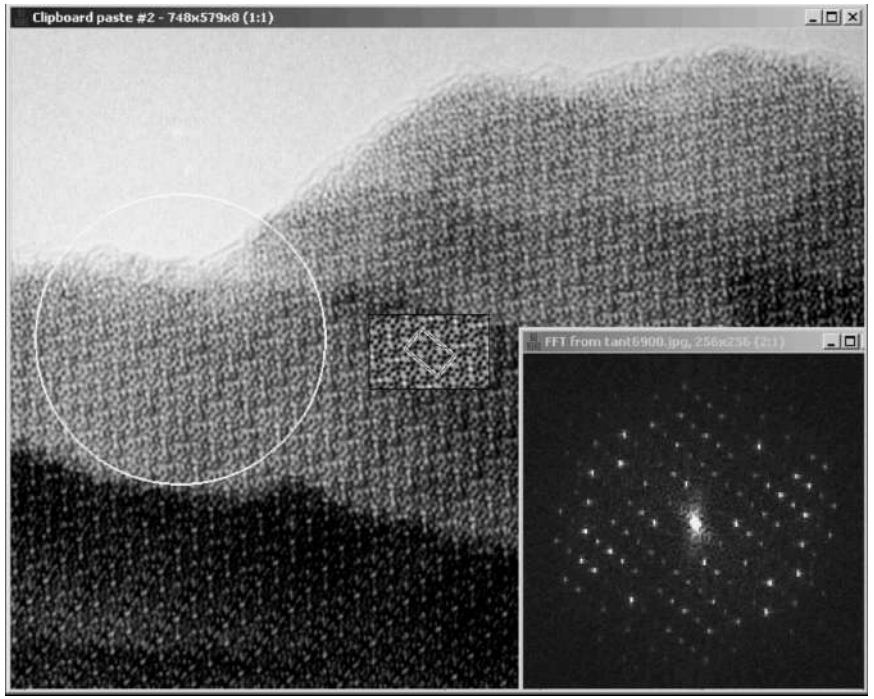
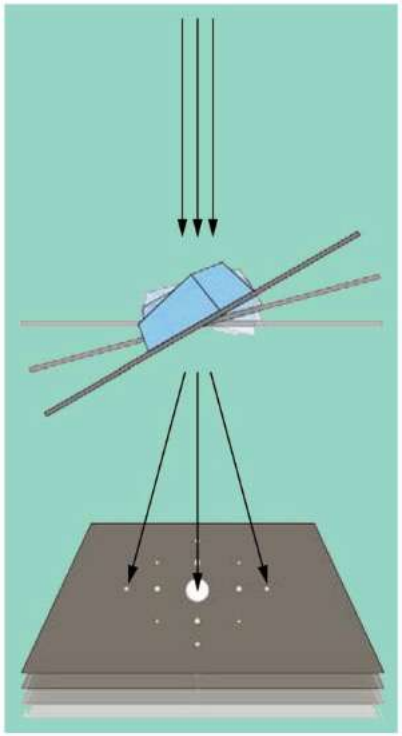
MicroED and 2D Crystallography

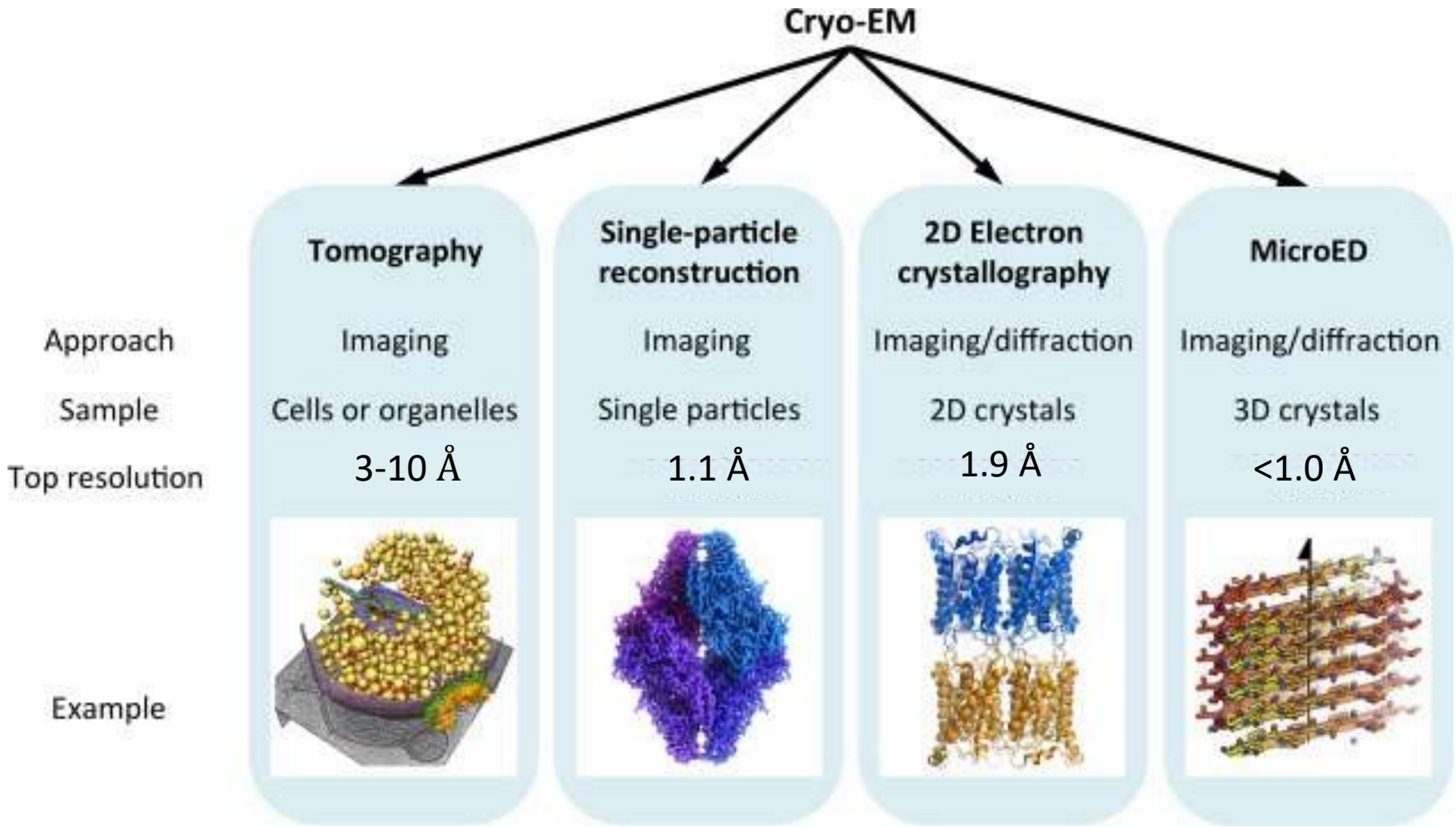
Jan. 31, 2022

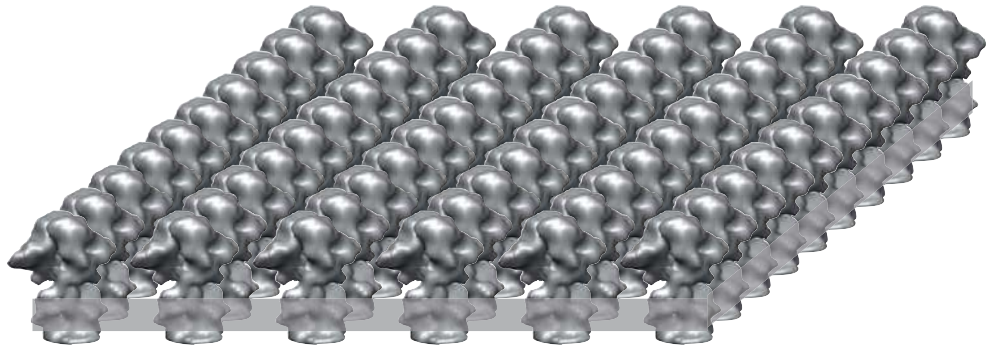
William Rice, NYU Langone





Best Resolution from EM Techniques

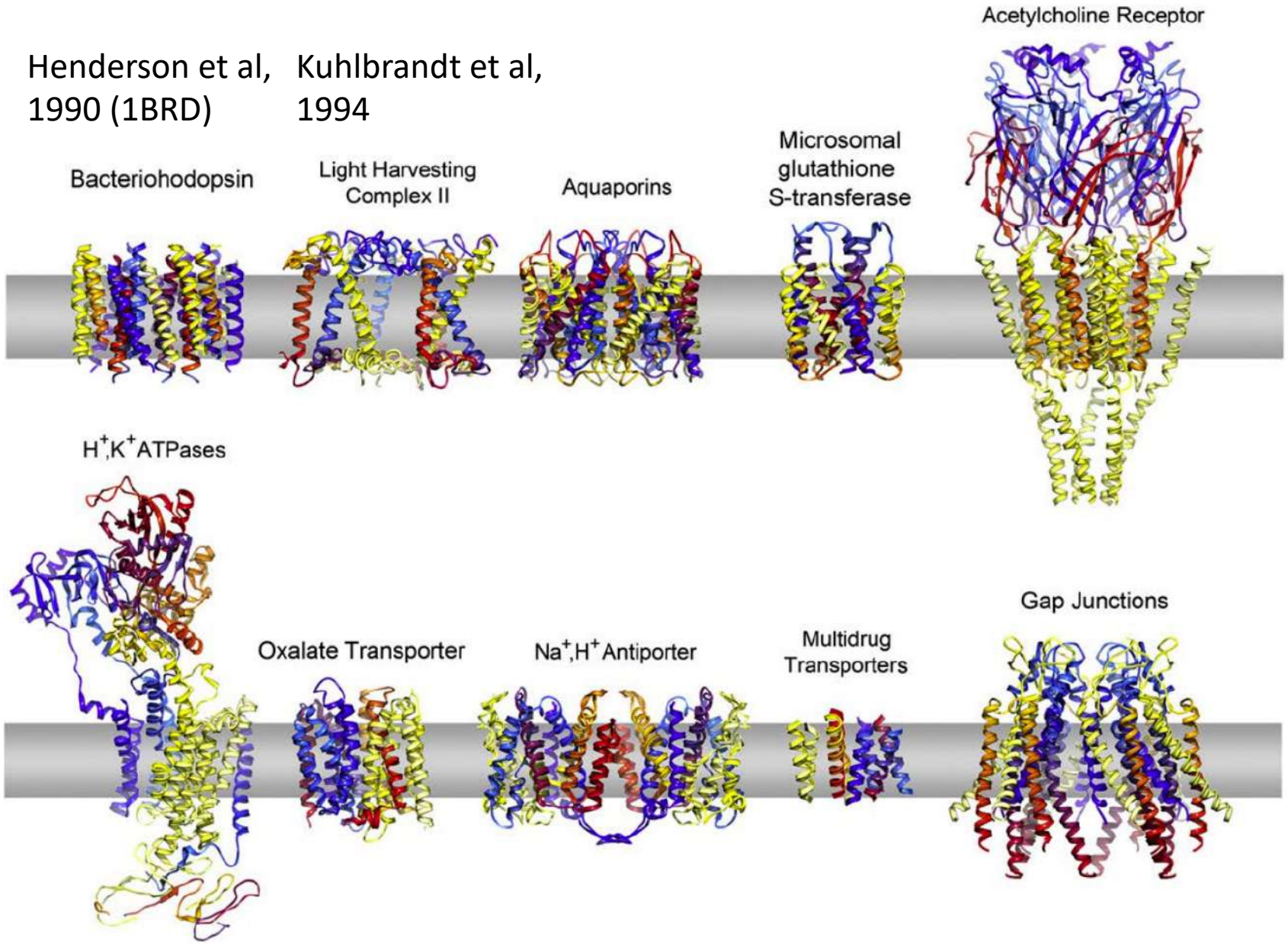




2D Crystallography

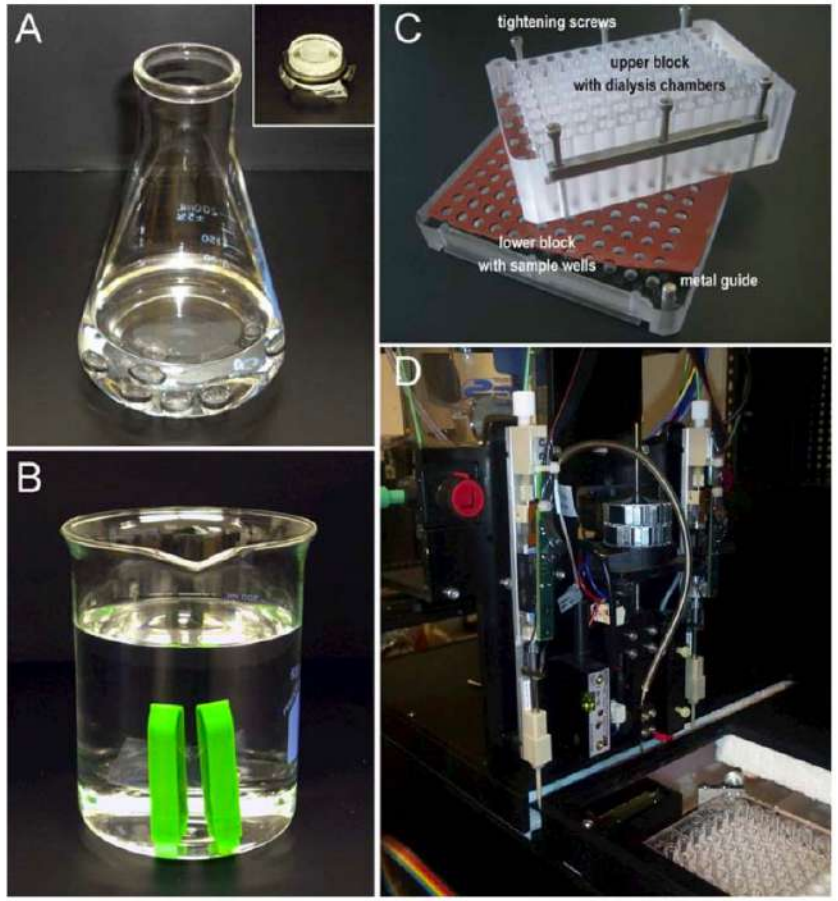
The first high resolution Cryo-EM method, mostly for membrane proteins

High Resolution Structures from 2D Crystallography



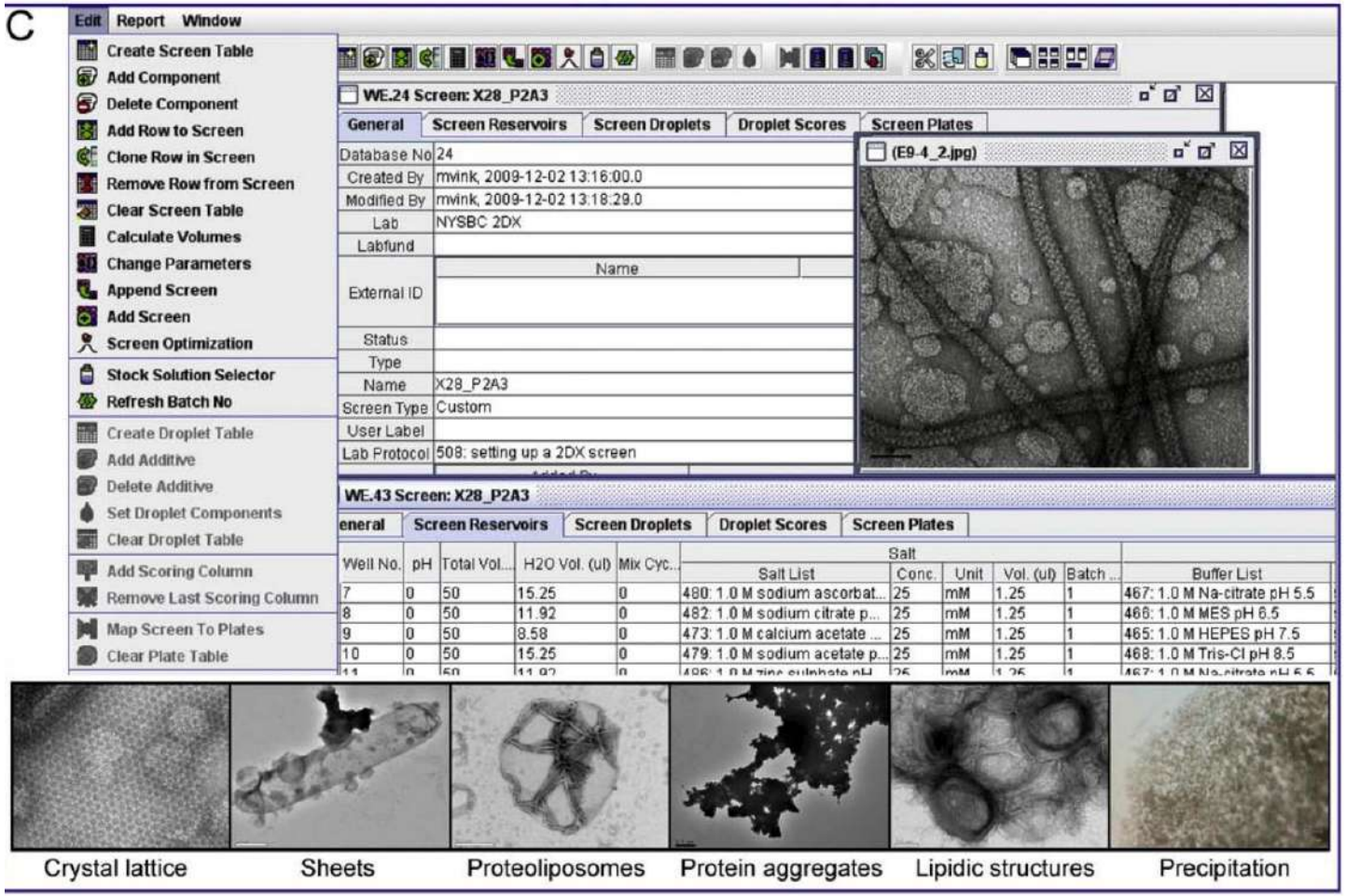
Wisedchaisri et al, 2011

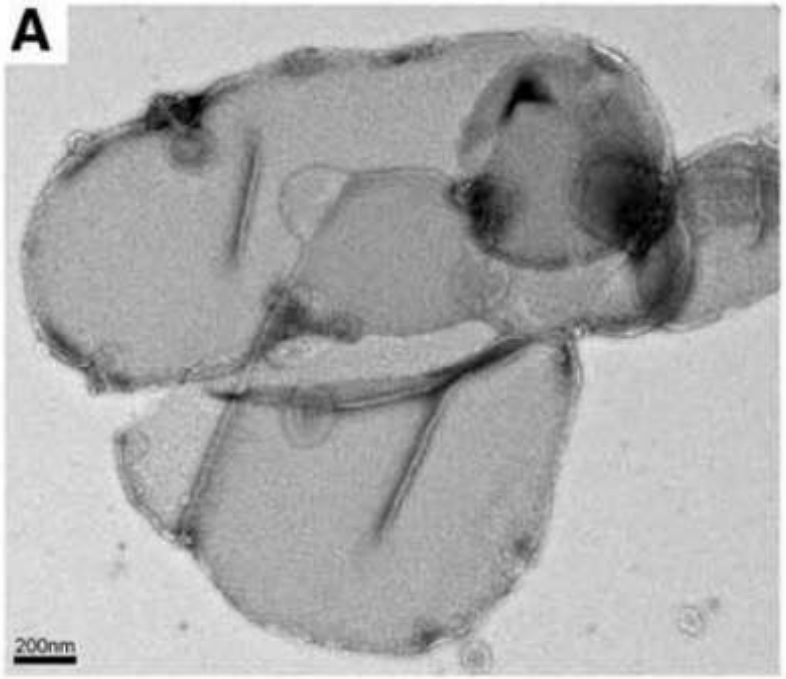
Preparation of 2D crystals: Remove detergent and put into lipid bilayer

