HHS Public Access

Author manuscript

Cell Rep Phys Sci. Author manuscript; available in PMC 2024 July 25.

Published in final edited form as:

Cell Rep Phys Sci. 2024 June 19; 5(6): . doi:10.1016/j.xcrp.2024.102007.

Reaching the potential of electron diffraction

Devrim Acehan[1,2,3], **Katherine A. Spoth**[1], **Gabrielle R. Budziszewski**[1], **M. Elizabeth Snell**[1], **Christopher S. Campomizzi**[1], **Miranda L. Lynch**[1,2], **Sarah E.J. Bowman**[1,2,4,*]

[1]Hauptman-Woodward Medical Research Institute, Buffalo, NY 14203, USA

[2]University at Buffalo, Jacobs School of Medicine and Biomedical Science, Department of Structural Biology, Buffalo, NY 14203, USA

[3]University of Rochester, School of Medicine and Dentistry, Department of Biochemistry and Biophysics, Rochester, NY 14642, USA

[4]University at Buffalo, Jacobs School of Medicine and Biomedical Science, Department of Biochemistry, Buffalo, NY 14203, USA

SUMMARY

Microcrystal electron diffraction (MicroED) is an emerging structural technique in which submicron crystals are used to generate diffraction data for structural studies. Structures allow for the study of molecular-level architecture and drive hypotheses about modes of action, mechanisms, dynamics, and interactions with other molecules. Combining cryoelectron microscopy (cryo-EM) instrumentation with crystallographic techniques, MicroED has led to three-dimensional structural models of small molecules, peptides, and proteins and has generated tremendous interest due to its ability to use vanishingly small crystals. In this perspective, we describe the current state of the field for MicroED methodologies, including making and detecting crystals of the appropriate size for the technique, as well as ways to best handle and characterize these crystals. Our perspective provides insight into ways to unlock the full range of potential for MicroED to access previously intractable samples and describes areas of future development.

INTRODUCTION

The fields of experimental structural biology and structural chemistry have under-gone explosive growth in the past decade, with multiple advances in all areas of structure determination. In X-ray crystallography, these advances include serial synchrotron techniques and X-ray free-electron laser (XFEL) sources, enabling time-resolved studies that provide molecular movies of biomolecular dynamics.[1,2] Developments in electron detectors, electron microscope optics, computer hardware, and image correction and analysis software have made the cryoelectron microscopy (cryo-EM) “resolution-revolution” possible.[3]

*Correspondence: sbowman@hwi.buffalo.edu.

AUTHOR CONTRIBUTIONS

Conceptualization, D.A., M.L.L., and S.E.J.B.; writing – original draft, D.A., K.A.S., G.R.B., C.S.C., M.L.L., and S.E.J.B.; writing – review & editing, D.A., K.A.S., G.R.B., M.E.S., C.S.C., M.L.L., and S.E.J.B.; funding acquisition, D.A., M.L.L., and S.E.J.B.

DECLARATION OF INTERESTS The authors declare no competing interests.

Single-particle cryo-EM methods are now reaching resolution limits that were previously obtainable only with diffraction-based methods[4,5] but without needing to crystallize the sample. Despite these developments, more conventional X-ray sources still have advantages, such as extremely fast data collection, efficient methods for fragment and small-molecule structures (structure-based drug design), and ease of obtaining high-resolution structural models for a wide range of molecule sizes. Most crystal-based structural data have been, and still are, collected using X-ray diffraction, at either home sources or synchrotrons. Finally, computational structure prediction tools such as AlphaFold2 (AF2), trained on the body of experimental protein structures, provide an additional advance in macromolecular structure determination.[6–8] These remarkable computational tools not only predict structures but also contribute to providing models for molecular replacement methods, largely solving the “phase problem” in X-ray crystallography.

A recent addition to the structural science toolbox is microcrystal electron diffraction, frequently called MicroED, three-dimensional (3D) electron crystallography, or 3D ED. MicroED combines aspects of crystallography, in that it is a diffraction-based technique requiring crystalline targets, and of EM, in that it uses an electron beam in a transmission electron microscope (TEM) setup to probe the sample on an EM grid. Previously, electron diffraction was primarily used for 2D crystals, which can be described as a single crystal layer embedded in a lipid bilayer or proteins forming sheets or tubular structures.[9] Early examples of electron diffraction usage include structures of bacteriorhodopsin[10,11] and aquaporin-0.[12] While these methods can result in high-resolution structures, the number of suitable samples forming such crystals remains extremely limited. Recently, there has been increased interest in electron diffraction applications for 3D crystals, where multiple layers of the molecule extend in all three dimensions. MicroED is a technique that has great potential to determine the structures of molecules from an impressive array of sizes, from macromolecules to peptides and small molecules. In this perspective, we highlight the unique features of MicroED that separate it from other structural methods, as well as the distinctive challenges and opportunities inherent in this emerging technology.

It is helpful to consider the primary distinctions between X-ray and electron diffraction for structure determination, as the difference in scattering between the two sources dictates the crystal sizes needed (Figure 1). These experiments generate data from the interactions of electrons or X-rays with the sample, causing cohesive diffraction and constructive interference due to the ordered array of molecules that form crystals (Figure 2). In X-ray methods, X-ray photons scatter off the electron clouds around individual atoms. Since the X-ray photons are uncharged and mass-less, this interaction is quite weak. Therefore, larger, macroscopic crystals are typically required to generate a sufficient signal. In electron diffraction, by contrast, the nonzero mass and negative charge of the interrogating electrons allow much stronger interactions with the sample. The electron scattering is dictated by the electrostatic potential, which combines contributions from the electron charge density and the nuclear charge density, such that the strong Coulombic interactions enable diffraction from much smaller-volume crystals. The short mean-free path of electrons, in comparison to X-rays, allows diffraction data to be collected from crystals that are thousands of times smaller than those needed for X-ray diffraction data. Electrons accelerated within common electron microscopes have more interactions than X-rays when traveling equal distances

within crystals, resulting in more diffraction and higher signals from small crystals. On the other hand, an electron traveling through a larger crystal may interact multiple times, resulting in noise or total signal loss from that electron. Indeed, these multiple scattering events were considered one of the primary obstacles to the utility of MicroED methods. The extent of this “dynamical diffraction” derives directly from the density, thickness, and intrinsic ordering of the crystal, as well as from the energy of the impinging electron and the angle of orientation of the crystal to the electron beam. In addition to enabling the measurement of full reflections more accurately, continuous rotation methods also assist with the latter feature.[13,14] While higher energies for the electrons reduce the likelihood of scattering in general, crystal size remains a fundamental influence on dynamical diffraction. Therefore, it is critical to have high-quality small crystals suitable for MicroED experiments.

There are various ways to generate crystals in the submicron size range required for MicroED. In one approach, large crystals are produced and manipulated in some way to make them small enough for the electron beam to penetrate. In another approach, crystals of the appropriate size are detected, harvested, and used directly. Small molecules are more likely to form suitable small crystals, which can be used directly for MicroED, whereas macromolecular crystals have proven more challenging to work with (Figure 3). While there have been advances for small crystal detection methods and in focused ion beam (FIB) milling, the crystal size requirement, especially for biomolecular crystals, continues to be a limiting factor in the widespread adoption of MicroED for macromolecular structure determination.

