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Serial Femtosecond Crystallography Opens New Avenues for Structural Biology

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Abstract

Free electron lasers (FELs) provide X-ray pulses in the femtosecond time domain with up to 10^{12} higher photon flux than synchrotrons and open new avenues for the determination of difficult to crystallize proteins, like large complexes and human membrane proteins. While the X-ray pulses are so strong that they destroy any solid material, the crystals diffract before they are destroyed. The most successful application of FELs for biology has been the method of serial femtosecond crystallography (SFX) where nano or microcrystals are delivered to the FEL beam in a stream of their mother liquid at room temperature, which ensures the replenishment of the sample before the next X-ray pulse arrives. New injector technology allows also for the delivery of crystal in lipidic cubic phases or agarose, which reduces the sample amounts for an SFX data set by two orders of magnitude. Time-resolved SFX also allows for analysis of the dynamics of biomolecules, the proof of principle being recently shown for light-induced reactions in photosystem II and photoactive yellow protein. An SFX data sets consist of thousands of single crystal snapshots in random orientations, which can be analyzed now “on the fly” by data analysis programs specifically developed for SFX, but de-novo phasing is still a challenge, that might be overcome by two-color experiments or phasing by shape transforms.

Keywords

Femtosecond crystallography; free electron lasers; GPCRs; membrane proteins; photosystem I; photosystem II

INTRODUCTION

Brief Summary of History of Structure Determination of Biomolecules

Structural biology unravels structural information of biomolecules with a high success rate as more than 100,000 structures of proteins have been determined to date, leading to usable understanding of their function. New method developments in X-ray crystallography, nuclear magnetic resonance, electron microscopy and atomic force microscopy have made a

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CONFLICT OF INTEREST

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huge impact in the field. This review will focus on new developments in X-ray crystallography made possible by the advent of free electron lasers. The history of X-ray crystallography spans more than a century from the first proof of X-ray diffraction by Laue and Bragg (for a historic review see Bragg [1]), the structure of the biomolecule penicillin by Dorothy Hodgkin [2], the first structure of a protein [3], the first structure of a membrane protein [4] and to the structure determination of large macromolecular complexes like the ribosome [5]. Important milestones in the method developments for X-ray crystallography have been on all ends of the structure determination pipeline, which range from the heterologous expression of proteins and automated crystallization screening, freezing of crystals, invention of synchrotron sources and microfocus beamlines, phasing with Se-MAD/SAD to the huge advancement in computational methods for structure determination, refinement and automated model building (for a review see Helliwell and Mitchell [6]).

With all these great method developments, structure determination of well-ordered single domain soluble proteins can now be achieved in high throughput. However, most proteins do not act on their own but form complexes, with many of these large complexes being located in biological membranes. Membrane proteins are an extremely important class of proteins. They are located in or are attached to biological membranes and catalyze the key reactions in respiration, photosynthesis, nerve function, transport of molecules in and out of the cell, and cell-to-cell communication to name just a few. They are of extremely high medicinal relevance. For example, they let us smell, taste, feel joy or anger, are responsible for hormone function, regulate our blood sugar level and blood pressure. 60% of all current drugs are targeted to membrane proteins. Despite the great importance of membrane proteins, their structure determination lags far behind soluble proteins with less than 600 membrane protein structures determined to date. One of the many challenges in the X-ray structure determination effort is the growth of large, well-ordered single crystals. Photosystem I, which consists of 36 proteins and 381 cofactors is an example of this challenge where it took 13 years from the first small crystals reported [7] to the determination of a 2.5 Å structure of photosystem I [8]. Another example of challenging constructs are G-protein coupled receptors (GPCRs). They are the most important class of human membrane proteins. The human genome encodes more than 500 different GPCRs and 40% of all human drugs currently approved interact with GPCRs. The first GPCR structure (bovine rhodopsin, involved in vision) was solved in 2000 [9] but it took 7 more years until the first structure of a human GPCR was solved in 2007 [10] using partial data from many of small crystals (20–50 µm) of the beta-adrenergic receptor in lipidic cubic phases collected at microfocus beamlines.

One of the major challenges of X-ray data collection on small crystals, which are often better ordered than large crystals (as they employ less long range disorder), is X-ray damage [11] caused by photo-ionization events, which lead to radical formation and photo-reduction events which finally damage the biomolecules and the translational order of the crystals.

Principle of Serial Femtosecond Crystallography

FELs produce ultrashort high intensity X-ray pulse with femtosecond pulse duration. The flux is extremely high (2×10^{12} photons/pulse) and they destroy thereby any solid material. In

2000 Neutze and Hajdu simulated the explosion of a protein in such a strong X-ray pulse and proposed that the molecule would explode in approx. 5–10 fs, i.e. if pulse duration could be limited to femtoseconds one may observe scattering of the molecule before it is destroyed [12]. The first high-flux FEL, FLASH, was built at DESY in Hamburg and operates at low energy. In 2006 Chapman and coworkers showed at FLASH that scattering from an image etched into a silicon-nitrate plate, observed in a single shot before it was destroyed, could be reconstituted from its scattering pattern (Fig. 1) [13]. However, many scientists doubted that the diffract-before-destroy principle would extend to biomolecules. The first high-energy FEL, the Linac Coherent Light Source (LCLS) was built in Stanford at SLAC and started operating at intermediate energy of 2 keV in 2009. Just 2 months after LCLS started operating the first diffraction experiments on single particles and nanocrystals were performed in Dec 2009 and provided the proof of principle that diffraction of biomolecules can be observed with FELs [14,15]. Virus particles and photosystem I crystals were delivered to the FEL beam at room temperature by a liquid injector with a gas dynamic virtual nozzle (GDVN) [16,17], which ensured that the sample is replenished between the X-ray shots. However, approximately only 1 out of 10,000 crystals was “hit” by the X-ray beam, which means that tens of milligrams of crystals were required for the collection of a full data set. Scattering of single particles from the mimivirus was observed to a resolution of approx. 300 Å¹ while serial femtosecond X-ray diffraction of the nanocrystals of the large membrane protein complex, photosystem I, was observed to 8 Å², with the resolution solely restricted by the energy of the FEL beam. These first experiments already provided the proof that serial femtosecond crystallography can overcome the problem of X-ray damage in crystallography [18]. One of the most exciting observations was that the diffraction patterns from nanocrystals showed shape transforms i.e. the Fourier transform of the size and shape of the object. It was already proposed in 1952 by Sayre [19] that shape transforms, should be observed if one would ever be able to observe diffraction from a crystal that it so small that the unit cells could be counted. By counting the fringes between the peaks observed in the photosystem I diffraction patterns one can determine the number of unit cells in one direction and full evaluation of just one of the shape transforms allows for the determination of the size and shape of the crystal (see Figure 2). In the future, when larger detectors with higher dynamic range (such as the AGIPD, currently under development) will become available, the shape transforms may allow for direct phasing [20] of SFX data of nanocrystals.

