Membrane protein structural biology has benefitted tremendously from access to micro-focus crystallography at synchrotron radiation sources. X-ray free electron lasers (XFELs) are linear accelerator driven X-ray sources that deliver a jump in peak X-ray brilliance of nine orders of magnitude and represent a disruptive technology with potential to dramatically change the field. Membrane proteins were amongst the first macromolecules to be studied with XFEL radiation and include proof-of-principle demonstrations of serial femtosecond crystallography (SFX), the observation that XFEL data can deliver damage free crystallographic structures, initial experiments towards recording structural information from 2D arrays of membrane proteins, and time-resolved SFX, time-resolved wide angle X-ray scattering and time-resolved X-ray emission spectroscopy studies. Conversely, serial crystallography methods are now being applied using synchrotron radiation. We believe that a context dependent choice of synchrotron or XFEL radiation will accelerate progress towards novel insights in understanding membrane protein structure and dynamics.

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Introduction
Much has been made of the challenge of recovering well diffracting membrane protein crystals due to their inherent instability when extracted from a biological membrane and their schizophrenic nature with both hydrophobic and hydrophilic surfaces [1]. Since membrane proteins are typically more difficult to crystallize than their soluble counterparts, considerable efforts have been made to maximize the data that can be collected from small, weakly diffracting crystals. A key initiative was the development of a dedicated micro-focus beamline at the ESRF from which diffraction data from micro-crystals of bacteriorhodopsin (bR) were collected almost twenty years ago [2] and which was also crucial in developing protocols for data collection from G protein-coupled receptors (GPCRs) [3–6].

X-ray free electron lasers (XFELs) differ fundamentally from synchrotron radiation. In an XFEL the efficiency with which the energy of electrons within an undulator are converted into X-rays is dramatically increased if the electron bunch is sufficiently short and the undulator sufficiently long such that a feedback mechanism (lasing) occurs [7]. Because of the lasing phenomenon, the peak X-ray brilliance (a parameter which describes how many photons can be focused through a small focal spot in a short period of time) achieved is nine orders of magnitude higher than that attained using synchrotron radiation and thus XFELs represent a revolutionary (rather than evolutionary) advancement of X-ray user facilities.

The first XFEL to lase near the Ångstrom wavelength was the LCLS in Stanford, USA [8] at which soft X-ray beamlines were initially commissioned [9] and experimental stations providing a wavelength \( \lambda \sim 1 \) Å soon followed [10]. The Japanese XFEL, SACLA in Harima, was the second XFEL to begin operation [11]. XFEL user facilities currently under construction are the PAL-XFEL in Pohang, South Korea; the European XFEL in Hamburg, Germany; and the SwissFEL in Villgen, Switzerland. All of these infrastructures will support experiments in material science, chemistry, physics, and life science.

To truly exploit the remarkable properties of XFELs for life-science, completely new experimental approaches are required beyond what has been successful using synchrotron radiation. Early life-science experiments using XFEL radiation mostly focused on proof-of-principle demonstrations of these new capabilities [1,2]. These include the development of serial femtosecond crystallography (SFX), where diffraction data is recorded from a continuous stream of microcrystals and complete data is recovered by merging thousands of diffraction images from thousands of microcrystals; demonstration of ‘damage-free’ diffraction data from radiation sensitive samples; demonstration of time-resolved SFX (TR-SFX) as a...
novel high-quality method in structural biology; the visualization of a light-induced ultrafast ‘protein quake’ using time-resolved wide angle X-ray scattering (TR-WAXS); and diffraction studies from 2D crystals at lower-resolution. In this review we summarize recent progress in these areas and emphasize how XFEL radiation has been used to probe membrane protein structure and dynamics.

