Cryo-EM:
3D Electron Microscopy

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CCP-EM, UK

Many of these slides were kindly provided by:
Prof. Helen Saibil (Birkbeck, London)
Dr. Rebecca Thompson (University of Leeds)

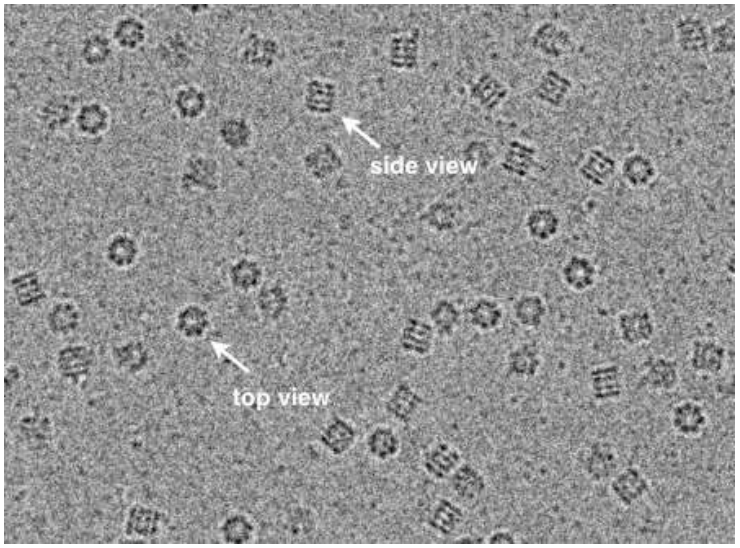
Lecture contents

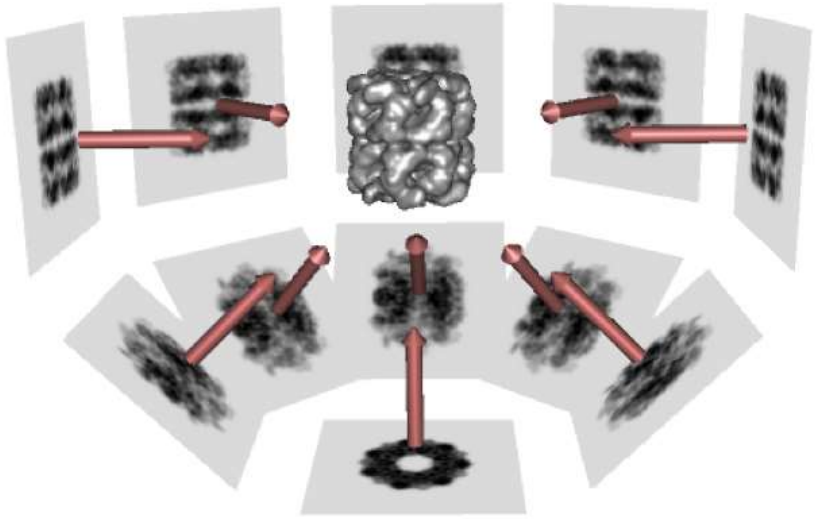
- Single particle cryo-EM
  - Why cryo-EM?
  - Sample preparation
  - Data collection
  - Data processing
- Other cryo-EM techniques
  - Tomography
  - Sub-tomogram averaging
  - FIB milling and correlative microscopy
  - Electron crystallography
- CCP-EM

Why cryo-EM?

Single particle cryo-EM overview

- Collect images of macromolecules frozen in ice
- Extract and orient particle images
- Reconstruct 3D volume
- In general, more particles => higher resolution (< 3 Å)





Macromolecular structure techniques

X-ray crystallography	• Needs crystals • Gives atomic resolution • Conformation may be affected by crystal lattice
NMR	• Gives near-atomic resolution • Can see dynamic processes • Small proteins by solution NMR • Larger complexes by selective labelling, solid state
Cryo-electron microscopy	• Resolution 2 – 20+ Å (depends on sample order and data volume) • Ordered assemblies or isolated particles • Can trap transient states and sort heterogeneity

Why cryo-EM?

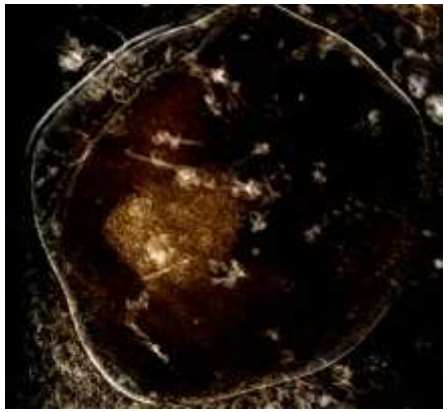
Key advantages:

- Directly image macromolecules in near-native state
- No need for crystals
- Can obtain structures of interesting targets:
  - Large molecular complexes
  - Multiple conformational states

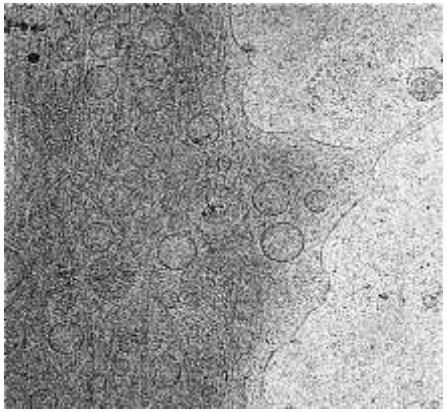
Structural biology from cells to molecules

Increasing biological complexity and integrity

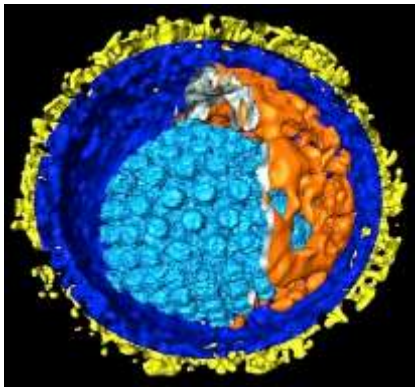
Fluorescence microscopy



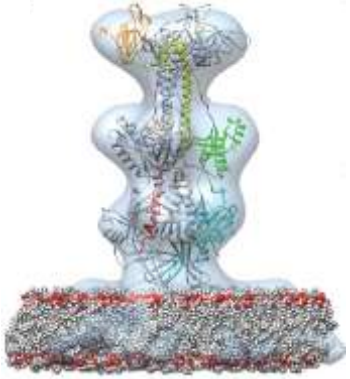
X-ray microscopy



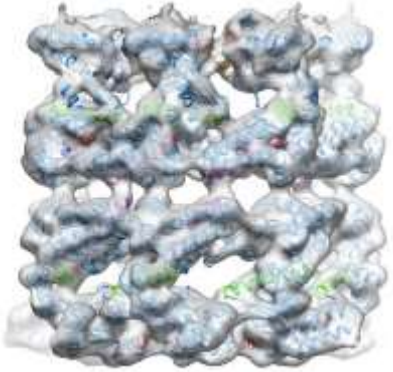
Cellular cryo-electron tomography

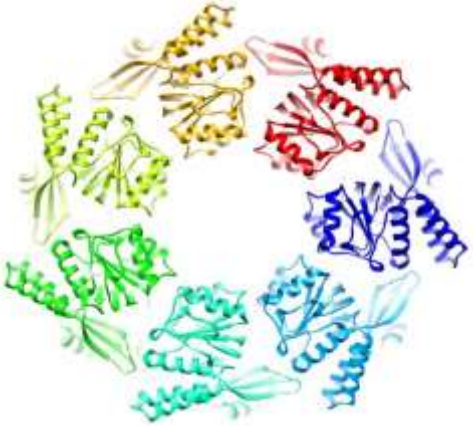


Cryo-electron tomography



Sub-tomogram averaging

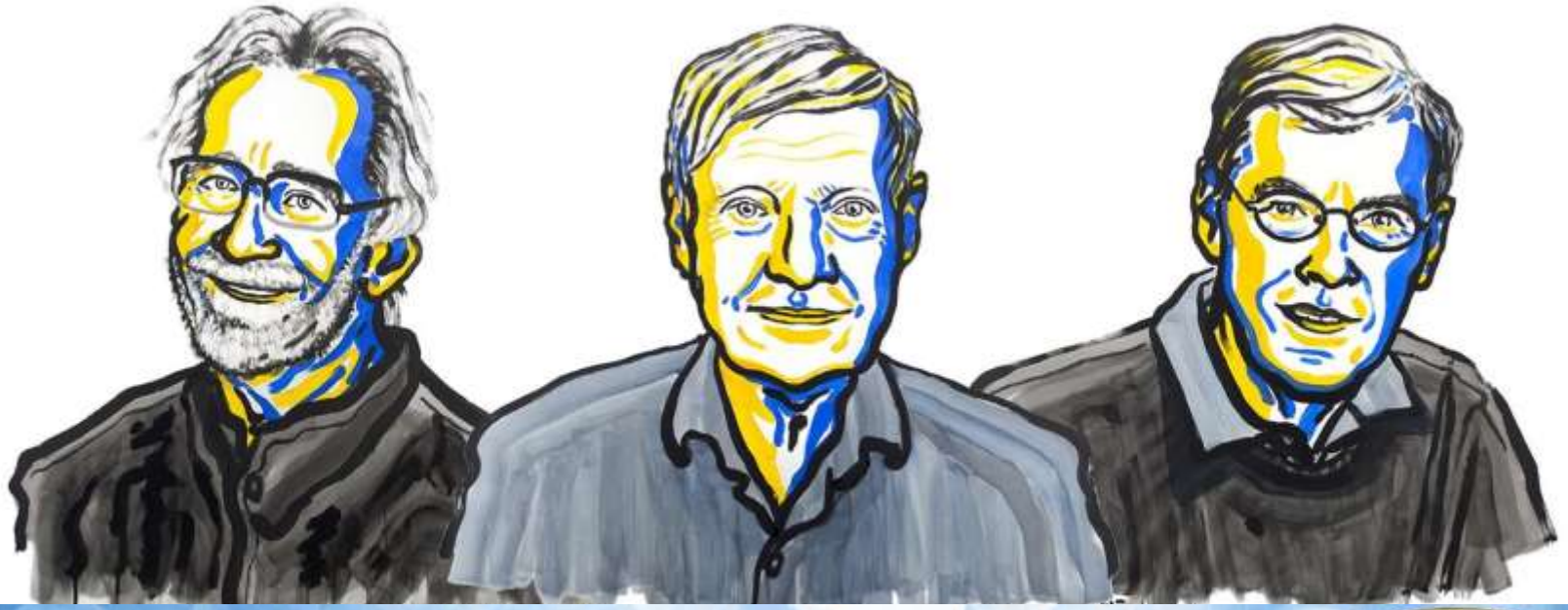




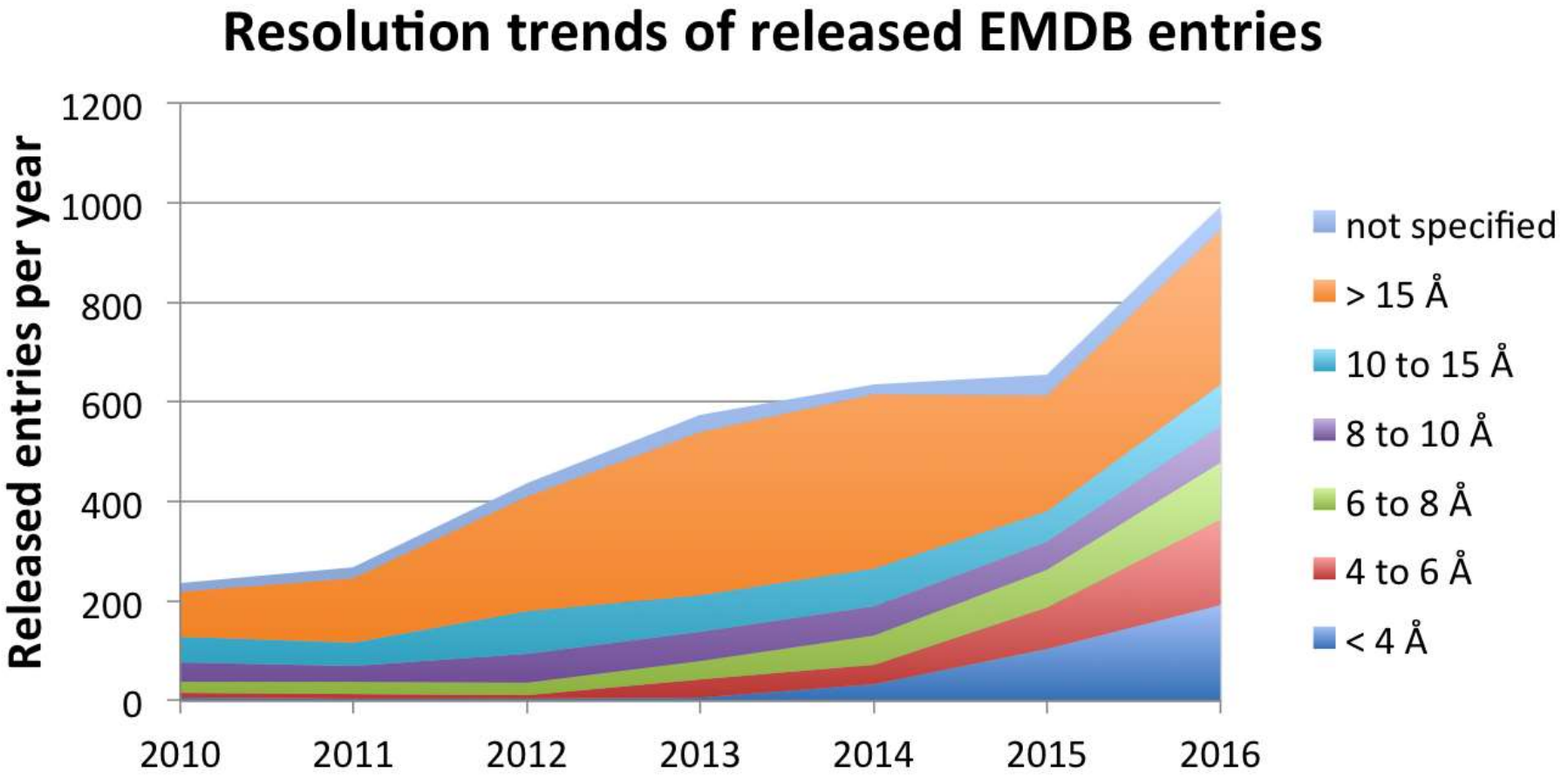
Single particle cryo-EM and X-ray crystallography

Increasing resolution

Why cryo-EM now?



Why cryo-EM now?



(EMDB: like the PDB, but for EM volumes)

Ardan Patwardhan, Acta Cryst. ***D73****:503–508*

Why cryo-EM now?

Key recent improvements:

- Better detectors
- Better microscopes
- Better algorithms

Sample preparation

Sample preparation

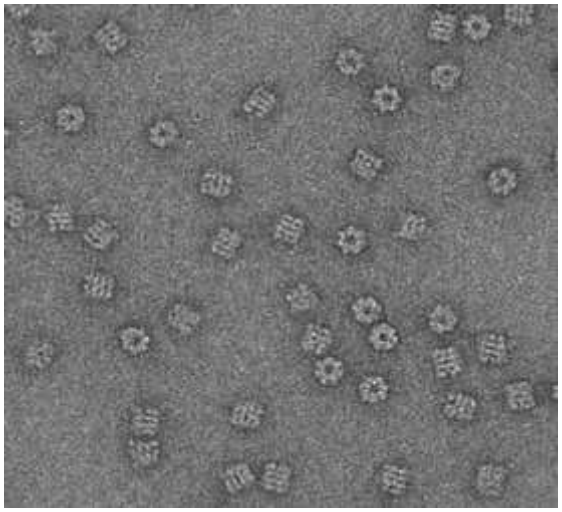
Starting material: aqueous solution of macromolecules

Traditional methods use heavy metal stains for contrast

Limited resolution: best is with “negative stain”, approx. 10–25 Å

Still very useful for quick and simple visualisation of molecules!

Negative Stain



Sample preparation

Starting material: aqueous solution of macromolecules

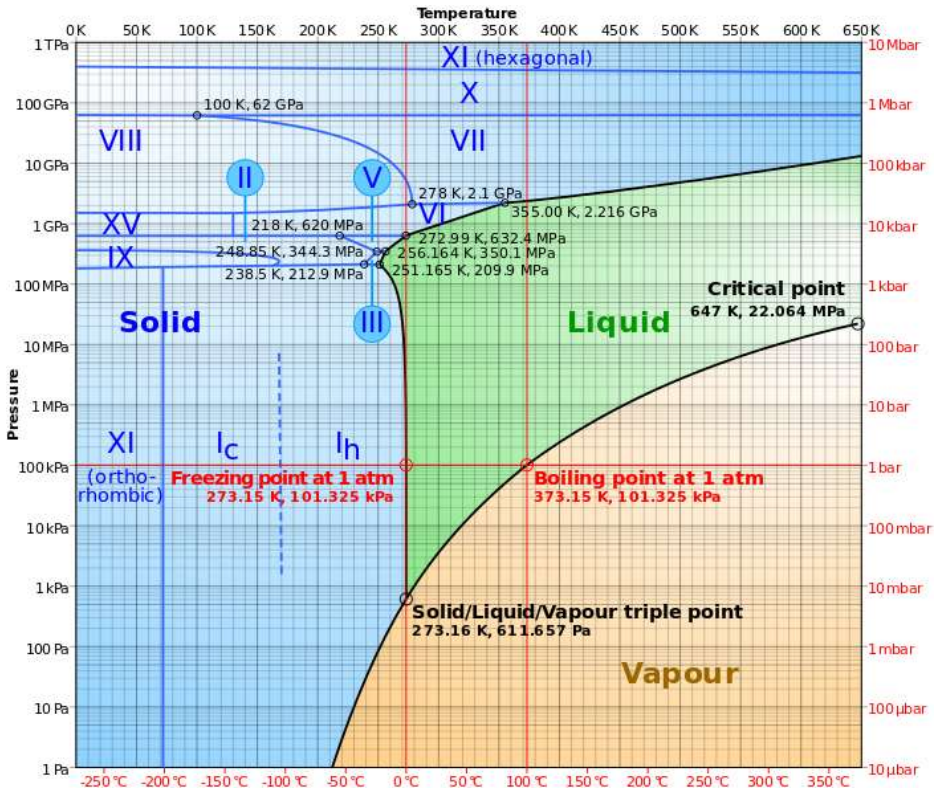
Traditional methods use heavy metal stains for contrast

Limited resolution: best is with “negative stain”, approx. 10–25 Å

For higher resolution, need to look at the molecules themselves, not heavy atoms nearby

=> cryo-preservation

Vitrification of water



Very rapid freezing (~10^6 °C/s)

So fast the water does not have time to crystallize

Water and specimen fixed in a vitreous, amorphous state

If cooling is too slow, or temperature is not kept below −137 °C, crystalline ice is formed

Negative stain vs.cryo EM

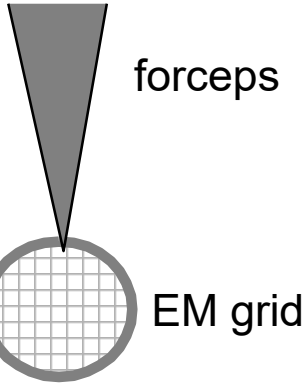
Negative staining

- Simple procedure
- Quick to check samples
- High contrast
- Dehydration
- Heavy metal salts
- Possible distortion, flattening

Cryo EM

- More complex preparation
- Longer time for checking samples
- Low contrast
- Native, hydrated state
- Near physiological conditions
- 3D structure preserved
- Rapid freezing can trap transient states

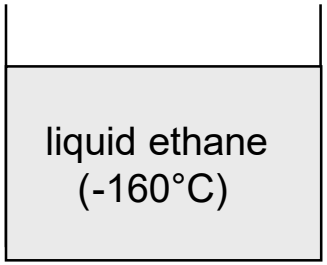
Sample preparation for cryo-EM



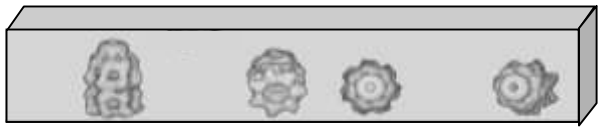
1. Add small volume of sample

2. Blot

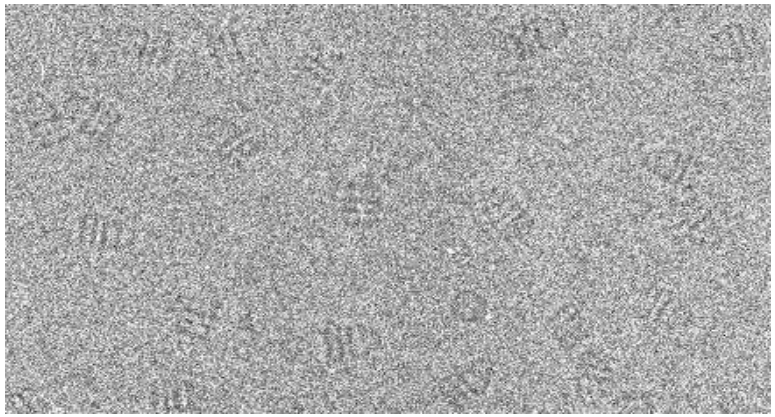
3. Plunge into liquid ethane



4. Keep the grid at liquid nitrogen temperature



Edge-on view of an unsupported part of the water layer



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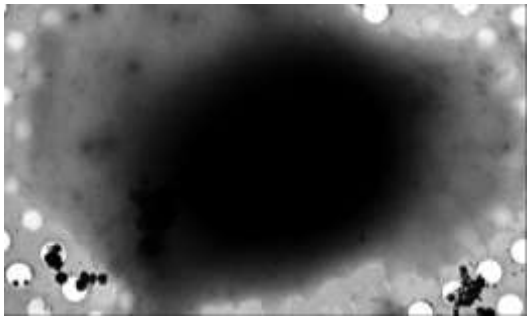
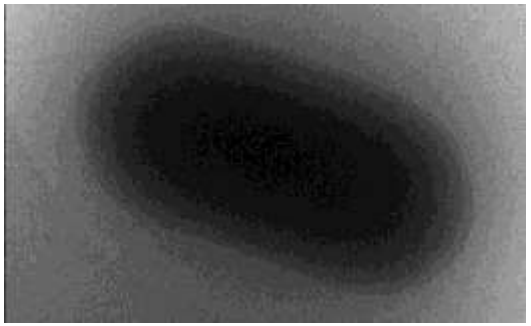
Sample preparation for cryo-EM

Plunge freezing can be used for a wide range of specimens

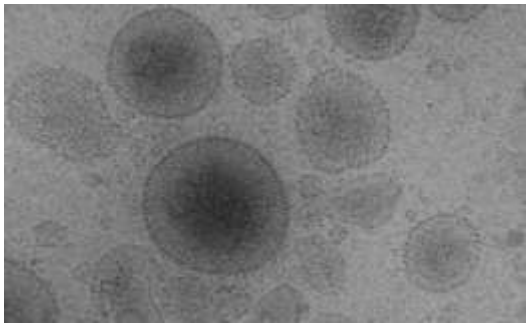
Eukaryotic cells
10-100 µm



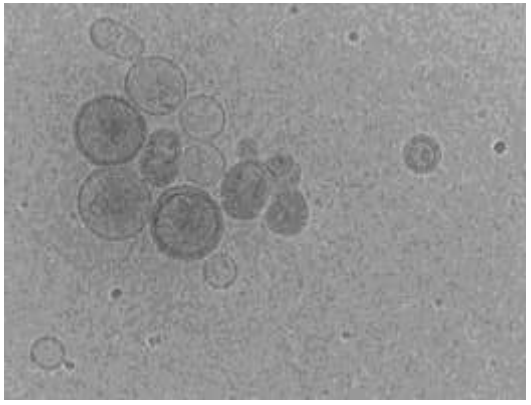
Prokaryotic cells
0.1-5 µm



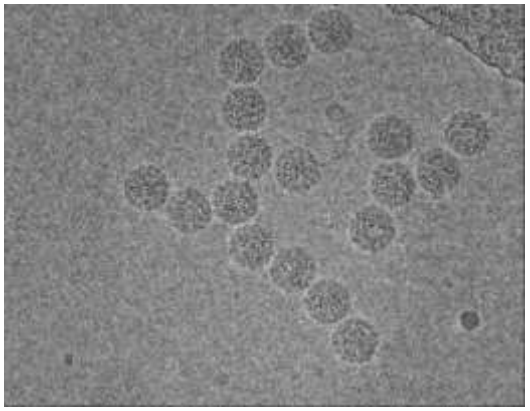
Isolated organelles
100 nm-2 µm



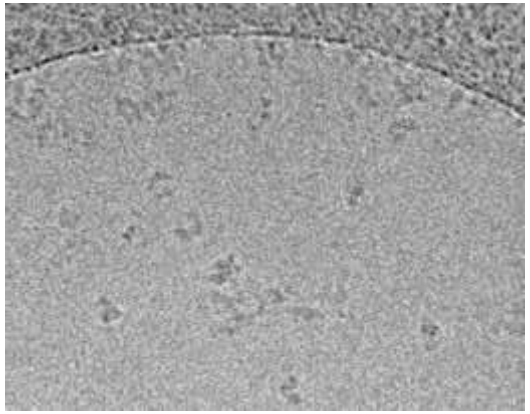
Synthetic liposomes
20 nm-500 nm



Viruses
20-400 nm



Macromolecular complexes
> 100 kDa



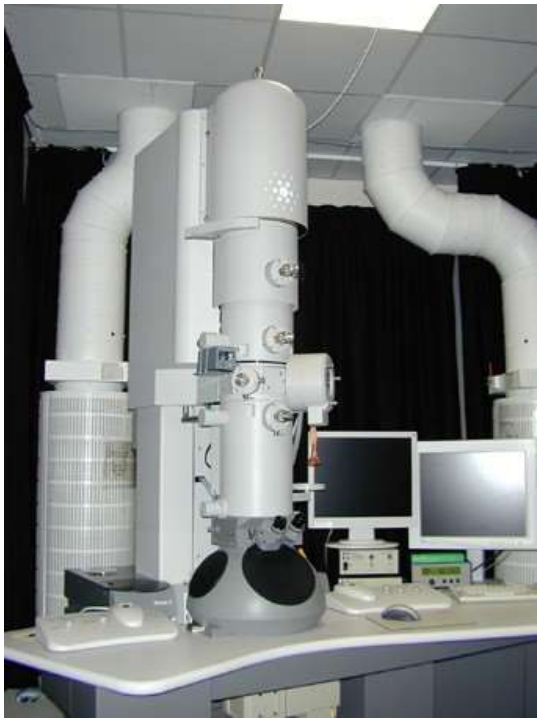
High pressure freezing

- For thicker specimens (e.g. thicker than 10 µm nuclear regions of cells, tissues up to 200 µm)
- Based on Le Chatelier principle, where the volume of water increases when it freezes.
- High pressure inhibits the expansion of water during freezing, thereby inhibiting crystallisation.

