

Time-resolved Serial Femtosecond Crystallography of Macromolecules

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Until the last decade, time-resolved crystallography of macromolecules was largely limited by the need for large crystals and the degradation of samples due to X-ray damage¹. The time resolution was also poor, as full rotation of the crystal was rarely possible during a single time point. As the first X-ray free electron lasers (XFELs) came online, rapid advances in both spatial and time resolution were made possible^{1,2}. The Linac Coherent Light Source was first brought online in 2009, followed by the SPring-8 Ångstrom Compact free electron laser in 2011, PAL-FEL and the European XFEL in 2017, and the SwissFEL in 2018.

XFELs use a single-pass magnetic undulator through which relativistic electrons are passed and “bunch” (Figure 1) together and emit X-ray radiation. This radiation promotes further “bunching” and emission alignment until the field is saturated. The resulting beam is transversely coherent and polarized, though it should be noted that the longitudinal coherence is poor, requiring extremely bright electron beams and high quality undulator arrays³.

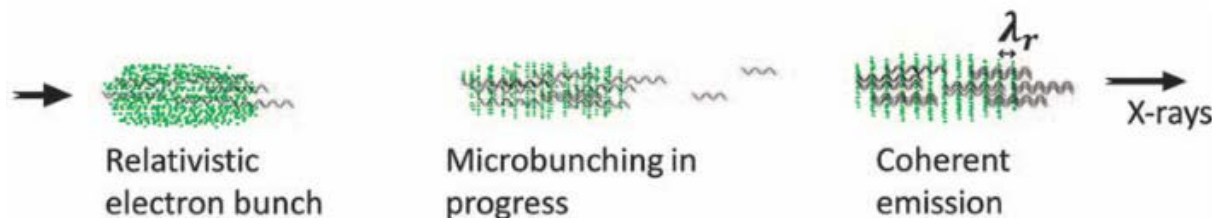


Figure 1. Cartoon depicting the “bunching” of relativistic electrons in the XFEL undulator field leading to coherent X-ray emission³.

Time-resolved crystallography using XFELs presents multiple advantages over the use of a synchrotron x-ray source. The time resolution possible at synchrotron sources is approximately 100 ps, while the time resolution possible with XFELs is sub-100 fs, depending on the apparatus. Additionally, the photon flux of XFELs is 9 orders of magnitude greater than the synchrotron sources and is quasi-monochromatic. One consequence of the increase in flux is that the absorption of energy from the X-ray pulses often induces structural damage and, in some cases, destruction of the crystal being analyzed. Such damage makes it necessary to use multiple crystals in a variety of orientations in order to obtain a full data set. Taking the above concept and applying the idea of “diffraction-before-destruction”⁴, in which it is assumed that the diffraction occurring from a fs X-ray pulse occurs before the structural damage becomes significant, it follows that an easy way to do this would be to present a “stream” of crystals to the XFEL beam. Often, these are micro- or nano- crystals, as the use of smaller crystal sizes allows for better penetration of the beam. This approach is termed serial femtosecond crystallography (SFX), where partial diffraction patterns are obtained from single crystals and then patched together to obtain a full pattern and eventual electron density map. Though a variety of delivery methods are available, common themes involve delivery of a solution (liquid jets) or slurry (“toothpaste” jets and superlube)³ of crystals in either their mother liquor or suspended in a viscous medium, respectively. Protein consumption in liquid jets is often on the order of hundreds of milligrams, and wastes 90% of sample, while the slurries

of and deposited crystals allow for a slower-moving stream where a greater percentage of the crystals are interrogated by the beam.

Two methods for reaction initiation in time-resolved studies have been well-proven. The first, photo-initiation as seen in pump-probe spectroscopy, “pumps” the samples with a visible wavelength, and then uses the XFEL beam as the probe. Prerequisites for this method of initiation are that the process to be observed can be photo-initiated, whether this is due to photo-induced isomerization as seen in studies of photoactive yellow protein, or the requisite cofactor or substrate is photocaged. The other dominant method is “mix-and-inject”⁵, where the cofactor or substrate is mixed with the crystal slurry and allowed to diffuse through the crystal. This has allowed for the study of enzymatic reactions. Recently, the mix-and-inject technique has been used to determine structures of riboswitch RNA reaction states⁶, as well as antibiotic interactions with β -lactamase⁷.

