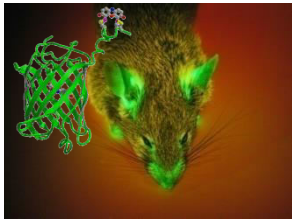
Microscopy Principle

History of Microscopy

1595: The first compound microscope built by Zacharias Janssen

1910: Leitz builds first "photo-microscope"

Video microscopy developed early 1980s



1994: GFP used to tag proteins in living cells

1600 1700 1800 1900 2000 2010

1680: Antoni van Leeuwenhoek awarded fellowship in th Royal Society fo his advances in microscopy

1934: Frits Zernike invents phase contrast microscopy

1955: Nomarski invents Differential Interference Contrast (DIC) microscopy

Super-Resolution light Microscopy

Introduction

Biochemical analysis is frequently accompanied by microscopic examination of tissue, cell or organelle preparations. Such examinations are used in many different applications, for example:

- To evaluate the integrity of samples during an experiment.
- To map the fine details of the spatial distribution of macromolecules within cells.
- To directly measure biochemical events within living tissues.

- **Light Microscope**
- **Electron Microscope**

- **Magnification**
- **Resolution**
- **Contrast**

$$d = 0.61\ \lambda / NA$$

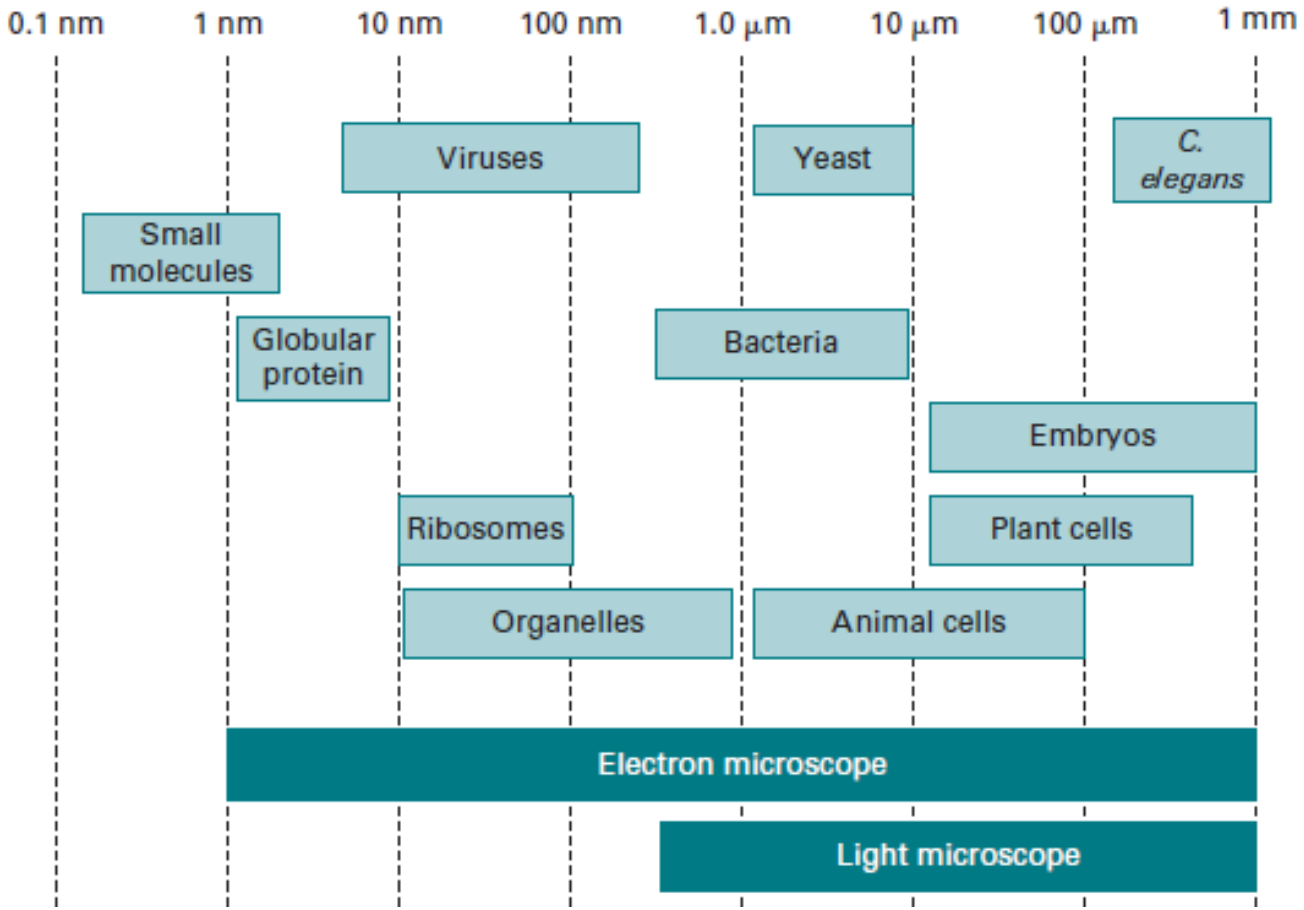
d- Minimum distance which can be resolve
λ – Wavelength of the light
NA – Numerical aperture

$$NA = n\ \sin\alpha$$

n – refractive index of the medium
α – angle between object and the objective lens

$$d = 0.61\ \lambda / n\ \sin\alpha$$

The relative sizes of biological specimens and the devices used to image them



Basic components of microscope

All modern light microscopes are made up of more than one glass lens in combination. The major components are-

- The condenser lens
- The objective lens
- The eyepiece lens

Each of these components is in turn made up of combinations of lenses, which are necessary to produce magnified images with reduced artifacts and aberrations.

Spherical aberration
chromatic aberration

This problem was encountered in the early microscopes of van Leeuwenhoek and Hooke. All modern lenses are now corrected to some degree in order to avoid this problem.

Continue..

The main components of the compound light microscope include a light source that is focused at the specimen by a condenser lens. Light that either passes through the specimen (transmitted light) or is reflected back from the specimen (reflected light) is focused by the objective lens into the eyepiece lens. The image is either viewed directly by eye in the eyepiece or it is most often projected onto a detector, for example photographic film or, more likely, a digital camera. The images are displayed on the screen of a computer imaging system, stored in a digital format and reproduced using digital methods.

