I.E OPERATION OF THE TEM

Choice of accelerating voltage
Choice of apertures
Specimen stage/holder
Choice of magnification
Focusing
Magnification calibration
Resolution tests
Image intensifiers/TV displays
Microscope maintenance
Photography

I.E OPERATION OF THE TEM

I.E.1 Choice of Accelerating Voltage

Most routine biological microscopy: 80 or 100kV

As kV is **increased** several factors are affected:

- Specimen penetration **increases:** means thicker specimens can be studied

- Amplitude contrast **decreases** (fewer elastically and inelastically scattered electrons stopped by objective aperture)

- Electron gun brightness **increases**

I.E OPERATION OF THE TEM

I.E.1 Choice of Accelerating Voltage

Additional effects of **increased** kV:

- Efficiency of screen phosphor **increases**

- Efficiency of most photographic emulsions **decreases**

- Inelastic scattering **decreases:** thus chromatic aberration effects **decrease** (get better resolution) & radiation damage **decreases** (longer specimen lifetime)

- Electron gun **more sensitive** to vacuum quality - high voltage stability **decreases**

- Resolution limit due to diffraction effects - improves because e^- λ **decreases**

I.E OPERATION OF THE TEM

I.E.1 Choice of Accelerating Voltage

Some Rules of Thumb:

- **80-100kV** preferred for biological specimens
  Reduces radiation damage & improves resolution compared to lower kV

- Use **small objective aperture** and/or use **staining** techniques to **increase specimen scattering contrast** rather than a lower kV

- **Turn filament current off** before switching on high tension
  Prevents filament damage

- The higher the kV selected, the longer the gun takes to stabilize
  Discharges take place due to the desorption of residual column gases

- **Pre-condition** gun at a voltage **higher** than you will use
  TEM will be more stable at the lower kV

I.E OPERATION OF THE TEM

I.E.2 Choice of Apertures

I.E.2.a Condenser Aperture

Factoids:

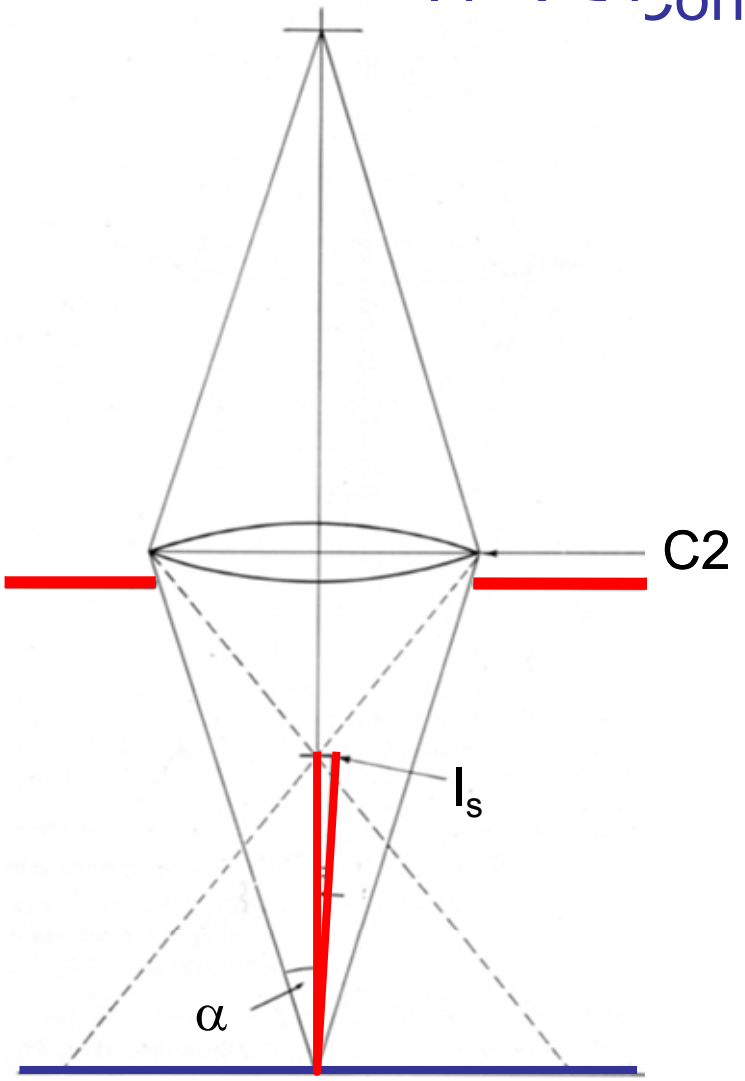
- Condenser aperture defines the maximum illumination aperture angle (2α) of the electron beam

- Smaller effective aperture achieved by **defocusing** condenser lens 2 (beam spread more widely)

  - As aperture size is **reduced**, beam size becomes smaller at crossover & total number of electrons $\Downarrow$.

I.E.2 Choice of Apertures

I.E.2.a Condenser Aperture



From Agar, Fig. 1.18, p.24

- **Condenser 2 aperture** defines the ∠ of illumination on specimen when C2 is focused on the specimen plane (C2 at crossover)

- When C2 strength is increased (*i.e.* over focused - crossover above specimen plane) the **angle of illumination** is defined by the illumination source crossover point I_s

- C2 lens **defocusing** reduces effective illumination aperture

I.E.2 Choice of Apertures

I.E.2.a Condenser Aperture

Factoids:

- Small apertures best for high resolution

  Beam will be **more coherent** & give **better phase contrast**

- Beam coherence "perfect" if radiation source is infinitely small (*i.e.* infinite distance from specimen)

  Effectively the case with smallest condenser apertures which select only a narrow band of paraxial electrons

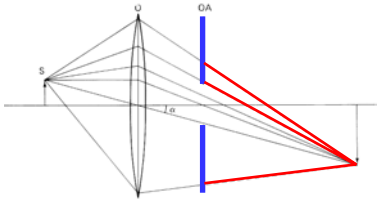
- Another way to improve coherence:

  Over focus **C1** lens

  C2 lens aperture will collect fewer e^- from the smaller, demagnified source

I.E.2 Choice of Apertures

I.E.2.b Objective Aperture



Factoids:

- Used to improve **scattering** contrast
- Sizes range from ~ **10 to 75 μm** (40-50 typical size used)
- The **smaller** the aperture the **>** the contrast
- Smaller apertures **improve** resolution limited by spherical and chromatic aberration but **reduce** resolution limited by diffraction
- Smaller apertures harder to align, keep aligned & more sensitive to contamination
- Ultra-thin, **self-cleaning** apertures eliminate many problems caused by contamination

  Scattered electrons heat aperture edge high enough to prevent the formation of contamination

I.E OPERATION OF THE TEM

I.E.3 Specimen Stage/Holder

Some Rules of Thumb:

- **Never use bare hands** to handle specimen holder tip
- Specimen grid must be **flat & secure** in holder for proper thermal contact
  - A bent grid can change the specimen height (∴ magnification)
  - May need to change course objective focus to get good image
- Specimen inserted into the microscope with:
  - **Filament turned off**
  - High voltage **on**
  - Gun and condenser apertures **aligned**
  - Magnification at a **low setting** suitable for locating good specimen

I.E OPERATION OF THE TEM

I.E.4 Choice of Magnification

Choice of which magnification to use depends on the nature of the experiments being performed

Several criteria used to establish a suitable magnification level

I.E OPERATION OF THE TEM

I.E.4 Choice of Magnification

I.E.4.a Low Magnification

- Low magnification (<10,000X) required to record a **large field of view** from a specimen in a single micrograph

- **Montage:** splicing together prints from several low magnification micrographs

  - If **very low** magnifications used, **pincushion** and **barrel** distortions could be significant & the prints won't line up.

I.E.4 Choice of Magnification

I.E.4.b Statistical Studies

For statistical studies (particle counting) use the **lowest** magnification at which the particles can be correctly identified.