THE BIG CRYSTAL APPROACH: MAKING BIG CRYSTALS SMALL

A range of techniques and various physical manipulations are used to generate small crystals from larger crystals. One method mechanically disrupts large crystals into the appropriate size range, often by crushing or vortexing. A similar strategy, referred to as seeding, is often employed for crystal optimization for X-ray diffraction experiments. In seeding, microscopic crystal fragments nucleate the growth of larger crystals. The crystal seeds are made from crushing or vortexing larger crystals, and an array of tools (seed beads, glass seeding tools, etc.) have been developed to fragment the crystals.[15–17] However, crystal fragmentation can yield un-predictable outcomes. Further, macromolecular crystals can be up to ~65% solvent,[18] resulting in weak intermolecular interactions that hold the crystal lattice together, making the crystals easily destroyed instead of just disrupted. Fragments may not retain the diffraction properties of the original large crystals. Finally, this method can result in a heterogeneous range of crystal sizes, which will complicate a MicroED experiment by requiring substantial effort to triage appropriately sized crystals on the grid. In an effort to exert greater control over the sizes of crushed crystals, seeding techniques have been developed to generate seed crystals more homogeneously by using small-diameter zirconium beads and higher vortexing speeds to efficiently fragment crystals to a narrow size range that can be fine-tuned by varying the bead size and vortexing speed.[19] This method improves control over the final average crystal size and could be adapted for experiments that require crystals of different orders of magnitude, including MicroED, serial synchrotron, or XFEL experiments.

Another protocol that has been employed to reduce crystal dimensions for macromolecular MicroED diffraction is cryo-FIB milling, which has been used to shape crystals already frozen onto cryo-EM grids. The cryo-FIB milling technique addresses major challenges for MicroED, including inappropriate sample thickness and complex sample media such as viscous mother liquor and lipidic cubic phase mixtures, which can obfuscate crystals on the grid. The milling approach first uses a cryogenic scanning electron microscope to identify suitable crystals following platinum sputter coating. The crystals are then roughly milled, finely milled, and polished with a suitable ion beam to create a crystal lamella suitable for electron diffraction.[20] These crystal lamellae can not only be tuned to a suitable thickness for electron diffraction but can also eliminate artifacts from the sample media.

All of the approaches using larger crystals to produce crystals small enough for MicroED still require generating the initial larger crystal, which remains a major bottleneck, especially in macromolecular crystallography.[21] These methods therefore limit the MicroED approach to crystals that can be generated as a larger crystal and manipulated into the correct size for the technique. This approach does not allow researchers to take full advantage of the less demanding crystal size requirements of the MicroED methods.

THE SMALL CRYSTAL APPROACH: METHODS TO DETECT AND HANDLE NANOCRYSTALS

Until recently, the useful size of macromolecular crystals for diffraction methods was >30 μm due to limitations in synchrotron beam brilliance and large beam sizes. However, as synchrotron sources have become brighter and beams smaller, and as more XFEL sources have come online, crystals down to 1–5 μm in size can now yield structurally useful information using these methods. Further, vanishingly small crystals (on the order of 200–600 nm thick) have found utility as a sample source for MicroED, which requires small, thin crystals to produce usable diffraction patterns. Approaches that harness the ability to produce nanocrystals without physically altering the crystal enable those samples that produce only small crystals, and are therefore not amenable to X-ray diffraction, to be analyzed using MicroED methods.

MicroED for small-molecule studies has seen substantial development because molecular compounds of interest readily form nanocrystalline powders or deposits that can be applied directly in dry form to EM grids. The emergence of MicroED has therefore been a boon to the fields of synthetic chemistry, pharmaceutical chemistry, and chemical engineering. Two key studies in 2018[22,23] unleashed an explosion of interest in using MicroED for small molecules.[24,25] The pharmaceutical community has increasingly adopted the technique, which enables the characterization of natural products and active pharmaceutical ingredients that had defied previous efforts (using nuclear magnetic resonance spectroscopy and single-crystal X-ray diffraction) for structure determination.[26,27] MicroED has also been used to structurally characterize metal-organic frameworks, a new class of microporous material increasingly of interest in materials chemistry.[28] For small-molecule crystals, it can be sufficient to use the material directly, as the samples are often already in a microcrystalline form. X-ray powder diffraction prior to attempting the MicroED experiment can be helpful

to verify crystallinity. Powder samples are deposited on a cleaned EM grid, which can be loaded into the microscope at room temperature to eliminate ice crystal growth that occurs when loading in cryogenic conditions. Nevertheless, small-molecule samples are often then cooled in the microscope vacuum and imaged at liquid nitrogen temperature to slow the rate of damage to the samples.[29]

Biomolecular crystals, with a much higher solvent content than small-molecule crystals, can be more difficult to detect and to handle for MicroED. There have been advances on this front, many of them initially developed for other diffraction-based techniques that also require very small crystals (serial synchrotron and XFELs) (Figure 1). Often, features in crystallization experiments that yield “cloudiness” are used as a way to predict the possible presence of nanocrystals.[30] Nanocrystals are too small to be visualized by light microscopes, making them difficult to detect and to differentiate from precipitate. Using TEM has revealed that many macromolecular crystallization experiments thought to contain protein precipitate actually contained protein nanocrystals.[31] An additional method to visualize nano- and microcrystals makes use of nonlinear optical imaging, specifically second harmonic generation (SHG) and ultraviolet two-photon excited fluorescence (UV-TPEF). SHG employs a high-intensity femtosecond laser that relies on a second-order coherence process for visualizing anisotropic materials. In ordered systems like chiral crystals, the result of the frequency doubling of light is highly selective for crystalline material, with optical properties that reduce sensitivity to optical scatter.[32,33] It is important to note that while achiral crystals will not produce an SHG signal, the vast majority of protein crystals should produce a signal.[34] To determine whether a crystalline material is protein, UV fluorescence can be utilized.[35] UV fluorescence relies on the intrinsic fluorescence of aromatic amino acids, primarily tryptophan. UV-TPEF has some advantages over traditional UV fluorescence in that it improves signal to noise predominately by allowing excitation and emission within a defined focal volume.[36] Notably, the same instrumentation with slight modifications can be used for both SHG and UV-TPEF measurements, and the combination of both imaging methods enhances selection of the nanocrystalline material (Figure 4).[37]

MicroED grids for crystals in solution are prepared similarly to other cryo-EM experiments. Usually, EM grids are made hydrophilic using a plasma cleaning system to improve the interaction of the aqueous solution, containing the microcrystals, with the grid surface.[38,39] A solution of microcrystals is deposited on an EM grid (Figure 4), which is then blotted and plunge frozen in a cryogen, typically liquid ethane.[39,40] The fast freezing rate of this method is used to prevent crystalline ice formation and to vitrify the water. The frozen grid is then loaded into a cryo-holder and inserted into the EM. Often, microcrystals have a preferred orientation on the flat EM grid, which, together with the limitations of the sample rotation within the microscope, can result in incomplete data.[41] Apart from developing sample stages allowing larger tilt ranges along multiple tilt axes,[42] sample grids allowing crystals to rest in different orientations, such as holey or lacey grids or less common grid supports like nanofibers,[43] may improve data completeness of MicroED experiments. It has recently been demonstrated that crystals can be grown directly on EM grids, eliminating the need for transferring crystals to the grid and potentially addressing the experimental difficulties encountered with preferred orientation. In this particular experiment, however, the crystals

were still larger than the submicron size needed for MicroED and required FIB milling prior to electron diffraction.[44]

EXPERIMENTAL CONSIDERATIONS

In any X-ray diffraction and EM technique, there is a balance between collecting sufficient data and collecting these data before the sample is heavily damaged or destroyed.[45] In a MicroED experiment, as the crystal is rotated continuously under the beam, later tilt angles will suffer a higher amount of beam-induced sample degradation. A recent study demonstrates that an exposure as little as 3 $e^{-}/Å^2$ may destroy the near-atomic resolution information (better than 2Å), and a decrease in diffracted intensities is observable with as little as 1 $e^{-}/Å^2$.[45] Because MicroED data are collected from a crystal composed of millions of molecules, a sufficient signal can be collected with an exposure rate as low as 0.01 $e^{-}/Å^2/s$, making it possible to collect continuous rotation datasets with a total exposure below 1 $e^{-}/Å^2$. Ultralow exposure rates permit the collection of MicroED data with electron counting detectors, leading to the first sub-Å MicroED structure from a protein crystal.[46] For crystals with preferential orientation on the grid, it can be beneficial to vary the starting angle over a number of crystals so that the highest-resolution information at the starting angle is collected across multiple orientations.

