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Snapshots of internal protein crystal architecture at the nanoscale

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ABSTRACT Macromolecular crystallography has historically inferred models of internal crystal architecture from reciprocal-space measurements of Bragg reflections. Nevertheless, direct real-space visualization of crystallographic disorder remains elusive, particularly at the nanoscale. Using a 15-nm probe, here we apply both ambient-temperature and cryogenic four-dimensional scanning transmission electron microscopy (4D-STEM) to map the topography of coherently diffracting domains (CDDs) in lysozyme and myoglobin microcrystals at length scales 100× finer than conventional X-ray and electron beams. Virtual dark-field images show that each Bragg peak arises from spatially distinct subvolumes representing smooth and continuous variations in local lattice orientation. Under sustained irradiation, protein CDDs undergo rearrangements spanning several micrometers of internal movement. Furthermore, pinpoint high-dose “impact crater” experiments reveal delocalized radiolytic damage propagating hundreds of nanometers from primary irradiation sites, behavior similar to small-molecule crystals but amplified in both rate and magnitude. Together, these results establish macromolecular microcrystals as dynamic assemblies whose internal architecture continuously reorganizes during irradiation, laying the foundation for realistic models of mosaicity directly informed by both real-space and reciprocal-space observations.

SIGNIFICANCE Protein crystallography forms the bedrock of modern structural biology. Nevertheless, our understanding of macromolecular crystal architecture has relied principally on diffraction-space analysis, leaving the real-space story underdeveloped. Here we apply scanning nanobeam electron diffraction with a fast camera to peer inside protein crystals at nanoscale resolution. Our approach unveils a hidden topography of imperfections invisible to the micron-scale probes typically used in X-ray crystallography. We also visualize the effects of electron-beam-induced radiolytic damage cascading through the lattice, with damage fronts propagating hundreds of nanometers from the point of incidence. These results reshape our understanding of macromolecular nanocrystals as conglomerates of coherently diffracting domains whose complex internal architecture undergoes a series of dynamic internal changes during exposure to ionizing radiation.

INTRODUCTION

X-ray crystallography has uniquely defined the inception and trajectory of structural biology,¹ delivering the first landmark structures of macromolecules such as proteins² and nucleic acids.³ Successful macromolecular structure determination depends on crystal quality, particularly on the degree of long-range order that governs diffraction to

higher Bragg angles. Nevertheless, macromolecular crystals typically contain significant imperfections that limit their diffracting power, often thwarting efforts aimed at high-resolution structure elucidation.⁴

Mapping the exact spatial distribution of these imperfections has historically proven challenging. Micron-scale X-ray beams⁵ average signal over vast numbers of unit cells, obscuring nanoscale heterogeneities. This limitation has spurred X-ray crystallographers to infer the presence of defects in real-space crystal architecture by analyzing reciprocal-space measurements of Bragg reflections.⁴ Specifically, the classical model of mosaicity^{6,7} conceptualizes crystals as patchworks of misaligned “mosaic blocks”

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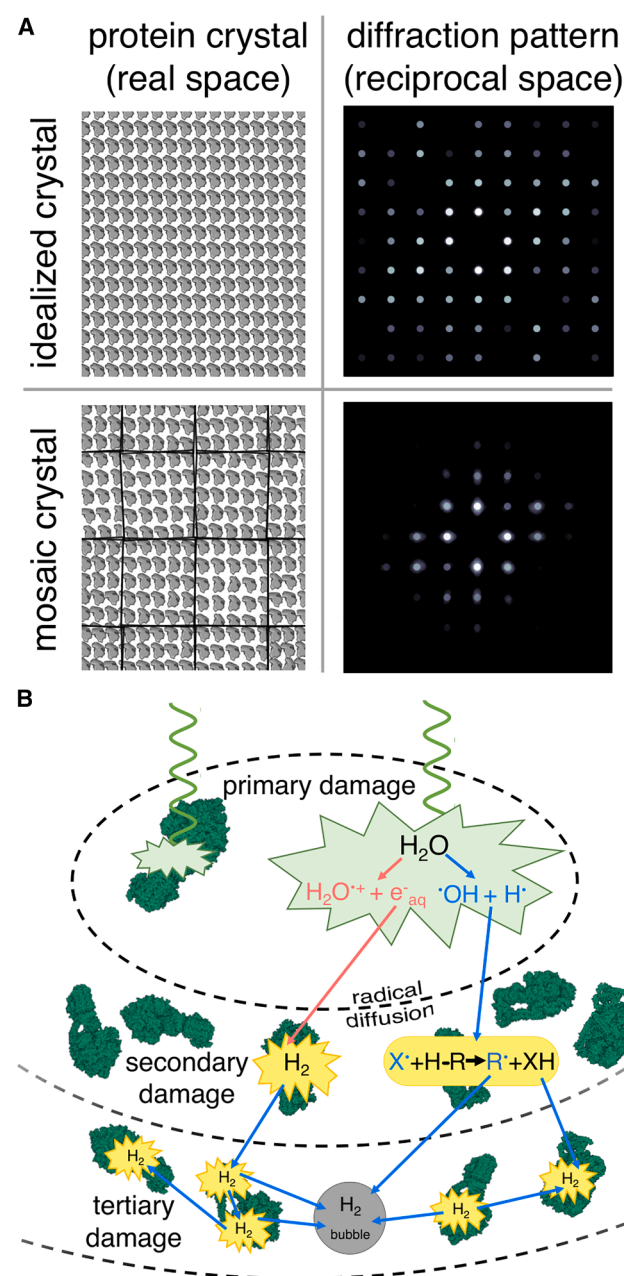


Figure 1. Models of mosaicity and radiation damage. (A) Schematic representations of an idealized, monolithic crystal (top row) and an imperfect, mosaic crystal (bottom row) alongside corresponding diffraction patterns (right column) simulated by the Fourier transform of the images on the left. The mosaic crystal contains several angularly misoriented subregions, introducing peak broadening effects in the Fourier transform. Molecule-to-molecule variations in packing and conformation between adjacent unit cells, represented as slight rotational jitter, also contribute to disorder. (B) Schematic representation of electron-beam-induced radiolytic damage, where incident quanta (green wave) scatter inelastically within the specimen (the primary inelastic collision, represented by the green polygon), resulting in ionization, electronic excitation, secondary electron emission, and subsequent radiolytic cleavage of covalent bonds into free radicals. These reactive radiolytic fragments diffuse away from the parent bond and inflict cascades of secondary damage via downstream radical chemistry (yellow), breaking distal bonds further removed from the

whose angular misorientation in real space produces peak broadening effects^{8,9} in reciprocal space (Figure 1A). However, typical structure-determination experiments cannot directly image the size, shape, and distribution of these putative mosaic blocks, and mosaicity makes no attempt to model the boundaries or discontinuities separating miniature crystallites.¹⁰ In other words, a real-space perspective is lacking. Consequently, reciprocal-space signatures such as distorted Bragg peak profiles have provided the major window into crystal imperfections, leaving our understanding of the underlying organization of nanoscale coherently diffracting domains (CDDs) underdeveloped.

