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Using synchrotrons and XFELs for time-resolved X-ray crystallography and solution scattering experiments on biomolecules

Matteo Levantino¹, Briony A Yorke², Diana CF Monteiro³, Marco Cammarata⁴ and Arwen R Pearson²



Time-resolved structural information is key to understand the mechanism of biological processes, such as catalysis and signalling. Recent developments in X-ray sources as well as data collection and analysis methods are making routine time-resolved X-ray crystallography and solution scattering experiments a real possibility for structural biologists. Here we review the information that can be obtained from these techniques and discuss the considerations that must be taken into account when designing a time-resolved experiment.

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Introduction

The relationship between the structure of proteins and their function is determined and controlled by their dynamic properties. From the process of polypeptide translation by the ribosome, through folding to form the functional protein and finally as the protein undergoes its particular function in the cell or across the cell membrane, molecular motions play a crucial role. A complete understanding of protein function therefore requires experiments that are able to probe both structural and dynamic properties. X-ray crystallography and solution scattering are routinely used methods to examine molecular structures (Figure 1). However, when used in a time-resolved manner, these methods can also enable the observation of functionally related structural changes

and dynamics over a broad range of spatial and temporal resolutions.

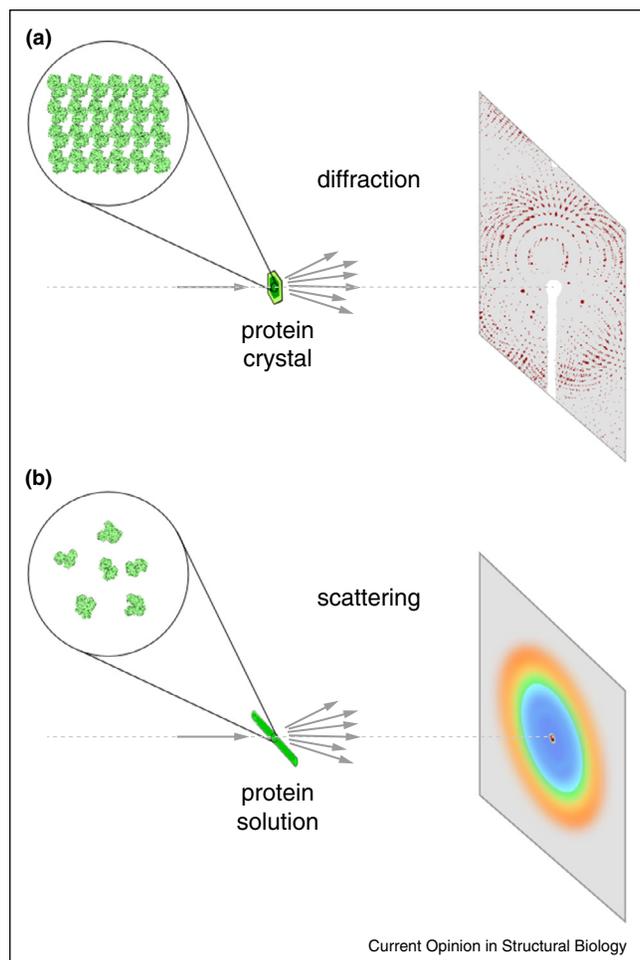
The time-scales of biologically relevant processes range from fs (10^{-15} s) to hours and so the design of a time-resolved experiment must be matched to the time-scale of the process of interest (Figure 2). For example, while an enzymologist might wish to focus on early steps in a reaction mechanism occurring on fs–ns time-scales, a molecular biologist might be interested in the large scale conformational rearrangements associated with ligand binding or signal transduction that occur in μ s–ms. Recent developments in X-ray sources, beamline instrumentation and data collection methodologies now provide structural biologists with a suite of experimental tools to simultaneously probe biomolecular structure and dynamics.

X-ray methods to study biomolecular structure and dynamics

Time-resolved X-ray crystallography is the technique that is currently able to provide the greatest spatial and temporal resolution over the whole structure of a protein. However, it is limited by the requirement for well diffracting protein crystals. Although great advances have been made in this field, crystallization remains an art rather than an exact science. Excitingly, the possibility of performing X-ray diffraction experiments on *in vivo* grown protein microcrystals has been recently demonstrated [1,2*]. However, major protein classes such as membrane proteins and intrinsically disordered proteins, both key players in the life of the cell, are difficult or impossible to crystallize. This challenge is compounded by the delicate nature of protein crystals. Large conformational changes can either destroy the crystalline lattice, making X-ray data impossible to collect, or be restricted by the crystal packing, preventing reaction progression in the crystal.

