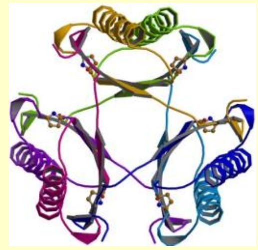
Image Formation

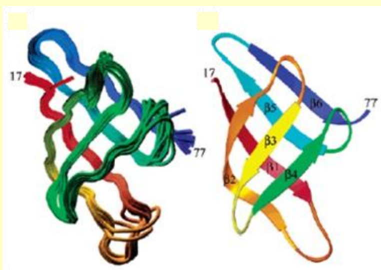
• Light Photography

$\lambda \sim$ **400 - 700 nm**

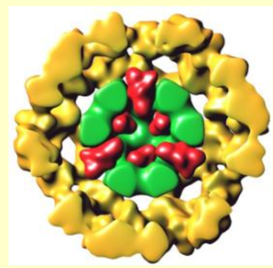


• X-Ray

$\lambda \sim$ **0.1 nm**



• **NMR**



• Electron Microscopy

$\lambda \sim$ **0.001 - 0.1 nm**

Microscopy: Goals for this unit

1. Basics: *Understanding differences of common microscopies*

TEM – Transmission Electron Microscopy

SEM - Scanning Electron Microscopy

STM - Scanning Tunneling Microscopy

AFM - Atomic Force Microscopy

Scanning Transmission EM

2. Cryo-EM (3D images of complex macromolecular assemblies)

- *single particles / heterogenous samples / image gallaries*
- *power of symmetry averaging to improve S/*N

3. Medical Imaging: *CAT / PET / MRI / Ultrasound*

Molecular Imaging Techniques

Electron Microscopy

TEM – Transmission Electron Microscopy

(Cryo EM)

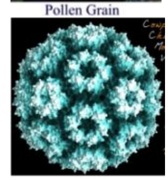
SEM – Scanning Electron Microscopy

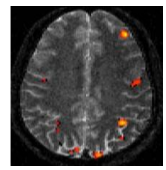
STM / AFM – Atomic Force Microsopy

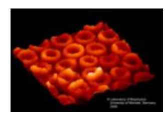
Medical Imaging Methods

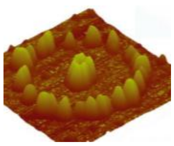
(CAT / PET / MRI / Ultrasound)

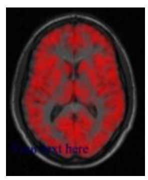

Pollen Grain

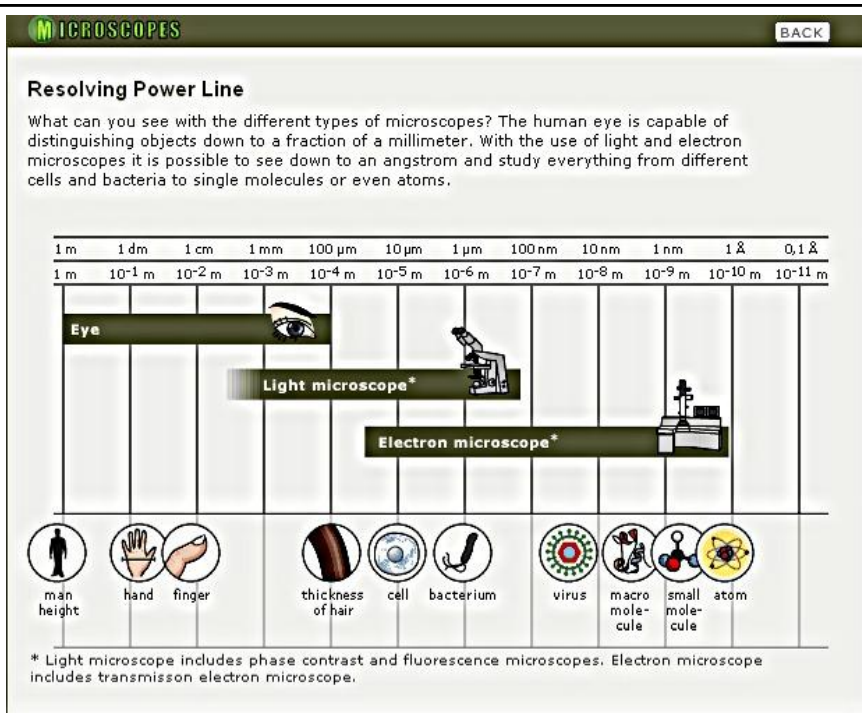












MICROSCOPES BACK

Time Line

14th century – The art of grinding lenses is developed in Italy and spectacles are made to improve eyesight.

1590 – Dutch lens grinders Hans and Zacharias Janssen make the first microscope by placing two lenses in a tube.

1667 – Robert Hooke studies various object with his microscope and publishes his results in Micrographia. Among his work were a description of cork and its ability to float in water.

1675 – Anton van Leeuwenhoek uses a simple microscope with only one lens to look at blood, insects and many other objects. He was first to describe cells and bacteria, seen through his very small microscopes with, for his time, extremely good lenses.

18th century – Several technical innovations make microscopes better and easier to handle, which leads to microscopy becoming more and more popular among scientists. An important discovery is that lenses combining two types of glass could reduce the chromatic effect, with its disturbing halos resulting from differences in refraction of light.

1830 – Joseph Jackson Lister reduces the problem with spherical aberration by showing that several weak lenses used together at certain distances gave good magnification without blurring the image.

