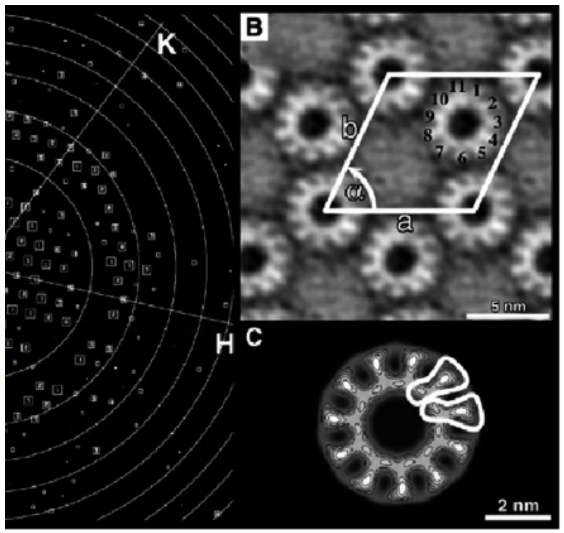
Structural Biology: Other techniques

For EM: some figs borrowed from Helen Saibil (Birkbeck College)

Electron diffraction

Theory similar to X-ray diffraction, but:

- electrons interact
  matter and therefo
  - are applicable or
    (2D crystals)
  - cause serious rad
    (limit to resoluti
- one can also use a
  an image that can
  transformed to giv



2D crystal

The Royal Swedish Academy of Sciences has decided to award **Jacques Dubochet**, **Joachim Frank** and **Richard Henderson** the Nobel Prize for Chemistry 2017 for "developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution".

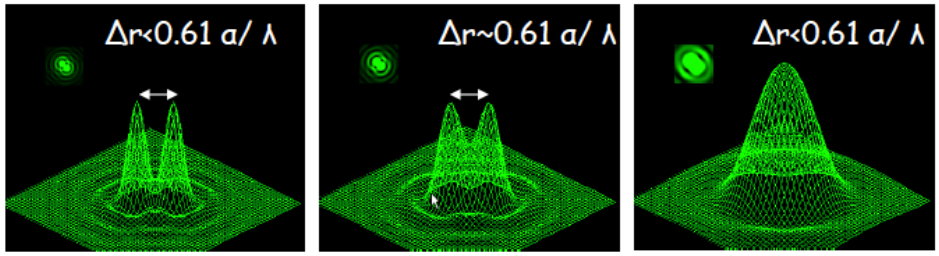
Using electrons?

Resolution is determined by the wavelength of the radiation used (Rayleigh criterion $\Delta R = 0.61\ \lambda/n\sin\theta$)

Rayleigh criterion:

$$\Delta r = 0.61\frac{\lambda}{\alpha}$$

a=aperture size from which we "see" an object
λ= wavelenght
for human eye, Δr~100μm (0.1mm)



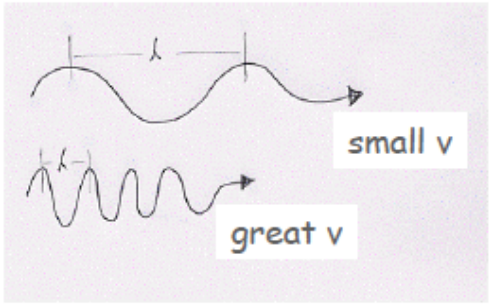
Visible light: ~ 400-750 nm, res limit ~ 170 nm

Using electrons?

De Broglie wavelenght:

Any object has a wave-like nature and its wavelenght is related to momentum p

$\lambda = h/p$ h=Plank constant (4×10^{-15}eV)
p=mass x velocity



Examples:

1) A man running at 10m/sec has a De Broglie wavelenght of 10^{-36}m
2) An electron travelling at the speed of light *c* has De Broglie wavelenght of 10^{-10}m

Since electrons can be treated as waves, is it possible to develop "electron optic", in analogy with light optic?

Yes, it is! But…why??

Using electrons?

$\lambda = h/p$ $\quad \lambda(nm) \sim 1.2/E^{1/2}\,(eV)$

Energy	Wavelenght (A)	
100eV	1.226	
200eV	0.867	Conventional SEM
500eV	0,548	
1keV	0,38	
30keV	0,0698	
100keV	0,037	Conventional TEM
200keV	0,0251	
300keV	0,0197	
400keV	0,0164	
1.25MeV	0,00735	

light wavelenght ~3500-7000A

⇩

In principle, EM should ensure spatial resolution 6 order of magnitude smaller than optical microscope

but

electromagnetic lenses aberrations limits our resolution

Using electrons?

λ of electrons is determined by their energy, i.e. by the voltage of the filament from which they are emitted:

300 kV $\rightarrow$ λ=0.002 nm

Can use magnetic lenses, and therefore obtain images (no phase problem)

However:

- need vacuum (e- have path length of few mm in air)
- large kinetic energy: specimen T can get very high
- e- are charged and interacts strongly with matter
  - need very thin specimen
  - serious radiation damage due to ionisation (Electron irradiation leads to the breaking of chemical bonds and creation of free radicals, which in turn cause secondary damage)

Electron microscopy

Optical Microscope:

✓ Light Source

✓ Lenses: system to change photon beam convergente (def. of refractive index)

✓ Sample

✓ Detection system

Electronic Microscope:

✓Electrons Source

✓ Lenses: system to change electrons beam convergente (def. of refractive index)

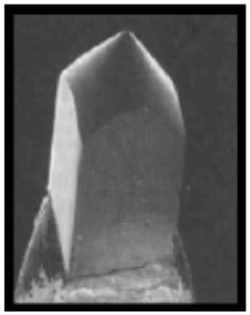
✓ Sample

✓ Detection system

Electron source

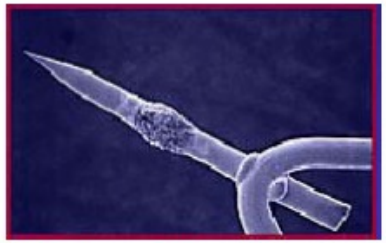
Thermoionic Source

W harpin



Lanthanum hexaboride (LaB_6)

Field Emission Gun (FEG)



Single oriented W crystal fine tip

To help electrons overcoming the potential barrier in the solid:

- Provide them with extra energy
- Lowering the barrier

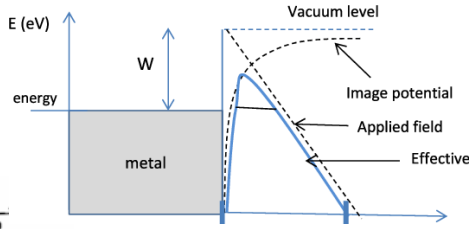
Electron source

Thermoionic sources:

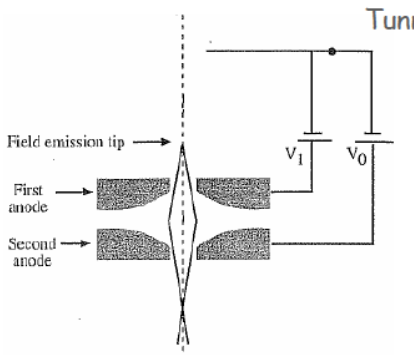
Current density $J \sim T^2 \exp(-\Phi/KT)$

Ideal material : high melting point and/or low Φ (LaB_6, W)

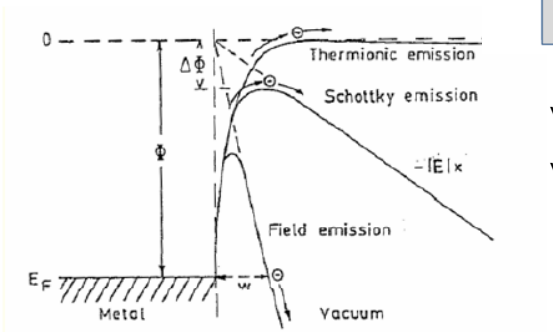
Field emission sources:
Intense electric field to lower the potential barrier



W tip **<0.1 µm**, application of a potential of 1 kV gives an electric field of 10^{10} V m^{-1} which is large enough to allow electrons to tunnel out of the sample.



Tunnel effect



V1: 2-3 V/nm electric field
V2: accelerates el. Up to 200 keV or more

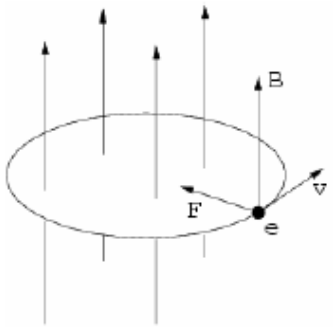
Electron optics

Magnetic lenses:

e^- with velocity **v** in a magnetic field B feels the Lorentz force $\vec{F} = e(\vec{v} \wedge \vec{B})$

Simpler case: constant **B**:

$\vec{v} \perp \vec{B}$

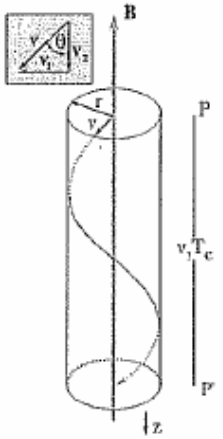


e^- follows a circular trajectory

No focusing!!

$\vec{v} \angle \vec{B}$

v can be divided into 2 components



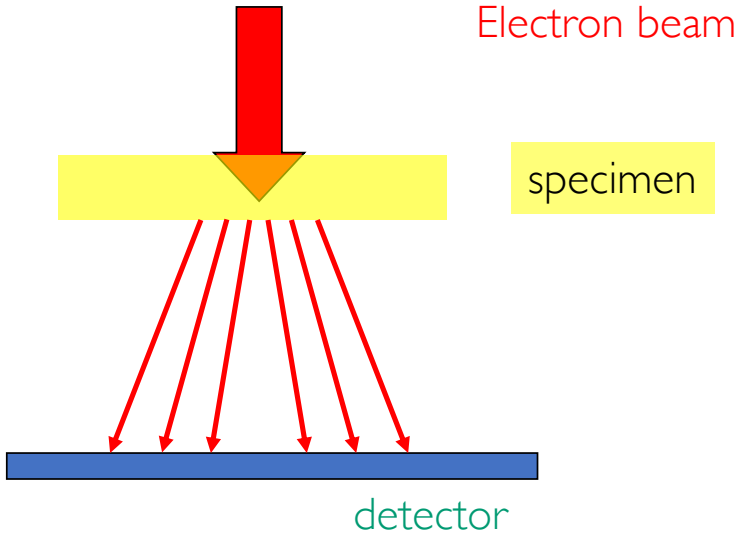
The trajectory follows a spiral

TEM vs SEM

Ernst Ruska,
Nobel Prize for Physics in 1986

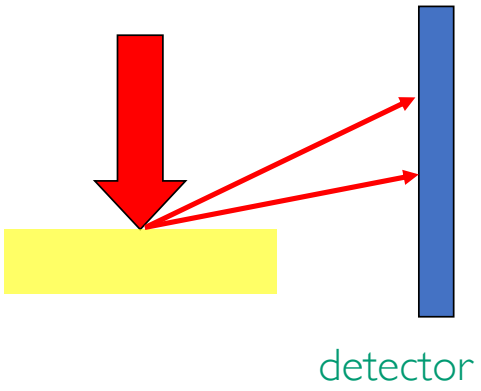
The sample itself is inserted into the path of the electrons, and for the best resolution must be extremely thin; a few nanometers. This is to maximise the number of transmitted electrons, and minimise multiple scattering events which make it more difficult to deduce information about the material.

