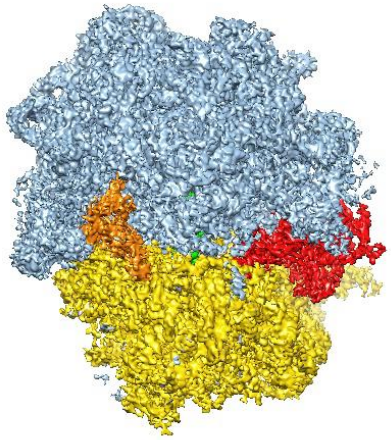
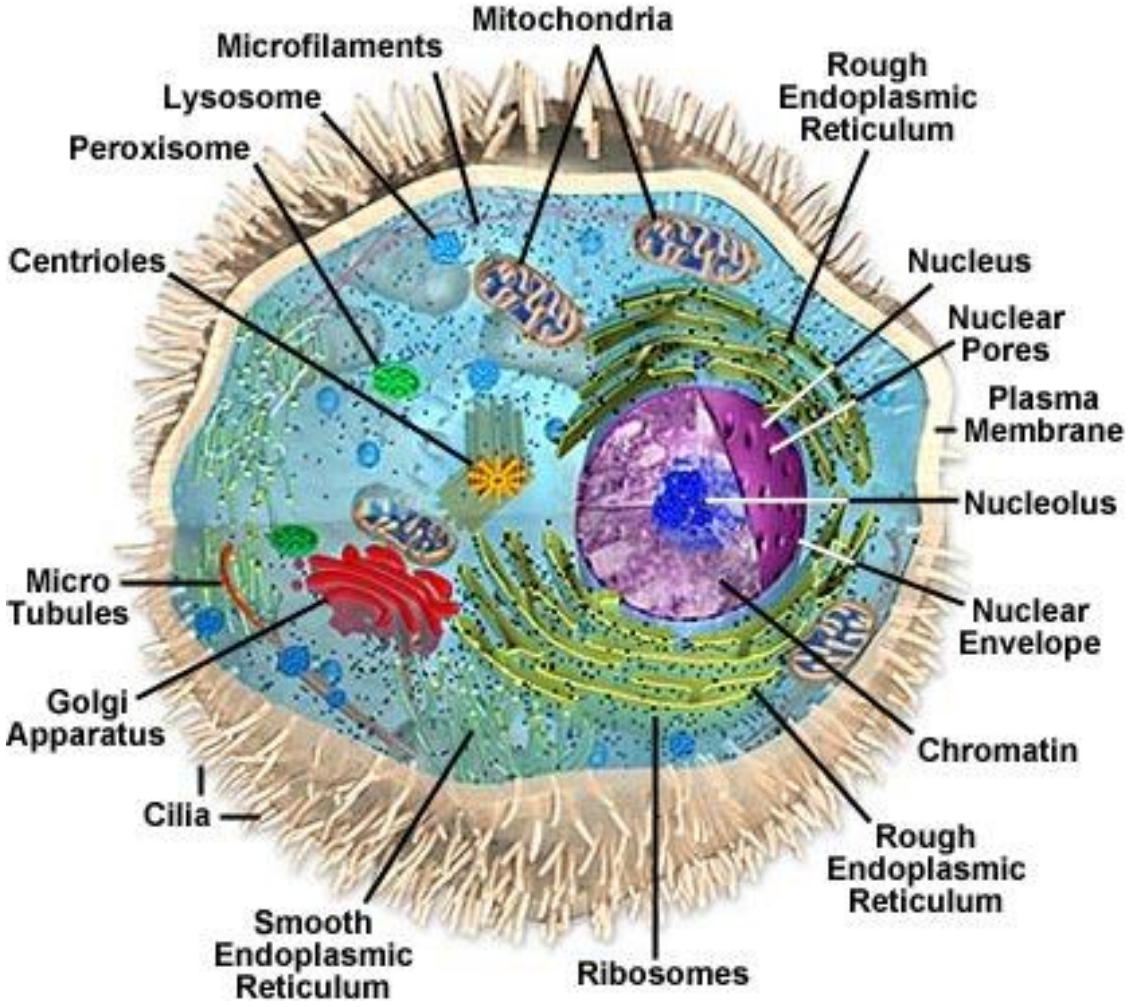
Single-particle Cryo-EM -- Visualization of Biological Molecules in their Native States



Joachim Frank
Department of Biochemistry and Molecular Biophysics
Department of Biological Sciences
Columbia University

Funding : National Institutes of Health

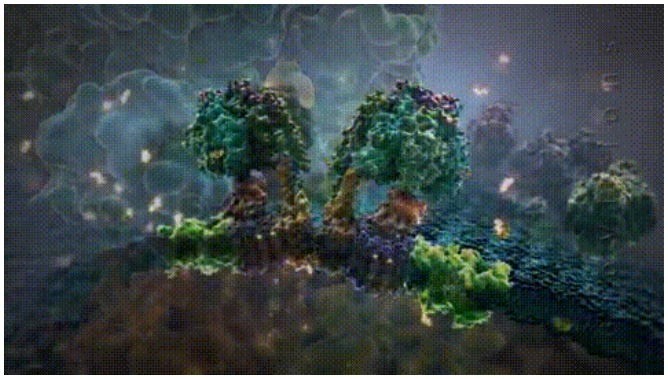

Mitochondria
Microfilaments
Lysosome
Peroxisome
Rough Endoplasmic Reticulum
Centrioles
Nucleus
Nuclear Pores
Plasma Membrane
Nucleolus
Micro Tubules
Nuclear Envelope
Golgi Apparatus
Chromatin
Cilia
Rough Endoplasmic Reticulum
Smooth Endoplasmic Reticulum
Ribosomes

M.W. Davidson

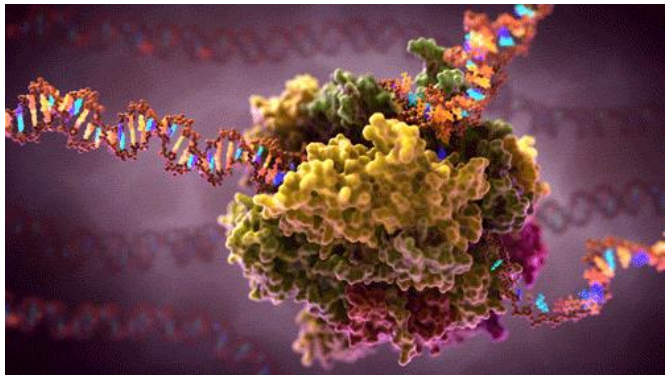
doi: 10.7554/eLife.19274

Crowded environment inside a cell, simulated on the Stampede supercomputer by Michael Feig of Michigan State University.
MD simulation shows a few ns of dynamics in atomistic detail of bacterial cytoplasm.

Molecular Machines in the Cell



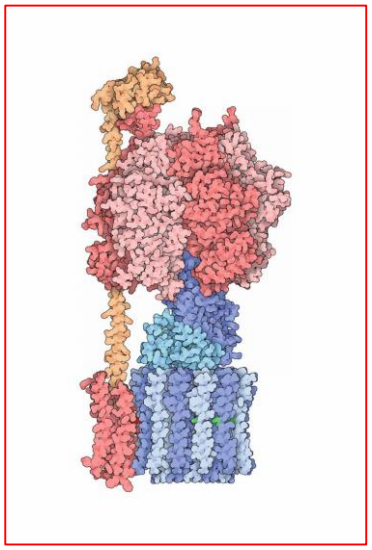
ATP Synthase



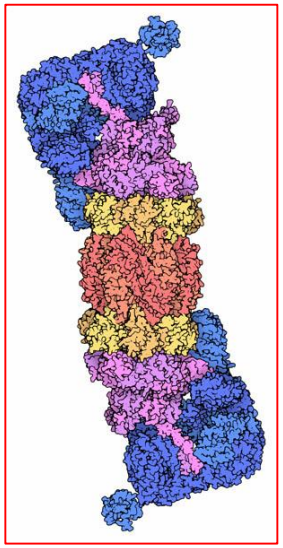
RNA Polymerase *(Art of the Cell)*

Bruce Alberts, Cell 1998

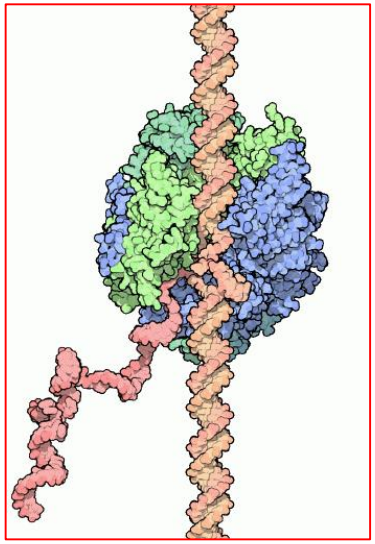
- Molecular machines: many molecules act in concert, in a processive way
- We wish to know the structures of all components but also the way they interact dynamically
- Reductionism: we study a subsystem in isolation (in vitro), hoping to approximate the processes in the environment of the cell
-



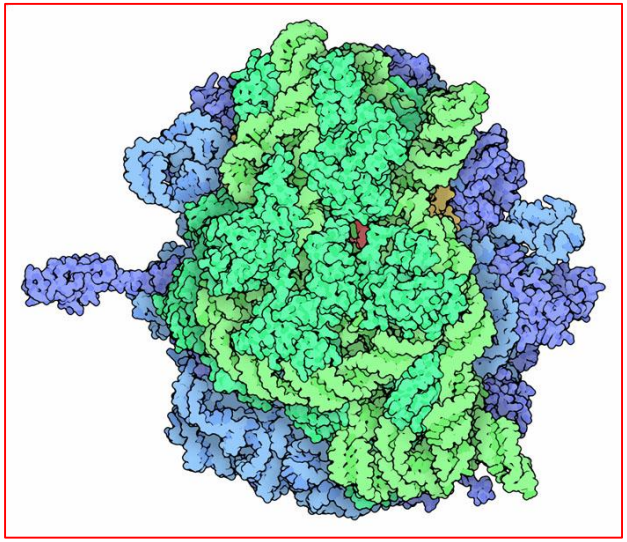
ATP Synthase:
makes ATP



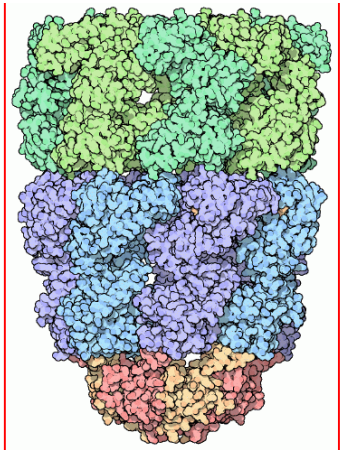
Proteasome:
recycles proteins



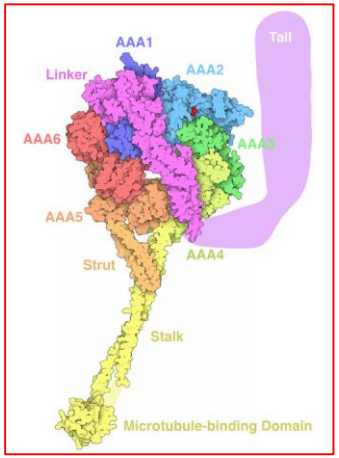
RNA Polymerase:
copies DNA → mRNA



Ribosome:
makes proteins

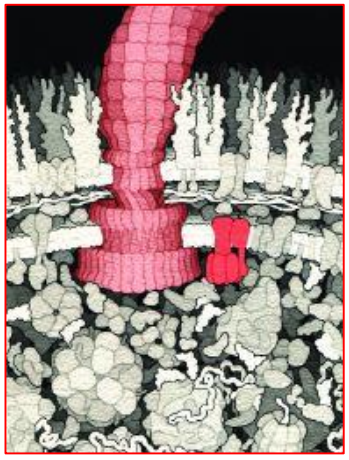


Chaperone:
folds proteins



Dynein:
transports molecules

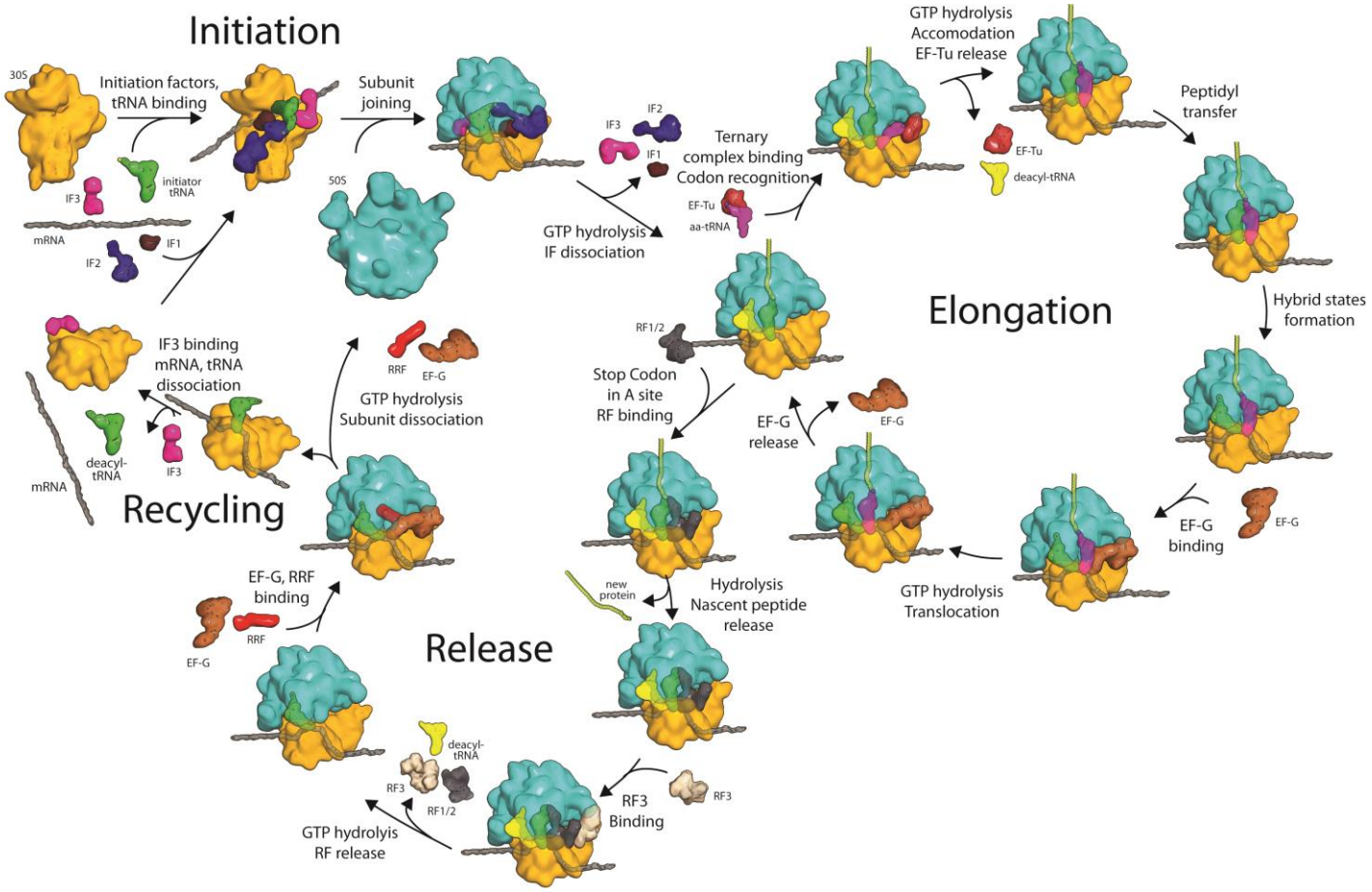
Spliceosome:
edits mRNA



Flagella motor:
rotates flagella

David Goodsell

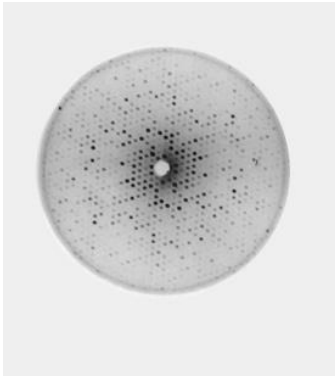
MULTIPLE STATES OF A MOLECULAR MACHINE: THE RIBOSOME

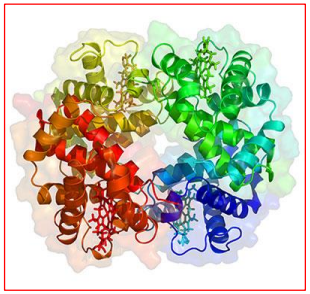


Schmeing and Ramakrishnan, Nature 2009

X-ray Crystallography

- Crystal: many copies of the molecule arranged in regular order.
- *Exposure to X-ray beam → diffraction pattern → structure determination.*
- X-ray beam must be high-intensity, crystal must be almost perfect.
- *To date ~ 140,000 structures solved by X-ray crystallography, available in public databanks.*
- Crystal packing → molecules not visualized in all conformations/binding states that important for function.
- *Many molecules do not form highly ordered crystals.*
- Sample quantity can be a big issue, as well.
-



