CHM 165,265 / BIMM 162 / BGGN 262 Spring 2013

Lecture Slides

Jan 24, 2013

CHM 165,265 / BIMM 162 / BGGN 262 Spring 2013

Announcements for Jan 24, 2013

Reading assignment for Tuesday: Lecture notes pp.124-195

Note: much of this material will NOT be covered in lecture

'Virtual' homework: Answers to first 4 sets posted outside NSB 4-105

Recitation session: Tomorrow 5:00-6:00 pm in York 4080A

TEM facility tour: NEXT WEEK Jan 28,29 (check web site)

CHM 165,265 / BIMM 162 / BGGN 262 Winter 2013

3D Electron Microscopy of Macromolecules

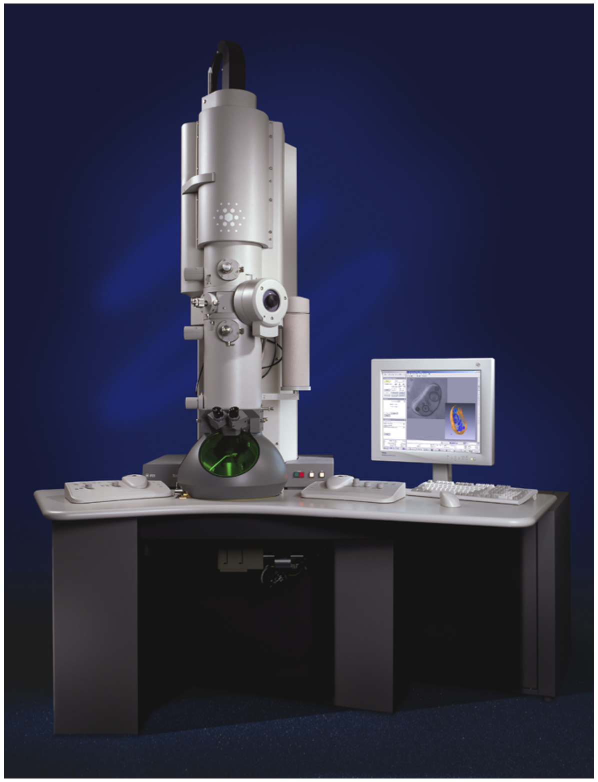
TEM Facility Tour

Where: 1510 Bonner Hall basement

When: Mon Jan 28 and Tue Jan 29th

Check class web site for details on dates, times, and directions to facility

Attendance is optional but 5 pts extra credit towards final grade will be awarded



FEI Technai Sphera
(200keV; LaB_6; LN_2)

To reserve and guarantee a time slot, email **nholson@ucsd.edu**
First come, first served.

I.C CONTRAST AND IMAGE FORMATION

KEY CONCEPTS FROM LECTURE #5

- TEM images do **NOT** give a **completely faithful** rendering of the density distribution (*i.e.* structure) of specimens

- Relationship between image and specimen is described by the **contrast transfer function** (**CTF**), which is characteristic of or influenced by:

  1) Specific TEM used (Obj lens C_s); 2) Conditions of imaging (defocus used); 3) Specimen

- **Microscope CTF** arises from the **objective lens focal setting AND** from the **spherical aberration** in the **objective lens**

$$CTF(\nu) = -\left\{\left(1 - F_{amp}{}^2\right)^{1/2} \bullet \sin\left(\chi(\nu)\right) + F_{amp} \bullet \cos\left(\chi(\nu)\right)\right\}$$

where $$\chi(\nu) = \pi\lambda\nu^2\left(\Delta f - 0.5C_s\lambda^2\nu^2\right)$$

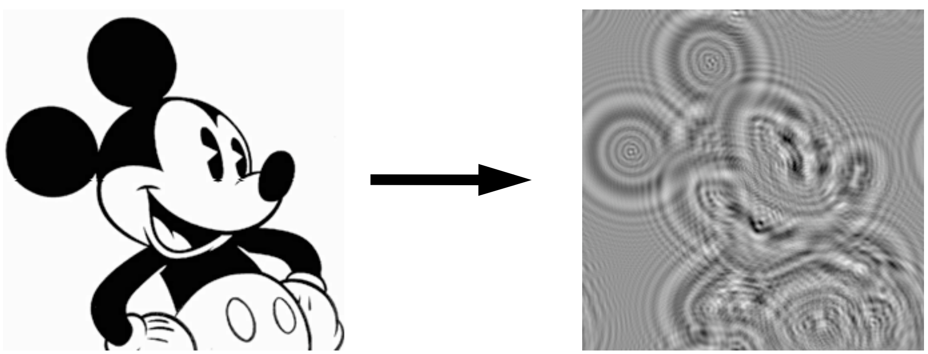
I.C CONTRAST AND IMAGE FORMATION

KEY CONCEPTS FROM LECTURE #5

- **Microscope** **CTF** arises from the **objective lens focal setting** **AND** from the **spherical aberration** in the lens

$$CTF(\nu) = -\left\{\left(1 - F_{amp}{}^{2}\right)^{1/2} \bullet \sin(\chi(\nu)) + F_{amp} \bullet \cos(\chi(\nu))\right\}$$

where $$\chi(\nu) = \pi\lambda\nu^{2}\left(\Delta f - 0.5C_{s}\lambda^{2}\nu^{2}\right)$$



Object **Image**

I.D ALIGNMENT/ADJUSTMENT OF THE TEM

MORE CONCEPTS FROM LECTURE #5

- TEM **alignment** affects resolving power and convenience of operation

- Goal of alignment: make optical elements of TEM **coaxial**

- Principle for aligning any electromagnetic lens based on image rotations caused by fluctuating the current (or voltage) in lenses

- **Small** changes in **objective** lens current used to focus electron images

I.D ALIGNMENT/ADJUSTMENT OF THE TEM

MORE CONCEPTS FROM LECTURE #5

Top Five Disturbances to Microscope Performance

- **Contamination** leads to astigmatism, drift, and decreased contrast (*Use anticontaminator*)

- **Image drift** and **mechanical instabilities** caused by instabilities in specimen holder, stage assembly, and specimen. (*Measure drift rate*)

- **Electrical** and **magnetic instabilities** (*Use high voltage to distinguish*)

- **Image astigmatism** (*"Experts" able to correct this*)

- **Focal drift** (*Micro-discharges in gun?*)

I.E OPERATION OF THE TEM

MORE CONCEPTS FROM LECTURE #5

What happens as V is increased?

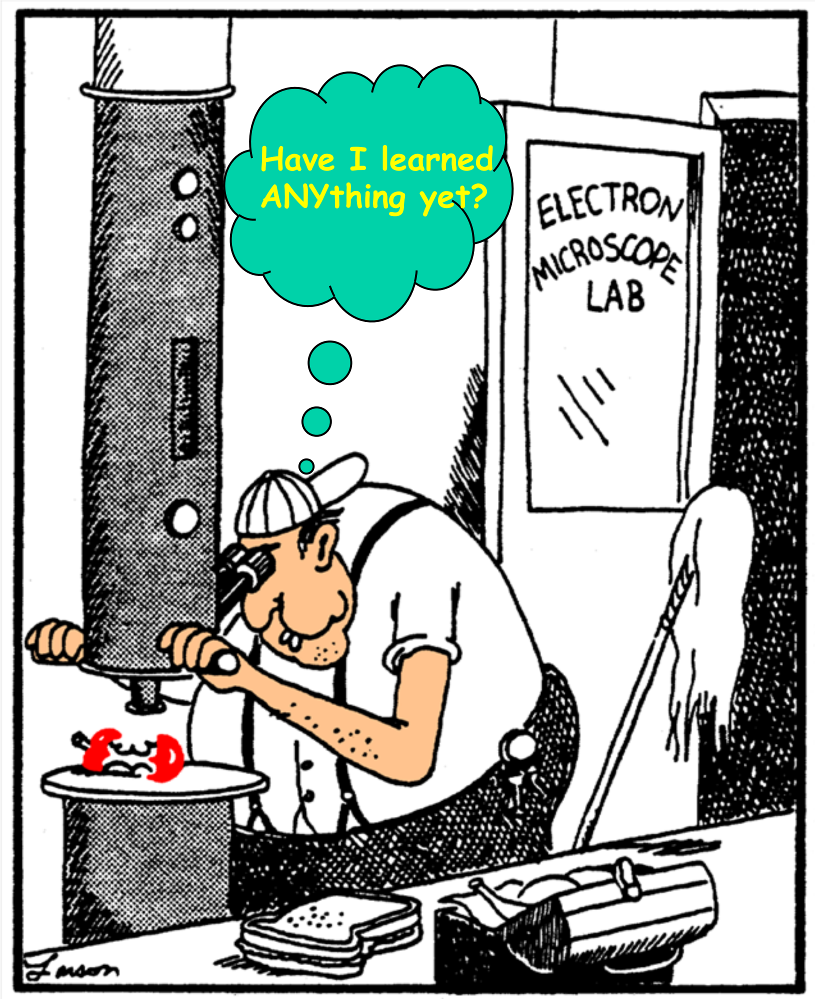
- Specimen penetration **in**creases
- Amplitude contrast **de**creases
- Resolution limit (diffraction) **improves**
- Inelastic scattering **de**creases
- Chromatic aberration **de**creases
- Radiation damage **de**creases
- Photographic emulsion efficiency **de**creases
- CCD image quality **de**creases
- Electron gun **more sensitive** to vacuum quality
- Electron gun brightness **in**creases
- Screen phosphor efficiency **in**creases

I.E OPERATION OF THE TEM

MORE CONCEPTS FROM LECTURE #5

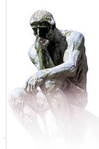
What happens as V is decreased?

- Specimen penetration **de**creases
- Amplitude contrast **in**creases
- Resolution limit (diffraction) **gets worse**
- Inelastic scattering **in**creases
- Chromatic aberration **in**creases
- Radiation damage **in**creases
- Photographic emulsion efficiency **in**creases
- CCD image quality **in**creases
- Electron gun **less sensitive** to vacuum quality
- Electron gun brightness **de**creases
- Screen phosphor efficiency **de**creases



TOPICS

- Principles of TEM
  - Electrons, lenses and optics
- Design of TEM
  - Components top to bottom
- Contrast and image formation
  - Electron scattering from object
- Optimizing TEM performance
  - Alignment assures 'best' images



- Operation of TEM
  - "What do all these buttons do?"
- Other modes of TEM
  - Many ways to 'observe' specimens
- Specimen preparation for TEM
  - Getting specimen ready
- Radiation damage
  - Less is better
- 3D reconstruction
  - Specimen 3D structure from 2D images

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 (analog and digital)

p-Flasher Question

The contrast transfer function of the TEM is affected by which of the following?

A. The defocus setting of the objective lens

B. Spherical aberration in the objective lens

C. The voltage and therefore wavelength of the imaging electrons

D. The coherence of the electron beam

E. All of the above

§ I: The Microscope

I.E Operation of the TEM

I.E.2 Choice of Apertures

- Condenser
- Objective

I.E OPERATION OF THE TEM

I.E.2 Choice of Apertures

I.E.2.a Condenser Aperture

Bottom Line: Smaller is better……up to a point.

Small apertures are best for **high resolution** imaging

Produces a **more coherent** beam and **better phase contrast**

BUT: As **condenser** aperture size is **reduced**, fewer electrons are available to illuminate the specimen

Harder to focus and correct astigmatism accurately

Need for longer exposure times

I.E OPERATION OF THE TEM

I.E.2 Choice of Apertures

I.E.2.b Objective Aperture

Bottom Line: Smaller is also better……up to a point.

Small apertures improve scattering/amplitude contrast

- The smaller the better, right?

Small apertures reduce spherical and chromatic aberrations

- The smaller the better, right?

BUT: As **objective** aperture size is **reduced**,

- Diffraction limited resolution gets worse (Airy disk gets larger)
- Small apertures are harder to align and keep aligned, and are more sensitive to effects of contamination

I.E OPERATION OF THE TEM

- ★ Choice of accelerating voltage
- ☆ Choice of apertures

Bottom Line: Smaller is better……up to a point.

Condenser: small apertures best for high resolution

Objective: small apertures improve scattering contrast and reduce spherical and chromatic aberrations

However:

Condenser: small apertures reduce illumination and hence make it harder to focus and correct astigmatism

Objective: small apertures lead to poorer diffraction limited resolution and are harder to align and keep aligned, and are more sensitive to the effects of contamination

Slide not shown in class lecture

I.E OPERATION OF THE TEM

- ★ Choice of accelerating voltage
- ★ Choice of apertures
- ☆ Specimen stage/holder
  - Hands off the merchandise!!!
  - Specimen grid: flat and secure
  - (pp.91-92, lecture notes)
- Choice of magnification
- Focusing
- Magnification calibration
- Resolution tests
- Image intensifiers/TV displays
- Microscope maintenance
- Photography (analog and digital)

I.E OPERATION OF THE TEM

- Choice of accelerating voltage
- Choice of apertures
- Specimen stage/holder
- Choice of magnification

Focusing

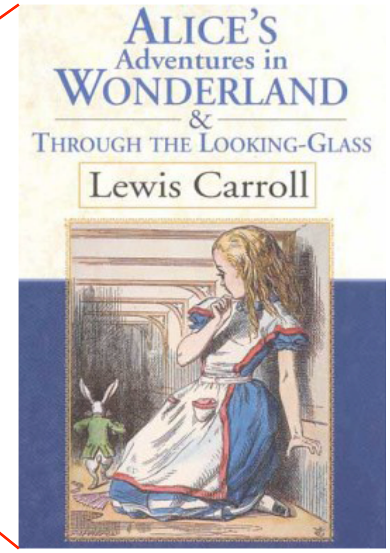
Magnification calibration

Resolution tests

Image intensifiers/TV displays

Microscope maintenance

Photography (analog and digital)



Is it appropriate?

- Field of view
- Radiation damage
- High resolution

I.E OPERATION OF THE TEM

I.E.4 Choice of Magnification

"Should I go higher or lower?"

Choice depends on the nature of the experiment

Many criteria for choosing a suitable magnification

I.E OPERATION OF THE TEM

I.E.4 Choice of Magnification

"Should I go higher or lower?"

Choose "low" (< 10,000X) to maximize field of view
(*i.e.* get the BIG picture)

Choose "high" (> 30,000X) to maximize resolution captured in the image (*i.e.* to see fine details)

I.E OPERATION OF THE TEM

I.E.4 Choice of Magnification

I.E.4.a Low Magnification (<10,000X)

- Necessary to capture 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 are used, **pincushion** and **barrel** distortions could be significant and the prints won't match correctly

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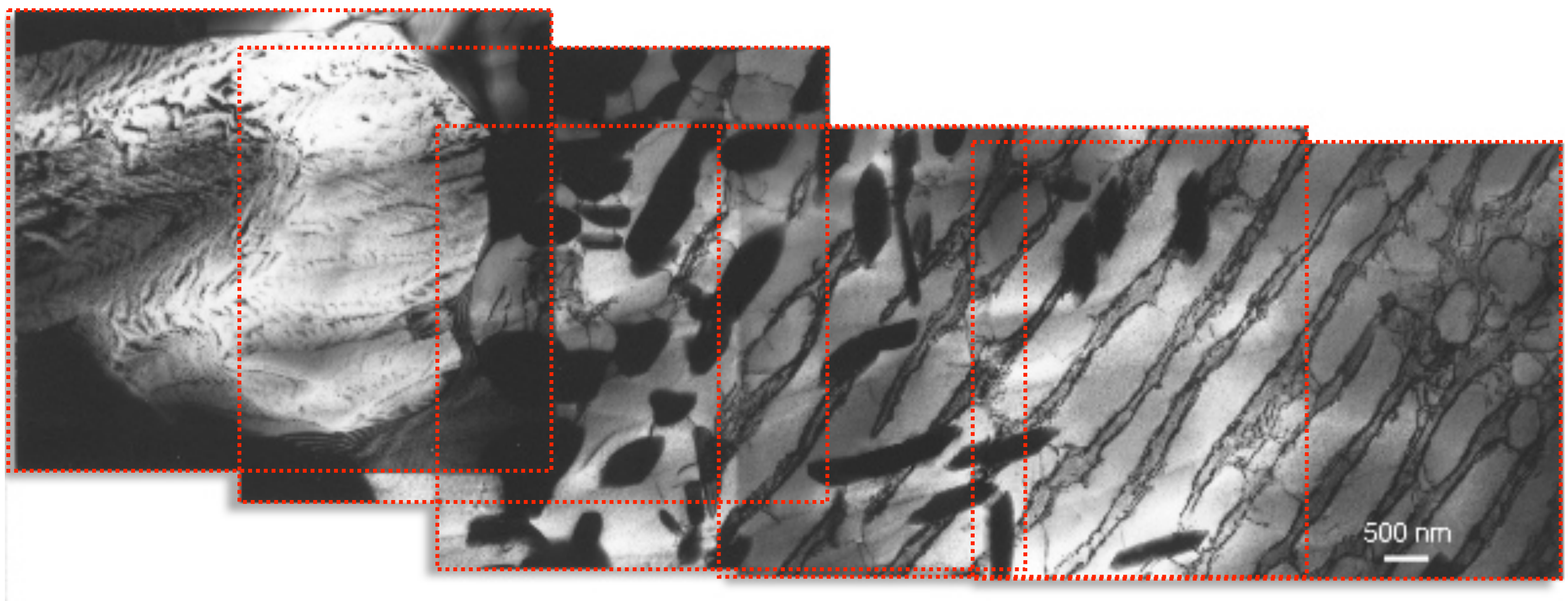
I.E.4 Choice of Magnification

I.E.4.a Low Magnification (<10,000X)

Use when need to capture a **large field of view** from a specimen in a **single** micrograph

Use if need to prepare a **montage**, where prints from several low magnification micrographs are spliced together



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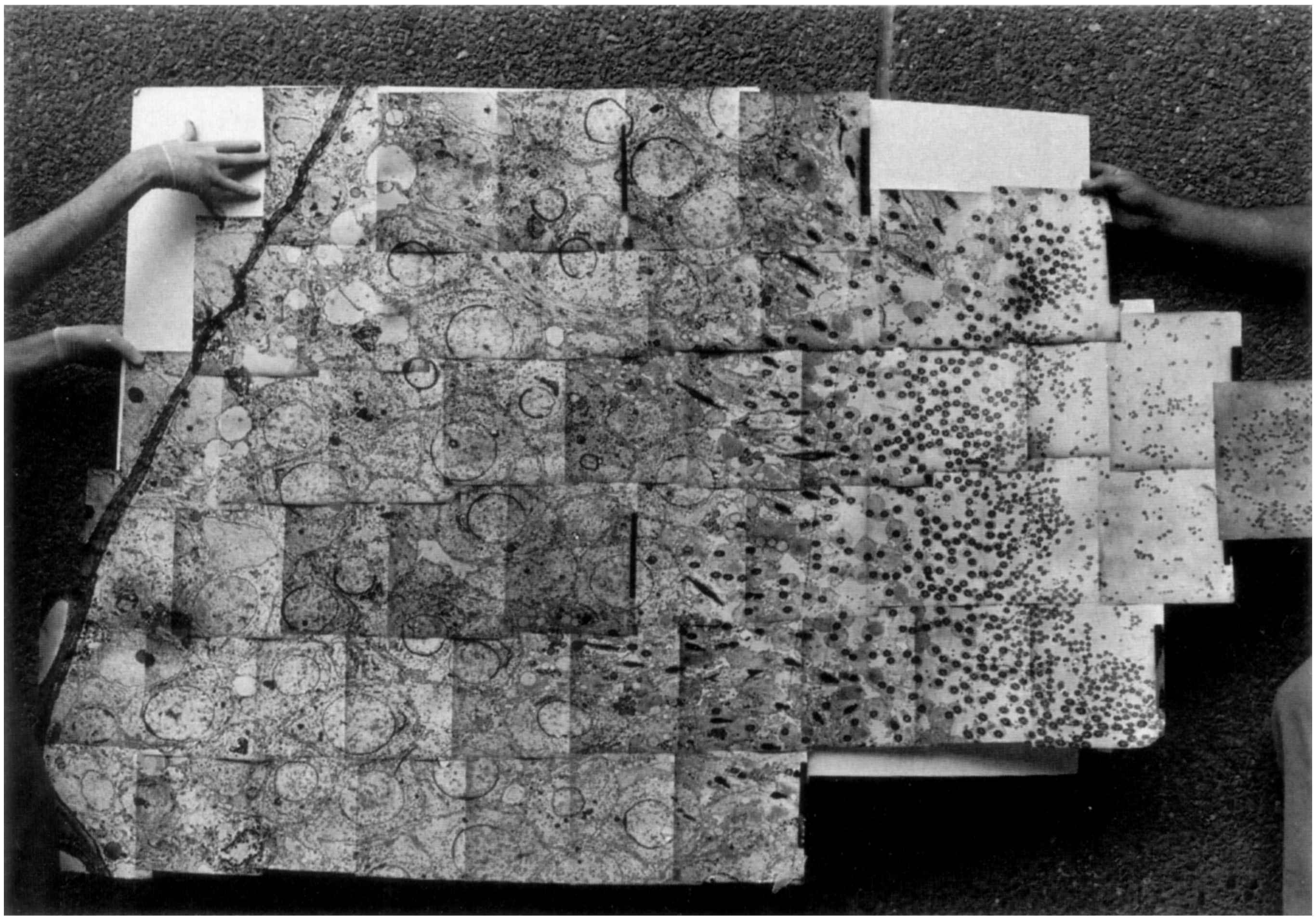
I.E.4 Choice of Magnification

I.E.4.a Low Magnification (<10,000X)

Montage of several overlapping micrographs to show extended view of tissue at high magnification