Transmission EM



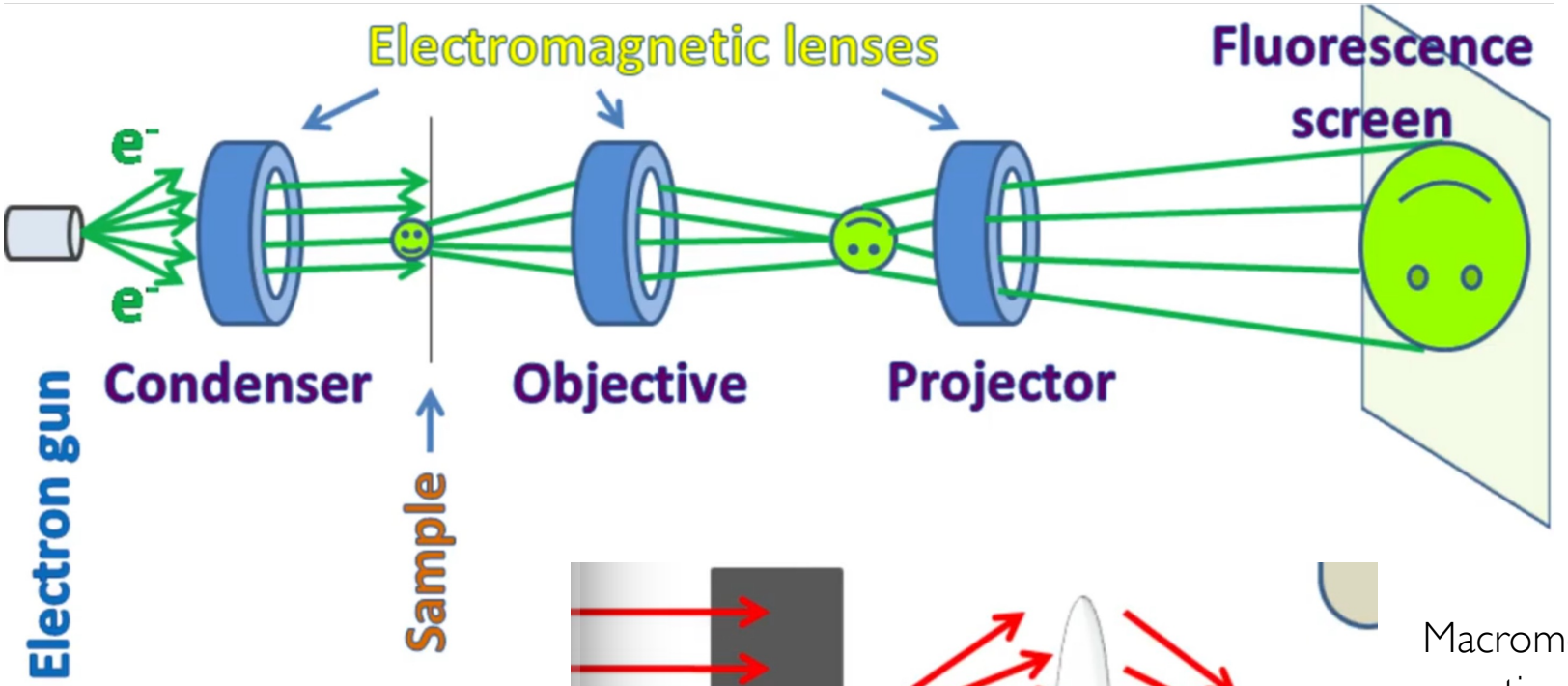
Detection of electrons behind the specimen (scattered e^-)

Scanning EM

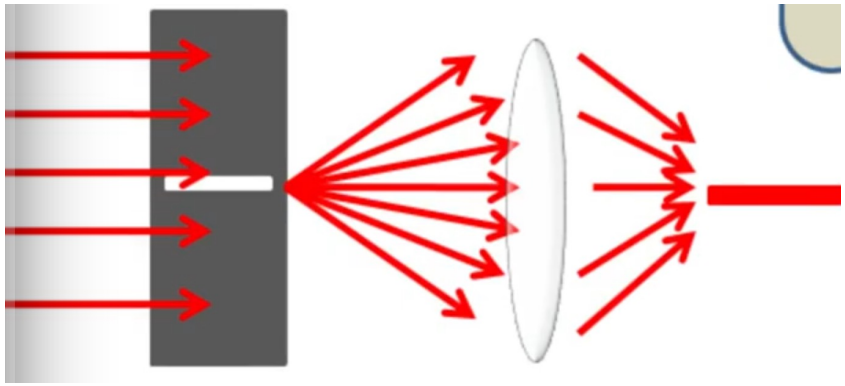


Detection of backscattered electrons (primary e^-) and generated electrons (secondary e^-)

Electron diffraction



Lens collects the diffraction pattern
Image is given by the interference pattern of diffracted light

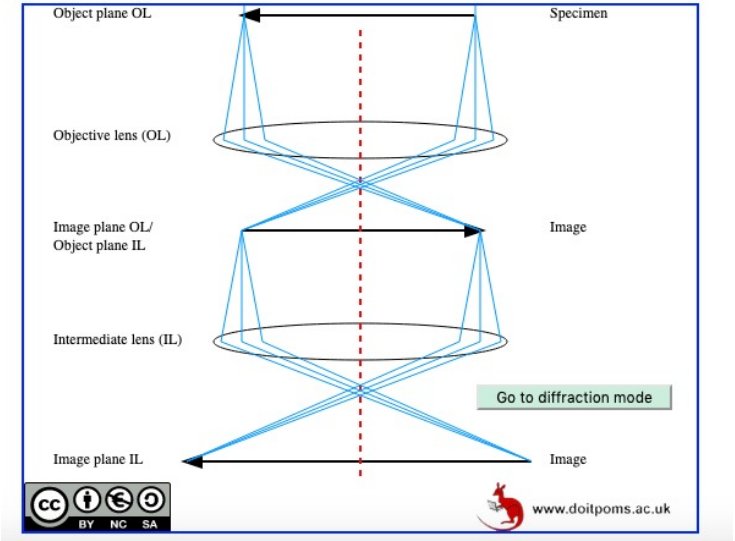


Macromolecules require negative staining with dried solution of heavy metal salt: increase diffraction contrast and preserves the sample in close-to-native state

Electron diffraction

The specimen sits right inside the objective lens and must therefore be small - typically less than 3 mm in diameter. It is necessary to align the specimen very accurately with the electron beam to achieve good imaging.

The objective lens takes electrons transmitted through the specimen and forms a diffraction pattern (in the back focal plane) and an image of the specimen (in the image plane).



https://www.doitpoms.ac.uk/tlplib/tem

In conventional TEM we have the option of magnifying either the image or the diffraction pattern by changing the settings of the intermediate lens.

In imaging mode, the microscopist focuses the intermediate lens onto the image plane of the objective lens to produce a magnified version of the image further along the optic axis and on the viewing screen. To view a diffraction pattern, the intermediate lens is adjusted so that its object plane coincides with the back focal plane of the objective lens, where the first diffraction pattern is formed. The diffraction pattern is then displayed on the viewing screen.

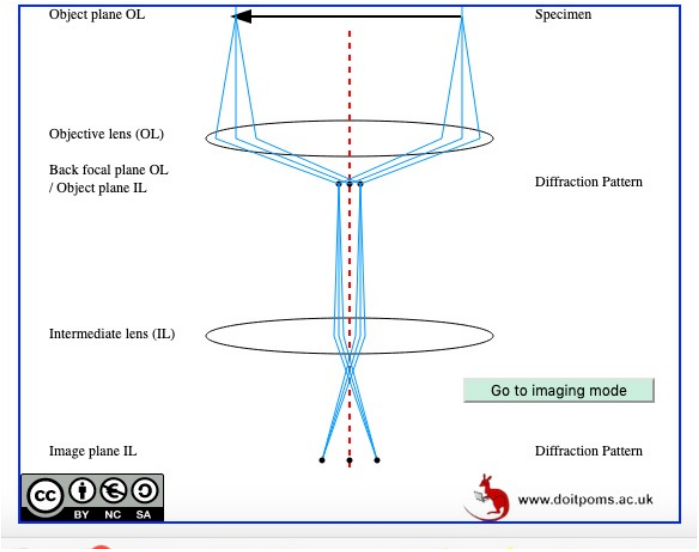


Image contrast

- Mass absorption contrast
  - On passing through matter, a beam of electrons is gradually attenuated. The degree of attenuation increases with the thickness of the specimen and its mass, so variations of mass and thickness across the sample give rise to contrast in the image.
- Diffraction contrast
  - Diffraction of electrons from Bragg planes causes a change in their direction of travel (elastic scattering). Hence, contrast can arise between adjacent grains or between different regions near the core of a dislocation.
- Phase contrast
  - Scattering mechanisms often cause a change in the phase of the scattered electrons, as well as a change in direction. Interference between electrons of different phase which are incident on the same part of the image will cause a change in intensity and give rise to contrast. This is normally only visible at high magnifications and for microscopes that can achieve atomic resolution (HRTEMs).

Image resolution

The resolution of an image is the smallest distance between two points at which they may be distinguished as separate. The resolution of perfect optical lenses is limited by diffraction effects (Rayleigh criterion).

Electron lenses are not perfect. They suffer from astigmatism, as well as chromatic and spherical aberrations, which arise from the spread of electron velocities in the beam, their angular distribution, and their distance for the optic axis as they travel through the magnetic field generated by the lenses.

Lens astigmatism is corrected by adjusting lens stigmators to compensate image distortions.
The effect of chromatic aberrations is seen when electrons travelling at different velocities experience a different Lorentz force as they cross the lens, and are focused at different distances along the optic axis. This degrades the resolution of the image. The effect can be reduced substantially by using a FEG electron source with a small energy spread. It is important to note that the beam energy distribution always broadens when electrons interact with the specimen through inelastic collisions. Hence small chromatic distortions are unavoidable in TEM images.

A lens is said to display spherical aberration when the field of the lens behaves differently for electrons travelling near the optic axis, and those travelling off-axis. The image resolution is degraded by $r_s = C_s\, a^3$, where C_s is the spherical aberration coefficient (usually expressed in mm), and a is the semi-angle subtended by the lens(aperture). Spherical aberration may be reduced by forming images just with electrons that travel close to the optic axis, i.e. minimising a, using a small aperture to exclude electron trajectories that cross the lens far from its centre.

Image resolution

Reducing the aperture size reduces the beam current and increase the diffraction experienced by the beam. There is, therefore, an optimum aperture size for the greatest resolution. The optimum resolution can be expressed as: $R_{opt} = \lambda^{1/4} C_s^{3/4}$.

Conventional TEMs can achieve resolutions of 0.2 nm, and hence allow imaging of atomic lattices. Aberration corrected TEMs, where additional electron-optic components are introduced to compensate for spherical and chromatic aberrations, can achieve point resolutions below 0.1 nm (in phase contrast images).