An alternative approach to study the dynamics of biological macromolecules is to monitor the time evolution of X-ray scattering by liquid solutions of the biomolecules of interest. This has recently emerged as a powerful method to investigate relevant biological reactions on systems that are either not easy to crystallize or undergo large scale conformational changes that cannot take place within a crystalline environment, such as quaternary structure

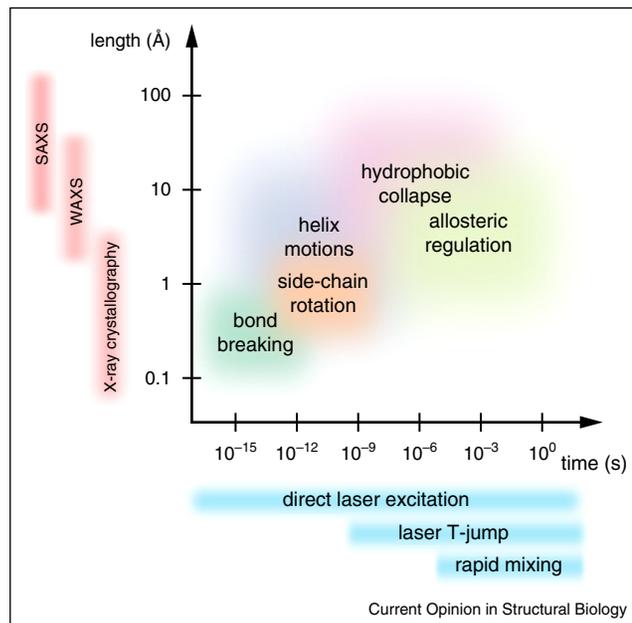
Figure 1



Time-resolved X-ray diffraction from protein crystals and scattering from protein solutions provide complementary information. Crystallography can yield atomic resolution information about the whole molecule, but requires that the crystal remains intact during the reaction. Solution scattering is extremely sensitive to structural changes with the advantage that crystals are not required. However, associating the scattering changes with specific structural rearrangements remains difficult.

rearrangements, macromolecular folding or protein aggregation. The ability to investigate such a large variety of biologically relevant processes comes at the expense of spatial resolution and unbiased retrieval of structural information. The X-ray scattering pattern from an ensemble of randomly oriented and unequally spaced macromolecules lacks the characteristic Bragg peaks of crystalline samples (Figure 1). Nevertheless, changes in the overall dimension/mass can be readily measured in the SAXS (small-angle X-ray scattering) region of the scattering pattern while higher resolution information (relative position of different subunits, domains, or secondary structure elements) has fingerprints in the WAXS (wide-angle X-ray scattering) region (Figure 3).

Figure 2



Conformational changes of biomolecules can be triggered with different methods (i.e. direct laser excitation, laser T-jump and rapid mixing) and monitored with time-resolved X-ray crystallography, wide-angle X-ray scattering (WAXS) or small-angle X-ray scattering (SAXS). The experimental technique and the triggering method must be matched with the time-scale and type of structural change to be investigated. Although crystallography can — in principle — be used to detect conformational changes extending up to tens-of-Angstroms, often only relatively small motions can be accommodated by the crystalline environment and thus be observed in a time-resolved experiment.

Designing a time-resolved experiment

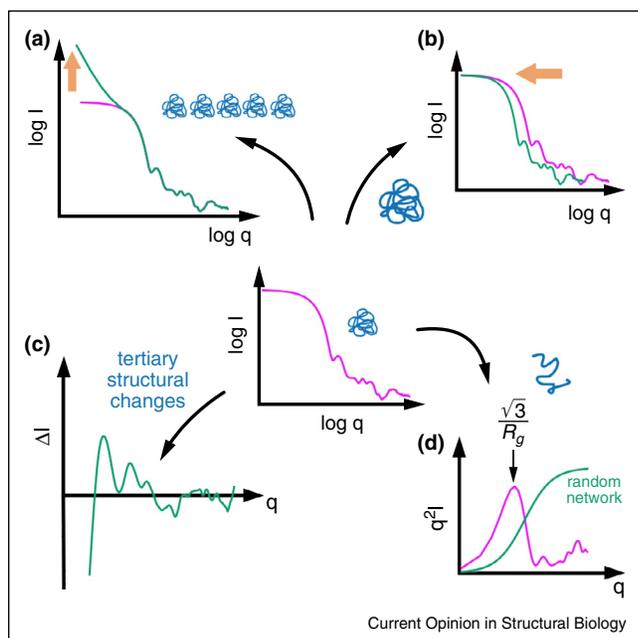
When planning a time-resolved experiment a number of factors must be taken into consideration. First, the time-scales of interest must be identified as these will define the X-ray source and experimental protocol required. Once the decision as to type of experiment and time-resolution required has been reached then the question of reaction initiation must be addressed.

Choice of source and experimental protocol

In any time-resolved experiment, the key consideration is signal to noise (S/N). The fundamental time-resolution of the experiment is determined by the time it takes to deliver enough photons onto the detector for a measurable signal to be obtained. How fast the required number of photons can be delivered depends on the brilliance of the X-ray source.

Third generation synchrotron radiation sources are able to provide X-ray pulses as short as ~ 100 ps and, by using polychromatic Laue radiation, can deliver 10^{10} photons per pulse at the sample. Such X-ray photon flux is sufficient to perform time-resolved experiments at time-scales

Figure 3



Schematic illustration of the X-ray solution scattering profile of a typical globular protein (central panel, in magenta) and qualitative signal changes (in green) as a result of (a) protein aggregation; (b) protein expansion; (c) protein tertiary structural changes; (d) protein unfolding. In the case of tertiary structural changes, expected difference signals in the WAXS region are of the order of $\sim 1\%$ and are thus better visualized as scattering difference patterns (ΔI versus q). The unfolding of globular proteins can be qualitatively assessed using a Kratky plot ($q^2 I$ versus q). In the case of globular proteins, the Kratky plot presents a peak at $q = \sqrt{3}/R_g$, where R_g is the protein radius of gyration.

as short as 100 ps. For lower time-resolutions, a train of 100 ps X-ray pulses rather than a single pulse can be used to probe the sample, thus increasing the S/N.

