Angular-split/temporal-delay approach to ultrafast protein dynamics at XFELs

Zhong Ren^{a,b,*} and Xiaojing Yang^{b,c}

^aRenz Research Inc., Westmont, IL 60559, USA, ^bDepartment of Chemistry, University of Illinois at Chicago, Chicago, IL 60607, USA, and ^cDepartment of Ophthalmology and Vision Sciences, University of Illinois at Chicago, Chicago, IL 60607, USA. *Correspondence e-mail: zren@uic.edu

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X-ray crystallography promises direct insights into electron-density changes that lead to and arise from structural changes such as electron and proton transfer and the formation, rupture and isomerization of chemical bonds. The ultrashort pulses of hard X-rays produced by free-electron lasers present an exciting opportunity for capturing ultrafast structural events in biological macromolecules within femtoseconds after photoexcitation. However, shot-to-shot fluctuations, which are inherent to the very process of self-amplified spontaneous emission (SASE) that generates the ultrashort X-ray pulses, are a major source of noise that may conceal signals from structural changes. Here, a new approach is proposed to angularly split a single SASE pulse and to produce a temporal delay of picoseconds between the split pulses. These split pulses will allow the probing of two distinct states before and after photoexcitation triggered by a laser pulse between the split X-ray pulses. The split pulses originate from a single SASE pulse and share many common properties; thus, noise arising from shot-to-shot fluctuations is self-canceling. The unambiguous interpretation of ultrafast structural changes would require diffraction data at atomic resolution, as these changes may or may not involve any atomic displacement. This approach, in combination with the strategy of serial crystallography, offers a solution to study ultrafast dynamics of light-initiated biochemical reactions or biological processes at atomic resolution.

1. Introduction

The ultrashort pulses of hard X-rays produced by free-electron lasers (XFELs) such as the Linac Coherent Light Source (LCLS; White *et al.*, 2015) present an exciting opportunity for capturing ultrafast structural events in molecules within femtoseconds after photoexcitation. LCLS pulses have been used to eject atomic inner-shell electrons (Young *et al.*, 2010), to create a solid-density plasma (Vinko *et al.*, 2012), to power a pulsed electron paramagnetic resonance spectrometer (Takahashi *et al.*, 2012), to image single particles of mimivirus (Seibert *et al.*, 2011) and to produce electron-density maps (Boutet *et al.*, 2012; Chapman *et al.*, 2011) and electron-density changes (Barends *et al.*, 2015; Tenboer *et al.*, 2014) of protein structures from single-crystal diffraction patterns. These recent advances have unraveled fundamental states and processes of light, matter and life. Among many approaches based on spectroscopic, scattering and imaging observations, X-ray crystallography promises direct insights into reaction mechanisms by revealing electron-density changes that lead to and arise from structural rearrangements such as electron and proton transfer and the formation, rupture and isomerization of chemical bonds. Crystallographic

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observations of these ultrafast atomic and molecular events would significantly advance our mechanistic understanding of physical, chemical and biological processes at a fundamental level (Miller, 2014).

1.1. 100 ps and sub-bunch time resolutions at synchrotrons

Prior to the availability of XFELs, significant research and development activities at synchrotrons had advanced the techniques of Laue diffraction (reviewed in Bourgeois *et al.*, 2007; Clifton *et al.*, 1997; Ren *et al.*, 1999) and time-resolved crystallography (Moffat, 2001), also known as photocrystallography (Coppens *et al.*, 2010). These methods were developed to capture structural events that last barely longer than the duration of a single electron bunch of a synchrotron, typically 100 ps. For example, the motion of a photo-oxidized Tyr residue in a photosynthetic reaction center that lies in the electron-transfer pathway was observed at a sub-millisecond time resolution (Wöhri *et al.*, 2010). A transient asymmetric state of dimeric hemoglobin, a simpler version of hemoglobin from invertebrates, was captured at a time resolution of 100 ps during the transition between two stable symmetric states (Ren *et al.*, 2012). Intermediate conformations that are far beyond the equilibrium states of the *p*-coumaric acid chromophore in photoactive yellow protein (PYP) were observed at a series of time delays starting from nanoseconds in previous studies (Ihee *et al.*, 2005; Ren *et al.*, 2001) and 100 ps in more recent studies (Jung *et al.*, 2013; Schotte *et al.*, 2012). Photoswitchable spin states and other light-induced structural changes were also observed in smaller organic molecules and complexes (Collet *et al.*, 2012; Makal *et al.*, 2012).

However, the interpretations of these transient structural species based on changes in electron densities have not been without controversy (Jung *et al.*, 2013, 2014; Kaila *et al.*, 2014; Schotte *et al.*, 2012). Two different scientific studies led by Anfinrud and Ihee presented inconsistent structural findings of short-lived intermediates in the photocycle of PYP, even though their experimental data were acquired with the same synchrotron apparatus using nearly identical experimental protocols. Each group had characterized multiple intermediate structures in the photocycle using data sets at good spatial and temporal resolutions of 1.6 Å and 100 ps, respectively. While qualitative interpretation followed by quantitative refinement of multiple transient species may play an important role in the reconciliation of apparently different findings, one agreement could be reached: that is, both atomic spatial resolution and ultrafast temporal resolution are important for unambiguous interpretation of transient species.

The notion that the best time resolution of synchrotron-based experiments is limited by a convolution of the pump and probe, and thus the electron bunch length of the synchrotron (Moffat, 2001), has been challenged by a slight modification of the timing protocol (Haldrup *et al.*, 2011). Usually, a pump pulse arrives at a sample well before an X-ray pulse that probes structural responses. The time resolution is largely dictated by the duration of the X-ray pulse despite the availability of much shorter pump pulses. Such a limitation can be

overcome if a shorter pump pulse is placed inside a longer X-ray pulse, which in effect partitions the X-ray pulse into leading and trailing periods. Only the trailing period, a fraction of the entire X-ray pulse, can probe light-induced structural changes, thereby achieving a sub-bunch time resolution. This seemingly trivial strategy of using overlapping pump and probe pulses provides an opportunity to capture ultrafast structural species with a lifetime shorter than an electron bunch of a synchrotron. However, the desired light structures are inevitably mixed with that of the dark state in the same diffraction image. This complication could be overcome, to a certain extent, by a computational approach developed to resolve structural heterogeneity (Ren *et al.*, 2013).