**Diffraction before destruction**

X-ray induced radiation damage is the process that limits the structural information that can be extracted from any protein sample [13] and a practical dose limit of 30 MGy at 100 K (1 Gray corresponds to one joule deposited per kilogram) has been reported for which useful structural information can be collected [14]. This limits the minimum size of a protein crystal from which useful structural information can be obtained such that, while it is now routine to collect data from protein micro-crystals of approximately 5 μm across using synchrotron radiation [6], it is not possible to collect a diffraction pattern from a single 2 dimensional crystal using synchrotron radiation. The exceptional peak brilliance of XFEL radiation means that it is possible to achieve a submicron focus when delivering approximately $10^{12}$ X-ray photons onto a sample within a few tens of femtoseconds. This corresponds to a dose of tens to hundreds of Mgy which induces a temperature jump of the order of 10 000 K in the time it takes for light to traverse a strand of hair! Although any sample is destroyed by the resulting explosion, molecular dynamics simulations predicted that if the X-ray pulse was sufficiently short (<50 fs) then X-rays would be scattered towards an X-ray detector before the protein responded structurally [15]. This concept has become known as ‘diffraction-before-destruction’ and was first demonstrated experimentally at a soft XFEL by imaging model objects in projection [16].

**SFX**

Nanocrystals of the membrane protein complex photosystem I were used to first demonstrate that interpretable X-ray diffraction data can be collected using focused XFEL radiation [17]. At that time the only suitable beamline available at the LCLS [9] operated at $\lambda \sim 6$ Å, making the large unit cell of photosystem I an ideal choice for this proof-of-principle study. In addition to developing software that could process these data, the key technical developments included the construction of an in-vacuum flexible X-ray detection environment [18] and a novel approach of delivering nanocrystals into the XFEL beam using a continuously flowing liquid microjet [19] (Figure 1). Despite the low-resolution of the data, molecular replacement succeeded and a SFX structure of photosystem I was solved at 8 Å resolution. A second membrane protein structure, a bacterial photosynthetic reaction center, was also solved by SFX using 6 Å radiation following lipidic sponge phase crystallization using batch setups [20]. Although these crystals were not isomorphous with any earlier crystal form, the SFX data at 8 Å resolution were of sufficient quality to allow a new crystal packing to be identified by molecular replacement.

An eagerly awaited demonstration that ‘diffraction-before-destruction’ holds true at atomic (2.1 Å) resolution

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**Figure 1**

Schematic of the setup for a serial femtosecond crystallography (SFX) experiment performed at an XFEL. A continuous stream of microcrystals is injected across the XFEL beam by a microjet (inset) and X-ray diffraction is recorded on an X-ray detector from each and every XFEL pulse. Reprinted with modifications from Boutet et al. [21].
followed shortly afterwards using lysozyme as a model system [21]. Despite each and every crystal receiving an X-ray dose of 33 MGy at room temperature, no evidence for radiation-induced damage of disulphide bonds was observed. Microcrystals of lysozyme in complex with a lanthanide complex were also used to demonstrate the potential of XFEL based SFX for phasing using single-wavelength anomalous scattering [22*]. The first novel structure solved with SFX was that of cathepsin B using microcrystals that grew during protein expression in insect cells [23]. Electron density from the X-ray structure [24*] revealed that a native peptide inhibitor which was otherwise lost during protein purification, was bound to cathepsin B in crystals grown in vivo. A 6 Å resolution SFX structure of the membrane protein complex photosystem II was also reported [25] for which the diffraction power of the microcrystals, rather than the XFEL wavelength, was the limiting factor.