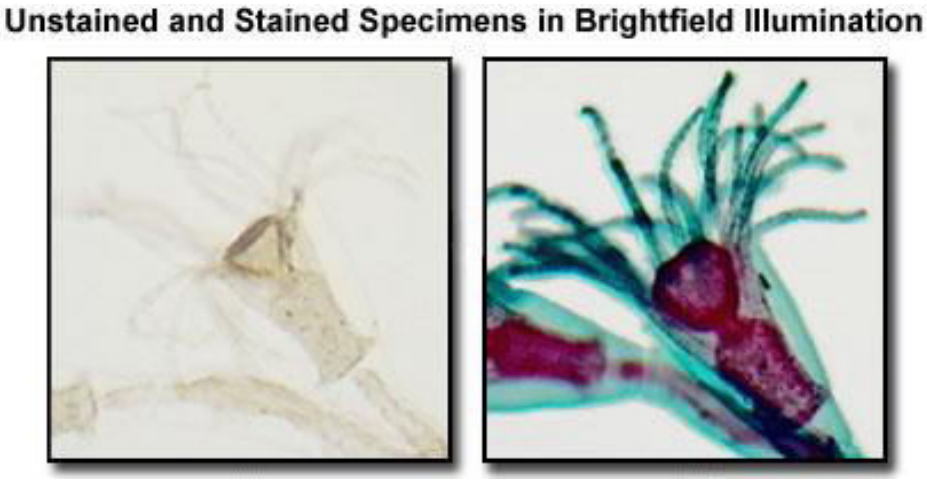
Continue..

The field of microscopy has undergone a renaissance over the past 20 years with many technological improvements to the instruments. Most images produced by microscopes are now recorded electronically using digital imaging techniques digital cameras, digital image acquisition software, digital printing and digital display methods. In addition, vast improvements have been made in the biological aspects of specimen preparation. These advancements on both fronts have fostered many more applications of the microscope in biomedical research.

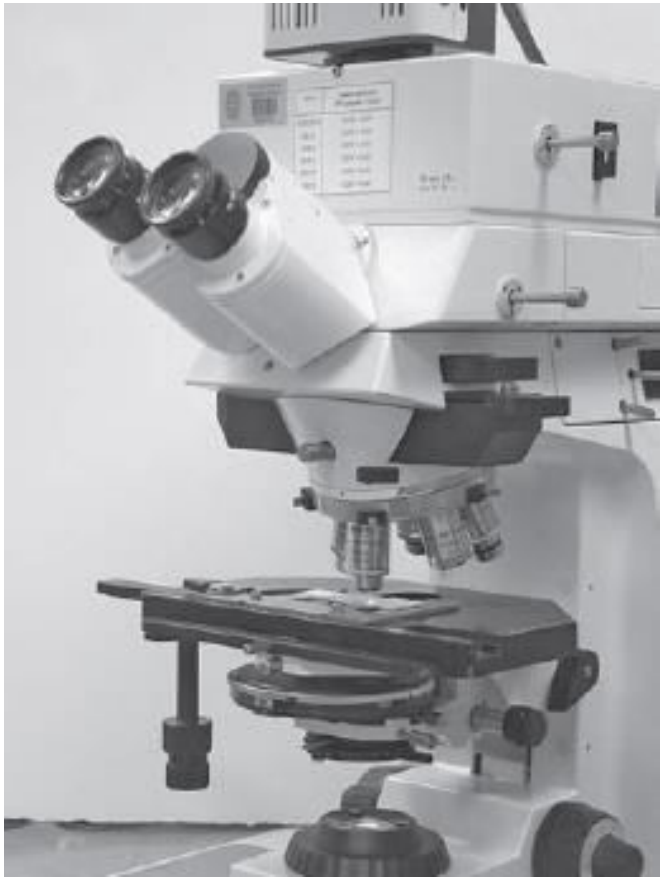
Bright field Microscopy

- Simplest technique to set up
- True color technique
- Proper technique for measurements
  - Dimensional or Spectral
- What is the problem with Bright-Field microcopy?

Low contrast
Staining is required



The part of the microscope that holds all of the components firmly in position is called the stand.



Upright light microscope

Inverted light microscope

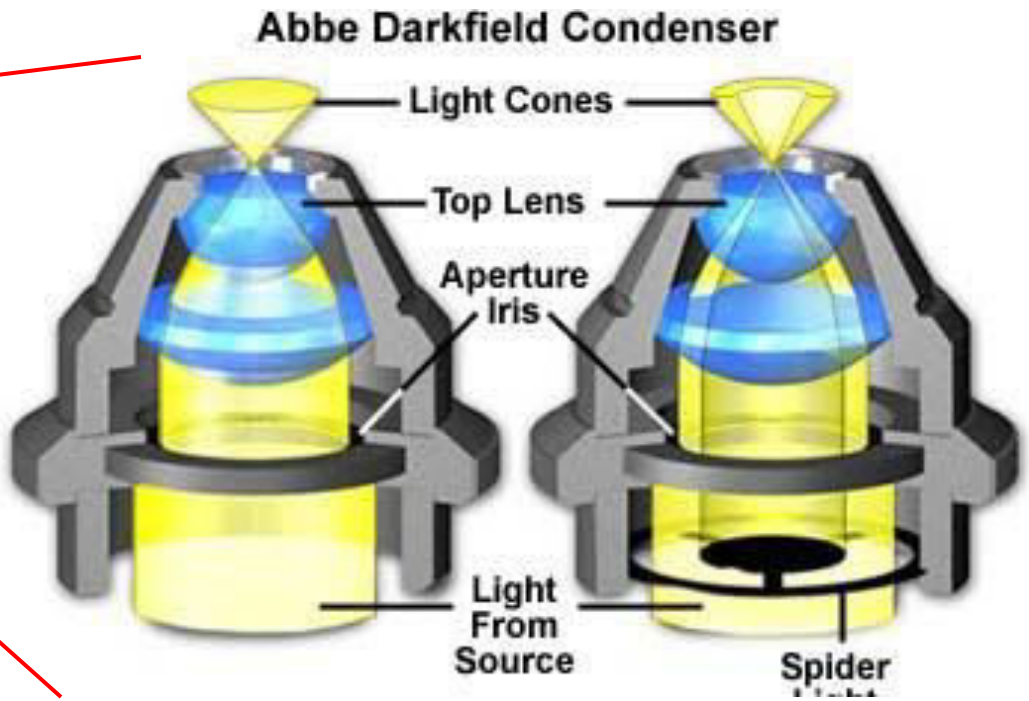
Inverted LM allows additional room for manipulating the specimen directly on the stage, for example, for the microinjection of macromolecules into tissue culture cells, for in vitro fertilization of eggs or for viewing developing embryos over time.

