

Orientation Reconstruction of Proteins Using Coulomb Explosions

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We solve the orientation recovery of a tumbling protein in the gas phase from single-event measurements of the spatial positions of its ions after an x-ray laser-induced explosion. We simulate diffracted x-ray signal and ion dynamics under experimental conditions and compare our method to conventional orientation recovery in single-particle imaging with x-ray free-electron lasers using only diffraction data. We reconstruct 3D diffraction intensities using orientations recovered from the ion signatures and retrieve the electron density with established phase-retrieval algorithms. We test our orientation recovery procedure on 56 proteins ranging from 14 to 52 kDa (1800 to 6500 atoms), achieving an angular error of around 5°. The resulting 3D electron-density reconstructions are compared to ground-truth volumes simulated at the same nominal resolution and achieve the resolution at the edge of the detector in conditions similar to current single-particle imaging setups. We investigate the reconstruction quality and demonstrate that ion data can be used for reliable orientation recovery of particles in single-particle imaging, achieving orientation on par with or better than currently used recovery techniques. This Letter shows the potential of ion detection for retrieving additional information from the sample fragmentation and boosting single-particle imaging with x-ray lasers in the cases where the diffraction signal is a limiting factor.

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Introduction—Proteins are central to life, carrying out a wide range of essential tasks, such as enzymatic catalysis, signal transduction, and molecular transport. Because structure and function are tightly linked, resolving protein structures at high resolution is a key step toward understanding biological processes at the molecular scale. Crystallography [1] and cryoelectron microscopy [2] are the workhorses of experimental structural biology [3], allowing the study of hundreds of thousands of molecules with angstrom resolution and, more recently, the development of neural network based computational tools such as AlphaFold [4], which has been proven to predict accurate protein structures directly from the amino-acid sequence. To image biologically relevant but rare conformations of single proteins, development of new methods is required.

Single-particle imaging (SPI) [5] with x-ray free electron lasers (XFELs) allows one to study individual, noncrystalline samples in gas phase, promising one day to achieve high-resolution 3D reconstructions for weak

scattering particles such as single proteins. In recent years, SPI has progressed considerably, with advances in sample delivery [6] and analysis methods [7], opening a new way for the understanding of structural heterogeneity in nanoparticles [8]. Though further development is required to fully realize its potential and to make it competitive with other imaging methods, SPI recently achieved a new milestone with the first observation of an x-ray diffraction pattern from a single-protein structure [9]. As outlined early in an SPI road map [10], one of the big challenges is the unknown orientation of the sample during delivery, as each sample can rotate freely while being delivered. So far, aligning molecules using laser-induced alignment [11] works only on small samples, outside the range of what can currently be imaged. Aligning large molecules such as proteins is still under active research, possibly using electric fields to align the dipole of the molecule during sample delivery [12–15]. Another approach is to use algorithms that can reconstruct 3D diffraction volumes from patterns with unknown orientation, including expand maximize compress (EMC) [16] along with common arc [17] and manifold embedding [18]. In practice, orientation retrieval is performed only using EMC, which is the most successful algorithm developed so far. Despite its frequent use, EMC comes at the cost of no proven convergence, and while the effect of noise can be somewhat mitigated [19] and symmetries of the sample can be taken into account to assist the convergence of reconstructions, high-resolution reconstructions necessitate ultimately hundreds

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of thousands of high-quality measurements [20], which is currently not feasible.

