基因體與蛋白質體醫學

基因產物在細胞之超微結構分析

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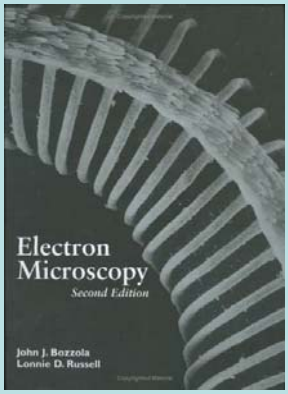
基因產物在細胞之超微結構分析

- 基因產物: DNAs, RNAs, Proteins
- 超微結構分析:
  - 電子顯微鏡 –TEM & SEM
    - Negative staining – DNAs, RNAs,
    - Immuno-electron microscopy – proteins,
    - Freeze fracture – intramembranous structures
    - Rotary shadowing – filaments (proteins)
  - Cryo-EM
  - 原子力顯微鏡 (Atomic force microscopy)

電子顯微鏡 –

1. TEM (transmission electron microscopy) 穿透式

2. SEM (scanning electron microscopy) 掃瞄式



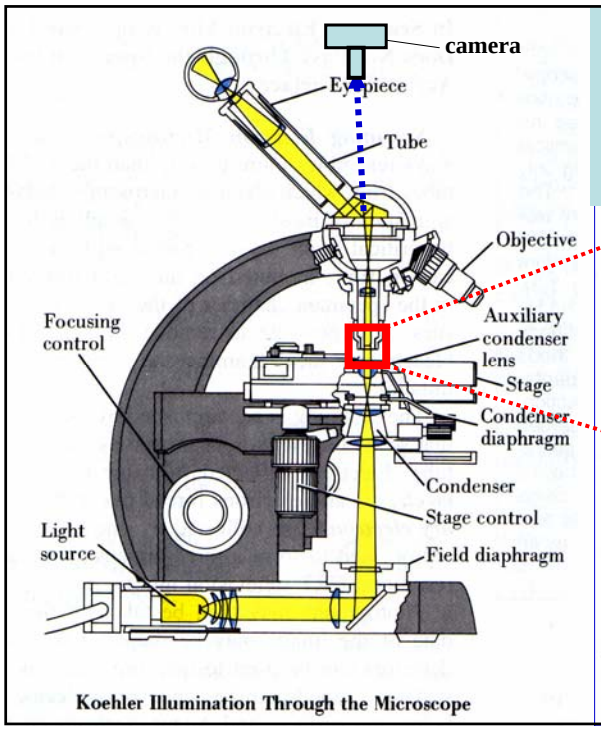
Reference:

1. Bozzola J.J. and Russel L.D. (1999) ***Electron microscopy*** *2nd* ed., Jones and Bartlett Publisher, Sudbury, Massachusetts, USA

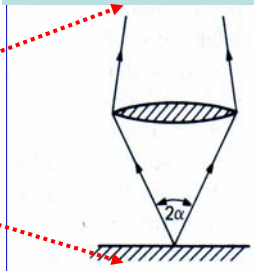
2. Sommerville J and Scheer U (1987) ***Electron microscopy in molecular biology, a practical approach.*** ILR Press, Oxford

Linear Equivalents		
1	Angstrom	= 0.1 nanometer
10	Angstroms	= 1.0 nanometer (**nm**)
		[formerly millimicron (m μ)]
1000	nanometers	= 1.0 micrometer (μ m)
		[formerly micron (μ)]
1000	micrometers	= 1.0 millimeter (mm)
1000	mililimeters	= 1 meter (m)

1. Resolution (resolving power)
2. *Numeral Aperture*
3. Focal depth
4. Field depth



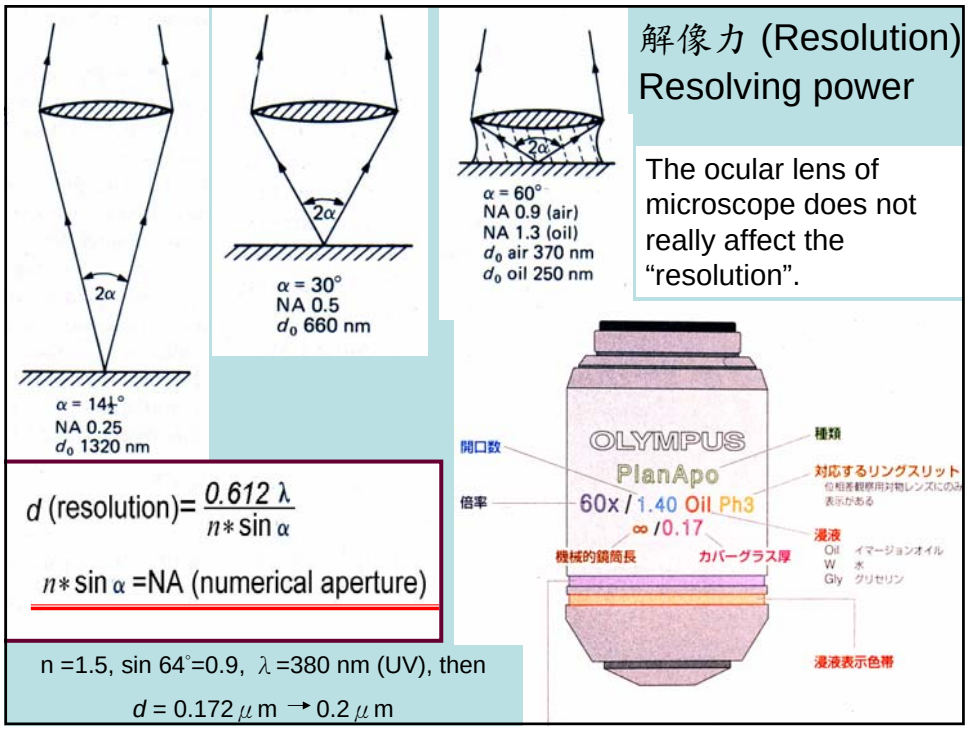
解像力 (Resolution) Resolving power



α = 30°
NA 0.5
d_0 660 nm

$$d\ (\text{resolution}) = \frac{0.612\,\lambda}{n * \sin\alpha}$$

$n * \sin\alpha$ =NA (numerical aperture)



解像力 (Resolution) Resolving power

The ocular lens of microscope does not really affect the "resolution".

$$d\ (\text{resolution}) = \frac{0.612\,\lambda}{n * \sin\alpha}$$

$n * \sin\alpha$ =NA (numerical aperture)

n =1.5, sin 64°=0.9, λ =380 nm (UV), then

d = 0.172 μm → 0.2 μm

Resolution of eye versus instrument	
	Distance between Resovable points
Human naked eyes	0.2 mm
Birght field microscope	0.2 μm
SEM	0.2 nm
TEM	0.2 nm
Theoretical	0.005 nm
Tissue section	1.0 nm

- 電子顯微鏡之構造與基本原理 (TEM及SEM)
- 電子顯微鏡標本之基本製作 (TEM切片及SEM標本)
- 電子顯微鏡術在細胞分子生物學之應用

TEM之構造與基本原理
(Principle of TEM)
TEM之標本製備
(Specimen preparation for TEM)

Properties of Electrons

Negative Charge

1. Capable of manipulation by a magnetic field (electromagnetic lenses)
2. Can be accelerated through a voltage differential

Tiny Mass

1. Will penetrate only ultrathin sections
2. Will be deflected by air particles (high vacuum in microscope column)

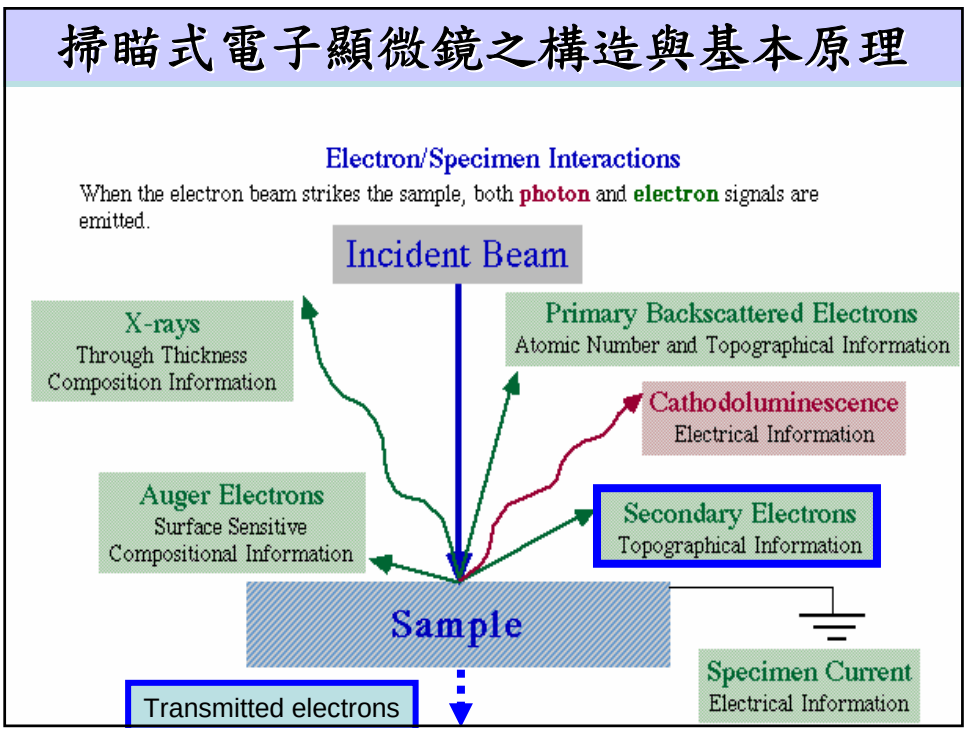
Wave and Particle Properties

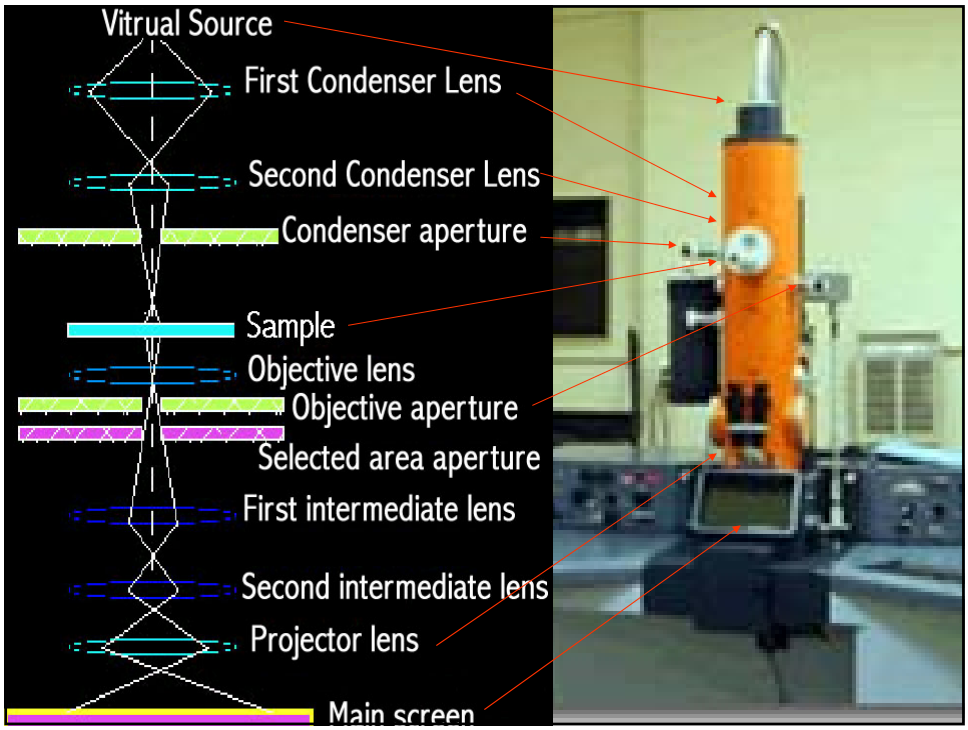
1. Wavelength of electrons (λ > .005 nm) provides for enhanced resolution over optical microscopes (λ @ 550 nm)
2. Electron particles deflected by heavy atoms (basis for contrast in electron Images)