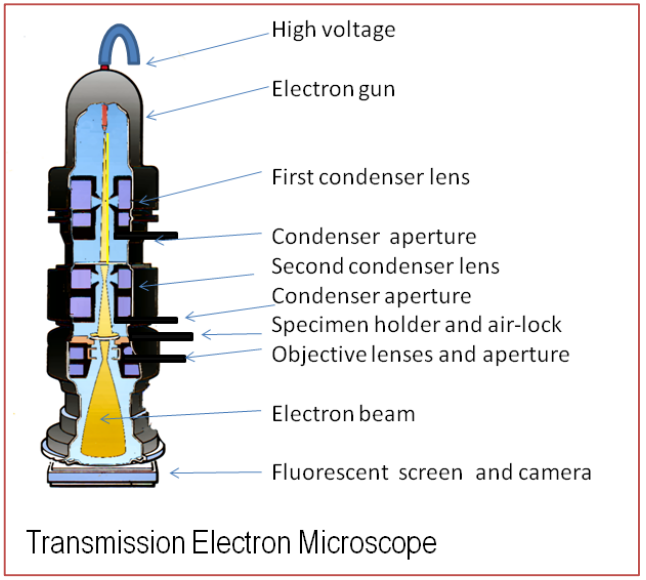


Max Perutz and John Kendrew with a model of hemoglobin, 1962

http://www.mfpl.ac.at/vips/max-f-perutz/

Electron Microscopy

- Electron microscopy can be used to solve molecular structures, as well.
- *Projection images formed at very high magnification, e.g. 30,000 x.*
- To reconstruct an object, many different views must be collected.
- *Sample must be very thin, electrons are readily absorbed by matter.*
- BUT: Electrons strongly damage the molecules -- need for low dose! 10-20 electrons/square Angstrom.
- *Images are very noisy (shot noise)*
-

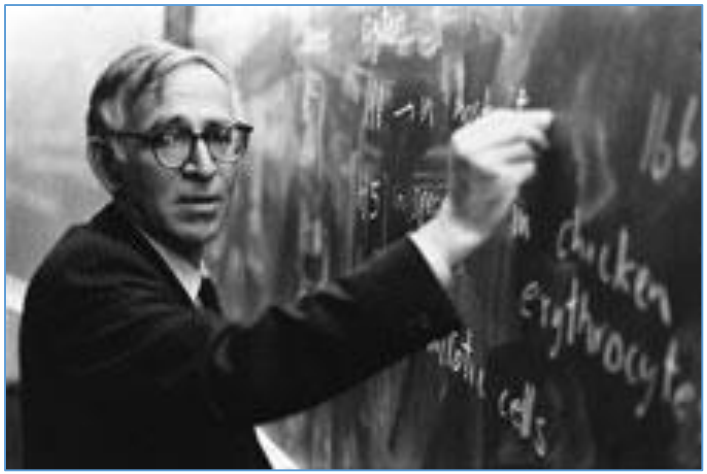


http://www.newworldencyclopedia.org/entry/File:Electron Microscope.png

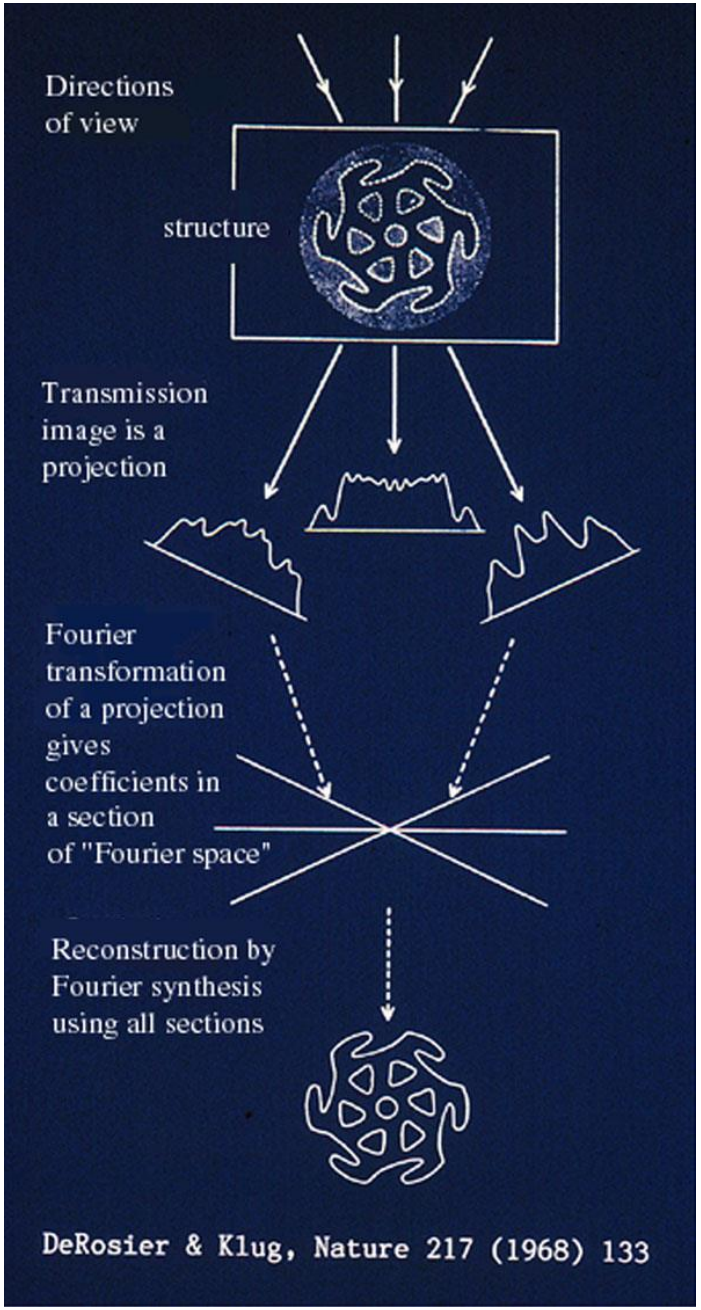
THREE-DIMENSIONAL RECONSTRUCTION:
STRUCTURES WITH HELICAL SYMMETERY
(negative staining used)

Pioneering work: 3D reconstruction of a bacteriophage tail using the Fourier-Bessel approach, 1968

Application of the Projection-Slice Theorem



Aaron Klug and David DeRosier, LMB/MRC Cambridge



THREE-DIMENSIONAL RECONSTRUCTION: VIRUSES WITH ICOSAHEDRAL SYMMETRY (negative staining used)

Tony Crowther

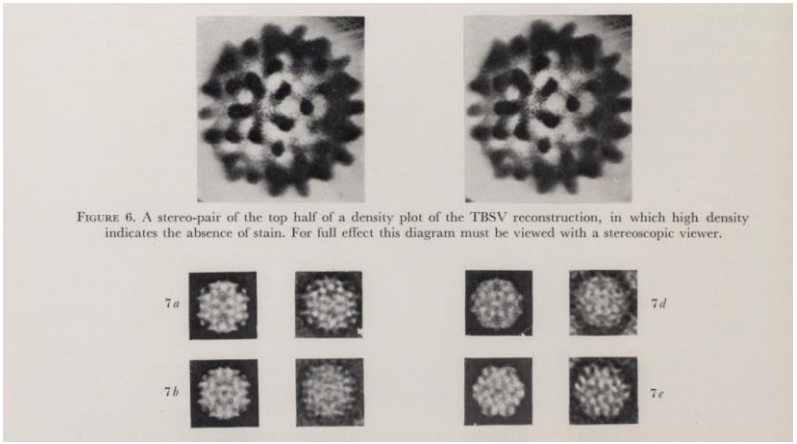


FIGURE 6. A stereo-pair of the top half of a density plot of the TBSV reconstruction, in which high density indicates the absence of stain. For full effect this diagram must be viewed with a stereoscopic viewer.

7*a* 7*b* 7*d* 7*e*

R. A. Crowther, Phil. Trans. Roy. Soc. 1971

THREE-DIMENSIONAL RECONSTRUCTION: STRUCTURES THAT FORM 2D CRYSTALS (glucose embedding used)

(Reprinted from Nature, Vol. 257, No. 5521, pp. 28–32, September 4, 1975)

Please don't throw away

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

A 7-Å resolution map of the purple membrane has been obtained by electron microscopy of tilted, unstained specimens. The protein in the membrane contains seven, closely packed, α-helical segments which extend roughly perpendicular to the plane of the membrane for most of its width. Lipid bilayer regions fill the spaces between the protein molecules.

THE purple membrane is a specialised part of the cell membrane of *Halobacterium halobium*[1]. Oesterhelt and Stoeckenius[2] have shown that it functions *in vivo* as a light-driven hydrogen ion pump involved in photosynthesis. It contains identical protein molecules of molecular weight 26,000, which make up 75% of the total mass, and lipid which makes up the remaining 25% (ref. 3). Retinal, covalently linked to each protein molecule in a 1:1 ratio is responsible for the characteristic purple colour[3]. These components together form an extremely regular two-dimensional array[4].

We have studied the purple membrane by electron microscopy using a method for determining the projected structures of unstained crystalline specimens[5]. By applying the method to tilted specimens, and using the principles put forward by De Rosier and Klug[6] for the combination of such two-dimensional views, we have obtained a three-dimensional map of the membrane at 7 Å resolution. The map reveals the location of the protein and lipid components, the arrangement of the polypeptide chains within each protein molecule, and the relationship of the protein molecules in the lattice.

Electron microscopy and diffraction

The purple membrane was prepared under normal conditions from cultures of *H. halobium*[3] and applied to the microscope grid in the presence of 0.5% glucose. The purified membranes are mostly oval sheets up to 1.0 μm in diameter and about 45 Å thick[4,7]. The array of molecules making up these sheets is accurately described[7] as an almost perfect crystal of space group P3 ($a = 62$ Å) with a thickness of one unit cell only in the direction of the c axis. A single membrane thus contains up to 40,000 unit cells; that is 120,000 protein molecules (three per unit cell).

These large periodic arrays from which electron diffraction patterns and defocused bright field micrographs are recorded[8] enable us to overcome the principal problem normally associated with high resolution electron microscopy of unstained biological materials; that is, sensitivity to electron damage[8]. Only a small number of electrons can pass through each unit cell before it is destroyed, but because of the large number of unit cells, the information in the diffraction patterns and micrographs is sufficient to provide a picture of the average unit cell. The micrographs recorded with such low doses of electrons appear featureless, since the statistical fluctuation in the number of electrons striking the plate is large compared with the weak phase contrast (<1%) produced by defocusing.

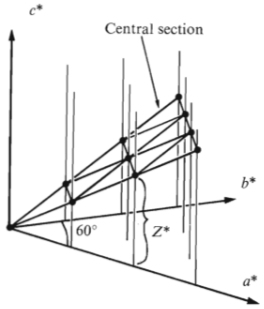
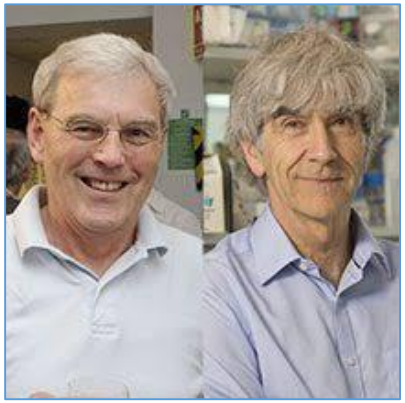


Fig. 1 Part of the three-dimensional reciprocal lattice showing the geometry of the lattice lines in the hexagonal space group P3. a^*, b^* and c^* are the reciprocal lattice vectors. a^* and b^* lie in, and c^* is perpendicular to the plane of the membrane. A central section which is perpendicular to the incident electron beam has been drawn through the lattice. The intersection of this central section with the reciprocal lattice is determined by the angle of tilt and the axis about which the membrane is tilted. Individual diffraction patterns and micrographs provide the amplitudes and phases in this section at the points shown. z^* represents the coordinate along the c^* direction of one of the points. The angle of tilt was measured to within 2° for each of the specially modified, tilted specimen holders, and the direction of the tilt axis on the photographic plate was established during operation of the microscope. However, estimates based on the geometry of the spacings of the lattice points (for high tilt angles), the variation of the degree of underfocus across the plate (for low tilt angles), and least squares refinement against data obtained at high tilt angles (for diffraction patterns) provided more accurate figures which were used in the calculation. The accuracy of measurement of both the amplitudes and phases depended on having sharp lattice lines. We therefore took care to ensure that, on the microscope grid, the membranes remained coherently ordered and flat to within 1/5°.

As a result, analysis of each micrograph by densitometry and computer processing[5] is required to combine the information from individual unit cells.

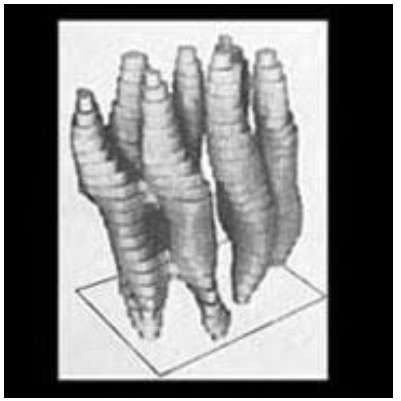
Solution of the three-dimensional structure of the purple membrane requires the determination of the amplitudes and phases in three dimensions of the Fourier terms into which it can be analysed. The diffraction pattern or Fourier transform of the membrane is not a three-dimensional lattice of points as is the case with a normal crystal, but since it is only one unit cell thick, a two-dimensional lattice of lines which are continuous in the direction of c^* (that is perpendicular to the membrane). A single electron diffraction experiment therefore



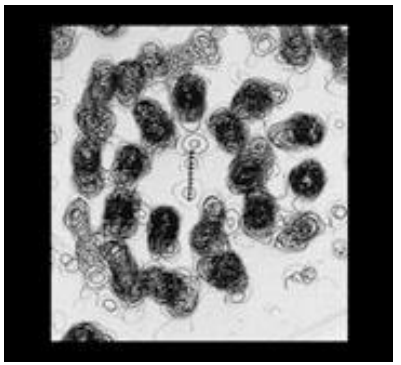
Richard Henderson and Nigel Unwin

Purple membrane
Protein

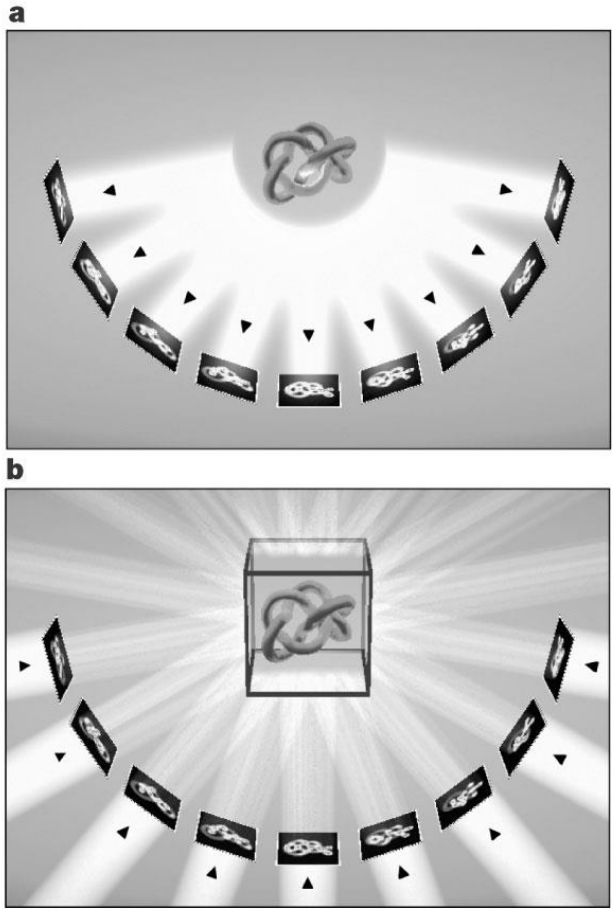
Bacteriorhodopsin



<u>Electron dose is spread over many repeats of the molecule in the crystal</u>



WHY CRSTALS?
3D Reconstruction of Asymmetrical Molecules by Electron Tomography

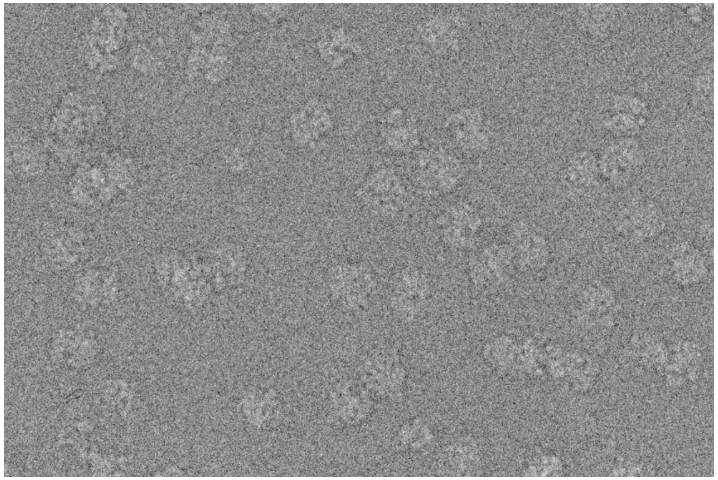


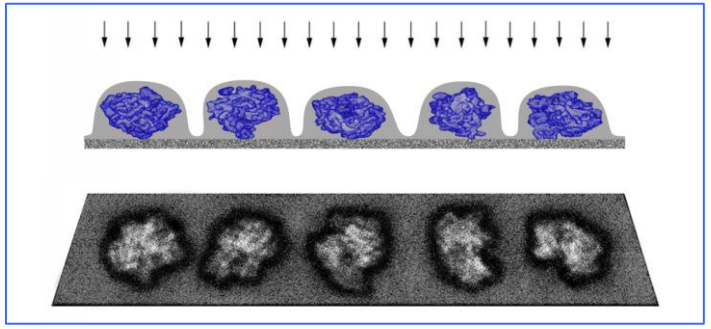
- Electron Tomography of single molecules
- Examples: fatty acid synthetase and ribosome
- BUT: Accumulated electron exposure exceeded 1000 e^-/A^2

Walter Hoppe (MPG Archive)

WHY CRYSTALS?
3D Reconstruction of Asymmetrical Molecules by Single-Particle Techniques – the Concept

- Single-particle techniques: structural information from images of single (i.e., unattached) molecules in many copies.
- *Molecules are free to assume all naturally occurring conformations.*
- Molecules are randomly oriented.
- *A single snapshot may already give us hundreds of particle views.*
- As we collect more snapshots, more orientations will be covered, until we have enough for reconstructing the molecule in three dimensions.
-





EM images can be aligned to within better than 3 Angstrom!

