Nat Methods. Author manuscript; available in PMC 2015 March 01.

Published in final edited form as:
Nat Methods. 2014 September ; 11(9): 927–930. doi:10.1038/nmeth.3043.

High-resolution structure determination by continuous rotation data collection in MicroED

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Abstract

MicroED uses very small three-dimensional protein crystals and electron diffraction for structure determination. An improved data collection protocol for MicroED called “continuous rotation” is presented. Here microcrystals are continuously rotated during data collection yielding improved data, and allowing data processing with MOSFLM resulting in improved resolution for the model protein lysozyme. These improvements pave the way for the implementation and application of MicroED with wide applicability in structural biology.

Producing large well-ordered crystals is a major bottleneck for protein structure determination by X-ray crystallography. Because large crystals are needed to withstand the negative effects of radiation damage during data collection, small micro and nanocrystals have generally been ignored and cast aside as unusable. Many difficult to crystallize targets never reach a usable size and are generally discarded; therefore methods that can facilitate structure determination from these small crystals would be exceedingly valuable. Advances with X-ray free-electron lasers (XFEL) have made it possible to use microcrystals to solve protein structures[1-3], however its current implementations require the collection of XFEL diffraction patterns from thousands, or even millions, of crystals.

Recently we reported the development of MicroED (micro electron diffraction) as a complementary method to XFEL. In MicroED, electron diffraction data are collected from extremely small, three-dimensional protein microcrystals for protein structure determination. In our previous work[4], diffraction data of lysozyme microcrystals were taken as a tilt series

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AUTHOR CONTRIBUTIONS B.L.N contributed to project design, conception, data collection, data analysis, manuscript writing, figure making.
D.S contributed to project design, conception, data collection, data analysis, manuscript writing, figure making.
A.G.W.L contributed to data processing and analysis in MOSFLM and manuscript writing.
T.G. contributed to project design, conception, data analysis, and manuscript writing.

ACCESSION CODES The final structure factors and coordinates for the structure solved from the two crystal data set were deposited in the Protein Data Bank with accession code 3J6K.

Declaration of competing interests: The authors declare no competing financial interests.

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of still exposures and these data were integrated and merged using in-house developed programs[5]. Following phasing and refinement, the final structure of lysozyme was solved to 2.9 Å and represented an important first step in the use of electron diffraction data to solve structures of biological material from nano and microcrystals.

Here we report a number of significant developments in MicroED. An improved data collection protocol was developed called “continuous rotation method for MicroED”. Here the electron diffraction data is collected as a movie as the crystal is continuously rotated by the microscope stage (a method that is analogous to X-ray crystallography where the crystal is oscillated using the goniometer). The collected data were processed by MOSFLM[6, 7], a well established data processing program for X-ray crystallography, which further improved the process of structure determination by MicroED. These enhanced data collection and processing strategies yielded more accurate diffraction data and allowed the structure of lysozyme to be solved at 2.5 Å resolution with significantly improved statistics relative to the original report.

In the original MicroED protocol[4], diffraction data were collected as a series of still exposures, each related by the tilt of the stage (0.1 - 1°) between exposures during data collection (Figure 1a and b). By nature still exposures mainly produce partial intensities, and while we showed that a data set solely composed of still exposures is able to produce a structure, some inaccuracies resulting from the partiality will be carried through the subsequent data processing steps unless scaling is performed.

In order to overcome this problem and improve the quality of the MicroED data, we sought to collect diffraction data as the crystals are continuously rotated which is more similar to how X-ray diffraction data is collected[8]. Our hypothesis was that this improved data collection method would yield more accurately measured intensities as reciprocal space is more finely sampled (Figure 1c and d). Such sampling would allow the use of standard X-ray data processing programs for integrating and scaling, further improving data quality and subsequent processing.

