Membrane proteins are the gatekeepers of cellular membranes where they act as enzymes, transporters, signaling receptors, or in energy conversion. Traditionally seen as a difficult field, the last decade has brought dramatic progress to membrane protein structural biology. Here, I describe recent advances in studying the conformational dynamics of membrane proteins by X-ray free electron lasers. By integrating sample efficient high viscosity injectors into pump probe setups, it has become possible to determine whole series of structural snapshots by time-resolved serial femtosecond crystallography and assemble them to molecular movies of proteins in action. According to the current studies on bacteriorhodopsin, photosystem II and nitric oxide reductase, I outline the technical challenges as well as new possibilities to study membrane protein function.

**Address**
Division of Biology and Chemistry – Laboratory for Biomolecular Research, Paul Scherrer Institut, 5232 Villigen, Switzerland

**Introduction**
Membrane proteins are molecular machines that rely on structural rearrangements to fulfill their function. Membrane transport proteins undergo defined conformational rearrangements to accept their substrate on one side of the membrane and release it on the other. Signaling by G protein-coupled receptors (GPCRs) depends on ligand-induced opening of the transmembrane-helical bundle to allow binding of kinases, arrestins and G proteins. In a subtler, but globally important way energy conversion in plant photosynthesis requires atomic rearrangements to transfer electrons from water to CO₂ in order to provide the oxygen and nutrients we all depend on. The ultimate goal of time-resolved crystallography is to capture molecular snapshots of all proteins at different stages of their functional cycles. Providing detailed insights into how proteins change over time and to relate these structural changes to their biological function remains one of the major challenges in modern structural biology.

X-ray free electron lasers (XFELs) opened exciting new opportunities to study the structure, the dynamics and the function of proteins. Modern XFELs have been particularly successful in studying GPCRs [1], a class of membrane proteins that is well known to yield only small weakly diffracting crystals. As the XFEL pulses are not only ultrabright (10e12 photons per pulse) but also ultrafast (10–50 fs), most forms of radiation damage can be avoided by the ‘diffraction before destruction’ principle [2]. Without radiation damage, there is no need for cryocooling, which, together with time-resolution up to the femtosecond range, predestines the new X-ray sources to study the dynamics of membrane proteins at near-atomic resolution. Two recent molecular movies of bacteriorhodopsin (bR) [3,4] together spanning eleven orders of magnitude in time (about 150 fs–8.33 ms) and high resolution dynamics of O=O bond formation in photosystem II [5] are wonderful examples for how the technology can be used to study membrane protein function.

New ways of triggering reactions in a pump-probe setup using photopharmacological switches [6], caged-compounds [7], electric fields [8] or temperature jumps [9] are in development to target a wide range of biologically relevant systems. The revival of time-resolved crystallography is further fueled by the completion of new XFELs. Initially only the Linac Coherent Light Source (LCLS, USA) provided a limited number of users with the possibility to conduct pioneering experiments. These were followed by exciting studies from the Spring-8 Ångstrom Compact Free Electron Laser (SACLA, Japan), and now the Pohang Accelerator Laboratory X-ray Free Electron Laser (PAL-XFEL, Korea), the European X-ray Free Electron laser (EuXFEL, Germany) and the Swiss Free Electron Laser (SwissFEL, Switzerland). Together with the increasing synergy of XFELs with upgraded synchrotrons, the time seems right to address pressing questions in structural biology with dynamic measurements at ambient temperature and unprecedented spatial and temporal resolution. In this article I will review future directions and the remaining challenges in sample preparation, crystal delivery, and trigger systems based on recent examples from the literature and with a particular emphasis on membrane proteins.
Lipidic cubic phase serial femtosecond crystallography

The dramatic increase in the peak brilliance of XFEL radiation sources created completely new opportunities in structural biology, but also new challenges that had to be overcome (Figure 1). A major hurdle was the need to develop new ways to collect and analyze X-ray data because each crystal is destroyed from the dose of a single XFEL pulse. The solution came from large international scientific collaborations measuring at LCLS and was termed serial femtosecond crystallography (SFX). The method relies on injectors providing a constant stream of...
sample to replace crystals in a serial fashion between every XFEL pulse. Yet each of these single shots provides only a small fraction of the information necessary to reconstruct the molecular structure of a protein. To create a coherent dataset, the measured intensities from several thousand crystals of varying size and random orientation are averaged and merged using Monte Carlo integration [10]. The SFX principle was established at low resolution (X-ray energy and geometry limited) using the membrane protein complexes photosystem I [11] and a photosynthetic reaction center [12], followed by high resolution structures of soluble model proteins like lysozyme [13] and provided new structural information from *in vivo* grown cathespin B crystals [14].