Summary of Recent Developments in SFX

One of the major questions asked after the first experiments were conducted was if the resolution of SFX would extend to atomic resolution. In 2011 the energy of LCLS was increased 9.4 keV which formed the basis for the determination of the first protein structure determination at near-atomic resolution [21]. Since these first experiments, further breakthroughs have been achieved. The first novel structure was determined by an SFX study where diffraction patterns were collected and the structure solved from crystals grown by overexpression of the protein CatB inside living insect cells [22].

In the last 2 years it was shown for the first time that SFX can also be applied to membrane proteins crystallized in lipidic environments (the lipidic cubic phase, LCP) [23,24], which

has been applied to determine structures of the highly medically relevant class of human membrane proteins, the G-protein coupled receptors (GPCRs) (for reviews see [25, 26]). A new type of injector has been developed for these highly viscous samples, which not only allows for the delivery of membrane proteins in LCP [24] but also reduces the sample amount required for one data set by 2 orders of magnitude. It has recently led to the structure determination of the angiotensin receptor that regulates the blood sugar level and is one of the major targets for drugs fighting hypertension [27]. Femtosecond crystallography also opens a new avenue for determination of protein dynamics. First experiments on the proof of principle for time resolved serial femtosecond nanocrystallography have been performed on the photosystem I-ferredoxin complex [28] and photosystem II nanocrystals [29,30]. In the work of Kupitz *et al.* conformational changes of the Mn_4O_xCa cluster and its protein environment were observed for the first time in the transition from the dark to the doubly excited state [29]. Very recently it was shown that time-resolved SFX (TR-SFX) studies extend to atomic resolution using the photoactive yellow protein as a model system [31]. These pioneering studies pave the way for dynamic molecular movies of membrane proteins “at work” in the future, including the determination of molecular movies of water splitting.

GROWTH AND BIOPHYSICAL CHARACTERIZATION OF NANOCRYSTALS

Since its inception, biomolecular crystallography has provided a challenge in the form of growing large, well-ordered crystals. This has led to many developments and improvements in the screening process resulting in a multitude of crystallogensis methods for large crystals. It has been empirically common during initial screening for showers of nanocrystals to be observed which, other than serving as a foundation for further screens to optimize single crystal growth conditions or seeding techniques, have had little use in structural discovery. Some proteins, particularly membrane proteins, have shown great resistance to the growth of large, well-ordered crystals while still exhibiting these showers during the pursuit. The introduction of serial femtosecond crystallography (SFX) has not only given purpose to these small crystals but has presented an opportunity to access previously unavailable proteins using crystallographic methods. This also necessitates new methods for growth and characterization of nano crystals, the development of which has advanced significantly since the first SFX experiments were successfully carried out on the massive photosystem I membrane protein complex in 2010 at the LCLS at SLAC [15].

Crystallogensis

Methods for growing nanocrystals are based on the same concepts that have been employed for macrocrystallography (MX), differing only in the objective. In the pursuit of SFX suitable crystals, it is useful to understand the manipulation of crystal growth via a phase diagram. In order for nucleation and/or crystal growth to occur, the protein necessarily must be in a supersaturated condition. Nucleation typically dominates in higher supersaturated conditions while crystal growth becomes more prominent in milder supersaturated conditions. As one approaches higher supersaturating conditions, nucleation events occur more rapidly and frequently. As a consequence, the plethora of nuclei rapidly depletes the solute concentration of protein, thereby minimizing time spent in the metastable zone and resulting in a shower of nanocrystals. This is in contrast to traditional MX where the aim is

to induce few nucleation events and allow the nuclei to spend as much time as possible in the metastable zone in order to form few large crystals. It is important to note that going too far into supersaturation with nuclei forming and growing too rapidly to allow energetically favorable ordered configurations can lead to amorphous precipitation.

Historically, showers of nanocrystals have been observed using various methods such as vapor diffusion and batch crystallization (shown in Fig. 3 or e.g. Rupp [32]). As such, these methods have also been employed in the quest for SFX sample preparation and, indeed, have shown success. It should be mentioned that the size at which a crystal is “too small” for traditional MX varies and the most relevant metric for which is the number of unit cells in a given direction. For example, photosystem I (unit cell $281 \times 281 \times 165.2 \text{ \AA}^3$) requires crystals on the order of half a millimeter at a synchrotron for comparable resolution to micron crystals at an XFEL (other parameters such as crystal quality and radiation damage obscure a general size limit). The hanging drop vapor diffusion method has already been successfully applied for diffracting crystals of the 30S ribosomal subunit [33]. One drawback for SFX studies, however, is the need for copious amounts of protein compared to MX, often needing milliliters of concentrated (on the order of 10^9 – 10^{11} crystals/mL, depending on crystal size, jet diameter etc.) sample. This provides the motivation for the scaling up of yields compared to historical methods, which is prohibitive for vapor diffusion methods that are highly dependent on a drop surface to volume ratio, limiting yield per setup. The batch method is quite suitable for this though, and has been effectively applied numerous times to SFX studies [15,21,31,34–36]. This technique is essentially unchanged from MX applications with no modification necessary for SFX preparation.