EM: electron detection

Phosphor screen: e^- induce fluorescence in ZnS screen that emits green light

Charged Coupled Devices (CCD): arrays of electrically isolated pixels. Every pixel accumulates an electric charge proportional to the intensity of the incident electron beam (digital images)

Photographic plates: photographic emulsions sensitive to electrons

EM: sample preparation

Samples for EM have to be carefully prepared:

- they have to be exposed to high vacuum and therefore fixed with special chemicals or frozen

- have to be prepared in extremely thin sections since electrons have limited penetrating power

- samples are often exposed to heavy metals since contrast depends on the atomic number (negative staining)
- also, negative staining mitigates electron induced sample damage since reagents containing heavy atoms, such as uranyl acetate, coat accessible surface making it less radiation-sensitive

Negative staining:

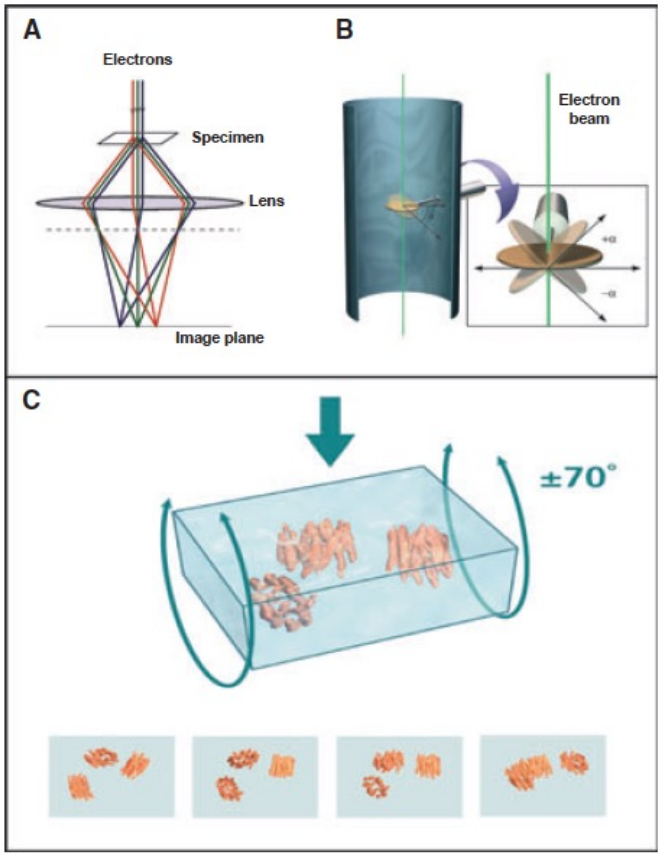
Aaron Klug (Nobel Prize for Chemistry in 1982)

the FEBS Journal
REVIEW ARTICLE

Cryo-electron microscopy – a primer for the non-microscopist

Jacqueline L. S. Milne[1], Mario J. Borgnia[1], Alberto Bartesaghi[1], Erin E. H. Tran[1], Lesley A. Earl[1], David M. Schauder[1], Jeffrey Lengyel[2], Jason Pierson[2], Ardan Patwardhan[3] and Sriram Subramaniam[1]

So why is it not possible to routinely image individual proteins, viruses and cells in their native state directly in an electron microscope at atomic resolution? The primary reason why this is challenging is because of the extensive damage that results from the interaction of electrons with organic matter. Electron irradiation leads to the breaking of chemical bonds and creation of free radicals, which in turn cause secondary damage [21–23]. One way to mitigate electron-induced sample damage is to use the method of negative staining, in which accessible molecular surfaces are coated with reagents containing heavy atoms, such as uranyl acetate, that are much less radiation-sensitive than organic matter. Because these stains do not penetrate into biological samples, they essentially make a cast of the specimen surface, a high-contrast 'relief' of the surface, albeit at the expense of internal structural information and with the potential for artifacts such as sample flattening. Nevertheless, this has been a common mode of specimen preparation for many decades in conventional transmission electron microscopy, and has been routinely used for visualization of cells, viruses and proteins, yielding, in the latter case, structures at resolutions of approximately 20–40 Å [24,25].



Cryo-EM

Developed starting from the '90s. The revolution started from 2013

- Fast freezing samples in non crystalline water

- Development of a new generation of electron detectors

MAPS (monolithic active pixel sensors) or direct electron detectors. El. Pass through a thin (10 micron) semiconductor membrane; the deposited energy is detected by electronics on the membrane. Backscattered el. are reduced by reducing support material. In this way the quantum efficiency of detecting (DQE) is high, means S/N from incoming signal is not much worsened by detector (ideally DQE = 1; = 0.3 in photographic films; 0.1 in CCD for high E el.

- Improved imaging processing procedures

Introduction of automated data acquisition

Trends in Biochemical Sciences January 2015, Vol. 40, No. 1

Cryo-EM

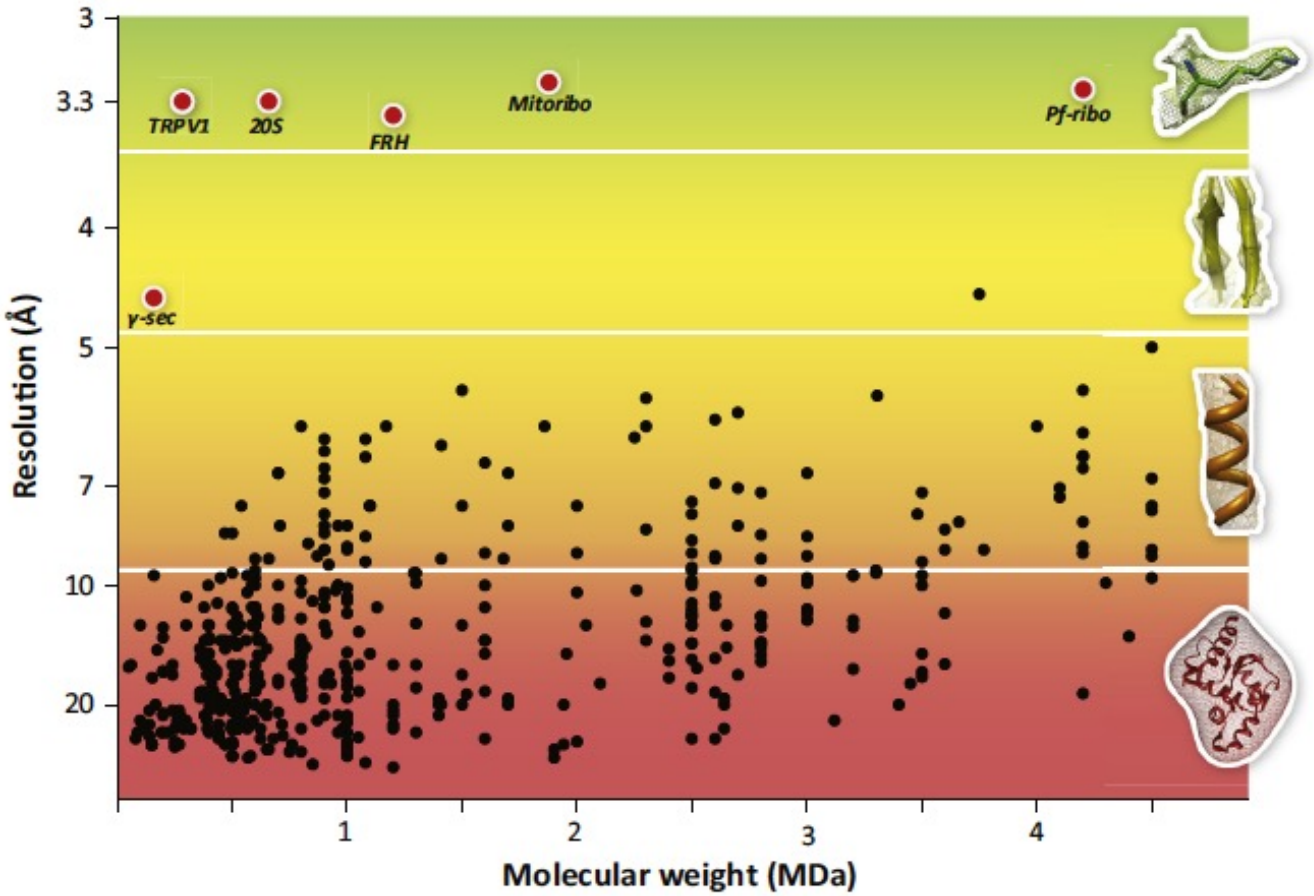


Figure 1. Revolutionary progress in cryo-electron microscopy (EM) single-particle analysis. The black dots represent single-particle cryo-EM structures that were released from the Electron Microscopy Data Bank (EMDB) between 2000 and 2012. The red dots are examples of recent progress in the field: γ-secretase (γ-sec), the transient receptor potential cation channel subfamily V member 1 (TRPV1), the 20S proteasome (20S), F420-reducing [NiFe] hydrogenase (FRH), the large subunit of the yeast mitochondrial ribosome (mitoribo), and the cytoplasmic ribosome of *Plasmodium falciparum* in complex with emetine (Pf-ribo). Whereas previously many structures only resolved protein domains (red area) or α helices (orange area), recent structures are detailed enough to distinguish β strands (yellow area) or even amino acid side-chains (green area).