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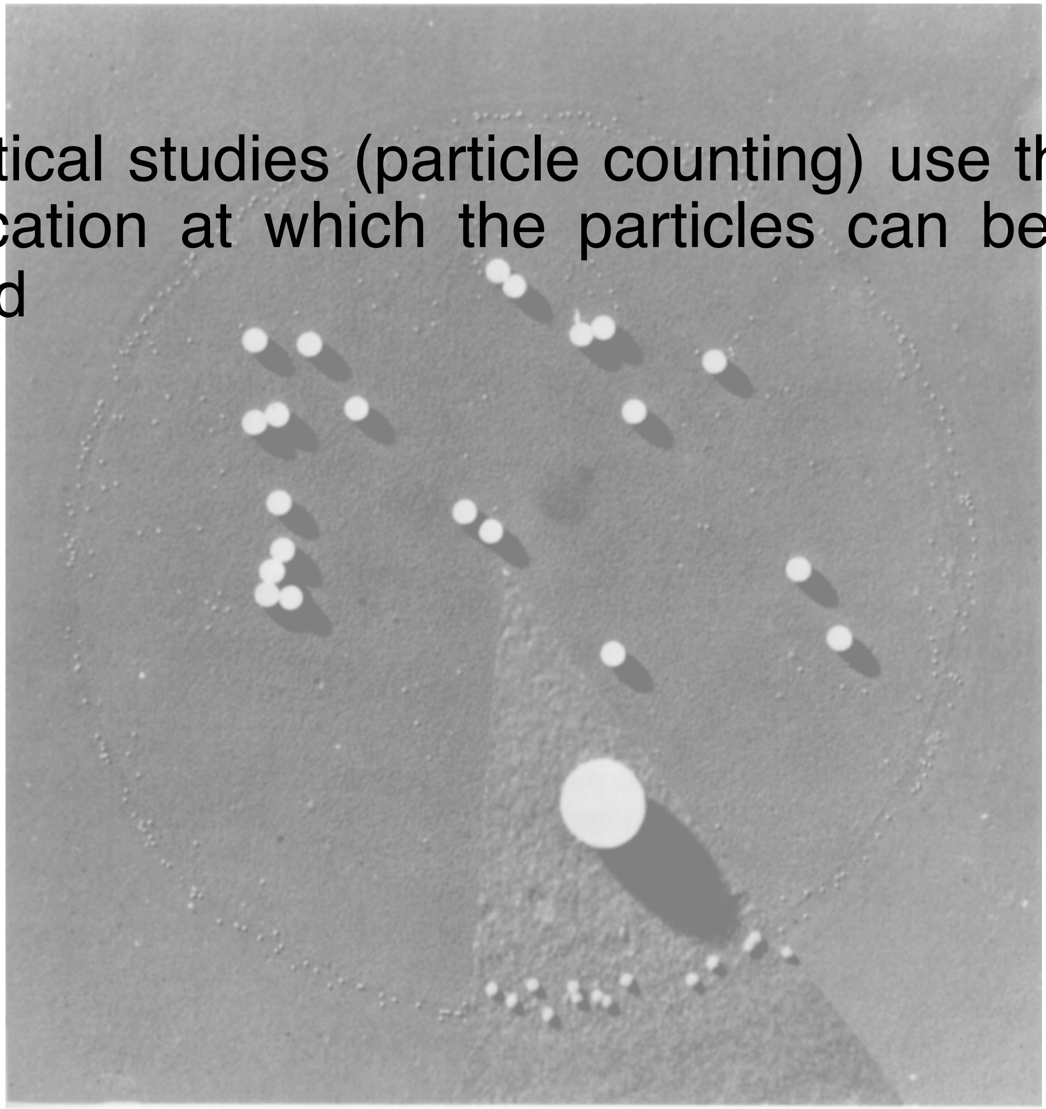
From Bozzola, 1st Ed., Fig. 8-14, p.233

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



From Hall, Fig. 10.65, p.359

Slide not shown in class lecture

I.E.4 Choice of Magnification

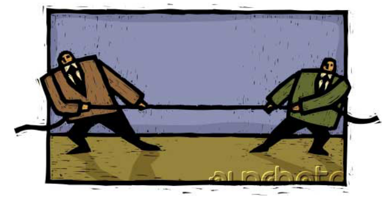
I.E.4.c Radiation Damage

Use **lowest** possible magnification **and** illumination level when **searching the grid** for a suitable region of a **radiation sensitive** specimen

Use **lowest** possible magnification **and** illumination level when **recording an image** of the desired area **but high enough** to capture desired level of **detail**

I.E.4 Choice of Magnification

I.E.4.d High Magnification



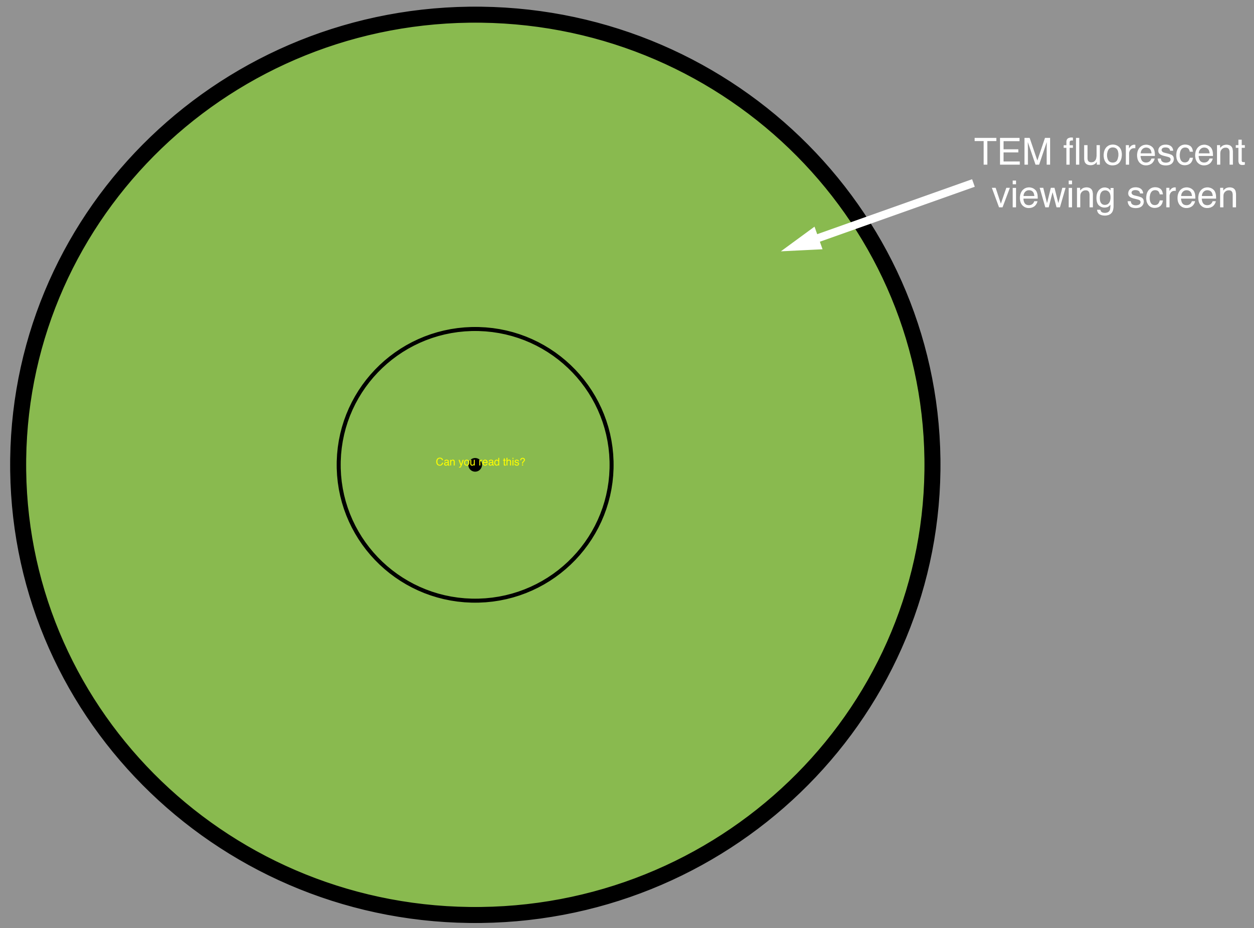
"High" magnification required to achieve **highest resolution** images (*i.e.* capture the finest specimen details)

Excessive ("empty") magnification:

Leads to unnecessary **radiation damage** because higher doses are needed to expose a photographic emulsion or CCD/DDD camera

