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Serial synchrotron crystallography for time-resolved structural biology

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The current state-of-the-art experiments in time-resolved structural biology are undoubtedly the recent extremely impressive results that are emerging from XFEL-based experiments. However, there is a large range of macromolecular systems where the biological interest is predominantly in the slower dynamics (μs–s), that produce well diffracting microcrystals, and for which synchrotron-based experiments are extremely well suited. The combination of microfocus X-ray beams and the development of a range of sample delivery platforms has now made routine millisecond time-resolved experiments at microfocus macromolecular crystallography beamlines a real possibility and is driving development of dedicated endstations for time-resolved serial synchrotron crystallography.

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Introduction

Time-resolved X-ray science is a dynamic, fast developing field that has the potential to revolutionise our understanding of life and materials sciences, providing insights into the details of the relationship between structure, dynamics and function. These relationships lie at the heart of modern medicine and biotechnology, underpinning fields from drug discovery to the generation of new green bio-inspired catalysts for use in consumer products. X-ray crystallography is particularly suited to study these questions, as it is able to provide atomic resolution and element specific information in the context of the entire macromolecular structure under study. Indeed, even in the early days of protein crystallography, pioneers such as Perutz were attempting to use reactions in crystals to understand biological mechanisms [1]. Interest in such experiments has dramatically increased over the last decade, partly due to the exciting new results becoming available from X-ray free electron laser sources and the 'resolution revolution' in electron microscopy [2,3]. In addition to this, the structural biology field is shifting towards the view that single, static structures do not tell the whole story, and can indeed be misleading when attempting to understand biological function and mis-function.

Despite this increased interest, time-resolved crystallographic experiments so far remain niche, and the province of a few expert groups around the world. This is because the experiments are complex and require a considerable amount of planning, preparation and effort to achieve [4]. These difficulties are compounded by the fact that facilities to carry out such experiments have, until very recently, been few and far between. The recent advances made possible by X-ray free electron laser (XFEL) sources, and the rapid cross-fertilisation of the concepts of serial crystallography to the synchrotron community has resulted in nearly all synchrotrons now offering some form of serial synchrotron crystallography (SSX) capability at microfocus macromolecular crystallography (MX) beamlines [5•]. This in turn has led a number of groups to explore the possibility of at least millisecond time-resolved SSX experiments at synchrotron sources, and such experiments are now finally beginning to transition from the demonstration stage into the realm of experiments that can be carried out by the non-expert user.

General considerations for a time-resolved crystallography experiment

All time-resolved experiments follow the same scheme, first proposed by Porter in the 1940s, where a reaction is initiated and then probed after a time-delay. This is repeated at a number of time-delays in order to build up a picture of the time-dependent changes in the system. The 'pump' could be a laser pulse that directly photo-activates the system, releases a photocage or produces a temperature or pH jump. Alternatively, for slower reactions or steps, rapid mixing with a ligand, substrate or altered buffer can also be used. Here the key parameter that must be considered is the time it takes for the added molecule or solution to diffuse through the crystal to each active site. In any pump-probe experiment the achievable time-resolution is determined by whichever is slower, the reaction initiation step or the time needed to obtain sufficient signal-to-noise in the probe measurement to detect the change that is being studied.

For time-resolved crystallographic experiments there is the added constraint that a sufficient number of different crystal orientations must be recorded to yield a complete dataset. To achieve this, the experiment must be repeated many times at each time delay, either allowing the sample to relax back to ground state between measurements or accumulating sufficient randomly oriented diffraction patterns from a huge number of different crystals (the serial approach).

