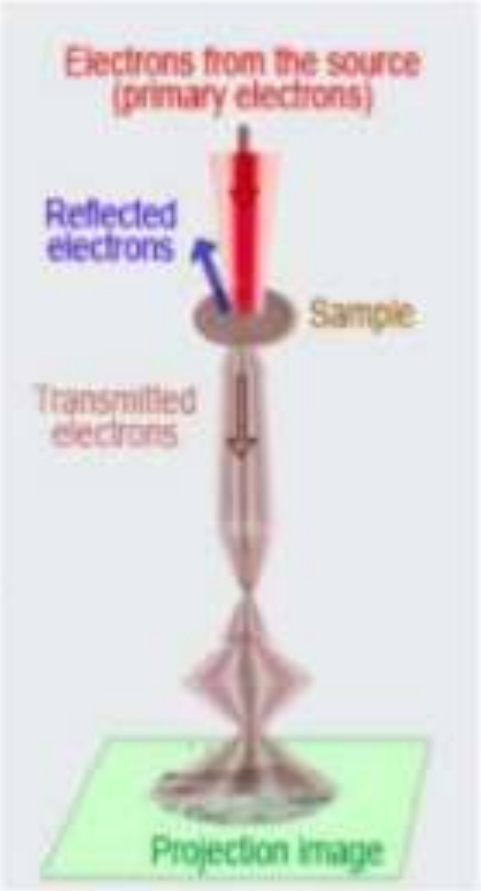
Bioanalytical techniques -3

(Graphics are collected from Internet)

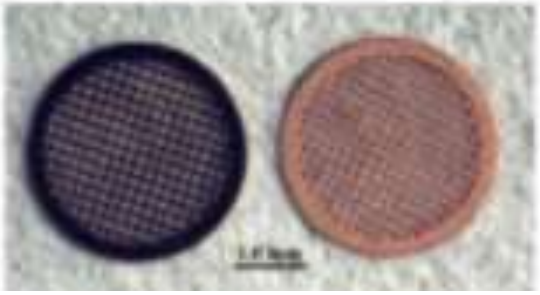
PRINCIPLE OF WORKING OF TEM

- Electrons possess a wave like character.
- Electrons emitted into vacuum from a heated filament with increased accelerating potential will have small wavelength.
- Such higher-energy electrons can penetrate distances of several microns into a solid.
- If these transmitted electrons could be focused - images with much better resolution.
- Focusing relies on the fact that, electrons also behave as negatively charged particles and are therefore deflected by electric or magnetic fields.



SAMPLE PREPARATION FOR TEM

- **Fixation -** fixed with chemical products (e.g. glutaraldehyde)
- **Rinsing and 'staining' -** treated with heavy metal compounds.
- **Dehydration -** washing with increasing ethanol concentration, followed by final wash in another a polar substance like propylene oxide.
- **Embedding in resin -** material is gradually infiltrated with the still unpolymerized resin .Little pieces of resin-infiltrated material are placed in small holders.
- **Trimming of resin block and ultrathin sectioning -** sections with a thickness of about 70 nm are cut with special knifes of cleaved glass . The cutting is done with a ultra-microtome.
- **Collection of sections on grid**



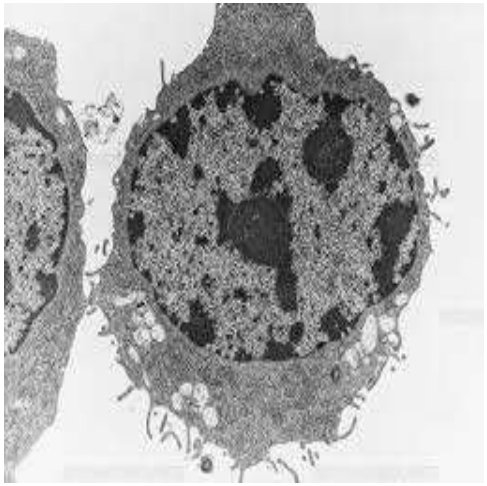
ADVANTAGES & DISADVANTAGES OF TEM

Advantages

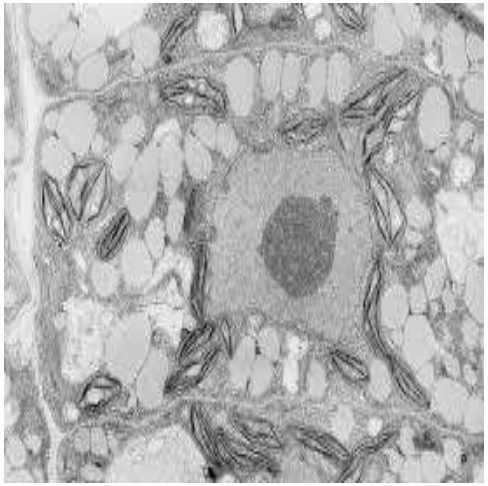
- TEMs offer very powerful magnification and resolution.
- TEMs have a wide-range of applications and can be utilized in a variety of different scientific, educational and industrial fields
- TEMs provide information on element and compound structure .
- Images are high-quality and detailed.

Disadvantages

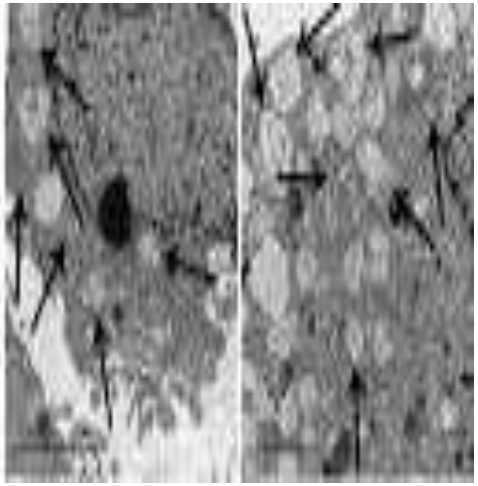
- TEMs are large and very expensive.
- Laborious sample preparation.
- Operation and analysis requires special training.
- Samples are limited to those that are electron transparent.
- TEMs require special housing and maintenance.
- Images are black and white .



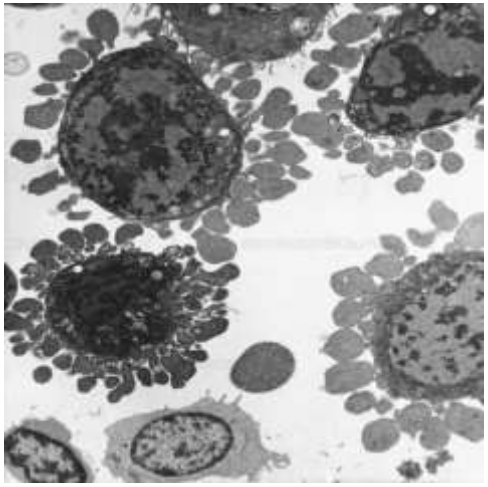
Animal cell



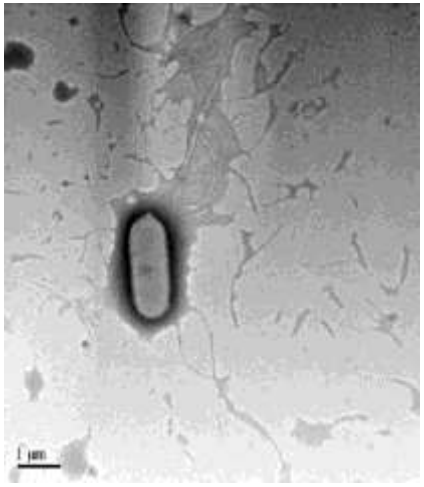
Plant cell



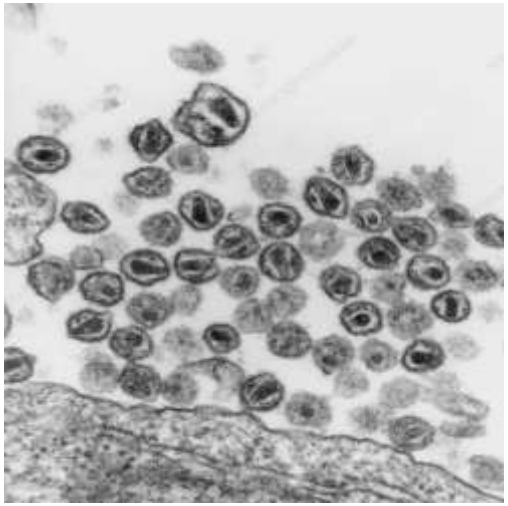
autophagy



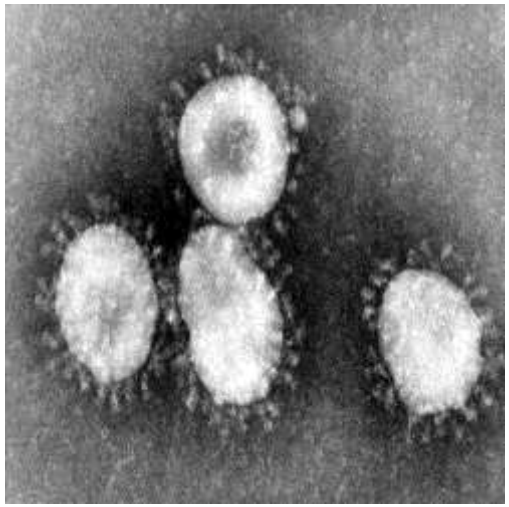
apoptosis



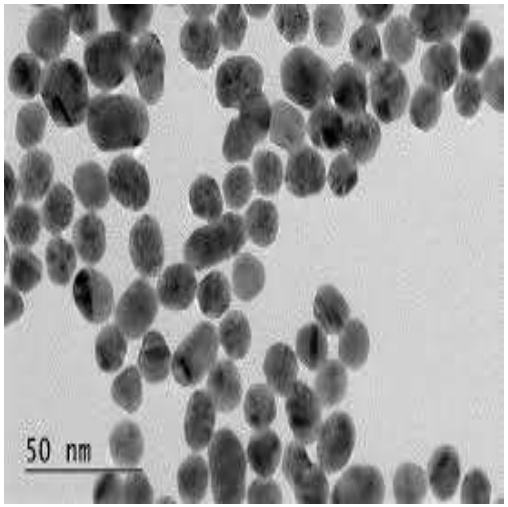
E.coli



HIV



Corona



Gold nanoparticles

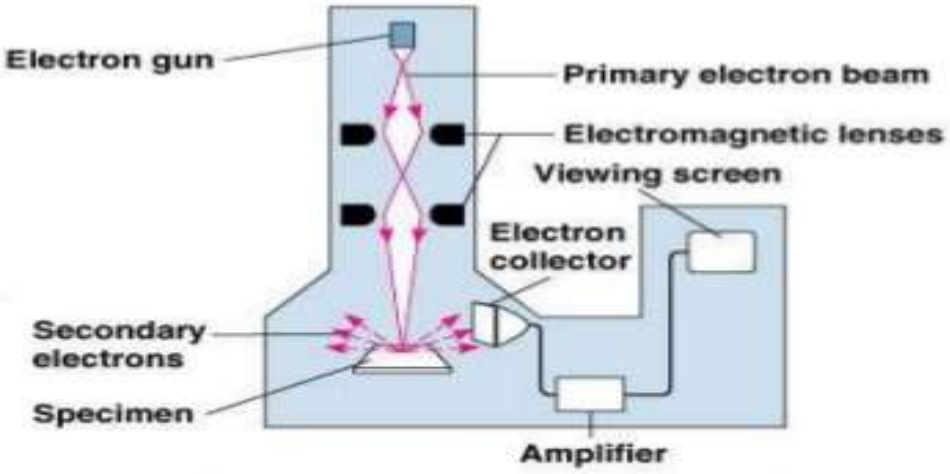
A **scanning electron microscope** (**SEM**) is a type of **electron microscope** that produces images of a sample by **scanning** the surface with a focused beam of **electrons**. The **electrons** interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample.

secondary electrons emitted by atoms excited by the electron beam are detected using a secondary electron detector (Everhart-Thornley detector). The number of secondary electrons that can be detected, and thus the signal intensity, depends, among other things, on specimen topography. SEM can achieve resolution better than 1 nanometer.

Scanning Electron Microscopy (SEM)

- An electron gun produces a beam of electrons that scans the surface of a whole specimen.

- Secondary electrons emitted from the specimen produce the image.



Nonconductive specimens collect charge when scanned by the electron beam, and especially in secondary electron imaging mode, this causes scanning faults and other image artifacts.

For conventional imaging in the SEM, specimens must be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge.

Non-conducting materials are usually coated with an ultrathin coating of electrically conducting material, deposited on the sample either by low-vacuum sputter coating or by high-vacuum evaporation.

Conductive materials in current use for specimen coating include gold, gold/palladium alloy, platinum, iridium, tungsten, chromium, osmium, and graphite. Coating with heavy metals may increase signal/noise ratio for samples of low atomic number (Z). The improvement arises because secondary electron emission for high-Z materials is enhanced.

An alternative to coating for some biological samples is to increase the bulk conductivity of the material by impregnation with osmium using variants of the OTO staining method (O-osmium tetroxide, T-thiocarbohydrazide, O-osmium).

Nonconducting specimens may be imaged without coating using an environmental SEM (ESEM) or low-voltage mode of SEM operation.

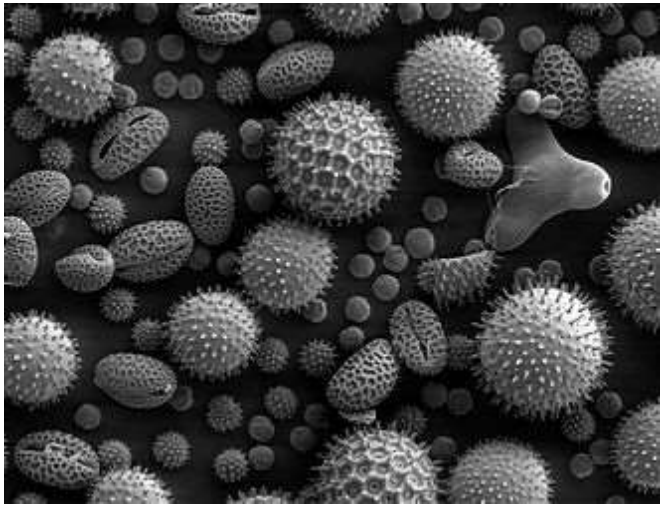
In ESEM instruments the specimen is placed in a relatively high-pressure chamber and the electron optical column is differentially pumped to keep vacuum adequately low at the electron gun.

The high-pressure region around the sample in the ESEM neutralizes charge and provides an amplification of the secondary electron signal.

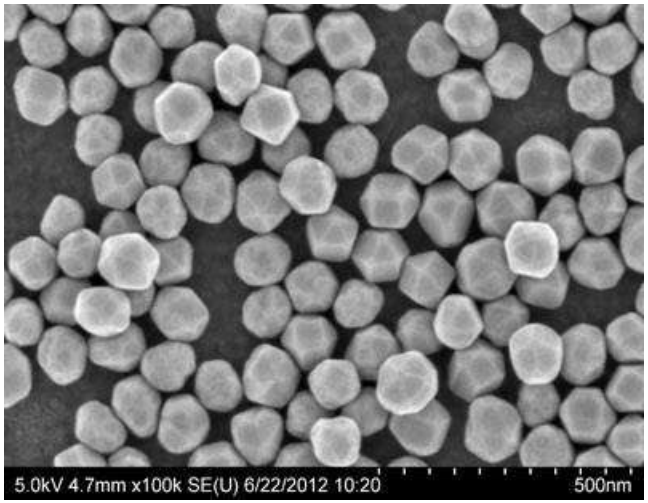
Low-voltage SEM is typically conducted in an instrument with a field emission guns (FEG) which is capable of producing high primary electron brightness and small spot size even at low accelerating potentials.

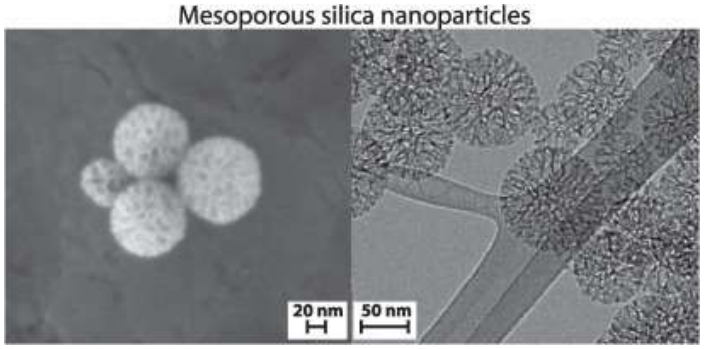
Images in SEM

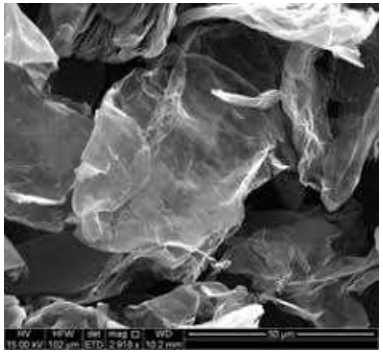
Pollen



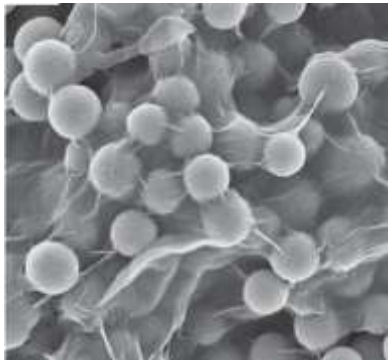
silver nanoparticles







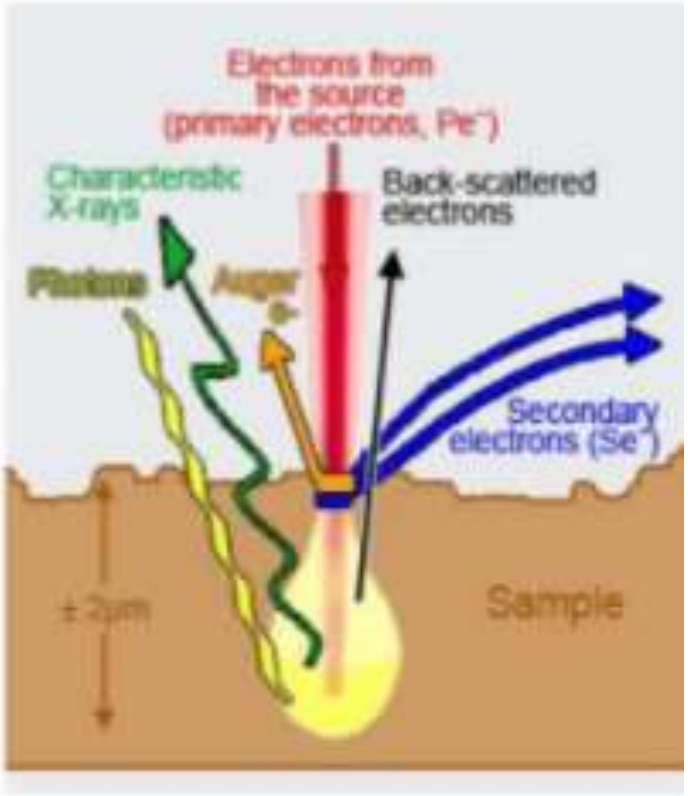
graphine



graphine wrapped Nanoparticles

PRINCIPLE OF WORKING OF SEM

- Incoming (primary) electrons
  - can be "reflected" (backscattered) from a bulk specimen.
  - can release secondary electrons.
- Primary electrons are focused into a small-diameter electron probe that is scanned across the specimen.
- Electrostatic or magnetic fields, applied at right angles to the beam, can be used to change its direction of travel.
- By scanning simultaneously in two perpendicular directions, a square or rectangular area of specimen (known as a **raster**) can be covered.
- Image of this area can be formed by collecting secondary electrons from each point on the specimen.



- When the beam touches the surface of the sample, it produces:
  - Secondary electrons (SE)
  - Back scattered electrons (BSE)
  - X - Rays...
- The emitted SE is collected by SED and convert it into signal that is sent to a screen which produces final image.
- Additional detectors collect these X-rays, BSE and produce corresponding images.

SEM SAMPLE PREPARATION

•Sample coated with a thin layer of conductive material.
•Done using a device called a "**sputter coater.**"
• Sample placed in a small chamber that is at a vacuum .
•Gold foil is placed in the instrument.
• Argon gas and an electric field cause an electron to be removed from the argon, making the atoms positively charged.
• The argon ions then become attracted to a negatively charged gold foil.
•The argon ions knock gold atoms from the surface of the gold foil.
• These gold atoms fall and settle onto the surface of the sample producing a thin gold coating.


Sputter coater


A spider coated in gold


13mm radius aluminium stubs

ADVANTAGES & DISADVANTAGES OF SEM

Advantages

- It gives detailed 3D and topographical imaging and the versatile information garnered from different detectors.
- This instrument works very fast.
- Modern SEMs allow for the generation of data in digital form.
- Most SEM samples require minimal preparation actions.

Disadvantages

- SEMs are expensive and large.
- Special training is required to operate an SEM.
- The preparation of samples can result in artifacts.
- SEMs are limited to solid samples.
- SEMs carry a small risk of radiation exposure associated with the electrons that scatter from beneath the sample surface.