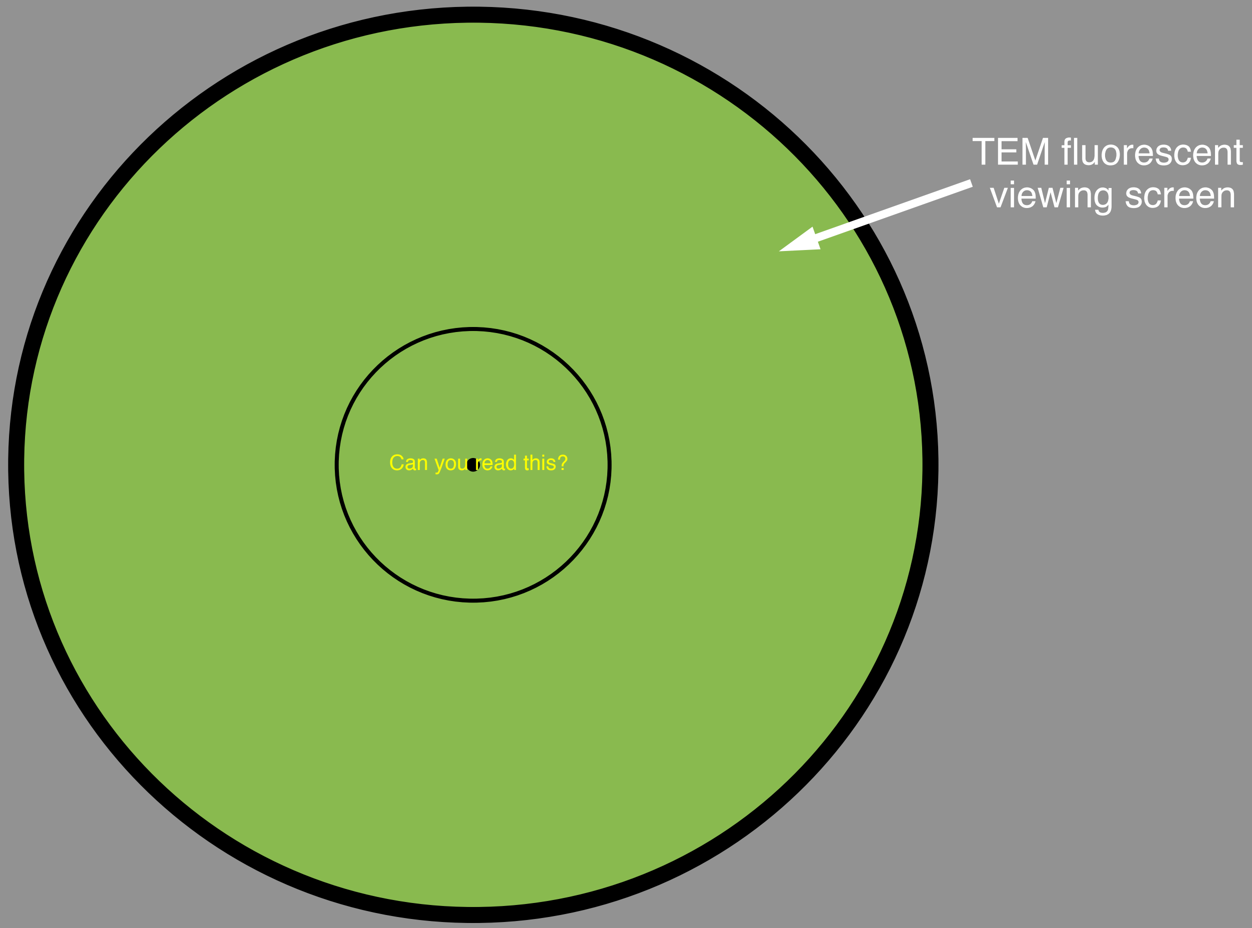
I.E.4 Choice of Magnification

I.E.4.d High Magnification



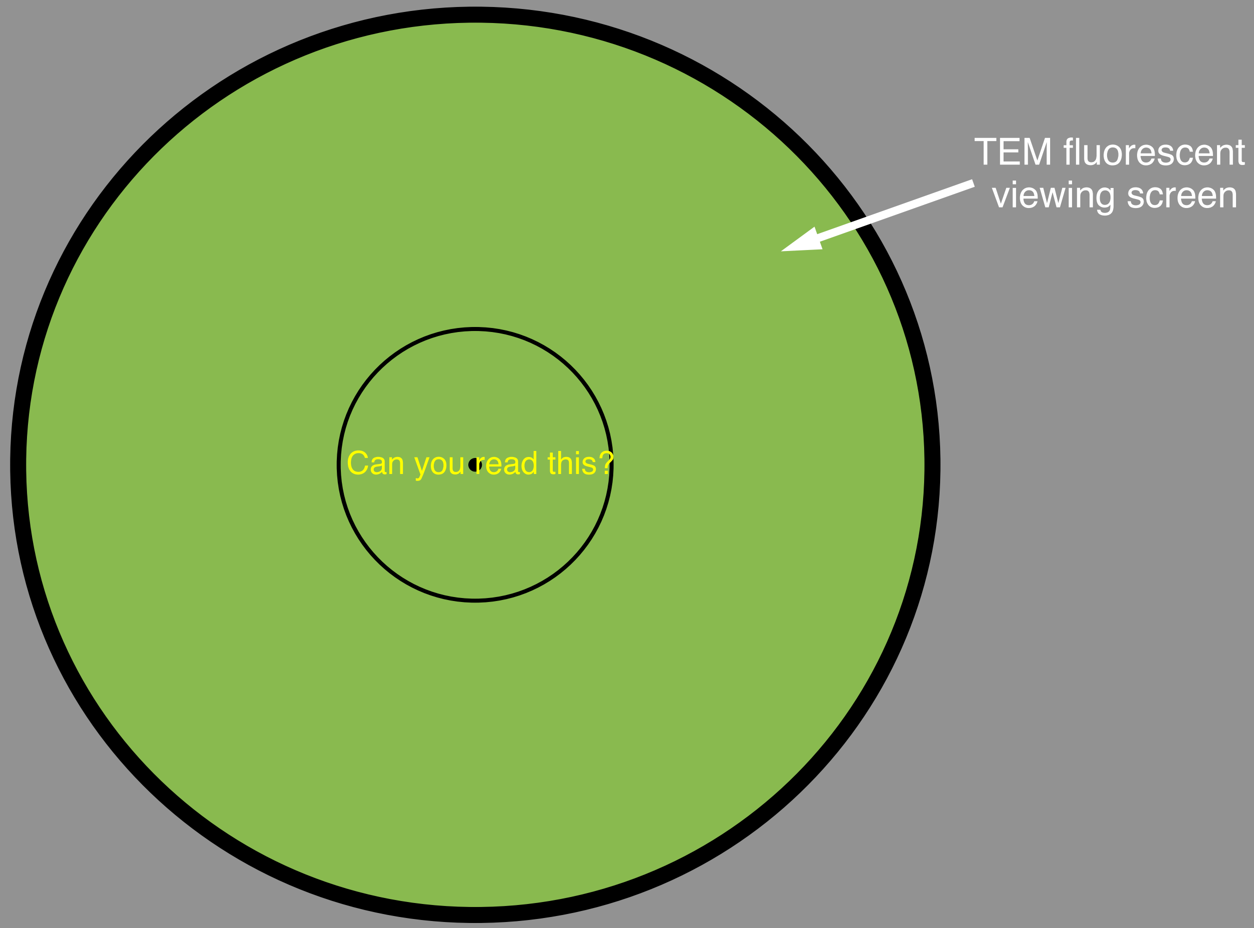
I.E.4 Choice of Magnification

I.E.4.d <u>High</u> Magnification



I.E.4 Choice of Magnification

I.E.4.d <u>High</u> Magnification

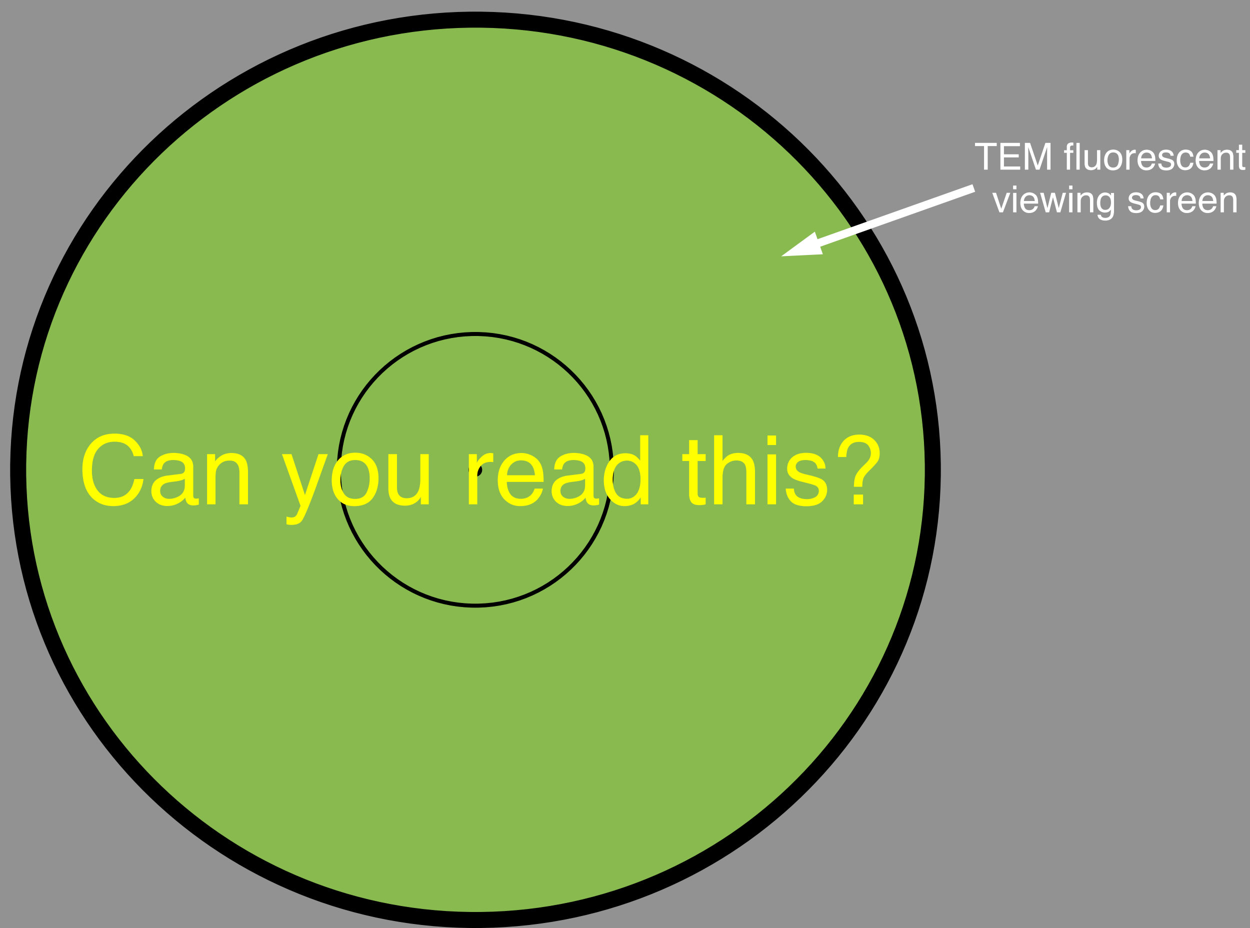


I.E.4 Choice of Magnification

I.E.4.d High Magnification

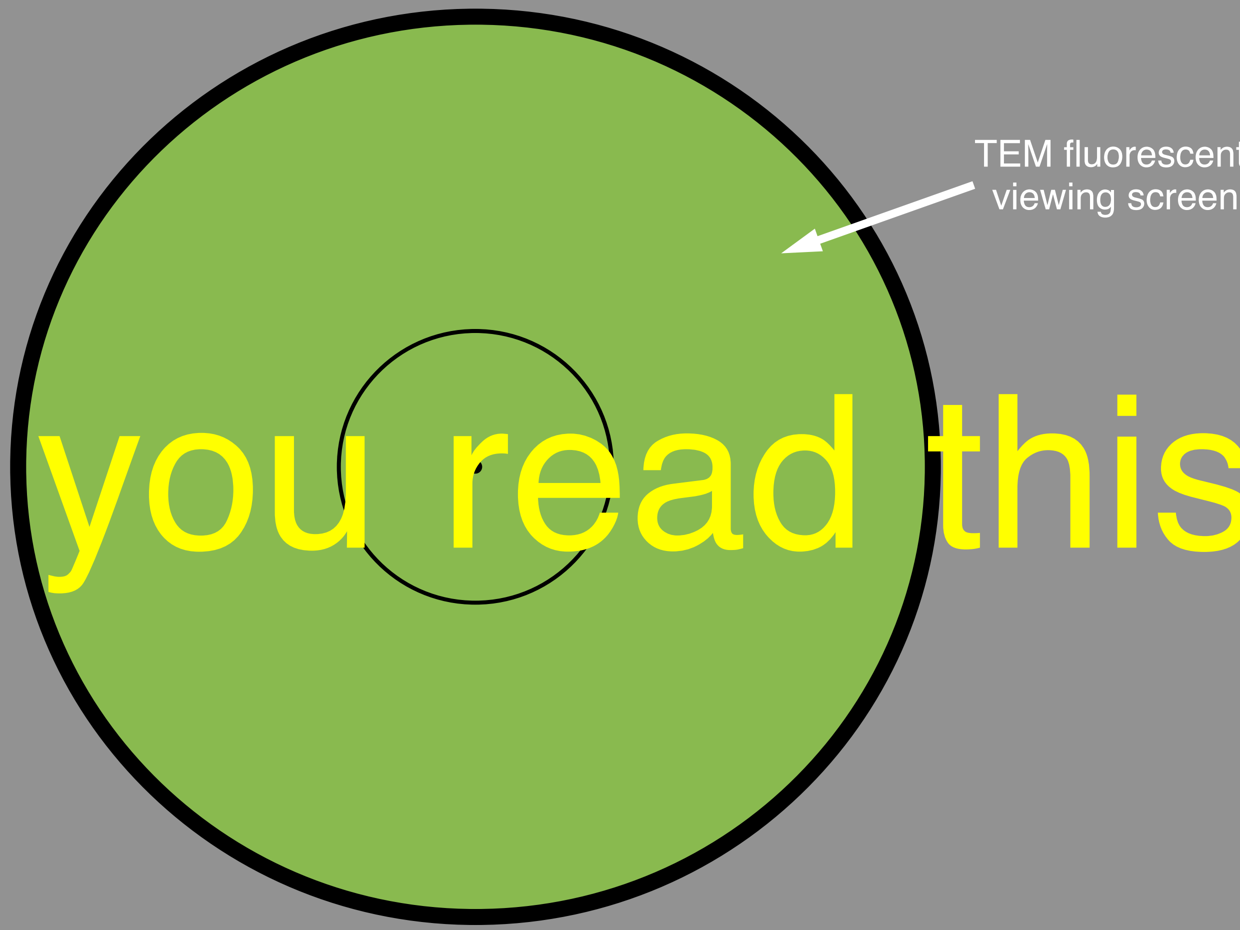
I.E.4 Choice of Magnification

I.E.4.d High Magnification



I.E.4 Choice of Magnification

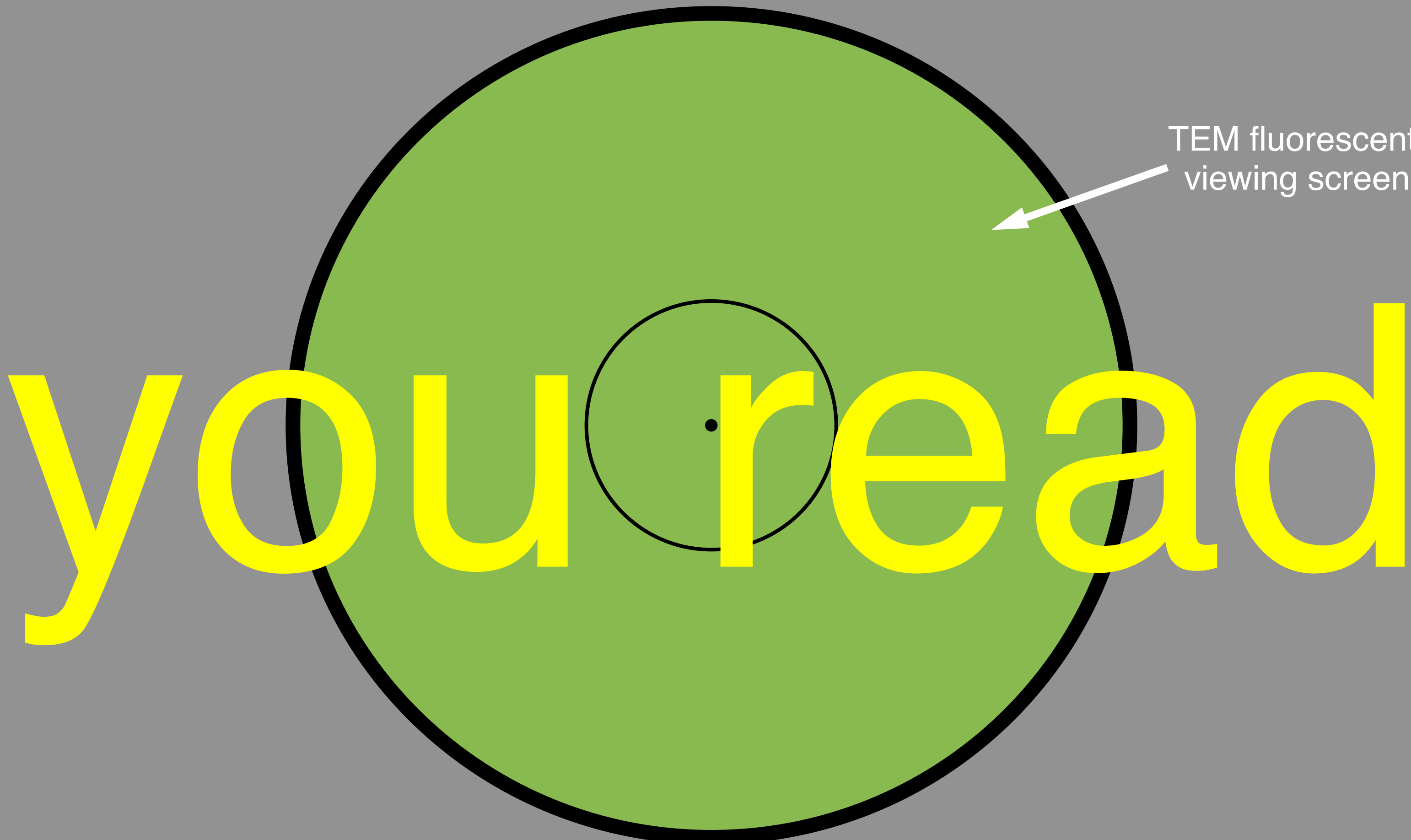
I.E.4.d High Magnification



Can you read this?

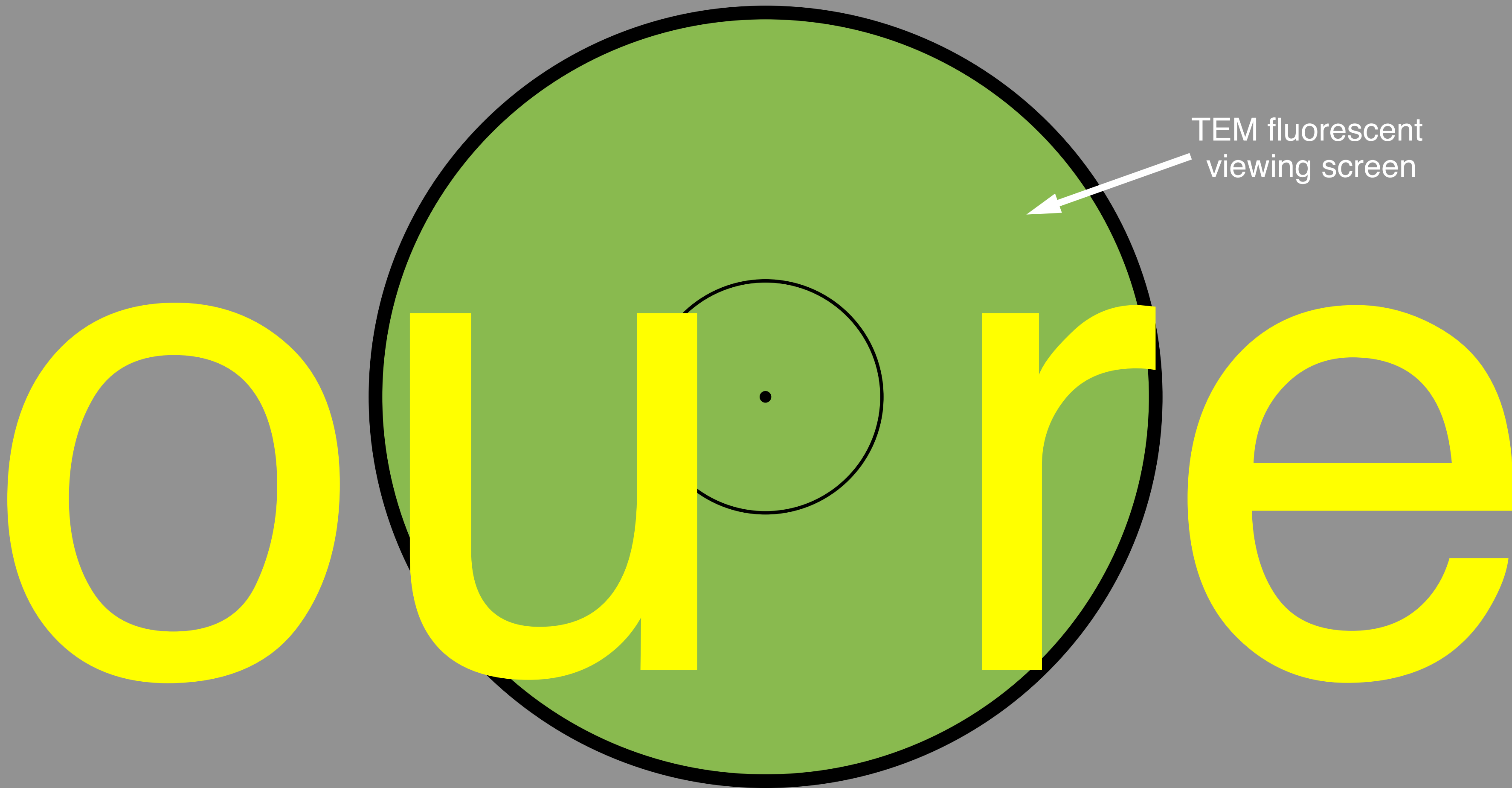
I.E.4 Choice of Magnification

I.E.4.d High Magnification



I.E.4 Choice of Magnification

I.E.4.d High Magnification



I.E.4 Choice of Magnification

I.E.4.d High Magnification

Resolution of photographic medium (or pixel size of CCD or DDD camera) ultimately determines the **maximum** magnification needed to record details at a ***predetermined or estimated*** resolution

Photographic emulsions can resolve image details **AT LEAST** as small as **20 μm**

Theoretical (*i.e.* potential) resolution of object details captured in a photographic image depends on image magnification according to:

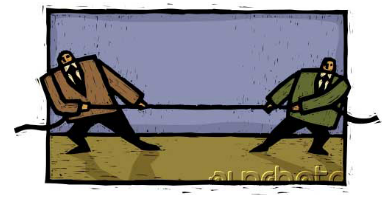
$$\text{Potential resolution} = \frac{20\ \mu m\ (\text{'pixel' size})}{\text{magnification}}$$

(captured on film)

NOTE: This is a conservative estimate since good photographic films can resolve details as small as 5-10 μm.

I.E.4 Choice of Magnification

I.E.4.d High Magnification

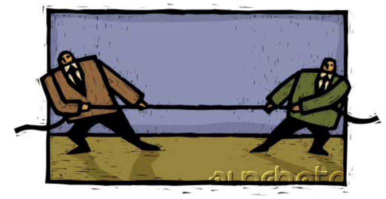


Potential resolution of object detail on film = 20 μm/magnification

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

Take home message:

Given the limited resolution that can be obtained in images of biological specimens, most “typical" microscopy is performed at magnifications **much higher than necessary** (and restricts specimen field of view)



I.E.4 Choice of Magnification

I.E.4.d High Magnification

Rule of Thumb:

Select **lowest** magnification consistent with required resolution and the recording medium used

NOTE: Each time magnification is doubled, a 4-fold increase in beam intensity is required at the specimen to maintain the same intensity level in the image

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 (analog and digital)

I.E OPERATION OF THE TEM

- Choice of accelerating voltage
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p-Flasher Question

At which one of the following objective lens focal settings does interference contrast complement aperture contrast?

A. Slight under focus

B. Slight over focus

C. Near focus

D. True focus

E. Exact focus

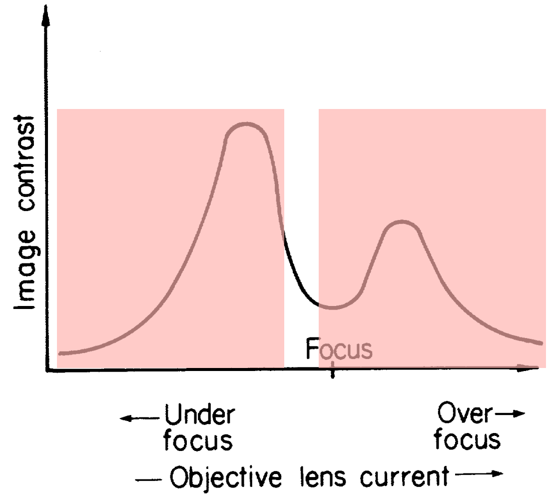
I.E OPERATION OF THE TEM

I.E.5 Focusing

- Desired focus setting is set by making **small** changes in **objective** lens current

- **True / near / exact / dead focus:** condition where, ideally, **no** Fresnel fringes will form at an image point

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



From Meek, 2nd ed., Fig. 5.3, p.100

I.E OPERATION OF THE TEM

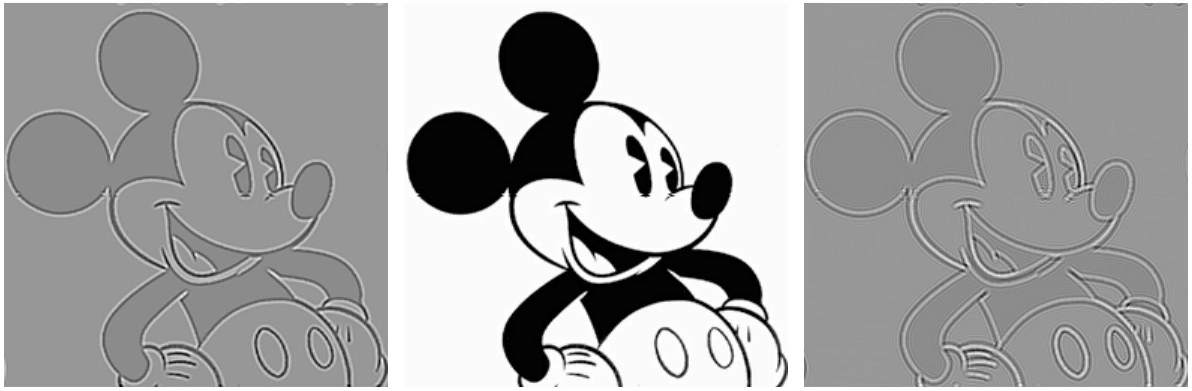
I.E.5 Focusing

- Desired focus setting is set by making **small** changes in **objective** lens current

- **True / near / exact / dead focus:** condition where, ideally, **no** Fresnel fringes will form at an image point

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

Here, aperture contrast will be **enhanced** with interference contrast (*i.e.* they work TOGETHER)

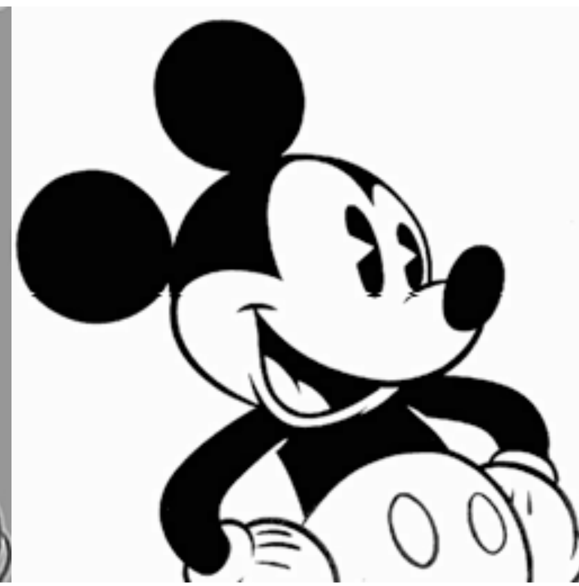


I.E.5 Focusing

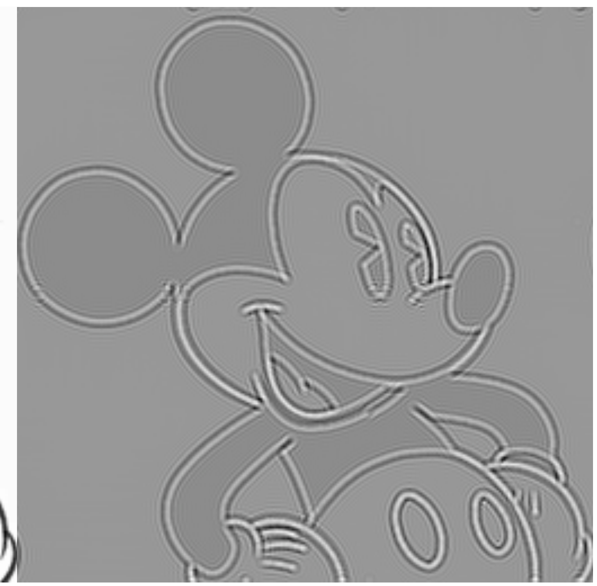
Slight degree of **underfocusing** gives optimum results

Here, aperture contrast will be **enhanced** with interference contrast (*i.e.* they work TOGETHER)

Slight underfocus
(5% Amplitude, 95% Phase)



In focus
(100% Amplitude)



Slight overfocus
(5% Amplitude, 95% Phase)

I.E.5 Focusing

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

Primary Methods

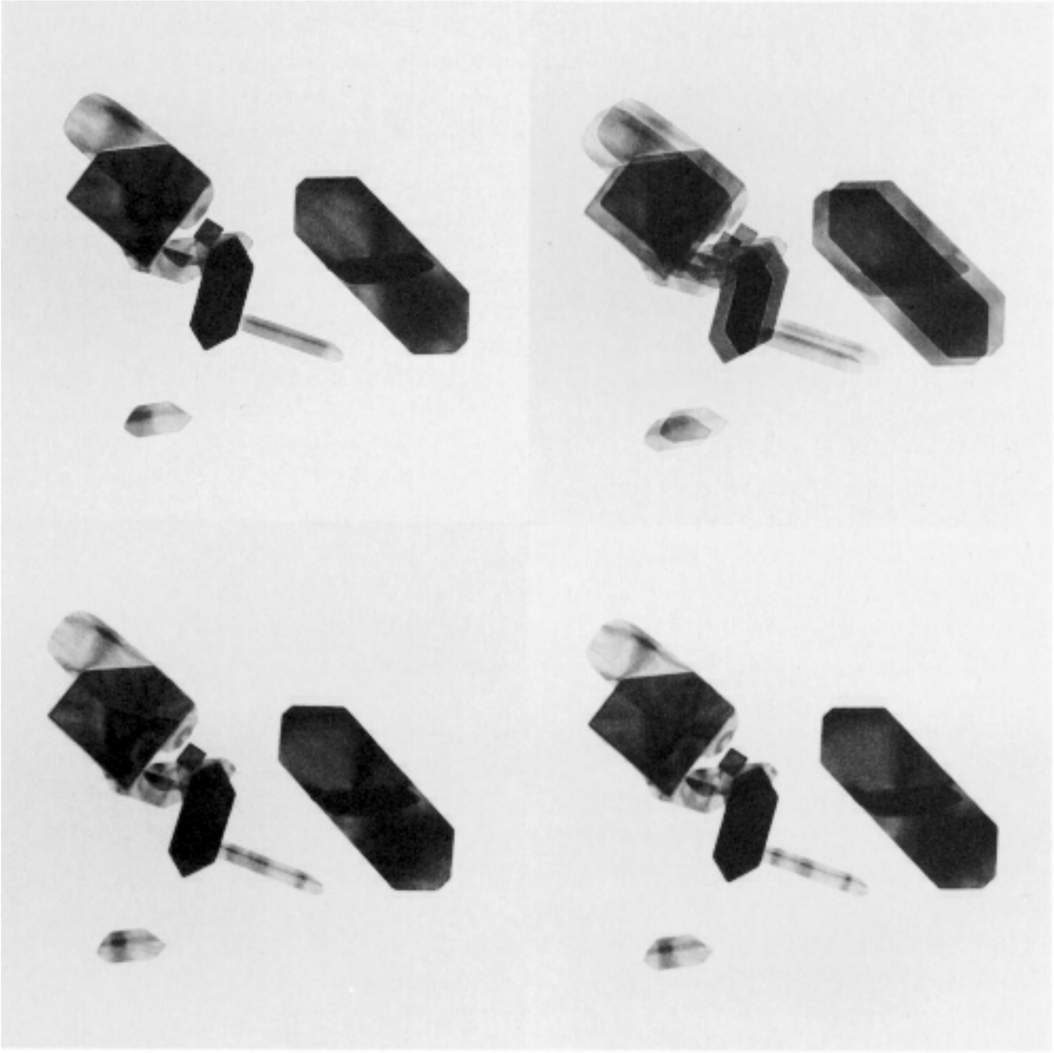
1. **Wobbler**
2. **Minimum Contrast**

(see p.93 of lecture notes)

I.E.5 Focusing

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

Focusing with a Wobbler Aid



Out of focus

In focus

Wobbler off   Wobbler on

From Watt, Fig. 2.11b, p. 30

I.E.5 Focusing

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

2. Minimum Contrast Method

(see p.93 of lecture notes)

A. Withdraw objective aperture

B. Focus objective lens to minimize image phase contrast
(i.e. try to make image “disappear”)

C. Reinsert objective aperture and record image

Not the most effective or recommended way to focus!

1) Objective aperture may become misaligned

2) Too crude for careful imaging esp. at high resolution

Slide not shown in class lecture

I.E.5 Focusing

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

Primary Methods

1. **Wobbler**
2. **Minimum Contrast**

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

Primary Method

Forget the specimen; use the support film!

I.E.5 Focusing

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

Forget the specimen; use the support film!

