Other TEM Imaging Modes

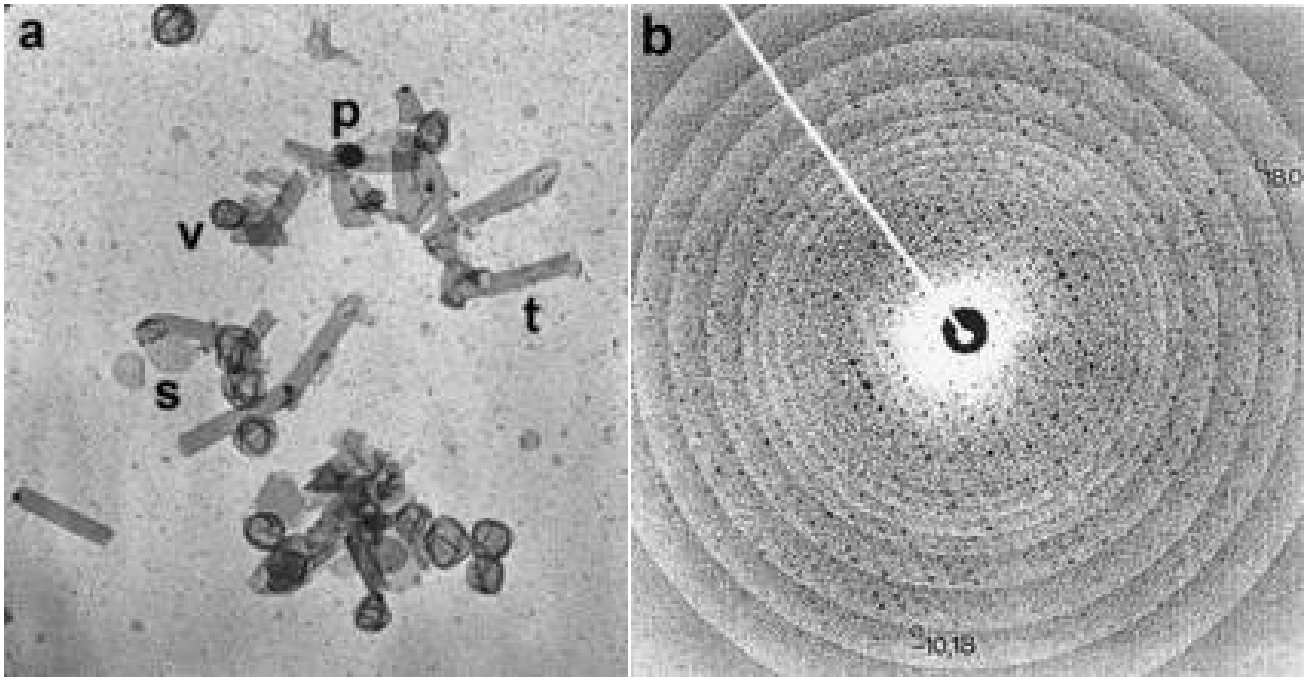
- **Electron diffraction**
- **X-ray microanalysis**
- **Energy loss spectroscopy**
- **Dark field microscopy**
- **Tilting & stereo microscopy**

Electron Diffraction (ED)

- Electron diffraction (ED) – used to identify crystalline substances - based on atomic plane spacing within the structure.
- Used to determine orientation relationships between crystalline phases in different materials (precipitates, coatings, etc.)
- Used to analyze defects in solids.
- Analysis can use a region as small as 5 nm.
- Used primarily in materials science as metals produce strong e^- diffraction patterns
- Can be used in biology if you have a crystalline substance. Biological specimens diffract weakly.

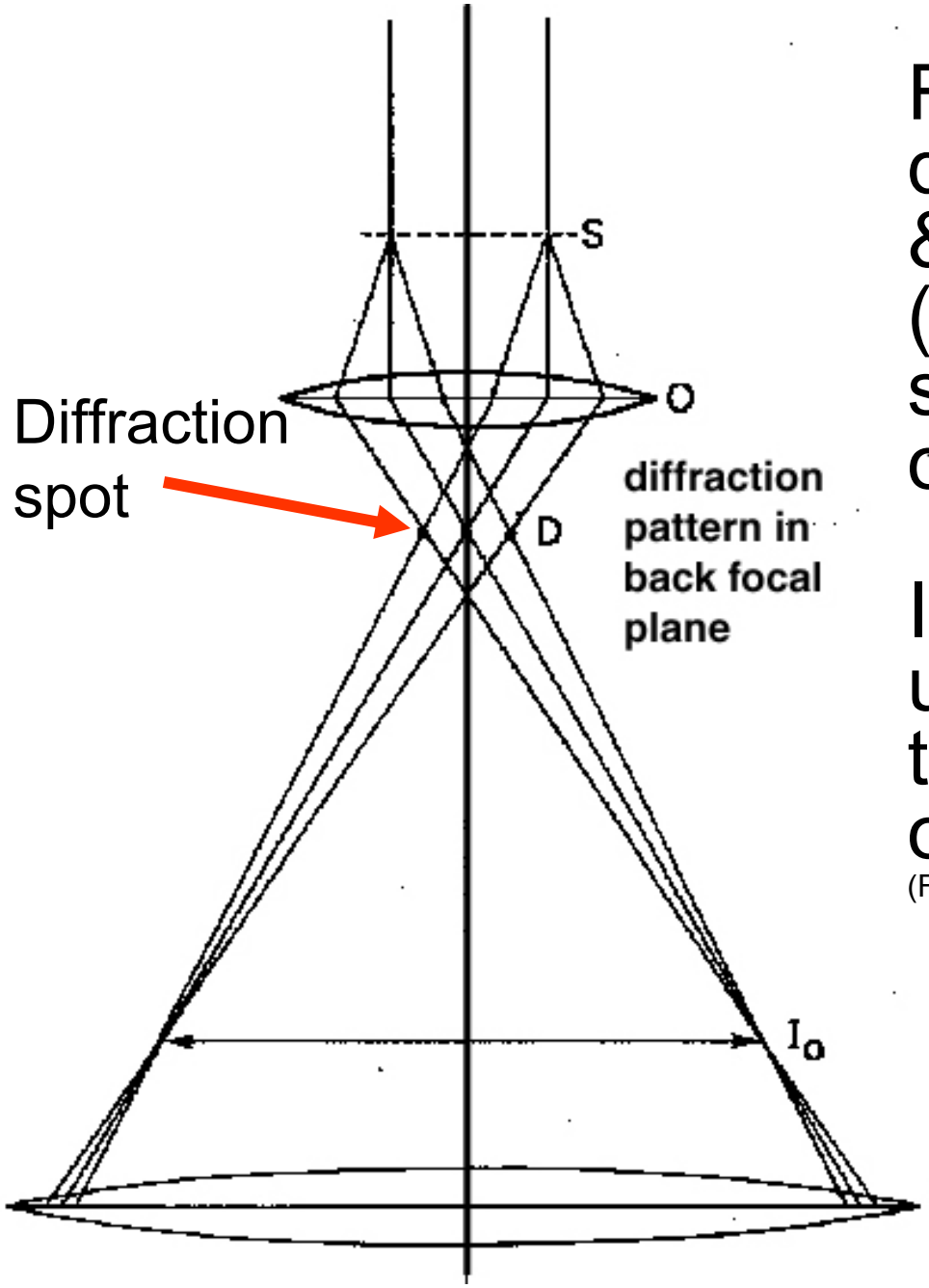
Rhodopsin, purified from bovine rod outer segments. Electron diffraction patterns of single layers (Fig. 1b) taken at 120 kV using a Phillips CM12 electron microscope and a large area, cooled CCD detector. All work was carried out at liquid nitrogen temperature.

Gebhardt Schertler. http://www2.mrc-lmb.cam.ac.uk/groups/GS/rhodopsin.html



Electron Diffraction

- Crystalline specimens diffract the e^- beam strongly through well-defined directions (given in angles, θ) dependent on e^- wavelength & crystal lattice spacing according to Bragg's law, $n\lambda = 2d\sin\theta$
  - n = integer
  - λ = electron wavelength
  - d = crystal lattice spacing between atomic planes
  - θ = angle of incidence & also of reflection



Formation of the diffraction pattern (D) & intermediate image (I_o) of a crystalline specimen (S) by the objective lens (O).

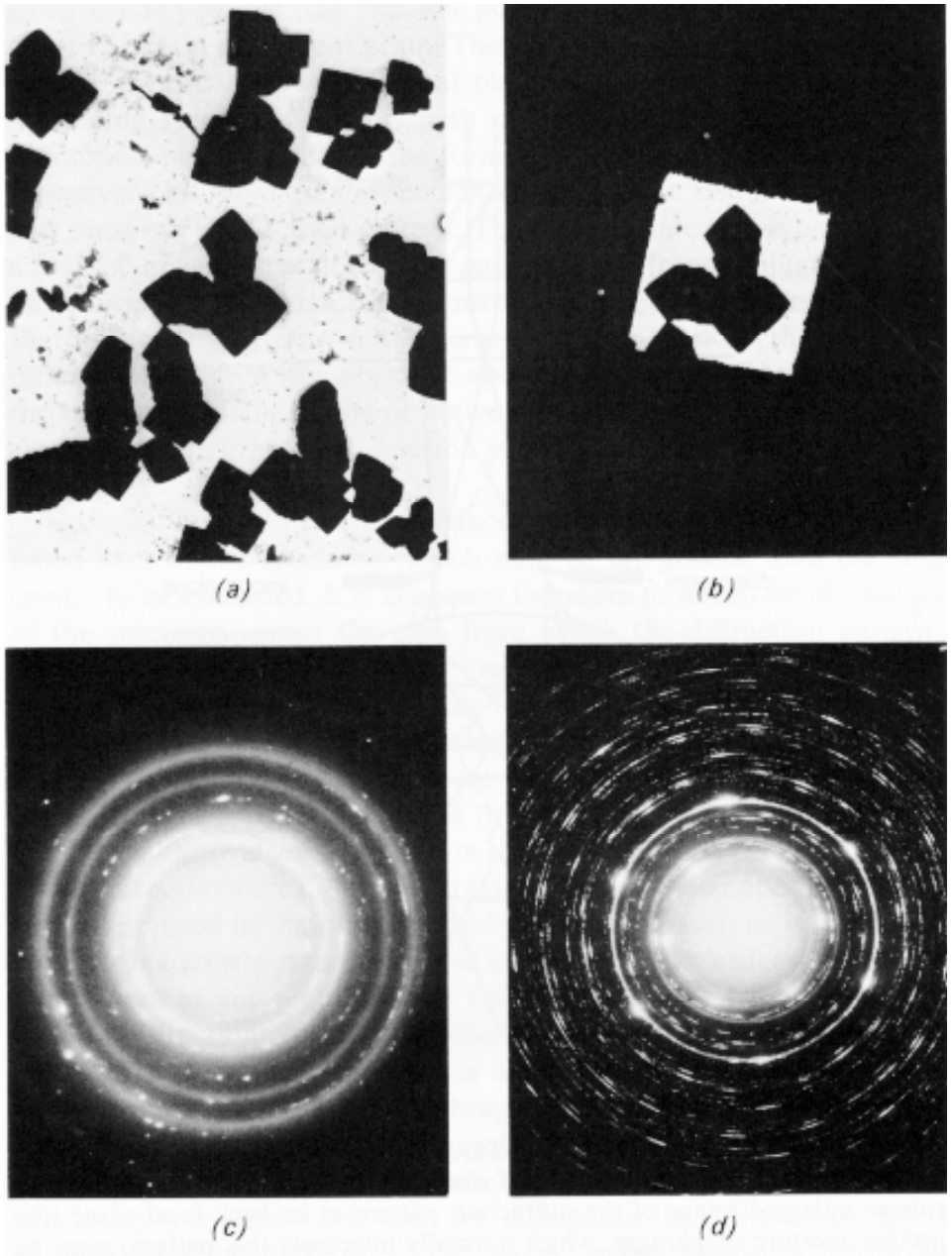
Intermediate lens (I) used to image either the intermediate image or diffraction pattern.

(From Agar, p.111)

Producing the diffraction pattern

- For optimum results - need constructive interference conditions to get a sharp diffraction patterns.
- Specimen illuminated with highly coherent beam (use a very small aperture of illumination).
- Under these conditions, all e^- striking a given spot on the specimen arrive from the approximate same direction. The objective aperture usually withdrawn while recording the pattern otherwise part of the pattern may be cut off.