Other sample preparation methods have been developed specifically for SFX since its inception. A free interface diffusion (FID) method (described in detail in Kupitz *et al.* [9]) has been employed which relies on a liquid-liquid interface of high protein and precipitant solutions. This can cause a shower of nanocrystals, which subsequently sink into the precipitant and away from free protein, essentially quenching crystal growth and providing a pellet of suitable crystals. This is very convenient for crystal density optimization of a hydrated suspension that will subsequently be delivered to an XFEL. A variant of this technique, which incorporates centrifugation of the setup, is also useful, allowing one to expedite the removal of the nanocrystals from the free protein and enabling influence on the size of the crystals at the point they are quenched. Nanocrystal FID techniques often lead to a very high degree of size homogeneity which is particularly useful in the subsequent data analysis (due to the Monte Carlo integration of diffraction snapshots [37]). Furthermore, control of this size is critical for time-resolved experiments (discussed below).

A highly unique technique for nanocrystal growth has been found recently in which the overexpression of some proteins in insect cells has resulted in crystallogenesis *in vivo* [22,38–41]. The overexpressed protein forms a natural crystal within the cell, causing the cell to deform around it. The nano crystals can either then be harvested from the cells after lysis [22] or, as was recently shown, unlysed cells can be used directly [38]. The crystals used in this method have proved to be perfectly suited for SFX studies with dimensions on the order of $1 \mu\text{m}^3$. Notably, the first *in vivo* SFX study resulted in the discovery of the inhibition mechanism of TbCatB, a critical protein involved in African sleeping sickness, by

revealing a fully glycosylated precursor complex structure to 2.1 Å [22]. This technique has the potential to solve structures in a native, cellular environment and possesses the added benefit to eliminate purification and recrystallization steps for suitable proteins. However, it must be noted that while this technique is currently being explored further, it has thus far only been shown to be effective on a limited set of proteins and its mechanism is poorly understood at present. Current research is probing the breadth to which this technique is viable.

Characterization of Nanocrystals

Identifying nanocrystals presents a particular challenge compared with large crystals since nanocrystals are, in many cases, indistinguishable from amorphous precipitate optically. Even birefringence is difficult to detect in many cases since the small size results in a highly attenuated signal. The advent of second-order nonlinear imaging of chiral crystals (SONICC) has proven to be highly beneficial to this end, having the ability to identify chiral crystals down to submicron levels [42,43]. As a caveat, not all crystals are SONICC active and experience severe attenuation of the second harmonic generated signal due to pseudoisometric crystallographic symmetry [44]. Furthermore, some precipitants can form chiral crystals and, thus, it is imperative to use another technique such as UV fluorescence to confirm the existence of protein in the detected crystals.

There are currently only two operational XFELs (LCLS in California, USA and SACLA in Japan) and, as such, beamtimes are highly competitive. This provides strong motivation for optimization of sample quality prior to an SFX experiment. The most important characteristics in this optimization are size distribution, density and, of course, diffraction quality. Size distribution for submicron crystals can be obtained using dynamic light scattering [29,45], Nanosight [33,46] or electron microscopy [47]. A new microfluidic technique even allows inert post crystallization sorting using dielectrophoresis [35]. Electron microscopy is an excellent characterization tool for nanocrystals, allowing predicted diffraction quality, analysis of size distribution and permitting comparison of samples at various stages in the sample preparation and subsequent experiment [47] (see Fig. 4). This is especially useful for protein crystals, being exceptionally sensitive to handling due to the high solvent content and low relative crystal contacts. Experimentally probing the diffraction quality prior to SFX can be done at traditional X-ray sources by obtaining powder diffraction. While the diffraction quality in an SFX is often higher than that obtained in a powder pattern, powder diffraction is the most certain way of verifying diffraction and serves as a relative metric for screening samples against each other.

SAMPLE DELIVERY AND X-RAY DATA COLLECTION AT FELs AND SYNCHROTRONS

The core idea behind SFX has been around since at least the 1980's [48] but only recently has the technology been available to put it into practice. Fig. 5 shows a common scheme for SFX experiments where a hydrated suspension of protein nanocrystals is jetted transversely towards a pulsed XFEL. Pulses last ~50 fs with 8–10 orders of magnitude higher peak brilliance than the most advanced synchrotron sources [49]. This allows enactment of the

“diffract before destroy” principle, outlined in Neutze *et al.* [20], whereby diffraction data is gleaned in a sufficiently short interval such that it is unaffected by the retarded intense radiation damage and subsequent vaporization of the crystal. Relying on random orientation and high redundancy of measured intensities, individually indexed images are then merged using Monte Carlo integration to obtain relative structure factor intensities [50–52]. Real-time hitfinding has been developed and implemented in order to assess the viability of a sample as it is running, allowing users to rapidly adjust factors like sample density and crystal batch on the fly [53,54]. In addition to allowing the utility of nanocrystals for data collection, this setup can provide a more biologically relevant environment (i.e. temperature, hydration) than can be commonly attained at a synchrotron. Although special setups exist for hydration control and room temperature collection at a synchrotron, radiation damage limits the breadth of use, especially for molecules with high-Z centers, frequent in active sites.