Dark field Microscopy

- Central Dark field via hollow cone
- Oblique Dark field via Illumination from the side
- Undeviated light (zero-order) blocked off so black background
- Only Scattered / Diffracted light visible
- Shows sub-resolution details, particles, defects etc. with excellent reversed contrast
- Good technique for live specimens
- Not for measurements (gives wrong size)

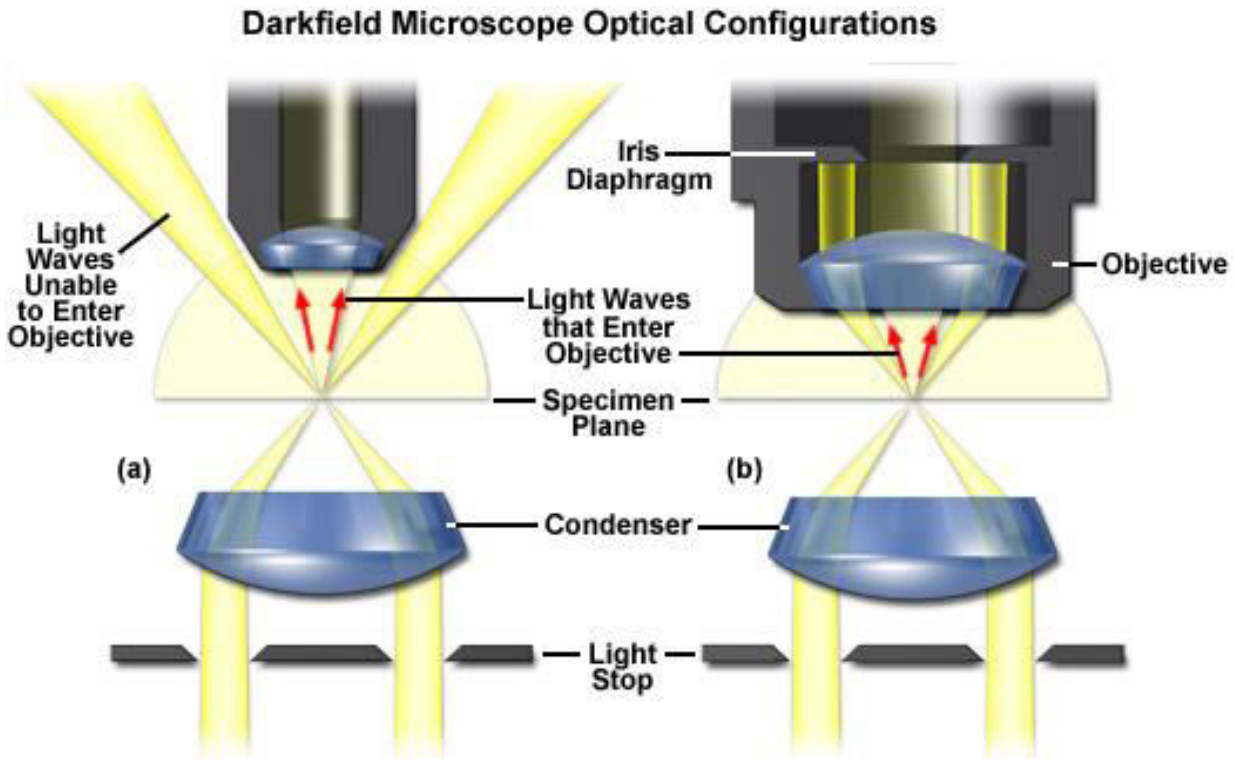
Dark field Microscopy

Darkfield Condenser



Dark Field Illumination

- Required conditions for Dark field
  - Illumination Aperture must be larger than objective aperture
  - Direct light must bypass observer



Phase contrast Microscopy

- Phase contrast is used for **viewing unstained cells growing in tissue culture and for testing cell and organelle preparations for lysis**.
- **Light that passes through thicker parts of the cell is held up relative to the light that passes through thinner parts of the cytoplasm.**
- It requires a **specialised phase condenser** and **phase objective lenses** (both labelled **ph**).
- **Each phase setting of the condenser lens is matched with the phase setting of the objective lens.**
- These are usually numbered as Phase 1, Phase 2 and Phase 3, and are found on both the condenser and the objective lens.

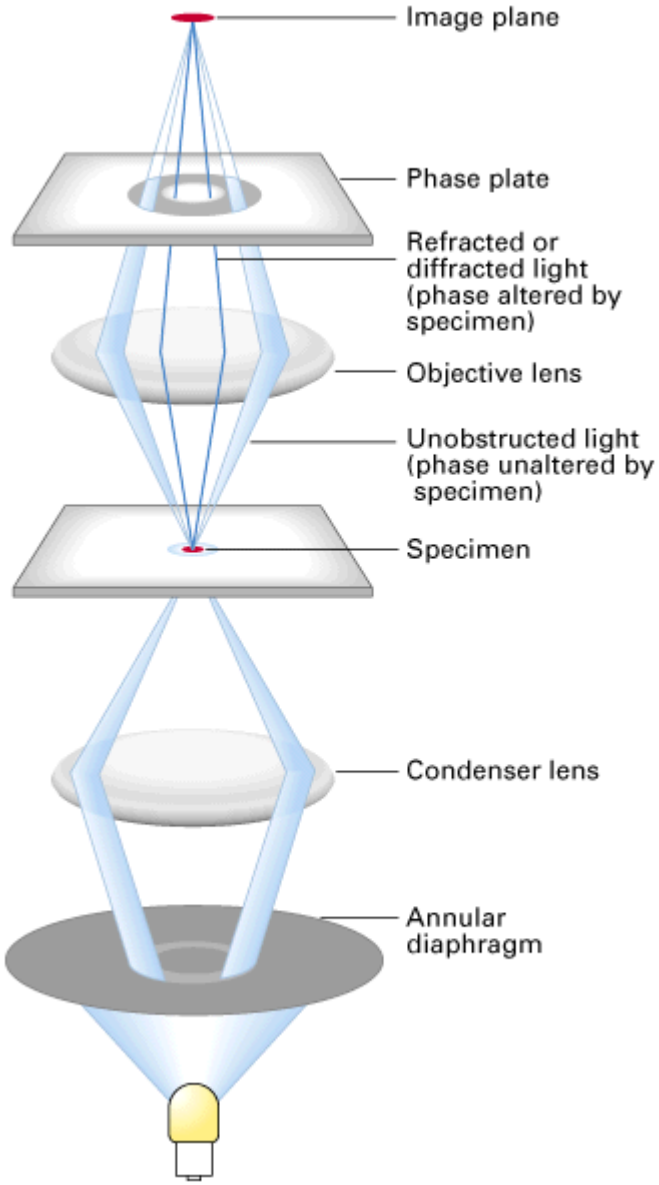
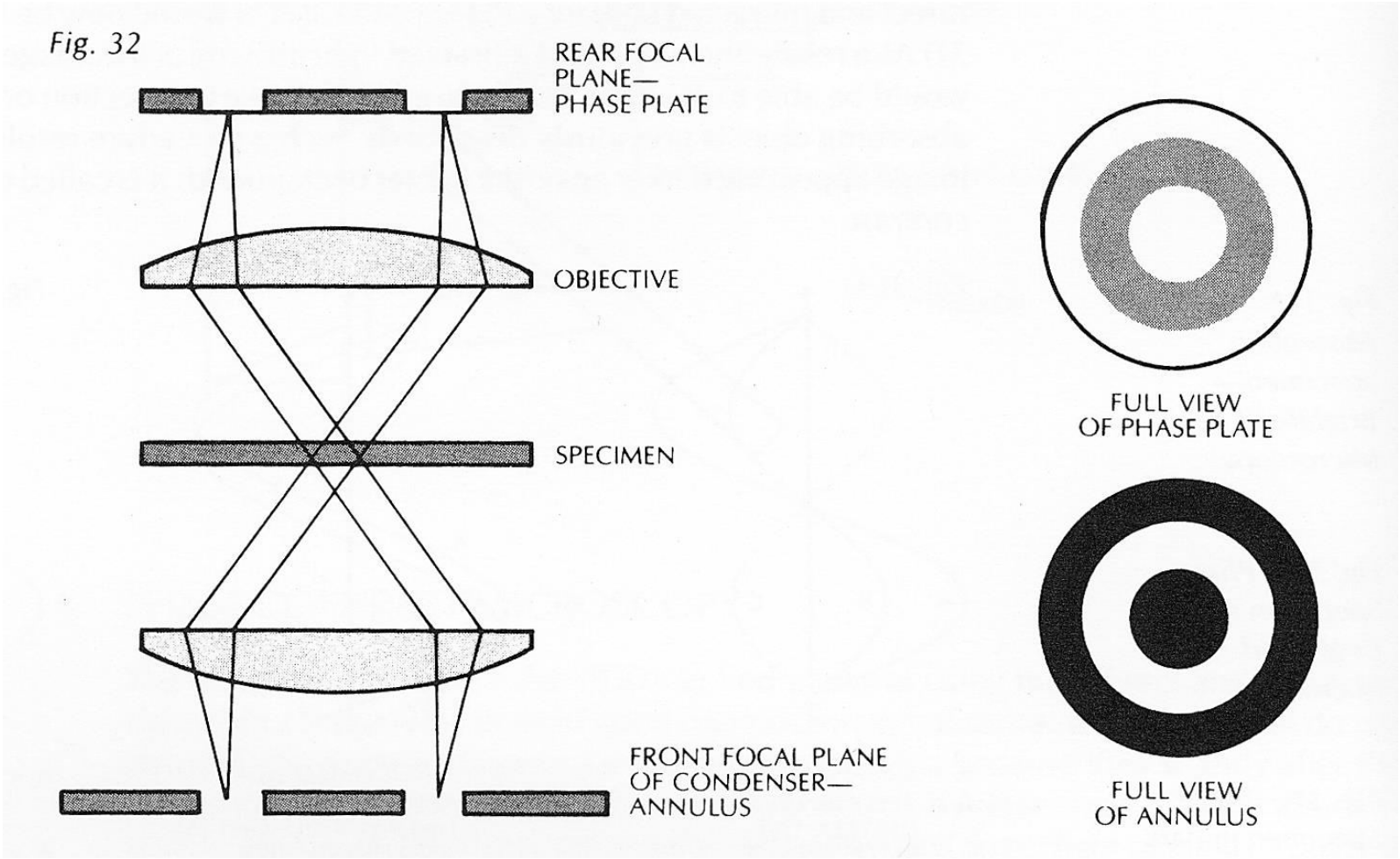


