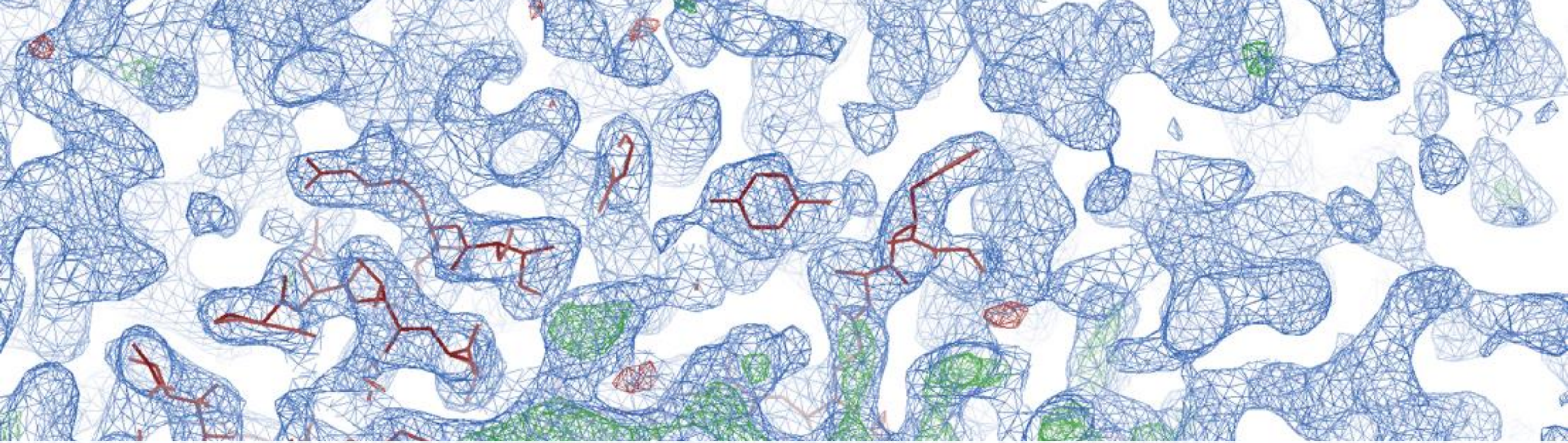


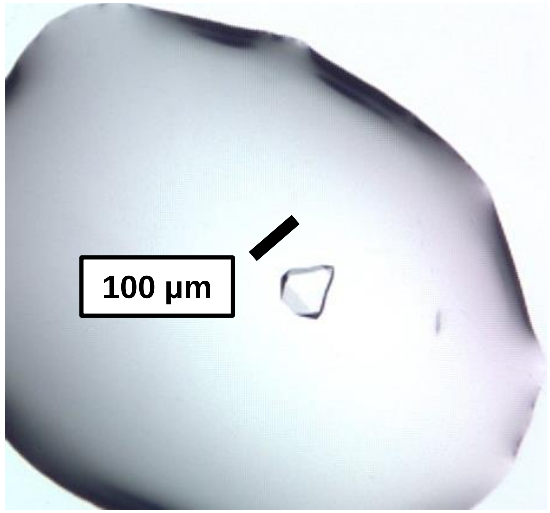
MicroED: EPU-D results, bottlenecks and future perspectives

Ieva Drulyte, Abhay Kotecha, Bart Buijsse, Lingbo Yu, Fanis Grollios, Hans Raaijmakers
Materials and Structural Analysis, EM Life Sciences

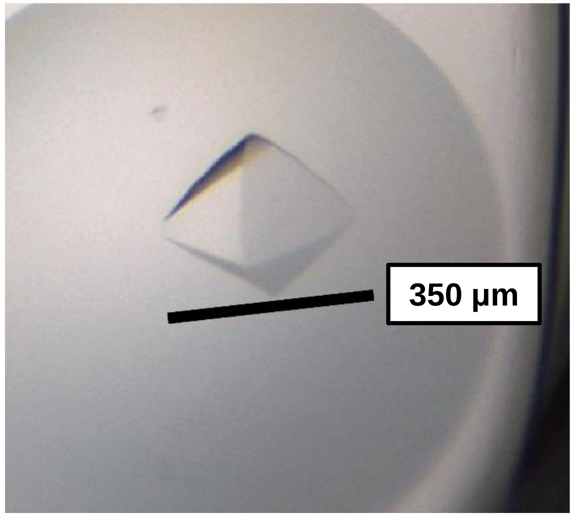
EMBO practical course: Image processing for cryo-electron microscopy
Birkbeck, London, September 6, 2019

Why is micro-electron diffraction useful?

X-ray diffraction requires large crystals

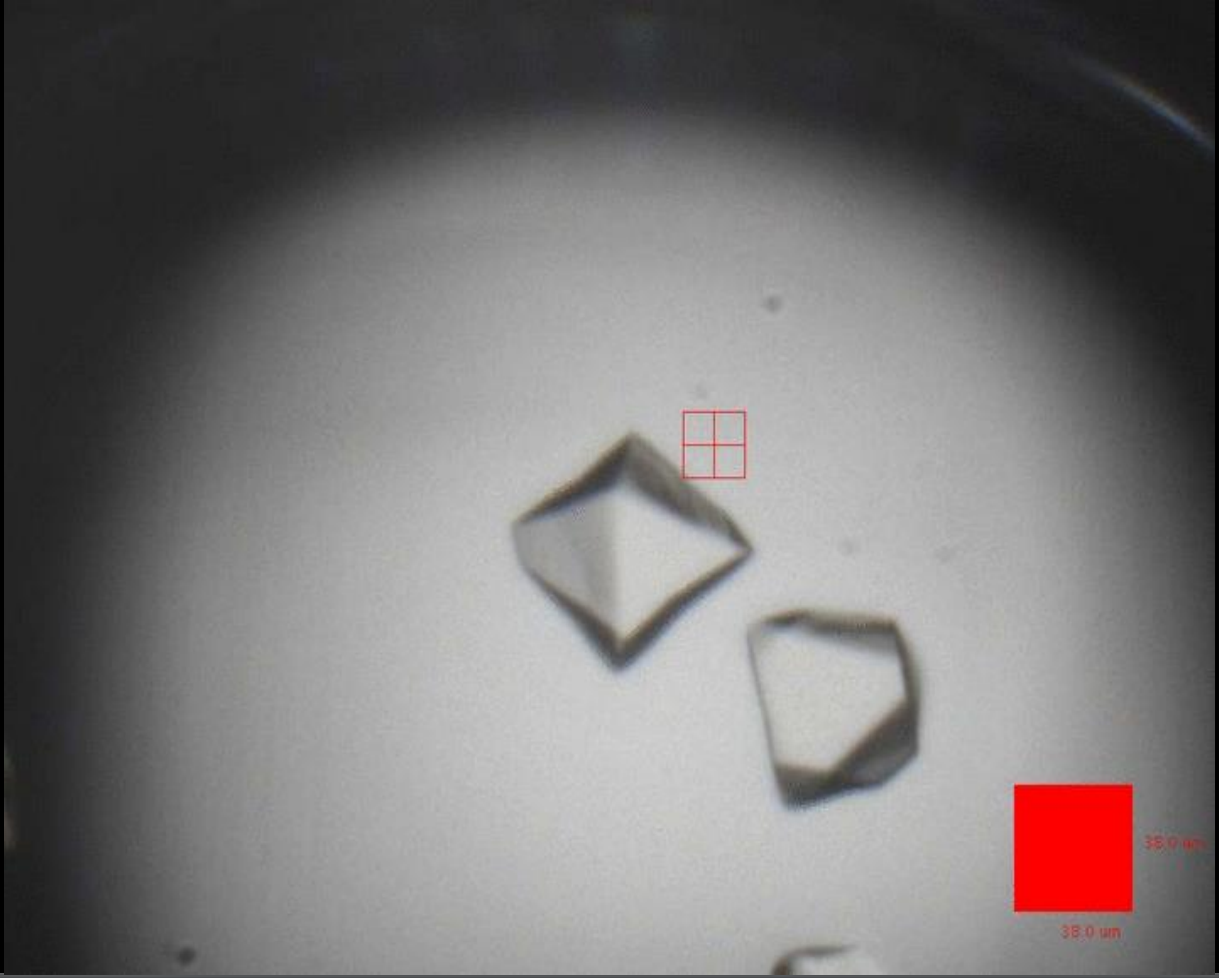


Crystals of FMDV



Crystals of IdmH

- Limiting factor for X-ray diffraction – big ordered protein crystals needed.
- 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.

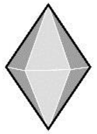

38.0 um
38.0 um

Electrons vs x-rays

	Electrons 80–500 keV	X-rays 1·5 Å	X-rays 30 Å
Ratio † (inelastic/elastic) scattering events	3	10	10^3–10^4
Mechanism of radiation damage	Secondary e^- emission	Photoelectric e^- emission	
Energy deposited per inelastic event	20 eV	8 keV	400 eV
Energy deposited per elastic event**	60 eV	80 keV	400 keV
Energy deposited relative to electrons			
(inelastic)	1	400	20
(elastic)	1	1000	10000

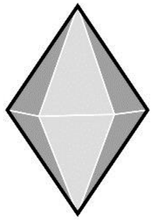
Henderson (1995) *Quart. Rev. Biophys.* **28,** 171

Why is microED useful?



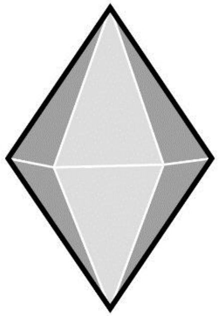
Avg size range:
50-500 nm

microED



Avg size range:
1-10 micron

Too small for XRD
Too large for MED



Avg size range:
10-100 micron

X-ray diffraction

Main microED applications for nanocrystals:

- Protein structures 1-3 Å resolution range
- Small organic molecules <1 Å resolution range

Main microED application for microcrystals:

- Protein structures after FIB-milling

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.

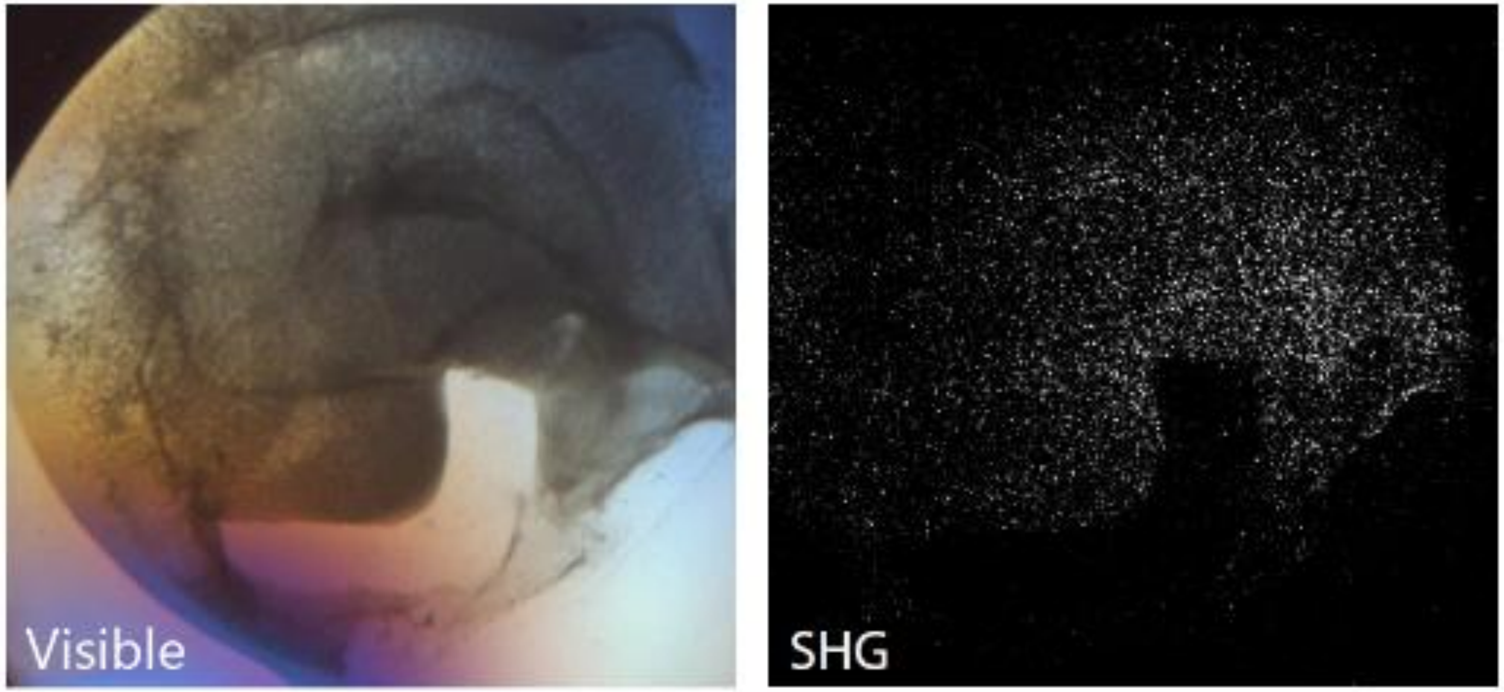
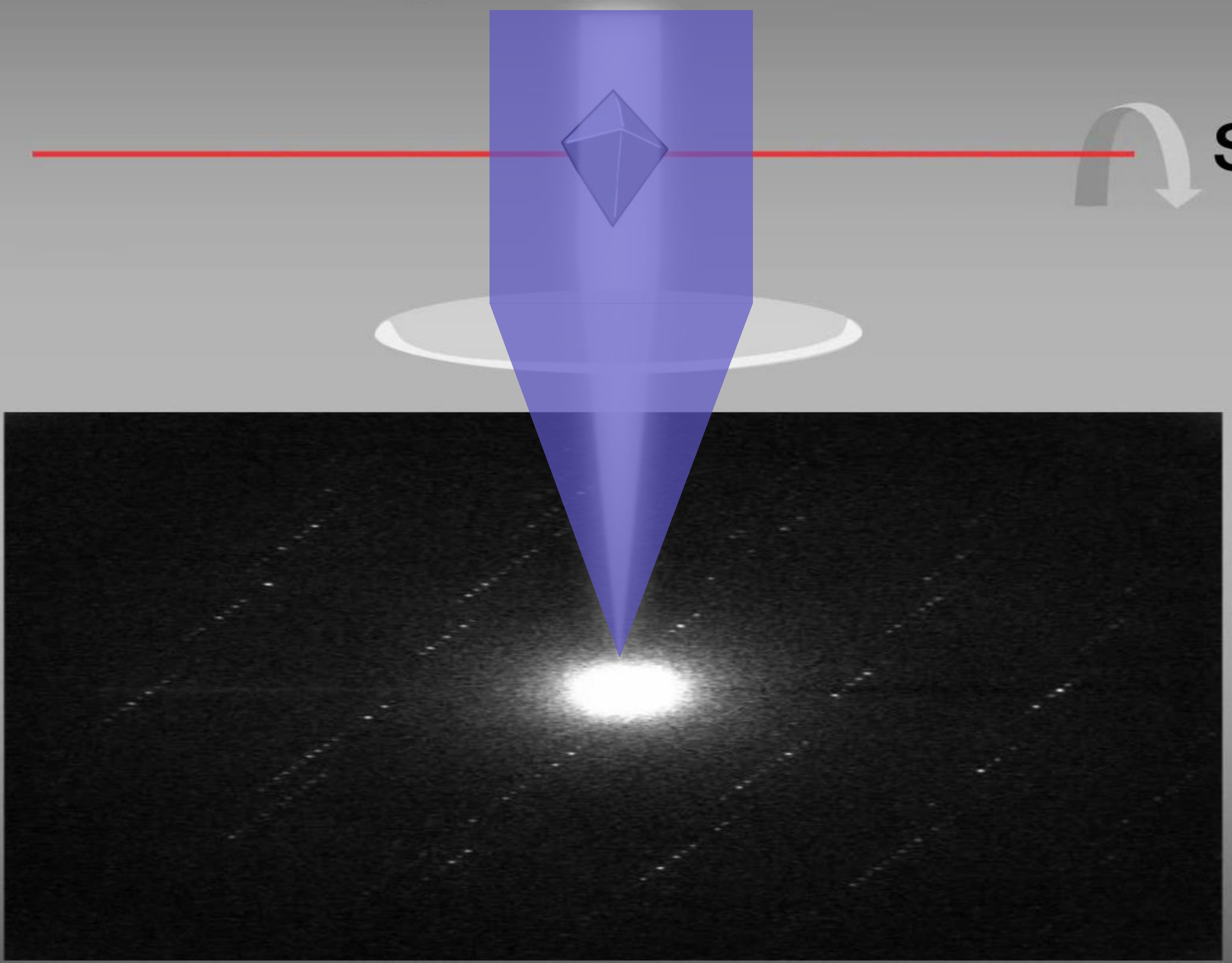


