Time-resolved crystallography using the Hadamard transform

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We describe a method for performing time-resolved X-ray crystallographic experiments based on the Hadamard transform, in which time resolution is defined by the underlying periodicity of the probe pulse sequence, and signal/noise is greatly improved over that for the fastest pump-probe experiments depending on a single pulse. This approach should be applicable on standard synchrotron beamlines and will enable high-resolution measurements of protein and small-molecule structural dynamics. It is also applicable to other time-resolved measurements where a probe can be encoded, such as pump-probe spectroscopy.

The ability to watch chemistry and biology happen has huge potential to add to our understanding of molecular function in many contexts. There is increasing interest in targeting specific protein conformational states as a route to higher drug specificity and selectivity1, and functional materials that respond to changes in their environment represent new possibilities for sensors and for energy and information storage2,3. All these research areas require a basic understanding of both structure and dynamics. High-resolution crystal structures now exist for many reactive molecules and macromolecules, providing static models from which to infer function. In many cases, however, the reaction mechanisms remain imperfectly understood, usually because intermediate species are too short-lived to observe by conventional diffraction methods. In contrast, spectroscopic methods make it possible to observe transient molecular species with lifetimes as short as femtoseconds4 but do not usually provide structural information, leaving the problem of relating structure to function unresolved. Time-resolved X-ray crystallography (TRX) has begun to meet these challenges5,6 but so far has provided high-resolution structural information only for short-lived (sub-millisecond) intermediates of a small number of proteins.

Although ultrafast methods for time-resolved spectroscopy are well established, this is not the case for crystallography. The current state-of-the-art methods for TRX are the Laue method7,8 (~100 ps) along with streak camera techniques and bunch slicing methods, for which ~1 ps or better time resolution has been obtained for molecular samples9,10. Such high time resolution can be achieved because there are sufficient photons in each single X-ray probe pulse that measurable diffraction data can be obtained, but these techniques require highly specialized instrumentation. Recent developments in X-ray free-electron lasers offer the possibility of femtosecond TRX11. Such high time resolutions cannot be achieved at a conventional monochromatic beamline for a macromolecular sample, however, because there are simply not enough X-ray photons in a period of less than 1 μs to obtain a usable diffraction pattern12.

The conventional way to study transient phenomena in spectroscopic or crystallographic experiments is to initiate the process and then probe the system at a later time, either with X-rays13 or visible or infrared (IR) photons4,14,15. In classical time-resolved experiments, a laser (pump) pulse is used for initiation and a single probe pulse follows every pump pulse after a series of predetermined time delays. To measure n time points, n pump-probe pairs are needed (Fig. 1a).

Here we present a Hadamard time-resolved approach. The Hadamard approach is a transform method that has been used in spectroscopy16 but has so far not been used in time-resolved measurements. In contrast to the conventional pump-probe method, in our Hadamard approach each pump pulse is followed by a sequence of probe pulses and the total signal from each sequence is recorded in a single measurement (Fig. 1b). The sensitivity of the experiment is therefore defined by the number of photons within the entire probe sequence, with the time resolution defined as the total probe sequence length divided by the number of pulses. This frees the achievable time resolution from its current dependence on source brilliance by summing time points across the probe sequence. It also results in an improved signal-to-noise ratio16 because of the larger number of photons recorded during each measurement.

As in classical pump-probe experiments, n pump-probe sequences are needed to measure n time points. The pattern of the probe sequence can be represented as rows of a $n \times n$ matrix (S) derived from a Hadamard sequence (Supplementary Figs. 1 and 2, Online Methods)16. As shown for the simplest case of three time points (Fig. 1b), each row of the matrix (and hence probe sequence) is obtained by a cyclic left shift by one element from the previous row (Fig. 1c).

