Nanoscale mosaicity revealed in peptide microcrystals by scanning electron nanodiffraction

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Changes in lattice structure across sub-regions of protein crystals are challenging to assess when relying on whole crystal measurements. Because of this difficulty, macromolecular structure determination from protein micro and nanocrystals requires assumptions of bulk crystallinity and domain block substructure. Here we map lattice structure across micron size areas of cryogenically preserved three-dimensional peptide crystals using a nano-focused electron beam. This approach produces diffraction from as few as 1500 molecules in a crystal, is sensitive to crystal thickness and three-dimensional lattice orientation. Real-space maps reconstructed from unsupervised classification of diffraction patterns across a crystal reveal regions of crystal order/disorder and three-dimensional lattice tilts on the sub-100nm scale. The nanoscale lattice reorientation observed in the micron-sized peptide crystal lattices studied here provides a direct view of their plasticity. Knowledge of these features facilitates an improved understanding of peptide assemblies that could aid in the determination of structures from nano- and microcrystals by single or serial crystal electron diffraction.
The physical and chemical properties of a crystal depend in part on its underlying lattice structure. Changes in the packing of macromolecules within crystals perturb this structure as is exemplified by crystal polymorphism. Packing rearrangements can also lead to deterioration of lattice order and limit the usability of a crystal for structural determination. Imperfections in protein crystals can in part be described by the mosaic block model, in which monolithic crystal blocks or domains tile to form a macro-crystal, but vary in size, orientation and/or cell dimensions. Because directly measuring mosaicity in protein crystals is inherently challenging, crystallographic software must estimate disparities in domain block size, shape and orientation per crystal, for full and partial Bragg reflections. Because these domains are vastly smaller than the typical illumination in diffraction experiments, they are modelled as a continuous, but bounded, spectrum of morphology/orientation. Mosaicity varies by crystal and is affected by crystal size, crystal manipulation and parameters for data collection. The challenge in accurately assessing these models in protein nanocrystals has been highlighted by analysis of diffraction measured using x-ray free electron lasers.

Direct views of a protein crystal lattice can be obtained by high-resolution electron microscopy (EM), facilitated by advances in high-resolution imaging and cryogenic sample handling techniques. Cryo-EM also reveals crystal self-assembly and, for two-dimensional protein crystals, shows natural variation between unit cells. Domain blocks can be identified in cryo-EM images of three-dimensional (3D) lysozyme nanocrystals, where Fourier filtering helps estimate the location and span of multiple blocks across a single crystal.

Macromolecular structures can be obtained from similar nanocrystals by selected area electron diffraction-based methods such as MicroED and rotation electron diffraction. Structures determined by MicroED or similar approaches range in size from small molecules to proteins, including a variety of peptides. In MicroED, frozen-hydrated nanocrystals are unidirectionally rotated while being illuminated by an electron beam to produce diffraction movies. These movies are processed by standard crystallographic software, and structures are determined and refined using electron scattering factors. Diffraction signal permitting structures from well-ordered crystals can be determined by MicroED with atomic resolution and mirror those obtained by microfocus x-ray crystallography. These structures represent an average over entire crystals or large crystal areas, due to the use of a selected area aperture during data collection.

In EM, greater control over illuminated areas is achieved by scanning transmission EM (STEM), which positions a focused electron beam (typically <1 nm) at discrete locations on a sample to produce images of sub-micron-thick biospecimens over large fields of view. A variety of sample properties can be probed by collecting electrons from different angular ranges, such as annular dark field detection with low and high scattering angle detectors (ADF, HAADF) and annular bright field detection (ABF) and differential phase contrast detection, providing access to different contrast mechanisms underlying these modalities. These approaches typically rely on monolithic detectors that integrate electrons over a specific angular range originating from the sample at each probe position and attribute the signal intensity to a point on the sample. These techniques have been successful in the 3D mapping of atomic features within imperfect crystals of radiation hard materials.

