BT 601: Analytical Biotechnology

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Lec-14

Microscopic Techniques

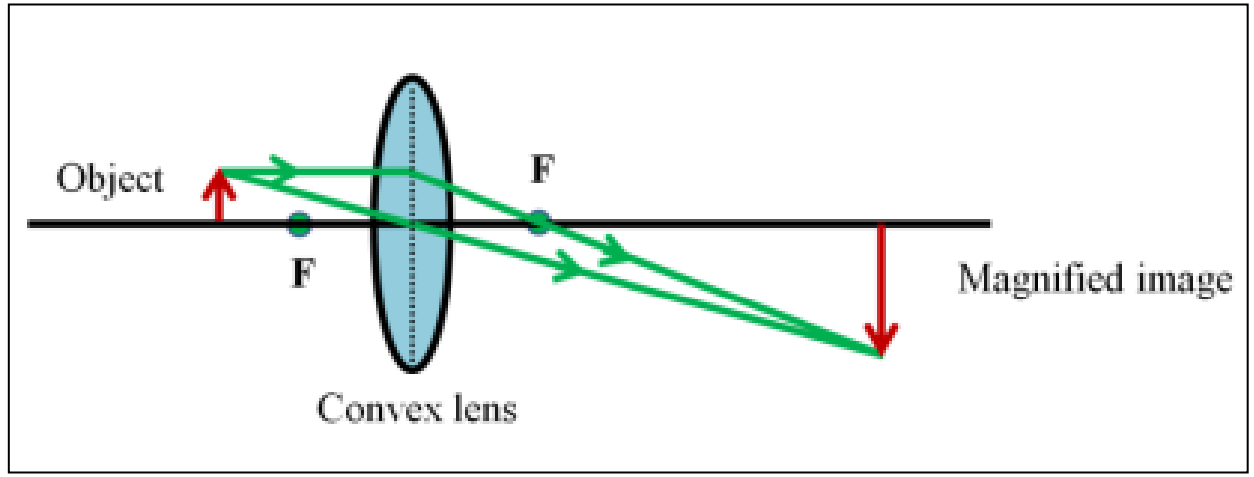
Light microscopy
Fluorescence microscopy
Confocal microscopy

Simple microscope

- **Microscopy** comprises of the tools that are used to see/image the microscopic objects and even macromolecules.
- **Light microscopy is the simplest form of microscope** that use **electromagnetic radiation** to achieve magnification.
- Light microscopy uses **glass** for bending and focusing the light.

A **convex lens is the simplest microscope**.

Example of simple microscope is **a magnifying glass that has a double convex lens with a short focal length**.



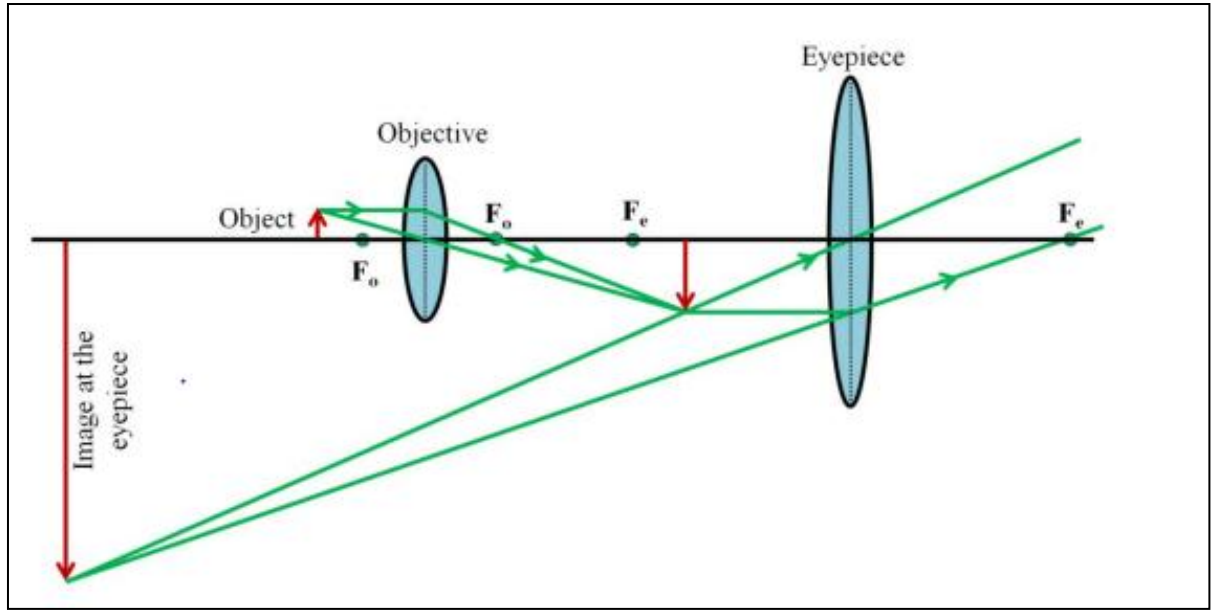
Magnification of an object by convex lens

Compound microscope

A microscope that uses **two lenses** to generate the magnified image of the object is called a **compound microscope**.

- The magnified image generated by one lens is further magnified by the second lens.
- Magnification of a compound microscope is the product of the **magnification caused** by the **objective and ocular (eyepiece) lenses:**

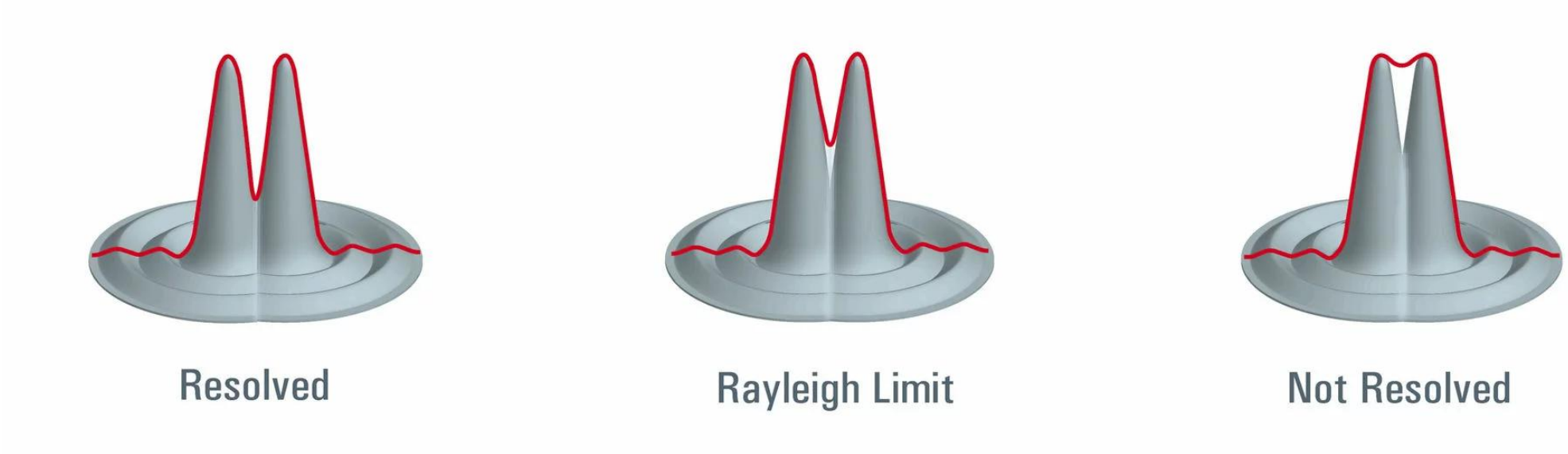
$$M_{final} = M_{objective} \times M_{ocular}$$



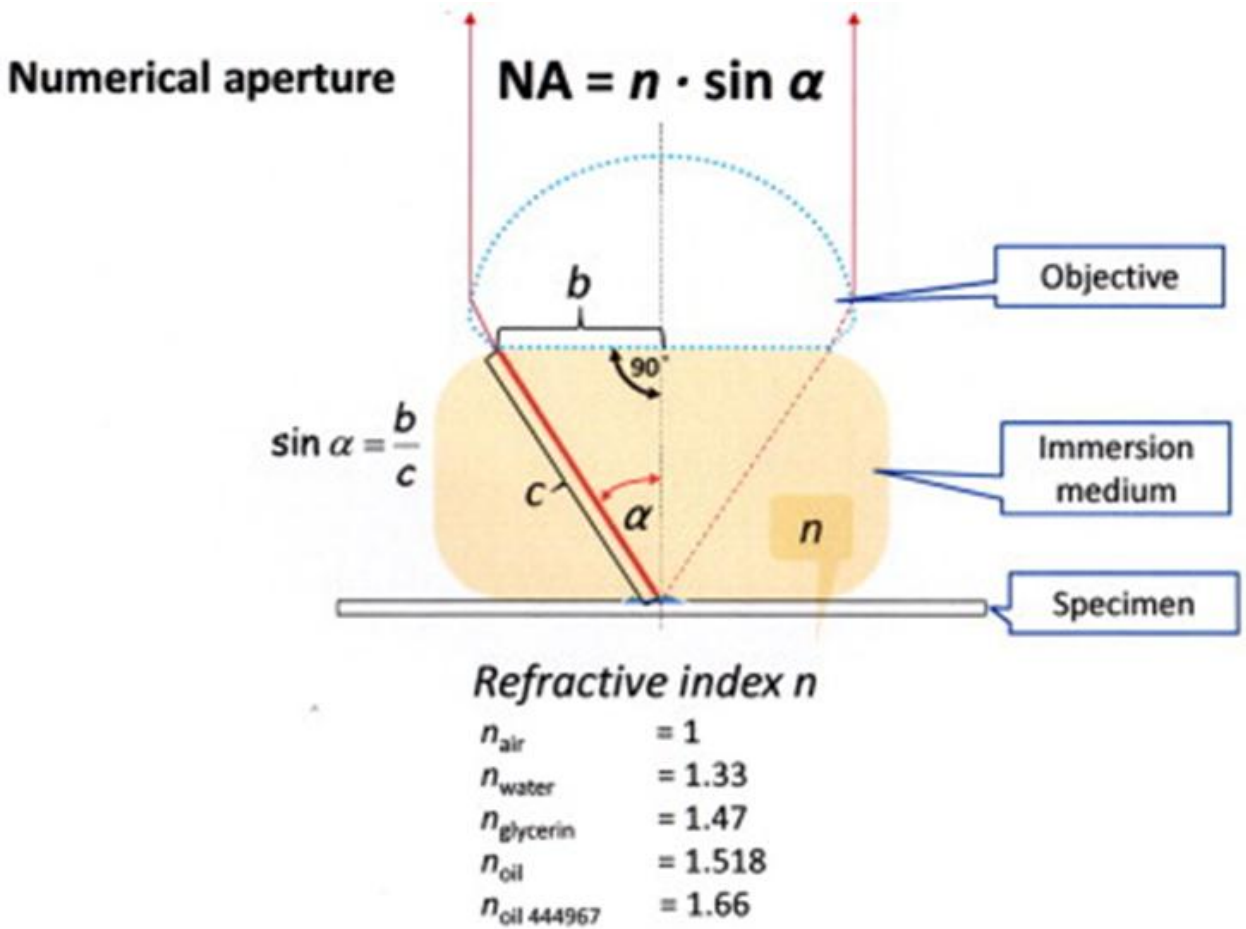
Ray optical diagram of a compound microscope

Resolution of microscope and Numerical Aperture

‘Resolution' is used to describe the minimum distance at which two distinct points of a specimen can still be observed.



Numerical Aperture (NA) is a dimensionless number that characterizes the range of angles over which the system can accept or emit light.



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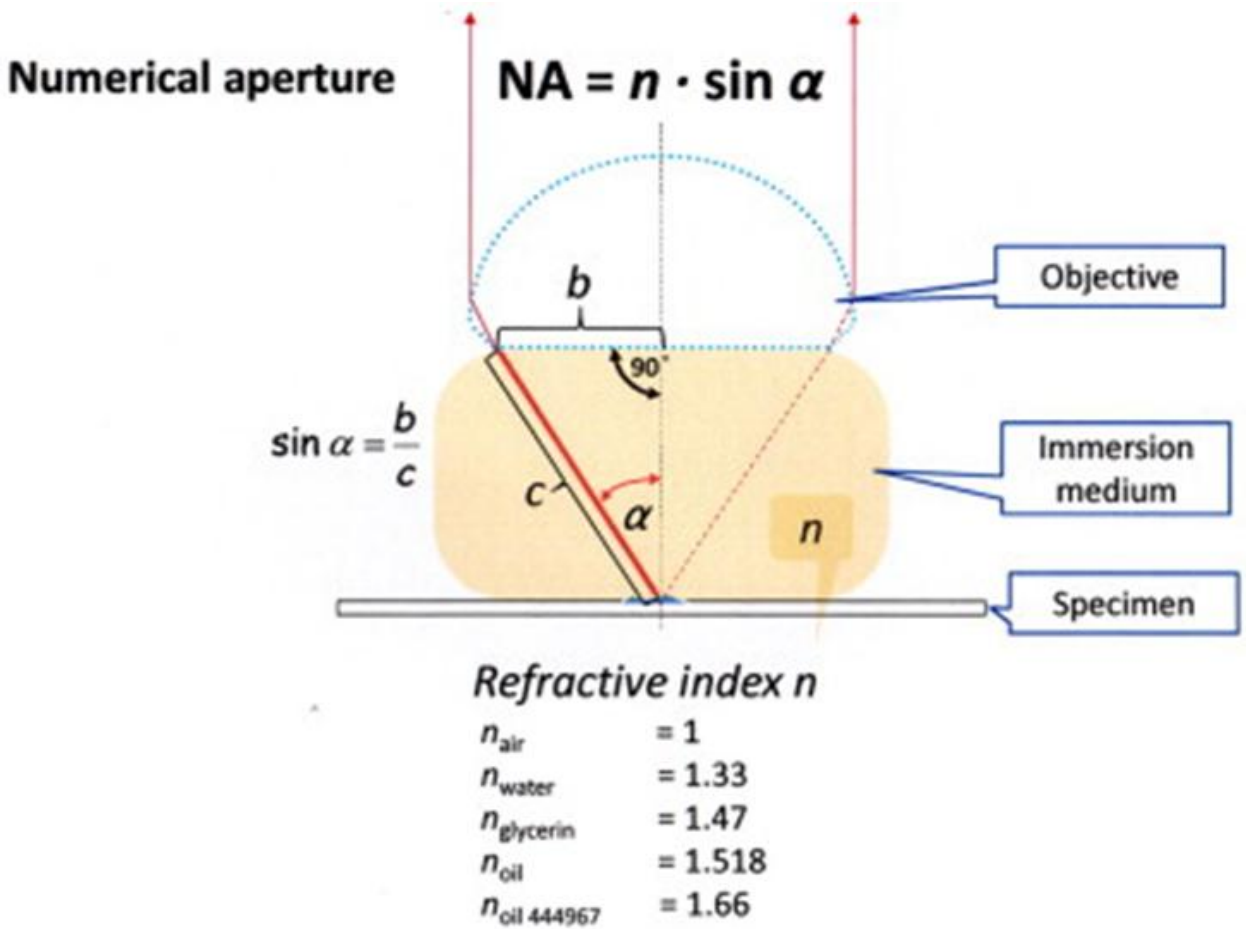


Resolution of microscope and Numerical Aperture

- Numerical aperture (NA) is related to the refractive index (n) of a medium through which light passes as well as the angular aperture (α) of a given objective (NA = n sinα).
- The resolution of an optical microscope is not solely dependent on the NA of an objective, but the NA of the whole system, taking into account the NA of the microscope condenser.
- Resolution is also related to the wavelength of light which is used to image a specimen; light of shorter wavelengths are capable of resolving greater detail than longer wavelengths.

There are 3 mathematical concepts which need to be taken into consideration when dealing with resolution:

- Airy discs
- Abbe's diffraction limit
- Rayleigh criterion

George Biddell Airy and 'Airy Discs' (1835)

- Airy wrote this paper very much from the view of an astronomer and in it he describes "the form and brightness of the rings or rays surrounding the image of a star as seen in a good telescope".
- An Airy disc is the optimally focused point of light which can be determined by a circular aperture in a perfectly aligned system limited by diffraction. Viewed from above **(Figure 1),** this appears as a bright point of light around which are concentric rings or ripples (more correctly known as an Airy Pattern)
- There are of course many points of light in a specimen as viewed with a microscope, and it is more appropriate to think in terms of numerous Airy patterns as opposed to a single point of light as described by the term '**Airy disc'.**

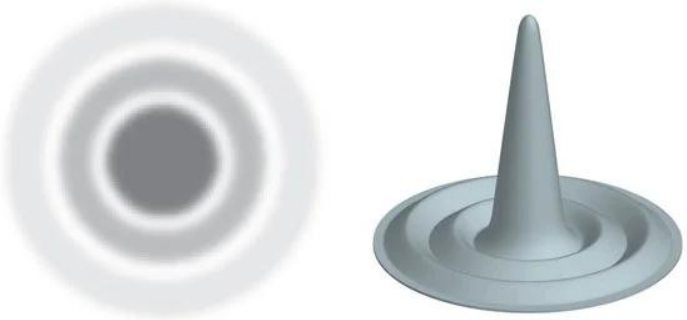


Figure 1: Typical phenomenon of an Airy Pattern, also known as Airy Disc, with its central maximum point of light and the encircling diffractive rings.