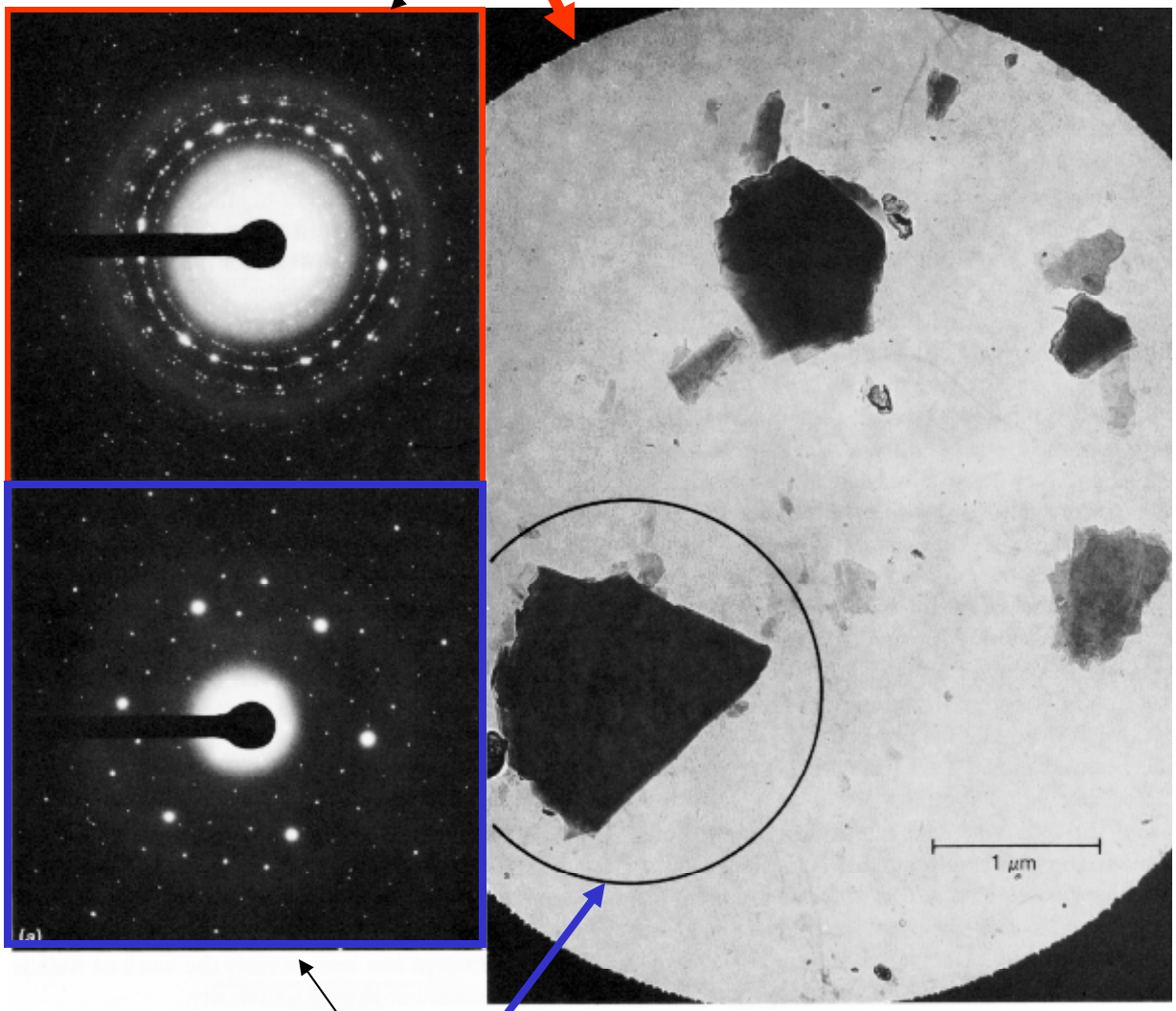
(a) crystalline material (b) area selected with area selection blades or diffraction aperture (c) source focused on the screen with the intermediate lens & condenser fully defocused & the objective aperture withdrawn. Pattern of dots & circles = diffraction pattern of the specimen. (c) Poor definition due to specimen thickness (NaCl crystals) & presence of carbon & Araldite supporting films. (d) Sharply-defined pattern unsupported aluminum oxide. (Meek 1st ed., p.288)



Selected area diffraction (s.a.d.).

Field of crystalline particles outlined by a large selection aperture (6 μm at specimen). ‘Spotty ring' type of diffraction pattern indicates crystals are oriented in different directions.

Using a smaller selecting aperture (2.3 μm at specimen) pattern formed from the chosen crystal alone. (Watt, p.124)

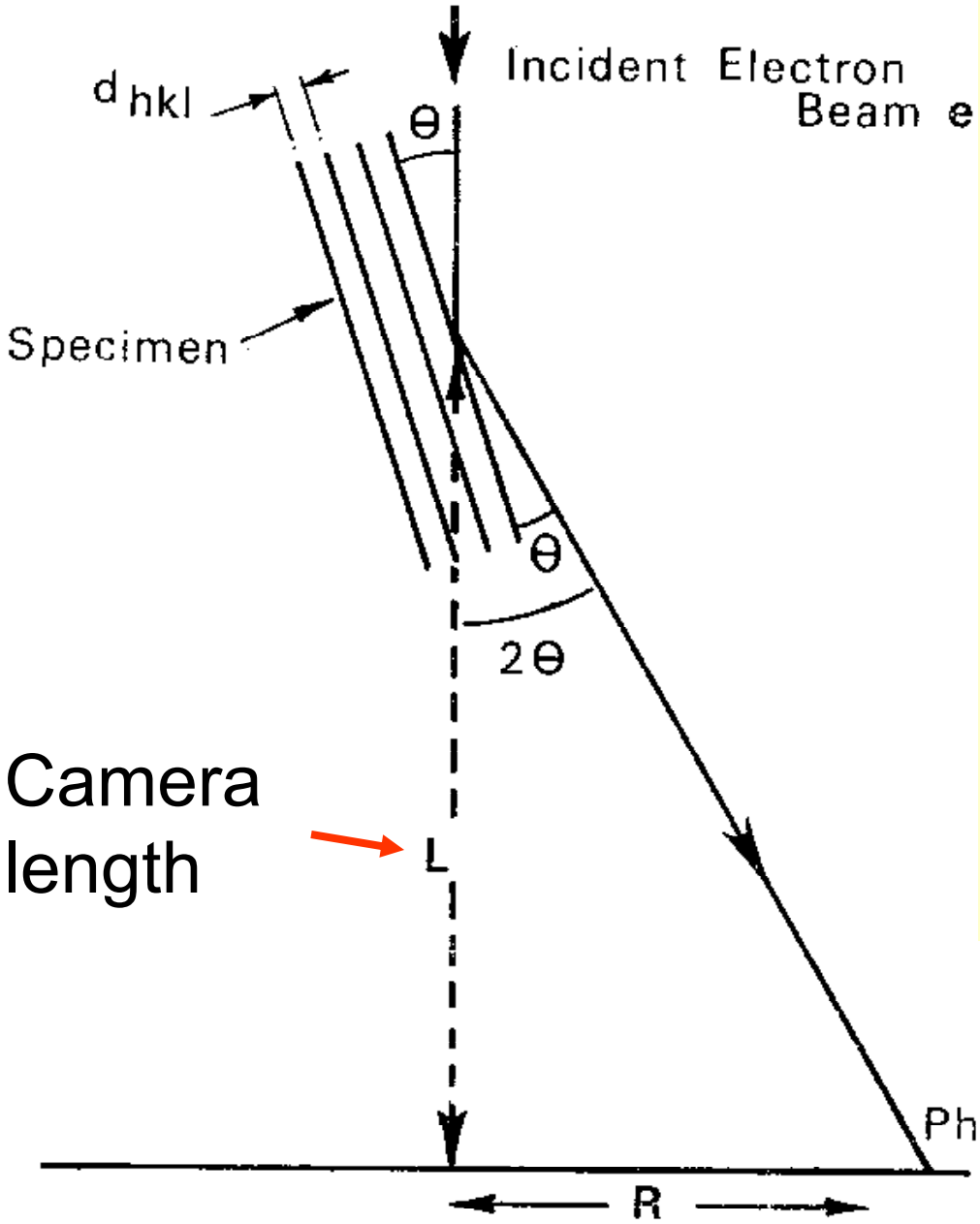


Camera length & camera constant

- Information about crystal composition can be determined from the diffraction pattern.
- Scope needs to be calibrated using a diffracting substance of known structure.
- Camera constant defined as λL, where L is the camera length (usually in mm; typically L = 300 mm.)
- When the camera constant is known, the *d* spacings in the crystal can be calculated when radius (*R)* is measured.

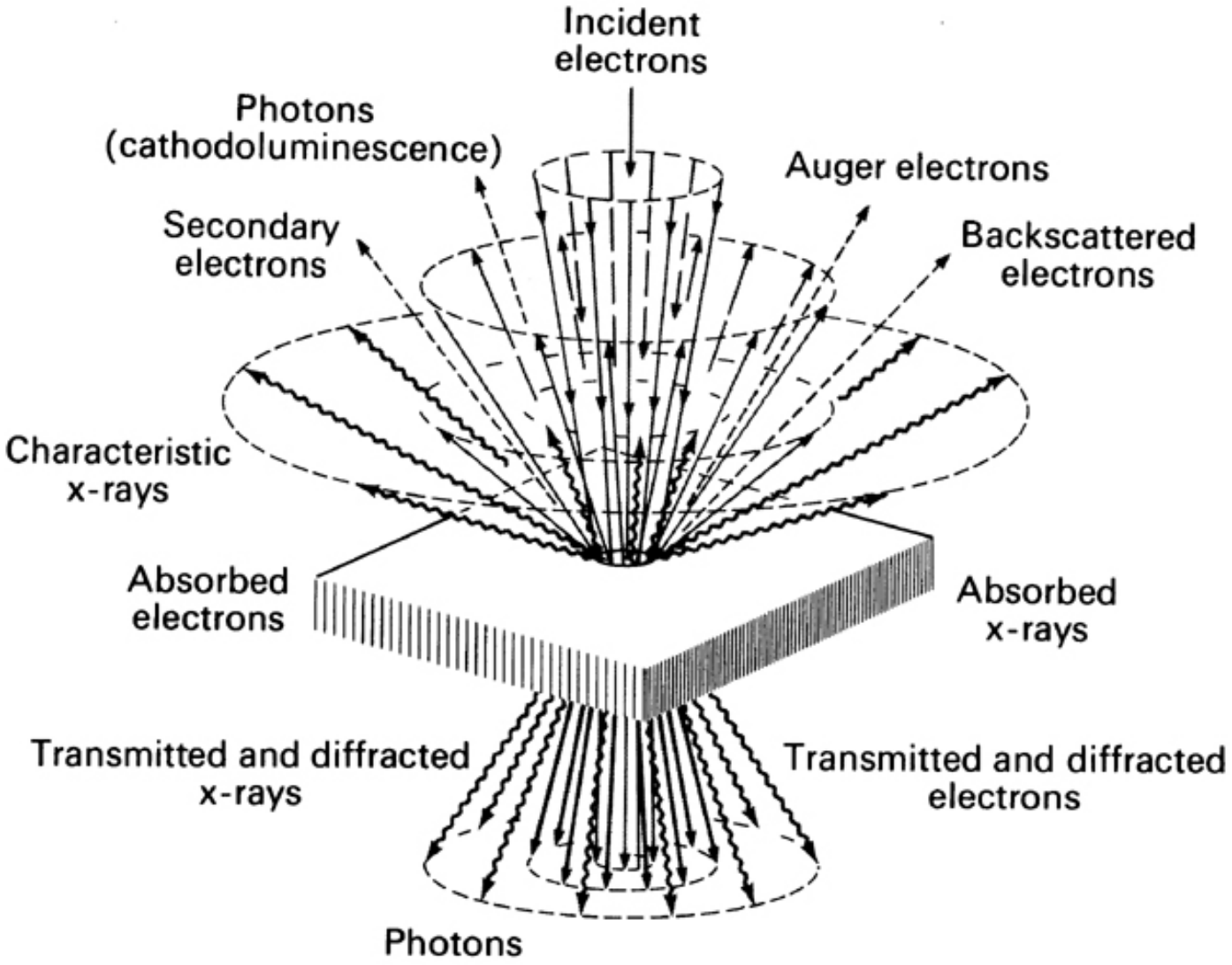
Calibration of camera length (*L*)

- If the system geometry & λ of the e^- beam are known exactly, the instrument constant, *L*, can be derived & spacings in the specimen can be calculated from measurement of the spots or rings in the diffraction pattern.
- However - in practice…

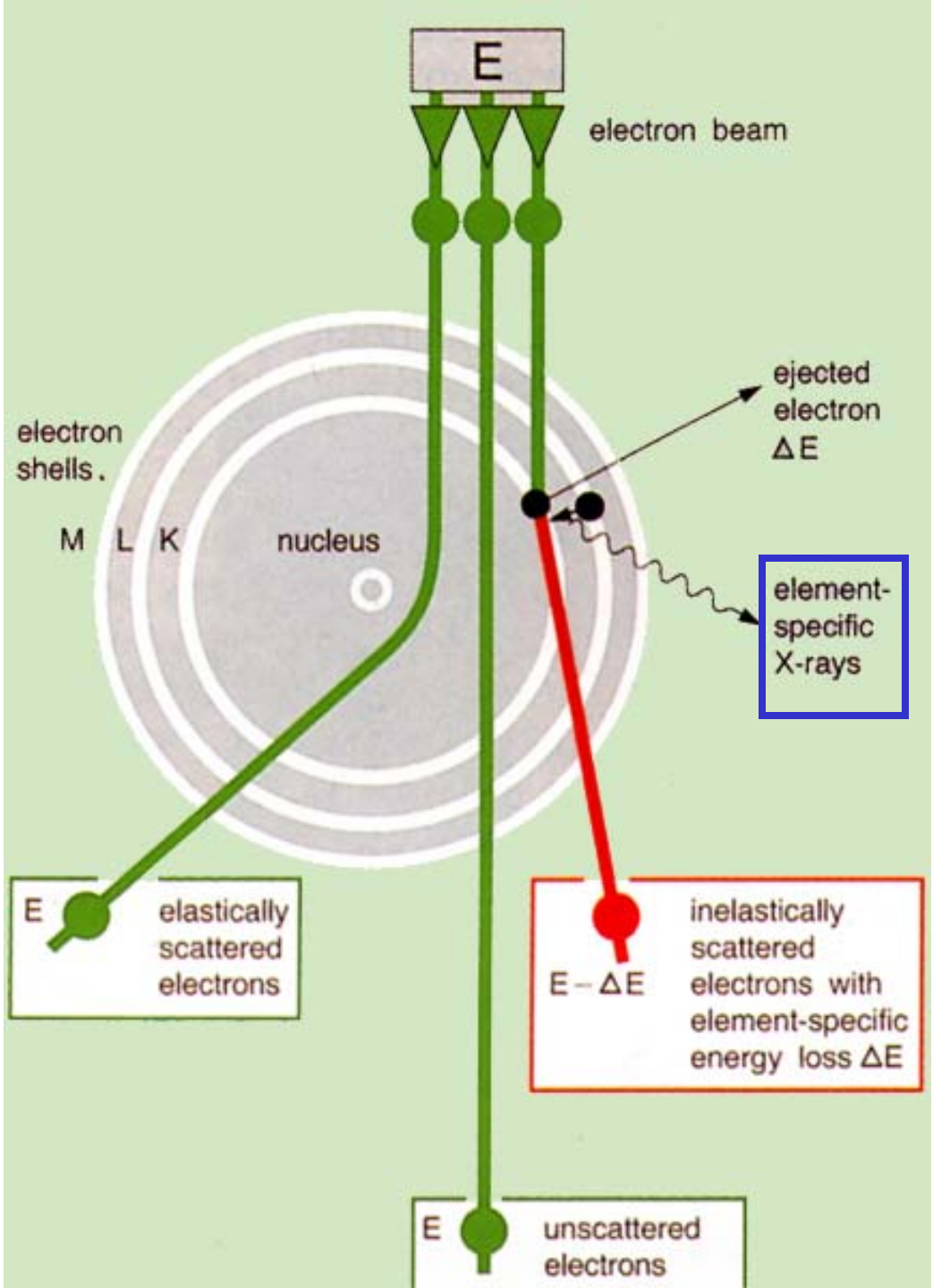


Instrument constant depends on lens currents & object position. Ideally calibration pattern should be taken at the same time as the unknown. For biological specimens -useful standard = catalase crystals

Elemental Analysis



Reaction and interaction of electron beam encountering a solid.