Time-resolved serial crystallography at synchrotrons

Serial experiments at synchrotrons come in many flavours, from approaches like mesh-and-collect that raster a microfocus X-ray beam across hundreds of microcrystals mounted in standard loop or mesh mounts, to true serial experiments where many thousands of crystals are delivered to the X-ray beam and each shot only once [5•,6•]. For time-resolved experiments it is this latter flavour of SSX that is of interest. While the achievable rate of reaction initiation is specific to each sample, the time needed for the probe is a direct function of the beamline chosen for the experiment. In practice, this has so far limited the achievable time-resolution to the ms domain for 3rd generation monochromatic microfocus beamlines with 10^{12}–10^{13} photons/s in their fully focussed beam. To achieve higher time-resolution, pink-beam serial crystallography at Laue beamlines offers the possibility to record data with time-resolution as short as 100 ps [7–9]. Further exciting possibilities are offered by the new microfocus beamlines under construction at the diffraction limited storage rings MAXIV and ESRF-EBS, where the decreased horizontal emittance will provide a large increase in flux in their fully focussed beams, allowing time-resolutions of at least microseconds, if not beyond.

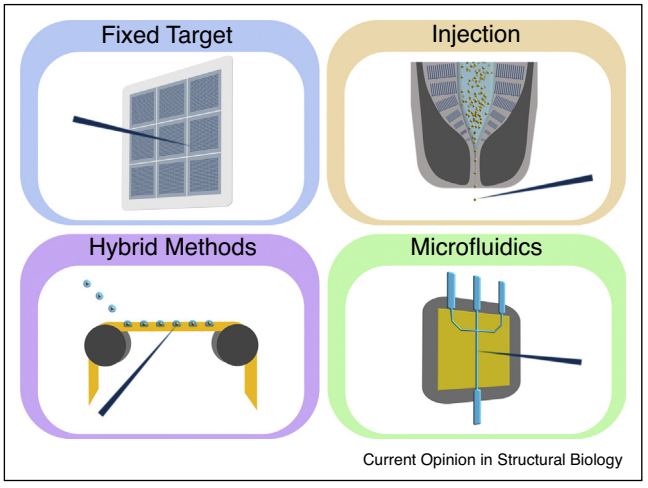
Sample delivery

Time-resolved SSX (TR-SSX) experiments require sample delivery methods that can efficiently deliver tens of thousands of crystals into the X-ray beam *and* allow for reaction initiation [6•,10,11••]. Reaction initiation is triggered either by a laser light pulse or by rapid mixing. As long as the crystals are small enough, small molecules can diffuse into the crystal within a few ms. The two most commonly used sample delivery platforms for TR-SSX to date are fixed targets (with photoactivation or rapid mixing) and high-viscosity injectors (with photoactivation), although promising first results for TR-SSX using microfluidics have recently been reported [12•]. Tape-drives have been very effectively used at XFELs for time-resolved measurements, however their uptake at synchrotrons has so far been limited to demonstration experiments [13] (Figure 1).

Data collection

Once a sample delivery method has been decided upon, the experimental design for the time-resolved experiment itself requires some thought [4]. A key consideration here are the time delays to be measured. For injector, microfluidics and hybrid approaches the reaction initiation is to some extent decoupled from the X-ray data collection, as the time delay is determined solely by the length of time it takes a crystal to travel from the site of reaction initiation to the X-ray beam. This allows adjusting the time delay by simply offsetting the point of reaction initiation relative to the point of X-ray data collection, in principle from minutes down to very short time scales. However, for fixed targets the matter is a little more complex, particularly for longer time-delays (> second) where using a simple pump-wait-probe approach to record tens of thousands of diffraction patterns can take several hours or even days, making a TR-SSX experiment impractical in a standard 24 hour shift. An elegant solution is the *hit-and-return (HARE)* approach

