PG01CBIT02: Bioanalytical Techniques and Instrumentation

Unit I

Visualization techniques: Principle of working and applications of bright field & dark field microscopy, phase contrast microscopy, fluorescence microscopy, confocal microscopy, scanning and transmission electron microscopy, scanning tunneling microscopy, atomic force microscopy. Principle and applications of cytophotometry and flow cytometry.

Unit II

Separation techniques: Basic principle and application of analytical and preparative centrifugation, settling time & velocity, types of rotor, sedimentation coefficient, relative centrifugal force (RCF) differential, density and ultracentrifugation. Principle and applications agarose and 2D gel electrophoresis. Capillary electrophoresis and its applications. Native-PAGE, SDS-PAGE Principle, methodology and applications of gel–filtration, ion–exchange and affinity chromatography; Thin layer and High-Performance Thin Layer Chromatography. Gas chromatography, High performance liquid chromatography and FPLC.

Unit III

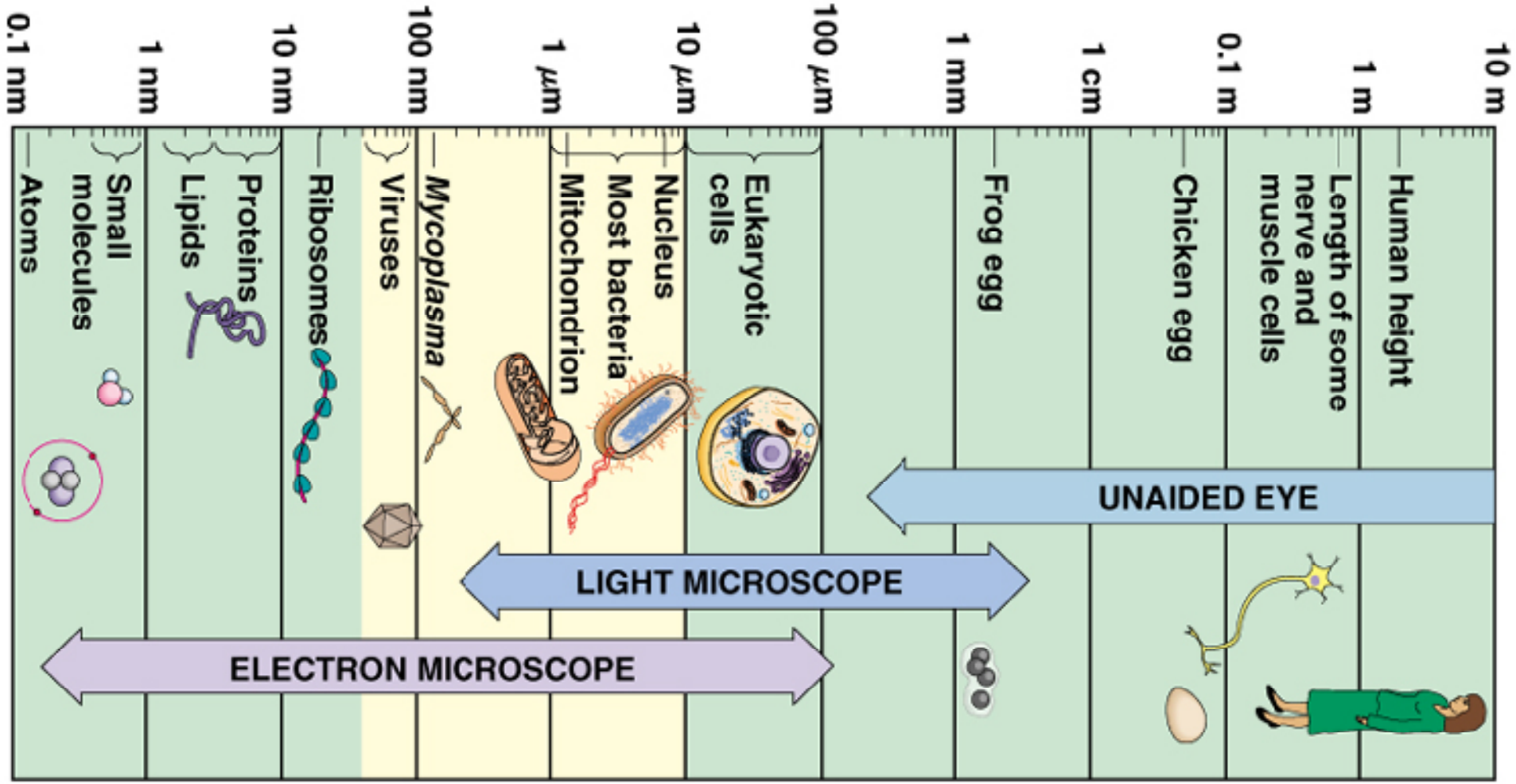
Spectropscopy Basic principle of electromagnetic radiation, instrumentation and applications of UV, Visible, IR (including FTIR and ATR), AAS, NMR, Mass, MALDI-TOF, fluorescence and CD spectroscopy.

Unit IV

Principle and applications of tracer technique in biology: Concept of radioactivity, rate of radioactive decay; units of radioactivity- uses of radioisotopes in life sciences and biotechnology; autoradiography; cerenkov radiation; radiation dosimetry; ionization and scintillation-based detection of radioactivity. Principle of biophysical methods used for analysis of biopolymer structure: X-ray diffraction.

Microscopy

Viewing System & Resolution



Human eye	100 µm (0.1 mm)
Light Microscope	1 µm (0.001 mm)
Electron Microscope	0.001 µm (10 Å)

INTRODUCTION

- **Microscope is the biggest tool for us to reveal the mystery and beauty of the unseen world.**

- **Antony van Leeuwenhoek (1632-1723)**

- **Robert Hook(1678)**

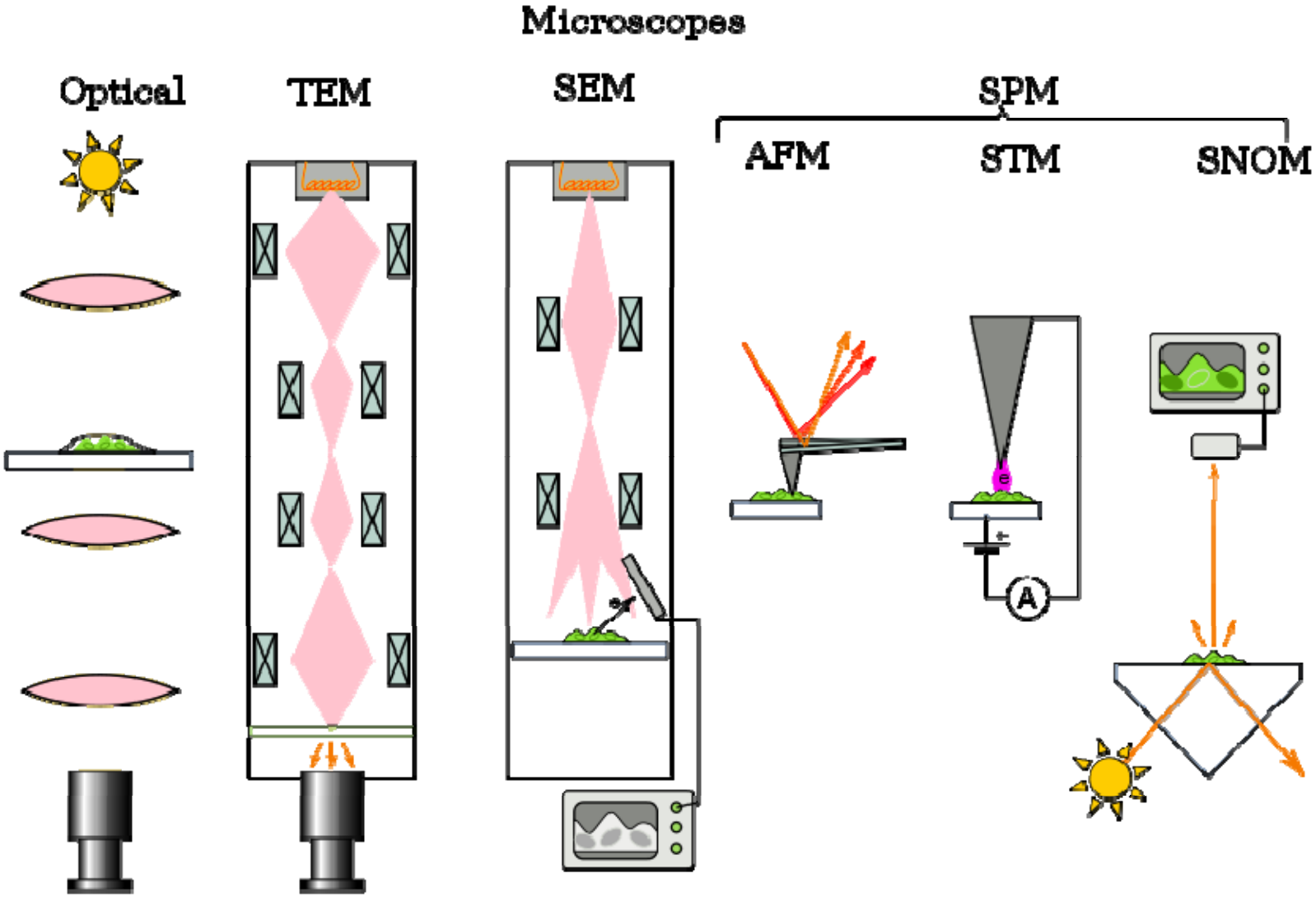
DEFINITION

- Microscope may be defined as an optical instrument consisting of a lens or combination of lenses for making an enlarged or magnified image of a minute object.
- Microscopes use lenses to bend and focus light rays to produce enlarged images of small objects.
- There are mainly two groups based on source of illumination.

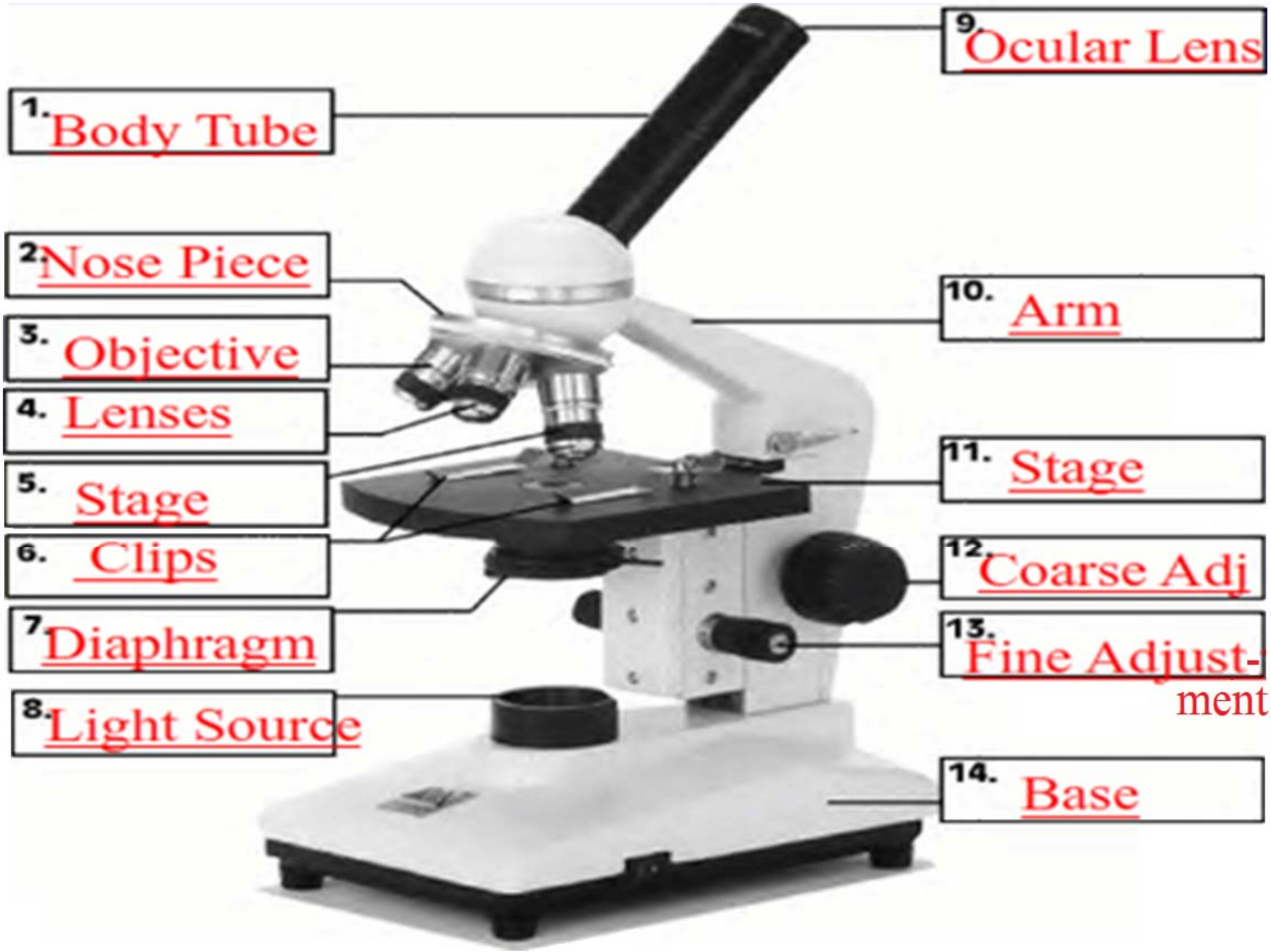
Microscopes

- Upright
- Inverted
- Köhler Illumination
- Dissecting (Stereoscopic)

"Microscope" was first coined by members of the first "Academia dei Lincei" a scientific society which included Galileo



Microscope: Micro = Gk. *"small"* + skopien = Gk. *"to look at"*


1. Body Tube
2. Nose Piece
3. Objective
4. Lenses
5. Stage
6. Clips
7. Diaphragm
8. Light Source
9. Ocular Lens
10. Arm
11. Stage
12. Coarse Adj
13. Fine Adjust-ment
14. Base

A microscope magnifies and resolve the image of an object that otherwise would be visible by naked eye.

The History

Many people experimented with making microscopes.
The first microscope was 6 feet long
The Greeks and Romans used “lenses” to magnify object over 1000 years ago.
Hans and **Zachrias Janssen** of Holland (1550’s) – 1st Compound Microscope.
Antony van **Leeuwenhock** and **Robert Hooke** made improvements by working on the lenses

Zacharias Jansen
1588 – 1631

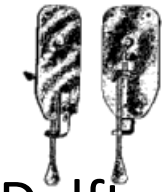
The “First”: Microscope

Antony van Leeuwenhock
1632 – 1723

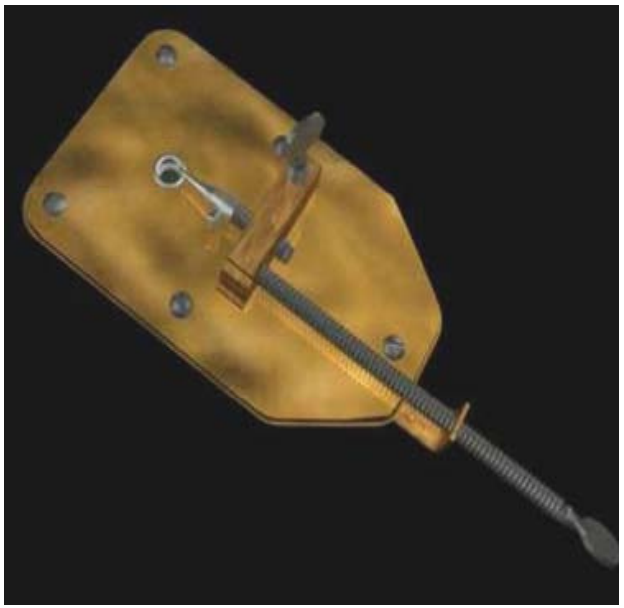
Robert Hooke
Microscope

Robert Hooke
1635 – 1703

Earliest Microscopes



- 1673 - **Antony van** Leeuwenhoek (1632-1723) Delft, Holland, worked as a draper (a fabric merchant); he is also known to have worked as a surveyor, a wine assayer, and
- Leeuwenhoek is incorrectly called "the inventor of the microscope" created a “simple” microscope that could magnify to about 275x, and published drawings of microorganisms in 1683 as a minor city official.
- In 1673, Leeuwenhoek began writing letters to the Royal Society of London - published in Philosophical Transactions of the Royal Society
- In 1680 he was elected a full member of the Royal Society, joining Robert Hooke, Henry Oldenburg, Robert Boyle, Christopher Wren



Magnification

Ratio of enlargement between the specimen and its image

Microscope brings small object closer to the observer by increasing the magnification of sample

To calculate magnification we multiply the power of each lens through which the light from the specimen passes.

To determine magnification

We just multiply the ocular lens by the objective lens

Ocular 10X objective 40 X = 10 x 40 = 400

So the object is 400 times “larger”

Resolution

Resolution is the ability to distinguish details in image.

It is limited by **Wavelength**, **Numerical Aperture (NA)**, **Optical defects** (Chromatic and Spherical aberration), **Image contrast**.

Ability of a lens to separate or distinguish small objects that are close together

Wavelength of light used is major factor in resolution

Shorter wavelength – greater resolution

Contrast

The difference between the brightness of various details in the object and the difference as compared with the background.

Sharpness

Sharpness implies distinct, realistic image detail and contrast. Sharp image require sufficient magnification, minimal effort to imagine and interpret the image.

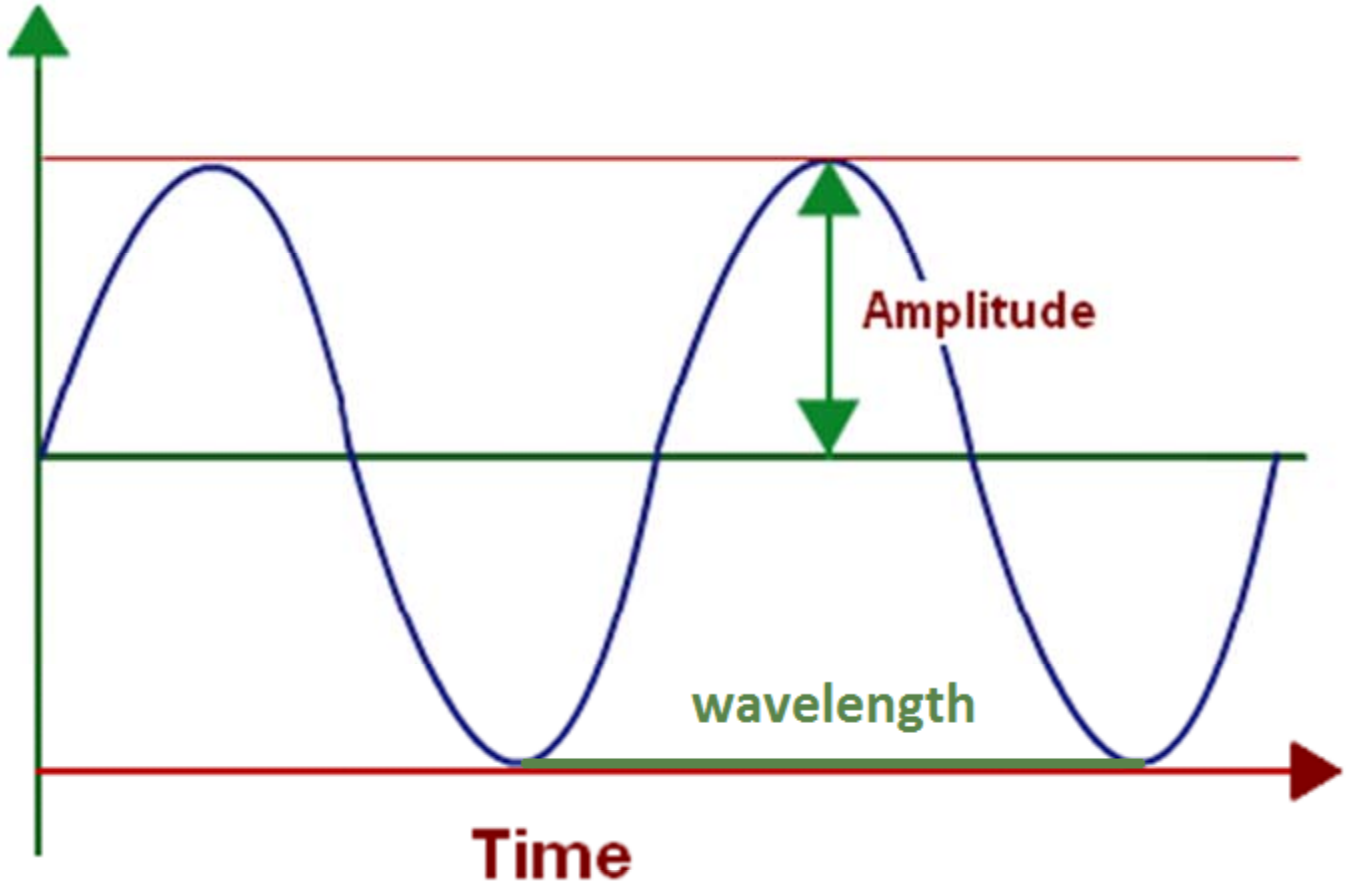
Objective lens have their magnification written on them

The Properties of Microscope Objective

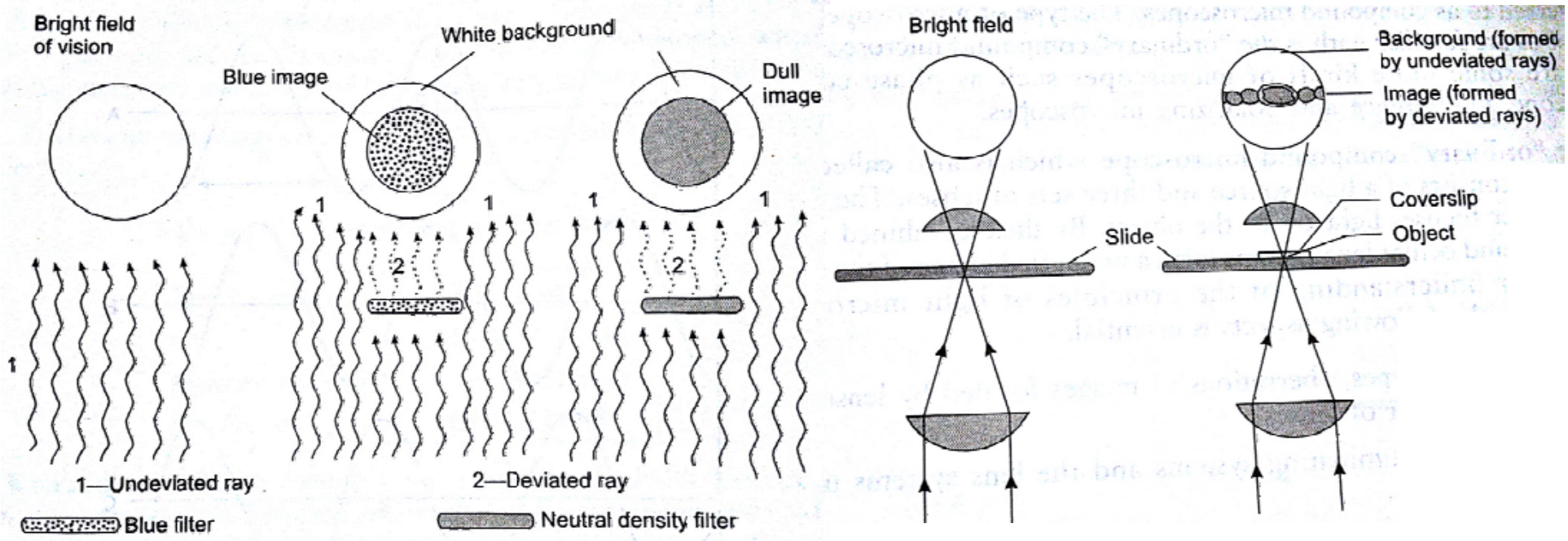
Property	Objective			
	Scanning	Low Power	High Power	Oil Immersion
Magnification	4x	10x	40-45x	90-100x
Numerical Aperture (NA)	0.10	0.25	0.55-0.65	1.25-1.4
Focal length	40 mm	16 mm	4 mm	1.8-2.0 mm
Working Distance	7-20 mm	4-8 mm	0.5-0.7 mm	0.1 mm
Approx Resolving Power with light of 450 nm	23 µm	0.9 µm	0.35 µm	0.18 µm

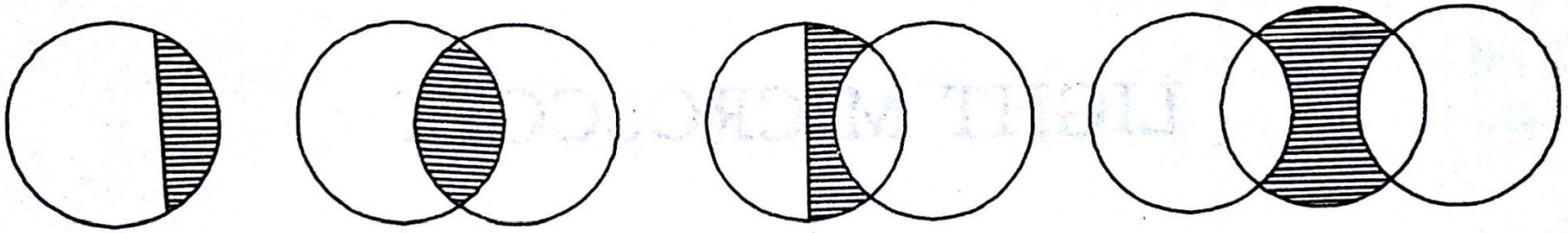
Working distance

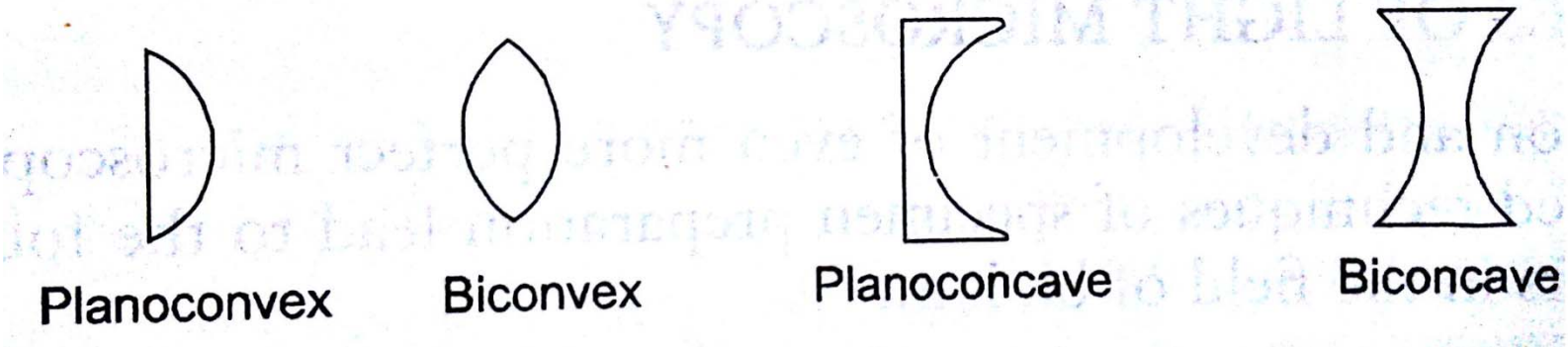
Distance between the front surface of lens and surface of cover glass or specimen

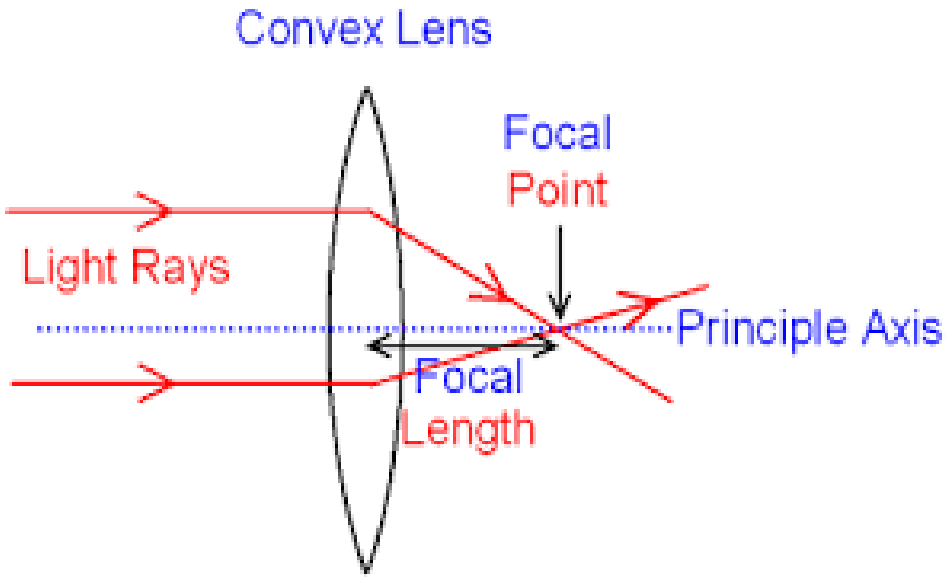


Light is a series of energy particles travelling at a speed of 186000 miles Sec^{-1} in a wavy snake like path

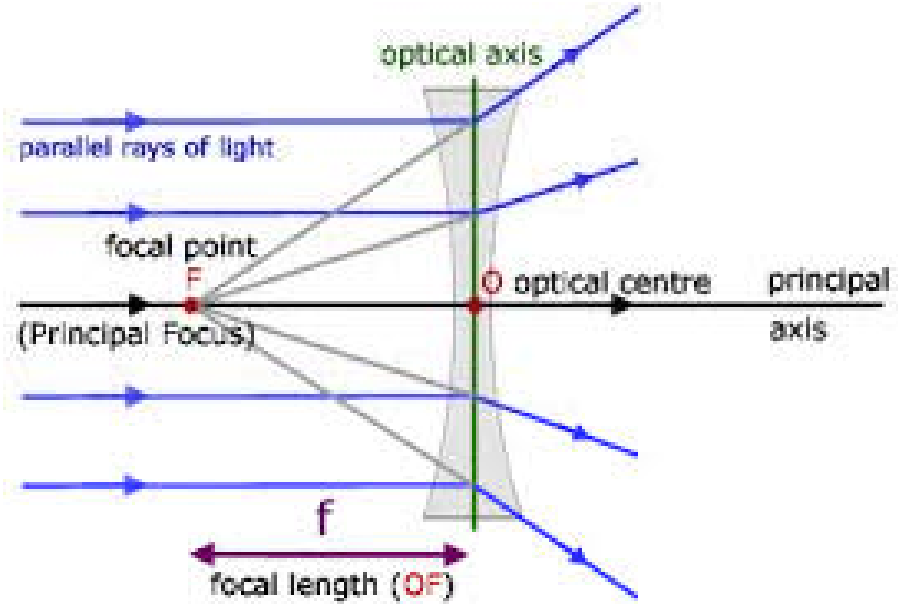

Bright field of vision
White background
Blue image
Dull image
1
2
1—Undeviated ray
2—Deviated ray
Blue filter
Neutral density filter
Bright field
Slide
Background (formed by undeviated rays)
Image (formed by deviated rays)
Coverslip
Object




Planoconvex
Biconvex
Planoconcave
Biconcave



Converging action of a convex lens



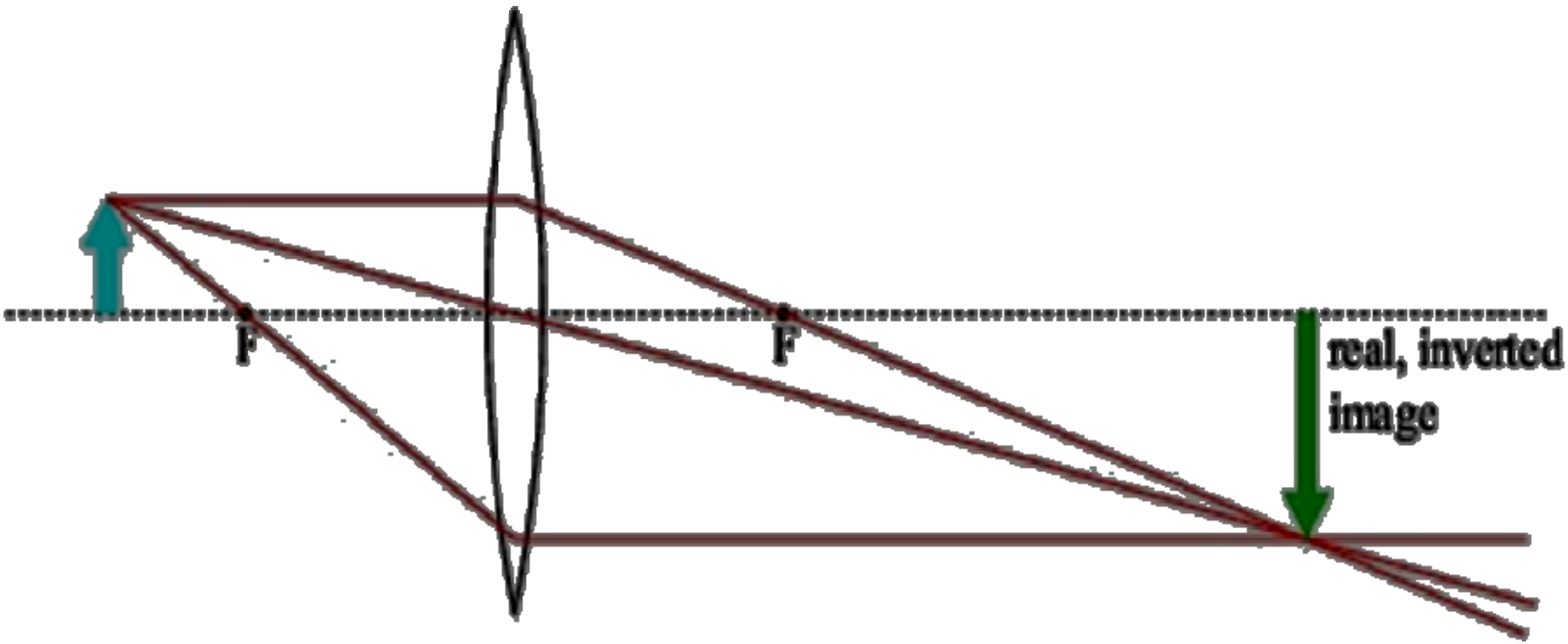
Diverging action of a concave lens

Position of the object	Position of the image	Relative size of the image	Nature of the image
At infinity	At focus F_2	Highly diminished, point-sized	Real and inverted
Beyond $2F_1$	Between F_2 and $2F_2$	Diminished	Real and inverted
At $2F_1$	At $2F_2$	Same size	Real and inverted
Between F_1 and $2F_1$	Beyond $2F_2$	Enlarged	Real and inverted
At focus F_1	At infinity	Infinitely large or highly enlarged	Real and inverted
Between focus F_1 and optical centre O	On the same side of the lens as the object	Enlarged	Virtual and erect

Different Varieties of Glasses

Type of glass	Constituents	Special use
Soft glass	$Na_2CO_3.CaCO_3.SiO_2$	Ordinary glass for window panes, test tubes, bottles, etc.
Hard glass	K_2CO_3, $CaCO_3$,SiO_2	For combustion tubes and chemical glassware
High refractive index glass (Flint glass)	Lead oxide, K_2CO_3	For making lenses cut glasses
Pyrex glass	Na_2CO_3, Al_2O_3, B_2O_3 or borax, sand	For high quality glass apparatus cooking utensils
Crook's glass	K_2CO_2, $PbCO_3$, CeO_2, sand	Absorbs ultra violet rays, for making lenses
Jena glass	Zinc and Barium Boro silicates	It is resistant to heat shock and common reagent. It is used for making good quality of glass wares

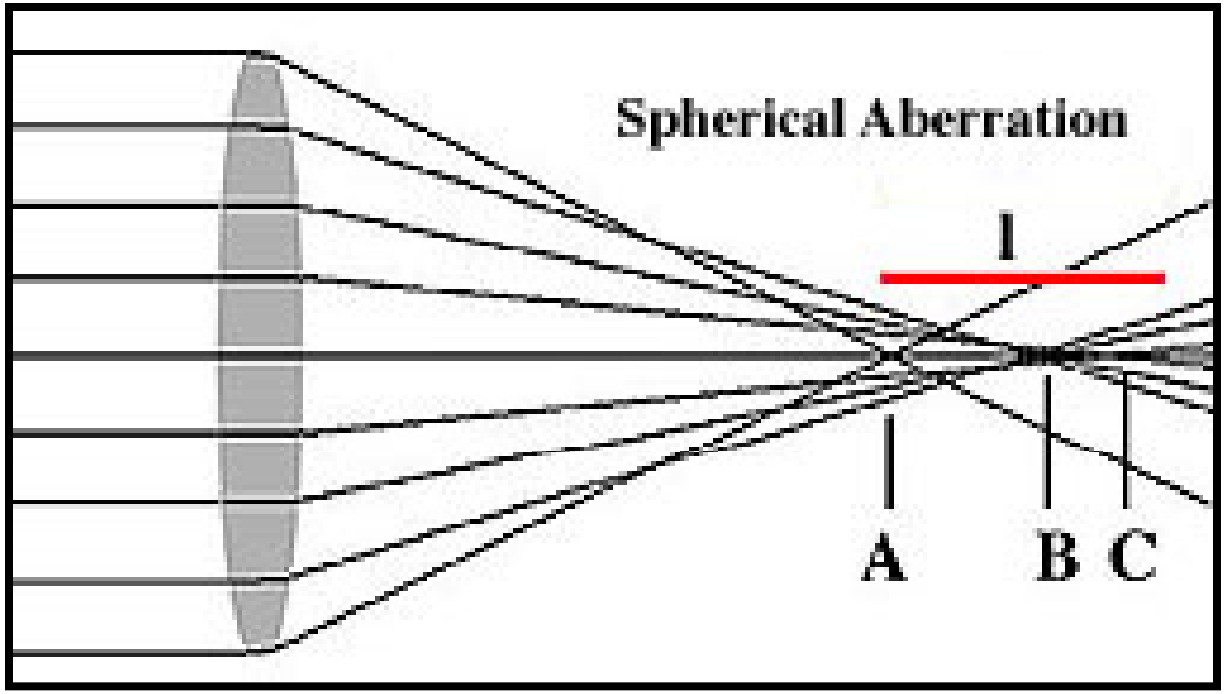
Converging (positive) lens: bends rays **toward** the axis. It has a **positive focal length**. Forms a **real inverted** image of an object placed to the left of the first focal point and an **erect virtual** image of an object placed between the first focal point and the lens.



Abberations

- Spherical aberration
  - Most severe
  - Immersion fluid
- Chromatic aberration
- Field curvature
- Astigmatism, coma

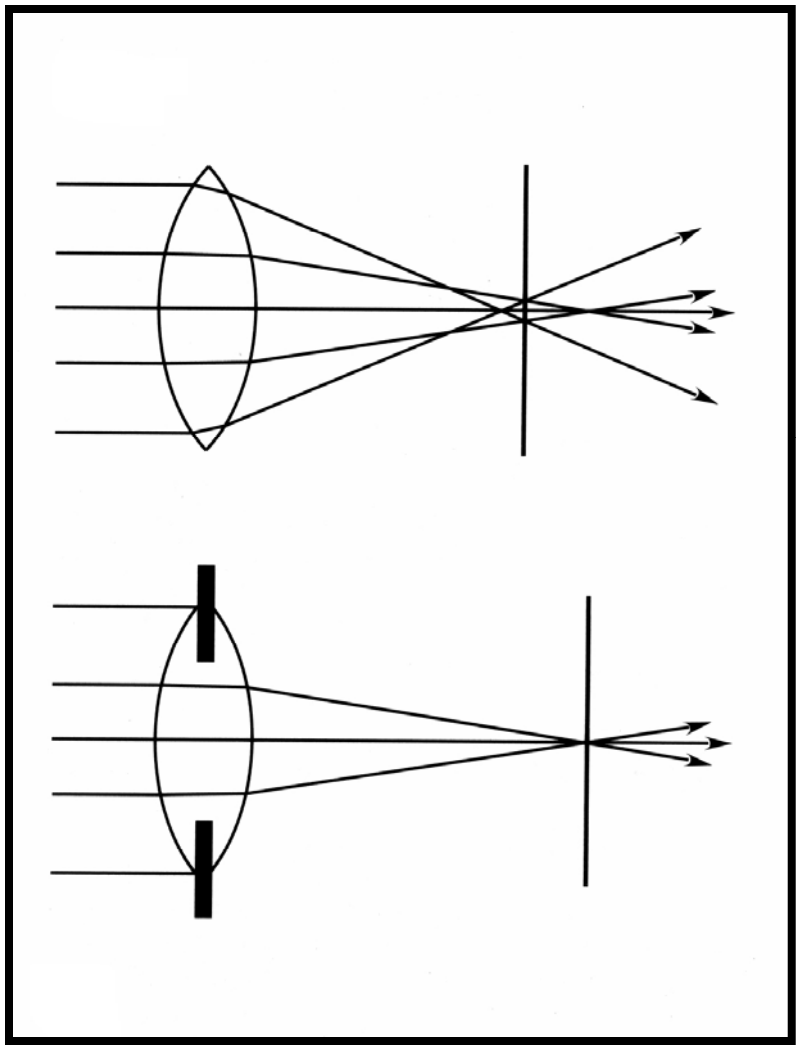
Lens Defects



It is due to the **refraction** of light.

The wavelengths enter and leave the lens field at different angles results in a defect known as spherical aberration. The result is that wavelengths are brought to different focal points .

Spherical aberration depends on the distance between the centre of the lens and its periphery.

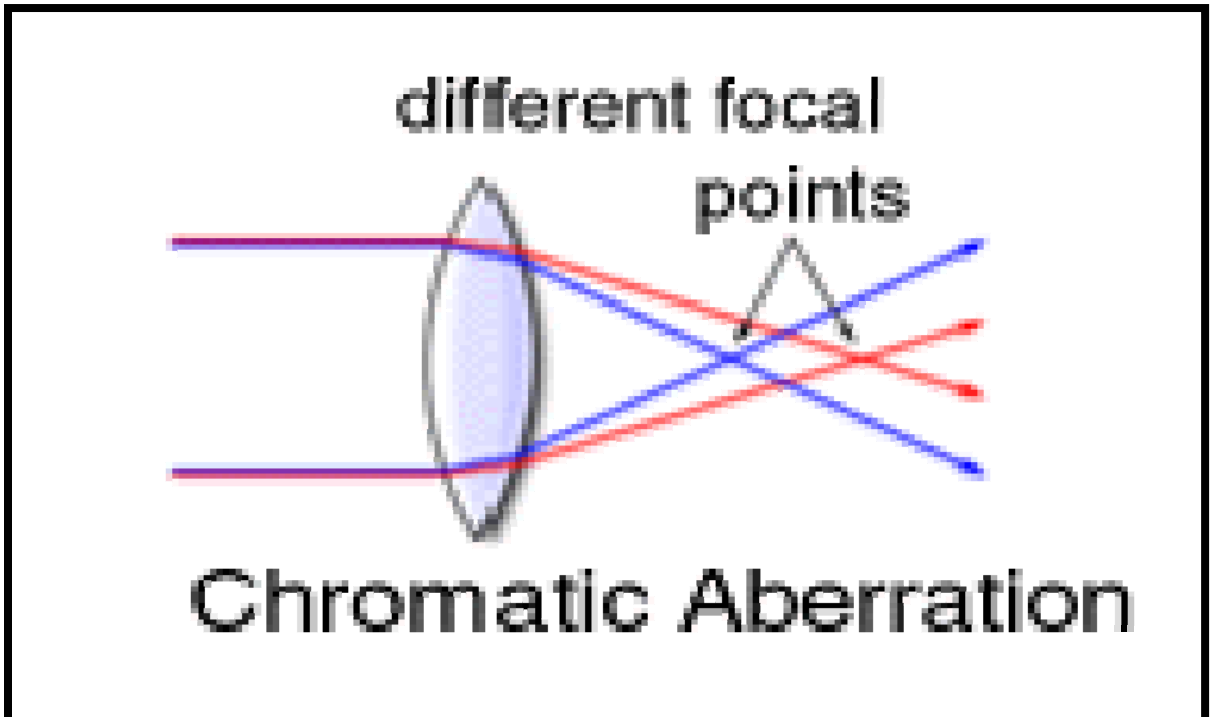


Spherical aberrations are worst at the periphery of a lens so a small opening aperture that cuts off the most offensive part of the lens is the easiest way to reduce the effects of spherical aberration but throws away a lot of the available illumination (i.e. pinhole camera)

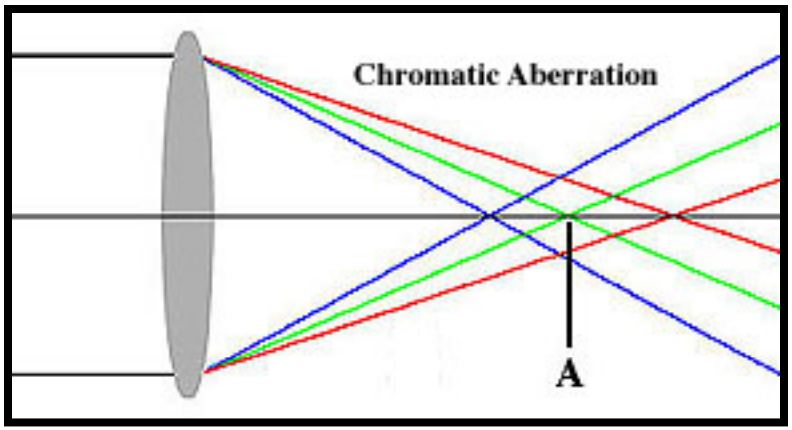
Lens Defects

Light rays enering a lens undergo dispersion at the same time when they undergo refrection.

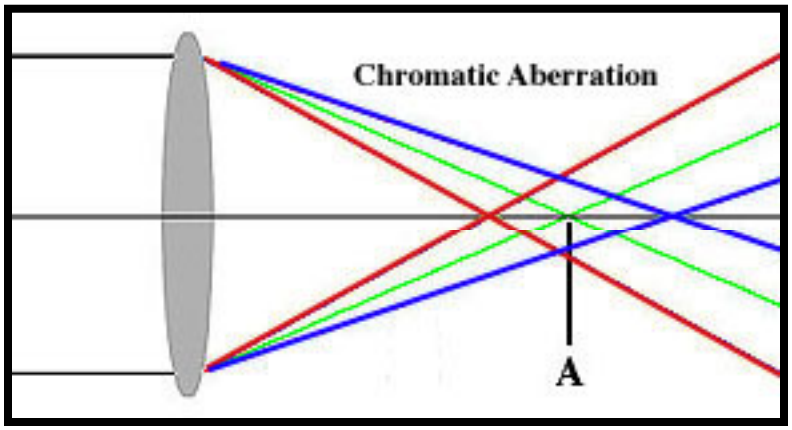
Since the focal length f of a lens is dependent on the strength of the lens, if follows that different wavelengths will be focused to different positions. Chromatic aberration of a lens is seen as fringes around the image due to a "zone" of focus.



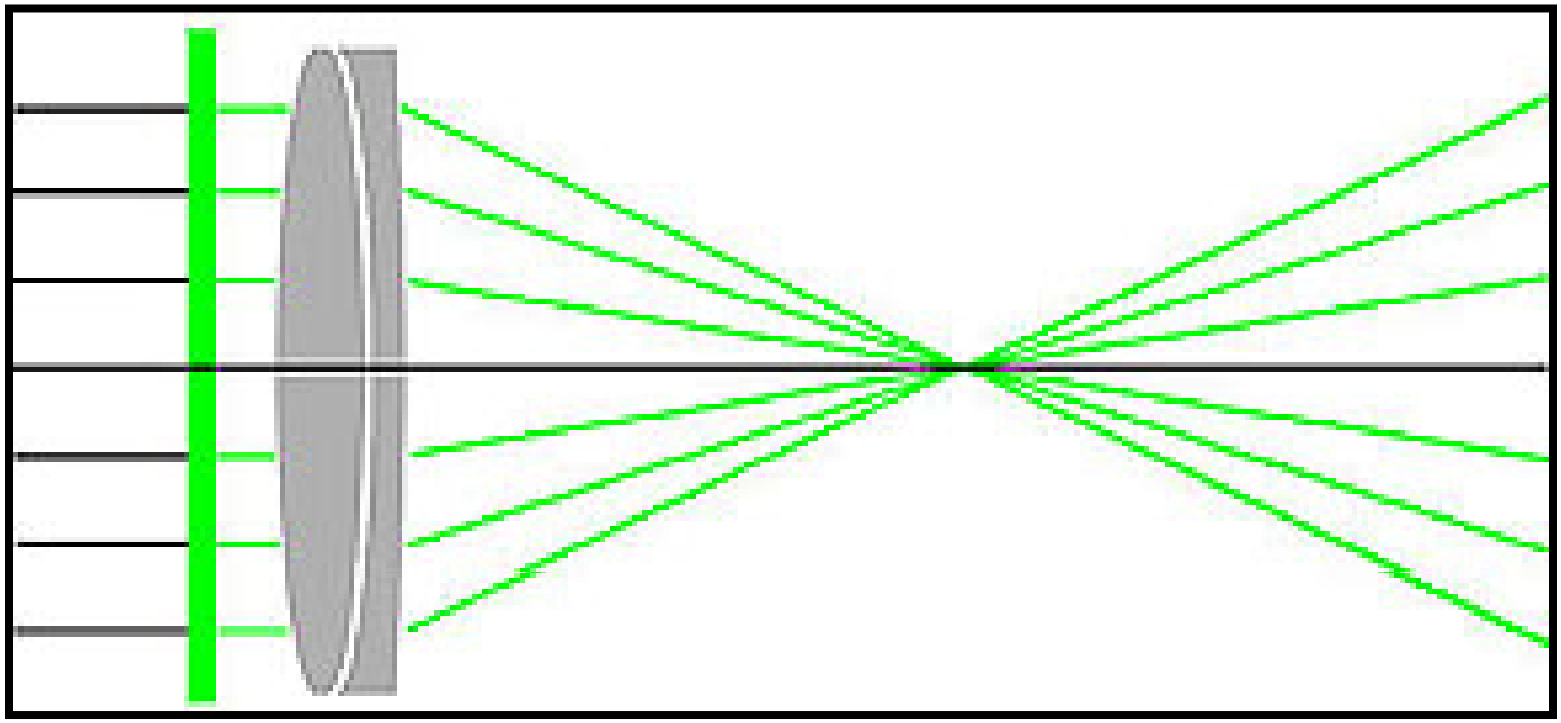
Lens Defects



In light optics wavelengths of higher energy (blue) are bent more strongly and have a shorter focal length



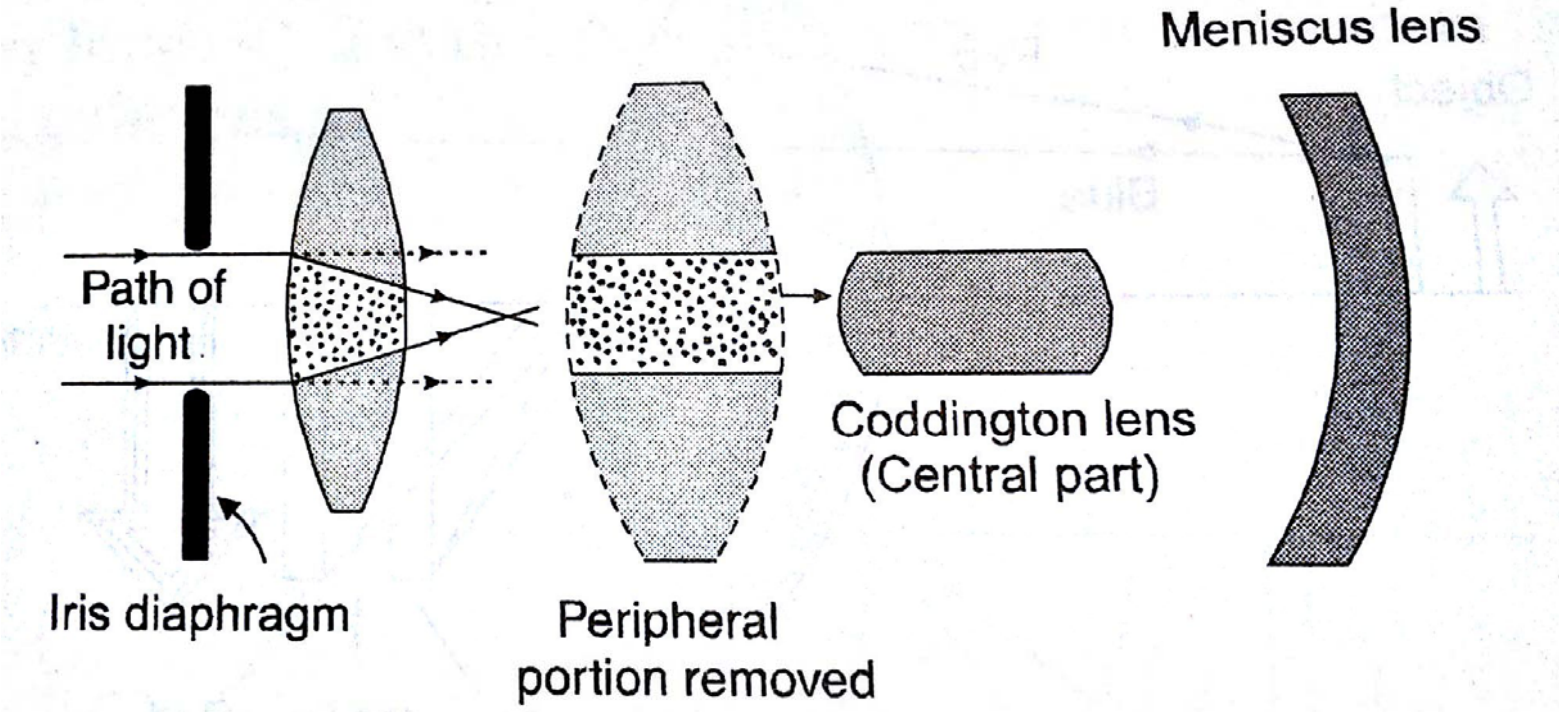
In the electron microscope the exact opposite is true in that higher energy wavelengths are less effected and have a longer focal length



The simplest way to correct for chromatic aberration is to use illumination of a single wavelength!

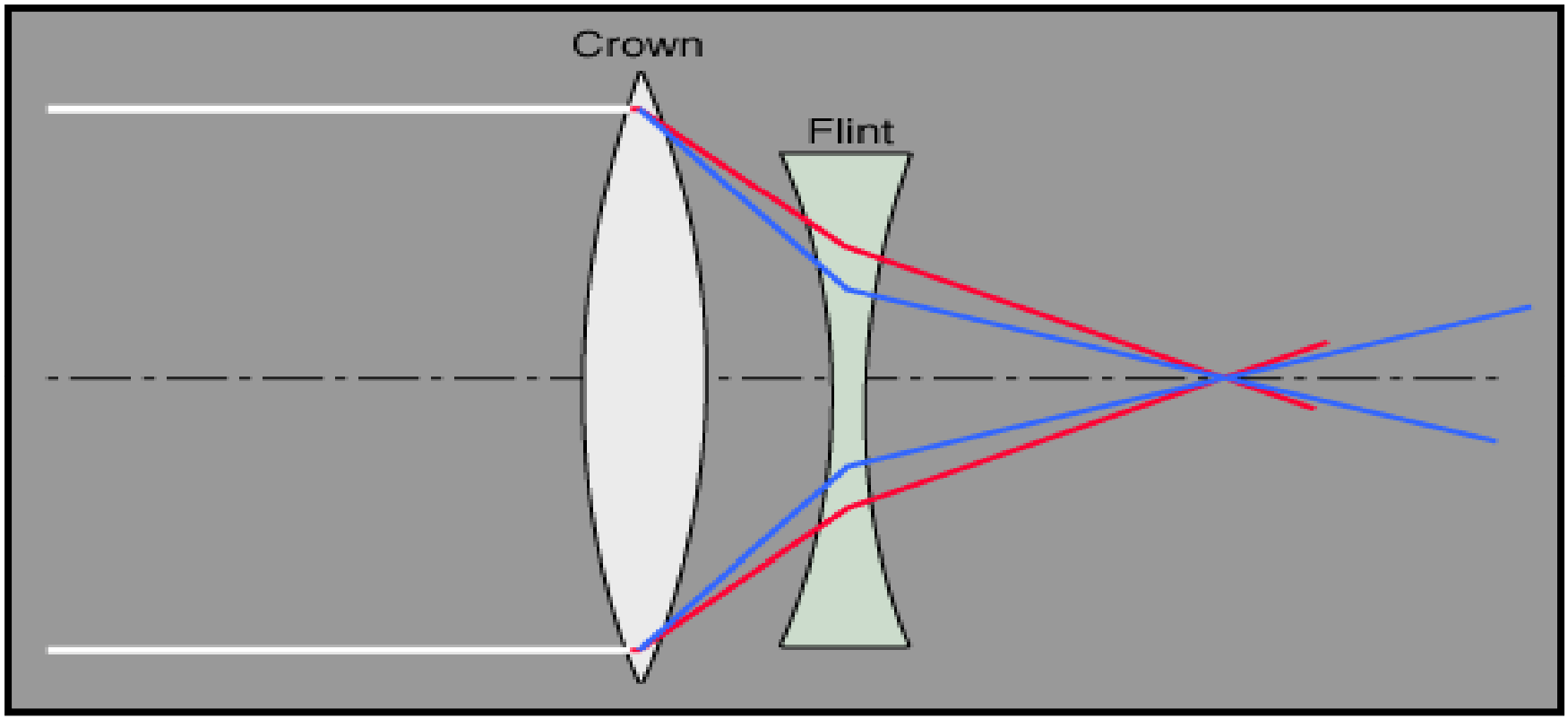
Such illumination is called monochromatic .