In contrast, a scanning nanobeam diffraction experiment records diffraction patterns on a two-dimensional pixelated detector at each scan position across a sample. Each $\text{Scan}_x \times \text{Scan}_y$ position of the scan has a $K_x \times K_y$ dimension in reciprocal space (diffraction image) resulting in a four-dimensional data set resulting in a four-dimensional data set (4DSTEM). These data can then be processed to reconstruct a real space image of the sample corresponding to specific features in the measured diffraction patterns from each scan point, resulting in a greater flexibility in the imaging contrasts obtainable from a single experiment. Cooling sensitive samples to cryogenic (liquid nitrogen) temperatures is advantageous to minimize the electron-induced radiation damage in such experiments. Using these methods, sensitive, semi-crystalline organic polymers have been investigated by 4DSTEM to reveal differential lattice orientation within thin films.

Here we analyse beam-sensitive 3D peptide nanocrystals at liquid nitrogen temperatures by 4DSTEM. Our findings address a lack of direct estimates of nanoscale lattice variation in biomolecular microcrystals by other diffraction methodologies and address their relationship to diffraction data quality. Our measurements reveal effects that may influence MicroED experiments and other nanocrystallography methods including those performed at x-ray free electron laser, or synchrotron-based micro- and nanocrystallography.

**Results**

**4DSTEM of 3D beam-sensitive peptide nanocrystals.** To assess nanoscale lattice changes within single peptide crystals, we generated two-dimensional maps of their lattice structure by 4DSTEM (Fig. 1). We evaluated 34 nanocrystals formed by a prion peptide segment with sequence QYNQNQNFV, whose structure has been previously determined (PDB ID 6AXZ) by MicroED to sub-A resolution. Crystals analysed by 4DSTEM were equivalent in shape and size to those evaluated by MicroED; most were needle shaped and several microns in their longest dimension, but less than a micron thick and wide (Supplementary Figure 1). Crystals that lay over holes on the quantifoil grid were found to produce the highest contrast signal and were chosen for analysis by 4DSTEM.

During 4DSTEM data collection, an approximately six nanometre electron beam (Supplementary Figure 2) was scanned coarsely across a grid, while regions of interest were identified by low-mag, low-dose STEM imaging. Fine scans were performed on up to 3-μm-long regions of single nanocrystals, while diffraction patterns were measured at each step using a direct electron detector (Fig. 1c). Beam parameters in 4DSTEM scans were comparable to those of prior studies (Supplementary Table 1), with an estimated fluence of $\sim 1 \text{ e}^- \text{Å}^{-2}$ in regions of the crystal exposed to the beam. For all 4DSTEM scan points, we applied the lowest dose necessary to detect Bragg reflections (Fig. 1d, e, Supplementary Figure 1). We confirmed that the dose was sufficiently low by measuring several overlapping scans from the same area of a single crystal and found that decay in the highest resolution reflections occurred only after multiple scans of the same area (Supplementary Figure 3). The probe dwell time and diffraction image exposure were synchronized and produced full-frame patterns at a rate of 400 s⁻¹ (Supplementary Figure 4).

These parameters yielded diffraction patterns whose resolution extended beyond 1.4 Å (Supplementary Figure 1), measured from only an estimated 1000–15,000 diffracting molecules given an effective illumination area of $6 \times 6 \text{ nm}$, a crystal thickness of 100–500 nm and unit cell constants of $a = 4.94$, $b = 10.34$, $c = 31.15$, $\alpha = 94.21$, $\beta = 92.36$, $\gamma = 102.2$. Bragg diffraction was observed only from within the bounds of the crystals, as identified by annular dark field STEM images (Fig. 1, Supplementary Figure 1, 3). Diffraction patterns integrated over micron-sized regions of single crystals matched MicroED patterns measured from a micron-sized selected area (Supplementary Figure 1).
observed at most Bragg reflections compared to standard counting, but improved estimates of central beam intensity (Supplementary Figure 5).

