Illuminating the secrets of crystals: Microcrystal Electron Diffraction in Structural Biology

Barringer, R.1,2 & Meier T.1

1 Centre for Structural Biology, Imperial College, London, UK
2 Corresponding author (r.barringer@imperial.ac.uk)

ABSTRACT (151 words)

X-ray crystallography (XRC) has been used to visualise biological macromolecules in exquisite detail for over 50 years, relying on a combination of mathematical principles to offer insight into atomic structures. Crystals can diffract various electromagnetic waves aside from the conventional X-ray, offering an alternative approach to crystallographic structural analysis. Microcrystal Electron Diffraction (MicroED) illuminates crystals with electron waves instead of X-rays. A specialised group in the USA have demonstrated that MicroED can give high-resolution structural data, successfully solving more than 17 unique structures to date. Complementary experiments from Europe offer support for the method, and as interest in the technique grows it is becoming apparent that MicroED is a powerful and accessible tool. How MicroED compares to XRC will be key to assessing it as a stand-alone crystallographic technique. This review presents a critical analysis of MicroED, with comments on theoretical and practical aspects and suggestions of further work and development.
INTRODUCTION

Life is dependent on the ability of cells to perform a myriad of functions alone or in communities as tissues. When cellular processes falter, diseases can arise depending on the aberrant process. Understanding these processes is therefore essential to understanding the cellular basis of disease pathology. Cellular functions depend on proteins, of which there are likely over 19,000 in humans (Ezkurdia et al, 2014), each with unique functions and interacting with various biomolecules (Rolland et al, 2014). Proteins act as nano-scale cellular ‘tools’ with functions that are intimately linked to their unique structure; the role that protein architecture plays in biomolecular interactions has a long history, most commonly typified in the mind of the public by the ‘lock-and-key’ hypothesis (Koshland, 1994). Since proteins are incredibly small (for example haemoglobin has a diameter of ~1/200,000th of a mm) (Erickson, 2009), structural studies require the use of complex experimental methods, denoting the field of ‘structural biology’. There are three main methods used to assess protein structure: Electron Microscopy (EM), Nuclear Magnetic Resonance (NMR), and X-ray Crystallography (XRC) (Curry, 2015), with XRC having the richest history of the three (Wilkins, 2013). This review focusses on a new crystallographic method (microrystal electron diffraction, MicroED) that uses electrons instead of X-rays, outlining a brief history of both and then presenting a critical comparison of theoretical and practical aspects.

A short history of crystallography

X-ray crystallography has been the primary macromolecular structural method for over 50 years (Jaskolski, Dauter & Wlodawer, 2014). The theory of the method is simple: a pure crystal containing exquisitely ordered repeating units of identical molecules are placed in the path of an X-ray beam, which is scattered by the molecules within the crystal to produce a constellation-style pattern (Figure 1). While beautiful to the naked eye, the symmetry and ‘brightness’ of each spot of the ‘diffraction pattern’ contains crucial structural information about the molecules that scattered the X-rays (Sweet, 1985). Crystallographers use diffraction patterns to elucidate the atomic structures of biomolecules, allowing us to gain an atomistic perspective of nature’s tools. X-rays were first used in crystallography by Max von Laue, who hypothesised that X-ray wavelengths are short enough to be diffracted by atoms within a 3-dimensional crystal (Eckert, 2012). Incoming X-rays excite the electrons surrounding atoms of the protein molecules in the crystal, and the X-ray energy is subsequently redistributed in all directions as a wave. In certain directions, the diffracted X-rays of each identical molecule interfere coherently (in-phase) to produce observable diffraction ‘spots’. The Bragg’s showed that these angles of observation depend on the X-ray wavelength and the spacing between molecules in the crystal (Bragg & Bragg, 1913), and that the characteristic ‘spotting’ of diffraction patterns results from these two properties. The spots are observed because the scattered X-rays of the repeating molecules only amplify when they overlap with each other in phase. This creates a ‘reciprocal lattice’ of spotting at certain scattering angles. The brightness of each spot results from the cumulative X-ray waves scattered from every atom of every molecule, overlapping in unique phases at each angle to give an average phase that produces a characteristic spot intensity (i.e. spot ‘brightness’). Each crystal therefore gives unique diffraction patterns (Figure 1) which are measured by crystallographers to determine the spatial location of the atoms that scattered the X-rays to visualise the protein structure (after inferring the phase information of each spot).