SFX data collected from microcrystals of a bacterial photosynthetic reaction center demonstrated that structural information from membrane proteins can be recovered using XFEL radiation up to a resolution where side-chains and co-factors can be built with confidence [26*] (Figure 2a,b). Since serial crystallography averages diffraction data from thousands of microcrystals, we frequently observed that higher quality electron density maps are achieved when compared with traditional single-crystal cryo-crystallography at the same nominal resolution. The SFX structure of the human serotonin receptor [27**], a GPCR activated by the neurotransmitter serotonin, was reported from data collected from microcrystals grown in a lipidic cubic phase (LCP) [28] and injected using a specially developed microjet [29**]. Although this GPCR structure was very similar to that solved from a single crystal using synchrotron radiation [30], there were some side-chains for which a different conformation could be assigned from the SFX data. Further SFX structures of GPCRs have emerged using XFEL radiation including the structure of an Angiotensin II type 1 receptor in complex with an antagonist [31**], the structure of a human β-opoid receptor with bound agonist or antagonist [32] and the long-awaited crystal structure of a GPCR in complex with arrestin [33**]. Indeed, the rhodopsin:arrestin complex is a major achievement that provides completely new structural insight into how arrestin interacts with GPCRs and thereby redirects cellular signaling processes to GPCR independent pathways. Larger membrane proteins such as P-type ATPases have also proven amenable to SFX [34]. These results clearly establish a framework from which SFX studies of membrane proteins using XFEL radiation can be expected to have major scientific impact into the future.

A major limitation of the liquid microjet [35] used in the first SFX studies is that the volumes of protein required are simply not realistic for all but a few select membrane protein targets. In order to overcome these limitations, a viscous LCP microjet consuming approximately two orders of magnitude less sample has been developed [29**]. This and similar systems, such as the High-Viscosity Extrusion (HVE) injector [36*] and an injection system using microcrystals suspended in a grease matrix [37*], make it possible to collect complete SFX diffraction data with 100 μl or less of microcrystals, which is well within the realms of what is commonly attained at most membrane protein structural biology laboratories. Important technical developments pertaining to membrane protein structure determination by XFELs have recently been discussed [38].

‘Damage free’ XFEL structures

A corollary of ‘diffraction-before-destruction’ is that ‘damage free’ crystal structures of proteins may be recovered using XFEL radiation. Both the lysozyme and photosynthetic reaction center structures solved by SFX showed continuous electron density across disulphide [21] or thioether [26*] bonds that are typically broken by radiation damage at a comparable radiation dose (~33 MGy) when using cryo-crystallography at a synchrotron source. Evidence of damage to the 4Fe–4S clusters of ferredoxin, however, was observed in SFX data collected at either side of the Fe absorption K-edge using an 80 fs X-ray exposure and a dose of 20–30 MGy per crystal [39*]. The high ionization cross-section of the 4Fe–4S cluster was suggested to explain its apparent radiation sensitivity, although the possibility of systematic (non-damage related) differences between the XFEL and synchrotron data could not be ruled out. This work highlights that it is not possible in principle for any X-ray diffraction study to be truly ‘damage free’ and in some cases caution should be applied when making such claims.

Another approach used a standard diffractometer at SACLA with the XFEL beam focused to ~2 μm². A 50 μm step grid scan consequently enabled a series of diffraction images to be collected from large crystals of the membrane protein complexes cytochrome c oxidase [40*] (9.9 MGy/exposure) and photosystem II (1.4 MGy/exposure) [41**] and diffraction data from many large crystals were merged (as has been applied in virus crystallography [42]) to recover a complete ‘damage-free’ diffraction data set. For cytochrome c oxidase, high quality electron density was observed for water molecules at the active site [40*]. For photosystem II, slightly shorter (<0.2 Å differences) manganese-to-manganese atomic separations were recovered within the oxygen evolving complex (OEC) Mn4CaO4 after structural refinement than were found in the PSII structure solved using synchrotron radiation [43]. This result therefore implied that the earlier structure solved using synchrotron radiation may have reflected a photo-reduced OEC, even at cryogenic temperatures. Although the $2F_{obs} - F_{calc}$ electron density
Figure 2