Perfect, monolithic crystals yield identical diffraction patterns regardless of where they were sampled in real space, producing sharp, point-like Bragg peaks (Figure 1A). Conversely, imperfect macromolecular crystals exhibit spatial variations in diffracting power and orientation that originate from smooth, continuous irregularities in the underlying lattice structure.¹¹ Crucially, these domains do not remain static under irradiation, as electron- or X-ray-beam-induced radiolytic damage continually degrades long-range order during data collection—thus broadening Bragg peaks and increasing mosaicity.^{12,13} Radiolysis is initiated via core- or valence-shell ionization events that break covalent bonds, generating low-energy secondary electrons and mobile free radicals.^{14–17} These reactive species proceed to travel through the specimen once formed (Figure 1B), inflicting more damage through cascades of secondary and tertiary reactions.^{18–21} While many conventional measurements have documented this deterioration by monitoring the fading or broadening of diffraction spots, the corresponding real-space evolution of macromolecular crystal architecture during radiolysis is poorly understood. Furthermore, the fundamental length scale at which Bragg diffraction transitions from monolithic to mosaic—effectively, the coherence length of individual mosaic blocks in protein microcrystals—remains inaccessible to measurement by broad-beam methods.

Here we employ four-dimensional scanning transmission electron microscopy (4D-STEM; Figure 2A) to bridge these gaps.^{22–24} By acquiring independent diffraction patterns at an array of probe positions separated by nanometer-scale steps, 4D-STEM generates dual-space data sets that simultaneously encode real- and reciprocal-space information at length scales matching expected mosaic block dimensions.⁹ We build on previous studies conducted using synchrotron X-ray topography^{10,25} and dark-field transmission electron microscopy (TEM) imaging,²⁶ both of which have yielded valuable insights into macromolecular crystal architecture. Our approach is differentiated from prior X-ray work by the significantly smaller probe size achievable using

point of origin. The accumulation of many such events leads to global decay of structural integrity (tertiary damage). A visible symptom of tertiary damage is the formation of H_2 gas bubbles.

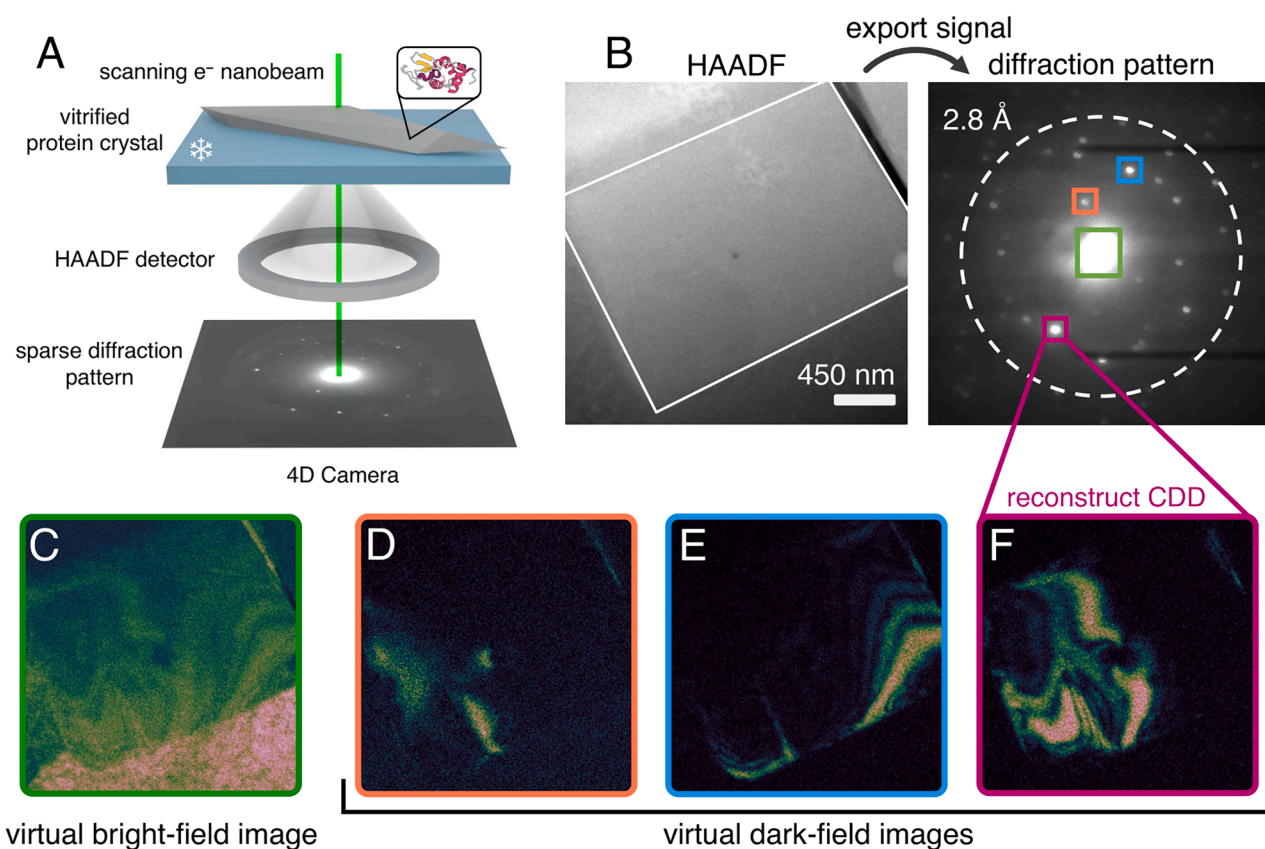


Figure 2. 4D-STEM data collection and processing. (A) Schematic of a standard cryo-4D-STEM experiment, with a scanning electron nanobeam impinging on a vitrified protein microcrystal. A separate high-angle annular dark-field (HAADF-STEM) image is acquired on a monolithic detector, whereas coherent Bragg signal is acquired on the 4D camera operating at 87,000 frames per second. (B) Workflow for generating a selected-area diffraction pattern by masking a specific subregion (white border; virtual selected-area aperture) of a simultaneously acquired HAADF-STEM image. (C) Virtual bright-field image reconstructed by masking the unscattered beam, marked by the green square (virtual objective aperture) in (B). (D–F) Virtual dark-field images reconstructed by masking specific Bragg reflections, marked by the orange (D), blue (E), and magenta (F) squares in (B), respectively.