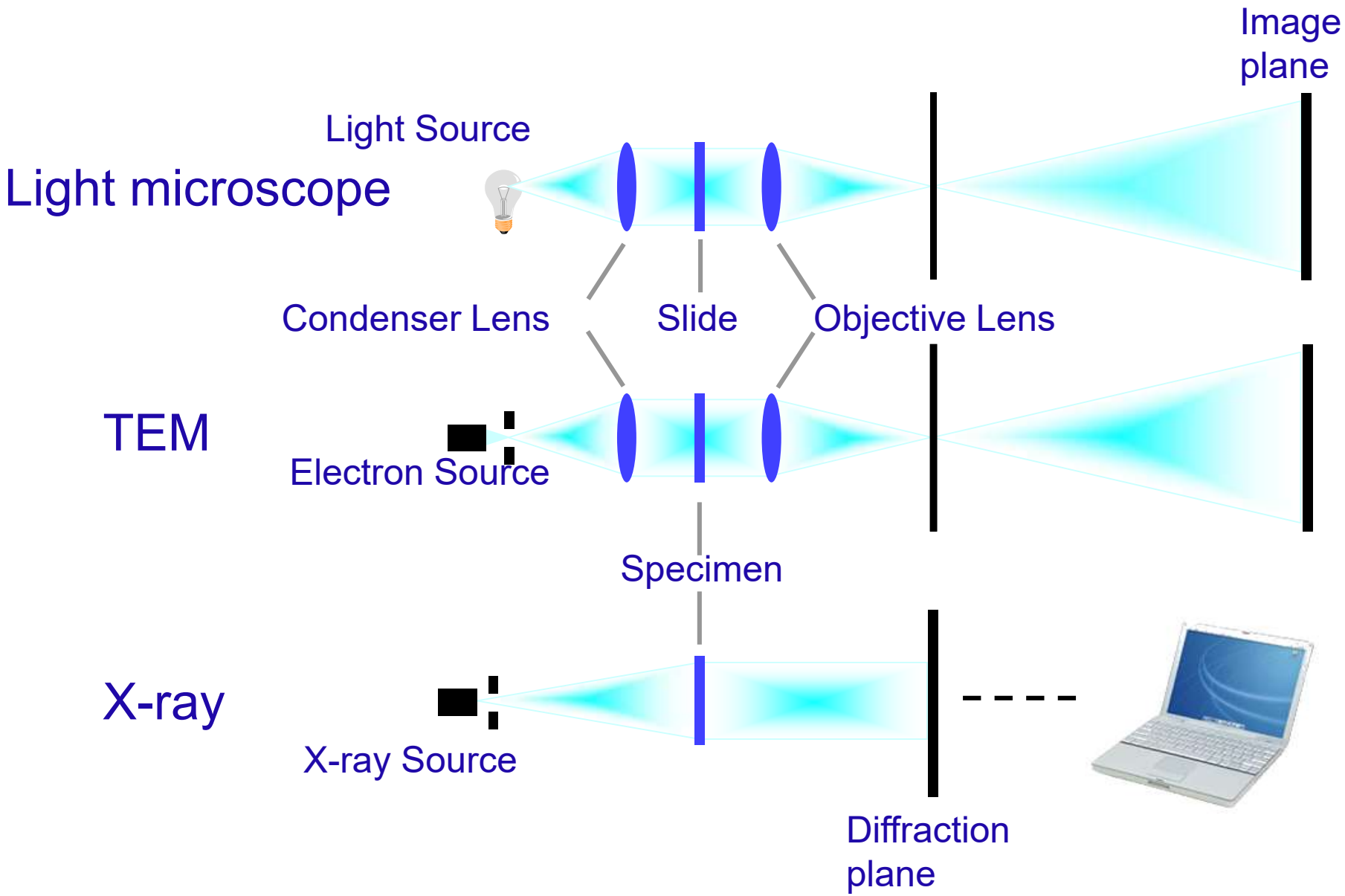
Collecting the data

Equipment

- Microscope
  - FEG
  - Cold trap
  - Cryo stage
- Cryo holder
- Detector

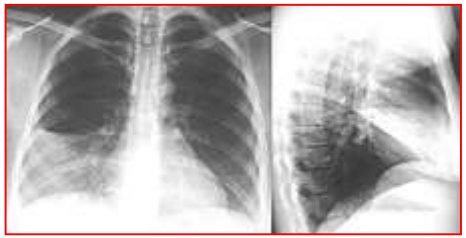


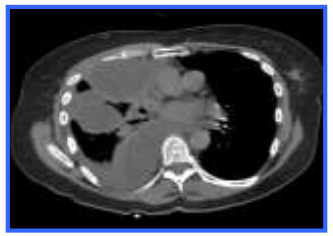
Similar principles



How is the EM image formed?

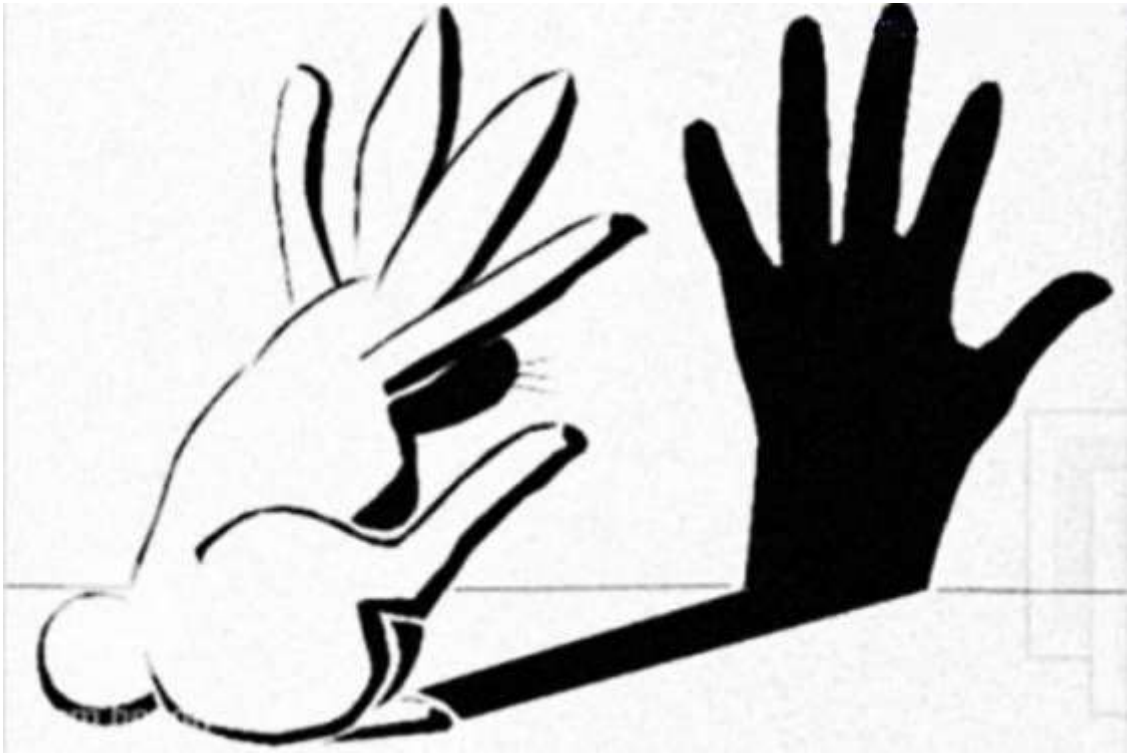
- Thin specimen scatters electrons
- Interference between scattered and unscattered electrons gives phase contrast image
- Image is 2D projection of original 3D object
- 3D structure can be determined from a set of views at different orientations
- Beam damage is the ultimate limit on resolution





TEM images are 2D projections

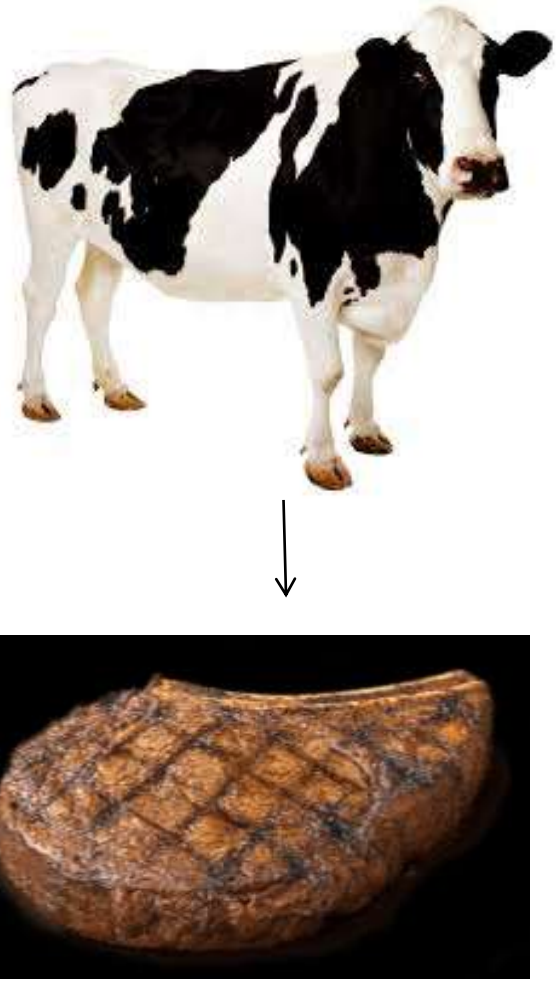
- but 3D information is important!



John V. Muntean

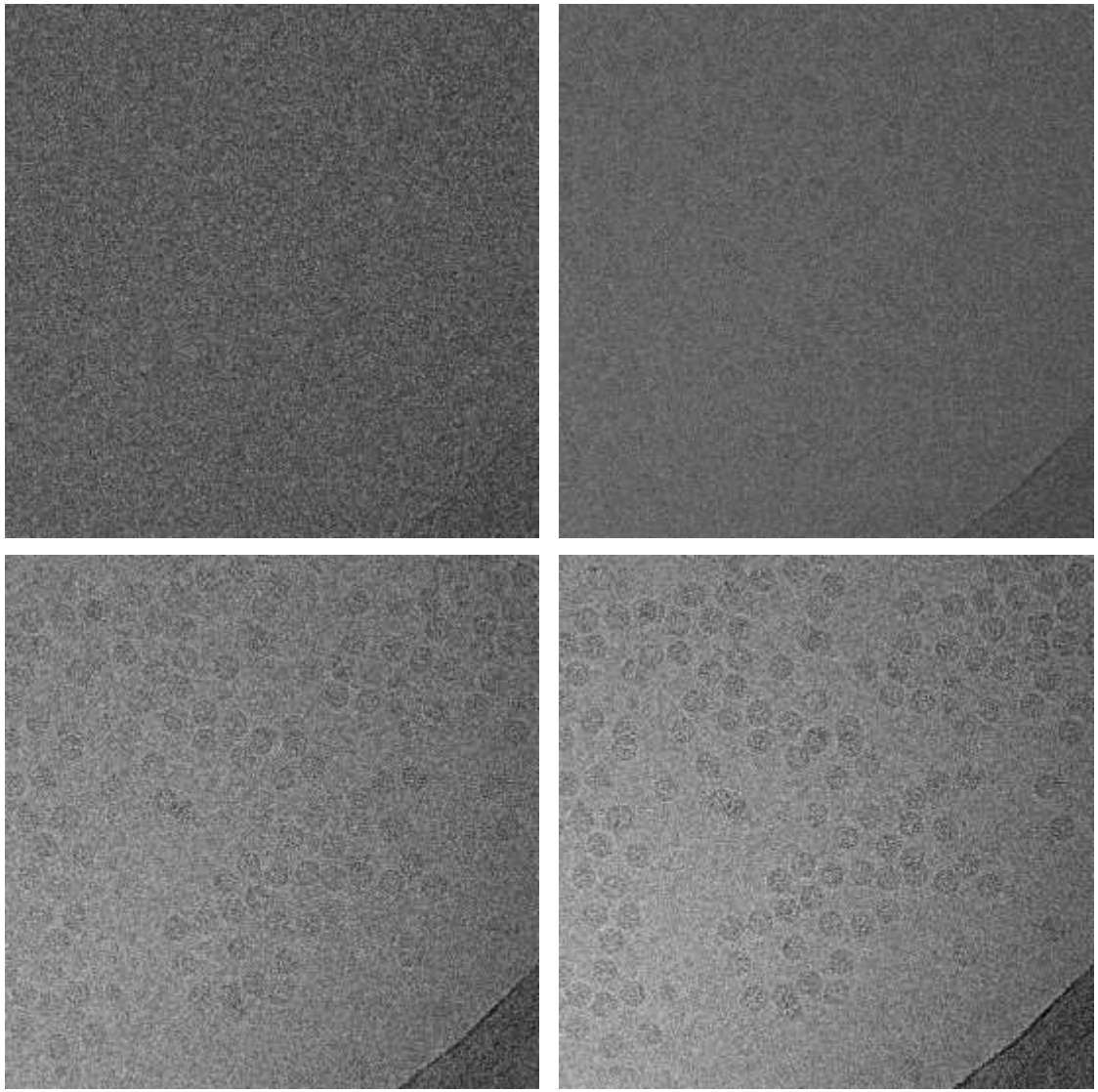
Radiation damage

- Biological material is radiation-sensitive
- Electrons used for imaging are high energy particles that can transfer energy to the specimen and cause radiation damage.
- High resolution information is lost first
- Need to minimise exposure to electron beam prior to imaging (“low dose” technique)
- Electron dose is very limited

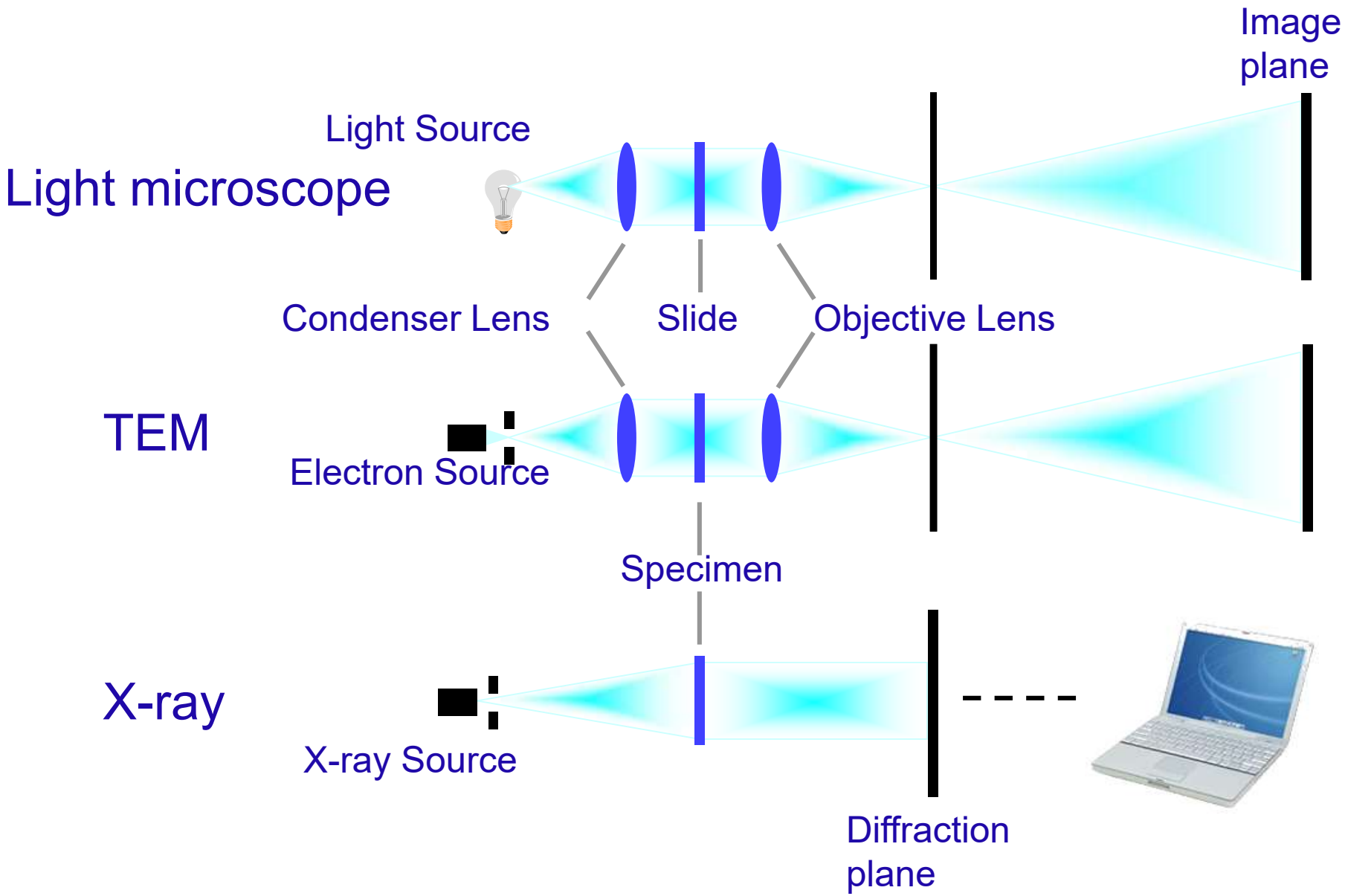


TEM images are noisy

- No electron dose = no signal
- Trade off between enough dose to see the specimen, but not so much you cause significant radiation damage and destroy high-resolution features
- New direct electron detectors have a huge impact



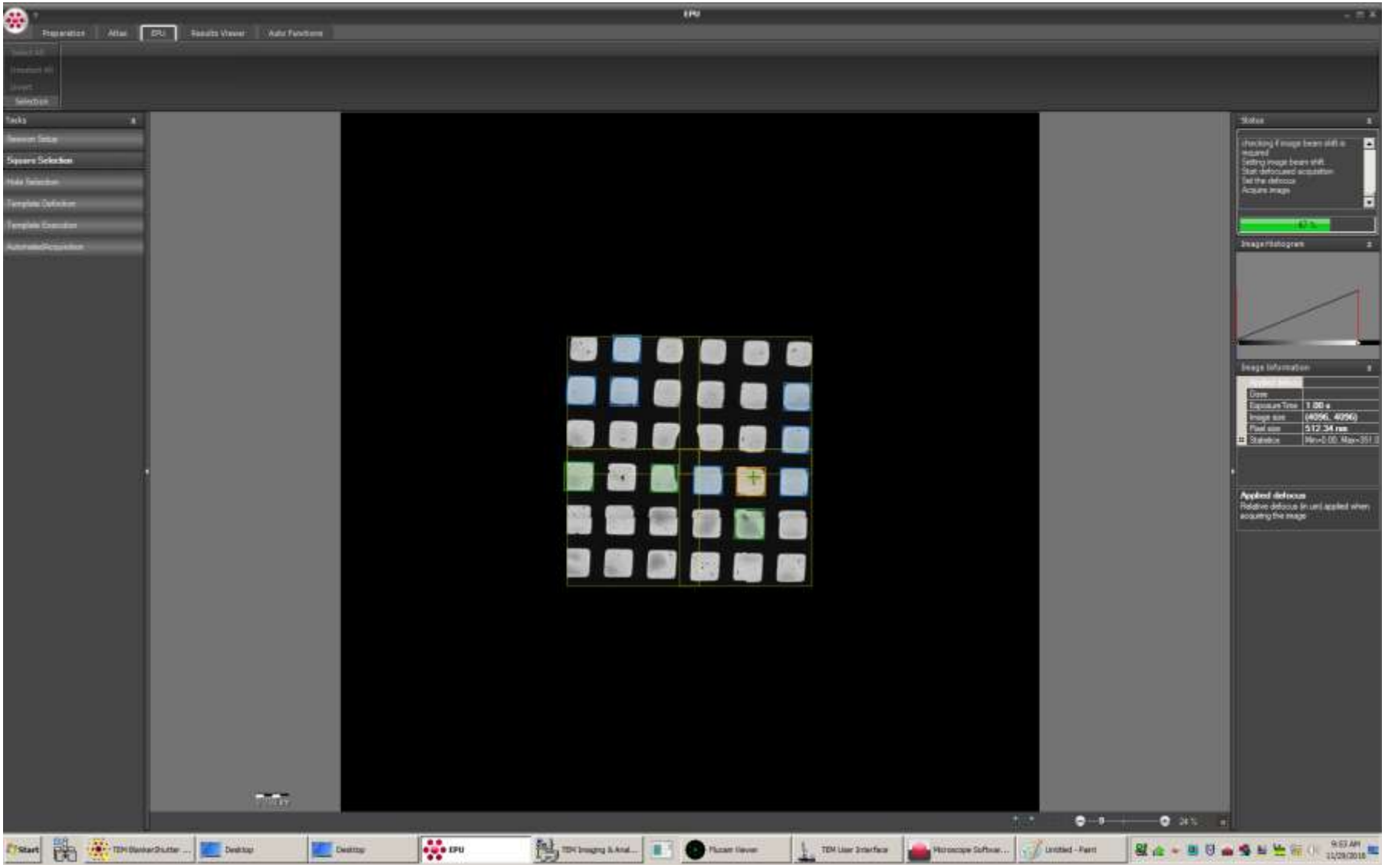
Imaging vs. Diffraction



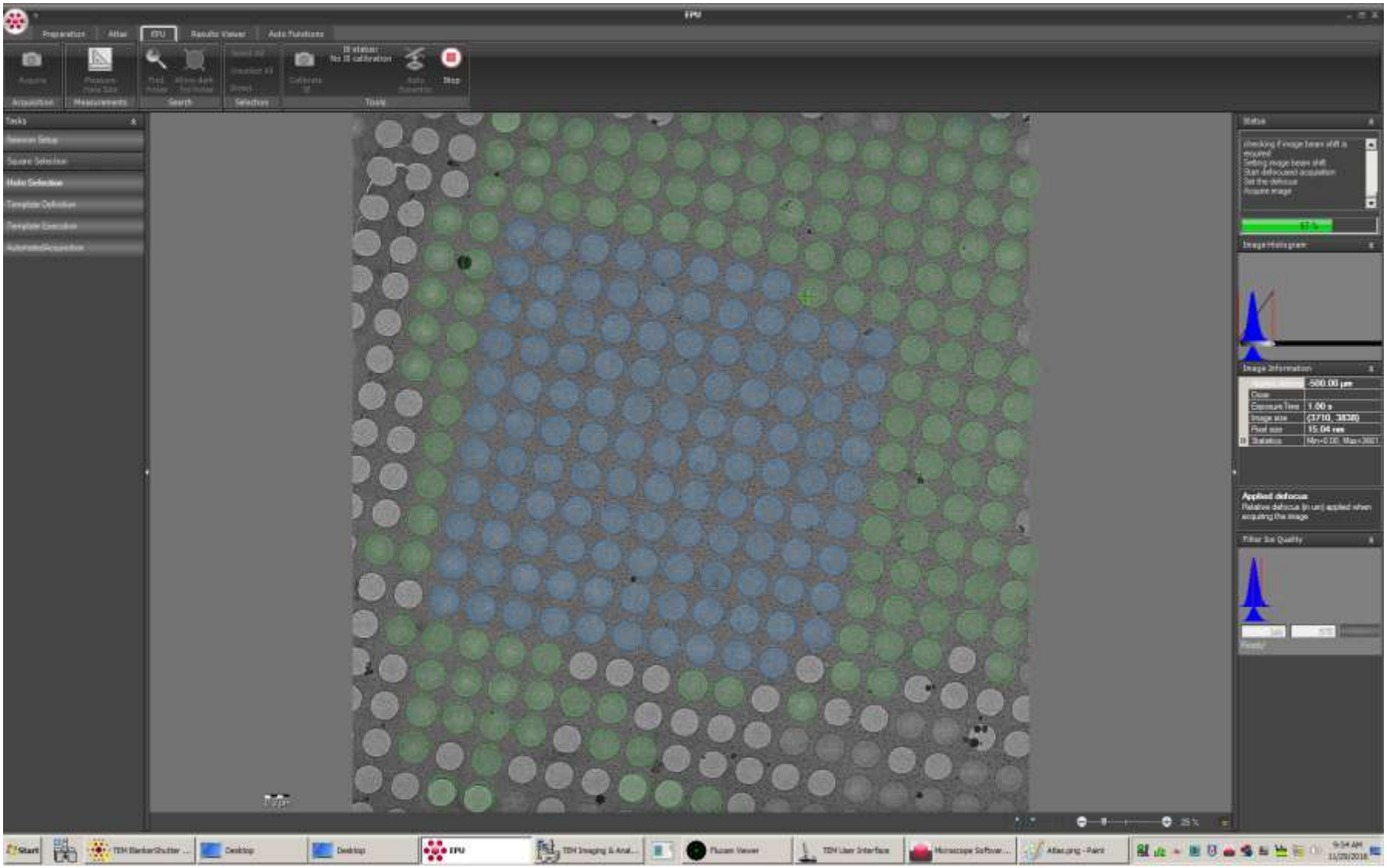
Imaging vs. Diffraction

- In diffraction, we measure intensities with high accuracy
  - Phase problem
- In cryo-EM imaging, we measure phases as well as intensities, but with a lot of noise
- Projection problem and noise problem are both solved in the same way – combining and averaging information from many images
- Need many images – automated data collection is important

