**TEJASVI NAVADHITAMASTU**

*"Let our (the teacher and the taught) learning be radiant"*

*Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose*

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# E –content

**Course:** M.Sc.
**Subject:** Biochemistry; Biotechnology, Microbiology, Environmental Science
**Topic:** Instrumentation and Analytical Techniques
Subtopic: **MICROSCOPY**
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# MICROSCOPY

- **A microscope (Greek: *micron* = small and *scopos* = aim)**
- **MICROSCOPE - An instrument for viewing objects that are too small to be seen by the naked or unaided eye**
- **MICROSCOPY - The science of investigating small objects using such an instrument is called microscopy**

Microscopy is to get a magnified image, in which structures may be resolved which could not be resolved with the help of an unaided eye.

## Magnification

- It is the ratio of the size of an object seen under microscope to the actual size observed with unaided eye.
- The total magnification of microscope is calculated by multiplying the magnifying power of the objective lens by that of eye piece.

## Resolving power

- It is the ability to differentiate two close points as separate.
- The resolving power of human eye is 0.25 mm
- The light microscope can separate dots that are 0.25μm apart.
- The electron microscope can separate dots that are 0.5nm apart.

## Limit of resolution

It is the minimum distance between two points to identify them separately.

It is calculated by Abbé equation.

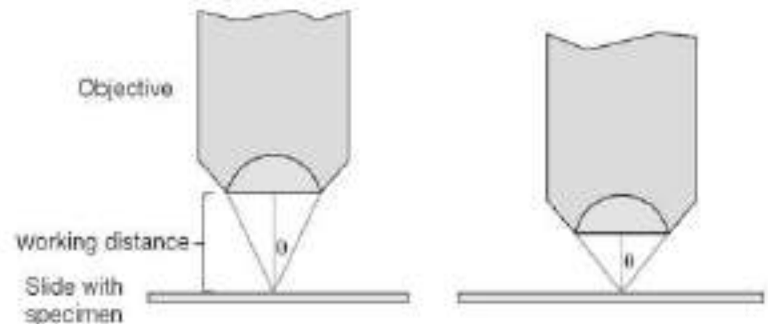
$$\text{Resolving power (R.P.)} = \frac{\text{Wavelength of light in nm}}{2 \times \text{Numerical aperture of objective lens}}$$

Limit of resolution is inversely proportional to power or resolution.

If the wavelength is shorter then the resolution will be greater.

## Working distance

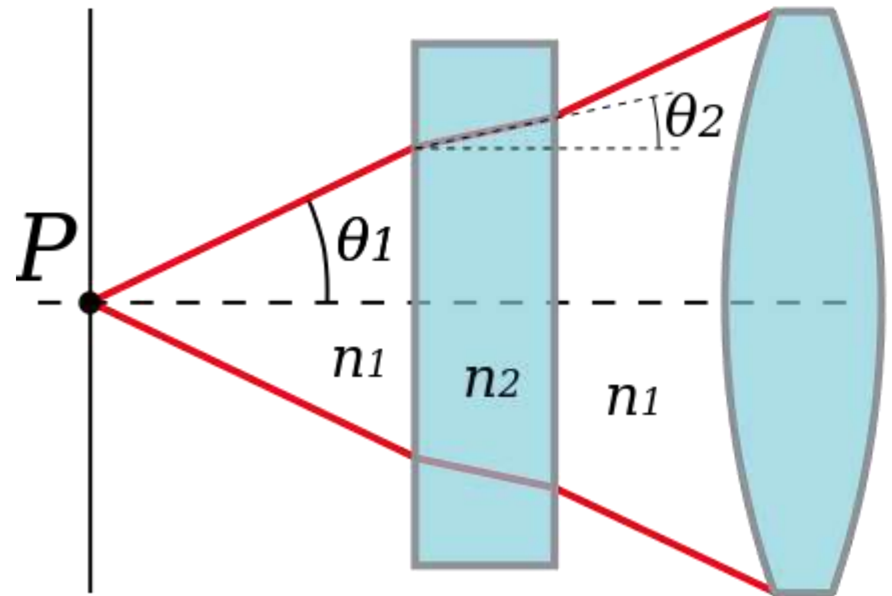
- It is the distance between the objective and the objective slide.
- The working distance decreases with increasing magnification.



In most areas of optics, and especially in microscopy, the numerical aperture of an optical system such as an objective lens is defined by

$$\mathrm{NA} = n \sin\theta,$$

where $n$ is the index of refraction of the medium in which the lens is working (1.00 for air, 1.33 for pure water, and typically 1.52 for immersion oil;[1] see also list of refractive indices), and $\theta$ is the maximal half-angle of the cone of light that can enter or exit the lens. In general, this is the angle of the real marginal ray in the system.




Due to Snell's law, the numerical aperture remains the same:

$$\mathrm{NA} = n_1 \sin\theta_1 = n_2 \sin\theta_2.$$

## Angular aperture

The **angular aperture** of a lens is the apparent angle of the lens aperture as seen from the focal point:

In a medium with an index of refraction close to 1, such as air, the angular aperture is approximately equal to twice the numerical aperture of the lens.[1]

## f-number

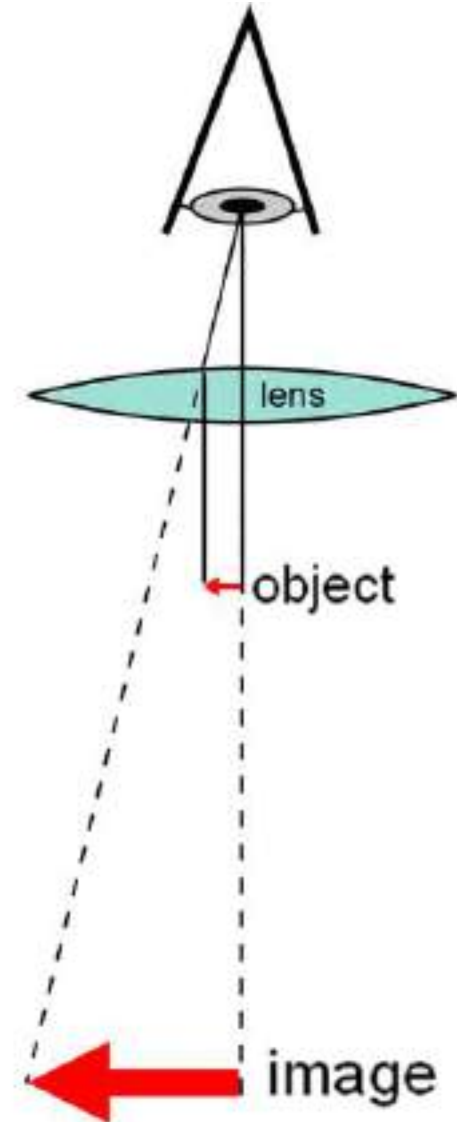
In optics, the **f-number** (sometimes called **focal ratio**, **f-ratio**, **f-stop**, or **relative aperture**[1]) of an optical system is theratio of the lens's focal length to the diameter of the entrance pupil.[2] I

# Light microscope

The **optical microscope**, often referred to as **light microscope**, is a type of microscope which uses visible light and a system of lenses to magnify images of small samples. Optical microscopes are the oldest design of microscope and were possibly invented in their present compound form in the 17th century. Basic optical microscopes can be very simple, although there are many complex designs which aim to improve resolution and sample contrast.

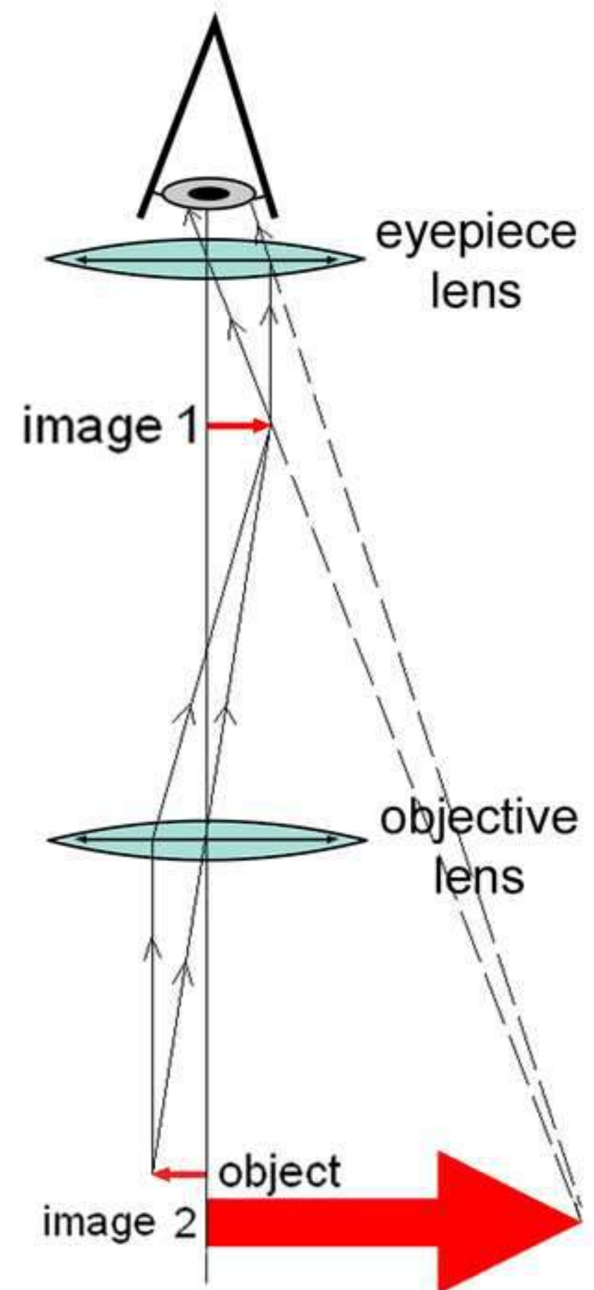
## SIMPLE MICROSCOPE

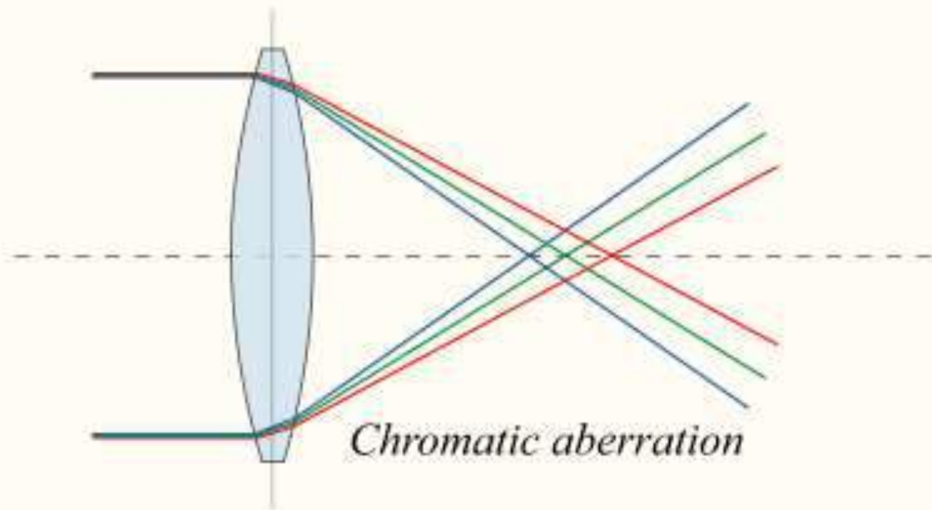
A **simple microscope** is a microscope that uses a lens or set of lenses to enlarge an object through angular magnification alone, giving the viewer an erect enlarged virtual image.[3][4] Simple microscopes are not capable of high magnification. The use of a single convex lens or groups of lenses are still found in simple magnification devices such as the magnifying glass.