I.E.4 Choice of Magnification

I.E.4.c Radiation Damage

With radiation sensitive specimens use the **lowest** possible magnification & illumination level when searching for a suitable specimen area

I.E.4 Choice of Magnification

I.E.4.c High Magnification

High magnification required to achieve **highest resolution** images

Excessive magnification leads to unnecessary **radiation damage** because higher doses are needed to suitably expose the photographic emulsion

I.E.4 Choice of Magnification

I.E.4.c High Magnification

Resolution of photographic medium ultimately determines the **maximum** magnification needed to record details at a *predetermined* resolution

Photographic emulsions can resolve image details at least as small as **20 µm**

The **theoretical potential** resolution of object details depends on image magnification according to:

Potential resolution = 20 µm/magnification

I.E.4 Choice of Magnification

I.E.4.c High Magnification

Best potential resolution = 20 µm/magnification

Magnification	Potential Resolution at Object (nm)
2,000	10.0
20,000	1.0
50,000	0.4
100,000	0.2

"Typical" microscopy performed at **excessive** magnifications given the limited resolution of the specimens (especially biological)

I.E.4 Choice of Magnification

I.E.4.c High Magnification

- **Photographic** magnification (usually <10x) does **not** increase image resolution

- **Only** makes it possible for the **eye** to resolve details

- Use of unnecessarily high magnification **restricts** specimen field of view

Rule of Thumb:

Select **lowest** magnification consistent with required resolution

Each time magnification is doubled, a 4-fold increase in beam intensity is required at the specimen to expose a micrograph

I.E OPERATION OF THE TEM

I.E.5 Focusing

- Image focusing affected by making **small changes** in **objective lens** current

- **True or near focus:** condition at which **no** Fresnel fringe is formed at an image point

- **Slight** degree of **underfocusing** gives optimum results

Scattering contrast is **enhanced** with interference contrast at slight underfocus

I.E OPERATION OF THE TEM

I.E.5 Focusing

I.E.5.a Low Magnification Focusing (< 15,000X)

Focusing with a Wobbler Aid

- Wobbler produces a cyclical deflection of the incident beam

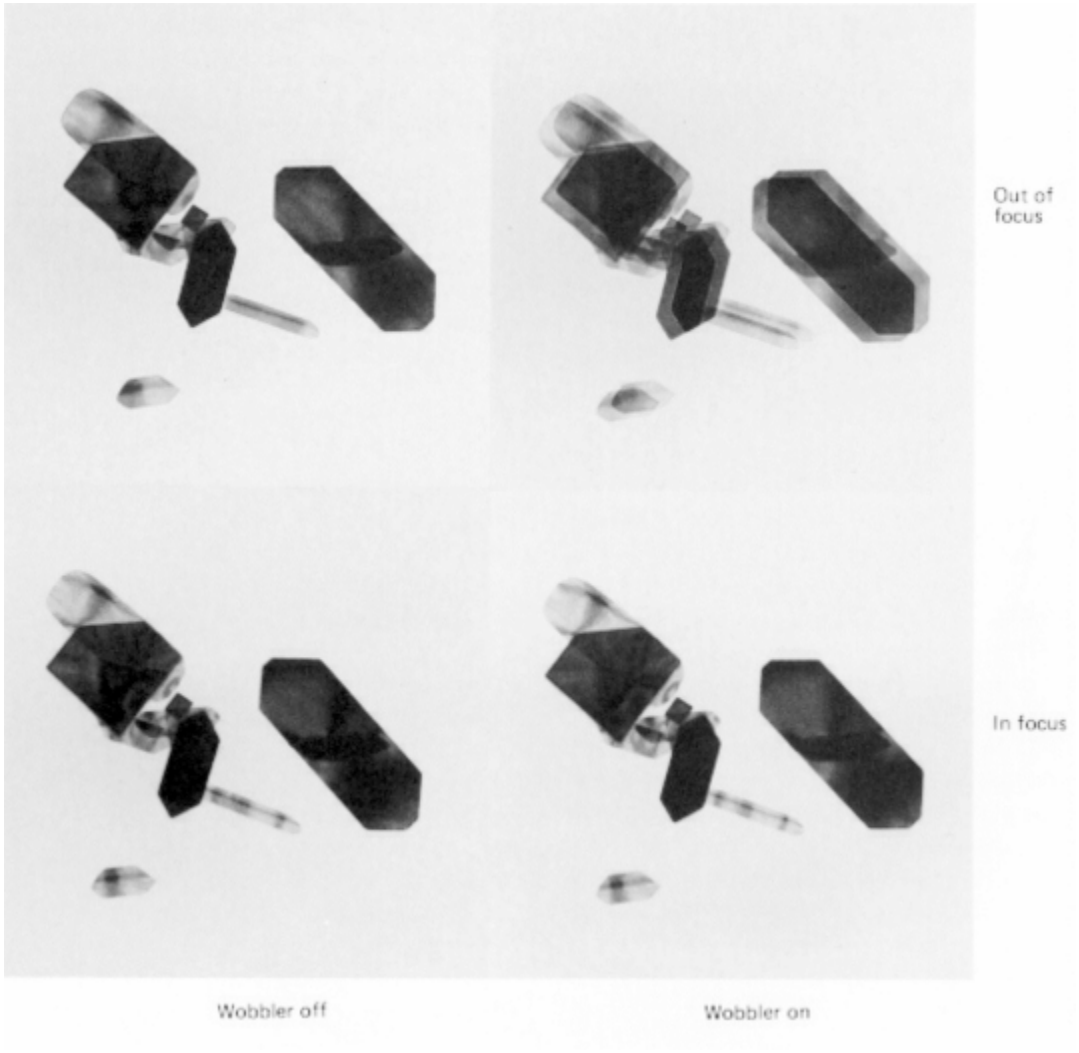
- Causes image movement when the objective lens is focused on a plane above or below the specimen

  - Image appears sharpest when objective lens is focused on the specimen plane

I.E.5 Focusing

I.E.5.a Low Magnification Focusing (< 15,000X)

Focusing with a Wobbler Aid



From Watt, Fig. 2.11b, p. 30

I.E.5 Focusing

I.E.5.a Low Magnification Focusing (< 15,000X)

Minimum Contrast Method

- **Withdraw** objective aperture and set the objective lens strength to give an image with **minimum contrast**

- **Reinsert** objective aperture before photography

- Works best with **thin** specimens because they show a dramatic drop in contrast near the in-focus position

- **Not** the most effective or recommended way to focus!

I.E.5 Focusing
I.E.5.b High Magnification Focusing (usually > 30,000X)

- Focus on **background support film**
  - "Structure" mainly due to phase contrast
- Best **NOT** to focus directly on the specimen, especially if it is a new and unfamiliar one
  - Support film well-defined, well-behaved "specimen" with a characteristic appearance at near focus settings
- Consider phase contrast granularity when interpreting pictures taken at high resolution
  - Granularity **erroneously misinterpreted** as genuine fine structure in the specimen
  - Record a **through-focal series**

I.E.5 Focusing

I.E.5.b High Magnification Focusing (usually > 30,000X)

Rules of Thumb:

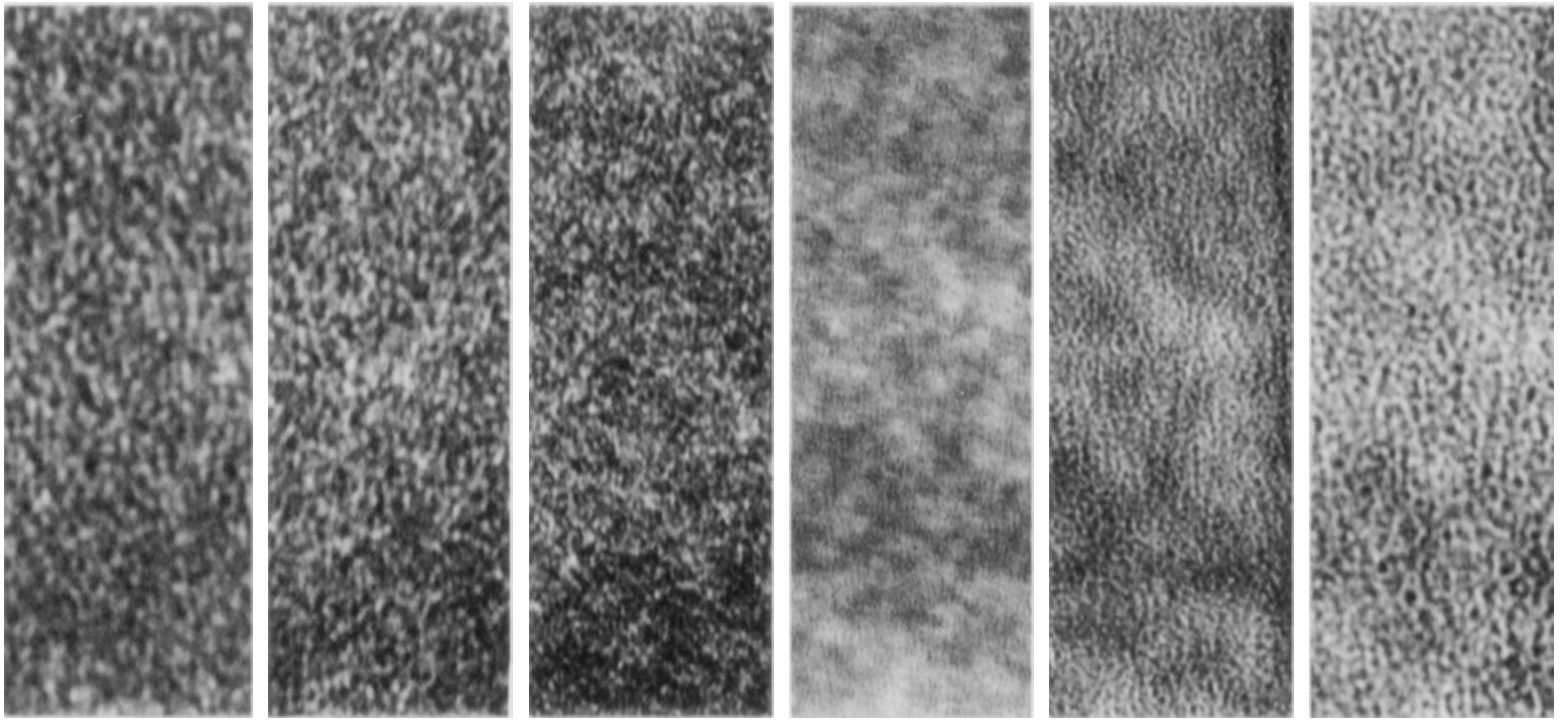
1. The greater the level of magnification, the more accurate the focusing must be

2. High contrast does **NOT** mean you have achieved a "sharp focus" condition

I.E.5 Focusing

I.E.5.b High Magnification Focusing

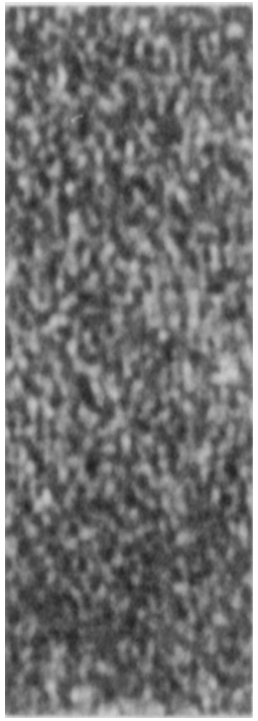
Through focus series of identical area of a thin carbon film lightly shadowed with platinum