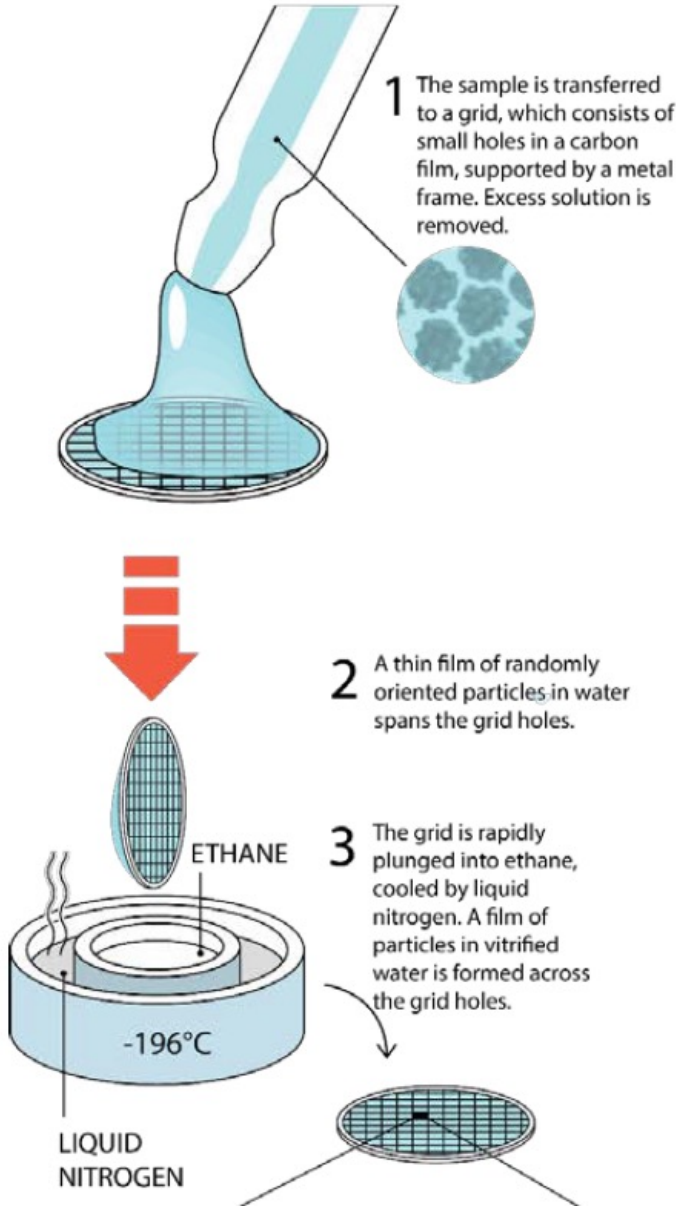
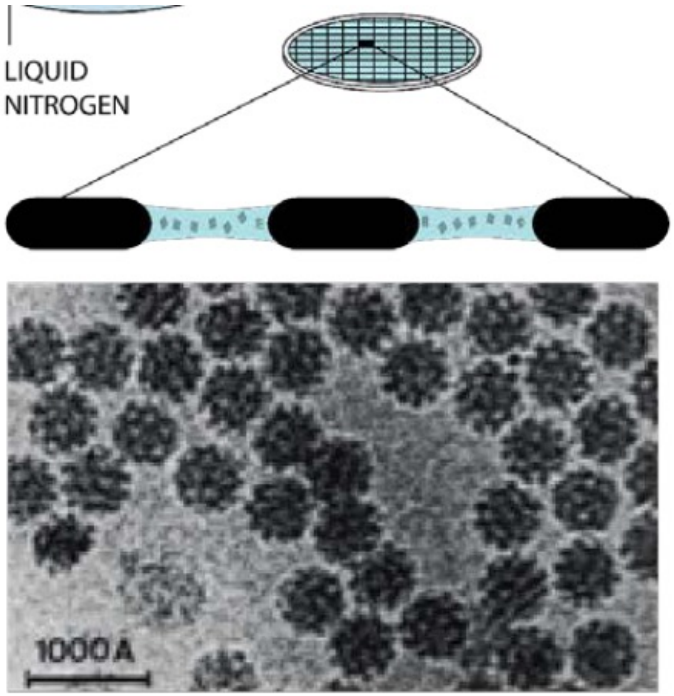
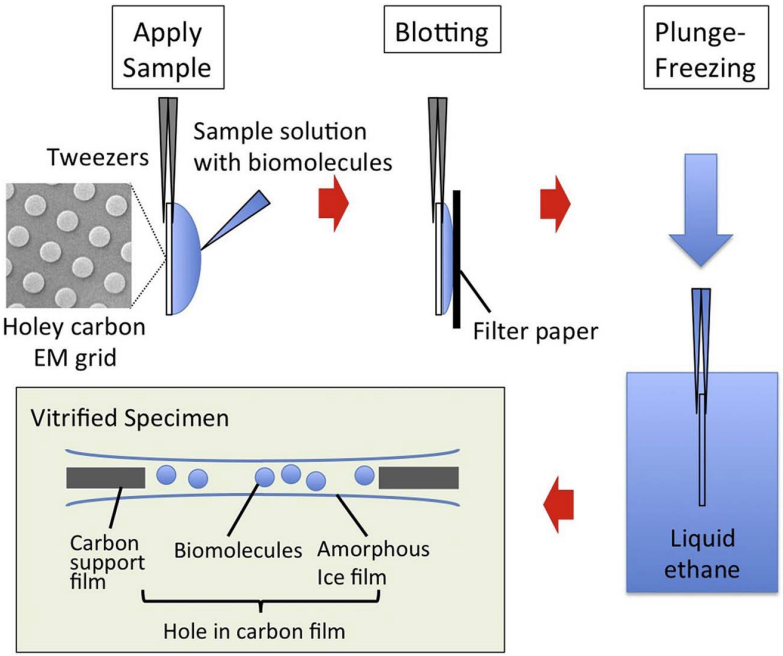
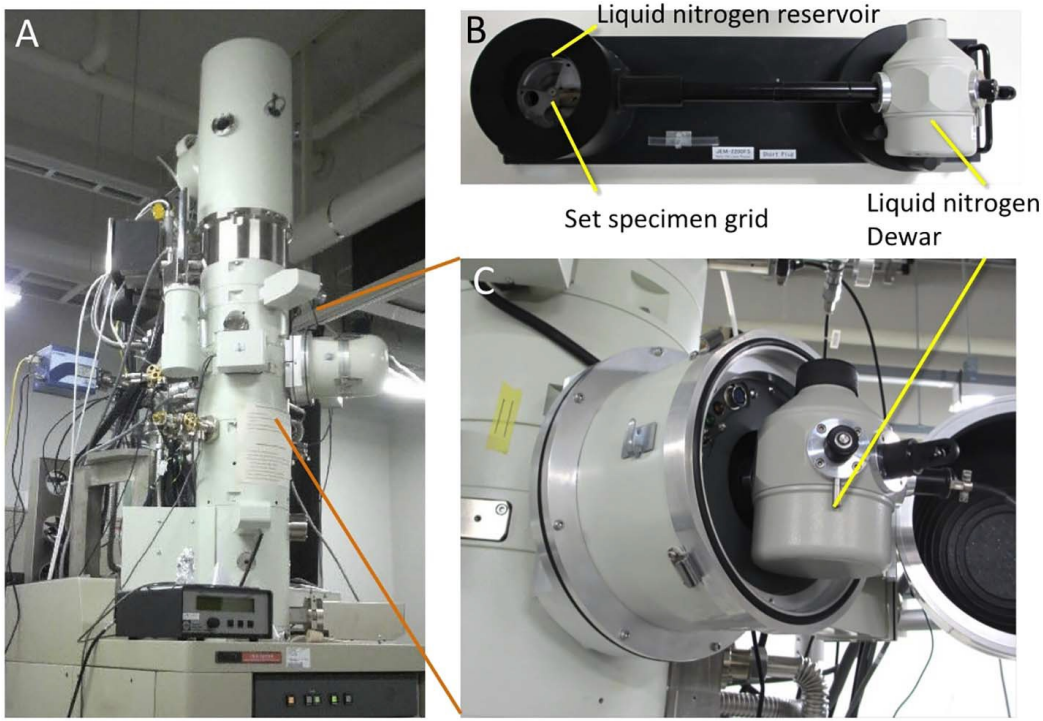


Fig.8. Sample-preparation procedure for cryo-EM. Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences. The

1984 Jacques Dubochet

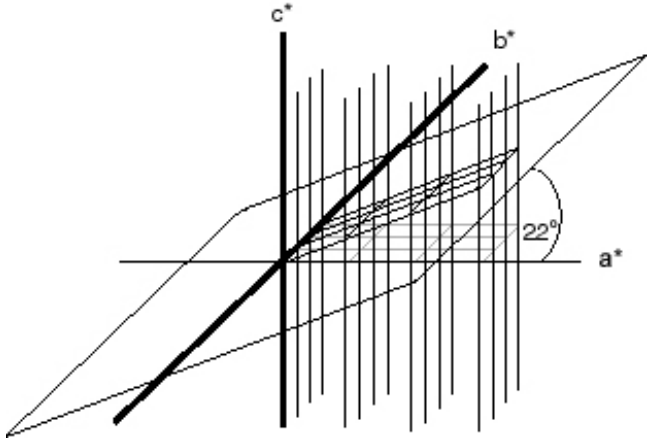


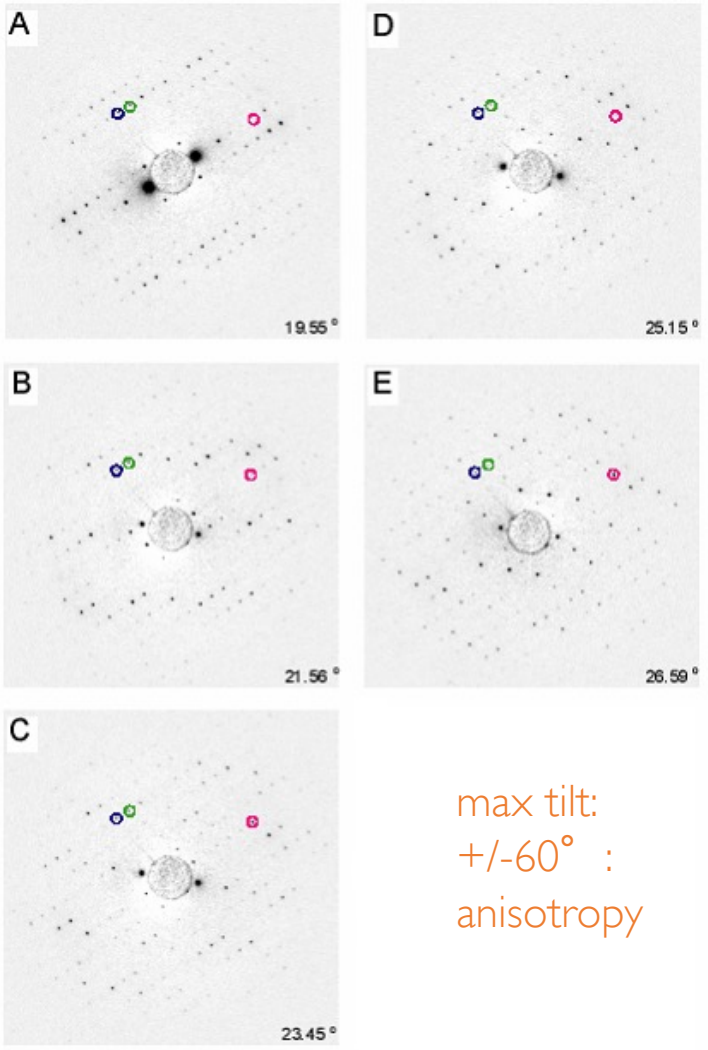

Apply Sample
Blotting
Plunge-Freezing
Tweezers
Sample solution with biomolecules
Holey carbon EM grid
Filter paper
Liquid ethane
Vitrified Specimen
Carbon support film
Biomolecules
Amorphous Ice film
Hole in carbon film


A
B
Liquid nitrogen reservoir
Set specimen grid
Liquid nitrogen Dewar
C

Cryo-EM

Initially only diffraction images from 2D samples (i.e. protein channel in lipid bilayers) were obtained



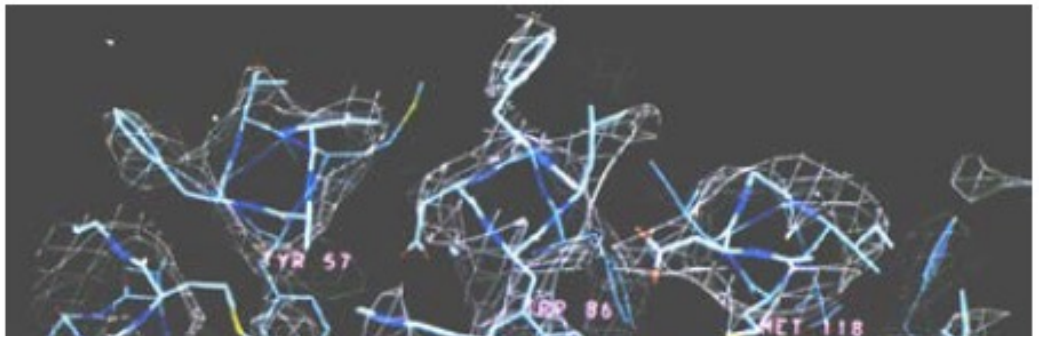


Tilt the 2D crystal to get information about the 3rd dimension

max tilt: +/-60° : anisotropy

Cryo-EM structures at high resolution

1990
Richard Henderson



The analysis of a large number of molecules in the 2D crystal is equivalent to averaging directly in the microscope. For non-periodic assemblies of symmetrical particles, the signal-to-noise ratio can be increased by averaging over the asymmetrical units. However, for the general case of non-periodic asymmetrical particles, the challenge was to determine the position and orientation of each particle in an image from weak signals. Once this could be achieved, averaging would be possible. However, such analyses would require computer power well beyond that available in 1990 (36).

The first high-resolution model of bacteriorhodopsin (31) was based on analysis of millions of protein molecules in a 2D crystal, which allowed the spread of the total electron dose over a large number of particles.

Five years after the publication of the high-resolution structure of bacteriorhodopsin

Large virus	300 M	Yes
Small virus	11 M	Yes
Ribosome	3·3 M	Yes
	1·4 M	Yes
Multimeric enzyme	420 K	Possibly
	180 K	Possibly
	52 K	Possibly
Small protein	18 K	No
Very small protein	7 K	No

Fig. 3. Extract from Table 2 in (37), which addresses the question: can single molecule alignment be carried out in practise? The answer is given to the right for a number of example proteins, listed to the left, with molecular weights (Da) in the middle column.

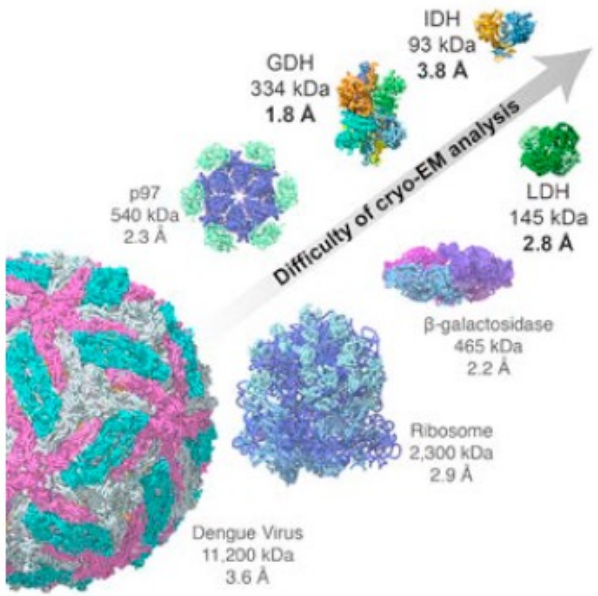


Fig. 4. Examples of structures determined using cryo-EM as of May 2016 [image from (38)]. The figure illustrates the conclusions from (37). Note that the smallest protein (64 kDa) determined to date using cryo-EM is haemoglobin (39).