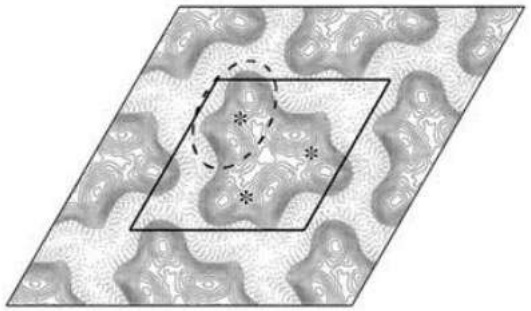


- A: dialysis buttons
- B: Dialysis tubing
- C: 96-well dialysis block
- D: Robot for cyclodextrin mediated detergent removal

2D crystal screening negative stain







Wisedchaisri et al, 2011

Cryo Imaging

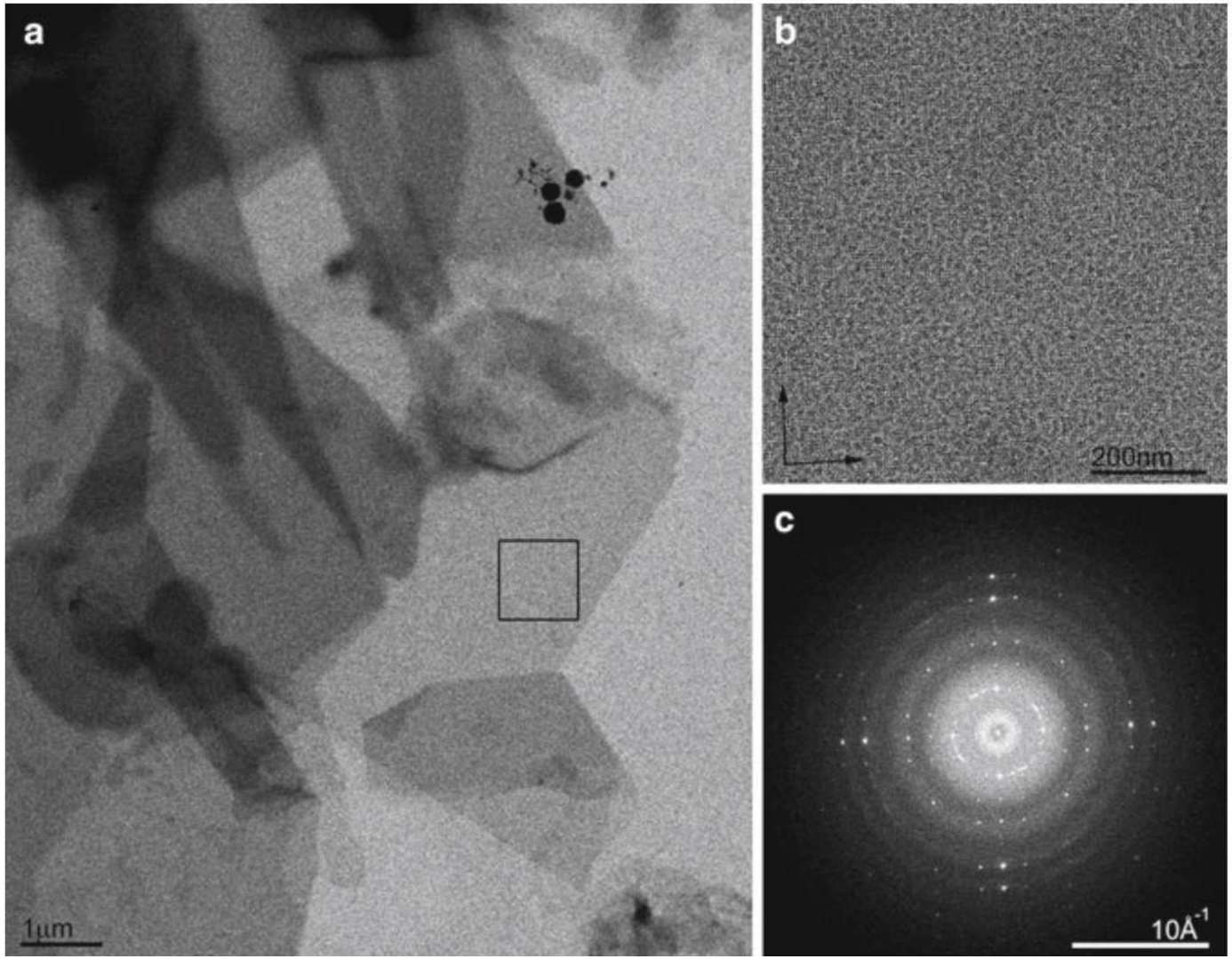
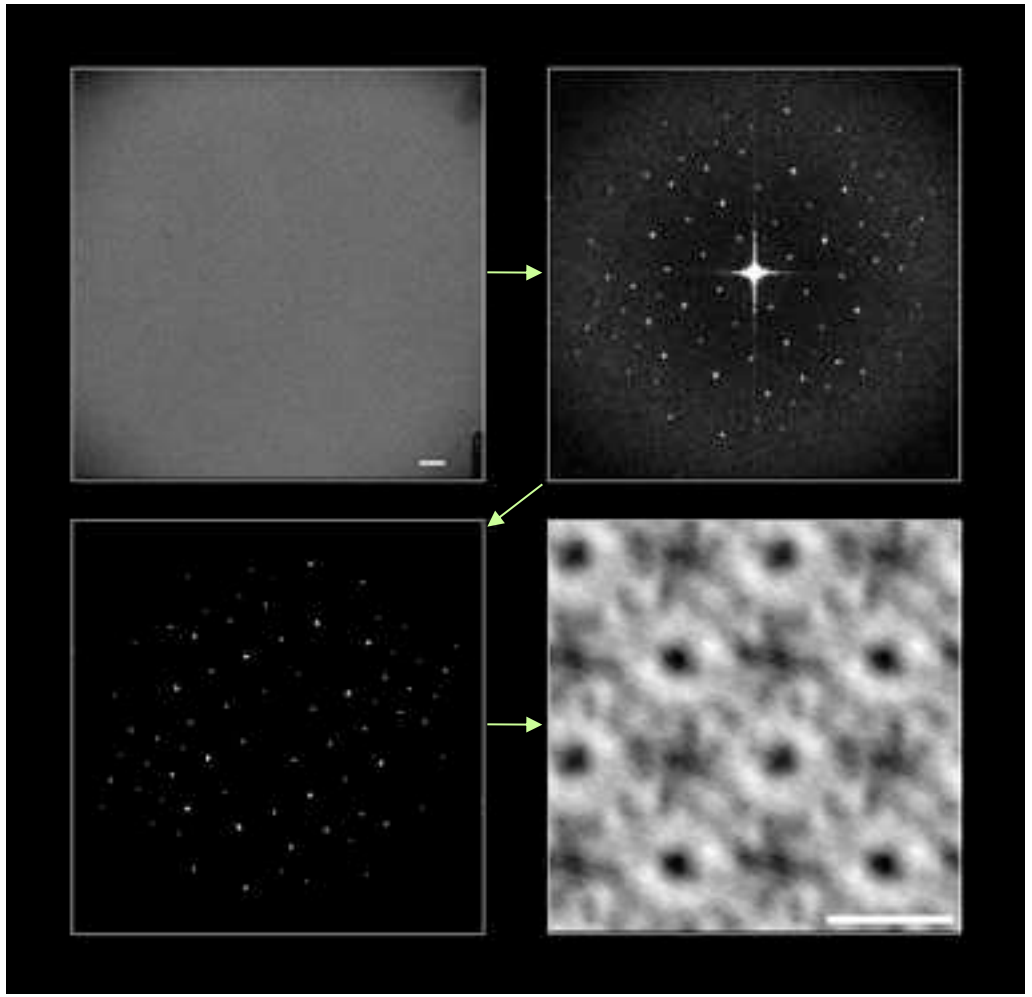


Fig. 3. Cryo EM of two-dimensional crystals. (**a**) Crystals of the water channel aquaporin-0 are large and have sharp edges attesting to the degree of order within. (**b**) High-resolution image of the crystal area highlighted by a *box* in (**a**). (**c**) Fourier transform of the image in (**b**) showing strong and sharp spots to ~6Å resolution. These crystals are ready for analysis by electron diffraction because the crystals appear uniformly *grey* on the grid. The spots in the Fourier transform are sharp and extend to ~6Å resolution without unbending. At this stage the sample should be frozen and the microscope setup should be changed to diffraction and data collected.

Gonen, 2011

Fourier analysis of images of 2D crystals

Original image

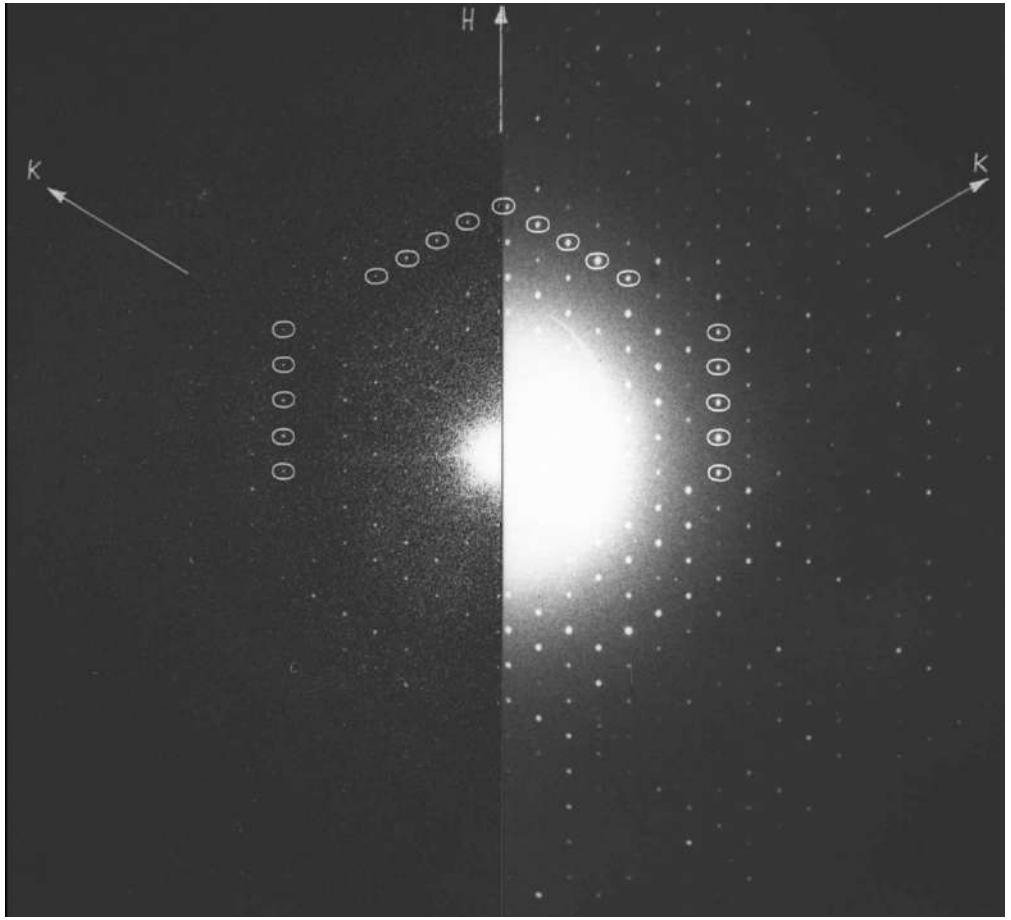


Fourier transform

Extraction and correction of Fourier components

Fourier synthesis

Diffraction amplitudes are better than image amplitudes



Calculated FFT Diffraction image

- Diffraction data can be collected directly
- Amplitudes are better
  - Not affected by CTF
  - Not affected by specimen movement
- Phases are lost
- Intensities are collected: Amplitude^2
- Dose can be very small

Diffraction data screening of 2D crystals

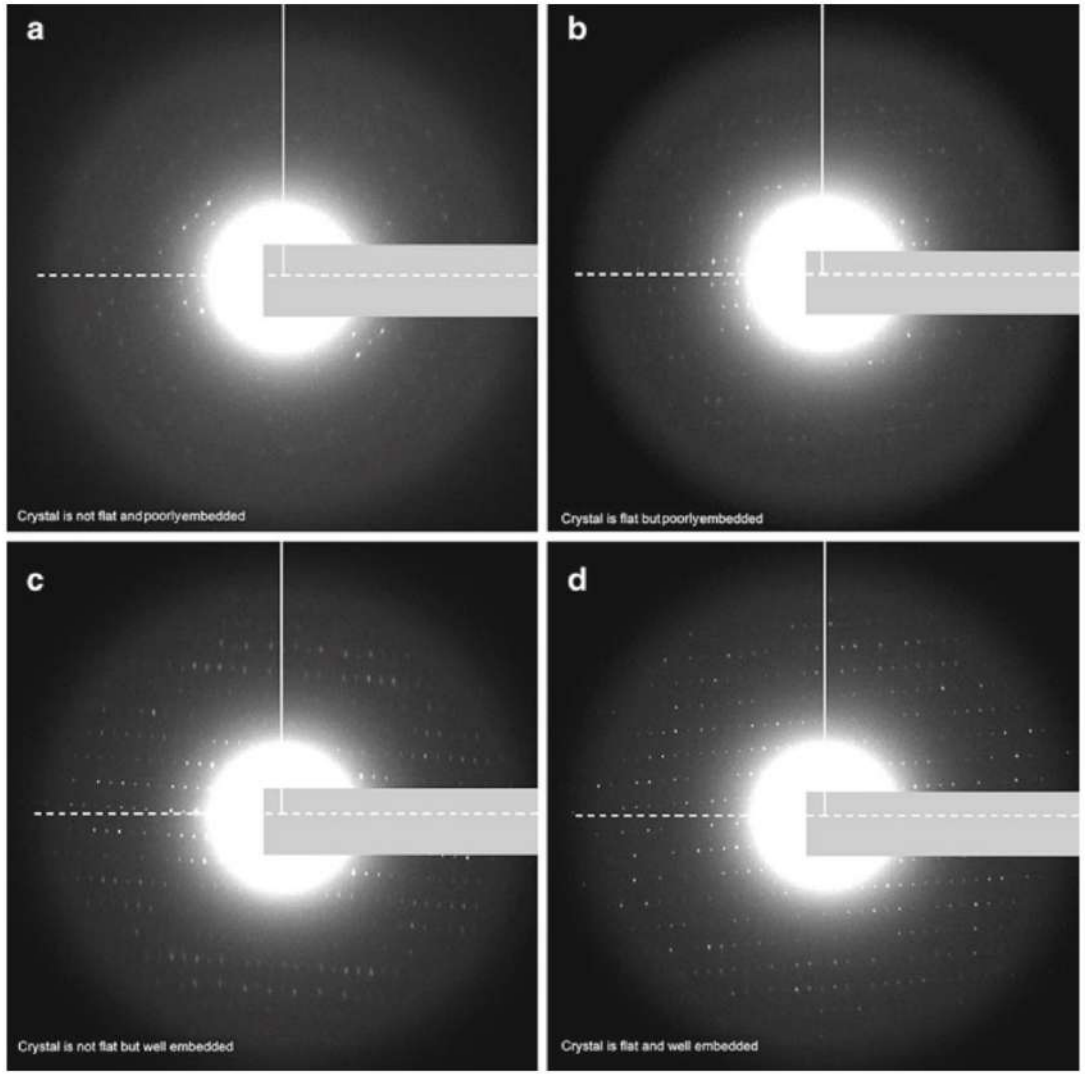
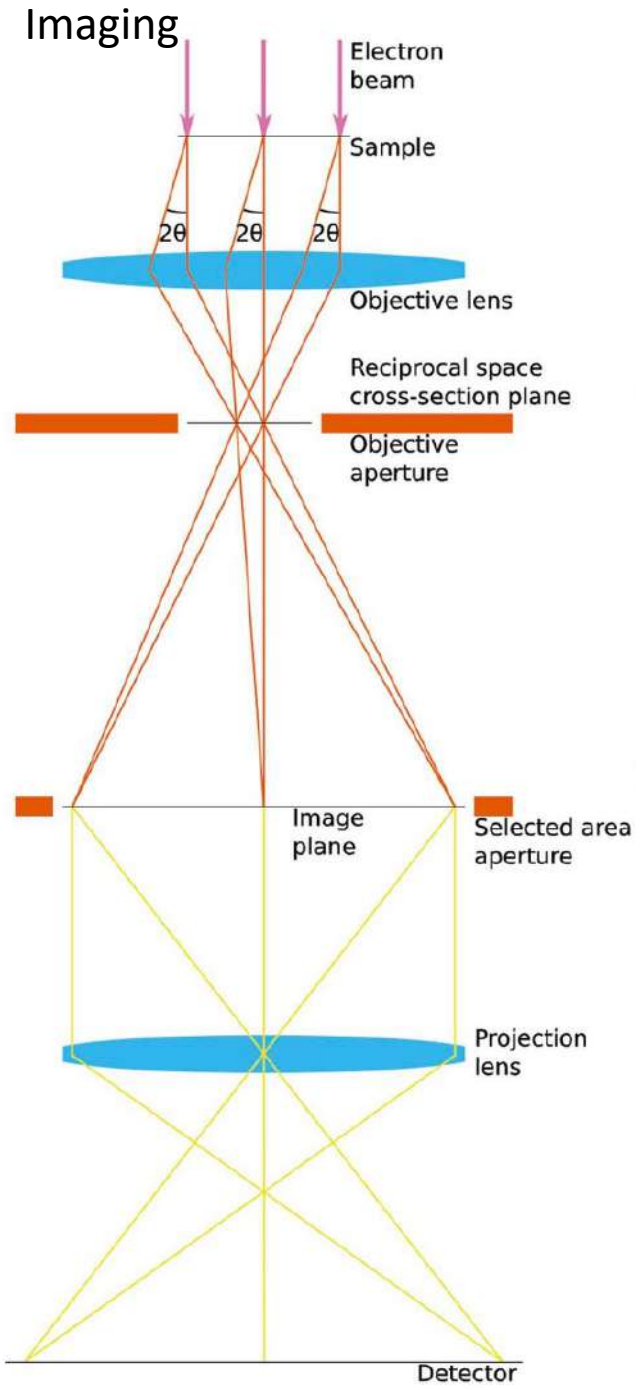