Ernst Abbe and 'Abbe's Diffraction Limit' (1873)

- In 1873, Abbe published his theory and formula which explained the diffraction limits of the microscope.
- Abbe recognized that specimen images are composed of a multitude of overlapping, multi-intensity, diffraction-limited points **(or Airy discs).**
- In order to increase the resolution, **$d = \lambda/(2NA)$,** the specimen must be viewed using either a shorter wavelength (λ) of light or through an imaging medium with a relatively high refractive index or with optical components which have a high NA (or, indeed, a combination of all of these factors).

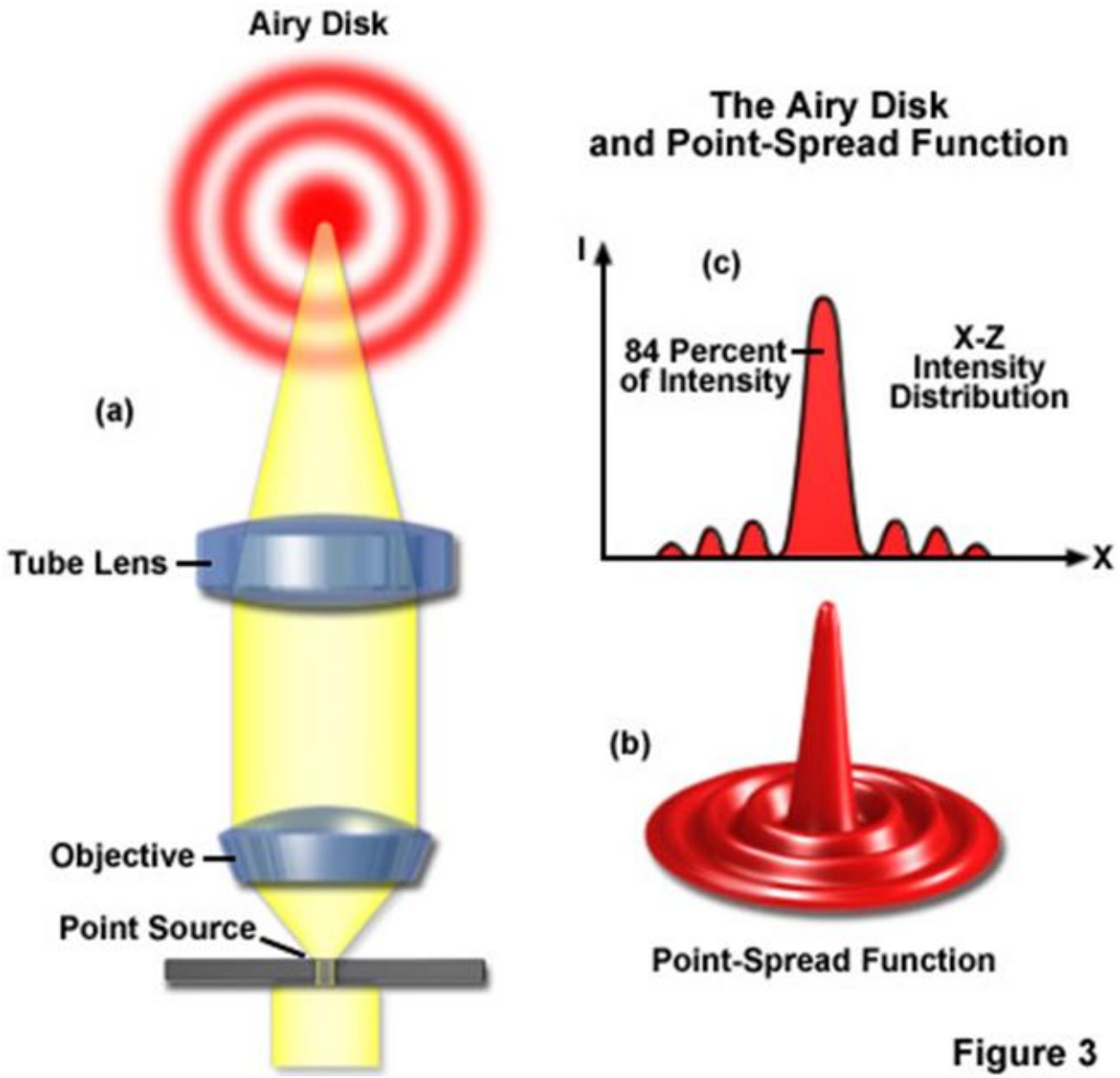

Airy Disk
(a)
Tube Lens
Objective
Point Source
The Airy Disk
and Point-Spread Function
I
(c)
84 Percent
of Intensity
X-Z
Intensity
Distribution
X
(b)
Point-Spread Function

Figure 3

John William Strutt and 'The Rayleigh Criterion' (1896)

- Rayleigh built upon and expanded the work of George Airy and invented the theory of the 'Rayleigh criterion' in 1896 .
- The Rayleigh criterion defines the limit of resolution in a diffraction-limited system, in other words, when two points of light are distinguishable or resolved from each other.
- Using the theory of **Airy discs,** if the diffraction patterns from two single Airy discs do not overlap, then they are easily distinguishable, **'well resolved'** and are said to meet the Rayleigh criterion. When the center of one Airy disc is directly overlapped by the first minimum of the diffraction pattern of another, they can be considered to be '**just resolved'** and still distinguishable as two separate points of light (Figure 2, mid). If the Airy discs are closer than this, then they do not meet the Rayleigh criterion and are '**not resolved'** as two distinct points of light.

The Rayleigh Criterion

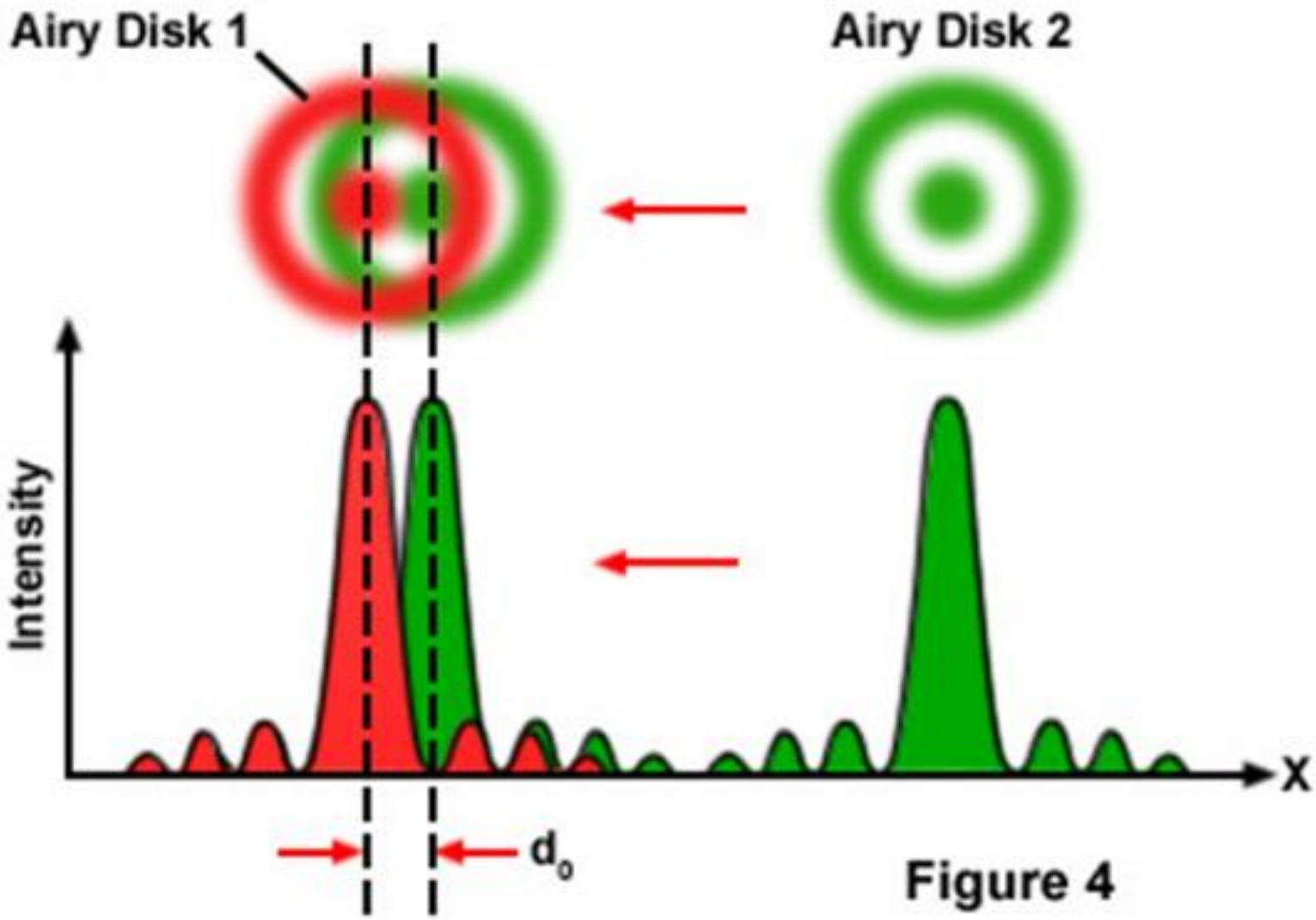


Figure 4

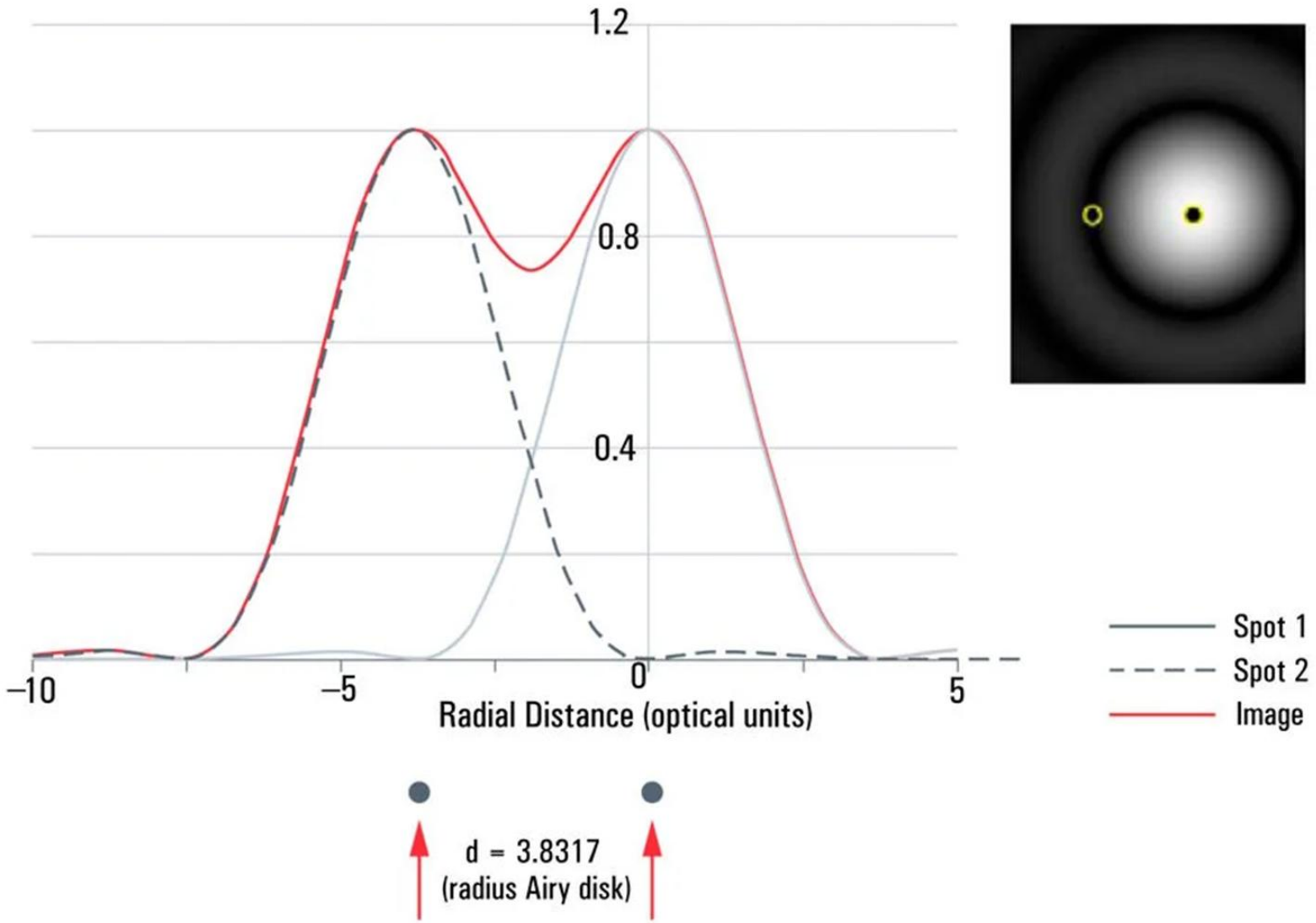
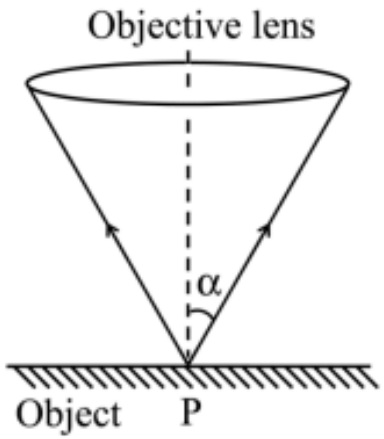


Figure 2: For the Rayleigh criterion, 2 spots (centers marked with yellow rings shown in inset image) are considered resolved if the center of one spot falls onto the first zero (dark ring) of the other spot's. The criterion is only valid for Airy-like disk.

