“Techniques in Cellular and Molecular Neurobiology”

International Master’s Degree in Neuroscience

Lesson 6

DIPARTIMENTO DI SCIENZE DELLA VITA

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MICROSCOPY and BIOLOGY

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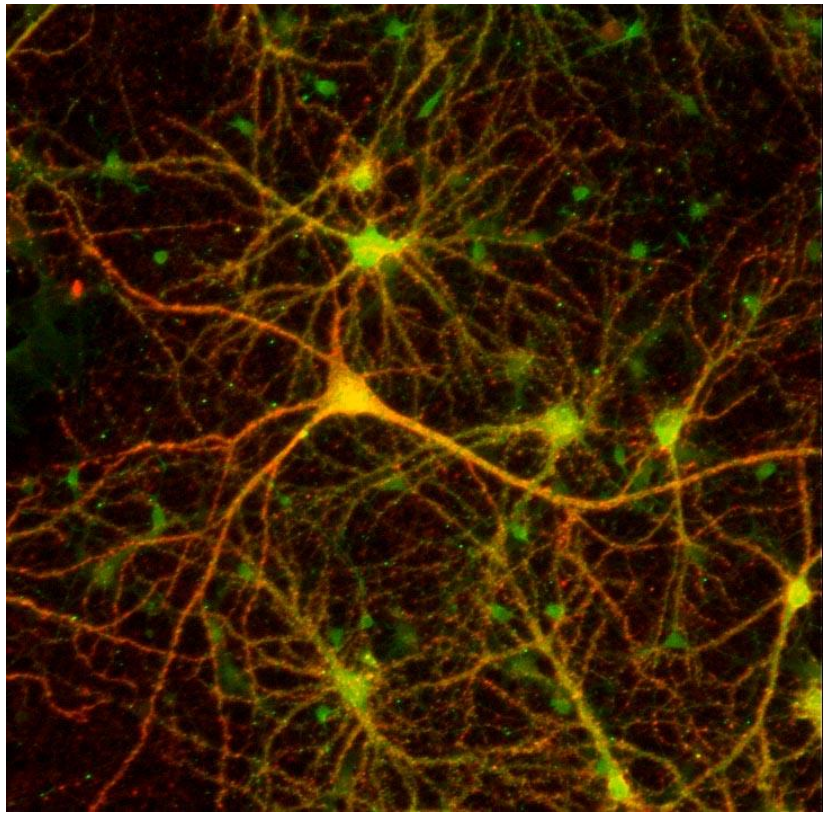

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Microscopy

After reading this chapter, you should be able to:

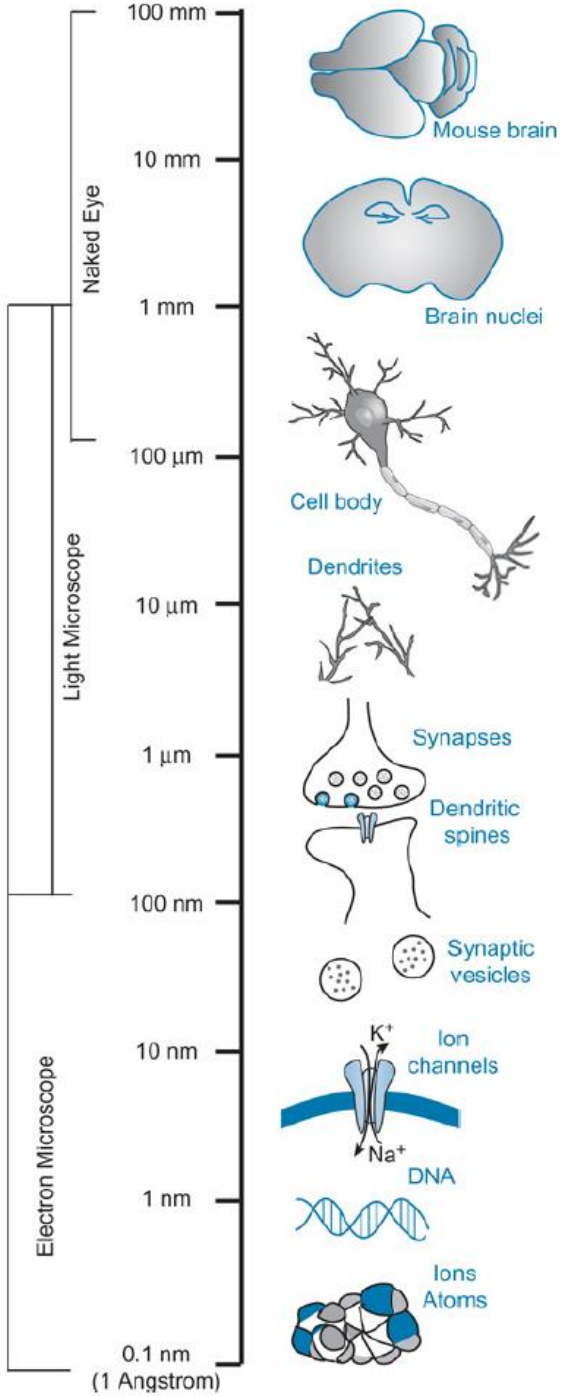
- Define magnification and resolving power, as well as the parts of a microscope that affect these parameters
- Describe the fundamental parts of a standard light microscope and how they manipulate light to magnify an image
- Compare the relative strengths and limitations of common forms of microscopy
- Discuss issues related to image preparation and analysis

MICROSCOPY

There are two important values to consider in microscopy:

Magnification refers to how much larger the sample appears compared to its actual size.

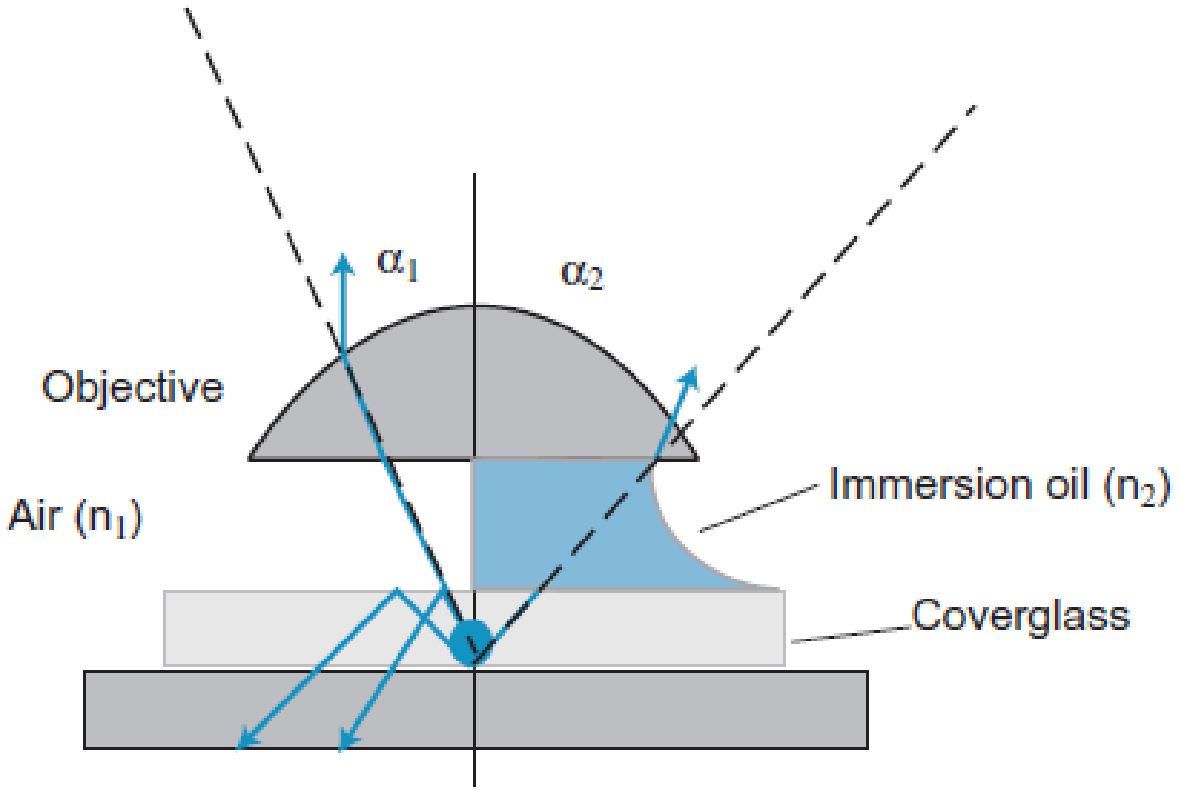
Resolution (or **resolving power**) refers to the minimum distance by which two points can be separated yet still be distinguished as two separate points.



Microscopy is not simply see “bigger”!!

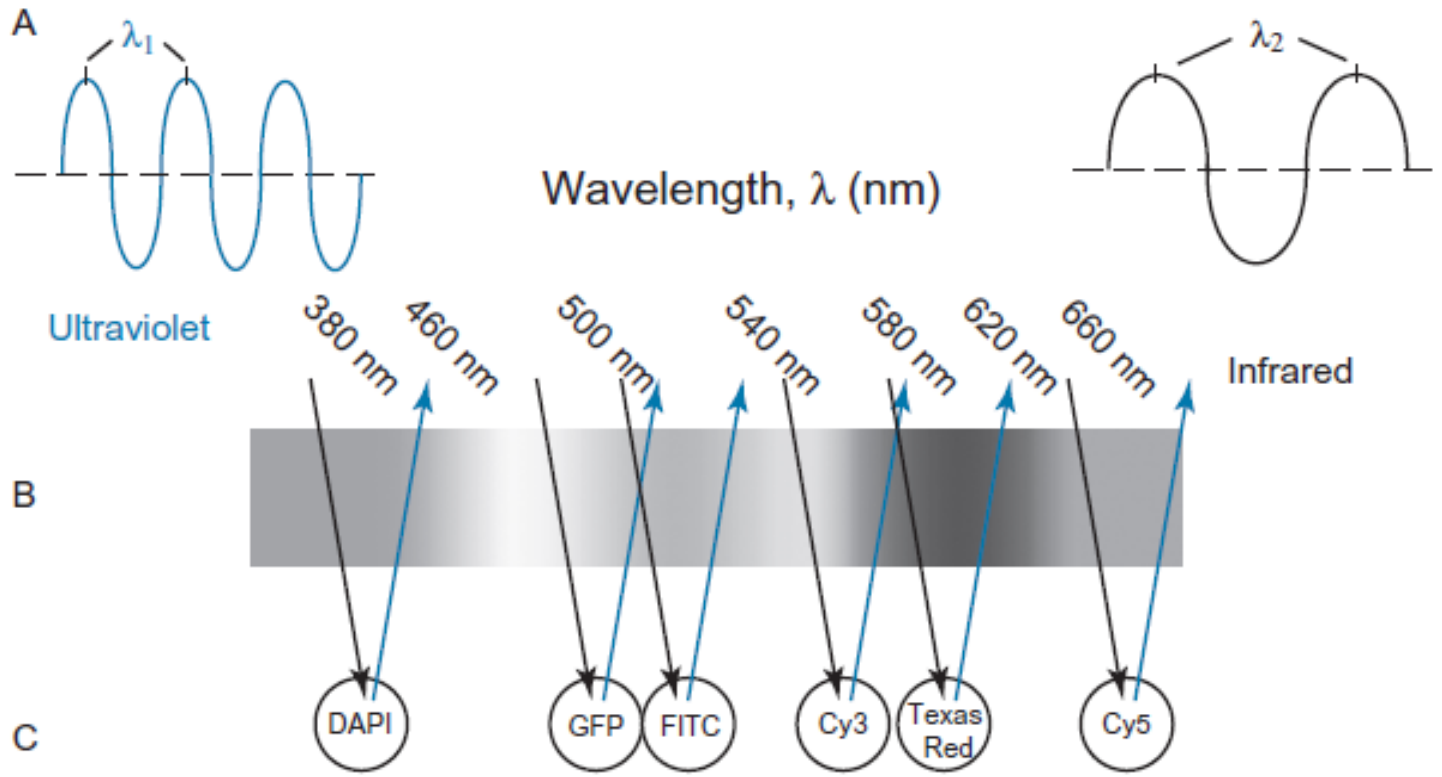
Location
Morphology
Intensity/Amount
Movement
Interactions
Etc.