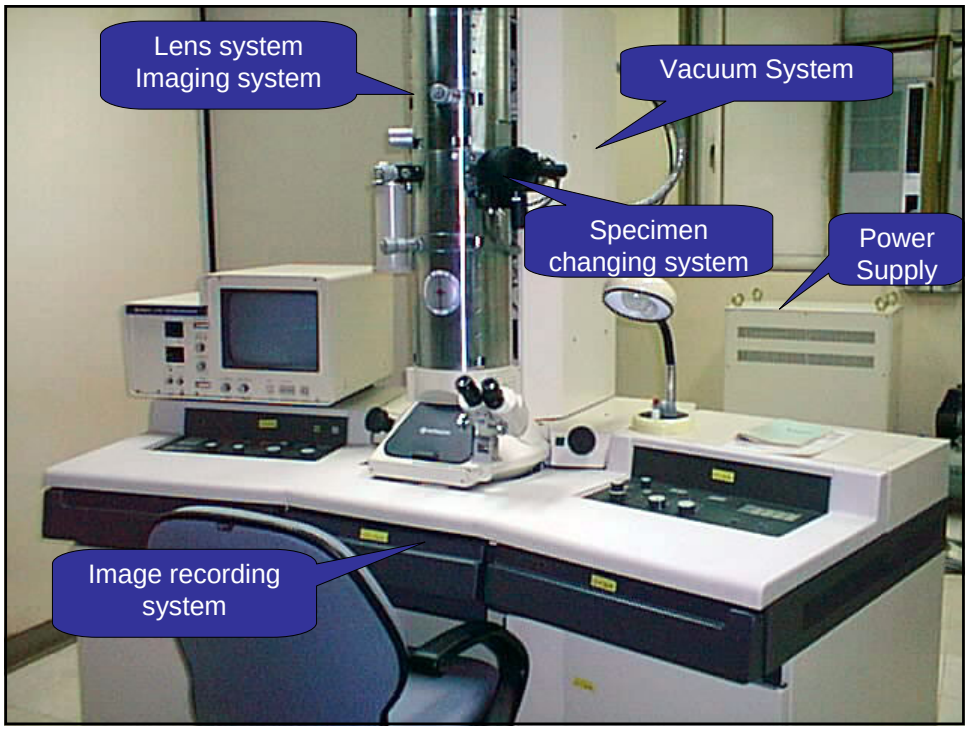
Imaging

1. Electrons are not directly detectable by human vision.
2. Electron images must be converted to photon image (phosphorescent screen) or recorded on photographic emulsions.

掃瞄式電子顯微鏡之構造與基本原理




Vitrual Source
First Condenser Lens
Second Condenser Lens
Condenser aperture
Sample
Objective lens
Objective aperture
Selected area aperture
First intermediate lens
Second intermediate lens
Projector lens
Main screen


Lens system
Imaging system
Vacuum System
Specimen
changing system
Power
Supply
Image recording
system

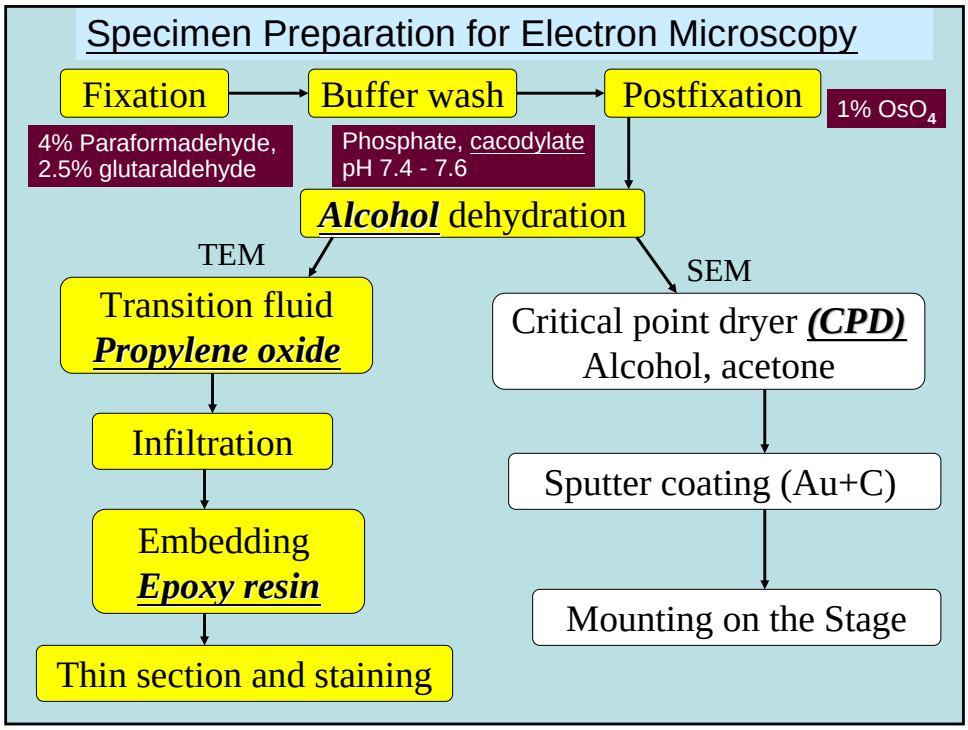


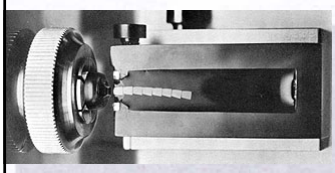
TABLE 2.1 General Tissue Preparation Scheme for Electron Microscopy

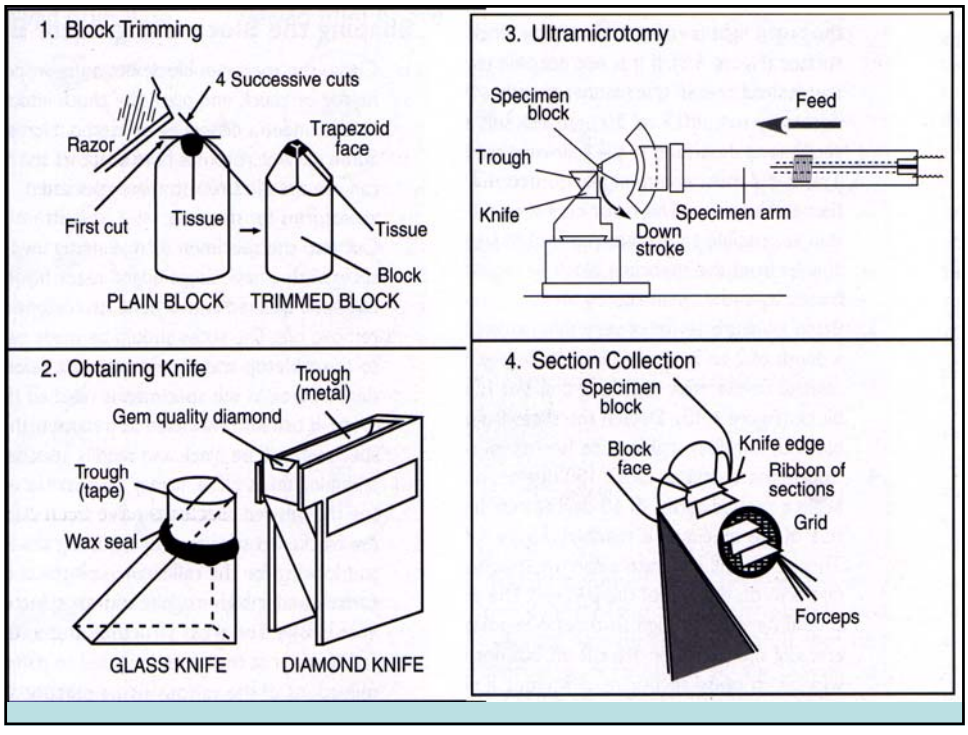
Activity	Chemical	Time Involved*
Primary Fixation	tissue is fixed with 2–4% glutaraldehyde in buffer	1–2 hr
Washing	buffer (three changes at 4°C, one of which may be overnight)	1–12 hr
Secondary Fixation	osmium tetroxide (1–2%: usually buffered)	1–2 hr
Dehydration	30% ethanol**	5 min
	50% ethanol	5–15 min
	70% ethanol	5–15 min
	95% ethanol (2 changes)	5–15 min
	absolute ethanol (2 changes)	20 min ea
Transitional Solvent	propylene oxide (3 changes)	10 min ea
Infiltration of Resin	propylene oxide: resin mixtures; gradually increasing concentration of resin	overnight–3 d
Embedding	pure resin mixture	2–4 hr
Curing (at 60°C)		1–3 d

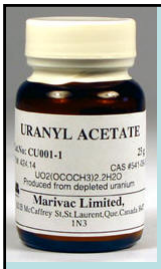
*The specified times do not include the time involved in preparation of chemicals.
**Some recommend omitting the 30% and 50% ethanol steps.

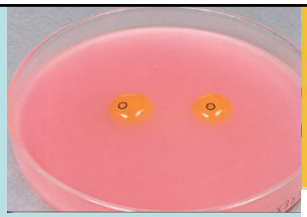
4% paraformaldehyde, Karnovsky fixative (2% paraform- & 2% glutar-aldehyde)

Ultra-microtome





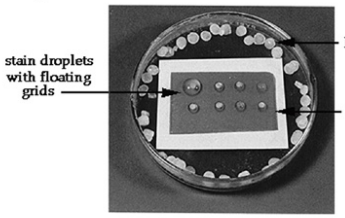




1. Uranyl acetate
2. Lead citrate

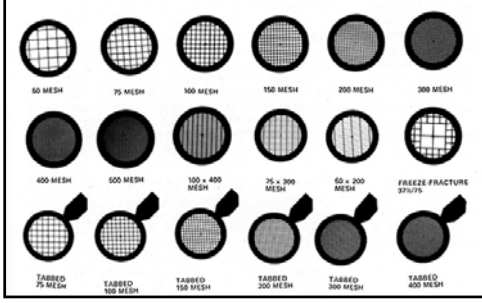
Staining Grids with Reynold's Lead Citrate

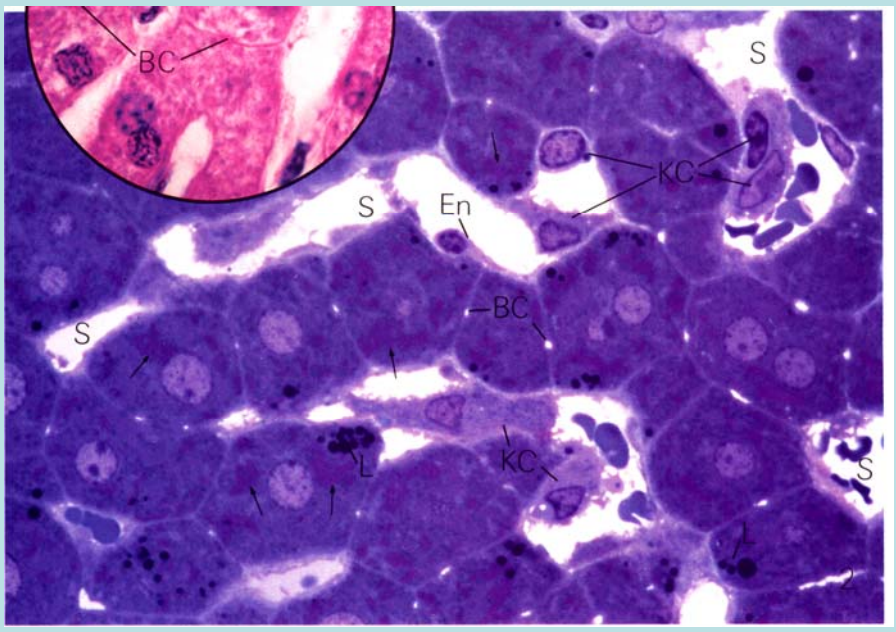
1. Freshly-prepared lead citrate is good for many months if kept in a stoppered volumetric flask and protected from CO_2.
2. Staining is accomplished by floating grids on droplets of lead citrate which have been added to a smooth surface of dental wax or teflon by syringe-filtration.
3. In order to prevent precipitation of stain by exposure to CO2, pellets of NaOH are added to the staining dish to scavage and CO2 from the atmosphere in the dish.



