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Advances and applications of microcrystal electron diffraction (MicroED)

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Abstract

Microcrystal electron diffraction, commonly referred to as MicroED, has become a powerful tool for high-resolution structure determination. The method makes use of cryogenic transmission electron microscopes to collect electron diffraction data from crystals that are several orders of magnitude smaller than those used by other conventional diffraction techniques. MicroED has been used on a variety of samples including soluble proteins, membrane proteins, small organic molecules, and materials. Here we will review the MicroED method and highlight recent advancements to the methodology, as well as describe applications of MicroED within the fields of structural biology and chemical crystallography.

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Keywords

Cryo-electron microscopy, Cryo-EM, Microcrystal electron diffraction, MicoED, Crystallography, Protein crystallography, Small molecule crystallography.

Introduction

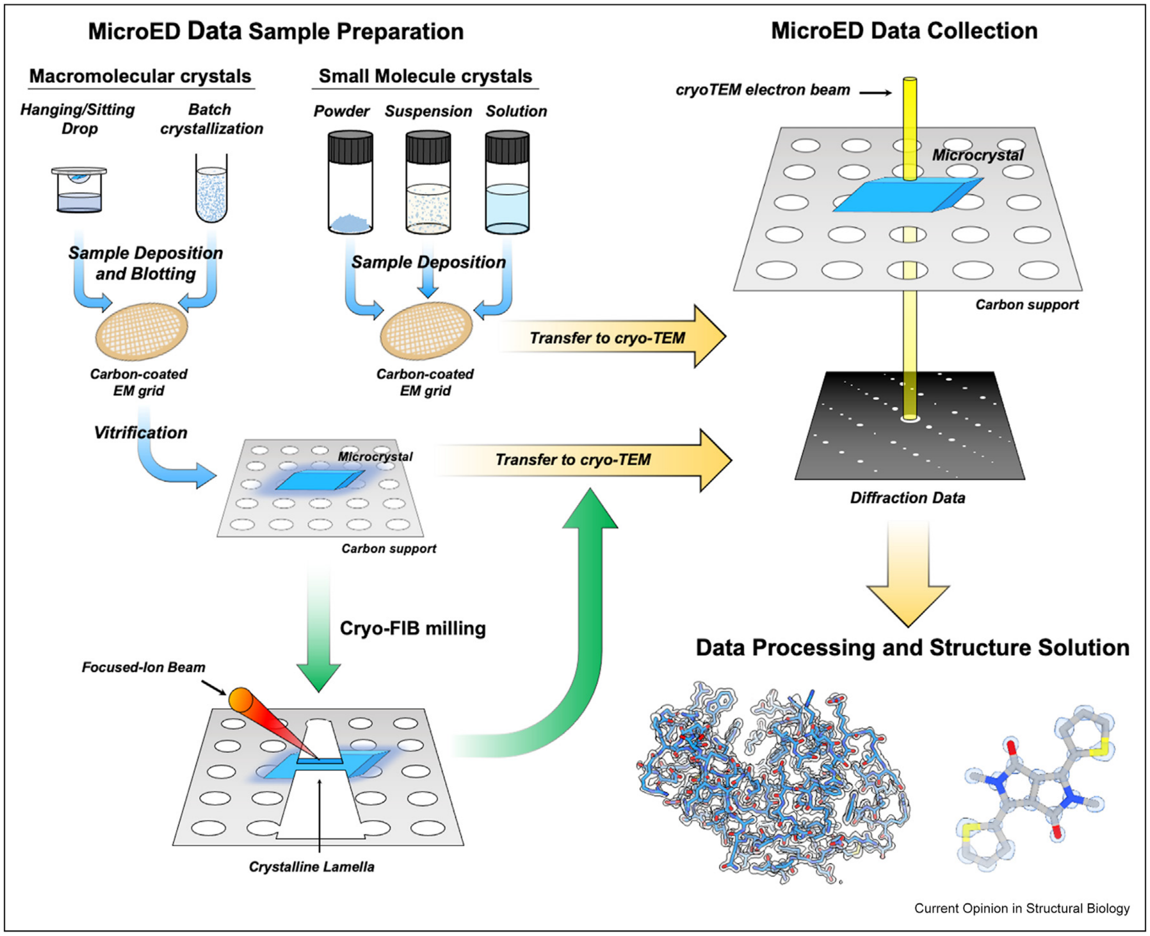
Crystallography has been, and to this day remains an incredibly successful method for the determination of high-resolution macromolecular and small molecule structures. While the vast majority of diffraction experiments in crystallography make use of X-rays, there is a key bottleneck associated with X-ray diffraction, namely the requirement of large well-ordered crystals. In many cases, the difficulty associated with growing relatively large high-quality crystals for X-ray experiments can set back the study of those target systems. Therefore, methods that can make use of smaller, and in many cases easier to obtain, crystals promise to be powerful tools for the structural biology and chemical crystallography communities.