X-ray free-electron lasers (XFELs) are an exciting new alternative if sub-picosecond time-resolution is needed. XFELs are able to produce extremely short (~ 10 fs) X-ray pulses with a number of photons $\sim 10^2$ – 10^3 times higher than the ~ 100 ps pulses produced at synchrotrons. Moreover, XFEL pulses can be focused down to $\sim 0.1 \mu\text{m}$ without significant reduction in the number of X-ray photons delivered to the sample. XFELs have been used to perform the first steady-state serial femtosecond crystallography (SFX) experiments on protein crystals [3,4]. In SFX a suspension of nano-to-micro crystals is delivered, usually via a liquid jet, into the path of the X-ray beam. Single diffraction patterns are recorded by a fast pixel array detector whenever an X-ray/crystal hit occurs and these patterns are then assembled into a complete dataset. Similar experiments have been shown to be feasible also at synchrotrons [5–8]. The main advantage of XFELs for dynamic studies is thus that they allow us to address scientific questions that require sub-ps time-resolution.

Several options for time-resolved data collection strategies exist. All are based on the well established classical pump-probe method, where the reaction initiation (pump) is followed by an X-ray probe (or probe series) after a time-delay. In the simplest case of a reversible system which relaxes back to its initial state in a reasonable time (within a few seconds) and without appreciable radiation damage buildup, this can be repeated many times to increase S/N, explore different time-delays and, for crystallography, all needed crystal orientations. In practice, even for readily reversible biological systems, such as naturally photoactive proteins, radiation damage is a significant problem, necessitating either a constant delivery of fresh sample or the dilution of radiation damage over a large sample volume for both scattering and diffraction experiments. An elegant modification for diffraction experiments is the Ratio method where a probe-pump-probe sequence is used and the ratio between the pre and post pump X-ray intensities [9,10], or between independent reference and post pump intensities [11], is determined for each reflection. A recently proposed alternative approach to classic pump-probe experiments is to use Hadamard transform based X-ray probe pulse sequences to improve the time-resolution of experiments using slow detectors or flux limited X-ray sources [12].

Reaction initiation

There are two requirements that must be considered for reaction initiation. First, the process must be triggered in a significant fraction of the sample and second, the triggering event must be faster than the process of interest.

Rapid mixing

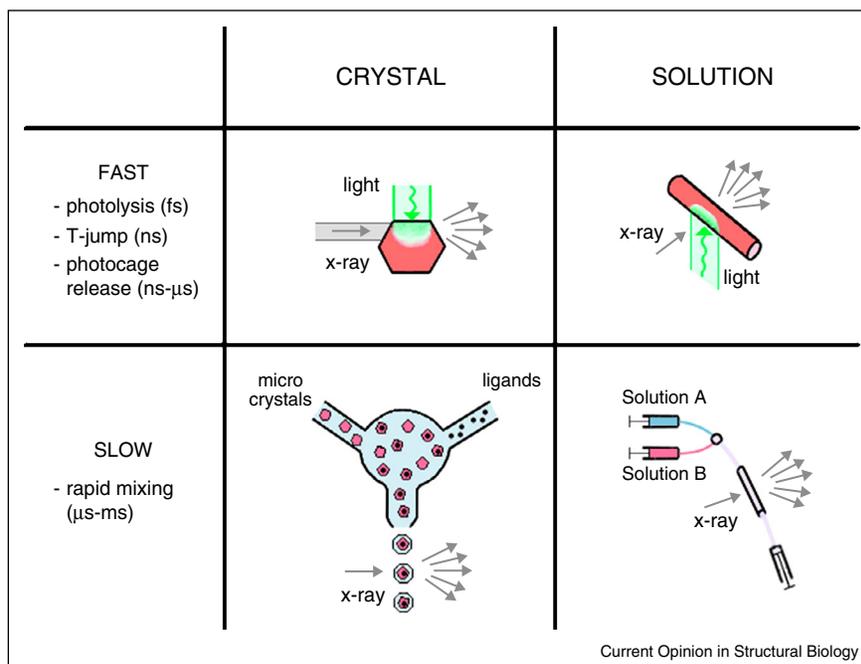
Classical stopped-flow and continuous-flow apparatus are available at several synchrotron beamlines dedicated to time-resolved X-ray scattering experiments,* and can be used to study reactions triggered by the rapid mixing of different solutions in the ms [13,14] or even μs time-scales if a continuous-flow apparatus with T-shaped micromixers [15] or jet-in-jet devices are employed [16,17]. In the case of protein crystals, the simplest approach to reaction initiation is to allow the substrate to diffuse into the crystal, where for micro or nano-crystals diffusion times are on the order of ms and μs respectively (Figure 4) [18].

Direct and indirect light activation

To achieve higher time-resolutions reactions must be triggered with a short laser light pulse. This can be used to either directly photoexcite a light sensitive reaction

* BL4-2 beamline at SSRL (SLAC National Accelerator Laboratory, USA); BioCAT 18ID beamline at APS (Argonne National Laboratory, USA); SAXS-1 beamline at LNLS (Brazil); ID02 beamline at ESRF (France); SWING beamline at SOLEIL (France); P12 BioSAXS beamline at PETRA III (DESY Hamburg, Germany); I22 beamline at Diamond Light Source (UK); BL45XU-SAXS beamline at Spring-8 (Japan).

