C22: Techniques in Structural Biology

Macromolecular structure determination by electron microscopy and image reconstruction

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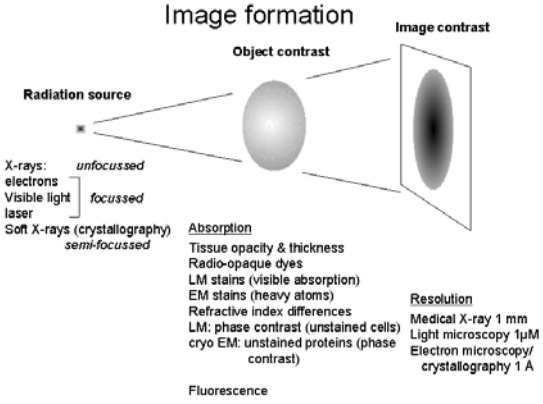
EM lecture 1

Methods for cellular and molecular structure determination
Scanning and transmission microscopies
Image formation
Projections and sections
What can be studied by transmission EM?
Image reconstruction from projections by tomography
Molecular structure methods
Negative stain and cryo EM

EM lecture 2

Single particles
Image processing
Methods for 3D reconstruction
Combining X-ray crystallography and cryo EM
Helical assemblies
2D crystals
Examples
References

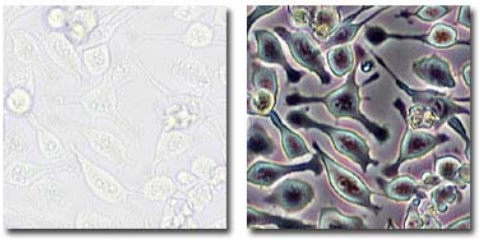
Image formation



3D cellular structure techniques

Light microscopy (phase contrast, fluorescence)	• Can be done on living cells • Thickness up to ~10 µm • Resolution limited by optical wavelength (200 nm)
Thin sectioning (electron microscopy)	• Fixation and plastic embedding • Mechanical damage • Thickness up to ~1 µm (high voltage) • Resolution limited by specimen preparation
Cryo sectioning or vitrification (rapid freezing) of thin cells	• Can preserve native cellular structure • State-of-the-art, not currently routine • Thin cells or sections < 1 µm • Resolution limited by radiation damage (4-5 nm)

Living Cells in Brightfield and Phase Contrast



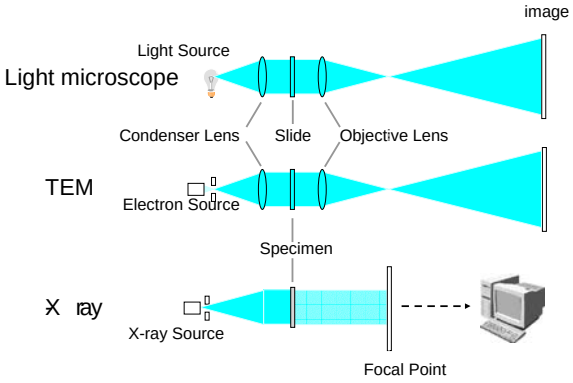
In phase contrast microscopy, small differences in scattering from transparent specimens are converted into intensity variations, to give better contrast

Protein structure techniques

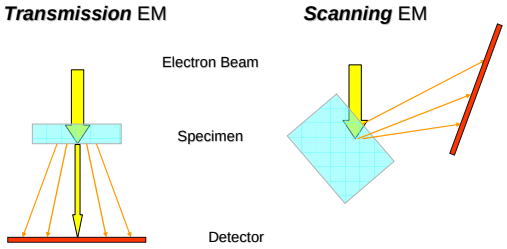
X ray crystallography	• Needs crystals • Gives atomic resolution • Conformation may be affected by crystal lattice
NMR	• Gives near-atomic resolution • Can see dynamic processes • Protein must not be too large (current limit ~80 kDa, TROSY ~800?)
Cryo electron microscopy	• Resolution 4 – 30 Å (depends on sample order and data volume) • Crystals, ordered assemblies or isolated particles • Can trap transient states