Electron crystallography

XRC requires large crystals, since X-rays interfere weakly with crystals, which accumulate damage and degrade over time, therefore requiring more matter to gain sufficient signal (Henderson, 1995). X-rays deposit 1333x more energy than electrons in to the crystal per elastic scattering (ES) event (single scattering of a wave without loss of amplitude or phase) (80keV vs 60eV per ES respectively) but electrons interact more frequently, degrading crystals very quickly (Glaeser, 1971). To accommodate for this challenge, 2 dimensional electron crystallography (2DEC) was developed in the 1970s, using thin crystals that reduce electron interaction, thereby reducing noise. The technique successfully determined the structure of purple membrane protein (today known as the light-driven proton pump “bacteriorhodopsin”) and the enzyme catalase (Unwin & Henderson, 1974) (Henderson & Unwin, 1975), and had the unique ability to form crystals of proteins within a lipid bilayer (an important property of proteins embedded in cellular membranes, Wisedchaisri, Reichow & Gonen, 2011), which was essential for the structural characterisation of the long-studied aquaporins in open/closed conformations (Andrews, Reichow & Gonen, 2008), typified by Aquaporin-0 to 1.9A (Gonen et al, 2005). Research continued well into the millennium (Nogales, Wolf & Downing, 1998) (Ruprecht et al, 2004) (Nettles et al, 2004) but waned over time, possibly due to quick crystal degradation (Zou & Hovmöller, 2008) and laborious crystallisation processes. Work attempted to probe the use of electrons on increasingly thicker 3 dimensional macromolecular crystals (3DEC) in the late 20th century onwards (Shi et al, 1995, Dorset, 1998), and in the new millennium Jan Pieter Abrahams gained a single Lysozyme diffraction pattern from a microcrystal using 3DEC (Georgieva et al, 2007) and wrote extensively onwards to create a bridge from 2DEC to 3DEC (Abrahams et al, 2009) (Abrahams, 2016). Unfortunately, the solo Lysozyme diffraction patterns provided a challenge as the orientation of the crystal lattice for each pattern was unknown, so merging data from multiple crystals was difficult. Smaller crystals and better detectors were able to improve signal-to-noise ratios (SNR) (Nederlof et al, 2011), and the group reduced the electron dose (to 0.1 e Å² s⁻¹), preventing degradation and acquiring more diffraction patterns per crystal (Nederlof et al, 2013), allowing orientation of patterns and overcoming the first hurdle of 3DEC.
Protein crystals give unique diffraction patterns due to the atomic arrangement of the protein (i.e. the structure of the macromolecule). For this reason, each crystal has a characteristic diffraction pattern that can be analysed to determine the atomic structure of the crystallised molecule. This is illustrated using the diffraction pattern of glycine modified mono-acetoacetyl insulin (from Lindsay & Shall, 1969 with permission), which presents a hexagonal star-shaped pattern, demonstrating the symmetry that is inherent in protein crystallography and diffraction patterns.