1.2. Static and time-resolved crystallography at XFELs

The strategy of using overlapping pump and probe pulses to enhance the time resolution has an intrinsic limitation. Ultrafast observations at sub-picosecond time resolution require much shorter X-ray pulses from self-amplified spontaneous emission (SASE) at XFELs. Here, 'ultrafast' is defined as a time resolution far beyond what can be achieved at a synchrotron source. The diffraction-before-destruction mode of data collection at XFELs (Chapman *et al.*, 2011) adopts a 'serial' experimental strategy that is more commonly used in particle physics, in contrast to conventional cryocrystallography, but on the other hand is well suited for studying irreversible biochemical reactions and biological processes in crystalline states. A direct extension from injector-based serial nanocrystallography has been proposed for time-resolved observations (Aquila *et al.*, 2012). Several pioneering experiments at LCLS have rapidly advanced the time resolution from a fraction of a second (Kern *et al.*, 2013) to a fraction of a millisecond (Kupitz *et al.*, 2014), to sub-microsecond (Tenboer *et al.*, 2014) and to sub-picosecond (Barends *et al.*, 2015; Pande *et al.*, 2016). Continued efforts are needed to fully exploit the time-resolving power contained in the ultrashort X-ray pulses from XFELs for a broader range of biological samples.

Here, we discuss several foreseeable challenges when the time resolution reaches the sub-picosecond regime and continues to approach the limit of XFELs: as short as a few femtoseconds. Our considerations mainly focus on the photosensitive systems of biological macromolecules, such as light-driven ion pumps, channels, photoreceptors and light-dependent DNA-repair enzymes, as well as protein complexes involved in light harvesting, photoprotection and photosynthesis. Direct observations of primary light-driven structural events in these important biological systems have far-reaching impact on biomedical, agricultural, environmental and energy sciences. Our discussion is confined to a sample-oriented perspective, from which a sufficiently high signal-to-noise ratio dictates whether any experimental and computational approaches are deemed feasible. We present several potential solutions to tackle the difficulties that might be encountered in attempts to observe ultrafast structural dynamics of biological macromolecules. Yet, we do not

pretend to offer a comprehensive plan that includes every design detail.

1.3. Challenges for ultrafast observations

Despite significant progress in time-resolved crystallography in recent years, wider applications to many important biological systems remain difficult even though light-sensitive crystals are available with good diffraction quality (Rinaldi *et al.*, 2012; Yang *et al.*, 2011; Zeng *et al.*, 2015). The major difficulty arises from the irreversible nature of many reactions or processes in crystalline states, which does not permit repetitive pump–probe cycles to accumulate signals and to complete a data set (Miller, 2014). Successful cases in time-resolved crystallography thus far are largely limited to those organic and a few protein systems that exhibit rapid dark reversion without obvious structural damage to the crystal lattices.

The second difficulty specific to XFELs arises from the shot-to-shot fluctuations inherent to SASE, the very process that generates the ultrashort X-ray pulses. At least two X-ray pulses are required to record single-crystal diffraction images to capture changes in electron densities before and after laser excitation. In an ultrafast experiment, the laser pump pulse is timed to fire after the first X-ray pulse probes the dark state, yet femtoseconds before the second X-ray pulse that probes the light state. A major source of noise arises from the fact that any two X-ray pulses produced by the stochastic SASE process have little correlation in their spectral, temporal and transverse profiles (Saldin *et al.*, 1998). As a result, subtle difference signals corresponding to light-induced structural changes could be severely masked by fluctuations in the X-ray source if two independent SASE pulses are used. The self-seeding scheme (Amann *et al.*, 2012) would greatly reduce the shot-to-shot fluctuation in the spectral profile: see below for a discussion on the use of seeded pulses.

The third difficulty originates from the expected small structural signal associated with the ultrafast time resolution of sub-picoseconds to femtoseconds. Here, the ‘small structural signal’ arises for three reasons (Ren *et al.*, 2013). (i) Small structural changes often refer to small atomic displacements that may occur picoseconds or even femtoseconds after photoexcitation. It is entirely possible that no atomic displacement has yet developed on such an ultrafast time scale. The only detectable changes may be the redistribution of electron density owing to charge separation (Voityuk *et al.*, 2014) or electron transfer (Zhong, 2015). (ii) Structural changes on an ultrafast time scale are likely to be very localized. That is to say, a large-scale protein quake has not yet propagated far enough at an early time point. (iii) The extent of reaction initiation following excitation by a brief pump pulse could be rather limited. In other words, only a small fraction of protein molecules in the crystal sample have the chance to enter a productive photochemical reaction or process upon excitation by the ultrashort pulse, while the majority of the molecules remain in the ground state. Localized small or no atomic displacements only in some protein molecules inevitably lead

to very limited structural signal. The first two causes of the small structural signal clearly suggest the use of larger crystals to give better signal-to-noise ratios. The last aspect, however, calls for smaller crystals or microcrystals that give rise to more uniform photolysis owing to their limited optical depth (Tenboer *et al.*, 2014). It is clear that sufficient spatial resolution is critically important for the unambiguous interpretation of ultrafast structural dynamics, in which numerical deconvolution of mixed structural species becomes necessary.

Lastly, ultrafast diffraction at XFELs leads to partial reflections, in which the integrated intensity of a Bragg reflection directly related to the crystal structure cannot be fully measured by a single pulse in an XFEL experiment. Partial intensities introduce errors into the Fourier synthesis, a process that produces electron-density maps. How to obtain accurate structural signal from these partial measurements is still a major challenge. Variable mosaic spread from crystal to crystal also affects the partiality of the reflections. We discuss partiality and possible solutions in the section on data processing (§2.2).

