SECM^4 Workshop – Day 1

Negative-staining transmission electron microscopy

Scott Stagg

Introduction

- Schedule
  - https://docs.google.com/spreadsheets/d/1zqyaKOycyOdzgmG7lsQ9R-LcOOgOXR8__bx8nfAJrIM/edit#gid=0
- Instructors
  - Scott Stagg
  - Hosna Rastegarpouyani
  - Anthony Warrington
  - David Boose
  - Behrouz Ghazi Esfahani
  - Ken Taylor
  - Xiaofeng Fu
  - Bing Wang
  - Hemant Goswami
  - Shirin Kamalaldinezabadi
  - Ruizhi Peng

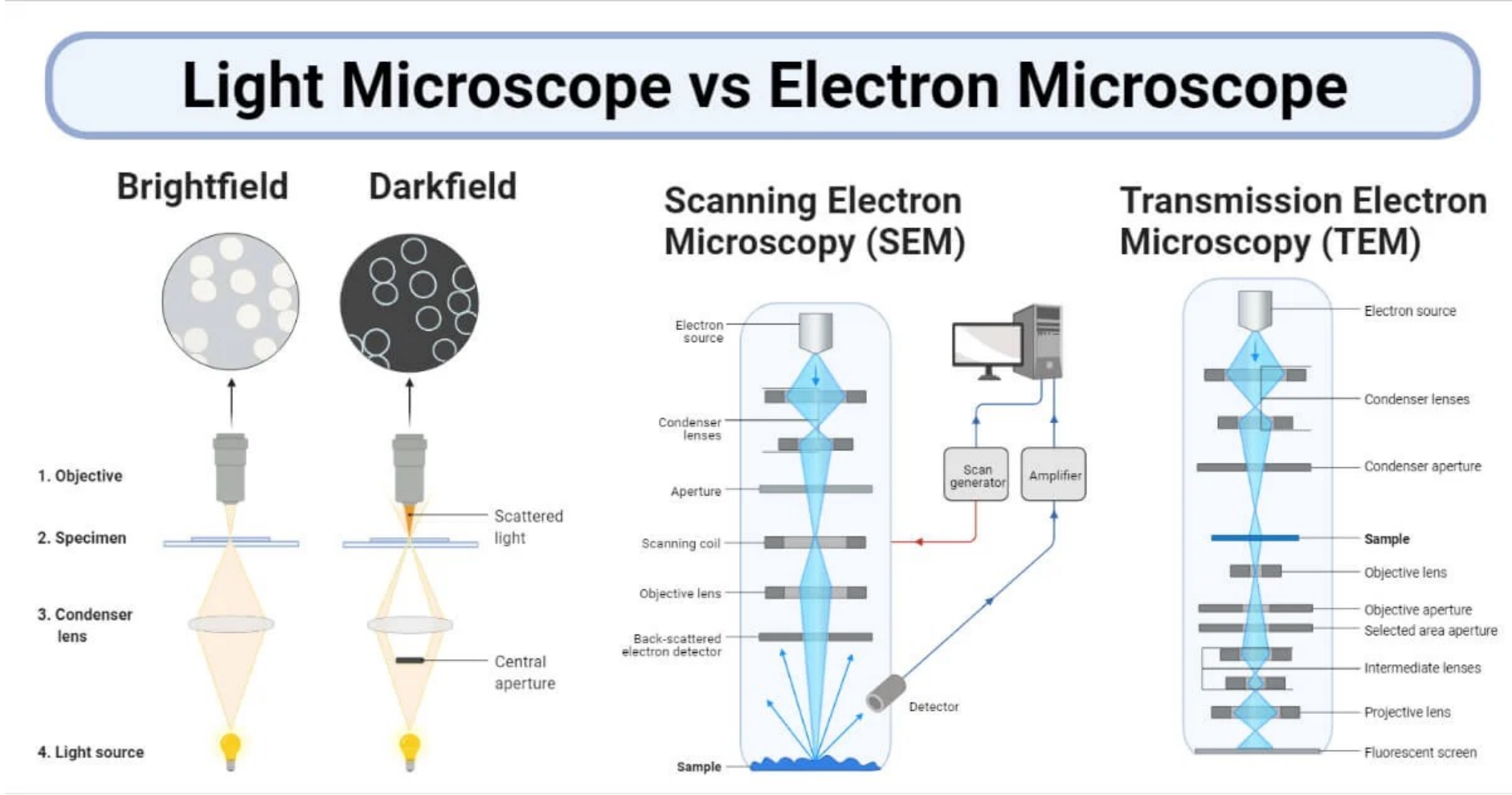
For a detailed introduction to cryo-EM practice and theory visit Grant Jensen's YouTube lectures

https://www.youtube.com/playlist?list=PLhiuGaXlZZenm7lu5qv_A59zEWkRKkBn5

Principles of Microscopy

- **Definition:** Microscopy is the science of magnifying and analyzing objects that are too small to be seen by the naked eye.
- **Evolution:** From simple magnifying glasses in the 16th century to advanced electron microscopes today, microscopy has continuously evolved.
- **Applications**: Essential in fields like biology, medicine, materials science, and nanotechnology, aiding in disease diagnosis, research, and material analysis.
- **Resolution:** Modern microscopes can visualize structures down to the atomic level, revealing intricate details of specimens.
- **Significance:** Microscopy bridges the gap between the macroscopic world we see and the microscopic world at the cellular or atomic level, enhancing our understanding of life and matte

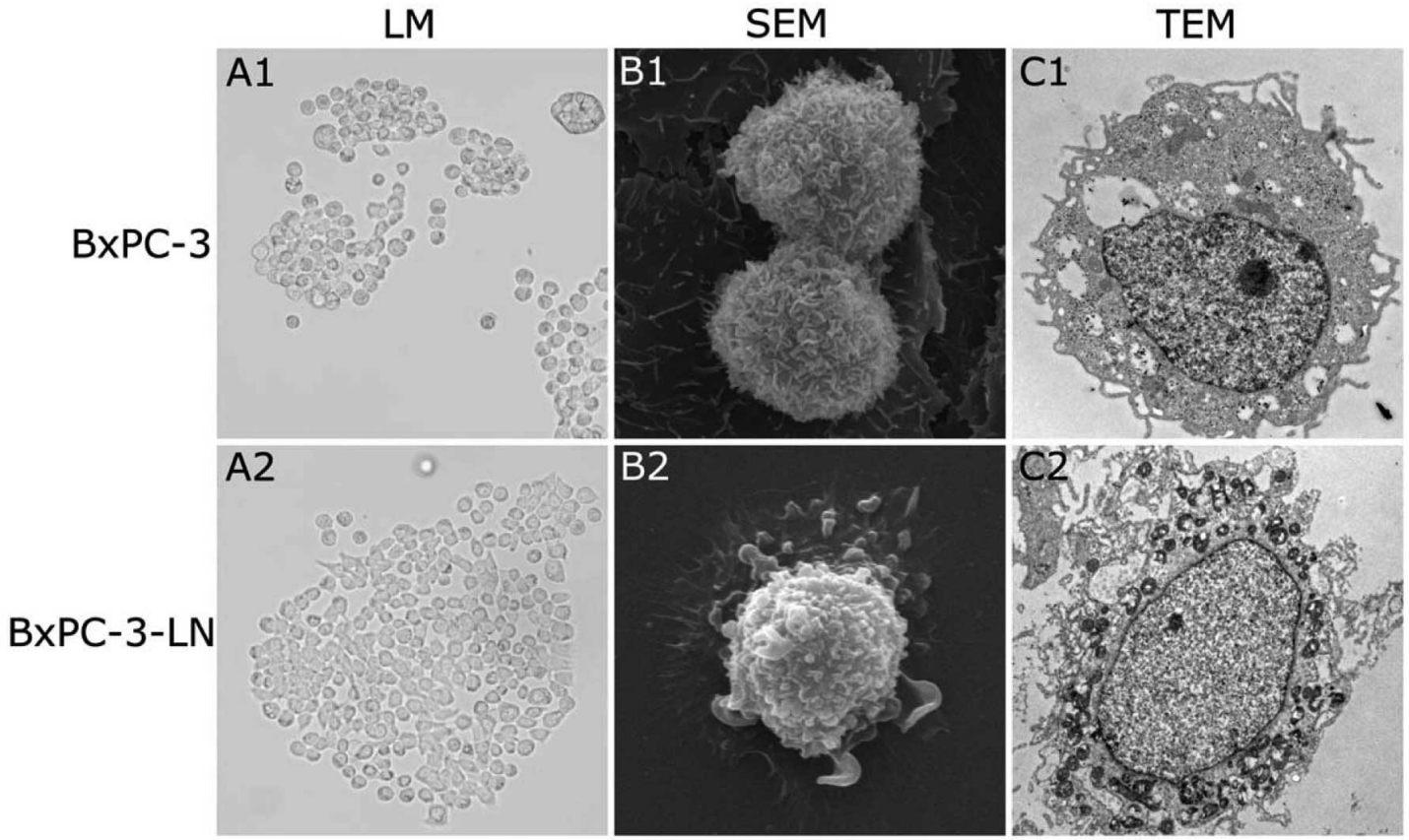
Types of Microscopes



Different types of images

Practical importance of imaging modalities:

- **LM** does not show cell morph. differences between healthy A1 and cancer line A2.
- **SEM** shows filo- and lamellipodia develop in B2 vs B1
- **TEM** shows increase in the number of secretory granules in C2 vs C1



Components of a Light microscope

- **Objective Lens:** Primary magnifying lens closest to the specimen.
- **Eyepiece (or Ocular Lens):** Lens through which the viewer observes, further magnifies the image.
- **Stage**: Platform where the specimen is placed.
- **Light Source:** Illuminates the specimen for observation.
- **Condenser:** Focuses light from the source onto the specimen.
- **Coarse & Fine Focus:** Knobs to adjust the clarity of the image.