Resolution of microscope



$$d_{min} = \frac{1.22\,\lambda}{2n\,\sin\alpha} = \frac{1.22\,\lambda}{2\,N.A.} = \frac{0.61\,\lambda}{N.A.}$$

d_{min} = minimum distance between point objects that can be resolved

λ = wavelength of the light source used

n = refrective index of the medium between the objective lens and the specimen

α = half of the objective angular aperture

N. A. = numerical aperture = $n\,\sin\alpha$

The **theoretical limit for d_{min}** for a light microscope operating in high refractive index (typically, n_{max} = 1.4 for the oil used in microscopy) is **~ 0.17 µm** (Assuming λ = 400 nm and sinα = 1)

- It is therefore an **intrinsic limitation of a light microscope** to **resolve the particles closer than ~0.17 µm.**

- It is evident that the **resolution can be increased** if the **wavelength of the source radiation is reduced**

Formulas

1. Firstly, it should be remembered that: $NA = n(\sin\alpha)$.
2. Abbe's diffraction formula for lateral (XY) resolution is:

   $d = \lambda/(2NA)$
3. The Rayleigh Criterion is a slightly refined formula based on Abbe's diffraction limits:

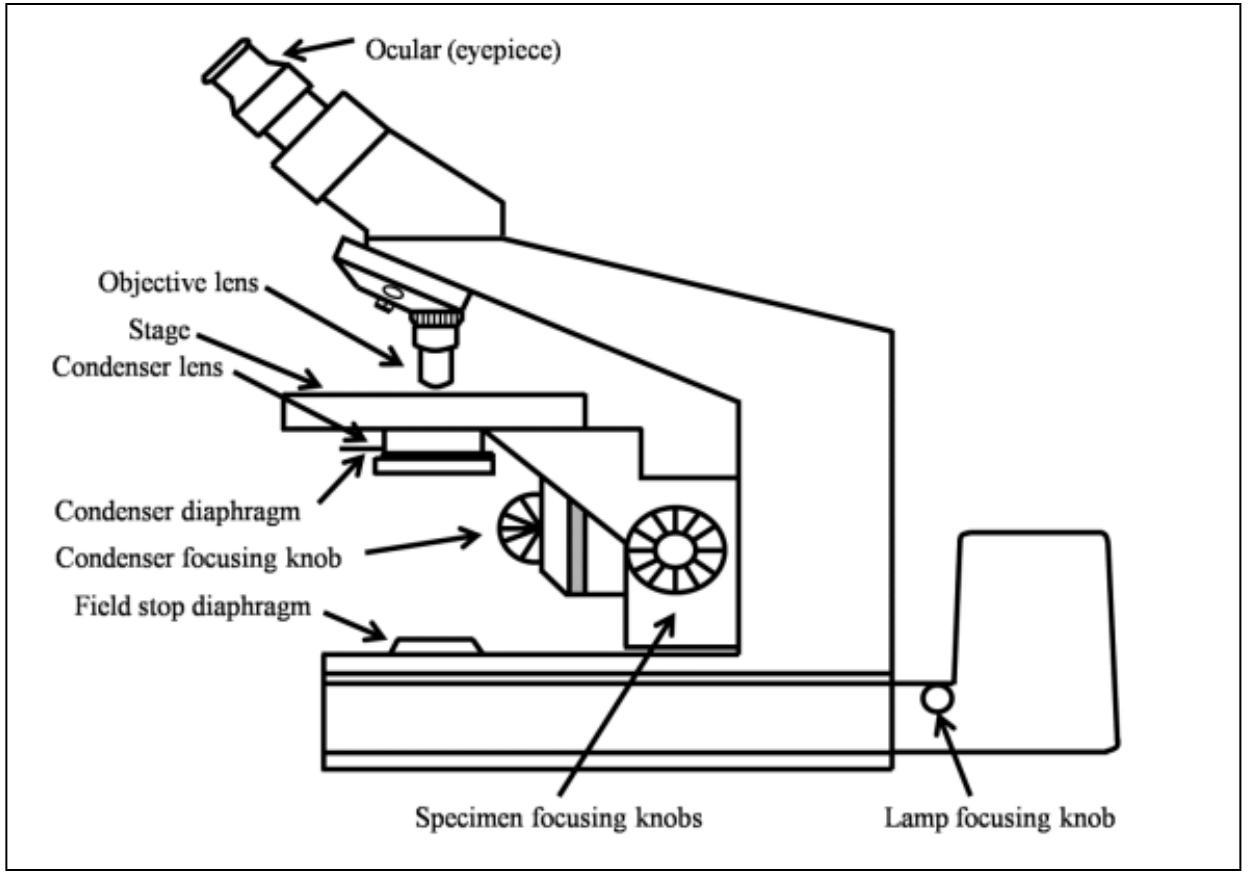
   $R = 1.22\lambda/(NA_{obj} + NA_{cond})$

Types of Fluorescent microscope

1. Upright
2. Inverted
3. Epifluorescent
4. Confocal

Parts of a light microscope

- The light is produced by a **lamp source.**
- Focused on the specimen by the **condenser**.
- The light **diffracted** by the sample is then collected by the **objective lens** that generates a real magnified image.
- This image is further **magnified by the eyepiece**.



Schematic diagram of a compound microscope showing its different components.

The Concept of Numerical Aperture for Objectives and Condensers

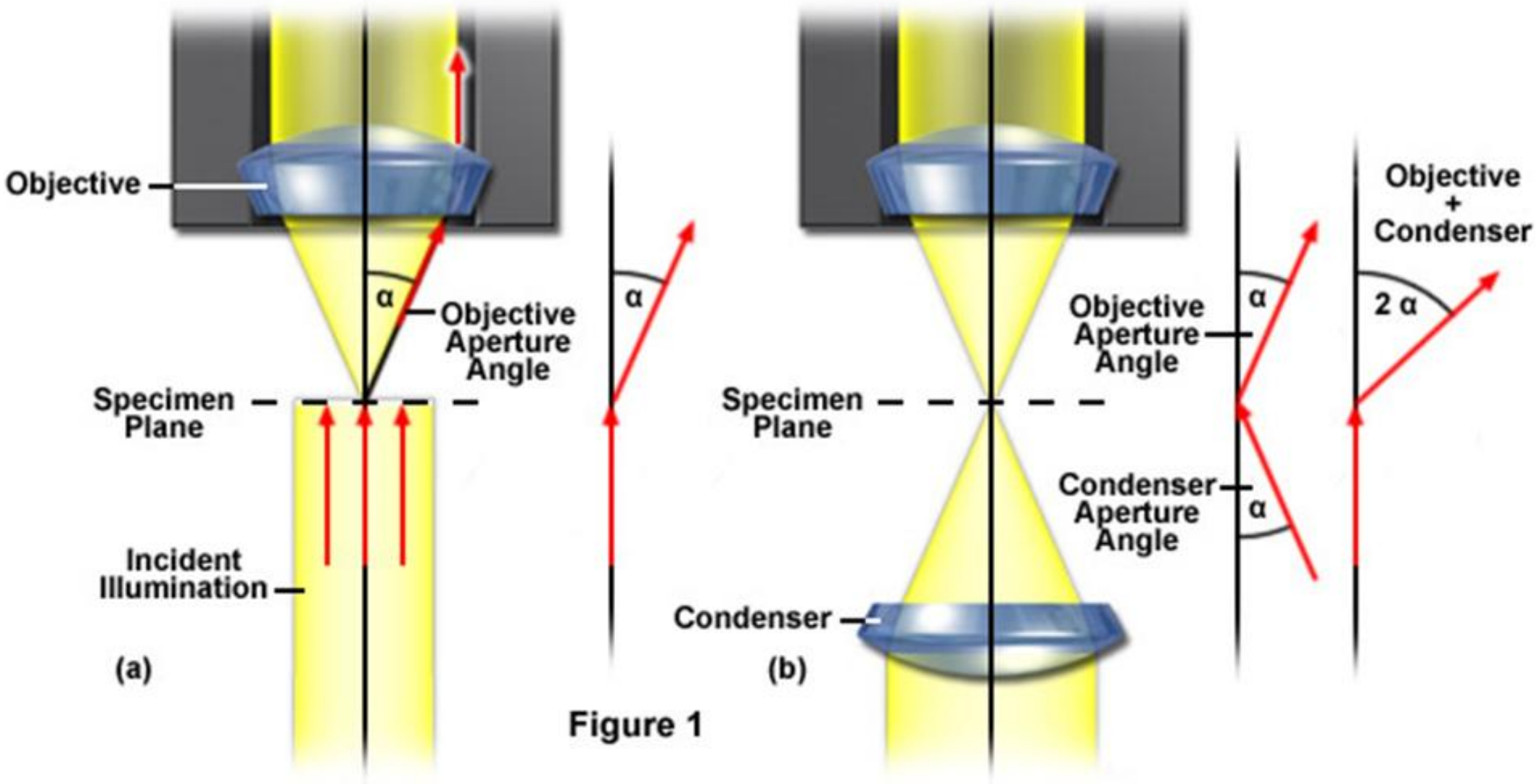
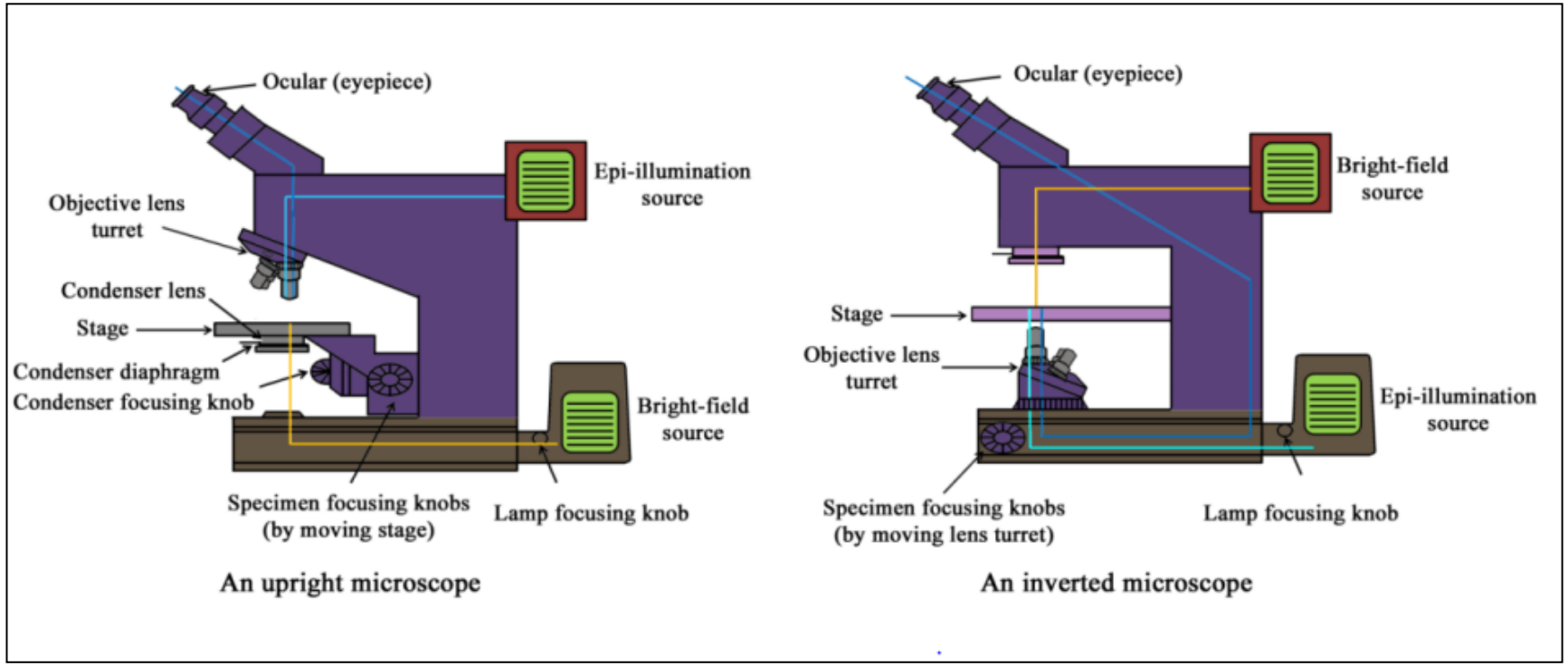


Figure 1

Inverted and upright microscope

Light microscopes come in two designs: upright and inverted

- In upright microscope, the objective turret is fixed and the image is focused by moving the sample stage up and down.
- In inverted microscope, the sample stage is fixed and objective turret is moved up and down to focus the final image.



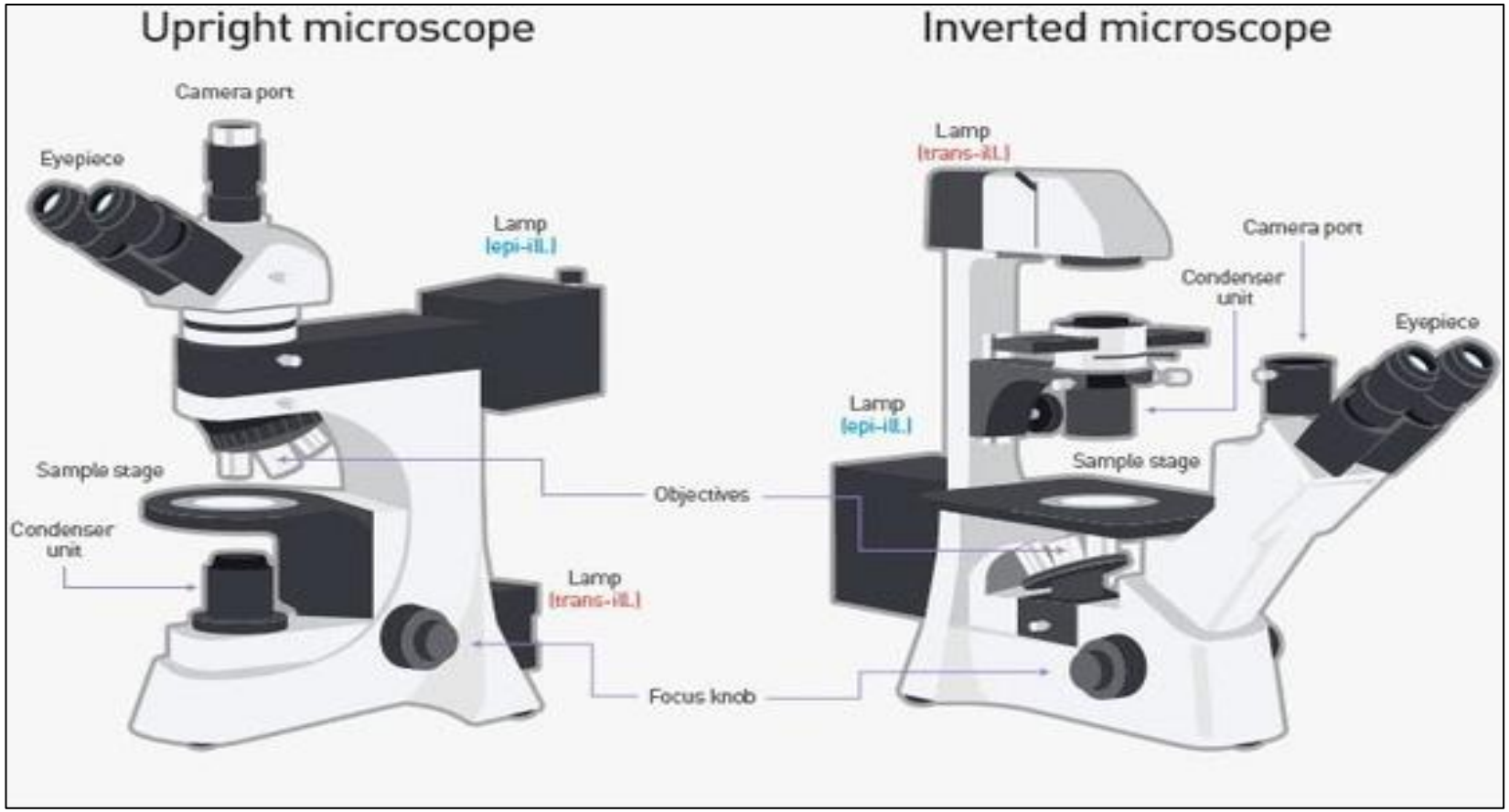
Design of upright (A) and inverted (B) microscopes

Inverted microscopes offer certain advantages over upright microscopes:

i. As the objective turret is at the bottom of the stage, the sample stage is more accessible allowing manipulations of the sample.

ii. The specimen need not be covered at the top by a coverglass.

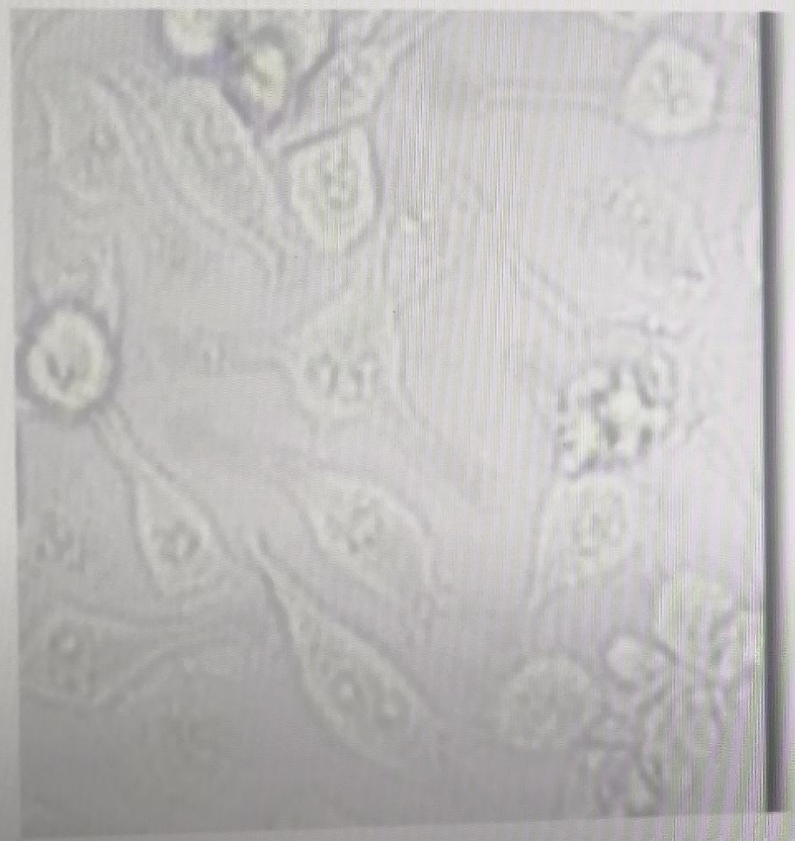
iii. Inverted microscopes enable to look at more samples in a short period of time, as does not require to adjust the focus every time the sample is changed