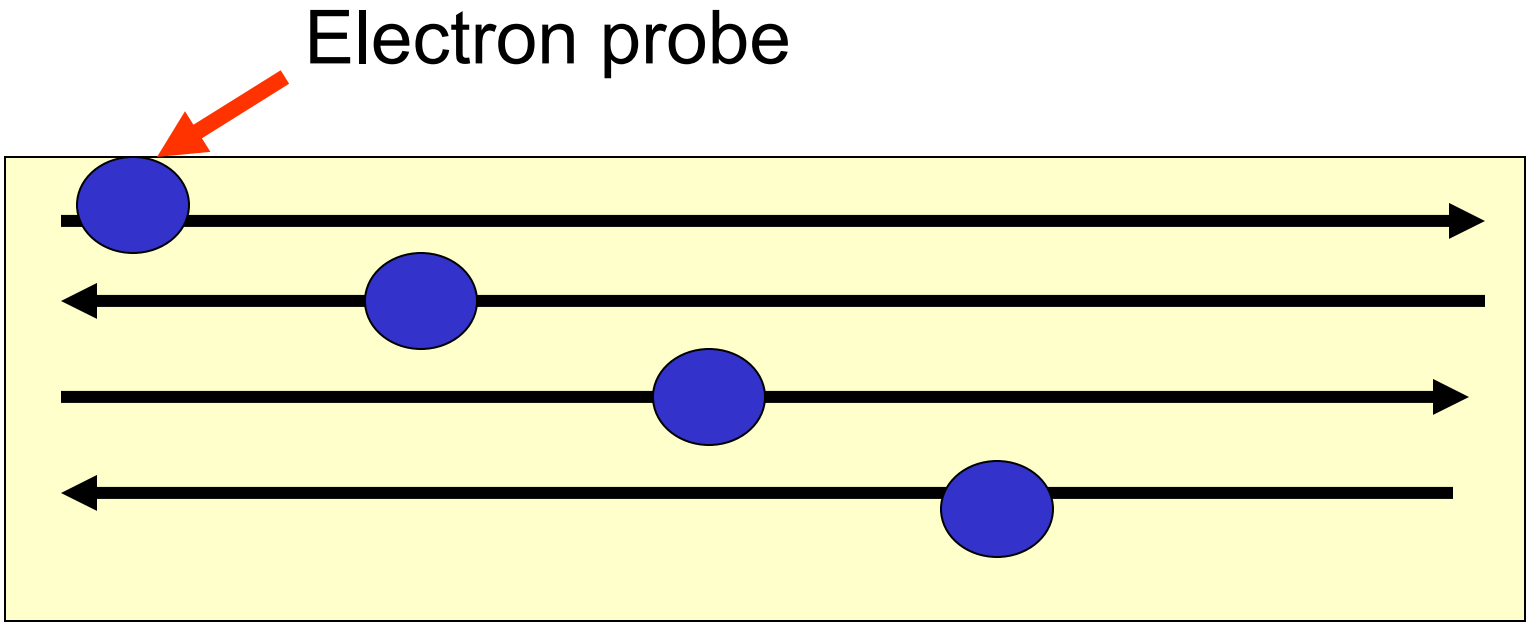
Elemental analysis based upon element specific X-ray generation or electron energy loss (EELS).

X-ray microanalysis

- Principal: When the e^- beam interacts with the specimen, e^- are boosted to a higher energy levels (orbital).
- When e^- decay back to a lower orbital they emit some of the energy as x-rays. The x-rays have a specific wavelength for each element & orbital.

X-ray microanalysis

- To analyze elements to a specific location requires the use of a scanning transmission electron microscope (STEM).
- Instead of the e^- beam illuminating the entire specimen, a small probe is used to scan back & forth across the specimen, & the image is formed line by line. (alternatively, the probe may be focused onto a small area of the specimen)



X-ray microanalysis: Possible to scan the sample with the probe, or focus the probe on a small area

Hitachi **HD-2000** STEM.

high resolution of 0.24 nm & maximum magnification of 2 million times.

X-ray microanalysis

- As the probe is scanned across the specimen surface & x-rays are generated, these are detected, & the location of different elements displayed by line scans or dot maps observed by overlaying STEM image with the x-ray map on a TV monitor (elemental mapping). Requires various software routines to select the x-ray energy levels of interest, so that only spectra of interest are displayed on the TV monitor.

X-ray microanalysis

- Specimen preparation: Biologists usually interested in lighter elements that are very mobile (Na, K, Ca). Difficult.
- Requires special preparation techniques. For light element analysis – need unfixed, quick frozen specimens. Samples cryosectioned, & frozen section transferred to a cold stage. Section maintained in the frozen state in the microscope by the cold stage.

X-ray microanalysis

- Because the amount of the element of interest may be very low in a thin section, long collection times may be necessary to collect a usable spectrum (as long as 20 minutes).
- Some usable information may be obtained from resin embedded sections for heavier elements that may be localized in an intracellular inclusion (Fe storage). Heavy metals (Pb, Cadmium)

X-ray microanalysis

- **Limits of detection** for analyzing elements from Na to U from a region of 100-200 nm diameter, range between 10^{-14} and 10^{-19} g.
- Low X-ray yield of low-Z elements of biological significance difficult to detect & quantitate.
- Due principally to electron probe lateral spread within specimen - X-rays generated from a greater volume than the probe spot size.

Electron Energy Loss Spectroscopy (EELS)

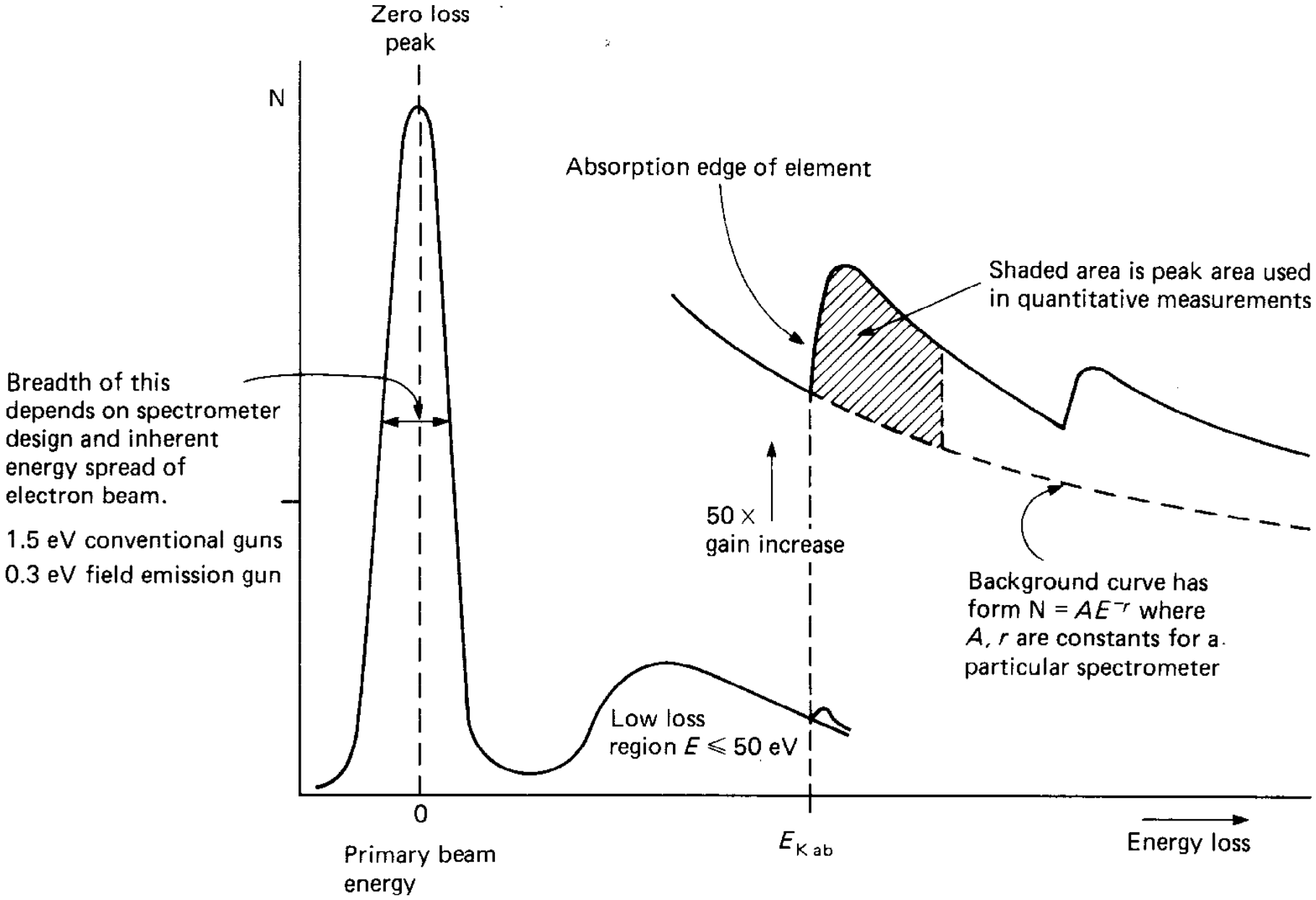
- Can be used to determine elemental composition & perform elemental mapping by selecting electrons with a specific loss energy.
- Can also be used to determine atomic bonding state & distinguish between different forms of the same element or compound