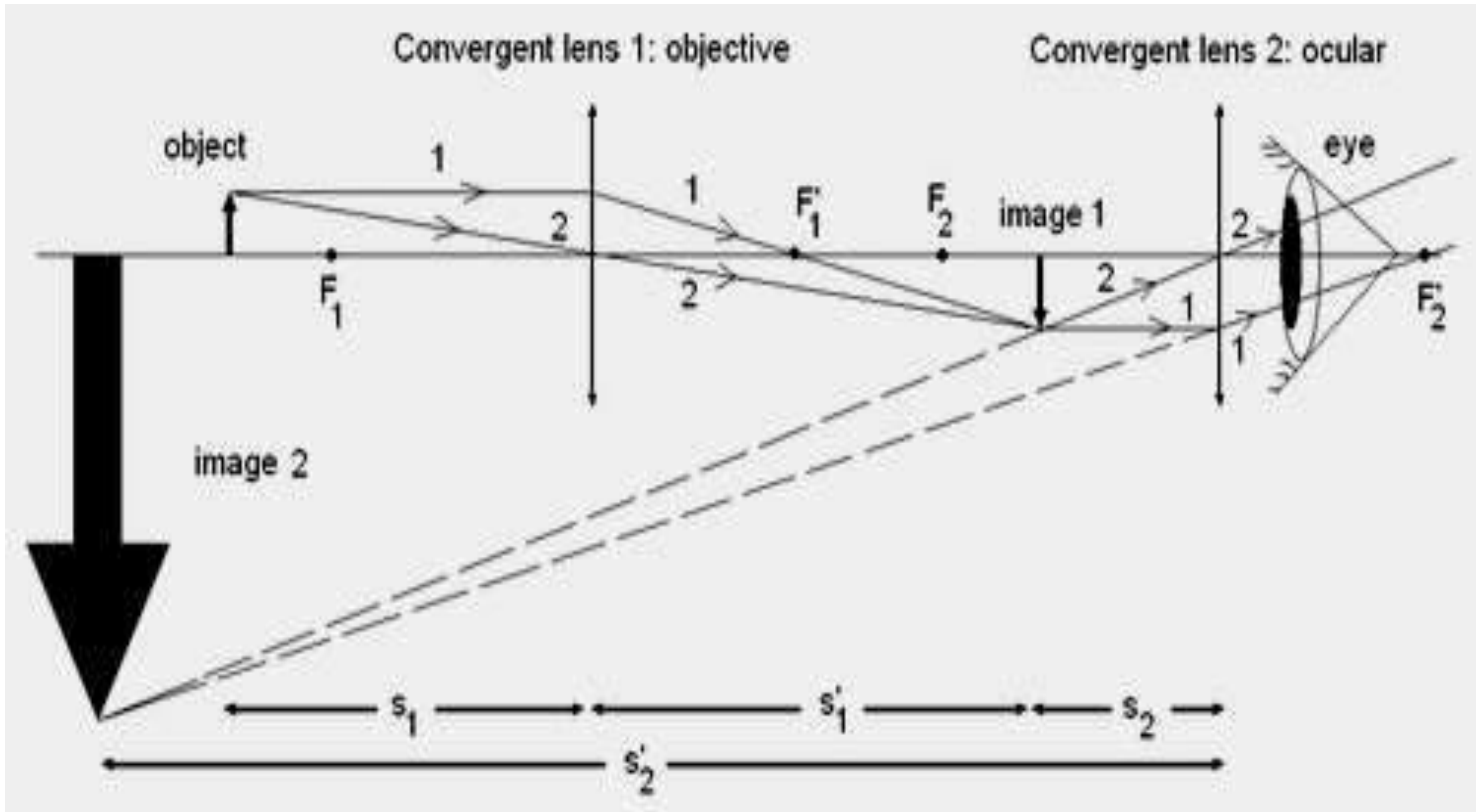
## COMPOUND MICROSCOPE

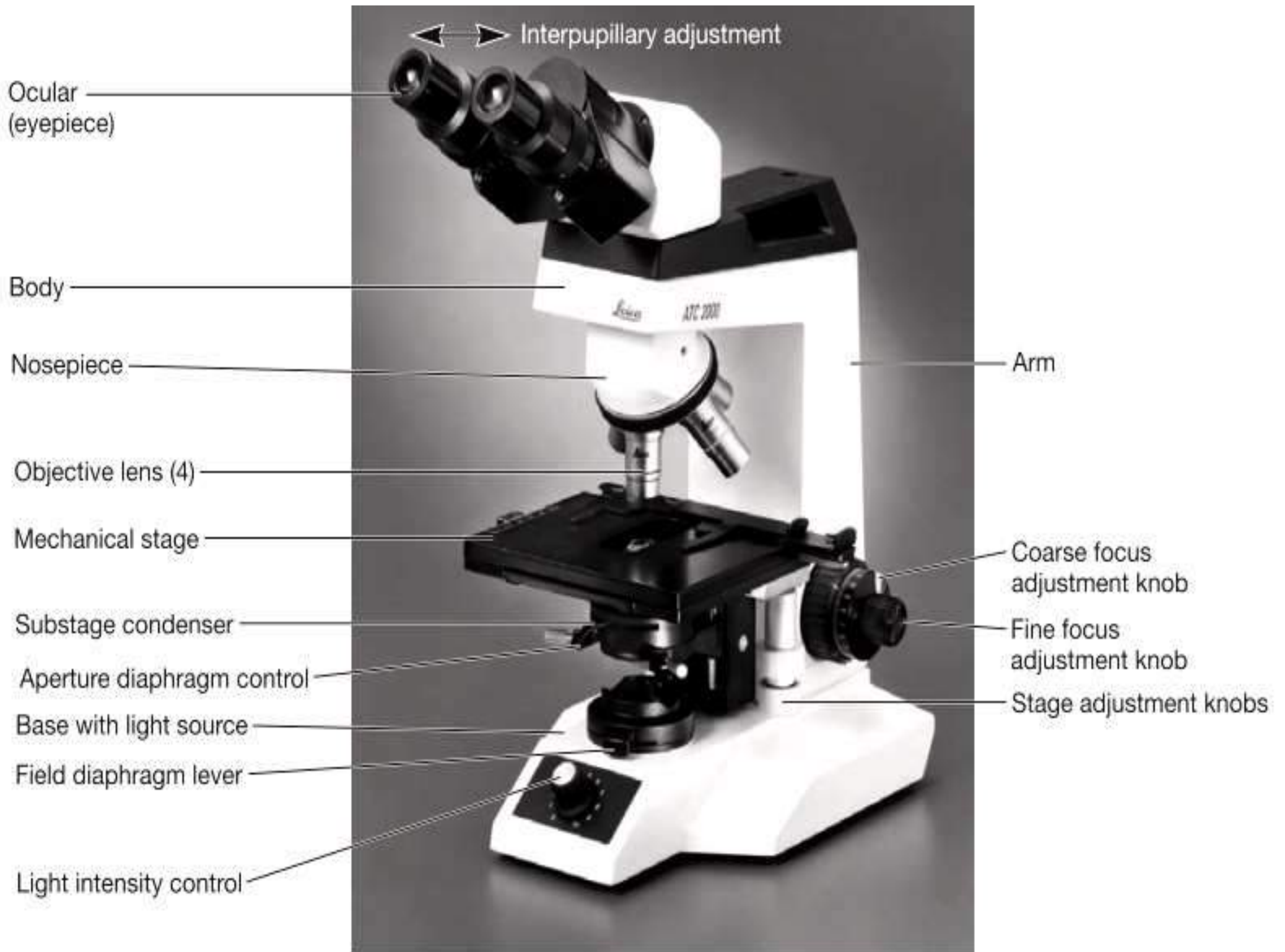
A **compound microscope** is a microscope which uses a lens close to the object being viewed to collect light (called the objective lens) which focuses a real image of the object inside the microscope (image 1). That image is then magnified by a second lens or group of lenses (called the eyepiece) that gives the viewer an enlarged inverted virtual image of the object (image 2).[5] The use of a compound objective/eyepiece combination allows for much higher magnification, reduced chromatic aberration and exchangeable objective lenses to adjust the magnification. A compound microscope also enables more advanced illumination setups, such as phase contrast.




Chromatic aberration

# OPTICAL PATH IN COMPOUND MICROSCOPE




Interpupillary adjustment
Ocular
(eyepiece)
Body
Nosepiece
Objective lens (4)
Mechanical stage
Substage condenser
Aperture diaphragm control
Base with light source
Field diaphragm lever
Light intensity control
Arm
Coarse focus
adjustment knob
Fine focus
adjustment knob
Stage adjustment knobs

## Parts of microscope

- Illuminator - This is the light source located below the specimen.
- Condenser - Focuses the ray of light through the specimen.
- Stage - The fixed stage is a horizontal platform that holds the specimen.
- Objective - The lens that is directly above the stage.
- Nosepiece - The portion of the body that holds the objectives over the stage.
- Iris diaphragm - Regulates the amount of light into the condenser.
- Base – Base supports the microscope which is horseshoe shaped.
- Coarse focusing knob - Used to make relatively wide focusing adjustments to the microscope.
- Fine focusing knob - Used to make relatively small adjustments to the microscope.
- Body - The microscope body.
- Ocular eyepiece - Lens on the top of the body tube. It has a magnification of 10× normal vision.