In addition to using low total exposure, optimizing crystal thickness is also an important consideration for beam-induced damage. The impact of inelastic scattering and multiple scattering increases exponentially for crystals thicker than the mean free path of an electron. Current microscope (with mean free path 200–300 nm) and detector technology dictate that the crystal thickness remains under twice the mean free path for high-resolution diffraction data.[47]

While it will not reduce the effects of beam-induced damage, energy filtering can improve diffraction patterns by removing most of the inelastically scattered electrons. This reduces low-frequency noise in the diffraction patterns and sharpens the diffraction spots in the case of electrons scattered both elastically and inelastically.[48] This is especially pronounced for protein crystals embedded in amorphous ice, due to the high inelastic scattering cross-section of electrons in ice. When compared directly with unfiltered data, structures solved using an energy filter show improved statistics.[49,50] With the inelastic signal removed, it becomes possible to investigate charge and bonding states in the model.[51]

Detector performance is an important consideration for MicroED experiments. A detector with minimal dead time is required to minimize missing angles between movie frames. Scintillator-coupled CMOS detectors, standard on many TEMs, are often sufficient, particularly for strongly diffracting small-molecule samples. Better performance can be achieved by hybrid pixel direct detectors like those commonly used in X-ray diffraction experiments. These detectors are optimized for diffraction experiments, combining fast readout with a high dynamic range and single-electron sensitivity. Counting detectors optimized for low-dose cryo-EM imaging also have produced high-resolution structures using MicroED. Here, experimental conditions must be carefully optimized, as a low electron exposure rate is necessary to minimize coincidence loss. Because these detectors

are so common in the field, they are currently the best option for many adopters of MicroED.

SOFTWARE CONSIDERATIONS

Currently, software used for MicroED data collection varies between laboratories based on microscope and detector combination. Minimally, data collection software needs to synchronize stage rotation and movie collection. Additional useful features could include map collection for crystal screening, saved microscope presets for imaging and diffraction, control over beam stop and apertures, batch data collection over a list of pre-selected crystal positions, and automatic recording of experimental parameters allowing the creation of input files for further processing. Some commonly used packages are SerialEM,[52–54] EPU-D (Thermo Fisher Scientific), Instamatic,[55] and Leginon.[26,56]

MicroED data processing has so far been done with software developed for X-ray diffraction data.[57–61] However, distortion in diffraction patterns due to the optics of the electron microscope can create difficulty in accurate determination of the lattice parameters.[62] This distortion can make it difficult to define input parameters for X-ray crystallography software. Some software pipelines have been developed that iteratively test inputs to find optimal values for each dataset.[63–65] Small-molecule processing is also possible in X-ray crystallography software. Software specifically for electron diffraction data has been developed and used extensively for these small-molecule samples.[66–68] Due to the limited tilt range accessible by a TEM stage, merging of multiple crystal datasets is usually required to increase completeness. Merging diffraction data from multiple crystals to optimize data quality is not new; developments in X-ray data experiments such as XFELs and serial synchrotron have assisted with multiple crystal merging in MicroED.[69] Additionally, automation in MicroED data collection can allow for the collection of hundreds of datasets in a single session. Clustering programs have been applied to determine the optimal datasets to merge.[63,70–73]

Nearly all small-molecule structures solved using MicroED were phased using direct methods. However, protein data have so far been phased almost exclusively using molecular replacement with a homologous structural model. Predicted structures from AF2 have been used for crystallographic phasing over the last several years to great success, and the first novel structures have recently been solved with AF2 models for MicroED.[74,75] Fragment-based phasing methods are also being investigated and have allowed ab *initio* phasing of peptides[76] and proteins, where an electron counting detector contributed to improved resolution.[46]

Standard refinement for X-ray crystallography does not account for dynamical scattering effects. While dynamical diffraction is prevalent in MicroED, its effects may be reduced by inelastic scattering and solvent scattering.[77] This enables the solution of protein structural models from relatively thick crystals using MicroED data with standard X-ray crystallography packages, although refinement statistics for these models tend to be worse than typical models from X-ray diffraction. Programs have been developed for the dynamic refinement of diffraction data based on Bloch wave calculations, which lead to improved

models,[78] and the ability to assign correct handedness of chiral compounds.[79] It has not been possible to apply these techniques to proteins, as the greater size and complexity of the molecule greatly increase the computational cost. A simpler general likelihood-based computational approach to assign a scaling factor based on estimated dynamical diffraction has been found to improve models for small molecules and for more weakly scattering proteins.[80,81]

FUTURE OUTLOOK

The ultimate goal of structural science is to relate molecular structure with molecular function. Given the 3D structure of a molecule, we can investigate molecular function and the impact of structural perturbations on function, as well as how molecules interact with one another. We are in the midst of an explosive growth period with new developments in ways to make use of diffraction-based structural methods such as MicroED. A major advantage of MicroED is the ability to use extremely small crystals. This same feature—the requirement for small crystals—is also a significant limiting factor in the widespread adoption of the technique. For small molecules, MicroED is making major inroads and generating structural details with astonishing rapidity, in part because purified samples are often already in a microcrystalline state inherently suitable for MicroED experiments. For macromolecules, the requirement for thin crystals has proven more difficult to address, although advances are being made in both using larger crystals to generate small crystals via techniques like FIB milling and developing new ways to detect and handle macromolecular nanocrystals directly.

While cryo-FIB milling approaches enable access to MicroED for larger crystals, more unexplored territory relies on developing new ways to see and work with submicron crystals. Crystal detection and handling methods continue to improve. Synergy between different diffraction-based methods is yielding advances in many directions. For example, microcrystal sample deposition onto EM grids for X-ray diffraction at the new nanofocus beamline VMXm at the Diamond Light Source[82] provides insight into different ways to prepare crystal samples on cryo-EM grids. These advances have great potential to enable structural investigation for samples that have previously been deemed intractable, as they may only produce small, un-detected but usable micro- and nanocrystalline material.

Similar to other structural techniques, individual samples will require optimization in terms of sample preparation, imaging, and analysis conditions. Nevertheless, it is important to develop methods and pipelines that can be applied to the majority of small crystal samples to improve sample throughput. With these pipelines in place, MicroED will become feasible for more samples that defy other structural methods.

As it stands, there are a number of limitations in the adoption of the MicroED technique. A limitation for many research groups is access to costly time on cryo-EM instruments. Ample microscope time is required to develop robust protocols for electron diffraction. Another limitation is access to suitable test samples for macromolecular MicroED. Diffraction from unadulterated macromolecular crystals is relatively untested, and cryo-FIB milling instrumentation for generating crystal lamellae is scarce. Further, a more unified, consistent,

and user-friendly data processing pipeline would make MicroED much more accessible. Data analysis pipelines being developed for multicrystal merging in serial synchrotron and XFEL diffraction experiments are informing on data pipelines for MicroED experiments. A unified file format output and experimental metadata from the instrument like those currently used for X-ray diffraction experiments would significantly enhance MicroED.[73]

Diffraction data collection using scanning TEM (STEM), in which the electron beam is focused to a much smaller size, enables the interrogation of smaller regions of crystals. STEM has been demonstrated for serial electron crystallography, where diffraction is collected at a single tilt and single probe position from many crystals.[83] 4D-STEM, in which the probe is scanned over the crystal as a diffraction pattern is collected from each scan position, allows for the investigation of nanoscale crystallinity and, when combined with discrete tilt series collection, enables structures to be solved from post-acquisition-defined subregions of crystals.[84–86] So far, continuous rotation has not been used with STEM probes, potentially because, with a nanometer-sized probe, it becomes more difficult to interrogate a consistent region of the crystal as it rotates. However, the STEM mode is not standard on popular dedicated cryo-EM instruments.

