Progress in electron cryo microscopy (cryoEM)
Modalities of biomolecular cryoEM

Electron crystallography

Single particle analysis

Electron tomography

Zeev-Ben-Mordehai and Grünewald. In Structural Glycobiology. 2013
• **Electron crystallography**
  – *(purified) objects arranged in an ordered lattice, i.e.*
  – *needs crystals, either 2D or small 3D*
    - Aquaporin structure at 1.9 Å Gonen, et al. Nature. 2005
    - Lysozyme from 3D micro-crystal at 2.9 Å Shi, et al. 2013
  
  • **Next: MicroED** (Gonen, et al.)

• **Single particle analysis (SPA)**
  – *(purified) objects arranged in many different random orientations*
  – *Particles are computationally aligned - ‘in-silico crystallisation’*
    - Mitochondrial Large Ribosomal Subunit at 3.2 Å Amunts, et al Science 2014

• **Electron tomography (ET)**
  – *unique biological objects, i.e. pleomorphic objects with a unique shape without symmetry*
  – *cells, organelles, subcellular structures, viruses, macromolecules*
Sample preparation for cryoEM

- Vitrification of biological objects
- structures in frozen hydrated state
- no fixation, staining, dehydration …
- densities relate directly to biological material (protein, lipid, nucleic acid …)
Move over X-ray crystallography. Cryo-electron microscopy is kicking up a storm in structural biology by revealing the hidden machinery of the cell.

By Ewen Callaway

The revolution will not be crystallized
Method of the Year 2015

The end of ‘blob-ology’: single-particle cryo-electron microscopy (cryo-EM) is now being used to solve macromolecular structures at high resolution.

The three-dimensional structure of a protein or protein complex provides crucial insights into its biological function. As a structure-determination technique, cryo-EM has played second fiddle to the higher-resolution approaches of X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. This is rapidly changing, however, thanks to recent technical advances that now allow near-atomic-resolution structures to be solved using cryo-EM. The time is right to celebrate single-particle cryo-EM as our Method of the Year.

For decades, X-ray crystallography has been the go-to approach for solving protein structures. However, many proteins—especially membrane proteins and protein complexes—are stubbornly difficult, if not impossible, to crystallize. Alternatives to traditional crystallography have had different limitations. For example, the technique of serial femtosecond crystallography, carried out using an X-ray free-electron laser (XFEL), requires a slew of easier-to-produce microcrystals rather than single large protein crystals, but the competition for preparation as well as sophisticated image-processing software tools, as discussed in a Primer on page 23.

The cryo-EM resolution revolution is really just beginning, as discussed in a Commentary by Robert Glaeser on page 28. The sensitivity enhancements in detector technology have spurred opportunities for the development of new and improved methods that will push resolution, applicability and usability even further. Though cryo-EM is especially suited for large protein complexes, those that have been studied so far represent mainly the low-hanging fruit. Practical, reproducible and general methods for sample preparation are strongly needed to extend cryo-EM’s applicability to structures that have so far resisted determination by any structural technique. Data analysis is also ripe for improvement: researchers want simple, reliable computational methods to go from raw two-dimensional images to three-dimensional protein structures, especially for examining structurally heterogeneous systems.
beamtime at a highly specialized XFEL is fierce. NMR spectroscopy is useful for solving the structures of small proteins, but it remains quite difficult to apply it to larger ones.

In contrast to crystallography, cryo-EM is particularly well suited for obtaining structural information for large protein complexes and for systems that exhibit multiple conformational or compositional states. Researchers in this initially small field have made steady advances to improve the resolution and, by extension, the biological applicability of cryo-EM over the past few decades. A brief history of the key milestones in cryo-EM is given in a Historical Commentary by Eva Nogales on page 24.

This once small field is now exploding. A new type of highly sensitive direct-detection camera that captures electrons directly is making leaps in achievable resolution possible. The first papers to exploit these new detectors were published in 2013, and 2014 saw the publication of several important, high-resolution structures solved by cryo-EM. In 2015, the 3-Å resolution barrier was breached in multiple studies—an unprecedented feat that has surprised even long-time cryo-EM practitioners.

