The **evolution, challenges,** & **promise** of **cryo-electron microscopy**

David DeRosier, 2024

The

evolution,

challenges,

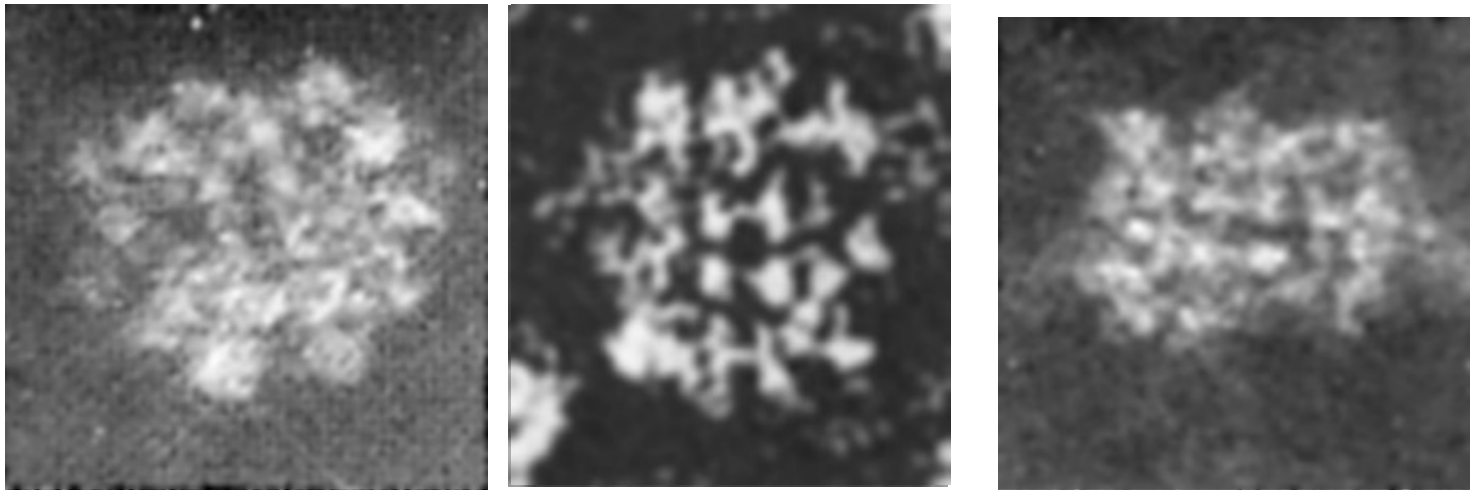
&

promise

of

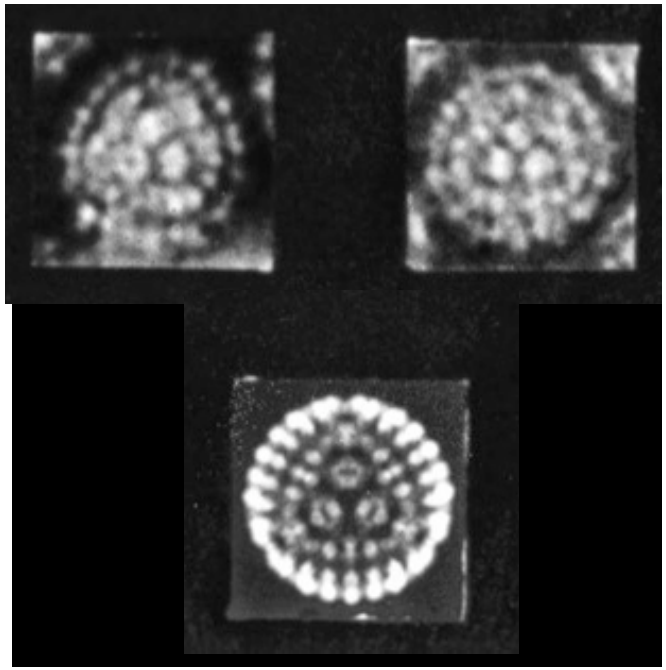
cryo-electron microscopy

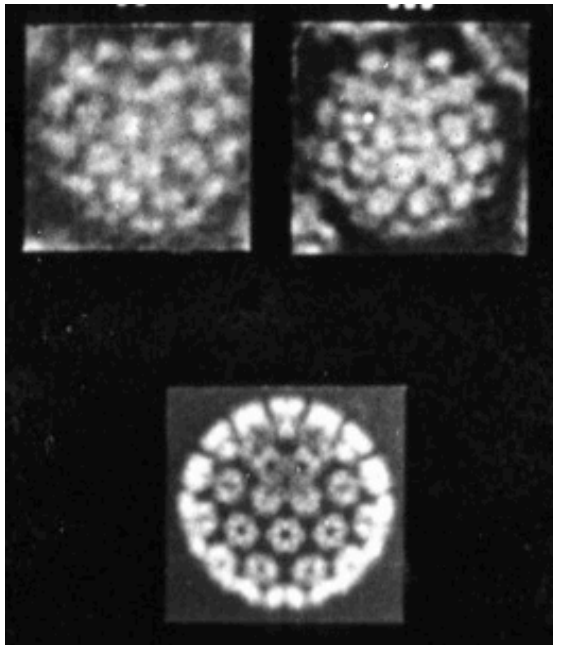
Image analysis once consisted of guessing the structure and building a simple model that *seemed* to account for the images.

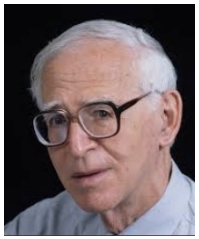


(L.J. Reed & D.J. Cox, The Enzymes, 1, 213-240, 1970)

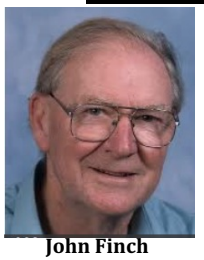
Klug and Finch simulated EM images of model structures and compared them in great detail to micrographs of human wart virus.







Aaron Klug

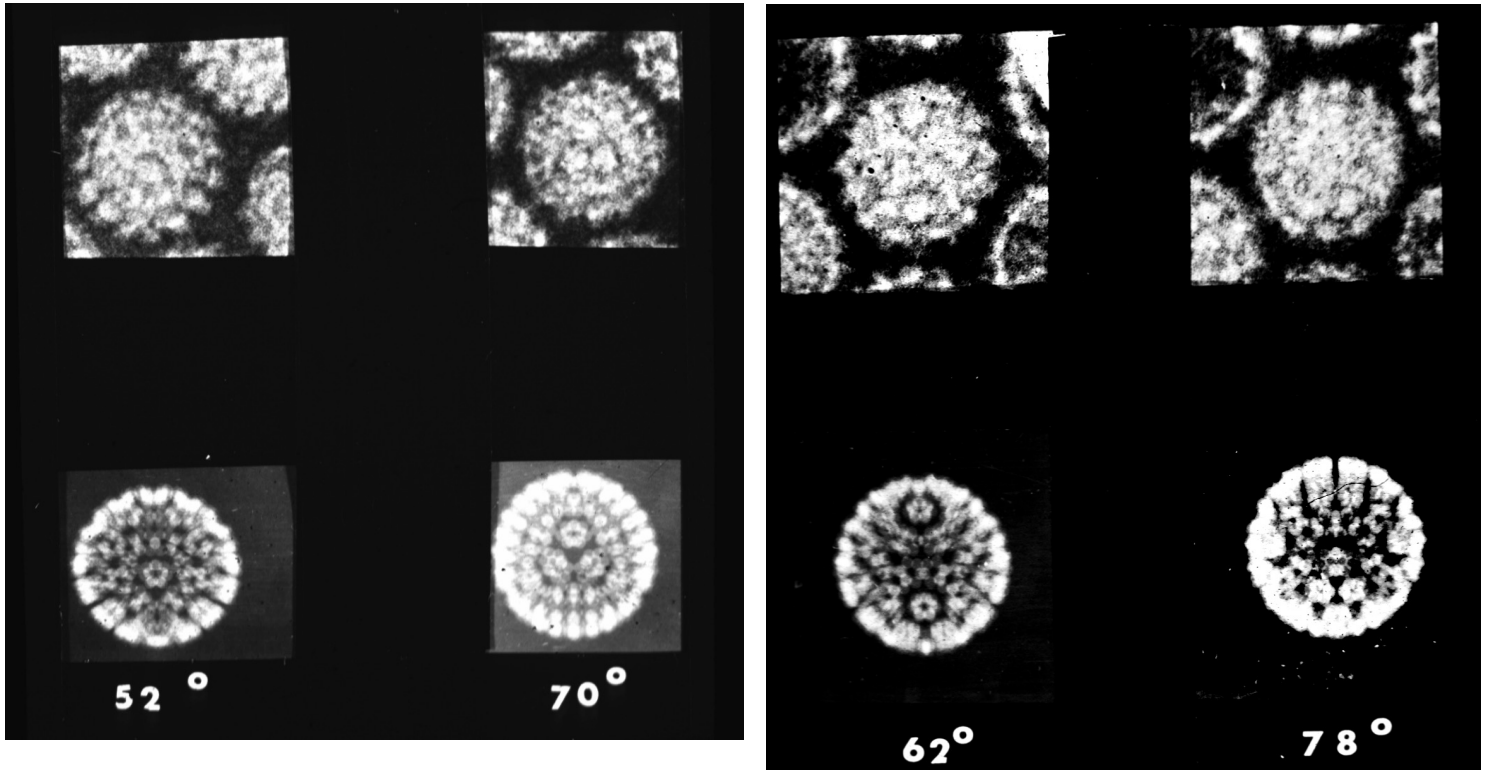


John Finch

A. Klug & J.T. Finch, J. Mol. Biol. 11, 403-423, 1965;A. Klug, J. Mol. Biol. 11, 424-431, 1965

They tilted the virus in the microscope and the model by the same amount and about the same axis to prove their model was unique.

Here are two ~17º tilts:



Structure determination: build a model, compare model to images, & tilt to prove uniqueness.

(*Reprinted from Nature, Vol. 217, No. 5124, pp. 130–134, January 13, 1968*)

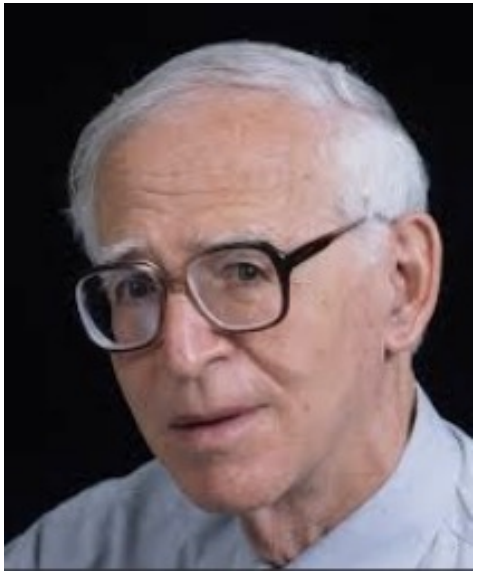
Reconstruction of Three Dimensional Structures from Electron Micrographs

by

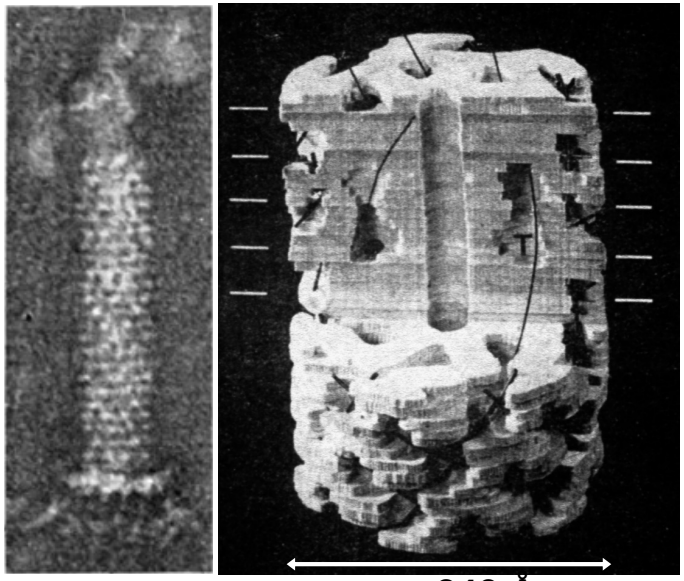
D. J. DE ROSIER
A. KLUG

MRC Laboratory of Molecular Biology, Hills Road, Cambridge

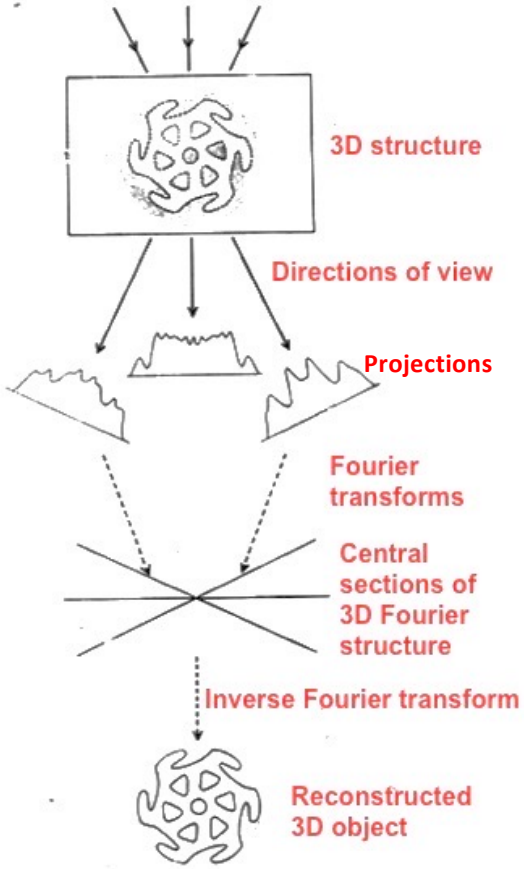
General principles are formulated for the objective reconstruction of a three dimensional object from a set of electron microscope images. These principles are applied to the calculation of a three dimensional density map of the tail of bacteriophage *T4*.