Lens Defects

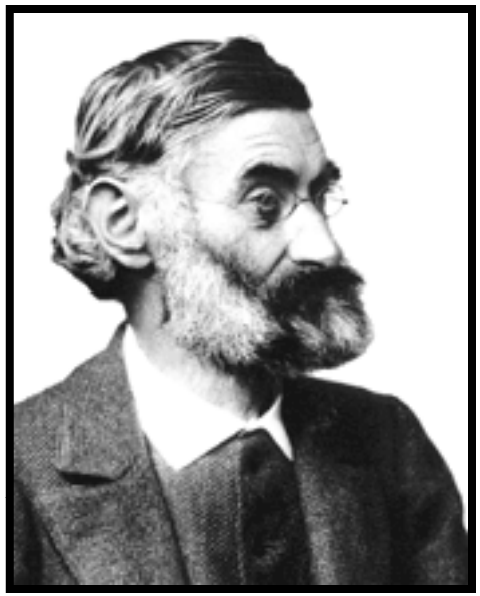


- Place the diaphragm in front of the lens so that only the centre of the lens is used.
- Use of Coddington lens. It is obtained by grinding a lens on each end of a cylindrical piece of glass (i.e. the central part of a biconvex lens is which the peripheral parts are removed.
- By using miniscus lens (i.e. lens with one surface is convex and other is concave

Lens Defects



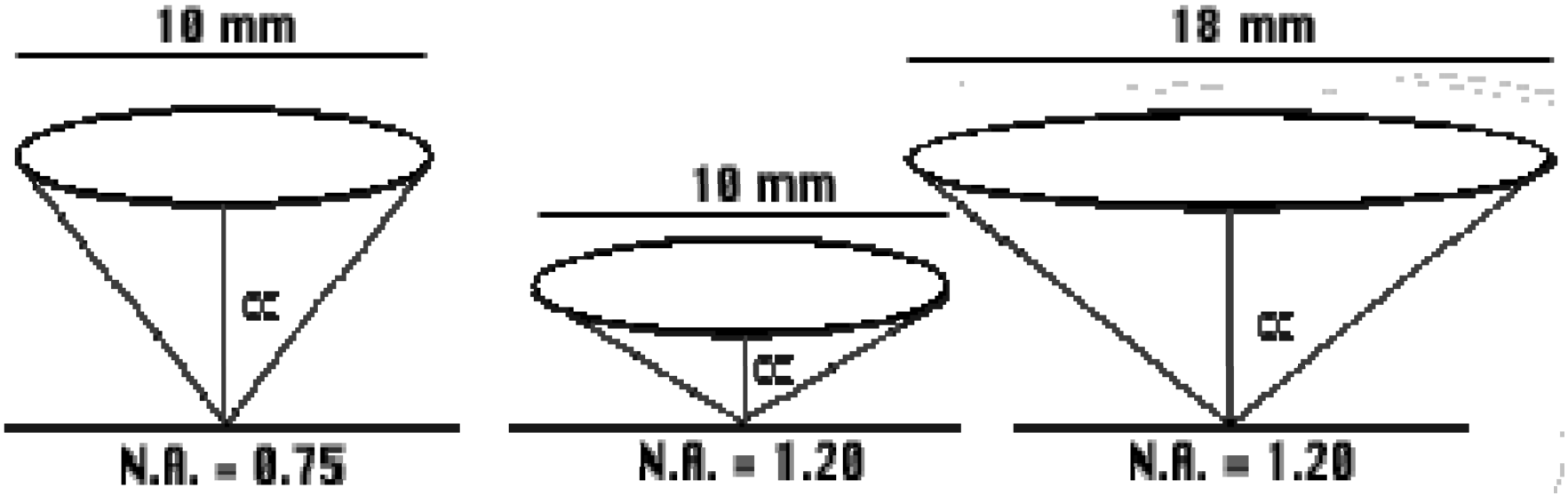
In light optics **chromatic aberration** can be corrected by combining a converging lens of one O.D. with a diverging lens of a different O.D. This is known as a "**doublet**" lens



Ernst Abbe
1840 - 1905

$$R.P. = \frac{0.61\,\lambda}{N.A.}$$

λ = wavelength of illumination
N.A. = n (sine α)
n = index of refraction
α = half angle of illumination



$$\text{Resolving Power } (\delta) = \frac{\lambda}{2n.\sin\alpha}$$

(Where, R = the separation distance or resolution, λ = illumination wavelength, n = referective index, α = one half of the objective angular aperture

$$\text{Resolving Power } (\delta) = \frac{0.5\lambda}{n\sin\alpha}$$

for air $n = 1$ and when water or oil is used then resolving distance is reduced to 1/n

$$\text{Resolving Power } (\delta) = h'\frac{0.61\lambda}{n'\sin u'}$$

$$\text{Resolving Power } (\delta) = \frac{0.61\lambda}{\text{NA}} \qquad (\text{Where } n\sin\alpha = \text{NA})$$

$$\text{Resolving Power } (\delta) = \frac{1.22\lambda}{2(\text{NA}_{(\text{obj})} + \text{NA}_{(\text{Cond})}}$$

In light microscopy the N.A. of a lens and therefore resolution can be increased by:

a) Increasing the half angle of illumination,

b) Increasing the refractive index of the lens by using Crown glass and

c) Decreasing the wavelength (λ) of illumination.

d) Distance resolved will decrease with increase in NA & decrease in λ

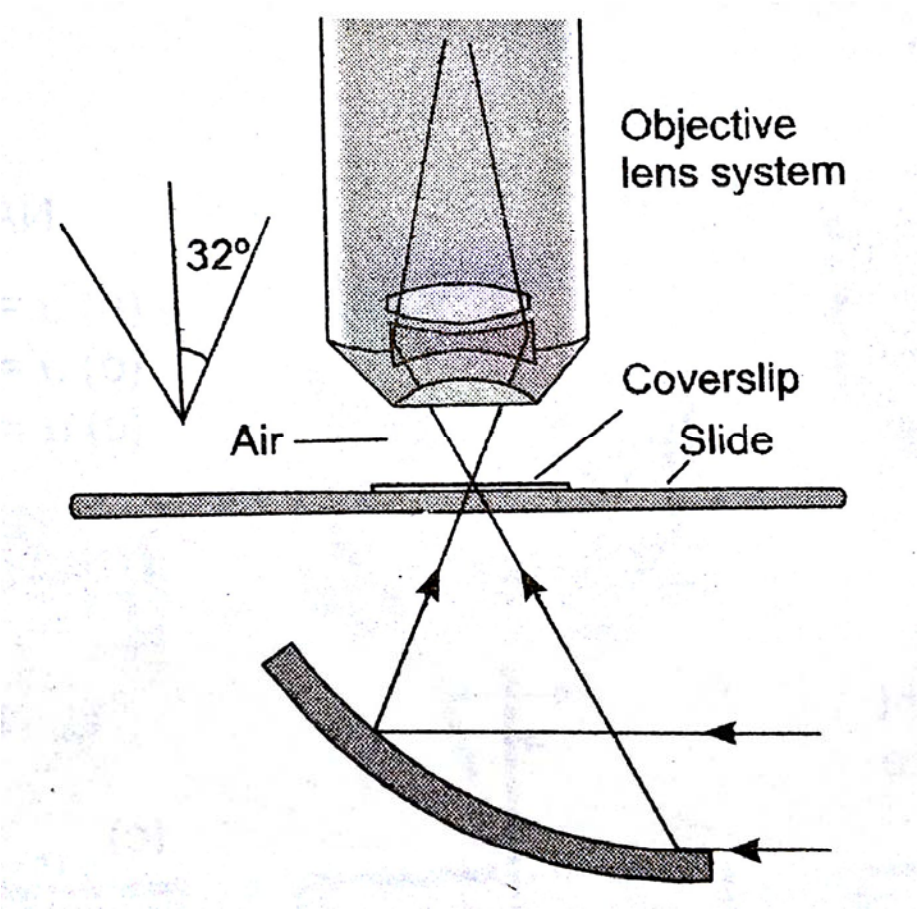
Factor determining the resolution in optical light microscope

1. Wavelength
2. Angular aperture of the objective lens (AA_{obj})
3. Correct alignment of the microscope optimal system
4. The refraction index in the object space between the objective front lens and the specimen
5. Objective numerical aperture (NA_{obj})
6. Types of specimen
7. Coherence of illumination
8. Degree of aberration correction
9. The contrast enhancing methodology applied in the optical system

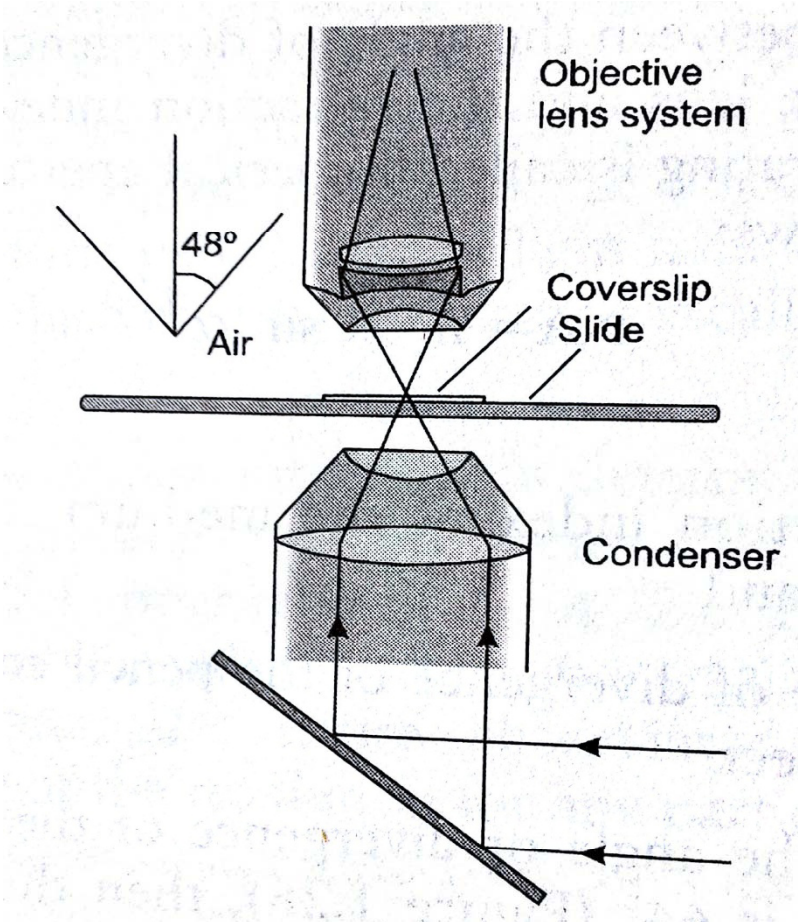
Method for enhancing the resolving power of a lens system

1. By using short wavelength
2. By using immersion oil in the light path
3. By using inclined light

Numerical Aperture (NA)



NA without the use of condenser



NA with substage condenser

If n=1 and $\alpha = 32^0$

Then N.A. = n (sine α) = 1 (sin32)

= 1 (0.62) = 0.62

If n=1 and $\alpha = 48^0$

Then N.A. = n (sine α) = 1 (sin48)

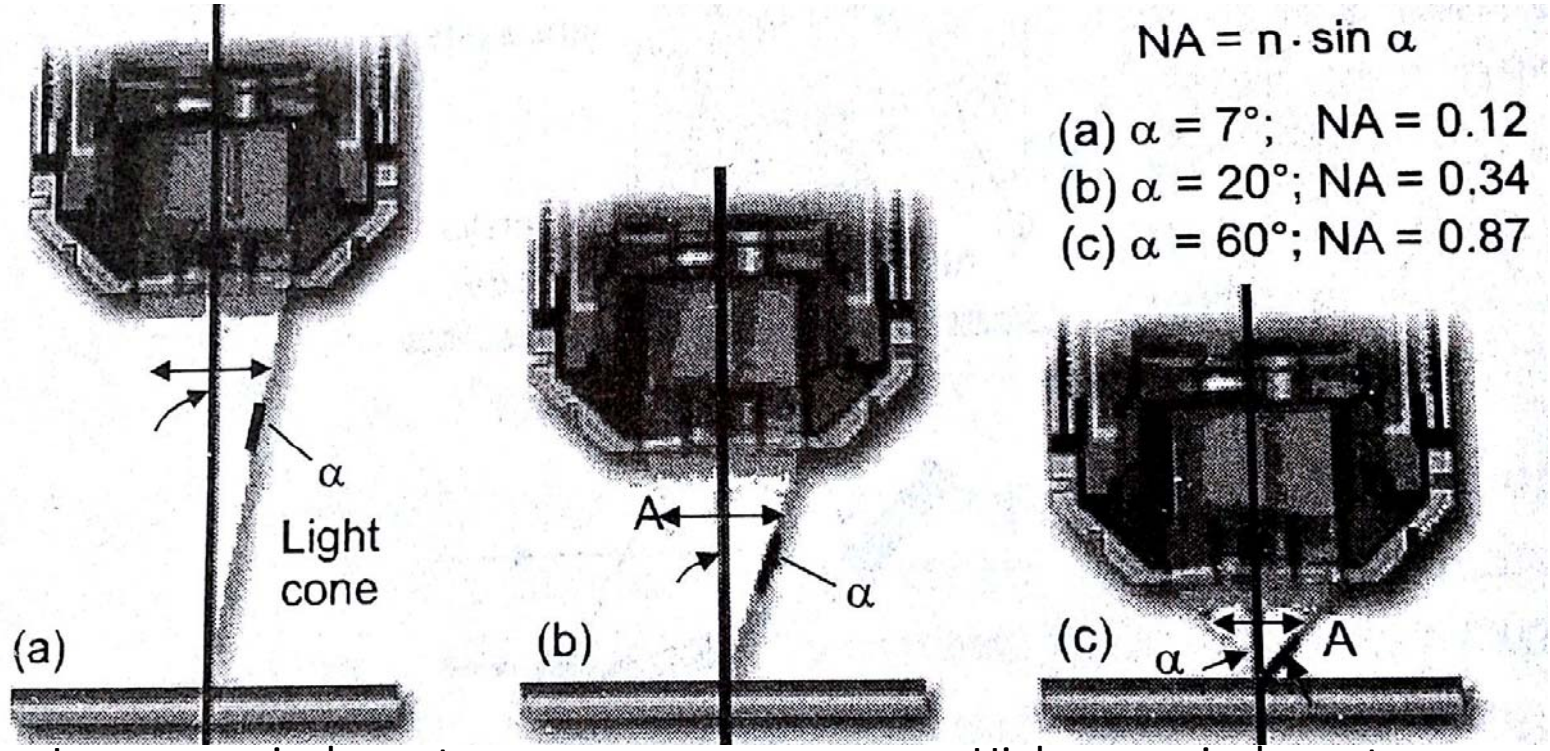
= 1 (0.80) = 0.80

Object Resolution

1. 40 x 1.3 N.A. objective at 530 nm light
2. 40 x 0.65 N.A. objective at 530 nm light

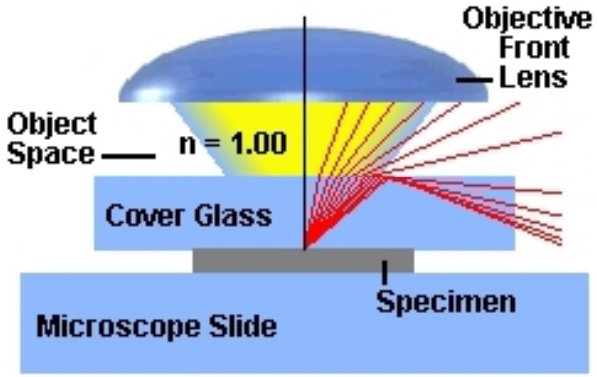
1. $\frac{\lambda}{2 \text{ x NA}} = \frac{0.00053}{2 \text{ x } 1.3} = 0.20 \text{ µm}$

2. $\frac{\lambda}{2 \text{ x NA}} = \frac{0.00053}{2 \text{ x } 1.3} = 0.20 \text{ µm}$

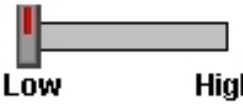


Low numerical aperture
Low value for a low resolution

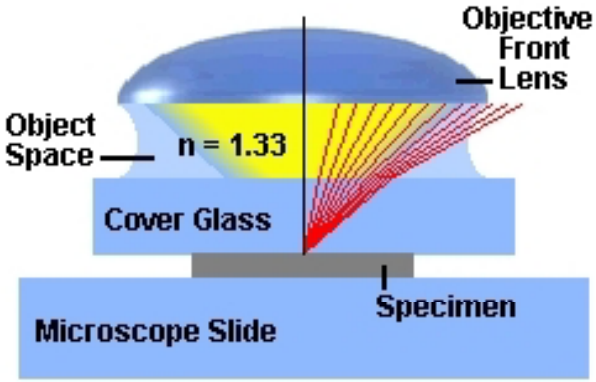
High numerical aperture
High value for a low resolution



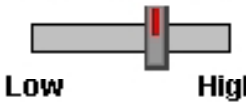
n = Refractive Index



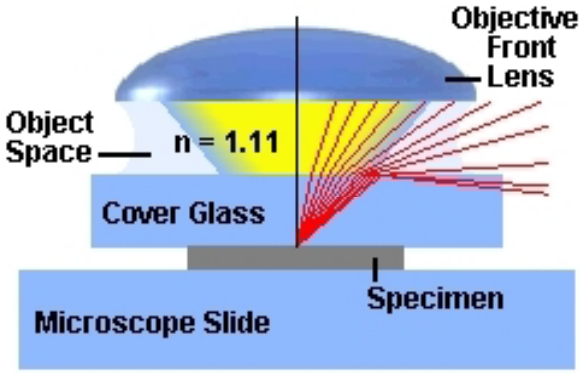
Numerical Aperture (NA) = n sin(θ)
NA = 1.00 sin (65°)
0.90 = 1.00 sin (65°)
θ = Angular Aperture = 65°



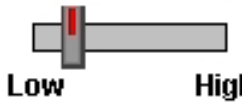
n = Refractive Index



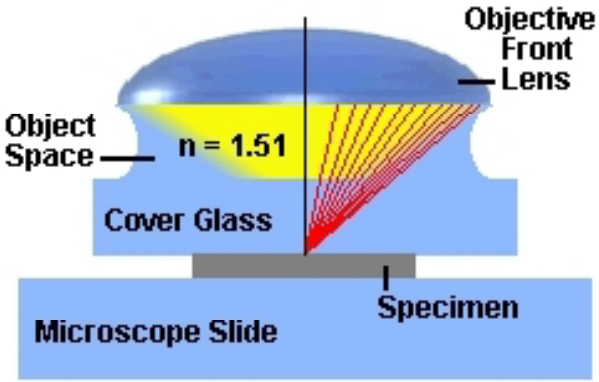
Numerical Aperture (NA) = n sin(θ)
NA = 1.33 sin (65°)
1.20 = 1.33 sin (65°)
θ = Angular Aperture = 65°



n – Refractive Index



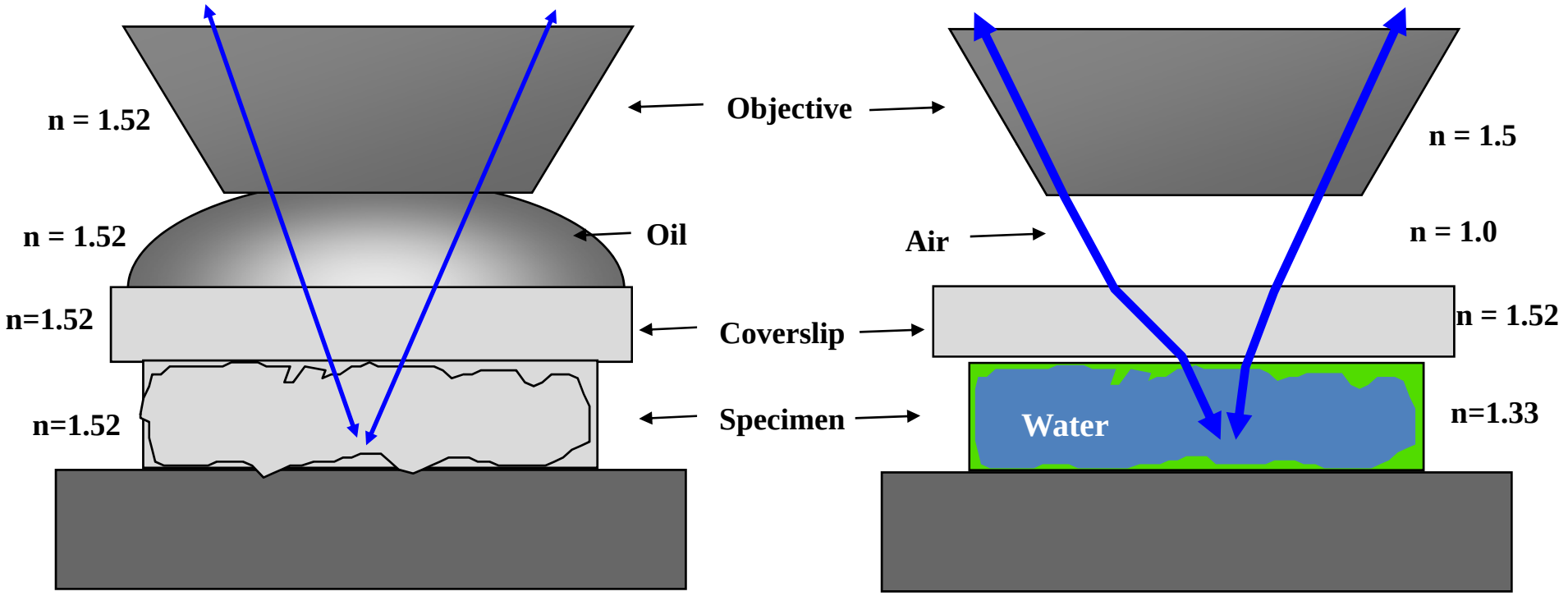
Numerical Aperture (NA) = n sin(θ)
NA = 1.11 sin (65°)
1.00 = 1.11 sin (65°)
θ = Angular Aperture = 65°

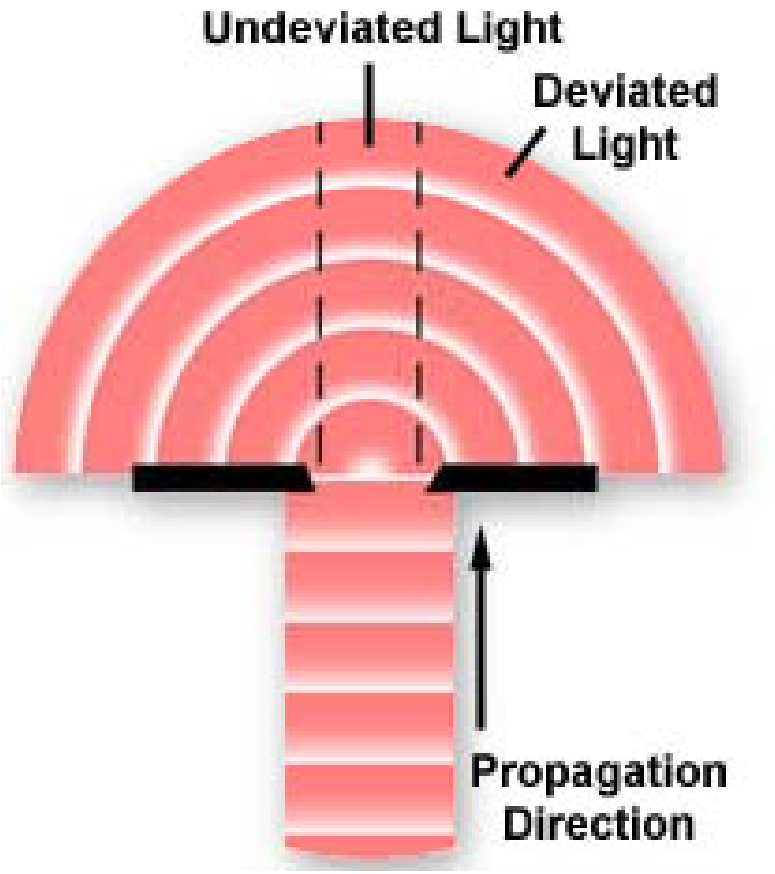


n = Refractive Index

Numerical Aperture (NA) = n sin(θ)
NA = 1.51 sin (65°)
1.38 = 1.51 sin (65°)
θ = Angular Aperture = 65°

Refractive Index



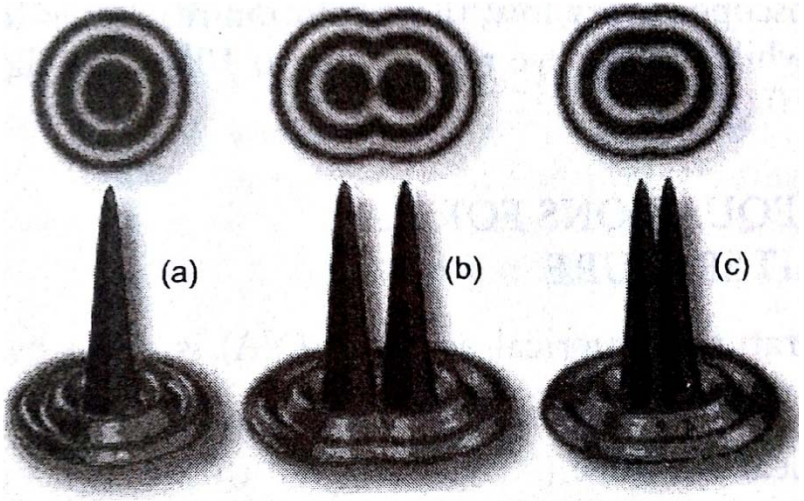


Light that passes both around and through the specimen undisturbed in its path is called **direct light** or **undeviated light**. The background light passing around the specimen is also undeviated light.

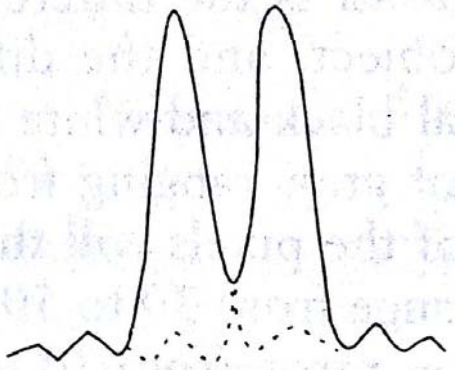
Some of the light passing through the specimen is deviated when it encounters parts of the specimen. Such deviated light is rendered one-half wavelength or 180^0 out of phase with the direct light that has passed through undeviated.

The one-half wavelength out of phase caused by the specimen itself enables this light to cause destructive interference with the direct light when both arrive at the intermediate image plane at the diaphragm of the eyepiece.

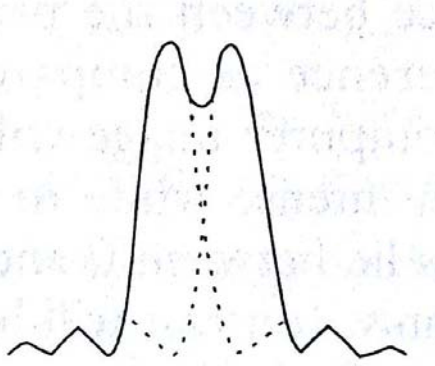
Airy disc and upper limit or resolution



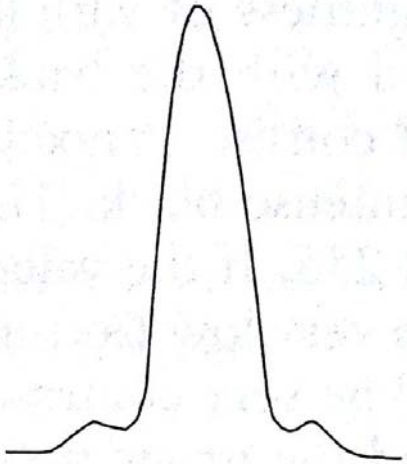
Intensity distributions



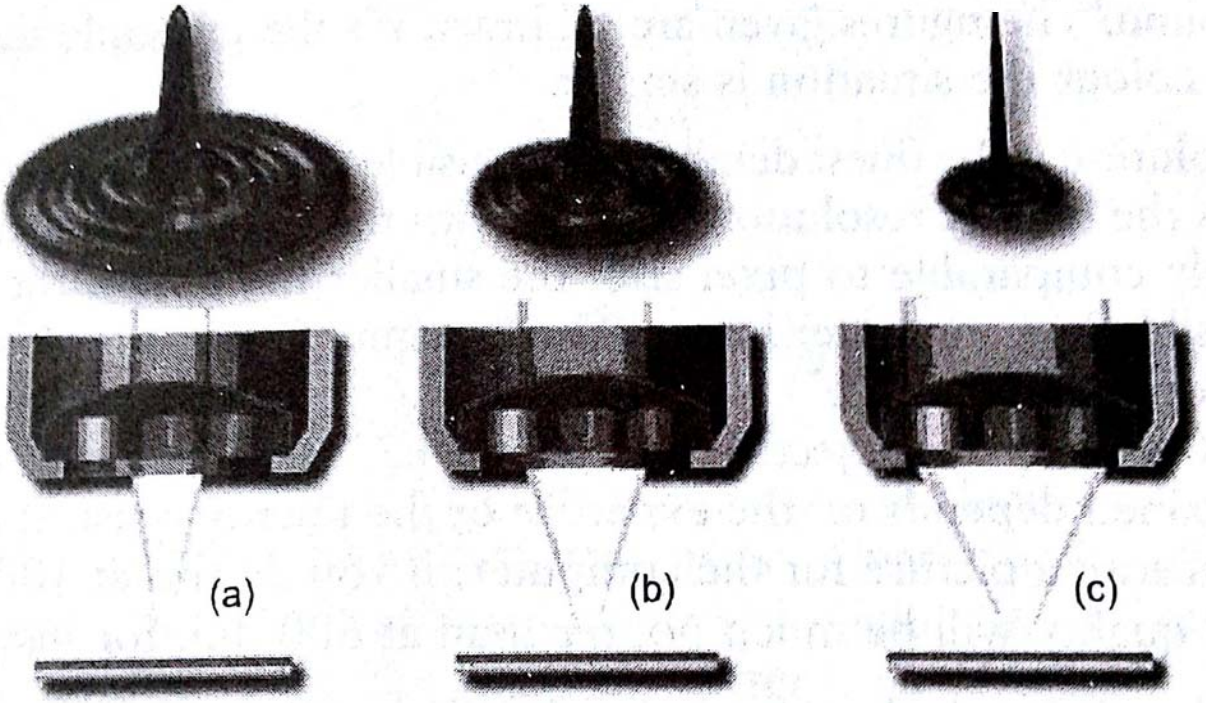
Resolvable



Rayleigh criterion

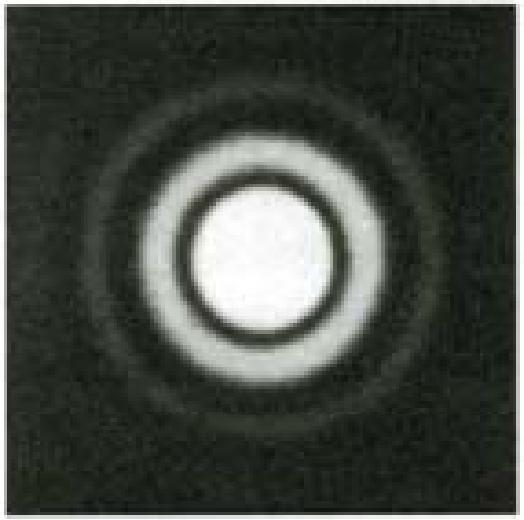


Unresolvable



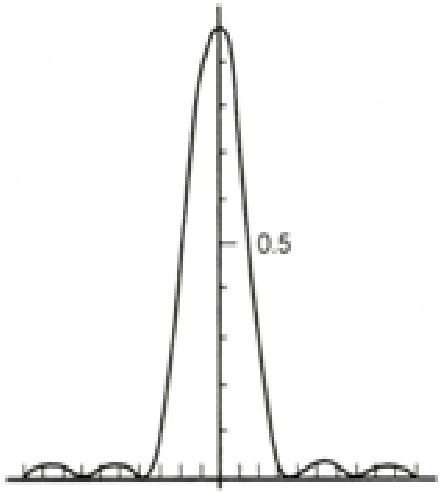
Airy disc

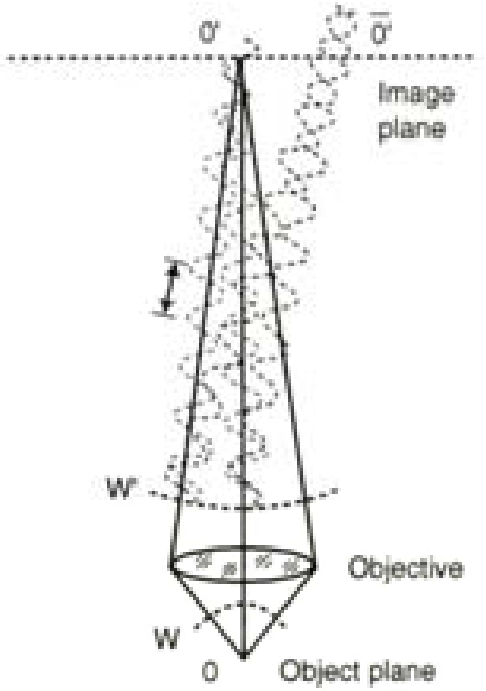
A



B

C





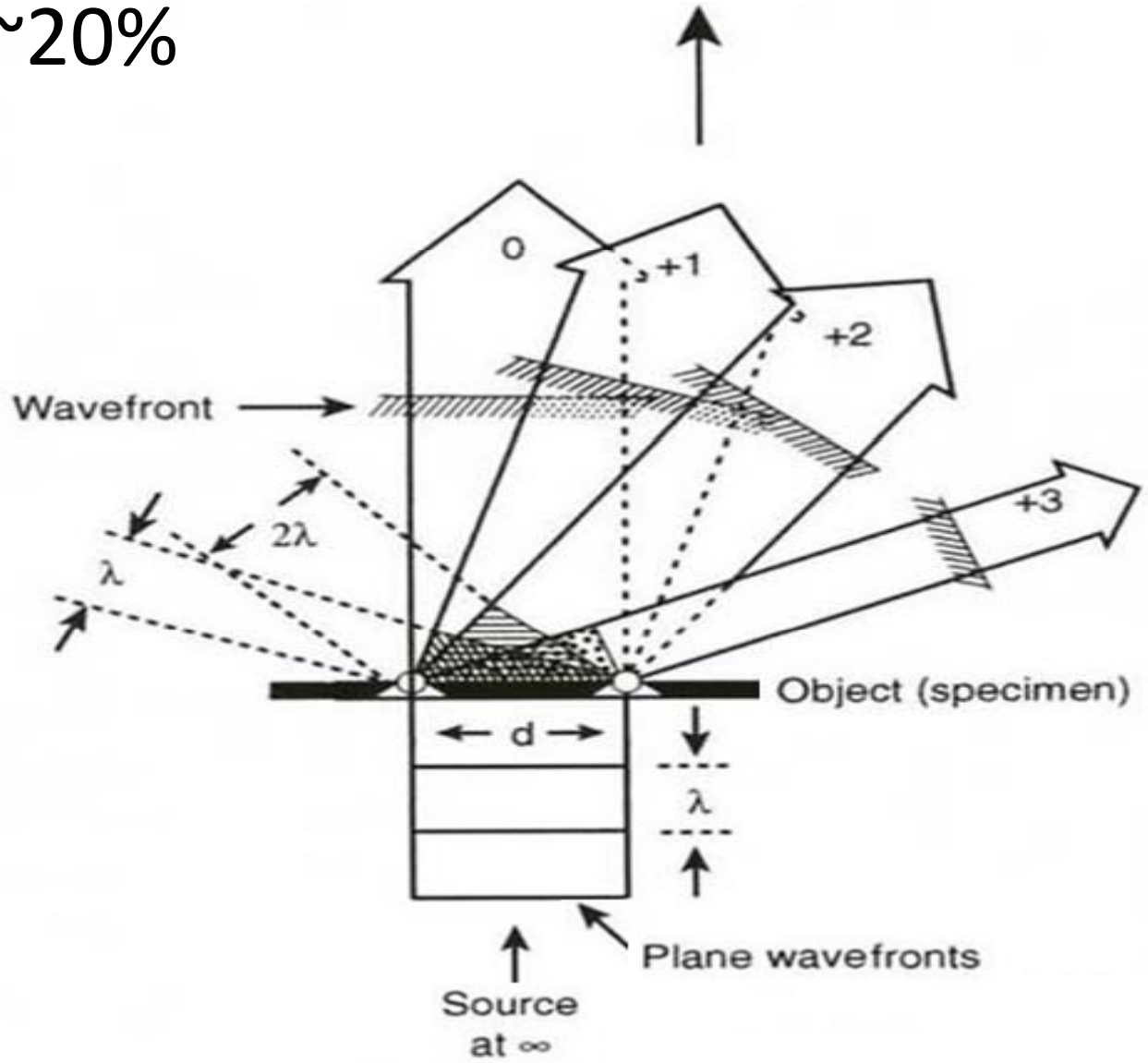
Lens Resolution

- Geometric optics predicts lenses of infinite resolution
- However, because of the phenomenon of diffraction, every point in the object is converted into an Airy disc
- Diameter of Airy disc:

$$D = \frac{1.22 \text{ x } \lambda}{n \sin\alpha},$$

$$\text{or, } D = \frac{1.22 \text{ x } \lambda}{NA}$$

We cannot resolve objects whose Airy discs overlap by ~20%



As a consequence, Abbe's rule is that d=λ/NA

The Abbe limit resolution is due to diffraction in numerical aperture of the lens

Other lenses

- Collector
- Condenser
  - Allow us to use point light sources instead of parallel illumination
  - Also (later) increase the resolution of the microscope
- Ironically, van Leeuwenhoek, who used simple non-compound, single-lens microscopes, was using the lens of his eye as a projection lens!

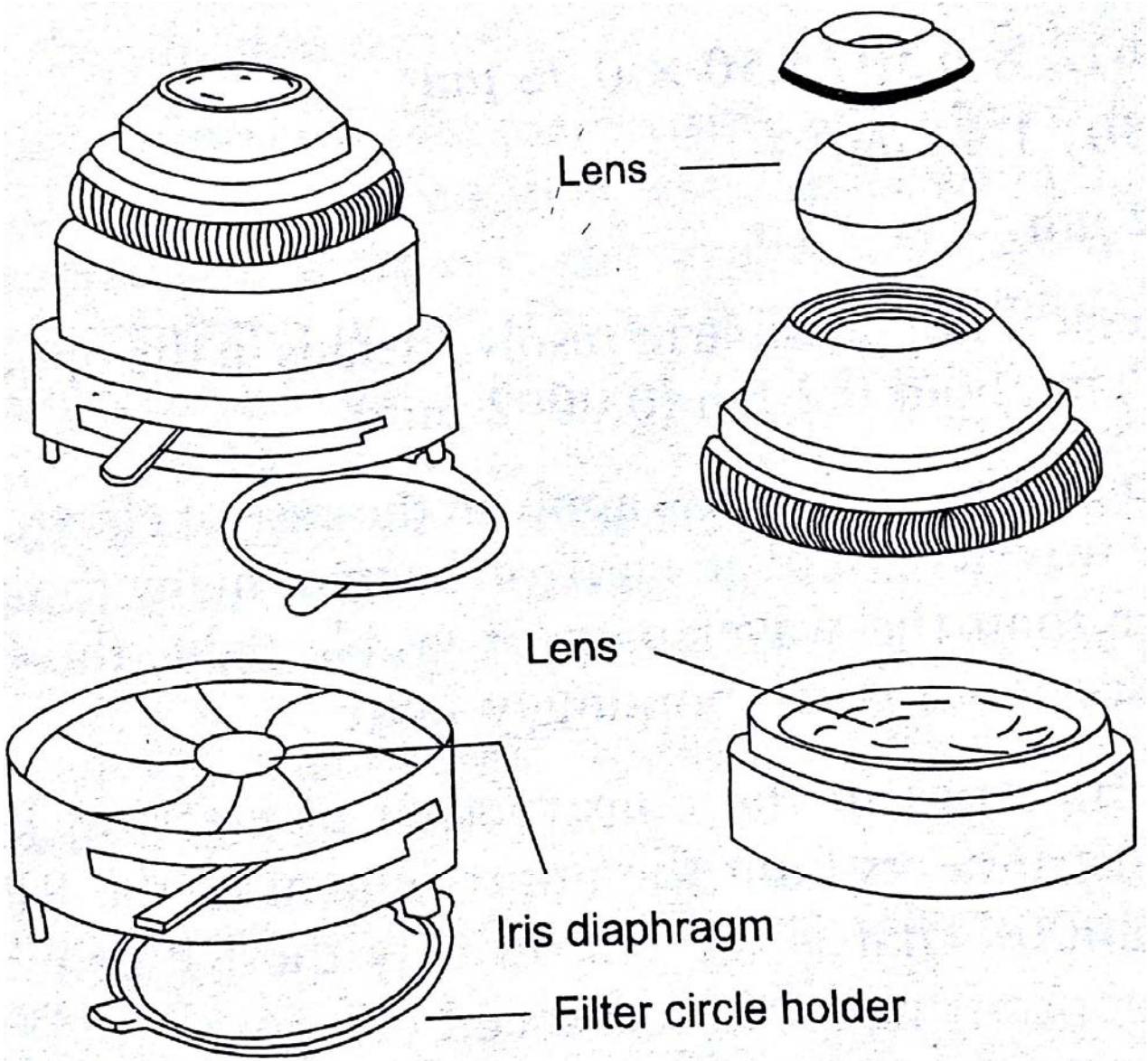
For a typical 1.3 NA lens at 525 nm, the limit of resolution is ~ 400 nm

- How to improve?
  - Larger NA (lenses, immersion fluid)
  - Shorter λ
- Add a condensor:

$$D = \lambda / (NA_{obj.} + NA_{cond.})$$

- So, for a 1.3 NA lens and condensor, D drops to ~200 nm

Condenser

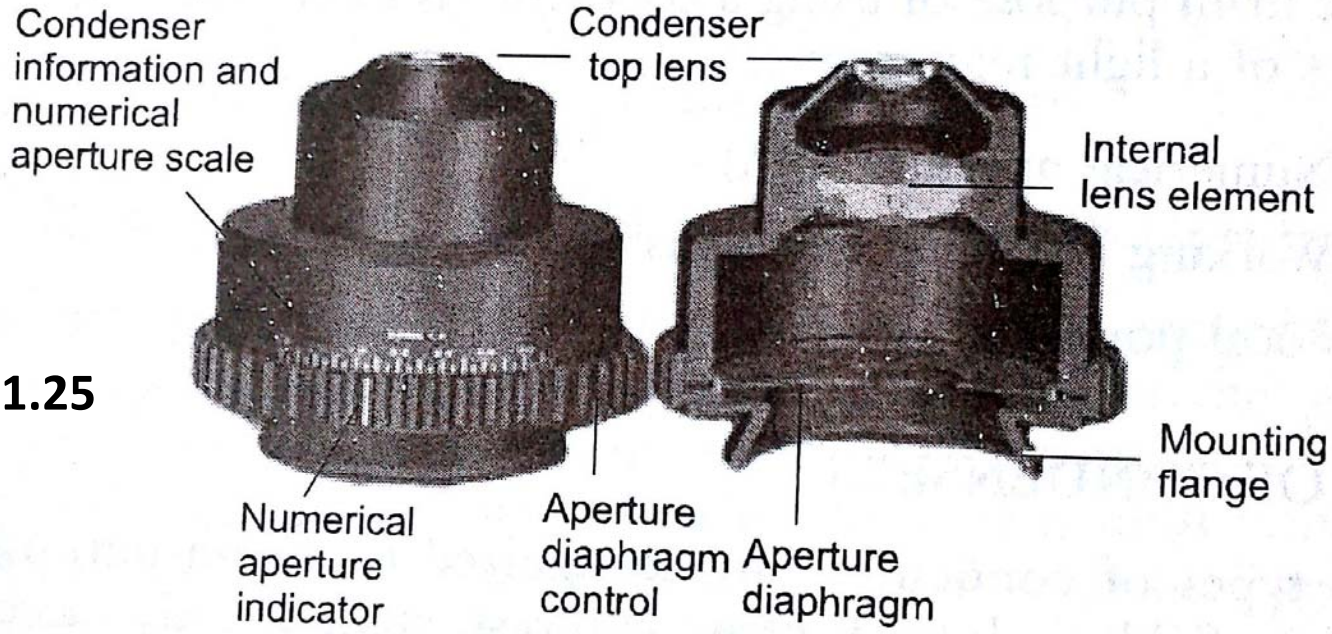


- It control NA
- By controlling NA resolution increases
- Enhance the working distance of the lens
- Enhance the focal point

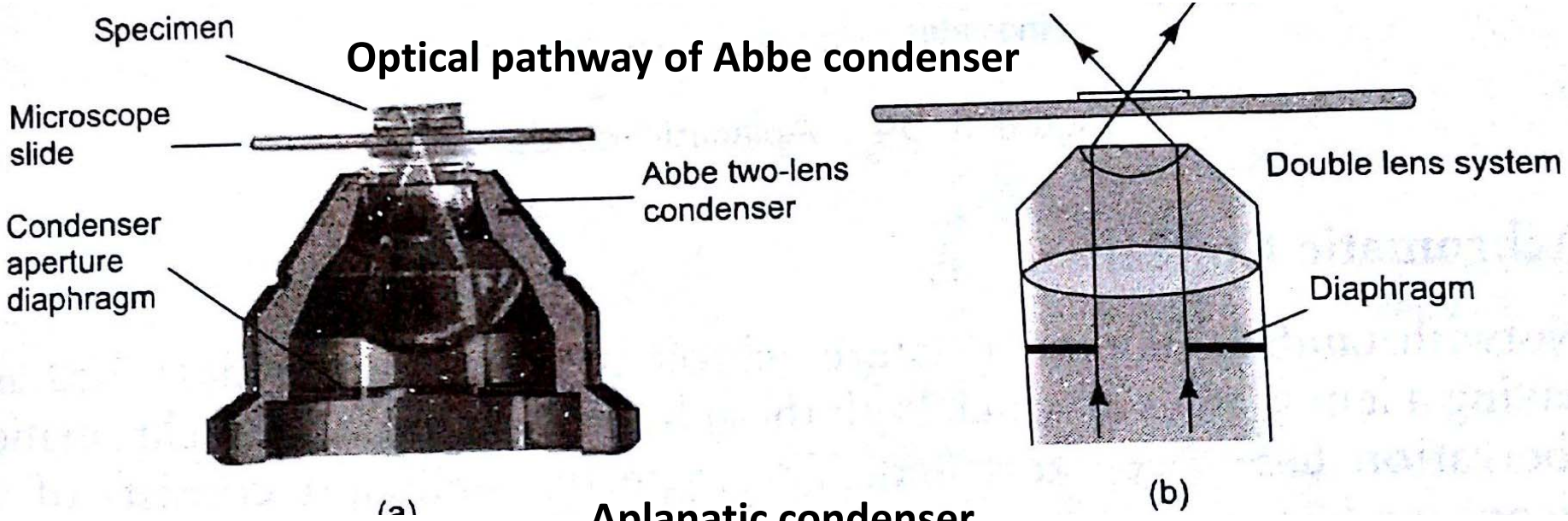
Types of Condenser

	Aberration	
	Spherical	Chromatic
• Abbe	-	-
• Aplanatic	+	-
• Achromatic	-	+
• Aplanatic Achromatic	+	+