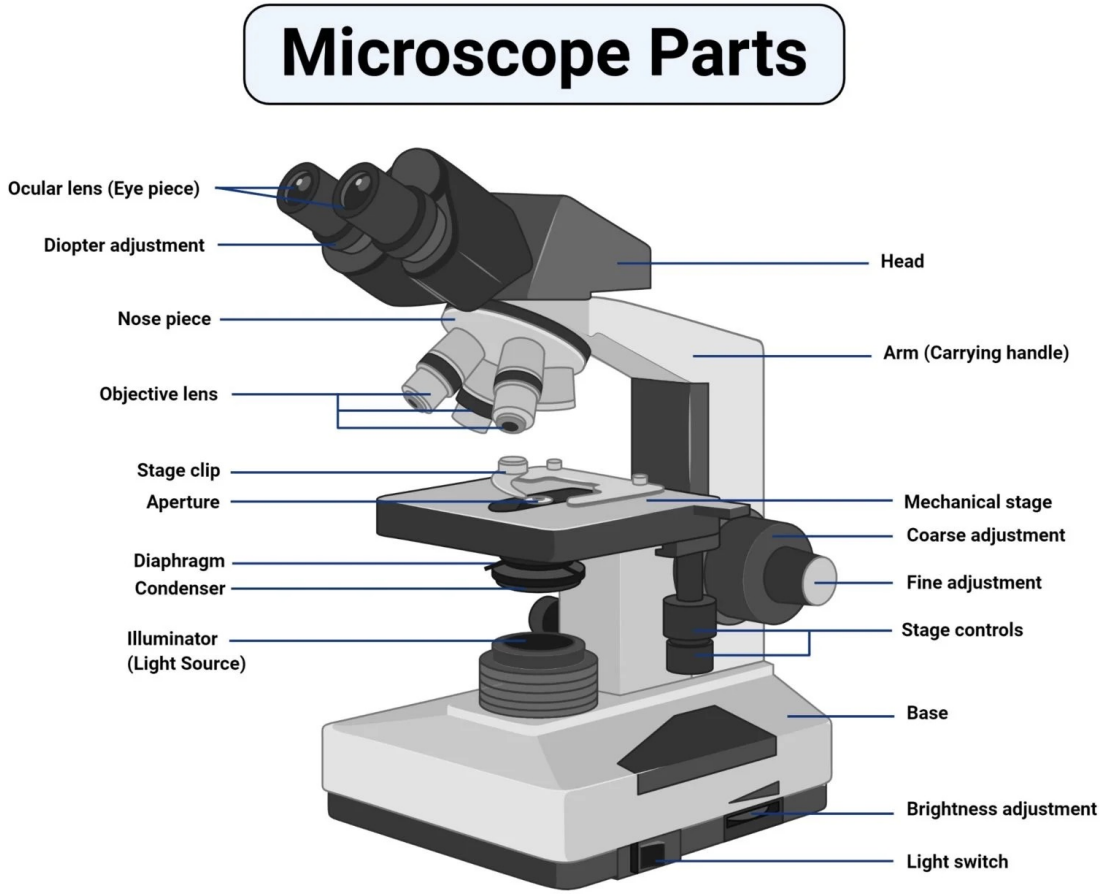
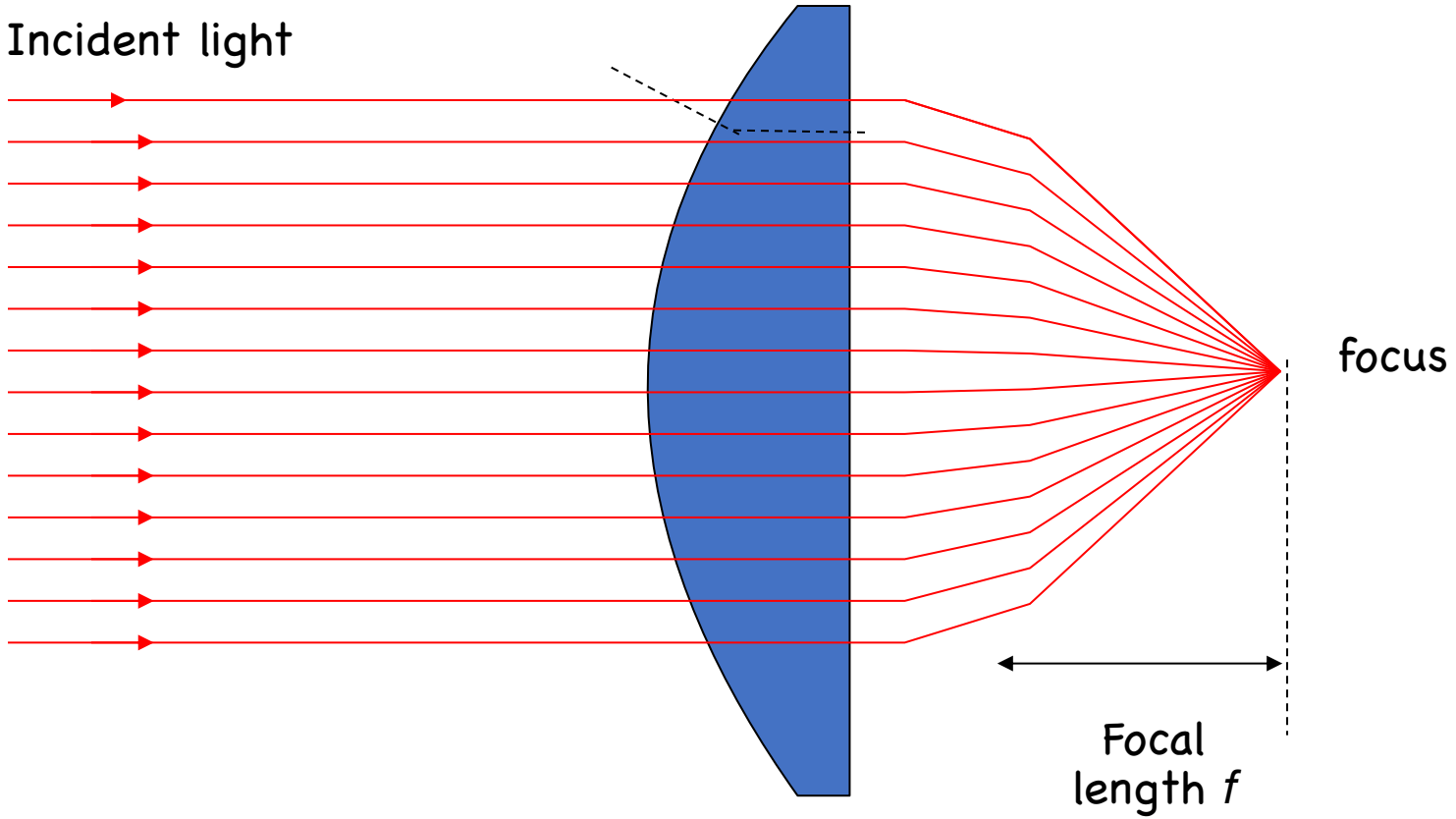
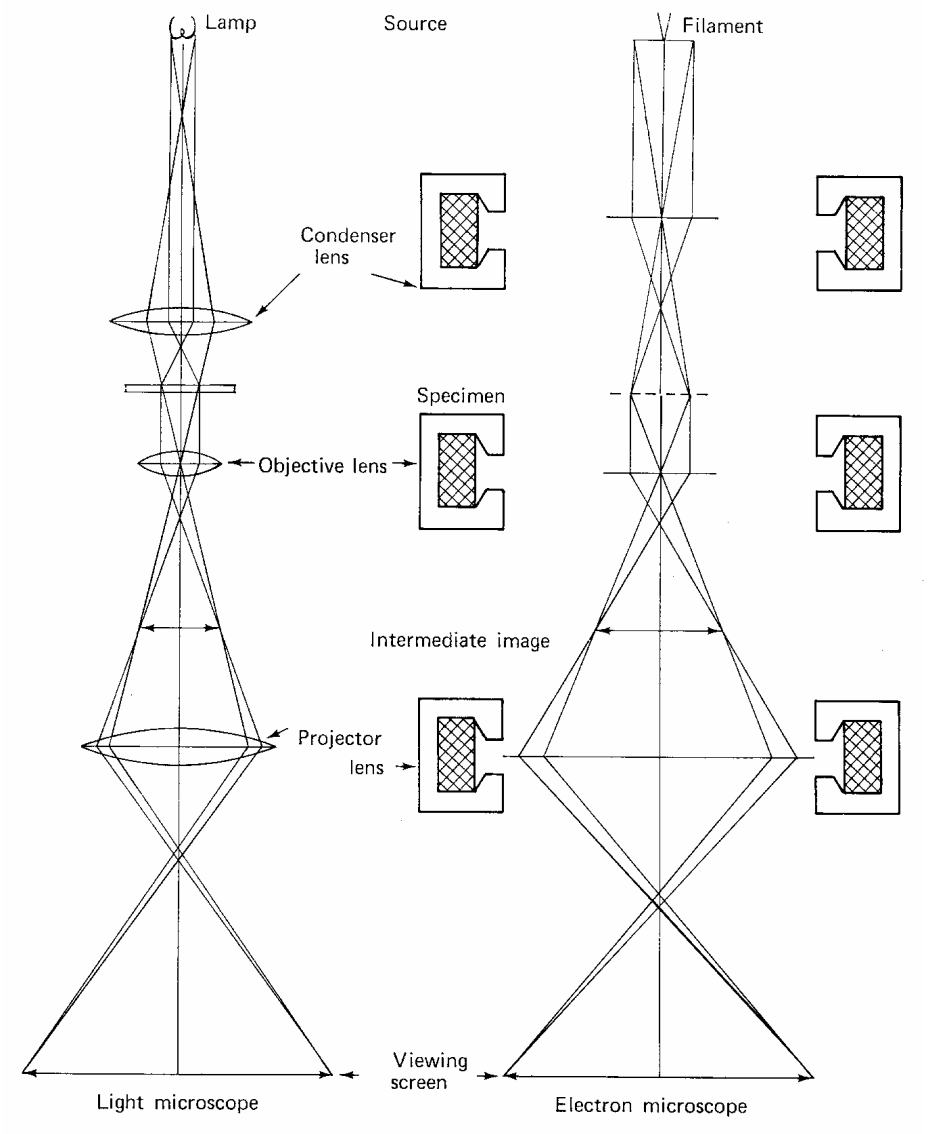


Figure: Parts of a microscope, Image Copyright © Sagar Aryal, www.microbenotes.com