The next important step in the development was to make the method applicable to proteins that cannot be produced in large quantities. The pioneering studies above made use of liquid injector systems [15] (relying on gas dynamic virtual nozzles [16]) to deliver crystal suspensions at a rate of at least 10 μl/min most of which is unfortunately wasted using first generation XFELs with a repetition rate around 100 Hz. At new sources such as the EuXFEL with up to 27 kHz (delivered in 10 trains per second with a 4.5 MHz repetition rate within each train) the liquid injectors remain a striking option to achieve very fast rates of data collection up to bursts in the megahertz range [17,18].

But the desire to increase efficiency has also triggered development of a variety of different injector systems and fixed target approaches (reviewed for example in [19†]). For membrane proteins and particularly GPCRs [1†], the high viscosity injector system [20]† has been the most successful. It allows crystals grown in lipidic cubic phase (LCP) to be extruded at a 200 times lower flow rate (about 0.05 μl/min) compared to liquid systems. The advantages are obvious: crystals need limited preparation before injection, the membrane protein remains in a lipidic environment and consumption is reduced so that single structures can be solved with as little as 200 μg of protein crystallized in about 10–20 μl of LCP. New methods allowed growing sufficient amounts of densely packed crystals in gas-tight syringes [21] or by hanging wires in Eppendorf tubes [3†] and to use alternative carrier media for crystals not directly grown in LCP [22–25]. Sample efficiency could further be improved by adapting the injector-based serial approach to synchrotron sources where crystals are not immediately destroyed [26*,27], a method called serial millisecond crystallography (SMX) with reference to serial femtosecond crystallography (SFX) at XFELs. At either synchrotron [28*,29] or XFEL [29], it is possible to collect native single-anomalous dispersion data of sufficient quality for *de novo* phasing of membrane proteins as demonstrated using the adenosine A2A receptor. A series of other phasing methods has been established using high viscosity injection at the XFEL with bR as an example [30]. Ligand soaking can be easily implemented into the workflow [28*,31] and room temperature structures in a lipidic environment are good representatives for the native conformation of a protein as was argued based on comparing SFX and cryo-structures of the human serotonin 5-HT2B receptor [32]. Another milestone was the structure determination of the signaling complex between visual rhodopsin and arrestin [33], which, at the time, could only be solved at low resolution using conventional synchrotron crystallography. Taking the propensity of GPCRs and many other pharmacological relevant membrane proteins to crystallize in LCP into account [34] the method is now well suited for applications in structure-based drug design on difficult targets.

**Time-resolved pump probe data collection**

The next logical step was to demonstrate how to observe structural intermediates at particular points in time and how to characterize the functional pathway of a membrane protein (Figure 2). Yet, despite the undiscutable high quality of the data that can be collected by LCP-SFX, it has long been debated if crystal extrusion by high viscosity injectors is suitable for time-resolved measurements. For one, high viscosity carrier media are not appropriate for mix and inject approaches [35,36] because of the long diffusion times and challenges in rapidly mixing viscous media such as LCP. Similar to previous approaches by the Laue method, time-resolved serial femtosecond crystallography (TR-SFX) relies on an optical pump laser to trigger conformational changes within the protein. Ideally, multiple snapshots collected at several pump probe delays can be assembled to molecular movies frame by frame. Using liquid injectors TR-SFX was favorably validated against Laue crystallography [37†] and extended to the subpicosecond time regime using photoactive yellow protein [38], myoglobin [39†] and a reversibly photoswitchable fluorescent protein rsEGFP2 [40]. These time-resolved studies represent important milestones, but suffered from an even higher sample demand compared to pure SFX, because each recorded time point requires additional sample and data of high multiplicity is needed to resolve small structure factor variations and calculate Fourier difference maps of conformational changes.

Slow crystal extrusion leads to low sample consumption; however, it was argued that extrusion with the high viscosity injector is already too slow to clear crystals between each pump-probe cycle. Simply increasing extrusion speeds and/or reducing the repetition rate of the pump laser established high viscosity injectors as a viable delivery system for time-resolved pump-probe measurements on bR [41], especially with the higher excitation levels from using a nanosecond laser [37*,42]. For efficient data collection, the extrusion needs to run continuously without clogging and with constant velocity to allow reliable collection of interleaving dark and light images. This can be achieved by carefully pretesting and
optimizing conditions using an offline injector setup [43] and/or by additives to increase consistent flow [3**,44].