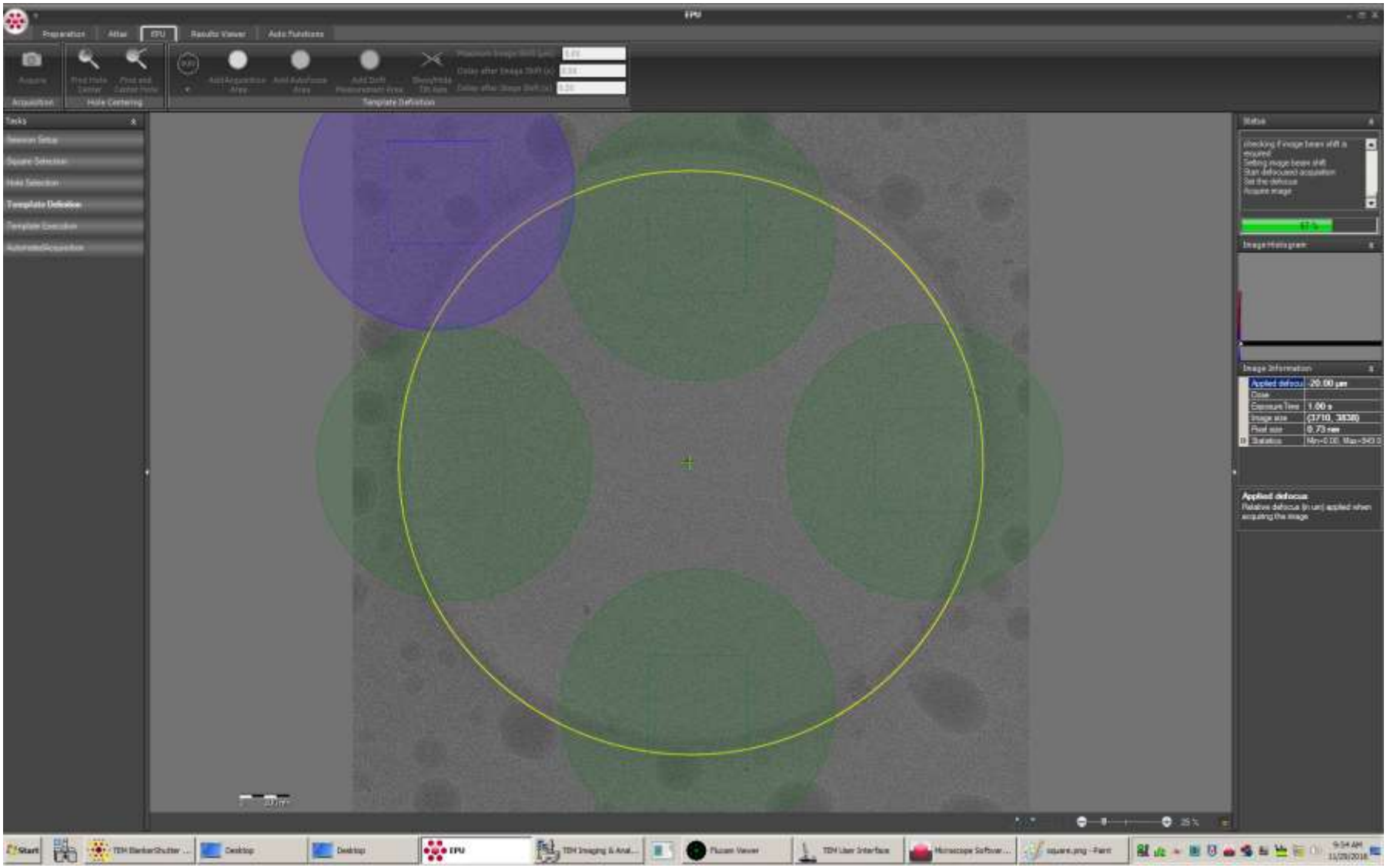
Automated data collection



Automated data collection

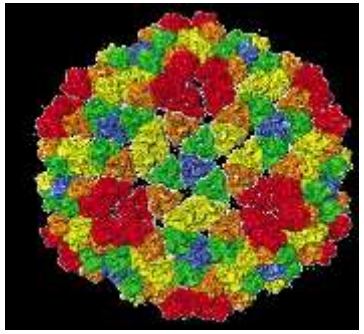


Automated data collection



Data processing

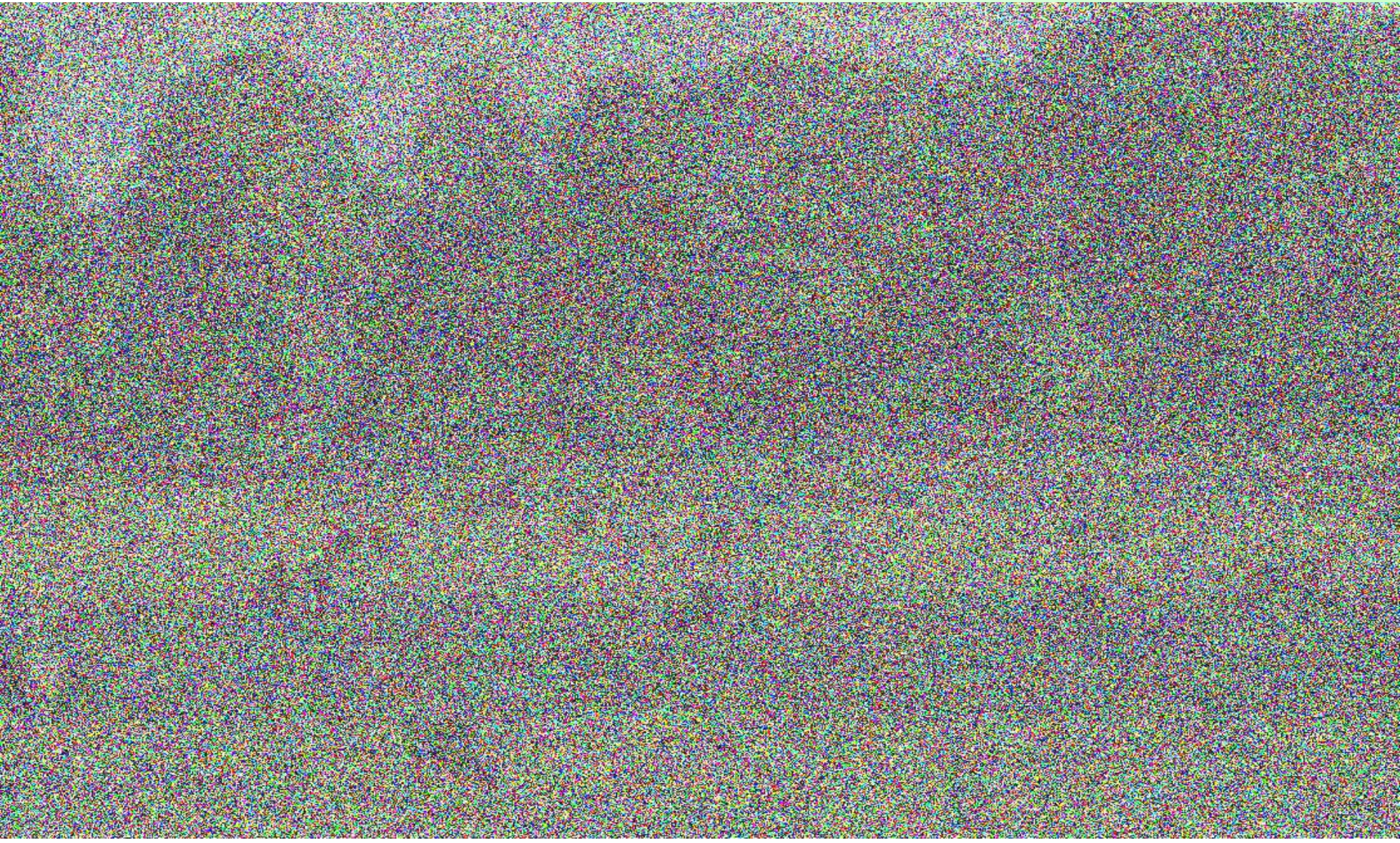
Single particles



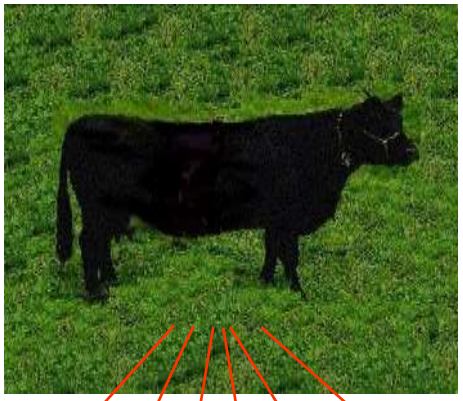
- Isolated macromolecular complexes
- Randomly oriented in solution
- Can be trapped in different reaction states by vitrification
- No crystallization or ordered assembly needed
- The position and orientation of each particle must be determined for 3D reconstruction
- The more particles used, the higher the resolution (<3 Å)
- Mixed states can be separated (“purification in the computer”)
- Ultimate limit to resolution from radiation damage
- Interpretation by atomic structure docking or direct determination of backbone

Single particles

Low signal:noise



raw images



3D starting model

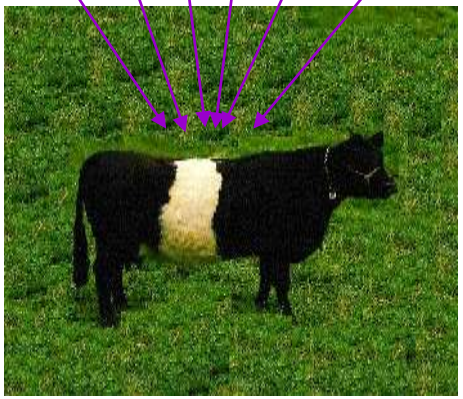
project

2D templates

Align and group into classes

2D class averages

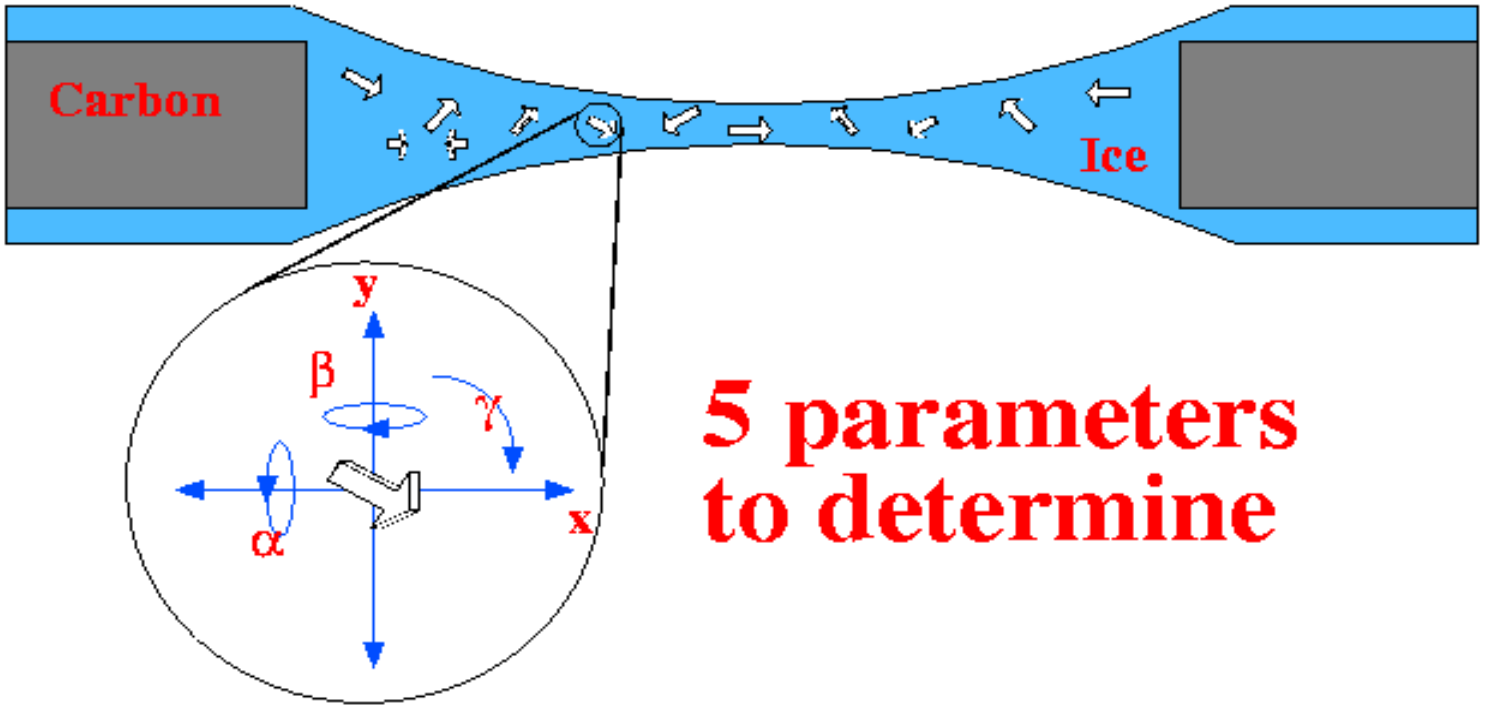
reconstruct



new 3D model

Finding orientations

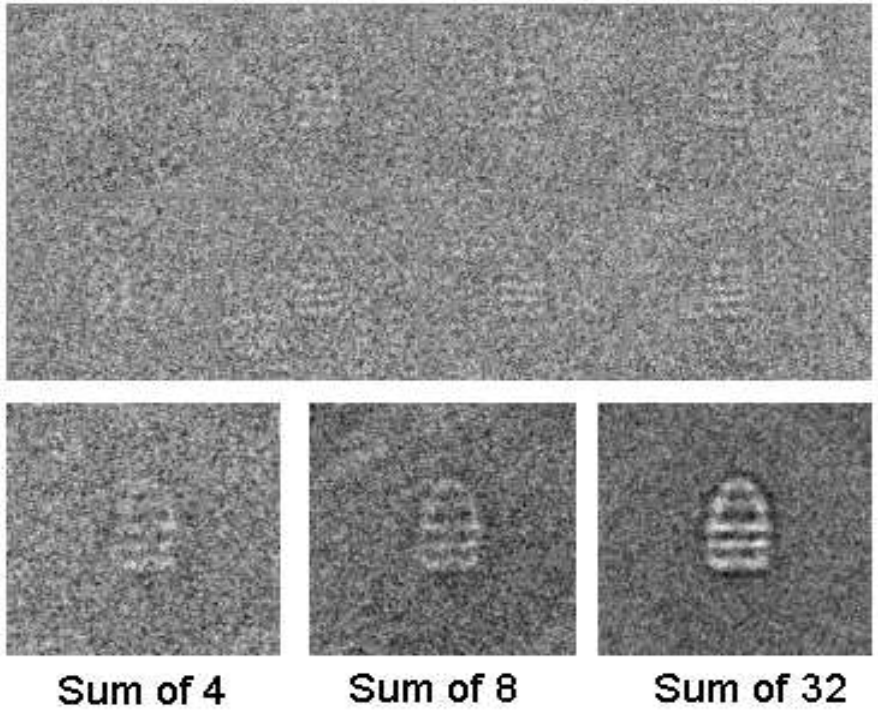
Single Particles in Ice



5 parameters to determine

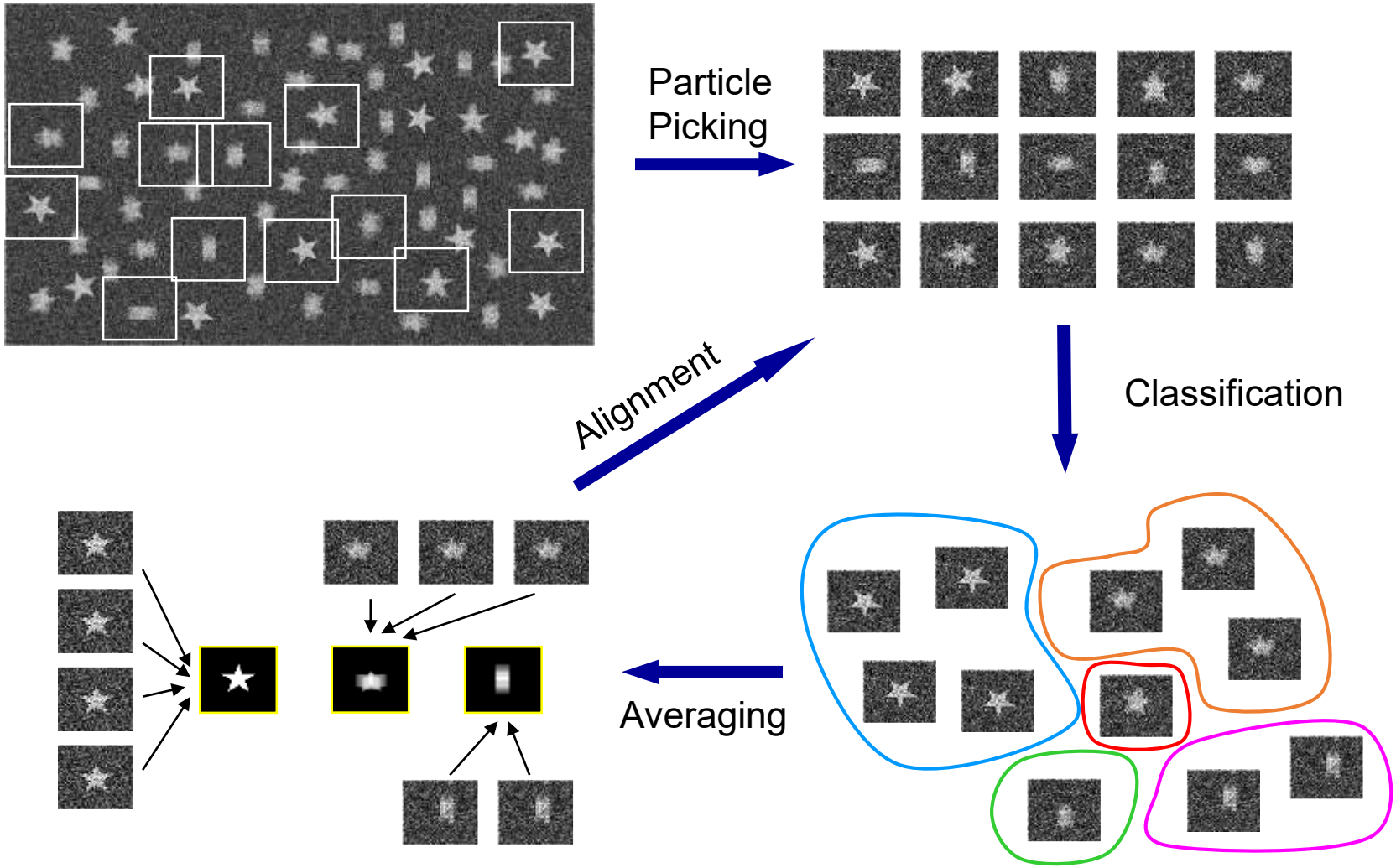
N. Grigorieff,
Brandeis Univ.

Averaging similar views improves the signal:noise ratio



Individual raw images

Single Particle Image Processing



3D reconstruction from projections

- Theory now 100 years old

SITZUNG VOM 30. APRIL 1917.

Über die Bestimmung von Funktionen durch ihre Integralwerte längs gewisser Mannigfaltigkeiten.

Von

JOHANN RADON.

"On the determination of functions from their integral values along certain manifolds"

- Rediscovery and practical application 50 years later

130 NATURE, VOL. 217, JANUARY 13, 1968

Reconstruction of Three Dimensional Structures from Electron Micrographs

by

D. J. DE ROSIER
A. KLUG

MRC Laboratory of Molecular Biology, Hills Road, Cambridge

General principles are formulated for the objective reconstruction of a three dimensional object from a set of electron microscope images. These principles are applied to the calculation of a three dimensional density map of the tail of bacteriophage T4.

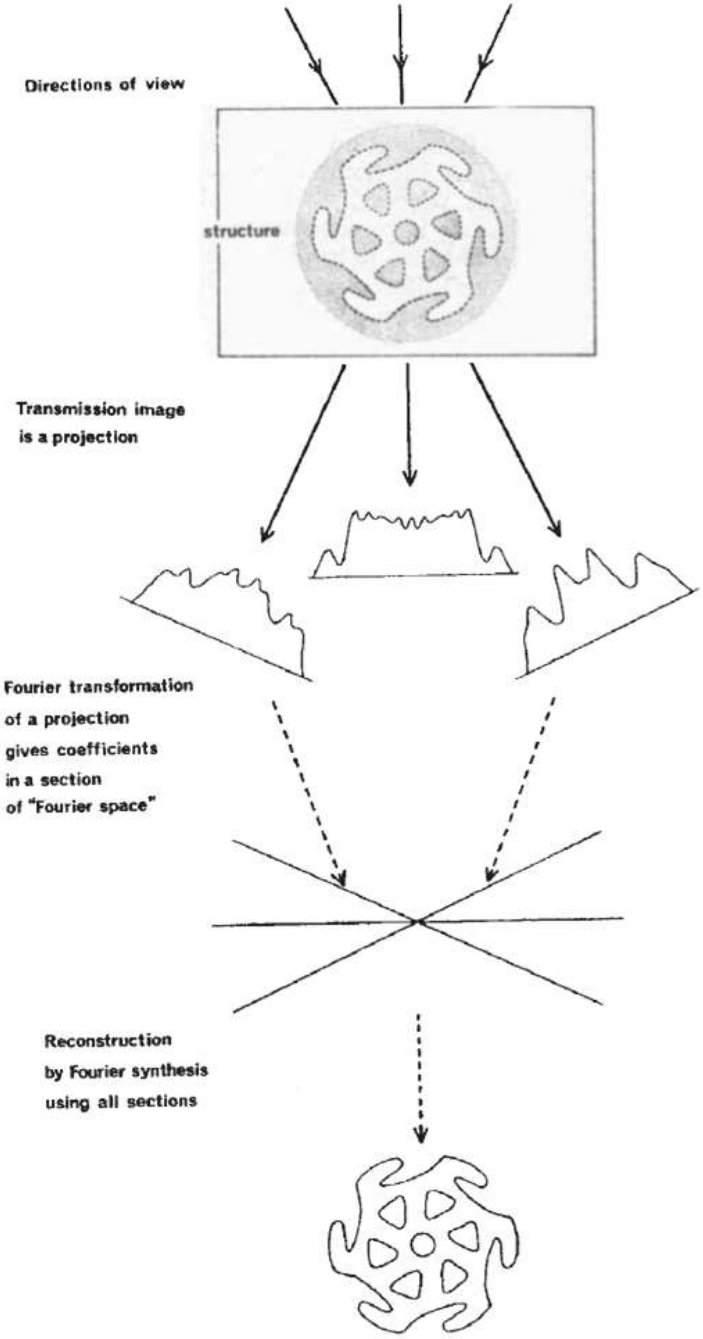
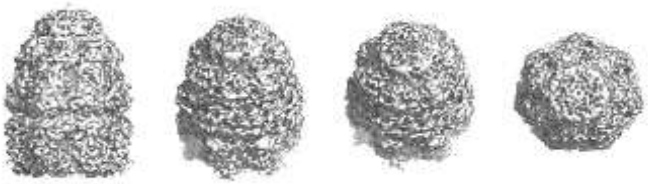
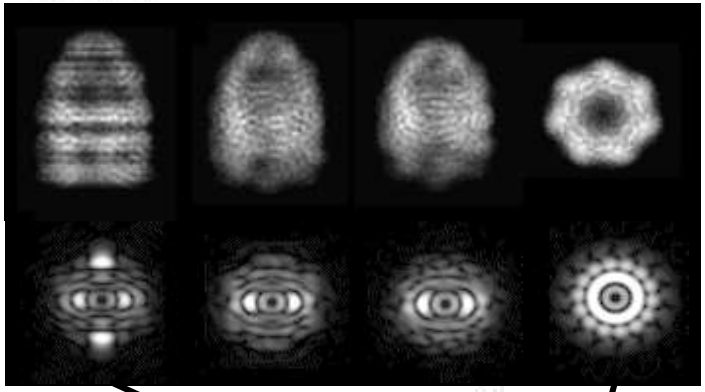


Fig. 6. Scheme for the general process of reconstruction of a structure from its transmission images.

3D reconstruction from 2D projections

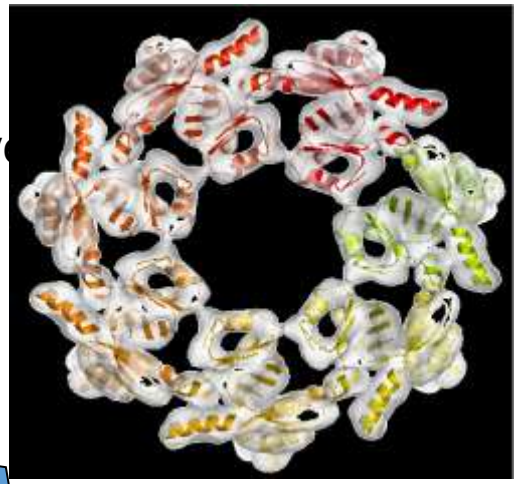


Molecular orientations



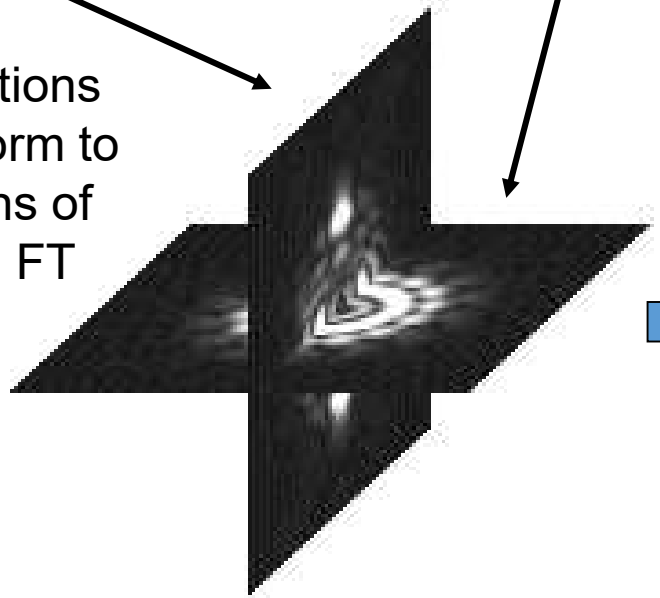
2D projections (observe images, without noise)

Calculated transforms



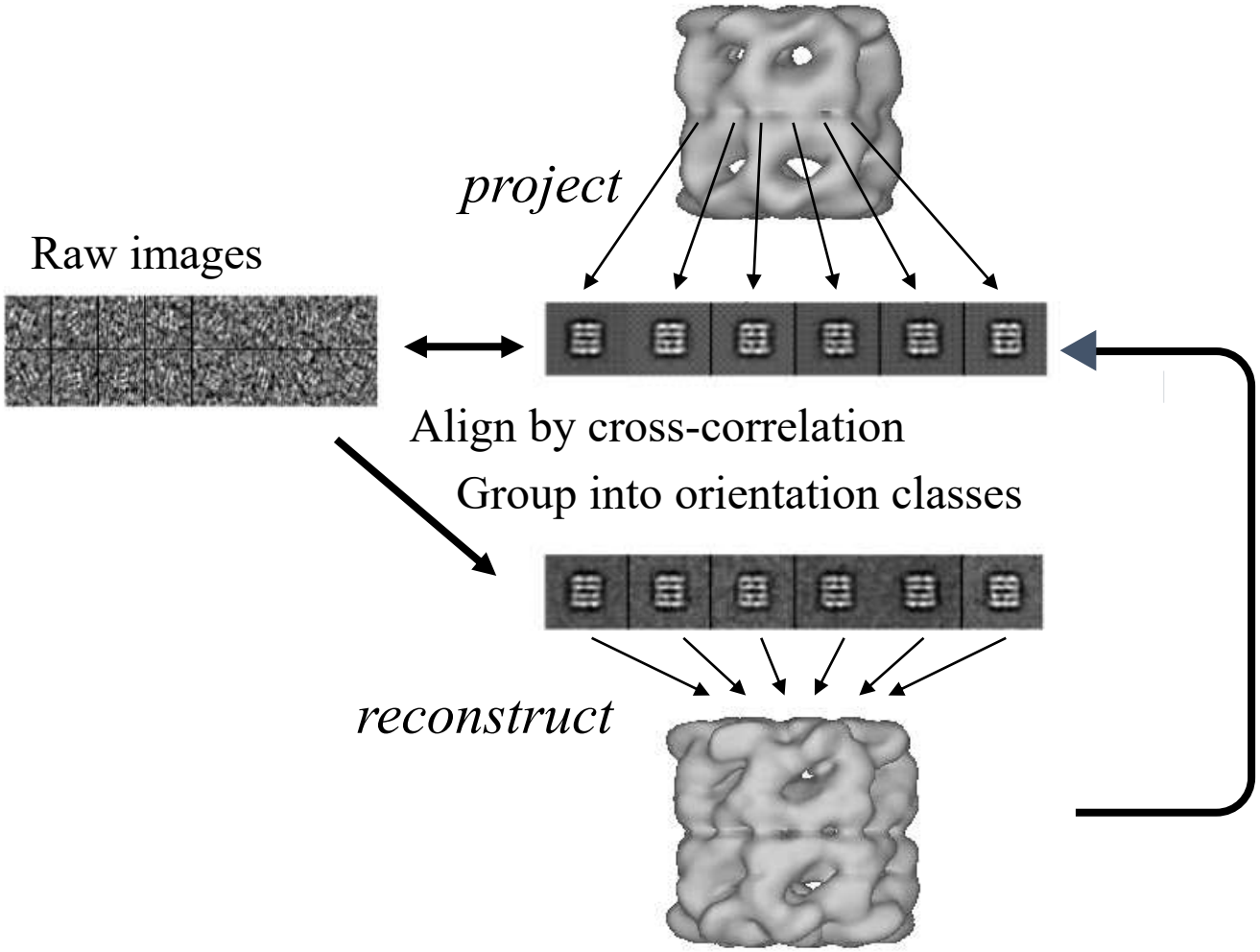
Section through map with fitted atomic structure

Projections transform to sections of the 3D FT

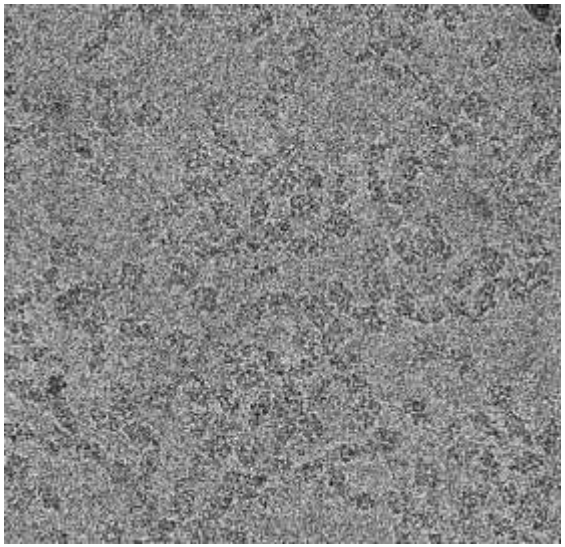


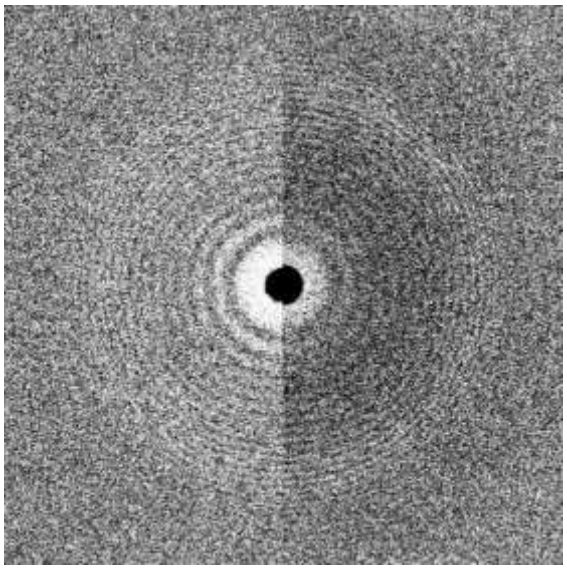
Inverse Fourier transformation gives the 3D density map