Image copyright: Formulatrix

Thermo Fisher SCIENTIFIC

Principle of micro-ED



3D structure

- Only few crystals needed
- Low dose imaging (1.5 - 3 $e^{-}/Å^{2}$)
- Cryo-conditions
- Provides high resolution

No special tools required

Vitrobot

Talos L120C

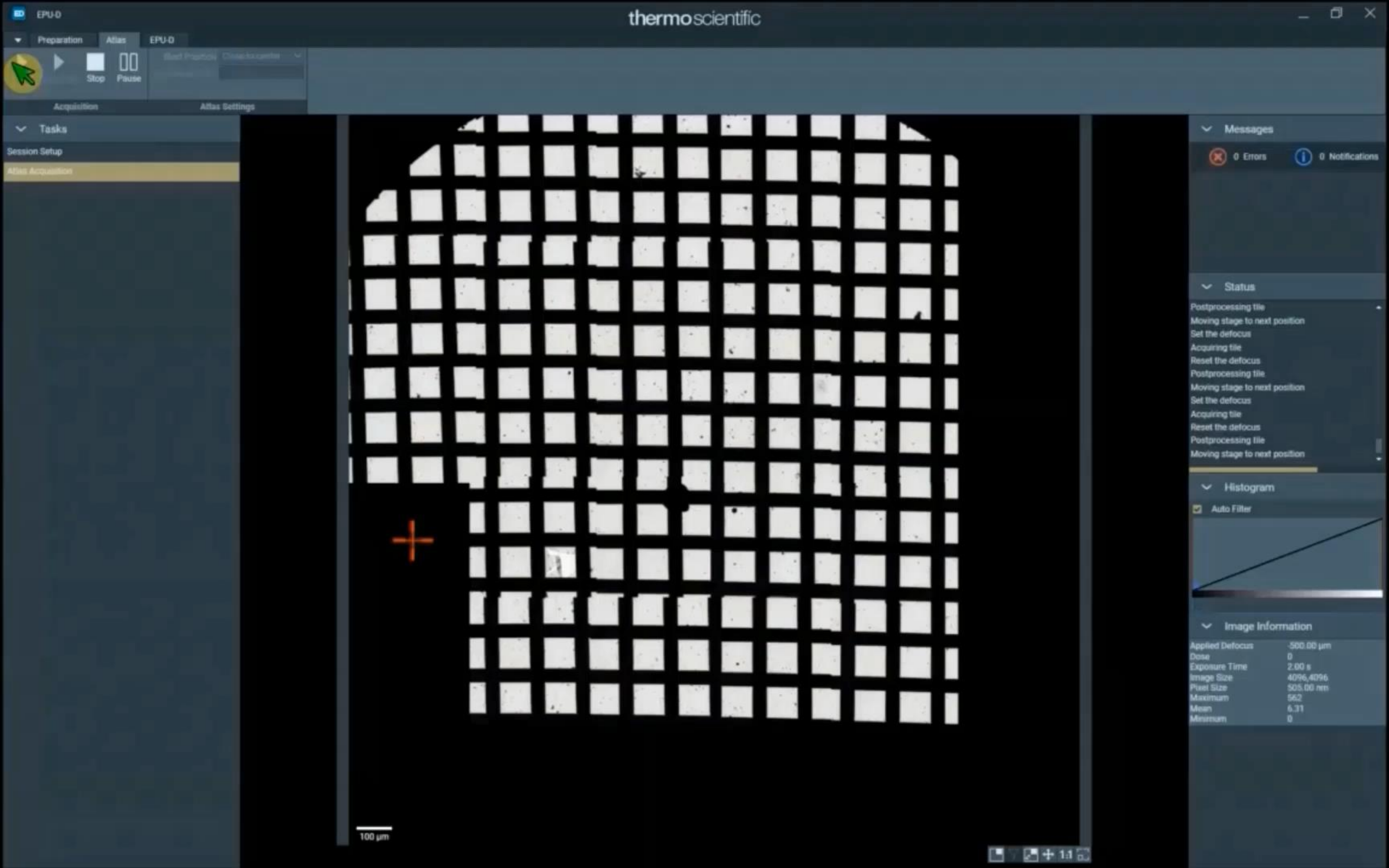
F200C

Glacios

Talos Arctica

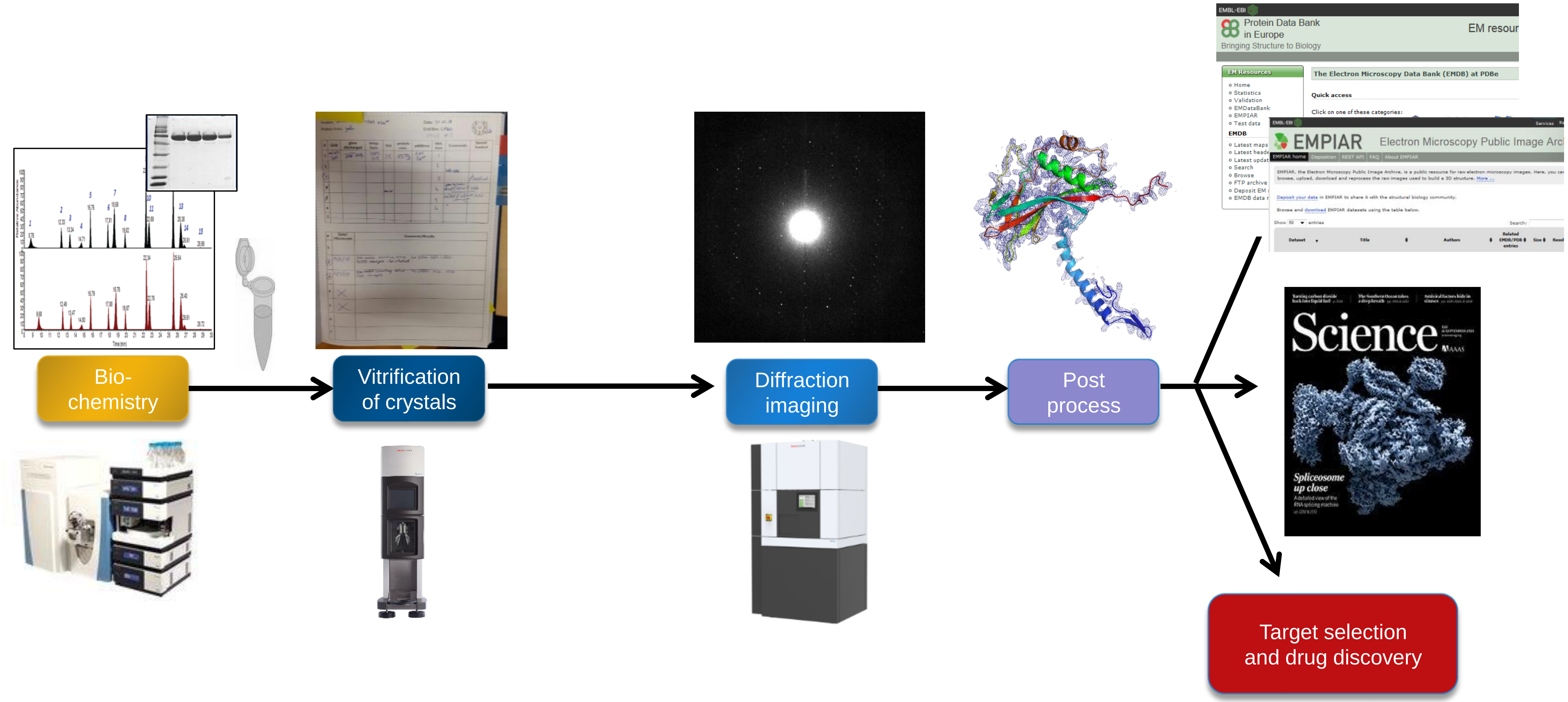
Krios

Aquilos


EPU-D
thermoscientific
Preparation
Atlas
EPU-D
Stop
Pause
Acquisition
Atlas Settings
Tasks
Session Setup
Atlas Acquisition
100 µm
Messages
0 Errors
0 Notifications
Status
Postprocessing tile
Moving stage to next position
Set the defocus
Acquiring tile
Reset the defocus
Postprocessing tile
Moving stage to next position
Set the defocus
Acquiring tile
Reset the defocus
Postprocessing tile
Moving stage to next position
Histogram
Auto Filter
Image Information
Applied Defocus -500.00 µm
Dose 0
Exposure Time 2.00 s
Image Size 4096,4096
Pixel Size 505.00 nm
Maximum 562
Mean 6.31
Minimum 0
1:1

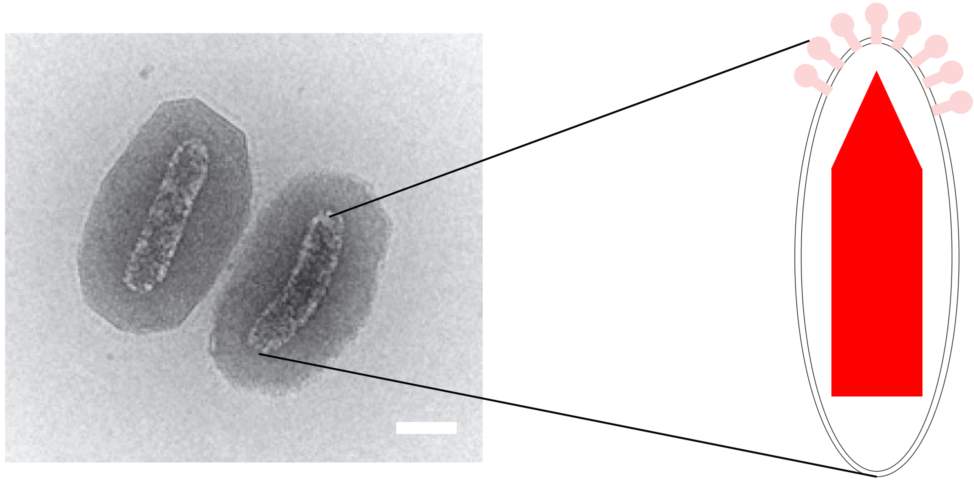
EPU-D application results

Application results I: mED of small (<1 µm) protein crystals



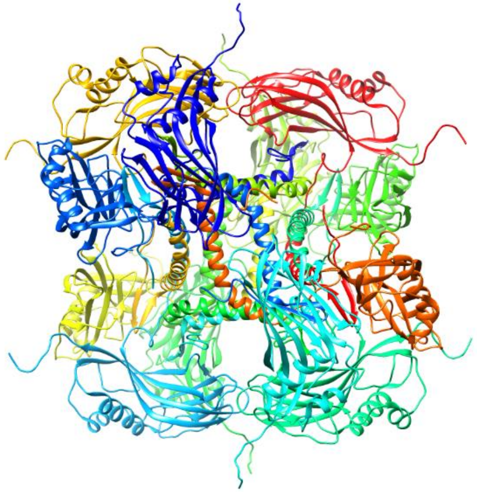
Application results I: nanocrystalline granulovirus