Under such optimized conditions TR-SFX using high-viscosity injectors is quite efficient. Sample consumption for the most recent study on bR was about 1.5 mg per 10 000 indexed patterns [4**]. This is a major improvement over pioneering TR-SFX experiments using the photoactive yellow protein [37**] where average sample consumption was with 74 mg per 10 000 indexed patterns.
about 50 times higher. High viscosity injectors thus have clear advantages when the amount of protein available is limited or when crystals are grown directly in LCP, as is the case for many membrane proteins.

The method is a clear advance over the classical Laue method which started time-resolved studies. The Laue method focused on a number of systems that have subsequently been studied using XFELs, generally validating results but with excellent quality and higher temporal resolution [38,39]. Despite its successes, Laue crystallography has to rely on larger, well diffracting crystals with low mosaicity that need to withstand multiple photoexcitations. The sole example for a membrane protein studied using the Laue method is the photoactivation center with no visible conformational changes at lower resolution [45] and rearrangements of a tyrosine close to the special chlorophyll pair at higher resolution [46]. However, a recently emerging theme is to adapt classical Laue approaches to serial crystallography using solid supports or injectors with polychromatic ‘pink beams’ [47]. At XFELs such a merger will increase data acquisition rates and the number of snapshots that can be collected from a given amount of sample and beamtime. At synchrotrons, the higher photonflux in comparison to monochromatic beams will reach shorter timescales. The combination could revive Laue diffraction of membrane proteins that had been mostly abandoned for freeze-trapping techniques to structurally characterize spectroscopic intermediates. Freeze-trapping does not provide real time-resolution though and this makes it difficult to separate overlapping intermediates and put them into sequence to understand whole cycles of activation (refer to Ref. [48] for a detailed comparison of TR-SFX and freeze-trapping studies on bR).

Mutations are another possibility to lock functional protein conformations (for example [49–51]), although such mutations by their very nature have to interfere with critical activation switches and are thus not without controversy.

In conclusion, there are a number of distinct advantages to study membrane proteins by TR-SFX. First, microcrystals of membrane proteins are arguably easier to grow than large ones and their low optical absorbance allows the proteins in them to be fully photoactivated. Second, in the serial approach each crystal is exposed only once – reducing the problem of laser-induced damage and permitting the study of non-reversible reactions. Third, the ultrafast pulses avoid X-ray induced damage and extend the achievable time resolution into the femtosecond regime. Fourth, the extreme averaging with multiplicity values in the hundreds allows accurate measurements. Last but not least, the protein can be embedded into a lipidic, membrane-like environment, where structural rearrangements can be studied at controlled ambient temperature and in real time.

### Current examples and future directions

The recent breakthroughs in dynamic structural biology of membrane proteins were pioneered by developments at SACLA [52], where the combination of high viscosity injectors (implementing modified versions of Ref. [20]) with a nanosecond pump laser [42] allowed investigation of the structural dynamics of bR [3**,5], photosystem II [5**] and demonstrated the use of caged substrates using a fungal nitric oxide reductase [7**]. First examples from LCLS [4**,41] show how the method can range into the ultrafast regime using femtosecond pump laser systems [53] (Figure 3).

Already the initial studies of bR activation were remarkably efficient and only 48 hours of beamtime were sufficient to resolve 13 structural snapshots from 16 ns to 1.75 ms after activation. Placing the pump-probe delays on a scale with equal logarithmic spacing resulted into a continuous evolution of structural features over time. Such an approach is preferable to targeting just a few predefined delays, as the rise and decay of structural intermediates can be deconvoluted more easily and compared to spectroscopic results. In the case of bR, the kinetics of activation within the crystal lattice and the native purple membrane are quite similar [3**,54]; however, that is unlikely to be the case for all systems and the ability to cover a wide range in time prevents missing major structural rearrangements. The transition between such states can further be of particular functional significance. Again proton pumping by bR is a great example, because a key functional step is the formation of a transient water mediated hydrogen-bonding network between the retinal Schiff base and the proton acceptor Asp85, which coincides in time with the primary proton transfer event between key intermediates of the photocycle [3**,48]. By targeting just single snapshots of the major spectroscopic intermediates such transient changes might have been easily missed.

The most recent bR study from LCLS extended our dynamic view of activation to the ultrafast regime [4**] which had been intractable by freeze-trapping experiments. At subpicosecond time scales the jitter between the optical pump laser and the X-ray probe becomes significant and has to be corrected based on measurements of the relative delay in each pump-probe cycle (using so called timing tools). Sequential windows of data within the first ps together with data collected at 10 ps and 8.33 ms yielded 20 snapshots of bR activation with a focus on the primary photochemical event of retinal isomerization and the immediate adaptation of the binding pocket. For refinement, the data were further grouped to resolve four characteristic stages of how retinal progresses along its isomerization trajectory. It was especially exciting to see how the engaging protein environment reacts already within the first 150 fs to charge redistributions along the retinal double bond system to catalyze an efficient and specific trans–cis isomerization.