* **1878** – Ernst Abbe formulates a mathematical theory correlating resolution to the wavelength of light. Abbes formula make calculations of maximum resolution in microscopes possible.

$$d = \frac{0.612\,\lambda}{n \sin \alpha} \sim \frac{\lambda}{2}$$

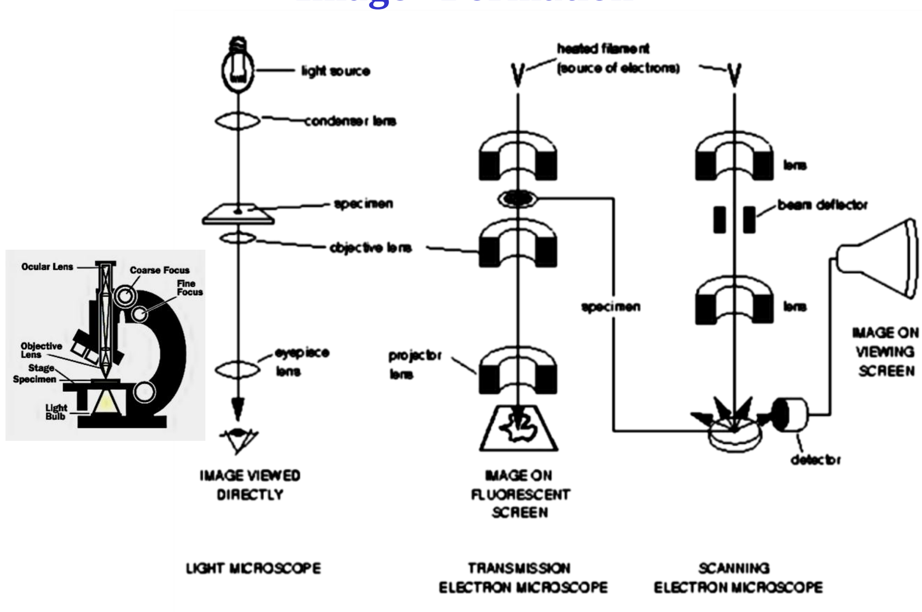
1903 – Richard Zsigmondy develops the ultramicroscope and is able to study objects below the wavelength of light.
The Nobel Prize in Chemistry 1925 »

1932 – Frits Zernike invents the phase-contrast microscope that allows the study of colorless and transparent biological materials.
The Nobel Prize in Physics 1953 »

* **1938** – Ernst Ruska develops the electron microscope. The ability to use electrons in microscopy greatly improves the resolution and greatly expands the borders of exploration.
The Nobel Prize in Physics 1986 »

* **1981** – Gerd Binnig and Heinrich Rohrer invent the scanning tunneling microscope that gives three-dimensional images of objects down to the atomic level.
The Nobel Prize in Physics 1986 »

Image Formation



TEM – Transmission Electron Microscope

Sample preparation is key to getting useful data.

Thinly sliced samples of fixed cells are stained with osmium tetroxide to provide contrast.

Individual particles are stained with uranyl acetate (negative stain).

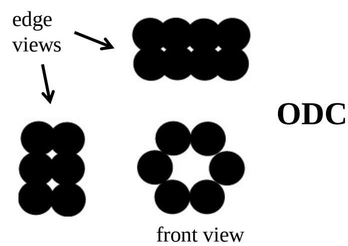
Cryo-EM involves examining particles in ice – low contrast so image averaging is required

Comparisons of the low-resolution structures of ornithine decarboxylase by electron microscopy and X-ray crystallography: the utility of methylamine tungstate stain and Butvar support film in the study of macromolecules by transmission electron microscopy. J. Electron Microsc. Tech. 1991 Jun;18(2):157-66. JK Stoops, C Momany, SR Ernst, RM Oliver, JP Schroeter, JP Bretaudiere, ML Hackert

Abstract

The structure of ornithine decarboxylase (Mr approximately 1.04 x 10^6) from *Lactobacillus 30a* was investigated by electron microscopy and x-ray crystallography. Electron micrographs showed the structure to be well preserved in methylamine tungstate stain. The molecules interacted little with the Butvar support film, yielding three unique projections: **a hexagonal ring** (**front view**) and **two rod-shaped projections (edge views**). Stereo pairs revealed a novel feature of the Butvar film in that some molecules were suspended in the stain in random orientations. Consequently, the relatedness of the hexagonal ring and the rod-shaped particles could be demonstrated since **some particle shapes interconverted when the stage was tilted +/- 45 degrees**. The **two edge views** were related by a 30 degrees rotation about the sixfold axis. Image averaging of the three primary views suggested a **dodecamer (point group symmetry 622) composed of two hexameric rings**, apparently in an **eclipsed** configuration.

To investigate the **structural organization of the complex**, the dissociation of the enzyme was studied by electron microscopy. The **dissociation process involved the initial breakage of the ring** followed by **separation of dimers** from the ring (one subunit from each of the two hexamers). **Thus, the dodecamer forms as a hexamer of dimers rather than a dimer of hexamers.**



Ornithine Decarboxylase

Judith A. Sharp and R. Malcolm Brown, Jr.
Department of Botany, The University of Texas at Austin, Austin, Tx.

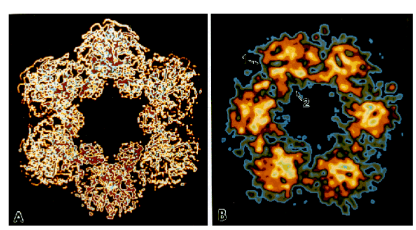
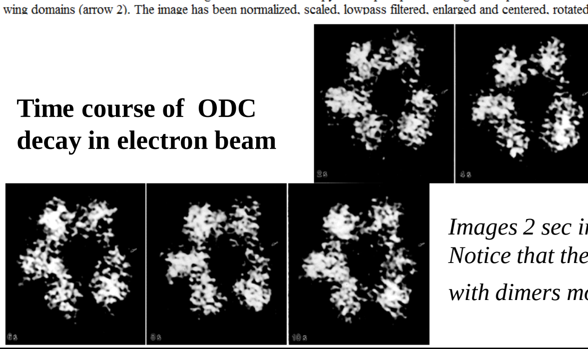
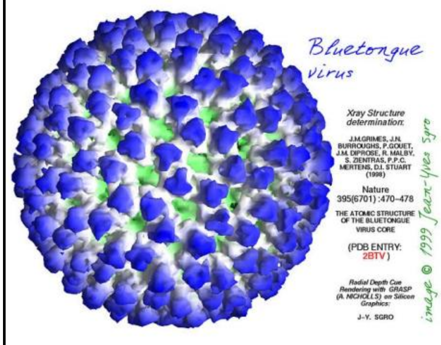