THE HISTORY OF MicroED

Low dose electron beams using 3D microcrystals

"Microcrystal Electron Diffraction" (MicroED, hereon used as a catch-all term for the method used by Gonen and Abrahams) was pioneered in the laboratory of Tamir Gonen, and initially used 3D Lysozyme microcrystals of $2 \times 2 \times 0.5\mu m$ to demonstrate proof of concept (Figure 2) (Shi et al, 2013), similar to the Abrahams group. The group also found that after $9e^-/\AA^2$ the diffraction patterns deteriorated, thus by using $\sim0.5\mu M^3$ microcrystals and a $0.01e/\AA^2s^{-1}$ dosage over 10 second exposures per angle, they collected 90 diffraction patterns per crystal, three times more than the Lysozyme crystals of Nederlof et al, 2013. A wavelength of 0.025Å (with a 200kV acceleration current) was used, shorter than typical XRC wavelengths ($\sim1\AA$). Consequently, the Bragg angles are smaller, showing more diffraction spots in a single illumination than XRC; the Ewald sphere (a mathematical tool to determine how many reciprocal lattice points are accessible from a single illumination) is so large that it presents a virtually flat surface, producing slices through reciprocal space instead of the lunes typically seen in XRC, therefore sampling more lattice points per illumination (Figure 3, Nannenga & Gonen, 2014). Crystals were rotated $1^\circ$ between illuminations by tilting the specimen stage, with each spot observed 34 times from multiple angles on average. Notably, due to the stationary crystal during illuminations, true intensities of each spot were lost as the slices through reciprocal space do not intersect each spot perfectly, leading to loss of intensity data. Due to the extensive sampling of each spot however, crude intensity approximations were made by assuming that the highest intensity of a spot represented the ‘true’ intensity, though future techniques would more accurately sample true intensities (see Continuous crystal rotation in MicroED). The Lysozyme structure was solved to 2.9Å, using Molecular Replacement (MR, a technique that derives phase information from prior experiments of the same molecule) (Cipriani et al, 2012).
Continuous crystal rotation in MicroED

The Gonen group introduced 'continuous-rotation' (CR)-MicroED: uninterrupted observation of diffraction data during continual crystal rotation, with frame rates of 4s/frame with 0.09°/s rotation, recording 0.36°/frame (Nannenga et al, 2014b), and the subsequent Lysozyme pattern compared better with XRC diffraction data than static-MicroED (2.5Å vs 2.9Å respectively, Figure 4). This is presumably because a) rotation allows reciprocal lattice points to properly intersect the slice through reciprocal space and b) rotating matter reduces beam contact time, reducing inelastic-scattering (IES, where incident electrons are deflected by atomic electrons and lose energy) and multiple-elastic-scattering (MES, where electrons elastically scatter multiple times) effects which contribute to spot intensities to give non-true values. This matched previous observations suggesting that precession imaging reduces MES (Georgieva et al, 2007). The group suggested that larger unit cells (the repeating unit of molecules throughout the crystal) and lower symmetry (the orientation of proteins within the unit cell) of the static vs CR Lysozyme structure (p2;2;2; vs p4;2;2) may increase noise due to lower protein/solvent ratio, increasing solvent contribution and lowering resolution. Further CR-MicroED work used XRC software to generate a Catalase structure to 3.2Å (Nannenga et al, 2014b), before the Abrahams group solved a Lysozyme structure to 2.1Å resolution, showing that CR-MicroED can be implemented by multiple groups (Clabbers et al, 2017).

The resolution breakthrough

As more MicroED macromolecular structures were solved (Yonekura et al, 2015) (Seidler et al, 2018), protocols were published outlining data collection and analysis (Hattne et al, 2015) (Shi et al, 2016) (Hattne et al, 2016) including microcrystal acquisition from macro-crystals using sonication, pipetting and vortexing (de la Cruz et al, 2017). The resolution breakthrough came with an α-synuclein structure at 1.4Å resolution, using MR that utilised β-strand motifs as a search model for this simple protein (Rodriguez et al, 2015) before the Sup35 amyloid core component (GNNQQNY, a heptapeptide) was elucidated to 1.1Å (Sawaya et al, 2016). This marked a turning point for MicroED; solving structures without prior XRC data (ab initio), an achievement previously applied only to organic compounds by the Abrahams group (van Genderen et al, 2016). More MR/direct structures appeared (Krotee et al, 2017), including the first novel macromolecule structure, TGF-βm-TβRII (de la Cruz et al, 2017) and when applied to inorganic crystals solved Au₄₁₋₄₆(p-MBA)₁₅ micro-clusters to 0.85Å via direct methods (Vergara et al, 2017) and zeolite crystals to 0.8Å – 1.0Å (by Tim Gruene’s group, associated with Abrahams) (Gruene et al, 2018). By using MicroED, more than 17 structures have been solved (Rodriguez, Eisenberg & Gonen, 2017), alongside recent structures of a prion protofibril (the PRPcore) to 0.75Å (Gallagher-Jones et al, 2018) the TDP-43 core to 1.4Å (Guenther et al, 2018), and the fused in sarcoma (FUS) protein amyloid forming core to 0.73Å.
Figure 3 Illumination using electrons creates slices (left) through reciprocal space instead of lunes typically observed in XRC (right).

