1 kHz fixed-target serial crystallography using a multilayer monochromator and an integrating pixel detector

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Reliable sample delivery and efficient use of limited beam time have remained bottlenecks for serial crystallography (SX). Using a high-intensity polychromatic X-ray beam in combination with a newly developed charge-integrating JUNGFRAU detector, we have applied the method of fixed-target SX to collect data at a rate of 1 kHz at a synchrotron-radiation facility. According to our data analysis for the given experimental conditions, only about 3000 diffraction patterns are required for a high-quality diffraction dataset. With indexing rates of up to 25%, recording of such a dataset takes less than 30 s.

1. Introduction

Serial crystallography (SX) is based on merging data from still diffraction patterns collected from several tens to several hundreds of thousands of crystals into a complete set of structure factors, which is then used for protein structure determination (Chapman et al., 2011; Schlichting, 2015). In contrast, conventional macromolecular X-ray crystallography is based on collecting a series of rotation photographs from one or more crystals, which are then merged into a complete dataset. By avoiding the need to rotate crystals over a certain angle increment during their exposure and requiring individual measurements of only minimal signal to background, SX thus allows almost arbitrarily short exposure times, making this method ideally suited for time-resolved experiments.

SX employs a variety of methods to deliver individual microcrystals to the X-ray beam for data collection (Grunbein & Kovacs, 2019; Martiel et al., 2019). For fixed-target SX experiments the microcrystals are immobilized on a solid support and systematically raster scanned through the X-ray beam. Fixed-target sample delivery has the advantage of being highly reliable, yields high hit rates and requires only minimal amounts of sample. This approach has thereby removed several bottlenecks for SX experiments at X-ray free-electron lasers (XFELs) and also at synchrotron facilities (Roedig et al., 2015, 2016, 2017; Hunter et al., 2014).

Fixed-target SX experiments at XFELs, such as the Linac Coherent Light Source in the US, are typically performed.
using single exposures from femtosecond X-ray pulses, which consequently destroy the sample because of their extremely high intensities of up to $10^{12}$ photons typically focused into a few micrometre spot. For a pulse duration short enough to outrun radiation damage and a given pulse fluence, the number of patterns required to complete a high-quality dataset of structure factors also depends on factors such as crystal size, symmetry, as well as the bandwidth of the radiation (White et al., 2013). With a bandwidth of 0.2%, experiments with pulses of these parameters usually require a few thousand still diffraction patterns to complete a high-quality dataset of structure factors.

By removing the requirement to obtain high signal-to-background data from individual crystals and by reducing the dose by spreading the exposure over many crystals, SX is also a compelling method at synchrotron sources. Using available beamlines, several such experiments have been conducted using monochromatic radiation (Stellato et al., 2014; Beyerlein et al., 2017). With an X-ray flux of $10^{12}$–$10^{13}$ photons $s^{-1}$ focused into a 10 $\times$ 10 $\mu$m spot, exposure times at these instruments are typically in the few milliseconds range, to obtain a sufficient diffraction signal from small crystals. Using a much smaller bandwidth of only 0.01%, SX experiments with monochromatic synchrotron radiation typically require more than tens of thousands of diffraction still images to obtain a complete high-quality dataset (Stellato et al., 2014).

Recently we demonstrated SX measurements at a synchrotron facility using a polychromatic ‘pink’ X-ray beam with exposure times of 100 ps only (Meents et al., 2017). Using a full harmonic of the undulator spectrum with a bandwidth of 5.7% [full width at half-maximum (FWHM)], still diffraction patterns from about 50 crystals were sufficient to obtain a high-quality dataset. In contrast to previous SX experiments using radiation of narrower bandwidth, processing of these data turned out to be challenging, with only 13% of patterns being amenable to indexing using available algorithms (Meents et al., 2017). This difficulty was attributed to the low-energy tail of the polychromatic beam, which extends to photon energies 20% lower than the peak energy, giving rise to the large number of Bragg spots observed in a diffraction pattern. Automatic indexing algorithms used for monochromatic radiation are not suitable for processing such patterns as it is impossible to distinguish which wavelength of the incident polychromatic beam diffracted into a particular spot and hence calculate its reciprocal-space coordinates. Available pink-beam indexing algorithms rely on finding ellipses in the diffraction-spots patterns and often fail when small or weakly diffracting crystals are used which is typically the case in SX experiments. Furthermore, no fully automatic data-processing suite is available for polychromatic single-crystal X-ray diffraction data, as the software requires prior knowledge of the unit-cell parameters and substantial manual input.

