Structure Determination by ELECTRON MICROSCOPY

STRENGTHS:
- Small amounts of material usually OK
- Purity often not a major issue
- Maximum size of particles not any problem

WEAKNESSES:
- Radiation damage
- Minimum size of the particle
- Limited resolution of reconstructed image
  (often in the range of 40, 20 to 10 Å resolution,
  rarely better than 5 Å, but there is a very recent example case of 3.8 Å for adenovirus, and an 1.9 Å structure for an aquaporin!)
High Resolution Electron Microscopy of Biomacromolecules

PRINCIPLES

Figure 3

The relationship between imaging and diffraction. A simplified schematic view of imaging and diffraction shows that the waves scattered by the specimen form a Fourier transform, observed as a diffraction pattern. The objective lens in a microscope recombines the scattered waves in an inverse transformation, forming the magnified image. In the diffraction experiment, only the diffracted intensities are recorded and the lost phase information must be retrieved by other methods.

What is the (theoretical) resolution of an electron microscope?

The lens aperture (expressed by its half angle, \( \alpha \)) may limit how many diffraction orders pass \( \Rightarrow \) resolution is limited.

Quantitation: If 2 image points are separated by a distance, \( d \), then their first diffraction order will occur at an angle, \( \theta \), which will permit the path difference for light waves diffracted from the two points to constructively interfere; this means that the path difference, \( d \), for the two waves must equal the wavelength, \( \lambda \), of the radiation, or
\[
\delta = d \sin(\theta) = \lambda
\]
where \( n \) is the index of refraction of the medium (Fig. 1b). The resolution \( \text{limit} \) is (approximately) equal to the \( d \) which corresponds to a first order diffraction at \( \theta = \alpha \) where \( \alpha \) is the aperture half angle of the objective lens. Substituting \( \alpha \) for \( \theta \) in the equation above gives:
\[
d = \frac{\lambda}{n \sin(\alpha)}
\]

\( n \sin(\alpha) \) is called the Numerical Aperture.

That is a nice formula for the resolution, but what are the values for the wavelength and the aperture?

De Broglie discovered that moving particles have a wave nature with
\[
\lambda = \frac{h}{mv}
\]
where \( h = \text{Planck's constant} \), \( m = \text{particle mass} \), and \( v = \text{particle velocity} \). Electrons have a charge and can be accelerated in an electric potential field as well as focused by electric or magnetic fields. An electron accelerated in a potential of \( V \) volts has kinetic energy
\[
\frac{1}{2}mv^2 = eV
\]
where \( e \) is the charge on the electron.

Solving for \( v \) and substituting into de Broglie's equation (and expanding in \( V \) to account for the fact that the electron mass is different when moving than when at rest):
\[
\lambda = \frac{\hbar}{\sqrt{2m_eV}}
\]

Thus, for \( V = 100,000 \text{ V} \) (a typical value) \( \lambda = 0.037 \text{ Å} \)
And for \( V = 1,000,000 \text{ V} \) (High Voltage EM) \( \lambda = 0.0087 \text{ Å} \)

What resolution can be expected?

Unfortunately, electron lenses are poor and are therefore designed with very small apertures, of say 0.02 radians, but the small wavelength \( \lambda \) comes to the rescue:

With \( \alpha = 0.02 \text{ radians} \) and \( \lambda = 0.04 \text{ to } 0.01 \text{ Å} \), and \( n=1 \), the formula
\[
d = \frac{\lambda}{n \sin(\alpha)}
\]
gives a resolution \( d = 2 \text{ to } 1 \text{ Å} ! \)

I.e. the theoretical resolution is quite high and is indeed achieved with electron microscopes.
II. Electron Microscope Design - All optics at less than $10^{-5}$ torr!

A. Source—Electron Gun

1. Variable Bias—hot tungsten hairpin filament—most common type (Fig. 2a). The filament is heated by a current, $I_f$, and electrons are drawn off by the potential difference between the filament and the anode. The filament is surrounded by a shield or gun cap (also called a Wehnelt) which is kept several hundred volts more negative than the filament by a variable bias resistor which connects the two. This potential difference and the design of the Wehnelt focuses the electrons at a crossover point between the front of the Wehnelt and the anode, this point is the effective source of illumination for the system. Brightness of the source increases as $I_f$ is increased up to a Saturation point when further current increases don’t increase brightness but does shorten filament life (Fig. 2b).