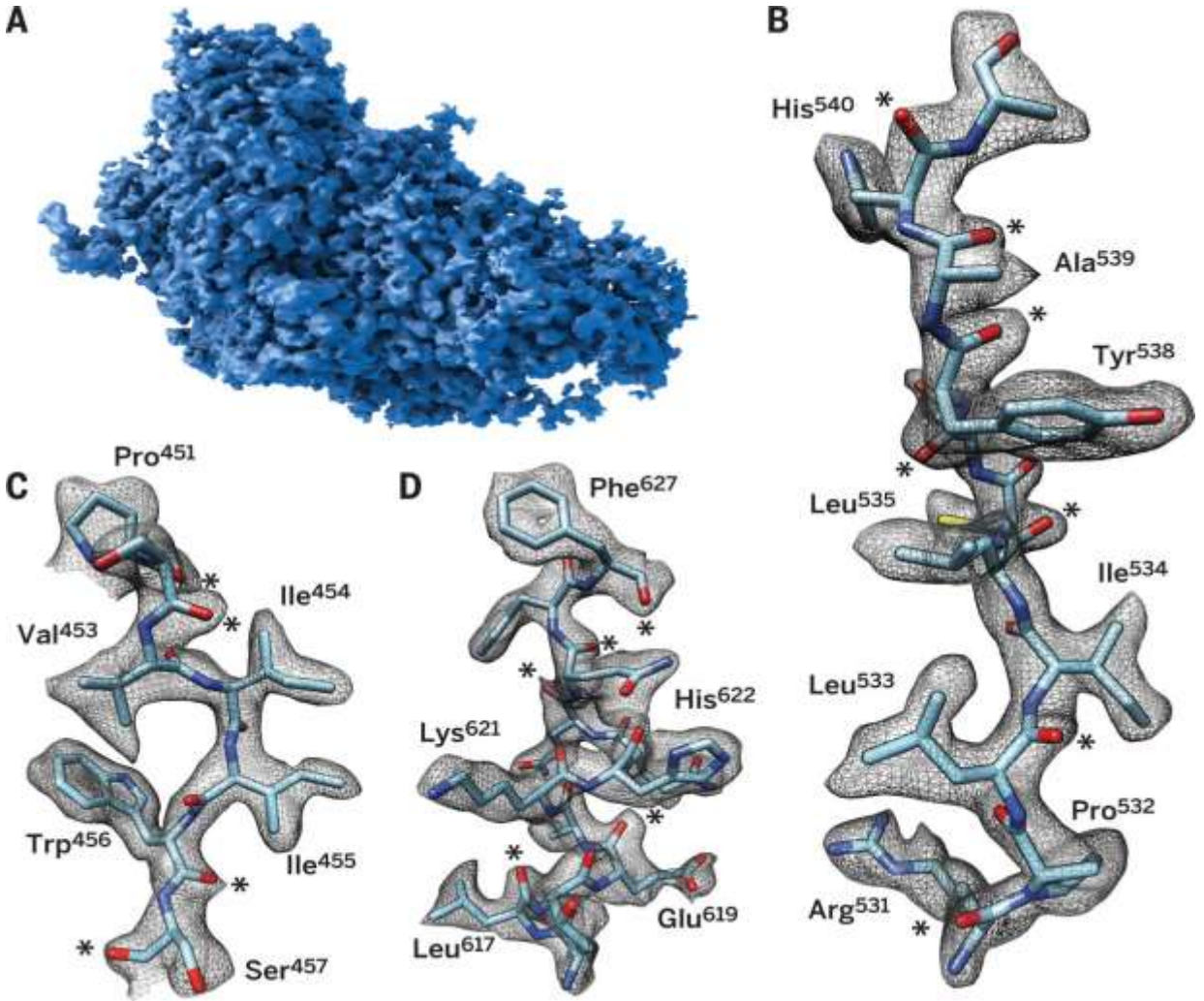
Projection matching/ Angular refinement



Cryo-EM: the resolution revolution

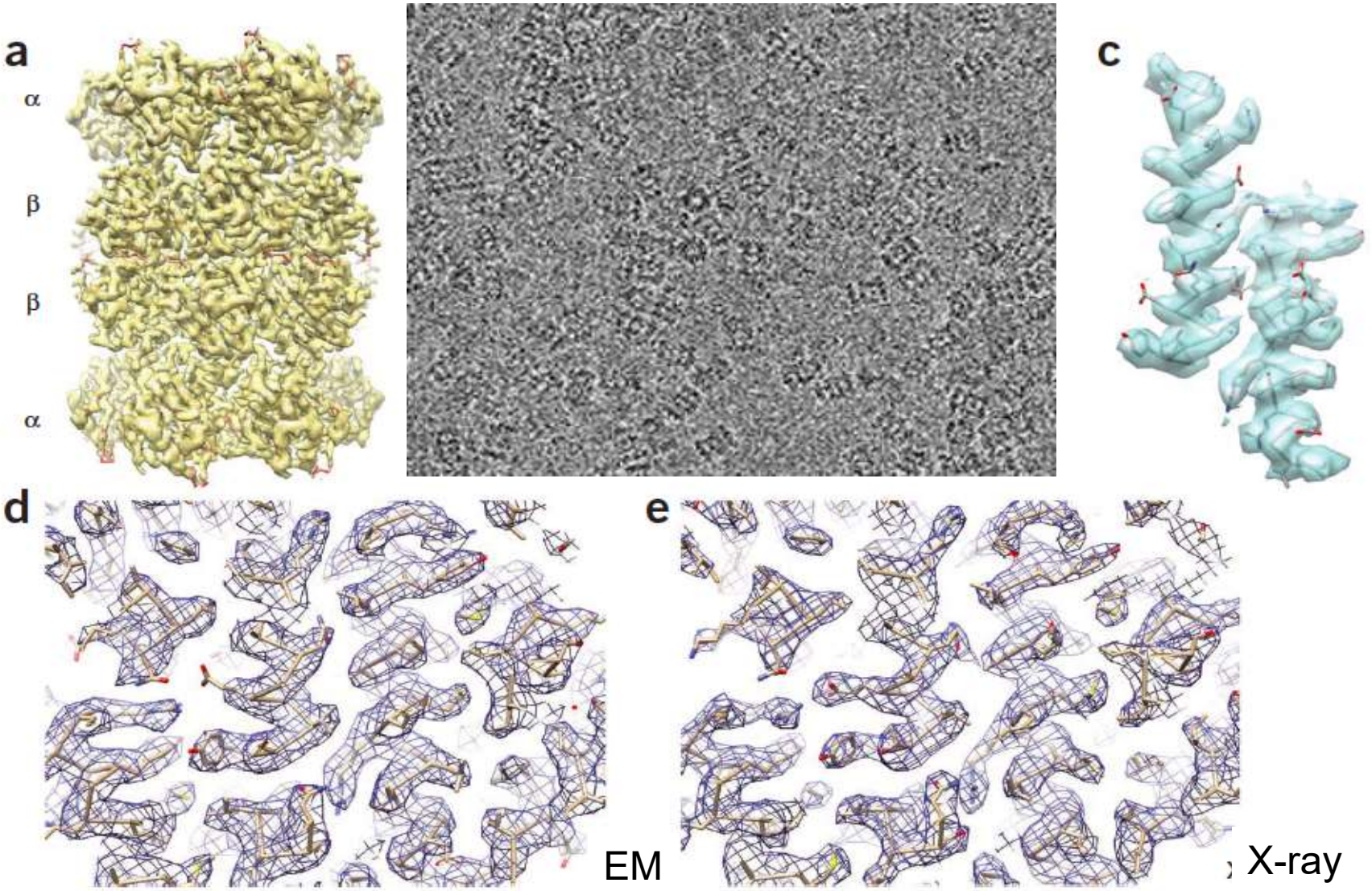






Bartesaghi et al (2015) Science 348, 1147

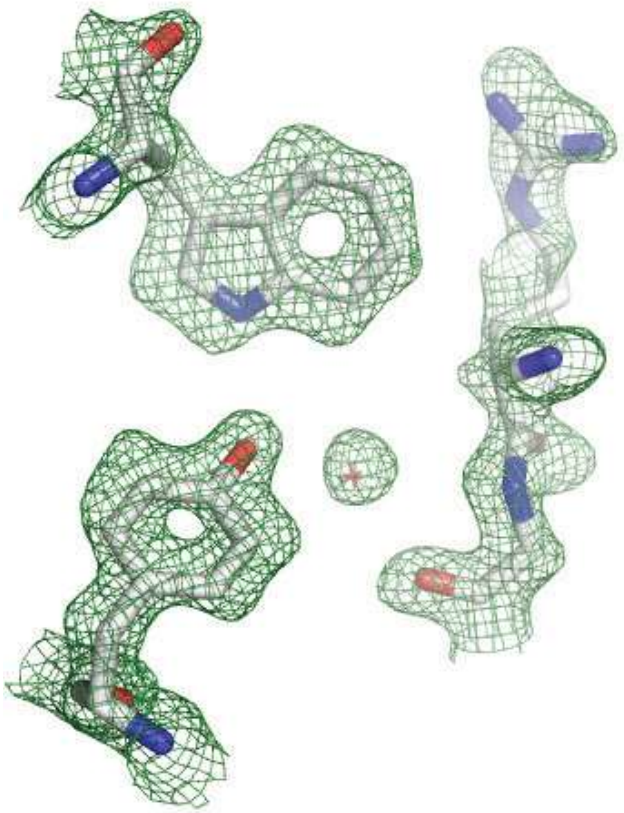
Single particle cryo EM vs X-ray: The proteasome at 3.3 Å



D7 symmetry

Li, Cheng et al, Nature Meth. 2013

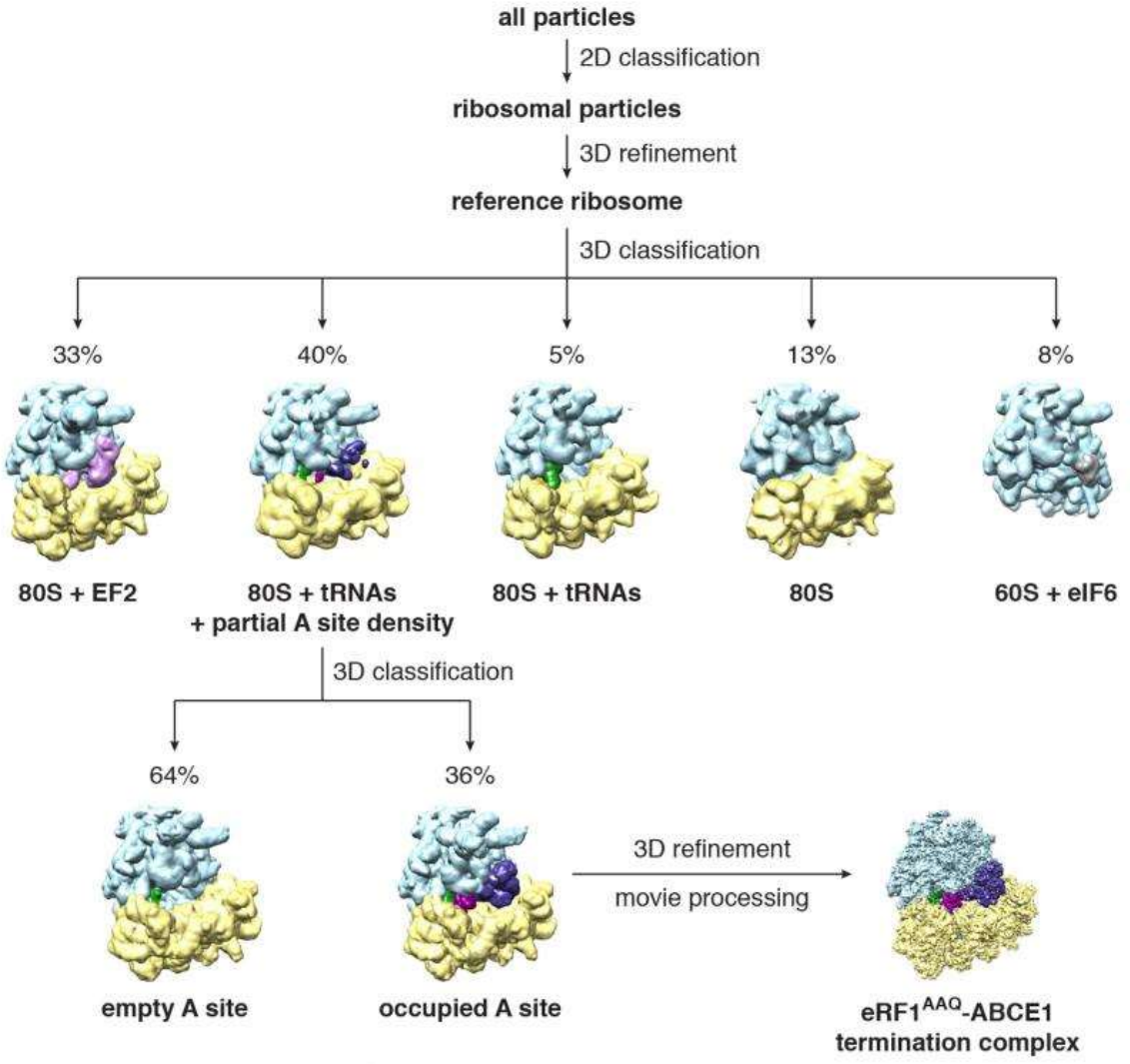
Cryo-EM: pushing the resolution limit



Apoferritin at 1.65 Å

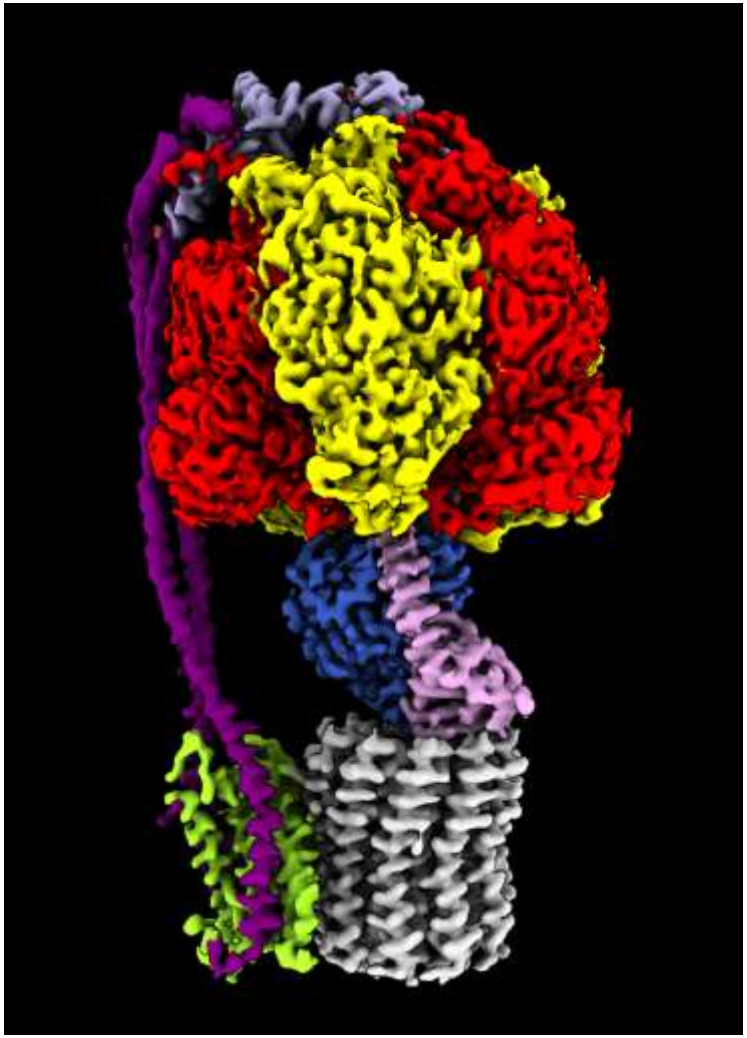
Zivanov et al., 2018. eLife 2018;7:e42166

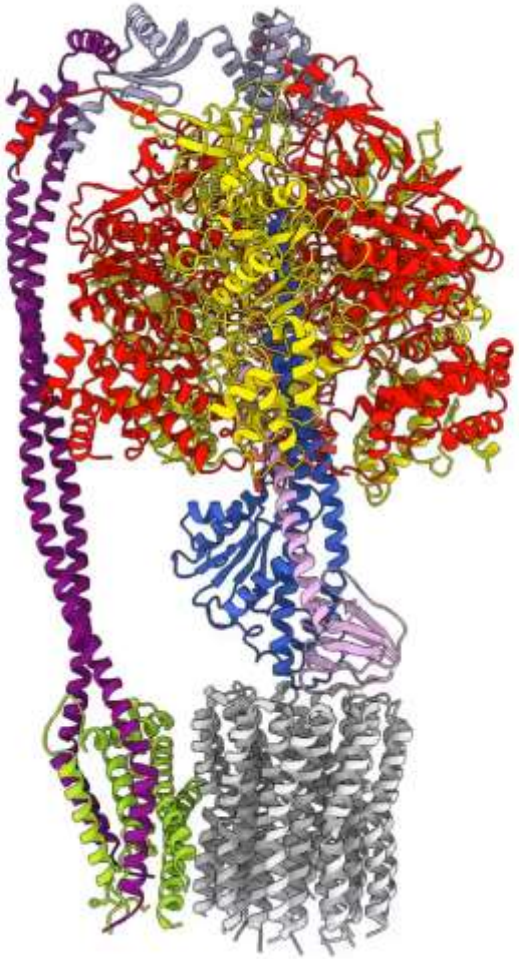
3D classification



Brown et al., (2018) Nature 524:493-496

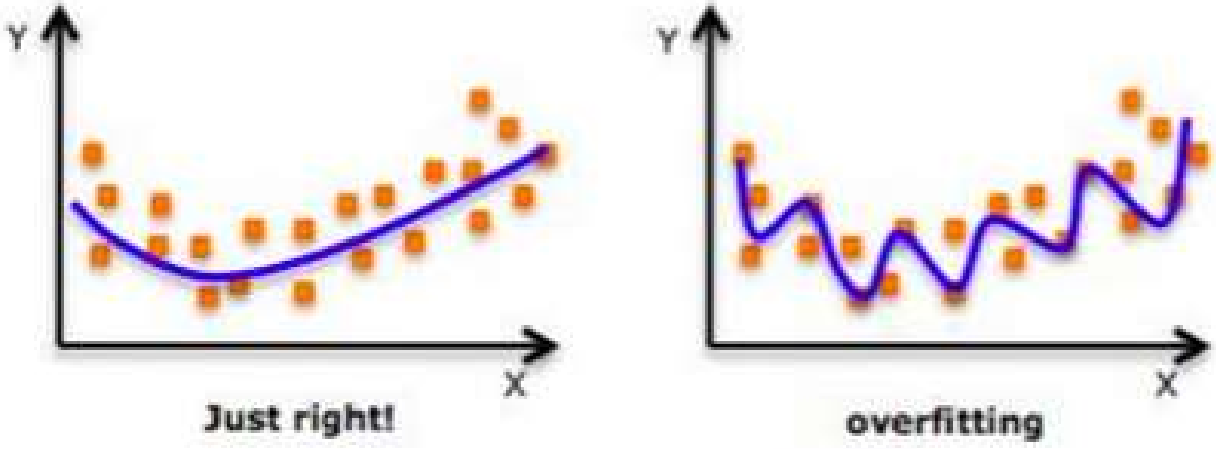
Dynamics from classification of conformational states





Guo, Suzuki & Rubinstein https://www.biorxiv.org/content/early/2018/11/06/463968

Validation in single particle reconstruction

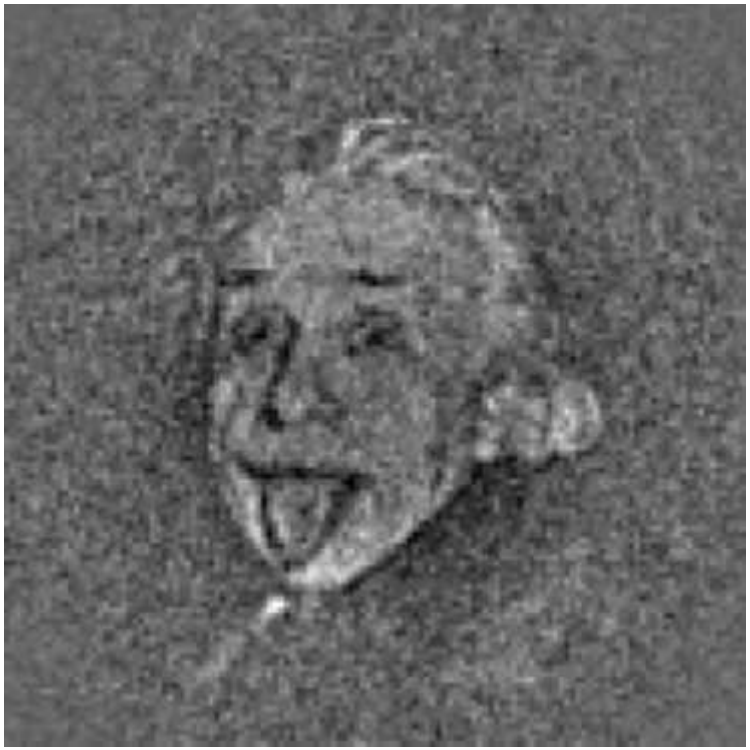


Three main ways overfitting can happen in single-particle analysis:

- Particle selection
- Particle alignment, orientation and masking
- Model fitting and building

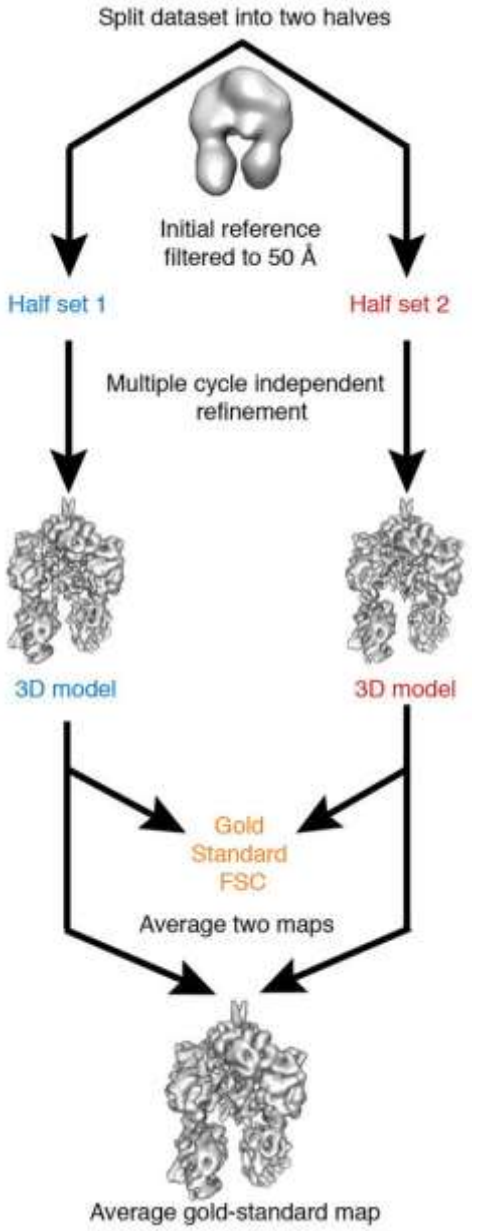
Overfitting in cryo-EM

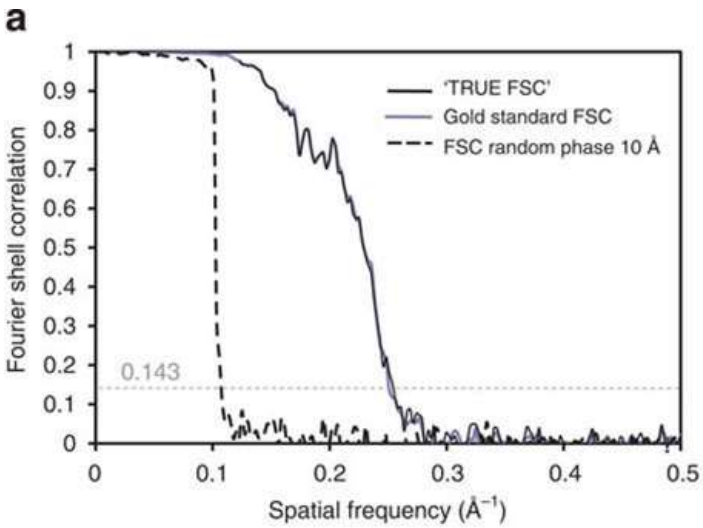
- Famous “Einstein from noise” phenomenon

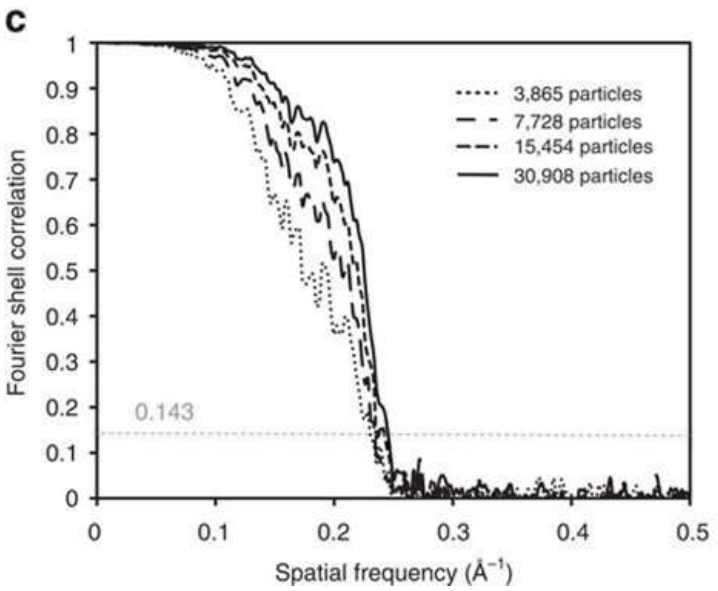


Avoiding overfitting

- Avoid influence of features from reference structure (low pass filtering or start with a simple shape, e.g. a sphere)
- Keep two half data sets independent
  - Similar idea as R_{free}
- Assess quality with Fourier Shell Correlation







Model validation: room for improvement

High-Resolution Cryo-EM Maps and Models: A Crystallographer's Perspective

Alexander Wlodawer,[1,4,*] Mi Li,[1,2] and Zbigniew Dauter[3]

Another observation common to almost all the deposited models based on high-resolution maps is that they seem to lack the final quality control. The presence of very doubtful multiple conformations of the side chains, poor geometry of the model in comparatively clear regions of the maps, location of the side chains outside of the clear density, or the occurrence of interatomic clashes may indicate the difficulty of manual inspection of these very large structures....

Nevertheless, more attention needs to be paid to such problems that are not easily solved by purely automated means.

- Take care with PDB models from cryo-EM – check any important details yourself!

