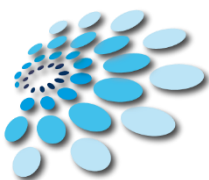


# “ Techniques in Cellular and Molecular Neurobiology ”

International Master's Degree in Neuroscience

Lesson 5



DIPARTIMENTO DI  
SCIENZE DELLA VITA

Gabriele Baj  
gbaj@units.it



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# Overview of the Course



## 1) Introduction

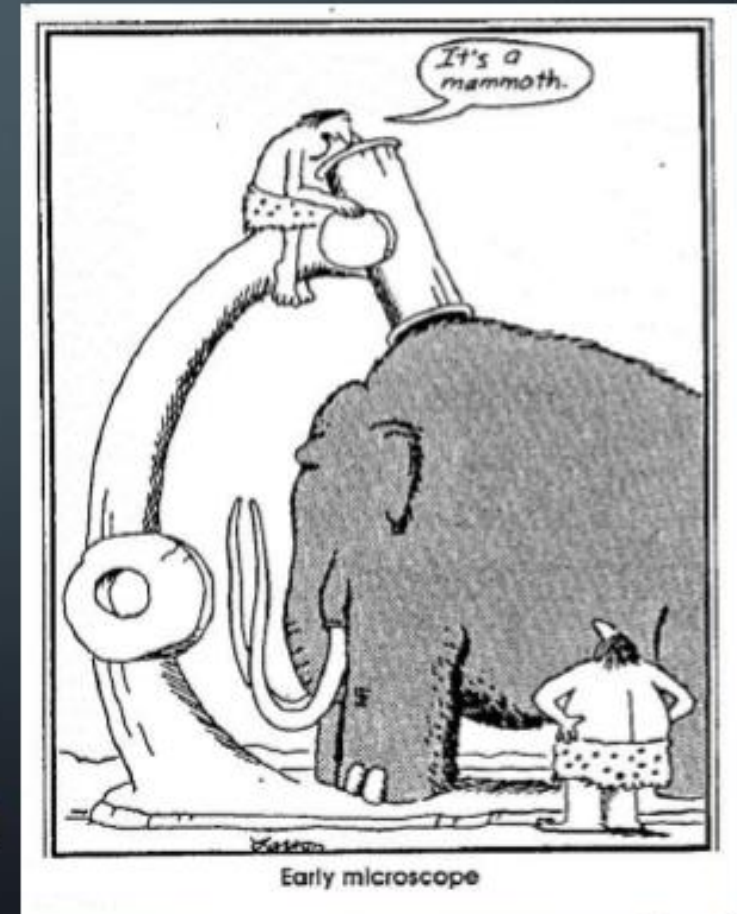
What Can You Learn with a Light Microscope?  
Early History of Microscopy

## 2) Image Formation

Lenses and Image Formation  
Microscope Imaging and Koehler Illumination  
Objectives and Eyepieces  
Diffraction and Point Spread Function

## 3) Resolution , What is Light?

How to Focus and setting up Koehler Illumination





# Overview of the Course



## 4) Contrast Generation for Transmitted Light

Darkfield and Phase Contrast Microscopy

Polarized Light and Polarization Microscopy

Differential Interference Contrast (DIC) Microscopy

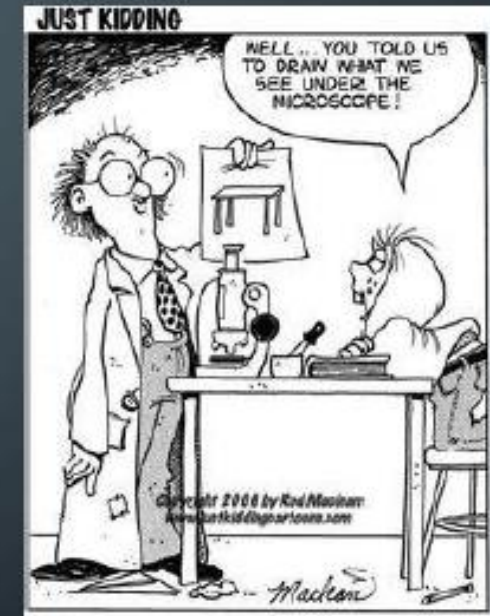
## 5) Fluorescence Microscopy

Introduction to Fluorescence Microscopy

Fluorescent Probes / Fluorescent Proteins

Optical Sectioning and Confocal Microscopy

Light Sheet Sectioning



# Overview of the Course



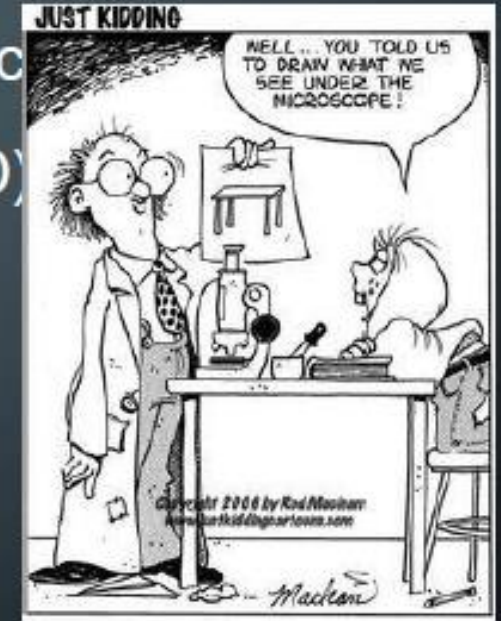
## 6) Super-Resolution:

Total Internal Reflection Fluorescence (TIRF) Microscopy

Overview and Stimulated Emission Depletion (STED) Microscopy

Localization Microscopy

Structured Illumination Microscopy (SIM)



## 7) Photobleaching and Photoactivation

Förster Resonance Energy Transfer (FRET) Microscopy

Fluorescence Lifetime Imaging Microscopy



# Overview of the Course



## 8) Designing a Fluorescence Microscopy Experiment

Labeling Proteins with Fluorescent Probes

Correlating Fluorescence with Electron Microscopy

## 9) Introduction to Digital Images

## 10) Quantitative Analysis of Biological imaging Microscopy

Cameras and Detectors I: How Do They Work?

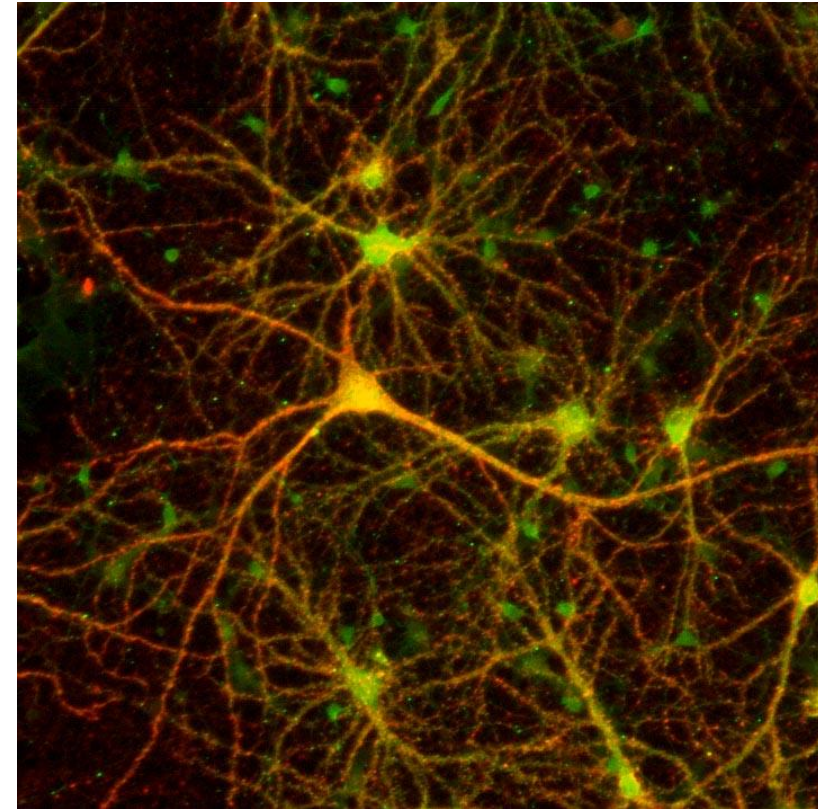
## 11) Image Analysis / Deconvolution Microscopy



# MICROSCOPY and BIOLOGY



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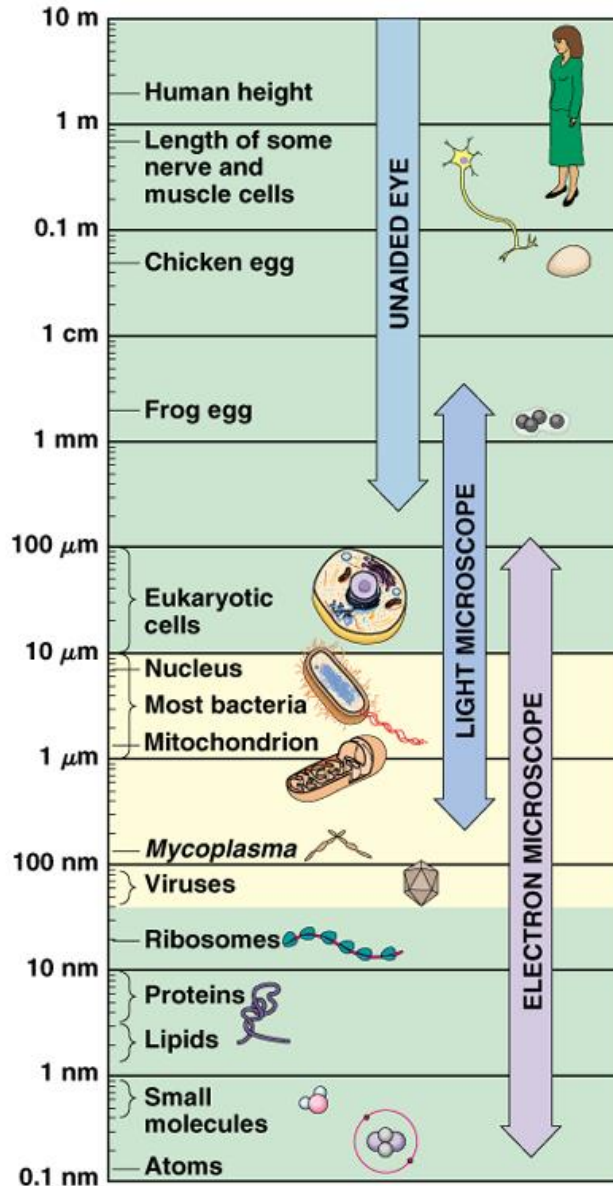


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# MICROSCOPY



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Microscopy is not simply see “bigger”!!

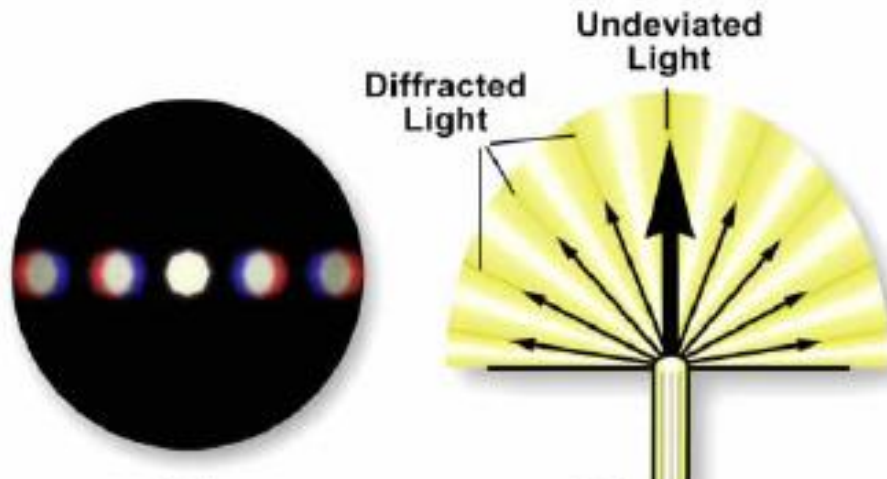
Location  
Morphology  
Intensity/Amount  
Movement  
Interactions  
Etc.



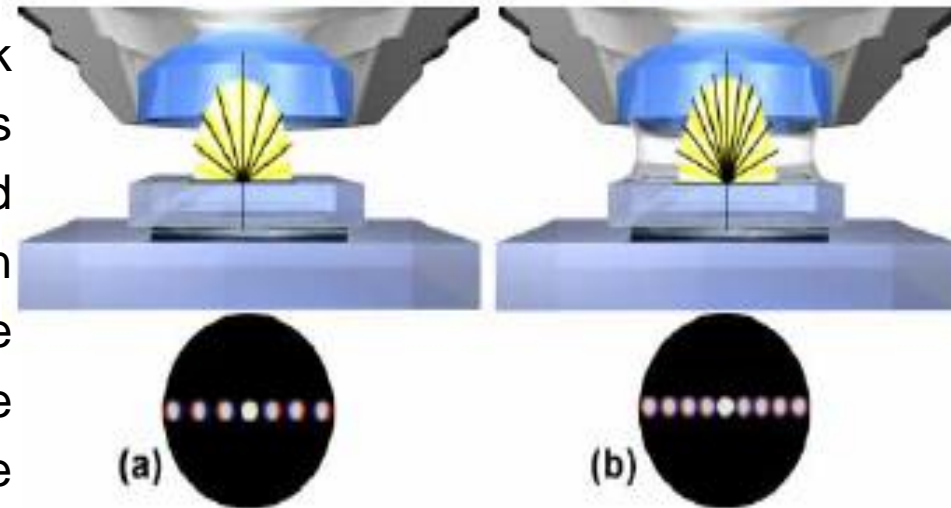
# Light in Microscopy



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(a) Conoscopic image of objective back focal plane diffraction spectra when air is the medium between the cover slip and the objective front lens. (b) Diffraction spectra when immersion oil of refractive index similar to glass is used in the space between the cover slip and the objective front lens.





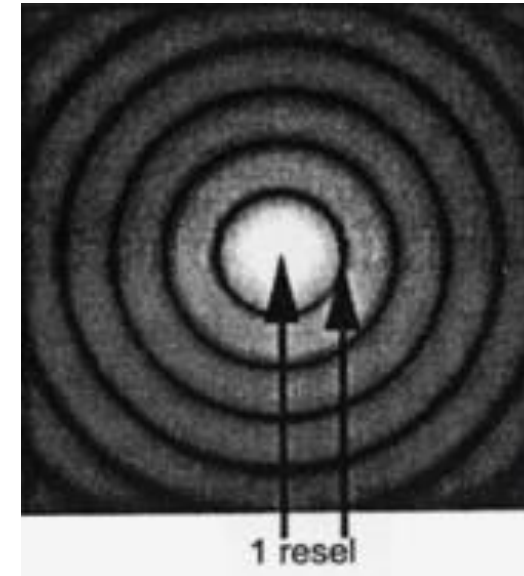
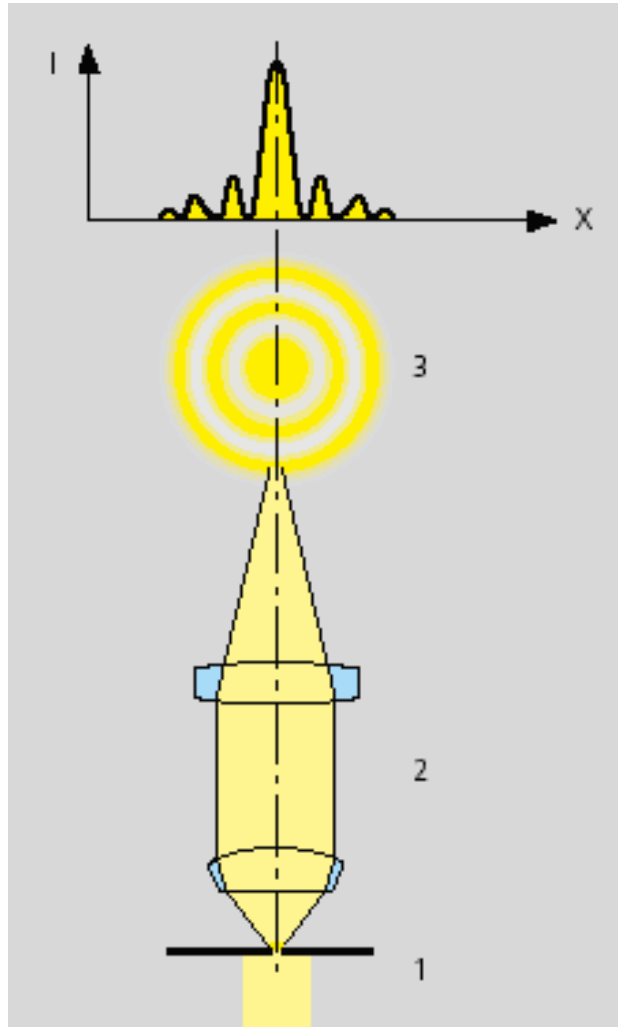
# Resolution in microscopy



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Point Spread Function (PSF).

PSF describes the probability for an area where a given point will appear.



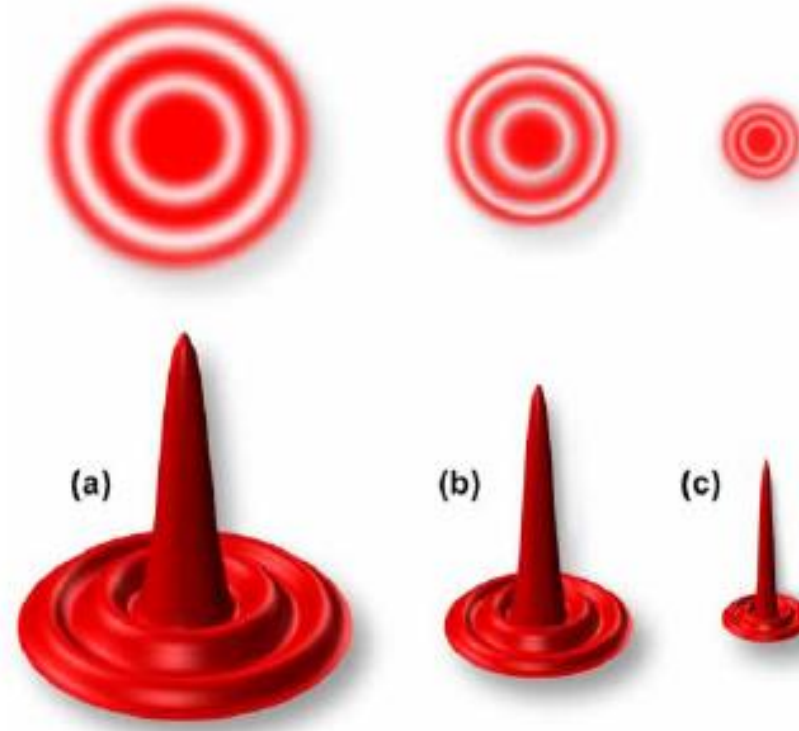
When single point resolution is concerned, the radius of the inner ring, as shown in the figure, is used to define the resolution, thus called resolution element (resel). When point-to-point resolution is concerned, the resolution is defined as the distance of the two points where the maximum of one point is just above the first minimum of the second point (Rayleigh criterion).