2. Pointed Filament — Brightness and coherence increased by decreasing size of the area from which electrons are drawn. Pointed filaments may be made from a hairpin filament with a small tungsten crystal or etched piece of tungsten wire welded onto it. A better solution is a pointed filament made of LaB$_6$; such a filament can be 100x brighter (usually more like 10x) than a normal hairpin filament.

3. Field Emission Gun (cold cathode) — A very fine tip which is usually not heated. Instead, electrons are drawn from the tip by a sharp potential drop (electrons “tunnel” out). This type of source is very difficult to construct and maintain partially because it requires ultra-high vacuum technology, but it is 10$^4$ x brighter than a conventional filament and produces a beam of electrons with a very small spread of energies (very monochromatic and very coherent).
Interactions of Electrons with Specimen

1. **Elastic Scattering** -- negligible loss of energy ==> don't damage and are scattered over relatively large angles.

2. **Inelastic Scattering** -- electrons lose energy (10's of eV's) which is transferred to electrons of the specimen forming ions and free radicals breaking chemical bonds. **This is the source of radiation damage which is the principal limitation to high resolution structure determination.**

Since inelastically scattered electrons have lost energy, their wavelength is altered ==> **chromatic aberration** which limits resolution with very thick specimens. The amount of energy lost depends upon the atom with which the electron interacts ==> **information on elemental composition.** Electrons are scattered inelastically over relatively narrow angles.

For biological specimens the Radiation Damage is a VERY serious problem – as explained on the next slide.
Radiation Damage

A. Signal-to-Noise (S/N) for High Resolution Imaging

1. In the ideal case, the S/N is determined by the statistics of electron counting; the signal is the number of electrons scattered from a particular resolution element (pixel) while the noise is the square root of the number of incident electrons for each resolution element (the statistical variation in number of incident electrons for each pixel). Thus, in principle any resolution (up to the instrumental limitation of 2-4 Å) can be achieved for any specimen if it is imaged with enough electrons.

2. Contrast is low for biological specimens because they are composed of relative light elements which scatter electrons weakly. Thus, to produce a 10 Å resolution of a typical biological specimen, one must image it with 250 electrons/Å² if total contrast is 2%.

B. How Sensitive Are Biological Specimens?

1. Specimens lose 10-30% of their mass during one exposure (10-100 el/Å²).
2. Protein crystals disordered after an exposure of 1-5 el/Å² at 0°C.
3. Enzyme activity is lost after an exposure of 0.05 el/Å².
4. Thus, it would appear that one cannot image biological specimens at high resolution because images produced with a low enough exposure to prevent severe radiation damage would have a S/N << 1.0. The situation is much worse in the case of analytical imaging since many more electrons are required to form an image (mean free path for high energy loss event is large). But there are some limited solutions to this dilemma described below.

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of Biomacromolecules

SPECIMEN PREPARATION
**Specimen Preparation**

Goals -- most techniques achieve only some of these goals.

1. **Make thin specimens which can exist in vacuum;** this usually means remove all H$_2$O. Thin specimens are required in order to:
   a. prevent absorption of electron beam
   b. reduce multiple scattering
   c. decrease chromatic aberration from inelastic electron scattering.

2. **Increase contrast--heavy atom stain**

3. **Decrease radiation damage**
   a. heavy atom stain distribution less sensitive to radiation
   b. less damage at low temperatures

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**Negative Stain** - particles (enzymes, viruses), membrane fragments.

1. Adsorb specimen to support film--thin carbon film over electron microscope grid.
2. Rinse with heavy atom solution--e.g. 1-2% uranyl acetate, uranyl formate, or phosphotungstate--draw off excess and dry.
3. As it dries, the stain forms a glassy electron dense replica of specimen. Stain-excluding areas (volumes) appear light in the image.