Figure 1



Overview of sample delivery methods for time-resolved serial synchrotron crystallography [5•,6•,10,11••,37•]. Clockwise from top left: **Fixed target** methods include any approach where the sample is immobilised on a target that is then rastered across the X-ray beam [7,9,14,24•,28••,29••,30•,31•,36,38–42]. Both photoactivation and rapid mixing are possibilities for reaction initiation using a fixed target. Fixed targets offer the additional advantage that crystals can be grown directly on the target, minimising damage to crystals during harvesting [43–46]. At monochromatic synchrotron beamlines high-viscosity **injectors** are used in combination with photoactivation [8,20,25••,47–49]. Because of the high viscosity, the sample traverses the microfocus X-ray beam within a few ms. In a pink-beam SSX experiment faster flowing liquid jets can be used due to the higher X-ray flux with, in principle, both photoactivation and rapid mixing possible. **Microfluidic devices** provide an attractive option for rapid-mixing based time-resolved experiments on monochromatic beamlines, where the closed channel maintains a stable flow, even at lower speeds [12•,50]. Photoactivation is, in principle, also possible. Tape drive approaches are a **hybrid method** where droplets of sample are 'written' onto a tape that is then passed in front of the X-ray beam [13]. This very flexible approach allows activation by both rapid mixing and light, as well as the use of environment chambers in which, for example, different gases can diffuse into the droplet containing the crystal [51•].

where a series of crystals are activated (*hit*) and then the target drives back (*return*) to allow X-ray data collection after the desired time-delay [14]. For longer time-delays more crystals are activated before the return step. In this way the total data collection time for a fixed target with ~25 k crystals remains the same, regardless of the time delay (up to 10 s of minutes).

Data processing and analysis

A number of software packages are now available for the processing of serial crystallographic data (from both synchrotrons and XFELs) that span the steps from hit finding to a final scaled and merged reflection file [15•,16•,17•]. Of particular note here are the recently developed *pinkindexer*, which is able to process pink-beam and Laue serial data, and the recent experimental demonstration of the use of the EMC algorithm for processing of data that are too weak to be indexed by other methods [18,19]. These packages are now beginning to make the transition from expert only use to incorporation into expert autoprocessing pipelines at beamlines that are able to provide users with relatively rapid feedback during data collection on the progression of the experiment [20–23]. Key here is the provision of easily understandable graphics that rapidly feedback, for example, on hit rate and resolution, as well as providing easily digestible details of space group, unit cell dimensions, and scaling statistics. It is not unusual in SSX experiments to see distinct crystal populations in the same sample [24•], and this can require additional data collection to ensure sufficient data for each time point are obtained. In addition, early detection of low hit rates allows on the fly troubleshooting in order to maximise the success of the experiment.

Moving from demonstration experiments to user experiments

Three recent reports published during 2019 have demonstrated both the potential and increasing accessibility of TR-SSX experiments. The first, by Standfuss *et al.*, reported a TR-SSX study of bacteriorhodopsin from 5 ms to 200 ms [25••]. This experiment was done at the Swiss Light Source on beamline X06SA, using a high-viscosity injector and laser excitation to trigger the bacteriorhodopsin photocycle. This, in combination with time-resolved SFX experiments [26,27], provides a nearly complete picture of the photocycle of bacteriorhodopsin, and allowed them to determine how the protein resets itself ready for the next photocycle by transporting a proton from the cytoplasmic side of the membrane to the retinal Schiff base via a transient water chain (Figure 2).

The second report by Pai *et al.* is a *tour de force* of mechanistic enzymology. They used a fixed target approach in combination with photodecaging of the substrate fluoroacetate and the *HARE* data collection scheme to follow the dimeric enzyme fluoroacetate dehalogenase (FAcD) through 4 turnover cycles, collecting data on beamlines P11 and P14 at PETRA III [14,28••]. They reported 18 timepoints, spanning 30 ms to 30 s, revealing the mechanism of the allosteric communication between the two enzyme subunits (Figure 3). Key to the success of this experiment was the soaking of the crystals with a photocaged substrate that could not bind the active site, but was at high concentration in the crystal (~50 mM).

