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Serial crystallography for structure-based drug discovery

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Abstract

Rational drug discovery has greatly accelerated the development of safer and more efficacious therapeutics, assisted significantly by insights from experimentally determined 3D structures of ligands in complex with their targets. Serial crystallography (SX) with X-ray free electron lasers (XFELs) has enabled structural determination using micrometer- or nanometer-size crystals. This technology, applied in the last decade to solve structures of notoriously difficult-to-study drug targets at room temperature, has now been adapted for use in synchrotron radiation facilities. Ultrashort time scales allow time-resolved characterization of dynamic structural changes and pave the road to study the molecular mechanisms by ‘molecular movie’. This article summarizes the latest progress in serial crystallography technology and deliberate its demanding applications in future structure-based drug discovery.

Keywords

X-ray free electron laser (XFEL); Serial femtosecond crystallography; Synchrotron-based serial crystallography; Structure-based drug discovery (SBDD); GPCR ligand swapping; Time-resolved serial crystallography

Membrane Protein Structural Studies with Serial Crystallography

In 1976, attention was brought up on how knowledge of a protein structure could help drug design [1]. In the following decades, ‘rational drug design’ has rapidly developed to become a mature cyclical process (Figure 1) and routinely used to design and optimize potential hits using detailed structural information on complexes of pharmaceutical targets and their ligands. Protein X-ray crystallography has proven to be the most effective tool for these studies. Structure determination at each cycle in the iterative process provides invaluable knowledge to the medicinal chemist to validate hypothesized molecular interactions of ligands with their targets and rationalize structure-activity relationships (SAR). Membrane

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proteins comprise approximately 30% of the proteome of eukaryotic cells [2] including human cells and due to the important and varied physiological roles that they play, are targets of ~60% of drugs currently in the market [3]. Although they are of critical biomedical value, only ~2% of the structures in the Protein Data Bank (PDB) are of membrane proteins. This limited access to structural information has retarded rational drug discovery progress. Several key technologies developed since 2000 have dramatically increased their number [4, 5]. The lipidic cubic phase (LCP) method and serial femtosecond crystallography (SFX) with X-ray free electron lasers (XFELs) are part of this technology breakthrough.

XFELs produce fully coherent and ultrashort X-ray pulses with a peak brilliance (i.e., number of photons/pulse), about ten orders of magnitude higher than 3rd generation synchrotron sources. In SFX [6], microcrystals are streamed in synchrony with femtosecond X-ray pulses which are short enough that diffraction is captured before significant radiation damage happens and is the basis for the concept of ‘diffraction-before-destruction’ [7–9]. It enables the use of small and weakly diffracting crystals, which are recorded in a ‘one crystal one shot’ mode. However, variations in beam intensity, crystal size and random orientations raised major questions on data quality [7]. Data collected in XFELs are still and mostly partial reflections per snapshot [10]. To mitigate these factors, sample delivery systems were, thus, optimized to synchronize crystal delivery with XFEL pulses, increasing high hit rates [11] and new strategies were also devised to index, integrate, and merge collected data [10]. Serial crystallography (SX) also enables time-resolved (TR) studies and the possibility of observing dynamic structural changes upon ligand binding or substrate interaction leading to the production of molecular movies and new insights on the mechanisms underlying the interaction/activation of ligands on target proteins [12]. Taken together, these developments in sample delivery, detector sensitivity, data collection methods, and data analysis software have boosted microcrystallography and TR studies to an unprecedented era (Figure 2). Beginning in 2014, efforts were made to adapt SX methods for synchrotron sources, with both monochromatic and polychromatic sources, providing more opportunities for advanced structural studies and addressing the lack of beamtime in XFEL facilities [13–17]. In this review, we will discuss the history, the latest developments, and applications of serial crystallography technology and elaborate on its impact on structure-based drug discovery (SBDD).

Innovative Developments to Facilitate Serial Crystallography

SX was initially developed in 2011 to collect diffraction data in the newly built XFEL facility, Linac Coherent Light Source (LCLS) at SLAC National Accelerator Laboratory in the United States. LCLS was the first hard X-ray free electron laser source launched in 2009 [7, 18]. This milestone was immediately followed by the commissioning of four other operational XFEL sources worldwide (Figure 3): the Spring-8 Angstrom Compact Free-Electron Laser (SACLA) in Japan built in 2011, the European-XFEL (EuXFEL) in Germany and the Pohang Accelerator Laboratory-XFEL (PAL-XFEL) in South Korea in 2017, and the Swiss-Free Electron Laser (SwissFEL) in Switzerland in 2018. In 2020, LCLS was upgraded to LCLS-II. Shanghai High repetition rate XFEL and Extreme light facility (SHINE) in China is scheduled to open in 2025.