The image shows that at an angle perpendicular to a crystal axis, every reciprocal lattice point within the plane was observed using electron sources (with a complete absence of lune formation), while the XRC experiment demonstrated circular lunes. Image from Nannenga & Gonen, 2014, with permission.

by a Chinese group (Luo et al, 2018). More MicroED to amyloid studies to 1.1Å are archived and awaiting publication (Hughes et al, 2017) as a study on HIV-1 Gag STD-SP1 binding of Bevirimat to 2.9Å (Purdy et al, 2017). Recent reviews highlight MicroED’s history and future improvements (Rodriguez, Eisenberg & Gonen, 2017), and an engaging review probing the mathematical and theoretical principles of the technique (Clabbers & Abrahams, 2018).

Challenges facing MicroED

Nannenga et al, 2014b noted that large crystals may contribute more MES events while separate work predicted that ≥0.1µm thickness would give unusable data (Subramanian et al, 2015). Surprisingly Proteinase K crystals of 0.1 – 1µm thickness acquired good resolution (up to Rwork = 0.2218) when studied by Martynowycz et al, 2017, whereby crystal disorder was suggested to explain the disparity between the experiment and predictions. Nonetheless, a negative correlation between crystal volume and resolution was observed; thick crystals absorbed electrons more frequently. This presents a problem; crystals must be large enough for observable ES, while minimising IES and MES. IES occurs 3x more than ES to add noise (Henderson, 1995), prompting Nannenga et al, 2014b to suggest energy filters for IES to improve SNR in line with other work (Yonekura, Mak-Yonekura & Namba, 2002) (Leis et al, 2008). Phasing methods present another challenge; acquiring phases is challenging for X-ray (Taylor, 2003), Electron (Dorset, 1997) and Neutron crystallography (Hauptman & Langs, 2003). XRC overcomes this using Isomorphous Replacement (IR: heavy metal soaking of crystals to off-set phases and infer the original phases) and anomalous scattering (AS: whereby X-ray wavelength is altered to deposit energy into heavy metals which off-set phases to infer original phases) (Hendrickson & Ogata, 1997), but MicroED has been unsuccessful in implementing IR/AS so far. Further work is required to develop MicroED phasing methods.
MicroED and XRC

Synchrotron facilities and X-ray free-electron lasers (XFELs) generate short X-rays useful for high resolution diffraction and yielded 0.65Å Lysozyme structures (Wang et al, 2007). Comparably, MicroED has acquired Lysozyme to 1.8Å using MR (de la Cruz et al., 2017), demonstrating that cutting edge XRC techniques are currently still superior to MicroED. However, MicroED requires a handful of crystals within an EM laboratory, whereas XFELs require many crystals and along with synchrotrons (that use a similar amount of crystals as MicroED) are located within specialised institutes that typically require booking ahead of time, (Shi et al., 2016). Considering that current MicroED methodology limits resolution to ~0.025Å compared to ~0.48Å in the highest resolution XRC structure which is Cambrin (Schmidt et al., 2011), MicroED may one day surpass XRC. When considering crystal acquisition, crystallisation is often the critical time-consuming bottleneck in XRC studies, and in cases where crystallisation fails to produce large crystals, MicroED might be applicable since these failed conditions often produce microcrystals (Stevenson et al., 2014) and (Stevenson et al., 2016). While previously regarded as a by-product of improper crystallisation, MicroED (and XFEL analysis) allows microcrystals to be useful for

Figure 4 Lysozyme diffraction data of CR MicroED, static-frame MicroED and XRC experiments