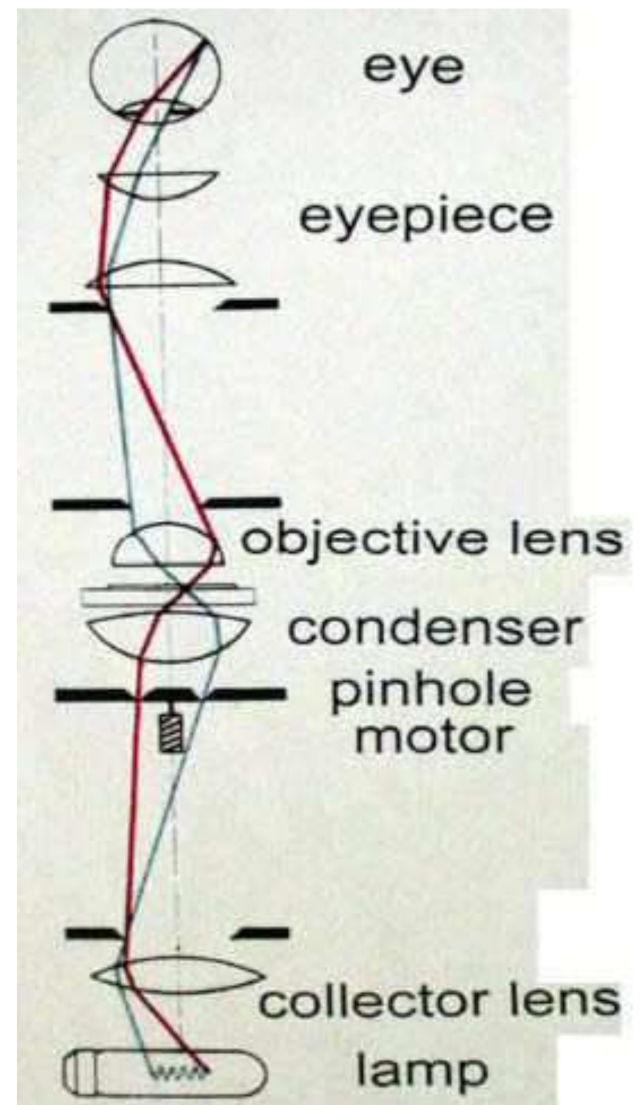
| | Objective | | |
|---|---|---|---|
| **PROPERTY** | **LOW POWER** | **HIGH POWER** | **OIL IMMERSION** |
| Magnification of objective | 10x | 40-45x | 90-100x |
| Magnification of eyepiece | 10x | 10x | 10x |
| Total magnification | 100x | 450 – 450x | 900 – 1000x |
| Numerical aperture | 0.25 – 0.30 | 0.55 – 0.65 | 1.25 – 1.4 |
| Mirror used | Concave | Concave | Plane |
| Focal length (Approx) | 16 mm | 4 mm | 1.8 – 2 mm |
| Working distance | 4 – 8 mm | 0.5 – 0.7 mm | 0.1 mm |
| Iris diaphragm | Partially closed | Partially opened | Fully opened |
| Position of condenser | Lowest | Slightly raised | Fully raised |
| Maximum resolution(Approx) | 0.9 μm | 0.35μm | 0.18μm |

# Bright Field Microscopy

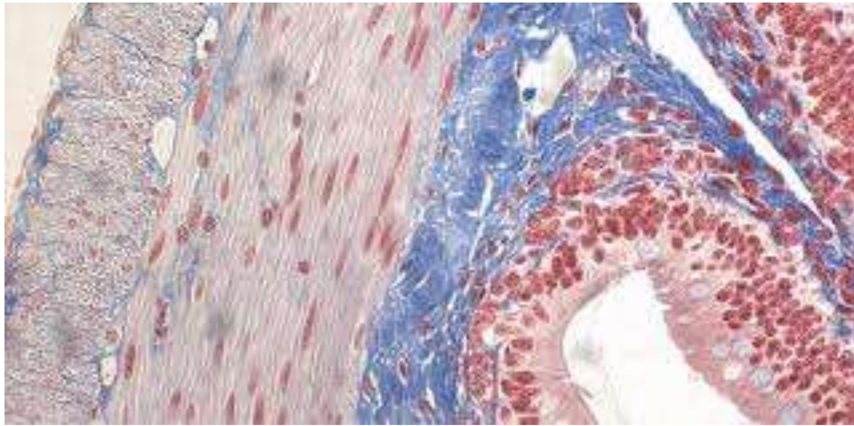
- Simplest optical microscopy illumination technique
- Uses visible light as source of illumination
    - *> the shorter the wavelength, the greater the resolution (blue is the best)*
- Contrast comes from absorbance of light in the sample, or from staining.
- When the diaphragm is wide open the image is brighter and contrast is low.

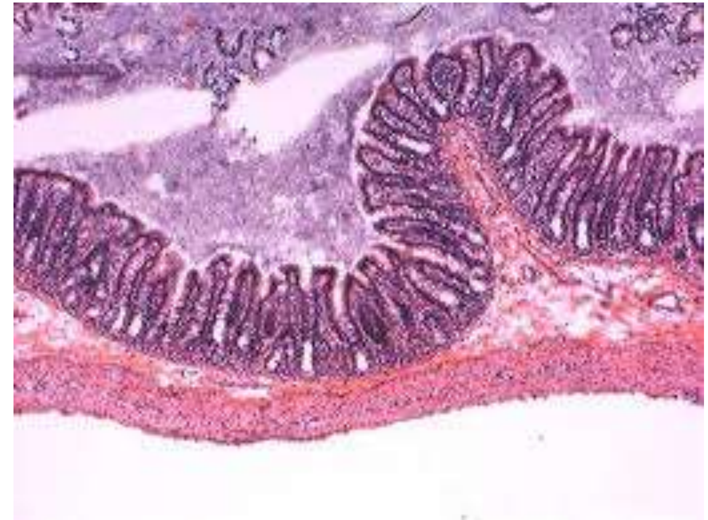
- Sources of illumination: *Lamp on the base*
- Types of image produced: *Relatively large internal structures and outline can be seen*
- Total Magnification: *(if 10x ocular magnification is used)*
  *Range: 10x-1000x*
- Resolution: *Up to 200nm (white light)*

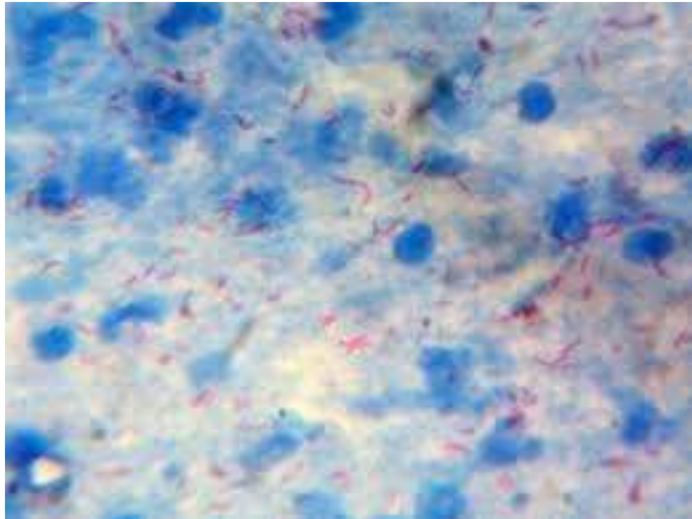
oculars
arm
revolving nosepiece
objective lenses
stage
iris diaphragm lever
stage manipulator knobs
coarse adjustment knob
fine adjustment knob
light source
base

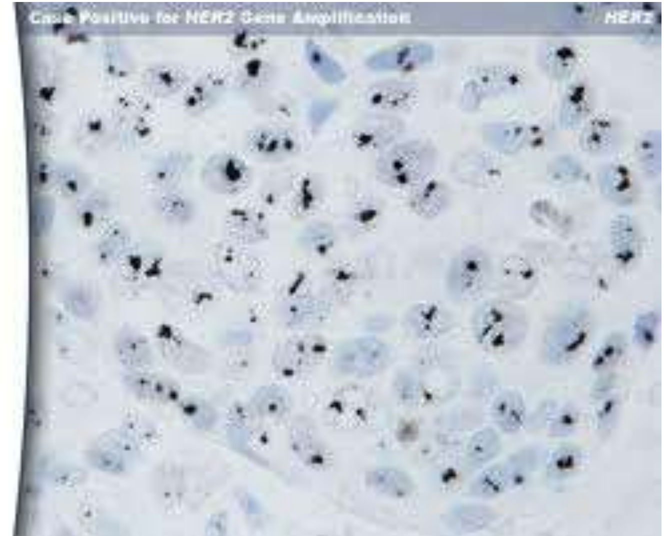



*Source: http://www.austincc.edu/biocr/1406/labm/ex3/prelab_3_8.htm*








Case Positive for HER2 Gene Amplification
HER2

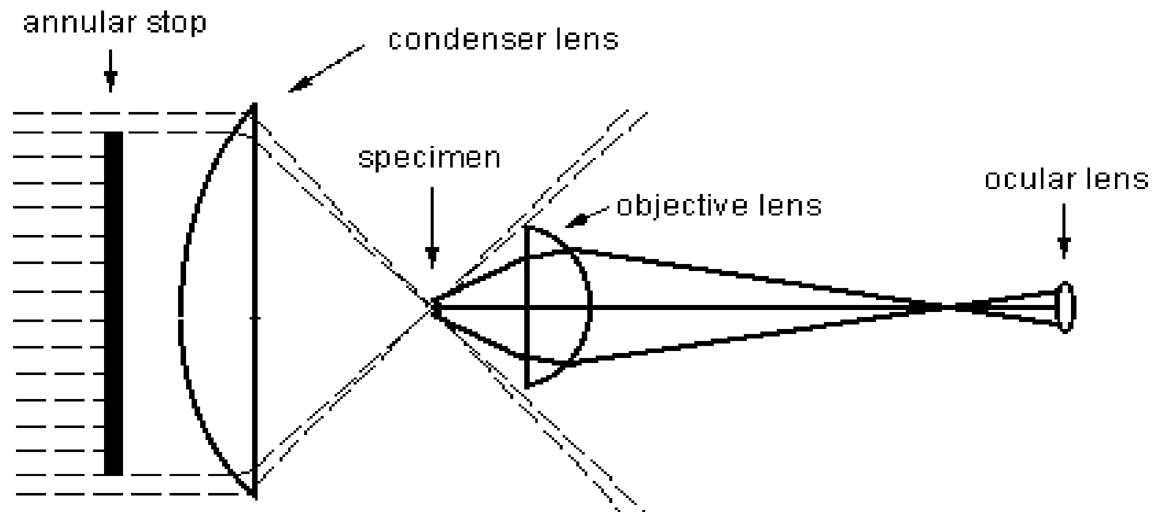
# Dark Field Microscopy

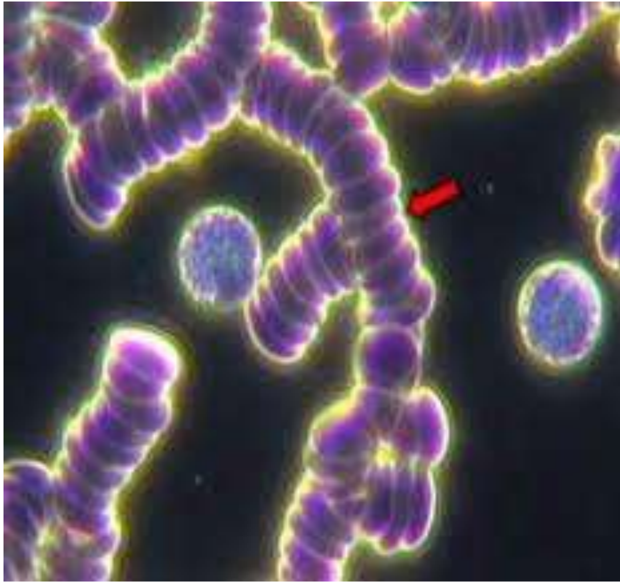
**Dark field microscopy** (dark ground microscopy) describes microscopy methods, in both light and electron microscopy, which exclude the unscattered beam from the image. As a result, the field around the specimen (i.e., where there is no specimen to scatter the beam) is generally dark.