The concept of ‘diffraction-before-destruction’ forms the underlying basis for the SX in which one crystal produces one useful ‘still’ diffraction pattern. Diffraction images produced by tens of thousands of crystals in random orientations and synchronously streamed with X-ray pulses are merged to complete one dataset. During the data collection process, a ‘hit rate’ is defined as images containing discernible diffraction patterns. The hit rate is monitored to ensure enough diffraction images with indexable Bragg peaks are captured. In order to minimize sample consumption and increase the diffraction hit rate, a diversity of sample delivery technologies have been developed (Figure 4). In the early days of SFX experiments, a gas dynamic virtual nozzle (GDVN) injection device was commonly used, with a relatively high sample consumption of ~10 $\mu\text{L}/\text{min}$ [19]. However, the GDVN injector is not optimal for viscous samples such as membrane protein crystals embedded in LCP [20–23]. In 2013, development and implementation of the high-viscosity extrusion (HVE) injector lead to the first high-resolution SFX structure of a human G Protein-Coupled Receptor (GPCR) which belongs to a family of notoriously challenging membrane proteins with massive pharmaceutical value [24]. The HVE injector [25] is now widely used with a variety of viscous samples with controlled flow rates of 0.001–0.3 $\mu\text{L}/\text{min}$, including but not limited to agarose [26], high-molecular weight poly (ethylene oxide) (PEO) [16], hydrogels [27], and hydroxyethyl cellulose [28]. Other delivery systems have now been introduced which are chosen depending on experimental requirements. These include double-flow focusing nozzle (DFFN) [29], mix-and-inject system (MISC) [30], microfluidic electric sample holder (MESH/coMESH) [31, 32], acoustic levitation [33], and fixed target methods (nylon loop [34], silicon chip [35], microfluidic chip device [36], and conveyor belt drive system [37]) (Figure 4).

In addition, variation in XFEL pulse intensity and focus as well as the random orientation of microcrystals in the interaction center are being addressed by continued development of advanced XFEL data analysis software to improve the accuracy of experimental structure factors [10]. Introduction of automated data collection, processing and refinement pipelines are now accelerating the hit-to-lead cycle in drug discovery with the high-throughput determination of several structures of complexes [38–41].

Monochromatic and Pink Beam Serial Crystallography at Synchrotrons

To address the shortage of XFEL facilities worldwide (5 fully functional ones, Figure 3), 3rd generation synchrotrons with higher flux density, multiple focusing optics, micro-focused beam, and upgraded detectors is becoming an alternative synchrotron analogue to SFX with XFELs. It was shown that room-temperature serial millisecond crystallography (SMX) with micro-focused monochromatic beamline at synchrotrons can outrun most secondary radiation damage [42, 43] and thus enable the use of microcrystals that are radiation sensitive; it also enables ultrafast TR-SX studies at sub-picosecond time resolution. The proof-of-principle SMX experiment was performed at PETRA III at DESY and yielded a 2.1 Å lysozyme structure from microcrystals streamed in a glass capillary [45]. The first room-temperature LCP-SMX experiments were used to solve the structure of bacteriorhodopsin crystals grown in LCP at the ESRF Microfocus Beamline (ID13) equipped with the HVE injector [15], in parallel with the structure determination of lysozyme with crystals reconstituted into LCP at the PXII beamline at the Swiss Light Source (SLS) [25]. Later, the

human adenosine A_{2A} receptor (A_{2A}AR) structure was resolved in an LCP-SMX study at GM/CA at the Advanced Photon Source (APS) in Argonne National Laboratory [16]. SMX with synchrotrons is currently under development to extend its use for time-resolved studies [46].

With monochromatic X-ray beam, only ‘still’ diffraction can be recorded with partial reflections in each pattern. By removing the monochromator and using only a mirror for high-energy cut-off with a moderate increase in bandwidth, a pink-beam is produced with a flux 100 times higher than monochromatic beam, producing high quality Laue diffraction requiring more multifaceted processing [14]. Following the first pink beam SX experiments by a fixed target approach at the BioCARS 14-ID beamline at APS [14], the first high-viscosity injector-based pink beam SX experiments were performed at the same facility [17]. The structures of A_{2A}AR and proteinase K (PK) were determined to 4.2 Å and 1.8 Å resolution using 24 and 4 consecutive 100 ps X-ray pulse exposures, respectively. Strong PK data were processed using existing approaches, whilst weaker A_{2A}AR data required an alternative strategy using CrystFEL. On top of the low signal-to-noise ratio in the A_{2A}AR study, the other major challenge is the outdated data processing software [14, 17]. Although it has been recently improved, processing time was quite excessive. The high beam flux and the use of Laue diffraction presents new opportunities for the TR experiments with microcrystals to study structural changes in real-time at pink beam synchrotron beamlines.

