Cryo-EM:
3D Electron Microscopy

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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?

Resolution trends of released EMDB entries

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

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

1. Add protein in buffer
2. Add heavy metal stain
3. Blot
4. Air dry

Carbon support film
Grid bars
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 ($\sim 10^6 ^\circ\text{C/s}$)

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 $-137 ^\circ\text{C}$, 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
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

Light microscope

- Light Source
- Condenser Lens
- Slide
- Objective Lens
- Specimen
- Image plane

TEM

- Electron Source
- Specimen
- Diffraction plane

X-ray

- X-ray Source
- Diffraction plane

X-ray Source
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!
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

Light microscope

- Light Source
- Condenser Lens
- Slide
- Objective Lens
- Image plane

TEM

- Electron Source
- Specimen
- Diffraction plane

X-ray

- X-ray Source
- Diffraction plane
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
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
Single Particle Image Processing

Particle Picking

Alignment

Classification

Averaging
3D reconstruction from projections

- Theory now 100 years old

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

- Rediscovery and practical application 50 years later
3D reconstruction from 2D projections

Molecular orientations

2D projections (observed images, without noise)

Calculated transforms

Projections transform to sections of the 3D FT

Section through map with fitted atomic structure

Inverse Fourier transformation gives the 3D density map
Projection matching/ Angular refinement

Raw images

Align by cross-correlation
Group into orientation classes

project

reconstruct
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

Li, Cheng et al, Nature Meth. 2013
Cryo-EM: pushing the resolution limit

Apoferitin at 1.65 Å

Zivanov et al., 2018. eLife 2018;7:e42166
3D classification

Dynamics from classification of conformational states
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 $R_{\text{free}}$
- Assess quality with Fourier Shell Correlation
Model validation: room for improvement

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!
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
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
The missing wedge problem:
Pairwise cross correlation must use only common regions of data

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

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Subtomogram averaging: single particle analysis in 3D

aligned to a reference

Use new reference for alignment. Iterate until reference is stable

averaged subtomograms (new reference)
Subtomogram averaging: GroEL

Tilt series

Reconstruction
GroEL reconstruction from 4600 sub-volumes

Resolution estimate

Fourier shell correlation

C1

C7

5.5 Å

8.7 Å

10.7 Å
Structures of the nuclear pore

1992, Hinshaw et al. X. l. (extracted) Negative Stain (RCT)
1993, Akey et al. X. l. (NE) Cryo EM (RCT)
1998, Yang et al. S. c. (extracted) Cryo ET (83 Å)
2003, Beck et al. D. d. (Nuc) Cryo ET (66 Å)
2003, Stoffler et al. X. l. (extracted) Cryo ET
2007, Beck et al. D. d. (Nuc) Cryo ET (58 Å)
2012, Maimon et al. Human (whole cell) Cryo ET (20 Å)
2013, Bui et al. Human (NE) Cryo ET (32 Å)
2015, Eibauer et al. X. l. (NE) Cryo ET (23 Å)

Use of direct electron detection and fit of high resolution structures

Assymetric unit based structure
Placement of Y-complex
Isotropic X.l. structure

3D structure
S. c. structure
Isotropic structure
Human structure

100 nm

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.

(Freeze-substitution makes life easier)
Sectioning vitrified cells by FIB milling

Specimens vitrified by plunge-freezing or high pressure freezing need to be $\ll 1$ 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

Kay Grunewald, Oxford
Multiscale imaging

- Single crystal X-ray diffraction & NMR
- Isolated particle EM tomography/sub tomogram averaging
- Averaging from cryo section tomography

Single particle EM

Cellular EM 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

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

Electron diffraction pattern of a 2D crystal
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

Henderson & Unwin (1975)

Noisy, low contrast image of crystal

2D projection density map

Phases (Image)

Model of 3D structure

Amplitudes (Electron diffraction)
3D electron crystallography ("MicroED")
What does the map tell us?

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

“Real” lattice

“unbending”

Perfect lattice
CCP-EM
Collaborative Computational Project for Electron cryo-Microscopy

Located at Research Complex at Harwell, UK

Alongside CCP4 core team – shared expertise between projects

Tom Burnley
Colin Palmer
Agnel Joseph
Martyn Winn
CCP-EM Activities

- Software suite
- Spring Symposium
- Training workshops
- Mailing list
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
The revolution will be televised
Symposium Proceedings

Acta Crystallographica Section D
STRUCTURAL BIOLOGY

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.
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
Contact details

Website: www.ccpem.ac.uk
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Twitter: @ccp_em
Email us: ccpem@stfc.ac.uk

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• Lecture courses and talks:
  • Caltech: http://cryo-em-course.caltech.edu/ and https://em-learning.com
  • CCP-EM Symposium: search “CCP-EM” on YouTube, or links on http://www.ccpem.ac.uk/