To collect diffraction data by the continuous rotation method, lysozyme microcrystals (Supplementary Figure 1) were grown, applied to an EM grid, and vitrified in liquid ethane as described previously[4]. The microcrystals were wedge-shaped, typically with the length and width of ~2 μm and the wedge thickness measured as ~0.1 μm - 0.6 μm within each individual crystal. The crystal thickness was measured in real space by analyzing the same crystal at different tilts in imaging mode. The grids were then loaded, assessed and diffracted at cryogenic temperatures using a 200 kV TEM equipped with an FEG. Once a crystal was selected, the compustage of the microscope was rotated at a constant rate of ~0.09° s^{-1}. As the crystal rotated, it was simultaneously exposed to the electron beam and the diffraction was recorded as a movie on a CMOS-based detector in rolling shutter mode with each frame covering a ~0.36° wedge (frame rate of 4 s per frame). Typically a total of ~44° of data were collected per crystal before reaching the total accumulated dose limit of $5e^{-}/Å^2$ (see Supplementary Results, Supplementary Video 1, and Supplementary Figure 2 for description of radiation damage assessment). With this procedure we were able to collect continuously rotating electron diffraction data sets with visible reflections beyond 2 Å

(Supplementary Figure 1, Supplementary Video 2) that could be merged and further processed.

One of the major factors for the widespread use of X-ray crystallography is the powerful and relatively user-friendly software that has been developed over many decades for efficient data processing. We sought to capitalize the work put in to such programs by processing the MircoED data with MOSFLM[6, 7], a widely used program for X-ray data integration and processing. Processing the MicroED continuous rotation data with MOSFLM was possible without making any changes to the software (Supplementary Figure 3), but some modifications to the standard procedure and processing parameters were required, as described in the Supplementary information.

Data from two crystals were collected and processed with their overall completeness to 2.5Å being 80% and 45% for crystals 1 and 2, respectively. It is important to note that the total angular range collected for both crystals was the same (44°), and the higher completeness for crystal 1 is the result of crystal orientation on the grid. Ultimately, data from either one or two crystals were processed with MOSFLM and merged to provide a complete dataset. The intensity data were merged, scaled, and converted to structure factor amplitudes using POINTLESS[9], AIMLESS[10], and CTRUNCATE within the CCP4 suite[11]. The data sets were truncated at 2.5 Å based on the merging statistics presented in Supplementary Table 1.

Molecular replacement (MR) was performed using PHASER[12] in order to determine phases with lysozyme PDB 1IEE[13] as a search model. We continued with refinement in PHENIX[14] using electron scattering factors to obtain models with good statistics and geometry for both data sets (Supplementary Table 1). The final refined structures show excellent agreement with the density map (Figure 2, Supplementary Video 3), with strong clear density for the peptide backbone as well as the side-chains and some well-ordered water molecules.

In order to check for model bias in the final structure, residues 27-36 of the final model were removed and the remaining model was used for MR with the original data. After refinement the resulting map showed strong interpretable density in the region where the model had been removed (Figure 2b) and the correct residues could be readily fitted into this density (Figure 2c and d) indicating low levels of bias from the search model.

Overall the quality of the MicroED data obtained by continuous rotation is better than the original still diffraction data that we reported previously (Figure 3). When the scaled amplitudes from continuous rotation data are compared with those from a lysozyme data set obtained by X-ray crystallography, the correlation coefficient between the continuous rotation data and the X-ray data was 0.76 overall (0.84 to 6.0 Å). This is a substantial improvement over the still diffraction data collected previously[4], which showed a correlation of 0.56 for the data overall (0.63 to 6.0 Å).