It has been recently reported that usable intensities can be obtained from sparse pink-beam diffraction data using the standard pipeline for monochromatic data processing (Martin-Garcia et al., 2019). A limitation of this approach is that only 10% of the diffraction patterns could be indexed and the valuable high-resolution diffraction spots could not be indexed and were not included in the structure refinement.

Our experience with the indexing of protein crystal diffraction obtained with polychromatic radiation, reported previously (Meents et al., 2017) and in this article, along with our attempts at indexing simulated diffraction patterns, have shown that existing auto-indexing algorithms tend to fail when the bandwidth increases to the point where there are significant peak overlaps. This is unsurprising given that those algorithms assume monochromatic radiation, but we have found that a good compromise between a high indexing success rate and high beam fluence is obtained with a bandwidth of about 2%, as long as the spectrum is approximately symmetric. Such a spectrum can be obtained at an undulator beamline using a multilayer monochromator, for example. This choice of bandwidth also produces a reasonably high proportion of Bragg peaks that are fully integrated and so accurate structure factors can be estimated from far fewer diffraction patterns than needed when using narrower bandwidth radiation, where most peaks are only partial reflections.

For example, beamline ID09 at the European Synchrotron Radiation Facility (ESRF) provides up to $10^9$ photons in a single 100 ps pulse when using a multilayer monochromator. Higher photon fluxes of more than $10^9$ photons can be achieved with microsecond exposure times. This appears to be an optimal compromise between flux and time resolution for investigating many biological processes.

A challenge for diffraction measurements with a high-flux pink beam is that suitable detectors are rarely available. Hybrid pixel-array photon-counting detectors have limited count rates of several megahertz (Broennimann et al., 2006). That is, in a 1 $\mu$s exposure, it is only possible to count a few photons, at most, in each pixel. Instead, integrating detectors are required. While charge-coupled device (CCD) detectors are an older technology than hybrid photon-counting detectors, with higher noise and slower readout, they are integrating and hence have remained standard detectors for synchrotron experiments using polychromatic X-rays. Recent developments of hybrid charge-integrating pixel detectors, such as the Cornell-SLAC hybrid Pixel Array Detector (CSPAD; Hart et al., 2012), the Adaptive Gain Integrating Pixel Detector (AGIPD; Allahgholi et al., 2016) and the adjusting gain detector for the Aramis User station (JUNGFRAU; Mozzanica et al., 2016), initially developed for XFELs, have also opened new opportunities for high-flux synchrotron experiments as they can handle this high flux and sustain high frame rates (Leonarski et al., 2018).

In this article, we describe fixed-target SX experiments performed at a frame rate of 1 kHz using a polychromatic synchrotron beam with a bandwidth of 2.5%, using a JUNGFRAU detector and a Roadrunner II goniometer for high-speed sample delivery (Roedig et al., 2017).

2. Experiments

Diffraction experiments were performed at beamline ID09 at the ESRF using the multilayer monochromator installed at the
instrument. The resulting energy spectrum of the X-rays used for the experiments is shown in Fig. 1 with an energy spread of 2.5% (FWHM) centered at a photon energy of 15.2 keV. The measured beamsize at the sample position was 60 × 60 μm.