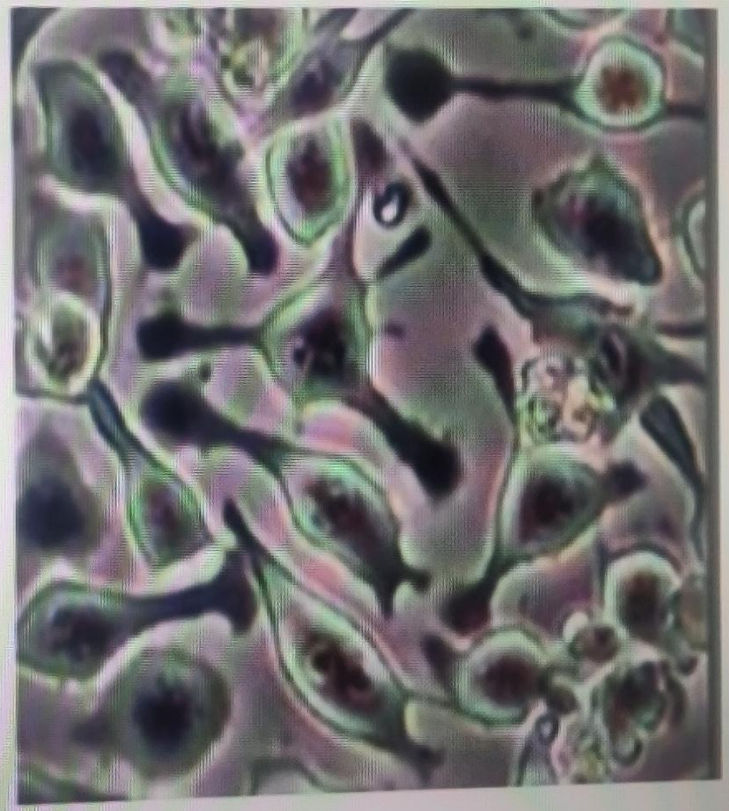
Comparison of upright and inverted microscopes

Concept of Bright Field, Phase Contrast and Dark Field microscopy

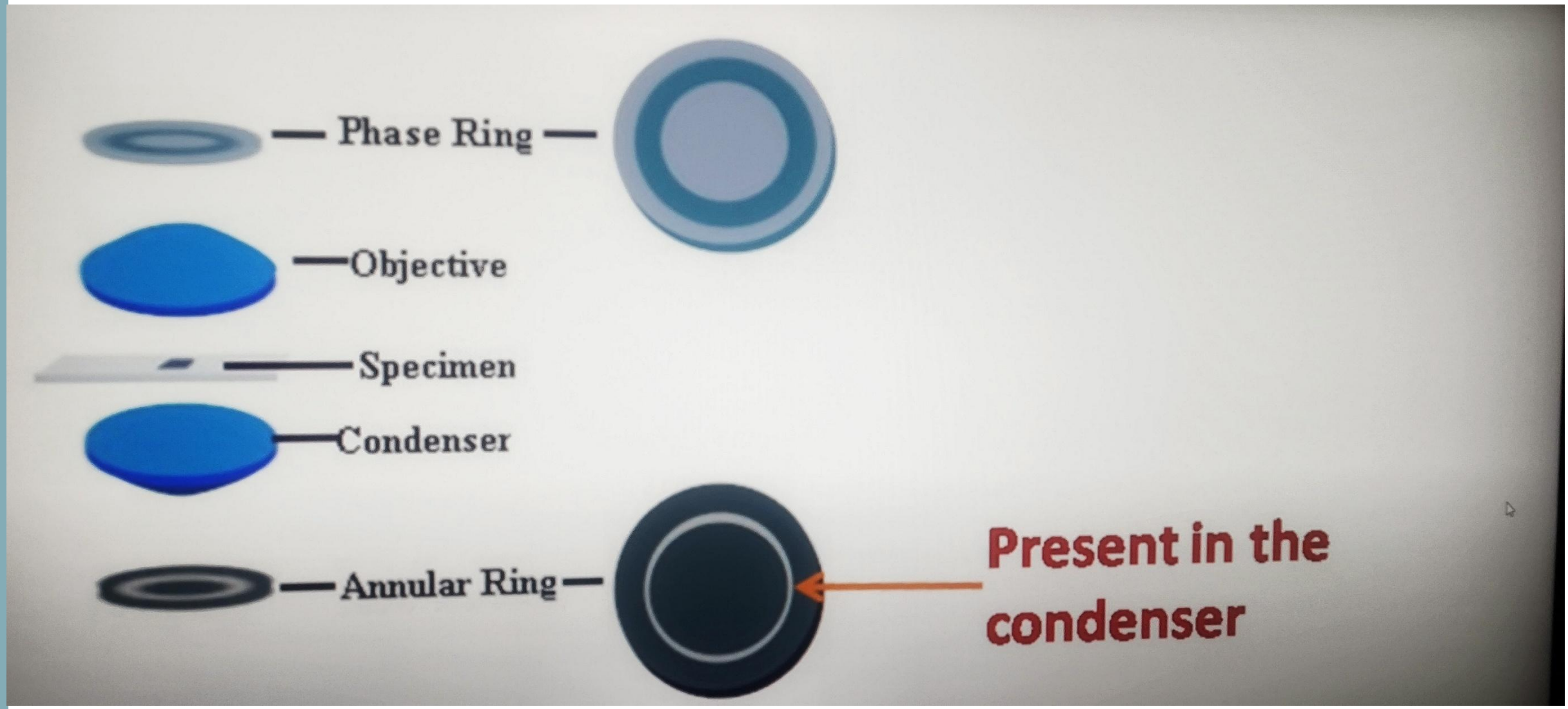
Bright field



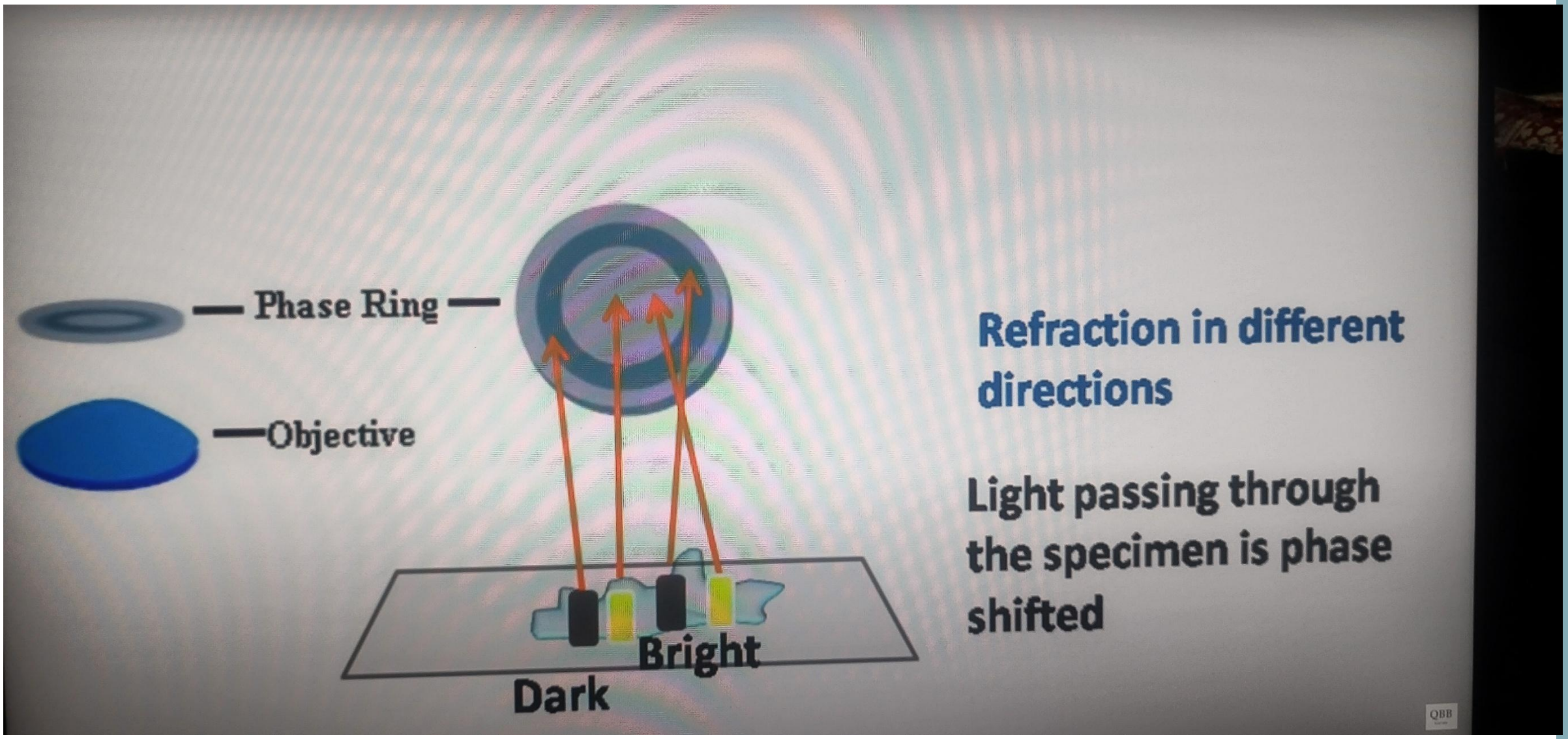
Phase contrast



Phase Contrast



Phase Contrast



Phase Specimens and Amplitude Specimens:

Specimens that do not possess much color **but a different refractive index that the surrounding mounting medium can be referred to as phase specimens as they cause a phase-shift in of the light.** The unaided human eye is not capable of detecting this phase shift. This phase shift is then converted into a brightness difference by the optics of the phase-contrast microscope. Bacteria are a good example here. They are nearly completely transparent but nevertheless appear darker or brighter (depending on the optics) than the background.

Amplitude specimens possess a color and are able to decrease the brightness of the passing light all on their own. These specimens are best observed using bright-field microscopy. Pigmented structures (such as chloroplasts) and specimens that are selectively stained are examples.

Many specimens are a combination of these two. Here the choice of the right kind of microscope is important in order to see that what one wants to see. Phase contrast microscopes will optically darken certain structures to the extent that it is not possible to see the natural color of the structure. In this case it is probably better to use bright field microscopy. Stained bacteria, for example, should be observed in bright field

Advantages and disadvantages of bright field and phase contrast microscopy

Advantages of bright-field microscopy:

- The optics do not change the color of the observed structures. Sometimes stains are used to make certain structures visible. The optics of a bright field microscope do not change these colors.
- Bright-field optics is generally cheaper than phase contrast optics
- Bright-field microscopy requires fewer adjustments before one is able to observe the specimens.

Advantages of phase contrast microscopy:

- It is possible to visualize certain structures that are otherwise invisible. This includes certain cell organelles which can not be seen well in bright field.
- Sometimes the phase contrast image subjectively looks better than a bright field image due to the details visible.

What is EPI fluorescence?

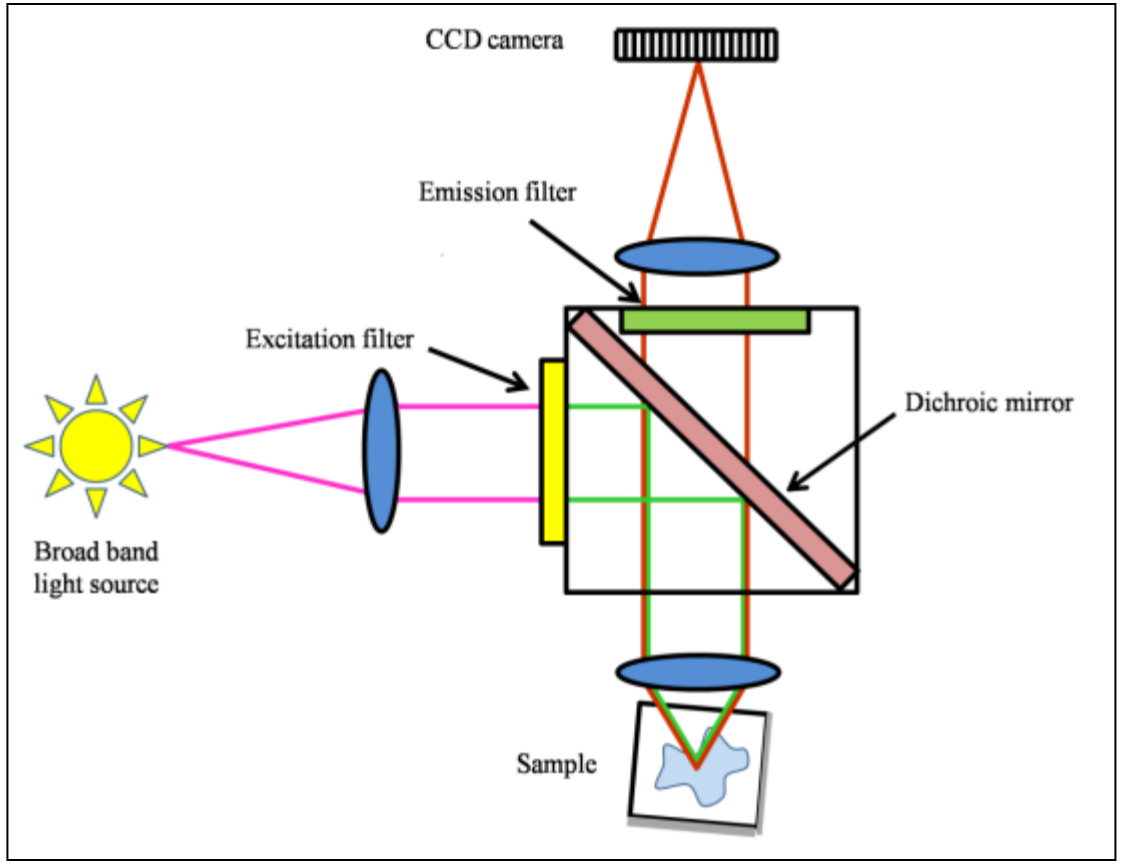
In epifluorescence microscopy, both the excitation and emission light travel through the same objective.
This arrangement—where both the illuminated and emitted light travels through the same objective lens—is referred to as epifluorescence microscopy, where "epi" is borrowed from the Greek to mean "same".

Epifluorescence microscope

Epifluorescence microscope is the **simplest of all fluorescence microscopes**.

In an epifluorescence microscope,

- The illumination of the specimen as well as the collection of the fluorescence light is achieved by a single lens (objective)
- This has become possible due to the incorporation of **dichroic mirror** in the optics. A dichroic mirror is largely **reflective for the light below a threshold wavelength and transmissive for the light above that wavelength**.
- Scattered light from the illumination source, goes back into the objective lens and to the detector or eye. Along the way, **another filter** blocks out the illumination light, so all that is left is the fluorescent light from the sample.



A diagram showing the optical path in an epifluorescence microscope.

Parts of Fluorescent microscope

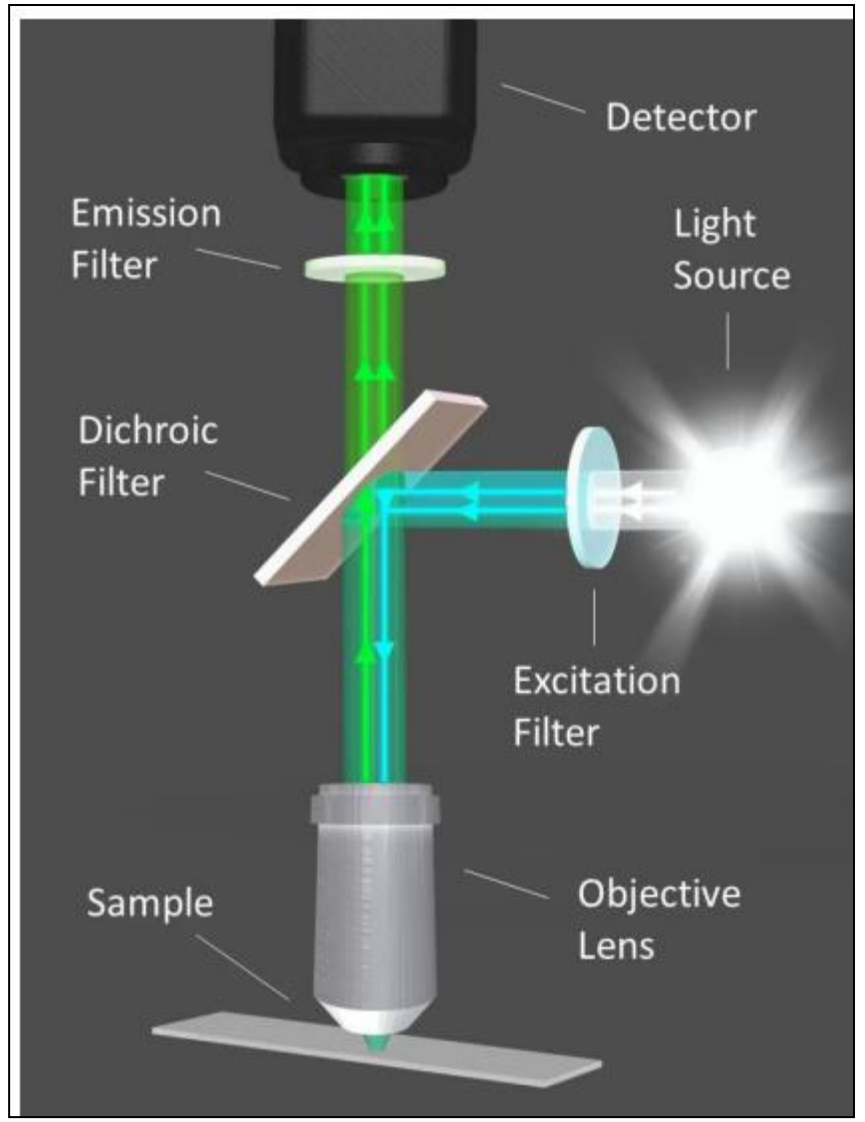
1. **Light source:** The microscope has a high power lamp source, usually a mercury or xenon arc lamp.