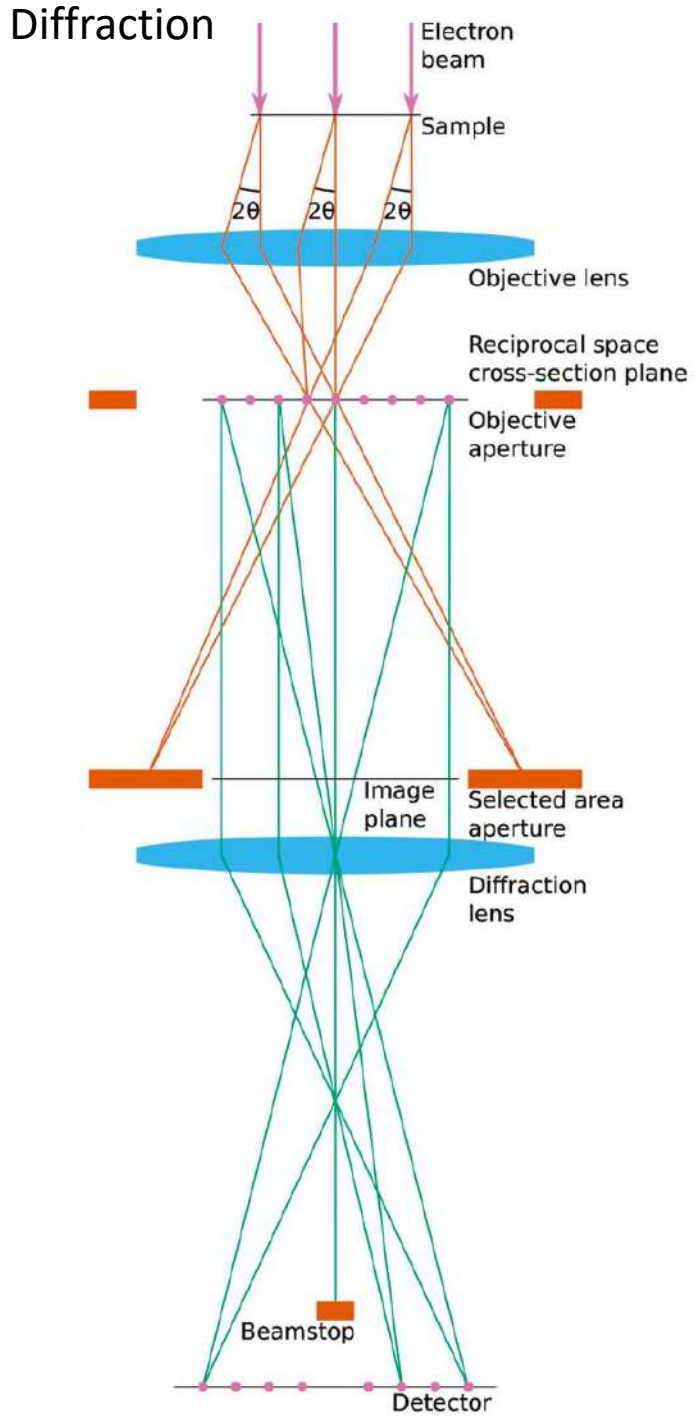
Fig. 4. Assessment of crystal flatness and embedding by electron diffraction. (**a**–**d**) The flatness of the crystal will affect the attainable resolution. If crystals are not flat the resolution will be cut off in the direction perpendicular to the tilt axis (tilt axis in *dashed line*, perpendicular to the tilt axis in *solid line*). Likewise, a well-embedded crystal will show strong and sharp spots in the diffraction pattern but a poorly embedded crystal will have limited resolution. (**a**) An example of a crystal that is not flat and also poorly embedded. No sharp spots are visible perpendicular to the tilt axis, indicating that this crystal is not flat. Only a limited number of spots are visible on the tilt axis, indicating that the crystal is not embedded properly and was damaged during grid preparation. (**b**) An example of a crystal that is flat but poorly embedded. It is flat because many spots are visible in all directions, even perpendicular to the tilt axis. The crystal, however, is poorly embedded because most of the spots are not sharp and have a weak intensity. (**c**) An example of a crystal that is not flat but is embedded well. The crystal is well embedded because most spots in the diffraction patters appear strong. However, the crystal is not flat because only the spots on the tilt axis are sharp, while the spots perpendicular to the tilt axis are smeared. (**d**) An example of a crystal that is both flat and well embedded. All of the spots in this diffraction pattern are intense and sharp. In all figures the beam stop is masked by the *grey rectangle*.

Gonen, 2013

Electron microscope setup for diffraction

Electron Optics





Hattne et al, 2015

Diffraction data

- Not affected by
  - Stage instabilities
  - Beam induced specimen movement, charging
  - Optical aberrations (CTF: defocus, astigmatism, Cs,…)
    - Except: diffraction astigmatism
  - Temperature instabilities
- Only intensities (amplitude^2) collected
  - Phase problem
- For 2D crystallography, collect images for phases and diffraction images for amplitudes

Importance of Phase and Amplitude

1

Calculate FFT Keep amplitude

2

Calculate FFT Keep phase

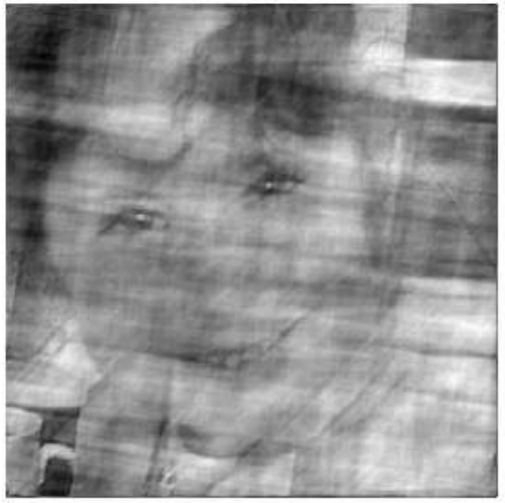
Most of the information is recorded in the phases

1

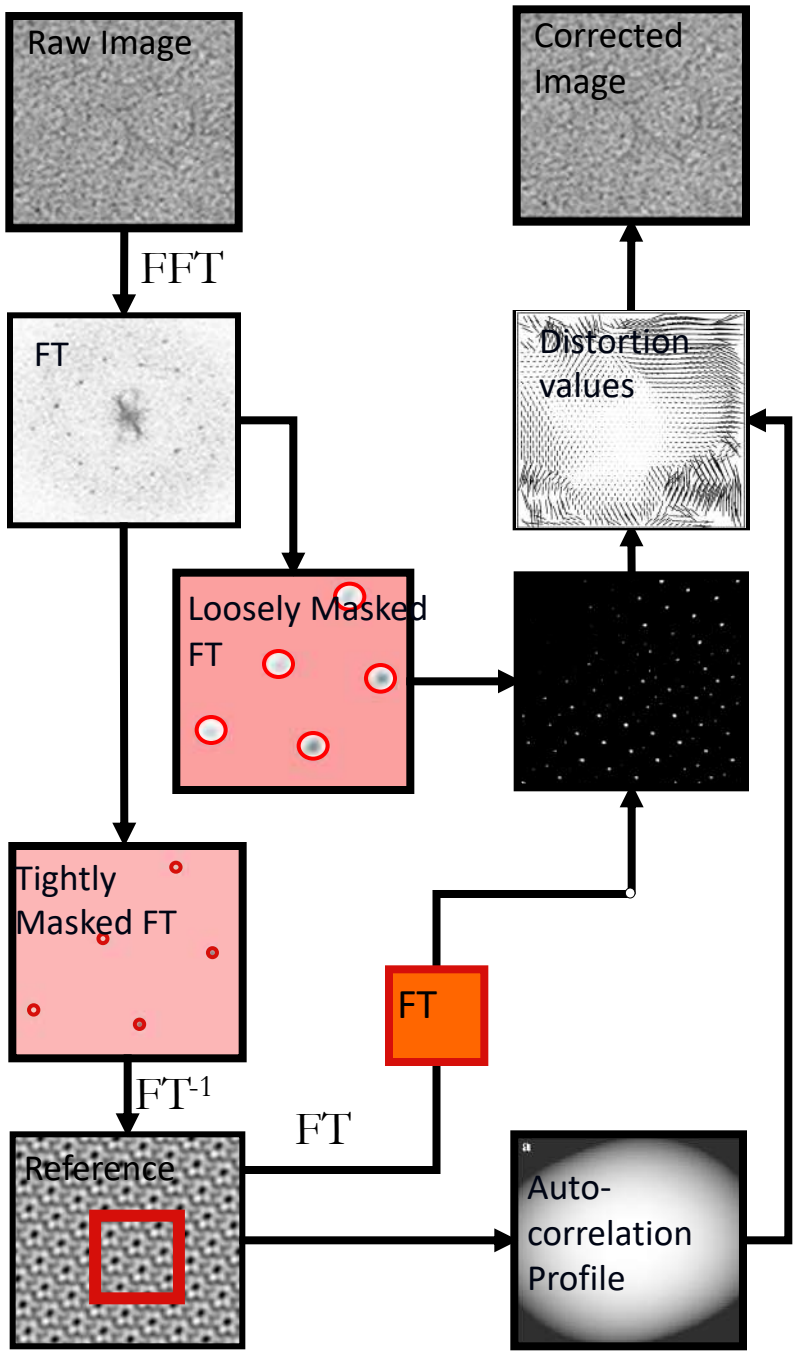
2

Amplitude of object 1
Phase of object 2

Inverse FT



Reconstituted image is dominated by phase

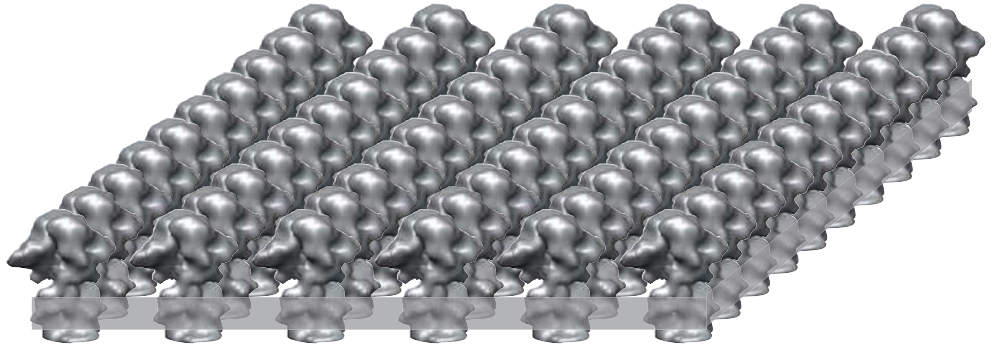


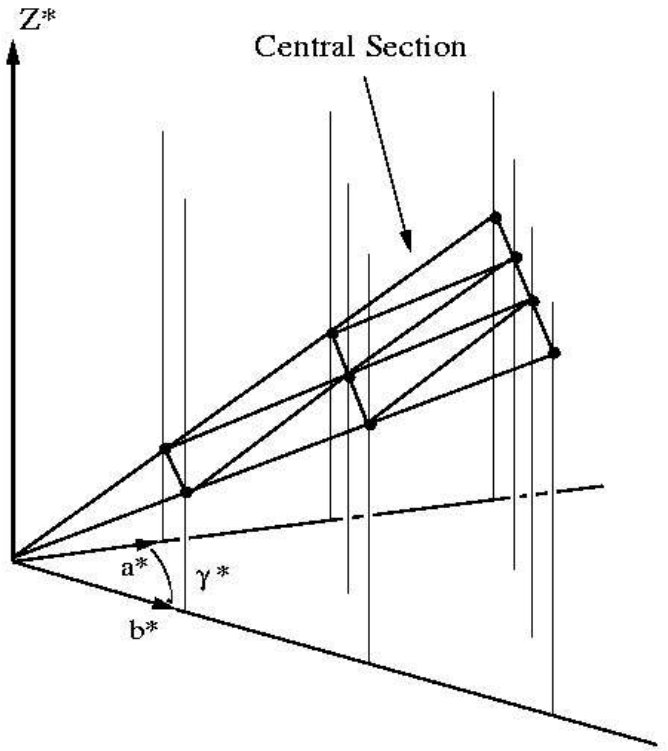
Unbending: Removal of lattice distortions

- Define a reference with good contrast
- Enhance the distortion at a similar contrast as the reference
- Generate Cross-Correlation map
- Create auto-correlation profile of the reference
- Quantify the distortion
- Make the correction

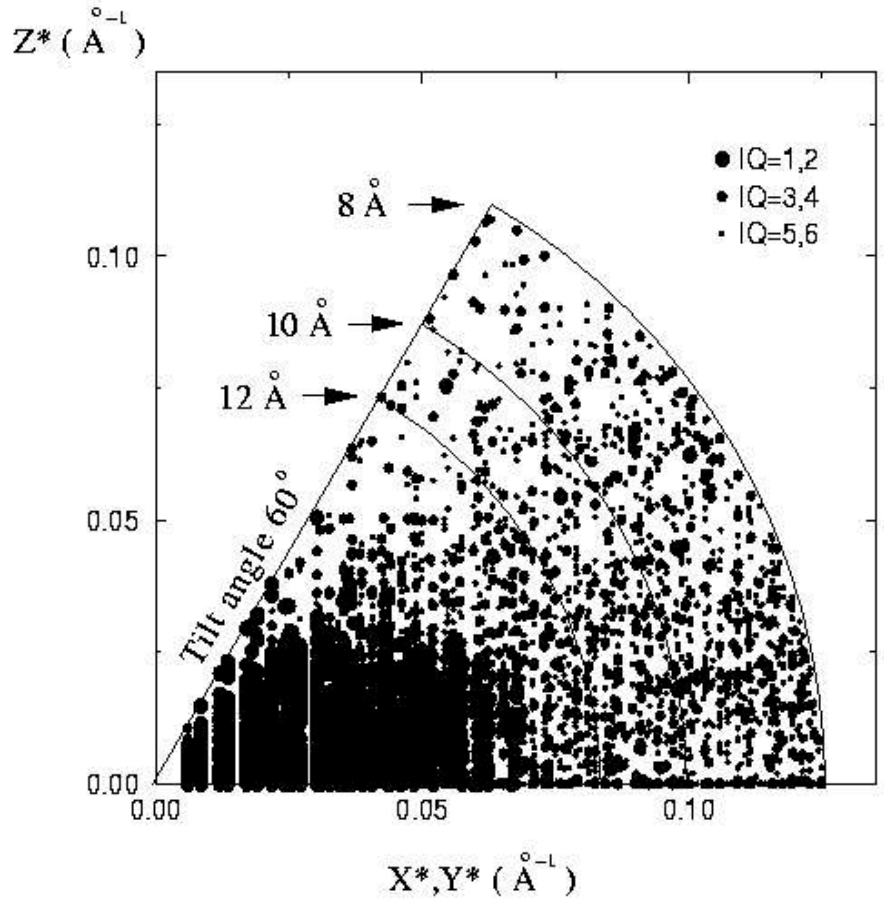
Anchi Cheng

For 3D information, we need to collect images of tilted crystals





Sampling of Fourier Space
after combination of all central sections



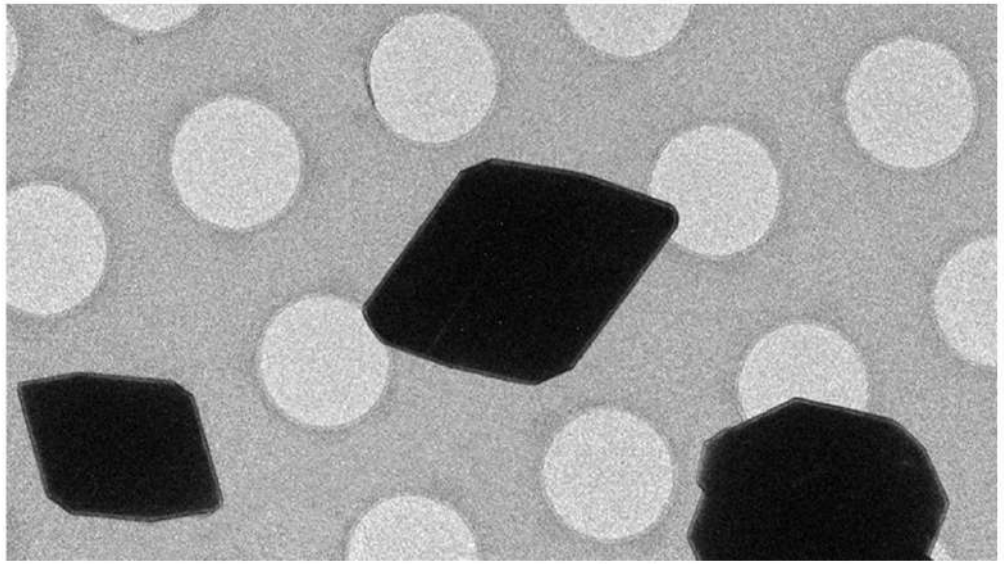
Difficulties in 2D crystallography

- Screening
  - Setting up conditions
  - Screening one by one
  - Large factorial surface (buffer, additives, lipid, detergent, speed of detergent removal)
- Samples
  - Need to be extremely flat over a large area
  - Need to be very well ordered
- Collection
  - Need to merge crystals at different tilts to get 3D reconstructions
  - Collect images as well as diffraction data
  - Hard to collect high quality tilted images
  - Manual collection
- Software
  - Difficult to use: until 2dx, command-line driven scripts

