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

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Molecular Machines in the Cell

- 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

Bruce Alberts, Cell 1998

ATP Synthase

RNA Polymerase (Art of the Cell)
• Ancient history, EM and X-ray crystallography
• 1975 Single particle techniques -- the concept
• 1975 – 1987 SPIDER, programs for averaging, classification, 3D reconstruction
• 1981 Dubochet’s discovery of vitreous ice
• 1987 First single-particle reconstruction – negative stain
• 1989 First single particle reconstruction – vitreous ice
• 1990 – 2012 Cryo-EM reconstructions with increasing resolutions up to 5.5 Angstrom
• 2012 Direct electron detection cameras hit the market
• 2012 – now “resolution revolution”
• TODAY: exponential increase in cryo-EM structure depositions
• Future: Time-resolved cryo-EM & Mapping of continuum of states using cryo-EM
X-ray Crystallography

- Crystal: many copies of the molecule arranged in regular order.
- Exposure to X-ray beam $\rightarrow$ diffraction pattern $\rightarrow$ structure determination.
- X-ray beam must be high-intensity, crystal must be almost perfect.
- To date $\sim$ 140,000 structures solved by X-ray crystallography, available in public databanks.
- Crystal packing $\rightarrow$ 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/
5% of all Nobel Prizes are related to X-ray crystallography. Half of these were for biomolecules
One of the First Hybrid Meetings!

Hirschegg, site of 1968 workshop on X-ray crystallography and EM of proteins organized by Walter Hoppe and Max Perutz.
Harold Erickson, Richard Henderson, Ken Holmes, Hugh Huxley, Nigel Unwin . . .
Conference site of the Hirschegg Meetings

Walter Hoppe with Max Perutz in Hirschegg
Basic incompatibility of biological imaging:

1) Electrons destroy biological matter. “Shooting with cannons at sparrows”

2) Electrons require vacuum to travel; biomolecules require an aqueous environment for their structure to be sustained

Solution:

- Low exposure
- Hydration chamber or ice embedding

→ Low exposure + averaging over many repeats of a molecule image
→ Hydration to keep molecule in native state: hydration chamber at room temperature -- or -- embedding in vitreous ice, cryo-EM
Limitations to significant information biological electron microscopy as a result of radiation damage 1,
Structure of Wet Specimens in Electron Microscopy

D. F. Parsons

Science
Published By:
American Association for the Advancement of Science

https://www.jstor.org/stable/1739696

Fig. 3. Electron diffraction pattern of a wet microcrystal of ox liver catalase recorded on No-Screen medical x-ray film at 200 kv. The projection was \( P2_2 \), symmetry and corresponds to an orthorhombic habit of catalase.
PLUNGE FREEZING/EMBEDDING IN VITREOUS ICE  1981

Molecules embedded in vitreous ice

Robert Glaeser 1976

Jacques Dubochet 1981

Plunge-freezer
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.

*BUT: Sample must be very thin, electrons are readily absorbed by matter.*

Electrons strongly damage the molecules -- need for low dose! 10-20 electrons/square Angstrom.

*Images are very noisy (shot noise)*
THREE-DIMENSIONAL RECONSTRUCTION: STRUCTURES WITH HELICAL SYMMETRY. 1968
(sample prep: negative staining)
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 (sample prep: negative staining) 1970

tomato bushy stunt virus

Figure 5. (a) Contour map of a reconstruction of tomato bushy stunt virus. (b) The same map (photographed using different illumination) with the $T = 3$ surface lattice (Caspar & Klug 1962) superimposed. Contours indicate the absence of stain. The principal morphological units lying on the strict and local twofold axes of the lattice are indicated by nuts while the subsidiary morphological units at the fivefold positions are marked by washers.

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.

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

Tony Crowther
THREE-DIMENSIONAL RECONSTRUCTION: STRUCTURES THAT FORM 2D CRYSTALS (glucose embedding). 1975

Three-dimensional model of purple membrane obtained by electron microscopy

Richard Henderson and Nigel Unwin

Purple membrane Protein

Bacteriorhodopsin

Electron dose is spread over many repeats of the molecule in the crystal
Why Crystals?
3D Reconstruction of Asymmetrical Molecules by Electron Tomography ~1968

- Electron Tomography of single molecules
- Examples: fatty acid synthetase and ribosome
- BUT: Accumulated electron exposure exceeded 1000 e⁻/Å²

Walter Hoppe (MPG Archive)
• 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.