XFEL based structural studies of the bacterial photosynthetic reaction center from Bl. viridis. (a,b), $2F_{\text{calc}} - F_{\text{obs}}$ electron density (blue, contoured at 1.0σ) and the $F_{\text{calc}} - F_{\text{obs}}$ electron density (green, contoured at 3.0σ) for the special pair (a) and a heme (b) cofactor of this integral membrane protein. The magnesium and iron atoms were removed from the model for this calculation. Reproduced from Johansson et al. [26] and both electron density maps were calculated from pdb entry 4AC5. (c) Time resolved wide angle X-ray scattering (TR-WAXS) difference data (laser on minus laser off) for the same reaction center following extreme multi-photon excitation of the cofactors. Experimental difference data (black) were split into four ‘basis spectra’ with time-dependent amplitudes (which are summed to give the red lines). Reproduced from Arnulf et al. [74]. (d) Cartoon representation of the ‘protein quake’ which emerged following structural refinement against one of these four basis spectra. The amplitude is exaggerated fourfold to illustrate the motion and the backbone is colored with the lightest tone representing the starting structure. In this model for the TR-WAXS difference data the epicenter of the quake was the expansion of the bacteriochlorophyll cofactors (red) due to rapid heating by fs laser pulse.
maps for both the synchrotron and XFEL data were similar (Figure 3b,c), it is interesting to note how the residual $F_{\text{obs}} - F_{\text{calc}}$ electron density is distinctly different, with strong positive features associated with Mn1D and Mn2C in XFEL data (Figure 3b) that are not visible for the synchrotron data (Figure 3c). Whether or not these observations may reflect anisotropic motions within the OEC or may arise from crystallographic technicalities (Fourier ripples) due to merging a finite number of partial reflections, we cannot say. The profound importance of photosystem II in biology, the enzyme which harvests light to generate oxygen means that subtle differences in the structure of the Mn$_4$CaO$_5$ cluster will be carefully scrutinized against X-ray absorption data and theoretical calculations [44].

In our view, high-resolution ‘damage-free’ crystal structures of complex membrane proteins solved with XFEL radiation are significant achievements with far reaching implications, although such claims require a certain degree of caution [39]. Since metal centers are incorporated into the active sites of numerous enzymes, XFEL radiation has the potential to impact on our structural understanding of chemical details of catalysis involving radiation sensitive metals.

**Serial millisecond crystallography (SMX)**

SFX was an inspired marriage of complementary technologies within a completely new context that rapidly delivered a novel approach to protein crystallography exploiting XFEL radiation. While radically different from
traditional data-collection approaches, serial crystallography has recently evolved full-circle to now also be applied using synchrotron radiation. A SMX structure of lysozyme \[45^*\] was recovered by flowing a slurry of microcrystals through a quartz capillary held in a focused X-ray beam of the Petra III synchrotron. An exposure of 3 ms per frame was achieved by using a rapid-readout X-ray detector \[46^*\] and diffraction data were collected from hundreds of thousands of randomly oriented microcrystals. In a related experiment, a similar quality SMX structure of lysozyme was recovered by embedding microcrystals within an LCP matrix and delivering them across an X-ray beam at the Swiss Light Source using a high-viscosity microjet \[36^*\]. Serial crystallography data have also been collected using synchrotron radiation from a slurry of microcrystals frozen on a cryo-loop \[47^*\], sandwiched at room temperature between two silicon nitride wafers \[48^*\], or on a semi X-ray transparent microfluidic chip \[49\].

An exciting potential for synchrotron based SMX to impact on membrane protein structural biology was demonstrated using LCP grown microcrystals of bR \[28^*\]. In an historical quirk, these SMX studies were performed at the same microfocus beamline of the ESRF as was used to pioneer microfocus cryo-crystallography \[2\]. The resulting 2.4 Å resolution SMX structure of bR \[50^*\] was of high quality (Figure 4) and is comparable to a similar resolution structure recovered from a single bR crystal at low temperature. Closer examination showed a slight perturbation of helices D, E and F away from helices A and B in the room temperature SMX structure relative to an equivalent single-crystal structure solved from data collected at cryogenic temperature.