For a Hadamard time-resolved experiment, the reaction is initiated and then the entire probe sequence (given by the first
The row of the \( S \) matrix) is recorded by the detector as a single image. This is repeated on a new sample (or on the same sample after relaxation), but with the probe sequence now defined by the next row of the \( S \) matrix, until all rows have been used. The resulting encoded signals from \( n \) excitations are collated to form a vector \( W \) of length \( n \). To obtain the time-dependent signal, \( I_t \), the probe sequence encoding is reversed by multiplying the vector \( W \) by the inverse of the matrix \( S \): that is, \( I_t = S^{-1}W \).

Hadamard encoding has not, to our knowledge, been previously applied to time-resolved experiments, but it has been used to improve the signal-to-noise ratio in optical and IR imaging and spectroscopy\(^{16}\), mass spectrometry\(^{17}\) and NMR spectroscopy\(^{18}\). The use of Hadamard sequences for time-resolved measurements is generally applicable to any time-resolved experiment in which the probe beam can be encoded, such as transient absorption spectroscopy. Here we demonstrate the validity of this approach by observing X-ray induced, time-dependent changes in thaumatin crystals\(^{19}\) in a Hadamard time-resolved crystallographic (HATRX) experiment.

The use of the Hadamard approach is independent of the manner in which the pulse sequence is generated. A detector-encoded HATRX experiment can be reproduced by selectively summing single images from a continuous series of evenly spaced exposures.

**Figure 1** | Comparison of classical pump-probe and HATRX methods. (a) The classical pump-probe method, showing three pump-probe time delays. (b) The simplest Hadamard pulse sequence to measure three time points. Note that in this experiment the detector is read out only at the end of the whole sequence. (c) The \( 3 \times 3 \) Hadamard \( S \) matrix illustrating how each row produces a single summed intensity for each reflection on the detector \( (w_1, \text{ etc.}) \) forming the vector \( W \).

**Figure 2** | Difference electron density maps showing the comparison of control and HATRX data for thaumatin. Maps are calculated as \( F_t - F_1 \) (where \( t = 2, 4, 7, 10, 13, 16, 19, 22, 25 \)) for the HATRX data (left) and the control data (right). The dose resolution of the experiment is 1.2 MGy, which was delivered in 200 ms. The maps are contoured at 3 r.m.s. deviations. Negative difference density is colored magenta and positive difference density is colored cyan. The disulfide bonds between Cys56 and Cys66 and between Cys71 and Cys77 are shown.
into HATRX images. This allows the HATRX electron density to be compared to that from a control time series acquired in the conventional manner. By doing this, we demonstrated that a HATRX experiment can successfully track time-dependent structural changes in the protein thaumatin with a nominal time resolution of 200 ms (Fig. 2).

Exposure of protein crystals to the microfocus X-ray beamline 124 (Diamond Light Source) results in fast, irreversible radiation damage that can be readily observed through progressive breakage of disulfide bonds. In our proof-of-principle HATRX experiment, the absorbed X-ray dose was used as a proxy for time, with the X-ray beam itself acting as the ‘pump’. We used a 3 × 3 S matrix, but to extend the dose (time) range probed, we repeated this 9 times to give a total of 27 time points. We collected diffraction data sets (wedges) consisting of 27 repeated images of the same 1° oscillation. We also recorded equivalent wedges of data from an independent set of crystals to provide a control data set with known intensities at each time point. The exposure time and incident flux were such that substantial radiation damage accumulated within each wedge, with the experimental dose limit reached by the final image.

For the control data, we created composite scaled data sets for each time point (Fig. 3a, Supplementary Table 1). To create the HATRX data sets, we assigned wedges an exposure sequence corresponding to a single line of the \( n = 3 \) S matrix (Fig. 3b) and summed them into 9 HATRX data sets encoding all 27 time points. Both the HATRX and the control images were integrated and scaled using standard methodology (Online Methods).

Each 3 × 3 HATRX experiment comprises three data sets, each encoded with one line of the S matrix (Supplementary Table 2). For each data set, we converted the intensities of all reflections into amplitudes and then collated these into a vector \( W \). This was transformed to yield three data sets now corresponding to three data sets with known intensities at each time point. The exposure time and incident flux were such that substantial radiation damage accumulated within each wedge, with the experimental dose limit reached by the final image.