3D structure determination of macromolecules

Technique		Sample	Resolution	Advantages	Disadvantages
X-ray crystallography		Molecule to virus	Atomic	High resolution; Well established, often routine	Lots of pure specimen, crystals. No phases
NMR		Small molecule	Atomic	High resolution, in solution	MW < 100 kDa, concentrated, isotopic labelling
Cryo EM	2D crystals	Molecule	Atomic/ molecular	Membrane proteins, Get phases	Need crystals, tilting, slow and difficult
	Symmetrical assemblies	Icosahedral virus, helix	Secondary structure	Native structure in solution, get phases, time resolution, separate mixtures	Limited resolution, but improving, ~7 Å
	Single particle	Large complexes	Molecular		



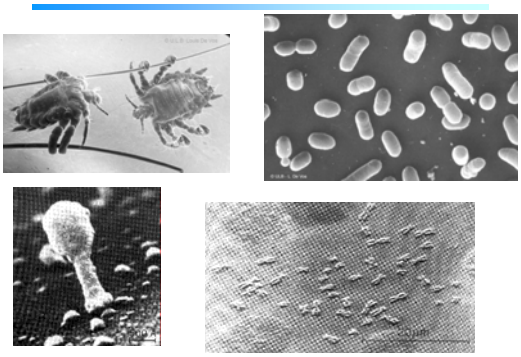
TEM vs. SEM



Detection of electrons behind the specimen (scattered e^-)

Detection of backscattered electrons (primary e-) and generated electrons (secondary e^-)

Scanning EM Examples



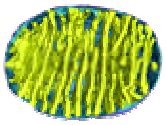
What can be studied by TEM?

2D crystals
(need tilted views for 3D)

Helical assemblies

Icosahedral viruses

Asymmetric particles

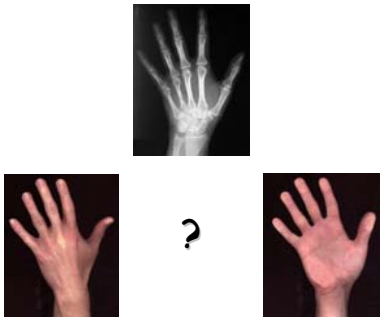


Whole cells or organelles
(need tilt series for 3D, unique objects)

How is the 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

A single projection image is insufficient to infer the 3-D structure of an object

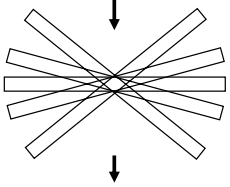


Tomography

3D structures can be reconstructed if a series of 2D projections is collected.

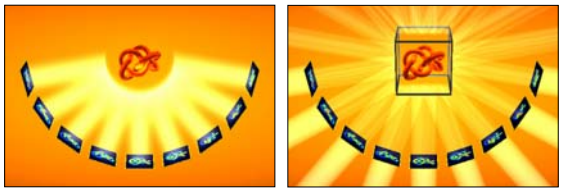
View direction

Multiple exposure of specimen at different tilt angles



Record images

Principle of Electron Tomography



3D-object => set of 2D-projections

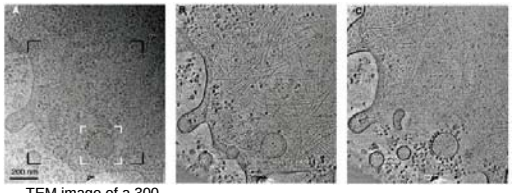
2D-projections => 3D-reconstruction

W Baumeister, MPI Martinsried

Reconstruction of whole cells or organelles by tomography

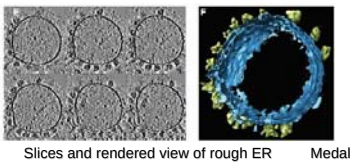
- Small pieces of tissue or thin, whole cells can be vitrified
- Cell regions up to 1 µm thick can be examined
- Many exposures of the same area - tilt series - because unique object
- Resolution 3-4 nm - main limit is radiation damage
  Also limitation on vertical resolution because maximum tilt ~70° - missing views from 70-90°
- 3D reconstruction by back projection