**Unstained Specimens** - how to preserve them in vacuum in order to obtain images of the actual specimen rather than the distribution of stain around the specimen

1. **Environmental stage**--maintain H$_2$O vapor around specimen by differential pumping; isolate specimen area with small apertures above and below so higher concentration of H$_2$O can be maintained around it. Seldom used.
2. **Freeze-dry or critical point dry**--to remove H$_2$O without totally disrupting the structure.
3. **Embed in glucose** (sugar) syrup by drying in thin layer of 1% glucose (as when negatively staining).
   a. -OH's of sugar mimic polar H$_2$O
   b. supports specimen from collapse during drying
   c. reduces contrast--glucose has density similar to protein. One alternative is to use heavy atom substituted sugars such as auro-thio-glucose.
4. **Frozen-hydrated specimens** -- best method
   a. freeze specimen in thin layer of vitreous (non crystalline) ice
   b. "natural" aqueous environment
   c. ice shows protein in positive contrast
   d. can irradiate with 5-10x more electrons at low temp
   e. requires special stages
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IMAGE PROCESSING

Image Processing--How can it help?

A. Image Restoration -- correct aberrations of the "phase contrast transfer function" (sometimes called the "point spread function" of the imaging system). Most easily accomplished by operation on the Fourier transform.

B. Signal Averaging -- improve low signal-to-noise ratio by adding images of identical structures together.

Remember: Low exposures required to prevent radiation damage in unstained specimens result in poor S/N in the Image

1. Average unit cells in a crystal -- Electron Crystallography
   a. Digitize and calculate the Fourier transform of the image which is non-zero only at points of a Reciprocal Lattice for perfect crystals. Spacings of the reciprocal lattice are the inverse of those of the crystal lattice.
   b. Reconstruct average image of all unit cells by inverse Fourier transformation (Fourier synthesis) using only information at Reciprocal Lattice points.
   (WH: Other averaging possibilities occur in helical and high-symmetry arrangements of the same subunit, see later slides)

2. Average individual particles by Correlation Methods and Correspondence Analysis.
Correspondence Analysis sorts images of individual particles into groups which are similar to one another. Images of individual particles which are similar in structure can be averaged together using Correlation methods to bring them all into a common alignment.
Three-Dimensional Reconstruction

1. Sorts out overlapping details in two-dimensional images.

2. Tilt specimen in electron microscope - but usually only by ±60° - like CAT scan and NMR imaging; obtain projections of structure at different angles.

3. Central Section Theorem -- the 2-dimensional Fourier transform of a projection of a 3-dimensional object is a Central Section (a section passing through the origin) of the 3-dimensional Fourier transform.
   a. Thus, one can determine the 3-dimensional Fourier transform by sampling it on central sections obtained from 2-dimensional images of tilted specimens.
   b. With enough central sections, the entire 3-dimensional transform can be determined to a limited resolution (determined by the number of central sections, the more central sections the higher the resolution). The 3-dimensional structure can be calculated by an inverse Fourier transform operation on the estimated 3-dimensional Fourier transform.

4. Again, Fourier methods are especially applicable to periodic objects because their transforms have special properties.
   a. Fourier transforms of 2-dimensional crystals (crystals which are periodic in two dimensions but only one unit cell thick in the third) are sampled on x,y plains but continuous along the nonperiodic z-axis.
   b. Specimens with helical symmetry have Fourier transforms which are non-zero only along layerlines oriented perpendicular to the helix axis. If one knows the nature of the helical symmetry, many Fourier transform central sections can be determined from a single image => 3-dimensional reconstruction to limited resolution from a single images.
   c. Icosahedral Particles (spherical viruses) have Fourier Transforms with special properties.

5. Other 3-dimensional reconstruction algorithms have and are being used: most common is Filtered Back-projection -- used in some CT scanners.

Figure 1 EM sample geometry. A variety of macromolecular assemblies, both ordered and as individual complexes, can be analysed by cryo-EM. Of the ordered assemblies, the 2D crystals are the ones which have yielded atomic resolution structures. However, they must be tilted to high angles to provide 3D data. The helical assemblies are only crystalline in one dimension and the varying orientation of the subunit around the helical axis is useful in providing a set of different views. No crystallization or naturally occurring order is necessary in the case of single particles (isolated complexes in solution), provided they are large enough to be detected and analysed. Single particles can range from highly symmetric, such as the icosahedral viruses, to asymmetric structures, such as the ribosome.

