Principles of TEM Image formation, particle detection from TEM images and noise handling

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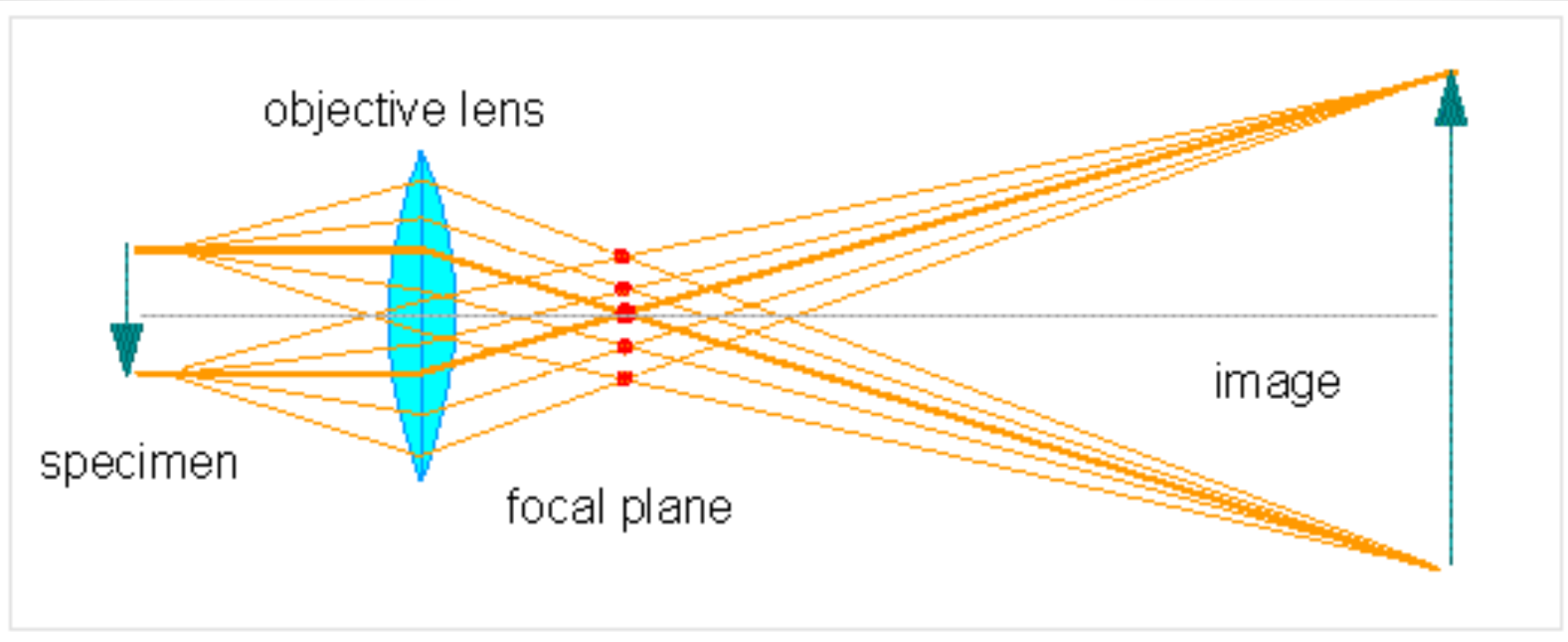
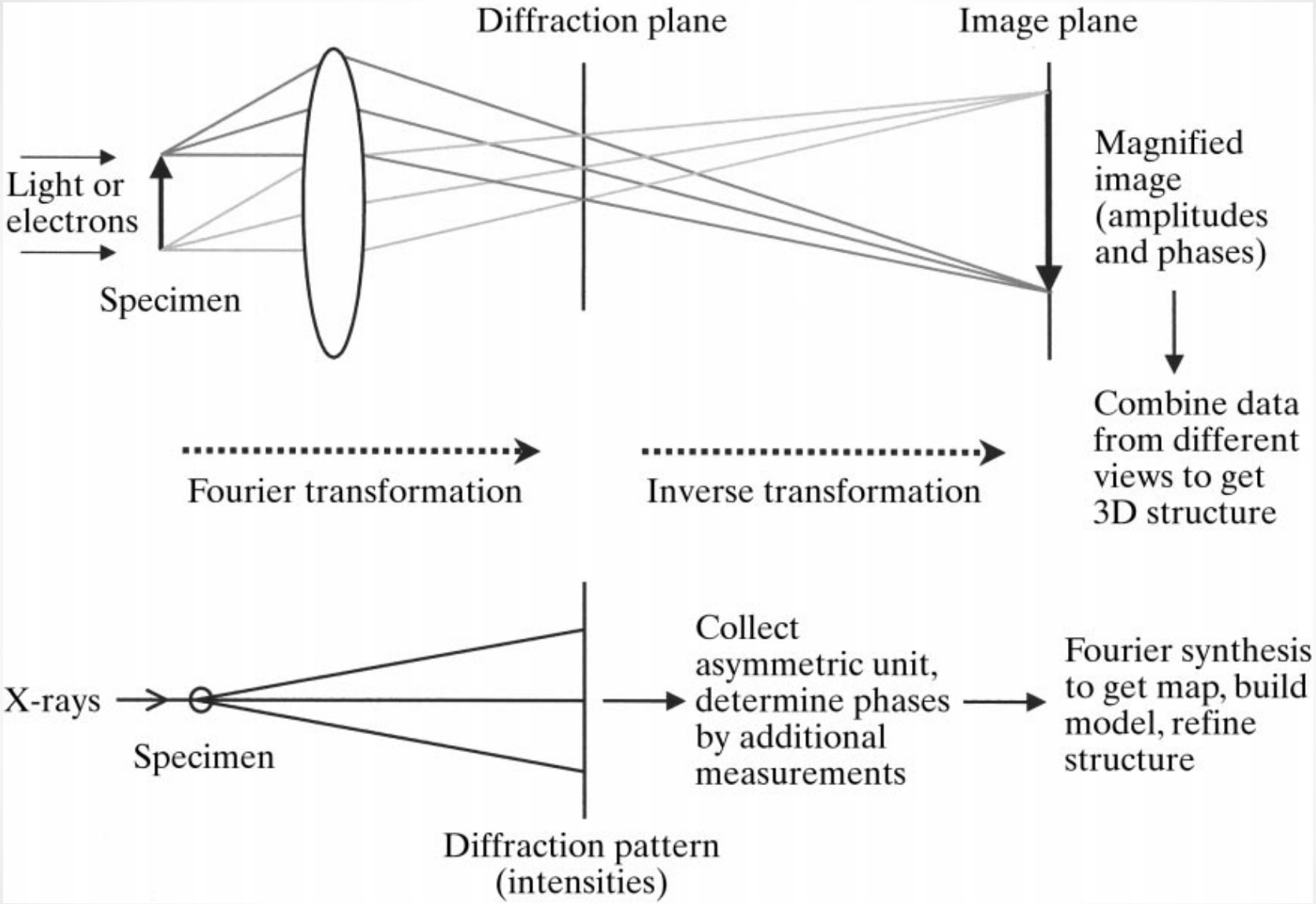
Principle of image formation



$L_1/L_2 = A_1/A_2$,

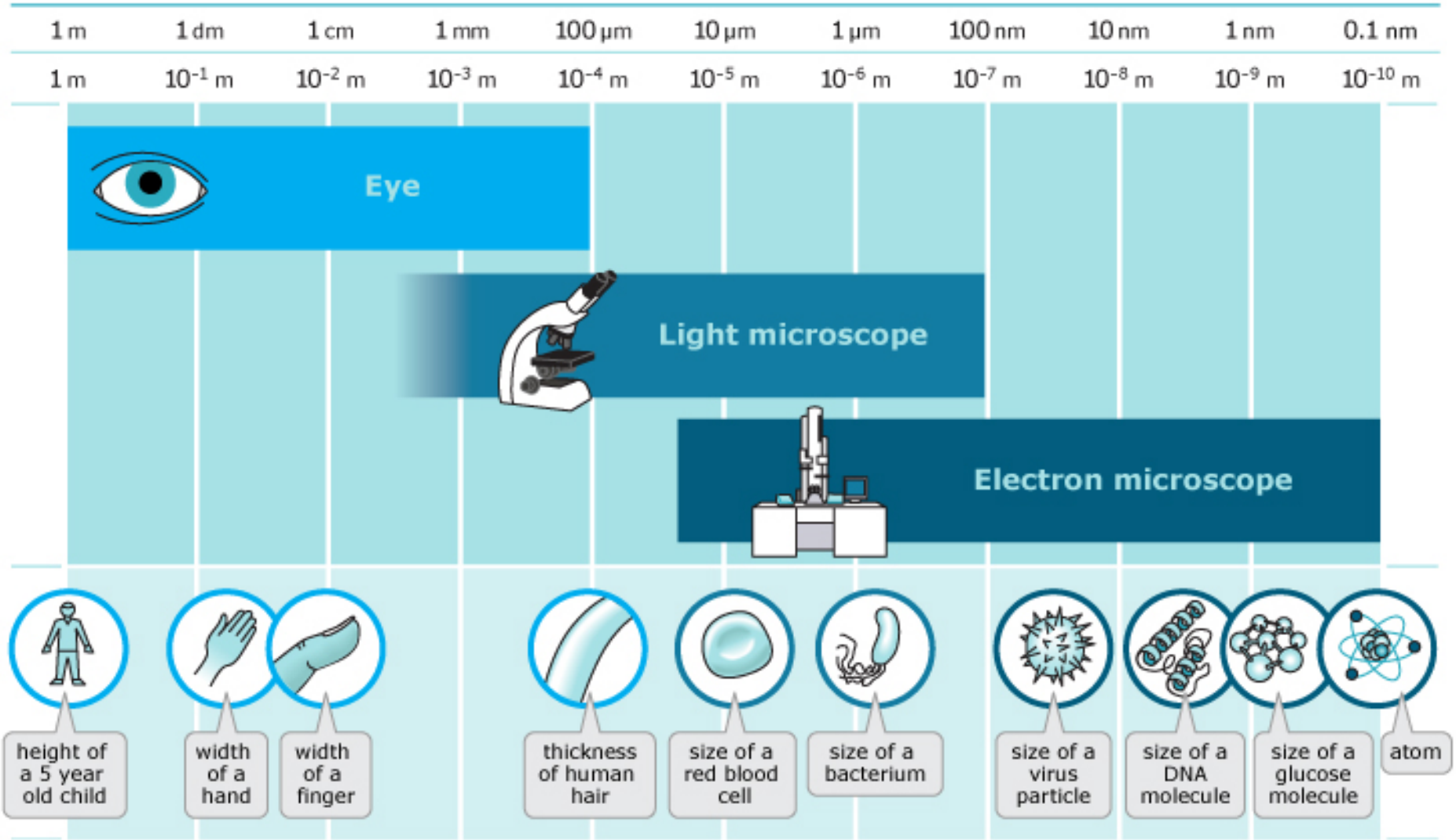
where, L_1 = distance of object from lens
L_2 = distance of object from image
A_1 = size of object
A_2 = size of image

Principle of image formation



Saibil H., Acta Cryst, 2000

Resolving power of microscopes



First electron microscope

Major discoveries:

- *Accelerated electrons behave like light in vacuum*
- *Travel in straight lines, wave like properties*
- *Wavelength 100,000 x shorter than visible light*
- *Electric and magnetic fields could be used to bend and focus electrons*

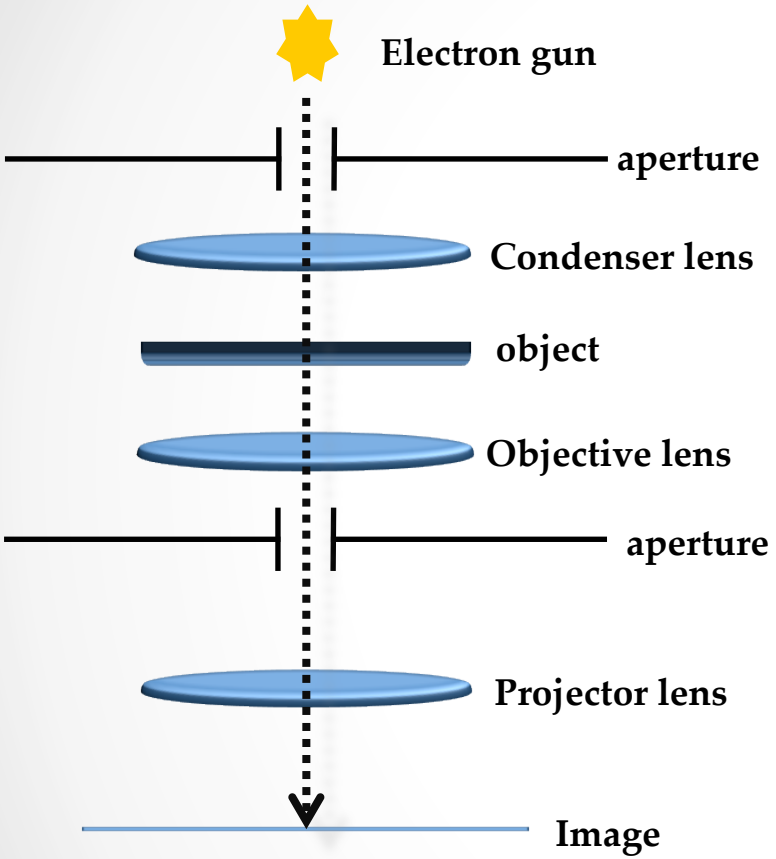
First electron microscope (TEM) designed and built by Ernst Ruska in 1931
Lens for electrons constructed in 1926 by H. Busch

Resolution ~ 100 nm

(~ 200 nm for modern light microscopes)

Current versions

Schematic of an electron microscope



Condenser lenses convert diverging electron beam into parallel beam

Objective aperture prevents electrons scattered at high angles from reaching image plane, improves contrast

Provides initial magnification (20-50x)

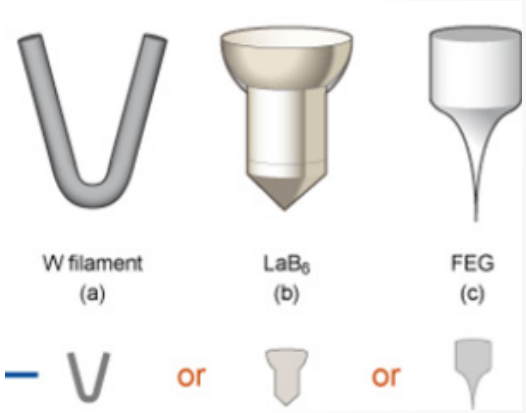
Aberration affects image formation

Intermediate and projector lenses further magnify image

Sources of electrons

Tungsten filament
- Heated to 2000-3000 °C
- Thermionic emission
- Electrons accelerated by electric field between anode and filament
- Energy distribution 2.5 eV
- 40-50 Kx magnification

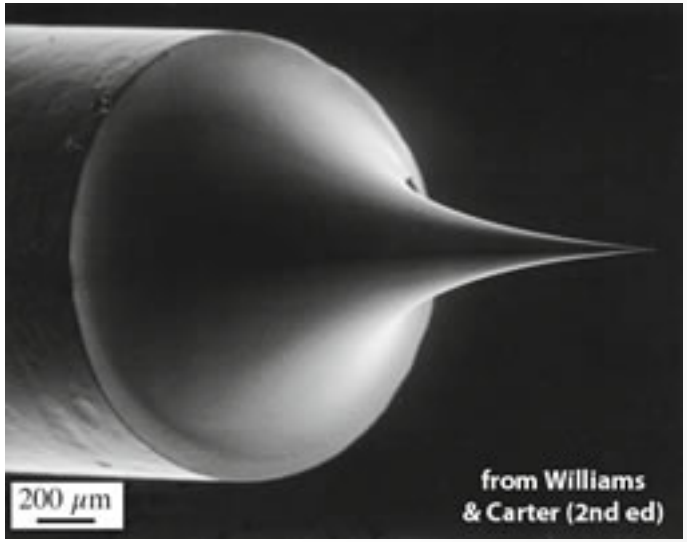
LaB_6 crystal
- Thermionic emission
- Electrons produced from crystal vertex
- Lower temperature required, lower work function
- Better brightness and lifespan compared to tungsten
- Require higher vacuum levels
- Energy distribution 1.5 eV
- 50-100 Kx magnification



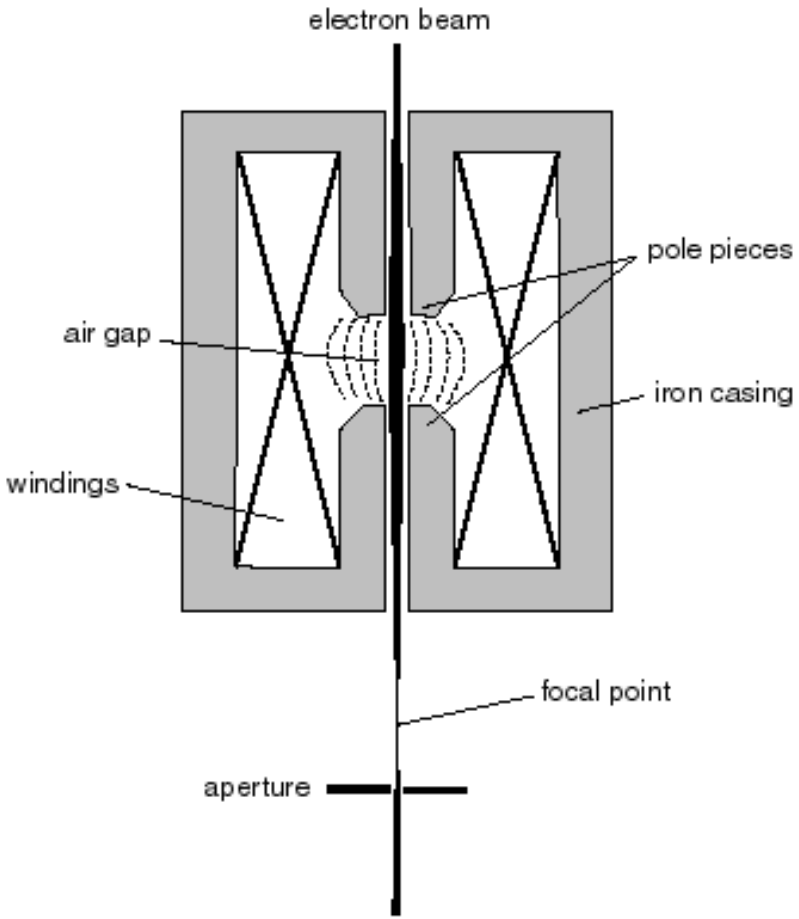
Sources of electrons

Schottkey type Field emission gun (FEG) source
- Single crystal tungsten tip sharpened to 10-25 nm diameter
- Coated with ZrO_2
- Thermally emitted electrons, extracted by strong potential gradient (field emission)
- Accelerated through 100-300 KV
- Extremely bright (~ 500x more than tungsten), very coherent
- Energy distribution 1.0 eV
- > 100 Kx magnification

Cold FEG
- No heating required
- Better brightness
- Energy distribution 0.25 eV
- More intense maintenance