With continuous rotation data the effects of dynamic scattering should be diminished, as was reported for electron diffraction using precession diffraction of thicker materials[15, 16]. Dynamic scattering (multiple elastic scattering events) in electron diffraction can redistribute primary reflection intensities as the primary scattered electrons scatter again elastically within the crystal, which can lead to a reduction in the accuracy of the measured

intensities[17]. We previously noted that dynamic scattering in still diffraction data of lysozyme contributed on average an error of ~5%[4]. This observation was based on intensity measurements for forbidden reflections in the still data set. The lysozyme crystals have $P4_32_12$, symmetry and systematic absences are expected along a* and b* at positions (2n +1,0,0). However, in the still data set weak reflections were observed at the positions where absences were expected (Supplementary Figure 4). We argued that these reflections originated from dynamic scattering events. By performing the same analysis as reported previously[4], it was clear these weak forbidden reflections along the a* and b* axis were reduced and contributed an average error of only 2.5% (SEM = 0.9%, n=16) in the continuous rotation data.

To conclude, we present here improved methods for collecting and processing MicroED data. By using continuous rotation, MOSFLM for data processing, and standard X-ray programs for merging and scaling, MicroED delivered more complete and accurate data at higher resolution relative to our original study[4], even from a single crystal. Additionally, the overall process from data collection to the refinement of the final structure was greatly simplified, streamlined and accelerated by using established software for crystallography.

The continuous rotation method described here is similar in principle to precession electron diffraction[18]. Improvements in data accuracy with precession electron diffraction have been reported in materials science due to the reduction in dynamic scattering effects and better intensity values as the reflections are integrated through the Bragg angle[15, 16]. The effects of dynamic scattering are diminished in precession electron diffraction because at any given point along the beam's precession path, the total number of allowed secondary scattering paths are reduced[16]. This decreases the intensity redistribution due to dynamic scattering as the reflections are integrated over the complete circular path of the precession beam. Because continuous rotation is a simplified version of precession diffraction, it is not surprising that we improved the MicroED data in comparison with data collected with diffractions stills[4].

While the improvements to MicroED reported here are significant, there still remain other areas that could further improve the performance and applicability of MicroED to the structural study of biological materials. One possibility is the use of faster detectors with real movie mode capabilities. While the rolling shutter mode on the CMOS camera we use is performing well, the data still suffers because of the readout time of the sensor. The rotation of the crystal during the readout time results in an angular gradient across an image that is hampering data integration at the highest resolution shell. At the same time, signal to noise is also an issue in rolling shutter mode. Improvements in detector speed or including new algorithms to better handle this error in MOSFLM should help to further improve the final data. Also, while we have attempted to reduce the effects of multiple elastic scattering by continuously rotating the sample, we have not yet addressed the errors associated with inelastic scattering. Inelastic scattering leads to high levels of background noise in thicker samples, and the use of an energy filter would greatly reduce the noise in our diffraction patterns[19]. By collecting data on a microscope equipped with an energy filter, the signal to noise ratio of the MicroED data should be significantly improved.

The continuous rotation method coupled with processing in standard X-ray crystallographic software makes MicroED a much more accessible method to all structural biologists already familiar with the suite of programs available for X-ray diffraction data. The only difference is the way in which the diffraction data is obtained – electrons *versus* X-rays. With such improvements and streamlining we believe that MicroED is quickly becoming a feasible method with wide applicability for solving structures of biological materials from extremely small crystals using an electron microscope.

METHODS

Collection of rotation electron diffraction data

Lysozyme microcrystals and EM samples for diffraction were prepared as described previously[4]. All electron diffraction was performed with intensity less than 0.01 $e^{-}/Å^2$ on a TEM operated at 200 kV, equipped with an FEG, and data were collected with 4k × 4k TVIPS TemCam-F416 CMOS camera in rolling shutter mode (15.6 μm pixel size). For continuously rotating diffraction data the stage of the microscope was set to rotate at 0.09° s^{-1} using the microscope's standard hardware and software. Crystals were located on the grid by searching in over-focused diffraction mode. When a crystal was found, an initial diffraction pattern was recorded to judge the quality of that particular crystal. If the crystal showed strong and sharp diffraction[20], the beam was blanked and the rotation of the stage was started. Once the stage began its rotation and had achieved a constant rate, the beam was unblanked and diffraction data were recorded at a constant frame rate of 1 frame per 4 s (0.36°/frame) using the camera's rolling shutter mode. Data sets of approximately 44° were collected for each crystal (total dose $<5e^{-}/Å^2$, see supplementary results for dose rate). For the radiation damage assessment, the stage was set at 0° and oscillated back and forth between −1° and 1° during the course of the continuous dosage experiment.