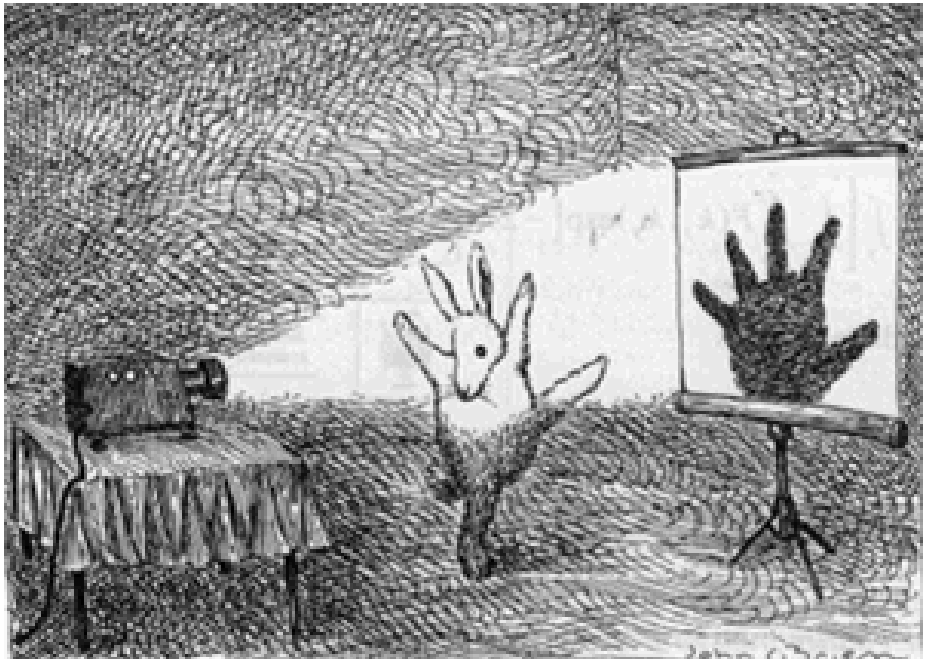
How is the image in single particle cryo-EM formed?

- thin specimen scatter electrons
- interference between scattered and unscattered electrons give phase contrast image
- image is 2D projection of original 3D object
- 3D structure can be determined from a set of views at different orientations
- radiation damage is the ultimate limit on resolution - to avoid destroying the sample, one uses very low doses, obtaining very noisy images

Therefore we have two problems

- getting a signal out of very noisy images
- getting a 3D structure out of 2D projections

From 2D projections to 3D models?

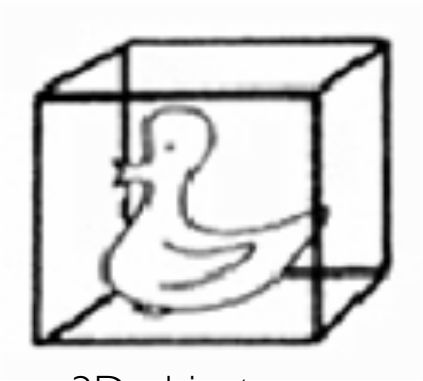


(The New Yorker, 1991)

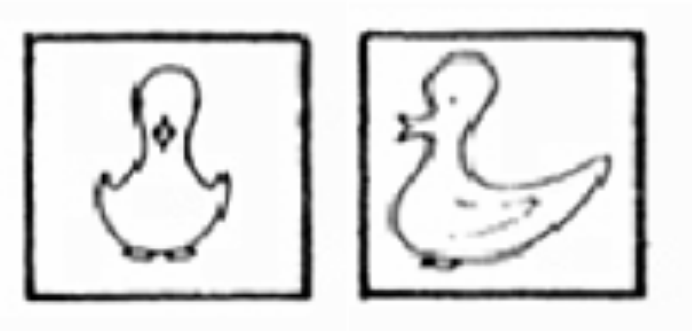
Obviously one projection is not sufficient to infer the structure of an object...

From 2D projections to 3D models

From a number of projections in different directions we can reconstitute the object - the more projections we have, the more details we can get.



3D object

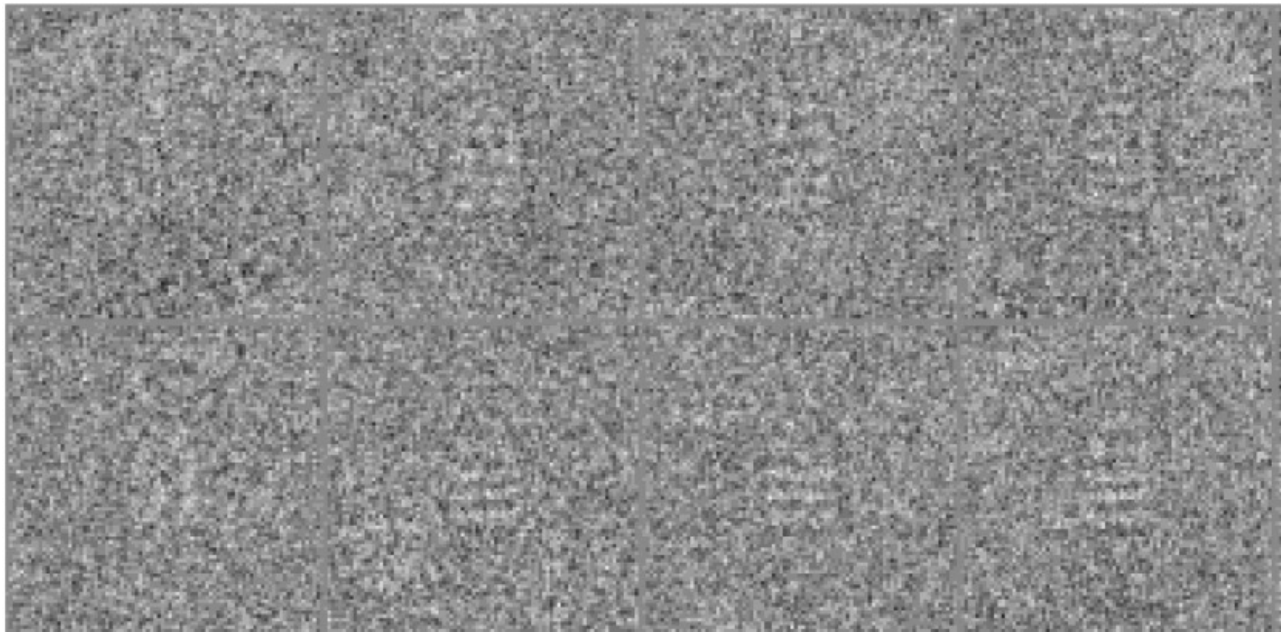


2D projections

The difficult step in 3D image processing is to determine the orientation angles (Euler angles) for each projection image.

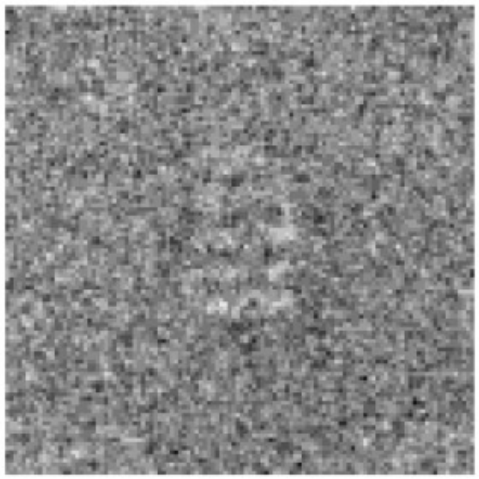
Single molecule images

A single protein molecule gives only a weak and ill-defined image.
Combine the information from many molecules so as to average out the random errors in the single images.



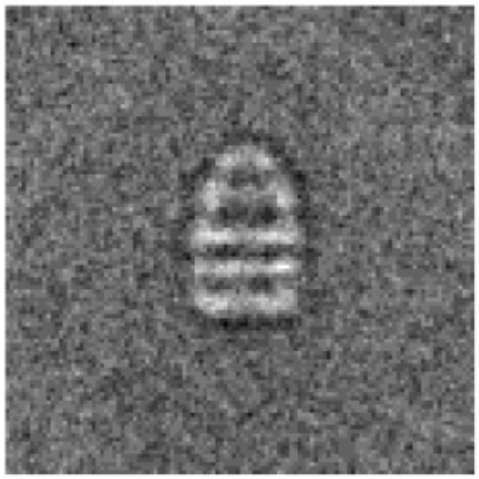
Individual raw images of a molecule in the same orientation

Single molecule averaged images



Sum of 4 images

Sum of 8 images



Sum of 32 images

Averaging large number of particles massively increases the signal/noise. However, we have to make sure we are averaging views with the same orientation!

Ensembles of single asymmetric particles in solution

A fundamental problem in studies of unstained, non-crystalline, asymmetrical, randomly oriented particles in solution is "*the alignment of features that are only faintly visible on a noisy background*" (20). In the mid-1970s, Frank addressed this problem in a study that became in many ways the starting point for future developments (20).

Frank and colleagues presented a method for aligning low-dose images of individual molecules using cross-correlation functions (20,41,42). A quantitative analysis of the problem was presented in 1977 (41). The analysis concluded that it would be possible to locate randomly positioned particles using non-destructive electron doses. Consequently, the implication was that it would be possible to average images of many radiation-sensitive particles to eventually obtain high-resolution data. The feasibility of the approach was illustrated in studies of negatively stained glutamine synthetase (43).