- 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

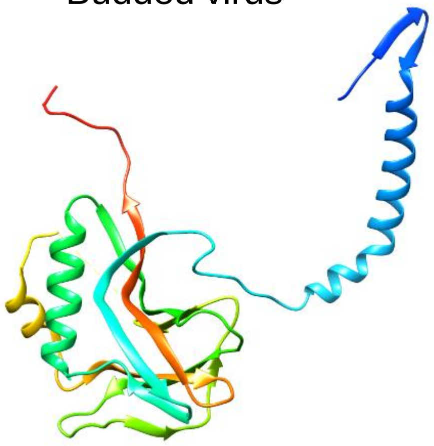


Occlusion bodies

Budded virus



Unit cell (12 granulins)



Granulin monomer

Application results I: nanocrystalline granulovirus

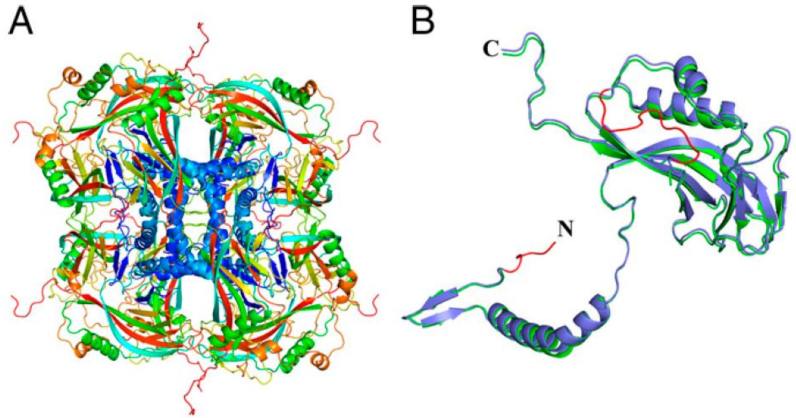
Structure solutions by X-ray crystallography:

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

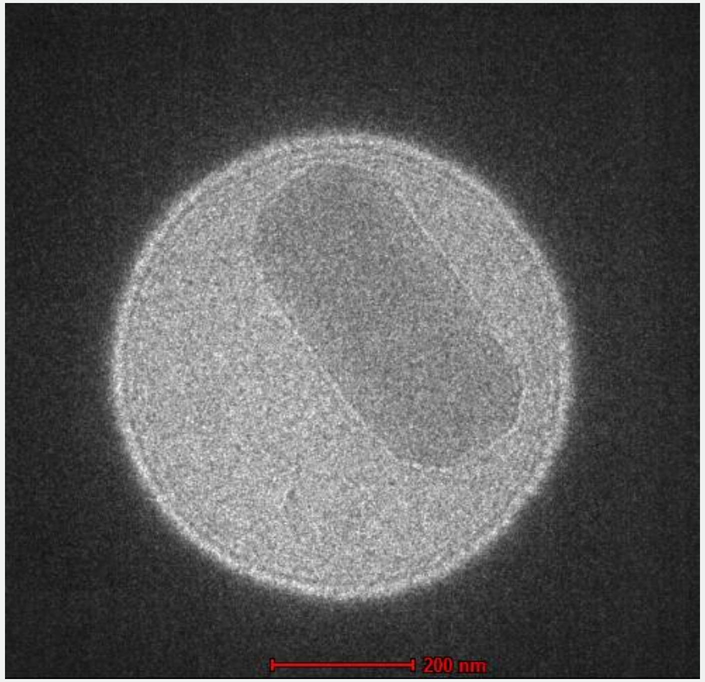
PNAS

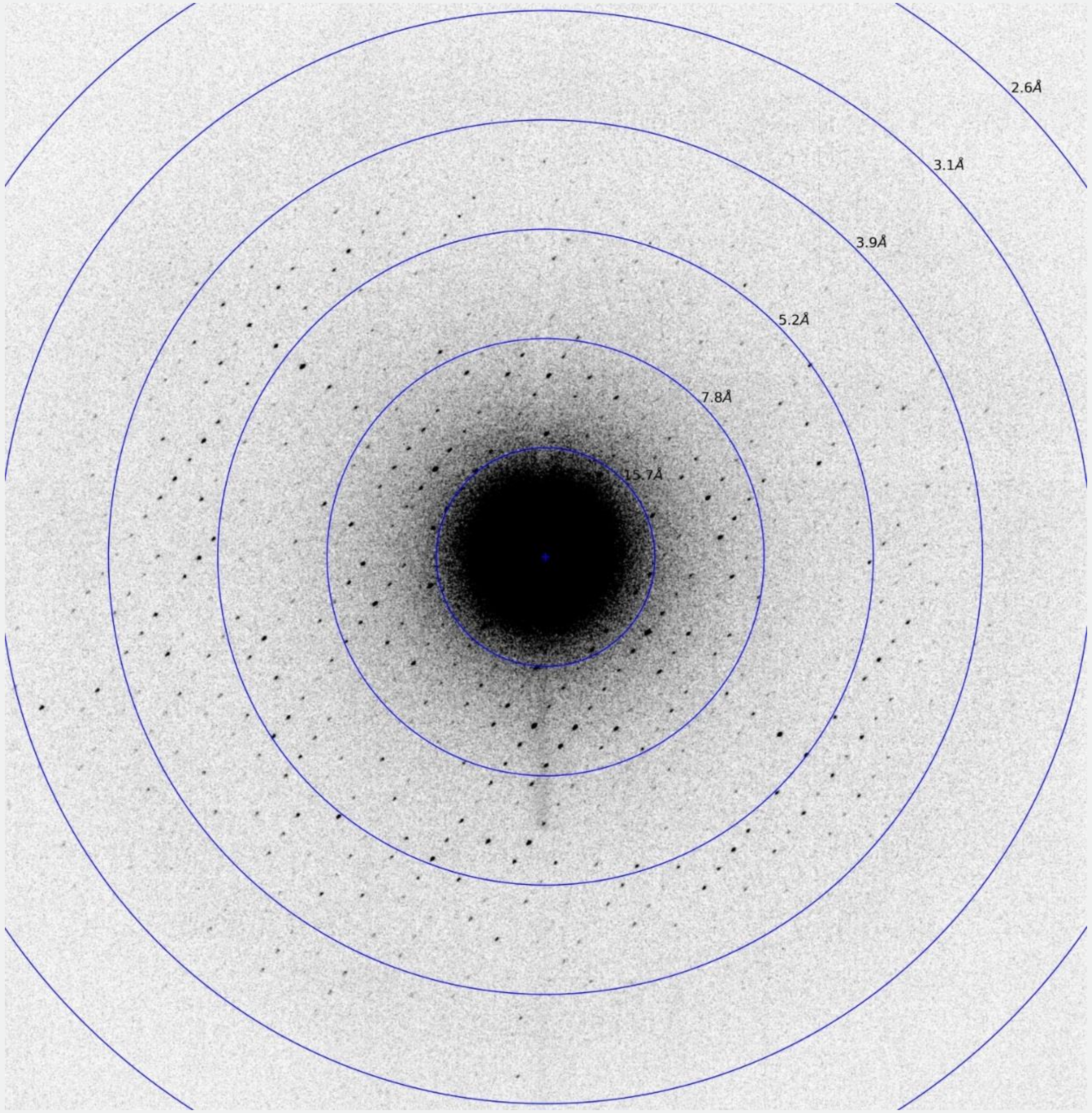
Atomic structure of granulin determined from native nanocrystalline granulovirus using an X-ray free-electron laser

Cornelius Gati[a,1], Dominik Oberthuer[a], Oleksandr Yefanov[a], Richard D. Bunker[b,2], Francesco Stellato[a], Elaine Chiu[b], Shin-Mei Yeh[b], Andrew Aquila[a,c], Shibom Basu[d,e,3], Richard Bean[a,c], Kenneth R. Beyerlein[a], Sabine Botha[f,4], Sébastien Boutet[g], Daniel P. DePonte[a,h], R. Bruce Doak[i,5], Raimund Fromme[d,e], Lorenzo Galli[a], Ingo Grotjohann[d], Daniel R. James[i], Christopher Kupitz[d,e,6], Lukas Lomb[f], Marc Messerschmidt[g,7], Karol Nass[a,8], Kimberly Rendek[d], Robert L. Shoeman[f], Dingjie Wang[i,9], Uwe Weierstall[e,i], Thomas A. White[a], Garth J. Williams[g,10], Nadia A. Zatsepin[e,i], Petra Fromme[d,e], John C. H. Spence[e,i], Kenneth N. Goldie[j], Johannes A. Jehle[k], Peter Metcalf[b,11], Anton Barty[a], and Henry N. Chapman[a,l,m,11]



Application results I: nanocrystalline granulovirus data collection





System:	*Talos Arctica*	*Camera length:*	***3.6 m***
Wavelength:	*0.025Å*	*Dose per frame:*	*0.06 $e^-/Å^2$*
Stage:	*single-tilt*	*Total frames:*	*25-50*
Camera:	*Ceta-D*	*Total dose:*	*1.5 -3.0 $e^-/Å^2$*
Sample temp :	*cryo*	*Rotation speed:*	*0.25 deg/s*
Optical mode:	*nanoprobe*	*Ang.increment:*	*0.5 deg*

Application results I: granulin density map at 2.8 Å ($2F_{obs} – F_{calc}$ omit map)

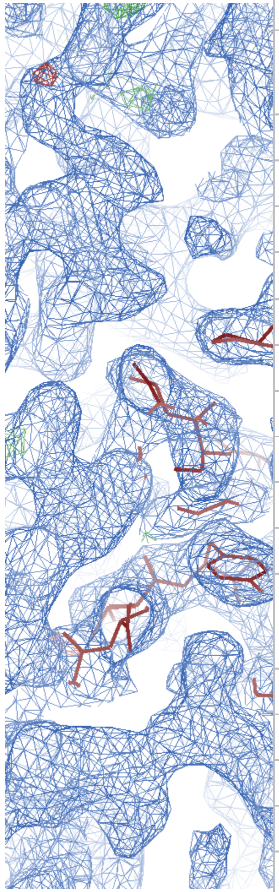
Processing:

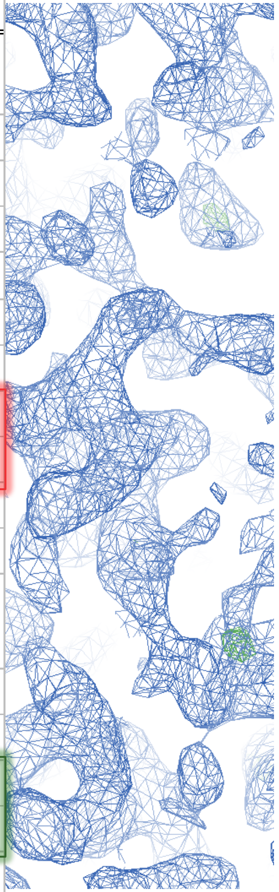
- Using DIALS indexing software (*Acta Cryst D* **74**, 506-518)

- 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)