Resolution Revolution

- With the development of direct detectors, electron counting, and better software such as Relion and Cryosparc, single particle analysis can routinely reach “near-atomic” or even atomic resolution on good samples
- Minimum sample size ~60 kDa for single particle analysis
- 2D crystallography has been abandoned for the most part, apart from helical analysis
- However, 3D crystallographic techniques appeared coincidentally at about the same time as direct detectors…

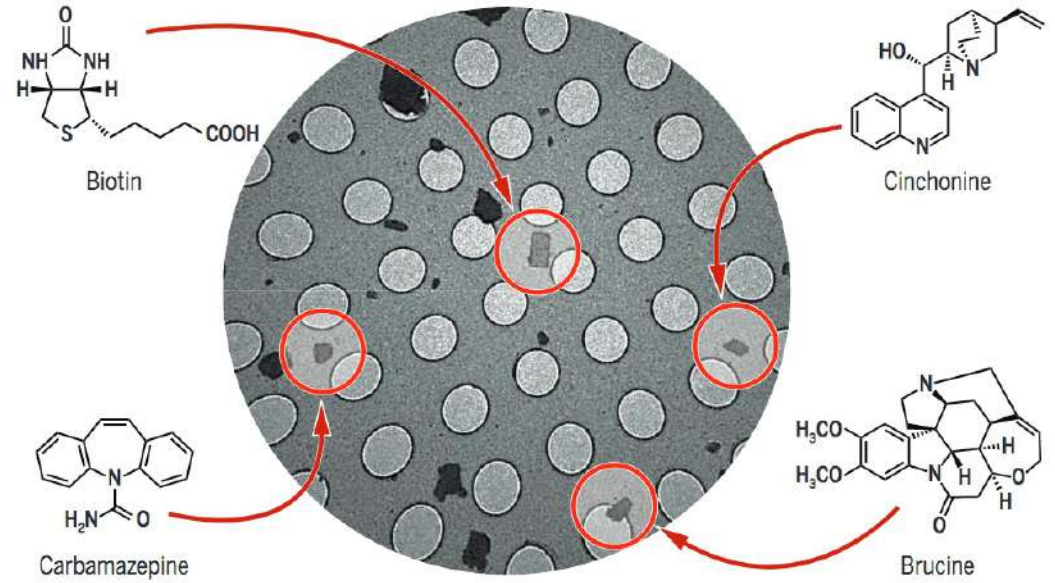
Molecular structures made simple



Structures can now be gleaned from micrometer-size crystals (black), seen here on an electron microscope slide. (GONEN LAB)

Structures from a mix of microcrystals

A new technique identified structures of four compounds from tiny crystals on an electron microscope slide.

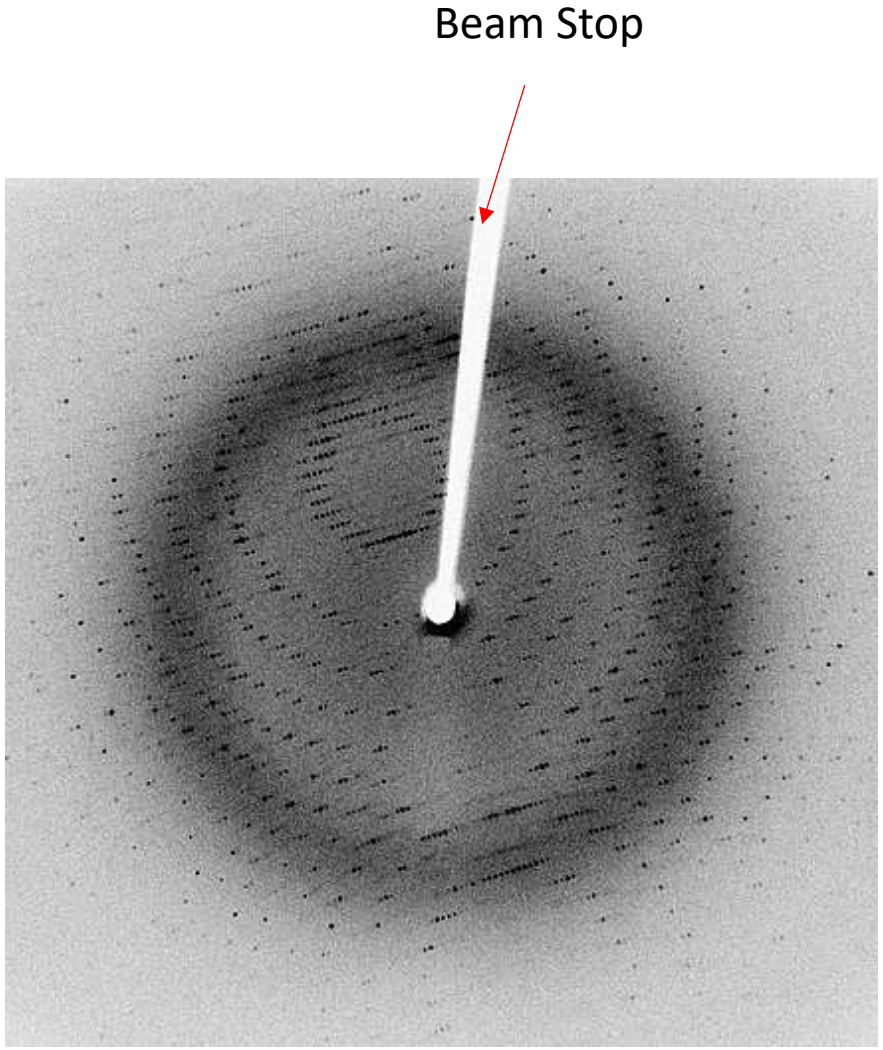


MicroED

Runner-up for Science's 2018 Breakthrough of the Year

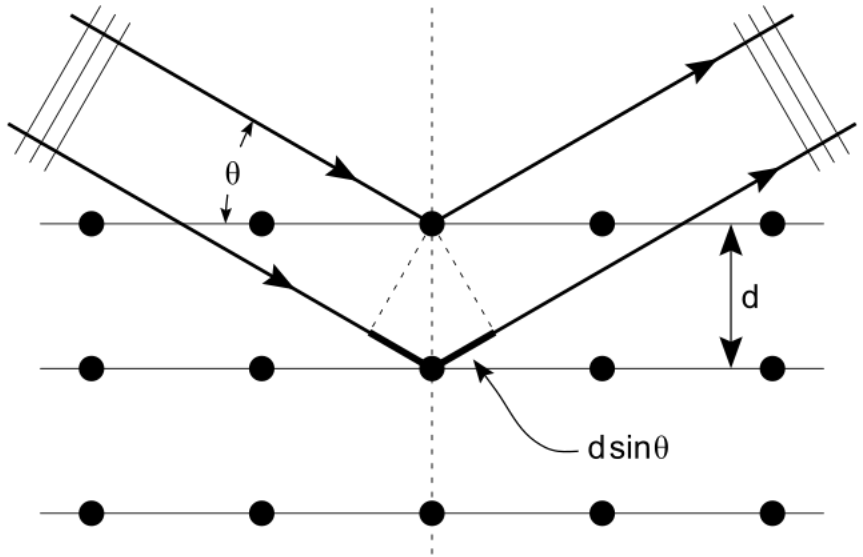
X-Ray Diffraction of crystals

- Mount crystal, expose to x-ray beam at defined wavelength
- Collect images of reflections on detector
- Only collect intensities and positions, not phases
- Rotate crystal (180 deg) to get all reflections
- From positions, get 3D lattice parameters
- Phasing
  - Ab initio (small, high resolution)
  - Heavy atom derivatives
  - MAD/SAD
  - Molecular replacement



Wavelengths

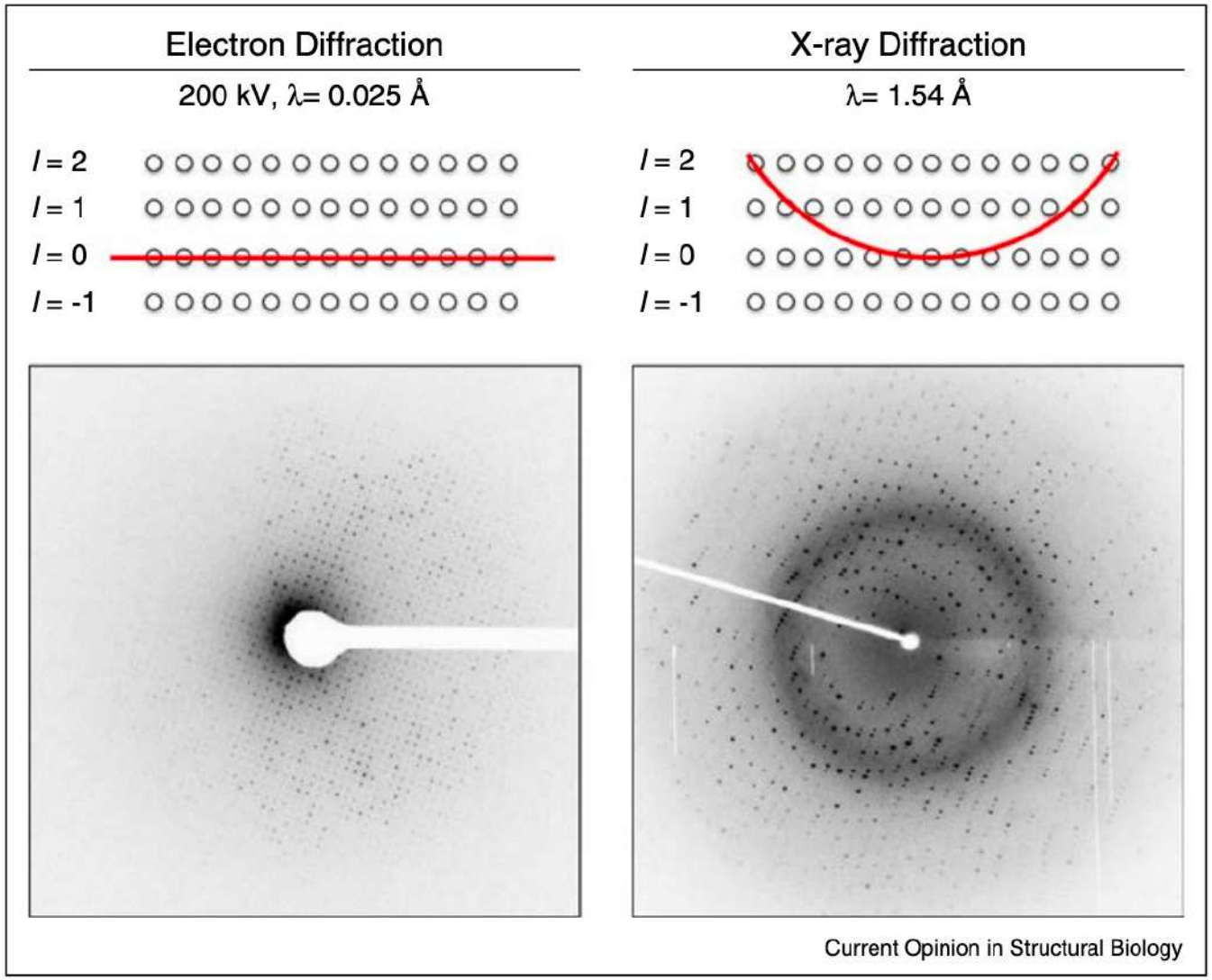
- X-ray
  - λ=70.9 pm (Ag Ka)
  - λ =154 pm (Cu Ka)
- EM
  - 80 keV: 4.18 pm
  - 120 keV: 3.35 pm
  - 200 keV: 2.51 pm
  - 300 keV: 1.97 pm



Bragg: nλ = 2d sin θ

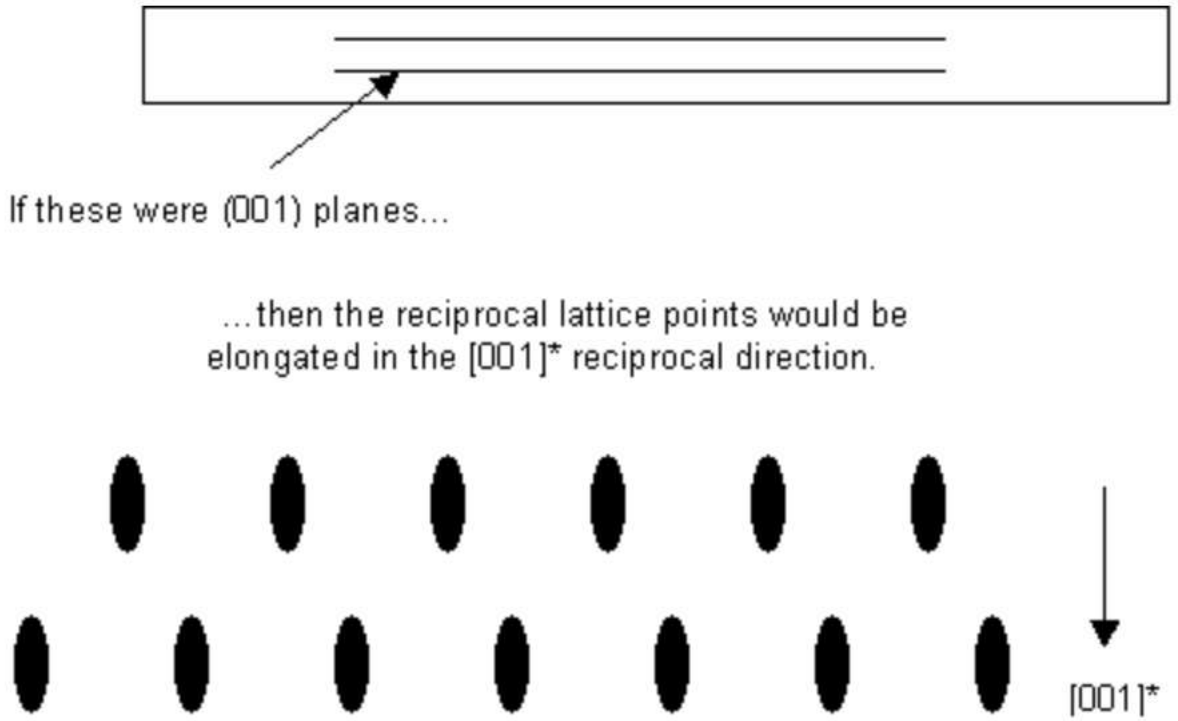
Ewald Sphere

https://www.doitpoms.ac.uk/tlplib/reciprocal_lattice/ewald.php



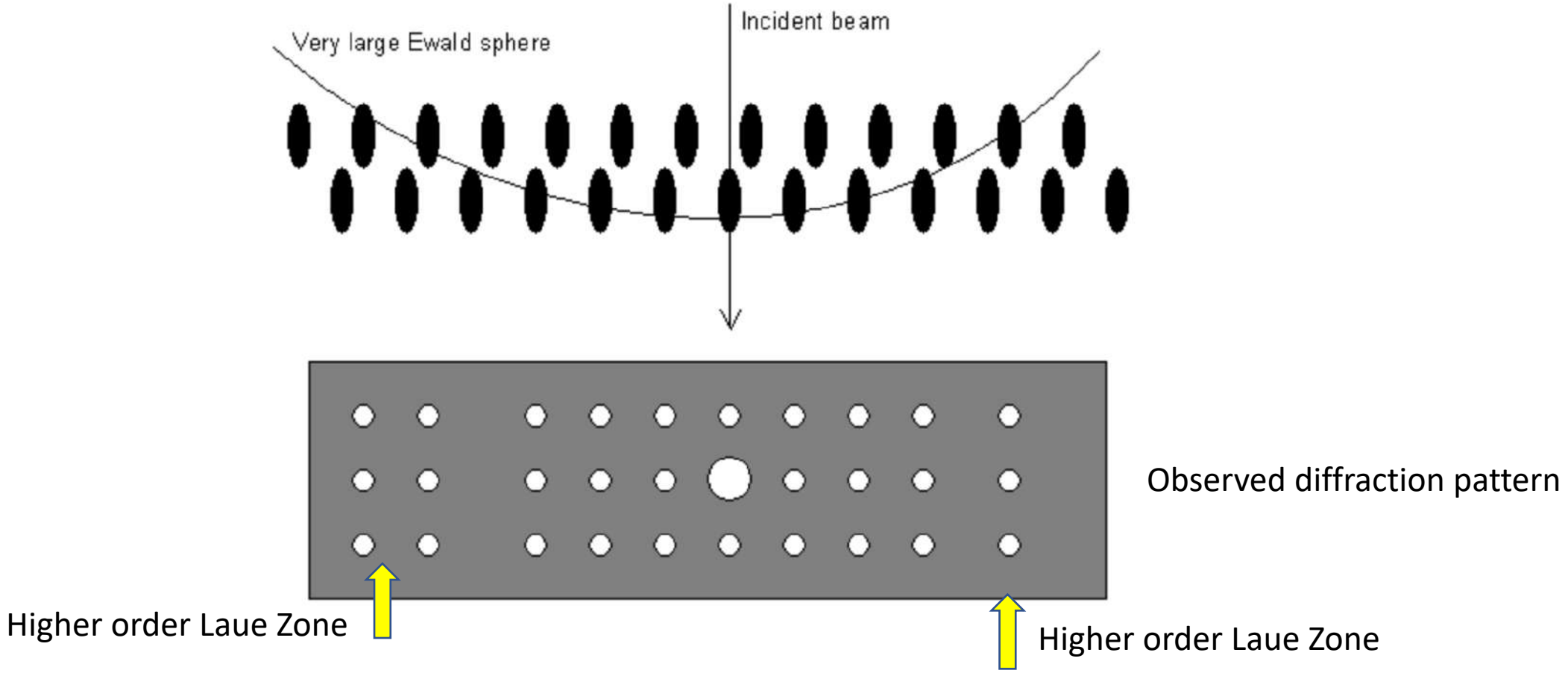
Comparison of diffraction data obtained from lysozyme crystals by electron diffraction and X-ray diffraction. Because the wavelength of the diffracting electrons is so short, the resulting Ewald sphere (left, red line) is essentially a plane when compared to the Ewald sphere for X-ray diffraction (right, red line). Diffraction only occurs when the Ewald sphere contacts a reflection in reciprocal space (top panels, white circles represent reflections in reciprocal space). Therefore, because the Ewald sphere is so flat, the patterns produced from electron diffraction (bottom left) appear as planar 2-dimensional slices through the 3-dimensional volume of reflections, whereas the patterns from X-ray diffraction (bottom right) appear as circular 2-dimensional projections of the sphere on the detector.