Figure 4



Techniques for triggering protein conformational changes either in crystalline or solution samples. Light activation: light pulses are used to photolyze a bond, rapidly increase the temperature of the solvent surrounding the protein, or to induce the release of a photocaged compound. Rapid mixing: a solution containing either protein crystals or solubilized protein can be mixed with a solution containing a substrate or a denaturant agent and then probed with X-rays to monitor any structural change occurring as a function of time.

[19] or to create a temperature jump [20] that initiates, for example, a folding or enzymatic reaction.

The simplest biological processes to investigate with time-resolved methods are those occurring in inherently light sensitive proteins, many of which can be activated efficiently with short (fs–ns) laser pulses. Unsurprisingly the majority of sub-ms time-resolved structural experiments to date have focused on such systems.

For non-naturally photoactivatable systems, either photolabile protecting groups can be chemically added to the natural substrate, or photocaged unnatural amino acids can be site-specifically incorporated into the protein itself [21^{*}]. However, this is non-trivial. Site-specific labelling of proteins is dependent on the reactivity and accessibility of the residues or requires genetic incorporation of the unnatural amino-acid (with only a few photocaged unnatural amino acids currently available), and the photocaging of substrates can be synthetically challenging [22]. Furthermore, the correct choice of photocaging scaffold is essential as this determines the time-scales accessible.

In all light activated reactions (whether natural or not) the major limitation of photoinitiation is the laser penetration through the sample (Figure 4). Again a balance must be struck between a high sample concentration, desirable to

provide good S/N (and unavoidable in a crystallographic experiment), and the need for the laser to uniformly excite the sample volume to be probed.

Data analysis

The result of an X-ray time-resolved experiment, either on a crystalline or solution sample, is usually a set of average multi-dimensional data (integrated Bragg peaks, scattering curves, etc.) at different time-delays from the reaction triggering event. The signal evolution at time-scales longer than few tens of ps, can usually be attributed to exchanges in the population of a finite set of different molecular species each with a well defined (not time-dependent) structure. In such cases, singular value decomposition (SVD) or global analysis (GA) are usually used to extract a limited number of ‘components’ describing the time-evolution of the experimental signals [23–26]. When a plausible kinetic model is available, it can be used to analyze the data in terms of a generalized GA approach[#] that retrieves the fingerprint of a given physical species on the basis of its predicted time-evolution [11,24,27]. It must be noted that at ultrafast

[#] While SVD or GA analysis are implemented in several commercial or freely available scientific software packages, a custom fitting routine that matches the kinetic model chosen to describe the observed kinetic changes is required in the case of generalized GA.

time-scales (from fs to ps) the very concept of population kinetics is of course meaningless and every single time point has to be treated as an independent component. The signal time-evolution in this time regime is typically due to structural relaxations within a given minimum of the system free-energy landscape. This corresponds to the observation of a protein within a given energy state dynamically changing its structure as opposed to the observation of an energy-activated transition between two different energy states.

Once the time-independent components have been found, the analysis usually proceeds very differently for crystallography and solution scattering. In both cases, several software packages able to perform standard data analysis have been developed and are freely distributed. Crystallographic data are analyzed in the same way as static data, starting with a known structure to phase the experimentally determined intensities, followed by examination of difference electron density maps to identify the structural changes that occur over time. In the case of solution scattering data, the basis patterns are usually analyzed in terms of global parameters (molecular mass, radius of gyration) or by *ab initio* methods [28]. However, the attribution of experimental time-resolved signals to specific biomolecular motions is usually difficult. This is only partly due to the intrinsic low spatial resolution of solution scattering data. Indeed, although it is conceptually impossible to retrieve a high resolution (all-atom) model of a biomolecule conformation using only its solution X-ray scattering pattern, the combination of TR-SAXS and TR-WAXS data with other detailed structural information either from complementary spectroscopic techniques or from computational approaches can greatly facilitate the structural interpretation of the data [29,30,31*].

Examples/case studies

Time-resolved X-ray crystallography

Time-resolved crystallographic studies allow the structural changes associated with bond breakage/formation, isomerization, side-chain rotation etc. to be visualized across the whole protein, this information when combined with spectroscopic data can provide compelling evidence for the mechanisms of enzyme catalysis and protein function. The dynamic motions that accompany chemical mechanisms, can be used to explain how the structure of the entire protein contributes to both the lowering of the activation energy required for enzyme catalysis and the structural rearrangements that enable signalling and energy transfer. Incomplete photoactivation and mixtures of states produced by parallel reaction pathways can introduce uncertainty during data analysis, by performing time-resolved experiments at multiple temperatures it is sometimes possible to separate the overlapping energy profiles of parallel processes to determine intermediate structures, energy barriers and kinetics.