Views of *Dictyostelium* cytoplasm from cryo tomography



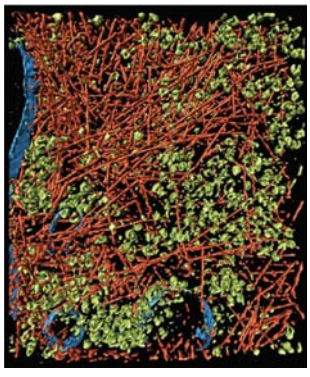
TEM image of a 300 nm thick region

Slices from the reconstruction



Slices and rendered view of rough ER

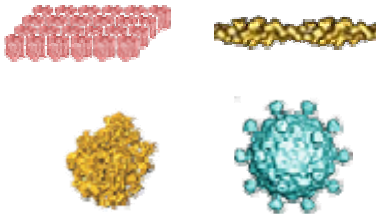
Medalia *et al.* (2002)



Rendered view of the actin network , membranes and macromolecular complexes *in situ*

Medalia *et al.* (2002)

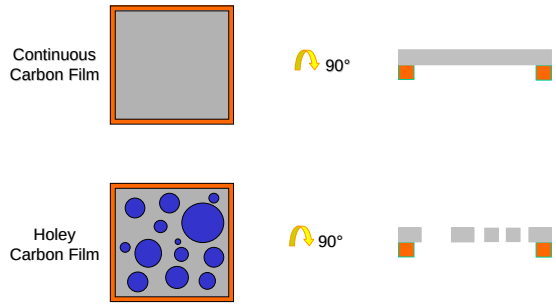
Molecular structure



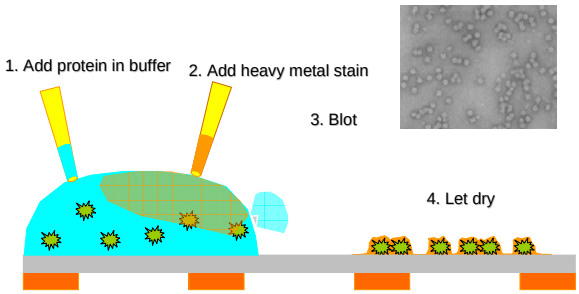
Negative stain vs.cryo EM

Negative staining	Cryo EM
• Simple procedure	• More complex preparation
• Quick to check samples	• Longer time for checking samples
• High contrast	• Low contrast
• Dehydration	• Native, hydrated state
• Heavy metal salts	• Near physiological conditions
• Possible distortion, flattening	• 3D structure preserved
	• Rapid freezing can trap transient states