# Light in Microscopy



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## Airy Disks and Resolution



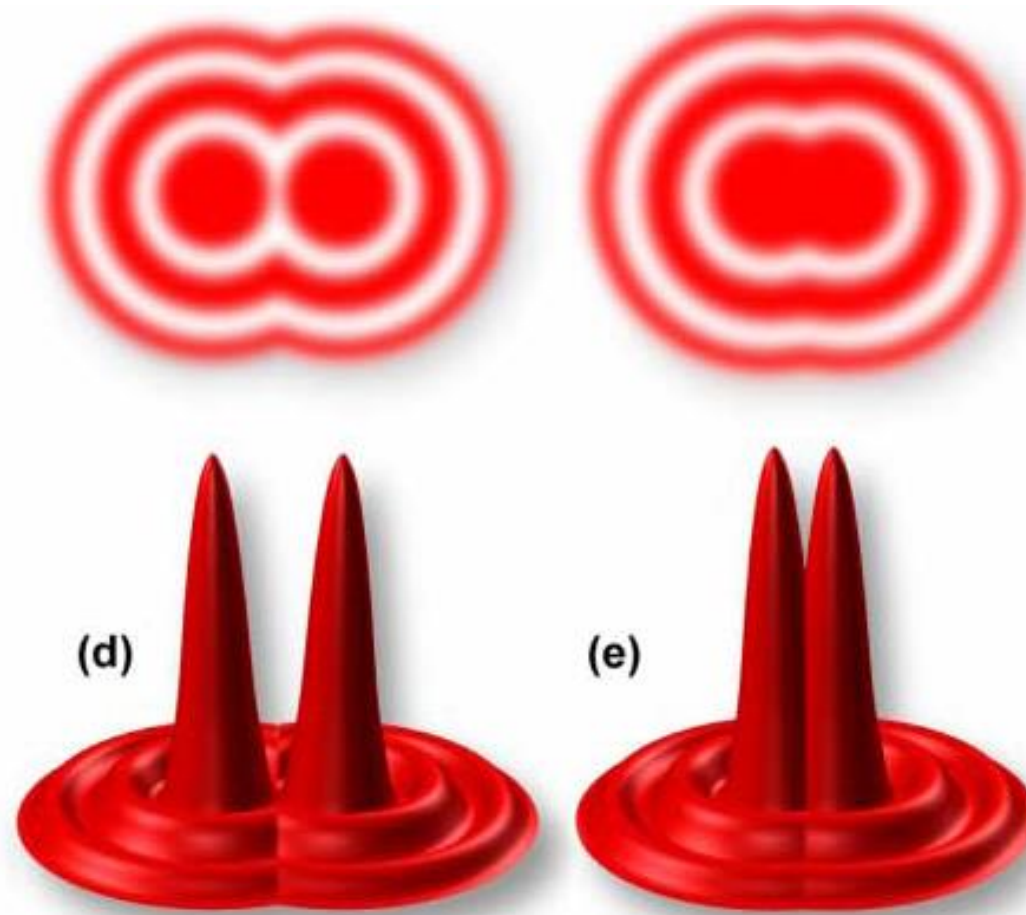
Airy disks and resolution. (a-c) Airy disk size and related intensity profile (point spread function) as related to objective numerical aperture, which decreases from (a) to (c) as numerical aperture increases.



# Light in Microscopy



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(e) Two Airy disks so close together that their central spots overlap. (d) Airy disks at the limit of resolution.

## Airy Disks and Diffraction Pattern Intensity Profiles

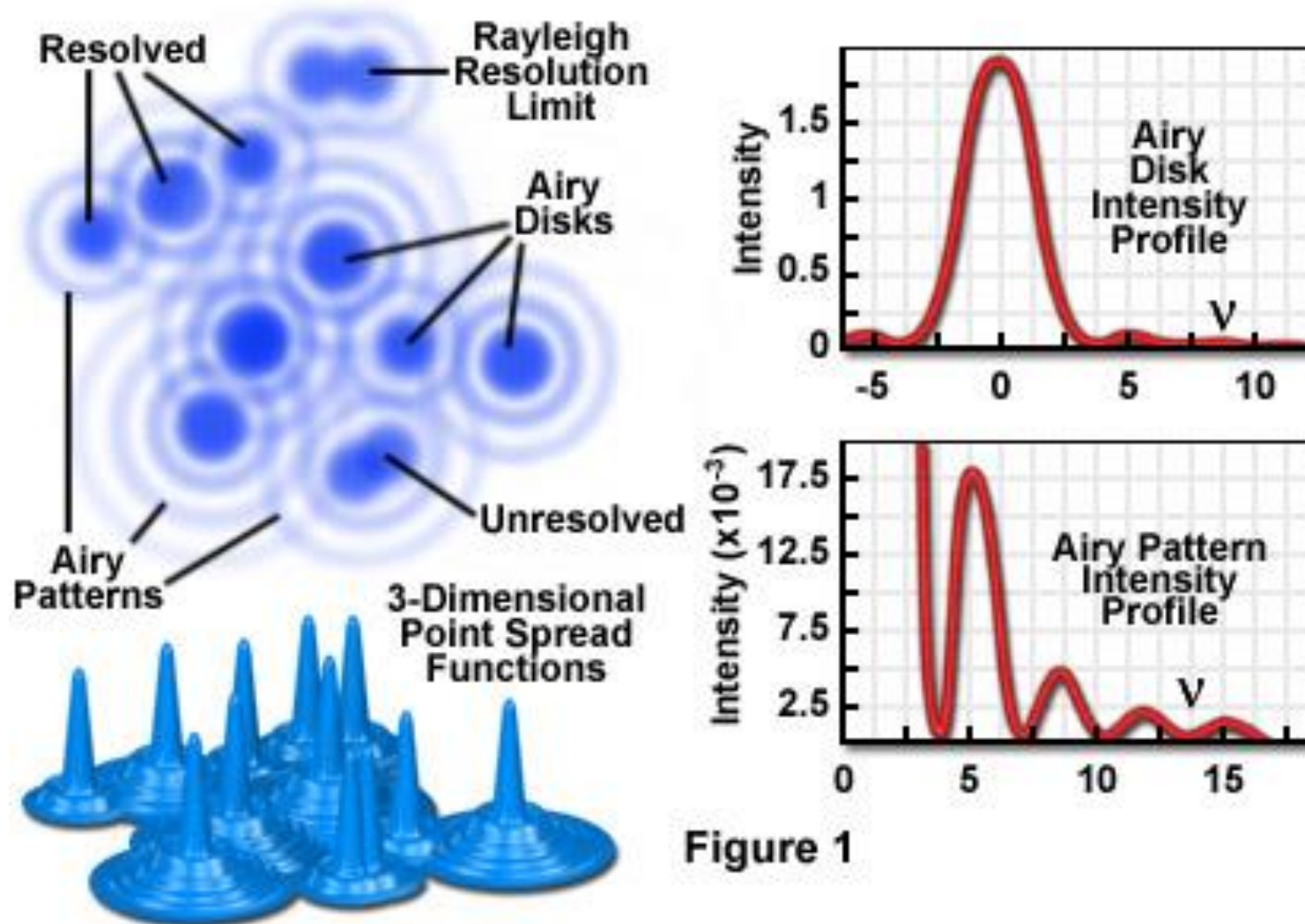


Figure 1



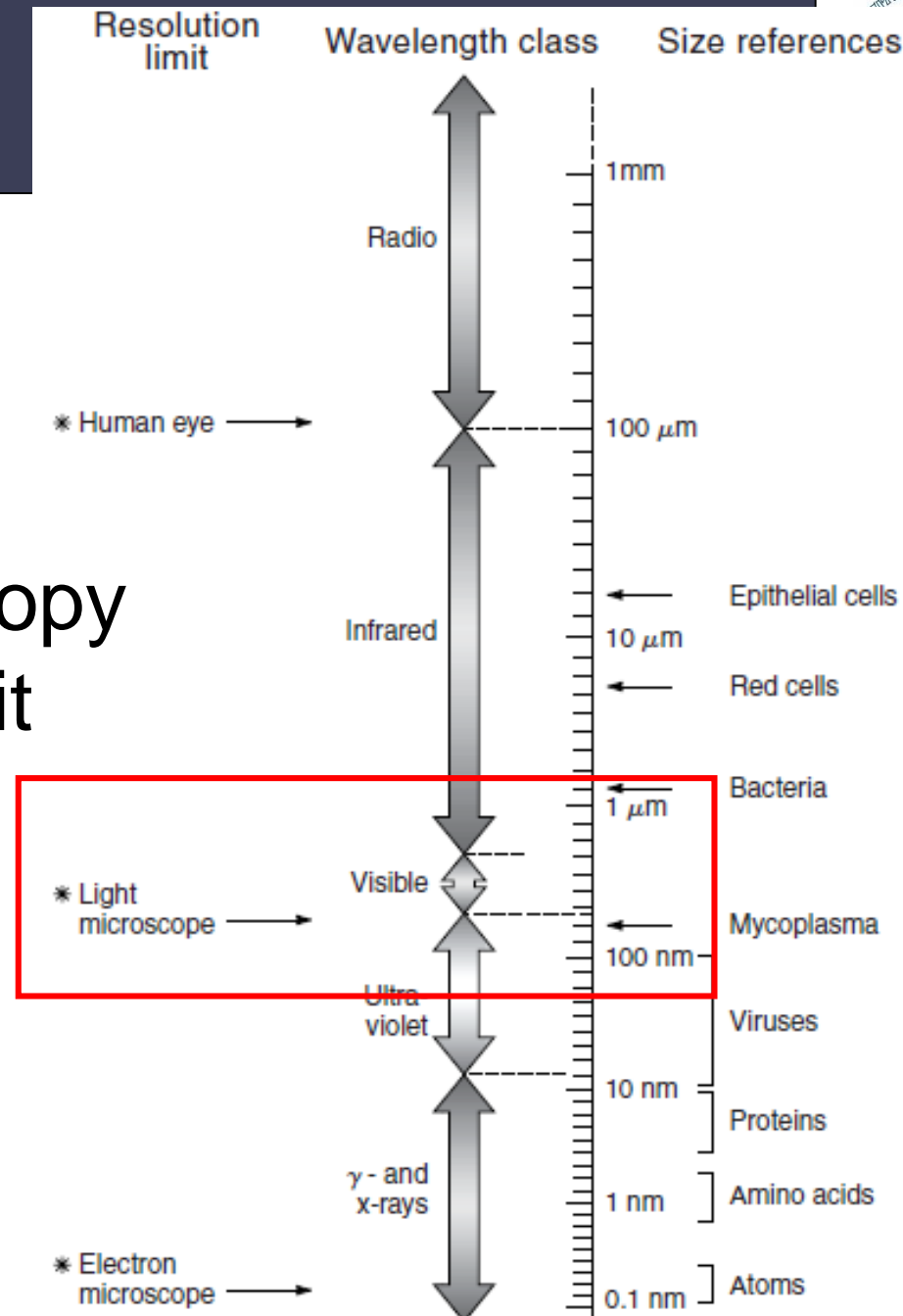
# Microscope Resolution



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- ability of a lens to separate or distinguish small objects that are close together
- wavelength of light used is major factor in resolution  
shorter wavelength  $\Rightarrow$  greater resolution

# Light microscopy resolution limit

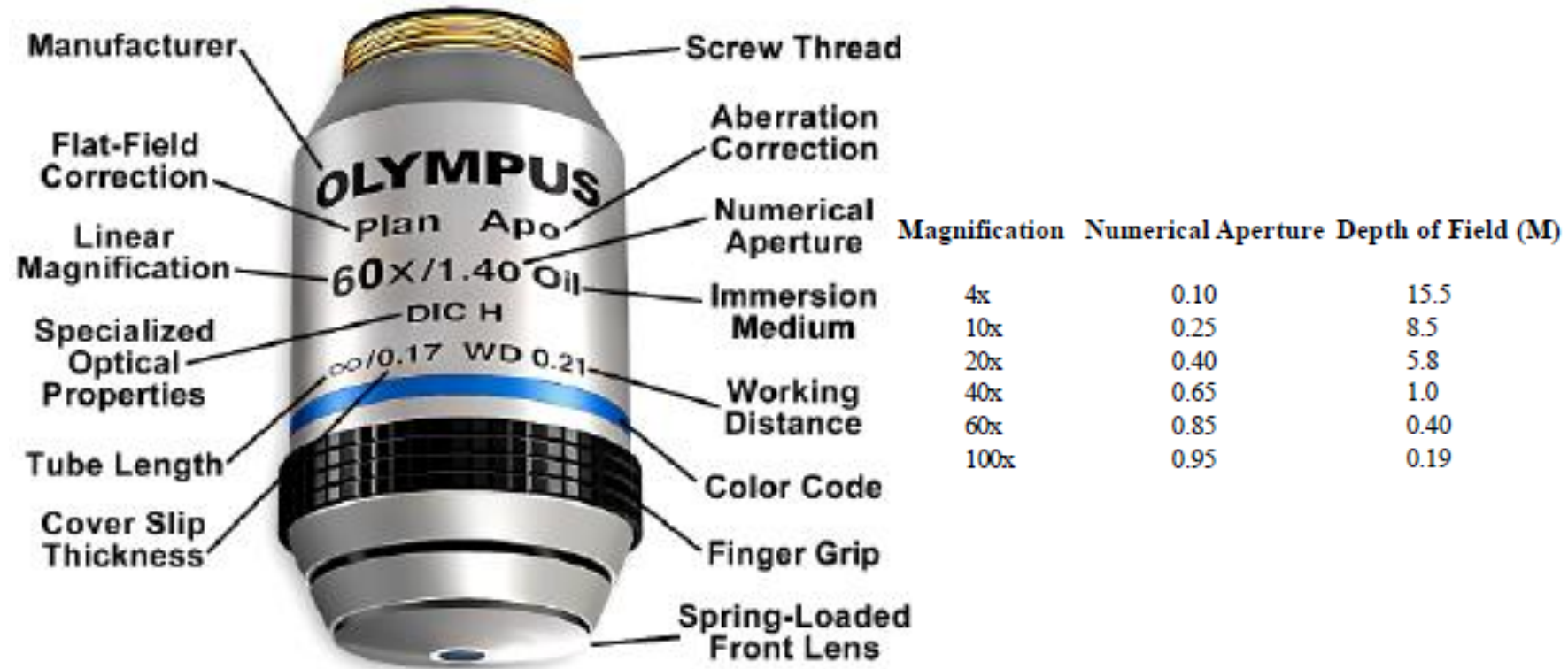




# Objective Specifications

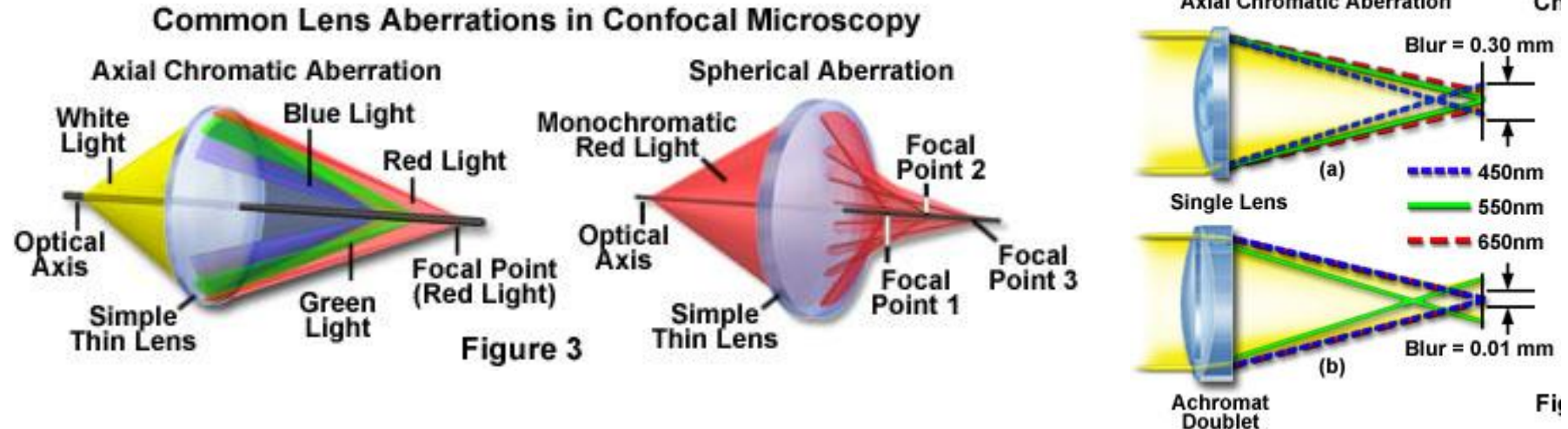


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Specifications engraved on the barrel of a typical microscope objective. These include the manufacturer, correction levels, magnification, numerical aperture, immersion requirements, tube length, working distance, and specialized optical properties.

# MANY WAVES – MANY “PROBLEMS”



Lens aberrations that affect objective performance may be divided into two categories, including those that are non-chromatic (invariable with wavelength), and the chromatic aberrations, which are wavelength dependent. The chromatic aberrations are characterized as either **lateral chromatic aberration** or **longitudinal chromatic aberration**, and the wavelength independent group includes **spherical** aberration, **coma**, **astigmatism**, **field curvature**, and **distortion**.

# Light in Microscopy



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## Corrections for Aberrations

Type	Spherical	Chromatic	Flatness Correction
Achromat	* <sup>b</sup>	2 <sup>c</sup>	No
Plan Achromat	* <sup>b</sup>	2 <sup>c</sup>	Yes
Fluorite	3 <sup>d</sup>	< 3 <sup>d</sup>	No
Plan Fluorite	3 <sup>d</sup>	< 3 <sup>d</sup>	Yes
Plan Apochromat	4 <sup>e</sup>	> 4 <sup>e</sup>	Yes

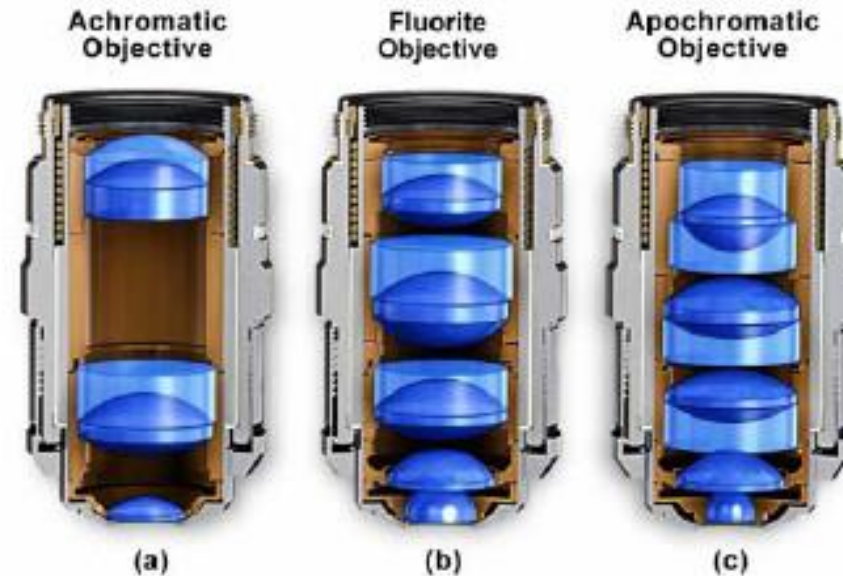
<sup>a</sup> Source: Nikon Instrument Group

<sup>b</sup> Corrected for two wavelengths at two specific aperture angles.

<sup>c</sup> Corrected for blue and red - broad range of the visible spectrum.

<sup>d</sup> Corrected for blue, green and red - full range of the visible spectrum.

<sup>e</sup> Corrected for dark blue, blue, green and red.