Diffraction patterns were recorded on a JUNGFRAU 1M pixel detector. The detector consisted of two individual 500 K pixel JUNGFRAU modules mounted on top of each other with a vertical gap of 2.8 mm in between them. With the given detector area of 77 × 80 mm and a minimum detector distance of 100 mm limited by geometrical restrictions at the instrument, we decided to offset the detector center horizontally by 26 mm with respect to the incident X-ray beam to allow collection of reflections up to 1.4 Å at the edge of the detector. For 1 kHz data collection, the integration period of the detector area of 77 × 80 mm and a minimum detector distance of 100 mm limited by geometrical restrictions at the instrument. The resulting energy spectrum of the X-rays used for the experiments is shown in Fig. 1 with an energy spread of 2.5% (FWHM) centered at a photon energy of 15.2 keV. The measured beamsize at the sample position was 60 × 60 μm.

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Crystals of the two model compounds, lysozyme and proteinase K, were directly grown on micropatterned silicon chips as described in more detail by Lieske et al. (2019) and in the Supporting information. Crystals of both proteins all had dimensions of 50 × 50 × 50 μm. A technical drawing of the silicon chips used for the experiment (also referred to as Roadrunner II chips) and the corresponding pore-pattern are shown in Figs. 2(a) and 2(b). For data collection, a Roadrunner chip with crystals was taken out of its crystallization chamber, the crystal-growth solution was removed through the pores by blotting the underside of the chip with filter paper (Roedig et al., 2016) and the chip was then mounted on the Roadrunner II goniometer installed at the beamline.

In contrast to instrumentation usually available at crystallography endstations, the Roadrunner II goniometer is equipped with a high-speed horizontal scanning stage (x axis) capable of scanning at speeds of up to 100 mm s⁻¹. This fast scanning axis is mounted on a yz translation stage allowing for it to be positioned vertically (the y direction) and along the X-ray beam direction (the z direction). This whole scanning unit can be rotated (by an angle ω) around the x axis, using a high-precision air bearing. A technical drawing of the Roadrunner II goniometer, as used for the experiment, is shown in Fig. 3.

Once mounted onto the scanning unit, each chip was aligned with respect to the X-ray beam with an in-line sample-viewing microscope, and the scanning grid was defined using the Roadrunner software. For subsequent data collection, each chip was continuously scanned through the X-ray beam in the horizontal direction with a constant velocity of 100 mm s⁻¹. With an X-ray pulse frequency of 1 kHz, generated by an X-ray chopper, this corresponds to a spatial separation of 100 μm between two shots, which is about twice the beamsize at the sample position. During an X-ray exposure of 1 μs, the crystal moves by only 100 μm, which is insignificant compared with the crystal and beam sizes. The scans started at the bottom-right corner of every chip. After a horizontal line scan was finished, each chip was moved down vertically by 100 μm to the next line, rotated by a small ω increment and then scanned along x in the reverse direction. This procedure was repeated for the whole chip. More details about the chip alignment and scanning procedure can be found in the work by Roedig et al. (2017).

All diffraction measurements were carried out at room temperature. Lysozyme diffraction data were collected at X-ray exposure times of 5 and 1 μs per crystal. Proteinase K data were collected only at an exposure time of 1 μs. With the beam parameters mentioned above and 3.5 × 10⁹ photons per
5 μs exposure and $7 \times 10^8$ photons per 1 μs exposure, these exposure times correspond to X-ray doses of 500 and 100 Gy, respectively. At these doses, data should not be affected by radiation damage or sample heating effects, even without cryogenic cooling (Roedig et al., 2016; Henderson, 1990). This was also confirmed experimentally by measuring multiple diffraction patterns at the same position of the chip and comparing the Bragg peaks at high resolution. Two example diffraction patterns of a lysozyme crystal and a proteinase K crystal are shown in Fig. 4. In total we collected diffraction data from ten chips. Scanning and data-collection parameters for every chip are provided in Table 1. The datasets, each from a separate chip, are labeled lys08 to lys15, and protK03 and protK04. On average, 36 000 diffraction patterns were collected per chip with a scanning time of about 150 s for an entire chip. This is longer than the 36 s of data-collection time because of the overhead of changing direction at the end of the scan. The hit fraction depends on the crystal-growth conditions. In the case of lysozyme crystals with 5 μs exposure, the average hit fraction was 30% and of these patterns 76% could be indexed, corresponding to an effective data-collection rate of 55 indexed patterns per second. For the lysozyme and proteinase K crystals measured with 1 μs exposure, the effective data-collection rate was lower with 28 and 9 indexed patterns per second, respectively, which is probably a result of a lower crystal density on the chip.

