

# Time-resolved methods in biophysics. 6. Time-resolved Laue crystallography as a tool to investigate photo-activated protein dynamics†

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When polychromatic X-rays are shined onto crystalline material, they generate a Laue diffraction pattern. At third generation synchrotron radiation sources, a single X-ray pulse of  $\sim 100$  ps duration is enough to produce interpretable Laue data from biomolecular crystals. Thus, by initiating biological turnover in a crystalline protein, structural changes along the reaction pathway may be filmed by ultra-fast Laue diffraction. Using laser-light as a trigger, transient species in photosensitive macromolecules can be captured at near atomic resolution with sub-nanosecond time-resolution. Such pump–probe Laue experiments have now reached an outstanding level of sophistication and have found a domain of excellence in the investigation of light-sensitive proteins undergoing cyclic photo-reactions and producing stiff crystals. The main theoretical concepts of Laue diffraction and the challenges associated with time-resolved experiments on biological crystals are recalled. The recent advances in the design of experiments are presented in terms of instrumental choices, data collection strategy and data processing, and some of the inherent difficulties of the method are highlighted. The discussion is based on the example of myoglobin, a protein that has traversed the whole history of pump–probe Laue diffraction, and for which a massive amount of data have provided considerable insight into the understanding of protein dynamics.

## 1. Introduction

A precise understanding of how a protein functions requires not only the knowledge of its three-dimensional structure at rest, but also of the subtle conformational changes that take place when the molecule is at work. Amongst the many techniques sensitive to the motions and dynamics of biological macromolecules, such as NMR, neutron scattering and from UV to IR spectroscopy, X-ray crystallography plays a central role.

Because many proteins are functionally active in the crystalline state, “kinetic” crystallography experiments can be performed, whereby turnover is deliberately induced in the crystal so as to generate one or several intermediates whose structures can be determined.<sup>1–3</sup> Several approaches in kinetic crystallography experiments have been developed, that can be divided into two main classes: in the first one, the lifetime of the intermediate is artificially prolonged so as to match the typical X-ray data collection time available from synchrotron beamlines employing monochromatic radiation. This can be achieved by generating steady state ac-

cumulation of a (rate limited) intermediate under continuous reaction triggering, or more commonly by physical trapping of the intermediate, usually based on fine cryo-temperature control. In contrast, in the second class, the X-ray exposure time is matched to the “genuine” lifetime of the intermediates. This approach is more elegant because no deliberate perturbation is made to the sample and because it allows one, at least in principle, to literally film proteins in action. That is, real kinetic experiments can be made, where not only the transient species are visualized at quasi-atomic resolution, but also their lifetime and inter-conversion rates are measured at ambient temperature. The approach is based on employing Laue crystallography, where diffraction patterns are obtained by exposing a static crystal to a polychromatic X-ray beam, contrary to the standard set-up where a rotating crystal is exposed to a monochromatic beam.<sup>4</sup> In this way, exposure times are shortened by three to four orders of magnitude relative to monochromatic experiments, opening access to the microsecond time domain at third-generation synchrotron radiation sources. As a microsecond is about the time it takes for an electron bucket to circulate around a synchrotron ring, it becomes possible to take advantage of the time-structure of synchrotron radiation to further reduce the effective X-ray exposure time: if the full synchrotron current is loaded into a single electron bucket, each  $\sim 100$  ps X-ray pulse generated by this bucket becomes sufficient to provide an interpretable diffraction pattern.

The sub-nanosecond time resolution provided by Laue diffraction has an expensive price. The “hot” polychromatic X-ray beam tends to generate poor quality diffraction data, damages the crystal, and is susceptible to induce unwanted chemistry in biomolecules. However, in the last few years, tremendous progresses have been achieved in the design of pump–probe Laue

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experiments. These methodological developments have mainly targeted light-sensitive proteins undergoing repeatable photocycles that involve short-lived intermediates in the 100 ps–100  $\mu$ s time range. Recent experiments on such samples have brought considerable mechanistic insight onto protein function,<sup>5–7</sup> sometimes providing detailed kinetic schemes on complex photo-reaction pathways, that would be inaccessible by trapping experiments.<sup>8–10</sup> In this paper, we describe the methodological improvements that have favored the emergence of this “niche of excellence” for the time-resolved Laue technique. As an application example, we summarize the input of the technique to the understanding of the structural dynamics of a myoglobin mutant.<sup>6,7,11</sup>

## 2. The challenges

Amongst the many challenges encountered in kinetic protein crystallography, a few are specifically relevant to time-resolved Laue experiments. A first issue consists in triggering the  $\sim 10^{14}$  molecules of the crystal uniformly and synchronously so that a major fraction of those molecules walk in pace along the reaction pathway. When timescales shorter than  $\sim$ ms are sought, light must be used as a trigger and questions related to penetration depth into optically dense crystals, unwanted photo-oxidation/ionization, and spurious non-linear effects must be considered. A second issue is to properly handle the structural heterogeneity implied

by incomplete reaction initiation and by the inherent distribution of conformational states adopted by individual molecules in the crystal. High-resolution diffraction data typically allow extracting at most two conformations from a single set of crystallographic structure factors, but not more. The question therefore arises as to how the set of individual time-independent intermediates can be retrieved from a series of time-dependant structures. A third issue is that, assuming intermediate states can be properly identified from crystallographic data, their physiological relevance must be assessed. That is, how to make sure that crystal packing constraints, crystallizing agents, physico-chemical parameters such as pH, temperature, viscosity or dielectric constant, and the X-rays themselves, do not alter the sample behavior in misleading ways?