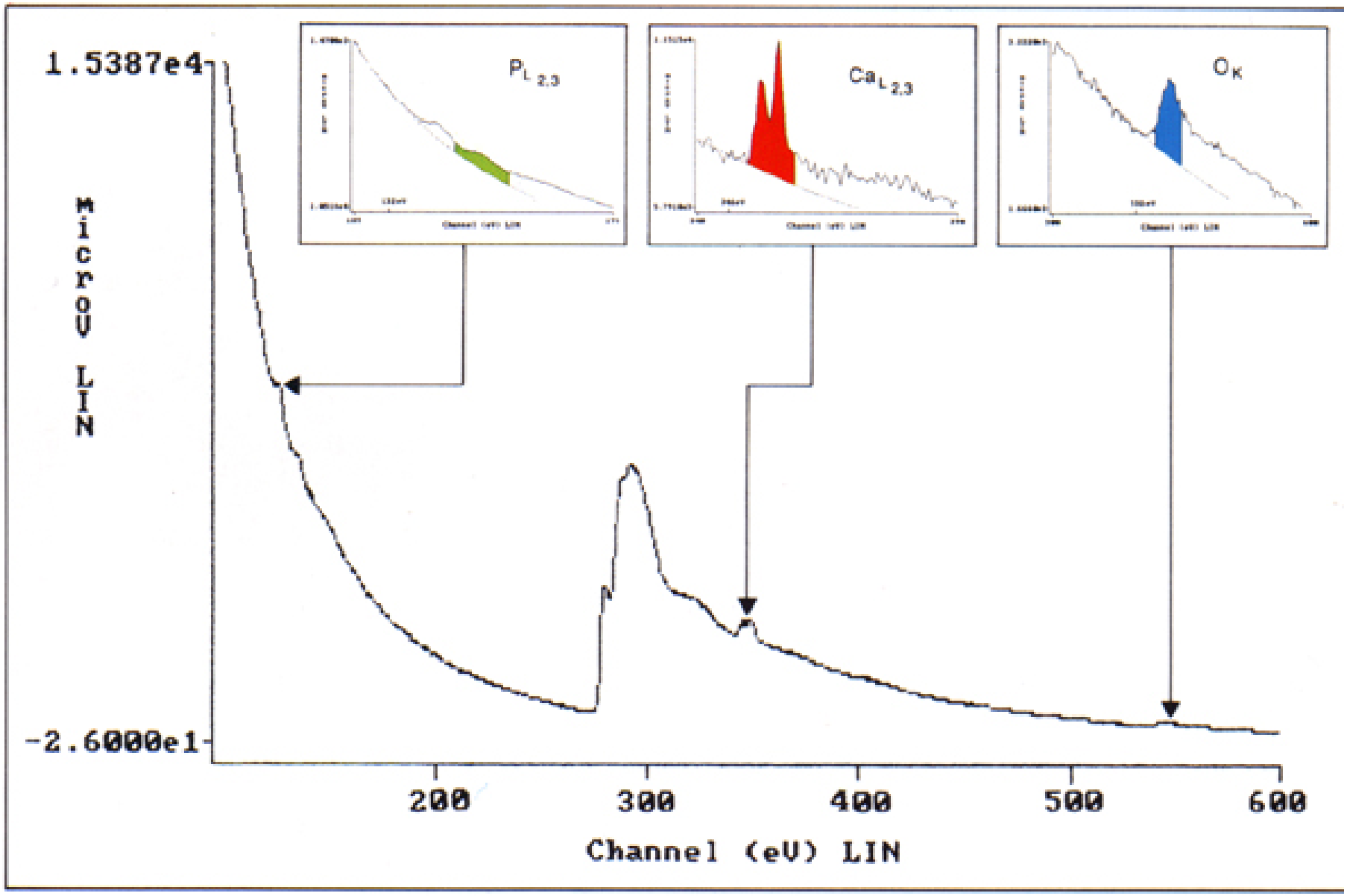
EELS

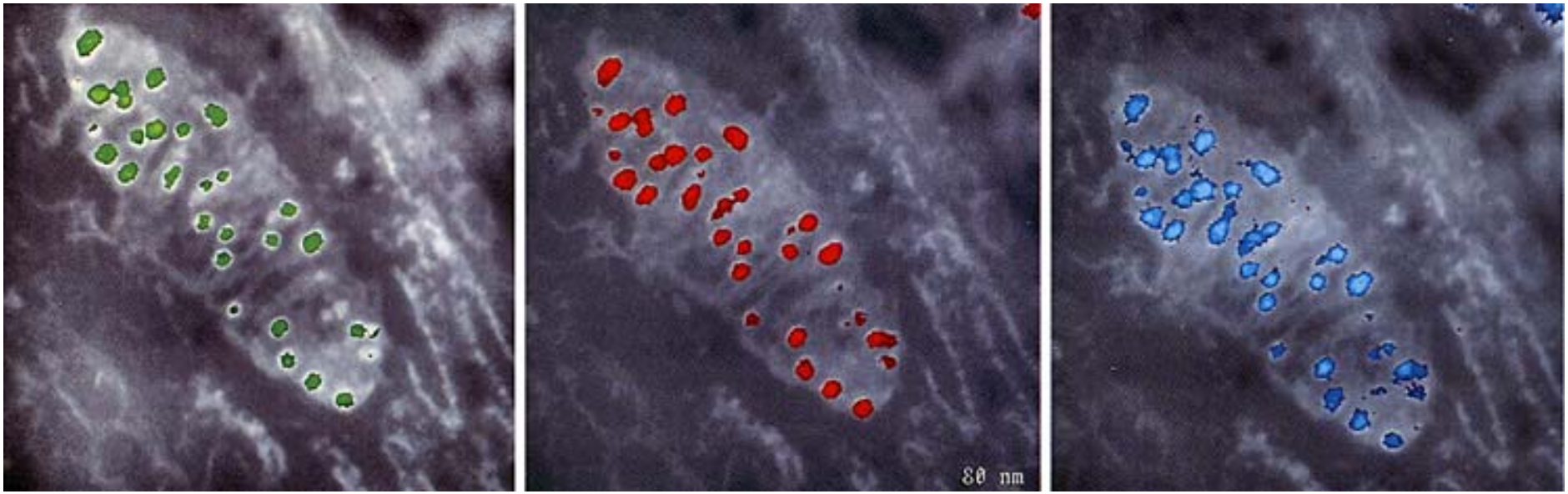
- Principle: When an e^- beam interacts with a specimen - for every characteristic K-shell x-ray photon generated, there will be an e^- which has lost the characteristic energy E_{Kab}.
- By measuring the energy losses through a localized region of the specimen it is possible to infer the specimen composition.
- EELS (Energy loss spectroscopy) - confined to relatively thin specimens (only 1 inelastic collision). ~ 30-50 nm for biological material @ 100kV.
- Most useful for light elements (e.g. Na & below).

EELS

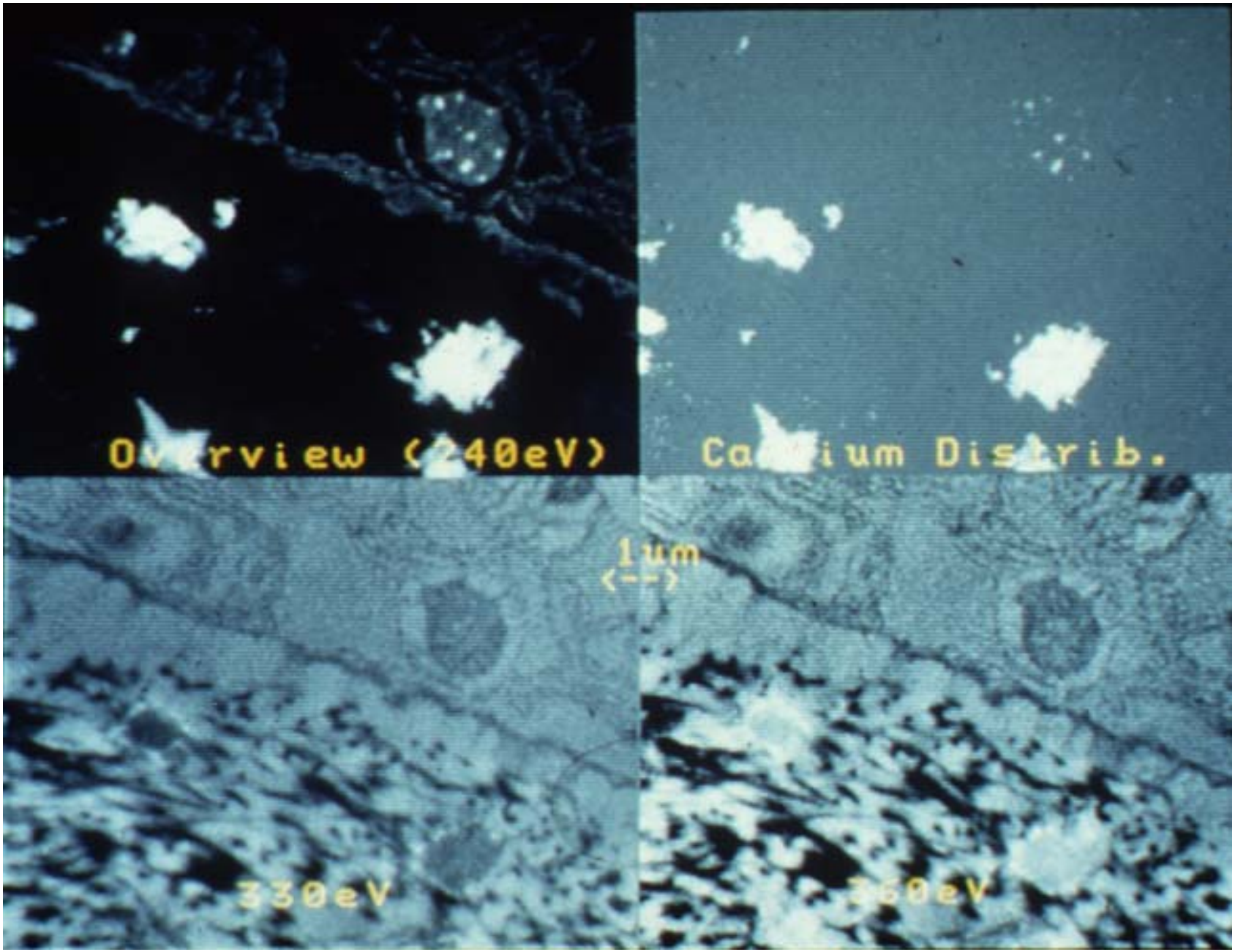
- Energy analyzer at bottom of the TEM column below the viewing screen, produces a spectrum of the energy distribution in the transmitted e^- relative to the primary beam energy.
- Most e^- in a spectrum found in the **zero loss peak** & in loss peaks involving interactions with electrons up to ~ 50eV loss.
- Beyond this a smoothly falling background has superimposed on it the ionization edges of atoms whose x-ray absorption energies are reached. These are the peaks used for analysis.


Zero loss peak
N
Absorption edge of element
Shaded area is peak area used in quantitative measurements
Breadth of this depends on spectrometer design and inherent energy spread of electron beam.
1.5 eV conventional guns
0.3 eV field emission gun
50 × gain increase
Background curve has form N = AE^{-r} where A, r are constants for a particular spectrometer
Low loss region $E \leqslant 50$ eV
0
Primary beam energy
$E_{K\,ab}$
Energy loss

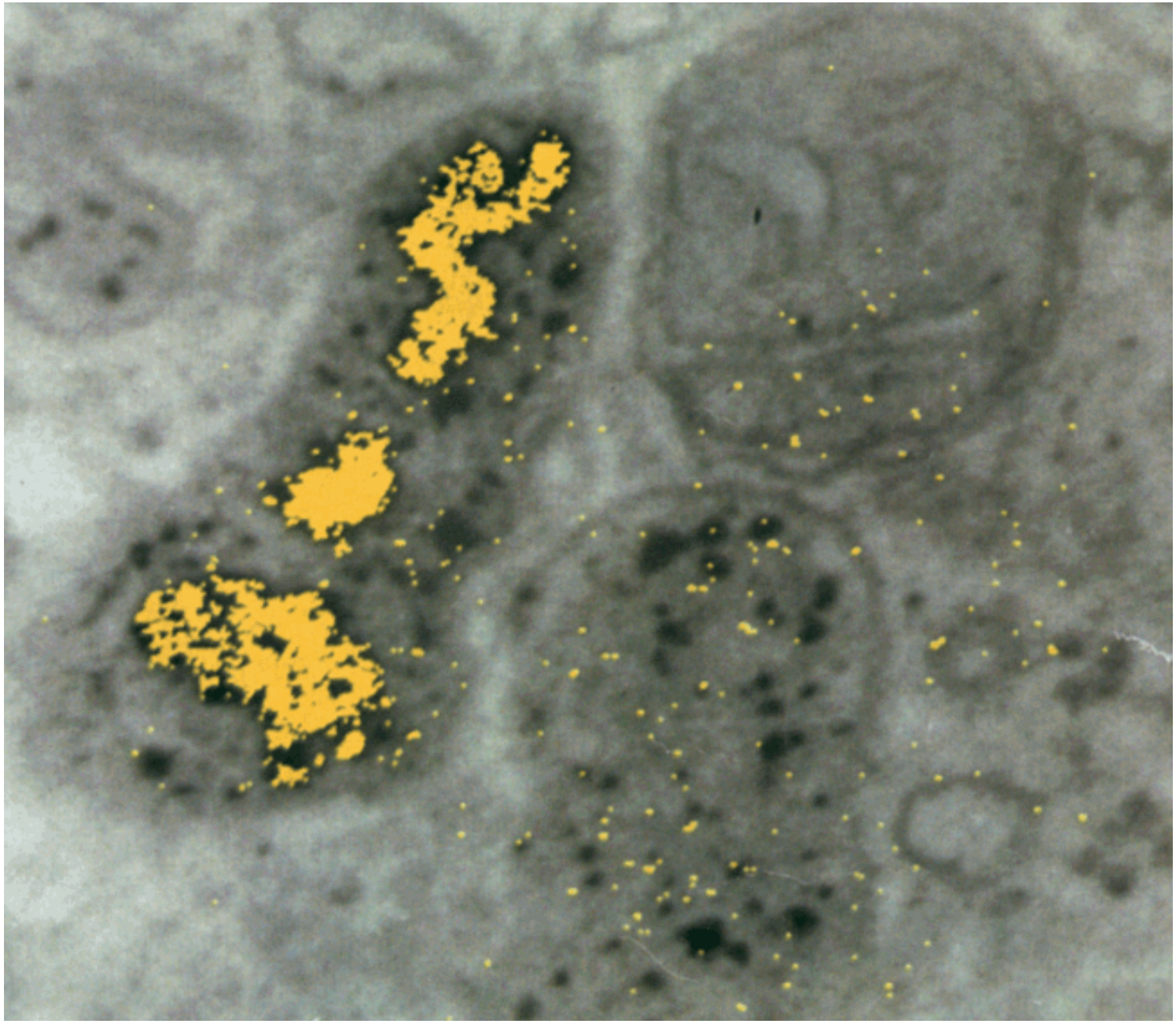

1.5387e4
MicroV LIN
-2.6000e1
200
300
400
500
600
Channel (eV) LIN
P L 2,3
Ca L 2,3
O K



Phosphorus, Calcium and Oxygen in a mitochondria (LEO) -EELS)



Calcium map - EELS



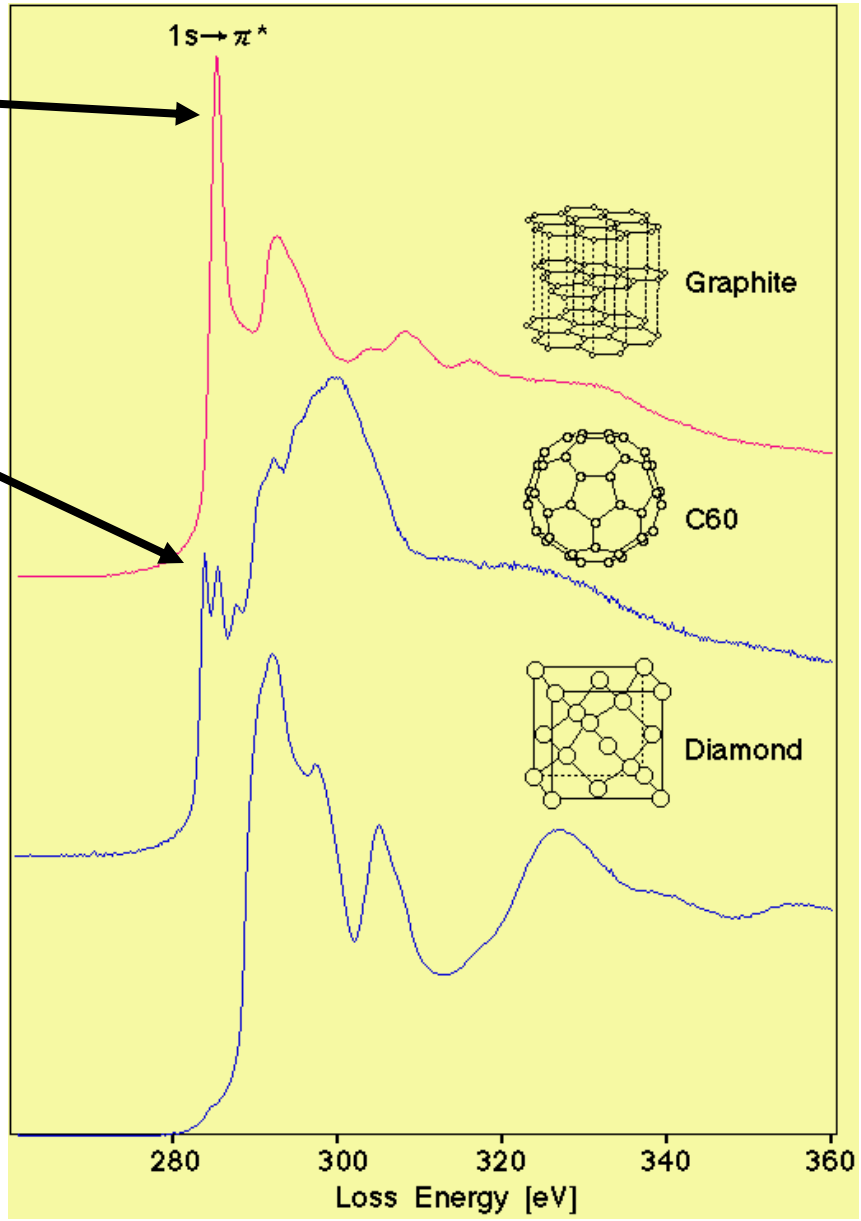
Vanadium chloride in phagolysosome - EELS

EELS can distinguish between bonding different states of matter

http://eels.kuicr.kyoto-u.ac.jp/eels.en.html

- Diamond, graphite & fullerene all have absorption peaks around 284 eV in EELS (carbon atom) and the difference in bonding state & local electronic state can be detected.