- Focus image based on the **appearance** of the **support film** (“sub-structure” seen is mainly just phase contrast Fresnel fringes)

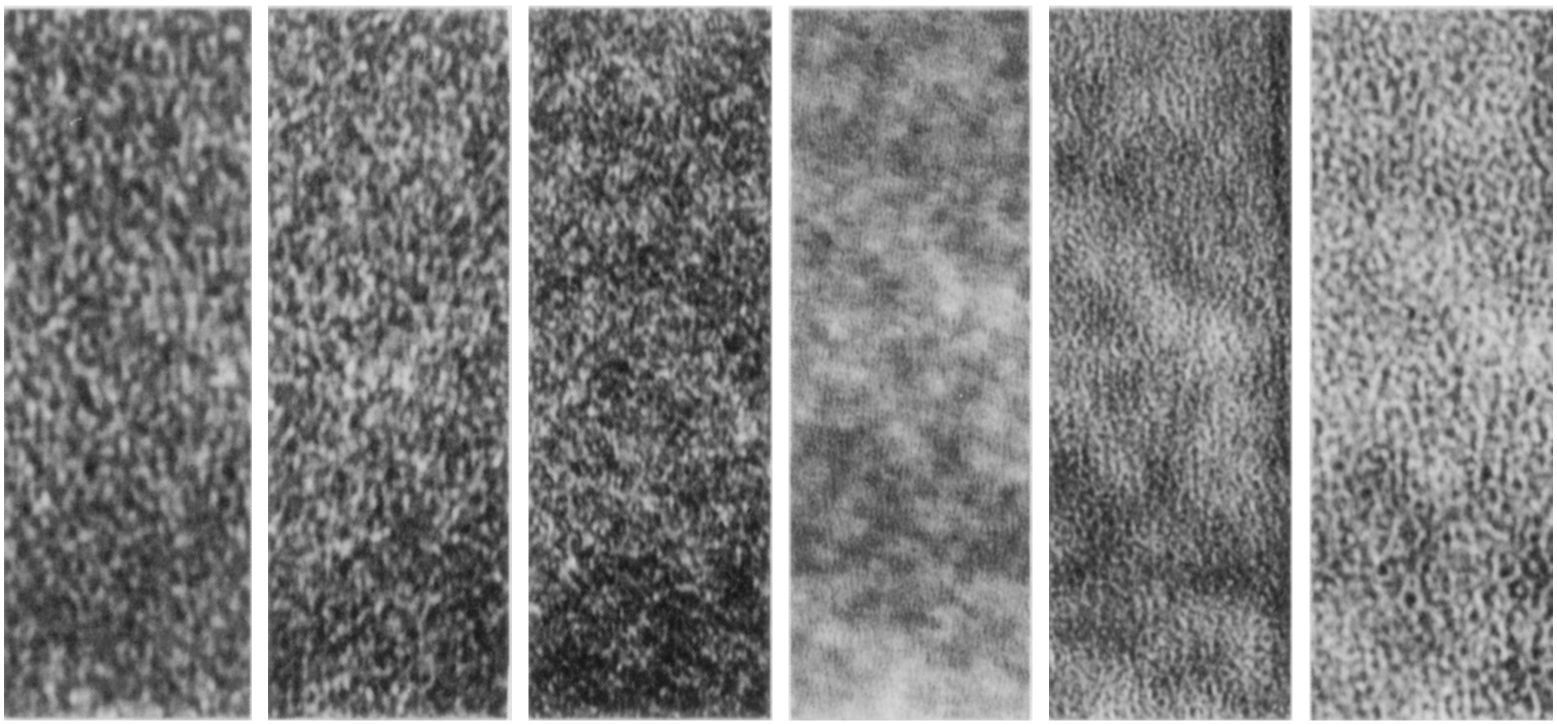
- Best **NOT** to judge proper focus based merely on the appearance of a specimen, especially a new or unfamiliar one

Support film is a predictable (well-defined, well-behaved) "specimen" with a characteristic appearance at near focus settings

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Through Focus Series:
Thin Carbon Film Lightly Shadowed with Platinum



-2.1 μm | -1.4 μm | -0.7 μm | near focus | 0.7 μm | 1.4 μm

← **Under focus** **Over focus** →

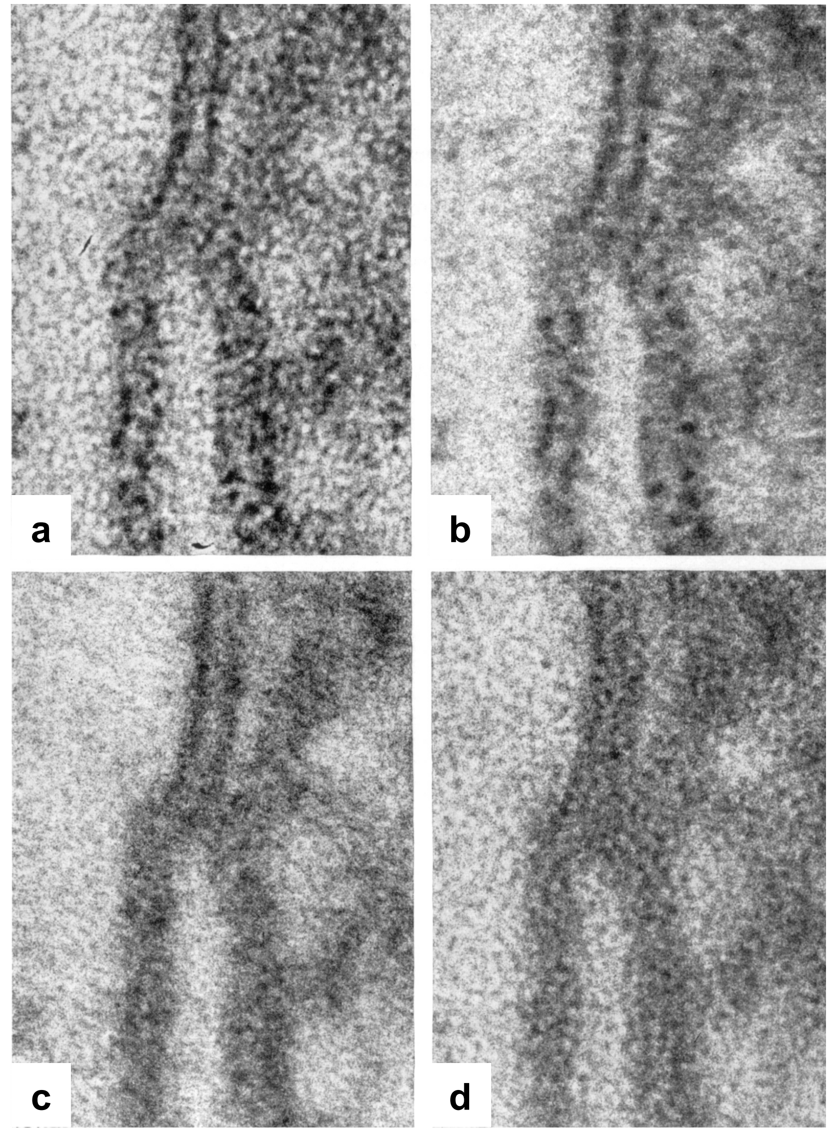
From Meek 1st ed., Fig. 11.1, p. 294

I.E.5 Focusing

I.E.5.b High Magnification Focusing

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

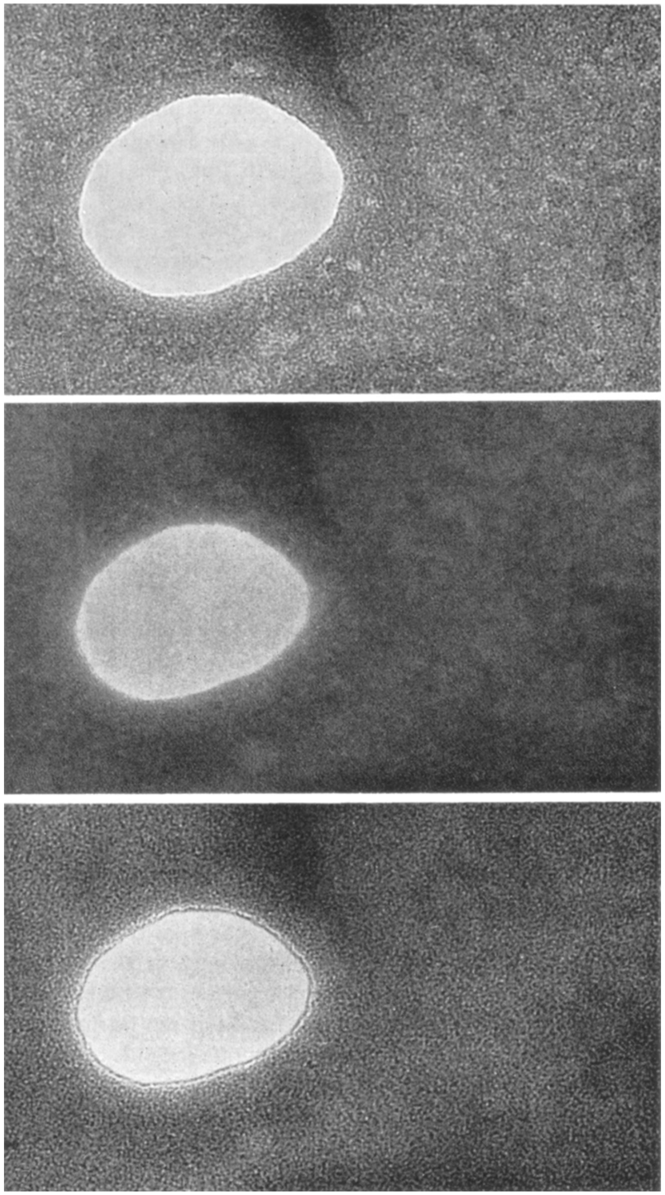
a) Under focus
b) Close to focus
c) Slightly over focused
d) Over focused



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

Bright line inside hole (objective lens too weak)

Near focus

Over focus

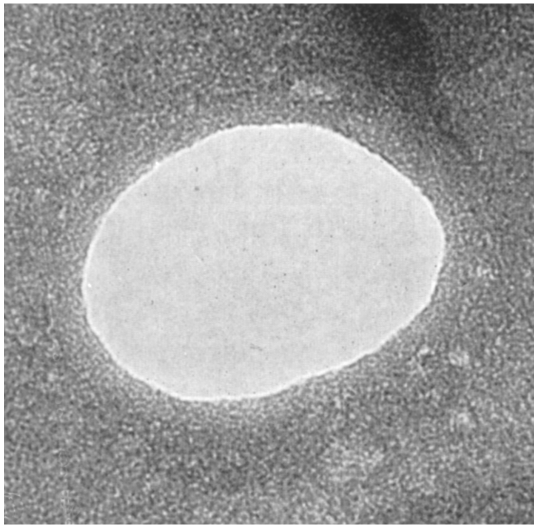
Bright line inside carbon film (objective lens too strong)

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a Thin Carbon Film



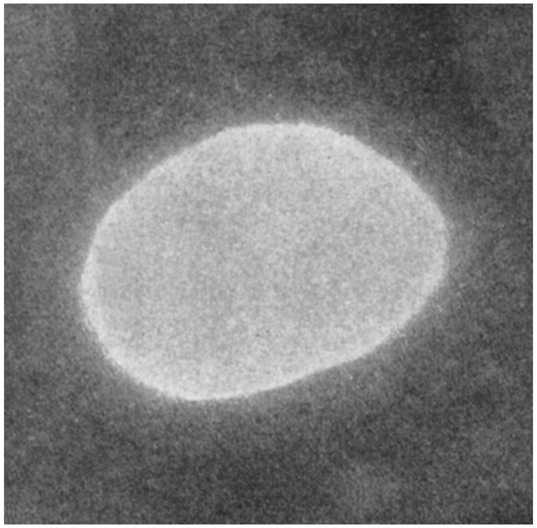
Under focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a Thin Carbon Film



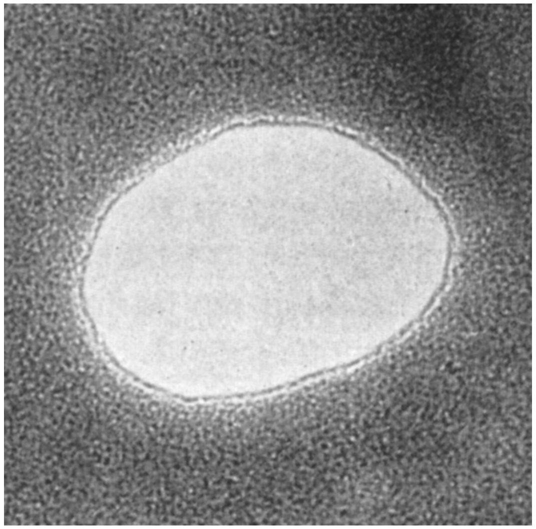
Near focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing

I.E.5.b High Magnification Focusing

Hole in a Thin Carbon Film

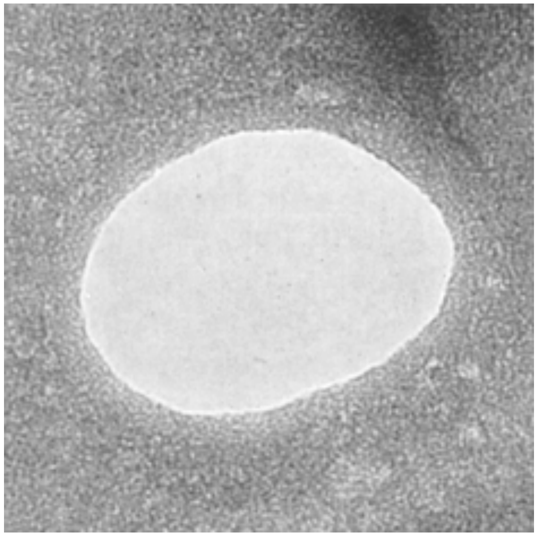


Over focus

From Agar, Fig. 4.11, p. 137

I.E.5 Focusing
I.E.5.b High Magnification Focusing

Hole in a Thin Carbon Film



Focus series
Animation

From Agar, Fig. 4.11, p. 137

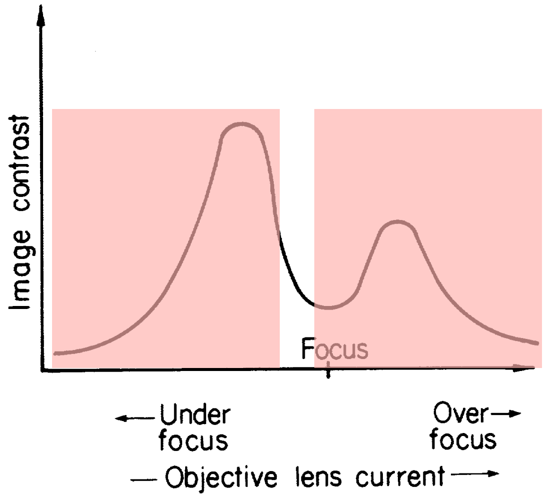
I.E.5 Focusing

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

Rules of Thumb to Live (and Die) By:

1. The higher the magnification, the **more accurately** the **image must be focused**

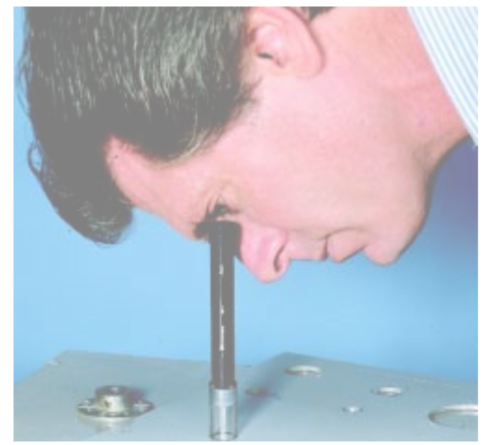
2. **High contrast** does **NOT** mean you have achieved optimum focus



From Meek, 2nd ed., Fig. 5.3, p.100

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 (analog and digital)



I.E OPERATION OF THE TEM

- Choice of accelerating voltage
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- Specimen stage/holder
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- Image intensifiers/TV displays
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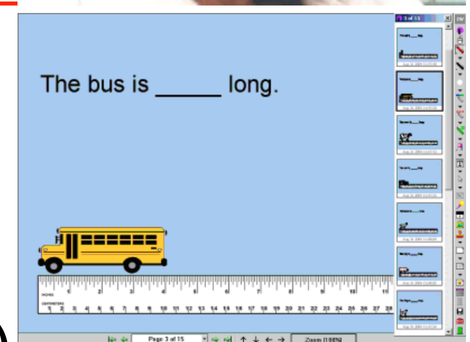
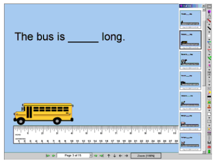




I.E OPERATION OF THE TEM

I.E.6 Magnification Calibration

Why be concerned?

~ 2-5% uncertainty in **nominal** magnification settings in modern TEMs

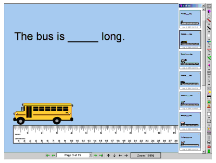
Must have an **independent** calibration of image magnification to measure specimen dimensions accurately

Why are nominal settings uncertain?

TEM lenses suffer from **hysteresis** and must be **normalized** to improve reproducibility (p. 32 of lecture notes), but there is no guarantee that actual image magnifications will precisely match the nominal settings.

What must be done?

Record images of **calibration standards** (specimens with known dimensions or spacings)



I.E OPERATION OF THE TEM

I.E.6 Magnification Calibration

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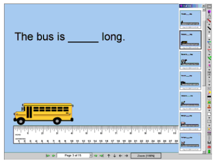
What must be done?

Record images of **calibration standards** (specimens with known dimensions or spacings)

Each specimen holder used must be calibrated separately

Slide not shown in class lecture



I.E OPERATION OF THE TEM

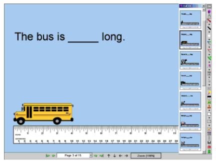
I.E.6 Magnification Calibration

Primary Calibration Standards

Polystyrene Latex Spheres

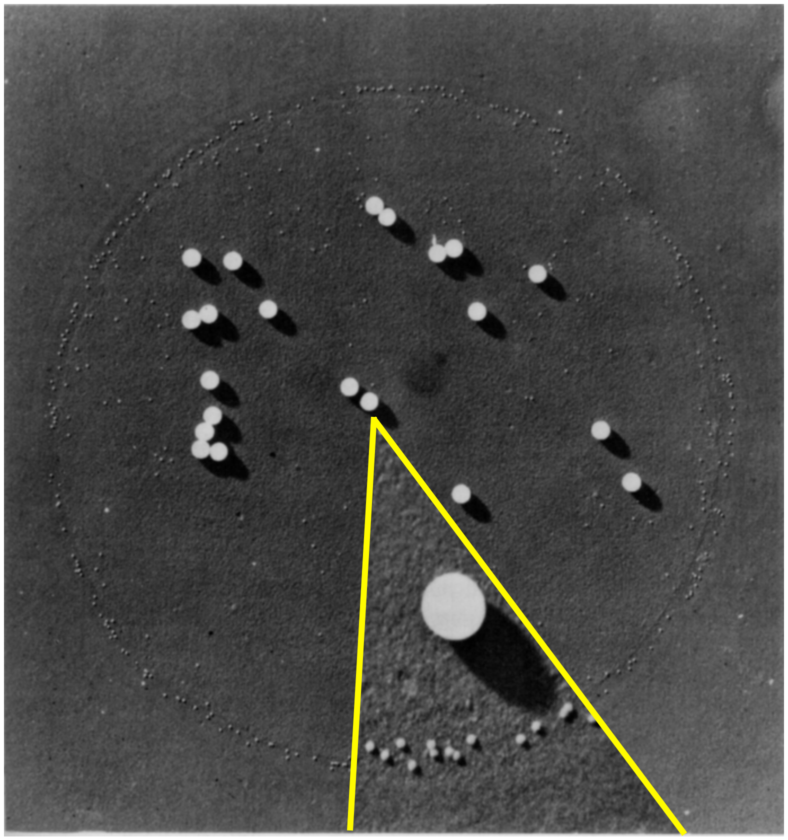
Diffraction Grating Replicas

Crystalline Specimens



I.E.6 Magnification Calibration

I.E.6.a Polystyrene Latex Spheres



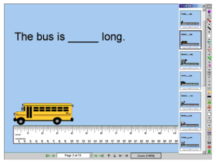
From Hall, Fig. 10.65, p.359

- Relatively **uniform** (come in various sizes ≥ 100 nm diameter)

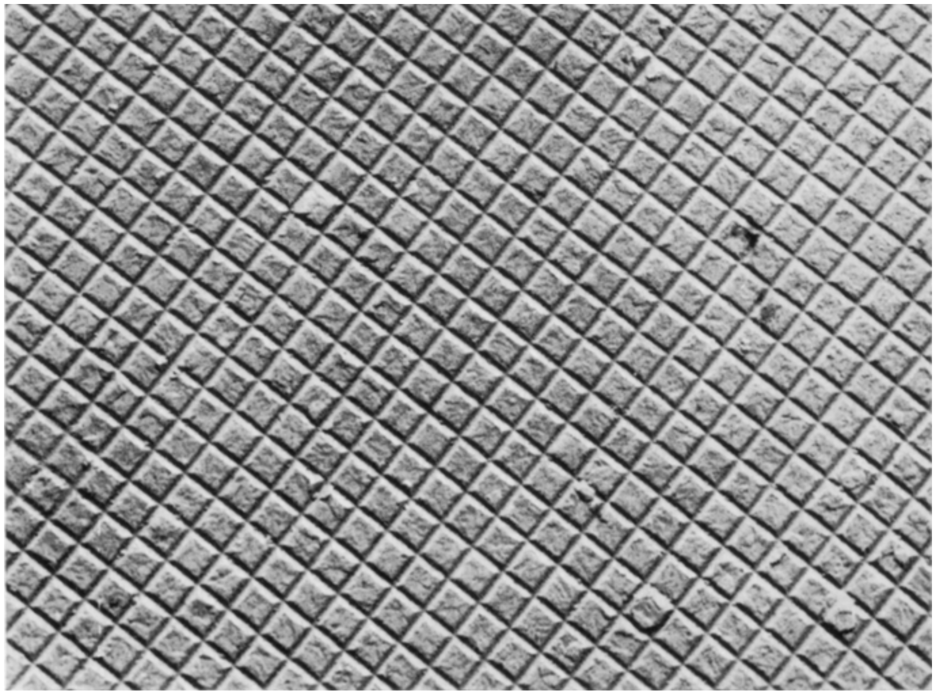
- Serve as **internal** calibration standards (**mix** with specimen sample and image together)

- **Accuracy** only **~5-10%**

- **Tedious:** need to measure large numbers of spheres



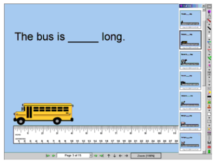
I.E.6 Magnification Calibration
I.E.6.b Diffraction Grating Replicas



From Agar, Fig. 5.9, p. 162

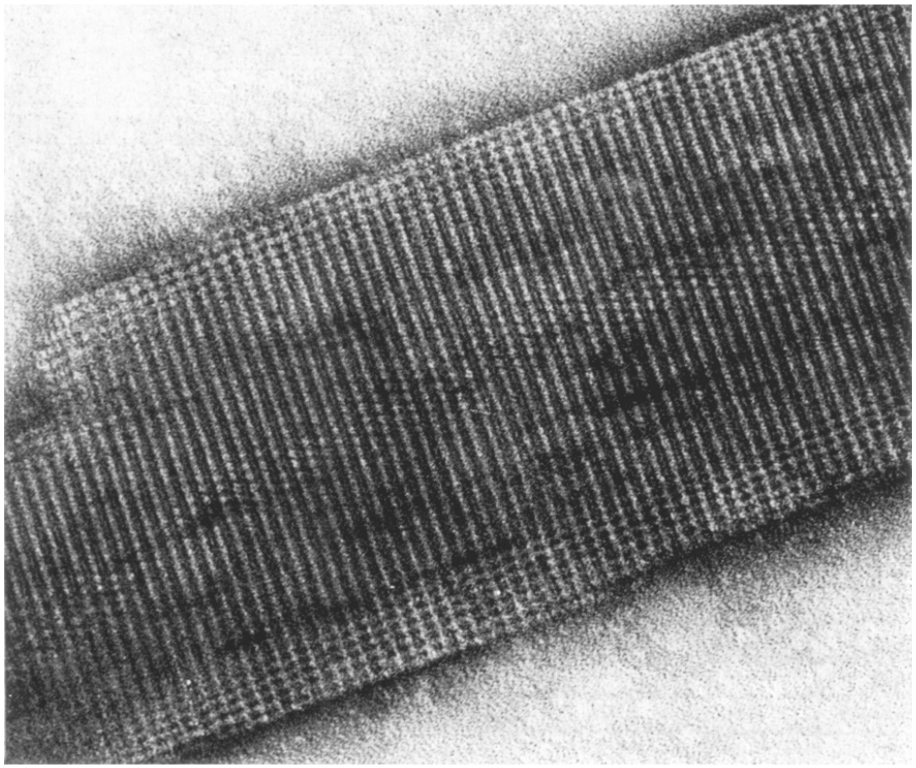
Replica of Cross-ruled Diffraction Grating (2160 lines/mm)