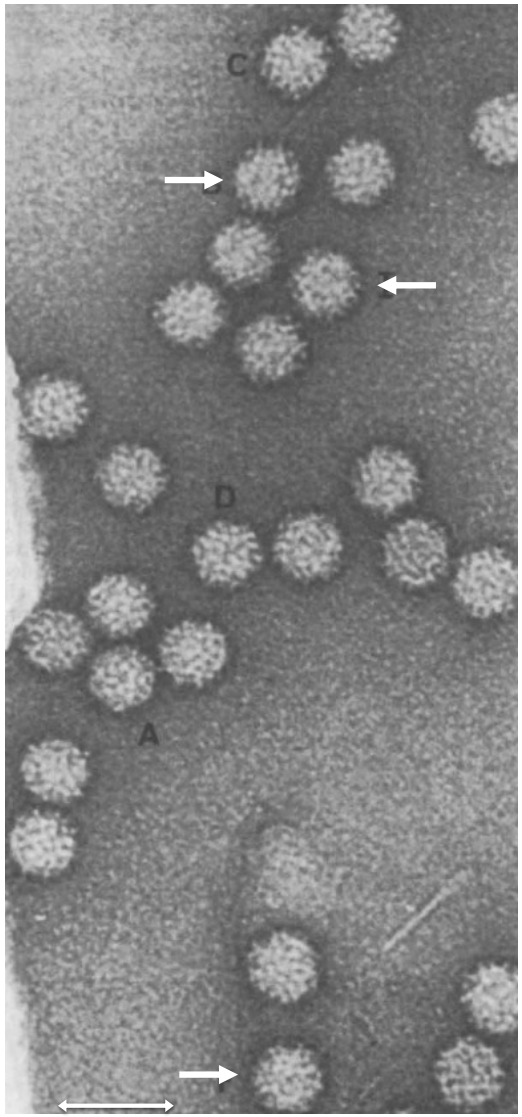
Aaron Klug



240 Å
resolution = ~35Å

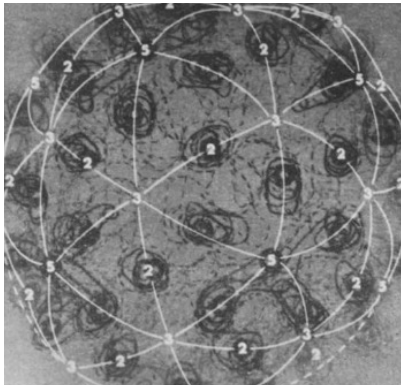


Now there was a general method for solving structures.



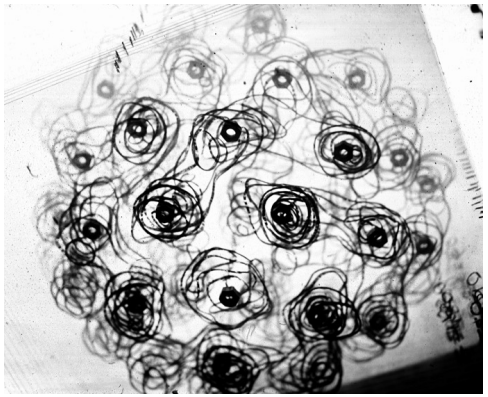
First single particle reconstructions by combining tilted views

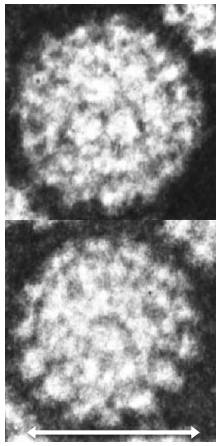
Tomato bushy stunt virus



resolution = ~28 Å

Human wart virus





resolution = ~60 Å

Tony Crowther

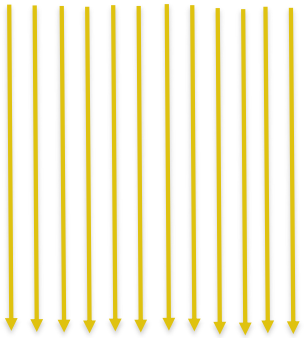
Linda Amos

Molecular resolution by EM!

R.A. Crowther, L.A. Amos, J.T. Finch, D.J. DeRosier, & A. Klug, Nature 226, 421-425, 1970.

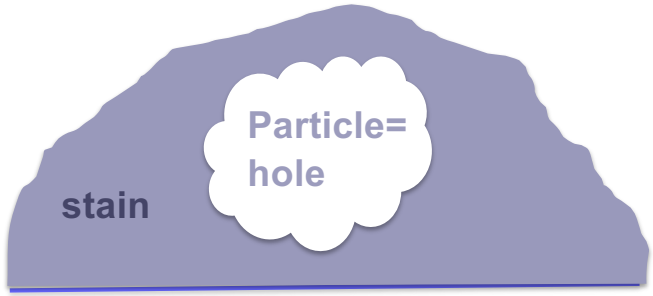
scale bars = 500 Å

3D reconstruction was limited to molecular resolution by the inherent nature of negative staining.

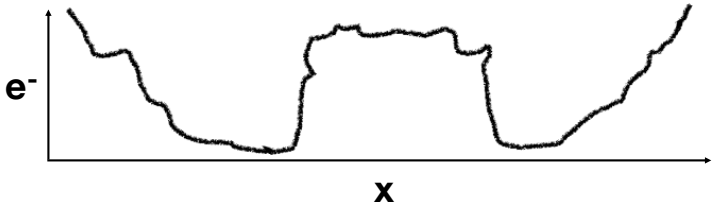


Electron beam

Uranium salts are good negative stains because they strongly scatter electrons providing amplitude contrast.

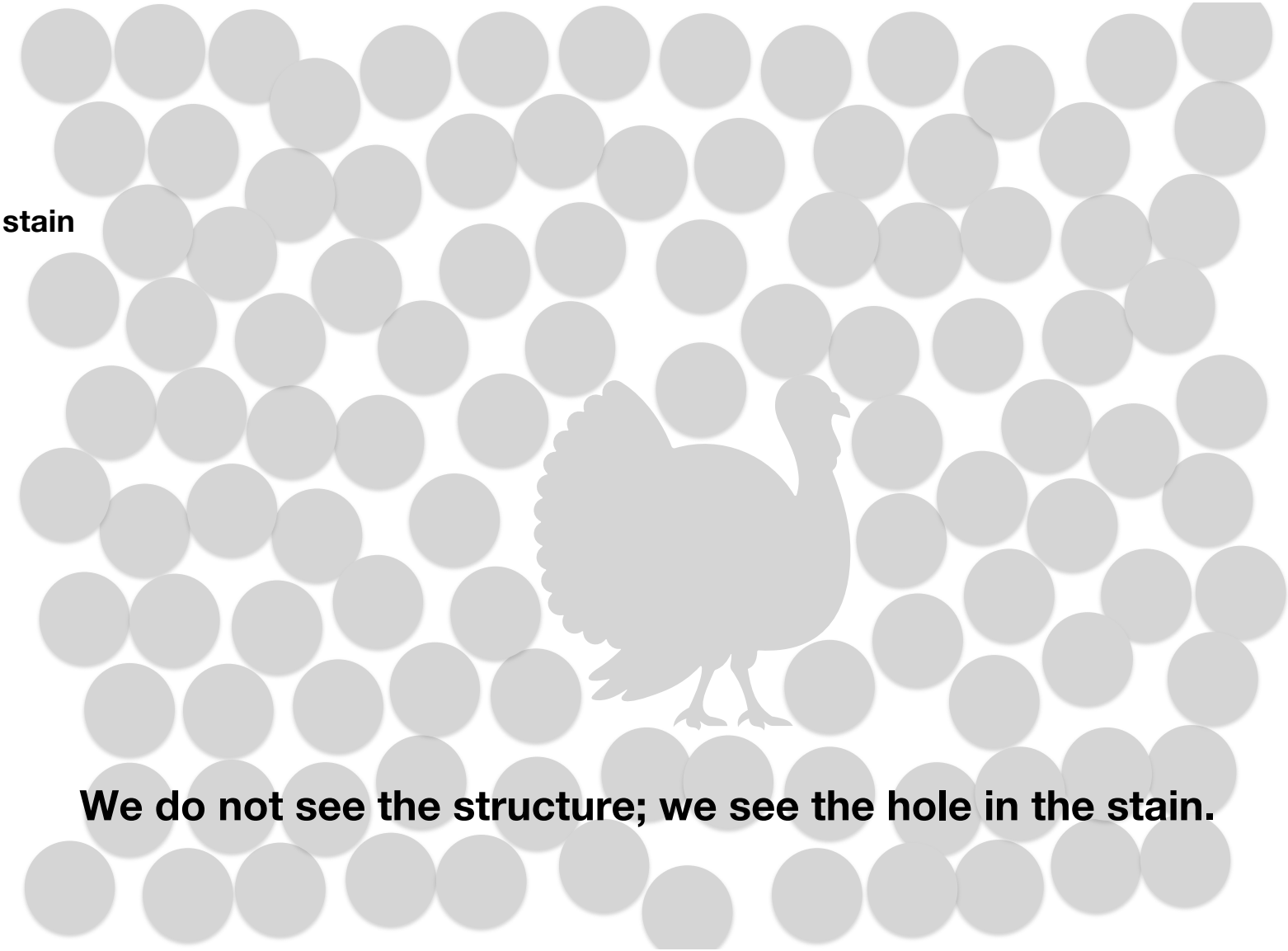


Number of electrons hitting image plane



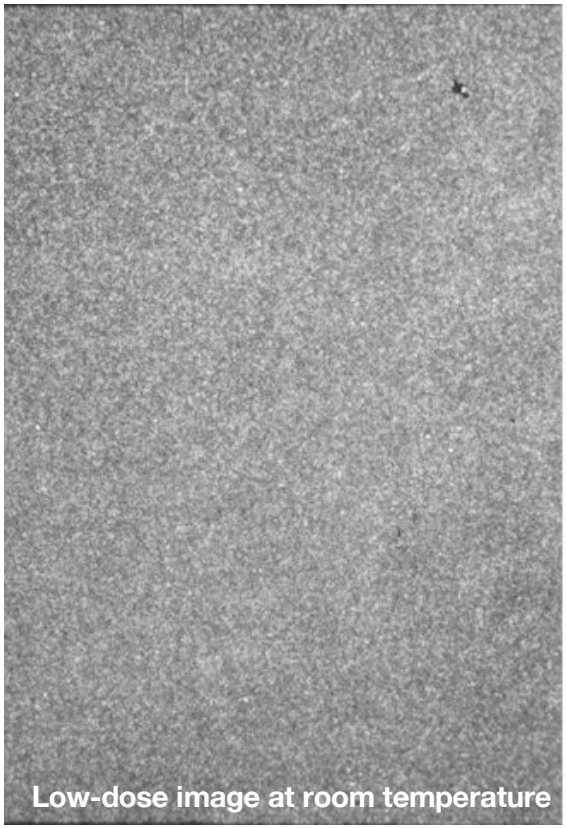
Resolution is limited because stain occurs as clumps about 1 nm in size.