Advantages of Serial Crystallography for Drug Discovery

The nature of SX and the various related innovative technology developments powers SBDD. The peak brilliance of XFEL pulses and the microfocus beam size enables the structure determination of those difficult-to-study targets, using micrometer- or nanometer-size crystals in a carrier material extruded by several newly developed sample delivery devices, and to a relatively higher resolution in most cases [22, 47–49]. For example, the first complex structure of rhodopsin bound to arrestin was determined with XFELs in 2015 [50], providing the first structural insights of the signal coupling of GPCR with arrestin that could enrich the knowledge of biased ligands for drug discovery. Microcrystals of two human angiotensin II receptors grown in LCP were used to determine the structure of AT₁R at 2.9 Å and AT₂R 2.8 Å resolution, relatively higher than the one using synchrotron [51, 52]. The highest resolution of GPCR structure, A_{2A}AR, was achieved by SFX technology at 1.7 Å [53]. Most significantly, the structure of the human smoothened receptor (SMO) in complex with cyclopamine could only be determined by SFX, as attempts to do synchrotron data collection resulted in poor diffraction quality and higher mosaicity, likely related to cryo-cooling [47]. More recently, structure determinations of 2 human melatonin receptors (MT₁ and MT₂) by SFX [54, 55] allow the virtual ligand screening from a library with over 150 million molecules to discover the new signaling mechanisms and pharmacology conferring the receptor selectivity [56].

In addition, the concept of ‘diffraction-before-destruction’ utilizes the ultrashort XFEL pulses to acquire diffraction data at room temperature, avoiding cryo-cooling process that freezes the protein molecules in a specific conformation. In 2013, a modified SFX data collection method using LCP injector resulted in the first room temperature GPCR structure,

human serotonin 5-HT_{2B} receptor bound with agonist ergotamine [24]. Compared with the synchrotron structure of 5-HT_{2B} collected at 100 K (PDB ID 4IB4), the largest structural deviation observed in its XFEL structure (PDB ID 4NC3) demonstrated an unique distribution of thermal motions, which are likely to be more physiologically relevant [24]. A more outstanding example is the δ -opioid receptor bound with a bi-functional peptide solved by XFEL with more atomic details on the binding site, therefore shed much more structural insights on the binding mechanism comparing to the structure from the synchrotron data, and deliver more accurate pharmacological profiles [57]. SX determines room temperature structure, which is more likely closer to the physiological condition and may more accurately represent the structural conformation in the native process with ligand interaction and activation.

Secondary radiation damage has been shown to produce alterations such as disulfide bond breakage or elongation, decarboxylation of Asp or Glu, C-S bond cleavage in Met, and more severely, reduction of metal clusters in proteins [58]. It has been demonstrated that such radiation damage can be minimized in XFEL data collection for radiation-sensitive biological systems. For example, SFX data of photosynthetic reaction center collected with 40 fs XFEL pulses of moderate energy (corresponding to a dosage of 33 MGy at cryogenic temperature per pulse), unveiled a covalent thioether bond, which was missing in a cryogenic synchrotron structure with 77 MGy dose [59]. Therefore, room temperature SX with minimized secondary radiation damage may preserve more specific chemical and structural information.

Multiple Ligands Structure Determination with Serial Crystallography

Structure-based virtual ligand screening has been widely used in rational drug discovery, involving the docking of potent ligands into a known protein target structure to score the ligand binding affinity to the drug target [60]. On top of this computational technique [61], soaking and co-crystallization experiments between drug target and multiple ligands are being explored to provide more detailed structural information on the drug binding modes and chemical optimization. In classic soaking experiment, a single crystal of unliganded (apo) protein is incubated with ligand solution at desired concentration for a few seconds to several days to achieve complete ligand exchange. However, ligand soaking has not been productive with the challenging proteins, including GPCRs, where co-crystallization is the most common technique when multiple ligands need to be investigated. However, co-crystallization of the same receptor with different ligands requires further optimization from construct design to crystallization. Such time and effort consuming process hinders SBDD toward the hit and lead optimization for important drug targets.

In 2019, a method based on SX was reported for the rapid determination of multiple co-crystal structures for the target GPCR in complex with several ligands. Human β_2 -adrenergic receptor (β_2 AR) was employed as a validating model protein in this development. 6 ligands ranging from inverse agonists, antagonists, and arrestin-biased agonists were screened and resulted in 8 room temperature SFX structures [62]. More importantly, β_2 AR in complex with carvedilol and propranolol are novel, not previously reported. In this ligand swapping method, a transient ligand, with relatively faster off-rate compared with the crystallization

timescale, is added to stabilize the receptor during purification and reconstitution into LCP. The proteinladen LCP is incubated with precipitant solution containing a large excess of the ligand of interest in a syringe, in which the ligand exchange occurs as well as the co-crystallization of receptor and the ligand of interest. Capitalizing on the LCP-SFX method, it is possible to collect a complete dataset at an XFEL facility within 2 hours for each ligand-receptor co-crystallization. The resulting complex structures were shown to be identical with those previously determined. This method is so called Complex-LCP, crystallization of membrane proteins using transient ligand exchange in LCP, which sets up the stage for SAR studies with accelerated pace.