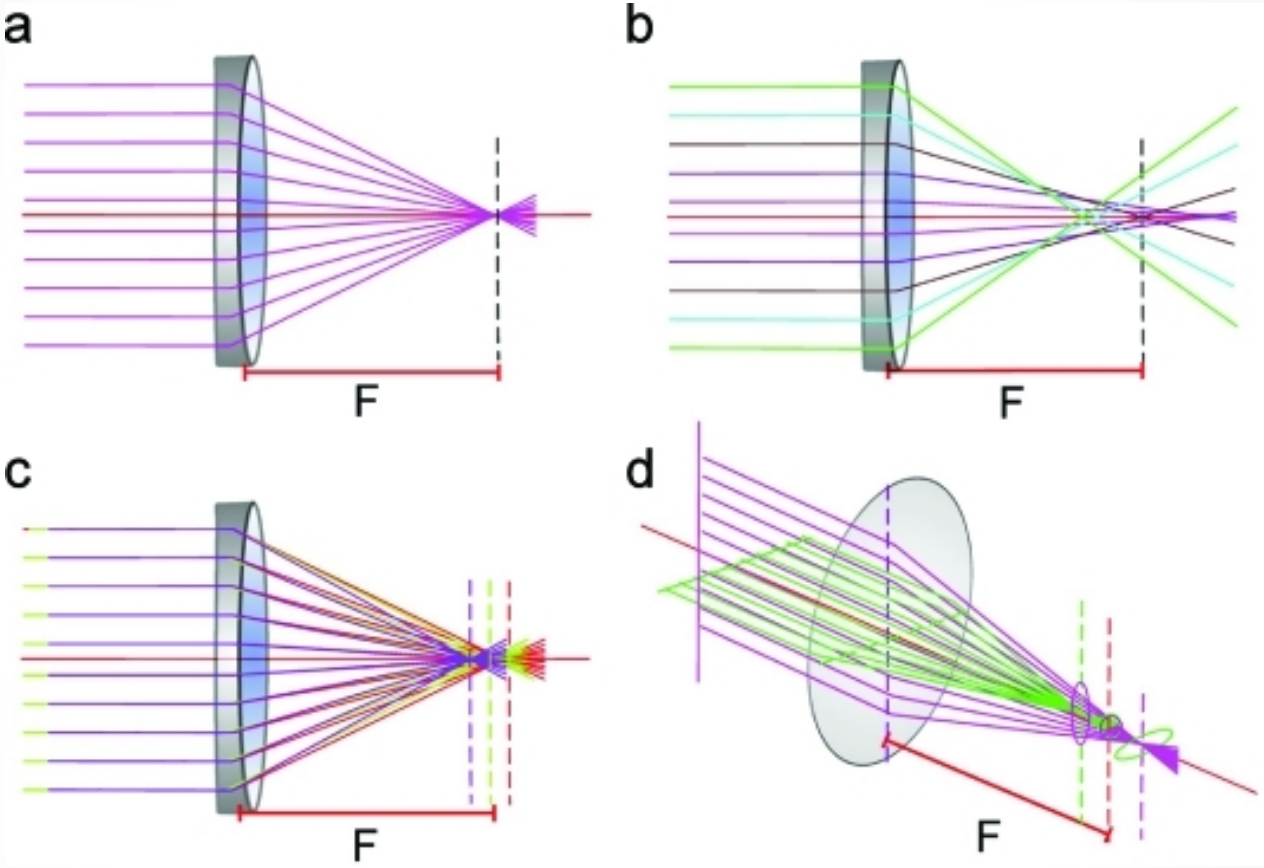
Lenses in electron microscopy



Electromagnetic lenses, varying current in coils alters lens power

Orlova and Saibil, Chem Rev, 2011

Lens aberrations



Orlova and Saibil, Chem Rev, 2011

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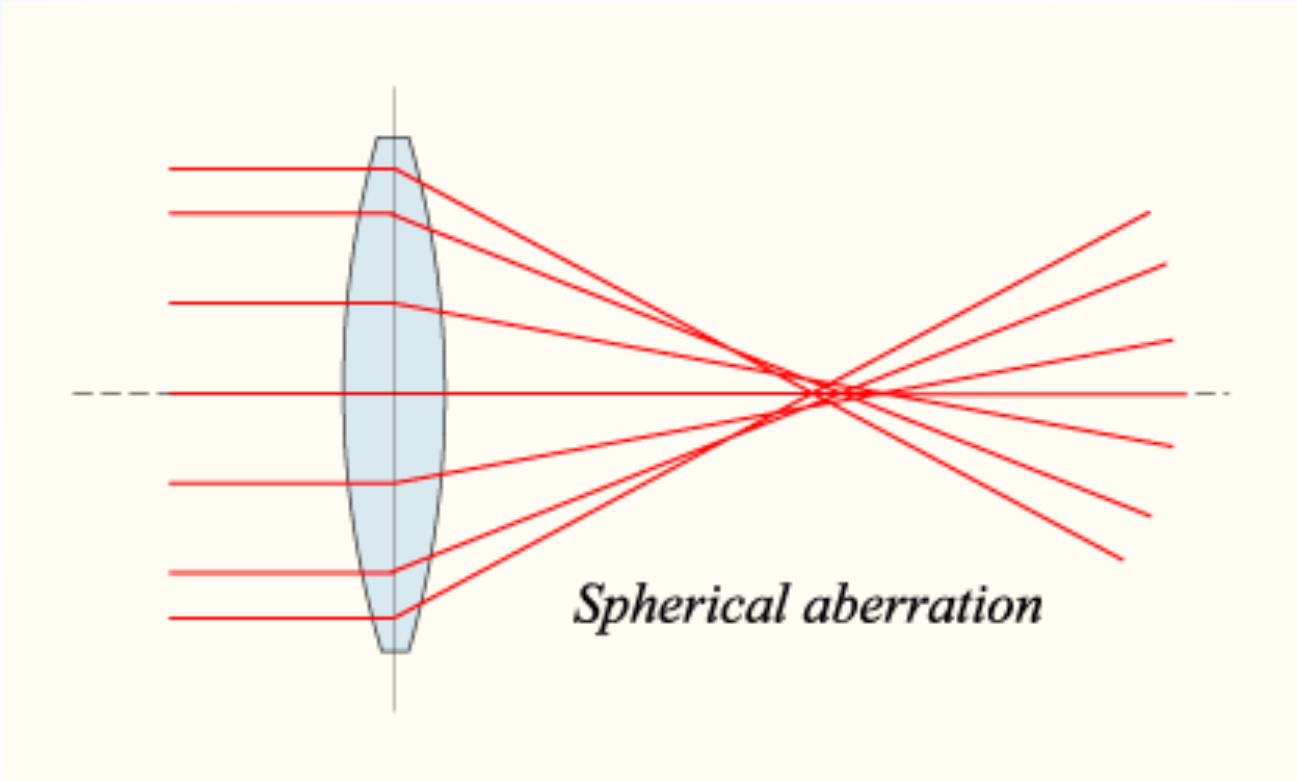
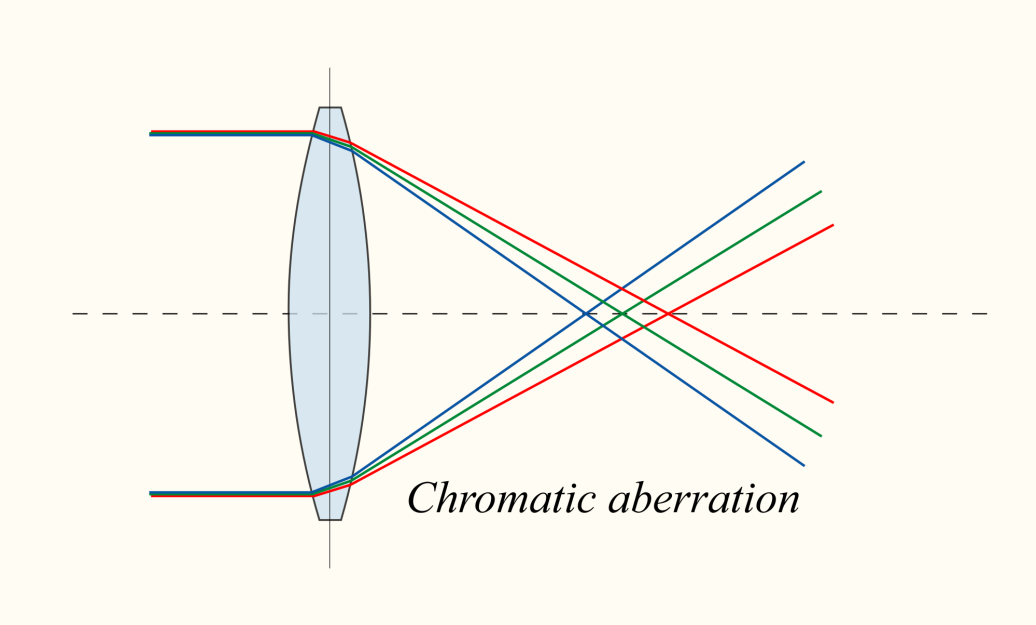


Spherical aberration

- Diffracted rays with higher angle of incidence converge before the focal point
- correction depends on lens design and manufacture

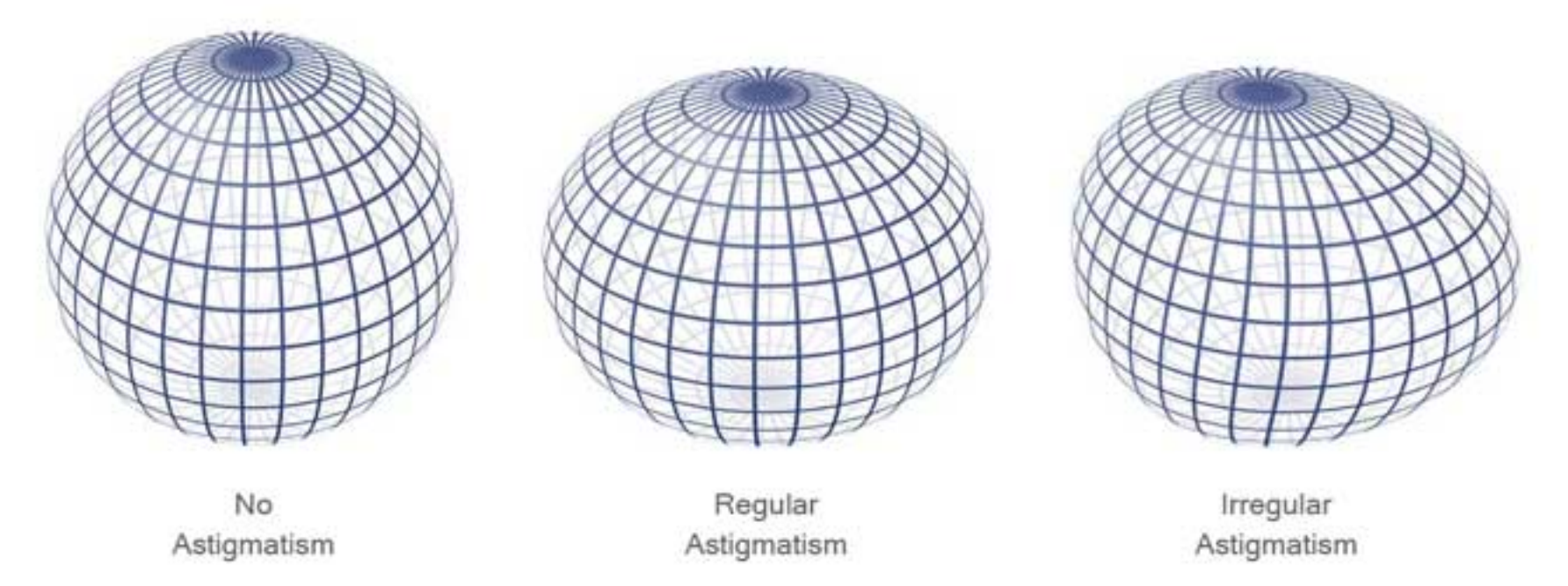
Lens aberrations



Chromatic aberration

- Longer wavelength rays focused more strongly
- Colored halos around images, blurs fine details
- Result of variation in electron energy
- Fixed by stable accelerating voltage

Lens aberrations



Astigmatism

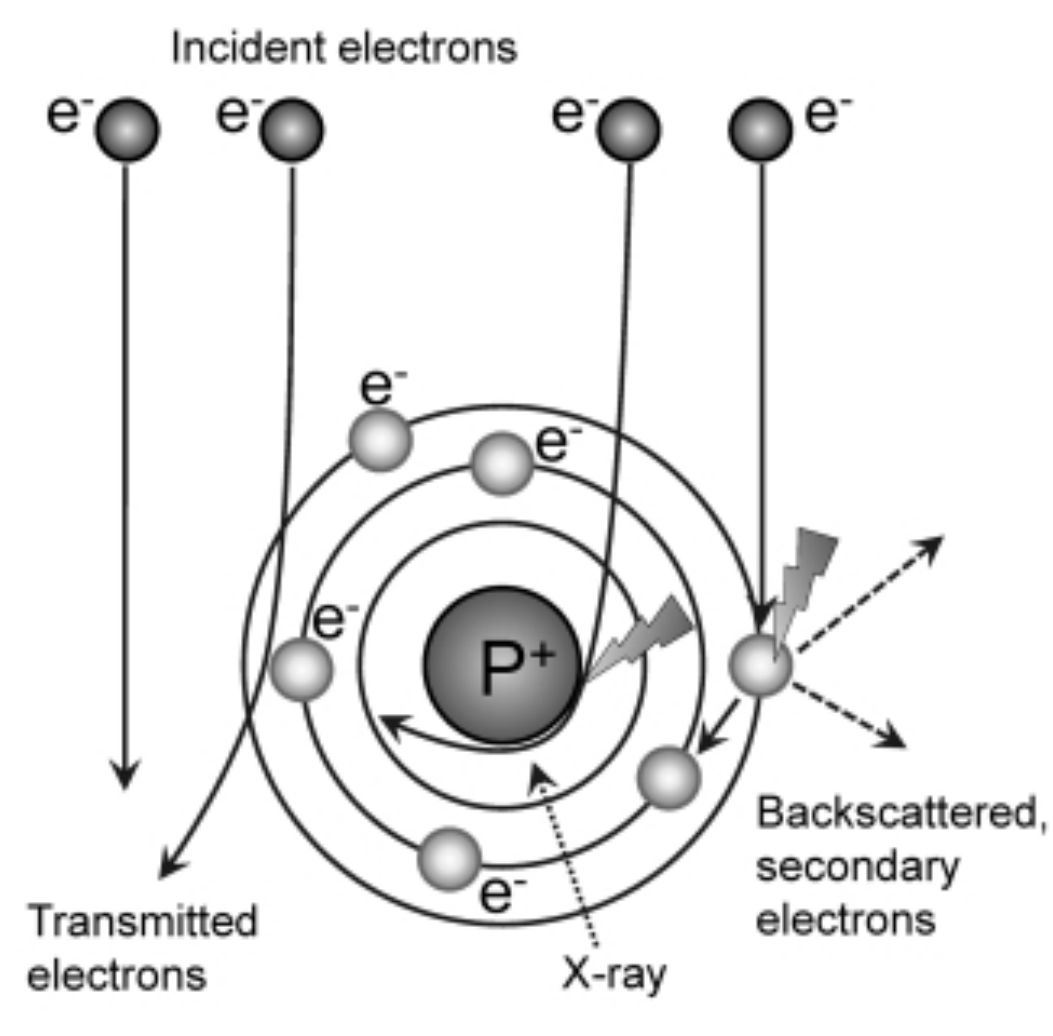
- Caused by asymmetric magnetic field in lenses
- Point becomes ellipse
- Compensated by stigmator coils

Interaction of electrons with samples

Upon elastic collision of electrons with atom, electrons will be scattered with no change in kinetic energy
- contribute to image formation

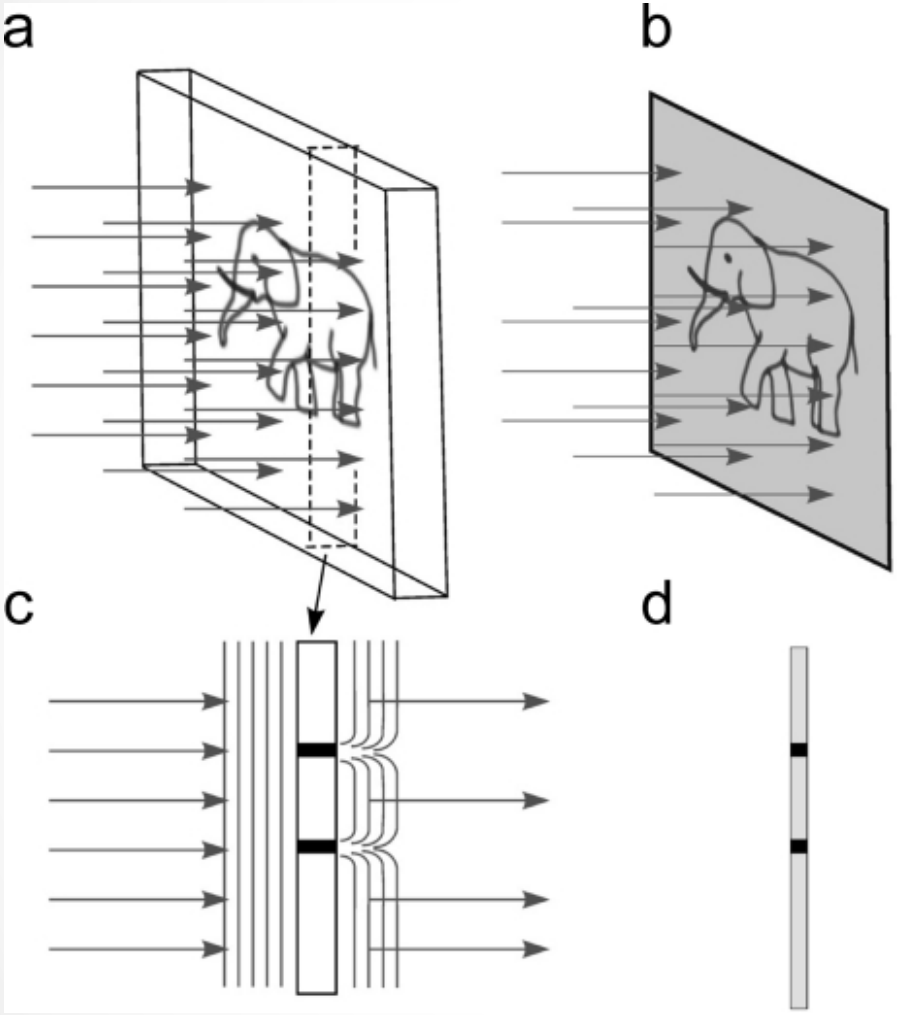
Upon inelastic collision of electrons with atoms, a part of the kinetic energy of electrons is transferred to the atom
- can ionize atoms, generate free radicals, alter chemical bonds, generate X-rays
- contribute to noise

