

# C22: Techniques in Structural Biology

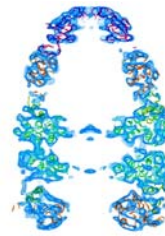
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## Macromolecular structure determination by electron microscopy and image reconstruction

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<http://people.cryst.bbk.ac.uk/~ubcg16z>



### EM lecture 1

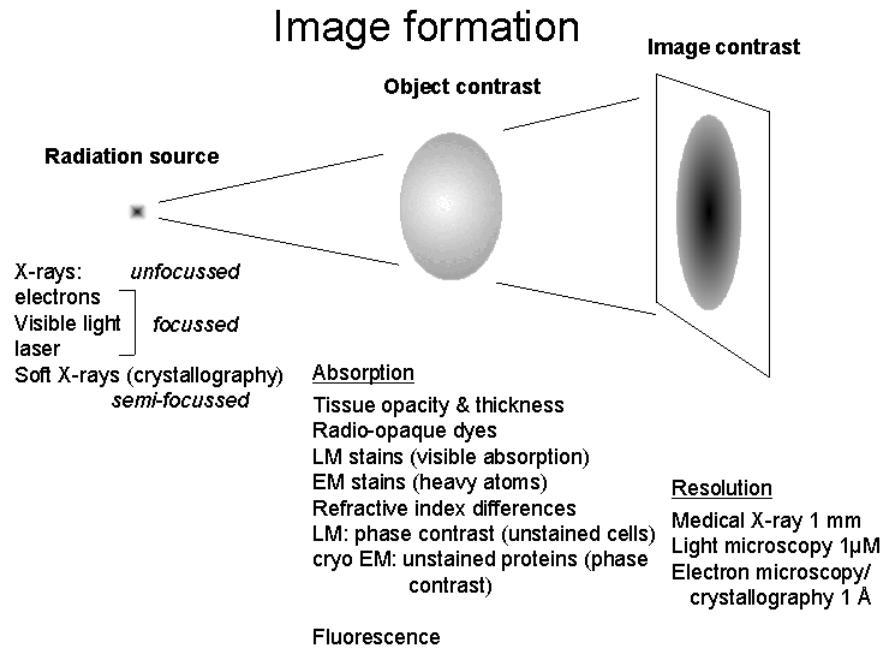
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Methods for cellular and molecular structure determination  
Scanning and transmission microscopies  
Image formation  
Projections and sections  
What can be studied by transmission EM?  
Image reconstruction from projections by tomography  
Molecular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

### EM lecture 2

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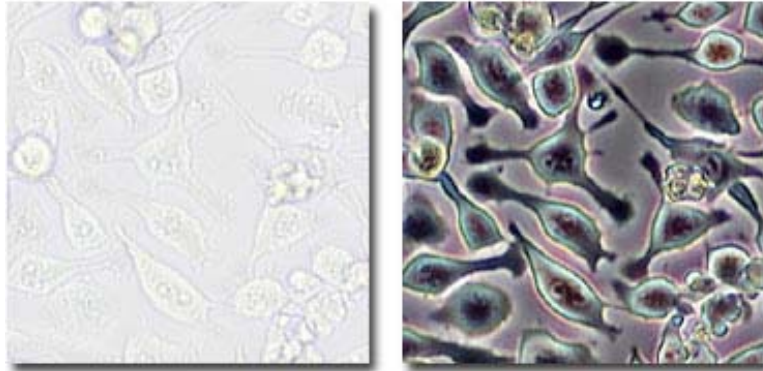
Single particles  
Image processing  
Methods for 3D reconstruction  
Combining X-ray crystallography and cryo EM  
Helical assemblies  
2D crystals  
Examples  
References



## 3D cellular structure techniques

- |  |   |
|--|---|
| <p>Light microscopy<br/>(phase contrast, fluorescence)</p>             | <ul style="list-style-type: none"> <li>• Can be done on living cells</li> <li>• Thickness up to ~10 <math>\mu</math>m</li> <li>• Resolution limited by optical wavelength (200 nm)</li> </ul>   |
| <p>Thin-sectioning<br/>(electron microscopy)</p>                       | <ul style="list-style-type: none"> <li>• Fixation and plastic embedding</li> <li>• Mechanical damage</li> <li>• Thickness up to ~1 <math>\mu</math>m (high voltage)</li> <li>• Resolution limited by specimen preparation</li> </ul>                                |
| <p>Cryo-sectioning or vitrification (rapid freezing) of thin cells</p> | <ul style="list-style-type: none"> <li>• Can preserve native cellular structure</li> <li>• State-of-the-art, not currently routine</li> <li>• Thin cells or sections &lt; 1 <math>\mu</math>m</li> <li>• Resolution limited by radiation damage (4-5 nm)</li> </ul> |

## 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

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### X-ray crystallography

- Needs crystals
- Gives atomic resolution
- Conformation may be affected by crystal lattice

### NMR

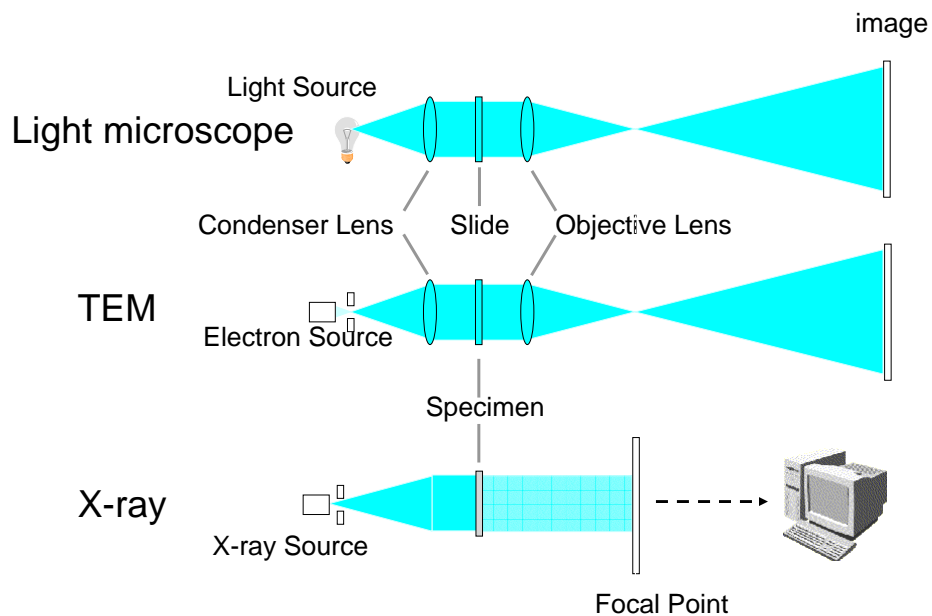
- Gives near-atomic resolution
- Can see dynamic processes
- Protein must not be too large (current limit ~80 kDa, TROSY ~800?)

### Cryo-electron microscopy

- Resolution 4 – 30 Å (depends on sample order and data volume)
- Crystals, ordered assemblies or isolated particles
- Can trap transient states

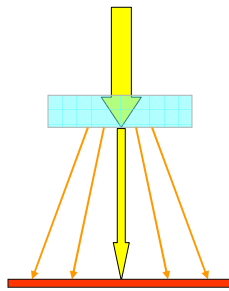
## 3D structure determination of macromolecules

Technique	Sample	Resolution	Advantages	Disadvantages	
<i>X-ray crystallography</i>	Molecule to virus	Atomic	High resolution; Well established, often routine	Lots of pure specimen, crystals. No phases	
<i>NMR</i>	Small molecule	Atomic	High resolution, in solution	MW < 100 kDa, concentrated, isotopic labelling	
<i>Cryo EM</i>	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		