2. Methods

Here, we present an integrated approach aiming to overcome the abovementioned hurdles that might hinder the crystallographic observation of ultrafast structural events for the mechanistic elucidation of many important biological systems. Specifically, we propose to employ an angular-split/temporal-delay strategy combined with serial crystallography. An SASE pulse is angularly split so that the slightly different incident angles of the split pulses cause two nearly identical diffraction patterns. Each pair of diffraction patterns is recorded on a single image that is subsequently read out once by an integrating area detector. These two split patterns can be readily distinguished during data processing, as similar split patterns from time-resolved Laue diffraction have successfully been processed (Ren *et al.*, 1996). A complete survey of reciprocal space will depend on a strategy of serial crystallography, in which a large number of split diffraction patterns are necessary. A temporal delay of picoseconds is inserted between the split pulses. When a laser pulse is precisely timed in between to initiate a photochemical reaction, these split pulses are able to record and contrast two distinct states before and after photoexcitation from the same crystal. Since both split pulses originate from a single SASE pulse, their properties remain largely in common. As a result, the noise arising from shot-to-shot fluctuations are self-canceling, which is crucial for accurate measurements of light-induced difference signals. We discuss in depth the foreseeable differences between the split pulses, their influence on data quality and potential solutions in data processing. It can be shown that the split diffraction patterns share some reflections with exactly the same partialities, even if these partialities are unknown. This set of reflections with equal unknown partialities allows straightforward data scaling between two split patterns, which is necessary to produce accurate difference signals. This ratio-metric approach circumvents direct comparisons between

Figure 1
A schematic illustration of the dichromator.

Figure 2
Temporal delay and angular split between split pulses. The temporal delay Δt is proportional to the separation of two dichromator crystals δ (2). Here, it is presented as picoseconds per centimetre separation as a blue mesh. The angular split $2\Delta\theta$ is proportional to the relative dispersion between two pulse energies (3). It is presented as milliradians per 1% of dispersion as a brown mesh (on the negative side). The zero plane is shown as a gray mesh. Specific examples are shown as red and green dots. See also Table 1.

diffraction images that are derived from different SASE pulses and/or different crystals, and hence is hugely advantageous in reducing two major sources of noise owing to the shot-to-shot fluctuations in SASE radiation and the natural variations in biological samples from crystal to crystal.

The split pulses are both monochromatic and are only picoseconds apart. These features are important to eliminate

the effect of physical movement of the sample between pulses and to reduce the effect of radiation damage initiated by the first pulse to a negligible level. Considering the radiation damage to protein crystals by XFELs at room temperature, a complete survey of reciprocal space is achieved by serial crystallography, in which many pairs of dark and light diffraction patterns are collected from a series of crystals in

random orientations. Nanocrystals and microcrystals are more conveniently delivered by liquid (Chapman *et al.*, 2011) and paste jets (Liu *et al.*, 2013). Crystallization devices and crystal-loading devices for high-throughput diffraction open the possibility of serial crystallography of macroscopic protein crystals (Mueller *et al.*, 2015; Pawate *et al.*, 2015; Perry *et al.*, 2014). Our plan also includes computational approaches that go hand in hand with the experimental strategies. Specifically, our ratiometric approach bypasses both experimental and computational integrations of full reflections including Monte Carlo integration, one of the current solutions to partial diffraction encountered in femtosecond crystallography (Kirian *et al.*, 2010, 2011).

2.1. Proposed dichromator for angular-split/temporal-delay pulses

Firstly, we propose a conceptual design for a dichromator instrument that produces angular-split/temporal-delay pulses (Fig. 1). The full spectral width of the raw, unseeded SASE radiation from LCLS is about 15–100 eV (Amann *et al.*, 2012; Emma *et al.*, 2010; Shu *et al.*, 2013). This is too wide to be considered as monochromatic radiation, but too narrow to be useful for polychromatic Laue diffraction. A pair of thin diamond or silicon crystals separated by a small distance δ could be used to select two monochromatic components of the SASE radiation a few tens of eV apart from each other. The first crystal reflects a higher energy component at a Bragg angle θ_1 and routes a monochromatic beam through a shorter path. The second crystal is set at a slightly greater Bragg angle $\theta_2 > \theta_1$, reflecting a lower energy component that routes another monochromatic beam through a longer path. It can be shown that the path length from a dichromator crystal to the point where two paths intercept and where a sample crystal shall be located is

$$p_{1|2} = \delta \frac{\sin 2\theta_{2|1}}{\sin 2\Delta\theta}, \quad (1)$$

where p_1 and p_2 are the path lengths from crystals 1 and 2 to the point of intercept, respectively. The difference in the beam-path lengths Δp is a function of both Bragg angles θ_1 and θ_2 and is proportional to δ , but is no greater than 2δ ,

Table 1
Selection of dichromator crystals.

First crystal	Second crystal	$\Delta E/E$ (eV)	Δt (ps) [†]	$2\Delta\theta$ (°)	Application
Diamond (111)	Diamond (111)	40/8000	9.0	0.23	Ultrafast time-resolved crystallography with SASE pulses
Diamond (220)	Diamond (220)	40/8000	22.1	0.45	
Diamond (220)	Diamond (220)	20/8000	22.1	0.22	
Diamond (220)	Diamond (220)	5/4951	46.8	0.97	
Diamond (111)	Germanium (220)	~0/8000	9.3	1.47	Ultrafast time-resolved crystallography with seeded pulses
Diamond (111)	Germanium (220)	~0/12000	4.2	0.94	
Diamond (111)	Germanium (220)	10/12658	3.8	0.86	SAD phasing at the absorption edge of Se with and without seeding
Diamond (111)	Germanium (220)	2.5/12658	3.8	0.88	

[†] Picoseconds per centimetre of δ .

$$\Delta p = \delta + p_2 - p_1 = 2\delta \frac{\sin \theta_1 \sin \theta_2}{\cos \Delta\theta} \approx \delta - \delta \cos(\theta_1 + \theta_2), \quad \theta_1 \lesssim \theta_2. \quad (2)$$