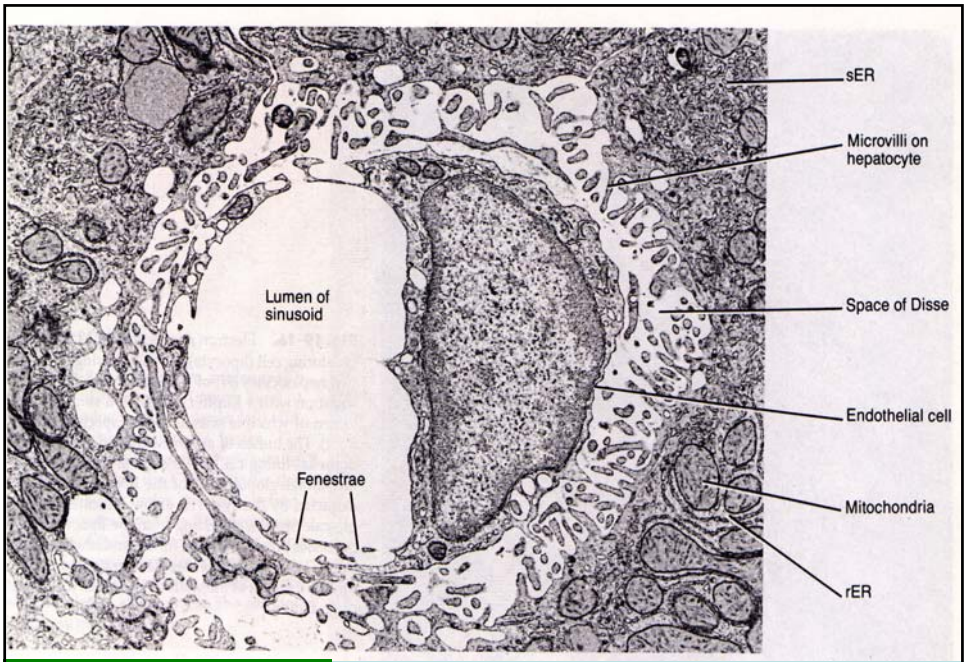
4. The grids should be placed sections-side down on the droplets and and stained for 2-5 mins. with the perti dish cover on to exclude atmospheric CO2.
5. After staining the grids should be washed in a jet of CO2-free, double distilled water while held securely with a forceps.
6. The grids should then be deposited with wicking onto a clean filter paper in a petri dish for drying (5-10 mins.)

Grid Types and Mesh Sizes





1 μ m thick plastic (epxoy resin) section, toluidine blue stain



Hepatic sinusoid 70nm thin section, uranyl acetate & lead citrate

- 電子顯微鏡之構造與基本原理 (TEM及SEM)
- 電子顯微鏡標本之基本製作 (TEM切片及SEM標本)
- 電子顯微鏡術在細胞分子生物學之應用

SEM之構造與基本原理
(Principle of SEM)
SEM之標本製備
(Specimen preparation)

Basic Principle & Components of Scanning Electron Microscope

1. Secondary electrons,
2. Scanning devices,
3. CRT – recording devices

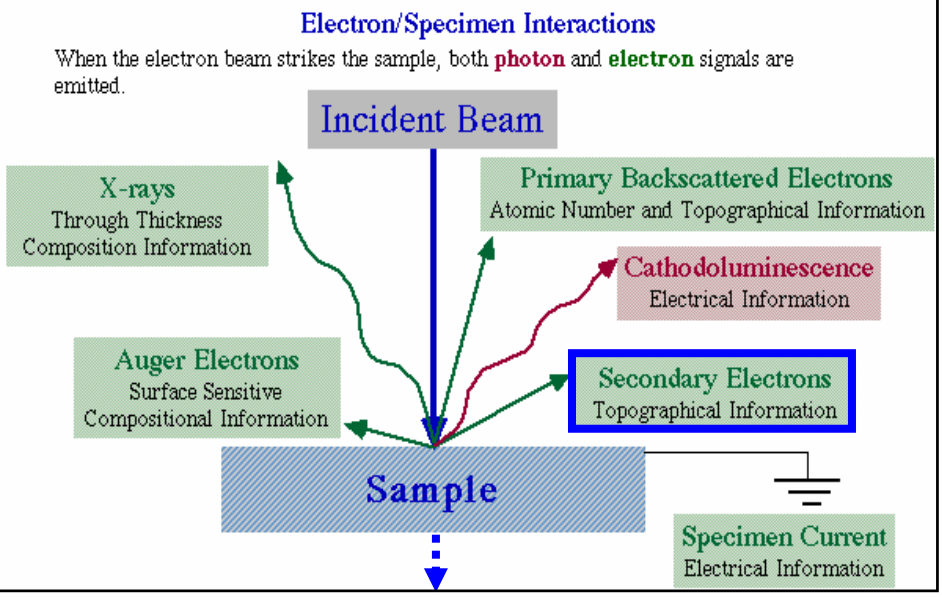
I. Main body (Column)
   1. Illuminating system
   2. Specimen chamber
   3. Image forming system
   4. Image recording system