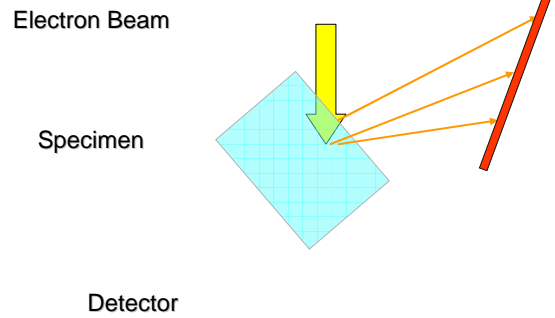
# TEM vs. SEM

## Transmission EM



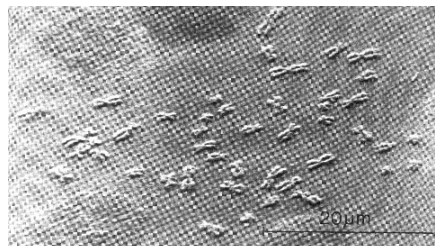
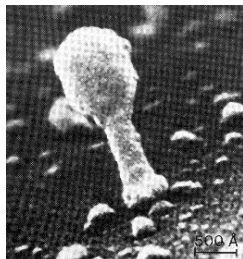
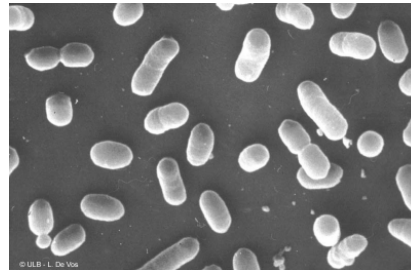
Detection of electrons behind the specimen (scattered e<sup>-</sup>)

## Scanning EM



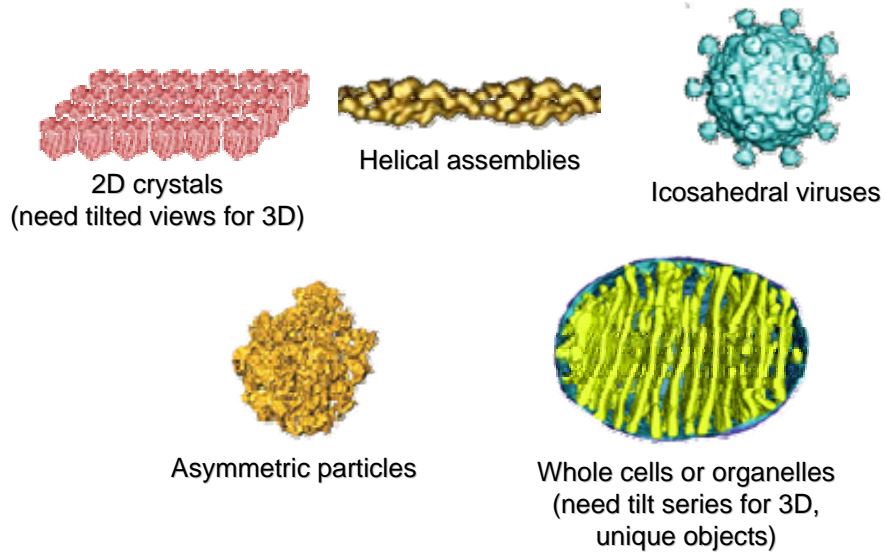
Detection of backscattered electrons (primary e<sup>-</sup>) and generated electrons (secondary e<sup>-</sup>)

## Scanning EM Examples



## What can be studied by TEM?

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## How is the image formed?

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- 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

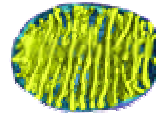
A single projection image is insufficient to infer the 3-D structure of an object

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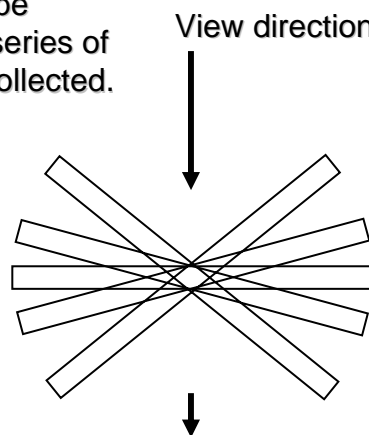
## Tomography

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

Multiple exposure of specimen at different tilt angles



Record images

## Principle of Electron Tomography



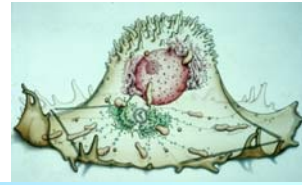
3D-object => set of 2D-projections



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  $\mu\text{m}$  thick can be examined

Many exposures of the same area - tilt series - because **unique object**

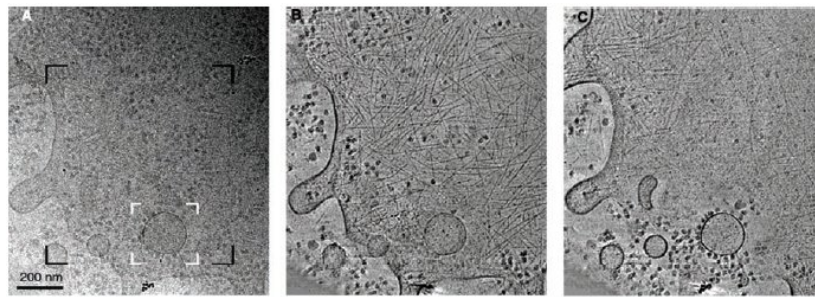
Resolution 3-4 nm - main limit is **radiation damage**

Also limitation on vertical resolution because maximum tilt  $\sim 70^\circ$  - missing views from  $70-90^\circ$

3D reconstruction by **back projection**

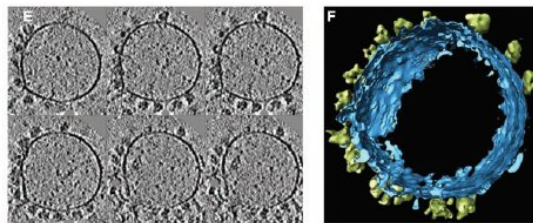


## Views of *Dictyostelium* cytoplasm from cryo tomography



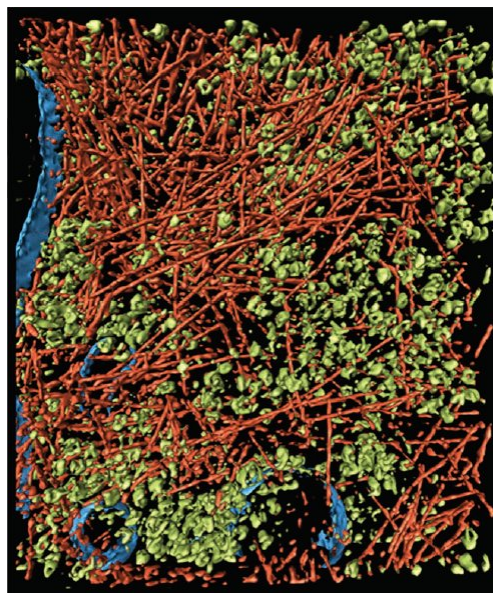
TEM image of a 300 nm thick region

Slices from the reconstruction



Slices and rendered view of rough ER

Medalia *et al.* (2002)

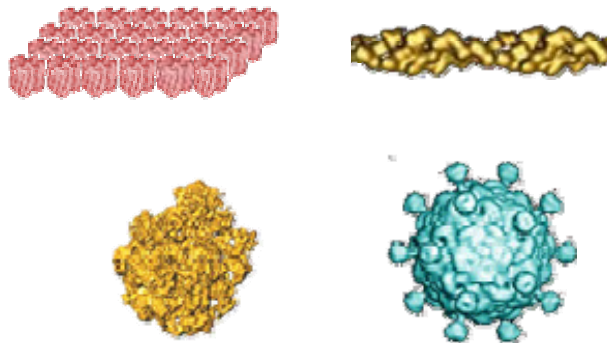


Rendered view of the actin network, membranes and macromolecular complexes *in situ*

Medalia *et al.* (2002)

# Molecular structure

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## Negative stain vs.cryo EM