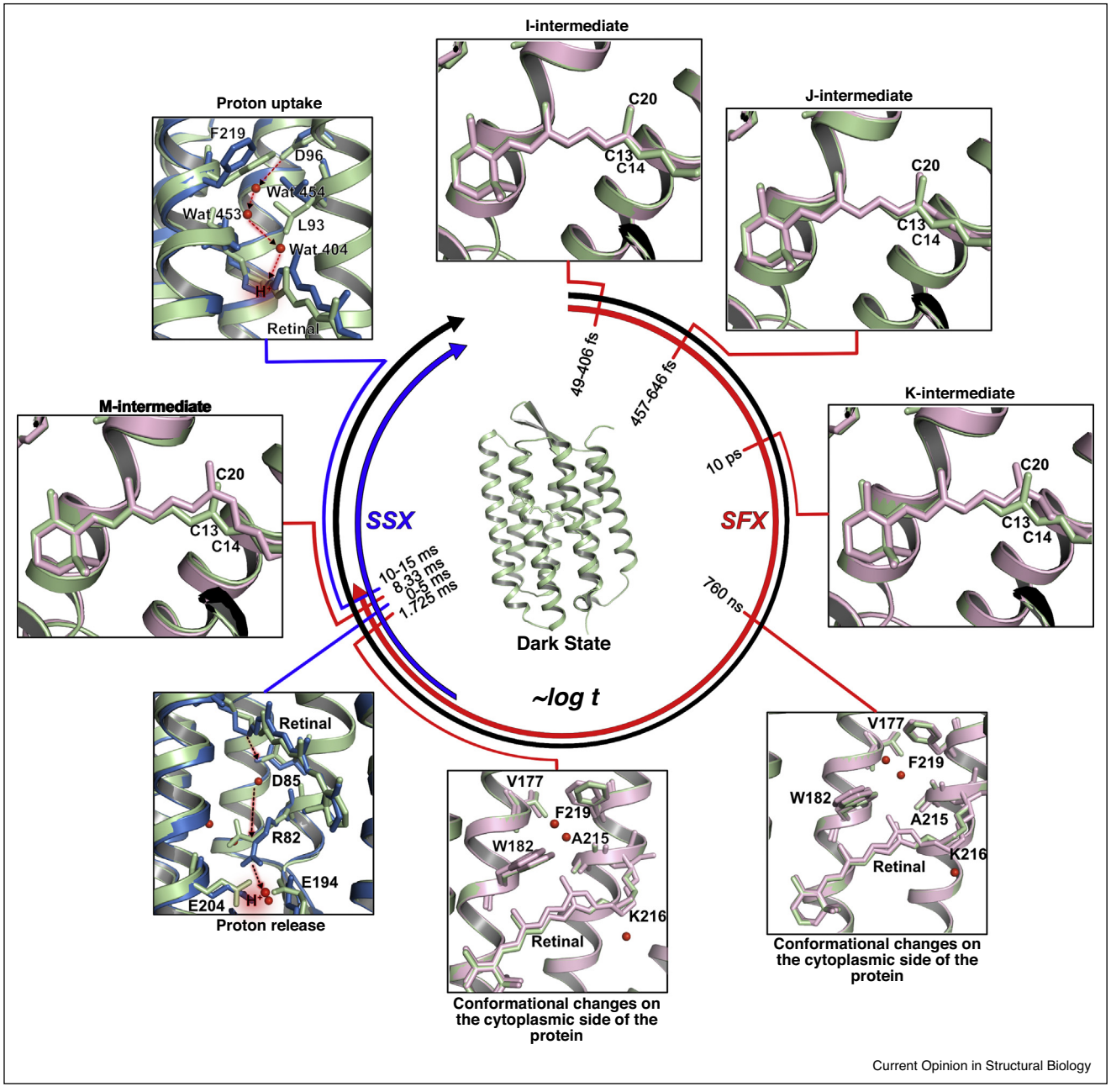
It is clear from both these studies that photoactivation can be coupled extremely efficiently to TR-SSX. However, photoactivation in general requires that either the protein or its substrate can be activated by light[3] . For non naturally light-activated systems, such as FAcD, this means photocaged substrates or ligands have to be used. Rapid mixing of ligand is, in principle, a much more general approach to reaction initiation, especially for TR-SSX of microcrystals where the rate of diffusion of ligand into the crystal is on the order of ms. The third study highlighted here shows the great potential for the combination of rapid-mixing and TR-SSX. In this report picolitre droplets are 'written' onto crystals already mounted on a fixed target using the so called *liquid application method for time-resolved analyses (LAMA)* [29••]. At room temperature the thermophilic enzyme xylose isomerase has an extremely low turnover rate, and this study takes advantage of this to visualise first glucose binding and then slow glucose ring opening, the first two steps in the reaction mechanism. In combination with *HARE* [14,30•], this approach has the potential to be a general tool for TR-SSX with millisecond time-resolutions. It is easy to set up and minimises the challenges for the novice user. In effect, all that is needed are a sufficient number of well behaved microcrystals (and a few microliters of the substrate) and they are ready to carry out a first time-resolved experiment.