From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing

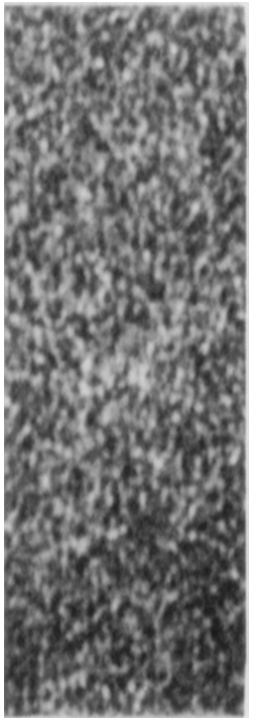


-2.1 µm

From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing

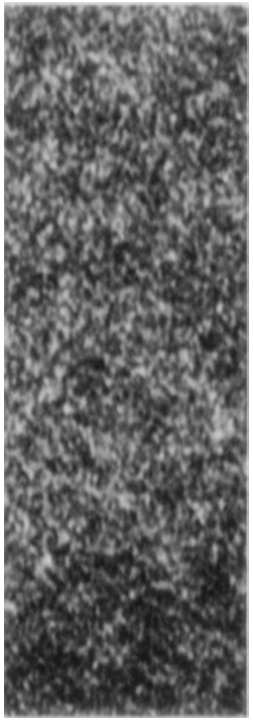


-1.4 µm

From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing

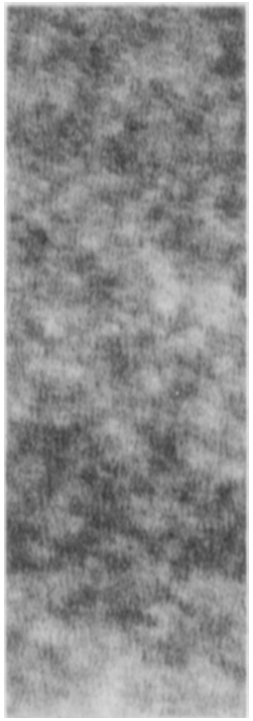


-0.7 µm

From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing



near focus

From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

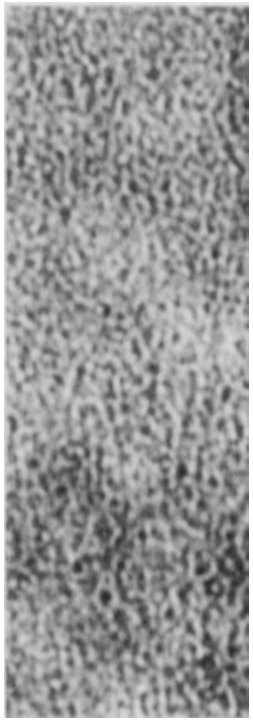
I.E.5.b High Magnification Focusing

0.7 μm

From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing



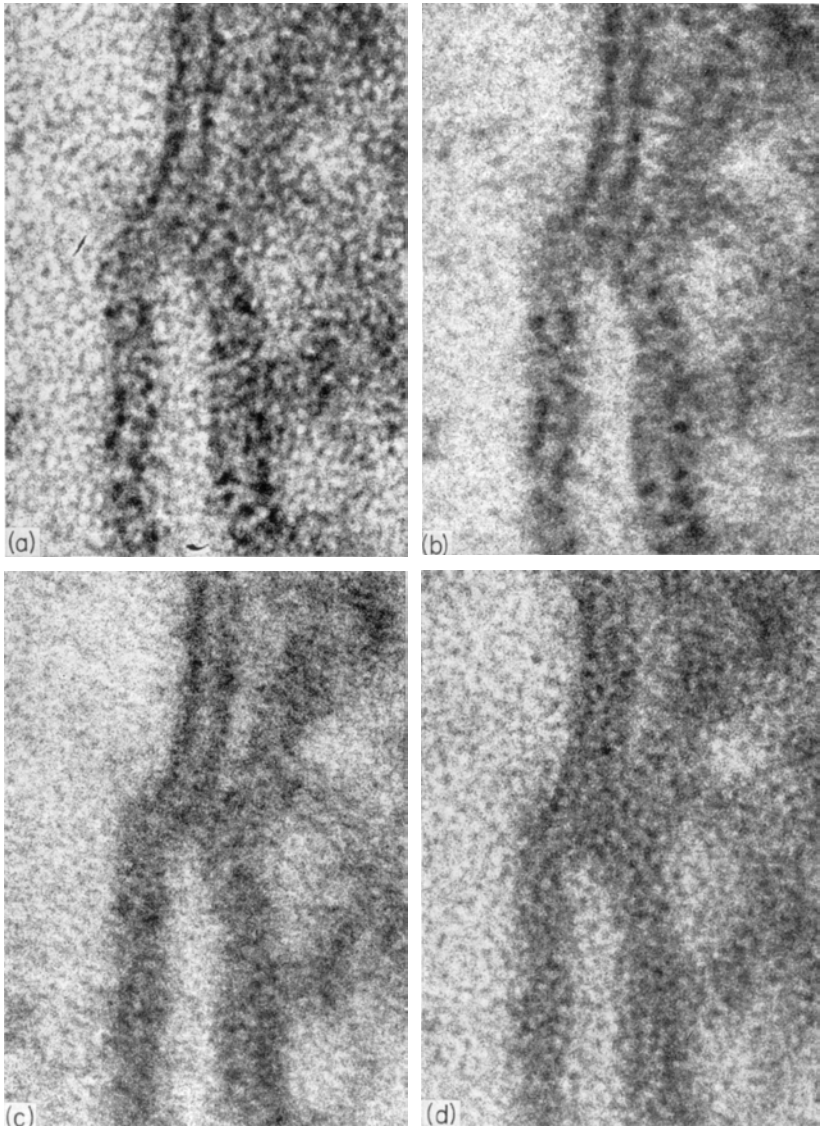
1.4 μm

From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Phase contrast effects on image of mitochondrial outer membrane in thin section



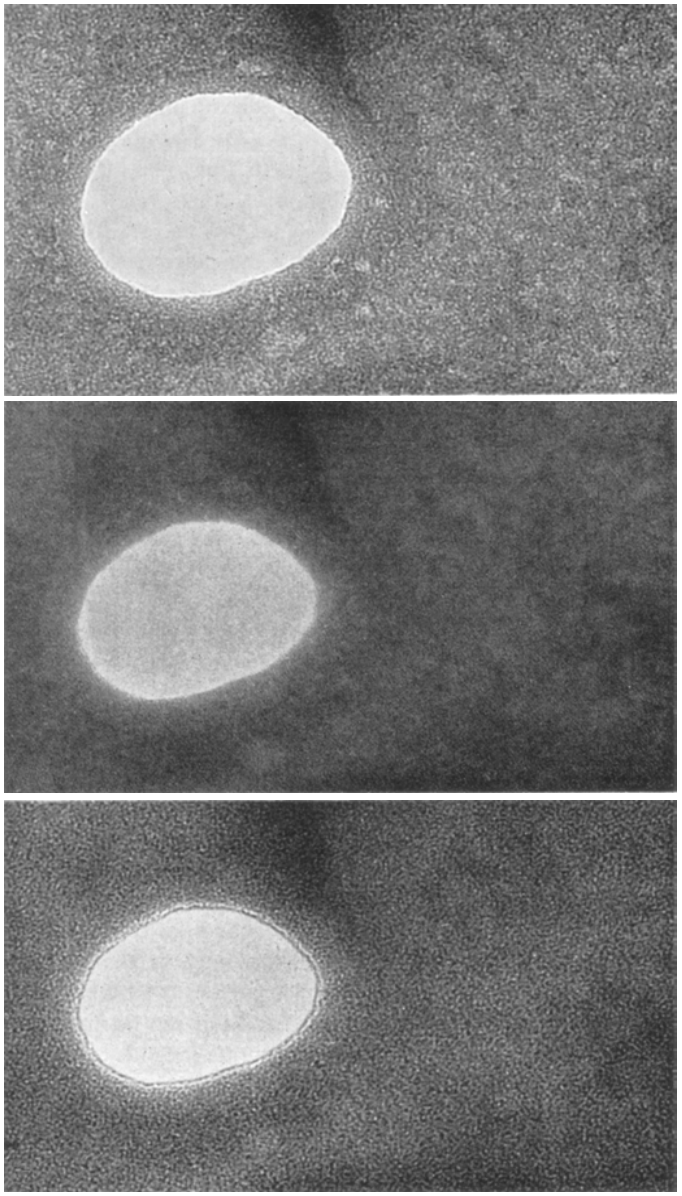
a) Under focus
b) Close to focus
c) Slightly over focused
d) Over focused

From Sjostrand, Fig. IV.13, p. 109

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a thin carbon film



Under focus

Near focus

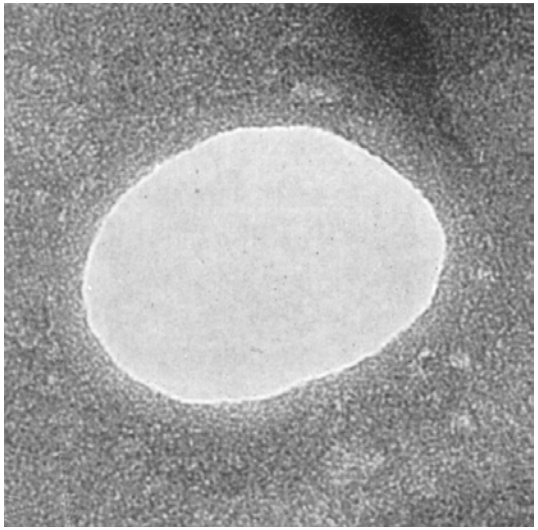
Over focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a thin carbon film



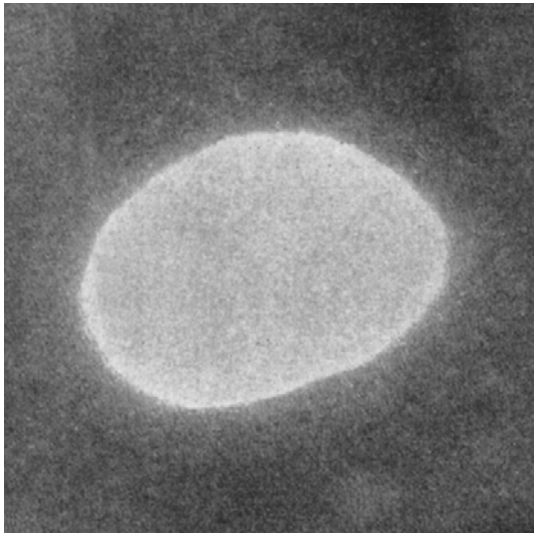
Under focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a thin carbon film



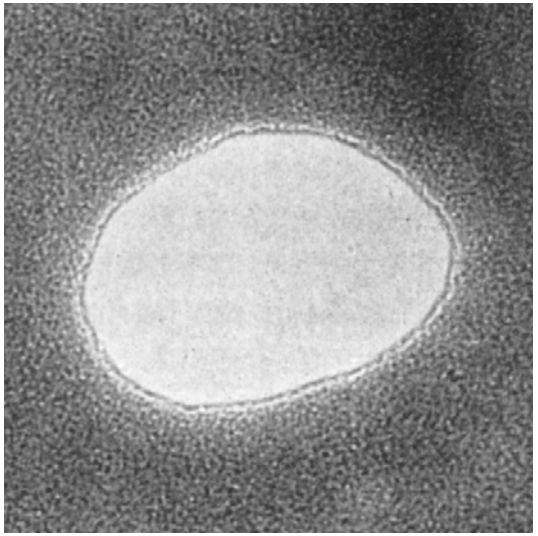
Near focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a thin carbon film