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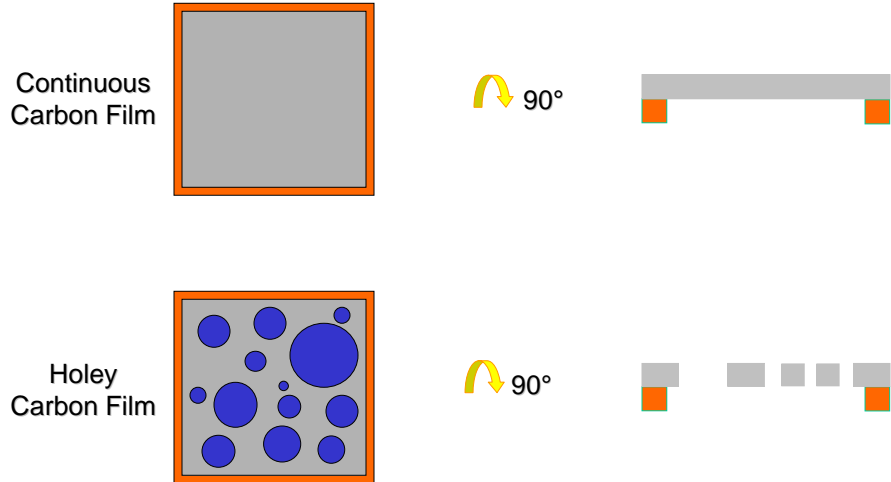
### Negative staining

- Simple procedure
- Quick to check samples
- High contrast
- Dehydration
- Heavy metal salts
- Possible distortion, flattening