Sample requirements

With the increasing number of SSX and TR-SSX studies now reported, it is possible to begin to define some rules of thumb with regard to sample quantities. For fixed target experiments using the HARE chip 200–400 μl of a crystalline suspension containing 1–1.5 × 10^6 crystals/mL is required for each chip when loading using a pipette [30•]. The total amount of crystal suspension used can be significantly reduced (3 μl) when using acoustic drop ejection for chip loading, at the price of a reduced diffraction hit rate [31•]. However, a word of caution is necessary here. In a TR-SSX experiment sufficient data must be collected to ensure that the dataset can be stably scaled and merged, and that there is sufficient signal-to-noise to see the often subtle structural changes associated with the reaction of interest. In practice a dataset of 5–10 thousand indexed diffraction patterns is usually sufficient to reveal time-dependent differences [32•].

[3] Laser induced temperature or pH jumps are also possible.

Figure 2

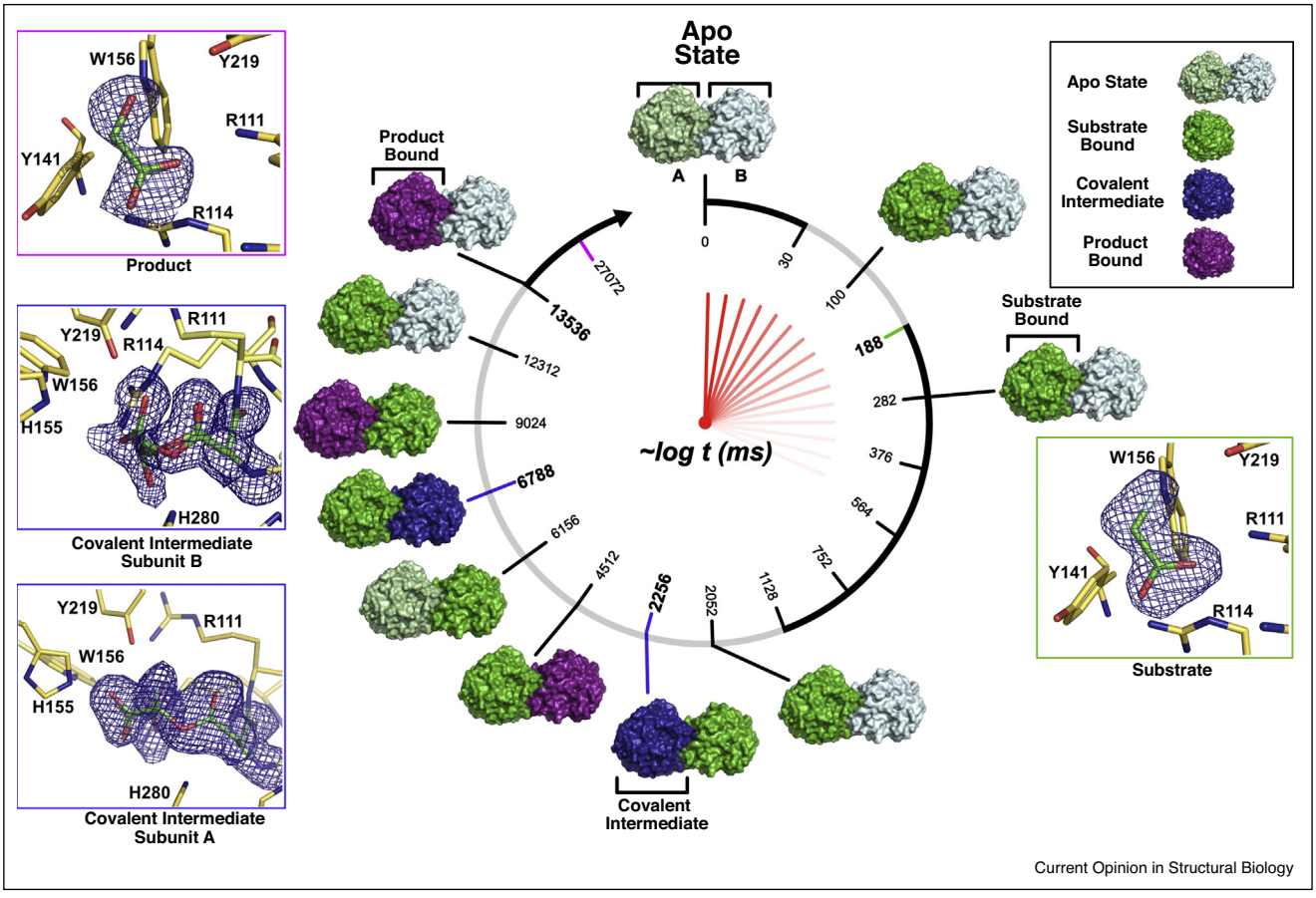


Schematic representation summarising the different states of bacteriorhodopsin (bR) captured using both TR-SSX [25••] and TR-SFX [26,27]. The central wheel indicates the time points where different states were identified, from fs–ms, with (SFX) measurements in red and (SSX) in blue. The SSX data were in fact collected over 200 ms, allowing calculation of a series of difference maps. Shown here are two time points where structurals changes associated with proton release and subsequent uptake for the next reaction cycle could be observed. The central secondary structure cartoon in green shows the resting dark state of bR. The outer panels show how key residues and the retinal cofactor (in stick representation, SFX in pink and SSX in blue) move relative to the dark state (green) during the different reaction steps of retinal isomerization, proton release and proton uptake Waters are shown as red spheres.

Future

