

Cryo-FIB thinning of protein microcrystals for MicroED

Bridging the gap in crystal size

Authors: Abhay Kotecha, Alexander Rigort, Alex Ilitchev

Introduction

Cryo-electron microscopy (cryo-EM) and diffraction are important methods for the analysis of biological structures at near-atomic resolutions. Most conventional atomic structure determination is performed via X-ray diffraction on synchrotrons; these experiments necessitate large, well-ordered protein crystals (50-100 μm). Their growth is a critical bottleneck that is either time-consuming or impossible. Special micro-focus beamlines can analyze crystals down to sizes of $\sim 10\text{-}50\ \mu\text{m}$, but these are susceptible to radiation damage, which destroys the crystal before a diffraction pattern can be acquired.

For the analysis of very small crystals (well below $1\ \mu\text{m}$ in size), electrons are more advantageous than X-rays because their interaction with matter is stronger, hence a stronger signal can be obtained. The technique of micro electron diffraction (MicroED)

is, therefore, well suited for the analysis of small nanocrystals, generating high-resolution 3D structures of small chemical compounds or biological macromolecules.

Rather than requiring a specialized synchrotron facility, MicroED experiments can be performed using cryo-EM equipment that fits in a standard laboratory. MicroED studies are performed in vacuum, reducing background scatter. Operation at cryogenic temperatures protects the sensitive crystal sample from radiation damage that would otherwise lead to rapid degradation. This approach also significantly shortens the sample preparation process, as only nanometer sized crystals must be grown. Due to their small size, these crystals can only be examined using MicroED, as they are too small to diffract with other methods.

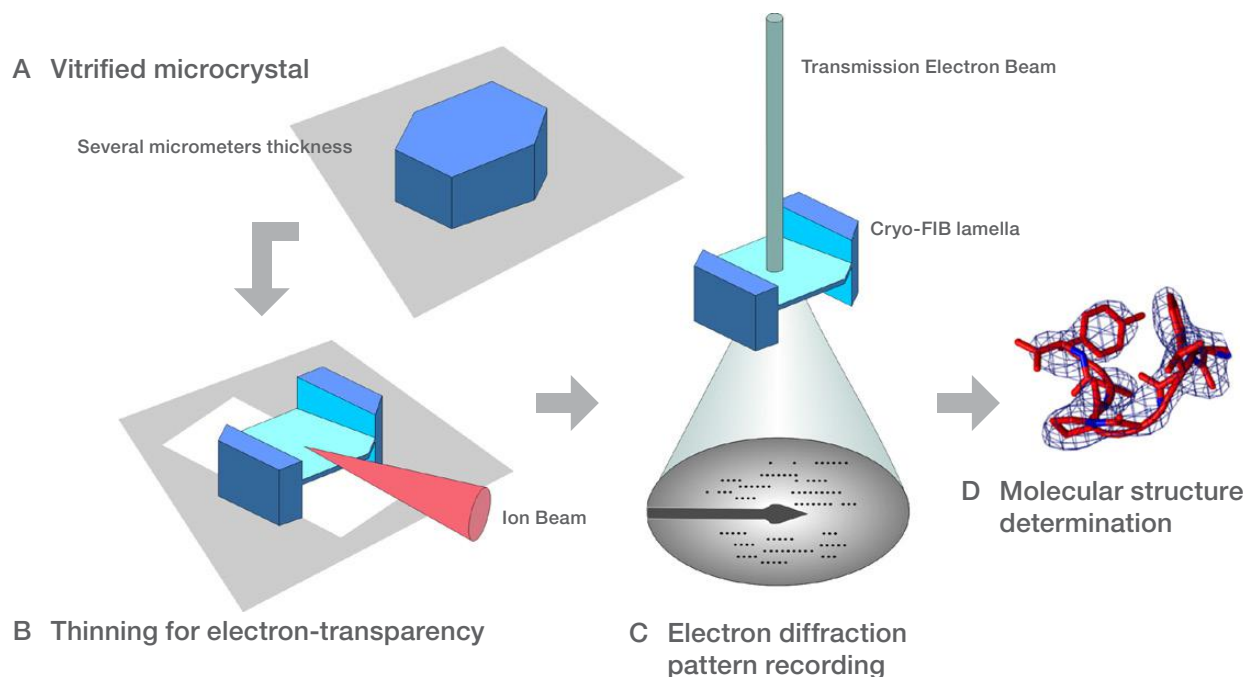


Figure 1. The process of thinning microcrystals with a cryo-focused ion beam for micro electron diffraction analysis.