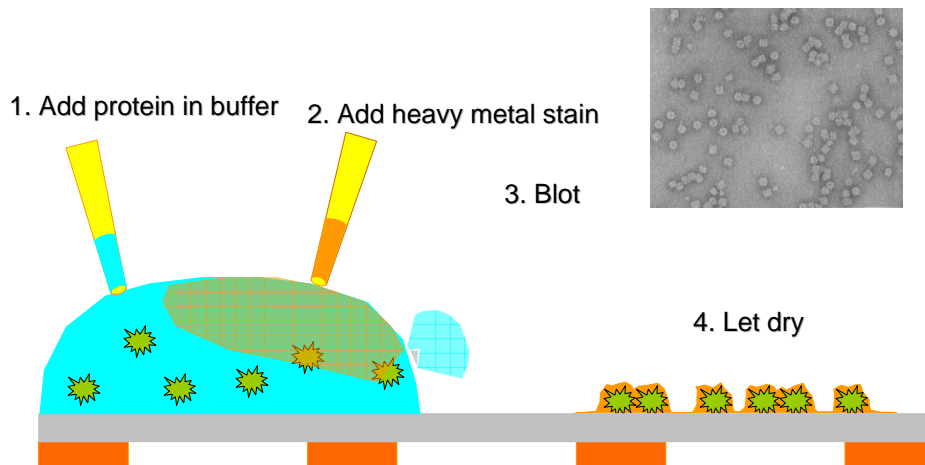
### Cryo EM

- 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

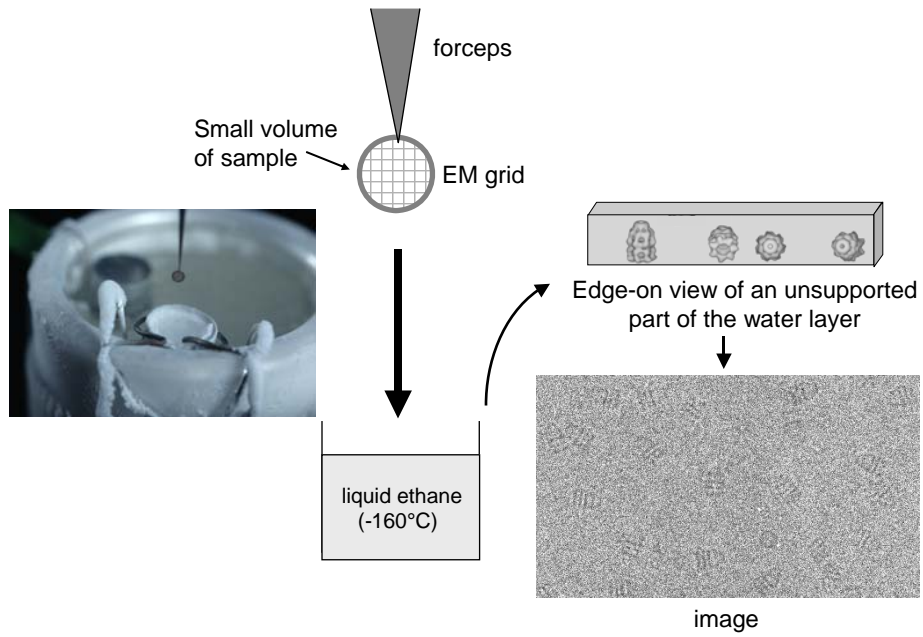
## Two Types of Carbon Support



## Negative Stain



## Sample preparation for cryo EM

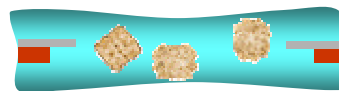


## The Specimen

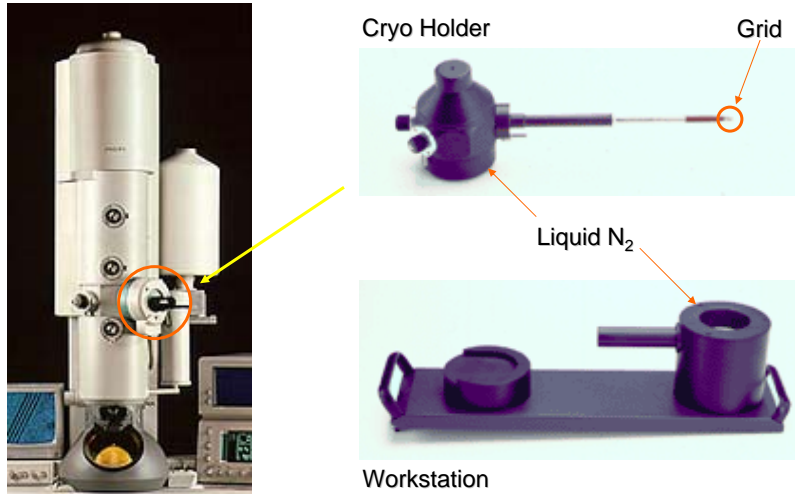
Heavy metal stain



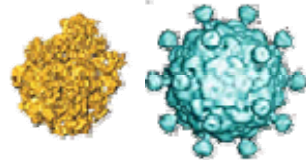
Vitreous ice



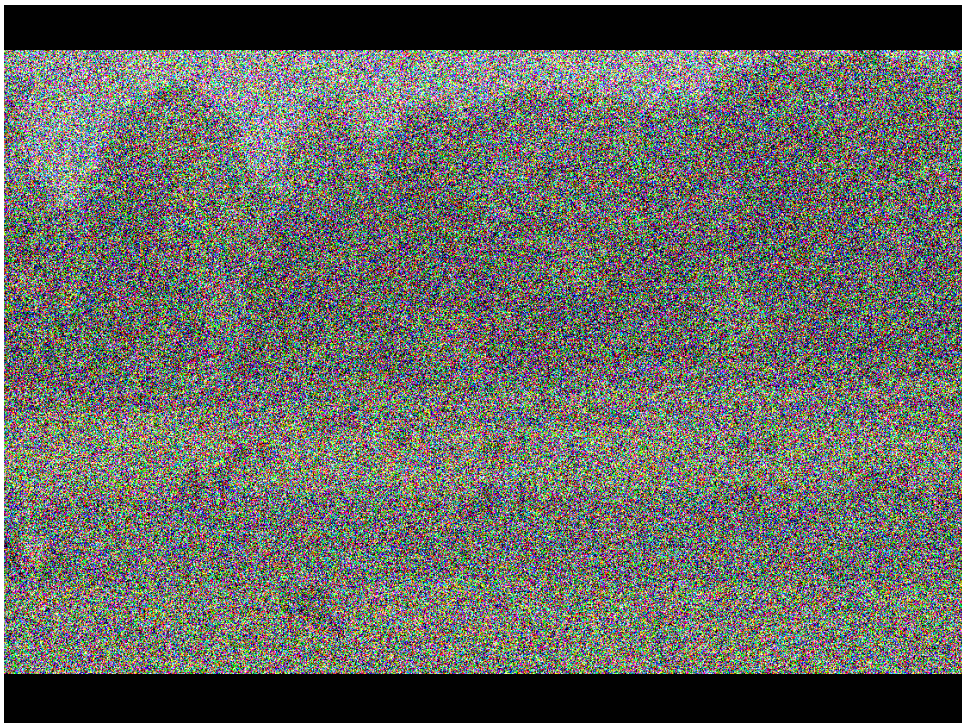
# Cryo-Transfer

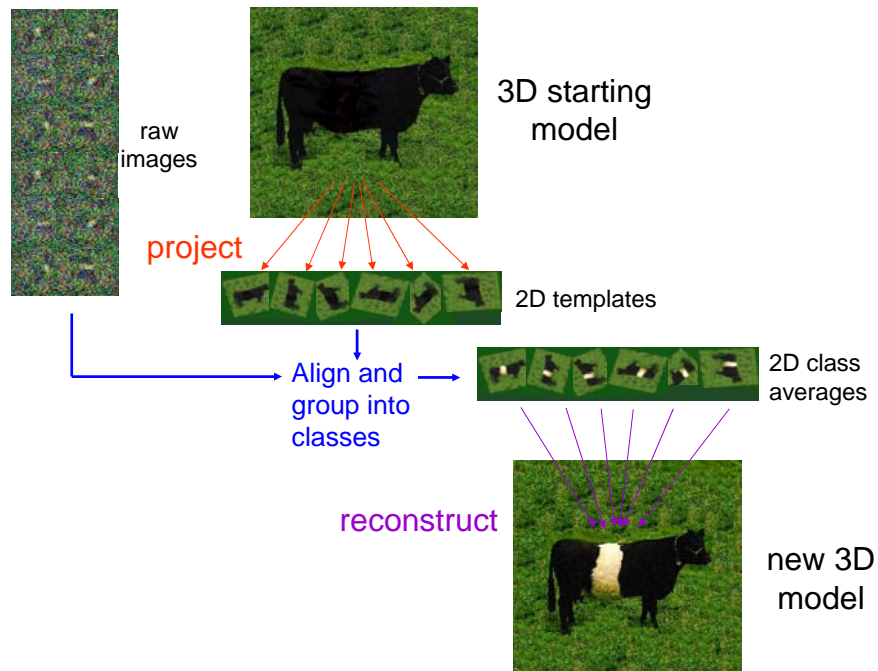


## Single particles



- 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”)

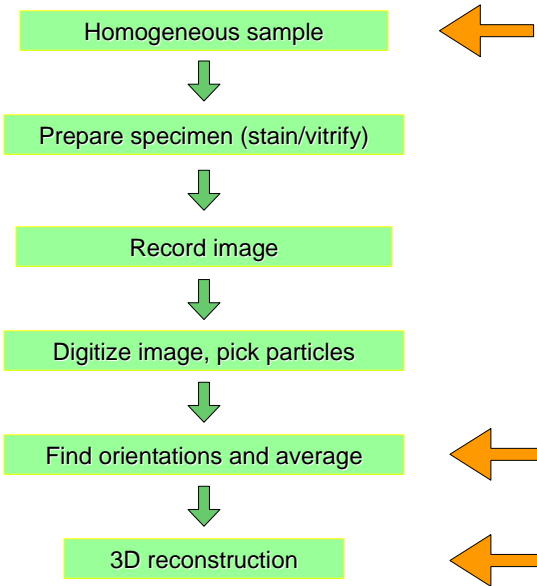




## Size limitations for single particle EM

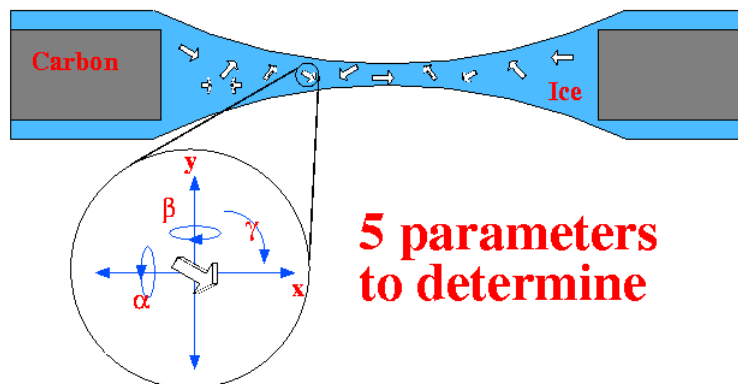
Type of molecule	M <sub>w</sub> (kDa)	Diameter (Å)	Single particle EM possible?
Large virus	300 000	900	Yes
Small virus	11 000	300	Yes
Ribosome	2 500	250	Yes
Multimeric enzyme	420	300	Yes
	180	75	Yes
	52	50	Negative stain only
Small Monomeric Protein	18	35	Negative stain?
Very small protein	7	25	No

## Steps in single particle structure determination



### Finding orientations

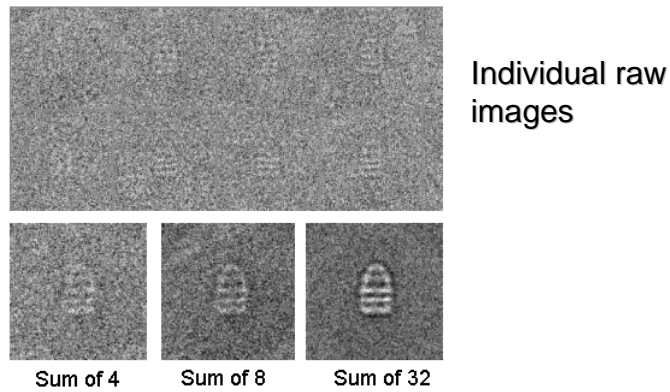
## Single Particles in Ice



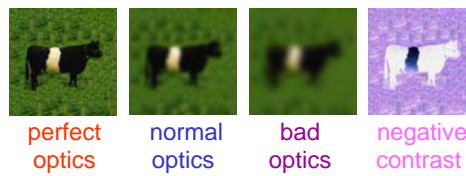
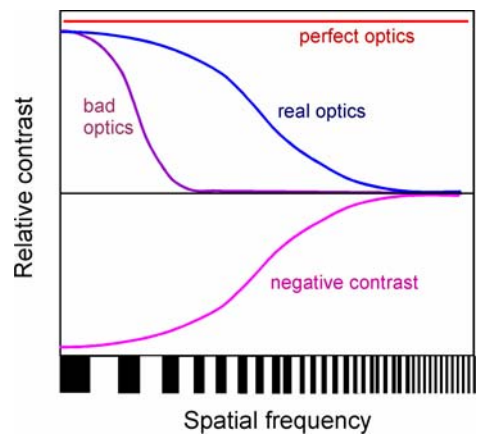
N. Grigorieff,  
Brandeis Univ.



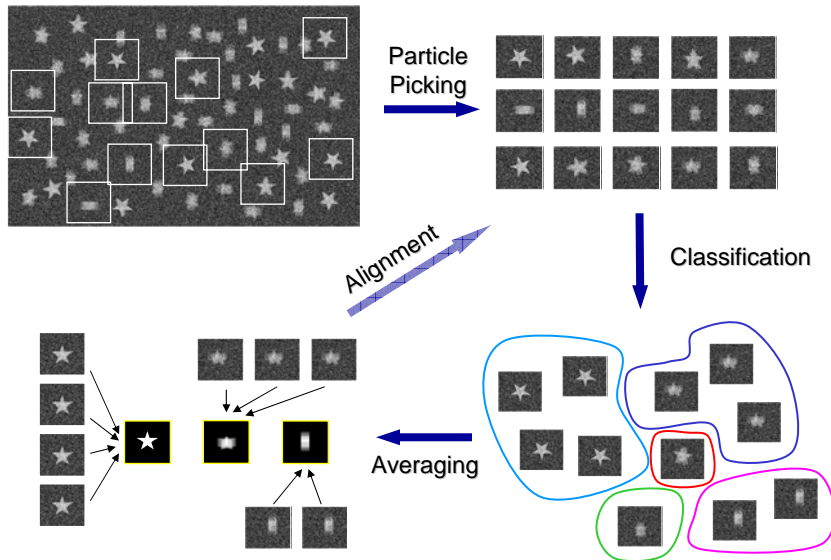
## Averaging similar views improves the signal:noise ratio