An outcome of single-particle experiments that is often overlooked is the ejected ions. In SPI, the x-ray pulse is intense enough to destroy the sample, but because the XFEL pulse is so short the interference can outrun the damage, and the pattern one measures is from the sample with the structure mostly intact, at least in theory. This is known as *diffraction before destruction* [21]. Collecting ions and photons simultaneously has already been performed experimentally to assess the hit rate of the beam on the sample [22] and to automate identification of hits [23]. Although the explosion is governed by many-body Coulomb dynamics, where small changes in the initial configuration of the sample could produce large changes in individual ion trajectories, the ensemble of ion positions should still preserve the underlying structural information. Recent studies have explored the structural information contained in the Coulomb explosion with different approaches, using COLTRIMS with momentum imaging [24] as well as machine learning aided investigations [25,26]. Building on this, earlier work established a progression of structural information accessible from the ions: that x-ray laser-induced explosions are reproducible [27,28]; that ion measurements can distinguish protein conformations differing by as little as a twist in the *beta*-hairpin loop of a viral capsid protein [29]; and that the resulting *explosion footprints* can be grouped by unsupervised machine learning [principal component analysis (PCA), t-stochastic neighbor embedding (t-SNE), clustering], establishing that ion maps in principle carry information about the structure and orientation of the sample [30]. This previous work has been carried out with our own code, MolDStruct [31], a Monte Carlo/molecular dynamics hybrid code. While intricate bond breaking is difficult to capture within a classical framework, the overall ion dynamics for high charge states in classical and density functional theory (DFT) calculations are shown to be consistent [32].

What none of these works achieved, however, is the recovery of the orientation itself: reproducibility and classification establish that the information *exists*, but the full rotation of the sample over $SO(3)$, the quantity actually required for any 3D reconstruction, remained unsolved.

Drawing upon these results, we here close that gap. We present an algorithm that recovers the *full* orientation of an exploding protein over all of $SO(3)$ from its ejected ions alone to an angular error of $\sim 5^\circ$. We show that these ion-derived orientations can be carried through diffraction-volume assembly and phase retrieval to yield an actual 3D electron density, demonstrating that ion-based orientation retrieval is accurate enough to reconstruct a structure. Because the orientation is obtained from an independent, real-space measurement, the approach is effective already with ~ 100 explosion footprints, making it complementary to established diffraction-only methods such as EMC in the data-limited regime in which single-protein SPI currently operates [9].

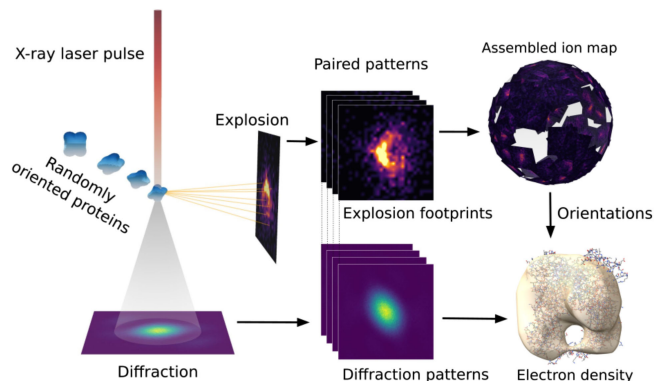


FIG. 1. Randomly oriented proteins are hit by an x-ray laser to produce single-shot diffraction patterns and ion explosion footprints. Patterns and footprints corresponding to each concurrent event are paired; we find the orientations of explosion footprints by mapping and assembling them on a spherical surface. These orientations are then applied to the diffraction patterns for reconstructing the 3D diffraction intensities space. Using phase-retrieval algorithms, we then reconstruct electron densities.

The workflow, schematically illustrated in Fig. 1, can be summarized as follows: (i) we simulate the Coulomb explosion and the diffraction signal from the same proteins in gas phase at different orientations in concurrence. Each protein structure is sampled from a vacuum equilibration simulation to mimic realistic dynamics. We then sample the simulations and pair the simulated explosion footprints with the corresponding diffraction patterns; (ii) we use the explosion footprints to determine the relative orientation of each measurement and then project and stitch the measurements onto a spherical surface to reconstruct a 4π sr ion map; (iii) we use each diffraction pattern with their corresponding predicted orientations to assemble a 3D diffraction volume and retrieve the electron densities using phase retrieval. Ultimately, this Letter addresses two scientific questions: (Q1) How well can we retrieve the orientation of a protein in gas phase solely from the ejected ions of its Coulomb explosion induced by an XFEL beam? (Q2) How robust is the predicted orientation from ion maps when reconstructing the 3D structure from coherent diffraction?