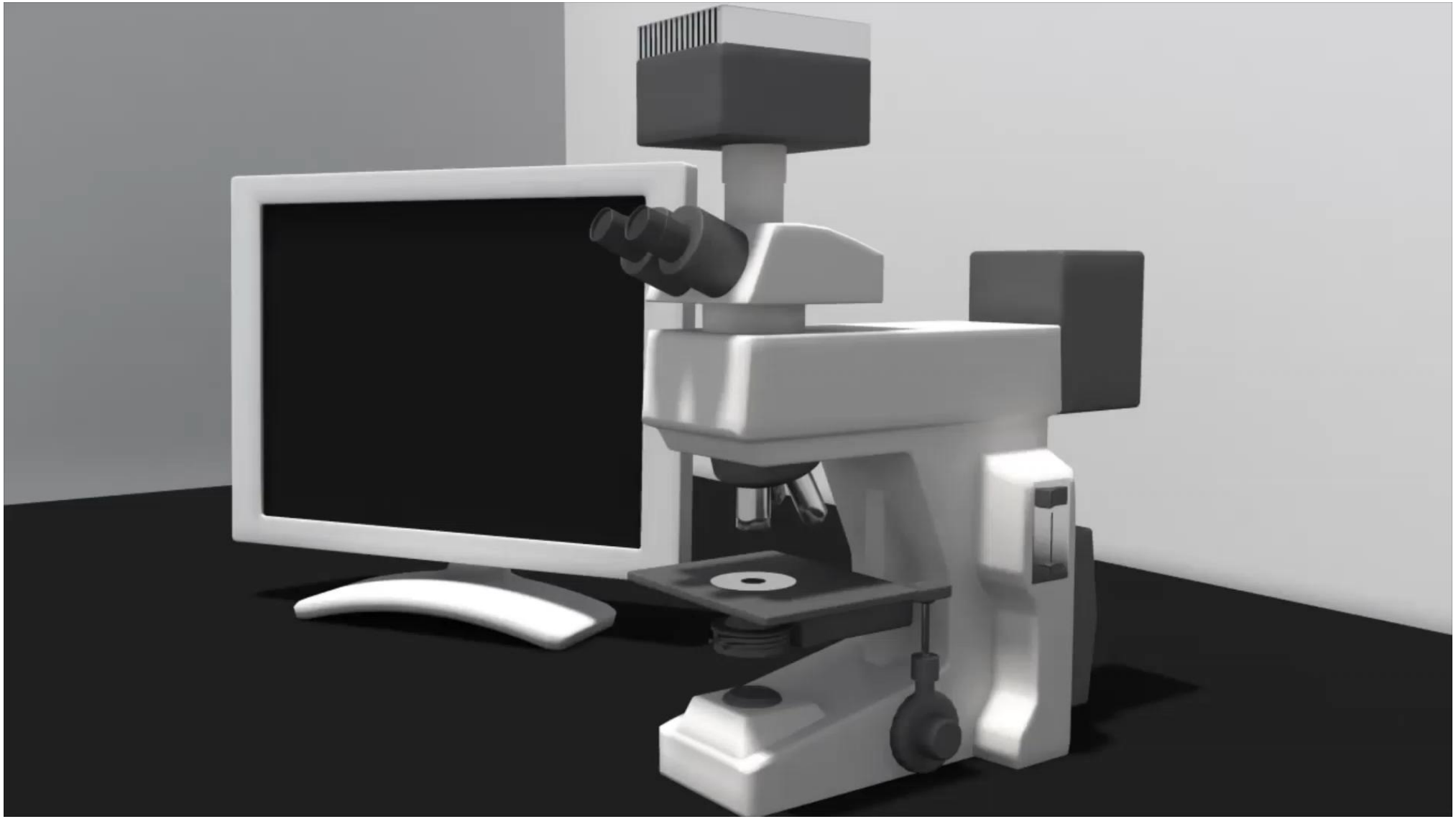
2. **An excitation filter** (bandpass filter) transmits the band of the excitation radiation. It minimizes the excitation of other sources of fluorescence.

3. **Emission filter:** It only passes those wavelengths that are emitted from a fluorophore.

4. **Dichroic mirror :** The excitation radiation is reflected by the dichroic mirror towards the **condenser/objective** lens that focuses the light on the specimen.

Light emitted by the fluorescent molecules (higher wavelength due to Stokes shift) is collected by the same lens and is transmitted by the dichroic mirror towards the **ocular lens**





Animation representing the working of a light and fluorescent microscope

Sample Preparation for Fluorescence Microscope

1. Biological fluorescent stains:

Use of small fluorescent dyes that can translocate across the biological membrane and bind to the cellular targets with high specificity.

Example: DAPI, Hoechst, Acrydine Orange for staining nucleic acids. Phalloidin used to stay actin fibres in mammalian cells.

2. Immunofluorescence:

For intracellular targets, however, the cells are usually fixed and permeabilized to allow the fluorescently labelled antibodies enter the cells.

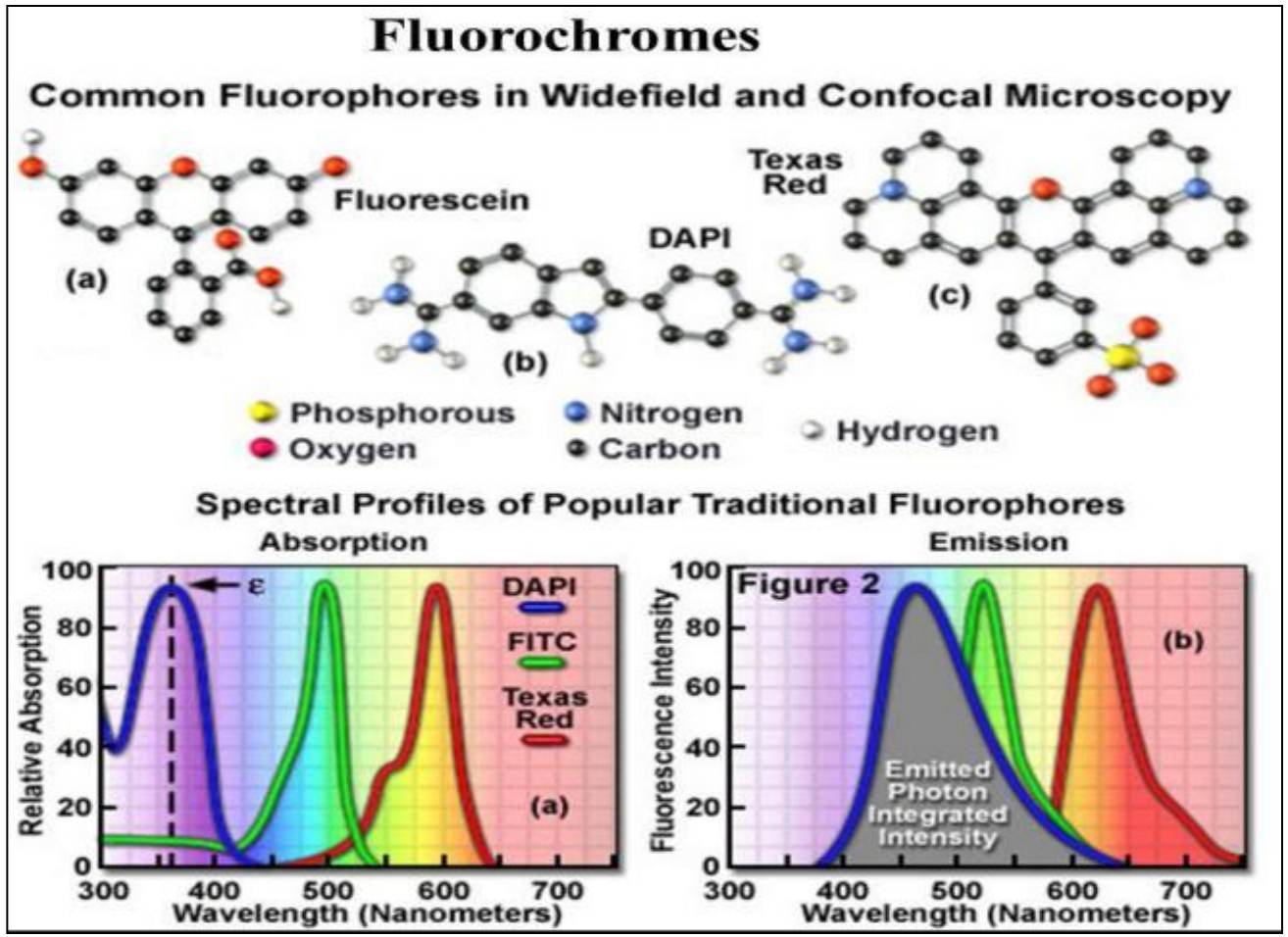
3. Florescent Proteins:

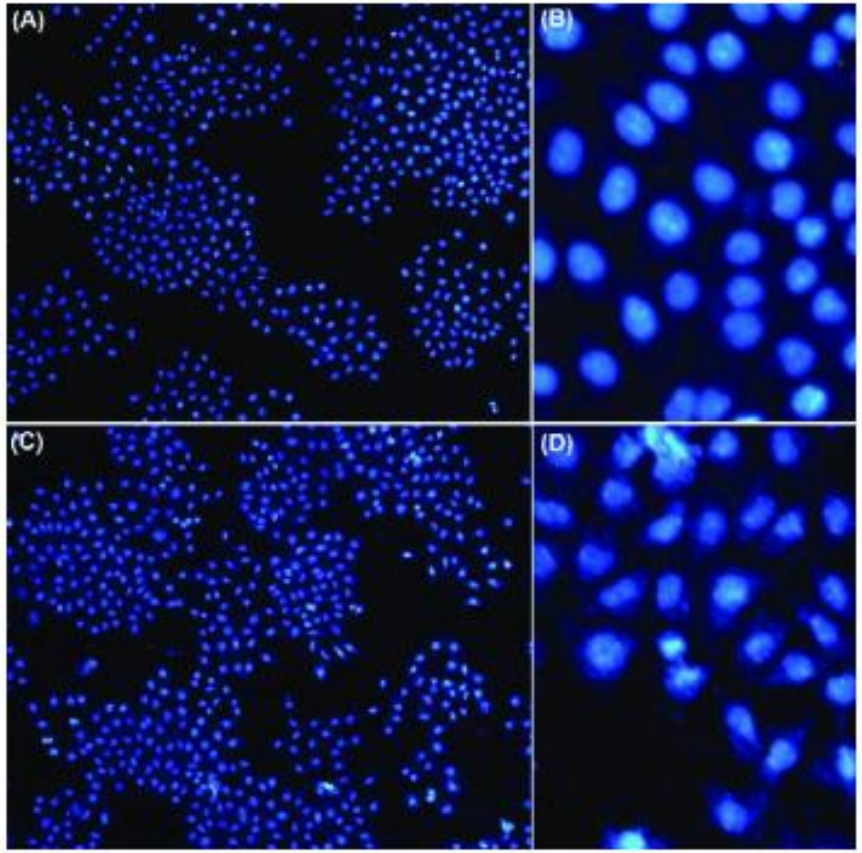
Proteins like GFP, YFP, RFP are used.

.

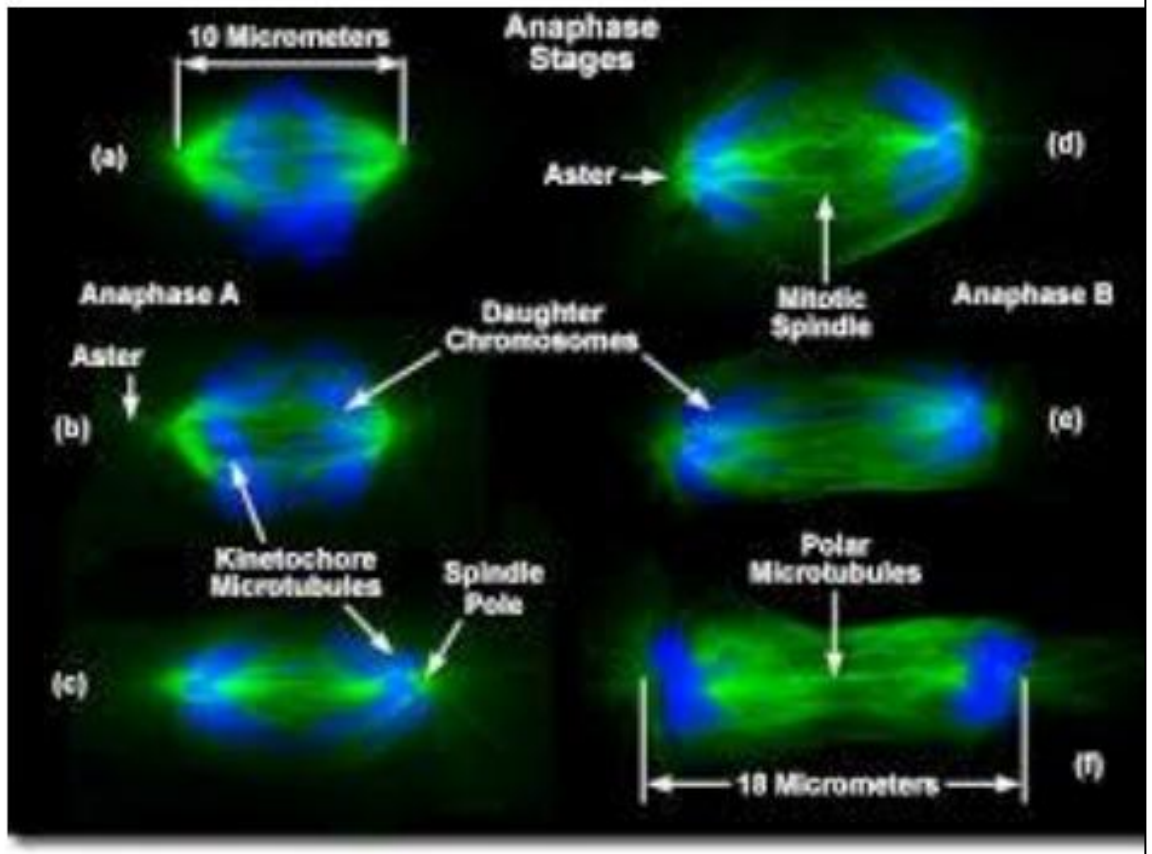
Fluorophore	Absorption maximum	Emission maximum
DAPI	345	460
Fluorescein isothiocyanate	492	520
Cyanine based dyes	~490 – 740 nm	506 to > 750 nm
Lissamine-rhodamine B	575	595
Texas red	596	620
BODIPY-based dyes	~500 – 600 nm	~500 to >750 nm

List of some fluorescent molecules/fluorophores routinely used for fluorescence microscopy with biological specimens.





DAPI (blue), in Hela cells.