Numerical aperture

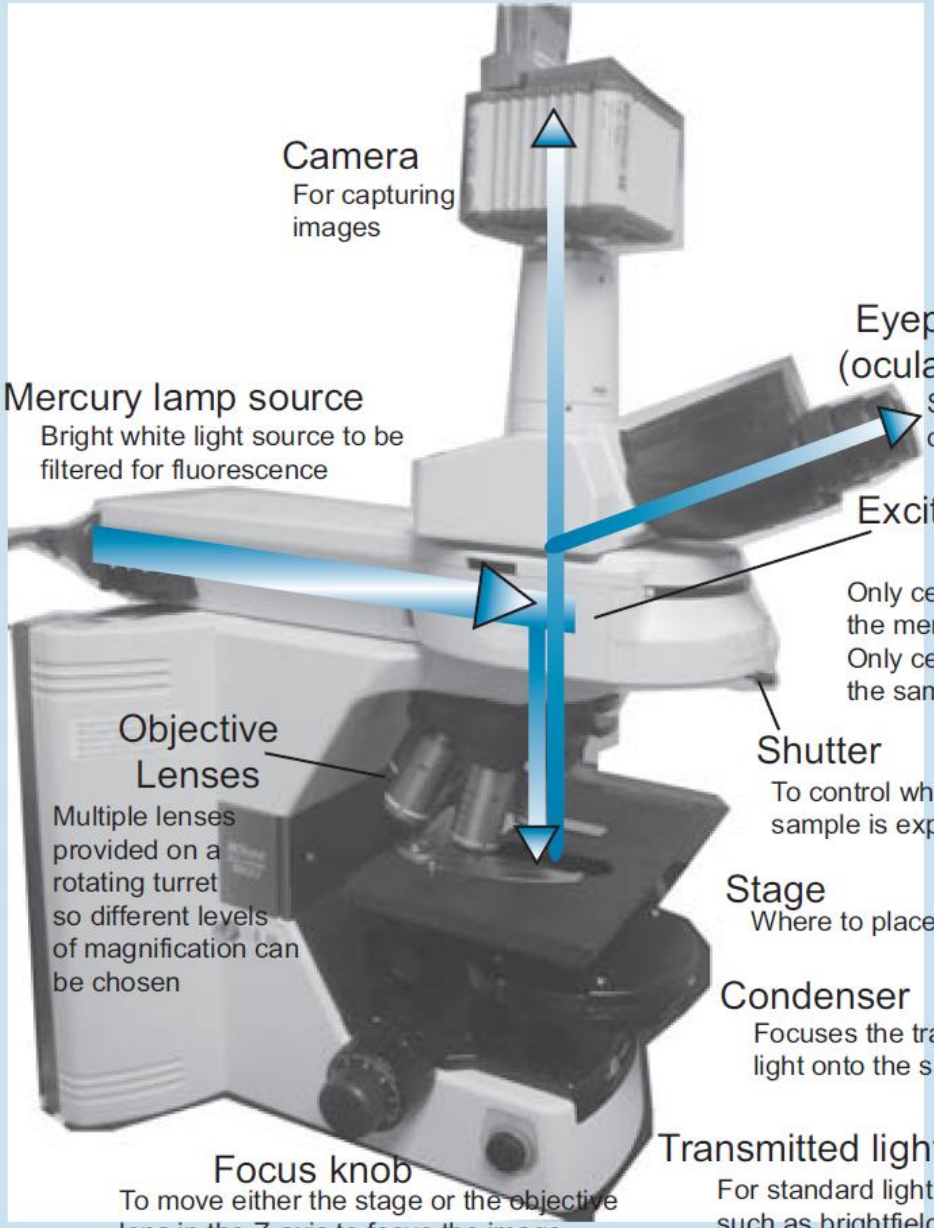


Numerical aperture and the angle of light entering the objective.
An objectivewith a higher NA collects more light rays, leading to a higher resolving power

Light wavelength and the visible spectrum



(A) The wavelength of light, λ , is the distance between two repeating units of a propagating light wave. (B) Light rays of different wavelengths appear as different colors to the human eye. Shorter wavelengths (λ 1) appear violet-blue, while longer wavelengths (λ 2) appear more red. (C) Approximate peak excitation (black) and emission (blue) wavelengths of commonly used fluorophores



The blue arrows indicate the light path for fluorescence.

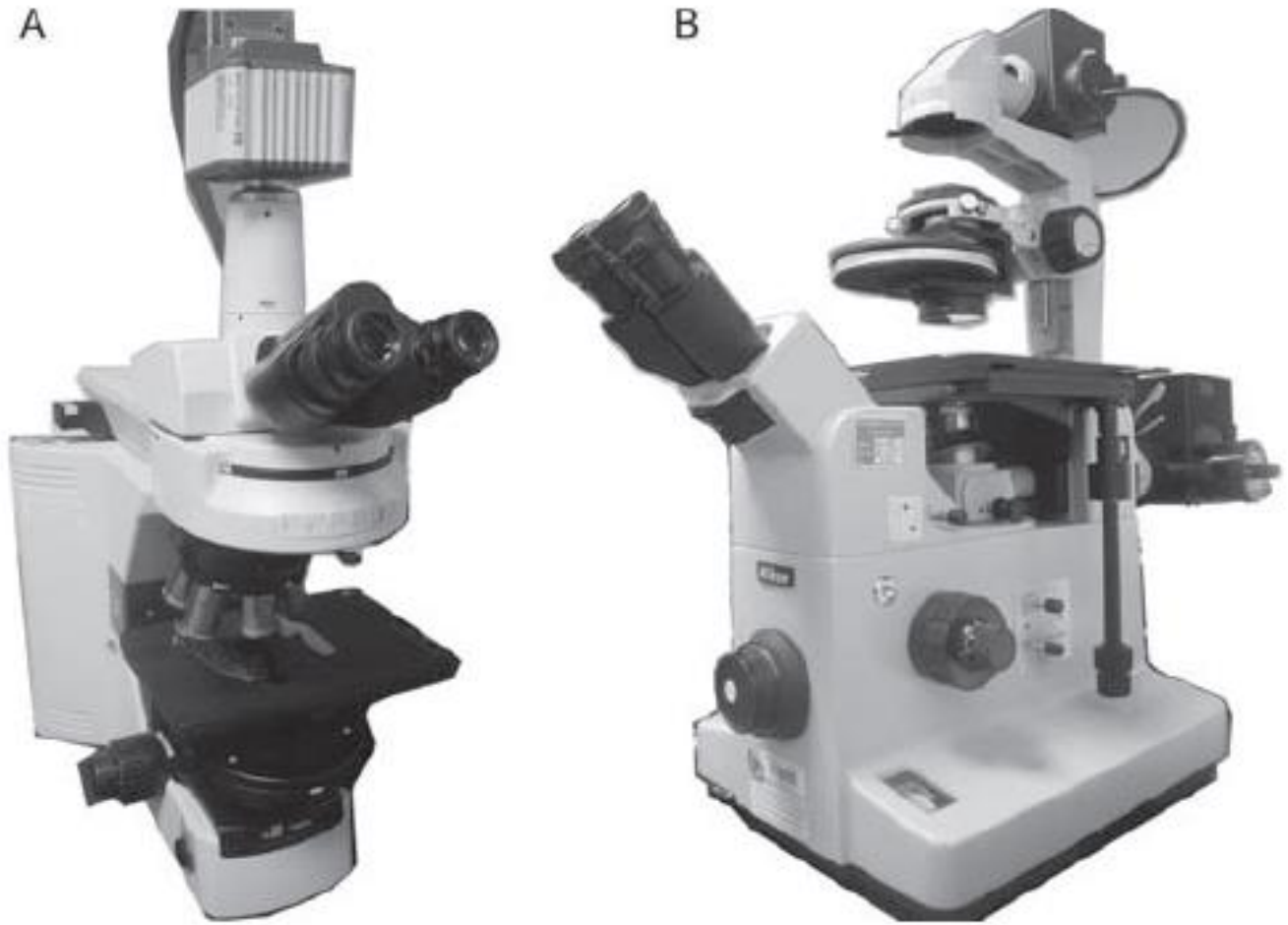


FIGURE 5.6 Upright versus inverted microscopes. **(A)** In an upright microscope, the specimen is placed just below the objective. **(B)** In an inverted microscope, the specimen is placed just above the objective, allowing for larger cell culture plates and access from above.

Four types of light microscopy used to analyze the same cell

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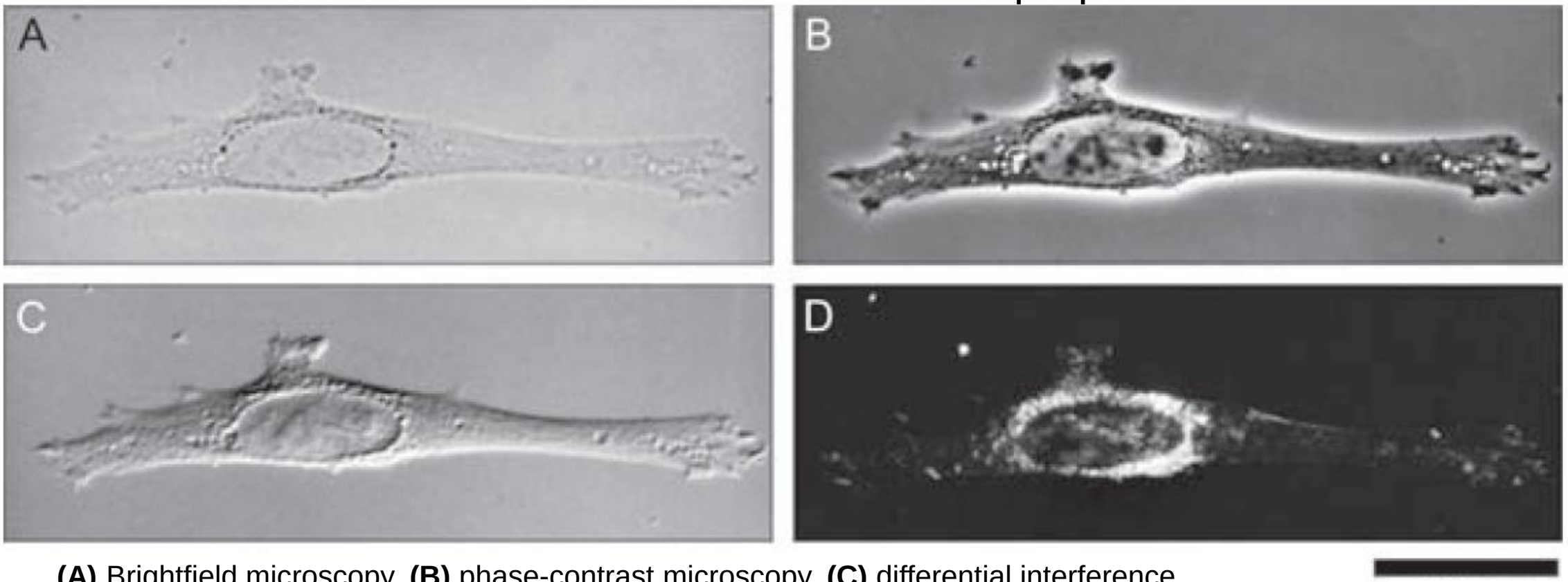
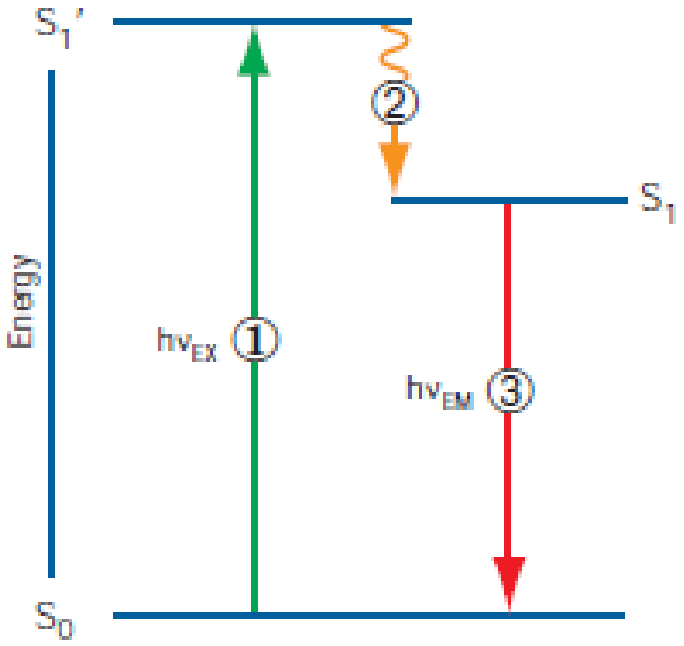
Various methods of light microscopy are used to enhance contrast and visualize details in unstained tissue preparations.



(A) Brightfield microscopy, **(B)** phase-contrast microscopy, **(C)** differential interference contrast (DIC/Nomarski) microscopy, and **(D)** darkfield microscopy.

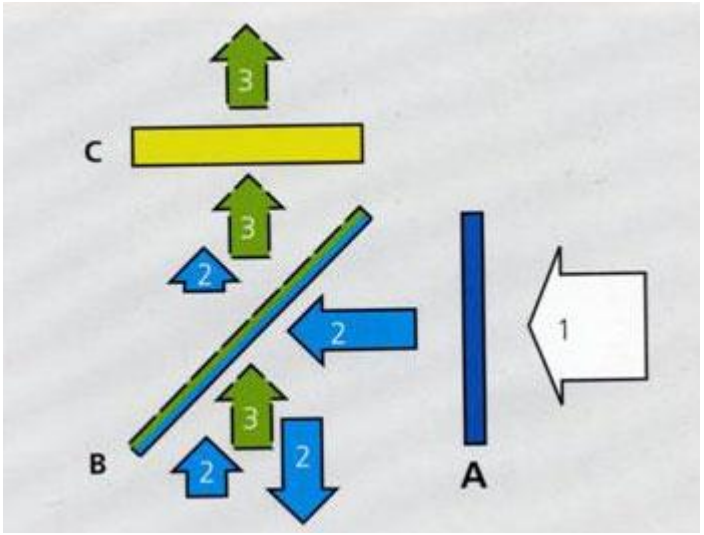
Fluorescence process



Jablonski diagram illustrating the processes involved in creating an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. ① Excitation; ② Vibrational relaxation; ③ Emission.

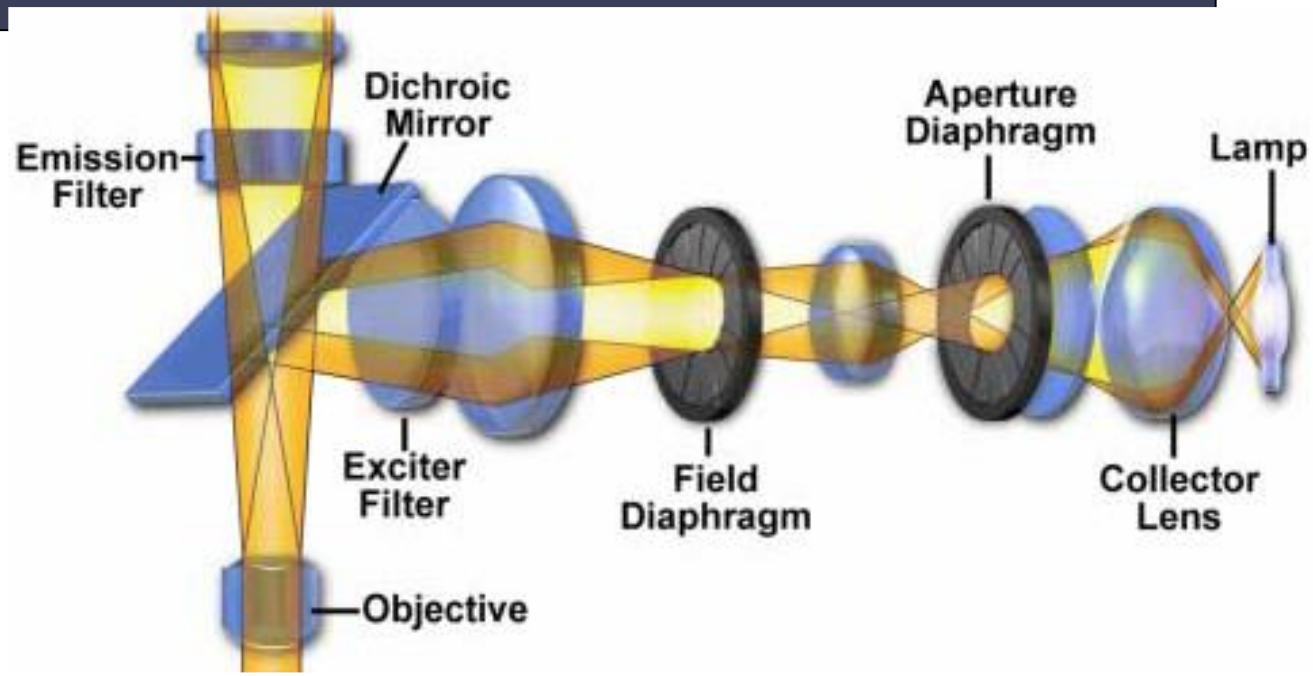
Filter-set and its choice, limitation

Arrow 1 represents the excitation light source of mixed wavelength. **Filter A** is the excitation filter, which is usually a band pass or long pass filter, allows light at certain wavelength range or wavelength longer than cut-off value to go through.