The (001) diffraction plane of Lysozyme using CR-MicroED (c), static-frame MicroED (a) and XRC (b) are shown. CR-MicroED shows the best agreement with the XRC diffraction pattern. The Pearson correlation coefficients (describing similarity between diffraction data sets) shown in (d) were 0.76 between CR-MicroED (c) and X-ray data (b), vs 0.56 for static-frame MicroED (a) and X-ray data (b). Image from Nannenga et al, 2014b, with permission.
structural studies, allowing crystallographers to make use of more crystallisation conditions. MicroED struggles to get high macromolecular resolution compared to small molecules, but further development will undoubtedly improve this by understanding how various properties affect resolution (see ‘Improvements for MicroED’). Additionally, IR-phasing methods have proved challenging in electron crystallography due to electron cloud shielding, requiring a dependence on MR from XRC studies or ab initio phasing methods (see ‘Overcoming the phase problem’). Although MicroED presents an exciting method with much potential, better understanding of the critical parameters that affect resolution and development of phasing methods is required to improve resolution.

**IMPROVEMENTS FOR MicroED**

**Rotation scope**

What challenges might MicroED overcome to increase macromolecular resolution? MicroED typically uses a ±70° crystal tilt, which becomes problematic depending on crystal symmetry; most crystals present with space groups (notations denoting the repeating symmetry of proteins within unit cells) of P2₁,2₁,2 and P₂₁ (Wukovitz & Yeates, 1995), giving unique diffraction spots over 180°. A ±70° tilt limits the accessible data to 140° from a single crystal (commonly known as the ‘missing wedge’ of electron microscopy, Bartesaghi et al, 2008). A 360° tilt (Barnard et al, 1992) might improve single-crystal sampling and resolution.

**Optimal crystal sizes**

Crystal volume is important, requiring sufficient volume to provide signal while excessively large crystals generate significant MES/IES, prompting Martynowycz et al, 2017 to suggest an upper limit of 500μm for crystal sizes for optimal diffraction. Protocols exist for acquiring microcrystals from larger (>500 μm) crystals (de la Cruz et al, 2017), but understanding why certain microcrystal volumes give optimal resolution is necessary. Interestingly, currently the highest resolution data derives from small unit cell crystals with complex symmetry (see Table 1), suggesting that perhaps the protein atomicity of a crystal (i.e. protein molecules per Å³) or high solvent content (e.g. when protein atomicity is low) contributes to volume/resolution relationships. It is already known that solvents generate noise (Bragg & Perutz, 1952) (Fraser, MacRae & Suzuki, 1978), and are typically disordered and not uniformly oriented in crystals (Weichenberger et al, 2015), which could increase noise when protein atomicity is low (as solvents occupy more volume). When reviewing MicroED structures (Table 1), an observable trend between protein atomicity and resolution appears (Figure 5). Unfortunately, this compares structures over years from many laboratories, so variability is likely significant, however Sawaya et al, 2016 analysed GNNQNY in two different symmetries with the higher symmetry crystal giving better resolution, providing clear evidence that atomicity affects resolution. Further research is required to identify the relationship between ES/IES/MES and microcrystal volume.

Reducing electron dosage – crystal rotation speed

Electrons deposit less IES per ES than X-rays and less energy per IES, but interact more frequently with matter, leading to quicker crystal degradation (Henderson, 1995). Electron dosage is kept below a critical threshold (Shi et al, 2013) and continuous rotation reduces electron exposure to different crystal locations (Nannenga et al, 2014a). Rotation speeds and framerates are a compromise between adequate sampling of diffraction spots while preventing spot overlap (Hattne et al, 2015). A faster rotation with a shorter frame rate might reduce electron dosage while preventing spot overlap. Rotation speeds varied over experiments, from 0.1%/s (Vergera et al, 2017) to 0.29%/s (de la Cruz et al, 2017), but Error! Reference source not found. shows that when comparing resolution to crystal rotation speed (Figure 6) and the frame scope (the angle covered by a single frame, Figure 7), there appears to be no apparent trend; different rotation speeds and frame scopes did not correlate with resolution. It is notable that this data is a collection of many years work from various laboratories and equipment, and therefore inter-laboratory and inter-assay variability likely contributes significantly to resolution quality, as mentioned in Optimal Crystal Sizes. Indeed, the Gonen group point out that the increase in resolution over their 3 Lysozyme structures is attributed to improved data collection and processing (Nannenga & Gonen, 2018). To date, no published work specifically dedicated to the effect of rotation speeds is available. Further research is required to probe rotation speed/frame rate contribution to diffraction patterns and resolution using identical crystals to control for all other variables.