A good detector is not everything, however. A successful cryo-EM study depends heavily on good sample preparation, and the advent of cryo-EM presents new challenges in sample preparation. Like any scientific field undergoing a period of rapid growth, cryo-EM has not been without growing pains, as discussed in a News Feature on page 19. Encouragingly, many countries are setting up national user facilities with high-end instrumentation, but the demand for the top instruments is currently outstripping their availability. Many researchers are eager to take advantage of the technology, but cryo-EM is not a push-button technique (at least, not yet). There are many complex steps in sample preparation and in data analysis, which researchers must take care to properly apply, document and validate in order to avoid making mistakes. Proper training of new cryo-EM practitioners is crucial. The field must also come to a consensus about what raw data should be made available to reviewers and upon publication of a cryo-EM structure, as well as devise practical solutions for storing such large data sets.

Do these developments in cryo-EM signify the end of crystallography? X-ray crystallography will remain a robust technique for solving structures of proteins that can be readily crystallized. But the recent advances in cryo-EM open up exciting opportunities to access previously unexplored biological space. This is where future development efforts are likely to be concentrated.

To our readers, a very happy new year!
Recent technical advances in cryoEM

- More stable microscopes, FEGs, parallel illumination …
- Advanced control and data collection software (EPU)
- **Direct electron detectors**
- Advances in processing software (Relion, Freealign …)
- *Ongoing: Phase plates, …*
Better Detectors

**Indirect detection** (creation of photons first)
- CCD cameras (charged coupled devices)
  - Fibre coupled CCDs
  - Lens coupled CCDs
  - Deceleration

**Direct detection (of electrons)**
- Pixel detectors
  - Active Pixel Sensors (APS)
  - CMOS based detectors

**Detector developments:** Most relevant, higher sensitivity and higher resolution in cryo-ET studies!
Direct detection ‘revolution’:
DD (Gatan K2) vs. Film vs. CCD

Detective Quantum Efficiency = \frac{SN_{\text{out}}^2}{SN_{\text{in}}^2}.

Nyquist frequency → Nyquist/Shannon sampling theorem i.e. double the pixel size.
Ability to do electron counting!

**Figure 2**
Detective quantum efficiency (DQE) is the critical metric for evaluating high-performance detectors, such as those used in structural biology EM. This plot shows the relative performance of four common detectors used in structural biology: film, a scintillator-based CCD and two types of direct detectors. The CCD camera does not fully match the performance of film. The charge integrating direct detector, offers a large performance increase over CCDs and gives a slight improvement over film. Whereas the detector with counting and super-resolution offers a substantial increase in performance over all other detector technology.
Principle:
Instead of single images take **movies** in electron counting
With same dose!

By **post-aligning the movie frames** to each other, **compensate for beam-induced motion** …
i.e. superior images, higher resolution features preserved!
Direct electron detector: TMV on carbon film

Image with beam-induced movement

Same image after sub-frame alignment

1.3 Å per pixel
150,000 asymmetric units; 38 frames; 1° angle step; 4 Å resolution at 0.5 FSC
**DD example 1: quality**

**FIGURE 6** 300 kV low-dose image of vitrified *Thermoplasma acidophilum* archael 20S proteasome at 25,000x, 2.2 e-Å and 2.2 μm defocus. Courtesy of Xiemin Li and Yiyan Cheng, UCSF.

**FIGURE 7** Thon rings from an ice embedded 20S proteasome sample before and after drift correction. Shown in the top panel is the FFT of a region of ice-embedded 20S proteasomes using the K2 Summit camera without drift correction. Below is an image of the same sample after correction for sample drift. The image was collected at 31,000x nominal magnification (2.4 Å per physical pixel and 1.2 Å per effective pixel) and 20 e-Å. Drift correction was applied as described in Li et al. Nature Methods 10:584–590, 2013.