Although serial crystallography achieves its full potential only at an XFEL, these results show that it is also a very promising method at synchrotron sources. By contrast to XFELs, synchrotron radiation is far more readily available to users and may become the primary instrument for serial crystallography in the coming years. Current trends towards increasingly brilliant synchrotron radiation facilities \[51\], dedicated microfocus crystallography stations \[6\], rapid readout X-ray detectors \[52,53\] and potentially the use of polychromatic radiation \[54\] should allow SMX data to be collected up to two orders of magnitude more rapidly than for these proof-of-principle demonstrations. SMX studies at synchrotron sources will also help prepare for additional XFEL based experiments and, for many instances, the resolution obtained at a synchrotron source may suffice for a given study.

### Potential impact of serial crystallography

Synchrotron or XFEL based serial crystallography with viscous-jets \[29^*\,36^*,37^*\] has several practical advantages over traditional crystallography: it avoids a tedious step of crystal fishing, it is a room-temperature technique and therefore avoids the need to search for optimal cryo-conditions, by continuously replacing crystals the accumulated effects of radiation damage are minimized, and time-resolved crystallography approaches are possible \[55^*\] since the microcrystals are at room-temperature. The impact of serial crystallography methods on the structural biology of GPCRs is becoming apparent \[27^*\,31^*,32,33^*\] and structural insights from this important family of pharmaceutical targets are expected to impact on future drug-development \[56\]. In combination with automated soaking technologies that screen drug-fragments \[57\], molecular agonists or antagonists, viscous jet-delivery technologies \[29^*,57\] seem likely to become an important tool in the repertoire of applied synchrotron based life-science. It is therefore not difficult to foresee crystalization and sample delivery technologies being combined with microfluidic tools \[49\] that facilitate crystal screening or micro-crystal soaking within a single streamlined automated process that is directly connected to a microfocus beamline. Such developments will greatly improve the efficiency with which structural information can be extracted from very small crystals and will assist serial crystallography to become mainstream in academia and industry.

#### 2D crystallography at an XFEL

2D-crystals consist of a single layer of an ordered array of membrane proteins, typically embedded in a lipid bilayer of a defined lipid mixture. This arrangement is closer to physiological conditions than a 3D crystal and provides accessibility to both sides of the protein for ligand interaction. Due to their small size, 2D-crystals suffer from severe radiation damage at synchrotrons and have therefore previously been examined using electron diffraction at cryogenic temperatures \[58–60\]. The main bottlenecks so far are technical difficulties in preparing well ordered 2D crystals, missing possibilities for correcting for lattice bending through image processing, the missing cone problem when collecting tilted data \[61\] and a lack of automation for 2D crystal production. The latter bottleneck will probably improve in the future as several groups now successfully move 2D crystallography towards automation \[62,63\]. There are relatively few cases where an identical protein crystallizes as both 2D and 3D crystals, but in the case of the Na+/betaine symporter \[64,65\] differences between the 2D and 3D structures were observed that may be functionally important. For other proteins such as bR, visual rhodopsin, photosystem II or the light harvesting II complexes, no significant structural differences arise between different crystallization conditions.

Diffraction-before-destruction is predicted to allow approximately four orders of magnitude more X-rays to be delivered onto a biological sample than for synchrotron radiation \[15\]. This opens up the possibility of measuring 2D diffraction at room temperature at XFELs, which potentially removes a number of technical obstacles.
Serial millisecond crystallography (SMX) and single 2D membrane protein crystal diffraction studies of bacteriorhodopsin (bR) using synchrotron and XFEL radiation respectively. (a) Overview of the 7TM helix structure of bR containing a covalently bound retinal chromophore. (b) Close up view of the 2.4 Å resolution 2F^{obs} - F^{calc} electron density map (blue, contoured at 1σ) around the retinal. The electron density map was calculated from pdb entry 4X31 [50\textsuperscript{[1]}]. (c) An XFEL single shot diffraction pattern of the two-dimensional bR crystal (purple membrane) obtained from solid support measurements [67\textsuperscript{[1]}]. (d) Sum of 106 independent XFEL diffraction patterns from purple membrane patches.