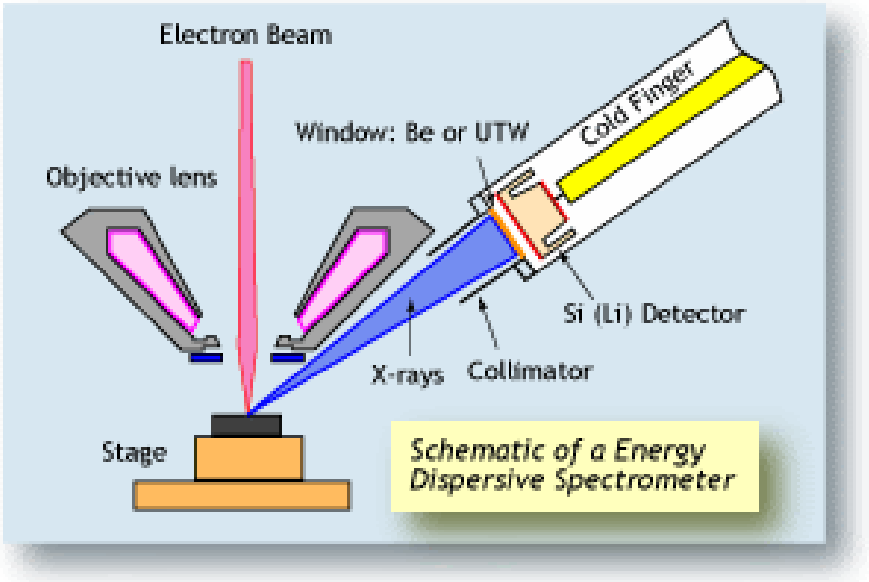
Differences between SEM and TEM

TEM	SEM
Electron beam passes through thin sample.	Electron beam scans over surface of sample.
Specially prepared thin samples are supported on TEM grids.	Sample can be any thickness and is mounted on an aluminum stub.
Specimen stage halfway down column.	Specimen stage in the chamber at the bottom of the column.
Image shown on fluorescent screen.	Image shown on TV monitor.
Image is a two dimensional projection of the sample.	Image is of the surface of the sample

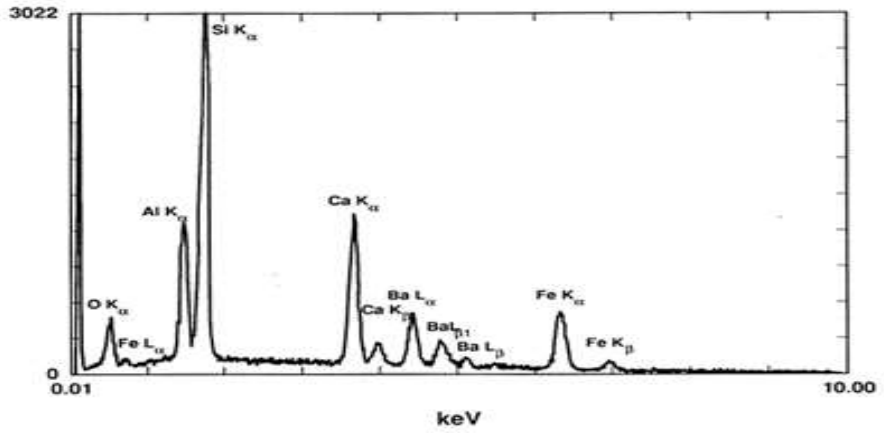
Electron microscopes often equipped with instrumentation for elemental analysis

- EDAX (Energy Dispersive Analysis of X-rays)

EDS Principle. Energy Dispersive X-ray Spectroscopy (**EDS** or **EDX**) is a qualitative and quantitative X-ray microanalytical technique that provides information on the chemical composition of a sample for elements with atomic number (Z) >3



Energy-dispersive X-ray spectroscopy (EDS, also abbreviated EDX or XEDS) is **an analytical technique that enables the chemical characterization/elemental analysis of materials**.



Scanning Electron Microscope

- Unlike the TEM, where the electrons in the primary beam are transmitted through the sample, the Scanning Electron Microscope (SEM) produces images by detecting secondary electrons which are emitted from the surface due to excitation by the primary electron beam.
- In the SEM, the electron beam is scanned across the surface of the sample in a raster pattern, with detectors building up an image by mapping the detected signals with beam position.

SPECIMEN

- For electron microscopy, tissue is fixed in 4% glutaraldehyde at 4°C for 4 hours.
- **Fixation** - In chemical fixation for electron microscopy, glutaraldehyde is often used to crosslink protein molecules and osmium tetroxide to preserve lipids.
- **Dehydration** - Organic solvents such as ethanol or acetone for SEM specimens or infiltration with resin and subsequent embedding for TEM specimens.
- **Embedding** - Resin (for electron microscopy) such as araldite or LR White, which can then be polymerised into a hardened block for subsequent sectioning.

SPECIMEN

- **Sectioning** - Typically around 90nm cut on an ultramicrotome with a glass or diamond knife. Glass knives can easily be made in the laboratory and are much cheaper than diamond, but they blunt very quickly and therefore need replacing frequently.
- **Staining** - uses heavy metals such as lead and uranium to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to the electron beam.
- By staining the samples with heavy metals, electron density is added to it which results in there being more interactions between the electrons in the primary beam and those of the sample, which in turn provides us with contrast in the resultant image.

Disadvantages of Electron Microscopy

- Electron microscopes are **very expensive to buy and maintain**.
- They are **dynamic rather than static in their operation**: requiring extremely stable high voltage supplies, extremely stable currents to each electromagnetic coil/lens, continuously-pumped high/ultra-high vacuum systems and a cooling water supply circulation through the lenses and pumps.
- As they are **very sensitive to vibration and external magnetic fields**, microscopes aimed at achieving high resolutions must be housed in buildings with special services.
- A **significant amount of training is required in order to operate** an electron microscope successfully and electron microscopy is considered a specialised skill.
- The **samples have to be viewed in a vacuum**, as the molecules that make up air would scatter the electrons. This means that the samples need to be specially prepared by sometimes lengthy and difficult techniques to withstand the environment inside an electron microscope.
- Recent advances have allowed some hydrated samples to be imaged using an environmental scanning electron microscope, but the applications for this type of imaging are still limited.

DYNAMIC LIGHT SCATTERING (DLS)

Clip slide

Dynamic light scattering (DLS) is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution.

SYNONYMS;

- Photon correlation spectroscopy
- Quasi-elastic light scattering.

It measures the dynamic properties like:

- Size distribution
- Hydrodynamic radius
- Diffusion coefficient

PRINCIPLE OF DLS;

DLS is used to analyze size range from a few nanometers to a few micrometers. This technique operates on the principle that particles move randomly in gas or liquid i.e. undergo Brownian motion (random motion). The movement (diffusion) of these particles is described by the Stokes-Einstein equation.

BROWNIAN MOTION;

- Brownian motion is the fundamental of this instrument.
- Brownian motion of the particle is random motion due to the bombardment by the solvent molecule surround them.

- Brownian motion of the particle related to size.
- It describes the way in which very small particles move in fluid suspension.

Brownian motion is influenced by

- Particle size
- Temperature
- Sample viscosity

STOKES-EINSTEIN EQUATION

$D = K_B T / 6\pi\eta R$

- D = diffusion constant
- K_B = Boltzmann's constant
- T = absolute temperature
- η = dynamic viscosity
- R = radius of sphere

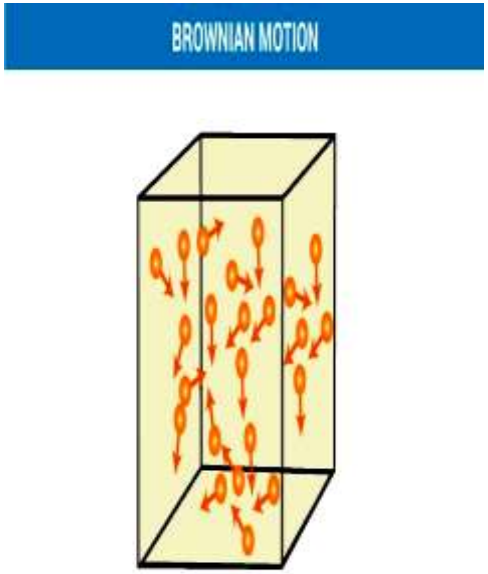
The diffusion (D) is equal to the product of Boltzmann's constant (k) divided by the hydrodynamic radius of the particle (R) of the particle and the shear viscosity of the solvent (η). Larger particles have a slower velocity and will have smaller coefficients of diffusion than larger particles.

- In DLS we measured the speed at which the particles are diffusing due to Brownian motion.
- Speed of diffusion is measured by measuring the rate at which the intensity of the scattered light fluctuates.
- Small particles causes the intensity to more fluctuate than larger.
- It measures the diffusion coefficient by using correlation coefficient.

Dynamic Light Scattering(DLS)

What is Dynamic light Scattering

- Particle size can be determined by measuring the random change in intensity of light scattered from suspension.
- It measure and interpolate the light scattering up to microsecond
- So it measure real time intensity, thus measuring the dynamic properties
  - Size distribution
  - Hydrodynamic radius
  - Diffusion coefficient



Application Of DLS

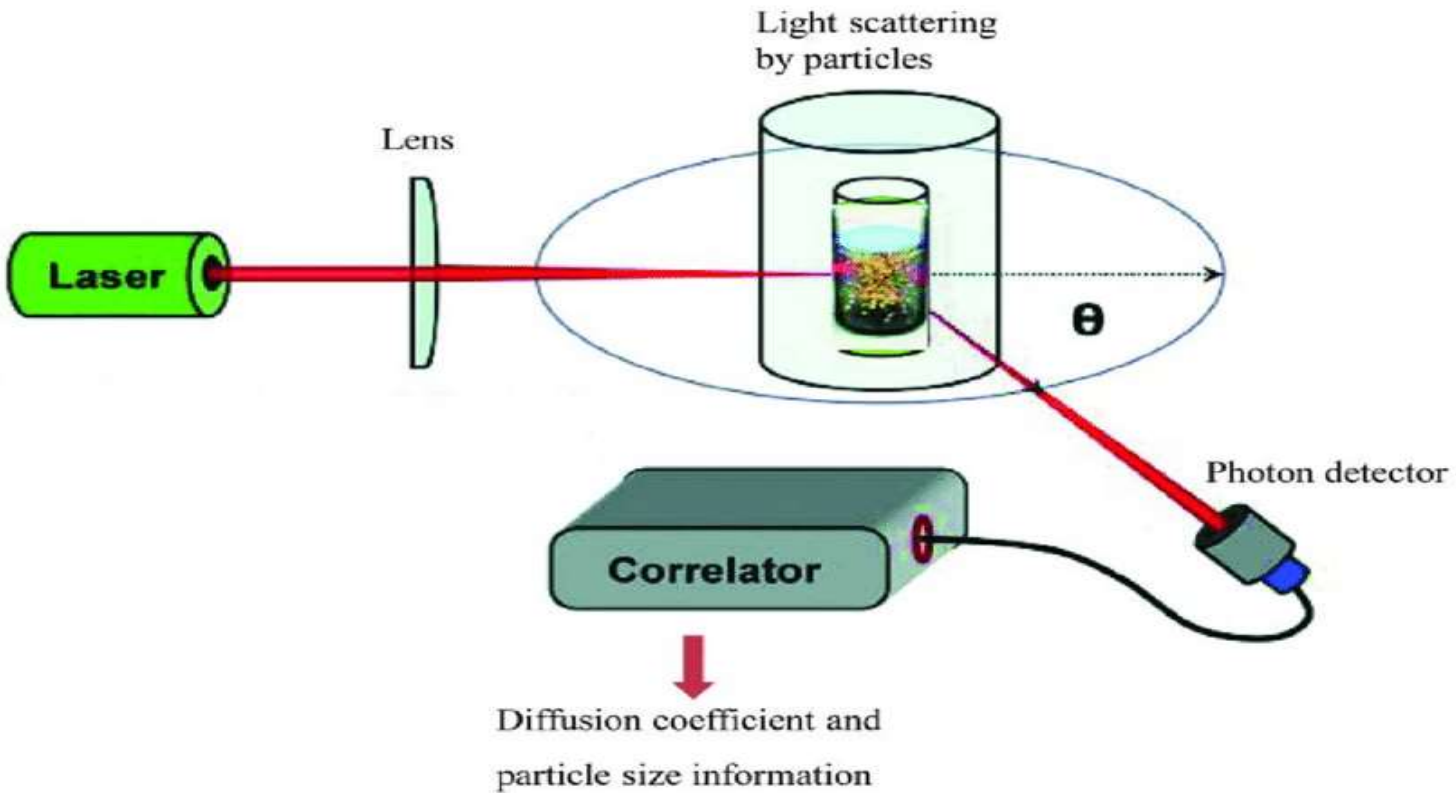
- We measure Hydrodynamic Size of nanoparticle ,protein and biomaterial
- We can also study stability of nanoparticles as function of time
- Good for detecting the aggregation of the particles

Other Then these

- Required small volume of sample
- Complete recovery of sample after measurement
- Sample preparation is not required for the measurement

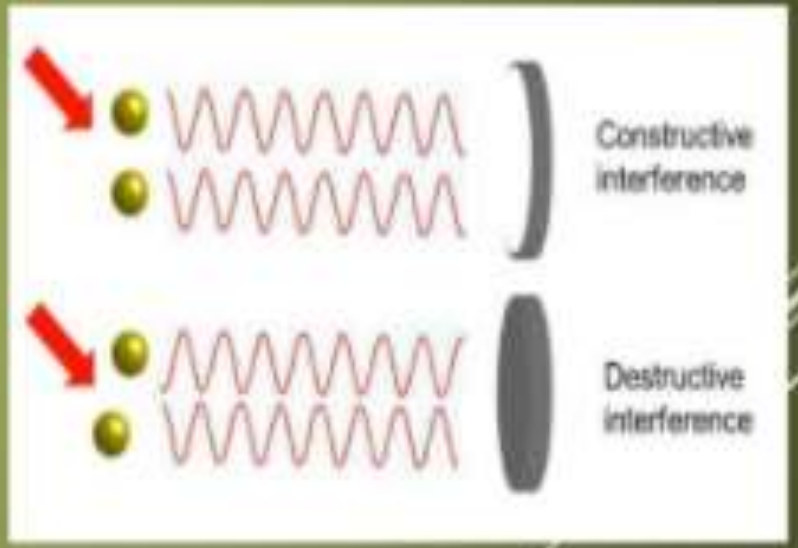
Dynamic light scattering (**DLS**) is a **technique** in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution

It **works** on the principle that when a beam of light (a laser) is scattered by a group of **particles**, the angle of light scattering is inversely proportional to **particle size** (ie. the smaller the **particle size**, the larger the angle of light scattering)



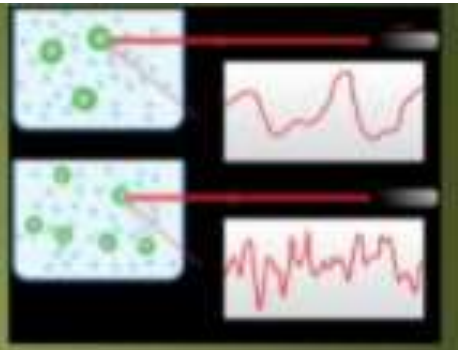
- In most DLS systems a laser (i.e. He-Ne) of known wavelength passes through a dilute sample in solution
- The intensity of scattered light is collected by a detector
- And deconvoluted by algorithms to determine the particle size distribution of the sample
- The amount of scattered light collected is dependent on refractive indices of the particle and solvent

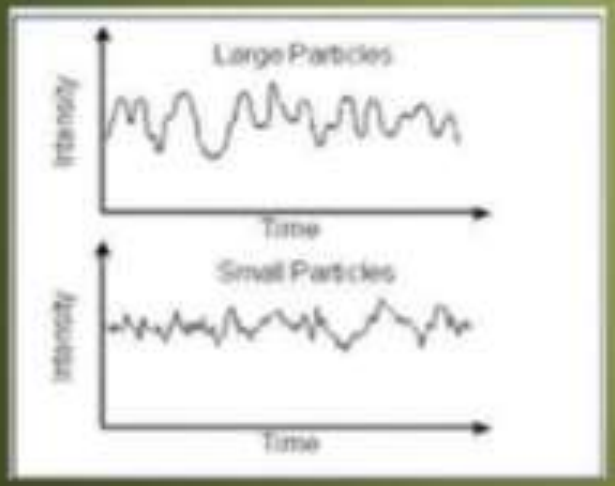
Before reaching the detector, the scattered light from individual particles experiences interference from those scattered by other particles all of which are moving randomly due to Brownian motion.
This results in random fluctuations in time.

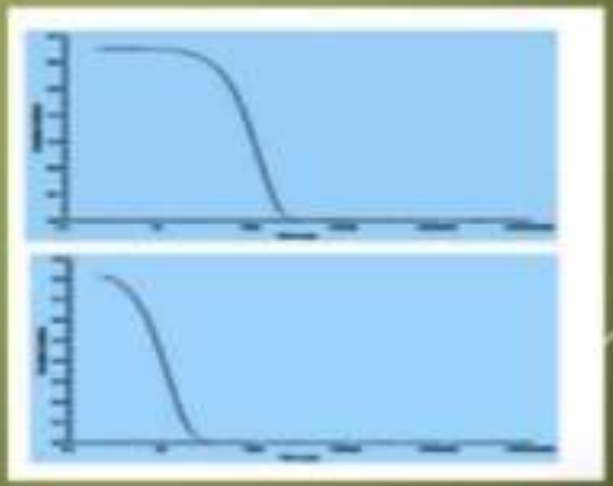


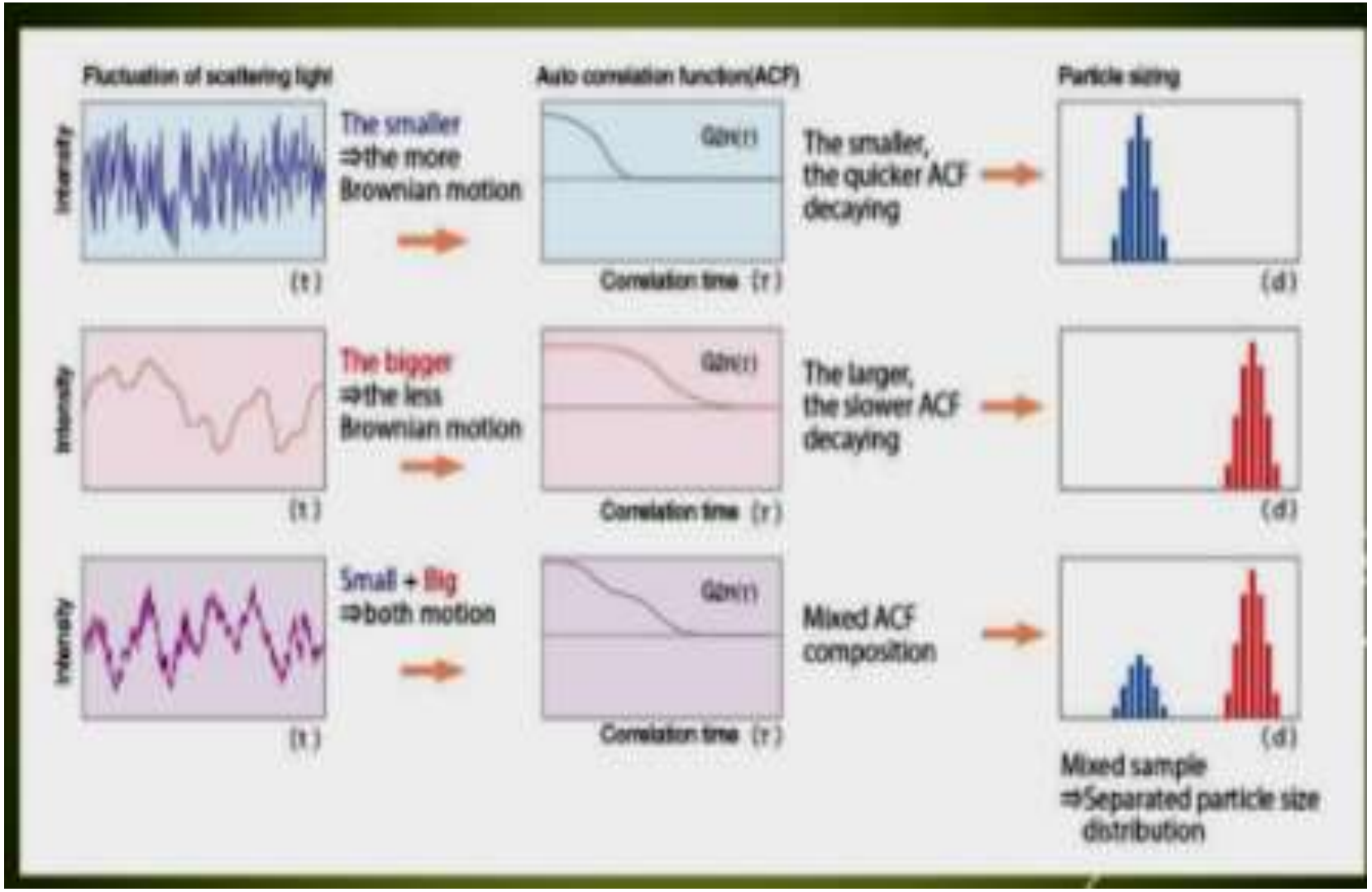
CORRELOGRAM

Large particles: smooth curve
Small particles: noisy curve








Fluctuation of scattering light
Auto correlation function(ACF)
Particle sizing
Intensity
(t)
The smaller
⇒the more
Brownian motion
G2(τ)
Correlation time (τ)
The smaller,
the quicker ACF
decaying
(d)
Intensity
(t)
The bigger
⇒the less
Brownian motion
G2(τ)
Correlation time (τ)
The larger,
the slower ACF
decaying
(d)
Intensity
(t)
Small + Big
⇒both motion
G2(τ)
Correlation time (τ)
Mixed ACF
composition
(d)
Mixed sample
⇒Separated particle size
distribution

APPLICATIONS

► **CHARACTERIZE SIZE OF VARIOUS PARTICLES;**

DLS is used to characterize size of various particles including proteins, polymers, micelles, vesicles, carbohydrates, nanoparticles, biological cells and gels.

► **AGGREGATION OF PARTICLES;**

This technique is best for detecting the aggregation of particles.

► **DETERMINATION OF EFFECTIVE DIAMETER;**

If the system is not disperse in size, the mean effective diameter of the particles can be determined. This measurement depends on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium.

► **DETERMINATION OF DIFFUSION COEFFICIENT;**

DLS essentially measures fluctuations in scattered light intensity due to diffusing particles, the diffusion coefficient of the particles can be determined.

▸DISPLAYS PARTICLE POPULATION;

DLS software of commercial instruments typically displays the particle population at different diameters. If the system is monodisperse, there should only be one population, whereas a polydisperse system would show multiple particle populations.

▸ANALYSIS OF STABILITY;

Stability studies can be done conveniently using DLS. In some DLS machines, stability depending on temperature can be analyzed by controlling the temperature in situ. We can study stability of Nano particles as function of time.

ADVANTAGES

Some of the advantages of DLS technology include

- Accurate,
- Reliable,
- Repeatable particle size analysis is 1 or 2 min
- Turbid samples can be measured directly
- It requires small volume of sample.
- Complete recovery of sample can be done after measurement.