Other exciting examples for the capabilities of X-ray lasers to study membrane biology are the recent studies of the oxygen evolving cluster in photosystem II. Water splitting and formation of atmospheric O₂ in this large
membrane complex are catalyzed by redox chemistry in a Mn₄CaO₅ cluster coordinated by a defining protein environment. The structure was known from conventional crystallography; however, doubts remained concerning radiation damage having changed the critical metal cluster. XFEL structures from frozen macrocrystals [55] and SFX at room temperature [56] shed new light on the problem, yet the dream of following the chemistry of
water splitting by dynamic crystallography remained only partially fulfilled at a low resolution [57**,58] where structural rearrangements remained obscure. The combination of crystal delivery with a high viscosity injector with two-flash photolysis provided the solution and direct evidence into how two oxygens are brought close enough together to allow formation of the O=O double bond [5**]. The final view of the electron and proton transfer reactions inside the water splitting complex may not have fully emerged yet. But without doubt TR-SFX has demonstrated how it can contribute to solving the puzzle.

The last example is a study on the binding of nitric oxide to a fungal reductase [7**]. This heme-binding enzyme is not a membrane protein but the study is included here because it is a demonstration of how high viscosity injectors can be used for time-resolved studies of soluble proteins by embedding crystals into alternative carrier media. In addition, it involves a photosensitive caged-compound instead of an inherent chromophore to trigger the reaction in a pump probe setup. Both are important prerequisites for a wide adaption of the technology as not every protein will readily grow into crystals within lipidic cubic phases and contain an efficient photochemical trigger. The fraction of proteins in the protein data bank with absorbing co-factors has been estimated to be as high as 20% [59], yet a wide adaption of TR-SFX will still have to rely more on synthetic triggers such as caged-compounds or azobenzene photoswitches. The conformational landscape of a protein can further be modified by temperature [8] or electric fields [9] and rapid jumps in these parameters have great promise as trigger systems for future time-resolved studies. Beam-time applications indicate the field is growing rapidly and follows up on the published studies with a range of interesting targets including light-gated ion-channels and transporters, or visual photoreceptors such as the GPCR rhodopsin. The conformational dynamics of proteins directly influence the affinity and binding kinetics of small molecular ligands and in the medium term we might expect applications of dynamic crystallography targeting a range of other pharmacologically relevant GPCRs.

Conclusions and remaining challenges
The development of serial crystallography at X-ray lasers has led to an increasing interest in time-resolved crystallography of membrane proteins. The accessible temporal range is stunning and reaches, when the equivalent orders of magnitude are expressed for accessible comparison in years, from now to long before even the solar system had been formed 4.5 billion years ago. Back to membrane biology the range is enough to cover ultrafast charge transfers and atomic motions up to water movements, rotamer changes and the rearrangement of transmembrane helices to open a channel or allow signaling. Time-resolved spectroscopy on crystals allows to determine the relevant time range where these changes occur and to verify results. Matching the scale and timing of motions to quantum chemical and molecular dynamics simulations will help improve theory by providing experimental feedback on atomic coordinates in time.

The range of applications will critically depend on the development of synthetic trigger systems and how much sample and data will be needed to achieve a scientific goal. Of course, this depends on many factors including the symmetry and diffraction power of the crystals, the efficiency of the photochemical trigger and the extent of conformational changes to resolve. As a rule of thumb, crystals should diffract well beyond 3 Å resolution with measurements below the nanosecond regime needing more sample and better data as changes tend to be small and excitation with a femtosecond laser is less efficient and more prone to induce photo damage. In contrast, even in the femtosecond range TR-SFX should be less susceptible compared to spectroscopic methods because crystals are optically dense and because averaging means only dominant changes are visible. More systematic studies are needed to understand photo damage under TR-SFX conditions and find the sweet spot where data are collected the best way. In some cases, large conformational changes could also be inhibited or destroy crystal order. Unlike crystals of bR those of visual rhodopsin for example quickly loose diffraction once exposed to light suggesting that only early intermediates are compatible with crystal packing. Unfortunately, this means some molecular movies will have to end with a cliffhanger, and it will be important to ask the right biological question. In this respect, cryo-electron microscopy could act as a complementary technique as it can resolve conformational ensembles of proteins, provided the differences are large enough to sort individual particles, but does not resolve the temporal dimension. Certainly not to be underestimated is the increasing synergy with next-generation synchrotron sources where samples can be tested and optimized before an XFEL experiment and where the implementation of time-resolved serial crystallography in the millisecond range and beyond is just a matter of time.

Conflict of interest statement
Nothing declared.

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