Interaction of electrons with samples



Orlova and Saibil, Chem Rev, 2011

Generation of contrast in images



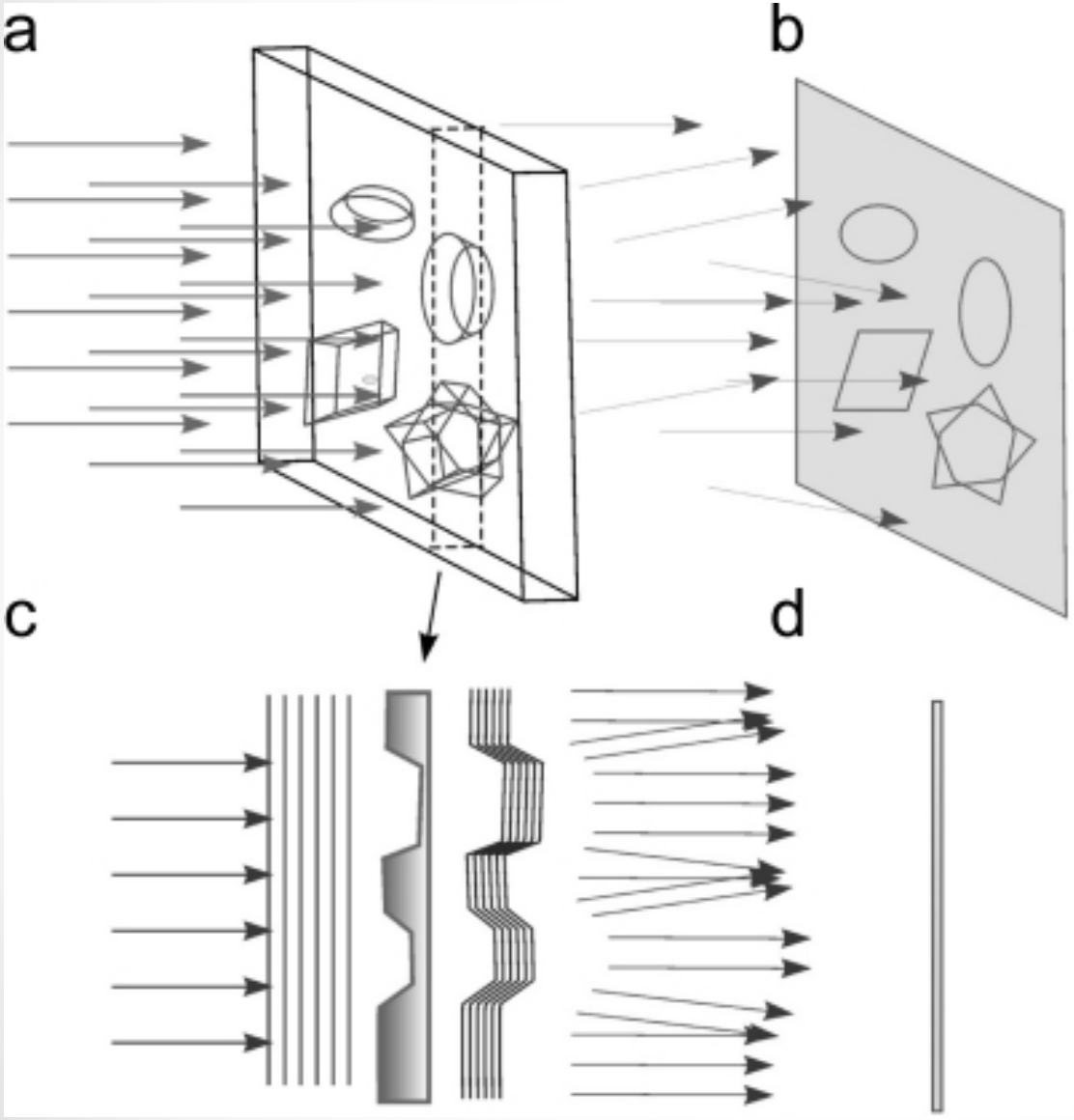
Amplitude contrast:

Part of beam absorbed by the sample
Produces image contrast

Problem: Biological samples do not absorb beam, rather deflect beam
Intensity difference very small

Orlova and Saibil, Chem Rev, 2011

Generation of contrast in images



Phase contrast-

Electrons undergo scattering at various angles

Have different path lengths throughout sample

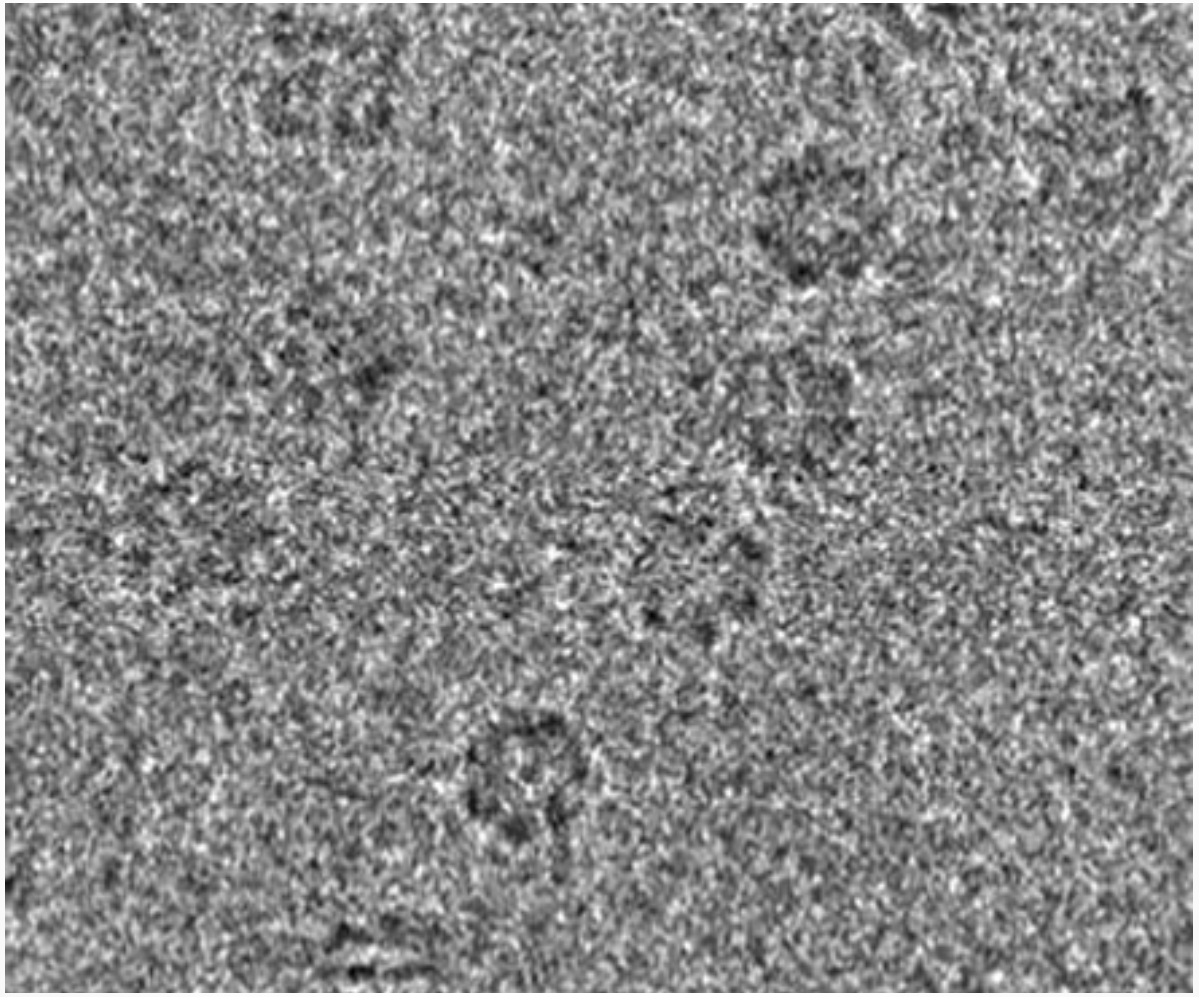
Emergent beam undergoes constructive or destructive interference with parallel beam

Phase variations may be converted to amplitude variations

Orlova and Saibil, Chem Rev, 2011

Generation of contrast in images

Biological samples consist of light atoms – C, H, O, N



Nagayama and Danev, Philo Trans Royal Soc B, 2008

Generation of contrast in images

Transparent object varies in refractive index or thickness

Amplitude of emergent wave remains same, phase differs

$T(x, y) = A_0 \, exp\,[i\phi(x, y)]$, $A_0 = 1$

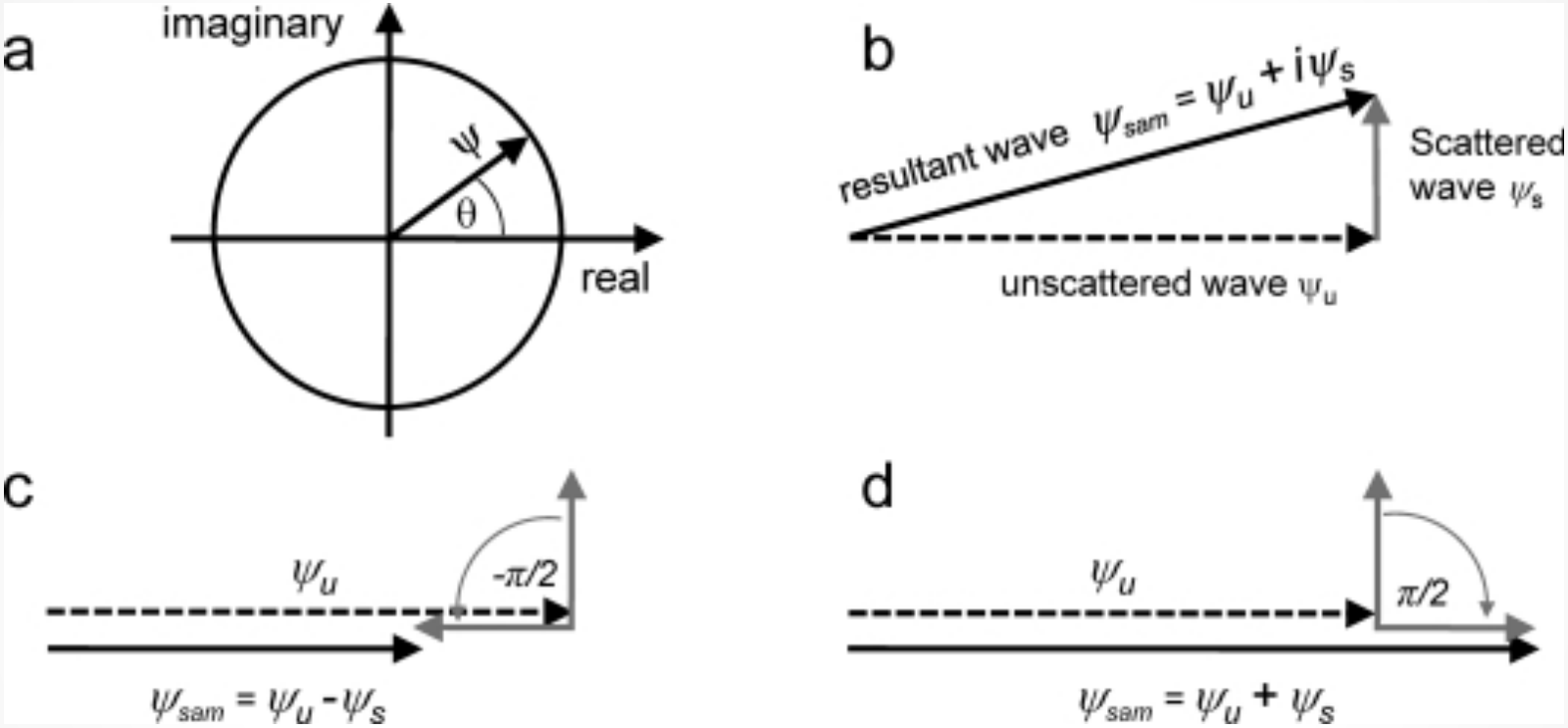
Representation of emergent wave (assuming sample is thin, and phase shift is small):
$exp\,[i\phi] \approx 1 + i\phi$ (weak phase object)

Therefore, $T(x, y) = 1 + i\phi$

Observed intensity: $I^2(x, y) = T^2(x, y) = 1 + i\phi \approx 1$

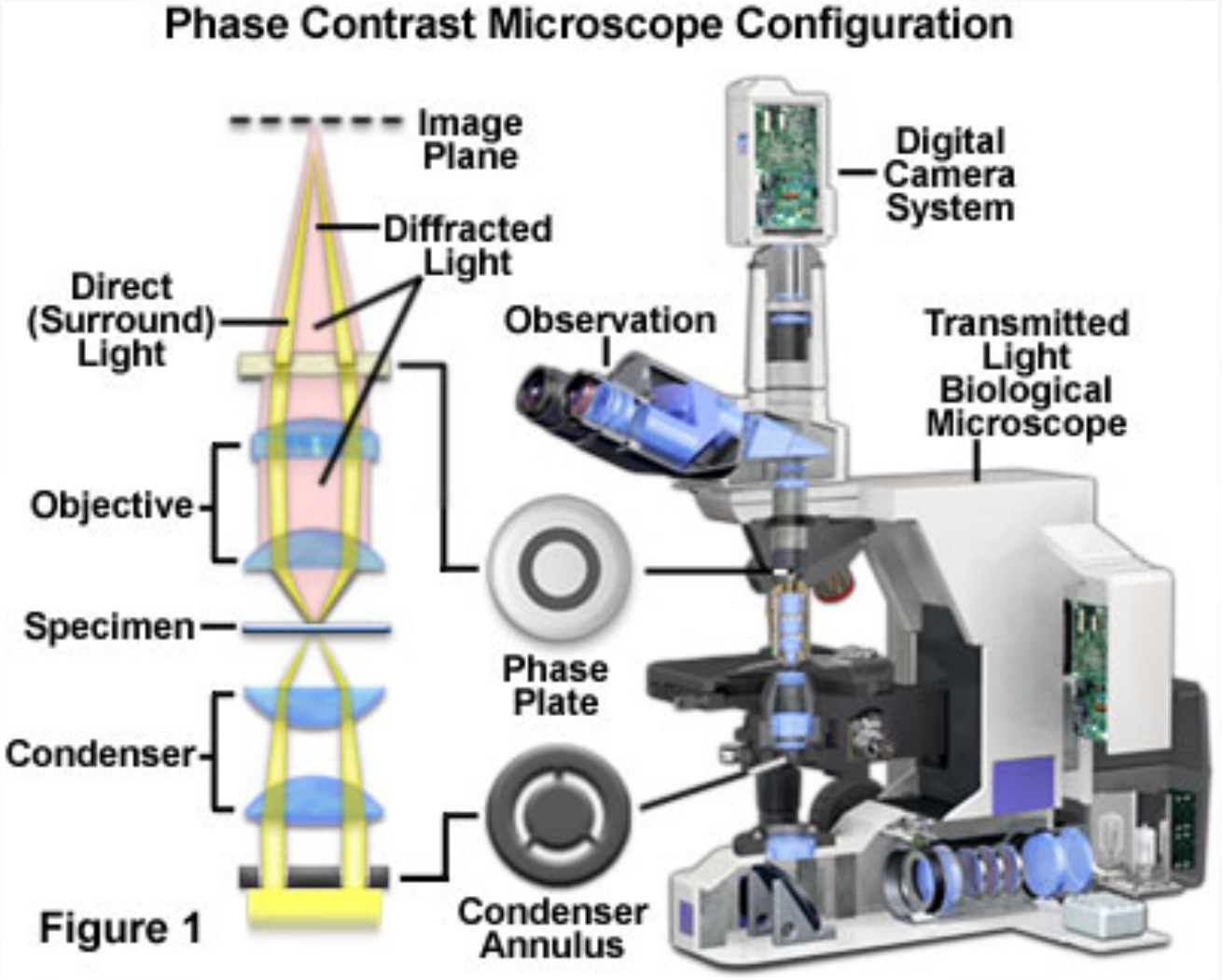
With additional phase shift of 90°, however, $I^2(x, y) = T^2(x, y) = (1 - \phi)^2 \approx 1 - 2\phi$

Phase contrast microscopy



Orlova and Saibil, Chem Rev, 2011

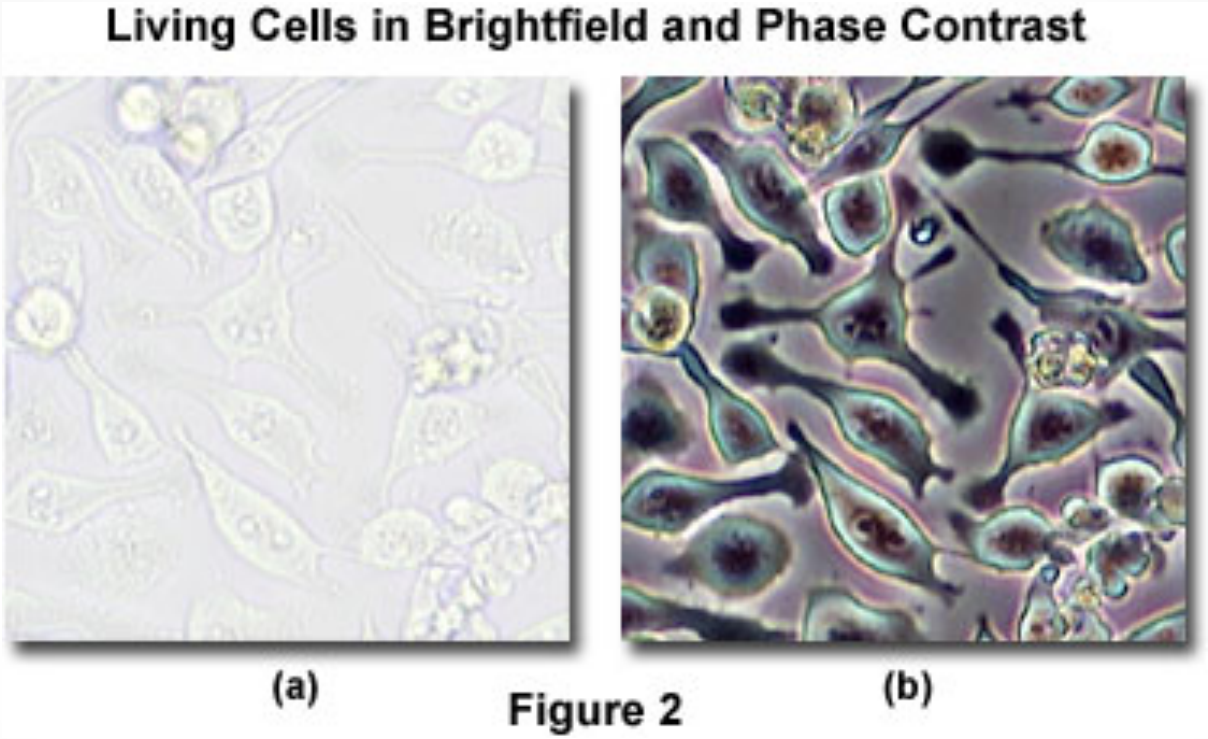
Generation of contrast in images



Fritz Zernike (1934) - Phase contrast microscopy
Phase plates introduced in the back focal plane of objective lens
Shifts phase of scattered waves by 90°, amplitude contrast

http://www.microscopyu.com/

Generation of contrast in images



Improvement in contrast of biological samples

http://www.microscopyu.com/

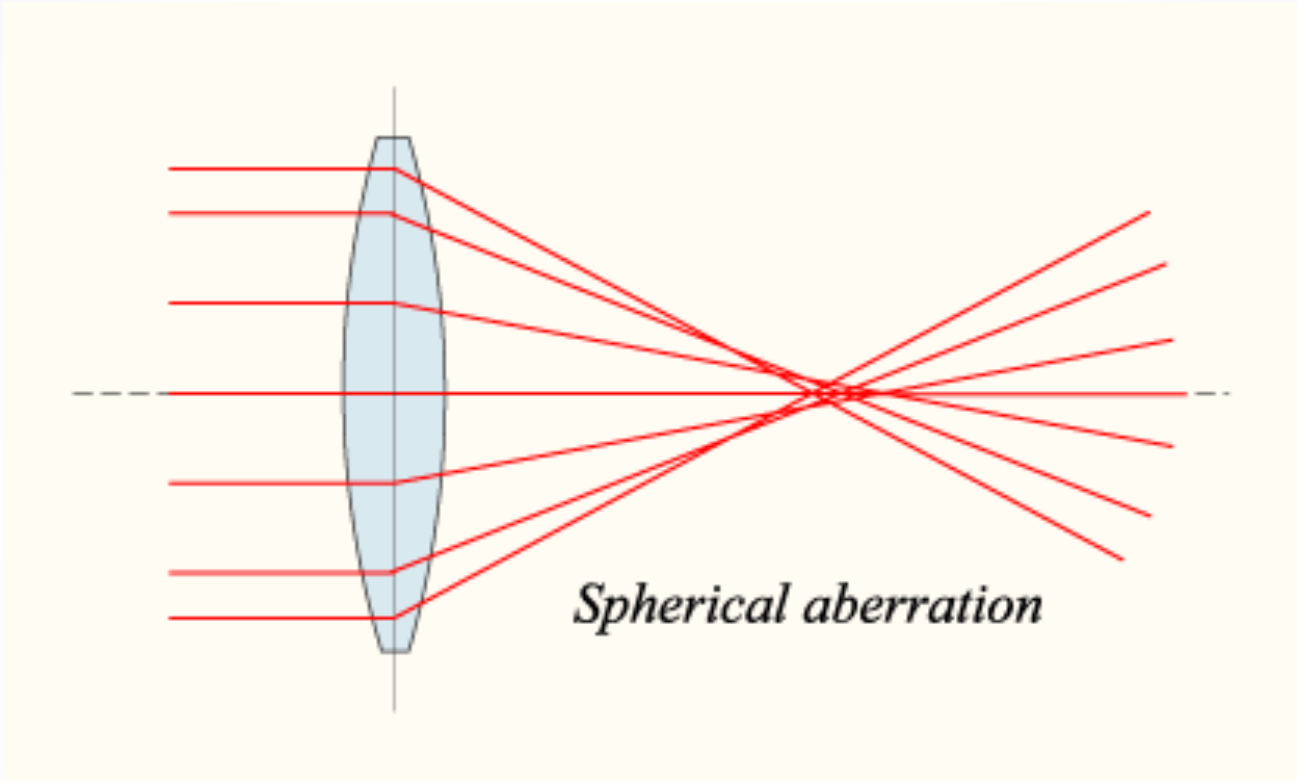
Generation of contrast in electron microscopy