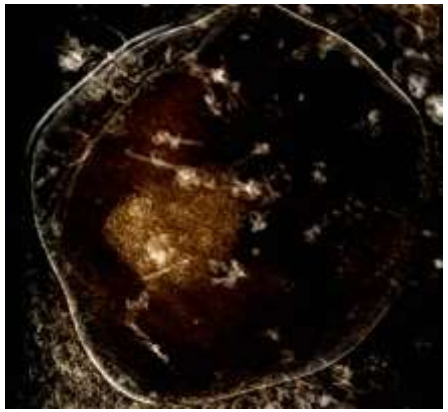
Wlodawer, Li & Dauter (2017) Structure 25:1589-1597

Other cryo-EM techniques: tomography

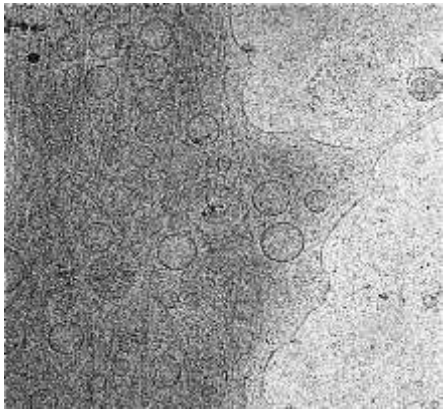
Structural biology from cells to molecules

Increasing biological complexity and integrity

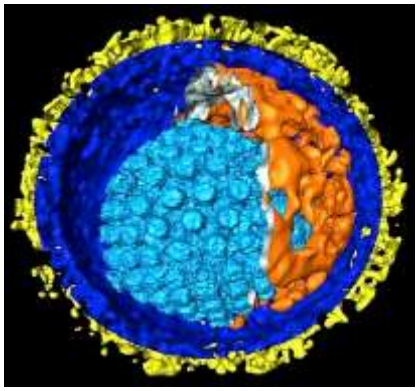
Fluorescence microscopy



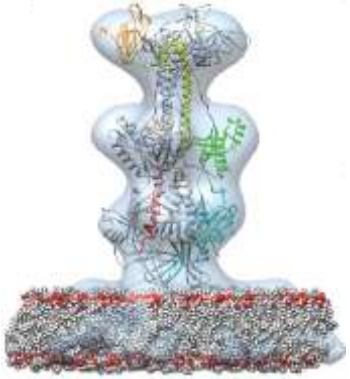
X-ray microscopy



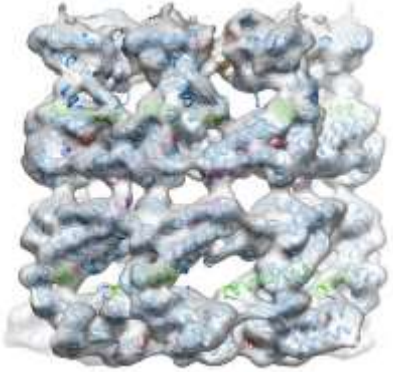
Cellular cryo-electron tomography

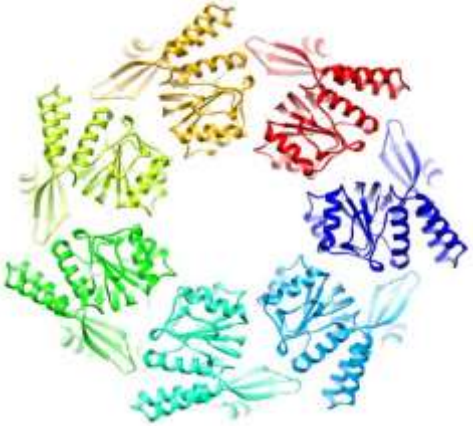


Cryo-electron tomography



Sub-tomogram averaging





Single particle cryo-EM and X-ray crystallography

Increasing resolution

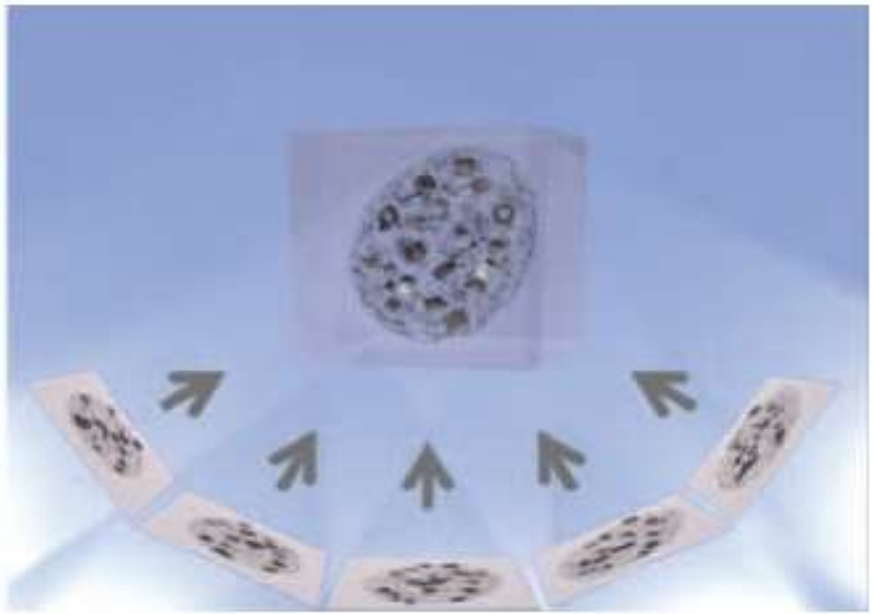
Principle of Electron Tomography

3D-object => set of 2D-projections

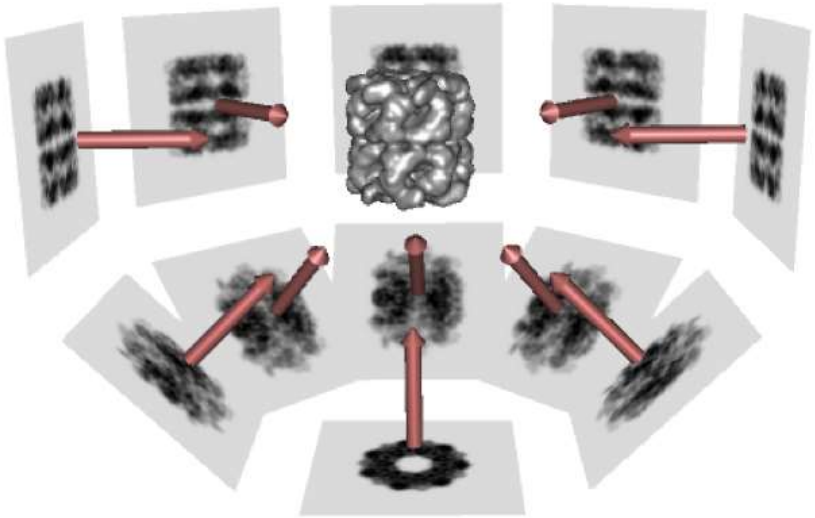
2D-projections => 3D-reconstruction

W Baumeister, MPI Martinsried

Same reconstruction process in tomography and single-particle cryo-EM

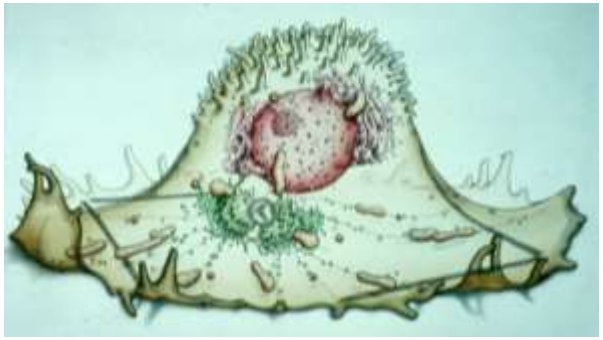


Tomography



Single particle analysis

Reconstruction of whole cells or organelles by tomography



Small pieces of tissue or thin, whole cells can be vitrified

Cell regions up to 0.5-1 μm thick can be examined

Many exposures of the same area - tilt series - because unique object

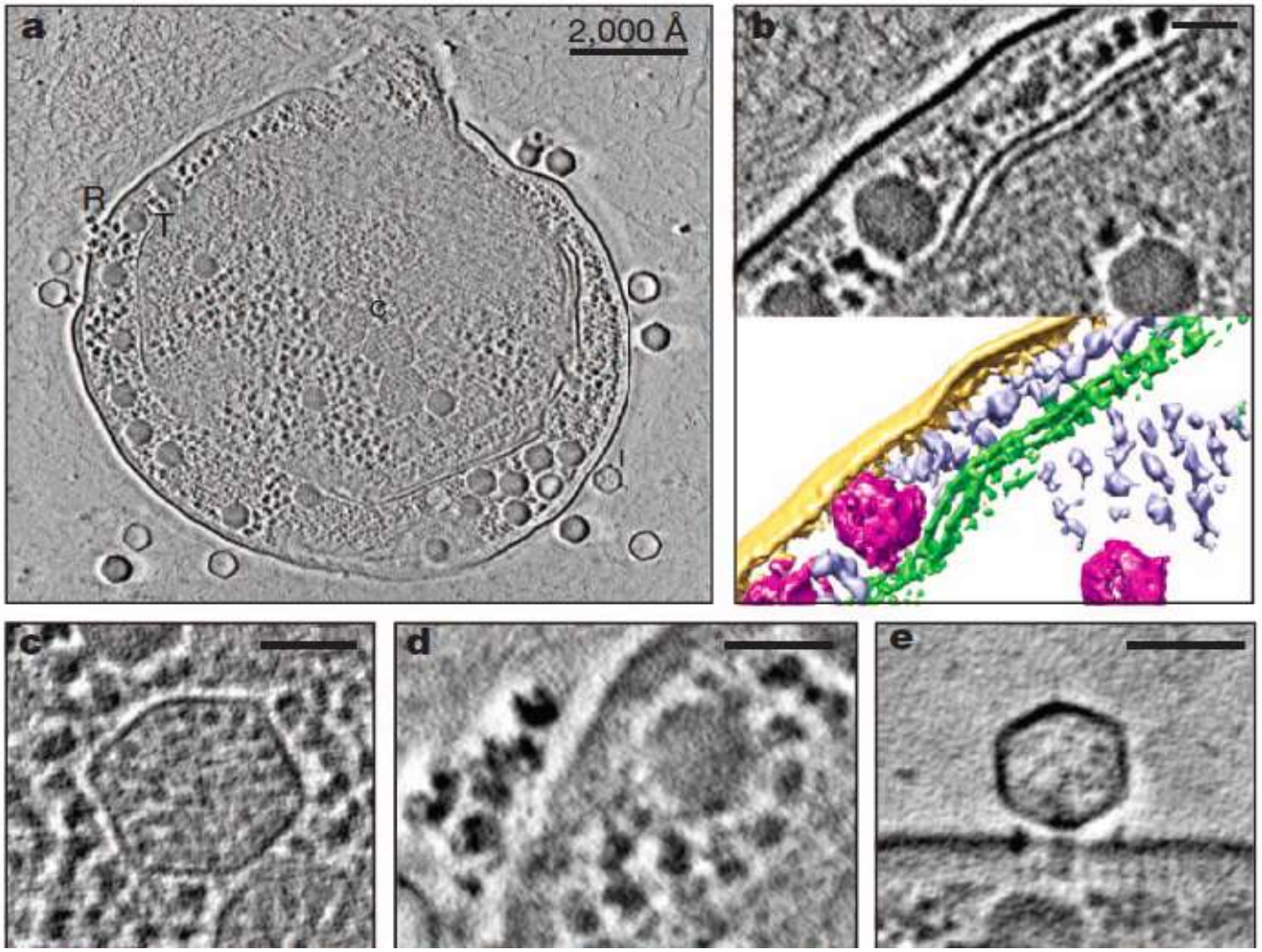
Resolution 1-3 nm - main limit is radiation damage

Sub tomogram averaging can now go to ~3 Å resolution

Limitation on vertical resolution because maximum tilt ~70° - missing views from 70-90°, can be filled in by averaging

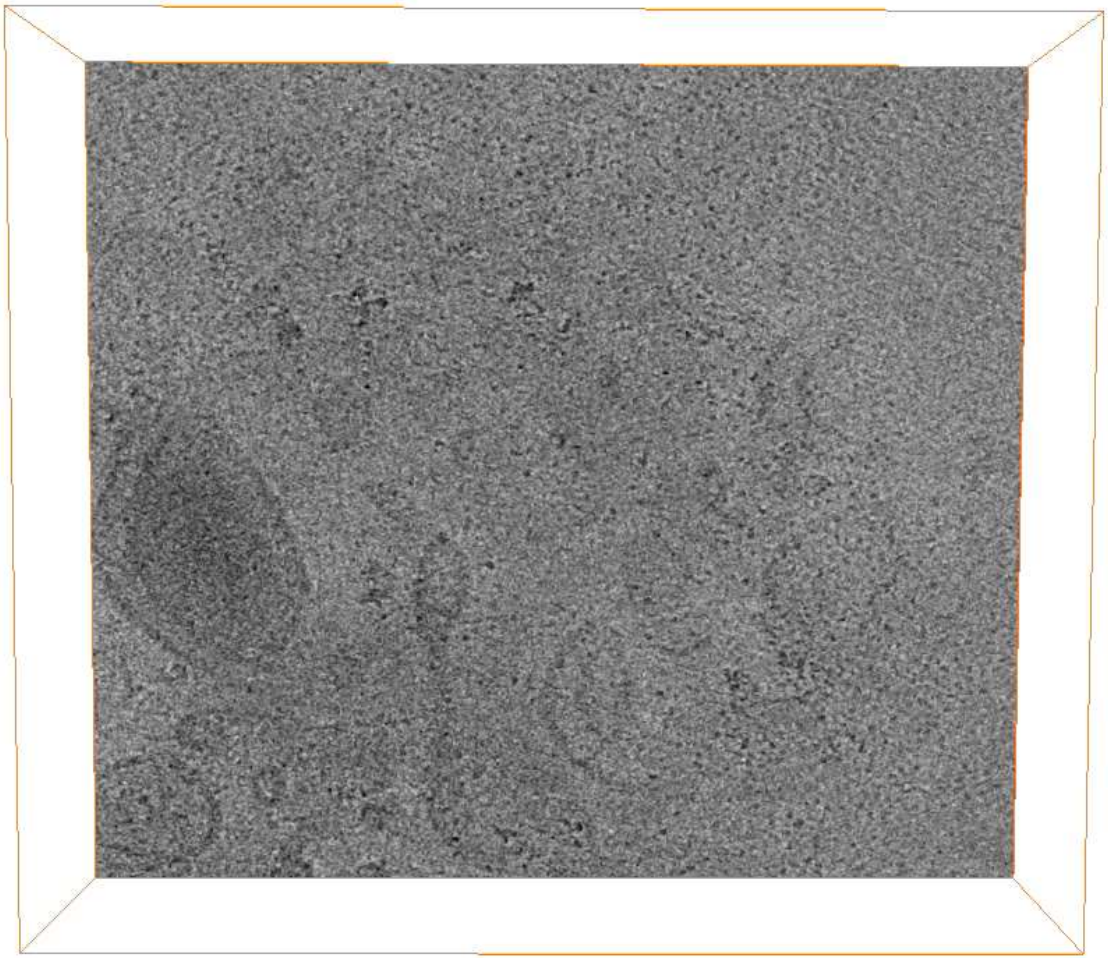
3D reconstruction by back projection

Cryo-tomography example



Dai, Chiu et al, Nature 2013

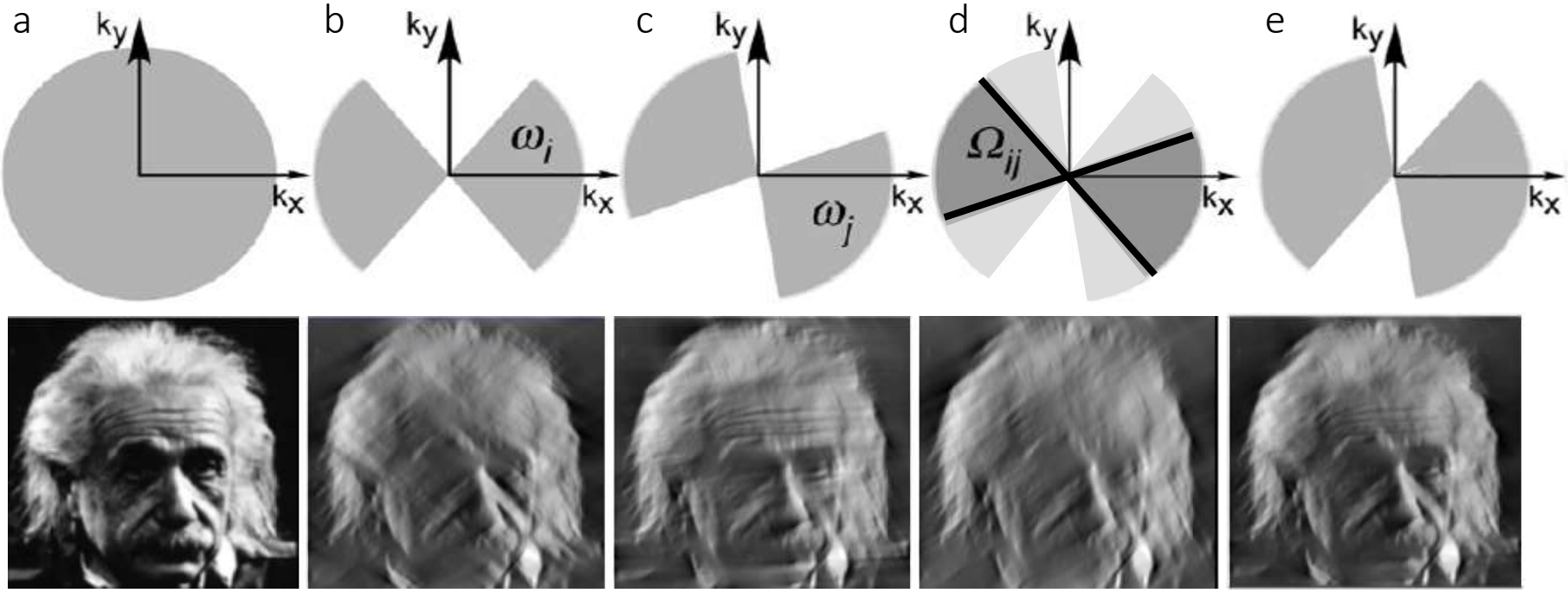
Tomogram segmentation



Amira

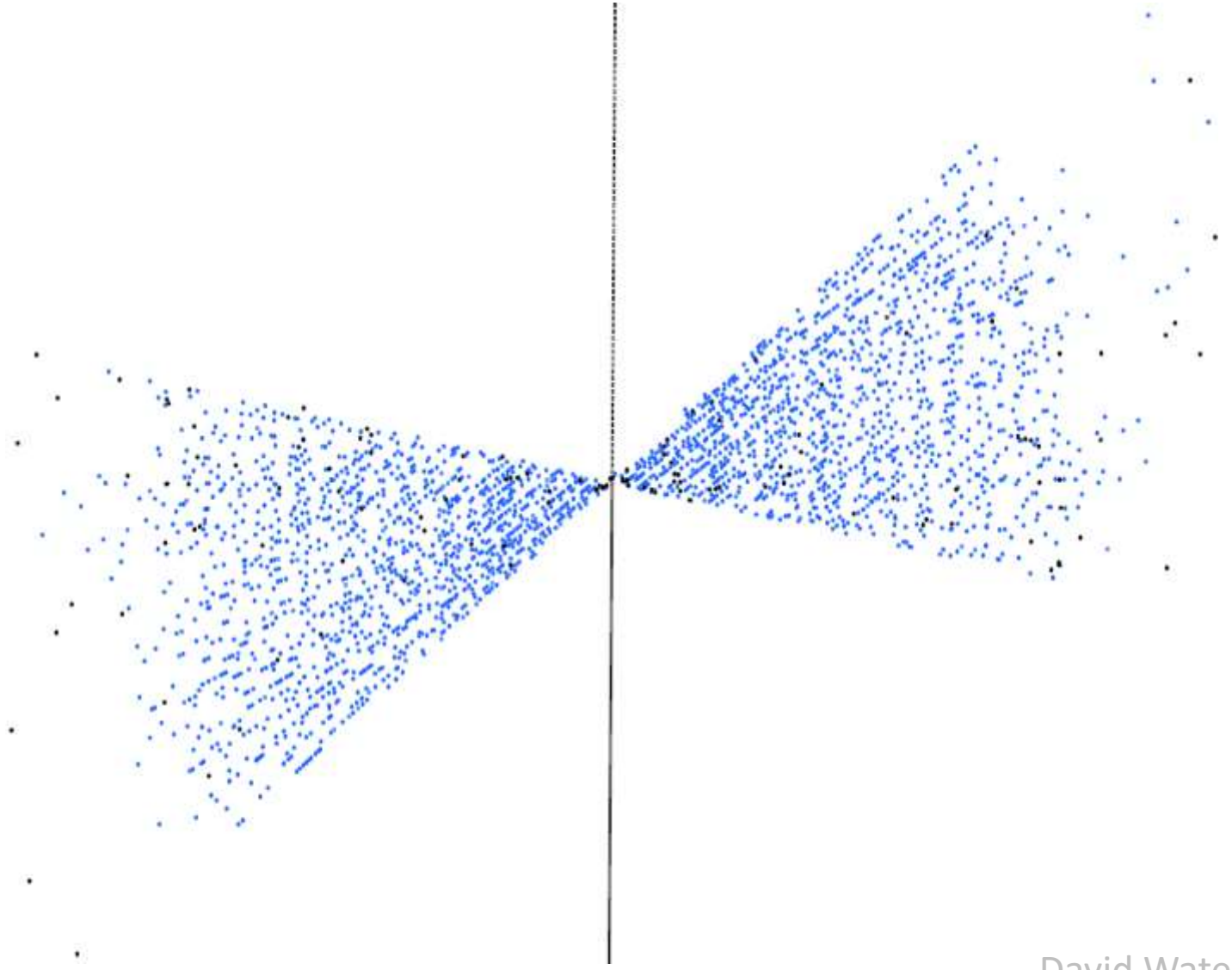
The missing wedge problem:

Pairwise cross correlation must use only common regions of data

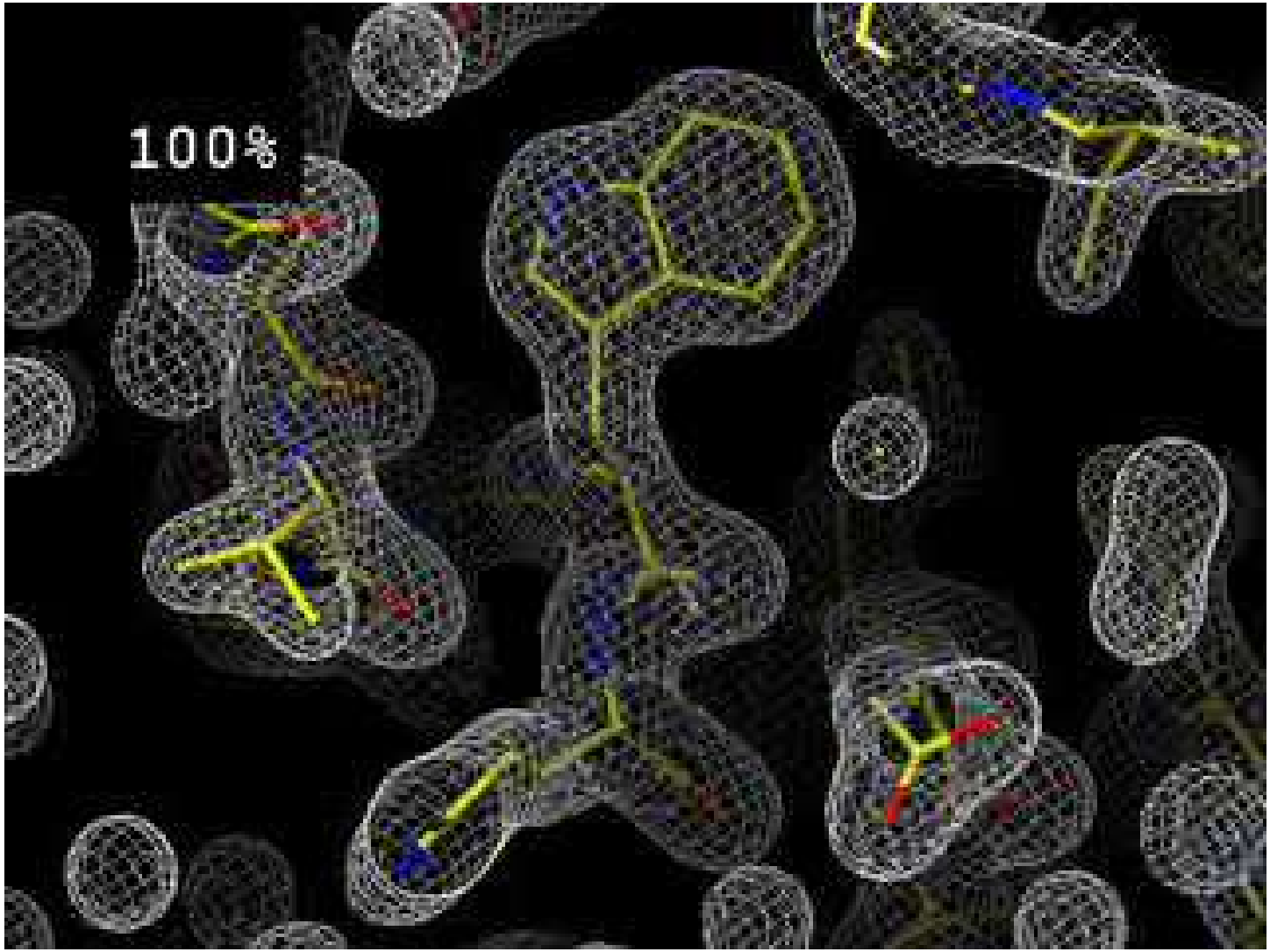


Classification of cryo-electron sub-tomograms using constrained correlation. Förster, F, Pruggnaller, S, Seybert, A, Frangakis, AS (2008) J. Struct. Biol. 161, 276–286

The missing wedge in crystallography

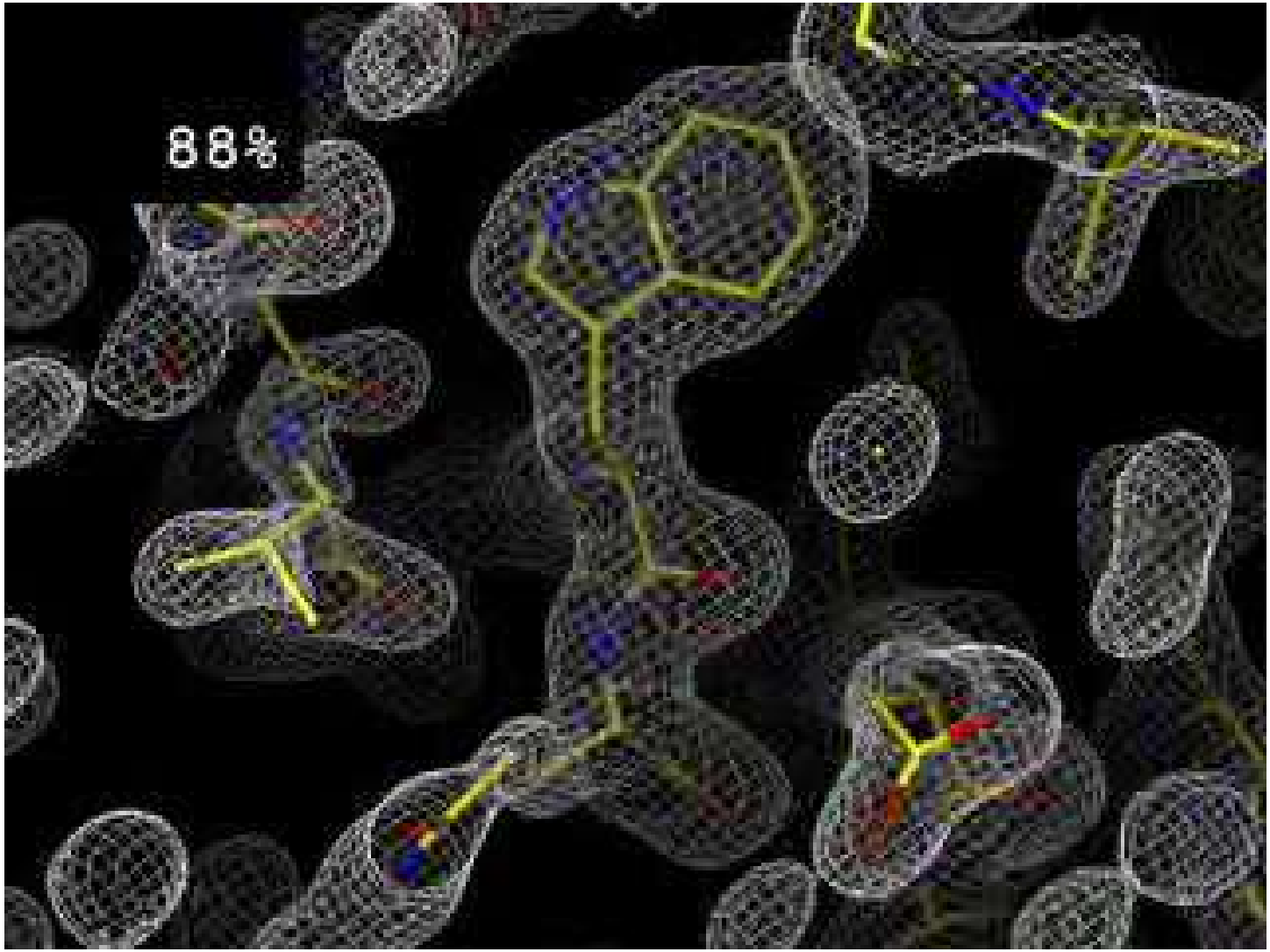


The missing wedge in crystallography



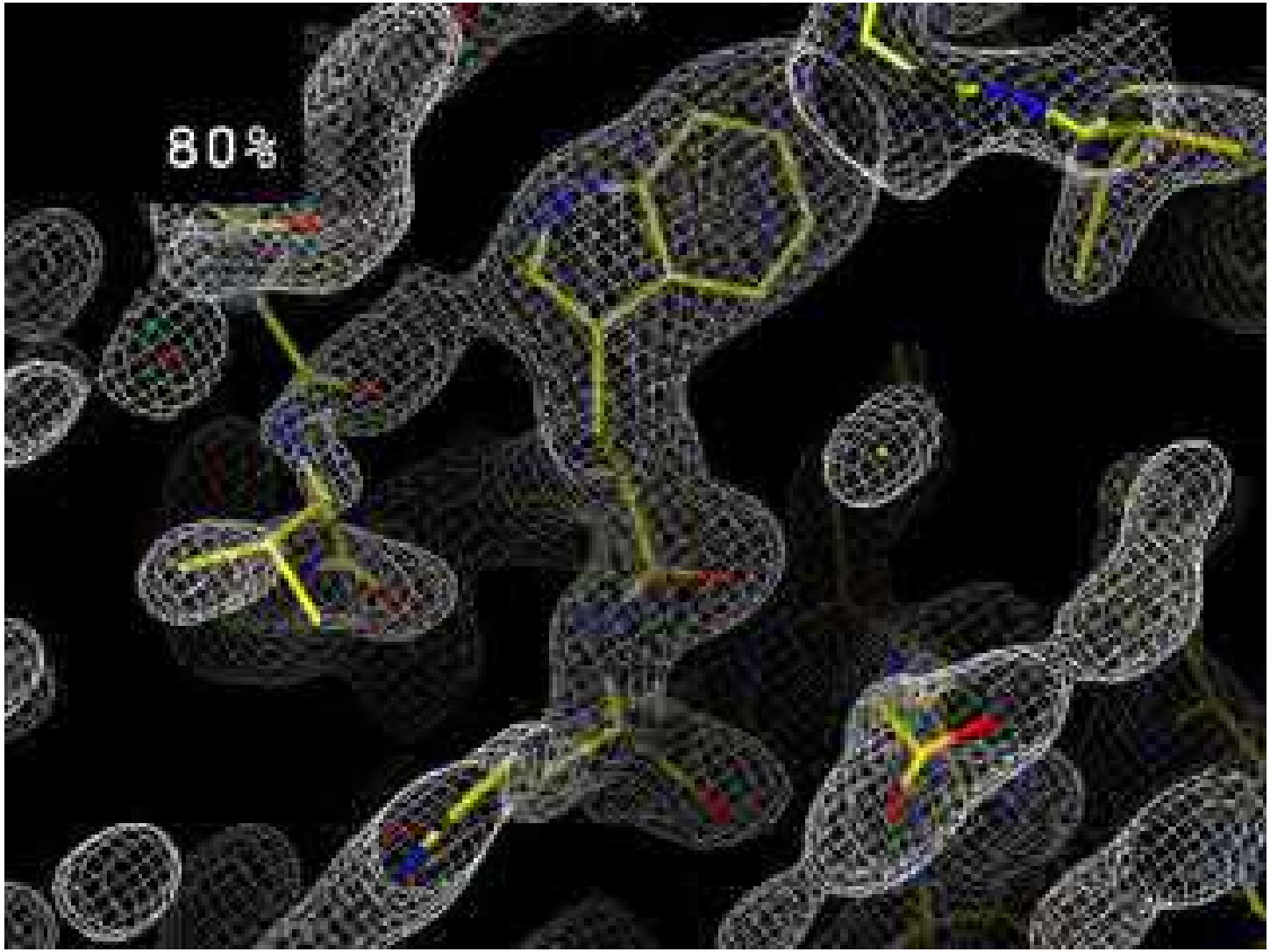
From a movie by James Holton: http://bl831.als.lbl.gov/~jamesh/movies/