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IMAGE RECONSTRUCTION from TWO-DIMENSIONAL CRYSTALS

Data-collection geometry for 2D crystals. Each lattice line will have a continuously varying amplitude and phase along its Z coordinate. The tilted views sample the lattice lines at different heights in Fourier space. Most of the 3D FT can be determined by combining these sections derived from different tilt views in projection. However, in EM of 2D crystals there is a missing cone of information because the maximum tilt angle of the crystal is limited to about 60 degrees.

Figure 2. Structure of the partially fluorinated Ni\(^{2+}\)-chelating lipids used in this study. The lipids were designed to spread at the air-water interface into monolayers in the fluid LE (liquid expanded) phase.

Lipids 1 and 2 differ by the length of the linker between the fatty chains and the NTA moiety.

Lipid 3 was used as a diluting lipid in the crystallization experiments.

Lipid 4 (Ni-NTA-DOGA) was used for comparative experiments.


A sophisticated method to generate 2D crystals (ctd.)

1. Specifically designed fluorinated Ni-NTA-DOGA lipids are spread at the air-water interface, the lipid monolayer remains stable for hours in the presence of detergents.

2. The His-tagged-protein in detergent with regular lipids is added to the solution.

3. Remove the detergent by adsorption.

4. The His-tags bind to the Ni-NTA-DOGA and concentrate the proteins near the surface with lipids between the proteins as in a biomembrane (at least that is the goal).

This leads to the possible formation of protein 2D arrays in a very biological way.
A sophisticated method to generate 2D crystals (ctd.)

Figure 5. 2D crystals of the Arabidopsis thaliana plasma membrane H+-ATPase grown on a layer of lipids 2 and 3 after removal of the detergent, dodecyl-β-D-maltoside, by adsorption to Bio-Beads1.

Crystals were stained with 2% (w/w) uranyl acetate as a contrasting agent.

(a) Overview of a large crystalline area.
(b) The rectangular crystal lattice is visible at higher magnification.

LeBeau et al
Two-dimensional crystallization of a membrane protein on a detergent-resistant lipid monolayer.

A sophisticated method to generate 2D crystals (ctd.)

Figure 6. (a) Structure factors of a 2D crystal of the Arabidopsis plasma membrane H+-ATPase. The image was obtained by electron cryo-microscopy and image processing of unstained 2D crystals. The size of the square and the number reflect the signal-to-noise ratio of the computed Fourier component (Henderson et al., 1986). The concentric rings indicate the zero positions of the contrast transfer function. The resolution at the edge of the plot is 9 Å.

b) Projection map of the plasma membrane H+-ATPase at 9 Å resolution calculated from merged amplitudes and phases from five independent crystals with P22121 symmetry. Solid lines indicate density above the mean. Negative contours are indicated by dotted lines. The unit cell contains four molecules and parameters are a = 151 Å, b = 139 Å and γ = 90 degrees. The monomer boundary cannot be defined with certainty in the projection map but comparison with the projected structure of the Neurospora enzyme suggests the outlined shape of the monomer.

J. Mol. Biol. 308, 639-647.
Electron cryo-crystallography at atomic resolution

Aquaporin 0 main chain surrounded by lipids

Individual waters and very well defined phenyl ring at 1.9 Å resolution

Layers of aquaporin molecules surrounded by very well defined lipids

High Resolution Electron Microscopy of Biomacromolecules

IMAGE RECONSTRUCTION from HELICAL OBJECTS
Reconstruction of Helical Particles

Fig. 2. Electron microscopy by negative staining of Rotavirus capsid VP6 assemblies. (a) 45-nm-diameter tubes; (b) 75 nm-diameter tubes.

Reconstruction of Helical Particles (ctd.)

ROTAVIRUS CAPSID PROTEIN HELICES

Fig. 6. Sections and three-dimensional representation of the reconstruction of the small (A and C) and large tubes (B and D). The three different contacts of each VP6 trimer are labeled d (D1 and D2), s and w (W) (lower case, small tube; upper case, large tube). Contact d (D1 and D2) displays a double density connectivity in contrast to contact s (single connectivity). Contacts d, D1 and D2 are similar. Contact w (W) is weak.
Reconstruction of Helical Particles (ctd.)