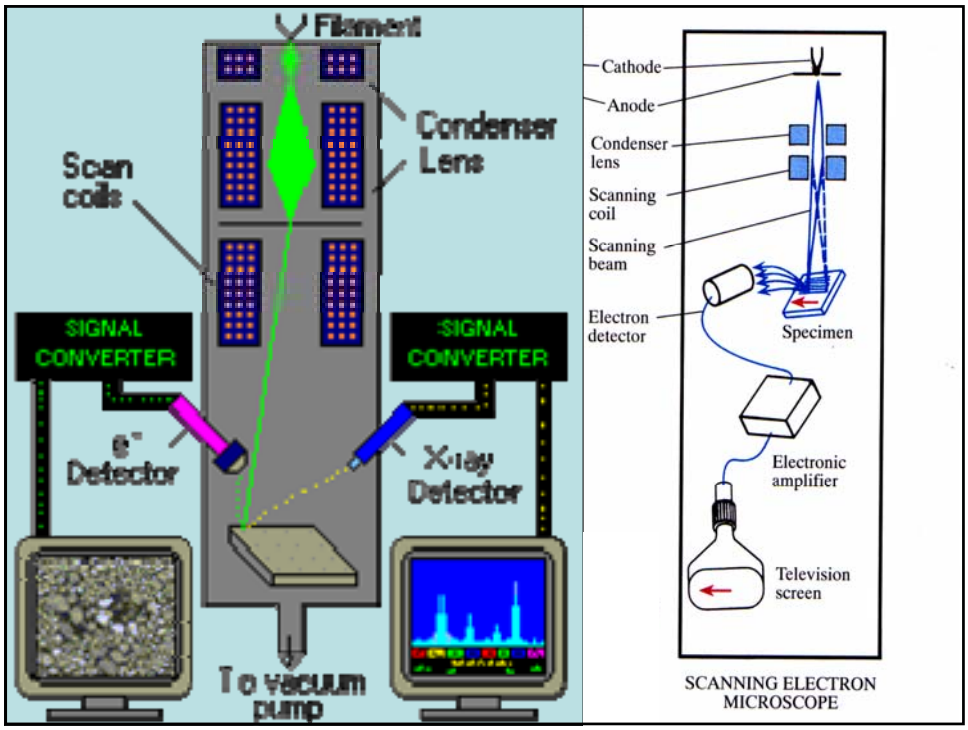
II. Vacuum System

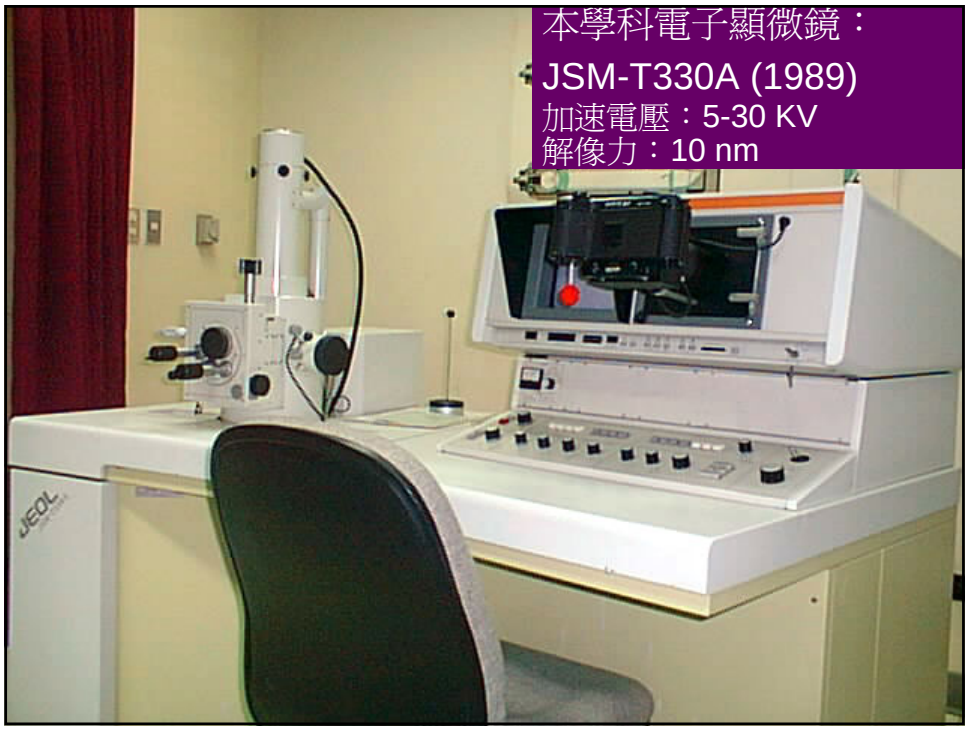
III. Electric System

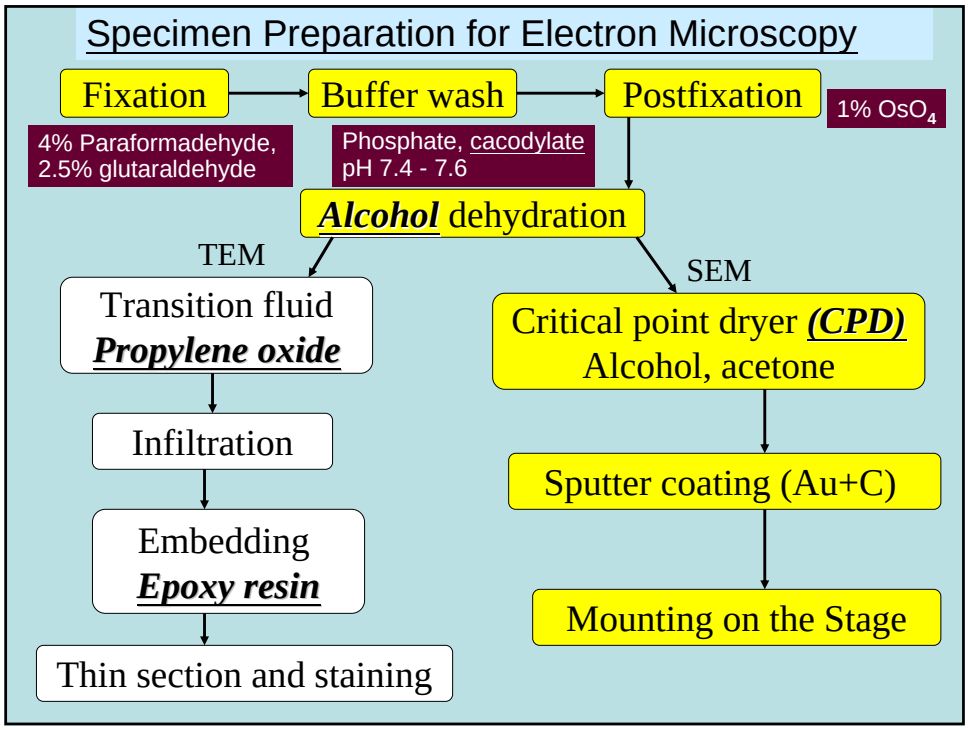
IV. Cooling system

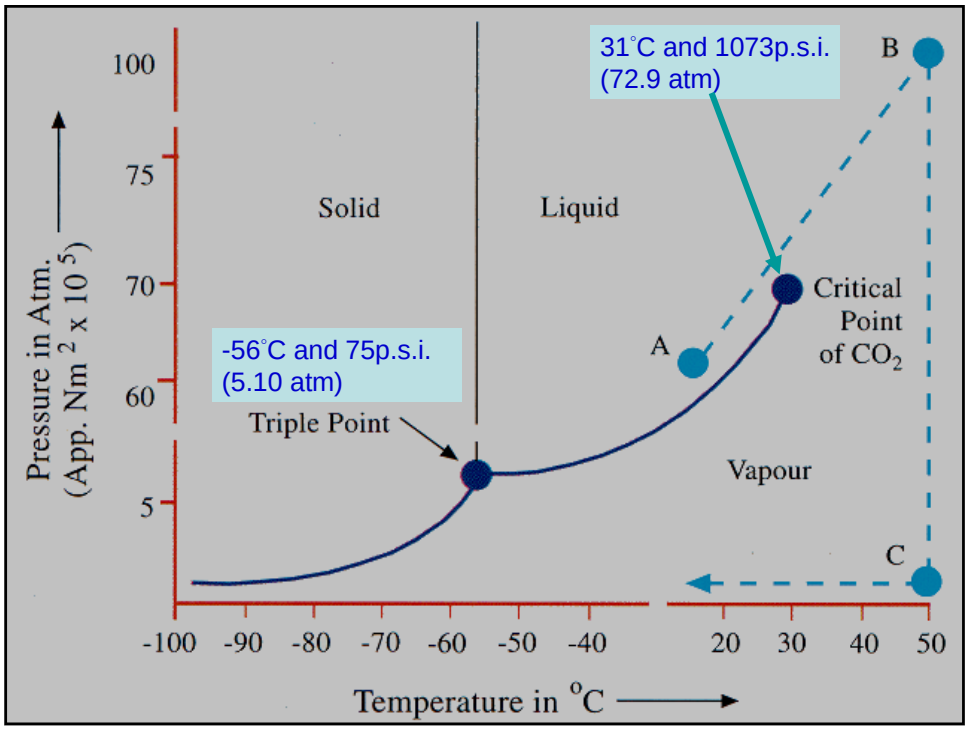
掃瞄式電子顯微鏡之構造與基本原理

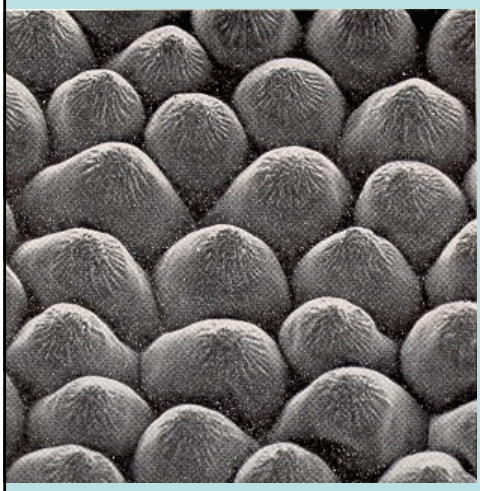


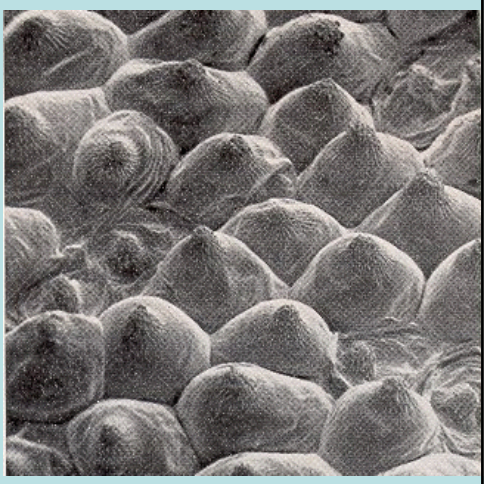

Filament
Condenser Lens
Scan coils
SIGNAL CONVERTER
SIGNAL CONVERTER
e⁻ Detector
X-ray Detector
To vacuum pump
Cathode
Anode
Condenser lens
Scanning coil
Scanning beam
Electron detector
Specimen
Electronic amplifier
Television screen
SCANNING ELECTRON MICROSCOPE


本學科電子顯微鏡：
JSM-T330A (1989)
加速電壓：5-30 KV
解像力：10 nm
JEOL


Specimen Preparation for Electron Microscopy
Fixation
Buffer wash
Postfixation
1% OsO4
4% Paraformadehyde, 2.5% glutaraldehyde
Phosphate, cacodylate pH 7.4 - 7.6
Alcohol dehydration
TEM
SEM
Transition fluid
Propylene oxide
Infiltration
Embedding
Epoxy resin
Thin section and staining
Critical point dryer (CPD)
Alcohol, acetone
Sputter coating (Au+C)
Mounting on the Stage


31˚C and 1073p.s.i.
(72.9 atm)
B
Solid
Liquid
Critical
Point
of CO2
A
-56˚C and 75p.s.i.
(5.10 atm)
Triple Point
Vapour
C
Pressure in Atm.
(App. Nm 2 x 10 5)
100
75
70
60
5
-100
-90
-80
-70
-60
-50
-40
20
30
40
50
Temperature in °C



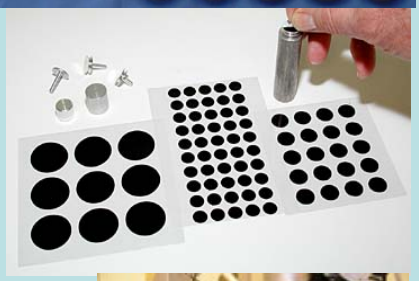


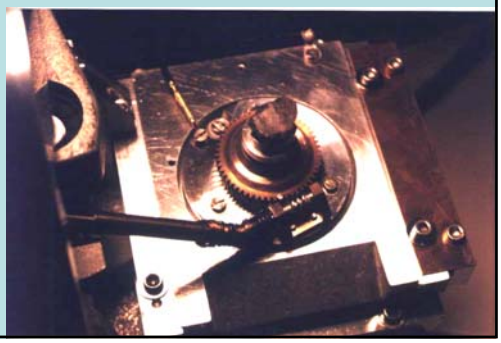
CPD dried

air dried

Critical Point Dryer

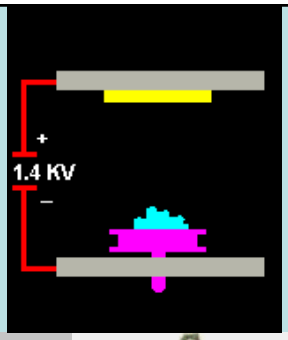
Stubs (specimen stage)

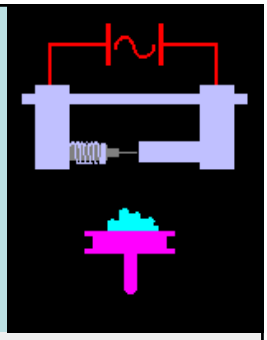


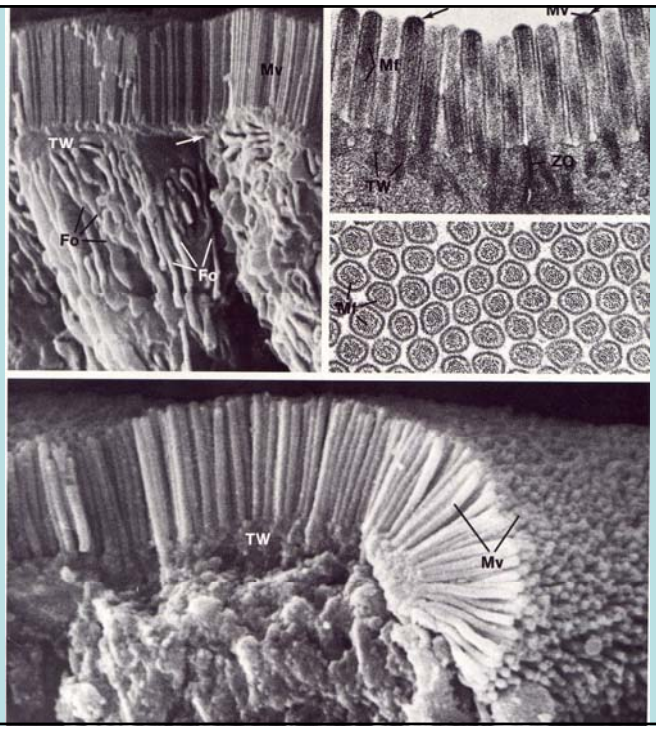


Sputter Ion Coater- Au

Evaporator --C

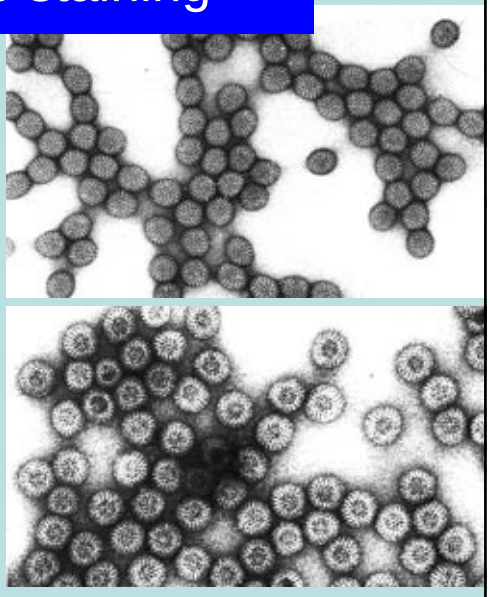






Negative staining

- A negative staining technique uses heavy metal salts to enhance the contrast between the background and the virion's image.