An excellent case study of photoinitiated TR-SFX is that of determining the structures of intermediates of the oxygen evolving complex (OEC) in photosystem II (PS II). First discovered by Joliot in 1969, a year later Kok proposed a mechanism for oxygen evolution⁸ (Figure 2a), wherein the OEC proceeds through 5 intermediate states, triggered by four photo-excitations of the system. In order to probe these states, it is necessary to use a time-resolved method, where the number of excitations is matched to the time point at which the system is probed. Figure 2b shows the pulse sequences and timing to access states S_1 through S_0 .

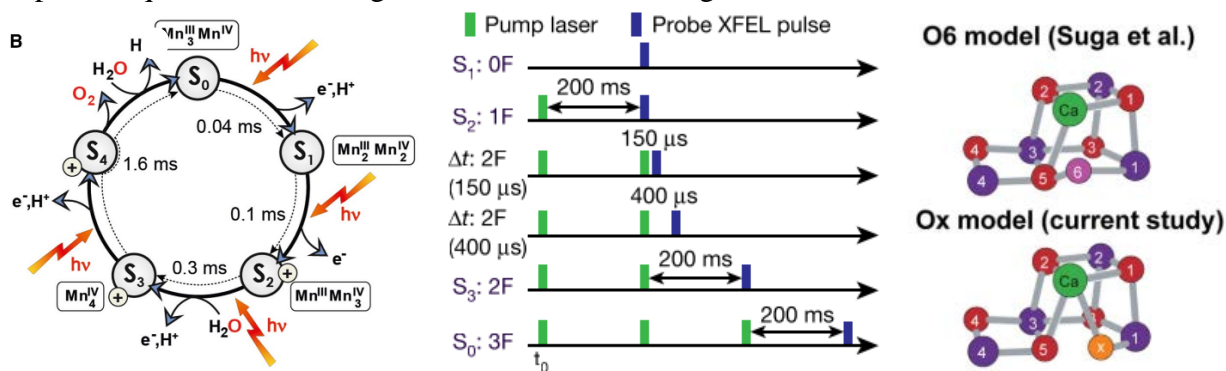


Figure 2. **a** Kok's cycle for O₂ evolution proceeding through states S₀ to S₄, showing light is required to move the mechanism forward⁹. **b** Timeline of pump-probe studies, showing pulse sequences needed to access each state¹⁰. **c** Structural models of the oxygen evolving complex comparing bonding of O6/Ox (purple/orange, respectively).

In 2017, time-resolved studies by Suga *et al*¹¹ showed evidence of a previously undetected insertion of an oxygen atom (O6/Ox, see Figure 2c) near the OEC forming a μ -peroxo bridge between Mn atoms during S₃. Claiming that the peroxo species was later released as O₂, this lent credence to more recently proposed mechanisms for O=O bond formation based in density functional theory calculations.¹² Time-resolved measurements taken by Suga *et al* had a 2.35 Å resolution; just a year later, Kern *et al* published time-resolved structures of OEC intermediates with an improved resolution of 2.08 Å¹⁰. This improvement in resolution was enough to better observe the formation of the supposed μ -peroxo bridge, but on structural refinement fitting with the peroxo model, they found a negative electron density correlated with oxygen atoms at the proposed O6 position and instead positive electron density near the Mn1 position. Refinement with Ox located nearer to Mn1 indicates an Ox-O5 distance of 2.1 Å, much longer than reported peroxo bond lengths. Additionally, the position of the Ox was nearly 1 Å away from that modeled by Suga *et al*. Kern *et al* determined this misinterpretation was likely due to the low resolution of earlier

data, as the electron density of the Ox-Mn1 bond was only clearly visible in the difference map at high resolution, and suggested that instead, the OEC is an open structure rather than a closed cubane, and the ligation of the Ca atom changes over the course of the reaction, which had not been considered previously. Overall, the advancements in XFEL instrumentation made in the course of just a year led to a shift in what was held for nearly a decade as the prevailing mechanism for O₂ generation.

While progress in SFX with XFELs has been immense in terms of time and spatial resolution for photo-initiated cyclic processes, there is much room for progress to be made in enabling the study of irreversible reactions. Time resolution using the mix-and-inject technique is still on the millisecond scale. New techniques for sample delivery to maximize the XFEL pulse hit rate. Additionally, in the realm of data analysis, work is being done to improve the algorithms for phasing and compilation of partial hits. Regardless, the field is still extremely promising and has the potential to lead to great mechanistic insight in biological processes.

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