Results—We simulated the x-ray laser-induced explosions with an XFEL pulse fluence of 3.6×10^7 photons/nm² (see Supplemental Material for details of simulations and shot-to-shot fluence impact [33]) on a set of 56 unique proteins, listed in Supplemental Material, Table 1 [33], and measured the ions on a virtual spatially resolved multichannel plate detector (MCP) with side length 80 mm, and 256×256 pixels, at 10 mm from the protein-laser interaction. This records a single-event time-integrated explosion footprint of the induced explosion, where there is no distinction made between atomic species. Each explosion footprint only contains a partial view of the ions, as only ions in the solid angle of the detector are

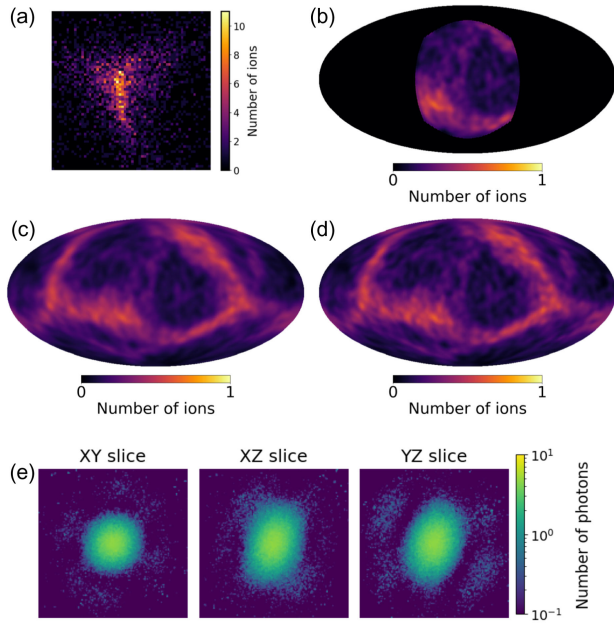


FIG. 2. Mapping and assembling the explosion and diffraction volume. We map the simulated Coulomb explosion footprints onto a unit sphere. After finding the relative orientation of the sample from the ion maps, we can retrieve the electron density in 3D using diffraction intensities. (a) Example of a binned simulated explosion footprint for an 80 mm MCP located at 10 mm from the interaction region. (b) Spherical projection of a single event, plotted using Mollweide view (noninteger intensities in the spherical projection arise from binning and Gaussian filtering). (c) Spherical map of the full explosion estimated from the predicted orientations. (d) Spherical map of the full explosion simulated from the sum of all the measurements when applying the exact orientations of the sample. (e) Orthogonal slices (XY, XZ, YZ) through the assembled diffraction volume. The color bar indicates the number of photons (log scale).

collected, Fig. 2(a). From the explosion footprint, one can index the hit locations of the ions on the detector, and from these we construct spherical projection maps using the Hierarchical Equal Area isoLatitude Pixelisation (HEALPix) algorithm for the pixelization of a 2-sphere [34,35], a method commonly used in cosmology and astronomy when working with spherical data. We display an example of an explosion footprint projected using HEALPix and displayed using a Mollweide projection in Fig. 2(b).

Using this procedure, we obtain the ion maps for random orientations in the laboratory frame. The next step is to find the relative orientation in the protein frame, thereby assembling a global *ion map* that corresponds to the total 4π sr solid angle of a complete sphere, covering the full angular distribution of the ions. To achieve this, we generate trial orientations via Sobol-based sampling [36,37], minimize the negative normalized cross-correlation between each pattern and a reference pattern selected

from the set, and then iteratively refine the reference (see Supplemental Material for details [33]). An example of a reconstructed ion map is shown in Fig. 2(c) and is compared to the ground truth assembled from known orientations as in Fig. 2(d).