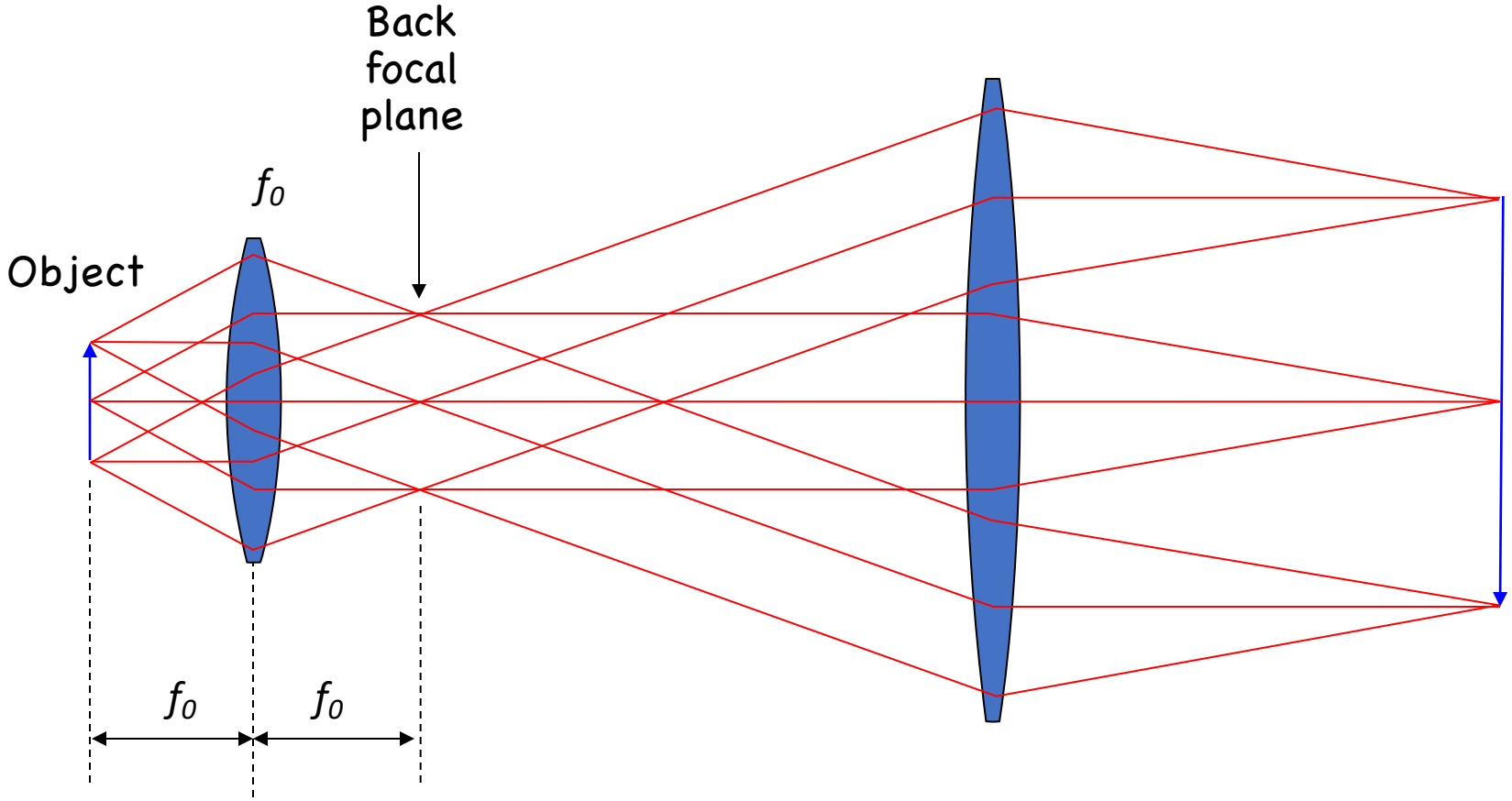
Glass lenses work by refraction



Light microsope vs Electron Microsope

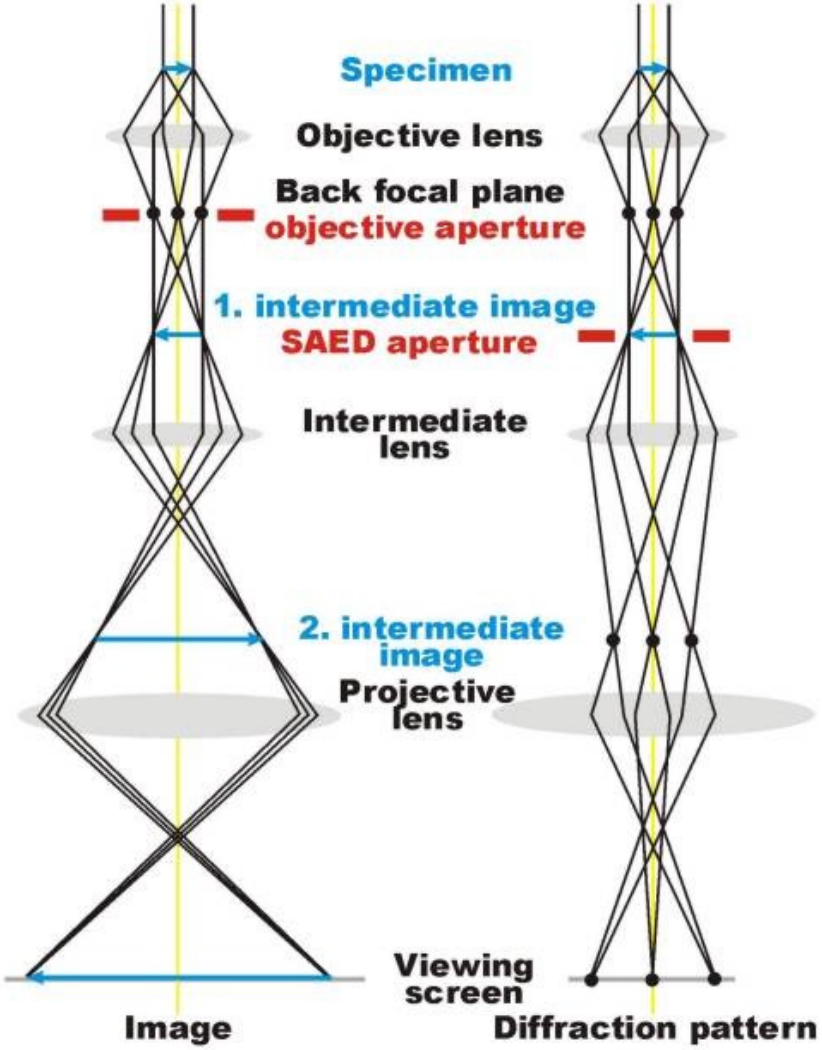


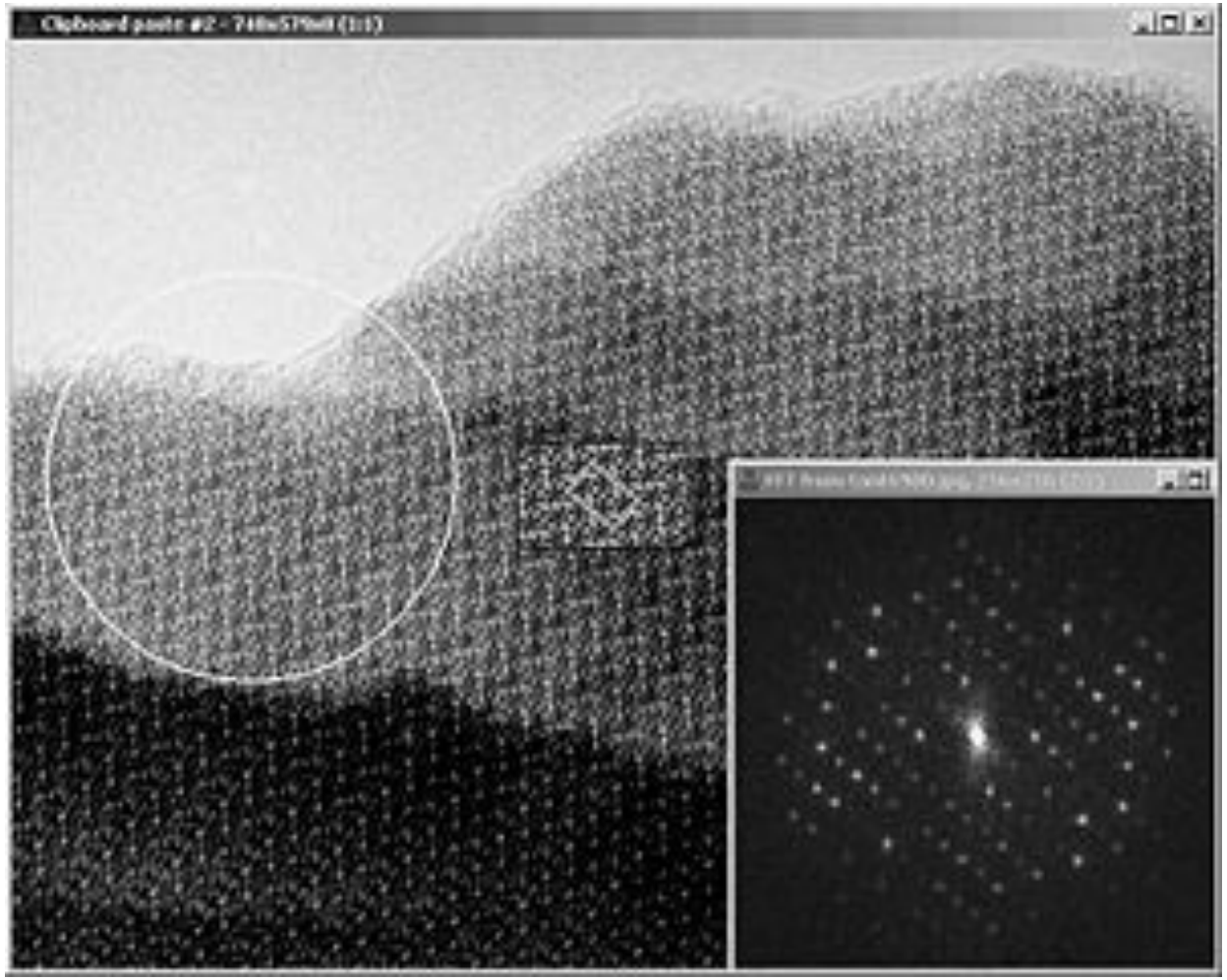
Significance of the Back focal plane



Rays that leave the object with the same angle meet in the objective's *back focal plane*

In TEM, you can choose to view images or diffraction patterns





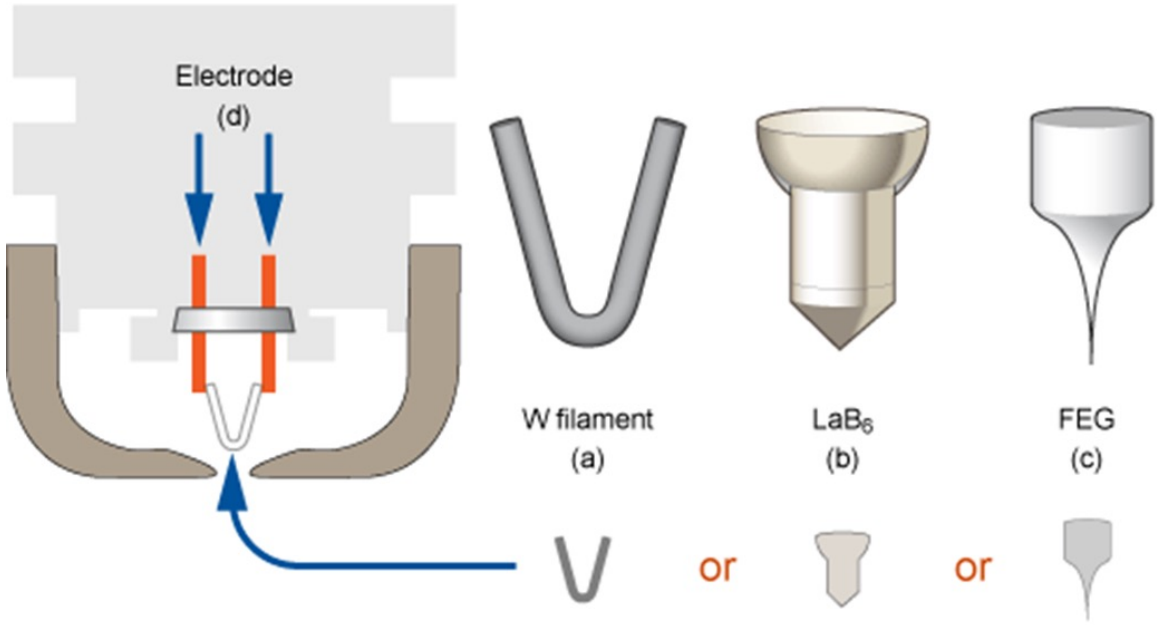
How Does a TEM Work?

- TEM uses electron beams for high-resolution imaging.

- **Principle**: Electrons pass through a thin specimen, creating an image.
- **Electron Source**: High-voltage gun emits the electron beam
- **Imaging**: Detected electrons form a magnified image on a screen
- **Applications**: Used in biology, materials science, and nanotechnology

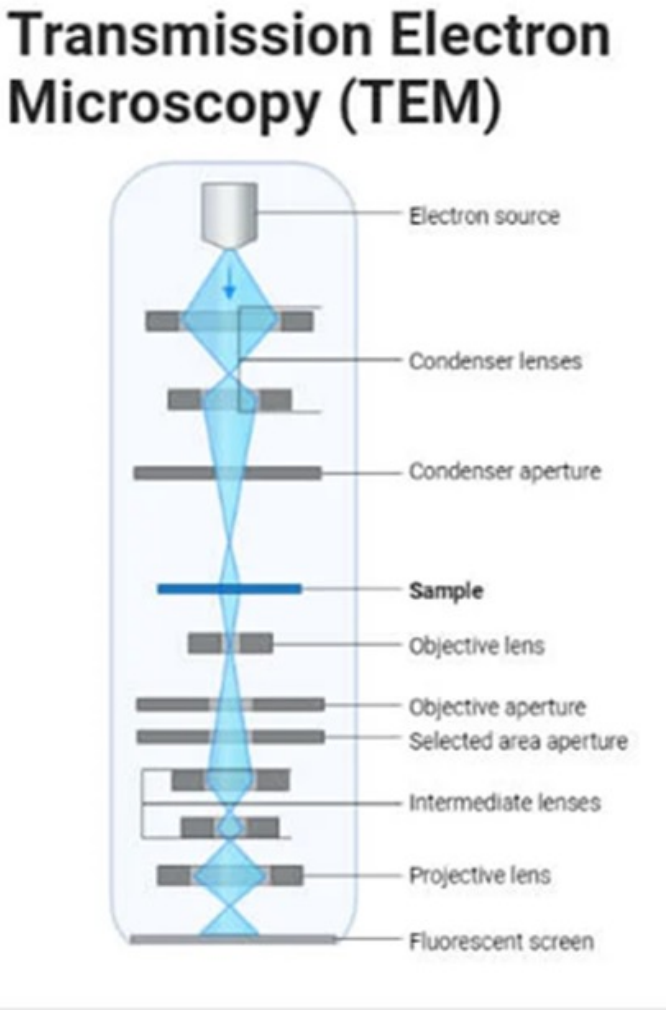
Electron sources

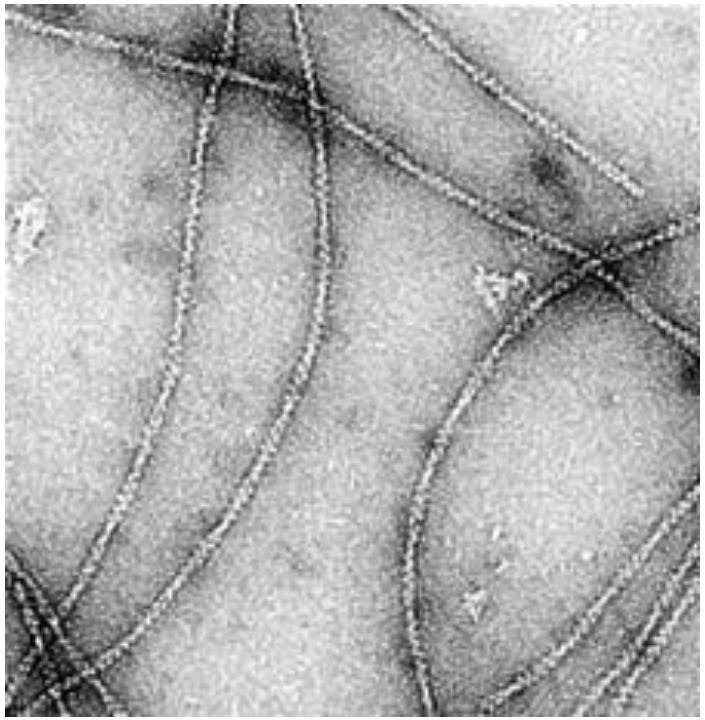
- **Thermionic** – works by heating up W filament and using high voltage to shoot electrons down column
  - Cheap, lower resolution, lower brightness
- **Field emission gun** – uses high voltage to rip electrons off of a thin drawn W tip
  - Expensive, higher resolution, higher brightness