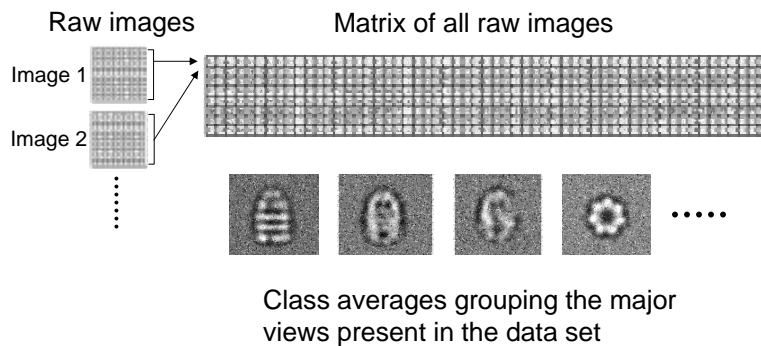
## Optical corrections: Contrast transfer



# Single Particle Image Processing

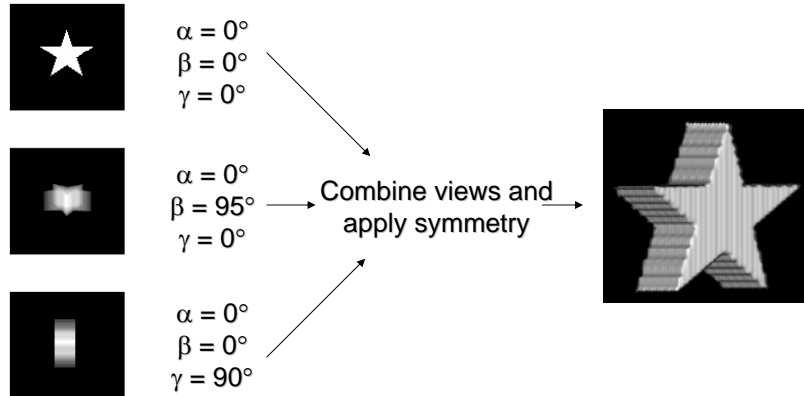


## Classification of images: Multivariate statistical analysis



## Angular reconstitution

Find angles by searching for common line projections



## 3D reconstruction: Conical tilt

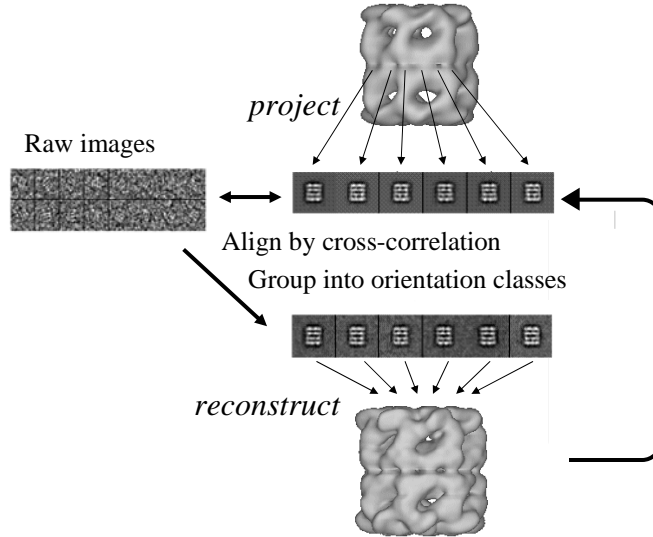


Pairs of images are recorded of the same field of particles at high tilt and untilted

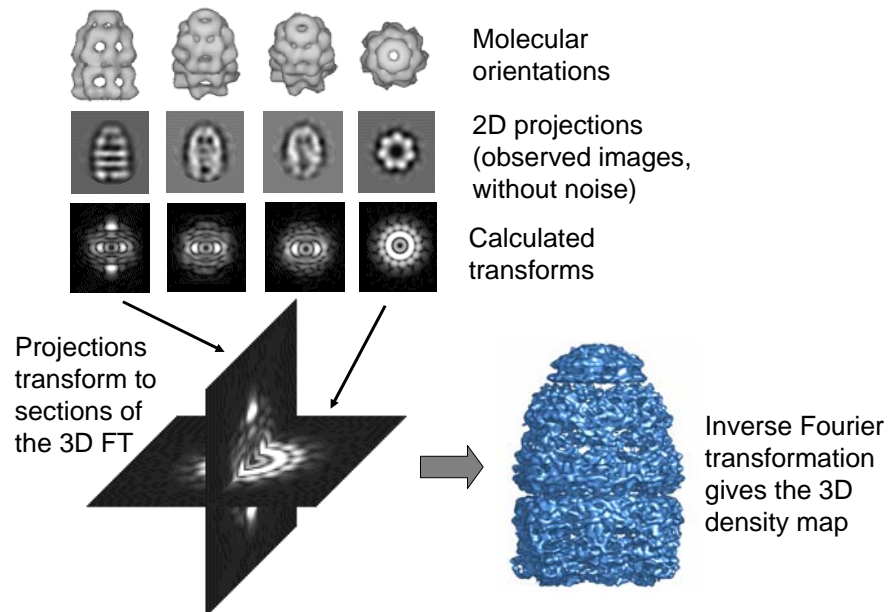
Orientations for 3D reconstruction are determined from the pairs of views - tilt angle is known

Frank (1998)

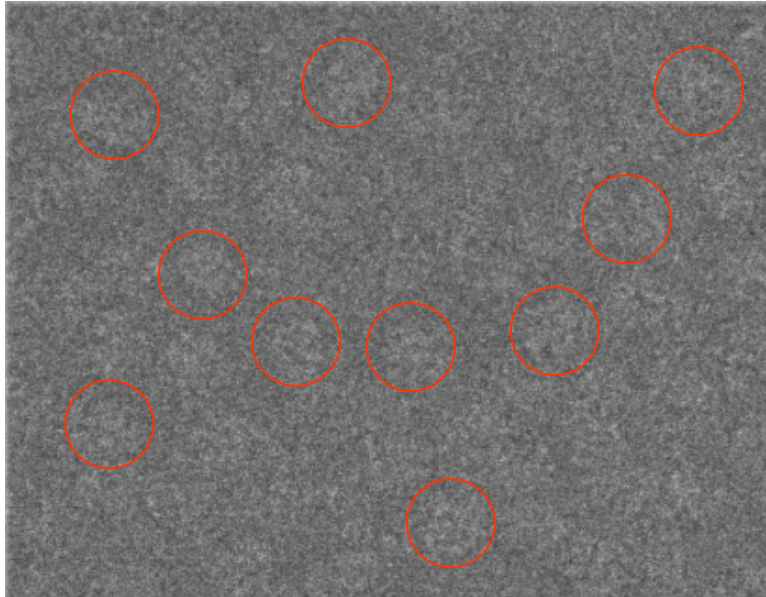
## Projection matching/ Angular refinement



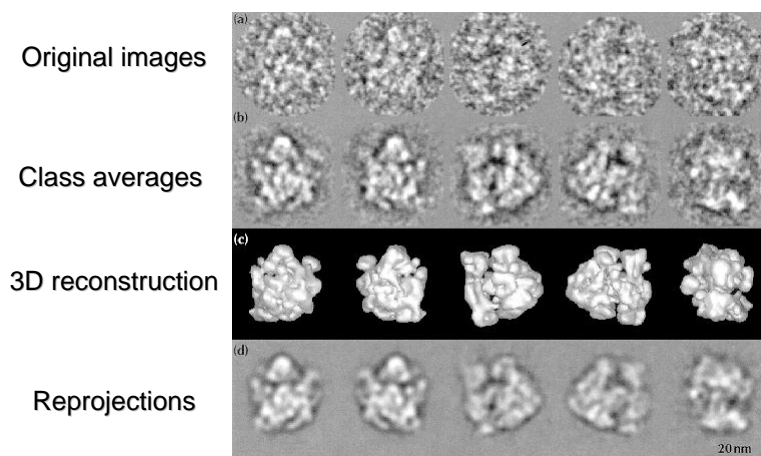
## 3D reconstruction from 2D projections