Reducing electron dosage – electron wavelength

The incident beam might be adjusted to reduce electron dosage; electrons with a shorter wavelength have been demonstrated to degrade crystals slower and improve longevity (Glaeser, 1971), and while MicroED uses an electron wavelength of 0.025Å, no work assessing wavelength effect on ES/IES/MES has been published. Electron exposure was assessed in Protease K and GSQNNF crystals however; high-resolution spots degraded at the 0.025Å wavelength (likely due to their typical low signal) with high resolution data (>2.0Å) being severely degraded at >3 e Å⁻² (Hattne et al, 2018). At low doses electrons were absorbed and shielded atoms from proper ES, e.g. at ≥0.9 e Å⁻² disulphide bridges deteriorated and broke at 5.78 e Å⁻², while glutamate/aspartate residues lost carboxyl density at ≥2 e Å⁻² which was absent at 5 e Å⁻², meaning electron dosage contributes to diffraction data quality and is an important element to control. Higher frequency electrons interact with matter less frequently (Birkhoff, 1958); therefore further research could build on the work of Hattne et al, 2018 to determine whether shorter wavelengths might reduce electron/crystal interactions, reducing dosage and IES/MES to improve resolution.
Overcoming the phase problem

2DEC IR techniques have been attempted, but shielding of metal nuclei by large electron clouds weakened IR interference (Ceska & Henderson, 1990), however some argued that heavy metal phase contributions can theoretically give sufficient information to solve phases (Burmester & Schroder, 1997). While MicroED has successfully used direct methods to process data (Sawaya et al., 2016, de la Cruz et al., 2017, Vergara et al., 2017, Martynowycz et al., 2017), these computational methods require resolution to be 1.2Å or better, (aka 'Sheldrick rule', Sheldrick 1990, Morris & Bricogne, 2003). Consequently, this method is currently limited to small molecules, which tend to give the highest resolution data (Taylor, 2010) as seen in the Sawaya et al., 2016 publication. Direct methods have been used on macromolecular data of ~2Å in XRC by prospective MR using archetypal α-helices/β-sheets as search models, solving the structure of a previously unknown 111-residue protein (Rodriguez et al., 2009). This may benefit MicroED, but may not be appropriate for macromolecules lacking sufficient α-helix and β-sheet structures. XRC also overcomes the phase problem using AS, requiring wave absorption by heavy atoms (Hendrickson & Ogata, 1997) an effect that electrons have been predicted to be capable of achieving (Burmester & Schroder, 1997). A breakthrough is needed in MicroED analogous to IR/AS to find dependable phasing methods for macromolecular crystals.

Improvements to equipment and data processing

Tim Gruene’s group is focussing on the development of electron detectors in MicroED, previously publishing a demonstration of a novel electron detector that samples smaller pixel areas and has shorter dead time between frames, reducing data loss from pixel-related overlap of intensities (Tinti et al., 2018). Furthermore, the Abrahams group applied new DIALS integration software successfully as they continue to optimise the equipment, technology and software involved in MicroED (Clabbers et al., 2018). This comes after previous work from the group utilising other detectors in MicroED (Clabbers et al., 2017) to increase SNR. The Gonen group noted that IES electrons may be filtered out of the diffraction data by using energy filters to remove IES (Nannenga et al., 2014b), but have seemingly not yet utilised this approach. More innovation and development of equipment and data processing to improve SNR would greatly improve resolution, and energy filters must be explored to determine their effect on SNR.