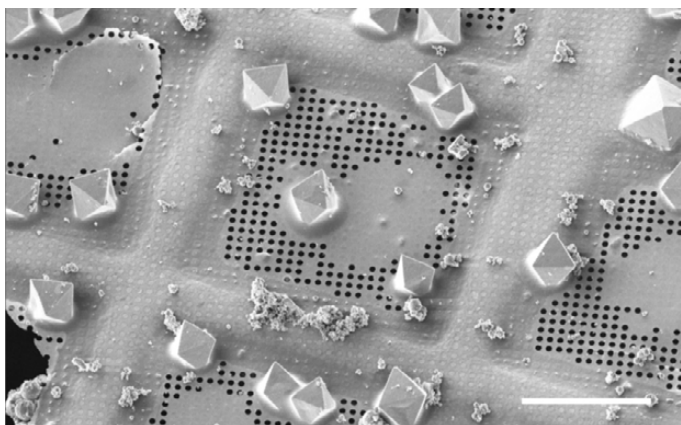


Figure 2. Secondary electron image showing vitrified apoferritin microcrystals attached to the holey carbon-film of an EM grid. The small tetragonal crystals are around 15-30 μm in size. Without cryo-FIB thinning they are too thick for electron diffraction in the cryo-TEM. Scale bar: 50 μm . Image courtesy of eBIC, Harwell, UK.

Unfortunately, the strong interaction with matter is also a limitation of electron diffraction (ED), since the electron beam is absorbed very strongly by only a few hundred nanometers of crystal thickness. For intermediate sized crystals (5-30 μm in size) there is a gap between the nanometer scale of ED and the micrometer scale of synchrotron X-ray diffraction; making such microcrystals accessible to electron diffraction experiments requires thinning the crystal sample to a suitable thickness.

A cryo-focused ion beam scanning electron microscope (cryo-FIB/SEM) can be used to perform precise milling; in this application note we describe how a Thermo Scientific™ Aquilos™ 2 Cryo-FIB can be used for micromachining crystals to appropriate sizes for MicroED analysis (Figure 1).

Vitrification of microcrystals

Microcrystals can be grown using standard methods and deposited on EM grids. For vitrification, grids are blotted and plunged into liquid ethane using a Thermo Scientific Vitrobot™ System. Figure 2 shows an overview image of vitrified tetragonal microcrystals of apoferritin on an EM grid. It is clear that the crystals are too thick to be examined by cryo-transmission electron microscopy (cryo-TEM). The strong scattering of the electrons would make it impossible to efficiently penetrate the crystals with the electron beam. The Aquilos 2 Cryo-FIB can be used for thinning the microcrystals to thicknesses that are suitable for MicroED investigation.

Thinning of micron-sized crystals using a cryo-FIB/SEM microscope

For a typical cryo-FIB experiment, an EM grid with vitrified microcrystals is mounted into a dedicated cryo-holder using a loading station and subsequently transferred onto the cooled (approx. $-180\text{ }^{\circ}\text{C}$) cryo-stage within the Aquilos 2 microscope. Target crystals are identified via secondary electron imaging (Figure 2). During the thinning process, termed “ion beam milling”, a focused beam of ions is progressively scanned over the specimen surface and the impacting ions expel surface layer atoms from their positions via collisions (also known as sputtering). This allows successive layer-by-layer material removal (Figure 3).