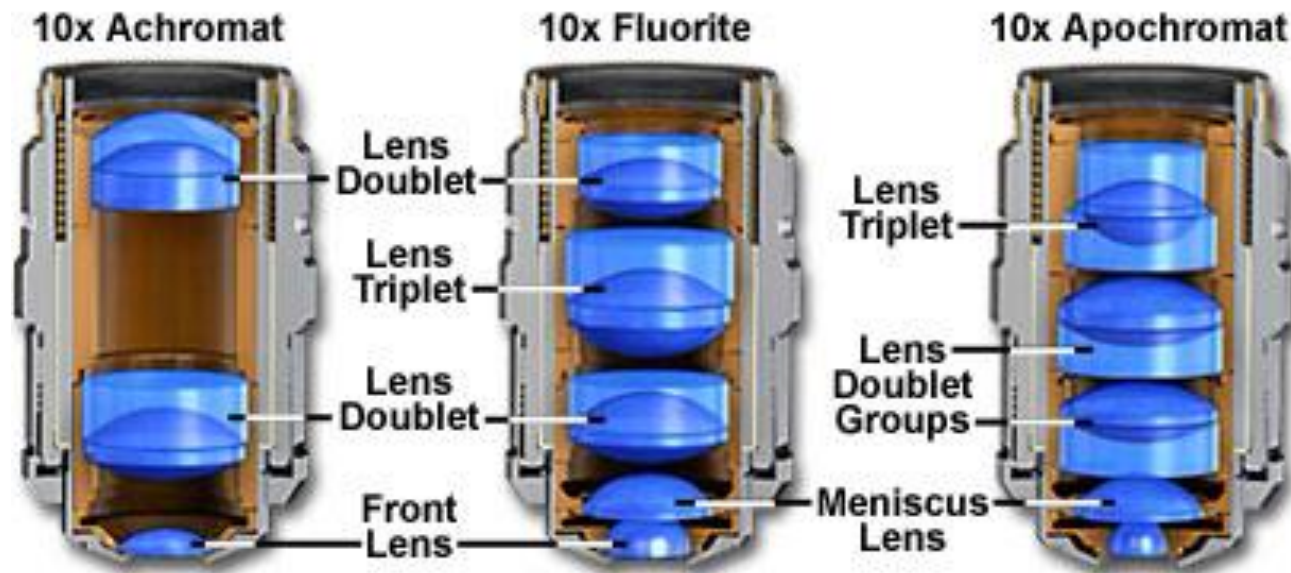




# Objective configuration



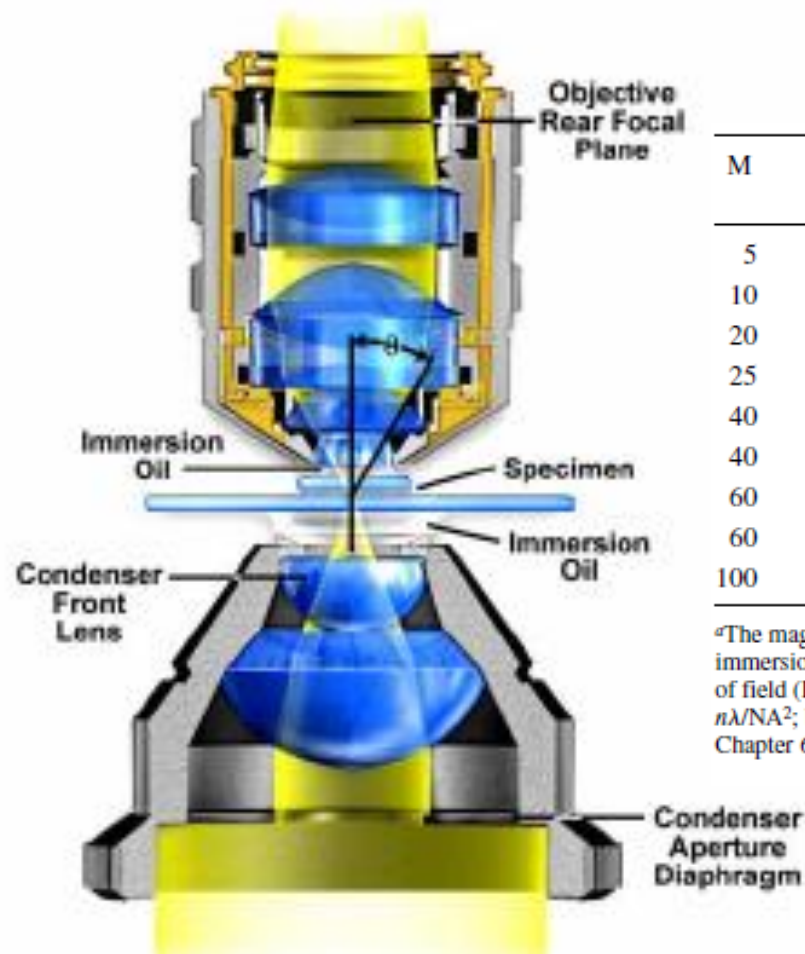
## Common Objective Optical Correction Factors



# Condenser/ objective configuration for optical microscopy



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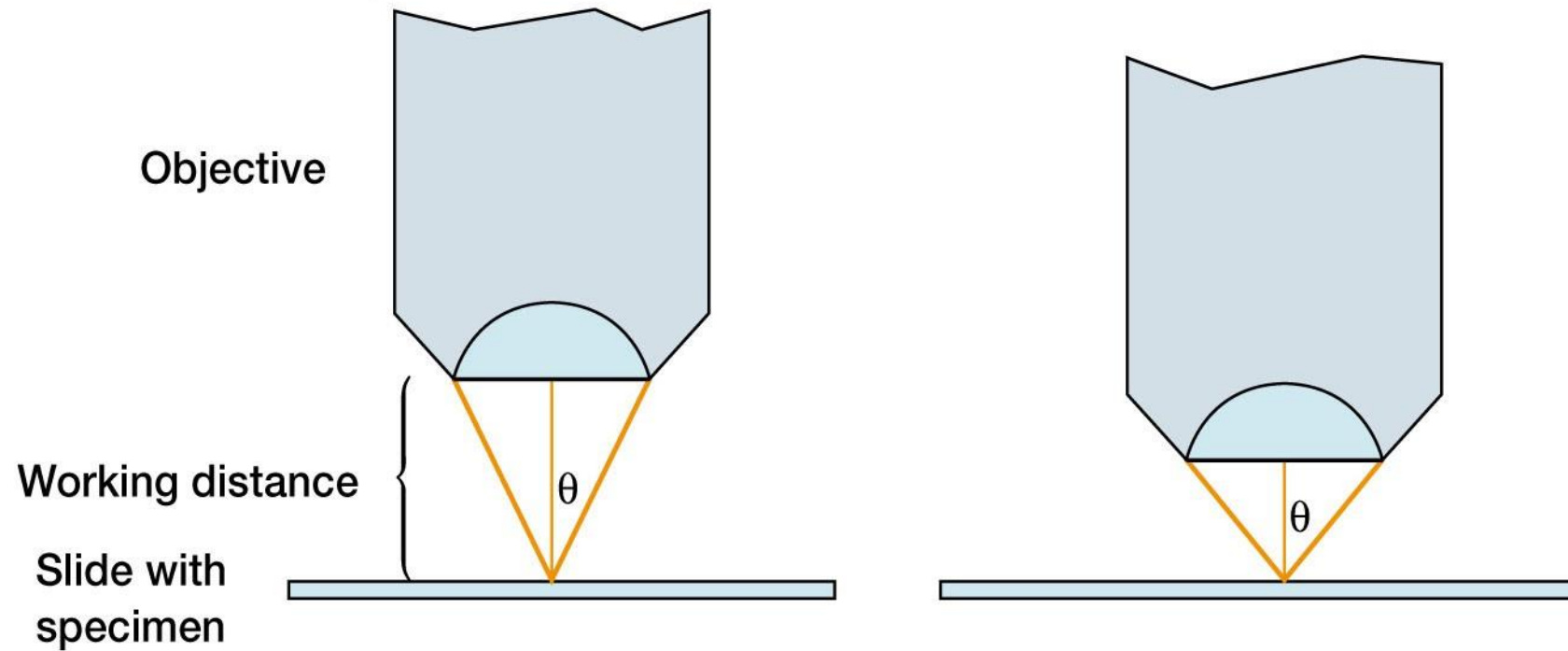
M	Type	Medium (n)	WD (mm)	NA	$d_{\min}$ ( $\mu\text{m}$ )	DOF ( $\mu\text{m}$ )	B
5	Achromat	1	9.9	0.12	2.80	38.19	0.1
10	Achromat	1	4.4	0.25	1.34	8.80	0.4
20	Achromat	1	0.53	0.45	0.75	2.72	1.0
25	Fluorite	1.515	0.21	0.8	0.42	1.30	6.6
40	Fluorite	1	0.5	0.75	0.45	0.98	2.0
40	Fluorite	1.515	0.2	1.3	0.26	0.49	17.9
60	Apochromat	1	0.15	0.95	0.35	0.61	2.3
60	Apochromat	1.515	0.09	1.4	0.24	0.43	10.7
100	Apochromat	1.515	0.09	1.4	0.24	0.43	3.8

<sup>a</sup>The magnification (M), type of lens design, refractive index (n) of the intervening medium (air or immersion oil), working distance (WD), numerical aperture (NA), minimum resolvable distance (d), depth of field (DOF), and brightness (B) are indicated. Terms are calculated as: wave-optical depth of field,  $n\lambda/\text{NA}^2$ ; brightness in epi-illumination mode,  $10^4 \text{ NA}^4/\text{M}^2$ . Resolution and depth of field are discussed in Chapter 6.

# Objective configuration



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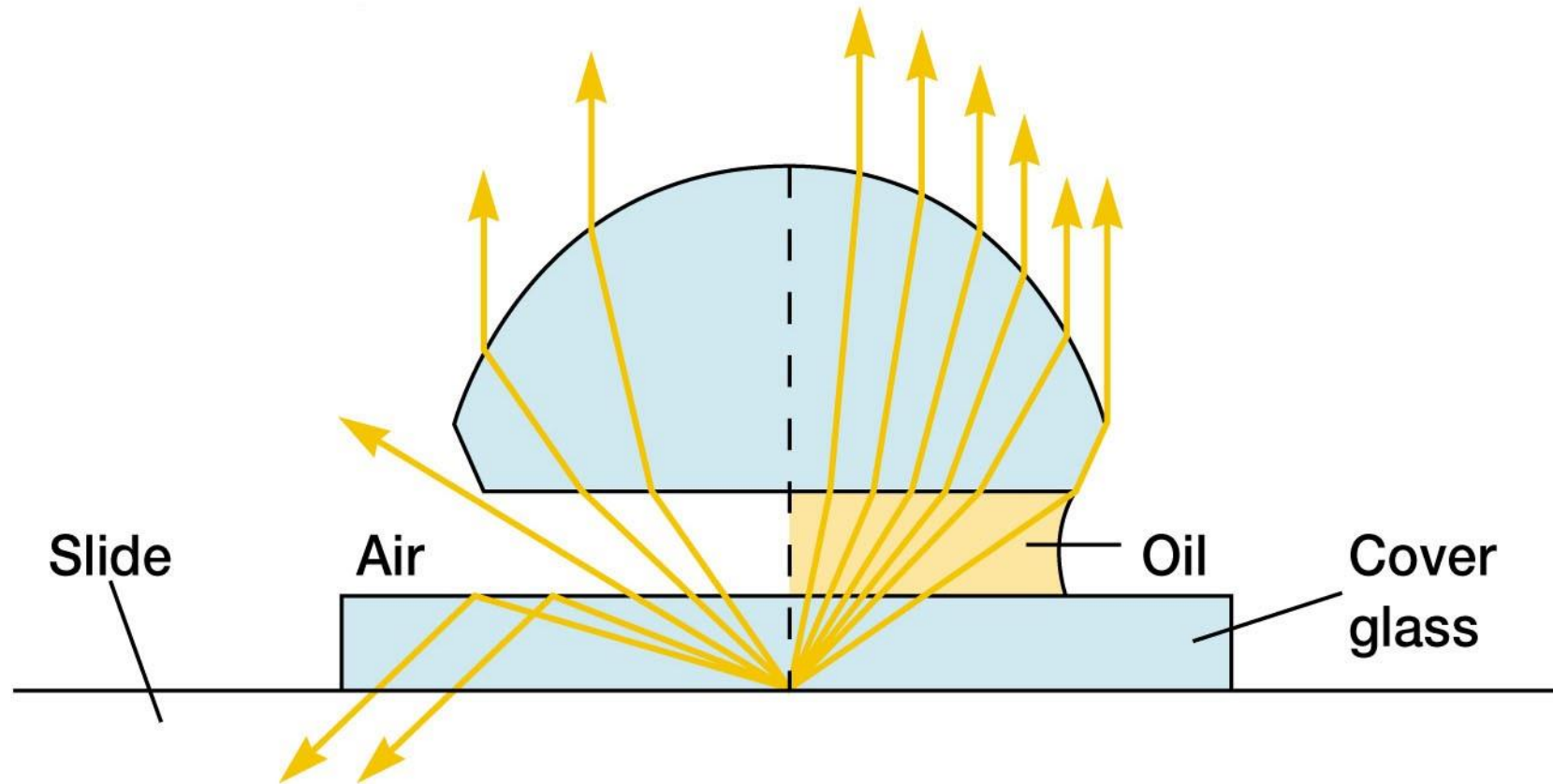




# Objective configuration



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# The Light Microscope



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- many types one target = contrast generation
  - bright-field microscope
  - dark-field microscope
  - phase-contrast microscope
  - DIC-microscope
  - fluorescence microscope

# The Bright-Field Microscope



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- produces a dark image against a brighter background
- has several objective lenses
  - parfocal microscopes remain in focus when objectives are changed
- total magnification
  - product of the magnifications of the ocular lens and the objective lens



# The Dark-Field Microscope



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- produces a bright image of the object against a dark background
- used to observe living, unstained preparations

# The Phase-Contrast Microscope



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- enhances the contrast between intracellular structures having slight differences in refractive index
- excellent way to observe living cells

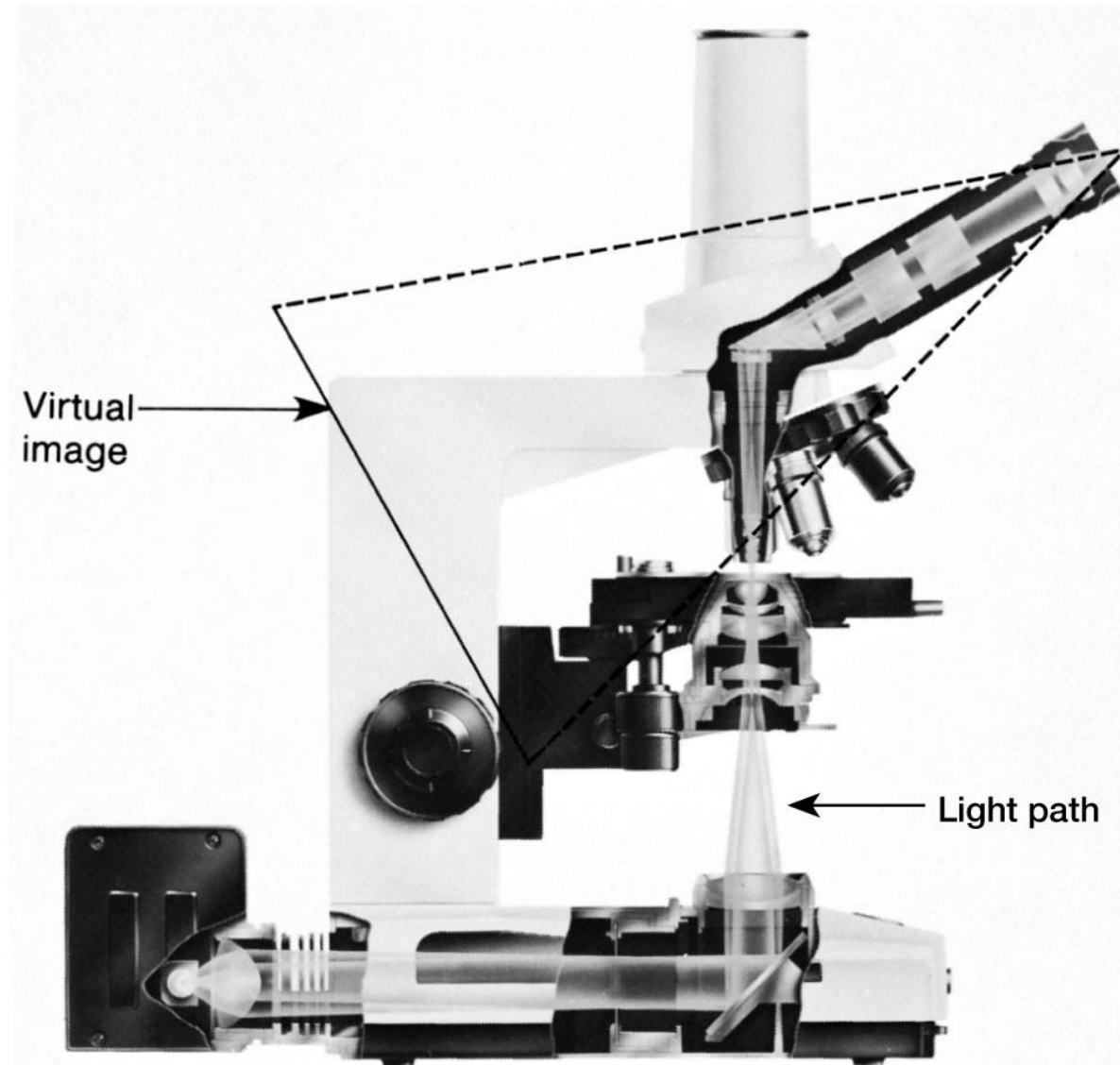
# The Differential Interference Contrast Microscope



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- creates image by detecting differences in refractive indices and thickness of different parts of specimen
- excellent way to observe living cells



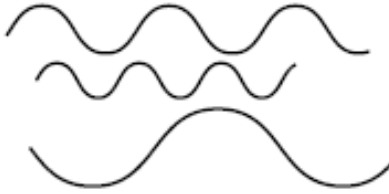


# Illumination is a critical determinant of optical performance in light microscope



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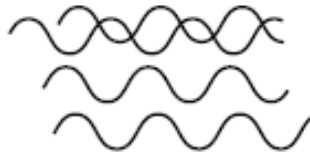
Polychromatic



Nonpolarized



Noncoherent

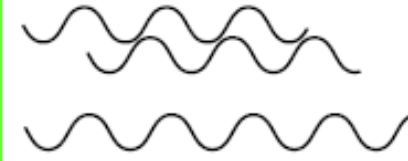


Divergent



“different lights”

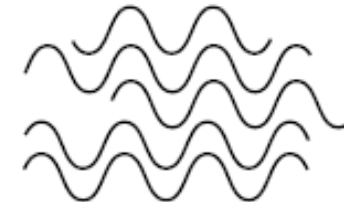
Monochromatic



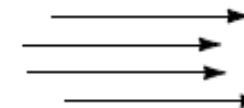
Linearly polarized



Coherent



Collimated



FLUO  
microscopy

CONFOCAL  
microscopy

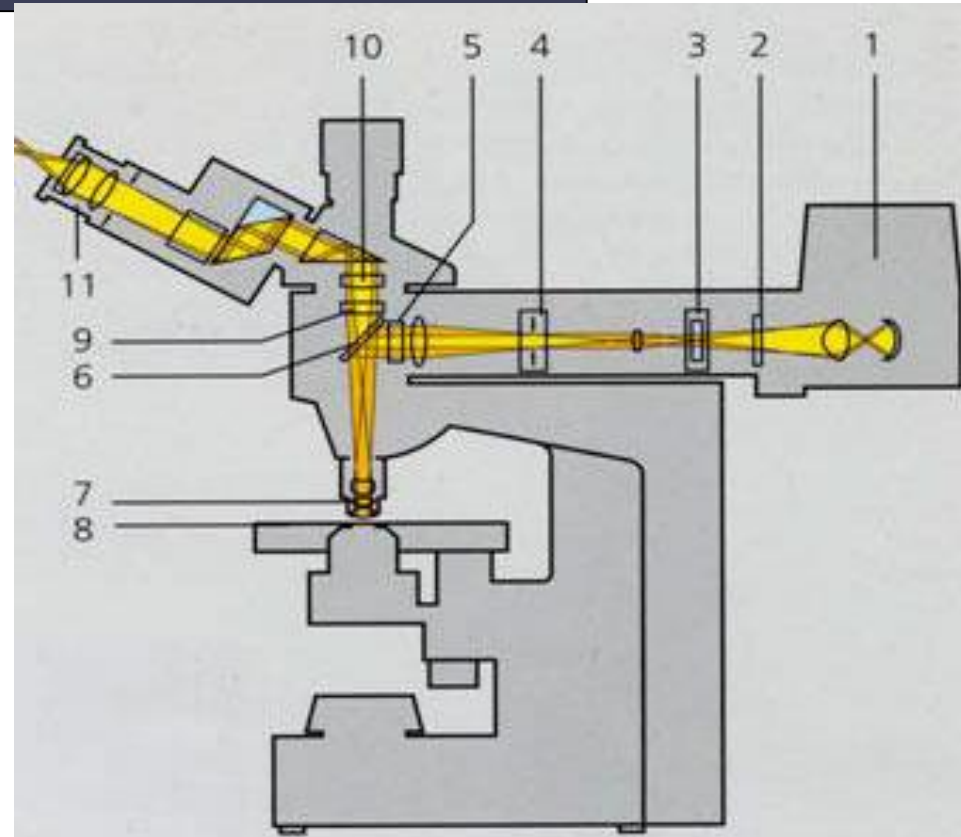
# Basics of conventional fluorescence microscope



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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).