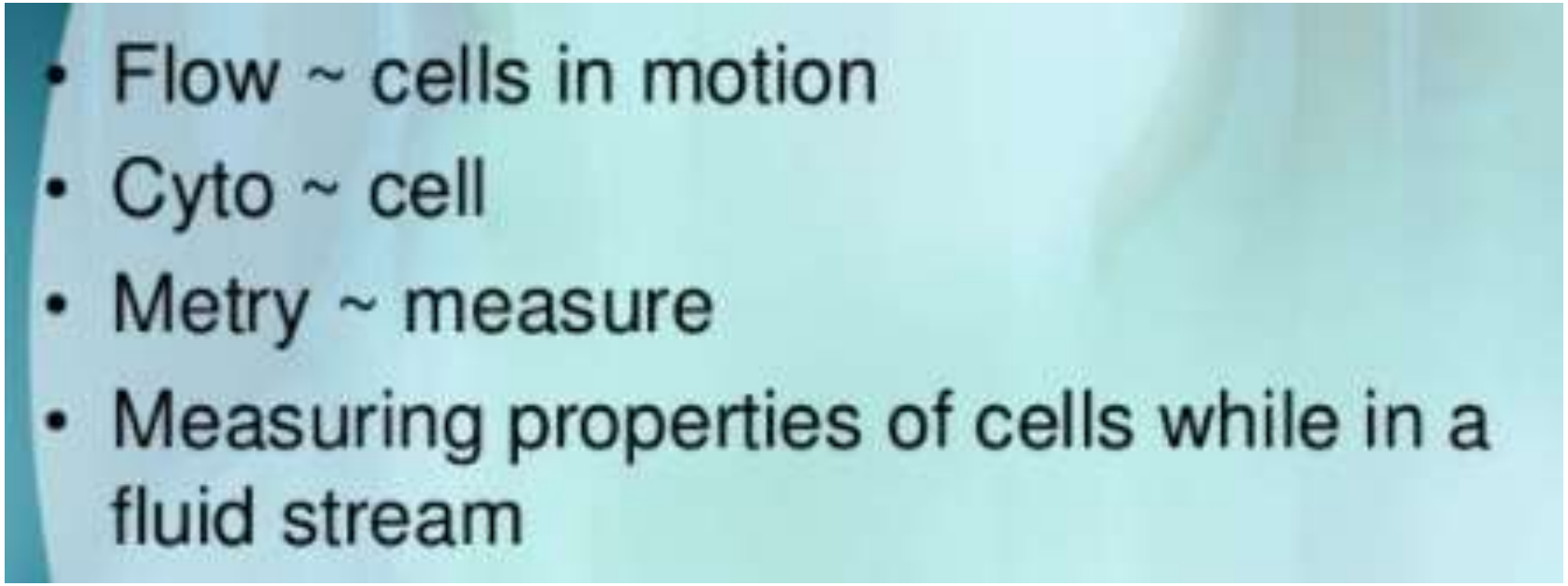
LIMITATIONS

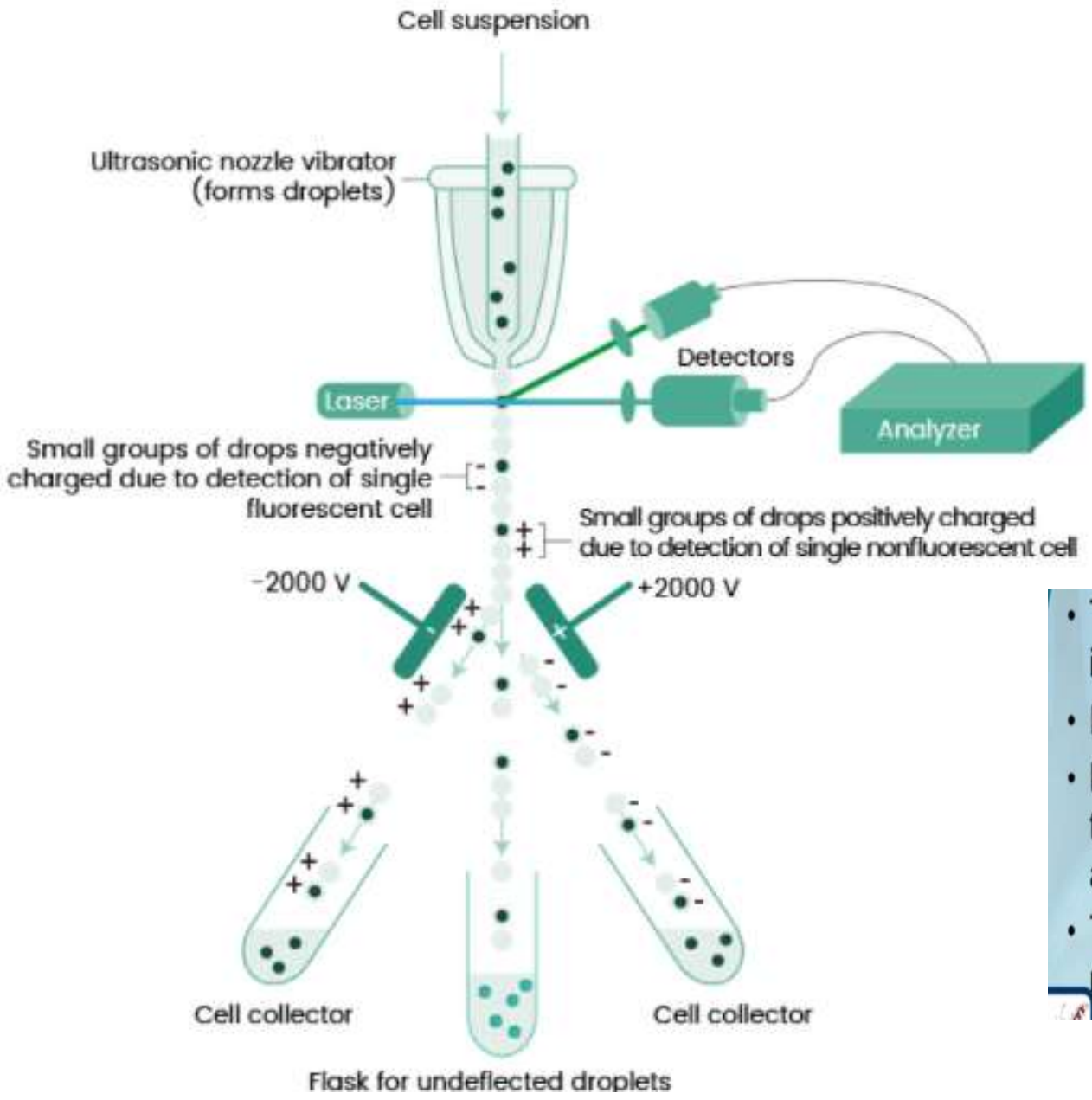
- We measure the hydrodynamic radius of the particle, not able to measure the actual size of the particle.
- The particles having size greater than 1000nm are not measured by this method.
- Size of solid particles are not measured by DLS.

Bioanalytical techniques -4

(Graphics are collected from Internet)

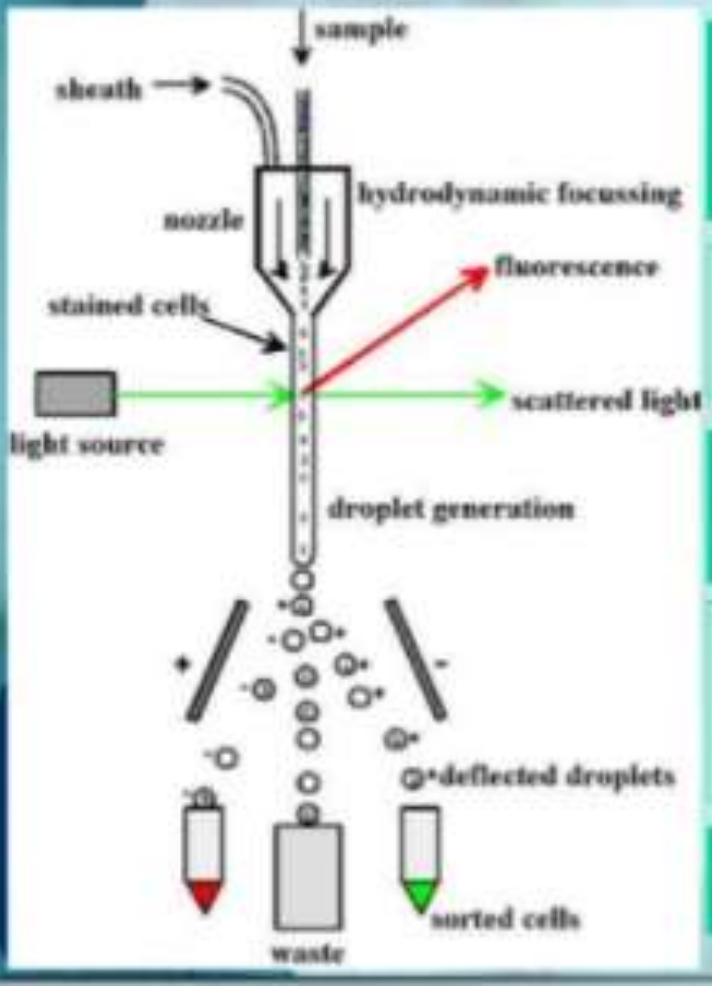
Fluorescence-activated cell sorting (**FACS**) is a technique to purify specific **cell** populations based on phenotypes detected by flow cytometry. This method enables researchers to better understand the characteristics of a single **cell** population without the influence of other **cells**.





- The cells from the sample tube are injected into the sheath stream
- Flow in a flow cell is laminar.
- Hydrodynamic focusing pushes the cells to line up single file along their long axis.
- The shape of the flow cell provides the means for hydrodynamic focusing.

Flow Cytometry Sorting Schematic



The nozzle/flow cell is vibrated by a transducer (converts electrical energy into mechanical energy) so it produces a stream breaking into droplets.

Laser interrogation and signal processing followed by sort decision: sort right, sort left, or no sort.

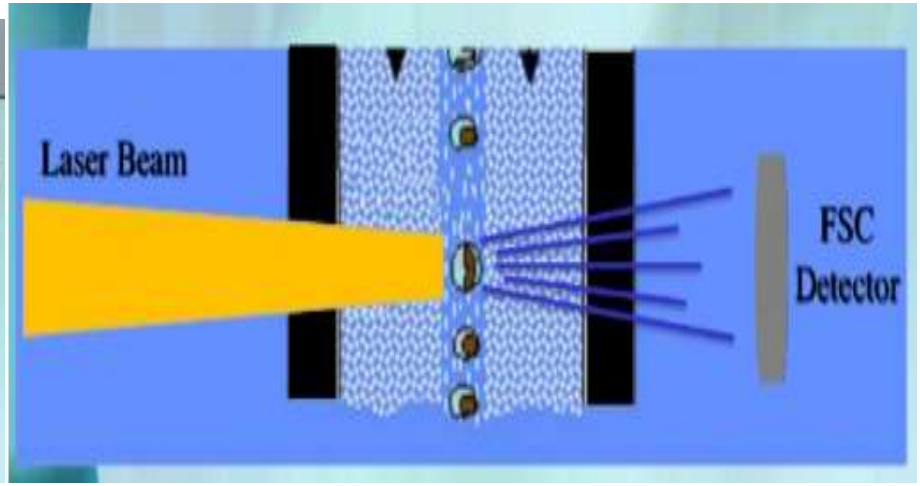
Electronic delay until cell reaches break off point. Then the stream is charged : + or -.

Charged droplets deflected by electrostatic field
from plates held at high voltage (+/-3000 volts).

Besides tubes can sort onto slides or multi-well plates.

FORWARD SCATTER

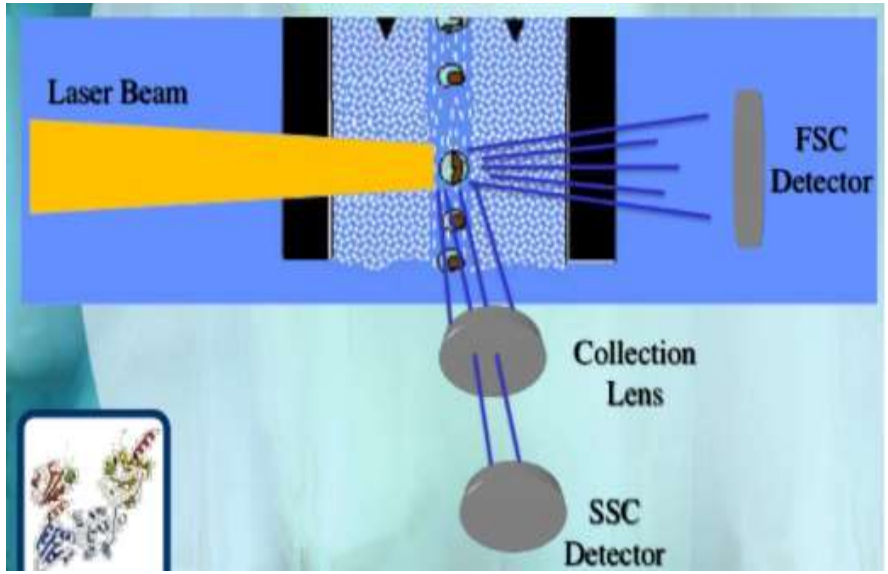
1. LIGHT SCATTER USED IS LOW ANGLE
2. SENSITIVE TO CELL SIZE AND SURFACE AREA
3. LIVE / DEAD DISCRIMINATION



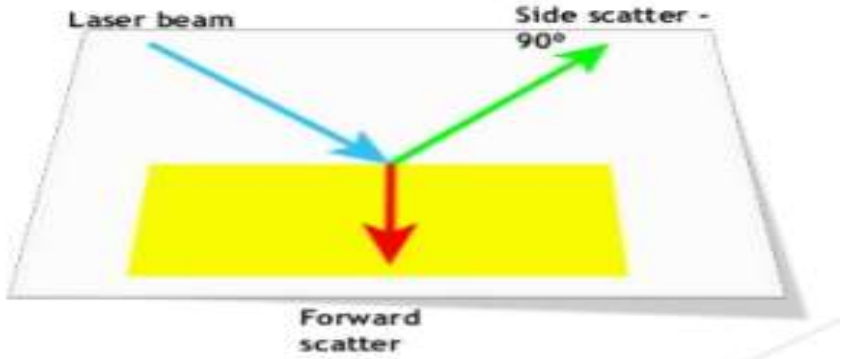
SIDE SCATTER

1. LIGHT SCATTER USED IS 90°
2. SENSITIVE TO INTERNAL STRUCTURES

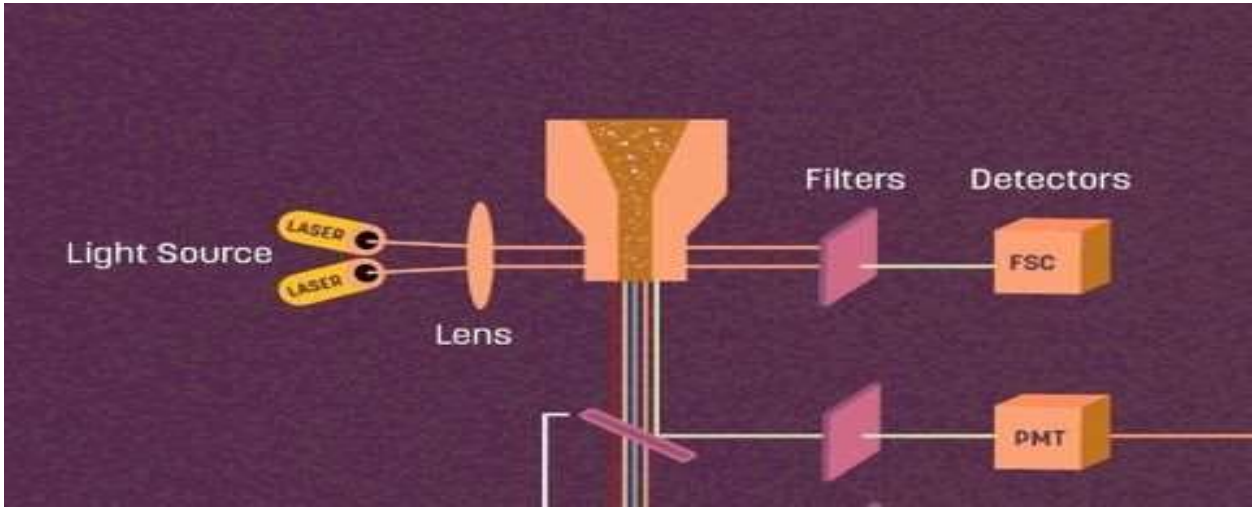
BEST ANALYSIS USING FORWARD AND SIDE SCATTER TOGETHER; ANALYSIS OF HETEROGENEOUS POPULATIONS



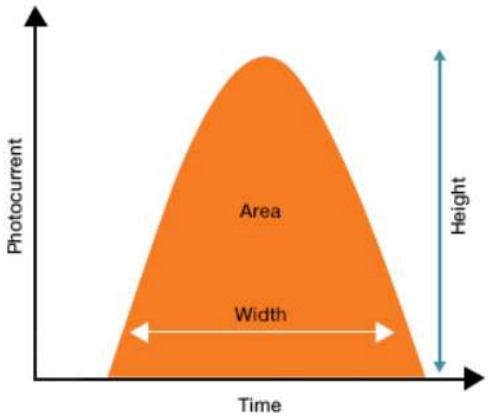
Light Collection



- Some Information Can Be Obtained
- FSC Correlates With Cell Size
- SSC Correlates With Internal Complexity
- To Distinguish Between 2 Cell types
  - A. Size Has To Be Different OR
  - B. Internal Complexity i.e amount of granules
- If These Two Parameters Are The Same, Then No Distinction Can Be Made



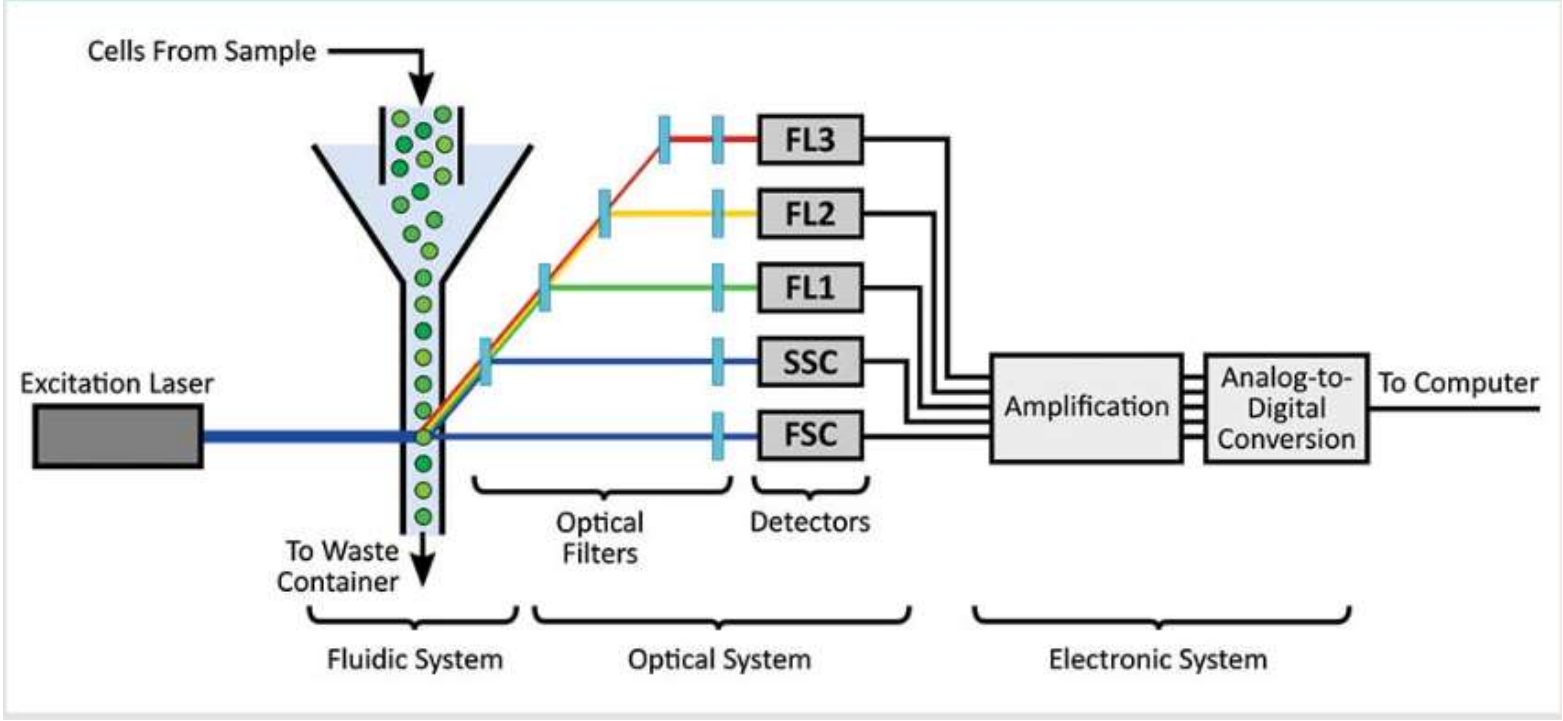
A dichroic can perform two functions. First, it allows specific wavelengths to pass in the forward direction. Second, it can reflect light at a 90° angle. This allows the light path to be passed through a series of filters. The precise choice and order of the filters can be arranged so that multiple signals can be detected simultaneously.



Height:	The maximum amount of current output by PMT.
Area:	The integral of the pulse.
Width:	The time interval during which the pulse occurs.

Signal intensity can be measured by either height or area.

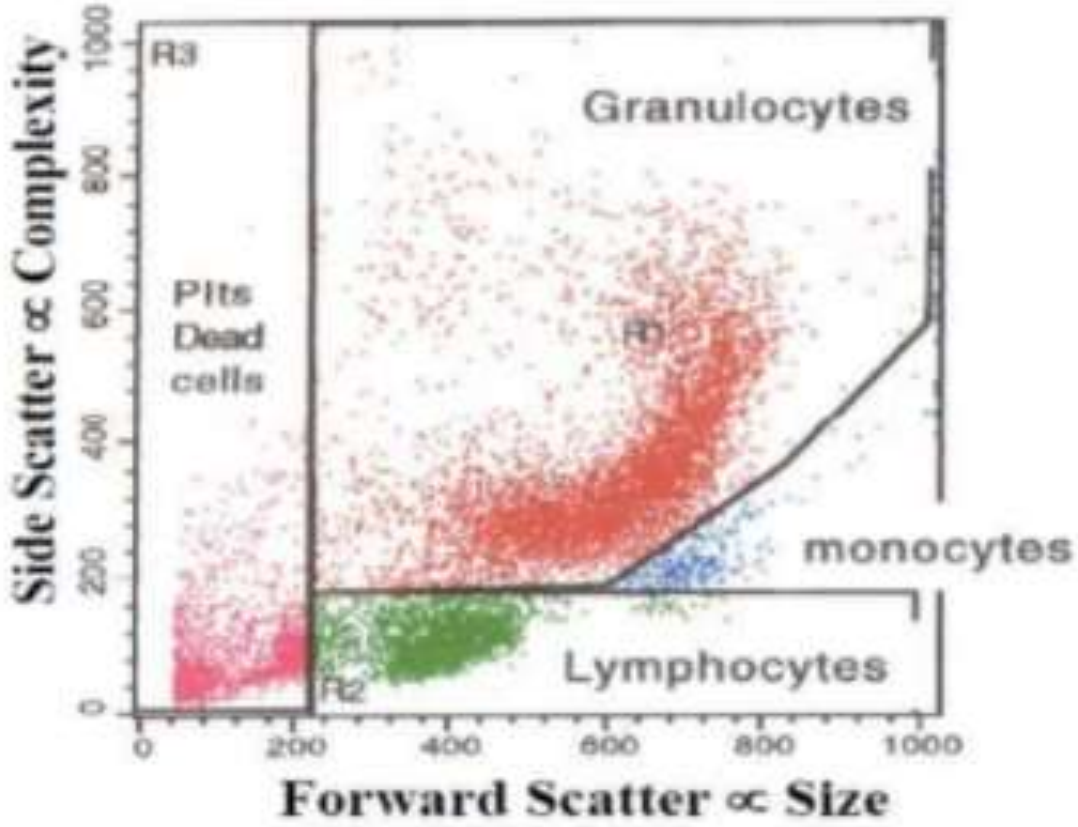
The width parameter measures the **time that the cell spends in the laser.**



- Fluorescent markers used to detect the expression of cellular molecules such as proteins or nucleic acids in a system.
- The fluorescent compound absorbs light energy over a range of wavelengths that is characteristic of that compound.
- This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level.
- The excited electron quickly decays to its ground state, emitting the excess energy in the form of fluorescence which is then collected by detectors.
- In a mixed population of cells, different fluorochromes can be used to distinguish separate subpopulations.
- The fluorescence pattern of each subpopulation, combined with FSC and SSC data, can be used to identify which cells are present in a sample and to count their relative percentages.
- The electronics system then converts the detected light signals into electronic signals that can be processed by the computer.