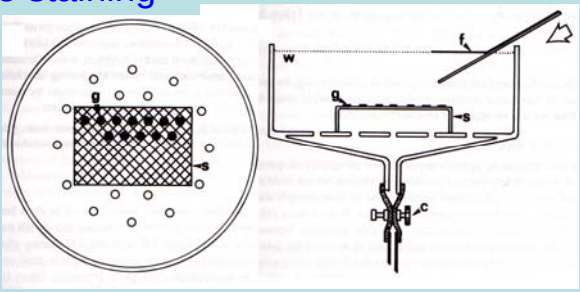
Negative stain of rotavirus

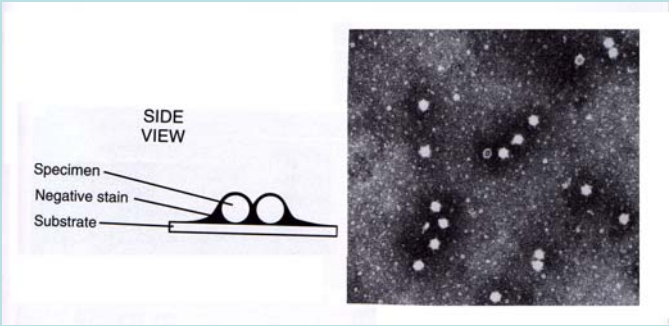
Negative staining

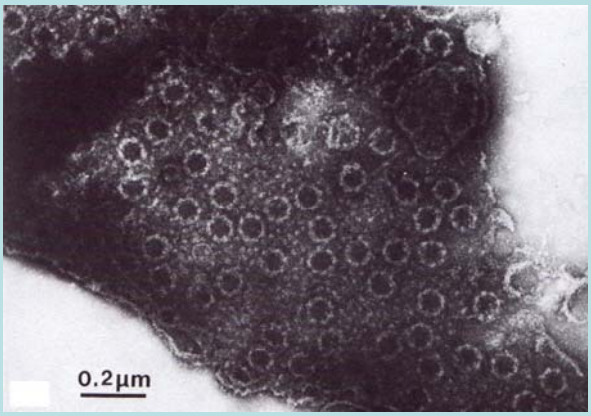
TABLE 5.1 Some Commonly Used Negative Stains

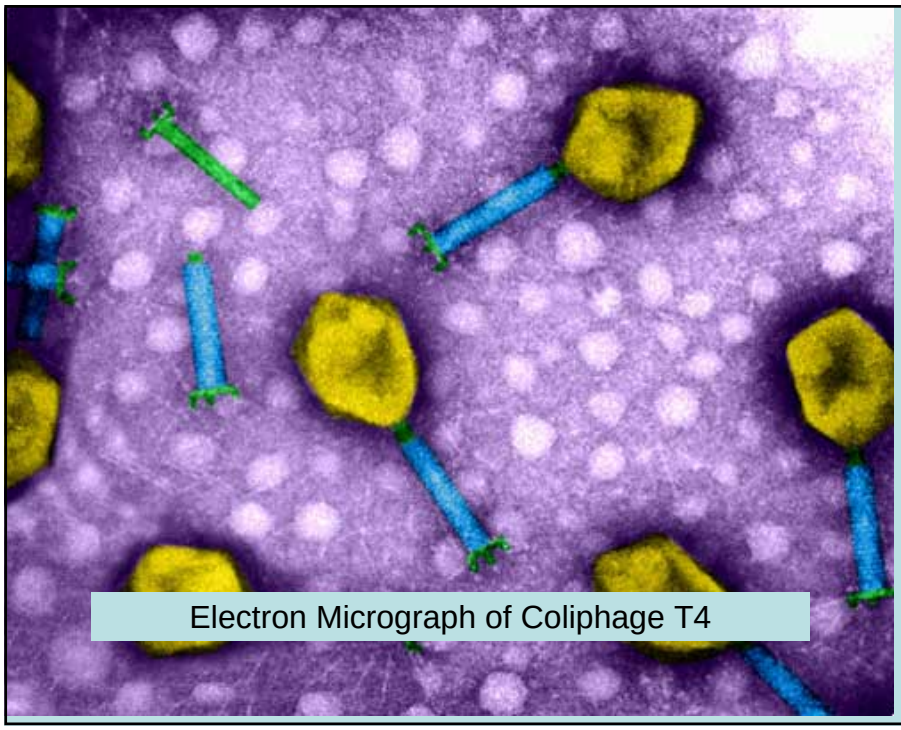
Salt	Preparation	Uses	Reference
Ammonium molybdate	1–3% aqueous	Membranes, enzyme subunits, cell fractions	Muscatello & Horne, 1968
Phosphotungstic acid (K-PTA)	0.5–2% aqueous, adjust pH to 5.5–8.0 with 1 M KOH	Viruses, bacteria, cell fractions, frozen sections, macromolecules (DNA, actin, enzymes, etc.)	Valentine & Horne, 1962; Horne, 1967
Uranyl acetate	0.5–2% aqueous	Same as above	Van Bruggen et al., 1960
Uranyl magnesium acetate	1% aqueous	Same as above	Valentine & Horne, 1962; Horne, 1967
Uranyl oxylate	12 mM uranyl oxylate + 12 mM oxalic acid. Mix equal parts and titrate to pH 6.5–6.8 with ammonium hydroxide	Small macromolecules	Mellama et al., 1967
Uranyl formate	0.5–2% aqueous solution adjust pH to 4.5–5.2 with ammonium hydroxide	Same as above	Leberman, 1965

1. Negative staining





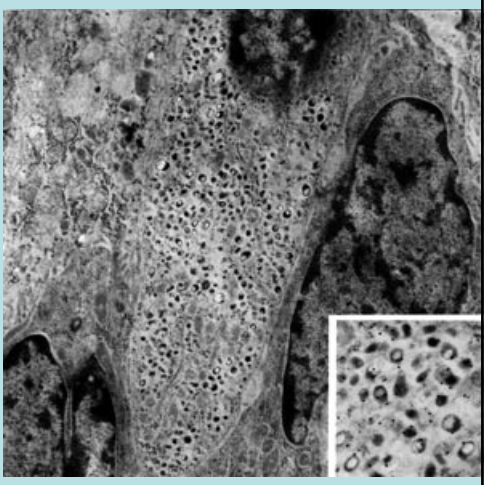




Electron Micrograph of Coliphage T4

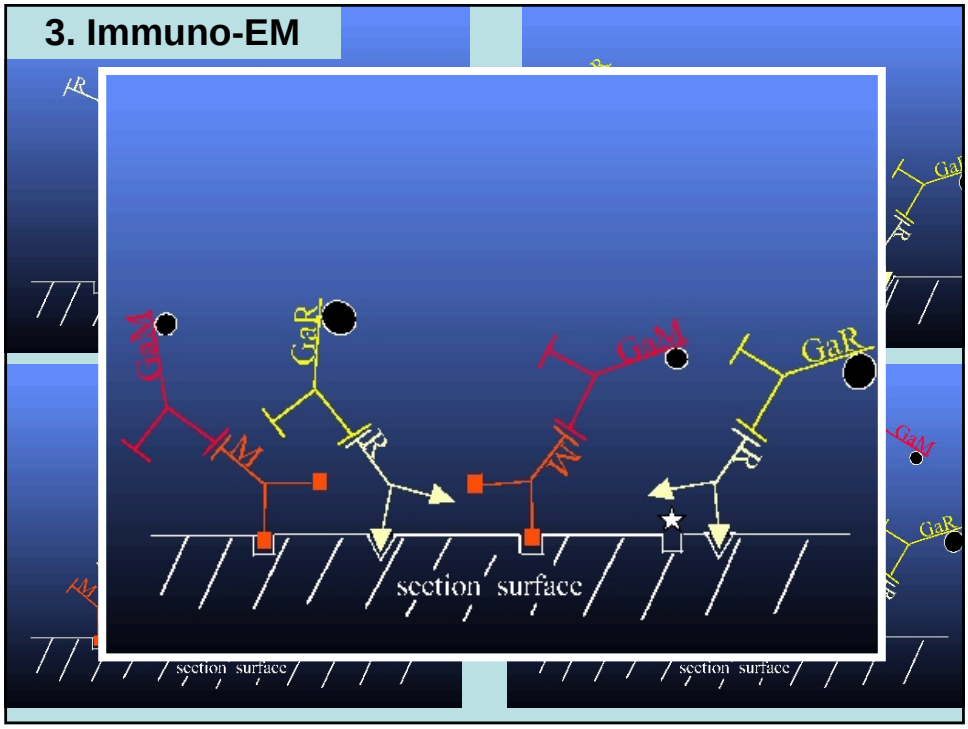
Immunoelectron microscopy

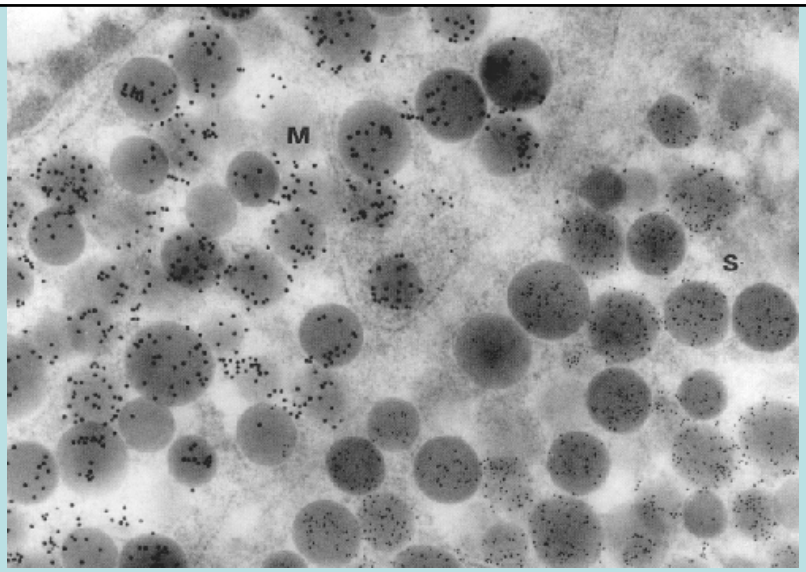
- This technique allows the investigator to identify antibody/antigen complexes that localize to a particular subcellular organelle or compartment by using the Protein A gold technique.



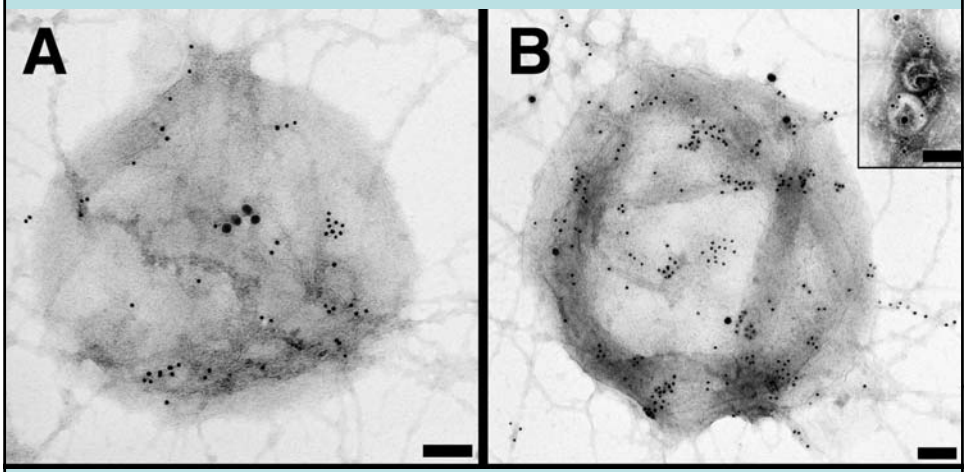
Protein A gold labeling of a prostatic endocrine-paracrine cell demonstrating localization of calcitonin (inset) to the neuroscretory granules.