Time-Resolved Serial Femtosecond Crystallography (TR-SFX) towards Molecular Movies

Previous time-resolved studies relied on large crystals mounted on a goniometer using Laue diffraction with polychromatic X-ray beams [63]. TR studies at synchrotrons were limited by the X-ray pulse length, which has been improved from milliseconds to picoseconds time scales in the last few decades [64]. Though light-activating system is a well-measurable target in TR studies, the size of the crystal is another limitation since molecules that absorb activating photons may result in a decrease in transmission with increasing path length. Microcrystals allow more synchronized reaction initiation throughout the crystal and all molecules within a single crystal can be activated by the pump system. The emerging SFX has become an ideal technique to study TR systems with the real-time capture of the protein conformational changes as a function of the X-ray laser emission delay (in the order of femtoseconds to microseconds) (Figure 2c) [65]. Therefore TR-SFX can provide relatively high resolution of a rest state of the structure and render molecular movies about structural transitions stimulated by photo-sources or chemical ligands, with the innovative systems such as pump-probe method and mix-and-inject system [66].

The first TR-SFX experiment was performed at LCLS with co-crystals of photosystem I (PSI) in complex with ferredoxin, whose diffraction data were then collected at the time delay of 5 to 10 μ s after photoexcitation. The conformational change in the shifting dark and pumped states within the PSI was revealed from the collection of several diffraction images of single crystals in the pulse duration around 70 fs [67]. In 2016, a ‘molecular movie’ of bacteriorhodopsin comprising 13 snapshots of conformational changes using 10-fs X-ray pulses at SACLA deciphered the native pathway for proton transfer at room temperature [68]. The photoactivation of the light-adapted bacteriorhodopsin microcrystals was demonstrated by the different diffraction pattern and electron density maps in several time delays between the light laser (pump) and x-ray pulse (probe) [68]. Furthermore, the study about drug resistance of many pathogens to most of the antibiotic with beta-lactam has been carried out with the assistance of SX. b-lactamase, a bacterial enzyme that is able to break the β -lactam ring of antibiotics such as penicillin by hydrolysis to inactivate the drug. After mixing with ceftriaxone, microcrystals of β -lactamase from *Mycobacterium tuberculosis* (BlaC) were probed by x-ray pulses and diffraction patterns were collected in the time delay from 30 ms to 2 s after mixing. TR-SFX revealed both the rest and activated state of β -lactamase including transitions and conformational change of the antibiotic cleavage

primarily associated with Ser-70 [69]. Additionally, the ultrafast conformational changes in the myoglobin with Fe-carbon monoxide bond was analyzed with the pump-probe method at LCLS [70]. Microcrystals of the myoglobin-carbon monoxide complex were injected into the XFEL beam and photolyzed by the optical laser in the duration of 150 fs, diffractions were then probed with time delays from 0.5 ps to 150 ps. Movements of the structural elements from the lighted to dark states were demonstrated from the molecular cartoon with thousands of electron density maps [70].

TR-SFX displays its huge potential on the dynamic studies of membrane proteins, including the insights on the structural basis, the knowledge about intermediates and conformational transitions and even the demonstrations of energy landscape associated with dynamic motion. In addition, the native physiological information can be obtained because laser illumination and data collection from the SFX setup both take place at room temperature.

Concluding Remarks and Future Perspectives

SX with high intensity and ultrashort x-ray pulses overcomes the barriers of traditional synchrotron approaches, including crystal size, cryo-cooling and harvesting. It also boosts structure determination efforts from microcrystallography to dynamic TR studies. The extension of SX from the original XFEL resources to synchrotrons opens the door for structure determination both static and dynamic, possibly accelerating the compound optimization process for drug discovery. With further development of more efficient and compatible sample delivery systems and automation, SX should evolving into a high-throughput process. Despite the successive evolution in SX, specific questions remain to be addressed (see Outstanding Questions).

SBDD has been hampered on some challenging targets, especially with human membrane proteins like GPCRs. With respect to the emerging fragment-based drug discovery (FBDD), a super-stabilized GPCR construct is difficult to obtain for structure determination with low affinity fragments. Soaking various ligands into crystals of unliganded proteins is a common method used in SBDD. However, GPCRs are highly dynamic in the unliganded state, making it next to impossible to crystallize with current approaches. Previous studies were more focused on structure-based docking and virtual ligand screening [71]. The development of a new method called 'Complex-LCP', taking advantage of LCP-SFX technology, bespeaks a bright future to determine multiple co-crystal structures of a target receptor in complex with a variety of ligands. More accurate structural insights on ligand binding mode and receptor activation mechanism can be unveiled to facilitate the drug development. Although the ligand exchange experiment may tolerate small conformational changes, co-crystallization should be performed if dramatic conformational changes are expected, like during receptor activation process.