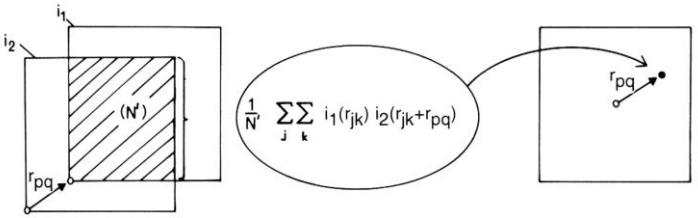
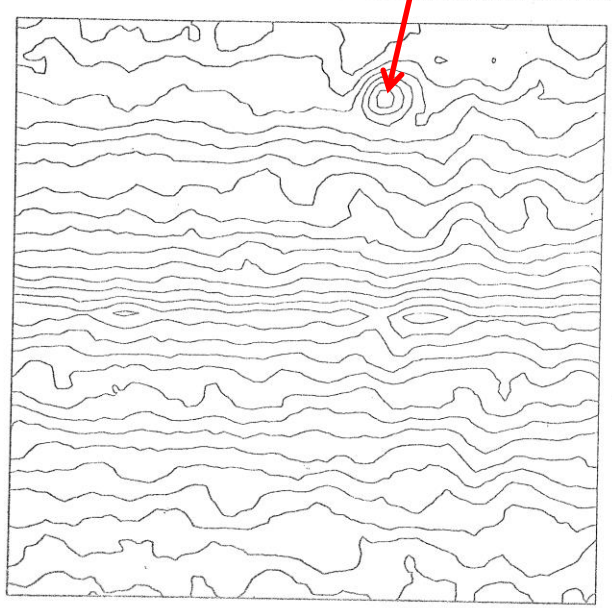


Fig. 3.8. Definition of the cross-correlation function. Image 1 is shifted with respect to image 2 by vector $\mathbf{r}_{pq}$. In this shifted position, the scalar product of the two images arrays is formed and put into the CCF matrix at position (p, q). The vector $\mathbf{r}_{pq}$ is now allowed to assume all positions on the sampling grid. In the end, the CCF matrix has an entry in each position. From Frank (1980). Reproduced with permission of Springer-Verlag, New York.

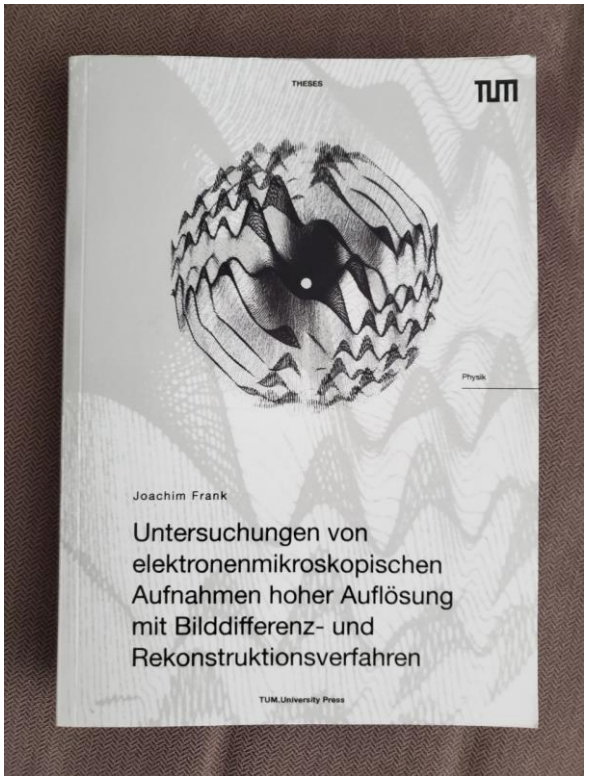


. 11a,b Graphitfolie; Korrelationsfunktionen
(ber Teilbereich). Höhenschichtlinien:
Abstand 0.006, von 0.0 bis 0.03
Abstand 0.001, von 0.147 bis 0.166

Cross-correlation function of
2 successive micrographs
of the same carbon film

J. Frank, Ph.D. thesis 1970

Dissertation at
Technical University Munich,
published in 2019,
49 years after completion



“Analysis of high-resolution electron micrographs using image difference and reconstruction methods”

Ultramicroscopy 1 (1975) 159–162

SHORT NOTE

AVERAGING OF LOW EXPOSURE ELECTRON MICROGRAPHS OF NON-PERIODIC OBJECTS

Joachim FRANK *
The Cavendish Laboratory, Free School Lane, Cambridge CB2 3RQ, UK

Received 20 October 1975

The investigation concerns the possibility of extending to non-periodic objects the low exposure averaging techniques recently proposed for non-destructive electron microscopy of periodic biological objects. Two methods are discussed which are based on cross-correlation and are in principle suited for solving this problem.

1. Introduction

Recent work on low exposure techniques combined with averaging [1–3] (called 'SNAP shot techniques' in [3]) shows that information can be retrieved from periodic biological objects at higher than conventionally available resolutions [4]. Unwin and Henderson [2] were able to achieve 7 Å image resolution, by re-6]. In these applications, the contrast of the individual marker atom image to be superposed is sufficient for straightforward alignment. However, the requirement of subminimum exposure poses a new problem: the alignment of features that are only faintly visible on a noisy background.

"If such methods (i.e., for averaging data from arrays of identical objects that are not periodic) were to be perfected, then, in the words of one scientist, "the sky would be the limit."

Arthur L. Robinson, Science 192 (1976) 360-363

CONDITIONS FOR ALIGNMENT OF TWO IMAGES OF A MOLECULE OF SIZE *D*

$$D \geq \frac{3}{c^2 dp_{crit}}$$

PARTICLE SIZE > 3 / [CONTRAST^2 x RESOLUTION (in Å) x CRITICAL ELECTRON DOSE]

Saxton & Frank, Ultramicroscopy 1977

Devil in the detail – *Problems to be solved:*

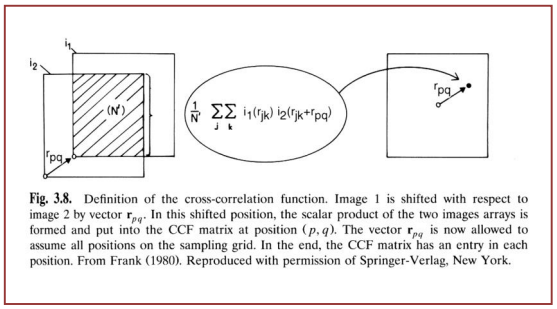
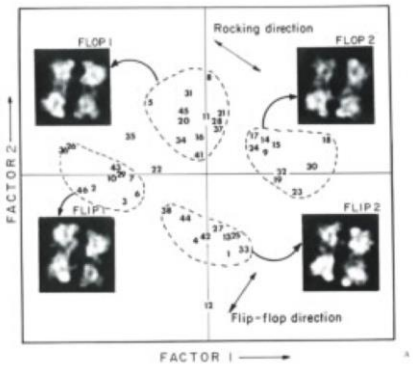


Fig. 3.8. Definition of the cross-correlation function. Image 1 is shifted with respect to image 2 by vector $\mathbf{r}_{pq}$. In this shifted position, the scalar product of the two images arrays is formed and put into the CCF matrix at position (p, q). The vector $\mathbf{r}_{pq}$ is now allowed to assume all positions on the sampling grid. In the end, the CCF matrix has an entry in each position. From Frank (1980). Reproduced with permission of Springer-Verlag, New York.

- ALIGN IMAGES
- ESTIMATE RESOLUTION OF AVERAGE
- SORT/CLASSIFY IMAGES
- FIND PROJECTION ANGLES
- RECONSTRUCT IN 3D



SPIDER -- Modular image processing program

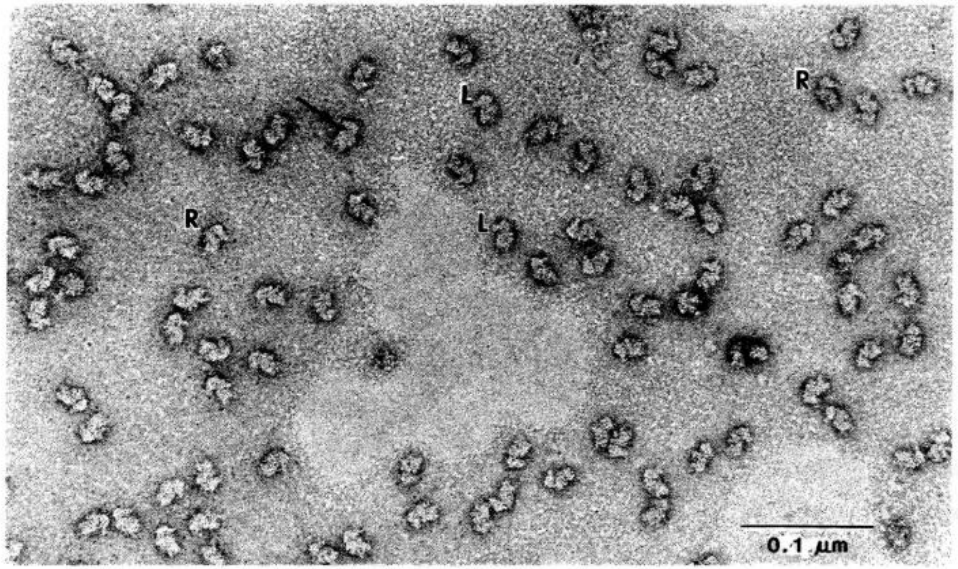
Toronto EM conference abstract 1978
Ultramicroscopy 1981

Some of the operations
(out of hundreds):

AC -- autocorrelation
CC – cross-correlate 2 images
FT -- Fourier transform
RT -- rotate
SH -- shift
WI -- window

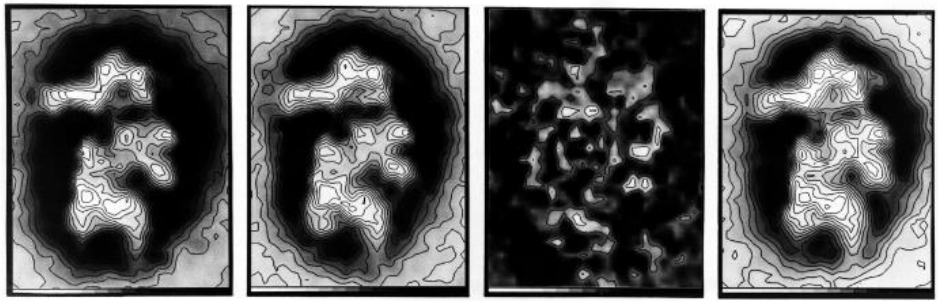
“WORKBENCH” FOR PROCESSING IMAGES

Alignment and averaging



Proof of concept

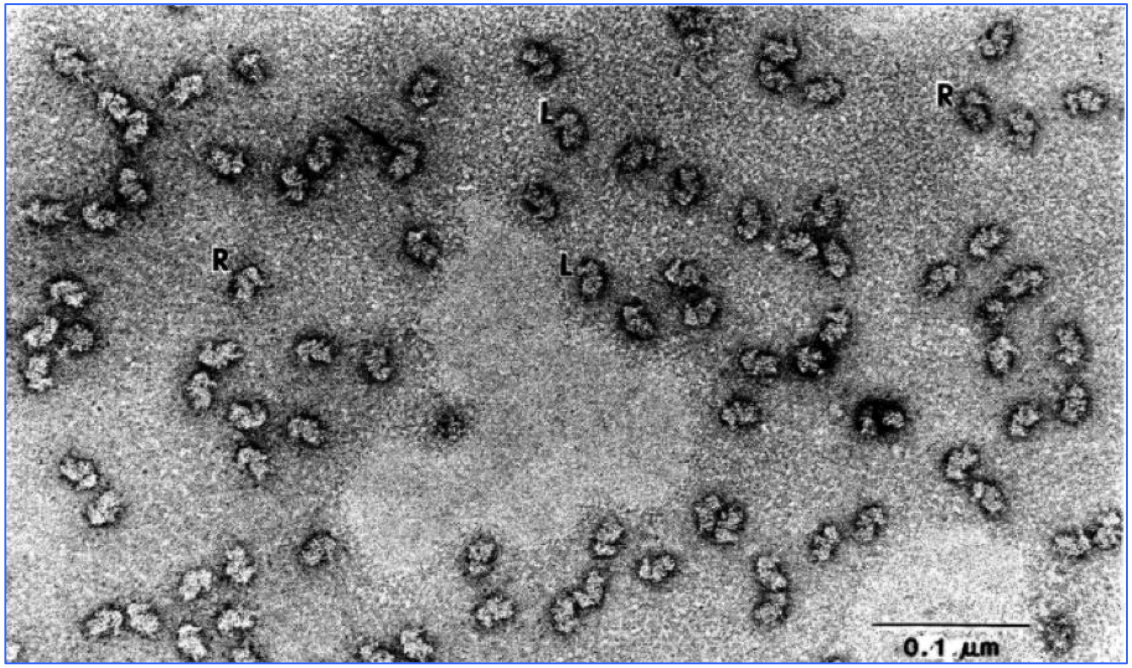
40S subunits of HeLa (human) Ribosomes



HALF-AVERAGES S.D. MAP AVERAGE

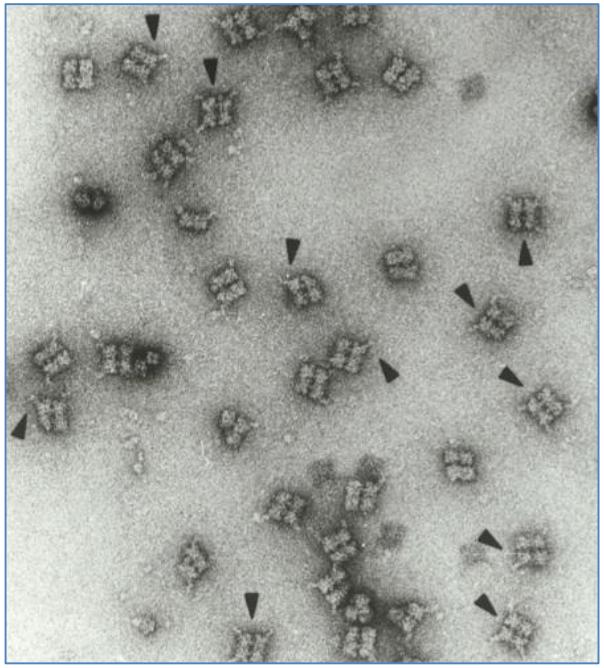
Frank et al., Science 1981

Problem of heterogeneity: molecules are in different orientations and conformations