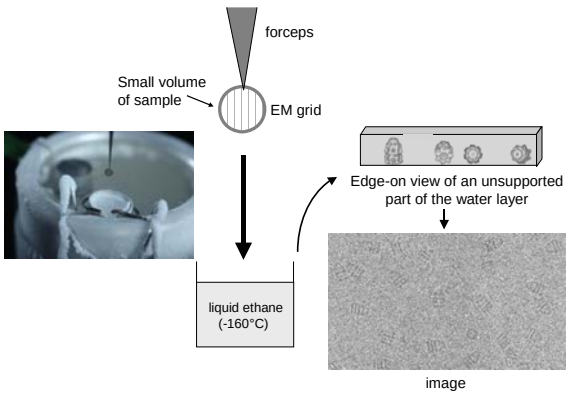
Two Types of Carbon Support



Negative Stain

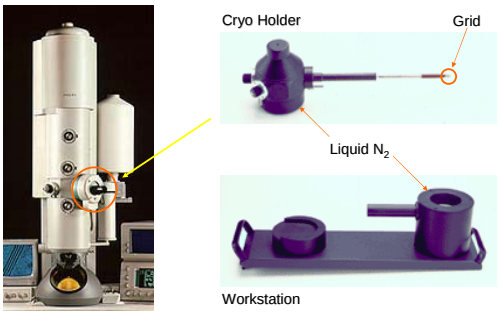


Sample preparation for cryo EM

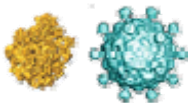


The Specimen

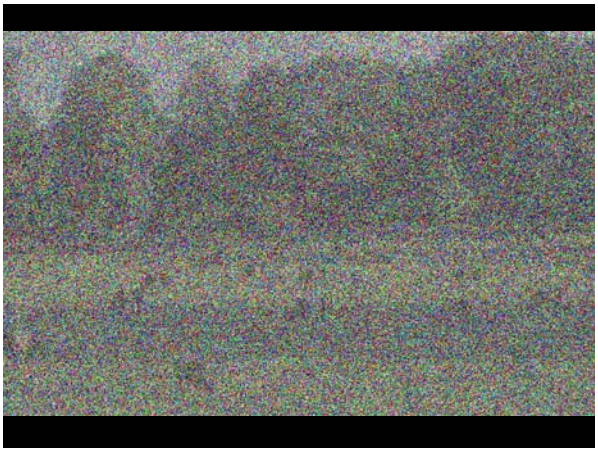
Cryo-Transfer

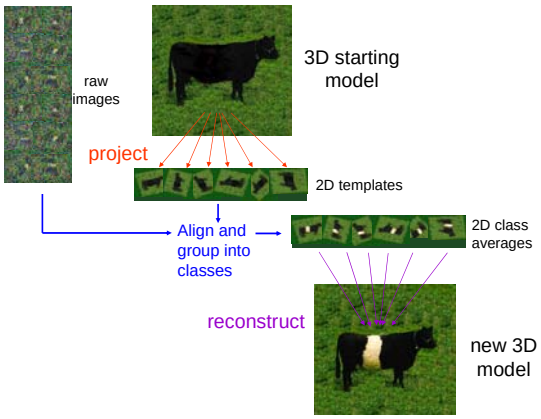


Single particles



- 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
- Mixed states can sometimes be separated ("purification in the computer")

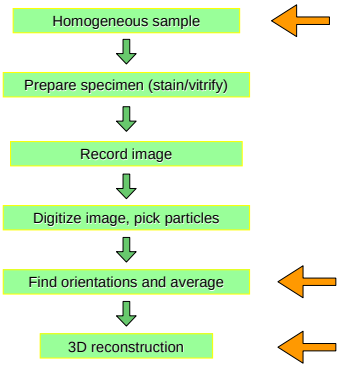




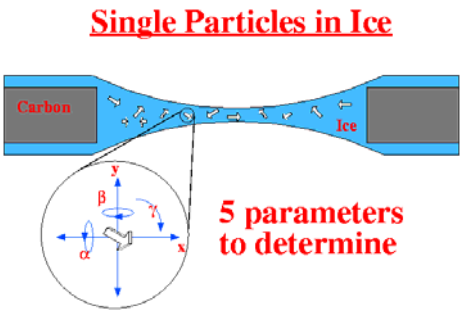
Size limitations for single particle EM

Type of molecule	M_w (kDa)	Diameter (A)	Single particle EM possible?
Large virus	300 000	900	Yes
Small virus	11 000	300	Yes
Ribosome	2 500	250	Yes
Multimeric enzyme	420	300	Yes
	180	75	Yes
	52	50	Negative stain only
Small Monomeric Protein	18	35	Negative stain?
Very small protein	7	25	No