Convenient calibration of low magnification TEM settings (~5,000-20,000X)



I.E.6 Magnification Calibration

I.E.6.c Crystalline Specimens

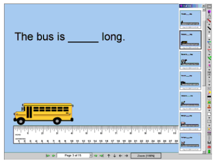


From Agar, Fig. 5.10, p. 163

Thin crystal of beef liver catalase, negatively-stained with ammonium molybdate

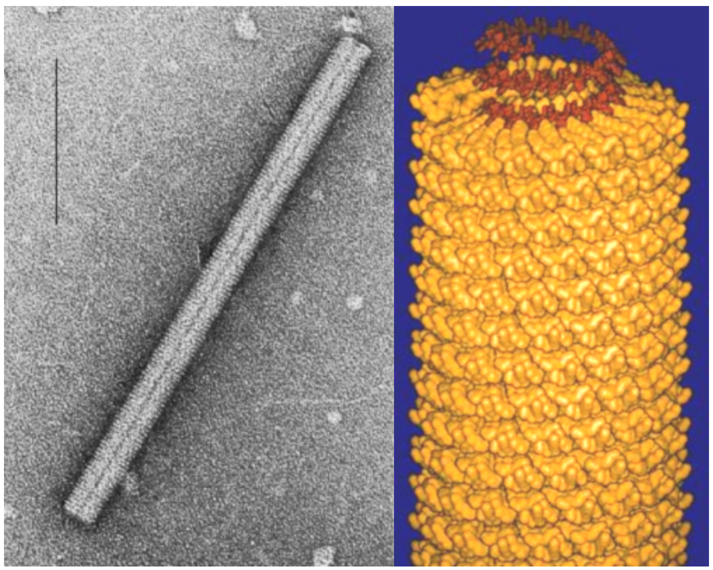
Periodicities: 8.75 x 3.43 nm

Convenient calibration of medium magnification TEM settings (~20,000-100,000X)

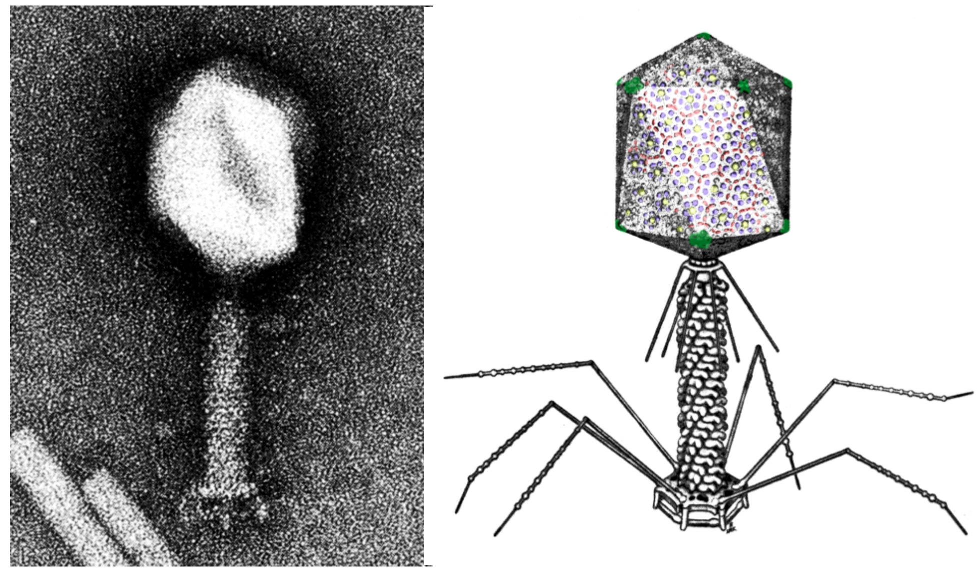


I.E.6 Magnification Calibration
I.E.6.c Crystalline Specimens

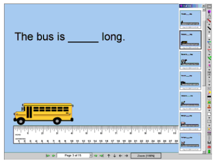
Several good **internal** calibration standards for biological specimens



Tobacco mosaic virus
(2.3 nm axial spacing)

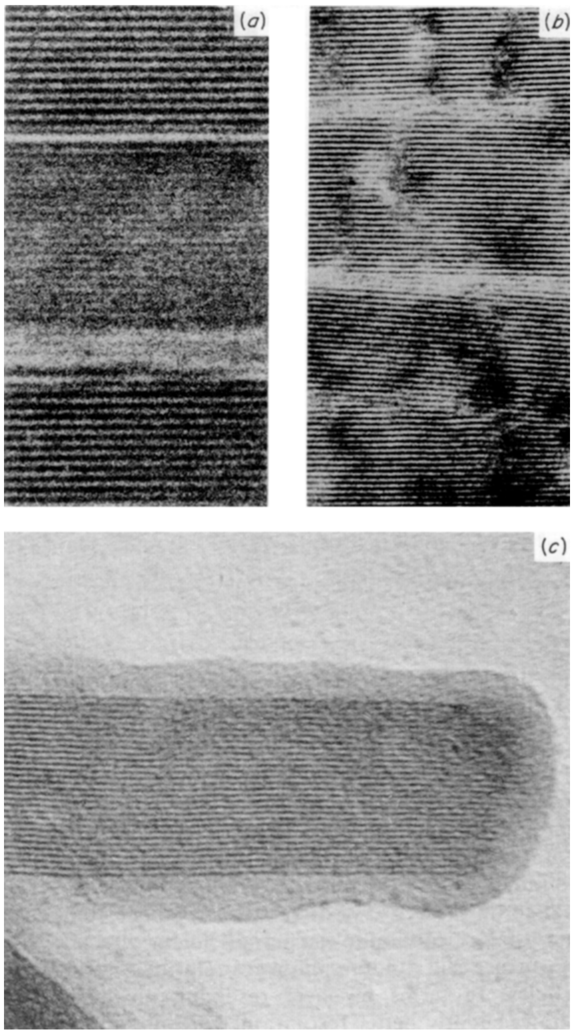


T4 bacteriophage tails
(3.9 nm axial spacing)



I.E.6 Magnification Calibration
I.E.6.c Crystalline Specimens

Calibration standards for high magnifications (>100,000X)



From Meek 1st ed., Fig. 12.6, p. 334

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

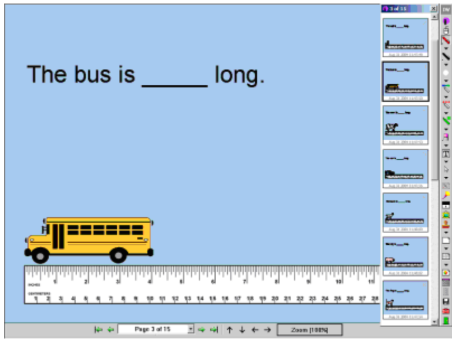
(b) K-chloroplatinate (**0.699 nm** spacing; but very susceptible to beam damage)

(c) Pt-phthalocyanine (**1.25 nm** spacing)

Slide not shown in class lecture

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 (analog and digital)



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 (analog and digital)

I.E OPERATION OF THE TEM

I.E.7 Resolution Tests

Recall: **Resolving power** of the TEM

Defined as the best possible performance as limited by the small aperture of objective lens

How does one check microscope performance?

- Record micrographs of suitable **test** specimens
- Measure **actual resolution** achieved **in the recorded image**

I.E OPERATION OF THE TEM

I.E.7 Resolution Tests

Primary Resolution Tests

Point Separation **(p.100 in lecture notes)**

Lattice Resolution

I.E.7 Resolution Tests

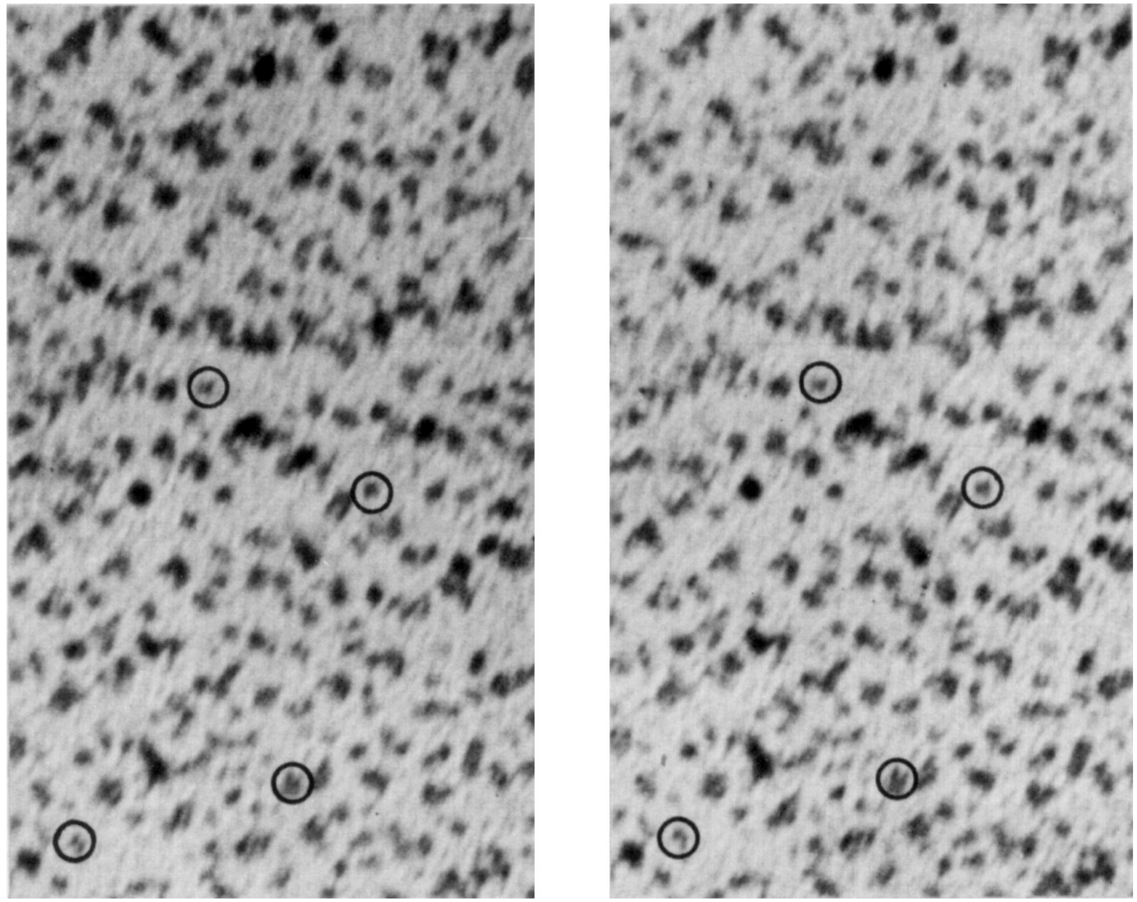
I.E.7.a Point Separation Test

Point Separation Resolution Measurement

Platinum-iridium on carbon substrate

Separation of < 1.0 mm for pairs of particles, imaged at ~ 2 million times, indicates resolving power of better than 5 Å

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



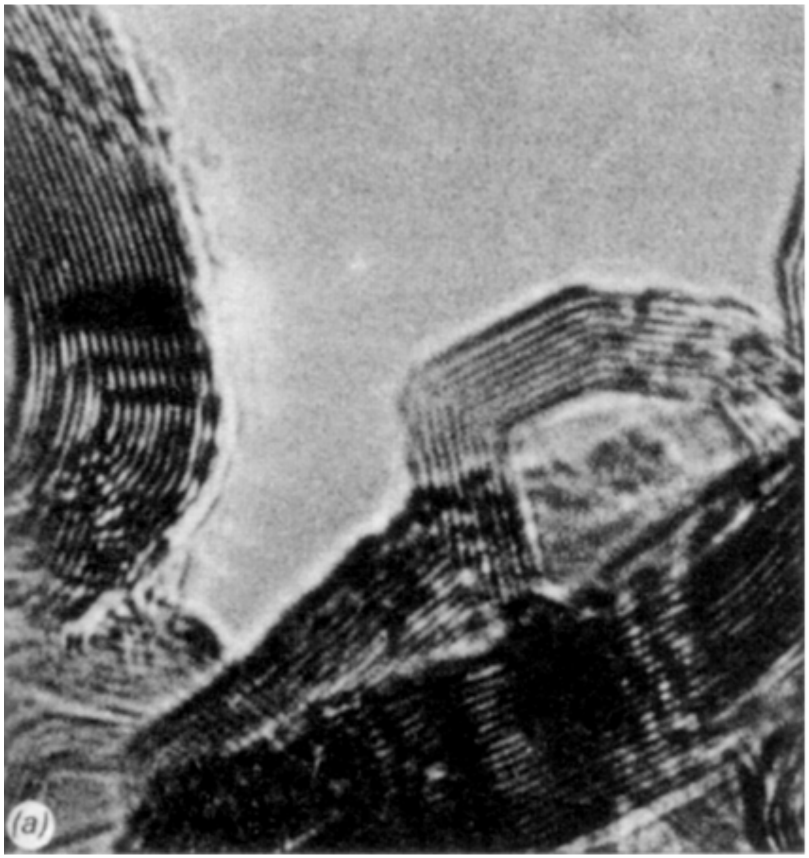
From Meek 1st ed., Fig. 12.5, p. 333

Slide not shown in class lecture

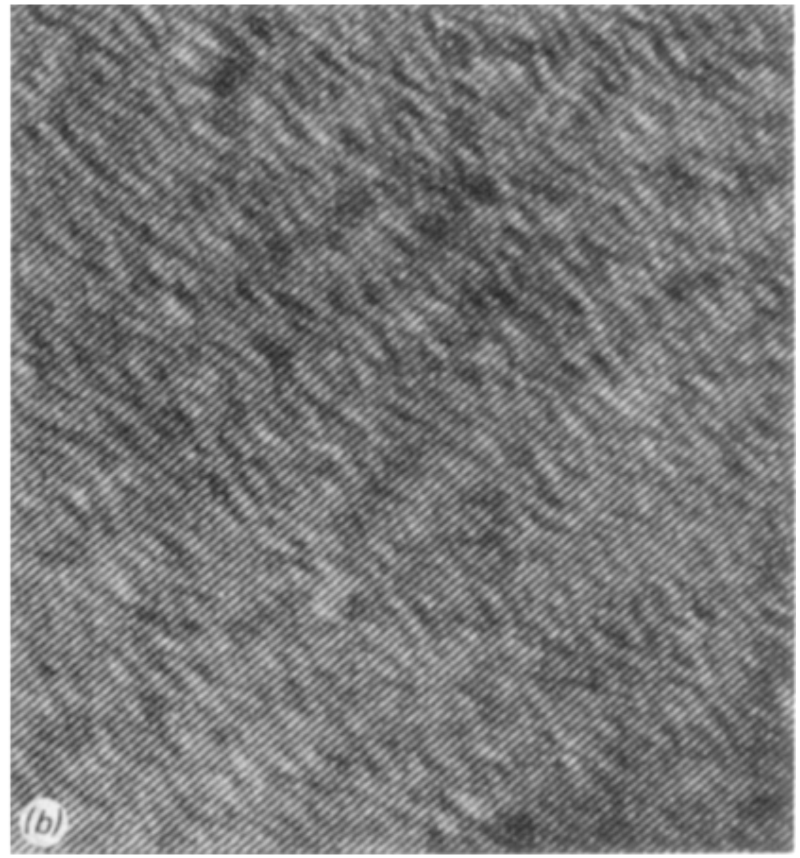
I.E.7 Resolution Tests

I.E.7.b Lattice Resolution Test

Crystal Lattice Spacings



Graphitized carbon
(lattice spacing = 3.4 Å)



Gold foil
(lattice spacing = 2.04 Å)

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

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 (analog and digital)

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 — (pp.102-103, lecture notes)
- Microscope maintenance
- Photography (analog and digital)

I.E OPERATION OF THE TEM

I.E.8 Image Intensifier / TV Displays

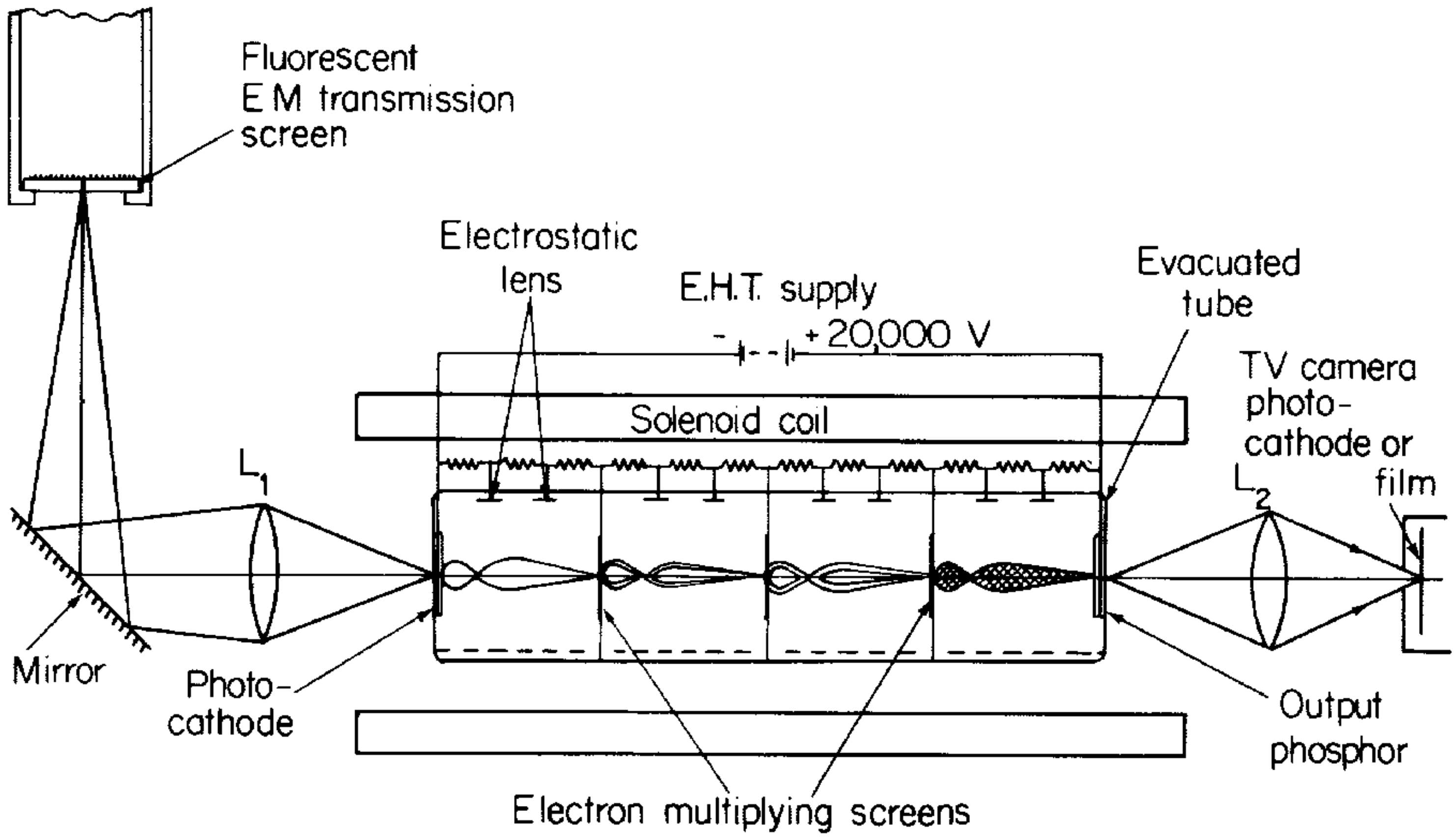


Diagram of TEM image intensifier developed by GEC-AEI Ltd..

From Meek 1st ed., Fig. 15.10, p. 401

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 — (pp.102-103, lecture notes)
- Microscope maintenance
- Photography (analog and digital)

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 (p. 103 lecture notes)
- Photography (analog and digital)

BOTTOM LINE: Pay big bucks for a service contract

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 (analog and digital)

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)

Photographic process
Optical density
Density related to exposure
Density/exposure curves
Contrast
Speed (sensitivity) of electron emulsion
Electron range in emulsion
Number of grains per electron
Graininess
Resolution (image spread - electron diffusion)

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)

- ☆ Photographic process
- Optical density
- Density related to exposure
- Density/exposure curves
- Contrast
- Speed (sensitivity) of electron emulsion
- Electron range in emulsion
- Number of grains per electron
- Graininess
- Resolution (image spread - electron diffusion)

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)

What's the Bottom Line?

Complete, faithful, and permanent record of details contained in the electron image

With film images in the TEM, the goal is to:

Maximize Density

Enhance Contrast

Reduce Noise

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)

KEY CONCEPT

Photographic emulsions respond **differently** to **electrons** and **photons**

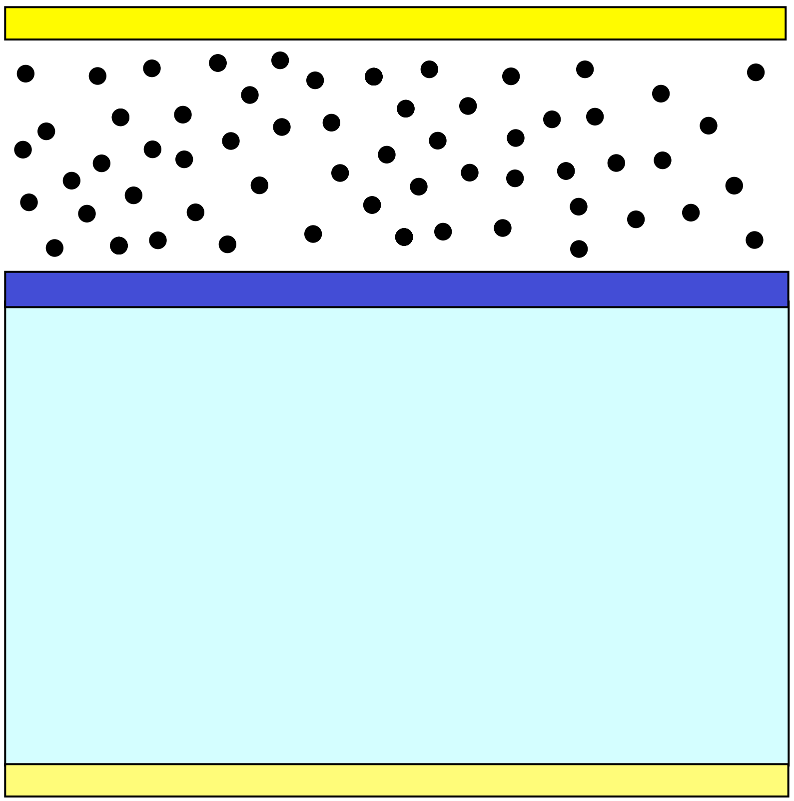
Electrons**: single-hit** process

Photons**: multiple-hit** process

I.E.10 Photography (Film)

I.E.10.a The Photographic Process

Structure of the Photographic Film

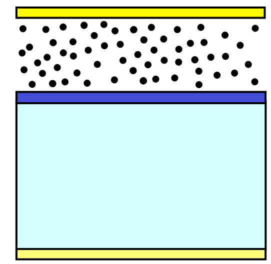


I.E.10 Photography (Film)

I.E.10.a The Photographic Process

What happens when electrons or photons hit an emulsion?