- Type of microscopy which is the exact opposite of a bright field microscope
- Dark background/field with the specimen being the only one illuminated.
- Used in observing unstained specimens
- Most microscopes have the potential to do dark field microscopy such as compound or stereomicroscopes.

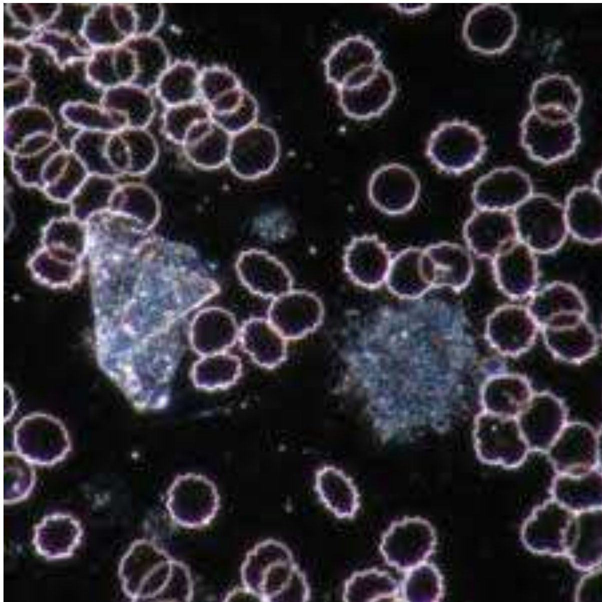
- Light source: Light bulb from a microscope
- Condenser type: Specialized to block most light from the source; contains an annular/patch stop which disperses the light in various directions, resulting to a “cone of light”
- Image formed: Dark background with illuminated specimen; may be inverted or not depending on microscope used

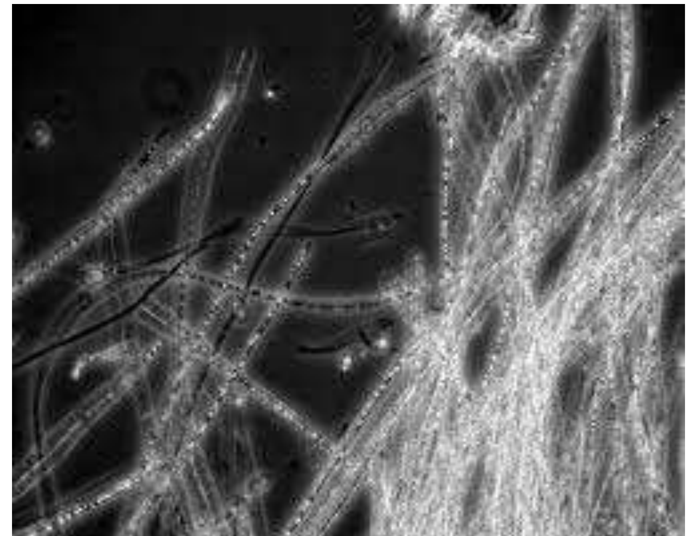
- Total Magnification: Can range from those of compound microscopes (10x to 1000x)





10 μm





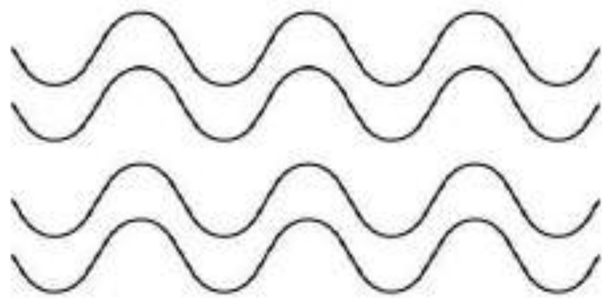
# Phase Contrast Microscopy

**Phase-contrast imaging** is a method of imaging that has a range of different applications. It exploits differences in the refractive index of different materials to differentiate between structures under analysis. In conventional light microscopy, phase contrast can be employed to distinguish between structures of similar transparency, and to examine crystals on the basis of their double refraction.

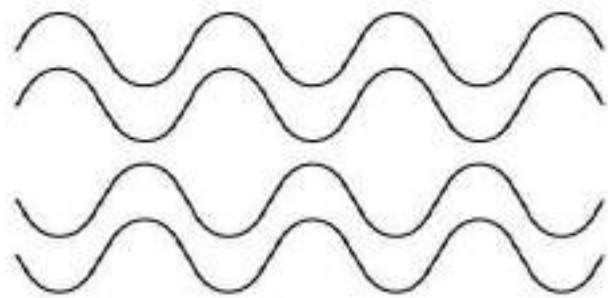
**Phase-contrast microscopy** is an optical-microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image.

# PRINCIPLE

- A unique part of the phase-contrast microscope, called the **phase-plate**, amplifies this change in phase to one-half wavelength
- When both the direct (undiffracted) and reflected (diffracted) types of light waves converge at the ocular lens, **constructive and destructive interference** occurs



Rays in phase



Rays out of phase

**(a)**

Phase plate

Bacterium

Ray deviated by specimen is 1/4 wavelength out of phase.

Deviated ray is now 1/2 wavelength out of phase.

**(b)**

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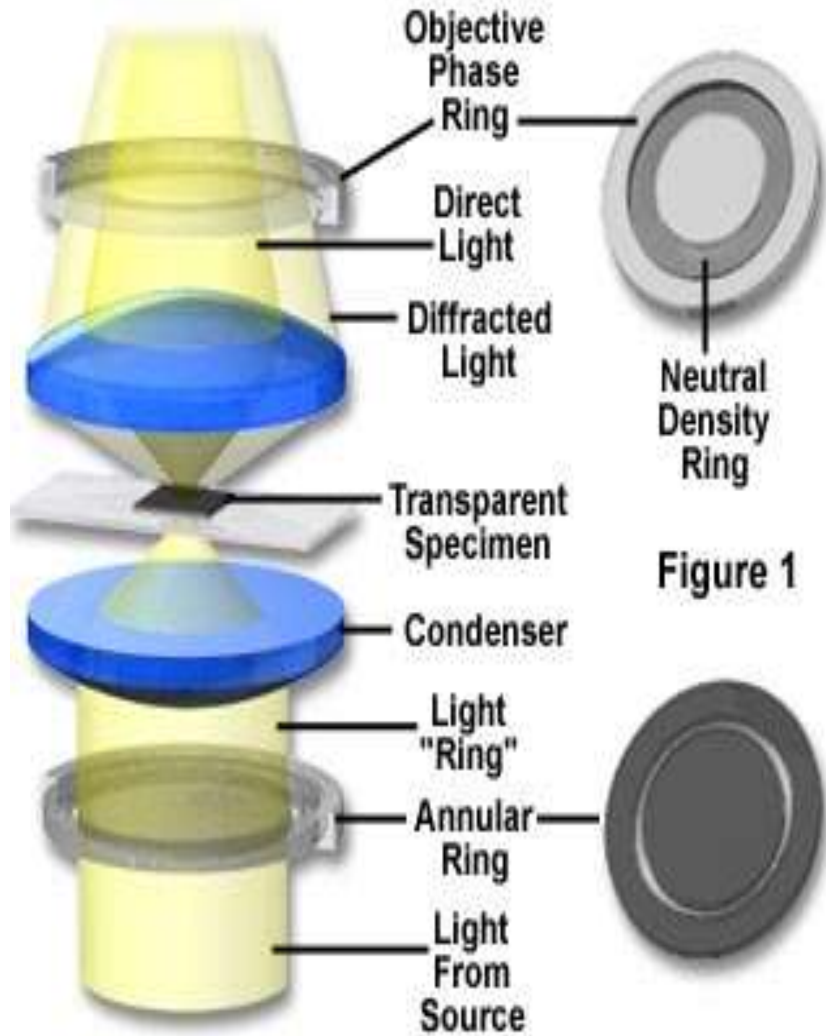



Image plane

Phase plate

Refracted or diffracted light (phase altered by specimen)

Objective lens

Unobstructed light (phase unaltered by specimen)

Specimen

Condenser lens

Annular diaphragm

In 1935 F.Zernike produced the phase contrast microscope. Phase-contrast microscope is also called as zernike microscope.

Phase-contrast microscope uses a special condenser and objective lenses.

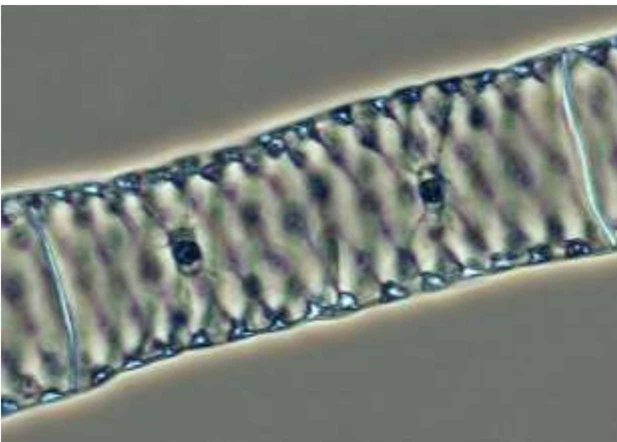
This condenser lens on the light microscope splits a light beam and throws the light rays slightly out of phase.

The separated beams of light then pass through and around the specimen, and small differences in the refractive index within the specimen show up as different degrees of brightness and contrast.

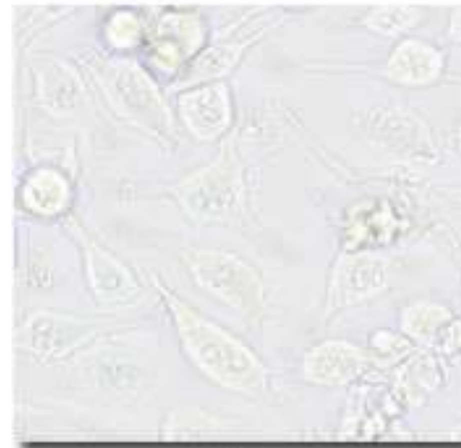
Uses:

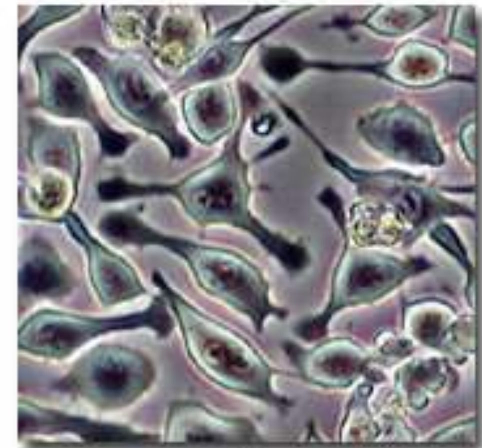
Phase-contrast microscopy is especially useful for studying microbial motility, studying eukaryotic Cells, determining the shape of living cells, and detecting bacterial components such as endospores and Inclusion bodies that contain poly--hydroxyalkanoates (e.g., poly-hydroxybutyrate), polymetaphosphate, sulfur, or other substances.