## Cryo EM image of ribosomes

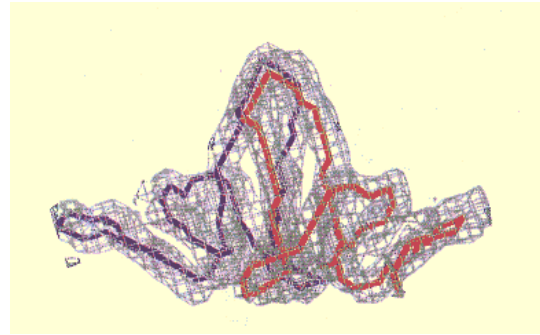
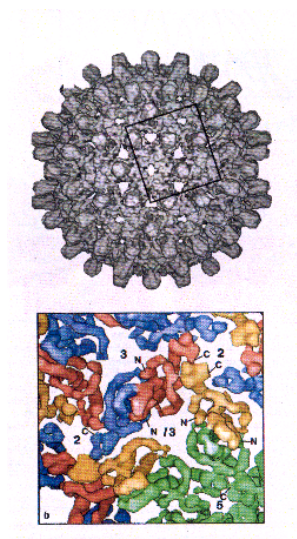


## Ribosome: Angular reconstitution



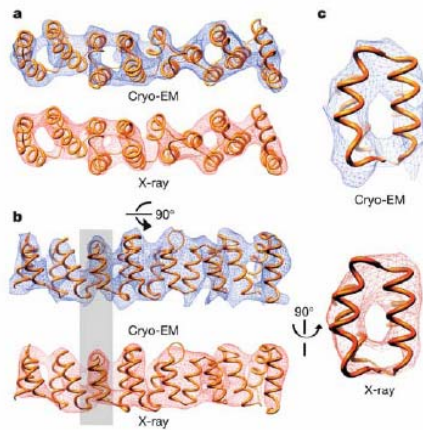
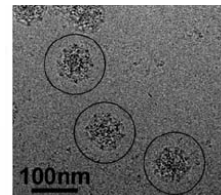
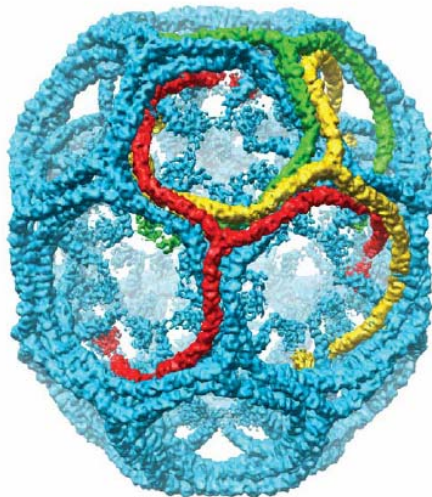
Stark *et al.* (1995)

## Hepatitis B virus at 7.5 Å resolution



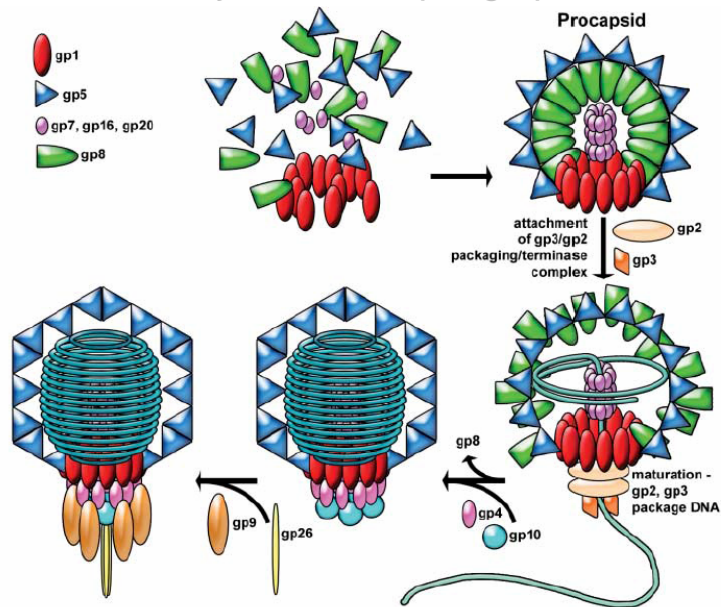
Boettcher et al. (1997)

## Clathrin at 8 Å resolution

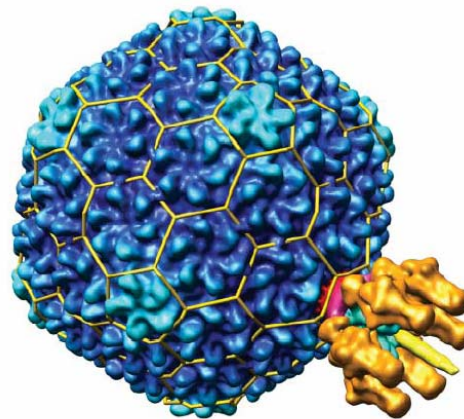
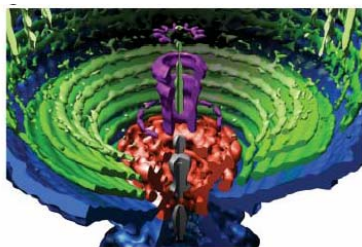
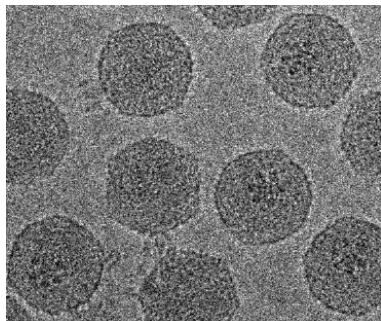


Fotin et al. (2004)

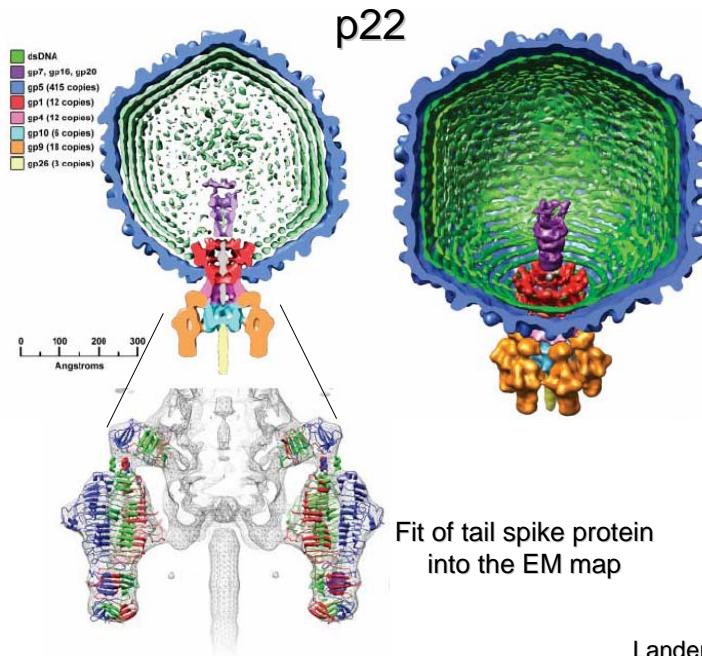
## Macromolecular machines: assembly of bacteriophage p22



## Cryo EM and asymmetric single particle reconstruction of phage p22

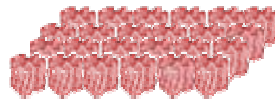


Lander *et al.* (2006)



Lander *et al.* (2006)

## 2D crystals

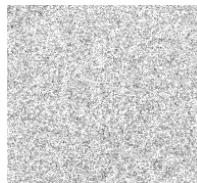


2D crystals contain a single layer of protein molecules

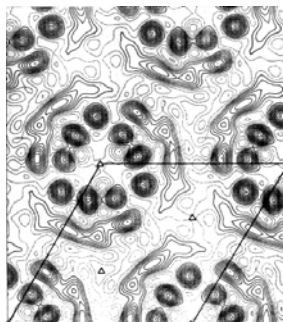
### Three-dimensional model of purple membrane obtained by electron microscopy