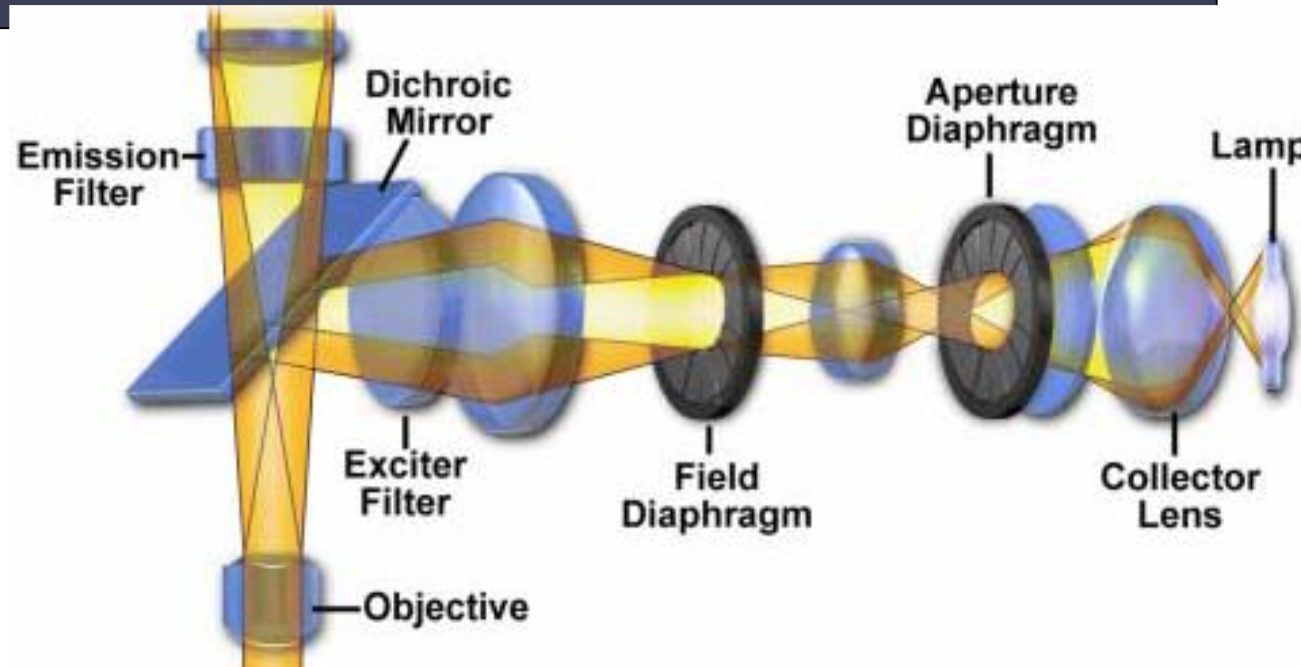
The fluorescence emission from the specimen comes back through the same path to BSP again. This time, the light should not be reflected away but passes through it and reaches emission filter(9). Being further filtered by emission filter, the emission light is either focused on the front focal plane of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



# Fluorescence Microscopy



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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.

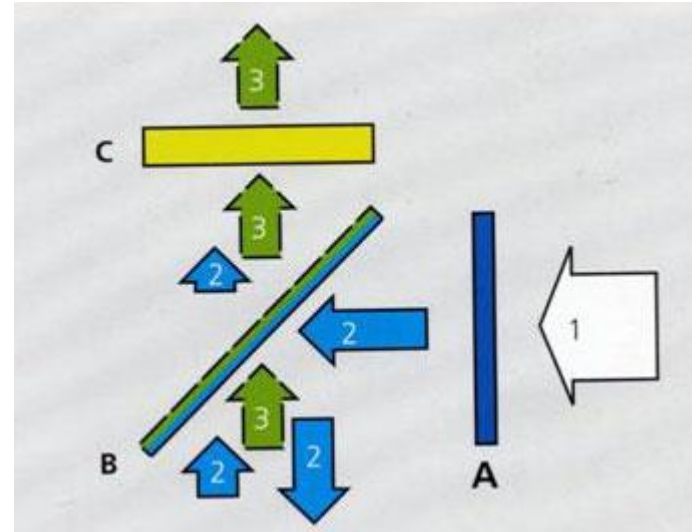


# Filter-set and its choice, limitation



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**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.

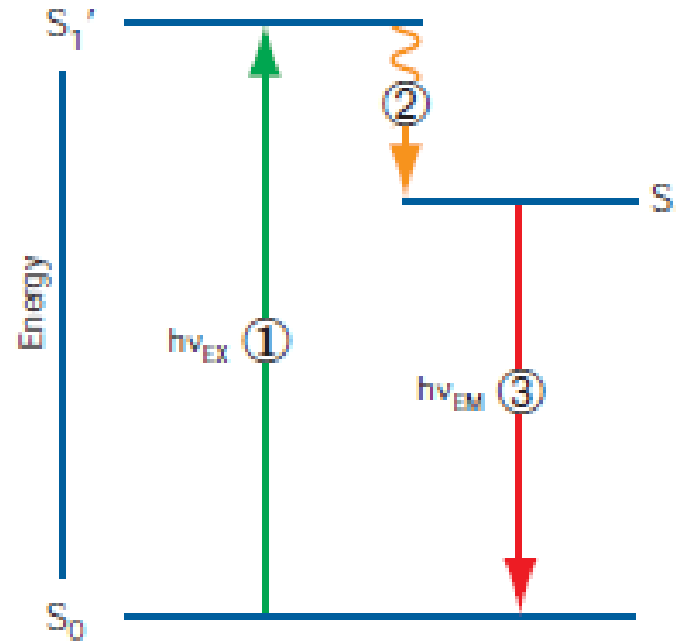


**Arrow 2** represents excitation light which passes through A and reaches the surface of **Beam Splitter B**, BSP is a special filter which reflects certain wavelength away but permit other wavelength to pass. A multiple-bands reflecting filter or neutral percentage splitter can be used for this role. 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 process



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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 CUBE



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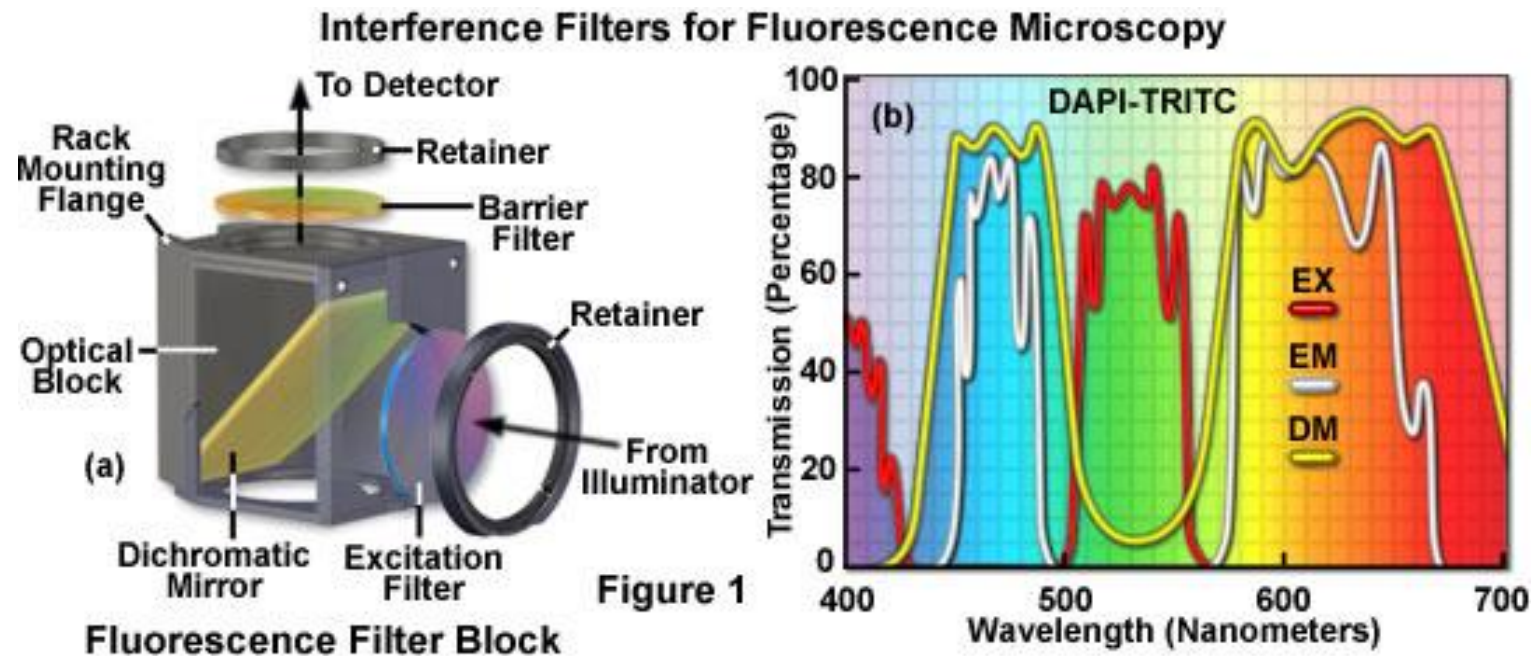
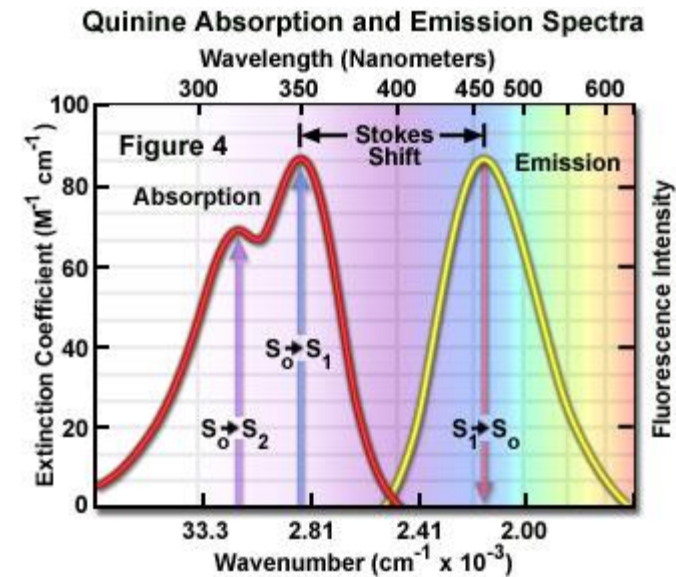
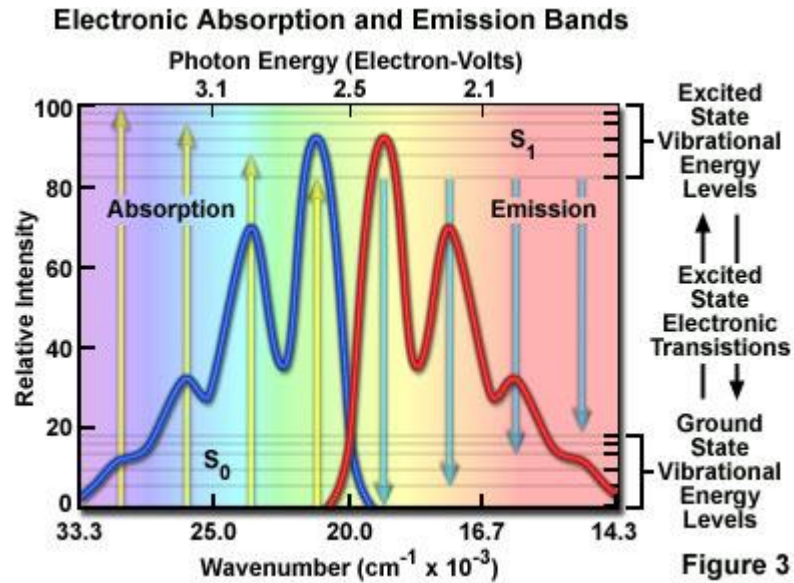


Figure 1

# Fluorescence Microscopy



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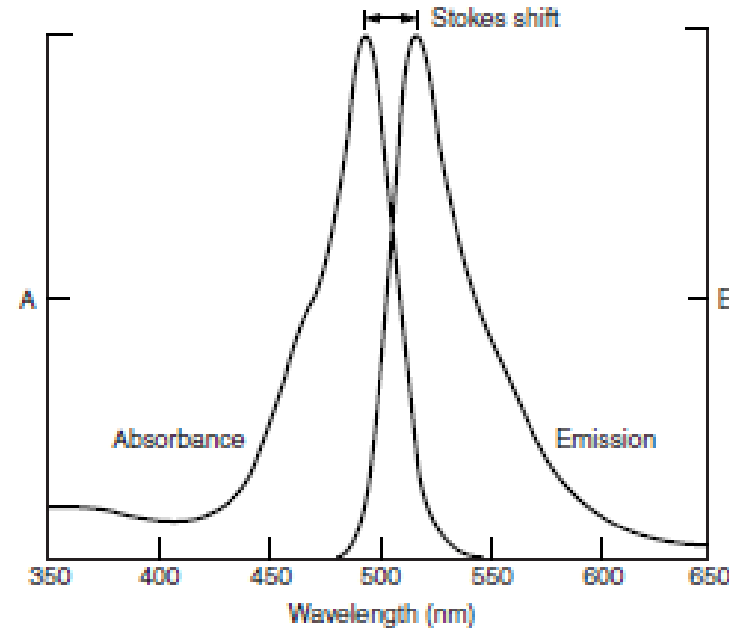




# Fluorescence Microscopy



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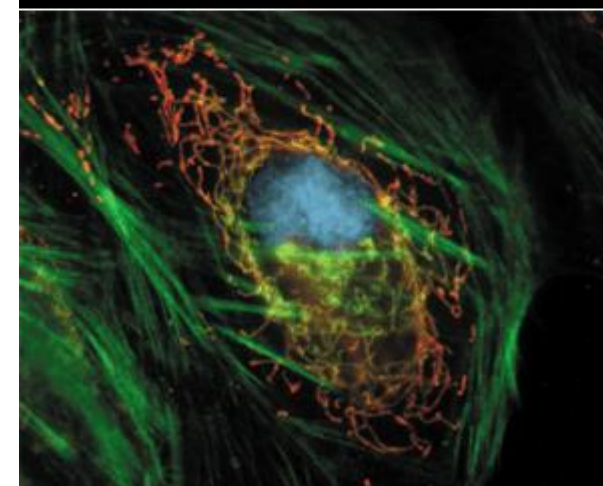
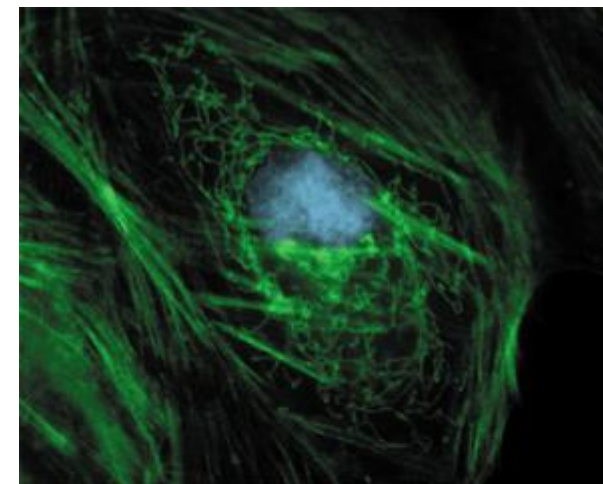
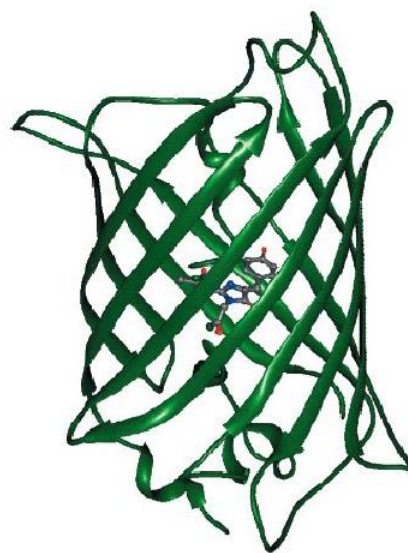
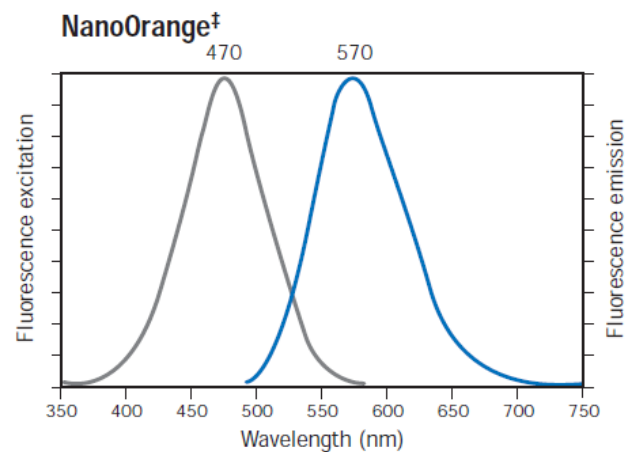
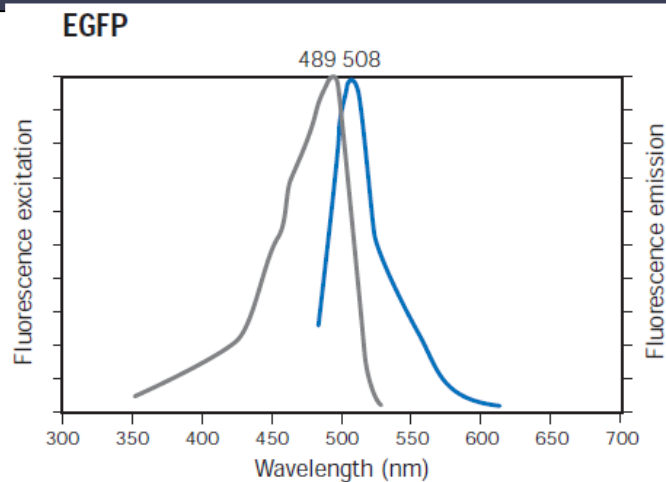


Normalized absorption and fluorescence emission spectra of fluorescein-conjugated IgG. Both spectra span a wide range of wavelengths. Fluorescein has an absorption/excitation peak at 492 nm, but is also stimulated by ultraviolet wavelengths. Fluorescein emission has a peak at 520 nm and looks yellow-green 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.