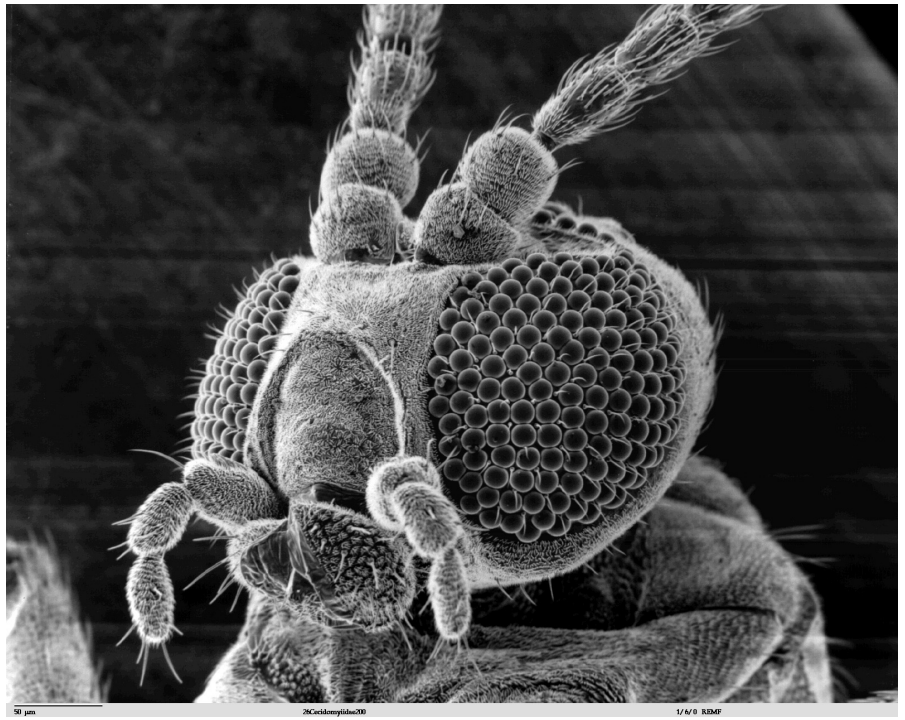
Resolution and brightness determined by electron source

TEM images are projection images



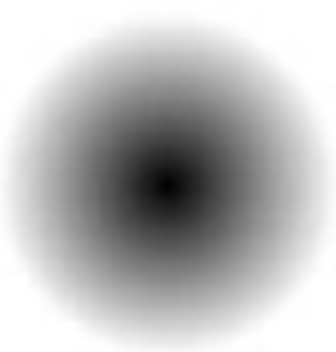


TEM projection through entire volume



SEM resolves only surface features

Electron micrographs are projection images

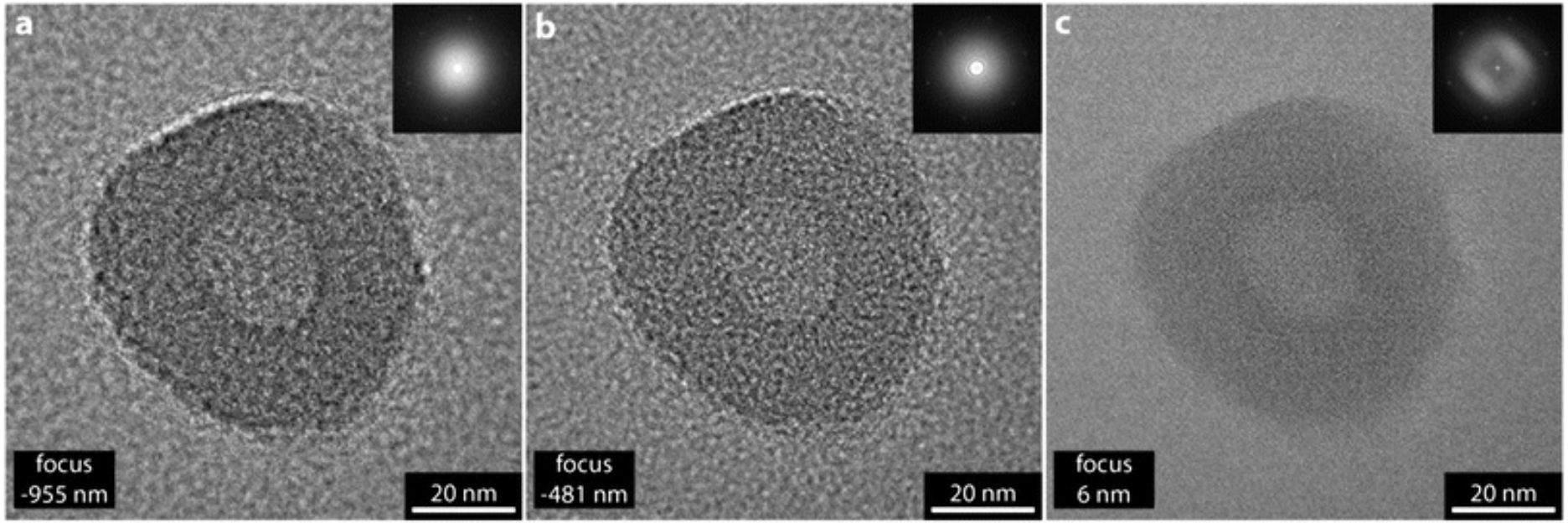


Projection of a solid sphere

Projection of a hollow sphere

Contrast Mechanism

- Contrast differentiates specimen from background.



Contrast in TEM is enhanced by **defocusing** the objective lens

Amplitude and phase contrast

- **Amplitude contrast** is produced by the loss of amplitude (i.e. electrons) from the beam
- **Phase contrast** originates from shifts in the relative phases of the portions of the beam which contribute to the image

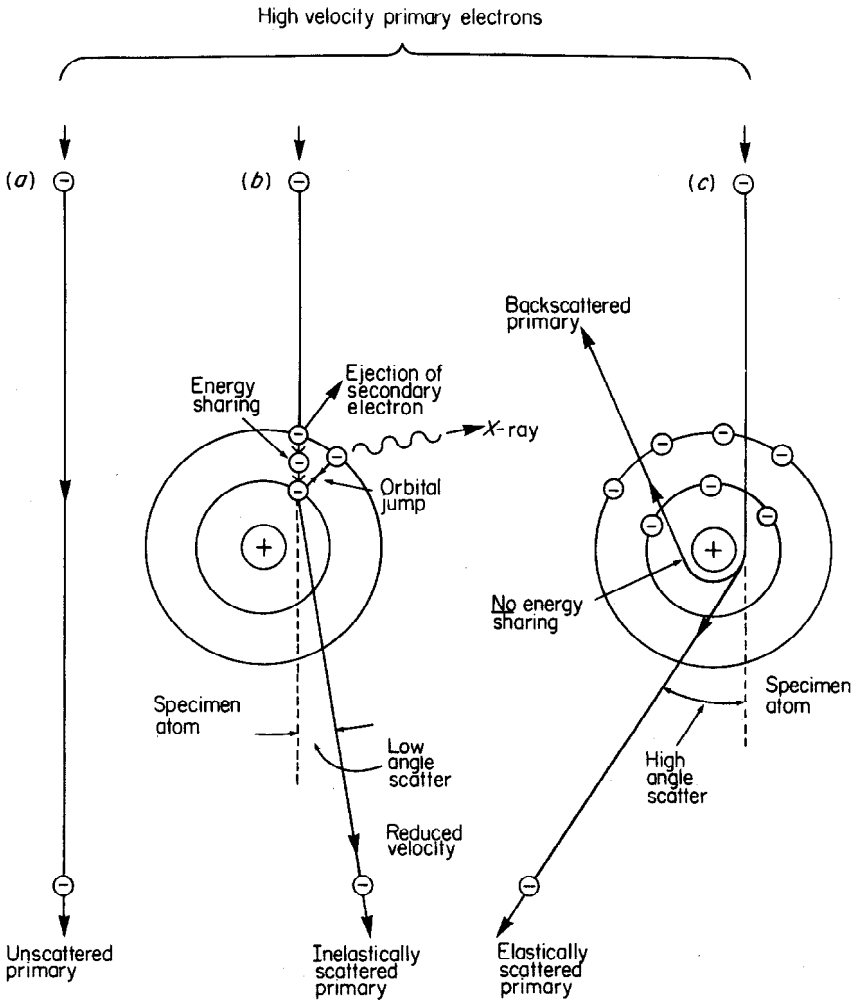
Contrast continued

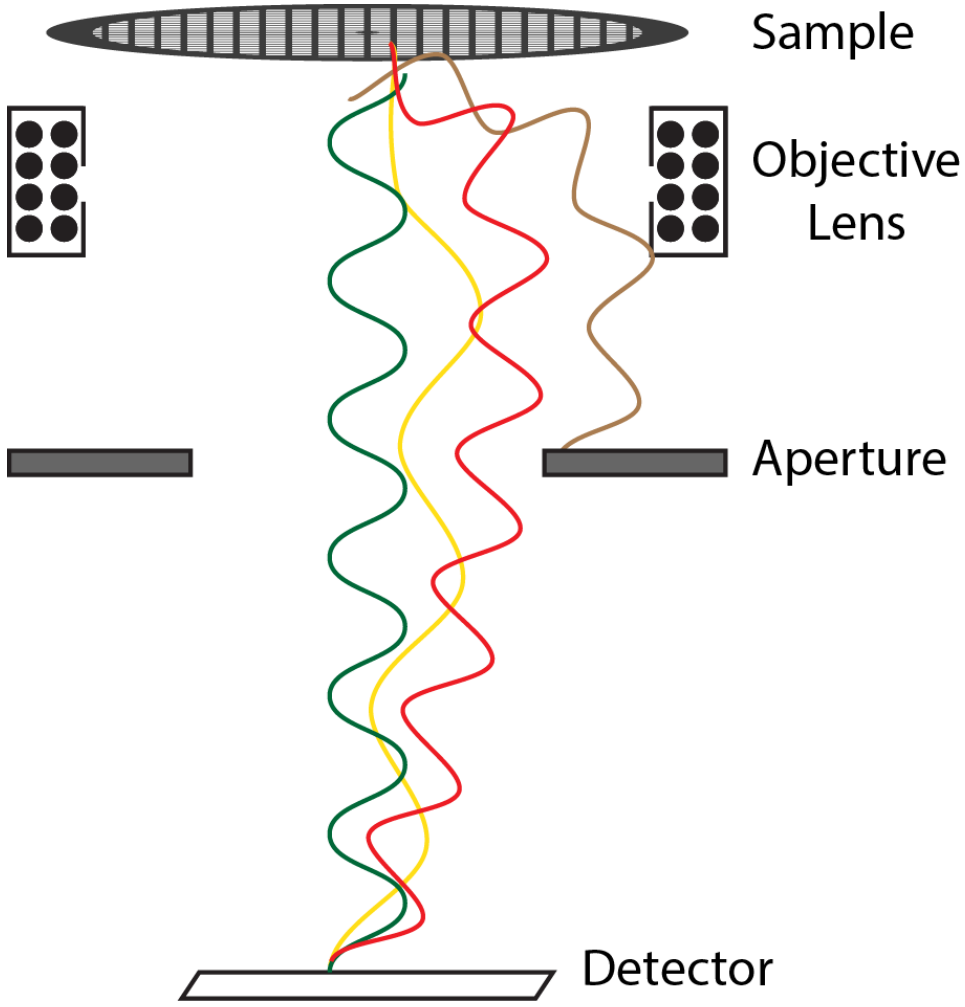
$$Percent\ contrast = 100 \times \frac{|I_o - I_b|}{I_b}$$

where I_o = intensity of the object point
I_b = intensity of the background adjacent to the object point

- In TEM, 7-10% of overall contrast comes from amplitude contrast
- 90-93% of contrast comes from phase contrast