3. Data analysis

Diffraction data were indexed and integrated using the CrystFEL software suite (White et al., 2012), which was modified and extended to handle diffraction data recorded with 2.5% X-ray bandwidth and merged into different (sub-) datasets (see the Supporting information). A typical indexed diffraction pattern of a lysozyme crystal is displayed in Fig. S1 in the Supporting information, which shows a good correspondence between diffraction peaks and predicted Bragg spot positions. In Figs. S2 and S3 we further provide a comparison of the spot prediction and the resulting CC* values of the datasets obtained by using modified and unmodified versions of CrystFEL. An improvement in the data quality arising from the CrystFEL modifications is clearly visible. Structure refinements for all generated datasets were carried out using PHENIX (Adams et al., 2010). PDB structures 6frt and 5kxv served as starting models for lysozyme and proteinase K (Wiedorn et al., 2018; Masuda et al., 2017).

The lysozyme diffraction datasets recorded with 5 μs exposure time (lys08, lys09, lys10) were further analyzed to
determine the dependence of analysis metrics on the number of diffraction patterns collected. These three chips provided a total of 24,344 indexed diffraction patterns. We created eight subsets from this group, consisting of 200 to 15,000 randomly

### Table 1

| Chip name | lys08 | lys09 | lys10 | lys11 | lys12 | lys13 | lys14 | lys15 | protK3 | protK4 |
|-----------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|
| Exposure time (μs) | 5 | 5 | 5 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| No. of horizontal scan points | 331† | 331† | 331† | 333† | 331† | 151‡ | 163‡ | 163‡ | 310‡ | 156‡ |
| No. of vertical scan points | 38396 | 34752 | 34423 | 38628 | 34754 | 3580 | 4707 | 3443 | 2538 | 640 |
| Total no. of scan points | 12209 | 12512 | 7489 | 5376 | 4621 | 937 | 4707 | 3443 | 2538 | 640 |
| No. of hits | 9238 | 8813 | 6293 | 5376 | 4621 | 937 | 28 | 28 | 17 | 5.1 |
| No. of indexed and merged hits | 12209 | 12512 | 7489 | 5376 | 4621 | 937 | 28 | 28 | 17 | 5.1 |
| Total scanning time (s) | 158 | 143 | 142 | 158 | 143 | 28 | 139 | 125 | 153 | 125 |
| Hits per second | 77 | 87 | 53 | 34 | 32 | 33 | 34 | 28 | 17 | 5.1 |
| Indexed patterns per second | 58 | 62 | 44 | 28 | 27 | 27 | 24 | 18 | 8.9 | 1.8 |
| Effective scanning rate (frames s⁻¹) | 243 | 243 | 243 | 244 | 243 | 130 | 137 | 131 | 231 | 132 |

† 15 scan points at the beginning and end of every line were used for acceleration and deceleration of the linear axis, so the total horizontal scanning range slightly exceeds the chip lengths. ‡ These chips were only partially scanned.