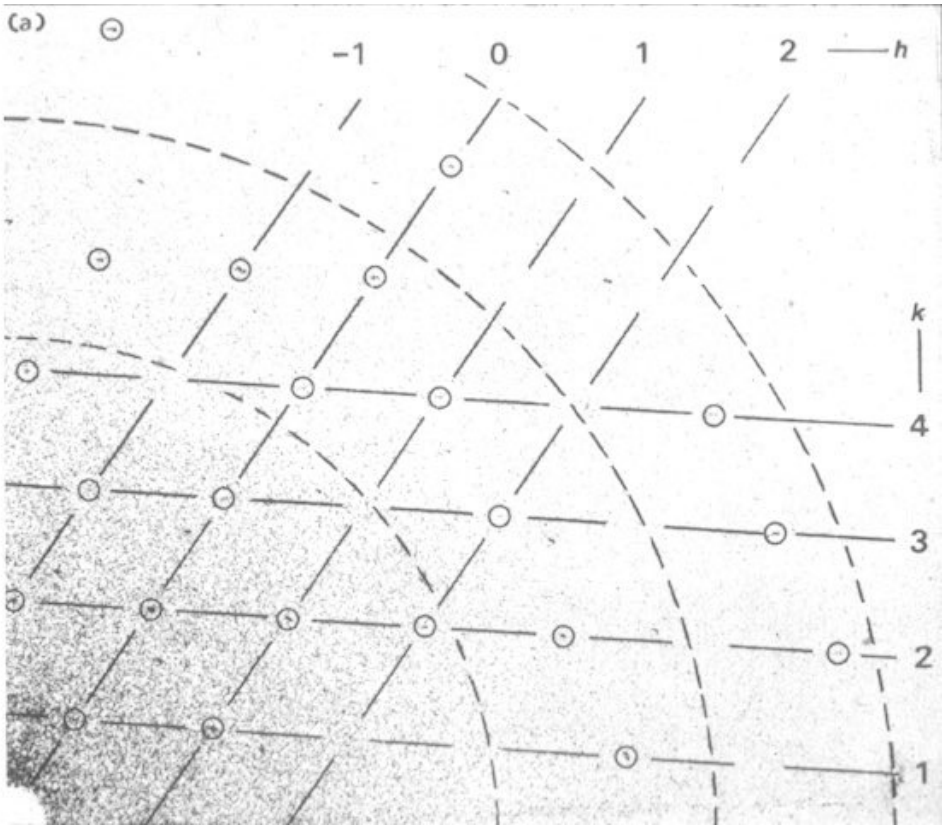
Clumps of stain



We do not see the structure; we see the hole in the stain.

Unwin and Henderson replaced negative stain with glucose, which preserved the protein in the vacuum of the microscope. The sample is a 2D crystalline membrane protein, bacterial rhodopsin.





Fourier transform of image.

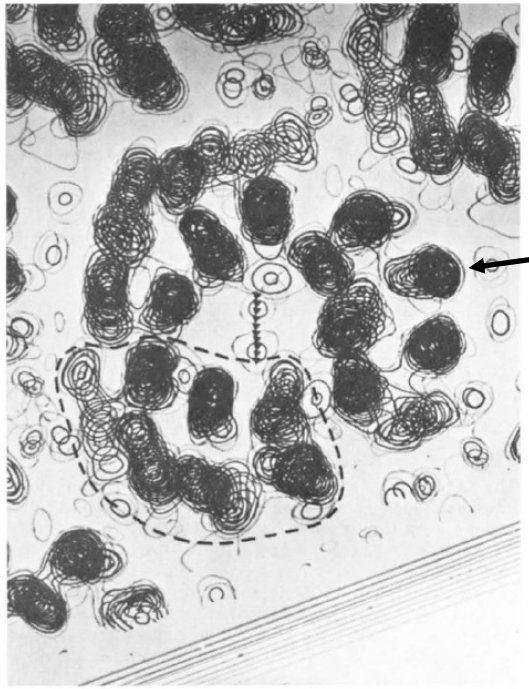
Even though not visible to our eyes, the structural features are preserved to at least ~ 7Å resolution.

Unwin PN, Henderson R. J Mol Biol. 1975 May 25;94(3):425-40.

They collected tilts and could see, in 3D, the protein instead of the hole in the negative stain.

This was also the first 3D map of a membrane protein!

Nigel Unwin and Richard Henderson



Each column of density is an alpha helix.

But … glucose is not a good embedding agent for single particles because the particles are 'hidden' in the equal-density glucose layer.

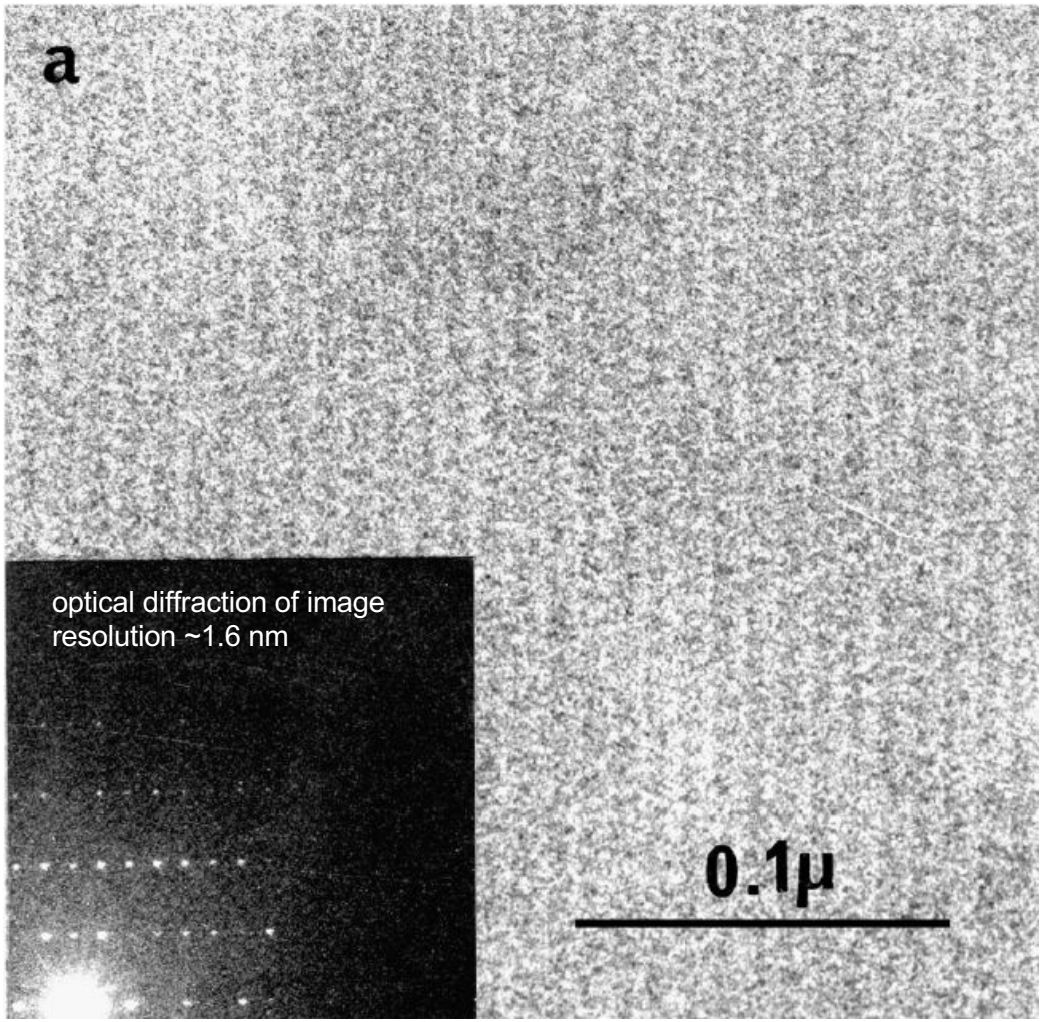
3D map of crystalline bacterial rhodopsin at 7Å resolution

Unwin PN, Henderson R. J Mol Biol. 1975 May 25;94(3):425-40.
Henderson R, Unwin PN. Nature. 1975 Sep 4;257(5521):28-32..

Ice is a better embedding agent than glucose because it is less dense than glucose.

Bob Glaeser

Ken Taylor



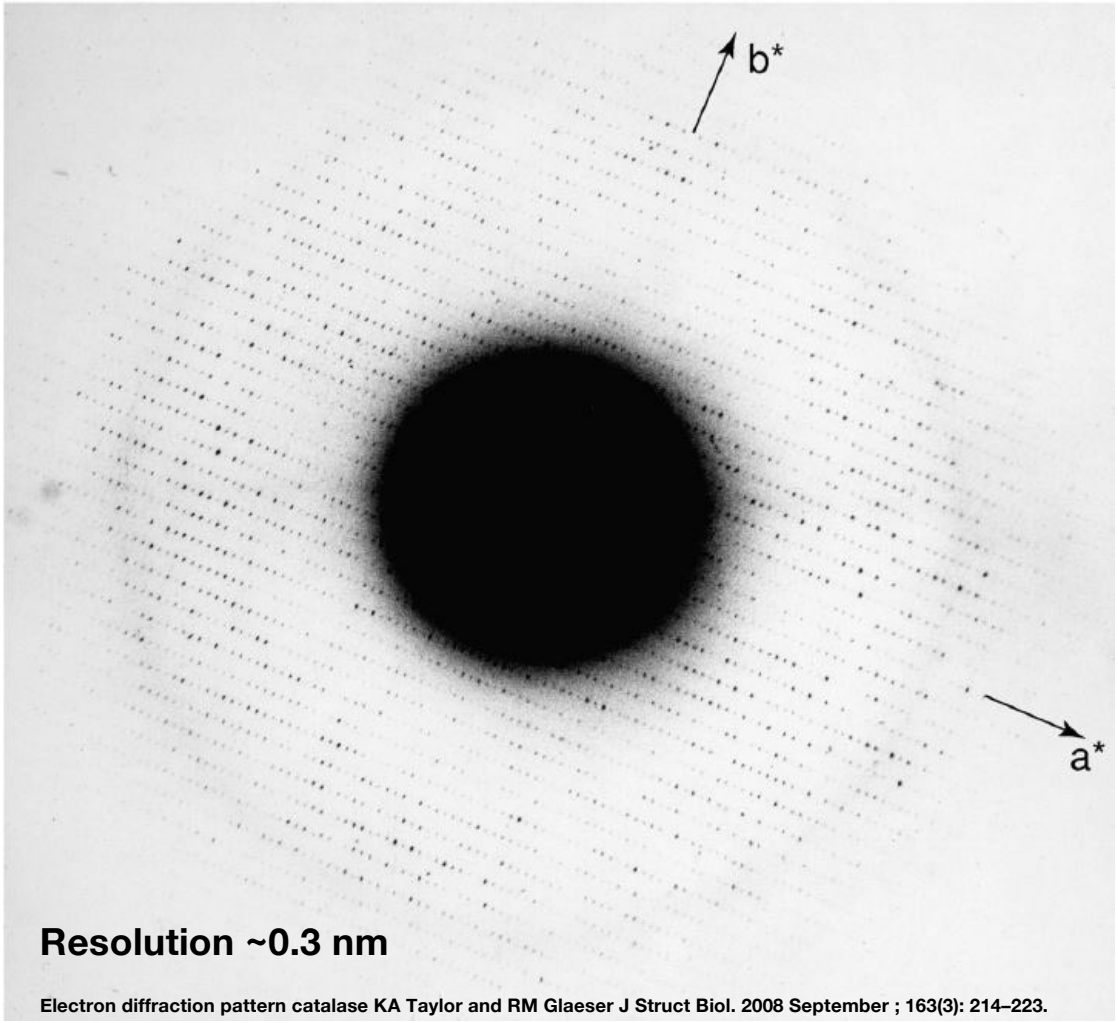
Frozen-hydrated specimens

Thin crystal of catalase in ice

Was the preservation of structural detail only as good as negative stain i.e.,16 Å?

Taylor KA. J Microsc.1978; 112:115–125.

No … the electron diffraction pattern shows preservation in ice is good to near atomic resolution.

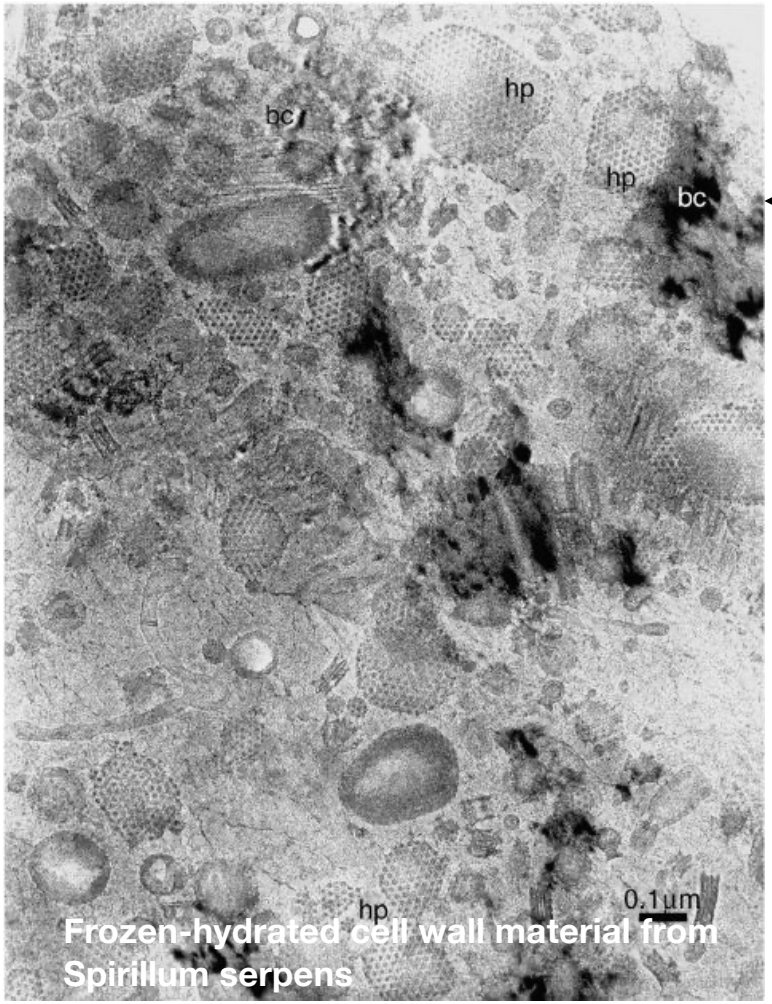


Electron diffraction pattern catalase KA Taylor and RM Glaeser J Struct Biol. 2008 September ; 163(3): 214–223.