Fig. 7. Fit of the X-ray atomic model into the reconstruction of the small (A and C) and large tubes (B and D). While (A) and (B) are side views of the tubes, (C) and (D) are sections centered at contact d (D) and passing through neighboring trimers.


High Resolution Electron Microscopy of Biomacromolecules

IMAGE RECONSTRUCTION from ICOSAHEDRAL OR OTHER HIGH-SYMMETRY OBJECTS
Icosahedral Symmetry – 60 subunits (or multiples thereof)

Approximate shape of an icosahedron

But the shape is irrelevant. The fact that all symmetry axes are known without any error, as well as that these intersect in exactly one point, the particle center, is the power of averaging this type of particles.

(In addition, these spherical particles are often quite sturdy)
Reconstruction from High-Symmetry Particles

Figure 2
(a) The capsid of hepatitis B virus as determined by 3D reconstruction to 9 Å resolution;
(b) a molecular model of the dimer of 149-residue subunits that serves as its building block. The sites of the three Reference points marked – the N terminus, the C Terminus and an internal peptide (residues 78–83) – Were determined by high-resolution cryo-electron Microscopy (cryo-EM) labeling techniques, (Ref. 26.)
The C-terminal peptide, residues 140–149, is an important determinant of capsid size, namely in specifying the relative amounts of 180-subunit and 240-subunit capsids that are assembled. It is also the attachment point of the basic domain, residues 150–183, that binds nucleic acid inside capsids made of the full-length protein. Courtesy of J. Conway.


Reconstruction from High-Symmetry Particles (ctd.)

Figure 4
Binding of poliovirus to its receptor (Pvr)23,24. Left: cryo-electron microscopic (cryo-EM) reconstruction of poliovirus (red) with the water-soluble ectodomain of Pvr (green) stoichiometrically bound23. In addition to revealing the binding interaction, the reconstruction aided the construction of a quasi-atomic model of Pvr (right), previously known to consist of three immunoglobulin-like domains. Domains 1 (which contacts the virus) and 2 are approximately aligned, whereas domain 3, which is closest to the cell surface, is offset at an angle of ~60°. The reconstruction clearly shows two carbohydrate moieties (arrows) attached to arginine residues on domain 2. Courtesy of D. Belnap.

High Resolution Electron Microscopy of Biomacromolecules

IMAGE RECONSTRUCTION from “SINGLE-PARTICLE ANALYSIS”


Left: A virus particle on a carbon support film is shown in schematic side view. The particle is outlined with good contrast by heavy-metal stain, but is somewhat flattened by the accompanying dehydration.

Right: It is preserved in the native hydrated state in the cryosample but the protein-ice contrast is very low. In order to maximize the contrast, the cryo-specimen is imaged over holes in the carbon support film.
Holey grids

Although the carbon film is very thin and usually presents no big problem in obscuring a sample, for high resolution studies of unstained biological macromolecules any interference on the beam can be problematic. For high resolution studies, holey grids are used in conjunction with freeze-sample (cryo) electron microscopy. Similar to normal carbon coated grids, holey grids are covered with a fine layer of carbon. However, as part of the preparation process, the carbon film is deposited in such a way that there are holes of a desired size in the carbon.

One of the purposes of these holes is to eliminate any absorption and scattering of the electron beam by the carbon film, which will generate noise and obstruct the signal. Because cryo EM does not use staining, any elimination of background noise is desirable. The holes also allow for "pockets" of solvent to form. **Within these pockets, the specimen remains fully hydrated, even when the sample has been frozen.**

![Holey grid image](http://www.snaggledworks.com/em_for_dummies/grids.html)

Vitrification of cryo-EM specimens and time-resolved cryo-EM

A cryo-EM grid with a thin film of solution is plunged into liquid ethane for vitrification. Time-resolved experiments are possible by spraying a reagent onto the grid just before it enters the ethane, using an atomiser. A time resolution of several milliseconds can be achieved. To the right is a schematic view of a part of the frozen water layer, with macromolecules trapped in different orientations. Bottom right, part of a cryo-EM image showing weak and noisy views of the complexes.