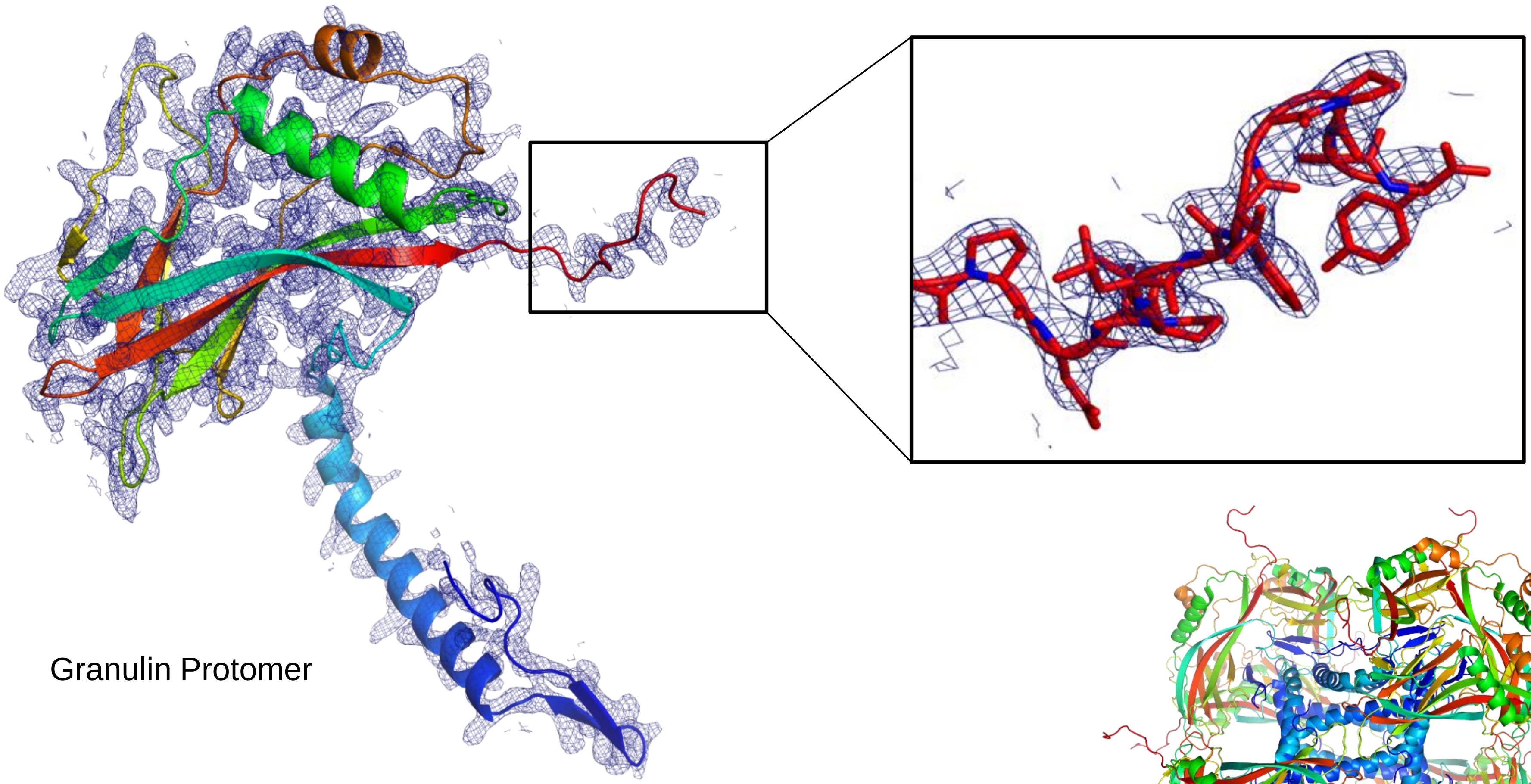




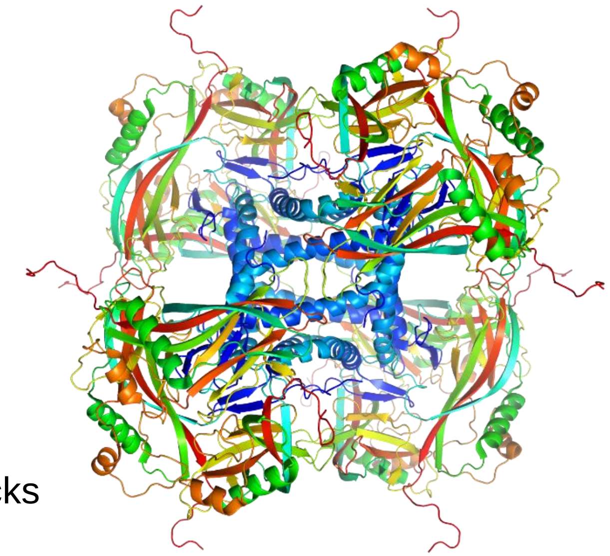
Phasing by ... anulovirus

	Single crystal	Five crystals
Data collection		
No. of crystals	1	5
Unit cell (Å)	*a* = *b* = *c* = 103.0	*a* = *b* = *c* = 103.6
Resolution range (Å)	27.5–3.0	27.7–2.8
Total reflections	9103 (278)	20356 (539)
Unique reflections	2622 (125)	4056 (214)
Multiplicity	3.5 (2.2)	5.0 (2.1)
Completeness (%)	67.9 (32.4)	88.0 (46.8)
Mean $I/\sigma(I)$	2.72 (1.49)	3.16 (0.65)
Wilson *B*-factor	48.0	47.5
R_{merge}	0.33 (0.48)	0.32 (0.75)
Refinement		
Reflections used in refinement	2616 (125)	3999 (214)
Reflections used for R_{free}	265 (15)	400 (22)
R_{work}	0.24 (0.27)	0.18 (0.32)
R_{free}	0.29 (0.32)	0.23 (0.49)
Protein residues	243	243
R.m.s bond lenghts(Å), angles (°)	0.005, 0.6	0.004, 1.0
Ramachandran plot (%)		
Favoured (%), Allowed, Outliers	96.7, 3.3, 0	95.9, 4.1, 0
Average protein *B*-factor (Å^2)	46.3	39.8

Application results I: granulin structure at 2.8 Å

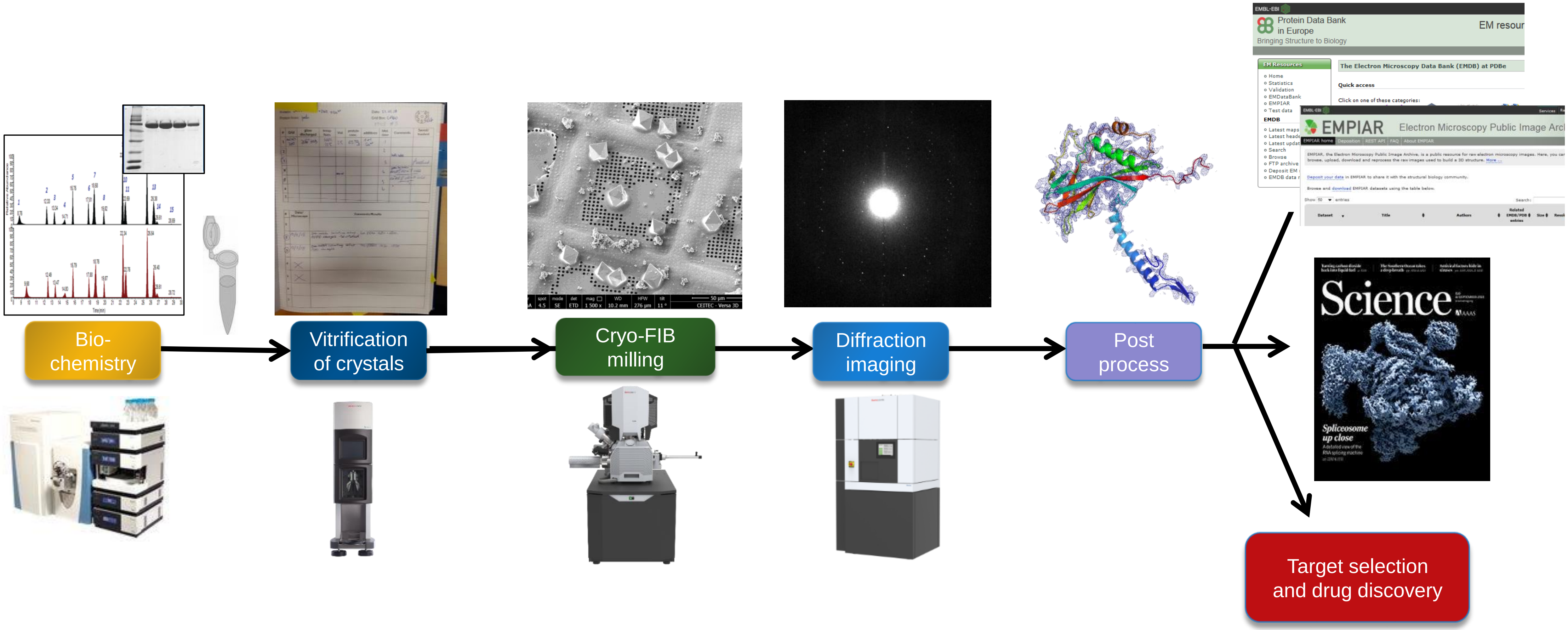


Granulin Protomer

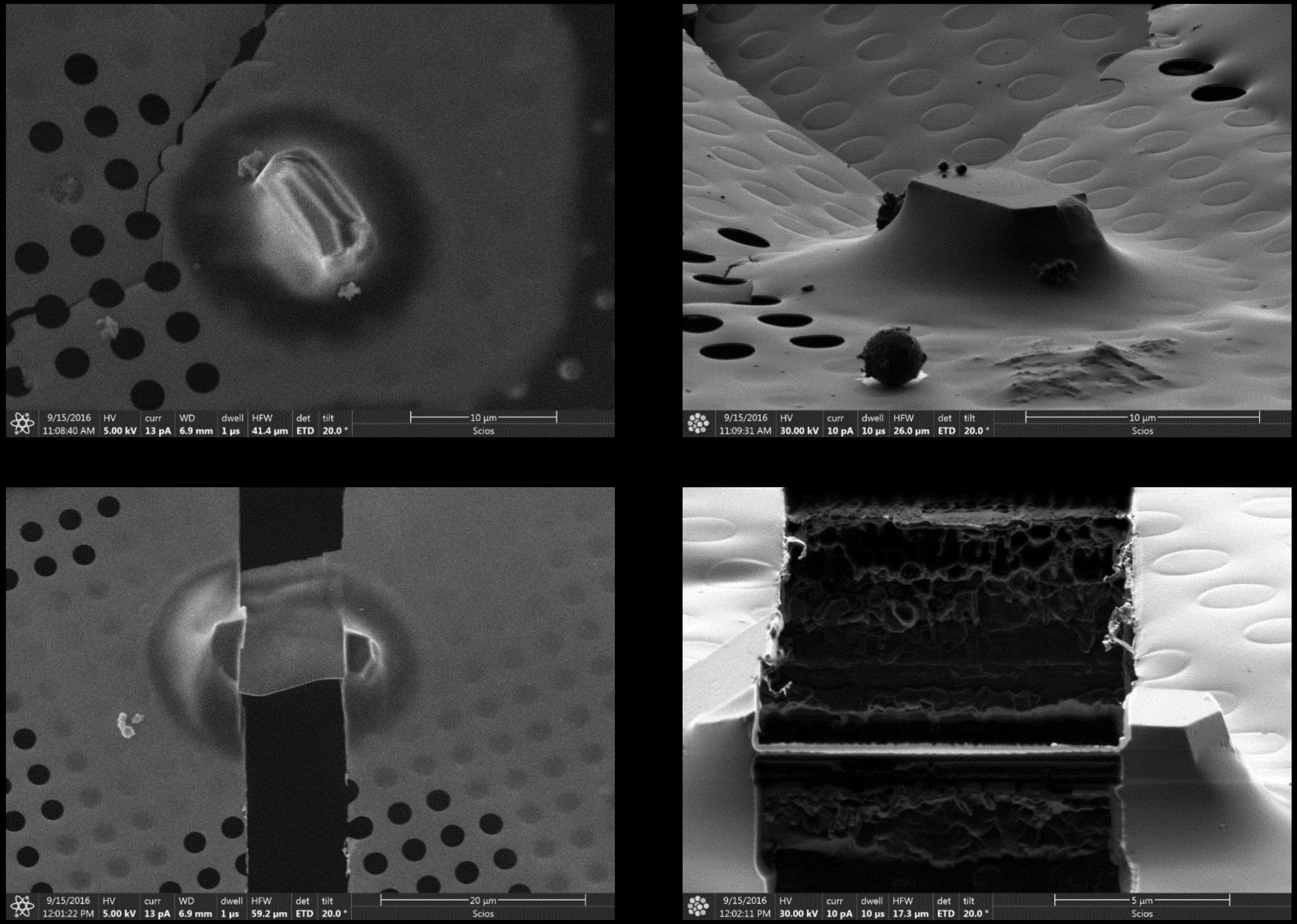


Biological unit; dodecameric building blocks

Application results II: FIB milling of intermediate size (1-30 µm) protein crystals