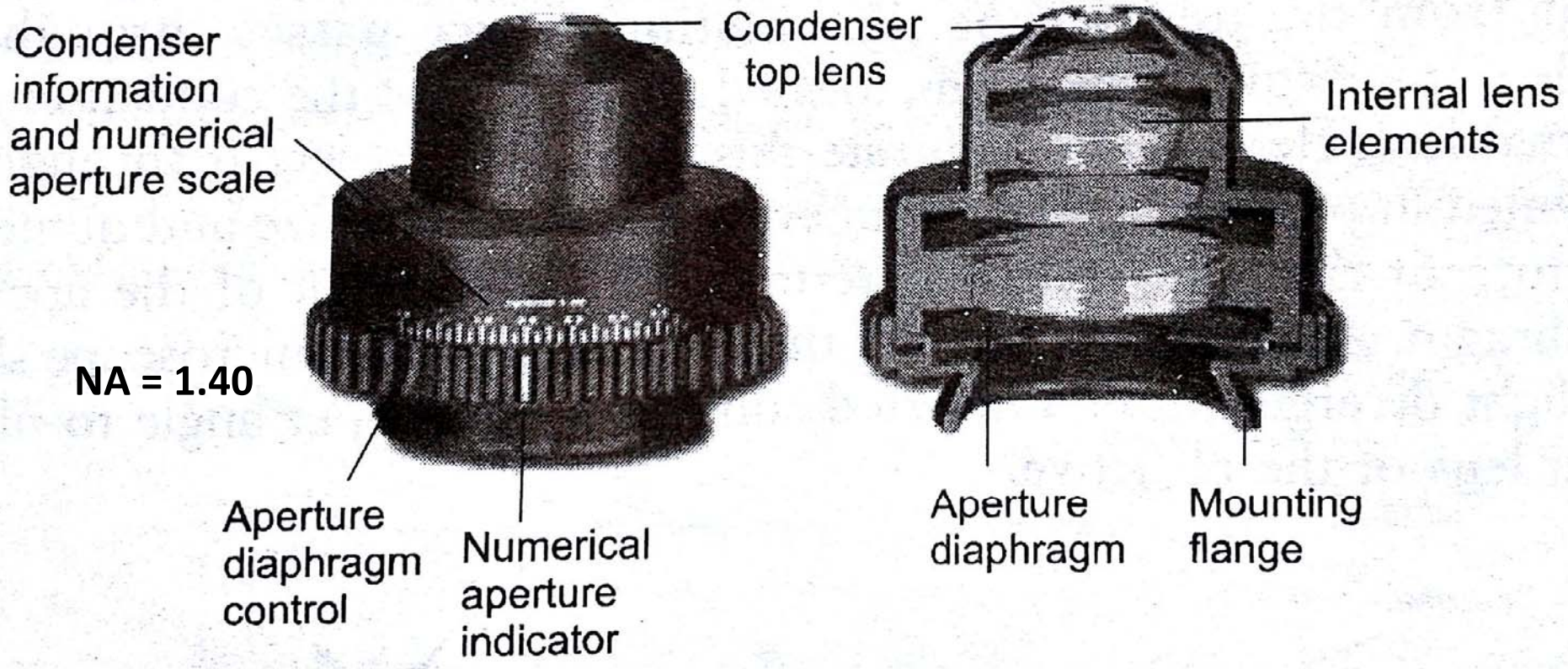
NA = 1.25



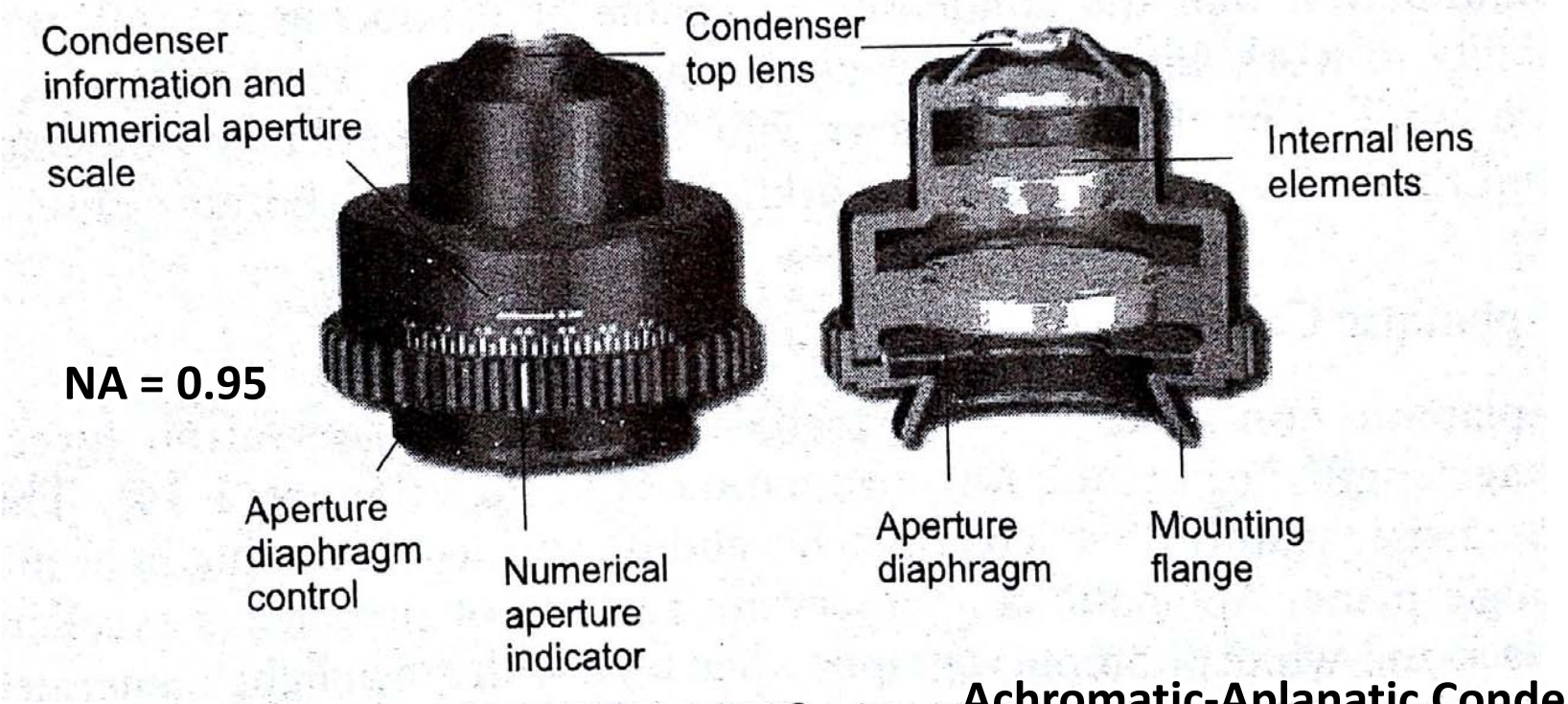
Optical pathway of Abbe condenser



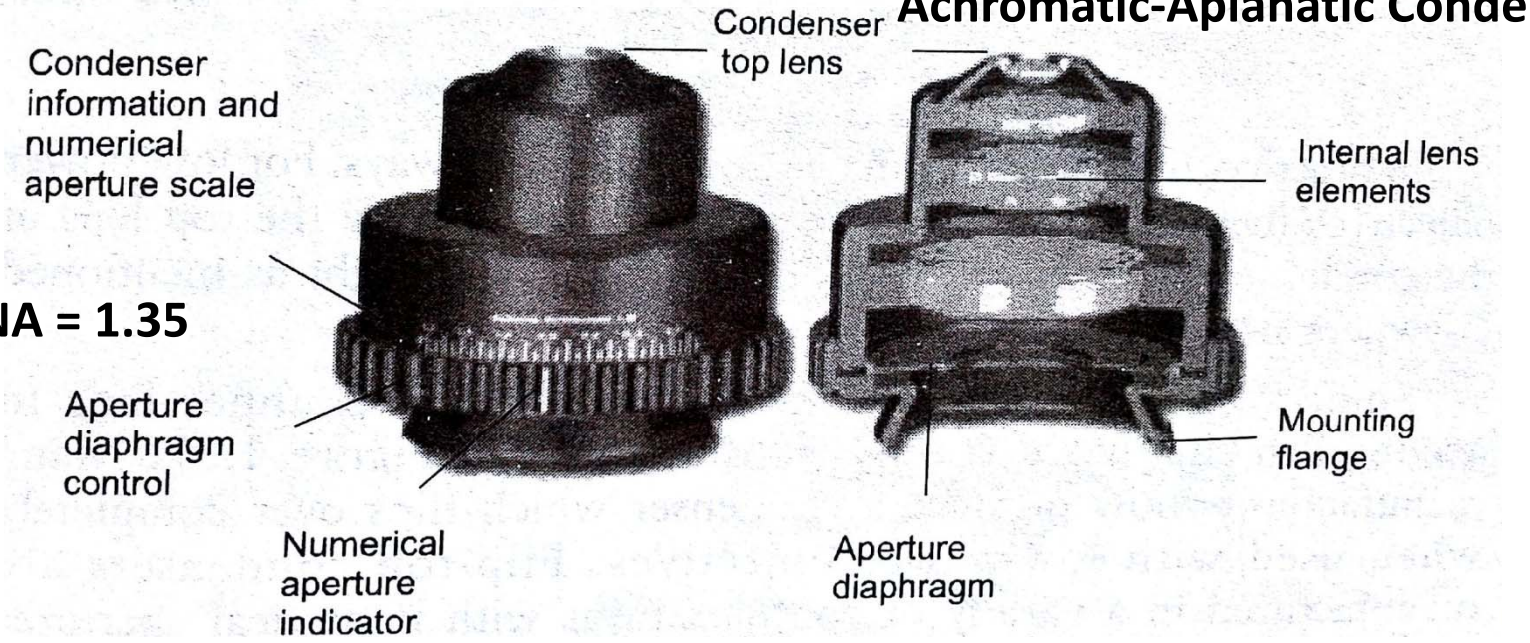
Aplanatic condenser



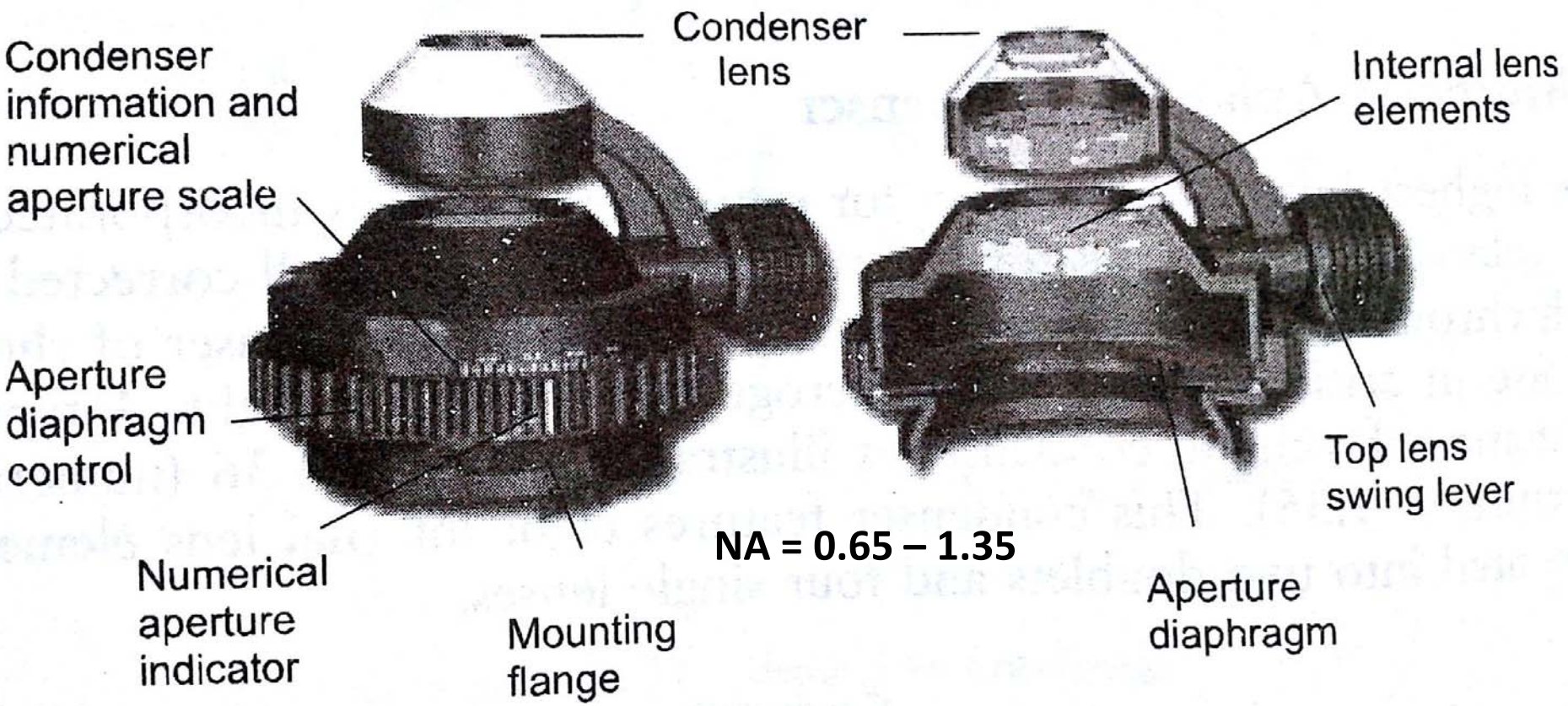
Achromatic Condenser



Achromatic-Aplanatic Condenser



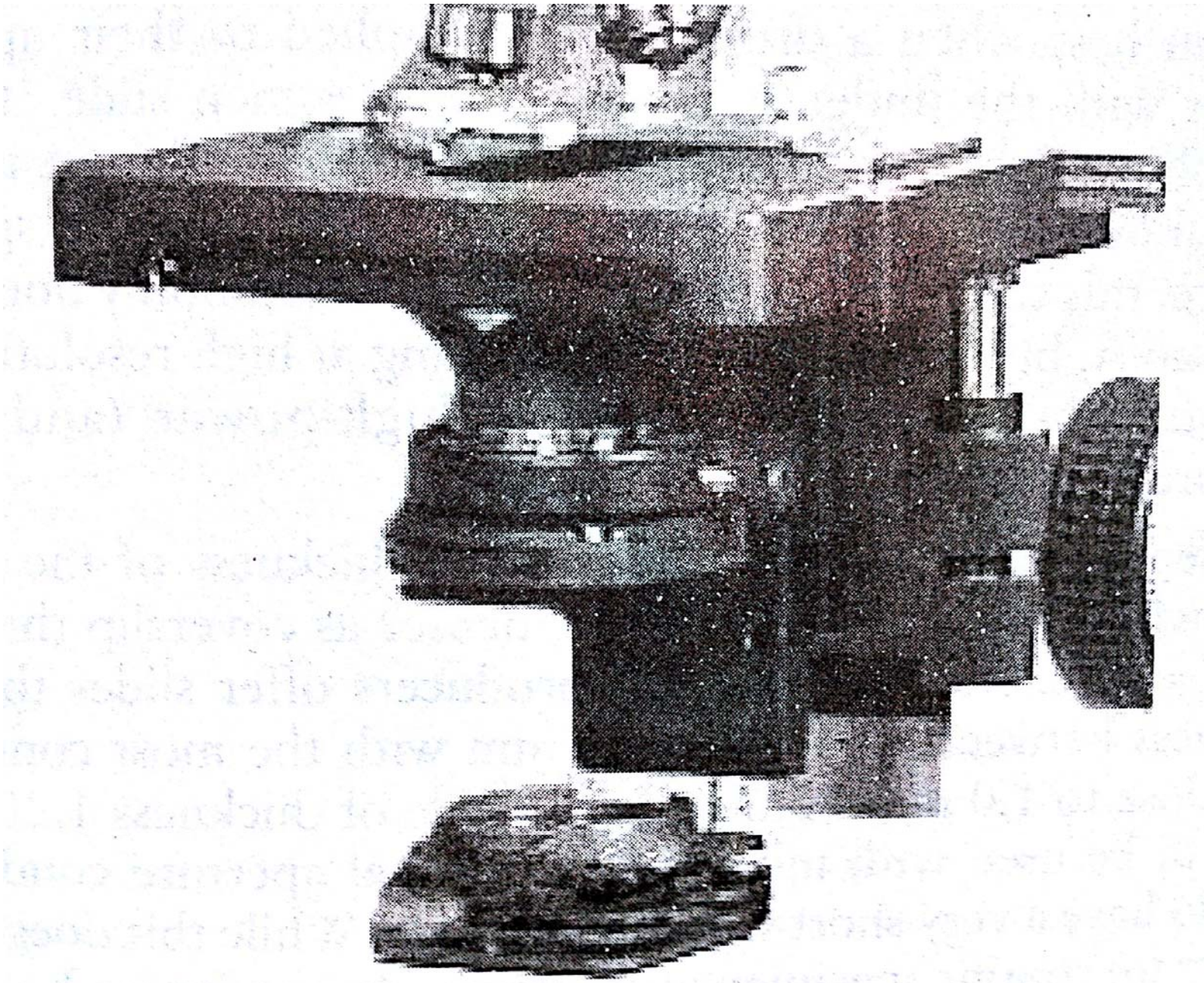
Swing-top lens Condenser



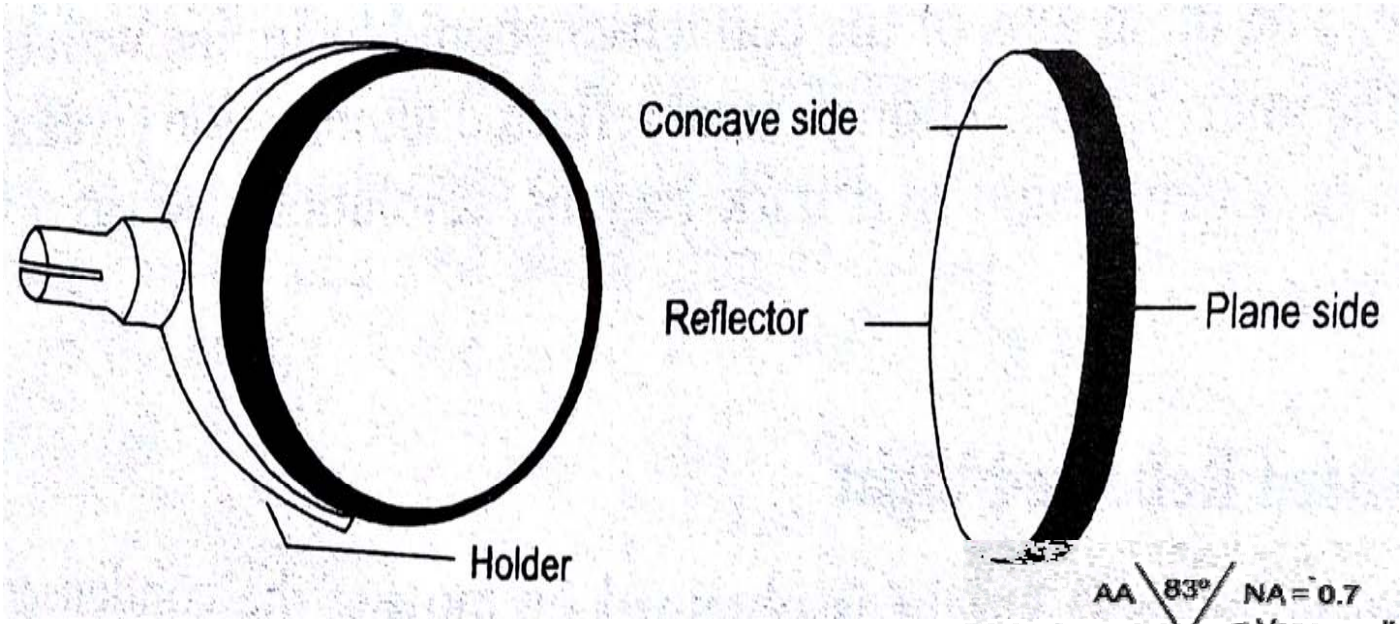
Immersion Condenser

Immersion condensers increases the NA greatly and make the lens system waterproof.

Movable and Fixed Condenser

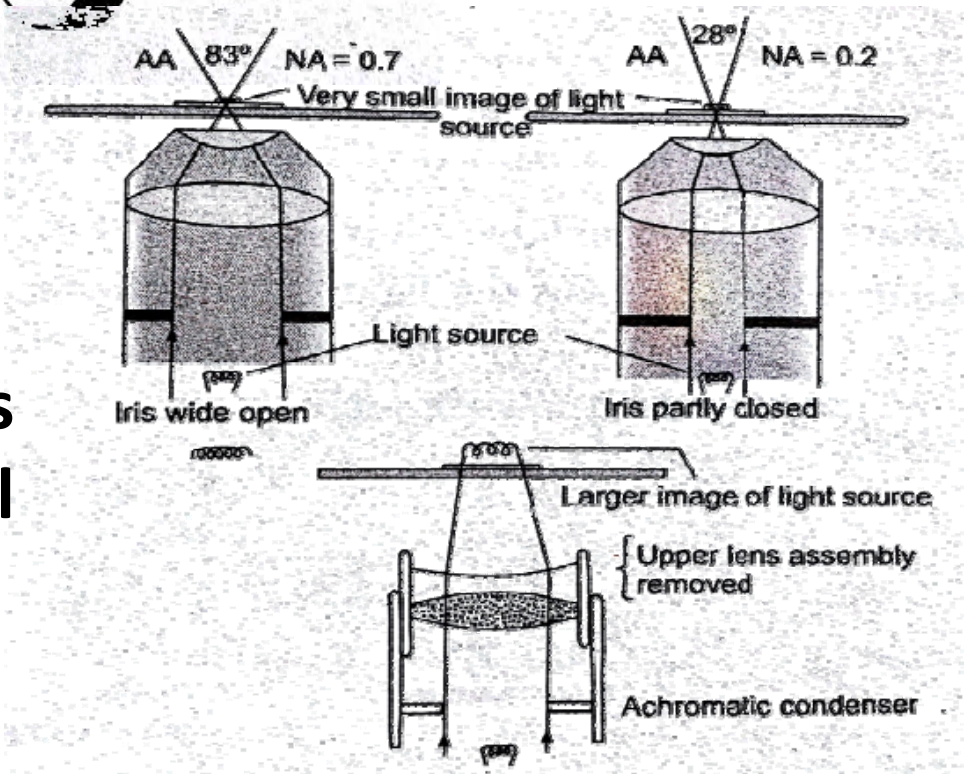


Illuminating System



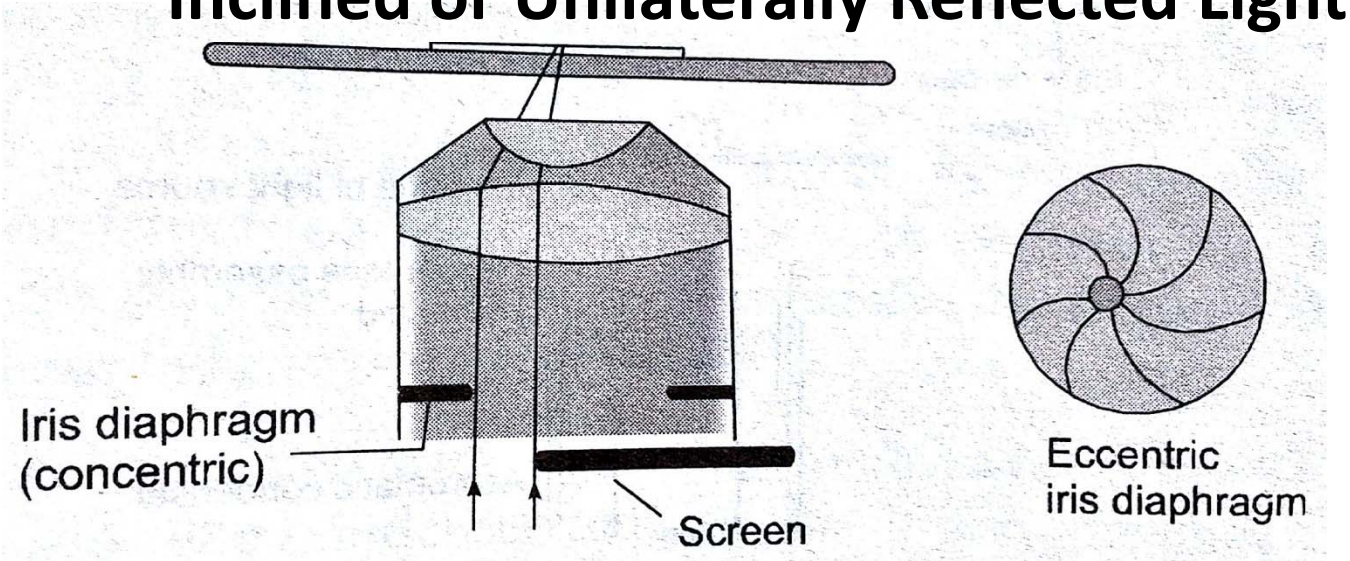
Axial Reflected Light

Use of condenser & Iris diaphragm to control illumination

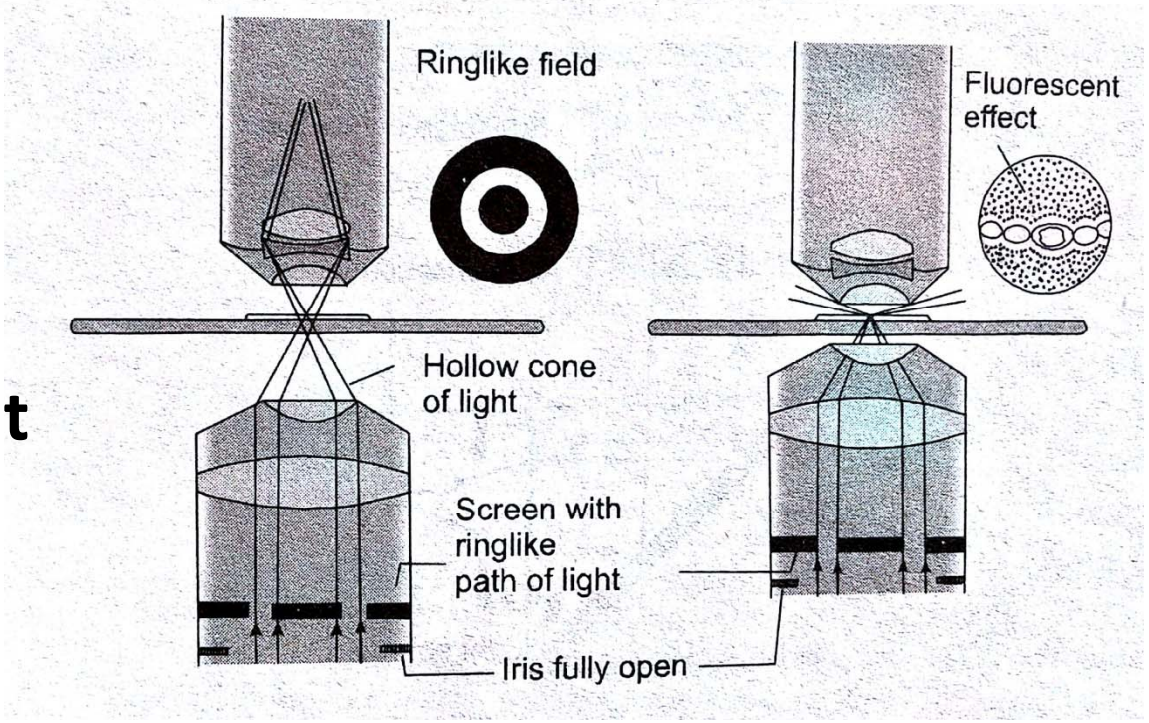


Condensed Reflected Light

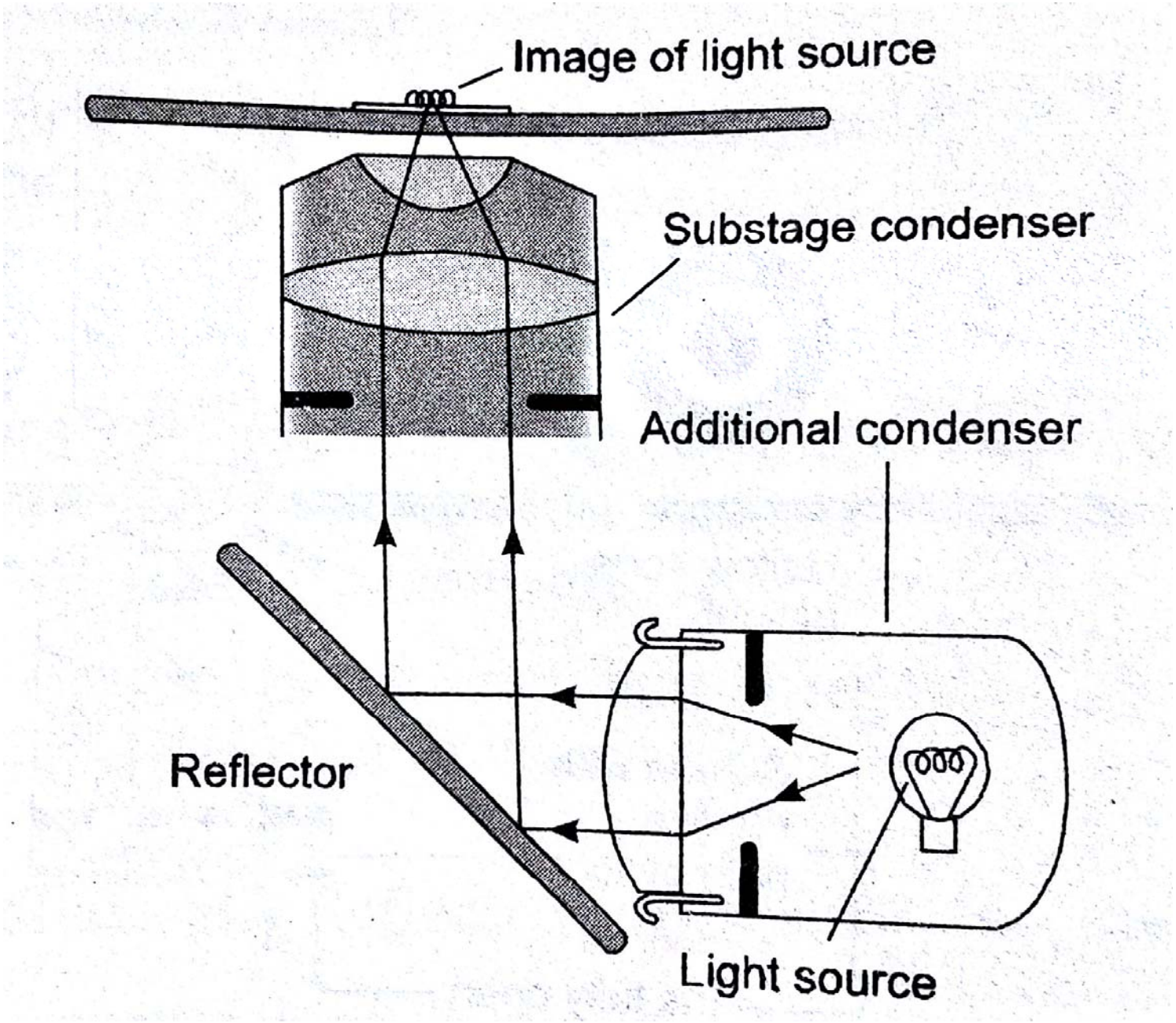
Inclined or Unilaterally Reflected Light



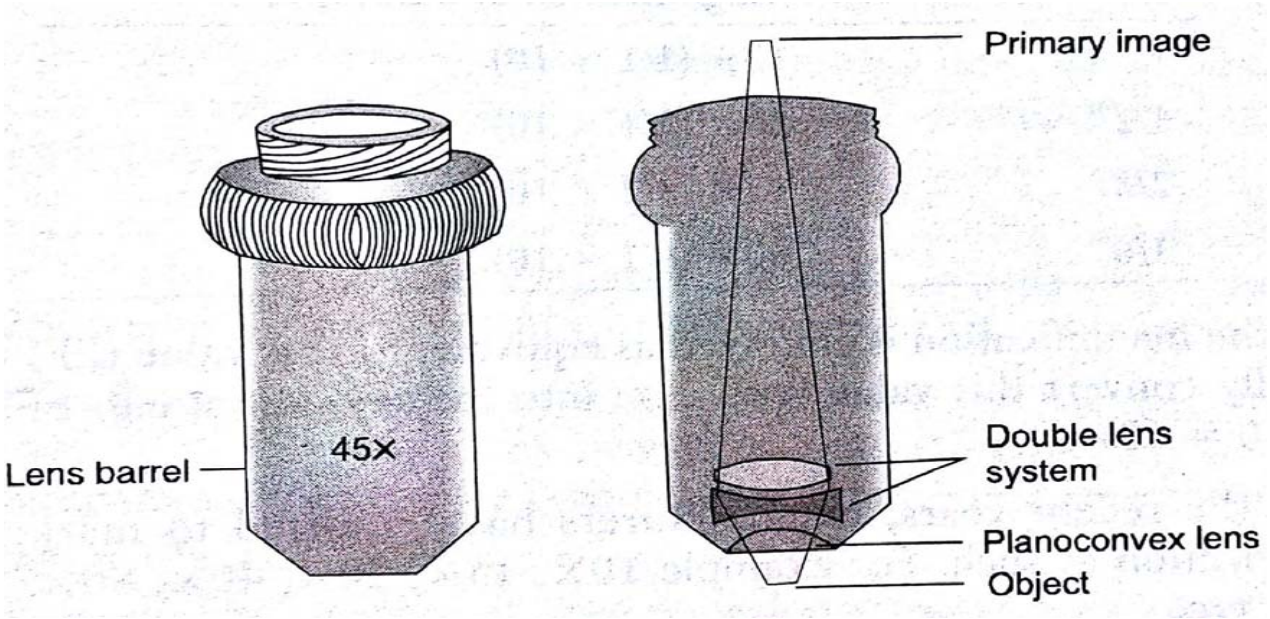
Circular Ringlike Light



Additional Condenser for Illuminating System



Lens Systems – Objective Lens System



3.5x	45x
4x	80x
10x	90x
15x	100x
20x	120x
40x	

Focal length of objective is inversely proportional to the magnification.
Majority of microscope has tube length 160 mm
FL at 10x = 160/10 = 16
FL at 45x = 160/45 = 3.6

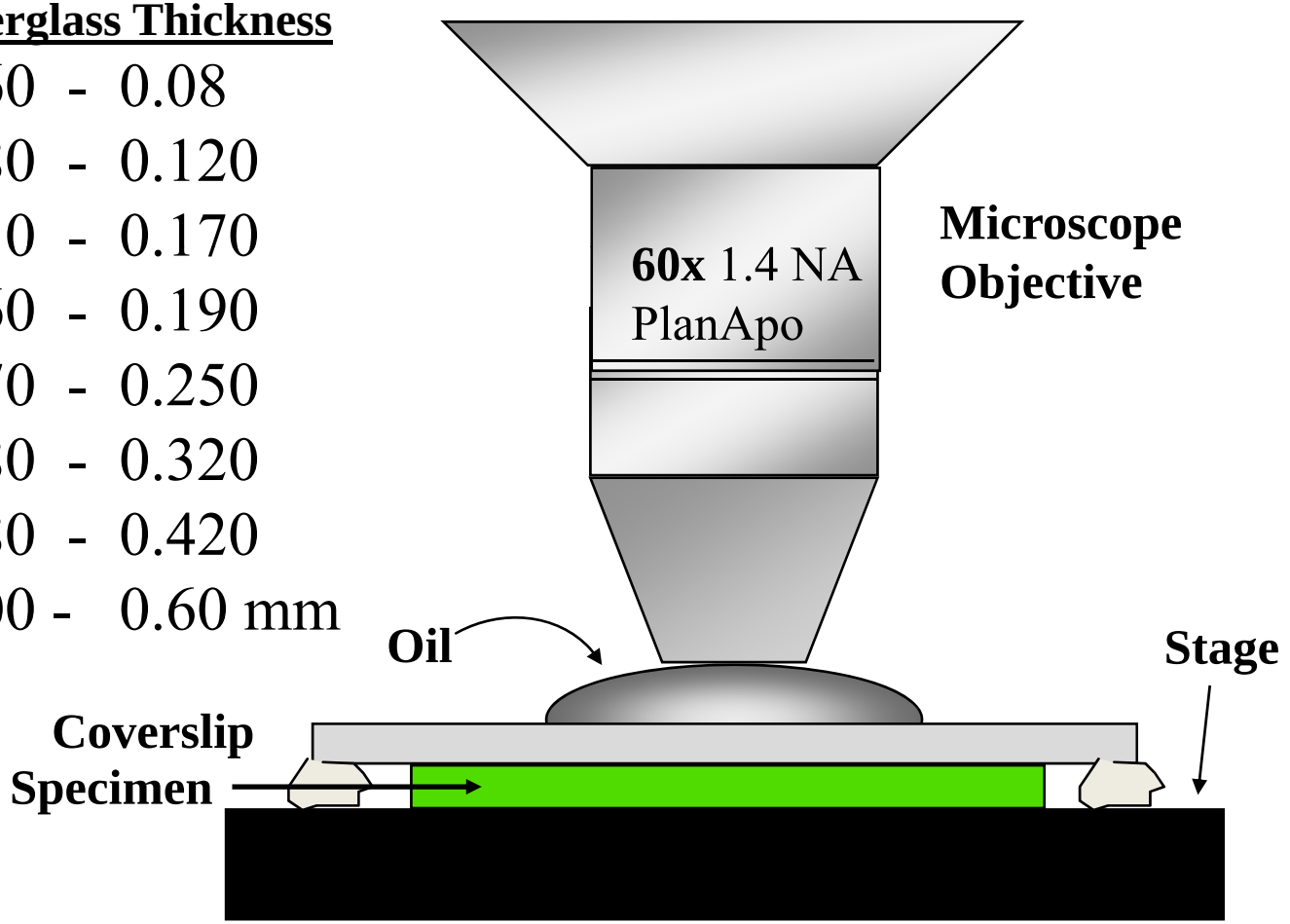
Equivalent Focal length	Magnification at a distance 10”
1”	10 x (1/1 x 10)
1/2”	20 x (2/1 x 10)
2/3”	15 x (3/2 x 10)
1/6”	60 x (6/1 x 10)

Reading an objective

Microscope Objectives

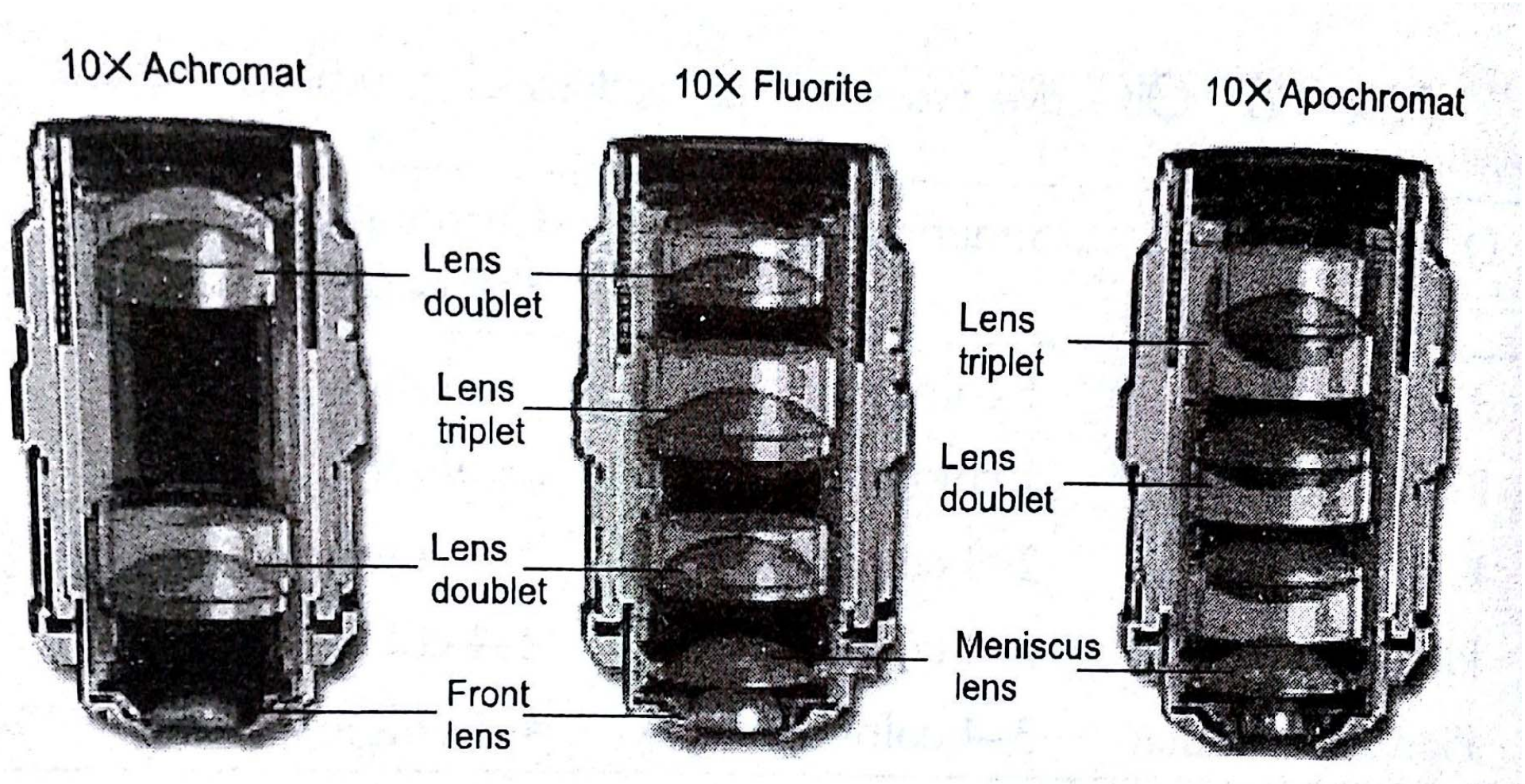
Standard Coverglass Thickness

#00 = 0.060 - 0.08
#0 = 0.080 - 0.120
#1 = 0.130 - 0.170
#1.5 = 0.160 - 0.190
#2 = 0.170 - 0.250
#3 = 0.280 - 0.320
#4 = 0.380 - 0.420
#5 = 0.500 - 0.60 mm

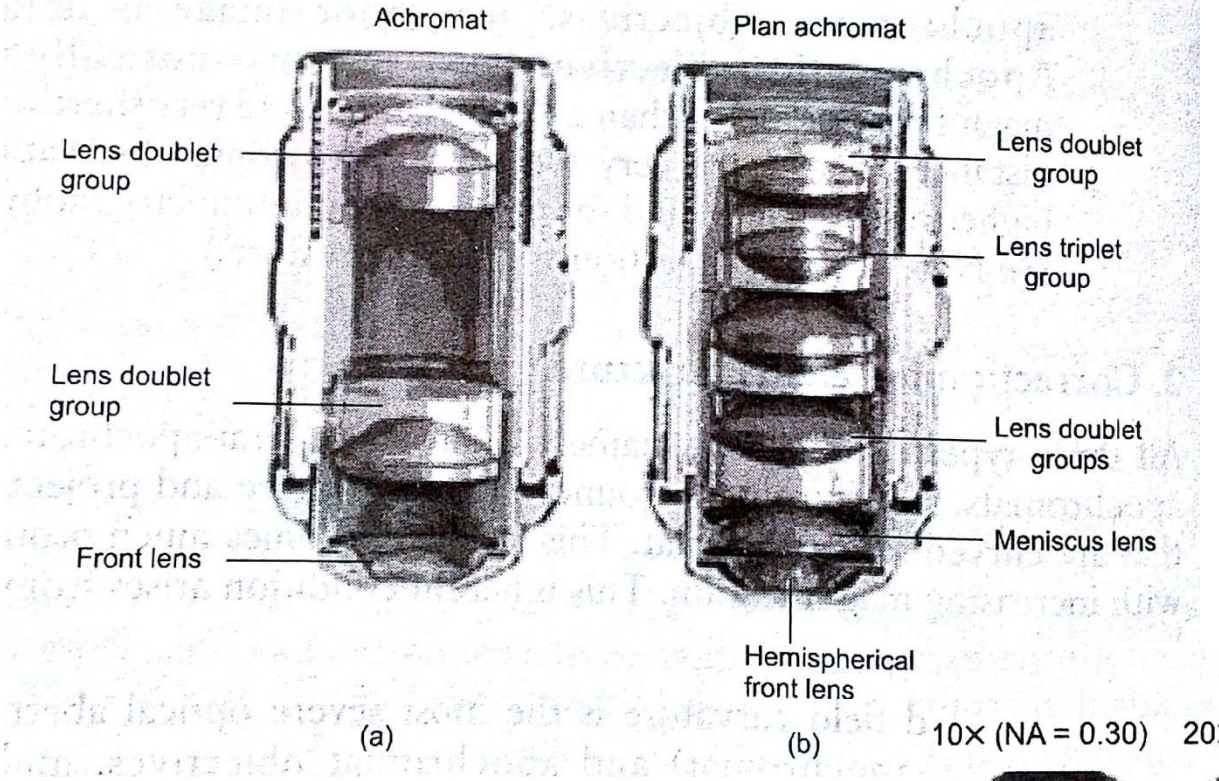


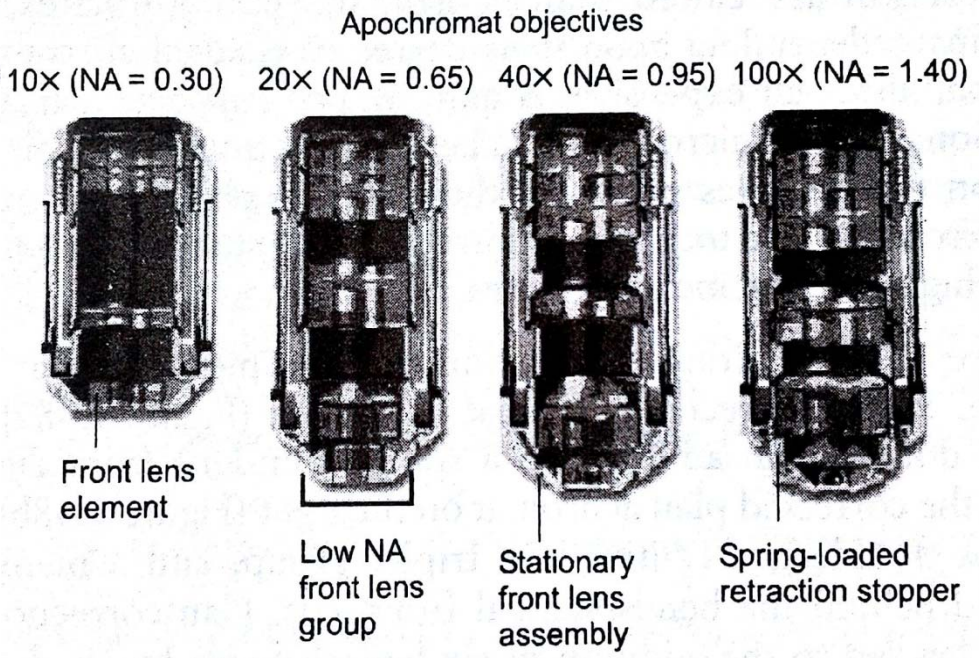
Correction in Optical Aberration

- Achromatic Objectives (Achromats)
- Semi-apochromatc Objectives (Fluorites)
- Apochromatic Objectives (Apochromats)

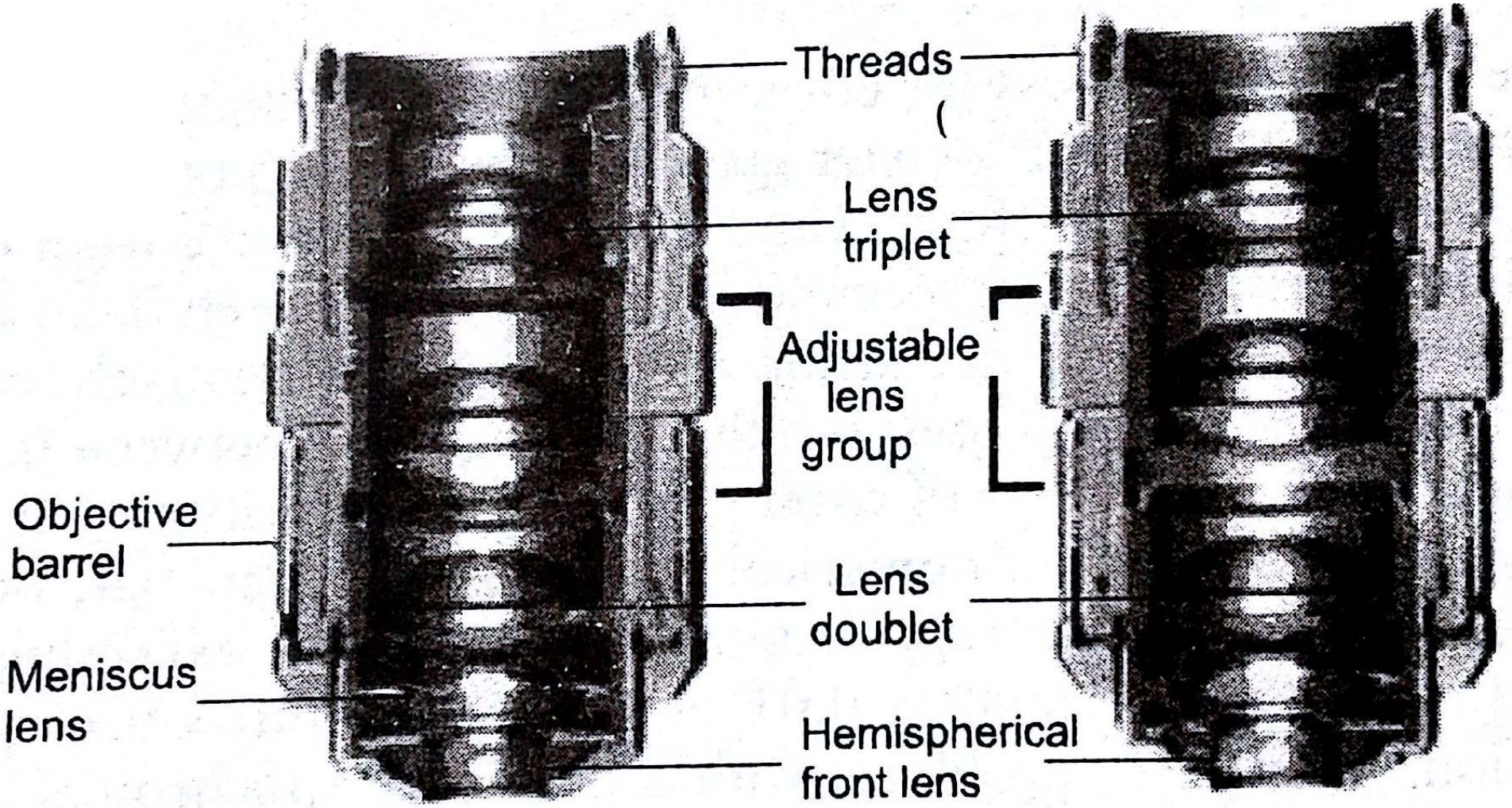


Lens Systems – Objective Lens System





Correction/Compensation for variations in Cover Glass



Collar adjusted for cover glass of thickness 0.20 mm

Collar adjusted for cover glass of thickness 0.13 mm

The issues between simple and compound microscope

- Simple microscopes could attain around 2 micron resolution, while the best compound microscopes were limited to around 5 microns because of chromatic aberration.

- In the 1730s a barrister named **Chester More Hall** observed that flint glass (newly made glass) dispersed colors much more than "crown glass" (older glass). He designed a system that used a concave lens next to a convex lens which could realign all the colors. This was the first ***achromatic lens***.

TYPES

- **<u>Light Microscope</u> : use sunlight or artificial light.**

1. Bright field microscope.
2. Dark field microscope.
3. Phase contrast microscope.
4. Fluorescence microscope.

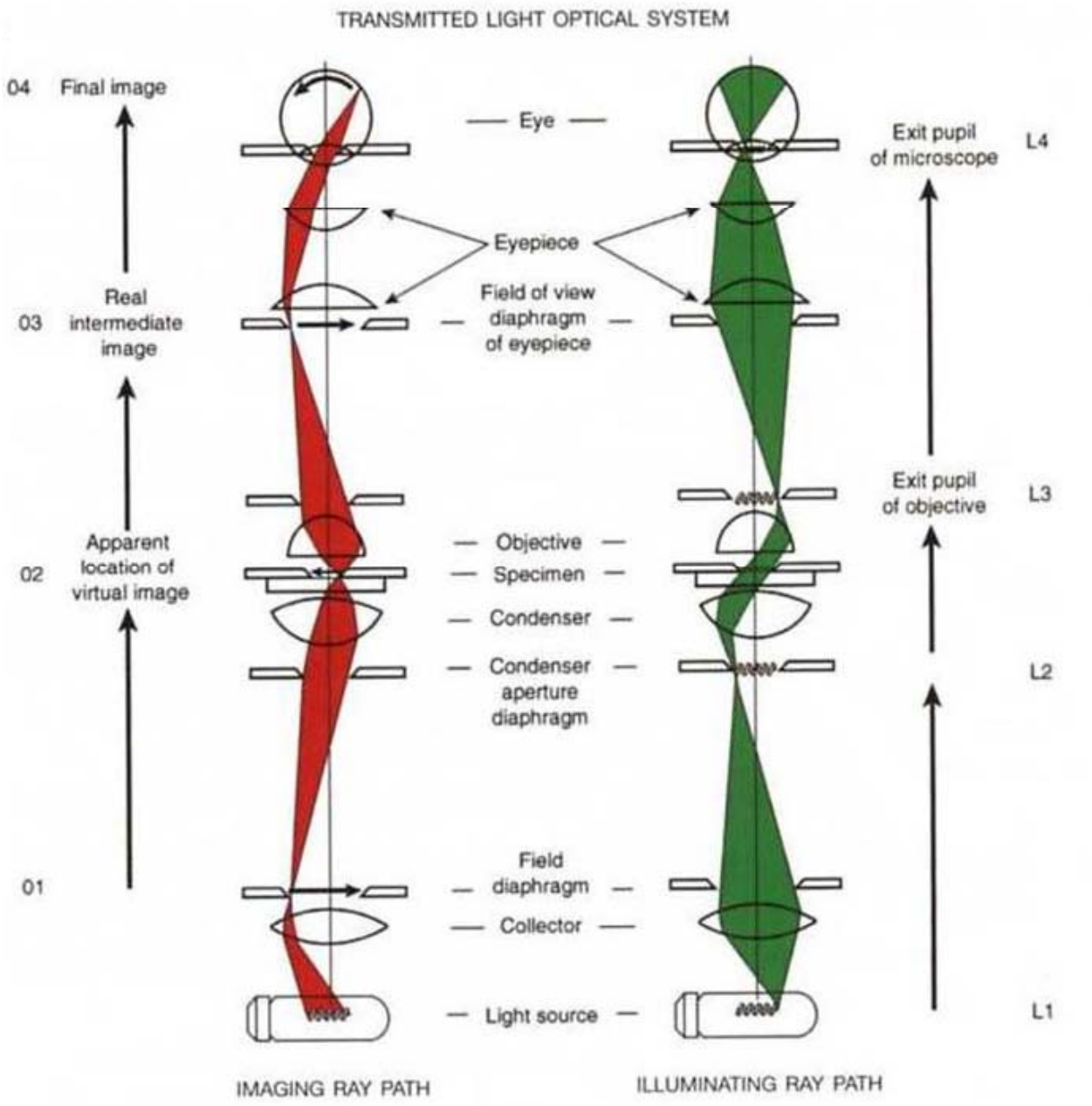
- **<u>Electron microscope</u> : use of electron.**

1. Transmission electron microscope.
2. Scanning electron microscope.

Compound Microscope

- The compound microscope uses at least two lens systems
  - The objective forms an intermediate real image of the object at the **objective tube length**
  - The ocular forms a virtual image of that intermediate image to the retina of the eye
  - If we are dealing with a photodetector, we must use a projection lens to form a real image from the intermediate image

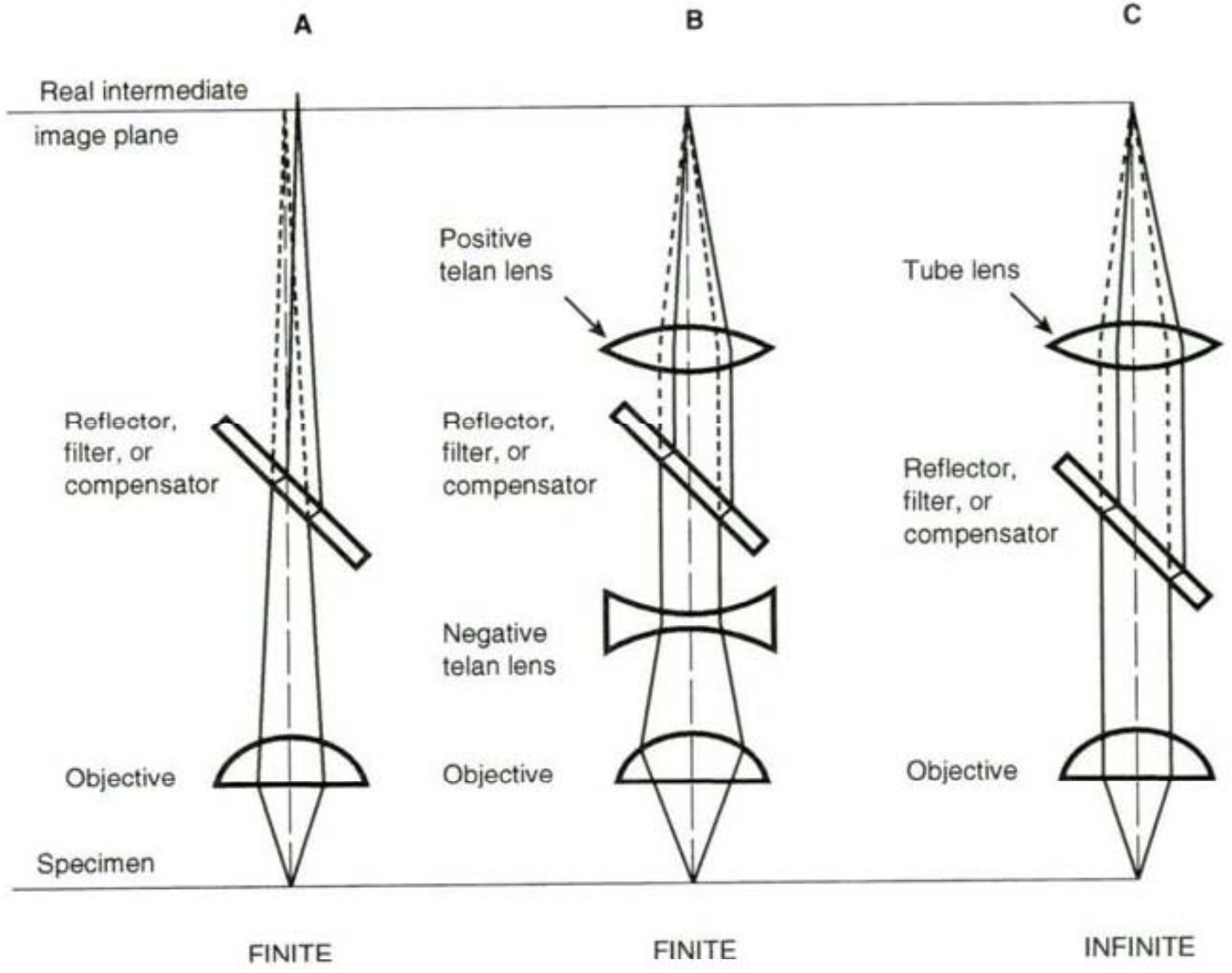
Ray Tracings in the microscope



Current microscope objective tend to be infinity corrected

- Infinite tube length
- Require an additional lens in objective to converge beam
- Advantages
  - Objectives are simpler
  - Optical path is parallel through the microscope body:

Infinitive correction



Benefits of infinity space. (A) Insertion of reflector or filter causes lateral and axial shift. (B) Tow telan lenses generate infinity space to climate shift. © Objective directly provides infinity space.

Types of Light Microscope.

- Bright-field Microscopy.
- Dark-field Microscopy.
- Phase contrast Microscopy.
- Fluorescence Microscopy.

WORKING OF COMPOUND MICROSCOPE

- Light is transmitted and focussed by mirror and condenser.

- Focussed light illuminate the object or specimen.

- The refracted light is collected by an objective where primary image of the object is formed, it is real,inverted enlarged image of the object.

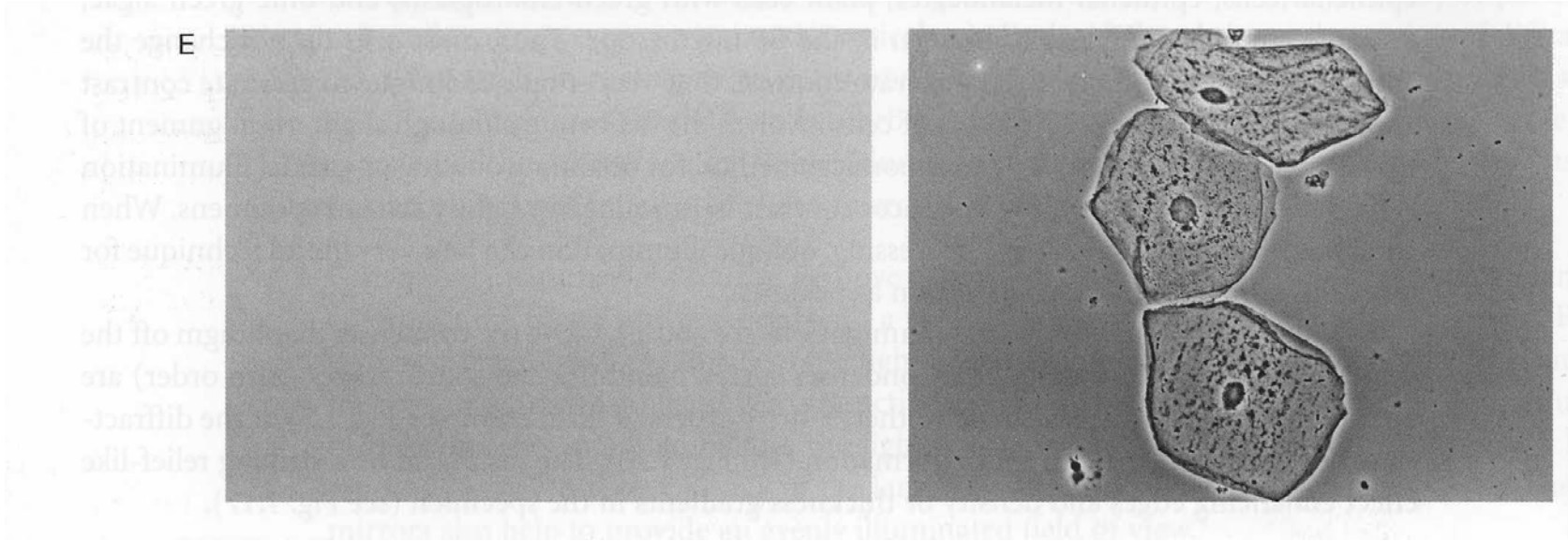
- The eyepiece further magnifies this primary image into virtual,erect enlarged image, this is the final image that lies above the stage.

APPLICATION

- Observation of morphology of microorganisms.
- Detection of cell structures.
- Observation of intracellular structures.
- Observation of motility.
- Measurement of size.
- Observation of blood smears.