Raw patterns collected on a K2 direct electron detector at 400 frames s\(^{-1}\) were processed using a workflow that involves background subtraction, normalization, thresholding, and pixel-level stepwise counting (Supplementary Figure 4). The hybrid counting approach was implemented as follows: We used the distribution of pixel intensities within an entire 4DSTEM scan to estimate background signal and categorize measured pixel intensities into counting bins corresponding to single or multiple electron counts (Supplementary Figure 4e, 5a). Based on this criterion, the majority of pixels in a pattern were zero valued (Supplementary Figure 4e); a small fraction (<1%) within the regions where Bragg reflections were expected received single counts and a lesser minority of pixels were assigned multiple counts corresponding to measurement of coincident electrons (Supplementary Figure 5a). Of the latter group, most events occurred within the region illuminated by the central (000) focused beam disc (Supplementary Figure 5a (insets)), particularly over thin sample regions or holes in the carbon support (Supplementary Figure 5b, c).

The range of signal counts in processed patterns spanned values from 1 to 35, but very few pixels were assigned values >10 (Supplementary Figure 5a). While the true degree of coincidence cannot be measured by this method, comparison of these patterns to those processed with a binary threshold shows an increase in dynamic range achieved by hybrid counting protocols (Supplementary Figure 4f, 5b, c). This process resulted in an estimate of 2612 ± 445 electrons per pattern, with 222 ± 146 electrons being localized to Bragg reflections. A small fraction of electrons that are uncounted scatter beyond the view of the pixelated detector and are detected by the HAADF. These measurements approximate the 3750 electrons expected to impinge upon a crystal region of this size.

Pixel-wise processing of data also allowed us to correct diffraction pattern shifts due to beam scanning using centre of mass alignment (Supplementary Figure 6). The majority of scans presented minimal shifts; some scans showed preferential drift of the diffraction pattern along a single orientation (Supplementary Figure 6c, d (inset)). The combined intensity of the central beam and Bragg reflections allowed us to estimate crystal thickness at each scan point (Supplementary Figure 7). This estimated thickness correlates well with features observed in annular dark field STEM images including empty regions within holes and the carbon support (Supplementary Figure 7).

Analysis of lattice structure in 4DSTEM scans across large areas of peptide crystals. To better understand changes in the pattern of Bragg reflections observed across different areas of a single crystal, we performed unsupervised classification of patterns in each scan (Fig. 2). Given the high sparsity of signal outside the central disk in individual diffraction patterns (Supplementary Figure 4, 5), we masked out the central beam to limit its influence on pattern classification (Fig. 2). This procedure revealed groups of patterns within a scan that shared a common lattice; patterns could be classified in this way for all measured crystals. The number of regions identified by classification varied between crystals and ranged from 4 to 20 with an average area of 1.6 × 10^5 ± 1.7 × 10^5 nm^2 (Supplementary Figure 8). In 2 of the 34 datasets analysed, the number of clusters was manually assigned because fewer than five clusters were reproducibly identified. The spatial distribution of clusters identified by this approach was consistent with changes in the pattern of Bragg reflections across
a scan, rather than other background signal or other diffuse scattering (Supplementary Figure 9).

The number and intensity of Bragg reflections varied across classified patterns (Fig. 3c, Supplementary Figure 10). Bragg reflections were weak or attenuated in regions where crystals appeared thickest and in thin regions where the central beam showed high counts (Fig. 3c, Supplementary Figure 10). The overall pattern of Bragg reflections differed across a single crystal, changing in a coordinated fashion at all resolutions. Diffraction patterns in a cluster appeared spatially linked within a crystal (Fig. 3b). This effect was reinforced by the requirement that patterns be assigned to a particular class. When mapped onto crystals, diffraction pattern clusters gave the appearance of nanodomains with definite boundaries (Fig. 3a, Supplementary Figure 9b). However, these boundaries were deceptive, since in reality the change in diffraction appeared more continuous (Supplementary Figure 10). To assess the true extent of angular reorientation, we performed a library-based indexing of patterns from four crystals obtained by cluster averaging (Supplementary Figure 11). These particular crystals were selected as they contained the necessary number of reflections per cluster to unambiguously assign a lattice orientation. Library indexing of patterns from these four crystals showed that changes in diffraction across clusters could be attributed to an average ±1° tilt of the lattice away from the mean orientation of the crystal (Fig. 3c). The minimum deviation observed in the analysed
crystals was ±0.5°, while the maximum deviation was ±4°. While the degree of variation in lattice tilt differs between crystals, lattice tilt was observed in all 34 crystals investigated in this study.