Uptake of the MicroED technique would benefit from instruments dedicated to electron diffraction that could overcome the current limitations of electron microscopes, with some solutions recently becoming commercially available. An ideal instrument could explore solutions to tilt limitations, incorporate new sample holders, and include detectors optimized for electron diffraction purposes. It may also include enhanced crystal detection capabilities using optical, nonlinear optical, TEM, and even fluorescence-based imaging of the areas of interest for determining the size and quality of crystals before setting up diffraction experiments in a more correlative fashion within the same instrument.

The promise of MicroED as an exciting new avenue to structure determination for both small molecules and for macromolecules has been demonstrated. But with this new frontier, new needs emerge for focused technology development, improved protocols and data processing pipelines, dedicated software tools, and taking all the steps necessary to move MicroED into the standard toolbox of structural scientists. MicroED has reached a level of maturity as a technique that warrants the community to come together to consolidate and establish data standards, formats and metadata requirements, and best practices for sample handling and data collection. We are at the brink of this realization of the enormous potential of MicroED; it is exciting to watch this burgeoning science make new contributions to our understanding of molecular structure.

ACKNOWLEDGMENTS

This perspective was supported by funding from Dr. Louis Skarlow Memorial Fund (S.E.J.B.) and National Institute of General Medical Sciences of the National Institutes of Health under award number R01GM141273 (S.E.J.B. and M.L.L.). For the electron diffraction image in Figure 2 and the TEM image of microcrystals in Figure 4, data were collected at the Hauptman-Woodward Medical Research Institute CryoEM Center. For the X-ray diffraction image in Figure 2, data were collected at Stanford Synchrotron Radiation Lightsource Beam Line 9-2. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract no. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research and by the National Institutes of Health, National Institute of General Medical Sciences (P30GM133894). The

contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NIGMS or NIH. All figures were generated using BioRender (currently available at BioRender.com).

REFERENCES

1. Orville AM (2020). Recent results in time resolved serial femtosecond crystallography at XFELs. Curr. Opin. Struct. Biol 65, 193–208. [PubMed: 33049498]
2. Barends TRM, Stauch B, Cherezov V, and Schlichting I (2022). Serial femtosecond crystallography. Nat. Rev. Methods Primers 2, 59. 10.1038/s43586-022-00141-7. [PubMed: 36643971]
3. Kühlbrandt W (2014). Biochemistry. The resolution revolution. Science 343, 1443–1444. [PubMed: 24675944]
4. Nakane T, Kotecha A, Sente A, McMullan G, Masiulis S, Brown PMGE, Grigoras IT, Malinauskaite L, Malinauskas T, Miehling J, et al. (2020). Single-particle cryo-EM at atomic resolution. Nature 587, 152–156. [PubMed: 33087931]
5. Yip KM, Fischer N, Paknia E, Chari A, and Stark H (2020). Atomic-resolution protein structure determination by cryo-EM. Nature 587, 157–161. [PubMed: 33087927]
6. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, et al. (2021). Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589. [PubMed: 34265844]
7. Baek M, DiMaio F, Anishchenko I, Dauparas J, Ovchinnikov S, Lee GR, Wang J, Cong Q, Kinch LN, Schaeffer RD, et al. (2021). Accurate prediction of protein structures and interactions using a three-track neural network. Science 373, 871–876. [PubMed: 34282049]
8. Lin Z, Akin H, Rao R, Hie B, Zhu Z, Lu W, Smetanin N, Verkuil R, Kabeli O, Shmueli Y, et al. (2023). Evolutionary-scale prediction of atomic-level protein structure with a language model. Science 379, 1123–1130. [PubMed: 36927031]
9. Glaeser RM, and Downing KH (1993). High-resolution electron crystallography of protein molecules. Ultramicroscopy 52, 478–486. [PubMed: 8116103]
10. Grigorieff N, Ceska TA, Downing KH, Baldwin JM, and Henderson R (1996). Electron-crystallographic refinement of the structure of bacteriorhodopsin. J. Mol. Biol 259, 393–421. [PubMed: 8676377]
11. Henderson R, Baldwin JM, Ceska TA, Zemlin F, Beckmann E, and Downing KH (1990). Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. J. Mol. Biol 213, 899–929. [PubMed: 2359127]
12. Gonen T, Sliz P, Kistler J, Cheng Y, and Walz T (2004). Aquaporin-0 membrane junctions reveal the structure of a closed water pore. Nature 429, 193–197. [PubMed: 15141214]
13. Nederlof I, van Genderen E, Li YW, and Abrahams JP (2013). A Medipix quantum area detector allows rotation electron diffraction data collection from submicrometre three-dimensional protein crystals. Acta Crystallogr. D Biol. Crystallogr 69, 1223–1230. [PubMed: 23793148]
14. Nannenga BL, Shi D, Leslie AGW, and Gonen T (2014). High-resolution structure determination by continuous-rotation data collection in MicroED. Nat. Methods 11, 927–930. [PubMed: 25086503]
15. Stura EA (1999). Seeding Techniques. In Crystallization of Nucleic Acids and Proteins: A Practical Approach, 2nd edition, Ducruix A and Giegé R, eds. (Oxford).
16. Luft JR, and DeTitta GT (1999). A method to produce microseed stock for use in the crystallization of biological macromolecules. Acta Crystallogr. D Biol. Crystallogr 55, 988–993. [PubMed: 10216295]
17. de la Cruz MJ, Hattne J, Shi D, Seidler P, Rodriguez J, Reyes FE, Sawaya MR, Cascio D, Weiss SC, Kim SK, et al. (2017). Atomic-resolution structures from fragmented protein crystals with the cryoEM method MicroED. Nat. Methods 14, 399–402. [PubMed: 28192420]
18. Matthews BW (1968). Solvent content of protein crystals. J. Mol. Biol 33, 491–497. [PubMed: 5700707]
19. Shoeman RL, Hartmann E, and Schlichting I (2023). Growing and making nano- and microcrystals. Nat. Protoc 18, 854–882. [PubMed: 36451055]