The ultrashort pulses and minimized radiation damage in XFELs enable the dynamic trace of structural changes on the time resolution upon the functional activation using TR-SFX. Latest progress in TR-SFX pave the way to the future studies on the spatial and temporal dynamics of pharmaceutical targets with the candidate drugs. Most biological drug targets are activated upon ligand binding in rapid time, therefore, the dynamic route that ligand

binds and dissociates provides important evidence for SBDD. Furthermore, the molecular movie enables the rational drug design to stabilize the intermediate conformations during activation. The current TR studies include, but not limited to, the pump-probe method for light-activating systems, mixing jet to image chemical dynamics with the reactions initiated by pH change or diffusing substrates [72], Cryojet or Cryostream used for reactions initiated by temperature jump, and electrode system for electric-field stimulated reaction [73–75]. Mix-and-inject of crystals with such fast-biological reactions could be challenging for TR studies, as the mixing efficiency and diffusion rate of substrates to crystal surface may not be fast enough, and accurate time resolution is difficult to be controlled and evaluated. Recent microfluidic system studies have displayed more precisely control of sample mixing for TR-SFX studies. The other idea is to chemically synthesize photo-switchable or photocaged ligands, so that the activation can be achieved by different wavelength light laser, for instance, pump-probe delivery system. Meanwhile, novel ligands design will be considered to transition an inverse agonist or antagonist to an agonist and thereby observing the dynamic activation process. The activation could be triggered by lasers for photocaged ligands as discussed above, or possibly by other approaches such as pH shifts, temperature changes, or even use of electric fields, according to the differentiated synthetic chemical groups.

Taken together, with all rapid developments in SX, this technology shows the great promising prospect in the structure-based drug discovery, especially in the direction of ‘Complex-LCP’ and the molecular movie of a functional process.

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References

1. Beddell CR et al. (1976) Compounds Designed to Fit a Site of Known Structure in Human Haemoglobin. *British Journal of Pharmacology* 57 (2), 201–209. [PubMed: 938794]
2. Almén M et al. (2009) Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. *BMC Biology* 7 (1).
3. Gong J et al. (2019) Understanding Membrane Protein Drug Targets in Computational Perspective. *Current Drug Targets* 20 (5), 551–564. [PubMed: 30516106]
4. Kloppmann E et al. (2012) Structural genomics plucks high-hanging membrane proteins. *Current Opinion in Structural Biology* 22 (3), 326–332. [PubMed: 22622032]
5. Grabowski M et al. (2016) The impact of structural genomics: the first quinquennial. *Journal of Structural and Functional Genomics* 17 (1), 1–16. [PubMed: 26935210]
6. Neutze R et al. (2000) Potential for biomolecular imaging with femtosecond X-ray pulses. *Nature* 406 (6797), 752–757. [PubMed: 10963603]
7. Chapman HN et al. (2011) Femtosecond X-ray protein nanocrystallography. *Nature* 470 (7332), 73–77. [PubMed: 21293373]
8. Spence JCH (2018) X-ray lasers for structure and dynamics in biology. *IUCrJ* 5 (Pt 3), 236–237.
9. Nass K (2019) Radiation damage in protein crystallography at X-ray free-electron lasers. *Acta Crystallographica Section D Structural Biology* 75 (2), 211–218. [PubMed: 30821709]