Arrow 2 represents excitation light which passes through A and reaches the surface of **Beam Splitter B**, BSP is a special filter which reflects certain wavelength away but permit other wavelength to pass. A multiple-bands reflecting filter or neutral percentage splitter can be used for this role. Wavelength falling into the reflecting band or shorter than the cut-off value will be stopped and reflected to the specimen. The fluorescence light emitted from specimen is illustrated as **Arrow 3**. The emission is longer than excitation wavelength and cut-off value of the BSP, so the returned emission can go through the BSP towards **emission filter C.**

Fluorescence Microscopy



Schematic diagram of the configuration of reflected light fluorescence microscopy. Light emitted from a mercury burner is concentrated by the collector lens before passing through the aperture and field diaphragms. The exciter filter passes only the desired excitation wavelengths, which are reflected down through the objective to illuminate the specimen. Longer wavelength fluorescence emitted by the specimen passes back through the objective and dichroic mirror before finally being filtered by the emission filter.

FILTER CUBE

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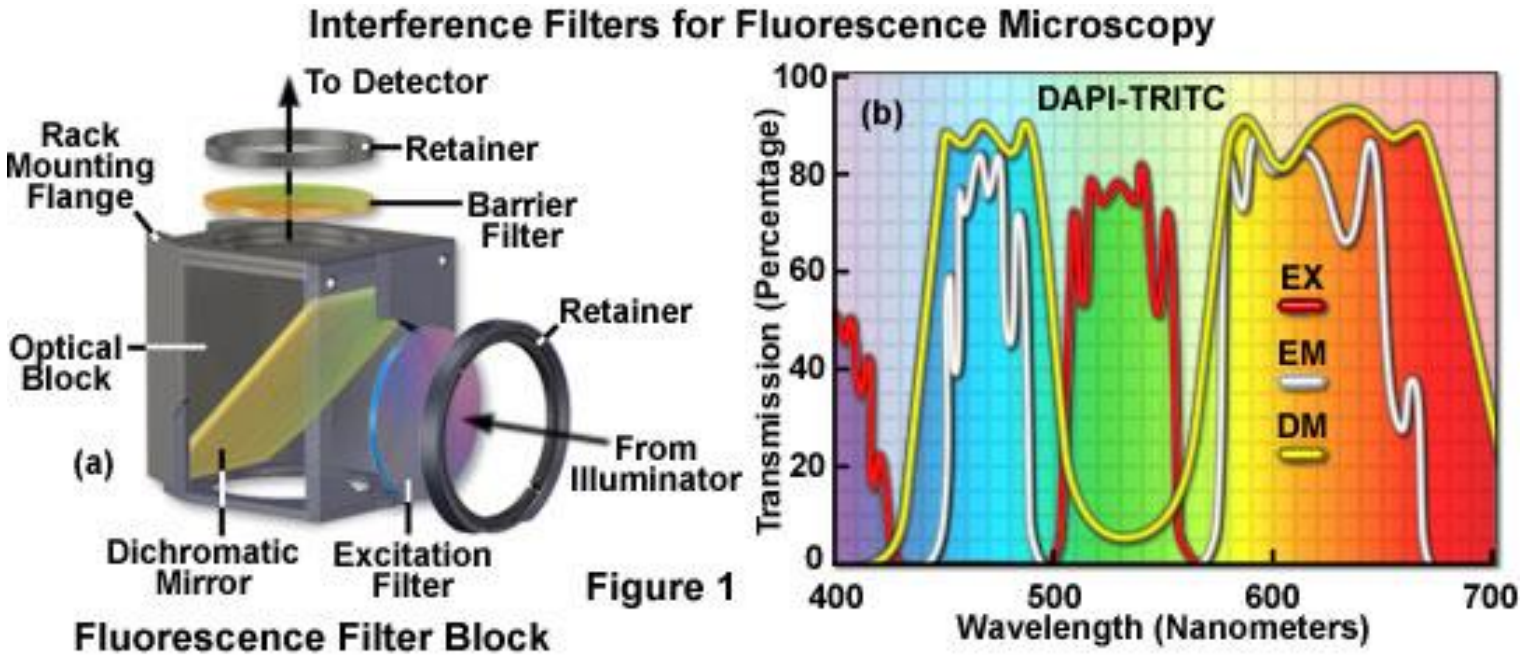
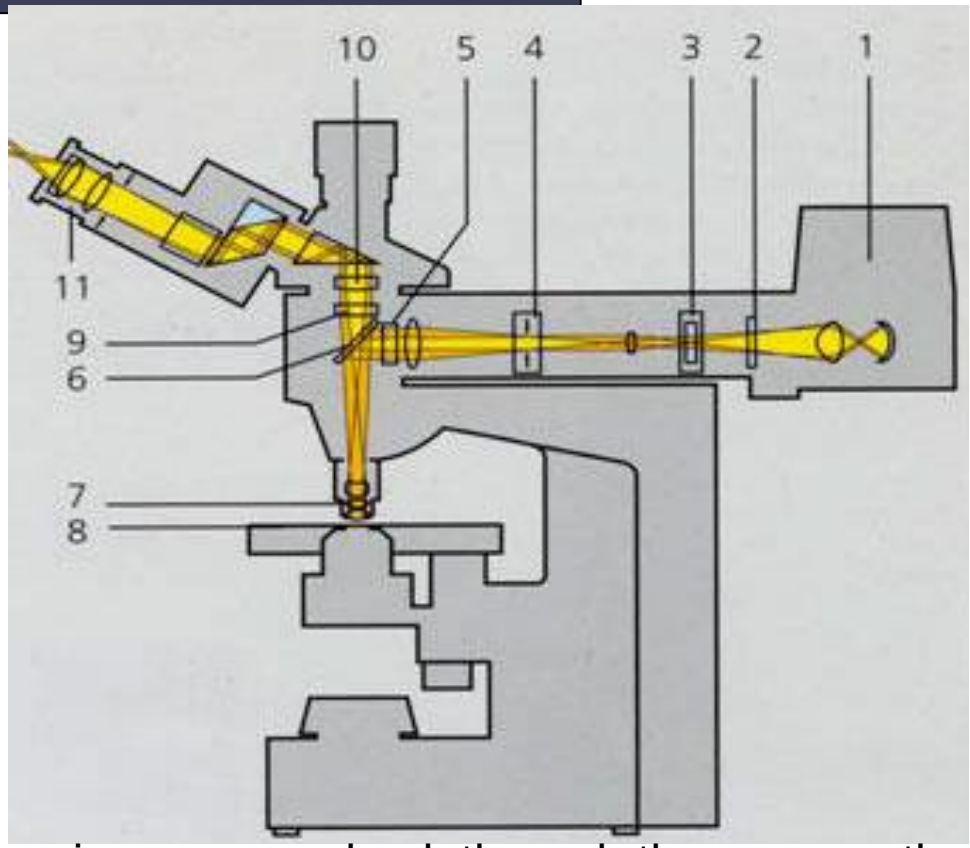


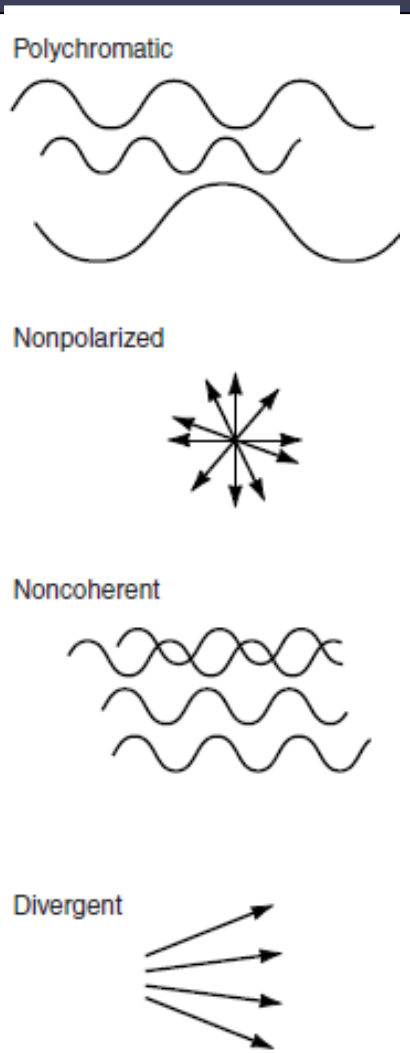
Basics of conventional fluorescence microscope

The light from source (1) pass through neutral density filter, aperture, field stop (2,3,4) for Köhler illumination adjustment, reaches excitation filter(5). After filtering away unwanted wavelength, excitation light goes further and reaches a special and important filter called beam splitter (6), BSP for short. The excitation light selected by BSP is reflected to the specimen via objective (7) and excites fluorophores within specimen (8).

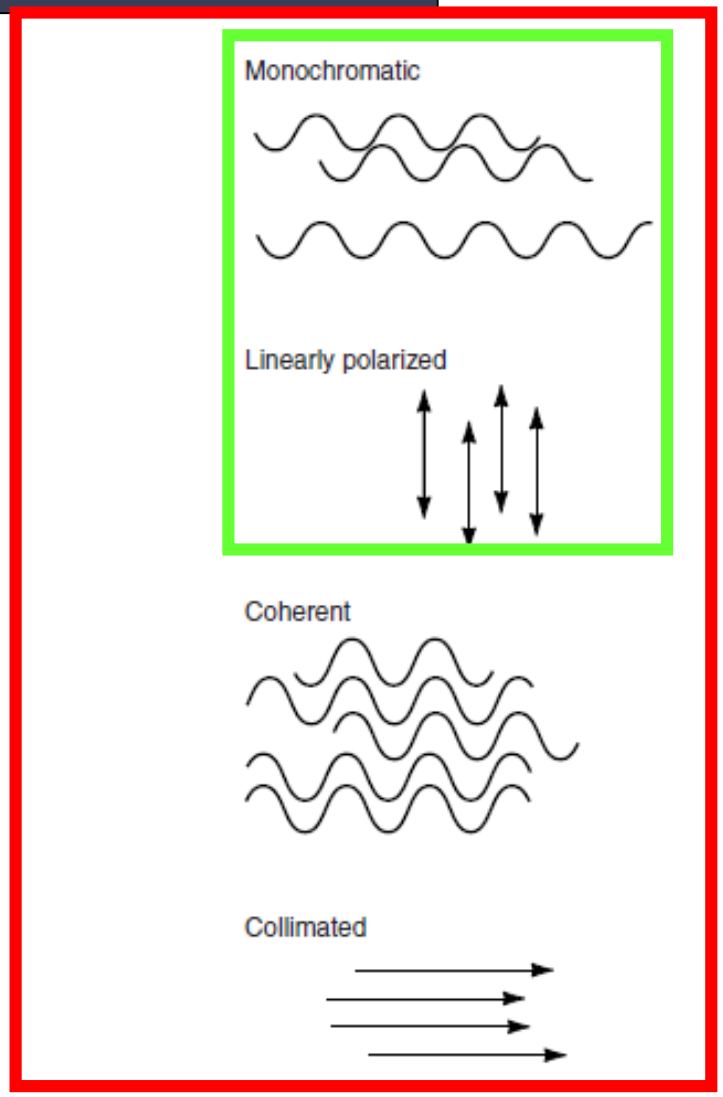


The fluorescence emission from the specimen comes back through the same path to BSP again. This time, the light should not be reflected away but passes through it and reaches emission filter(9). Being further filtered by emission filter, the emission light is either focused on the front focal plane of binocular or projected to infinite by tube lens in case of infinite corrected objectives(10). The final image is further magnified by binocular(11).

Illumination is a critical determinant of optical performance in light microscope



"different lights"



FLUO microscopy

CONFOCAL microscopy

Fluorescence Microscopy

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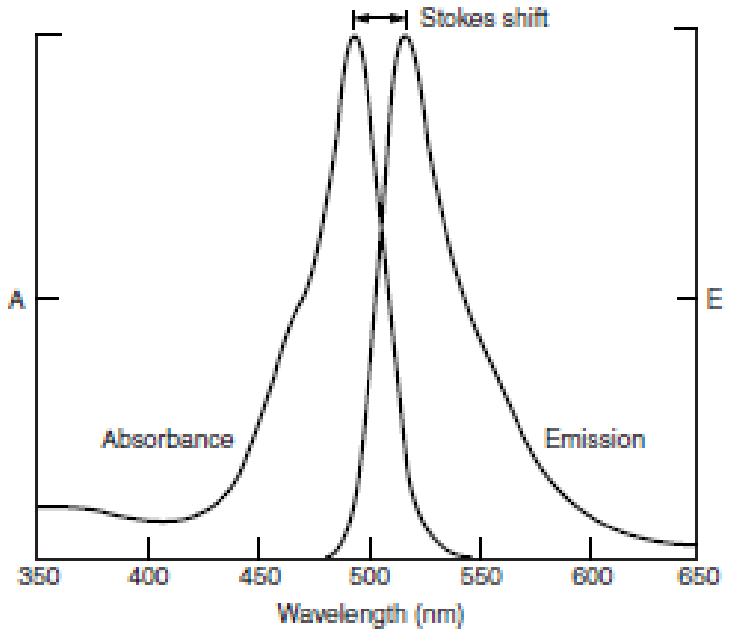

Normalized absorption and fluorescence emission spectra of fluorescein-conjugated IgG. Both spectra span a wide range of wavelengths. Fluorescein has an absorption/excitation peak at 492 nm, but is also stimulated by ultraviolet wavelengths. Fluorescein emission has a peak at 520 nm and looks yellow-green to the eye, but actually fluoresces at wavelengths ranging from blue to red. The difference in nanometers between the excitation and emission maxima is called the Stokes shift.