Combination of various factors generate contrast

1) Spherical aberration
2) Defocus
3) Apertures

Induce phase shift, cut off inelastically scattered electrons

Generation of contrast in electron microscopy



Spherical aberration

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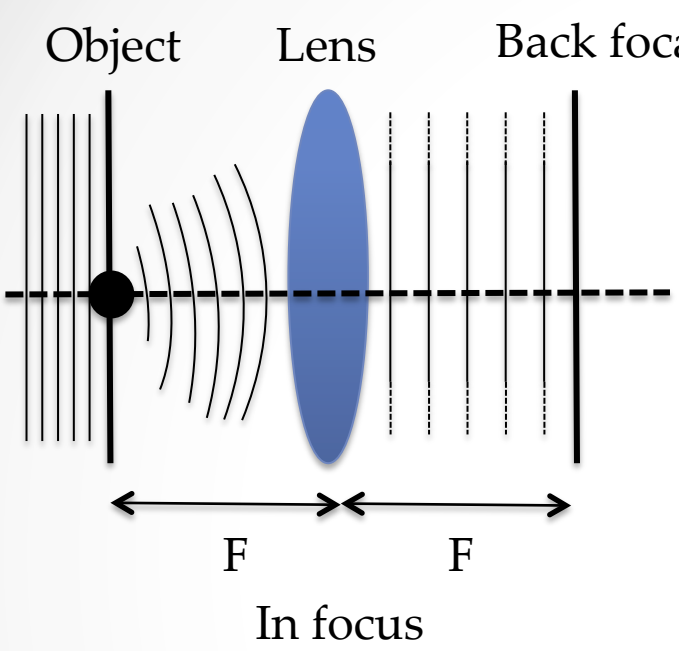
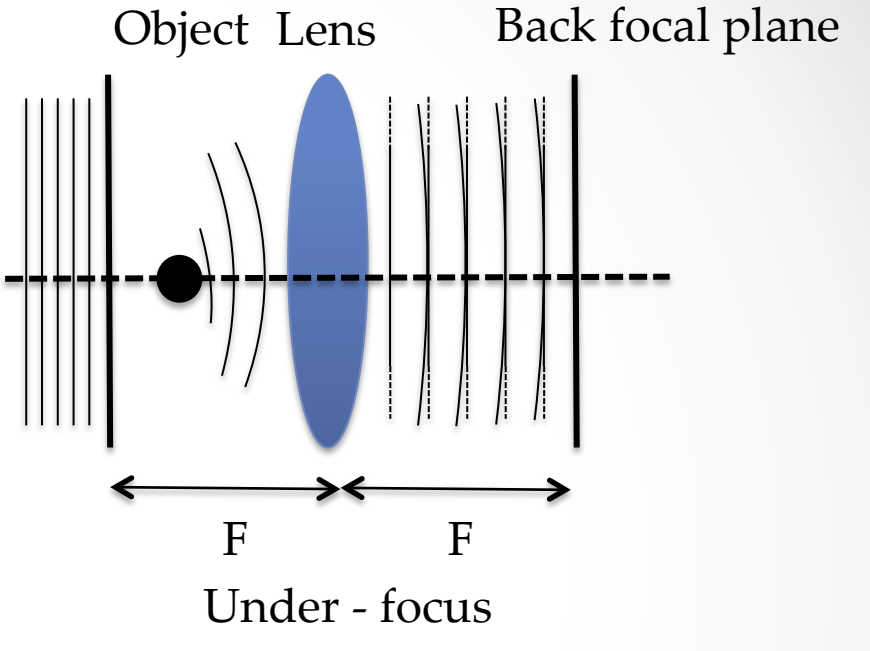
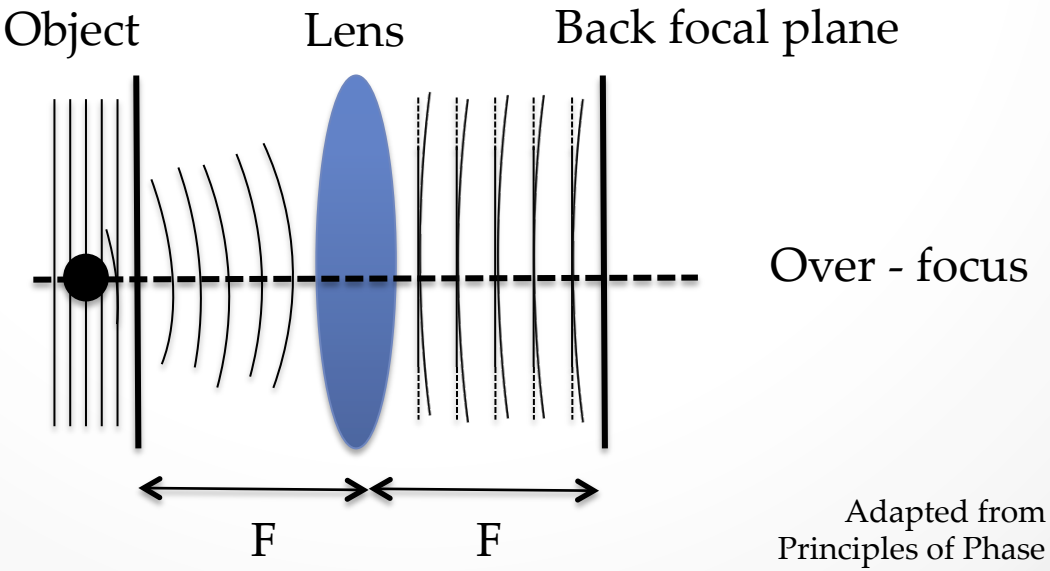
- correction depends on lens design and manufacture

Generation of contrast in electron microscopy

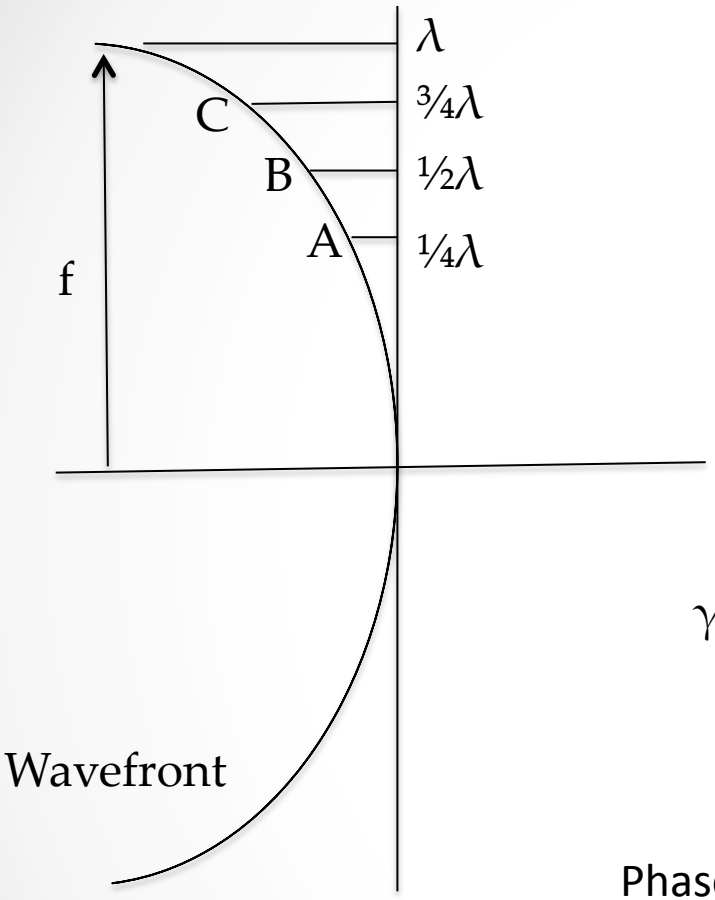


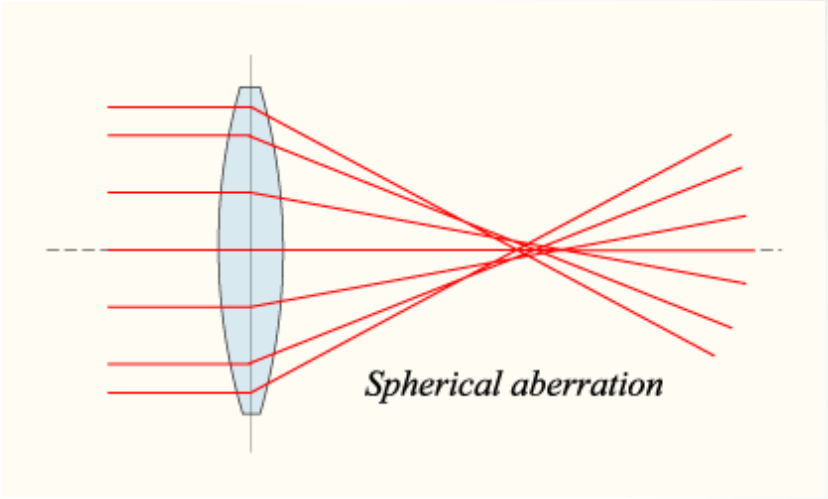




Adapted from Marin van Heel,
Principles of Phase Contrast Microscopy

Generation of contrast in electron microscopy





$$\gamma(\vec{R}) = -2\pi\left(\tfrac{1}{2}\Delta\lambda\,\vec{R}^2 - \tfrac{1}{4}\,C_S\,\lambda^3\,\vec{R}^4\right)$$

Phase shift

Defocus

Radial frequency

Spherical aberration

Defocus and spherical aberration together cause phase shift at the back focal plane
Contrast generation

Adapted from Marin van Heel,
Principles of Phase Contrast Microscopy

Microscope properties affecting image formation

Lens aberrations

Coherence of source

Drift

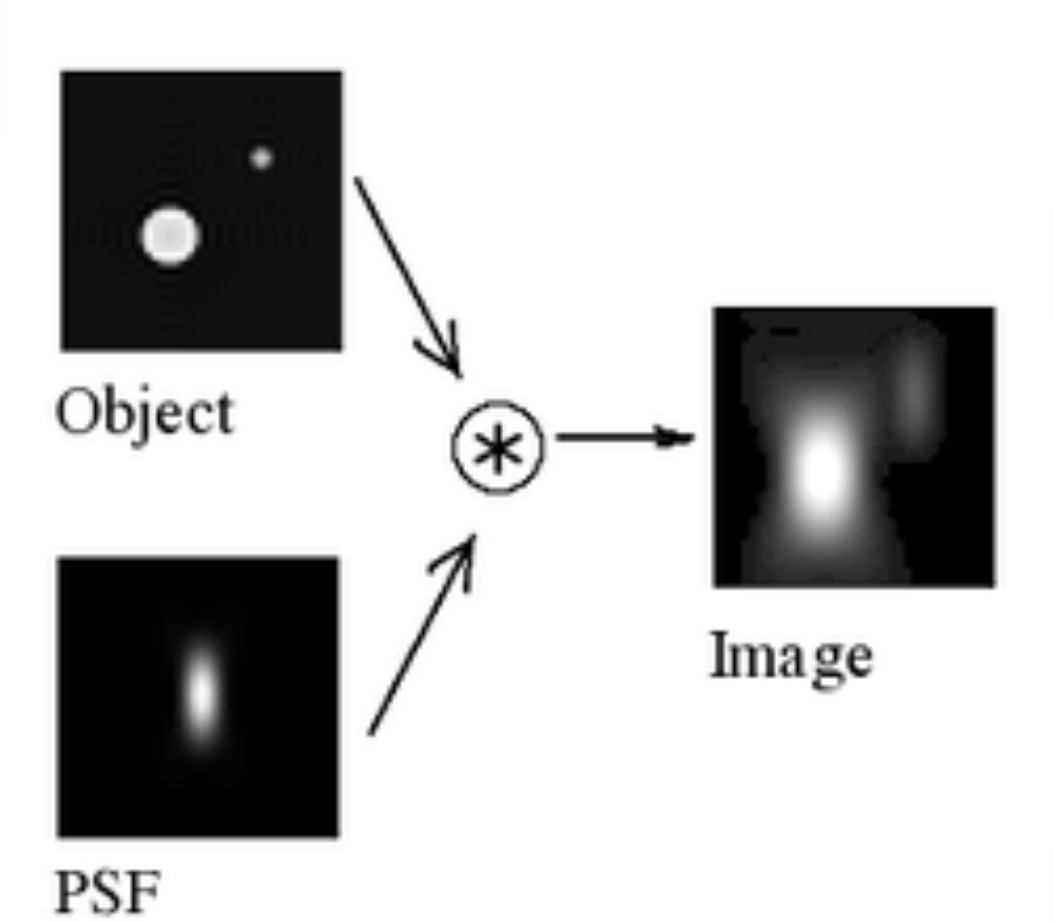
Quality of ice

Alterations in lens current

Quantum noise

Instrumental or environmental instability

Microscope properties affecting image generation



Point Spread Function (PSF) represents microscope aberrations
Convolution of Object (FT) with PSF (FT) generates image
Imperfections transferred to image

Orlova and Saibil, Chem Rev, 2011

Microscope properties affecting image generation

Microscope properties affecting image generation

Fourier Transform of PSF = Contrast Transfer Function (CTF)

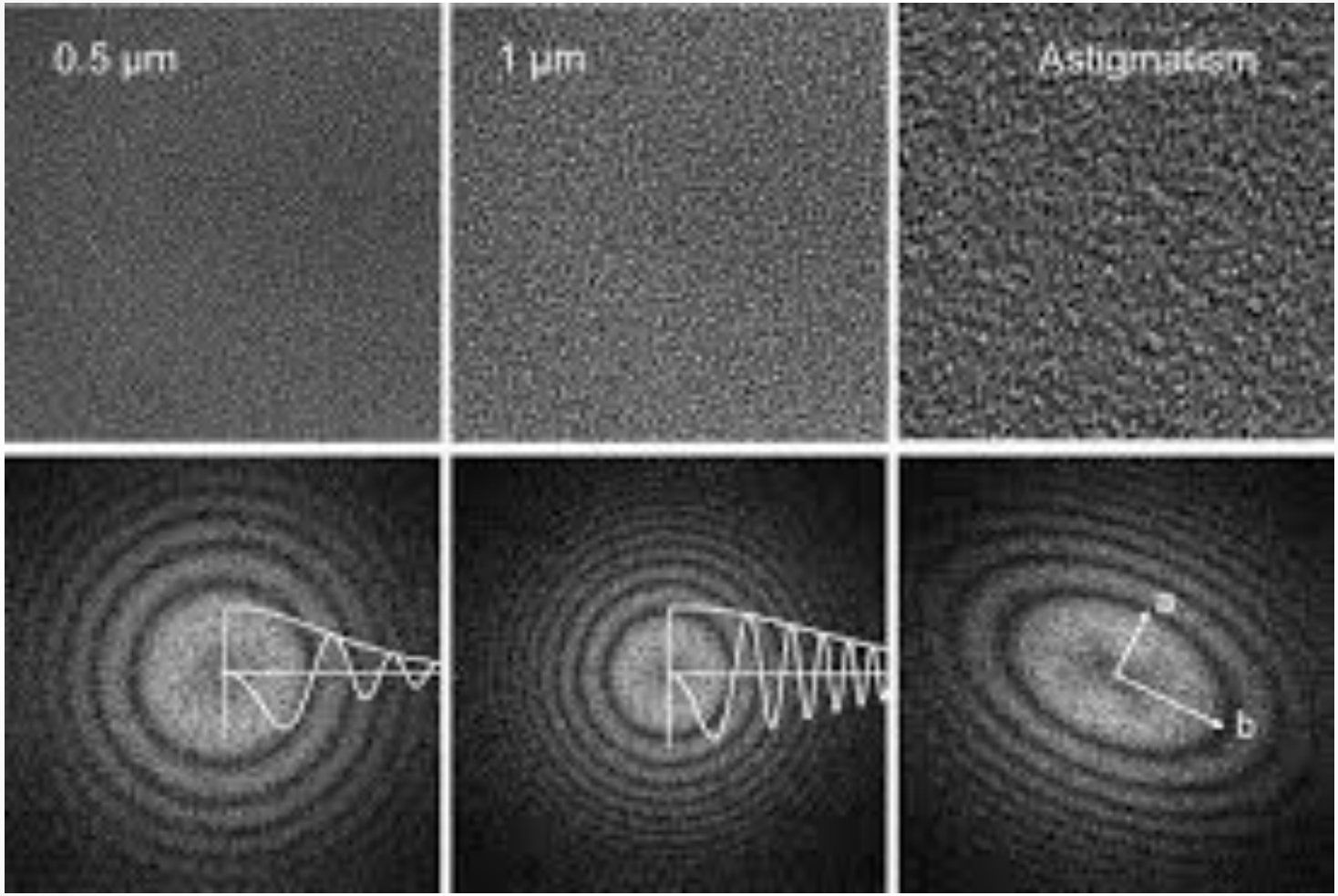
Describes the imaging properties of the objective lens