Multivariate statistical analysis is used to sort the vectors

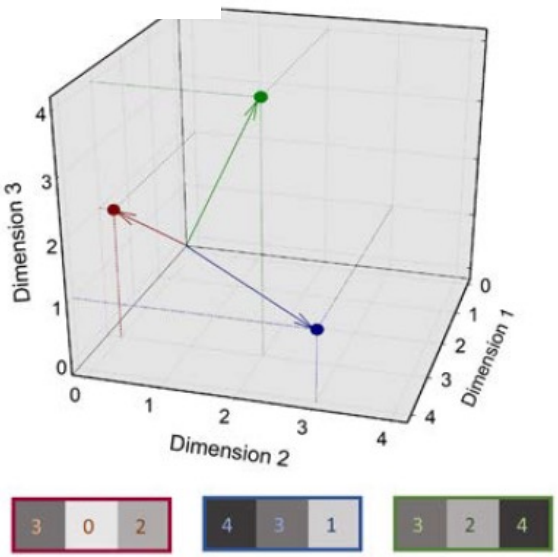
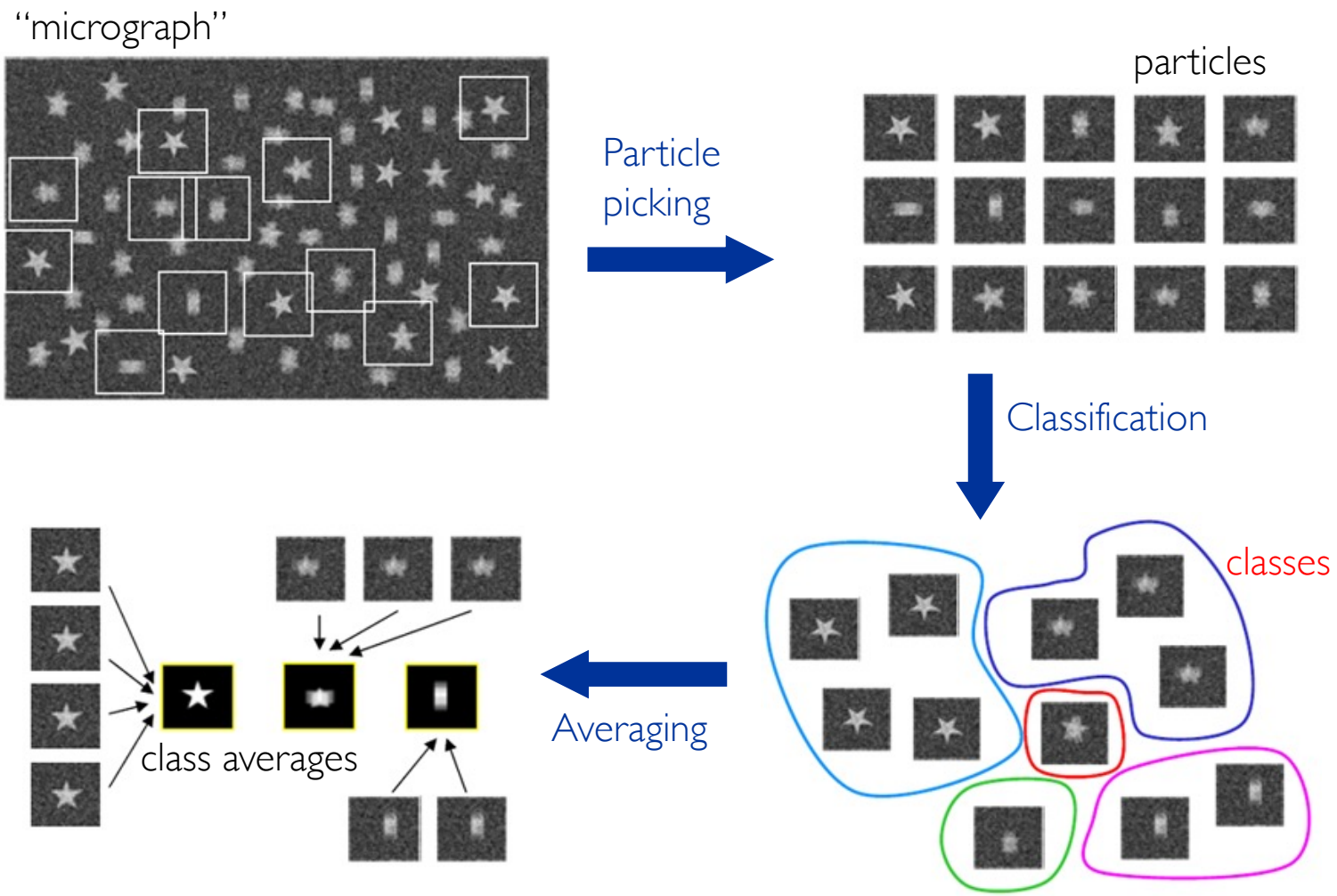


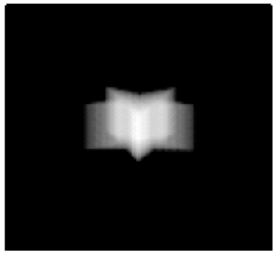
Image processing

Joachim Frank

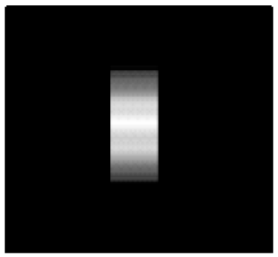


Angular reconstitution

α=0°
β=0°
γ=0°



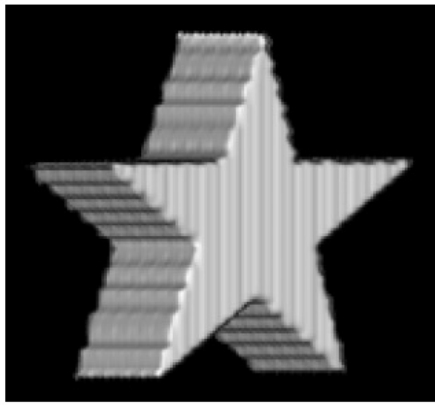
α=0°
β=95°
γ=0°



α=0°
β=0°
γ=90°

1. Define the Euler angles for each class average

2. Combine all views (and apply symmetry if present)

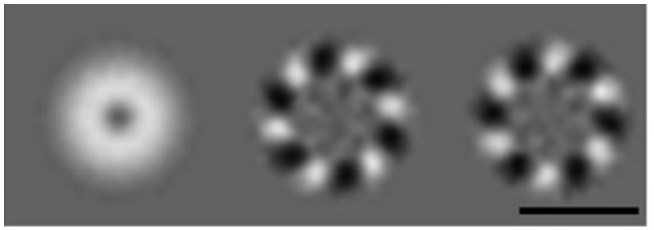


The process can be further refined by projecting the model obtained and use the projections to break the original classes into smaller classes, and assigning more precise angles.

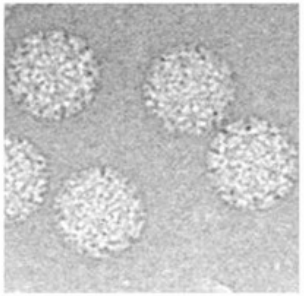
Symmetric objects

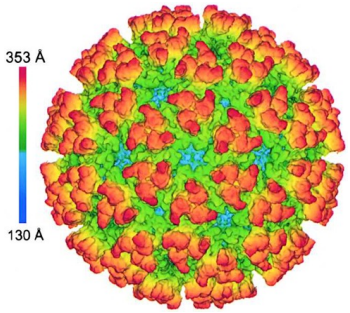
Process is easier when the molecule contains symmetry:
- less particles are required to get the same accuracy
- easier to determine the initial orientations (top, side, etc)
- can detect the symmetry before solving the structure

The presence of symmetry can be detected in the images, before determining the 3D structure

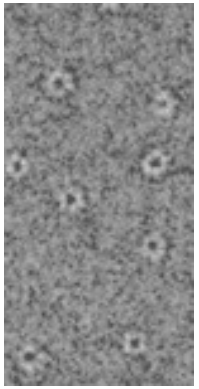


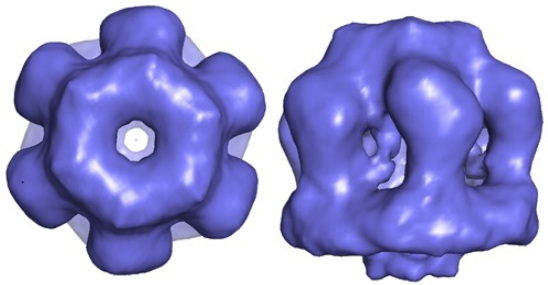
A virus



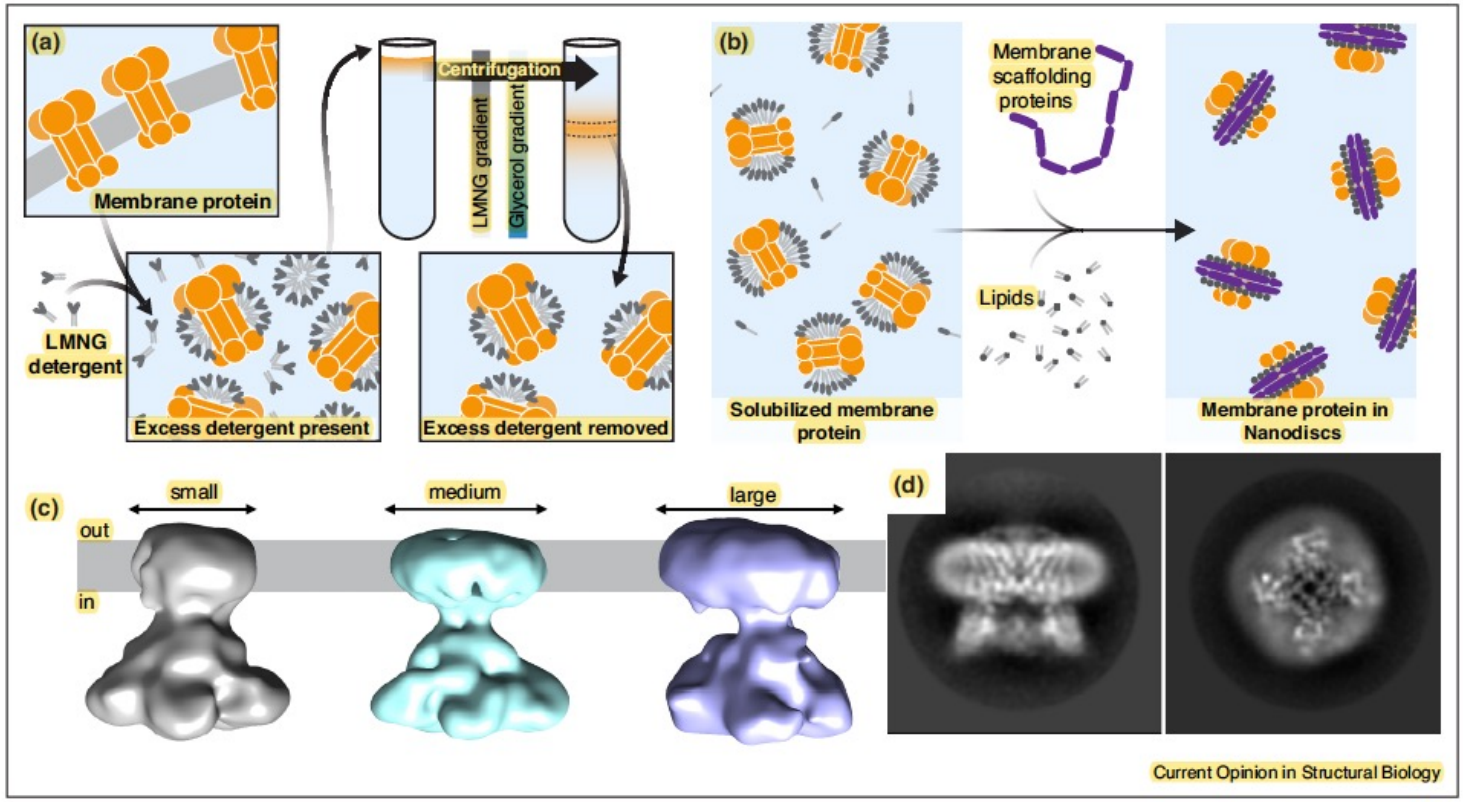


A hexameric helicase





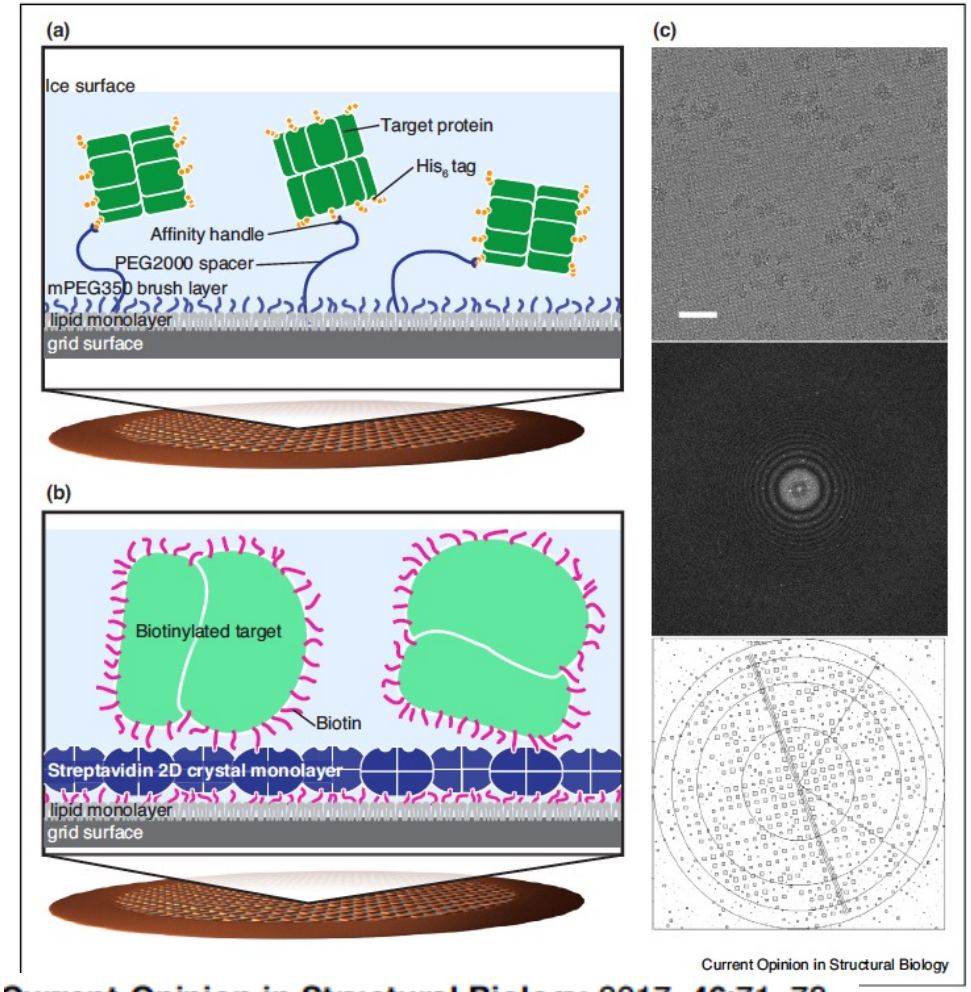
Cryo-EM challenge: membrane proteins



Membrane proteins removed from the native lipid environment via detergent can destabilize.