R. Henderson & P. N. T. Unwin

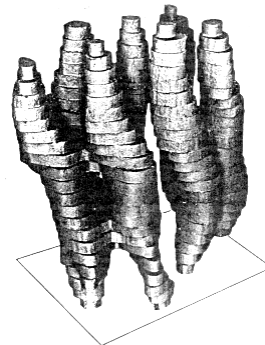
Noisy, low contrast image of crystal



2D projection density map



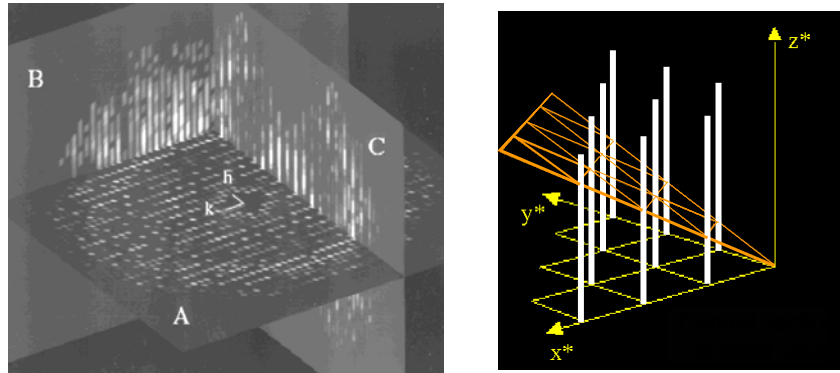
Model of 3D structure



Henderson & Unwin (1975)



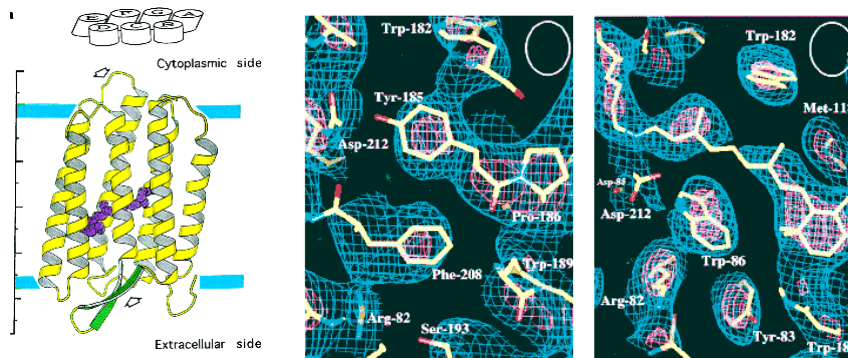
## Tilting of 2D crystals to get 3D data



3D electron diffraction intensity data for tubulin

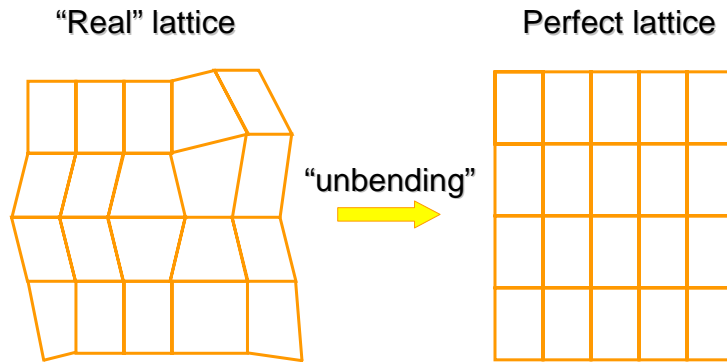
Nogales *et al.* (1997)

## Refined structures of bacteriorhodopsin

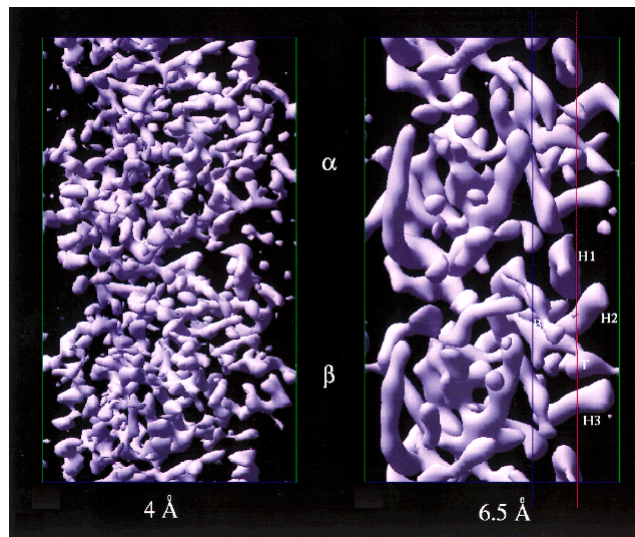


Grigorieff *et al.* (1996)

# Unbending

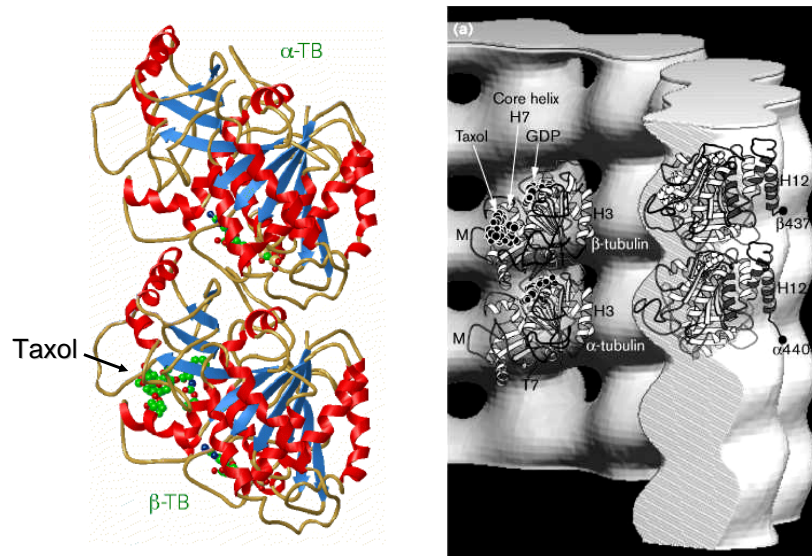


## Tubulin (from 2D crystals)



Nogales *et al.* (1997)