Data processing and structure refinement

Raw MicroED data was converted into an SMV file format, which could be read by MOSFLM. Diffraction data were indexed, integrated, merged, scaled and prepared for refinement using MOSFLM v7.1.0[6] and the graphical interface iMOSFLM v1.0.7[7], POINTLESS[9], and AIMLESS[10]. In the scaling and merging step, the refinement of the standard deviation correction factors in AIMLESS was unstable and these were set to SDFAC=1.5, SDB=0.0, SDADD=0.03. Phases were obtained by molecular replacement using PHASER[12] with lysozyme PDB ID: 1IEE[13] as a search model (LLG = 673 and TFZ = 24.3 for multiple crystal data set; LLG = 821 and TFZ = 8.5 for single crystal data set), and the molecular replacement solutions were refined using electron scattering factors in PHENIX[14] using a 5% free data set.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ACKNOWLEDGMENTS

The authors wish to thank J. Hattne, F.E. Reyes, D. Olbris, H. Tietz, and M. Stumpf for helpful discussions. Work in the Gonen lab is supported by the Howard Hughes Medical Institute. A.G.W. Leslie is supported by the Medical Research Council (U105184325) and BBSRC (BB/F020384/1) and CCP4.

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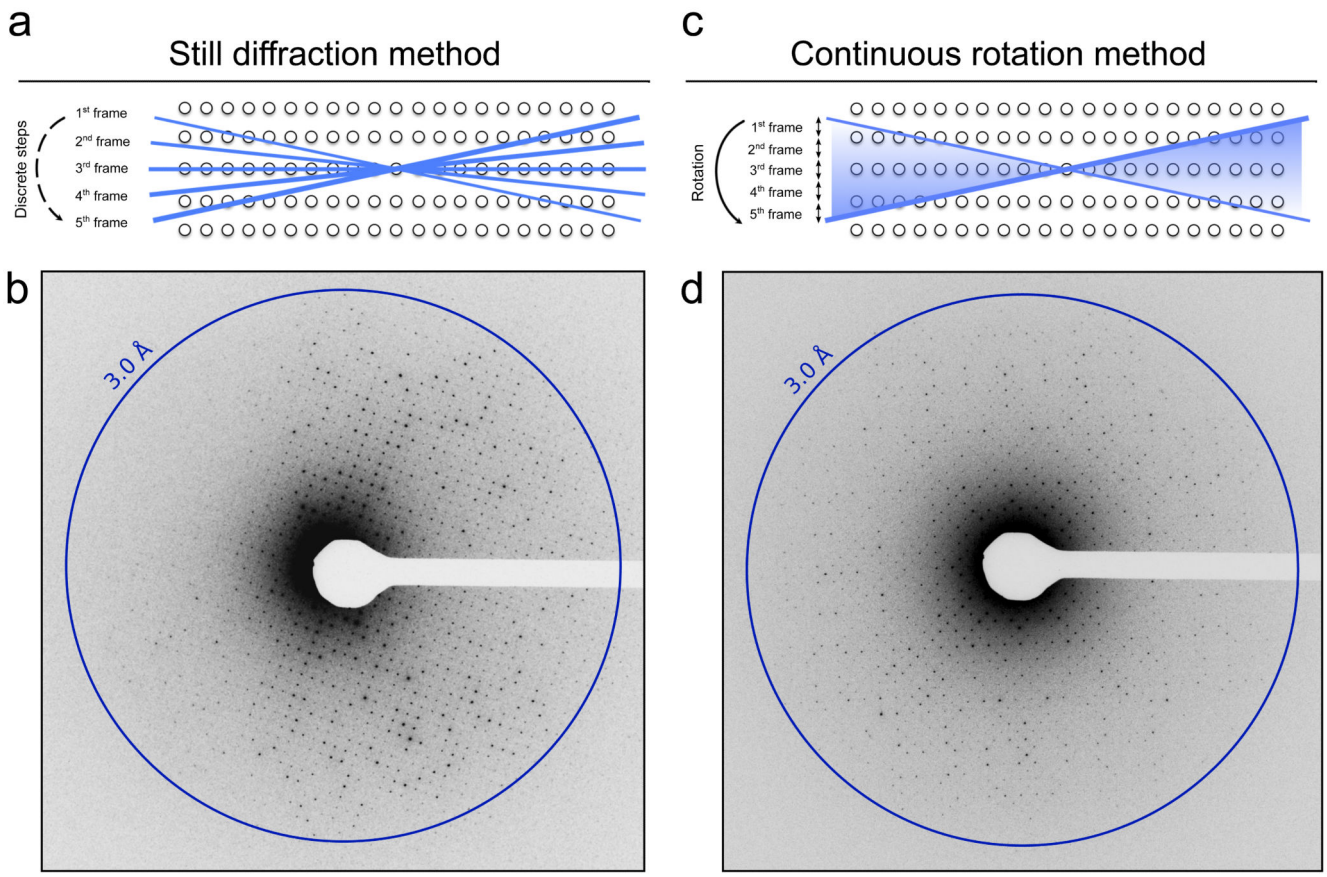

