MicroED: EPU-D results, bottlenecks and future perspectives

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Materials and Structural Analysis, EM Life Sciences

EMBO practical course: Image processing for cryo-electron microscopy
Birkbeck, London, September 6, 2019
Why is micro-electron diffraction useful?
X-ray diffraction requires large crystals

- Macromolecular crystallography (MX) beamlines require crystals ~30-100 μm.
- Microfocus MX beamlines makes it possible to analyze smaller (<10 μm in case of nanofocus beam) crystals; however, small crystals are often more prone to radiation damage.
## Electrons vs X-rays

<table>
<thead>
<tr>
<th></th>
<th>Electrons 80-500 keV</th>
<th>X-rays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 Å</td>
<td>30 Å</td>
</tr>
<tr>
<td>Ratio † (inelastic/elastic) scattering events</td>
<td>3</td>
<td>10 $10^3$–$10^4$</td>
</tr>
<tr>
<td>Mechanism of radiation damage</td>
<td>Secondary e⁻ emission</td>
<td>Photoelectric e⁻ emission</td>
</tr>
<tr>
<td>Energy deposited per inelastic event</td>
<td>20 eV</td>
<td>8 keV 400 eV</td>
</tr>
<tr>
<td>Energy deposited per elastic event**</td>
<td>60 eV</td>
<td>80 keV 400 keV</td>
</tr>
</tbody>
</table>
| Energy deposited relative to electrons  
(inelastic)  
(elastic) | 1 1 | 400 20 1000 10000 |

Why is microED useful?

MicroED

Main microED applications for nanocrystals:
- Protein structures 1-3 Å resolution range
- Small organic molecules <1 Å resolution range

Too small for XRD
Too large for MED

Main microED application for microcrystals:
- Protein structures after FIB-milling

Avg. size range: 50-500 nm

X-ray diffraction

Avg. size range: 10-100 micron
Growing sub-micron crystals...

- Some protein crystal detection systems developed for X-ray crystallography (especially for lipid cubic phase crystallography) can detect sub-micrometer size crystals.

![Visible](image1)

![SHG](image2)

Image copyright: Formulatrix
Principle of micro-ED

- Only few crystals needed
- Low dose imaging (1.5 - 3 e⁻/Å²)
- Cryo-conditions
- Provides high resolution
No special tools required

Vitrobot

Aquilos

Talos L120C  F200C  Glacios  Talos Arctica  Krios
EPU-D application results
Application results I: mED of small (<1 μm) protein crystals

Bio-chemistry → Vitrification of crystals → Diffraction imaging → Post process

Target selection and drug discovery
Application results I: nanocrystalline granulovirus

- Belong to the Baculoviridae family, a group of insect viruses
- They form occlusion bodies (OBs) to protect the virus when outside of host
- OBs have a protective crystalline coat
- Granulin is a 29 kDa protein forming the asymmetric unit cell on this coat
- There are 9000 unit cells per virion creating a thickness of ~250 nm
Application results I: nanocrystalline granulovirus

Data collection:

- **Synchrotron**: data from 21 recombinant 5 μm crystals → 1.7 Å resolution
- **XFEL (2017)**: data from 83,000 native crystals → 2.0 Å resolution

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**Atomic structure of granulin determined from native nanocrystalline granulovirus using an X-ray free-electron laser**

Cornelius Gati1, Dominik Oberthuer1, Oleksandr Yefanov1, Richard D. Bunker1,2, Francesco Stellato3, Elaine Chiu3, Shin-Mei Yeh3, Andrew Aquila3, Shihom Basu1,2,3, Richard Bean1,2, Kenneth R. Beyerlein1, Sabine Botha1, Sébastien Boutet1, Daniel P. DePonte1,2, R. Bruce Doak3,4, Raimund Fromme3,4, Lorenzo Galli3, Ingo Groth Johann3, Daniel R. James1, Christopher Kupitz4,2, Lukas Lomb1, Marc Messerschmidt3,4, Karol Nass1, Kimberly Rendek4, Robert L. ShoeMan1, Dingjie Wang1, Uwe Weierstall3, Thomas A. White4, Garth J. Williams3,9,10, Nadia A. Zatsepin4, Petra Fromme3,4, John C. H. Spence1, Kenneth N. Goldie1, Johannes A. Jehle4, Peter Metcalf1,11, Anton Barty4, and Henry N. Chapman3,11.
Application results I: nanocrystalline granulovirus data collection