Associated with flash-cooling sensitive crystals. In proof-of-principle studies using the membrane protein bR, 2D diffraction data were recorded to 8 Å resolution [66\textsuperscript{[1]}; (Figure 4c) which could be improved to 7 Å by merging diffraction data records from multiple crystals [67\textsuperscript{[1]}; (Figure 4d). Optimized experimental conditions and improved data processing will allow higher resolution to be reached if the crystal quality is adequate. XFEL based data-collection will also improve the crystal lattice distortion problem since ‘diffraction-before-destruction’ (by contrast to electron radiation) will outrun problems associated with the massive ionization of the sample distorting a 2D crystal lattice and the greater penetration depth of X-rays should help alleviate the missing cone problem. Moreover, computational methods that address the missing cone problem have been developed for electron crystallography and may also be applied to XFEL data [61]. While at present it may be difficult for XFEL based 2D membrane protein crystallography to approach a resolution that is competitive with 3D...
crystallography, the potential for improvement is high by developing more efficient approaches to sample delivery, merging of 2D diffraction data, and computational routines to extract diffraction data at various tilting angles which would allow the reconstruction of the 3D structure of the membrane protein in a lipid environment. Thus the advent of XFEL based 2D crystallography illustrates yet another completely new avenue for pursuing membrane protein structural biology that has the potential to have significant scientific impact in the long term. In particular, it opens up the possibility of structural characterization of membrane proteins interacting with large binding partners on the intracellular and extracellular side in the 2D array or the exiting possibility of kinetic and dynamic studies carried out at room temperature with 2D crystals.

**TR-SFX and WAXS**

One very exciting new opportunity of XFEL radiation is that its short pulse duration ($\leq 100$ fs) opens up the possibility to perform time-resolved diffraction and scattering studies on an ultrafast time-scale. Time-resolved Laue diffraction of light-sensitive proteins using synchrotron radiation is a mature method that relies upon both dark and light-activated data being collected from the same crystal [68,69]. Because SFX does not allow multiple X-ray diffraction images to be collected from a single crystal, there were legitimate concerns as to whether or not the quality of SFX diffraction data would allow time-dependent changes in electron density to be resolved. The first attempt at TR-SFX showed that light induced the disordering of crystals of the photosystem I:ferredoxin complex [70] but these data did not allow any difference density to be calculated. A major breakthrough came with the presentation of TR-SFX data from microcrystals of photoactive yellow protein 10 ns and 1 µs after photo-activation [55**]. The resulting difference electron density maps (laser on minus laser off) were superior to similar maps calculated from data collected using synchrotron radiation, presumably because a higher population of the photoexcited species could be attained in microcrystals than for larger crystals and because of the power of multi-crystal averaging of diffraction data. SFX [25] and TR-SFX studies have also been pursued on the membrane protein photosystem II, but the resolution attained to date has not been beyond 4.5 Å [71*,72,73], which is not sufficient to convincingly reveal light-induced changes in electron density [55**]. The lower resolution of microcrystals relative to larger crystals (1.95 Å resolution) [41**] implies that the small-size of the crystals used in the TR-SFX studies may have limited the resolution, although the careful control of the crystallization process needed to achieve well diffracting crystals of PSII [43] does not rule out future improvements using microcrystals. One interesting and impressive technical development was the simultaneous measurements of X-ray emission spectroscopy (XES) and diffraction from microcrystals of PSII, which enabled the oxidation state of the OEC Mn$_4$CaO$_5$ to be observed thereby confirming that the photoexcitation laser flash had successfully initiated the reaction and that the OEC was not photo-reduced by X-rays [71*,72].