STEM, enabling spatial resolution $100\times$ finer than comparable X-ray topographs. Furthermore, 4D-STEM is distinguished from prior electron microscopy work by its ability to reconstruct both dark-field images and selected-area diffraction patterns (Figure 2B) using custom-shaped virtual apertures.²⁷ In contrast, conventional TEM relies on manually maneuvering fixed physical apertures to chase rapidly decaying Bragg signal during the experiment itself. Under fixed-orientation, near-parallel-beam conditions, 4D-STEM permits direct visualization of all nanoscale regions that satisfy the Bragg condition within the acceptance range defined by the convergence angle (0.07 mrad) of the probe (Figures 2C–2F).

Using a custom-built 87-kHz frame-rate direct electron detector,²⁸ here we apply both ambient-temperature and cryogenic 4D-STEM to visualize the internal architecture of microcrystals formed by the model proteins lysozyme and myoglobin. We find that individual Bragg reflections arise from spatially distinct, often nonoverlapping CDDs. Subdividing these CDDs into smaller nanoscale sections produces no significant changes in the anisotropy of Bragg

peak profiles. Moreover, under sustained electron irradiation, these domains undergo rearrangements spanning hundreds of nanometers. Through pinpoint high-dose “impact crater” experiments,²³ we also visualize propagating fronts of amorphization extending several hundred nanometers from initial irradiation sites. These results demonstrate that secondary radiolytic processes produce delocalized damage over length scales relevant to macromolecular electron crystallography and single-particle cryogenic electron microscopy (cryoEM).

MATERIALS AND METHODS

Materials

Hen egg white lysozyme and equine heart myoglobin were purchased from Sigma-Aldrich and used without further purification. Stock solutions of pH 4.5 sodium acetate trihydrate (1.0 M; Hampton Research HR2-789), pH 7.0 sodium acetate trihydrate (4.0 M; Hampton Research HR2-763), sodium nitrate (7.0 M; Hampton Research HR2-661), and

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ammonium sulfate (3.5 M; Hampton Research HR2-541) were diluted with Milli-Q water to the desired molarity. Sterile 2.0-mm zirconium oxide beads were purchased from Next Advance.

Crystallization of lysozyme seeds

Crystallization procedures were adapted from literature precedent.^{29,30} Hen egg white lysozyme was dissolved in an aqueous solution of 0.05 M sodium acetate trihydrate (pH 4.5) to a concentration of 20 mg mL⁻¹ in a 1.0-mL Eppendorf tube. The protein solution was mixed in a 1:1 ratio with a precipitant solution containing 0.4 M sodium nitrate and 0.05 M sodium acetate trihydrate (pH 4.5). The mixture was incubated at 4°C for 1 day, after which an opaque suspension became visible. The tube was then allowed to rest at RT for 1 week, after which large, colorless crystals were observed at the bottom.

Crystallization of myoglobin seeds

The crystallization procedure reported by Sherwood et al.³¹ was followed without modification. Equine heart myoglobin was dissolved in an aqueous solution of 3.0 M ammonium sulfate and 0.1 M sodium acetate (pH 7.0) to a concentration of 8 mg mL⁻¹ in a 1.0-mL Eppendorf tube. The tube was left undisturbed at RT for 1 week, after which reddish-brown crystals were observed.

Crystallization of microcrystals

The rapid mixing crystallization (RaMiC) protocol developed by Hofer et al. was adapted.^{32,33} Seed crystals were transferred into a tube containing 5–6 zirconium beads and vortexed in short bursts. In another tube, the solution was mixed at a 2:1:1 ratio with solutions containing modified concentrations of protein and precipitant. For lysozyme, the concentrations of protein and precipitant were doubled to 40 mg mL⁻¹ and 0.8 M sodium nitrate, respectively. For myoglobin, the precipitant concentration was tripled while the protein concentration remained unchanged. The mixture was vortexed in bursts to promote secondary nucleation through crystal fragmentation, resulting in a dense suspension of microcrystals. Crystals of all sizes were visible when browsing the grid at low magnification in the TEM. Data were collected on crystals that were thin enough to allow for a visible diffraction pattern, $\lesssim 250$ nm.

Grid preparation

The procedure described by Hofer et al.^{32,33} was adapted. Quantifoil holey carbon grids were dipped in 1% Tween-20 (Sigma-Aldrich) and then blotted from the metallic side until visibly dry to make the grid hydrophilic. Then, 3 μ L of crystal slurry was deposited onto the grid, which

was then blotted again from the metallic side until visibly dry. For vitrified samples, the grid was then hand-plunged into liquid ethane and then cryo-transferred into the STEM.

4D-STEM data collection

All 4D-STEM experiments were conducted at an accelerating voltage of 300 kV on the double-aberration-corrected TEAM 0.5 instrument located in the National Center for Electron Microscopy at Lawrence Berkeley National Laboratory. Before data acquisition, the incident beam current was reduced to the detection threshold of the fluorescent screen ammeter (<40 pA) by using the monochromator focus as a continuously adjustable gun lens. Beam flux was estimated from previous reference measurements using a Faraday cup and an ammeter under similar conditions. A near-parallel probe was formed by using a custom 10 μ m C2 aperture (Norcada) to implement a narrow semiconvergence angle of 0.07 mrad. This produces a beam profile with an approximately 15 nm FWHM. Using a custom DigitalMicrograph script, multiscan 4D-STEM data were acquired with the 4D camera operating at 87-kHz frame rate. Each scan is composed of 512 $\times$ 512 probe positions over a 2.3- μ m field-of-view with a probe step size of 4.6 nm.

RESULTS AND DISCUSSION

Protein crystals consist of coherently diffracting domains resembling bend contours

We first conducted a single, low-fluence (0.6 $e^- \text{\AA}^{-2}$), parallel-beam 4D-STEM scan at 85 K on vitrified microcrystals of triclinic lysozyme and monoclinic myoglobin, using a 15-nm full width at half maximum (FWHM) probe. In both cases, we found that each Bragg reflection originates from a smaller, distinct fraction of the overall coherently diffracting volume (Figure 2). For instance, placing virtual apertures around individual Bragg reflections in lysozyme reveals mostly nonoverlapping, roughly parallel CDDs (Figures 3C–3E and 3H–3J). Follow-up experiments on nearby lysozyme crystals replicated this behavior (Figure S1). Myoglobin, by comparison, exhibits arc-like CDDs thinner and more heterogeneous in shape (Figures 3M–3O and 3R–3T), forming an interlocking pattern. To visualize the contributions from all subregions producing coherent Bragg diffraction at this orientation, we summed the signal from all selected Bragg peaks to create a composite virtual dark-field (vDF) image (Figures 3G, 3Q, and S2). We found that the composite vDF image in lysozyme revealed a more contiguous diffracting volume (Figure 3G), whereas in myoglobin the boundaries of individual CDDs remained discernible (Figure 3Q).