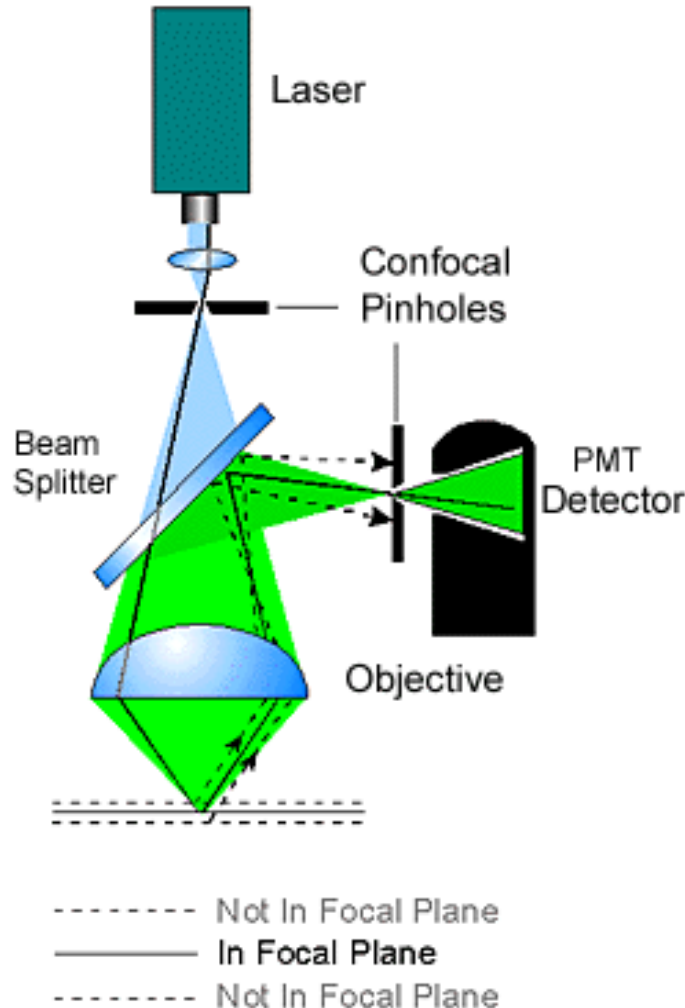
# FLUORESCENT PROTEINS AND PROBES



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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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## Widefield versus Confocal Point Scanning of Specimens

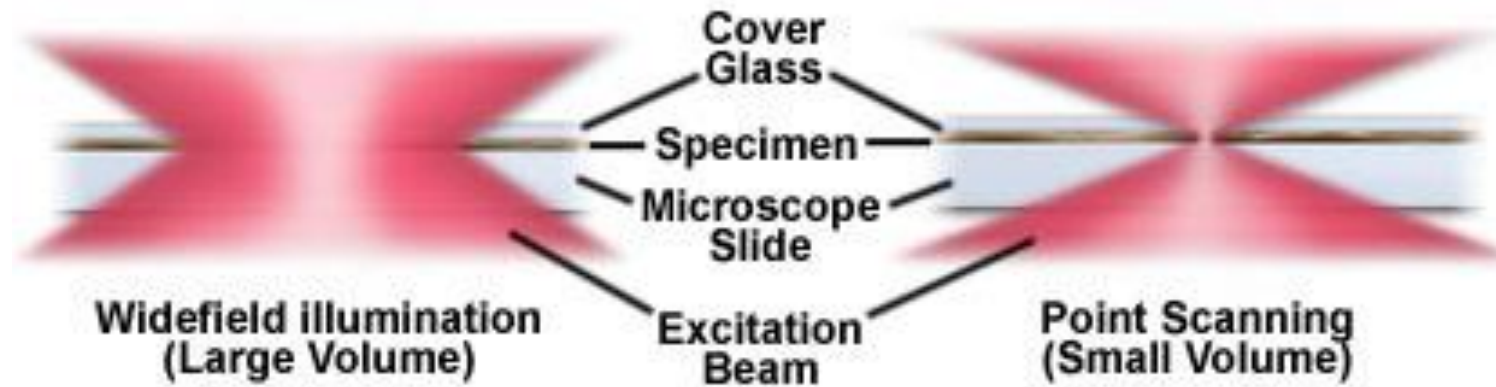


Figure 4



# Laser scanning confocal Microscope (LSCM)



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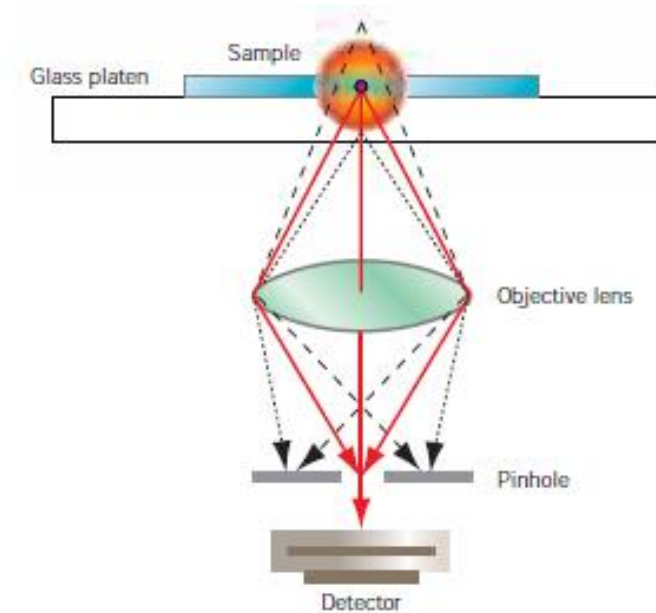


Illustration of confocal optics. Fluorescence from the sample is collected by a objective lens and directed toward a pinhole aperture. The pinhole allows the emitted light from a narrow focal plane (red solid lines) to pass to the detector, while blocking most of the out-of-focus light (black dashed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

# What is confocal?



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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 suppression of out-of-focal-plane light, suppression 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 superimpose 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.

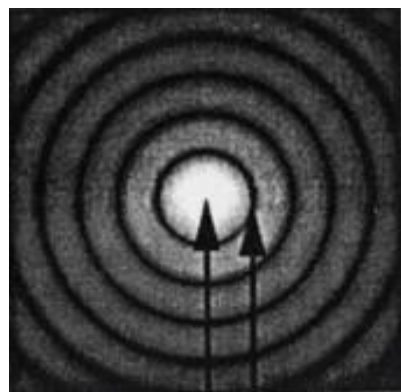
# What is confocal?



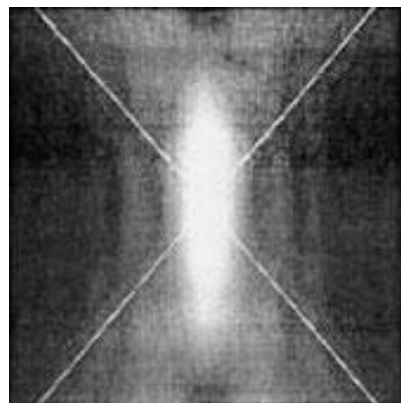
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Standard Microscope

Lateral

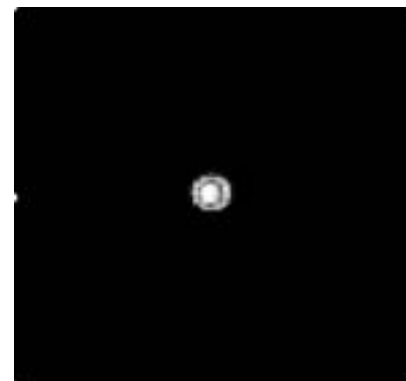


Axial



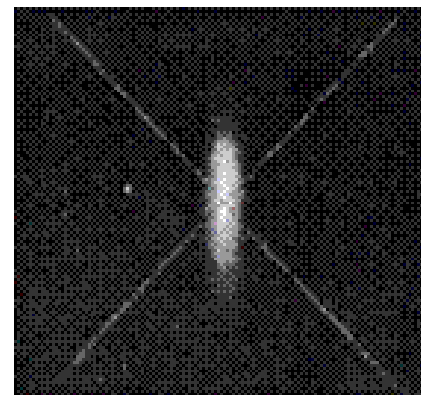
Confocal Microscope

Lateral



with pinhole

Axial



with pinhole

# Lateral and Axial Resolution in confocal system



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## RESEL

$$\text{Res}_{\text{lateral}} = \frac{0.51 \lambda_{\text{em}}}{NA}$$

50 -100 nm with interpolation

$$\text{Res}_{\text{lateral}} = \frac{0.37 \lambda_{\text{ex}}}{NA}$$

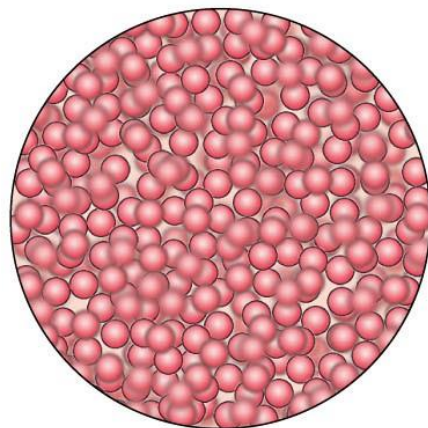
100 -150 nm with interpolation



# What is confocal?

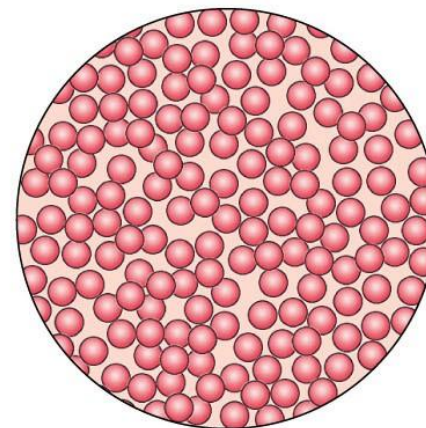
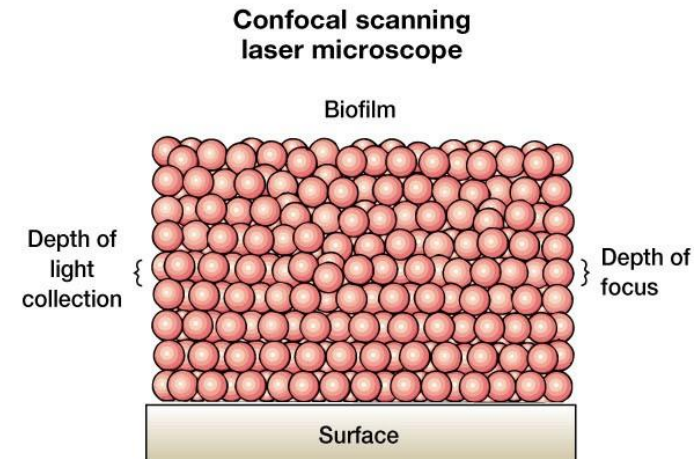


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(a)

Image in field of view



(b)

Image in field of view

# Laser scanning confocal Microscope (LSCM)



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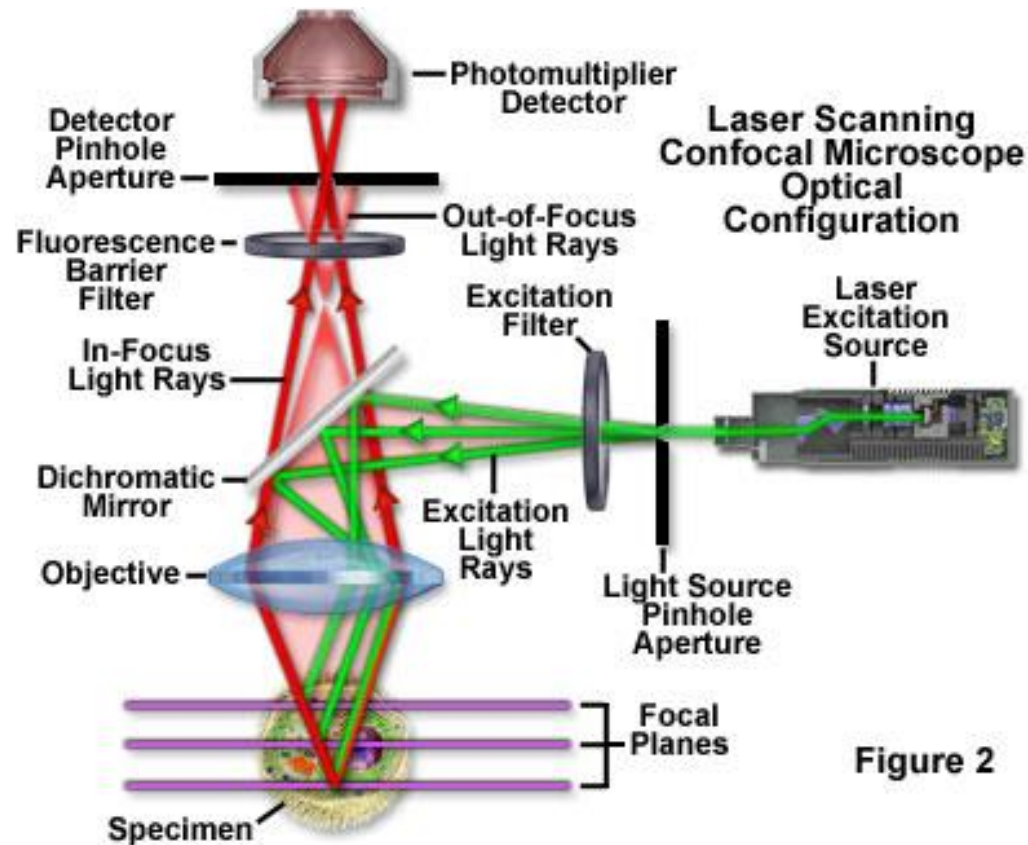


Figure 2

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

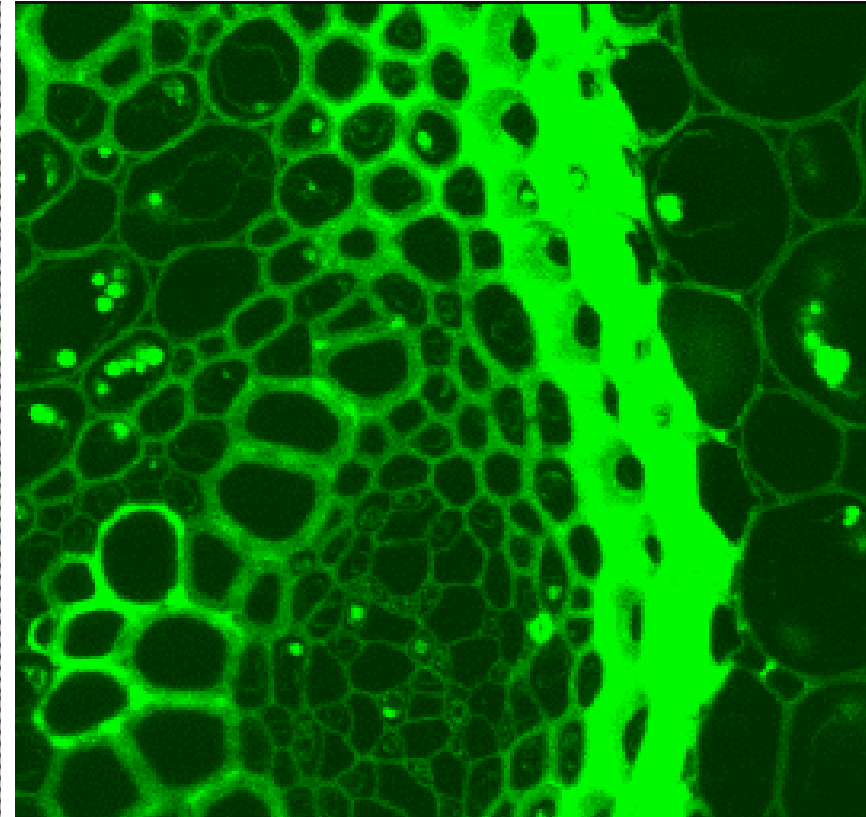
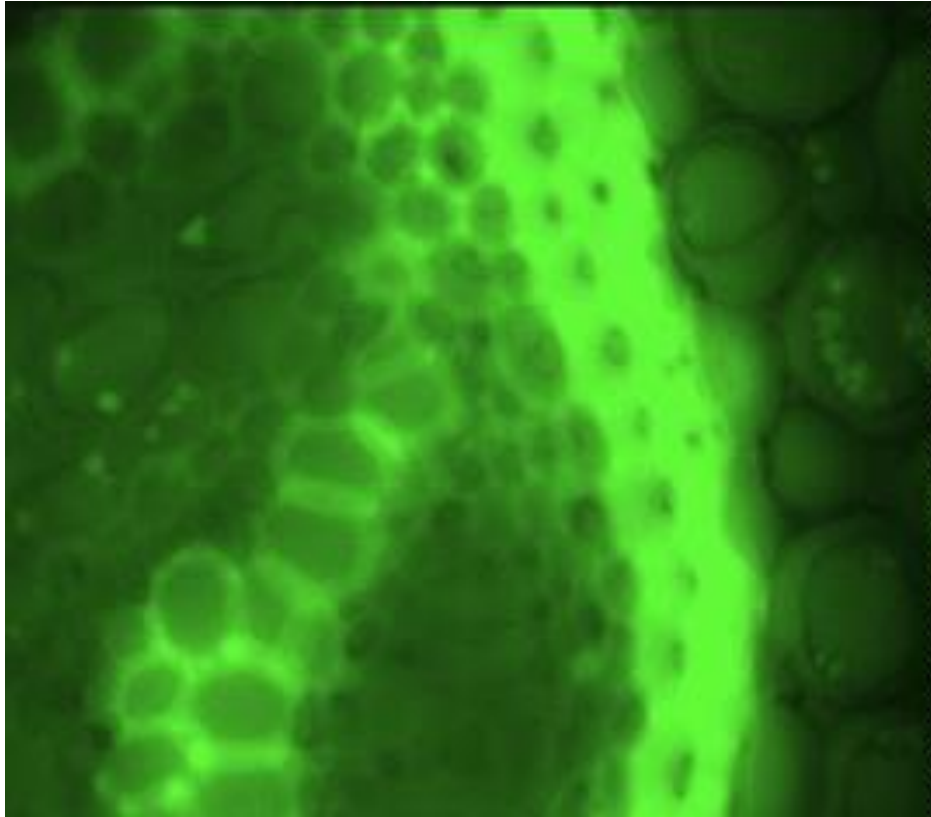
Detecting pinhole is used to get rid of out-of-focus signal. PMTs are used as detecting device. The main advantage of this type is its combination of good image quality, versatile functionality and reliability.