TECHNICAL REQUIREMENTS AND METHOD PROCEDURE

How challenging is MicroED from a technical point of view? Can the typical electron microscopy department implement the necessary adaptations for MicroED experiments? A comprehensive method guide was recently outlined by Nannenga & Gonen, 2018, reporting that standard equipment used in single particle analysis (EM, tilting stage, carbon grids and liquid ethane vitrification) are sufficient for MicroED, making the method readily accessible for many EM equipped departments or facilities. MicroED requires a high frame-rate camera fast enough to capture individual reflections and prevent spot overlap, and high speed electron detectors to minimise between-frame readout times which do not sample the continually rotating crystal, including CMOS, hybrid pixel array and potentially direct electron detectors (as used previously by the Abrahams group, van Genderen et al., 2016). A comprehensive procedural MicroED protocol was also outlined by Shi et al., 2016, including the necessary equipment, materials, and sample preparation, data acquisition and processing. Briefly, the process involves crystallisation and microcrystal identification, before calibrating electron dosage, stage height and tilt parameters and setting up data collection processes. A trouble shooting guide is included in the Nannenga & Gonen, 2018 publication and an outline of a home-made device to ensure constant crystal tilt is included in Shi et al., 2016. Data can be processed and refined using standard XRC software, and structural models can be built using COOT, as outlined by Hattne et al., 2015. Thus, aside from an appropriate camera and a home-made tilt controller, MicroED is readily available to any department equipped for single particle analysis, with supporting protocols and guides to aid researchers.

MicroED IN THE WIDER COMMUNITY

The structural biology community increasingly is utilising MicroED in structural studies. A group in Stockholm published Lysozyme structures to 2.2Å by merging multiple datasets and commented on MicroED’s main challenges: 1) crystal degradation and 2) goniometric imperfections in crystal rotation (Xu et al., 2018). The first use of MicroED to study amyloid fibrils outside of the Gonen group was published by Chinese researchers, solving the (FUS) amyloid core to 0.73Å (Luo et al., 2018). MicroED was applied to chemical crystallography by Japanese researchers to complement solid-state (crystalline) NMR of L-histidine in order to probe crystal lattice parameters (Oikawa et al., 2017). Other groups are attempting to improve the technique; Australian researchers assessed the effect of small micocrystals (with more incomplete unit cells on the surface) on Bragg spot broadness and theoretical adjustments necessary to properly integrate diffraction spots (Williams et al., 2017). A US researcher suggested that maps generated from MicroED might be utilising sub-optimal MR methods as structure factors don’t take negative contributions from IES into account (Wang, 2017) and Korean researchers reviewed the history of electron crystallography from 2DEC to MicroED (Lee, Chung & Jung, 2017).
The table shows MicroED experiments that used crystals of four proteins (Proteinase K, Lysozyme, Catalase and the hepta-peptide GNNQQNY) under different crystal rotation speeds, frame rates, crystal unit cells and volumes, along with the resultant resolution of the final structures. References to relevant publications are given in the table. The relationship of resolution plotted against protein atomicity (density within the unit cell, defined as the volume per molecule), crystal rotation speed and frame scope (the total angle sampled by a single frame) are shown in Figure 5, Figure 6 and Figure 7, respectively.

| Molecule   | Crystal rotation speed (°/s) | Frame rate (s/frame) | Rotation per frame (°) | Space group | Molecules per unit cell | Unit Cell Volume (Å³) | Volume per molecule | Resolution (Å) | Reference                  |
|------------|------------------------------|----------------------|------------------------|-------------|------------------------|----------------------|-------------------|----------------|---------------------------|
| Proteinase K | 0.090                        | 4.0                  | 0.360                  | p4_2_2_2    | 8                      | 452897               | 56612             | 1.60           | de la Cruz et al, 2017    |
|            | 0.089                        | 5.1                  | 0.454                  | p4_2_2_2    | 8                      | 452267               | 56533             | 1.71           | Hattne et al, 2018        |
|            | 0.090                        | 4.0                  | 0.360                  | p4_2_2_2    | 8                      | 457776               | 57222             | 1.75           | Hattne et al, 2016        |
| Lysozyme   | 0.090                        | 4.0                  | 0.360                  | p4_2_2_2    | 8                      | 215821               | 26978             | 1.80           | de la Cruz et al, 2017    |
|            | 0.152                        | 0.5                  | 0.076                  | p2_1_2_1    | 4                      | 228046               | 57012             | 2.11           | Clabbers et al, 2017      |
|            | 0.450                        | 2.0                  | 0.900                  | p2_1_2_1    | 4                      | 222650               | 55663             | 2.20           | Xu et al, 2018             |
|            | 0.090                        | 4.0                  | 0.360                  | p4_2_2_2    | 8                      | 212691               | 26586             | 2.50           | Nannenga et al, 2014b     |
|            | N/A                          | 10.0                 | N/A                    | p4_2_2_2    | 8                      | 219373               | 27422             | 2.90           | Shi et al, 2013            |
| Catalase   | 0.09                         | 6.0                  | 0.540                  | p2_1_2_1    | 4                      | 2125468              | 531367            | 3.20           | Nannenga et al, 2014a     |
|            | 0.75a                        | 2.0a                 | 1.500a                 | p2_1_2_1    | 4                      | 2466129              | 616532            | 3.20           | Yonekura & Maki-Yonekura, 2016 |
| GNNQQNY    | 0.30                         | 2.0                  | 0.600                  | p2_1_2_1    | 4                      | 4625                 | 1156             | 1.05           | Sawaya et al, 2016        |
|            | 0.30                         | 2.0                  | 0.600                  | p2_1_2_1    | 2                      | 2726                 | 1363             | 1.10           | Sawaya et al, 2016        |