A complementary technology, time-resolved wide angle X-ray scattering (TR-WAXS), was used at an XFEL to observe light-driven motions in a bacterial photosynthetic reaction center that arise within picoseconds. Proof-of-principle studies were performed using extreme multi-photon excitation of this photosynthetic protein and the TR-WAXS data (Figure 2c) revealed a global protein motion (Figure 2d) that arose with a half-rise of 1.4 ps and decayed with a half-life of 44 ps [74**]. One elegant aspect of TR-WAXS is that time-dependent changes in the X-ray scattering are sensitive to both changes in protein structure (typically seen as oscillations visible for 0.1 Å$^{-1} \leq q \leq 1.0$ Å$^{-1}$, Figure 2c) and, for wider angles (1.5 Å$^{-1} \leq q \leq 2.5$ Å$^{-1}$), changes in the packing of the solvent, which correlates with its temperature. Thus TR-WAXS data simultaneously measured the dynamics of a protein conformational change and the propagation of heat from the cofactors (which were heated by the pump-laser pulse) through the protein and into the solvent, convincingly demonstrating that an ultrafast ‘protein quake’ like motion propagated through the protein more rapidly than thermal diffusion. Similar TR-WAXS studies of light-induced motions in samples of myoglobin with bound carbon monoxide (CO) [75] also revealed ultrafast protein motions. These motions originated from the active site, at which the ligating bond of CO to the active-site heme was broken by flash-photolysis, and propagated rapidly throughout the protein. Quite remarkably, these data showed coherent global protein oscillations with a period of 3.6 ps that were damped on a time-scale of tens of picoseconds. These structural findings herald the beginning of a new field of ultrafast structural biology that has only become accessible due to the ultrafast properties of XFEL radiation.

**Conclusions**

The breath taking development of XFEL sources that generate femtosecond X-ray pulses with peak brilliance one billion times that available from synchrotron radiation has generated tremendous excitement across many scientific disciplines. Membrane protein structural biologists have exploited these new opportunities to study protein structure and dynamics in completely novel ways. We can now routinely collect ‘damage free’ structural data from micron or submicron scale crystals, perform time-resolved studies of protein dynamics on an ultrafast time scale, and a promising technique of XFEL based 2D membrane protein diffraction is emerging. With XFEL structural biology now five years of age [12*], an increasing trend is that XFEL sources are being used when they offer a competitive advantage over more conventional synchrotron radiation or single particle cryo-electron microscopy.
approaches. As the field moves from proof-of-principle studies towards the search for novel scientific discoveries, truly breakthrough science will build from the creative input of individuals who understand where the key technical advantages lie and how these complementary structural techniques can be used in synergy to tackle the most challenging scientific questions in structural biology.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

24. Demonstration that SFX data at an XFEL is of sufficient quality to allow heavy-metal phasing in a model system.
30. First demonstration that XFEL based SFX can recover a structure of a membrane protein at a resolution where side-chains are easily assigned.
32. Development of an LCP injector suitable for XFEL based SFX studies of membrane proteins grown in lipidic phases.
35. Determination of the structure of a previously unknown GPCR with XFEL radiation. This structure of a highly relevant pharmacological target highlights the importance of the new technology for molecular pharmacology.
38. SFX structure of a GPCR in complex with arrestin solved using XFEL radiation. Arresting binding block further G-protein mediated signaling and is therefore of tremendous interest in pharmacology.

37. Sugahara M et al.: Grease matrix as a versatile carrier of • proteins for serial crystallography. *Nat Methods* 2015, 12:61. Demonstration of another high-viscosity injector suitable for XFEL based SFX. In this case microcrystals are immersed in grease to increase viscosity before being delivered across a focused XFEL beam.


39. Nass K et al.: Indications of radiation damage in ferredoxin • microcrystals using high-intensity X-FEL beams. *J Synchrotron Radiat* 2015, 22:225. An important contribution to the debate as to whether or not SFX structures represent ‘damage free’ structures. These data indicate that heavy metal sites in ferredoxin show signs of X-ray damage when intense 80 fs pulses are used to collect data.

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45. Stellato F et al.: Room-temperature macromolecular serial • crystallography using synchrotron radiation. *IUCrJ* 2014, 1:204. First demonstration of SMX using synchrotron radiation. In this case data were collected from crystals of lysozyme flowing through an X-ray capillary.


First direct measurement of an ultrafast protein-quake whereby ultrafast protein motions in a photosynthetic reaction center were induced by a laser flash and followed by X-ray scattering using XFEL radiation.