The photoisomerization and subsequent structural rearrangement of photoactive yellow protein (PYP) has been extensively studied using synchrotron based time-resolved crystallography (most recently [11,32]). Discrepancies between density functional theory calculations and the mechanism deduced from the analysis of time-resolved data have since been highlighted [33,34]. It may be possible to address these discrepancies with ultra-fast experiments using an FEL. Indeed, recent time-resolved SFX experiments on PYP have shown that high-resolution difference electron density maps show improved detail when compared to those obtained from Laue diffraction data [35**]. This has enabled the analysis of the relative occupancies of two intermediate states (pR_1 and pR_2) at 1 μ s. Serial time-resolved crystallography has also been applied to photosystem I (PSI) [36] and photosystem II (PSII) [37,38]. For PSI, changes in diffraction were observed at 5 and 10 μ s following photoexcitation matching the time-scale of electron transfer from PSI to ferredoxin. The FEL data also allowed the observation of changes in the oxygen evolving complex in PSII indicating binding of a second water molecule during the photocycle.

Time-resolved X-ray solution scattering

A straightforward application of time-resolved X-ray scattering is the study of large conformational changes. These can be monitored by changes in the macromolecule radius of gyration (R_g) from the analysis of the SAXS scattering profile at very small angles in combination with rapid mixing techniques in which a protein solution is combined for example with a substrate solution or a denaturant one [14,15,39].

Time-resolved scattering has also been used in protein aggregation studies. The formation of protein aggregates is naturally and easily detectable in the SAXS region of the scattering pattern [40–42]. However, the large sample heterogeneity (distribution of molecular species with different sizes simultaneously present in solution) can limit the data analysis and makes the data interpretation challenging.

Smaller, faster structural changes, such as allosteric motions or tertiary local changes in protein conformation can also be very effectively studied using time-resolved X-ray scattering [27,43–44,45*,46]. We will illustrate this using the prototypical model system for allosteric proteins, human hemoglobin (Hb). TR-WAXS, in combination with laser photolysis, was used to study the kinetics of the R \rightarrow T transition in solution [24,47]. The changes in the relative position of the Hb subunits associated with the R \rightarrow T transition are evident in the WAXS region of the X-ray scattering patterns and the time-resolved data can be accurately described in terms of linear combinations of the deoxyHb (T-state) WAXS pattern and a HbCO (R-state) WAXS pattern [24,48]. Interestingly,

the rate of the Hb allosteric transition was shown to be of the order of $\sim 1 \mu\text{s}$, that is much faster than previously assumed on the basis of time-resolved optical spectroscopic data [49]. Moreover, in spite of the high heterogeneity that characterizes a cell suspension, it was possible to observe the Hb allosteric transition within intact red blood cells thus demonstrating the feasibility of in-cell TR-WAXS experiments [50].

The advent of XFELs has opened the possibility of investigating the structural dynamics of proteins with fs resolution [51,52]. The extension of time-resolved X-ray scattering techniques to the use of XFELs is straightforward, as demonstrated by a TR-WAXS study on the *Blastochloris viridis* photosynthetic reaction center [53*]. A subpicosecond motion of the reaction center, reminiscent of the so-called ‘protein-quake’ proposed by Frauenfelder and coworkers for myoglobin (Mb) already 30 years ago [54] was observed, although under conditions of extensive multiphoton absorption by the protein chromophores [53*]. More recent experiments on carbonmonoxy Mb [55*] showed without any ambiguity that protein helices are indeed able to move in the ps time-scale. The strain released by the heme chromophore after mild laser photoexcitation conditions is transferred to the Mb polypeptide chain within a few ps and triggers a damped oscillation of the entire protein. Although synchrotron TR-WAXS experiments clearly indicated that structural changes were occurring within 100 ps [27], their characterization has only been possible with XFELs.

Summary

With the recent developments in X-ray sources (micro-focus beamlines and FELs), high throughput sample delivery methods and data analysis protocols time-resolved X-ray crystallography and solution scattering are becoming increasingly accessible to the wide community interested in investigating biologically relevant macromolecular conformational changes. However, the major obstacle that remains is the challenge of reaction triggering in systems whose mechanisms are not naturally photoinduced. This will only be overcome by bringing together the expertise of X-ray and instrumentation method developers with that of chemists and biologists to develop new and interesting methods of initiation for research questions that will benefit from time-resolved data.

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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