FLUORESCENT PROTEINS AND PROBES

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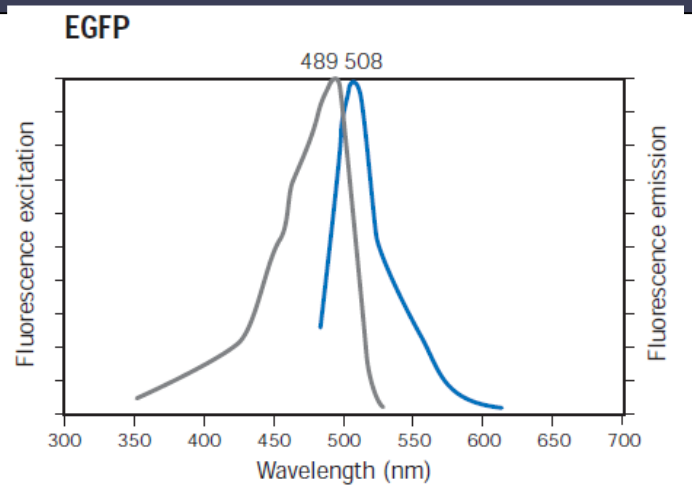
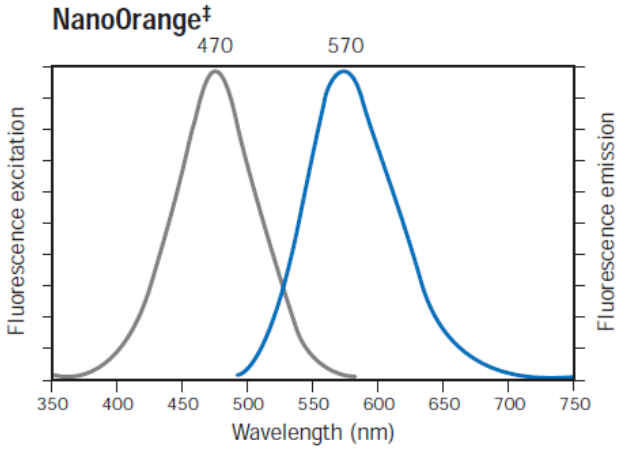
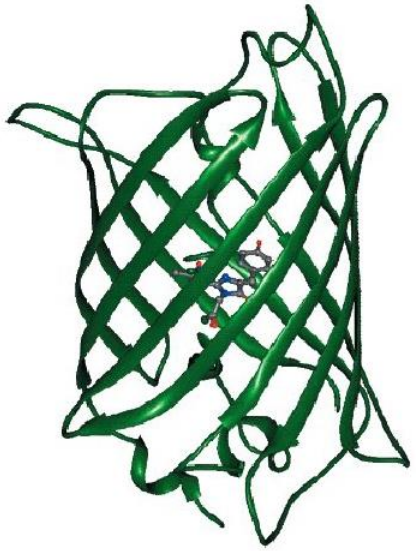
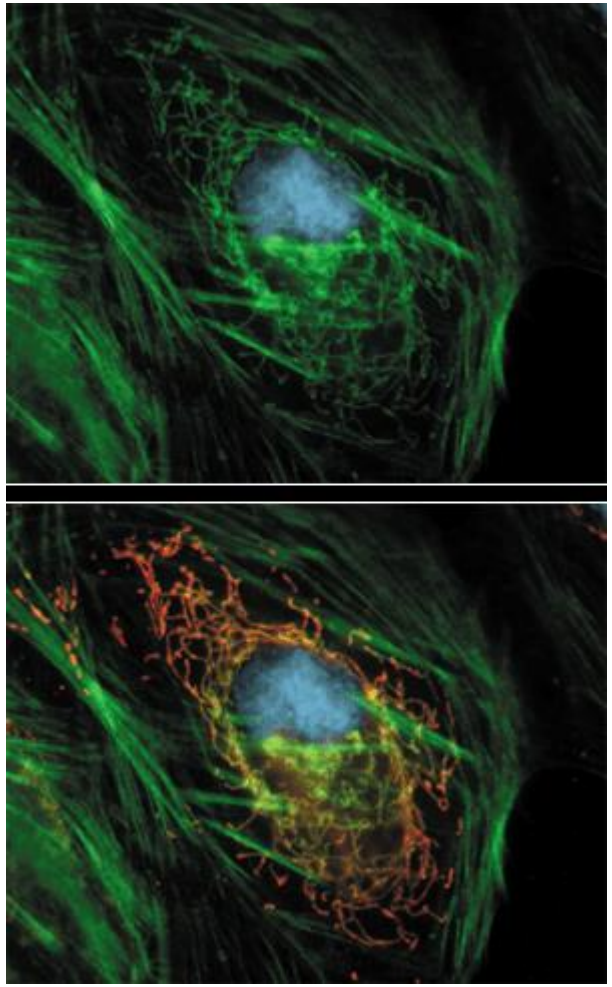
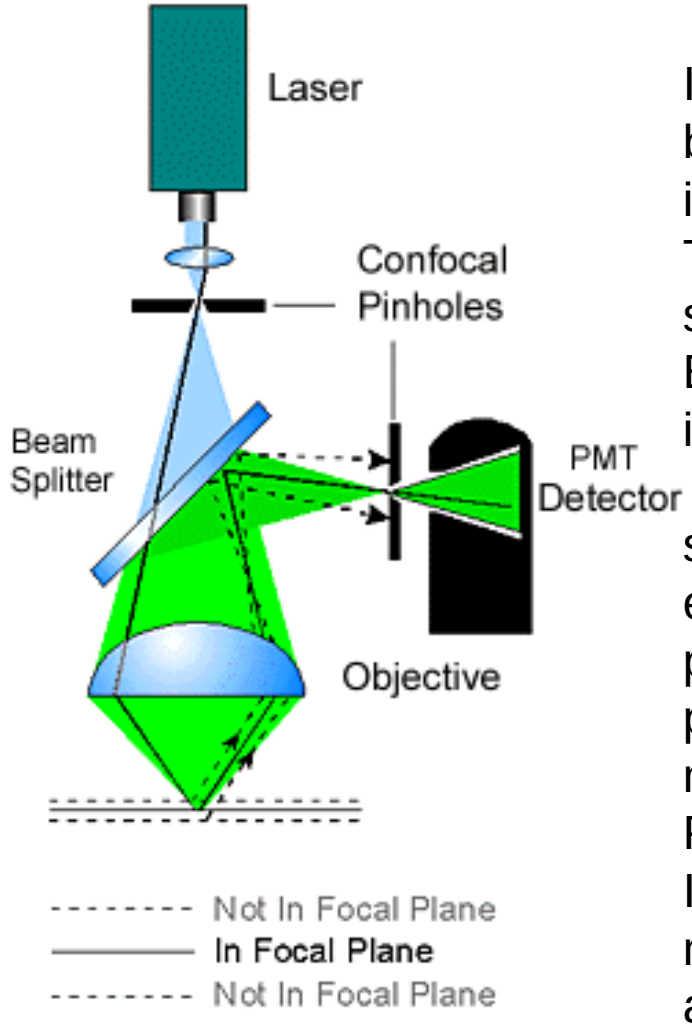








What is confocal?



In practical, a point-like light source is achieved by using a laser light passing through a illumination pinhole.
This point-like light source is directed to the specimen by a beam splitter (or AOBS in Leica's BSP-free system) to form a point-like illumination in the specimen.
The point-illumination move or scan on the specimen by the help of a scanner. The reflected emission light from specimen's focal plane passes through the detecting pinhole and form point-like image on detector PMT (photon multiply tube).
PMT converts detected photon into electron. It is possible to amplify weak signal by manipulating the voltage (gain) on the tube. It is also possible to cut off background signal by set certain threshold (Offset) on the tube.

What is confocal?

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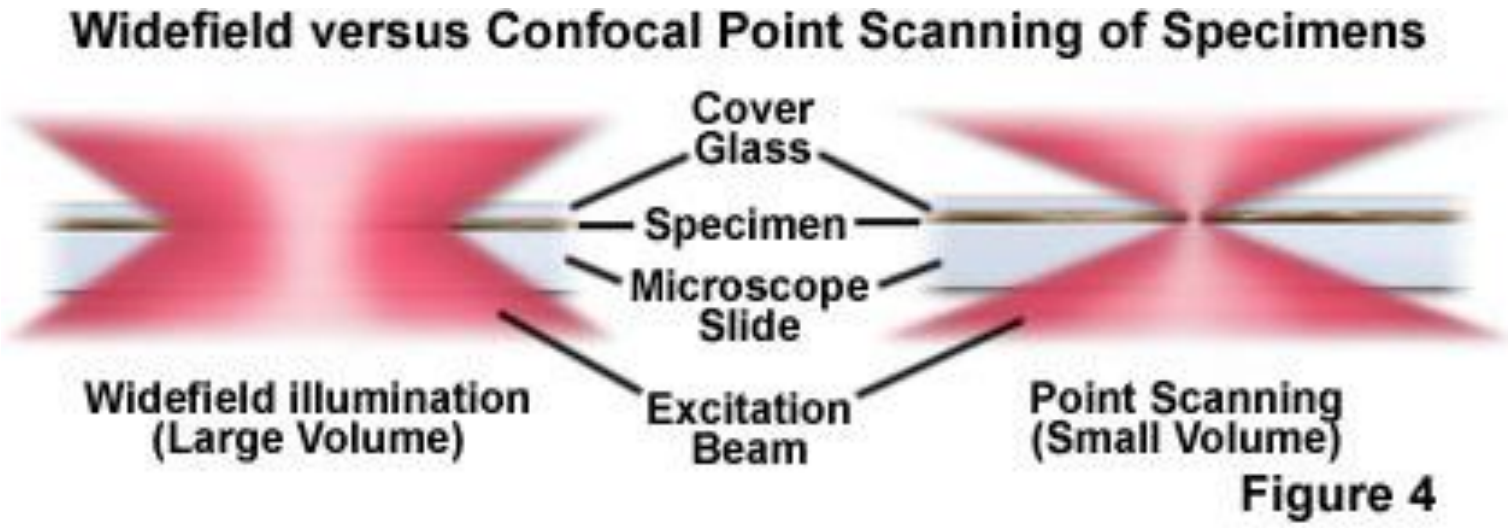

Figure 4

Laser scanning confocal Microscope (LSCM)

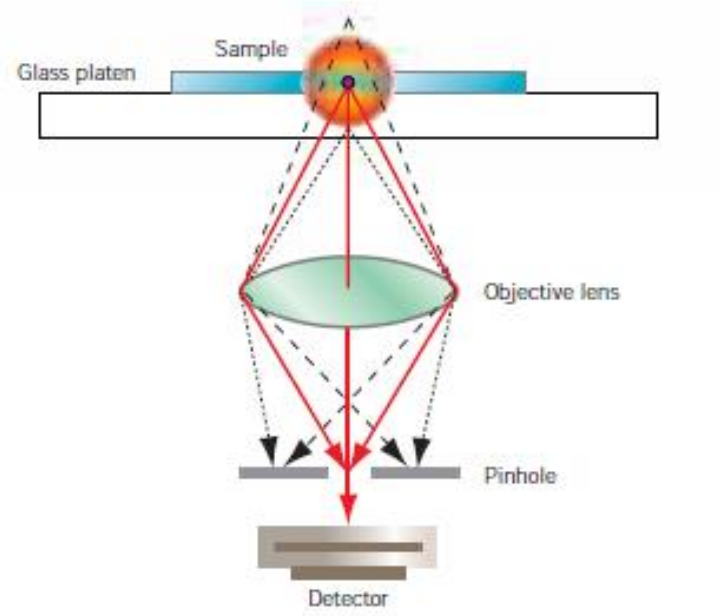

Illustration of confocal optics. Fluorescence from the sample is collected by a objective lens and directed toward a pinhole aperture. The pinhole allows the emitted light from a narrow focal plane (red solid lines) to pass to the detector, while blocking most of the out-of-focus light (black dashed lines).

What is confocal?

1. A point light source for illumination
2. A point light focus within the specimen
3. A pinhole at the image detecting plane

These **three points** are **optically conjugated together** and aligned accurately to each other in the light path of image formation, this is **confocal**.

1. Confocal effects result in supression of out-of-focal-plane light, supression of stray light in the final image
2. Confocal images have following features:
3. void of interference from lateral stray light: higher contrast.
4. void of supperimpose of out-of-focal-plane signal: less blur, sharper image.
5. images derived from optically sectioned slices (depth discrimination)
6. Improved resolution (theoretically) due to better wave-optical performance.

PROBLEMS

confocal effect is obtained at a cost of reduced detecting volume (total signal amount), increase vulnerability to noise, reduced dynamic range.

What is confocal?

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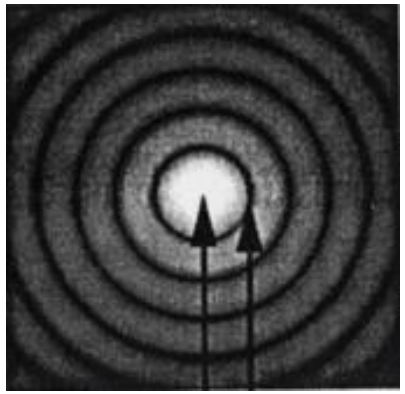
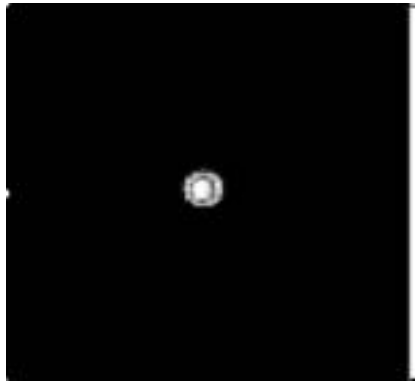
Standard Microscope

Confocal Microscope

Lateral

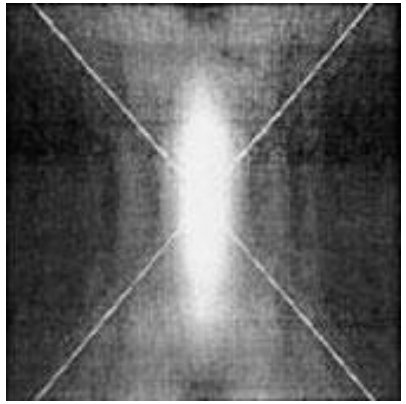


Lateral

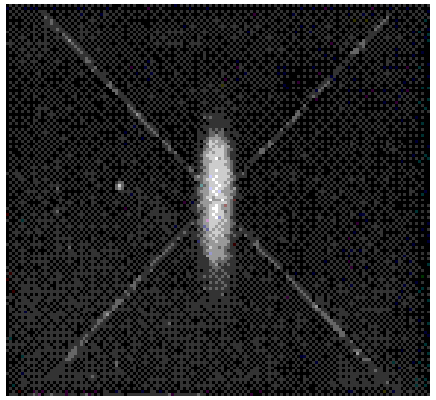


with pinhole

Axial



Axial



with pinhole

Lateral and Axial Resolution in confocal system

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$$Res_{lateral} = \frac{0.51\lambda em}{NA}$$

RESEL

50 -100 nm with interpolation

$$Res_{lateral} = \frac{0.37\lambda ex}{NA}$$

100 -150 nm with interpolation

What is confocal?