Frank et al., Science 1981

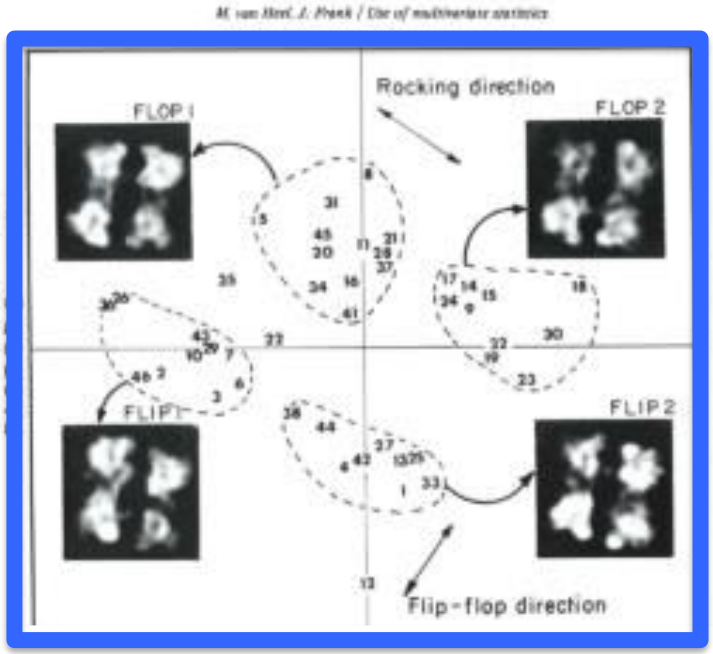
L and R views (flip and flop) of HeLa ribosomes



N. Boisset, thesis 1987

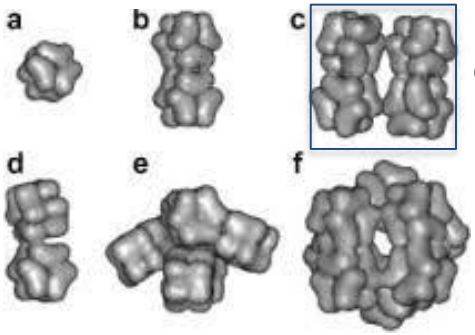
flip and flop views of hemocyanin

Multivariate analysis of aligned molecule images



Van Heel and Frank, Ultramicroscopy 1981

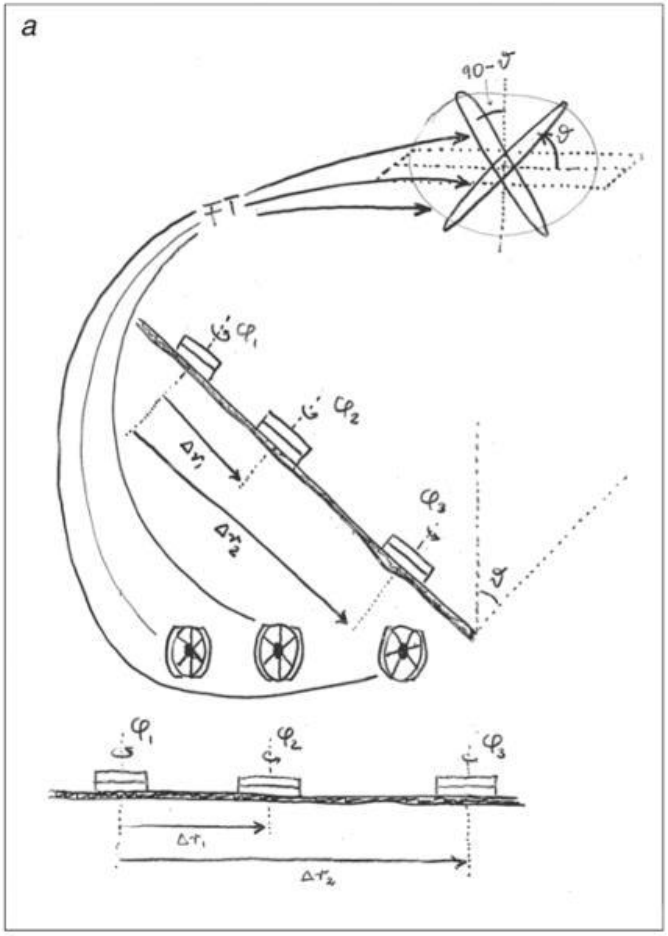
FLIP/FLOP and Rocking positions



dodecamer

Hemocyanins of Arthropods are oligomers of a basic unit

RANDOM-CONICAL RECONSTRUCTION – PRINCIPLE



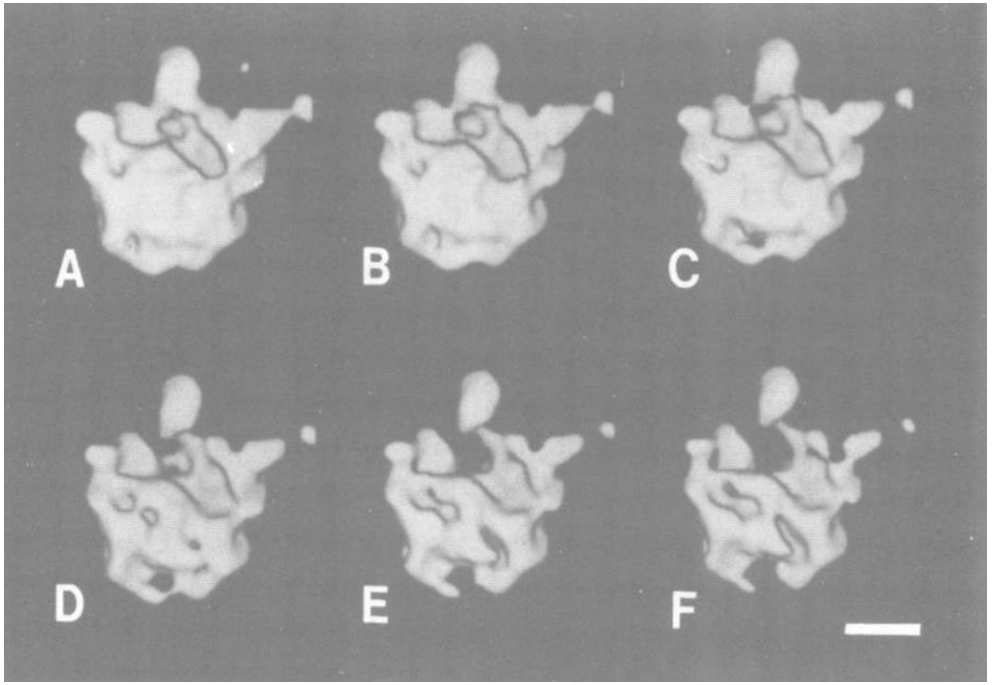
J. Frank, overhead 1979

RANDOM-CONICAL RECONSTRUCTION – PRINCIPLE

(FANCY VERSION)

J. Frank, American Scientist 1998

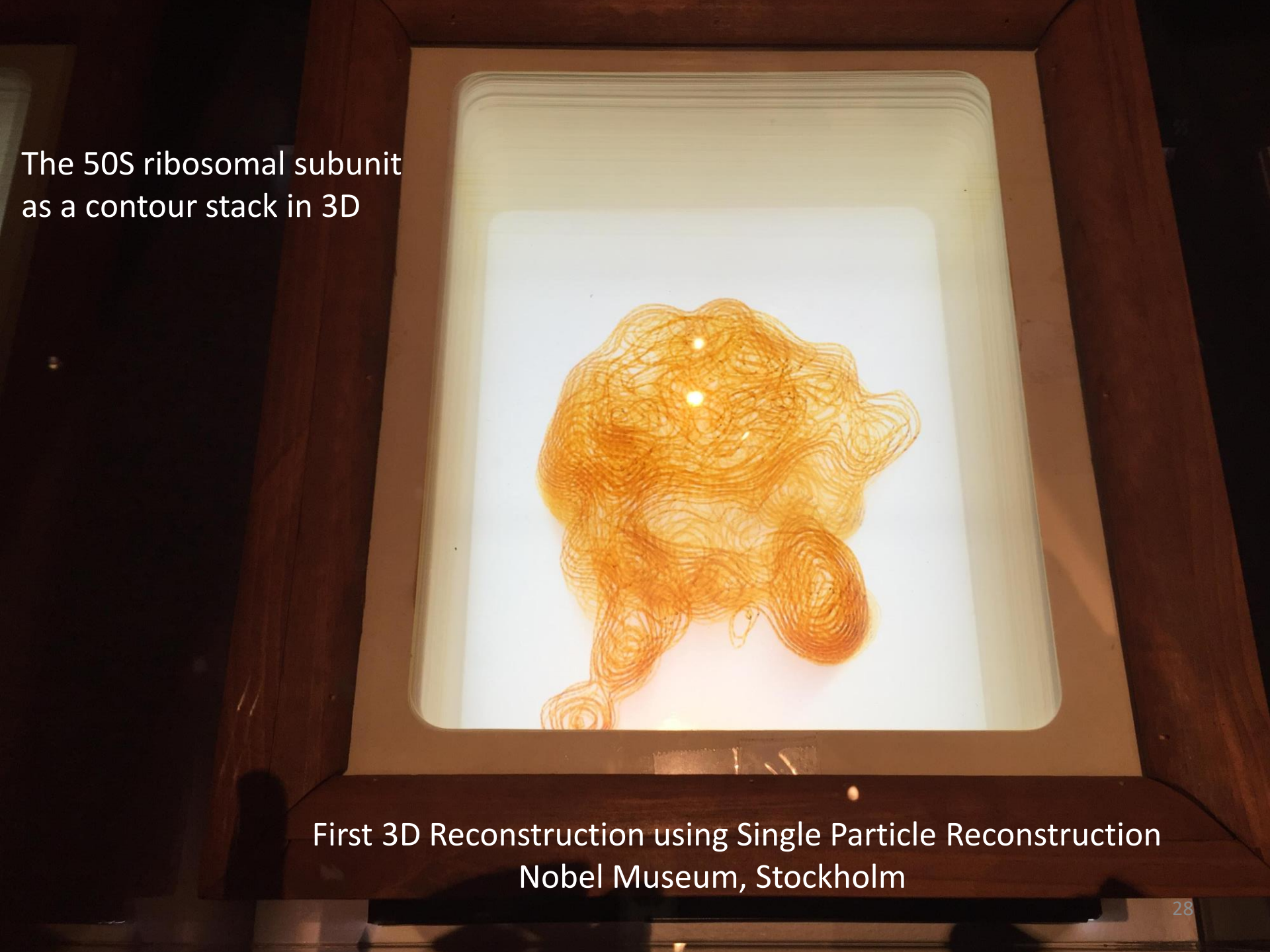
RECONSTRUCTION OF 50S RIBOSOMAL SUBUNIT FROM *E. COLI* RIBOSOME



Radermacher et al., EMBO J. 1987

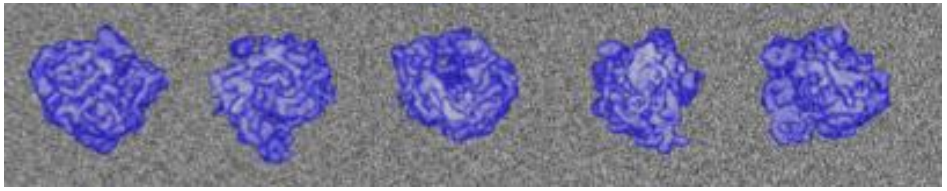

Nicholas Boisset
Raj Agrawal
Pawel Penczek
Rasmus Schroeder
Bob Grassucci
Martin Kessel
Michael Radermacher
ART STUDIO
Carmen Mannella
Manjuli Sharma
Jose-Maria Carazo
Montserrat Samso

The 50S ribosomal subunit
as a contour stack in 3D



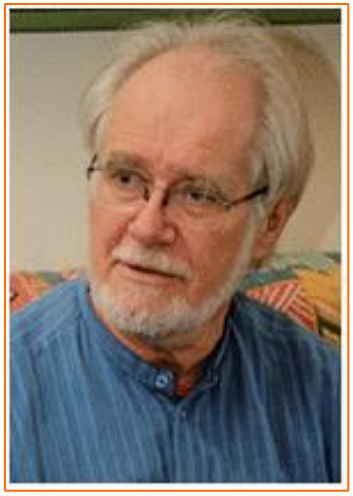
First 3D Reconstruction using Single Particle Reconstruction
Nobel Museum, Stockholm