Over focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Stigmating

'Line drawing' method of objective lens stigmating

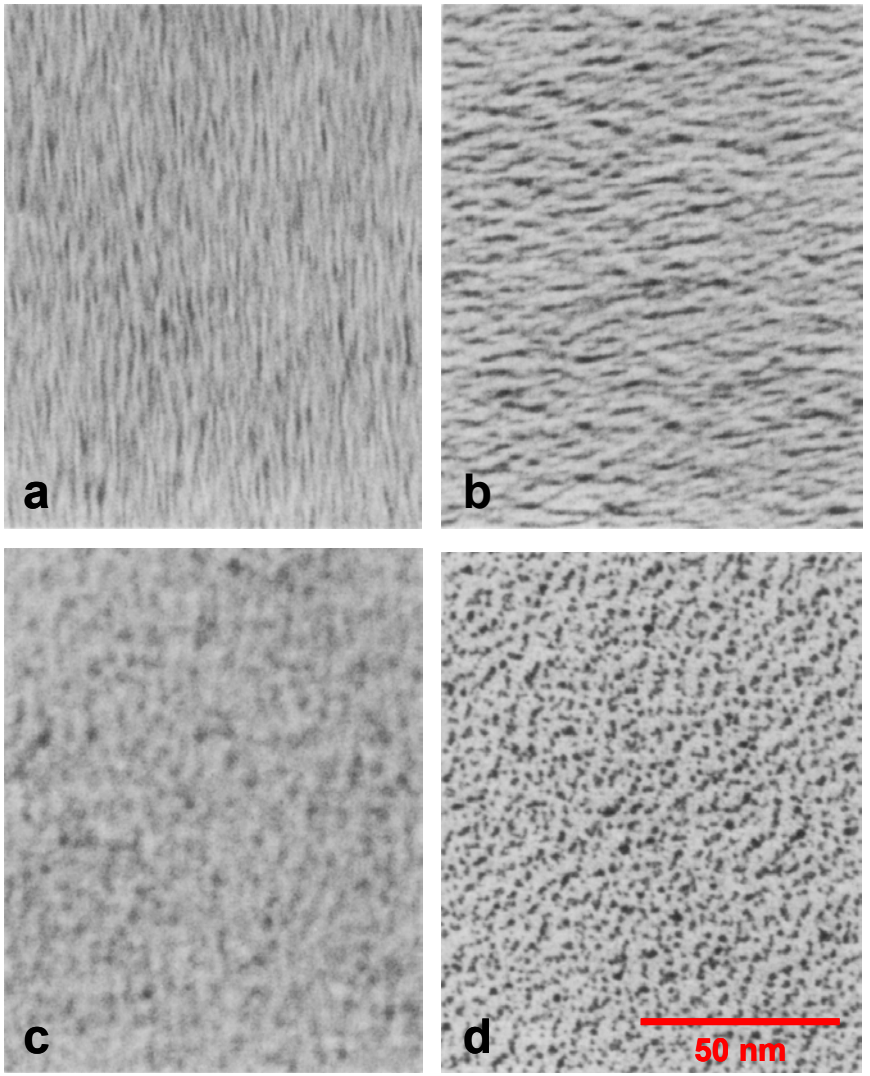
Specimen: Pt-Ir on carbon substrate

a) Astigmatic lens, underfocused.
Astigmatic lines run N-S

b) Same area, slight overfocused.
Astigmatic lines run E-W at 90° to (a)

c) Same area at 'jump-over point' of best focus with the astigmatic lens

d) Same area at best focus with astigmatism corrected to better than 10 Å.



From Meek 1st ed., Fig. 11.4, p. 304

I.E OPERATION OF THE TEM

I.E.6 Magnification Calibration

- Because of **hysteresis**, actual lens strength depends on whether the current arrives at a given value from a higher or lower value

- Nominal magnification settings in modern TEMs may have an uncertainty of **~ 2-5%** or more

- Most TEM have a means to **normalize** magnification by cycling the lenses in a standard way to improve reproducibility to **~ 2% or better**

- Essential to have an **independent** magnification calibration

- A given calibration is **only valid** for the specimen holder being used

- Magnification is calibrated with **reference specimens** with **known dimensions or spacings**

I.E.6 Magnification Calibration

I.E.6.a Polystyrene Latex Spheres

- Polystyrene latex spheres of uniform size

- Good **internal** calibration standards

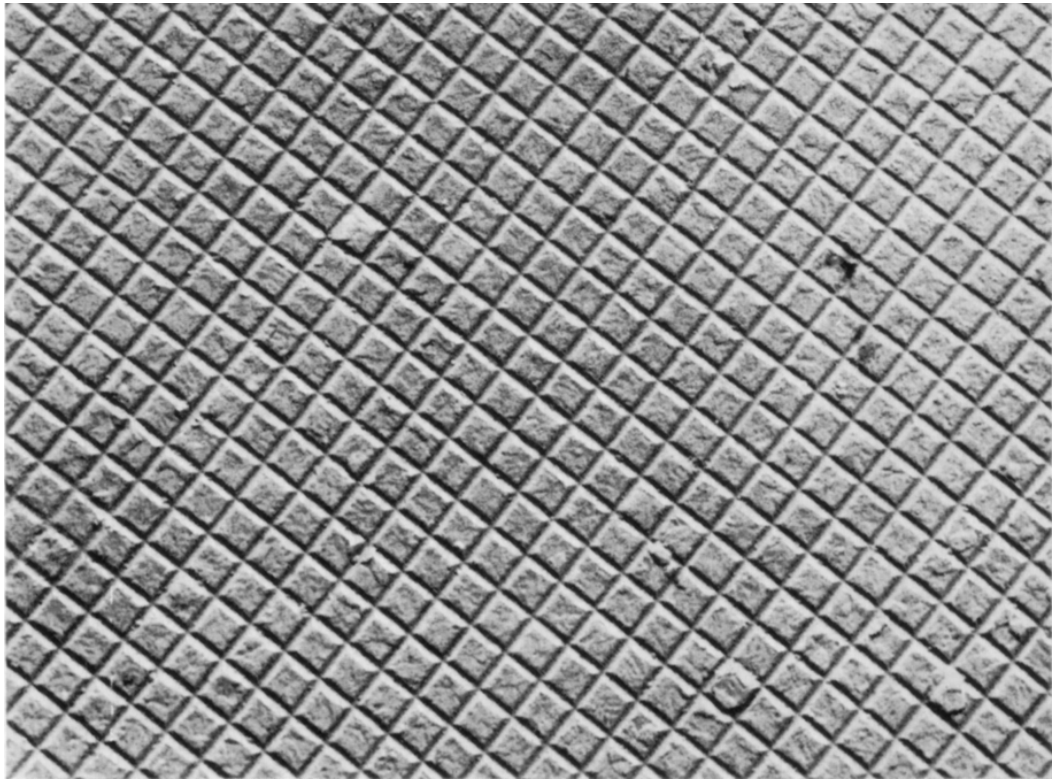
- **Add to** & **photograph with** a specimen sample

  - Accurate to **5-10% or better** if a large number are measured in the absence of contamination

I.E.6 Magnification Calibration

I.E.6.b Diffraction Grating Replicas

Replica of Cross-ruled Diffraction Grating (2160 lines/mm)