20. Martynowycz MW, and Gonen T (2021). Protocol for the use of focused ion-beam milling to prepare crystalline lamellae for microcrystal electron diffraction (MicroED). STAR Protoc. 2, 100686. [PubMed: 34382014]
21. Lynch ML, Snell ME, Potter SA, Snell EH, and Bowman SEJ (2023). 20 years of crystal hits: progress and promise in ultrahigh-throughput crystallization screening. Acta Crystallogr. D Struct. Biol 79, 198–205. [PubMed: 36876429]
22. Gruene T, Wennmacher JTC, Zaubitzer C, Holstein JJ, Heidler J, Fecteau-Lefebvre A, De Carlo S, Müller E, Goldie KN, Regeni I, et al. (2018). Rapid Structure Determination of Microcrystalline Molecular Compounds Using Electron Diffraction. Angew. Chem. Int. Ed. Engl 57, 16313–16317. [PubMed: 30325568]
23. Jones CG, Martynowycz MW, Hattne J, Fulton TJ, Stoltz BM, Rodriguez JA, Nelson HM, and Gonen T (2018). The CryoEM Method MicroED as a Powerful Tool for Small Molecule Structure Determination. ACS Cent. Sci 4, 1587–1592. [PubMed: 30555912]
24. Newman JA, Iuzzolino L, Tan M, Orth P, Bruhn J, and Lee AY (2022). From Powders to Single Crystals: A Crystallographer's Toolbox for Small-Molecule Structure Determination. Mol. Pharm 19, 2133–2141. [PubMed: 35576503]
25. Brown A, and Clardy J (2018). Tiny Crystals Have Big Potential for Determining Structures of Small Molecules (Nature Publishing Group UK). 10.1038/d41586-018-07756-5.
26. Bruhn JF, Scapin G, Cheng A, Mercado BQ, Waterman DG, Ganesh T, Dallakyan S, Read BN, Nieusma T, Lucier KW, et al. (2021). Small Molecule Microcrystal Electron Diffraction for the Pharmaceutical Industry-Lessons Learned From Examining Over Fifty Samples. Front. Mol. Biosci 8, 648603. [PubMed: 34327213]
27. Saha A, Nia SS, and Rodríguez JA (2022). Electron Diffraction of 3D Molecular Crystals. Chem. Rev 122, 13883–13914. [PubMed: 35970513]
28. Banihashemi F, Bu G, Thaker A, Williams D, Lin JYS, and Nannenga BL (2020). Beam-sensitive metal-organic framework structure determination by microcrystal electron diffraction. Ultramicroscopy 216, 113048. [PubMed: 32570132]
29. Andrusenko I, and Gemmi M (2022). 3D electron diffraction for structure determination of small-molecule nanocrystals: A possible breakthrough for the pharmaceutical industry. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol 14, e1810. [PubMed: 35595285]
30. Nannenga BL, and Gonen T (2019). The cryo-EM method microcrystal electron diffraction (MicroED). Nat. Methods 16, 369–379. [PubMed: 31040436]
31. Stevenson HP, Makhov AM, Calero M, Edwards AL, Zeldin OB, Mathews II, Lin G, Barnes CO, Santamaria H, Ross TM, et al. (2014). Use of transmission electron microscopy to identify nanocrystals of challenging protein targets. Proc. Natl. Acad. Sci. USA 111, 8470–8475. [PubMed: 24872454]
32. Kissick DJ, Wanapun D, and Simpson GJ (2011). Second-order nonlinear optical imaging of chiral crystals. Annu. Rev. Anal. Chem 4, 419–437.
33. Haupert LM, and Simpson GJ (2011). Screening of protein crystallization trials by second order nonlinear optical imaging of chiral crystals (SONICC). Methods 55, 379–386. [PubMed: 22101350]
34. Wampler RD, Kissick DJ, Dehen CJ, Gualtieri EJ, Grey JL, Wang H-F, Thompson DH, Cheng J-X, and Simpson GJ (2008). Selective detection of protein crystals by second harmonic microscopy. J. Am. Chem. Soc 130, 14076–14077. [PubMed: 18831587]
35. Judge RA, Swift K, and González C (2005). An ultraviolet fluorescence-based method for identifying and distinguishing protein crystals. Acta Crystallogr. D Biol. Crystallogr 61, 60–66. [PubMed: 15608376]
36. Madden JT, DeWalt EL, and Simpson GJ (2011). Two-photon excited UV fluorescence for protein crystal detection. Acta Crystallogr. D Biol. Crystallogr 67, 839–846. [PubMed: 21931215]
37. Miller RD, Iinishi A, Modaresi SM, Yoo B-K, Curtis TD, Lariviere PJ, Liang L, Son S, Nicolau S, Bargabos R, et al. (2022). Computational identification of a systemic antibiotic for gram-negative bacteria. Nat. Microbiol 7, 1661–1672. [PubMed: 36163500]
38. Aebi U, and Pollard TD (1987). A glow discharge unit to render electron microscope grids and other surfaces hydrophilic. J. Electron. Microsc. Tech 7, 29–33. [PubMed: 3506047]

39. Shi D, Nannenga BL, de la Cruz MJ, Liu J, Sawtelle S, Calero G, Reyes FE, Hattne J, and Gonen T (2016). The collection of MicroED data for macromolecular crystallography. Nat. Protoc 11, 895–904. [PubMed: 27077331]
40. Shi D, Nannenga BL, Iadanza MG, and Gonen T (2013). Three-dimensional electron crystallography of protein microcrystals. Elife 2, e01345. [PubMed: 24252878]
41. Glaser RM, Tong L, and Kim S-H (1989). Three-dimensional reconstructions from incomplete data: Interpretability of density maps at “atomic” resolution. Ultramicroscopy 27, 307–318. [PubMed: 2749922]
42. Myasnikov AG, Afonina ZA, and Klaholz BP (2013). Single particle and molecular assembly analysis of polyribosomes by single- and double-tilt cryo electron tomography. Ultramicroscopy 126, 33–39. [PubMed: 23376404]
43. Wennmacher JTC, Zaubitzer C, Li T, Bahk YK, Wang J, van Bokhoven JA, and Gruene T (2019). 3D-structured supports create complete data sets for electron crystallography. Nat. Commun 10, 3316. [PubMed: 31346178]
44. Gillman C, Nicolas WJ, Martynowycz MW, and Gonen T (2023). Design and implementation of suspended drop crystallization. IUCrJ 10, 430–436.
45. Hattne J, Shi D, Glynn C, Zee C-T, Gallagher-Jones M, Martynowycz MW, Rodriguez JA, and Gonen T (2018). Analysis of Global and Site-Specific Radiation Damage in Cryo-EM. Structure 26, 759–766.e4. [PubMed: 29706530]
46. Martynowycz MW, Clabbers MTB, Hattne J, and Gonen T (2022). Ab initio phasing macromolecular structures using electron-counted MicroED data. Nat. Methods 19, 724–729. [PubMed: 35637302]
47. Martynowycz MW, Clabbers MTB, Unge J, Hattne J, and Gonen T (2021). Benchmarking the ideal sample thickness in cryo-EM. Proc. Natl. Acad. Sci. USA 118, e2108884118. 10.1073/pnas.2108884118.
48. Yonekura K, Maki-Yonekura S, and Namba K (2002). Quantitative comparison of zero-loss and conventional electron diffraction from two-dimensional and thin three-dimensional protein crystals. Biophys. J 82, 2784–2797. [PubMed: 11964264]
49. Gemmi M, and Oleynikov P (2013). Scanning reciprocal space for solving unknown structures: energy filtered diffraction tomography and rotation diffraction. tomography methods 228, 51–58.
50. Yang T, Xu H, and Zou X (2022). Improving data quality for three-dimensional electron diffraction by a post-column energy filter and a new crystal tracking method. J. Appl. Crystallogr 55, 1583–1591. [PubMed: 36570655]
51. Yonekura K, Kato K, Ogasawara M, Tomita M, and Toyoshima C (2015). Electron crystallography of ultrathin 3D protein crystals: atomic model with charges. Proc. Natl. Acad. Sci. USA 112, 3368–3373. [PubMed: 25730881]
52. Mastronarde DN (2005). Automated electron microscope tomography using robust prediction of specimen movements. J. Struct. Biol 152, 36–51. [PubMed: 16182563]
53. de la Cruz MJ, Martynowycz MW, Hattne J, and Gonen T (2019). MicroED data collection with SerialEM. Ultramicroscopy 201, 77–80. [PubMed: 30986656]
54. Takaba K, Maki-Yonekura S, and Yonekura K (2020). Collecting large datasets of rotational electron diffraction with ParallEM and SerialEM. J. Struct. Biol 211, 107549. [PubMed: 32544623]
55. Cichocka MO, Ångström J, Wang B, Zou X, and Smeets S (2018). High-throughput continuous rotation electron diffraction data acquisition via software automation. J. Appl. Crystallogr 51, 1652–1661. [PubMed: 30546290]
56. Cheng A, Negro C, Bruhn JF, Rice WJ, Dallakyan S, Eng ET, Waterman DG, Potter CS, and Carragher B (2021). Leginon: New features and applications. Protein Sci. 30, 136–150. [PubMed: 33030237]
57. Kabsch W (2010). XDS. Acta Crystallogr. D Biol. Crystallogr 66, 125–132.
58. Clabbers MTB, Gruene T, Parkhurst JM, Abrahams JP, and Waterman DG (2018). Electron diffraction data processing with DIALS. Acta Crystallogr. D Struct. Biol 74, 506–518. [PubMed: 29872002]