- Constructive interference corresponds to bright spots in the field of view
- Destructive interference corresponds to dark spots



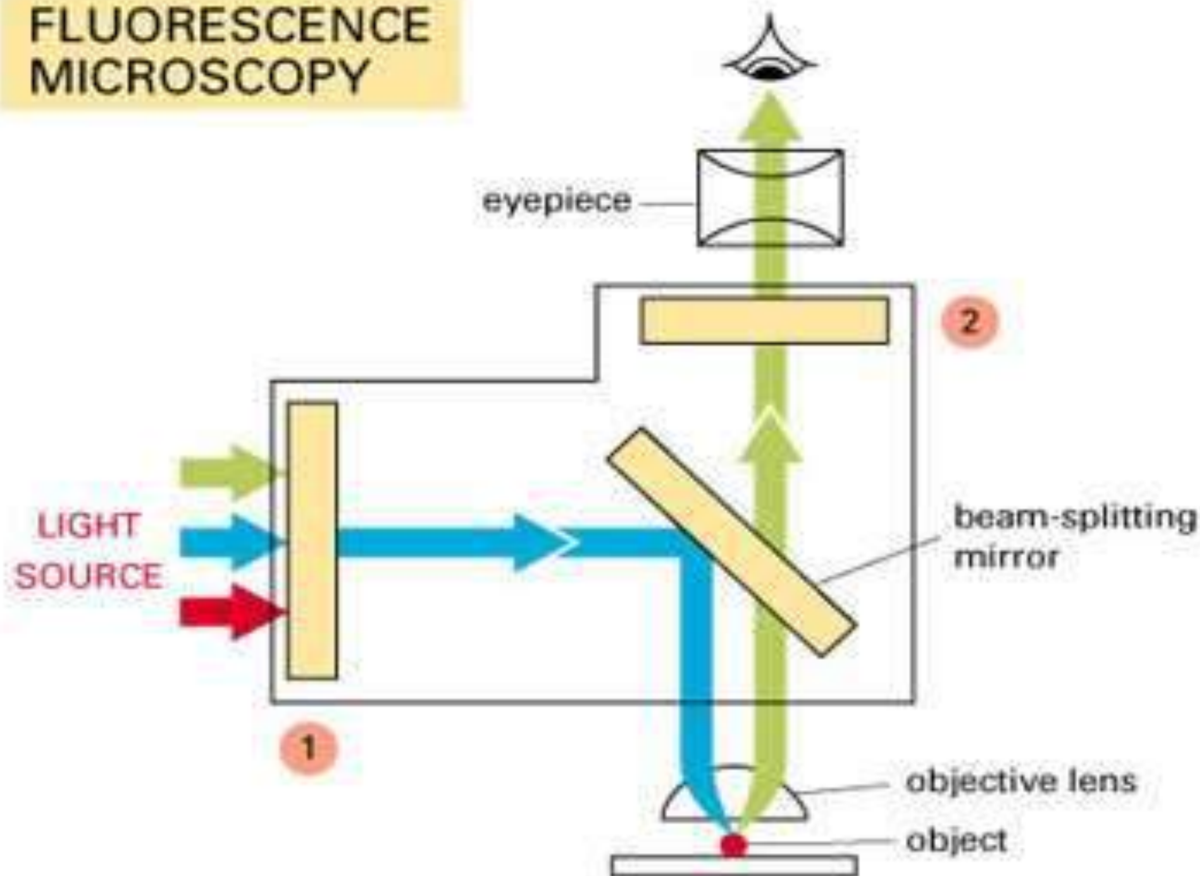
Living Cells in Brightfield and Phase Contrast





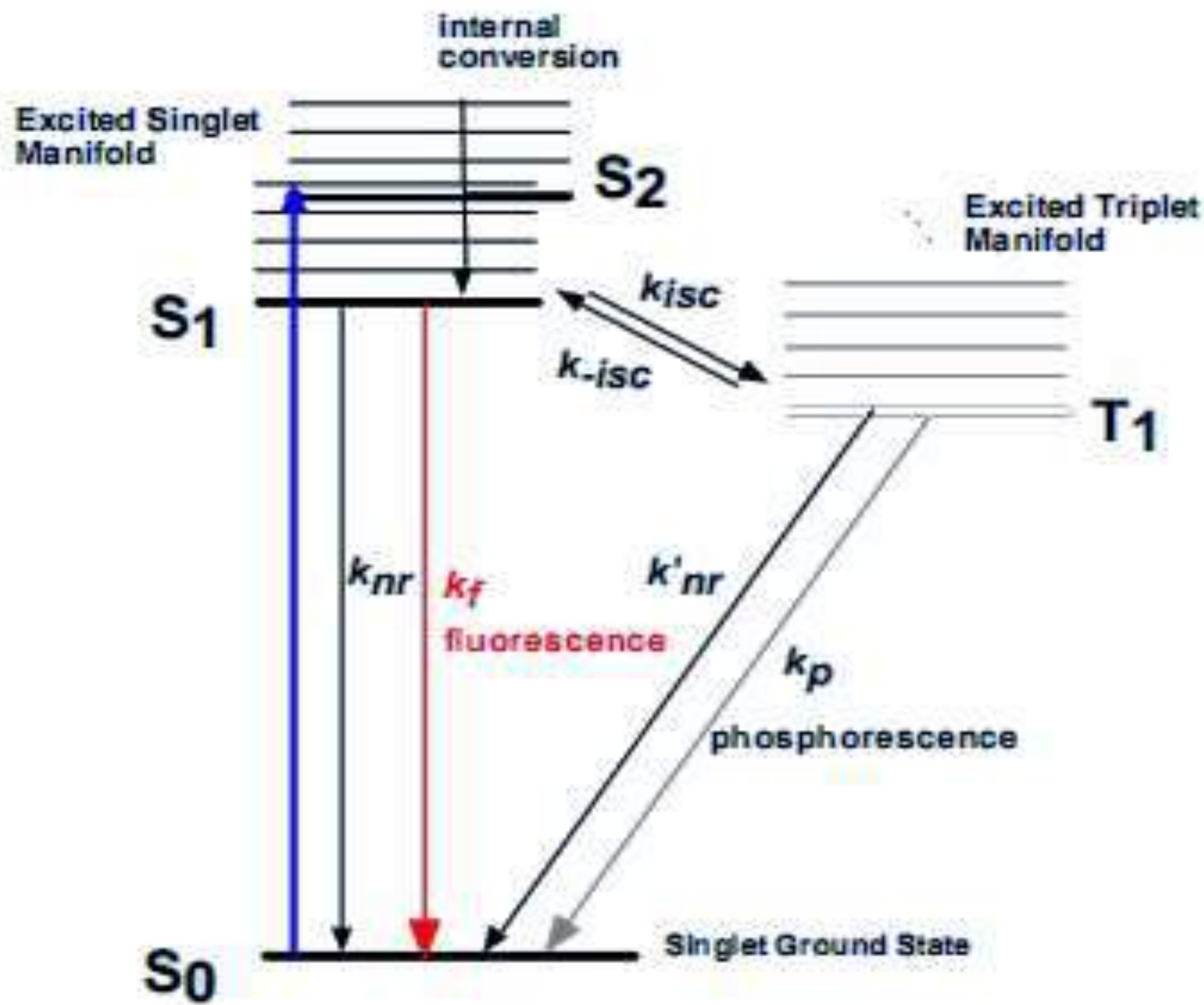
(a) (b)

Figure 2




Fluorescent dyes used for staining cells are detected with the aid of a *fluorescence microscope*. This is similar to an ordinary light microscope except that the illuminating light is passed through two sets of filters. The first ( 1 ) filters the light before it reaches the specimen, passing only those wavelengths that excite the particular fluorescent dye. The second ( 2 ) blocks out this light and passes only those wavelengths emitted when the dye fluoresces. Dyed objects show up in bright color on a dark background.

# Jablonski Diagram



# Fundamental Concepts Underpinning Fluorescence Microscopy

Jablonski Energy Diagram

Excitation (Absorption) $10^{-15}$ Seconds

Internal Conversion and Vibrational Relaxation ($10^{-14}$ - $10^{-11}$ Sec)

Fluorescence ($10^{-9}$ - $10^{-7}$ Sec)

Intersystem Crossing

Quenching

Non-Radiative Relaxation

Excited Singlet States

$S_2$ 5 4 3 2 1 0

Vibrational Energy States

Internal Conversion

$S_1$ 5 4 3 2 1 0

Delayed Fluorescence

Excited Triplet State ($T_1$) 5 4 3 2 1 0

Intersystem Crossing

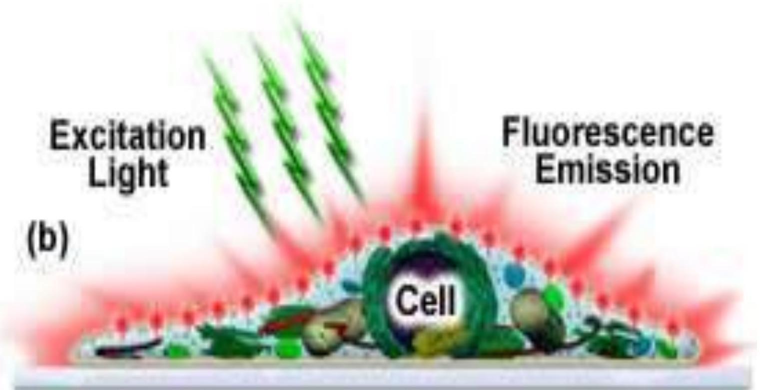
(a)

Non-Radiative Relaxation (Triplet)

Phosphorescence ($10^{-3}$ - $10^{2}$ Sec)

$S_o$ 5 4 3 2 1 0

Ground State



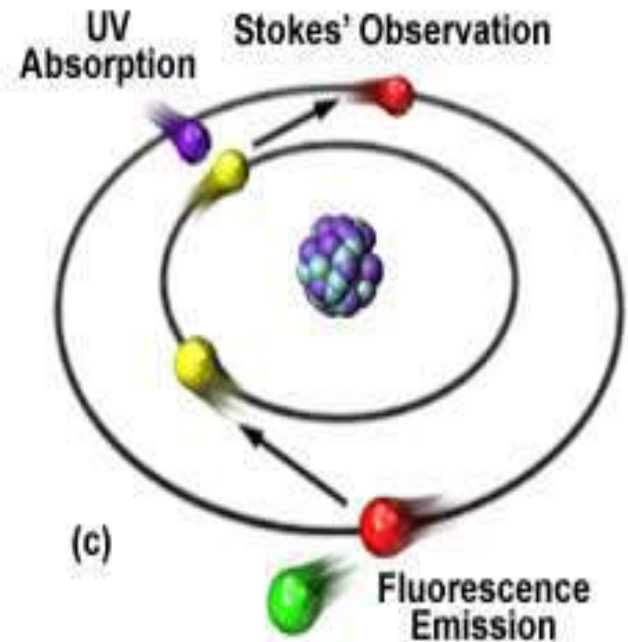




Figure 1

A fluorescence microscope is basically a conventional light microscope with added features and components that extend its capabilities.