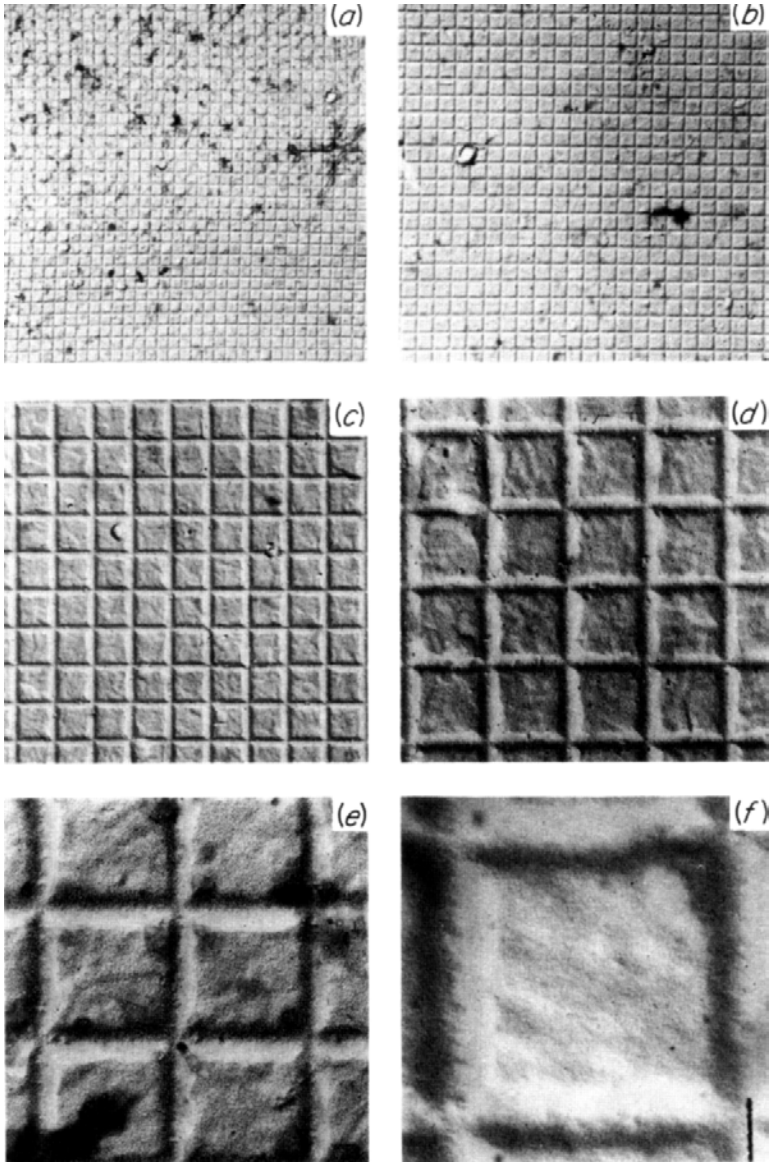


Convenient for low magnification calibrations (5-20,000X)

From Agar, Fig. 5.9, p. 162

I.E.6 Magnification Calibration

I.E.6.b Diffraction Grating Replicas



Magnification calibration with a shadowed diffraction grating replica

A square grating (2,160 lines/mm) taken at nominal instrumental magnifications of:

(a) 3,000
(b) 5,000
(c) 10,000
(d) 20,000
(e) 30,000
(f) 60,000

From Meek 1st ed., Fig. 12.7, p. 336

I.E.6 Magnification Calibration

I.E.6.c Crystalline Specimens with Known Lattice Spacings

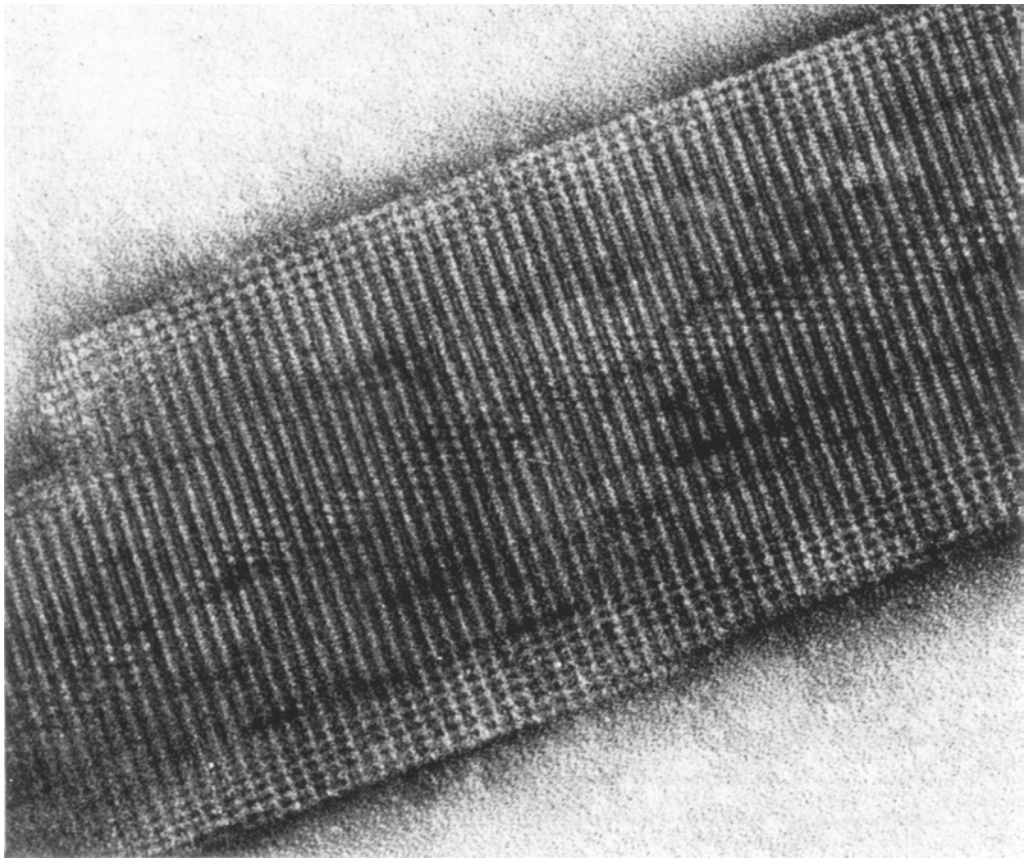
Internal calibration standards for biological specimens examined at medium to high magnification.

- Beef liver catalase (8.75 x 6.85 nm spacings)
- Tobacco mosaic virus (2.3 nm axial spacing)
- T4 bacteriophage tails (3.9 nm axial spacing)

I.E.6 Magnification Calibration

I.E.6.c Crystalline Specimens with Known Lattice Spacings

Beef live catalase crystal negatively-stained with ammonium molybdate



Spacing between lines: 8.75 nm
Periodicity along lines: 3.43 nm
Nominal magnification ~150,000X