Figure 3

A simulation of diffraction patterns produced by angularly split pulses. The photoactive crystal of a plant UV-B photoreceptor (Zeng *et al.*, 2015), a potential project for ultrafast diffraction at XFELs, is used for this simulation up to a resolution of 2 Å. The unit-cell parameters are $a = 112.4$, $b = 75.8$, $c = 188.7$ Å, $\beta = 96.0^\circ$ in a monoclinic space group. The blue pattern is a dark reference. The red pattern captures light-induced changes. The energy dispersion between the split pulses is 40 eV at 8 keV; that is, 0.5%. Both dichromator crystals are diamond (111). Most reflections, if not all, are present in both patterns owing to the small horizontal angular split of 0.23° . If an area detector of 300×300 mm is located 200 mm away from the sample crystal, the separation of spots in the dark–light pairs ranges from 0.8 to 1.7 mm with an average of greater than 1 mm, equivalent to an average angular separation of 0.3° . If an MX300-HS area detector (Rayonix, Evanston, Illinois, USA) is selected (78×78 µm per pixel), a dark–light pair is 13 pixels apart on average, which is adequate for the data-processing software to distinguish. It is important to note that this simulation considers the smallest angular separation among many possibilities discussed here. Other dichromator crystals are expected to produce equivalent or greater separations (Table 1).

Therefore, the higher energy pulse arrives at the sample sooner than the lower energy pulse. By timing a pump laser pulse between these two split X-ray pulses, a delay time as short as femtoseconds could be achieved (Bionta *et al.*, 2014).

For example, if we choose diamond (111) for both crystals in the dichromator, the Bragg angles are about 22° at 8 keV. Two diamond crystals 1–10 cm apart from each other would result in a

range of temporal split Δt of 9–90 ps (Fig. 2), which caps the upper bound of the accessible pump–probe delay range and is beyond what can be normally achieved at a synchrotron. Any detectable physical motions of a sample crystal within 100 ps would require the crystal to move orders of magnitude faster than the speed of sound or tumble at billions of revolutions per minute. In other words, the sample crystals can be considered to be completely stationary during the period between two split pulses, even when they are delivered by liquid or paste jets. The sample position is 0.7–7 m away from the dichromator crystals (1), which would provide a sufficient working distance for a collimator assembly, sample-positioning stages and spindle, sample-viewing camera and beam stop. The difference in beam-path lengths Δp and the temporal delay Δt are proportional to the distance δ between the two dichromator crystals (2), which determines the overall size of the instrument. Longer delays that require a larger instrument would be less attractive since synchrotron-based technologies are likely to be more effective.

2.1.1. Angular split. It is very important to offset these two split pulses by a small angle $2\Delta\theta$ of a fraction of a degree. Such a small angular offset gives rise to two very similar diffraction patterns on the same integrating area detector with easily distinguishable spots (Fig. 3). The blue pattern from the higher energy pulse occurs first before a pump laser pulse and serves as a dark reference. The red pattern from the lower energy pulse occurs after the pump pulse and hence captures photo-induced changes. Without the angular offset, the dark and light patterns would superimpose exactly on the detector space and thus be indistinguishable. The intensity change within each pair of spots can be scaled directly as they are obtained from a single SASE pulse, but no comparison is needed between two images from different SASE pulses in this ratiometric method (see §2.2 for data processing). It can be shown that the angular split is proportional to the energy dispersion ΔE between the split pulses,

$$2\Delta\theta = \frac{2\Delta\lambda/\lambda}{(4d^2/\lambda^2 - 1)^{1/2}} = \frac{-2\Delta E/E}{[4d^2 E^2/(12398.52)^2 - 1]^{1/2}}, \quad (3)$$

where E is the photon energy in eV and λ is the corresponding wavelength. d is the d -spacing of the selected dichromator crystal plane in Å. Both the energy dispersion ΔE and the angular split $2\Delta\theta$ between the split pulses remain constant

during an experiment, in which the two crystals of the dichromator are stationary. The energy dispersion ΔE is limited by the bandwidth of the raw, unseeded SASE radiation. An energy dispersion of 40 eV at 8 keV (0.5%) is expected to allow an angular split of 0.23° by diamond (111) crystals (Table 1). A larger angular split of 0.45° is possible with diamond (220) at higher Bragg angles. Alternatively, the same angular split can be achieved from a smaller energy dispersion of 20 eV by diamond (220). A full consideration of the choice of crystals requires detailed simulations.

2.1.2. Spectral and temporal properties. The finest independent spectral feature of SASE radiation is about 0.13–0.30 eV apart according to both theoretical simulations and experimental observations (Makita *et al.*, 2015; Zhu *et al.*, 2012). Each crystal of the dichromator would integrate multiples of such fine elements, as their energy resolutions are significantly wider than the SASE elements. Thus, it is unlikely that all of the independent spectral elements have nearly zero intensity simultaneously, resulting in a pulse dropout, although uneven intensities are expected from the monochromated split pulses (Supplementary Movie S1). It is expected that the intensity ratio between two split pulses fluctuates from shot to shot and therefore from image to image. The next section on data processing describes a scaling procedure to correct for uneven intensities between two split pulses. The design considerations of the dichromator includes a coarse energy resolution that would lower the chance of dropouts. It is entirely possible that the split pulses do not have unimodal spectra, and these spectra differ from each other and vary from one SASE pulse to the next (Supplementary Movie S1).

Figure 4

The effect of photon energy on the location of a diffracted ray on the detector. (4) is plotted assuming that a Rayonix MX300-HS detector is located 200 mm from the sample. The value of the derivative dp/dE is converted to pixels of $78 \mu\text{m}$ per spectral element of 0.2 eV. For example, at $\theta = 10^\circ$ the effect of a fine spectral element is only 0.026 pixels at 8 keV and 0.017 pixels at 12 keV, which is two orders of magnitude too small to be differentiated by the detector pixels.