Steps in single particle structure determination

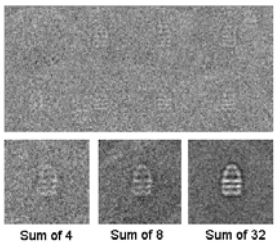


Finding orientations



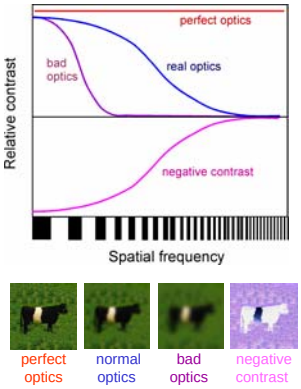
N. Grigorieff,
Brandeis Univ.

Averaging similar views improves the signal:noise ratio

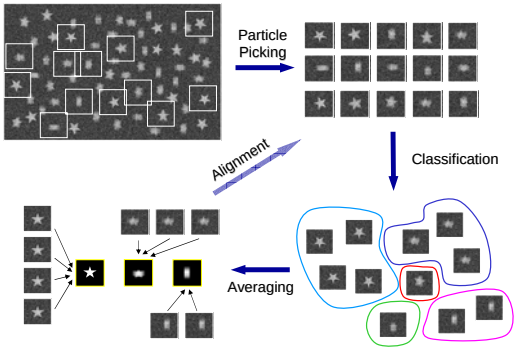


Individual raw images

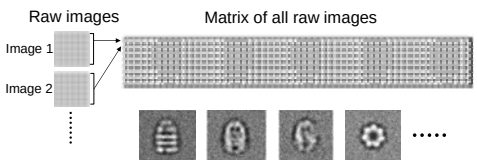
Optical corrections: Contrast transfer



Single Particle Image Processing



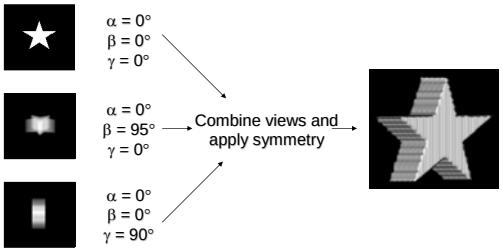
Classification of images: Multivariate statistical analysis



Class averages grouping the major views present in the data set

Angular reconstitution

Find angles by searching for common line projections



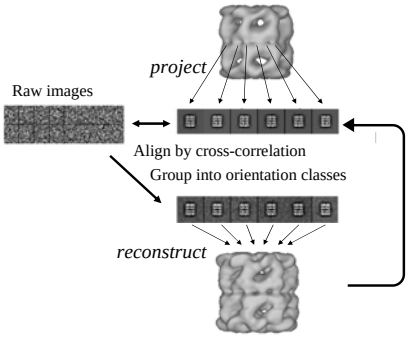
3D reconstruction: Conical tilt

Pairs of images are recorded of the same field of particles at high tilt and untilted

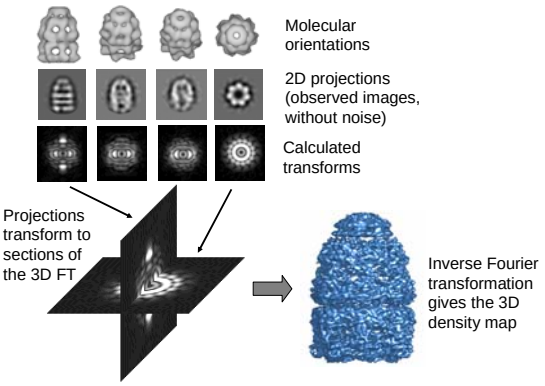
Orientations for 3D reconstruction are determined from the pairs of views - tilt angle is known

Frank (1998)