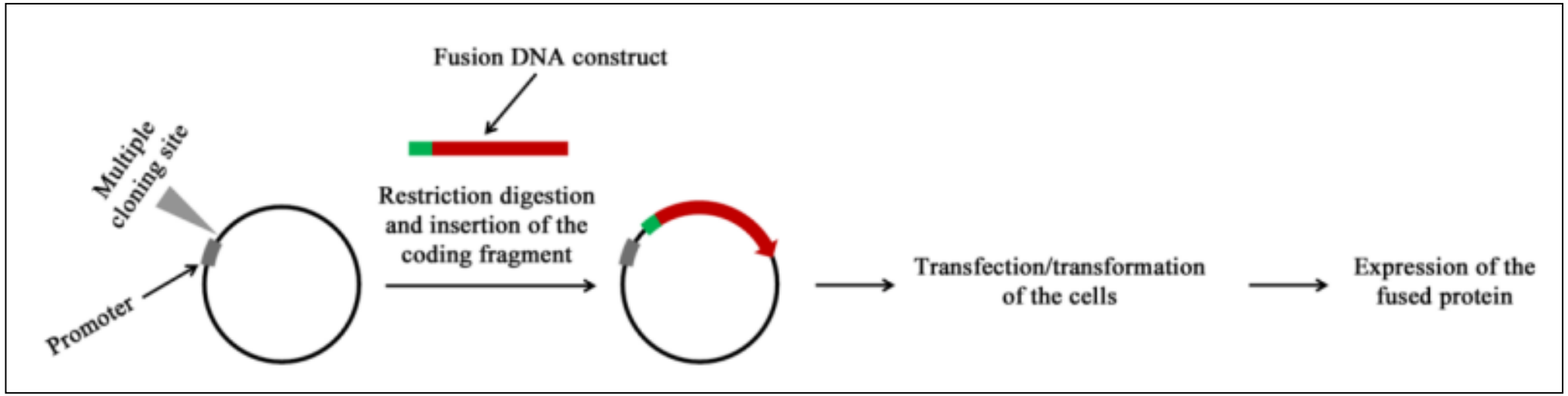


Live Cell imaging of Mitosis, DAPI (Blue- nucleus) , Actin(Green-Microtubules)

Fluorescent protein

Discovery of **green fluorescent protein (GFP)** and developments of its variants with different spectral properties has made it possible to selectively label the proteins inside the cell using molecular cloning.

- It is possible to put the GFP (or its variant) tag at either ends of the protein.



Strategy for selectively labeling a protein in a cell.

The **cDNA for the protein under study is fused with that of cDNA of GFP** or any of its variants.

Confocal Laser Scanning Microscope (CLSM)

The principle of confocal imaging was patented in 1957 by Marvin Minsky

Emergence of Confocal microscopy

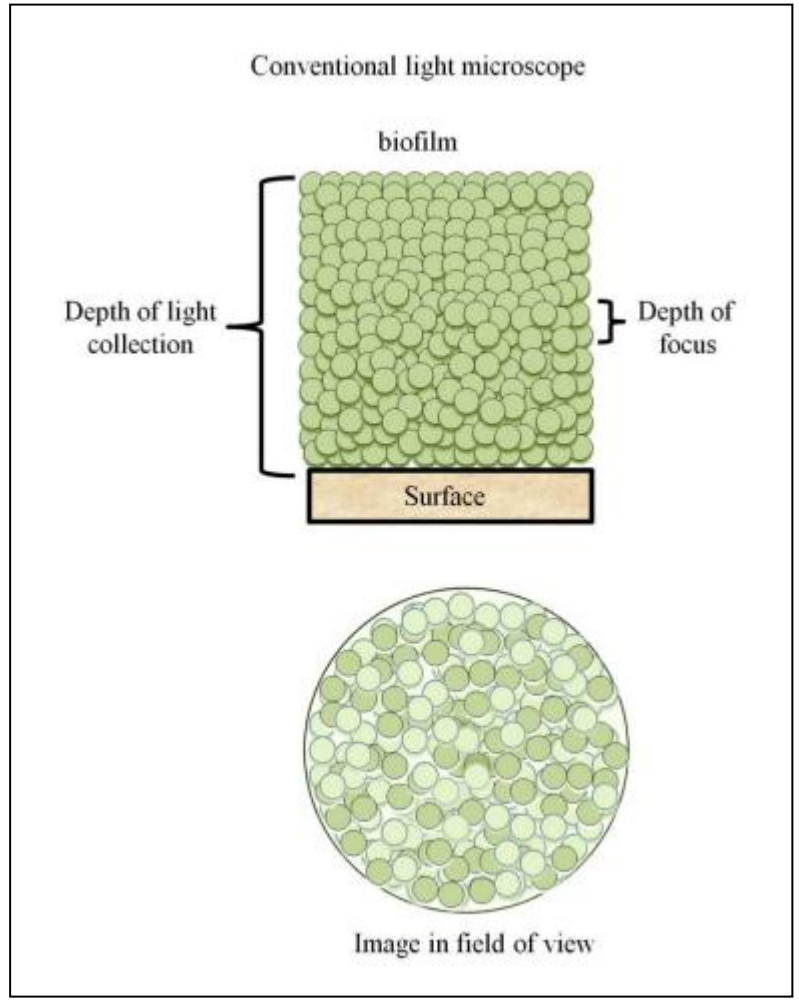
Confocal microscope, which **uses optical sectioning** to get better resolution of the fluorescent image.

Problem with by conventional fluorescence microscopy

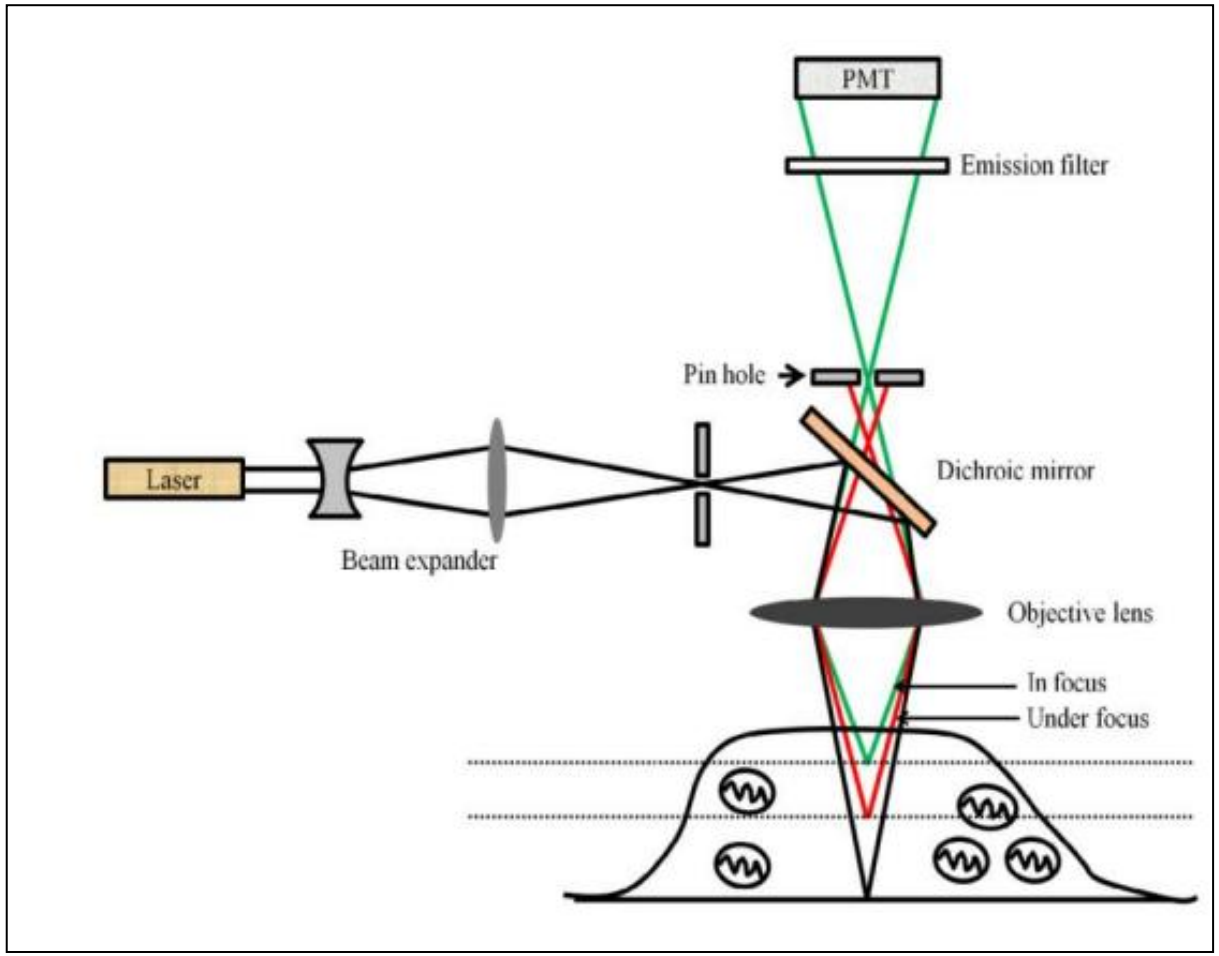
When **thick biological specimen** is studied by conventional fluorescence microscopy, the light is emitted by the entire illuminated volume of sample and **the out of focus light results in higher background intensity** and affects the image contrast.

Small modification in fluorescence microscope allows **collection of fluorescence from a thin section of the sample**, resulting in a sharp image

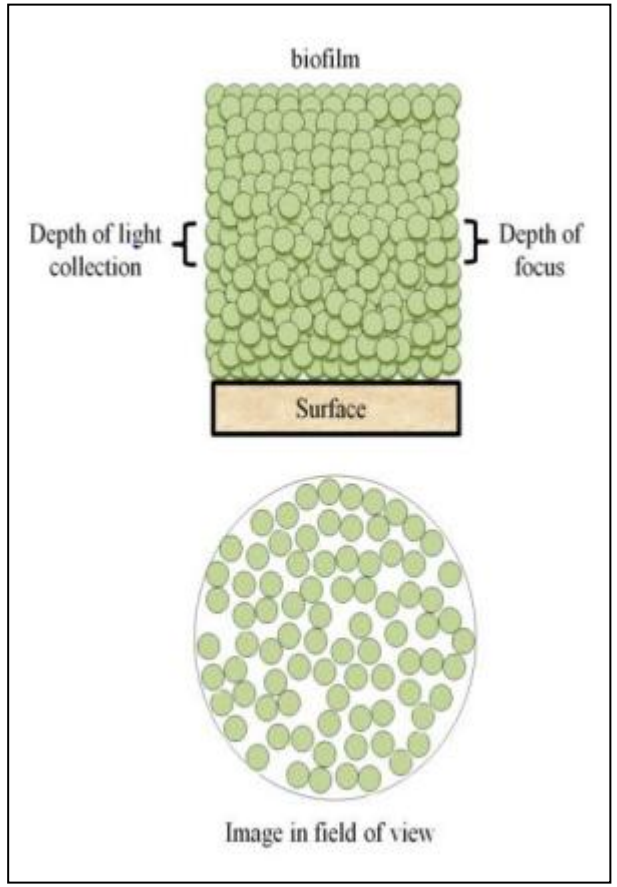
This **rejection of out-of-focus light by using a pinhole** is the principle behind confocal microscopy



Imaging of a thick sample using conventional wide-field microscopy

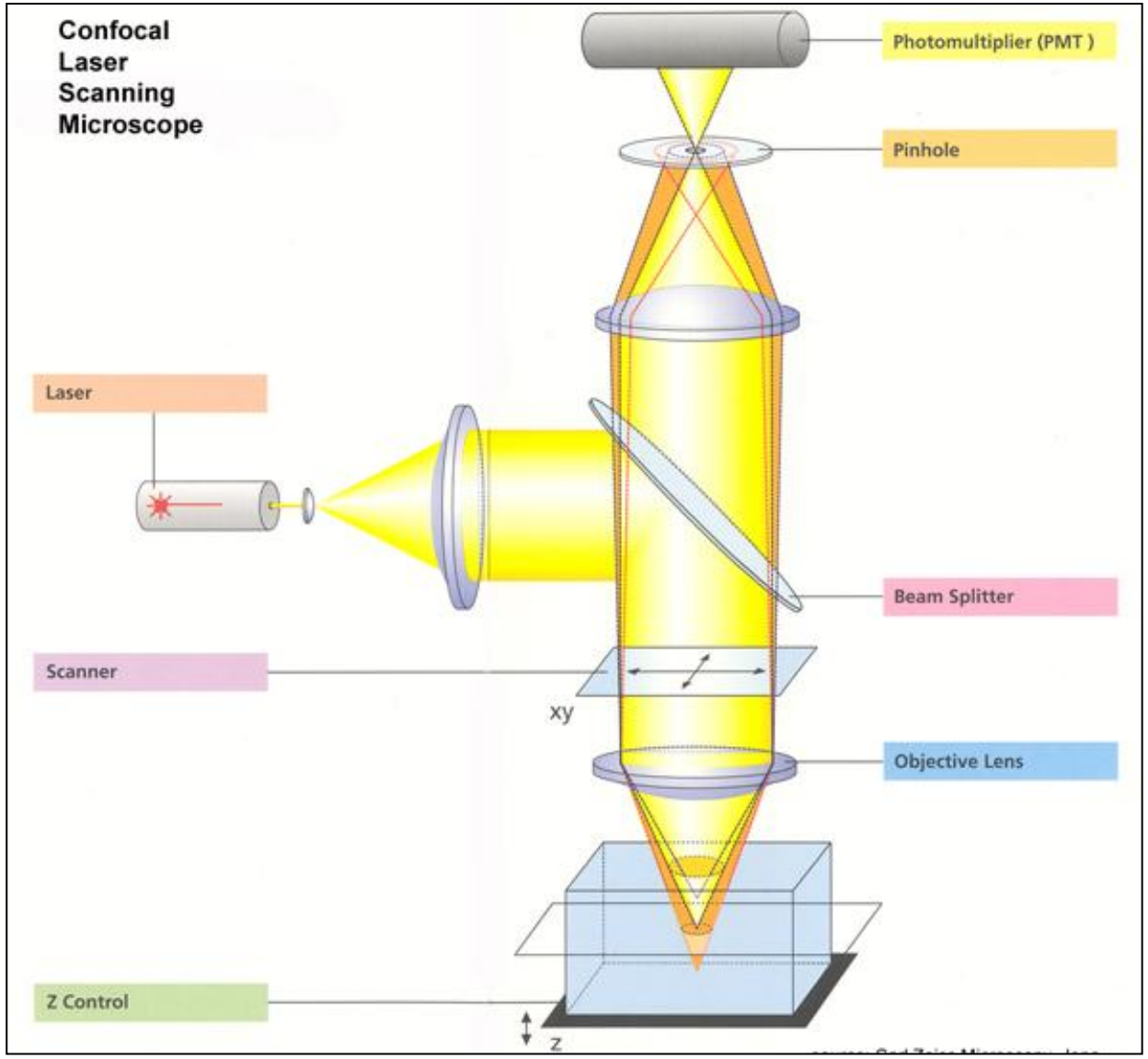


Optical diagram of a confocal laser scanning microscope; **the pinhole rejects the light coming from non confocal planes**



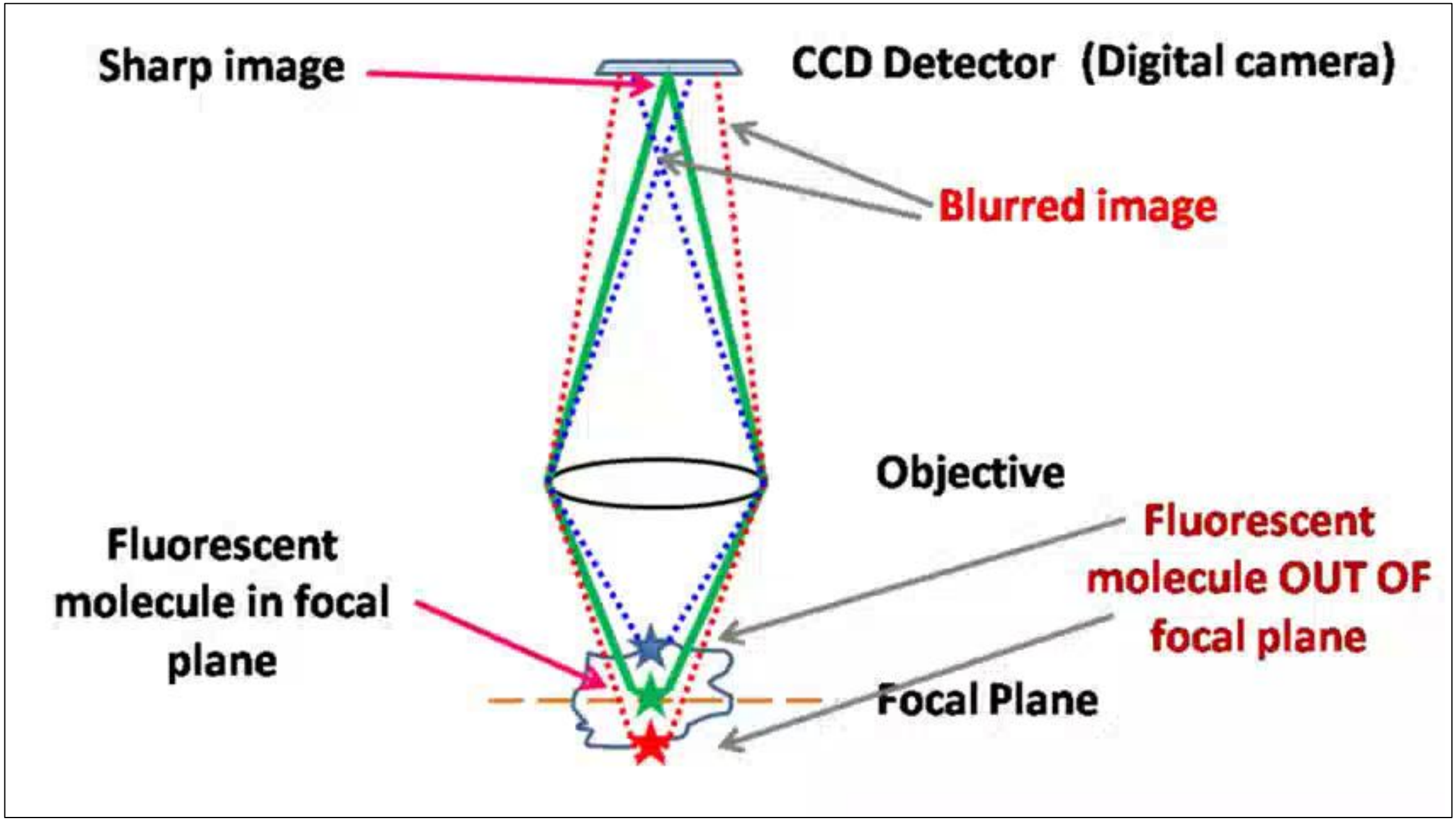
Imaging of a thick sample using confocal microscopy. Thin section of sample is achieved using pinhole, Avoids background intensity