Frozen-hydrated specimen / freeze-plunging / vitreous ice / cryo-EM

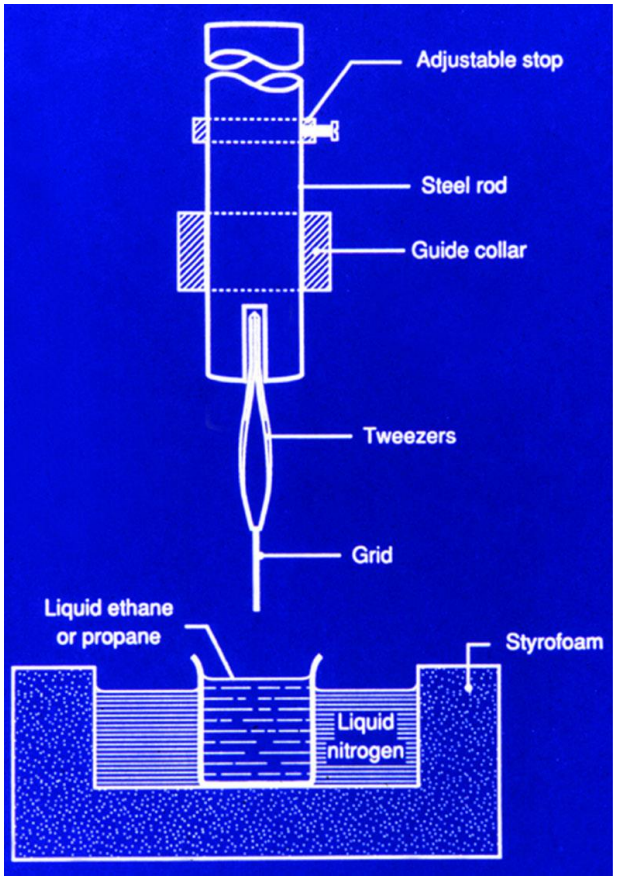


Molecules embedded in vitreous ice

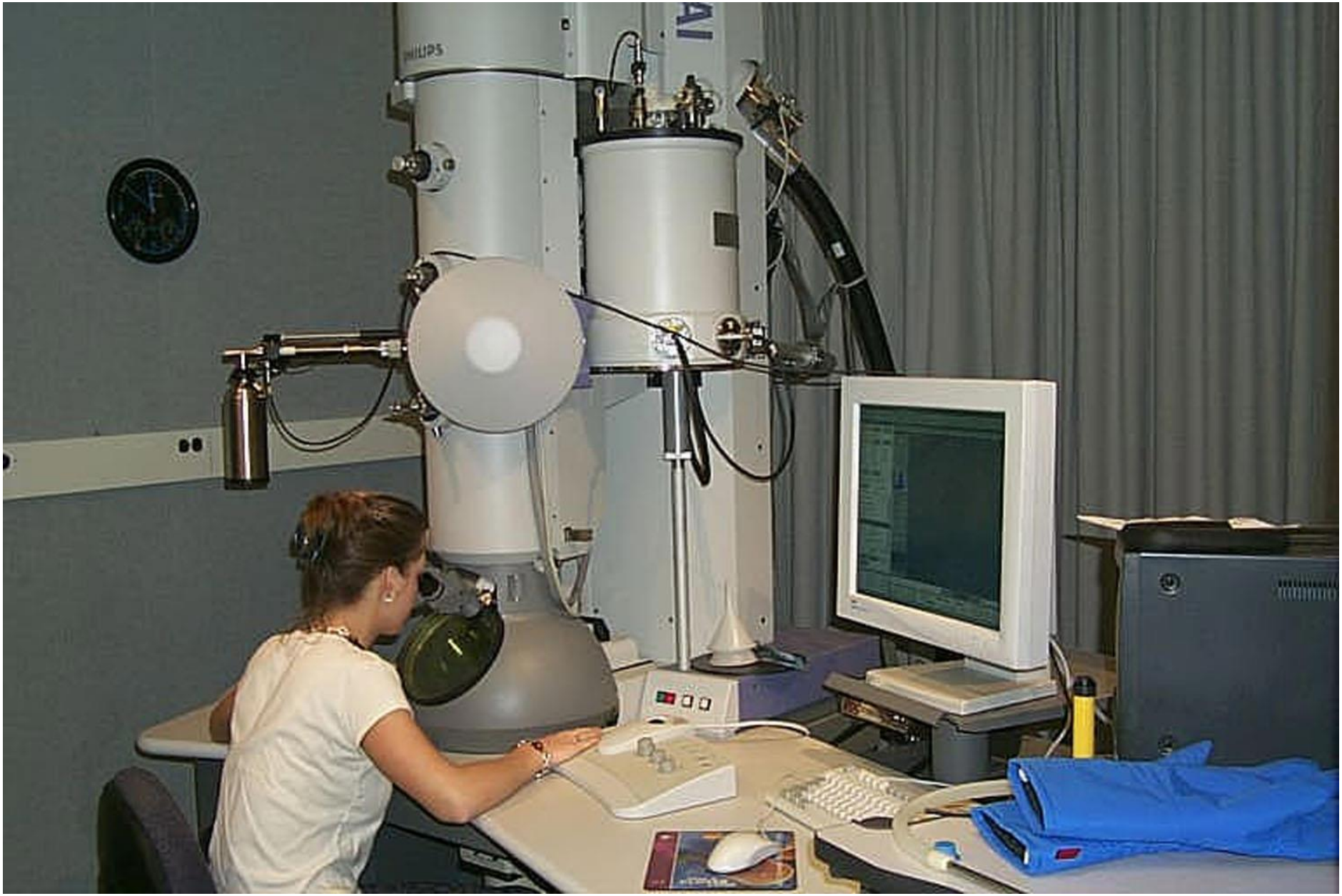
Robert Glaeser
1976



Jacques Dubochet
1981

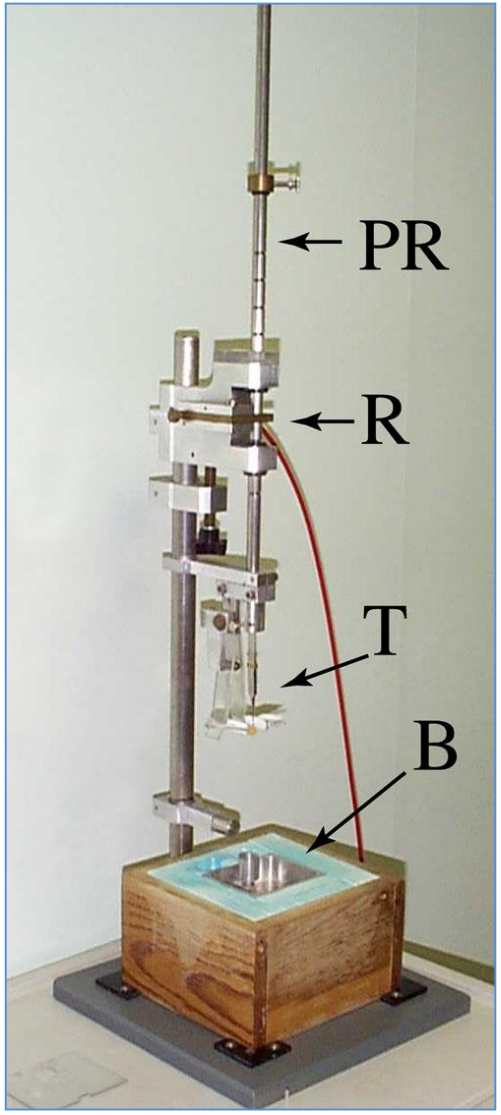


Plunge-freezer

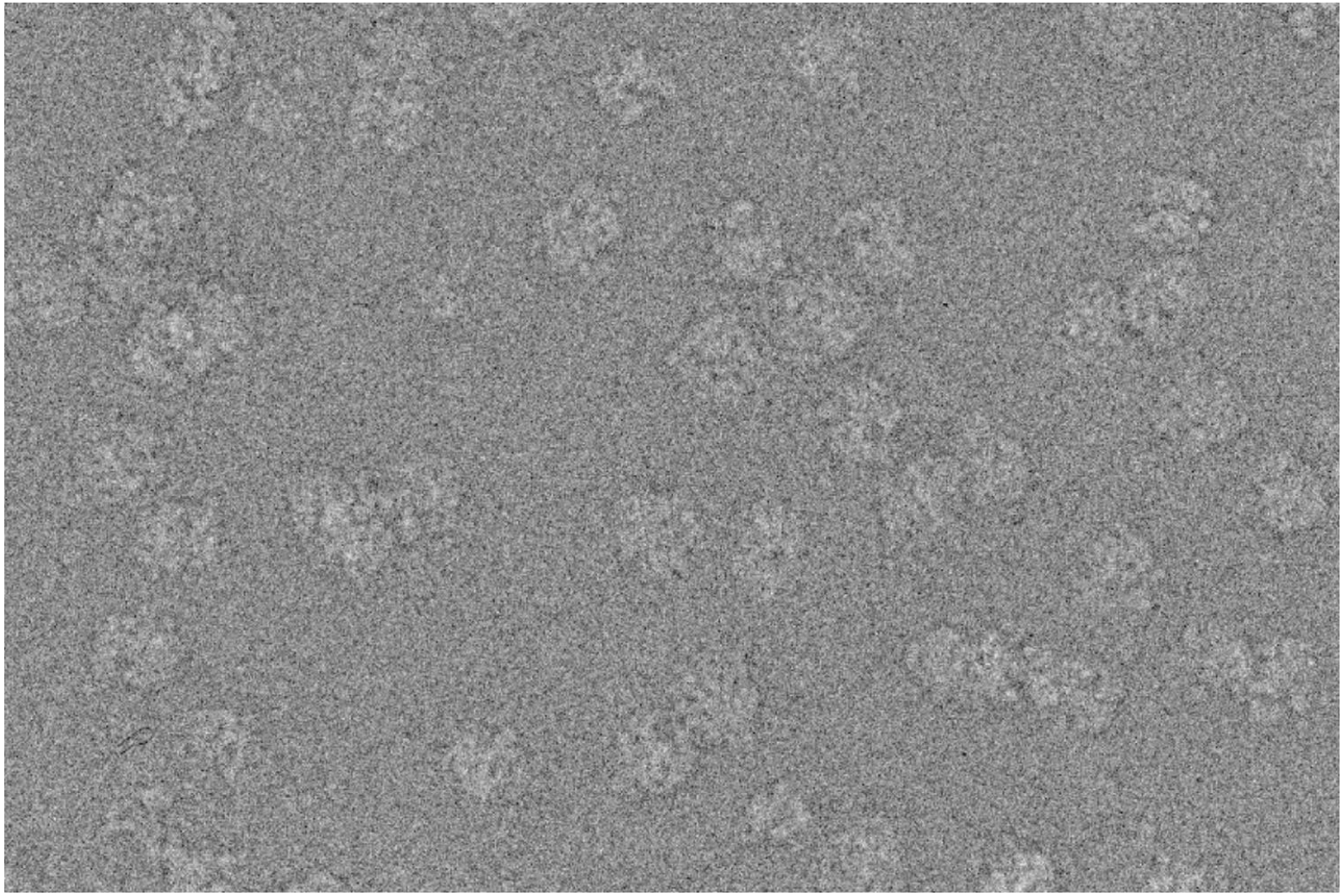

PHILIPS

Plunge-Freezer

manual

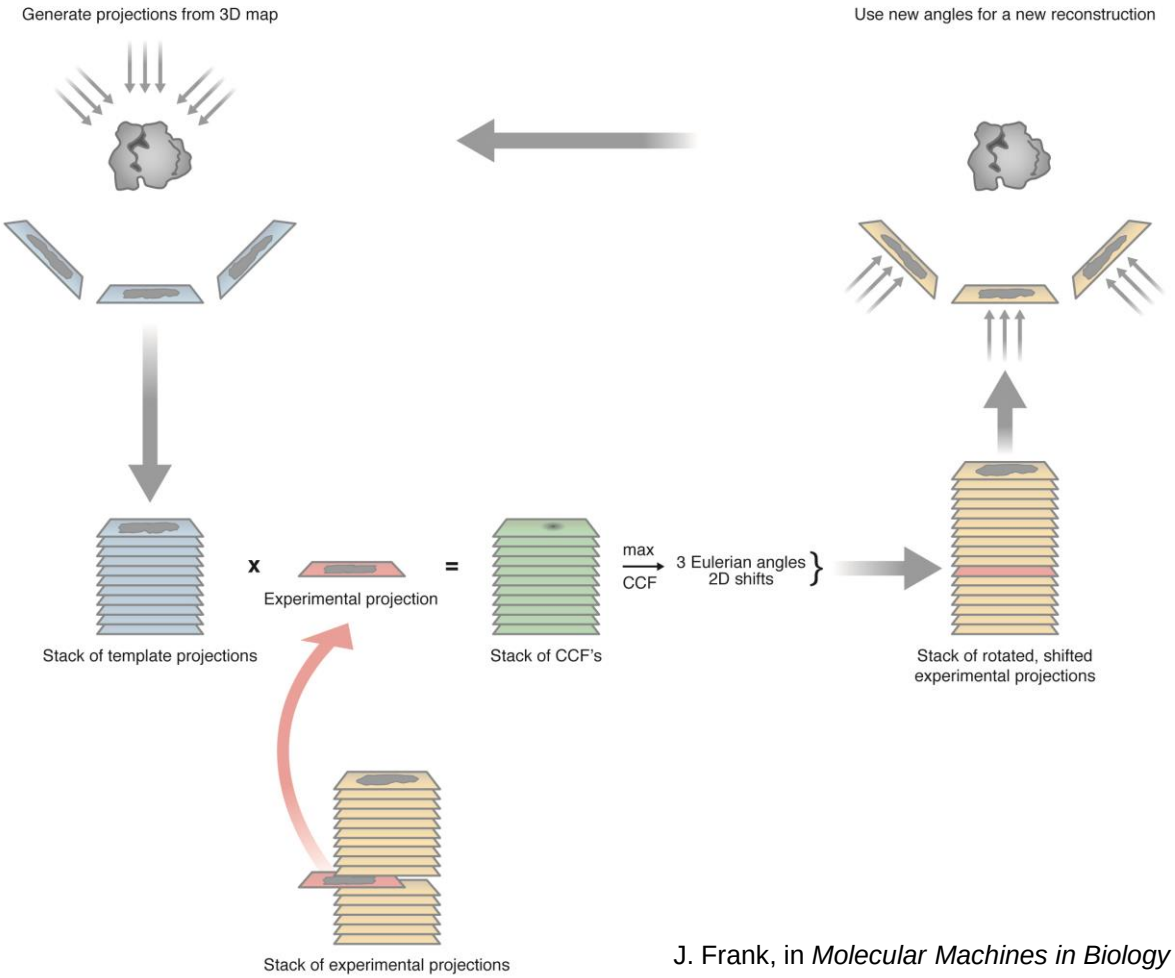


automated, climatized



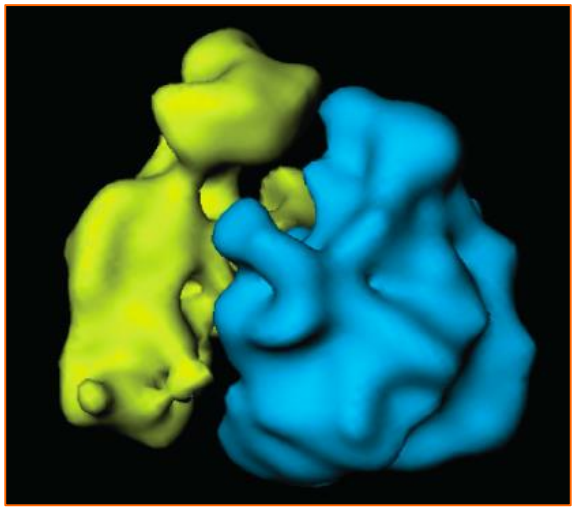
ribosomes, recorded on film

Iterative angular refinement



J. Frank, in *Molecular Machines in Biology* 2011

E. coli ribosome

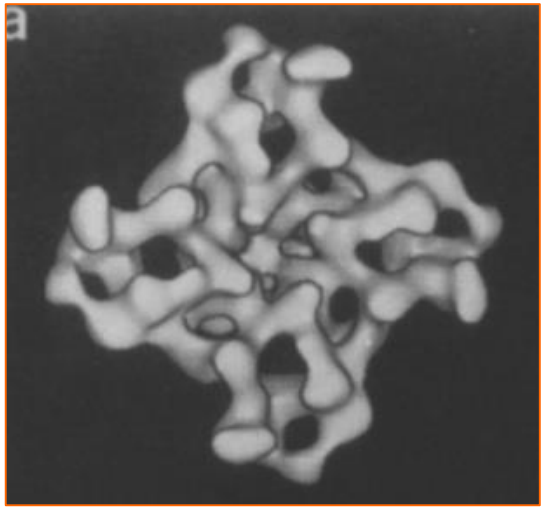


Frank et al., Nature 1995

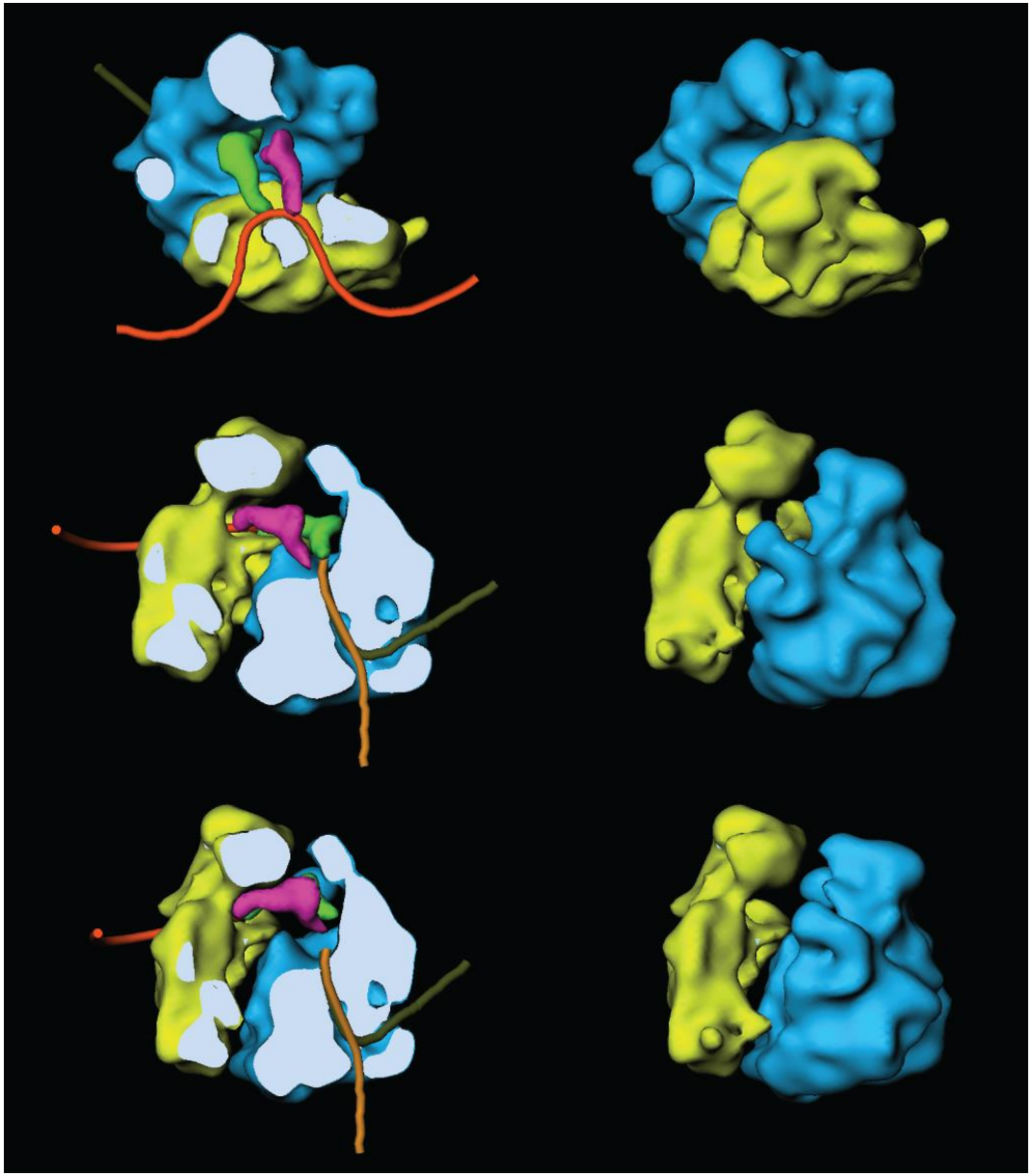
Octopus hemocyanin

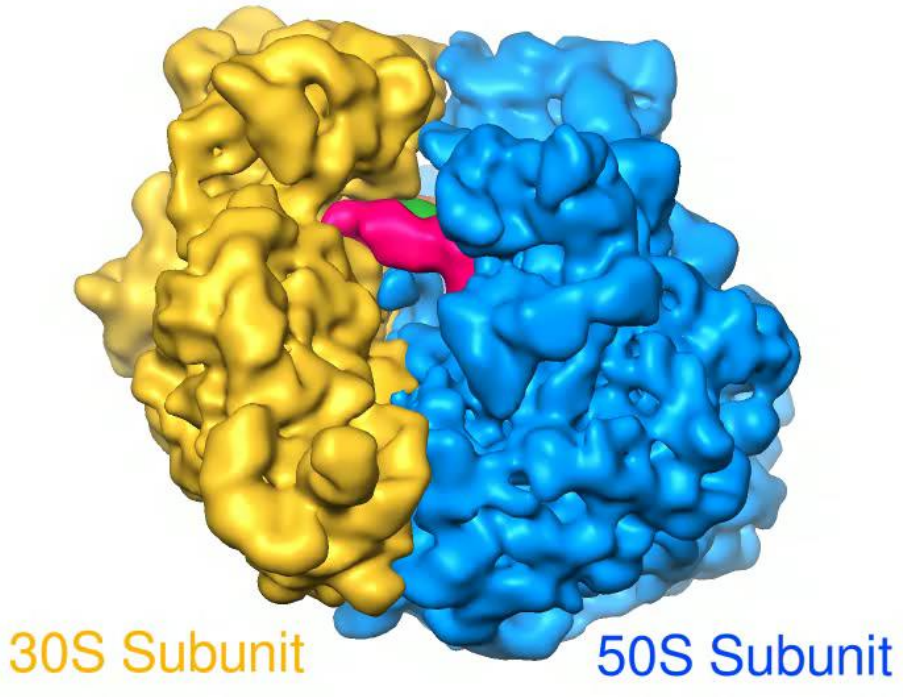
Lambert et al., 1994

Calcium Release Channel



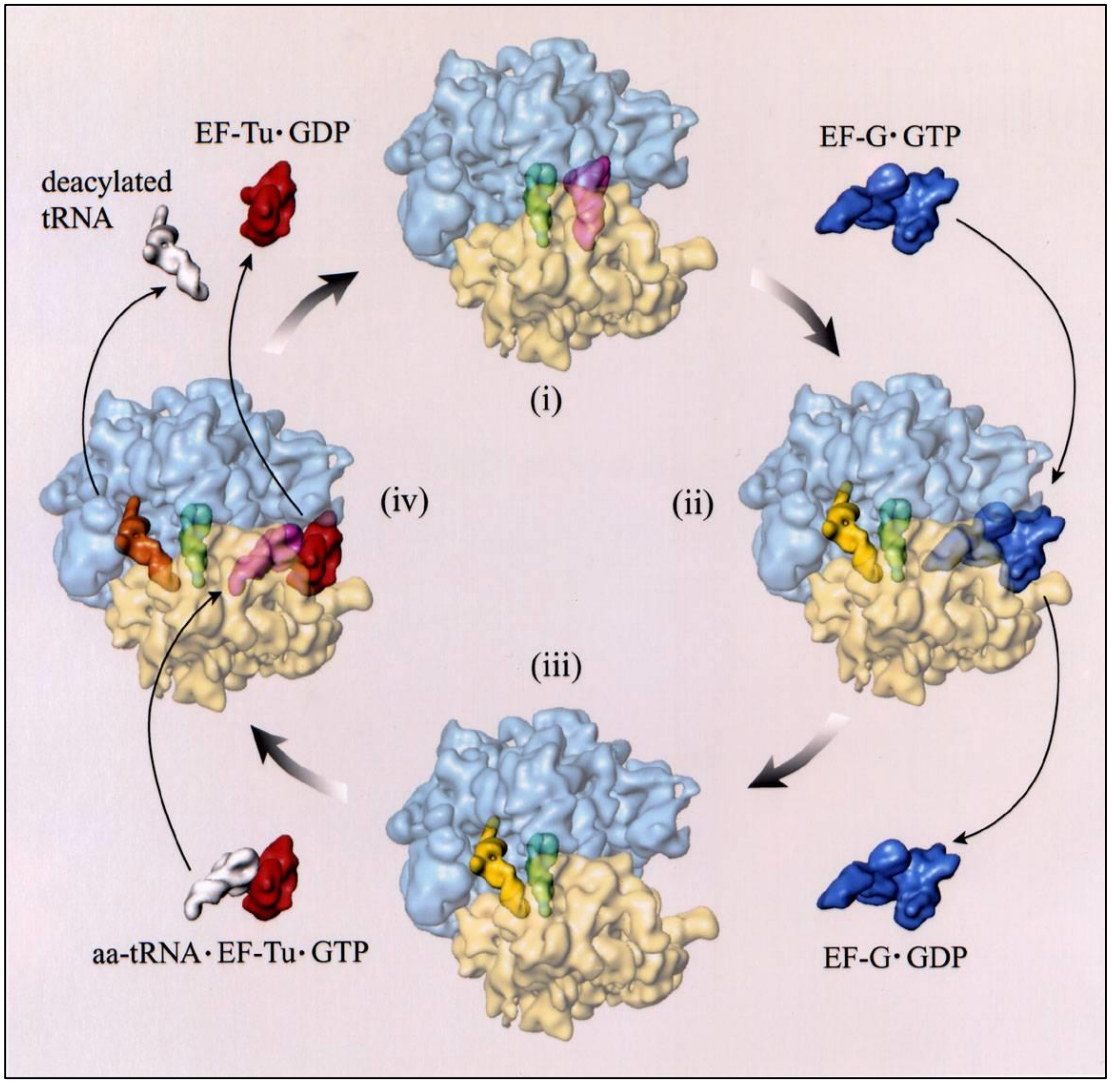
Radermacher et al., 1994




30S Subunit
50S Subunit

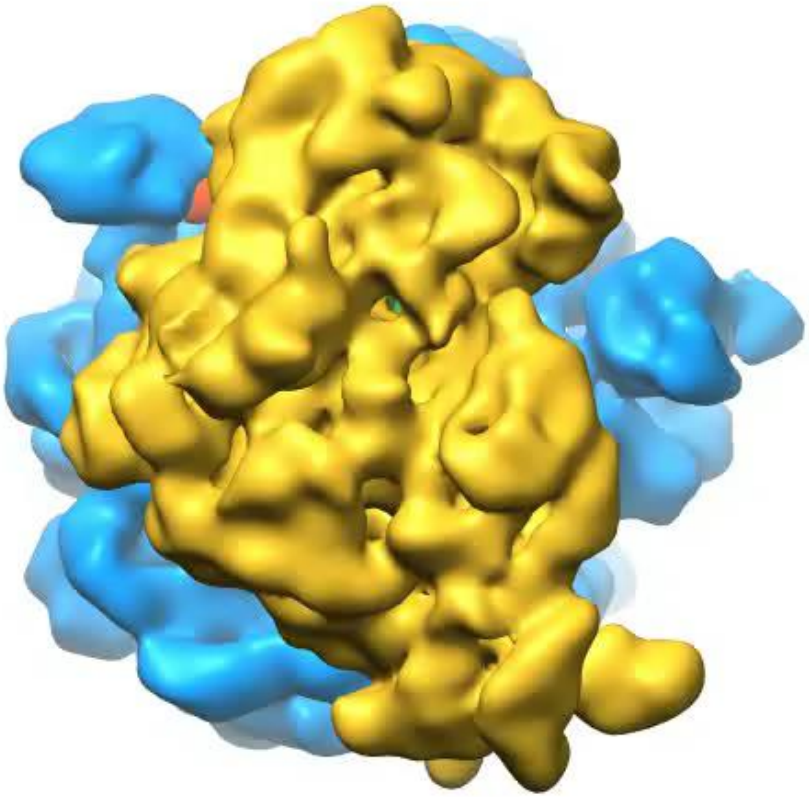
Elongation Cycle (for adding each amino acid)

Decoding



Translocation