To benchmark our orientation retrieval procedure, we explore five parameters: (a) sample size and mass; (b) number of explosion footprints used; (c) solid angle covered by the detector, which can be controlled by changing the detector-sample distance; (d) *detection coefficient*, which simulates the efficiency of the detector and corresponds to the ratio of detected ions and the total number of ions expected within the solid angle; and (e) signal-background ratio (S/B). The baseline parameters are 200 footprints, 1.0 detection coefficient, no background, and a covered field of view of 42% of the sphere, as assumed in our simulations with a detector-sample distance of 10 mm for an 80 mm $\times$ 80 mm MCP. The dataset corresponds to simulations of 56 different protein structures. The results are all shown in Figs. 3(a)–3(e), which correspondingly show the best orientation achieved as a median error on the total number of iterations of our algorithm for the different benchmarks. Each plot varies only one parameter at a time, while the remaining baseline parameters are held fixed. There is a clear trend [see Fig. 3(a)], connecting molecular mass and stable convergence of the algorithm. Running the algorithm for different proteins [Fig. 3(a)], we see clearly that the bigger proteins are the easier to align; this could be attributed to ion trajectories being more well defined and reproducible as discussed in [28,30], and all proteins bigger than 3000 atoms successfully converge.

We focus on four structures from the Protein Data Bank (PDB) for tests: (b), (c), (d), and (e); viral CASP8 and FADD-like apoptosis regulator (2BBR [38]), outer surface protein A (2OL6 [39]), mBaoJin green fluorescent protein (8QDD [40]), and DNA gyrase subunit B (1KIJ [41]). The choice of these four proteins allows us to probe the performance of the algorithm across a wide molecular mass range sufficient for stable convergence while also identifying molecules with different structural features—small and compact, elongated, barrel-shaped, and dimer—hence with different signatures in the global ion map. When varying the number of images [see Fig. 3(b)], we quickly reach successful reconstructions, although quite unstable until 100 images; for a small number of images, we might not completely sample the sphere and have few overlapping images, making reconstruction difficult. For 100 and more images, the error remains low and stable. In the plot, we show as a baseline the lowest error achieved with EMC on the same four proteins with 400 patterns; our procedure outperforms orientation retrieval on diffraction patterns, reaching a lower error and requiring less data.

Figure 3(c) shows that the most compact molecules, 2BBR and 8QDD, are also the ones that require a small