Laue experiments at the sub-nanosecond timescale introduce additional difficulties linked to the signal to noise issue. Short timescales mean small structural changes in addition to low occupancy of the excited states. Therefore, high-resolution diffraction data are needed to detect those motions. At the same time, short exposure times mean few available X-ray photons, resulting in a poor signal to noise ratio in difference electron density maps.

The solutions to all these problems start with high-quality crystals, optimized reaction triggering schemes, and maximization of the photon flux. However, tuning of the X-ray bandpass, design of the data collection strategy, and sophistication of data processing also play crucial roles. In the following, we describe how the above mentioned challenges are currently tackled.

### 3. The Laue diffraction technique

#### Laue diffraction geometry

The Laue technique is at the same time the simplest and the most complex X-ray diffraction method. It is simple because, basically, it is sufficient to hold a crystal within the raw X-ray beam of a standard generator to generate a meaningful pattern.<sup>12</sup> This is essentially the setup used by Max Von Laue in 1912 to determine the structure of zinc sulfide. However, recording a complete Laue data set from a photo-activated protein crystal at 1.5 Å with 100 ps time-resolution is a rather different story, requiring considerable sophistication in instrumentation<sup>13–15</sup> and a great deal of expertise to evaluate the data.<sup>16</sup> The principles of Laue diffraction have been discussed extensively in several seminal papers,<sup>16–19</sup> and only three main conclusions are recalled below.

Firstly, let's consider an X-ray bandpass  $\Delta\lambda = \lambda_{\max} - \lambda_{\min}$ , where  $\lambda_{\max}$  and  $\lambda_{\min}$  are the maximum and minimum wavelengths at which significant X-ray flux is produced. The volume of the reciprocal space collected in a single Laue exposure is proportional to  $\Delta\lambda$  and is given by:

$$V_{\text{Laue}} = (\pi/4)(1/d^4)\Delta\lambda \quad (1)$$

where  $d$  is the resolution of the data. Assuming the lowest crystallographic symmetry  $P1$ , the fraction  $f$  of reciprocal space recorded in a single frame amounts to  $f = 3/8\Delta\lambda/d$ . It is immediately seen that a large fraction of data can be recorded in a single shot, e.g. with  $\Delta\lambda = 1$  Å and  $d = 1.5$  Å,  $f = 25\%$ . This has formed the basis for developing Laue diffraction as the method of choice for time-resolved studies, but as will be outlined later, severe pitfalls counterbalance this key advantage.

Secondly, for a reflection with Miller indices  $\mathbf{h} = (h, k, l)$  characterized by a structure factor  $F(\mathbf{h})$ , the recorded Laue integrated intensity  $I(\mathbf{h})$  is given by:

$$I(\mathbf{h}) = [I_0(\lambda)D(\lambda)A(\lambda)] [L \exp(-B/2d^2)] F^2(\mathbf{h}) \quad (2)$$

The Lorentz factor  $L$  and the damping term  $\exp(-B/2d^2)$  due to the “temperature factor”  $B$  depend on the resolution  $d$  of reflection  $\mathbf{h}$ , but not on the stimulating X-ray wavelength  $\lambda$ . In contrast, the available photon flux  $I_0(\lambda)$ , the detector response  $D(\lambda)$  and the absorption coefficient  $A(\lambda)$  strongly depend on  $\lambda$ . Therefore, to extract  $F(\mathbf{h})$ , the parameter of interest carrying structural information, wavelength normalization needs to be carried out: the term  $I_0(\lambda)D(\lambda)A(\lambda)$  must be evaluated and scaled out along the whole available X-ray spectrum. Wavelength normalization is the most specific aspect of Laue diffraction in terms of data reduction and requires sophisticated procedures to be performed accurately.<sup>18,20</sup>

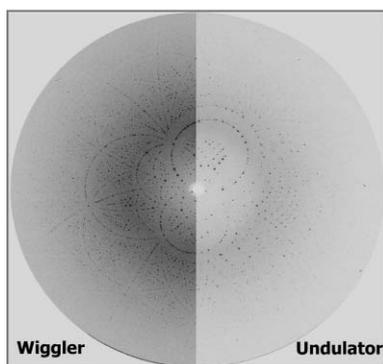
Thirdly, for a crystal with mosaic spread  $\eta$ , the size of a Laue spot on the detector is defined by

$$\begin{aligned} s_{\text{rad}} &= 2\eta(D/\cos^2 2\theta) + x/\cos 2\theta \\ s_{\text{azi}} &= 2\eta(D \sin \theta/\cos 2\theta) + x \end{aligned} \quad (3)$$

where  $s_{\text{rad}}$  and  $s_{\text{azi}}$  are the radial and azimuthal spot size,  $D$  is the crystal to detector distance,  $\theta$  is the Bragg angle,  $x$  is the linear size of the crystal and the X-ray beam divergence is neglected. As an example, for  $\eta = 0.5^\circ$ ,  $\theta = 10^\circ$ ,  $D = 100$  mm and  $x = 0.1$  mm,  $s_{\text{rad}}$  and  $s_{\text{azi}}$  amount to 2.1 mm and 0.4 mm, respectively. Therefore, Laue spots get severely elongated in the radial direction in the presence of mosaic spread or if crystal disorder is induced by e.g. reaction initiation or light-induced thermal expansion. This explains why a successful time-resolved Laue experiment requires excellent crystals and gentle reaction triggering.