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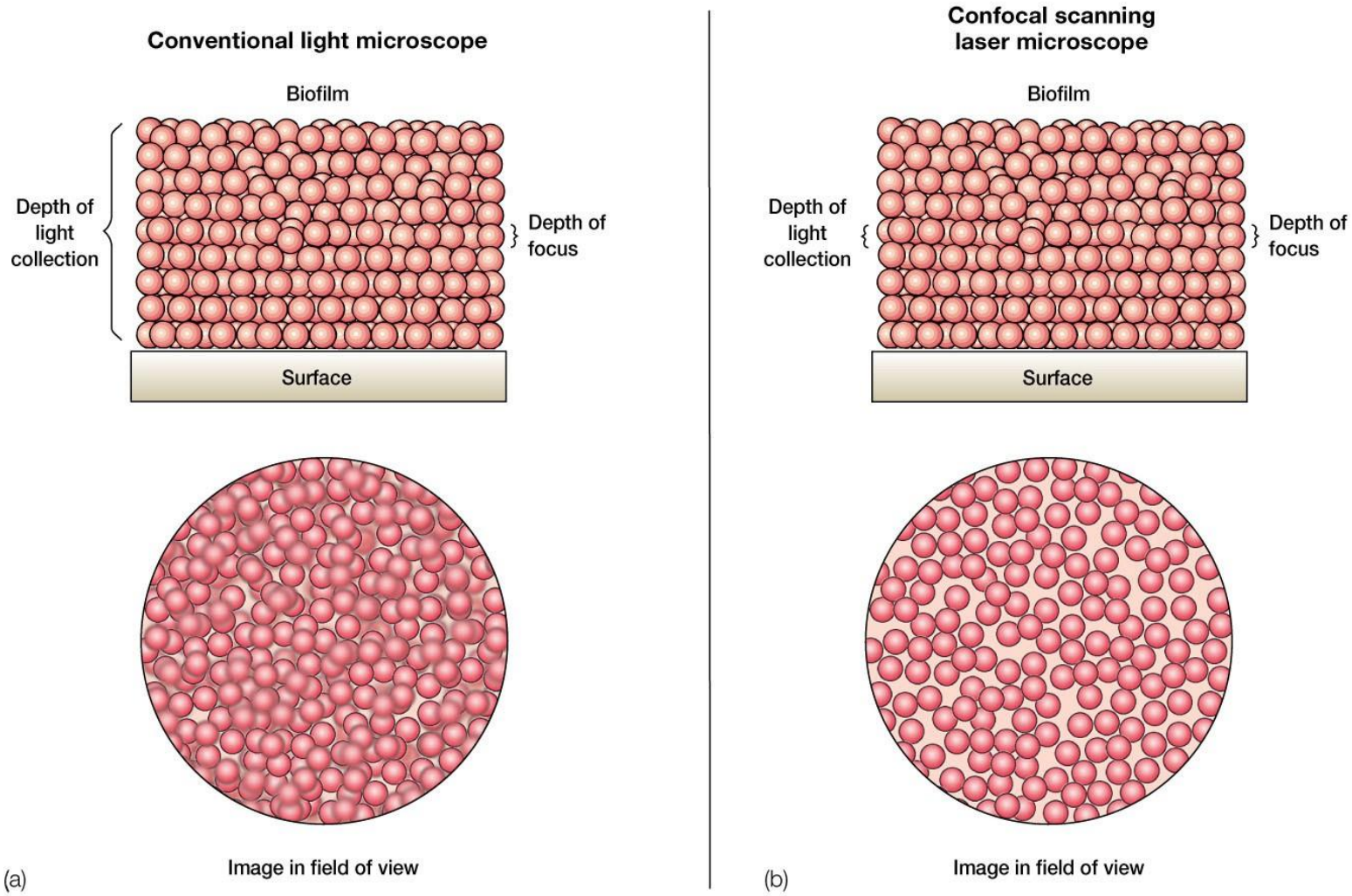

Laser scanning confocal Microscope (LSCM)

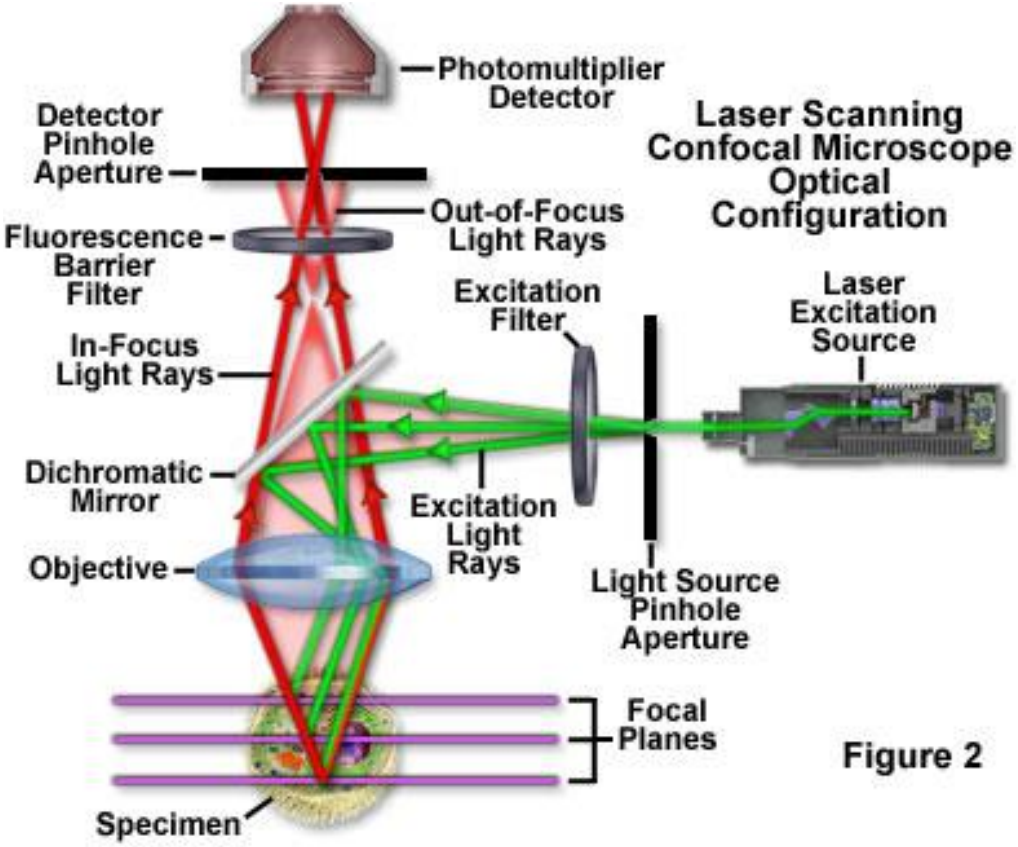

Figure 2

It utilizes laser and illumination pinhole to get point-like light source illumination on the specimen.

Detecting pinhole is used to get rid of out-of-focus signal. PMTs are used as detecting device.
The main advantage of this type is its combination of good image quality, versatile functionality and reliability.

CONVENTIONAL vs CONFOCAL

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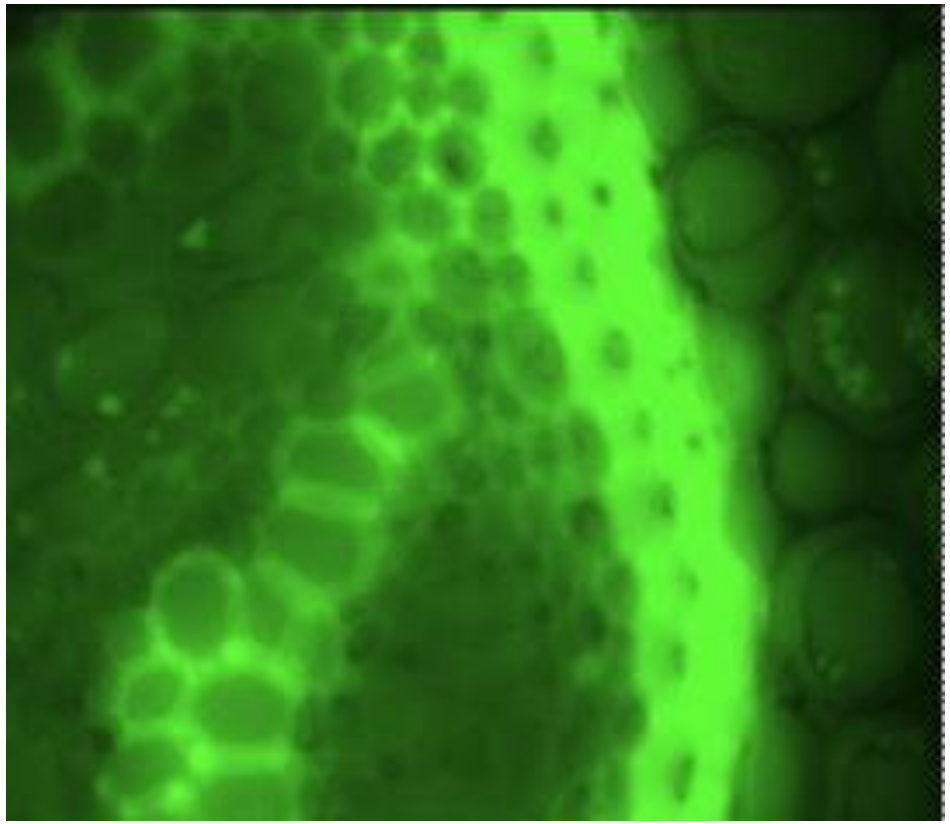
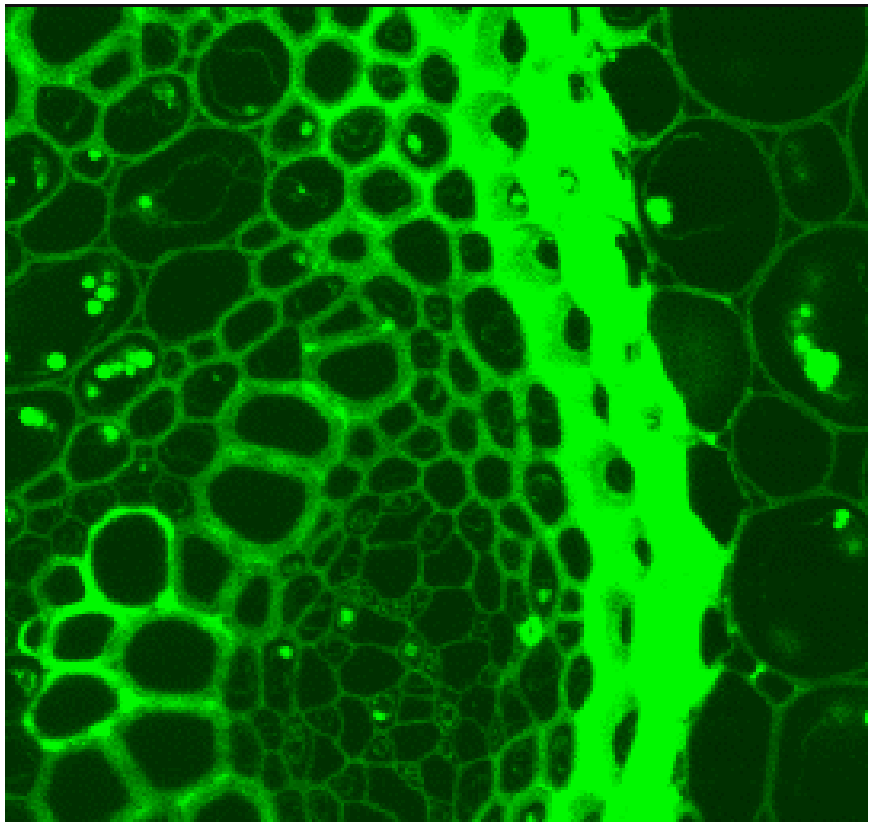
DETAILS