EPU-D application results II: micro-crystals (5-7µm) of lysozyme

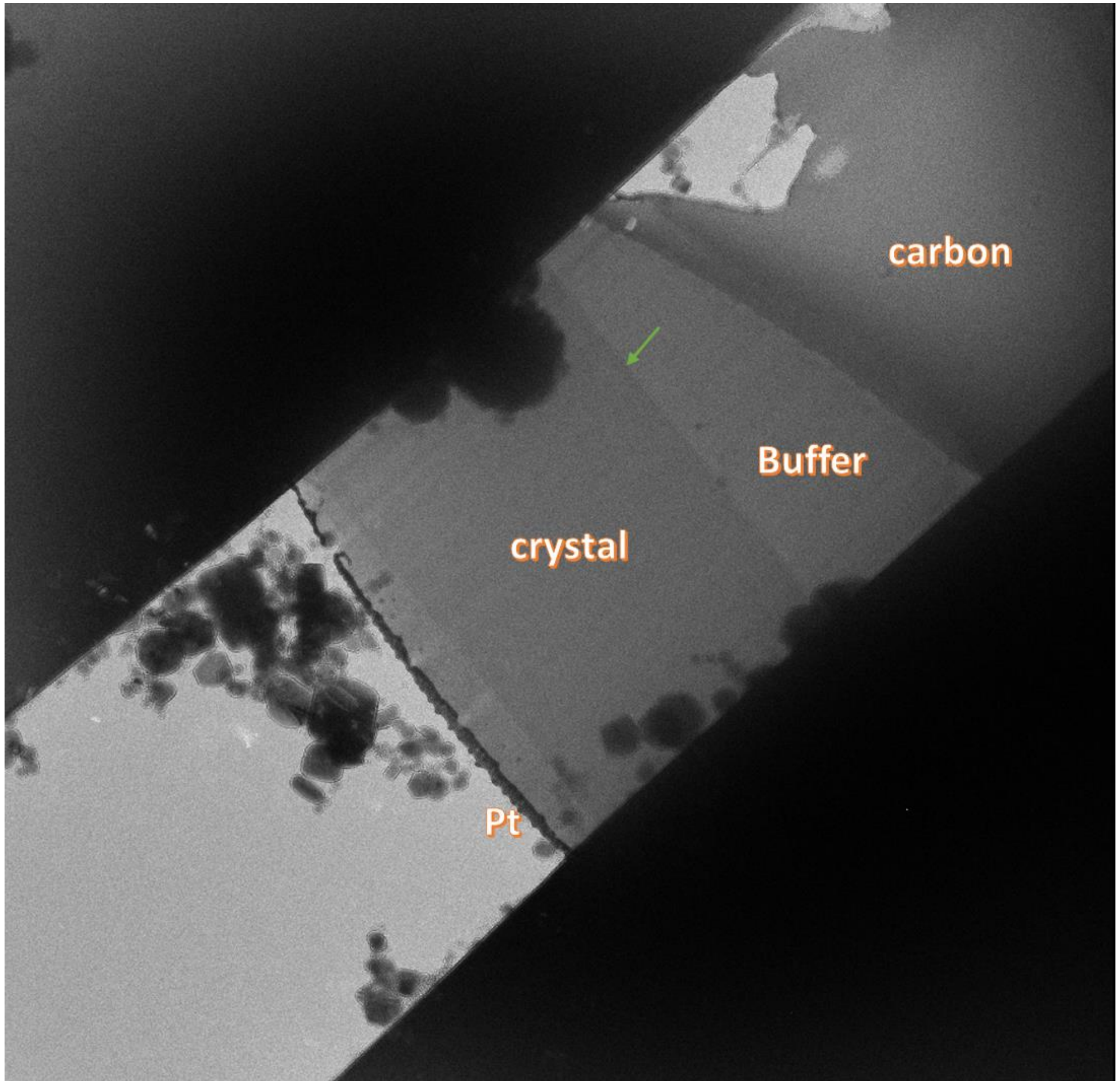


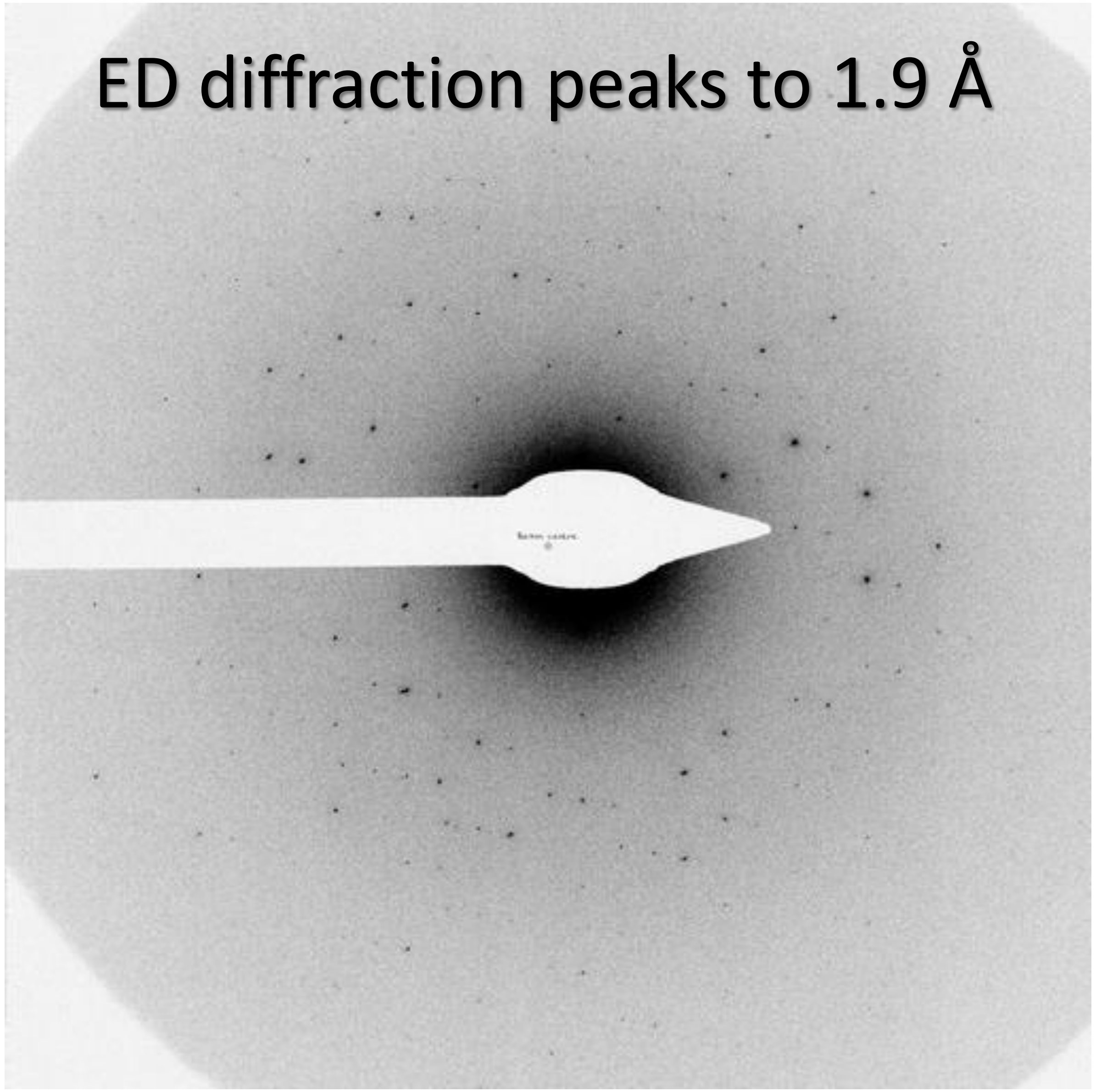
SEM **Ion**

- Lysozyme crystal
- Single crystal milling
- Lamella thickness 200 nm
- Milling time: 10-45 min

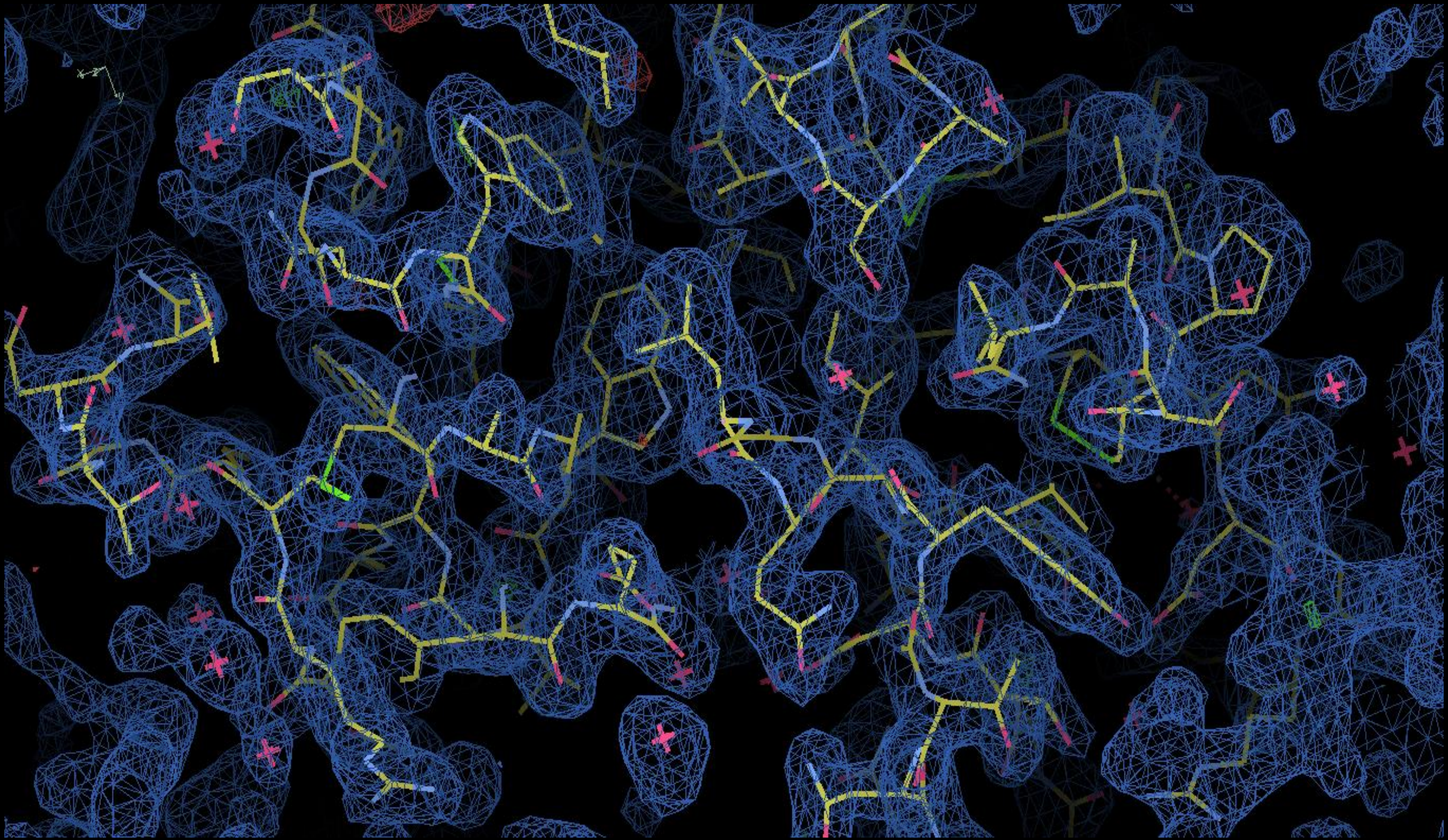
Thermo Fisher SCIENTIFIC

EPU-D application results II: TEM on lyzozyme crystal lamella



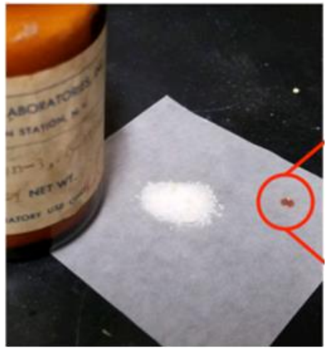


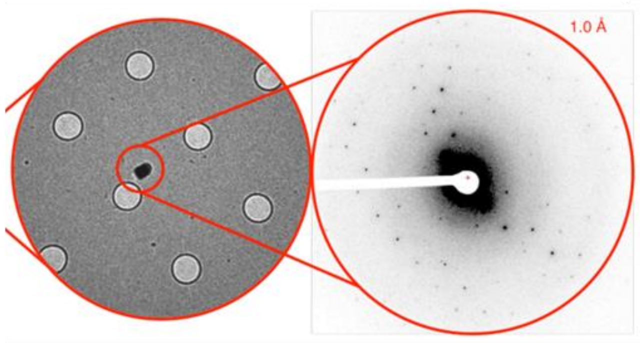
EPU-D application results II: lysozyme map (1.9 Å)

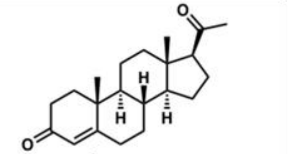


Duyvesteyn, Kotecha et al (2018) *PNAS* 115 (38), 9569-9573

EPU-D application results III: pharmaceutical molecule structure determination







1, progesterone

drug discovery

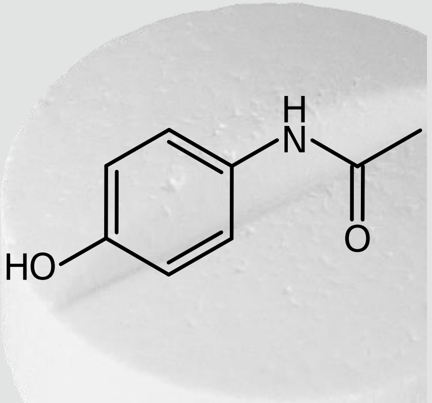
Chemistry → Diffraction imaging → post process → drug discovery / Science

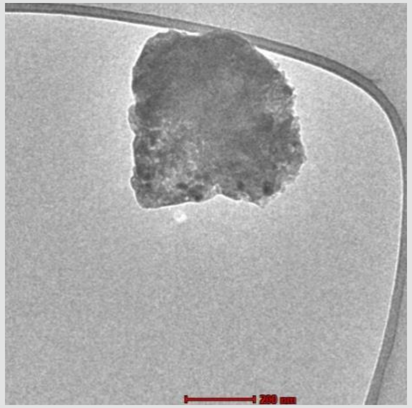
Progesterone at
1 Å resolution
Diameter of grid
holes – 1 µm

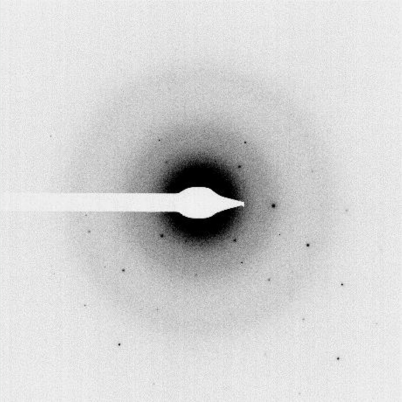
Powder	Grid – nanocrystals	Full MicroED dataset	Structure
~10^{-1} g	~10^{-15} g	< 3 min	~10 min

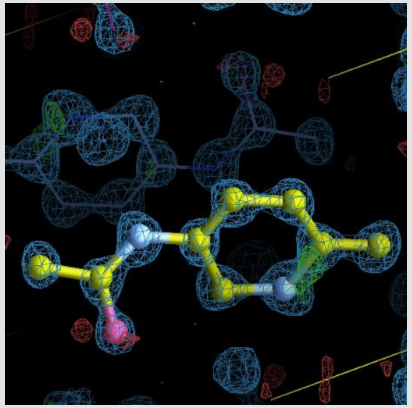
Jones et al (2018) *ACS Central Science* **4** (11), 1587-1592

EPU-D application results III: paracetamol structure determination









Sample

Ground paracetamol tablet

Prep.