Multiple fine elements selected by each dichromator crystal are confined within ~ 1 eV depending on the width of its rocking curve. These fine elements are too close in wavelength to generate distinct sets of reflections. Instead, they are effectively integrated into a monochromatic wavelength even when the smallest mosaic spread of a protein crystal is encountered. That is to say, the multimodal spectra of the split pulses can be safely considered as discrete dichromatic wavelengths (Supplementary Movie S1), which greatly simplifies the scaling process, as we propose below. One advantage of a seeded pulse over an SASE pulse is that occasional dropouts are minimized or eliminated and the spectral stability of the split pulses is further improved. However, a dichromator in combined use with self-seeding requires a different selection of dichromator crystals, as discussed below.

It can be shown that the location at which a diffracted ray is intercepted by the active surface of an area detector depends very little on whether this X-ray photon comes from one of the fine spectral elements or its adjacent element. Given that this location has a distance ρ from the direct-beam center on the detector with a normal setting,

$$\frac{d\rho}{dE} = -\frac{2M \tan \theta}{E \cos^2 2\theta}, \quad (4)$$

where M is the sample-to-detector distance and θ is the Bragg angle of the diffracted ray. At a typical Bragg angle in protein crystallography ($\theta < 15^\circ$), the location at which a diffracted ray falls onto the detector varies a diminishingly small amount as a function of the multiple fine elements in each split pulse (Fig. 4). Therefore, the multimodal fine structure in the fluctuating spectra of hard X-ray split pulses will be completely integrated into the detector pixels, and has a minimal effect on the data quality.

Furthermore, multiple fine elements selected by each dichromator crystal arrive at the sample position at different times. However, this temporal spread is capped by the duration of the parent SASE pulse. Although each split pulse may be far shorter than its parent SASE pulse, the effective duration of a split pulse approaches that of its parent SASE pulse for a data set derived from a serial crystallographic experiment. In this sense, the lower bound of the achievable time resolution is limited by the convolution of an SASE pulse and a pump laser, with an additional uncertainty from a timing jitter.

Each monochromated pulse carries only a small fraction of the photons in the original SASE pulse. Therefore, the split pulses are less destructive than their parent SASE pulse. Our previous experience at LCLS shows that a monochromated pulse with a defocused beam matching the size of a macroscopic protein crystal could produce sufficient diffraction signals without significant damage to the crystal (van Thor *et al.*, 2014). That is to say, the first higher energy pulse would safely produce a dark reference pattern before the pump–probe pulses. Although underfocused monochromatic pulses have successfully produced multiple diffraction images from an individual macroscopic crystal, it is not yet clear whether a tightly focused monochromatic pulse will terminate the

diffraction from a microcrystal or nanocrystal (Barty *et al.*, 2011). Nevertheless, the first pulse, whether defocused or focused, is intended only for a dark reference. However, its adverse effect will accumulate over time until the second pulse. Undesired structural changes or disorder may mix with the light-induced changes under investigation. Therefore, the temporal delay between two split pulses is an effective time window that should be set to be barely long enough to capture the desired ultrafast events but to screen out the effect of the first pulse as much as possible. See §3.2 for variable separation between the dichromator crystals.

2.2. Data processing

In this section, we propose computational methods that are complementary to our experimental strategies to process a large number of split diffraction patterns. We have previously demonstrated how to process four-way splitting diffraction patterns recorded on a single image in time-resolved Laue diffraction experiments. Multiple split patterns are readily distinguishable by software (Ren *et al.*, 1996). Some inadvertently overlapping spots can be deconvoluted (Ren & Moffat, 1995). Here, we focus on how to treat average and instantaneous partialities.

2.2.1. Average partiality. Femtosecond crystallography at XFELs differs markedly from conventional diffraction experiments, both monochromatic oscillation and polychromatic Laue diffraction. In monochromatic oscillation, X-rays of a fixed wavelength interact with an oscillating crystal to produce an integrated intensity while a reflection satisfies the diffraction condition as its corresponding reciprocal-lattice point crosses the Ewald sphere. In Laue diffraction, X-rays within a broad wavelength range interact with a stationary crystal. No crystal motion is required since the wavelength range is sufficiently wide to achieve fully integrated intensities. In both methods, partial reflections need special treatment (Ren *et al.*, 1999). They result from incomplete experimental integration and are often found at two ends of an oscillation range or a wavelength spectrum. In these conventional methods, only a small number of reflections are partial and they can be readily corrected by computational methods during data processing. In femtosecond crystallography at XFELs, in contrast, almost all reflections are partial, because the ultrashort exposure time does not permit any crystal motion and the bandwidth, on the other hand, is too narrow to cover full reflections. This situation becomes even more severe when self-seeding is applied, as the seeded pulses are essentially monochromatic. Monte Carlo integration, one of the solutions to partiality in femtosecond crystallography, relies on a statistical average of a large number of such partials (Kirian *et al.*, 2010, 2011). The underlying assumption is that the average partiality does not vary sharply from reflection to reflection when all reflections are measured with high redundancy. It can be shown that the average partiality is a tangent function of the Bragg angle θ and proportional to the relative bandwidth $d\lambda/\lambda$,

$$d\theta = \frac{d\lambda}{\lambda} \tan \theta. \quad (5)$$

This function varies slowly at low resolution but more and more sharply at higher resolution (Fig. 5). This suggests that Monte Carlo integration of XFEL data at high resolution requires additional correction according to this partiality function (5). Nevertheless, the accuracy of integrated intensity critically depends on knowing the instantaneous partiality of each measured reflection even if the average partiality is properly corrected. Solving instantaneous partialities for all spots remains difficult and could be computationally intensive, if it is solvable at all.