Electron diffraction through a thin crystal



https://www.doitpoms.ac.uk/tlplib/diffraction-patterns/laue.php

Electron diffraction through a thin crystal



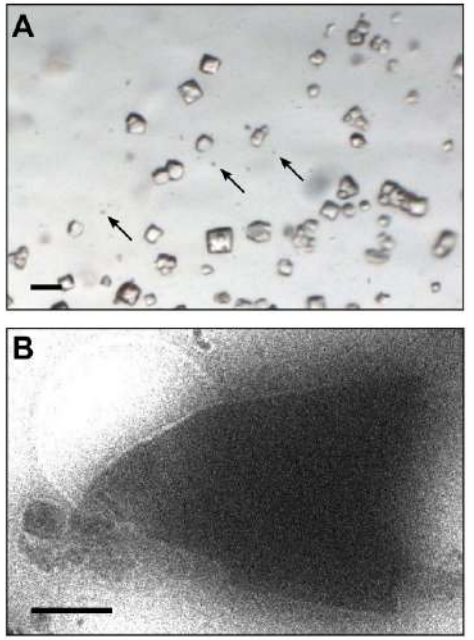
https://www.doitpoms.ac.uk/tlplib/diffraction-patterns/laue.php

Limitations of X-ray crystallography

- Approximately 30% of proteins that crystallize do not produce crystals large enough for x-ray diffraction experiments
  - Rupp, 2004; Quevillon-Cheruel et al., 2004
- XFEL?
  - Need many crystals
  - Expensive experiment

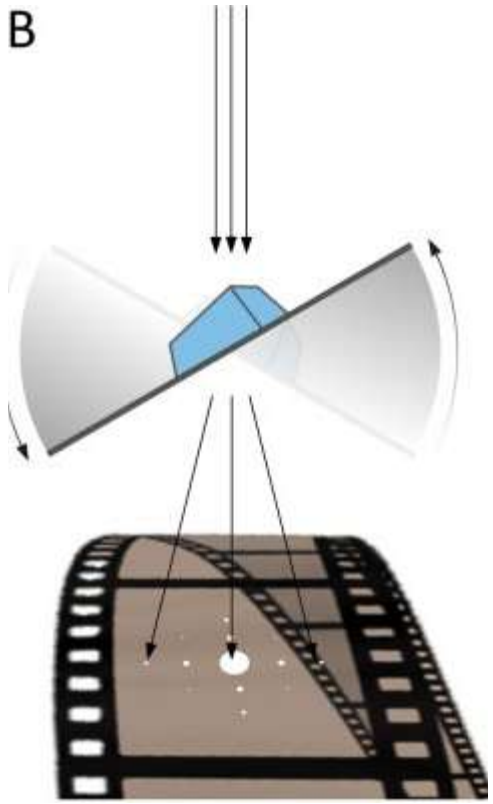
Original implementation (Shi et al, 2013)

- Image single images at various tilts (1 deg increment)
- Reflections recorded in this manner are generally partial reflections
  - Needed in-house scripts to index the data and group symmetry-related reflections
- Lysozyme at 2.9 Å resolution
  - 200 keV on TVIPS F416 CMOS detector
- Apply solution with crystals to grid
- Blot with Vitrobot

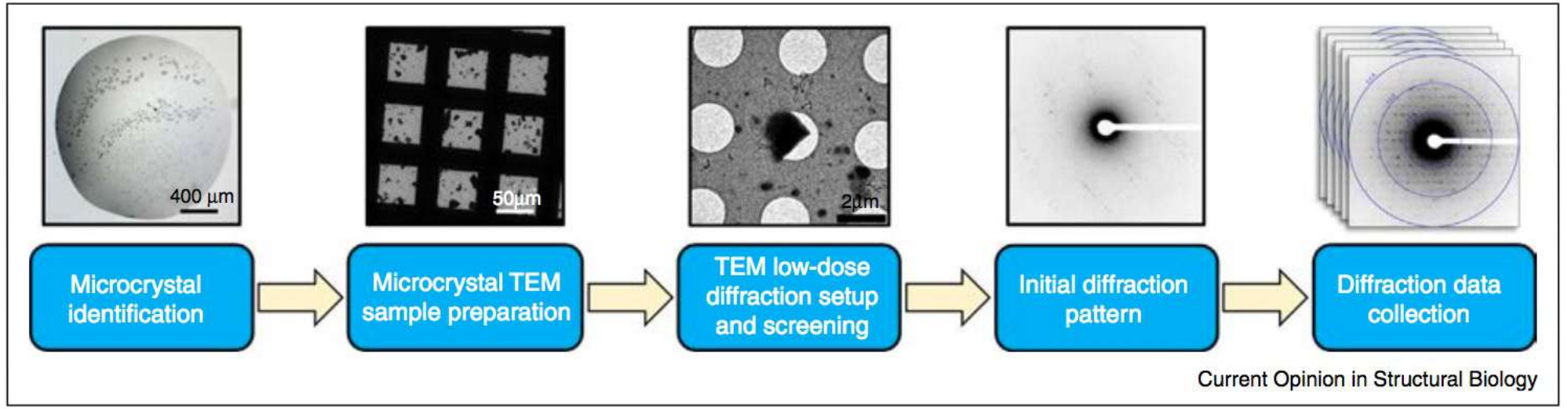


Better Data Collection: Continuous rotation

- Rotate stage at continuous rate
- Rotate to coordinate with exposure time
- Camera needs to be in continuous “rolling shutter” mode
- High rotation rate: increases the recorded reflection fraction on each frame
  - Too high: spot overlap
- Low rotation rate: makes weaker, high resolution reflections more visible
  - Too low: too few spots per image



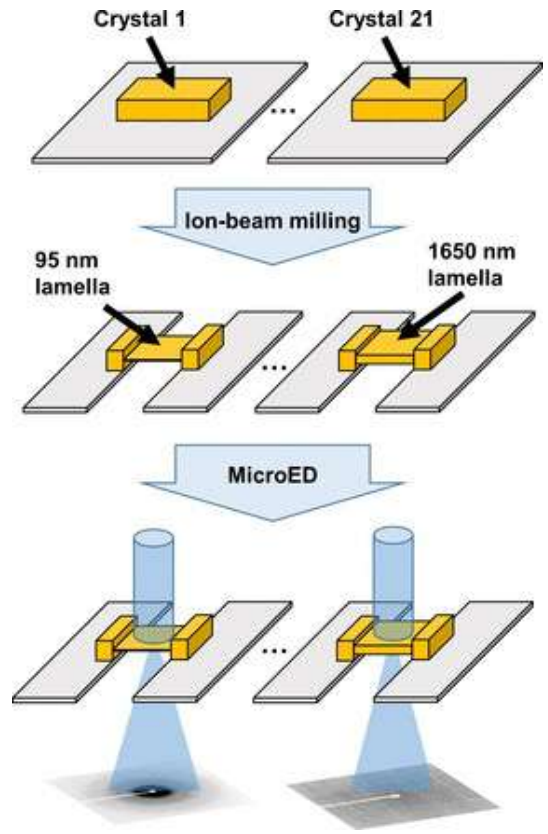
Workflow Overview



Nannenga and Gonen, 2014

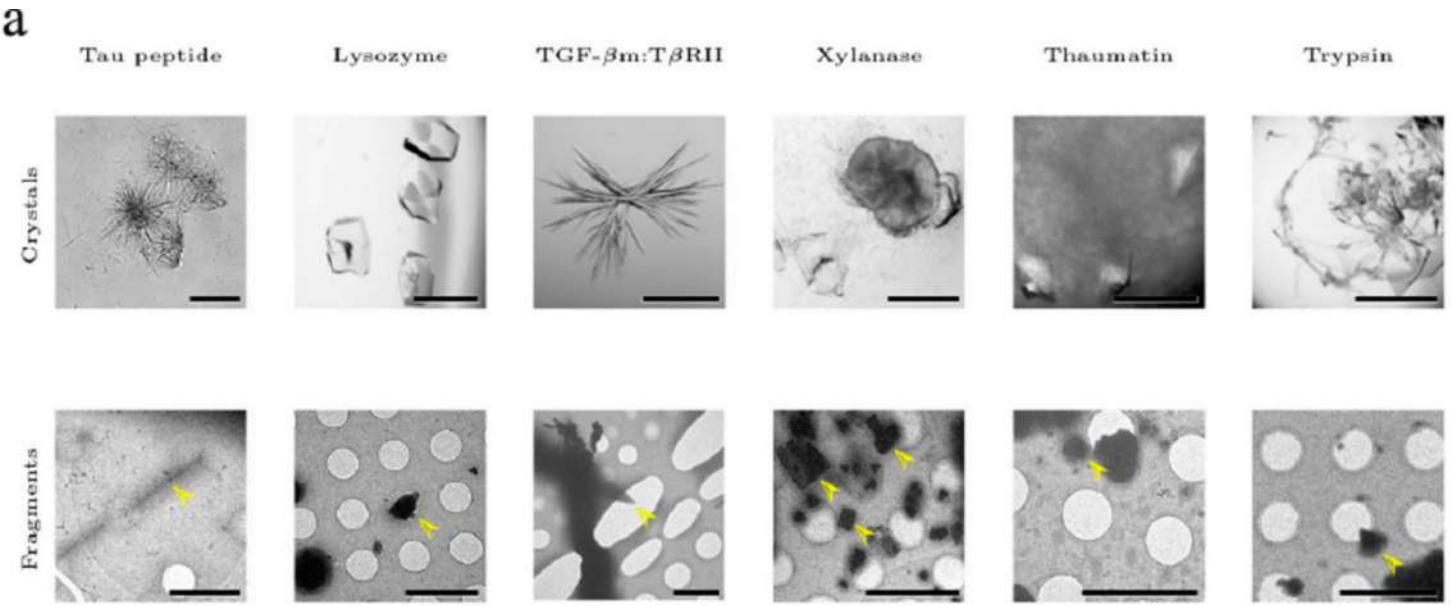
Crystal Thickness

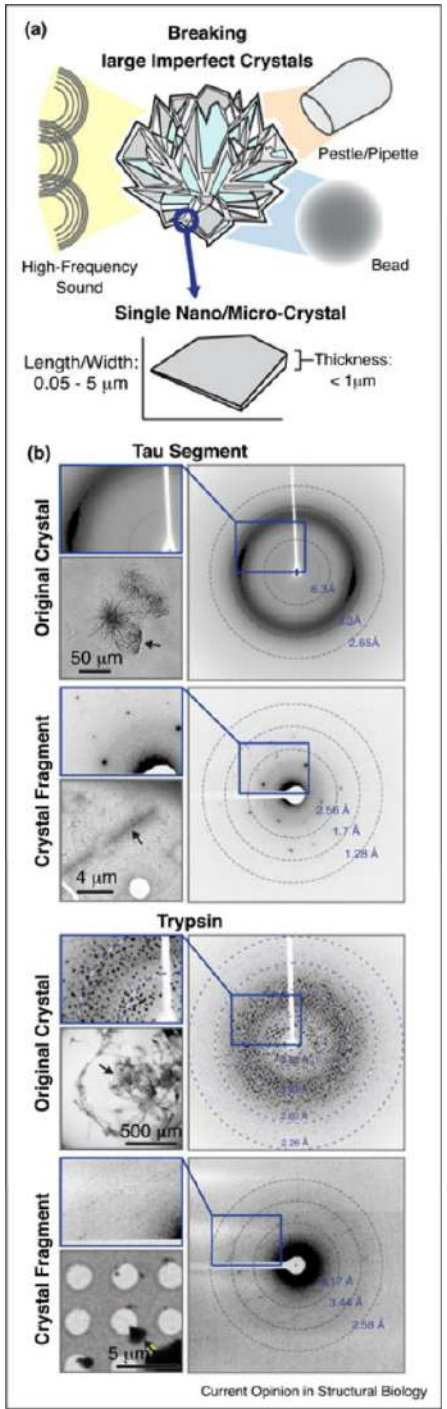
- Lysozyme: crystals thicker then 500 nm unusable
- Martynowycz MW, Clabbers MTB, Unge J, Hattne J, Gonen T. Benchmarking the ideal sample thickness in cryo-EM. Proc Natl Acad Sci U S A. 2021 Dec 7;118(49):e2108884118. doi: 10.1073/pnas.2108884118. PMID: 34873060; PMCID: PMC8670461.
- Maximum usable thickness ~ 2X mean free path of electrons
  - 120 kev: 430 nm
  - 200 kev: 540 nm
  - 300 keV: 640 nm