Figure 1. Data collection strategies in MicroED

(a-b) The initial data collection strategy termed “Still Diffraction” consists of rotating the stage in discrete steps between exposures. This provides data in the form of 2D slices through the 3D reciprocal space (a), and while this is sufficient for structure determination, the data are inherently incomplete because most reflections are only partially recorded (b). (c-d) The improved “Continuous Rotation” method for MicroED samples the reciprocal space continuously as the crystal is rotated (c), which yields much more complete and accurate measurements of reflection intensities (d). In the examples shown here originating from two different crystals, the reflections on the left side of the still diffraction are missing due to crystal orientation while they are present in the continuous rotation data because reciprocal space is being more completely sampled (c *versus* d, respectively).

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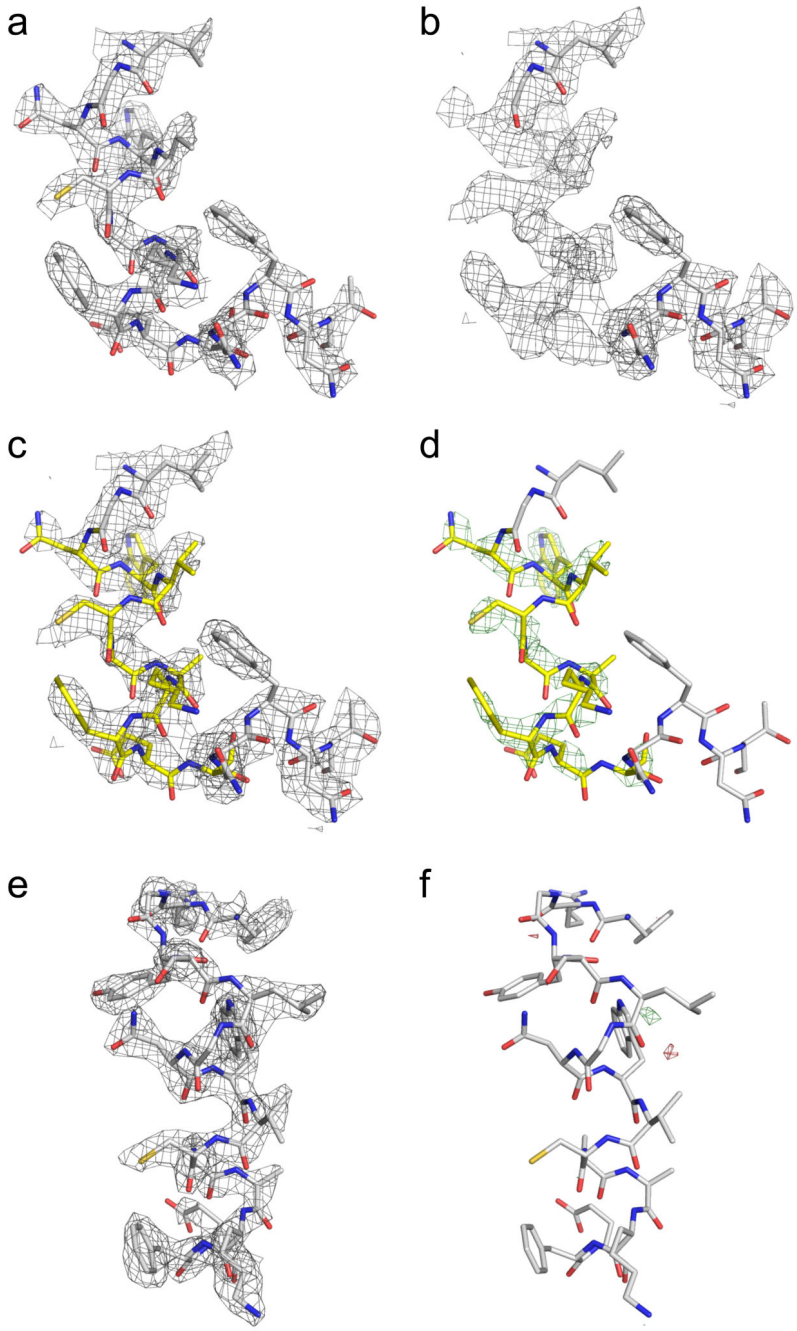