Analysis of lattice structure in HRTEM images of peptide crystals. To obtain context for the spatial distribution of changes in lattice structure in peptide crystals we observed by 4DSTEM, we performed HRTEM of similar crystals in search of similar effects.20 High-resolution cryo-EM on crystals of QYNNQNNFV with a total dose of 27.8 e⁻⁻/Å² per image (Supplementary Figure 12a, c) allowed us to directly visualize the lattice in these crystals at approximately 2.3 Å resolution (Supplementary Figure 12b, d). We processed these images by single-particle cryo-EM protocols, identifying image sub-regions for unsupervised clustering, analogous to the procedure used for 4DSTEM pattern classification (Supplementary Figure 13).

Fourier transforms of whole crystal images showed clear variation in Bragg reflections when different crystallites were imaged or when comparing crystalline features to empty regions within holes or regions of carbon support (Supplementary Figure 12, 13). Cluster averages also showed differing images (Figure 13c, f, Supplementary Figure 14a-d), but quantifiable differences between clusters were difficult to detect in either real or reciprocal space (Supplementary Figure 14a-h). This is potentially due to the influence of crystal thickness and defocus across the image that make true changes in 3D lattice character difficult to discern (Supplementary Figure 13).

Discussion
To reveal the lattice substructure within beam-sensitive 3D crystals, we performed 4DSTEM on peptide nanocrystals at cryogenic temperatures. In contrast to semi-crystalline polymers crystals, we performed 4DSTEM on peptide nanocrystals at

The changes in diffraction we observe across micron-sized regions of peptide nanocrystals point to inhomogeneities across a single crystal. These inhomogeneities did not necessarily correspond to those observed in HAADF images alone, as it appears that only sub-regions of crystals produce strong, measurable diffraction. Differences in diffraction are not only diagnostic of regions with strong and weak diffraction within individual crystals but also point to changes in the orientation of the lattice within a crystal. This nanoscale reorientation in sub-micron regions of a single nanocrystal is difficult to detect by methods that make use of selected area electron diffraction, as well as those that integrate diffraction from whole crystals. This is especially true after data from multiple crystals is merged64,65, a requirement for both serial crystallography and most MicroED experiments63.

The real space resolution of a map produced by 4DSTEM with a step size of 20 nm is contrasted with the real space size of the selected area aperture in MicroED of 500–1000 nm, allowing much finer sampling of lattice variation. Lattice reorientation at this scale in macromolecular nanocrystals may explain the discrepancy in dynamical scattering observed from crystals of this thickness by MicroED, compared to what would be expected by simulation from perfect crystals.66

The nanoscale lattice reorientation observed by 4DSTEM differs from conventional domain blocks. Whereas conventional mosaic blocks have been modelled as a continuous, semi-random distribution of orientations, we observe a gradual, progressive change in orientation as a function of spatial localization. Presently, our library-based orientation assignment acts on the average diffraction over clustered patterns and requires known cell constants, but improved interpretation of single sparse patterns and ab initio indexing of electron diffraction patterns may alleviate one or both of these limitations. Despite present limitations, nano-scale lattice changes in our crystals are more readily identifiable from 4DSTEM scans than from HRTEM images of similar nanocrystals owing to the higher contrast of Bragg peaks captured by diffraction. Transforms of sub-regions within a 3D lysozyme nanocrystal have shown similar lattice differences across a single crystal.20 However, in that case, the changes were also potentially attributable to differences in defocus across the crystal or to changes in the level of dynamical scattering across non-uniformly thick areas of the crystal.20

Interpretation of our own images of frozen-hydrated peptide nanocrystals presents similar challenges (Supplementary Figure 12, 13). 4DSTEM allows a quantitative classification of lattice orientations within a crystal and can reveal more subtle changes than those identified in HRTEM images of similar crystals. This difference may point to a greater sensitivity to detection of lattice changes by 4DSTEM at even lower doses than those required for HRTEM.