Ag atom **specks** form in the Ag-halide crystal



Photons: Required energy is **~ 30eV**

10 photons needed (each photon has ~2.5eV)

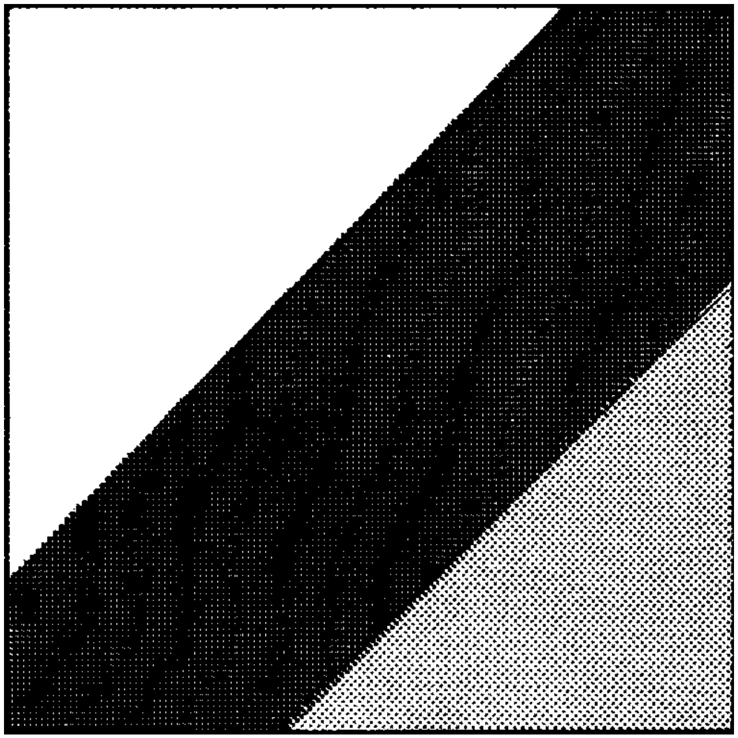
Electrons: Required energy is **~ 500eV**

1 e^- in a 100keV beam **more than sufficient** !!!

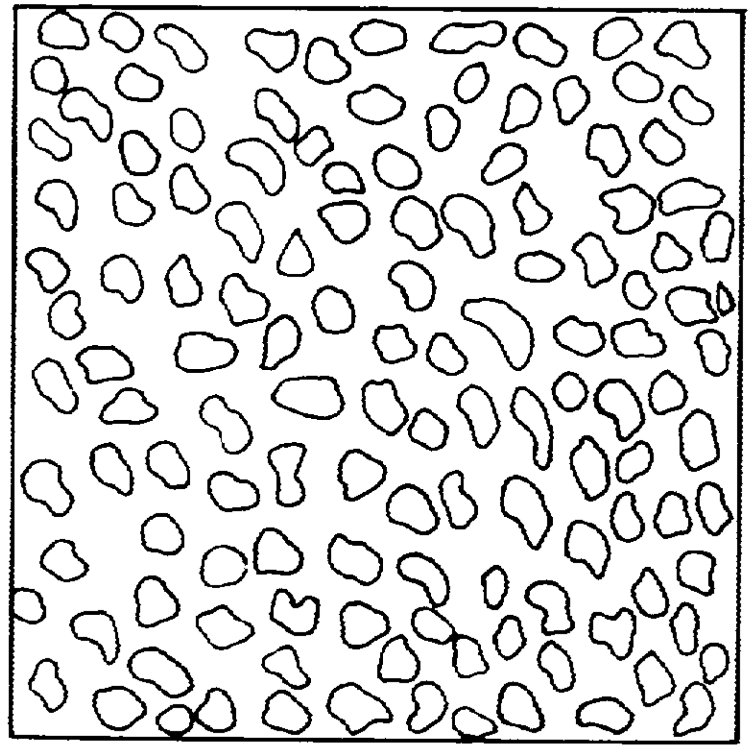
Exposure of film to e^- is a **single-hit** process

I.E.10 Photography (Film)

I.E.10.a The Photographic Process



Electron image
(Very high mag and small field of view)

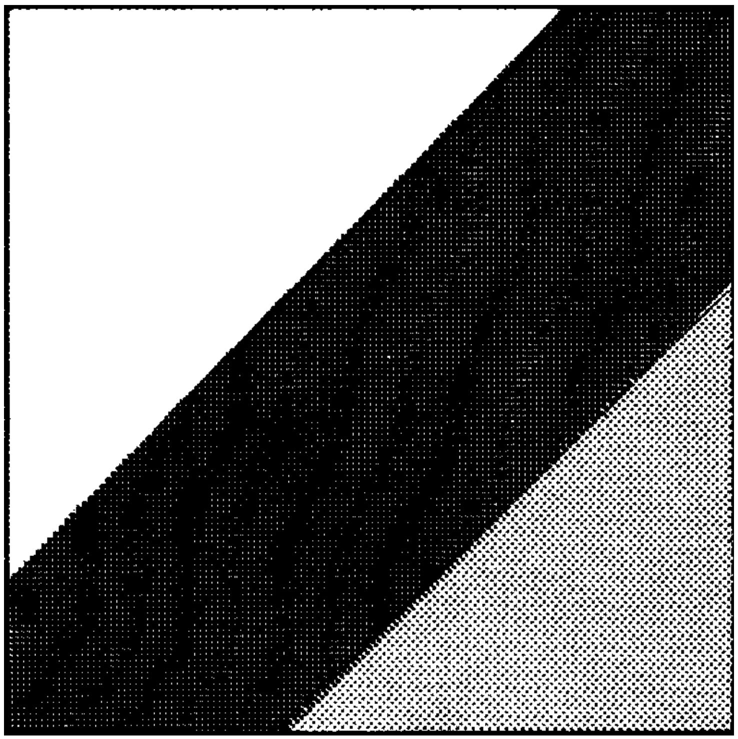


Unexposed emulsion
(Area same as image on left)

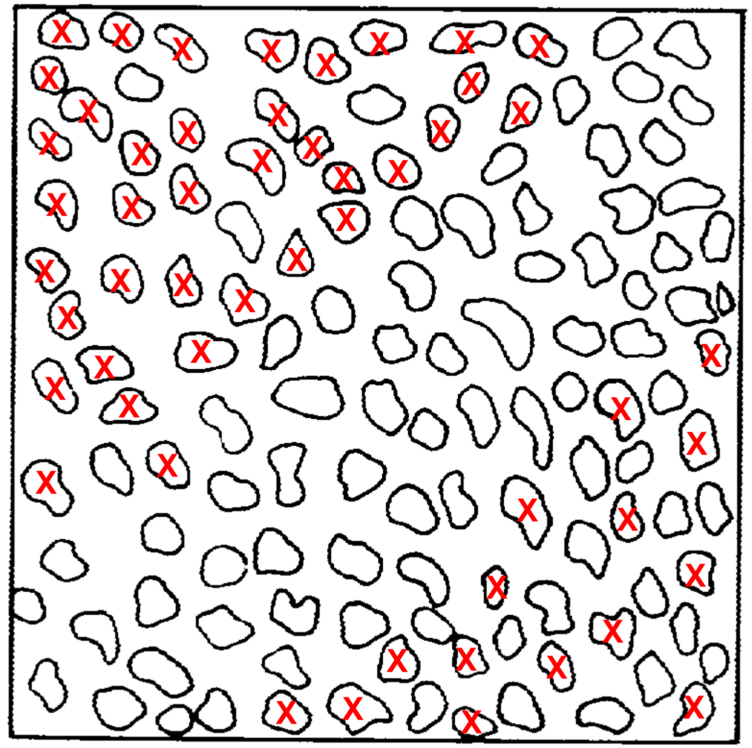
Adapted from Slayter, Fig. 21-1, p. 162

I.E.10 Photography (Film)

I.E.10.a The Photographic Process



Electron image
(Very high mag and small field of view)

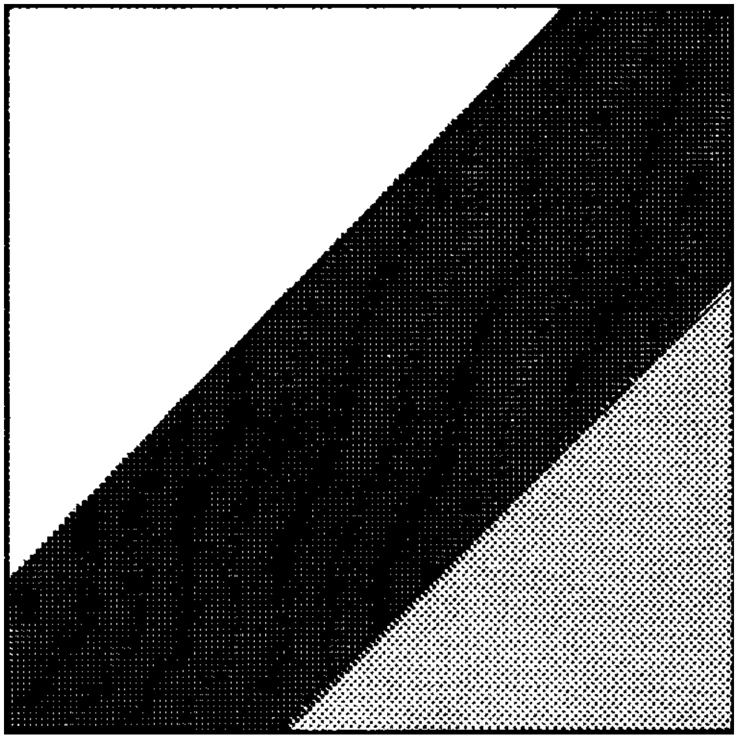


Exposed but
unprocessed emulsion

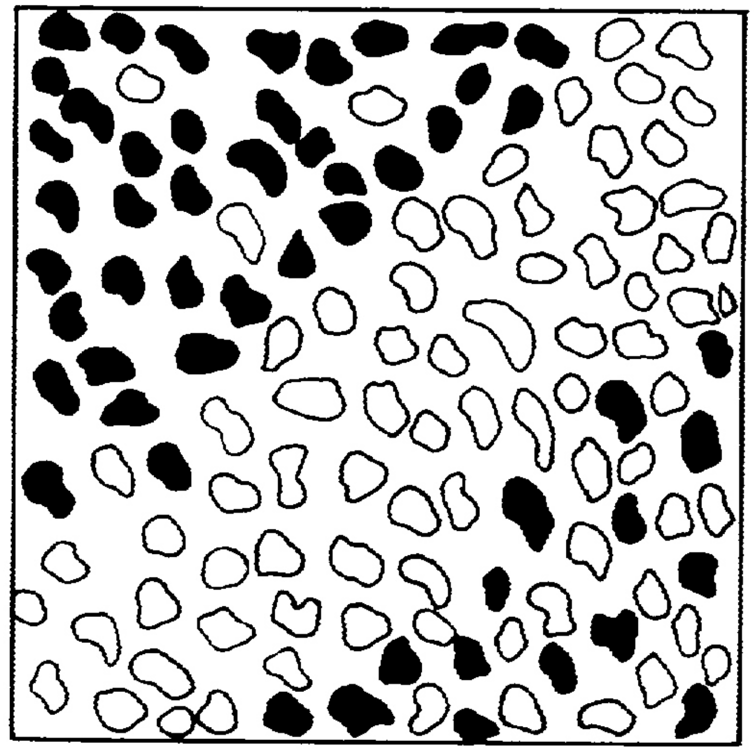
Adapted from Slayter, Fig. 21-1, p. 162

I.E.10 Photography (Film)

I.E.10.a The Photographic Process



Electron image
(Very high mag and small field of view)

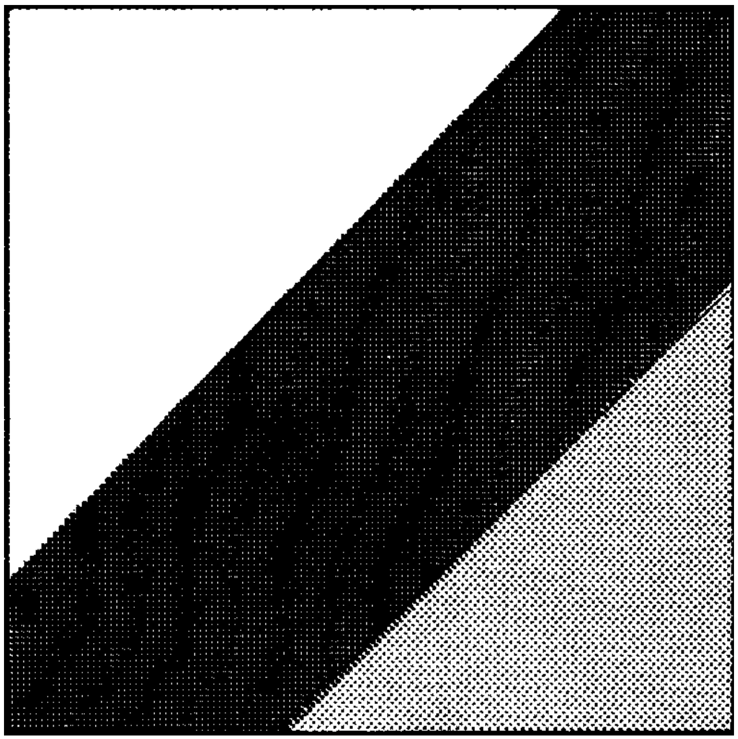


Developed but
unfixed emulsion

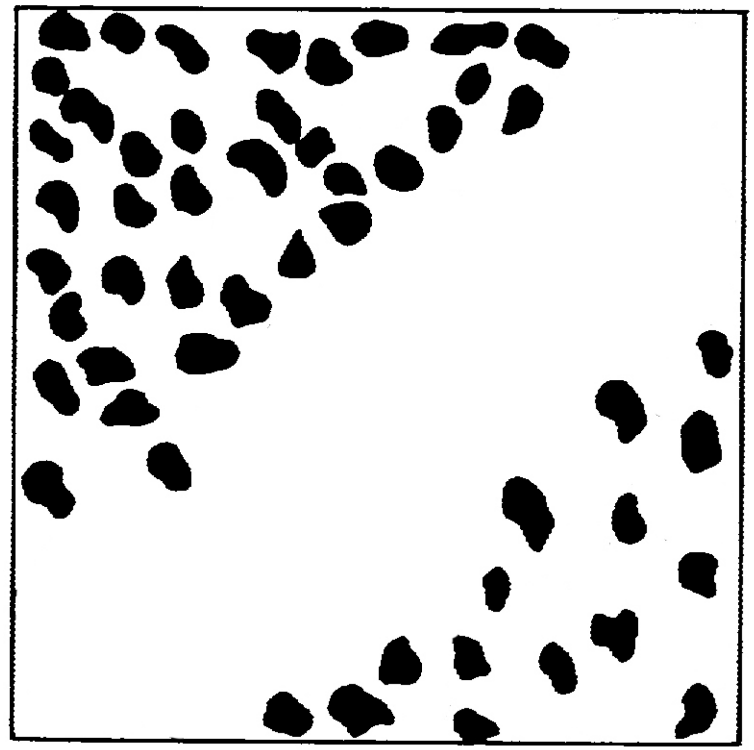
Adapted from Slayter, Fig. 21-1, p. 162

I.E.10 Photography (Film)

I.E.10.a The Photographic Process



Electron image
(Very high mag and small field of view)

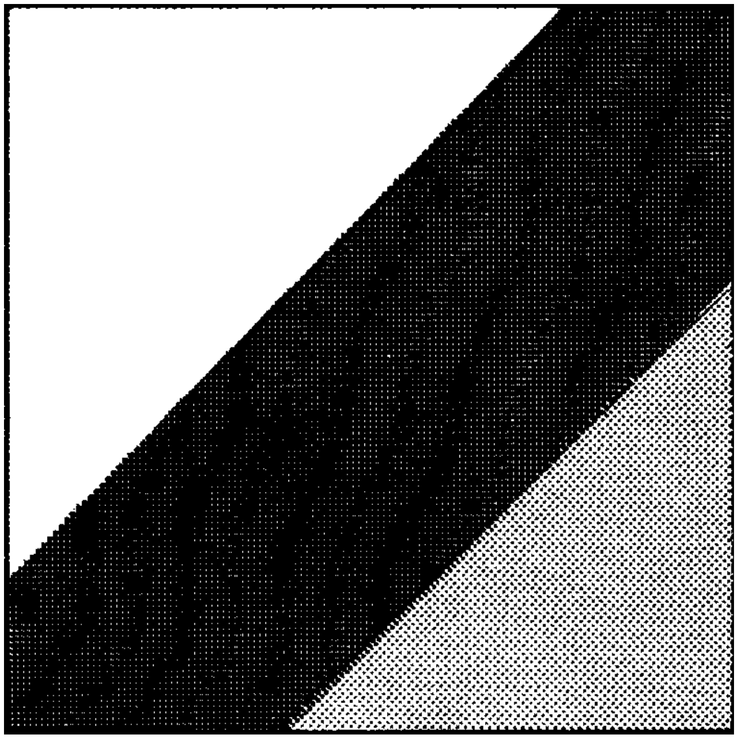


Final photographic image ("negative") after fixation

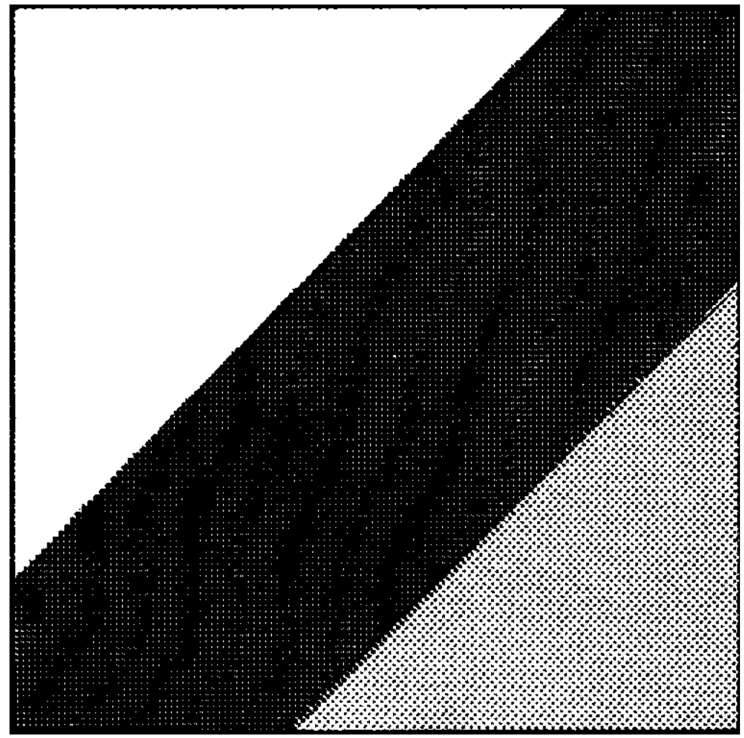
Adapted from Slayter, Fig. 21-1, p. 162

I.E.10 Photography (Film)

I.E.10.a The Photographic Process



Electron image
(Very high mag and small field of view)

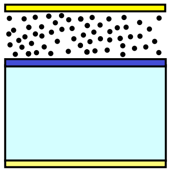


Electron image

Adapted from Slayter, Fig. 21-1, p. 162

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)



- Photographic process
- Optical density
  - Density related to exposure
  - Density/exposure curves
  - Contrast
  - Speed (sensitivity) of electron emulsion
  - Electron range in emulsion
  - Number of grains per electron
  - Graininess
  - Resolution (image spread - electron diffusion)

I.E.10 Photography (Film)

I.E.10.b Optical Density (OD) of the Processed Emulsion

Optical Density:

Quantitative measure of how **black** the photographic emulsion gets when exposed to radiation

Definition:

$$OD = \log_{10}(I_o / T)$$

I_o = intensity of **incident** radiation

T = intensity of **transmitted** radiation

I.E.10 Photography (Film)

I.E.10.b Optical Density (OD) of the Processed Emulsion

OD: quantitative measure of blackening of the emulsion

$$OD = \log_{10}(I_o / T)$$

EXAMPLES:

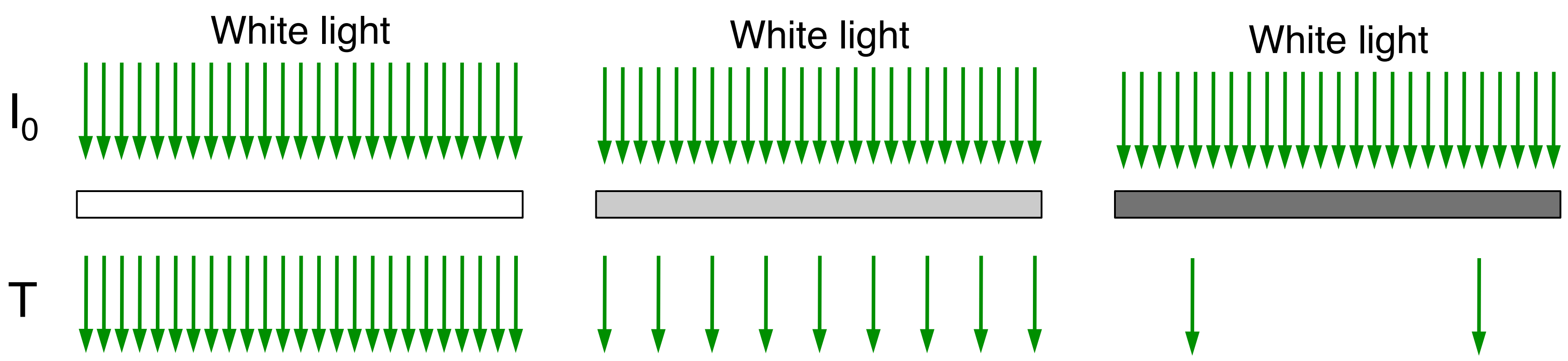
$\boldsymbol{OD = 1.0}$ for any portion of the photographic negative that transmits **10%** of the incident light ($\boldsymbol{T = 0.1}$)