Another consistent feature we observed was the spatial proximity between CDDs corresponding to Friedel mates. As shown in Figure S3, five pairs of Bragg reflections

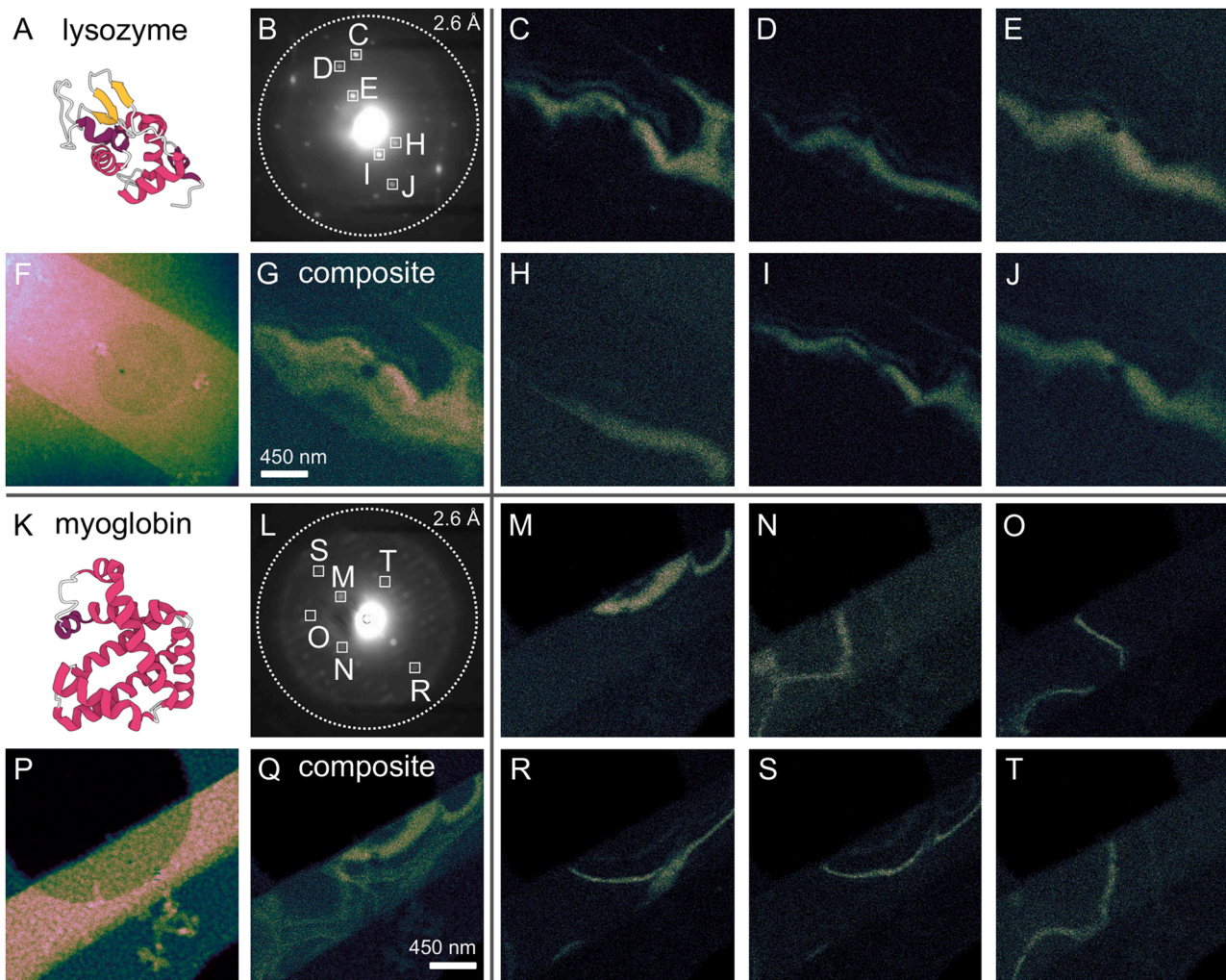


Figure 3. Anatomy of internal protein crystal architecture at the nanoscale. (A and K) Ribbon diagrams of lysozyme and myoglobin, respectively. (F and P) HAADF-STEM images of triclinic lysozyme and monoclinic myoglobin microcrystals, respectively, each overlaying a holey carbon support. These images were acquired simultaneously alongside each 4D-STEM scan. (B and L) Selected-area diffraction patterns generated by creating real-space virtual apertures via thresholding the HAADF-STEM images shown in (F) and (P). White squares correspond to reciprocal-space virtual apertures enclosing specific Bragg reflections. (C–E and H–J) Virtual dark-field images reconstructed from the reciprocal-space virtual apertures in (B). (M–O, and R–T) Virtual dark-field images reconstructed from the reciprocal-space virtual apertures in (L). (G and Q) Composite virtual dark-field images summing the Bragg signal in (C)–(E) and (H)–(J) and (M)–(O) and (R)–(T), respectively.

activated CDDs very close to their symmetry-equivalent counterparts, with several exhibiting partial overlap. In the solid-state TEM literature, similar behavior has been attributed to bend contours.³⁴ Although the origin of these imperfections is unclear, both intrinsic and extrinsic forces could play some role. Intrinsic factors include defects “baked in” during crystal growth—e.g., due to local variations in supersaturation, salt concentration, or pH²⁵—whereas extrinsic factors include *ex post facto* deformations introduced by vortex-assisted fragmentation with metal beads,³² plunge-freezing,³⁵ surface tension, or mechanical stress exerted by contact with the grid support.³⁶

Each CDD’s apparent size is filtered through the angular acceptance of the probe (0.07 mrad or 0.004°). In principle, increasing the semiconvergence angle would relax this

fairly stringent Bragg-condition tolerance, allowing more locally misoriented regions to contribute to the selected reflection. To test this, we acquired convergent-beam 4D-STEM data under otherwise identical irradiation conditions, using a wider semiconvergence angle (0.57 mrad or 0.03°) (Figure S4). Surprisingly, the CDDs reconstructed from the 0.57 mrad data set exhibited comparable lateral dimensions to some of our parallel-beam scans and did not obviously broaden or merge relative to the 0.07 mrad condition. This suggests that the “genuine” mosaic spread of these microcrystals—i.e., the floor at which the wandering of the local lattice orientation matches the beam divergence—is closer to the parallel-beam condition than the convergent-beam condition, consistent with previous rocking-curve measurements of mosaicity in lysozyme.^{25,37,38}

Within these constraints, our goal became to distinguish between two scenarios: a single, continuously monolithic domain versus a collection of smaller, misaligned mosaic blocks whose superposition produces the observed CDD. If the latter were true, subdividing the CDD should yield progressively sharper Bragg peaks as fewer misoriented blocks contribute to each subregion.^{6,9,25} This is related to the distinction between “macroscopic” and “microscopic” mosaicity discussed in Malkin and Thorne¹¹ and Dobrianov et al.²⁵ To probe internal disorder *within* the reconstructed CDDs, we selected a single low-resolution reflection in both lysozyme and myoglobin. We partitioned each CDD into 16 equally sized subregions (Figures S5A and S5D), each normalized to have the same total diffracting power. With these masks in hand, we fit 2D Gaussian functions to the Bragg reflections arising from each subregion to extract their centroids and standard deviations (σ ; see [supplemental information](#)). We compared these values to their counterparts obtained from the undivided CDD (Figures S5B, S5C, S5E, and S5F).