Cell sorting using FACS

Scatter Plot



Applications

- Cell size.
- Cytoplasmic granularity.
- Cell surface antigens (phenotyping).
- Apoptosis.
- Intracellular cytokine production.
- Intracellular signalling.
- Gene reporter (GFP).
- Cell cycle, DNA content, composition, synthesis.
- Bound and free calcium.
- Cell proliferation (BRDU and CFSE)
- Cell sorting, single cell cloning (clonecyt)

TUMOR DNA STAINING

SINGLE CELL SUSPENSION

↓

PERMEABILIZE WITH METHANOL

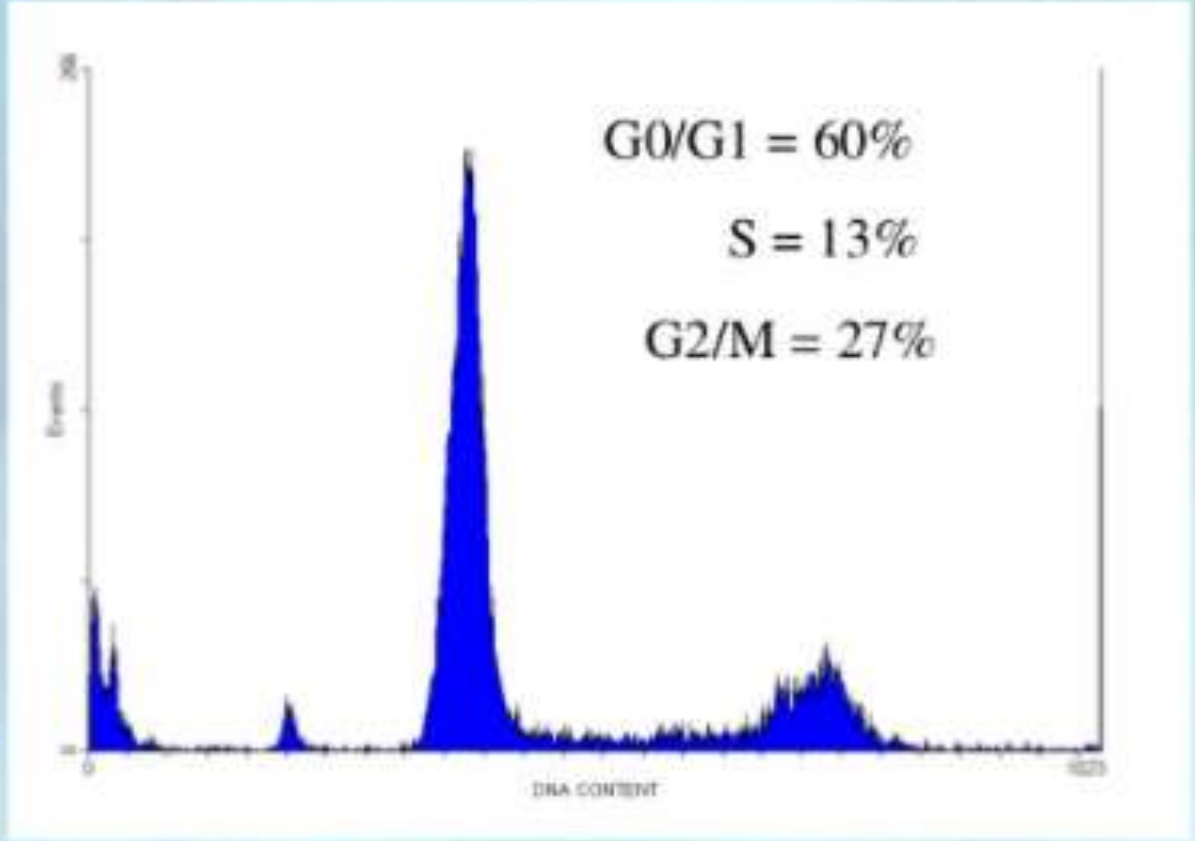
↓

DIGEST DS RNA WITH RNASE

↓

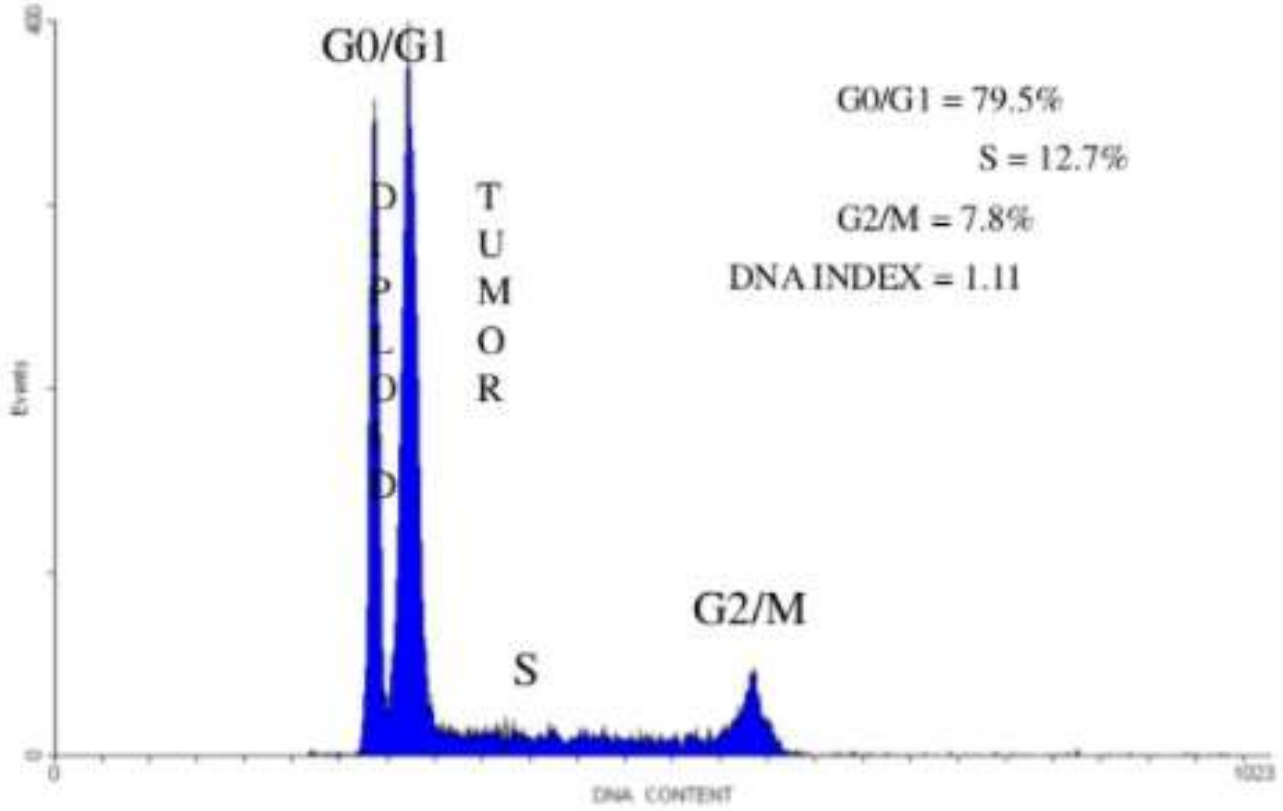
STAIN DNA WITH PROPIDIUM IODIDE

Cell Cycle Analysis



DNA CONTENT

CELL CYCLE ANALYSIS



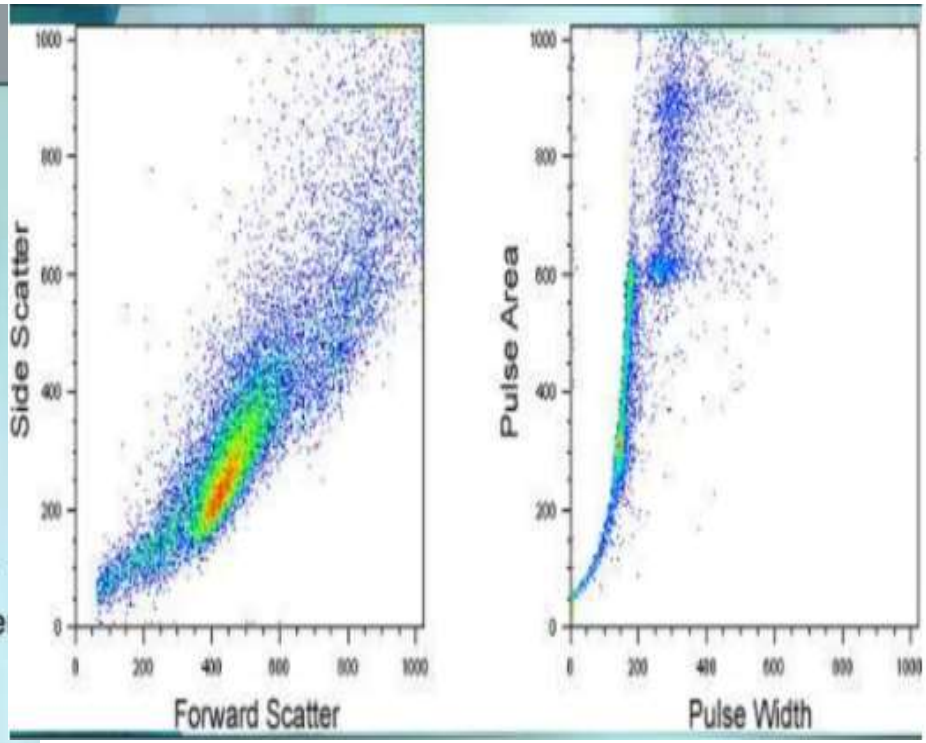
DATA PARAMETERS

1. FORWARD LIGHT SCATTER
2. 90° OR SIDE SCATTER
3. FLUORESCENCE EMISSION

Expected results

While running the cytometer, the following plots should be displayed:

1. Forward and side scatter to identify the cells
2. Pulse shape analysis to identify clumps and doublets (this can be pulse area vs. pulse width or pulse area vs. pulse height depending on cytomete
3. Forward scatter vs. PI signal; PI histogram.



- *No Apoptosis = Cell Viability*

  Cells that are negative for both Annexin V and the vital dye have no indications of apoptosis: PS translocation has not occurred and the plasma membrane is still intact.

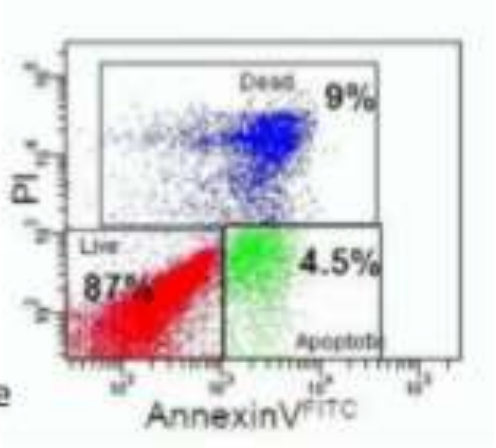
  Early Apoptosis

  Cells that are Annexin V-positive and vital dye-negative, however, are in early apoptosis as PS translocation has occurred, yet the plasma membrane is still intact.

- *Late Apoptosis or Cell Death*

  Cells that are positive for both Annexin V and the vital dye are either in the late stages of apoptosis or are already dead, as PS translocation has occurred and the loss of plasma membrane integrity is observed.

  When measured over time, Annexin V and a vital dye can be used to monitor the progression of apoptosis: from cell viability, to early-stage apoptosis, and finally to late-stage apoptosis and cell death.



- Thus, the combined use of cationic dyes (e.g. PI) with annexin V allows the discrimination between:
  - ➢Live cells = Annexin V negative/PI negative
  - ➢Early apoptotic cells = Annexin V positive/PI negative
  - ➢Late apoptotic = Annexin V positive/PI positive
  - ➢Necrotic cells = Annexin V negative/PI positive

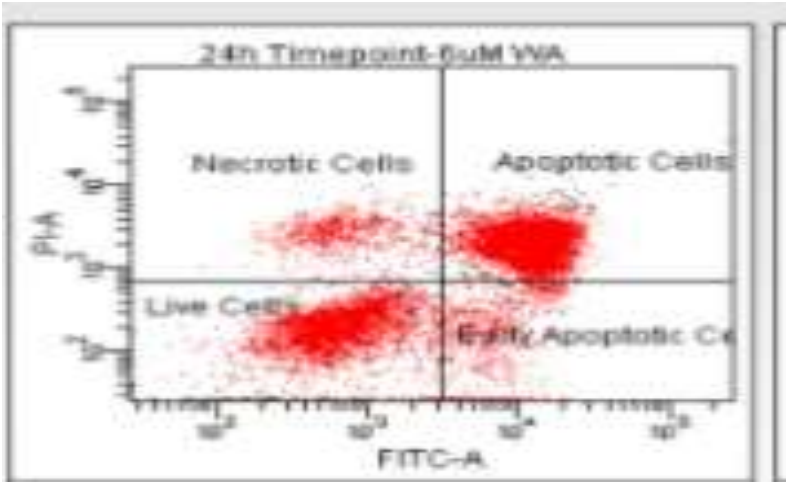
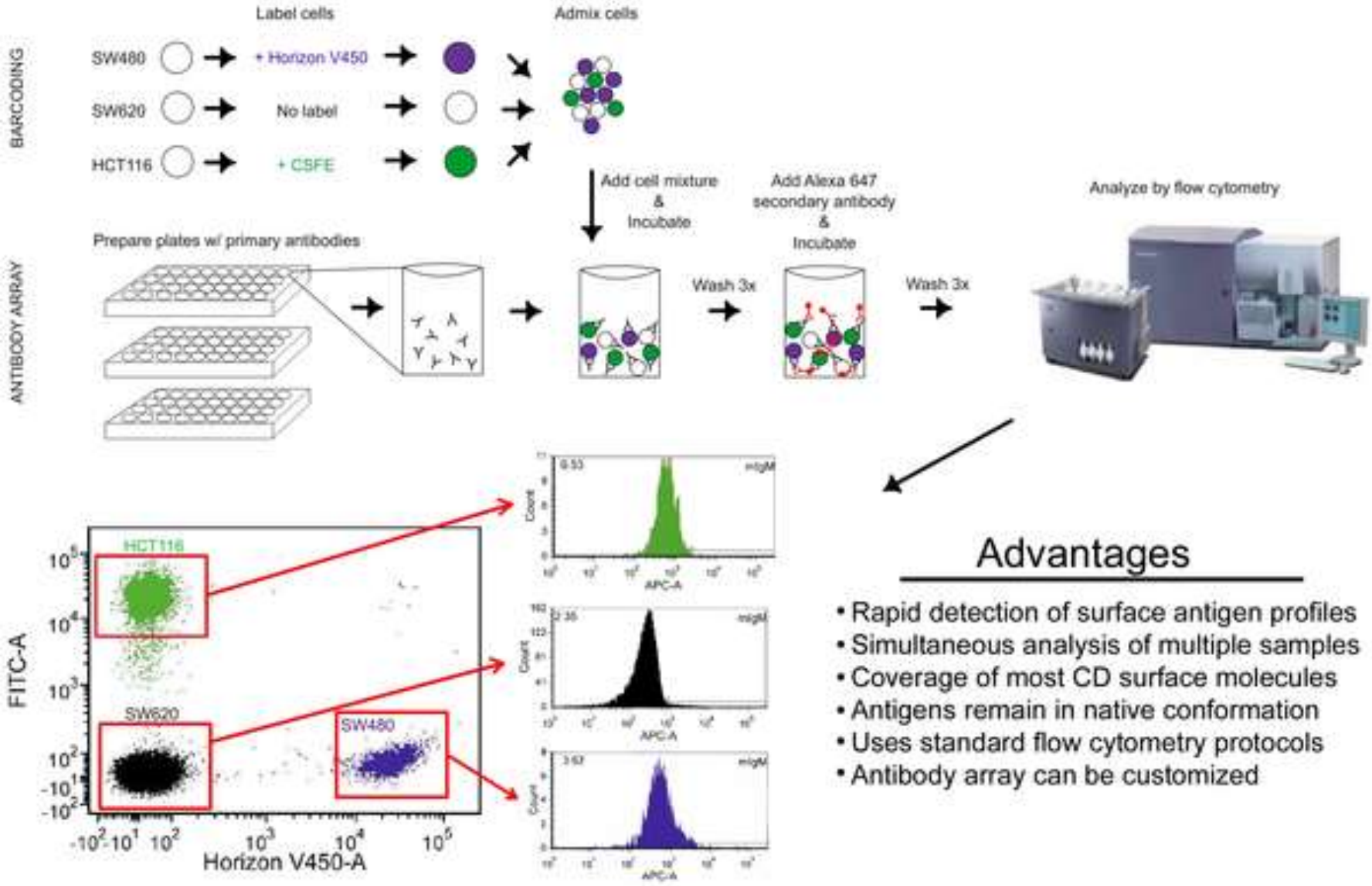


Figure 1. Diagram of experimental methods used for multiplexed barcoded antibody array.



Advantages

- Rapid detection of surface antigen profiles
- Simultaneous analysis of multiple samples
- Coverage of most CD surface molecules
- Antigens remain in native conformation
- Uses standard flow cytometry protocols
- Antibody array can be customized

The three cell lines were labeled with or without intracellular dye prior to admixing the cells into a single pool. The cells were then aliquoted into each well for antibody labeling. The contents of each well were then processed on a flow cytometer. The identity of each cell line was determined based on fluorescence intensity. The appropriate gates were drawn allowing for simultaneous analysis for each antibody. Histograms for mouse IgM isotype control are shown

By combining monoclonal antibodies with flow cytometry, researchers are able to identify tumor antigens for diagnostic and treatment purposes. For example, flow cytometry has been historically used to detect the expression of CD56 in the diagnosis of chronic myelomonocytic leukemia (CMML)

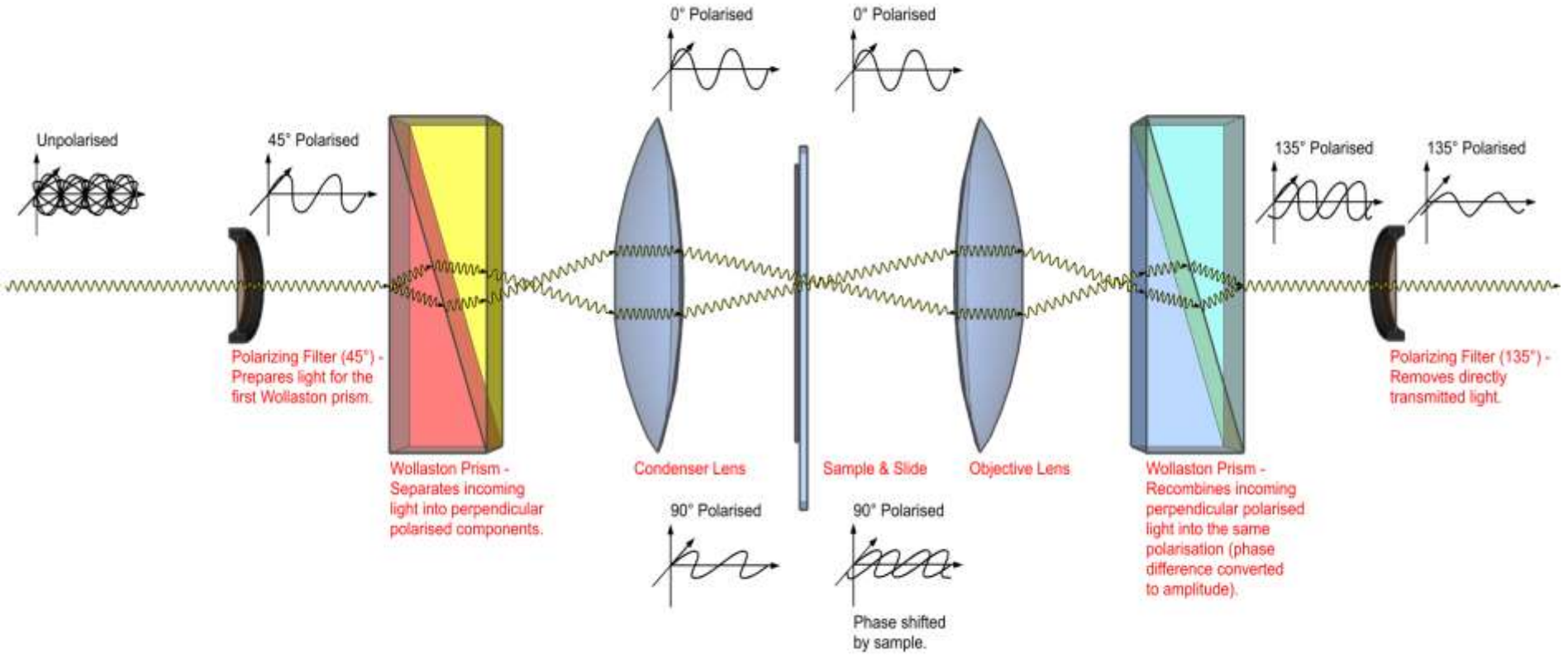
It can detect RNA (for blood cancers) and specific tagged surface markers (for lymphoid and myeloid neoplasms). The presence of DNA aneuploidy and a high proportion of S-phase tumor cells may indicate tumor malignancy.

Bioanalytical techniques -2

(Graphics are collected from Internet)

Differential interference contrast (DIC) microscopy, also known as Nomarski interference contrast (NIC) or Nomarski **microscopy**, is an optical **microscopy** technique used to enhance the contrast in unstained, transparent samples.

DIC works by separating a polarized light source into two orthogonally polarized mutually coherent parts which are spatially displaced (sheared) at the sample plane, and recombined before observation.