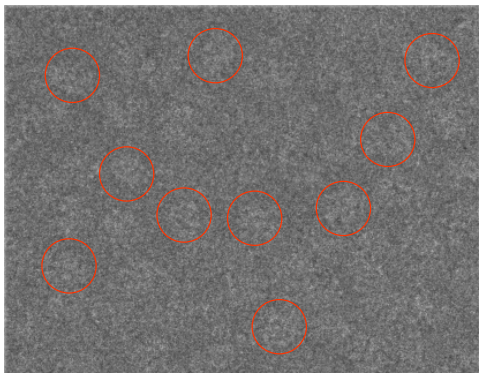
Projection matching/ Angular refinement



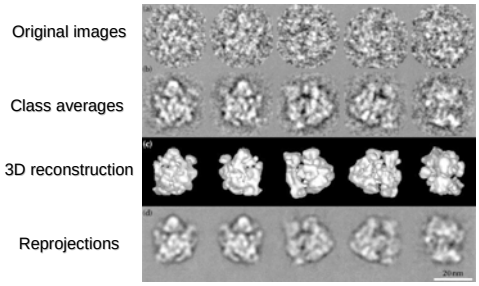
3D reconstruction from 2D projections



Cryo EM image of ribosomes

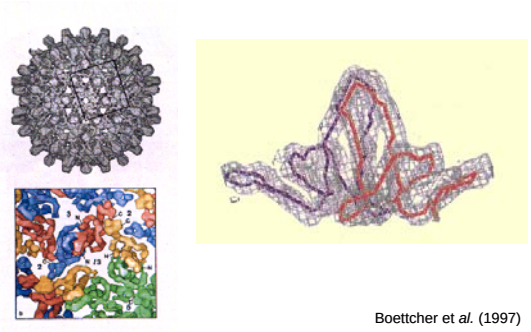


Ribosome: Angular reconstitution



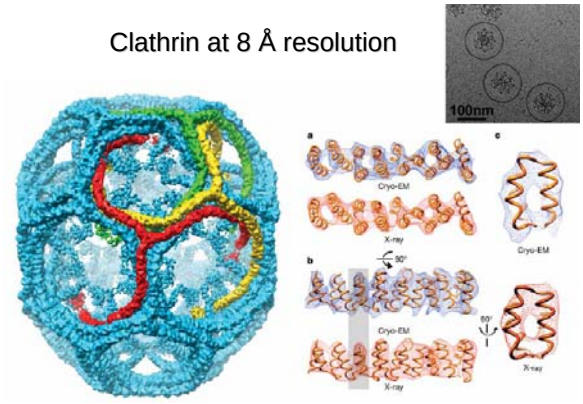
Stark *et al.* (1995)

Hepatitis B virus at 7.5 Å resolution



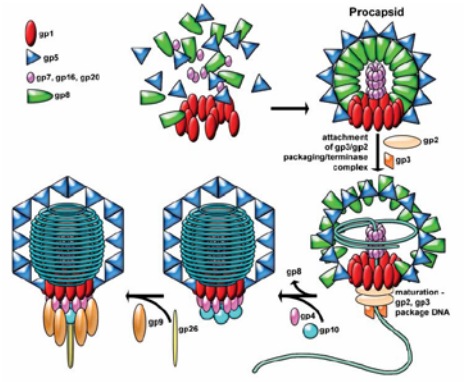
Boettcher et *al.* (1997)

Clathrin at 8 Å resolution

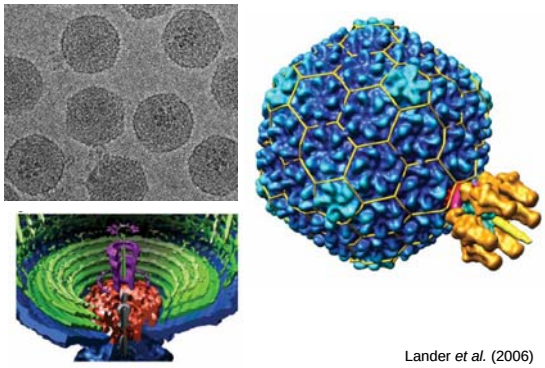


Fotin *et al.* (2004)