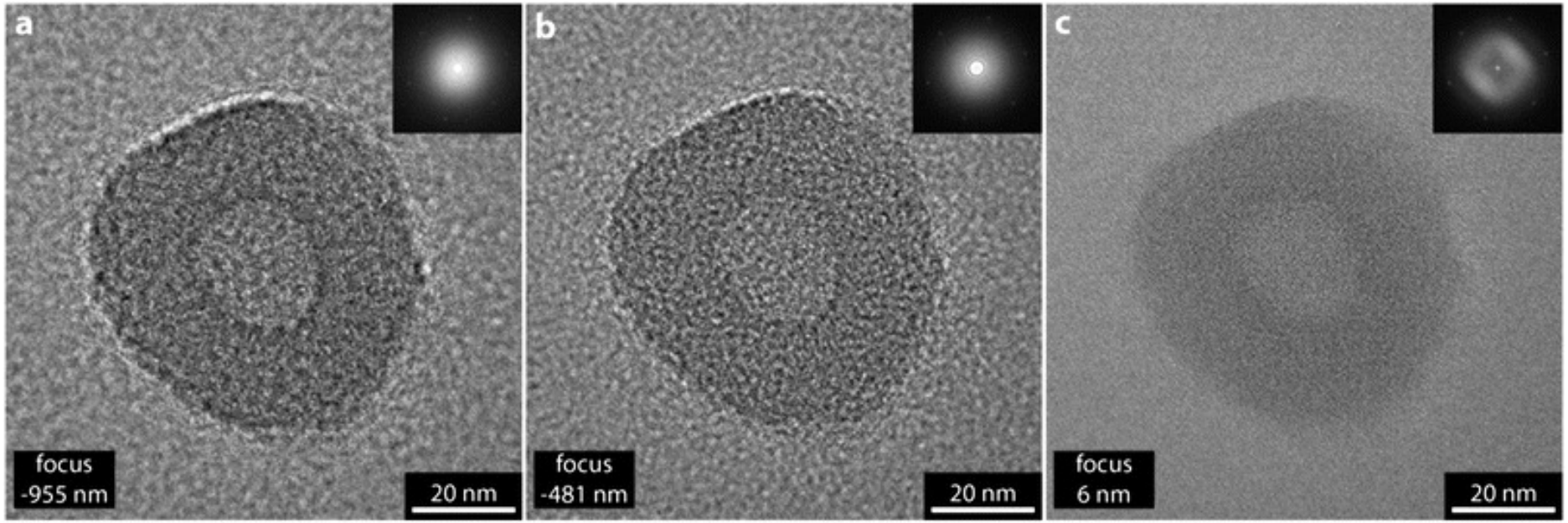
Electron scattering generates contrast





Contrast Mechanism

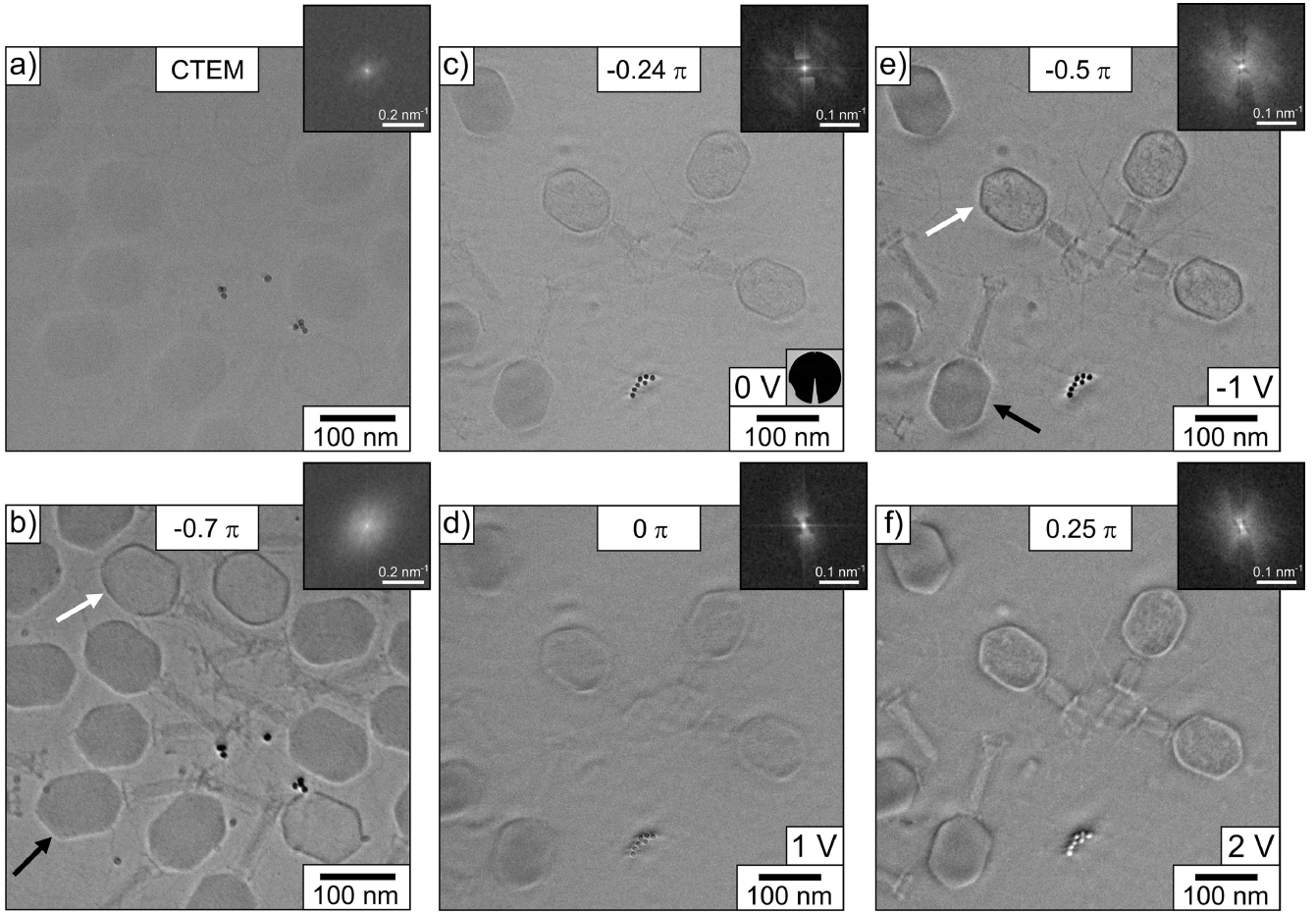
- Contrast differentiates specimen from background.



Contrast in TEM is enhanced by **defocusing** the objective lens

Phase contrast can be enhanced by **defocus** or by use of a **phase plate**

- **Phase Contrast:** shifting electron phases relative to unscattered beam
- Phase plates can be used to increase the phase shift
  - Ideal additional phase shift is $\pi/2$
- Multiple different types of phase plates
  - All work by placing phase plate in back-focal plane and influencing the scattered vs. unscattered beam
  - Zernike – layer of carbon with tiny hole for unscattered beam
  - Volta – layer of carbon where charge builds up on unscattered beam
  - Laser – super intense standing wave focused on unscattered beam
  - Zach – electrostatic potential (wire with voltage) influences unscattered beam



a) TEM without PP, b) Volta PP, phase shift of −0.7 π, c-f) Zach-PP-TEM series with varying voltages & phase shifts

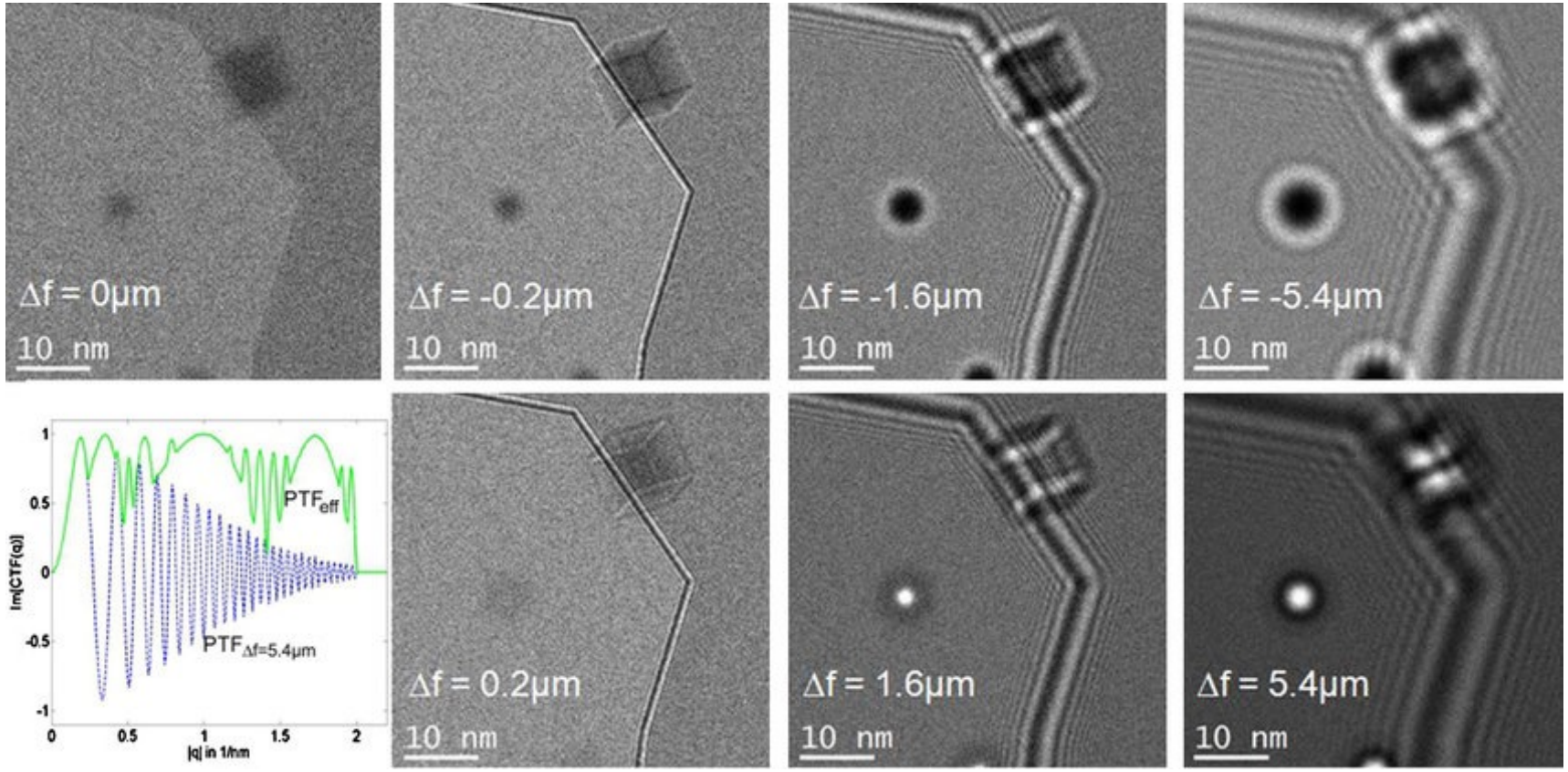
CTF (Contrast Transfer Function)

- **CTF Defined:** CTF (Contrast Transfer Function) describes how an imaging system modulates contrast at different spatial frequencies (resolutions)
- **Role in Microscopy:** CTF affects how microscopes interpret and display specimen details
- **Correction Importance:** Without CTF correction, images can have artifacts or missing information
- **Application:** Crucial in electron microscopy to achieve accurate, high-resolution images
- **Significance:** Understanding and correcting CTF ensures reliable and clear microscopic observations

Effects of CTF

Defocus:

- It refers to the extent to which an imaging system is out of focus. In electron microscopy, it's the distance between the plane where the microscope is focused and the actual plane of the specimen.