1. Redecke L, Nass K, DePonte DP, White TA, Rehders D, Barty A, Stellato F, Liang M, Barends TRM, Boutet S *et al.*: **Natively inhibited *Trypanosoma brucei* cathepsin B structure determined by using an X-ray laser.** *Science* 2013, **339**:227-230.
2. Gallat F-X, Matsugaki N, Coussens NP, Yagi KJ, Boudes M, Higashi T, Tsuji D, Tatano Y, Suzuki M, Mizohata E *et al.*: **In vivo crystallography at X-ray free-electron lasers: the next generation of structural biology?** *Philos Trans R Soc Lond B: Biol Sci* 2014, **369**:20130497.
- The ability to grow crystals directly within the expressing cell is extremely exciting and offers a route towards the crystallization of macromolecules and complexes that are difficult to extract and purify. This paper provides an excellent review of the current progress in *in vivo* crystallography.
3. Chapman HN, Fromme P, Barty A, White TA, Kirian RA, Aquila A, Hunter MS, Schulz J, DePonte DP, Weierstall U *et al.*: **Femtosecond X-ray protein nanocrystallography.** *Nature* 2011, **470**:73-77.
4. Schlichting I: **Serial femtosecond crystallography: the first five years.** *IUCrJ* 2015, **2**:246-255.
- This comprehensive review of the developments in macromolecular crystallography using FEL sources is an excellent starting point for readers new to the field.
5. Gati C, Bourenkov G, Klinge M, Rehders D, Stellato F, Oberthür D, Yefanov O, Sommer BP, Mogk S, Duszchenko M *et al.*: **Serial crystallography on in vivo grown microcrystals using synchrotron radiation.** *IUCrJ* 2014, **1**:87-94.
6. Stellato F, Oberthür D, Liang M, Bean R, Gati C, Yefanov O, Barty A, Burkhardt A, Fischer P, Galli L *et al.*: **Room-temperature macromolecular serial crystallography using synchrotron radiation.** *IUCrJ* 2014, **1**:204-212.
7. Botha S, Nass K, Barends TRM, Kabsch W, Latz B, Dworkowski F, Foucar L, Panepucci E, Wang M, Shoeman RL *et al.*: **Room-temperature serial crystallography at synchrotron X-ray sources using slowly flowing free-standing high-viscosity microstreams.** *Acta Crystallogr Sect D* 2015, **71**:387-397.
8. Coquelle N, Brewster AS, Kapp U, Shilova A, Weinhausen B, Burghammer M, Colletier J-P: **Raster-scanning serial protein crystallography using micro- and nano-focused synchrotron beams.** *Acta Crystallogr Sect D* 2015, **71**:1184-1196.
9. Coppens P, Pitak M, Gembicky M, Messerschmidt M, Scheins S, Benedict J, Adachi S, Sato T, Nozawa S, Ichiyanagi K *et al.*: **The RATIO method for time-resolved Laue crystallography.** *J Synchrotron Radiat* 2009, **16**:226-230.
10. Coppens P, Fournier B: **New methods in time-resolved Laue pump-probe crystallography at synchrotron sources.** *J Synchrotron Radiat* 2015, **22**:280-287.
11. Schotte F, Cho HS, Kaila VRI, Kamikubo H, Dashdorj N, Henry ER, Graber TJ, Henning R, Wulff M, Hummer G *et al.*: **Watching a signaling protein function in real time via 100-ps time-resolved Laue crystallography.** *Proc Natl Acad Sci USA* 2012, **109**:19256-19261.
12. Yorke BA, Beddard GS, Owen RL, Pearson AR: **Time-resolved crystallography using the Hadamard transform.** *Nat Methods* 2014, **11**:1131-1134.
- This paper describes a new method of time-resolved data collection where the time-dependent information is integrated onto the detector using an encoded Hadamard probe pulse sequence. Recording of data with all permutations of the pulse sequence allows the time-dependent signal to be recovered by a simple transformation. The method provides a route to fast time-resolved data collection using slow sources and/or detectors.
13. West JM, Xia J, Tsuruta H, Guo W, O’Day EM, Kantrowitz ER: **Time evolution of the quaternary structure of *Escherichia coli* aspartate transcarbamoylase upon reaction with the natural**