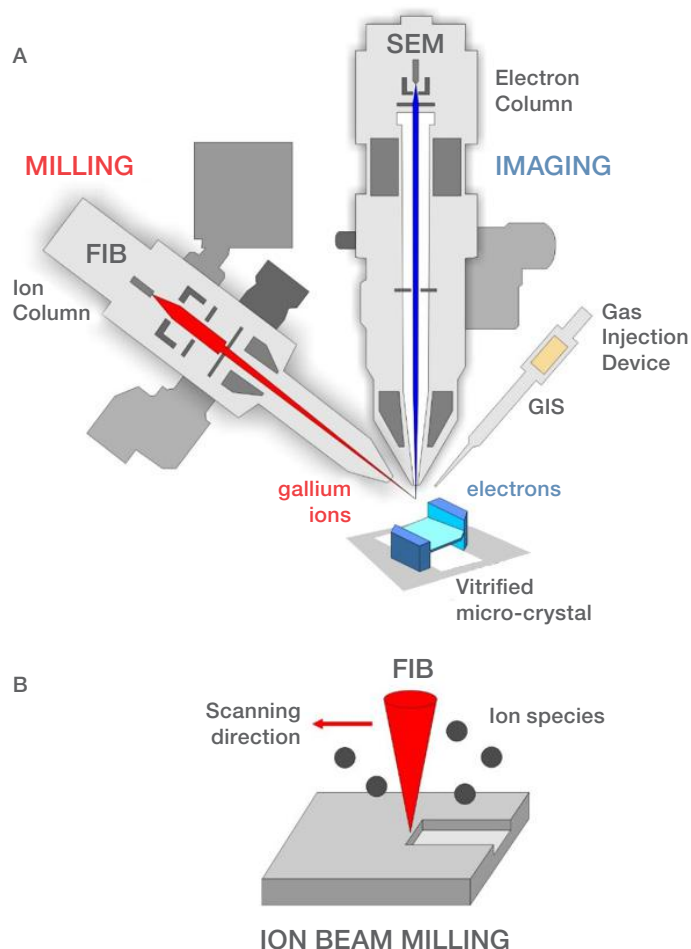


Figure 3. (a) Cross-sectional drawing of ion and electron beam columns of a dual-beam FIB/SEM microscope. The frozen microcrystal sample is thinned by the ion beam generated in the ion beam column. The process can be observed and controlled with the electron beam. (b) Principle of ion beam thinning: A beam of focused ions is directed and scanned across the sample surface. Sample atoms are removed in a layer-by-layer fashion via sputtering processes.

To prepare a thin electron-transparent lamella from a microcrystal, the ion beam must remove material above and below the target site (See Figure 1, Step 2). Ion beam thinning is performed directly on the grid and the sample can remain there for subsequent diffraction analysis in the cryo-TEM. This “on-the-grid” preparation method avoids the need for further manipulation steps such as lift-out of the thin and fragile cryo-lamella [1].

Figure 4 shows the milling of a microcrystal cryo-lamella from lysozyme crystals. The milling process starts with an overview image of the designated target crystal, oriented in the direction of the incident ion beam (Figure 4a). Two rectangular milling patterns, an upper and lower pattern, mark the regions that are to be removed; these flank the area intended for lamella preparation. After the ion beam sputtering operation is finished, a $\sim 200\text{ nm}$ thin cryo-lamella remains, which is laterally supported on both sides (Figure 4b). A top view of the resulting lamella shows that the area is now electron-transparent (Figure 4c).

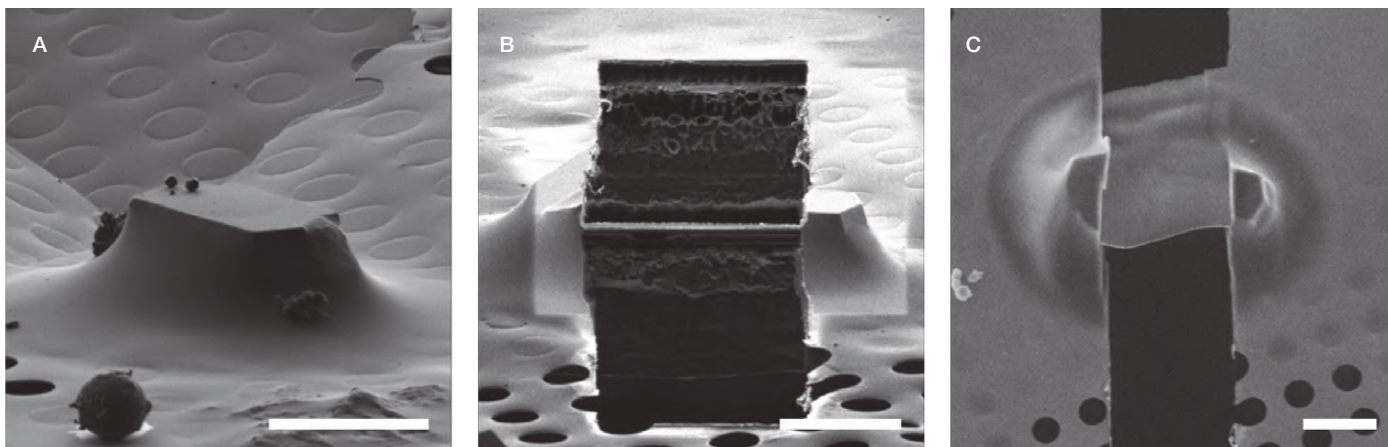


Figure 4. The making of a thin “on-the-grid” cryo-lamella from a lysozyme microcrystal. (a) Microcrystal before cryo-FIB thinning. (b) After preparation of an ~200 nm thin electron-transparent cryo-lamella. (c) Top view of the lamella shown in (b). Scale bars: 5 μm (a-c). Image courtesy of Division of Structural Biology (STRUBI), Oxford, UK.