The Bright-Field Microscope

- Produces a dark image against a brighter background
- Resolving power – 0.2µm
- Staining takes advantage of chemical differences
- Generally only useful for stained biological specimens
- Unstained cells are virtually invisible



Brightfield Phase contrast

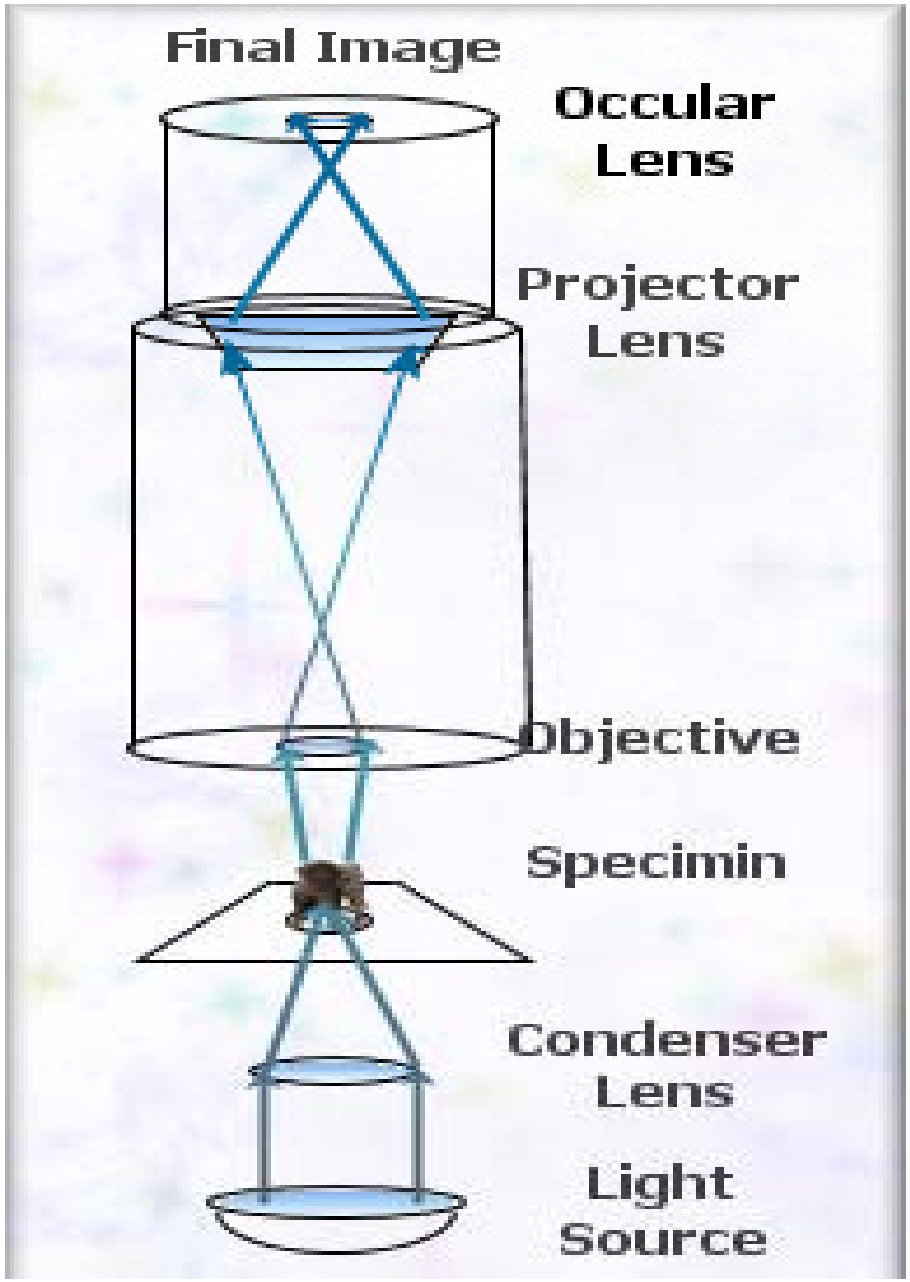
- Higher N.A
- Better light generation
- Better Resolution

- Shorter the Wavelength
- Better Resolution.

⦿ The ordinary microscope is called as a bright field microscope.

⦿ It forms dark image against bright background.

How Image is formed



- Image is created by objective and ocular lenses working together.
- Light from illuminated specimen is focused by the objective lens creating enlarged image within the microscope.
- The ocular lens further modifies the primary image.
- Total magnification is calculated by magnification by objective multiply by magnification by eyepiece.
- Ex : 45x X 10x =450x

Advantages

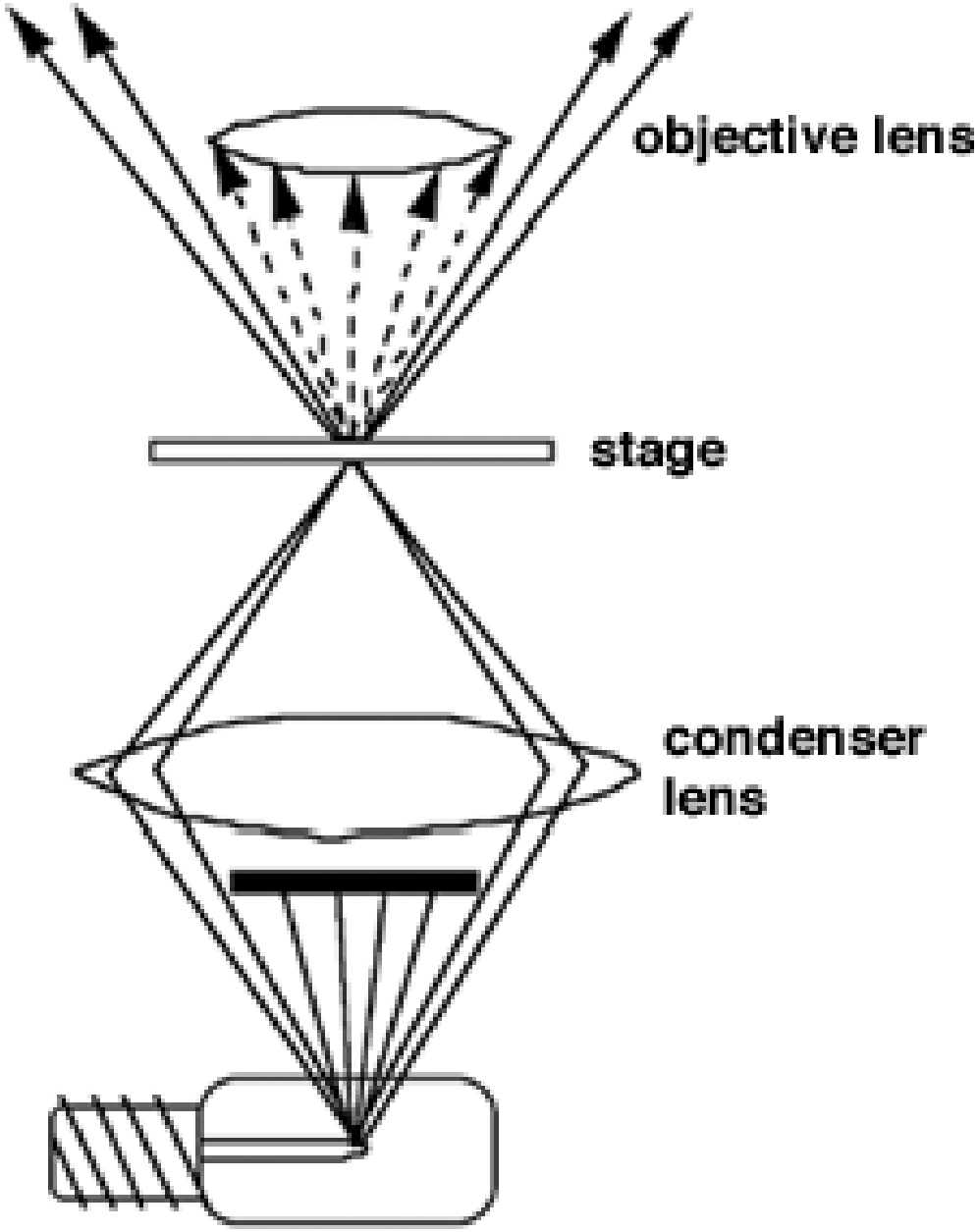
Bright field compound microscopes are commonly used to view live and immobile specimens such as bacteria, cells, and tissues. For transparent or colorless specimens, however, it is important that they be stained first so that they can be properly viewed under this type of a microscope. Staining is achieved with the use of a chemical dye. By applying it, the specimen would be able to adapt the color of the dye. Therefore, the light won't simply pass through the body of the specimen showing nothing on the microscope's view field

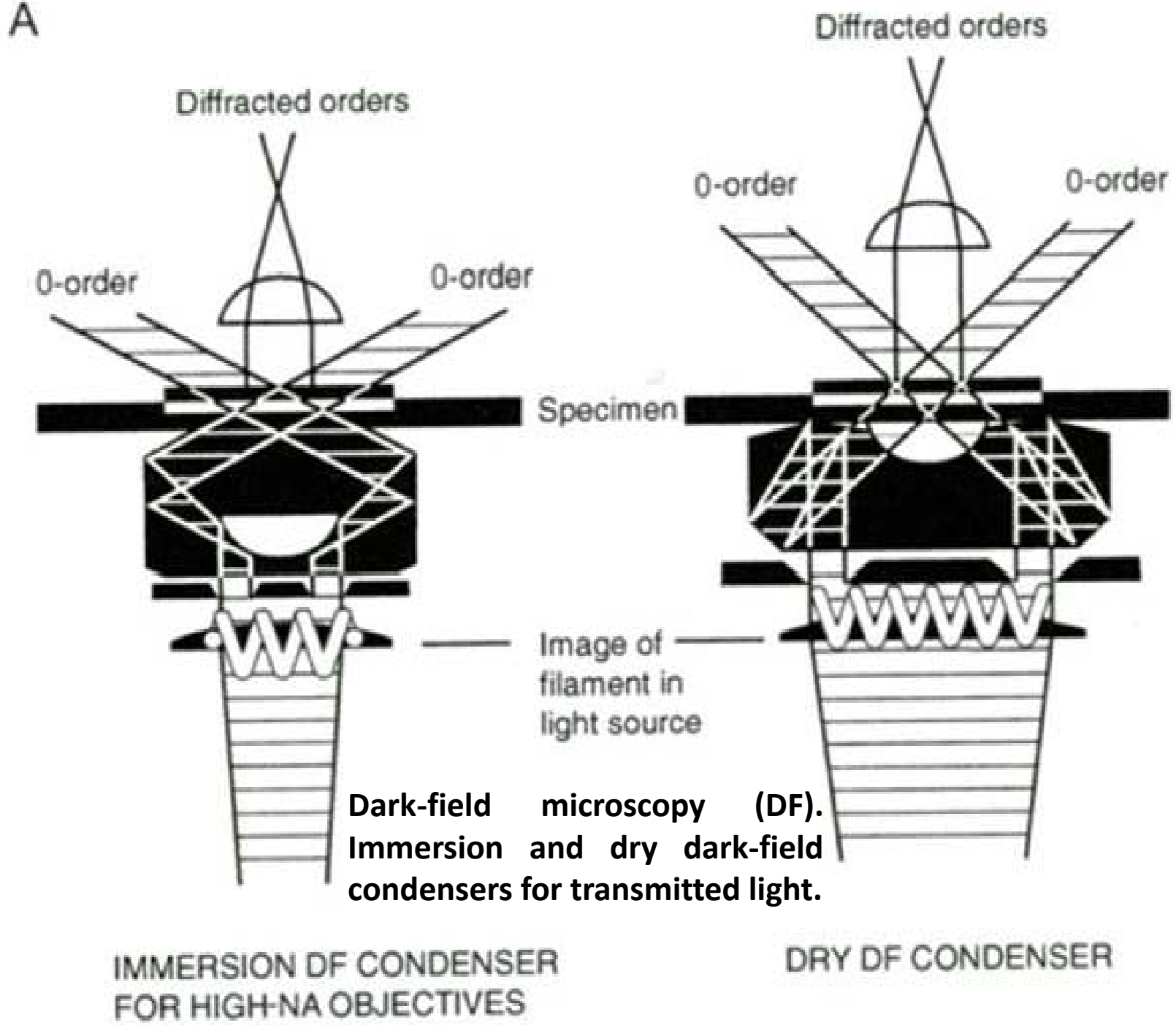
The Dark-Field Microscope

- Produces a bright image of the object against a dark background
- Used to observe living, unstained preparations
- Resolving power – 0.2µm
- Good for viewing in the natural environment

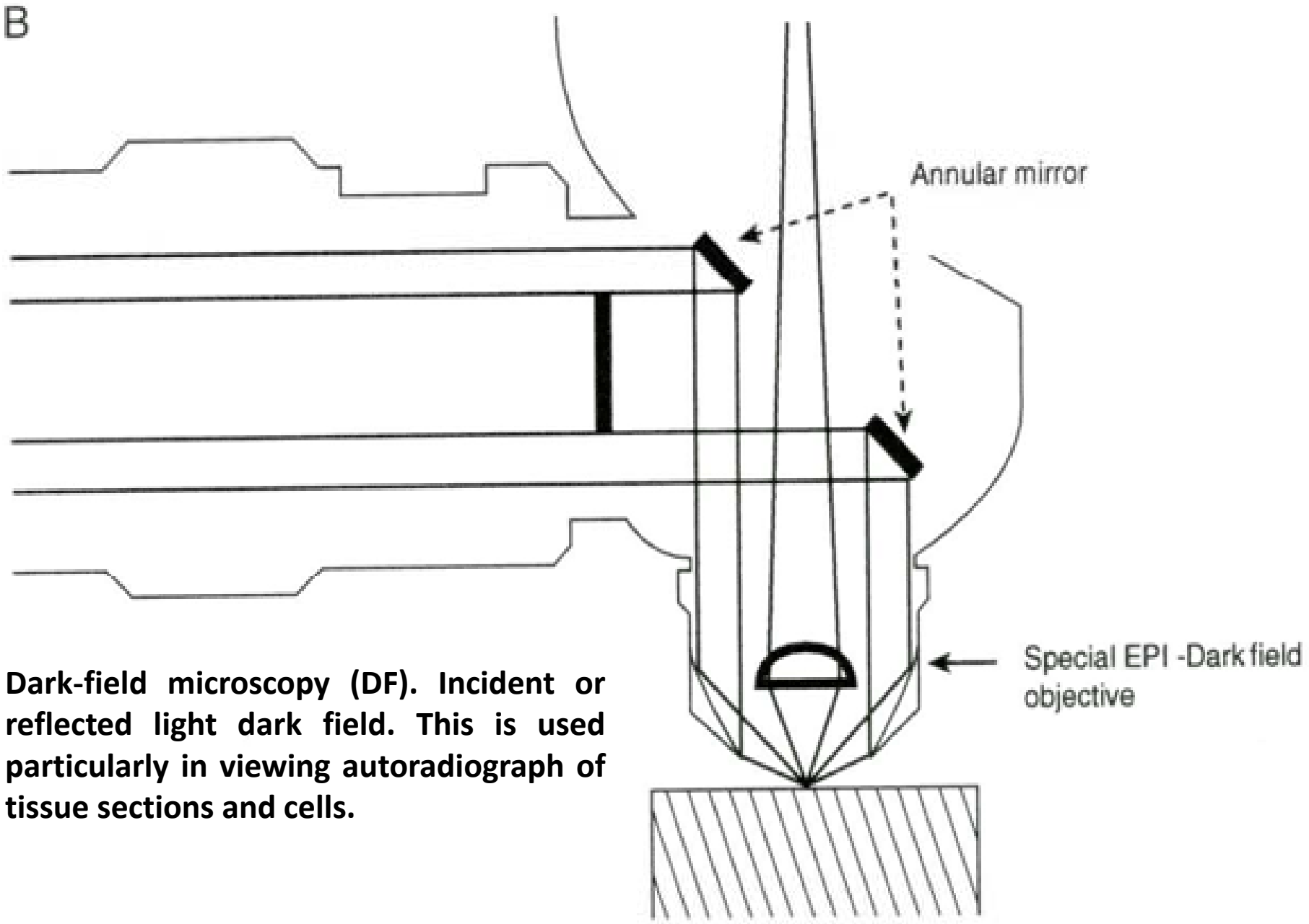
- Dark field microscopy allows viewer to observe living unstained cell and organisms simply by changing the way in which they illuminate the object.
- A hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective.
- Only light that has been reflected or refracted by the specimen forms the image
- The field surrounding specimen appears dark while the object brightly illuminated.
- The dark field microscope can revel considerable internal structure in larger eukaryotic microorganism.

How image formed in dark field microscopy





Dark-field microscopy (DF). Immersion and dry dark-field condensers for transmitted light.



Dark-field microscopy (DF). Incident or reflected light dark field. This is used particularly in viewing autoradiograph of tissue sections and cells.

Advantages

- The advantage of darkfield microscopy also becomes its disadvantage: not only the specimen, but dust and other particles scatter the light and are easily observed

- For example, not only the cheek cells but the bacteria in saliva are evident.

- The dark field microscopes divert illumination and light rays thus, making the details of the specimen appear luminous.

- Dark field light microscopes provide good results, especially through the examination of live blood samples.

- It can yield high magnifications of living bacteria and low magnifications of the tissues and cells of certain organisms.

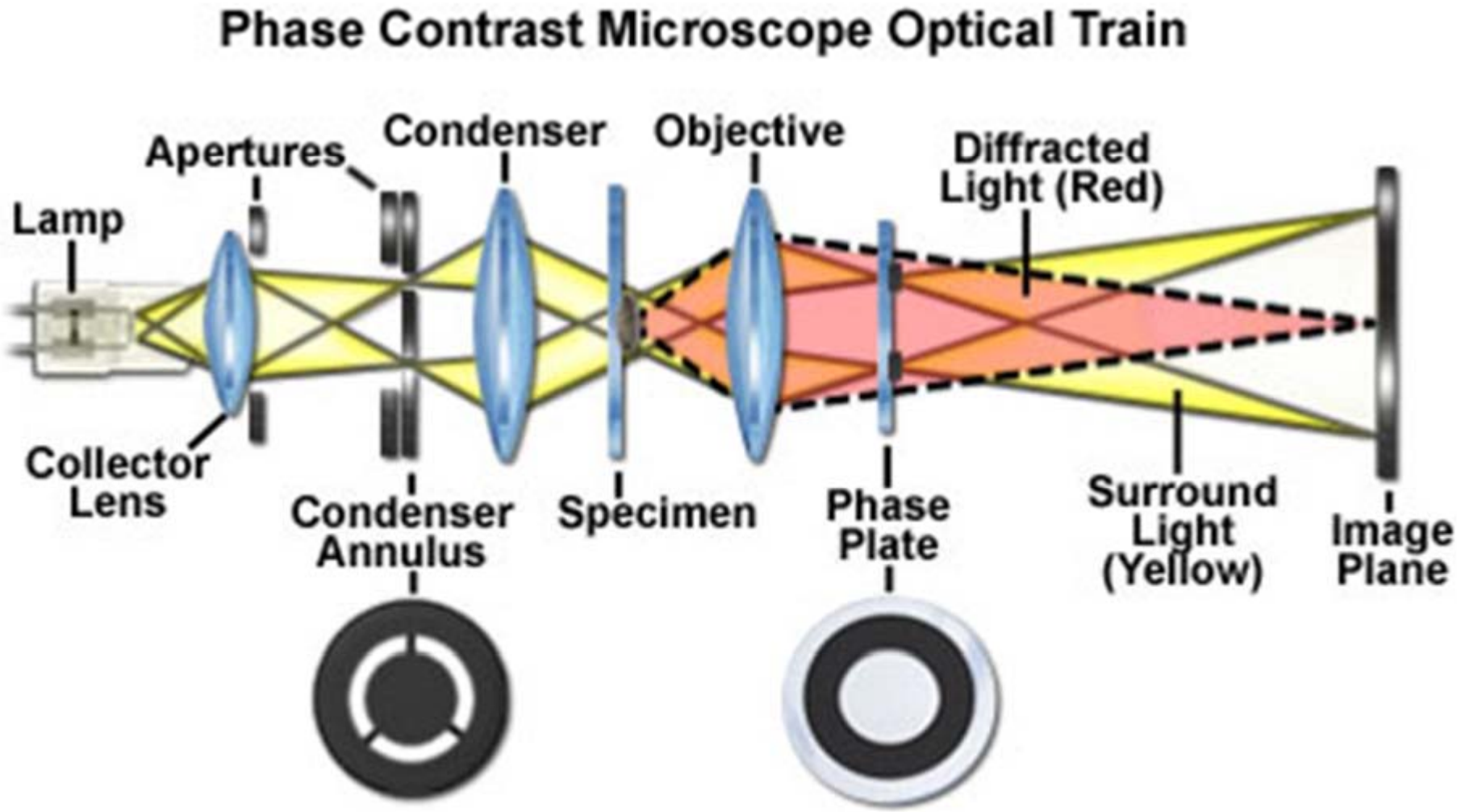
- Certain bacteria and fungi can be studied with the use of dark field microscopes.

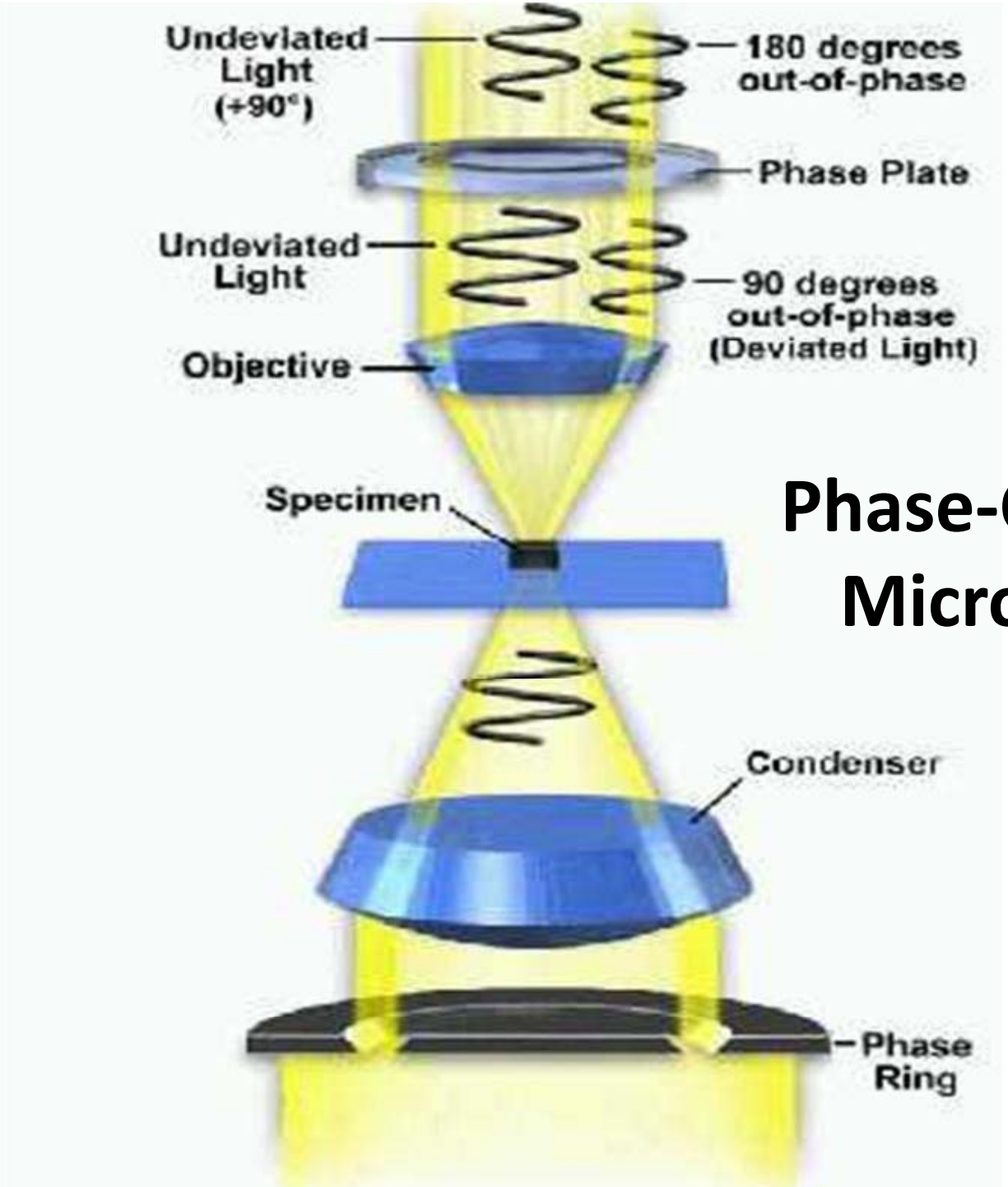
Phase-Contrast Microscopy

Phase contrast microscopy is an optical illumination technique in which small phase shifts in the light passing through a transparent specimen are converted into amplitude or contrast changes in the image.

The technique was invented by Frits Zernite in the 1930s for which he received the Nobel prize in physics in 1953 .

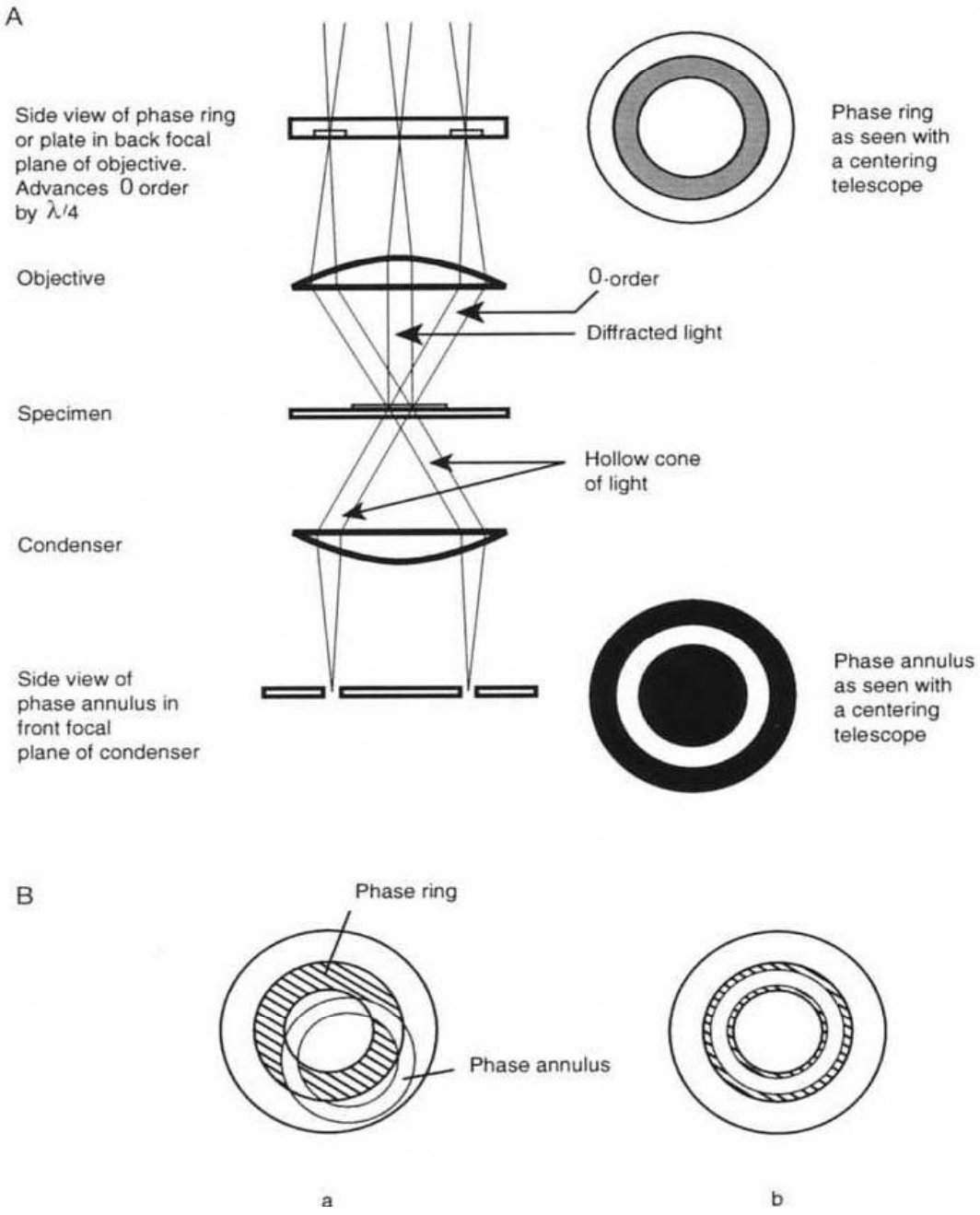
- Enhances the contrast between intracellular structures having slight differences in refractive index
- Excellent way to observe living cells
- Combines bright and dark field microscopy
- Diffracted light from hollow cone passed through special objective lens
- Resolving power – 0.2 µm
- Good for viewing living specimens


Phase Contrast Microscope Optical Train
Lamp
Apertures
Condenser
Objective
Diffracted Light (Red)
Collector Lens
Condenser Annulus
Specimen
Phase Plate
Surround Light (Yellow)
Image Plane

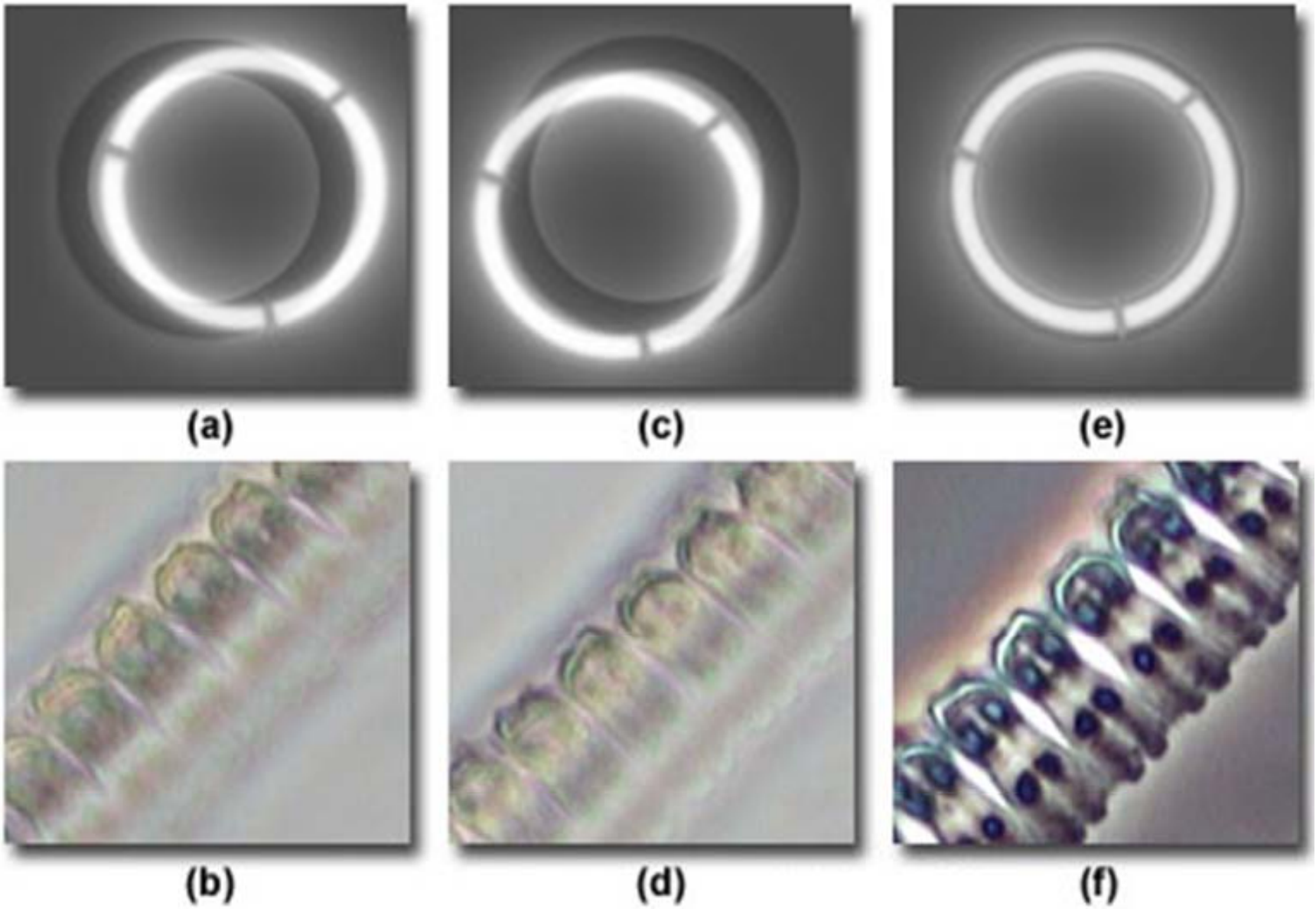


Phase-Contrast Microscopy

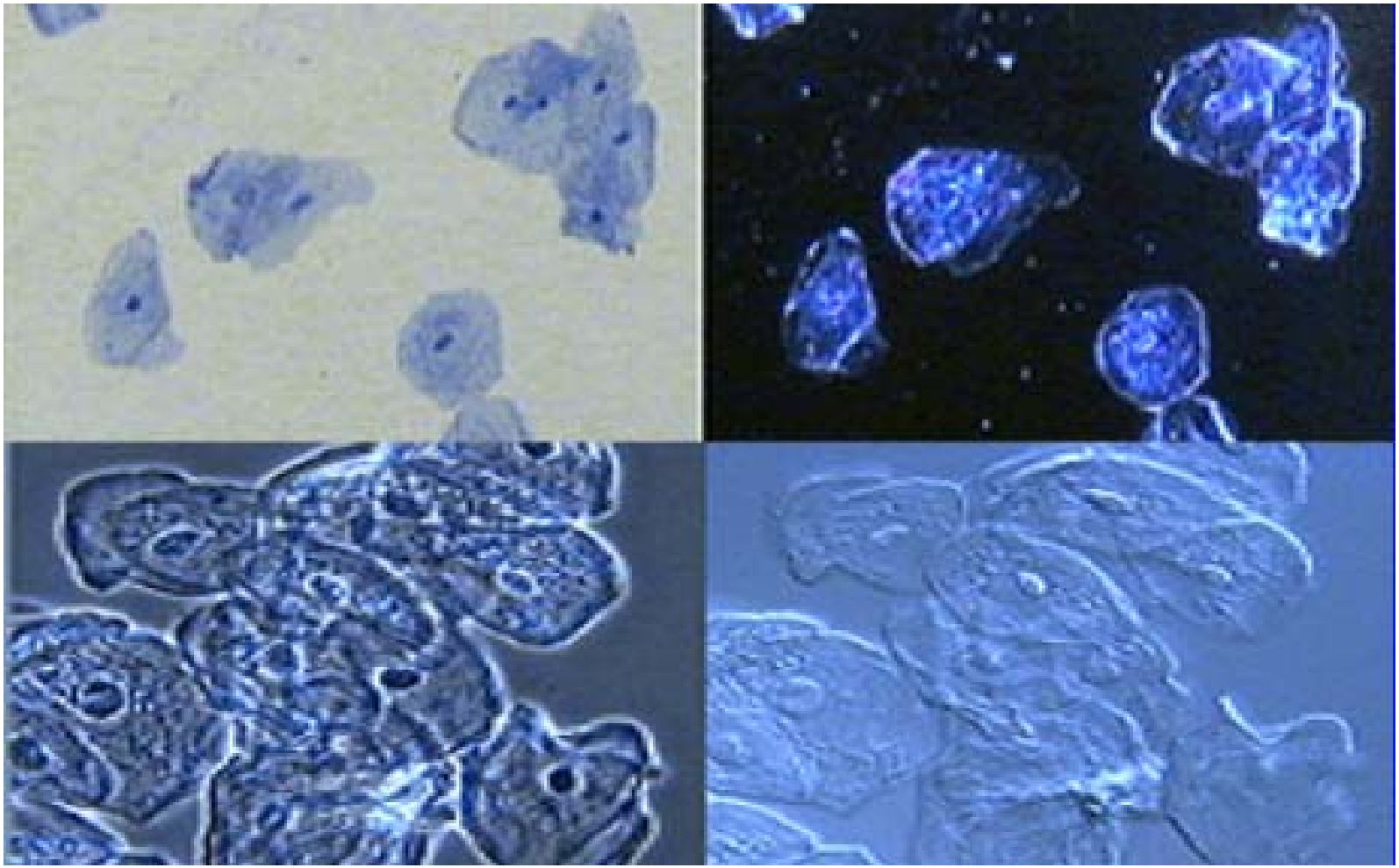
Phase contrast



Phase Contrast Optical System Alignment



Bright Field **Dark Field**



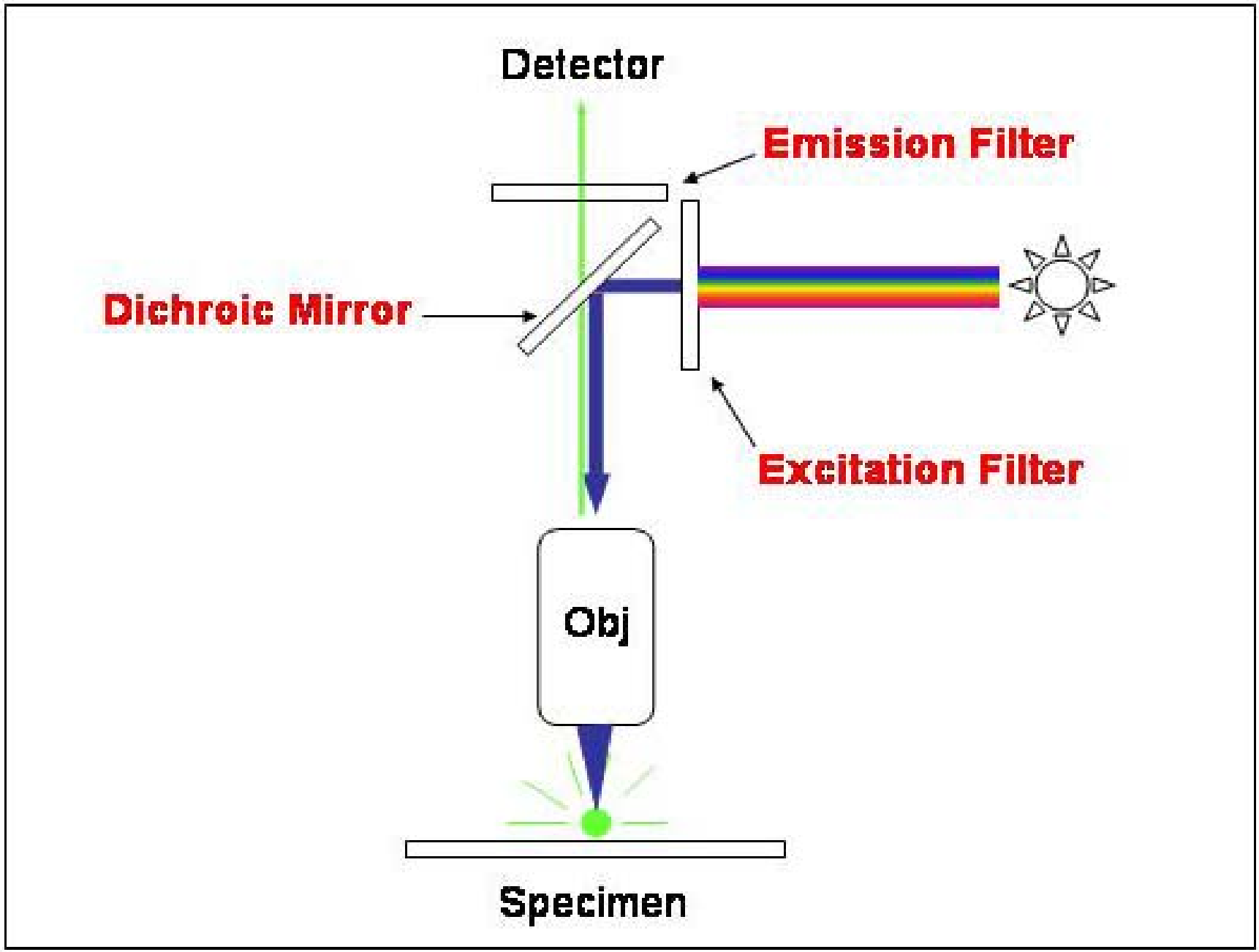
Phase Contrast

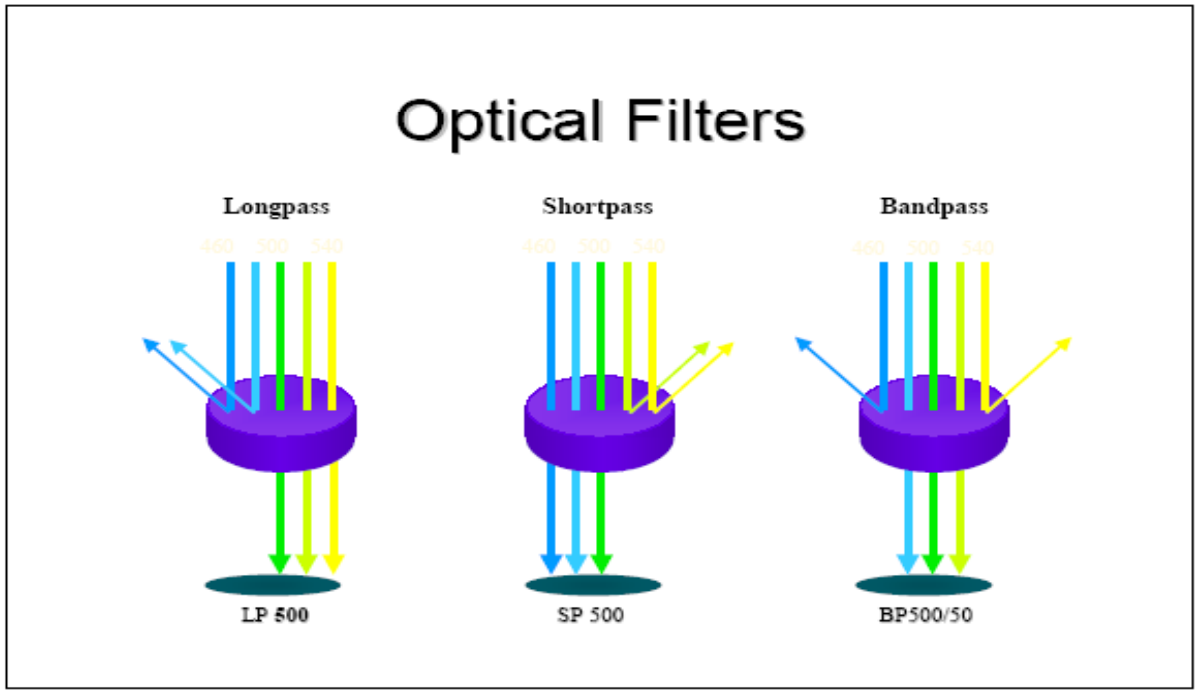
Application

- Applications for phase contrast microscopy equipment range from the study of living biological specimens, medical applications, study of live blood cells, and other biological and science applications
- Most commonly used to provide constrast of transparent specimens such as living cells or small organisms.
- Phase contrast microscopy is used in study of living cells and tissues.
- Microbes and parasites can be study .
- Useful in observing cells cultured in vitro during mitosis.

Fluorescent Microscope

- A component of interest in the specimen is specifically labeled with a fluorescent molecule called a fluorophore
- The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light).
- Typical components of a fluorescence microscope are the light source (xenon arc lamp or mercury-vapor lamp), the excitation filter, the dichroic mirror and the emission filter.
- Exposes specimen to ultraviolet, violet or blue light
- Specimens usually stained with **fluorochromes**
- Shows a bright image of the object resulting from the fluorescent light emitted by the specimen


Detector
Emission Filter
Dichroic Mirror
Excitation Filter
Obj
Specimen

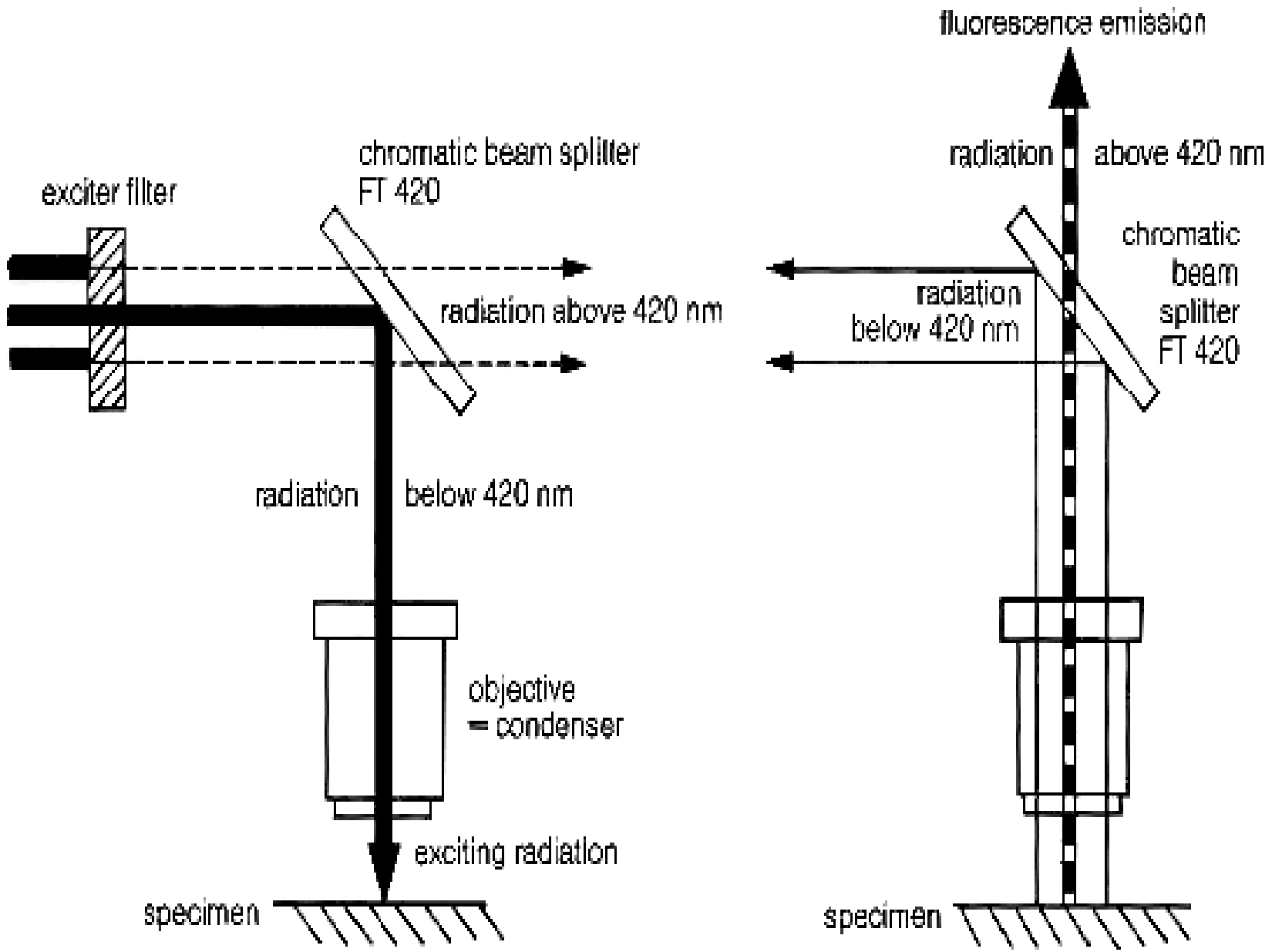


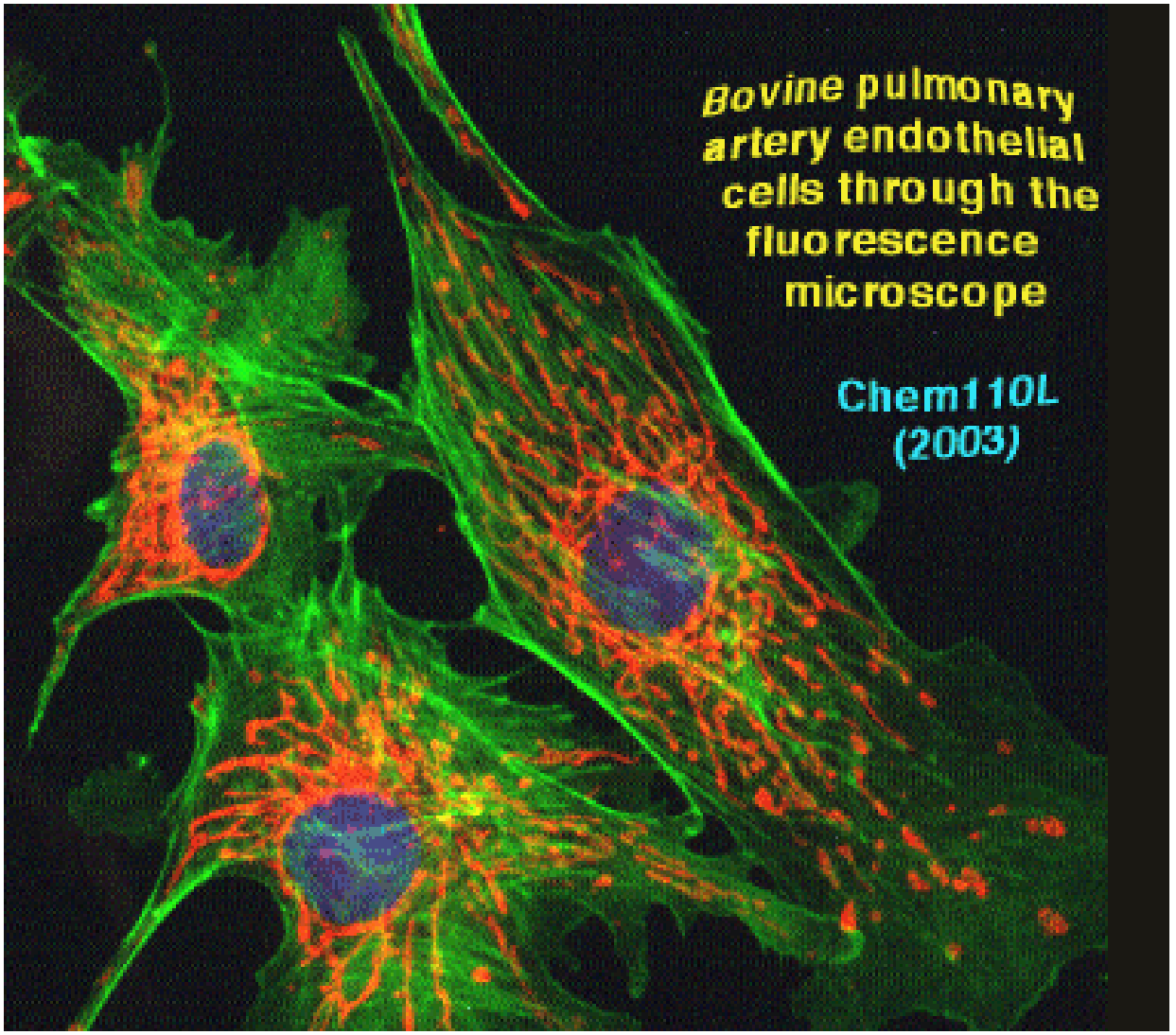
Long Pass filters: Transmit wavelengths above cut – on wavelength

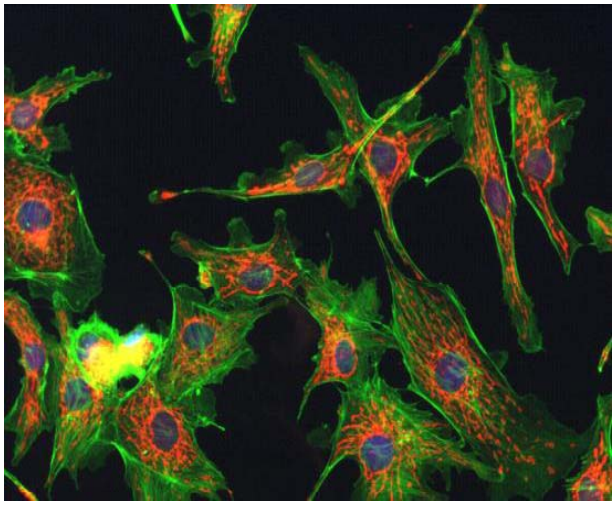
Short Pass filters: Transmit wavelengths below a cut – off wavelength

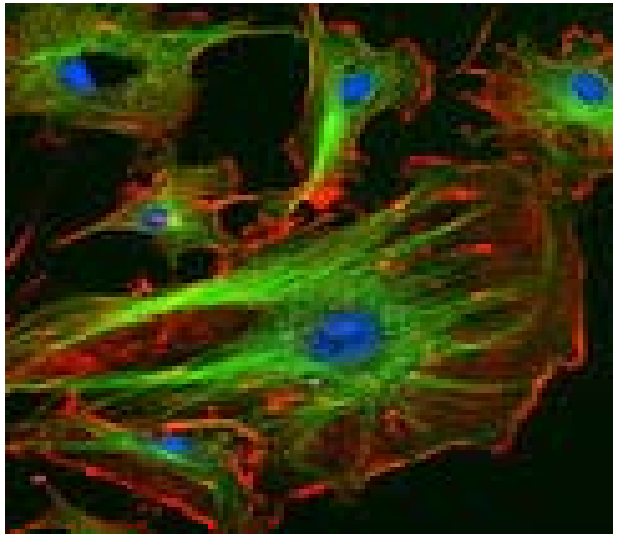
Band Pass filters: Transmit wavelengths in a narrow range around a specified wavelength

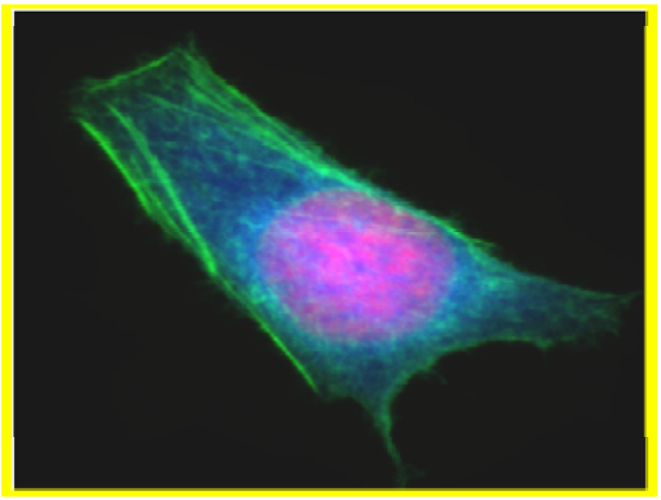
Dichroic Filters Function as Long pass Filters with Minimal Absorption Losses

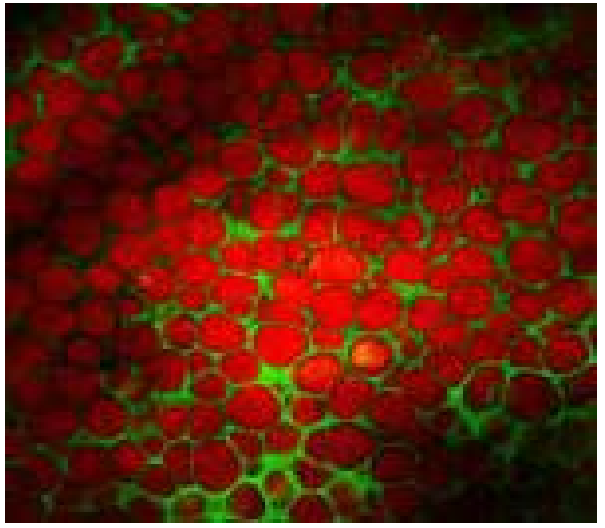

exciter filter
chromatic beam splitter
FT 420
radiation above 420 nm
radiation
below 420 nm
objective
= condenser
exciting radiation
specimen
fluorescence emission
radiation
above 420 nm
chromatic
beam
splitter
FT 420
radiation
below 420 nm
specimen


Bovine pulmonary artery endothelial cells through the fluorescence microscope
Chem110L (2003)









Applications

- Fluorescence microscopy is a critical tool for academic and pharmaceutical research, pathology, and clinical medicine.