#### The “pink Laue” concept

One of the key advances introduced in the last few years is the so-called “pink Laue” concept. This concept is based on the realization that the broad X-ray bandpass (up to several octaves) provided by wiggler insertion devices at synchrotron sources results in too many pitfalls. It was observed that a much better trade-off between data quality and single-shot coverage of the reciprocal space could be achieved by employing tailored undulator devices that provide considerable brightness but only a narrow  $\sim 3\%$  bandpass.<sup>21</sup> The advantages in using such a “pink” bandpass are immediately apparent in Fig. 1 and can be explained by the following arguments. Firstly, while the intensity of Laue diffraction spots are essentially independent of  $\Delta\lambda$ , the X-ray induced noise at every pixel of the detector scales with the bandpass. Therefore, the available signal to noise ratio is significantly improved when the bandpass is decreased. Of course, this is at the expense of reciprocal space coverage. With  $\Delta\lambda = 0.1$  Å and  $d = 1.5$  Å, the fraction  $f$  of data recorded in a single frame in space group  $P1$  amounts to only 2.5%, meaning that, depending on space group symmetry, 10–40 frames will be necessary to collect a complete data set. The sparse reciprocal lattice points stimulated in a pink Laue experiments nevertheless result in significant simplifications of the diffraction images because “harmonic” and “spatial” overlaps are much reduced.<sup>16</sup> Although these pitfalls can nowadays be properly handled with sophisticated numerical procedures even when broad

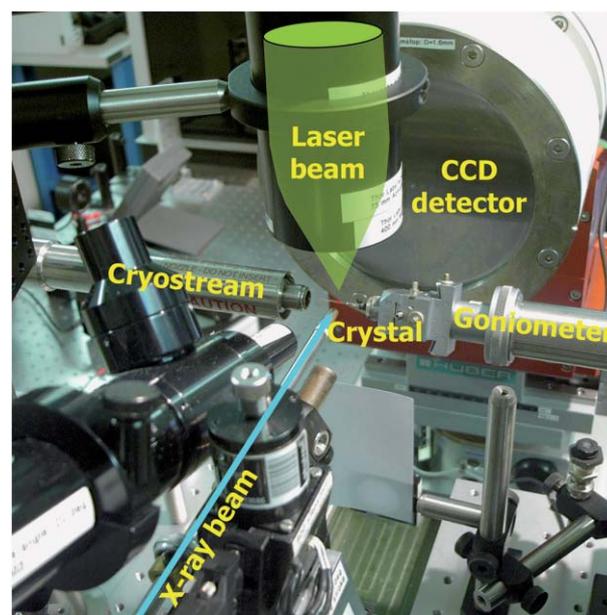


**Fig. 1** Composite Laue image of a myoglobin crystal. The left part of the image corresponds to half of a diffraction pattern recorded using a wide bandpass wiggler insertion device. The right part of the image corresponds to half of a diffraction pattern recorded using a narrow bandpass undulator insertion device. The improvement in background noise obtained with the “pink” Laue geometry is evident, as is the reduction in the number of stimulated reciprocal lattice points.

X-ray spectra are used,<sup>22,23</sup> their diminution in the pink Laue geometry provides much better raw data. In addition, with a pink beam, low-resolution data are not lacking severely, reducing the “low-resolution hole” typical of wiggler data and known to cause discontinuities in electron density maps. Moreover, a pink Laue beam decreases the heat-load on the X-ray beamline, resulting in a better stability of the optical elements. It also reduces the X-ray dose absorbed by the sample per image, with a potential beneficial effect on radiation damage. As a consequence of all these advantages, there now exists a consensus for using the pink Laue concept with undulator devices. It should however be kept in mind that narrow bandpass undulators introduce three difficulties: a more delicate indexing of the diffraction patterns, the presence of “partial” Laue spots, and, more importantly, a larger laser damage in pump–probe experiments because of the increased number of crystal orientations required to collect complete crystallographic data. While recent software has already started to address the first two points (see below), the third one is a more fundamental issue that may only be compensated for by more gentle laser excitation schemes or if the X-ray flux is boosted in such a way that the need for data averaging (*i.e.* the number of pump–probe sequences necessary to collect data of sufficient signal to noise ratio per crystal orientation), is decreased by the same amount. These pitfalls will need to be tackled thoroughly if very narrow bandpasses are to be used, *e.g.* in the case of future X-ray free electron laser (XFEL) sources (0.1% bandpass).

### Pump and probe sub-nanosecond Laue experiments

Modern time-resolved Laue diffraction experiments are of the “pump and probe” type: a laser pulse triggers the reaction of interest within the crystal and is rapidly followed by an interrogating X-ray pulse providing diffraction data from the excited molecules with sub-nanosecond time-resolution (Fig. 2). The X-ray pulse is typically generated by a single electron bucket circulating around the synchrotron storage ring. The “pump and probe” sequence needs to be repeated many times to (1) build-up sufficient signal to noise ratio for each individual diffraction pattern, (2) collect diffraction patterns at different crystal orientations so as to