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We calculated \( F_{\text{HATRX}} - F_{\text{CONTROL}} \) electron density maps for each time point, and these showed no substantial difference features at 3 r.m.s. deviations. However, \( F_{\text{HATRX}} - F_{\text{CONTROL}} \) electron density maps for both the HATRX and control data show similar clear electron density changes at the disulfide bonds, as expected, with increasing X-ray dose (Fig. 2, Supplementary Fig. 3). These results demonstrate that the HATRX method produces reliable four-dimensional electron density maps, which are simultaneously highly resolved in both space and time.

A comparison of the expected signal can be made using estimates of the known flux from a monochromatic X-ray beamline. For example, beamline 124 delivers \( \times 10^{12} \) photons per second. Assuming an elastic scattering efficiency of 0.1% for an image with 100 spots there are \( 10^7 \) photons scattered per spot per second (assuming for illustration that spots are of equal intensity). If we record 100 1-μs time points over a 100-μs range, then with classical pump-probe each spot will contain only 10 photons. In contrast, when the HATRX approach is used, each spot will contain approximately 500 photons because the HATRX sequence is approximately half on and half off (Supplementary Fig. 2).

There are two contributions to the improvement in signal to noise. The first is the absolute number of photons recorded for each time point. Consider the simplest \( n = 3 \) experiment. In the classical pump-probe approach, each time-dependent measurement is made once. However, with the HATRX approach, the encoded measurement is repeated three times, with each time point measured twice, resulting in a doubling of the signal recorded. Consequently, the mean square error associated with the measurement is reduced by a factor \( (n + 1)^2 / 4n = n/4 \), and so the signal-to-noise ratio is increased by a factor of \( (n + 1)^2 / 4n = n/2 \) relative to conventional experiments (Online Methods).

Although we have used a sequence of probe pulses that are equally separated in time, the method will also work if the probe pulses are logarithmically spaced, allowing a wide range of time scales to be observed in a single experiment. The experiment measures the total intensity from a sequence and is independent of the time stamp of each pulse.

An additional advantage of HATRX, specific to crystallography, is that the scaling of data from multiple crystals and time points need only be done once (as the same sample population progresses in time). In contrast, in a serial crystallography pump-probe experiment the data must be scaled first for each time point over all crystal orientations and then again over time.

Experimentally, it should be possible to generate Hadamard pulse sequences for HATRX at synchrotron sources in a number of ways, such as by using a rotating disc shutter encoded with a sequence or by deflecting electron bunches out of the synchrotron electron beam. An alternative approach would be to use a pixel array detector gated to record according to the Hadamard pulse sequence but reading out only after the sequence is completed (Online Methods).

HATRX will enable fast TRX experiments on both reversible and irreversible processes at synchrotron sources where currently the flux density achievable at the sample is the limiting factor. Though we demonstrated HATRX here with millisecond
time-resolution, we anticipate that it can practically be extended to shorter time-scales (Online Methods). This will greatly increase the applicability of the method. In addition, the method’s applicability to any experimental technique where the probe can be encoded makes this a general tool for dynamic studies.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. The low-dose thaumatin structure refined against HATRX summed data time point 1 is available from the Protein Data Bank under the accession code 4C3C.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
G.S.B. and B.A.Y. originally proposed the application of the Hadamard transform to time-resolved experiments. B.A.Y., R.L.O. and A.R.P. devised the proof-of-principle crystallographic experiment and collected the data. B.A.Y., R.L.O., G.S.B. and A.R.P. all processed data, wrote software and jointly wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Generation of S matrices. Hadamard matrices \( H \) are square and of size \( 4m \), where \( m \) is a positive integer; they contain only 1 and \(-1\) as elements and have the property \( H^T H = U \), where superscript \( T \) indicates the transpose and \( U \) the unit diagonal matrix. To convert an \( m \times m \) Hadamard matrix into an \( n \times n \) S matrix, the first row and column are ignored (as they contain only 1s in the Hadamard matrix) and then the changes \( 1 \rightarrow 0 \) and \(-1 \rightarrow +1\) are made.