2.2.2. Common partialities between split spots. Since the angular split between two pulses is proportional to their energy dispersion (3), the center of the Ewald sphere corresponding to the second pulse follows a certain trajectory in an Ewald construction (Fig. 6a). It can be shown that this trajectory is a spiral curve in the plane of the split angle in the reciprocal space, denoted the xy plane,

$$\begin{cases} x = -\frac{1}{\lambda + \Delta\lambda} \cos \frac{2\Delta\lambda}{(4d^2 - \lambda^2)^{1/2}}, \\ y = -\frac{1}{\lambda + \Delta\lambda} \sin \frac{2\Delta\lambda}{(4d^2 - \lambda^2)^{1/2}} \end{cases}. \quad (6)$$

The center of the Ewald sphere moves along a slightly curved section of the spiral (Fig. 6a). If viewed along the X-ray beam, the two Ewald spheres of the split pulses fit each other better on the right side (reciprocal coordinate $y < 0$ in equation 6 and Fig. 6) than on the left side (reciprocal coordinate

Figure 5
Correction function for average partiality (5). Average partiality is plotted as a function of the Bragg angle for 1% bandwidth. The inset is an Ewald construction with two Ewald spheres corresponding to λ and $\lambda + d\lambda$. The three black wedges share a constant angle and represent the mosaic spread of a sample crystal at different spatial resolutions. Severe partiality occurs at low resolution. The average partiality gradually eases at high resolution. There will be no partial reflections if the resolution reaches back-reflection angles.

$y > 0$ in equation 6 and Fig. 6). Therefore, paired diffraction spots on the right side of the detector space are more likely to exhibit comparable instantaneous partiality. Two spots in a pair on the left half of the image are expected to differ more in partiality. The goodness of fit between the two Ewald spheres of the split pulses offers an important weighting function in the data-processing protocols proposed below (Fig. 6*b*). On the loop where two Ewald spheres intersect with each other (Fig. 6), *i.e.* where the surface in gold crosses the zero plane in blue, the partialities of equivalent reflections from two split patterns are equal, even if they remain undetermined, that is two equal unknowns. The split patterns are guaranteed to share similar partialities everywhere near and inside the loop. Knowing this fact opens the possibility of accurate comparison between two partial intensities without integration.

Although the ratio between the intensities of two split pulses may vary from image to image (Supplementary Movie S1), it will remain constant for all reflection pairs on a single image and thus can be accurately determined using a least-squares scaling procedure based on hundreds of pairs of reflections (Fig. 3). The likelihood of common partiality (Fig. 6*b*) will be used to improve the accuracy of the scaling procedure. That is to say, a subset of the split reflections near and inside the loop on the right side of the diffraction image share nearly identical partialities and their collective intensity change largely reflects the difference in the overall intensities of the split pulses. Once the relative intensities of the split pulses have been normalized, the remaining difference between two spots in a pair can be mostly attributed to structural changes if the pair is located on the right side of the image. However, if the pair is on the left side, the remaining difference is likely to be caused by both difference in instantaneous partiality and structural changes.

Our analysis of the diffraction geometry of the dichromator demonstrates that some split spots share identical, although unknown, partialities. Knowing the distribution of common partialities in the detector space allows us to express ratio-metric changes in structure-factor amplitudes in the absence of definitive solutions to instantaneous partialities and integrated intensities. At the very least, this will apply to a significant subset of reflections on each image that share nearly equal partialities.

2.2.3. Determination of instantaneous partialities. Nevertheless, the determination of instantaneous partialities and hence integrated intensities may still be desirable. The split patterns collected from macroscopic crystals at high spatial resolution enable accurate geometric refinement of the orientation matrix, unit-cell parameters and mosaic spread of each sample crystal, since the wavelengths of both pulses and their split angle are precisely known. For example, the simulated split patterns show a zone of reflections in the blue pattern but very few reflections in the red pattern near the upper left corner (Fig. 3). These sources of information are critical for geometric refinement, which eventually leads to the accurate determination of instantaneous partialities of all diffraction spots. Refined partialities can be further validated by the likelihood function of common partiality (Fig. 6*b*).

After scaling of pulse intensity and the correction of instantaneous partialities, the remaining intensity differences expressed as ratios (Coppens *et al.*, 2009) are merged into a complete data set. Redundant measurements of these intensity ratios are then averaged under a weighting scheme, where the weight depends on the location of the corresponding spots in the detector space (Fig. 6*b*). It is noteworthy that integrated intensities after partiality correction are the byproducts of this ratiometric procedure and are not required for the synthesis of difference Fourier maps.

Figure 6
Trajectory of the Ewald sphere center. (a) Ewald construction of angular split pulses. The Ewald sphere for the first pulse of higher energy is in blue. The second pulse of lower energy is simulated at realistic and exaggerated energy dispersions of 1 and 10% from the first pulse. Their Ewald spheres are in red and green, respectively. The three colored arrows mark the directions of the corresponding pulses. The trajectory of the center of the Ewald sphere follows the spiral curve in black. (b) The probability of different partialities in a pair of split spots. This probability is proportional to the difference Δr between two Ewald spheres corresponding to two split pulses (a). The difference Δr is plotted as a surface in gold. The right side portion ($y < 0$) of the surface rises slightly above the zero plane in blue. The other area falls quickly into negative values.

2.2.4. Time-series processing. In principle, a difference Fourier map can be synthesized once a complete data set is available at an isolated sub-picosecond time point. In reality, such a map could still be very noisy. Structural interpretation based on data from a single time delay could be biased or even impossible because of commonly occurring structural heterogeneity; that is, two or more structural species are simultaneously present at the time point of measurement (Tenboer *et al.*, 2014). Joint analysis of a time series is not only desirable to unscramble the mixture, but is also necessary to identify and reduce noise along the dimension of time (Ren *et al.*, 2013).

3. Results and discussion

The aim of angular split/temporal delay is to reduce the noise in the difference signal through careful experimental design so that burdens on posterior analysis are significantly alleviated. This is based on the fact that fully integrated intensities, which are very difficult to obtain in femtosecond crystallography, are not necessary for generating difference structural signal. The angular split is designed to overlay the dark reference pattern and photo-perturbed pattern side by side so that direct contrast in dark–light difference signals can be recorded directly. More importantly, massive averaging, which is a major source of error and inefficiency, is no longer needed in this approach.