Sample Delivery Jets

The most successful sample deliver technique for SFX to date is the GDVN [16,55], having solved structures of multiple soluble and membrane proteins [15,21,22,28,29,31, 38]. This setup uses a pressurized jet of suspended crystals delivered from a capillary with a diameter on the order of tens of microns. This stream is then focused down to $\sim 1\ \mu\text{m}$ by an enveloping coaxial inert gas stream (see Fig 6a–c). This allows a relatively large diameter for the flowpath, avoiding clogging, as well as providing a protective sheath for the stream to avoid the formation of ice in vacuum. An HPLC can be used to pressurize the sample and provide a reliable flowrate, useful in avoiding upstream radiation damage from the plasmified interaction region and enabling precision on time-resolved studies.

One drawback to the GDVN is that most of the sample is essentially wasted with most of it flowing through the interaction region between the femtosecond pulses. While current repetition pulse rates will certainly be improved upon in future projects such as the European XFEL [56], current rates (LCLS is 120 Hz, SACLA 30 Hz) provide motivation for sample preservation. Two avenues to address this are to slow down the jet or eliminate unused sample between pulses. The stability of the jet is directly related to the viscosity and, as such, injector technology and viscous media have been developed to exploit this. Gel-like media for growing crystals predates the use of SFX [57] with multiple successes, especially of membrane proteins, using *in meso* crystallization (e.g. Pebay-Peyroula, Rummel [59] or Katona, Andreasson [58]) within a lipidic cubic phase (LCP) [57]. In response to this, and the fact that many proteins are still size limited in their growth within LCP, the LCP injector was developed. This injector uses a hydraulic stage and Teflon plugs to amplify pressures up to $>2,000\ \text{psi}$ to exude the LCP and, similar to the GDVN, uses a co-flowing gas to focus the LCP [60] (see Fig. 6d). This provides a flow rate of $1\text{--}300\ \text{nL min}^{-1}$ as compared to $\sim 20\ \mu\text{L min}^{-1}$ for the GDVN. This has been particularly successful in solving SFX structures of GPCRs [23,27,61,62].

Not all proteins are compatible with LCP, however, as it must be held at a constant temperature of $20^\circ\ \text{C}$ in order to maintain the cubic phase, may hinder crystallization of large complexes due to steric constraints owing to curved lipid bilayers, and is incompatible with some precipitants, notably ammonium sulfate [63–65]. Thus, development of other

viscous media as suitable carriers for minimized sample consumption in SFX have been underway and have recently made significant progress. A grease-matrix media enabled the SFX structures of four different soluble proteins to atomic resolution [66]. An agarose based medium has been shown to allow the mixing of preexisting nanocrystals into the gel, representing both soluble and detergent stabilized membrane proteins with a lower background than other viscous media [63]. A full structure of the phycocyanin was produced in addition to diffraction of photosystem II, a large photosynthetic membrane complex. Both proteins exhibited diffraction consistent with previous SFX and MX experiments [67].

Petroleum jelly was also revealed to be a suitable carrier for microcrystals in serial crystallography [65]. The interesting thing about this medium is that it was tested at a microfocus synchrotron beamline. Indeed, with crystals on the larger side of SFX suitability and the smaller side of MX, the proof of principle for serial crystallography at a synchrotron has been shown [40]. One of the first reported studies of this kind was in Stellato, Oberthur [68] where microcrystals of lysozyme were used as a model protein and flowed through a thin layered capillary in a high PEG solution to prevent settling. LCP has also proven to be capable of use in serial MX, shown in static structure determination of lysozyme [65] and even millisecond time-resolved studies of bacteriorhodopsin [69]. While the line between the SFX and serial synchrotron crystallography is dependent on many parameters, often sample specific, SFX retains the advantage on very small crystals with the ability for comparable resolution from crystals 1–2 orders of magnitude smaller than that needed for microfocus beamlines. It also has the edge on ultrafast time-points, though it is currently much more difficult to obtain beamtime.

Fixed Target Serial Crystallography

Another development that has been jointly beneficial for SFX and MX is that of fixed-target serial crystallography. In this case the jet is replaced by a chip with multiple crystal containing regions. These have been made of X-ray transparent materials like Kapton foil [70–72], low Z polymer microfluidic chips [73] and silicon nitride [74–76] which allow the deposition or growth of randomly oriented microcrystals on the surface (see Fig. 7 for example). The stage upon which the fixed targets are mounted can then be rapidly moved or rotated to accommodate serial data collection. Data sets have been collected using this method for both SFX [74–76] and MX [71,72] experiments. Influence from SFX to MX by way of serial methods exemplify the symbiotic relationship between the two fields and new avenues for room temperature, damage minimized structures to be obtained.

SCIENTIFIC HIGHLIGHTS OF STRUCTURE DETERMINATION WITH SFX

The First Proof of Principle of SFX with Photosystem I as a Model System

Before the first SFX experiment was performed there was a lot of skepticism questioning whether the feasibility of structure determination based on nanocrystals of proteins with femtosecond X-ray pulses was possible. Major critical points were based on the following three challenges: 1) Would the diffract before destroy principle be valuable for biomolecules? 2) Could diffraction from nanocrystals even been observed, taking into account that the crystals contain only hundreds or thousands of proteins compared to

trillions of molecules in a large single crystal? While the flux would be 10^{12} higher than at a synchrotron, the pulse duration would be 10^{14} to 10^{15} times shorter. 3) How could data evaluation and structure determination be performed from thousands of still diffraction patterns of randomly oriented crystals?