Confocal Microscopy

- Confocal scanning laser microscope
- Laser beam used to illuminate spots on specimen
- Computer compiles images created from each point to generate a 3-dimensional image

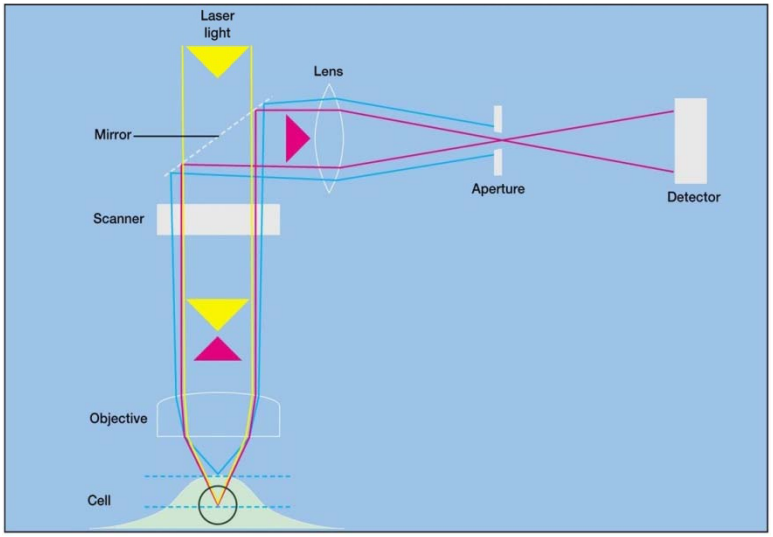
History of Confocal Microscopy

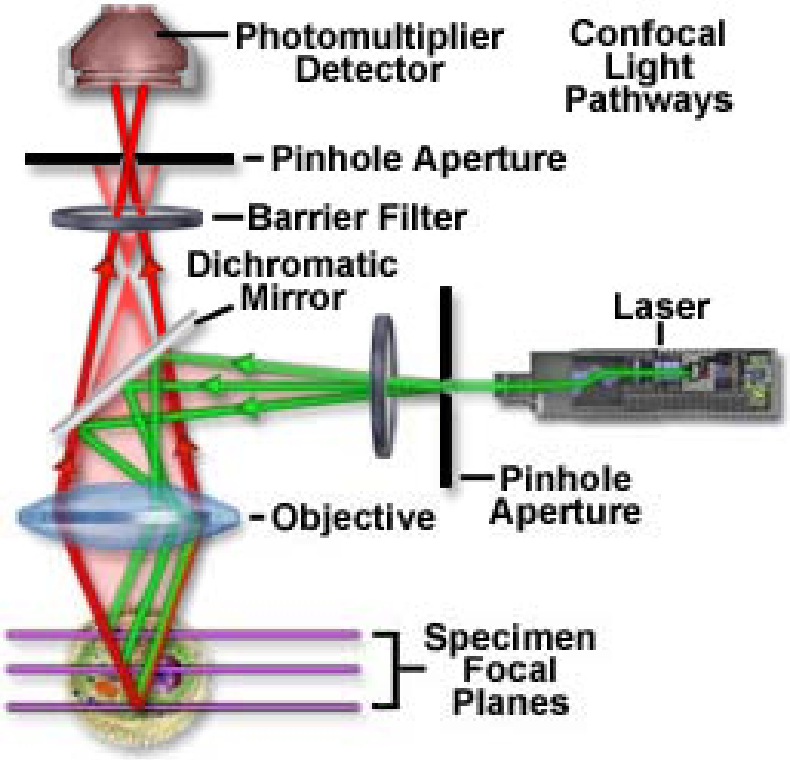
- Two investigators at Cambridge, **Brad Amos and John White** were attempting to look at the mitotic divisions in the first few divisions in embryos of *C. elegans*.
- They were doing antitubulin immunofluorescence and were trying to determine the cleavage planes of the cells, but were frustrated in their attempt in that the majority of the fluorescence they observed was out of focus no matter how much they adjusted the focus.
- They looked at the technique called **confocal imaging** which was **first proposed by Nipkow** and pioneered by a **postdoc at Harvard named Minsky** who made the first stage scanning confocal microscope **in 1957**. His microscope was commercially unfeasible because the technology needed to produce useful images was not available at the time.
- **In 1986-87**, a confocal microscope with the capabilities of producing very useful images could be built by combining the technologies of the laser, the computer, and microelectronics. **Amos and White** built the first prototype incorporating the technologies and obtained much better in-focus confocal images of the *C. elegans* embryos.

Basic Principle and Microscope Design

- The principle of confocal imaging was patented by Marvin Minsky in 1957.
- In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source. Due to the conservation of light intensity transportation, all parts of specimen throughout the optical path will be excited and the fluorescence detected by a photodetector or a camera.
- In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images.
- As only one point is illuminated at a time in confocal microscopy, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel scanning lines) in the specimen.
- The thickness of the focal plane is defined mostly by the square of the numerical aperture of the objective lens, and also by the optical properties of the specimen and the ambient index of refraction.

Basic Principle and Microscope Design





- "**Confocal**" is defined as "**having the same focus**." this means that the final image has the same focus as or the focus corresponds to the point of focus in the object. **The object and its image are "confocal**."
- The confocal microscope eliminates this out-of-focus information by means of a confocal "pinhole" situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light to be imaged.
- A Laser beam as a source of light
- Photomultiplier Detectors
- Pinhole Apertures
- A computer attached to the microscope for the storage and display of memory
- Dichromatic mirror

Working of a confocal Microscope

- The point is a diffraction limited spot on the specimen and is produced either by imaging an illuminated aperture situated in a conjugate focal plane to the specimen or more usually by focusing a parallel laser beam.
- With only a single point illuminated, the illumination intensity rapidly falls off above and below the plane of focus as the beam converges and diverges, thus reducing excitation of fluorescence for interfering objects situated out of the focal plane being examined.
- Fluorescent light (i.e. signal) passes back through the dichroic reflector and then passes through a pinhole aperture situated in a conjugate focal plane to the specimen. Any light emanating from regions away from the vicinity of the illuminated point will be blocked by the aperture, thus providing further attenuation of out-of focus interference.

Working of a confocal Microscope

- Light passing through the image pinhole is detected by a **photodetector**. Usually a computer is used to control the sequential scanning of the sample and to assemble the image for display onto a video monitor.
- Most confocal microscopes are implemented as imaging systems that couple to a conventional microscope.
- Most confocal imaging systems provide adjustable pinhole blocking apertures. This enables a tradeoff to be made in vertical resolution and sensitivity. **A small pinhole gives the highest resolution and lowest signal and vice versa**.
- The **photodetectors** and most commonly used are **photomultipliers**. These have reasonable sensitivity in the blue regions of the spectrum but markedly fall of in sensitivity in the red.

Laser Systems for Confocal Microscopy

- The lasers commonly employed in laser scanning confocal microscopy are **high-intensity monochromatic light sources**, which are useful as tools for a variety of techniques including Optical trapping
  - Lifetime imaging studies
  - Photobleaching recovery
  - Total internal reflection fluorescence.
- In addition, lasers are also the most common light source for scanning confocal fluorescence microscopy, and have been utilized, although less frequently, in conventional widefield fluorescence investigations.

Interference Filters for Fluorescence Microscopy

- The performance of high-resolution fluorescence microscopy imaging systems and related quantitative applications, especially as applied in living cell and tissue studies, requires precise optimization of fluorescence excitation and detection strategies.
- Fluorescence microscopy techniques could not have advanced so dramatically in recent years without significant developments in every dimension of the current state of the art, including the optical microscopes, the biology and chemistry of fluorophores, and perhaps most important, filter technology.
- The utilization of highly specialized and advanced thin film **interference filters** has enhanced the versatility and scope of fluorescence techniques, far beyond the capabilities afforded by the earlier use of gelatin and glass filters relying on the absorption properties of embedded dyes.

Resolution and Contrast in Confocal Microscopy

- All optical microscopes, including conventional widefield, confocal, and two-photon instruments are limited by fundamental physical factors in the resolution that they can achieve.
- In a perfect optical system, resolution is limited by **numerical aperture** of the optical components and by the **wavelength** of the light, both incident and detected.
- The concept of resolution is inseparable from contrast, and is defined as the minimum separation between two points that results in a certain contrast between them.
- Contrast is determined by the **number of photons** collected from the specimen, the **dynamic range** of the signal, **optical aberrations** of the imaging system, and the **number of picture elements (pixels)**

Non-Coherent Light Sources for Confocal Microscopy

- The **traditional illumination** system in the modern widefield microscope utilizes a **tungsten-halogen** source for transmitted light and a **short-arc lamp** for fluorescence excitation.
- Various lasers have been utilized as a light source for widefield observation, but the laser is being used in confocal microscopy.
- The **objective** is the most critical component of the system in determining the information content of the image.
- The **contrast, resolution** of fine specimen detail, the **depth** within the specimen from which information can be obtained and the **lateral extent** of the image field are all determined by the design of the objective.
- The crucial imaging component serves as the **illumination condenser** and is often required to perform with high precision at a wide range of wavelengths and at very low light levels without introducing unacceptable image-degrading

Confocal Microscope Scanning Systems

- Confocal imaging relies upon the sequential collection of light from spatially filtered individual specimen points, followed by electronic signal processing and ultimately, the visual display as corresponding image points.
- The point-by-point signal collection process requires a mechanism for scanning the focused illuminating beam through the specimen volume under observation.
- Three principal scanning variations are commonly employed to produce confocal microscope images. Fundamentally equivalent confocal operation can be achieved by employing

    1. A laterally translating specimen stage coupled to a stationary illuminating light beam (**stage scanning**),

    2. A scanned light beam with a stationary stage (**beam scanning**), or by maintaining both the stage

    3. Light source stationary while scanning the specimen with an array of light points transmitted through apertures in a spinning **Nipkow disk**.

Electronic Imaging Detectors

- Over the past several years, the rapidly growing field of fluorescence microscopy has evolved from a dependence on traditional photomicrography using emulsion-based film to one in which electronic images are the output of choice.

- The imaging device is one of the most critical components in fluorescence microscopy **because it determines at what level specimen fluorescence may be detected**, the relevant structures resolved, and/or the dynamics of a process visualized and recorded.

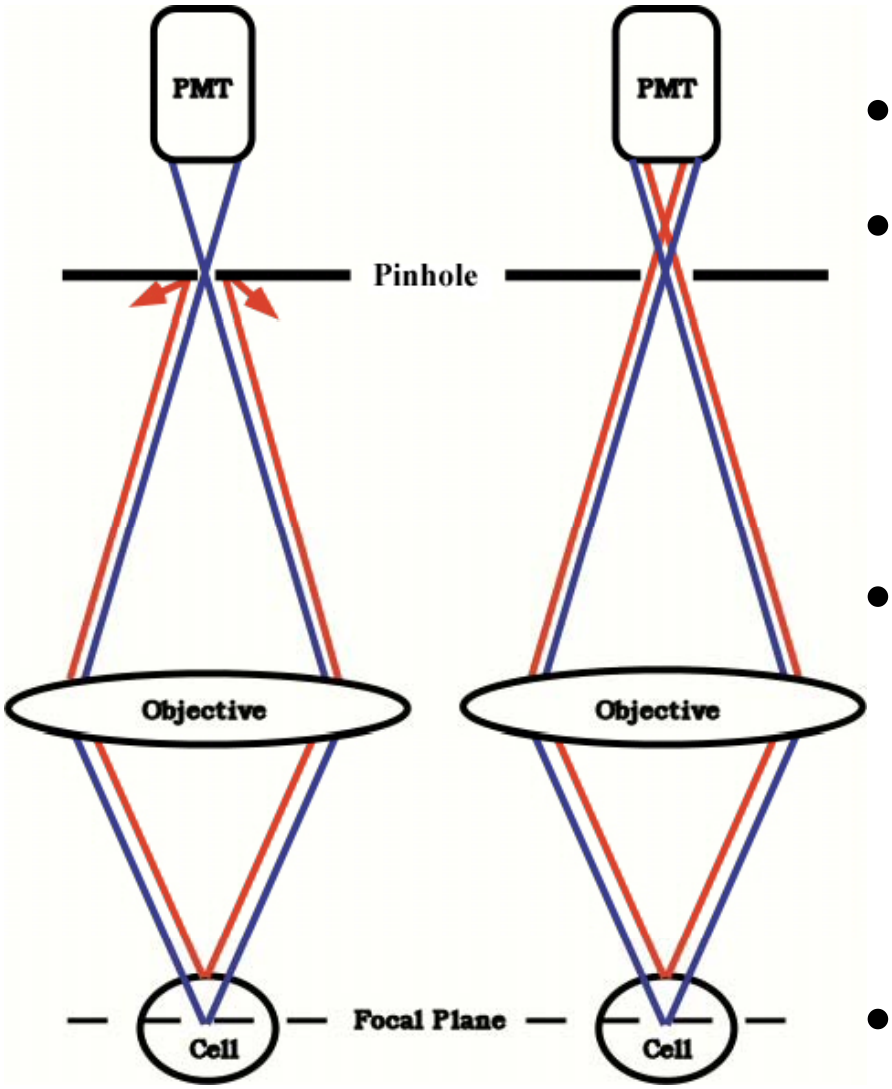
Electronic Light Detectors

- In modern widefield fluorescence and laser scanning confocal optical microscopy, the collection and measurement of secondary emission gathered by the objective can be accomplished by several classes of photosensitive detectors, including **photomultipliers**, **photodiodes**, and **solid-state charge-coupled devices** (**CCDs**).
- In confocal microscopy, fluorescence emission is directed through a pinhole aperture positioned near the image plane to exclude light from fluorescent structures located away from the objective focal plane, thus reducing the amount of light available for image formation.
- The exceedingly low light levels in confocal microscopy necessitate the use of highly sensitive photon detectors that do not require spatial discrimination, but instead respond very quickly with a high level of sensitivity to a continuous flux of varying light intensity.

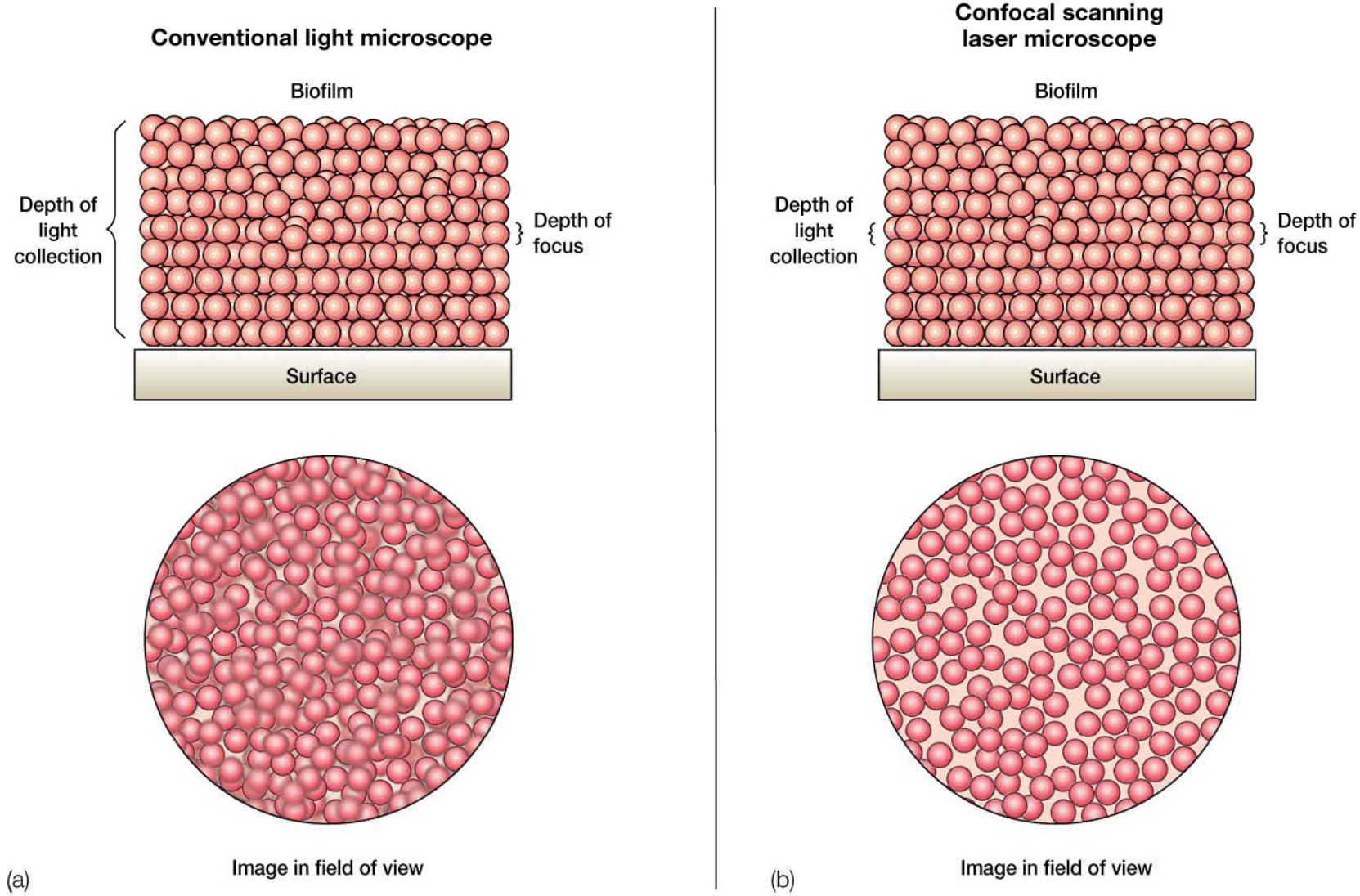
Important features of Confocal Microscope

- The capablity of isolating and collecting a plane of focus from within a sample, thus eliminating the out of focus "haze" normally seen with a fluorescent sample. Fine detail is often obscured by the haze and cannot be detected in a non-confocal, fluorescent microscope.
- The confocal microscope has a stepper motor attached to the fine focus, enabling the collection of a series of images through a three dimensional object. These images can then be used for a two or three dimensional reconstruction.
- Double and triple labels can be collected with a confocal microscope. Since these images are collected from an optical plane within the sample, precise co-localizations can be performed. In a fluorescent microscope a small part of a sample may be in focus but we can look at the entire object (i.e. we view what is in focus as well as what is out of focus).
- With the confocal microscope, the z-resolution, or optical sectioning thickness, depends on a number of factors: the **wavelength** of the excitation/emission light, **pinhole size**, **numerical aperture** of the objective lens, **refractive index** of components in the light path and the **alignment** of the instrument.

Important features of Confocal Microscope



- The effect of the pinhole, or iris diaphragm, on the thickness of the optical plane that is collected.
- The pinhole and focal plane in the sample are at conjugate planes of focus.
- The small pinhole opening in the diagram on the left enables data collection from a thin optical plane within the specimen. Points that are out of the plane of focus (red) will have a different secondary focal plane thus, most of the data is deflected.
- Although some of the out-of-focus light enters the photomultiplier tube (PMT) in the figure on the left, the intensity is too dim to be visualized. All of the data at the plane of focus is collected (blue). In this manner, the confocal microscope can collect only the data from within the focal plane.
- The larger pinhole opening in the figure on the right allows both in-focus and out-of-focus data to be collected.


Conventional light microscope
Biofilm
Depth of light collection
Depth of focus
Surface
Image in field of view
(a)
Confocal scanning laser microscope
Biofilm
Depth of light collection
Depth of focus
Surface
Image in field of view
(b)

Applications in Confocal Microscopy

- The broad range of applications available to laser scanning confocal microscopy includes a wide variety of studies in **neuroanatomy** and **neurophysiology**, as well as morphological studies of a **wide spectrum of cells and tissues**.
- In addition, the growing use of new **fluorescent proteins** is rapidly expanding the number of original research reports coupling these useful tools to modern microscopic investigations.
- Other applications include **resonance energy transfer**, **stem cell research**, **photobleaching studies**, **lifetime imaging**, **multiphoton microscopy**, **total internal reflection**, **DNA hybridization**, **membrane and ion probes**, **bioluminescent proteins** and **epitope tagging**.

Chameleons: Calcium Ion Probes

- Chameleons are a new class of indicators for calcium ion concentrations in living cells, which operate through a conformational change that results in fluorescence resonance energy transfer (**FRET**) in the presence of calcium ions.
- In the past, fluorescent probes, such as Fura-2, Indo-1, and Fluo-3 were very popular for measuring fluctuations in calcium ion concentrations within living cells.
- In 1997, **Dr. Atsushi Miyawaki** (of the Riken Brain Science Institute in Wako, Japan) developed a novel probe for calcium ion measurement. This probe consists of an artificial protein modified from green fluorescent protein (**GFP**), and was named **Chameleon** (after the chameleon reptile).
- The chameleon molecular structure is modeled as a fusion product between two fluorescent proteins (having differing excitation and emission characteristics), calmodulin (**CaM**), and the calmodulin-binding domain of myosin light chain kinase (**M13**).
- Calmodulin is capable of binding with free calcium ions and the M13 chain can bind with calmodulin after it has bound the calcium ions. The genes of these four proteins are joined linearly, and the fusion genes are expressed in a variety of cells.

Advantages of Confocal Microscopy over Conventional Microscopy

1. Light rays from **outside the focal plane** will not be recorded.
2. Defocusing **does not create blurring**, but gradually cuts out parts of the **object as they move away from the focal plane**. Thus, these parts become darker and eventually disappear. This feature is called **optical sectioning**.
3. True, three-dimensional data sets can be recorded.
4. Scanning the object in x/y-direction as well as in z-direction (along the optical axis) allows viewing the objects from all sides.
5. Due to the small dimension of the illuminating light spot in the focal plane, **stray light** is minimized.
6. By image processing, many slices can be superimposed, giving an extended focus image which can only be achieved in conventional microscopy by reduction of the aperture and thus sacrificing resolution.

Limitations of Confocal Microscopy

- Point-scanning microscopes, when used with high numerical aperture lenses, have an inherent speed limitation in fluorescence. This arises because of a limitation in the amount of light that can be obtained from the small volume of fluorophore contained within the focus of the scanned beam (<micron^3).
- At moderate levels of excitation, the amount of light emitted will be proportional to the intensity of the incident excitation. However, fluorophore excited states have significant lifetimes (in the order if a few nanosecond).
- As the level of excitation is increased, the situation eventually arises when most of the fluorophore molecules are pumped up to their excited state and the ground state becomes depleted. At this stage the fluorophore is saturated and no more signal may be obtained from it by increasing the flux of the excitation source.

Limitations of Confocal Microscopy

- Most commercial scanning beam confocal microscopes have laser excitation sources that give around 10 mw in the major spectral lines.
- Power level will cause saturation giving image degradation when the spectral line is near the excitation peak of the fluorophore being used (e.g. the 488 nm argon line and fluorescein) and a high numerical aperture lens used (>1.0 NA)
- Better images will be obtained by reducing the power by a factor of 10 or 100. This limits the speed which an image with a given signal-to noise ratio can be acquired. Typically, about 5 -10 seconds of integration is required with an average immunofluorescence preparation.

Co-localization of Fluorophores in Confocal Microscopy

- During the examination and digital recording of **multiply labeled** fluorescent specimens, two or more of the emission signals can often overlap in the final image due to their close proximity within the microscopic structure.
- This effect is known as **co-localization** and usually occurs when fluorescently labeled molecules bind to targets that lie in very close or identical spatial positions. The application of highly specific modern synthetic fluorophores and classical immunofluorescence techniques, coupled with the precision optical sections and digital image processing horsepower afforded by confocal and multiphoton microscopy, has dramatically improved the ability to detect colocalization in biological specimens.

ELECTRON MICROSCOPY

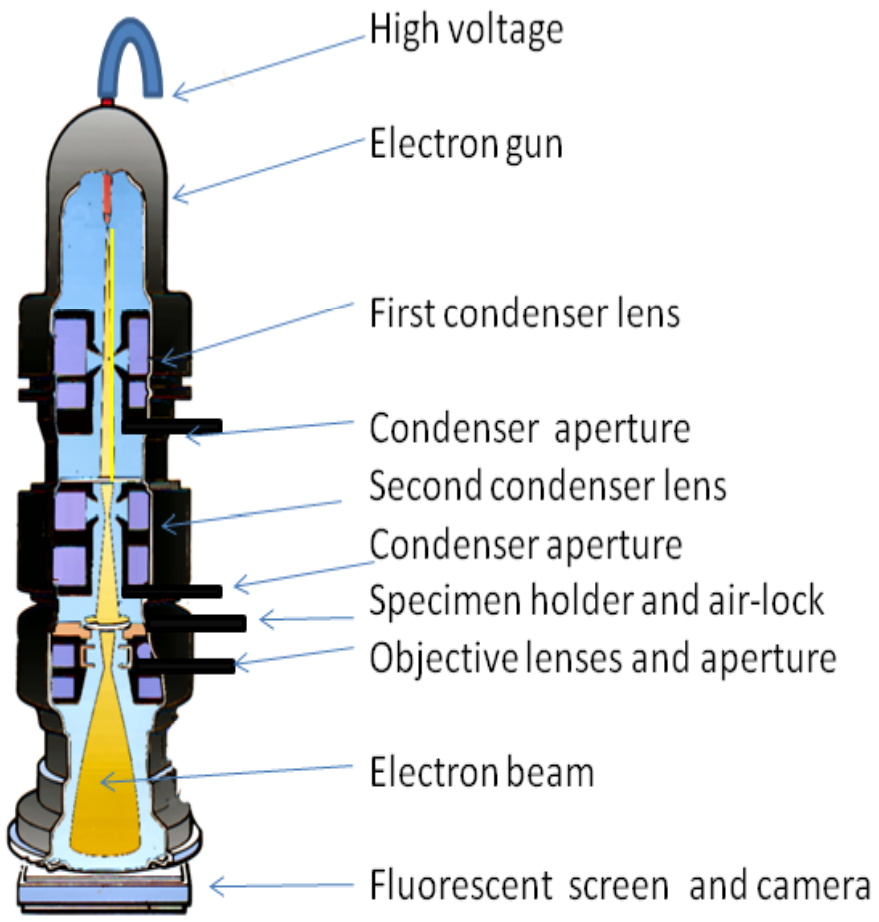
INTRODUCTION

- Electron microscope is a type of microscope that uses a particle beam of electrons to illuminate a specimen & create a highly-magnified image.
- Co-invented by Germans, Max Knoll and Ernst Ruska in 1931

- Electron microscopes have much greater resolving power than light microscopes & can obtain much higher magnifications of up to 2 million times, while the best light microscopes are limited to magnifications of 2000 times.

- Can be used to study the Topography, Morphology, Composition & Crystallographic Information.

Structure



Transmission Electron Microscope

Structure

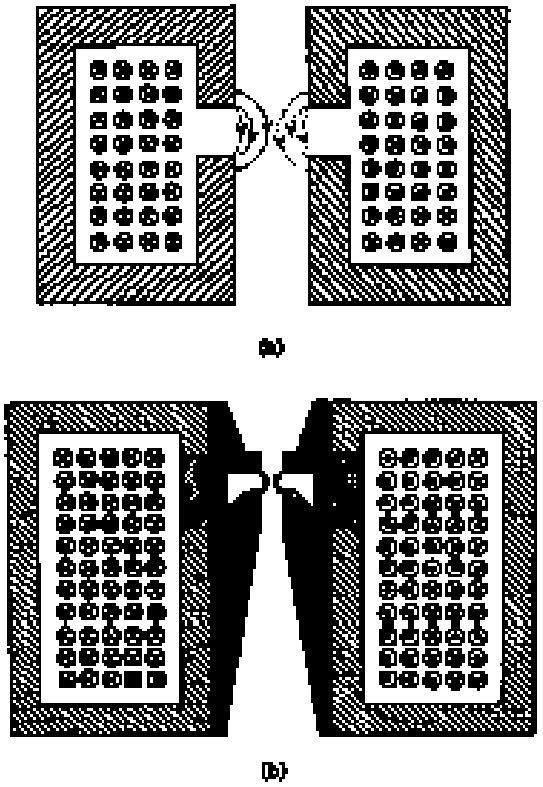
- Electron Gun: 2 types of guns- Thermionic Emission Gun & Field Emission Gun.
- Thermionic: Electrons emitted from heated filament (tungsten, Lanthanum Hexaboride). Most common, cheap & ultra high vaccum not required.
- Field Emission: Strong electron field used to extract electrons from filament. High vaccum needed.

Structure

- **Electromagnetic lens:**

  An electromagnet designed to produce a suitably shaped magnetic field for focusing & deflection of electrons in electron optical instruments.

- A strong magnetic field is generated by passing a current through a set of windings. This field acts as a convex lens in case of electron microscope.



Structure

- **First condenser lens:** The first lens (controlled by "spot size knob") largely determines the "spot size"; the general size range of the final spot that strikes the sample.

- **Second condenser lens:** The second lens(controlled by the "intensity/ brightness knob" changes the size of the spot on the sample; changing it from a wide dispersed spot to a pinpoint beam.

Structure

- The other parts include condenser aperture, objective lens, objective aperture, selected area aperture (to examine diffraction patterns), Intermediate lens (magnifies initial image formed by objective lens) & projector lens.

TYPES

- There are 2 types of electron microscopes:

  Transmission Electron Microscope:

- Process is carried out under vaccum to avoid friction.
- The "Virtual Source" at the top represents the electron gun, producing a stream of monochromatic electrons. The usual potential is around 10000 – 15000V.

Electron Microscopy

- Electrons are scattered easily.
- The specimen used in electron microscopy must be **extremely thin** (ultrathin, i.e., **1 nm to 10 nm**).
- Electrons are scattered even by gas molecules
- Very **high vacuum and the samples must be completely dry** and otherwise **non-volatile**.
- Thus, **living cells** which are **wet cannot be viewed** in electron microscope.
- Better than 50 pm **resolution** in annular dark-field imaging mode
- Magnifications of up to about 10,000,000×
- Scanning : 3D image.

Summary of TEM

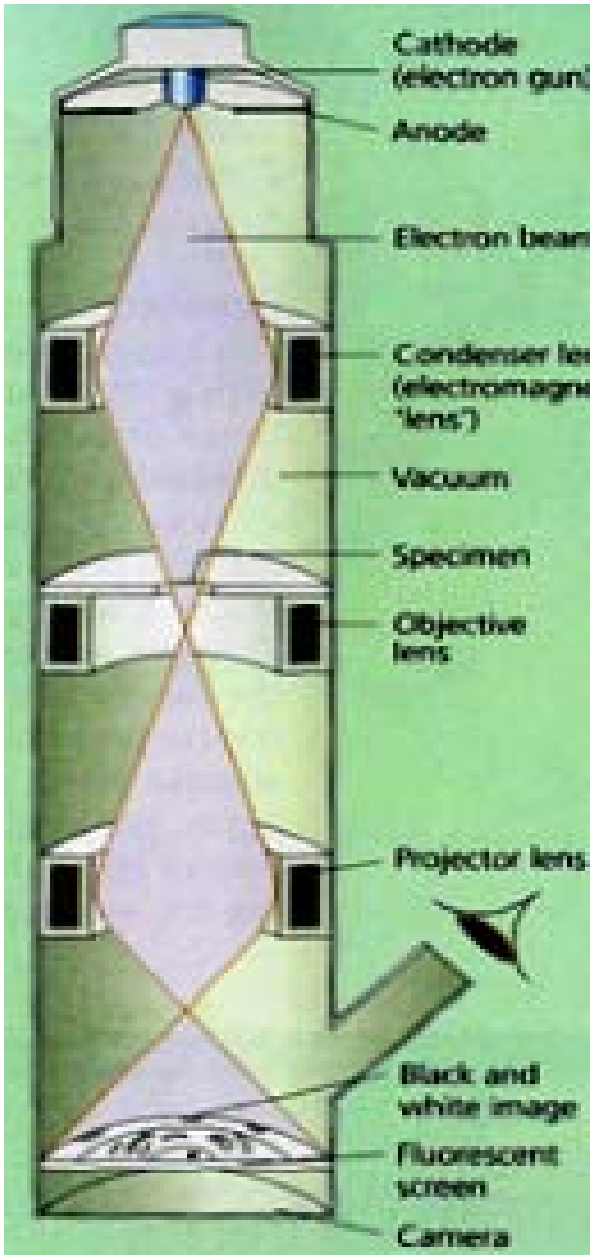
In TEM

The electrons pass through the specimen

Specimen needs to be extremely thin 10 nm to 100 nm

TEM can magnify objects up to 500000 times

TEM has made it possible to see the details of the discover new organelles



The electron microscopy uses the much shorter wavelengths of electrons to achieve resolution as low as 3 Å with a usual working range between 5 to 12 Å.

In the electron microscope electromagnetic coils (i.e., magnetic “lenses) are used to control and focus a beam of electrons accelerated from a heated metal wire by high voltages in the range of 20000 to 100000 volts.

The wavelength of an electron depends on the magnitude of the voltage and may be 0.01 Å or less.

The electron of the beam are scattered by a specimen placed in the path of the beam.

Electrons that do manage to pass through the specimen are focused by an objective coil (‘lens’) and a final magnified image is produced by a projector coil or ‘lens’.

The final image is viewed directly on the fluorescent screen or is recorded on photographic film to produce electron micrograph. This type of electron microscope is called transmission electron microscope (TEM).

The electron microscopy uses the much shorter wavelengths of electrons to achieve resolution as low as 3 Å

Compound light microscope, in which image formation depends primarily upon differences in light absorption.

Electron microscope forms image as a result of differences in the way electrons are scattered by various regions of the object.

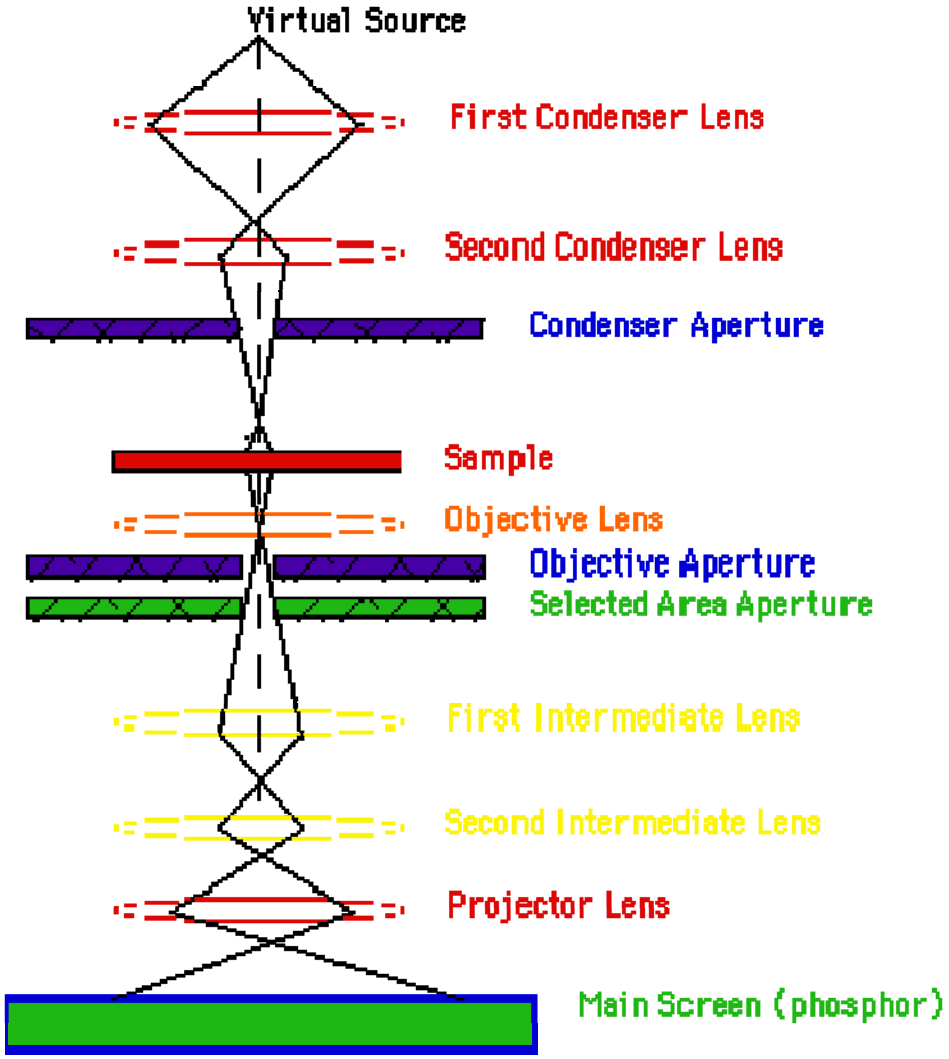
Electrons have a very low penetrating power that is they are easily scattered by objects in their paths.

The degree to which electrons are scattered is determined by the thickness and atomic density of the object.

Regions of high density (possessing atoms of high atomic number) scatter electrons more that region of lesser density and consequently appear darker in the final image.

TEM

- This stream is focused to a small, thin, coherent beam by the condenser lenses 1 & 2.
- The beam is restricted by condenser aperture knocking out high angle electrons.
- The beam strikes the specimen and parts of it are transmitted.



TEM

- This transmitted portion is focused by the objective lens into an image.
- The Objective & Selected Area metal apertures restrict the beam.
- The image is passed down the column through the intermediate and projector lenses, being enlarged all the way.
- The image strikes the phosphor image screen & light is generated, allowing the user to see the image. The darker areas represent areas that fewer electrons were transmitted (thicker or denser). The lighter areas represent areas that more electrons were transmitted (thinner or less dense)

TEM

- A TEM images using the electrons that pass through it- Unscattered Electrons, Elastically Scattered Electrons , Inelasticity Scattered electrons.
- TEM transmits electrons through a sample that has been cut so that it is only a few molecules thin & it reveals internal details of sample.
- Good resolution power up to 0.2 – 0.3nm.

SCANNING ELECTRON MICROSCOPE

History

- TEM constructed in 1931
- Von Ardenne first STEM in 1938 by rastering the electron beam in a TEM
- Zworykin et al. 1942, first SEM for bulk samples
- 1965 first commercial SEM by Cambridge Scientific Instruments

Resolution at that time ~ 50 nm : Today < 1 nm

Morphology only at that time : Today analytical instrument

What is SEM?

- It is a microscope that produces an image by using an electron beam that scans the surface of a specimen inside a vacuum chamber.

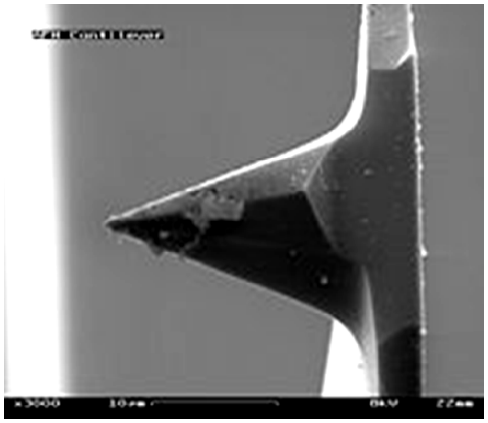
What can we study in a SEM?

- Topography and morphology
- Chemistry
- Crystallography
- Orientation of grains
- In-situ experiments:
  - Reactions with atmosphere
  - Effects of temperature

"Easy" sample preparation!!

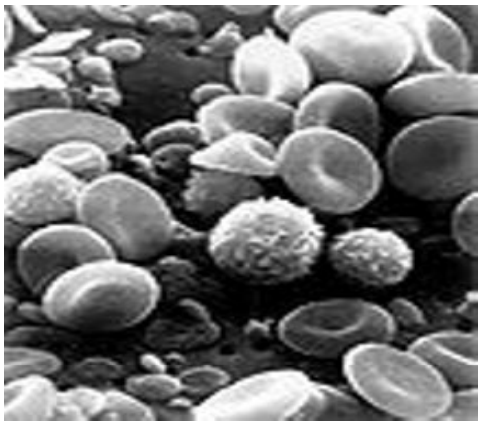
"Big" samples!

What does it looks like….

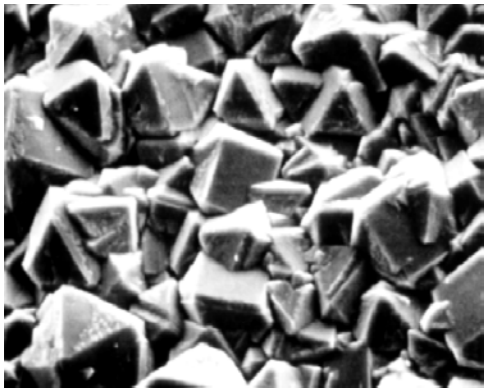


AFM Cantilever Tip

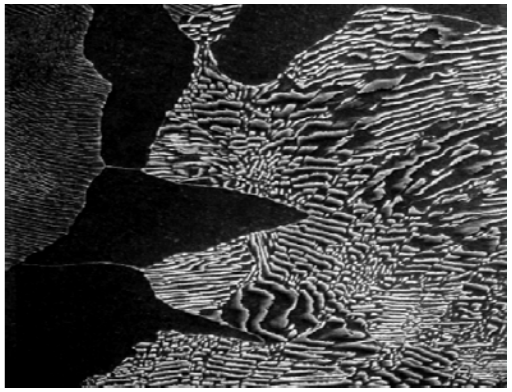
Ant Head



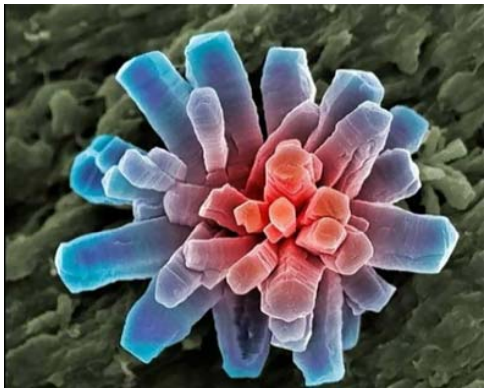
Blood Cells



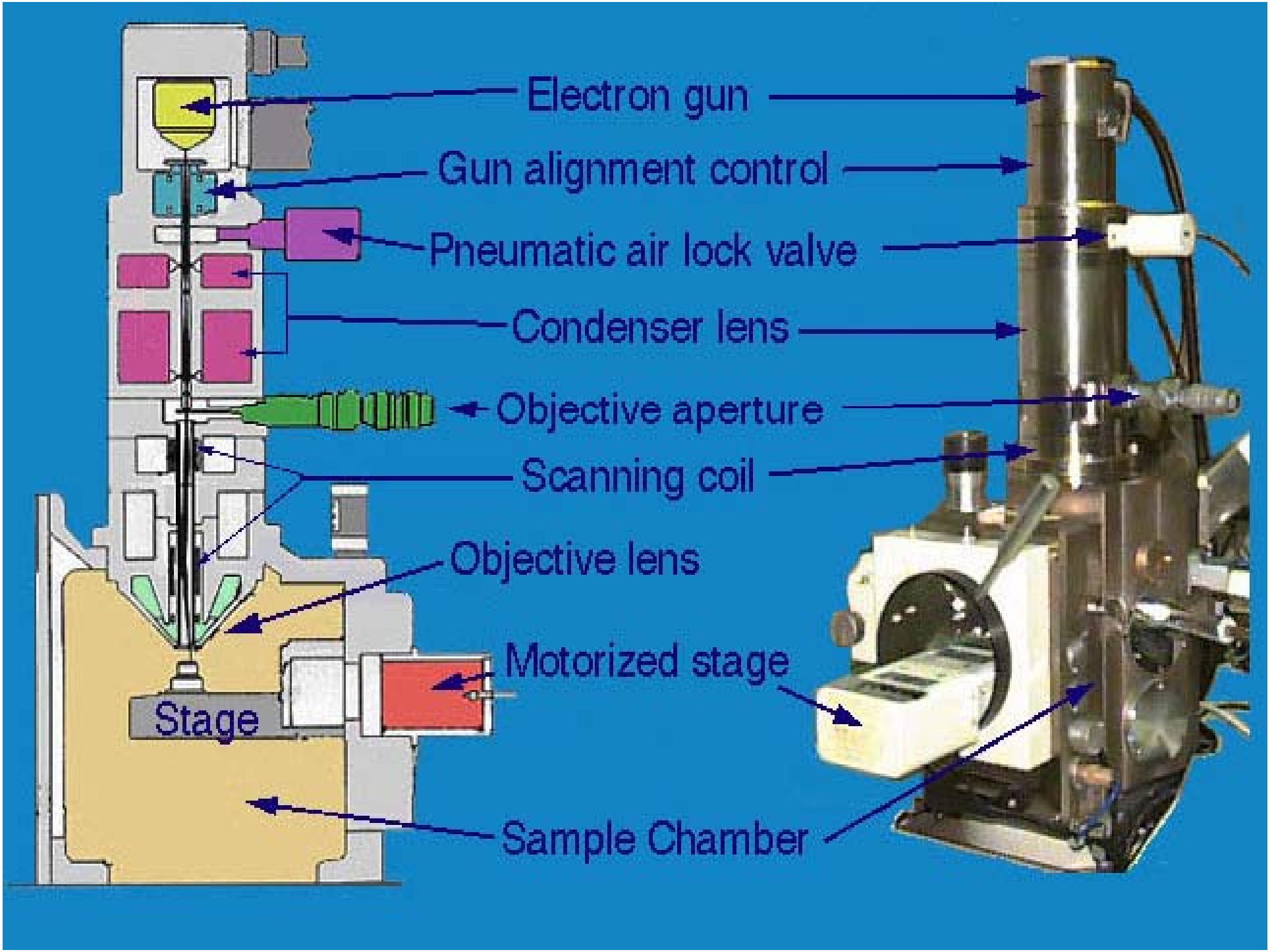
Diamond Thin Film (Numerous Multifaceted Micro-crystals)

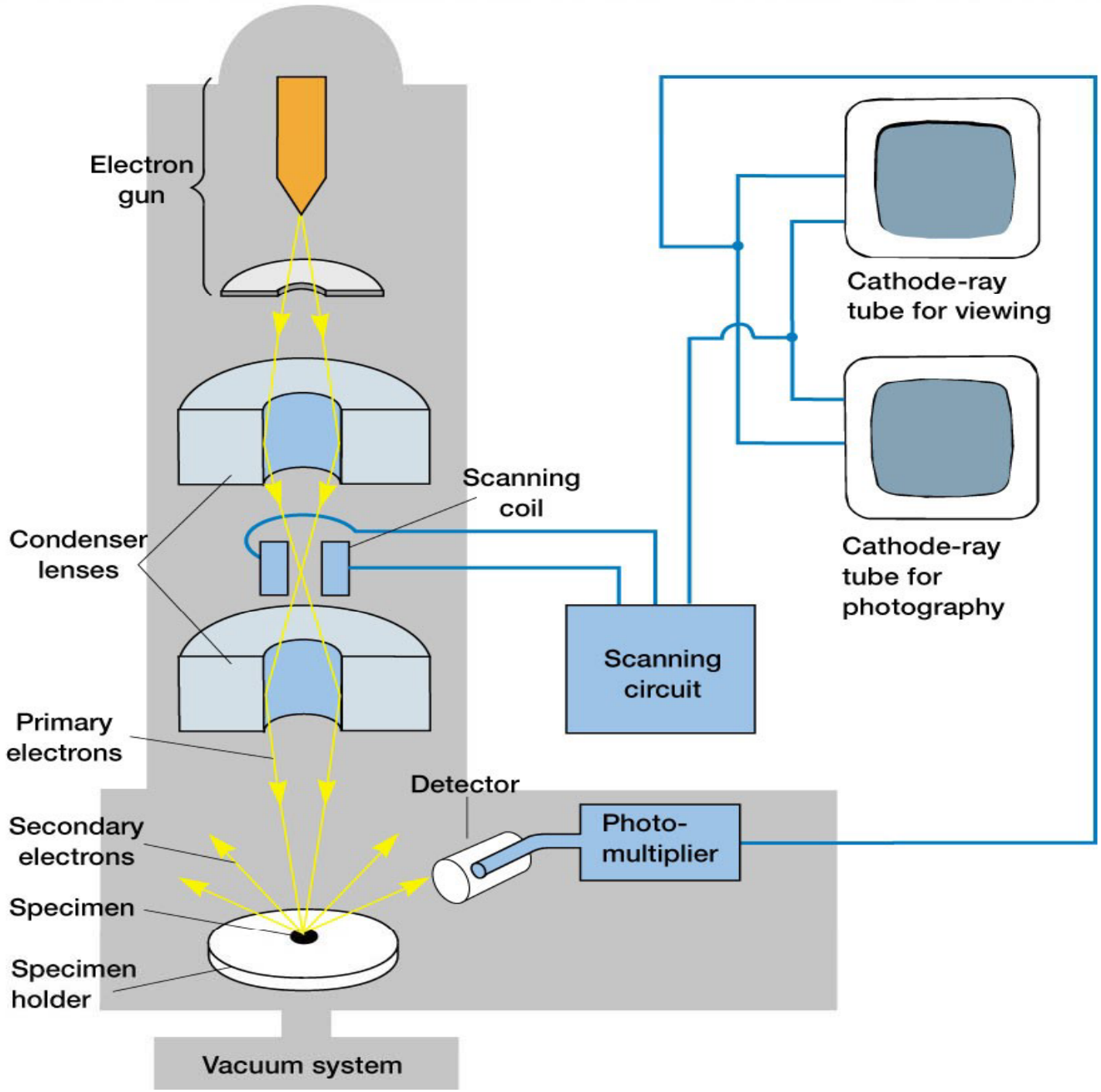


Microstructure of a plain carbon steel that contains 0.44 wt% of carbon



Calcium Phosphate Crystal


Electron gun
Gun alignment control
Pneumatic air lock valve
Condenser lens
Objective aperture
Scanning coil
Objective lens
Motorized stage
Stage
Sample Chamber


Electron gun
Condenser lenses
Scanning coil
Scanning circuit
Cathode-ray tube for viewing
Cathode-ray tube for photography
Primary electrons
Detector
Photo-multiplier
Secondary electrons
Specimen
Specimen holder
Vacuum system

SCANNING ELECTRON MICROSCOPE

- **The 1st scanning electron microscope (SEM) debuted in 1938 by Von Ardenne with the first commercial instruments out around 1965.**
- **In this case electrons are not used to directly image the specimen, but to excite it in such a way that it gives out secondary electrons which are collected by detectors & used to form the image.**

SEM

- A set of coils then "scan" or "sweep" the beam dwelling on points for a period of time determined by the scan speed (usually in the μs range)
- The Objective lens, focuses the scanning beam onto part of the specimen desired.
- When the beam strikes the sample (for few μs) interactions occur inside the sample and are detected by release of electrons.

SEM

- The beam is then constricted by the condenser aperture eliminating some high-angle electrons
- The second condenser lens forms electrons into a thin, tight, coherent beam & is controlled by "fine probe current knob"
- The objective aperture further eliminates high-angle electrons from the beam

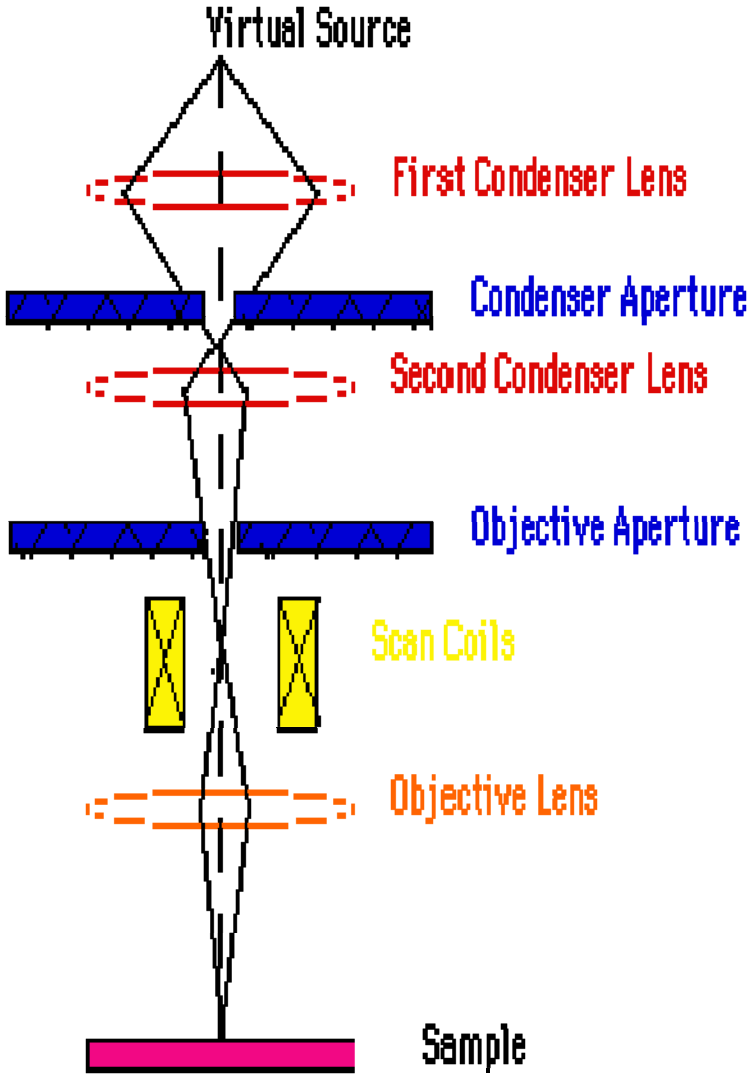
SEM

- Before the beam moves to its next dwell point these instruments count the number of interactions and display a pixel whose intensity is determined by this number (the more reactions the brighter the pixel).

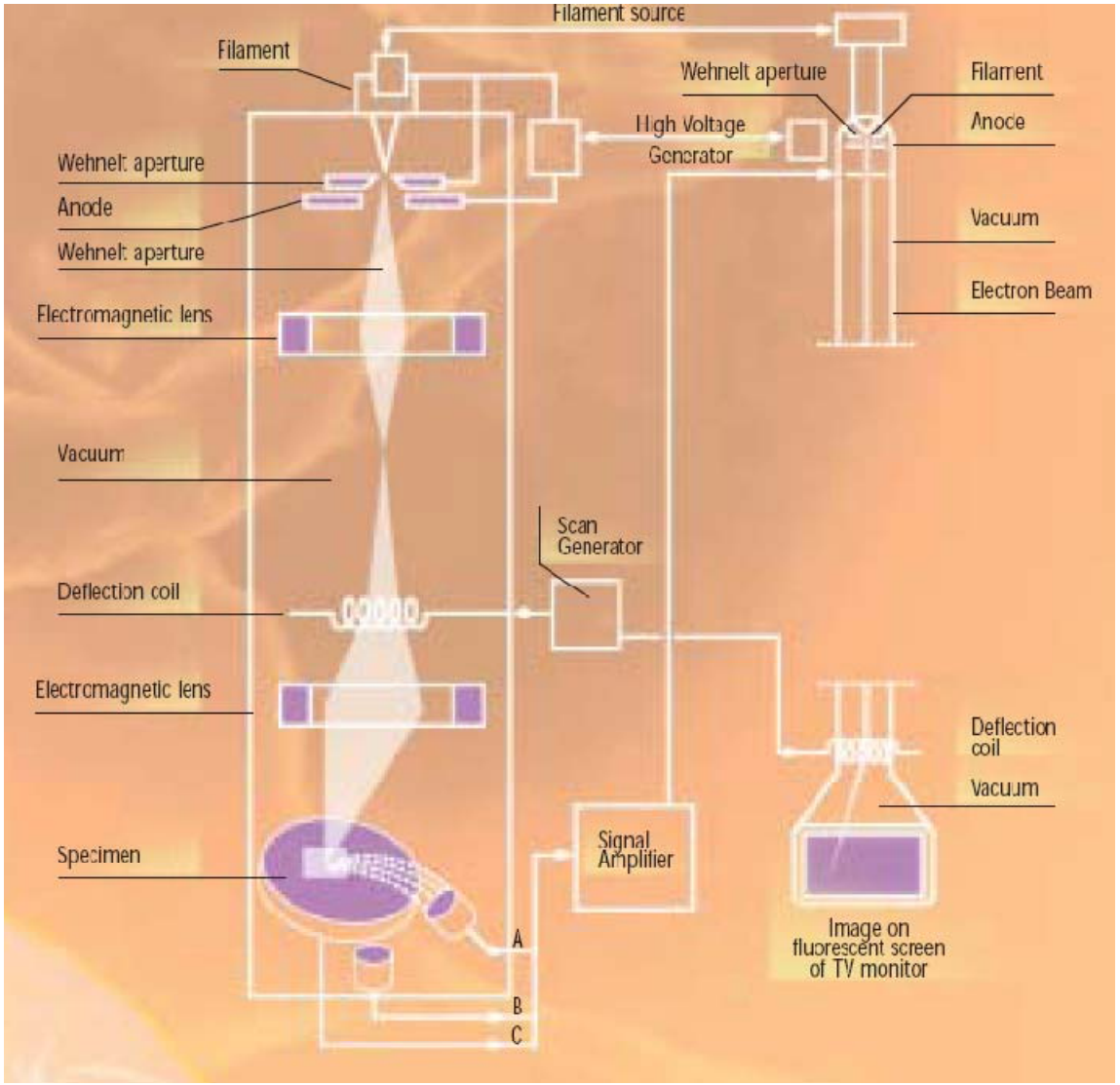
- The interactions lead to release of – secondary electrons, backscattered electrons & X- rays.