The missing wedge in crystallography



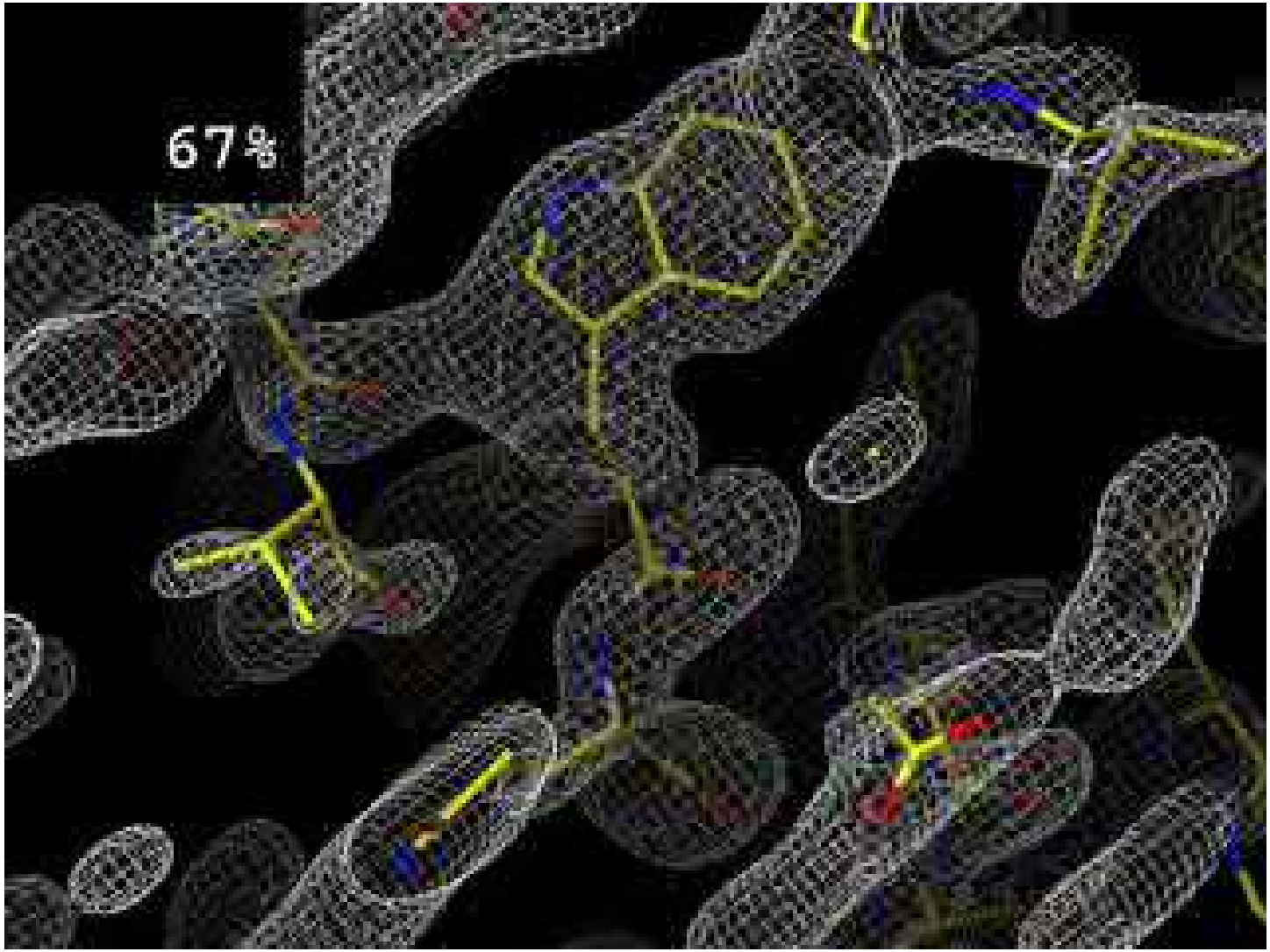
From a movie by James Holton: http://bl831.als.lbl.gov/~jamesh/movies/

The missing wedge in crystallography



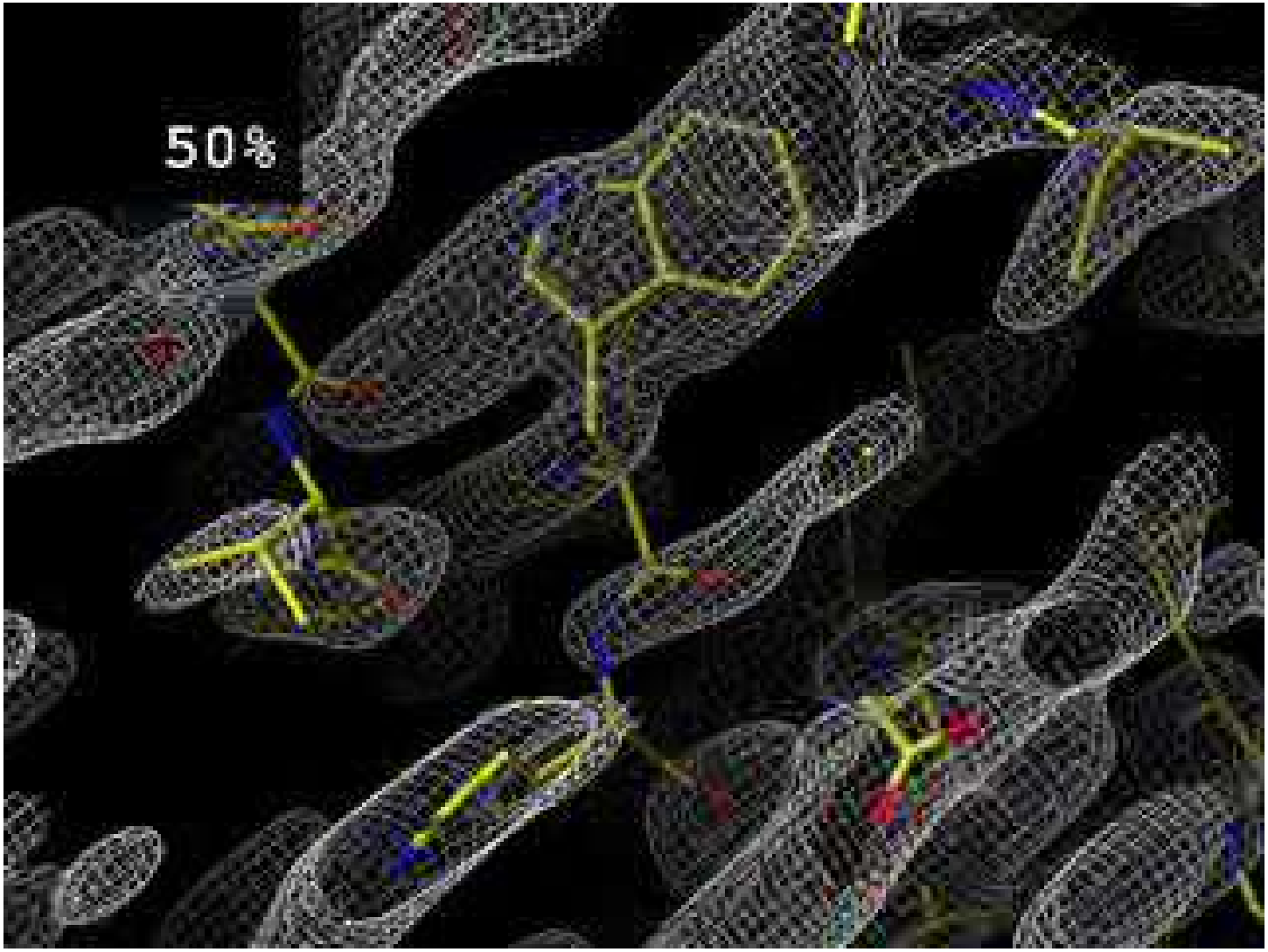
From a movie by James Holton: http://bl831.als.lbl.gov/~jamesh/movies/

The missing wedge in crystallography



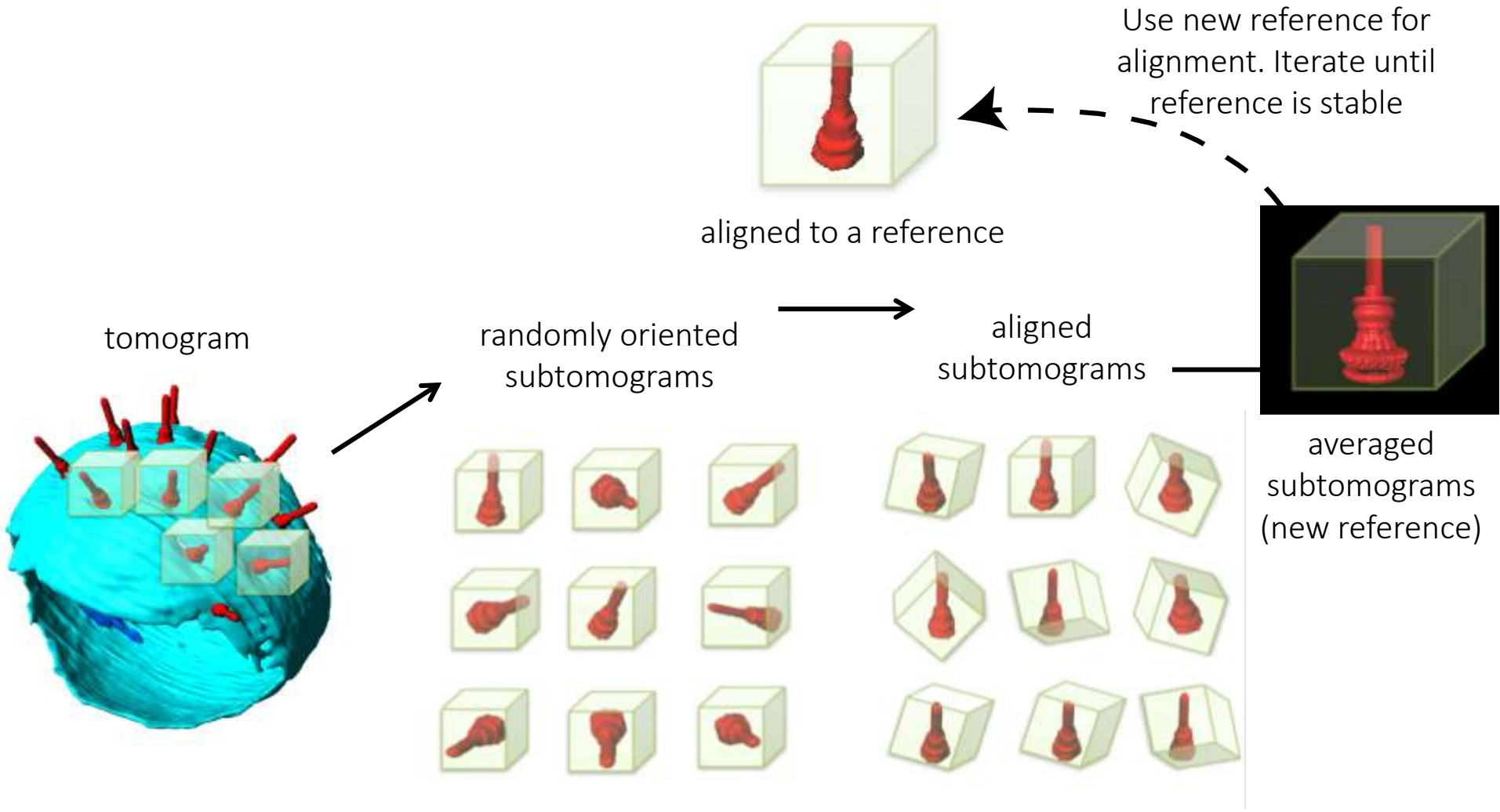
From a movie by James Holton: http://bl831.als.lbl.gov/~jamesh/movies/

The missing wedge in crystallography

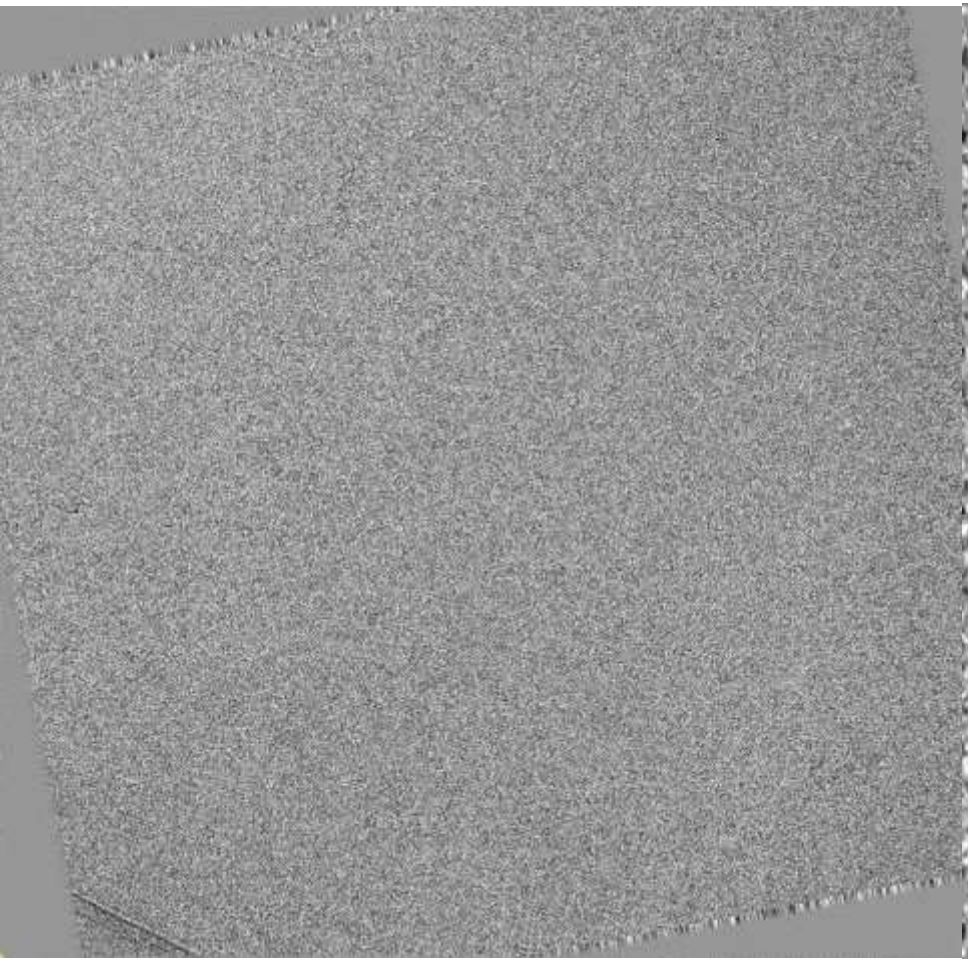


From a movie by James Holton: http://bl831.als.lbl.gov/~jamesh/movies/

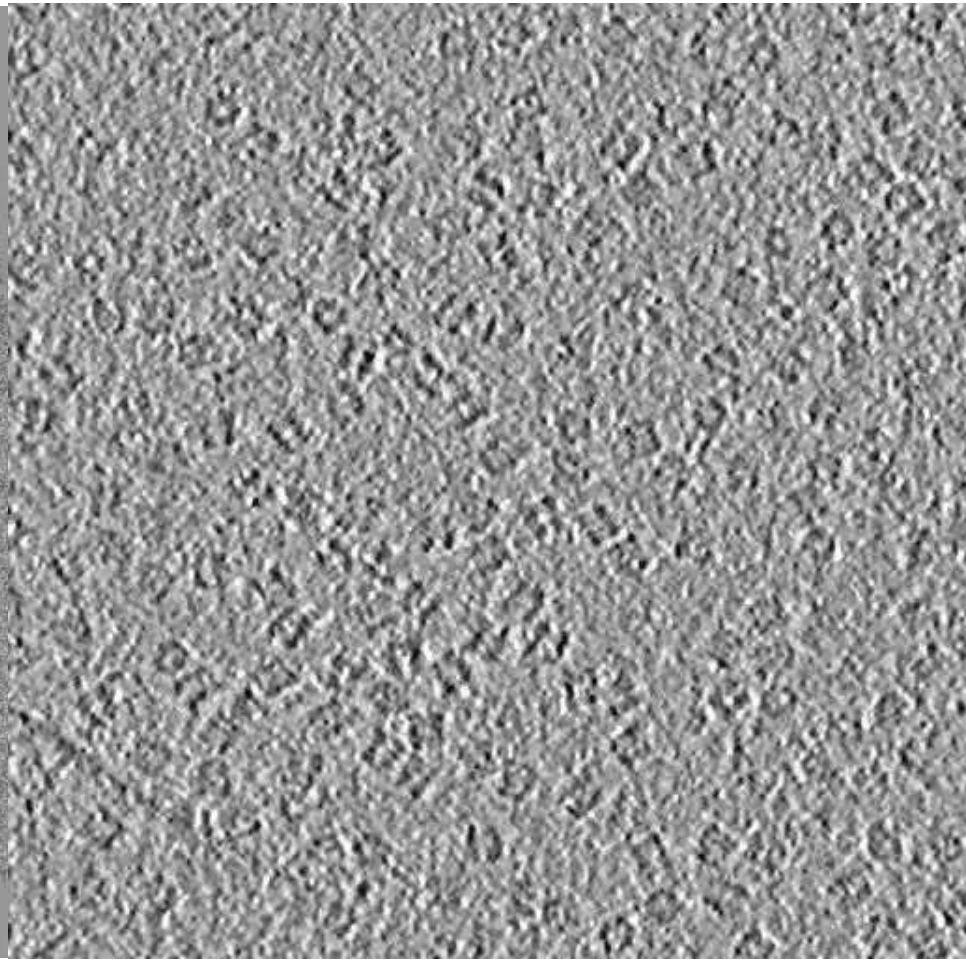
Subtomogram averaging: single particle analysis in 3D