59. Battye TGG, Kontogiannis L, Johnson O, Powell HR, and Leslie AGW (2011). iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr. D Biol. Crystallogr 67, 271–281. [PubMed: 21460445]
60. Sheldrick GM (2008). A short history of SHELX. Acta Crystallogr. A 64, 112–122. [PubMed: 18156677]
61. Dolomanov OV, Bourhis LJ, Gildea RJ, Howard JAK, and Puschmann H (2009). OLEX2: a complete structure solution, refinement and analysis program. J. Appl. Crystallogr 42, 339–341.
62. Brázda P, Klementová M, Krysiak Y, and Palatinus L (2022). Accurate lattice parameters from 3D electron diffraction data. IUCrJ 9, 735–755.
63. Wang B, Zou X, and Smeets S (2019). Automated serial rotation electron diffraction combined with cluster analysis: an efficient multi-crystal workflow for structure determination. IUCrJ 6, 854–867.
64. Powell SM, Novikova IV, Kim DN, and Evans JE (2021). AutoMicroED: A semi-automated MicroED processing pipeline. bioRxiv. 10.1101/2021.12.13472146..
65. Unge J, Lin J, Weaver SJ, Sae Her A, and Gonen T (2023). Autonomous MicroED data collection enables compositional analysis. Preprint at ChemRxiv. 10.26434/chemrxiv-2023-8qvwg.
66. Wan W, Sun J, Su J, Hovmöller S, and Zou X (2013). Three-dimensional rotation electron diffraction: software RED for automated data collection and data processing. J. Appl. Crystallogr 46, 1863–1873. [PubMed: 24282334]
67. Palatinus L, Brázda P, Jelínek M, Hrdá J, Steciuk G, and Klementová M (2019). Specifics of the data processing of precession electron diffraction tomography data and their implementation in the program PETS2.0. Acta Crystallogr. B Struct. Sci. Cryst. Eng. Mater 75, 512–522.
68. Kolb U, Gorelik T, and Otten MT (2008). Towards automated diffraction tomography. Part II–Cell parameter determination. Ultramicroscopy 108, 763–772. [PubMed: 18282662]
69. Sauter NK (2015). XFEL diffraction: developing processing methods to optimize data quality. J. Synchrotron Radiat 22, 239–248. [PubMed: 25723925]
70. Yamashita K, Hirata K, and Yamamoto M (2018). KAMO: towards automated data processing for microcrystals. Acta Crystallogr. D Struct. Biol 74, 441–449. [PubMed: 29717715]
71. Winter G (2009). xia2: an expert system for macromolecular crystallography data reduction. J. Appl. Crystallogr 43, 186–190.
72. Takaba K, Maki-Yonekura S, Inoue I, Tono K, Hamaguchi T, Kawakami K, Naitow H, Ishikawa T, Yabashi M, and Yonekura K (2023). Structural resolution of a small organic molecule by serial X-ray free-electron laser and electron crystallography. Nat. Chem 15, 491–497. [PubMed: 36941396]
73. Waterman DG, Frisina N, Owen CD, Winter G, and Nunes P (2023). A standard data format for 3DED/MicroED. Structure 31, 1510–1517.e1. [PubMed: 37536337]
74. Danelius E, Porter NJ, Unge J, Arnold FH, and Gonen T (2023). MicroED Structure of a Protoglobin Reactive Carbene Intermediate. J. Am. Chem. Soc 145, 7159–7165. [PubMed: 36948184]
75. Shiriaeva A, Martynowycz MW, Nicolas WJ, Cherezov V, and Gonen T (2023). MicroED structure of the human vasopressin 1B receptor. bioRxiv. 10.1101/2023.07.05.547888.
76. Richards LS, Flores MD, Millán C, Glynn C, Zee C-T, Sawaya MR, Gallagher-Jones M, Borges RJ, Usón I, and Rodriguez JA (2023). Fragment-Based Ab Initio Phasing of Peptidic Nanocrystals by MicroED. ACS Bio Med Chem Au 3, 201–210.
77. Latychevskaia T, and Abrahams JP (2019). Inelastic scattering and solvent scattering reduce dynamical diffraction in biological crystals. Acta Crystallogr. B Struct. Sci. Cryst. Eng. Mater 75, 523–531.
78. Palatinus L, Petříček V, and Corrêa CA (2015). Structure refinement using precession electron diffraction tomography and dynamical diffraction: theory and implementation. Acta Crystallogr. A Found. Adv 71, 235–244. [PubMed: 25727873]
79. Klar PB, Krysiak Y, Xu H, Steciuk G, Cho J, Zou X, and Palatinus L (2023). Accurate structure models and absolute configuration determination using dynamical effects in continuous-rotation 3D electron diffraction data. Nat. Chem 15, 848–855. [PubMed: 37081207]

80. Clabbers MTB, Gruene T, van Genderen E, and Abrahams JP (2019). Reducing dynamical electron scattering reveals hydrogen atoms. Acta Crystallogr. A Found. Adv 75, 82–93. [PubMed: 30575586]

81. Blum TB, Housset D, Clabbers MTB, van Genderen E, Bacia-Verloop M, Zander U, McCarthy AA, Schoehn G, Ling WL, and Abrahams JP (2021). Statistically correcting dynamical electron scattering improves the refinement of protein nanocrystals, including charge refinement of coordinated metals. Acta Crystallogr. D Struct. Biol 77, 75–85. [PubMed: 33404527]

82. Crawshaw AD, Beale EV, Warren AJ, Stallwood A, Duller G, Trincao J, and Evans G (2021). A Sample Preparation Pipeline for Microcrystals at the VMXm Beamline. J. Vis. Exp 10, 3791–62306.

83. Bücker R, Hogan-Lamarre P, Mehrabi P, Schulz EC, Bultema LA, Gevorkov Y, Brehm W, Yefanov O, Oberthür D, Kassier GH, and Dwayne Miller RJ (2020). Serial protein crystallography in an electron microscope. Nat. Commun 11, 996. [PubMed: 32081905]

84. Gallagher-Jones M, Bustillo KC, Ophus C, Richards LS, Ciston J, Lee S, Minor AM, and Rodriguez JA (2020). Atomic structures determined from digitally defined nanocrystalline regions. IUCrJ 7, 490–499.