Important parts of a Confocal Microscope



Parts of Confocal microscope

- **Light source & illumination:** Light sources used in confocal microscopes are **lasers.** Microscope works in epi-illumination mode. The laser beam is spread by **diverging lens.**
- The **expanded laser light** is reflected by the **dichroic mirror** on the objective that focuses the light as an **intense diffraction-limited spot on the sample.**
- The fluorescence from the illuminated spot is collected by the **objective and sent to the eyepiece/camera/detector through a pinhole aperture.**
- **Pinhole aperture:** The fluorescence light emitted by the illuminated sample is **focused as the confocal point at the pinhole**. Any light coming from below or above the focal plane is blocked by the pinhole plate. **Emission filter:** The light that passes through the pinhole is filtered by the emission filter before it reaches the detector


Sharp image
CCD Detector (Digital camera)
Blurred image
Objective
Fluorescent molecule in focal plane
Fluorescent molecule OUT OF focal plane
Focal Plane

Principle of Confocal Microscope

For confocal microscope, **point illumination** is the principle working mechanism

1. Laser beam is used and one mirror tilts the beam in the **X direction**, the other in the **Y direction**. Together, they tilt the beam in a **raster fashion.**
2. The beam is then focused by the objective lens to a **plane** above the objectives onto the sample. The specimen normally lies between the camera lens and the perfect point of focus, known as the **plane of focus.**
3. Using the laser from the microscope, the laser scans over a plane on the specimen (**beam scanning**) or by moving the stage (**stage scanning**).
4. This light then passes through a semi-transparent mirror which reflects it away from the laser and **toward the detection system.**
5. The first object in the detection system is the pinhole aperture, which cuts off signals that are out of focus, thus allowing only a small central portion of the light from illuminated spot to enter the light detector.
6. A detector then will measure the illumination producing an image of the optical section.
7. **3D objects** can be visualized by **scanning several optical planes** and stacking them using a suitable microscopy deconvolution software (z-stack).

Applications of Microscopy

1. **Specimen identification and quality:** The simplest application of microscopy is to observe the given sample to identify the different its components
2. **Cell counting:** Counting of cells using a hemocytometer utilizes light microscopy
3. **Classification of bacteria:** Differential staining of the bacterial cell wall by Gram staining method is the basis of classifying the bacteria into Gram positive and Gram negative.
4. **Microscopic analysis of body fluids:** Microscopic analysis of blood samples is routinely used to determine the blood cell count, to detect the microbial infection, and to identify any changes in the cellular structures.
5. **Histopathology** (study of anatomical changes in the tissues): A number of stains are available and the choice of stain depends on the histological features one needs to study.

Applications of Microscopy

6. **Cellular membranes and intracellular structures:** Probes that specifically bind to the cellular organelles like nucleus, mitochondria, and lysosomes are commercially available. e.g. DAPI for DNA staining.

7. **Membrane proteins:** Fluorescently labeled membrane proteins can be studied

8. Bacterial pathogen detection: Could be identified after staining them with fluorescent antibodies

9. **Live cell imaging:** Inverted microscopes allow direct visualization of the cultured cells.

10. **Protein dynamics and localization study.**

11. **Co-localization of the proteins:** Confocal images are recorded for the study, where two proteins are labelled with two different fluorophores.

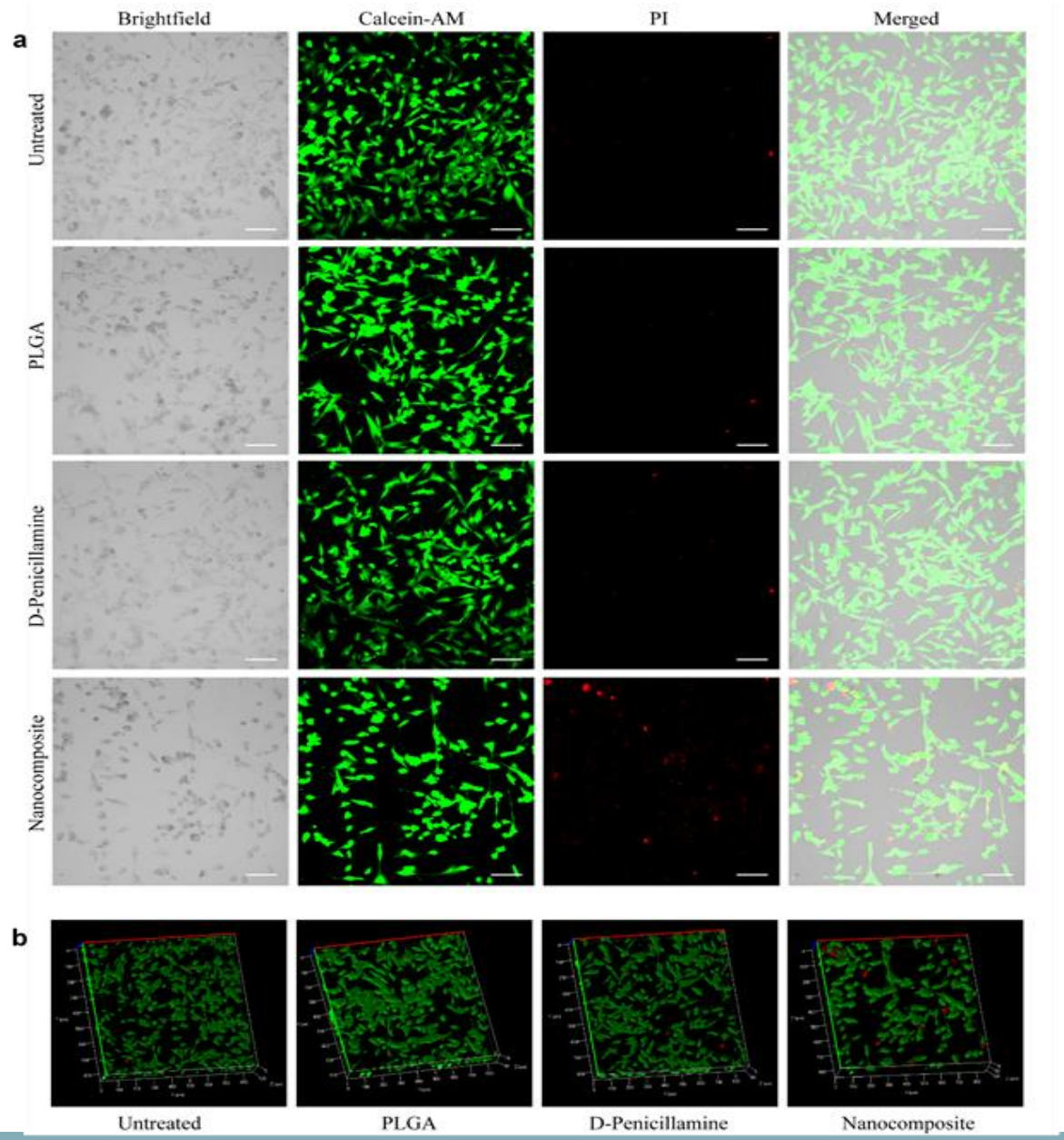
Advantages of Fluorescence Microscopy

1. Used to study dynamic behaviour exhibited in **live-cell imaging.**
2. Can **trace the location of specific protein** in the cell.
3. Due to **high sensitivity**, it can detect 50 molecules per cubic micrometer.
4. Allows **multicolour staining** of the specimen.

Disadvantages of Fluorescence Microscopy

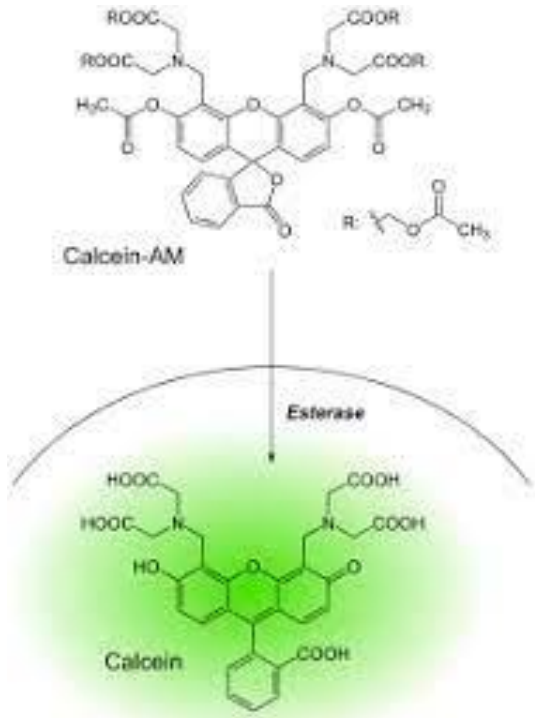
1. **Photo-bleaching** causes fluorophores to lose their ability to fluorescence.
2. Fluorescent molecules can **generate reactive chemical species** during the illumination process, which enhances the **phototoxic effect**.
3. Sample preparation is **costly**, **requires staining** with fluorescent dyes.

Live-dead cell visualization



a) Live-dead cell visualization of Monolayer culture using **calcein-AM/propidium iodide (PI)** dual staining. Green fluorescence by calcein-AM refers to live cells, whereas red fluorescence by PI refers to dead cells.

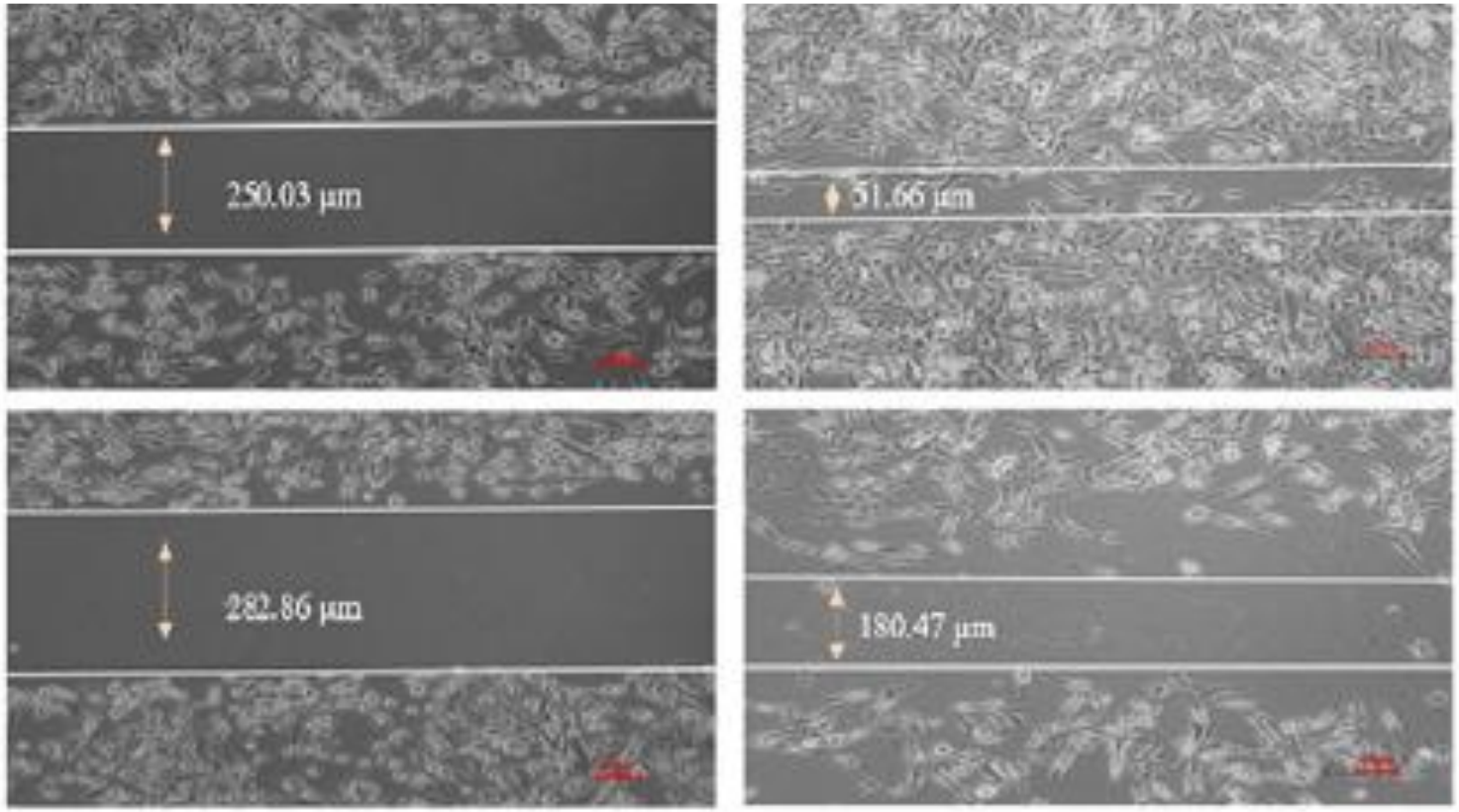
b) (b) Z-stack projections of live dead cell imaging of monolayer culture.