# CONVENTIONAL vs CONFOCAL



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## DETAILS

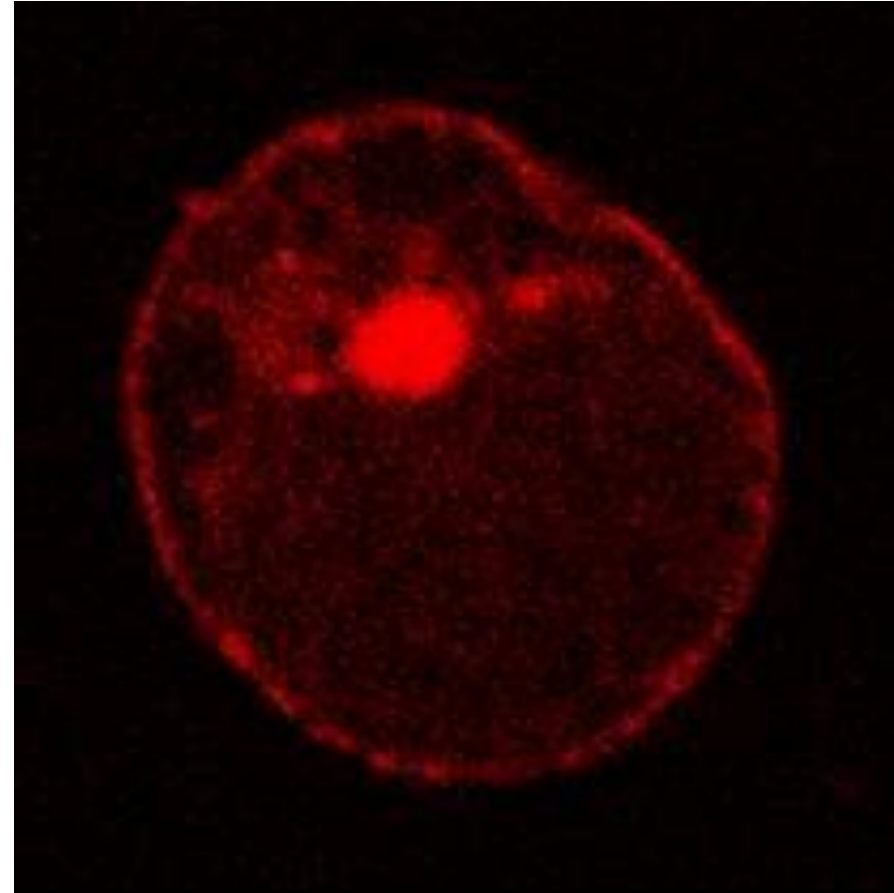
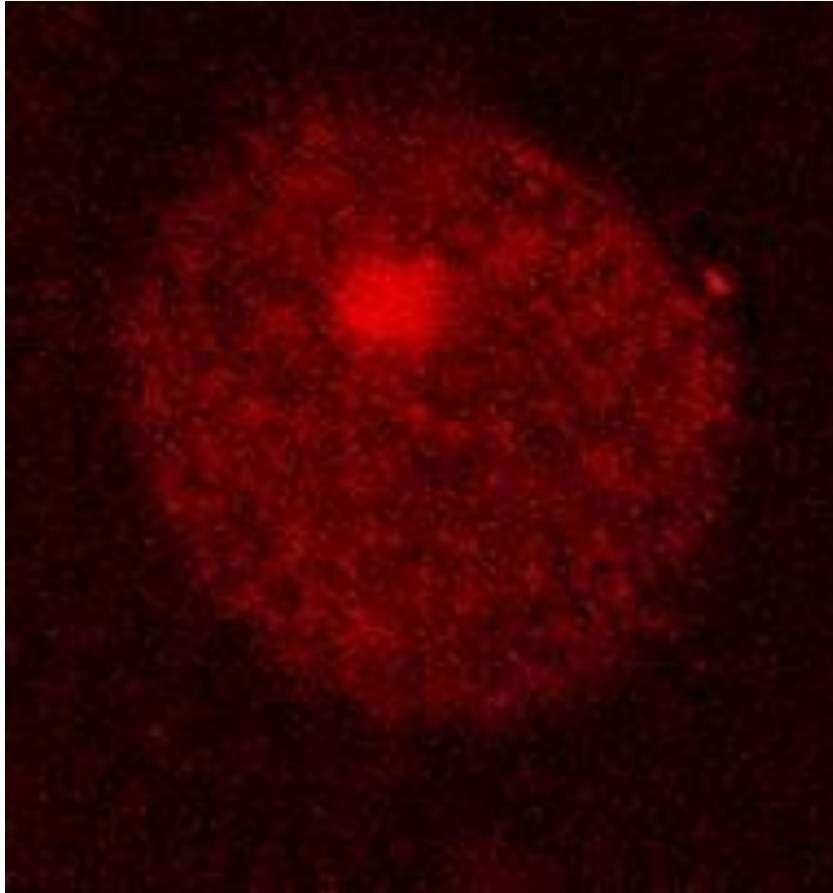


# CONVENTIONAL vs CONFOCAL



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## MOLECULES LOCALIZATION





# CONVENTIONAL vs CONFOCAL



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Confocal and Widefield Fluorescence Microscopy

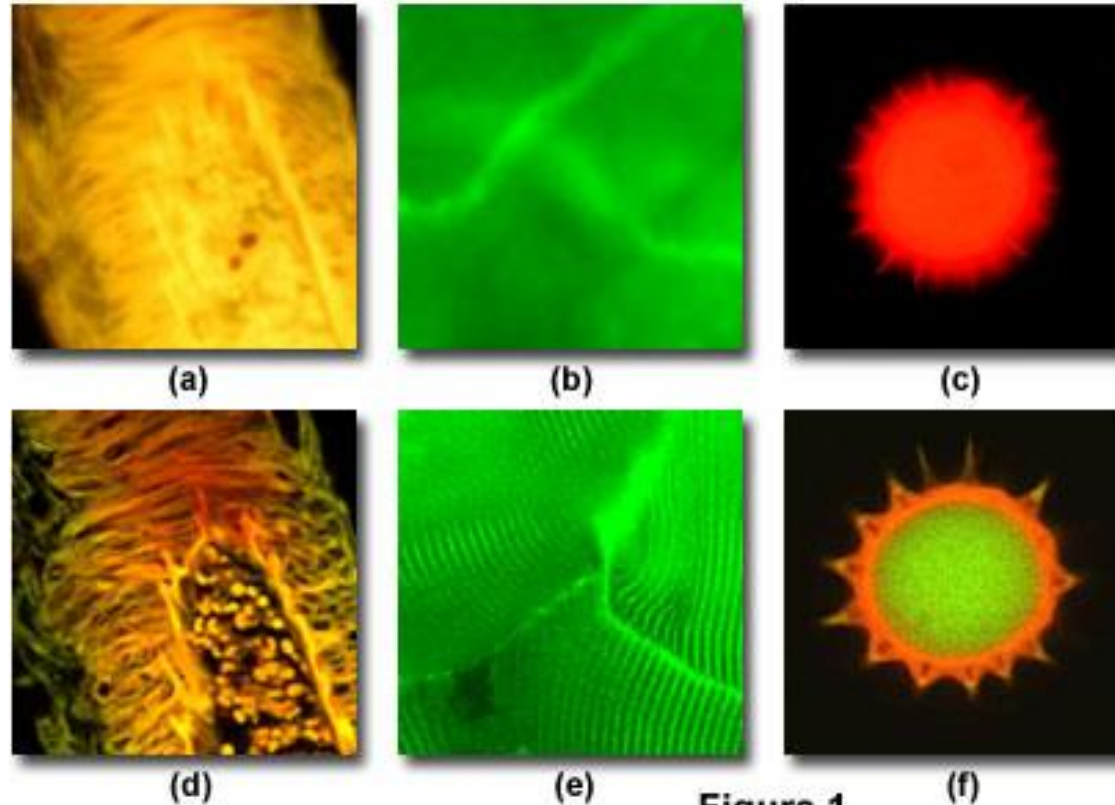


Figure 1

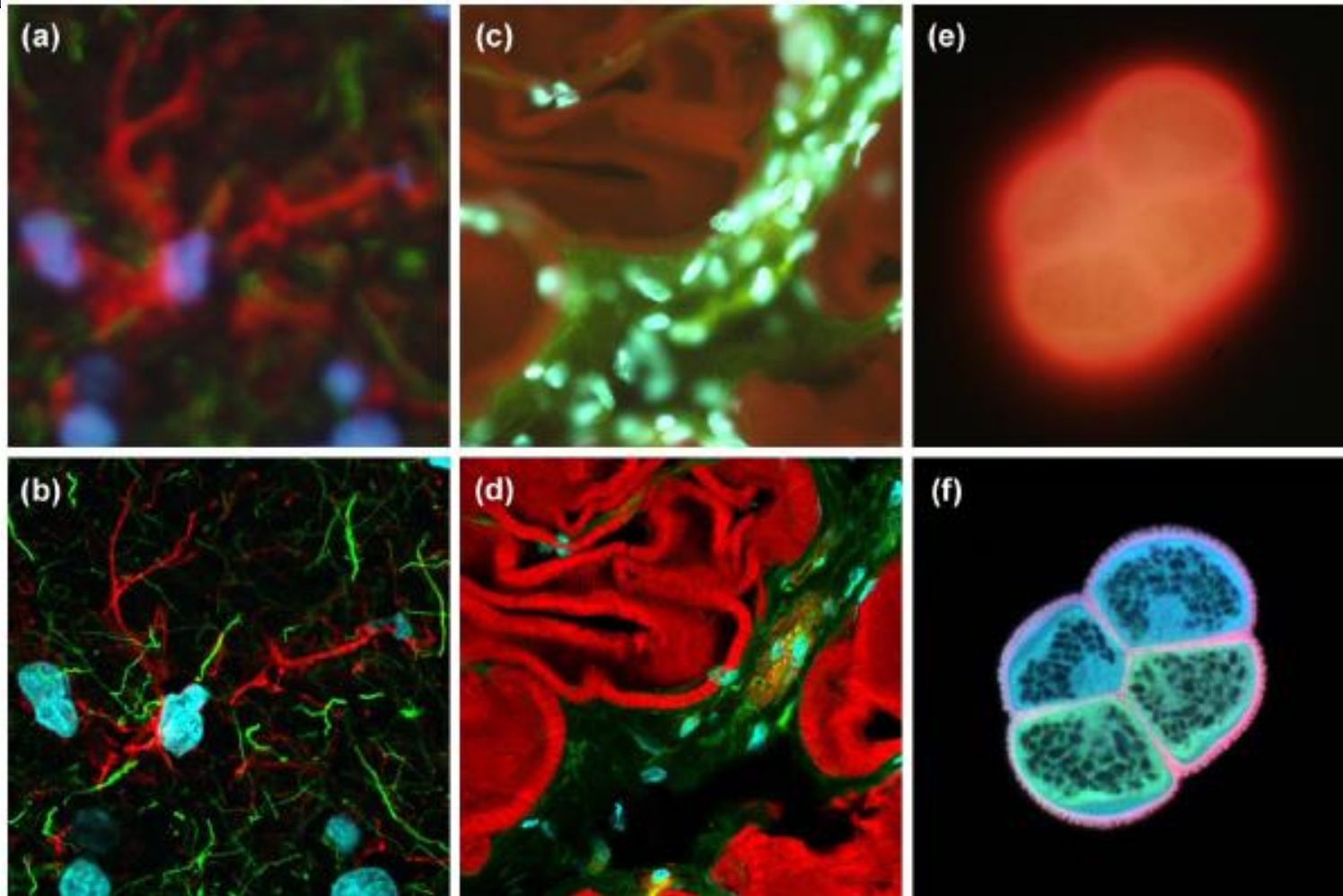
In a conventional widefield optical epi-fluorescence microscope, secondary fluorescence emitted by the specimen often occurs through the excited volume and obscures resolution of features that lie in the objective focal plane.



# CONVENTIONAL vs CONFOCAL



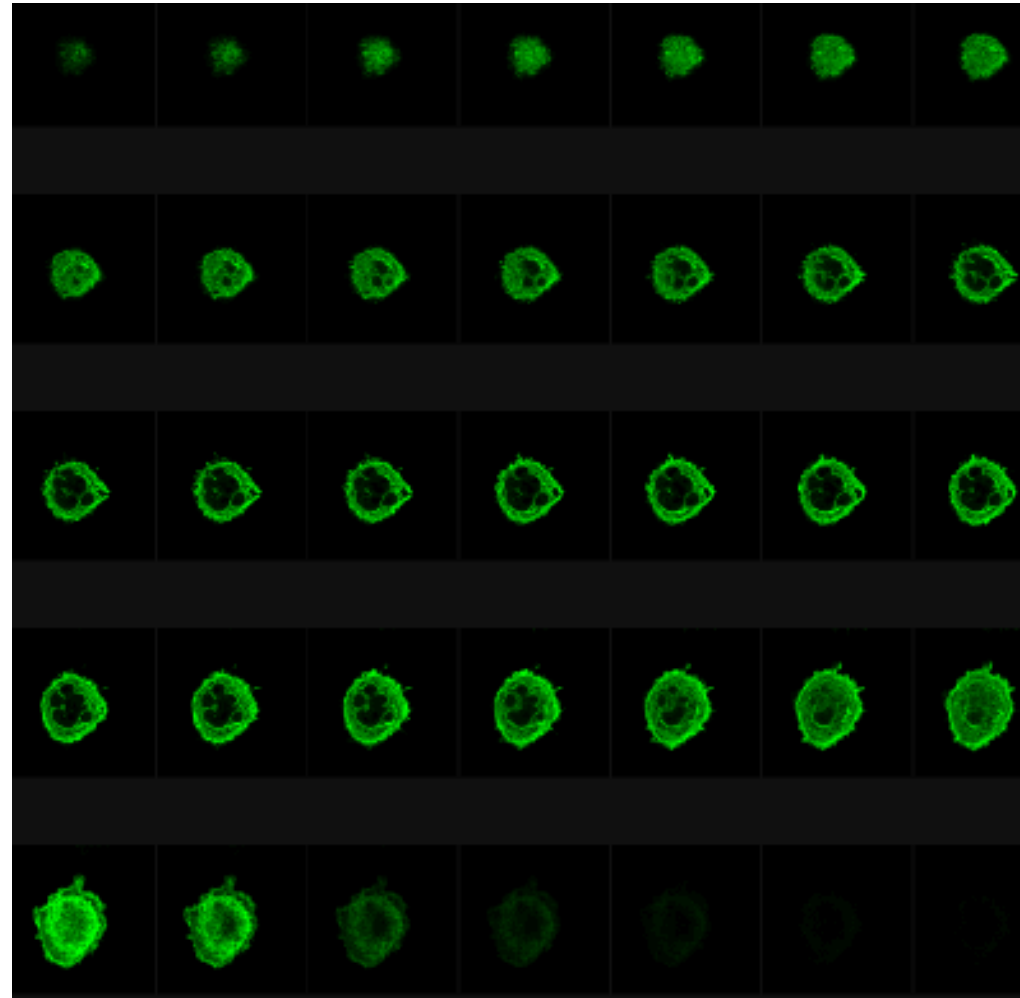
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Confocal Microscopy is the only way to get a true Z-stack for 3-D reconstruction from light microscope.



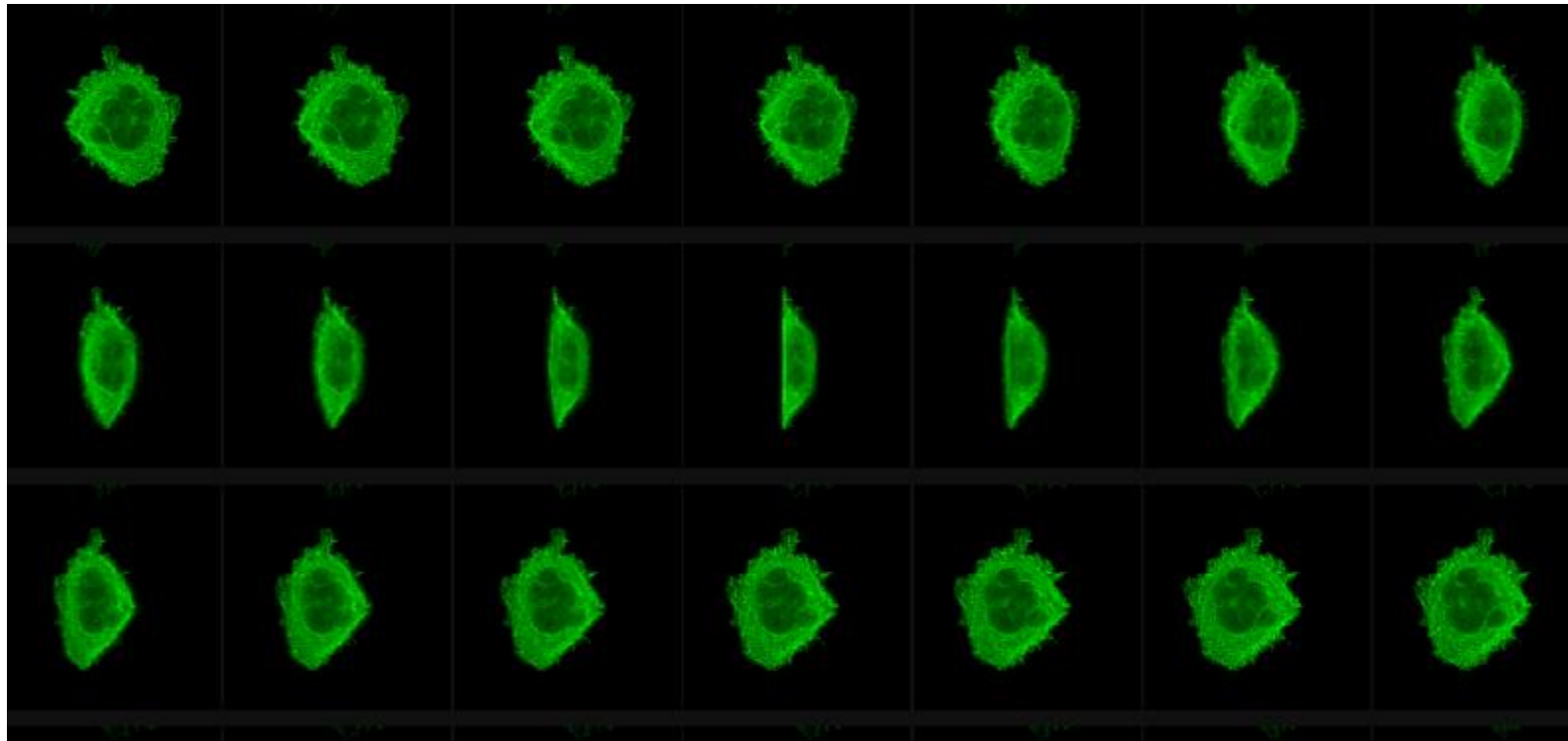
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Confocal Microscopy is the only way to get a true Z-stack for 3-D reconstruction from light microscope.



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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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Pollen Grain Serial Optical Sections by Confocal Microscopy