<table>
<thead>
<tr>
<th>System:</th>
<th>Talos Arctica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength:</td>
<td>0.025Å</td>
</tr>
<tr>
<td>Stage:</td>
<td>single-tilt</td>
</tr>
<tr>
<td>Camera:</td>
<td>Ceta-D</td>
</tr>
<tr>
<td>Sample temp:</td>
<td>cryo</td>
</tr>
<tr>
<td>Optical mode:</td>
<td>nanoprobe</td>
</tr>
<tr>
<td>Camera length:</td>
<td><strong>3.6 m</strong></td>
</tr>
<tr>
<td>Dose per frame:</td>
<td>0.06 e/Å²</td>
</tr>
<tr>
<td>Total frames:</td>
<td>25-50</td>
</tr>
<tr>
<td>Total dose:</td>
<td>1.5 -3.0 e/Å²</td>
</tr>
<tr>
<td>Rotation speed:</td>
<td>0.25 deg/s</td>
</tr>
<tr>
<td>Ang.increment:</td>
<td>0.5 deg</td>
</tr>
</tbody>
</table>
Application results I: granulin density map at 2.8 Å ($2F_{\text{obs}} - F_{\text{calc}}$ omit map)

**Processing:**
- Using DIALS indexing software (Acta Cryst D 74, 506-518)
- Structure refinement with CCP4 suite

**Collaboration:**
- Dominik Oberthür (CFEL, Hamburg)
- Richard Bunker (FMI Basel)
- **David Waterman (DIALS, DLS)**
- Abhay Kotecha, Bart Buijsse, Lingbo Yu, Michael Janus (Thermo Fisher Scientific)

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Single crystal</th>
<th>Five crystals</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of crystals</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>$a = b = c = 103.0$</td>
<td>$a = b = c = 103.6$</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>27.5–3.0</td>
<td>27.7–2.8</td>
</tr>
<tr>
<td>Total reflections</td>
<td>9103 (278)</td>
<td>20356 (599)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>2622 (125)</td>
<td>4056 (214)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.5 (2.2)</td>
<td>5.0 (2.1)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>67.9 (32.4)</td>
<td>88.0 (46.8)</td>
</tr>
<tr>
<td>Mean $\langle l / l \rangle$</td>
<td>2.72 (1.49)</td>
<td>3.16 (0.65)</td>
</tr>
<tr>
<td>Wilson $B$-factor</td>
<td>48.0</td>
<td>47.5</td>
</tr>
<tr>
<td>$R_{\text{merge}}$</td>
<td>0.33 (0.48)</td>
<td>0.32 (0.75)</td>
</tr>
</tbody>
</table>

**Refinement**
- Reflections used in refinement | 2616 (125) | 3999 (214) |
- Reflections used for $R_{\text{free}}$ | 265 (15) | 400 (22) |
- $R_{\text{work}}$ | 0.24 (0.27) | 0.18 (0.32) |
- $R_{\text{free}}$ | 0.29 (0.32) | 0.23 (0.49) |

- Protein residues: 243
- R.m.s bond lengths (Å), angles (°): 0.005, 0.6 / 0.004, 1.0
- Ramachandran plot (%): Favourable (96.7, 3.3, 0) / 95.9, 4.1, 0
- Average protein $B$-factor (Å$^2$): 46.3 / 39.8

Phasing by molecular replacement using PDB map of granulovirus.
Application results I: granulin structure at 2.8 Å

Granulin Protomer

Biological unit; dodecameric building blocks
Application results II: FIB milling of intermediate size (1-30 μm) protein crystals
EPU-D application results II: micro-crystals (5-7µm) of lysozyme

- Lysozyme crystal
- Single crystal milling
- Lamella thickness 200 nm
- Milling time: 10-45 min
EPU-D application results II: TEM on lysozyme crystal lamella

ED diffraction peaks to 1.9 Å
EPU-D application results II: lysozyme map (1.9 Å)

Duyvesteyn, Kotecha et al (2018) PNAS 115 (38), 9569-9573
EPU-D application results III: pharmaceutical molecule structure determination

Progesterone at 1 Å resolution
Diameter of grid holes – 1 μm

EPU-D application results III: paracetamol structure determination

**Sample**
Ground paracetamol tablet

**Prep.**
Lacey carbon grid, Cryo- temperature

**Acquisition**
81 x 1.0° x 1 sec
0.88 Å

**Analysis**
Dials → shelxt
49.3% complete
EPU-D application results III: paracetamol structure at 0.9 Å
Small molecule mED – example from literature

- mED can be used for quality control: for confirming the “intended” structure

- Heterogenous powder containing four compounds

Challenges and future perspectives
Protein crystal mED: crystals too large

Apo ferritin crystals...
Small molecule mED: ice and salt contamination

- Small molecule, salt and ice crystals all have similar unit cells and therefore their diffraction patterns look similar making it is hard to distinguish between the three during crystal screening and data collection.
- Worst case scenario: 50 datasets collected on a customer sample were all salt.