The \( n \times n \) S matrices are generated according to a method described by Harwit and Sloane \(^1\). These matrices contain only 0s and 1s. The first row of an S matrix is generated using the following (quadratic residue) procedure: first, the prime numbers, \( n \), are found for which \( n = 4m + 3 \) is true and where \( m \) is any positive integer \( \geq 0 \). The first few permissible values are \( n = 3, 7, 11, 19, 23, 31, 43, 47, 59, \ldots \) Next a vector \( (va) \) of length \((n - 1)/2\) is defined, where each element value is equal to the square of its index. A new vector \( (vb) \) is calculated where each element is the integer remainder of the corresponding element in \( va \) divided by \( n \), plus \( i \) : i.e., \((j \mod n) + 1\). The first row of the S matrix is calculated as a vector \( (S1) \) of length \( n \), with the first element equal to \( 1 \). Each subsequent element is made equal to \( 1 \) if its index is in \( vb \); otherwise its value is zero. To construct the S matrix, \( S1 \) is rotated by one position to the left to give the next row \( S2 \); \( S2 \) is then rotated by one position to the left to give \( S3 \); and this is repeated \( n \) times, resulting in an S matrix of order \( n \) (Supplementary Fig. 1).

For \( n = 3 \), the \( S1 \) sequence is \( 1 \ 1 \ 0 \) and this is the first row of the S matrix. Other rows are obtained by left cyclic rotation of the S row. For \( n = 7 \), the sequence is \( S1 = 1 \ 1 \ 1 \ 0 \ 1 \ 0 \ 0 \), and for \( n = 11 \), \( S1 = 1 \ 1 \ 1 \ 0 \ 1 \ 1 \ 0 \ 0 \ 0 \ 1 \ 0 \). The S matrices for \( n = 3, 7, 11 \) are shown diagrammatically in Supplementary Figure 2.

The inverse of an S matrix can be calculated by direct inversion, but more simply as

\[
S^{-1} = \frac{2}{n+1}(2S^T - I)
\]

where \( I \) is the all-1s matrix. Besides the multiplicity term \( 2/(n + 1) \), matrix \( S^{-1} \) contains \(-1\) where the S matrix contains \(0\).

In the main text it is stated that \( I_1 = S^{-1}W \), where \( I_1 \) is the intensity of a spot at time \( t \). To show that this is proportional to the change in signal, let \( f(t) \) describe how a measurable property of the molecule varies in time, e.g., X-ray spot intensity or transient absorbance. As we sample at points \( i = 1 \ldots n \), this gives a set of discrete signals \( f_i \) which will form a column vector \( F = [f_1, f_2, \ldots, f_n]^T \). In the HATRX method, the sampling points are defined by a sequence of probe pulses given by rows of the S matrix with elements \( s_{ji} \). The signal \( w_j \) from one sequence, measured after each pump event, is summed on the detector and is

\[
w_j = \sum_{i} f_i s_{ji}
\]

Together with signals from the other sequences, these form a column vector

\[
W = [w_1, w_2, \ldots]^T
\]

The signal is obtained as \( I = S^{-1}W \), and to show that this is the same as \( F \), if we let \( \sigma_j \) be the elements of \( S^{-1} \), then expanding gives

\[
I = \eta \begin{bmatrix}
\sigma_{11} & \sigma_{12} & \ldots & w_1 \\
\sigma_{21} & \sigma_{22} & \ldots & w_2 \\
\vdots & \vdots & \ddots & \vdots \\
\end{bmatrix}
\]

where \( \eta = 2/(n + 1) \).

The first term is

\[
I_1 = \eta \sum_{j} \sigma_{1j} w_j = \eta \sum_{j} \sigma_{1j} \sum_{i} f_i s_{ji}
\]

This is evaluated by inserting the values for \( s_{ji} \) (0 or 1) from the S matrix and \( \sigma_{ij} \) (±1) from \( S^{-1} \). Thus with \( n = 3, s_{1,3} = 1,1,0 \), and \( \sigma_{1,3} = (1,1,-1)/2 \), hence

\[
I_1 = [(f_1 + f_2) + (f_1 + f_3) - (f_2 + f_3)]/2 = f_1
\]

and similarly for the other terms, \( I_2 \) and \( I_3 \). This is true for all valid \( n \) and hence \( I = F \).