The measured σ and centroid values remained similar across all 16 subregions, closely matching the value obtained from the full CDD (just under 0.1 mrad; Figures S5C and S5F). This suggests that further subdivision does not isolate smaller misoriented constituents within the angular sensitivity of the experiment. Instead, each CDD reconstructed by placing a virtual aperture around a single Bragg reflection behaves as a monolithic unit. In sum, the observed CDDs match the description of “macroscopic” mosaicity presented in Fig. 13 of Dobrianov et al.,²⁵ where the dimensions of the crystal roughly match the characteristic length scale at which the local lattice orientation fluctuates. Furthermore, the individual Bragg peaks in Figure S5 show no significant variations in ellipticity or anisotropy as a function of where the CDD is sampled. This suggests comparable levels of “microscopic” mosaicity^{11,25}—i.e., disorder due to cell-to-cell variations in molecular conformation and packing—within the subdivided domains. We were unable to estimate peak broadening perpendicular to the Ewald sphere surface (e.g., using classical rocking-curve analysis) because TEM goniometer imprecision and rapid radiolytic damage preclude such fine-slicing measurements in 4D-STEM.

Radiolysis causes internal rearrangement of CDDs in protein crystals

As the diffracting power of a crystal fades, so do the intensities of Bragg reflections. In the aggregate—i.e., the sum of all diffracted intensities—this pattern is generally monotonic. Nevertheless, in some cases, the intensities of individual Bragg peaks can decay nonmonotonically, as observed in synchrotron X-ray studies of protein crystals.^{13,39} In previous 4D-STEM work on small-molecule crystals, we showed that vDF movies reconstructed from these reflections reveal CDDs rearranging into morphologies that temporarily better fulfill the Bragg condition *en route* to amorphization.²³

We set out to probe whether crystals of these two model proteins exhibited similar behavior. Unlike small-molecule crystals, a substantial fraction of the scattering volume of macromolecular crystals consists of disordered solvent,⁴⁰ typically ranging between 40% and 60%. Furthermore, under vitrified conditions, each crystal is embedded in glassy ice. We hypothesized that both internal solvent channels and the surrounding vitreous ice matrix could act as radiosensitizers, amplifying the effects of radiolysis by serving as an added reservoir of reactive free radicals (particularly $H\cdot$). Another salient difference is the looser packing intrinsic to macromolecular self-assembly. To test these hypotheses, we acquired a time series of consecutive 4D-STEM scans on triclinic lysozyme under vitrified conditions at 85 K.

We found that vDF movies reconstructed from nearly any Bragg reflection showed consistent behavior: radiolysis causes CDDs to undergo internal rearrangement. As irradiation proceeds, radiolytic damage continually changes the local orientation and strain field of the crystal lattice. Whenever this variation exceeds the angular sensitivity set by the semiconvergence angle of the probe (0.07 mrad), the vDF contrast associated with a selected Bragg peak relocates accordingly. When viewed from a fixed vantage point, this manifests as the apparent movement of the CDD across the body of the crystal (Figure 4). Although this does not connote literal translation of a physical domain, we use “movement” as an intuitive metaphor to describe how different subregions satisfy the selected Bragg condition as lattice disorder evolves, particularly because each CDD tends to maintain a specific shape and morphology throughout its lifetime. Importantly, this reconfiguration is internal—concurrently acquired high-angle annular dark-field (HAADF-STEM) images (Figure S6) confirm that there is no accompanying gross translation or rotation of the specimen. In Figures 4D and 4E, for instance, the two CDDs travel approximately 500 nm between consecutive scans as their corresponding Bragg reflections decay monotonically. In parallel, the trajectories followed by other CDDs involve marked expansion in their diffracting volumes, leading to nonmonotonic decay patterns (Figures 4F and 4G). In these cases, we see fleeting surges in Bragg peak intensity, similar to behavior exhibited by the solvent-free small-molecule crystals biotin and $Ni(dppf)Cl_2$.²³

The principal distinction, however, lies in the magnitude of the CDD rearrangements observed. For instance, the trajectories of CDD decay in biotin²³ involved comparatively minimal perturbation to initial shape and morphology. In comparison, lysozyme CDDs traveled nearly double that distance between consecutive scans. This discrepancy is striking given that the 4D-STEM experiments in Figure 4 were acquired at cryogenic temperatures, whereas our previous work on biotin was conducted at room temperature (RT). Furthermore, lysozyme and biotin share roughly the same elemental composition, indicating behavior not reflected by traditional metrics such as inelastic cross-sections. In