Can be used to describe the influence of factors on image quality

$$F\{\Psi_{obs}(\vec{r})\} = F\{\Psi_{sam}(\vec{r})\} . CTF(\vec{R}) . E(\vec{R})$$

$$F\{PSF(\vec{r})\} = CTF(\vec{R}) . E(\vec{R})$$

Microscope properties affecting image generation



Effect of defocus and astigmatism on CTF
Acts as a band pass filter

Orlova and Saibil, Chem Rev, 2011

Imaging cryo samples – Low dose mode

Incident electrons generate heat

Biological samples degrade

Area to be imaged not exposed until the image is taken

Focusing and alignment done on a different site

Electron dose – 5 -10 electrons/$Å^2$

Signal to noise ratio (SNR) very low

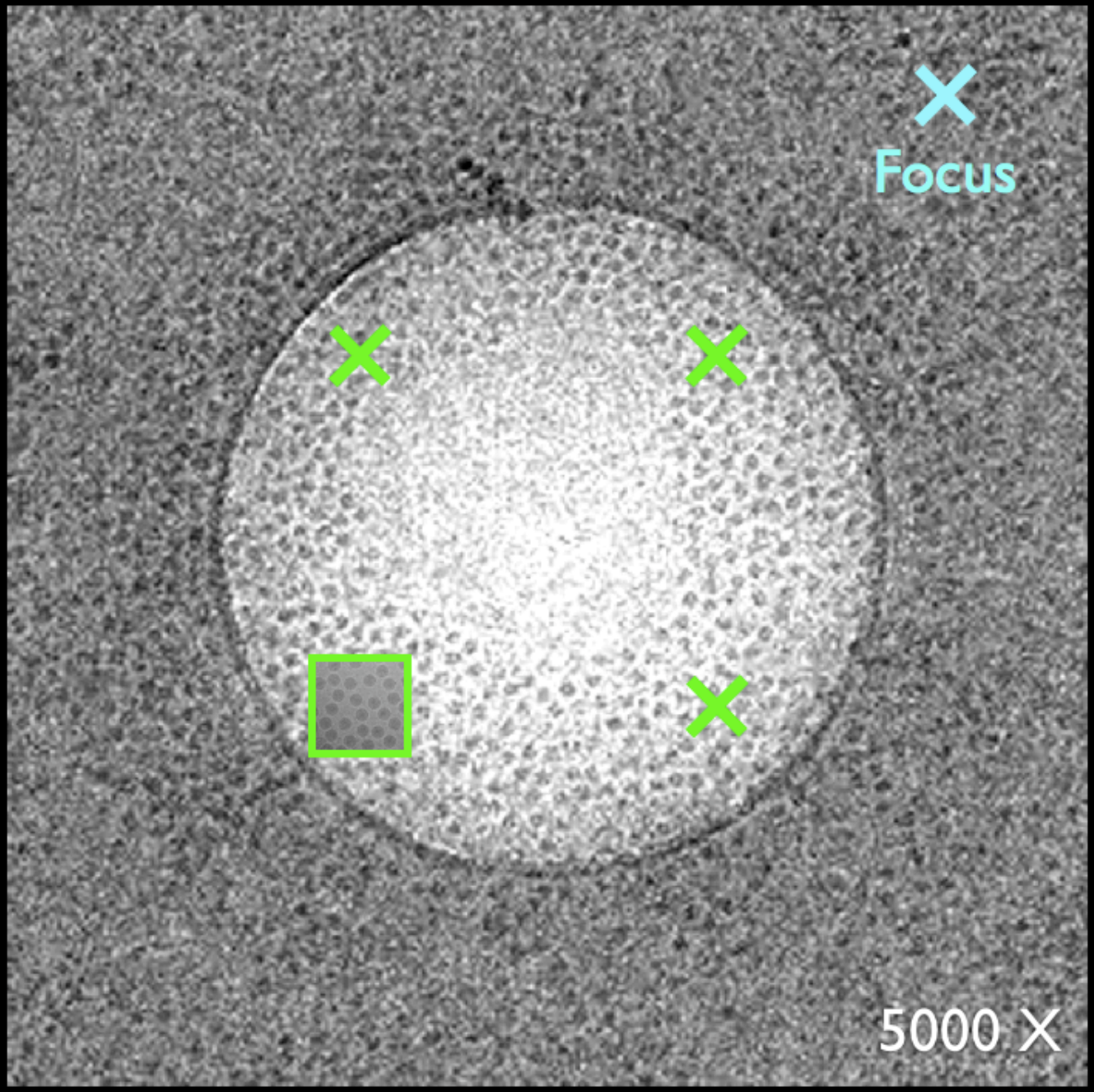


Photo Credit: Gabriel Lander, TSRI

Methods for contrast and SNR improvement

Sample level: Cryo-negative staining

Microscope level: phase plate, energy filters, aperture size, defocus

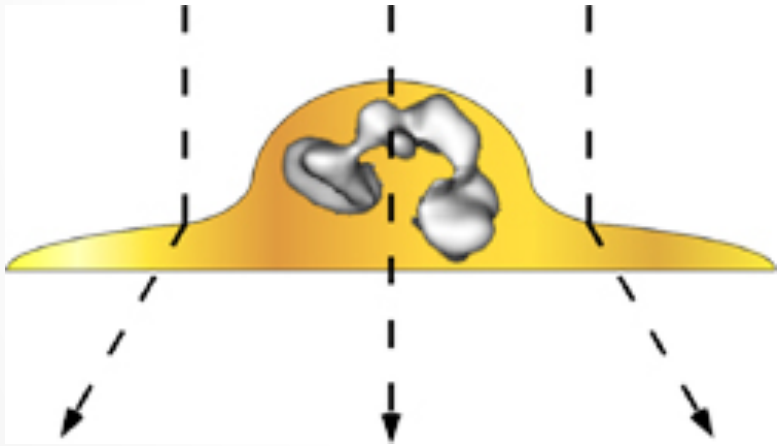
Data collection level: direct detectors, automated collection

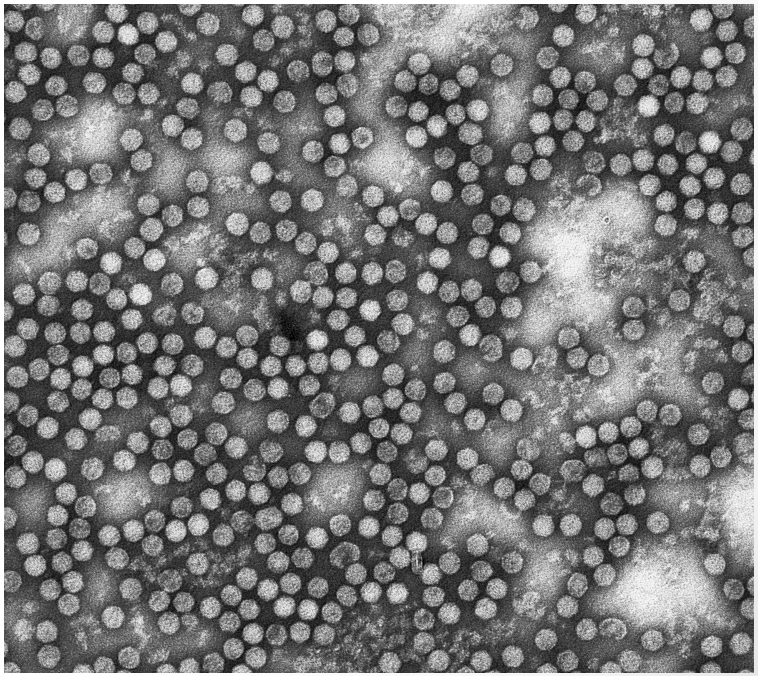
Negative staining with heavy metal

Very small amount of electrons absorbed by biological samples

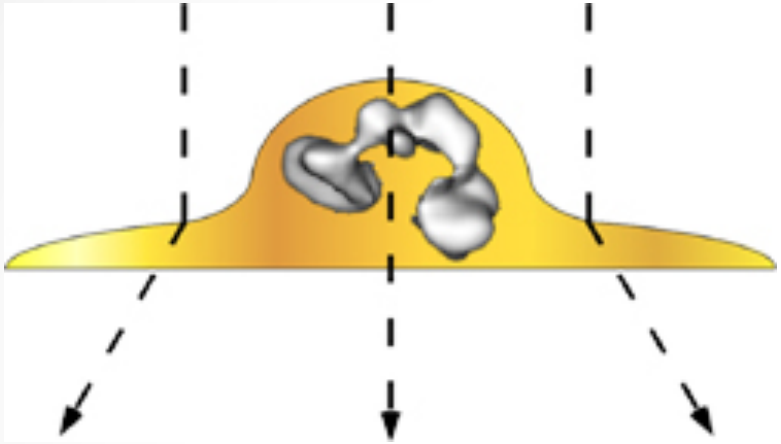
Heavy metal salt, that absorbs electrons fairly easily, used for negative staining

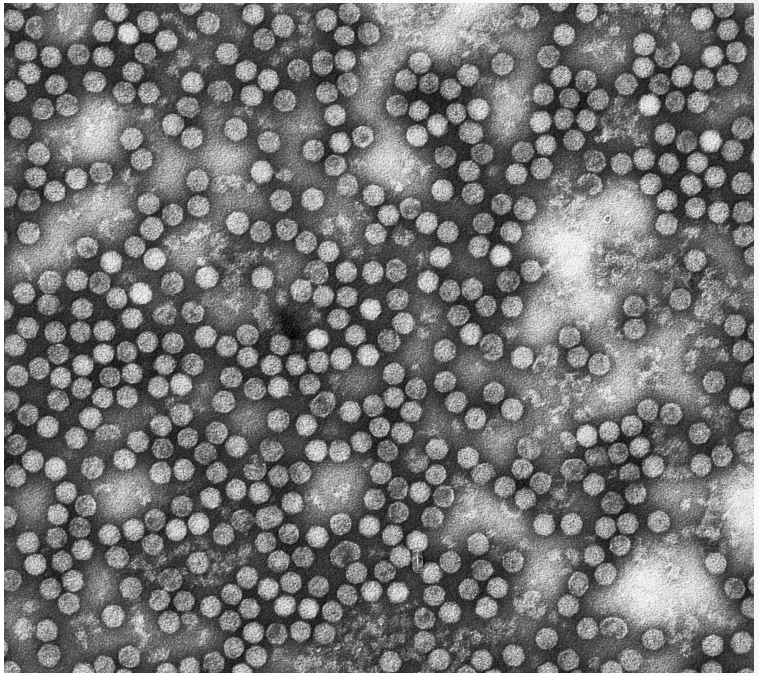
Uranium, tungsten, molybdenum, vanadium, lead





Negative staining with heavy metal





Drying step required

May cause dehydration-related damage

Formation of artifacts

Only surface features visible, low resolution

Structural details of external or internal regions not available

Possible to get low resolution reconstructions

Samples may have preferred orientation on continuous carbon film

Methods for contrast and SNR improvement

Sample level: Cryo-negative staining

Microscope level: phase plate, energy filters, aperture size, defocus

Data collection level: direct detectors, automated collection

Cryo-negative staining

Prevalent method developed by Marc Adrian in 1998

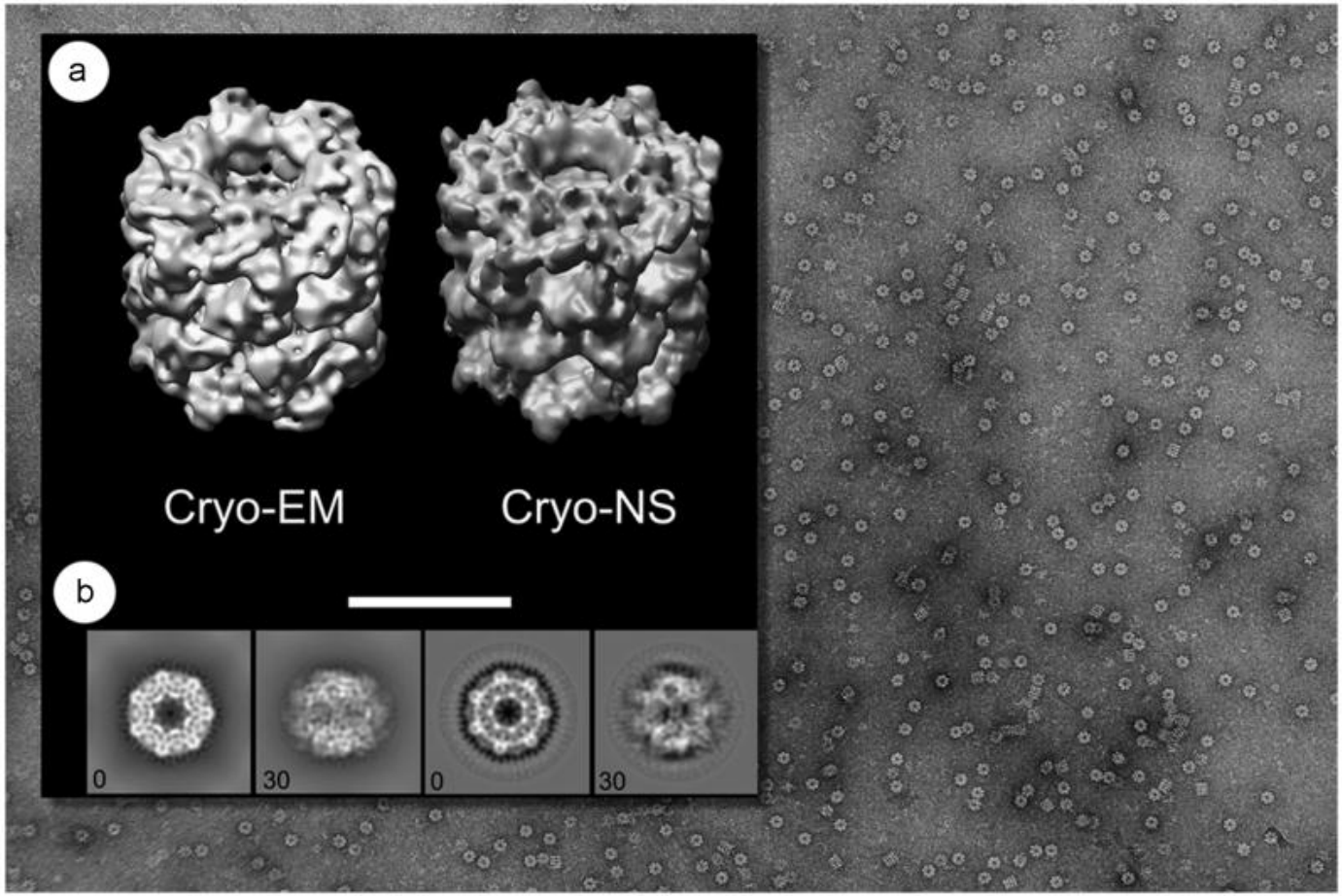
A thin layer of Au/Pd on one side of grid – allows sample spreading