The sharp peak at absorption edge corresponds to the excitation of carbon K-shell electron (1s electron) to empty anti-bonding pi-orbital. It is not observed for diamond, because of no pi-electron in it.

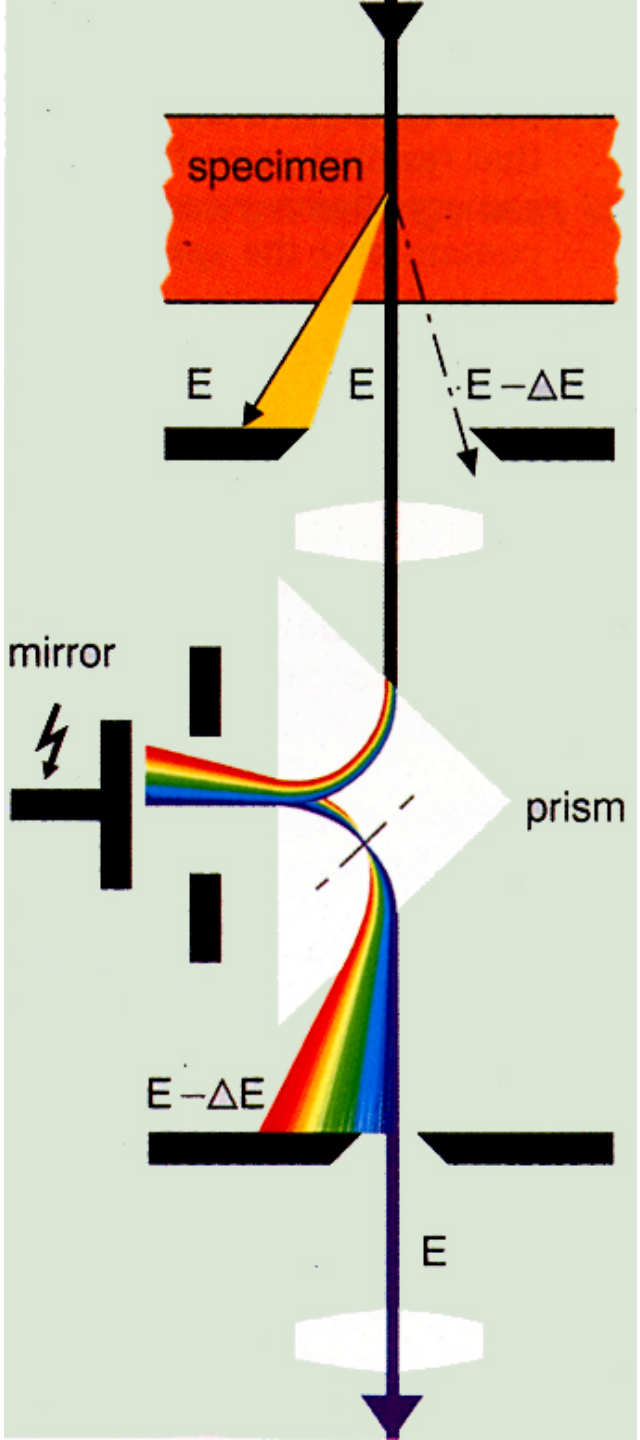


http://eels.kuicr.kyoto-u.ac.jp/eels.en.html

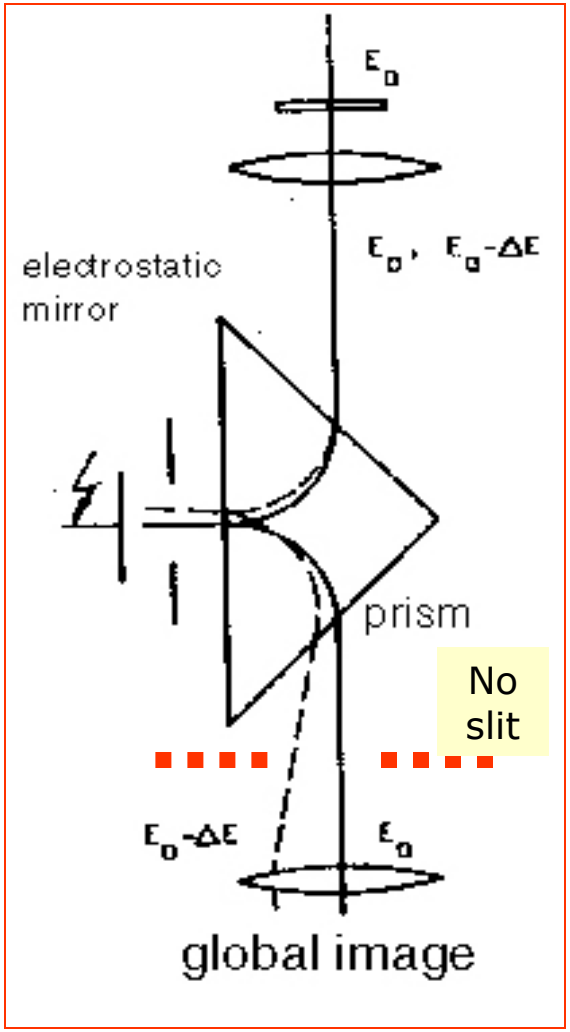
LEO 922 Energy filtering TEM

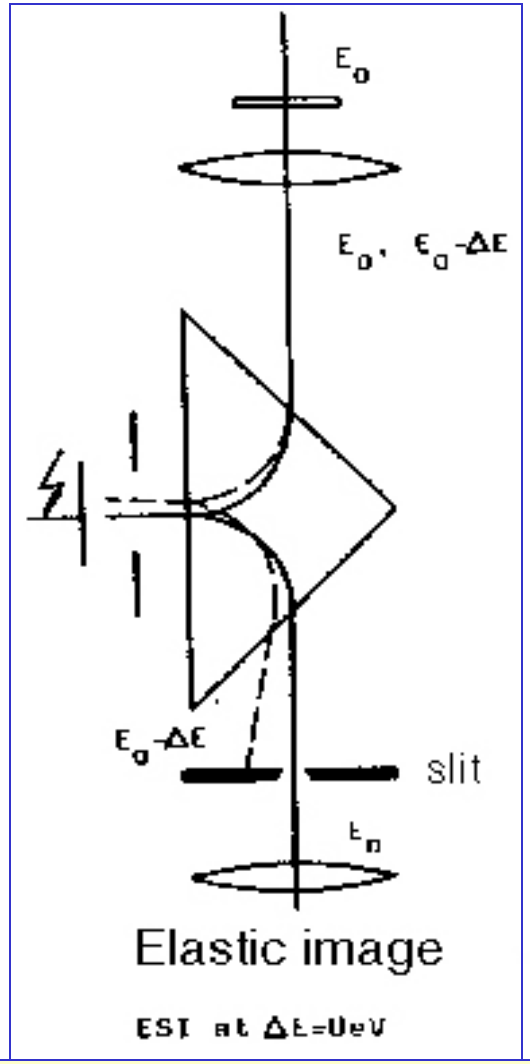
Microscopes can be fitted with energy filtering detectors or they may come as part of an integrated design

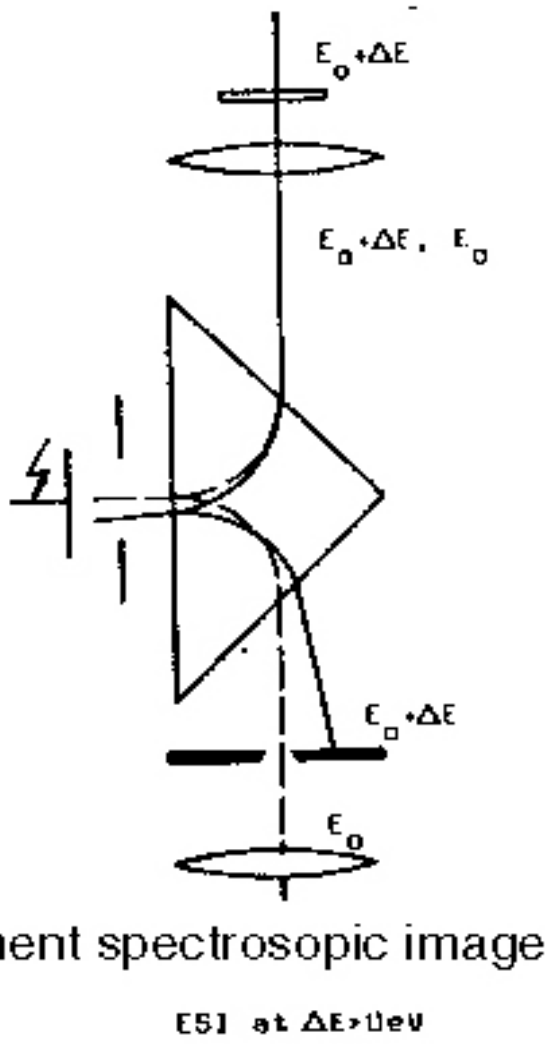
http://www.leo-usa.com/



EELS-LEO: e^- deflected in the magnetic field of the prism, then reflected in the electrostatic field of the mirror & again deflected in the magnetic prism. Deflection of the e^- in the magnetic field is directly proportional to the speed. Spectrometer disperses the e- in the energy dispersive plane according to their energy. In the energy dispersive plane is an energy selecting slit or diaphram that may be removed or adjusted to image elastically or inelastically scattered e^-.

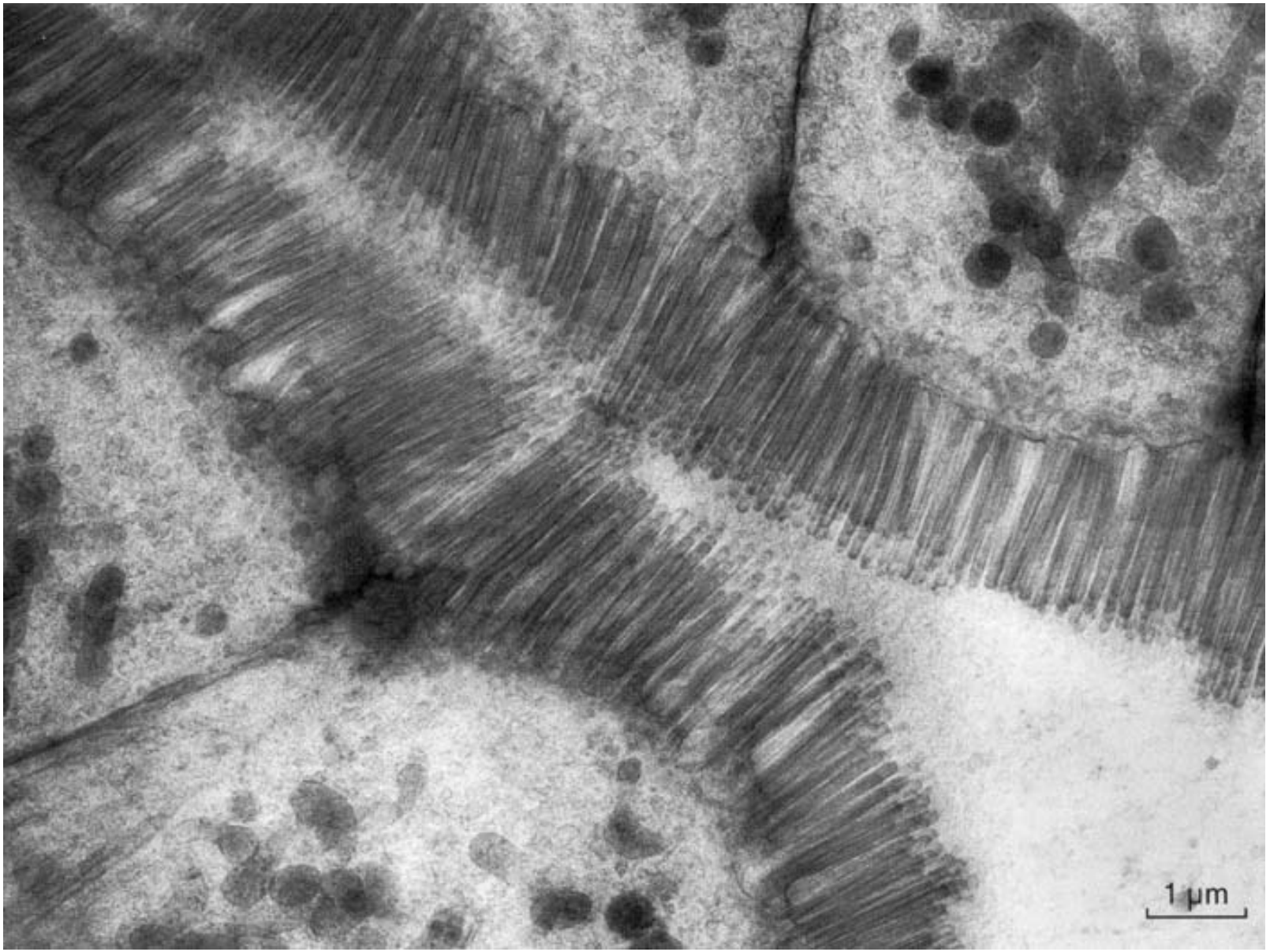






Comparable to a conventional TEM image

Only the unscattered & elastically scattered e^- imaged = **elastic brightfield image**.
Used to examine specimens up to 1μm thick at low kV (80-100 kV) & also thin unstained specimens.