**FIGURE 8** Final 3D reconstruction of the *Thermoplasma acidophilum* archael 20S proteasome. (a) 3D density map of 20S proteasome filtered to a resolution of 3.3 Å. (b) Two different views of asymmetrical α- and β-subunits segmented from the 3D density map in (a). The main chain can be traced throughout the entire map. (c) Two α-helices segmented from the α- and β-subunits showing clear density for the majority of side chains. (d) Portion of the cryo-EM density map showing clear side chain densities. The docked atomic structure was refined to fit the density map by a molecular dynamic flexible fitting procedure. (e) The same portion of a 2Fo – Fc map of 3.4 Å crystal structure calculated using the atomic structure (PDB: 1PMA).

6.6Å reconstruction of the Eukaryotic translation initiation complex from 5143 particles (3% initial data set)

Heterogeneous initial 130k particle data set.

Maximum likelihood classification.

5143 (3% initial!) particles = 6.6Å reconstruction.

Not interested in 40,727 particle P/E tRNA 4.3Å reconstruction….

Data acquired on Polara equipped with an FEI Falcon II.

Cellular cryoET

- dose fractionation theorem: very low signal to noise
- beam penetration limit under low dose conditions: ~1µm
  keep your specimen thin! (200-500nm)
- Crowther criterion: \( d \sim \pi \frac{D}{N} \)
Cellular electron cryo tomography: complexes in situ
Michael Grange

FASCINATING INSIGHTS:
CELLULAR ELECTRON CRYO TOMOGRAPHY
Wellcome Trust Centre for Human Genetics
OXFORD PARTICLE IMAGING CENTRE
DIVISION OF STRUCTURAL BIOLOGY
Nuffield Department of Clinical Medicine (NDM)

Deciphering molecular virus - host interactions by an \textit{integrated} structural cell biology approach

Increasing biological complexity and integrity

Fluor. microsc.
X-ray microsc.
Cellular cryoET
CryoET
Sub-tomo

Increasing resolution

µm
nm
Å

Proteomics

Spatio-temporal

SPA cryoEM /
Crystallography
Structure - function: An artists view ...

David S. Goodsel
Rendered in watercolor on Arches paper.
Published in Moran, L.A. and Scrimgeour K.G. (1994) "Biochemistry"
Neil Patterson Publishers /Prentis Hall, North Carolina.
© 1994 Neil Patterson Publishers
http://mgl.scripps.edu/people/goodsell
Modalities of biomolecular cryoEM

- Electron crystallography
- Single particle analysis
- Electron tomography (ET)

Ideal for pleomorphic objects

Note: cryoET is just a fraction of field; much larger community of ET of plastic embedded specimens

Cellular tomography:
Concept of cell as a giant supramolecular assembly
Macromolecules in their native cellular context!
Cryo electron tomography
Architecture of mature HSV-1 virions

Herpes simplex virus 1 ‘life cycle’
Virus entry

- Fusion process?
- Dynamics, which ‘pole’ first?
- Fate of glycoprotein spikes and tegument
Dedicated experimental sub-systems to address different levels of complexity

Interaction with adherent cells
Entry into parts of cells
Fusion with liposomes
Isolated virions
Recombinant fusion proteins

... towards ‘molecular gymnastics’ of glycoproteins
HSV-1 multicomponent fusion machinery: known crystal structures of glycoproteins

Krummenacher et al.  
*EMBO J.* 2005. 24:4144

Chowdary et al.  
*NSMB.* 2010. 17:882

Heldwein et al.  
*Science.* 2006. 313:217
Cell-derived vesicles as display platform for full length membrane proteins

- full-length protein
- anchored with correct topology in genuine membranes
- detergent free / chemical free / virus free
- MS: protein of interest is the only membrane protein


Two forms of full-length gB on membrane

Vesicle fusion: Successive computational slices of a single tomogram.