Slurry of ammonium molybdate as staining solution

Quick dip in stain on parafilm, dried for 1-3 s, plunge freezing

Some dehydration expected

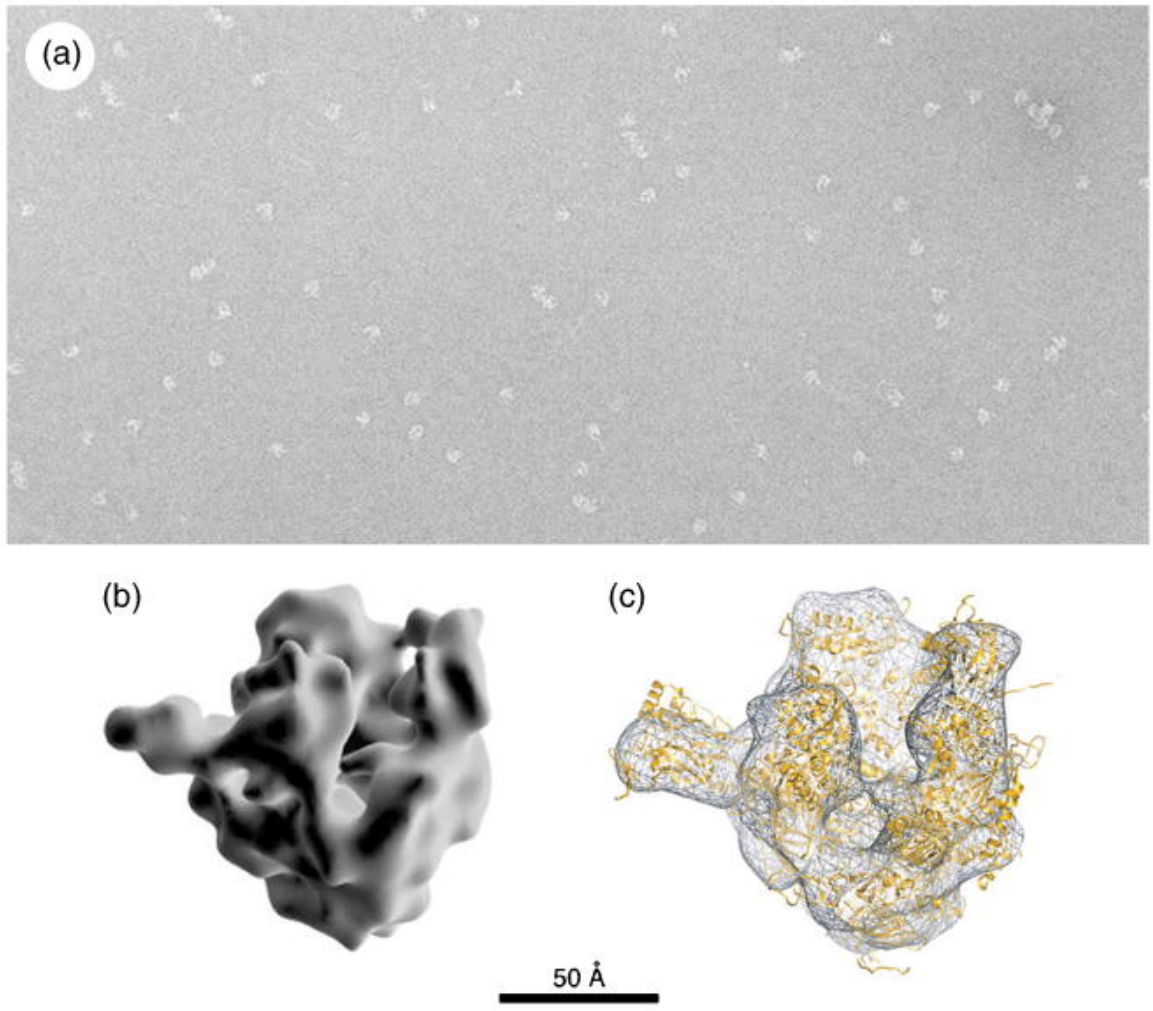
Cryo-negative staining



Reconstructions of GroEL frozen with and without stain

De Carlo and Harris, Micron, 2011

Cryo-negative staining



RNA polymerase solved with cryo-negative staining

De Carlo and Harris, Micron, 2011

Methods for contrast and SNR improvement

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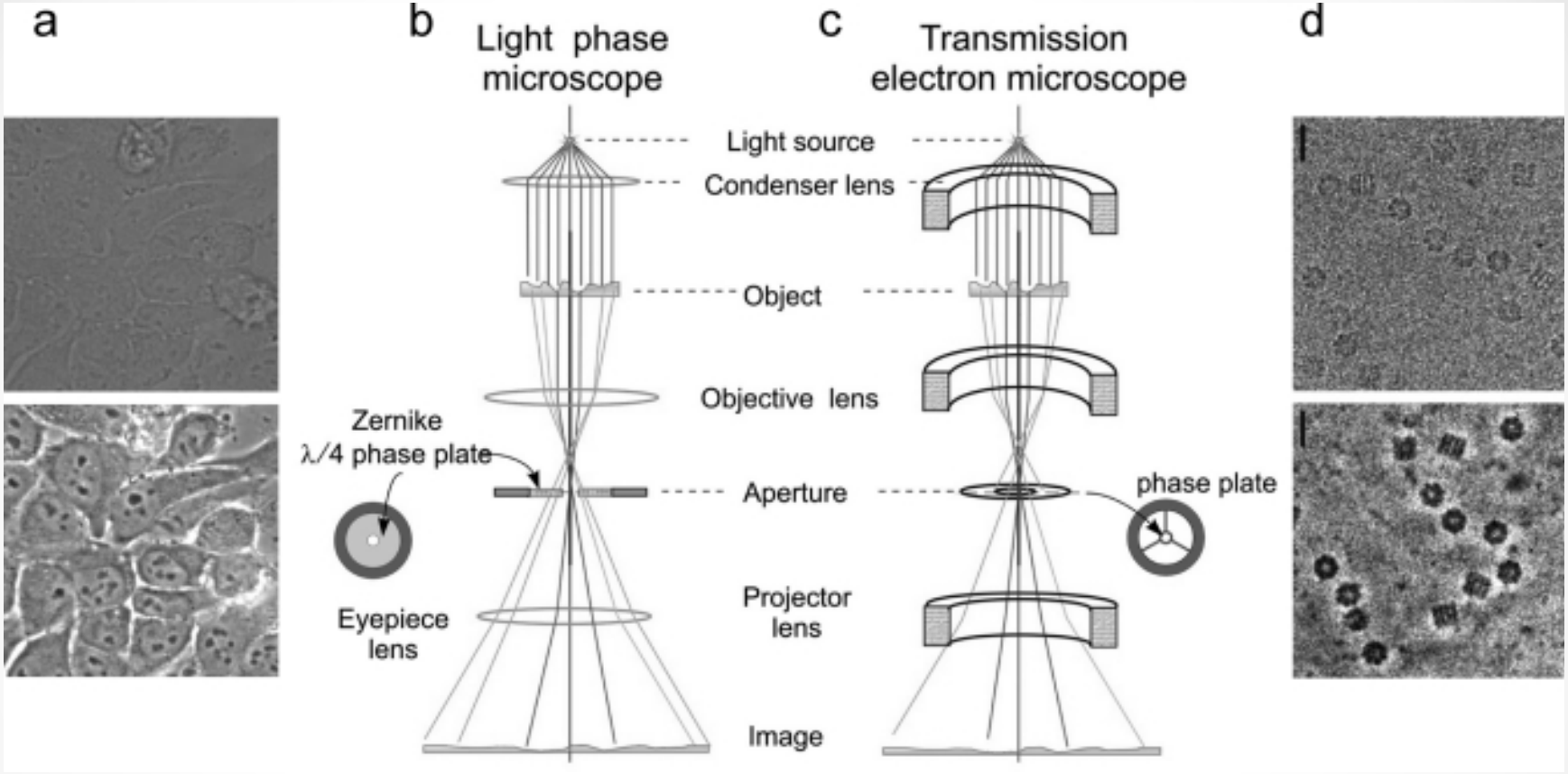
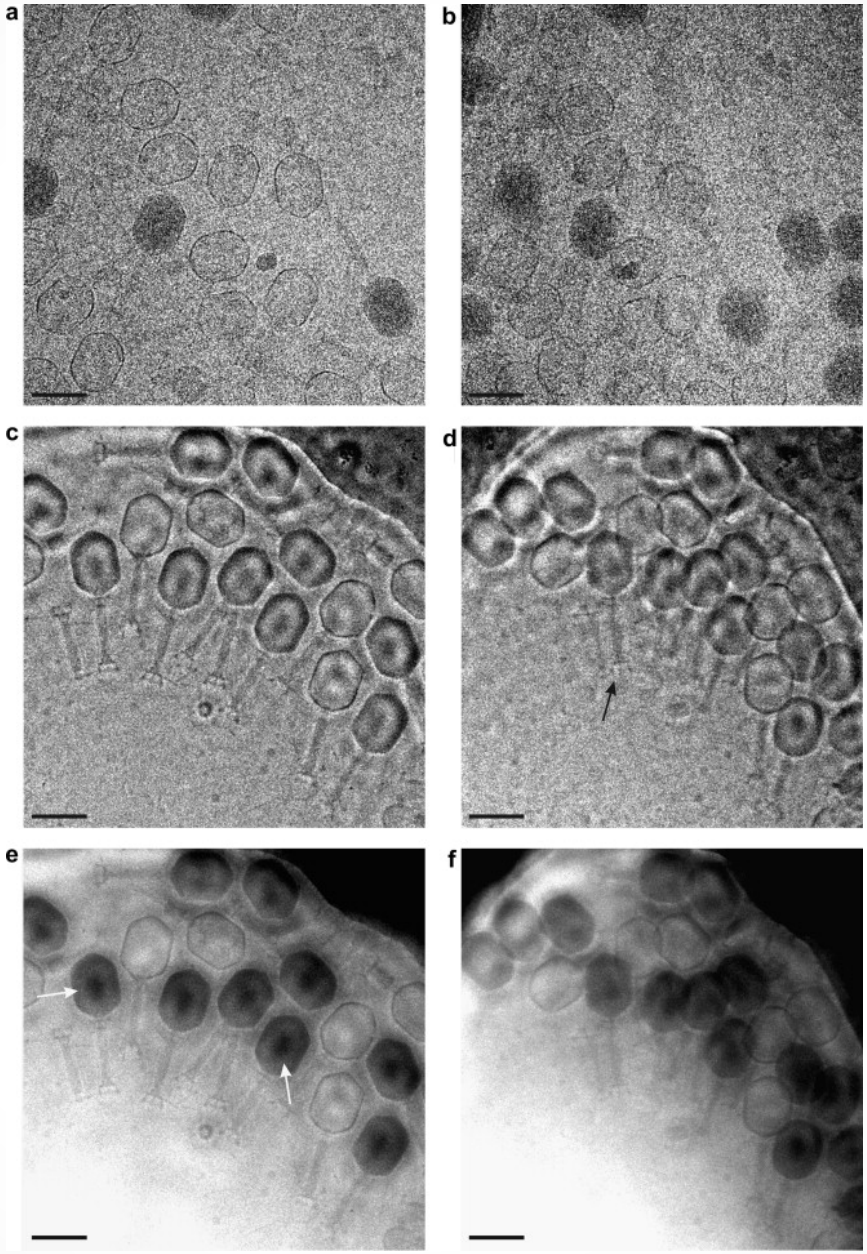
Introduction of phase plate



Phase plates introduced in the back focal plane of objective lens
Shifts phase of scattered electrons by 90°
Contrast improved upon combination with unscattered electrons
"Invisible" phase contrast converted into "recordable" amplitude contrast

Orlova and Saibil, Chem Rev, 2011

CryoET using Zernike phase plate



Danev *et al.*, J Struct Biol, 2010

Energy filtering

Removal of inelastically scattered electrons

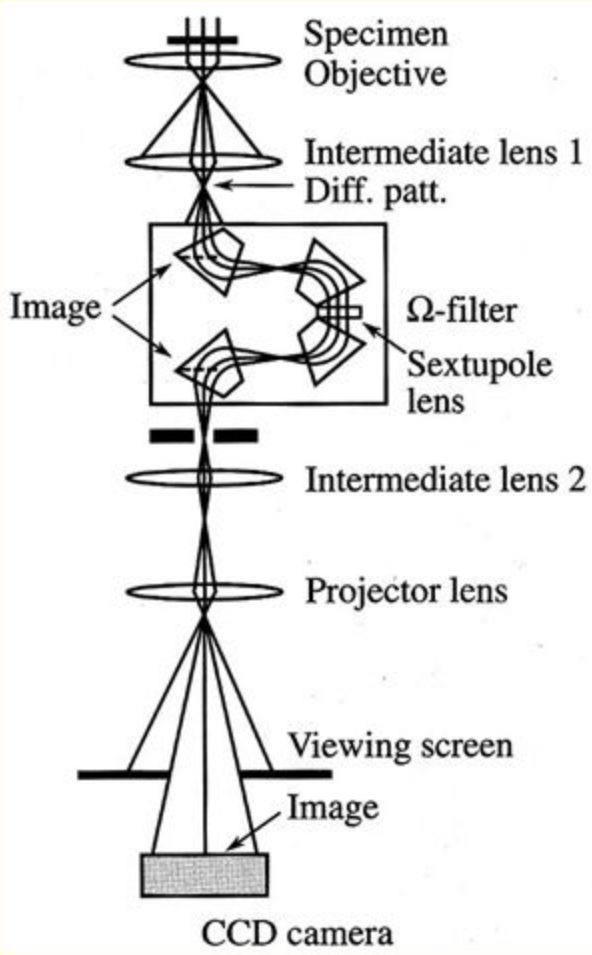
Lower energy, longer wavelength

Chromatic aberration, electrons focused in different planes

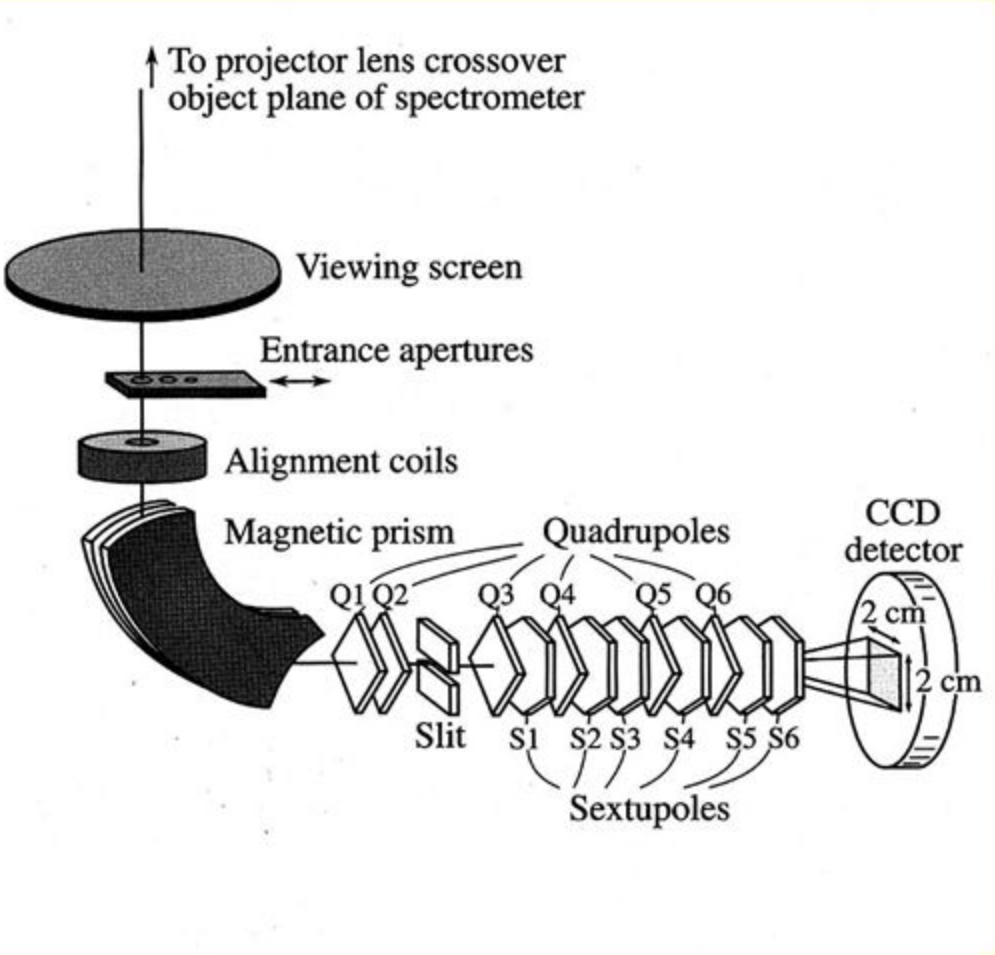
Causes blurriness in image

Removed by in-column or post-column filtration

EFTEM: In-Column and Post-Column Energy Filters



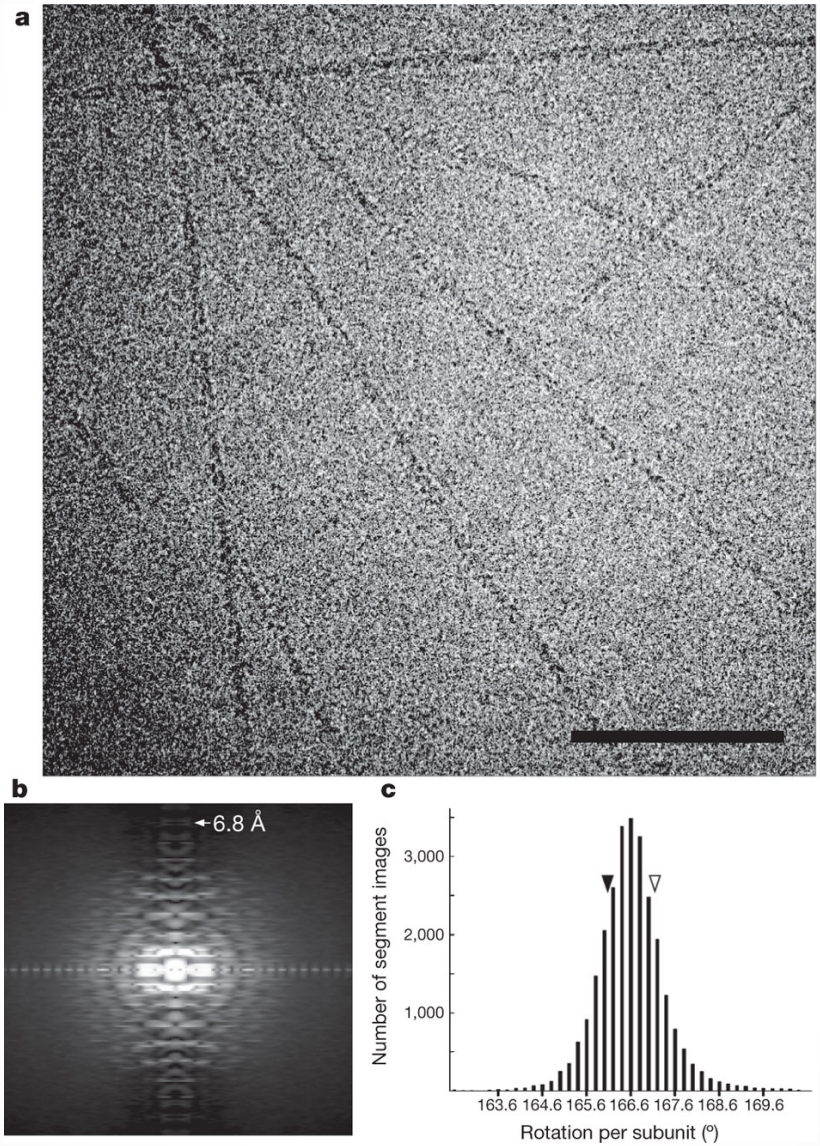
Omega Filter



Gatan Imaging Filter (GIF)

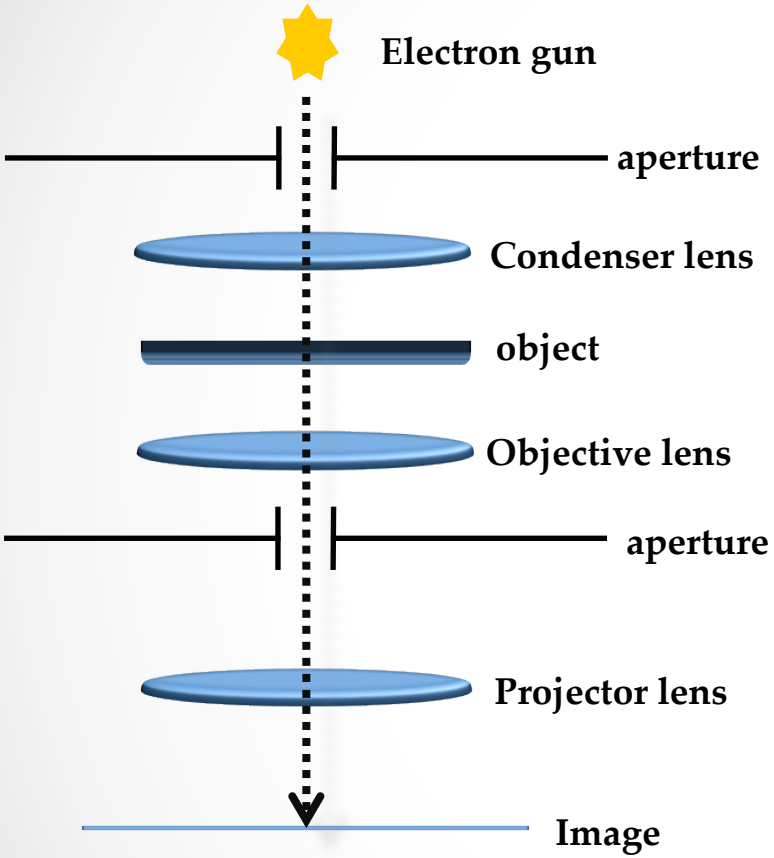
From Williams and Carter, *Transmission Electron Microscopy*, Springer, 1996

Imaging of actin filaments using a Ω type energy filter



Fujii *et al.*, Nature, 2010

Controlling apertures



Condenser lenses convert diverging electron beam into parallel beam

Objective aperture prevents electrons scattered at high angles from reaching image plane, improves contrast

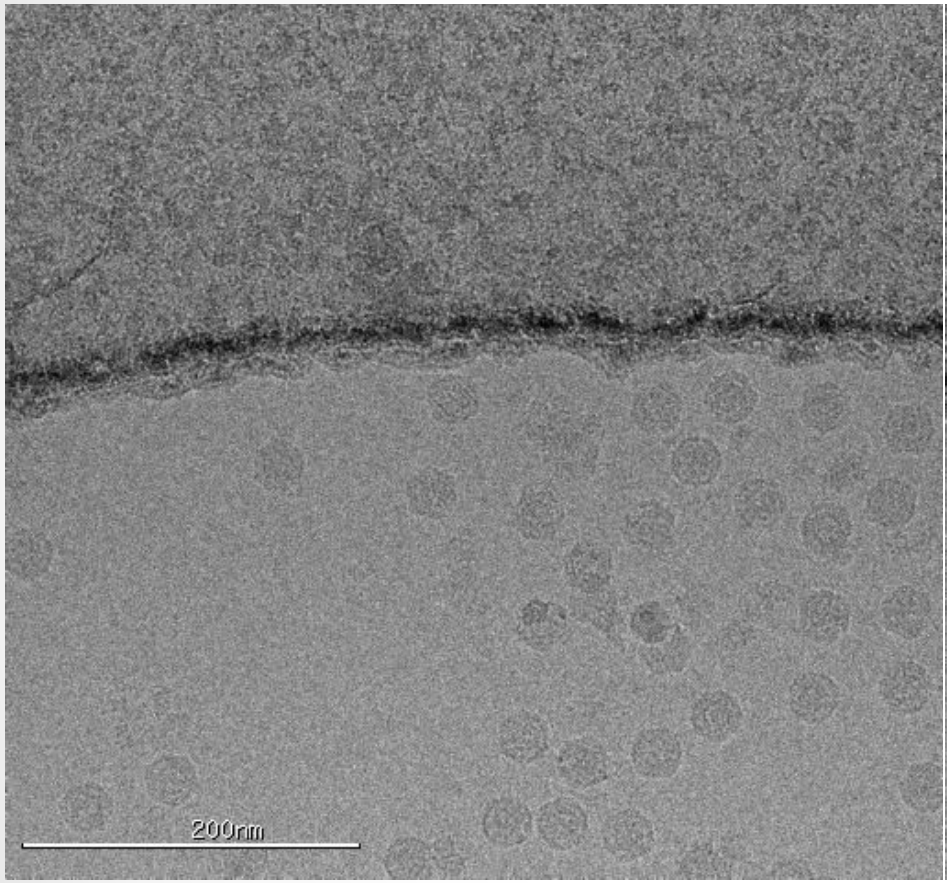
Provides initial magnification (20-50x)