10. White TA et al. (2013) Crystallographic data processing for free-electron laser sources. *Acta Crystallographica Section D Biological Crystallography* 69 (7), 1231–1240. [PubMed: 23793149]
11. Martiel I et al. (2019) Strategies for sample delivery for femtosecond crystallography. *Acta Crystallographica Section D Structural Biology* 75 (2), 160–177. [PubMed: 30821705]
12. Standfuss J and Spence J (2017) Serial crystallography at synchrotrons and X-ray lasers. *IUCrJ* 4 (2), 100–101.
13. Gati C et al. (2014) Serial crystallography on in vivo grown microcrystals using synchrotron radiation. *IUCrJ* 1 (Pt 2), 87–94.
14. Meents A et al. (2017) Pink-beam serial crystallography. *Nature Communications* 8 (1).
15. Nogly P et al. (2015) Lipidic cubic phase serial millisecond crystallography using synchrotron radiation. *IUCrJ* 2 (Pt 2), 168–76.
16. Martin-Garcia JM et al. (2017) Serial millisecond crystallography of membrane and soluble protein microcrystals using synchrotron radiation. *IUCrJ* 4 (Pt 4), 439–454.
17. Martin-Garcia JM et al. (2019) High-viscosity injector-based pink-beam serial crystallography of microcrystals at a synchrotron radiation source. *IUCrJ* 6 (Pt 3), 412–425.
18. McNeil B (2009) First light from hard X-ray laser. *Nature Photonics* 3 (7), 375–377.
19. DePonte DP et al. (2008) Gas dynamic virtual nozzle for generation of microscopic droplet streams. *Journal of Physics D: Applied Physics* 41 (19).
20. Cherezov V and Caffrey M (2007) Membrane protein crystallization in lipidic mesophases. A mechanism study using X-ray microdiffraction. *Faraday Discussions* 136.
21. Lan Z et al. (2018) Overview of Biochemical Assays in Lipidic Cubic Phase. *Trends Biochem Sci.*
22. Stauch B and Cherezov V (2018) Serial Femtosecond Crystallography of G Protein-Coupled Receptors. *Annu Rev Biophys* 47, 377–397. [PubMed: 29543504]
23. Al-Sahouri Z et al. (2018) The Lipid Cubic Phase as a Medium for the Growth of Membrane Protein Microcrystals. In *X-ray Free Electron Lasers*, pp. 87–107.
24. Liu W et al. (2013) Serial femtosecond crystallography of G protein-coupled receptors. *Science* 342 (6165), 1521–4. [PubMed: 24357322]
25. Botha S et al. (2015) Room-temperature serial crystallography at synchrotron X-ray sources using slowly flowing free-standing high-viscosity microstreams. *Acta Crystallogr D Biol Crystallogr* 71 (Pt 2), 387–97. [PubMed: 25664750]
26. Conrad CE et al. (2015) A novel inert crystal delivery medium for serial femtosecond crystallography. *IUCrJ* 2 (4), 421–430.
27. Kováčová G et al. (2017) Viscous hydrophilic injection matrices for serial crystallography. *IUCrJ* 4 (4), 400–410.
28. Sugahara M et al. (2017) Hydroxyethyl cellulose matrix applied to serial crystallography. *Scientific Reports* 7 (1).
29. Oberthuer D et al. (2017) Double-flow focused liquid injector for efficient serial femtosecond crystallography. *Scientific Reports* 7 (1).
30. Stagno JR et al. (2016) Structures of riboswitch RNA reaction states by mix-and-inject XFEL serial crystallography. *Nature* 541 (7636), 242–246. [PubMed: 27841871]
31. Sierra RG et al. (2012) Nanoflow electrospinning serial femtosecond crystallography. *Acta Crystallographica Section D Biological Crystallography* 68 (11), 1584–1587. [PubMed: 23090408]
32. Sierra RG et al. (2015) Concentric-flow electrokinetic injector enables serial crystallography of ribosome and photosystem II. *Nature Methods* 13 (1), 59–62. [PubMed: 26619013]
33. Tsujino S and Tomizaki T (2016) Ultrasonic acoustic levitation for fast frame rate X-ray protein crystallography at room temperature. *Scientific Reports* 6 (1).
34. Cohen AE et al. (2014) Goniometer-based femtosecond crystallography with X-ray free electron lasers. *Proceedings of the National Academy of Sciences* 111 (48), 17122–17127.
35. Hunter MS et al. (2014) Fixed-target protein serial microcrystallography with an x-ray free electron laser. *Scientific Reports* 4 (1).