$\boldsymbol{OD = 2.0}$ for any portion that transmits only **1%** of the incident light ($\boldsymbol{T = 0.01}$)

I.E.10 Photography (Film)

I.E.10.b Optical Density (OD) of the Processed Emulsion

$$OD = \log_{10}(I_o / T)$$



OD = 0

($T = 100\% \ I_0$)

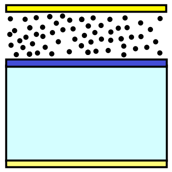
OD = 1

($T = 10\% \ I_0$)

OD = 2

($T = 1\% \ I_0$)

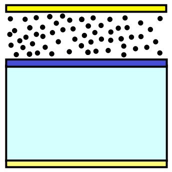
I.E OPERATION OF THE TEM



I.E.10 Photography (Film)

- ★ Photographic process
- ★ Optical density
- ☆ Density related to exposure - lecture notes p.106
- ☆ Density/exposure curves - lecture notes pp.106-107
- ☆ Contrast - lecture notes p.107
- ☆ Speed (sensitivity) of electron emulsion - lecture notes p.108
- ☆ Electron range in emulsion - lecture notes pp.108-109
- Number of grains per electron
- Graininess
- Resolution (image spread - electron diffusion)

I.E OPERATION OF THE TEM



I.E.10 Photography (Film)

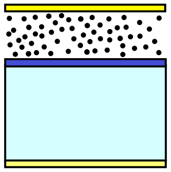
- ★ Photographic process
- ★ Optical density
- ★ Density related to exposure
- ★ Density/exposure curves
- ★ Contrast
- ★ Speed (sensitivity) of electron emulsion
- ★ Electron range in emulsion
- ☆ Number of grains per electron

  Graininess

  Resolution (image spread - electron diffusion)

I.E.10 Photography (Film)

I.E.10.h Number of Grains / Electron



Each **electron** in the image likely hits **more** than 1 Ag halide crystal as it passes into and through the emulsion

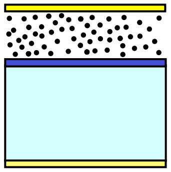
~1 halide crystal for each 2 μm of emulsion thickness

~ 10 grains per electron track for 20 μm thick emulsion

Each **electron** hits and passes through **several** halide crystals, losing some energy in each crystal

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)

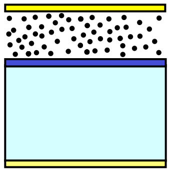


- ★ Photographic process
- ★ Optical density
- ★ Density related to exposure
- ★ Density/exposure curves
- ★ Contrast
- ★ Speed (sensitivity) of electron emulsion
- ★ Electron range in emulsion
- ☆ Number of grains per electron

  Graininess

  Resolution (image spread - electron diffusion)

I.E OPERATION OF THE TEM



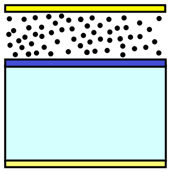
I.E.10 Photography (Film)

- ★ Photographic process
- ★ Optical density
- ★ Density related to exposure
- ★ Density/exposure curves
- ★ Contrast
- ★ Speed (sensitivity) of electron emulsion
- ★ Electron range in emulsion
- ★ Number of grains per electron
- ☆ Graininess - lecture notes pp.109-110

Resolution (image spread - electron diffusion)

I.E.10 Photography (Film)

I.E.10.i Graininess



Statistical phenomenon caused by **"electron noise"**

Random distribution of e^- "particles" in the beam

Graininess:

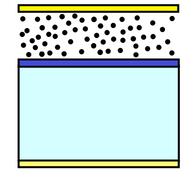
- **NOT a defect** in the photographic emulsion

- Product of **two random processes**

Slide not shown in class lecture

I.E.10 Photography (Film)

I.E.10.i Graininess



Graininess is the product of 2 random processes

1. **Random arrival** of electrons at the film

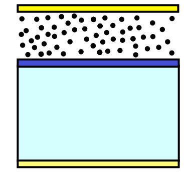
   Over **large** areas of an electron image, radiation appears **uniform**

   Over **small** regions, radiation distribution is **NOT uniform**

2. Granularity in the **emulsion**

Slide not shown in class lecture

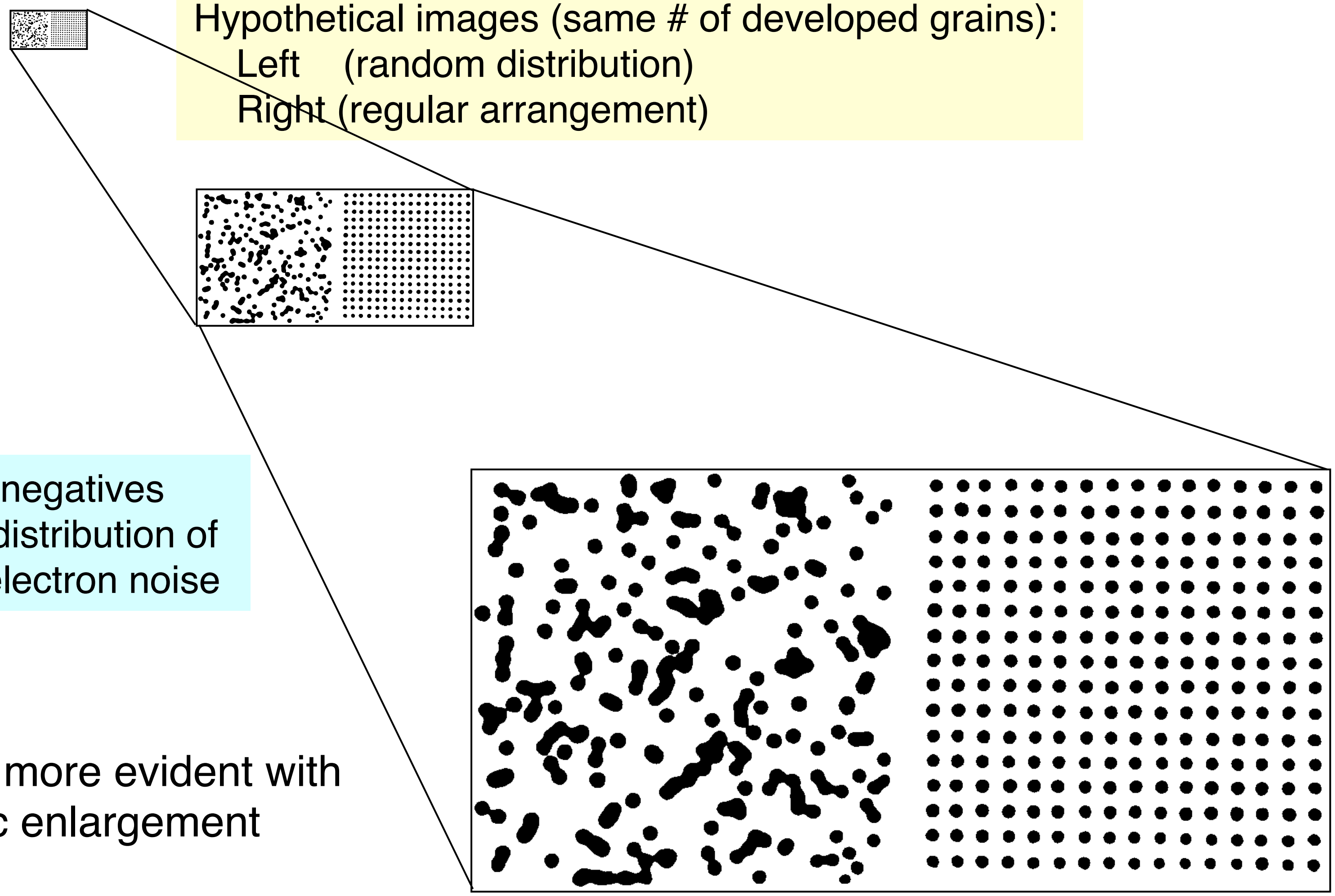
I.E.10 Photography (Film)



I.E.10.i Graininess

Graininess is the product of 2 random processes

Hypothetical images (same # of developed grains):
Left (random distribution)
Right (regular arrangement)



Recorded TEM negatives show random distribution of grains due to electron noise

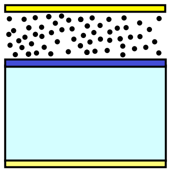
Graininess is more evident with photographic enlargement

From Agar, Fig. 7.6, p. 201

Slide not shown in class lecture

I.E.10 Photography (Film)

I.E.10.i Graininess



Graininess is the product of 2 random processes

1. **Random arrival** of electrons at the film

   Over **large** areas of an electron image, radiation appears **uniform**

   Over **small** regions, radiation distribution is **NOT uniform**

2. Granularity in the **emulsion**

   Results from variations in:

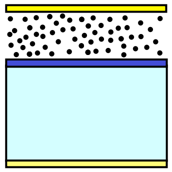
   - # of halide crystals hit by each electron in the image
   - Area over which exposed crystal grows during development

Graininess in TEM images mainly caused by #1

Slide not shown in class lecture

I.E.10 Photography (Film)

I.E.10.i Graininess



Ways to Reduce Graininess

To increase *S*/*N*, **need to increase "exposure"**

1. Use **more electrons** when recording images ("routine" TEM)

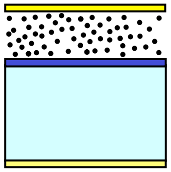
2. Use **image processing**: average many images together
   (effectively increases the # of electrons per image point)

3. Use **film development strategies**
   Have minimal effect on e- noise but can reduce grainy appearance

Slide not shown in class lecture

I.E OPERATION OF THE TEM

I.E.10 Photography (Film)



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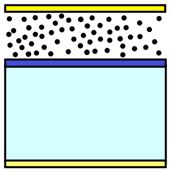
Resolution (image spread - electron diffusion)

I.E OPERATION OF THE TEM



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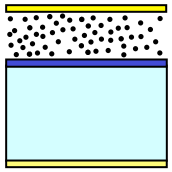
I.E.10 Photography (Film)

I.E.10.j Resolution - Image Spread



Emulsions Have Limited Resolution

Electron **tracks** through the emulsion **don’t follow straight lines** perpendicular to the surface

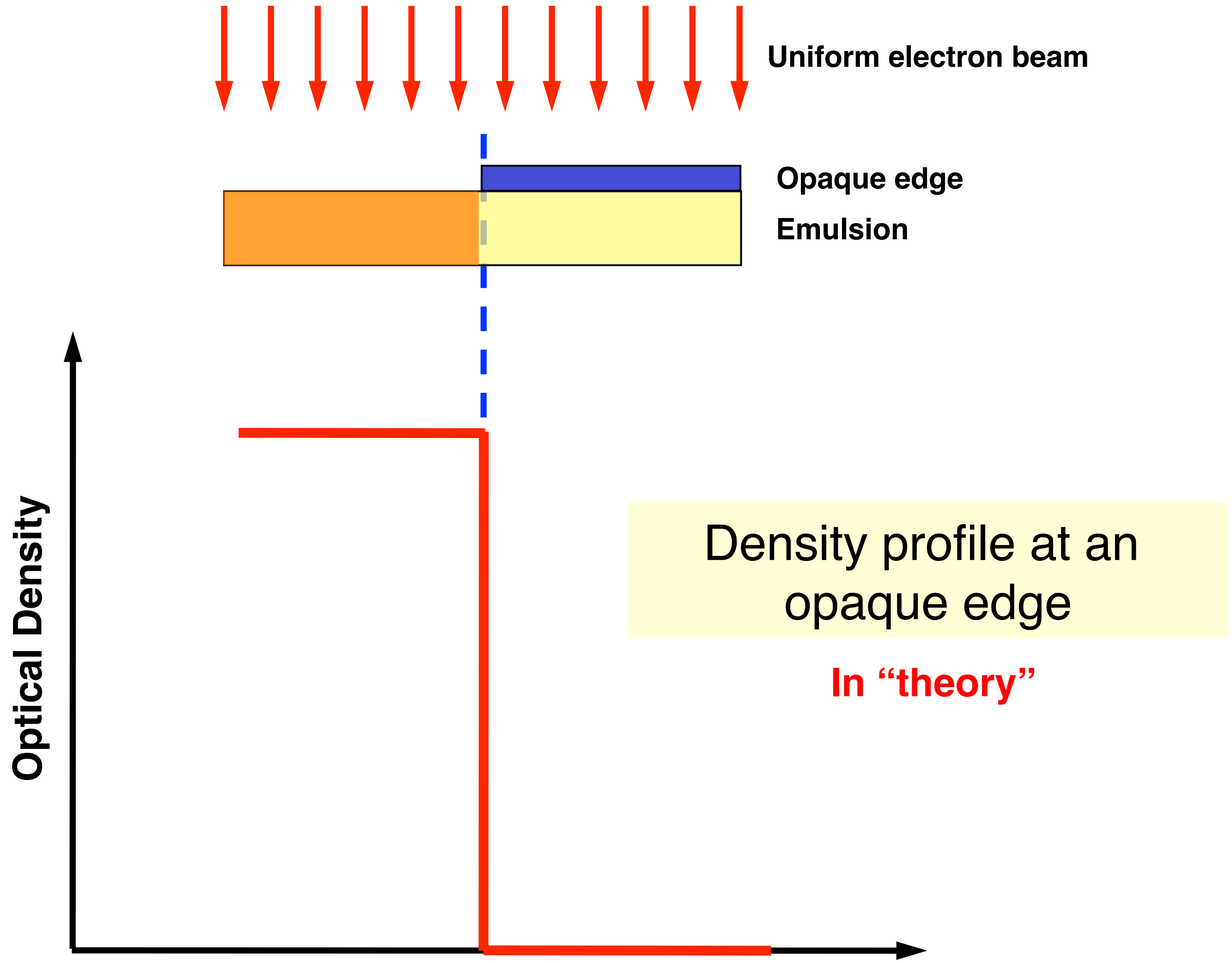
Electrons **scatter away** from the incident direction by interacting with atoms in the Ag halide crystals

Sideways scatter of electrons in the emulsion is called **“electron diffusion”**

∴ **Image details** are **spread out** and **contrast** is **reduced**

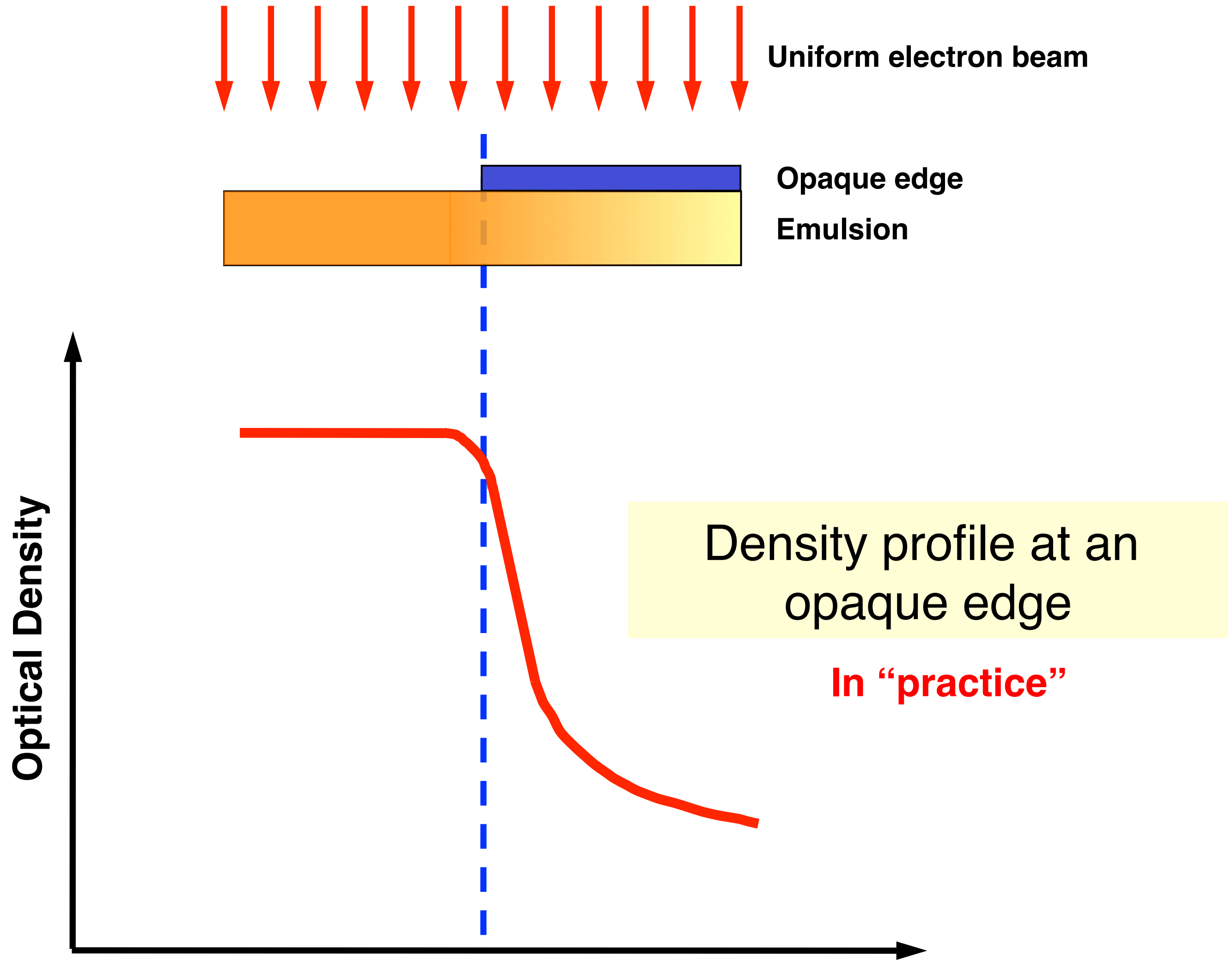
I.E.10 Photography (Film)

I.E.10.j Resolution - Image Spread



I.E.10 Photography (Film)

I.E.10.j Resolution - Image Spread



I.E.10 Photography (Film)

I.E.10.j Resolution - Image Spread

What's the Bottom Line?

Resolution in the FINAL (*i.e.* **recorded**) image is always **POORER THAN** the resolution achieved in the **electron** image

The solution?

Use magnification high enough to make sure details in the electron image are captured by the emulsion

But not too high!!! (restricted field of view, excessive damage, etc.)

I.E.10 Photography (Film)

I.E.10.j Resolution - Image Spread

Seems like everything affects resolution

Size of lens aperture (diffraction effects)

Lens aberrations (spherical, chromatic, asymmetry)

Defocus effects (phase contrast Fresnel fringes)

Emulsion grain size

Electron diffusion in emulsion

And yes, you can expect some more…

§ I: The Microscope

I.E Operation of the TEM

→ I.E.10 Photography (Film)

→ I.E.11 Digital Photography (CCD)

I.E OPERATION OF THE TEM

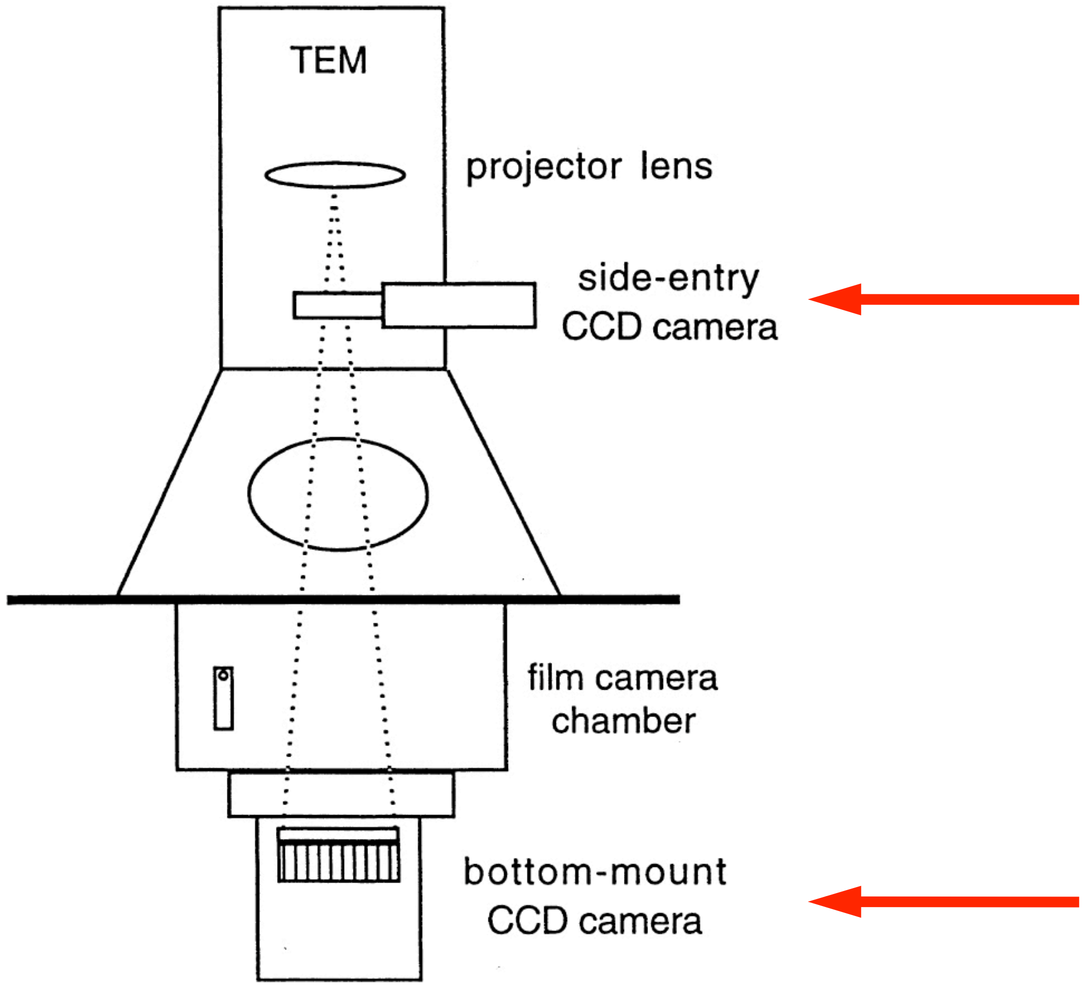
I.E.11 Digital Photography (CCD)

CCD Detectors/Cameras

CCD = Charge Coupled Device

I.E OPERATION OF THE TEM

I.E.11 Digital Photography (CCD)



From Fan & Ellisman [2000], p.2

I.E.11 Digital Photography (CCD)

Quotable Quotes

Film  Digital

I.E.11 Digital Photography (CCD)

Quotable Quotes

…likely consequence of these trends is that TEM film cameras and EM photographic darkrooms will ultimately be relegated to technical museums. As a practical example, this entire paper was prepared without any use of silver-halide-based photographic emulsions, and its authors have no intention of ever stepping into a photographic darkroom again.