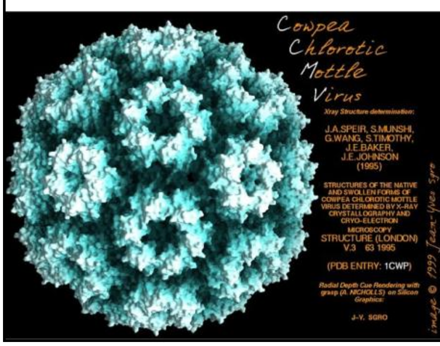
Fig. 2: A comparison of the structure of ornithine decarboxylase as resolved by X-ray crystallography and HRTEM. (A) Computer model of ornithine decarboxylase from the X-ray projection structure (courtesy, M.L. Hackert). The view depicted is along the intramolecular axis. The six dimers that make up the active enzyme aggregate to form a dodecamer with hexagonal symmetry. Also note areas of differential scattering within each dimer. The image has been converted to a color scale (x 5,200,000). **(B)** HRTEM structure of ornithine decarboxylase. The hexagonal symmetry of the dodecamer is represented, as well as substructural information within each dimer. Differential scattering information near the pyridoxal phosphate binding site is present to some degree in each dimer (arrow 1). Also present are the wing domains (arrow 2). The image has been normalized, scaled, lowpass filtered, enlarged and centered, rotated in 60o increments, and converted to a color scale

Time course of ODC decay in electron beam



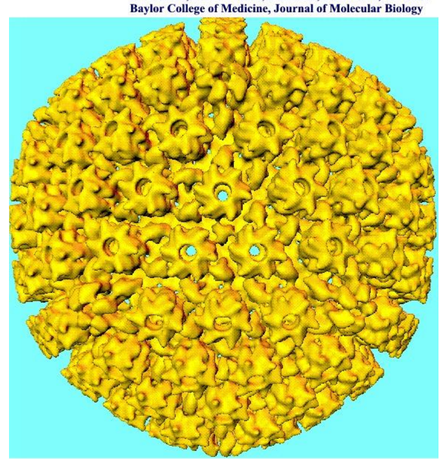
Images 2 sec intervals during 10 sec exposure: Notice that the dodecamer begins to dissociate with dimers moving / changing orientations.



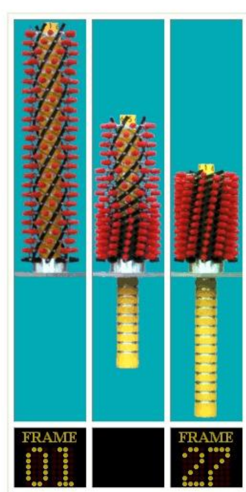


Herpes Simplex Virus-1 A-capsid from 400kV Spot-scan Electron Cryomicroscopy

Z. H. Zhou, B.V.V Prasad, J. Jakana, F.R. Rixon, W. Chiu
Baylor College of Medicine, Journal of Molecular Biology



T4 bacteriophage tail sheath motility



http://www.sb.fsu.edu/~caspar/animation/anim1sm.html

SEM - Scanning Electron Microscope

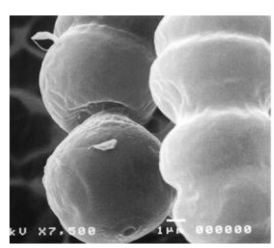
Scanning electron microscope (SEM)

With SEM, the sample is coated with metal atoms before imaging. This provides a conducting surface that prevents the accumulation of charge on the sample (a charged sample would deflect incoming electrons).

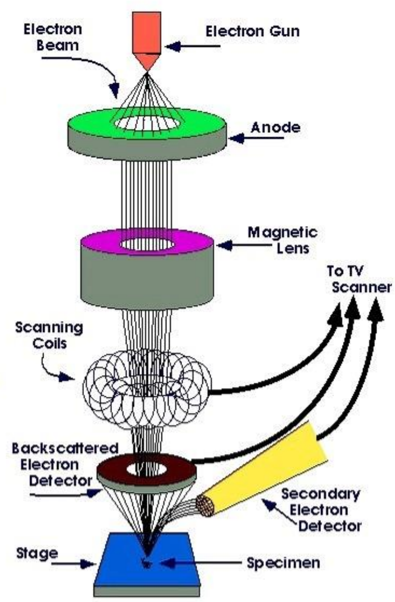
To form the SEM image, a focused beam of electrons is reflected from the surface of the sample and collected on an imaging screen.

A view of the surface of the sample is obtained, without any information on the sample interior.

SEM, algae :

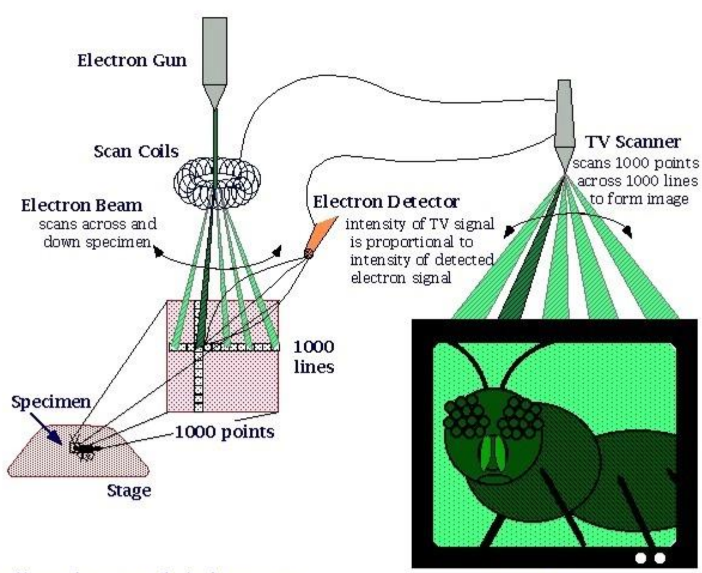


The SEM uses electrons instead of light to form an image. A beam of electrons is produced at the top of the microscope by heating of a metallic filament. The electron beam follows a vertical path through the column of the microscope. It makes its way through electromagnetic lenses which focus and direct the beam down towards the sample. Once it hits the sample, other electrons (backscattered or secondary) are ejected from the sample. Detectors collect the secondary or backscattered electrons, and convert them to a signal that is sent to a viewing screen similiar to the one in an ordinary television, producing an image.



http://mse.iastate.edu/microscopy

How an Image is Produced



http://mse.iastate.edu/microscopy

TV Screen

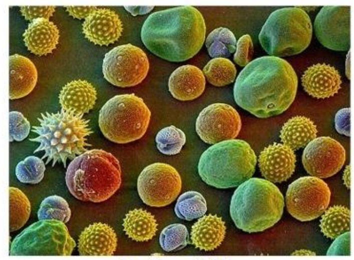
SEM Images

http://mse.iastate.edu/microscopy

Chigger Mite

Deer Tick

Pollen Grain



Pollen Mix

http://www5.pbrc.hawaii.edu/microangela/index.html

"Seeing" as the Blind Person "Sees"

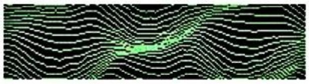
The microscope can be regarded as an extension of the human eye. But sight is not the only sense we use to orientate us in our surroundings, another is touching and feeling.