MicroED data acquisition from cryo-FIB-thinned microcrystals

MicroED data is acquired on intermediate voltage (200-300 keV) cryo-TEMs, using electrons as the incident beam. It is important that the milled crystal maintains its diffraction capacity, as is the case for cryo-FIB prepared lamellas (Figure 5).

Vitrified EM grids containing microcrystal lamellae are loaded at liquid nitrogen temperature from the Aquilos 2 Cryo-FIB into a cryo-TEM (e.g. a Thermo Scientific Glacios™, Talos™ or Krios™ TEM). There, the lamellae sites are first identified with low-magnification imaging (Figure 5a) and the diffraction quality of the milled crystals is analyzed by collection of an initial diffraction pattern (Figure 5b). Two different approaches have been reported for diffraction image recording.

1. Record individual images as stills separated by defined tilts of the grid (i.e. using 0.5° increments) [2].
2. Rotate the stage at a constant rate (i.e. 0.3-0.5 degrees per second), while the scattered electron signal is being recorded with the detector [3,4]. This common approach is termed “continuous rotation” and reports indicate that it results in structures with high completeness and good refinement statistics.

For MicroED data detection a camera, optimized for low-dose diffraction data collection, is required. Both charge-coupled device (CCD) or complementary metal-oxide-semiconductor

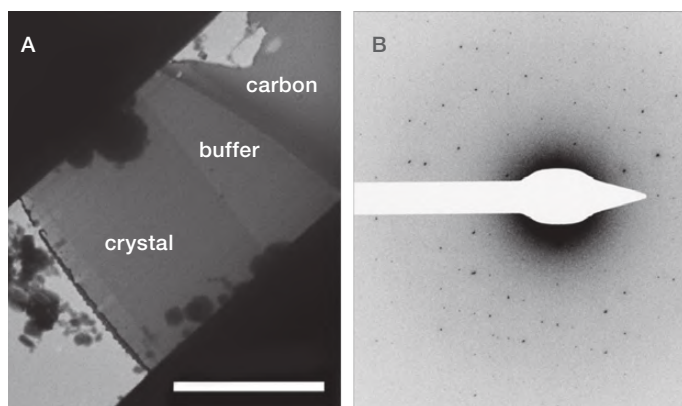


Figure 5. Cryo-lamella (a) and corresponding electron diffraction pattern (b) of the frozen, hydrated lysozyme crystal. The edges of the image correspond to Bragg spacings of ~1.8 Å. Scale bar: 3 μm (a). Modified from [2] with permission.

(CMOS) cameras have been used for MicroED data acquisition. Compared to CCD-based detectors, however, CMOS-based cameras (such as the Thermo Scientific CETA-D™ Camera) are superior due to their greatly increased read-out speeds and resistance to blooming effects. The CETA-D CMOS Camera, used here, has a high sensitivity and allows for reliable detection of high-resolution, low-intensity diffraction peaks. Its high signal-to-noise ratio allows for accurate measurement of the integrated peak intensity. Both elements are prerequisites for obtaining high-resolution structural information.

Structure determination from FIB-thinned microcrystals

The lysozyme crystal lamella can diffract up to a 1.9 Å resolution. For improved, more complete diffraction data, multiple datasets from the same or different crystals are collected and merged.

ED data can be processed with standard crystallography software (e.g. DIALS [5] and XDS [6]) for indexing, scaling and merging, but the software packages must be optimized to use electron scattering factors. The lysozyme dataset, although not isomorphous, still compares well with previous data collected from lysozyme X-ray diffraction (CC = 74%). Additionally, a simple rigid-body refinement with no manual intervention (performed in PHENIX software [7]) proved to be comparable to the X-ray data (Protein Data Bank (PDB) ID 5WR9). The quality of the data can be judged from the 1.9 Å electron potential map shown in Figure 6. The retention of high-resolution diffraction demonstrates that cryo-FIB milling preserves structural information within the lamellae, and there is no significant damage to the underlying crystalline lattice by the ion beam.

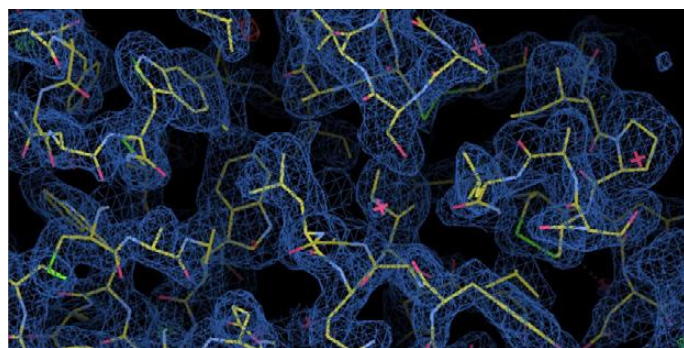


Figure 6. A section of the lysozyme electron potential map determined for FIB-thinned protein crystals. Image courtesy of Division of Structural Biology (STRUBI), Oxford, UK.