Zeev-Ben-Mordehai et al. (PNAS, 2016)
Sub-tomogram averaging of gB-bilayer complex

1) initial model calculated by averaging all particles, then iterative averaging:
2) View vector refinement
3) Angle around the spike’s long axis refined
3) Symmetry detection and subsequent application
3) refinement of emerging angle
Two forms of full-length gB on membrane

Vesicle fusion: Successive computational slices of a single tomogram.
Structure interpretation

inside

membrane

outside

inside
**Model building of pre-fusion conformation**

3D puzzle, with the following constrains:

- 3 globular domains
- D1 and D2 are connected with short linkers
- D3 forms a central 3 helix bundle in the centre, major refolding in VSVG
- D5 mainly lack secondary structure elements, connects to MPR leading to the TM
Hierarchical constrained density-fitting of domains

Zeev-Ben-Mordehai et al. (PNAS, 2016)

Anna Hernandez  Daven Vasishtan  with Maya Topf (Birkbeck)

Domain I

Chimera  ADP-EM  GMFit  MultiFit

- top 30 models
- top 30 models
- top 30 models
- top 30 models

120 models

Clustering

- 50 models
- top 10 models

Filtering

- backbone clashes between D1 protomers

- 7 models

Assessment using domain II

(Extended Data Figure 10)

Domain I

Superposition of D1+D2 (hybrid model) on D1 fit

- 7 models
- (a,b)

D2 ensemble generation

- ≤ 60° random rotation around D1+D2 linkers
- 250 models each

Filtering

- backbone clashes between D1/D2 protomers
- linker distances

- Local refinement of top 4 models
- (c)

Domain IV

D4 ensemble generation

- 210 models

Filtering

- backbone clashes with D1+D2

- 4 models
Model of pre-fusion conformation

fusion loops apart and most distal to the membrane

Zeev-Ben-Mordehai et al. (PNAS, 2016)
Validation by mapping insertion mutation data

Analysis of mutations permitting and non-permitting 5-aa insertions (Lin and Spear 2007) or fluorescence protein insertions (Gallagher et al 2014) and subsequent plasma membrane expression.

(sphere: glycosylation site N398)

Suggested model: Proposed domain rearrangement

**pre-fusion**
- metastable
- triggered by gD and/or gH/gL

**extended intermediates**
- fusion loops come together to form a hydrophobic patch
- domains 5 and MPR extend
- domains 3 extend and form coil-coiled

**post-fusion**
- zippering of domains 5

Zeev-Ben-Mordehai et al. (PNAS, 2016)
Interaction of HSV-1 fusion machinery components?

Zeev-Ben-Mordehai et al. (PNAS, 2016)
Summary for part on herpesvirus fusion protein

- Multicomponent fusion machinery
- Mechanistic understanding requires studying different levels of complexity
- Membrane protein analysis requires context of native membrane
- Extracellular vesicle display system made a metastable pre-fusion state accessible
- Model for this conformation build by hierarchical constraint density fitting
- Validation by existing mutational and antibody data
- Novel conformation with fusion loops exposed
- Revised simple model
- New gB interaction surface …
Nuclear egress of herpesvirus capsids
Specimen thinning

Cryo-immobilization:

Focused-ion-beam (FIB) milling

Plunge-freezing (< 5 µm)

High pressure-freeze-planting (200-300 µm)

CEMOVIS

Cryosectioning (< 138 K)

CryoEM / Tomography

Sample thickness limit for cryoET: < 1 µm
Primary envelopment of nuclear capsids

Christoph Hagen

Projection images of vitreous sections of trypsinised Vero cells cryo-immobilised 16 h post-infection with HSV-1 K26GFP at MOI 10; nominal section feed: 30 nm; compression: 47 % (corrected)


Rendered volume of live 3D SIM data from nuclei of BK/EFN/UL31/34gfp cells, infected with PrV Bartha strain 765 (tagged with mRFP-SCP/VP26), MOI 100, 8 h p.i.
Live cell 3D SIM: tracking the dynamics of vesicles (UL31/34GFP)

Michael Grange / Lothar Schermelleh

Increasing biological complexity and integrity

Increasing resolution

Fluor. microsc.

X-ray microsc.