Figure 4

Polychromatic diffraction patterns of (a) a lysozyme crystal from the chip lys08 recorded at beamline ID09 with a JUNGFRAU 1M detector with an exposure time of 5 μs and (b) a proteinase K crystal from the chip protK04 with an exposure time of 1 μs. Magnified areas of the diffraction images indicated by a red square in images (a) and (b) are shown in (c) and (e). Images (d) and (f) show even higher magnifications of the areas indicated in (c) and (e) and highlight the achievable low background-scattering levels around the Bragg reflections at 3.1 Å in the case of lysozyme (d) and 3.6 Å in the case of proteinase K crystals (f).
selected lysozyme diffraction patterns, plus the full dataset, which were all individually processed using CrystFEL. From these, structure refinements were then carried out with PHENIX (Adams et al., 2010) which quantified data completeness and the correlation with the calculated structure factors, CC*, as a function of resolution, as shown in Figs. 5(a) and 5(b). Further metrics of some of the subsets are given in Table 2, and the free R factor, $R_{	ext{free}}$, is plotted in Fig. 6.

As seen in Figs. 5(a) and 5(b), all datasets containing more than 500 diffraction patterns show almost 100% completeness up to a resolution of 2.3 Å. Here, completeness is defined as the fraction of reflections in the resolution shell that have been integrated at least once regardless of their intensity. With increasing numbers of merged patterns, this metric extends to higher resolution. The completeness of the dataset containing all 24 344 frames remains close to 100% for resolutions of up to 1.7 Å. The dependence of CC* on the number of patterns exhibits a different behavior: only the datasets containing more than 2 500 patterns show CC* values larger than 0.95, which then falls off at resolutions higher than 2.3 Å. Again, the dataset containing all of the patterns shows the highest CC*, which also extends to the highest resolution. Interestingly, the datasets consisting of a smaller number of diffraction patterns show a low CC* not only for the high-resolution reflections but also for the low-resolution reflections.

As seen in Fig. 6, $R_{	ext{free}}$ first decreases rapidly with the increasing number of patterns, from 0.35 for 200 patterns to about 0.21 for 1 500 merged patterns. Beyond this number of patterns there is little improvement in this metric which decreases only slightly to 0.175 when all 24 000 patterns are included. Both the low CC* values for the low-resolution reflections and the relatively high $R_{	ext{free}}$ values for datasets consisting of less than 1 500 diffraction patterns can be better understood with reference to the Ewald construction of diffraction as illustrated in Fig. S4. The limiting spheres of the minimum and maximum wavelengths of the polychromatic radiation define a volume of reciprocal space where reflections occur. To the first order, this volume can be thought of as a wedge. At low resolution, the wedge is thinner than the peak width, giving mainly partial reflections that contribute to a variance in their measured intensities. This situation is similar to the case of monochromatic radiation. As the resolution increases, so too does the width of the wedge, which eventually is broader than the peak width. At this resolution and higher, reflections are predominantly fully recorded, giving measurements with less variance. These reflections therefore need measurements from fewer patterns to achieve a given confidence.

Example electron-density distributions around an Arg128 residue for some different subsets of the 5 μs lysozyme measurements are shown in Fig. 7. Whereas for the densities determined from 750 and 1 500 merged diffraction patterns only one conformation is visible [Figs. 7(a) and 7(b)], merges from 3 000 and all 24 344 patterns clearly reveal the occupation of a second conformation of residue 128. For other electron-density regions of the lysozyme structure we observe a similar trend of additional conformations appearing (see Figs. S5–S7). This is consistent with the observation of a relatively strong decrease of the $R_{	ext{free}}$ values for merges from 250 to 3 000 patterns and only a moderate further decrease when more diffraction patterns are considered.