36. Coquelle N et al. (2015) Raster-scanning serial protein crystallography using micro- and nano-focused synchrotron beams. *Acta Crystallogr D Biol Crystallogr* 71 (Pt 5), 1184–96. [PubMed: 25945583]
37. Beyerlein KR et al. (2017) Mix-and-diffuse serial synchrotron crystallography. *IUCrJ* 4 (Pt 6), 769–777.
38. Barty A et al. (2014) Cheetah: software for high-throughput reduction and analysis of serial femtosecond X-ray diffraction data. *Journal of Applied Crystallography* 47 (3), 1118–1131. [PubMed: 24904246]
39. White TA et al. (2016) Recent developments in CrystFEL. *Journal of Applied Crystallography* 49 (2), 680–689. [PubMed: 27047311]
40. Sauter NK (2015) XFEL diffraction: developing processing methods to optimize data quality. *Journal of Synchrotron Radiation* 22 (2), 239–248. [PubMed: 25723925]
41. White TA (2014) Post-refinement method for snapshot serial crystallography. *Philosophical Transactions of the Royal Society B: Biological Sciences* 369 (1647).
42. Owen RL et al. (2012) Outrunning free radicals in room-temperature macromolecular crystallography. *Acta Crystallographica Section D Biological Crystallography* 68 (7), 810–818. [PubMed: 22751666]
43. Warkentin M et al. (2012) Global radiation damage: temperature dependence, time dependence and how to outrun it. *Journal of Synchrotron Radiation* 20 (1), 7–13. [PubMed: 23254651]
44. Redecke L et al. (2013) Natively inhibited *Trypanosoma brucei* cathepsin B structure determined by using an X-ray laser. *Science* 339 (6116), 227–230. [PubMed: 23196907]
45. Stellato F et al. (2014) Room-temperature macromolecular serial crystallography using synchrotron radiation. *IUCrJ* 1 (4), 204–212.
46. Weinert T et al. (2019) Proton uptake mechanism in bacteriorhodopsin captured by serial synchrotron crystallography. *Science* 365 (6448), 61–65. [PubMed: 31273117]
47. Weierstall U et al. (2014) Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography. *Nat Commun* 5, 3309. [PubMed: 24525480]
48. Liu W et al. (2014) Femtosecond crystallography of membrane proteins in the lipidic cubic phase. *Philosophical Transactions of the Royal Society B: Biological Sciences* 369 (1647).
49. Batyuk A et al. (2016) Native phasing of x-ray free-electron laser data for a G protein coupled receptor. *Sci Adv* 2 (9), e1600292. [PubMed: 27679816]
50. Kang Y et al. (2015) Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* 523 (7562), 561–567. [PubMed: 26200343]
51. Zhang H et al. (2015) Structure of the Angiotensin Receptor Revealed by Serial Femtosecond Crystallography. *Cell* 161 (4), 833–844. [PubMed: 25913193]
52. Zhang H et al. (2017) Structural basis for selectivity and diversity in angiotensin II receptors. *Nature* 544 (7650), 327–332. [PubMed: 28379944]
53. Weinert T et al. (2017) Serial millisecond crystallography for routine room-temperature structure determination at synchrotrons. *Nat Commun* 8 (1), 542. [PubMed: 28912485]
54. Johansson LC et al. (2019) XFEL structures of the human MT2 melatonin receptor reveal the basis of subtype selectivity. *Nature* 569 (7755), 289–292. [PubMed: 31019305]
55. Stauch B et al. (2019) Structural basis of ligand recognition at the human MT1 melatonin receptor. *Nature* 569 (7755), 284–288. [PubMed: 31019306]
56. Stein RM et al. (2020) Virtual discovery of melatonin receptor ligands to modulate circadian rhythms. *Nature* 579 (7800), 609–614. [PubMed: 32040955]
57. Fenalti G et al. (2015) Structural basis for bifunctional peptide recognition at human δ opioid receptor. *Nature Structural & Molecular Biology* 22 (3), 265–268.
58. Weik M et al. (2000) Specific chemical and structural damage to proteins produced by synchrotron radiation. *Proceedings of the National Academy of Sciences* 97 (2), 623–628.
59. Johansson LC et al. (2013) Structure of a photosynthetic reaction centre determined by serial femtosecond crystallography. *Nat Commun* 4, 2911. [PubMed: 24352554]
60. Cavasotto C and Orry W, A. (2007) Ligand Docking and Structure-based Virtual Screening in Drug Discovery. *Current Topics in Medicinal Chemistry* 7 (10), 1006–1014. [PubMed: 17508934]

61. Vera J et al. (2014) istar: A Web Platform for Large-Scale Protein-Ligand Docking. PLoS ONE 9(1).
62. Ishchenko A et al. (2019) Toward G protein-coupled receptor structure-based drug design using X-ray lasers. IUCrJ 6 (6), 1106–1119.
63. Hajdu J and Johnson LN (1990) Progress with Laue diffraction studies on protein and virus crystals. Biochemistry 29 (7), 1669–78. [PubMed: 2184884]
64. Schotte F et al. (2012) Watching a signaling protein function in real time via 100-ps time resolved Laue crystallography. Proceedings of the National Academy of Sciences 109 (47), 19256–19261.
65. Kupitz C et al. (2014) Serial time-resolved crystallography of photosystem II using a femtosecond X-ray laser. Nature 513 (7517), 261–265. [PubMed: 25043005]
66. Spence JCH (2017) XFELs for structure and dynamics in biology. IUCrJ 4 (4), 322–339.
67. Aquila A et al. (2012) Time-resolved protein nanocrystallography using an X-ray free electron laser. Optics Express 20 (3).
68. Nango E et al. (2016) A three-dimensional movie of structural changes in bacteriorhodopsin. Science 354 (6319), 1552–1557. [PubMed: 28008064]
69. Olmos JL et al. (2018) Enzyme intermediates captured “on the fly” by mix-and-inject serial crystallography. BMC Biology 16 (1).
70. Barends TRM et al. (2015) Direct observation of ultrafast collective motions in CO myoglobin upon ligand dissociation. Science 350 (6259), 445–450. [PubMed: 26359336]
71. Beuming T and Sherman W (2012) Current Assessment of Docking into GPCR Crystal Structures and Homology Models: Successes, Challenges, and Guidelines. Journal of Chemical Information and Modeling 52 (12), 3263–3277. [PubMed: 23121495]
72. Tripathi S et al. (2012) pH Dependence of the Photoactive Yellow Protein Photocycle Investigated by Time-Resolved Crystallography. Biophysical Journal 102 (2), 325–332. [PubMed: 22339869]
73. Šrajer V and Schmidt M (2017) Watching proteins function with time-resolved x-ray crystallography. Journal of Physics D: Applied Physics 50 (37).
74. Schmidt M et al. (2013) Protein energy landscapes determined by five-dimensional crystallography. Acta Crystallographica Section D Biological Crystallography 69 (12), 2534–2542. [PubMed: 24311594]
75. Hekstra DR et al. (2016) Electric-field-stimulated protein mechanics. Nature 540 (7633), 400–405. [PubMed: 27926732]