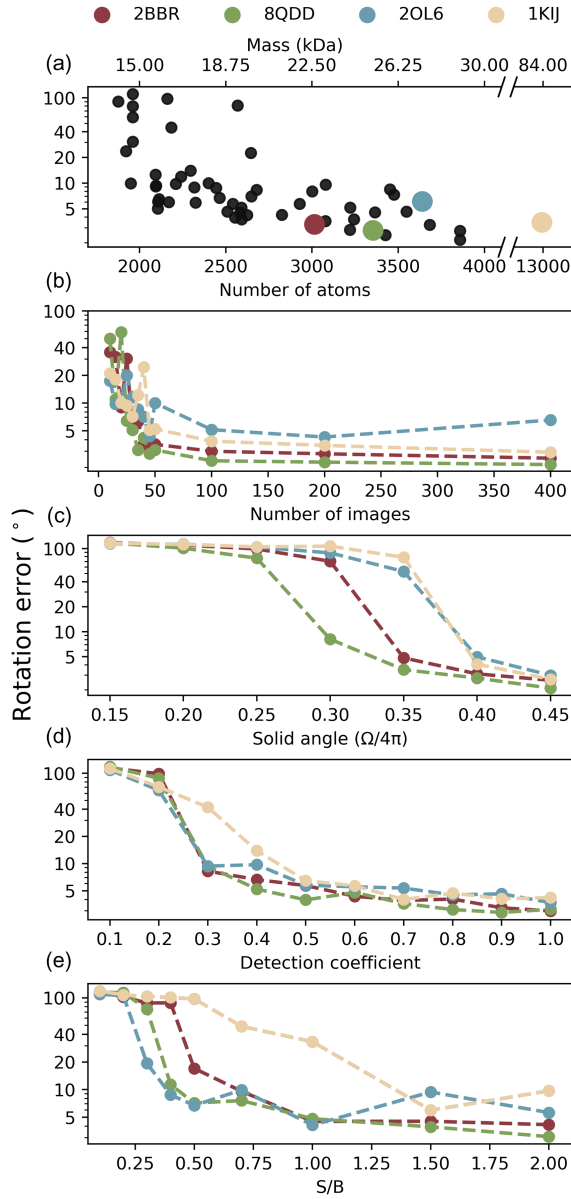


FIG. 3. Benchmark of the orientation recovery algorithm against the (a) number of atoms, (b) number of explosion footprints, (c) solid angle covered by the detector, (d) detection coefficient (the chance that an ion that hit the detector is recorded), and (e) signal-to-background ratio (S/B), obtained by superimposing an isotropic residual-gas ion background on each explosion footprint prior to orientation retrieval. Rotation errors (degrees) are shown on the y axis (log scale), and the benchmarks are carried out on the four representative proteins ordered by increasing molecular mass. In cases (a), (c), (d), and (e), the number of explosion footprints is 200.

coverage of solid angle for reliable convergence; on the axis, we scale the solid angle by a factor of $(1/4\pi)$ to clarify what ratio of the sphere needs to be covered. From the solid angle, we see that we need a setup such that around 35% to 40% of the solid angle is covered, which could be achieved by combinations of detector-sample distance and detector

sizes of currently used MCP detectors. For the detection coefficient [see Fig. 3(d)], the error remains low up until we only detect around 30% of the ions. Similarly for the signal-to-background ratio [S/B; Fig. 3(e)], where we superimpose an isotropic residual-gas ion background onto each footprint, the error remains low down to $S/B \approx 1-1.5$, i.e. even when the detector records nearly as many background ions as protein ions.

The robustness down to $\sim 30\%$ detection coefficient and to $S/B \sim 1$ suggests that orientation information is encoded in global anisotropic features of the explosion footprint rather than in individual ion trajectories. This is further confirmation that the orientation information is kept in global features; if each sample does not cover a large enough patch of the sphere, or if the background drowns out the anisotropic signal, these global distribution features can no longer be correlated between patches, the explosion footprints, of the total ion map.

Using the same x-ray exposure conditions for the ion explosion simulations, we simulated the corresponding diffraction patterns for our benchmark protein structures using Condor [42]. We simulate a detector of 1024 by 1024 pixels, 200 μm pixel size, downscaled by a factor of 8, and positioned at 50 cm from the interaction region, which limits the best achievable resolution to 2.2 nm calculated at the corner of the detector, comparable to experimental setups currently achievable for SPI at XFEL facilities [43–46]. In Fig. 4(a), we show the best performance for each benchmark PDB structure of 10 EMC instances. Given the small size of the proteins and the low resolution, we can assume D_2 symmetry of the sample, which further lowers the final rotational error of EMC. Under the given experimental setup and parameters, we note that, even for 400 patterns, EMC does not reach below 15° error. As a rule of thumb, orientation recovery is successful if the median angular error is less than 10° , consistent with thresholds adopted in other EMC-based reconstruction studies [47–49]. It is important to note that the smallest theoretical angular error of EMC depends on the sampling resolution parameter n , which sets how finely the space of quaternions is discretized [16]: increasing n reduces the angular spacing but also increases the number of sampled orientations and, thus, the computational cost that scales approximately as n^3 . For this study, we set $n = 20$, which corresponds to a theoretical smallest error of 2.7° . In contrast, the ion detector has an angular resolution of 3.8° for the central pixels and 1.8° on the outer pixels. This results in the ion measurements being sampled more finely than EMC, and hence the angular resolution in this comparison is also limited by the EMC discretization. The angular spacing affects the fluctuations in the convergence of EMC [16,49] and limits the best achievable resolution when using phase retrieval. With our results, in Figs. 3(a) and 4(a), we show that it is possible to achieve low errors, comparable to the theoretical optimum with EMC, even