Figure 2. Final refined structure of lysozyme at 2.5 Å from Continuous Rotation MicroED data

(a-d) A representative region of the final refined structure of lysozyme originating from the 2-crystal data set is shown, with the $2F_{obs}$-F_{calc} density map (a; contoured at 1.0σ) showing well defined density around the backbone and sidechains. The final 3D structure is also shown in Supplementary Video 3. To test any potential model bias, residues 27 through 36 were removed from the final refined model and the incomplete model was used to phase and refine the original data. The $2F_{obs}$-F_{calc} map (contoured at 1.0σ) without the deleted residues (b) shows clearly defined density for both backbone and sidechains where the missing

residues (shown in yellow) could easily be placed (c). The F_{obs}-F_{calc} map (contoured at 3σ) also shows very strong density for the deleted residues (d). The strong density for the missing residues in the $2F_{obs}$-F_{calc} and F_{obs}-F_{calc} maps indicate the final map does not suffer from model bias. (e-f) Final refined structure of lysozyme at 2.5 Å resolution using data originating from a single crystal. The $2F_{obs}$-F_{calc} density map (e; contoured at 1.0σ) shown around residues 20-35 shows well-defined density around both backbone and sidechains. The F_{obs}-F_{calc} (f; contoured at ±3σ) shows no clear differences between the observed data and the model.