Taylor KA, Glaeser RM. Science. 1974 Dec 13;186(4168):1036-7.

The good news: frozen water embedding make particles visible.

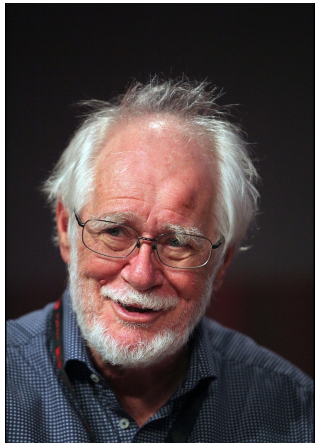
The bad news: ice crystals alter contrast and disrupt structures.



Frozen-hydrated cell wall material from Spirillum serpens

bc = bend contour, which shows the ice is crystalline

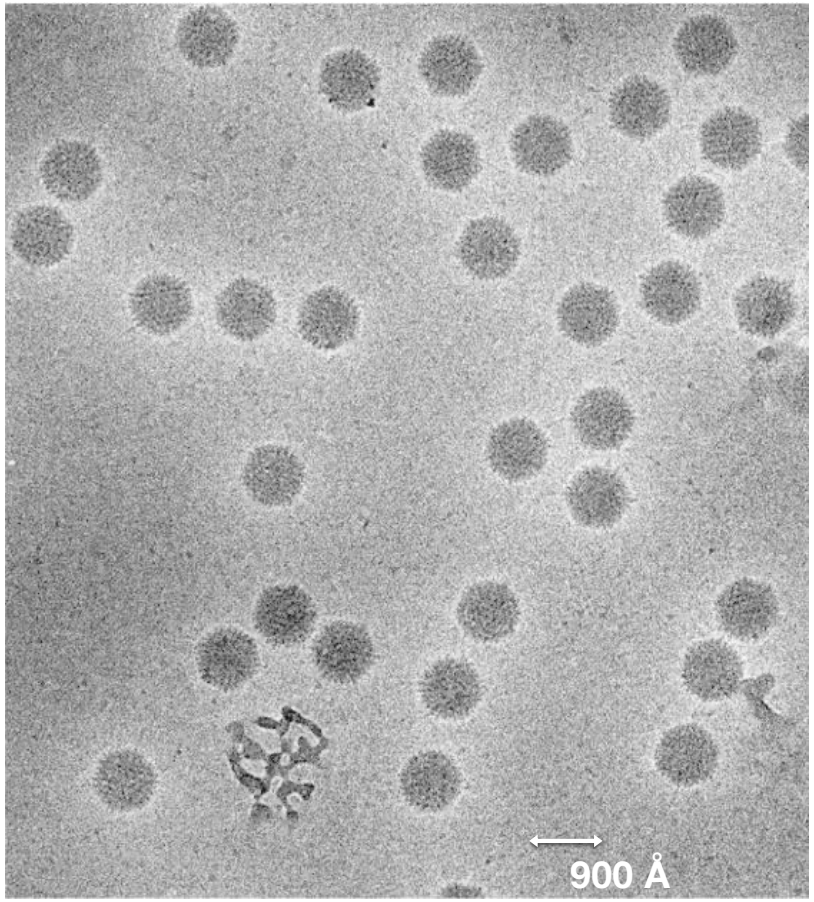
Taylor KA. Structure determination of frozen, hydrated, crystalline biological specimens. J Microsc.1978; 112:115–125.



Jacques Dubochet

Plunge freezing into liquid ethane (T<-140 C) produces vitreous as opposed to crystalline ice.

Adenovirus in amorphous (vitreous) ice

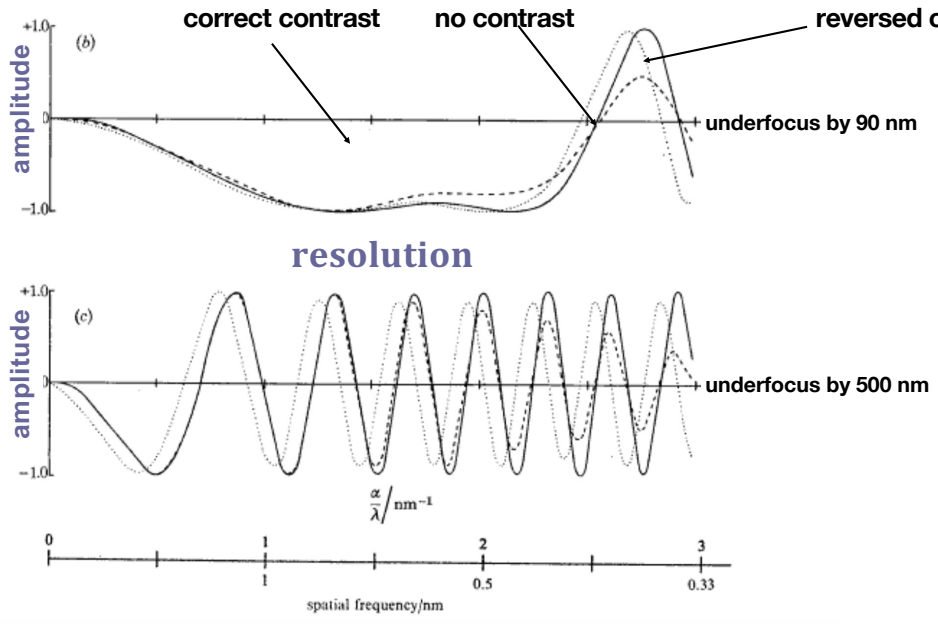


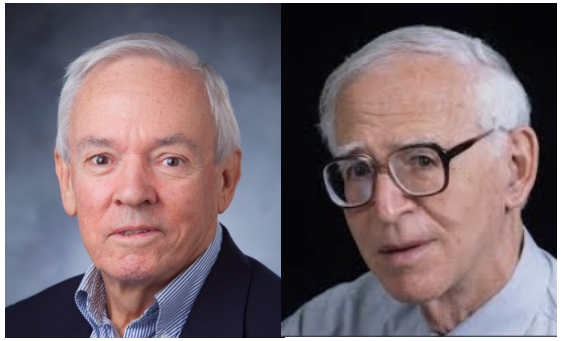
Adrian M, Dubochet J, Lepault J, McDowall AW. Cryo-electron microscopy of viruses. Nature. 1984; 308:32–36.

Particles in focus in vitreous ice are transparent: no contrast!

Defocusing produces phase contrast but it alters the information in the image.

Contrast transfer function (CTF)



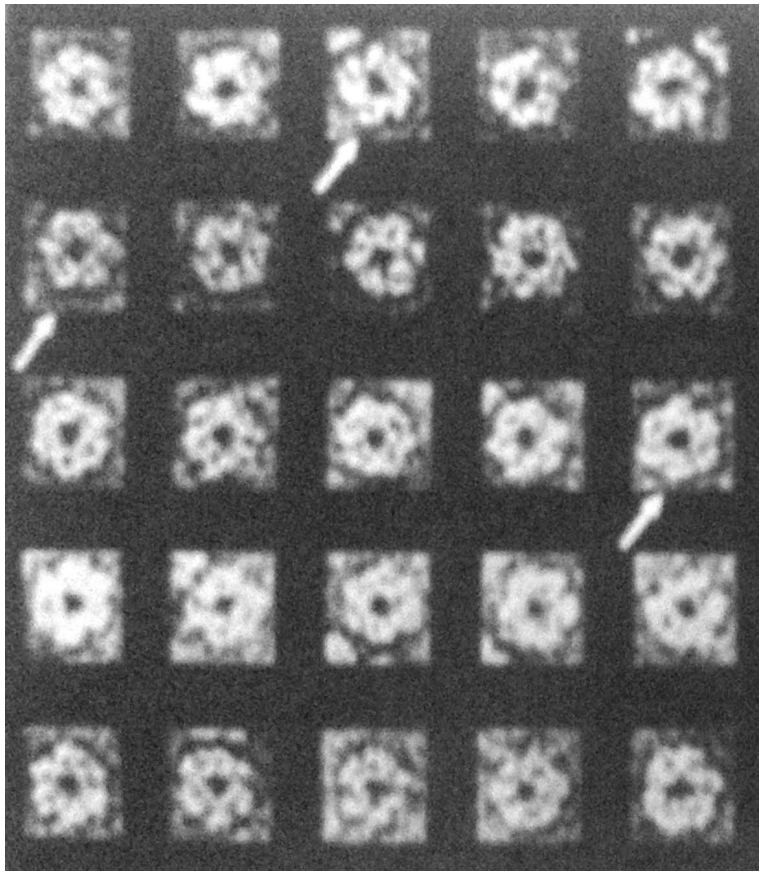


Harold Erickson **Aaron Klug**

Erickson and Klug showed how image contrast is generated by defocus, how it is altered by defocus, and

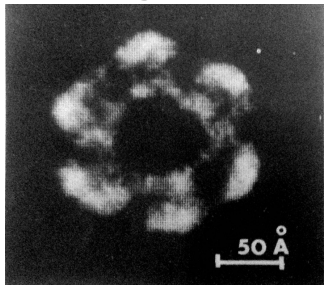
H. P. Erickson and Aaron Klug. Measurement and compensation of defocusing and aberrations by Fourier processing of electron micrographs Phil Trans Roy Soc Lond B Volume 261Issue 837 p 105-118 , 1971.

Single particle averaging using Frank's SPIDER, the first general, scriptable software package

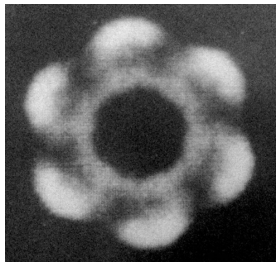


Joachim Frank

Averaged image



Rotationally averaged



Frank, J., Goldfarb, W., Eisenberg, D., and Baker, T.S. (1978). Reconstruction of glutamine synthetase using computer averaging. Ultramicroscopy 3, 283-290.

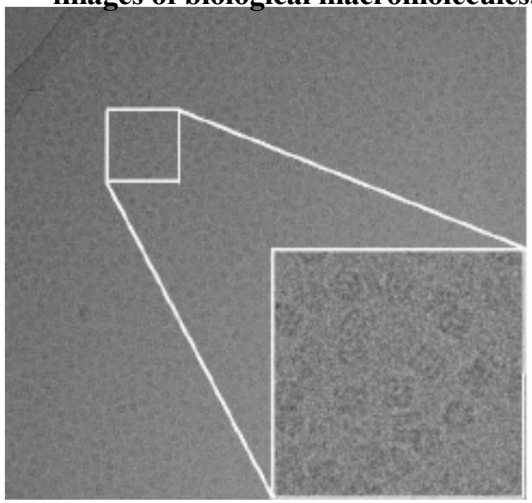
Multivariate statistics, introduced into SPIDER by Frank and van Heel, allowed them to sort particle images into classes and produce class averages.

Class averages can sort images by orientation, conformation, and composition!

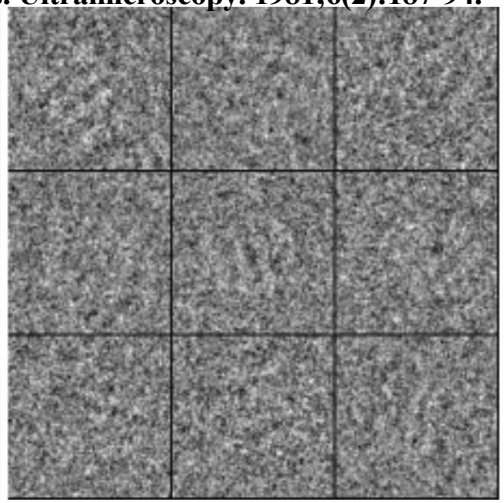
Joachim Frank

Marin van Heel

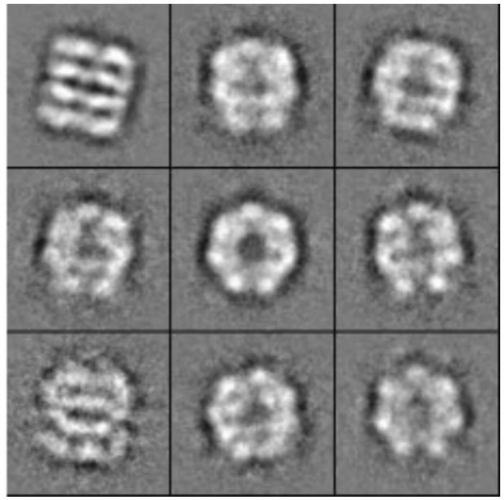
van Heel M, Frank J. Use of multivariate statistics in analysing the images of biological macromolecules. Ultramicroscopy. 1981;6(2):187-94.