From Agar, Fig. 5.10, p. 163

I.E.6 Magnification Calibration

I.E.6.d Very High Magnification

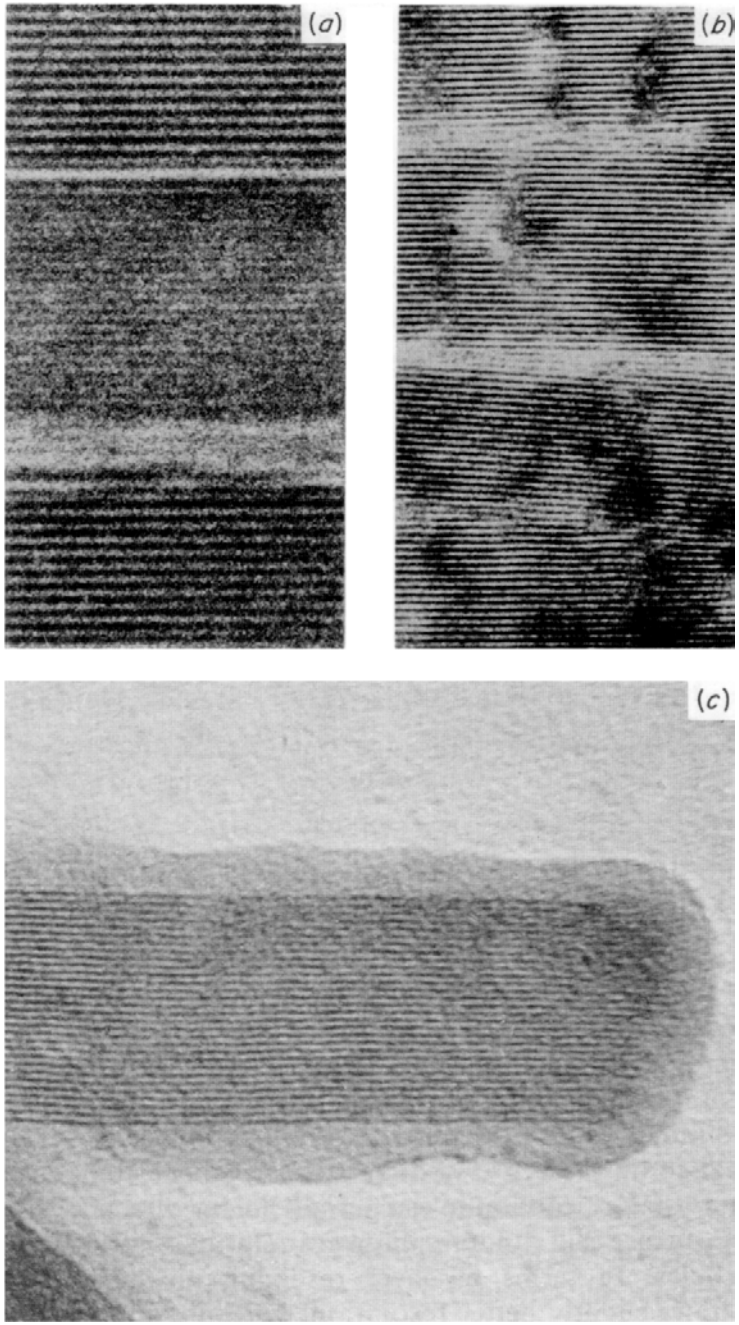
Crystal Lattice Plane Resolution Measurement

At very high magnification (>100,000X), a number of calibration standards are useful

a) Cu-phthalocyanine with **0.98** and **1.26 nm** lattice spacings

b) K-chloroplatinate with **0.699 nm** spacing. Very susceptible to beam damage

c) Pt-phthalocyanine crystal with **1.25 nm** lattice spacing.



From Meek 1st ed., Fig. 12..6, p. 334

I.E OPERATION OF THE TEM

I.E.6 Magnification Calibration

Cautionary Notes:

Measurements of spacings in micrographs should be made **directly from the micrograph** and **NOT from a photographic enlargement**

- Enlarger may introduce distortions

- Photographic papers can shrink or stretch with time

I.E OPERATION OF THE TEM

I.E.7 Resolution Tests

Recall: Resolving power of the microscope = best possible performance as limited by built-in instrumental parameters.

Record micrographs of a suitable test specimen & then measure the **actual resolution** achieved **in the image**

I.E.7 Resolution Tests

I.E.7.a Point Separation Test

- Photographic image of small particles is searched for two particles which can **just be distinguished** as separate

- Distance between centers defines the resolution

- **Two successive** photographs at same focal setting are needed and the **same** particles must be located on **both** micrographs to ensure electron noise is negligible

  - **Several pairs** of particles in **different orientations** are needed to demonstrate that astigmatism or other image defects are insignificant

I.E.7 Resolution Tests

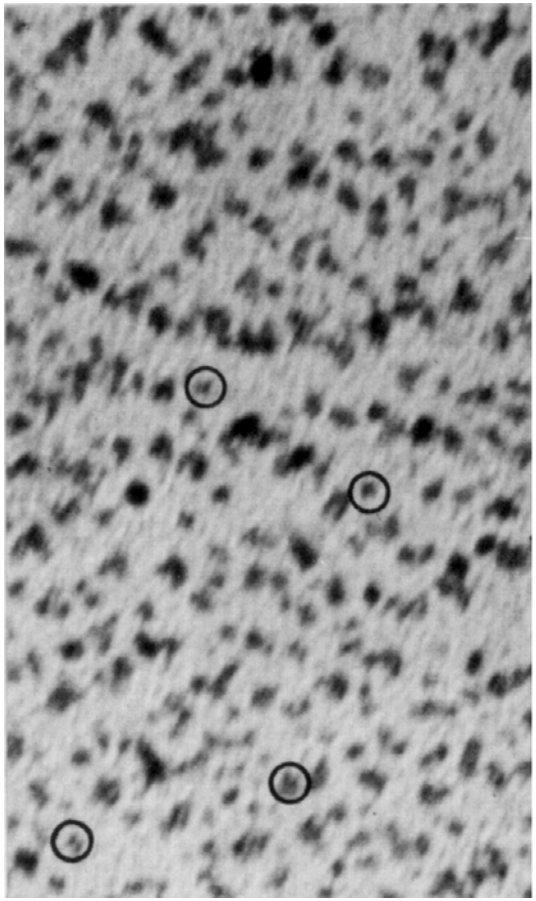
I.E.7.a Point Separation Test

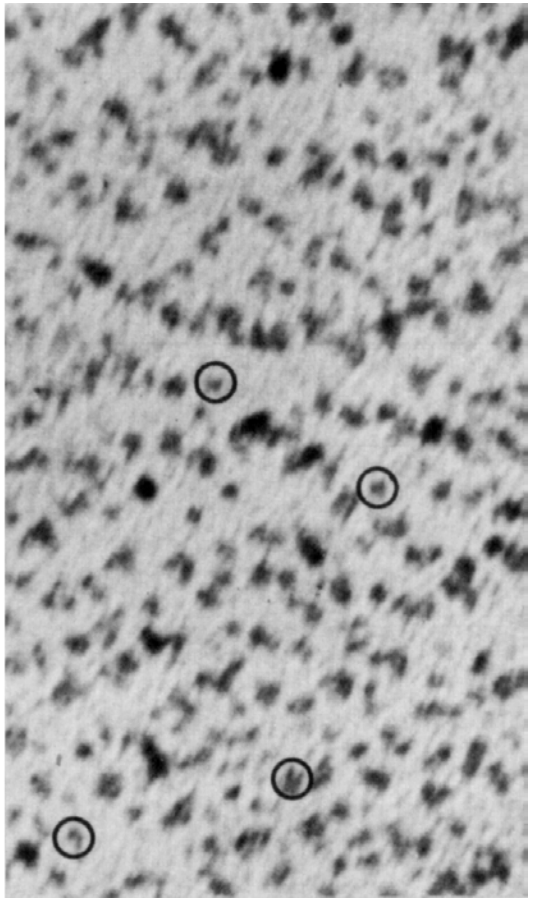
Point Separation Resolution Measurement

Specimen: platinum-iridium on carbon substrate

Pairs of particles show spacings of < 1.0 mm (at the overall magnification of ~ 2,000,000X indicates resolving power of better than 5 Å

Two micrographs must be recorded to ensure that random phase granularity is not measured by mistake for actual particles