- substrates and a slow, tight-binding inhibitor. *J Mol Biol* 2008, **384**:206-218.**
14. Konuma T, Kimura T, Matsumoto S, Goto Y, Fujisawa T, Fersht AR, Takahashi S: **Time-resolved small-angle X-ray scattering study of the folding dynamics of barnase.** *J Mol Biol* 2011, **405**:1284-1294.
 15. Akiyama S, Takahashi S, Kimura T, Ishimori K, Morishima I, Nishikawa Y, Fujisawa T: **Conformational landscape of cytochrome c folding studied by microsecond-resolved small-angle X-ray scattering.** *Proc Natl Acad Sci USA* 2002, **99**:1329-1334.
 16. Trebbin M, Krüger K, DePonte D, Roth SV, Chapman HN, Förster S: **Microfluidic liquid jet system with compatibility for atmospheric and high-vacuum conditions.** *Lab Chip* 2014, **14**:1733-1745.
 17. Wang D, Weierstall U, Pollack L, Spence J: **Double-focusing mixing jet for XFEL study of chemical kinetics.** *J Synchrotron Radiat* 2014, **21**:1364-1366.
 18. Schmidt M: **Mix and inject: reaction initiation by diffusion for time-resolved macromolecular crystallography.** *Adv Condens Matter Phys* 2013, **2013**:167276.
 19. Trincão J, Hamilton ML, Christensen J, Pearson AR: **Dynamic structural science: recent developments in time-resolved spectroscopy and X-ray crystallography.** *Biochem Soc Trans* 2013, **41**:1260-1264.
 20. Kubelka J: **Time-resolved methods in biophysics. 9. Laser temperature-jump methods for investigating biomolecular dynamics.** *Photochem Photobiol Sci* 2009, **8**:499-512.
 21. Lang K, Chin JW: **Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins.** *Chem Rev* 2014, **114**:4764-4806.
- This comprehensive review describes the currently available tools for residue- and site-specific labelling of proteins. It focuses on the genetic incorporation of unnatural amino acids but also discusses the chemoselective modification of both natural and unnatural amino acids for the introduction of a diverse range of probes, including photocages.
22. Lee H-M, Larson DR, Lawrence DS: **Illuminating the chemistry of life: design, synthesis, and applications of "caged" and related photoresponsive compounds.** *ACS Chem Biol* 2009, **4**:409-427.
 23. Schmidt M, Rajagopal S, Ren Z, Moffat K: **Application of singular value decomposition to the analysis of time-resolved macromolecular X-ray data.** *Biophys J* 2003, **84**:2112-2129.
 24. Cammarata M, Levantino M, Wulff M, Cupane A: **Unveiling the timescale of the R-T transition in human hemoglobin.** *J Mol Biol* 2010, **400**:951-962.
 25. Malmerberg E, Omran Z, Hub JS, Li X, Katona G, Westenhoff S, Johansson LC, Andersson M, Cammarata M, Wulff M *et al.*: **Time-resolved WAXS reveals accelerated conformational changes in iodoretinal-substituted proteorhodopsin.** *Biophys J* 2011, **101**:1345-1353.
 26. Kim KH, Oang KY, Kim J, Lee JH, Kim Y, Ihee H: **Direct observation of myoglobin structural dynamics from 100 picoseconds to 1 microsecond with picosecond X-ray solution scattering.** *Chem Commun (Camb)* 2011, **47**:289-291.
 27. Cho HS, Dashdorj N, Schotte F, Graber T, Henning R, Anfinrud P: **Protein structural dynamics in solution unveiled via 100-ps time-resolved X-ray scattering.** *Proc Natl Acad Sci USA* 2010, **107**:7281-7286.
 28. Blanchet CE, Svergun DI: **Small-angle X-ray scattering on biological macromolecules and nanocomposites in solution.** *Annu Rev Phys Chem* 2013, **64**:37-54.
 29. Ramachandran PL, Lovett JE, Carl PJ, Cammarata M, Lee JH, Jung YO, Ihee H, Timmel CR, van Thor JJ: **The short-lived signaling state of the photoactive yellow protein photoreceptor revealed by combined structural probes.** *J Am Chem Soc* 2011, **133**:9395-9404.
 30. Chen P, Hub JS: **Validating solution ensembles from molecular dynamics simulation by wide-angle X-ray scattering data.** *Biophys J* 2014, **107**:435-447.
 31. Björling A, Niebling S, Marcellini M, van der Spoel D, Westenhoff S: **Deciphering solution scattering data with experimentally guided molecular dynamics simulations.** *J Chem Theory Comput* 2015, **11**:780-787.
- This paper describes the use of time-resolved X-ray scattering difference patterns to simultaneously restrain and guide non-equilibrium molecular dynamics simulations. Although a meaningful comparison with ensemble measurements requires that calculated scattering patterns are averaged over many different independent simulations and the quality of the force field ultimately determines the accuracy of the structural model at the atomic level, we foresee that this kind of approach will enhance the structural interpretation of (time-resolved) solution X-ray scattering data.
32. Jung YO, Lee JH, Kim J, Schmidt M, Moffat K, Srajer V, Ihee H: **Volume-conserving trans-cis isomerization pathways in photoactive yellow protein visualized by picosecond X-ray crystallography.** *Nat Chem* 2013, **5**:212-220.
 33. Kaila VR, Schotte F, Cho HS, Hummer G, Anfinrud PA: **Contradictions in X-ray structures of intermediates in the photocycle of photoactive yellow protein.** *Nat Chem* 2014, **6**:258-259.
 34. Jung YO, Lee JH, Kim J, Schmidt M, Moffat K, Srajer V, Ihee H: **Reply to 'contradictions in X-ray structures of intermediates in the photocycle of photoactive yellow protein'.** *Nat Chem* 2014, **6**:259-260.
 35. Tenboer J, Basu S, Zatspein N, Pande K, Milathianaki D, Frank M, Hunter M, Boutet S, Williams GJ, Koglin JE *et al.*: **Time-resolved serial crystallography captures high-resolution intermediates of photoactive yellow protein.** *Science* 2014, **346**:1242-1246.
- This paper presents the first high resolution structures of PYP determined by time-resolved serial crystallography at a XFEL. Structures were determined at 10 ns and 1 μ s time-delays after photoisomerisation and clean and readily interpretable electron density maps were obtained.
36. Aquila A, Hunter MS, Doak RB, Kirian RA, Fromme P, White TA, Andreasson J, Arnlund D, Bajt S, Barends TR *et al.*: **Time-resolved protein nanocrystallography using an X-ray free-electron laser.** *Opt Express* 2012, **20**:2706-2716.
 37. Kern J, Alonso-Mori R, Tran R, Hattne J, Gildea RJ, Echols N, Glöckner C, Hellmich J, Laksmono H, Sierra RG *et al.*: **Simultaneous femtosecond X-ray spectroscopy and diffraction of photosystem II at room temperature.** *Science* 2013, **340**:491-495.
 38. Kupitz C, Basu S, Grotjohann I, Fromme R, Zatspein NA, Rendek KN, Hunter MS, Shoeman RL, White TA, Wang D *et al.*: **Serial time-resolved crystallography of photosystem II using a femtosecond X-ray laser.** *Nature* 2014, **513**:261-265.
 39. Arai M, Kondrashkina E, Kayatekin C, Matthews CR, Iwakura M, Bilsel O: **Microsecond hydrophobic collapse in the folding of *Escherichia coli* dihydrofolate reductase, an alpha/beta-type protein.** *J Mol Biol* 2007, **368**:219-229.
 40. Vestergaard B, Groenning M, Roessle M, Kastrop JS, Van De Weert M, Flink JM, Frokjaer S, Gajhede M, Svergun DI: **A helical structural nucleus is the primary elongating unit of insulin amyloid fibrils.** *PLoS Biol* 2007, **5**:1089-1097.
 41. Oliveira CLP, Behrens MA, Pedersen JS, Erlacher K, Otzen D, Pedersen JS: **A SAXS study of glucagon fibrillation.** *J Mol Biol* 2009, **387**:147-161.
 42. Ortore MG, Spinozzi F, Vilasi S, Sirangelo I, Irace G, Shukla A, Narayanan T, Sinibaldi R, Mariani P: **Time-resolved small-angle X-ray scattering study of the early stage of amyloid formation of an apomyoglobin mutant.** *Phys Rev E* 2011, **84**:061904.
 43. Andersson M, Malmerberg E, Westenhoff S, Katona G, Cammarata M, Wöhri AB, Johansson LC, Ewald F, Eklund M, Wulff M *et al.*: **Structural dynamics of light-driven proton pumps.** *Structure* 2009, **17**:1265-1275.
 44. Kim TW, Lee JH, Choi J, Kim KH, Van Wilderen LJ, Guerin L, Kim Y, Jung YO, Yang C, Kim J *et al.*: **Protein structural dynamics of photoactive yellow protein in solution revealed by pump-probe X-ray scattering.** *J Am Chem Soc* 2012, **134**:3145-3153.

