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Crystallographers have for a long time been interested in structural changes. This interest has been, for example, directly in a crystal by combining crystallography with photochemistry to drive the changes in nitrophenol (Coppens & Schmidt, 1965) or via the diffusion of additive molecules into a protein crystal (Wyckoff *et al.*, 1967). Both these studies took place on home-laboratory X-ray sources. The study of structural changes of biological macromolecules in solution was pioneered at the DESY synchrotron facilities in the EMBL Outstation Hamburg by Koch & Bordas (1983). Three-dimensional electron microscopy of macromolecular assemblies is well suited for the visualization of biological molecules in their native state (Frank, 2006). In terms of time-resolution, very fast, nanosecond, timescales were initially addressed by visible light spectroscopy. A notable study was the nanosecond recombination time courses measured by photolyzing O_2, NO, CO, methyl, ethyl, *n*-propyl, *n*-butyl and *tert*-butyl isocyanide complexes of sperm whale myoglobin with a 30 ns laser pulse (577 nm) at pH 7 at 20°C (Gibson *et al.*, 1986). This new volume edited by Moody and Kwan is in the trusted *Methods in Enzymology* book series. It comes closely after the book by Moffat & Lattman (2023). These two volumes thus represent a maturity of time-resolved structural biology methodologies. However, it is still a fast-moving topic, with dedicated new beamlines being unveiled regularly [see, for example, Mikolajek *et al.* (2023) and Orlans *et al.* (2025)]. Also, exploration of body-temperature (37°C) structural studies of biological macromolecules by all biophysical methods is expanding at pace (Brink *et al.*, 2025).

The above historical outline sets the scene for my review of the book edited by Moody and Kwon, which comprises chapters titled *The growth of microcrystals for time resolved serial crystallography*, *Use of fixed targets for serial crystallography*, *Sample efficient approaches in time-resolved X-ray serial crystallography*, *Complementary X-ray emission spectroscopy using drop-on-demand tape-drive systems*, *Sample delivery for structural biology at the European XFEL*, *Experimental approaches for time-resolved serial femtosecond crystallography at PAL-XFEL*, *Time-resolved IR spectroscopy for monitoring protein dynamics in microcrystals*, *Multiplexing methods in dynamic protein crystallography*, *Processing serial synchrotron crystallography diffraction data with DIALS* and *Time-resolved scattering methods for biological samples at the CoSAXS beamline, MAX IV Laboratory*.

Do these chapters, separately and as a collection, satisfy the book's aims? Quoting from the *Preface*:

> *The application and development of the methods of structure determination of biological macromolecules and their complexes has not only given us unprecedented insight into biological processes, but it has also had a far-reaching impact on the understanding and treatment of disease (as recognized by many Nobel prizes). However, following the changes that take place over time – such as the turn-over of a substrate by an enzyme, conformational changes upon binding, and changes in redox state – present additional challenges." ... "Deconvolution of the mixtures of different structures is a daunting prospect. If the energy profile of the reaction can be exploited,* ***an intermediate***

> ***with lower energy levels might be persuaded to accumulate*** *(indeed it may be possible to trap some intermediate states by cryo- or other means). Even when we cannot determine the structures of all the steps in a reaction, the* ***power of the difference Fourier lets us see what has moved over time****.*

The aspects emphasized here in the *Preface*, and which I have highlighted in bold, are not given individual chapters. Also, the 'power of the difference Fourier' requires good isomorphism of a protein crystal as time progresses. The utility of individual refined structures for each time point is then a major strength provided that the precision of the atom positions is estimated, as they can be with the *Online DPI* (Kumar *et al.*, 2015) based on Cruickshank (1999) or assessment by 'brute-force' multiple workflows of difference maps and/or refined models based on the same raw data set, or even replicate experiments as compute engines become ever more powerful at our central facilities beamlines.

Chapter 1 is on the growth of microcrystals for time-resolved serial crystallography by a world-leading authority, Professor Alex McPherson, and will prove an invaluable guide, one identified at The Royal Society UK XFEL conference in 2024 as an especial challenge. That said, where the aim is elucidating function as well as structure, conditions for crystallization can take one far away from the *in vivo* chemical conditions. Preserving the latter can prevent large crystals forming but still yield microcrystals. This is not discussed because the field does not regard functional assays after finding the crystallization conditions as a necessity, but in this book it is surely essential. Its inclusion would have significantly enriched the discussion, particularly for those aiming to connect crystallization conditions to functional relevance.