Krivanek & Mooney *Ultramicrosc.* 49[**1993**]95

Direct digital recording with slow-scan CCD cameras […] provides a significant advantage over film such as higher sensitivity and dynamic range. […] performance characteristics […] challenge the dominance of film as the recording medium for many scientific imaging requirements. […] These cameras are revolutionizing the way microscopists think about addressing problems of data acquisition …

Fan & Ellisman *Ultramicrosc.* 52[**1993**]21

…data acquisition in the electron microscope will increasingly be with electronic detectors. …CCDs are not yet suitable for high resolution imaging.

Faruqi, Henderson, & Subramaniam *Ultramicrosc.* 75[**1999**]235

…digital imaging systems appear to be poised to replace film in TEM.

…a much larger array size will be needed to match the large field of view and the great details typically contained in a piece of TEM film.

Fan *et al., Ultramicrosc.* 84[**2000**]75

CCDs are increasingly replacing photographic film as the recording medium of choice in electron microscopy.

Meyer *et al., Ultramicrosc.* 85[**2000**]9

… traditional and still the most common recording medium is photographic film …

Koeck *Microsc. Res. & Tech.* 49[**2000**]217

So where do we stand today?

Film has already been replaced by CCD cameras in many applications

Film and CCDs are in the process of losing the next "battle" against DDDs

DDD = Direct Detection Device

(measures electron events directly)

I.E.11 Digital Photography (CCD)

Advantages of Recording Images by CCD

- **Immediate** image access (no tedious film development)

- Much **larger dynamic range** than film

- Strict **linear response** with electron dose

- Amenable to all types of **automated** experiments

  Auto-focusing and astigmatism correction
  Auto-imaging
  Electron tomography
  Electron holography
  Protein electron crystallography
  Telemicroscopy
  etc. etc.

I.E.11 Digital Photography (CCD)

Disadvantages of Recording Images by CCD

- **Poorer "pixel" resolution** (15 μm vs. ~ 5-10 μm for film)

- Limited number of pixels, hence **small field of view**

  Affordable CCDs have $1k^2$ or $2k^2$ pixel arrays

  Film is comparable to 16,000 by 20,000 pixels

- High upfront cost (~ $200K for high quality $4k^2$ camera)

I.E.11 Digital Photography (CCD)

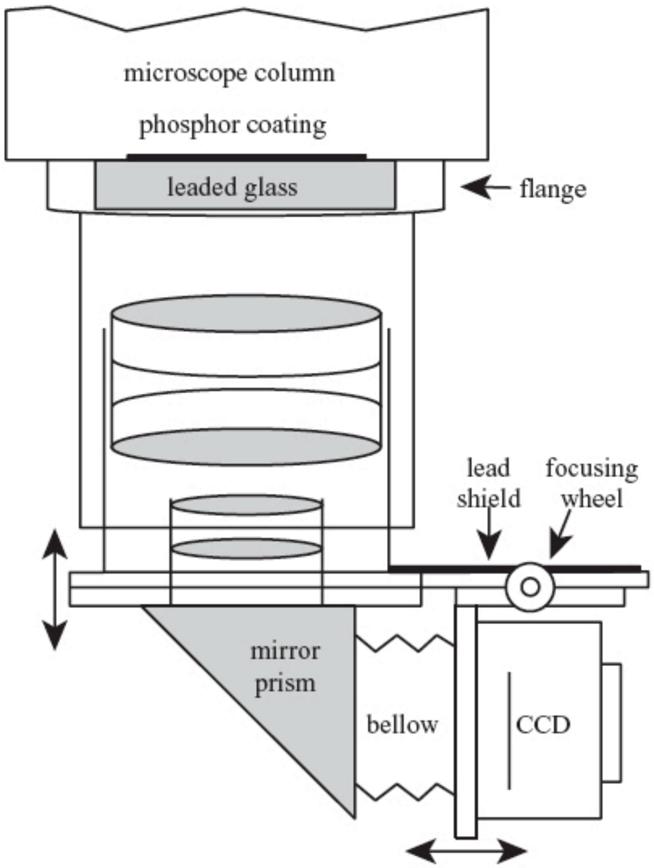
CCD Topics (Woulda, Coulda, Shoulda…)

- CCD design (lens or fiber optic coupling)
- Mount in TEM (in-line or retractable or off-line)
- Scintillators (YAG; P43 GOS; red P20 phosphor)
- Dark current; gain normalization; readout noise; Peltier cooling
- Pixel size; pixel resolution (PSF: point spread function); pixel binning
- MTF (modulation transfer function of camera)
- Nyquist frequency/sampling
- DQE (detective quantum efficiency of electron detection)
- Dynamic range
- Distortion

I.E.11 Digital Photography (CCD)

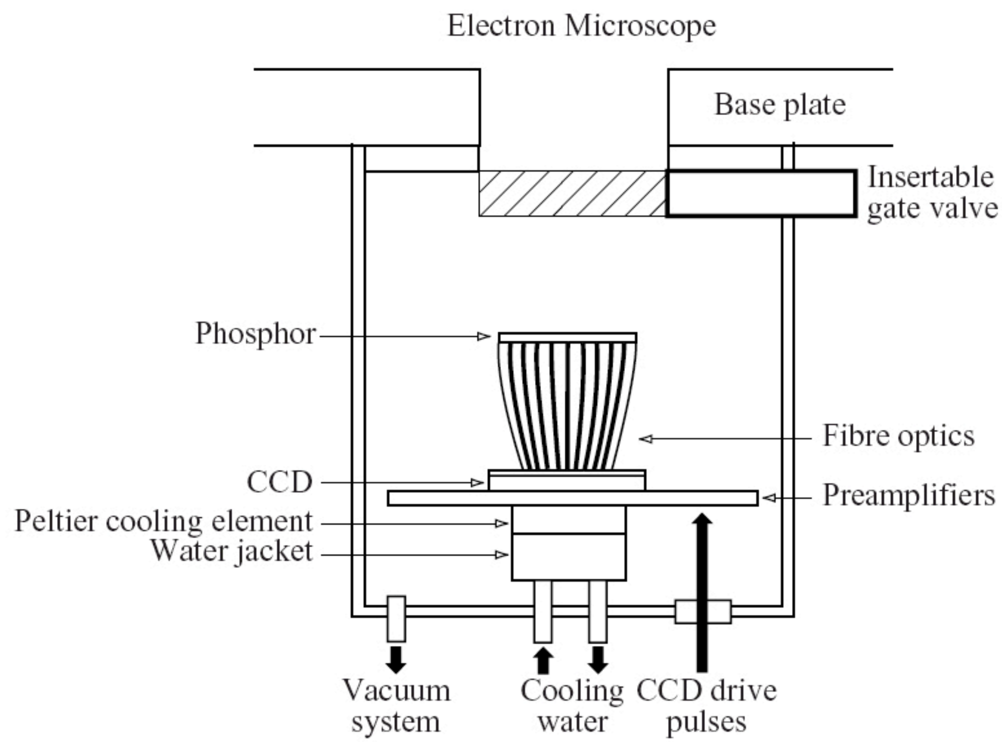
Two Basic CCD Designs

Lens-Coupled



From Fan & Ellisman [1993], p.22

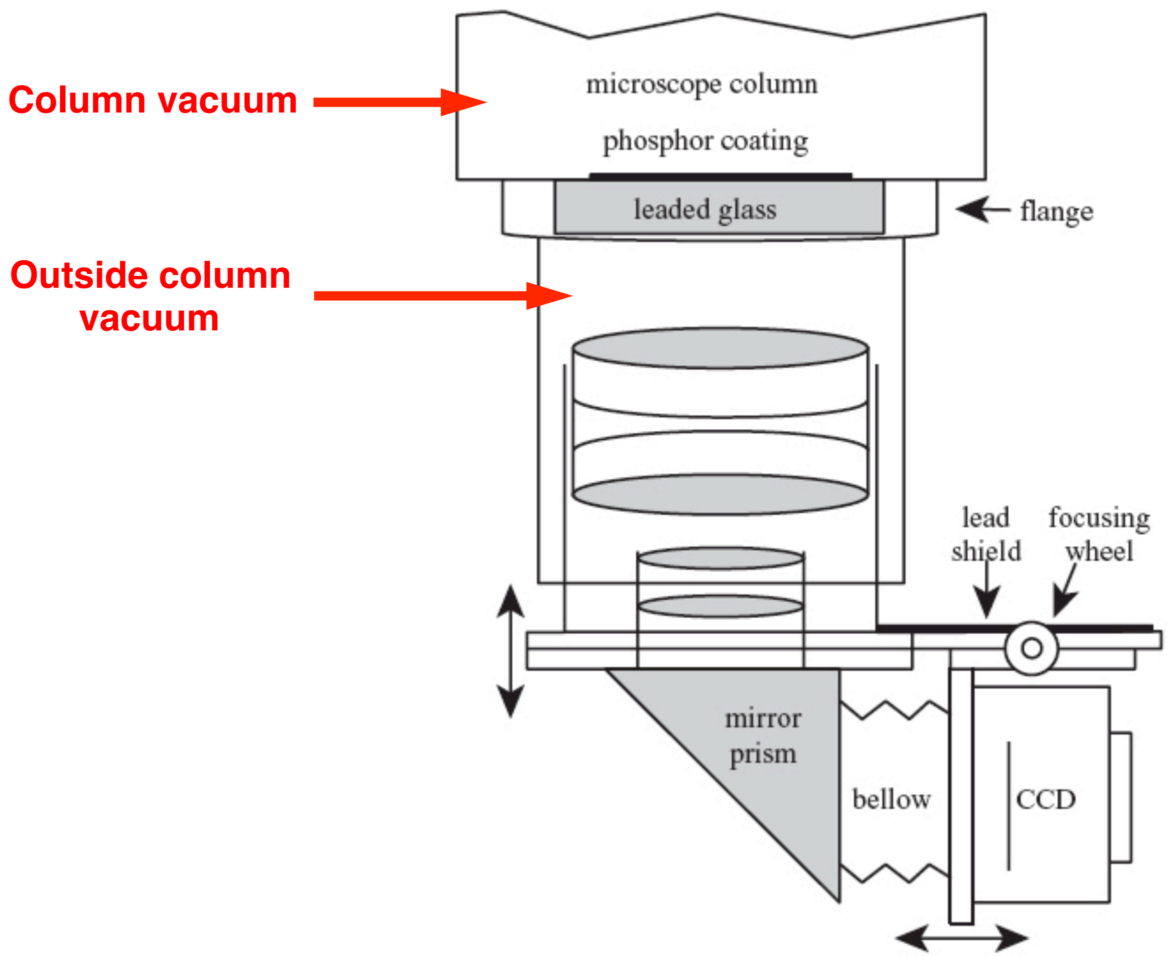
Fiber Optic-Coupled



From Faruqi & Andrews [1997], p.234

I.E.11 Digital Photography (CCD)

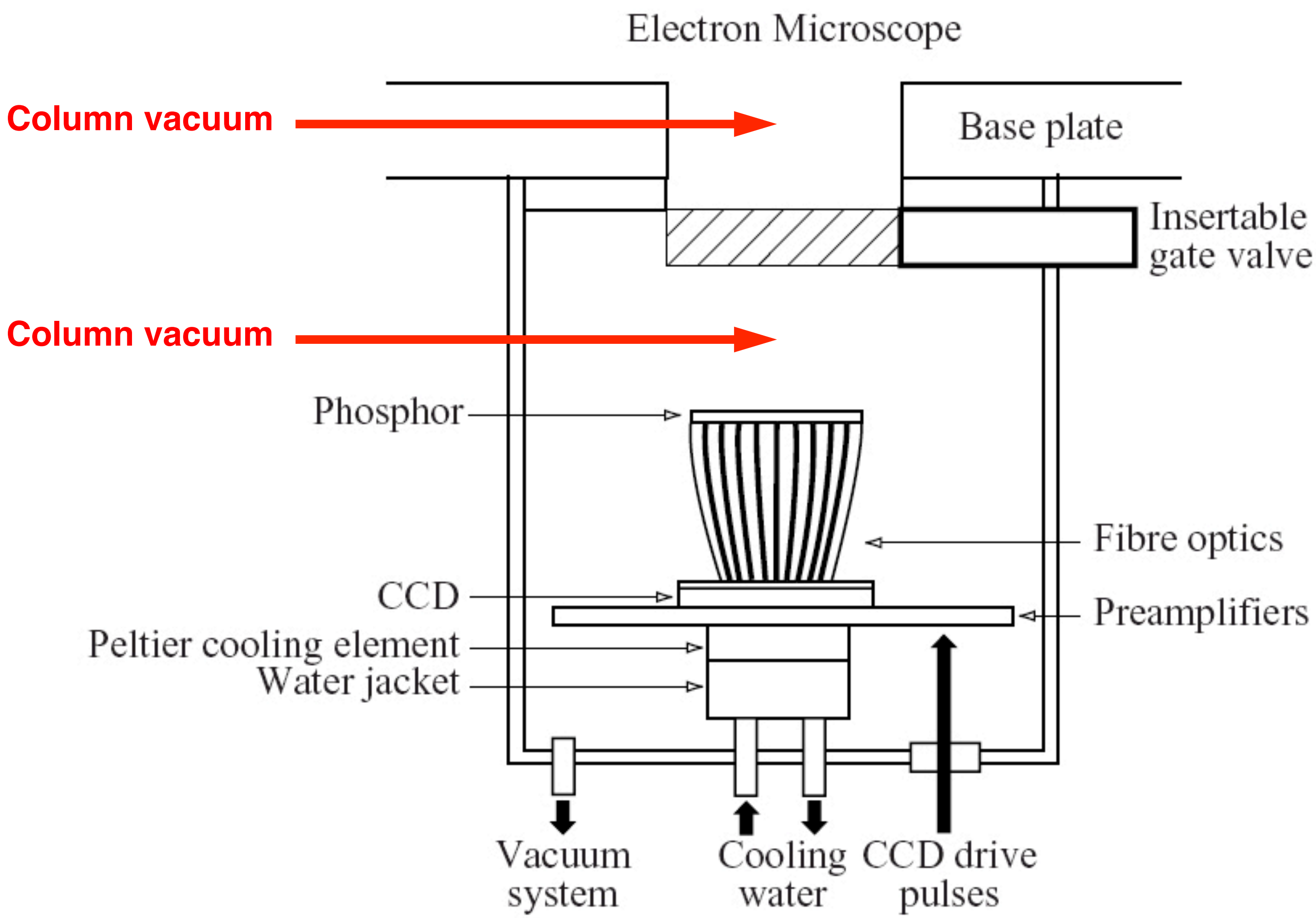
Lens-Coupled CCD



From Fan & Ellisman [1993], p.22

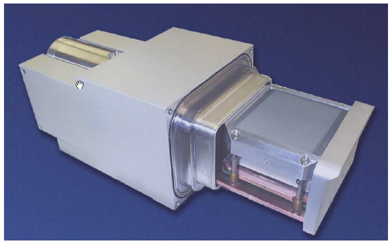
I.E.11 Digital Photography (CCD)

Fiber Optic-Coupled CCD



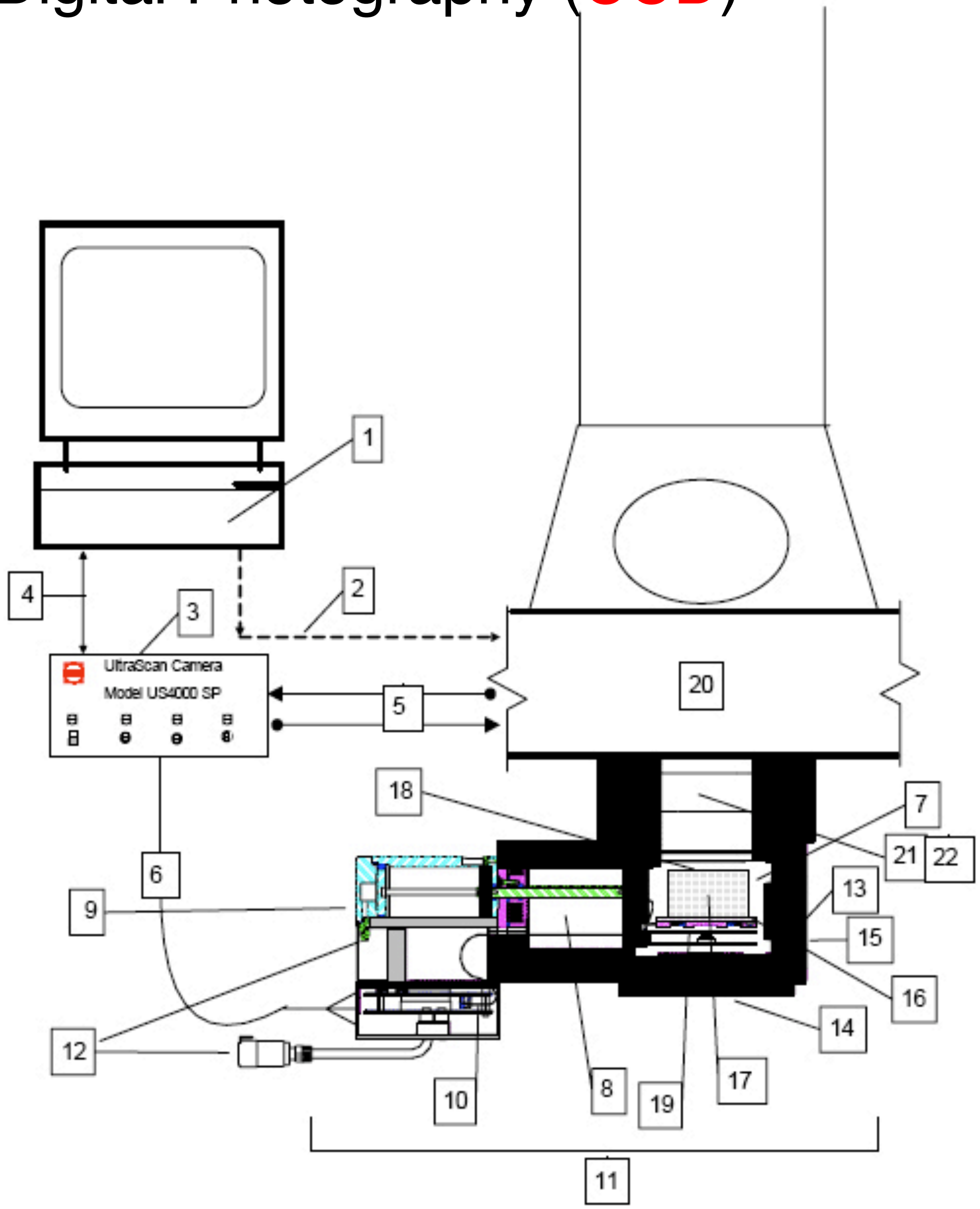
From Faruqi & Andrews [1997], p.234

I.E.11 Digital Photography (CCD)



UltraScan System

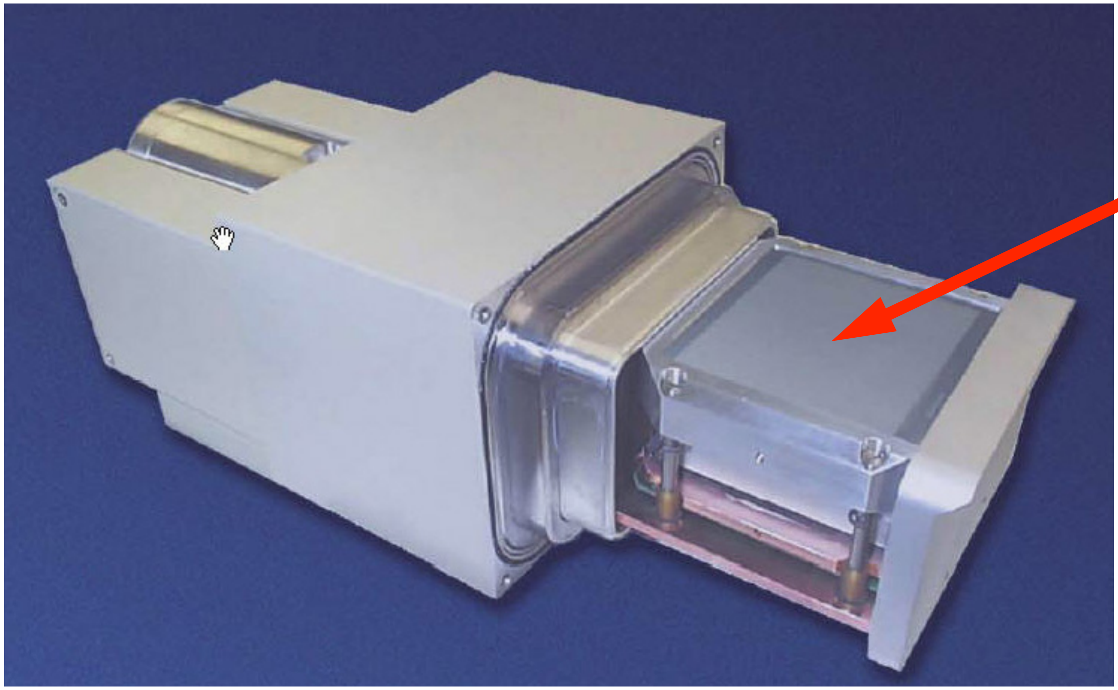
1. Computer
2. TEM-interface cable (optional)
3. Controller
4. Camera-interface cable
5. Shutter-interface cable
6. Camera-interface cable
7. UltraScan vacuum chamber
8. Drive assembly
9. Pneumatic inlets
10. Preamp assembly
11. UltraScan camera module
12. Coolant connections
13. X-ray shielding
14. Blanking flange
15. Accessory port
16. Charge-coupled device
17. Fiber optic plate
18. Electron scintillator
19. Peltier cooler
20. Electron microscope
21. Microscope vacuum
22. UltraScan flange



From Gatan, Inc. Ultrascan 4000 Userguide, p.48

I.E.11 Digital Photography (CCD)

Microscopy with a CCD



Scintillator

CCDs like the 16 megapixel Gatan Ultrascan™ (4080 x 4080 15 μm pixels) can produce high quality digital images in the TEM

http://www.gatan.com/imaging/ultrascan.html