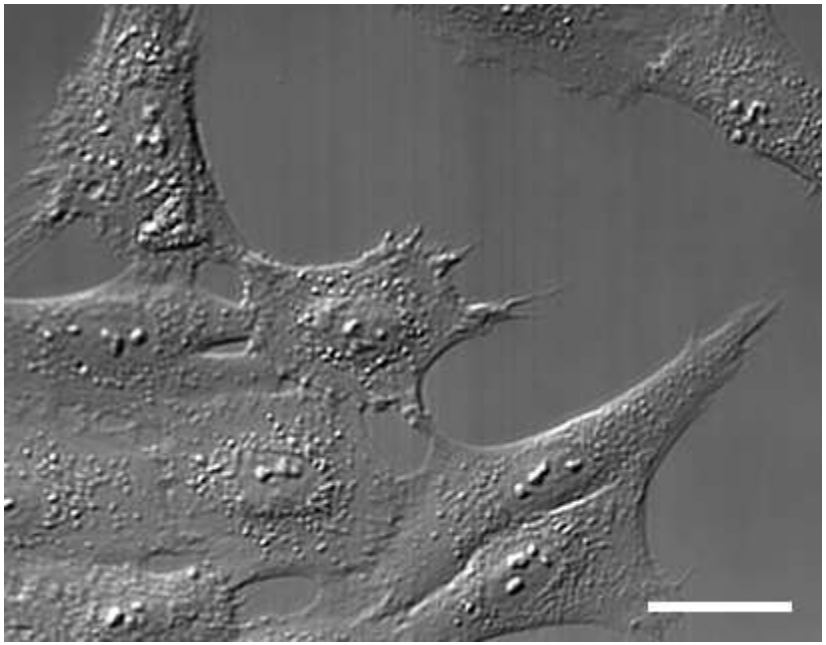
An advantage of DIC is that the specimen will appear bright in contrast to the dark background.
This system is relatively easy to incorporate with an existing brightfield microscope.

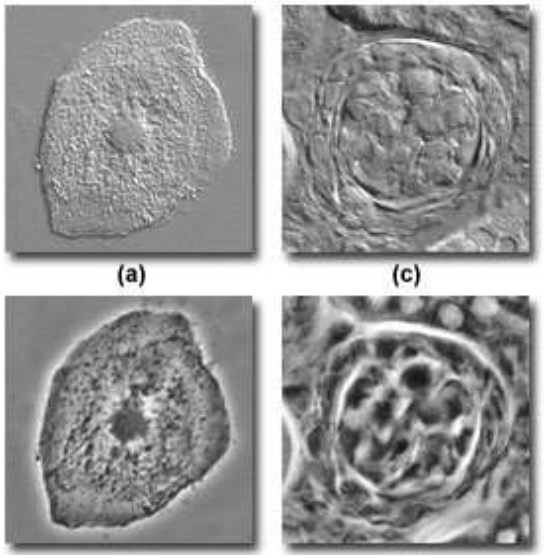
Two of the short comings of the phase contrast method are the fact that the specimen must be very thin and a halo is produced in the viewing field.
No halo effect occurs with differential interference contrast and it can be used to produce very clear images of thick specimens.

It can also be used in conjunction with digital imaging systems to add further definition to the image.

Differential interference contrast imaging can be used in conjunction with fluorescence microscopy to provide a better fluorescence image and to pinpoint specific areas on a specimen before switching to the fluorescence mode to further examine the object.

A major advantage of the differential interference contrast technique is in examining living specimens when normal biological processes might be impeded by normal staining procedures.
A drawback to this type of imaging is that the three-dimensional image of a specimen may not be accurate. The enhanced areas of light and shadow might add distortion to the appearance of the image.





DIC (no halo)

Phase contrast

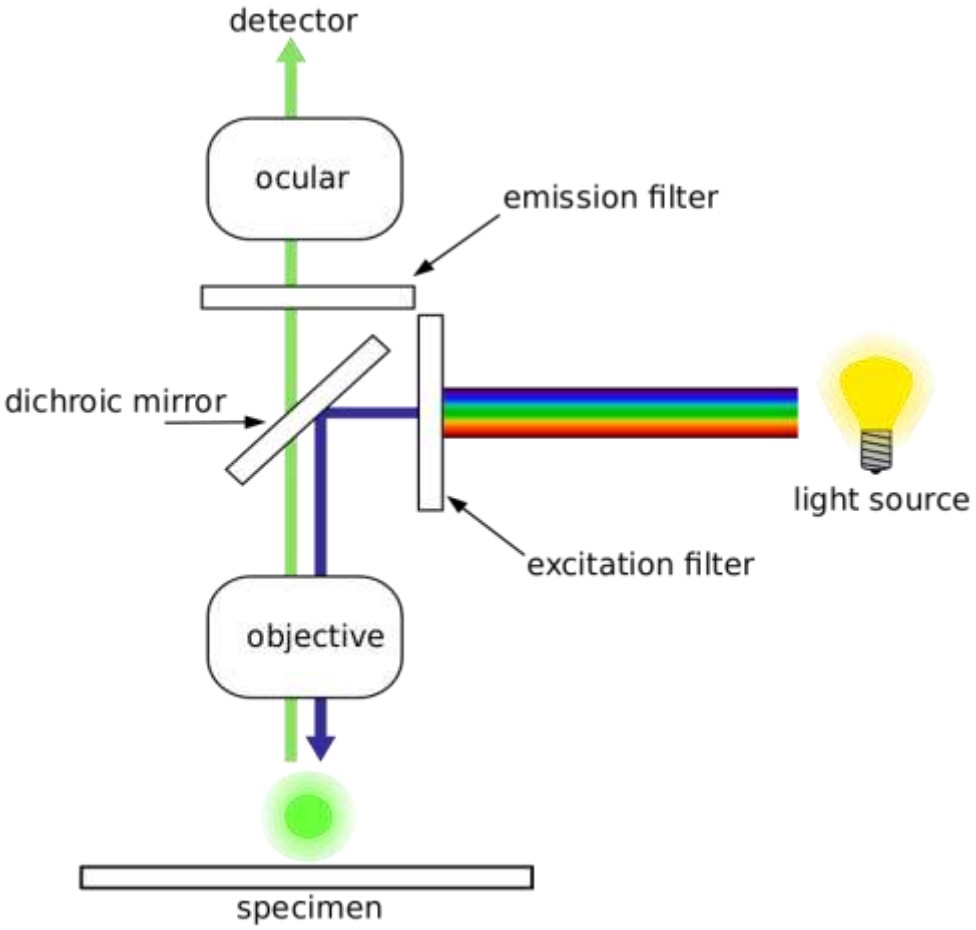
Fluorescence Microscope

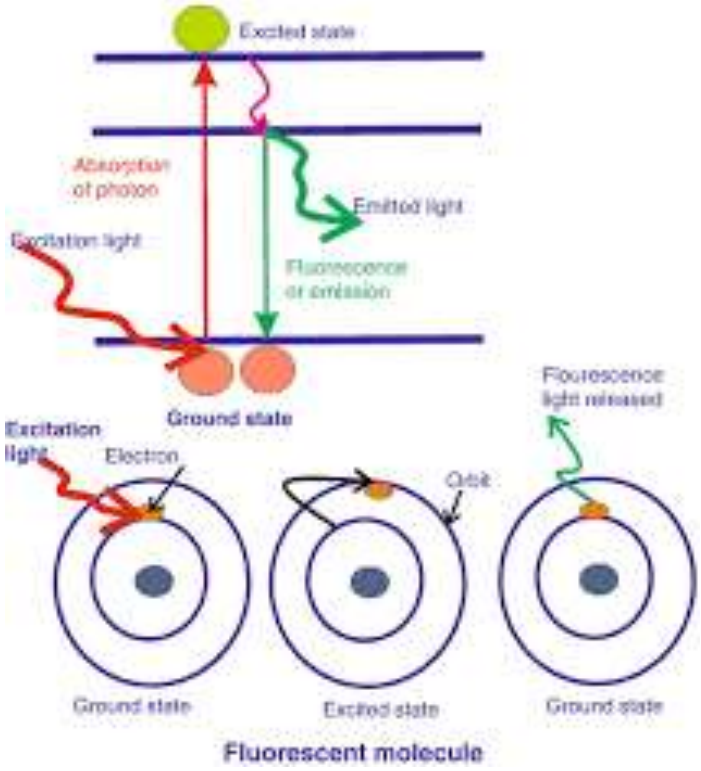
Fluorescence microscopy is an imaging technique **used** in light **microscopes** that allows the excitation of fluorophores and subsequent detection of the **fluorescence** signal.

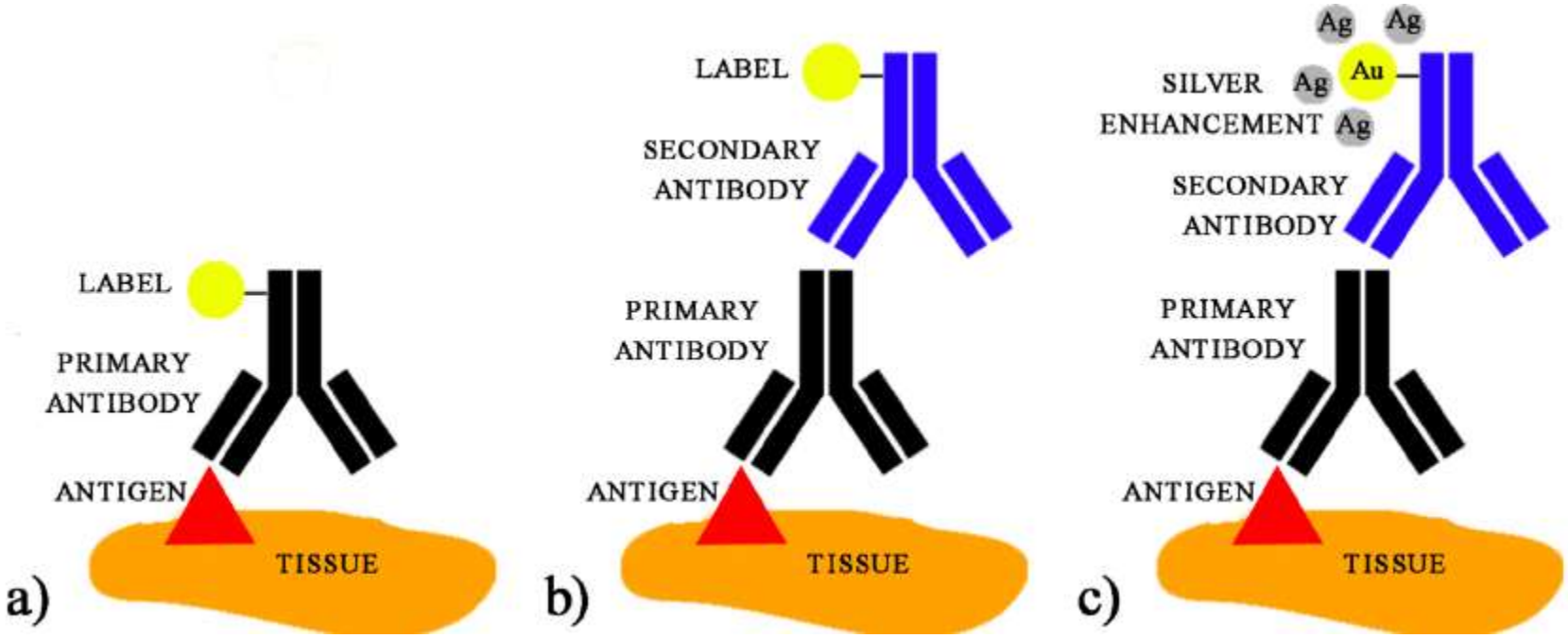
The basic task of the **fluorescence microscope** is to let excitation light radiate the specimen and then sort out the much weaker emitted light from the image.

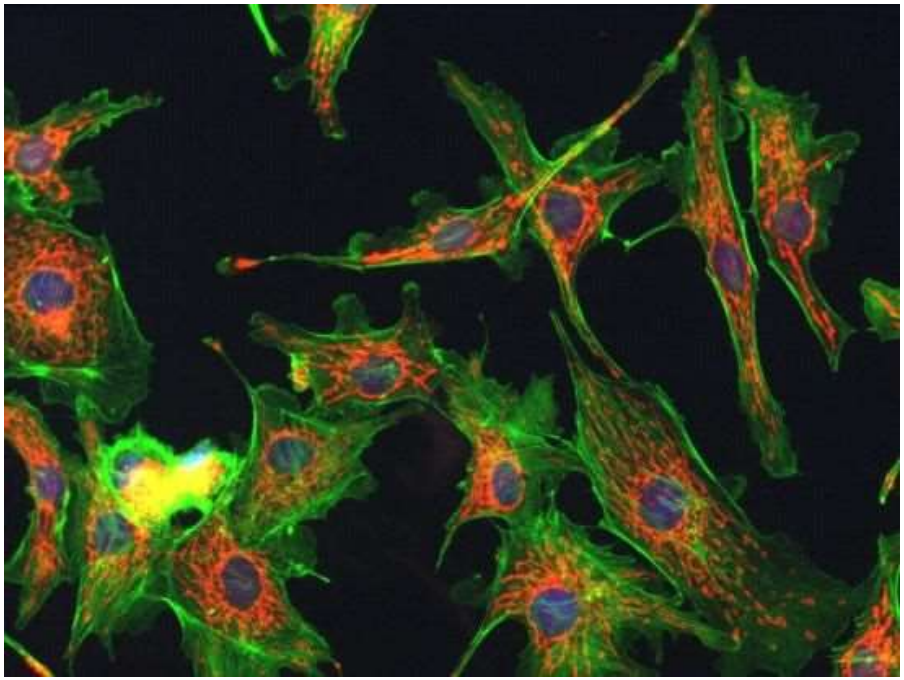
The radiation collides with the atoms in your specimen and electrons are excited to a higher energy level. When they relax to a lower level, they emit light.

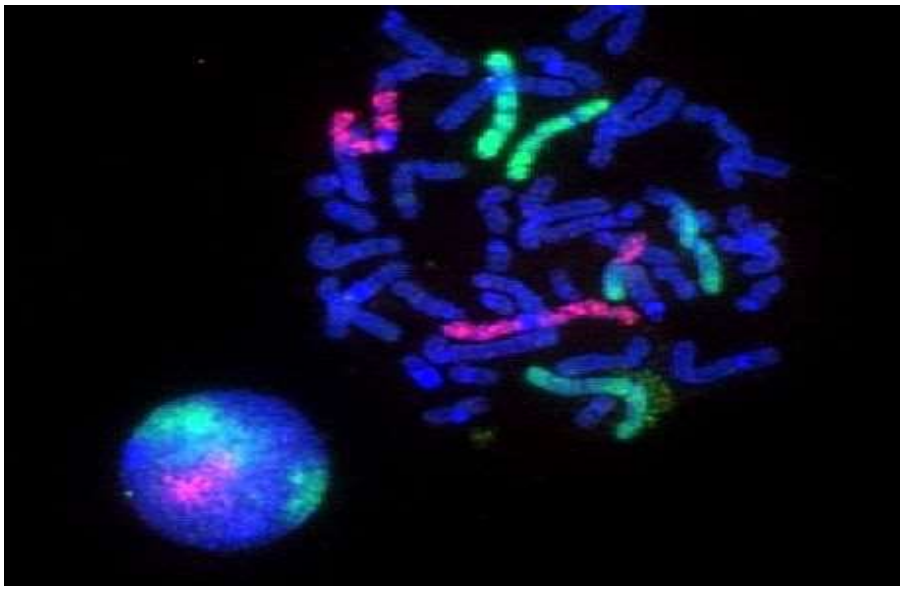
Basic Principle






LABEL
PRIMARY ANTIBODY
ANTIGEN
TISSUE
a)
LABEL
SECONDARY ANTIBODY
PRIMARY ANTIBODY
ANTIGEN
TISSUE
b)
Ag
Ag
SILVER ENHANCEMENT
Ag
Au
Ag
SECONDARY ANTIBODY
PRIMARY ANTIBODY
ANTIGEN
TISSUE
c)





Thegreatest **disadvantage** in **fluorescent microscopy** is the photobleaching and you cannot focus your specimen for much time at higher magnification (as intense light is required) for more time. And also it needs a quite a sophisticated instrumentation as well as lots of experimental optimization.

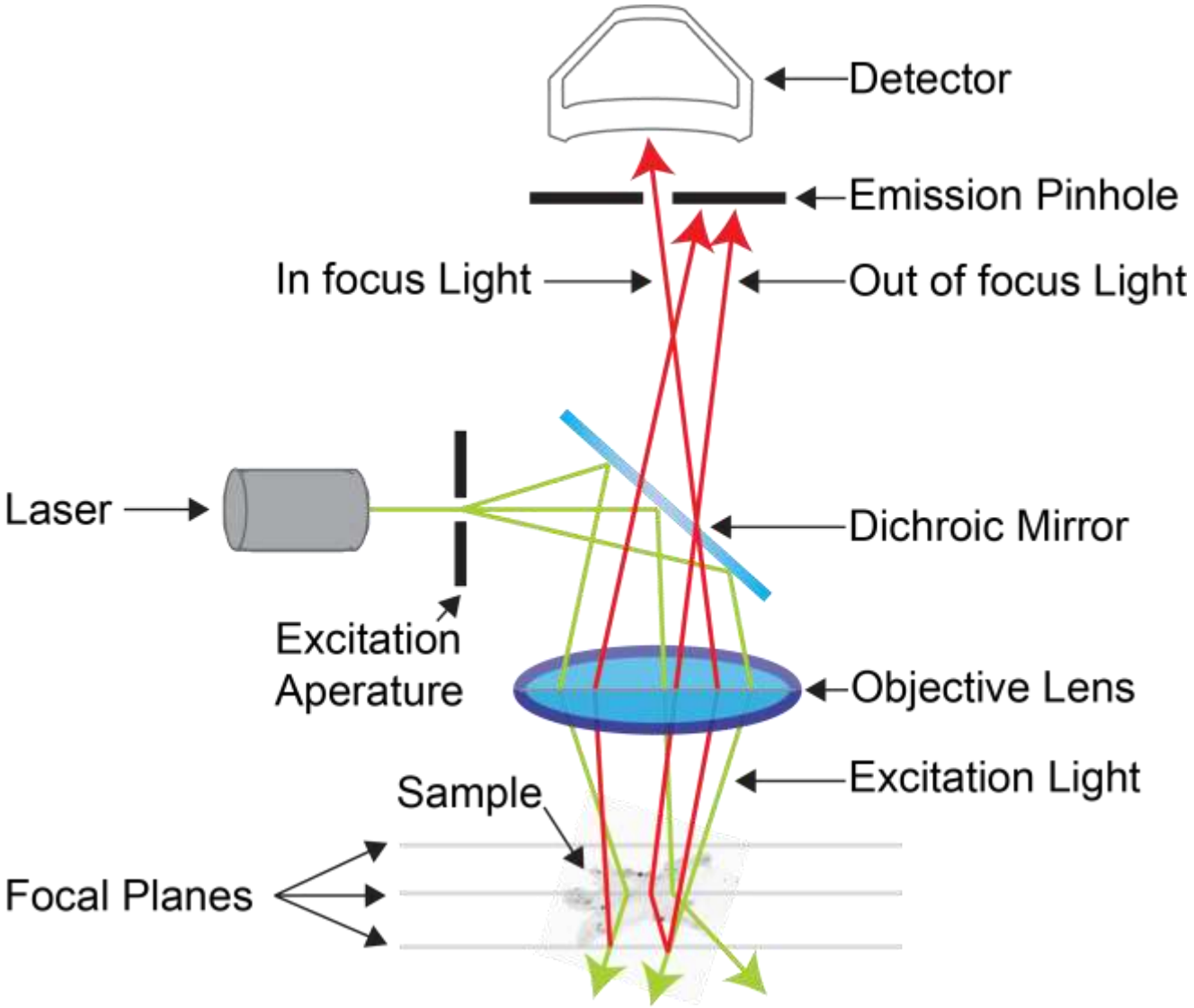
- Conventional fluorescence microscopy has two major limitations.
- First, the physical process of cutting a section destroys material, and so in consecutive (serial) sectioning a small part of a cell's structure is lost.
- Second, the fluorescent light emitted by a sample comes from molecules above and below the plane of focus; thus the observer sees a blurred image caused by the superposition of fluorescent images from molecules at many depths in the cell.
- The blurring effect makes it difficult to determine the actual three-dimensional molecular arrangement.

Confocal Microscope

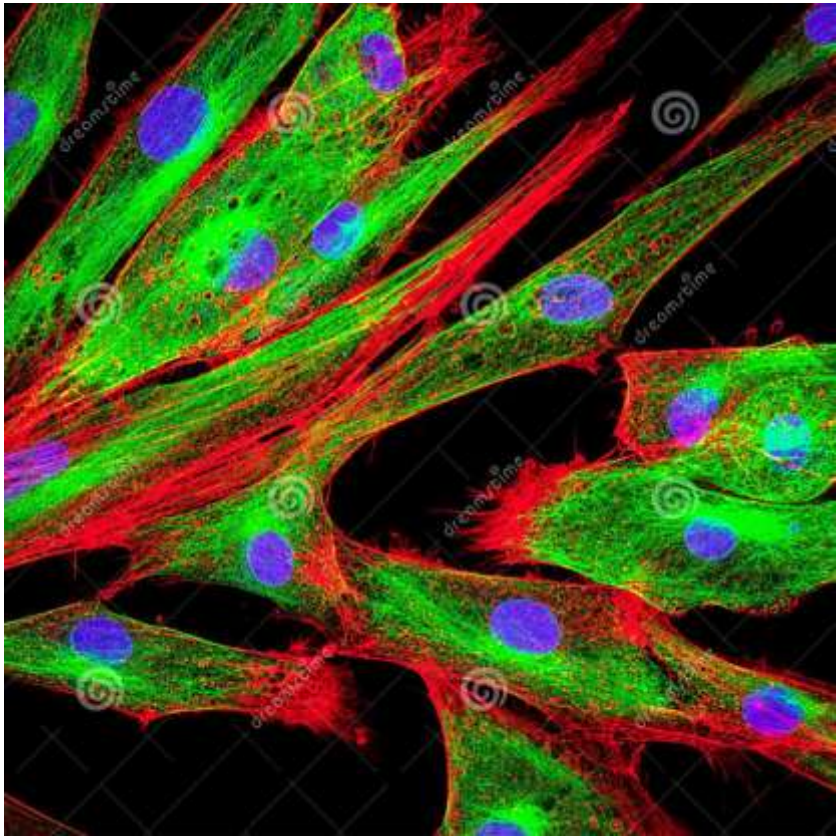
Confocal microscopy, most frequently **confocal** laser scanning **microscopy** (CLSM) or laser **confocal** scanning **microscopy** (LCSM), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation.