3.1. Existing split-and-delay and self-seeding protocols

A split-and-delay instrument is crucial for time-resolved experiments on ultrafast time scales. Time-resolved protein crystallography is no exception. Without split-and-delay, the period between two consecutive SASE pulses is six to nine decades longer compared with the temporal delay of the proposed dichromator. If two consecutive pulses shoot randomly oriented sample crystals for dark and light measurements, the resulting diffraction patterns are entirely different and thus have little chance of sharing the common reflections that are needed to produce difference signals directly (Barends *et al.*, 2015; Pande *et al.*, 2016). If a steadily held sample crystal is used to produce two similar diffraction patterns for dark and light measurements from two consecutive SASE pulses, many undesirable events, such as radiation damage from the first pulse, may develop and accumulate before the arrival of the second pulse. Possible artifacts during the time spanning many decades include thermal expansion of the sample crystal and its surrounding liquid and sample slippage in its mounting device, in addition to many physical and chemical processes associated with radiation damage and other redox reactions triggered by the first X-ray pulse. In contrast, the temporal delay produced by the dichromator effectively screens out these possible artifacts yet provides a time window that is just long enough to capture the desired dark–light difference signals. This time window determined by the distance between two dichromator crystals could be made adjustable (see §3.2 for variable separation between dichromator crystals).

The existing split-and-delay lines designed and implemented for various purposes are not directly adequate for protein crystallography when both ultrafast time resolution and atomic spatial resolution are in demand. Temporal splitting of VUV and soft X-ray pulses has been demonstrated using mirrors (Castagna *et al.*, 2013; Wöstmann *et al.*, 2013). The split-and-delay mirrors for soft X-rays are essentially operating at very small θ_1 and θ_2 (equations 1 and 2), and hence generate a small path difference. In our dichromator design, large Bragg angles are used to produce two well separated X-ray pulses.

An eight-crystal split-and-delay instrument and other related instruments designed for coherence measurements produce co-propagating split pulses of hard X-rays (Röling *et al.*, 2013; Roseker *et al.*, 2011). Therefore, diffraction patterns before and after a pump laser pulse would overlap right on top of each other if such an instrument were used for single-crystal diffraction. Although it is possible in principle to digitally unscramble dark and light diffraction intensities from a sum recorded on the detector, the large uncertainties associated with such numerical solutions could easily mask any subtle difference signal when spatially separate observations of dark and light diffractions are not available. A different strategy to split a hard X-ray pulse has been demonstrated by an electron-beam chicane at SACLA with two very different energies (Hara *et al.*, 2013). Although the split pulses co-propagate in the same beam without any angular split, two diffraction patterns produced by the temporally split pulses of very different energies could still be distinguishable even when they are recorded on the same image. However, these two patterns will not share many common reflections and thus it is hard to measure intensity changes directly except for a few reflections at very low resolution. If two split pulses of hard X-rays differ only slightly in energy, a small subset of diffraction spots may appear split in detector space owing to the mosaic spread of protein crystals. These split spots will inevitably encounter two different partialities. Both soft and hard X-ray pulses femtoseconds apart with slight color difference have been produced at LCLS (Lutman *et al.*, 2013; Marinelli *et al.*, 2015).

In summary, these established protocols are designed for specific goals but are not amenable to ultrafast dynamic observations by protein crystallography. In ultrafast time-resolved crystallography, we have to simultaneously take into consideration X-ray wavelengths, a range of time delays, differentiation between the dark and light diffraction patterns, partiality and, last but not least, complexity in execution. Our proposed dichromator instrument in the photon beam (not in the electron beam) offers a relatively simple strategy to generate both angular split and temporal delay simultaneously.

The self-seeding scheme (Amann *et al.*, 2012) would indeed minimize the shot-to-shot fluctuation in the spectral profile and thus reduce noise. We have considered the potential signal-to-noise improvement in great detail (van Thor *et al.*, 2014). Yet self-seeding alone is not a strategy to cancel noise that originates from factors other than the spectral profile.

Since the small bandwidth in SASE radiation is compressed into nearly a single energy as a result of self-seeding, severely partial measurements from the monochromatic still diffraction demand even more aggressive integration and thus greater sample consumption to suppress rising error levels owing to extreme partials. Therefore, self-seeding alone poses additional challenges while easing some others (Neutze, 2014). Nevertheless, self-seeding and our proposal here are not mutually exclusive. On the contrary, a combined use of self-seeding and angular split/temporal delay could be even more advantageous in achieving noise cancelation, eliminating pulse dropout and maintaining stable relative intensities between two split pulses. If the seeded pulse is desirable, the two dichromator crystals must be of different types so that the second crystal reflects (nearly) the same energy but at a greater Bragg angle (Table 1). For example, diamond (111) and germanium (220) as the first and second crystals, respectively, would result in an angular split of 1.47° at 8 keV or of 0.94° at 12 keV.

3.2. Dichromator implementation

Many design considerations will be guided by scientific aims and have to suit the conditions on a specific beamline. We do not intend to discuss every design requirement in this paper. The diamond monochromator for LCLS self-seeding (Shu *et al.*, 2013) could be a good starting point for the dichromator proposed here. The dichromator is behind a shutter in the experimental hutch and it receives an SASE pulse every time a new sample crystal is aligned and ready for diffraction. The frequency of the dichromator exposure during routine data collection is estimated to be between 1 and 120 Hz. Therefore, the average power that the dichromator crystals would receive is rather low, and the overall heat load will not be a concern. On the other hand, the two dichromator crystals require an additional motion control to ensure that the split pulses intercept at the sample position (spatially but not temporally).

A more sophisticated design would consider variable separation between two dichromator crystals so that the pump–probe delay range is adjustable. Since the temporal delay between two split pulses is proportional to the distance δ between these crystals (Fig. 1; equations 1 and 2), it is desirable to set δ to be only large enough for the planned pump–probe delay range during an experiment. An excessively large distance δ , and thus a pump–probe delay range that is much longer than necessary, opens the possibility of undesired physical and chemical processes interfering with the designed experiment. The intended dark–light difference signals contaminated with these systematic errors are often difficult to troubleshoot. This is yet another important aspect of our signal-to-noise concerns that argues for the benefit of split pulses over two consecutive SASE pulses (see §3.1).