Aberration affects image formation

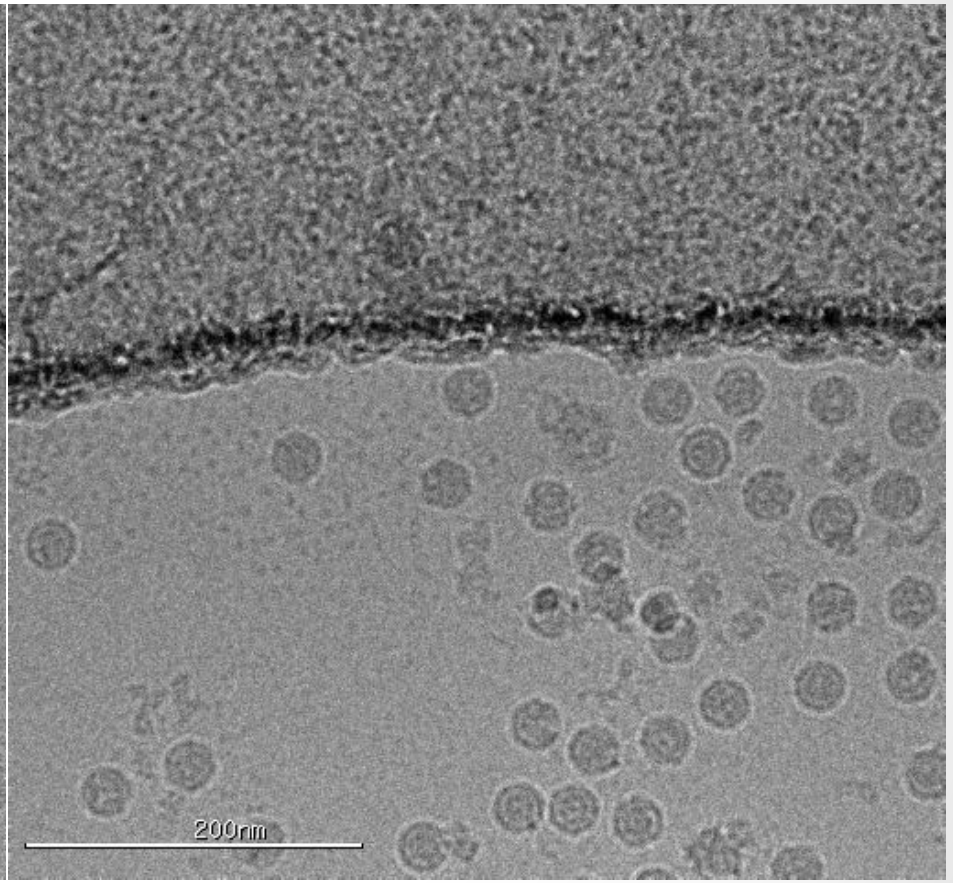
Intermediate and projector lenses further magnify image

Orlova and Saibil, Chem Rev, 2011

Images collected at different defocus values



-1 μm defocus



-6 μm defocus

Image courtesy Rebecca Taurog and John E. Johnson, The Scripps Research Institute

Methods for contrast and SNR improvement

Sample level: Cryo-negative staining

Microscope level: phase plate, energy filters, aperture size, defocus

Data collection level: direct detectors, automated collection

Detection system

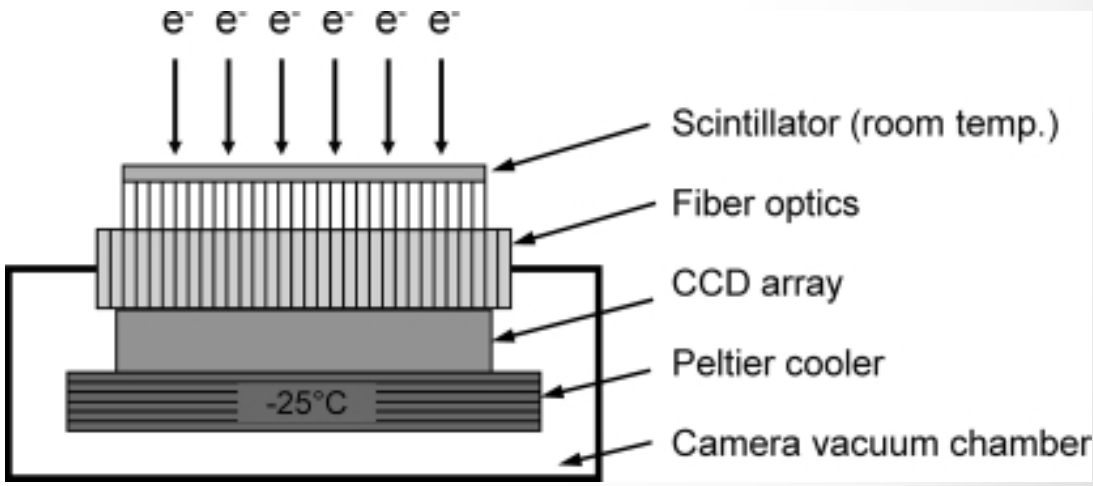
CCD camera

Incident electrons converted to photons

Fiber optics transfer image to charge coupled device sensor

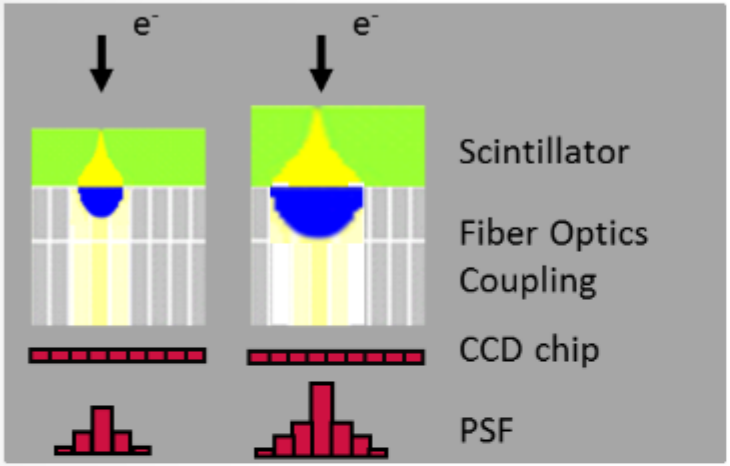
Photons generate electric charge

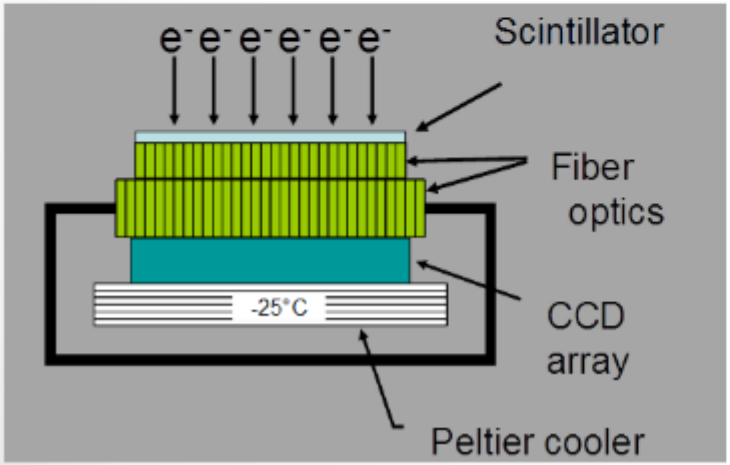
Charge converted to pixel for readout



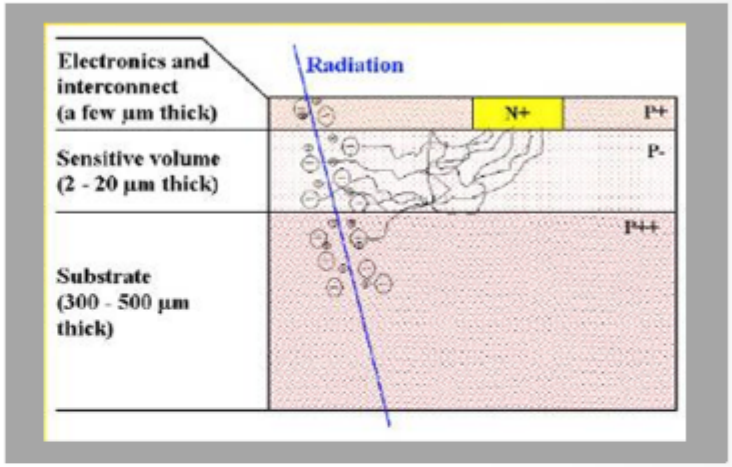
Direct Electron Detector

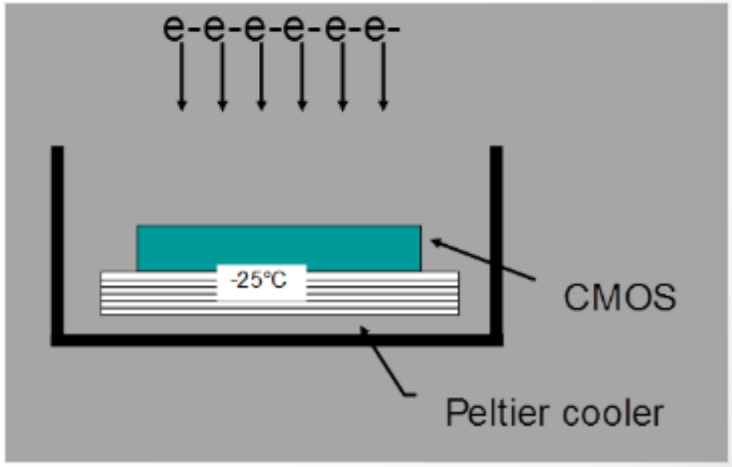
CCD: multi stage conversion of electron energy via fiber or lens optics





CMOS: direct conversion of electron energy without fiber or lens optics





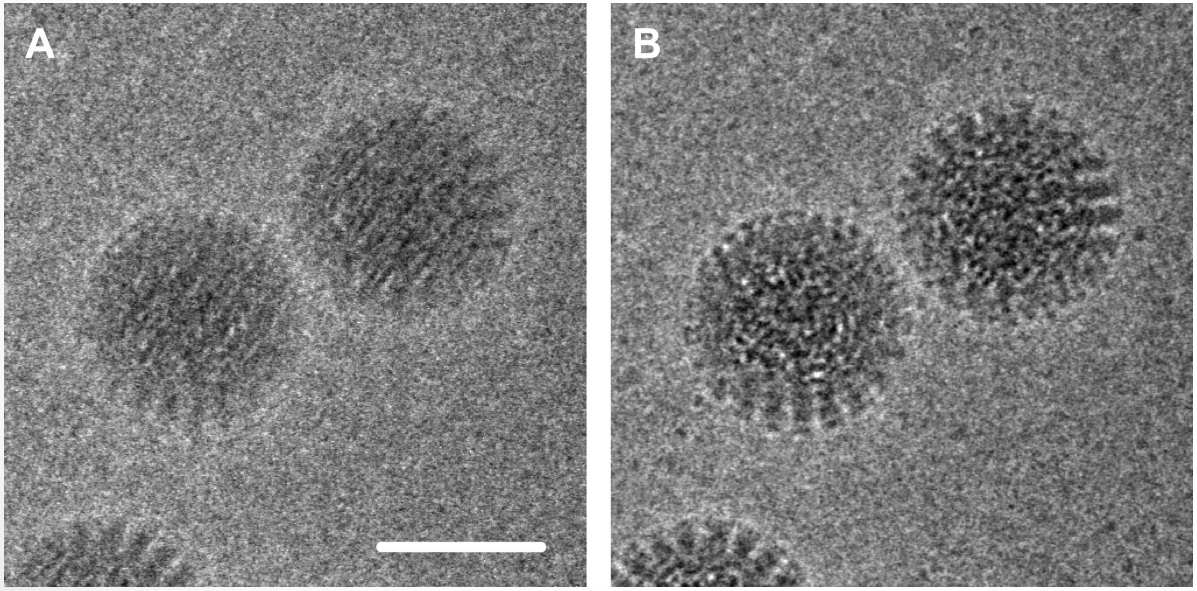
Advantages of Direct Electron Detector

Direct counting of electrons

Reduced noise from detector

Fast frame rate, correction of beam induced movement possible

Subframe alignment



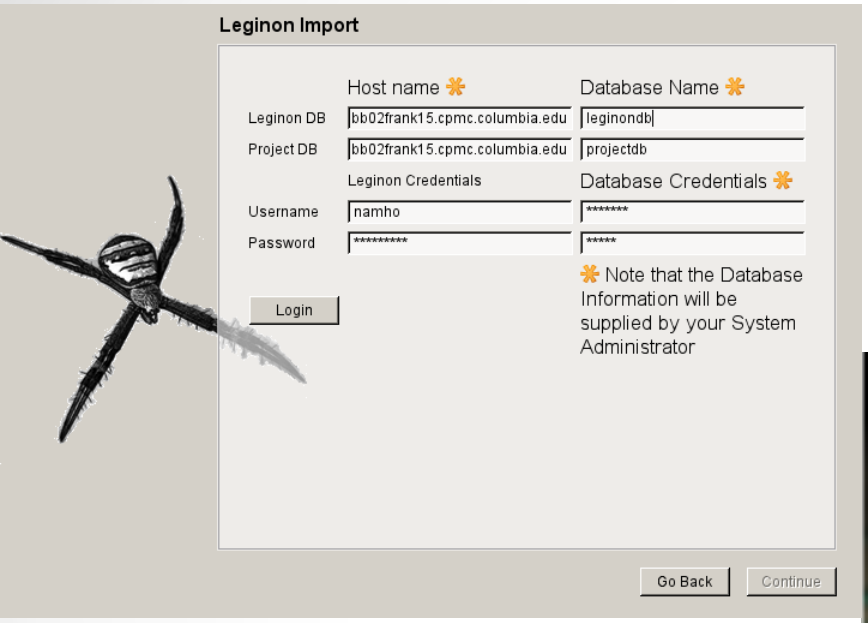
Grigorieff, eLife, 2013

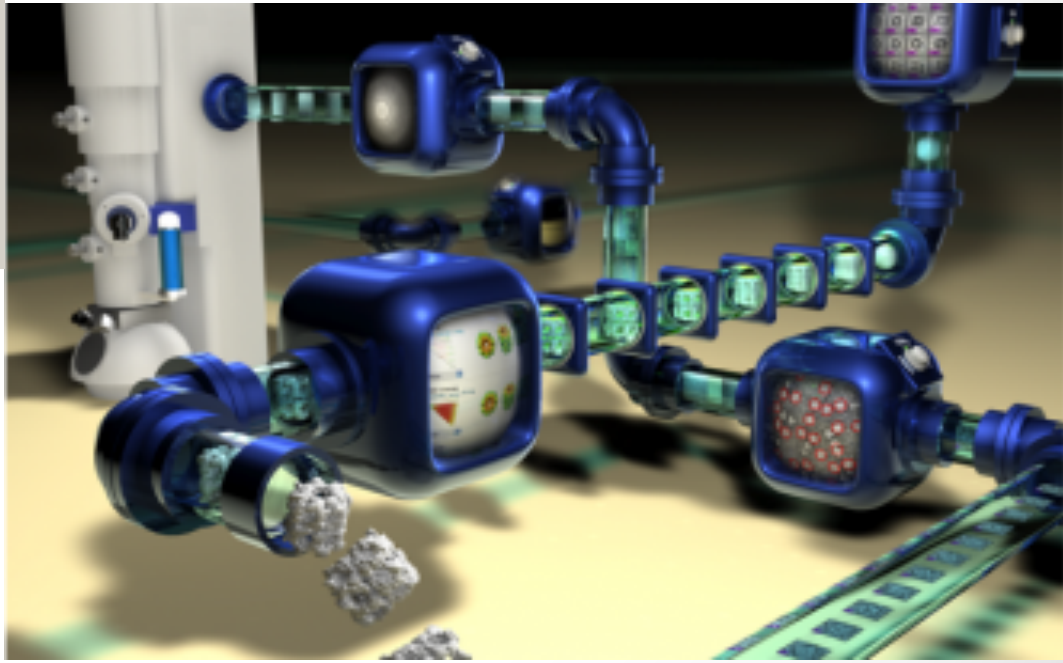
Computer controlled data collection

Automation of repetitive operations: Searching for suitable areas for imaging
Lens setting, stage movement
Low dose operation
Large dataset collection
Basic image FTs

Typically, overview images collected
cross-correlated with manually collected images
High-mag recording after low-mag searches

