a suitable, controllable time delay by a probe event[13, 17-19]. A structural change in the molecules in the crystal is triggered by a short laser pulse or other means (e.g. temperature jump, pH jump, voltage jump or diffusion of a key reactant) and probed by X-ray pulses of appropriate duration. The time resolution is dependent on the duration of the two pulses, pump or probe, and on jitter in the time delay between them. High peak power, fs pulsed lasers are readily available today for pumping or triggering reactions in photosensitive systems. The best time resolution is thus limited by the duration of the probe X-ray pulses, around 100 ps FWHM at all conventional synchrotron sources. Fast time-resolved experiments (100 ps to 100 μs time resolution) require the Laue diffraction technique, in which a stationary crystal is illuminated by polychromatic X-rays. Conventional monochromatic, rotating crystal techniques can be used when a time resolution of ms or longer will suffice. Reaction initiation is a critical step for the success of time-resolved experiments and has to be tailored to the particular biological system under investigation. Synchronization electronics establish and vary the time delay between the pump and probe pulses. Individual X-ray probe pulses or longer multi-pulse trains must be selected by a fast X-ray shutter train from the continuous X-ray pulse sequence emitted by the synchrotron. The data sets in a time-resolved experiment are large since they span a four-dimensional space: the intensities over the unique volume in

reciprocal space (hkl) and the time range over which the reaction proceeds (t). Typically, three or four complete data sets are acquired per decade of time, uniformly spaced in log t; a total of 30 to 50 time points is not unusual. Data are also acquired with high redundancy to permit the accurate determination of both the mean structure amplitudes |F(hkl,t)| and their variance σ^2 (hkl,t). The variance is essential to the implementation of data weighting schemes[14, 20, 21]. Indeed, since the time-dependent signal is small in magnitude and error-prone, the entire experiment depends on the elimination of systematic error and the minimization of random error. Finally, analysis methods such as singular value decomposition[20] are needed that ultimately lead to the deduction of mechanism(s) that are compatible with the structural data and to the identification and refinement of structural intermediates.

Current State-of-the-Art for producing time-resolved information-

BioCARS at sector 14 of the APS is the only facility in the USA equipped for studying the dynamics of biological, macromolecular systems on time scales of 100 ps or longer, using the techniques of time-resolved crystallography that BioCARS scientists pioneered. The recently upgraded BioCARS 14-ID-B beamline surpasses all other such facilities in the world in both its X-ray and associated laser capabilities. Although the double, collinear undulator sources and X-ray optics were designed and optimized for both polychromatic (Laue) and monochromatic experiments on single crystals of biological interest, very recent data shows that excellent small angle and wide angle X-ray scattering data can be obtained on protein solutions (SAXS and WAXS; P. Anfinrud and colleagues, unpublished) and fibers (G. Stubbs, M. Reedy, T. Irving and colleagues, unpublished). Likewise, excellent data have been obtained that addresses problems in time-resolved chemical crystallography (P. Coppens and colleagues, unpublished), and both static and time-resolved data arising from thermal diffuse scattering in silicon and gallium arsenide (D. Reis and colleagues, unpublished). Thus, the BioCARS sector is widely suited to time-resolved experiments, and the range of science conducted there may evolve more broadly.

Technologies needed to reach the next frontier-

1) Time resolution- Ultrafast X-ray pulses- One would like to be able to explore the structural course of ultrafast biochemical reactions such as those that occur in the earliest stages of photosynthesis, or in response to absorption of a photon of blue or red/far red light in a signaling photoreceptor. Ultrafast spectroscopies suggest that such reactions extend into the fs time range. There is a limited number of such reactions or systems, which all originate in the absorption of a photon, but they are critical to understanding photosynthesis and light-dependent signaling. Today's time resolution is set by the X-ray pulse duration at around 100 ps, which is in turn set by the RF characteristics and the long-term requirements on electron bunch stability in all circular storage rings. So, the structural bases of these ultrafast reactions are completely inaccessible at present.

Schemes have been advanced to generate much shorter X-ray pulses of a few ps duration via the introduction of "crab cavities" that would employ strong perturbation of individual electron bunches in the storage ring (refs). These X-ray pulses would be highly suitable for exploring ultrafast reactions, always provided that there were

sufficient X-ray photons in the shorter pulse to enable a good diffraction image to be obtained with one (or at most ~100) X-ray pulse. The figure of merit for recording a Laue diffraction pattern is photons/(pulse. mm^2. 0.1% bandpass), and should comfortably exceed 10^8 to generate a good Laue pattern from a quite strongly-scattering crystal. We support R&D on the installation of crab cavities in the APS and on the means to achieve this figure of merit. Other schemes such as laser-based pulse slicing of the synchrotron pulse perturb only a small fraction of the electrons in the bunch and hence fall short of this figure of merit by orders of magnitude. But here too, R&D may suggest alternative approaches.

2) Next generation detectors- Most APS macromolecular crystallography beamlines currently use CCD detectors that impose a 1-2 second readout time during which the X-ray shutter must be closed. We expect that over the next 5–10 years these detectors will be replaced with pixel array detectors that allow the collection of entire data sets without closing the shutter. This technology is currently available in a form factor well suited for macromolecular crystallography (PILATUS 6M) and, when implemented on a beamline, makes the sample changer, crystal centering, and the ability to process data the throughput bottlenecks.

Such a detector will replace the present "pump once – probe one time point" with a "pump once – probe many time points" approach. Pixel array detectors offer great promise since they are designed to record diffraction patterns at several time delays after a single reaction initiation event. A serious limitation, however, of existing and proposed pixel array detectors is that they have a minimum inter-frame dead time ~200 ns, just too long for recording rapid reactions. These require a minimum inter-frame time of 153.4 ns, the inter-bunch time in 24-bunch mode of operation of the APS. Much R&D is required before such ideal, large area, ultrafast pixel array detectors could be in routine use.

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