In the method of fixed-target SX using a humidified gas stream to prevent the crystals from drying out, one consideration to be addressed is that the unit-cell parameters of the room-temperature crystals may vary, depending on their positions on the chip. For example, the unit-cell volumes of all the indexed lysozyme crystals from chip lys09 are found to be normally distributed, with a mean value of 242 400 Å³ and a standard deviation of 1 400 Å³ (see Fig. S8). This is about 1.5 times the standard deviation obtained from similarly prepared lysozyme crystals also measured at room temperature but in solution, in a liquid jet (Wiedorn et al., 2018). In that case, measurements at the European XFEL yielded unit-cell volumes that were normally distributed with a mean of 237 100 Å³ and a standard deviation of 900 Å³. The spatial
distribution of the unit-cell volume of crystals on the lys09 chip is plotted in Fig. 8. The unit-cell volume varies between 241 000 and 245 000 Å³, a relative change of about 1.6%, diminishing from the top-left corner to the bottom-right corner of the chip [see Figs. 8(b) and 8(c)]. This variation appears to be caused by an uneven humidity inside the chamber, which encloses the chip when it is measured. The chip is moved inside this humidity chamber when it is scanned. Figs. 3(c) and 3(d) show the start and end positions of the scan. Even though every crystal is measured in the same location within the chamber (the X-ray beam position), during the entire scan (and during alignment) the chip experiences a gradient of humidity because of the flow of humid air from one side of the chamber to the other.

Another effect that can be seen in Fig. 8(c) is an oscillation of the unit-cell volume in the y direction with a periodicity of two rows and a magnitude of 0.5% of the average unit-cell volume. This magnitude matches the overall gradient experienced in the longitudinal direction. Since the total horizontal line scan takes only about 0.3 s but the deceleration at the end of the line, vertical movement and acceleration at the beginning of the next line takes about 1 s, the crystals might have enough time to shrink while the chip stays in the right part of the chamber, where humidity is lower, and then partially recover when the chip is again in the left part, where humidity is higher. Another explanation for this variation could be an oscillation of the next line takes about 1 s, the crystals might have enough time to shrink while the chip stays in the right part of the chamber, where humidity is lower, and then partially recover when the chip is again in the left part, where humidity is higher. Another explanation for this variation could be a systematic shift of the stage in the z direction depending on the scan direction. However, we verified that this was not the case since the chip stays within the few micrometre depth-of-focus of the in-line microscope throughout the scan.

Structure refinements carried out with datasets containing merged intensities from different areas of the chip did not reveal any significant structural changes. Despite the systematic changes in unit-cell volume in a scan, the structures of lysozyme determined with the method of fixed-target SX with microsecond exposure times at a synchrotron are of similar quality to the recent structure determination carried out at the European XFEL (Wiedorn et al., 2018; Grünbein et al., 2018) using femtosecond exposure times. It should be noted here that with conventional crystallography of large...
lysozyme single crystals, much higher resolutions of up to 0.94 Å have been achieved (Sauter et al., 2001).

4. Discussion
Using the approach of high-speed fixed-target SX in combination with the new JUNGFRAU integrating pixel detector, we were able to collect a complete high-quality diffraction dataset at a frame rate of 1 kHz in less than 150 s. By using the method of on-chip crystallization (Lieske et al., 2019), sample-preparation efforts were minimal as no additional crystal handling or manipulation steps such as pipetting are required. The crystals can be directly measured after removal of the mother liquor by blotting. The total time for preparation and measurement of one chip, including blotting, mounting of the chip on the goniometer, definition of the scan grid and data collection was about 10 minutes. After aligning the setup and establishing the data-collection procedure, we were able to measure ten chips in one hour, which directly translates into at least ten structure determinations.

In contrast to other sample-delivery methods, here the entire membrane area is systematically scanned through the X-ray beam guaranteeing that most of the material on the chip is exposed and contributes to the dataset. With about 10,000 crystals per chip with average dimensions of 50 μm this corresponds to a total amount of 1.6 mg of sample per chip, which is a fairly large amount of protein for a structural biology project. The main reason for using crystals of this size was to match the X-ray beam size of about 60 μm at ID09 at that time. The applied X-ray doses were as low as 100 Gy for the 1 μs exposure times, which is only about 16 times the LD₅₀ dose for human beings of 6 Gy and more than five orders of magnitude less than typical doses of 50 MGy in cryocrystallography (Meents et al., 2010; Owen et al., 2006). This highlights the potential of the method for investigations of the undamaged structure of redox-sensitive metalloproteins at unprecedented low dose levels (Beitlich et al., 2007; Yano et al., 2005; Corbett et al., 2007).