Lacey carbon grid, Cryo- temperature

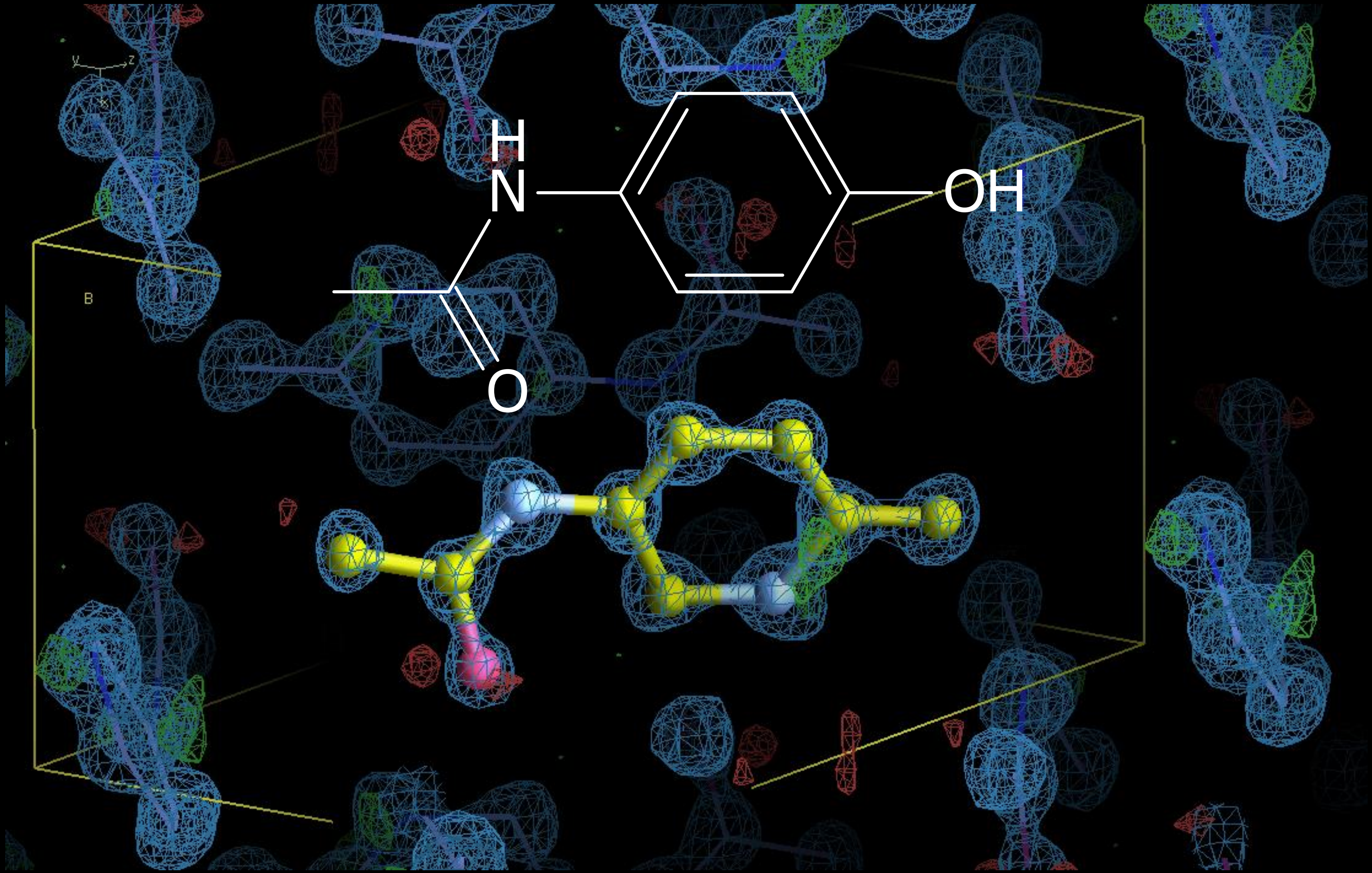
Acquisition

81 x 1.0° x 1 sec
0.88 Å

Analysis

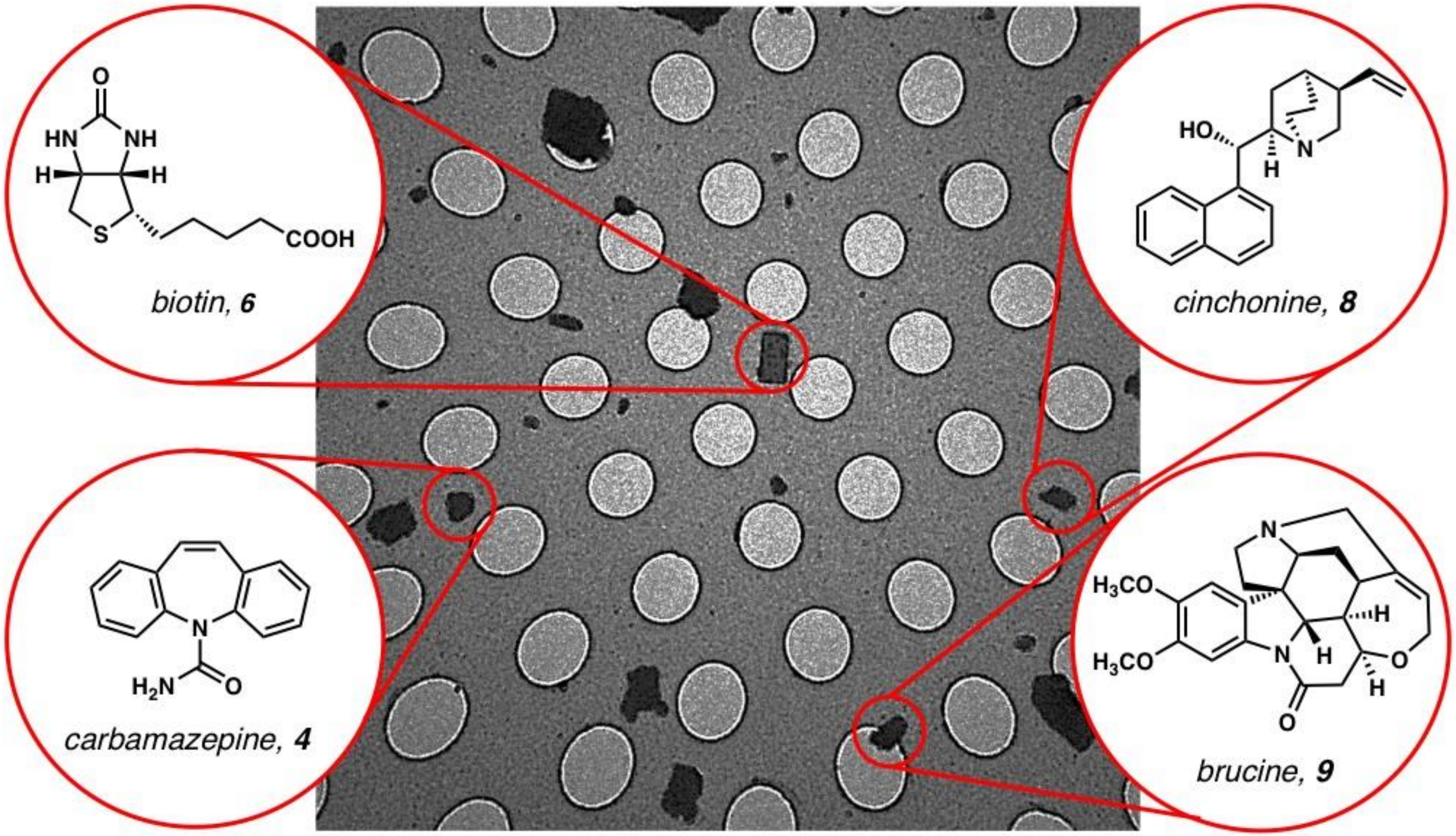
Dials → shelxt
49.3% complete

EPU-D application results III: paracetamol structure at 0.9 Å



Small molecule mED – example from literature

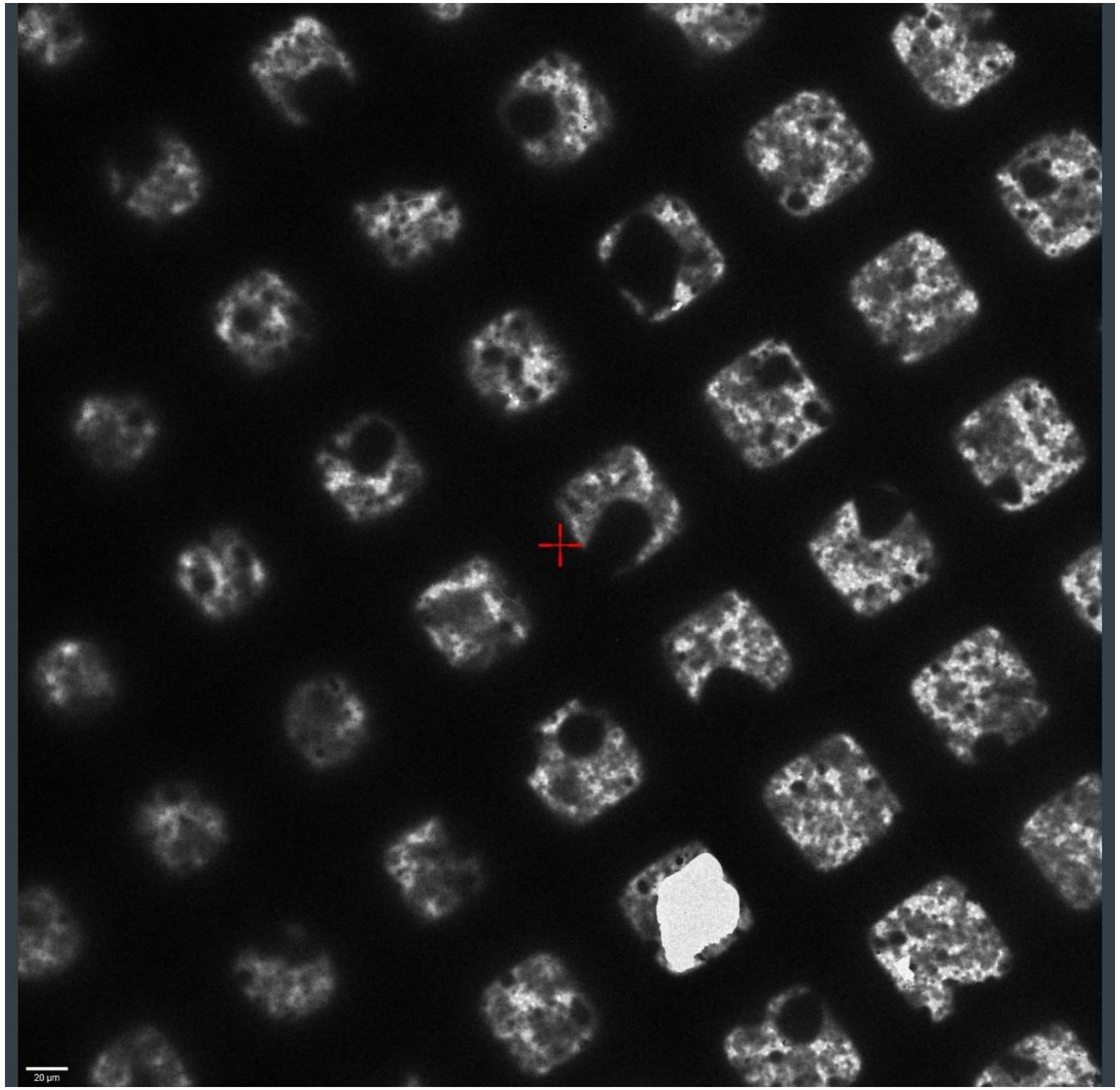
- mED can be used for quality control: for confirming the “intended” structure
- Heterogenous powder containing four compounds