The first SFX experiments were performed in December 2009, just 2 months after the start of the first run at LCLS and PSI, the most complex membrane protein crystallized so far was chosen as a model system. This choice appears at a first glance extremely adventurous but several factors lead to the immediate success of the first SFX experiments with Photosystem I: Photosystem I, as large and complex membrane protein is a prototype for all difficult to crystallize proteins and was therefore the ultimate target for SFX. The nanocrystals had been characterized by powder diffraction, dynamic light scattering and SONICC [77,78]. PSI is crystallized at low ionic strength, thereby formation of salt or PEG crystals in the interaction chamber was avoided and X-ray diffraction data on PSI nanocrystals were collected for 2 days without clogging of the injector until the LCLS data storage space of 23 Terabytes was reached. Due to the large size and unit cell constants, PSI crystals of 100–1000 nm size contained only 3–30 unit cells in each cell dimension, which allowed us to detect shape transforms on the back detector (see Fig. 1). Due to the large unit cell dimensions, there were sufficient reflections on the individual snapshot SFX diffraction patterns of the front detector to allow for indexing. The data evaluation strategy was already developed based on Monte Carlo integration and virtual diffraction patterns of Photosystem I had been simulated [51]. These factors all lead to the success of the first experiments and the proof of principle for SFX [15].

SFX Protein Structures Solved from In Vivo Grown Crystals

The most unexpected new development in biology with FELs was the finding that nanocrystals can be grown by overexpression of proteins in living insect cells [22]. The in vivo growth of crystals was first shown for the CatB, the protease that degrades red blood cells in patients suffering from sleeping sickness [79]. The CatB crystals were isolated from the cells and directly used for structure determination with SFX. The structure of CatB determined at 2.1 Å by SFX with its N-terminal inhibitor peptide [22] (highlighted as one of the 9 front runners for “breakthrough of the year 2012 in Science) is also of high medicinal importance as CatB is a potential drug target against sleeping sickness that affects 3 million patients each year worldwide. The picture of the in vivo grown crystals and the structure of CatB with its N-terminal inhibitor peptide, determined by SFX, are shown in Fig. 8. Since this first report, nanocrystals have also been shown from multiple other proteins, including membrane proteins (J. Love and P. Fromme, unpublished). Insects seem to be especially suited for in vivo crystal growth as reports also include crystal growth in the gut of living cockroaches [80,81]. Recently the first SFX structure of crystals grown inside a bacterium has been reported [38], where data were collected on isolated crystals and on crystals inside the living cells.

However, classic inclusion bodies, as they are frequently observed in *E. coli*, are not crystalline and consist in most cases of amorphous precipitate. Experiments are currently

underway to explore the mechanism of in vivo crystal growth in more detail with the goal to specifically target proteins to the pathways that lead to growth of crystals inside the cell.

STRUCTURE DETERMINATION OF GPCRs BY SFX

G-protein coupled receptors are one of the most important membrane proteins in the human body and are of outstanding relevance as 40% of all current drugs in the US are targeted to GPCRs. It is amazing that the GPCRs, which all consist of 7 transmembrane helices, show incredible large variations in function and substrate binding and play a role in so many different processes in the cell like signaling, tasting, hormone binding, mood control, blood pressure control etc. Also many proteins involved in cancer are GPCRs [82,83]. GPCRs have a high intrinsic flexibility and are often unstable in detergents. The crystallization in lipidic cubic phases, originally developed by Landau and Rosenbusch [57] was a breakthrough for the crystallization of this important class of human membrane proteins and more than 20 GPCR structures have been solved just in the last few years in LCP (for example see [27,61,62,84–86]). This also includes the first structure of a GPCR with a G-protein [87], for which B. Kobilka and Robert J. Lefkowitz received the Nobel Prize in 2012.

Crystal growth in LCP and data collection on these crystals is challenging especially as crystals rarely reach large sizes and are very sensitive to X-ray damage. While large amounts of small crystals (1–5 μm in size) are very frequently observed it takes often months or even years to grow crystals large enough for data collection at synchrotron beamlines. Furthermore, these larger crystals often suffer from high mosaicity and weak diffraction quality.

The really small crystals, however, are often better ordered and are the ideal target for SFX studies. As the LCP has a consistency of toothpaste, it could not be delivered with the jet developed for liquid samples, but a new injection system was developed for direct delivery of crystals in LCP [24], which allows for collection of a full data set with just 20–50 μl of crystals in LCP using 0.3 to 0.5 mg of protein. A new toolset has been developed to grow the nano/microcrystals of GPCRs in syringes from where they can be directly introduced into the sample chamber of the LCP injector. A challenge for the initial SFX experiments in LCP was the high phase transition temperature of monooleine (MAG 9-9) of 18°C, which led to partial transition of the LCP into a lamellar phase during injection due to the evaporative cooling effect of the high vacuum. This problem has been overcome by the use of lipid with lower phase transition temperatures (like MAG 7-9 or MAG 7-7) (Caffrey, Li [64]).

The first structure of a GPCR by SFX was the beta-adrenergic receptor, which had been previously solved by standard methods using synchrotron radiation. The comparison of the room temperature SFX structure and synchrotron structure (100 K) showed an improved electron density based on the SFX data sets, where missing side chains became visible, new salt-bridges were identified and even the backbone showed differences in some parts of the protein (see Fig. 9) [23]. The contrasts seen in the backbone are likely artifacts of cryogenic temperatures but the side chains and salt bridges cannot be ruled out as deriving from radiation damage. Since this breakthrough, four more important GPCR structures have been solved by SFX, including the smoothened receptor bound with its inhibitor cyclopamine

[24]. Cyclopamine, a toxin found in corn lilies, was identified in the 1950 as the cause of birth defects in sheep, leading to the birth of one-eyed lambs that also suffered from lethal brain malfunction. The structure unraveled that cyclopamine does not bind to the membrane intrinsic region of the receptor but is bound to the extracellular loop regions, which are thereby significantly altered in their conformation. While cyclopamine has a very dramatic lethal effect in the embryonic development, derivatives of this drug are currently under development as anti-cancer drugs that target the Hedgehog pathway [88].