The **"finger" in this case is a very fine needle**. Needle's movements in the vertical direction as it traverses the surface → topographical map

Two breakthroughs –

1. The so-called **tunnelling effect** - a method for keeping the tip of the needle at a very small and exact constant distance from the surface was developed, thus eliminating the mechanical contact between the needle and the surface. This involves **applying a potential between the needle tip and the surface so that an electric current flows between the needle and the surface without actually touching them**, provided that the tip of the needle and the surface are close enough together.
2. To produce extremely fine needles so that **the tip consists of only a few atoms**.

Preparation of Specimen » **Photo Gallery »**

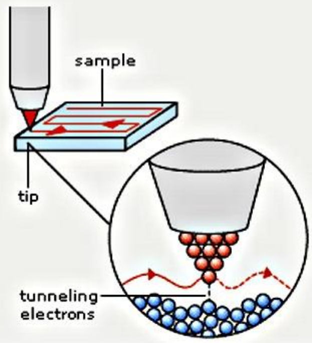


Try the Simulator! »
You need Macromedia Shockwave Player 8.5 to drive the microscope. Go to the **help page** to download the plug-in.

Important in Many Sciences
The study of surfaces is an important part of physics, with particular applications in semiconductor physics and microelectronics. In chemistry, surface reactions also play an important part, for example in catalysis. The STM works best with conducting materials, but it is also possible to fix organic molecules on a surface and study their structures. For example, this technique has been used in the study of DNA molecules.

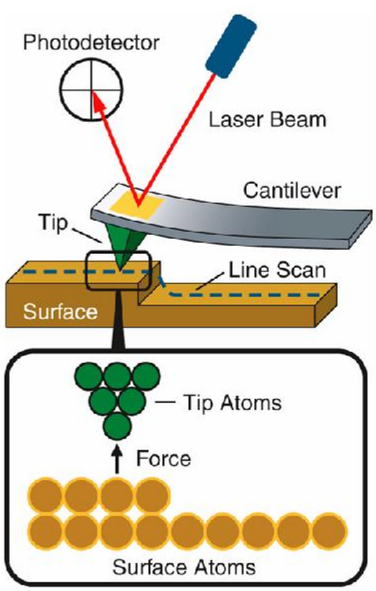
STM, *S*canning *T*unneling *M*icroscope (1981), is an excellent technique, but STM is ***limited to imaging conducting surfaces***

→ **AFM (~1986)**



Related Laureates:
The Nobel Prize in Physics, 1986
- Gerd Binnig and Heinrich Rohrer »

AFM – surface topography

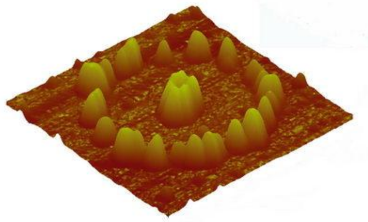


***Atomic Force Microscopy* (AFM)** is often called the "Eye of Nanotechnology" - a high-resolution imaging technique that can resolve features as small as **an atomic lattice** in the real space

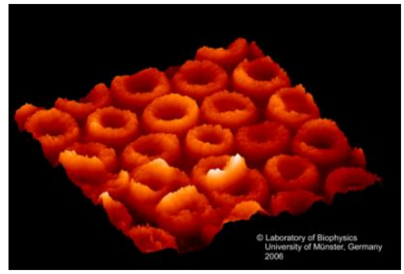
AFM works by bringing a **cantilever tip** in contact with the surface to be imaged. The amount of bending, measured by a laser spot reflected on to a split photo detector, can be used to calculate the force. **By keeping the force constant while scanning the tip across the surface, the vertical movement of the tip follows the surface profile and is recorded as the surface topography by the AFM.**

AFM has much **broader potential** and application because it can be used for **imaging any conducting or non-conducting surface**.

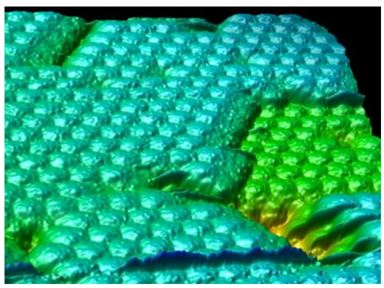
Examples of AFM Images



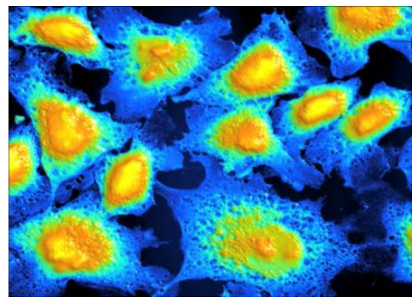
Self-assembled nanoparticles on a pre-patterned substrate – “chemical lithography”. Photo credit: Prabhakaran, et al.



Red blood cells



Virus Crystal

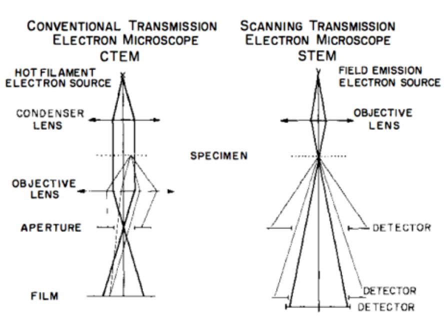


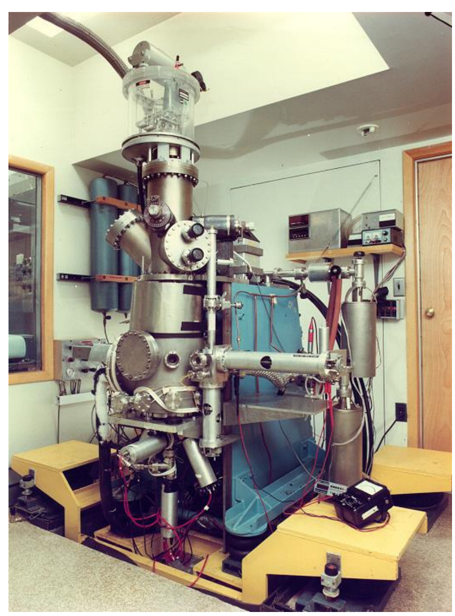
Chinese Hampster Ovary Cells

Scanning Transmission EM

1. Sample is applied to a carbon film and dried.
2. In the vacuum chamber, the sample is scanned with a focused beam of electrons
3. The scattering intensity is recorded to create an image.