Two correction over fluorescence microscope

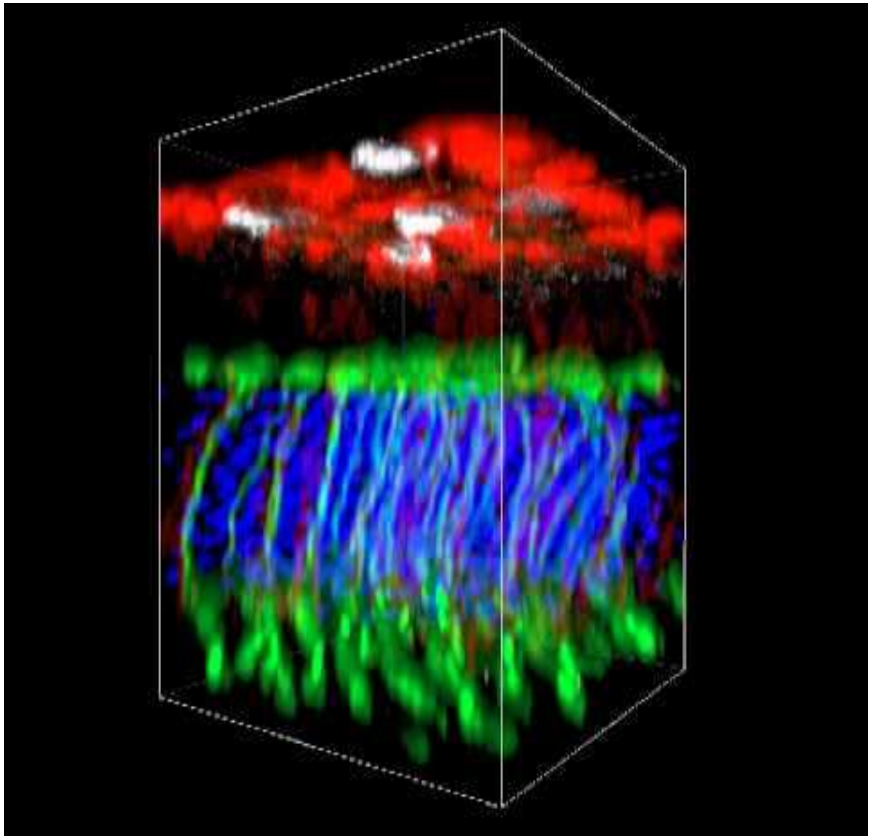
1. Introduction of pinhole at the conjugate foci poision of the lens to elliminate out Of focus light
2. Single point iraadiation and scan by introduction of laser.


Detector
Emission Pinhole
In focus Light
Out of focus Light
Laser
Dichroic Mirror
Excitation
Aperature
Objective Lens
Excitation Light
Sample
Focal Planes

Confocal images



human cells



3d View of cells

Laser scanning confocal microscopy

Advantages

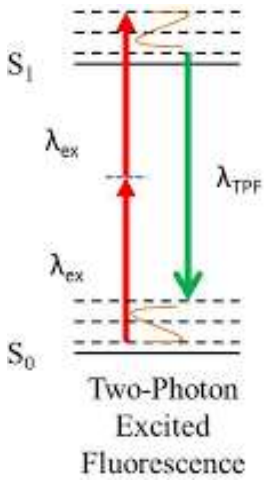
- Reduced blurring of the image from light scattering
- Optical sectioning of thick specimens
- Detection uses highly sensitive photomultipliers, improving signal to noise ratio
- Z-axis scanning enabling generation of 3D datasets
- Magnification can be adjusted electronically

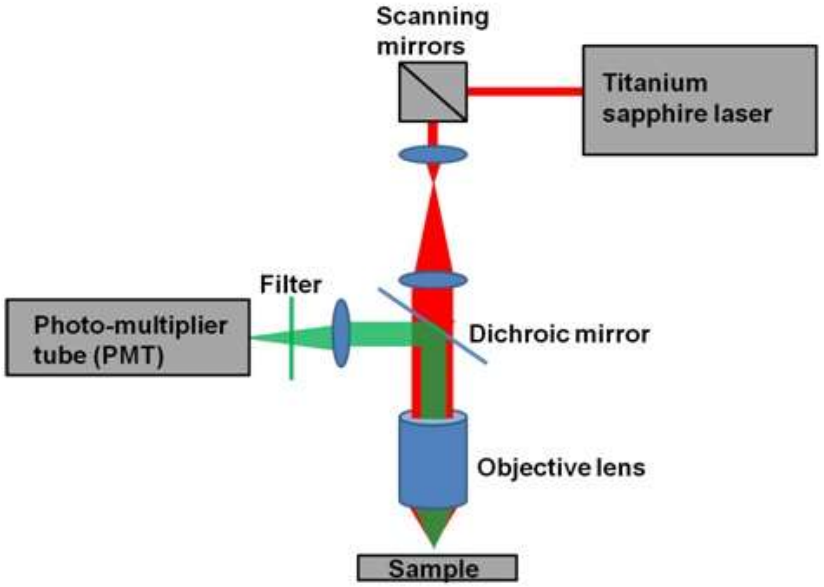
Disadvantages

- Slow scan speeds
- Limited use in dynamic tracking studies
- Photobleaching from laser excitation
- Lasers may damage living cells, limiting use in live cell studies
- Lower resolution than camera detection

Multiphoton Microscope

Multiphoton microscopy (MPM) is regarded as the method of choice for imaging of living, intact biological tissues on length scales from the molecular level through the whole organism





The principal advantages of two-photon microscopy are reduced phototoxicity, increased imaging depth, and the ability to initiate highly localized photochemistry in thick samples.

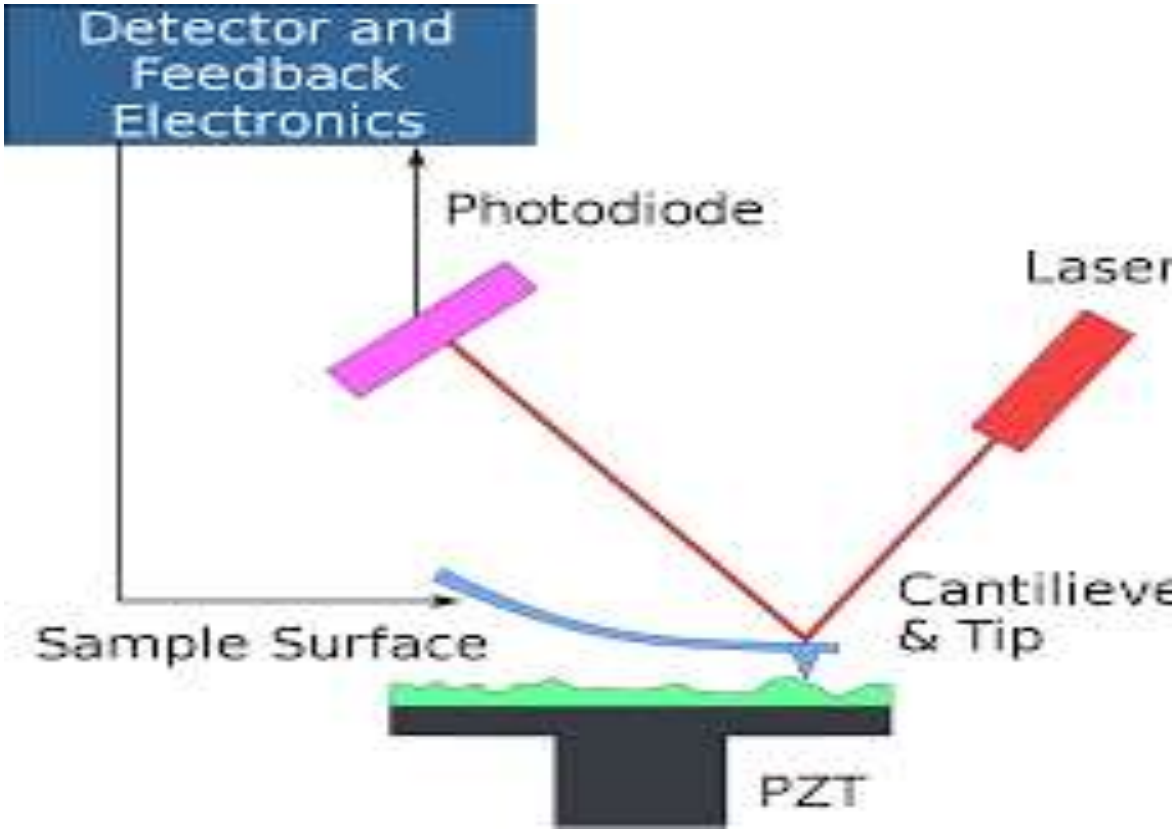
Atomatic force microscope

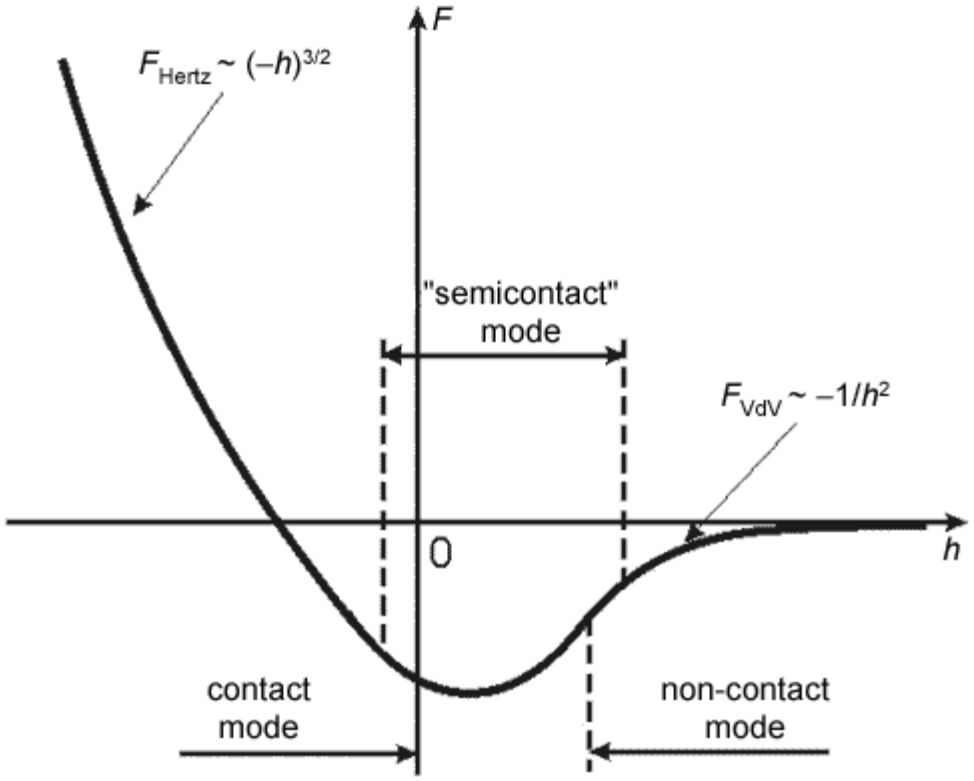
Atomic force microscopy (**AFM**) or **scanning force microscopy** (**SFM**) is a very-high-resolution type of scanning probe microscopy (SPM), with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit.

AFM Working **Principle**. The **AFM principle** is based on the cantilever/tip assembly that interacts with the sample; this assembly is also commonly referred to as the probe. ... The up/down and side to side motion of the **AFM** tip as it scans along the surface is monitored through a laser beam reflected off the cantilever.

Atomic-force microscopy (**AFM**) is a surface scanning technique that has sub-nanometer scale resolution. ... **AFM** is **used** widely to collect data on various mechanical, functional and electrical properties at the nanoscale as well as for topography (surface) studies.

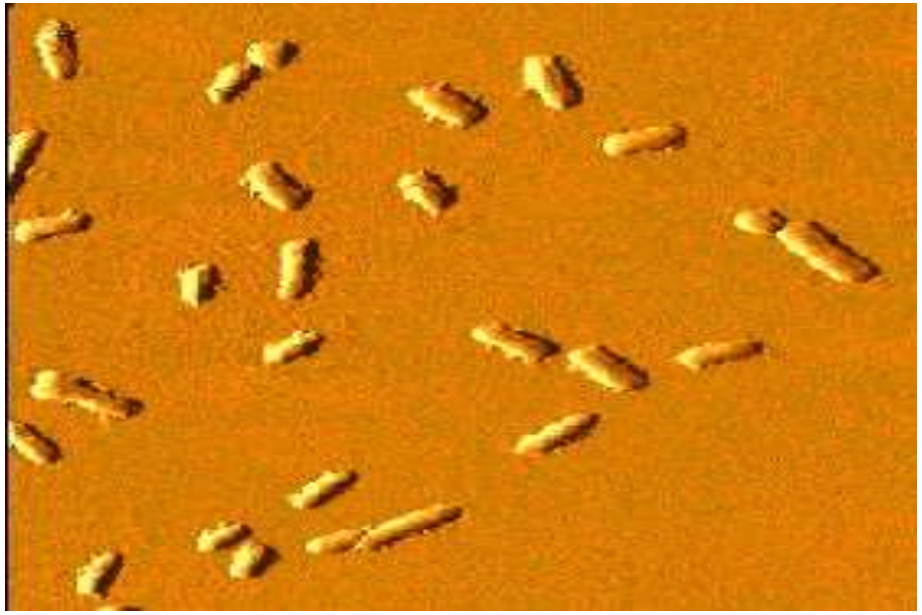
The probe mounted on the AFM performs the scan on the sample in a raster fashion. The movement of the microcantilever over the sample is carried out by the piezoelectric scanner, which comprises piezoelectric material that expands and contracts according to the applied voltage. There are several modes of operation for scanning and mapping surface. These modes include non-contact, contact and intermittent contact modes. These three modes of operation differ from each other, basically, by the tip and sample distance.

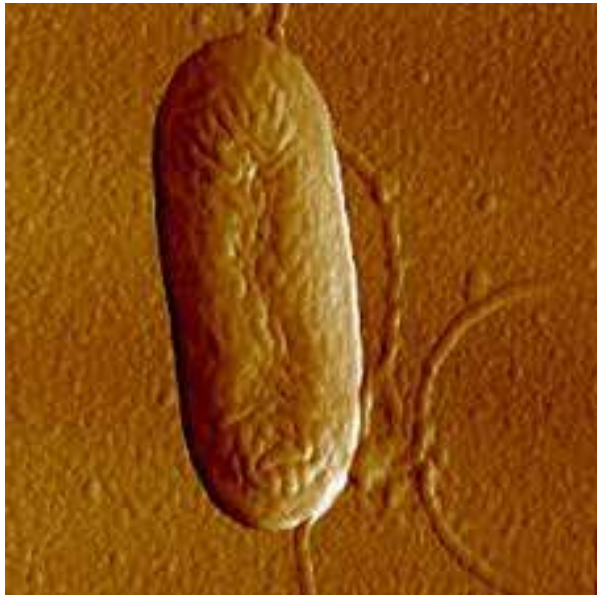


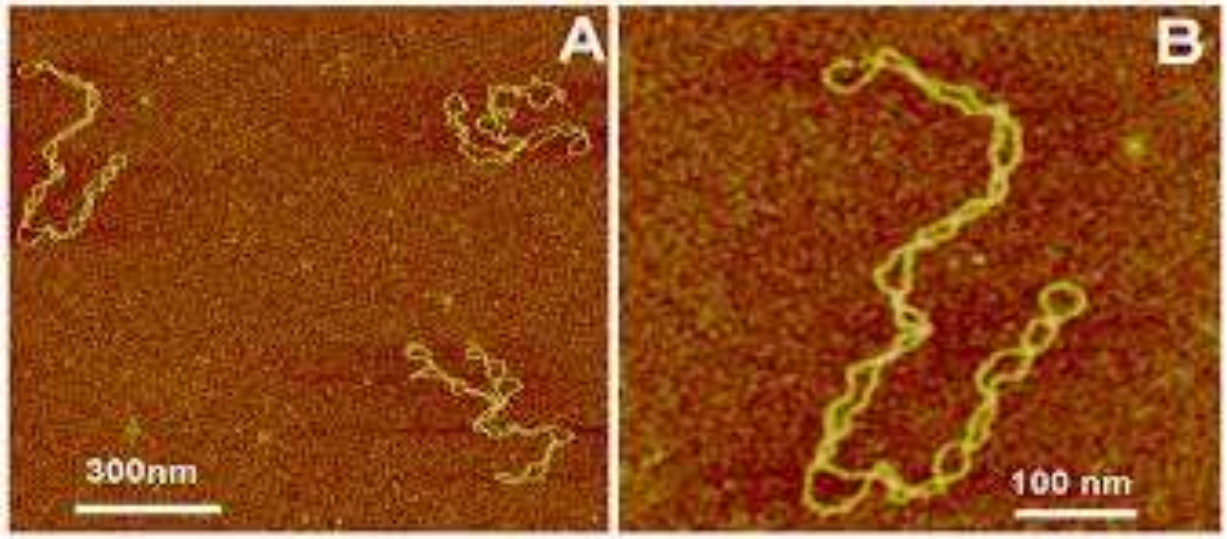


Depending on tip-sample separation during scanning, three modes of an atomic force microscope are available 1) contact, 2) non-contact, 3) "semicontact" which is intermediate between contact and non-contact.

In the **contact mode** the probe tip directly touches the sample surface during scanning. In the **non-contact mode** the probe is far enough and do not touch the surface. In the **semicontact mode** the intermittent contact occurs. The last two AFM modes are needed to implement modulation (or vibration) techniques.






A
300nm
B
100 nm

Advantages and Disadvantages of AFM

- Easy sample preparation
- Accurate height information
- Works in vacuum, air, and liquids
- Living systems can be studied
- Limited vertical range
- Limited magnification range
- Data not independent of tip
- Tip or sample can be damaged

Many more new types of microscope are now available

Super-resolution microscopy, in light microscopy, is a term that gathers several techniques, which allow images to be taken with a higher resolution than the one imposed by the diffraction limit

A **total internal reflection fluorescence microscope** (**TIRFM**) is a type of microscope with which a thin region of a specimen, usually less than 200 nanometers can be observed.

Electron Microscopy

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

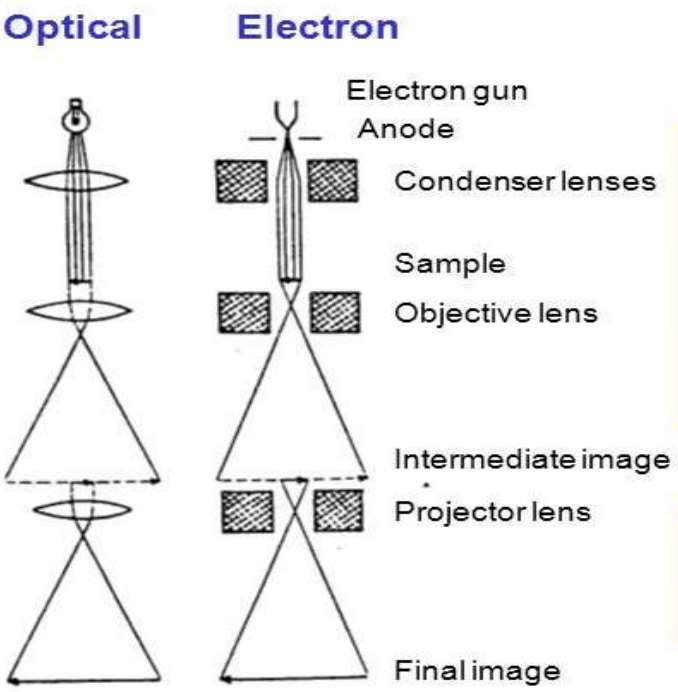
Working **Principle**: An **electron microscope** uses an '**electron** beam' to produce the image of the object and magnification is obtained by 'electromagnetic fields'; unlike light or optical **microscopes**, in which 'light waves' are used to produce the image and magnification is obtained by a system of 'optical lenses'.

Electrons are such small particles that, like photons in light, they act as waves. A beam of **electrons** passes through the specimen, then through a series of lenses that magnify the image. The image results from a scattering of **electrons** by atoms in the specimen

The Wavelength

- Resolving power of EM is from **Wave properties of electrons**
- Limit of **resolution is indirectly proportional to the wavelength** of the illuminating light
- ie, longer the wavelength, lesser is the resolution

Transmission Electron Microscopy (TEM)
Similarity of Optical and Electron Microscope

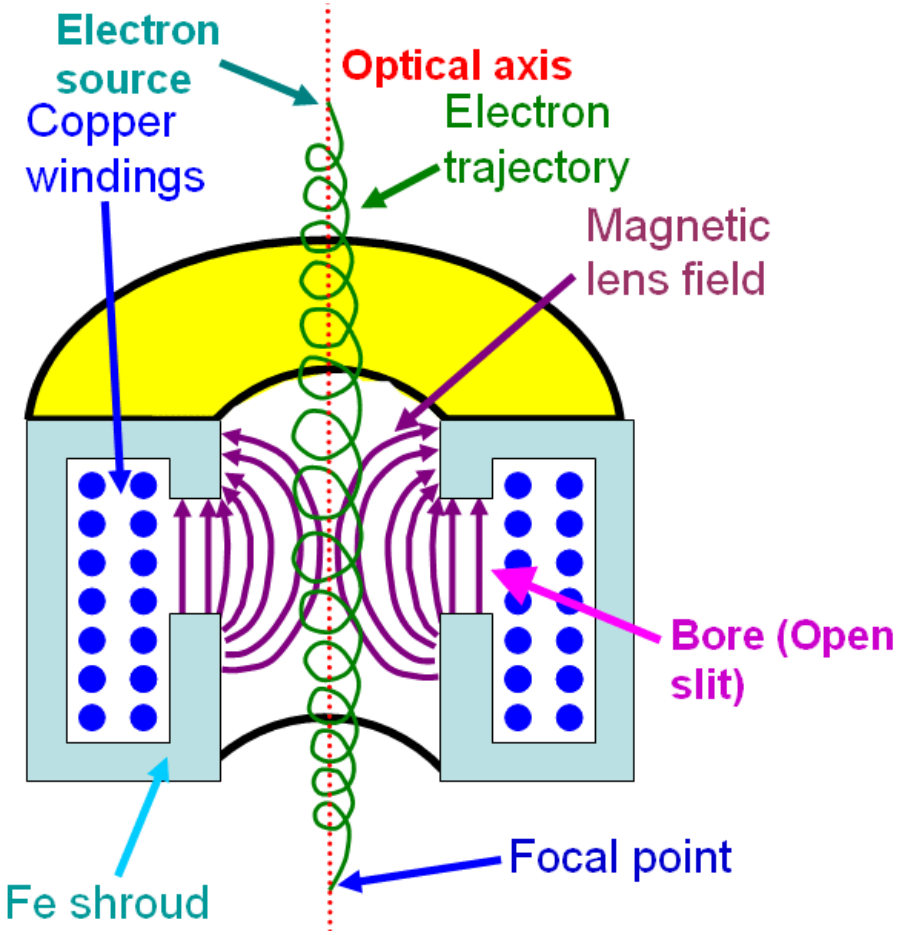


Essential difference:

wavelength of electrons: $3.7 \cdot 10^{-3}$ nm at 100 keV

wavelength of light: 400 - 700 nm

Consequently: resolution of electron microscope higher



In a **magnetic field** the force is always at right angles to the motion of the **electron** (Fleming's left hand rule) and so the resulting **path** of the **electron** is circular .

Charged particles move in circles at a constant speed if projected into a **magnetic field** at right angles to the **field**.

Electron Gun

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

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.

Motion in electric and magnetic fields

Magnetic fields

The force (F) on a wire of length L carrying a current I in a magnetic field of strength B is given by the equation: $F = BIL$.
But $Q = It$ and since $Q = e$ for an electron and $v = L/t$ you can show that :
Magnetic force on an electron = $BIL = B[e/t][vt] = Bev$ where v is the electron velocity

In a magnetic field the force is always at right angles to the motion of the electron (Fleming's left hand rule) and so the resulting path of the electron is circular (Figure 1).