**Fig. 2** Picture of the pump–probe Laue set-up on beamline ID09-B at the ESRF.

properly sample the reciprocal space, and (3) obtain structural information at different pump–probe delays to scan the time-dimension. As a consequence, the reaction under study must be photo-activatable and needs to relax back to the dark resting state in seconds or less, so that many cycles can be performed on the same sample within reasonable experimental time. The dark structure must be recorded from all crystals investigated so that conformational changes in “illuminated” states, monitored through the computation of structure factors differences relative to the dark state, are not biased by systematic errors such as a lack of isomorphism between different crystals. Thus, the crystal must sustain repeated laser and X-ray irradiations without rapid loss of diffraction quality. Overall, the method is best suited to study photo-cycles in light-sensitive proteins that provide excellent quality and radiation-hard crystals. Considering that about 5000 cycles (and  $\sim 1.5$  h of experiment) are needed to generate a complete set of data in a 6-fold symmetry space-group with ten different time-delays (31 crystal orientations, 16 averages per image, setting of beamline ID09-B at the ESRF), it is easy to understand that single-turnover (non-cyclic) reactions may not presently be investigated by this technique. This unfortunately precludes the use of caged-compounds<sup>24</sup> as well as the study of enzymes naturally activated by light such as DNA photolyase or protochlorophyllide-oxidoreductase. However, considerable insight into the complex dynamics of photoactive yellow protein,<sup>8</sup> myoglobin<sup>5–7,9,25,26</sup> and hemoglobin<sup>10</sup> could be obtained using the technique, and a vast field of investigations is still ahead for the study of many biologically relevant light-sensitive systems.

### The laser-pump

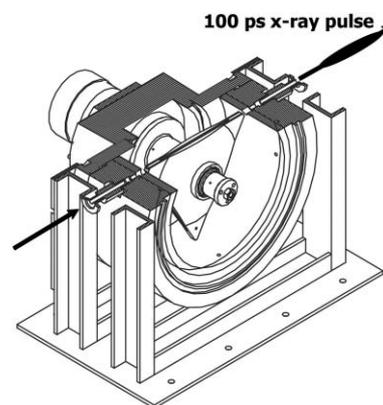
Providing efficient and homogeneous reaction initiation by laser-light within a protein crystal is one of the most challenging issues of time-resolved Laue experiments and is often the key determinant for success or failure. In choosing the laser wavelength, energy and pulse-duration, one must consider the high optical density of

colored protein crystals, minimize overall heating and temperature gradients throughout the crystal, and avoid oxidation/ionization events or non-linear absorption effects. Practical pitfalls also combine to reduce the effective photolysis yield achievable in crystals. Amongst these are: imperfect alignment of the laser beam, X-ray beam and crystal; loss of photolysis light due to Fresnel reflections on capillary and crystal surfaces; effect of non-random orientation of the absorbing groups within the crystal unit cell (potentially implying a dependence of photolysis yield on crystal orientation). With pulses in the 100 ps to ns regime, photolysis yields between 10% and 40% are typically obtained, while pulses in the fs regime tend to create enormous problems due to non-linear absorption effects (P. Anfinrud, personal communication). On beamline ID09B of the ESRF, laser pulses of typically 40  $\mu$ J energy, 100 ps duration and with wavelengths spanning the 480–520 nm range are typically used.<sup>14</sup>

### The X-ray-probe

The X-ray beam suitable for sub-nanosecond Laue diffraction on biological molecules should cope with the following criteria: the pulse duration must match the time-resolution of the experiment and its repetition rate the relaxation time of the biological process under study. The X-ray flux per pulse should be maximized to reduce the need for averaging, a time-consuming process generating laser-induced radiation damage. The X-ray beam should be stable and well focused onto the part of the crystal illuminated by the laser. The X-ray spectrum should be centered on a wavelength that suits the detector sensitivity and size, and the X-ray bandpass should be of a few percents, with smooth variations and minimum contamination by higher harmonics. To achieve these goals, specific configurations have been retained: synchrotron rings are operated in special modes (so called “single-bunch” or “few-bunches” modes), where one or few bunches carry several mA of current so that each of them generate a very intense  $\sim$ 100 ps X-ray pulse when traversing an insertion device, at a repetition rate in the Mhz range. At the ESRF, the 4-bunch mode mostly used for time-resolved Laue experiments consists of 4  $\sim$ 100 ps bunches of 10 mA each, separated in time by 705 ns. With the advent of a new generation of X-ray choppers (see below), other filling modes of the storage ring can be used for single-pulse applications, such as the 16-bunches mode or the so-called hybrid mode, that offer better compatibility with the requirements of other beamlines and therefore can be programmed more often than the 4-bunch mode. To generate the X-ray pulses, *in vacuo* mono-harmonic undulators have been designed. These devices deliver tremendous brilliance, with most photons emitting in a single tooth-shaped bandpass with a sharp energy cut-off, while second and third-order bands provide much reduced intensity. On beamline ID09B of the ESRF, a mono-harmonic “U17 undulator” is used, that generates  $\sim$ 100 W of radiation at 6 mm gap and 40 mA ring current (4-bunch mode), through a 3% X-ray bandpass (at FWHM) peaking at 0.83 Å. This insertion device provides  $\sim$ 10<sup>10</sup> photons pulse<sup>-1</sup> focused at sample position into an elliptical spot of 60  $\mu$ m height and 105  $\mu$ m width<sup>27</sup> (M. Wulff, personal communication). It is interesting to note that under such conditions, if 1 photon out of 10<sup>5</sup> is elastically scattered, and the crystal contains 10<sup>14</sup> molecules, only 1 molecule out of one billion will contribute to diffraction.

To allow the selection of properly timed X-ray pulses on the sample, a series of X-ray shutters are used in combination, forming critical components of the beamline. Opening times in the microsecond range are provided by high-speed choppers rotating at about a kHz about an axis perpendicular to the X-ray beam (Fig. 3). Such devices attain supersonic velocities at the rotor edge and therefore must be operated *in vacuo*. Phase-locking to the storage ring clock ensures synchronization to the X-ray beam with ns mechanical jitter, an amazing achievement made possible by the use of magnetic bearings. Clever design (tapering) of the opening channel and combination with a tightly focused X-ray beam allow the selection of a range of opening times at fixed rotating speed. Opening times down to  $\sim$ 100 ns have been achieved. On the other hand, opening times in the ms range are provided with more regular shutters. These shutters are essential to slow down at will the repetition rate of the pump–probe experiment (typically down to the 1 Hz range for proteins), and also to minimize heat-load on the beamline optical components, a key issue to ensure beam positional and flux stability.