The electron crystallographic method of microcrystal electron diffraction (MicroED) is one such method that is able to bypass the requirement of large crystals by making use of the strong interaction of electrons, which relative to X-rays is less damaging per useful elastic scattering event [1]. Since the early work on MicroED in 2013 and 2014 [2,3], the method has been used to determine a wide variety of structures including membrane proteins, soluble proteins, peptides, organic molecules, and materials [4,5]. Here we will describe the MicroED technique and recent advances to the methodology, and highlight recent applications of MicroED to various fields of structural research.

MicroED methodology and recent advancements

The MicroED workflow is a combination of cryo-EM and crystallography, with sample preparation and data collection being familiar to those in the cryo-EM field, and data processing and structure solution being familiar to researchers with a crystallography background. MicroED and electron crystallography details and procedures have been published in detail elsewhere and readers are encouraged to refer to these more detailed works [6–13]; however, here we will only briefly describe the MicroED workflow (Figure 1). The workflow for biomolecular crystals begins by depositing the sample on a carbon-coated EM grid, followed by the removal of excess sample solution by blotting and rapid plunge freezing into liquid ethane. This method is analogous to what is commonly performed for single-particle cryo-EM. For small molecule samples, the sample can be added to the surface of the grid by directly applying powdered sample, adding a crystalline slurry followed by solvent evaporation or blotting, or by direct crystallization on the surface of the grid [14–20]. Once the samples are loaded into the cryo-TEM, diffraction data are collected from the microcrystals on the grid surface. The initial MicroED data collection strategies were inspired by decades of work performed on 2D protein crystals. In 2D electron crystallography, each crystal, consisting of a single or double layer of protein, produces a single diffraction pattern, and

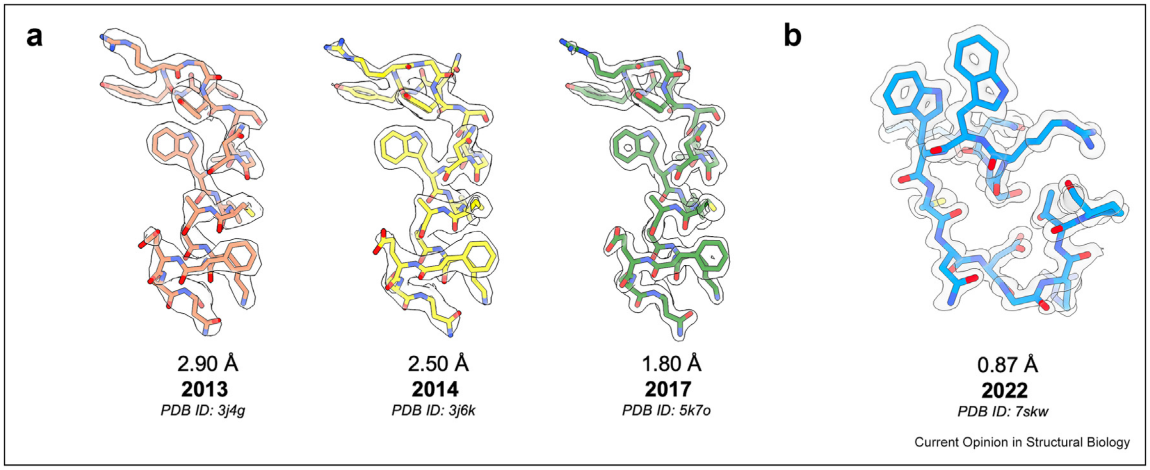
Figure 1



The MicroED workflow. The workflow for macromolecular crystals begins by transferring the crystals from crystallization experiments (e.g. hanging drop, sitting drop, batch crystallization) to the surface of a carbon-coated EM grid. The samples are then blotted and vitrified using manual or automatic plunge freezing setups. At this point, the microcrystals may be loaded into the cryo-TEM for MicroED data collection, or can be further processed using cryo-FIB milling (green arrows) prior to transfer into the cryo-TEM. For small molecule samples, the microcrystals are transferred to the grid by different approaches depending on the form of the sample. Dried powder can be directly applied to the surface of the grids. Microcrystals in suspension are applied to the grids and the solvent is then blotted away or allowed to evaporate from the surface. Finally, samples may be crystallized directly on the grid by applying the sample in solution and allowing evaporation to occur. For both macromolecular and small molecule crystals, data are collected by continuous-rotation MicroED data collection. The resulting data are processed, and structures are determined and refined, using standard crystallographic programs.