Cryo EM image



Particle images cut out from micrograph



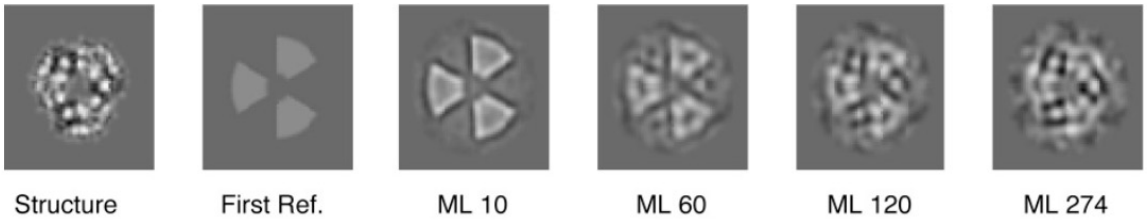
average of images in each class

Mallick, S. Agarwal, S., Kriegman, D., & Belongie, Serge 2006 Vision in the Small: Reconstructing the Structure of Protein Macromolecules from Cryo-Electr

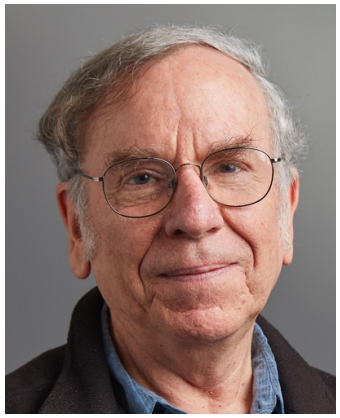
Maximum-likelihood

Particle images are added into every class average but weighted by the probability they belong to that class.

Cross correlation alignment and averaging



Maximum-likelihood alignment and averaging

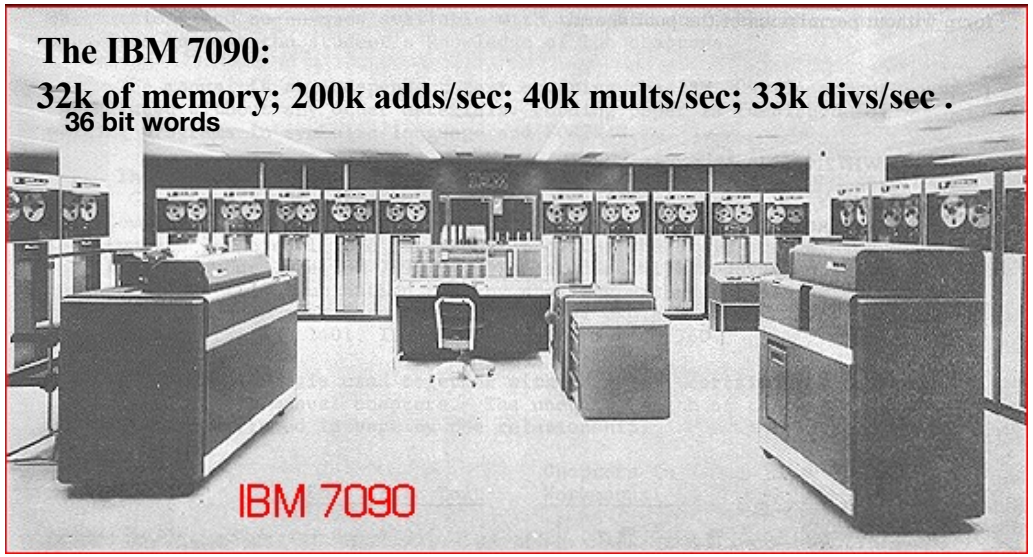


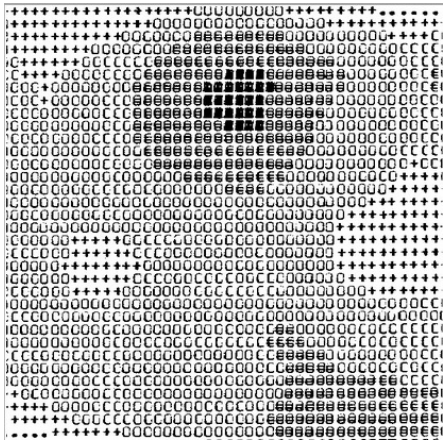
Fred Sigworth

Maximum-likelihood prevents iterative alignment from bias and from falling into local minima.

Sigworth, F. J. A maximum-likelihood approach to single-particle image refinement. J. Struct. Biol. 122: 328-339, 1998.

An obvious important development: faster computers with more memory and graphics.





Visualizing digital images by overprinting to generate pseudo densities.

http://employees.oneonta.edu/baumanpr/geosat2/RS%20History%201960-2000/RS-History-1960-2000.htm

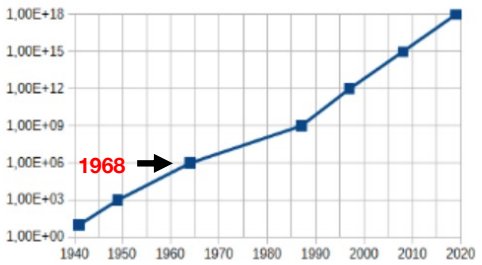
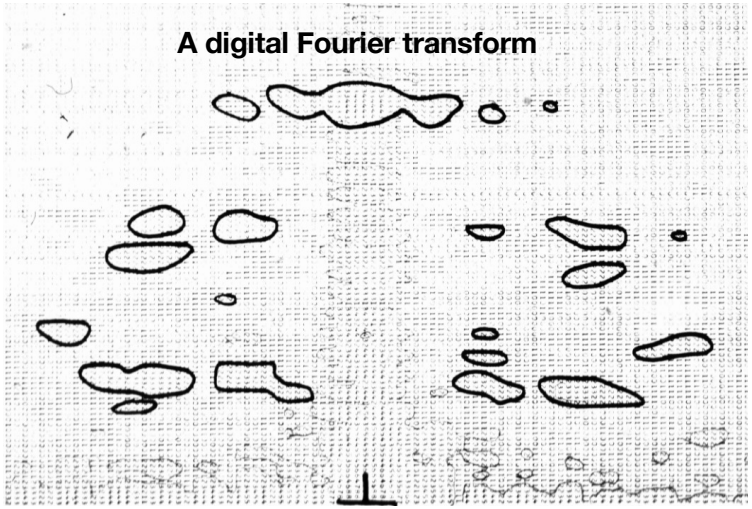


Figure 1: Computer Performance Evolution Over Time (FLOPs)

Amplitudes scaled from 0 to 9.

Phases printed in 10 degree intervals using character A to Z and 0 to 9.



https://study.com/academy/lesson/using-mips-flops-as-computer-performance-parameters.html

Many, many, many improvements in software since the MRC software in 1970 and Joachim Frank's single par

There are now many software packages available to use singly or in series.

Many improvements in the electron microscope.

Multiple-grid, stable cryo EM stages

Higher voltage, which is good for thicker specimens but probably not for very thin ones.

Field emission guns with better coherence, which dropped structural resolution from 7 to about 4 Å.

Direct electron detectors with speed and sensitivity, which further improved resolution to 2 Å and better.

Energy filters, which removed inelastically scattered electrons thus improving contrast.

1933

Invented by Ernst Ruska

Wikipedia

1968

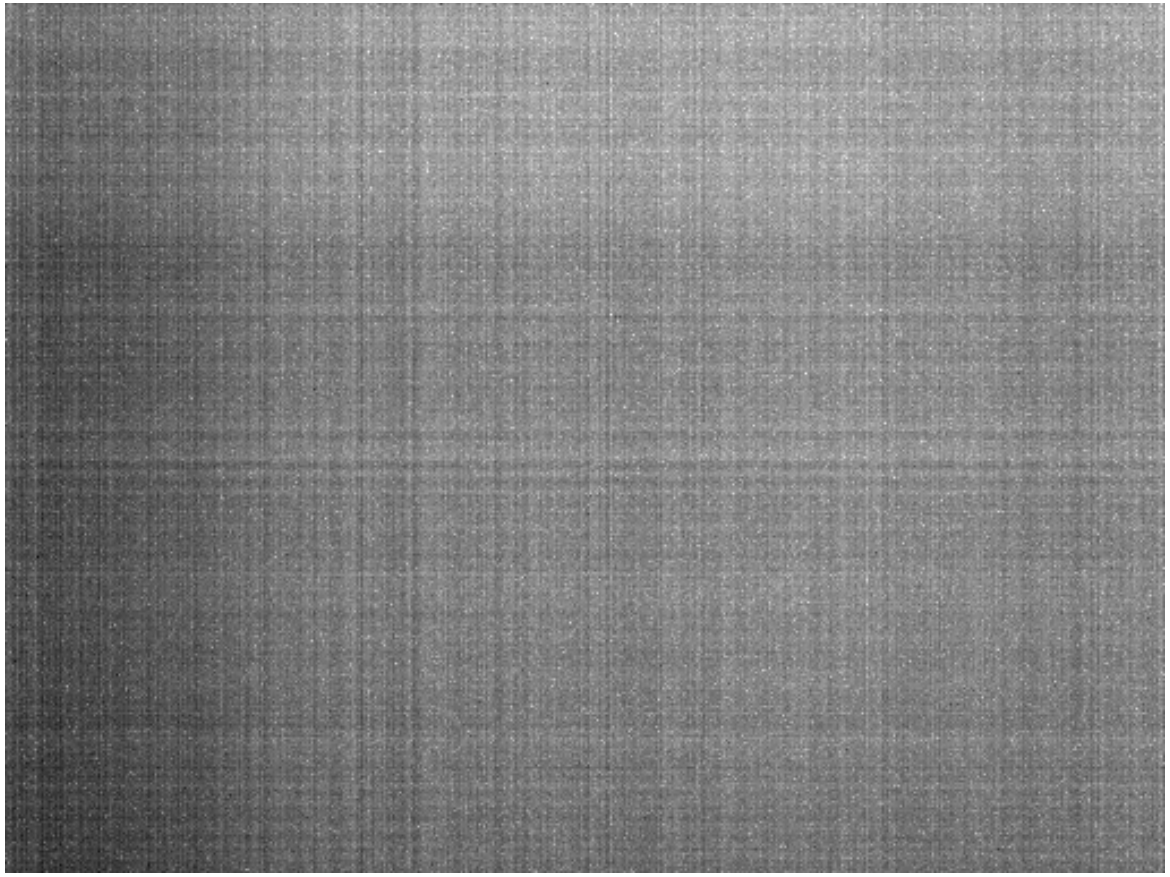
https:/collection.sciencemuseumgroup.org.uk/objects/co8648182/main-component-of-siemens-elmiskop-1-electron-microscope-electron-microscope

2024

https://www.thermofisher.com/us/en/home/electron-microscopy/products/transmission-electron-microscopes/krios-g4-cryo-tem.html

Beam induced motion (BIM) prevented resolutions below 4A.

The direct electron detectors made it possible to break a single exposure into a movie of many frames and correct for the motion.

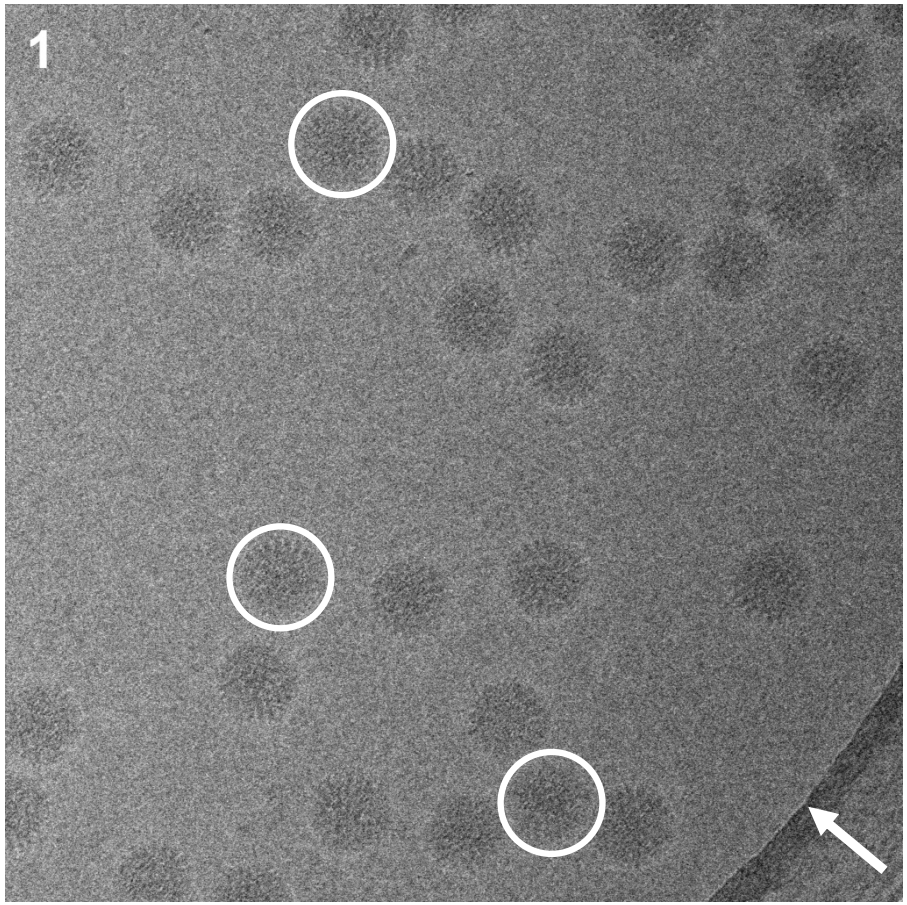


Recorded with direct electron detector DE-12 (Direct Electron)

Frame rate = 40 fps
Dose/frame = 0.5 $e^-/Å^2$
Duration = 1.5 s
No. of frames = 60
Total dose = 30 $e^-/Å^2$

J Struct Biol. 2012;177:630-7. Beam-induced motion of vitrified specimen on holey carbon film.Brilot AF, Chen JZ, Cheng A, Pan J, Harrison SC, Potter CS, Carragher B, Henderson R, Grigorieff N.