CONVENTIONAL vs CONFOCAL

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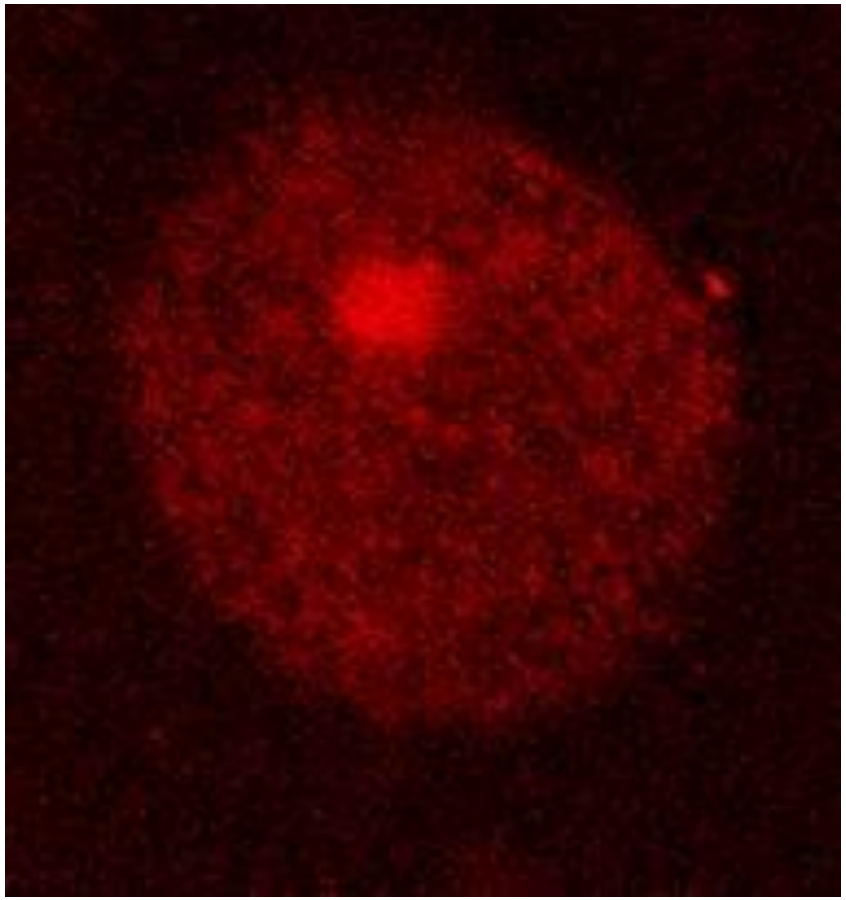
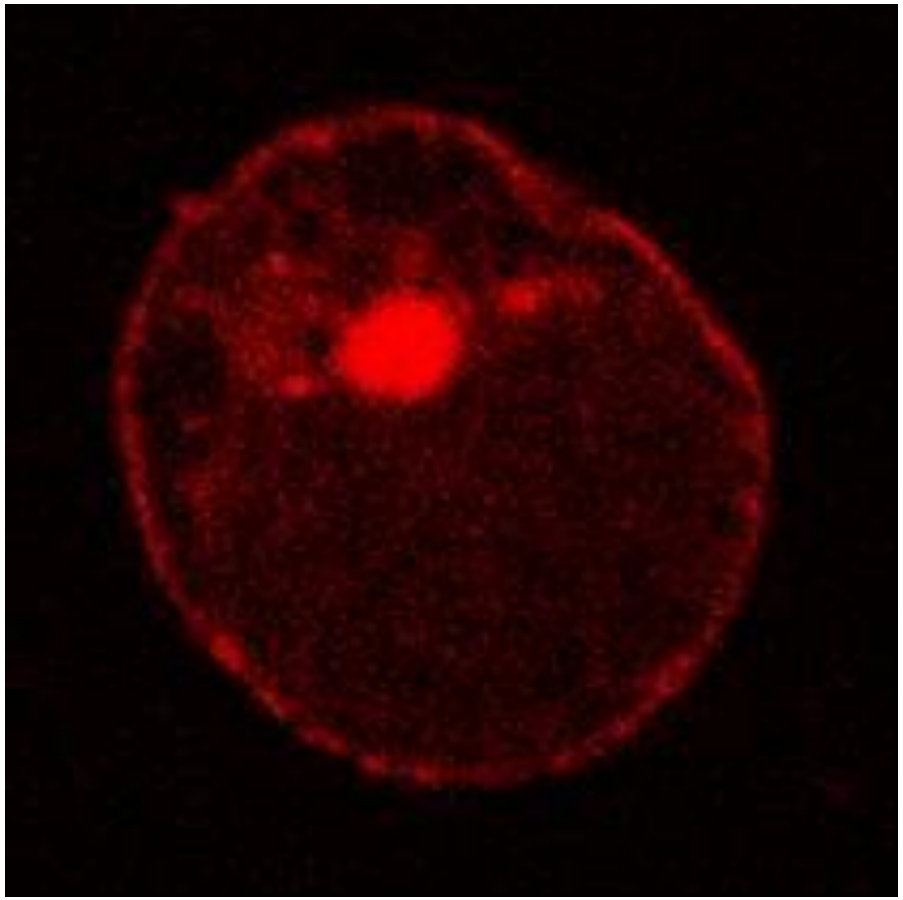
MOLECULES LOCALIZATION





CONVENTIONAL vs CONFOCAL

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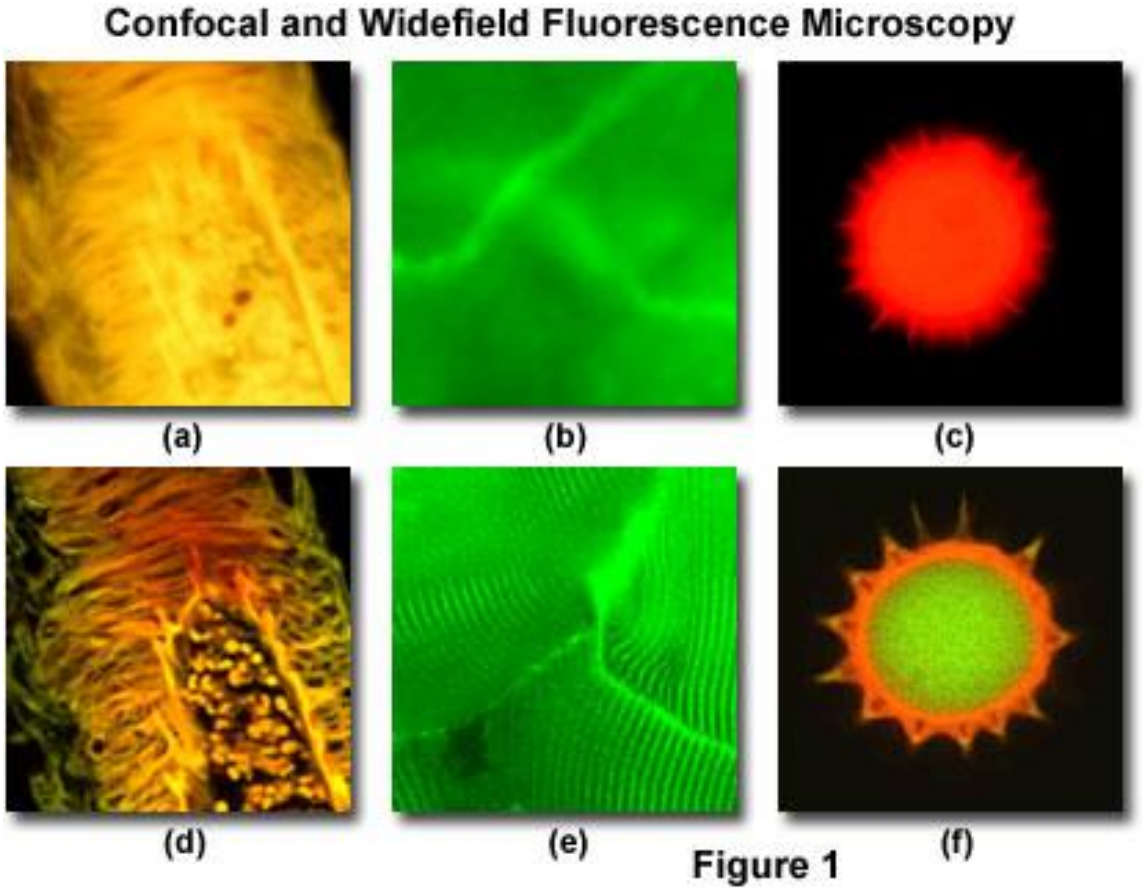

Figure 1

In a conventional widefield optical epi-fluorescence microscope, secondary fluorescence emitted by the specimen often occurs through the excited volume and obscures resolution of features that lie in the objective focal plane.

CONVENTIONAL vs CONFOCAL

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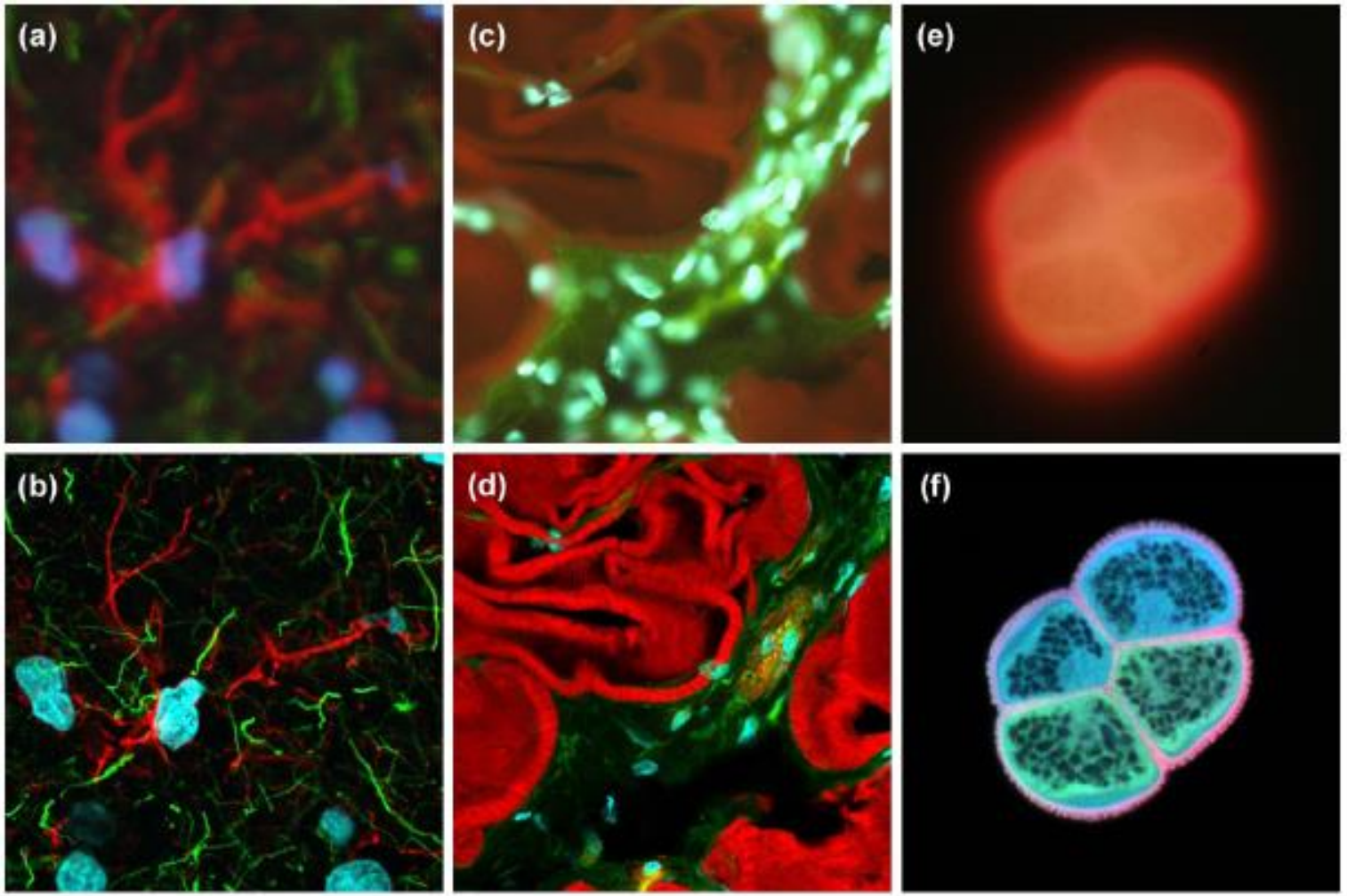
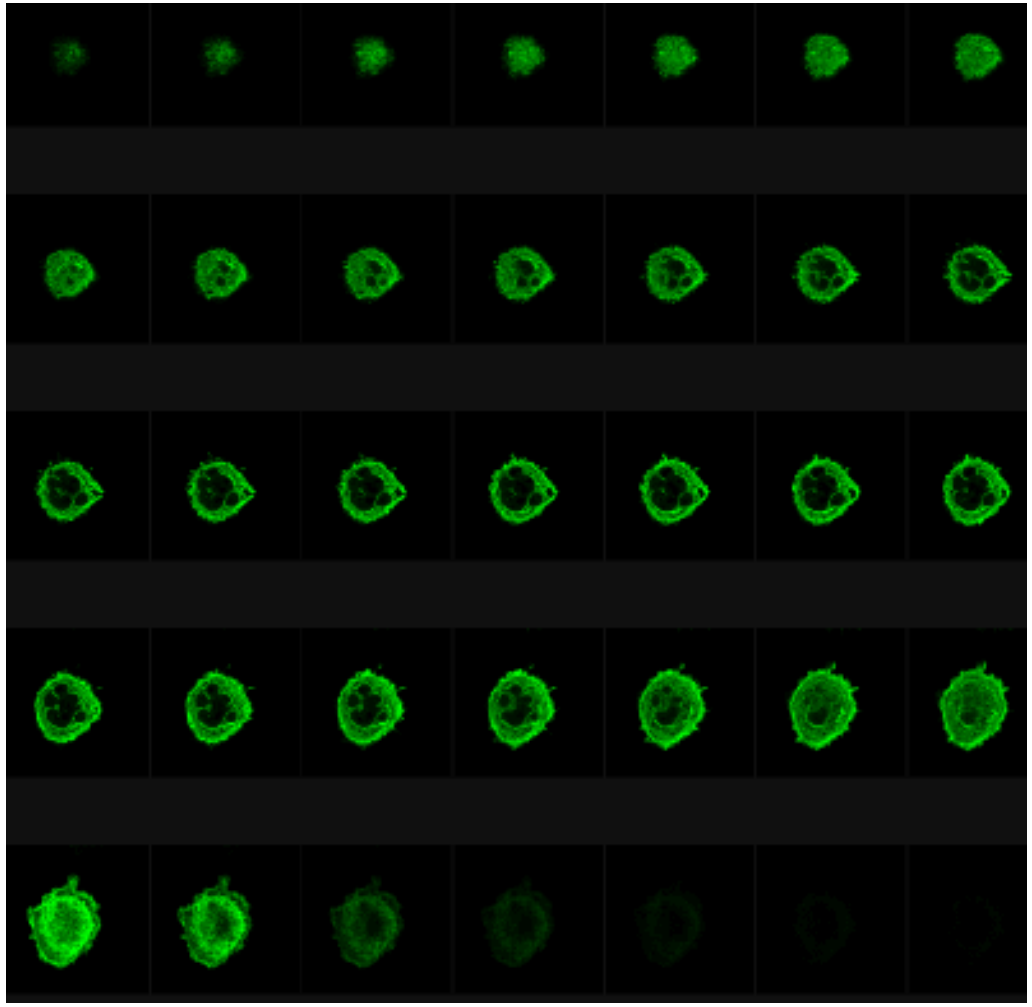


Confocal Microscopy is the only way to get a true Z-stack for 3-D reconstruction from light microscope.