45. Takala H, Björling A, Berntsson O, Lehtivuori H, Niebling S, Hoernke M, Kosheleva I, Henning R, Menzel A, Ihalainen JA *et al.*: **Signal amplification and transduction in phytochrome photosensors.** *Nature* 2014, **509**:245-248.

This paper reports exciting insights into the mechanism of signal transduction by bacteriophytochrome from *Deinococcus radiodurans*. The paper highlights how the combination of X-ray crystallography, time-resolved X-ray solution scattering, and MD simulations can be used to overcome the limitations of crystallographic-only studies when dealing with large amplitude protein motions.

46. Malmerberg E, M Bovee-Geurts PH, Katona G, Deupi X, Arnlund D, Wickstrand C, Johansson LC, Westenhoff S, Nazarenko E, Schertler GFX *et al.*: **Conformational activation of visual rhodopsin in native disc membranes.** *Sci Signal* 2015, **8**:ra26.
47. Levantino M, Spilotros A, Cammarata M, Schiro G, Ardiccioni C, Vallone B, Brunori M, Cupane A: **The Monod-Wyman-Changeux allosteric model accounts for the quaternary transition dynamics in wild type and a recombinant mutant human hemoglobin.** *Proc Natl Acad Sci USA* 2012, **109**:14894-14899.
48. Cammarata M, Levantino M, Schotte F, Anfinrud PA, Ewald F, Choi J, Cupane A, Wulff M, Ihee H: **Tracking the structural dynamics of proteins in solution using time-resolved wide-angle X-ray scattering.** *Nat Methods* 2008, **5**:881-886.
49. Hofrichter J, Sommer JH, Henry ER, Eaton WA: **Nanosecond absorption spectroscopy of hemoglobin: elementary processes in kinetic cooperativity.** *Proc Natl Acad Sci USA* 1983, **80**:2235-2239.
50. Spilotros A, Levantino M, Schirò G, Cammarata M, Wulff M, Cupane A: **Probing in cell protein structural changes with time-resolved X-ray scattering.** *Soft Matter* 2012, **8**:6434.

51. Spence JCH, Weierstall U, Chapman HN: **X-ray lasers for structural and dynamic biology.** *Rep Prog Phys* 2012, **75**:102601.
52. Neutze R: **Opportunities and challenges for time-resolved studies of protein structural dynamics at X-ray free-electron lasers.** *Philos Trans R Soc Lond B: Biol Sci* 2014, **369**:20130318.
53. Arnlund D, Johansson LC, Wickstrand C, Barty A, Williams GJ, Malmerberg E, Davidsson J, Milathianaki D, DePonte DP, Shoeman RL *et al.*: **Visualizing a protein quake with time-resolved X-ray scattering at a free-electron laser.** *Nat Methods* 2014, **11**:923-926.

This paper reports on the first experiment at an X-ray free-electron laser using time-resolved WAXS to study the conformational changes of a photosynthetic reaction center after light excitation. Selection of suitable frames from non-equilibrium MD simulations in conditions close to the experimental ones, i.e. extensive multiphoton absorption by the protein chromophores, was used to interpret the observed time-resolved WAXS signals in terms of a picosecond protein expansion.

54. Ansari A, Berendzen J, Bowne SF, Frauenfelder H, Iben IE, Sauke TB, Shyamsunder E, Young RD: **Protein states and proteinquakes.** *Proc Natl Acad Sci USA* 1985, **82**:5000-5004.
55. Levantino M, Schirò G, Lemke HT, Cottone G, Glowonia JM, Zhu D, Chollet M, Ihee H, Cupane A, Cammarata M: **Ultrafast myoglobin structural dynamics observed with an X-ray free-electron laser.** *Nat Commun* 2015, **6**:6772.

This paper demonstrates the power of the short FEL X-ray pulses in combination with time-resolved X-ray scattering to probe ultrafast structural rearrangements in proteins. By monitoring the signal changes in both the WAXS and SAXS regions, direct evidence of myoglobin proteinquake has been obtained. Myoglobin structure was also shown to undergo low frequency damped oscillations in the picosecond time-scale.