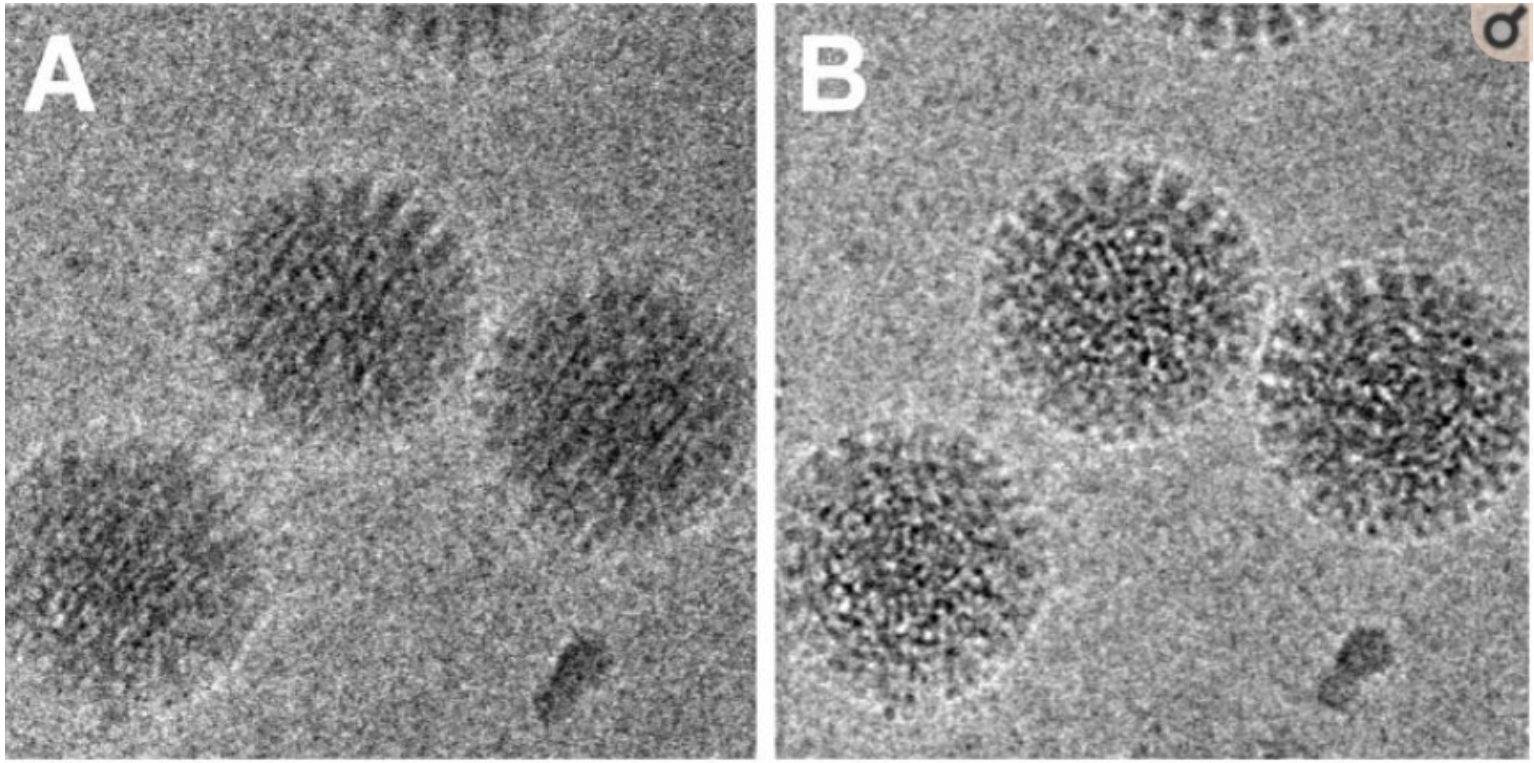
10-Frame Averages made from the movie better reveal the motion.



Each averaged frame corresponds to 0.25 s.

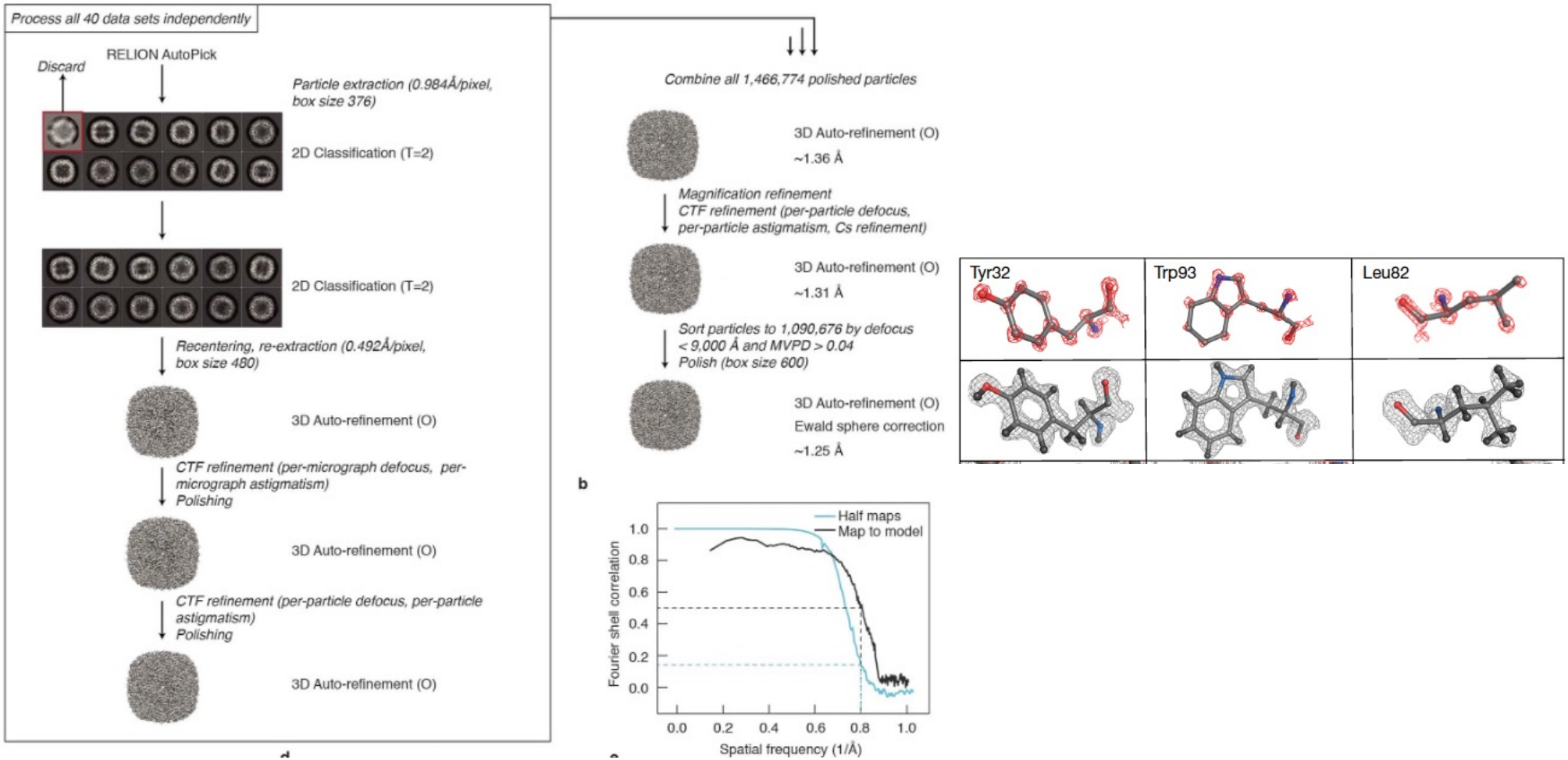
Dose/frame = 5 $e^-/Å^2$

Uncorrected for motion **Corrected for motion**



The direct electron detectors have all but eliminated the loss of resolution due to BIM.

The resolution revolution combining all the improvements: a 1.25 Å resolution map of apoferritin

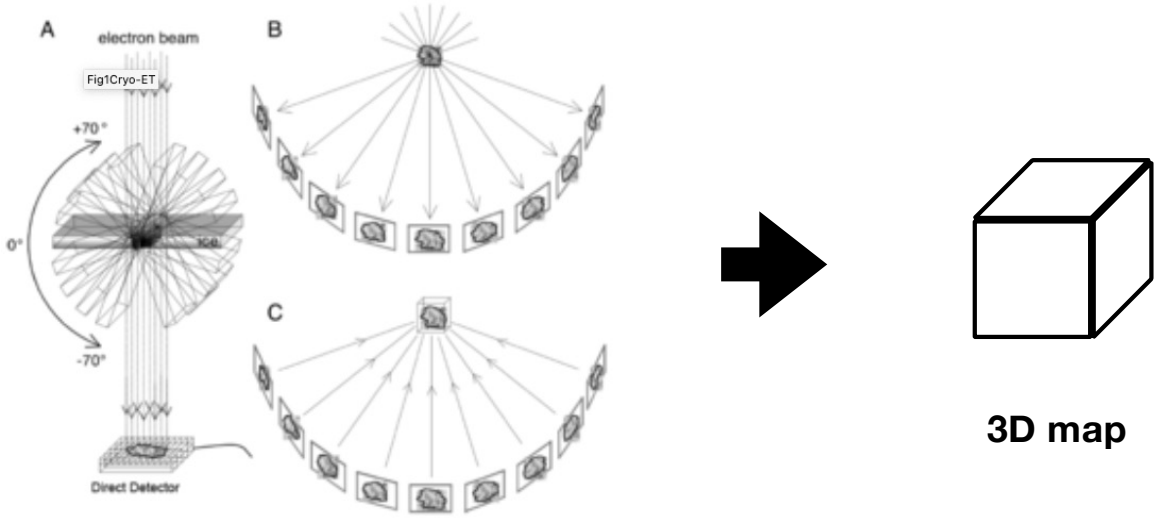


Yip KM, Fischer N, Paknia E, Chari A, Stark H. Atomic-resolution protein structure determination by cryo-EM. Nature. 2020;587:157-161.

Cryo electron tomography (Cryo-ET)

We turn from looking at single particles to looking at cells or organelles.

We can generate a tomogram (3D map) by tilting the sample from ~-60 to ~+60

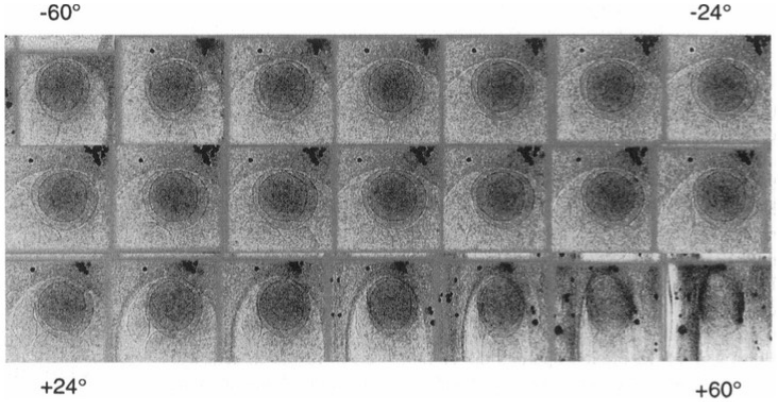


Steven A, Belnap D. Electron microscopy and image processing: an essential tool for structural analysis of macromolecules. Curr Protoc Protein Sci. 2005 Dec;Chapter 17:17.2.1-17.2.39.

Cryo-ET of lipid vesicles in ice!