Computer controlled data collection





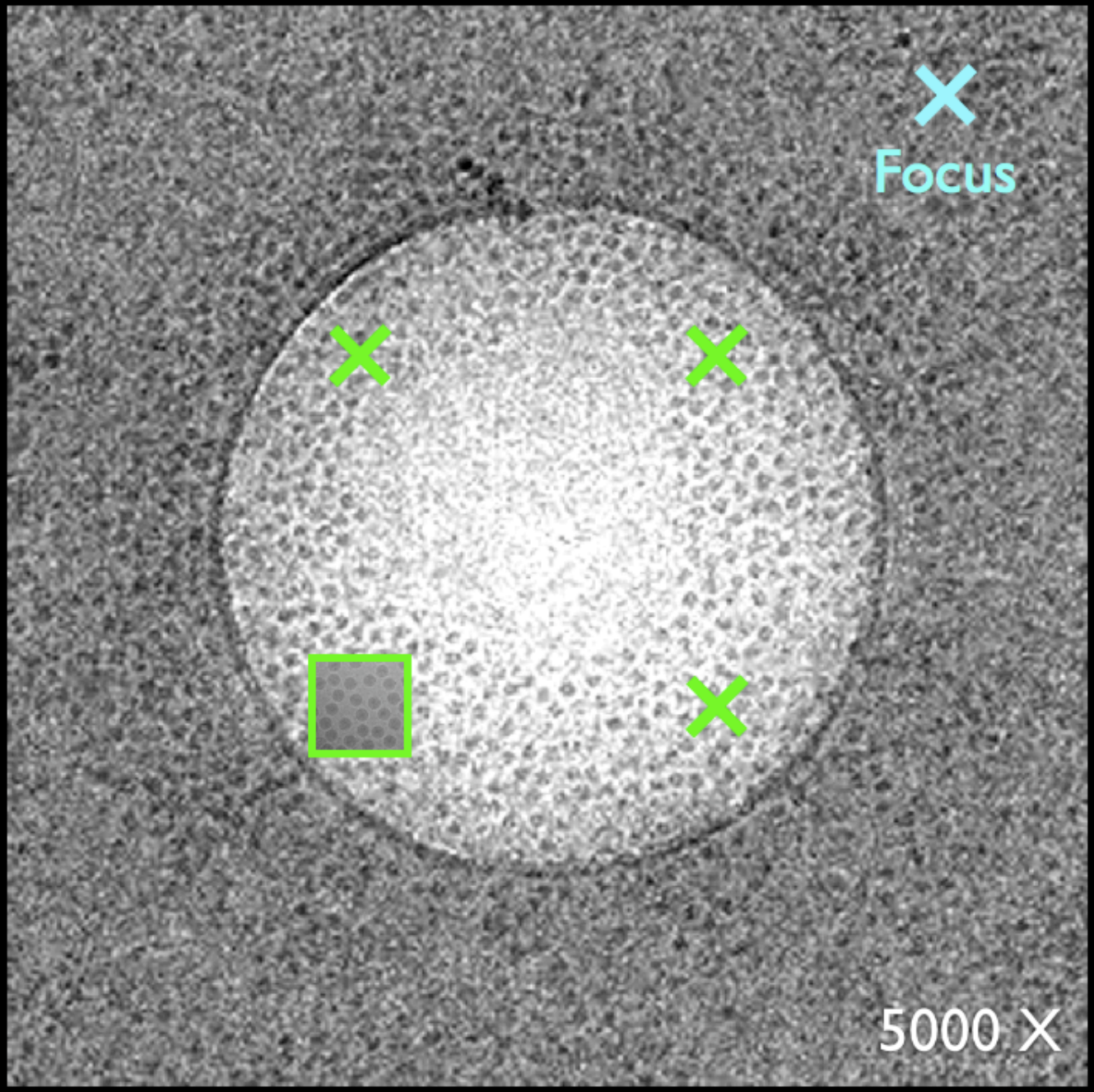
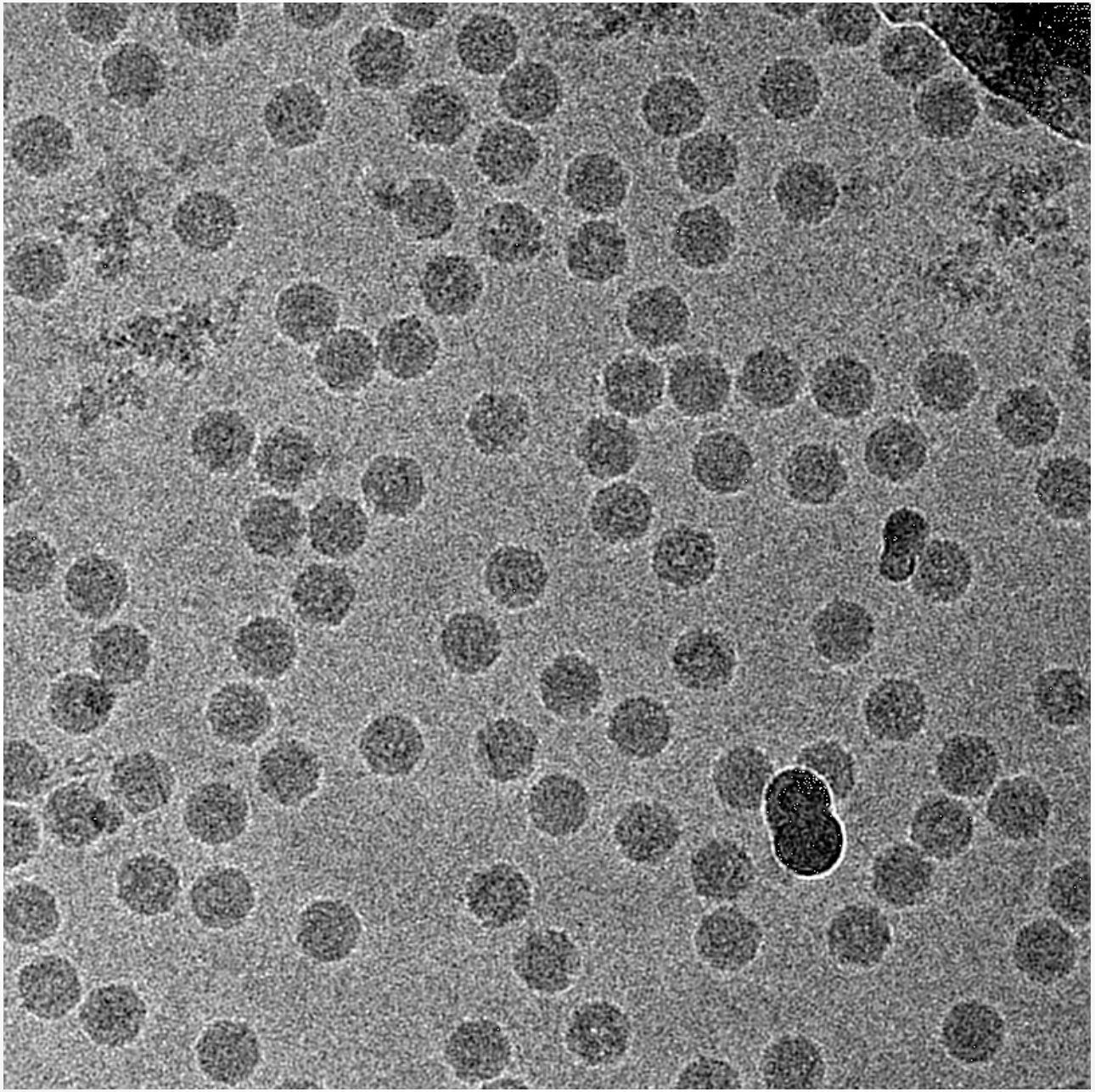
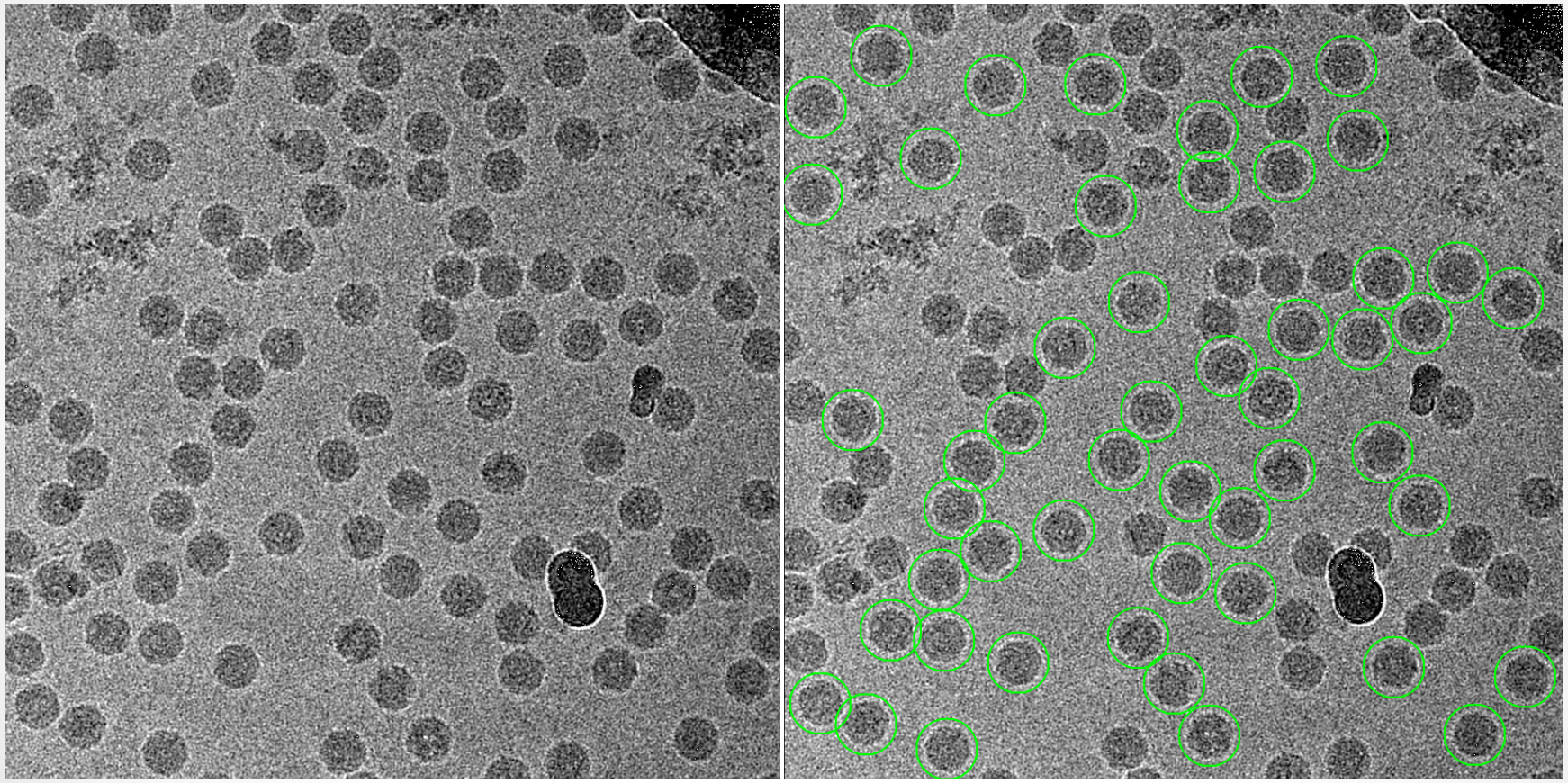


Photo Credit: Gabriel Lander, TSRI

Particle Picking



Particle Picking



Particle Picking

Template matching:

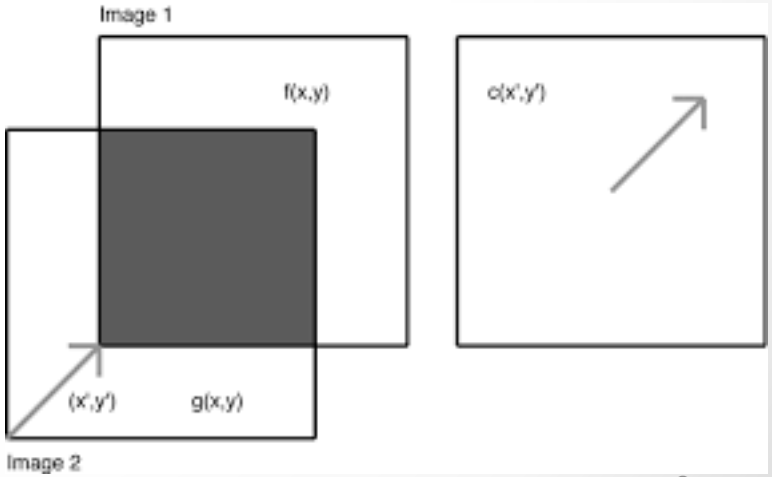
Match between image and reference image scored

Cross-correlation based methods

Sensitive to variations in spatial frequency

Multiple references required to account for different views

Rotationally averaged references/azimuthally averaged particle image



Nicholson and Glaeser, J Struct Biol, 2001

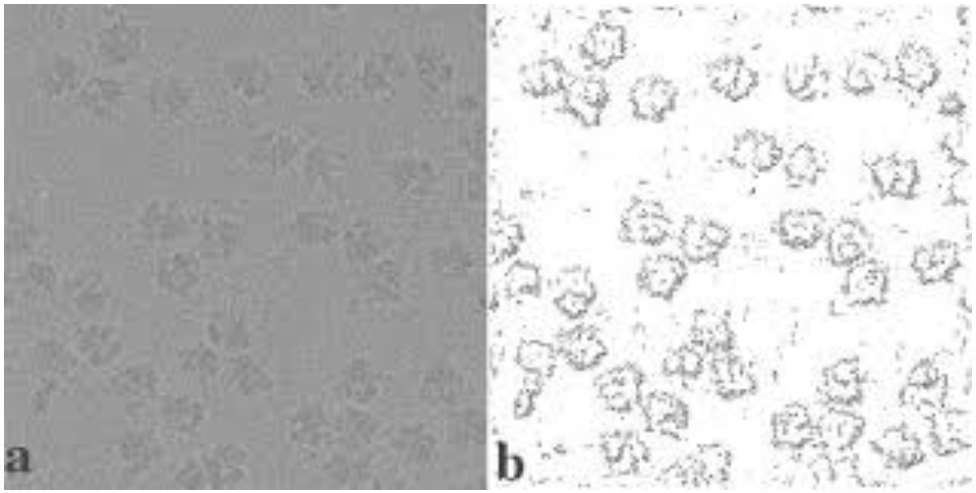
Particle Picking

Edge detection:

Identification of blobs in image, assignment of labels to adjacent pixels

Too close or too large “bounding boxes” rejected

Somewhat insensitive to spatial frequency



Nicholson and Glaeser, J Struct Biol, 2001

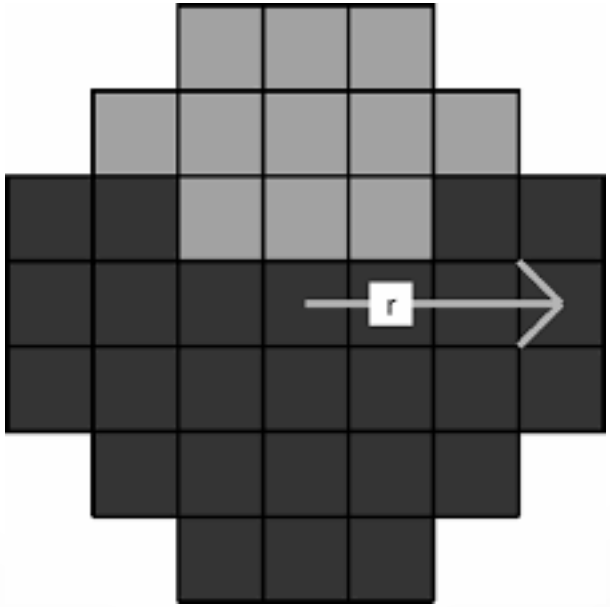
Particle Picking

Intensity comparisons:

Objects with uniform internal density selected

Image subjected to horizontal-vertical scan to identify clusters

Post-processing checks



Nicholson and Glaeser, J Struct Biol, 2001

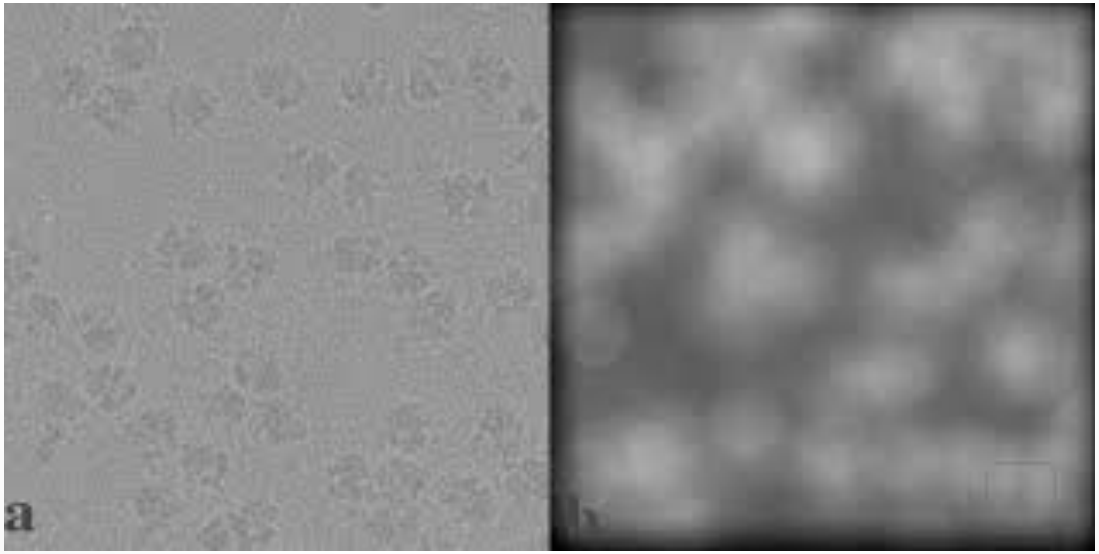
Particle Picking

Texture based methods:

Computes local variance over small area

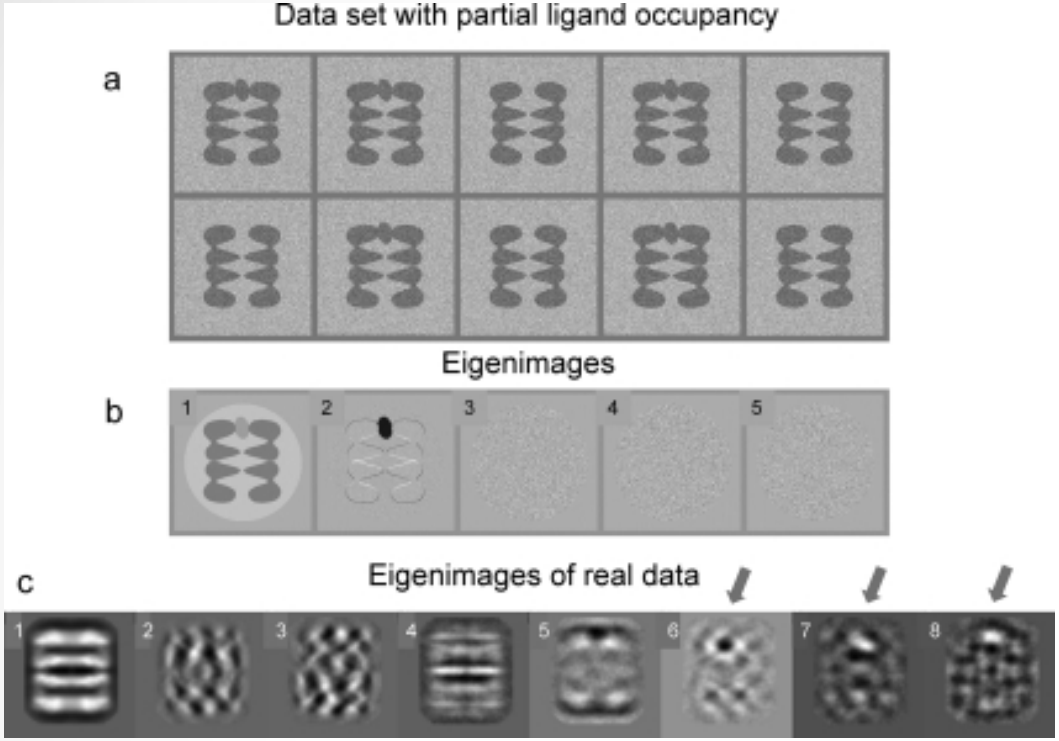
High values of local variance indicate presence of object

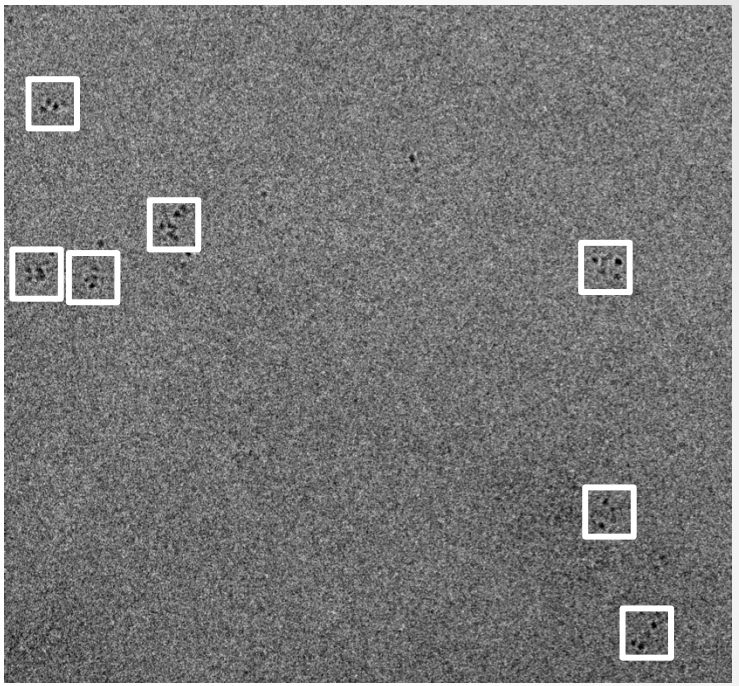
Also detects aggregates/contaminants

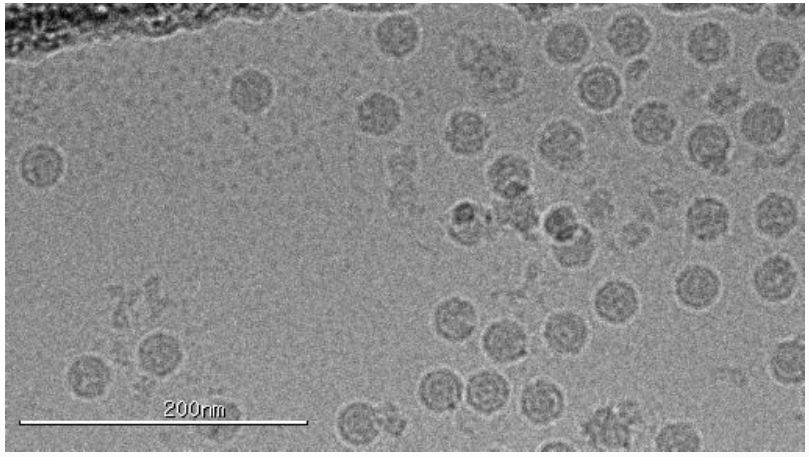


Nicholson and Glaeser, J Struct Biol, 2001

Problems – heterogeneity, contaminants, background noise







Orlova and Saibil, Chem Rev, 2011

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