Cellular cryoET

CryoET

Sub-volume averaging

Spatio-temporal

Proteomics

SPA / Crystallography

µm

nm

Å
Earth-based photographs taken by Petr Chlanda (Dec. 2009)

Beamline of Gerd Schneider and colleagues

Soft x-ray microscopy / tomography at BESSY II
Vesicle formation from the nuclear membrane is induced by coexpression of two conserved herpesvirus proteins

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Communicated by Patricia G. Spear, Northwestern University Feinberg School of Medicine, Chicago, IL, February 26, 2007 (received for review January 5, 2007)

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adherent cell on support film

nucleus
Herpesvirus infection (PrV-Bartha)

Soft X-ray TXM tomogram (left: reconstructed volume; right: rendered volume with negative contrast) of a vitreous nucleus of a EFN-R cell infected with PrV Bartha strain UL35gfp (GFP-VP26), MOI 100, 15 h p.i. (carbon hole diameter: 2 µm).

Correlation of fluorescence and soft X-ray cryo microscopy

GFP-labeled viral protein

FluoSpheres

X-ray tomography – 3D

X-ray tomography – slice

Viral budding sites on extension of nucleoplasmic reticulum ‘tubes’

Conventional cryoFM vs. CryoSTORM
Super-resolution cryoFM

- **vitrification** preserves specimen at level of resolution analysed
- ‘**blinking’**: reversible photo bleaching of standard fluorescent proteins at ~80K suitable for single molecule localization microscopy
  - **cryo stage stability** for stochastic single molecule super-resolution
  - average single molecule localization accuracy ~ 40 nm
  - **resolution of ~ 125 nm** (i.e. 3-5x improvement)
  - using a LWD objective, i.e. non-dipping lens!

Kaufmann et al. (2014) *NanoLetters*
Cryo-immobilization:

- **Focused-ion-beam (FIB) milling**

Plunge-freezing (< 5 µm)

High pressure-freezing (200-300 µm)

Specimen thinning for in situ approach

- CEMOVIS
- Cryo-sectioning (< 138 K)

**CryoEM / Tomography**

Sample thickness limit for cryoET: < 1 µm
Milling rationale: accessing any cellular area

Vitrified eukaryotic cell

MTOC

ion milling direction

nucleus
golgi

actin

(slide courtesy of Jürgen Plitzko)
Focused Ion Beam – FIB

FEI Quanta 3D FEG dual beam FIB/SEM instrument as installed at the MPIB

Thanks to Jürgen Plitzko and Felix Bäuerlein for early access to technology.
Cryo-ET of FIB milled specimens

FIB milling (A before; B after)

TEM projection (high-pass filtered)

thinned region (Cryo-SEM; top view)

Specimen transfer to TEM

slice from 3D-reconstruction

NEC in situ: cryoET of lamellae prepared by FIB milling

Cryo-ET reconstruction from a FIB lamella of BK/EFN/UL31/34gfp cell nominal lamella thickness: 300 nm.

Thanks to Jürgen Plitzko and Felix Bäuerlein (MPIB) for early access to technology.

Selected tomogram slices and scheme

Sub-tomogram averaging, and initial modelling of the NEC

- 2 layers
- different hexagonal symmetry

Shape and stoichiometry of the pUL31-34 heterodimer within NEC

- SAXS data
- Soluble heterodimer
- UL34 truncated
- No membrane anchor

- Fitting into EM map

Architectural basis for constrained curvature formation

Curvature induction

Interaction regions between partners (UL31/UL34)

very tight interaction, very stable complex!

Zinc finger domain

- Zinc finger domain stabilizes structure
- Mutations impact function

- **In situ** (cellular assay)
- **In vitro** (in GUVs)

Collaboration with Thomas Mettenleiter and Wolfram Antonin labs
Crystal structure based model and fit

Increasing biological complexity and integrity

Soft X-ray microscopy

Increasing resolution

Fluorescence microscopy (life-cell SIM, cryoSMLM)

Cellular cryoET (FIB-SEM prepared)

Fitted SAXS model

Curved lattice fit

Crystallography $U_{L31/34}$ heterodimer
Collaborative network

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