The lattice tilts we identify may ultimately represent the result of various physical phenomena: they may be intrinsic properties of the crystals themselves, limited to only a subset of macromolecular crystals and affected by or result from sample preparation procedures. Current methods in cryoEM sample preparation, for example, may exert forces potent enough to distort crystal lattices and affect molecular structures.67 Lastly, by allowing nanoscale (20 nm) interrogation of lattice structure in macromolecular crystals, 4DSTEM offers a potential way to shrink the number of molecules required for structure determination by electron diffraction from single or serial crystal data.
Methods

Preparation of crystals. Crystals were grown as previously described. Briefly, synthetic peptide (GenScript) with sequence QYNQNNFV at a purity of 98% was dissolved in ultrapure water to a final concentration of 3.5 mM. QYNQNNFV crystals were formed in batch by mixing dissolved peptide solution 1:1 with a buffer solution composed of 10% MPD (RS)-2-methyl-2,4-pentanediol and 0.1 M MES (2-N-morpholinoethanesulfonic acid) at pH 6.0.

4DSTEM data collection strategy. Crystal suspensions were diluted two-fold in water or crystallization buffer before being dispensed onto holey carbon grids (Quantifoil 2/4, #300 copper; Ted Pella Inc.) and allowed to air dry. Samples were introduced into the TEAM I microscope (FEI Titan) with a Gatan 636 Cryo holder, and cooled to liquid nitrogen temperatures throughout all data acquisition. The TEAM I microscope was operated at 300 kV in STEM mode with a convergence half-angle of 0.5 mrad resulting in a ~6 nm convergent beam (Supplementary Figure 2).

Crystals of interest were identified using annular dark-field STEM at low magnification (Fig. 1a). For this, the electron fluorescence (dose, $e^{-}\mu m^{-2}$) was limited to a fluence of < 0.01 $e^{-}\mu m^{-2}$ to minimize diffusion decay due to radiation damage, starting with parameters identified for other beam-sensitive organic lattices. Imaging was performed by scanning a focused beam with a dwell time of 1 μs over a 512 x 512 scans across 14 x 14 μm fields of view, corresponding to an electron fluence < 0.01 $e^{-}\mu m^{-2}$. We chose single crystals or crystal bundles visible at low magnification in annular dark field STEM images for further evaluation by 4DSTEM (Fig. 1a-c).

4DSTEM data were collected by scanning the 6 nm probe over the sample in two-dimensional scan using a 2.5 ms per step dwell time and a 20 nm step size. The electroadic lens above the probe-forming aperture was adjusted such that the overall fluence per scan step was ~1 $e^{-}\mu m^{-2}$. Diffraction patterns were recorded using a Gatan K2 LS direct electron detector (GATAN), with a 1792 x 1920 pixel detection and an effective camera length of 575 mm (Fig. 1a). Diffraction in 4DSTEM patterns was minimally occluded by the angular dark field detector. Typical scans consisted of 1000 to 10,000 diffraction patterns captured at 400 frames s$^{-1}$; datasets were ~5–30 GB in size.

4DSTEM dose estimation. The total dose imparted per 4DSTEM scan step was estimated as follows: The convergent probe was imaged in TEM mode at high magnification using a Gatan US1000 CCD detector with a exposure time of 10 s and the same as that used for data acquisition (Supplementary Figure 2). Using a nominal conversion rate of 3 counts per $e^{-}\mu m^{-2}$ at 300 kV, we estimated the total number of electrons within the probe to be 1.5 x 10$^{9}$ e$^{-}$. This number calculated from the image of the probe agrees with the screen current estimate for a similar gun lens setting. The diameter of the probe at FWHM was measured to be 6 nm (Supplementary Figure 2) and, given an exposure time of 0.0025 s, the final dose per scan step was estimated as ~1 $e^{-}\mu m^{-2}$. The calculated dose above is different than that calculated from the field of view which would have been reported as an order of magnitude less due to the real space 'pixel size' of 15–20 nm.