Image plane
Phase plate
Refracted or diffracted light (phase altered by specimen)
Objective lens
Unobstructed light (phase unaltered by specimen)
Specimen
Condenser lens
Annular diaphragm

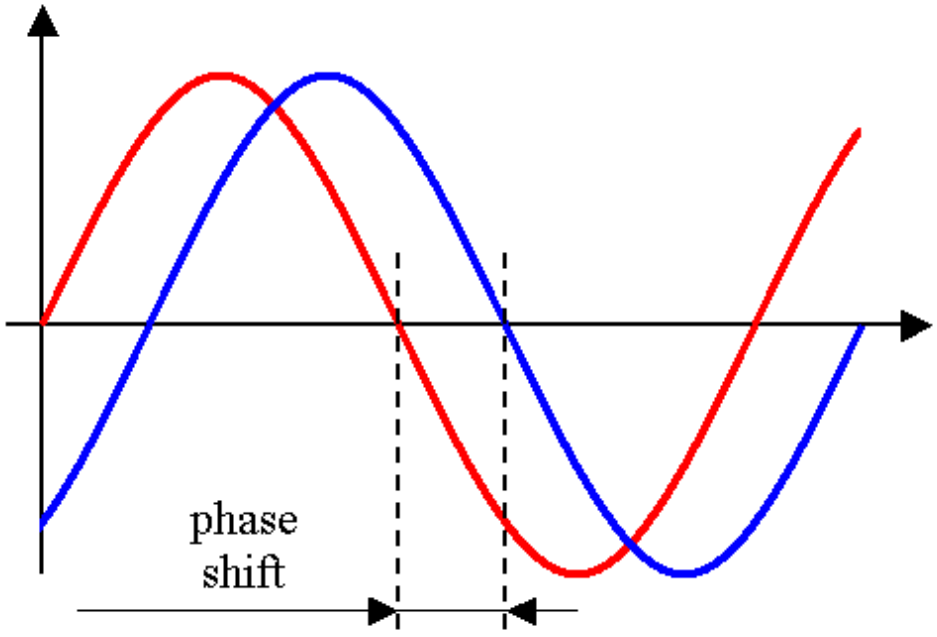
Phase Contrast Microscopy



F. Zernike, 1930s

Characteristics of a wave

- Phase shift is any change that occurs in the phase of one quantity or in the phase difference between two or more quantities.
- Small phase differences between 2 waves cannot be detected by the human eye but can be enhanced optically.



Phase contrast illumination

- Revolutionary technique for live cell imaging
- Used today in almost every tissue culture lab
- Depends on phase shift for contrast
- Dutch scientist Frits Zernike was awarded the Nobel Prize for his discovery

The Nobel Prize in Physics 1953
Frits Zernike

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The Nobel Prize in Physics 1953

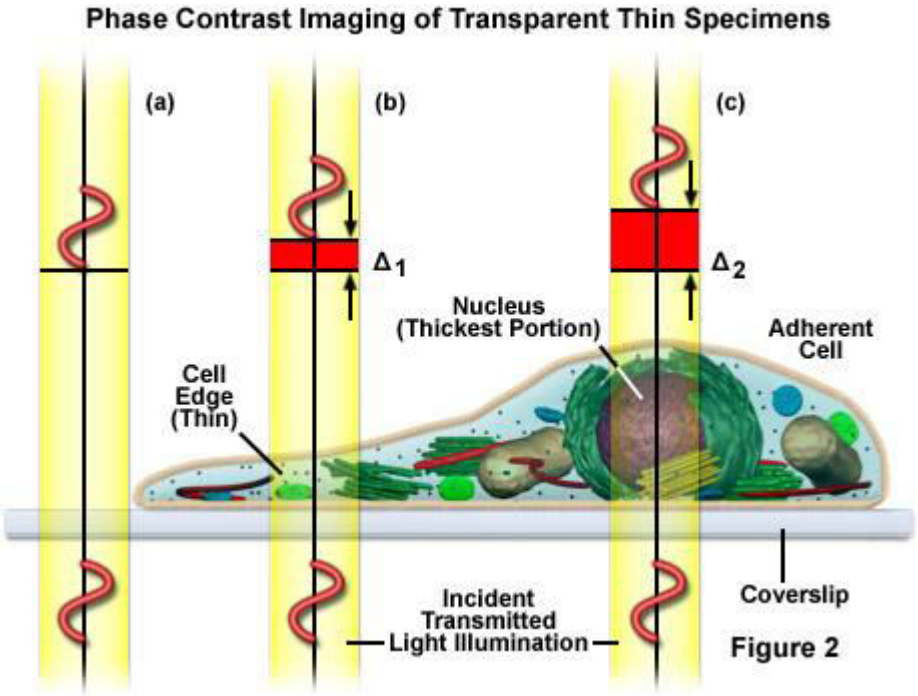
Frits (Frederik) Zernike

Prize share: 1/1

The Nobel Prize in Physics 1953 was awarded to Frits Zernike *"for his demonstration of the phase contrast method, especially for his invention of the phase contrast microscope"*.