multiple crystals are then collected at various stage tilt angles and merged together to provide a complete 3D structure of the protein [21]. In MicroED, rather than each crystal providing a single diffraction pattern, ultra-low doses are used to collect multiple diffraction patterns as the stage is rotated in the electron beam. When this is performed using a continuous rotation of the stage and continuous readout of the camera, data sets are collected that are analogous to those collected by single crystal X-ray diffraction experiments. This method of continuous-rotation data collection is currently the default mode of MicroED data collection [3,22]. This same approach of continuous electron diffraction data collection and stage rotation has also been analogously referred to as continuous rotation electron diffraction (cRED) [23] or 3D electron diffraction (3DED) [24,25]. With minor modification, MicroED data are processed using standard X-ray data processing programs, and the resulting structures are refined using standard X-ray crystallography refinement packages. Using these data collection and processing procedures, MicroED has determined many high-resolution structures from extremely small microcrystals. However, continued method development is still key to drive MicroED forward. Past improvements have greatly improved the throughput and quality of MicroED structures (Figure 2), and there are several recent advancements to the procedures, hardware, and software that promise to continue to advance the method.

Figure 2



MicroED methods advancement has greatly improved structure quality. (**a**) The first structure determined by MicroED was of tetragonal hen egg-white lysozyme in 2013. Data was collected using discrete tilts of the stage and the structure was determined to 2.90 Å. A short time later in 2014, continuous-rotation MicroED data collection was introduced, which improved data quality and allowed data processing to be performed using standard crystallographic programs. This advancement led the lysozyme structure to now be determined to 2.50 Å. Following continued MicroED methodology improvements, the structure of lysozyme was determined to 1.80 Å in 2017. (**b**) Recently, the powerful combined effect of several new MicroED method improvements, including cryo-FIB milling and data collection with direct electron detectors, was used on triclinic lysozyme crystals, which are known to diffract to higher resolution. The use of improved methodology and very high-quality crystals facilitated the structure determination of triclinic lysozyme to 0.87 Å resolution. Together these lysozyme studies show how critical methods development has been, and will continue to be, for MicroED. The structures displayed in (A) are of lysozyme residues 19 to 37 and residues 58 to 70 in (B), and the potential maps have been contoured to 1.5σ.

One recent area of methods advancement for MicroED has been in sample handling and preparation, especially when working with microcrystals in viscous solutions. As many crystallization conditions contain viscous components (e.g. high-molecular weight PEGs), it makes blotting the excess solution away very difficult. Often it isn't possible to simply dilute the crystallization solution, so working with hard-to-blot samples is a common difficulty in MicroED. One recently described approach for overcoming this barrier is to use a pressure-assisted device for removing excess buffer from the grid [26]. This setup, called Preassis, is better equipped for viscous samples by controlling the sample thickness on the grid, and can be used on a range of crystallization buffers. Additionally, because the excess solution is removed in a controlled manner through the grid, it can increase the quantity of crystals that are found on the surface of the grid following sample preparation.

Another major development in sample preparation for MicroED analysis has been the implementation of cryo-focused ion beam/scanning electron microscope (cryo-FIB/SEM) instruments to the MicroED workflow. A cryo-FIB/SEM can be used to mill unwanted material from the sample using the FIB and leave the remaining crystalline material relatively undamaged for subsequent MicroED data collection. Cryo-FIB milling has been used to create thin crystalline lamella within the cryo-FIB/SEM that are then transferred to a cryo-TEM for high-resolution MicroED data collection and structure determination [8,10,27–29]. One benefit of cryo-FIB milling for MicroED is it reduces the need for blotting the excess sample buffer from the EM grid. In standard MicroED sample preparation, to identify the location of crystals on the grid, the surrounding buffer must be removed such that the electron beam in the cryo-TEM is able to penetrate the sample, which is on the order of tens of nanometers. In contrast, when using cryo-FIB instruments, the thickness of the crystallization buffer must only be reduced to a level that allows the clear outlines of the crystals to be seen by the SEM and FIB within the cryo-FIB instrument. The FIB is then used to remove any excess material prior to MicroED data collection. The major advantage of cryo-FIB milling of microcrystals is that it allows MicroED analysis of crystals that are too thick for MicroED (greater than approximately 500 nm) yet are still too small (less than approximately 2–10 μm) for analysis by conventional X-ray crystallography. Even when used on crystals that are already thin enough for MicroED, cryo-FIB milling can improve data quality by removing excess material above and below the crystal, thereby setting the crystal thickness to an optimal value. Recently, crystalline lamella of varying thicknesses were tested at different cryo-TEM accelerating voltages to investigate the ideal thickness for MicroED experiments [30]. It was found that lamella up to twice the inelastic mean free path of the electron beam produced high-quality diffraction data, which serves as a guide for the creation of lamella from new samples.