The Vitrobot™, produced by Maastricht Instruments, is computer controlled to simplify and automate the vitrification of liquid samples.

http://www.snaggledworks.com/em_for_dummies/feesea.html

The freezing chamber of Vitrobot™ with a grid suspended before freezing, and after dunking into liquid ethane (bottom).

Interactive particle selection

Interactive particle selection using WEB (Frank et al., 1996). WEB is one of a number of programs that are available for interactive particle selection. The micrograph image is displayed on the monitor by the program, and the user points and clicks with the mouse on the particles that can be seen in the image. WEB and other similar software record the coordinates of the interactively selected particles in a file. The red circles are located at the positions of particles previously selected by the user (and assist the user in not making multiple selections of the same particle).

Towards automatic particle detection
(To the best of my knowledge not yet beating human detection)

An example of the effectiveness of edge detection.

(a) A micrograph showing ice-embedded ribosome particles. The micrograph was part of a dataset made available to participants at the “Single Particle Reconstruction from Electron Microscope Images” course held at the Pittsburgh Supercomputing Center on July 21–24, 1999. Comparable data are available as part of the SPIDER software distribution (Frank et al., 1996).

(b) An edge image obtained by applying the Shen–Castan edge detector (Shen and Castan, 1992) to the image in (a). The edge image was computed using the Shen program. Source code for the Shen program together with other image processing software is available from Parker (1997).


Flow chart of a typical “single particle” reconstruction

Experimental projections are first selected from electron micrographs. These particles are then centered, aligned, and classified. From the centered/aligned/classified particles, a preliminary model is generated. To refine this solution, the model is used to better align and classify the original particles. A new model is generated from the refined data, and so on until a satisfactory solution is reached.

http://www.snaggledworks.com/em_for_dummies/reconstruction.html
Single particle analysis, image processing and 3D reconstruction: a flow chart summarising the key steps.

The principle of 3D reconstruction from 2D projections

A set of four different molecular orientations, displayed as rendered iso-surfaces, are shown above their corresponding 2D projections. Real cryo-EM images are very noisy versions of these projections. The Fourier transform of each projection, shown below the projections, is a section through the 3D Fourier transform of the structure (DeRosier & Klug, 1968). The 3D transform is represented by two intersecting transform sections, derived from the side and end views of the structure. Once enough sections are available, the full 3D transform can be interpolated and inverse transformed into a 3D density map. The map is shown with the backbone of the structure to emphasize the fact that the full 3D density is obtained, including internal features. Surface rendering is generally used to represent such maps when the resolution is insufficient to provide an atomic model of the structure.
Refinement by projection matching.
A 3D map is projected into a set of different orientations to create reference images. Each raw image in the data set is translationally and rotationally aligned to each reference image in turn and is assigned the orientation of the one giving the highest correlation coefficient. The aligned images are grouped and averaged and these averages are used to create an improved 3D map. The procedure is iterated until the maps converge. Reprojections of the maps are compared to the preceding set of class averages to check for consistency.

Three-dimensional map of the archaeal thermosome holoenzyme as reconstructed from cryo-electron micrographs.

The thermosome is a hexadecameric chaperone, and the crystal structures of the subunits are modelled into the molecular envelope.

The beauty of electron microscopy is that the reconstructed images have often sufficient resolution to position structures of components solved at higher resolution with crystallography or nmr methods.
ELECTRON TOMOGRAPHY

Key paper:
“Electron Tomography: towards visualizing the molecular organization of the cytoplasm”.
Wolfgang Baumeister

Electron Tomography (ET)
The Principle

a. Collect 2D-images of projections in different directions through 3D-object

b. Reconstruct 3D-object from 2D-projections

(Radiation Damage the main enemy: high resolution requires many projections!)

Fig. 2. Segmented surface rendering of a **single** virion tomogram after denoising. 
(A) Outer surface showing the distribution of glycoprotein spikes (yellow) protruding from the membrane (blue).
(B) Cutaway view of the virion interior, showing the **capsid** (light blue) and the **tegument "cap"** (orange) inside the **envelope** (blue and yellow). pp, proximal pole; dp, distal pole. Scale bar, 100 nm.

The End of BC530

High Resolution Electron Microscopy