Phase contrast illumination

- Cells have higher η than water
  - Light moves slower in higher η
  - Light has shorter λ
- Light will be phase-retarded

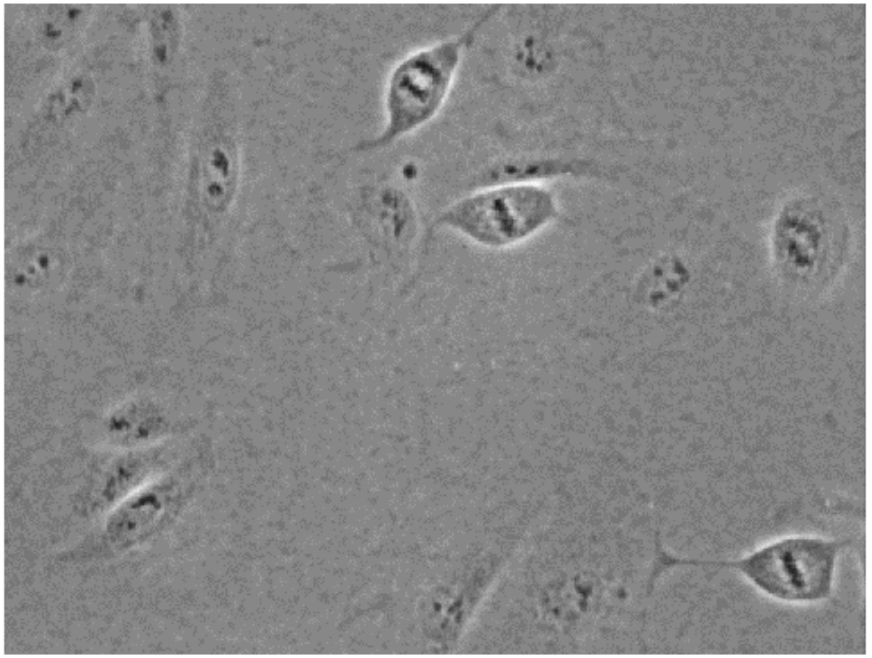


- For unstained (live) specimens
- Good depth of field
- Easy alignment (usually pre-aligned)
- Reduced resolution (small condenser NA)
- Not good for thick samples

Phase Contrast Images

Brightfield

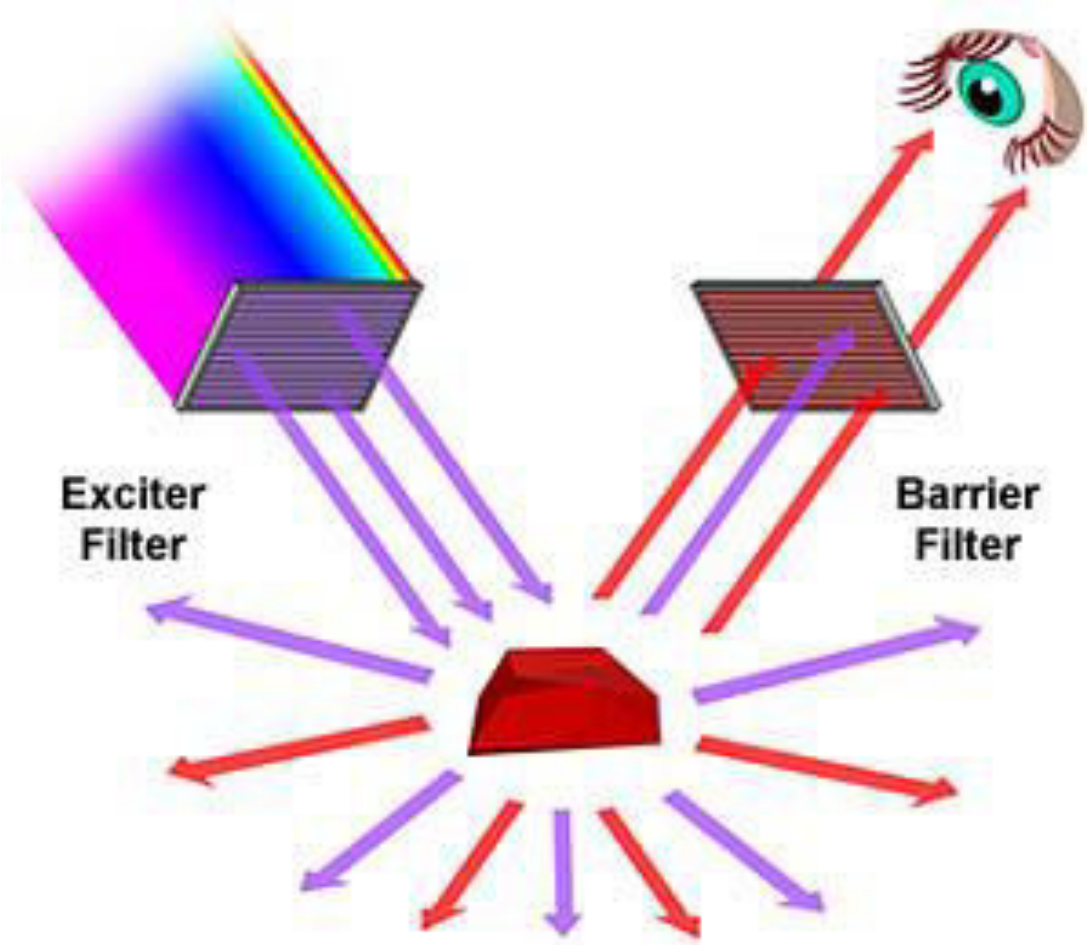
Phase Contrast



Fluorescence microscopy

- Fluorescence microscopy is currently the most widely used contrast technique since it gives superior signal-to-noise ratios for many applications.
- The most commonly used fluorescence technique is called **epi-fluorescence light microscopy**, where 'epi' simply means 'from above'.
- Here the light source comes from above the sample, and the objective lens acts as both condenser and objective lens.
- Fluorescence is popular because of the ability to achieve highly specific labeling of cellular compartments.