**conventional microscope**

✓ uses light to illuminate the sample and produce a magnified image of the sample.

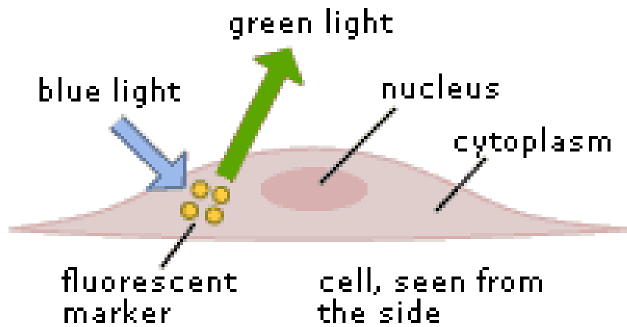
**fluorescence microscope**

✓ uses a much higher intensity light to illuminate the sample

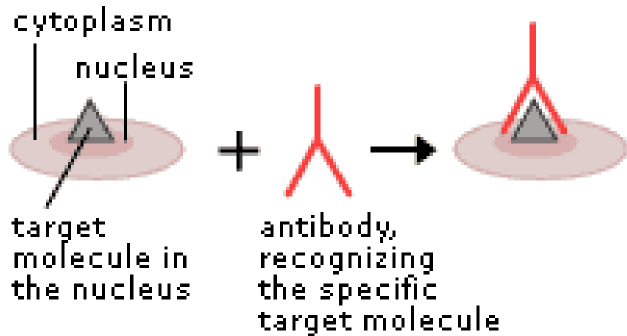
✓ This light excites fluorescence species in the sample, which then emit light of a longer wavelength.

✓ A fluorescent microscope also produces a magnified image of the sample, but the image is based on the second light source

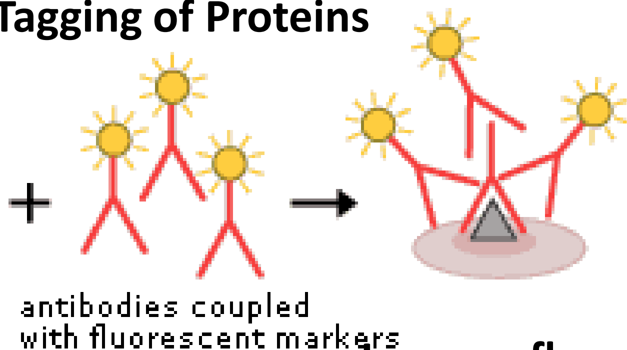
# Preparation of Specimen




**Fluorescent Dyes**




**Tagging of Proteins**




**Immunofluorescence**

## Techniques

### Fluorescent Dyes

- taken up by the cells
- incorporated and concentrated in specific subcellular compartments
- living cells are then mounted on a microscope slide and examined in a fluorescence microscope.

### Tagging of Proteins

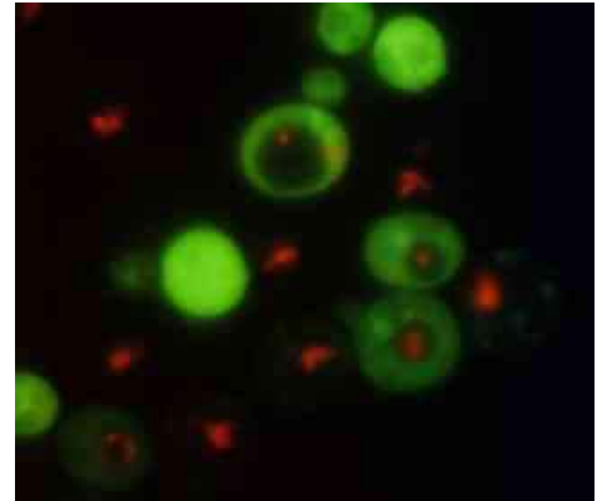
- modify cells so that they create their own fluorescing molecules
- the location of that protein can be studied. It is also possible to watch the movements of the proteins and its interactions with other cellular components inside the cell

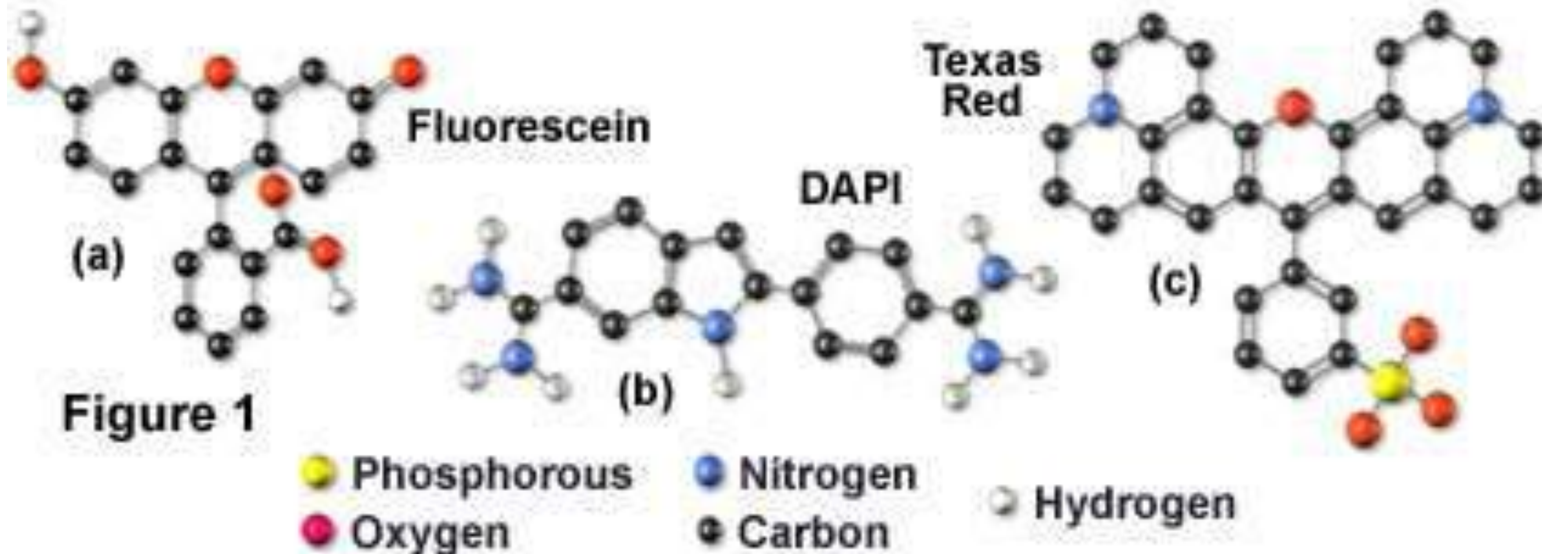
### Immunofluorescence

- use of antibodies to which a fluorescent marker has been attached.
- recognize and bind selectively to specific target molecules in the cell

# TYPES OF FLUOROPHORES USED

- fluorescein,
- DAPI,
- propidium iodide
- green fluorescent protein (GFP)
- Texas Red

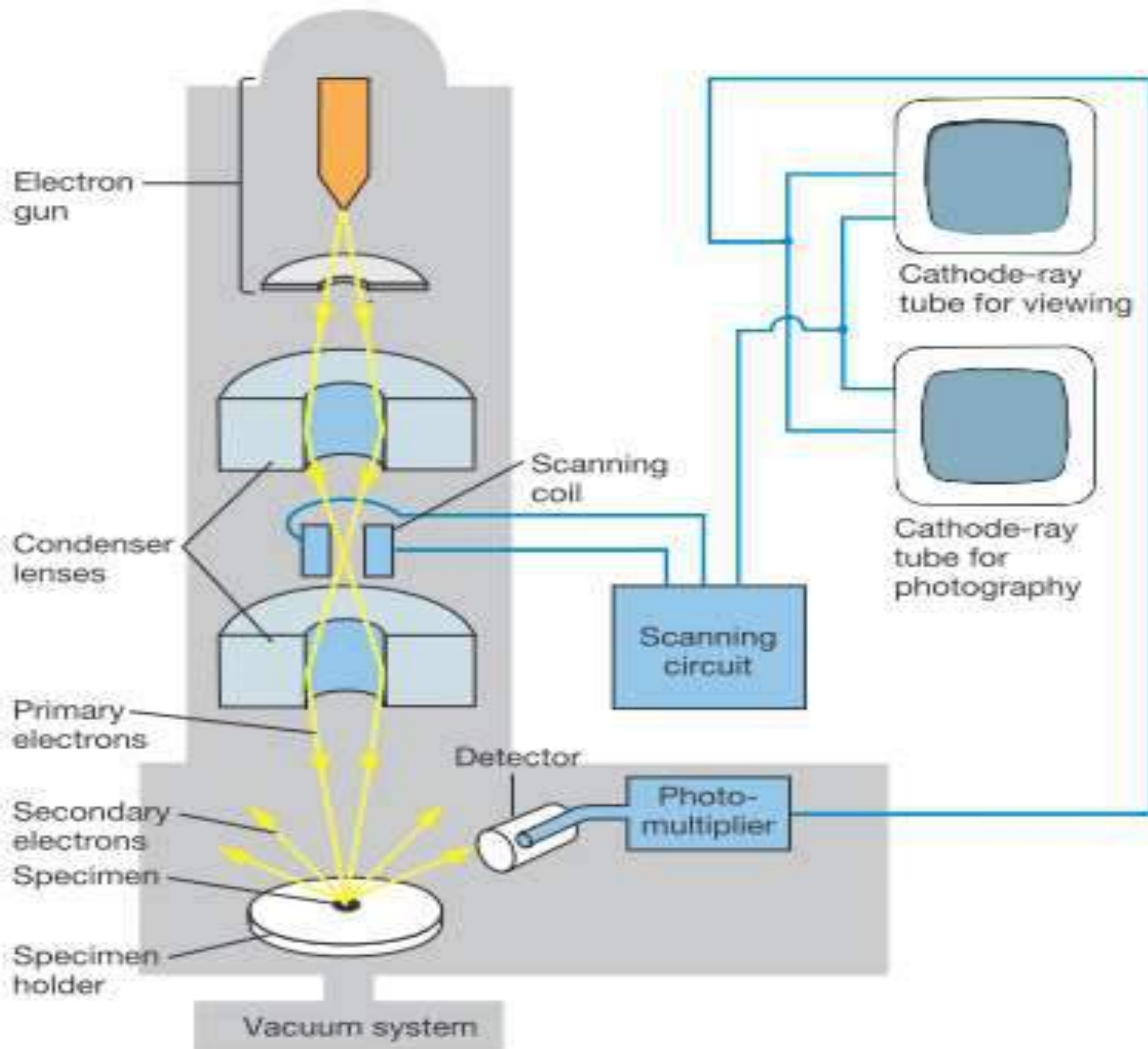




# Electron Microscopy

# Scanning Electron Microscope

- Illumination: electrons
- Magnification: ~100,000x
- How it works: Detect electrons back-scattered by the sample.
- Image: Monotone (but may be color enhanced), 3-D surface of specimen