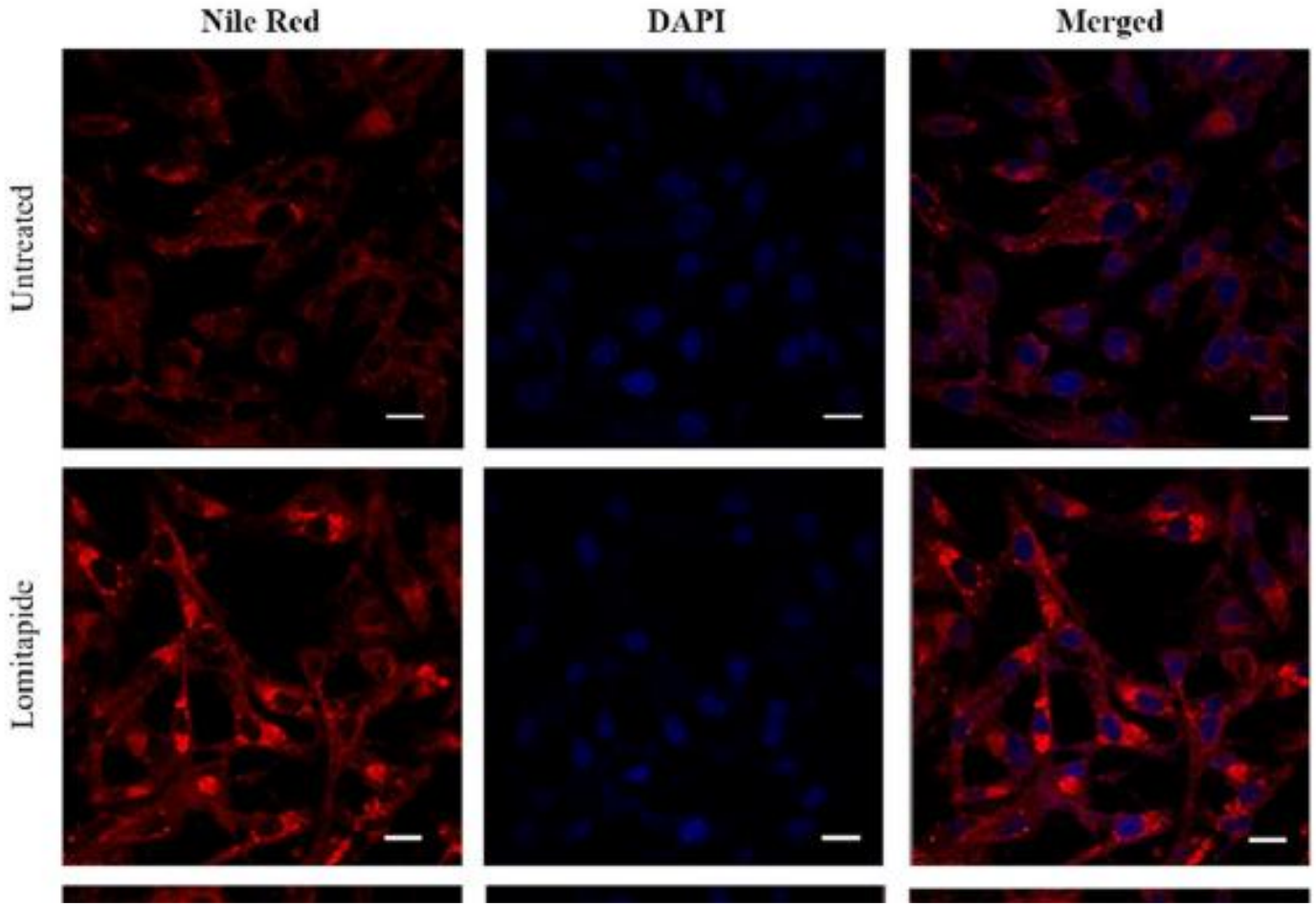
Scratch or Wound Healing Assay

Untreated cancer Cells

Drug treated Cancer Cells

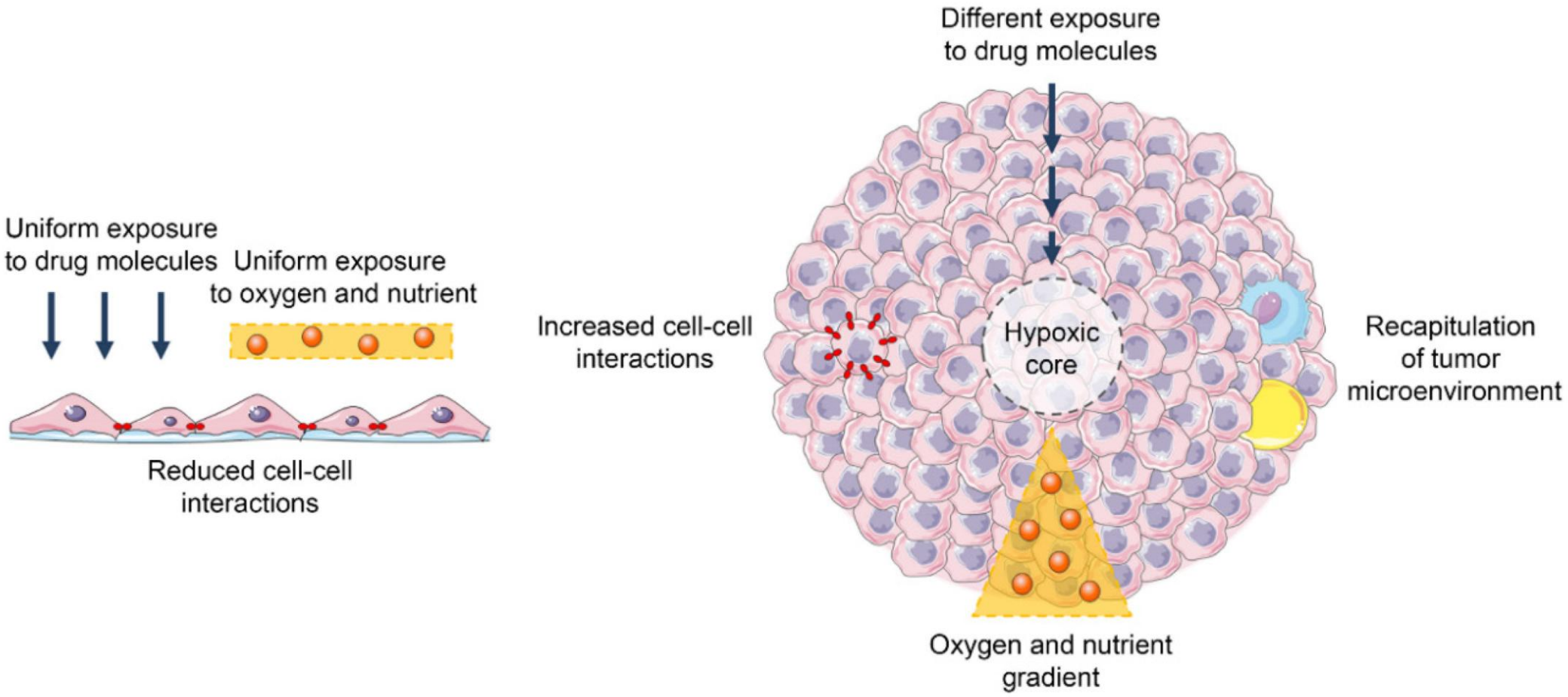


Nile Red staining of lipid droplets in Cancer Cells



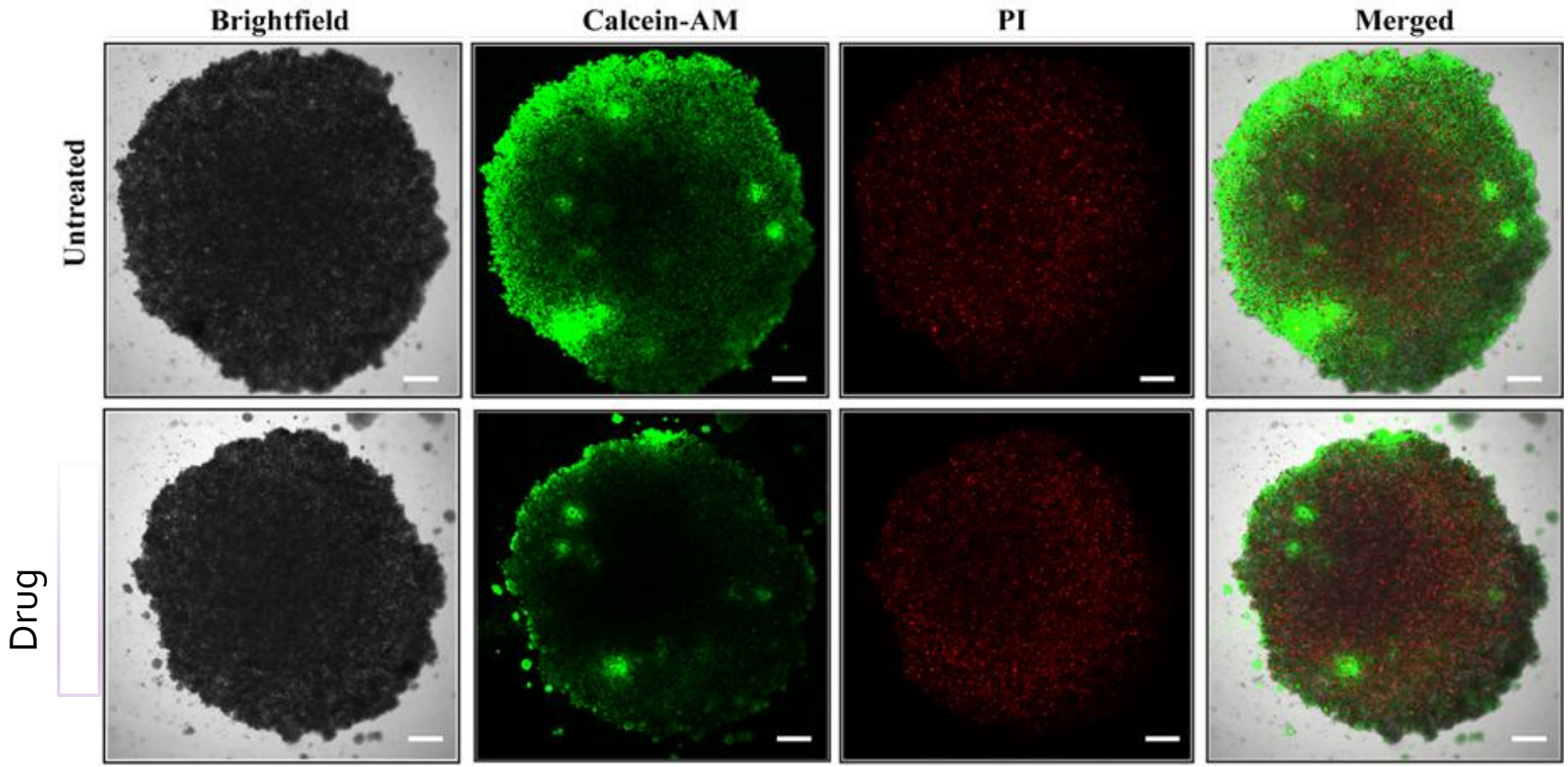
A fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI). It stains nucleus Blue

Tumor Spheroids

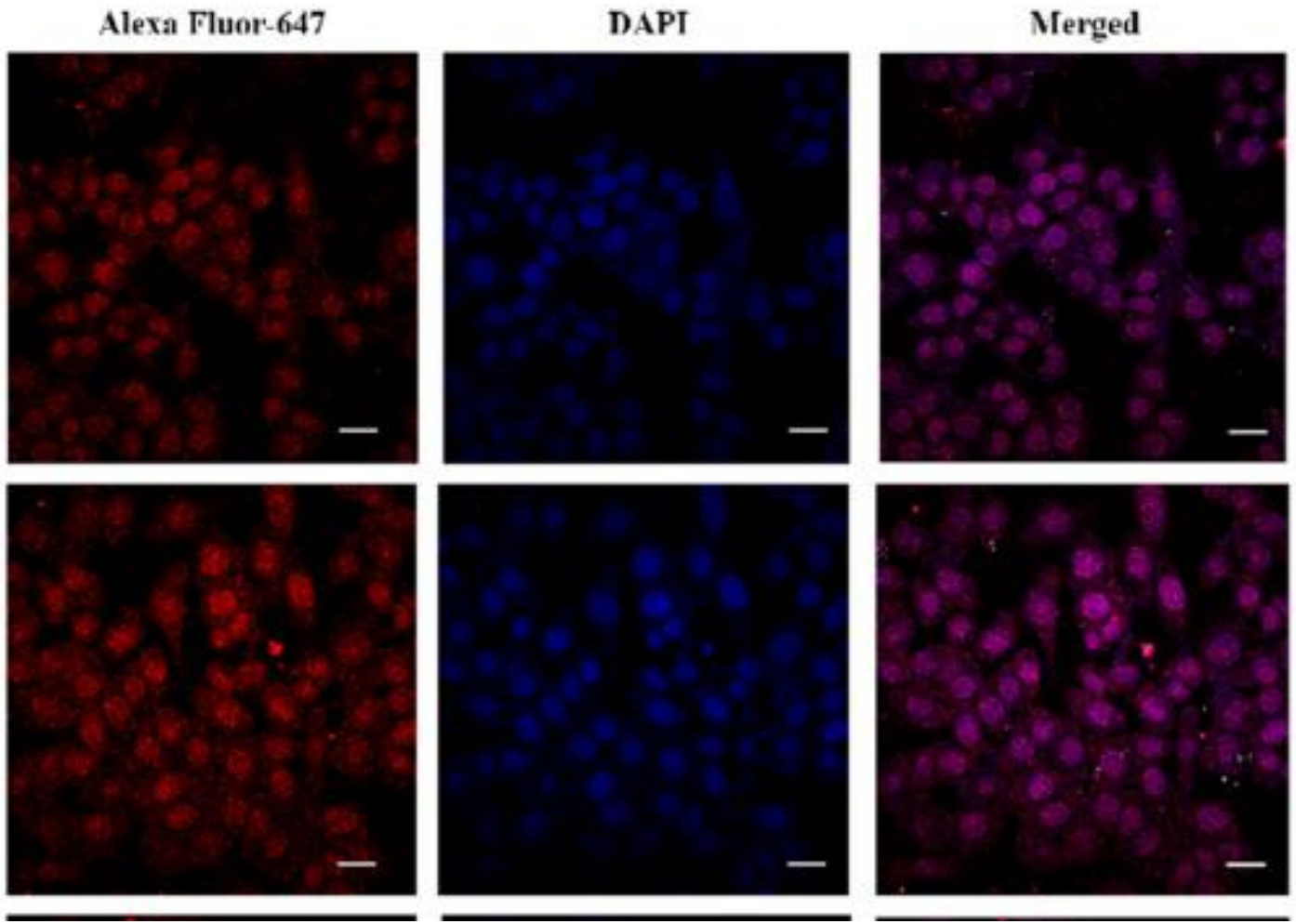


Imaging of Live and dead cell population In Tumor Spheroids

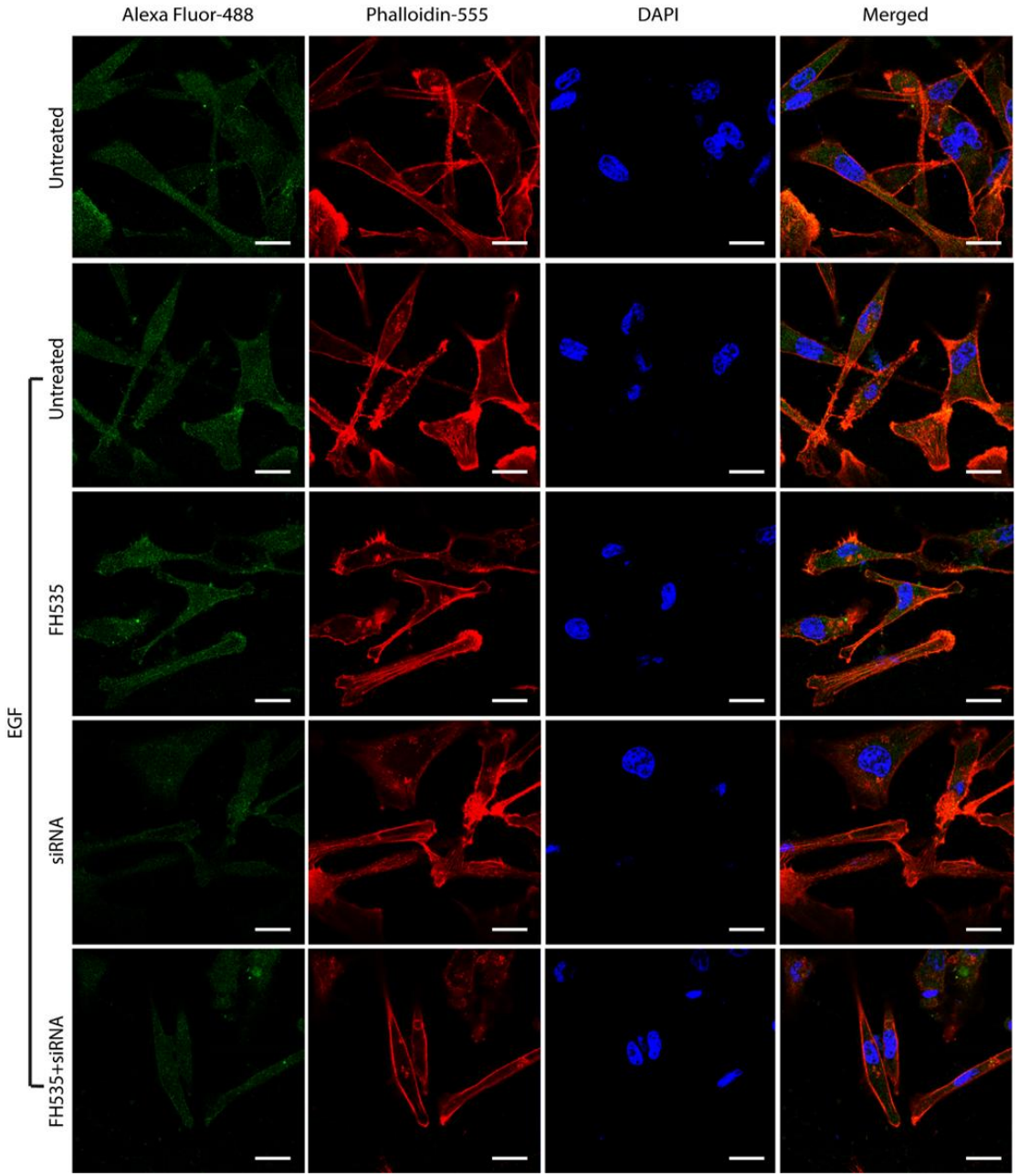
Live cells are denoted by green fluorescence, whereas dead cells are denoted by red fluorescence. Breast cancer spheroids were treated with drugs.



Immunocytochemistry of LC3 in Cancer cells



LC3 has been widely used to monitor the number of autophagosomes as well as autophagic activity.



1. Confocal images of monolayer cultures immune-stained with anti-phosphor-AKT antibody visualized by Alexa Flour-488.
2. Actin cytoskeleton were stained with Alexa Flour-555 conjugated phalloidin.
3. Nuclei were stained with DAPI.

THANK YOU