Basic principle

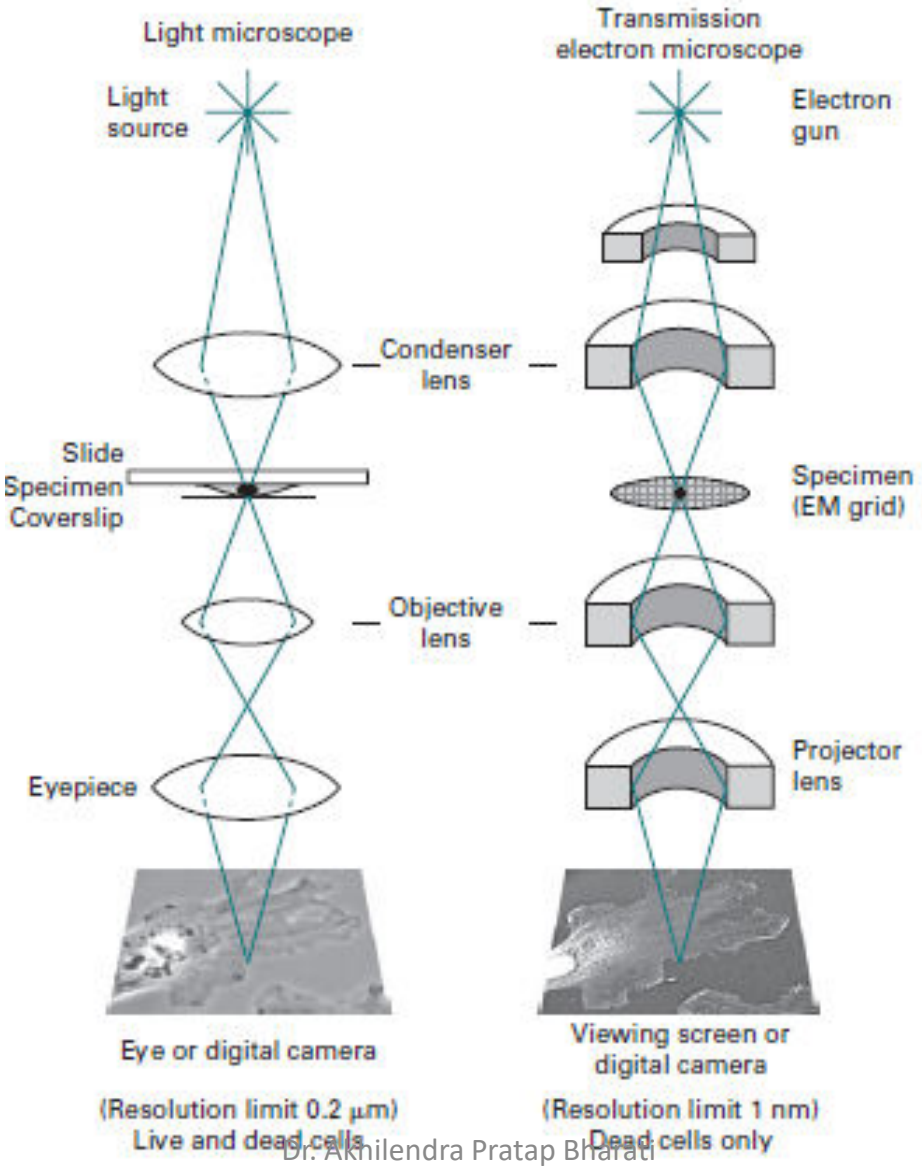


Different fluorescence dye used in microscopy

Dye	Excitation max. (nm)	Emission max. (nm)
Commonly used fluorophores		
Fluorescein (FITC)	496	518
Bodipy	503	511
CY3	554	568
Tetramethylrhodamine	554	576
Lissamine rhodamine	572	590
Texas red	592	610
CY5	652	672
Nuclear dyes		
Hoechst 33342	346	460
DAPI	359	461
Acridine orange	502	526
Propidium iodide	536	617
TOTO3	642	661

Ethidium bromide	510	595
Feulgen	570	625
Calcium indicators		
Fluo-3	506	526
Calcium green	506	533
Reporter molecules		
CFP (cyan fluorescent protein)	443/445	475/503
GFP (green fluorescent protein)	395/489	509
YFP (yellow fluorescent protein)	514	527
DsRed	558	583
Mitochondria		
JC-1	514	529
Rhodamine 123	507	529

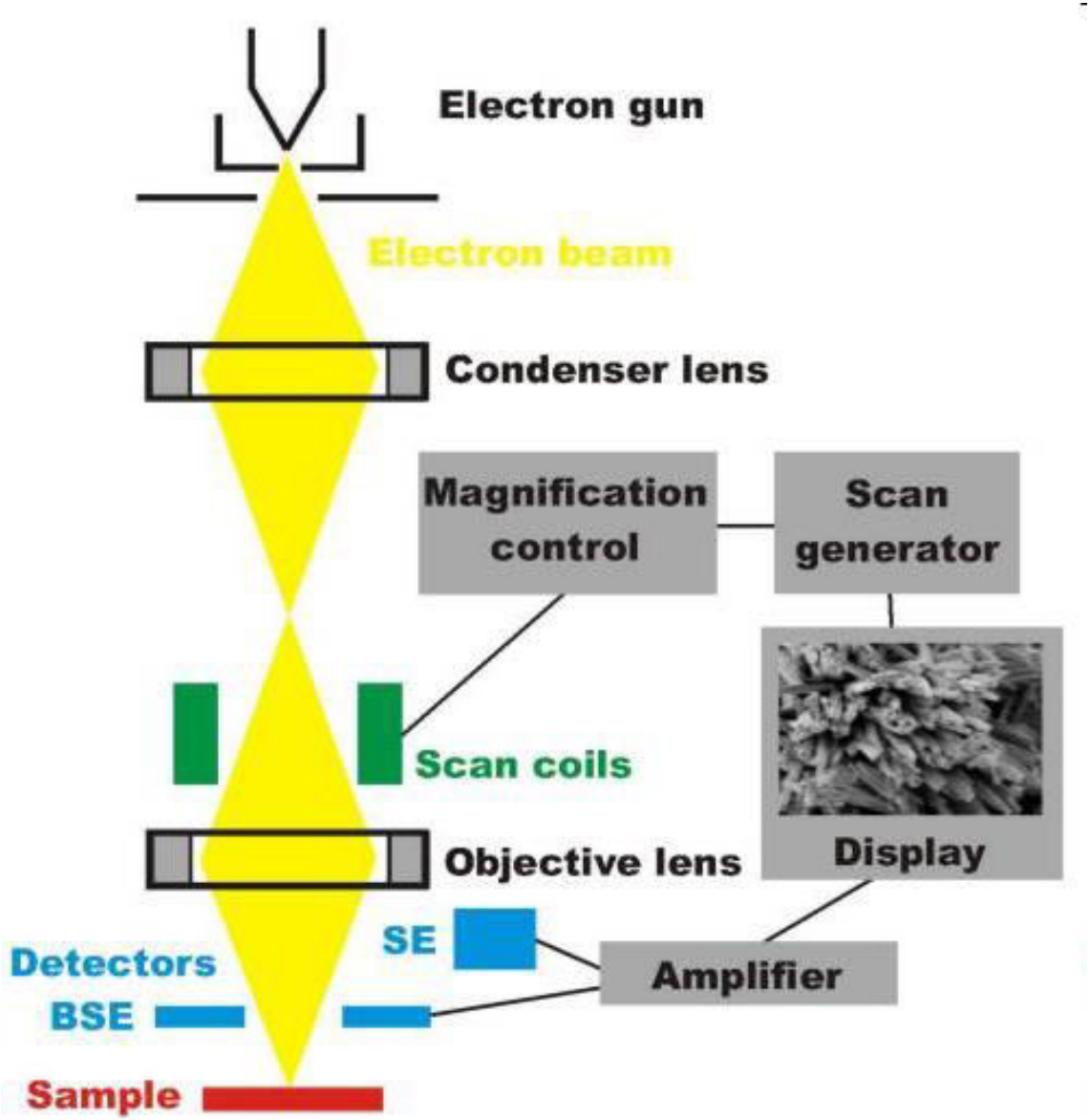
Comparison of Light with electron microscopy