DISCOVERY OF RATCHET-LIKE MOVEMENT DURING TRANSLOCATION

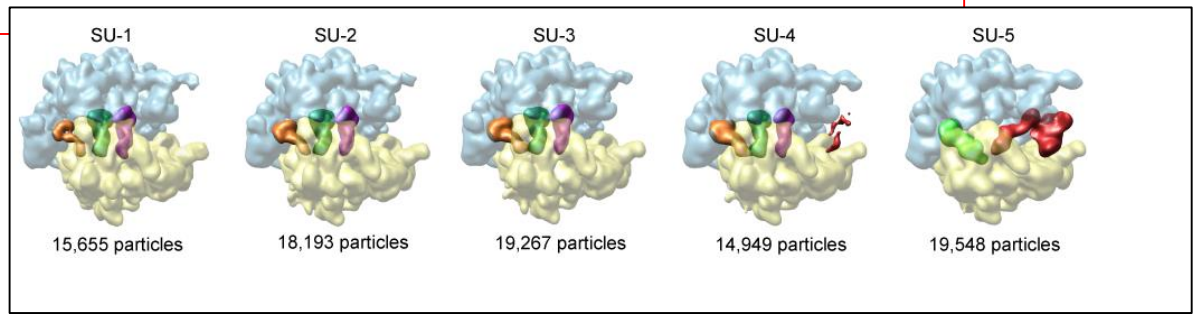


Frank and Agrawal, Nature 2000; Valle et al., Cell 2003

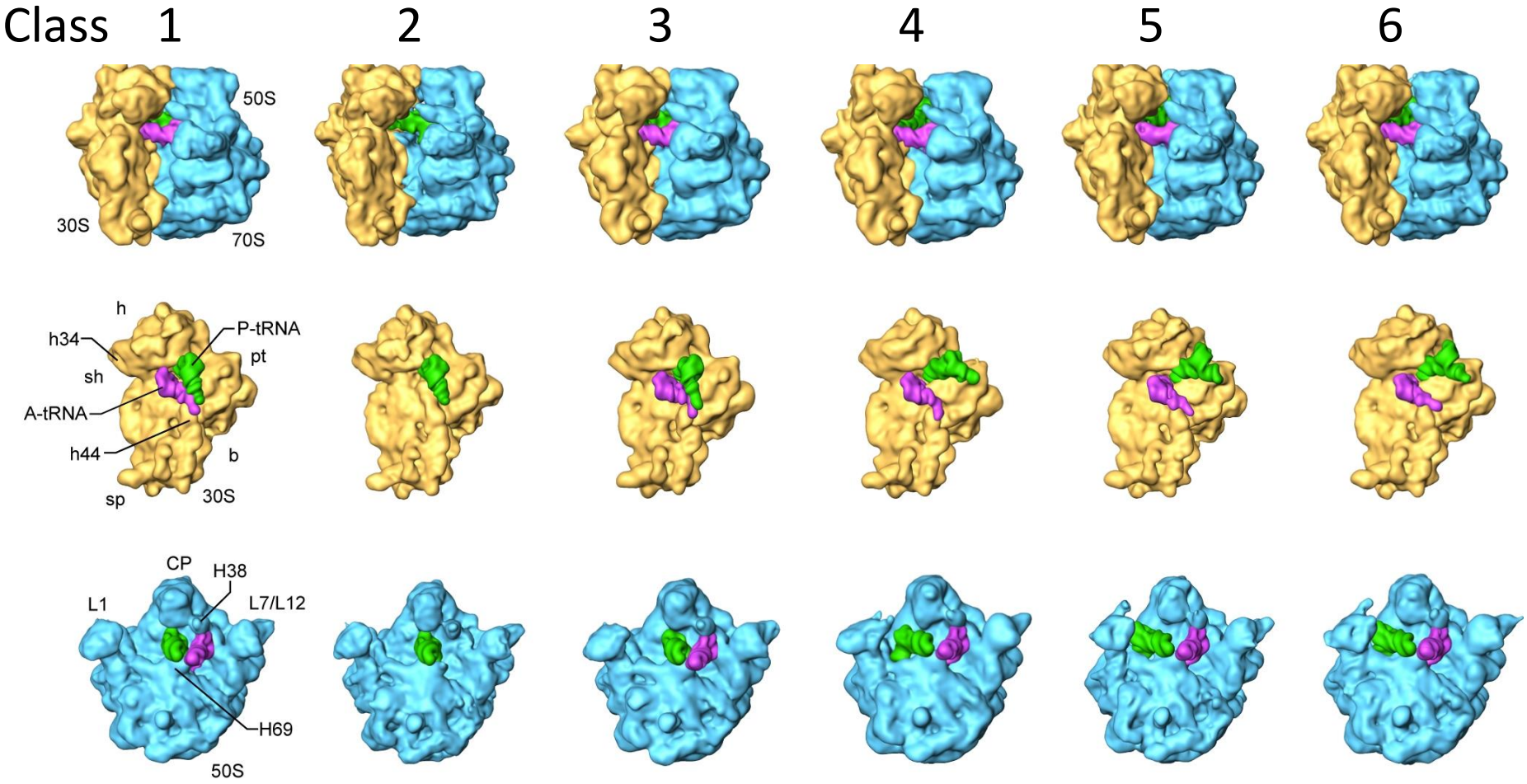
MAXIMUM LIKELIHOOD METHODS OF CLASSIFICATION

S.H.W. Scheres, H. Gao, M. Valle, G.T. Herman, P.P.B. Eggermont, J. Frank & J.M. Carazo (2007). "Disentangling conformational states of macromolecules in 3D-EM through likelihood optimization." *Nat. Methods*, 4, 27-29.

S.H.W. Scheres (2012). "A Bayesian View on Cryo-EM Structure." J. Mol. Biol. 415, 406-418.



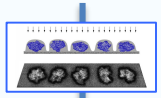
"**STORY IN A SAMPLE**" -- intermediate states in the ratchet-like motion and hybrid tRNA positions in the absence of EF-G



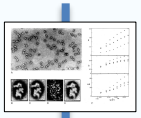
Agirrezabala et al., PNAS 2012

MILESTONES IN SINGLE-PARTICLE RECONSTRUCTION

1975 Concept

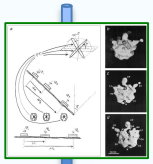


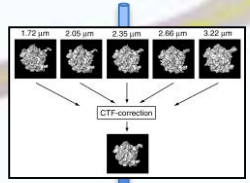
1978 Alignment via CCF
Resolution estimation



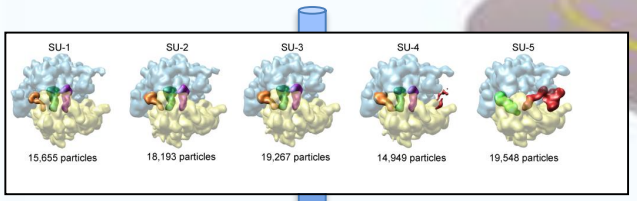
1981 Multivariate statistical analysis
2D classification