Image processing and hybrid counting of 4DSTEM data. All data processing was performed using custom scripts written in the MATLAB (MathWorks) programming language. Patterns belonging to a single area scan were jointly processed using 10 micrographs where crystalline ice was present using the program MotionCorr271. Initial estimates of anisotropic magnification (Fig. 1a, inset) were made using 10 micrographs where crystalline ice was present using the program mag-distortion-estimate22. These parameters were used to correct for anisotropic magnification in MotionCorr22. Frames were subsequently aligned without using patches, dose-weighted, and down-sampled by two.

Correction of diffraction shift in 4DSTEM scans. The horizontal and vertical shift of diffraction patterns induced by beam tilt during these relatively low magnification scans was corrected by tracking the centre of mass of the transmitted beam. As the signal to noise in a single pattern was not sufficient to calculate this accurately, we separated the problem into two steps, independently correcting the x- and y-scan and then applying the overall correction.

Lattice mapping in 4DSTEM and HRTEM. Before lattice mapping individual diffraction patterns were binned 8-fold to increase their SNR. The central beam was then masked out to remove the influence of transmission from the lattice mapping; we noticed that without this step the lattice maps produced matched variations in the thickness of the crystal too closely. We used $k$-means clustering to sort diffraction patterns from a single 4DSTEM dataset into different clusters based on their Euclidian distance from the average pattern within a particular cluster:

$$\arg\min_{\mu} \sum_{i=1}^{K} \sum_{x \neq \mu} ||x - \mu||^{2},$$

where $K$ is the number of clusters determined using an implementation of the $G$-means algorithm with $a = 0.001$23. $S$ is an individual cluster, $\mu_{S}$ is the average pattern of all within the cluster $S$, and $x$ is an individual diffraction pattern within the 4DSTEM dataset. The $k$-means++ algorithm was used to initialize $\mu_{S}$ for each cluster24. The algorithm was stopped when either 100 iterations were performed or when the within-cluster sum of squares stagnated. Clusters where the beam passed over vacuum showed little signal when the primary beam was excluded, preventing distances between patterns in this cluster from showing meaningful difference. These distances were therefore removed from the distance matrix by setting all pairwise distances to a distance of 1 to the closest cluster. To circumvent this, we included a break in our implementation of $G$-means that stopped assignment of clusters once at least 80% of the assigned clusters were
found to be Gaussian by the Anderson–Darling statistic. This substantially improved convergence of the algorithm.

For real space images collected by HRTEM, a similar procedure was followed with the following modifications. First 128 × 128 sub-images were cropped from the larger HRTEM images with no overlapping regions. We estimated K by the elbow method\(^23\), which we found to be more stable than the Anderson–Darling statistic for the noising imagery data. The clustering step was applied as above, with the inclusion of a 5 × 5 pixel-wise search to account for potential misalignments between the two images under consideration.

**Indexing of lattices in 4DSTEM cluster averages.** To assess the underlying lattice reorganization that could account for the changes in diffraction observed from the clustering, we indexed the cluster averages via library matching\(^3\) (Supplementary Figure 11). A library of nano-beam electron diffraction (NBED) patterns were simulated with PRISM\(^76\) (using the known crystal structure of QYNNQNVF. We simulated expected NBED patterns arising from a 40 × 40 nm region of a perfect crystallography and the largest HRTEM images with no overlapping regions. We estimated K by the elbow method\(^23\), which we found to be more stable than the Anderson–Darling statistic for the noising imagery data. The clustering step was applied as above, with the inclusion of a 5 × 5 pixel-wise search to account for potential misalignments between the two images under consideration.

To match the experimental patterns to the simulated library, a similar procedure was followed for real space images collected by HRTEM. For each class average the Fourier transform was computed to Erickson and Klug\(^77\). For each class average the lattice orientation can be found at: https://github.com/marcusgj13/4DSTEM_dataAnalysis.

**Data availability.** The MATLAB scripts for data pre-processing, clustering, and orientation assignment can be found at: https://github.com/marcusgj13/4DSTEM_dataAnalysis.

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J.A.R and M.G.-J. conceived the project. J.A.R directed the work. C.G., M.G.-J. and J.A.R. collected data. M.G.-J., C.O., K.C.B., K.C.M., D.R.B and J.A.R. analysed the data. M.G.-J. and J.A.R. wrote the Article, with input from all authors.

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