Chapter 2 is on the use of fixed targets for serial crystallography. It is packed full of practical experience gained at synchrotron XFEL beamlines on optimizing sample preparation, experiment design and the best use of beamtime during data-collection sessions. Special precautions for working with air- or light-sensitive proteins are described. A range of structural artefacts due to X-rays are described, but what is also needed is a description of functional changes during an experiment (Bras *et al.*, 2021). Progressive changes of unit-cell parameters are discussed as a diagnostic of reactions proceeding in crystals, although the impact of non-isomorphism on difference Fourier maps could benefit from a more detailed treatment.

Chapter 3 describes sample-efficient approaches in time-resolved X-ray serial crystallography and complementary X-ray emission spectroscopy using drop-on-demand tape-drive systems. A breakthrough for use of the serial methods, which *de facto* could not use stroboscopic reversible changes, and their use with 'real systems' rather than lysozyme or thaumatin microcrystal test systems, is the liquid drop-on-demand apparatus pioneered by Orville and coworkers described in this chapter. These allow a large variety of time-resolved experiments such as pump–probe, anaerobic and/or O_2 gas mixing or active drop-on-drop mixing experiments. Furthermore, this sample-delivery approach can be used with complementary spectroscopic methods. Again, this chapter is replete with great practical experience. To the biochemist as user all of these details, along with those in Chapter 2, may be admirable but may appear technically intensive at first glance, potentially presenting a learning curve for new entrants to the field. The chapter mentions raw data archiving, but at the facility's data store for the measuring team, not open for 'reproducibility scrutiny' by the editor and the referees of a submitted article, let alone the readers of a final publication. That requires the extra step of the measuring team placing their raw diffraction data underpinning a publication in a separate repository (Maia, 2012). Given the challenges of evaluating the significance of structural changes often at low occupancy, and quite possibly diluted further in mixtures of states, broader raw diffraction data accessibility would further strengthen reproducibility and scholarly engagement for the readers of a given study.

Chapter 4 is about structural biology at the European XFEL. It is emphasized that for time-resolved experiments, small and uniformly sized crystals are essential to ensure consistent reaction starting times and light intensities. The authors also describe the means to reduce sample consumption while utilizing a megahertz repetition rate of the X-ray pulses at the European XFEL. It is again a chapter packed full of practical experience. The Scientific Data Policy of the European X-Ray Free-Electron Laser Facility GmbH (EuXFEL) is especially interesting, striking a thoughtful balance between the opportunity for open data sharing and the practicalities of archiving large-scale data, as well as ensuring reproducibility and giving access to historic measurements for new software to the best extent possible. The relevant EuXFEL documents can be found at https://www.xfel.eu/sites/sites_custom/site_xfel/content/e51499/e141242/e141245/xfel_file234453/ScientificDataPolicy2023_eng.pdf and https://www.xfel.eu/sites/sites_custom/site_xfel/content/e51499/e141242/e141245/xfel_file234455/Quality_of_data_services_01.2024_draft_eng.pdf.

Chapter 5 is a report of the Korean XFEL covering the experimental approaches for time-resolved serial femtosecond crystallography there. The authors emphasize that to successfully collect time-resolved serial femtosecond crystallography data, understanding the characteristics of the XFEL facility and beamline that will be used, the available equipment and the experimental approach specific to the beamline is critical. They mention that factors such as temperature, humidity and air conditions can influence the crystal structure, highlighting the significance of reproducibility in this demanding experimental context and suggest that beamtime allocation be made for the replication of results. This chapter describes determining the time zero and data collection at different time-delay points, with delays ranging from a few femtoseconds to picoseconds being the most challenging.

The authors of Chapter 6 are from the University of Hyogo in Japan, rather than a central facility, and it is on time-resolved infra-red (IR) spectroscopy for monitoring protein dynamics in microcrystals. The method described reports on the vibrations associated with polar bonds in amino-acid side

chains, cofactors and ligands. Such complementary probes to the precise time-resolved crystal structures add insights into the accuracy of the results. They neatly emphasize that data interpretation and extrapolation to the solution state is often not straightforward as the *in crystallo* environment is not the same as it is in solution. They also directly compare time-resolved IR measurements of the dynamics between crystalline and solution conditions. This is clearly an important chapter, particularly if the biochemical *in vitro* conditions align closely with the *in vivo* context. This bridge between structural and cell biology is a complicated one and is explored by Nogales & Mahamid (2024).