In the case that the detector causes a random measurement error, \( \epsilon \), and assuming \( \epsilon \) has mean of zero and variance \( \sigma^2 \), then \( w_j = \sum_i f_i s_{ji} + \epsilon_j \), and hence for the \( k \)th point

\[
I_k = \eta \sum_{j} \sigma_{kj} \left( \sum_{i} f_i s_{ji} + \epsilon_j \right) = f_k + \eta \sum_j \epsilon_j
\]

The variance of a quantity \( ce \), where \( c \) is a constant, is \( c^2 \) times the variance of \( \epsilon \), and as the variance of a sum of independent random variables is the sum of the individual variances, the variance or mean square error of \( I_k \) is \( 4n \sigma^2/(n + 1)^2 \), i.e., this is reduced by \((n + 1)^2/4n \), which is the expression used in the main text.

Crystallization and data collection. Thaumatin is a protein that contains eight disulfide bonds and is well characterized in radiation damage studies\(^{19}\). Thaumatin crystals were prepared as follows. The protein in ddH\(_2\)O (40 mg/ml) was crystallized in sitting-drop plates in a 2:1 mixture (4 µl protein solution) with 0.05 M ADA pH 6.8, 0.6 M K/Na tartrate and 20% v/v glycerol. The crystals (10–20 µm along the longest edge) were then mounted on a polyimide mesh (MiTeGen) and cryo-cooled to 100 K in liquid nitrogen. All diffraction data were collected on beamline I24 at the Diamond Light Source (Didcot, UK) at 100 K, with a beam size of 10 × 10 µm\(^2\) and an incident flux of 1.5 × 10\(^{12}\) photons per second. Wedges of data were collected from 264 microcrystals. These wedges contained 27 images taken over a repeated oscillation of 1°: each image had an exposure time of 200 ms. The absorbed dose was calculated using RADDOSE\(^{21}\), and the total absorbed dose was 29 MGy over the 27 images.

Generation and processing of Hadamard series data. A subset of 31 crystals was used for the control data set. The diffraction data were integrated using XDS\(^{22}\) and then scaled together (but not merged) into a single .mtz file where the image numbers

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associated with each reflection were retained. The data from all image 1s were then scaled together using AIMLESS\textsuperscript{23} to produce a data set for time (dose) point 1. This was repeated for each time point to yield 27 time-resolved data sets. The merging statistics for the resulting data sets are shown in Supplementary Table 1.

The data wedges from the remaining 233 crystals were randomly split into three approximately equal groups by successively assigning wedges to a group, merging and scaling. Wedges that improved completeness were kept in that group, wedges that duplicated regions of reciprocal space already sampled were assigned to a different data set and poorly merging wedges were discarded. An exposure sequence corresponding to a line of the $3 \times 3$ $S$ matrix was assigned to each group to determine which images would be summed for the HATRX analysis (Supplementary Fig. 4). The required images were then summed using the program SUMSUB\textsuperscript{24} (http://code.google.com/p/disp/) to yield a new HATRX-encoded image that could then be indexed and integrated as normal (Fig. 3b). Scripts used for the creation of the HATRX data can be found in Supplementary Software 1 and 4.

The resulting HATRX images were indexed and integrated using XDS and then scaled using AIMLESS. Some poorly merging wedges from each group were discarded, resulting in 72, 76 and 70 wedges finally used for each HATRX group (Supplementary Table 2). The intensities were converted to amplitudes using TRUNCATE\textsuperscript{25}, and the $F$ and SigF for each reflection were collated into the vector $W$ for each of the 9 HATRX data sets.