SEM

- The "Virtual Source" at the top represents the electron gun, producing a stream of monochromatic electrons.
- The stream is condensed by 1st condenser lens (controlled by "coarse probe current knob"). This lens is used to both form the beam and limit the amount of current in the beam.



Components of the instrument



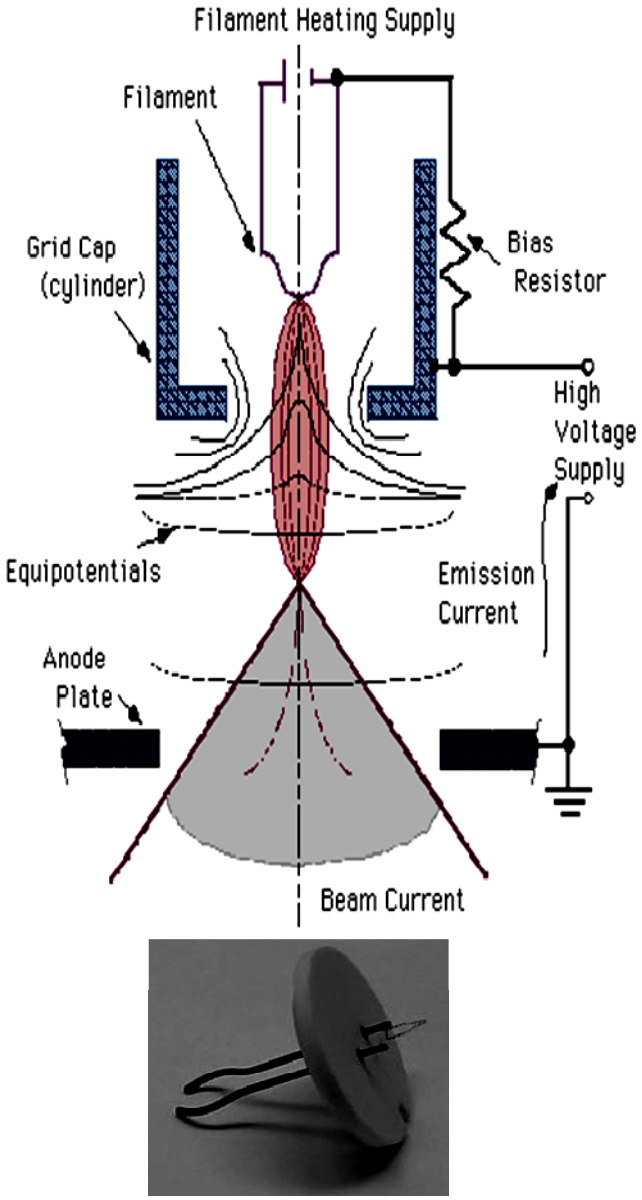
- electron gun (filament)
- electromagnetic optics
- scan coils
- sample stage
- detectors
- vacuum system
- computer hardware and software (not trivial!!)

SEM components

1. Electron optical column consists of:
   - electron source to produce electrons
   - magnetic lenses to de-magnify the beam
   - magnetic coils to control and modify the beam
   - apertures to define the beam, prevent electron spray, etc.
2. Vacuum systems consists of:
   - chamber which “holds” vacuum, pumps to produce vacuum
   - valves to control vacuum, gauges to monitor vacuum
3. Signal Detection & Display consists of:
   - detectors which collect the signal
   - electronics which produce an image from the signal

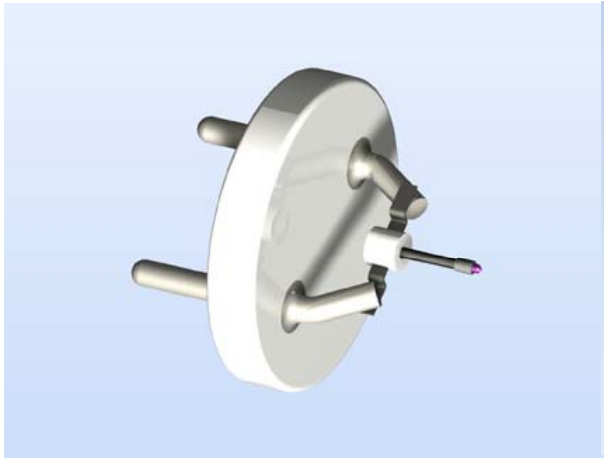
Electron guns

- We want many electrons per time unit per area (high current density) and as small electron spot as possible
- Traditional guns: thermionic electron gun (electrons are emitted when a solid is heated)
  - W-wire, LaB_6-crystal
- Modern: field emission guns (FEG) (cold guns, a strong electric field is used to extract electrons)
  - Single crystal of W, etched to a thin tip

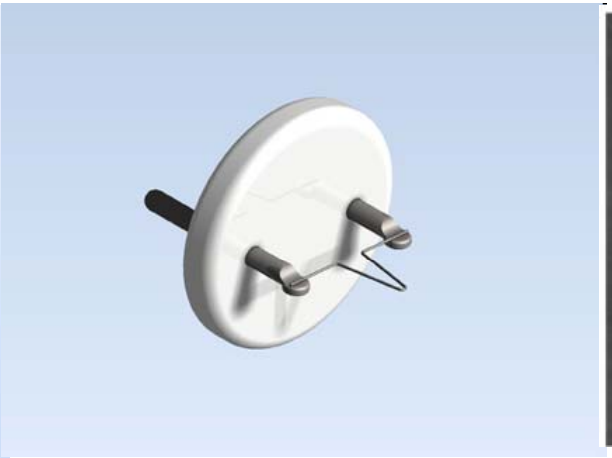


Electron guns

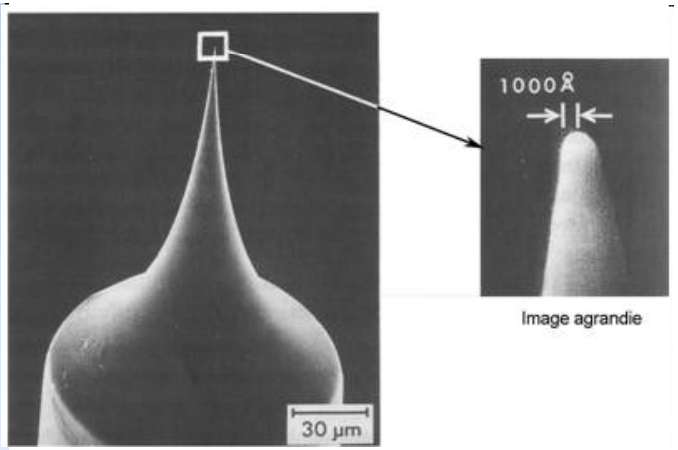
- With field emission guns we get a smaller spot and higher current densities compared to thermionic guns
- Vacuum requirements are tougher for a field emission guns



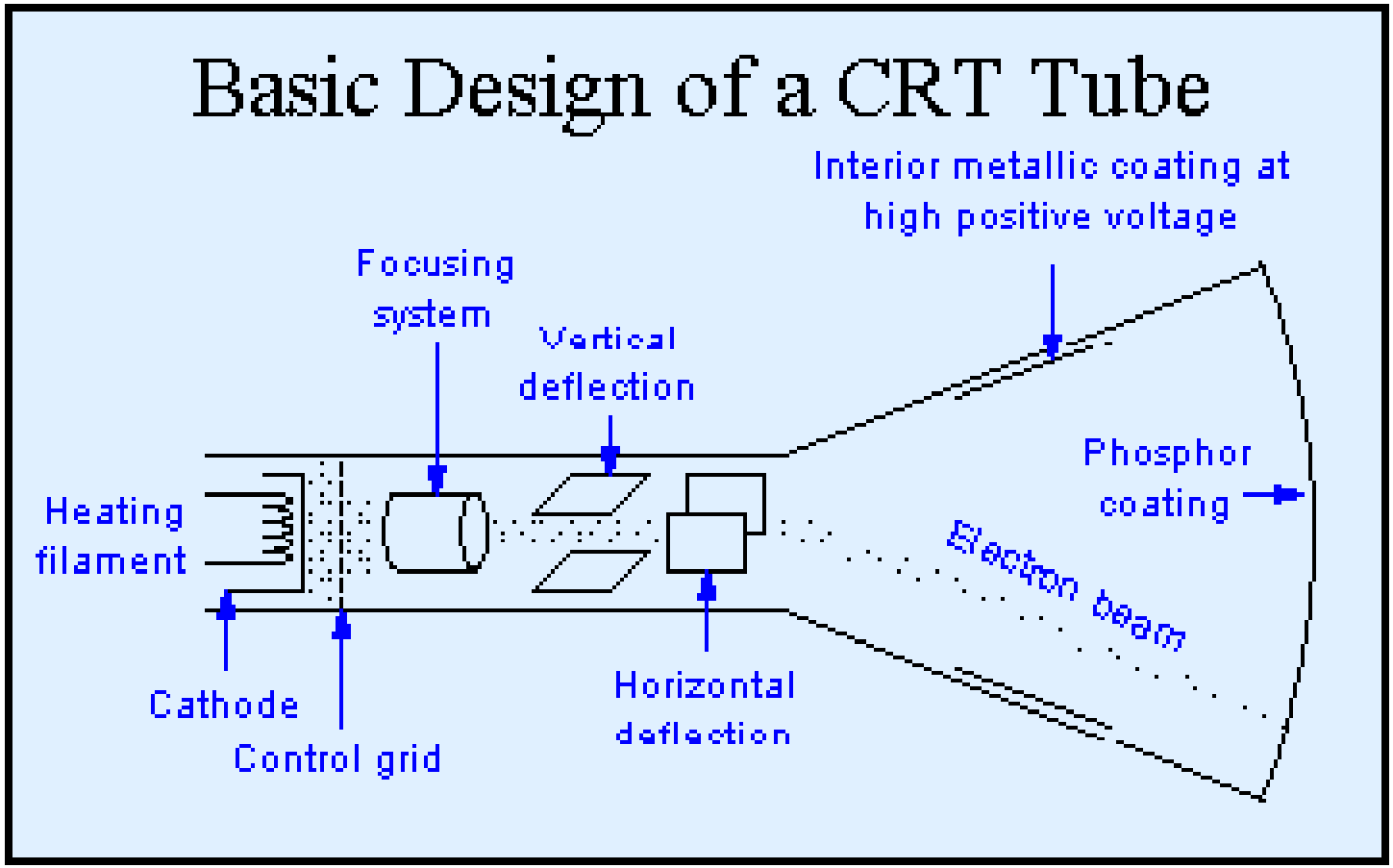
Single crystal of LaB_6



Tungsten wire

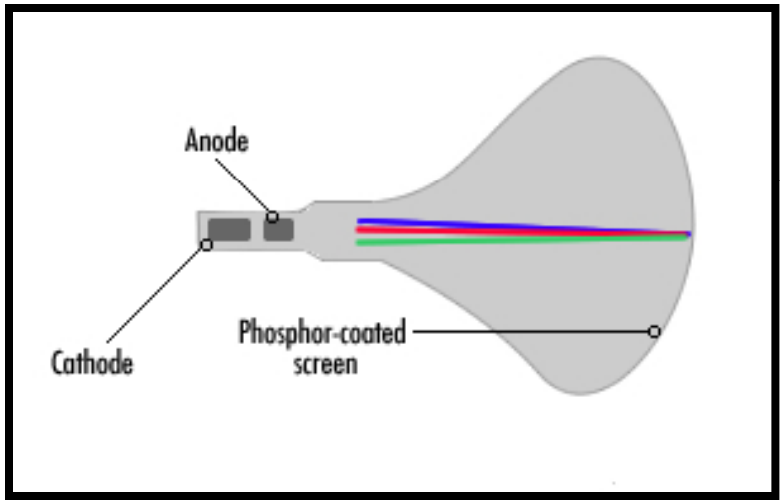


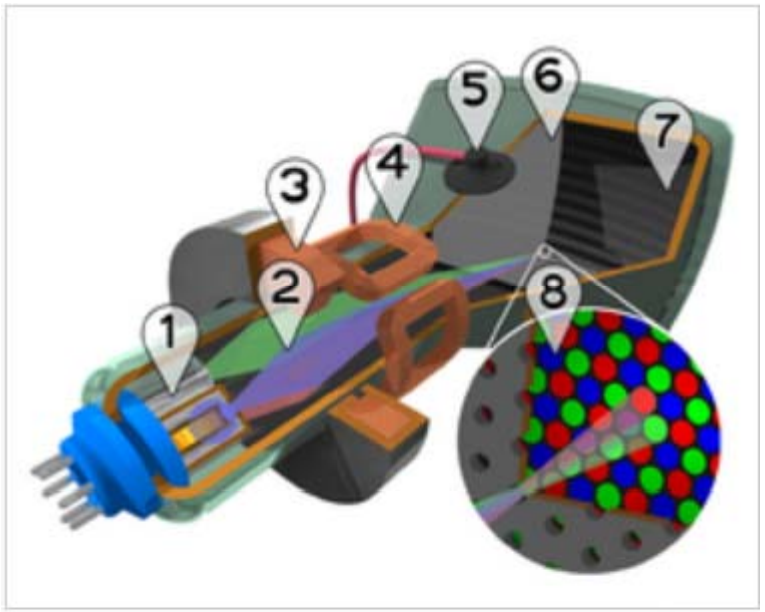
Field emission tip



Cathode Ray Tube (CRT) accelerates electrons towards the phosphor coated screen where they produce flashes of light upon hitting the phosphor. Deflection coils create a scan pattern forming an image in a point by point manner

Color CRT?





Cutaway rendering of a color CRT:

1. Three Electron guns (for red, green, and blue phosphor dots)

2. Electron beams

3. Focusing coils

4. Deflection coils

5. Anode connection

6. Mask for separating beams for red, green, and blue part of displayed image

7. Phosphor layer with red, green, and blue zones

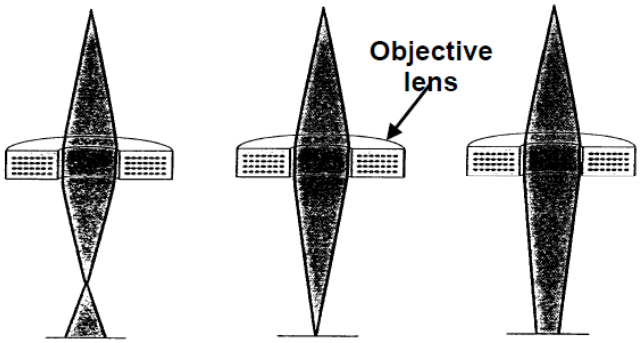
8. Close-up of the phosphor-coated inner side of the screen

The objective lens

- **The objective lens controls the final focus of the electron beam by changing the magnetic field strength**

- **The cross-over image is finally demagnified to a ~ 10nm beam spot which carries a beam current of approximately 10^{-9} - 10^{-13} A.**

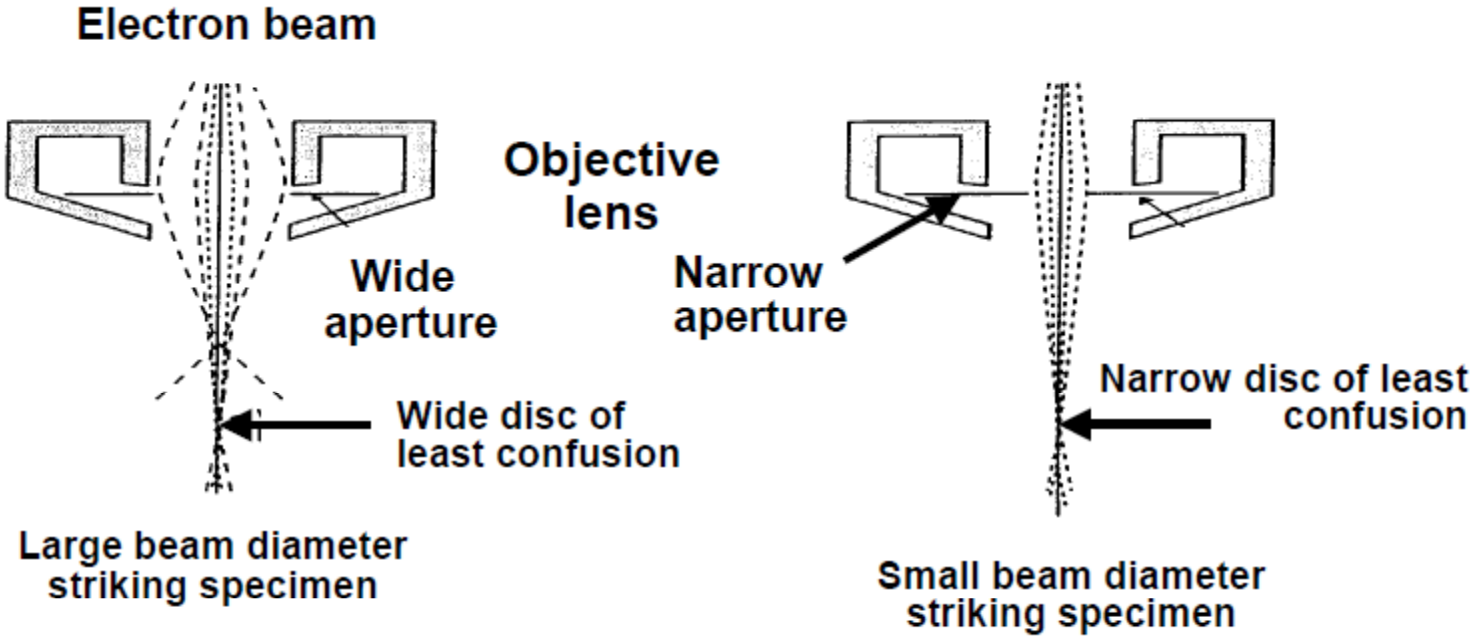
By changing the current in the objective lens, the magnetic field strength changes and therefore the focal length of the objective lens is changed.



The objective lens aperture

- **Since the electrons coming from the electron gun have spread in kinetic energies and directions of movement, they may not be focused to the same plane to form a sharp spot.**

- **By inserting an aperture, the stray electrons are blocked and the remaining narrow beam will come to a narrow "Disc of Least Confusion"**

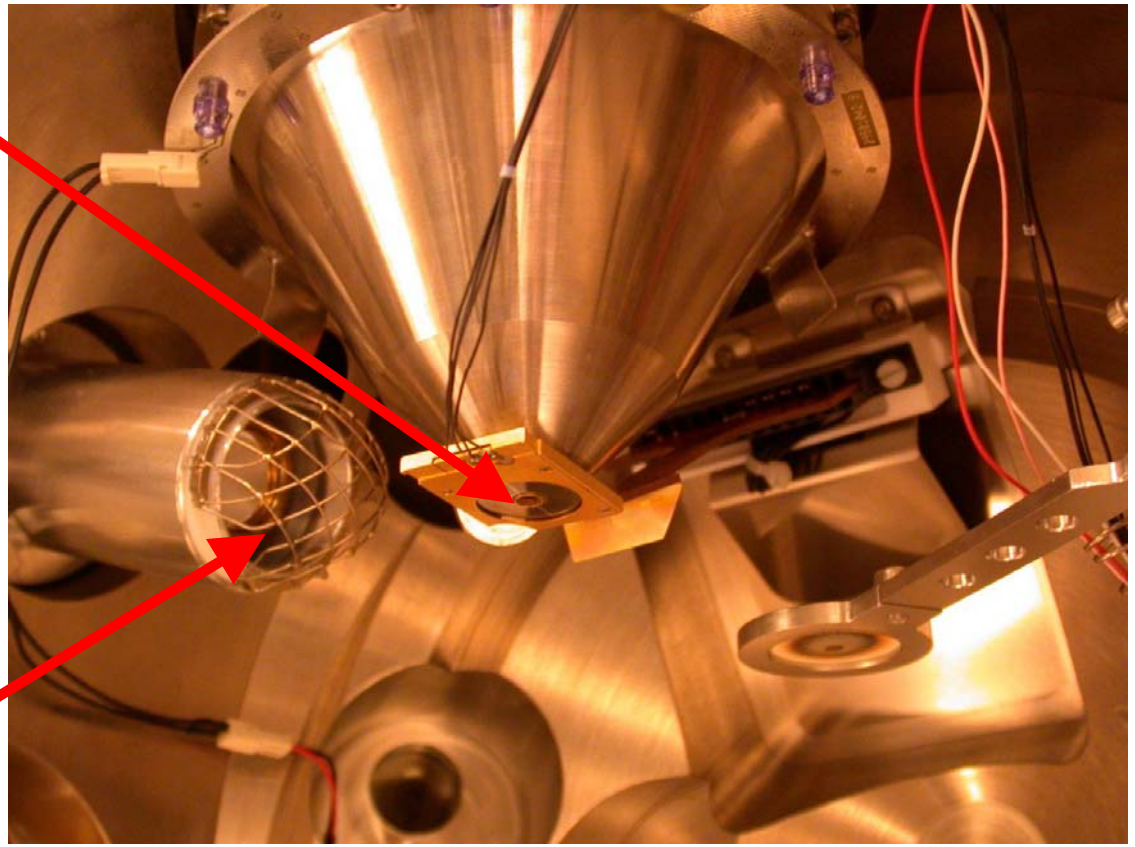


Aperture in SEM: either to limit the amount of electrons or enhance contrast

Detectors

Our traditional detectors

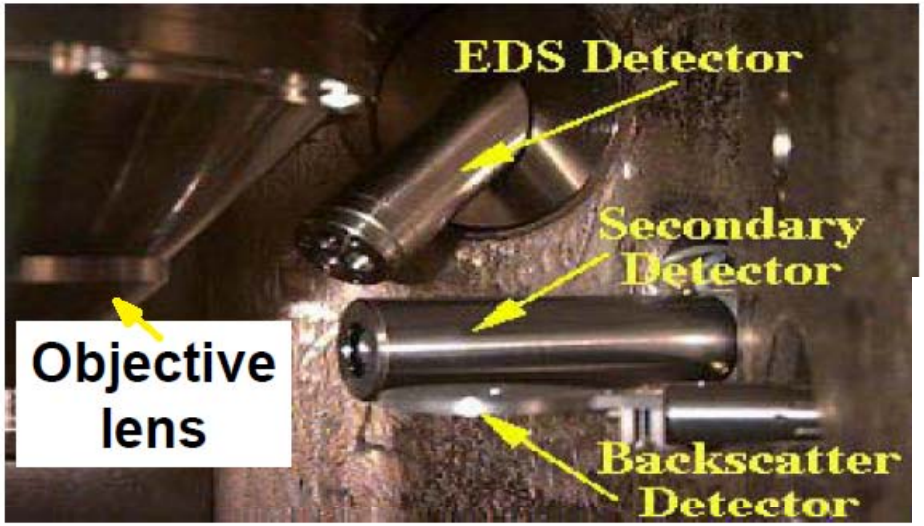
Backscattered electron detector:
(Solid-State Detector)



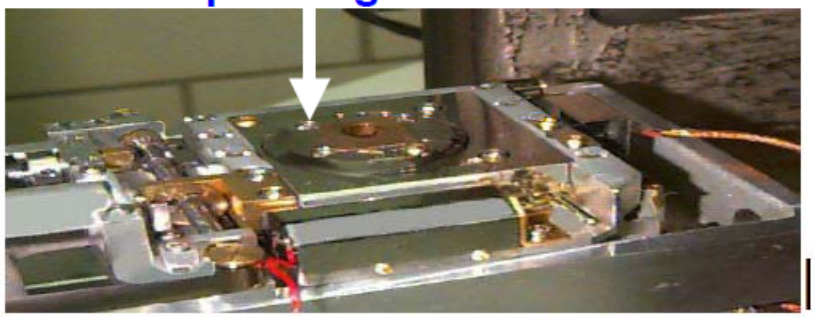
Secondary electron detector:
(Everhart-Thornley)

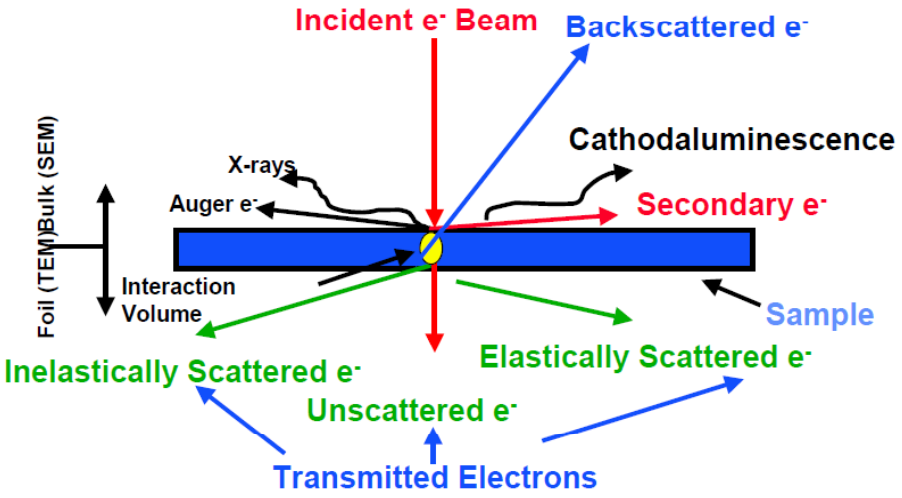
- Secondary electrons: Everhart-Thornley Detector
- Backscattered electrons: Solid State Detector
- X-rays: Energy dispersive spectrometer (EDS)

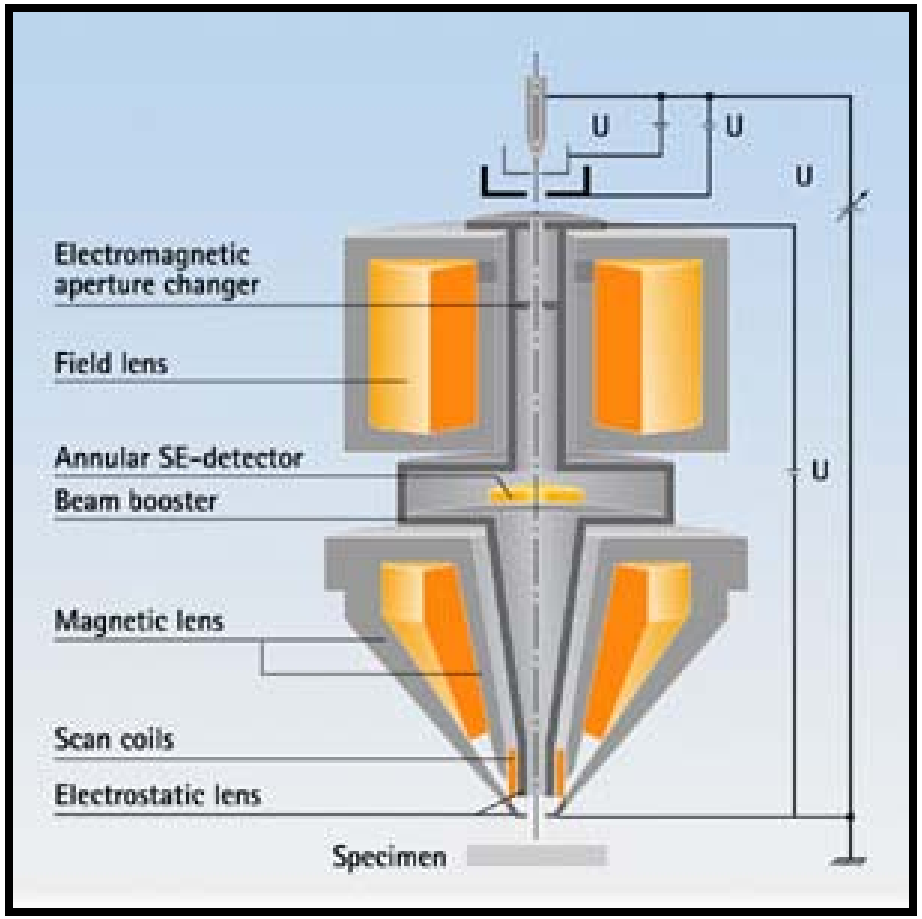
Detector and sample stage



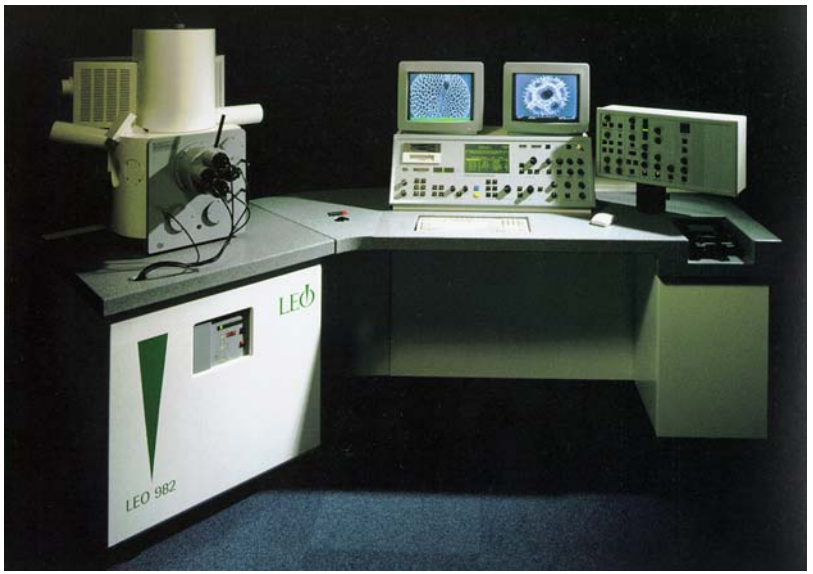
Sample stage





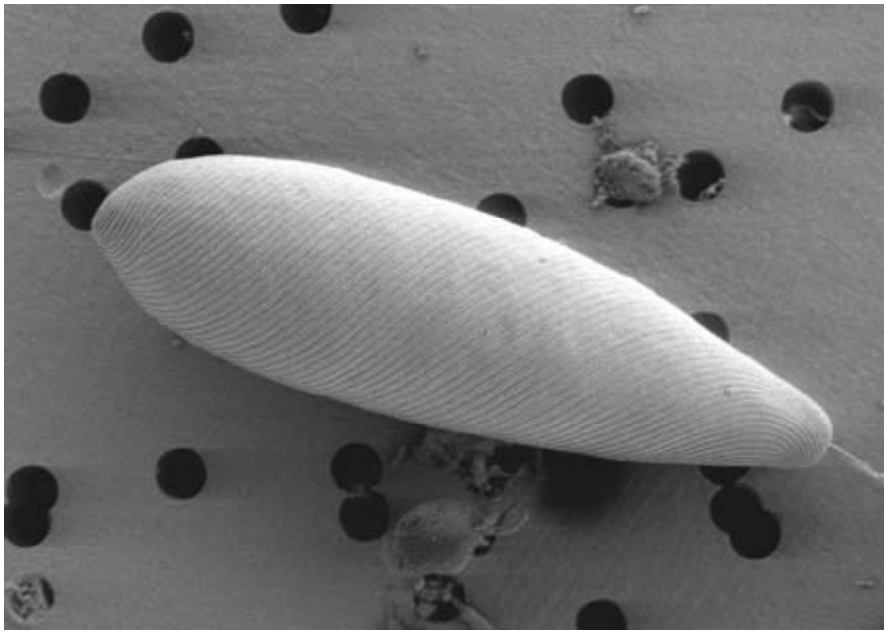


LEO Gemini Column

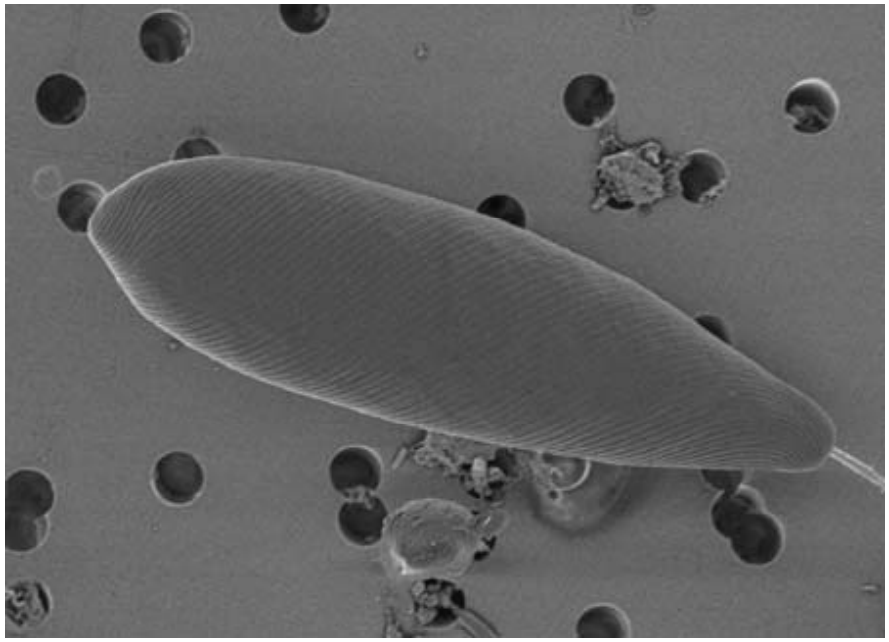


A detector placed within the column is known as an “in-lens” detector and produces a very different image compared to a conventionally located detector

Secondary Electron Detector

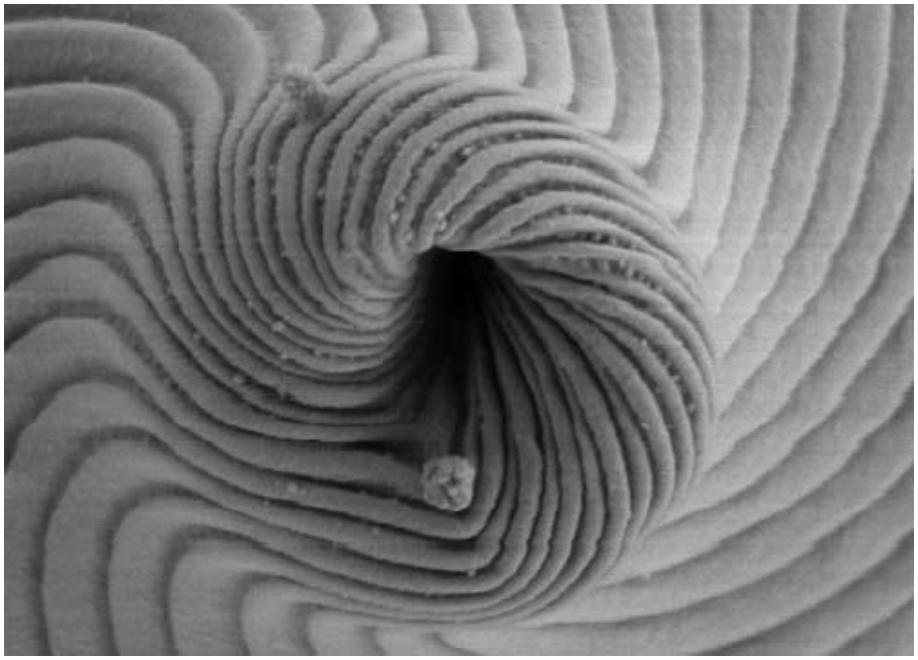


Side Mounted

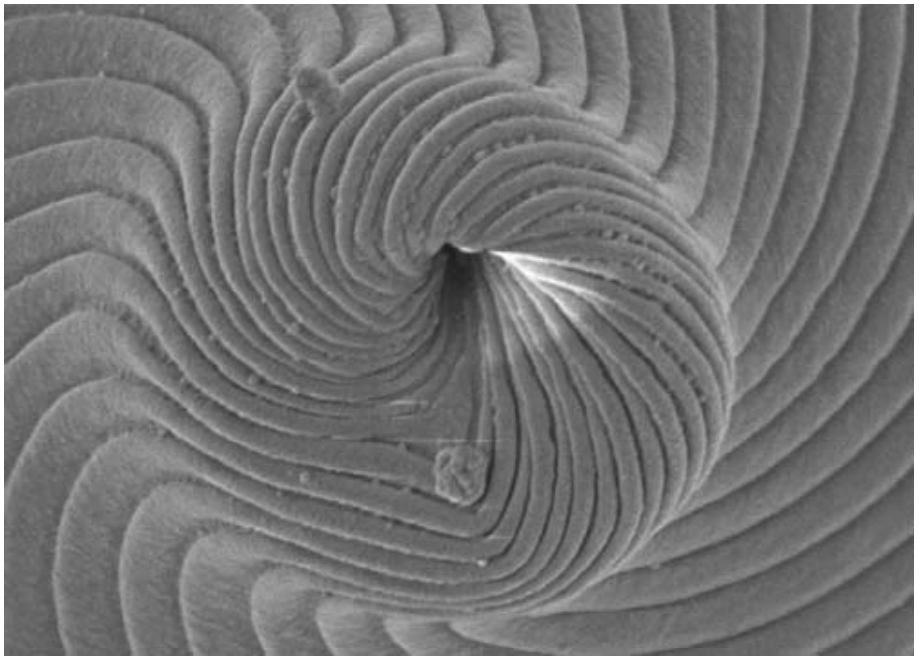


In-Lens

Secondary Electron Detector

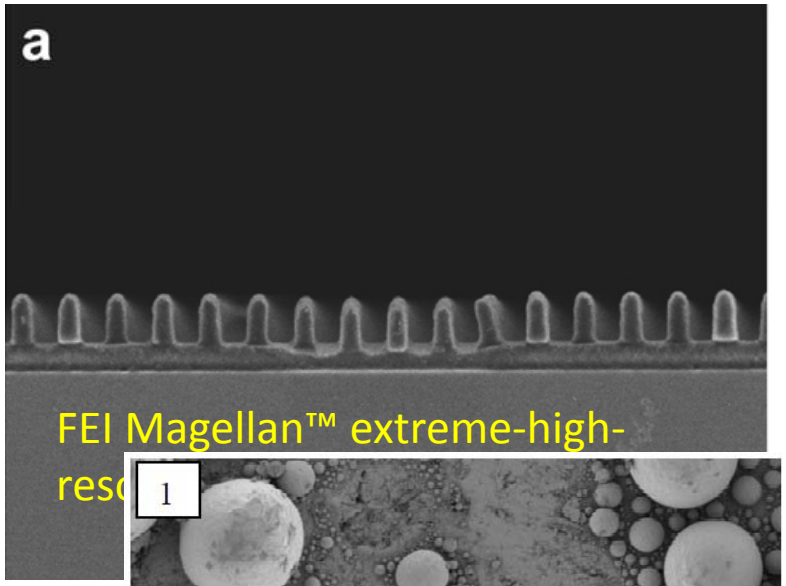


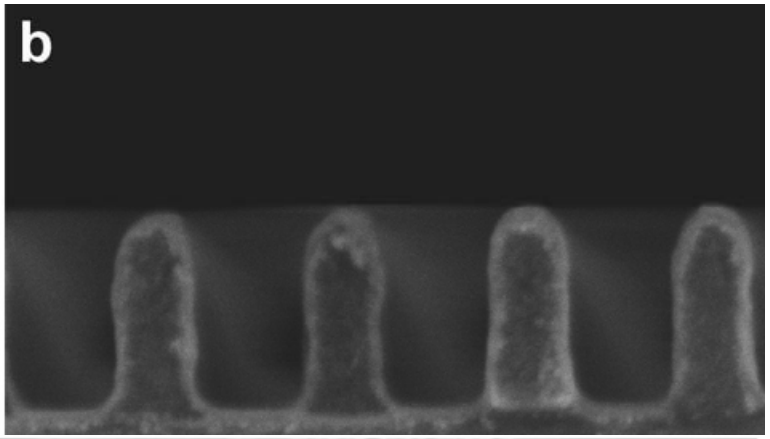
Side Mounted

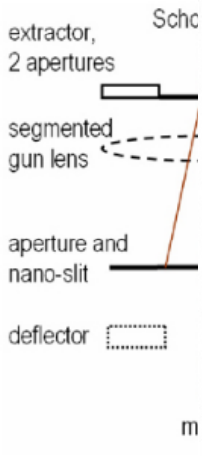


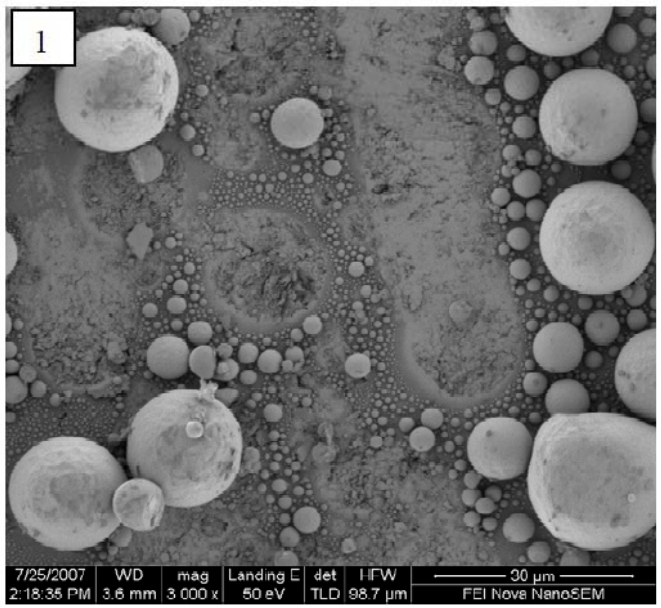
In-Lens

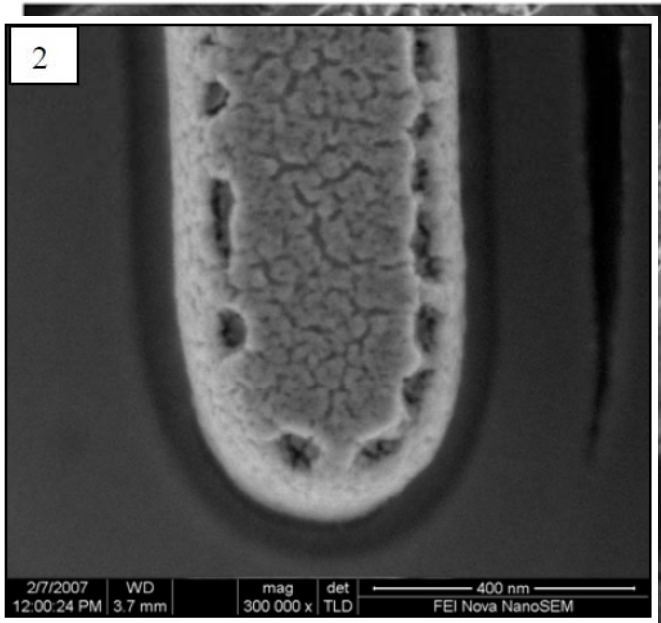
Beam deceleration











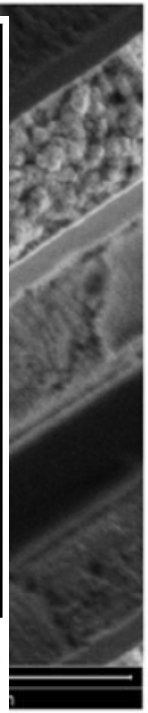


Figure 2. *The thr*
on-axis mode, (2
Schottky-FEG: Sc

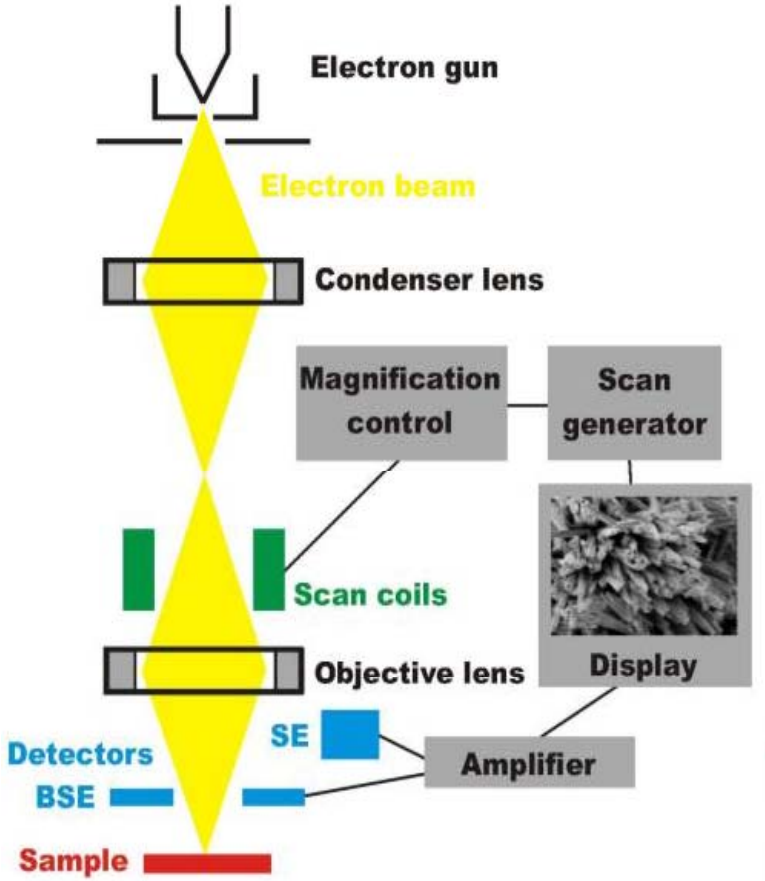
Fig 1. Image of tin balls using a primary electron landing energy E_L = 50 eV

Fig 2. UHR image of a depassivated integrated circuit imaged with an effective primary beam energy of 1 keV revealing fine surface detail

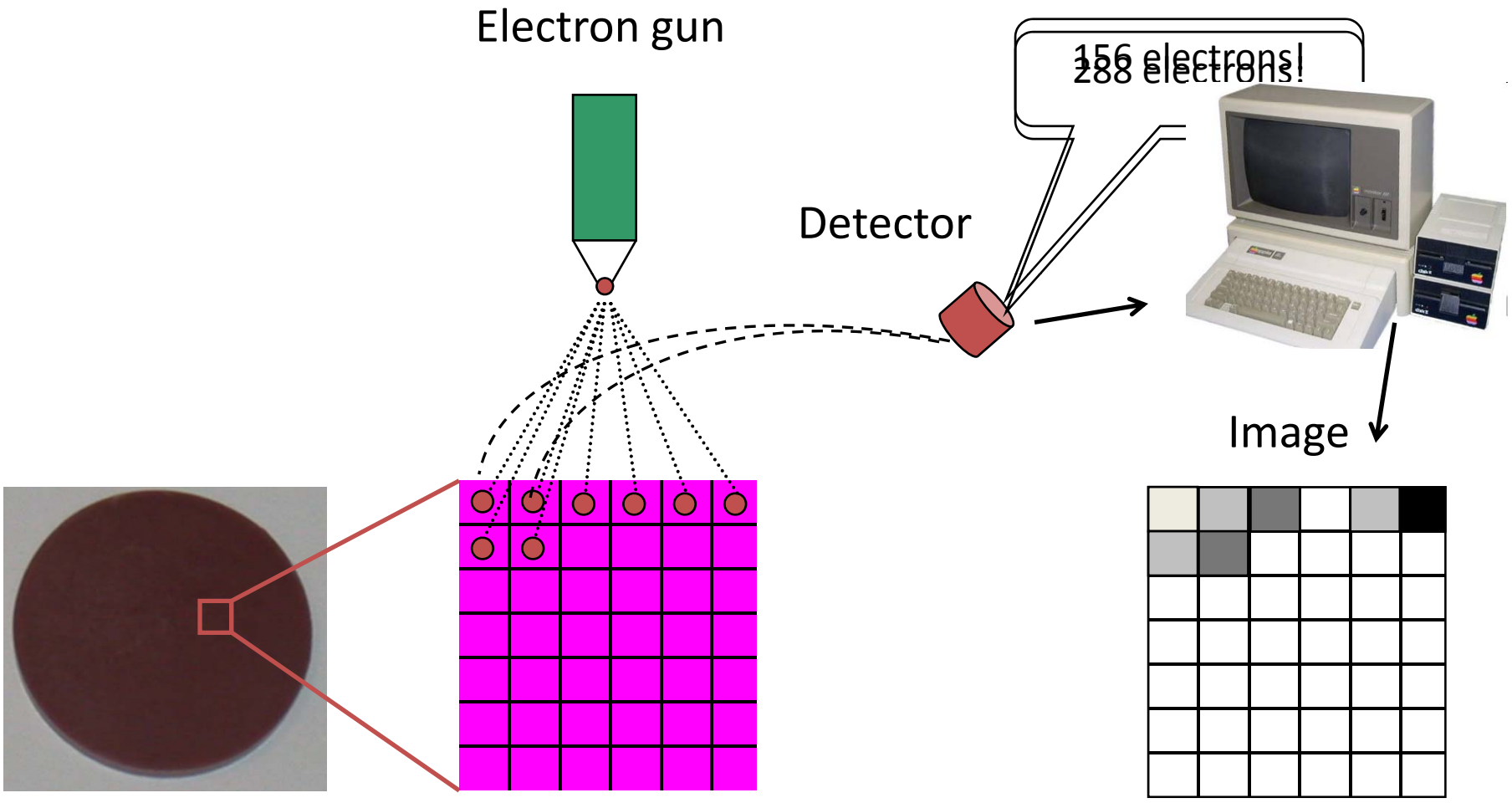
Figure 3. *XHR SEM image of a deprocessed semiconductor device image with a beam voltage of 50V (combines UC mode with beam deceleration).*

HOW THE SEM WORKS?

- The SEM uses electrons instead of light to form an image.
- A beam of electrons is produced at the top of the microscope by heating of a metallic filament.
- The electron beam follows a vertical path through the column of the microscope. It makes its way through electromagnetic lenses which focus and direct the beam down towards the sample.
- Once it hits the sample, other electrons (backscattered or secondary) are ejected from the sample. Detectors collect the secondary or backscattered electrons, and convert them to a signal that is sent to a viewing screen similar to the one in an ordinary television, producing an image.

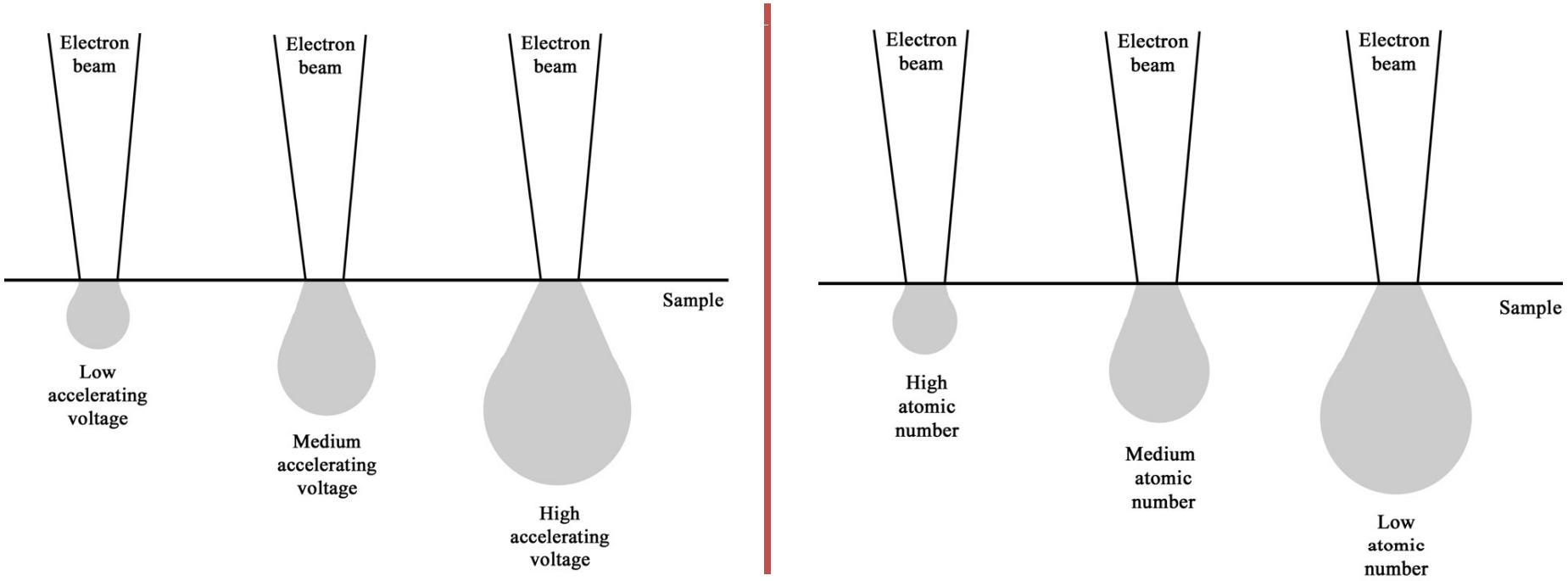


How do we get an image?

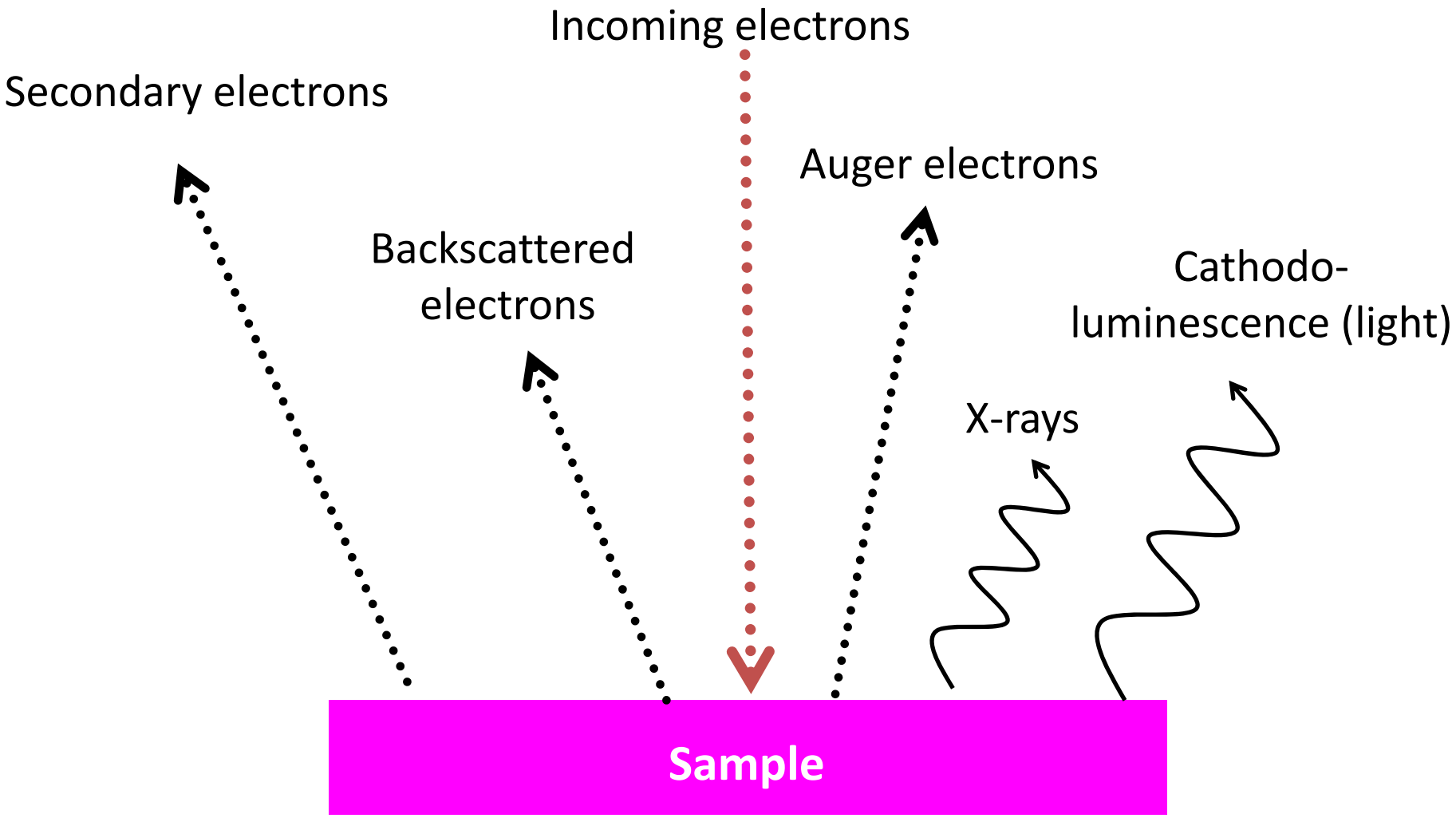


Electron beam-sample interactions

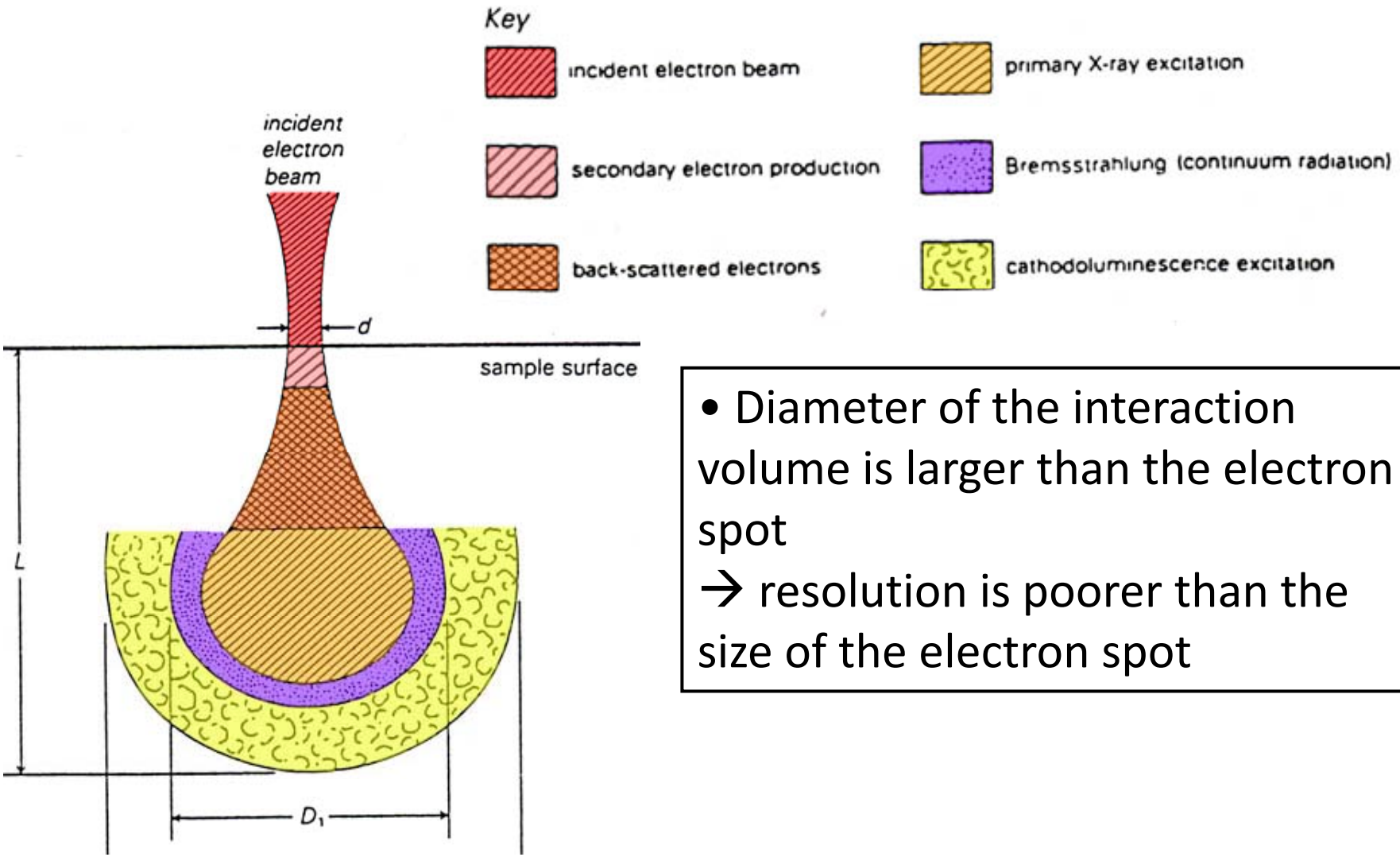
- The incident electron beam is scattered in the sample, both elastically and inelastically
- This gives rise to various signals that we can detect (more on that on next slide)
- Interaction volume increases with increasing acceleration voltage and decreases with increasing atomic number



Signals from the sample



Where does the signals come from?