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

## Pros

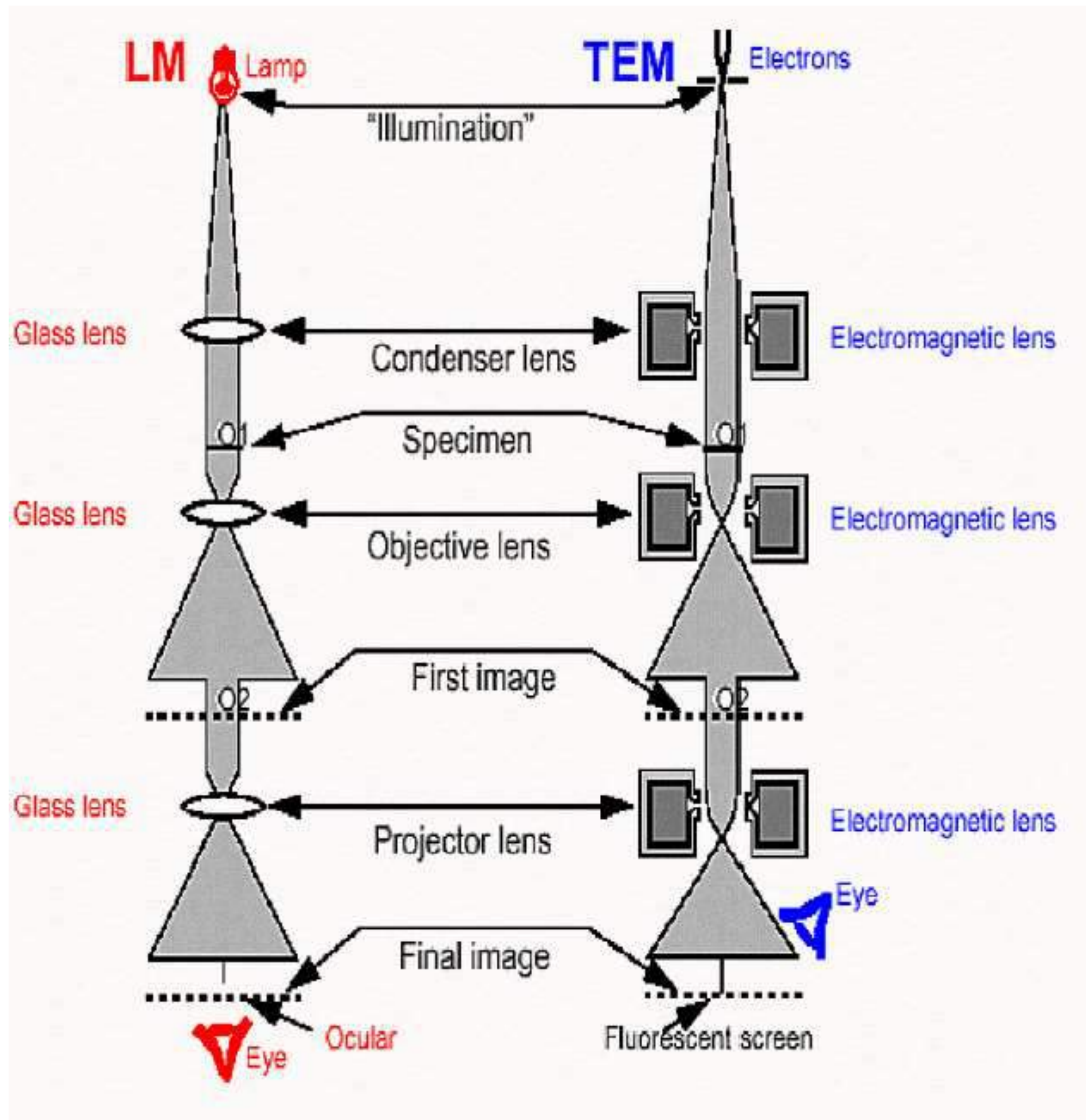
- High magnification
- High resolution
- Shows the surface of specimen

## Cons

- Needs specimen to be in vacuum
- Needs living cells and tissues and whole, soft-bodied organisms to be treated, usu. coated w/ gold film
- No color
- Cannot examine live specimen
- Really. Big. And Expensive. Equipment.

# Transmission Electron Microscope

- Illumination: electrons
- Magnification: ~100,000x
- How it works: Detect electrons scattered as they move through the sample.
- Image: Monotone (but may be color enhanced), 2-D structure of specimen




Source: http://www.lab.anhb.uwa.edu.au/hb313/main_pages/timetable/lectures/Image6.gif

## Pros

- High magnification
- High resolution
- Shows small structures that cannot be seen under light microscopes

## Cons

- Needs specimen to be in vacuum
- Needs specimen to be covered in gold film
- Specimen <100nm thick (obviously cannot observe live specimen)
- No color
- Really. Big. And. Expensive. Equipment

# COATING OF SAMPLES

- Coating of samples is required in the field of electron microscopy to enable or improve the imaging of samples.

- Creating a conductive layer of metal on the sample inhibits charging, reduces thermal damage and improves the secondary electron signal required for topographic examination in the SEM.

- Fine carbon layers, being transparent to the electron beam but conductive, are needed for x-ray microanalysis, to support films on grids and back up replicas to be imaged in the TEM.

- The coating technique used depends on the resolution and application.

# Light Vs Electron microscope

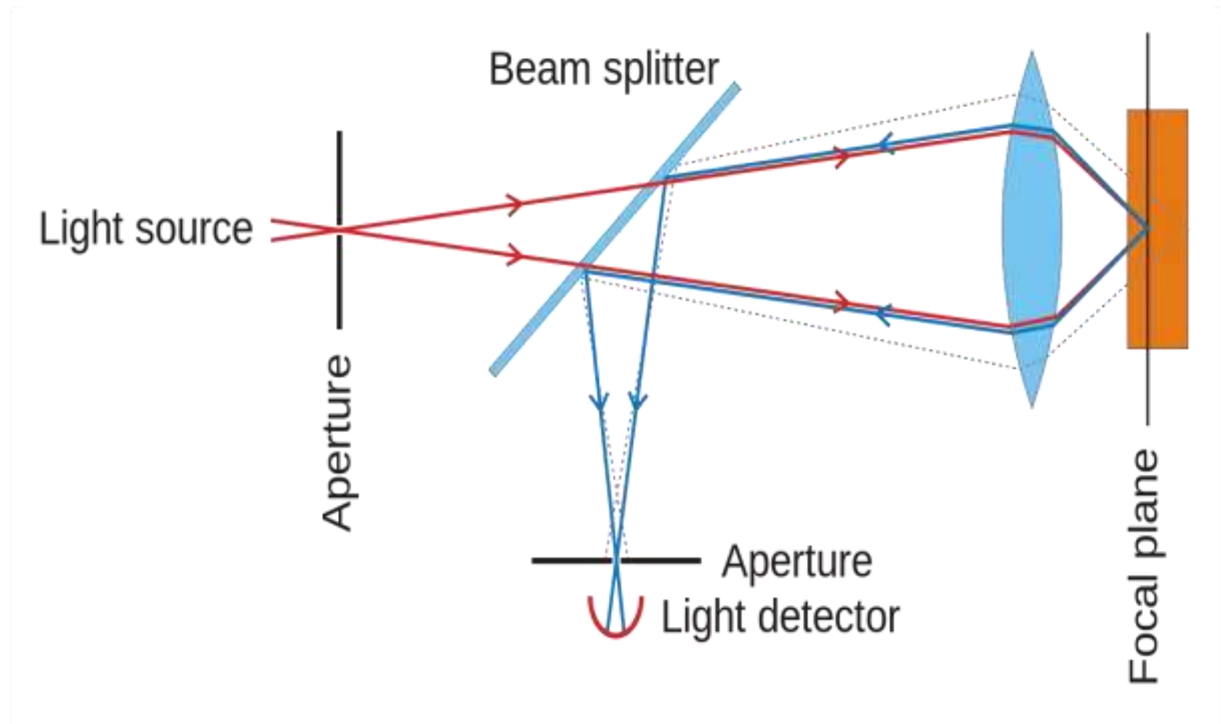
| Characteristic | Light or Optical | Electron (Transmission) |
|---|---|---|
| Useful magnification | 2,000× | 1,000,000 or more |
| Maximum resolution | 200 nm | 0.5 nm |
| Image produced by | Visible light rays | Electron beam |
| Image focused by | Glass objective lens | Electromagnetic objective lenses |
| Image viewed through | Glass ocular lens | Fluorescent screen |
| Specimen placed on | Glass slide | Copper mesh |
| Specimen may be alive | Yes | No |
| Specimen requires special stains or treatment | Not always | Yes |
| Colored images produced | Yes | No |

| Microscope | Important Features |
|---|---|
| **Visible light as source of illumination** | |
| Bright-field | Common multipurpose microscope for live and preserved stained specimens; specimen is dark, field is white; provides fair cellular detail |
| Dark-field | Best for observing live, unstained specimens; specimen is bright, field is black; provides outline of specimen with reduced internal cellular detail |
| Phase-contrast | Used for live specimens; specimen is contrasted against gray background; excellent for internal cellular detail |
| **Ultraviolet rays as source of illumination** | |
| Fluorescent | Specimens stained with fluorescent dyes or combined with fluorescent antibodies emit visible light; specificity makes this microscope an excellent diagnostic tool |
| **Electron beam forms image of specimen** | |
| Transmission electron microscope (TEM) | Sections of specimen are viewed under very high magnification; finest detailed structure of cells and viruses is shown; used only on preserved material |
| Scanning electron microscope (SEM) | Scans and magnifies external surface of specimen; produces striking three-dimensional image |

# CONFOCAL MICROSCOPY

**Confocal microscopy** is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light.[1]

It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.



## Basic concept of confocal Microscopy

The principle of confocal imaging was patented in 1957 by Marvin Minsky[2][3] and aims to overcome some limitations of traditional wide-field fluorescence microscopes.

In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source.

All parts of the specimen in the optical path are excited at the same time and the resulting fluorescence is detected by the microscope photodetector or camera including a large unfocused background part.