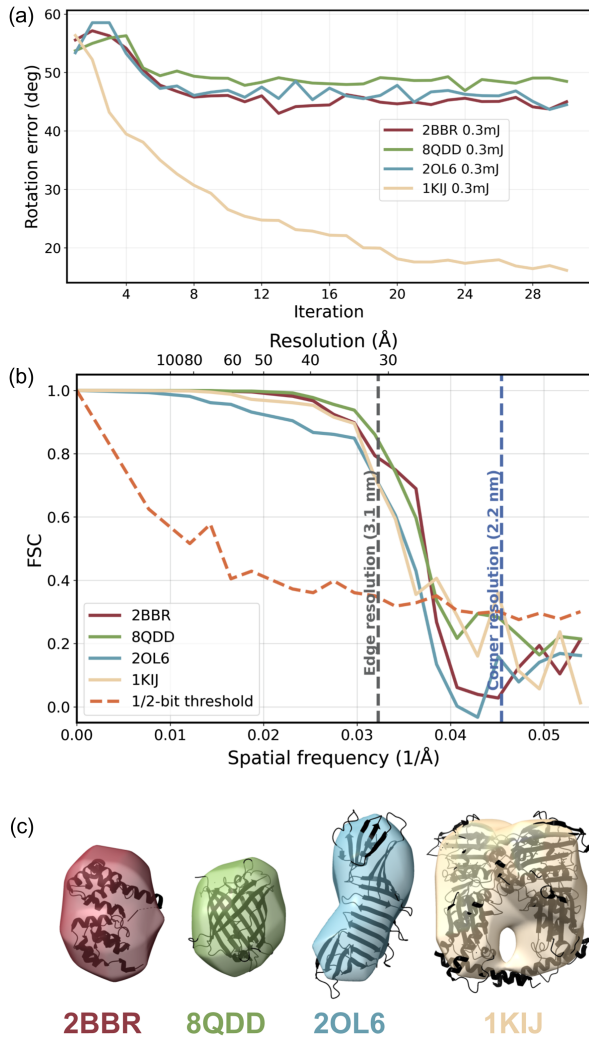


FIG. 4. SPI data analysis using diffraction patterns. (a) Orientation error as a function of EMC iteration using 400 diffraction patterns for four representative proteins. Orientation errors are reported modulo D_2 (crystallographic 222). (b) Fourier shell correlation (FSC) plot. The dashed orange line indicates the 1/2-bit FSC threshold, and the vertical dashed lines mark, respectively, the edge and the corner resolutions (3.1 and 2.2 nm). The achieved reconstruction resolutions, determined from the 1/2-bit FSC criterion, are 26.3 Å for 2BBR, 27.0 Å for 2OL6, 26.1 Å for 8QDD, and 24.9 Å for 1KIJ. All reconstructions achieve a resolution better than the edge resolution limit of 30.5 Å. (c) Representative reconstructed 3D volumes with embedded protein models. The density map was visualized in ChimeraX using an isosurface threshold of 30% of the map intensity range [50].

under conditions where EMC would otherwise fail to converge; without a converged EMC, the diffraction intensity volume cannot be reliably phased, and accurate real-space electron-density reconstruction is, therefore, not possible.

Figure 4(c) shows the four benchmark proteins and their electron densities obtained after phase retrieval (RAAR

[51] and error reduction [52], implemented in Hawk [53], with support updated via Shrinkwrap [54]) on the assembled 3D diffraction intensities using orientations as predicted in Fig. 3(a). We then use the Fourier shell correlation (FSC) [55] criterion to estimate the resolution of our reconstructions, as in Fig. 4(b). With ground truth available from the PDB files, we compare directly to the electron densities estimated at the corner resolution with ChimeraX [56] rather than using half-sets. The resolution can be estimated with the half-bit criterion [57], but, in this setting, our reconstructions are very small volumes, and the FSC remains mostly flat and close to 1 even when close to the edge of the detector. It might be that there is little independent high-frequency noise to decorrelate the maps, and this indicates that we are achieving the best reconstructions we can with the current detector setup.

Discussion and conclusion—With regard to the questions posed in the introduction: (Q1) We can find the orientation of the sample from ion maps down to $\sim 5^\circ$, and we achieve the best possible resolution in electron density reconstruction with the given diffraction parameters. (Q2) We require fewer ion measurements compared to diffraction measurements, down to 50–100 ion maps for reliable convergence, at the expense of collecting at least one third of the entire explosion.