Last year 2 more GPCR structures have been solved with SFX. In February 2015, the δ -opioid receptor structure was published in the form of several co-crystal structures with peptide inhibitors [61] (see Figure 10). This receptor controls emotions and pain, and is the target of several narcotic drugs. The peptide inhibitors may serve as treatment to combat pain and may also help fighting against drug addiction.

The most recent GPCR structure solved by SFX is the angiotensin receptor [27] (see Fig. 11). No structure has been reported previously for this important protein. The angiotensin receptor is of extremely high medical relevance as it controls blood pressure. The structure of the receptor has been solved as a co-crystal structure with an important drug (ZD&155) for the fight against hypertension. This structure will be of great importance for the further improvement of drugs against hypertension by structure-based drug design.

TIME-RESOLVED SFX, TOWARDS MOLECULAR MOVIES OF PROTEINS AT WORK

One of the most exciting areas in the context of SFX is time-resolved studies of proteins in action. Time-resolved SFX (TR-SFX) holds the potential to view a reaction happening in real time, creating a 'molecular movie'. This will lead to breakthroughs in our understanding of the mechanistic dynamics of biomolecules, allowing unprecedented progress in fields such as drug design and alternative energy. To accomplish this, TR-SFX can utilize pump-probe experiments for light activated complexes by 'pumping' the sample jet with a laser prior to interaction with the XFEL. With precision of the flowrate and geometry, different time points can be obtained by varying the delay time between the photoexcitation, or pump, and the X-ray diffraction, or probe. One can imagine a similar process for enzymatic reactions where a substrate is diffused through the nanocrystals with a calculated mixing time prior to the probe. Though Laue methods exist currently for time-resolved MX, they are currently limited by methods of reaction activation, especially hindered by the size of the crystals when attempting to obtain homogeneity of states when pumped optically or by diffusion of a substrate. This technique is further limited practically to reversible reactions, a problem bypassed by TR-SFX due to the constant replenishment of fresh sample.

One highlight of TR-SFX is recent work done on unravelling the mechanism behind energetic water splitting in oxygenic photosynthesis. This is done by the protein photosystem II through a cyclic reaction, known as the Kok cycle, whereby two water molecules are reduced to molecular oxygen, protons and electrons by the catalytic oxygen evolving complex (OEC) through a series of 5 S-states. In each sequential step, an electron is extracted with a water substrate being fully oxidized after evolution to the S₃ and S₀ states

respectively. This was an immediate target for initial TR-SFX experiments due to its high and broad impact, having been the subject of two of the first such experiments carried out [29,30]. In Kupitz, Basu [29] a pump probe experiment was performed where structures of the dark S_1 and doubly excited S_3 states were solved to 5 Å and 5.5 Å respectively. This was done by illuminating a liquid jet of nanocrystals twice, allowing 210 μs and 570 μs delays between each to allow the evolution of states before diffracting. In doing so, movement in the OEC and nearby loop region electron densities were observed. This is shown in Fig. 12, with the simulated annealing omit maps for the two states overlaid upon the 1.9 Å dark state OEC structure. This marks a monumental step for the field of solar energy as well as an exhibition of the potential that TR-SFX possesses. The differences between the SFX structure and the MX high resolution structure also highlight the advantage that radiation damage is outrun even for atoms with high Z, bypassing local radiation damage as well as global.

Since SFX deals with femtosecond pulses, it has the potential to push time-resolution limits to scales that were previously unattainable using MX Laue diffraction time-resolved methods, which are currently constrained to the hundreds of picosecond regime in the optimal case [89,90]. Using photoactive yellow protein (PYP) as a model protein, Tenboer and coworkers were able to compare intermediate structures in the PYP photocycle obtained from TR-SFX to known intermediates attained using Laue methods [31,90]. In this study, a 1 μs and a 10 ns were obtained in addition to the dark structure showing the isomerization of the chromophore (see Fig. 13). Structures were solved up to 1.6 Å on crystals < 10 μm³. The small nature of these nanocrystals allows for full penetration and incorporation of the pump laser. This is in contrast to synchrotron experiments where repeated pumping causes strain in the crystals due to it being focused on an area smaller than the crystal [31]. Furthermore, in order to cause initiation of the photoreaction, higher energy pulses are needed to fully penetrate the crystal, a constraint loosened in SFX. In fact, the observed extents of reaction initiation totaled up to 40% for SFX as compared with typical Laue rates around 10–15% [90]. This paves the way forward for imaging ultrafast reactions with SFX to atomic resolution.

So far, published TR-SFX work has only been applied to photoactivated reactions but work on substrate induced reaction methods has already begun. One of the most promising and exciting ways to do this would be a jet that allows mixing of substrate and crystallized enzyme on the fly. SFX is certainly advantageous in this since the small nature of the crystals allows for fast enough diffusion times to theoretically allow sub-millisecond temporal resolution (1x2x3 μm crystals are on the order of 150 μs [91]). This regime could allow the study of most biologically relevant enzymes. Mixing injectors have already been proposed, exemplified by the double-focusing mixing jet built by the same group that pioneered the GDVN [92]. Shown in Fig. 14, this design employs an inner capillary that would flow protein crystals to a mixing region where an outer capillary can deliver a substrate, pH change, etc. to initiate the reaction. Reaction times could be varied by translation of the inner capillary to manipulate the time at which mixing occurs prior to interaction with the XFEL. This mixed jet is then focused by an inert gas similar to the GDVN and LCP injectors.

Alternative to this method, so called ‘caged substrates’ could be introduced whereby a protein is co-crystallized with its substrate in an inactive form that can be induced by photolysing the ‘cage’ and allowing the reaction to proceed. This would bypass the limits imposed by diffusion times and allow down to nanosecond initiations [91]. This technique has already been demonstrated at synchrotrons and could introduce a way to increase homogeneity and extend temporal resolution for SFX by use of a pump-probe style TR-SFX setup, although this remains untested at present date.