Subtomogram averaging: GroEL

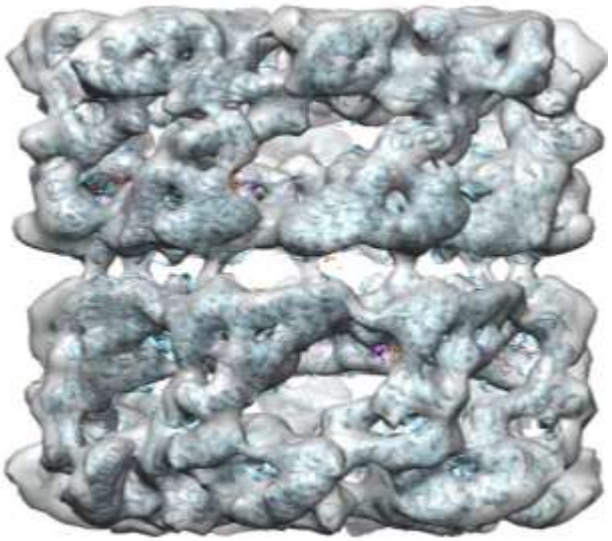


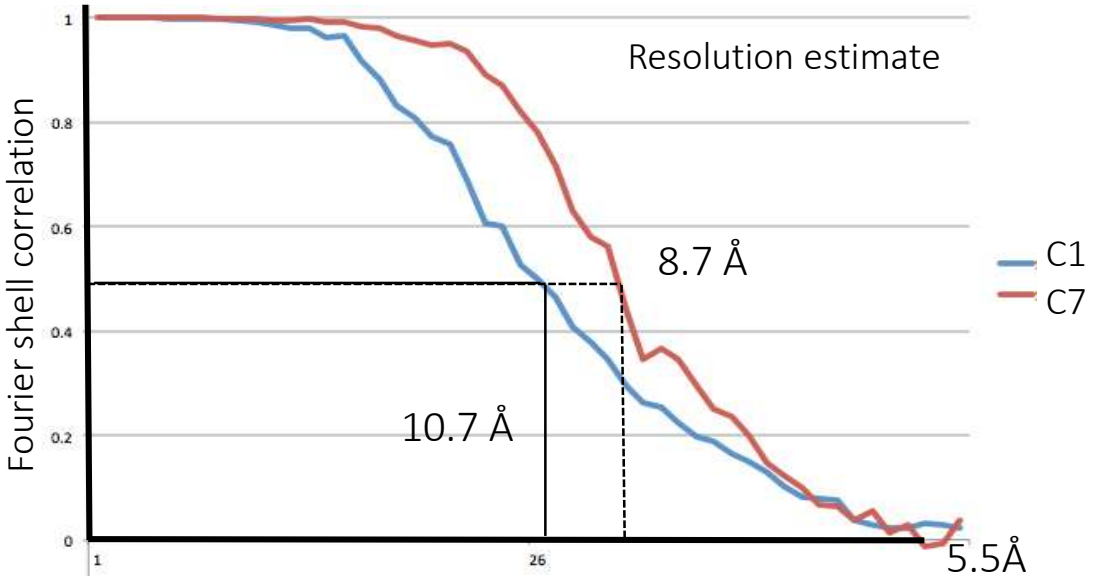
Tilt series

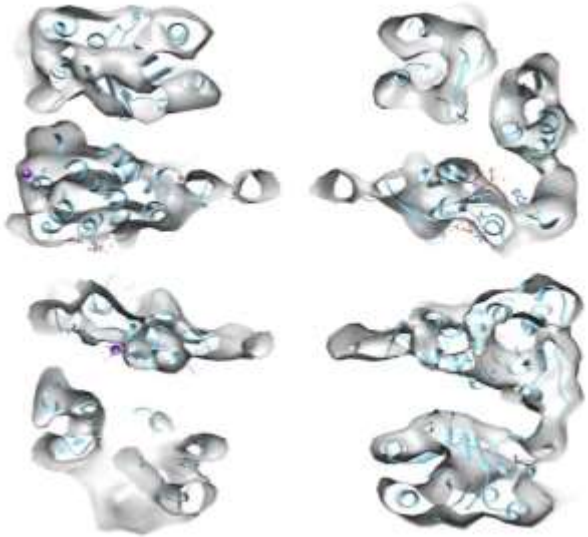


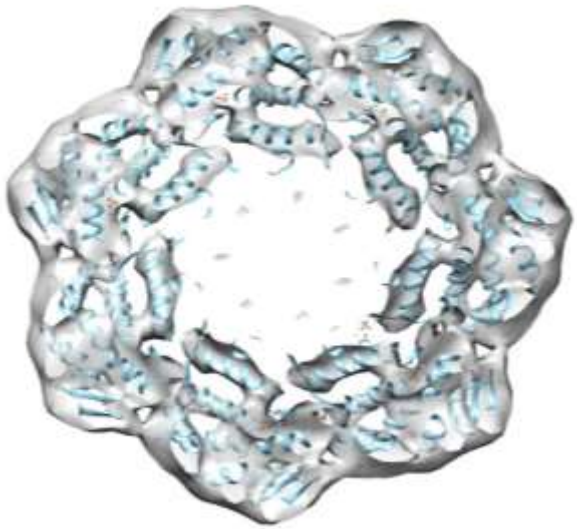
Reconstruction

GroEL reconstruction from 4600 sub-volumes

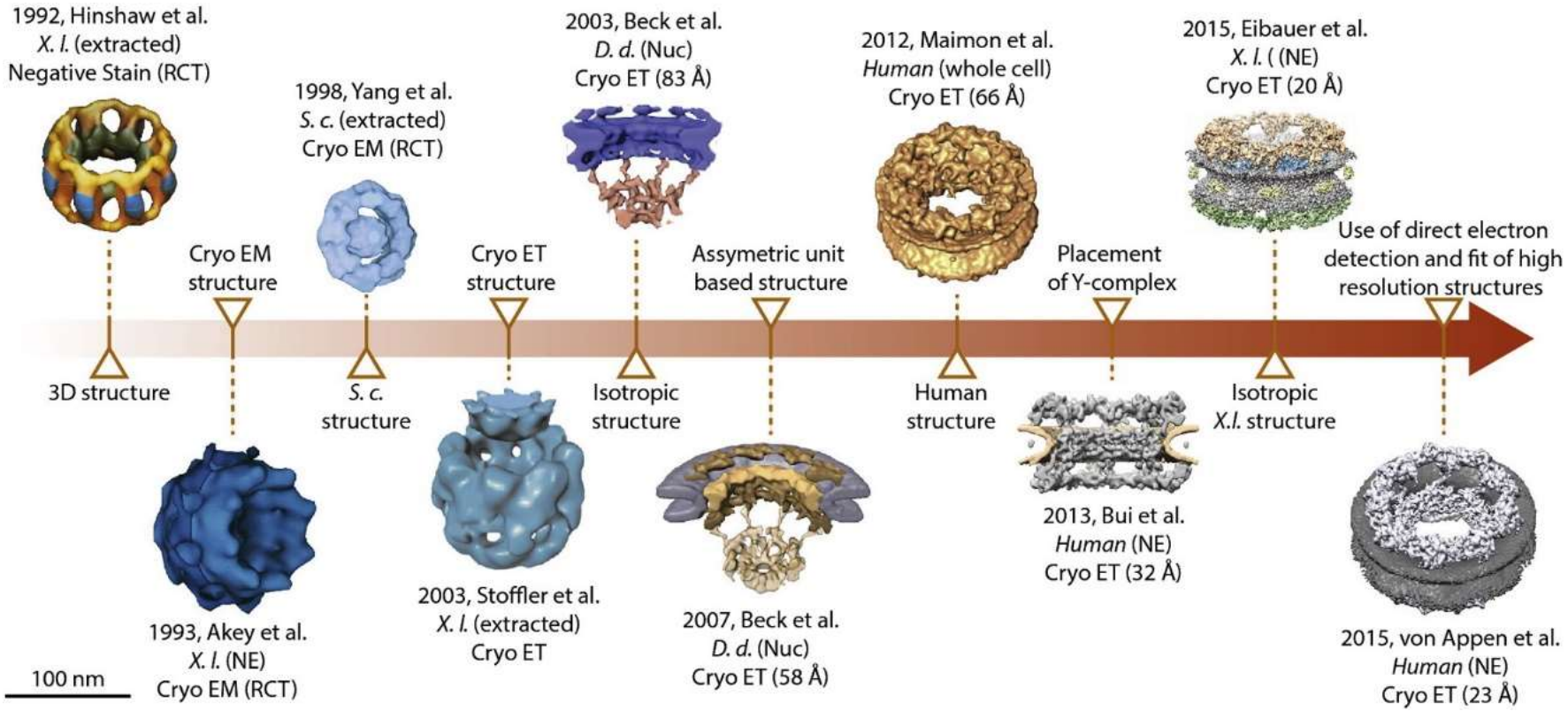






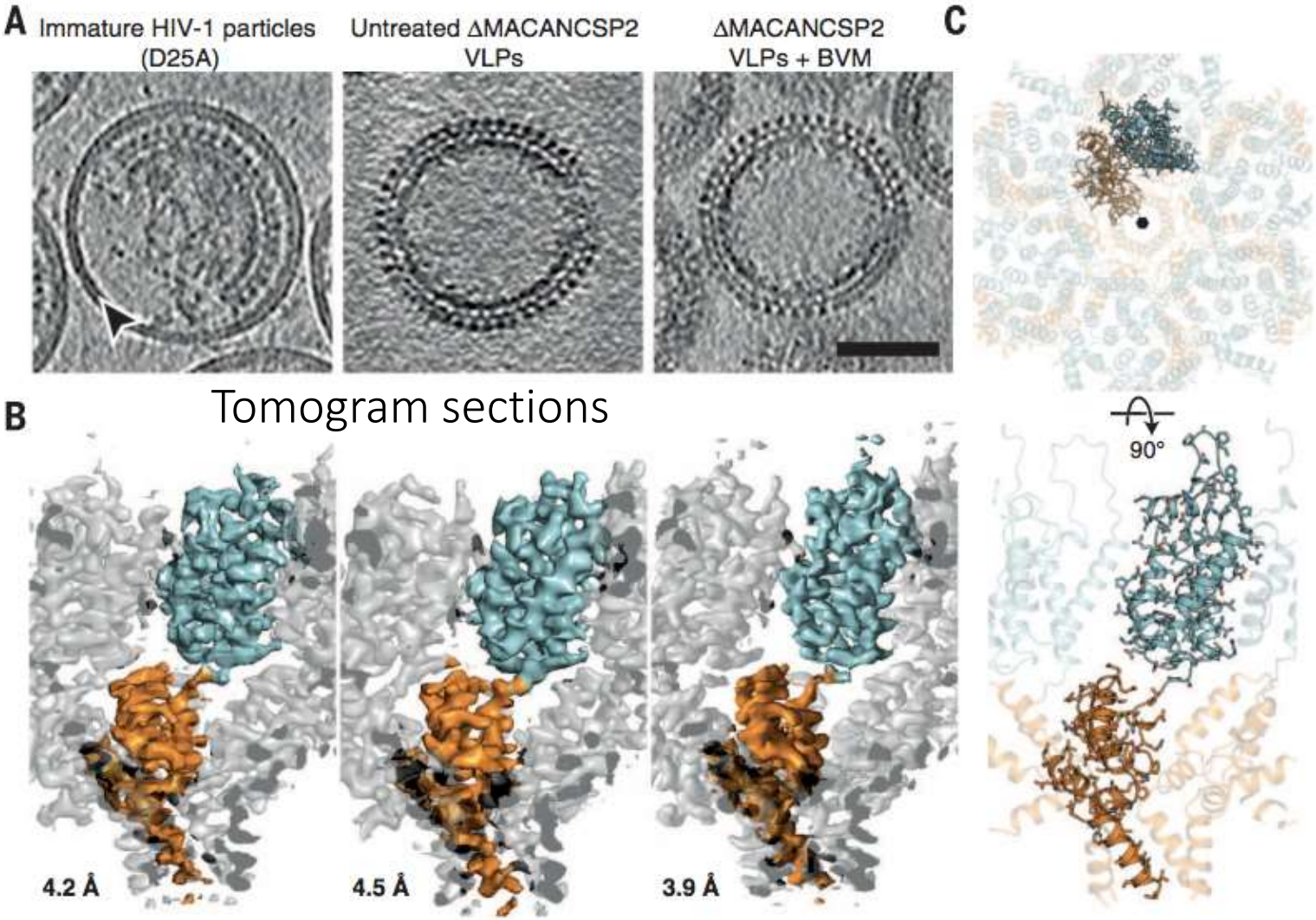


Structures of the nuclear pore



von Appen & Beck (2016)

Immature HIV capsid protein lattice at 4 Å resolution



Schur et al, 2016

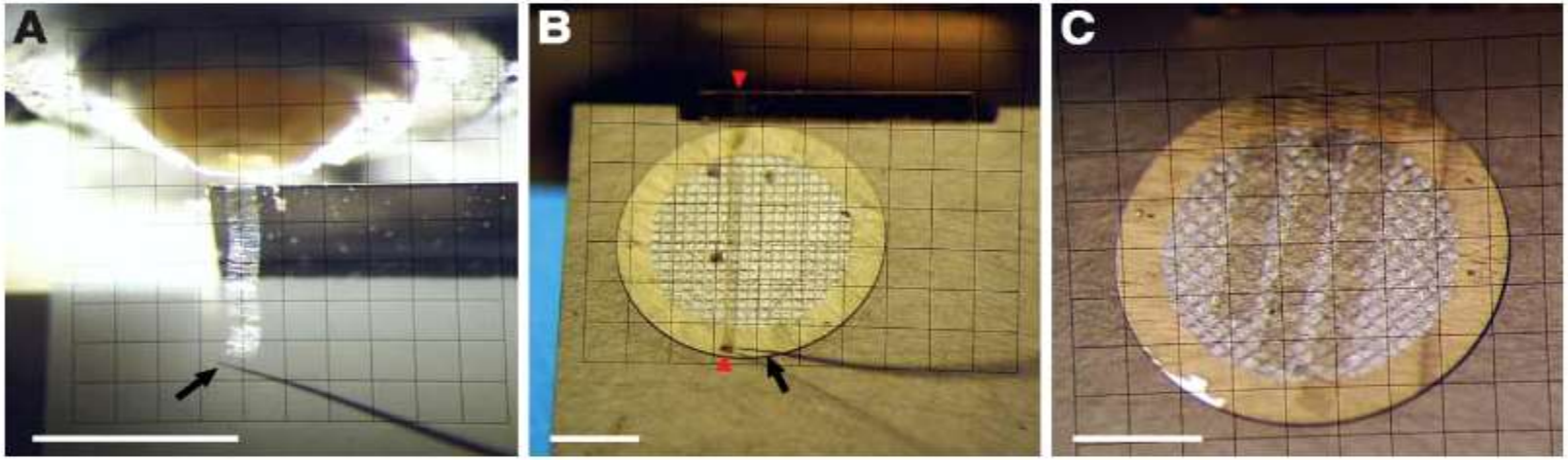
Tomography of vitrified cell sections

Cells can be grown on EM grids and plunge frozen, but only thin regions can be imaged

Cell paste or small piece of tissue (100-200 mm thick) can be vitrified in a high pressure freezer

Cryo sections (J. Dubochet) 50-100 nm thick can be cut in a cryo microtome and imaged for tomography

McIntosh, J Microsc 2006

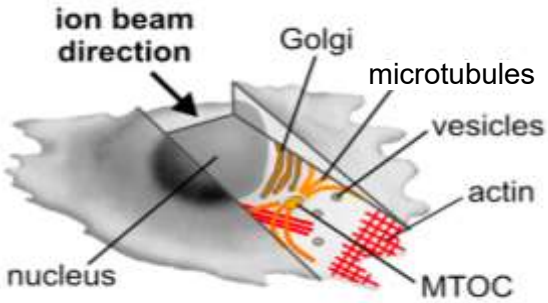


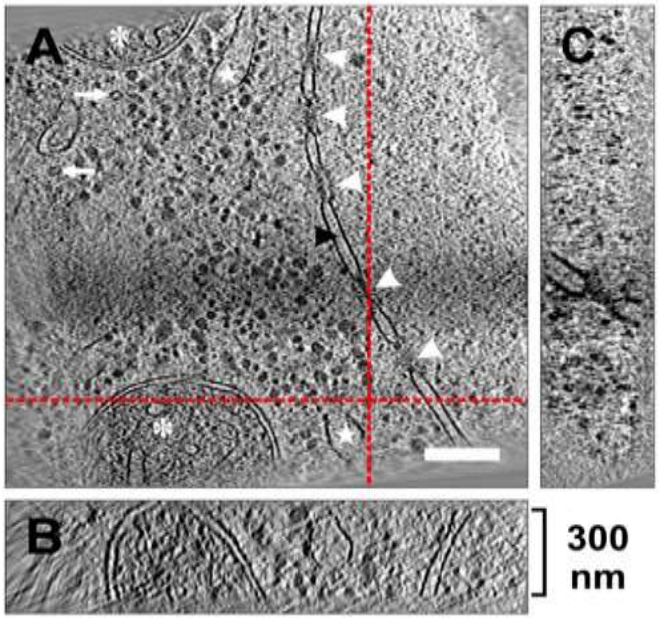
(Freeze-substitution makes life easier)

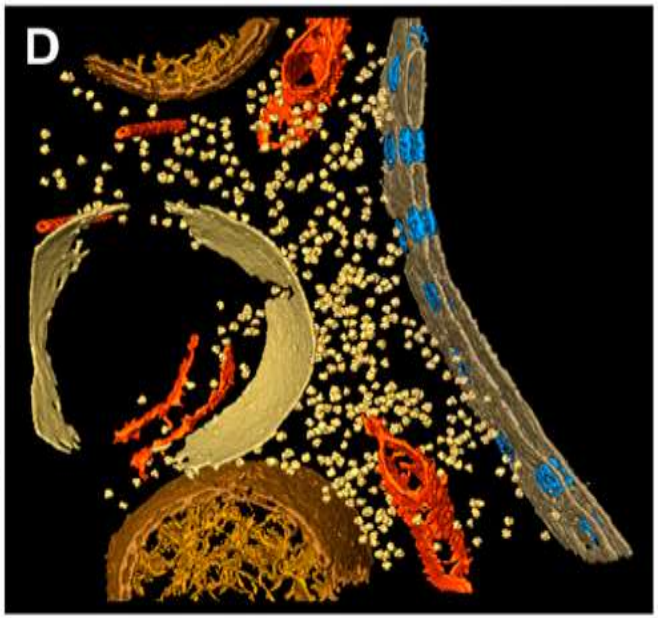
Sectioning vitrified cells by FIB milling

Specimens vitrified by plunge-freezing or high pressure freezing need to be <<1 micron thick

Focussed ion beam milling: Access to native, undistorted cell and tissue sections

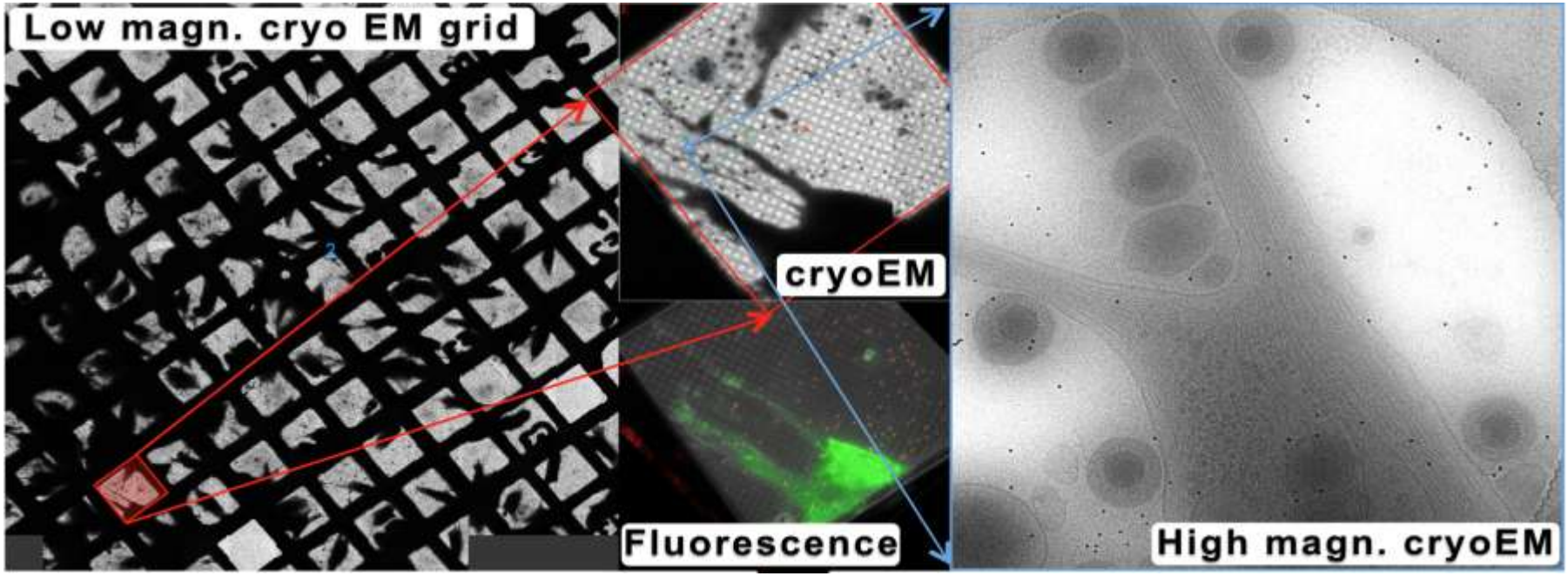






Rigort, Baumeister et al, PNAS 2012

Correlative microscopy:
cryo fluorescence ➔cryo EM



cryo fluorescence

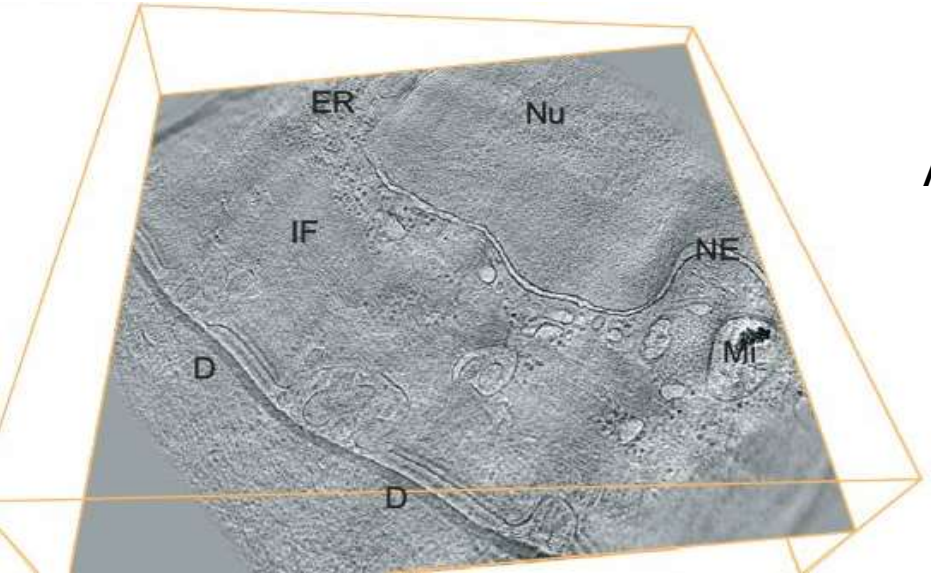
Kay Grunewald, Oxford

Multiscale imaging

Single crystal X-ray diffraction & NMR

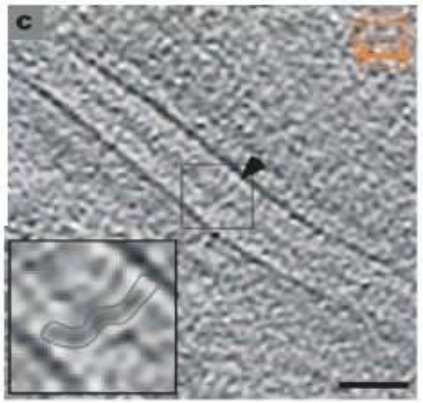
Single particle EM

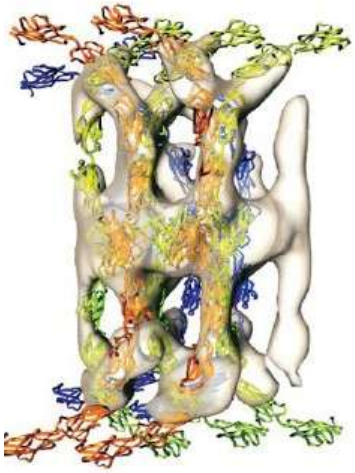
Isolated particle EM tomography/ sub tomogram averaging



Cellular EM tomography

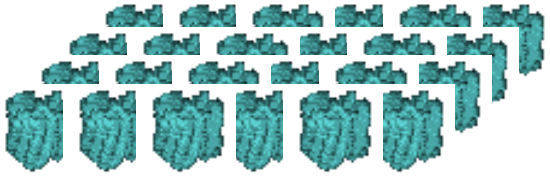
Averaging from cryo section tomography





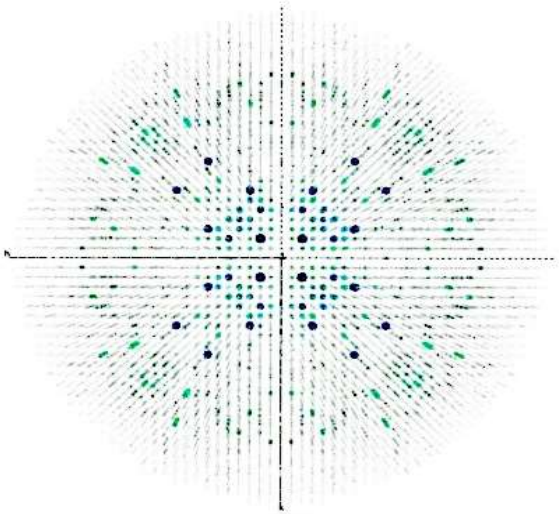
Other cryo-EM techniques

TEM in structural and cellular biology

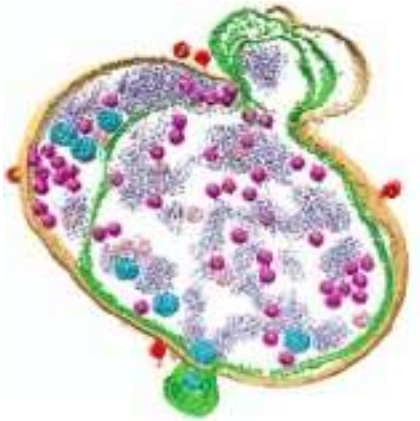


2D crystals

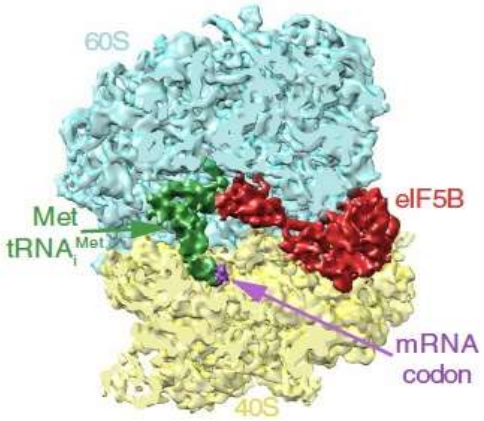
Electron crystallography
(views at different tilts)



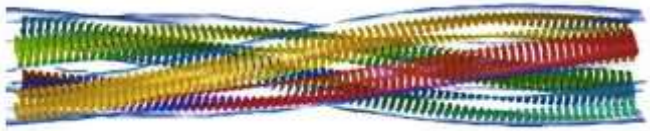
Microcrystal (<1 μm)
electron diffraction



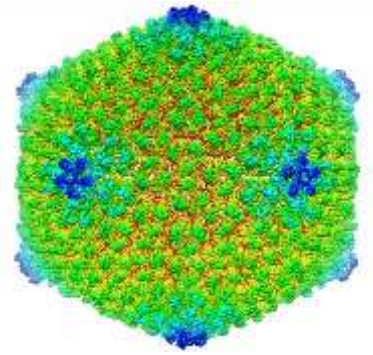
Whole cells or organelles
(**tomography** of unique objects,
cumulative irradiation)



Asymmetric **single particles**

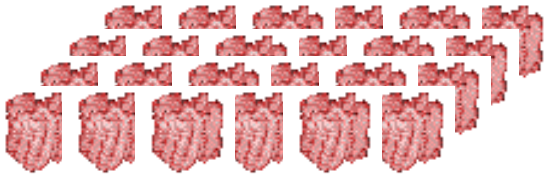


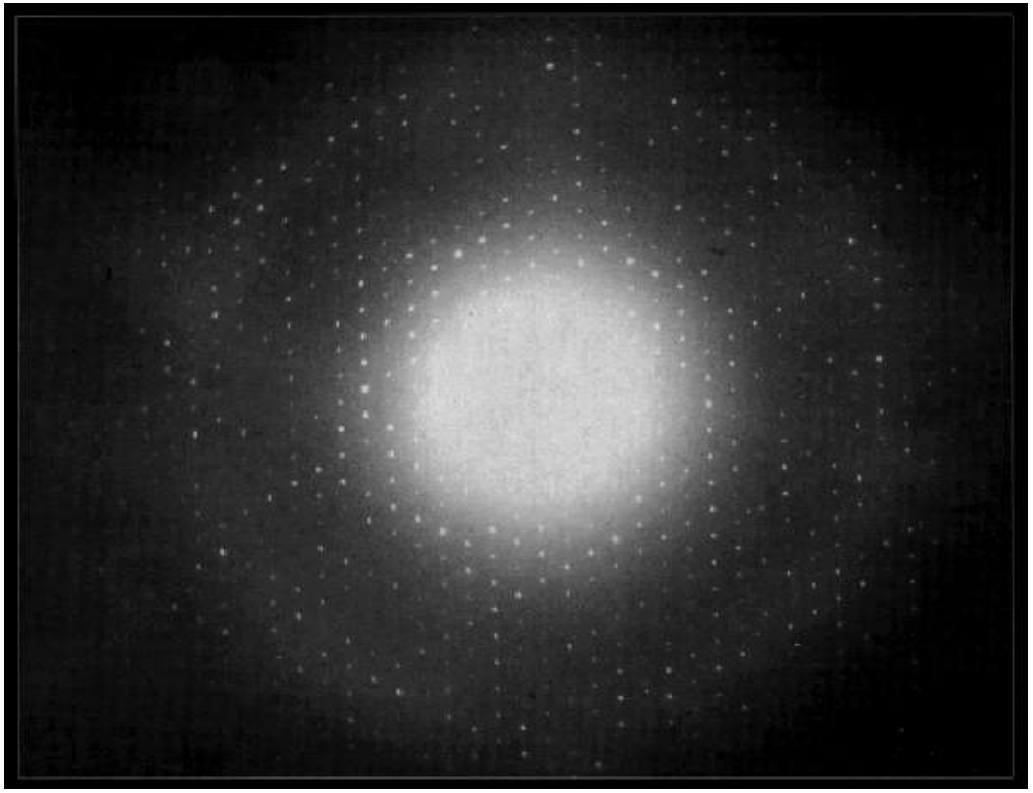
Helical assemblies



Icosahedral viruses

2D crystals



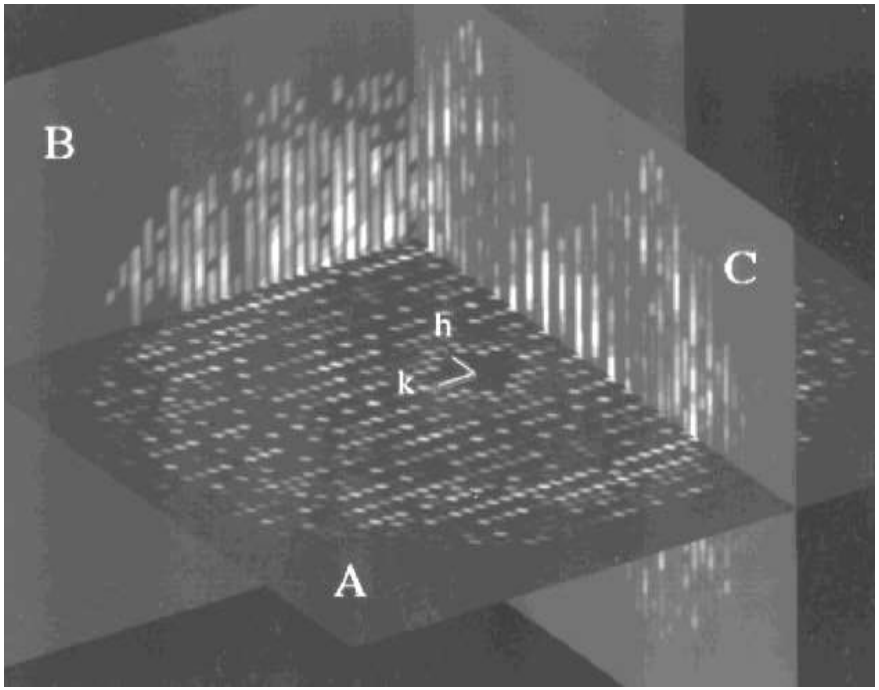


Electron diffraction pattern of a 2D crystal

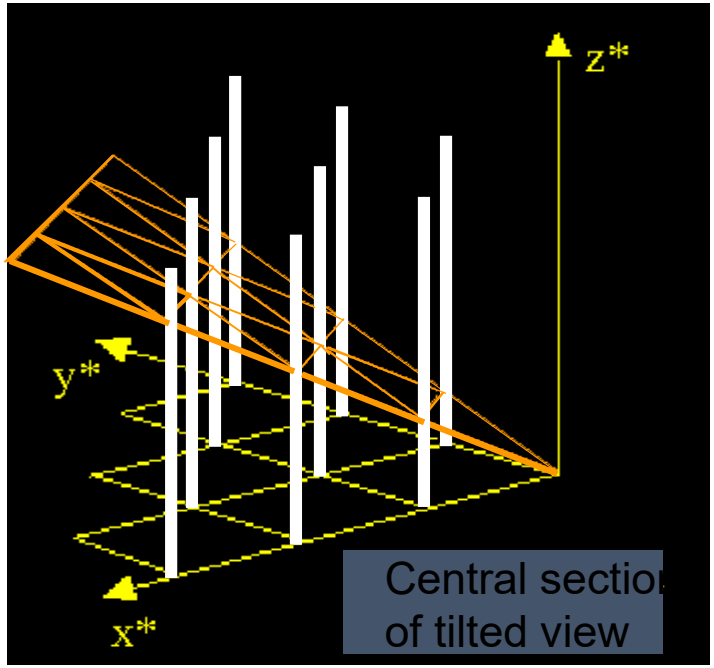
2D crystals contain a single layer of protein molecules

Electron diffraction can be recorded directly in the microscope, or the diffraction pattern can be computed from the image