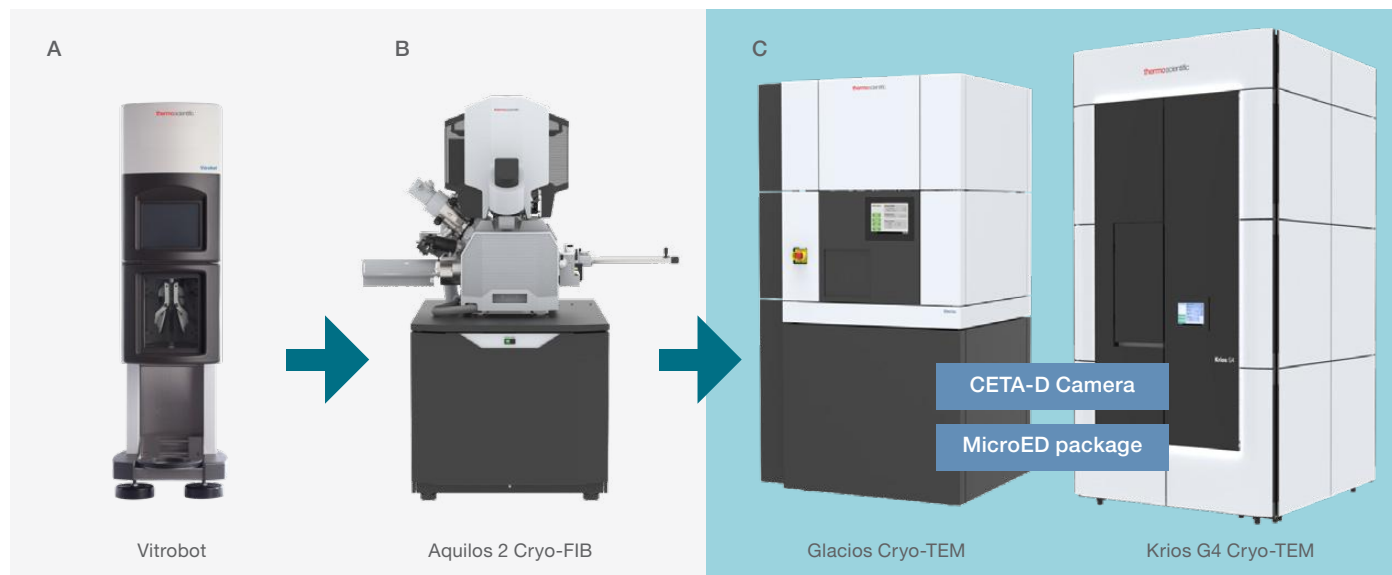


Figure 7. Suite of available instruments for MicroED. The Aquilos 2 Cryo-FIB is used for micromachining of intermediate sized microcrystals. MicroED can be performed on both 200 keV Glacios and 300 keV Krios G4 Cryo-TEM systems.

Suite of instruments for combining cryo-FIB with MicroED

Thermo Scientific's dedicated MicroED package ensures that a cryo-TEM is ready for electron diffraction experiments (Figure 7); whether it be a new cryo-TEM or a retrofit on an existing unit. The package includes:

- The CMOS-based CETA-D Camera
- Thermo Scientific EPU-DTM Software for screening and automated data acquisition
- An optimized smaller beamstop to mask the central beam without blocking low-resolution diffraction spots
- An optimized aperture set that maximally suppresses unwanted background signal from the area surrounding the crystal
- A set of dedicated electron-optical settings to enable optimal camera utilization while benefiting from best sample-beam geometry

Role of cryo-FIB for MicroED sample preparation

The use of cryo-FIB technology for thinning intermediate sized crystals considerably extends the available range of crystal sizes for MicroED analysis [8,9]. It also helps reduce the time spent screening for suitable nanocrystals; crystals of any size can be used and thinned by milling, thereby streamlining sample preparation. Large crystals with a thickness of several micrometers, which would otherwise not be suitable for MicroED, can now be thinned to a thickness of 100-300 nm using the cryo-FIB. This enables structure determination from crystals that were previously considered too large for these experiments. Since many sample production methods produce crystals that are too small for X-ray analysis and too large for

MicroED, cryo-FIB sample preparation opens up a new range of accessible samples. The technique can therefore bridge the gap in crystal size between synchrotron micro-crystallography and electron diffraction analysis of nanocrystals.

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