Larger (imperfect) crystals

→Break them up

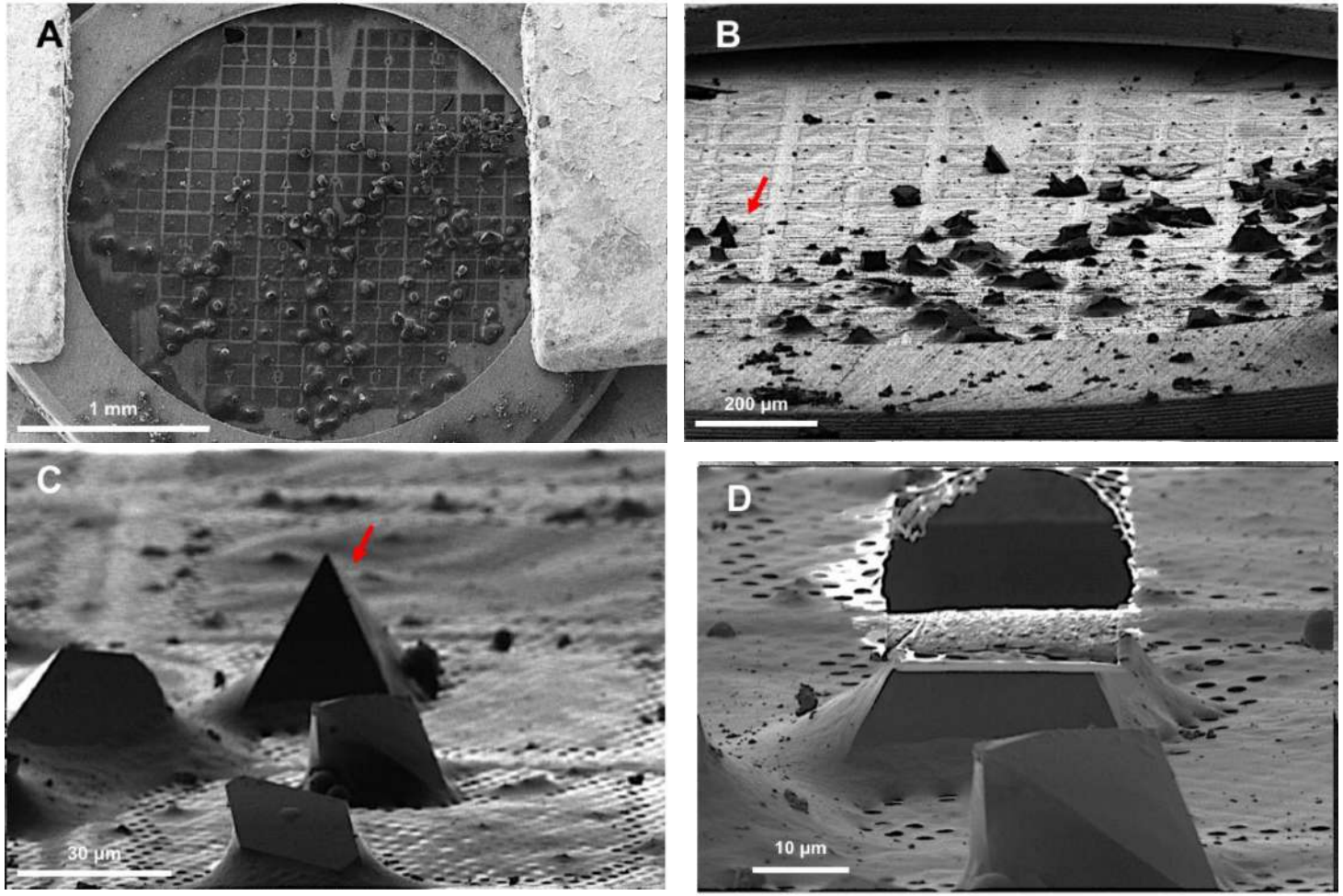




Nannenga and Gonen, 2014
de la Cruz et al, 2017

Larger (imperfect) crystals

→Use a FIB to thin them

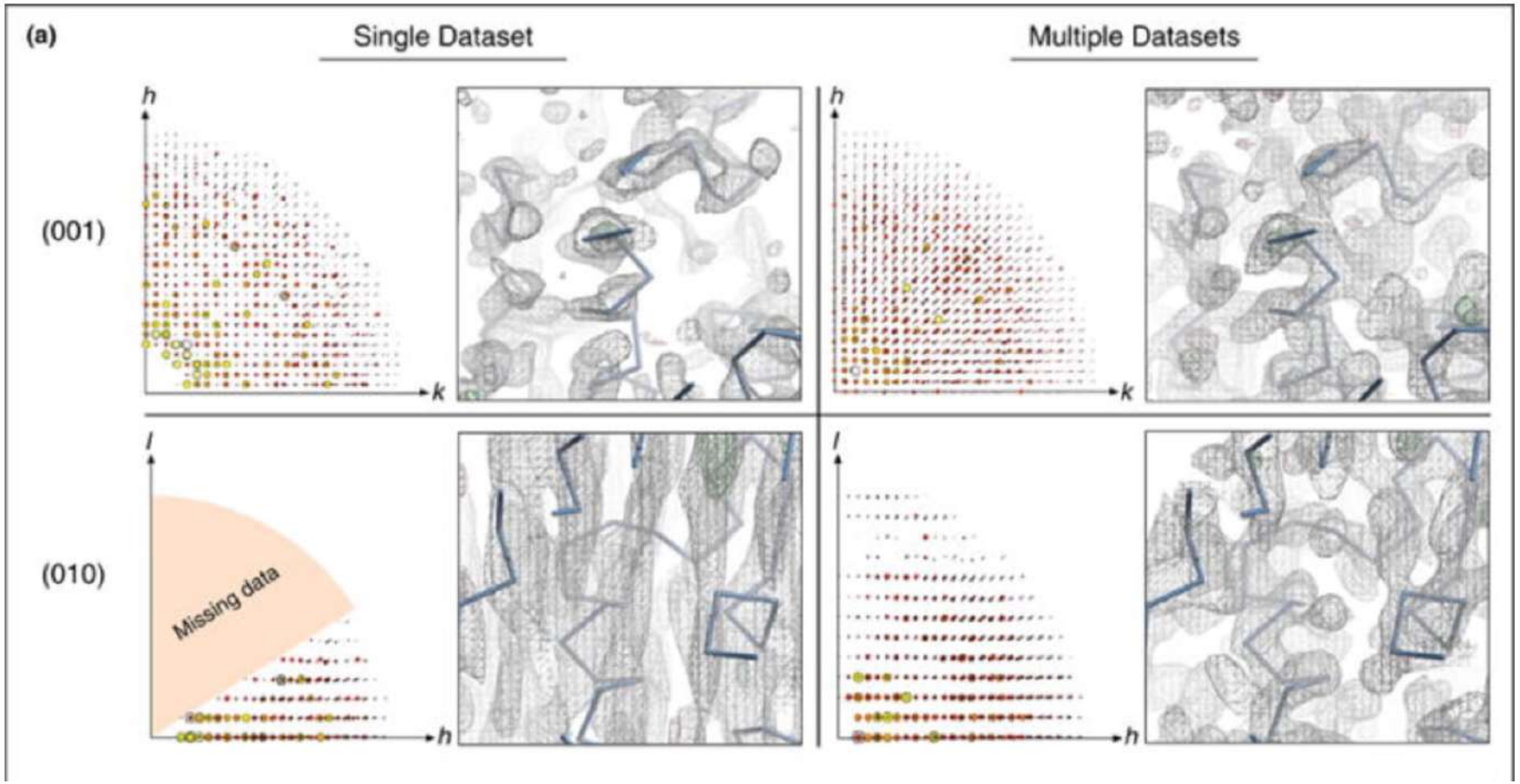


Martynowcyz et al, 2018

Data Collection: more specifics

- To date, most MicroED data was collected on a CMOS camera in “rolling shutter” mode
- Continuous readout of microscope parameters disabled
- In processing software, need to define
  - Beam Center
    - May not be center of image
    - May change due to microscope instabilities
  - Rotation rate of stage – angle and range of each frame
    - Need to record starting angle and direction (clockwise/counter-clockwise)
  - Virtual sample-detector distance
    - Calibrate from powder diffraction pattern of gold or graphite
- Conversion of movie to SMV (Super Marty View) format
  - Supported by x-ray software such as DIALS, MOSFLM, XDS
- Electron flux very low: 0.01-0.05 $e/Å^2/s$

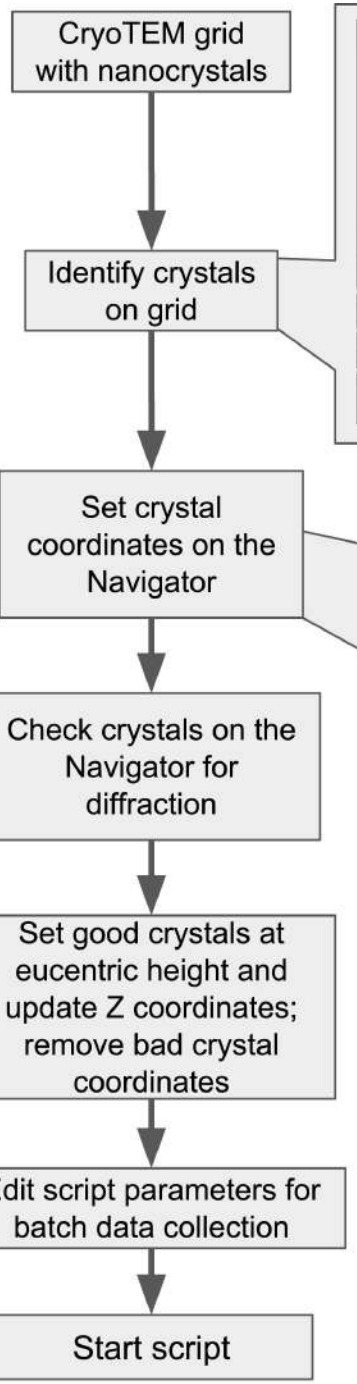
Missing Wedge (-60 to +60 degrees)

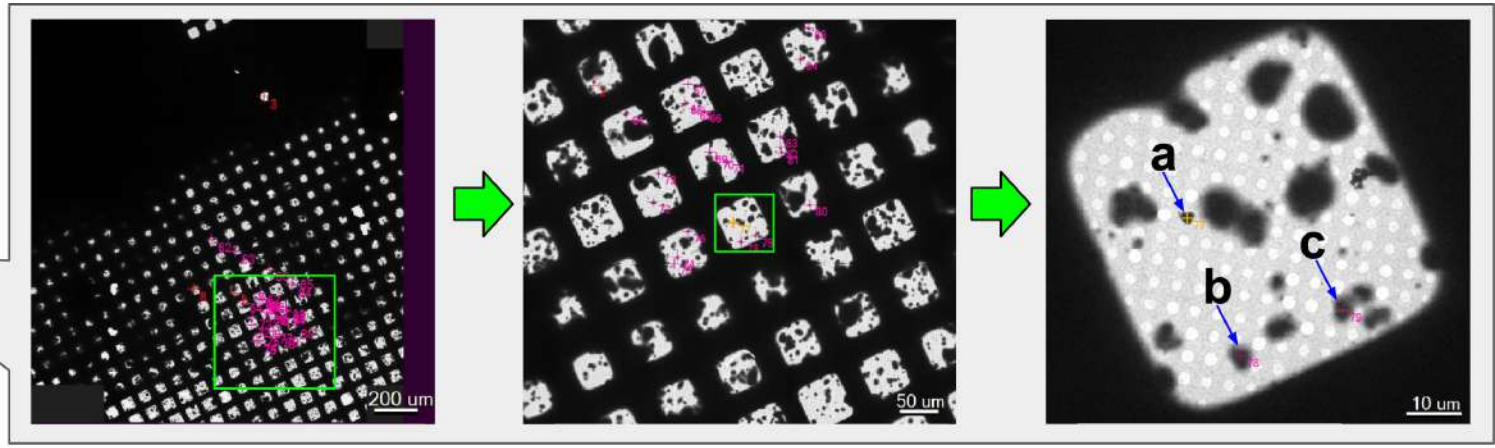


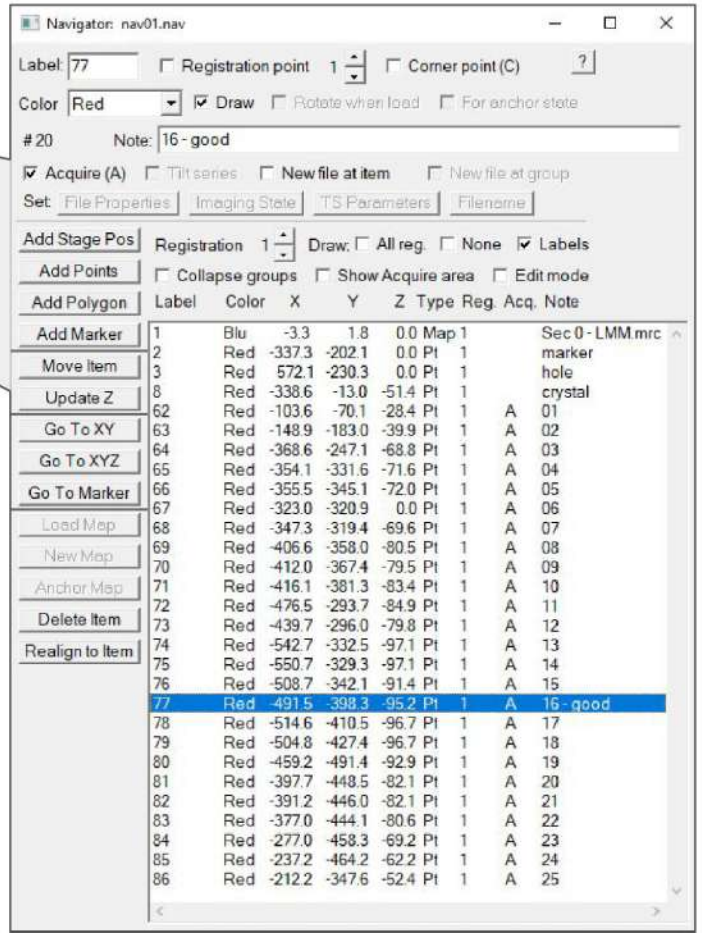
Nannenga and Gonen, 2014

Automated Collection

- SerialEM Script
- Leginon App
- Commercial: TFS
- In-house app (?)







de la Cruz et al., 2019

Processing

- Movies need to be converted to a format readable by X-ray crystallography packages such as DIALS (Waterman et al 2013), MOSFLM (Leslie and Powell, 2007), and XDS (Kabsch, 20100
- Super Marty View (SMV) can be read, conversion tools to SMV format exist
  - Interpretation of gain (ratio of variance to mean intensity in background regions)
  - Dead or hot pixels need to be flagged
  - Diffraction spots need to be in linear response region
- Most standard software needs a configuration file for the camera and microscope: camera length, wavelength, tilt axis

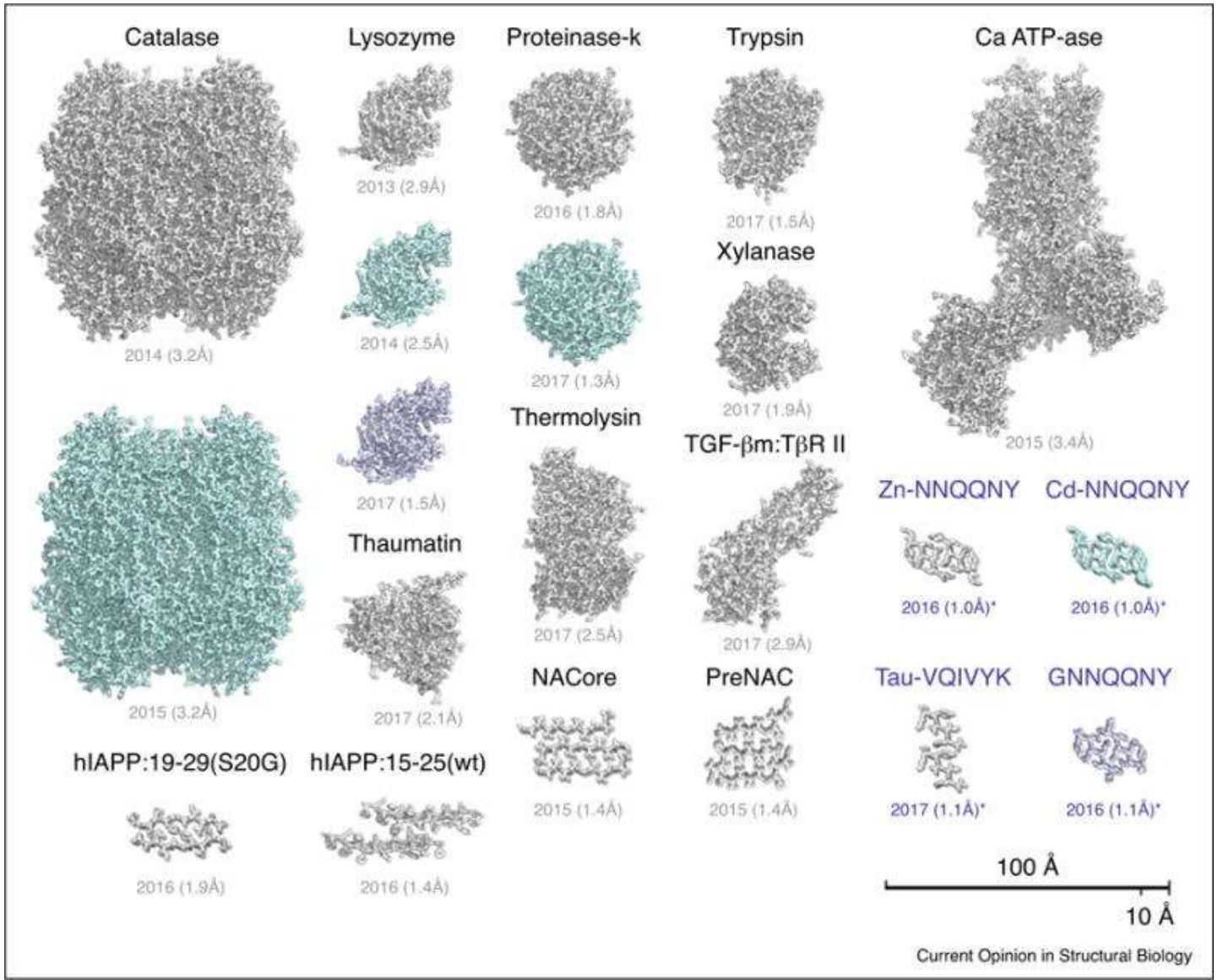
Crystal lattice determination (Indexing)

- More challenging than X-ray crystallography
- deBroglie wavelength 1/50 that of X-ray
  - Ewald sphere less curved so spots fall within an almost planar wedge of reciprocal space
- Autoindexing relies on the 3D lattice to get spacing and orientation correct
- 5-10 images covering ~20° of continuous rotation are often enough to work
- Geometry of collection is less well defined
  - Calculate approximate orientation by multiplying frame time by rotation rate
  - Throw residual errors into the “mosaicity” of the crystal: error sink

Phasing

- For protein structures, phasing was done through molecular replacement
- Standard X-ray crystallography tools
- CNS, Phaser, phenix.refine, REFMAC all have electron scattering factors built in
- Ab initio phasing: works well for small molecules
  - Need diffraction to 1.4 Å or better

Structures Solved by MicroED



Proteins (almost) all already solved by other means

Curr Opin Struct Biol. 2017 Oct; 46: 79–86.

Novel Structures

Solving a new R2lox protein structure by microcrystal electron diffraction

Hongyi Xu[1,*,†], Hugo Lebrette[2,†], Max T. B. Clabbers[1,†], Jingjing Zhao[1], Julia J. Griese[2,3], Xiaodong Zou[1,*] and Martin Högbom[2,*]

[1]*Department of Materials and Environmental Chemistry, Stockholm University, 10691 Stockholm, Sweden.*

[2]*Department of Biochemistry and Biophysics, Stockholm University, 10691 Stockholm, Sweden.*

[3]*Department of Cell and Molecular Biology, Uppsala University, 75124 Uppsala, Sweden.*

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↵† These authors contributed equally to this work.