85. Gallagher-Jones M, Ophus C, Bustillo KC, Boyer DR, Panova O, Glynn C, Zee C-T, Ciston J, Mancia KC, Minor AM, and Rodriguez JA (2019). Nanoscale mosaicity revealed in peptide microcrystals by scanning electron nanodiffraction. Commun. Biol 2, 26. [PubMed: 30675524]

86. Saha A, Pattison A, Mecklenburg M, Brewster A, Ercius P, and Rodriguez JA (2023). Beyond MicroED: Ab Initio Structure Elucidation using 4D-STEM. Microsc. Microanal 29, 309–310. [PubMed: 37613469]

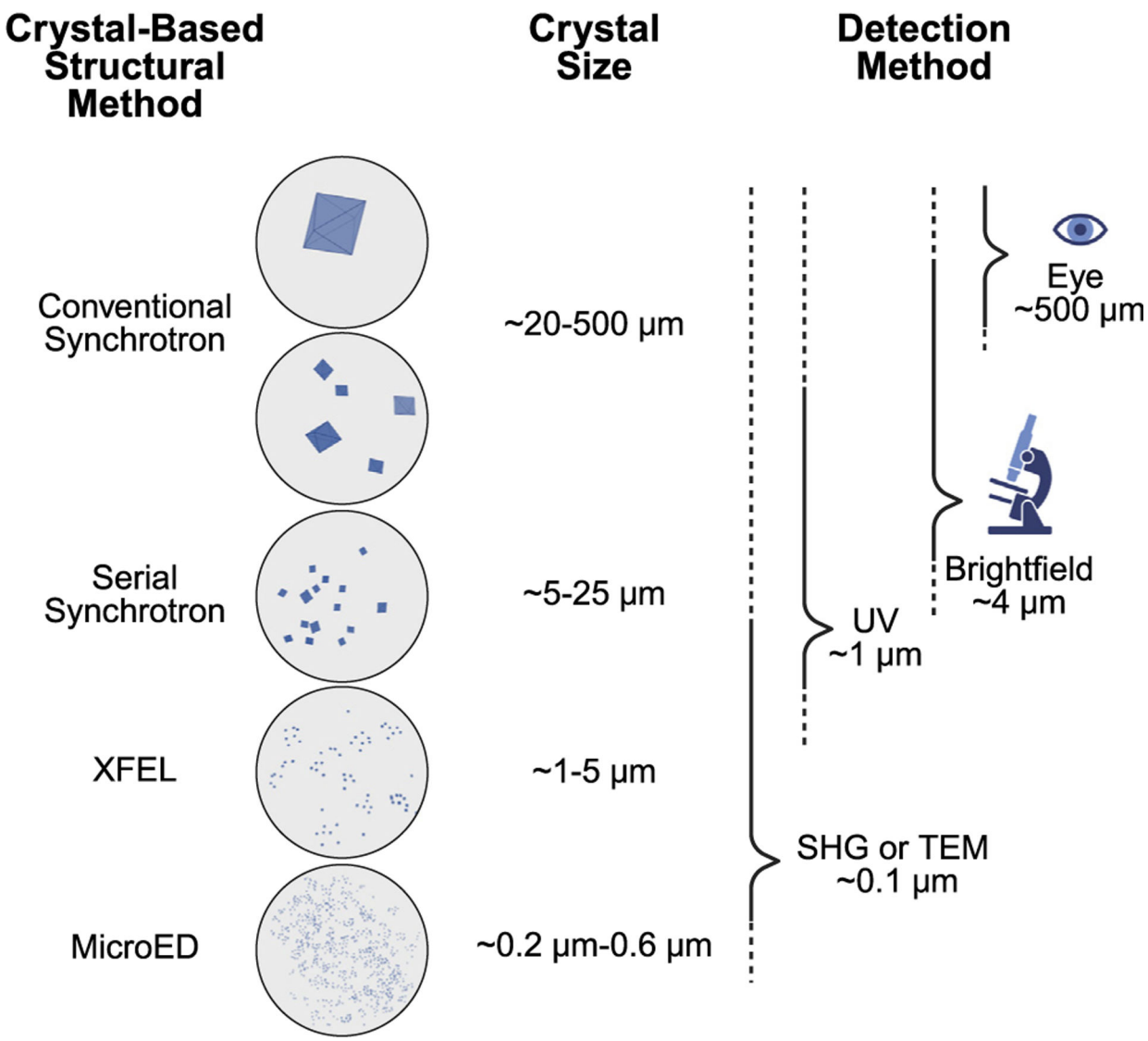

Figure 1. Schematic showing different diffraction-based structural methods and crystal detection techniques for a range of crystal sizes

Different diffraction-based methods (left) are appropriate for crystals in different size ranges. Representative crystal size ranges (middle) and available detection techniques (right) are shown. Micrometer-sized crystals in the size range suitable for conventional X-ray diffraction experiments, at either a home source or a synchrotron, can be seen by eye or with readily available bright-field microscopes. Micron-sized crystals are used for more advanced techniques like serial synchrotron and XFELs. When the crystals are even smaller, in the submicron size regime, electron beam sources can be used to collect diffraction data. While larger crystals can be detected by eye or light microscopy, smaller crystals suitable for advanced X-ray and electron diffraction experiments often require other methods such as TEM or nonlinear optical imaging techniques.