3. Immuno-EM





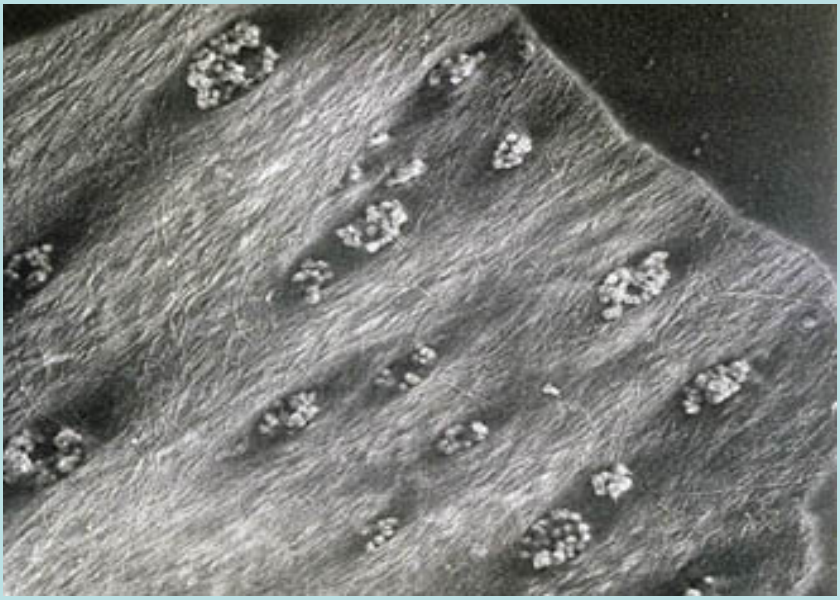
The portion of the cell on the left (M) is a 'mammotroph' cell; note the presence of the larger gold particle size over the prolactin-containing secretory granules. The other cell profile (S) is a 'somatotroph' cell; note that the growth hormone-containing secretory granules are labelled with the smaller gold particles.



Vesicle fraction from squid axoplasmic cells were subjected to gold-bead-conjugated kinesin or myosin V proteins. Small beads are kinesin, larger beads are myosinV

Rotary shadowed replicas

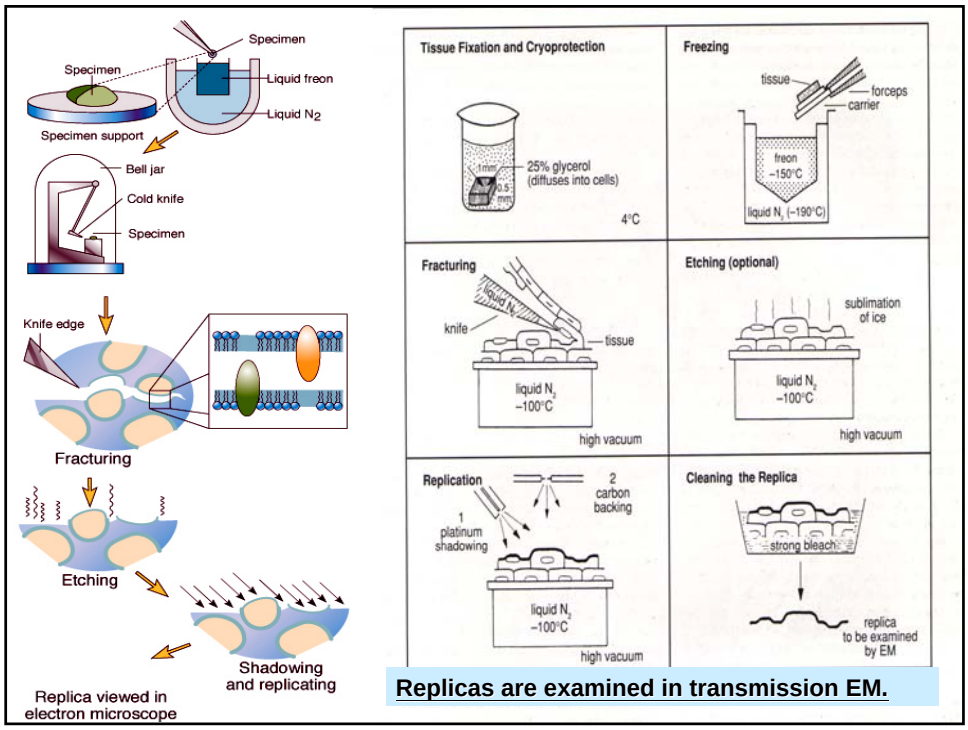
- Rotary shadowed replicas allow surface detail to be imaged at high resolution. The sample is dried and then a metal such as platinum is evaporated onto the surface in a vacuum. The source of the metal lies at an angle to the sample surface, so the thickness of the accumulated metal varies with the surface topography. This produces a 3-d effect when the metal replica is viewed in the TEM.



Rotary shadowed replicas

Freeze-fracture

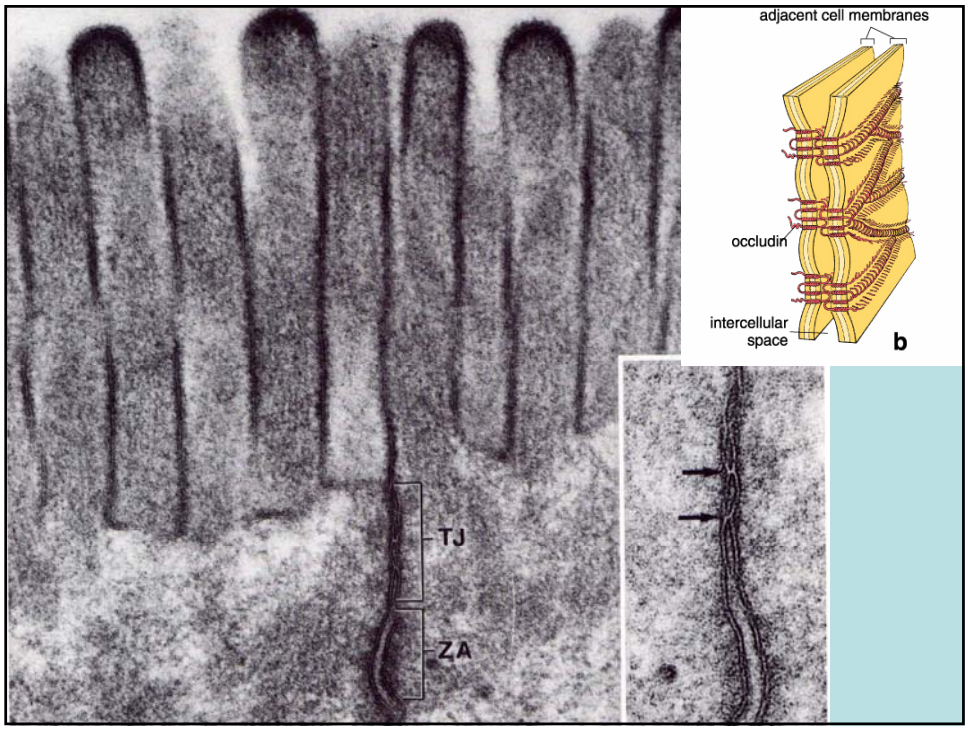
- Freeze-fracture reveals intracellular details in 3-d. Samples are frozen rapidly in liquid nitrogen and fractured to reveal internal structure. The fracture surface is etched under vacuum and shadowed with metal. The resulting replica contains fine morphological detail and is particularly useful for studies of lipid bilayers.



Replicas are examined in transmission EM.


P
E

轉180度


adjacent cell membranes
occludin
intercellular space
b
TJ
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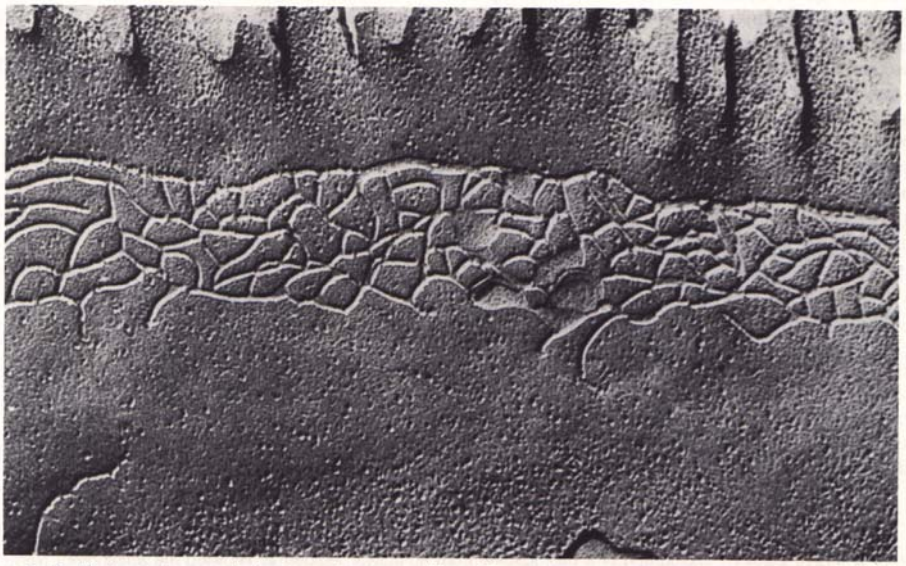
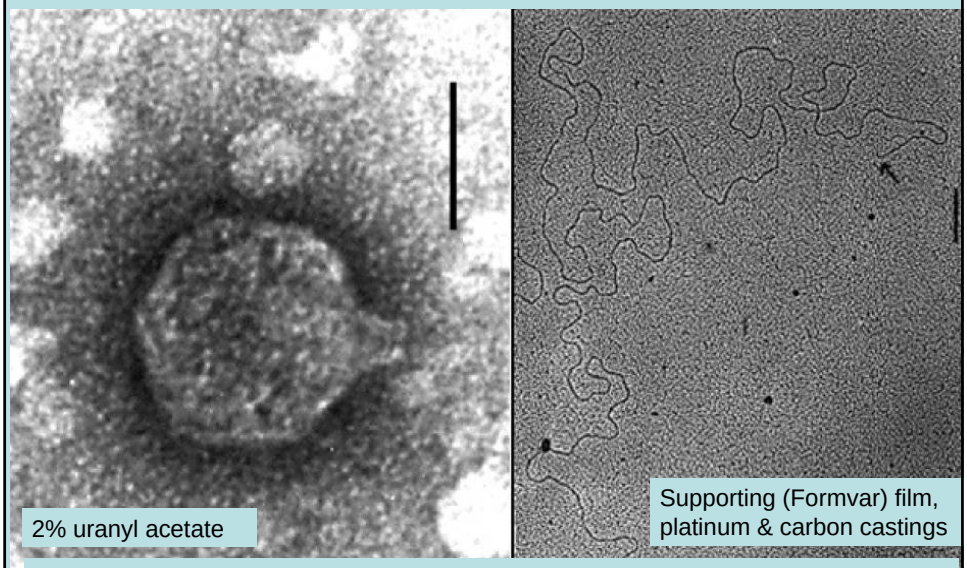


igure 2–10. Electron micrograph of a freeze-fracture replica of the zonula occludens of intestinal epithelium. A reticular