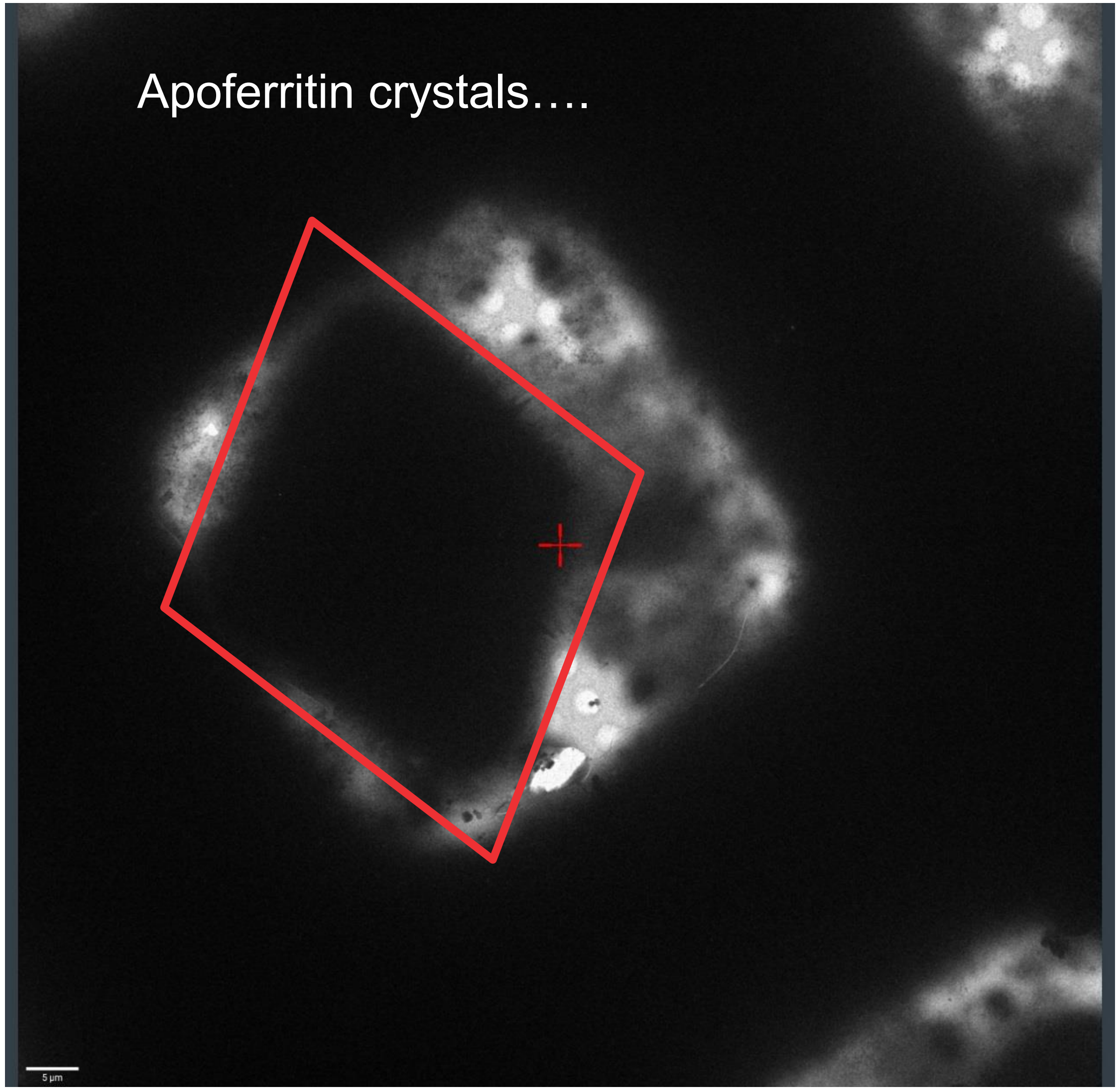


Jones et al (2018) *ACS Central Science* **4** (11), 1587-1592

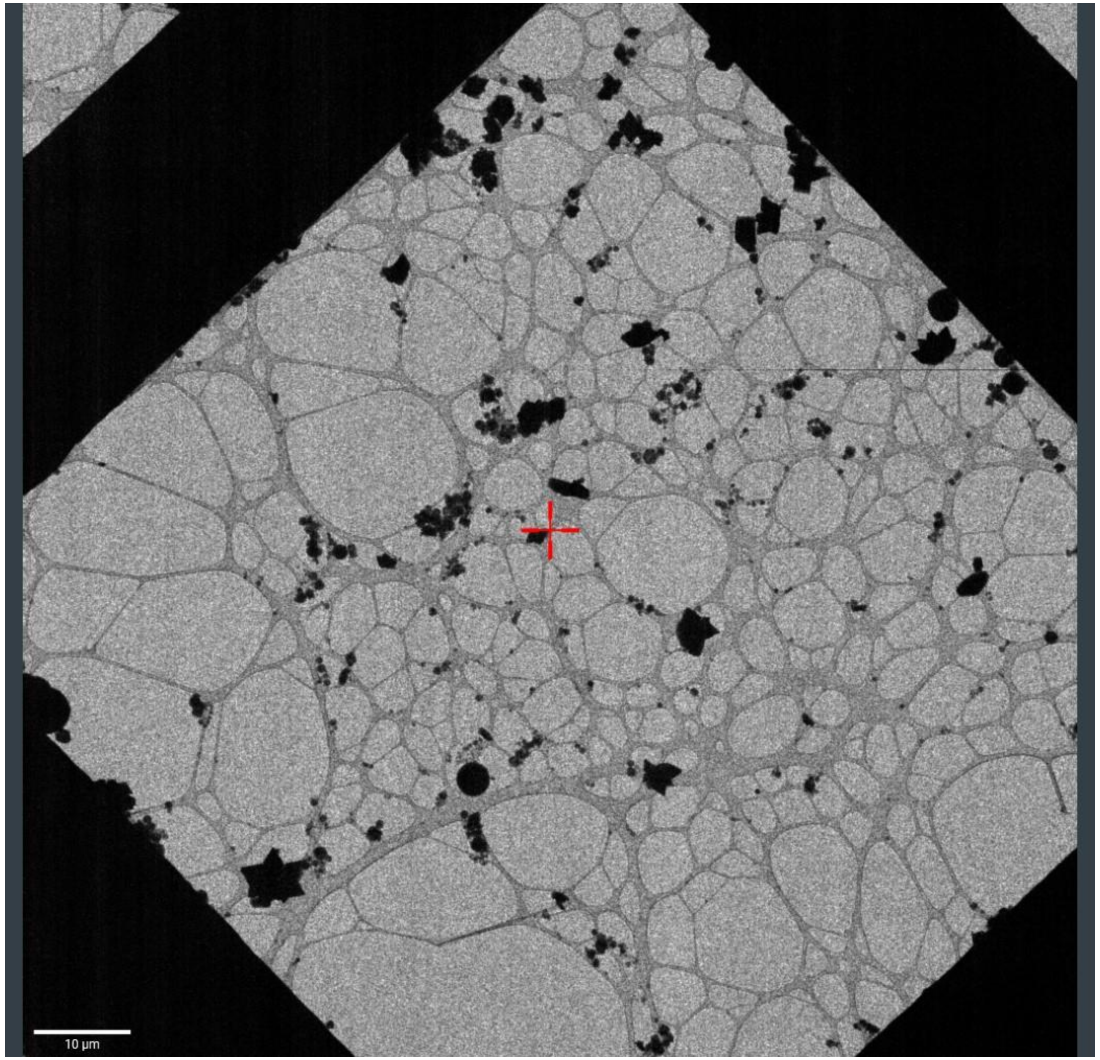
Challenges and future perspectives

Protein crystal mED: crystals too large





Small molecule mED: ice and salt contamination



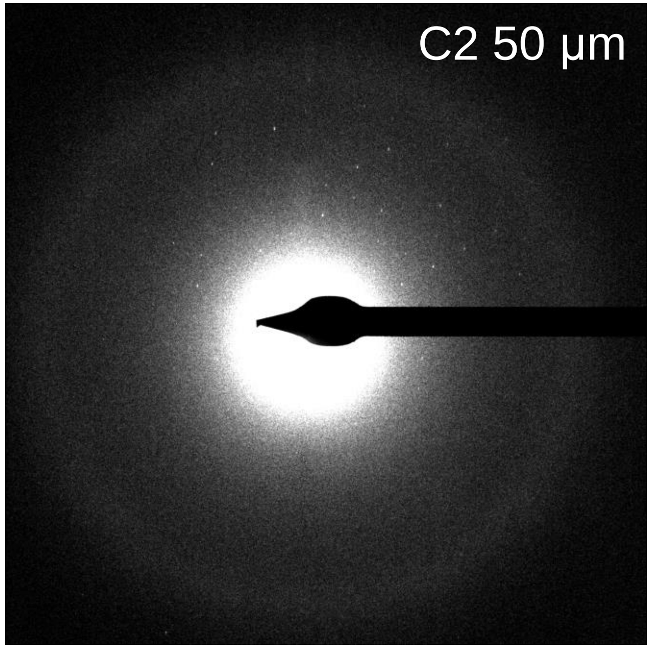
- Small molecule, salt and ice crystals all have similar unit cells and therefore their diffraction patterns look similar making it is hard to distinguish between the three during crystal screening and data collection.
- Worst case scenario: 50 datasets collected on a customer sample were all salt.

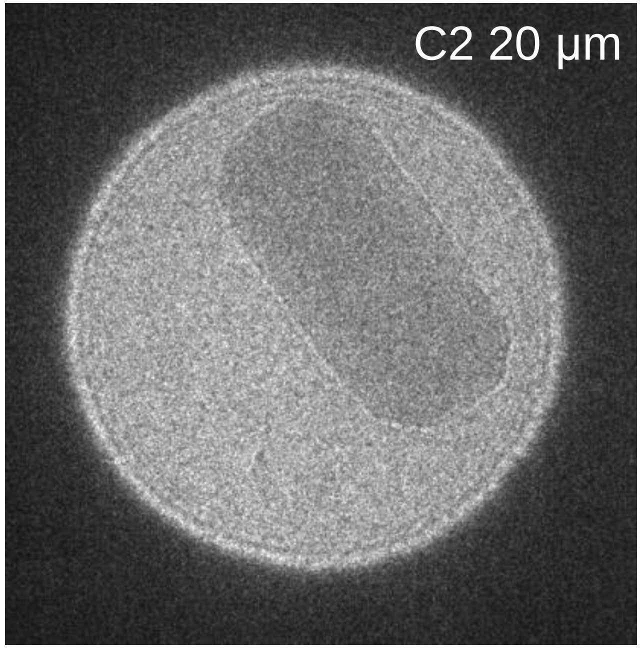
→ Better annotation autoprocessing needed

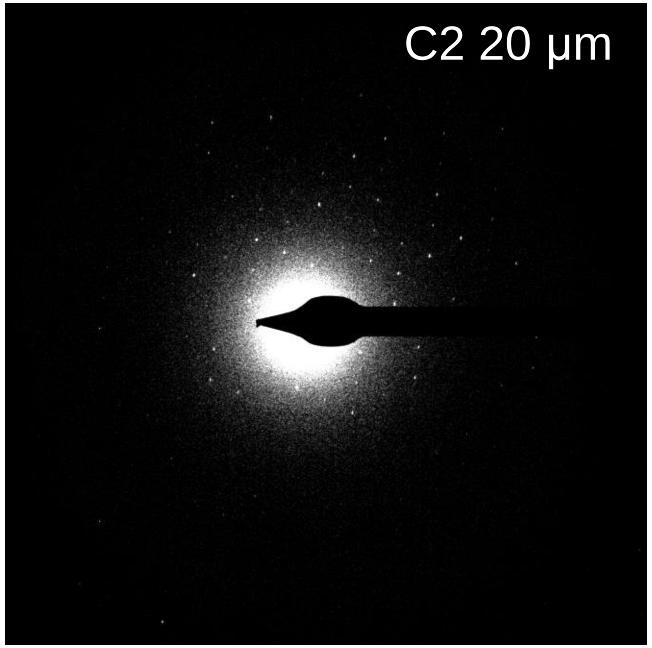
Noise in the diffraction images

- 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

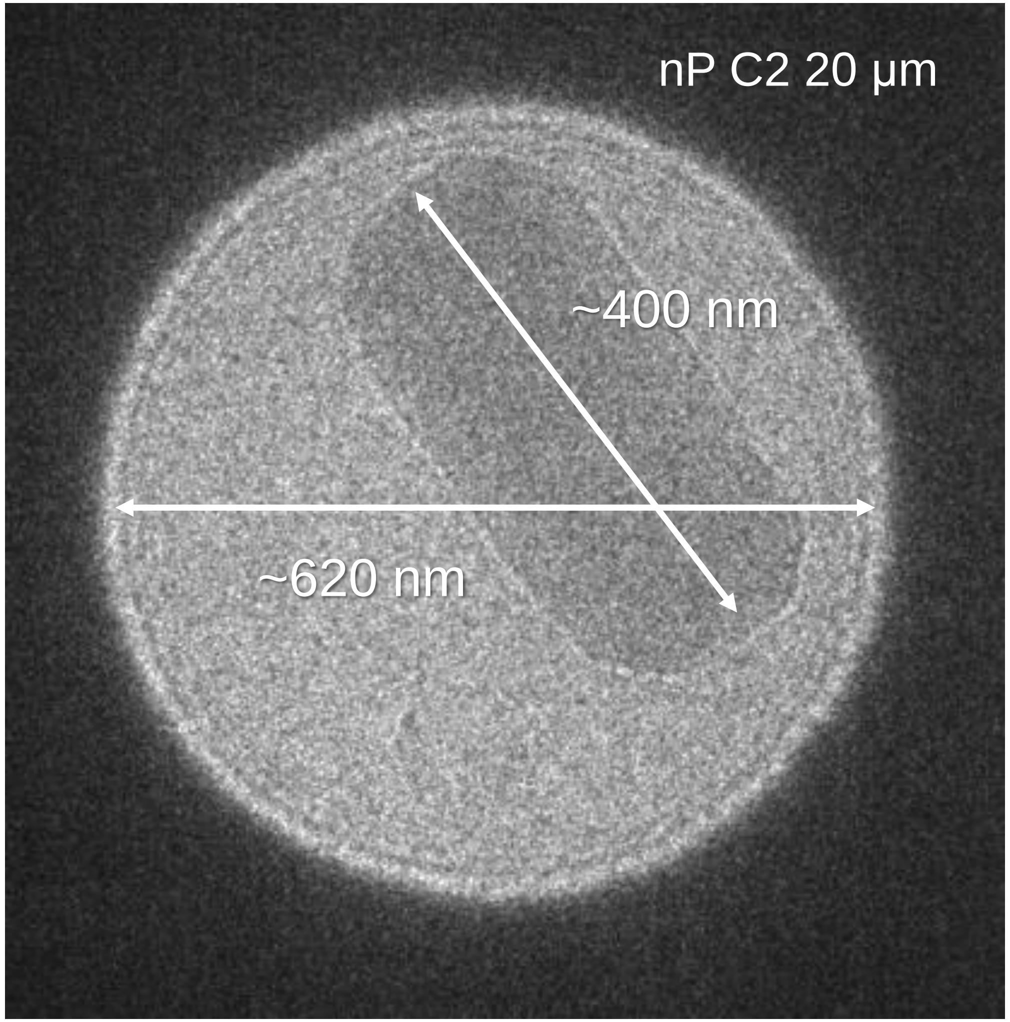






- Crystal needs to stay in a small field of view during stage rotation.
- Autoloader has an advantage over side-entry holder.
- Eucentricity specification for Talos is 2, 2, 4 µm and for Krios 1,1,3 µm (-70 to +70 degrees).
- Tilting above 50 degrees is more unpredictable, the crystal might drift out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

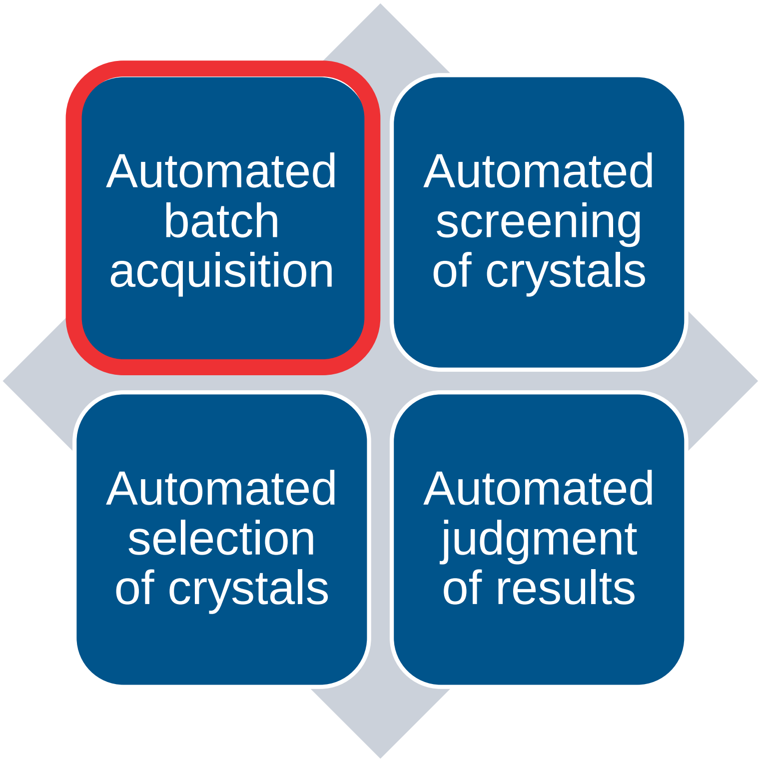
- Use a larger C2 aperture to compensate
- Collect multiple datasets to allow data merging