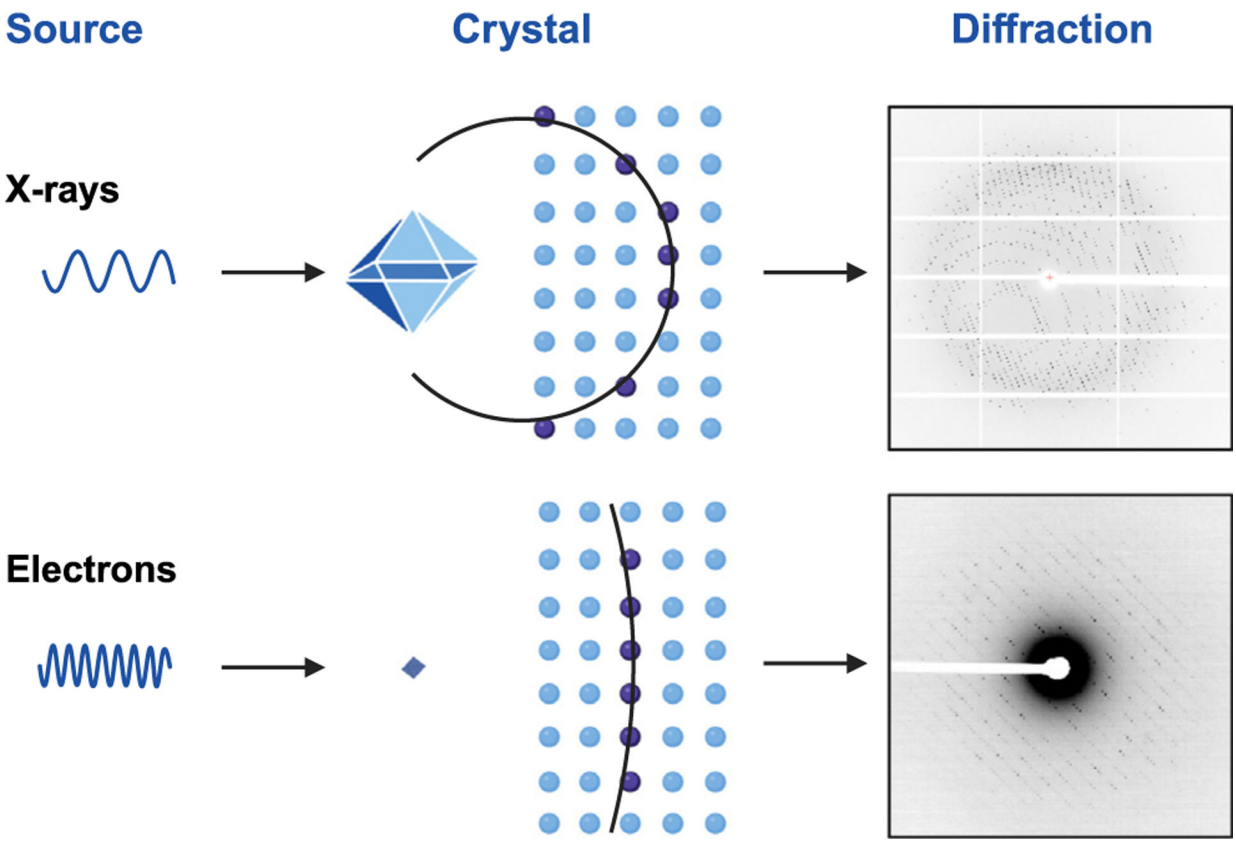

Figure 2. Comparison of X-ray and electron crystallography

When incident X-ray or electron waves interact with the crystal lattice planes and interfere constructively, they form diffraction spots (Bragg reflections, shown as blue dots in reciprocal space). The detector captures the signal from the reflections that intersect with the Ewald sphere, thus satisfying the Bragg condition (spots colored dark blue). The Ewald sphere radius (drawn as black line) is reciprocal of the incident wavelength. For X-rays from a copper source, the wavelength is 1.54 Å, producing diffraction patterns that include information about all three reciprocal space dimensions (*h,k,l* indices) in a single image. For electrons from a 200 kV TEM, the wavelength is 0.025 Å, resulting in diffraction patterns that only contain information froma single plane (capturing only two reciprocal space indices) on the flatter Ewald sphere.

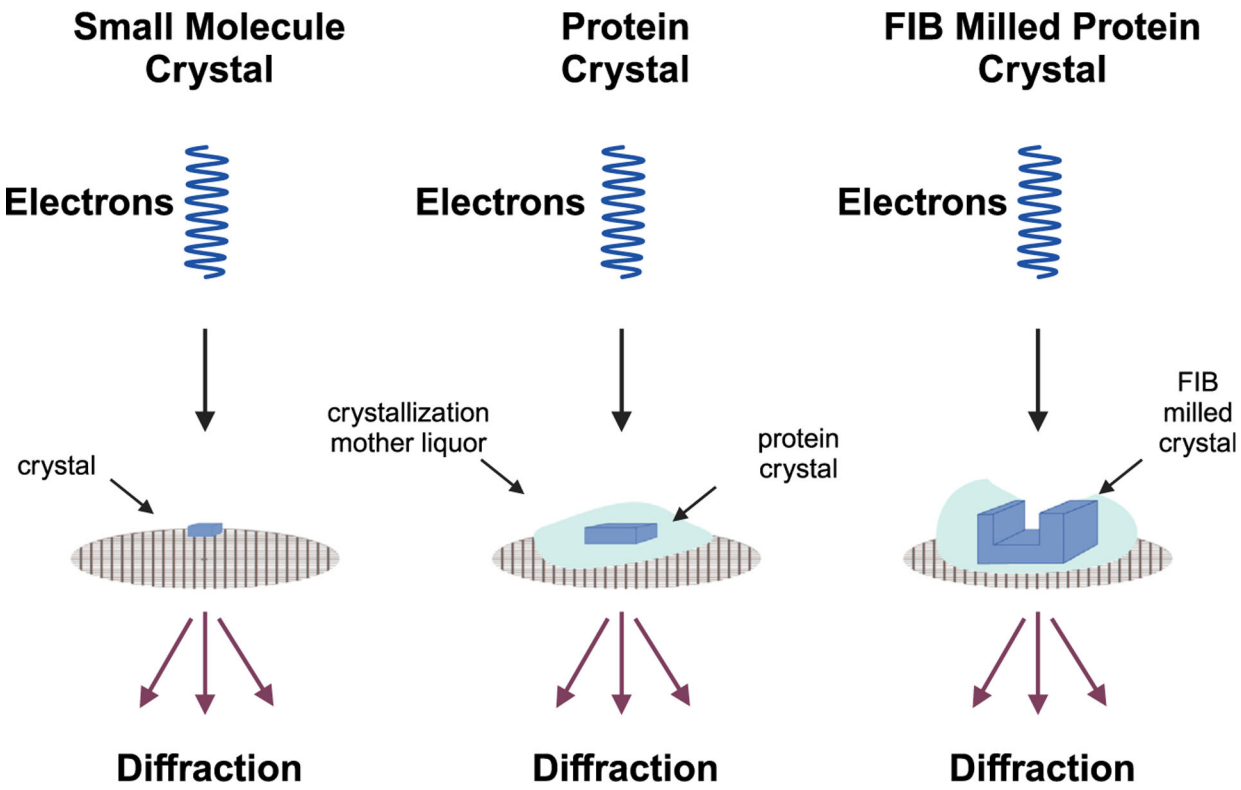

Figure 3. Sample preparation for electron diffraction experiments can be modified for different crystal samples

Small molecules are naturally more likely to form some nanocrystals. In general, the right conditions need to be found to form protein crystals. If the crystals are thin enough for an electron beam, they can be frozen on EM grids and used directly for data collection. FIB milling is a popular option for thinning larger crystals frozen on grids before collecting electron diffraction data.

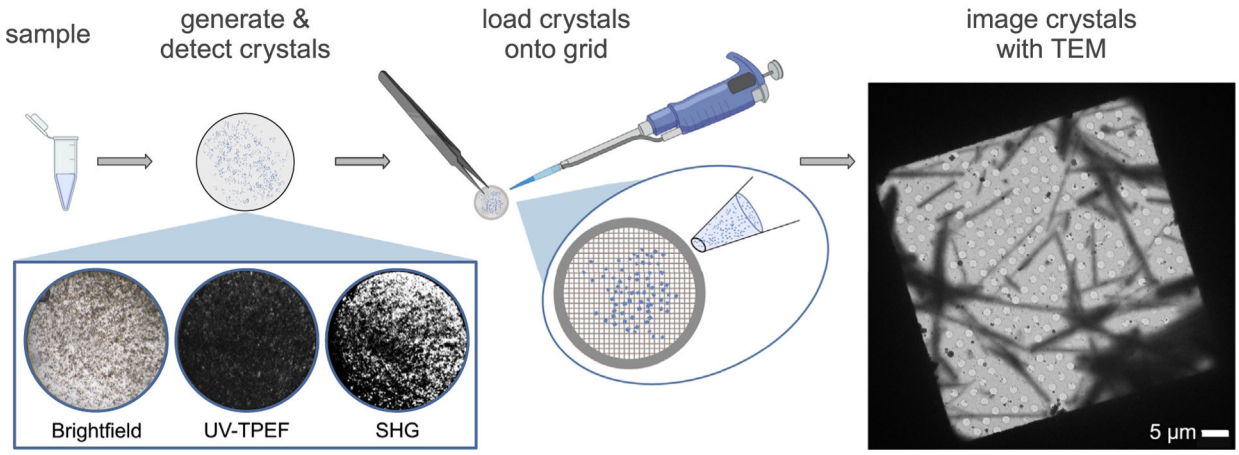

Figure 4. Schematic showing a pipeline for nanocrystal generation, detection, and deposition to the EM grid

A protein or peptide sample is incubated in batch or on vapor diffusion plates with the crystallization conditions to form small crystals. Protein-rich crystalline material can be detected using the combination of SHG and UV-TPEF (inset figure adapted from Miller et al.34; for scale, the diameter of the well and image field of view is 0.9 mm). The sample is loaded onto EM grids (depicted as a pipette transfer in the schematic; there are other transfer methods possible). The sample frozen on the EM grids could then be further screened with TEM to ascertain the size (scale bar: 5 μm) and quality distribution of the crystals before collecting electron diffraction data using the same cryo-EM.