轉180度

Negative staining

2. DNA spread and Shadow-casting



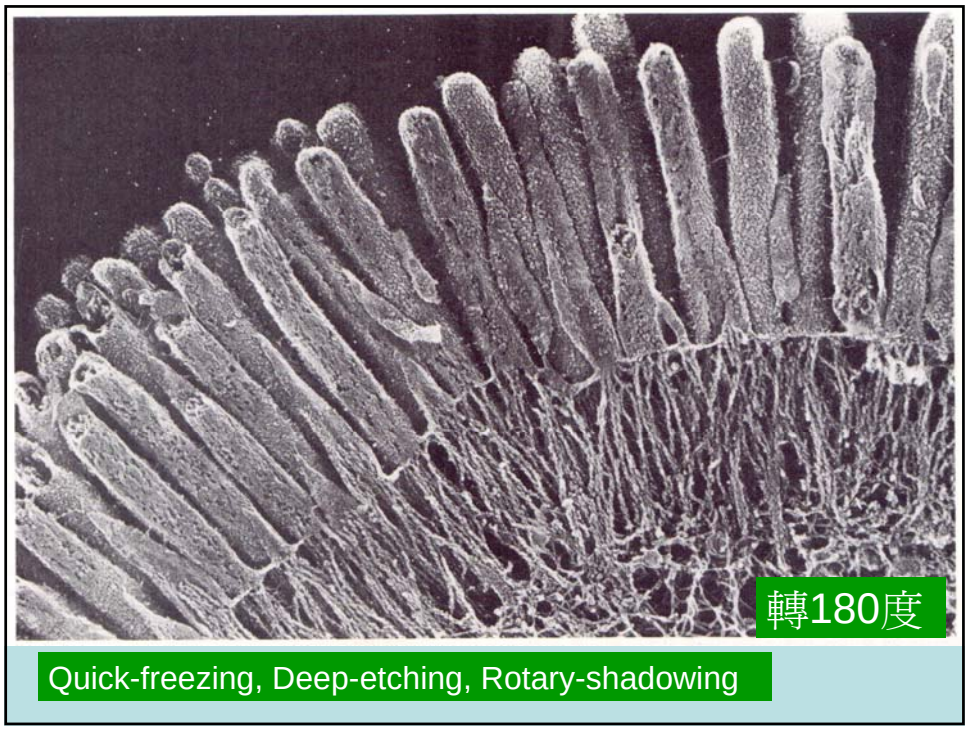
Electron microscopic analysis of vibriophage N5 virion morphology (2% uranyl acetate) and DNA structure (DNA mounted on the grid by Kleinschmidt's technique).

Quick-freeze, deep-etch

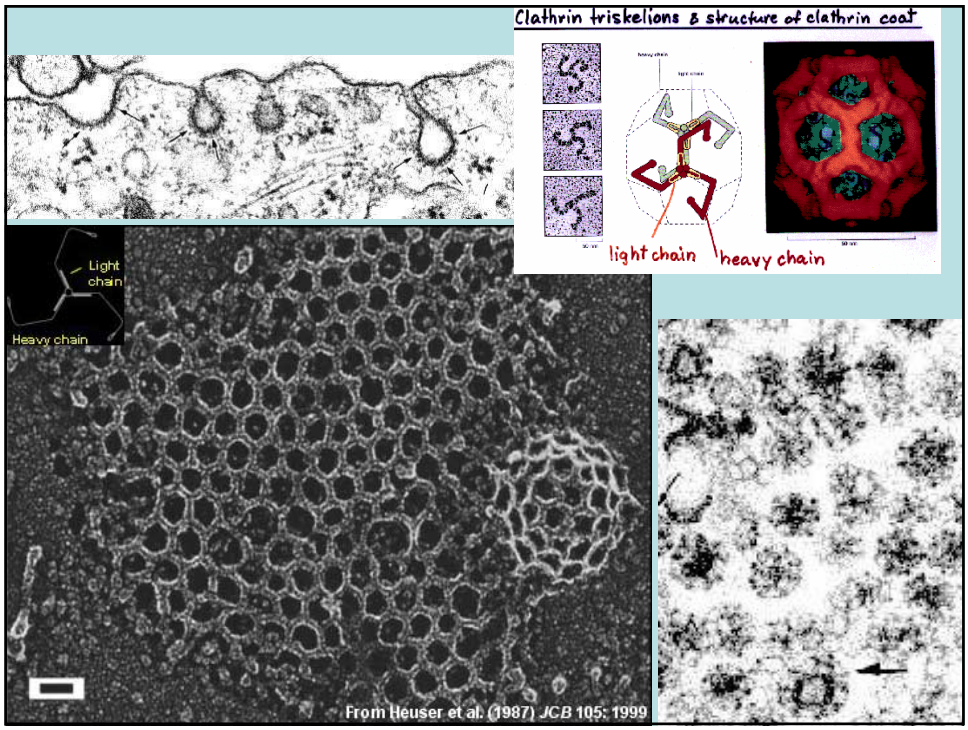
- Quick-freeze, deep-etch preparation for rotary shadowing. Samples are rapidly frozen in liquid nitrogen and transferred to a vacuum device in which surface ice is sublimed. This effectively etches the sample surface, revealing the preserved 3-d structure of the hydrated material. A rotary shadowed surface replica can then be obtained.

Shadow-casting

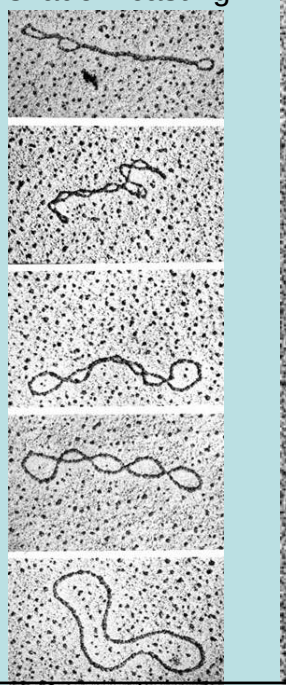
- Freeze-fracture
  - Unidirectional shadowing (↙ 45° Pt, ↓ 90° C)
  - Etching is optional
- Shadow-casting – in cells or tissues
  - Freeze-fracture,
  - deep-etching and
  - rotary shadowing – (all directions) – for cells, tissues & **macromolecules,**

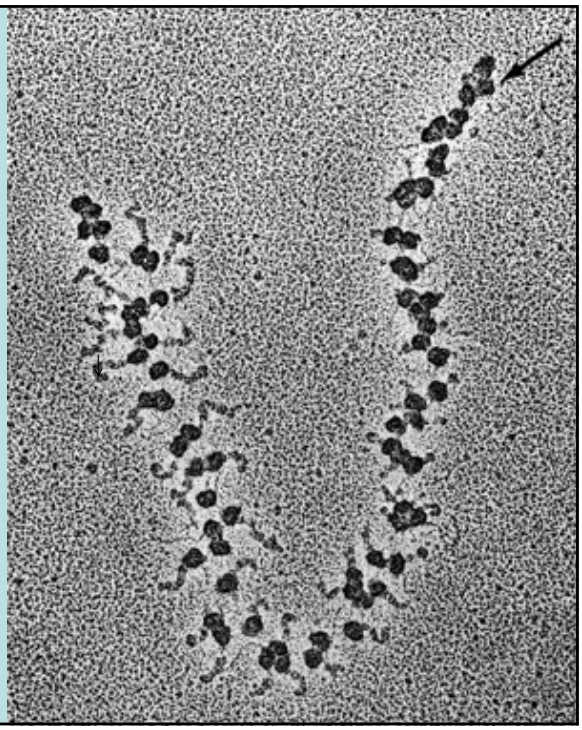


Quick-freezing, Deep-etching, Rotary-shadowing



Simple shadow-casting

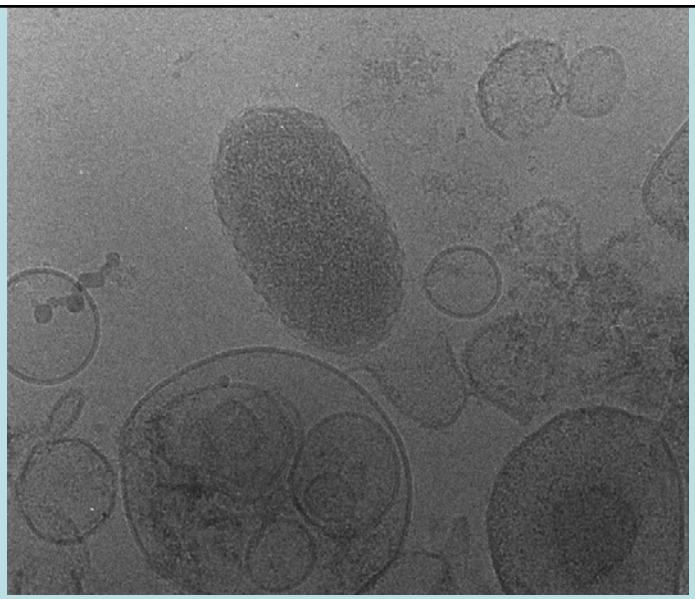




Cryo-EM

- Quick freeze,
- Cryo-ultracut
  - Thin section at freezing temperature (-196^{o}C)
  - Pick up on a formvar-coated copper grid
  - Metal-contrast staining or immunostaining
- Examine in an EM

1. Cryo-EM



This picture shows an animal virus called ORF, imaged by cryo-EM

Cyro-EM

1. Cryo-Electron Microscopy (cryo-EM) is a technique for getting 3D structures of biological molecules from an electron microscope.
2. Single-particle cryo-EM gathers many images of the same particle (molecule or molecular complex) in various orientations, and then uses something like computed tomography to reconstruct the 3D structure.
3. Cryo-EM routinely determines structures to about 15 Angstrom resolution, but with enough images (100,000 or more) resolutions of 7 or 8 Angstroms should be attainable.
4. So a key problem is automatically finding the particle images inside the micrographs.

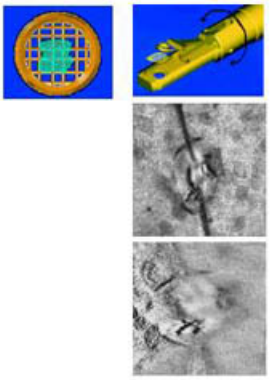
Cryo-EM (3D EM – electron tomography)

- The 3D structure of an object in its entirity is derived from a series of 2D images recorded of an objected tilted over various tilt angles. The data collection is tedious and involves **tilting** of the specimen in the electron microscope, correcting for **lateral shift** and **change of defocus**, followed by taking an **image.** This cycle is repeated, typically, over +/- 70 degrees, with 2 degree tilt increments.