What is coming to the micro-ED workflow

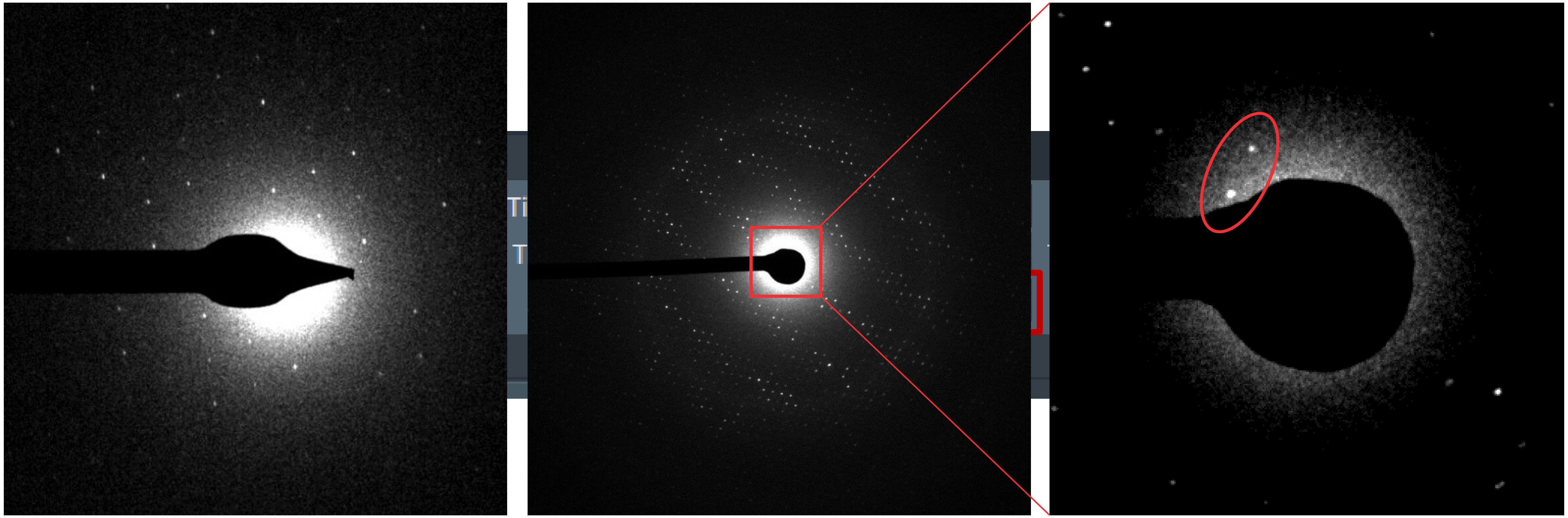
- Reproducible cryo sample preparation for microED
- MicroED-specific data processing step integration into the existing crystallography packages
  - DIALS and CCP4i2

Next steps in EPU-D development



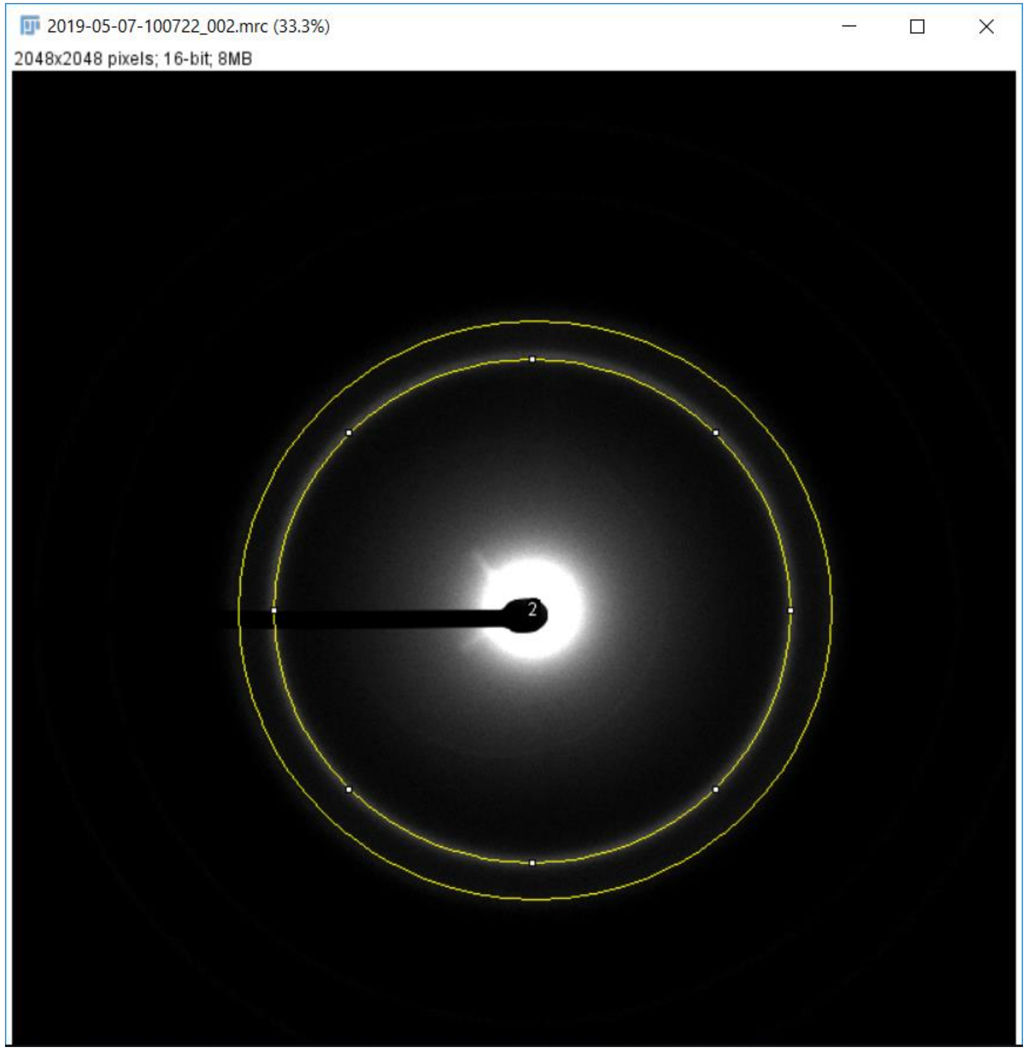
Practical aspects

1. For optimum stage linearity, do not exceed tilt speed of ≤ 1 deg/second (for our setup).
2. Smaller beam stop gives better results.



Practical aspects

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/Å^2/s but varies with different samples.



EM-learning.com: new learning platform that features over 70 hours of videos

EM-University
ELECTRON MICROSCOPY

Ieva Drulyte

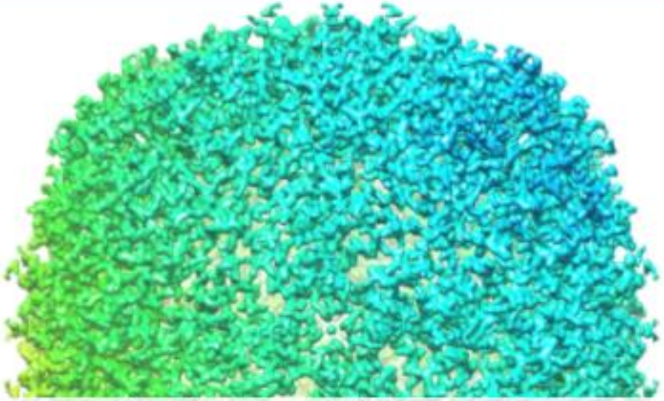
Introduction video

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

Credit video

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



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-asses 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.

Go to course

Thank you for your attention!

Any questions?

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Back-up slides

EPU-D: microED acquisition software

ThermoFisher SCIENTIFIC

Thermo Fisher's MicroED Solution

❶ Ceta-D camera

- Optimized for diffraction application: increased accuracy and sensitivity
- Compatible with TEM sample screening
- Compatible with bottom-mount filter (retractable)

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

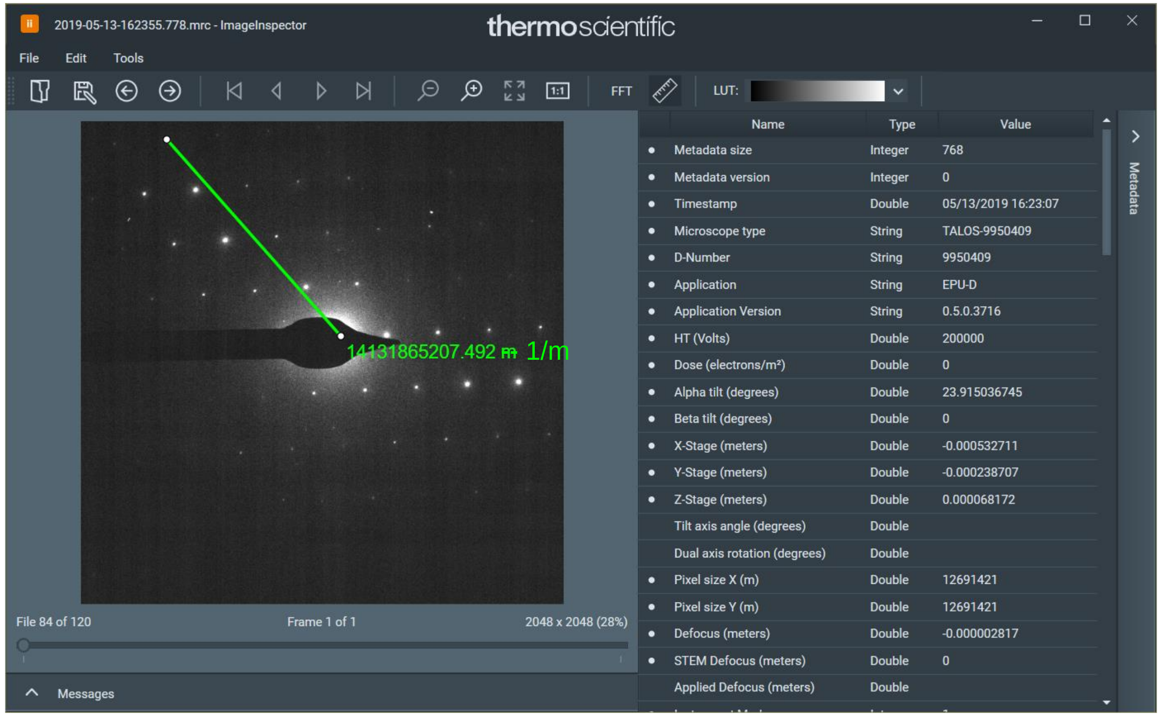
Future Developments for EPU-D

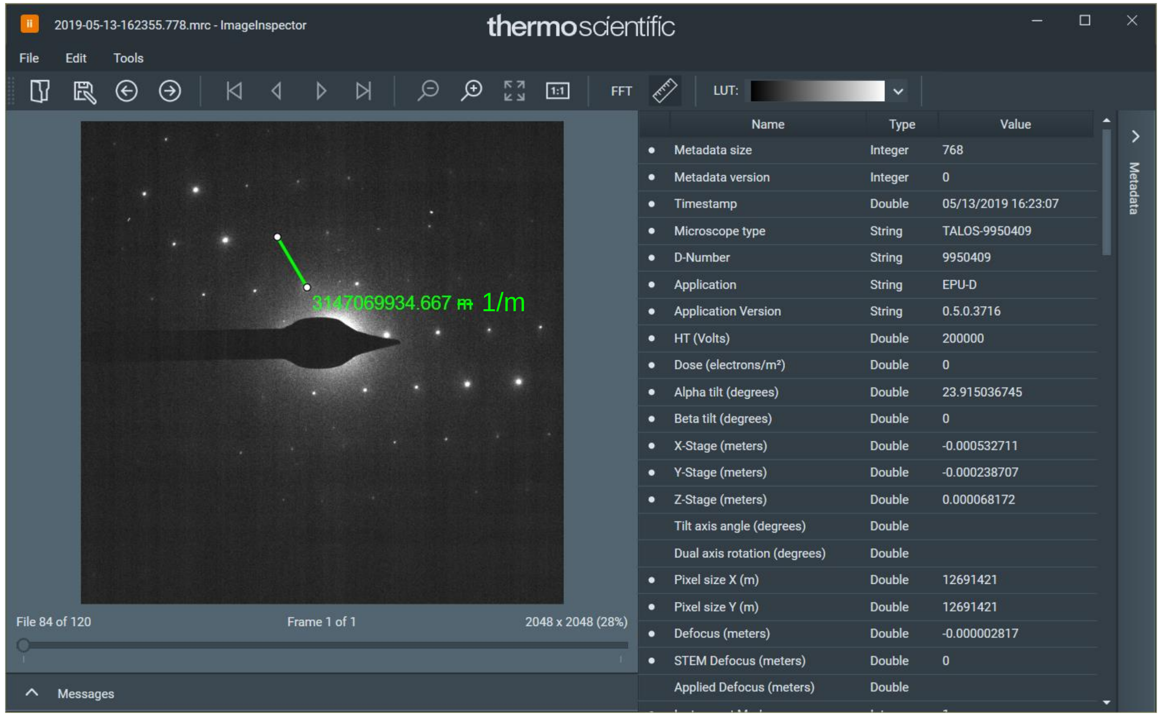
- Automated batch acquisition
- Automated screening of crystals
- Automated selection of crystals
- Automated judgment of results

Check resolution, unit cell dimension

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

- 3147,069,934 1/m
- 1/3147 1/um = 3.178e-, ~3A





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

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