Why Crystals?
3D Reconstruction of Asymmetrical Molecules by Single-Particle Techniques – the Concept 1975
EM images can be aligned to within better than 3 Angstrom!

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

J. Frank (1970) “Analysis of high-resolution electron micrographs using image difference and reconstruction methods”
SHORT NOTE

AVERRAGING OF LOW EXPOSURE ELECTRON MICROGRAPHS OF NON-PERIODIC OBJECTS

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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.
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*:

- ALIGN IMAGES
- CTF CORRECTION
- 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
The Ribosome – its role in the development of Single-Particle Techniques

Miloslav Boublík
Roche Institute, Nutley, NJ
In the beginning, there was the Lake model:
3D reconstruction by eye,
inferrred from EM images
1970s
Alignment and averaging of single-particle images

40S subunits of HeLa (human) Ribosomes

Proof of concept

Frank et al., Science 1981
Problem of heterogeneity: molecules are in different orientations and conformations

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

flip and flop views of hemocyanin

Frank et al., Science 1981

N. Boisset, thesis 1987
Multivariate analysis of aligned molecule images

FLIP/FLOP and Rocking positions

Hemocyanins of Arthropods are oligomers of a basic unit

Van Heel and Frank, Ultramicroscopy 1981
How to Find the Angles of Projection

Via bootstrap:

*Random-conical tilt reconstruction*

1979

1986/87
Random-Conical Tilt Reconstruction (Principle)

J. Frank, overhead 1979
Random-Conical Tilt Reconstruction (Principle – Fancy Version)

J. Frank, American Scientist 1998
First single-particle 3D reconstruction 1987

Radermacher et al., EMBO J. 1987

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

First 3D Reconstruction using Single Particle Reconstruction
Nobel Museum, Stockholm
Frozen-hydrated specimens / Plunge-freezing / Vitreous ice / Cryo-EM

Molecules embedded in vitreous ice

Robert Glaeser 1976

Jacques Dubochet 1981

Plunge-freezer
Plunge-freezers

--------- 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
E. coli ribosome 1995
Elongation Cycle (for adding each amino acid)

Decoding

Translocation
MAXIMUM LIKELIHOOD METHODS OF CLASSIFICATION


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

Class 1                  2                   3                   4                  5                  6

Agirrezabala et al., PNAS 2012
MILESTONES IN SINGLE-PARTICLE RECONSTRUCTION

- 1975 Concept
- 1978 Alignment via CCF
- 1986 Determine orientation 3D reconstruction
- 1981 Multivariate statistical analysis 2D classification
- 1996 CTF correction via Wiener filter
- 2007 Max likelihood 3D classification
- 2013
Resolution of single-particle cryo-EM was limited by the inferior quality of the recording medium

Best resolution from recording on film: 5.5Å
NEW ERA (SINCE 2012): DIRECT ELECTRON DETECTING CAMERAS

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

Detection Quantum Efficiency (DQE):
(how good is the recording device in capturing every single electron?)

![Graph showing DQE values for different single-electron detecting cameras](image)

Ribosomes, recorded on K2 GATAN direct electron detection camera
Elongation Factor G mutant H94A bound to the ribosome

nr 70S--P-E
nr 70S--EF-G—P-E
r 70S—EF-G—P/E
r 70S—P/E

50,000  90,000  35,000  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
Just in Time: high-resolution single-particle cryo-EM and the new pandemics

2012

2012 – 2015

MERS

2015 – 2016

Zika

2012 – 2015

2014 – 2016

Ebola

2015 – 2016

2019 – 2020

SARS-Cov2

2020 - ????
Fig. 4: Cryo-EM reconstructions of Fab–spike complexes and visualization of neutralizing epitopes on the spike surface.

From: Potent neutralizing antibodies against multiple epitopes on SARS-CoV-2 spike
Future directions

- Higher spatial resolution
- Time resolution
- Microfluidics
- State resolution
- Machine learning
- Energy landscape
Conclusion -- Single-particle cryo-EM: A new era in structural biology

• No need for crystals!
• *Compared to X-ray cryst., 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