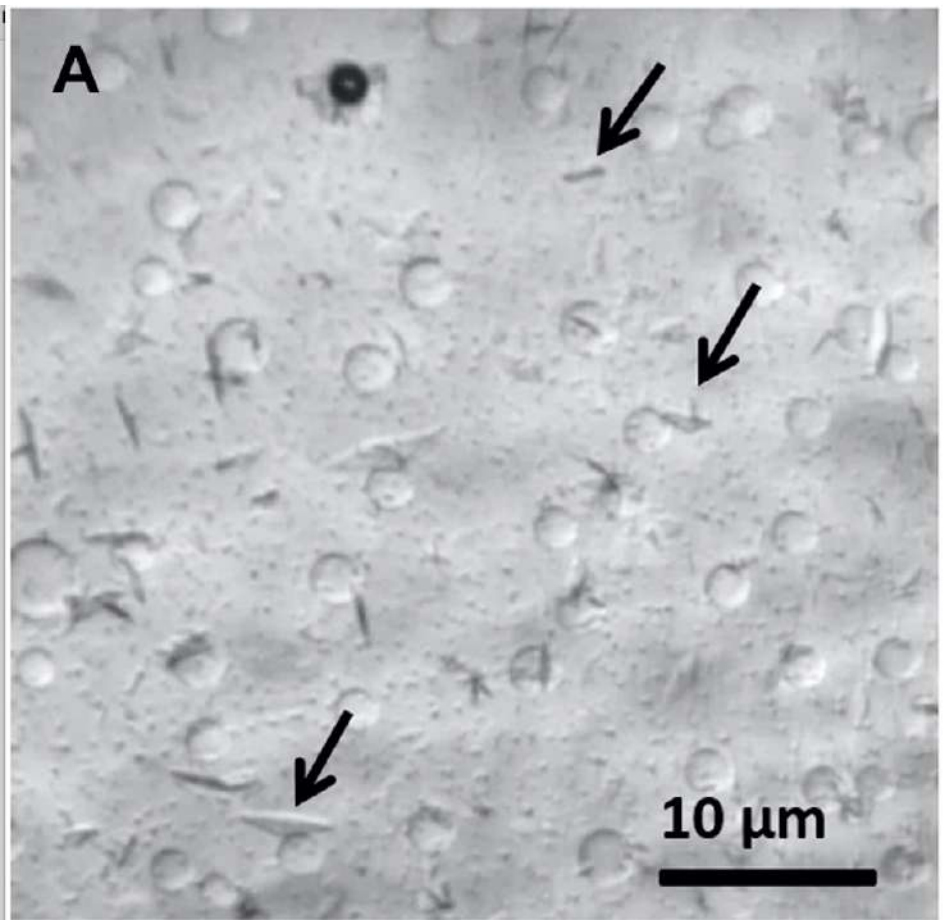
– Hide authors and affiliations

Science Advances 07 Aug 2019:
Vol. 5, no. 8, eaax4621
DOI: 10.1126/sciadv.aax4621

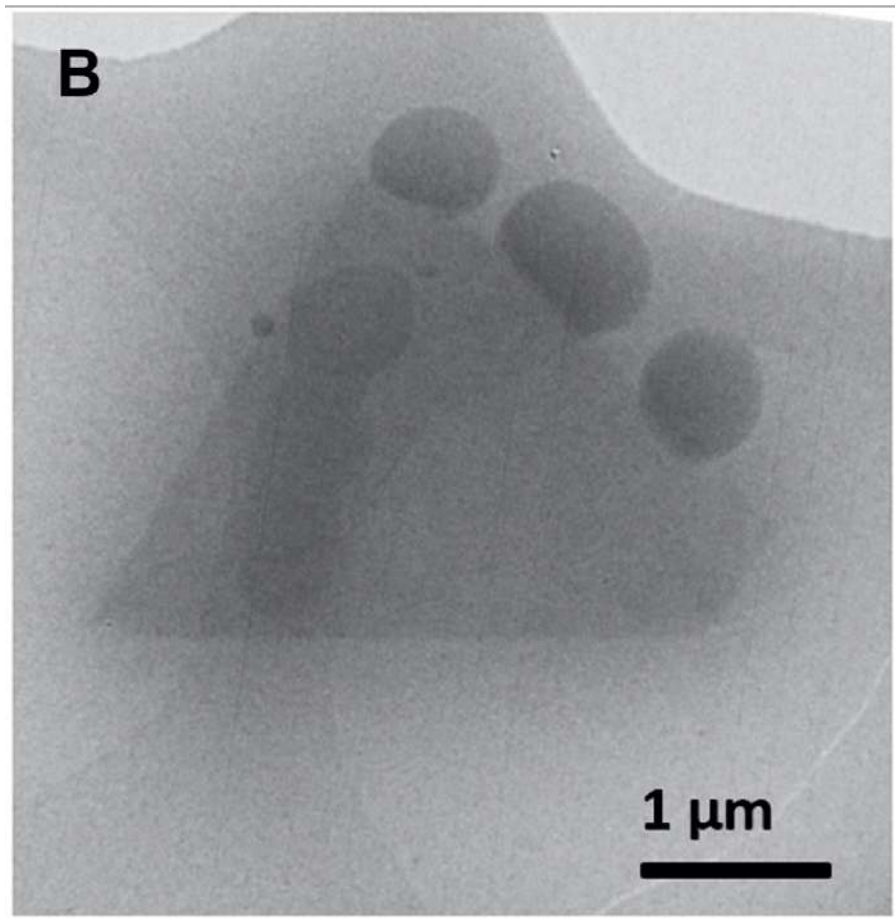
Continuous rotation method
JEM J2100 (200 keV, LaB6 filament)
Timepix hybrid pixel detector
Conventional software: XDS , phasing with phaser (used protein with 35% sequence identity), refinement (phenix.refine)

Solving a new R2lox protein structure by microcrystal electron diffraction

Hongyi Xu[1,*,†], Hugo Lebrette[2,†], Max T. B. Clabbers[1,†], Jingjing Zhao[1], Julia J. Griese[2,3], Xiaodong Zou[1,*] and Martin Högbom[2,*]



44% PEG 400; manual backside blot



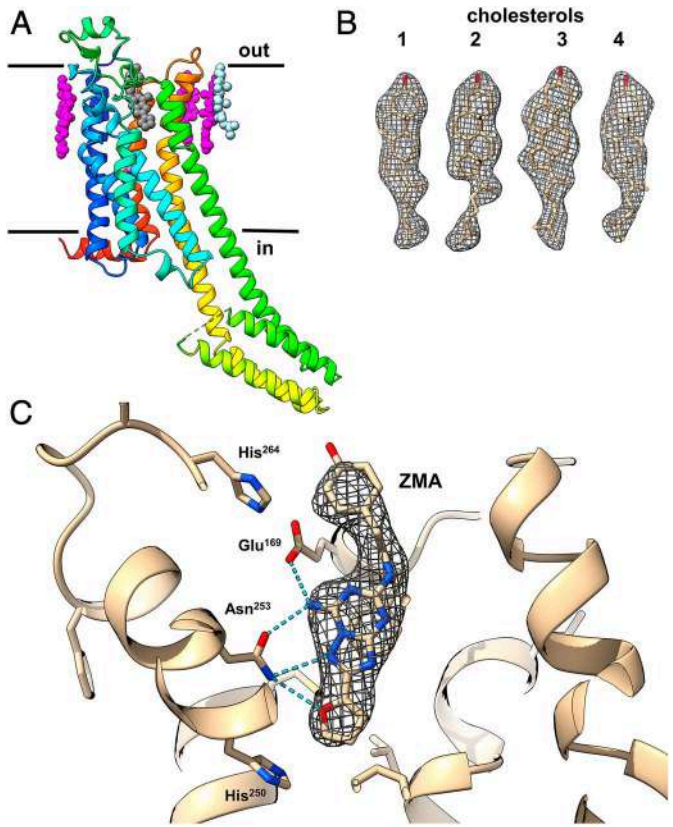
Thickness < 0.5 µm
Plate-like crystals had preferred orientation

MicroED structure of the human adenosine receptor determined from a single nanocrystal in LCP

Michael W. Martynowycz, Anna Shiriaeva, Xuanrui Ge, Johan Hattne, Brent L. Nannen…
+ See all authors and affiliations

PNAS September 7, 2021 118 (36) e2106041118; https://doi.org/10.1073/pnas.2106041118

Edited by Yifan Cheng, University of California, San Francisco, CA, and approved July 20, 2021 (received for review March 29, 2021)



- The gel-phased lipidic cubic phase (LCP) was converted to the liquid-like sponge phase by mixing the LCP with a sponge phase-inducing agent
- Thick crystals were thinned using FIB milling

Small Molecules

ACS central science

ACS AuthorChoice

Research Article

Cite This: *ACS Cent. Sci.* 2018, 4, 1587−1592

http://pubs.acs.org/journal/acscii

The CryoEM Method MicroED as a Powerful Tool for Small Molecule Structure Determination

Christopher G. Jones,[†,#] Michael W. Martynowycz,[‡,#] Johan Hattne,[‡] Tyler J. Fulton,[§] Brian M. Stoltz,[*,§] Jose A. Rodriguez,[*,†,∥] Hosea M. Nelson,[*,†] and Tamir Gonen[*,‡]

[†]Department of Chemistry and Biochemistry, [‡]Howard Hughes Medical Institute, David Geffen School of Medicine, Departments of Biological Chemistry and Physiology, and [∥]UCLA-DOE Institute, University of California, Los Angeles, California 90095, United States

[§]The Warren and Katharine Schlinger Laboratory of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, United States

Supporting Information

Small Molecules

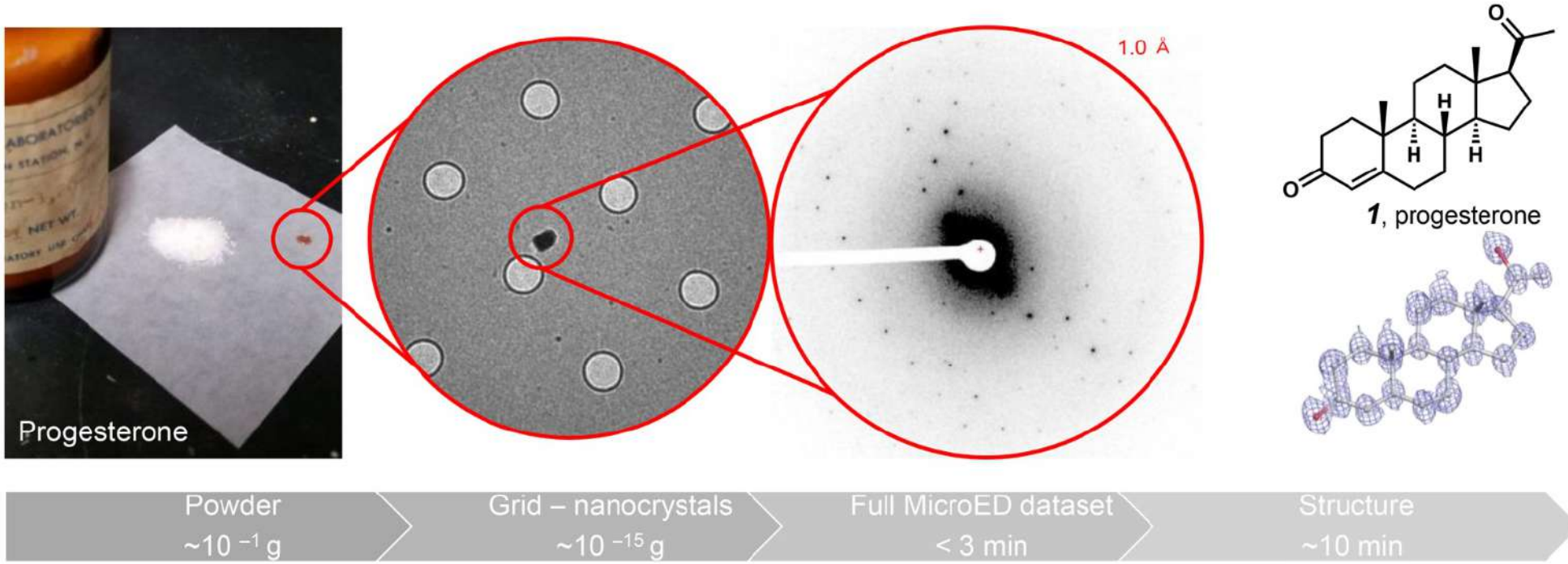
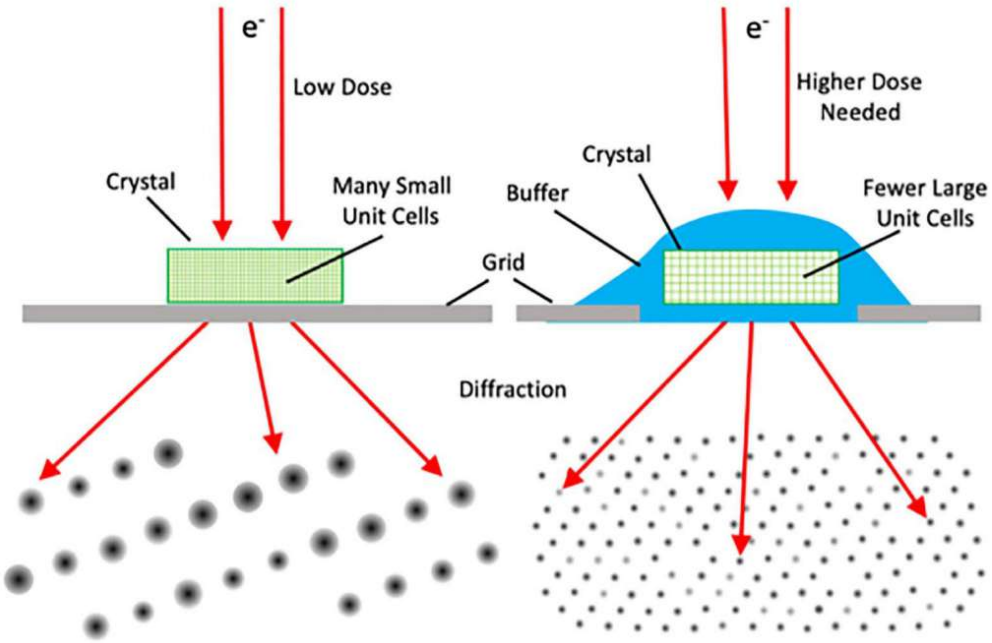


Figure 1. Process of applying MicroED to small molecule structural analysis. Here commercial progesterone (**1**) was analyzed, and an atomic resolution structure was determined at 1 Å resolution. Grid holes are 1 μm in diameter.

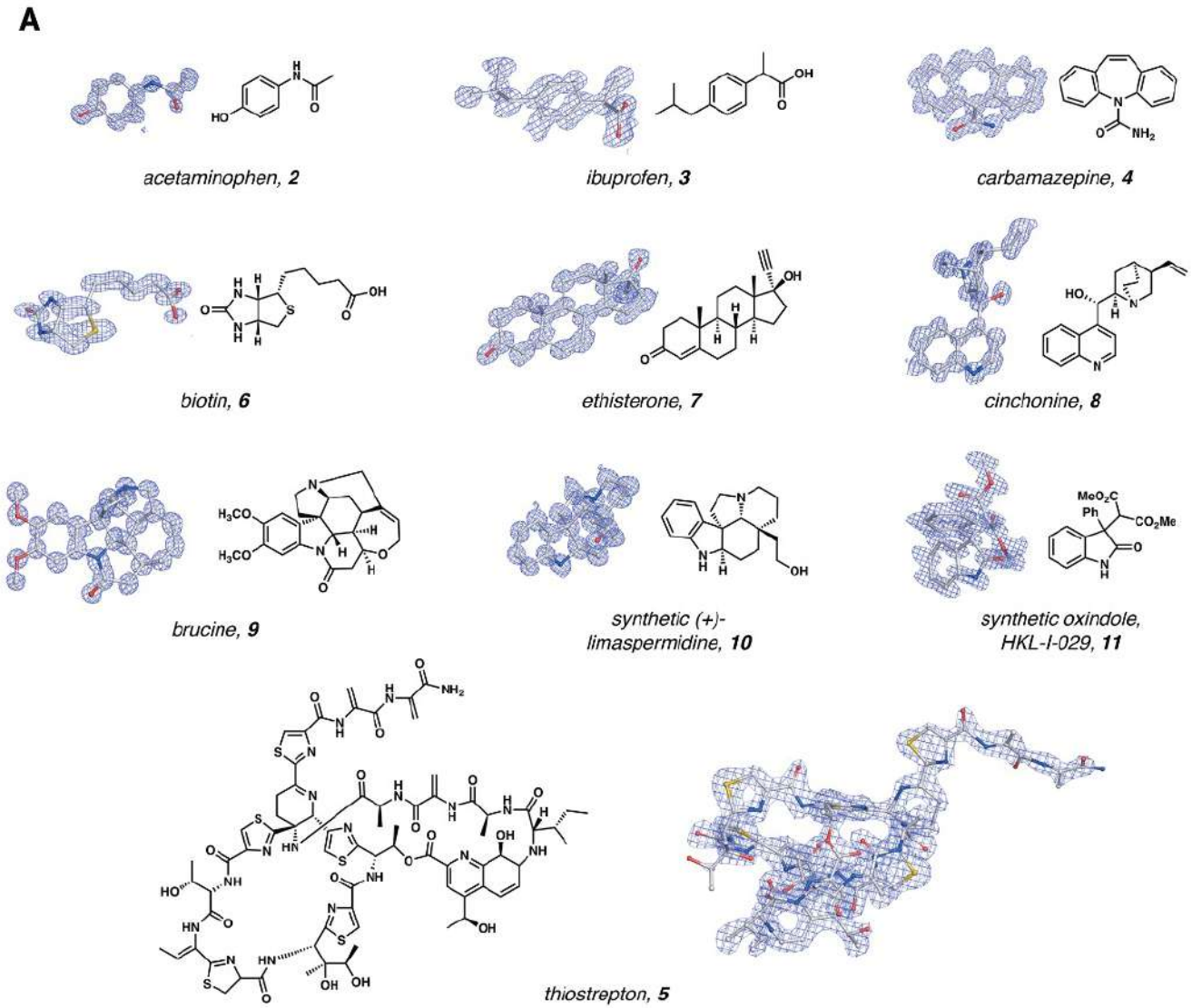
Jones et al, 2018

Sample prep for small molecules is easier than for proteins or peptides



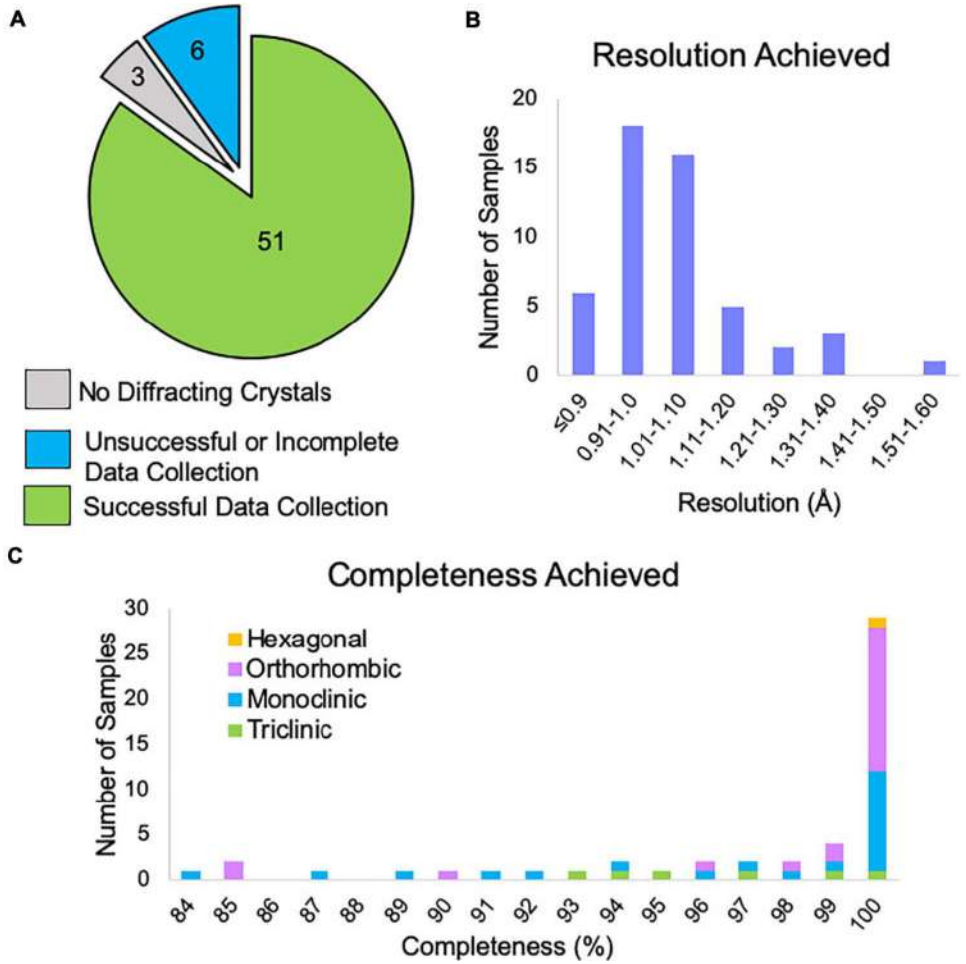
Bruhn JF, Scapin G, Cheng A, Mercado BQ, Waterman DG, Ganesh T, Dallakyan S, Read BN, Nieusma T, Lucier KW, Mayer ML, Chiang NJ, Poweleit N, McGilvray PT, Wilson TS, Mashore M, Hennessy C, Thomson S, Wang B, Potter CS, Carragher B. Small Molecule Microcrystal Electron Diffraction for the Pharmaceutical Industry-Lessons Learned From Examining Over Fifty Samples. Front Mol Biosci. 2021 Jul 12;8:648603. doi: 10.3389/fmolb.2021.648603. PMID: 34327213; PMCID: PMC8313502.