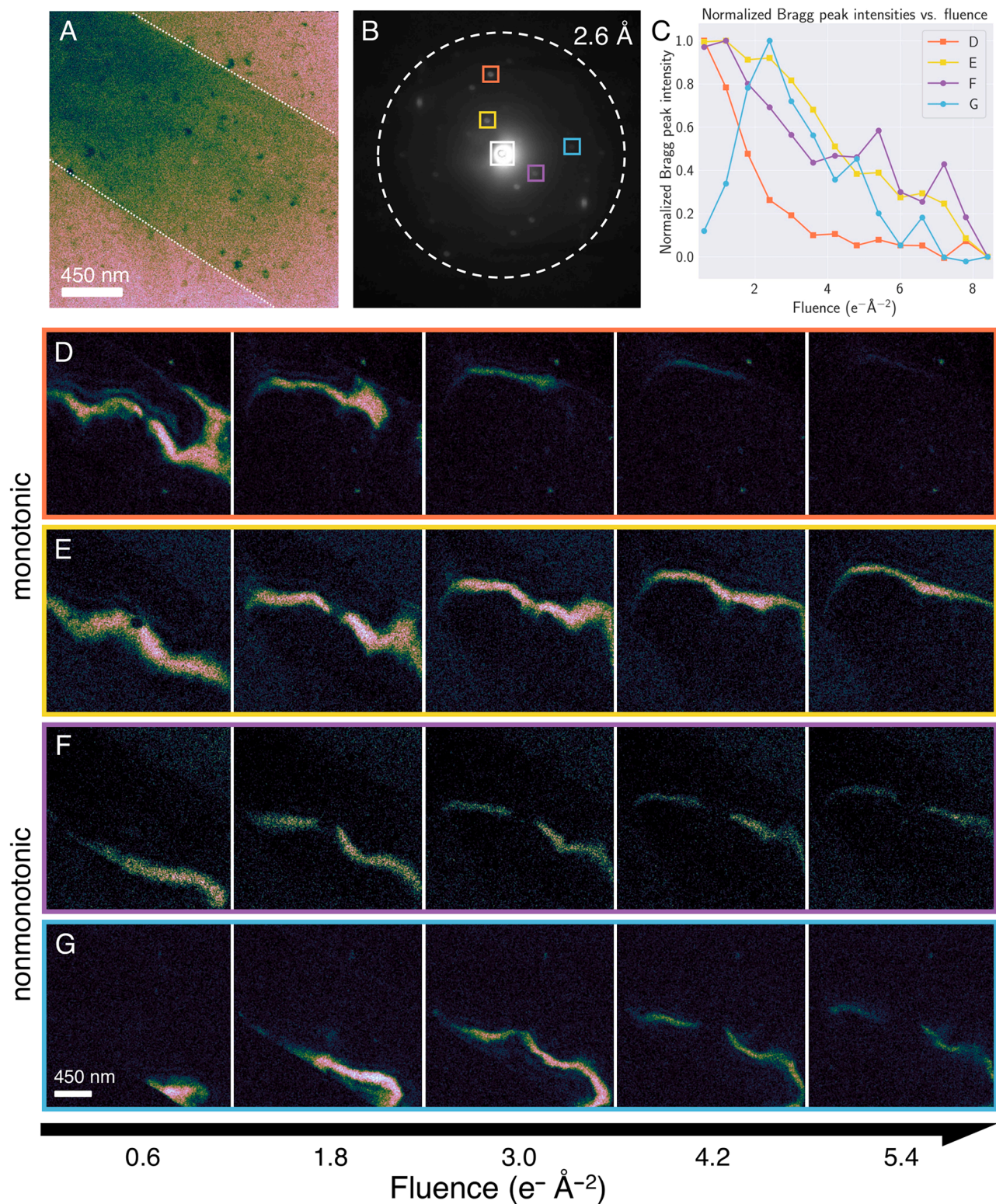


Figure 4. Comparison between virtual dark-field images reconstructed from monotonically and nonmonotonically decaying reflections in vitrified lysozyme at 85 K. (A) Virtual bright-field image of a vitrified lysozyme crystal, reconstructed from the unscattered beam (white box in B). (B) Selected-area diffraction pattern exported from the region outlined in white in (A), showing Bragg reflections extending to 2.6-Å resolution (dashed circle). Colored boxes indicate virtual reciprocal-space apertures used for tracking intensity decay: monotonic (orange and yellow) and nonmonotonic (purple and blue). (C) Intensities of selected Bragg reflections plotted as a function of accumulating electron fluence, normalized by maximum value. (D and E) Montage of virtual dark-field images reconstructed from the monotonically decaying Bragg reflections marked by the orange and yellow apertures in (B). (F and G) Montage of virtual dark-field images reconstructed from the nonmonotonically decaying Bragg reflections marked by the purple and blue apertures in (B).

addition to solvent-driven radiosensitization, another possible cause lies in the fundamental mechanical properties of the two systems. Hydrated macromolecular crystals possess elastic moduli around $8\times$ lower than their small-molecule counterparts, largely due to weaker intermolecular bonding and disordered solvent content.⁴¹ These intrinsic differences may confer greater flexibility enabling larger-scale CDD rearrangements.

Tracking distant secondary damage from spatially nonuniform irradiation

Repeated electron irradiation at a single probe position produces expanding fronts of amorphization extending hundreds of nanometers, previously referred to as “impact craters.”²³ These phenomena represent cascades of secondary radiolytic damage that propagate outward from primary ionization sites. We sought to determine whether protein crystals—species much more radiosensitive than the organometallic complexes used in our initial demonstration—exhibit analogous behavior, and whether this sensitivity accelerates crater emergence and expansion. Although macromolecular crystals also decay via radiolysis, the length scales over which these cascades travel in beam-sensitive biological materials remain unexplored. Furthermore, their behavior as a function of temperature is poorly understood.

To generate the impact crater, we placed the 15-nm probe at the center of a lysozyme crystal and irradiated this dwell point for approximately 200 ms in between consecutive 4D-STEM scans²³ at an incident fluence of $0.4\text{ e}^{-}\text{\AA}^{-2}$. Our first foray was conducted at RT. We immediately observed significant delocalization of radiolytic damage, with an impact

crater 150 nm in diameter—already $10\times$ bigger than the FWHM of the probe—visible after a single experiment (Figures 5A and S7). Diffraction patterns exported from flanking subregions (Figure 5B) confirmed that Bragg signal was still robust outside the crater (Figures 5C and E), whereas Bragg reflections within the crater were extinguished (Figure 5D).

Interestingly, this behavior was replicated under vitrified conditions at 85 K, where a slightly smaller 140-nm crater emerged after a single scan ($0.6\text{ e}^{-}\text{\AA}^{-2}$; Figures 5F–5J). This result was unexpected, since the highly reactive hydroxyl radical ($\cdot\text{OH}$)—long considered a major proponent of radiolytic damage at RT^{42,43}—is considered immobilized below 100 K.⁴⁴ Therefore, we initially anticipated little to no crater formation under cryogenic conditions. The first wave of bond cleavage and radical formation is initiated by low-energy secondary electrons (SEs) generated by the primary inelastic event.^{45,46} However, low-energy SEs associated with plasmon losses in TEM (typically 25 eV or lower in energy) have very short mean free paths ($<5\text{ nm}$) smaller than the FWHM of the probe.^{47,48} These circumstances differentiate TEM from related precedent in the X-ray literature, where high-energy keV-scale photoelectrons can deposit energy over significantly longer micron-scale distances.²⁰

Since the crater dimensions observed here (140 nm) exceed the floor set by SE action alone, our results suggest that the next step in the cascade—i.e., the generation of multiplying free radicals¹⁶—plays a consequential role in propagating damage farther away from the dwell point. The existence of the crater at both RT and 85 K also implicates a radical species that can cause delocalized damage at both temperatures. One plausible candidate is the hydrogen