Macromolecular machines: assembly of bacteriophage p22

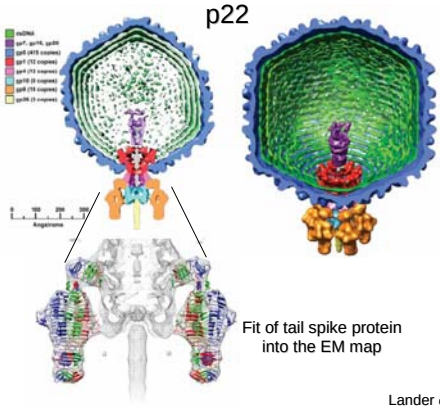


Cryo EM and asymmetric single particle reconstruction of phage p22



Lander *et al.* (2006)

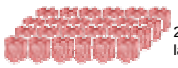
p22



Fit of tail spike protein into the EM map

Lander *et al.* (2006)

2D crystals

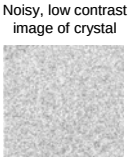


2D crystals contain a single layer of protein molecules

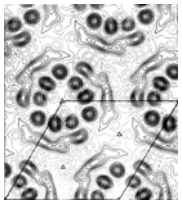
Three-dimensional model of purple membrane obtained by electron microscopy

R. Henderson & P. N. T. Unwin

Noisy, low contrast image of crystal



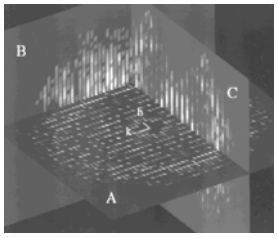
2D projection density map

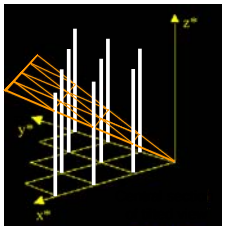


Model of 3D structure

Henderson & Unwin (1975)

Tilting of 2D crystals to get 3D data

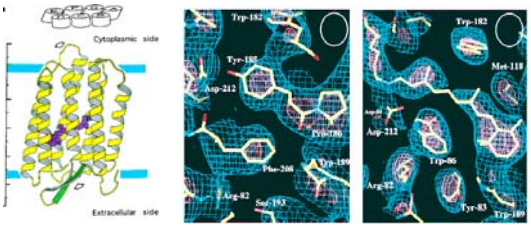




3D electron diffraction intensity data for tubulin

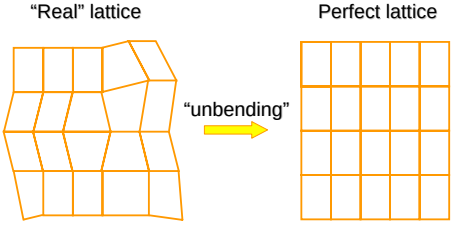
Nogales *et al.* (1997)

Refined structures of bacteriorhodopsin

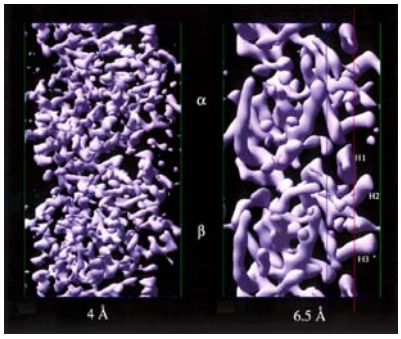


Grigorieff et *al.* (1996)

Unbending

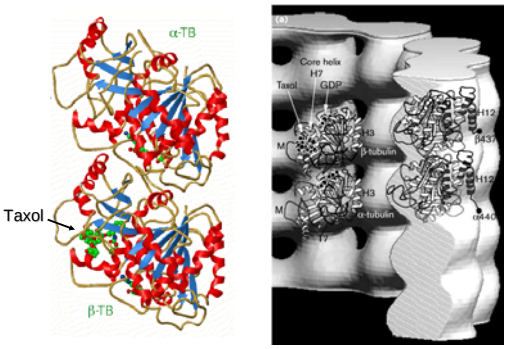


Tubulin (from 2D crystals)