Overall, the full potential for time-resolved molecular movies has begun to be realized with the advent of SFX and, as the field progresses, could conceivably change the paradigm of structural biology as we know it.

THE CRYSTALLOGRAPHIC CHALLENGE OF SFX DATA EVALUATION

Two dominant programs have been developed for the SFX data evaluation. The program Cheetah [93] performs the detector calibration, background correction and sorts the “hits” and “non-hits” for the millions of snapshots in the SFX data sets. Further data evaluation, which includes peak selection and indexing, is performed by the SFX data evaluation program CrystFEL [50,94]. Both programs are Unix-based and are freely available from the server at the CFEL institute at DESY and include user-friendly step-by-step online tutorials. The data evaluation method development is constantly being improved and new features and options (like reconstruction of the peak profiles) are added and made available to all users in form of updates. The large majority of SFX structures published to date have been evaluated using Cheetah and CrystFEL [12,15,21,23,27,29,31,33,35, 36,38,61,64,67,74,76,79,81,95–98]. A second, alternate program has been developed and applied for SFX data evaluation (cctbx.xfel) [99]. Even if overall data evaluation statistics are similar (see [38] for a study where both programs were used for evaluation of the same SFX data sets), there are inconsistencies in data evaluated with cctbx.xfel (like unreasonably high I/σ values and too low B factors compared to the $CC_{1/2}$ values) which have to be resolved in the future.

A further challenge of data evaluation of SFX is the unambiguity of indexing for polar space groups. New algorithms have been developed to solved the indexing unambiguity of polar crystals in serial crystallography [100] and have been successfully applied for the evaluation of the first atomic resolution time resolved SFX study [31].

Monte Carlo integration averages the measured intensity of the reflections_{hkl} over all the experimental variables like fluctuation of peak intensity, size of crystals, number of molecules in the X-ray beam and variations in background. For accurate determination of structure factors, a redundancy of at least 50 is therefore required. However this number might be reduced significantly if the partiality of each reflection could be taken into account. In principle, the partiality of each reflection can be determined with knowledge of the exact orientation of the individual SFX snapshot in the Ewald sphere. Currently, algorithms are developed and are in the process of implementation in SFX data evaluation programs.

De novo phasing is still a challenge for SFX as standard routine methods as SAD and MAD cannot be easily applied to SFX. The main challenge is the large fluctuation of the intensity

(up to a factor of 4 shot to shot variation) and the slight variation of the energy of each FEL X-ray pulse, which makes it very difficult to detect small changes in intensity with the high accuracy, required for SAD and MAD. A seeded FEL beam can be used to reduce the shot to shot variation of the FEL beam [101]. A de novo phasing method currently being developed involves two-color experiments that utilize two pulses of different energies that are femtoseconds apart, resulting in femtosecond MAD-style experiments where the shot to shot variation can be incorporated (see [101] for details). Also, a seeded FEL beam can be used to reduce the shot to shot variation of the FEL beam [102]. However, the use of the seeded beam comes with two major drawbacks: seeding is only successful in 5–20% of the FEL pulses and the seeded FEL pulses feature a 90% reduction of the photon flux. A recent study of SFX with lysozyme has not revealed significant advantages in data statistics for the seeded versus non-seeded SFX mode of operations [101]. A further drawback is that Semethionine cannot yet be used for phasing as the Se absorption edge at 12.6 keV cannot yet be reached by LCLS with reasonable photon flux.

To compensate for these difficulties, the first report of a structure determination with experimentally determined phases used the heavy metal (Gd) bound to a very small protein (lysozyme) [36] for their studies. A new method for phasing uses radiation-damage-induced phasing (RIP) for the phasing of SFX data [95].

Data collection at XFELs poses unique challenges in itself and advances in detector technology have evolved to meet these challenges. The Cornell-SLAC Pixel Array Detector (CSPAD) is the current detector used at CXI, LCLS and was developed to manage the short timescales of data collection while providing good S/N and a large dynamic range. It consists of 2.3 megapixels, each $110 \times 110 \mu\text{m}^2$ and able to handle 2700 photons/pixel in low gain mode or single photon capability in high mode [103]. This is crucial since the combinations of heterogeneity in crystal size/quality and pulse intensity fluctuations require a range of scalability. The data acquisition system at SLAC is able to write with <1% dead time [103], necessary to cope with the large amounts of data collected (up to 4 TB/hour) [104]. One challenge particular to FELs is that charges arrive concurrently, causing a massive voltage spike that the electronics need to have time to respond to. Highly intense signals can be an issue, leading to partial charge collection if the detector is not given enough time to respond. This was an observed issue in the first few years but has been addressed with upgrades and is now less prone to this error [105].

At SACL in Japan, a multi-port CCD (mpCCD) detector has been implemented, building off the CCD design popular at synchrotrons. It overcomes the problem that CCD's typically encounter in the form of fast readout rates needed by XFEL sources (>60 Hz). Damage is also a constant concern, with an annual fluence estimated to be on the order of 10^{14} photons/ mm^2 at SACL [106]. This is particularly a problem for CCDs and in order to combat this, the sensor is cooled to -20°C which drastically decreases current leakage [106].

With second generation FELs projected to come online in the near future, newer challenges for detectors are constantly arising and must be met as beam technology develops alongside. The Adaptive Gain Integrating Pixel Detector (AGIPD) has been developed for the unique

characteristics of the European XFEL facility that is currently scheduled to become operational in 2017. The European XFEL source has a 4.5 MHz pulse repetition that consists of 10 pulse trains per second each containing 2700 pulses separated by 220 ns. In order to manage this ultrafast data acquisition, the detector has a dynamic range from 10^4 photons at 12.4 keV (low gain mode) to single photon counting (high gain mode) [107]. It is capable of matching 4.5 MHz in its frame rate but can only store 352 of the 2700 images in any given pulse train¹⁰⁸. In order to maximize practical data, it relies on an on-the-fly veto signal that allows images from earlier in the pulse train to be overwritten before the readout is performed between pulse trains [108].