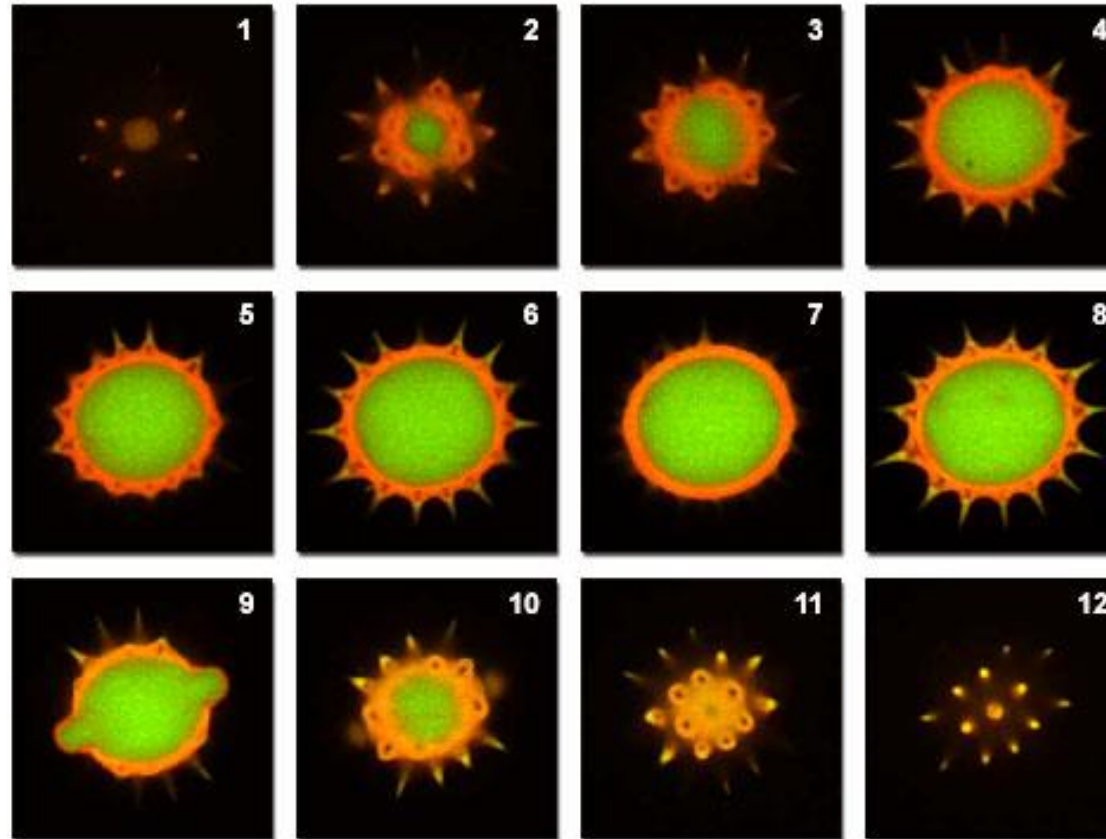
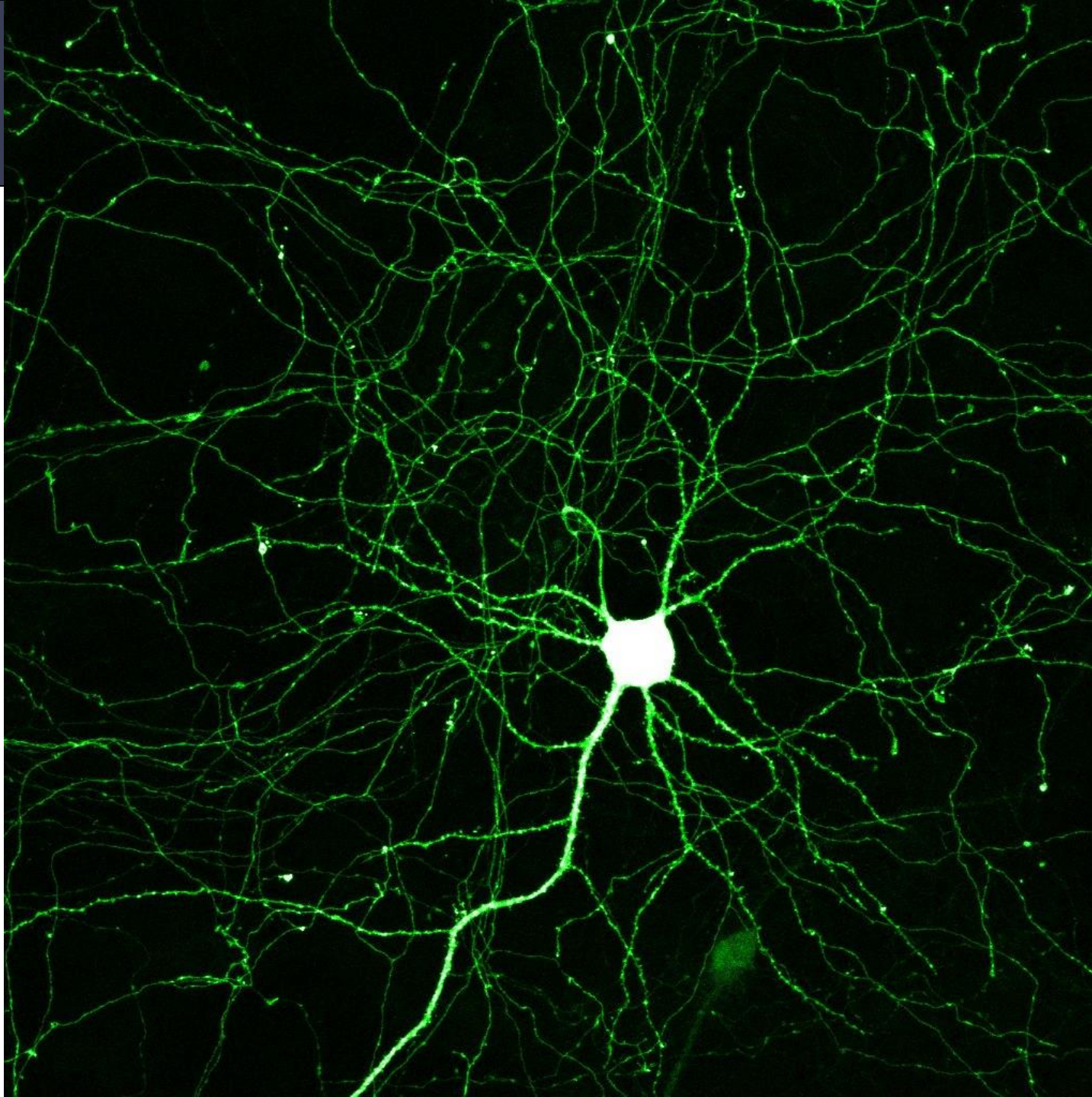


Figure 6





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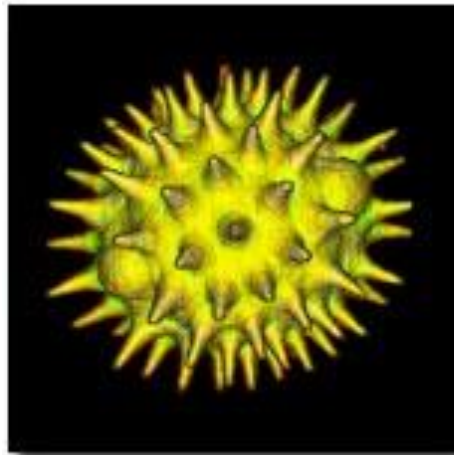


# 3D CONFOCAL rendering

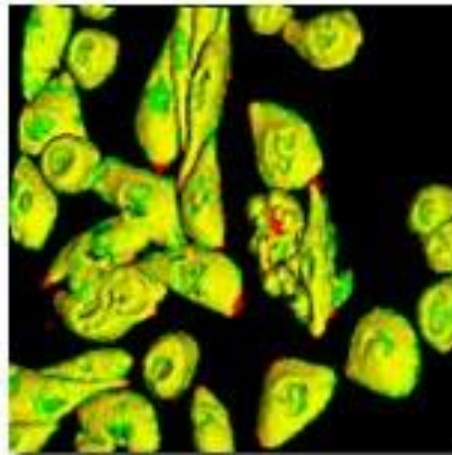


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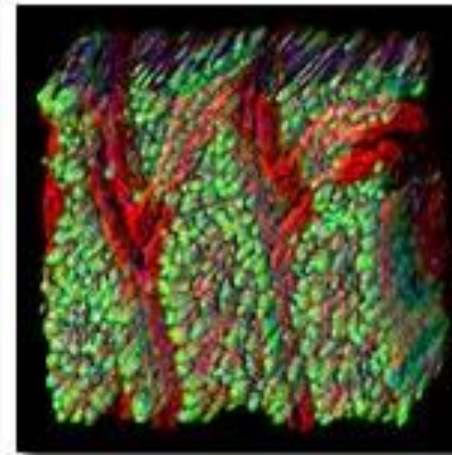
Three-Dimensional Volume Renders from Confocal Optical Sections



(a)

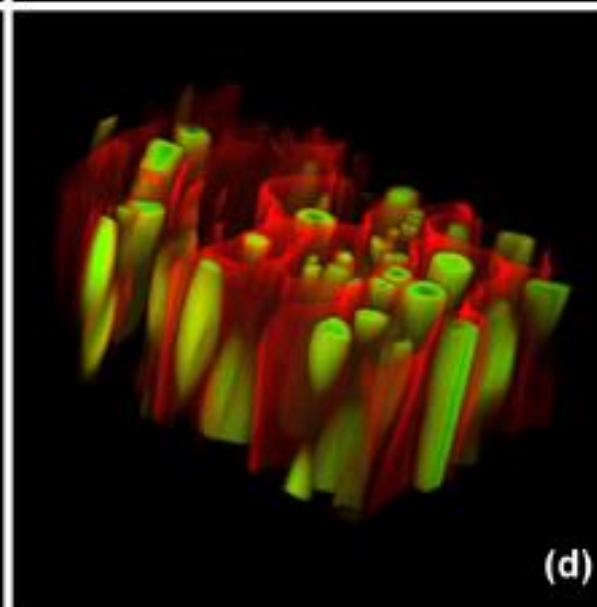
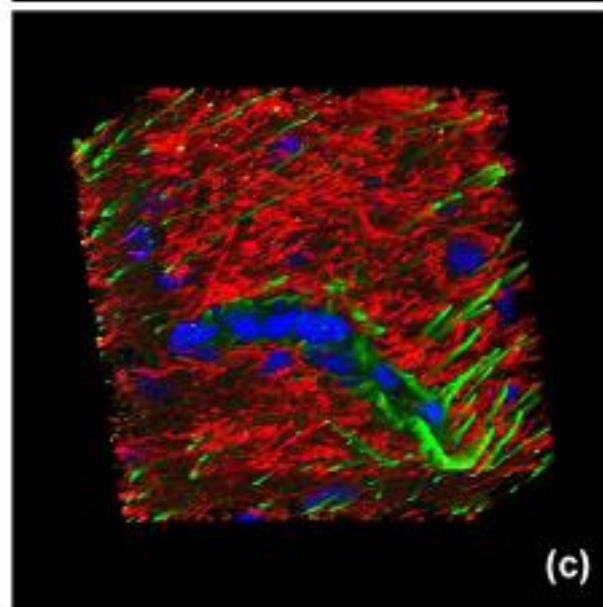
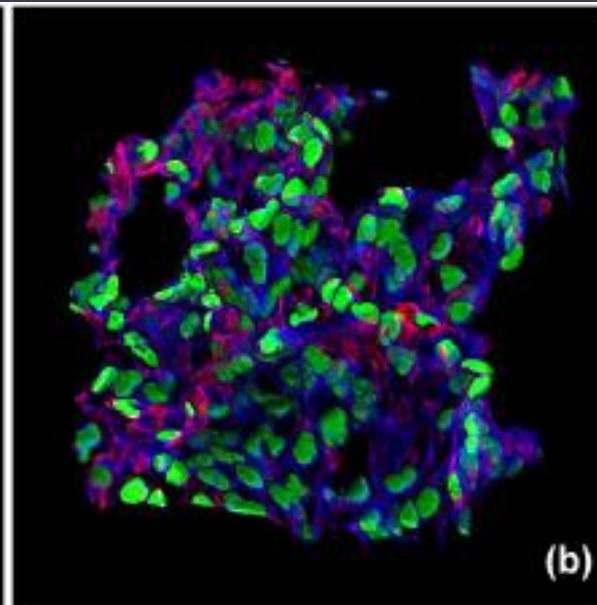
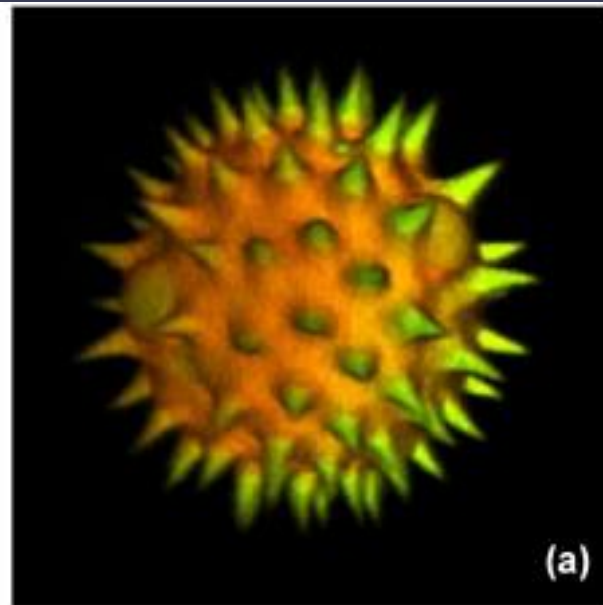


(b)



(c)

Figure 7



# Spectral Bleed-Through Artifacts in Confocal Microscopy



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## Simultaneous and Sequential Line Scanning in Confocal Microscopy

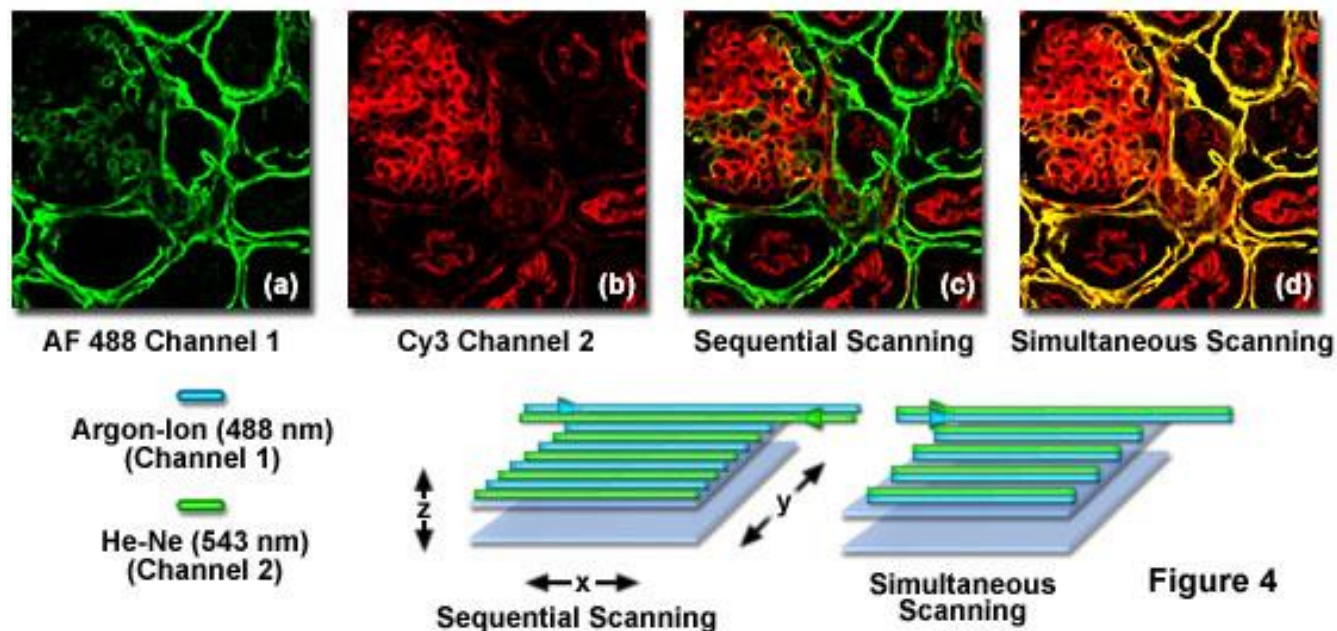


Figure 4

## Bleed-Through Management in Fluorescence Protein Imaging

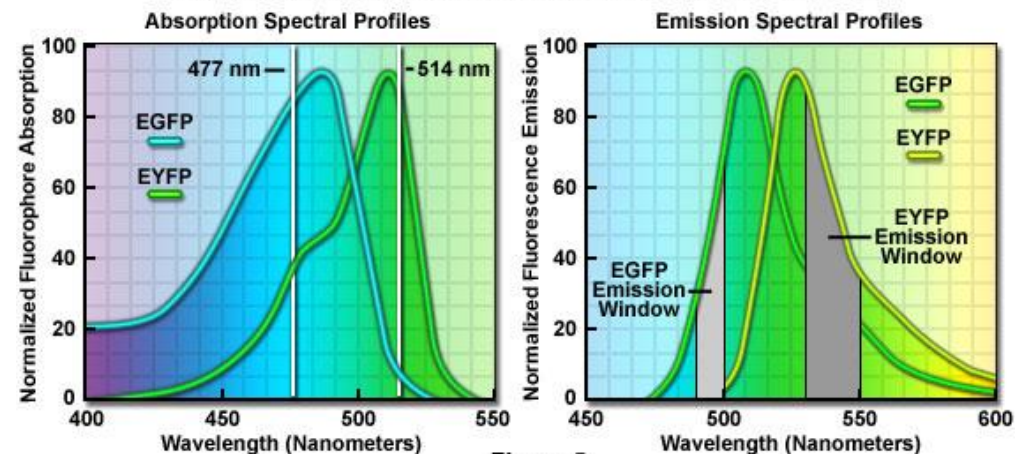


Figure 5

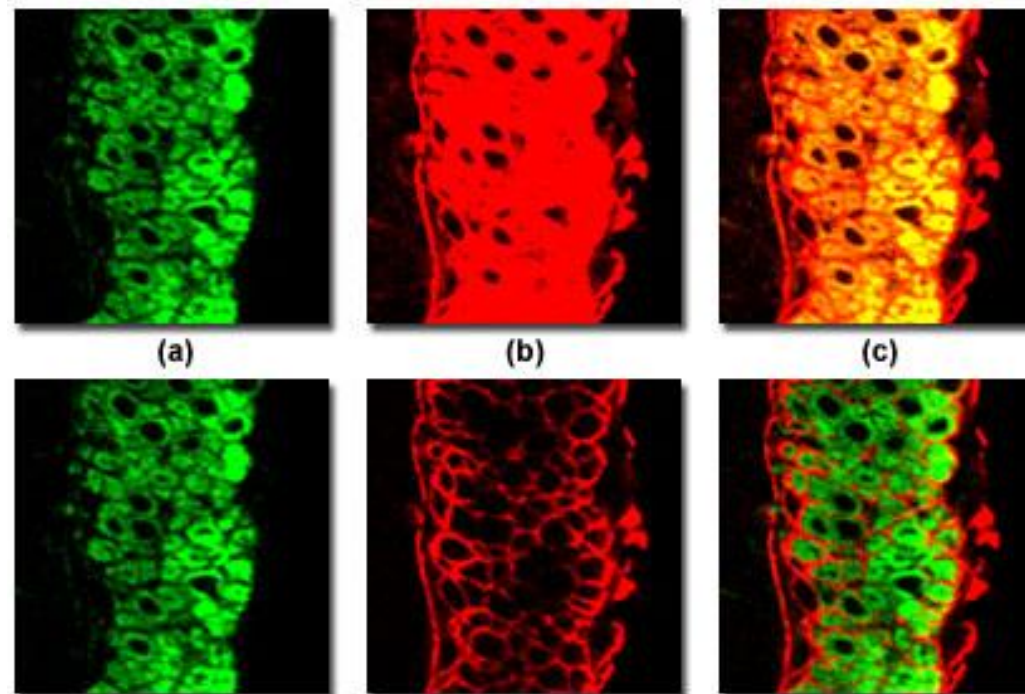


# Spectral Bleed-Through Artifacts in Confocal Microscopy



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## Fluorophore Emission Bleed-Through in Confocal Microscopy



## Spectral Overlap in Paired Alexa Fluor Probes

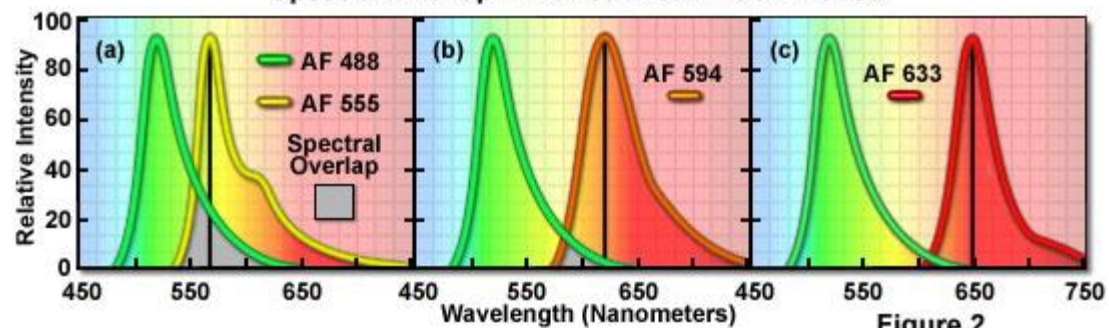


Figure 2

# CONFOCAL SETTINGS



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Pixel Size Effect on Contrast