Multiplication of these vectors (for both $F$ and SigF) by the inverse of the $S$ matrix

$$
\begin{bmatrix}
I_1 \\
\vdots \\
I_n
\end{bmatrix} = S^{-1}
\begin{bmatrix}
w_1 \\
\vdots \\
w_n
\end{bmatrix}
$$

yielded three data sets corresponding to three time (dose) points. This was repeated for the next eight sets, i.e., batch groupings of (4,5,6), (7,8,9), etc., to yield 27 time-resolved data sets (Supplementary Fig. 4). Scripts used for the HATRX transformation can be found in Supplementary Software 2 and 3.

Within the error of the experiment, the control and HATRX data were indistinguishable (Supplementary Table 3). The same was true for data treated as for a 7-period HATRX experiment (7 groups of 25 crystals used, i.e., batch groupings of (1–7), (8–14), etc. to yield 4 HATRX images; data not shown), although in this case the data completeness was insufficient to allow for map calculation (<60%).

Phases were calculated using PDB 1KWN\textsuperscript{26} as a starting model. The model was refined using REFMAC5 (ref. 27) against the HATRX time point 1 data and the resulting phases used for all further map calculations (Supplementary Table 4). Maps derived from the control and HATRX data were then compared by the calculation of difference maps: i.e., $F_{\text{HATRX}} - F_{\text{TRAD1}}$ for $1 \leq n \geq N$; ($F_{\text{HATRXN}} - F_{\text{HATRX1}}$) versus ($F_{\text{TRADN}} - F_{\text{TRAD1}}$).

Possibilities for the encoding of HATRX sequences at a synchrotron. The demonstration experiment reported in this paper has a time resolution of 200 ms. It is perhaps useful to consider the possibilities for encoding HATRX sequences on a synchrotron such as Diamond Light Source.

Encoding can be done using a pixel array detector (or ICCD camera) gated\textsuperscript{28} to record according to a specified Hadamard pulse sequence but reading out data only at the end of each probe sequence. This differs from traditional approaches in that the beam itself is not shuttered at high speed but instead the detector is selectivity read out. The advantage of such an approach is that it requires minimal modification to the beamline itself.

We have demonstrated the feasibility of this approach by collecting flat-field images using defined pulse sequences and comparing these to images collected normally. This shows that gating of a pixel array detector using a Hadamard sequence results in the expected number of counts, with the detector ‘active’ and ‘blind’ for the correct period of time (Supplementary Table 5).

A potential drawback of this approach for samples susceptible to X-ray induced damage is that although the detector is only counting the desired pulses, the sample is ‘continuously’ exposed and the total absorbed dose over the entire HATRX sequence should thus be considered when designing the experiment. For pulses logarithmically separated in time, a slower shutter linked to the same timing could be used to reduce the absorbed dose during the longer gaps toward the end of the sequence.

An alternate approach, and one that is already implemented on the Laue beamlines at the APS, ESRF and Photon Factory, is the use of ultrafast choppers to directly slice single bunches\textsuperscript{29–31}. Such ultrafast single-slot choppers can deliver opening times as short as 100 ns. This enables them to slice single bunches as long as the machine is operating in a fill mode where there is $>100$ ns between bunches. However, such shutters are not appropriate for a fast HATRX experiment as they have a repetition rate of the order of a millisecond\textsuperscript{29–31}. A similar shutter operating at the same rotation frequency, but containing a pattern of slots\textsuperscript{32}, would deliver better time resolution. A shutter patterned in such a way as to deliver a logarithmic pulse series could span timescales from several hundred nanoseconds to milliseconds. A final, interesting possibility is to directly encode the HATRX sequence into the ring bunch structure, for example, by using a kicker magnet as already demonstrated at the ALS\textsuperscript{33}. The maximum frequency of a single kicker magnet is MHz (the ring repetition rate), but additional kicker magnets would enable better time resolution, at the expense of major modifications to the machine.

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Corrigendum: Time-resolved crystallography using the Hadamard transform

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In the version of this article initially published, the Figure 2 legend misidentified the control data as on the left and the HATRX data as on the right. The error has been corrected in the HTML and PDF versions of the article.