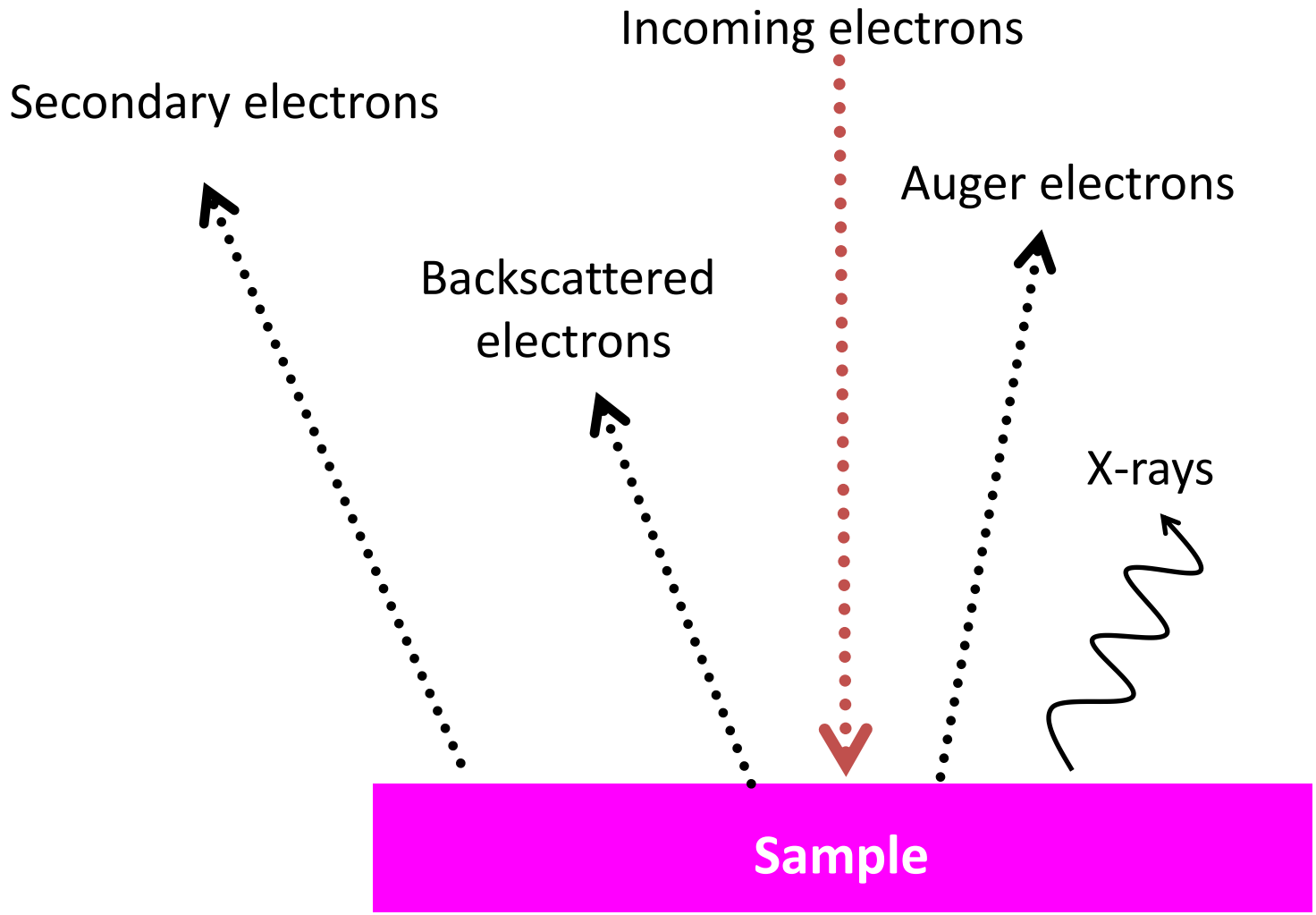
Electron Microscope vs. Light Microscope

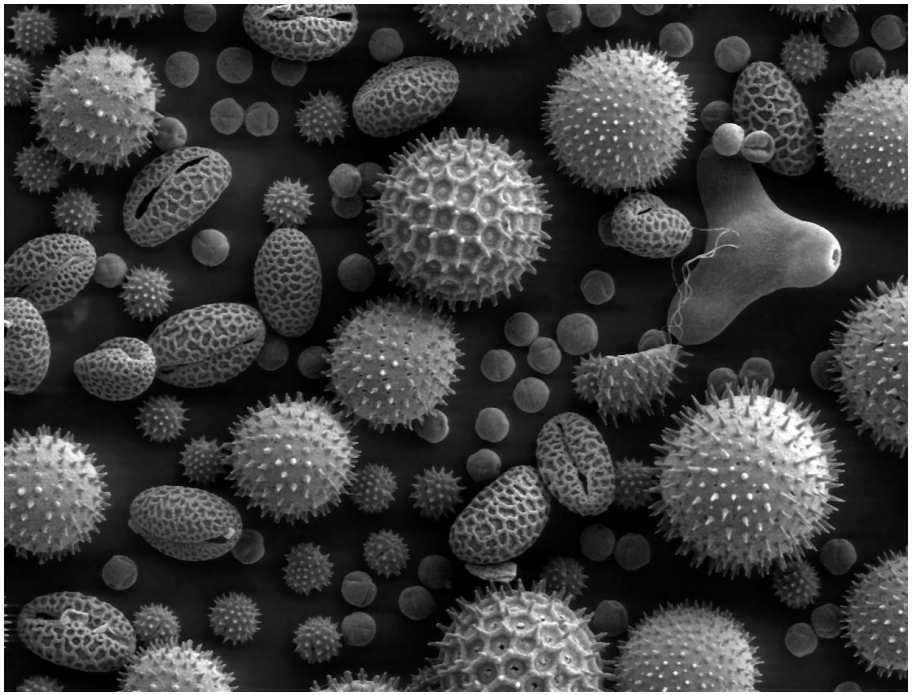
Electron Microscope	Light Microscope
➢ High resolution, higher magnification (up to 2 million times). ➢ View the 3D external shape of an object (SEM). ➢ 2 different types of electron microscopes: scanning electron microscopes (SEM) and transmission electron microscopes (TEM).	➢ Useful magnification (only up to 1000-2000 times). ➢ 3D external shape is not visible by optical microscopy. ➢ There are several modification of light microscopy (dissecting microscopes).