**Fig. 3** Representation of the high-speed rotating chopper of beamline ID09-B of the ESRF. The triangular rotor is seen in its vacuum chamber in the “open” position, that is when the channel cut along one of the rotor edge coincides with the X-ray beam path.

Synchronization between the laser and X-ray pulse is realized by sophisticated electronics providing ps accuracy. At beamline ID09B of the ESRF, all instruments are slaved to the master synchrotron bunch clock at 352 MHz. The pump–probe delay is adjusted by a digital delay generator and can be monitored thanks to a high-speed gallium arsenide photoconductor sensitive to both X-ray and laser lights and positioned close to the sample position.<sup>14,28</sup>

### Data collection strategy

Time-resolved Laue experiments are time-consuming experiments for which limited synchrotron beamtime is available. Therefore, the data collection strategy in the four-dimensional space should be carefully planned. As kinetic processes for molecular ensembles are exponential in essence, the time dimension should be sampled equidistantly on the logarithmic scale. One major concern is to avoid systematic errors in the diffraction data that may bias the observed structural changes along the time axis. Such systematic errors may occur as a result of X-ray induced crystal degradation, laser-induced photo-oxidation/ionization or instrumental pitfalls

causing for example a drift in the extent of reaction initiation. To minimize the impact of these errors, time is used as the “fast variable”: at a given crystal orientation, all the diffraction images at the investigated pump–probe delays (including a dark state image) are collected and then the crystal is rotated and the process repeated.<sup>8</sup> The calculation of difference electron density maps between different time-points then mostly subtracts out irreversible structural changes caused by X-ray or laser light. Such data collection scheme spreads out errors along the time-axis, but does not eliminate them. The quality of the diffraction data should therefore be followed regularly throughout the course of the experiment, ideally by “on the fly” data processing (see below) but at least by visual inspection of the Laue images. X-Ray damage will progressively deteriorate the quality of the diffraction patterns, whereas laser induced damage may result in crystal slippage, buckling or even cracking, resulting in transient or permanent spot streakiness and sometimes leading to complete loss of diffraction. A large number of fresh and high-quality crystals should therefore be made available. To obtain the most homogeneous reciprocal space coverage from a single crystal with limited lifetime, the spindle orientation should not be incremented linearly, but instead should follow a “gap filling” algorithm that samples reciprocal space more homogeneously. This is important with the pink Laue strategy, where a large number of different crystal orientations are needed to collect complete data, meaning an increased number of laser stimulations (and associated damage) per unit reciprocal space volume.

Finally, crystals used for Laue experiments are usually mounted in capillaries. This is important to avoid sample drying and to maintain sufficient pressure when gaseous ligands are used, such as CO in the case of globin proteins. As transient temperature jumps are caused by laser and X-ray radiation, it is appropriate to cool the sample moderately (*e.g.* 10 °C) to favor the rapid evacuation of excess heat and avoid sample drying that might result from distillation of the mother liquor located nearby the sample.<sup>14</sup>

### From Laue patterns to electron density maps to chemical mechanisms

The analysis of Laue data consists of two main parts: reduction of diffraction images to structure factor amplitudes and electron density maps; and analysis of the latter to extract quantitative information on intermediate states and rate constants. Fig. 4 summarizes the elementary steps involved. In the last years, Laue data reduction software has become quite mature, providing accuracy, automation and speed. Spatial and harmonic overlaps are now deconvoluted computationally on a routine basis.<sup>20,22,23</sup> On-the-fly assessment of data quality during the course of the experiment is now also available, thanks to efficient programming and fast computers. Three main packages are currently available, that all offer advantages and disadvantages. Precognition<sup>29</sup> offers a high level of sophistication over all processing steps but is not freely available; TReX (F. Schotte *et al.*, unpublished results), running on Windows, proposes excellent indexing algorithms and is extremely fast, but provides only basic wavelength normalization; PrOW/CCP4 combines excellent crystallographic software available on the CCP4 suite but relies on the identification of nodal spots for indexing.<sup>21</sup>

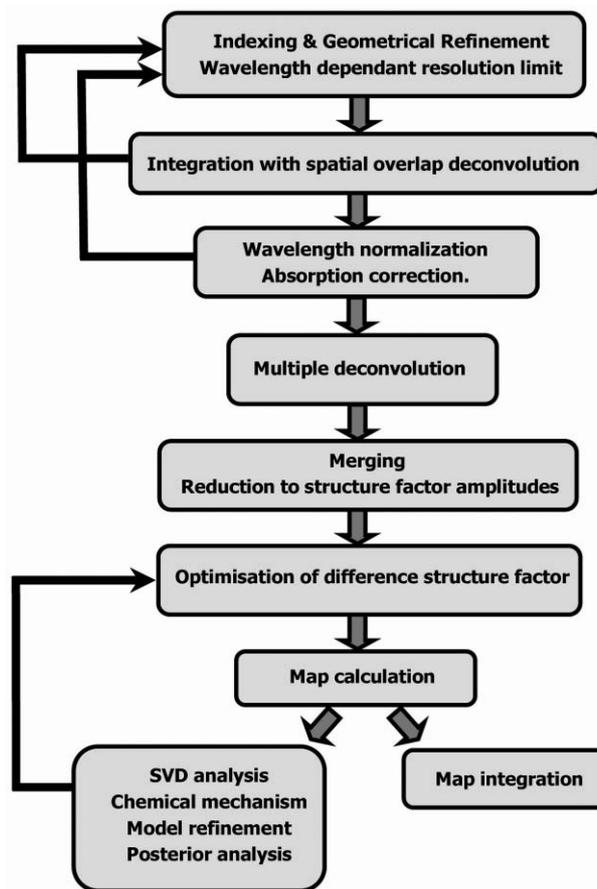


Fig. 4 Elementary steps of Laue data processing.