A central limitation of this study is that no single-protein ion dataset exists yet, so direct experimental validation of ion-based orientation retrieval is currently not possible. The approach is nonetheless experimentally grounded and can be validated incrementally. Simultaneous collection of ions and photons at FELs has already been demonstrated [22,23], and real-space molecular geometry has been retrieved experimentally from Coulomb explosion imaging of small molecules [25,26,58] and from COLTRIMS momentum imaging [24]. A natural validation path can, therefore, be extrapolated from these experimentally mature methods toward single-particle ion detection.

On the explosion-model side, we rely on a single software for simulating the ion dynamics and the reproducibility of the explosion, though other codes could equally be tested. Within this model, we are restricted to comparatively small samples, under the assumption that the freed electrons leave the sample; larger samples would instead retain a fraction of their electrons and undergo a more isotropic explosion. We have likewise considered only a single fold per protein, with thermal motion included, under the assumption that distinct conformations can be prepared or separated experimentally, for example, by mass spectrometry.

A further limitation lies in the orientation retrieval method itself, which uses a number of parameters that are held fixed across the whole dataset, among them the level of explosion-pattern smoothing, the number of bins in the HEALPix projection, and the pairwise overlap or similarity threshold used to accept or reject an orientation.

Tuning these parameters per protein yields better results, which becomes necessary for proteins below ~ 2500 atoms. For example, with such tuning, we successfully retrieved the orientation of the small protein ubiquitin (PDB-ID 1UBI [59], ~ 1200 atoms).

On the imaging side, we have assumed a fixed detector geometry, matching possible conditions at SQS instrument of the European XFEL. The amount of background reaching the detector is largely set by the sample-injection system. Moving from the injection systems typically used in SPI today [9] to mass-spectrometry-based (MS) injection would further suppress the residual-gas background. Combining SPI and MS has not been experimentally explored to date, but MS injection systems for SPI are being developed [60]. In our tests, orientation recovery remains successful down to a signal-to-background (S/B) ratio of about 1.0–1.5, which for the proteins considered corresponds to roughly 5000–10000 background ions, with an even higher tolerance for larger proteins. To conservatively gauge the background expected in practice, consider a $10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$ focus (well above the actual 200 nm sample focus) and residual gas ionized over a 200 μm range centered at the focus: at a pressure of 10^{-6} mbar, this volume ($10 \times 10 \times 200\text{ }\mu\text{m}^3$) contains $\sim 5 \times 10^2$ gas molecules (assuming an ideal gas), which, being mostly diatomic, yields on the order of 1000 background ions. Since this overestimate stays within the tolerated S/B, the residual-gas background is not a limiting factor. The background could be reduced further by exploiting the kinetic-energy difference between sample and background ions since the former originate from a highly charged environment: one could gate the detector to record only the fast sample ions or apply a deflection voltage that diverts only the slow background ions.

Heterogeneity classification and conformational studies are still open problems and active research areas in SPI. While the latest developments push toward smaller samples and higher resolution, it will become increasingly important to sort and classify heterogeneous datasets. As shown in publications related to this work [27,29,30], the spatial position of ions *postdestruction* in SPI contains sufficient information to disentangle small conformational changes. Rare conformational states would also less frequently be probed by diffraction, leading to less data, which can be a bottleneck for current SPI data analysis. In this Letter, considering nonheterogeneous structures with sampled thermal motion, we provide a pathway to orientation retrieval using explosion fingerprints and very scarce measurements. This work can help access structures that would not otherwise be possible to analyze. The fact that explosion footprints have been shown to be unique for each protein [29] hints at the possibility of mapping explosion footprints to structure. This, combined with the results described here, provides a strong scientific case for ion collection during SPI. A link between structures and ion

maps would allow structural features to be determined with tabletop lasers, completely omitting the diffraction.

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Data availability—The source code is available from [61]. All structure files are available from [62]. Additional data that support the findings of this article are available upon request.

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