Current Opinion in Structural Biology 2017, 46:71–78

Cryo-EM challenge: membrane proteins



Current Opinion in Structural Biology 2017, **46**:71–78

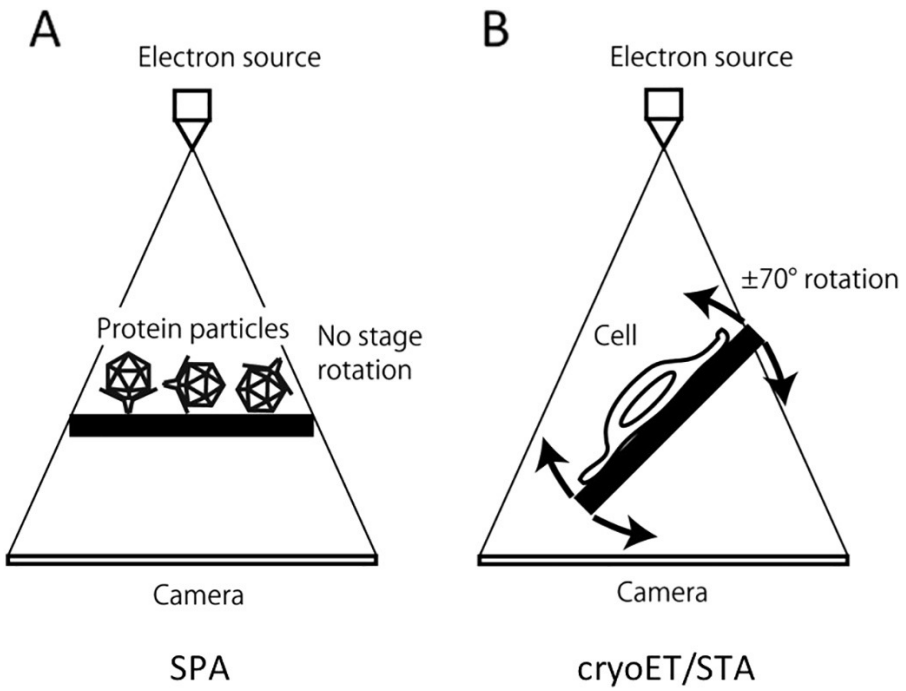
Interaction forces with the grid can also destabilize target proteins. Interfacial forces can promote dissociation of labile complexes, or affecting the distribution over the grid. Chemical modification and bioaffinity modification of the grid is introduced.

Cryo-EM tomography

Review

Cryo-electron microscopy for structural analysis of dynamic biological macromolecules☆

Kazuyoshi Murata[a,*], Matthias Wolf[b,*]



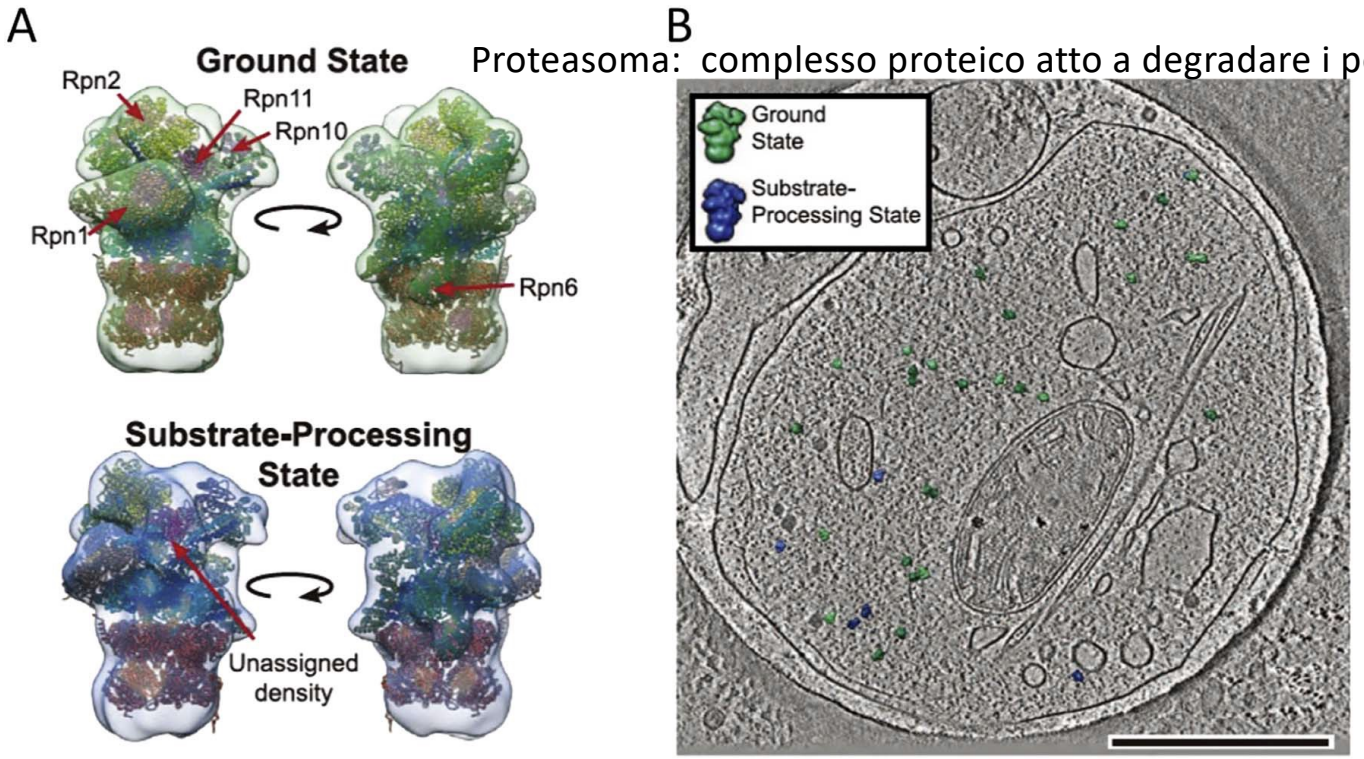
STA: subtomogram averaging

2D projections of heterogeneous particles are collected by tilting the specimen stage, and 3D tomograms are calculated using weighted backprojection or other reconstruction algorithms. Selected subsets of the tomogram, "subtomograms" containing individual particle volumes are picked, aligned, classified and averaged.

SPA: single particle analysis

the images of randomly orientated homogeneous particles are recorded at low electron exposure (dose). Then, particle images selected from digitized images are iteratively aligned against a reference and averaged.

Cryo-EM tomography



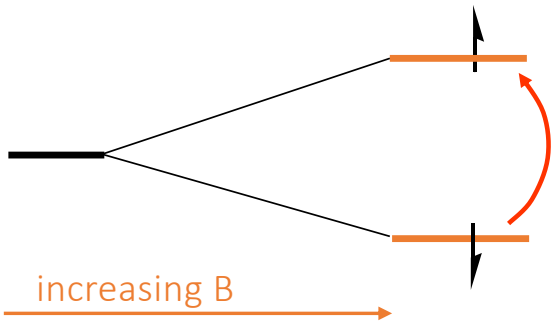
Proteasoma: complesso proteico atto a degradare i peptidi

Contrast-enhanced cryo-electron tomography shows dynamic states of 26S proteasome in their functional and unperturbed cellular environment (in situ).
A) Mammalian 26S proteasome subunits after sub-tomogram averaging and 3D classification with fitted atomic models in two biologically significant states. B) One tomogram slices through an annotated 3D atlas of a cell with individual orientations and conformational states of 26S proteasomes.

Other techniques:
nuclear magnetic resonance

Nuclear magnetic resonance (NMR)

Certain nuclei (^{1}H, ^{13}C, ^{15}N, ^{31}P) have angular momentum. In high magnetic fields we can separate the energy levels of nuclei with different spin angular momentum.



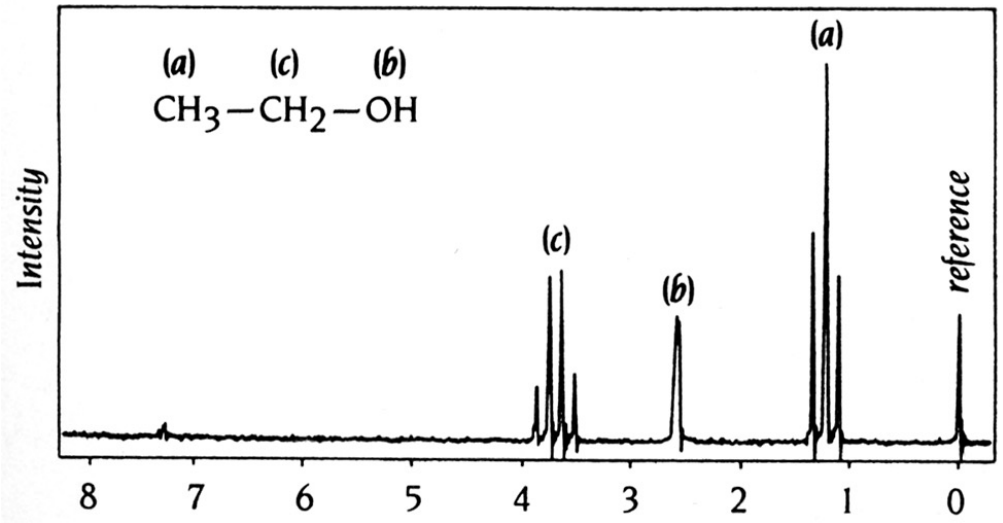
the spin will align along the field, but absorption of electromagnetic radiation of appropriate frequency (radio) induces a transition.

When the nuclei revert to their equilibrium state they emit radiation that can be measured. The exact frequency of the emitted radiation depends on the environment of the nucleus.

NMR: chemical shifts

The exact frequency of the emitted radiation depends on the environment of the nucleus. Nuclei in different chemical environment will absorb at slightly different frequencies. These different frequencies (relative to a reference signal) are called chemical shifts.