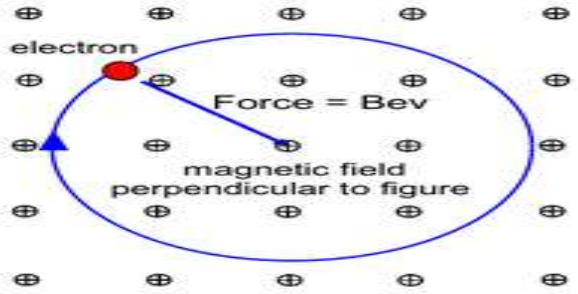


Figure 1

Therefore :

magnetic force = $Bev = mv^2/r$ = centripetal force

$[Ber]/m = v$

and so you can see from these equations that as the electron slows down the radius of its orbit decreases.

Charged particles move in circles at a constant speed if projected into a magnetic field at right angles to the field.
Charged particles move in straight lines at a constant speed if projected into a magnetic field along the direction of the field.

Figure 2 shows a 3D diagram of and electron moving at right angles to a uniform magnetic field.

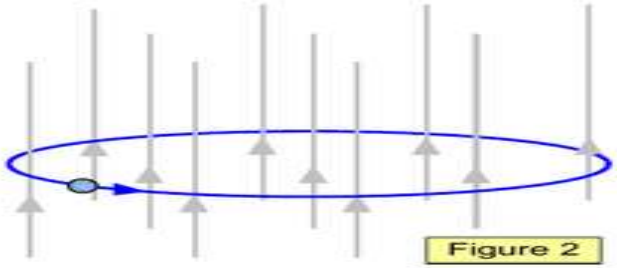


Figure 2

If the electron enters the field at an angle to the field direction the resulting path of the electron (or indeed any charged particle) will be helical as shown in figure 3. Such motion occurs above the poles of the earth where charges particles from the Sun spiral through the Earth's field to produce the aurorae.

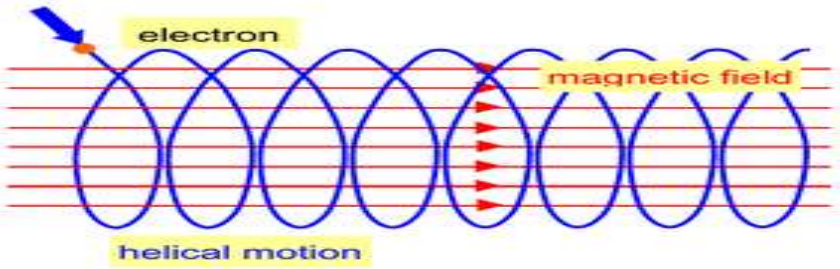
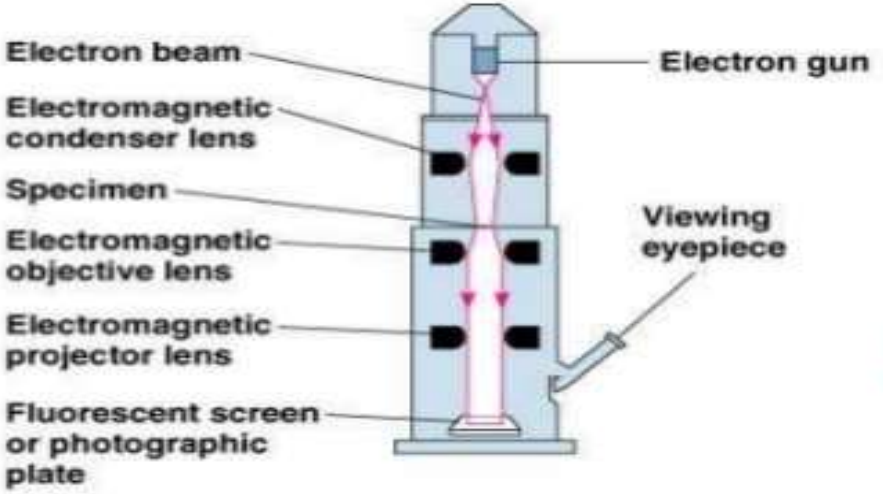


Figure 3

Transmission Electron Microscopy (TEM)

- **Ultrathin sections of specimens.**
- **Light passes through specimen, then an electromagnetic lens, to a screen or film.**
- Specimens may be stained with heavy metal salts.

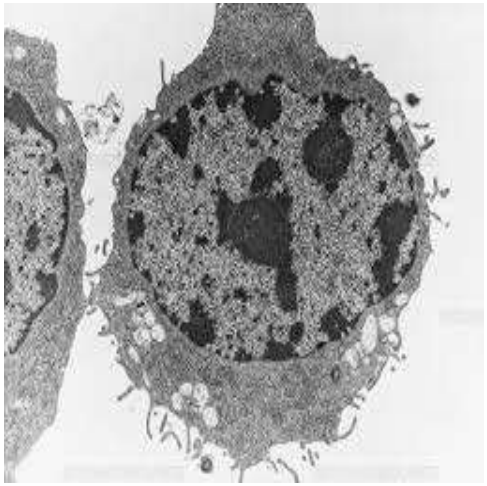


TRANSMISSON ELECTRON MICROSCOPE

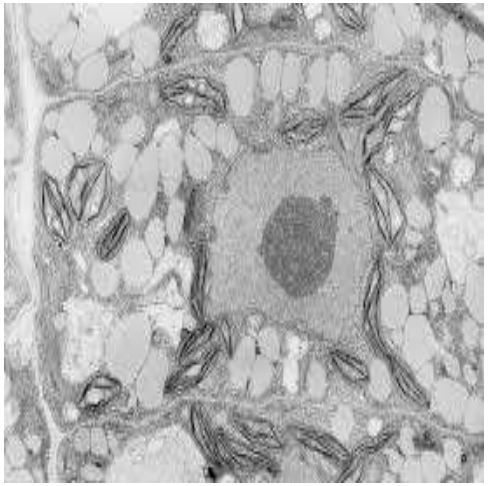
- This transmitted portion is focused by the objective lens into an image.
- The Objective & Selected Area metal apertures restrict the beam.
- The image is passed down the column through the intermediate and projector lenses, being enlarged all the way.
- The image strikes the phosphor image screen & 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)

TRANSMISSION ELECTRON MICROSCOPE

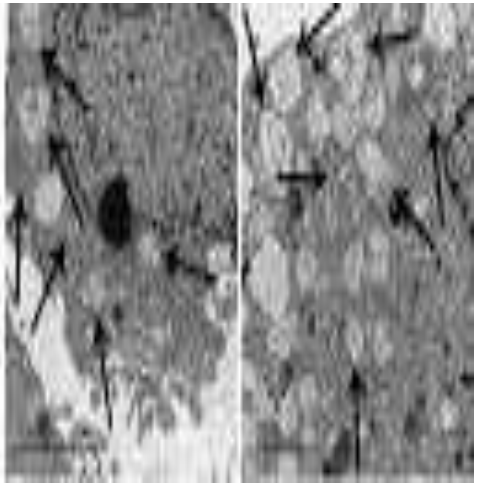
- Transmission electron microscopy (TEM) involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses. The electron beam that has been partially transmitted through the very thin (and so semitransparent for electrons) specimen carries information about the structure of the specimen.
- The spatial variation in this information (the "image") is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a monitor or computer.
- Transmission electron microscopes produce two-dimensional, black and white images.
- Resolution of the TEM is also limited by spherical and chromatic aberration, but a new generation of aberration correctors has been able to overcome or limit these aberrations.



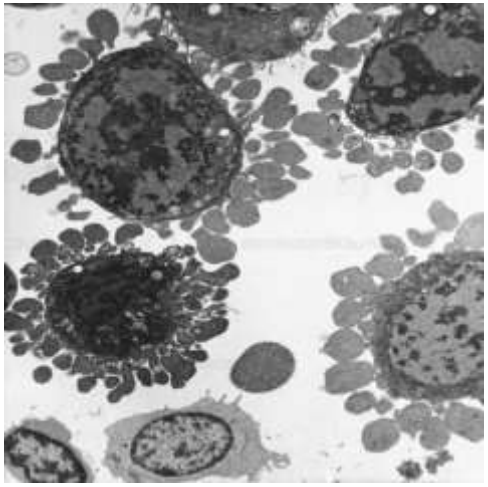
Animal cell



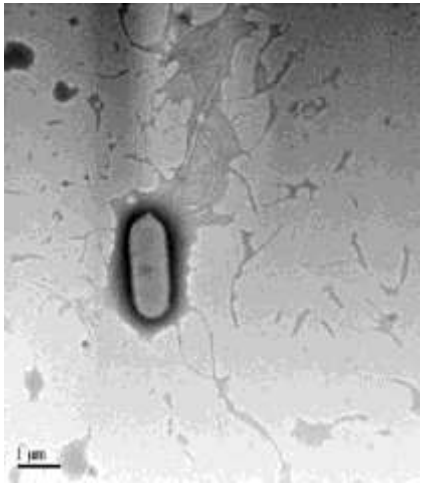
Plant cell



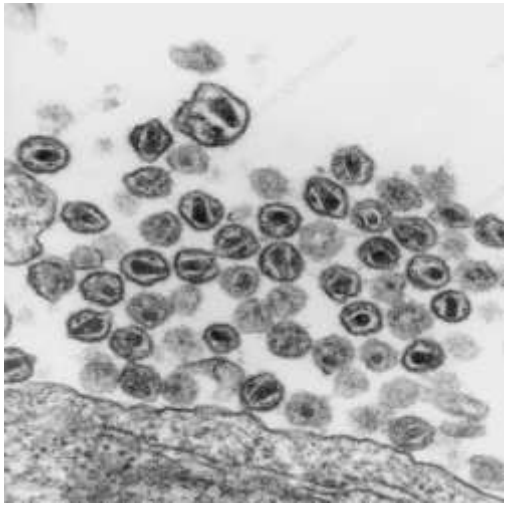
autophagy



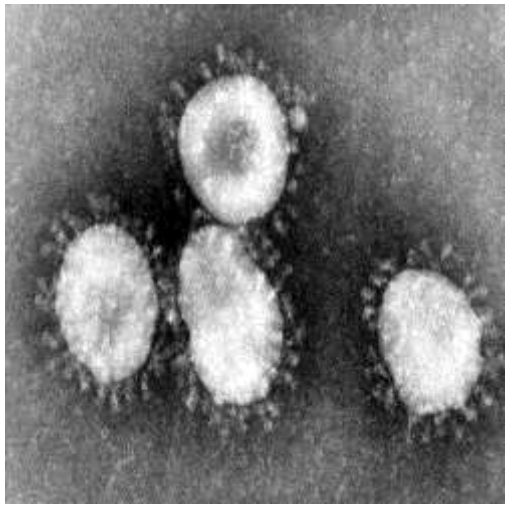
apoptosis



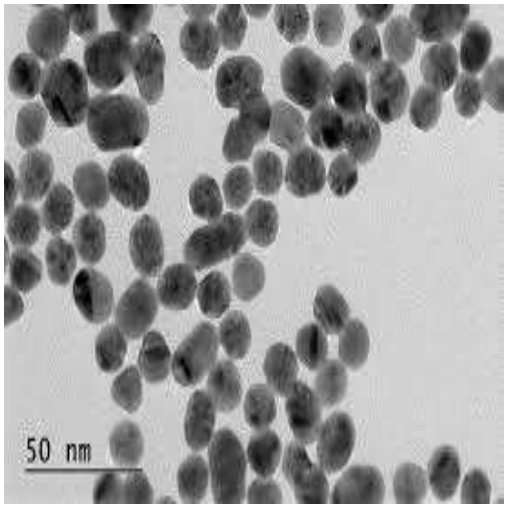
E.coli



HIV



Corona



Gold nanoparticles

A **scanning electron microscope** (**SEM**) is a type of **electron microscope** that produces images of a sample by **scanning** the surface with a focused beam of **electrons**. The **electrons** interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample.

secondary electrons emitted by atoms excited by the electron beam are detected using a secondary electron detector (Everhart-Thornley detector). The number of secondary electrons that can be detected, and thus the signal intensity, depends, among other things, on specimen topography. SEM can achieve resolution better than 1 nanometer.

Bioanalytical techniques -1

(Graphics are collected from Internet)

Microscopy :is the term of using microscopes to view objects and areas of objects that cannot be seen with the naked eye (objects that are not within the resolution range of the normal eye)

There are three well-known branches of Microscopy

- **optical,**
- **electron,**
- **scanning probe microscopy**

Optical or light microscopy involves passing visible light transmitted through or reflected from the sample through a single lens or multiple lenses to allow a magnified view of the sample. The resulting image can be detected directly by the eye, imaged on a photographic plate, or captured digitally.

The single lens with its attachments, or the system of lenses and imaging equipment, along with the appropriate lighting equipment, sample stage, and support, makes up the basic light microscope.

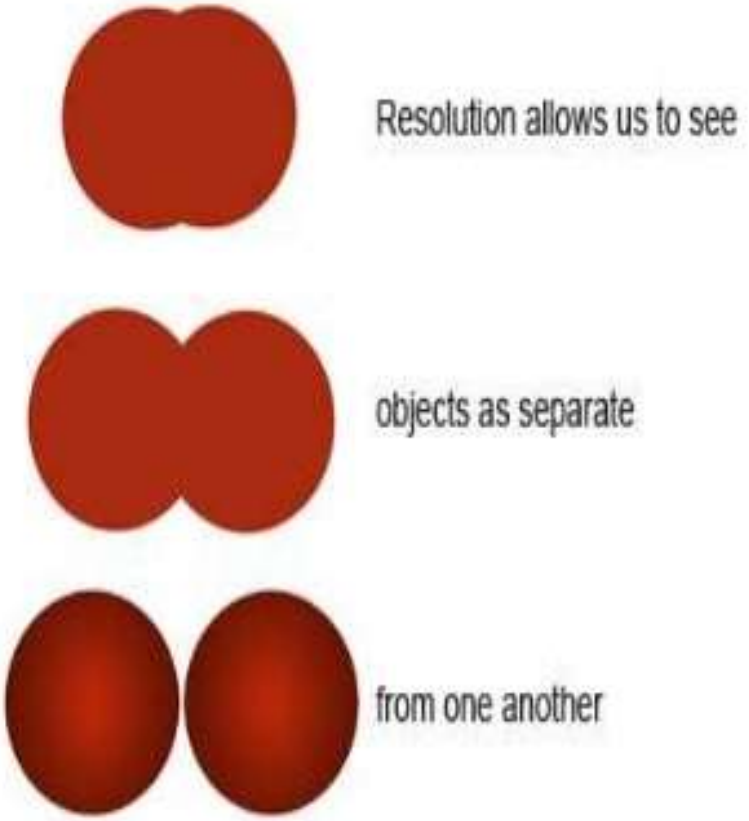
The most recent development is the digital microscope, which uses a CCD camera to focus on the exhibit of interest.

Vocabulary

- **Magnification** – larger image
- **Resolution** – clearer image
- **Numerical Aperture** – light gathering capacity of a lens
- **Working Distance** – the distance from the bottom of an objective to the in-focus area of an object (distance between specimen and lens)

Resolution

- Resolution is defined as the ability to distinguish two **very small** and **closely-spaced** objects as separate entities.
- Resolution is best when the distance separating the two tiny objects is small.
- Degree to which detail in specimen is retained in magnified image.
- Resolving power-
  - Unaided eye – 0.1 mm apart
  - Microscope - 0.2 µm apart



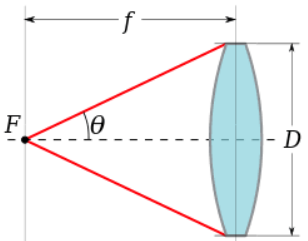
The limit of resolution (or resolving power) is a measure of the ability of the objective lens to separate in the image adjacent details that are present in the object. It is the distance between two points in the object that are just resolved in the image.
Thus an optical system cannot form a perfect image of a point.

Smaller means better device

Numerical Aperture

- NA is light gathering capacity of objective

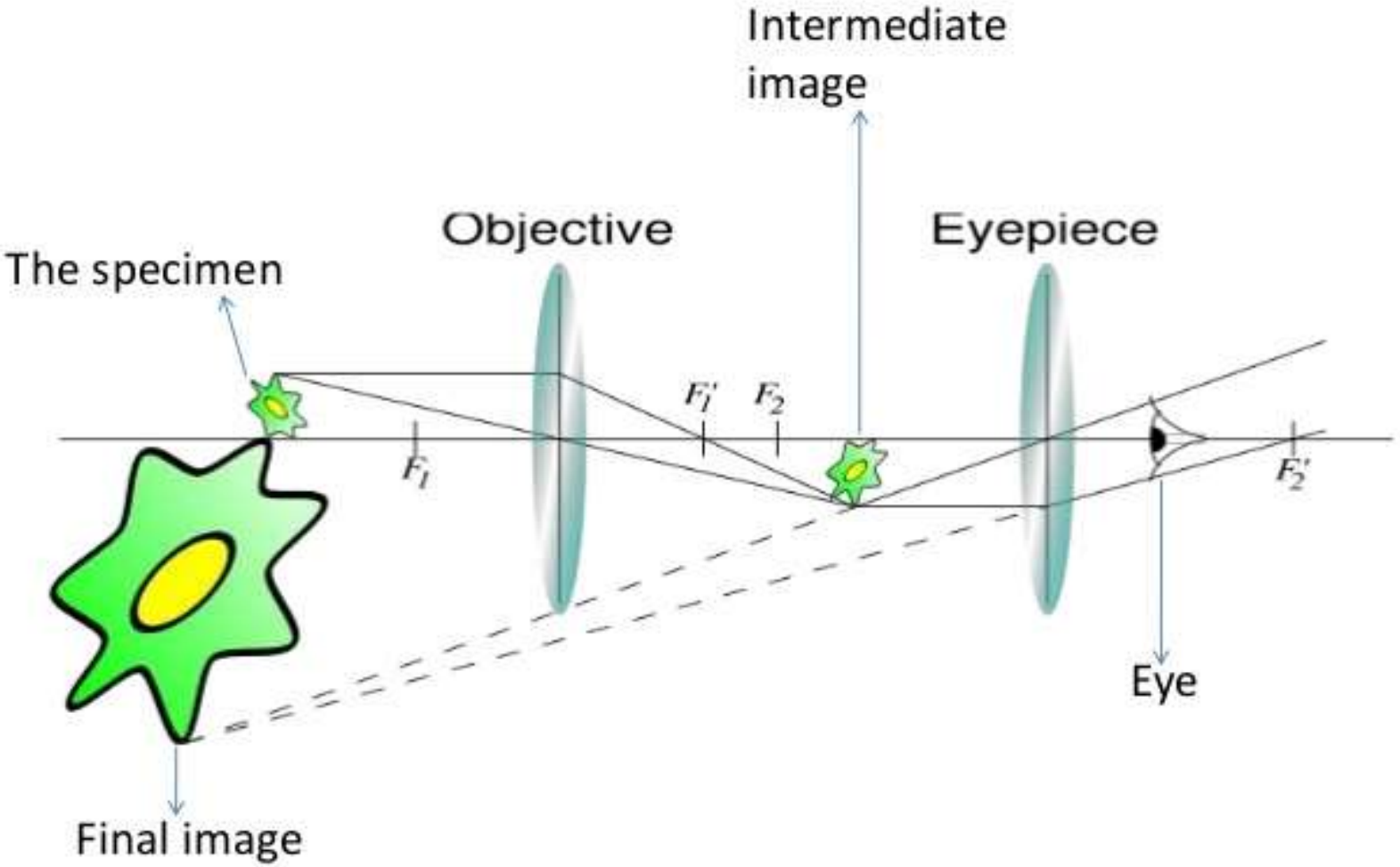
- Limit of resolution = $\frac{0.61\lambda}{NA}$ → Wavelength of illumination



- NA (Numerical Aperture) = n sinα → Aperture angle
  - ↓ Refractive index of air or liquid between specimen and lens

- The N.A. of each objective lens is inscribed in the metal tube, and ranges from 0.25-1.4
- The higher the N.A., the better the light-gathering properties of the lens, and the better the resolution.

Formation of image :



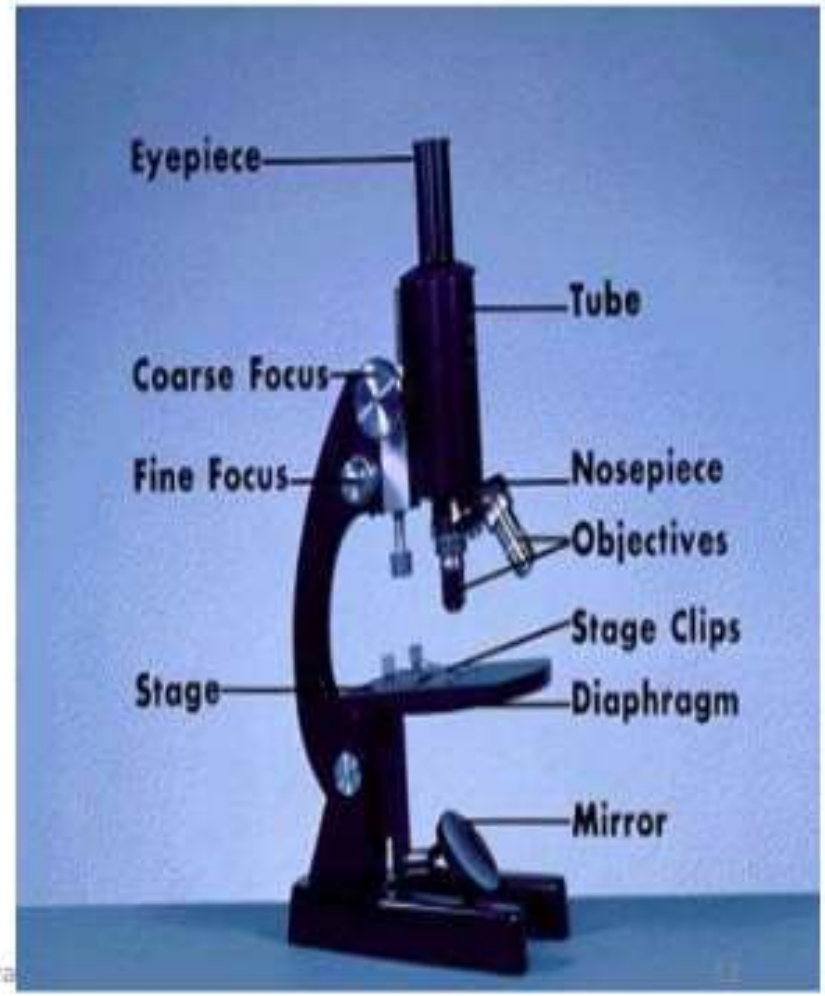
Modern Compound Microscope

The microscope is consists of:

•mechanical system which supports the microscope,

•an optical system which illuminates the object under investigation

•light passes through a series of lens to form an image of the specimen.