Scattering intensity is proportional to mass

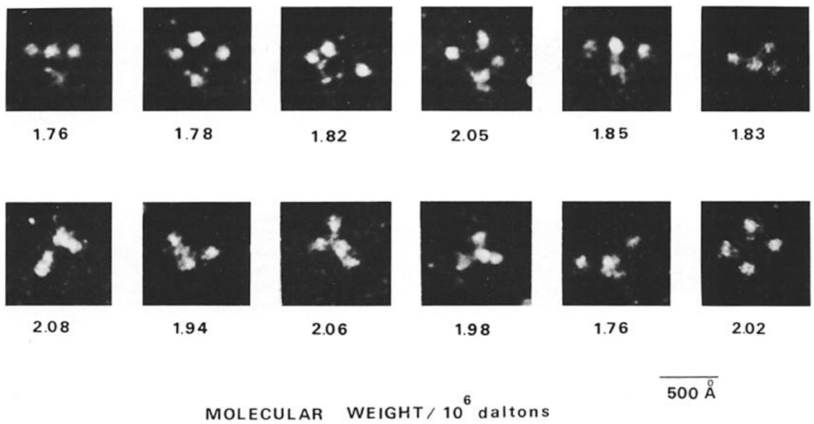




Brookhaven National Lab - STEM

Simultaneous measurement of mass and observation of structure

Studies showed that dynein had 3 head and a net mass of 2×10^6 daltons



Johnson and Wall, Structure and Molecular Weight of the Dynein ATPase, J. Cell Biol. (1983) 96, 669-678

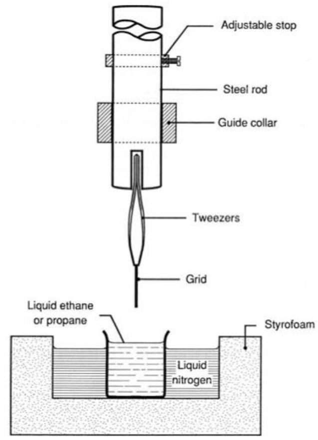
Electron cryo-microscopy (cryo-EM)

- versatile technique to visualize the 3D structure of macromolecules and their assemblies
- atomic structure of assemblies with molecular masses as low as ~200 kDa (for example, a membrane protein) or as high as hundreds of MDa (for example, a large virus).
- assemblies can be visualized single-fold, thus avoiding the need for crystals. This "single-particle" approach requires extremely small amounts of material, typically only a few tens of picomoles
- apply the single-particle technique to assemblies that are difficult to study by more traditional techniques such as X-ray crystallography and nuclear magnetic resonance (NMR). For example, membrane proteins are usually too large for NMR analysis or are difficult to crystallize for X-ray crystallography

JEOL 1200 cryo-EM

Freezing the samples protects them from radiation damage by the electron beam.

Cryo-EM samples are frozen in "vitrified" ice. This is glass-like ice, rather than crystalline ice. Vitrified ice can be obtained by flash-freezing the samples at liquid nitrogen temperatures.



Stewart M. 1991. Transmission electron microscopy of vitrified biological macromolecular assemblies. In *Electron Microscopy in Biology—A Practical Approach*, ed. JR Harris, pp. 229–42. Oxford,

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FEBS 25209

FEBS Letters 505 (2001) 199–205

Minireview

Single particle macromolecular structure determination via electron microscopy

Pamela A. Thuman-Commike*

QED Labs, 1190 S. Bascom Ave., Suite 208, San Jose, CA 95128, USA

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Edited by Amy M. McGough

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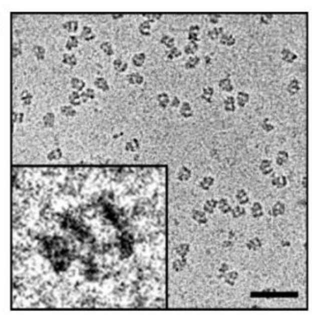
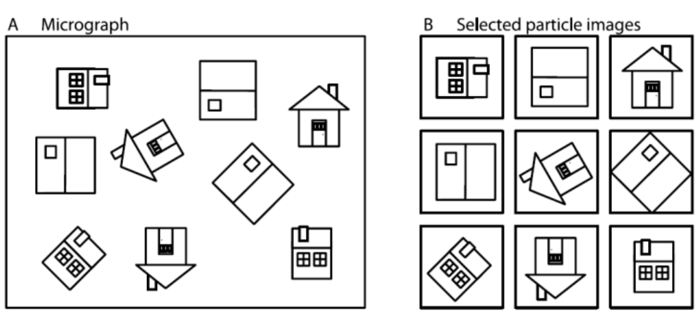
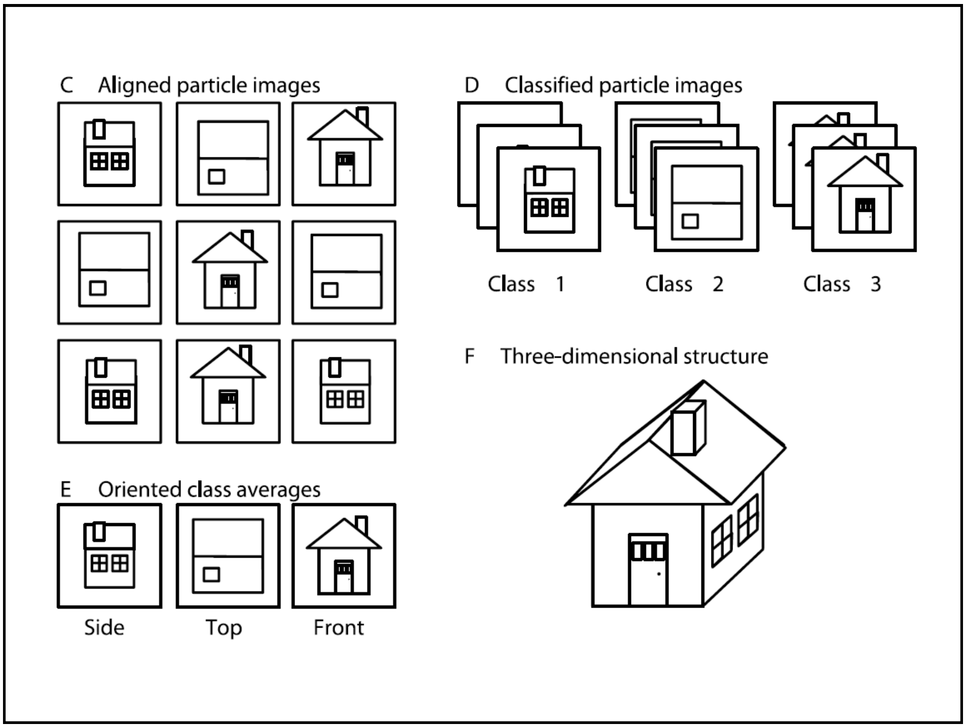