## Tubulin fitted into microtubules

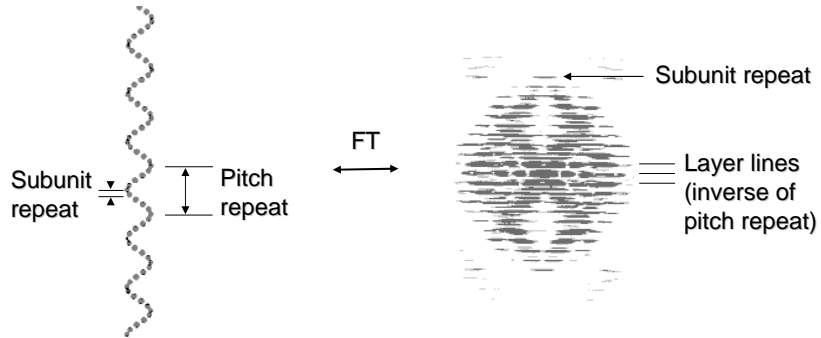


## Helical arrays



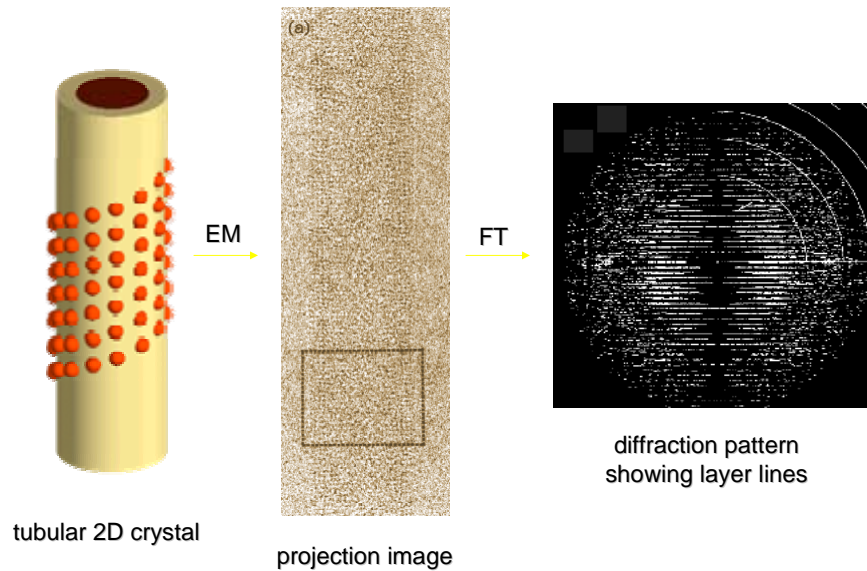
- Some samples form filaments or tubes with helical symmetry
- Identifying the repeat and lattice of the helix allows full 3D model to be generated
- All orientations of the sample are available hence no missing cone
- Examples are: [nicotinic acetylcholine receptor](#), [actin](#), [kinesin](#), [flagellin](#)

# Helical reconstruction

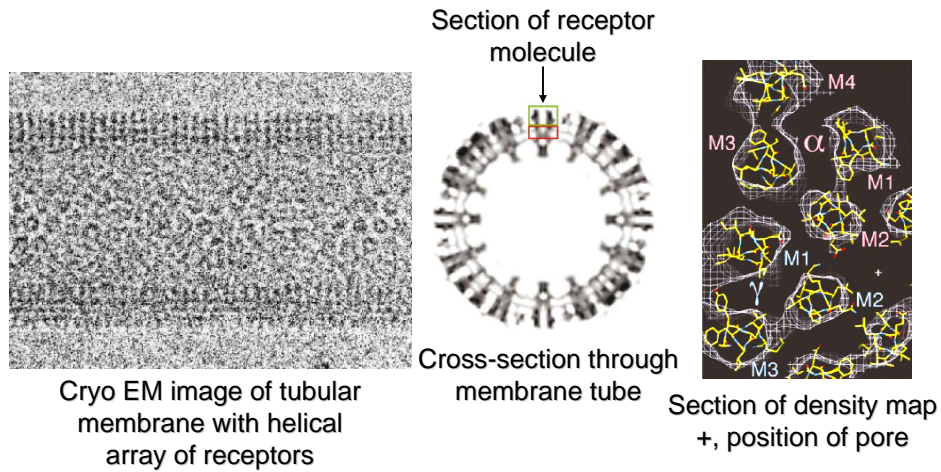


A helix can be considered as a 1D crystal, since it has a repeating structure along the axis, giving rise to a set of layer lines in the diffraction pattern. If the symmetry of the helix is known, a full 3D reconstruction can be calculated from the untilted filament transform, since the subunit is imaged at different angles about the filament axis.

# Helical diffraction

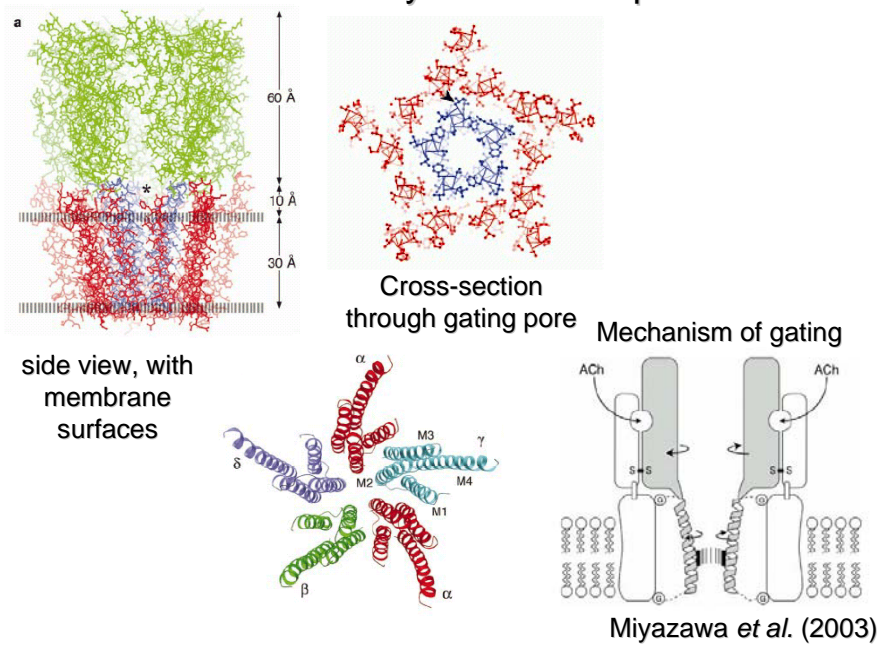


## Tubular crystals of acetylcholine receptors

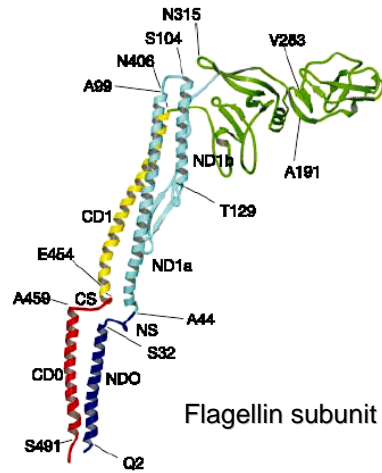
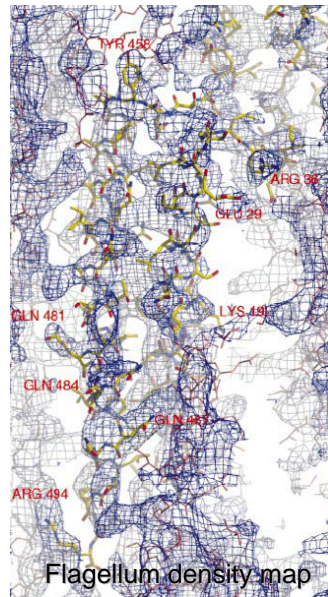


Miyazawa *et al.* (2003)

## Acetylcholine receptor structure

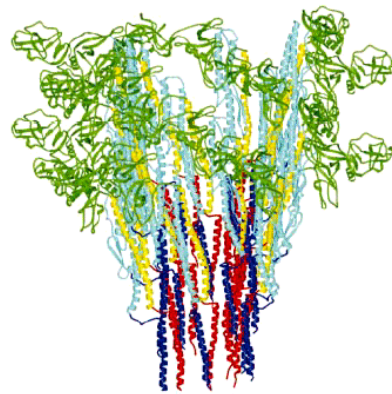
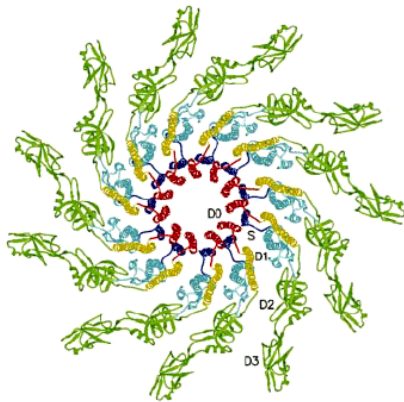


## Helical reconstruction of bacterial flagella



Yonekura et al (2003)

## Structure of bacterial flagella



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

## Electron microscopy references

1

### Reviews

- Hawkes, P. & Valdre, U. (1990) Biophysical Electron Microscopy, Academic Press
- Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowell, A.W. & Schultz, P. (1988). *Quart. Rev. Biophys.* 21, 129-228.
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- Saibil, HR (2000) Macromolecular structure determination by cryo-electron microscopy. *Acta Cryst. D* 56, 1215-1222.
- Chui, W, Baker, M, Almo, S (2006) Structural biology of cellular machines. *Trends in Cell Biol* 16, 144-150.
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### Cellular tomography

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