Wolfgang Baumeister

Tilt series



Slice from the tomogram

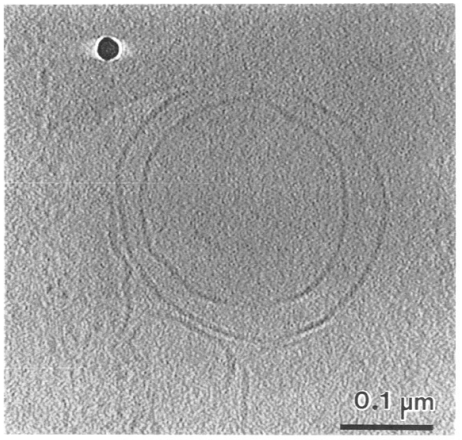
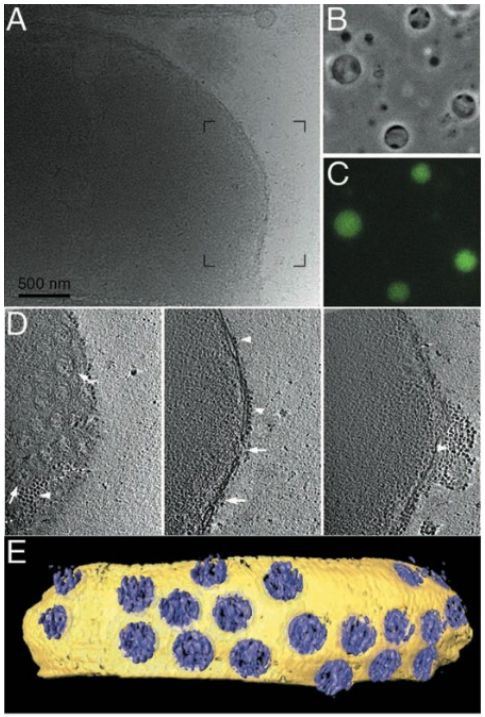


FIGURE 4 Central *x-y* section through the 3D reconstructed volume at full resolution, with frequency cutoff at $(5\ nm)^{-1}$.

Tomography is possible with frozen hydrated samples!

Dierksen K, Typke D, Hegerl R, Walz J, Sackmann E, Baumeister W. Three-dimensional structure of lipid vesicles embedded in vitreous ice and investigated by aut

Sub tomogram averaging, the 3D version of 2D single particle analysis.

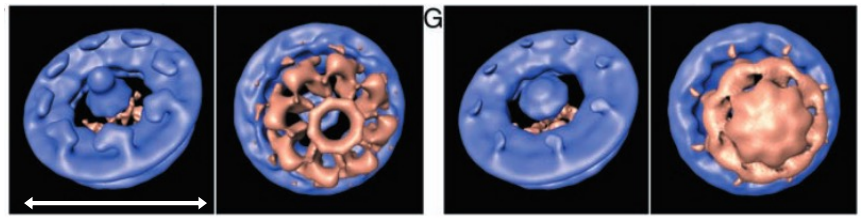


Cryo ET of 2 μ intact nucleus

Resolution ~9 nm

Sub tomogram averages

CF class **LR class**



1250 Å

Sub tomogram averaging: best resolution to date is ~3Å!

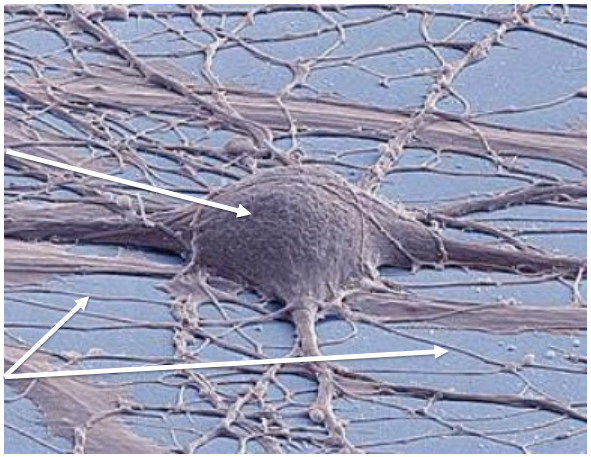
Walz J, Typke D, Nitsch M, Koster AJ, Hegerl R, Baumeister W. Electron Tomography of Single Ice-Embedded Macromolecules: Three-Dimensional

Beck M, Förster F, Ecke M, Plitzko JM, Melchior F, Gerisch G, Baumeister W, Medalia O. Nuclear pore complex structure and dynamics revealed by cryoelectron tomography. Science. 2004 Nov 19;306(5700):1387-90.

While cryo-ET could look into cells, it was limited by the penetrating power of the EM.

The cell body is too thick to get an electron beam through.

Filopodia, for example, are thin enough to get the electron beam through.



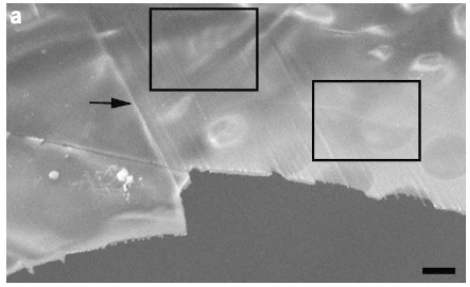
This is a scanning electron micrograph (false color) of a human induced pluripotent stem cell-derived neuron. Credit: Thomas Deerinck, UC San Diego

Focused Ion Beam milling of frozen-hydrated E. coli can make a sample thin enough for viewing in the EM.

Mike Marko and Mui

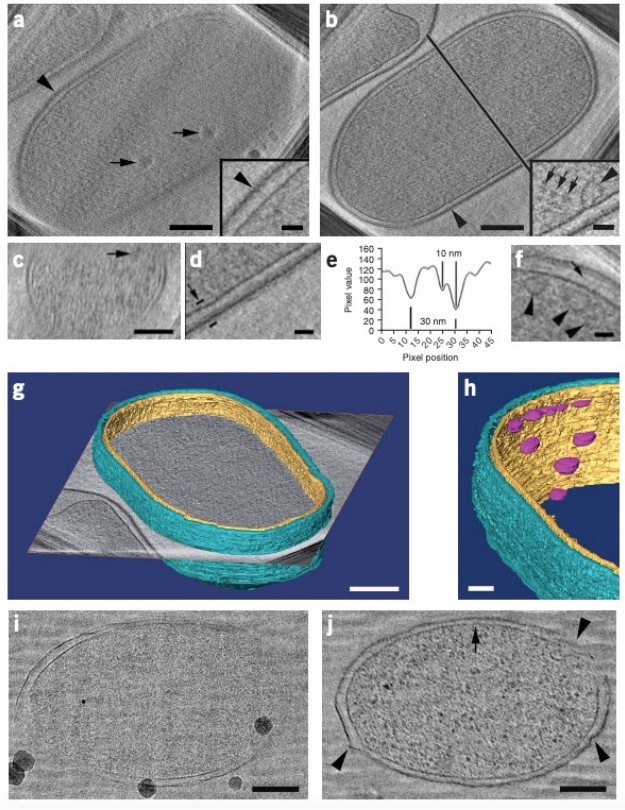
Varano, C. (2021). Mike Marko: Preserving the Past and Shaping the Future. *Microscopy Today, 29*(1), 56-57. doi:10.1017/S1551929520001741

Lamella

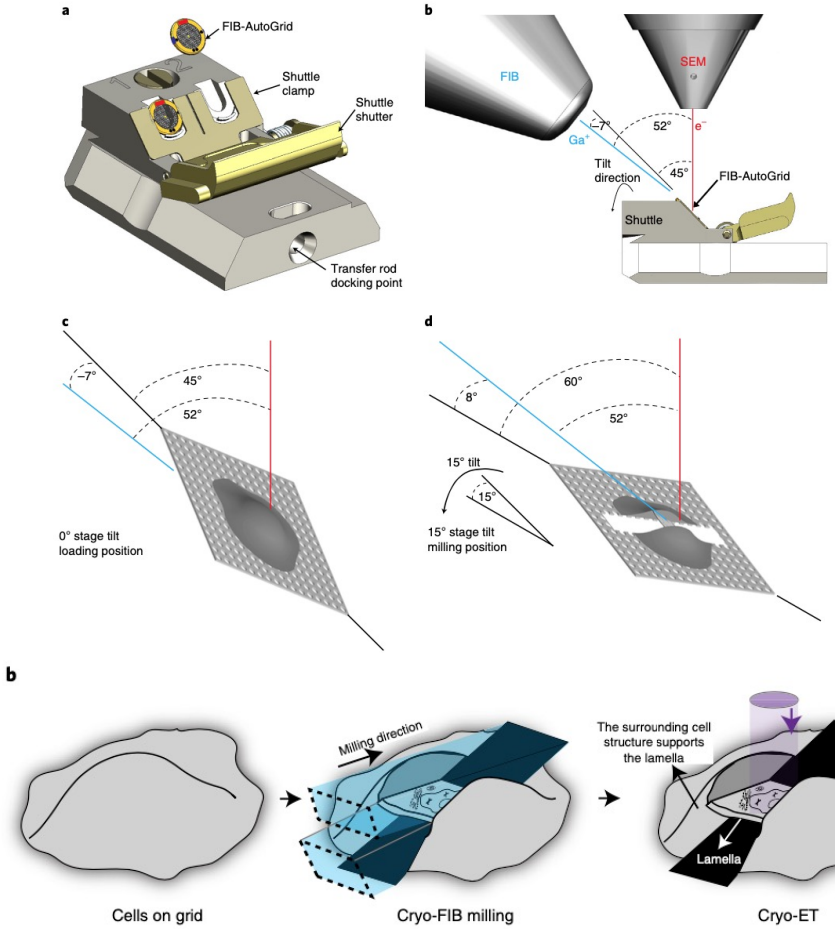


About 500 nm thick

Tomogram with segmentation



Marko M, Hsieh C, Schalek R, Frank J, Mannella C. Focused-ion-beam thinning of frozen-hydrated biological specimens for cryo-electron microsc



FIB-SEM as practiced today

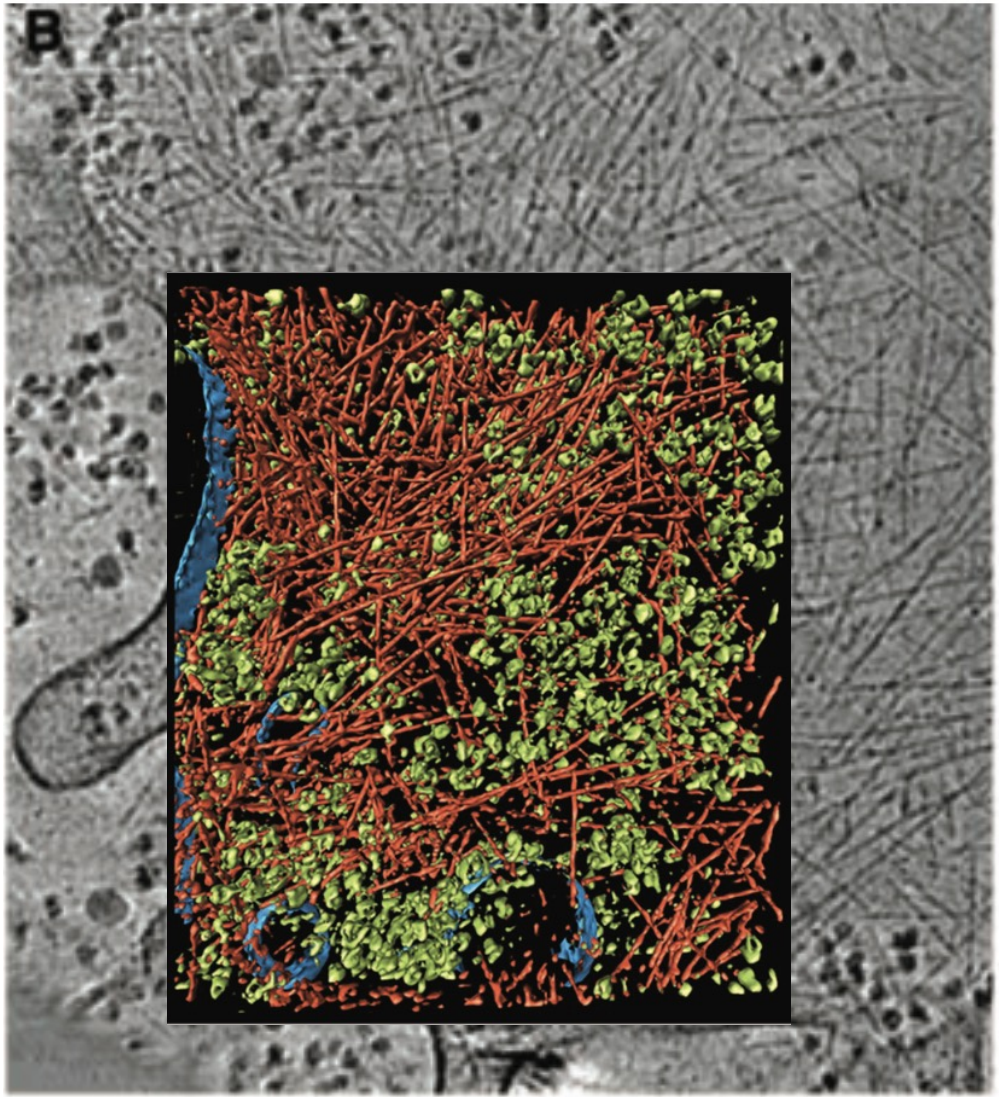
Thick specimens can be thinned and subjected to cryoET!

Wagner FR, Watanabe R, Schampers R, Singh D, Persoon H, Schaffer M, Fruhstorfer P, Plitzko J, Villa E. Preparing samples from whole cells using focused-ion-b

But how can we find our structures in a tomogram?

Medalia et al. introduced template matching. Using templates derived from the data bases, they located structures of interest in a tomogram.