P.A. Thuman-Commike/FEBS Letters 505 (2001) 199–205

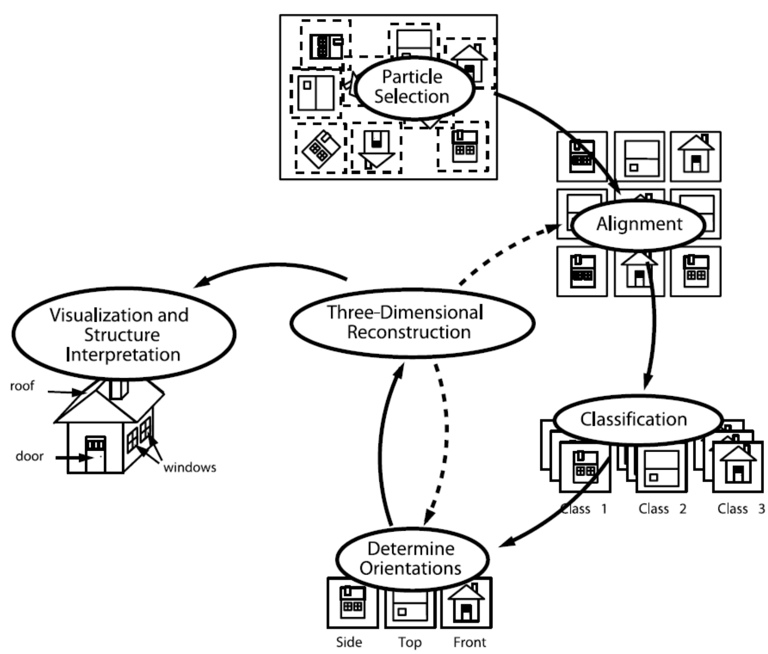
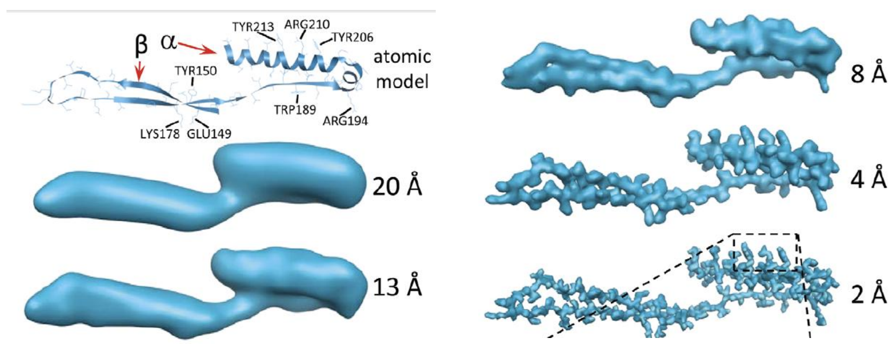


Table 1

Near-atomic resolution structures by single-particle cryoEM[a]

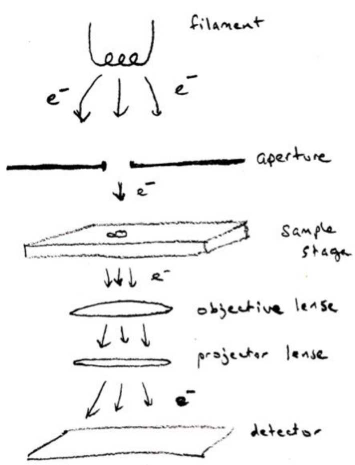
Complex [Ref.]	Sample temperature	CCD/film	Software	CPU hrs[b]	Subunits averaged	Effective resolution (Å)	Modeling method
CPV [18••]	LN	CCD	IMIRS [45]	$\sim 10^3$	7.69×10^5	3.8	O [57]
ε15 phage [17••]	LH	Film	EMAN [43]	$\sim 10^6$	1.2×10^6	4.5	Coot [58]
GroEL [20••]	LH	Film	EMAN [43]	NA	2.86×10^5	4.2	Coot [58]
Rotavirus [19••]	LN	Film	FREALIGN [44]	$\sim 10^5$	6.55×10^6	3.8	None

[a] CryoEM images were all recorded using 300 keV electrons generated by field emission guns. LN: liquid nitrogen; LH: liquid helium; NA: not available.
[b] CPU hours are estimated based on either information from the papers cited or through personal communication.



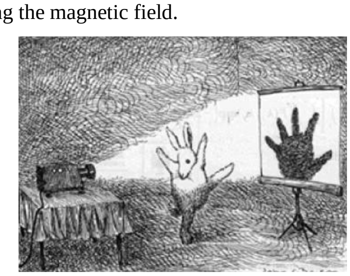
Cryo-EM is a type of TEM. That is, cryo-EM is a transmission method.

TEM schematic. Electron beam is generated at a hot metal filament.
"Lenses" are magnetic fields (compare with optical microscope).



A single wavelength of electrons is used (remember, electrons have wavelength!). $\lambda = \frac{h}{p}$

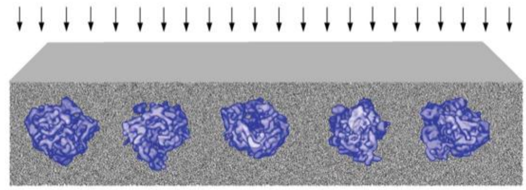
The amount of magnification is controlled by varying the shape of the objective lens. This is similar to optical microscopy, where magnification is varied by switching to a lens with a different curvature. In TEM, the lens is a magnetic field; the shape of the TEM lens is changed by varying the magnetic field.