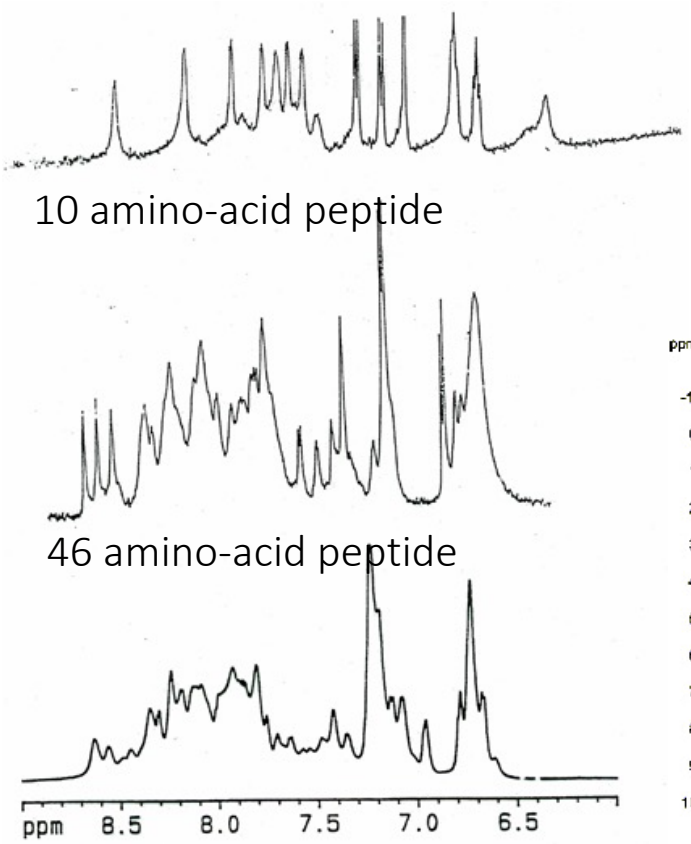
^{1}H-NMR spectrum of ethanol



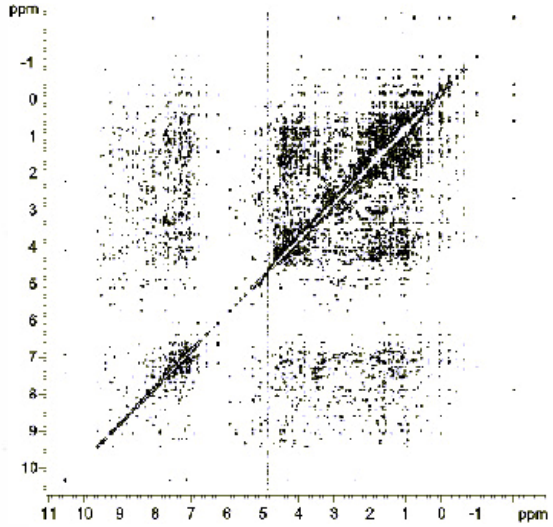
Chemical shifts for all the hydrogens in this small molecule: hydrogen that are part of the CH_3, or CH_2, or OH group have different shifts.

NMR and peptides

1D NMR spectra of proteins contain such a large number of overlapping peaks as to be uninterpretable.



We need to use pulse sequences with specific shapes and time intervals to allow magnetization transfer between nuclei and, therefore the detection of nuclear-nuclear interactions through space (NOESY).



One problem is overlap between peaks: labelling the protein with ^{13}C and ^{15}N help to resolve overlap in the proton dimension.

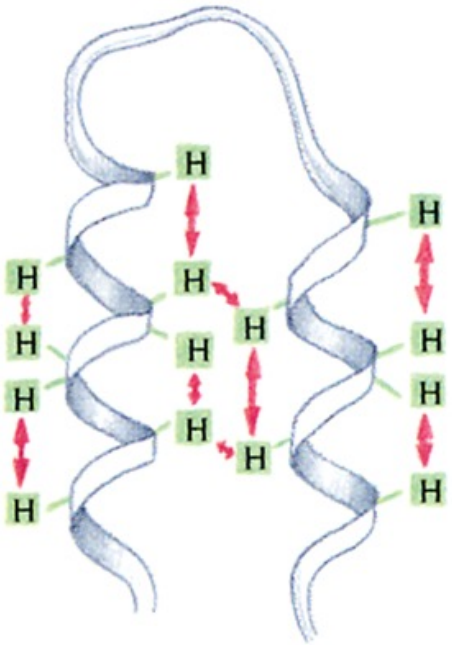
NMR: mapping distances

The tricky thing here is doing the assignment, i.e establishing the identity of the 2 H that give rise to that particular peak. Very labour-intensive. (Wüthrich made a major contribution - Nobel price in 2002).

One problem is overlap between peaks: labelling the protein with ^{13}C and ^{15}N help to resolve overlap in the proton dimension.

The intensity of a NOESY peak is r^{-6} ∝ the distance is determined according to intensity of the peak. The intensity-distance relationship is not exact, so usually a distance range is used.

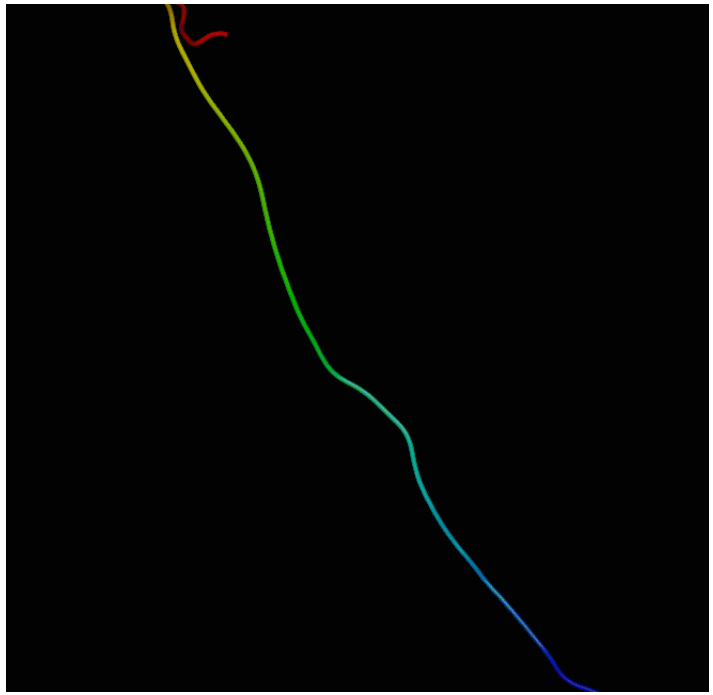
We can basically map distances between pairs of atoms: the NMR spectra by specifying which pairs of atoms are close together in space, contain three-dimensional information about the protein molecules.



NMR refinement

The experimentally determined distance restraints (together with the protein stereochemical constraints) can be used as input for the structure determination.

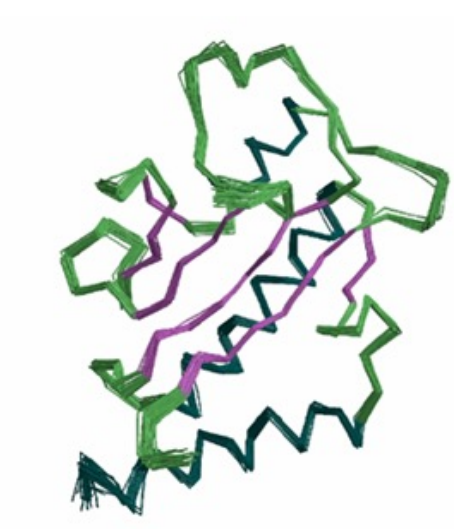
Algorithms start from an unfolded polypeptide and attempt to satisfy as many of the restraints as possible, in addition to general properties of proteins such as bond lengths and angles.
As in crystallography, this is done by converting them into energy terms and thus minimizing the energy.

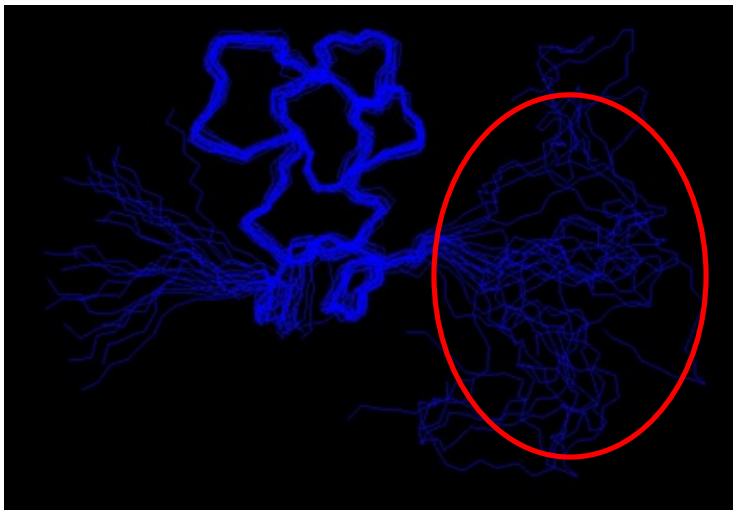


Repeat many times and compare results.

NMR structure

The process ias repeated many times and results in an ensemble of structures that, if the data were sufficient to dictate a certain fold, will converge.

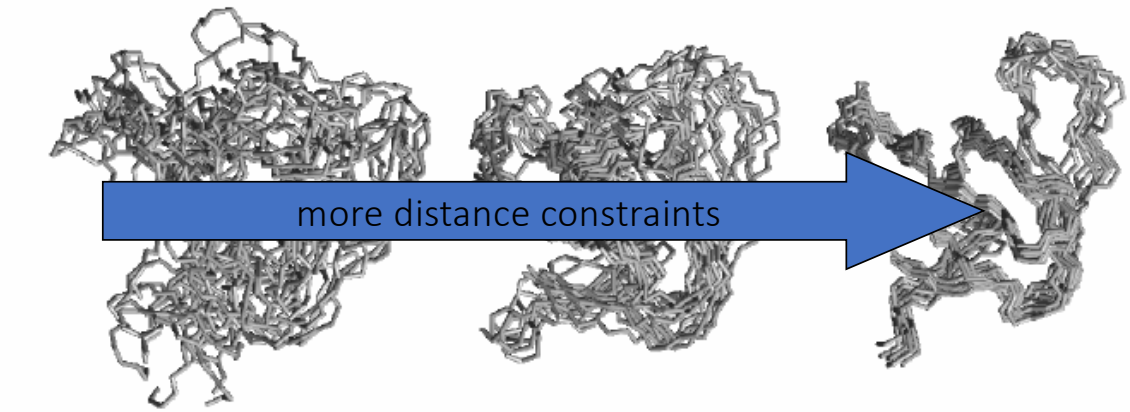




intrinsic disorder or lack of data?

Often it is claimed that NMR structures visualise the “dynamic” nature of proteins: however it is difficult to discriminate between flexibility and lack of data... (there are sophisticated ways of addressing this, but they are not used on the “average” NMR structure).

Accuracy of the structure?



There is a direct correlation between the quality of the NMR structure and the number of distance constraints:
more constraints → higher the precision of the structure

No corresponding equivalent to the R-factor (let alone R-free) or resolution

Accuracy is measured by the RMS deviation of the ensemble:
- “high resolution” : RMSD < 1A
- “medium resolution” : RMSD < 2A

Warning! strongly depends on which programme was used for the refinement!

NMR and protein size

Traditionally NMR is limited to relatively small proteins or protein domains.

Problems resolving overlapping peaks - alleviated by the introduction of isotope labelling and multidimensional experiments.

A more serious problem is the fact that in large proteins the magnetization relaxes faster, which means there is less time to detect the signal, causing the peaks to become broader and weaker, and eventually disappear.

Two techniques have been introduced to attenuate the relaxation: transverse relaxation optimized spectroscopy (TROSY) and deuteration of proteins.

No labelling:	< 15 kDa
$^{13}C/^{15}N$:	< 20 kDa
$^{13}C/^{15}N$ and deuteration:	< 40 kDa
Deuteration + TROSY:	up to 100 kDa??