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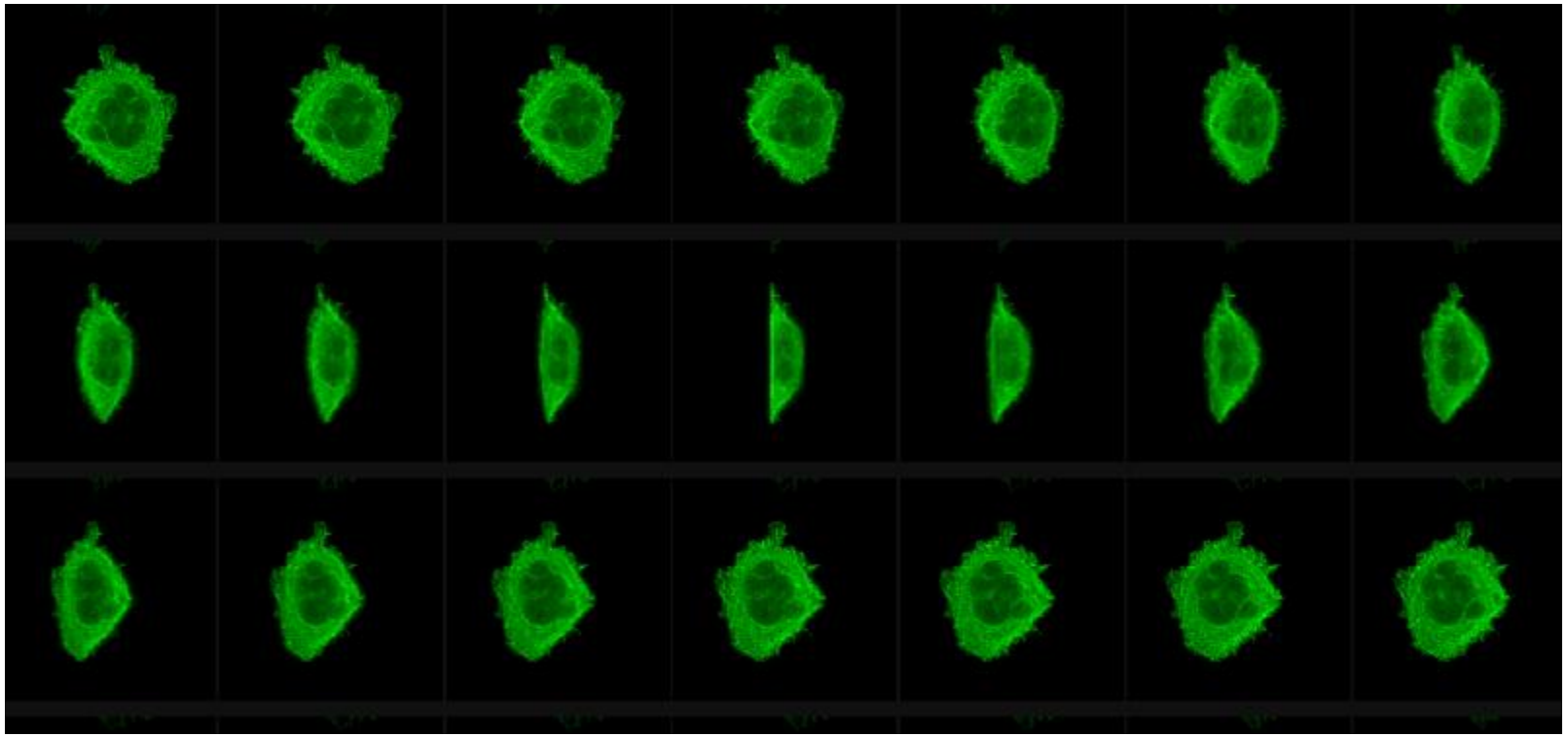

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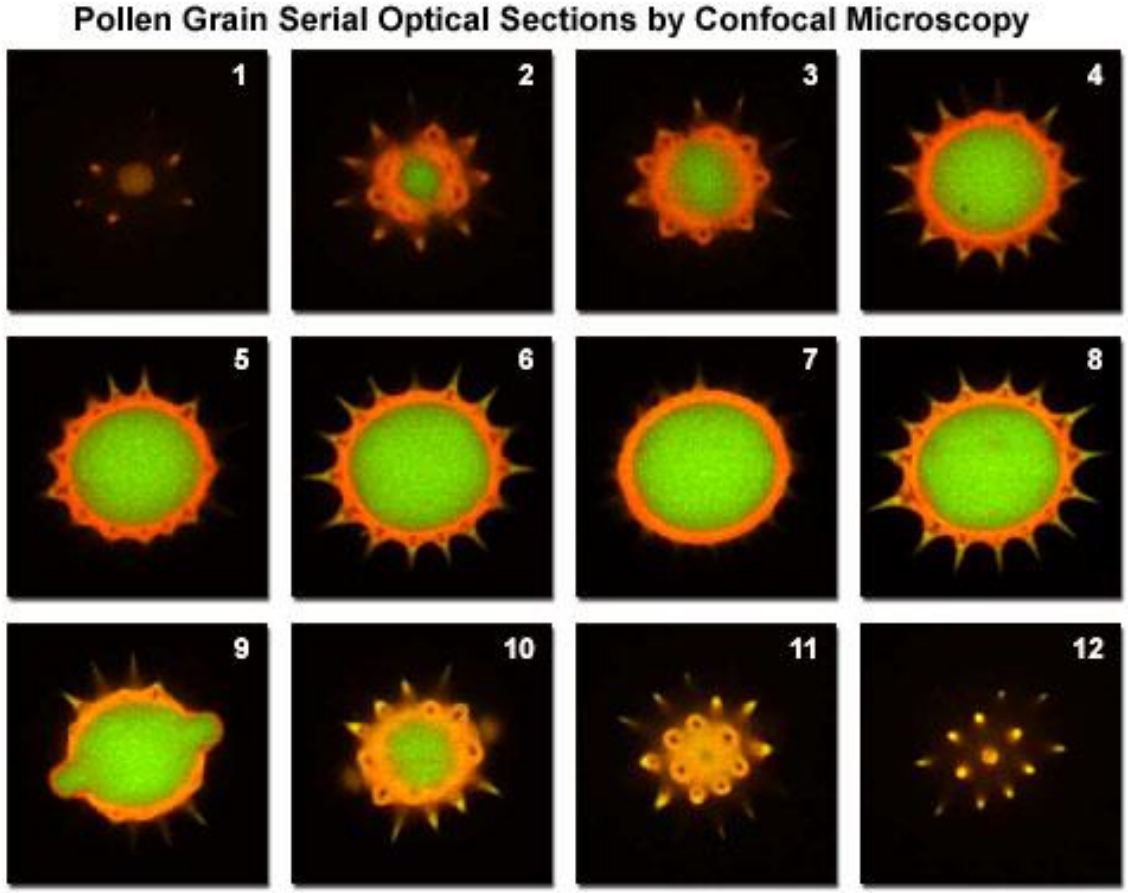
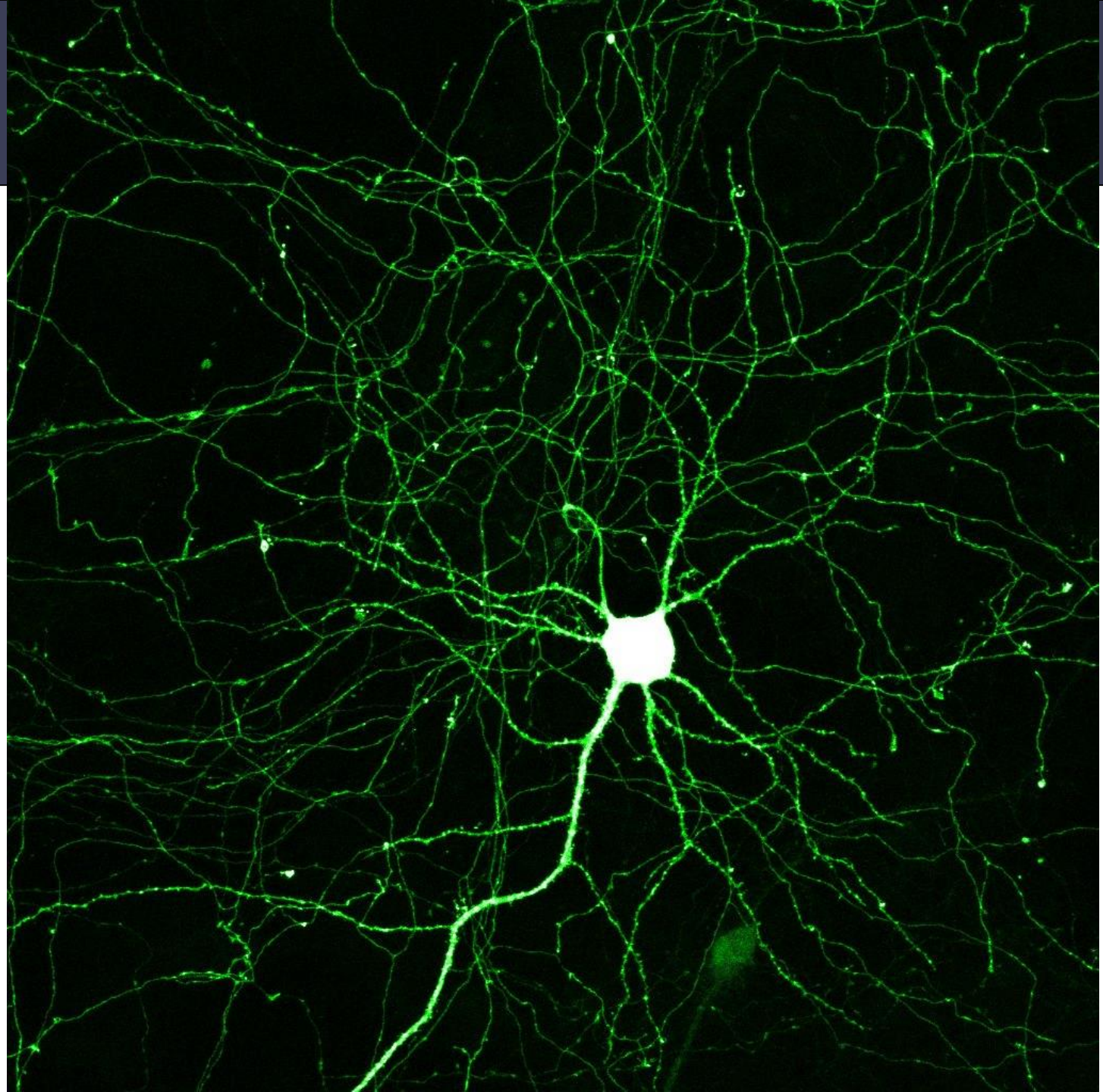


Figure 6



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3D CONFOCAL rendering

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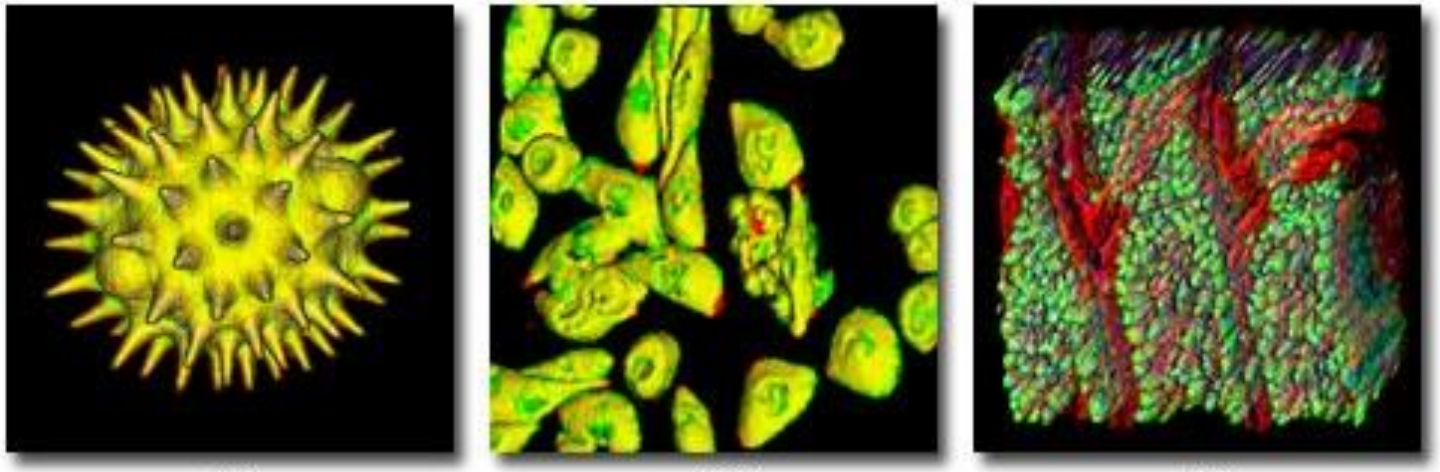
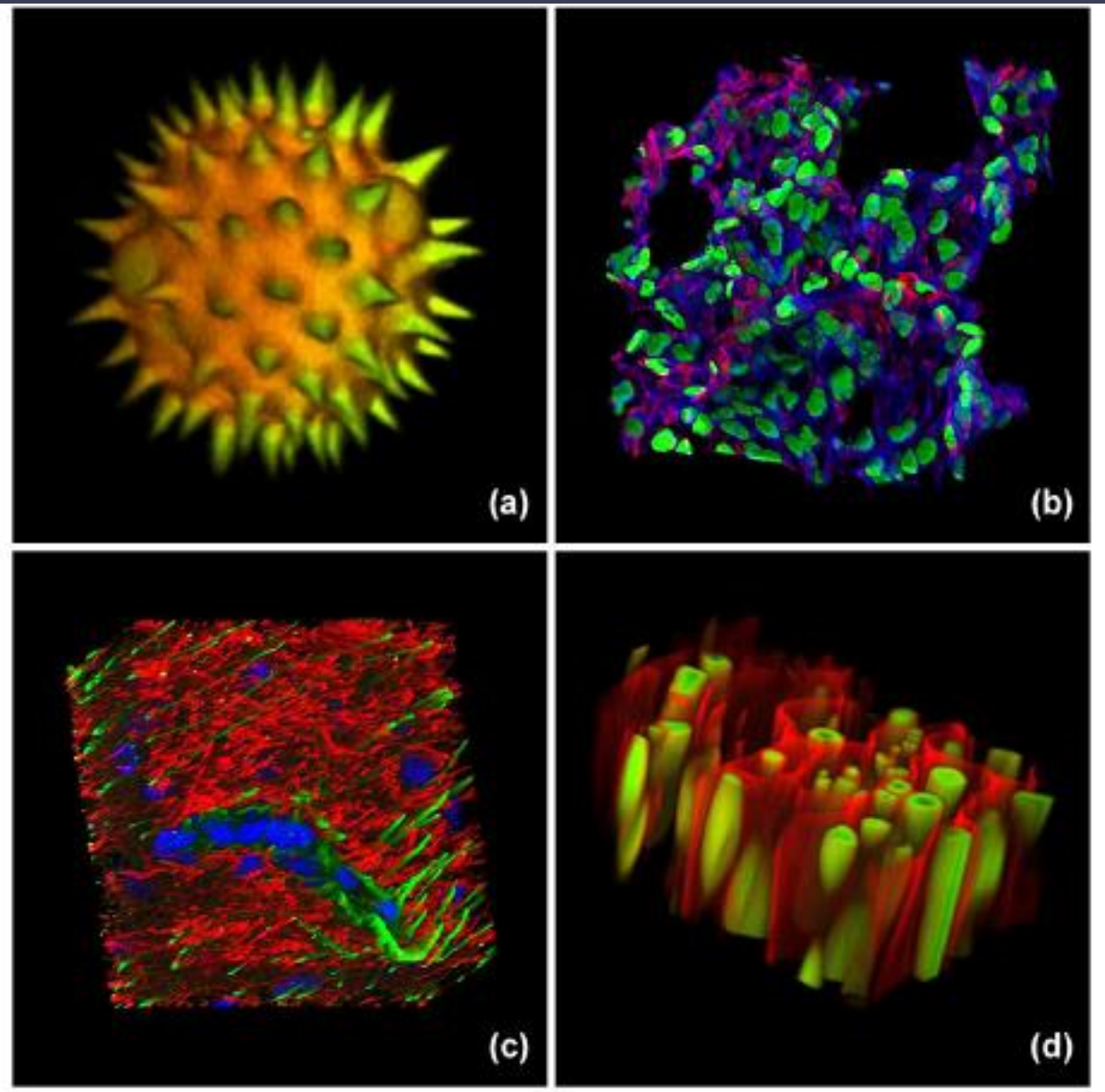
Three-Dimensional Volume Renders from Confocal Optical Sections