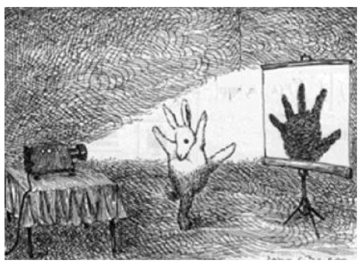
http://cryoem.berkeley.edu/~nieder/em_for_dummies/images/microscope_column.png

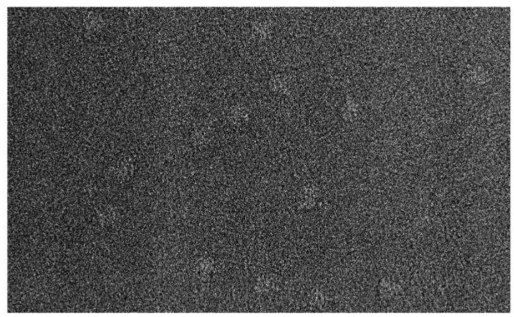
1) Collect transmission micrographs.

Ribosomes in vitrified ice =>



A transmission image of ribosomes in vitrified ice =>.

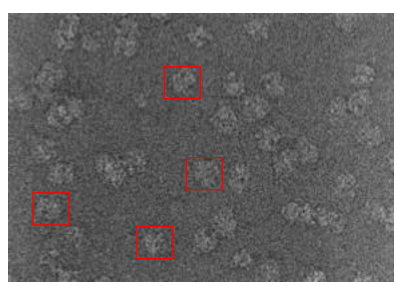




Annu. Rev. Biophys. Biomol. Struct. 2002. 31:303–19

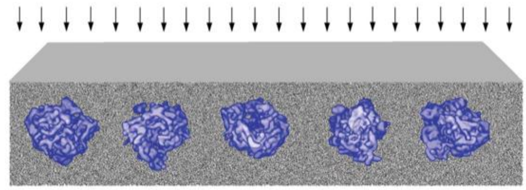
2) Select images of individual particles.

Ribosomes in transmission image =>



Annu. Rev. Biophys. Biomol. Struct. 2002. 31:303–19

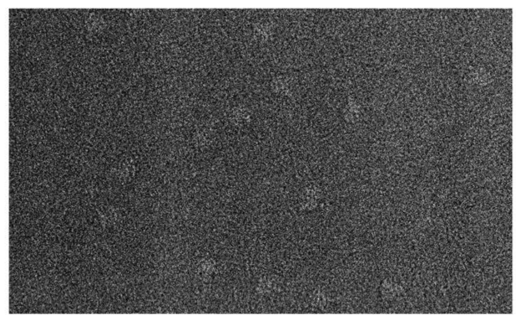
Improvement in image quality comes through the averaging of thousands of similar projections of the object being imaged. Determining the angular orientations for each set of projections is a difficult part of the process.



Ribosomes in vitrified ice =>

Sample platform may be tilted (by typically 20 to 45 degrees) to change the projections in a known way.

Images are projections of the ribosomes in various orientations =>



Annu. Rev. Biophys. Biomol. Struct. 2002. 31:303–19

3) Image reconstruction.

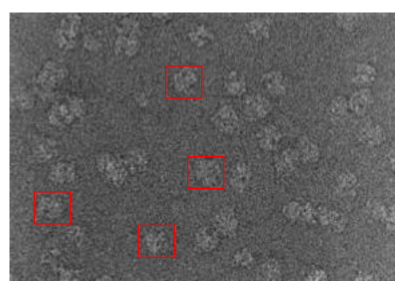
Identify particles in similar orientations, average their densities to get an initial improved image for each projection.

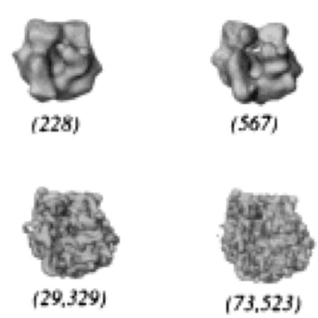
Estimate angular orientations for each set of projections (this is a hard part).

Make an initial image reconstruction:
Essentially, answering the question:
What distribution of electron density would account for the observed projections?

Iteratively refine the orientations of the particles, and average up to thousands of projections to improve the final image.

The cryo-EM image reconstruction method depends on having many (thousands) of images to average.





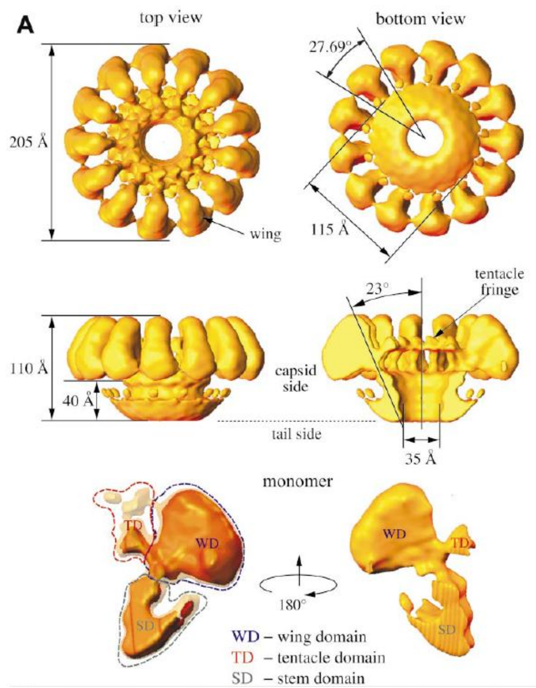
Annu. Rev. Biophys. Biomol. Struct. 2002. 31:303–19

Structural visualization of the bacteriophage SPP1 portal protein Nature Struct. Biol. 6, 842-846 (1999).