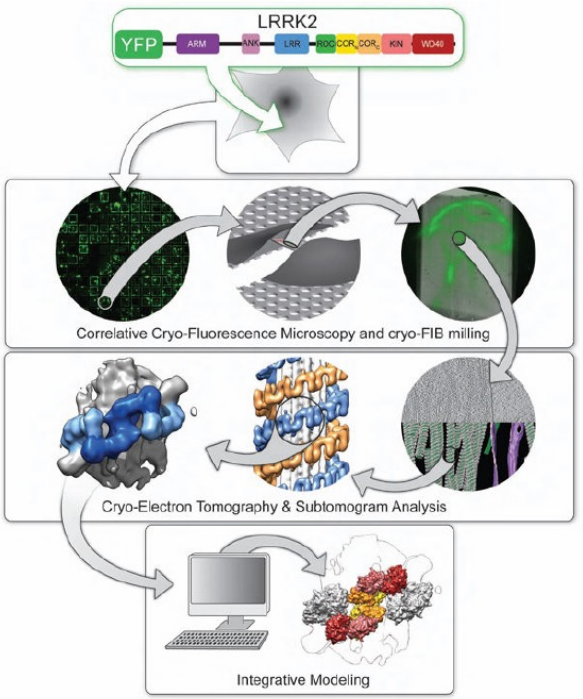
Having located membranes, filaments, and ribosomes in this 3D image, they replaced each located structure by a colored version of itself.

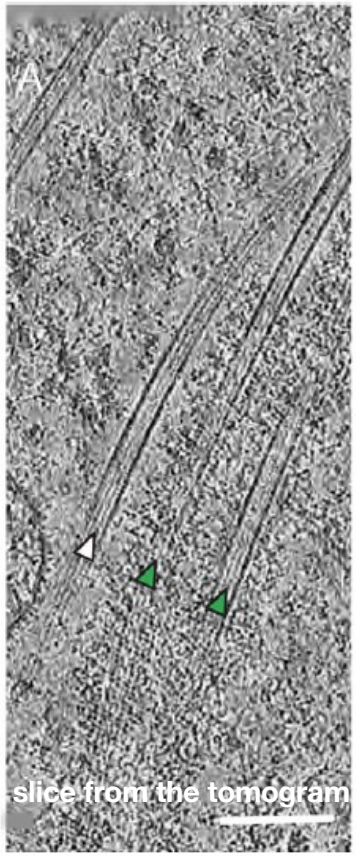


Medalia O, Weber I, Frangakis AS, Nicastro D, Gerisch G, Baumeister W. Macromolecular architecture in eukaryotic cells visualized by cryoelectron tomography. Science. 2002 Nov 8;298(5596):1209-13. doi: 10.1126/science.1076184. PMID: 12424373.

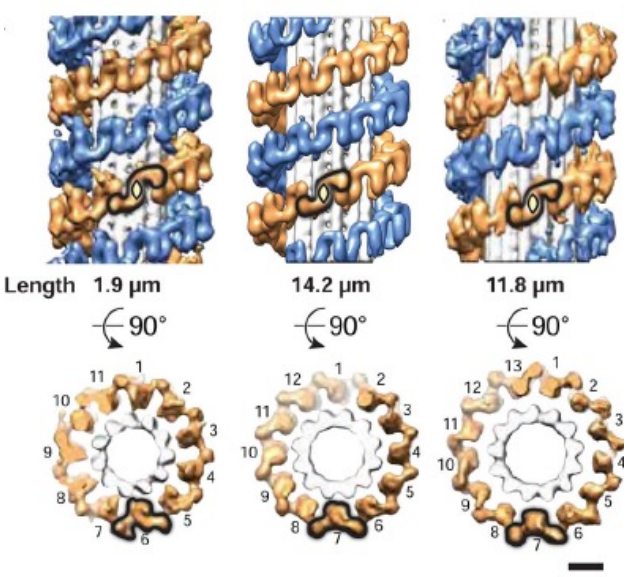
The two works below are a perfect example of using FIB milling, cryo-ET, subtomogram averaging, and modelin

work flow

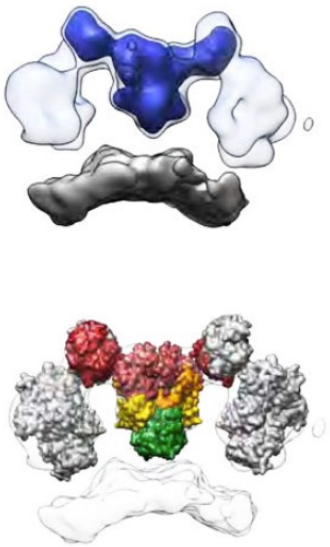




subtomogram averages of 3 classes



modeling to get 3D protein structure



Watanabe R, Buschauer R, Böhning J, Audagnotto M, Lasker K, Lu TW, Boassa D, Taylor S, Villa E. The In Situ Structure of Parkinson's Disease-Linke

Deniston CK, Salogiannis J, Mathea S, Snead DM, Lahiri I, Matyszewski M, Donosa O, Watanabe R, Böhning J, Shiau AK, Knapp S, Villa E, Reck-Peter

The **evolution,** **Challenges,** & **promise** of **cryo-electron microscopy**

David DeRosier, 2024

Challenges we can correct or (partially) control:

Electron damage to the specimen.

Underfocus phase contrast and astigmatism, which affect amplitudes and phases.

Digitization and boxing of the image, which affects amplitudes and phases.

Beam induced motion, which limits resolution.

Interpolation in digital image, which reduces high resolution amplitudes.

Image distortion by the lenses, which affects phases.

Beam tilt, which alters phases.

Insufficient depth of field, which alters amplitudes and phases.

Lack of plane parallel illumination, which alters phases.

Things we might not be able to control but we should be aware of:

Multiple scattering, which alters amplitudes and phases.

Inelastically scattered electrons, which add to the background.

Lack of coherence of the electron beam, which limits resolution.

Challenges in cryo electron tomography:

The tilt angle is limited to ~60 degrees (giving rise to the missing wedge from 60 to 120 degrees).

The incremental step in angle is limited by dose (giving rise to missing wedges between between slices).

The milling beam damages the outer layers of a lamella.

We are limited to a small volume of the cell (serial sectioning is not possible).

The process of milling and imaging is slow.

Our structure of interest is not in the lamella.

Lamellae are lost when moving from the FIB-SEM to the EM.

Ice contamination obscures details.

The **evolution, Challenges,** & **promise** of **cryo-electron microscopy**

David DeRosier, 2024

Some recent advances that hold promise:

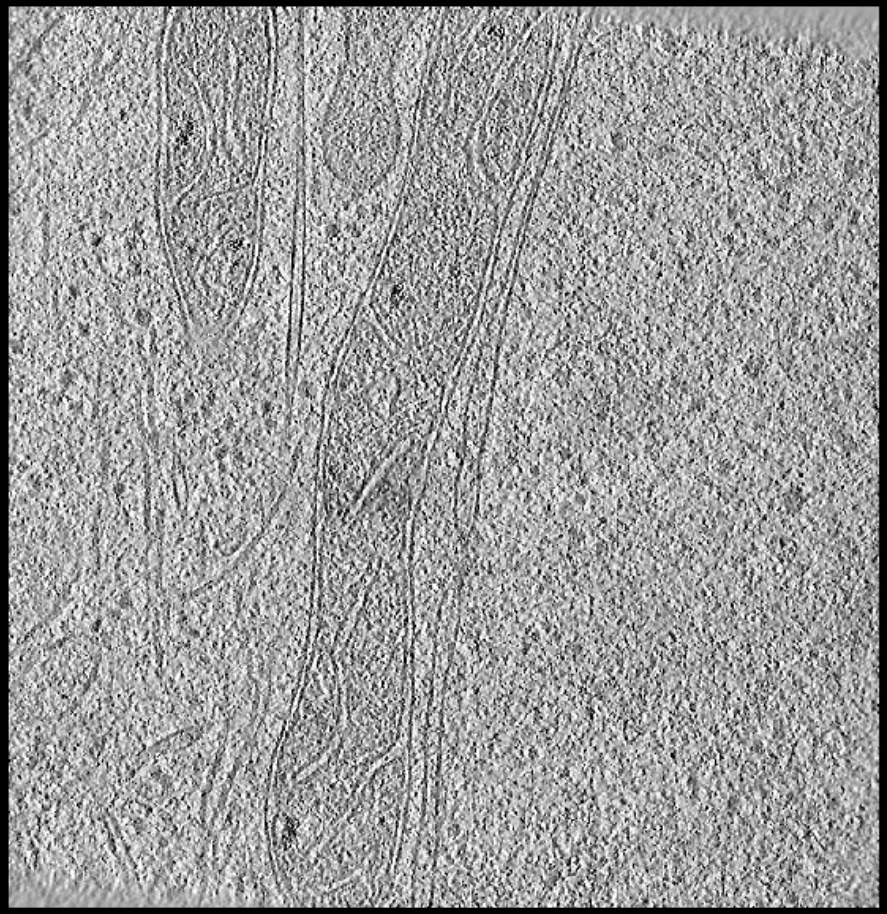
The laser phase plate, which is still experimental, provides almost perfect, stable phase contrast. No need to c

The development of clonable tags that are easily recognized by template matching in images and tomograms.

AI or ML aided image analysis in particle picking, clustering, segmenting and more. It is still being developed

The combined use of super-resolution cryo light microscopy with cryo electron tomography, which would give us more precise locations of our target molecules.

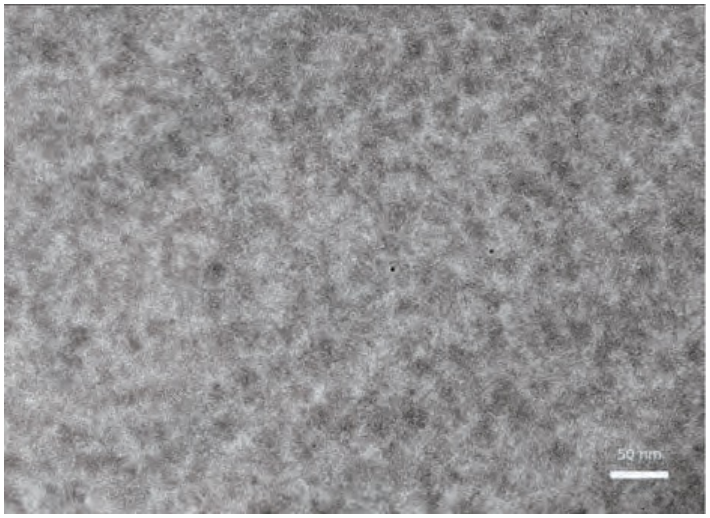
To give you a better feel for the amazing technology of today even with all its challenges, here is a vi



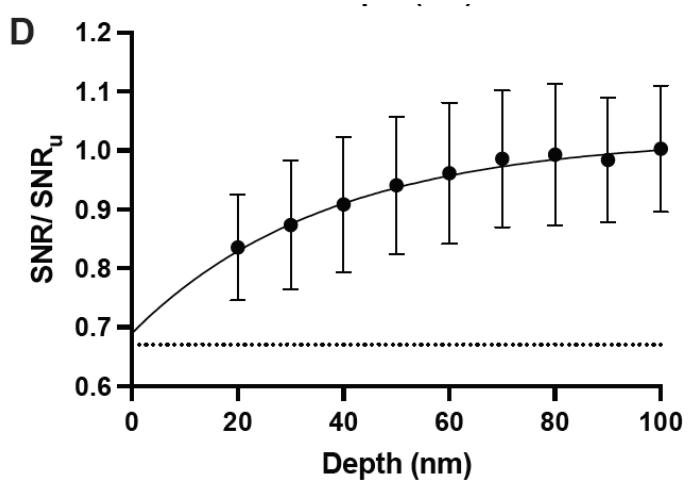
Wagner, F.R., Watanabe, R., Schampers, R. *et al.* Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomogra

We over estimate what will be done in the short term and under estimate what will be done in the long term.
(Bob Glaeser quotes this - the originator of the expression is unclear).

FIB damages particles near the faces of the lamella



Lamella from yeast packed with ribosomes



The rise of SNR with distance from the lamella face

Lucas BA, Grigorieff N. Quantification of gallium cryo-FIB milling damage in biological lamellae. Proc Natl Acad Sci U S A. 2023 Jun 6;120(23):e23(

What we can expect or hope for in single particle cryo-EM to get us all to <2Å:

A stable phase plate may allow us to determine structures of mw = 12,500 (Henderson limit).
Update: The laser phase plate provides almost perfect, stable phase contrast.

A simple reliable method for loading samples onto grids with thin ice, no denaturation at the air water inter

Complete automation from grid preparation to atomic model.

Easy access from images to the energy landscape of conformational forms; we want to visualize images and distributions of conformations within our structures.

What we can expect or hope for in cryo ET: complete segmentation of all structures in a tomogram.

Better lamella production: limited damage from FIB-SEM, no ice contamination, and no broken lamellae.

Localization of fluorescent markers with an error <~10nm and transfer of coordinates to the FIB-SEM for milling and to the EM images for segmentation.

Structural tags as opposed to fluorescent tags for structures of interest.

Identification and location of structures of interest with a tomogram.