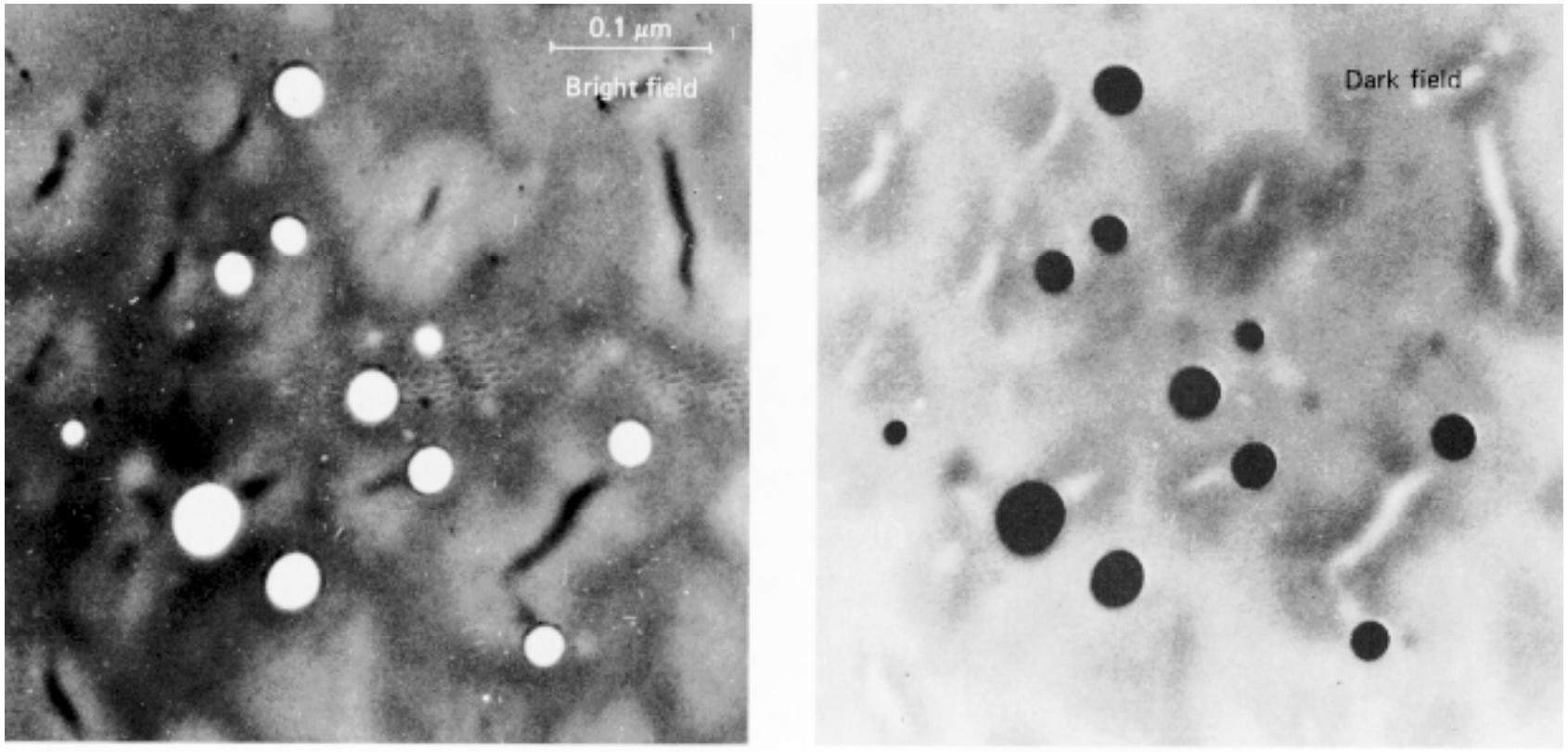
Elastic brightfield image: 1µm thick section, unstained, 80 kv (Leo)

EELS-Leo

- Energy selecting slit may be also used to select inelastically scattered e^-. The amount of energy loss due to inelastic scattering is element specific
- EELS is more sensitive than X-ray microanalysis, & the collection times are considerably reduced. Collection times of less than 1 minute are typical. Specimen preparation is similar to that for x-ray microanalysis (<u>but must be thinner!)</u>

Dark Field Microscopy

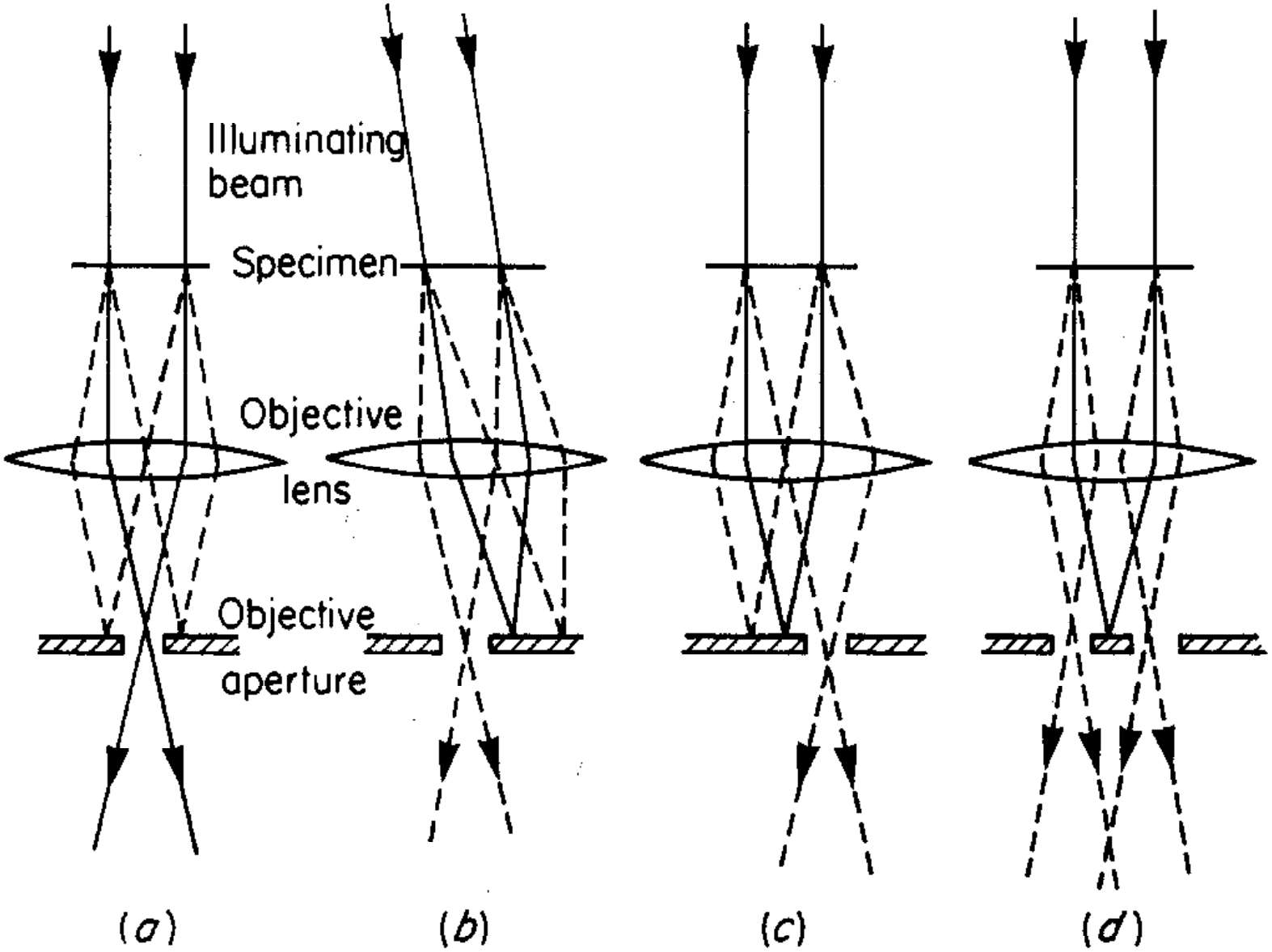
- **Conventional TEM = Bright field EM**
- Image formed only from the scattered electrons = **dark field** image.
- Viewing screen is dark unless there is specimen present to scatter electrons.
- Dark field images = considerably higher contrast than bright field images although the intensity is greatly reduced.
- Darkfield STEM provides even greater contrast than TEM



A holey carbon film in bright & dark field. Since one image is formed with electrons missing from the other, they are negatives of one another. (From Watt, p.126)

Methods of forming dark field images

- Lateral displacement of the objective aperture to intercept main unscattered electrons
  -Poor quality image - aperture accepts off-axis e^- subject to larger aberrations (spherical & chromatic) than those on the optic axis.
- e^- beam tilted so that diffracted beam of interest travels down the objective lens axis.



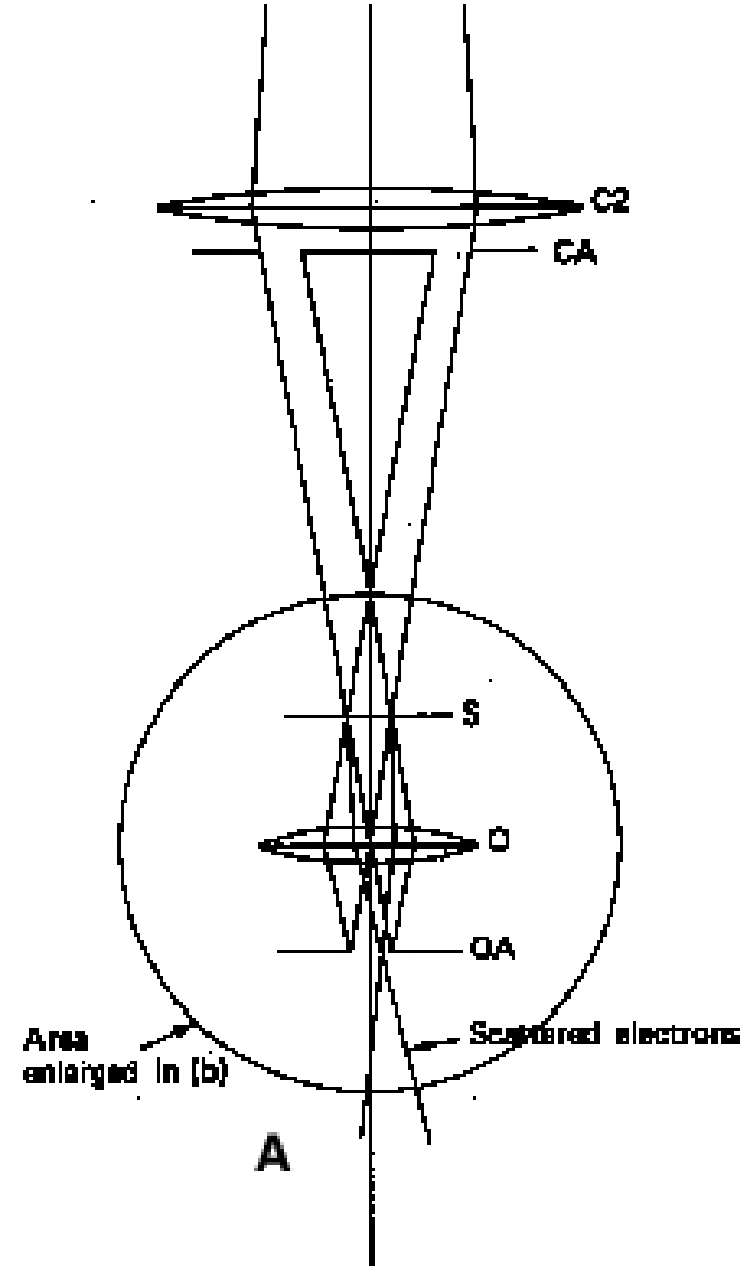
(a) Normal brightfield (b) beam tilt (c) aperture displacement (d) beam stop aperture

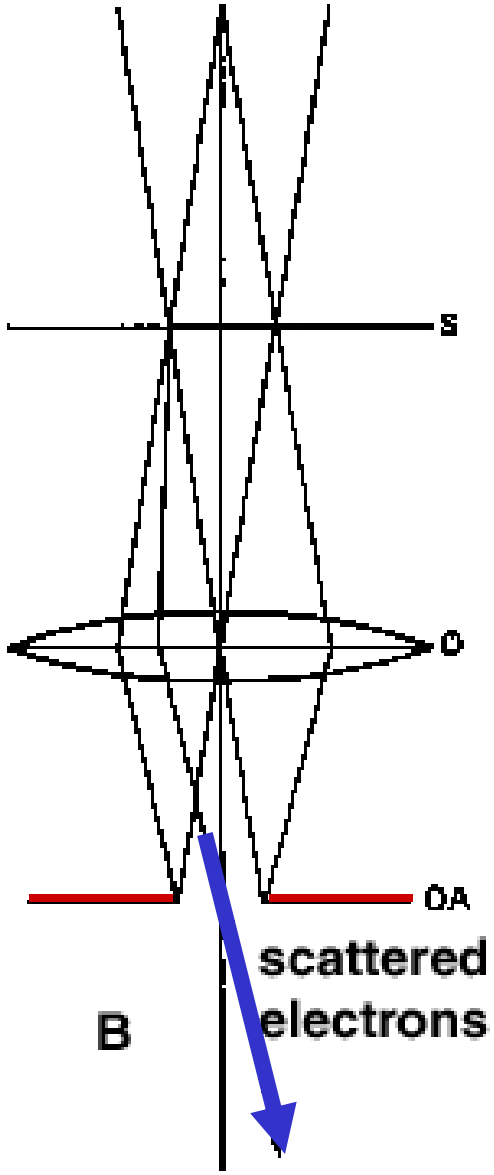
Strioscopic dark field

- Beam stopped by either a physical stop on the objective aperture or by using an annular condenser aperture
- Beam stop aperture - normal obj. aperture with a fine wire welded across center - allows only scattered e- to form image. (Useful @ high voltage -1000 kV) where contrast is low. Details which may be nearly invisible in bright field appear in good contrast using this technique.
- Drawback - due to objective aperture asymmetry - astigmatism correction for the bright field does not hold in dark field. Astigmatism correction in dark field is difficult.

Strioscopic dark field

- Annular condenser aperture forms a hollow beam that illuminates specimen evenly when the C2 is focused. In the absence of a specimen, the conical illumination forms a ring in back focal plane of the obj. lens.
- Unscattered e^- beam intercepted by obj.aperture. Diameters of the obj aperture & annular cond. aperture must be chosen so the hollow illuminating beam is correctly intercepted in the back focal plane of the objective lens. Scattered e^- which pass through the obj. aperture will form a high quality dark field image.