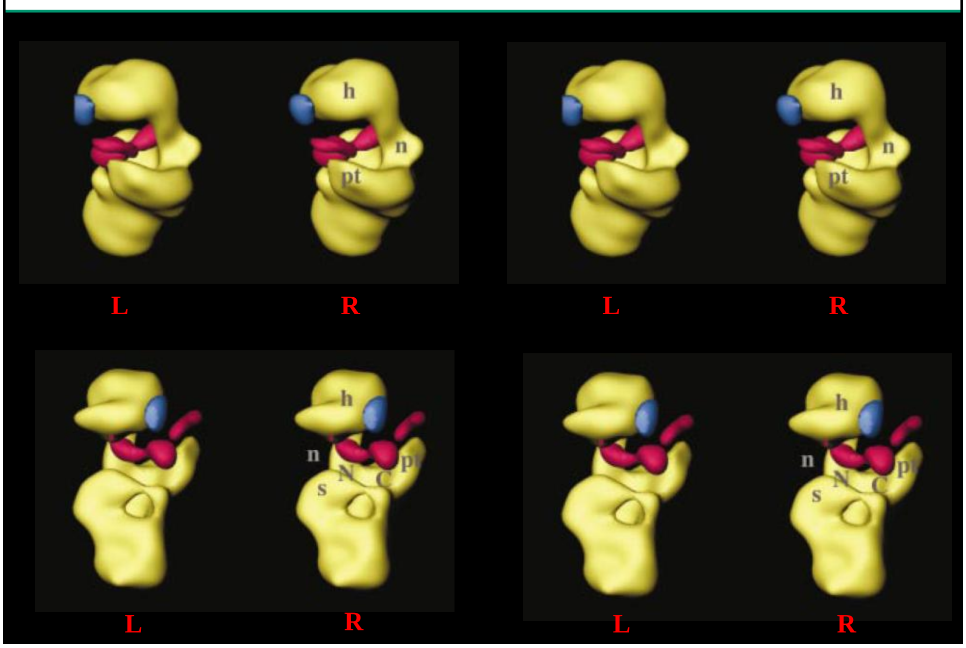
Top row: displayed surface is determined by calculating the appropriate mass of the structure given a threshold value.

Middle row: computationally manipulated views to reveal areas otherwise hidden.

Bottom row: computationally extracted component of the structure.



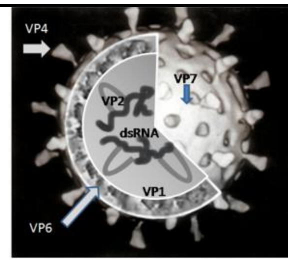
Stereo views of the 30S ribosome subunit bound to translational initiation factor IF3 with positive (magenta) and negative (blue) differences identified Proc. Natl. Acad. Sci. USA 96, 4301-4306 (1999).



Rotavirus – the most common cause of **severe diarrhea** among infants and young children.

Capsid / icosahedral symmetry

diameter of 80 nm (800 A)
inner capsids - diameter of about 50 nm
core - diameter of ~35 nm
genome is 16500-21000 nucleotides



Rotavirus genes and proteins

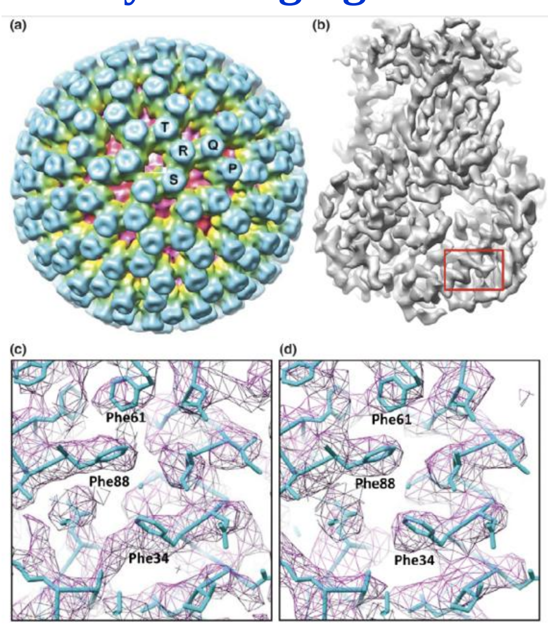
RNA Segment (Gene)	Size (base pairs)	Protein	Molecular weight kDa	Location	Copies per particle	Function
1	3302	VP1	125	At the vertices of the core	<25	RNA-dependent RNA polymerase
2	2690	VP2	102	Forms inner shell of the core	120	Stimulates viral RNA replicase
3	2591	VP3	88	At the vertices of the core	<25	Guanylyl transferase mRNA capping enzyme
4	2362	VP4	87	Surface spike	120	Cell attachment, virulence
5	1611	NSP1	59	Nonstructural	0	5'RNA binding
6	1356	VP6	45	Inner Capsid	780	Structural and species-specific antigen
7	1104	NSP3	37	Nonstructural	0	Enhances viral mRNA activity and shut-offs cellular protein synthesis
8	1059	NSP2	35	Nonstructural	0	NTPase involved in RNA packaging
9	1062	VP7[1] VP7[2]	38 and 34	Surface	780	Structural and neutralisation antigen
10	751	NSP4	20	Nonstructural	0	Enterotoxin
11	667	NSP5 NSP6	22	Nonstructural	0	ssRNA and dsRNA binding modulator of NSP2

Power of Symmetry Averaging

Rotavirus structure reconstructed from *8400 particles*: power of 13-fold nonicosahedral averaging.

(a) Rotavirus DLP

(b) *Trimer of VP6 after 13-fold averaging (13 copies of VP6 in a.u.).* A slab of density (red rectangular in (b)) superimposed with the X-ray model before (c) and after (d) averaging.



Summary of Cryo-EM:

Cryo-EM is a way of generating almost-atomic-resolution images of biomolecules, without the need for crystals.

It is a transmission EM method.

Samples are frozen in vitrified ice, to reduce damage from the electron beam.

There is low image contrast, so many thousands of projections must be averaged to generate an image of high quality.

Typical resolutions are typically 7 to 20 Å, so the method is most appropriate for particles that are 100 to 1000 Å in diameter.

What ultimately limits resolution? Ability to prepare identical particles for averaging; patience to average very large number of low-contrast images.

3 to 4 Å resolution may be achievable by cryo-EM, through the averaging of millions of images, tracking movements associated with decay by recording movie frames / correct.

Regular lattice, helical arrangement, or high molecular symmetry (viruses) in of particles enables obtaining higher resolution images.