From Meek 1st ed., Fig. 12.5, p. 333

I.E.7 Resolution Tests

I.E.7.b Lattice Resolution Test

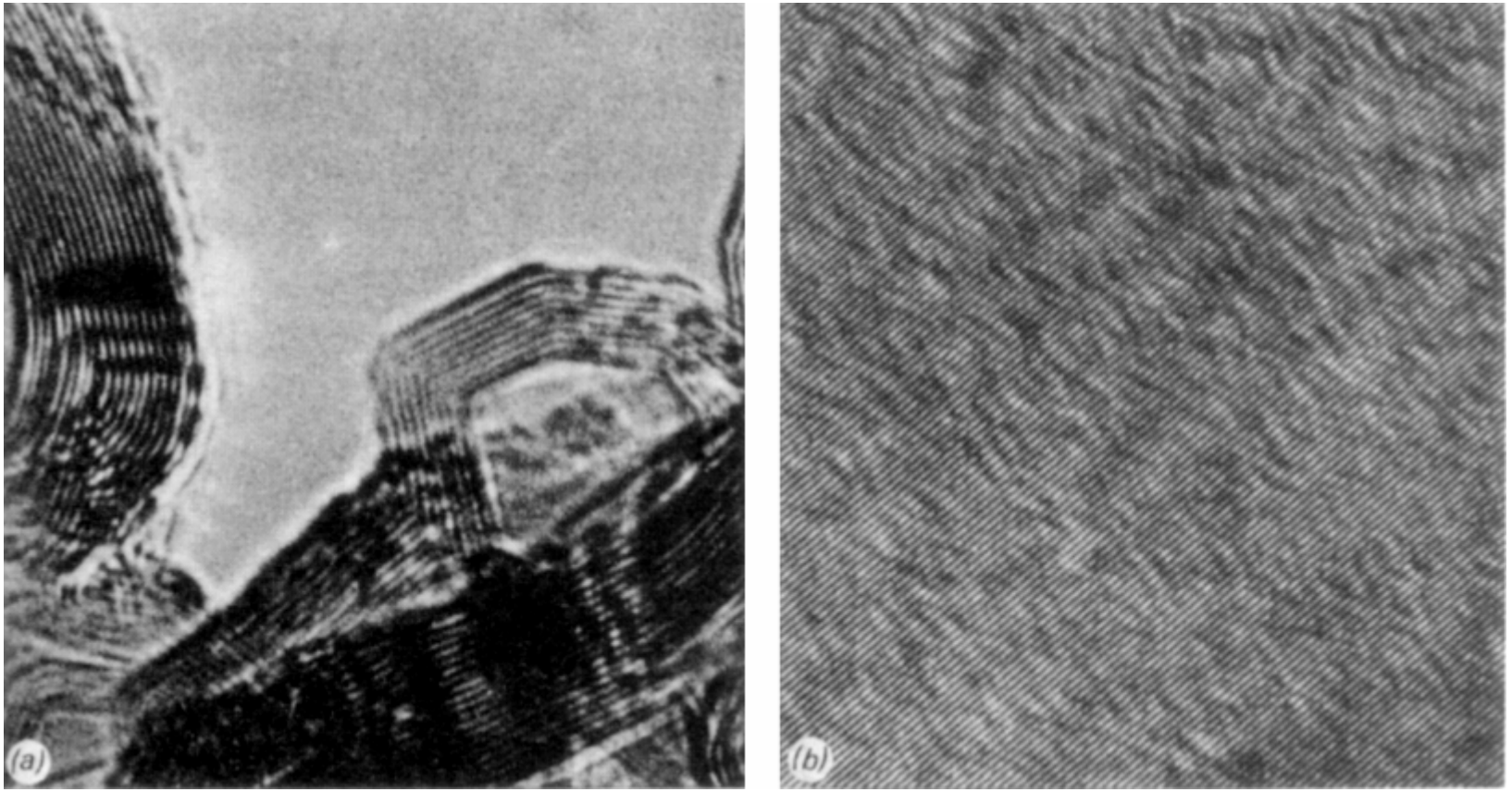
Specimen: suitably-oriented thin crystal with accurately known lattice spacings (*e.g.* from X-ray data)

- Best to use a specimen that has lattice planes oriented in more than one direction

  - **Partially graphitized carbon black:** has lattice planes (0.34 nm) that curve in many directions within a small region

I.E.7 Resolution Tests

I.E.7.b Lattice Resolution Test



Crystal lattice spacings in (a) graphite and (b) gold foil. Graphite lattice spacing = 3.4 Å. Gold lattice spacing = 2.04 Å. Magnification in book ~2,500,000X.

From Meek 2nd ed., Fig. 13.8, p. 317

I.E.7 Resolution Tests

I.E.7.b Lattice Resolution Test

- Lattice line test generally regarded as **stability test** rather than a guide to the performance with other specimens
- Particle separation & lattice plane resolution provide complementary information about TEM performance
- Resolution of point objects **poorer** than lattice planes partly due to the greater ease of identifying rows of molecules in a crystal compared to separate points in a random structure
- Contrast in **crystal** images mainly from **interference** (phase) effects
- Contrast in images of **point objects** primarily a **scattering** (amplitude) effect

I.E.7 Resolution Tests

I.E.7.c Fresnel Fringe Test

Visualization of the overfocused Fresnel fringe at the edges of a hole in a thin carbon film provides a **convenient check** of TEM performance

Measure the width of finest Fresnel fringe still visible around the entire circumference of a hole in the specimen

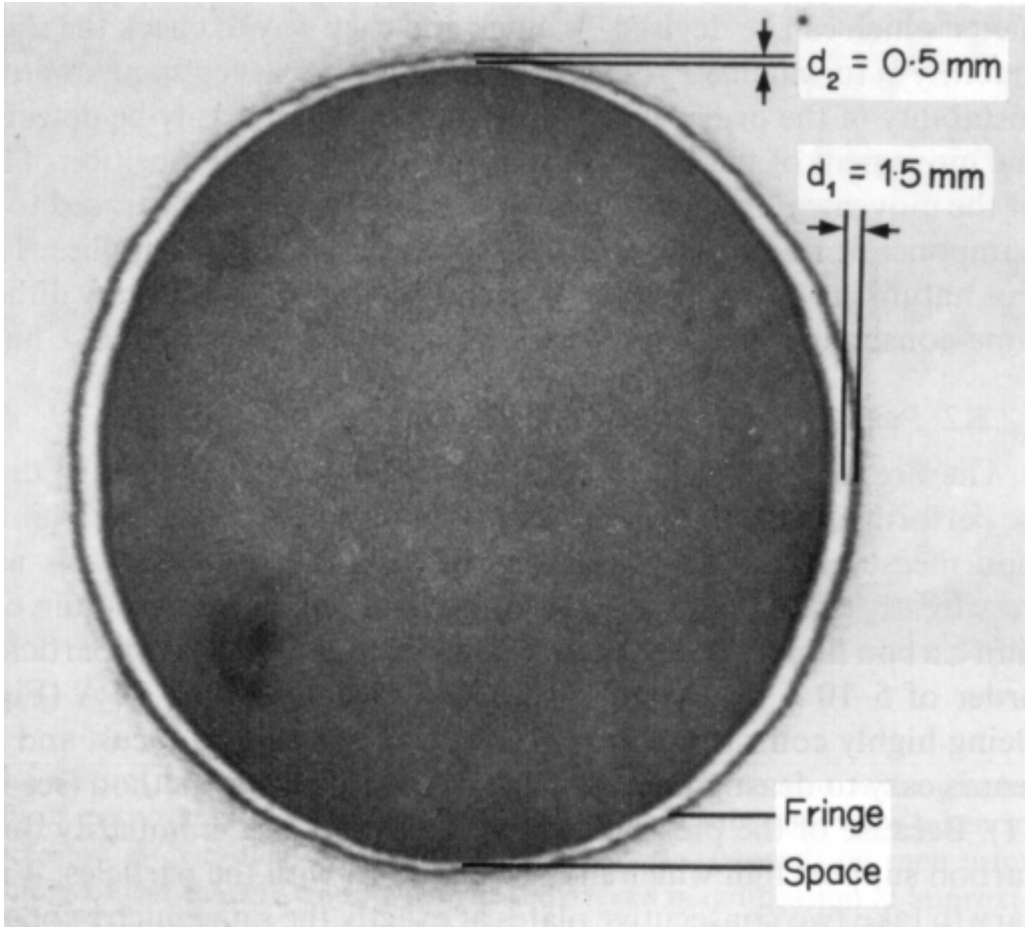
I.E.7 Resolution Tests

I.E.7.c Fresnel Fringe Test

Micrograph of hole in a carbon film with deliberately introduced astigmatism

Resolving power = difference between the d1 & d2 distances

The difference of 1.0 mm at a magnification of 500,000X indicates **2 nm** instrument resolving power



From Meek 1st ed., Fig. 12.4, p. 331

End of Sec.I.E.7