5. 3D EM – electron tomography

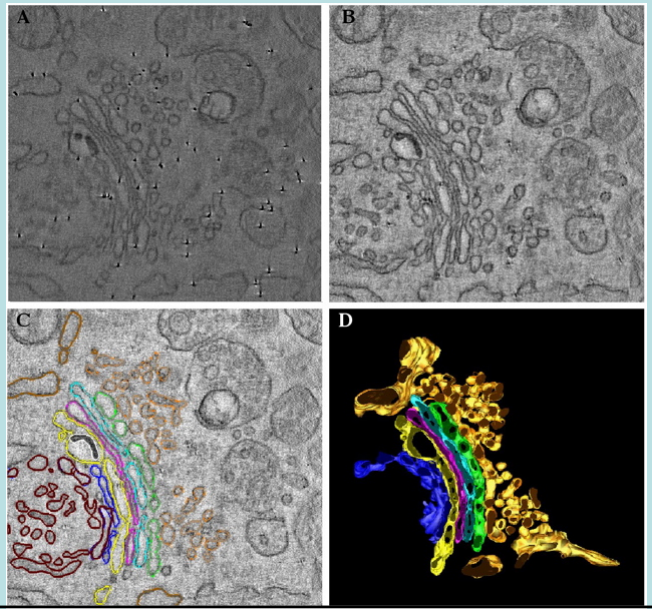
Basic steps electron tomography

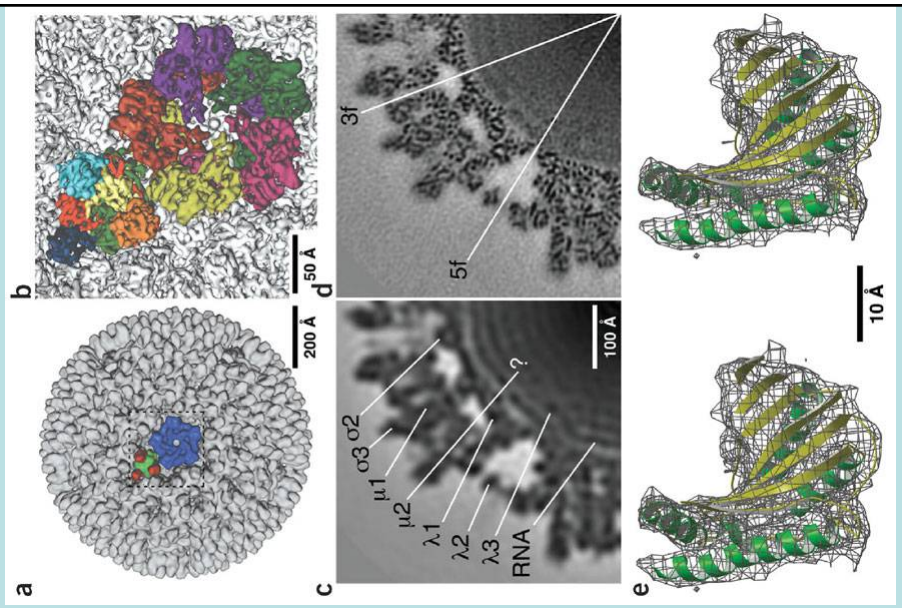
- Specimen preparation
  - Chem. fix or fast freezing
  - Plastic embedding
  - Semi-thin section (250 nm)
- Microscopy
  - Acquisition of tilt series (2D images)
- Image Processing
  - Alignment of the series of 2D images
  - Reconstruction of a 3D volume
  - Visualization, interpretation, modeling



resolution = pi * thickness of sample / number of projections.

Cellular Compartments





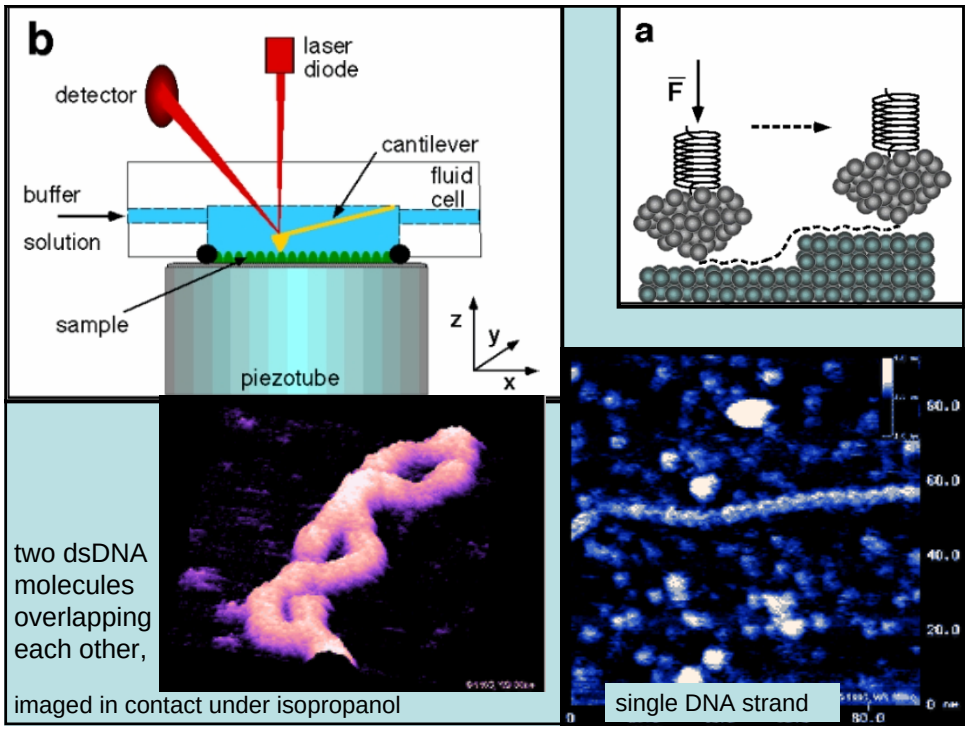
Nature Structural Biology **10**, 1011 - 1018 (2003)
Reovirus polymerase 3 localized by cryo-electron microscopy of virions at a resolution of 7.6 Å. By:Xing Zhang et al.,

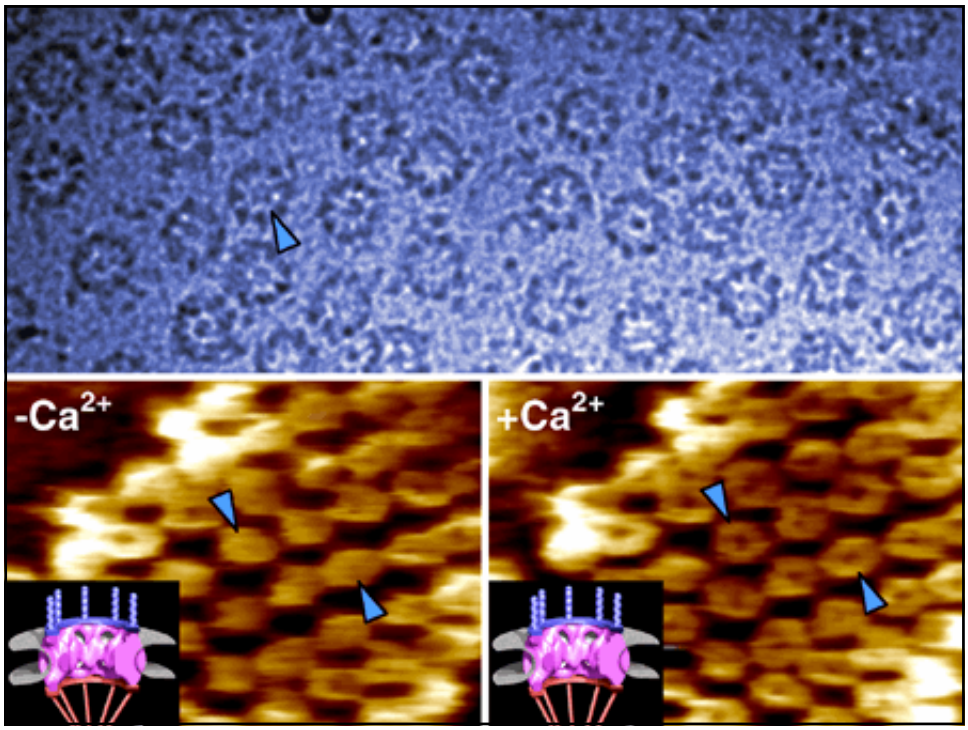
Atomic force microscope

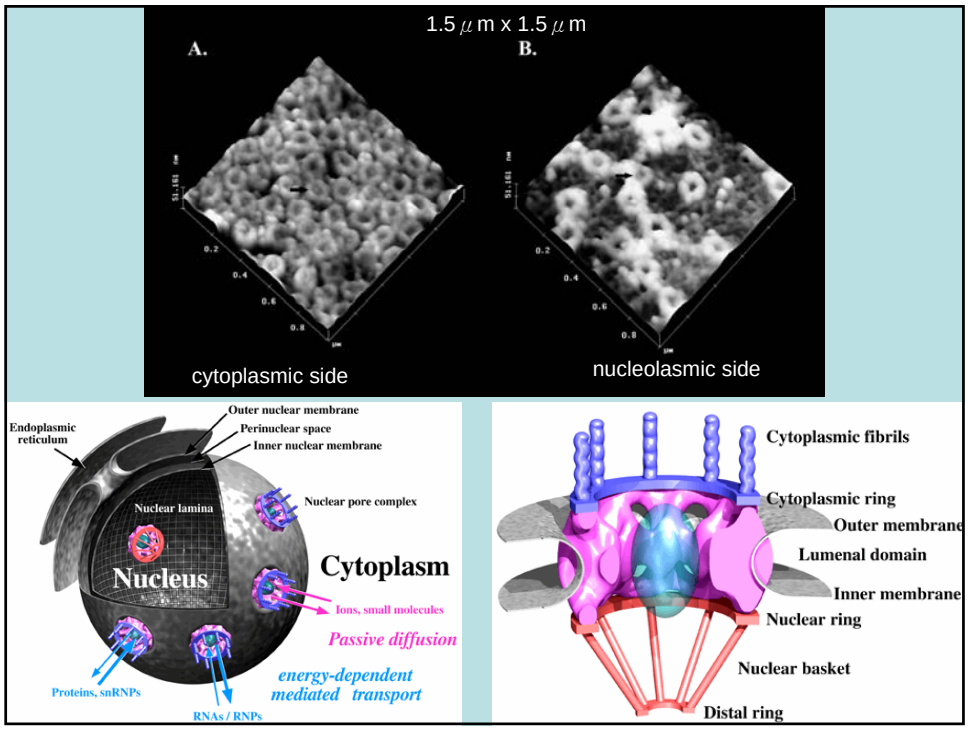
- The **atomic force microscope** (AFM) is one of the **most powerful tools** for determining the **surface topography** of **native biomolecules** at **subnanometer resolution**

- the AFM allows biomolecules to be imaged not only under **physiological conditions**, but also while **biological processes** are **at work**.

Because of the **high signal-to-noise** (S/N) **ratio**, the detailed topological information is not restricted to crystalline specimens. Hence single biomolecules without inherent symmetry can be **directly monitored** in their **native environment**




-Ca2+
+Ca2+


1.5 μm x 1.5 μm
A.
B.
cytoplasmic side
nucleolasmic side
Endoplasmic reticulum
Outer nuclear membrane
Perinuclear space
Inner nuclear membrane
Nuclear lamina
Nuclear pore complex
Nucleus
Cytoplasm
Ions, small molecules
Passive diffusion
energy-dependent mediated transport
Proteins, snRNPs
RNAs / RNPs
Cytoplasmic fibrils
Cytoplasmic ring
Outer membrane
Lumenal domain
Inner membrane
Nuclear ring
Nuclear basket
Distal ring

SUMMARY

- **電子顯微鏡之構造與基本原理 (TEM及SEM)**
  - Resolution, components of EM, accelerating voltage,
  - Transmitted electons (TEM), secondary electrons (SEM)
  - Rotary pump & pirani guage (10^{-1} atm) , diffusion pump & penning guage (10^{-4} atm)
  - Tungsten filaments, LaB_6, cold field emission,
- **電子顯微鏡標本之基本製作 (TEM切片及SEM標本)**
  - Thin sections (TEM) double staining, negative staining, spreading (virus particle)
  - Critical point dryer (CO_2), ion coater,
- **電子顯微鏡術在細胞分子生物學之應用**
  - Immuno EM – colloid gold labeled-antibody
  - Freeze-fracture, intramembranous particle (integral protein),
- Cryo-Electron Microscopy -
  - spicemen tilt, lateral shift, change focus, image, image analysis
- 原子力顯微鏡 AFM (atomic force microscopy)