CTF image correction

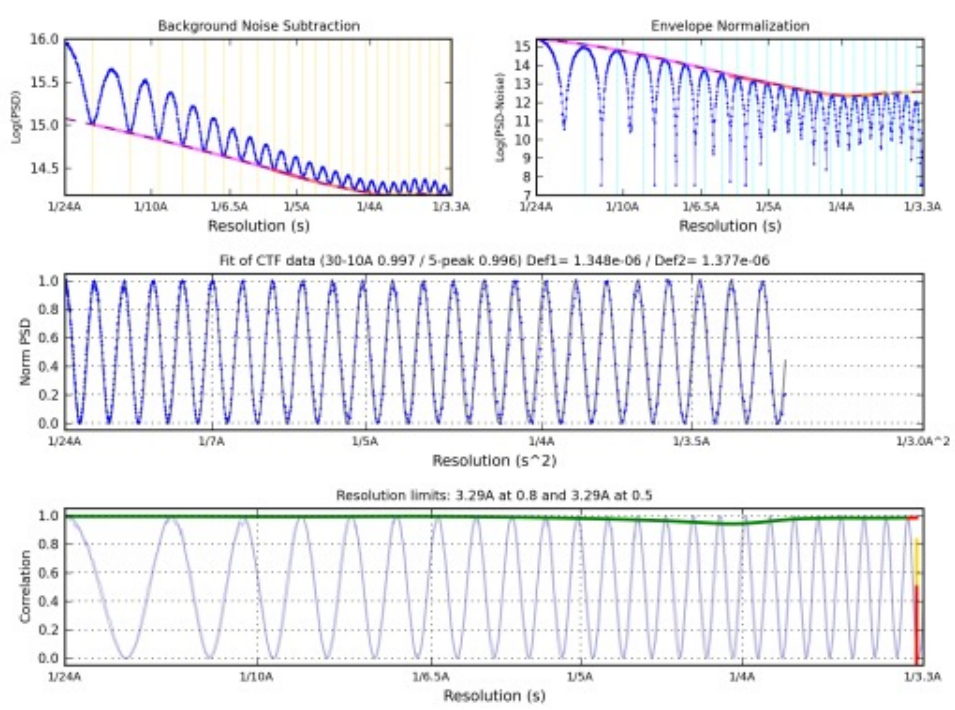
Image clarity: CTF correction ensures that the images produced by TEM are sharp and clear, free from distortions.

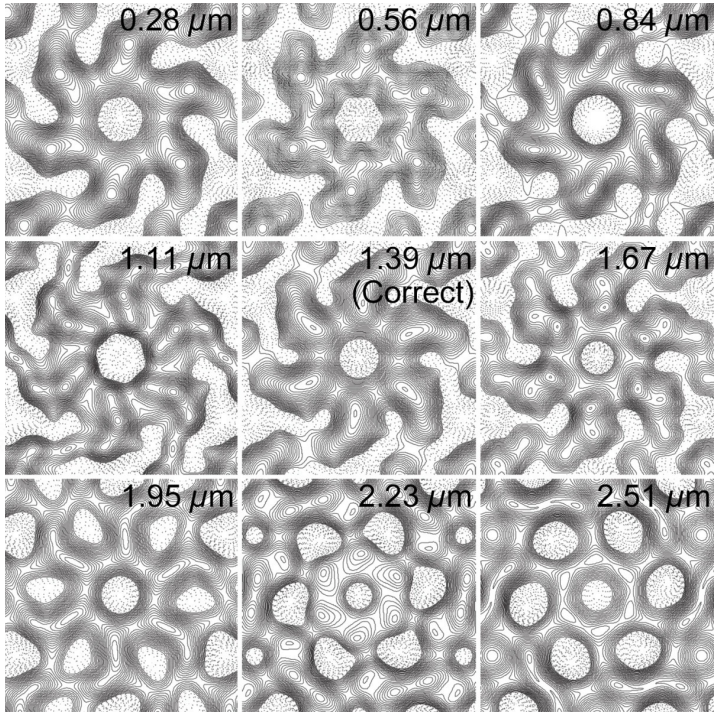
Accurate representation: Without CTF correction, the TEM images might not truly represent the specimen's actual structure.

Eliminate artifacts: CTF can introduce unwanted visual artifacts. Correcting it removes these, ensuring genuine observations.

High-resolution imaging: For TEM to achieve its potential in high-resolution imaging, CTF correction is essential.

Reliable data: CTF correction ensures the data from TEM is trustworthy

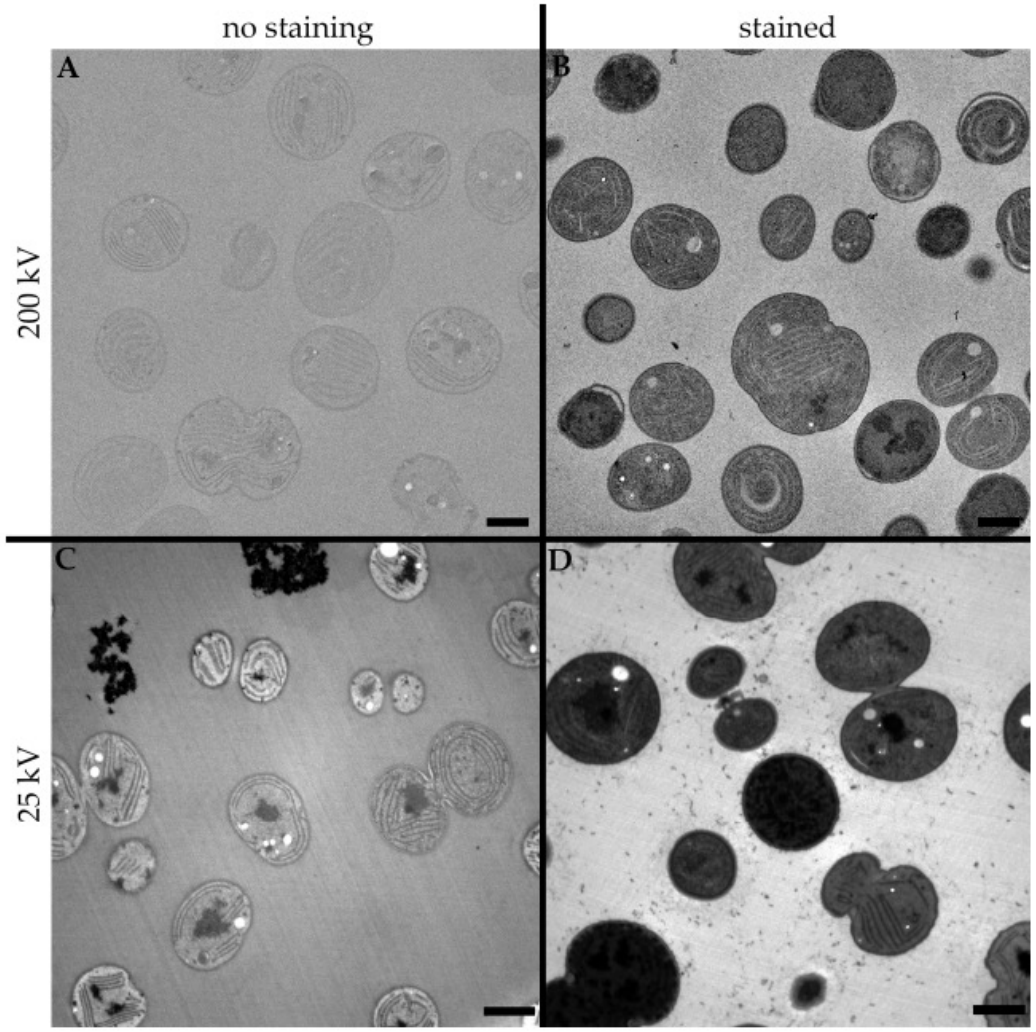




Jeong, HS.,. et al.. J Anal Sci Technol 4, 14 (2013)

Enhancing Contrast

- Staining
  - usage of heavy-atom dyes like uranyl-formate (UF), uranyl-acetate (UA), lead citrate
- Low voltage (amplitude)
  - limits the penetration of electron beam making the object less electron-transparent



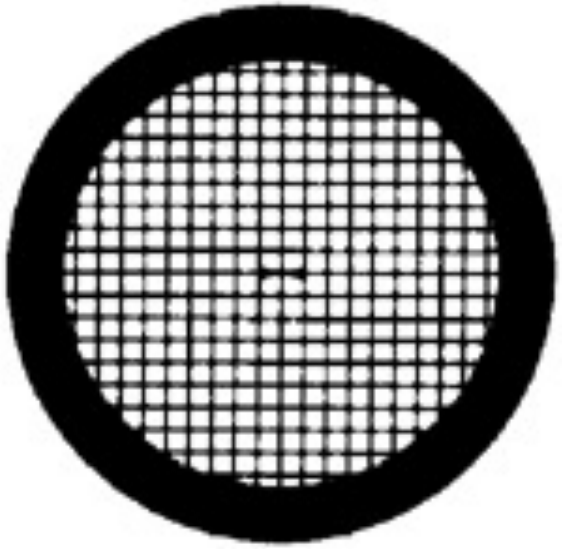
https://www.delong.cz/publications/uranyless-low-voltage-transmission-electron-microscopy-a-powerful-tool-for-ultrastructural-studying-of-cyanobacterial-cells

Principles of Negative Staining

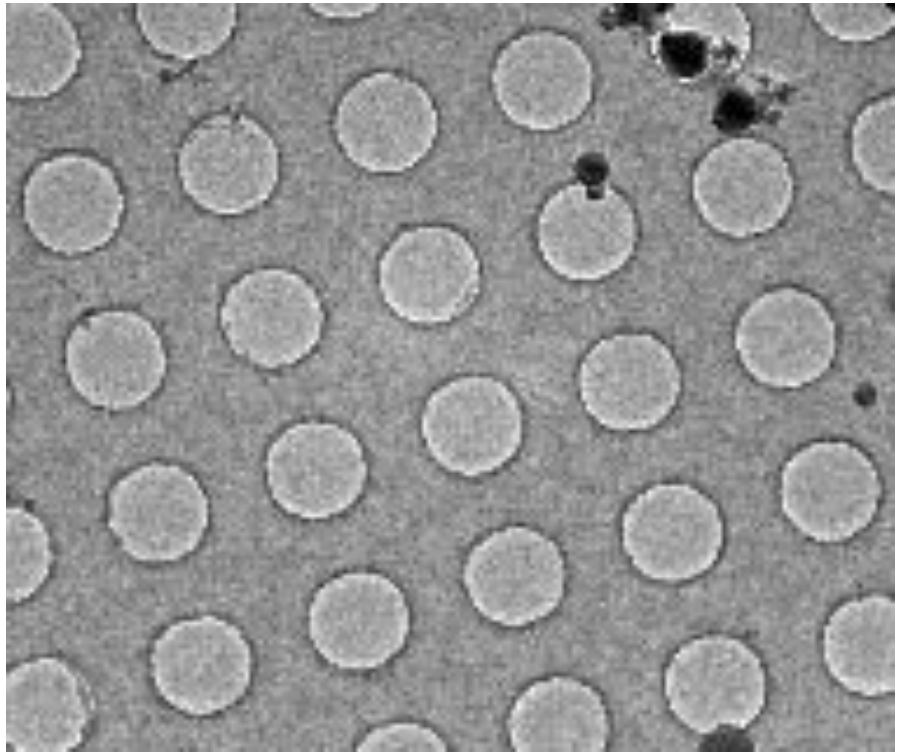
Negative staining is a microscopy technique that contrasts a specimen against a dark background, enhancing its visibility.

- **Staining Medium:** Typically heavy metal salts that scatter electrons or absorb light.
- **Specimen Contrast:** The background gets stained, not the specimen, making the specimen stand out.
- **Minimal Damage:** Gentle on specimens, preserving their natural state.
- **Rapid Technique:** Offers quick sample preparation and visualization.
- Applications: Ideal for observing thin specimens like viruses and bacterial flagella.