This leads to a very attractive alternate method for phasing of SFX data that has been proposed [20] which is based on the shape transforms (see Fig. 2) [15]. Shape transforms are Fourier transforms of the size and shape of the object. While the object is the same for all reflections on one image, the shape transforms are influenced by the phases. Shape transform phasing may become the method of choice when new detectors (currently under development at DESY and the Swiss XFEL) with high numbers of pixels/detector and high dynamic range (pixel-by-pixel dynamic gain setting) will become available in the next 2 years.

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LIST OF ABBREVIATIONS

CFEL	Center for Free-Electron Laser Science
DESY	Deutsches Elektronen-Synchrotron
FEL	free electron laser
FID	free interface diffusion
GDVN	gas dynamic virtual nozzle
GPCR	G-protein coupled receptor
HPLC	high performance liquid chromatography
LCLS	linac coherent light source
LCP	lipidic cubic phase
MAD	multi-wavelength anomalous dispersion
MX	macrocystallography
PEG	polyethylene glycol
PSI	photosystem I
RIP	radiation damage induced phasing SACLA

SAD	single anomalous dispersion
SFX	serial femtosecond crystallography
SONICC	second order non-linear imaging of chiral crystals
TEM	transmission electron microscopy
TR-SFX	time-resolved serial femtosecond crystallography
XFEL	X-ray free electron laser

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Figure 1. First femtosecond X-ray diffraction and reconstruction of the image from soft X-ray single-shot femtosecond X-ray diffraction of an object (reproduced from [1]) with modifications.

Figure 2.

Results from the first serial femtosecond crystallography experiments that used Photosystem I as a model system.

A) the liquid jet extruding from the nozzle, with glow from plasma formed at the interaction region. **B)** front detector diffraction pattern that indicates strong X-ray diffraction to the edge of the detector at 8 Å. **C)** Image from the back detector that unravels shape transforms and fringes between the Bragg peaks. **D)** structure of Photosystem I at 8 Å resolution derived from the SFX data. (Images from [2] with modifications)

Figure 3.

Representation of a phase diagram showing pathways of various crystallization methods. It is typical to obtain smaller crystals as one proceeds deeper into the nucleation zone. Note that precipitant concentration can be substituted for any relevant phase space parameter such as temperature or pH.

TEM images of protein crystals

Figure 4.

The use of TEM as tool for nanocrystal diffraction quality characterization. (a) shows lysozyme and (b) shows GFP tagged RNA polymerase II. Left and right images show crystals before and after injection respectively. Insets show Fourier transforms of images. Reproduced from [3].

Figure 5.

Typical setup for an SFX experiment where a room temperature, hydrated jet containing nanocrystals is introduced to an XFEL beam with 50 fs pulses. These snapshot diffraction patterns are then captured serially by a detector. Reprinted from [4].

Figure 6. GDVN shown in full (**a**), the glued nozzle alone (**b**), and in action jetting PSI crystals (**c**). The LCP injector schematic is shown in (**d**). Reprinted from [5] and [6].

Figure 7.

Setup for a raster scan style fixed target experiment. (a) shows the initial setup, (b) shows the addition of the first silicon nitride layer, (c) shows the addition of crystals in solution, (d) shows the application of the second silicon nitride layer and (e) shows the sealing of the crystals using an Araldite resin. Originally printed in [7].

Figure 8. Structure from *in vivo* crystals determined with a Free Electron Laser (left) crystals of the protease CatB in living insect cells (bottom left) structure of CatB determined by serial femtosecond crystallography from the *in vivo* grown crystals (right) (Images from [8] and [9] with modifications).

Figure 9. Comparison of the structures of the human serotonin receptor solved by SFX and synchrotron radiation. The SFX structure is better defined and shows alternate backbone and side chain conformations.
(Images from [10] with modifications)

Figure 10. SFX structure of the opioid receptor solved by SFX with peptides bound. The figure is reproduced from [11].

Figure 11.

Structure of the angiotensin receptor solved by SFX in lipidic cubic phases, the figure shows a schematic picture of the LCP, the injector and detector in addition to the structure of the receptor with the drug ZD&155 bound.

The figure is reproduced from [12].

Figure 12.

Electron density maps of the dark S_1 -state (**a**) and doubly illuminated S_3 -state (**b**). Both maps are overlaid upon the OEC structure from Umena, Kawakami *et al.* (2011). (**b**) shows potential movement of the CD and AB loops (yellow arrows) and an increase of the distance from the Mn₃O_xCa cubane structure by the dangler Mn. Originally published in [13].

Time-resolved electron densities compared between SFX and Laue

Figure 13.

The dark state structure of PYP solved by SFX (**A**) and Laue (**D**). These are superimposed on the respective time resolved difference electron density maps (**B**, **C**, **E**, **F**). The chromophore and coordinating residues are labelled in (**A**). Arrows in (**B**) highlight the double bond about which the chromophore isomerizes. Comparable time points were taken at 10 ns using SFX (**B**), 32 ns using Laue (**E**) and 1 μ s using SFX (**C**) and Laue (**F**). Purple and red structures correlate to the pR₁ and pR₂ structures involved in the PYP photocycle, showing the ability to resolve inhomogeneous states within a time point. Originally published in [14].

Figure 14.

Schematic of a double focusing mixing jet in which two liquids are mixed prior to jetting and focused with an inert gas. The mixing time delay prior to interaction with the XFEL can be modulated by moving the inner capillary. Originally published in [15].