The growth in SSX experiments has been facilitated by the willingness of beamline staff to accommodate users wishing to install SSX sample delivery platforms into their endstations, in addition to now relatively routine raster scanning SSX experiments for crystals mounted in loops or in crystal trays [recent implementations include 20,33,34•,35,36]. Indeed, many beamlines are now investing in dedicated infrastructure for SSX, with high viscosity injectors an increasingly common endstation

Figure 3



Schematic summarising the TR-SSX study of FAcD [28••]. The central wheel indicates the time points recorded in the experiment. The space filling models illustrate the state of the two FAcD subunits at each time point. The inset panels show the 2Fo-Fc electron density within the active site for the bound substrate and product, as well as for the covalent intermediate in the two subunits.

component [8,20,25••,35]. The number of beamlines actively developing TR-SSX is smaller, but not insignificant. Not unsurprisingly, the Laue beamlines ID09 at ESRF and 14ID at APS are already well placed for these experiments [7–9]. Excitingly, a number of dedicated endstations for (TR-)SSX are in operation or under development that will provide SSX and TR-SSX as standard configurations to the user. These include I24 at Diamond Light Source (operational), the T-REXX endstation in the second hutch of the EMBL beamline P14 at PETRA III (operational), MicroMAX at MAXIV (under development) and ID29-SSX at ESRF-EBS (commissioning).

Summary

TR-SSX is currently transitioning from being a niche technique into becoming a widely accessible method for the general structural biologist. The use of rapid mixing techniques for experiments with millisecond time resolutions offers an alternative to photoactivation and has opened the door to millisecond time-resolved studies of a wide range of systems. Faster experiments will still require considerable preparatory work from users, particularly in determining the optimal method for reaction initiation, but the infrastructure, data collection controls and data processing tools are becoming more and more accessible to the non-expert.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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Very interesting sample delivery approach that would be also suitable for use on a synchrotron beamline. Particularly exciting here are the possibilities to trigger reactions by diffusion of gases such as O_2.