(a) (b) (c)

Figure 7


(a)
(b)
(c)
(d)

CONFOCAL and DNA

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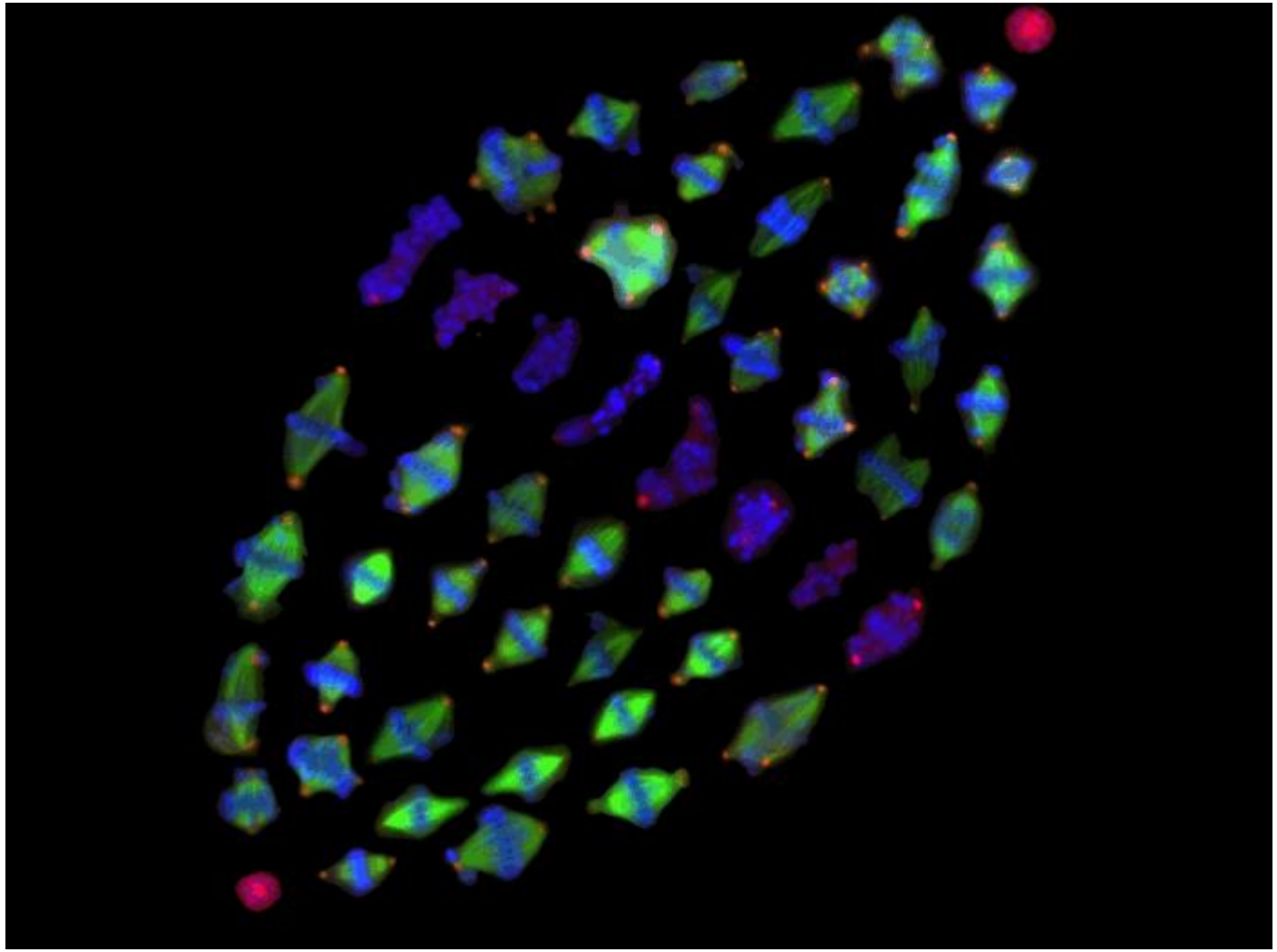

CONFOCAL and DNA

CONFOCAL and DNA

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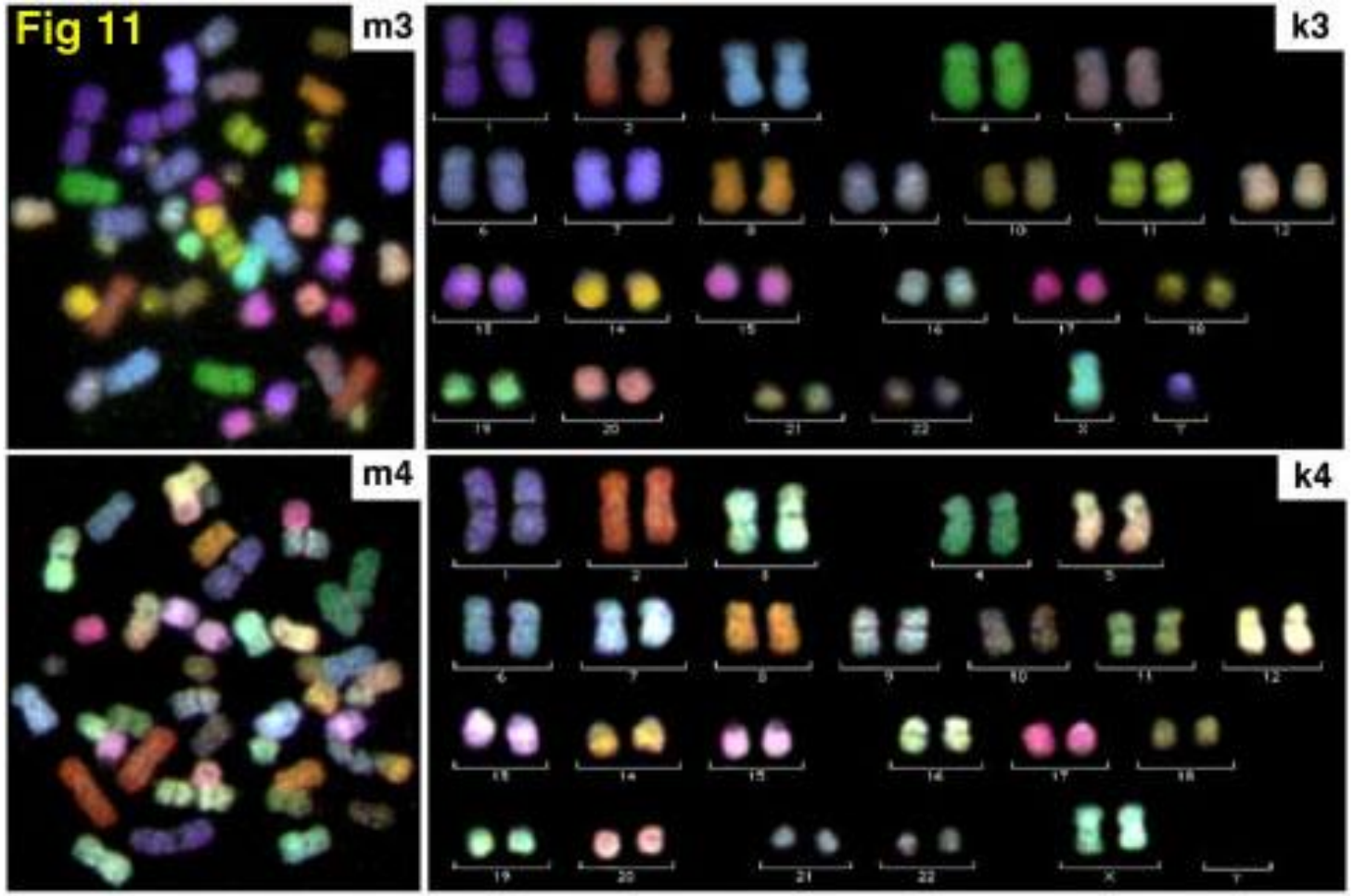

Comparison of Different Forms of Microscopy

	Description	Advantages	Disadvantages	Common Use In Neuroscience
Brightfield	Light transmitted through specimens. Contrast generated by natural pigmentation or added dyes.	Simple and inexpensive. Different-colored dyes can be used.	Most cells and tissues are transparent, so they can be difficult to see. Adding dyes to visualize structures usually requires fixation and sectioning.	Fixed samples processed using detection reagents that produce colored by-products (see Chapter 6).
Phase-Contrast	Contrast generated by changes in index of refraction because of variation in organelle densities.	Can be used on live cells. Doesn't require added chemicals or tissue processing.	Out-of-focus signals.	Tissue culture cells.
Darkfield	Specimen illuminated from the side at an oblique angle. Only scattered light is imaged.	Can be used on live cells. Sensitive. High signal-to-noise ratio. No added chemicals necessary.	Cannot see structures that do not scatter light.	Samples processed for radioactive in situ hybridization (see Chapter 6).
DIC/Nomarski	Optical methods used to exaggerate changes in index of refraction, enhancing contrast at the edges of objects. Has a 3D appearance.	Can be used on live cells. Can create thin optical sections. Can create 3D reconstructions from thin optical sections.	Can only image single, thin focal plane at a time.	Tissue culture cells and tissues.

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Epifluorescent	Specimen is illuminated by excitation wavelengths and emits light from excited fluorophores throughout entire thickness.	(Advantages of all forms of fluorescent microscopy) Can be used to detect specifically labeled fluorescent molecules. Multiple fluorophores can be imaged in same sample. High signal-to-noise ratio. Sensitive. (Advantage of epifluorescence over other forms) Simpler and faster image collection methods than other forms of fluorescent microscopy (confocal, two-photon).	Out-of-focus fluorescence can cause blurry pictures that make structures difficult to resolve.	Thin physical sections of fluorescently stained tissue or cells. Time-lapse fluorescence imaging in cells.
Confocal	Out-of-focus illumination is blocked by the use of pinhole apertures, so only in-focus emitted light is collected.	Optical sectioning ability creates sharp, in-focus images.	Intense laser illumination can cause photobleaching and phototoxicity. Long scan time.	Detecting fluorescence in thick tissues or small organisms (e.g., *Drosophila*). Time-lapse fluorescence imaging in thicker tissues (e.g., slice cultures) or for small structures (e.g., synaptic vesicles).

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Two-Photon	Fluorophores in a thin focal plane are selectively excited by effectively absorbing the combined energy of two photons that cannot excite fluorophores on their own.	Longer wavelengths of laser illumination allow deeper penetration of fluorescence excitation. Reduced photobleaching and phototoxicity.	Expensive equipment.	*In vivo* imaging of intact organisms. Long-term fluorescence imaging.
TEM (Transmission Electron Microscopy)	Electron beams transmitted through ultrathin sections.	Nanometer resolution.	Cannot be used on live cells. Harsh processing conditions can cause artifacts. Requires specialized equipment.	Ultrastructure of cells. Synapse structure.
SEM (Scanning Electron Microscopy)	Detect secondary electrons scattered off surface of sample.	Provides 3D topological information.	Cannot be used on live cells. Harsh processing conditions can cause artifacts. Requires specialized equipment.	Topography of cells and tissues.
ET (Electron Tomography)	Rotate specimen to take TEM images from multiple perspectives and create 3D reconstruction.	Provides 3D organization information.	Cannot be used on live cells. Requires intensive computation to reconstruct TEM views.	3D cellular ultrastructure and organization.

LINKS and TUTORIALS

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http://www.microscopyu.com

tutorials

http://www.microscopyu.com/tutorials/java/objectives/immersion/index.html

http://www.microscopyu.com/tutorials/java/virtual/confocal/index.html

http://www.olympusfluoview.com/theory/index.html

tutorials

http://www.olympusfluoview.com/java/confocalsimulator/index.html

http://unitsconfocal.googlepages.com/

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