Chapter 7 is on multiplexing methods in dynamic protein crystallography derived from the Hadamard transform, a method carried over from the laser spectroscopists. This multiplexing technique allows the use of the more widely available monochromatic synchrotron macromolecular beamlines rather than XFEL or Laue beamlines. This raises the question of whether broader accessibility of beamlines is really the constriction of time-resolved structural studies. However, anticipating this point, the authors adroitly emphasize that multiplexing offers a means to increase the signal of structural change to improve the signal-to-noise ratio (SNR), a definite challenge, as I remark on elsewhere in this review. Furthermore, importantly, they tackle the accuracy of the electron-density map calculated from the set of intensities affected by errors inherent in the measurement process, their indexing, integration and scaling procedures, sample non-isomorphism and the phases used in the Fourier transform. The authors make clear that multiplexing requires that each sample remains stationary in the beam for long enough to collect multiple exposures. This chapter very nicely embeds the practical details in a clear understanding of the principles. In their outlook, they make the good point that multiplexing is a way to extend the time resolution of a variety of time-resolved synchrotron experiments without requiring higher fluxes. Thus, in my view, this could now be extended to neutron macromolecular crystallography with the provision of an expanding number of global instruments, as also covered recently in the *Methods in Enzymology* series (Moody, 2020).

Chapter 8 is about the processing of serial synchrotron crystallography diffraction data with *DIALS*, a popular software package. It is currently one of six systems available. This is heavy in mathematical detail, and it is somewhat surprising that such a comprehensive treatment has not appeared in the previous literature, making this a particularly welcome contribution. The addition of reciprocal-lattice partiality uncertainty is a nice development. Non-isomorphism is considered as part of the data-reduction process. A very good level of detail is presented of processing example data sets with which a new user can compare their results.

Chapter 9 again takes us to the solution state with a description of the MAX IV time-resolved biological SAXS/WAXS ('CoSAXS') beamline. It spans millisecond experiments to those over several seconds. This chapter is also packed full of practical experience at this well equipped beamline. They emphasize combining SAXS with spectroscopic tools. The various case studies across a range of experiment types will be invaluable to guide future users at this and other instruments worldwide. Fig. 23 is a nice image for the whole book, the caption to which is *Timescales which can be accessed by laser pump–probe and time-resolved structural methods for biology compared with those explored by simulations. For comparison, the timescale of structural dynamics in proteins is included.*

Overall, what do we have here in this volume? It is derived from a splendid conference of the same title, but the themes of various speakers did not result in a chapter. The volume has a good representation of the crystallization, instrumentation and software up to the modern day as well as beamlines with case studies. However, consistent with historical trends in the field, there remains limited representation of model systems designed to accumulate key biochemical intermediates for detailed structural investigation. (For such an example, see Fig. 2 in Niemann *et al.*, 1994). The crystallography and spectroscopy diagnostic methods are working but, putting on my synchrotron facility director's hat, how can the field further broaden its user base? To which, putting on my beamline scientist's hat, I would reply that, as per the introduction, there are many decades of publications on *in operando* crystal structure studies. Secondly, beamtime is now also required for checking for artefacts due to extreme laser-beam intensities for photo-crystallography by performing power titration controls as advocated by a leader in the field, Professor Dr Ilme Schlichting (Barends *et al.*, 2024). Libraries will of course ensure that this new *Methods in Enzymology* volume is available. Meanwhile, cryoEM, which does not require crystals obviously, but is restricted to freeze times of approximately milliseconds, is regularly providing interesting snapshots of structural dynamics, for example comparing body-temperature (37°C) and room-temperature function differences of a Ca^{2+}-activated ion channel (Hu *et al.*, 2024); it is missing as a theme in this book. Macromolecular crystallography offers clear precision indicators, and for the real-time characterization of reactions complemented by in solution probes is making steady progress. Nonetheless, the field continues to be shaped by enduring standards such as the 'David Blow test' set at The Royal Society Discussion meeting of 1992 (Blow *et al.*, 1992) who preferred a series of conventional static crystal structures for the study of enzyme mechanism. This is much the same view as the chemical crystallographers, who prefer harnessing the great numbers of static crystal structures in the Cambridge Structure Database to understand dynamic conformational changes (see, for example, Bürgi, 2002). However, for full mechanistic understanding, real-time structure and dynamics clearly continues to be an ongoing pursuit across both research communities [see, for example, Catlow (2021) and Imura *et al.* (2025)].

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