Sample is supported by thin layer of carbon on a copper grid



2 mm

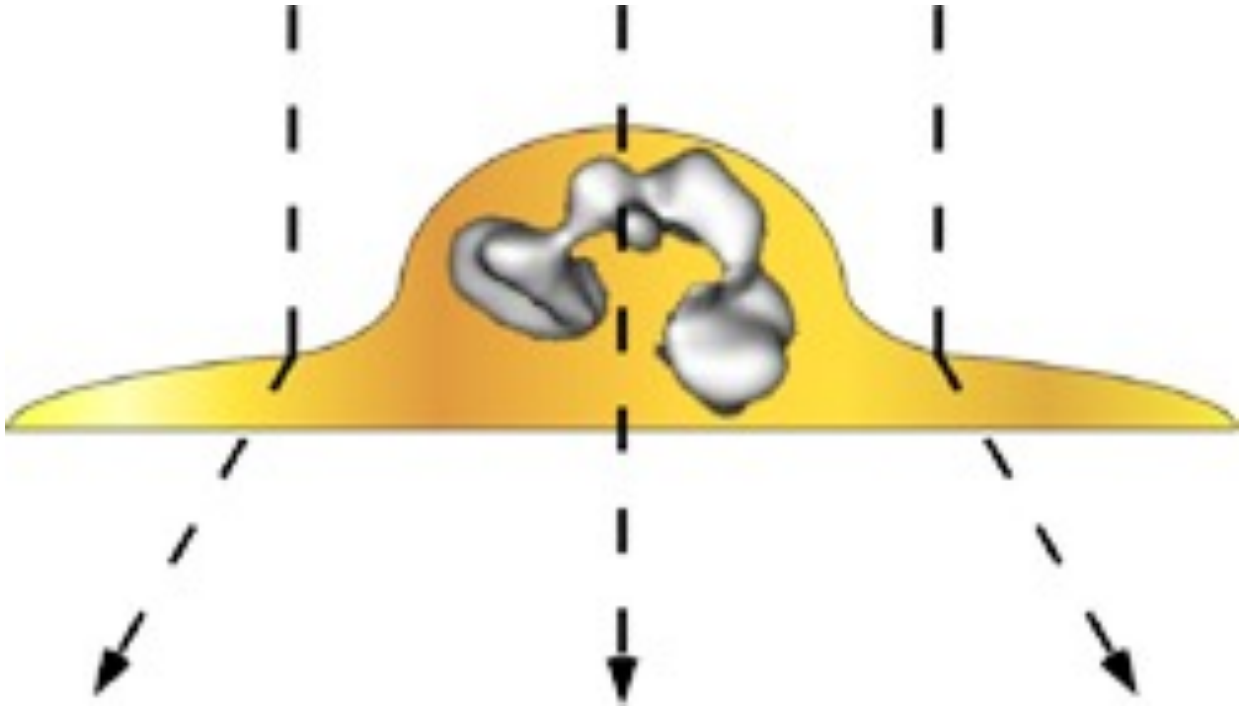


Carbon can be continuous or holey

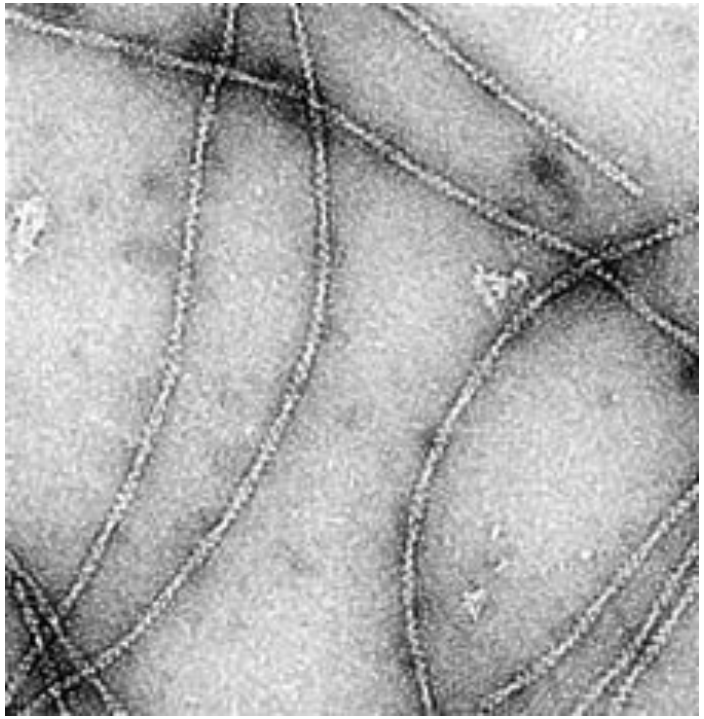
Practical aspects of Negative Staining

- **Sample Preparation**:
  - Purify the sample of interest (e.g., proteins, viruses, nanoparticles).
  - Ensure the sample is in an appropriate buffer solution.
- **Grid Preparation**:
  - Choose a suitable TEM grid, typically carbon-coated.
  - Glow-discharge the grid to make it hydrophilic (this step enhances sample adherence).
- **Sample Application**:
  - Place a small drop (typically 3-5 µL) of the sample onto the center of the grid.
  - Allow the sample to adhere for a set time (usually 1-2 minutes).
- **Blotting**:
  - Gently blot away excess liquid using filter paper, ensuring the sample remains on the grid.
- **Staining**:
  - Apply a drop of negative stain (commonly uranyl acetate or phosphotungstic acid) to the grid.
  - Allow the stain to interact for a set time (usually 1 minute).
  - Blot away excess stain gently.
- **Drying**:
  - Let the grid air-dry in a dust-free environment. This can take several minutes to hours.
- **TEM Imaging**:
  - Load the stained grid into the TEM.
  - Adjust the microscope settings for optimal contrast and focus.
  - Acquire images of the negatively stained sample.

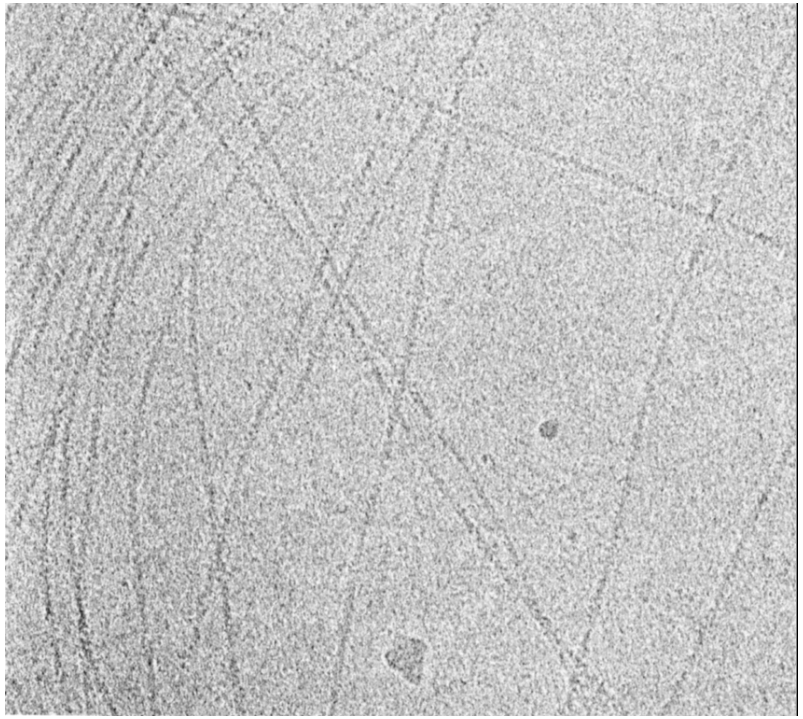
Negative staining



Negative staining vs. cryoEM

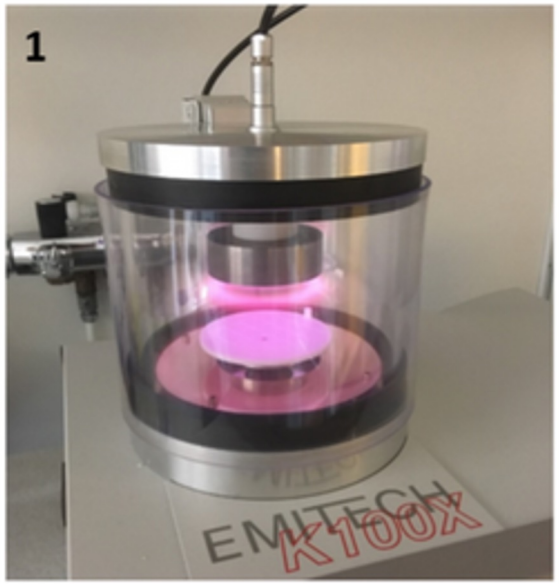


Negatively stained actin



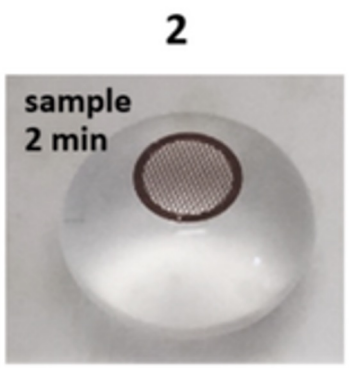
Actin in vitreous ice

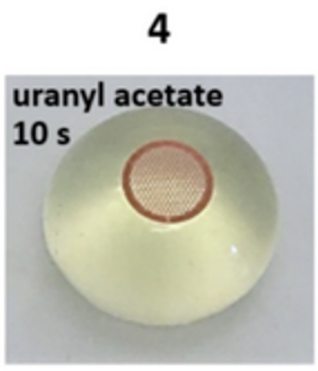
General workflow of Negative Staining

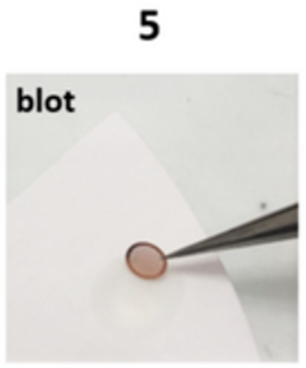


PROCEDURE:

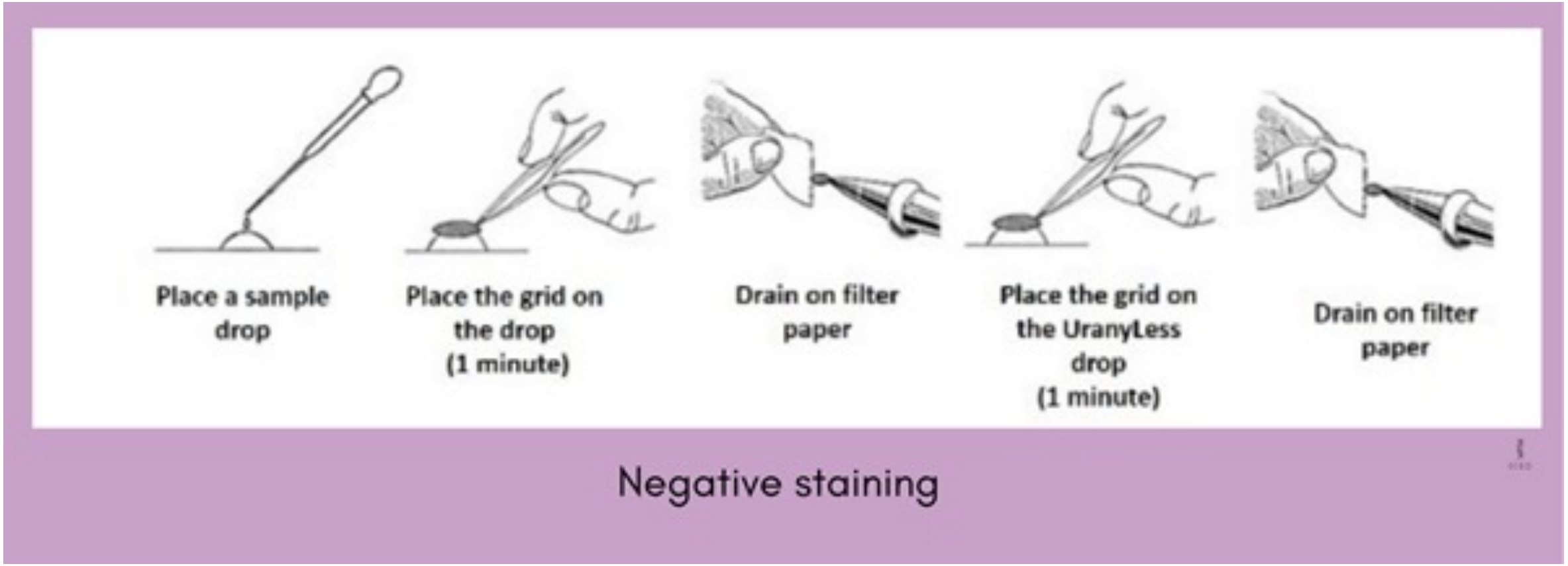
1. Glow discharge a formvar and /or carbon coated grid to make the film hydrophilic
2. Incubate the grid on a droplet of sample suspension
3. Blot excess sample away
4. Incubate grid on a drop of heavy metal stain for 10s
5. Blot the stain away and dry the grid for 30 minutes before imaging