Small Molecules



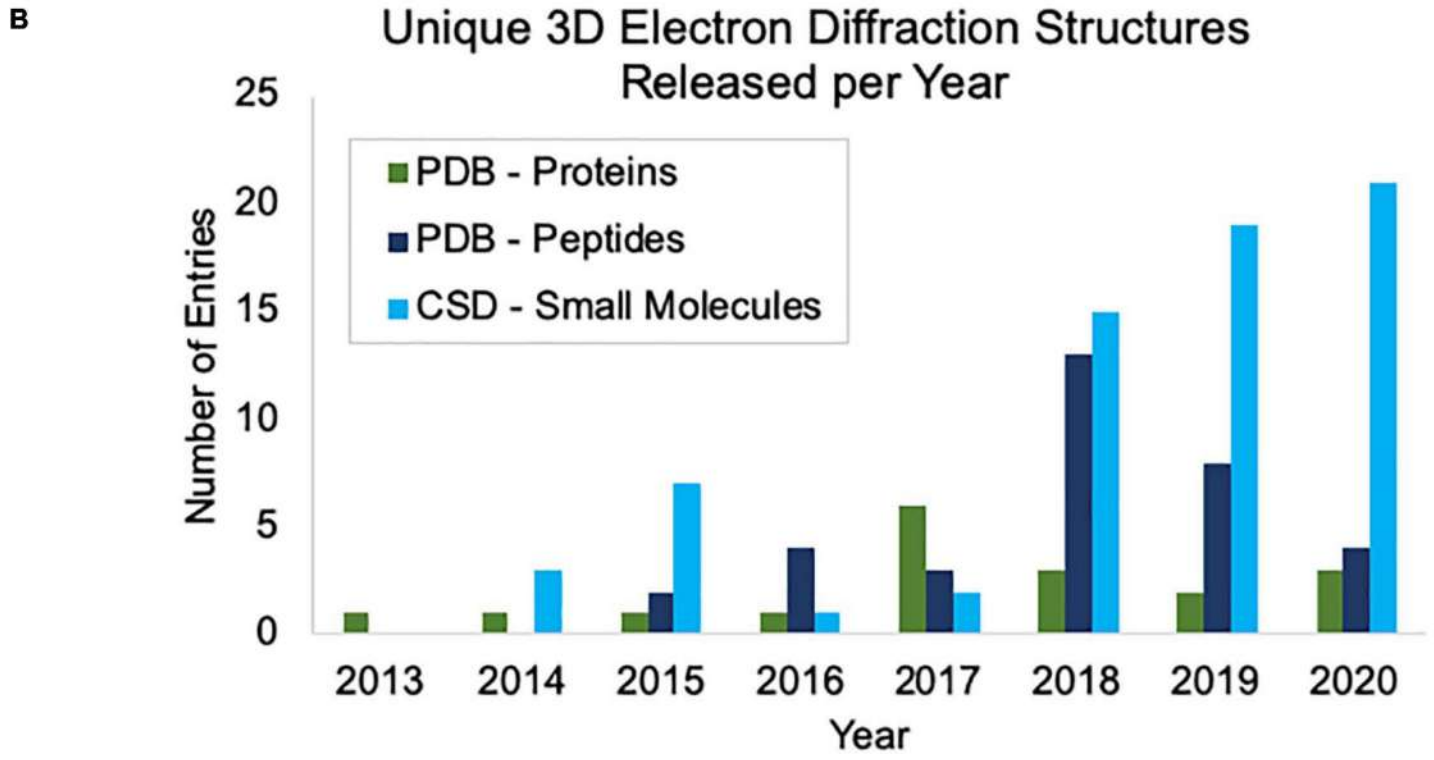
Jones et al, 2018

Collection of over 50 small molecule samples



Bruhn JF, Scapin G, Cheng A, Mercado BQ, Waterman DG, Ganesh T, Dallakyan S, Read BN, Nieusma T, Lucier KW, Mayer ML, Chiang NJ, Poweleit N, McGilvray PT, Wilson TS, Mashore M, Hennessy C, Thomson S, Wang B, Potter CS, Carragher B. Small Molecule Microcrystal Electron Diffraction for the Pharmaceutical Industry-Lessons Learned From Examining Over Fifty Samples. Front Mol Biosci. 2021 Jul 12;8:648603. doi: 10.3389/fmolb.2021.648603. PMID: 34327213; PMCID: PMC8313502.

Unique structures solved by MicroED

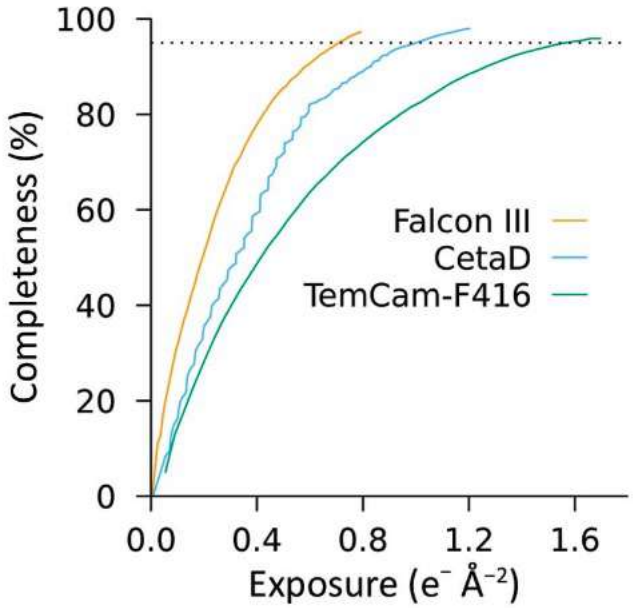


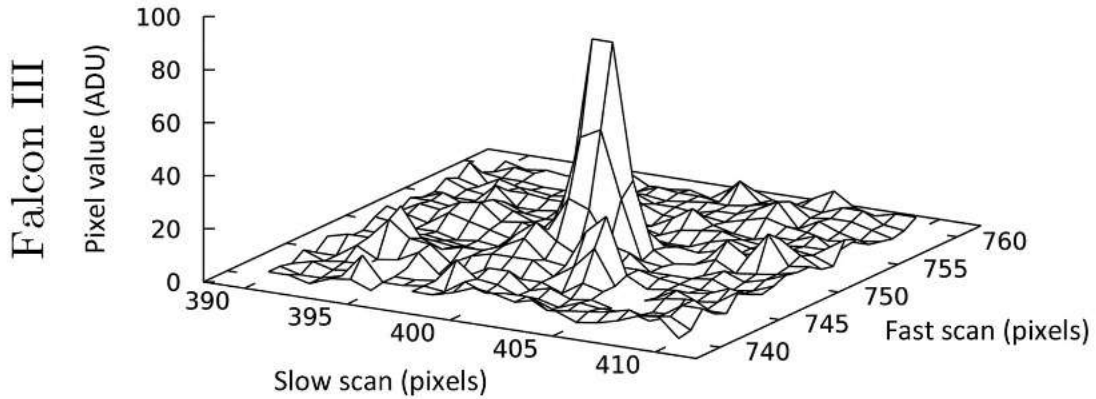
Bruhn JF, Scapin G, Cheng A, Mercado BQ, Waterman DG, Ganesh T, Dallakyan S, Read BN, Nieusma T, Lucier KW, Mayer ML, Chiang NJ, Poweleit N, McGilvray PT, Wilson TS, Mashore M, Hennessy C, Thomson S, Wang B, Potter CS, Carragher B. Small Molecule Microcrystal Electron Diffraction for the Pharmaceutical Industry-Lessons Learned From Examining Over Fifty Samples. Front Mol Biosci. 2021 Jul 12;8:648603. doi: 10.3389/fmolb.2021.648603. PMID: 34327213; PMCID: PMC8313502.

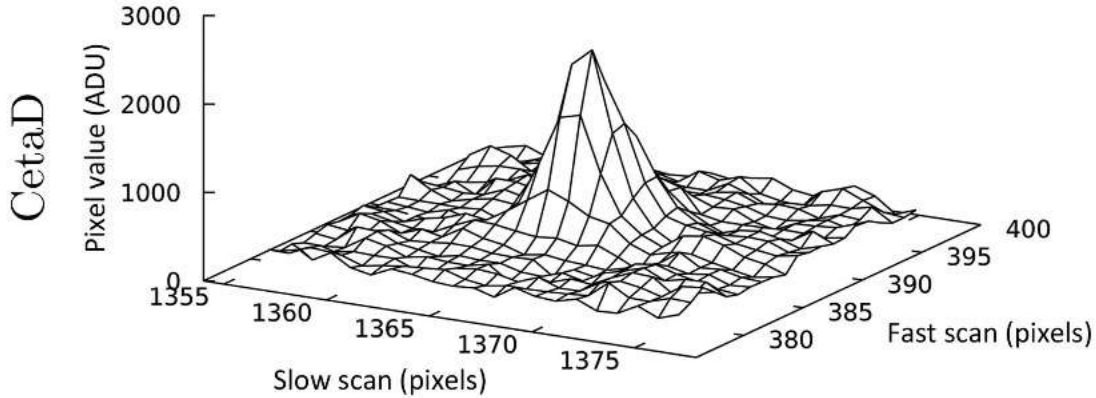
CMOS Detectors

- High dynamic range
- Fast readout low dead time (continuous rotation)
- TVIPS F416: 4kx4K CMOS detector with scintillation layer
  - Rolling shutter mode at 2kx2k available
- Ceta : 4Kx4K CMOS detector with rolling shutter mode
- CetaD: 4Kx4K CMOS detector with thicker scintillation layer than standard Ceta camera

Falcon III for Detection

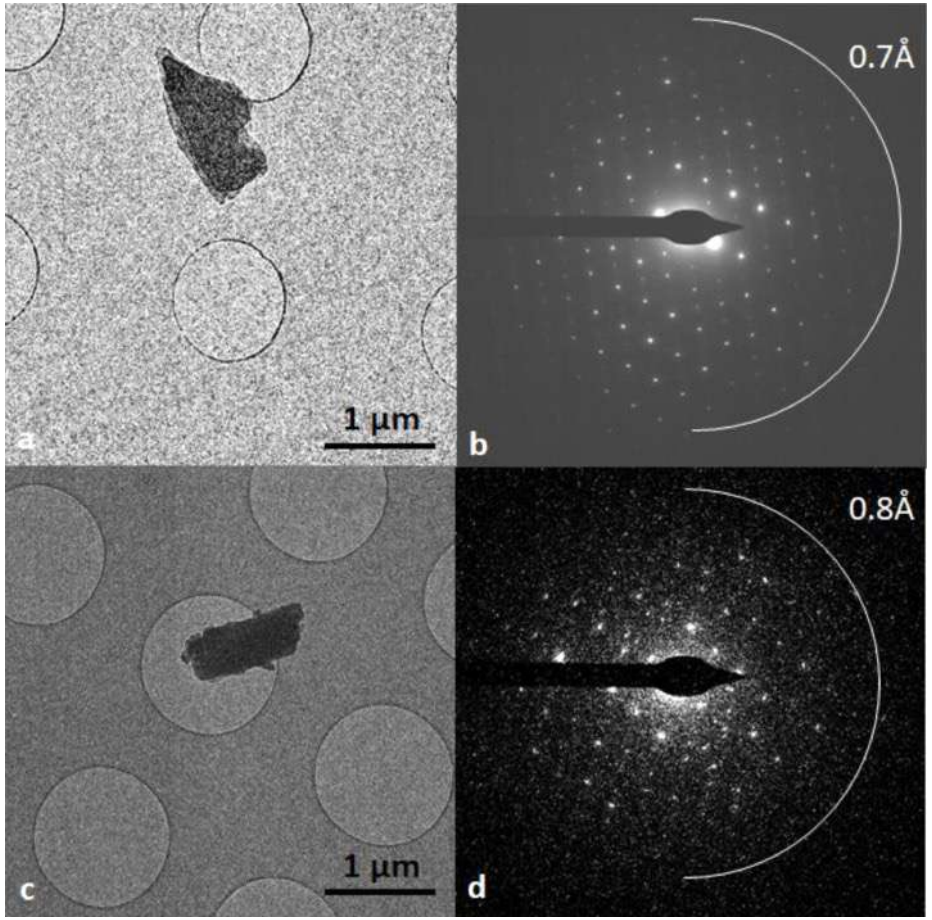






Hattne J, Martynowycz MW, Penczek PA, Gonen T. MicroED with the Falcon III direct electron detector. IUCrJ. 2019 Aug 17;6(Pt 5):921-926. doi: 10.1107/S2052252519010583. PMID: 31576224; PMCID: PMC6760445.

DE64 for Detection



Biotin

L-serine

Zhou W, Bammes B, Mitchell PG, Betz K, Chiu W. Electron crystallography of chiral and non-chiral small molecules. Ultramicroscopy. 2022 Jan;232:113417. doi: 10.1016/j.ultramic.2021.113417. Epub 2021 Oct 19. Erratum in: Ultramicroscopy. 2022 Jan 25;:113474. PMID: 34695647; PMCID: PMC8794741.

Hybrid Detectors

- EIGER (Tinti et al., 2018)
- Medipix (Nederlofetal., 2013)
- Timepix (van Genderenet al., 2016)
- Small size and large point spread function: dedicated diffraction cameras
- Only 256 x 256, up to 1024 x 1024 by merging 4

Micro ED Summary

- Advantages
  - Mid-level microscope (200 keV, FEG better but not essential)
  - Highest resolution yet achieved by cryo-EM technique
  - Sample prep for small molecules is relatively simple
- Disadvantages
  - Crystals must be thin and randomly oriented
  - Sample prep and screening for proteins is difficult
    - FIB milling complicates even more
  - High quality camera and stage are essential
  - Phasing problem: molecular replacement needed for most protein structures
  - Processing may require more expertise than is generally needed in x-ray crystallography

Recommended Recent Protocols

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- Bruhn JF, Scapin G, Cheng A, Mercado BQ, Waterman DG, Ganesh T, Dallakyan S, Read BN, Nieusma T, Lucier KW, Mayer ML, Chiang NJ, Poweleit N, McGilvray PT, Wilson TS, Mashore M, Hennessy C, Thomson S, Wang B, Potter CS, Carragher B. Small Molecule Microcrystal Electron Diffraction for the Pharmaceutical Industry-Lessons Learned From Examining Over Fifty Samples. Front Mol Biosci. 2021 Jul 12;8:648603. doi: 10.3389/fmolb.2021.648603. PMID: 34327213; PMCID: PMC8313502.
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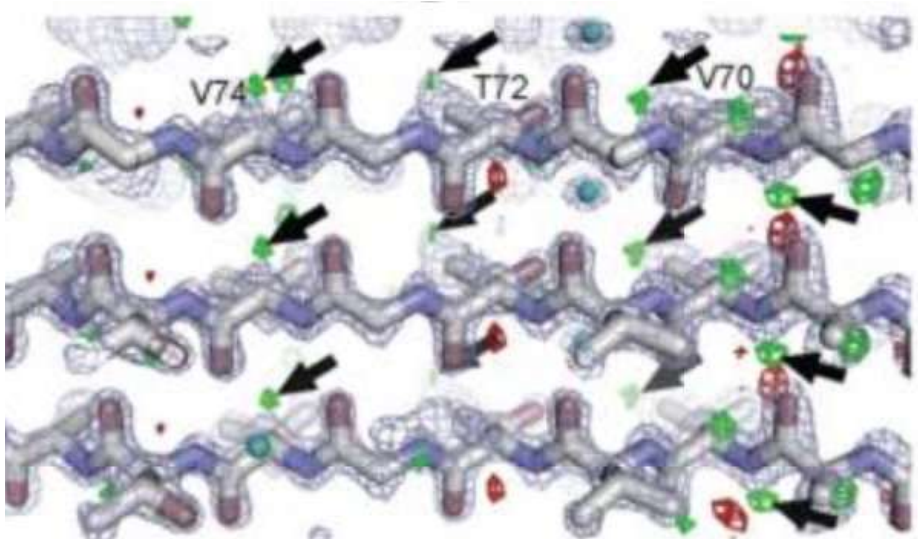
Questions



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