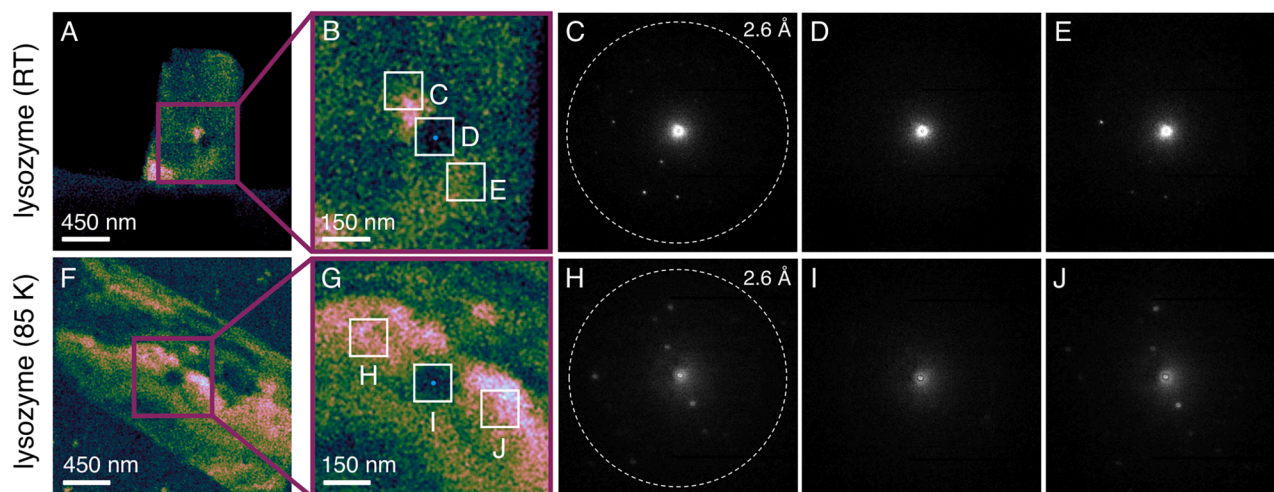


Figure 5. Zoomed-in view of the loss of diffraction signal within the expanding impact crater. (A and F) Composite virtual dark-field images reconstructed from all discernible Bragg reflections. (B and G) Zoomed-in view of the impact crater, with one real-space virtual aperture (D and I) placed directly over the epicenter (cyan dot) and two flanking apertures located approximately 150 nm (C and E) and 200 nm (H and J) away, respectively. The cyan dot represents both the point of incidence and the size of the probe (15 nm at FWHM). Diffraction patterns (C and E, H and J) show the corresponding Bragg signal exported from the labeled regions. In both cases, all Bragg reflections have been extinguished inside the burgeoning crater (D and I), but several remain observable outside its reach (C and E, H and J).

atom ($\text{H}\cdot$), a well-known by-product of radiolysis.^{16,49,50} In particular, H_2 gas formation via both X-ray and electron beam-induced radiolysis is well documented in frozen-hydrated specimens at cryogenic temperatures, driven by $\text{H}\cdot$ intermediates whose recombination or abstraction chemistry yields H_2 .^{49,51,52} Furthermore, unlike $\cdot\text{OH}$, the smaller $\text{H}\cdot$ faces minimal steric barriers to diffusion. Future vibrational electron energy-loss spectroscopy experiments will test this hypothesis by spectroscopically tracking radiolytic products as a function of dose and temperature. Finally, another plausible contributor to crater expansion is mechanical: since radiolytic cleavage of chemical bonds within the irradiated area changes the volume occupied by the damaged molecules, the resultant strain could disrupt the bordering regions.

We then attempted to map the expansion of the nascent impact crater by acquiring consecutive 4D-STEM scans, repeating our pinpoint-irradiation protocol between experiments. Unfortunately, tracking crater growth frame-by-frame was complicated by the CDD rearrangements described earlier. To image the crater accurately, active CDDs should ideally continue to surround the point of incidence during decay. However, the underlying mosaic often shifted significantly before we initiated the next scan, preventing consis-

tent visualization. Moreover, unlike in $\text{Ni}(\text{dppf})\text{Cl}_2$,²³ vDF images reconstructed from single Bragg reflections in lysozyme typically did not contain enough signal to visualize the crater, and several reflections fluctuated in and out of the Bragg condition. This necessitated the use of composite vDF images to monitor crater growth.

Despite these setbacks, we ultimately collected two dose series—one at RT and one under vitrified conditions at 85 K—in which CDDs coincided with the crater site long enough to observe some evolution (Figure 6). Interestingly, we found that crater expansion did show some temperature dependence, plausibly reflecting the reduced propagation of damaging free radicals at 85 K. At RT, the burgeoning crater doubles in size from 150 nm to 300 nm between the first two frames, whereas the 85-K crater grows more slowly from 140 nm to 200 nm in diameter (Figure S7). Unfortunately, later scans were difficult to interpret due to the aggressive rate of decay and accompanying CDD rearrangement, particularly at RT. Global diffraction decay was otherwise unremarkable, with Bragg reflections at 85 K lasting approximately $2.5\times$ longer than at RT as expected (Figure S8).

We could, however, draw a straightforward comparison with previous experiments on the organometallic complex