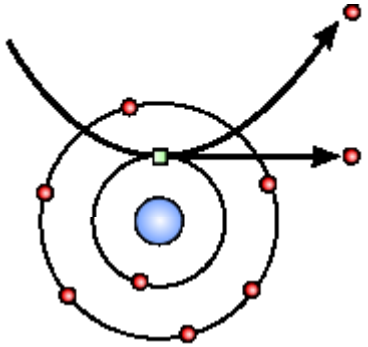


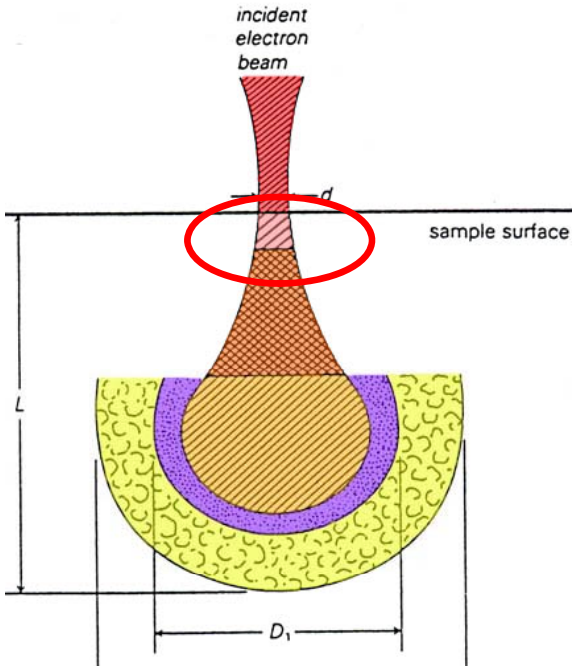
- Diameter of the interaction volume is larger than the electron spot

→ resolution is poorer than the size of the electron spot

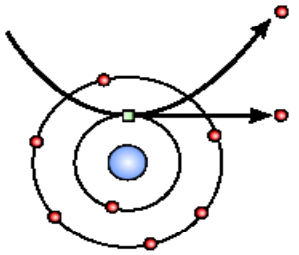
Secondary electrons (SE)

- Generated from the collision between the incoming electrons and the loosely bonded outer electrons
- Low energy electrons (~10-50 eV)
- Only SE generated close to surface escape (topographic information is obtained)
- Number of SE is greater than the number of incoming electrons
- We differentiate between SE1 and SE2





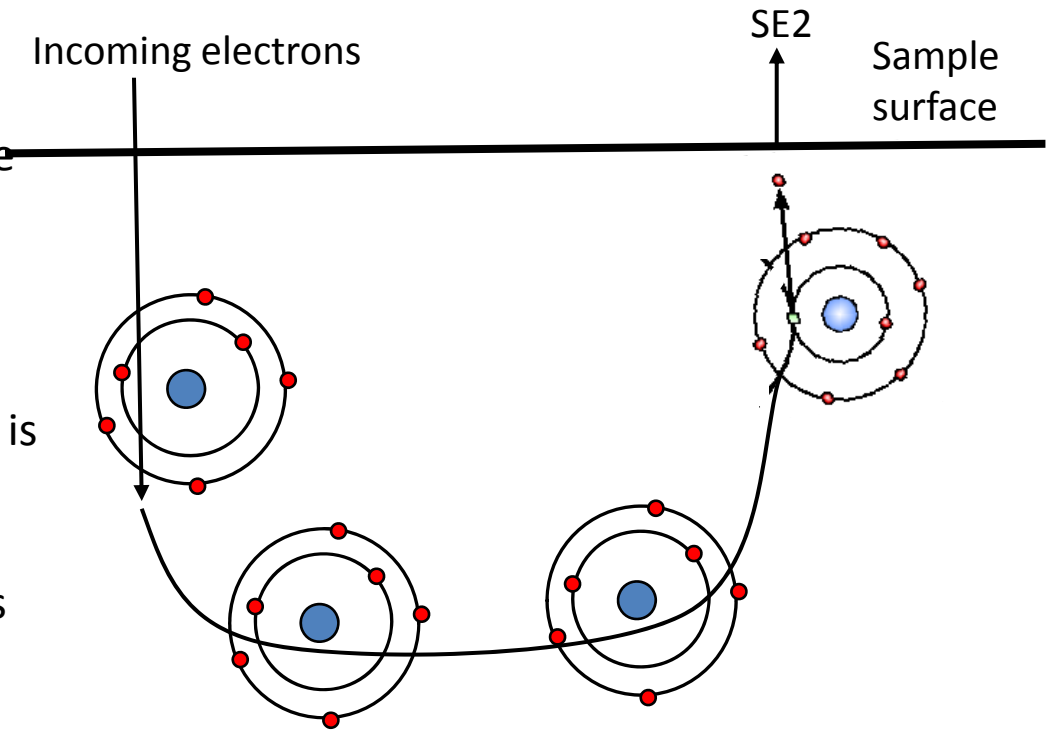
SE1



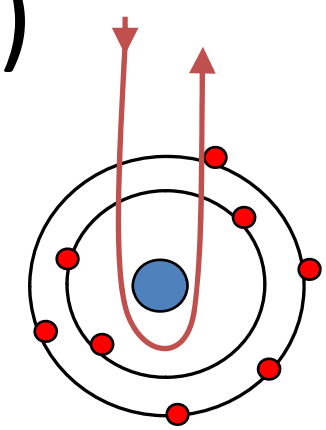
- The secondary electrons that are generated by the incoming electron beam as they enter the surface
- High resolution signal with a resolution which is only limited by the electron beam diameter

SE2

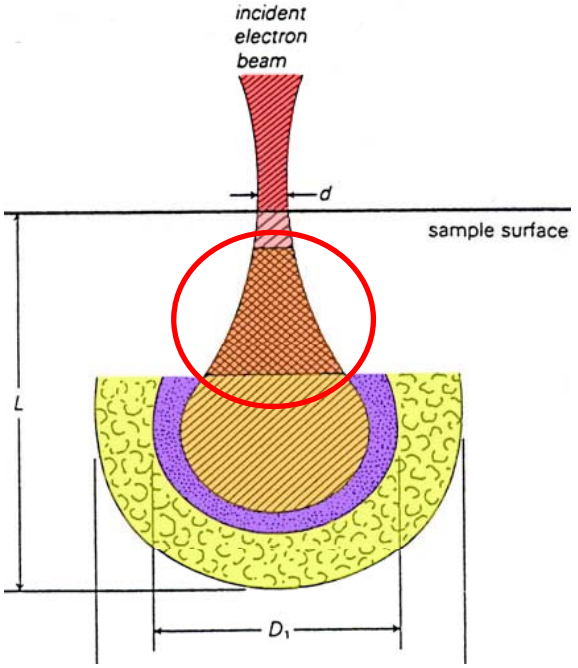
- The secondary electrons that are generated by the backscattered electrons that have returned to the surface after several inelastic scattering events
- SE2 come from a surface area that is bigger than the spot from the incoming electrons → resolution is poorer than for SE1 exclusively



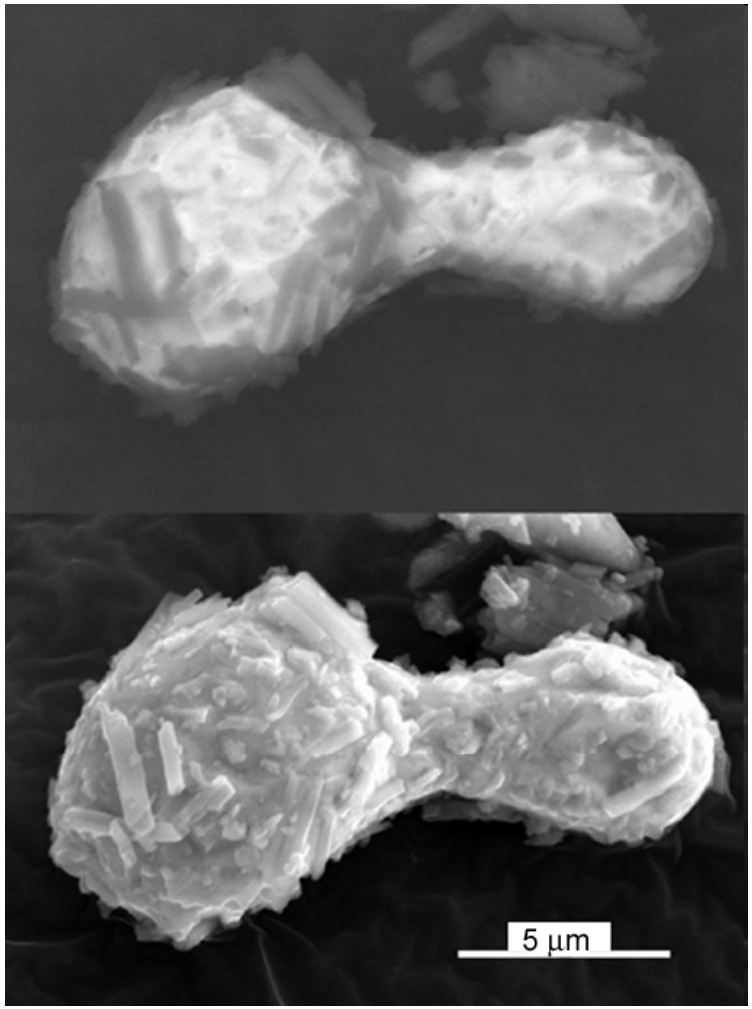
Backscattered electrons (BSE)



- A fraction of the incident electrons is retarded by the electro-magnetic field of the nucleus and if the scattering angle is greater than 180° the electron can escape from the surface
- High energy electrons (elastic scattering)
- Fewer BSE than SE
- We differentiate between BSE1 and BSE2



BSE vs SE



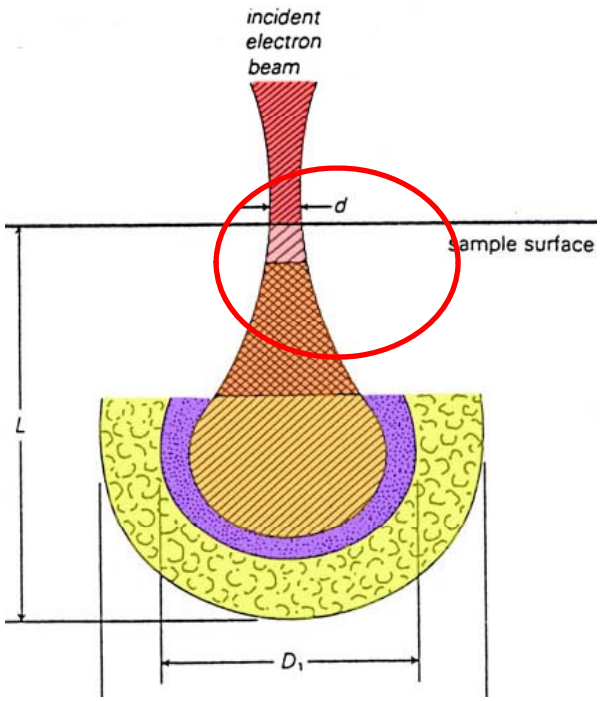
- SE produces higher resolution images than BSE
- By placing the secondary electron detector *inside* the lens, mainly SE1 are detected

  Resolution of 1 – 2 nm is possible

X-rays

- *Photons* not electrons
- Each element has a *fingerprint* X-ray signal
- Poorer spatial resolution than BSE and SE
- Relatively few X-ray signals are emitted and the detector is inefficient

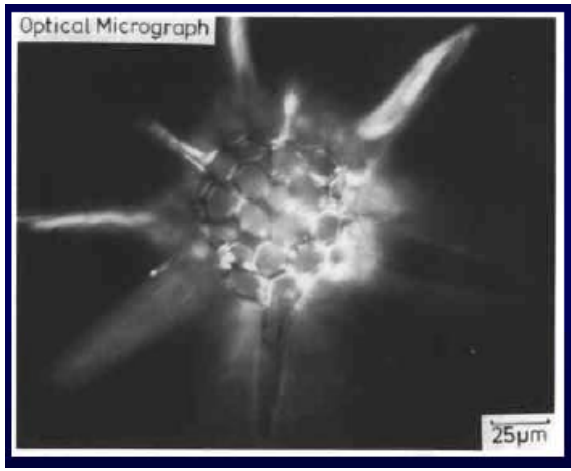
  → relatively long signal collecting times are needed

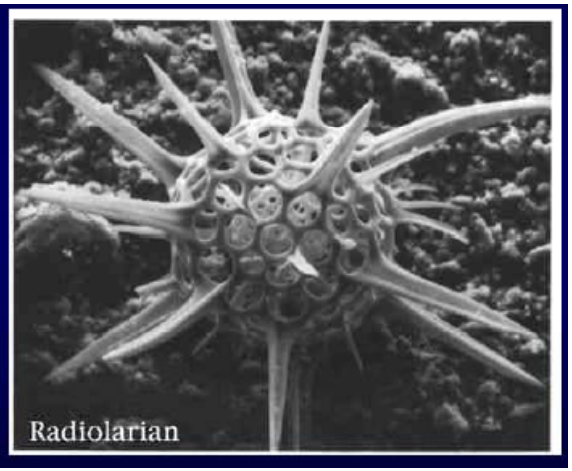


Some comments on resolution

- Best resolution that can be obtained: size of the electron spot on the sample surface
  - The introduction of FEG has dramatically improved the resolution of SEM's
- The volume from which the signal electrons are formed defines the resolution
  - SE image has higher resolution than a BSE image
- Scanning speed:
  - a weak signal requires slow speed to improve signal-to-noise ratio
  - when doing a slow scan drift in the electron beam can affect the accuracy of the analysis

Depth of Field





Magnification	Depth of Focus	
	Optical	SEM
10	60 µm	1000 µm
100	8 µm	100 µm
1,000	0.2 µm	10 µm
10,000	-	1 µm

Resolution

Resolution is the ability to resolve two closely spaced points. While you may have to be at a high magnification to see small features, resolution is NOT the same as magnification.

One way to improve resolution is by reducing the size of the electron beam that strikes the sample:

$$d_{min} = 1.29 C_s^{1/4} \lambda^{3/4} [7.92 (iT/J_c) x 10^9 + 1]^{3/8}$$

at low current:

$$d_{min} = 1.29 C_s^{1/4} \lambda^{3/4}$$

J_c = current density of the source, λ = electron wavelength

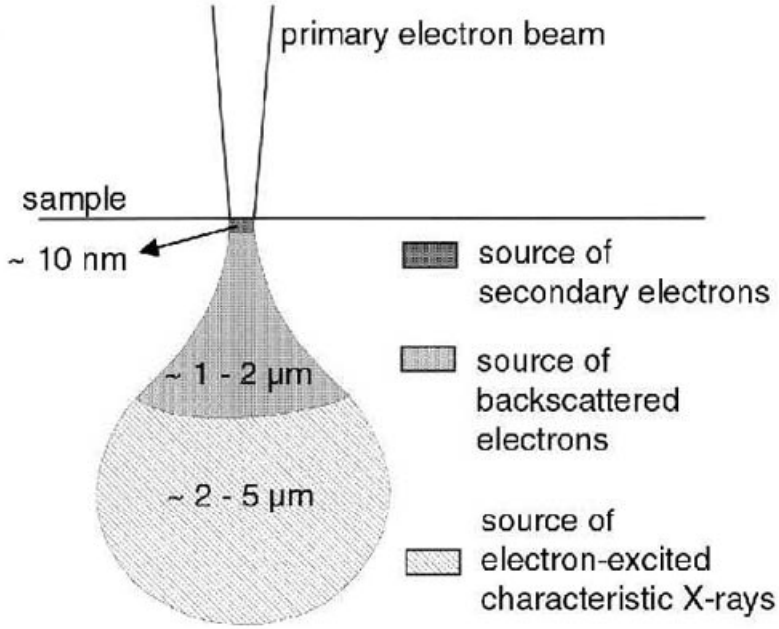
C_s = spherical aberration, i = current, T = temperature,

Resolution

Can increase the resolution by:

- **Increasing the strength of the condenser lens**
- **Decreasing the size of the objective aperture**
- **Decreasing the working distance**

How these changes affect the depth of field?



STEM in SEM

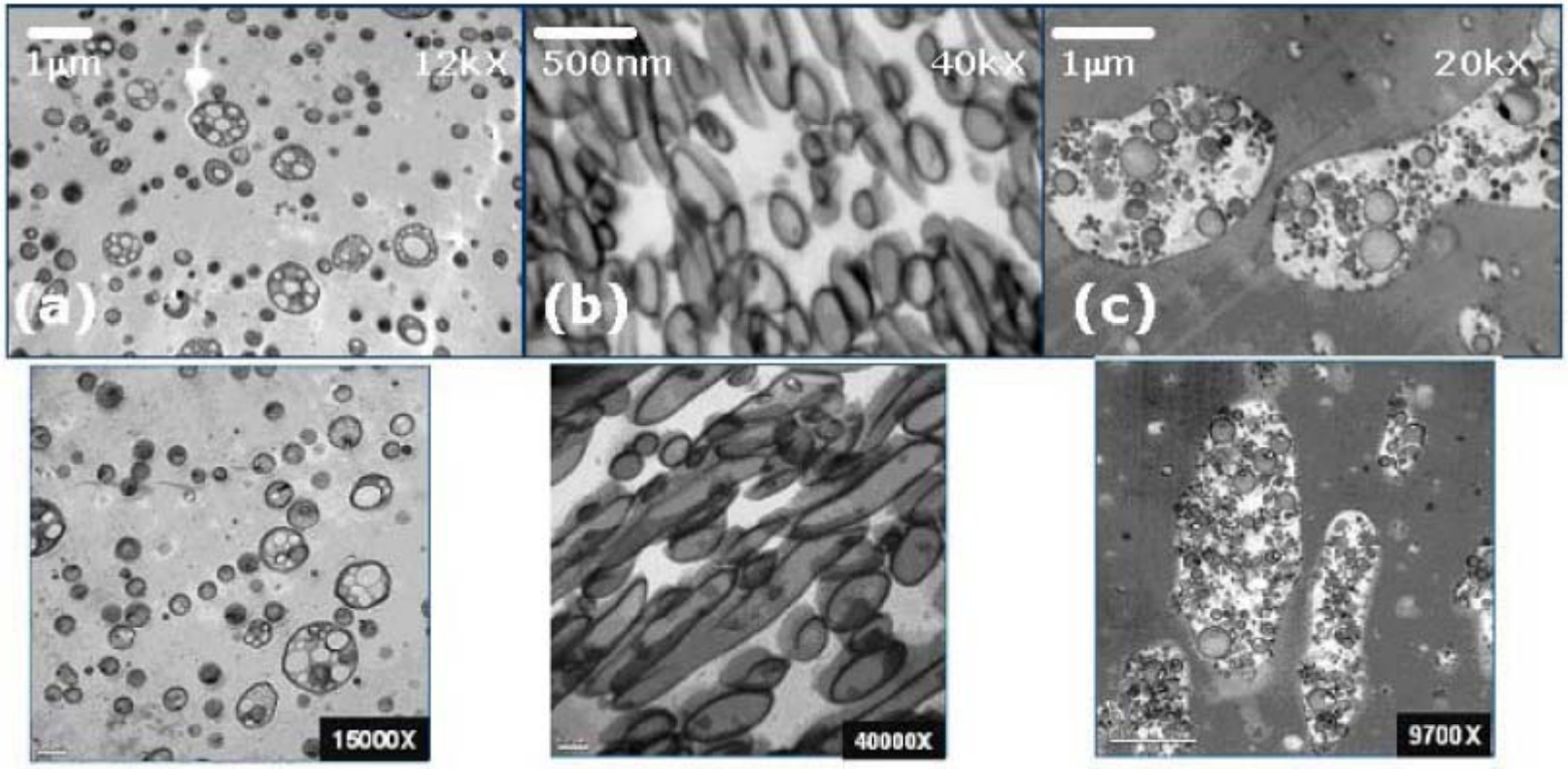


Figure 1: Morphology of various polymer blends obtained by STEM-in-SEM (top row) and TEM (bottom row): (a) SAN-ABS; (b) PP-PPE (c) PC-SAN-ABS

SEM artifacts

- ***Damage*** is an unexpected and irreversible change in the object and can occur before and during microscopy. In many cases, damage is very obvious in an image. However, sometimes the damage is les immediately obvious.

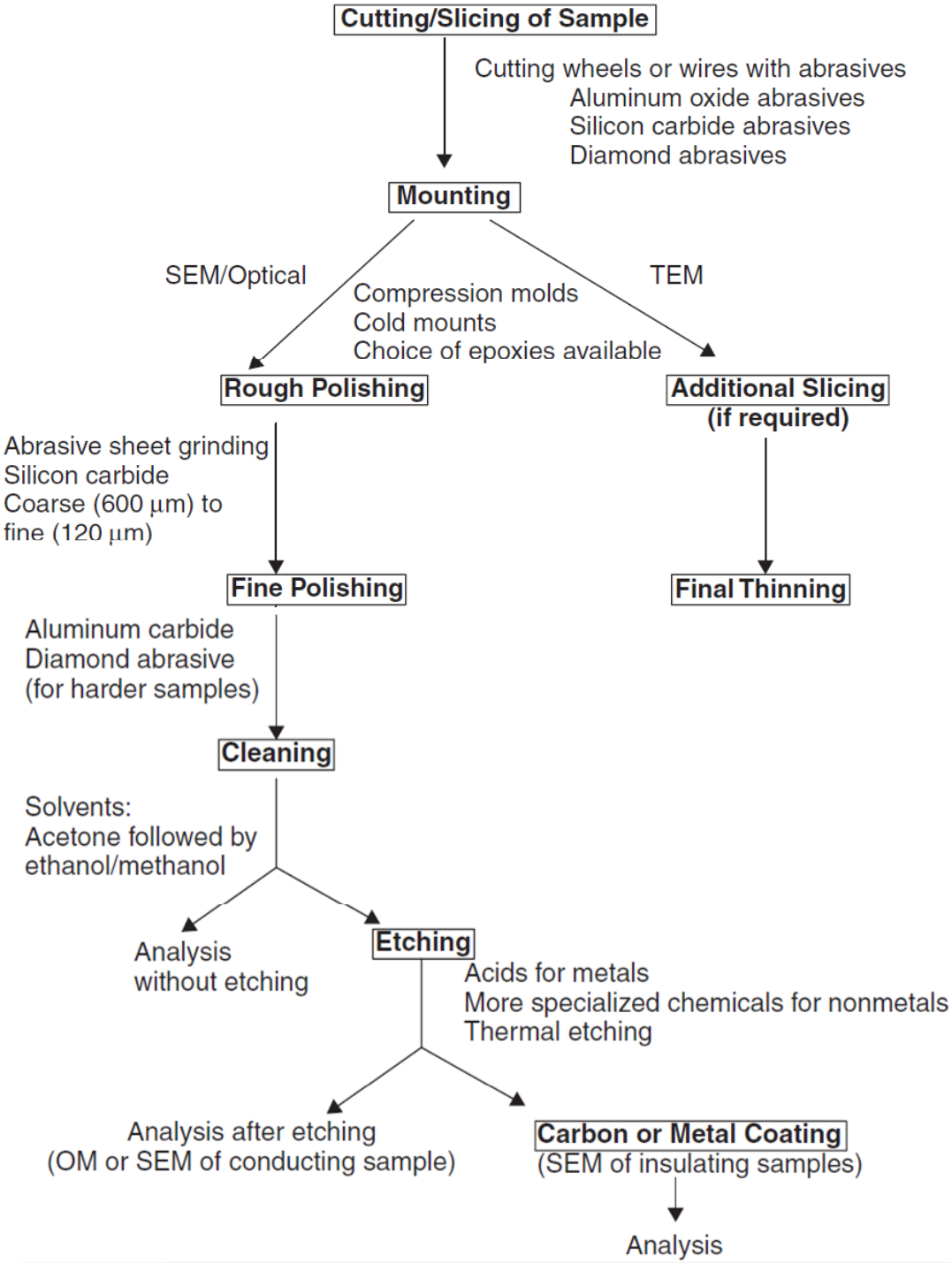
- ***Artifacts*** are percieved structural distortions or misrepresentative chemical changes to the original object that arise as a consequence of the techniques used in preparing objects for subsequent microscopy and analysis. Artifacts are frequently not immediately obvious.

Sample Preparation
for
SEM and TEM

	Light Microscope	Electron Microscope
Light Source	visible light for illumination and optical (glass) lenses to magnify specimens.	A beam of electrons to form an image of a specimen. EM is operated in the vacuum and focuses the electron beam and magnifies images with the help of electromagnetic lenses.
Magnification	Approximately 10 to 1,000 times their original size,	Depending on the instrument used, specimens can be magnified roughly between 10 and 100,000 times in SEM and between 500 to 500,000 times in TEM
λ	When compared to the wavelengths of visible light (λ = 400 nm to 700 nm).	The electron microscope takes advantage of the much shorter wavelength of the electron (e.g., λ = 0.005 nm at an accelerating voltage of 50 kV)
Resolving power	Increase with increase in numerical aperture and vice-versa Can be increased 1. By using short wavelength 2. By using immersion oil in the light path 3. By using inclined light	When the accelerating voltage is increased in EM, the wavelength decreases and resolution decreases. Increasing the velocity of electrons results in a shorter wavelength and increased resolving power.

Preparatory steps of specimens for EM

1. Surface cleaning
2. Fixation
3. Rinsing
4. Dehydration
5. Drying
6. Mounting
7. Coating or Embedding
8. Cutting
9. Staining


Cutting/Slicing of Sample
Cutting wheels or wires with abrasives
Aluminum oxide abrasives
Silicon carbide abrasives
Diamond abrasives
Mounting
SEM/Optical
Compression molds
Cold mounts
Choice of epoxies available
TEM
Rough Polishing
Additional Slicing
(if required)
Abrasive sheet grinding
Silicon carbide
Coarse (600 μm) to
fine (120 μm)
Fine Polishing
Final Thinning
Aluminum carbide
Diamond abrasive
(for harder samples)
Cleaning
Solvents:
Acetone followed by
ethanol/methanol
Analysis
without etching
Etching
Acids for metals
More specialized chemicals for nonmetals
Thermal etching
Analysis after etching
(OM or SEM of conducting sample)
Carbon or Metal Coating
(SEM of insulating samples)
Analysis

Sample Preparation for SEM

Cleaning the surface of the specimen

Variety of unwanted deposits –

Dust,
Silt,
Detritus (debris)
Media components, or
Other contaminants.

The specimen should be quickly rinsed in a suitable buffered solution of the appropriate

pH,
Temperature
Osmotic strength close to the location from which the specimen has been removed/collected.

The surface of mollicutes is to carefully rinse them three times for 10 min in 0.1 M cacodylic acid buffer (pH 7.3) at room

Stabilizing the specimen

There are various ways but typically done with fixatives.
It can be achieved by

Perfusion and microinjection

Immersions or Vapours

Using various **fixatives** including **aldehydes**, **osmium tetroxide**, **tannic acid**, or **thiocarbohydrazide**.

For mollicutes, a simple chemical fixation by immersing the specimen in a **1.5% glutaraldehyde** solution prepared in **0.1 M cacodylic acid buffer** (pH 7.3) and incubated at 4 ^{o}C overnight appears in most cases sufficient.

The use of a **postfixative** (e.g., **osmium**) improve bulk conductivity of the specimen, but does not necessarily provide a better stabilization of mollicutes prepared for SEM.

Rinsing the specimen

After the fixation step, samples must be rinsed in order to remove the excess fixative.

For Microbes: The specimens should be rinsed in **0.1 M cacodylic acid buffer** (pH 7.3), 1 time for 10 min

3 times for 20 min at 4 ^{o}C.

Note: Some of the samples can be stored in this EM buffer for several months because the buffer contains **arsenic** which inhibits the growth of unwanted microorganisms in the specimen container. However, **cacodylic acid buffer** can be used to preserve at least for a monthly if the samples are to be stored in this buffer for longer periods of time.

Dehydrating the specimen

The dehydration is typically performed with either a graded series of **acetone** or **ethanol**.

The dehydrating of mollicutes for SEM includes the immersion of the specimens in

50% acetone for 5 min

70% acetone for 10 min

80% acetone for 10 min

90% acetone for 15 min

100% acetone (dried with $CaCl_2$) twice for 20 min at 4 ^{o}C.

This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions.

Drying the specimen

The specimens must be dry or the sample will be destroyed in the electron microscope chamber.

Many electron microscopists consider a procedure called the **Critical Point Drying (CPD)** as the gold standard for SEM specimen drying.

The specimen is treating by numerous times using **liquid carbon dioxide** as the transitional fluid.

Carbon dioxide is removed after its transition from the liquid to the gas phase at the critical point, and the specimen is dried without structural damage.

Simple Desiccation (SD) technique is used to air-drying procedure after fixation, rinsing, and dehydration of the mollicutes. SD is not easy to do and there is the risk that specimens **collapse**, **flatten**, or **shrink** uncontrollable under these conditions. Although SD is faster and cheaper.

Mounting the specimen

After the mollicutes have been **cleaned**, **fixed**, **rinsed**, **dehydrated**, and **dried**, specimens must be mounted on a holder that can be inserted into the scanning electron microscope.

Samples are typically mounted on **metallic** (**aluminium**) stubs using a **double-sticky tape**.

It is important to decides on the best orientation of the specimen on the mounting stub before attaching it.

A re-orientation proves difficult and can result in significant damage to the sample.

Coating the specimen

The coating of specimen is done to **increase its conductivity** in the scanning electron microscope and to prevent the **build-up of high voltage charges** on the specimen by conducting the **charge to ground**.

Typically, specimens are coated with a thin layer of approximately 20 nm to 30 nm of a **conductive metal** (e.g., **gold**, **gold-palladium**, or **platinum**).

The samples are also coated with **sputtercoater** apparatus.

After all the steps are performed, the investigator is ready to view the mollicutes in the scanning electron microscope. This is the moment when the mycoplasmologist will find out whether or not the multi-step sample preparation for SEM was successful. It is important to perform to perfection in order to achieve SEM images that can be interpreted without the influence of **artifacts** caused by specimen handling.

Sample Preparation for TEM

Cleaning the surface of the specimen

As discussed earlier, the best way to clean the surface of mollicutes from contaminants is to carefully rinse them three times for 10 min in 0.1 M cacodylic acid buffer (pH 7.3) at room temperature.

Primary fixation of the specimen

Mollicutes can be chemically prefixed by immersing the specimens in a 1.5% glutaraldehyde solution prepared in 0.1 M cacodylic acid buffer (pH 7.3) and incubated at 4 oC overnight.

Rinsing of the specimen

In order to remove excess glutaraldehyde from the samples, the mollicutes should be subjected to a thorough but carefully conducted rinsing procedure. Specimens can be washed in 0.1 M cacodylic acid buffer (pH 7.3), starting with one time for 10 min, and then three times for 20 min at 4 ^{o}C.

Cleaning the surface of the specimen

As discussed earlier, the best way to clean the surface of

Secondary fixation of the specimen

It helps for preserving the structure of the specimen with no alterations from the living state. There is always the risk that potentially destructive autolytic processes begin altering the sample before fixation takes effect.

It is also important for protecting the specimen during steps such as embedding, sectioning, and exposure to the TEM electron beam which operates at higher accelerating voltages.

Minimizing the risks of artefact induction includes the selection of the most appropriate fixation protocol for a particular specimen.

Mollicutes can be successfully stabilized for TEM investigation by postfixation with 1% osmium tetroxide prepared in 0.1 M cacodylic acid buffer (pH 7.3) for 1.5 hrs at room temperature (immersion fixation).

Dehydrating the specimen

For TEM investigation, mollicutes can be dehydrated in a graded series of ethanol. Dehydration of mollicutes in 50% ethanol for 5 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 15 min, and 99.9% ethanol (dried with a 4-mesh molecular sieve) twice for 20 min at room temperature.

This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions.

Infiltration of the specimen with a transitional solvent

The ethanol is not miscible with the plastic embedding medium thus may be most suitable for TEM investigation of mollicutes. The replacement of the dehydration solution by another intermediary solvent (i.e., propylene oxide) is thus necessary.

This process is essentially an alcohol substitution.

The immersion of mollicutes in propylene oxide twice for 20 min at room temperature is sufficient before attempting to embed the specimens in a resin.

Infiltration with resin and embedding the specimen

Mollicutes can be embedded in a variety of different media depending on the use (e.g., conventional TEM or immuno TEM).
For conventional TEM of mollicutes, the epoxy resin Durcupan ACM is quite suitable.
Immersion of mollicutes in propylene-oxide/Durcupan-ACM (1:1; v/v) at room temperature overnight (use gloves and a fume hood, and leave the specimen container open for the propylene oxide to evaporate).
The next day, the specimens should be immersed in a freshly prepared Durcupan ACM mixture (pure) and left for 2 hrs at room temperature.
A second Durcupan ACM mixture (pure) is then prepared and used as the embedding medium (free of air bubbles).
Polymerization of the epoxy mixture can be achieved by placing the specimens in a drying cabinet for 2 days at 40 ^{o}C and for an additional 2 days at 60 ^{o}C.
Leaving the samples after heat polymerization for an additional 1-2 weeks at room temperature can improve the subsequent cutting experience as the resin blocks continue to harden during this time.

Sectioning and staining of the specimen

The procedure for cutting specimens into semithin (0.5 µm to 2 µm) and ultrathin sections (about 70 nm to 90 nm) is known as microtomy and ultramicrotomy.

Semithin sections are typically stained with toluidine blue for 1 min on a hot plate (70 ^{o}C to 90 ^{o}C), examined by LM, and used for identifying the specimen within the resin block before proceeding with ultramicrotomy.

Ultrathin sections are typically stained with uranyl acetate followed by lead citrate.

Virtually every step can affect the quality of the final electron micrograph. It is therefore important that the plans and executes every step in great detail.

Most of the chemicals used in EM are dangerous thus training in the proper use of all equipment and reagents in the EM laboratory is essentially required.

The investigator must be aware of potential hazards such as fire, chemical, electrical, and physical associated with these items.

Factors influencing the EM studies of the interaction between mollicutes and host cells:

Euthanasia procedures for laboratory animals needs to be carefully reviewed as some drugs can affect the function and structure of ciliated cells of respiratory epithelium.

The procedures for the establishment of cell cultures and explant cultures can influence the EM data (e.g., tracheal explants prepared with scalpels show on electron micrographs more damage than those prepared with razor blades).

Media used for the culture of mollicutes and explant/cell cultures need to be carefully selected (a poorly maintained tracheal explant or mollicutes taken from poorly frozen stocks will most likely reveal significant cell damage on electron micrographs).

Some eukaryotic cells (e.g., respiratory epithelial cells) have surface-exposed structures (e.g., cilia and microvilli) that can be easily damaged during handling; this damage may be later falsely attributed to the infection with mollicutes).

Infection of some mollicutes on respiratory epithelium results in the accumulation of significant amounts of mucus and cell debris which requires a balanced washing protocol of the specimens to determine attachment characteristics of the mollicutes and cytopathology induced

Figure 1: SE Micrograph of respiratory tracheal epithelium in-vitro infected with *Mycoplasma* mobile strain 163 K. Mollicute attached to epithelial cells and caused significant damage (deciliation and exfoliation of epithelial cells). Magnification: 1,000x; accelerating voltage: 6.2 kV; sputter-coating: gold.

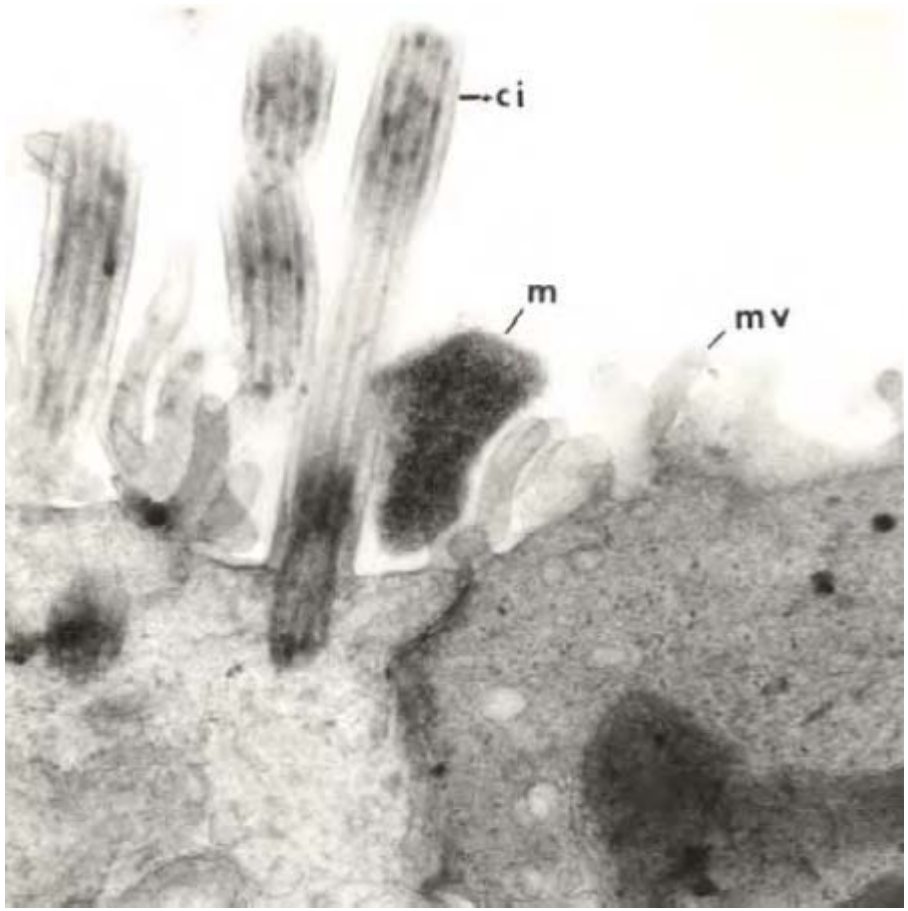
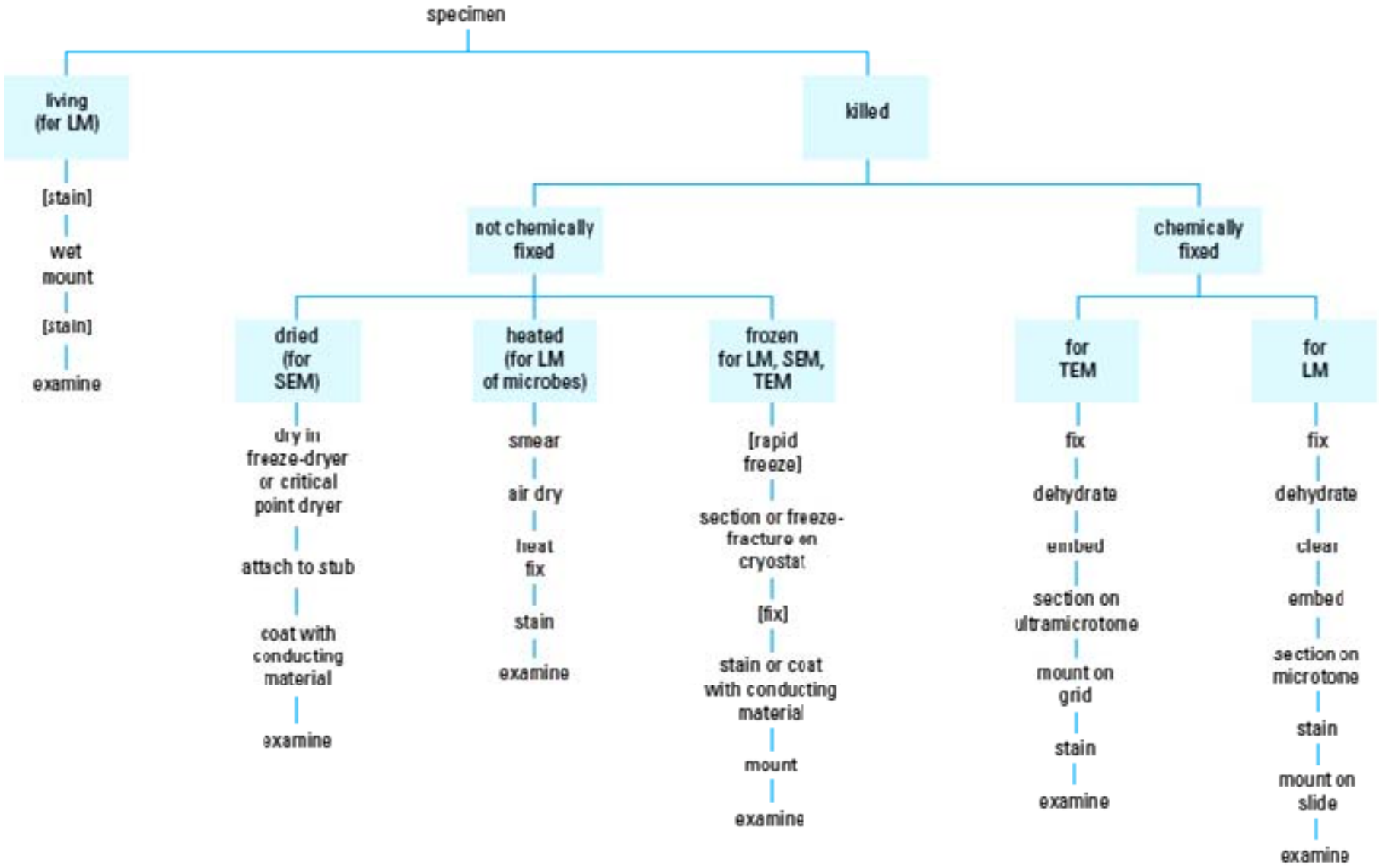


Figure 2: Transmission electron micrograph of the attachment of M. mobile to epithelial cells. Magnification: 33,800x; accelerating voltage: 80 kV; ci: cilia; m: *mycoplasma*; mv: microvilli.


specimen
living (for LM)
[stain]
wet mount
[stain]
examine
killed
not chemically fixed
dried (for SEM)
dry in freeze-dryer or critical point dryer
attach to stub
coat with conducting material
examine
heated (for LM of microbes)
smear
air dry
heat fix
stain
examine
frozen for LM, SEM, TEM
[rapid freeze]
section or freeze-fracture on cryostat
[fix]
stain or coat with conducting material
mount
examine
chemically fixed
for TEM
fix
dehydrate
embed
section on ultramicrotome
mount on grid
stain
examine
for LM
fix
dehydrate
clear
embed
section on microtome
stain
mount on slide
examine

Scanning Probe Microscopy

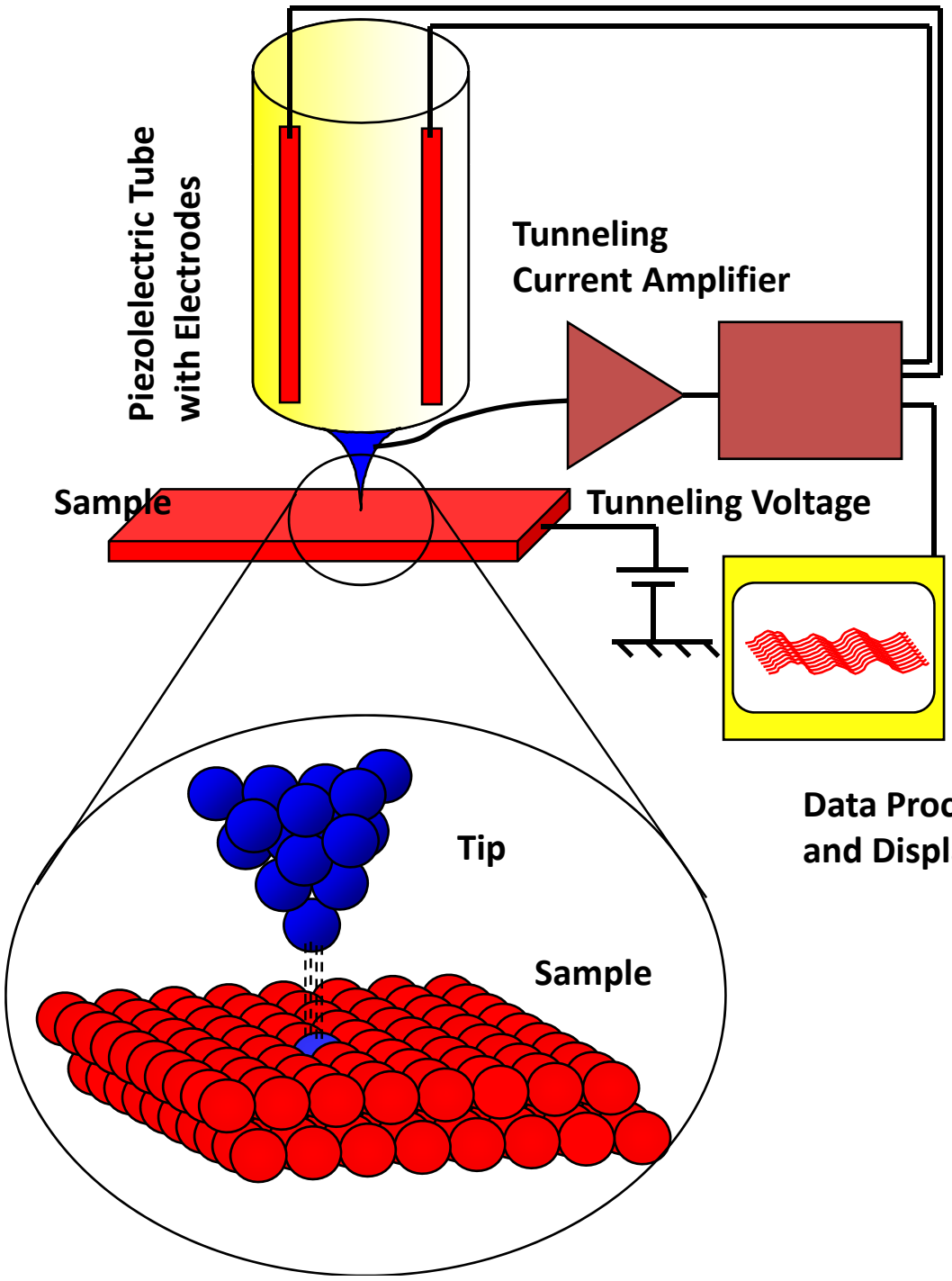
- scanning tunneling microscope
  - steady current (tunneling current) maintained between microscope probe and specimen
  - up and down movement of probe as it maintains current is detected and used to create image of surface of specimen

Scanning Probe Microscopy

- atomic force microscope
  - sharp probe moves over surface of specimen at constant distance
  - up and down movement of probe as it maintains constant distance is detected and used to create image

Scanning Tunneling Microscopy (STM)

Scanning Tunneling Microscopy (STM)



Fundamental process:

Electron tunneling

Electron tunneling

Elastic

Energy conservation during the process
Intial and final states have same energy

Inelastic

Energy loss during the process
Interaction with elementary excitations
(phonons, plasmons)

1D

Planar Metal-Oxide-Metal junctions

3D

Scanning Tunneling Microscopy

Rectangular barriers

Planar Metal-Oxide-Metal junctions

3D

Scanning Tunneling Microscopy

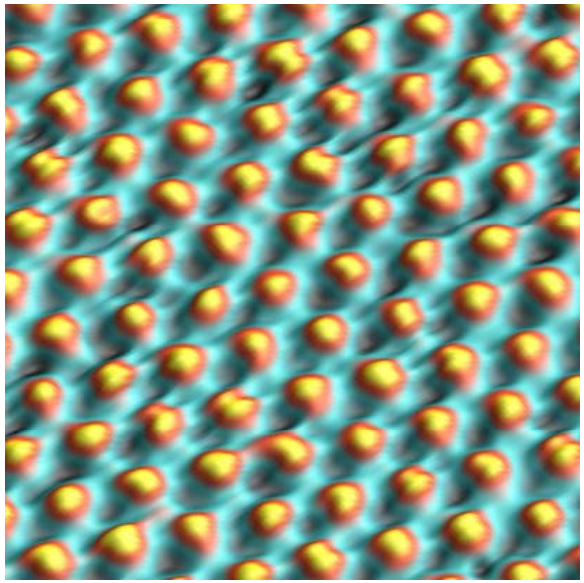
Time independent

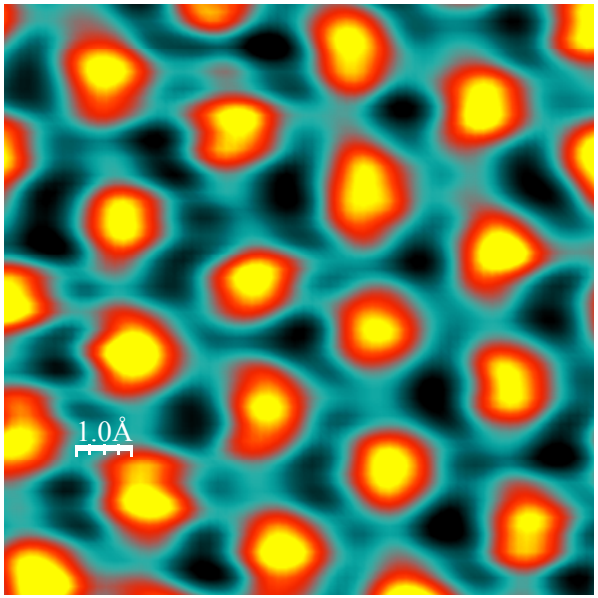
Matching solutions of TI Schroedinger eq

Time-dependent

TD perturbation approach:
$\psi(t)$ + first order pert. theory

STM: atomic resolution

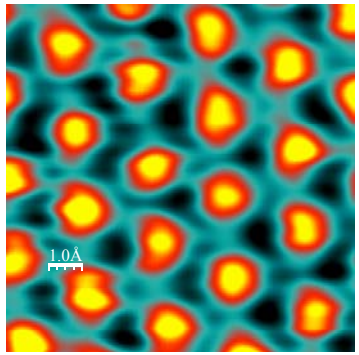




We observe features with a spatial resolution better than 0.1 nm
much lower of the tip curvature radius
Smaller than spherical approximation of the tip wave functions (0.8 nm)

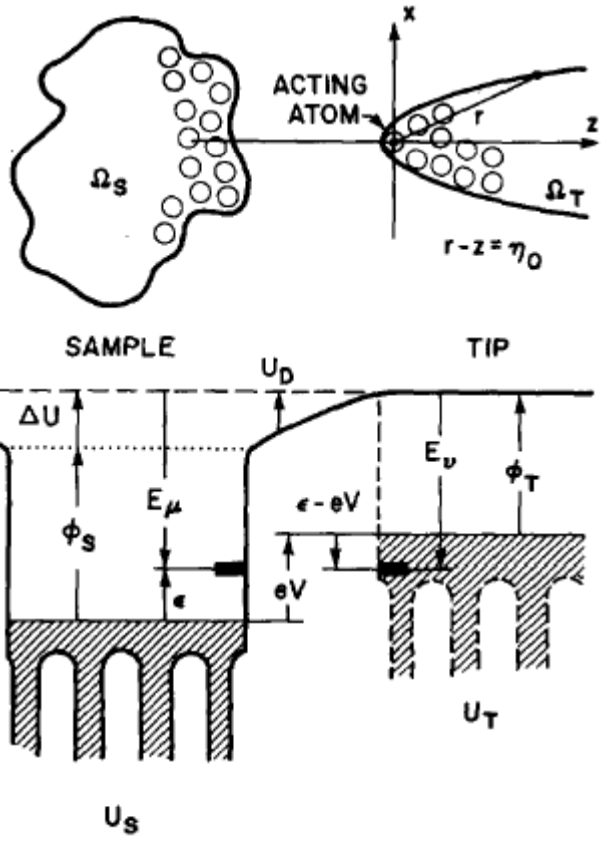
Model failing to explain the most important feature
of the STM: atomic resolution

STM: atomic resolution



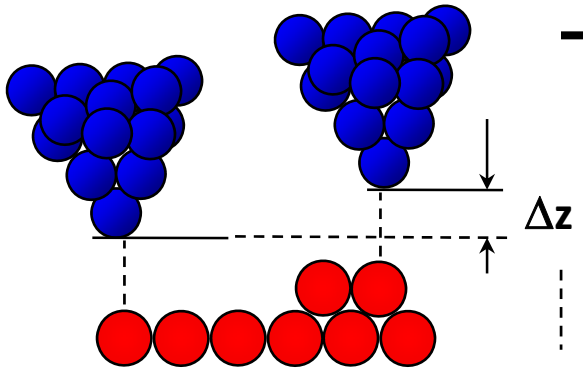
Accuracy of **perturbation theory**:
depends critically on the choice of the unperturbed wave functions, or the unperturbed **Hamiltonians**.
For 3D tunneling the choice of unperturbed Hamiltonians is not unique. This is especially true for higher biases, in which the potential in the tunneling gap is not flat.