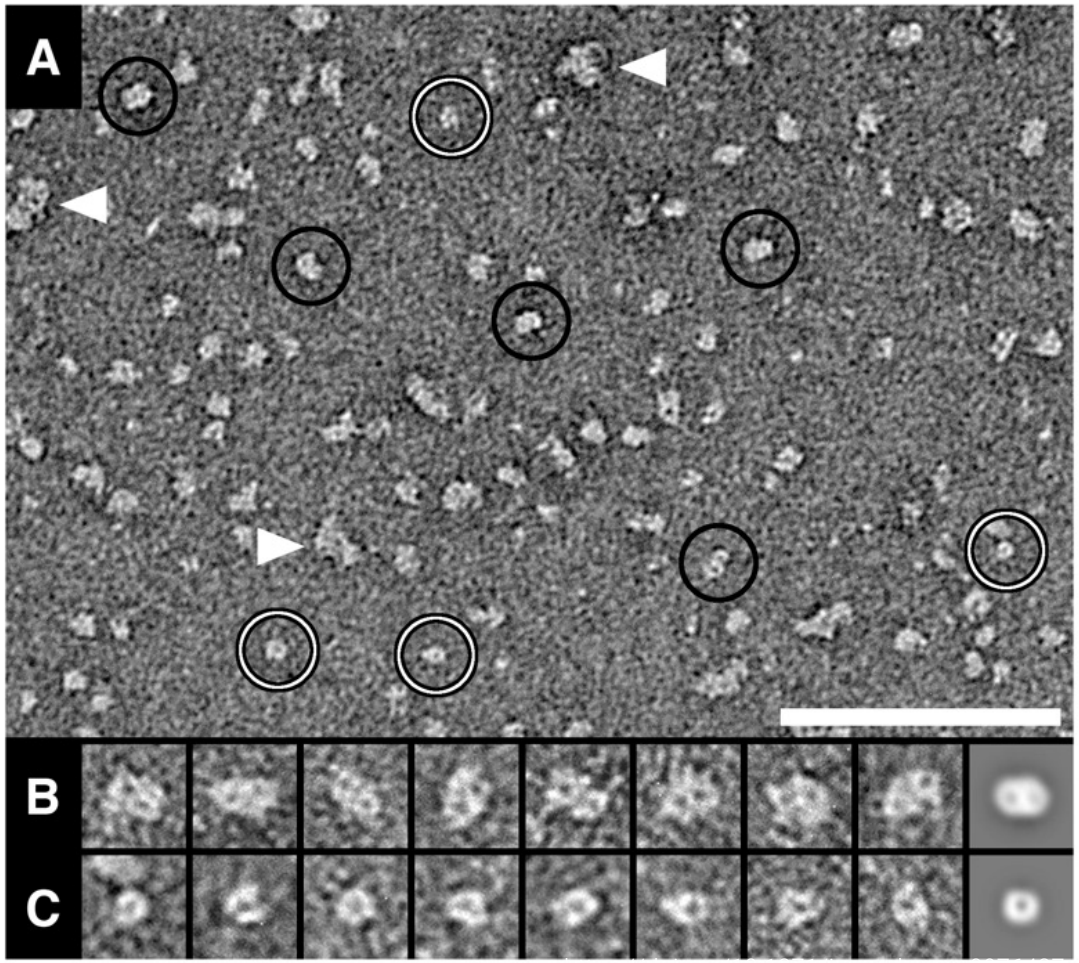


Practical aspect of Negative Staining

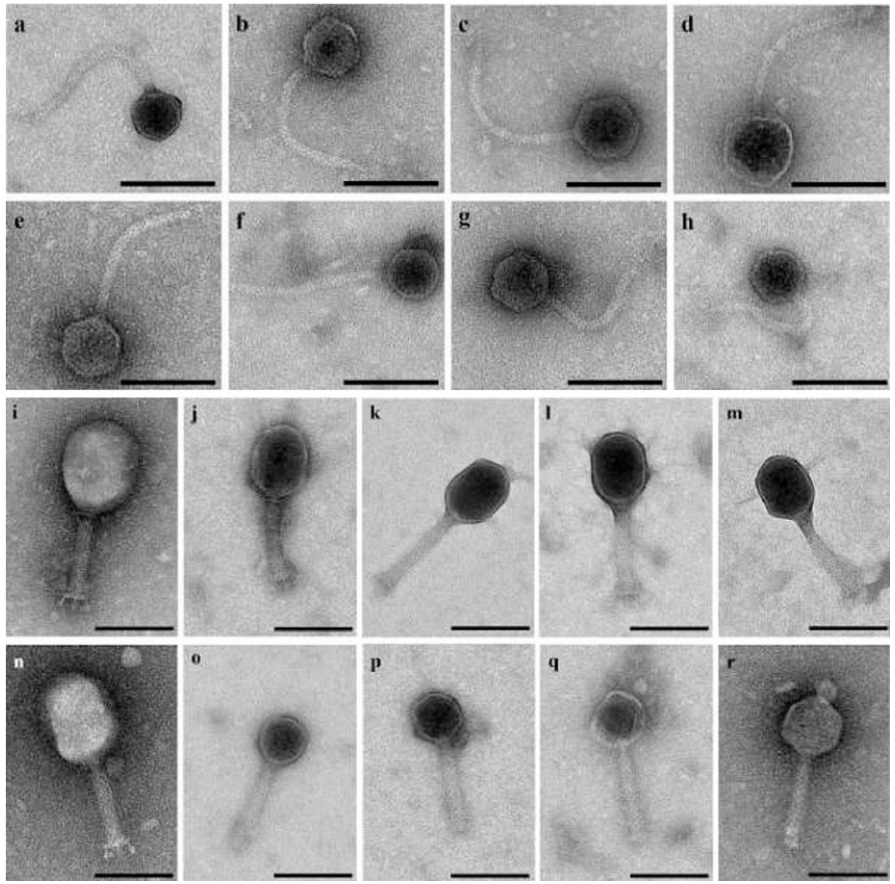


Negative Staining – the “running a gel” of cryo-EM

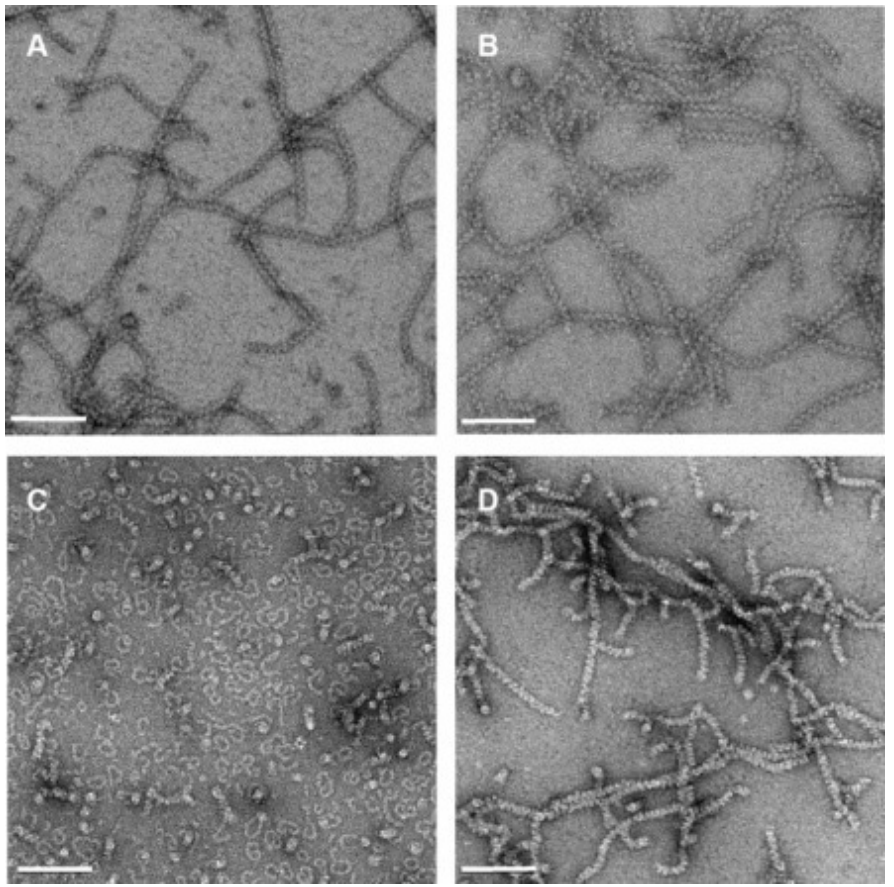
- **Particle Sizes**: Gauge dimensions and size variations
- **Particle Orientations**: See different angles and facets
- **Morphological Details**: Observe surface features and shape
- **Aggregation State**: Check for clusters or assemblies
- **2D Projections**: Get flat views for initial analysis
- **3D Reconstructions**: Create spatial models from 2D views
- **Sample Heterogeneity**: Assess the presence of different structural states or conformations within the sample.



Negative Staining of viruses and filaments



Negative staining electron microscopy images of Siphoviridae and Myoviridae coliphages



Negative staining EM micrographs of hRAD51 filaments

https://doi.org/10.1038/s41598-020-72177-2
asas

Different Negative Stains

Phosphotungstic Acid (PTA)

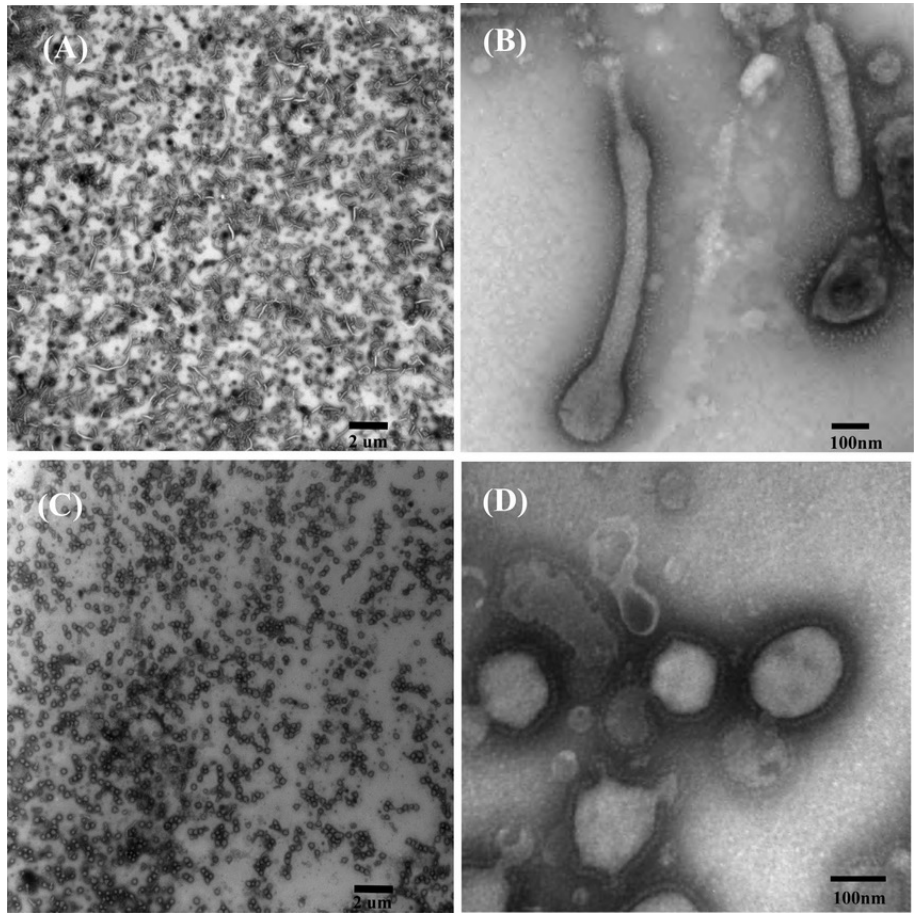
- **Use**: Stains viruses, bacteria; electron microscopy.
- **Pros**: Good contrast; pH adjustable.
- **Cons**: Can cause aggregation; potential sample damage

Uranyl Acetate

- **Use**: Negative staining in TEM; thin sections.
- **Pros**: High contrast; stable; easy prep.
- **Cons**: Mildly radioactive.

Nanowire Staining

- **Use**: Visibility of nanowires; materials science.
- **Pros**: Enhances nanoscale contrast.
- **Cons**: Specific to nanomaterials; not for bio samples.



Negatively stained Virus-Like-Particles (VLPs) (A, B) PTA-ebola nano-VLPs; (C, D) Murine Leukemia VLPs with Ebola virus glycoprotein on their surface.

Questions?