Using smaller polychromatic X-ray beams, soon available at several beamlines such as the MAX IV in Sweden, the ESRF in France and the Advanced Photon Source (APS) in the US, will allow a tremendous reduction in the amount of sample required for a pink-beam structure determination. Reducing the
beam area from $60 \times 60 \, \mu m$ used here to $10 \times 10 \, \mu m$ while retaining the same number of photons per pulse will, on the one hand, increase the dose by a factor of 36 from 100 Gy to 3.6 kGy for a 1 $\mu$s exposure, but, on the other hand, should allow the collection of datasets of similar quality using only 1.6/36 mg = 44 $\mu$g of sample. X-ray doses of 20 kGy are still well below the room-temperature dose limit of about 300 kGy, and an amount of 44 $\mu$g corresponds to one single crystal with dimensions of 330 $\mu$m, a crystal size typical for structure determination with X-ray tubes in the laboratory (Roedig et al., 2016).

As can be seen in Fig. 4, the background-scattering levels obtained in our measurements are very low, with the vast majority of the pixels having zero counts. This is a result of our low-background experimental setup (Meents et al., 2017) in combination with the single-photon sensitivity of the JUNG-FRAU detector. This leads to an improved signal-to-noise level of the data compared with that obtained with contemporary CCD detectors and thereby a higher overall data quality than usually achievable in conventional crystallographic experiments, especially for high-resolution reflections. Our fixed-target approach involves ‘naked’ crystals on the membrane, which are easily accessible for external manipulation. This makes the method ideally suited for ligand-binding studies and laser pump–probe experiments. Since any sealing of the chips with Mylar or Kapton foil is avoided, ligand solutions can be applied in situ to the crystals on the chip using, for example, micro-droplet generators. With crystals several micrometres in size, which will be measurable at beamlines with smaller beams and higher fluence to what we demonstrated here, diffusion times and hence the achievable time resolution in such experiments should be in the few millisecond range (Schmidt, 2013). Laser excitation of the crystals could be performed using illumination through the in-line sample-viewing microscope. Here again, the absence of any sealing material avoids unwanted reflections and scattering of the pump laser light that might otherwise cause unwanted pre-exposure of the neighboring crystals.

In comparison with diffraction experiments with polychromatic X-rays using the full 5% bandwidth of an undulator harmonic (Meents et al., 2017), the spectrum produced by the multilayer monochromator is approximately symmetric, which greatly facilitates data processing. The indexing and data-integration procedures developed and established here will be integrated into an upcoming version of the CrystFEL data-processing suite, and will be published elsewhere.

With an achievable time resolution in the microsecond range and even down to below a nanosecond with single bunches (Meents et al., 2017), the opportunities for time-resolved experiments at synchrotrons should be further developed. Many SX experiments currently performed at XFEL sources tend to use crystals that are large enough to give measurable diffraction signals at high-intensity synchrotron beamlines. Our method represents an attractive alternative for such experiments.

The method of pink-beam SX at synchrotron sources allows the collection of ultra-low-dose datasets of proteins from small crystals. This should enable almost damage-free structures of redox-sensitive proteins. It avoids cryopreparation and allows the collection of data at physiologically relevant temperatures for the study of conformational flexibility (Fraser et al., 2011) and time-resolved measurements of structures undergoing triggered reactions. With data-collection times of less than a minute for a complete dataset, it is further well suited for time-efficient and systematic screening of pharmaceutical compounds. In the future, we will perform time-resolved measurements by this method to study irreversible processes such as enzyme reactions. Automatic exchange of chips with a
robotic arm in combination with fully automatic identification of the scanning grid should further increase the throughput of our approach.

5. Related literature

The following references are cited in the Supporting information for this article: Afonine et al. (2012); Cammarata et al. (2009); Chen et al. (2010); Emsley et al. (2010); Göries et al. (2016); McCoy et al. (2007); Redford et al. (2018); Urzhumtseva (2009); White et al. (2016); Yefanov et al. (2015).

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