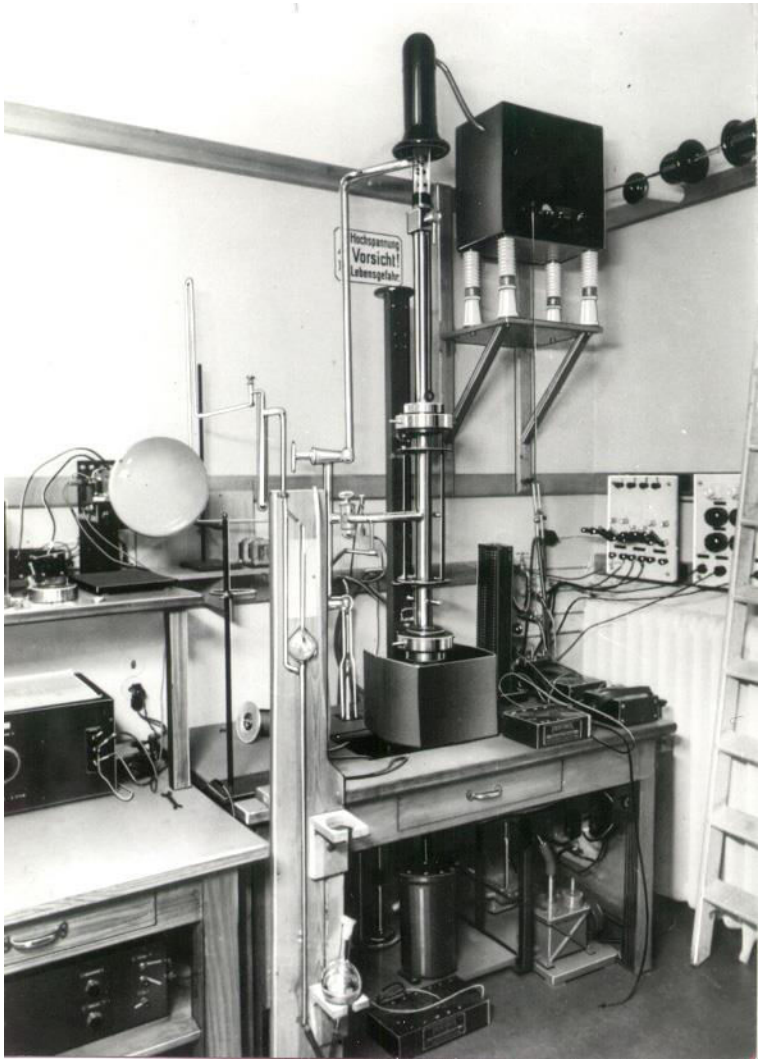
SEM (canning electron microscopy)



Signals from the sample





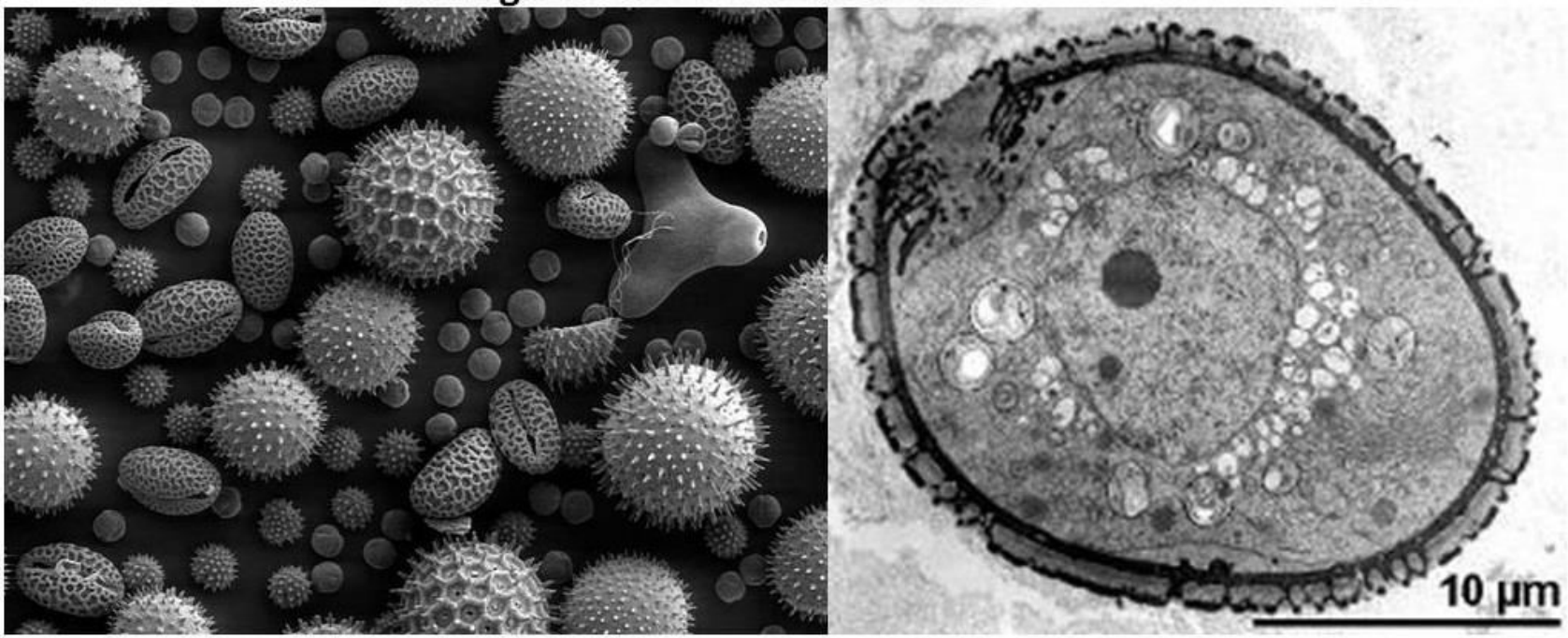

Hochspannung
Vorsicht!
Lebensgefahr

Transmission Electron Microscope (TEM)

- Electrons scatter when they pass through thin sections of a specimen.
- Transmitted electrons (those that do not scatter) are used to produce image.
- Denser regions in specimen, scatter more electrons and appear darker.

- Gun emits electrons
- Electric field accelerates
- Magnetic (and electric) field control path of electrons
- Electron wavelength @ 200KeV: $2x10^{-12}$ m
- Resolution normally achievable @ 200KeV: 2×10^{-10} m 2Å

Pollen grain under SEM and TEM



Scanning Electron Microscope (SEM) vs Transmission Electron Microscope(TEM)