Tilting of 2D crystals to get 3D data



3D electron diffraction intensity data for tubulin



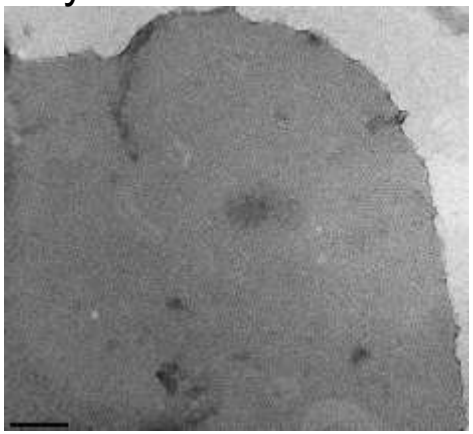
Nogales *et al.* (1997)

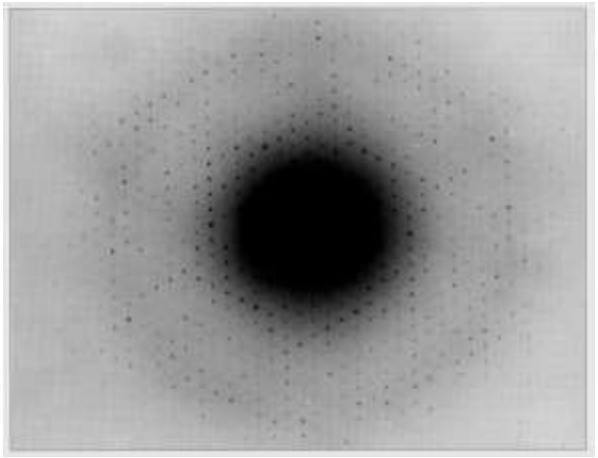
Three-dimensional model of purple membrane obtained by electron microscopy

R. Henderson & P. N. T. Unwin
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Henderson & Unwin (1975)

Noisy, low contrast image of crystal





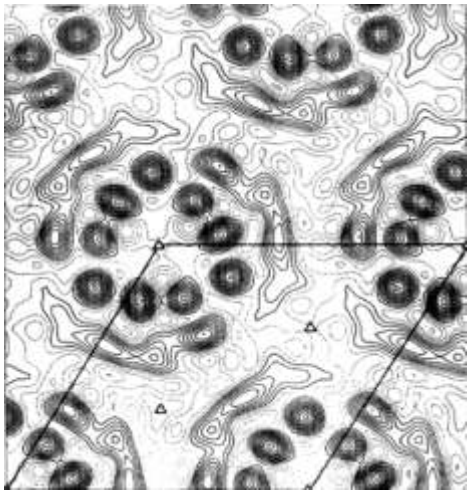
Phases

(Image)

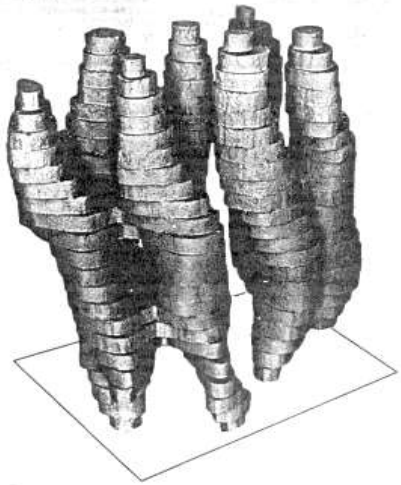
Amplitudes

(Electron diffraction)

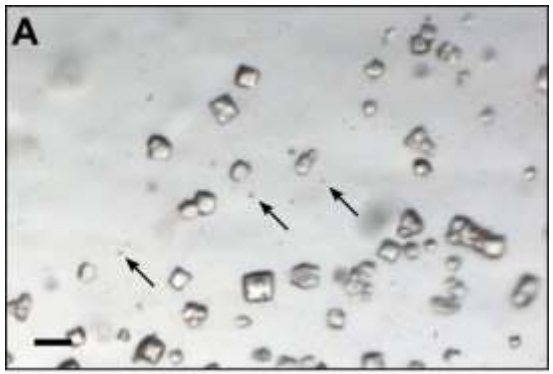
2D projection density map

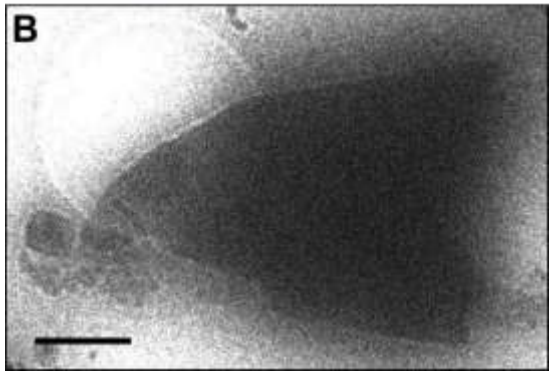


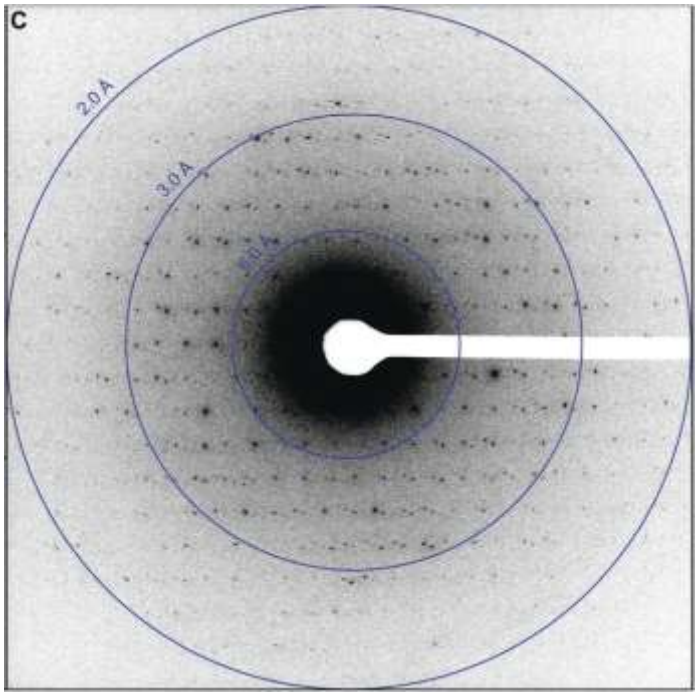
Model of 3D structure

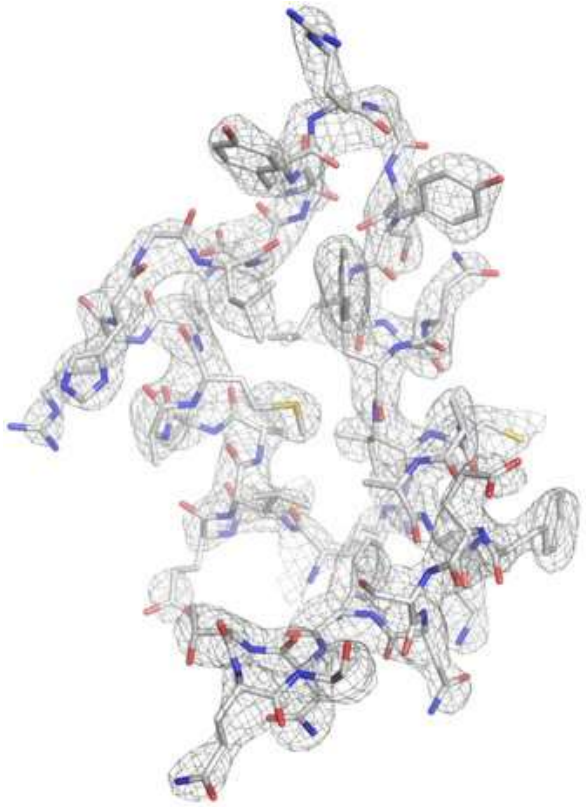


3D electron crystallography (“MicroED”)







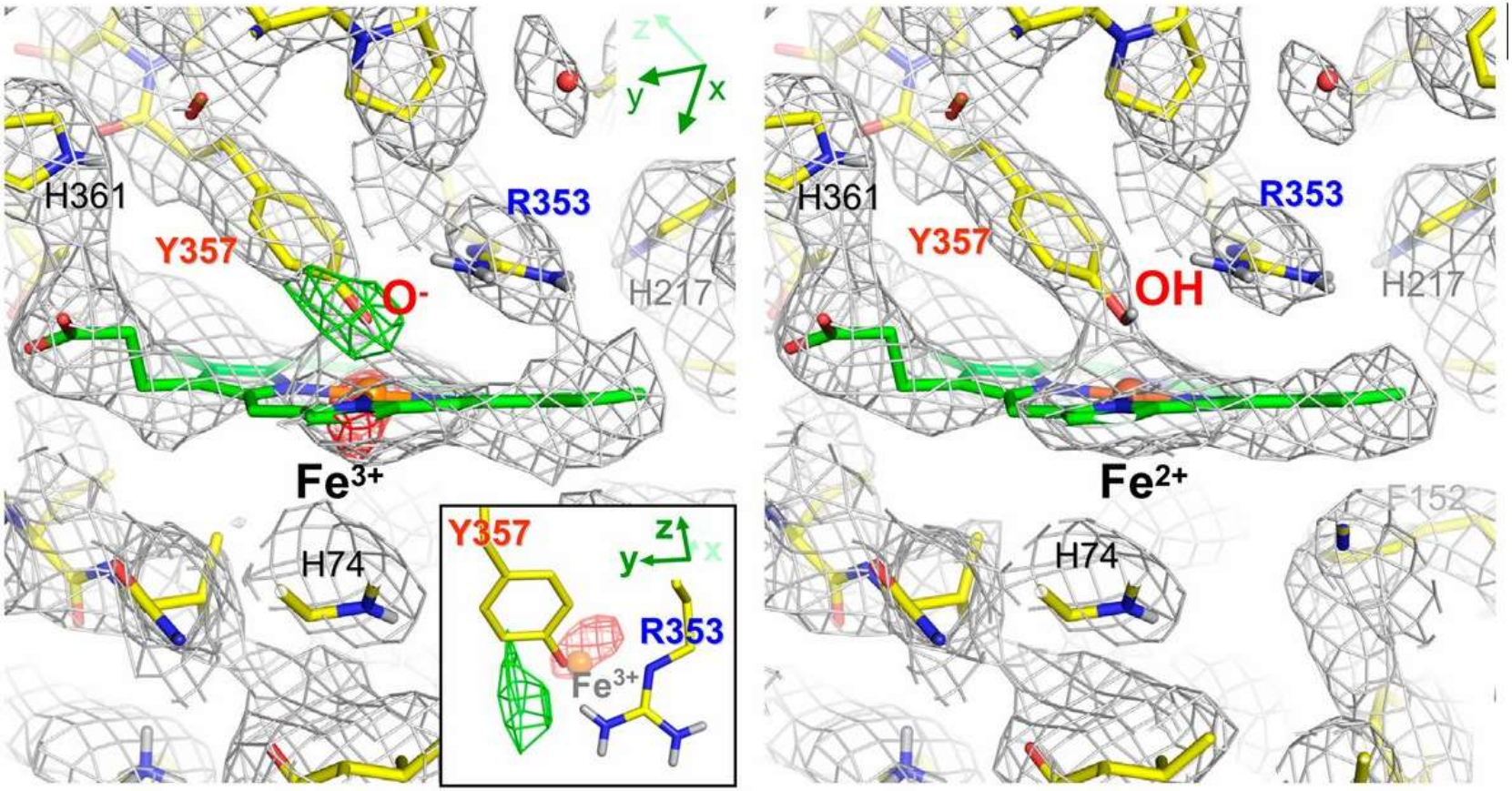


$2F_{obs} - F_{calc}$

Shi et al. (2013) eLife 2:e01345

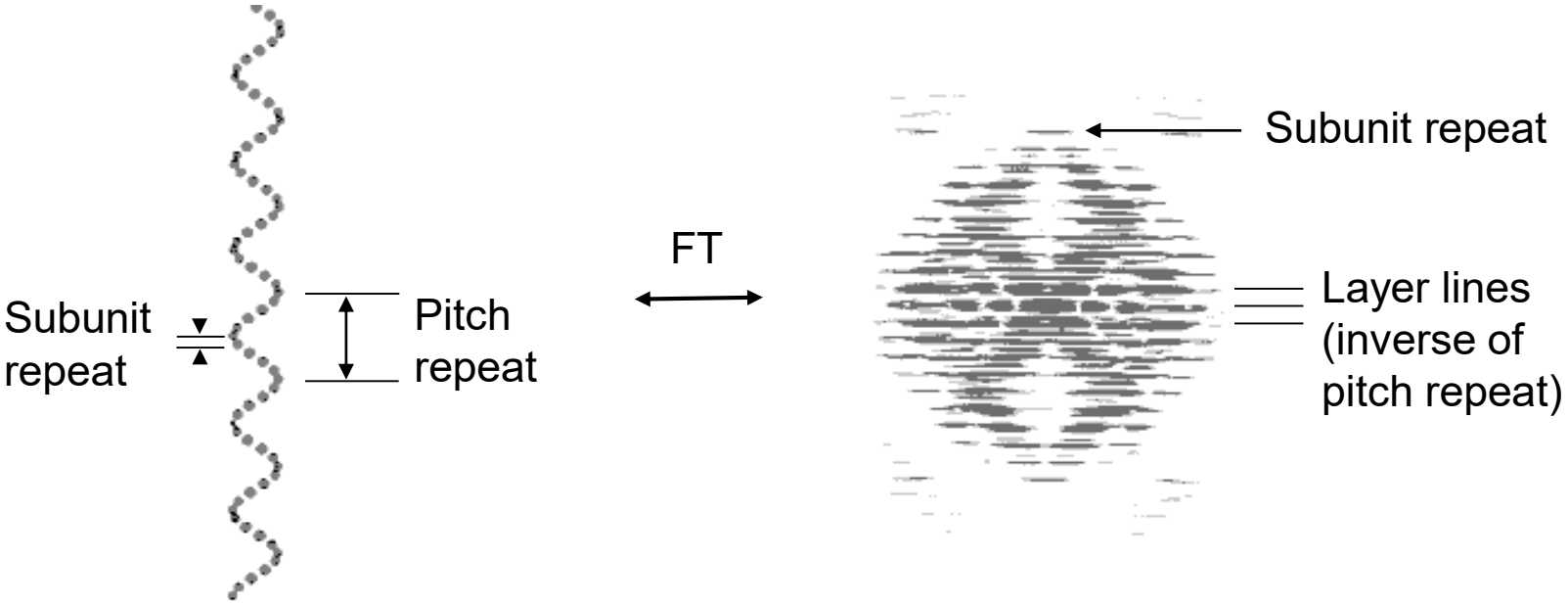
What does the map tell us?

- Electrons measure Coulomb potential, not electron density
- Therefore we can see charge states



Yonekura et al. PNAS 2015;112:11:3368-3373

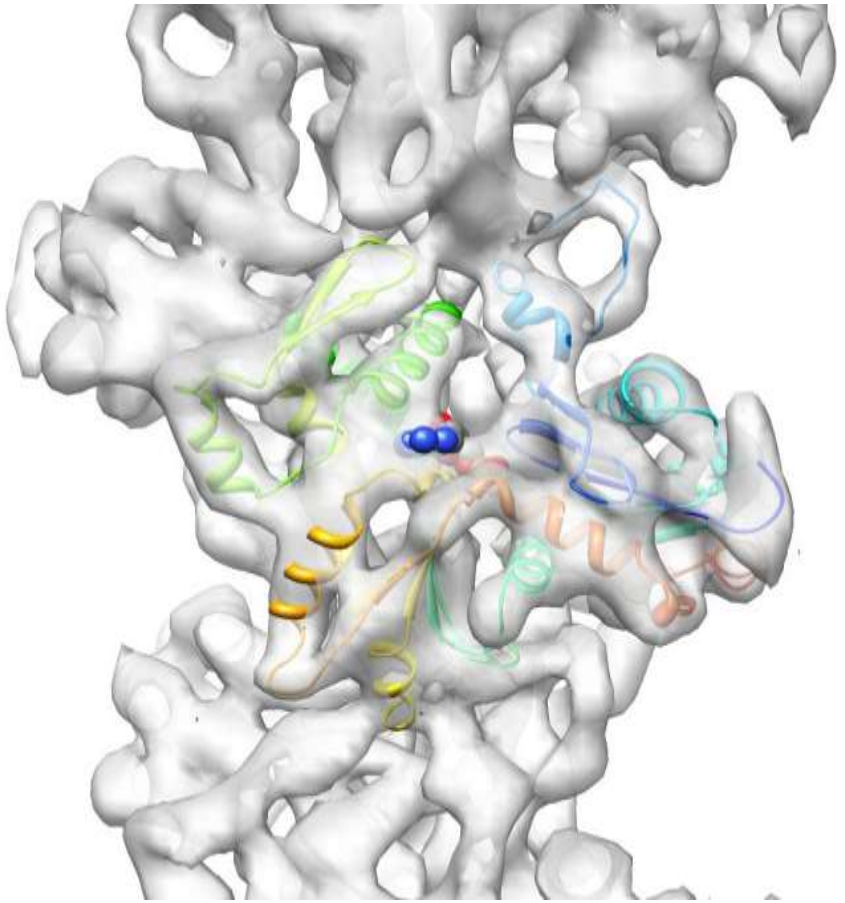
Helical reconstruction



A helix can be considered as a 1D crystal, since it has a repeating structure along the axis, giving rise to a set of layer lines in the diffraction pattern. If the symmetry of the helix is known, a full 3D reconstruction can be calculated from the untilted filament transform, since the subunit is imaged at different angles about the filament axis. Examples are: nicotinic acetylcholine receptor, actin, kinesin, flagellin.

EM structure of filamentous actin

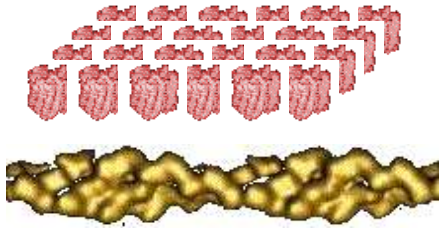
Cryo-EM image and
helical diffraction



Cryo-EM map with a fitted subunit

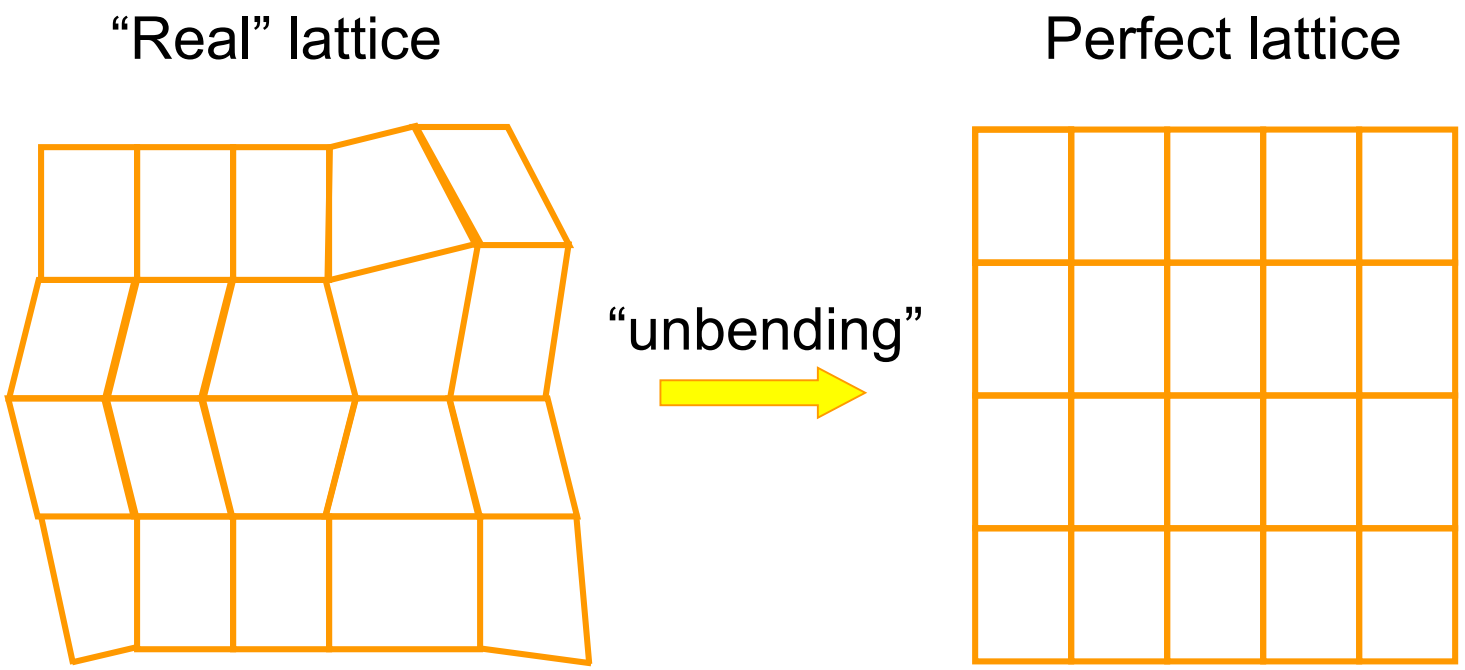
Fujii *et al.* (2010)

Ordered assemblies



Biological complexes often occur in repeating structures such as helical filaments, or can be induced to form 2D arrays. They can be reconstructed using the symmetry of the assembly but usually there is some disorder. Therefore, local deviations are detected by cross-correlation and corrected, combining the single particle strategy with symmetry-based reconstruction to improve the resolution. Lattice “unbending” is one such approach.

Unbending



CCP-EM

Science & Technology Facilities Council

Collaborative **C**omputational **P**roject for **E**lectron cryo-**M**icroscopy

Located at Research Complex at Harwell, UK

Alongside CCP4 core team – shared expertise between projects


Tom Burnley


Colin Palmer


Agnel Joseph

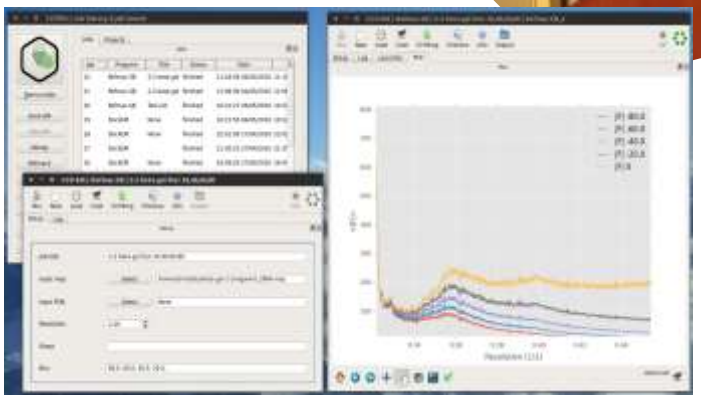

Martyn Winn

Research Complex at Harwell

Science & Technology Facilities Council

CCP-EM Activities

- Software suite
- Spring Symposium
- Training workshops
- Mailing list



Science & Technology Facilities Council

CCP-EM software suite

Suite of utilities for EM data processing

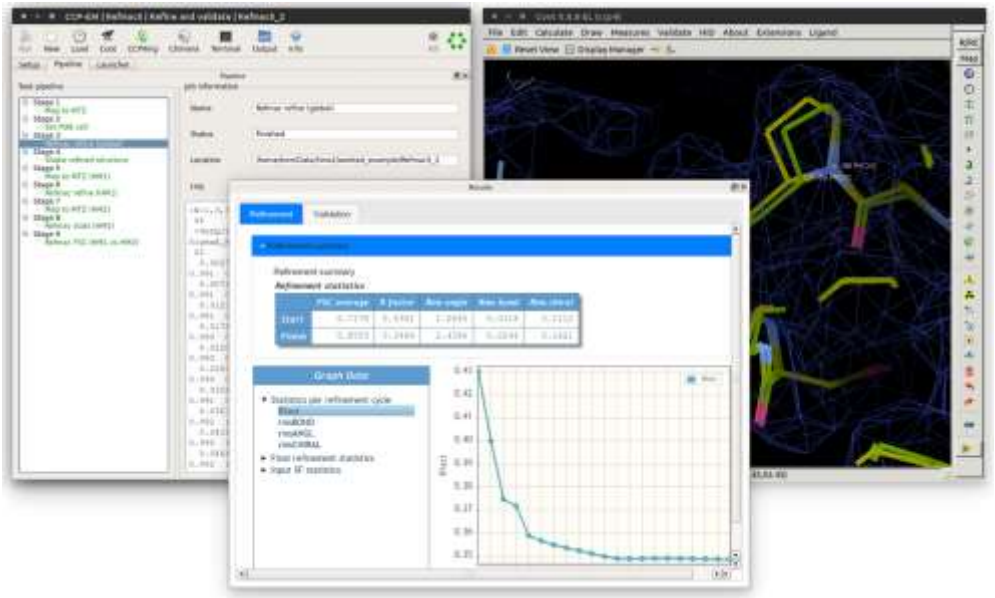
Uses EM functionality of several CCP4 tools (Buccaneer & Nautilus, Molrep, Refmac)

Initial focus on model building

Download from ccpem.ac.uk

Linux & Mac

Free for academic use



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The revolution will be televised

https://www.youtube.com/playlist?list=PLFEB3YHuxu11iA_RJRGh

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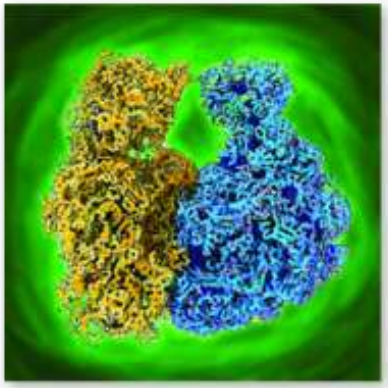
ISSN: 2059-7983

June 2017 issue

Proceedings of the CCP-EM Spring Symposium

Edited by Tom Burnley, Paula da Fonseca and Randy Read



Cover illustration: Cryo-EM has undergone a major `resolution-revolution'. It has helped advance our understanding of a key biological macromolecule, the ribosome. Ribosomes have shared a progressive journey with cryo-EM; in the development of the method and use of the method to understand ribosome structural biology (Javed *et al.*, p. 509). The cover shows a bacterial ribosome map (with independently painted subunits) to highlight the near-atomic details that can be resolved using the current technology thus driving biology forward.

Tweets by @ActaCrystD

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TJ McCorvie
@Norvey19

Playlist of the CCP-EM Spring Symposium 2016 presentations. Worth checking out: youtube.com/playlist?list=...

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Our contact details can be found here or see us at one of these meetings.

International School on Biological Crystallization

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CCP-EM Workshops

- 10+ workshops since 2014
  - CCP-EM software and others

- Single particle reconstruction
- Model building
- Subtomogram averaging

- Annual ‘Icknield’ high resolution model building workshop

- *See CCP-EM mailing list for announcements*

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Twitter: @ccp_em

Email us: ccpem@stfc.ac.uk

CCP-EM papers:

Collaborative Computational Project for Electron cryo-Microscopy. Acta Cryst. D71, 123-126, 2015

Recent developments in the CCP-EM *software suite. Acta Cryst.* D73, 469-477, 2017

CCP-EM

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Cryo-EM resources

- Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowell, A.W. & Schultz, P. (1988). Cryo-electron microscopy of vitrified specimens. Quart. Rev. Biophys. 21:129-228.

- Jensen, G.J., Ed. (2010). Methods in Enzymology
  481, Cryo-EM, Part A: Sample Preparation and Data Collection
  482, Cryo-EM, Part B: 3-D Reconstruction
  483, Cryo-EM, Part C: Analyses, Interpretation, and Case studies

- Frank, J (2006) Three-dimensional electron microscopy of macromolecular assemblies. Oxford University Press.

- Orlova, EV & Saibil, HR (2011) Macromolecular structure determination by cryoelectron microscopy. Chem. Rev. 111:7710-7748.

- Henderson, R (2015) Overview and future of single particle electron cryomicroscopy. Arch Biochem Biophys 581:19-24.

- Nogales, E. & Scheres, S. H. W. (2015) Cryo-EM: A unique tool for the visualization of macromolecular complexity. Mol Cell 58:677-689.

- Lecture courses and talks:
  - Caltech: http://cryo-em-course.caltech.edu/ and https://em-learning.com
  - MRC-LMB: ftp://ftp.mrc-lmb.cam.ac.uk/pub/scheres/EM-course/
  - CCP-EM Symposium: search “CCP-EM” on YouTube, or links on http://www.ccpem.ac.uk/