- The unperturbed Hamiltonian minimizes the error introduced by neglecting the higher terms in the perturbation series.
- The tip states are invariant as the bias changes, simplify calculations.
- Easier estimation of bias distortion because the bias only affects the sample wave function, thus can be treated perturbatively



Constant current imaging

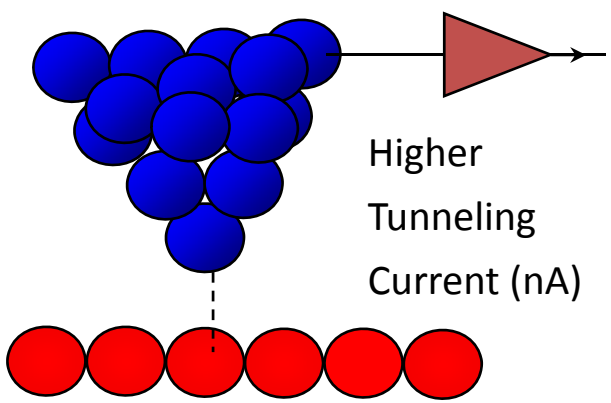
Unchanged
Tunneling
Current (nA)



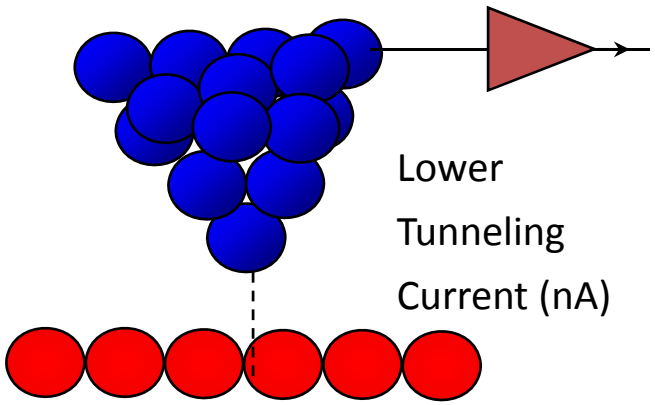
Unchanged
Tunneling
Current (nA)

Typical working mode

Constant height imaging



Higher
Tunneling
Current (nA)



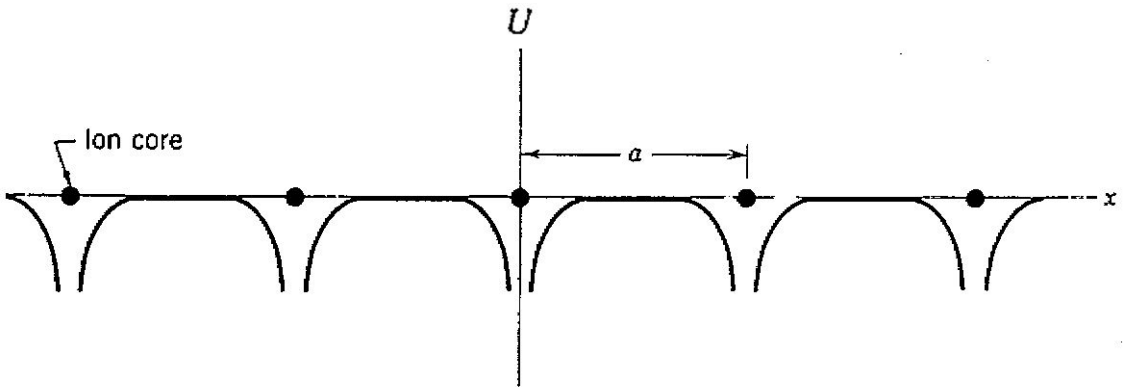
Lower
Tunneling
Current (nA)

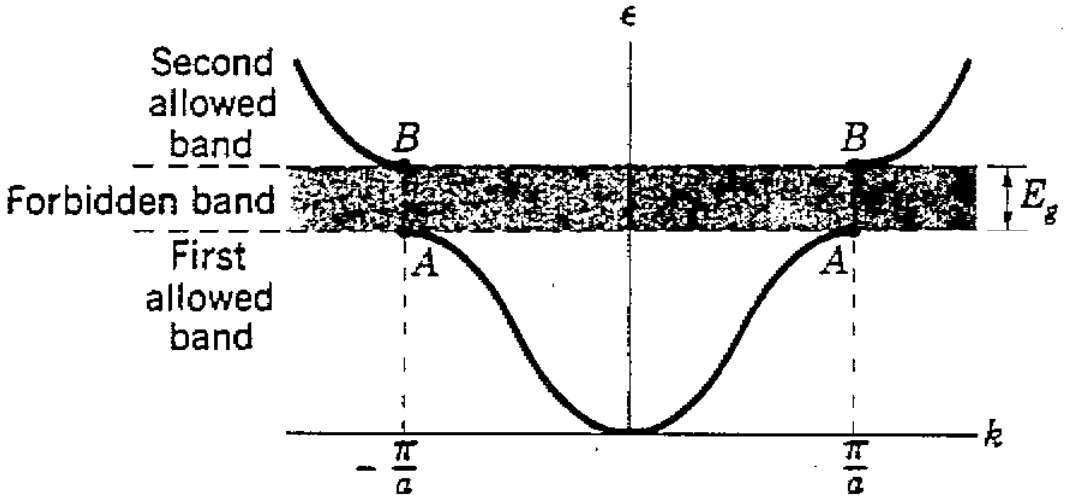
Applied only on very flat regions

Constant current imaging

Imaging: spatial configuration and energy dependence of electron states (LDOS) need not to correspond in any simple way to the atomic positions

Example: linear lattice

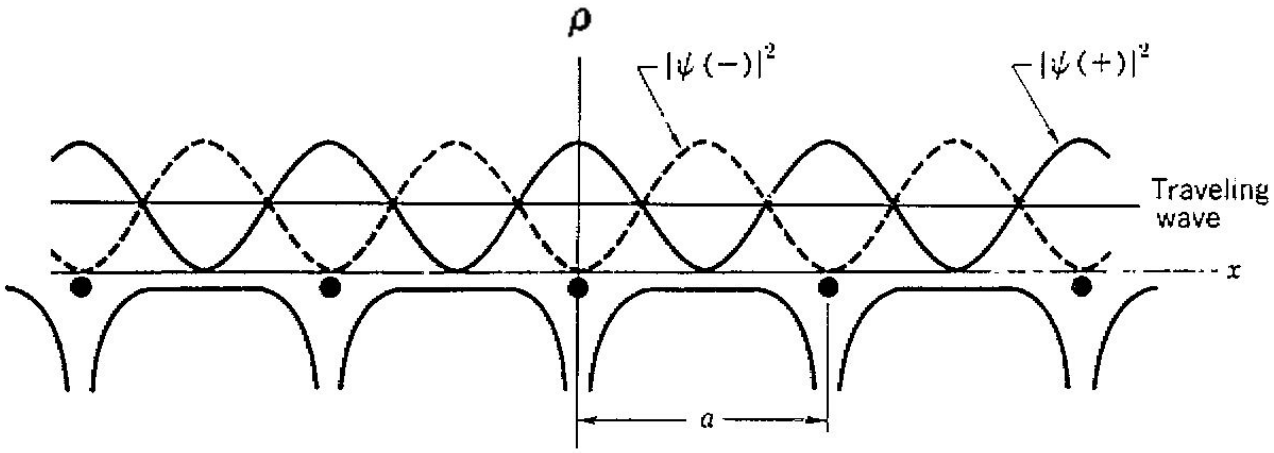




At the Bragg reflection the potential gives rise to a forbidden energy region
The band gap

Constant current imaging

Imaging: spatial configuration and energy dependence of electron states (LDOS) need not to correspond in any simple way to the atomic positions



Charge density ON atomic positions

Charge density BETWEEN atomic positions

In the image always topographic AND electronic features

Tunneling Spectroscopy

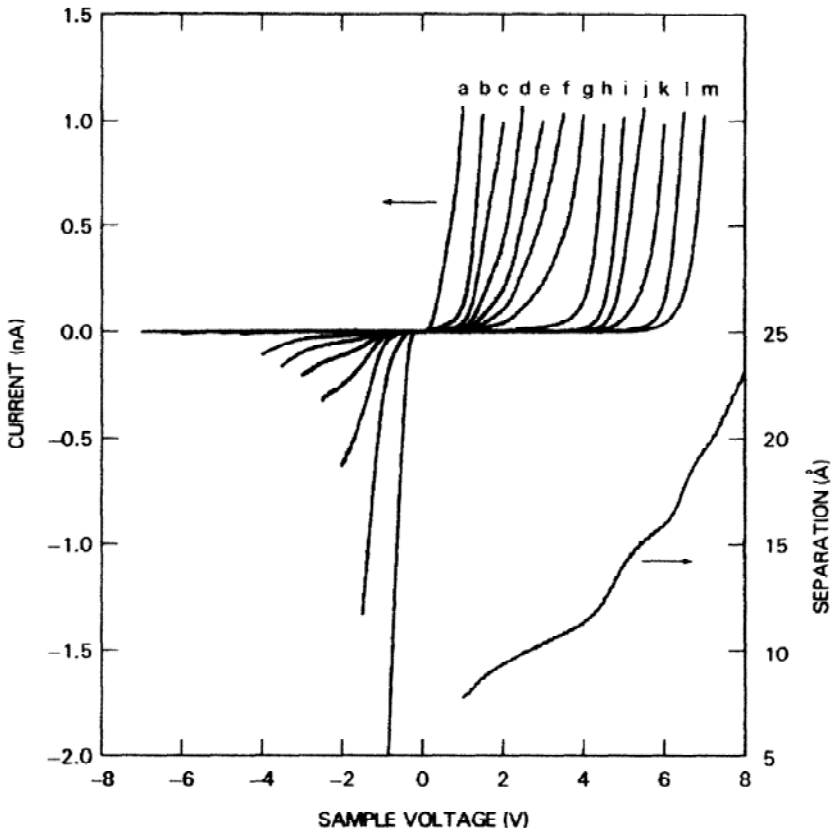
Acquiring STS spectra

Sample and hold technique

Stop the tip on a location
Disable feedback
Scan V and monitor I

Taken at different initial measuring conditions, i.e. different tip-sample distances

Si(111)-(2x1)

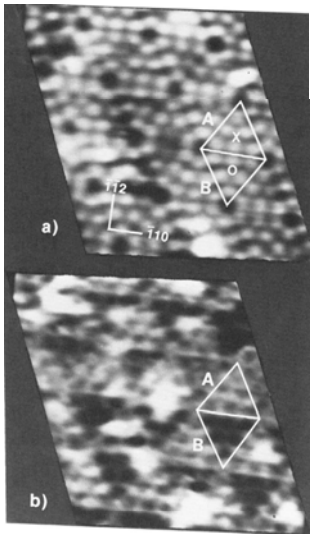


Tunneling Spectroscopy

Obtaining STS images

dI/dV with lock in

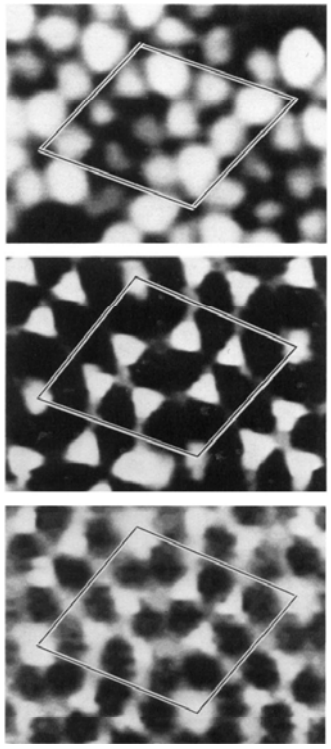
Apply modulation
Collect dI/dV while scanning simultaneously at each point



DOS at the set point of imaging condition
Emphasize one state
Possible only in stable tunneling conditions (not in band gap)

Current-imaging tunneling spectroscopy (CITS)

Feedback on only 30% of the time
Collect dI/dV at fixed separation



-0.35 V

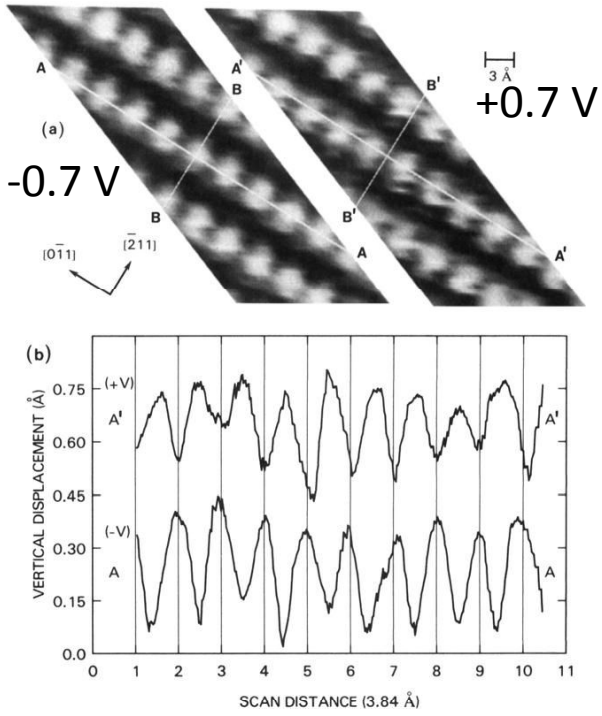
-0.8 V

-1.7 V

Need to be done at V following topography of nuclei

Voltage-dependent imaging

Integrate over an energy interval at state onset



Spatial relationship between occupied and unoccupied states

Scanning Tunneling Microscopy (STM)

Design and instrumentation

Approach mechanism

Enables the STM tip to be positioned
within tunneling distance of the sample

High precision scanning mechanism

Enables the tip to be rastered above the surface

Control electronics

Control tip-surface separation
Drive the scanning elements
Facilitate data acquisition.

Vibration isolation

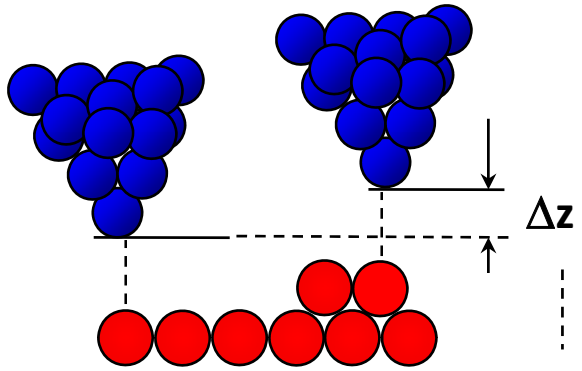
The microscope must be designed to be insensitive
or isolated from ambient noise and vibrations.

Scanning Tunneling Microscopy (STM)

Design and instrumentation

Vibration isolation

It is essential for successful operation of tunneling microscopes.



This stems from the exponential dependence of the tunneling current on the tip-sample separation.

Typical surface corrugation is 0.1 ÷ 0.01 nm or less

tip - sample distance must be maintained with an accuracy of better than 0.001 nm = 1 pm

Design criteria:

The system response to external vibrations and internal driving signals is less than the desired tip sample gap accuracy throughout the bandwidth of the instrument.

STM sensitivity to external and internal vibrational sources:

Structural rigidity of the STM itself

Properties of the vibrational isolation system

Nature of the external and internal vibrational sources

Scanning Tunneling Microscopy (STM)

Design and instrumentation

Approach mechanism

Enables the STM tip to be positioned
within tunneling distance of the sample

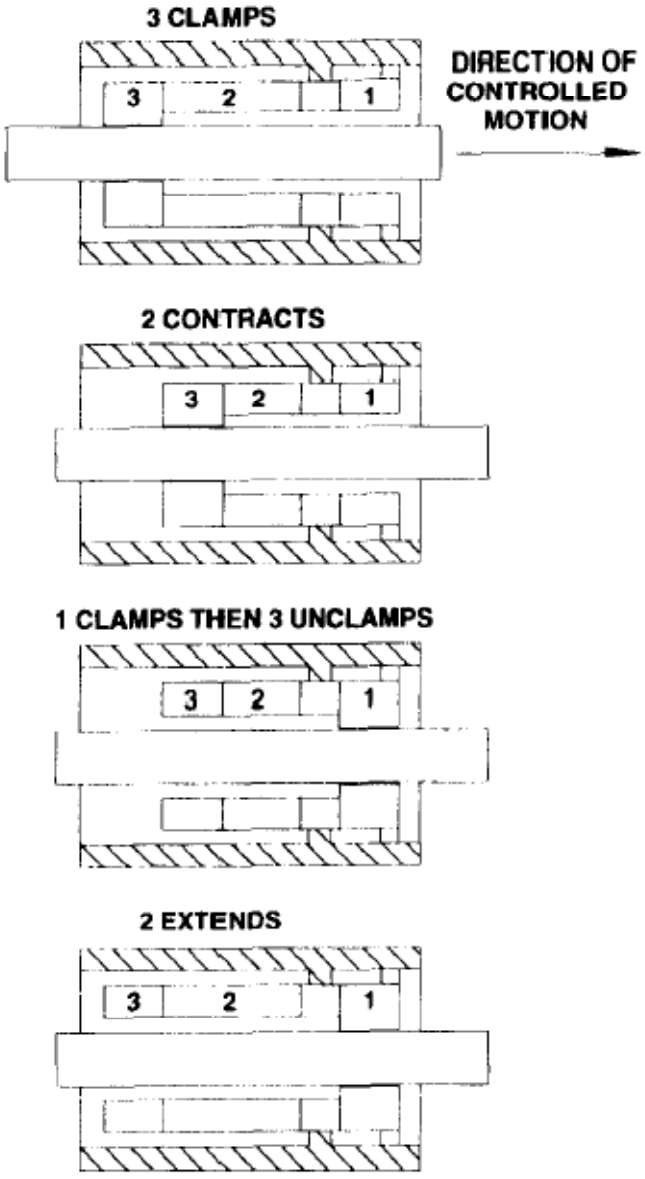
Coarse motion devices to bring the tip
and the sample into tunneling range

Inchworm stepper motor

Compact dimensions and high ν_m,
Vacuum compatibility
Reliability
High mechanical resolution.

Scanning Tunneling Microscopy (STM)

Design and instrumentation



Operating principle

Three piezoelectric elements

Outer elements 1 and 3 contract and clamp the motor to the shaft

The center element 2 contracts along the shaft direction

These elements operate independently

the motor can move relative to the shaft if the shaft is fixed

the shaft can be moved relative to the motor if the motor is fixed

In this example the motor is held fixed and the shaft is moved

To move the shaft one step towards the right

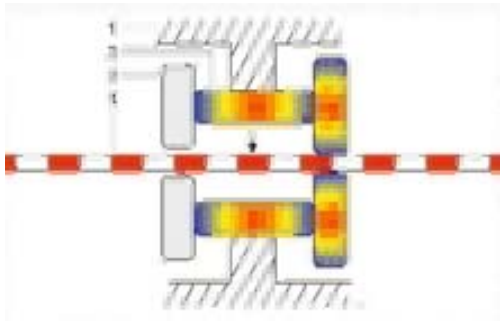
3 is clamped and 1 is unclamped

2 contracts and the shaft is then moved towards the right

1 is then clamped and element 3 is unclamped

2 is extended to its original length

Similar to those used to climb a rope.



Scanning Tunneling Microscopy (STM)

Design and instrumentation

The resonance frequency of the scanning element is an important factor in determining the data acuisition speed data, since it has its own T

For $\nu_{scan} < \nu_{se}$ the scanner responds uniformly to the drive voltage.

For $\nu_{scan} \sim \nu_{se}$ the amplitude of the scanners motion may increase dramatically

For $\nu_{scan} > \nu_{se}$ the mechanical response falls off.

ν_{se} of the scanning element may be as high as 100 kHz

ν_m is usually substantially lower (1-10 kHz)

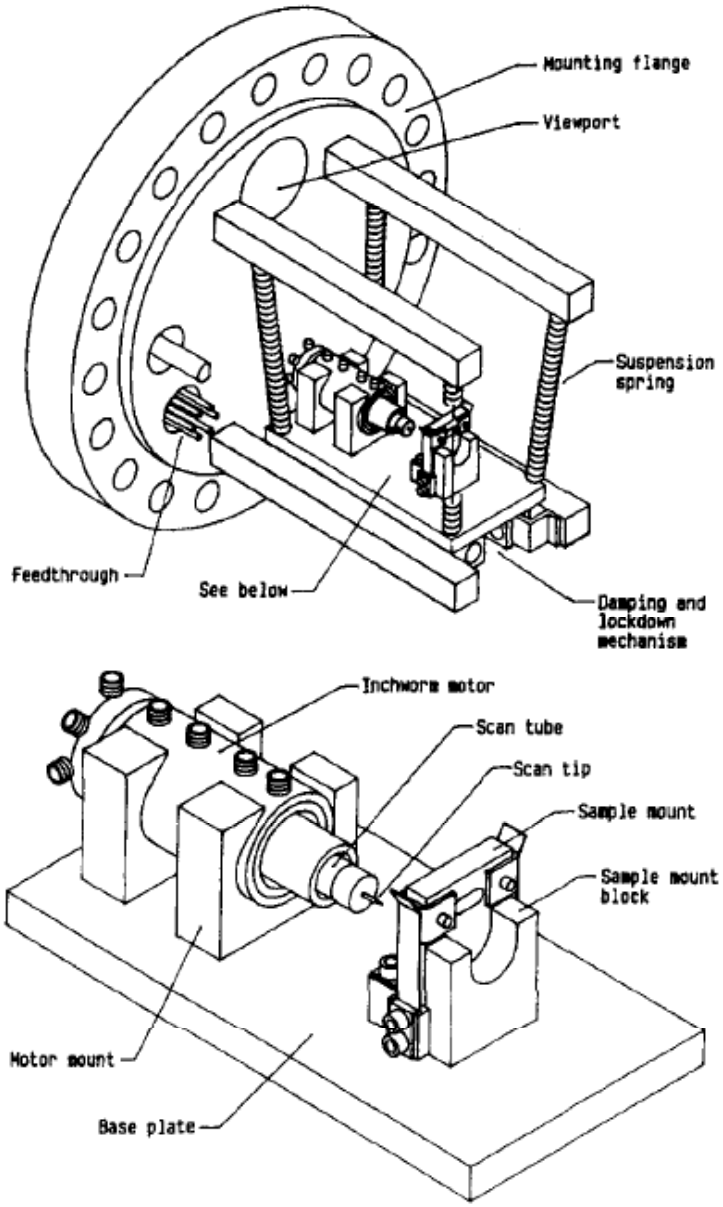
So scanning speed is limited much below 1 kHz

1 frame: 400 lines

2 lines /s = 0.5 Hz

Total 200 s

Limits: feedback loop gain

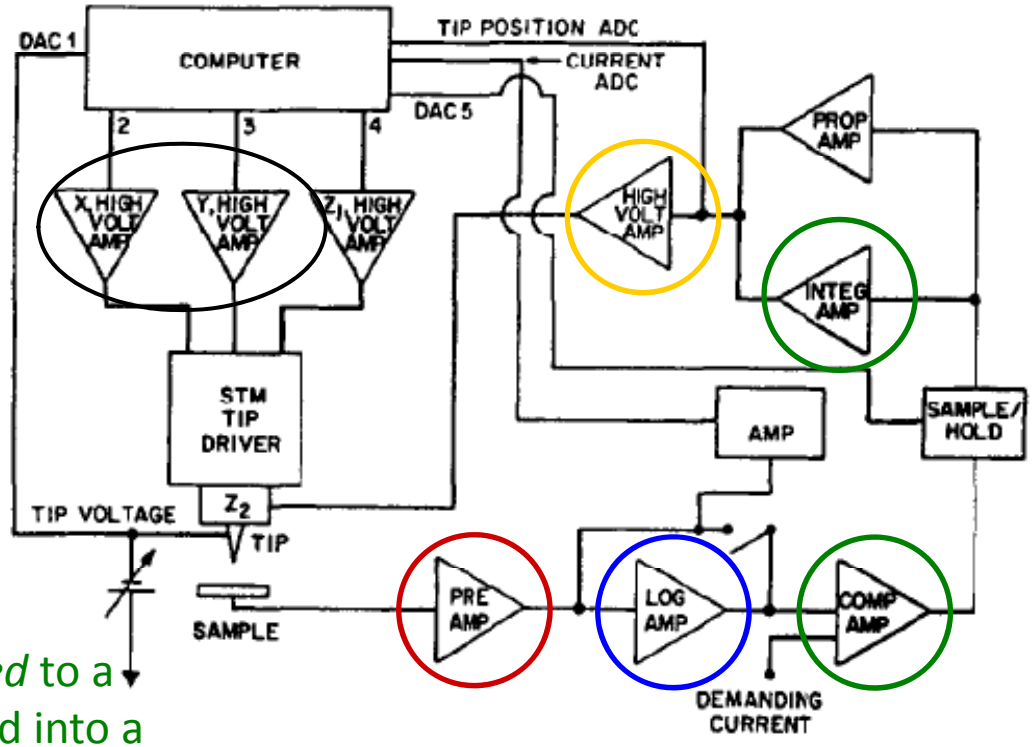


Scanning Tunneling Microscopy (STM)

Control electronics

Design and instrumentation

Control tip-surface separation
Drive the scanning elements
Facilitate data acquisition.



I is measured by a *preamplifier* with a variable gain of 10^6-10^9 V/A and variable τ_c to limit the bandwidth below the primary mechanical ν_m

The preamp is located as close to the tip as possible to minimize noise

The tunneling current is linearized by a *logarithmic amplifier*

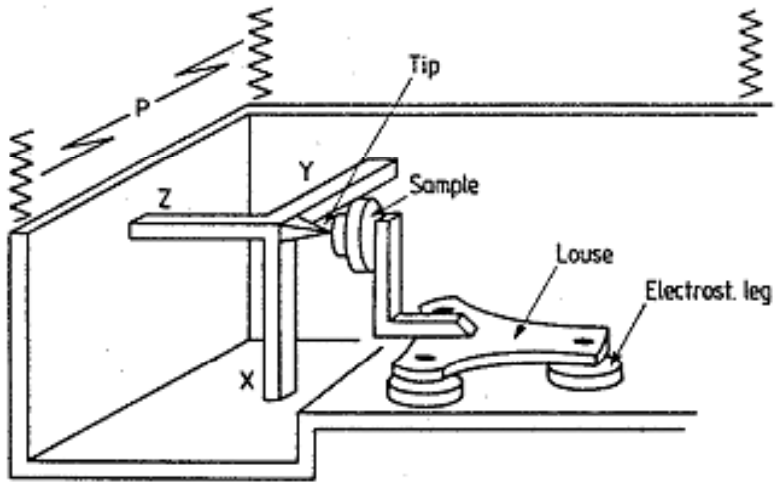
The tunneling current is then *compared* to a set-point, with the difference signal fed into a feedback amplifier that has an *integrating amplifier* with variable time constant.

The feedback signal is then amplified by a *high voltage amplifier,* the output of which is applied to the z-piezo to maintain the tunneling current at the desired set-point.

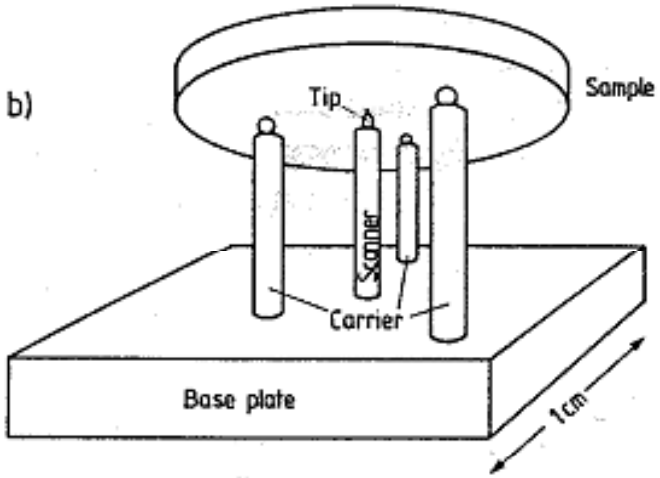
The x- and y-piezos are connected to *high voltage amplifiers,* which amplify slow scan (x) and fast scan (y) sweep signals generated by PC controlled DACs.

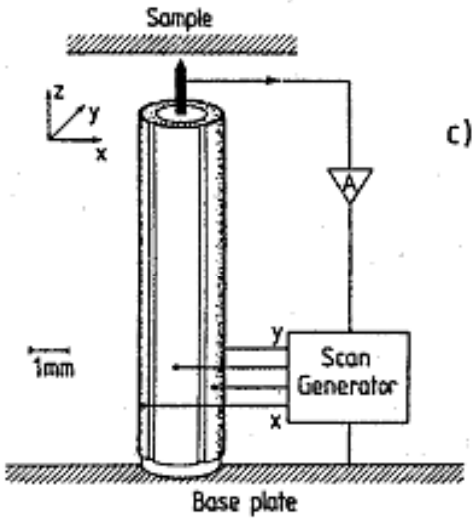
Scanning Tunneling Microscopy (STM)

Examples of STM Apparatus



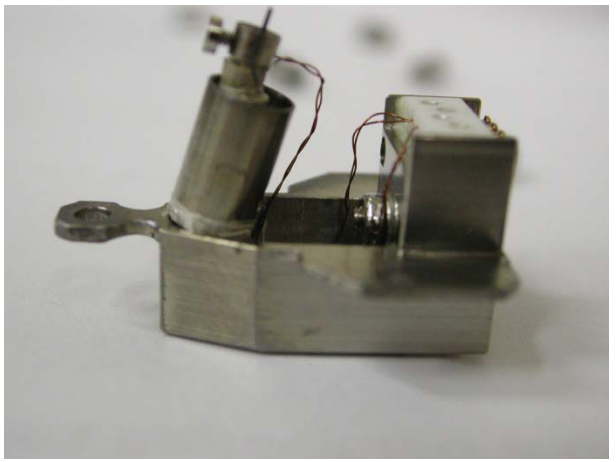
a)



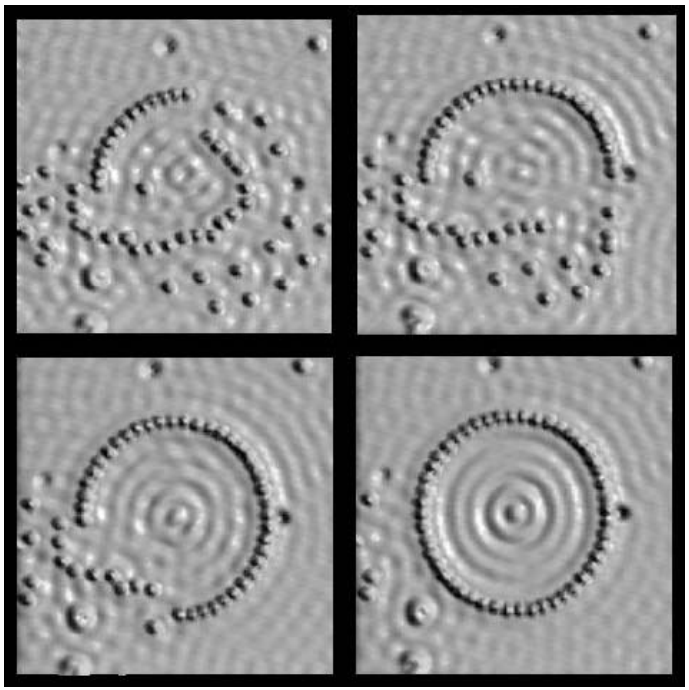


c)

STM scanner



Nanomanipulation
Quantum Corrals



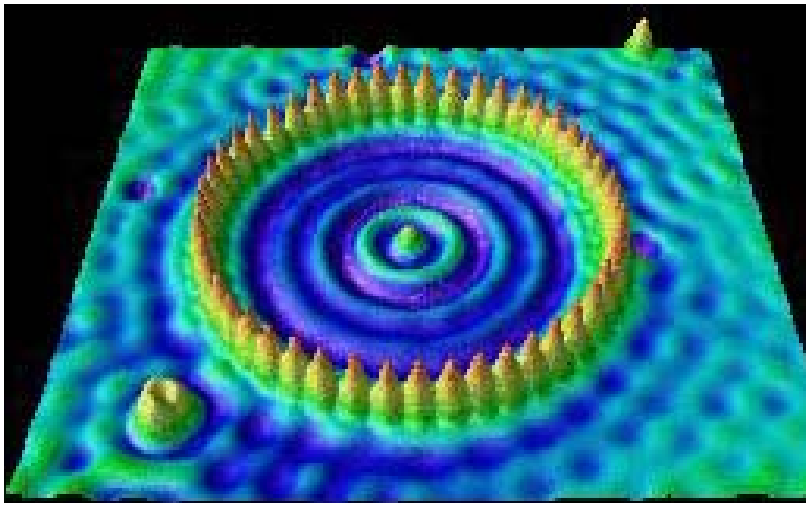
Fe atoms on Cu(111)

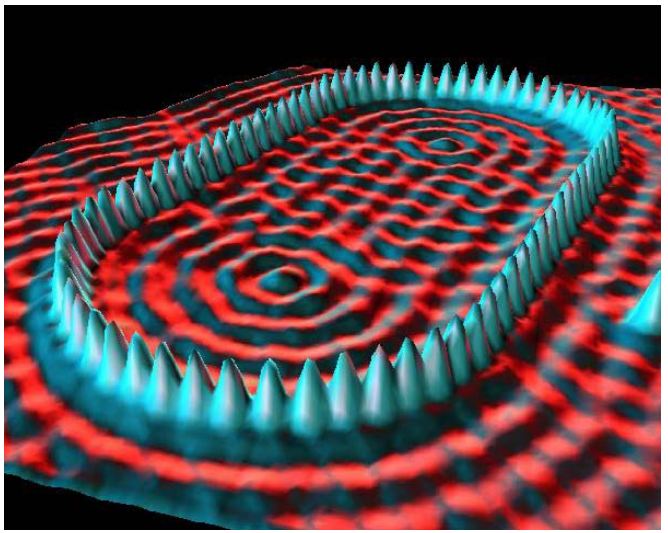
Nanomanipulation

Quantum Corrals are fabricated by manipulating atoms adsorbed at a solid surface to give a specific shape to the corral.

The STM tip is used to lift and put down the atomic units.

Peculiar effect related to Quantum Corrals
Formation of a two-dimensional electronic gas (standing waves) confined within the corral.



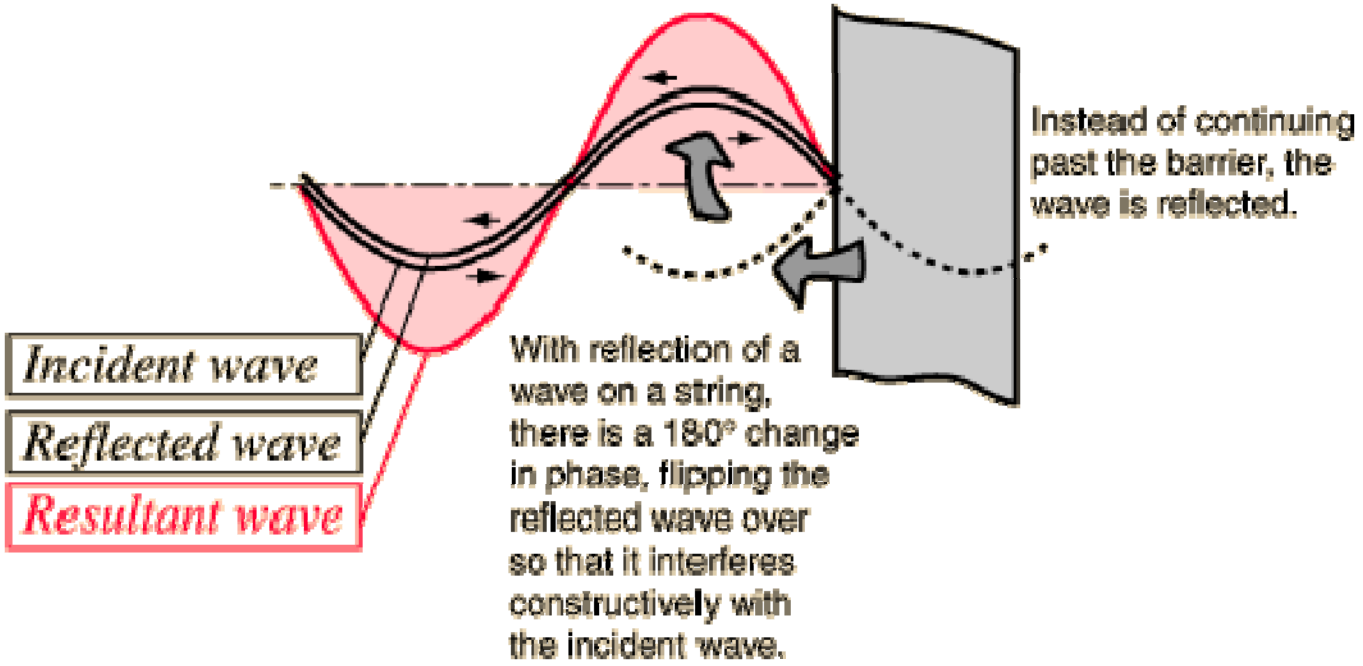


Standing waves

In general the standing waves are particular modes of vibrations in extended objects like strings.

These standing wave modes arise from the combination of reflection and interference such that the reflected waves interfere constructively with the incident waves.

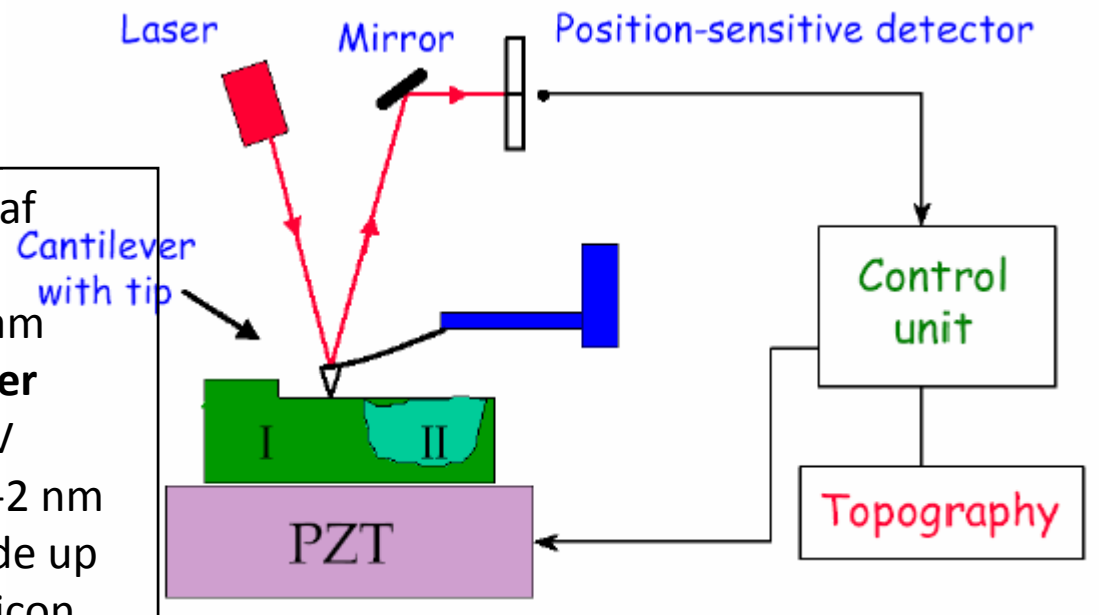
The waves must change phase upon reflection. Under these conditions, the medium appears to vibrate in regions and the fact that these vibrations are made up of traveling waves is not apparent - hence the term "standing wave".



Atomic Force Microscopy (AFM)

AFM basics

Basic idea:
Surface-tip interaction
Response of the cantilever



Thin flexible leaf spring or a microscale beam called **cantilever** (0.1mm long, V shaped with 1-2 nm sharp tip), made up of silicon or silicon nitride (S_3N_4)

Contact Mode

Tapping Mode

Non-Contact Mode

Mode of operation

i) **Contact or Static mode:** the tip is within a few Å of the surface and the repulsive force between the tip and the sample surface is kept constant during scanning by maintaining a constant deflection
ii) **Non-contact or Dynamic mode:** the distance between the tip and the surface is much larger in the range of 2 – 30 nm.

Vander Walls forces extends above the adsorved fluid layer from 1 nm – 10 nm and during scanning the interaction between the tip and the sample surface modifies the amplitude, phase or the resonance frequency of the vibration and provides information about the sample's characteristics.

Dynamic mode called the *intermittent contact mode or tapping mode* (tapping AFM) provides better image. The cantilever oscillates near 100 – 200 nm to facilitate modulate of the distance between the tip and the sample surface.

Advantages of AFM over SEM

i) AFM provides better resolution and a three dimensional surface profile is obtained compared to a two dimensional projection is SEM

ii) The instrument can be operated in ambient air and even in liquid environment without the requirement of vacuum, and hence, living organisms can be studied.

iii) Electrically non-conducting samples, such as biological samples and polymers can be studied and the samples do not require any pre-treatment.

iv) AFM can view and provide the small surface area of the sample (150 μm^2), whereas SEM require much larger area of the order of mm^2.

v) AFM can also be used as **force spectroscopy** to determine the distance curves of forces operating at nanoscale with a resolution better than 0.1 nm. Different types of forces can be measured like Van Der Waals forces, Casimir forces, dissolution forces in liquids, single molecular stretching, rupture forces and so on.

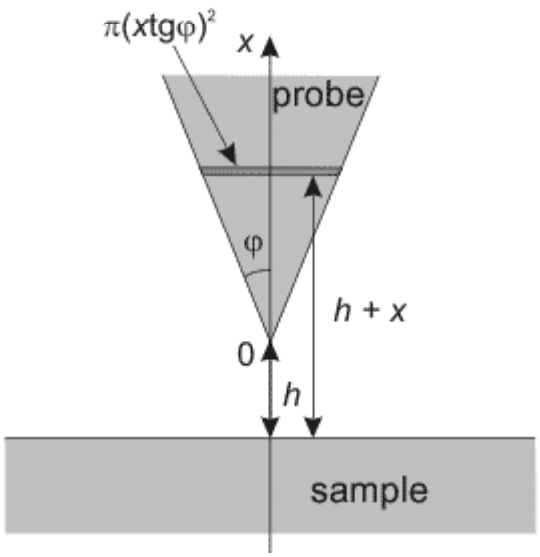
Origin of forces

Origin	Potential energy	
Fluctuation of the electronic clouds around a molecule, dipole formation	$U = -\frac{2\mu^4}{3kTr^6}$	**Keesom Dipole forces**
Fluctuation of the electronic clouds around a molecule, dipole formation, interaction of the dipole with a polarizable atom or molecule	$U = -\frac{\alpha\mu_{ind}^2}{r^6}$	**Debye Dipole forces**
Fluctuation of the electronic clouds around the nucleus, dipole formation with the positive charge of nucleus. Interaction of the dipole with a polarizable atom	$U = -\frac{3}{4}\frac{h\nu_i\alpha_1\alpha_2}{r^6}$	**London Dipole force**
Large overlap of core wavefunction of different molecules	$U_R = \frac{C_2}{r^{12}}$	**Born repulsive interatomic forces**

Origin of forces

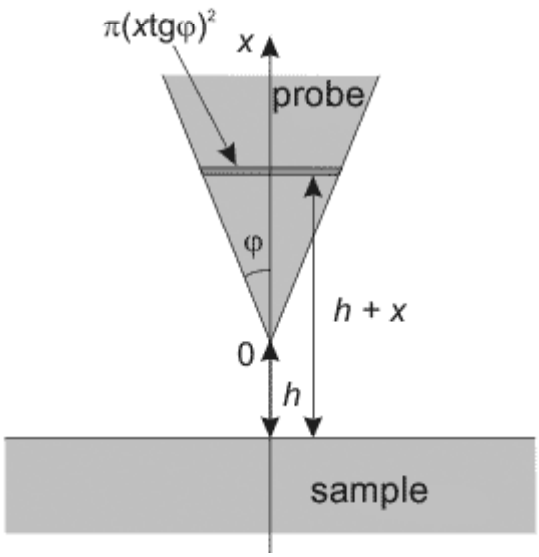
The force must be calculated for each shape

Conical probe



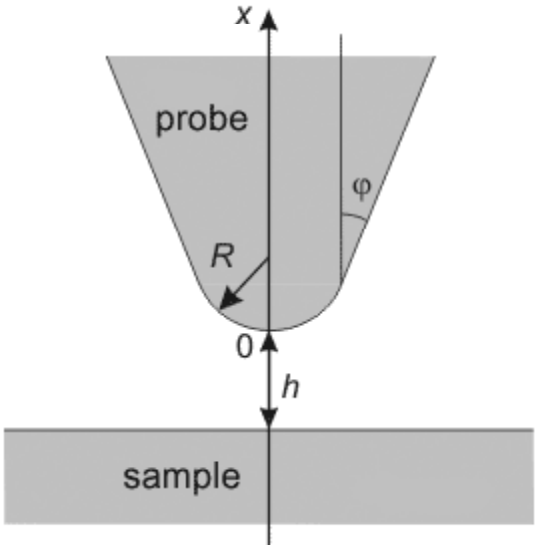
Tip radius r << h

Pyramidal probe



Tip radius r << h

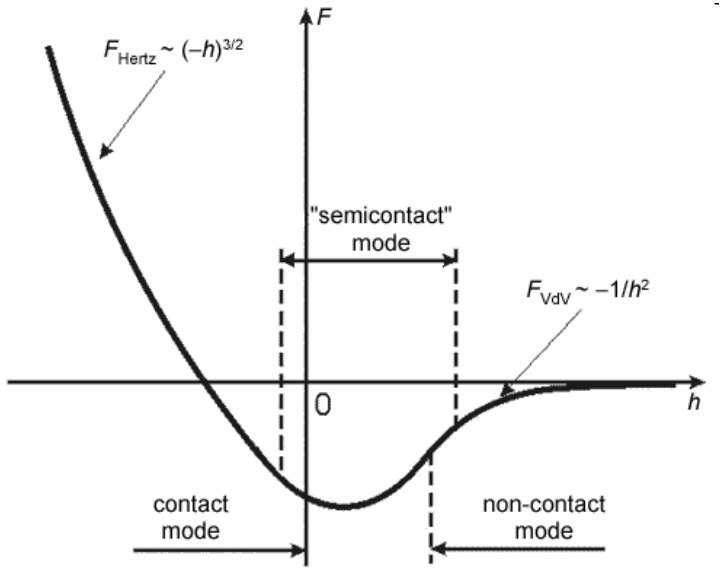
Conical probe rounded tip



For r >> h

Origin of forces

Adhesion forces



Middle range where attraction forces ($-1/r^6$) and repulsive forces ($1/r^{12}$) act

adhesion

It originates from the short-range molecular forces.

two types

- probe-liquid film on a surface (capillary forces)
- probe-solid sample (short-range molecular electrostatic forces)

electrostatic forces at interface arise from the formation in a contact zone of an electric double layer

Origin for metals

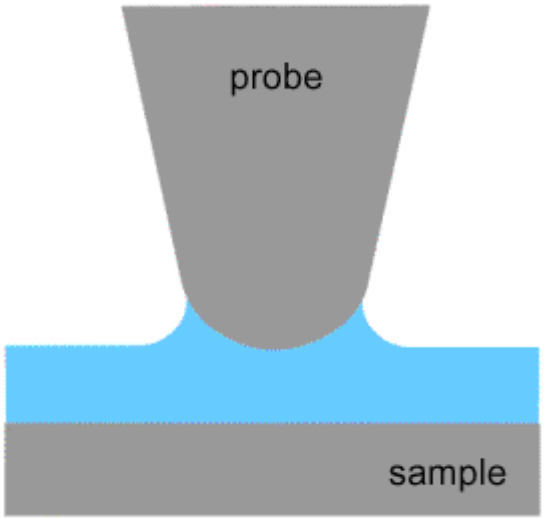
- contact potential
- states of outer electrons of a surface layer atoms
- lattice defects

Origin for semiconductors

- surface states
- impurity atoms

Origin of forces

Capillary forces



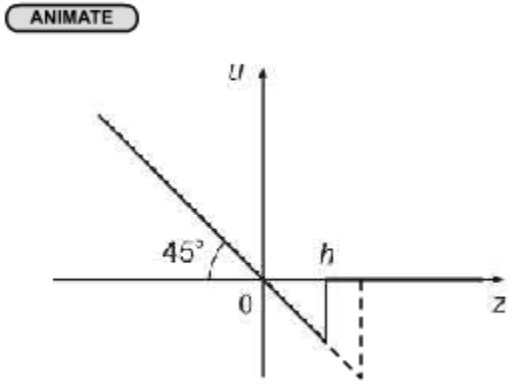
- Cantilever in contact with a liquid film on a flat surface
  The film surface reshapes producing the "neck“

The water wets the cantilever surface:
The water-cantilever contact (if it is hydrophilic)
is energetically favored as compared to the water-air contact

$$F \approx 10^{-9} N$$

Similar to VdW force

Consequence: hysteresis in approach/retractio



Operation modes

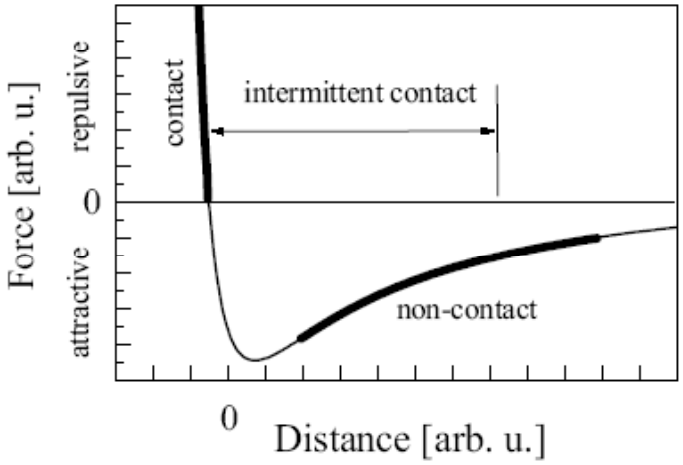
Contact Mode

Static cantilever

Tapping Mode

Non-Contact Mode

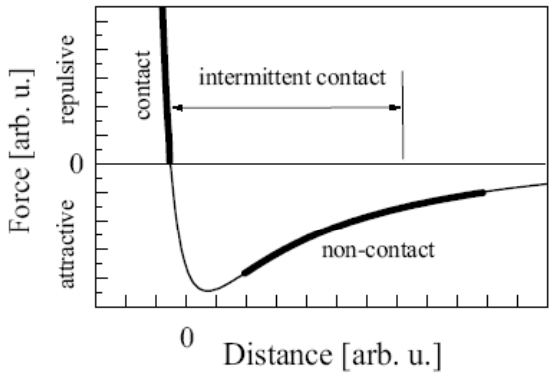
The cantilever is forced to oscillate



Tapping: Amplitude modulation (AM-AFM)
Non-contact: Frequency modulation (FM-AFM)

AM-AFM: a stiff cantilever is excited at free resonance frequency
The oscillation amplitude depends on the tip-sample forces
Contrast: the spatial dependence of the amplitude change is used as a feedback to measure the sample topography
Image = profile of constant amplitude
FM-AFM: the cantilever is kept oscillating with a fixed amplitude at resonance frequency
The resonance frequency depends on the tp-sample forces
Contrast: the spatial dependence of the frequency shift, i.e. the difference between the actual resonance frequency and that of the free cantilever
Image = profile of constant frequency shift.
Experiments in UHV: FM-AFM
experiments in air or in liquids: AM-AFM
Operation in non-contact or intermittent contact mode is not exclusive of a given dynamic AFM method

Contact mode



The tip is brought “to contact” with the surface
until a preset deflection is obtained.
Then the raster is performed keeping deflection
constant.

Equiforce surfaces are measured

Info on lateral dragging forces can be obtained

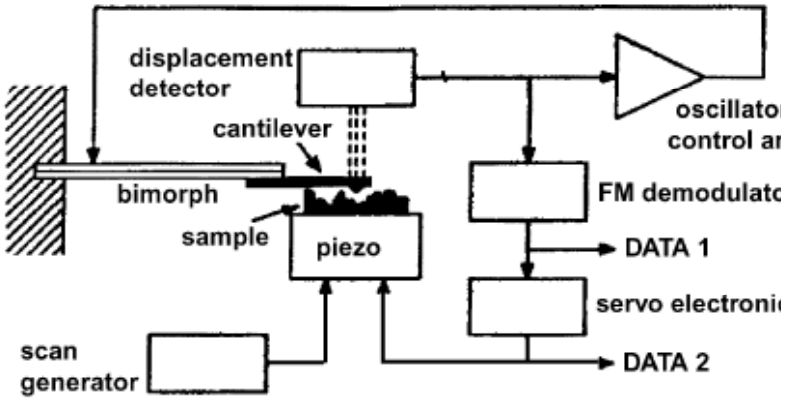
Drawbacks:
The download force of the tip may damage the sample
(expecially polymers and biological samples)

Under ambient conditions the sample is always covered
by a layer of water vapour and contaminants,
and capillary forces pull down the tip, increasing the tip-surface force
and add lateral dragging forces

Operation modes: FM

Frequency modulation

The signal used to produce the image comes from the direct measurement of cantilever resonance frequency (that depends on the tip–surface interaction)



$\neq$ from AM mode, the cantilever is kept oscillating at its current resonant frequency (different from ω_0 due to the tip–sample interaction) with a constant amplitude A_0

The driving signal of the cantilever oscillation is generated through a feedback loop where the a.c. signal coming from the PSD is amplified and used as the excitation signal

An automatic gain controller keeps the vibration amplitude constant

In FM-AFM, the spatial dependence of the $\Delta\omega$ induced in the cantilever motion by the tip-sample interaction is used as the source of contrast

During the scan, the tip–sample distance is varied in order to achieve a set value for $\Delta\omega$.

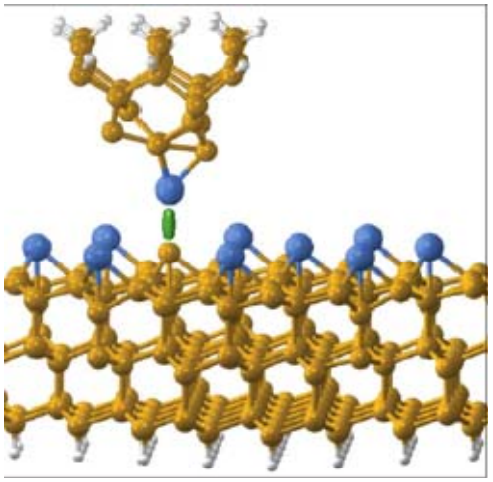
The topography represents a map of constant frequency shift over the surface.

Operation modes: FM

- long-range (LR) electrostatic
- vdW attractive
- repulsive contact Hertzian

Appropriate to explain main features of AFM
but cannot provide an explanation for the atomic resolution

Example: LR vdW interaction
Not dominated by the interaction of the tip atoms closer to surface
Depends on the macroscopic shape of the tip
How could provide the lateral resolution needed?



Atomic contrast relies on a significant lateral variation of the tip–surface interaction on an atomic length scale

This can only be provided by short-range (SR) interactions.

Operation modes: FM

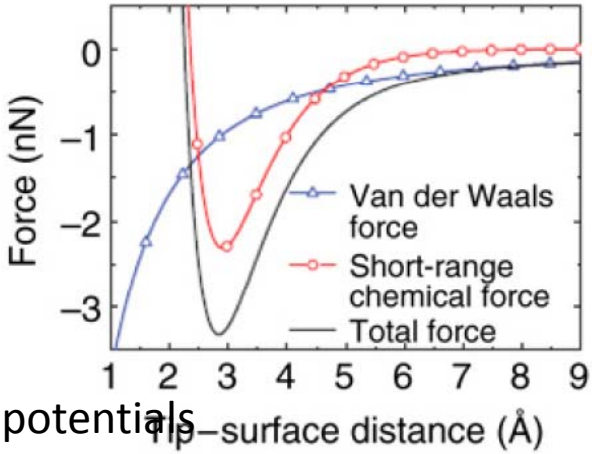
Origin of atomic resolution

Semiconductor surfaces

candidate force: covalent bonding interactions

In semiconductor surfaces and in the tip there are undercoordinated atoms with unsaturated bonds

They can contribute to the total tip–surface interaction and provide atomic resolution.



Covalent interaction: approximated by exponentially decaying potentials

The dependence on orbital overlap explains the exponential variation from d and makes it suitable for atomic resolution

Different from vdW interaction: related to the overlap of the atomic wavefunctions

Insulators (alkali halides and oxides) and oxidised tips

All the dangling bonds are saturated

Confined microscopic electric field around the oxygen tip apex provides the key to the lateral variation of the interaction

It is due to electrons taken from the surrounding Si atoms located on the strongly localised O 2p wavefunctions

The normal displacements of the surface ions + the related surface polarisation due to the strongly localised electric field are responsible for the atomic resolution in these materials