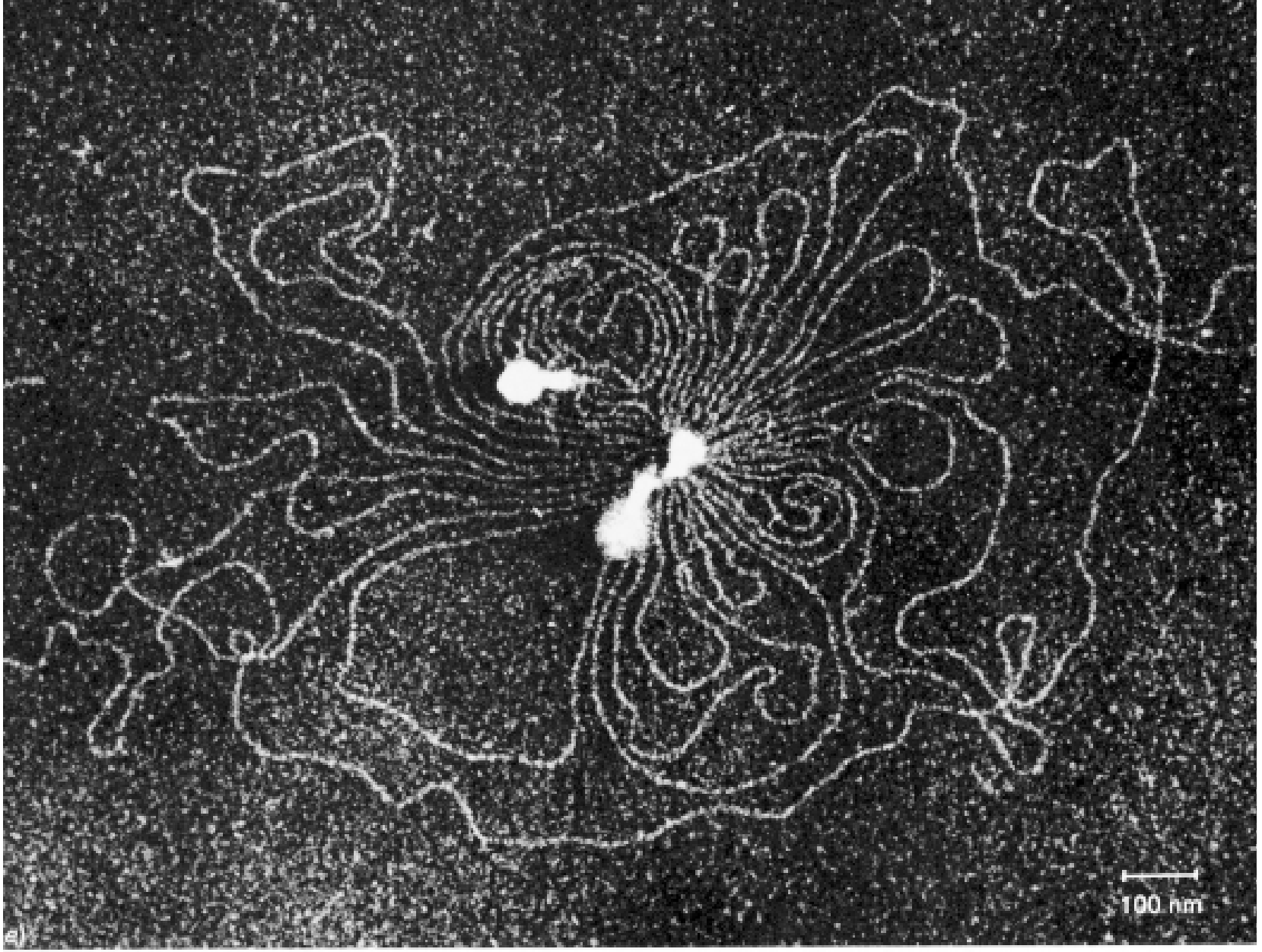
Annular cond. aperture (CA) forms hollow beam - illuminates specimen (S) evenly when the C2 is focused. Unscattered e- stopped on OA edge, but scattered e- pass through the aperture. (Agar, p.119)

Darkfield - annular Cond aperture

- Advantage of annular C method - symmetrical illumination about the axis - no danger of asymmetric charging of the objective aperture, & astigmatism change.
  - Annular cond. aperture may be replaced by a normal aperture to provide a bright field image for astigmatism correction (remains unchanged when the annular aperture is reintroduced).

Darkfield

- High contrast for molecules with very low contrast such as DNA
- Crystalline objects - diffraction spots selected in back focal plane of the objective lens in order to form a dark field image only from the e^- scattered by a chosen set of crystal planes.
- Problems - difficult to focus & correct for astigmatism-no phase contrast present
- Objective aperture transmits only a fraction of the scattered beam ∴ low image brightness & longer exposure times necessary. Therefore, specimens subjected to greater levels of radiation damage.



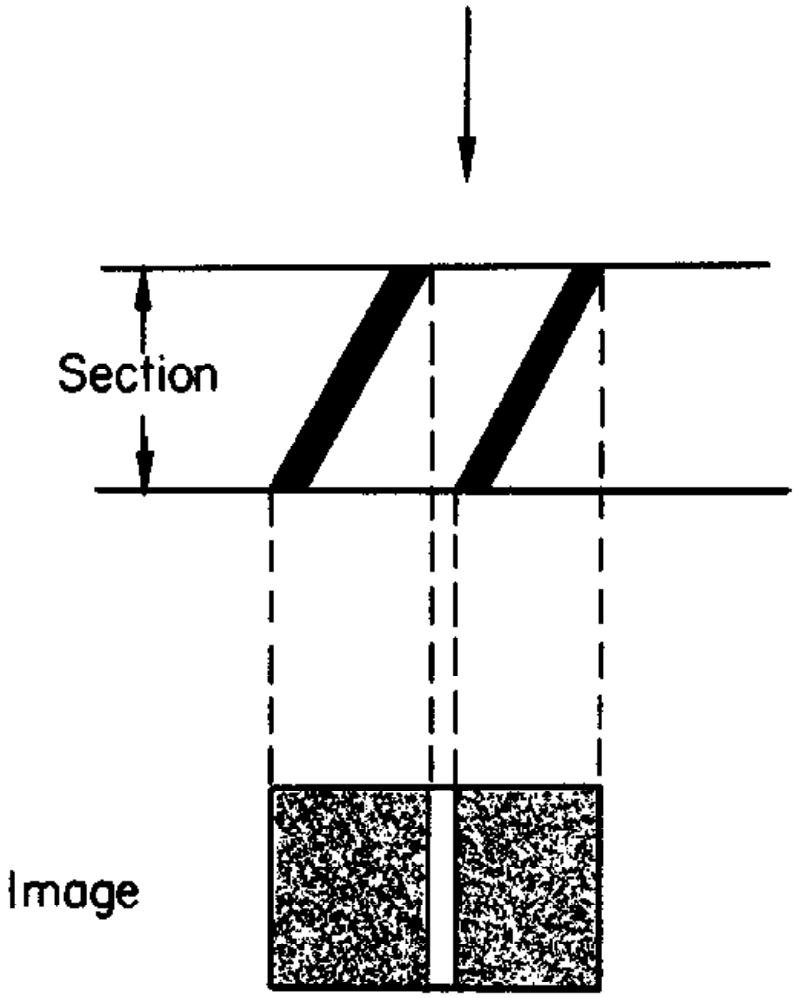
Strioscopic dark field micrograph (100kV) of unshadowed & unstained DNA extracted from T4 bacteriophage (Watt, p.130)

Tilting and Stereo Microscopy

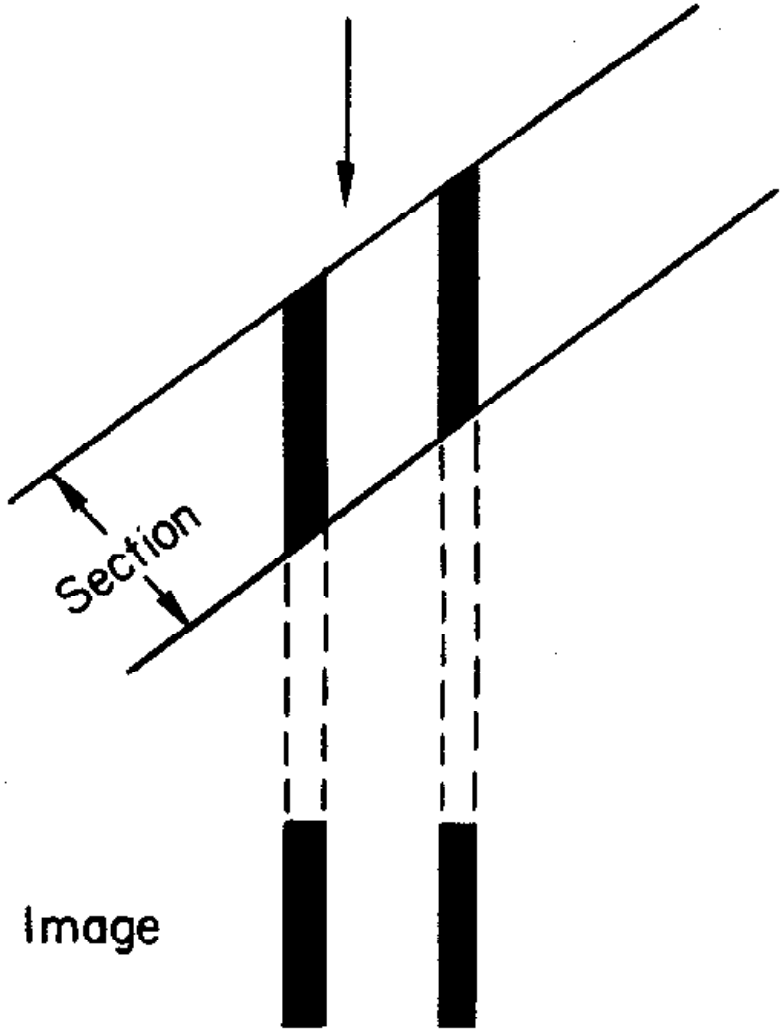
- **Stereoscopy -** visualization of objects in three dimensions.
- Based on the fact that the observer's two eyes have different viewpoints & the brain processes the information from these two sensors to give a mental impression of length, breadth & depth.
- Because of binocular vision, we are able to fuse the two images we see from slightly different viewpoints to obtain information about the third dimension (depth).

Tilting for interpretation

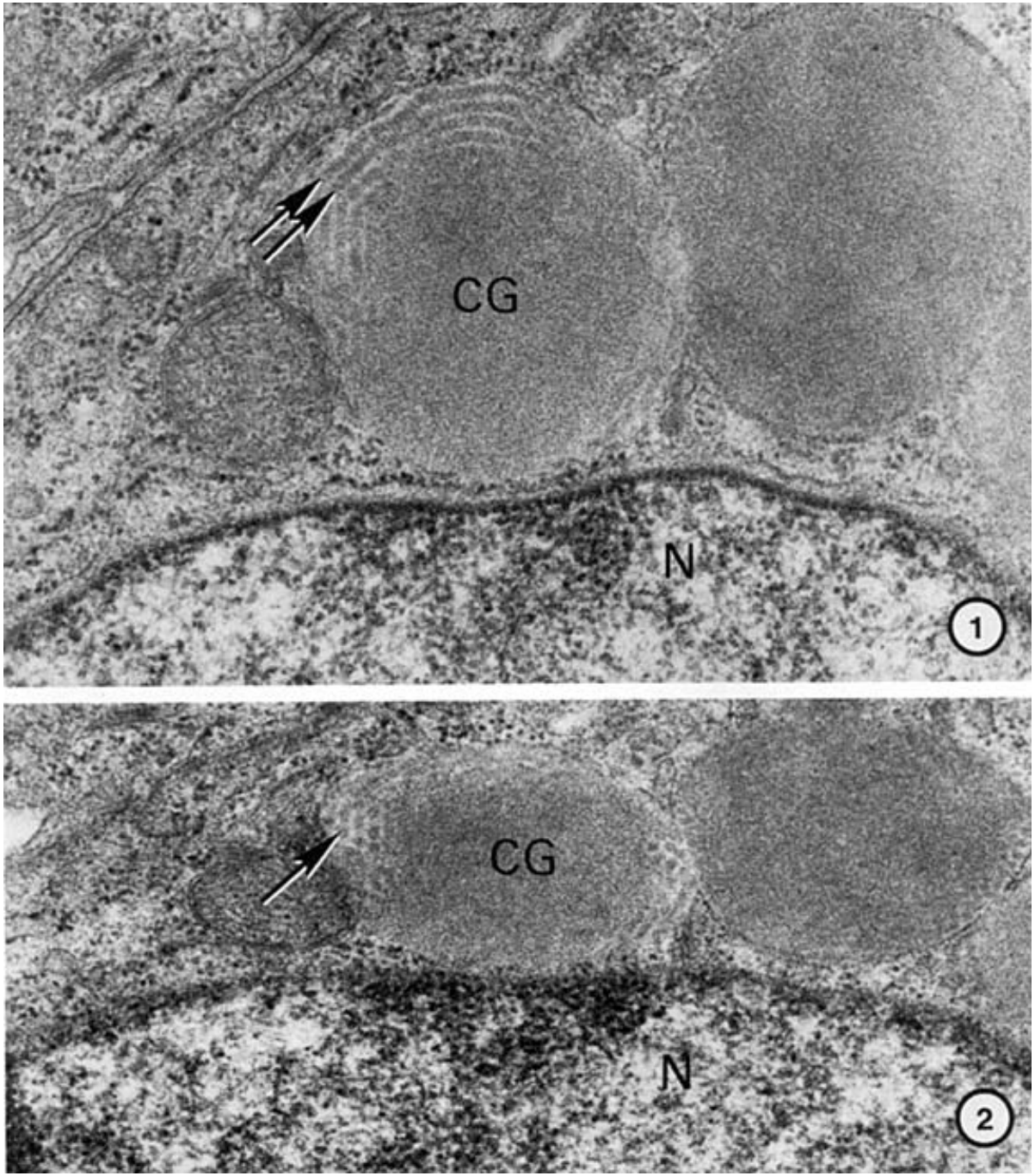
- Important method for sorting out 3-D structures.
- For specimens of moderate thickness, the tilt angle required to obtain the stereo effect is normally less than 10°.
- Only for very thin objects does a high magnification & a high tilt angle (up to 60°) become necessary.
- Goniometer tilt stage facilitates the examination of crystalline specimens where it is possible to orient the crystal with different crystallographic planes in the viewing direction.