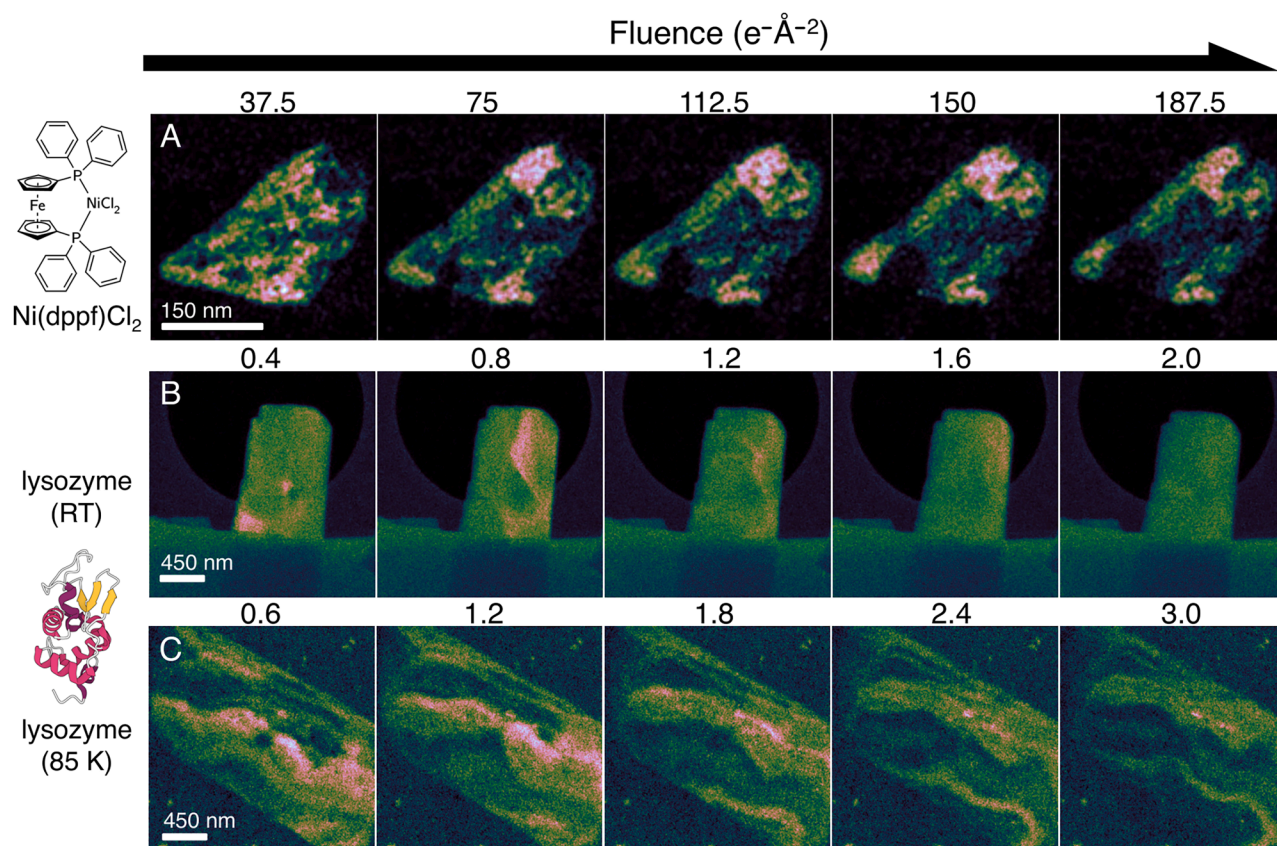


Figure 6. Comparison of impact crater expansion in $\text{Ni}(\text{dppf})\text{Cl}_2$, RT lysozyme, and vitrified lysozyme. (A) Montage of virtual dark-field images reconstructed from a single 4.5-Å reflection in $\text{Ni}(\text{dppf})\text{Cl}_2$ (data taken from Saha et al.²³), (B) RT lysozyme, and (C) vitrified lysozyme at 85 K. Both lysozyme series consist of composite virtual dark-field images reconstructed from all visible Bragg reflections.

Ni(dppf)Cl₂. Ni(dppf)Cl₂ required 187 e[−] Å^{−2} at 200 kV to produce a 75-nm crater—approximately half the size of the crater seen in [Figures 6B, 6C, and S7](#). This disparity suggests that each primary inelastic scattering event in lysozyme produces substantially more secondary radiolytic damage. In radiation chemistry terms, this result is consistent with an elevated *G*-value⁵³ in lysozyme; i.e., the radiolytic yield of reactive, mobile free radicals per input MGy is likely lower in Ni(dppf)Cl₂. We also observed that the HAADF-STEM response at the crater site differed between ambient-temperature and vitrified conditions ([Figure S6](#)). Previously, in Ni(dppf)Cl₂, repeated irradiation produced a reproducible increase in HAADF intensity at the dwell point, which we attributed to accumulation of carbonaceous contamination.²³ Lysozyme at RT displayed roughly the same behavior, with a slight increase in HAADF signal at the epicenter of the impact crater ([Figures S6A and S6B](#)). Under vitrified conditions, however, the crater site showed a net loss of HAADF signal, despite comparable cumulative fluence to RT ([Figures S6C and S6D](#)). Similar behavior was observed in control experiments conducted on pure ice ([Figure S6E](#)).

Two factors could account for these differences. First, Ni(dppf)Cl₂ is aromatic, with every carbon atom belonging to a delocalized π -system that absorbs and redistributes excitation energy to confer radioresistance.⁵⁴ In proteins, only Phe, Tyr, and Trp possess aromatic side chains, leaving the majority of the remaining C–H, N–H, and O–H bonds in the polypeptide backbone and other side chains susceptible to irreversible radiolytic scission and cross-linking. Second, radiolysis of the surrounding ice generates SEs and free radicals that attack protein molecules, amplifying secondary damage cascades beyond what protein self-ionization would produce. Since HAADF intensity scales with mass-thickness, reduced signal under vitrified conditions suggests local mass loss not present at RT—possibly thinning or void formation as highly concentrated radiolysis products escape into vacuum as volatile species. This is consistent with our hypothesis that the vitreous ice layer could act as a radiosensitizer.

CONCLUSION

Mosaicity has served as a framework for interpreting the reciprocal-space signatures of crystal imperfection since Darwin.⁶ Nevertheless, mosaic blocks in protein microcrystals have remained largely theoretical constructs, with their size, shape, and spatial distribution inferred from peak profiles and rocking widths. The experiments presented here offer a path toward grounding these models in direct observation. This capability opens the door to mosaicity models parameterized by real-space measurements; for example, domain size distributions and boundary morphologies could be extracted from 4D-STEM data and used to refine or constrain reciprocal-space fitting. Similarly, the magnitude of damage-induced lattice reorientation observed in consec-

utive 4D-STEM experiments could inform the development of EM-specific correction factors for integrated intensities.⁵⁵ Practically, 4D-STEM could serve as an assay for macromolecular microcrystal quality, enabling direct comparison of mosaic architecture across crystallization conditions or growth environments.

More broadly, as cryoEM pushes toward higher resolution, characterizing the spatial footprint of radiolytic damage in vitrified solvent will become increasingly important. Our observation that secondary damage propagates hundreds of nanometers from each exposure site suggests that radiolysis may compromise adjacent regions before imaging—an effect unaccounted for in dose management strategies ([Figure S9](#)). Looking forward, chemically informed mitigation strategies that target radical mobility warrant exploration. For instance, vitrification in D₂O, perdeuterated protein expression, or inclusion of radical scavengers could each reduce radiolytic damage propagation while preserving sample integrity. We anticipate that the combination of real-space and reciprocal-space information afforded by 4D-STEM will provide a powerful lens for dissecting radiolytic damage mechanisms in beam-sensitive biological specimens.

DATA AND CODE AVAILABILITY

All 4D-STEM data sets shown here are publicly accessible on Zenodo⁵⁶ and viewable using DuSC_explorer,⁵⁷ our open-source Python-based software.

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AUTHOR CONTRIBUTIONS

S.S.N. and A.S. contributed equally. Conceptualization: S.S.N., A.S., P.E., and M.M. Methodology: S.S.N., A.S., J.C., P.E., and M.M. Investigation: S.S.N., A.S., J.C., and P.E. Formal analysis: S.S.N., A.S., and M.M. Writing—original draft: S.S.N. and A.S. Writing—review and editing: S.S.N. and A.S., with feedback from all authors. Funding acquisition: Z.H.Z., P.E., and M.M.

DECLARATION OF INTERESTS

Z.H.Z. is a cofounder of Nanomega cryoAI.

SUPPORTING CITATIONS

Reference Scherrer⁵⁸ appears in the supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.bpj.2026.08.008>.

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