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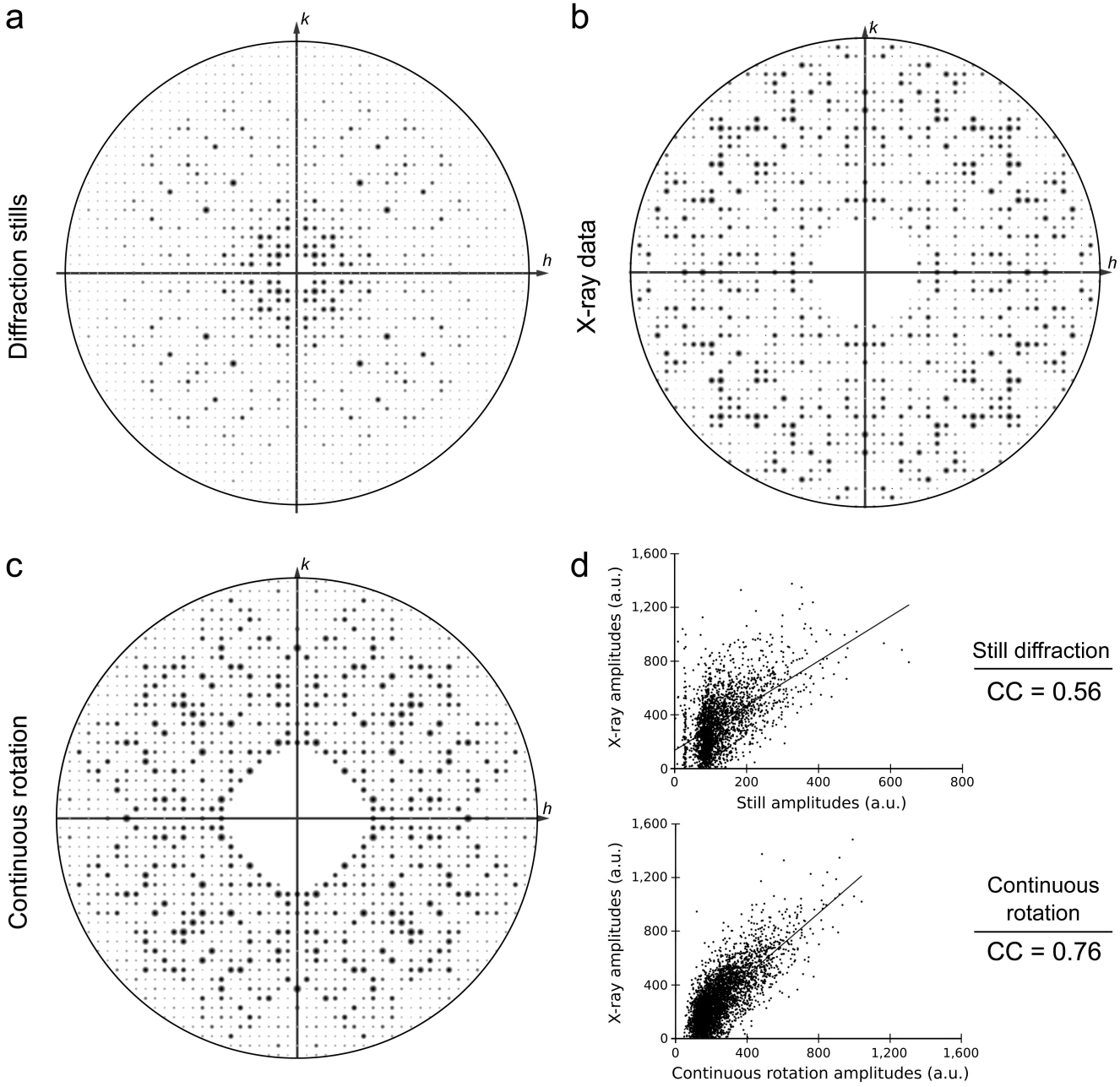

Figure 3. Continuous Rotation improves MicroED data quality

(a-c) Views of the (001) plane show that the intensities from still diffraction data (a) exhibit less variation between high and low intensity reflections when compared to X-ray data (b) and the data collected from continuous rotation (c). Data is displayed using VIEWHKL within the CCP4 suite[11]. (d) The lysozyme MicroED data collected as diffraction stills previously[4] was compared with a data set collected by X-ray crystallography (top). While the data is moderately correlated (Pearson correlation coefficient of 0.56 for all data to 2.9 Å, n = 2,466), there is some spreading of the data. When continuous rotation MicroED data is compared with X-ray data (bottom) it is clear that the two are much more correlated

(Pearson correlation coefficient of 0.76 for all data to 2.5 Å, n = 3,950) and the spread of the data is narrower relative to the analysis done with diffraction stills. Overall, this analysis shows that the continuous rotation method in MicroED yields data with high quality compared with X-ray data.

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