With the advent of narrow bandpass Laue crystallography, some complications in data reduction have been added. Firstly, indexing algorithms based on the manual identification of nodal spots (spots with low Miller indices that stand at the intersection of several conics) are often prone to failure, as these spots do not contrast out in pink Laue images. New auto-indexing routines based on the recognition of entire conics have already been implemented in the packages Precognition and TReX, and prove excellent robustness for bandpasses of a few percents. Secondly, the prediction of the crystal resolution limit needs to be made wavelength dependant. Indeed, the successful observation of reflections at a given resolution is strongly dependant on the available X-ray flux at the stimulating wavelength. Therefore, the use of a unique resolution limit for predictions throughout the entire spectrum would result in substantial degradation of the data quality. Efficient algorithms for assessment of the “wavelength dependant resolution limit” are available in the current packages such as in PrOW.<sup>21</sup> Thirdly, mono-harmonic undulators make wavelength normalization more difficult because of the presence of a sharp edge on the high energy side. Although satisfactory normalization is currently provided by modelling with Chebyshev polynomials, new ideas such as based on non-linear  $\lambda$ -mapping are emerging that should greatly enhance the accuracy of the process (Z. Ren, personal communication). Finally, as each diffraction spot from a mosaic crystal is composed of contributions from a range of wavelengths, normalization by a single scale factor is in principle not accurate. Moreover, the fraction of incompletely

recorded spots (“partials”) increases dramatically as the X-ray bandpass decreases. While these difficulties are often ignored with current mono-harmonic insertion devices, they will have to be dealt with extensively to analyze patterns derived from XFELs successfully.

Once structure factor amplitudes have been obtained for all investigated time delays, difference Fourier maps are calculated that reveal structural modifications along the reaction cycle. These maps are computed from difference structure factor amplitudes, using phases from the dark state, an efficient procedure that retrieves about half of the true signal despite the lack of true difference phases.<sup>30</sup> As subtle structural changes at short time delays lead to small difference amplitudes with high noise, it is advisable to maximize the signal to noise ratio of the difference maps by using weighting schemes derived from the *a priori* knowledge of error distributions and Wilson statistics, following Bayesian theory.<sup>31</sup> When the amount of time-points is sufficient (~3 per decade), SVD analysis (see below) also provides an efficient noise filter.

If only a qualitative interpretation of the data is sought, it can be advantageous to produce maps extrapolated to full photolysis so as to artificially suppress the contribution from the non-excited fraction of the crystal. Smart map rendering procedures may then be applied<sup>14</sup> that allows the eye to easily follow the subtle motions of individual atoms in spectacular movies.

Quantitative evaluation of the data, on the other hand, is made in real space using the set of raw difference Fourier maps as a starting point. Depending on the amount of time points, and on the investigated chemical mechanism, two approaches can be followed.

The first approach consists in integrating the density features at the locations of interest so as to assess the evolution of their electron content over time. Error estimates can be obtained by integrating noisy regions in the same way, or by computing standard deviations from repeated measurements on various crystals. Several procedures can be followed for map integration. Summation can be performed within masks generated by supplying atomic coordinates of the region of interest, specifying a radius of integration around given coordinates.<sup>9</sup> Alternatively, the search for density features may not be restricted to masks (which imply a certain level of *a priori* knowledge) but rather follow a connectivity search algorithm<sup>6</sup> for grid points standing above a threshold  $\sigma$  level. The first method appears more suitable when motions are well localized, but produces noisier results when these are weak and less well characterized.

The time evolution of integrated density features may then be fitted by kinetic schemes, as has been achieved successfully in several studies.<sup>9,10</sup> However, the method suffers from a fundamental limitation, stemming from the fact that several conformational states may coexist at any time in the crystal. Therefore, the density features do not necessarily represent a single species, but rather a combination of several species. This is why refinement of structural models from data at single time-points should only be attempted with great care.<sup>6</sup>

To address this key problem, the technique of singular value decomposition (SVD) familiar to the spectroscopists has been generalized to time-resolved crystallography.<sup>32,33</sup> The method works well for data that are free of systematic errors, although the results are always noisier than in spectroscopy. SVD deconvolution