1986 Determine orientation
3D reconstruction



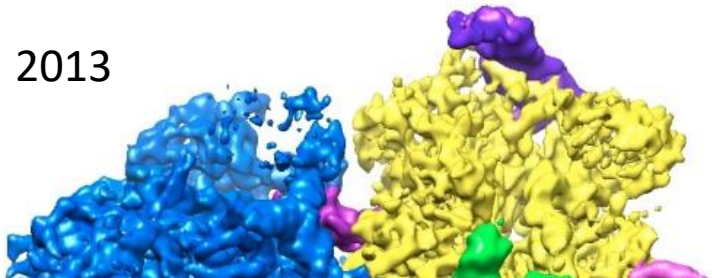


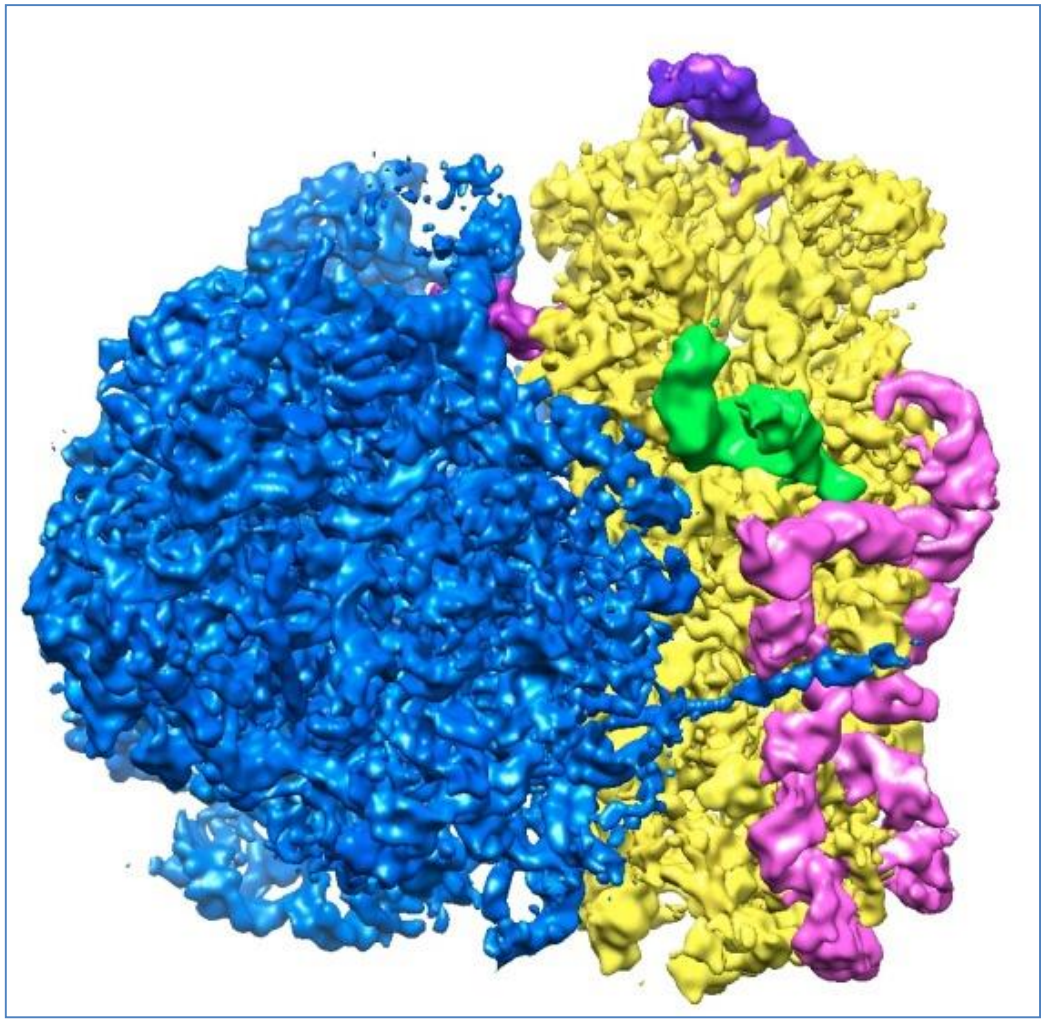
1996 CTF correction via Wiener filter



2007 Max likelihood 3D classification

2013





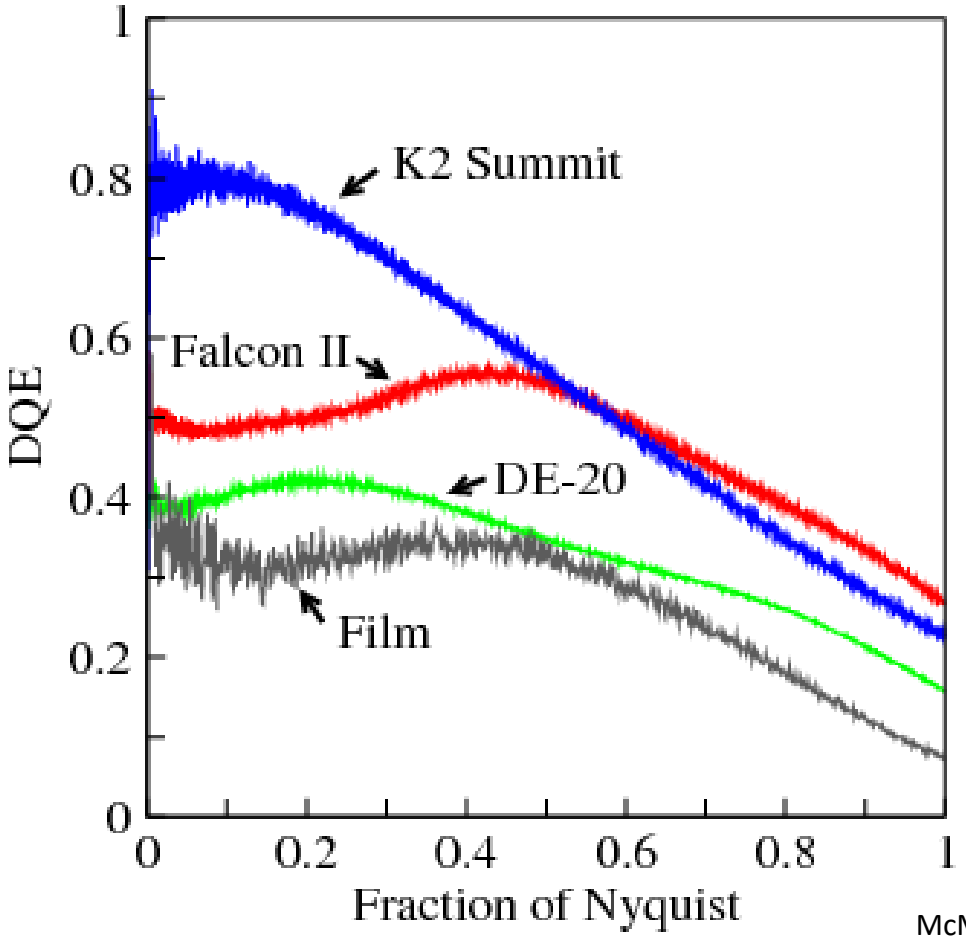
Hashem et al., Nature 2013

Best resolution from recording on film: 5.5Å

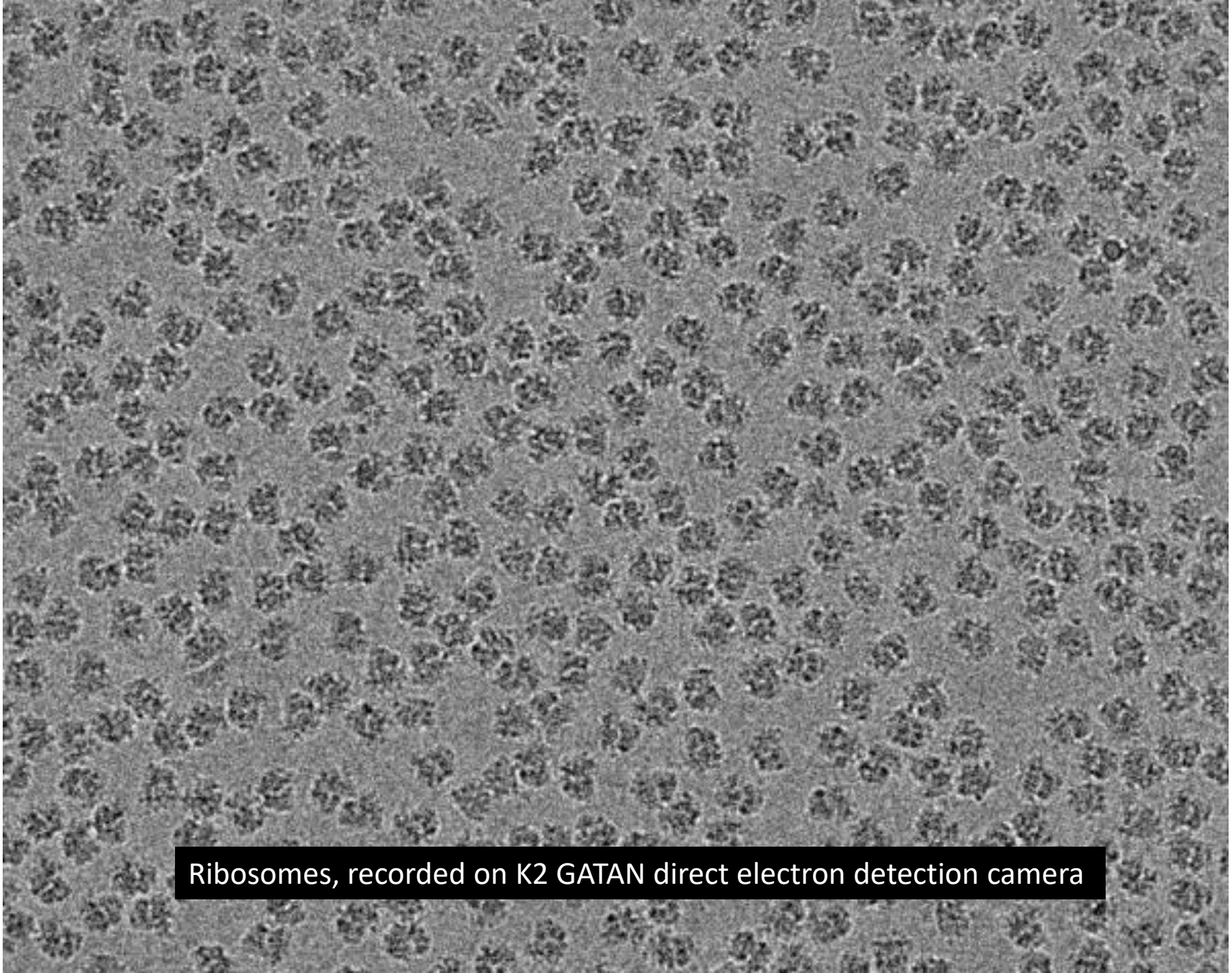
New era (since 2012): *New single-electron detecting cameras*

Detection Quantum Efficiency (DQE):

(how good is the recording device in capturing every single electron?)

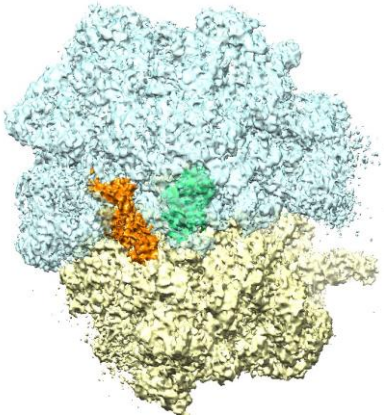


McMullan, G. et al. arXiv:1406.1389


Ribosomes, recorded on K2 GATAN direct electron detection camera

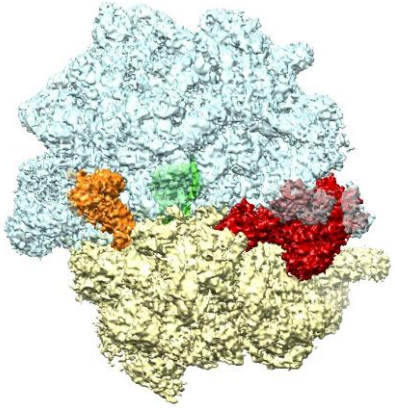
Elongation Factor G mutant H94A bound to the ribosome

nr 70S--P-E



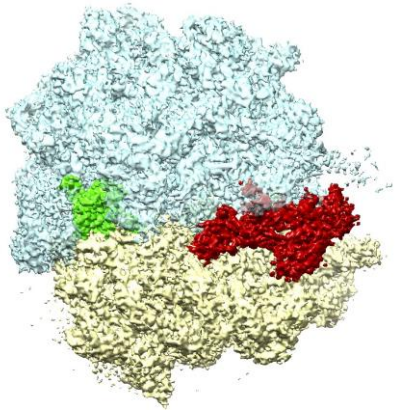
50,000

nr 70S--EF-G—P-E



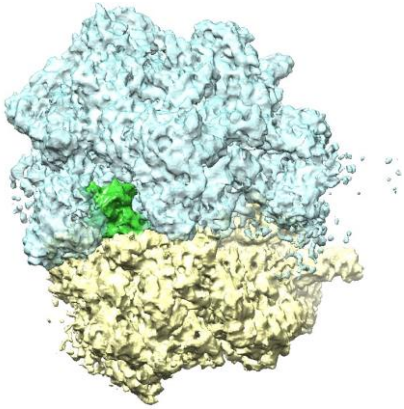
90,000

r 70S—EF-G—P/E



35,000

r 70S—P/E



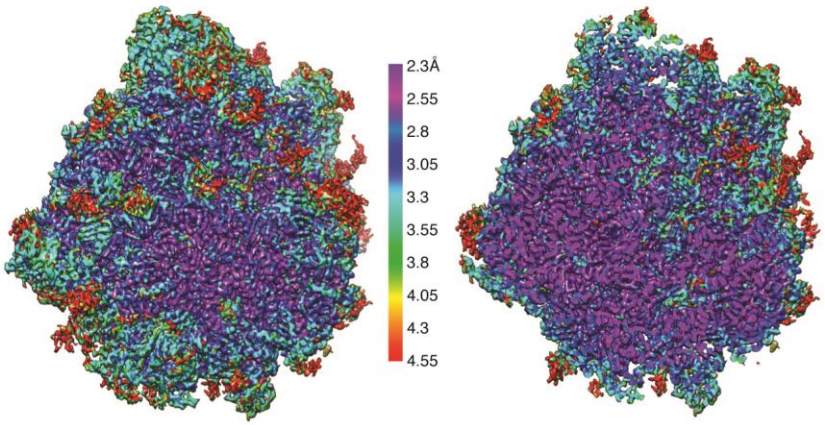
15,000

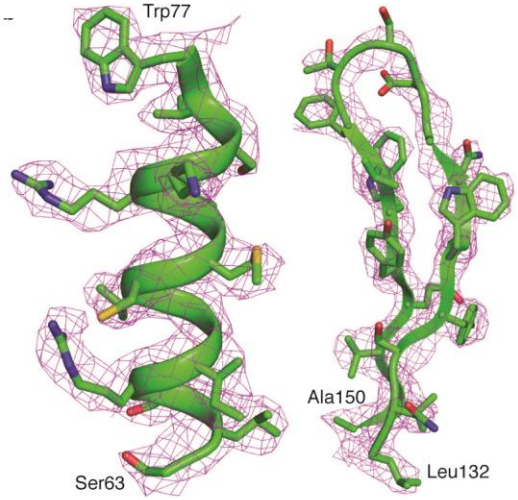
Example for maximum likelihood 3D classification
Multiple states in the same sample

Li et al., Science Advances 2015

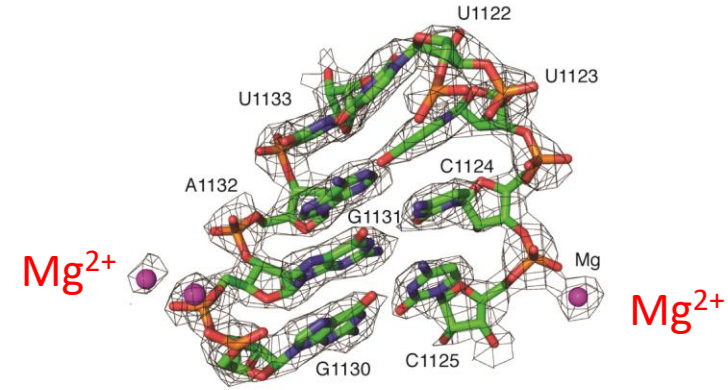
T. cruzi ribosome large subunit at 2.5 Å