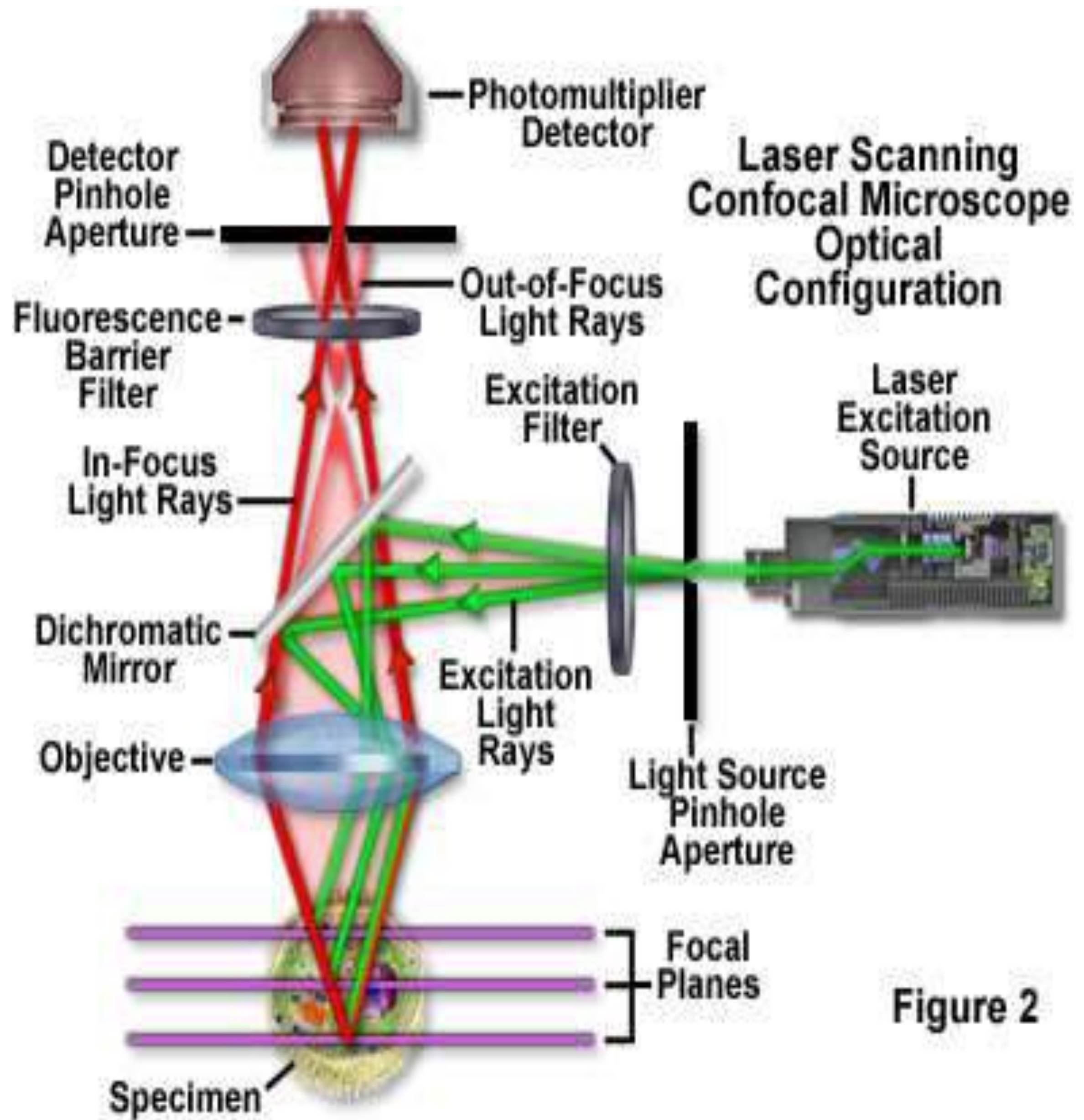
In contrast, a confocal microscope uses point illumination (see Point Spread Function) and a pinhole in an optically conjugate plane in front of the detector to **eliminate out-of-focus signal** - the name "confocal" stems from this configuration. As **only light produced by fluorescence very close to the focal plane can be detected**.

# Principle of confocal microscopy

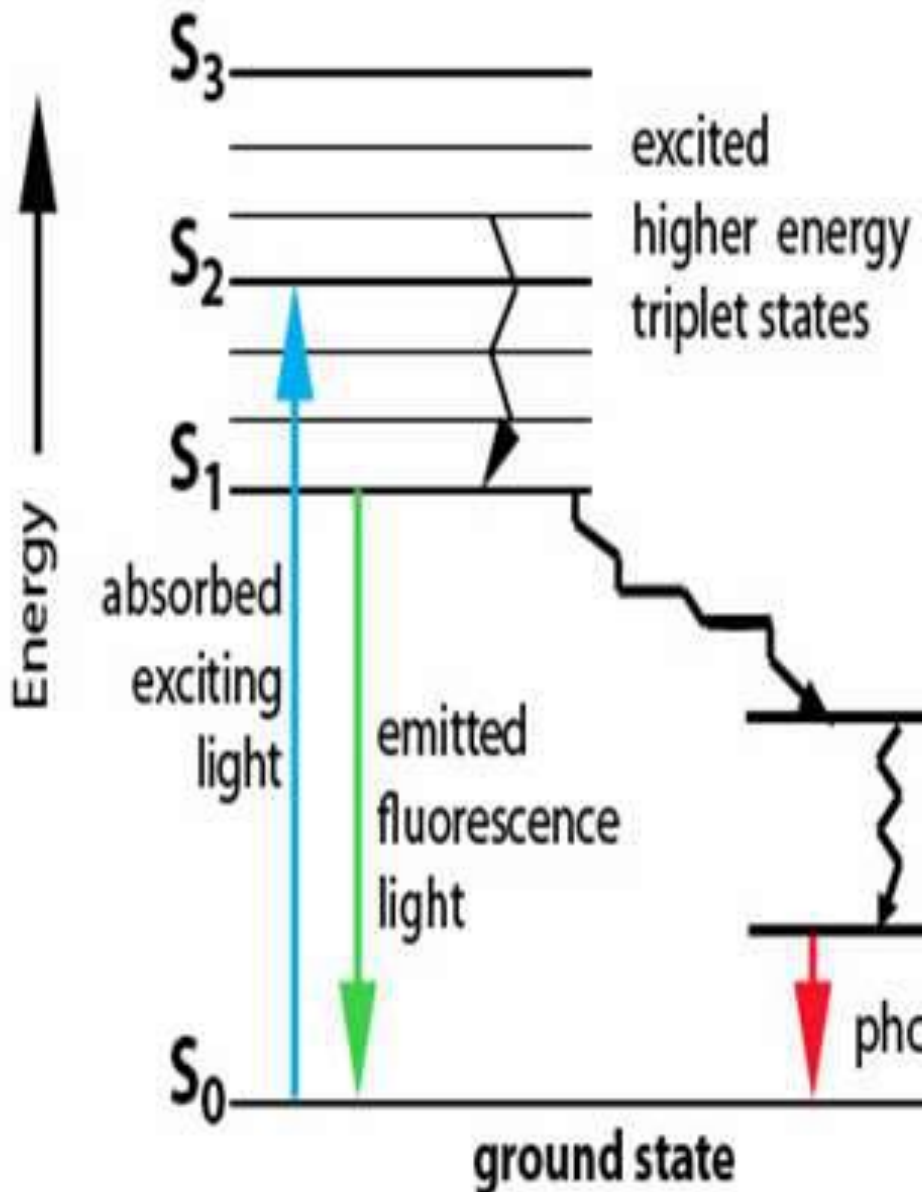
In confocal microscopy two pinholes are typically used:

- A pinhole is placed in front of the illumination source to allow transmission only through a small area
- This illumination pinhole is imaged onto the focal plane of the specimen, i.e. only a point of the specimen is illuminated at one time
- Fluorescence excited in this manner at the focal plane is imaged onto a confocal pinhole placed right in front of the detector
- Only fluorescence excited within the focal plane of the specimen will go through the detector pinhole
- Need to scan point onto the sample

Dye in the specimen is excited by the laser light & fluoresces. The fluorescent (green) light is descanned by the same mirrors that are used to scan the excitation (blue) light from the laser beam→ then it passes through the dichoric mirror→ then it is focused on to pinhole→ the light passing through the pinhole is measured by the detector such as photomultiplier tube. For visualization, detector is attached to the computer, which builds up the image at the rate of 0.1-1 second for single image.



# Alexander Jablonski Diagram




- Light from the excitation filter excites the fluorochoromes to a higher energy state
- From the high state it declines slowly releasing energy
- Transition between absorption & emission

# Importance

Better resolution
Cells can be live or fixed
Serial optical sections can be collected

A confocal microscope creates **sharp** images of a specimen that would appear otherwise blurred with the conventional microscope –this is achieved by excluding most of the light from the specimen, but not from the microscope's focal plane.

The image obtained has better contrast & less hazy .

In confocal microscopy, a series of thin slices of the specimen is assembled to generate a 3-dimensinal image.

The specimen is everywhere illuminated axially, rather than at different angles, thereby avoiding optical aberrations--→entire field of view is illuminated uniformly.

The field of view can be made larger than that of the static objective by controlling the amplitude of the stage movements.

# HOW DOES A CONFOCAL MICROSCOPE WORK

Confocal microscope incorporates 2 ideas :

1. Point-by-point illumination of the specimen.
2. Rejection of out of focus of light.

Light source of very high intensity is used—Zirconium arc lamp in Minsky's design & laser light source in modern design.

1. Laser provides intense blue excitation light.
2. The light reflects off a dichoric mirror, which directs it to an assembly of vertically and horizontally scanning mirrors.
3. These motor driven mirrors scan the laser beam across the specimen.
4. The specimen is scanned by moving the stage back & forth in the vertical & horizontal directions and optics are kept stationary.

# LIMITATIONS OF CONFOCAL MICROSCOPY

**1.Resolution** : It has inherent resolution limitation due to diffraction. Maximum best resolution of confocal microscopy is typically about 200nm.

**2.Pin hole size** : Strength of optical sectioning depends on the size of the pinhole.

**3.Intensity of the incident light**.

**4.Fluorophores :**

a)The fluorophore should tag the correct part of the specimen.

b)Fluorophore should be sensitive enough for the given excitation wave length.

C)It should not significantly alter the dynamics of the organism in the living specimen.

**5.Photobleaching**

**References**


1. Satyanarayana, U. “Biochemistry” 5th Edition, Elsevier India 2017, ISBN: 9788131249406
2. Voet D, Voet J. .G “Biochemistry”, 4th Edition, John Wiley & Sons, Inc.,2010. ISBN: 978-0-470-57095-1
3. David L. Nelson; Michael M. Cox “Lehninger Principles of Biochemistry” Seventh Edition, Macmillan 2017, ISBN:9781464187957
4. Wilson, K., Walker, J. (eds.); Cambridge University Press, Cambridge, 2000, 784 pp., ISBN 0-521-65873-X (paperback)



**All the original contributors of the concept and findings published are gratefully acknowledged while preparing the e-content for the students of Biochemistry and allied sciences**