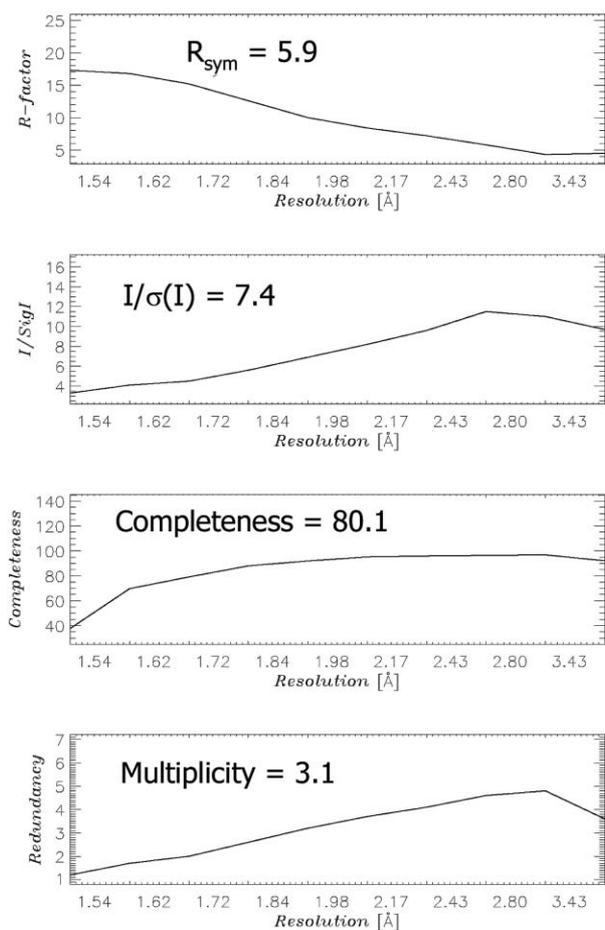
allows extracting from raw difference density maps the significant “left” and “right” singular vectors, where “significant” means “associated to singular values standing above the noise level”. These left and right singular vectors represent a linear combination of the true time-independent intermediates and of their time-dependant concentrations, respectively. A crucial task consists in finding out what this linear combination is. To do so, a plausible kinetic model describing the time evolution of each intermediate needs to be proposed and fitted to the data. If such a procedure is successful, both the structure of the individual intermediates and a consistent kinetic scheme governing their inter-conversion can be suggested (although not proven) and compared *e.g.* to spectroscopic data.

Efficient software for SDV analysis of 4-dimensional crystallographic data has been written (SVD4TX; Y. Zhao and M. Schmidt, unpublished results) although only exponential kinetic schemes can so far be fitted to the data, which may be inadequate when kinetic averaging is not fulfilled (*e.g.* stretched exponential behaviour in ultra-fast processes) or when bimolecular recombination takes place.

Finally, it should be noted that the extraction of time-independent intermediates from time-dependant electron density maps may be approached by other methods than SVD analysis, such as based on “cluster analysis”.<sup>34</sup>

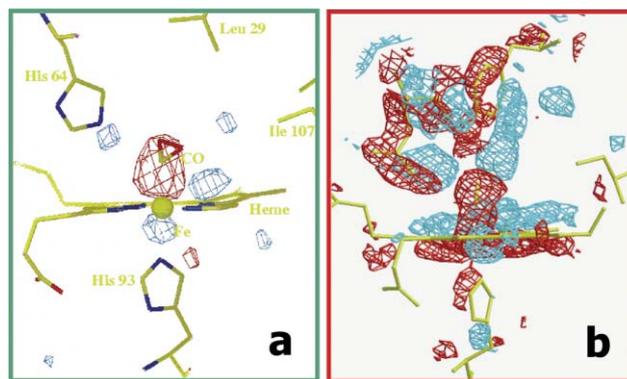
#### 4. Application on a myoglobin triple mutant

Myoglobin is the paradigm to investigate macromolecular conformational dynamics because it is a simple globular heme protein displaying a photosensitivity of the iron–ligand bond. Upon laser photo-dissociation of carbon monoxide, a non-equilibrium population of protein structures is generated that relaxes over a broad time-range extending from picoseconds to milliseconds. This process is associated to migration of the ligand to hydrophobic cavities in the matrix, which have been assigned a functional role as buffer sites. Time-resolved Laue diffraction has been successfully used in pioneering studies to depict the sequence of structural events associated with this extended relaxation.<sup>25,26</sup> Of special interest is how small changes induced by point mutations in the myoglobin distal cavity affect the conformational changes leading from the ligand-bound to the deoxy state of the protein. Outstanding results were obtained in the last few years in the case of the L29F mutant,<sup>5</sup> the L29W mutant<sup>9</sup> and wt-Mb.<sup>14</sup> We have studied a triple mutant called Mb-YQR (Leu29(B10)Tyr, His64(E7)Gln, Thr67(E10)Arg), which was originally engineered to mimic the hemoglobin of pig roundworm *Ascaris suum*.<sup>6,7,35</sup> This mutant displays very little geminate recombination and slows down ligand rebinding by approximately one order of magnitude relative to wild-type Mb, thereby extending the window of time over which conformational changes can be monitored. Furthermore, outstanding crystals of the CO-bound protein could be obtained, making YQR-Mb an excellent candidate for time-resolved Laue diffraction. These crystals grew in batch conditions and under CO pressure, an efficient way to provide well-ordered samples fully occupied with the ligand-bound protein. By applying the advanced pump–probe Laue methodology described above to these highly diffracting and radiation hard samples, diffraction data of outstanding quality could be obtained. An example of data collection statistics is given in Fig. 5. The evolution of



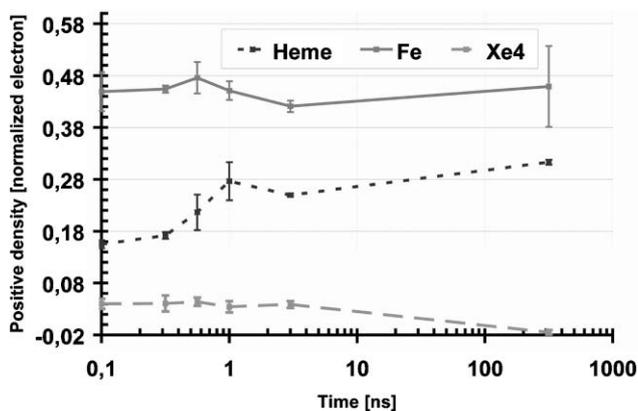
**Fig. 5** Typical data collection statistics from a single-pulse pink Laue data set recorded on a photolysed YQR-myoglobin crystal diffracting to a maximum of 1.5 Å resolution.