Outstanding Questions

- What is the future direction to develop more efficient and compatible sample delivery systems in order to increase the throughput of serial crystallography experiments?
- How to improve the data processing strategies for weak and sparse serial Laue diffraction patterns collected in pink beam serial crystallography?
- How to evaluate and control the data quality collected at synchrotron sources comparable to those from XFELs?
- How to overcome the radiation damage in SMX experiments at synchrotrons?
- What is the future development to enable time-resolved serial crystallography studies at synchrotrons in micro- to sub-microsecond resolution, the typical time scale of biological processes?
- How to synthesize physiologically functional photo-switchable or photocaged ligands and evaluate dynamic structural information of their interactions with pharmaceutical targets?

Highlights

- X-ray free electron lasers (XFELs) outrun radiation damage to enable room-temperature structure determination of challenging proteins with microcrystals, overcoming the limitations of conventional crystallography to power up structure-based drug discovery (SBDD).
- Latest developments in sample delivery system and data analysis strategies have facilitated serial crystallography.
- Ultrafast XFEL pulses allow time-resolved studies at sub-picosecond scale, providing new structural insights into conformational dynamics of ligands with pharmaceutical targets.
- The evolution of experimental operation from XFELs to synchrotron beamlines boosts the applicability and accessibility of serial crystallography.
- Complex- lipidic cubic phase (LCP) technology has been applied in serial crystallography to determine multiple structures of ligand-protein complexes, shortening the timelines for structure determination and accelerating the application in SBDD.

Trends in Pharmacological Sciences

Figure 1. Rational Drug Discovery Workflow.

Yellow box are tasks for target validation; green box elucidates the process of hit identification; blue box demonstrates the hit-to-lead optimization process; red box stands for the lead to drug verification. Magenta highlighted boxes represent the impact of X-ray crystallography in drug discovery.

Abbreviations: 3D, three-dimensional; FBDD, fragment-based drug discovery; HTS, high throughput screening; SBDD, structure-based drug discovery; LBDD, ligand-based drug discovery; MD, molecular dynamics; SAR, structure-activity relationship; ADME, absorption, distribution, metabolism, and excretion.

Figure 2. Statistics on Protein Structure Determination by Serial Crystallography.

a) Growth in the number of protein structures determined with SFX at XFELs (blue bars) and with SX at synchrotrons (red bars), deposited in Protein Data Bank (PDB); b) Unique structures determined with SX of soluble proteins (green bar), and membrane proteins (yellow bars), deposited in PDB; c) Publications of static structure determination (magenta bars) and time-resolved studies (orange bars); d) Numbers of structures of membrane proteins and soluble proteins determined by conventional crystallography and SX. Abbreviations: SFX, serial femtosecond crystallography; XFEL, X-ray free electron laser; SX, serial crystallography

Figure 3. Synchrotron Radiation (available for serial crystallography) and XFEL Facilities Distribution Worldwide.

6 XFEL facilities are shown as in pictures with the year of launch. 13 synchrotron radiation facilities available for the use of serial crystallography are represented in the red dots.

Abbreviations: ALS, Advanced Light Source; APS, Advanced Photon Source; CHES, Cornell High Energy Synchrotron Source; NSLS-II, National Synchrotron Light Source II, ESRF, European Synchrotron Radiation Facility; Sirius, the new Brazilian synchrotron light source; Soleil, an acronym for ‘Optimized Light Source of Intermediate Energy to LURE’; DESY, Deutsches Elektronen Synchrotron; MAX IV, a next-generation synchrotron radiation facility in Lund, Sweden; Spring-8, Super Photon ring-8 GeV.

Figure 4. X-ray Free Electron Laser Generation and SFX Experimental Set Up.

a) free electron laser is generated by a travel path of the linearly accelerated electrons through an undulator, focused by K-B mirrors to a size of micrometers, then delivered to the interaction with microcrystals. Diffraction data were recorded by a fast read-out detector as ‘one crystal, one hit’ mode for the final structure determination. A variety of b) injector-based sample delivery devices and c) fixed-target sample delivery systems are represented. Abbreviations: K-B, Kirkpatrick-Baez; LCP, lipidic cubic phase; HVE, high-viscosity extrusion; CSPAD, cornell-SLAC pixel array detector; GDVN, gas dynamic virtual nozzle; DFFN, double-flow focusing nozzle; MISC, mix-and-inject system; MESH, microfluidic electric sample holder