Nogales *et al.* (1997)

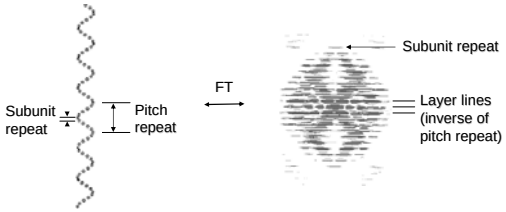
Tubulin fitted into microtubules



Helical arrays

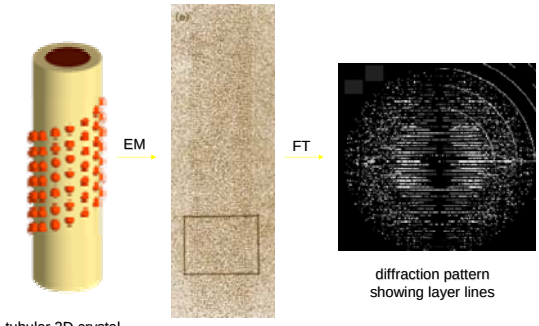
- Some samples form filaments or tubes with helical symmetry
- Identifying the repeat and lattice of the helix allows full 3D model to be generated
- All orientations of the sample are available hence no missing cone
- Examples are: nicotinic acetylcholine receptor, actin, kinesin, flagellin

Helical reconstruction



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.

Helical diffraction

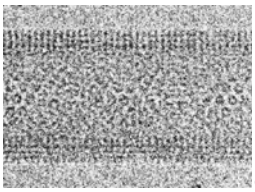


tubular 2D crystal

projection image

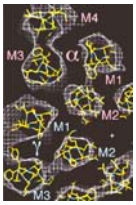
diffraction pattern showing layer lines

Tubular crystals of acetylcholine receptors



Cryo EM image of tubular membrane with helical array of receptors

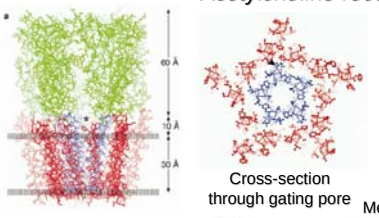
Cross-section through membrane tube



Section of density map +, position of pore

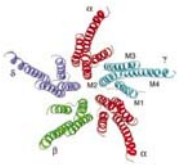
Miyazawa *et al.* (2003)

Acetylcholine receptor structure

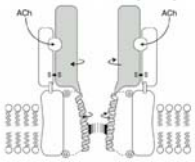


side view, with membrane surfaces

Cross-section through gating pore



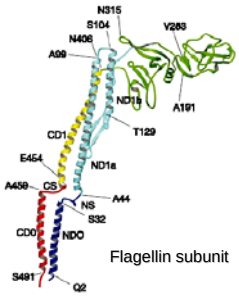
Mechanism of gating



Miyazawa *et al.* (2003)

Helical reconstruction of bacterial flagella

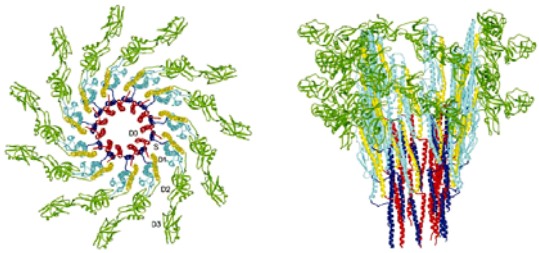
Flagellum density map



Flagellin subunit

Yonekura et al (2003)

Structure of bacterial flagella



Changes in packing lead to changes in twist that power the motions in bacterial swimming

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