*Note: for this Catalase experiment, various frame rates and rotation speeds were used from 0.5-1.0° over 1-3 seconds per frame median values of these ranges are displayed in the table.*
Figure 5 Resolution vs molecular crystal atomicity from MicroED structures of common proteins

MicroED structures of Proteinase K (blue), Lysozyme (red), Catalase (green) and GNNQQNY hepta-peptide (purple) are plotted to correlate structure resolution to unit cell atomicity, i.e. volume per protein molecule. Crystals with higher symmetry and tighter packing have less volume per molecule, and higher atomicity. More densely packed cells with higher atomicity give higher final structure resolution. All experimental information is derived from Table 1.

Figure 6 Resolution vs crystal rotation speed from MicroED structures of common proteins

The relationship between resolution and crystal rotation speed of all Table 1 molecules are shown above, colour coded for atomicity. Very high atomicity = 1000-1500 Å³/molecule, High atomicity = 25000-30000 Å³/molecule, Medium atomicity = 55000-60000 Å³/molecule, Very low atomicity = 500000-650000 Å³/molecule.

Figure 7 Resolution vs frame scope from MicroED structures of common proteins

The relationship between resolution and frame scope (angle sampled per camera frame) of all Table 1 molecules is shown above, colour coded for atomicity. Very high atomicity = 1000-1500 Å³/molecule, High atomicity = 25000-30000 Å³/molecule, Medium atomicity = 55000-60000 Å³/molecule, Very low atomicity = 500000-650000 Å³/molecule.
CONCLUSION

MicroED is an exciting method utilising short wavelengths that offer better theoretical resolution limits than XRC, while simultaneously sampling more reciprocal space per illumination. While modern macromolecular EM work typically relies on single particle analysis without phasing methods or crystals (Orlova & Saibil, 2004), MicroED offers significantly higher resolution; a characteristic that makes crystallographic methods so popular (Wilkins, 2013). MicroED offers an affordable and accessible XRC alternative by utilising EMs commonly used in many structural laboratories with minor adjustments. CR-MicroED and XRC diffraction software have improved the method, but more work is required to characterise the effect of crystal volume, unit cell packing, electron wavelength, crystal rotation speed, frame rate capture and the use of energy filters on resolution. While MicroED works very well with small molecule crystals, its use on larger molecules lacks comparable resolution, likely due to solvent/protein ratios of large unit cells with low symmetry crystals creating noise. For high macromolecular resolution, MicroED depends on MR from XRC studies. To be a standalone technique, MicroED must develop phasing methods for macromolecular datasets. Computational methods have somewhat addressed this challenge at resolutions of 1.2Å and 2.0Å, but macromolecules typically give lower resolutions than 1.2Å and even at 2.0Å may not present sufficient secondary structures to utilise the phasing method, therefore phasing is particularly troublesome and must be addressed by developing innovative phasing methods. As it stands, MicroED is a powerful technique for small molecules, elucidating these structures in unprecedented detail and might perhaps be considered better than XRC in terms of resolution and practicality. Further work and innovation will be essential for solving the MicroED phase problem and producing the next series of breakthroughs for this impressive technique.

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