completeness, redundancy and  $R_{\text{sym}}$  (or  $I/\sigma(I)$ ) as a function of resolution is quite typical of Laue data collected with a monochromatic undulator and to which a “wavelength dependant resolution limit” procedure has been applied. It is striking to compare the resulting difference Fourier maps with those obtained 12 years ago on the same beamline ID09B of the ESRF, but using a wiggler insertion device, suboptimal data processing, poorer crystals and less reliable laser photolysis schemes (Fig. 6). Quantitative integration of difference electron density maps at time points between 100 ps and 3 ms provided a number of insights concerning Mb-YQR relaxation. Motions of the distal E-helix and of the CD-turn were found to lag significantly (100–300 ns) behind local rearrangements around the heme such as heme tilting, iron motion out of the heme plane and swinging of the mutated residues Tyr29(B10) and Gln64(E7), all of which occur promptly ( $\leq 3$  ns). Over the same delayed time range, CO was observed to migrate from the so-called Xenon4 cavity distal to the heme to the Xenon1 cavity proximal to the heme, suggesting a correlation between ligand migration and large scale structural relaxation of the protein, in line with previous low temperature experiments.<sup>36,37</sup> It was also observed that in Mb-YQR the photo-dissociated ligand populates the Xenon4 binding cavity within 100 ps, accounting for the absence of fast geminate recombination



**Fig. 6** Progress made in the quality of pump-probe Laue data over the years: (a) difference electron density map Mb\* (photolysed state, 3 ns after laser flash) – MbCO (dark state) obtained in 1995 on wt-MbCO; (b) equivalent map obtained in 2004 on the triple mutant YQR-MbCO. The maps are shown at levels of  $+3.0 \sigma$  (blue) and  $-3.0 \sigma$  (red). Dark state models are shown in yellow (in slightly different orientations in (a) and (b)). The improvement in (b) is believed to be primarily due to the better crystallographic data and only marginally due to a change in photolysis yield, although wt-Mb and YQR-Mb are known to display different geminate recombination yields.

(Fig. 7). Another interesting finding was that the heme relaxation towards the deoxy configuration is heterogeneous, with a slower phase occurring on the  $\sim$ ns timescale (Fig. 7). This damping of the heme response was attributed to a transient strain exerted by the E-helix *via* the CD-turn onto heme pyrrole C. These results, together with those from other groups, and when bridged with kinetic data derived from spectroscopy, provide a frame to understand the functional properties of myoglobin based on its structural dynamics behaviour. However, the time-resolved diffraction data on Mb-YQR also reveal key obstacles of such experiments: precise



**Fig. 7** Time dependence of integrated difference electron densities associated to the iron atom, the heme (without iron atom), and the CO in the Xenon4 site in YQR-Mb. The numerical values reflect the integral of the positive electron density beyond  $3.0 \sigma$  and are corrected for variations in photolysis yield. They are normalized so that the negative bound-CO feature is assigned a value of 1. Error bars correspond to twice the weighted standard deviations between 4 equivalent data sets. The prompt motion of the CO into the Xenon4 cavity is clearly seen. Whereas the iron atom also moves within 100 ps (and certainly much faster), the response of the heme molecule is somewhat slower, with a phase developing only on the ns timescale.

kinetic schemes could not yet be extracted from the data, because the signal to noise was impeded by limited photolysis yield (<~20%), and more fundamentally, because ensemble techniques such as crystallography are inadequate (despite deconvolution strategies such as SVD analysis) to accurately disentangle the highly heterogeneous structural landscape of myoglobin.

#### 4. Conclusions

Time-resolved Laue diffraction has undergone a renaissance in the last few years due to a number of instrumental and methodological breakthroughs. The technique is particularly suitable for studying short-lived intermediates in light-sensitive proteins that can be repeatedly excited and provide excellent and radiation-hard crystals. New investigations will focus on photo-receptors such as photosynthetic reaction centers<sup>38</sup> or phycobiliproteins.<sup>39</sup> Heme-based proteins, amongst which neuroglobin that provides a molecular mechanism for neuroprotection, truncated hemoglobins that have recently received attention for their possible role in oxidation catalysis or oxygen scavenging, or oxygen sensors such as FixL that initiate signal transduction pathways<sup>40</sup> are potential candidates of high biological relevance for time-resolved studies. Fluorescent proteins of the green fluorescent protein (GFP) family are also fascinating systems that carry a large interest as markers for cellular microscopy and perform intricate photocycles of fundamental interest.

The underlying assumption of kinetic crystallography is that the crystalline state does not alter the conformational freedom of proteins. The larger the structural changes involved, the less valid this assumption might be. Therefore, complementary spectroscopic techniques that can be applied both in the solution and in the crystalline states are often essential to validate the structural results.<sup>41,42</sup> For pump-probe Laue experiments, the availability of ultra-fast microspectrophotometers connected to the same pumping light source as used for X-ray diffraction may therefore add considerable value to the experimental setup. Molecular dynamics simulations are also highly complementary to sub-nanosecond crystallographic data.<sup>43</sup> Individual pathways that are difficult to extract from electron density maps may be obtained by computer simulations that themselves are validated when the reconstructed average pathway accounts well for the X-ray data. In the future, new insight in protein dynamics might arise from a combination of time resolved Laue crystallography and single molecule spectroscopy, awaiting for ultra-fast single molecule diffraction experiments at X-ray free electron laser facilities.

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