Liu et al., PNAS 2016

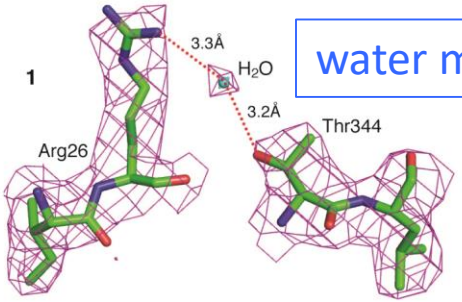




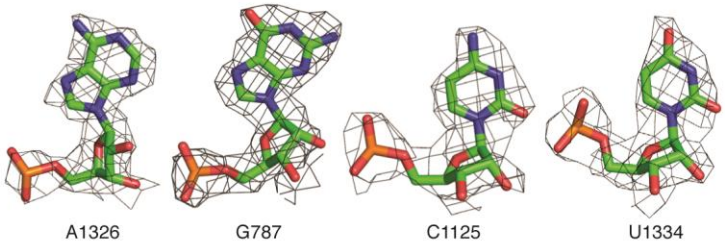
α-helix 2 strands of β-sheet

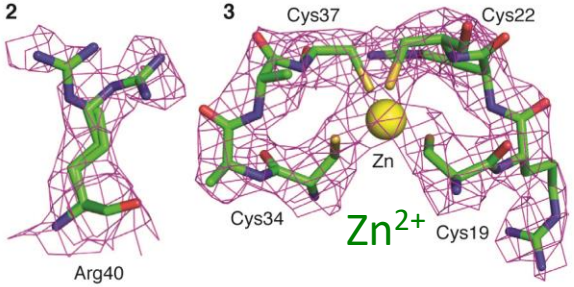


Mg^{2+} Mg^{2+}

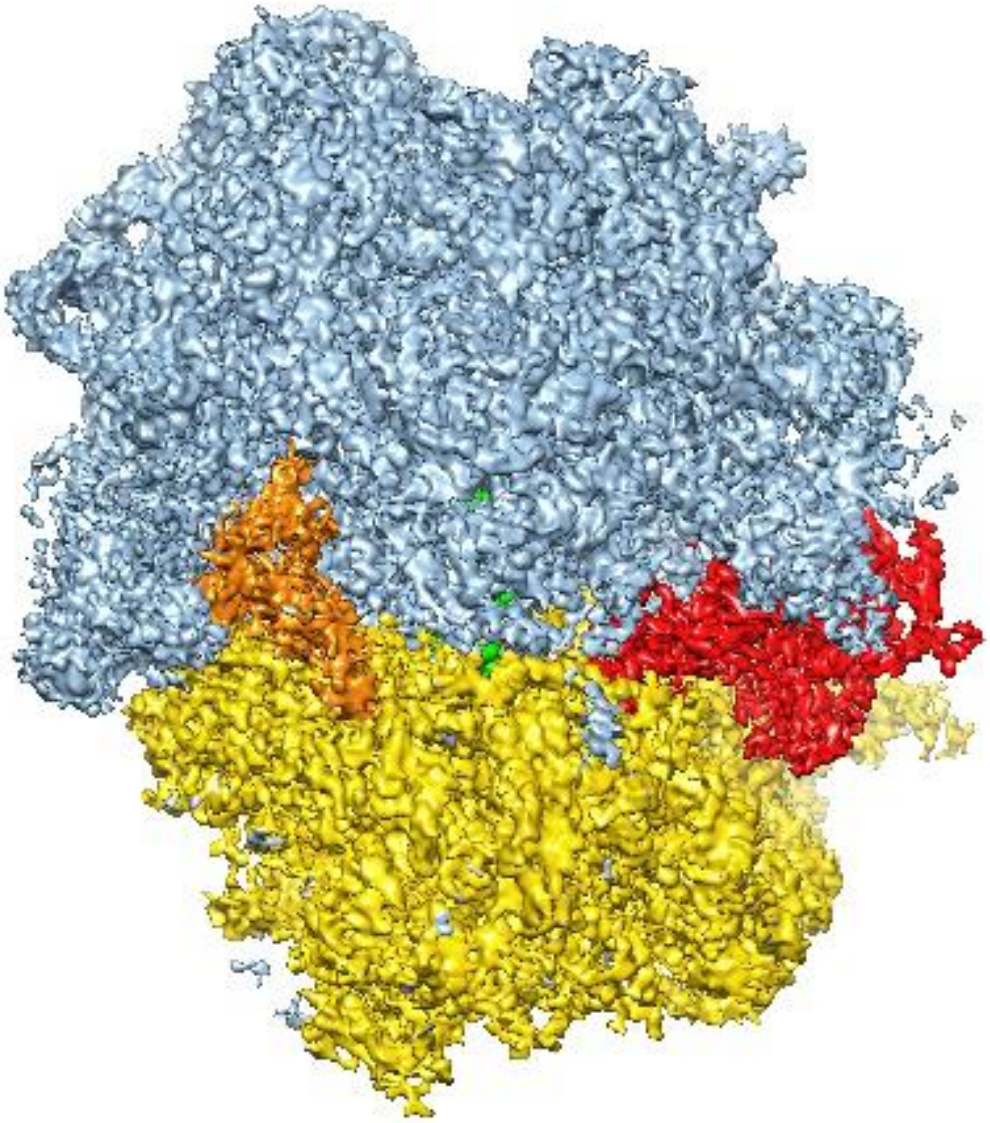


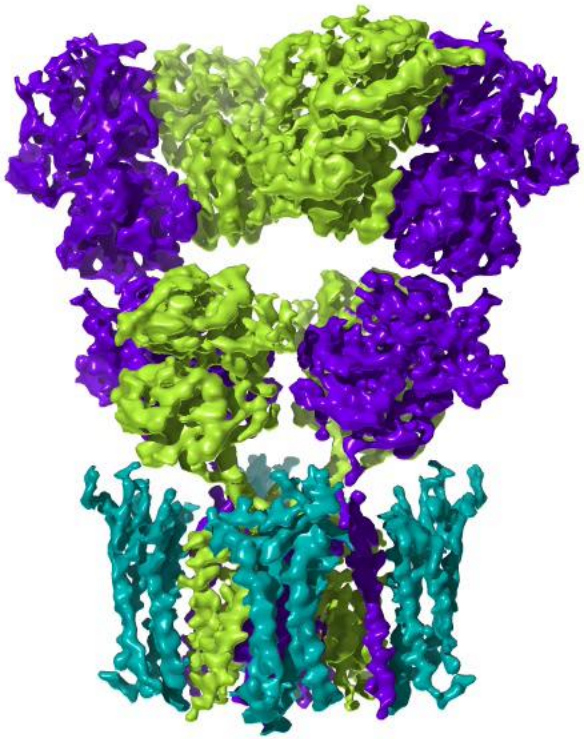
water molecule!





Zn^{2+}



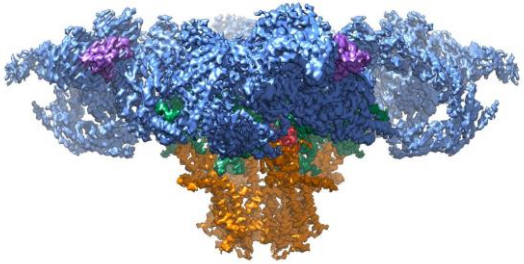


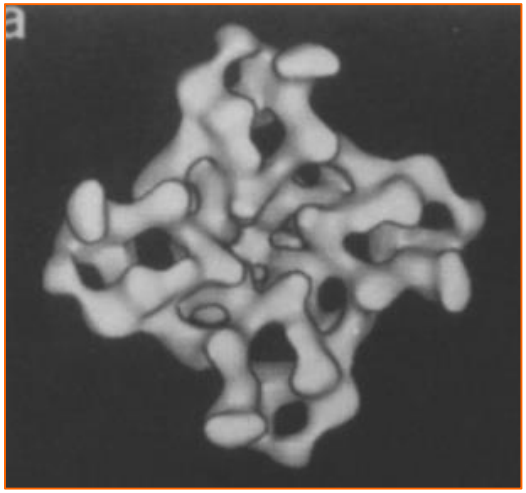
AMPA Receptor
Twomey et al., Nature 2017

Ryanodine Receptor

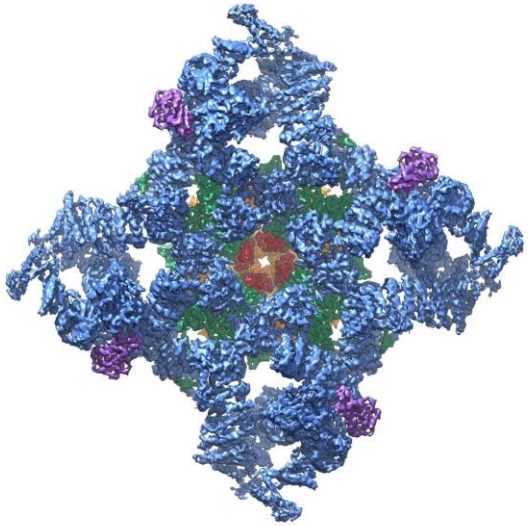
Zalk et al., Nature 2015

Des Georges et al., Cell 2016



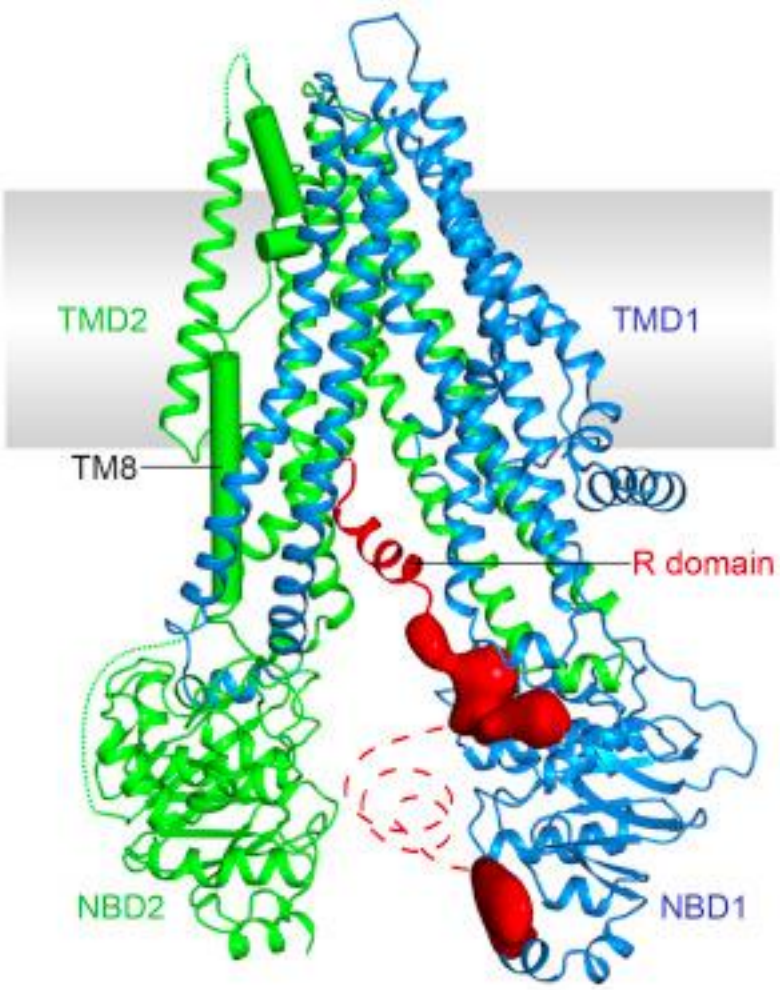


Radermacher et al., J. Cell Biol.
~30Å



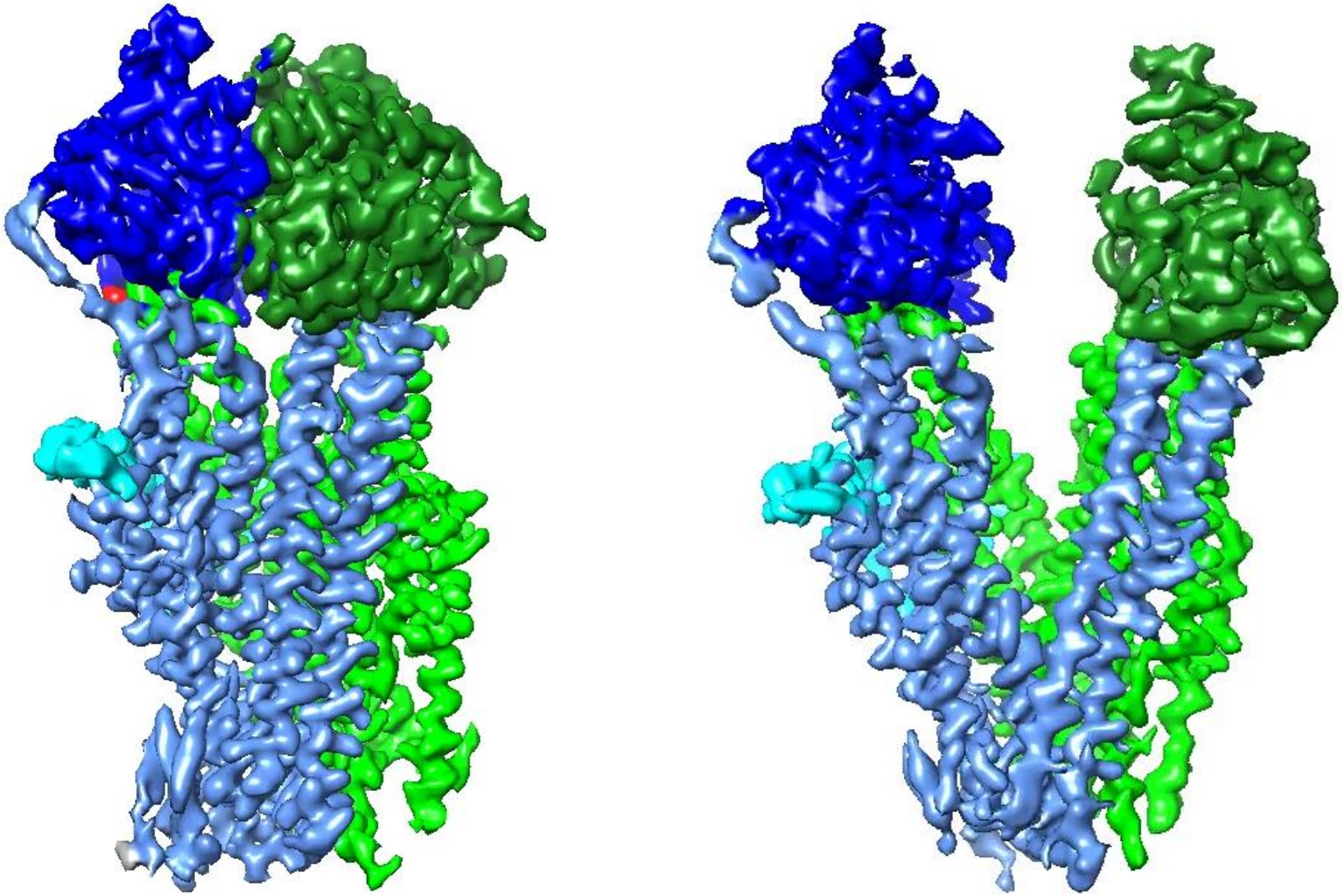
Des Georges et al., Cell 2016
3.6Å

CYSTIC FIBROSIS



Molecular Structure of the Human CFTR Ion Channel. Liu et al. Cell 2017

Open and Closed states of mutant 508del-hCFTR



Voribiev, Wang, & Yang et al.

From: Wynn, Ralph T.
Sent: Wednesday, October 25, 2017 1:55 PM
To: Frank, Joachim
Subject: Congratulations and Thank You

Dr. Frank

I want to add to the chorus of congratulations that you have no doubt received since the Nobel was announced.

I have a special reason to send a thank you as well, my seven year old grandson Jackson was diagnosed with CF

as a newborn in 2010. I have attached news that we received this week regarding his 10 months on combination therapy.

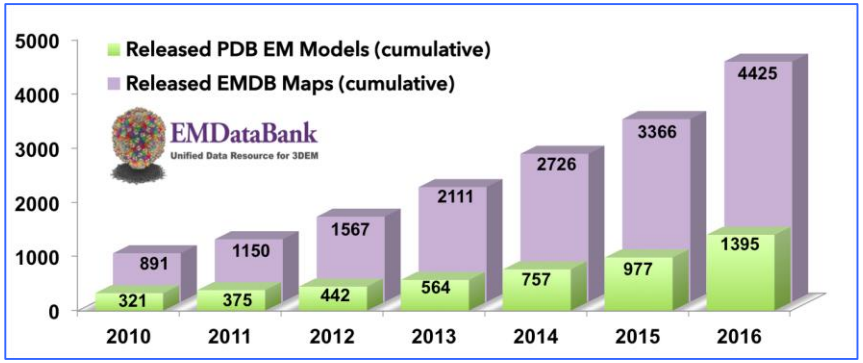
Just wanted to share this as a small expression of my (and Jackson's) gratitude.

Ralph

Ralph T. Wynn, MD, FACR
Associate Clinical Professor, Director of Breast Imaging
Department of Radiology, Columbia University Medical Center

Conclusion -- Single-particle cryo-EM: A new era in structural biology

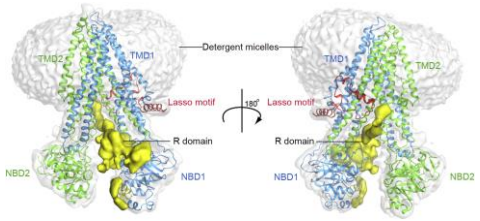
- No need for crystals!
- *Very small sample quantity needed*
- Resolution in the 3-4 Å range now routinely achievable
- *Multiple structures retrieved from the same sample → clues on function*
- Molecules in close-to-native conditions
- *Solving structures of membrane proteins much easier than with X-ray crystallography*
- Huge expansion of structural data base relevant for Molecular Medicine



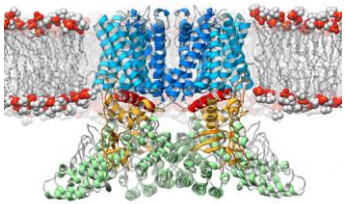
Impact in Biology and Molecular Medicine

The number of molecules with relevance to human health that can now be investigated is enormous. Among these:

1) Transmembrane proteins with particular biomedical significance, such as ion channels and receptors

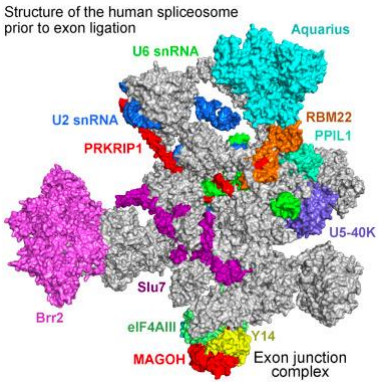


CFTCR
Zhang & Chen
Cell 2016



Pain receptor
Zubcevic et al.
NSMB 2016

1) Large molecular assemblies such as the spliceosome, which edits the genetic transcript



Spliceosome
Zheng et al.
Cell 2017

Thanks to students
(too numerous to list)
and to my collaborators . . . . . .

Special thanks to my wife,
Carol Saginaw, for 37 years
of unwavering support!


Cool microscopy captures life in atomic detail.