Better annotation autoprocessing needed
Noise in the diffraction images

- Strong diffraction pattern requires background suppression
- Small illuminated area (small cond. apt. or 3-condenser lens system) helps

Use such condenser aperture so that the field of view is only a little bit larger than the crystal
Stage eucentricity

- Crystal needs to stay in a small field of view during stage rotation.
- Autoloader has an advantage over side-entry holder.
- Eucentricity specification for Talos is 2, 2, 4 μm and for Krios 1,1,3 μm (-70 to +70 degrees).
- Tilting above 50 degrees is more unpredictable, the crystal might drift out of the field of view during diffraction data collection.

- Use a larger C2 aperture to compensate
- Collect multiple datasets to allow data merging
What is coming to the micro-ED workflow

- Reproducible cryo sample preparation for microED
- MicroED-specific data processing step integration into the existing crystallography packages
  - DIALS and CCP4i2
1. For optimum stage linearity, do not exceed tilt speed of $\leq 1$ deg/second (for our setup).
2. Smaller beam stop gives better results.
1. For optimum stage linearity, do not exceed tilt speed of ≤ 1 deg/second (for our setup).
2. Smaller beam stop gives better results.
3. Correct for diffraction lens astigmatism.
4. Important to know the real camera length in diffraction. If in doubt, take gold diffraction image using data collection parameters.
5. Do not forget to center the beam underneath the beam stop!
6. Keep in mind the radiation damage. Recommended total dose ≤ 3 e/Å²/s but varies with different samples.
EM-learning.com is a new learning platform that features over 70 hours of theoretical lectures and videos. It is created in collaboration with online education expert Prof. Grant Jensen (Caltech) and serves as an introduction to the field and is intended for participants of all levels. Upon completion, you will have a fundamental knowledge of cryo-EM, get tips and tricks to overcome sample preparation challenges and valuable practical advices on the cryo-EM workflow.

We would like to thank Grant Jensen, Matthijn Vos, Caltech film crew, Wendela Vuurberg, Innostrat and a number of Thermo Fisher Scientific colleagues to make this project happen.

Single Particle Analysis

The full cryo-EM SPA course will train users with alternating theory and practical demonstration videos in more depth with the possibility to self-assess the acquired knowledge with a test. The course will cover all aspects from cryogenic sample preparation, microscope design and operation, cameras, data acquisition, etc. in a logical order over different modules.
Thank you for your attention!

Any questions?

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Back-up slides
EPU-D: microED acquisition software

EPU™
Automated Data Acquisition Software for Single Particle Workflow
thermo scientific

EPU-D
Automated Data Acquisition Software for Micro Electron Diffraction Workflow
thermo scientific

TOMO
Automated Data Acquisition Software for Electron Tomography Workflows
thermo scientific
**Thermo Fisher’s MicroED Solution**

**Future Developments for EPU-D**

1. **Ceta-D camera**
   - Optimized for diffraction application: increased accuracy and sensitivity
   - Compatible with TEM sample screening
   - Compatible with bottom-mount filter (retractable)

2. **MicroED package**
   - EPU-D for data acquisition
   - Modified beam stop
   - Small C2 aperture
   - MicroED lens series

- Automated batch acquisition
- Automated screening of crystals
- Automated selection of crystals
- Automated judgment of results
Check resolution, unit cell dimension

- 14131,865,207 1/m
- 1/14132 1/um = 7.08e-5 um, ~0.7Å

- 3147,069,934 1/m
- 1/3147 1/um = 3.178e-, ~3Å
Large Ewald sphere

- Harder to perform 2D indexing of the spots due to a large Ewald sphere
- Especially problematic for merging datasets if the crystal has many isoforms or when multiple lattices are present

\[ s_0 = \frac{1}{\lambda} \]

\[ \theta \]

\[ S \]

1/d

- Collect as large a sweep as possible
- Only use the beginning of the dataset for reconstruction