Types of Light Microscopy

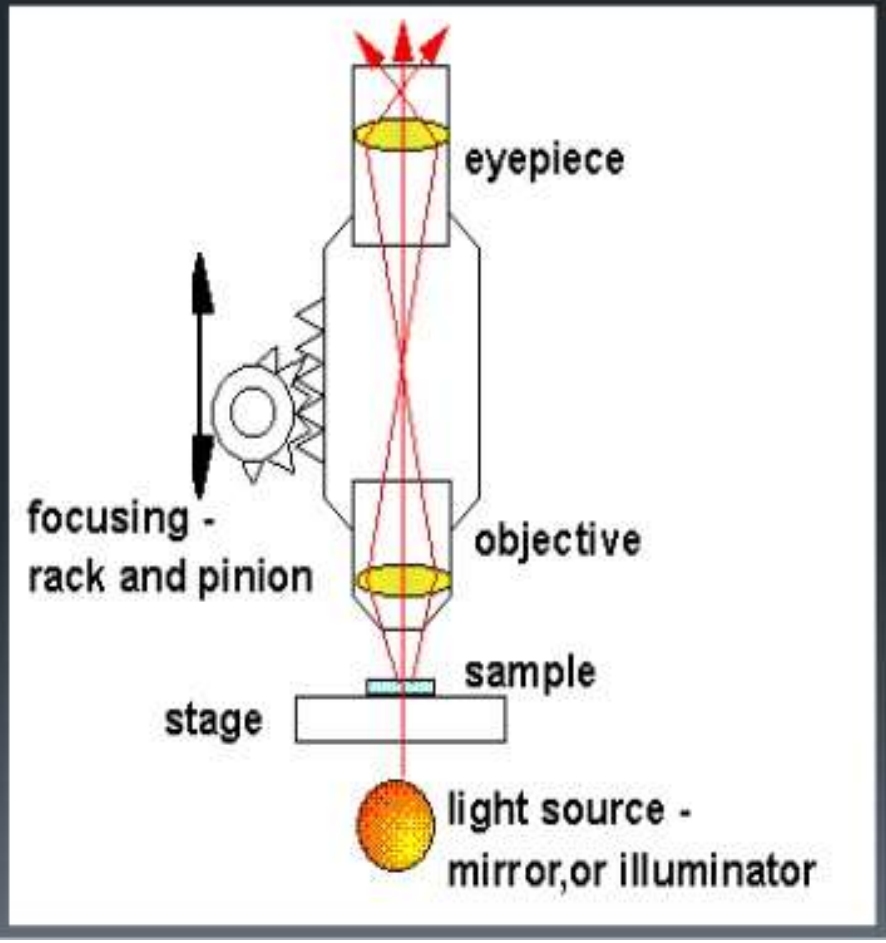
A. Bright field microscopy.

B. Dark field microscopy.

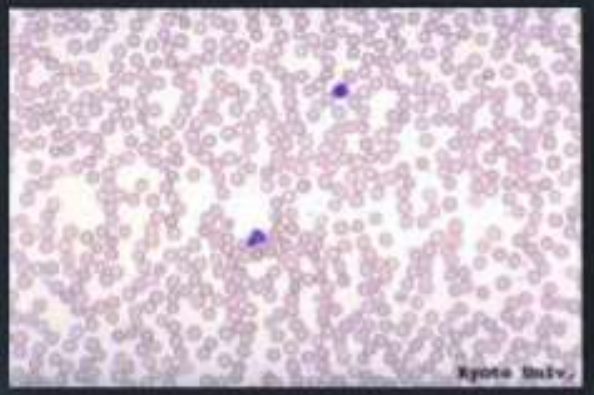
C. Fluorescence microscopy.

D. Phase contrast microscopy.

A. Bright field microscopy



Stained preparation/ slides



B. Dark filed microscopy

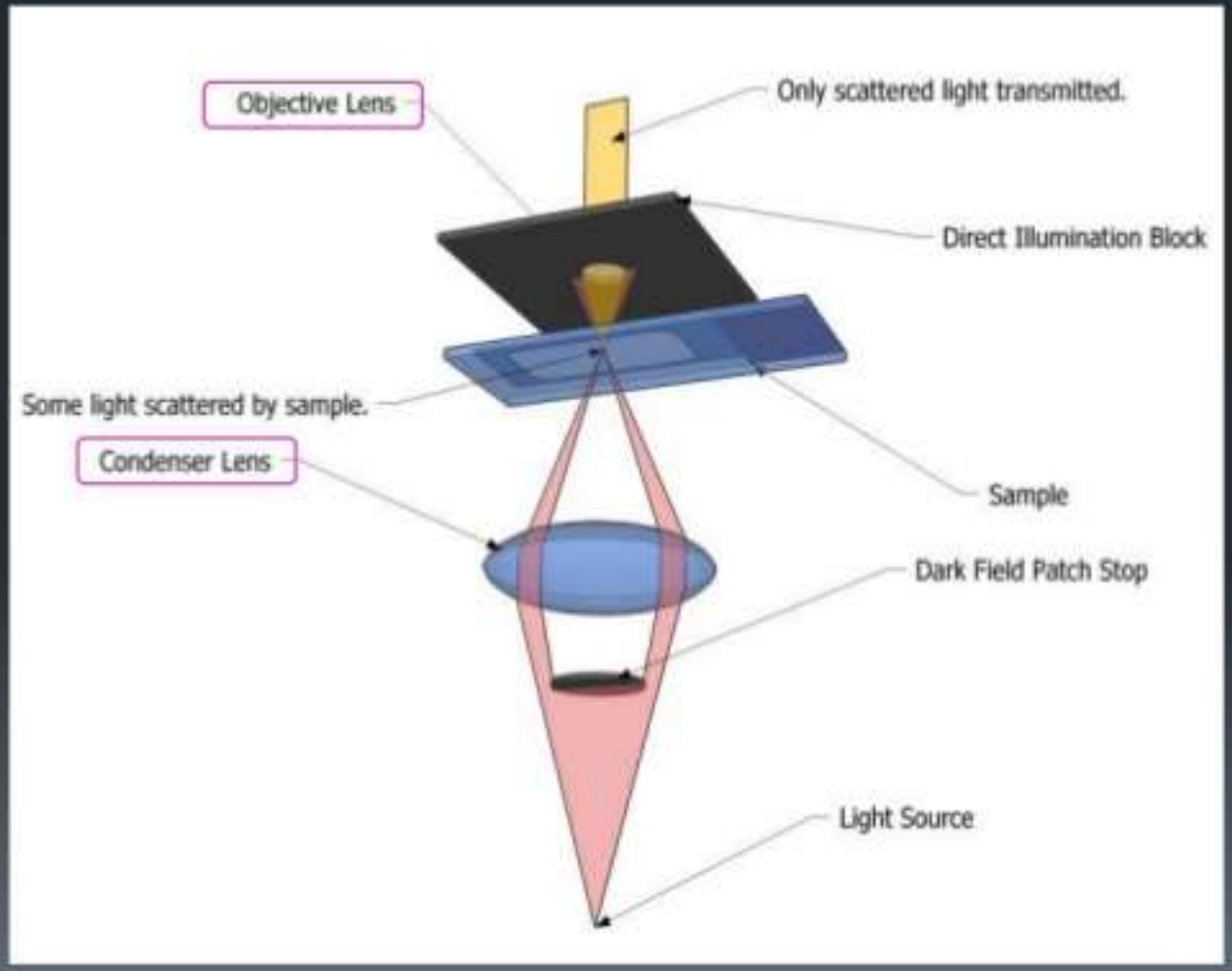


Image formation By brightfield and dark field

Aberrations

- **Spherical aberration:**

  Light rays hitting periphery will be more refracted than the rays hitting centre of lens.

- **Chromatic Aberrations:**

  White light of passing through simple lens, each wavelength will be refracted to different extent.

  Blue brought to a shorter focus than red, results in a un-sharp image with color fringes.

Corrected with Achromatic and Apochromat lens

Bright field microscopy is the conventional technique. It is suitable for observing the natural colors of a specimen or the observation of stained samples. The specimen appears darker on a **bright** background.

Darkfield microscopy shows the specimens **bright** on a **dark** background

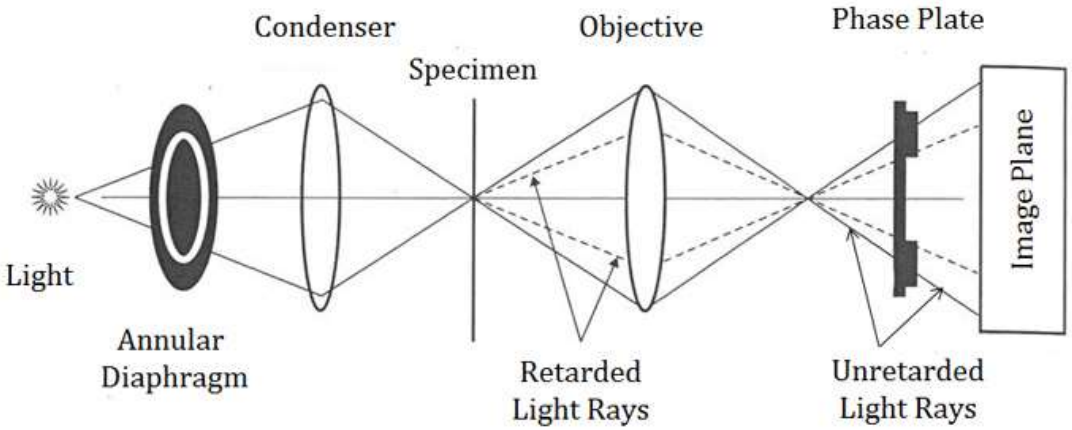
- The useful magnification of Light microscope is limited by its resolving power.
- The resolving power in limited by wavelength of illuminating beam.
- Resolution is determine by certain physical parameters like wave length of light and light generating power of the objective & condenser lens.
- Higher N.A Better light generation Better Resolution Shorter the Wavelength Better Resolution.

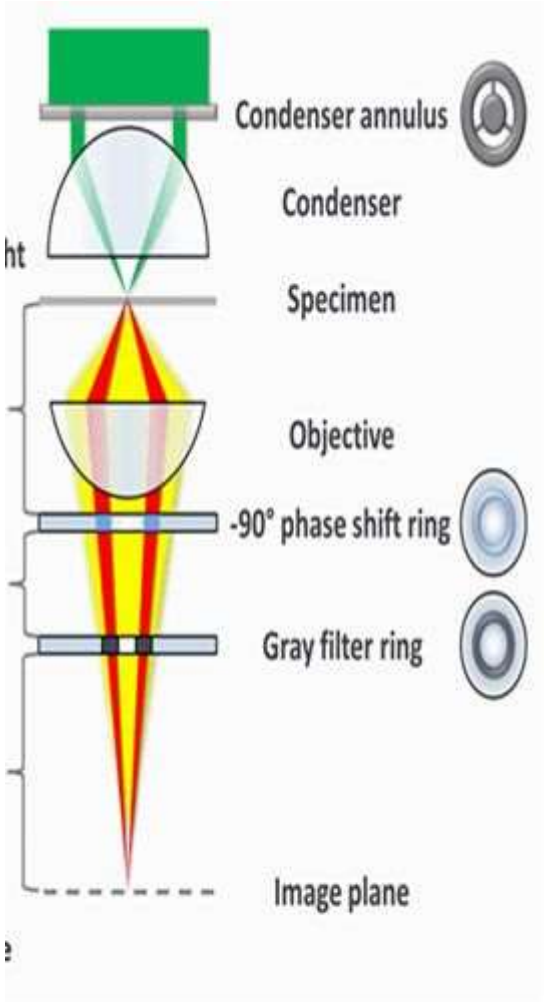
PHASE CONTRAST MICROSCOPY

- By converting the phase differences, between light passing through a specimen and that passing through the surrounding medium, into amplitude (brightness) differences, phase contrast microscopy provides a difference in brightness between the object and the background, which the eye can then see.

The **phase contrast microscopy** is based on the **principle** that small **phase** changes in the light rays, induced by differences in the thickness and refractive index of the different parts of an object, can be transformed into differences in brightness or light intensity.

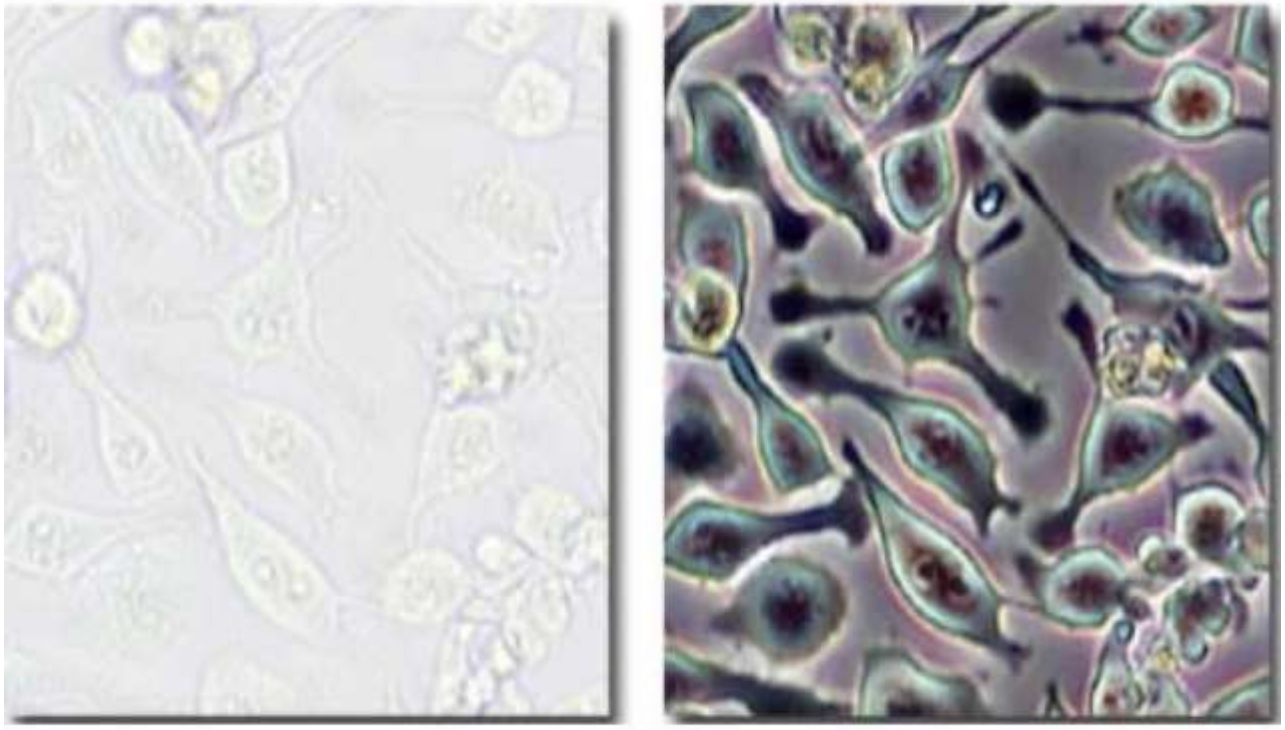
Phase Contrast Microscope





Optical Path of Phase Contrast Microscope

Living Cells in Brightfield and Phase Contrast



Brightfield Phase contrast

Advantages

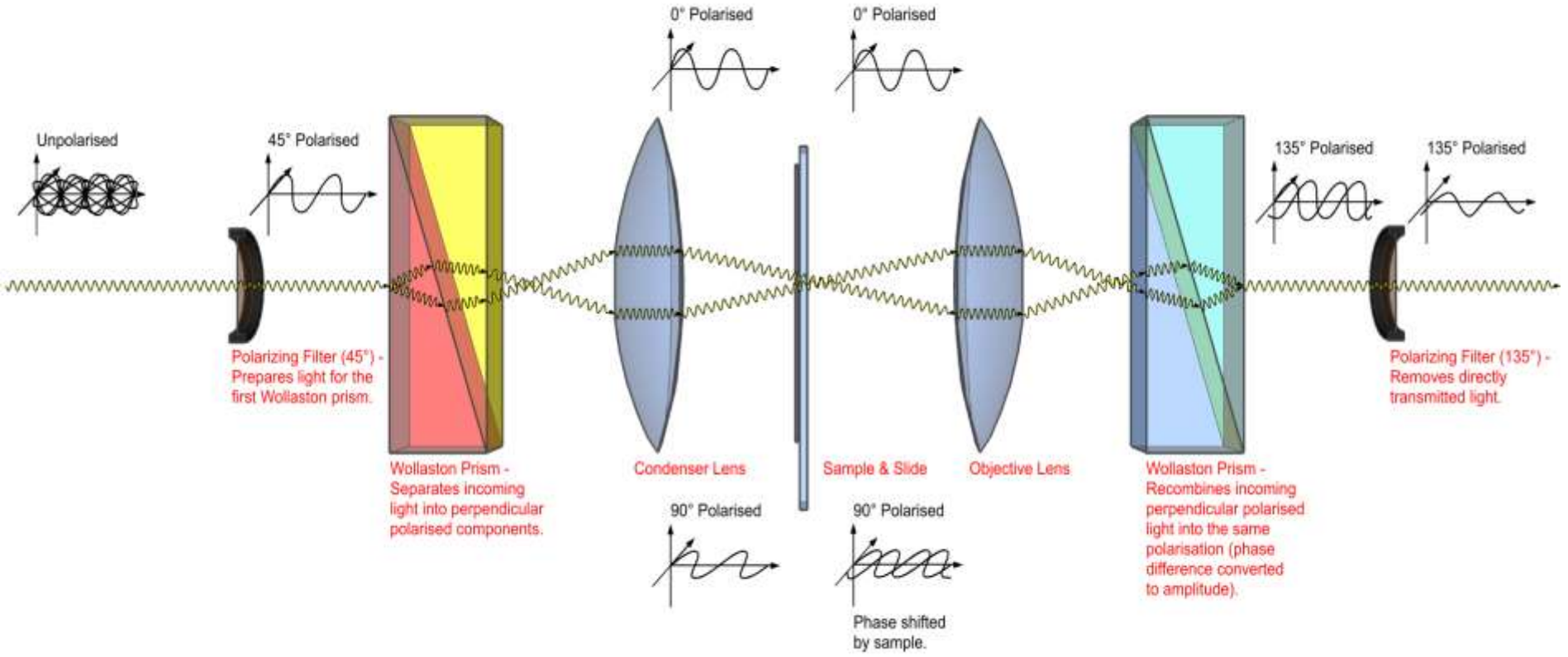
1. Small unstained specimens such as a living cell can be seen.
2. It makes Highly Transparent objects more visible.
3. Examining Intracellular components of living cells at relatively high resolution.
   eg: The dynamic motility of Mitochondria,
   mitotic chromosomes & vacuoles.
4. It made it possible for Biologists to study living cells and how they proliferate through cell division.

Disadvantages and **limitations of phase contrast**:

Annuli or rings limit the aperture to **some** extent, which decreases resolution. This method of observation is not ideal for thick organisms or particles. Thick specimens can appear distorted

Differential interference contrast (DIC) microscopy, also known as Nomarski interference contrast (NIC) or Nomarski **microscopy**, is an optical **microscopy** technique used to enhance the contrast in unstained, transparent samples.

DIC works by separating a polarized light source into two orthogonally polarized mutually coherent parts which are spatially displaced (sheared) at the sample plane, and recombined before observation.



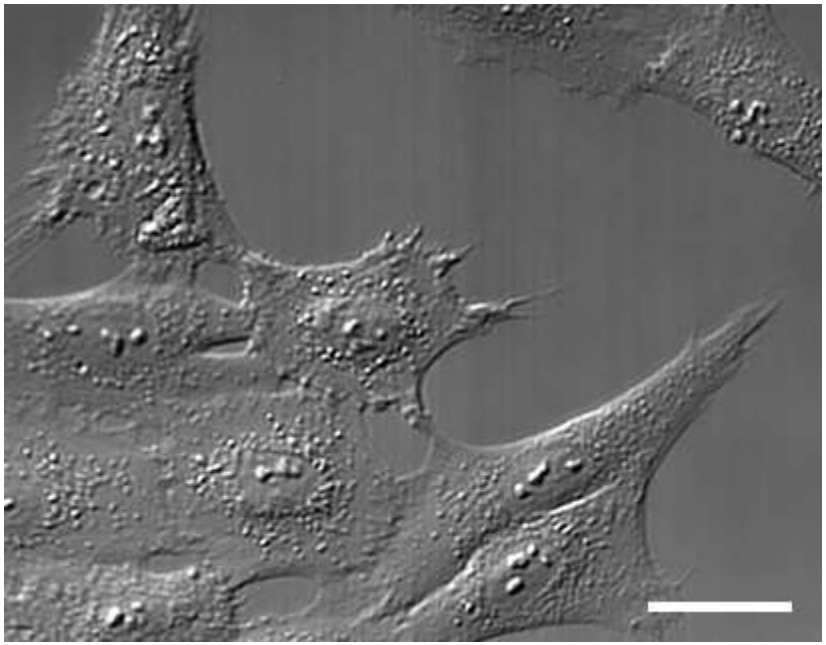
An advantage of DIC is that the specimen will appear bright in contrast to the dark background.
This system is relatively easy to incorporate with an existing brightfield microscope.

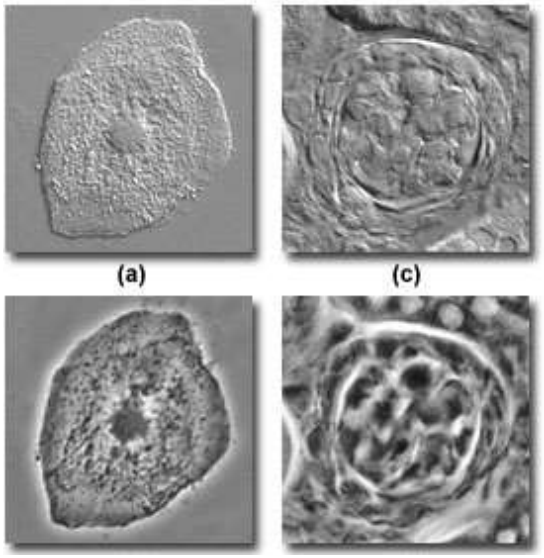
Two of the short comings of the phase contrast method are the fact that the specimen must be very thin and a halo is produced in the viewing field.
No halo effect occurs with differential interference contrast and it can be used to produce very clear images of thick specimens.

It can also be used in conjunction with digital imaging systems to add further definition to the image.

Differential interference contrast imaging can be used in conjunction with fluorescence microscopy to provide a better fluorescence image and to pinpoint specific areas on a specimen before switching to the fluorescence mode to further examine the object.

A major advantage of the differential interference contrast technique is in examining living specimens when normal biological processes might be impeded by normal staining procedures.
A drawback to this type of imaging is that the three-dimensional image of a specimen may not be accurate. The enhanced areas of light and shadow might add distortion to the appearance of the image.





DIC (no halo)

Phase contrast

Fluorescence Microscope

Fluorescence microscopy is an imaging technique **used** in light **microscopes** that allows the excitation of fluorophores and subsequent detection of the **fluorescence** signal.

The basic task of the **fluorescence microscope** is to let excitation light radiate the specimen and then sort out the much weaker emitted light from the image.

The radiation collides with the atoms in your specimen and electrons are excited to a higher energy level. When they relax to a lower level, they emit light.