To use the proposed dichromator in a focused beam, the focusing optics have to be located upstream. Therefore, the focal length must be greater than the path lengths from the dichromator crystals to the sample (1), and the focal depth must be greater than the difference in path lengths (2). These parameters such as focal spot size, focal length and depth must

be considered jointly with specific designs of the dichromator in the context of the driving scientific project.

3.3. Experimental measurements of anomalous scattering for phase determination

Although experimental phasing for macromolecular crystal structures and time-resolved observation of structural dynamics are seemingly unrelated, they both critically depend on the accurate measurement of intensity changes. Phase angles are not measured directly but are computed from intensity changes that are either owing to anomalous scattering near an absorption edge of a heavy atom, such as in single-wavelength and multi-wavelength anomalous diffraction (SAD/MAD), or modifications to a structure, such as heavy-atom derivatives and specific radiation damage (Banumathi *et al.*, 2004; Ravelli *et al.*, 2003; Son *et al.*, 2011). In this sense, experimental phasing is quite similar to the observation of structural dynamics, except for those proposed phasing protocols based on inter-Bragg scattering (Miao & Rodriguez, 2014; Spence *et al.*, 2011). It has been demonstrated that a Gd derivative of lysozyme produced sufficient phasing power from 60 000 diffraction images using microcrystals and SASE pulses at 8.5 keV (Barends *et al.*, 2013). This derivative with two Gd atoms per asymmetric unit produced strong anomalous signals of 5–20% up to a resolution of 2.1 Å. It remains to be seen whether unknown protein structures can be determined using XFEL data.

Here, we propose to take advantage of the dichromator to measure anomalous scattering signals if the X-ray photon energy can be tuned to cover an absorption edge of a selected heavy atom such as Se in selenomethionine, a commonly used substitute for S in protein structure determination. Such an alternative application of the dichromator could be even more attractive than our original intent for ultrafast dynamics. In this scenario, two split pulses should be calibrated to set the low energy at the inflection point and the high energy above the absorption edge in order to capture anomalous signals. However, the temporal delay between the pulses should be minimized. Therefore, the dichromator crystals in such an application must be carefully considered. Given diamond (111) and germanium (220) as the dichromator crystals, an angular split of 0.86° and a temporal delay of within 300 fs can be achieved at the absorption edge of Se (Table 1). Such side-by-side comparison of split patterns at two key energies allows the accurate measurement of anomalous differences. This approach is particularly useful for *ab initio* phasing of membrane proteins using serial crystallography, in which Friedel or Bijvoet mates cannot be measured from the same crystallite by monochromatic still diffraction.

The energies of the inflection and peak points of Se atoms in a protein environment differ only by 2–3 eV (Evans & Pettifer, 2001), where the scattering factors f' and f'' reach their minimum and maximum values, respectively, and thus warrant the most significant phasing signals from anomalous scattering. The current 1 eV bandwidth of the seeded pulse is slightly too narrow to span both the inflection and peak

energies. If the seeded bandwidth can be adjusted to cover both energies in future developments, the self-seeding scheme would be very attractive in this application given the pulse stability produced by self-seeding (Table 1). However, such experiments in reality demand an exquisite precision of control, because both the absolute X-ray energy and the energy dispersion between the split pulses can tolerate very little error. Small deviations from the targeted energies will diminish anomalous signals, as measurements of the Friedel and Bijvoet mates are not readily available for each individual crystal. We believe that the potential benefits from such a combined use of the dichromator and a self-seeding scheme are sufficiently significant to warrant a more detailed study of this possibility.

4. Conclusions

We present a plan with multiple scientific and technical components aiming at the direct crystallographic observation of ultrafast structural dynamics at atomic spatial resolution and with sub-picosecond temporal resolution. This scientific goal has been one of the major driving forces for the development of XFELs. Our proposal centers on efforts to enhance signal-to-noise ratios in space and time in order to achieve the best possible spatial and temporal resolutions. A dichromator instrument is proposed to split a single SASE pulse such that the first pulse probes the dark reference state and the second probes the light-induced state from the same sample crystal. Dark and light split patterns are laid out side by side for direct comparison enabled by the built-in angular split. The signal-to-noise ratio of the resulting difference maps will be significantly enhanced as a result of self-canceling two major sources of noise: shot-to-shot fluctuations in SASE radiation and natural variations in biological sample from crystal to crystal. The period between the split pulses is insufficient for a sample crystal to develop any macroscopic motion. This seemingly trivial coincidence effectively circumvents the otherwise tough problem of crystal immobilization when a biological sample is not frozen. The dark reference is taken just before the arrival of a pump pulse, which screens out the prolonged effects of radiation damage and other unintended redox reactions initiated by the first pulse. Underfocused split pulses are expected to safely produce dark and light patterns picoseconds apart from a macroscopic crystal without a significant adverse effect accumulating on the second pattern. However, the extent of structural damage captured by the second pattern remains to be seen if tightly focused split pulses are delivered to a microcrystal or nanocrystal even when both pulses are monochromated.

We also outline computational approaches to identify a subset of dark–light pairs with similar partialities before their precise values are determined. A scaling procedure to normalize pulse intensities based on this subset of split spots is expected to be reliable and accurate. This ratiometric approach allows more efficient data collection in four-dimensional space, with time being the extra dimension, instead of massive averaging to overcome errors associated

with unknown partialities. The ultimate determination of instantaneous partiality for each recorded reflection will depend on whether accurate geometric refinement can be achieved, which may be more feasible with split patterns at high resolution. Successful implementation of this plan will advance the capability of protein crystallography at XFELs for the direct observation of ultrafast photochemical events in proteins.

5. Related literature

The following reference is cited in the Supporting Information for this article: Schneidmiller & Yurkov (2013).

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