(c)

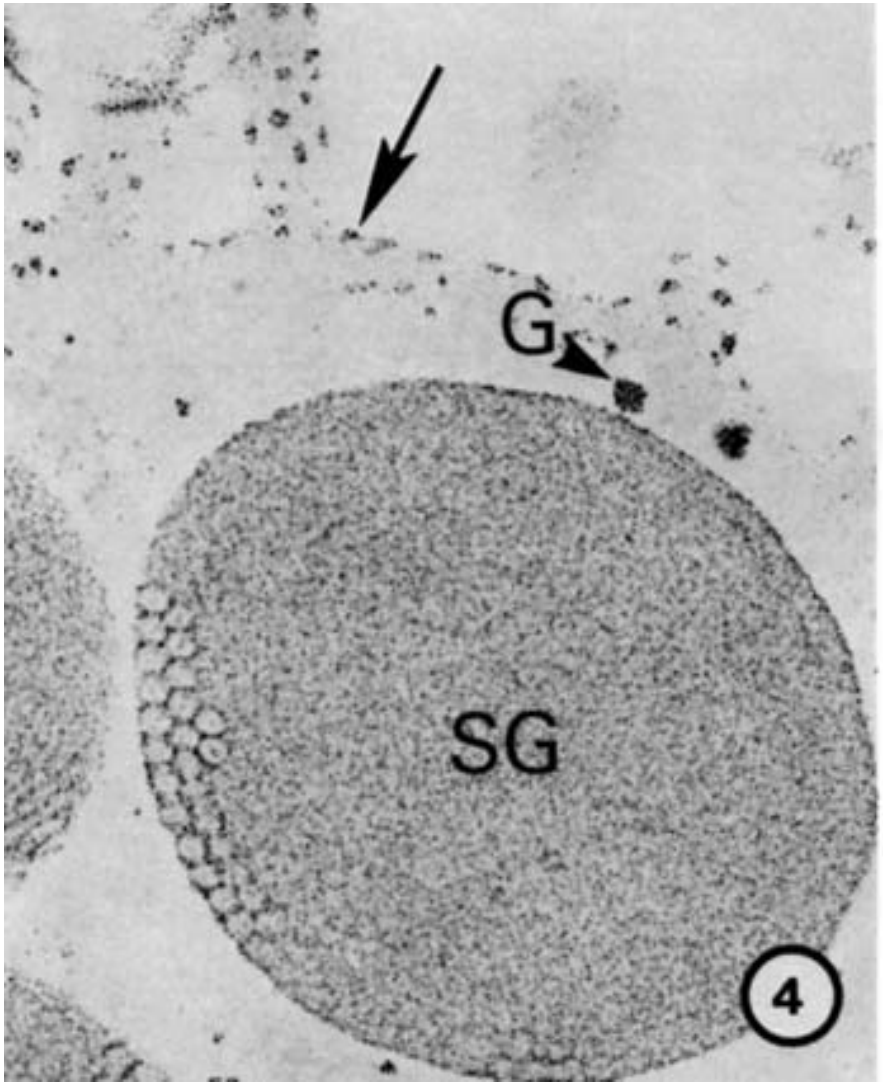


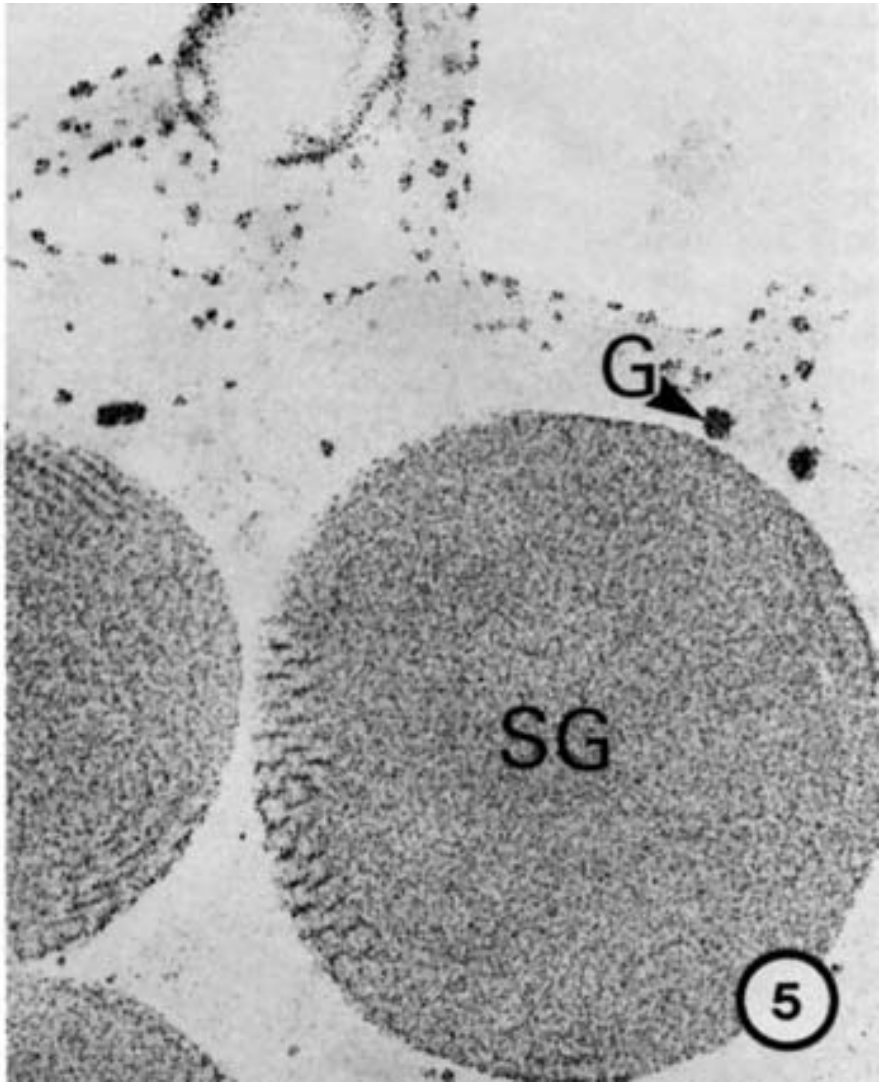
(d)



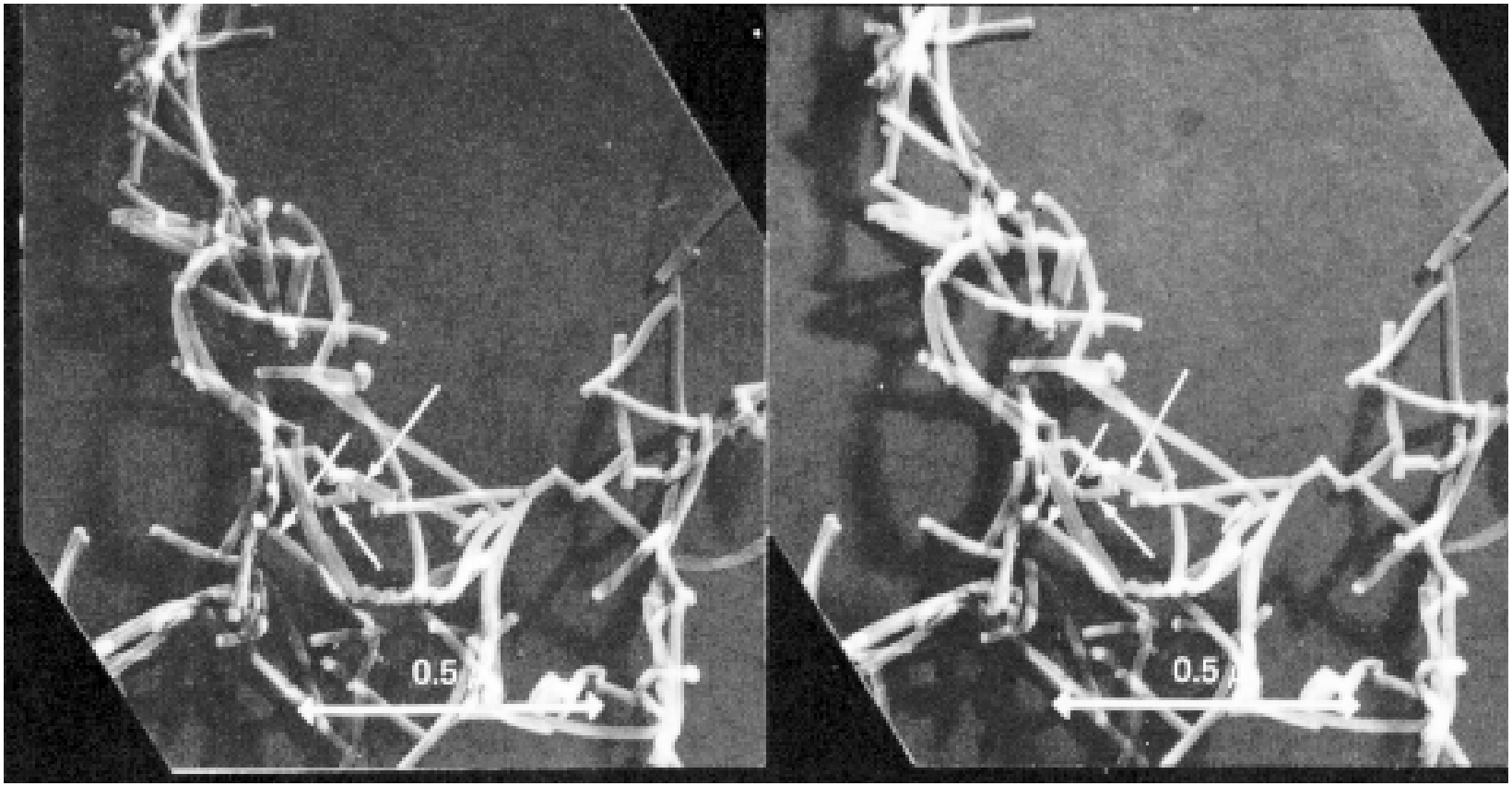
High-angle tilt ~45°

Turek, Anat. Record, 203:329, 1982

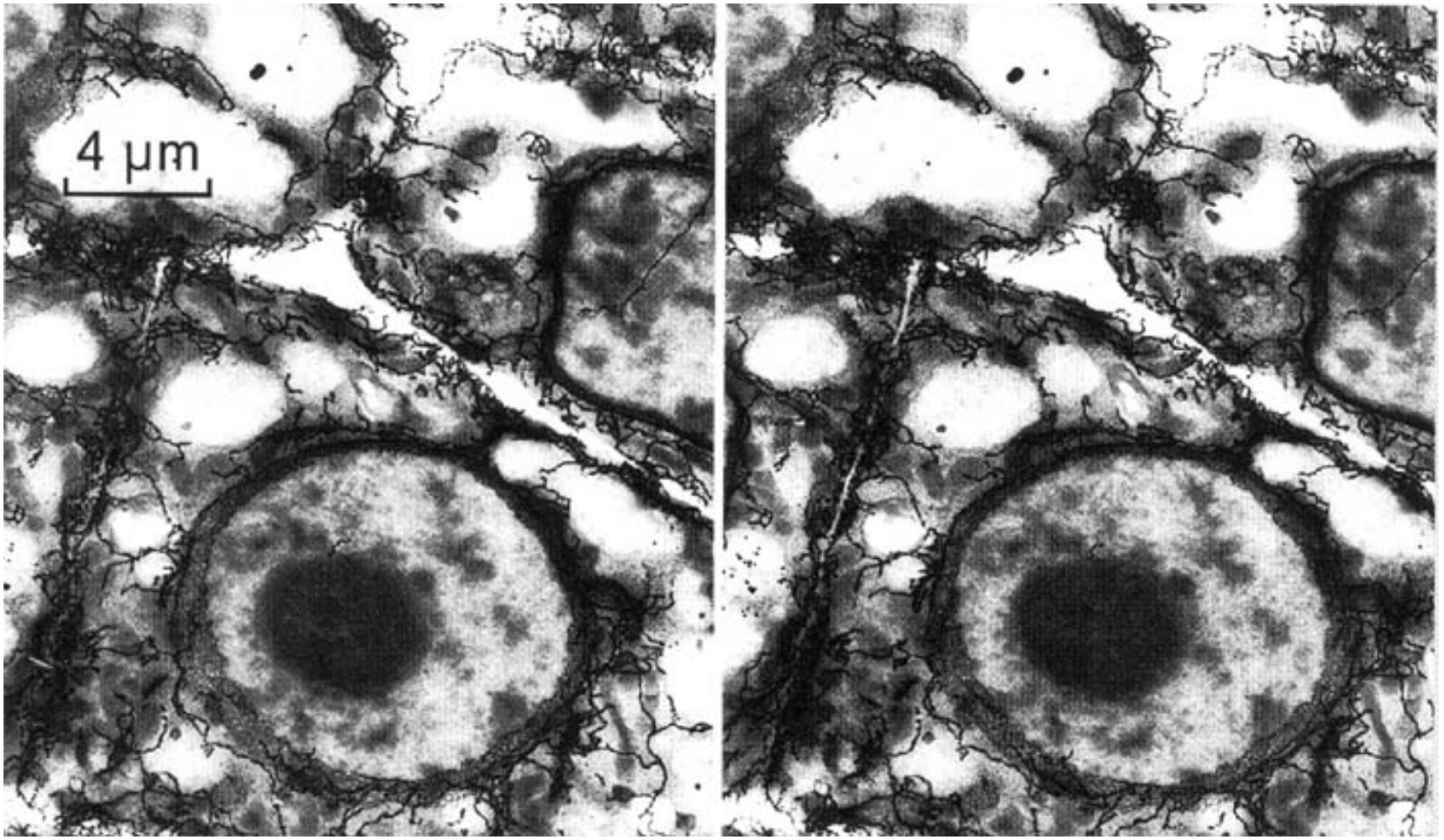




Turek, Anat. Record,
203:329, 1982



Stereoscopic pair of transmission micrographs of freeze-dried tobacco mosaic virus particles showing protein subunits. (From Watt, p.157)



Stereoscopic pair using thick specimen

Red-green or red-blue anaglyphs for stereo viewing