Another area of recent methods improvement for MicroED has been the implementation of new cameras for data collection. The collection of continuous-rotation data requires a camera with high frame rate and the nature of diffraction data requires that the camera also have a high dynamic range. Early MicroED work was performed on high-speed CMOS-based cameras, and these cameras are still standard for MicroED work. Recently, the use of direct electron detectors and hybrid pixel detectors have been tested and used for the collection of MicroED data on macromolecular crystals [31–35]. In the case of direct electron detectors commonly used for single-particle cryo-EM, it has been shown that the data collected by these detectors is superior to MicroED data collected by standard CMOS-based cameras, and they can be operated in counting mode at very high speeds [32,33,36].

In addition to the areas described above, electron diffraction provides unique opportunities and challenges that are being studied and implemented for MicroED studies. Electron crystallography and electron microscopy produce maps of the electrostatic potential of the sample, rather than the electron density produced in X-ray crystallography. Therefore, electron diffraction data is more sensitive to charge and hydrogens [37–40], and the accurate modeling and application of these effects is an area of active development. The analysis of dynamic scattering effects is an additional area of open investigation in the electron crystallography community. While the dynamical effects have been shown to be low and not preclude structure determination with MicroED, modeling dynamic scattering for small molecules has been shown to improve structure quality from electron diffraction data sets [41,42], and very excitingly can be used to determine absolute stereochemistry [42,43].

Applications of MicroED

The continued development of MicroED has been essential to driving the method forward and has facilitated its use in several new areas of structural research. MicroED was initially developed using model crystals of proteins with known structure, but recent years have seen the method applied to new protein samples that had resisted other structure determination methods. In 2019, MicroED was used to determine the structure of a novel R2-like ligand-binding oxidase from *Sulfolobus acidocaldarius* from microcrystals that were too small for conventional X-ray diffraction [31]. In 2022, MicroED was used to determine the structure of an engineered protoglobin variant from *Aeropyrum pernix* that is capable of enzymatic carbene transfer. Crystals of the *A. pernix* protoglobin variant had resisted structure determination by other methods until MicroED was employed on the sample [44]. The novel structure was determined by MicroED to 2.1 Å, which shed light on the conformation of the active site and the mechanism of this novel engineered enzyme. More recently, MicroED was employed on this same system to solve the structure of a bound reactive intermediate, and this new structure shed further light on the mechanism of the engineered enzyme [45].

An area of focus for MicroED methods development is the application of the technique to membrane proteins. Membrane proteins often form very small crystals, which makes MicroED a promising tool for membrane protein structural biology. In 2020, MicroED was used to determine the structure of a mutant voltage-dependent anion channel from crystals that were grown in bicelles [46]. In this work, the use of cryo-FIB milling was essential to the preparation of high-quality crystalline samples for MicroED data collection. An important class of membrane proteins are the G-Protein coupled receptors (GPCRs). Many GPCRs are crystallized in the gel-like lipidic cubic phase (LCP); however, the high viscosity of the LCP matrix makes microcrystals embedded in LCP very difficult to work with. Recent work has demonstrated that the LCP can be converted to less viscous phases that are compatible with MicroED analysis, and when coupled with cryo-FIB milling, high-resolution structures of GPCRs can be obtained [47,48].

Another exciting area of MicroED application is in the field of chemical crystallography. As with macromolecular crystals, electron crystallography has been shown to be capable of accurate structure determination from very small crystals of organic molecules and materials, and electron diffraction methods have been developed and employed for structural studies [14–16,19,25,49–55]. The application of MicroED to small molecule crystallography allows rapid structure determination, often directly from the as-synthesized material, without the need for additional crystallization trials. This greatly enhances the structure determination throughput of novel small molecule samples. Recently, electron diffraction has been successfully used to determine the structures of a variety of organic molecules, including, but not limited to, pharmaceuticals [11,16,56], enzymatic and natural products [57–59], organic semiconductors [18,60], crystalline polymers [61,62], and framework materials [17,20,63–66]. The use of electron diffraction on very small nanocrystals has also been shown to be capable of determining multiple structures from chemical mixtures as well as determining the structure of distinct crystal polymorphs within a sample [60,67].

MicroED has also been shown to be highly complementary to other structural analysis techniques. In one example, grazing-incidence wide-angle X-ray scattering (GIWAXS) was used alongside MicroED to determine both the crystal structure and the orientation of organic semiconductors in thin films [18]. In this work, crystals were grown directly on the surface of the carbon-coated

TEM grid using conditions analogous to what was used to grow the thin films for GIWAXS, helping to ensure consistency between the crystal forms analyzed by MicroED and those seen in the films.

Conclusions

MicroED is emerging as a powerful tool for the fields of structural biology and chemical crystallography. This has been due in large part to the constant improvements made to the MicroED methodology since its initial development. In the future, as the community of researchers making use of MicroED grows, and with continued focused method development, the MicroED method will see further improvements that will ultimately enhance the method's throughput and final high-resolution structure quality. These continued applications and advances will further establish MicroED as an essential tool for high-resolution structure determination.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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