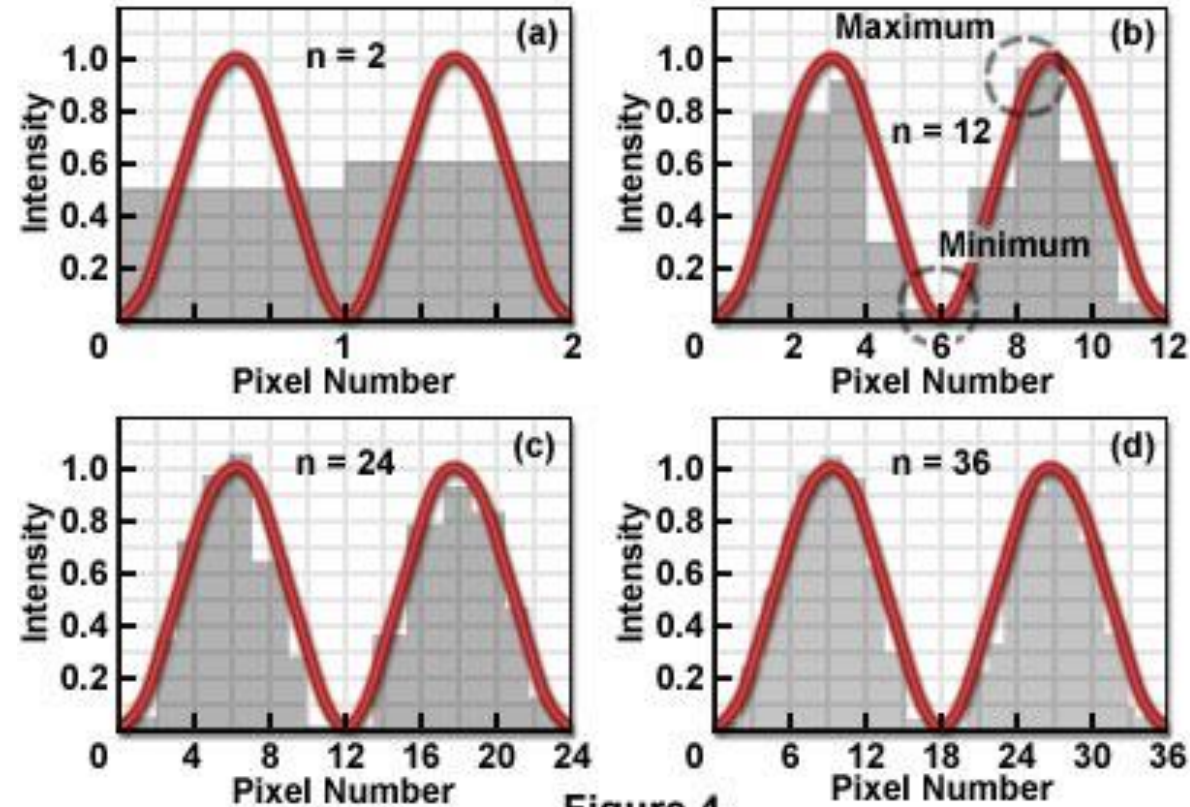


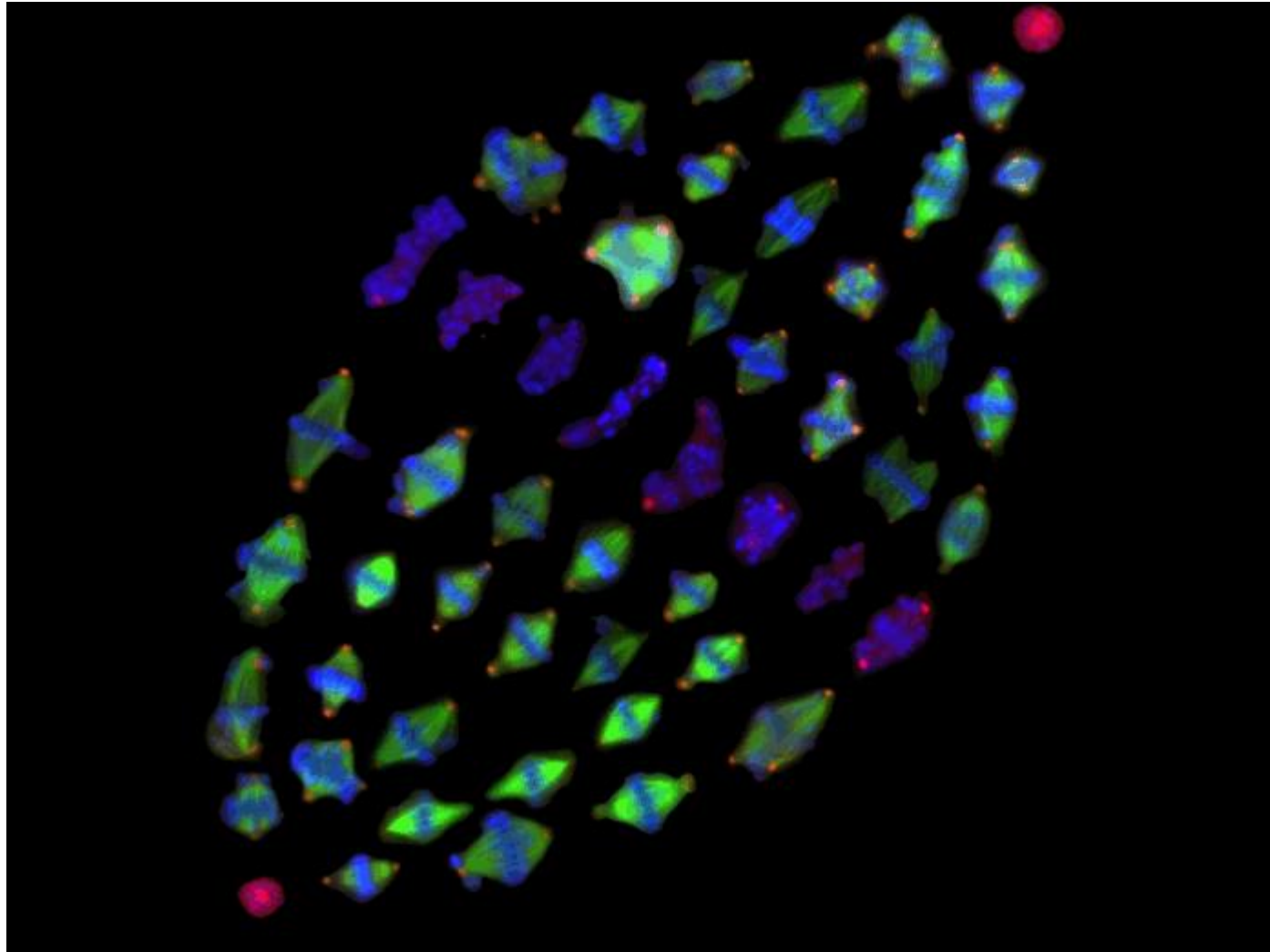
Figure 4



# CONFOCAL and DNA



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# CONFOCAL and DNA



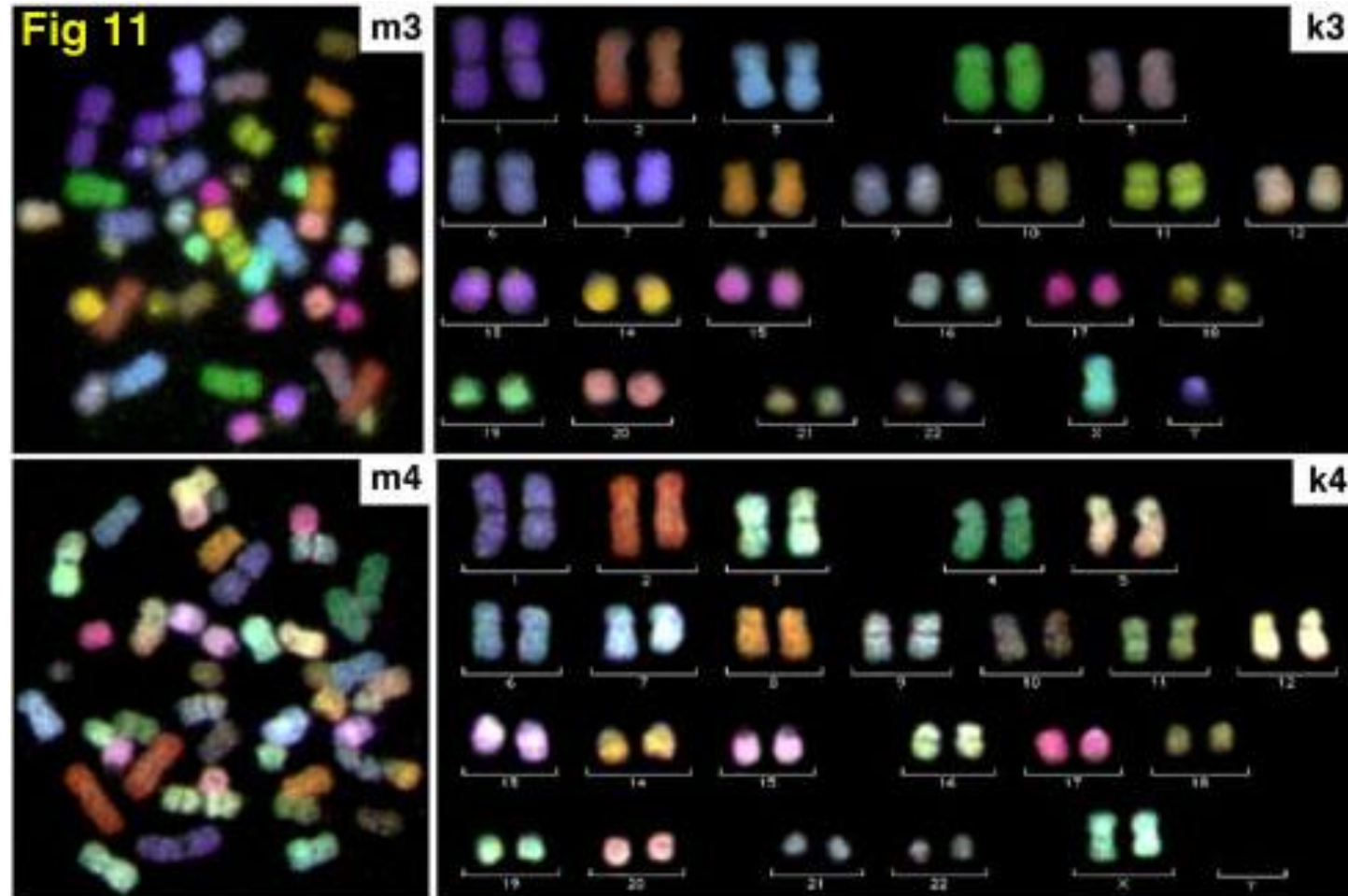
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# CONFOCAL and DNA



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# 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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# The Dark-Field Microscope



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- produces a bright image of the object against a dark background
- used to observe living, unstained preparations

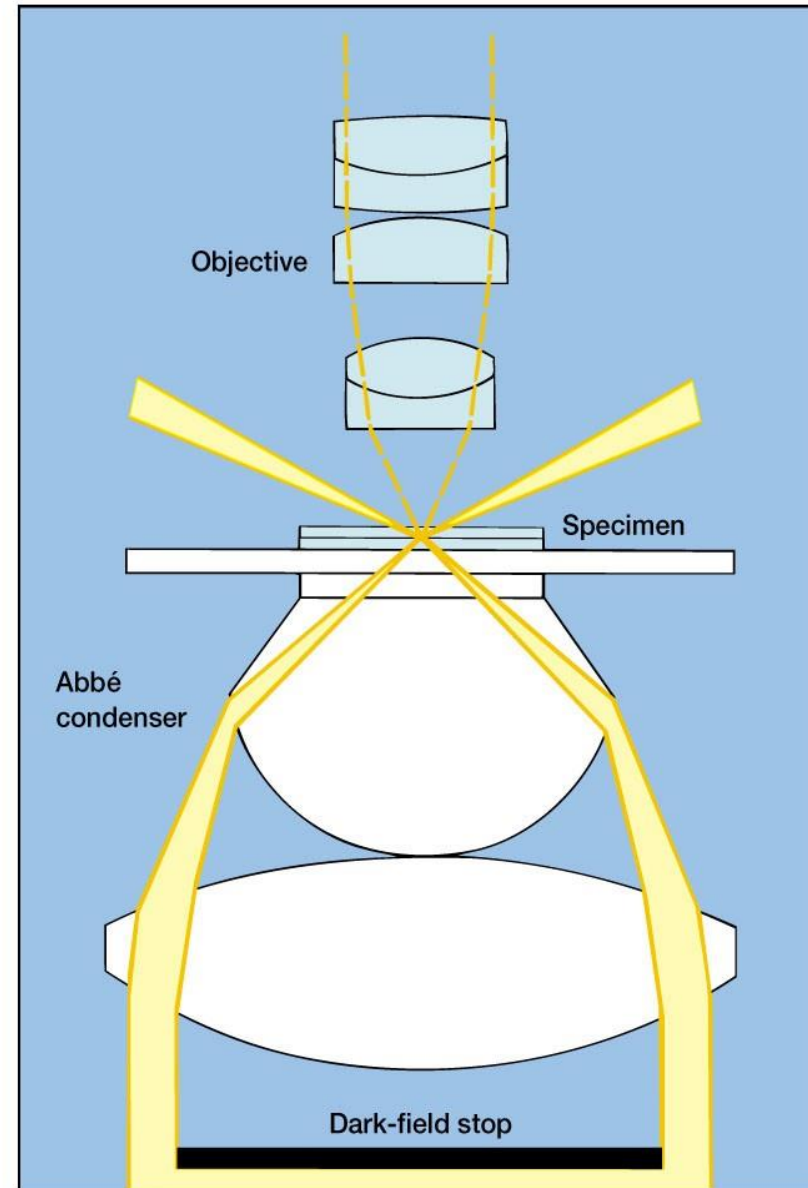


Figure 2.7b



# The Phase-Contrast Microscope

- enhances the contrast between intracellular structures having slight differences in refractive index
- excellent way to observe living cells

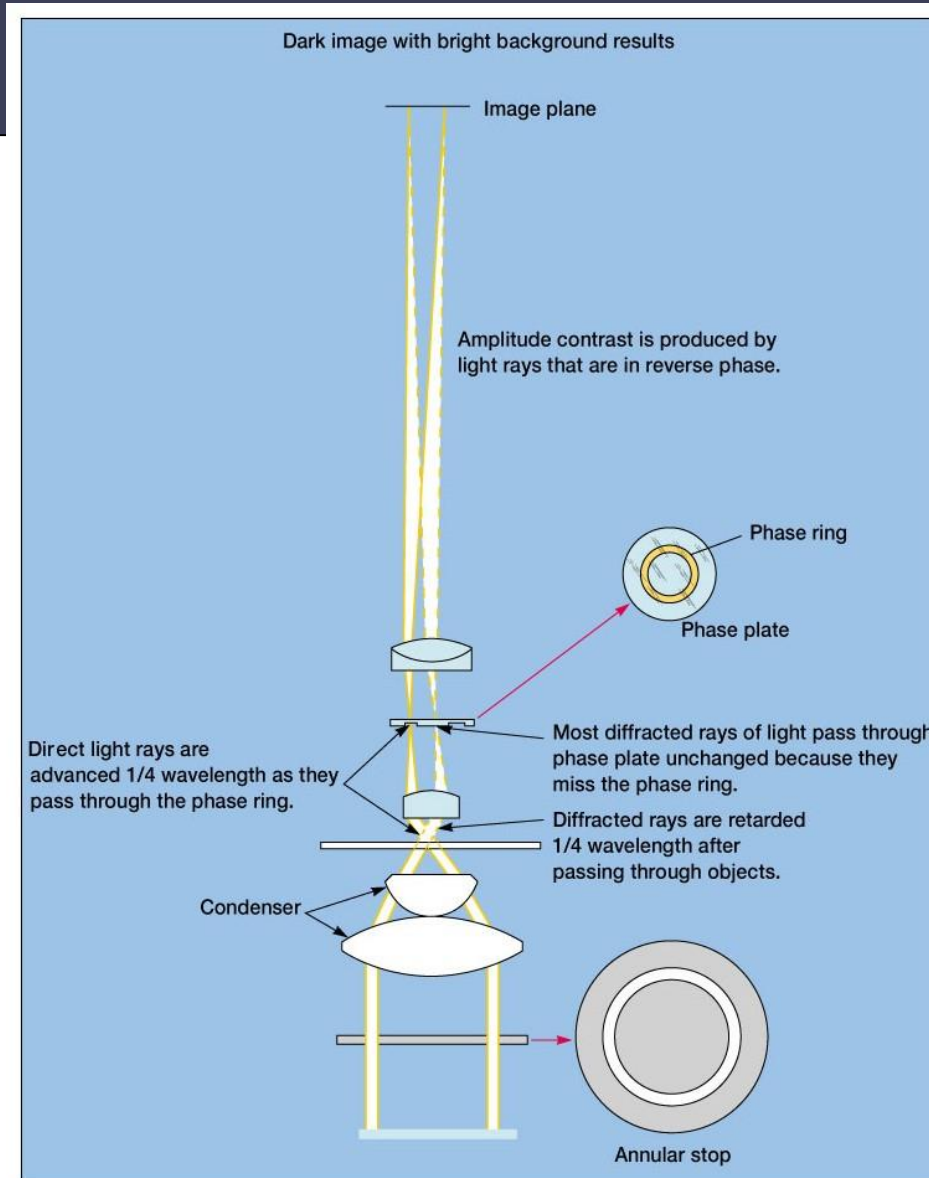


Figure 2.9

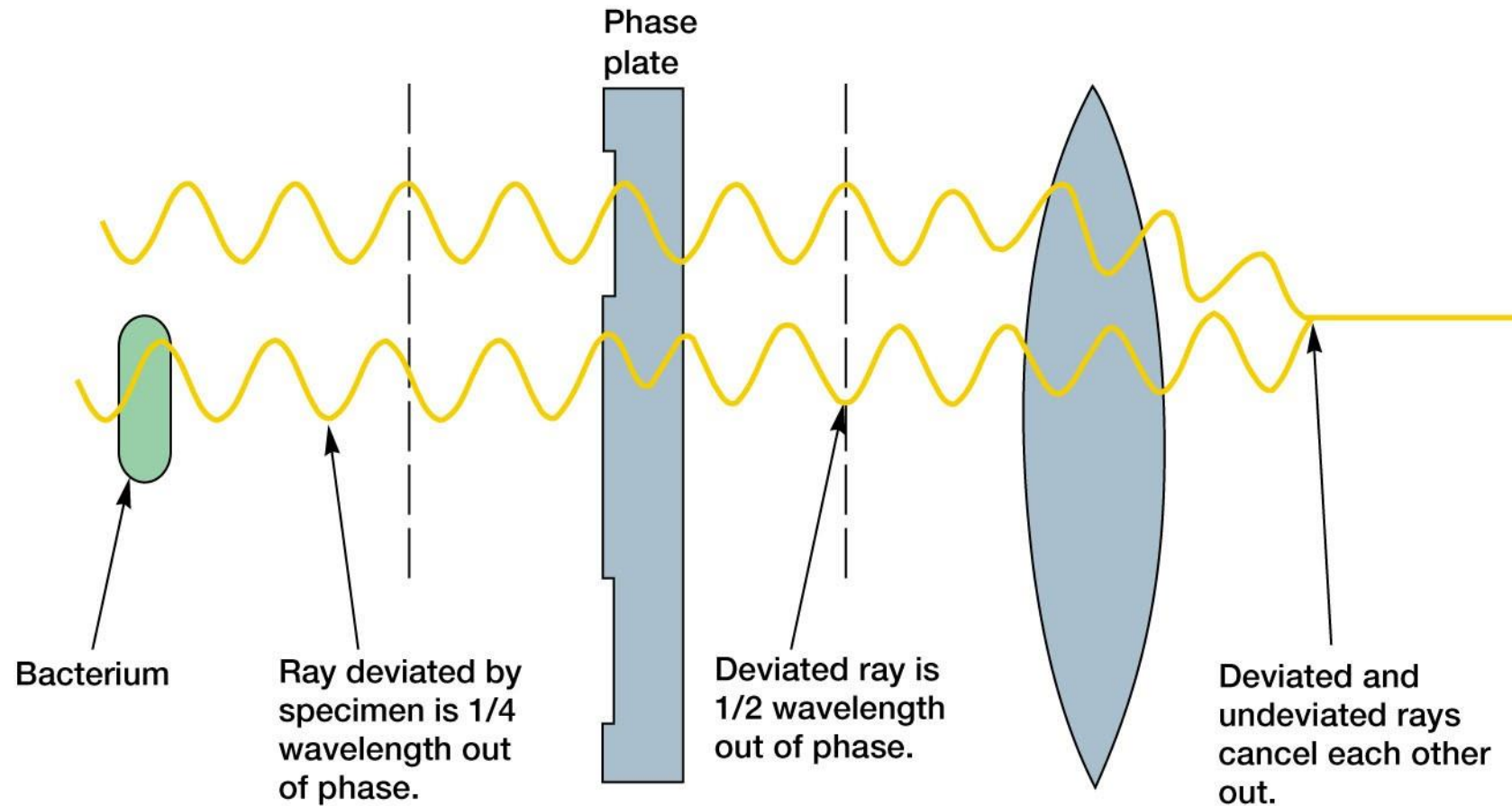


Figure 2.10





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